Journal of Pharmaceutical Sciences

JANUARY 1975

VOLUME 64 NUMBER 1

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FRAGILE: HANDLE WITH CARE

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After identifying the particular compound with the most favorable therapeutic index from among various possible homologs, analogs, congeners, salt forms, and the like, the next phase involves the complex process of studying and selecting ingredients and manufacturing processes that will result in the most effective dosage form for administering that drug entity to the patient.

Unfortunately, there has sometimes been a mistaken belief that once the elegant tablet, capsule, or other dosage form has been formulated in a pure, bioavailable, and uniformly potent form, the job was over. That is, there was little need for further concern as to the safety and effectiveness of the product.

It is now recognized, however, that the best of products—if not properly packaged to protect its integrity and quality—may undergo changes due to light, moisture, air, or volatilization, which very well might render the product unsafe or reduce its effectiveness, or both.

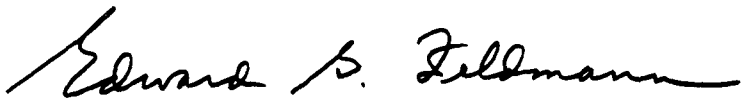
As in most situations, this problem has been the result of various factors including inadequate research, carelessness, faulty communications, and so on. But one of the major stumbling blocks has been lack of an objective test procedure to conduct the necessary measurements to determine the suitability of a particular container. And in the absence of a test procedure that permits quantitative measurements of reasonable accuracy and precision, it is obvious that no meaningful and enforceable standards or specifications could be developed relative to such container characteristics.

What was probably the first breakthrough in this field came about fifteen years ago with the development and adoption by the official compendia of a specific test procedure and a standard specification for the assessment of light protection afforded by glass containers, based upon spectrophotometric measurements. This procedure and standard subsequently were extended to cover plastic containers also.

But the problem of container "tightness" remained. A well-intentioned effort was made by the compendia to define a "well-closed container" and a "tight container," and to distinguish them from each other as well as from containers affording lesser protection; however, everyone recognized that this effort was more of a pious hope than a scientific standard.

Consequently, the more recent pioneering work of the National Formulary—along with the cooperative involvement of the United States Pharmacopeia and the Food and Drug Administration—has resulted in a very significant step forward. We refer, of course, to the new test procedure, and moisture permeability standards, that appear in the new editions of the compendia and concerning which FDA is currently in the process of establishing regulations to facilitate effective implementation.

In essence, this development serves to plug what has been a gaping hole in our overall system of providing high quality drug products to the patient. As a result, we soon shall be able to rest better assured that the drug product at the point of use by the patient is far more likely to be as safe and as effective as it was at the time of its original manufacture or preparation.



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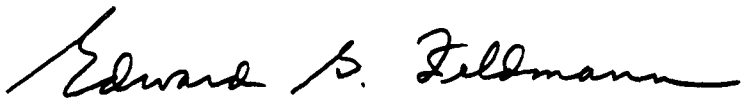

Antiviral Activity of Hederin

A recent communication by G. S. Rao *et al.*[1] mentioned that antiviral activity has not been previously reported for the triterpenoid saponins. However, an article, "La Hederina, Un Nuevo Antibiotico" (Hederin, A New Antibiotic), by A. I. Calabrese was published in 1949[2] and is listed in the *Quarterly Cumulative Index Medicus* (1949, p. 233). Hederin was the subject of my thesis for the Professor degree in toxicology at the University of Buenos Aires, Faculty of Medicine, in which I described its pharmacological, bactericidal, virostatic, antitumor, and toxicological activity.

Alberto I. Calabrese
Facultad de Medicina
Universidad de Buenos Aires
Buenos Aires, Argentina

Received September 17, 1974.

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Bioequivalency and Noise

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Perhaps the conspiratorial climate of the nation's capital, amplified by the continuing Watergate scenario, has managed to penetrate the pharmaceutical bastion on Constitution Avenue. The members of the Panel are all distinguished medical and pharmaceutical scientists who, no matter how faulty or correct their recommendations will prove to be, are blatantly slandered by such speculation—and all the more so since it appears in a reputable scientific journal.

One issue later, in October, the same editorialist provides a welcome touch of wry amusement in a Journal that rarely raises a smile when he informs us that the APhA has complained about the Academy of Pharmaceutical Sciences "speaking the wrong language and shouting besides." Time will inevitably tell us who was speaking the wrong language concerning the important issue of bioequivalency but as to who was guilty of shouting, we no longer need to wait.

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January 27–31
American Association for the Advancement of Science, Annual Meeting, Americana Hotel and City Squire, New York, N.Y.

February 25
Stability and Content Uniformity in Pharmaceuticals, Annual Analytical Symposium, Toronto Section, Canadian Institute of Chemistry, Toronto, Canada (Contact T. J. Carrothers, Bristol-Myers Products Canada Ltd., 106 N. Queen St., Toronto, Ontario M8Z 2E2, Canada)

February 28–March 2
World Congress on Electrocardiology, Cardiological Society of India, Bombay, India (Contact A. B. Mehta, World Congress on Electrocardiology, Department of Electrocardiology, L.T.M. Medical School, Sion, Bombay 400022, India)

March 9–13
Society of Toxicology, Williamsburg Lodge, Williamsburg, Va. (Contact R. E. Scala, Esso Research and Engineering Co., P.O. Box 45, Linden, NJ 07036)

March 14–16
National Medicolegal Symposium, American Medical Association and American Bar Association, MGM Grand Hotel, Las Vegas, Nev. (Contact AMA Office of the General Counsel, 535 N. Dearborn St., Chicago, IL 60610)

March 20–22
American Society for Clinical Pharmacology and Therapeutics, Annual Meeting, Washington Hilton Hotel, Washington, D.C.

April 6–11
American Chemical Society, National Meeting, Philadelphia, Pa. (Contact Meetings Department, American Chemical Society, 1155 16th St., N.W., Washington, DC 20036)

April 19–24
APhA Annual Meeting, San Francisco, Calif.

April 27–May 2
American Society for Microbiology, New York Hilton Hotel, New York, N.Y. (Contact R. W. Sarber, 1913 Eye St., N.W., Washington, DC 20006)

April 28–30
Pharmaceutical Manufacturers Association, Annual Meeting, Boca Raton, Fla.

May 1–4
American Society of Internal Medicine, Mayflower Hotel, Washington, D.C. (Contact William Ramsey, 535 Central Tower Building, 703 Market St., San Francisco, CA 94103)

May 2–3
American College of Clinical Pharmacology, Chalfonte-Haddon Hall, Atlantic City, N.J. (Contact Ms. Barbara Renaud, 2 E. 103rd St., New York, NY 10029)

May 2–4
Association of Clinical Scientists, Governors Inn, Research Triangle Park, N.C. (Contact F. W. Sunderman, Jr., M.D., University of Connecticut School of Medicine, P.O. Box G, Farmington, CT 06032)

May 4–5
American Federation for Clinical Research, Chalfonte-Haddon Hall, Atlantic City, N.J. (Contact C. B. Slack, 6900 Grove Road, Thorofare, NJ 08086)

May 5
American Society for Clinical Investigation, Chalfonte-Haddon Hall, Atlantic City, N.J. (Contact H. E. Williams, M.D., Room 4401, San Francisco General Hospital, San Francisco, CA 94110)

May 11–14
The Proprietary Association, Annual Meeting, The Greenbrier, White Sulphur Springs, W.Va.

May 22–24
International Congress on Patient Counseling, Amsterdam, The Netherlands (Contact K. Ellison Davis, Excerpta Medica Foundation, P.O. Box 1126, Jan van Gaelnstr, 335, Amsterdam, The Netherlands)

May 26–30
International Conference on Prostaglandins, Florence, Italy (Contact Dr. G. C. Folco, Scientific Secretary, Institute of Pharmacology and Pharmacognosy, University of Milan, via Andrea Del Sarto, 21-20129, Milan, Italy)

June 15–19
American Medical Association, Annual Meeting, Atlantic City, N.J. (Contact Dr. E. B. Howard, 535 N. Dearborn St., Chicago, IL 60610)

June 16–18
International Symposium on Mass Spectrometry in Biochemistry and Medicine, Alghero, Sardinia, Italy (Contact A. Frigerio, Istituto di Ricerche Farmacologiche, Mario Negri, Via Evitrea 62, 20157 Milan, Italy)

June 18–20
Drug Information Association, Annual Meeting, Sheraton Boston Hotel, Boston, Mass.

July 13–18
International Congress on Clinical Chemistry, Toronto, Ontario, Canada (Contact Dr. F. H. Sims, Women's College Hospital, Toronto, Ontario, Canada)

September 1–5
International Congress of Pharmaceutical Sciences, Dublin, Ireland (Contact Congress Secretary, 35th International Congress of Pharmaceutical Sciences, 44 Northumberland Road, Ballsbridge, Dublin 4, Ireland)

October 1–5
American College of Apothecaries, Annual Meeting, Four Seasons Sheraton, Toronto, Ontario, Canada

November 16–20
American Public Health Association, Annual Meeting, Chicago, Ill.

November 16–20
APhA Academy of Pharmaceutical Sciences, National Meeting, Marriott Motor Hotel, Atlanta, Ga.

November 29–December 4
American Medical Association, Clinical Meeting, Honolulu, Hawaii

November 30–December 5
American Chemical Society, National Meeting, Mexico City, Mexico (Contact Meetings Department, 1155 16th St., N.W., Washington, DC 20036)

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REVIEW ARTICLE

Cyclic Nucleotide Phosphodiesterases: Properties, Activators, Inhibitors, Structure–Activity Relationships, and Possible Role in Drug Development

M. SAMIR AMER ˣ **and WILLIAM E. KREIGHBAUM**

Keyphrases □ Phosphodiesterases, cyclic nucleotide—properties, activators, inhibitors, structure–activity relationships, possible role in drug development, review □ Cyclic nucleotide phosphodiesterases—properties, activators, inhibitors, structure–activity relationships, possible role in drug development, review □ Structure–activity relationships—cyclic nucleotide phosphodiesterases, review □ Nucleotide phosphodiesterases, cyclic—review of properties, possible role in drug development

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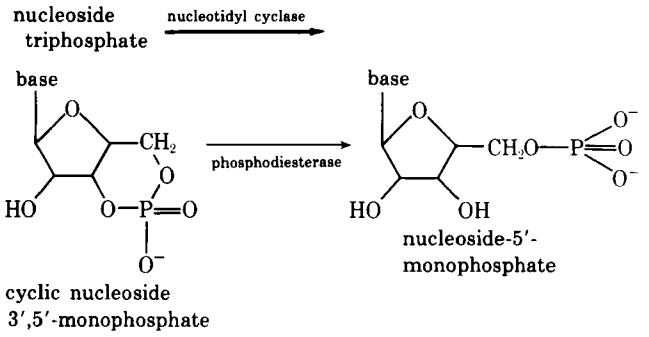
The central importance of cyclic nucleotide phosphodiesterases in drug development stems from the significant role that they must play in the control of intracellular cyclic nucleotide levels coupled with their apparent sensitivity to a large variety of chemical compounds (1, 2). Although two review articles on phosphodiesterases appeared recently (3, 4), aspects of these enzymes directly related to drug development have largely been ignored.

The purposes of this review are to cover these aspects in greater detail and to evaluate critically most major trends in this rapidly expanding area. However, due to the immense explosion in cyclic nucleotide research, an arbitrary selection of published reports had to be made. Many additional references may be found in the previous review articles (3, 4).

Phosphodiesterase catalyzes the apparently nonreversible (5) hydrolysis of the 3′-bond in the cyclic nu-



Scheme I

cleotides to produce their noncyclic 5′-monophosphate derivatives (Scheme I). Phosphodiesterase-catalyzed hydrolysis is the major physiological pathway for the termination of the intracellular effects of the cyclic nucleotides; there is not much support for other pathways for the degradation of cyclic AMP (6, 7).

Abnormal levels or forms of phosphodiesterase are associated with, and appear to contribute to, a number of disease states. Furthermore, various hormones may act by modulating phosphodiesterase activity in their target tissues. The enzyme also appears to be directly involved in the regulation of the chemotactic response of cellular slime molds (8–15) and may be implicated in receptors for taste (16–18), dopamine (19), and light (20) as well.

In a number of situations, the intracellular levels of the cyclic nucleotides and their related effects appear to be correlated closely with the magnitude of phosphodiesterase activity. The enzyme also translates into cellular events the actions of many drugs. It appears that phosphodiesterase represents more than just an off-switch for the cyclic nucleotide system and that it shares with the cyclases, the enzymes catalyzing the formation of the cyclic nucleotides, the important function of the delicate control of the intracellular cyclic nucleotide levels and consequently their myriad of important effects (21–23).

Although the cyclases, at least theoretically, present a suitable and possibly superior locus for the modulation of intracellular cyclic nucleotide levels, they presently occupy a position secondary to phosphodiesterases in efforts to develop new drugs that act *via* the cyclic nucleotide system. This stems primarily from the strict structural requirements for compounds that can directly affect cyclase activities. This may be due, at least in part, to the highly discriminative nature of these enzymes, since they appear to be primarily designed to distinguish only a limited number of the natural information-transferring molecules (hormones), depending upon the tissue in question. Only close structural relatives of those ligands can directly influence cyclase activity, thus severely limiting the range of chemical classes that can be developed for the purpose.

This limitation, however, may not include the compounds that can specifically block the stimulatory effects of the natural hormones on the cyclase enzymes. Here, a slightly wider selection of chemical entities may allow for the development of still better and possibly more selective blockers of hormonal effects on the cyclase enzymes (24). Conversely, phosphodiesterases, owing to their apparent sensitivity to a wider selection of chemical structures, present a more fertile field for development of new drugs with wider ranges of activity and selectivity. Since the cyclic nucleotides appear to be involved in nearly all facets of cellular activity in both healthy and disease states, there is no limit to the range of pharmacological effects that phosphodiesterase modifiers may produce, hence the central importance of phosphodiesterase for modern drug development.

This review is divided into two main sections. The first section describes the general aspects of the phosphodiesterase enzymes. The second section deals primarily with the activators and inhibitors of the enzyme and structure–activity relationships of the latter.

GENERAL CONSIDERATIONS

Phosphodiesterases occur widely in biological systems. They are present in nearly all mammalian tissues (3, 4, 25, 26), with the possible exceptions of red blood cells (27) and isolated rat adrenal cells (28). These two cellular systems provide excellent models for studying cyclic nucleotide synthesis uncomplicated by the interference of phosphodiesterases. These enzymes have been found also in bacteria (29–32), yeast (33), insects (34–37), higher plants (38–42), and several marine organisms (43, 44). In general, they are found wherever the cyclic nucleotides exist. Phosphodiesterase activity is detected early in embryonic development (20, 45–47), increases up to maturity, seems to decrease with age (48–50), and varies in activity with the cell cycle (51).

The central nervous system (CNS) seems to contain the highest levels of phosphodiesterase activity (27, 35, 52–55), a good portion of which appears to be particulate and may be associated with nerve endings, postsynaptic membranes (56–58), and synaptosomes (59–61). The enzyme from almost all other tissues is usually soluble, although there are definite indications that it may be at least partly particulate in intact cells and is solubilized during the isolation procedures. The particulate nature of phosphodiesterase and its possible plasma membrane localization are of the utmost importance to the design of drugs that can modulate the activity of the enzyme. The localization of the enzyme on the outside of cellular membranes is an obligatory assumption to provide necessary support for the effects on the enzyme activity in intact cells of some large peptide derivatives that cannot cross cellular membranes, *e.g.*, insulin–cepharose complexes that appear to act extracellularly (62, 63).

The general and kinetic properties of phosphodiesterases were recently reviewed (3, 4). Most studies in this area were carried out on the soluble enzymes, with few investigations of the particulate phosphodiesterases. Whether the soluble phosphodiesterases are identical structurally and/or functionally to the

particulate forms is not known. Thus, it may prove dangerous to extrapolate data obtained with the soluble enzymes to the particulate forms. This point cannot be overstressed, since the particulate forms may represent the more important *in vivo* forms of the enzyme and appear to respond differently to drugs.

Soluble phosphodiesterases are remarkably stable enzymes in the crude form, with an approximate molecular weight in the range of about 100,000–500,000 (3, 39, 64–66). They require a divalent cation for activity, preferably Mg^{+2}, and have a pH optimum between 7.5 and 8.5 (44, 67–71). Some unusual metal-ion requirements were reported for specific rat liver (72) and *Escherichia coli* enzymes (29). Some plant enzymes appear to have widely different properties and varying sensitivities to pH, ions, and inhibitors (30, 42, 73, 74). Plant phosphodiesterases also appear to exhibit unusual sensitivity to phosphate (73, 75) and can preferentially hydrolyze the 5′-bond of cyclic AMP to produce the 3′-monophosphate nucleoside, in obvious contrast to animal phosphodiesterases (42, 74, 76–78).

With mild techniques of isolation, phosphodiesterases seem to be associated with the cyclase complexes, appearing usually in the same fraction as the cyclases (56, 79–81). The association between phosphodiesterases and nucleotidyl cyclases can still be detected in partially purified preparations of the latter (82). It is possible that at least one phosphodiesterase form may represent a part of the cyclase complex. Phosphodiesterase contamination often presents a difficulty in cyclase assays, necessitating the addition of large quantities of theophylline to inhibit phosphodiesterase activity.

Kinetically, the enzyme presents a complex picture even when present in a reasonable state of purity. This observation led to the suggestion that the enzyme exhibits negative (79, 83) or even positive (84, 85) cooperative behavior. Such a conclusion, however, cannot be convincingly reached before greater purification of the enzyme is achieved.

Preparation, Purification, and Assay—The methods used for the preparation, purification, and assay of phosphodiesterase were recently reviewed (86, 87). These methods generally follow the leading efforts from the laboratory of Earl Sutherland, where the first methods to prepare, partially purify, and assay the enzyme were developed (52). The method selected for the assay of phosphodiesterase should be sensitive enough to measure the activity of the important, low K_m forms of the enzyme. Since the enzyme appears to exist in at least two forms, varying widely in K_m values, the substrate levels used in the assay do largely determine which form of the enzyme is being assayed.

There are several good methods for the assay of phosphodiesterase activity (88–91) (for review, see 3, 4, 86, 87, and 92). Although the colorimetric and titrimetric methods are simpler and more convenient to use than other methods, their low sensitivity makes them unsuitable for the assay of the high affinity, low K_m forms of the enzyme.

The most widely used methods at present are based on the use of labeled cyclic nucleotides as substrates. The noncyclic nucleotides formed by the action of the enzyme are converted into nucleosides by the addition of a nucleotidase (crude snake venom). These nucleosides are then separated and counted as a measure of phosphodiesterase activity (59, 93, 94). These methods are precise and sensitive enough to allow measurement of the enzyme activity at very low substrate concentrations similar to those encountered *in vivo*. They suffer, however, from the disadvantage of high blank values. Chromatography on more selective ion-exchange resins produces better separation of the labeled nucleosides, improving their recovery and allowing increased sensitivity and lower blank values (95, 96).

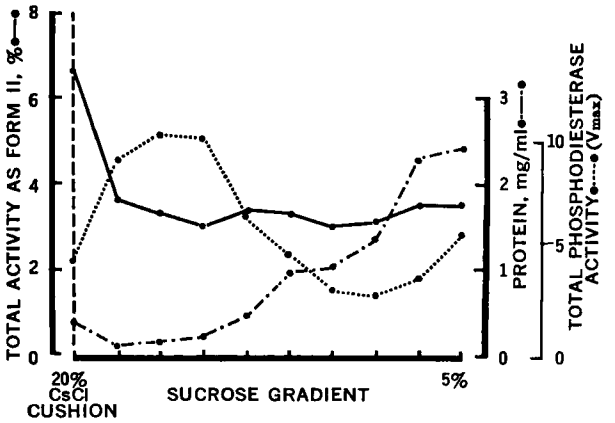


Figure 1—*Sucrose gradient fractionation of platelet whole homogenate. The fractions were collected by puncturing the bottom of the tube and were dialyzed against 0.05* M *potassium phosphate buffer, pH 7.5, and analyzed for protein and for phosphodiesterase activity. Phosphodiesterase assays were carried out at 15 substrate concentrations covering the range from* 3×10^{-8} *to* 8×10^{-3} M *to allow for the calculation of the relative proportion of activity attributable to either form of the enzyme. The percentage of the total activity present as Form II was calculated after the method of Cleland (138); (v) is expressed in μmoles hydrolyzed/0.5 ml/10 min at 30°.*

Histological assays have also been described for the study of phosphodiesterase (97), as have methods for the localization of the enzyme in tissues (98, 99).

Different Forms of Phosphodiesterase—Multiple forms of phosphodiesterase have been shown to exist under a wide variety of conditions and from a large number of sources (47, 55, 100–111). These forms appear to differ mainly in their substrate affinities (65, 92, 102, 112–119), heat and cation sensitivities (14, 101, 120–124), substrate specificities (59, 101, 125, 126), chromatographic and electrophoretic mobilities (102, 109), subcellular localization (20, 21, 59, 127), and, possibly, function (20, 128). The presence of multiple forms of the enzyme may explain its frequently anomalous kinetic behavior. It also allows for speculation as to the mode and manner in which these forms may be involved in the moment-to-moment control of phosphodiesterase activity and, consequently, the moment-to-moment control of the basal cyclic nucleotide levels.

The separation of the different forms of the enzyme has been accomplished by various methods (66, 102, 106, 129, 130). Sucrose gradient fractionation ap-

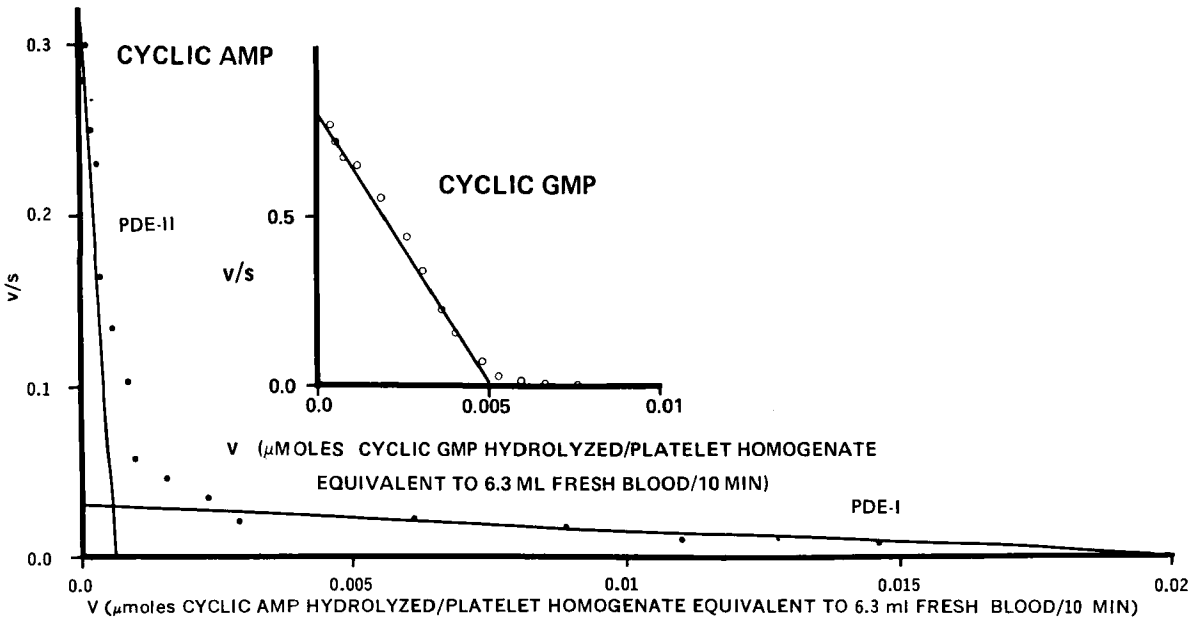


Figure 2—*Kinetics of hydrolysis of cyclic AMP and GMP by platelet whole homogenate. Enzyme assays were carried out on the peak tubes obtained by chromatography (DEAE) representing activities of Forms I and II. v = initial velocity (μmoles hydrolyzed/2 mg protein/min). s = substrate concentration (millimoles).*

pears to represent the simplest, most gentle, and convenient method for the crude separation of the different forms (79, 115). An example of the use of this method on platelet homogenates is shown in Fig. 1. Other methods may produce slightly "cleaner" fractions but generally do not improve the resolution significantly (115, 129).

Different Forms in Same Cell Line—Most investigators in this area agree that two main forms of the enzyme appear to exist in any homologous tissue preparation, multiple forms possibly being an artifact of the heterogeneity of the tissues used (119, 131). A case in point is the work of Uzunov and Weiss (106, 132) who found at least six forms of the enzyme in rat brain. However, when homologous preparations of rat astrocytoma cells were examined, only two forms were found (133).

The two forms differ in a number of aspects but primarily in their affinity for the substrates. Since there are two naturally occurring substrates for these enzymes, cyclic adenosine-3′,5′-monophosphate (cyclic AMP) and cyclic guanosine-3′,5′-monophosphate (cyclic GMP), two forms of the enzyme may exist for each substrate. Some tissues, however, appear to contain one form of the enzyme with a definite substrate specificity (134–137). For example, human blood platelets and rat brain seem to contain only one cyclic GMP phosphodiesterase (88, 115) (Fig. 2).

The resolution of the two forms is best achieved by kinetic analysis (81, 111, 129) at a wide range of substrate concentrations, particularly if coupled by physical separation. Table I summarizes the kinetic parameters of the enzyme forms present in a number of tissues obtained by kinetic analysis combined with chromatography[1] (129). The methods used are quite simple and can easily detect the presence of the two forms of the enzyme.

The presence of multiple forms of the enzymes stimulated speculation as to which, if either, of the two forms is more important in the control of the intracellular cyclic nucleotide levels. Since the intracellular levels of the cyclic nucleotides are in the micromolar range or lower (21, 139), the low K_m, high affinity phosphodiesterase (Form II) is favored for this role. In most tissues, Form II has a K_m value close to 10^{-6} M (4, 88), at least two orders of magnitude lower than that of the high K_m, low affinity Form I (3).

Only recently has the importance of Form II in the control of intracellular cyclic nucleotide levels in intact cells become more apparent (140). In tissues from a variety of hypertensive rats, the intracellular levels of cyclic nucleotides are inversely correlated with the Form II activity present (141–143). No such correlation existed with Form I activity, which was also measured in these same studies. The role, if any, played by Form I is open to question; studies utilizing high substrate concentrations that would preferably measure Form I activity are of questionable significance. At the present time, studies utilizing low substrate concentrations, in the micromolar range, that would preferentially reflect Form II activity appear to be more valuable because they may reflect the activity of the enzyme most probably involved in the *in vivo* control of intracellular cyclic nucleotide levels.

The significance of the presence of two main enzymes with two widely varying affinities for the hydrolysis of either cyclic nucleotide is not entirely clear. There are indications that the two forms are interconvertible. This makes it possible to speculate that the two dimorphs of phosphodiesterase could provide adequate means of controlling phosphodies-

[1] DEAE.

Table I—Cyclic AMP and Cyclic GMP Phosphodiesterases from a Number of Tissues[a]

Tissue	Cyclic AMP (cAMP)				Cyclic GMP (cGMP)				Total V_m cAMP / Total V_m cGMP
	K_m[b] Values, M		V_m[c]	% V_mII[d]	K_m[b] Values, M		V_m[c]	% V_mII[d]	
	I	II			I	II			
Human blood platelets[e]	6.1×10^{-4}	3.2×10^{-6}	0.025	4.2	1.6×10^{-5}		0.009	—	2.84
Monkey brain (sediment)	1.7×10^{-2}	1.7×10^{-5}	0.081	30.3	1.4×10^{-4}		0.019	—	4.20
Monkey brain (supernate)	3.0×10^{-4}		0.282	—	3.2×10^{-4}		0.478	—	0.59
Rabbit brain (sediment)	6.6×10^{-3}	5.6×10^{-5}	0.130	21.1	1.7×10^{-4}	3.7×10^{-6}	0.073	19.4	1.78
Rabbit brain (supernate)	1.9×10^{-3}	9.8×10^{-5}	0.390	8.7	6.6×10^{-5}	2.9×10^{-6}	0.122	7.8	3.20
Rabbit fundic mucosa	4.7×10^{-4}	1.2×10^{-6}	0.139	14.3	2.7×10^{-4}	5.4×10^{-7}	0.185	13.7	0.75
Rabbit gallbladder	4.0×10^{-3}	6.0×10^{-6}	0.059	14.6	4.0×10^{-3}	3.0×10^{-7}	0.028	12.6	2.10
Rabbit heart	1.7×10^{-2}	4.1×10^{-6}	0.104	13.0	2.0×10^{-4}	3.3×10^{-6}	0.023	21.4	4.47
Rabbit intestines	5.9×10^{-5}	1.0×10^{-6}	0.048	26.6	4.6×10^{-4}	3.4×10^{-6}	0.137	4.5	0.35
Rabbit kidneys	5.1×10^{-4}	5.5×10^{-6}	0.456	5.7	2.2×10^{-3}	2.0×10^{-5}	0.328	5.9	1.39
Rabbit liver	6.9×10^{-5}	4.0×10^{-7}	0.358	3.1	7.2×10^{-5}	4.0×10^{-6}	0.416	19.5	0.86
Rabbit pyloric mucosa	2.7×10^{-3}	8.7×10^{-6}	0.117	24.3	1.1×10^{-5}	1.2×10^{-6}	0.041	17.2	2.85

[a] Cyclic AMP and cyclic GMP phosphodiesterase activities were determined (102) in 4000 ×*g* supernates at 15 substrate concentrations (129). The incubation mixture contained ^{3}H-cyclic AMP or ^{3}H-cyclic GMP (2×10^{-11}–2×10^{-6} mole), 0.9 μmole $MgSO_4$, and 25 μmoles tromethamine buffer at pH 7.5 in a total volume of 0.5 ml. [b] Michaelis–Menten constant. [c] Enzyme maximal velocity = μmoles hydrolyzed/30 min at 30°/20 mg wet tissue. [d] Percent of total V_m as Form II, calculated as described by Cleland (138). [e] 10,000 ×*g* supernate of platelet homogenates was used. Platelets from 6.3 ml of blood were used per assay.

terase activity *in vivo*. Form I may represent a large store of the enzyme which is quickly convertible to the higher affinity Form II when the need arises. It may also serve the function of handling sudden surges of cyclic nucleotide concentrations (114). Form II would be the form normally concerned with the control of the *in vivo* cyclic nucleotide levels.

Most of the reported phosphodiesterase activity in different tissues was determined at relatively high substrate concentrations and would, under most experimental conditions, represent the form that is probably less important *in vivo* (Form I). This led to the somewhat misleading conclusion that phosphodiesterase activity may be 100 times the adenylyl cyclase activity in some tissues (52, 139, 144–147).

In a number of studies, the particulate nature of Form II was clearly stressed (12, 32, 79–81, 85, 98, 115, 148–156). Its presence on the external surface of cells has been well documented (156–158). This, in combination with its apparent calcium and hormonal sensitivity (140, 156, 159–161), supports the possible role of Form II as a hormonal receptor. The effects of calcium ions on phosphodiesterase activity from rabbit liver are shown in Fig. 3. As can be seen, no effect of calcium ions on Form I activity is observed in contrast to the large stimulatory effect on Form II activity. There are some reports, however, of an inhibitory effect of calcium ions on phosphodiesterase activity in certain tissues (119, 162).

The presence of soluble and particulate forms of phosphodiesterase should lead to a reconsideration of the role of the enzyme in the control of cyclic nucleotide levels. The occurrence of soluble and particulate forms of the same enzyme differing in kinetic parameters is not unknown. For example, malate dehydrogenase occurs in both soluble and particulate forms in *Neurospora* (163). It could be hypothesized that the active form of phosphodiesterase is membrane bound and in equilibrium with a soluble, low affinity form. Association or dissociation from the membranes would represent activation or inactivation of the enzymes, respectively. The membranous site for the attachment of the enzyme could then represent a receptor site for the action of ligands affecting intracellular cyclic nucleotide levels by activating or inactivating phosphodiesterase. More gentle methods for the preparation and assay of phosphodiesterase should be used to avoid the transfer of activity to the soluble fraction and the consequent obliteration of induced effects. Some recent studies with insulin support this mechanism (156, 164, 165).

The structural relationships between the two main forms of the enzyme are far from clear. Studies on the molecular structure of the two forms must await further purification of the different phosphodiesterases. Furthermore, it is not completely settled whether cyclic AMP hydrolysis is carried out by the same set of enzymes that also catalyzes the hydrolysis of other cyclic nucleotides. There are some indications in certain tissues that one of the two forms of the enzyme may be an artifact of the isolation procedures employed (126, 149, 166).

The importance of the two forms of the enzyme is underlined by the apparent absence of one form in human glial cell tumor (167) and in transformed cells (168).

From the point of view of drug development, the sensitivities of the two forms of phosphodiesterases to activators or inhibitors appear to be different (120, 135, 169–173). This is a very important point, since it is doubtful that agents acting on only the high K_m forms of the enzymes would be useful. It appears that screening compounds for possible activity against phosphodiesterases should stress the importance of the low K_m forms, since these are likely to be the more critical. An impressive example of drug selectiv-

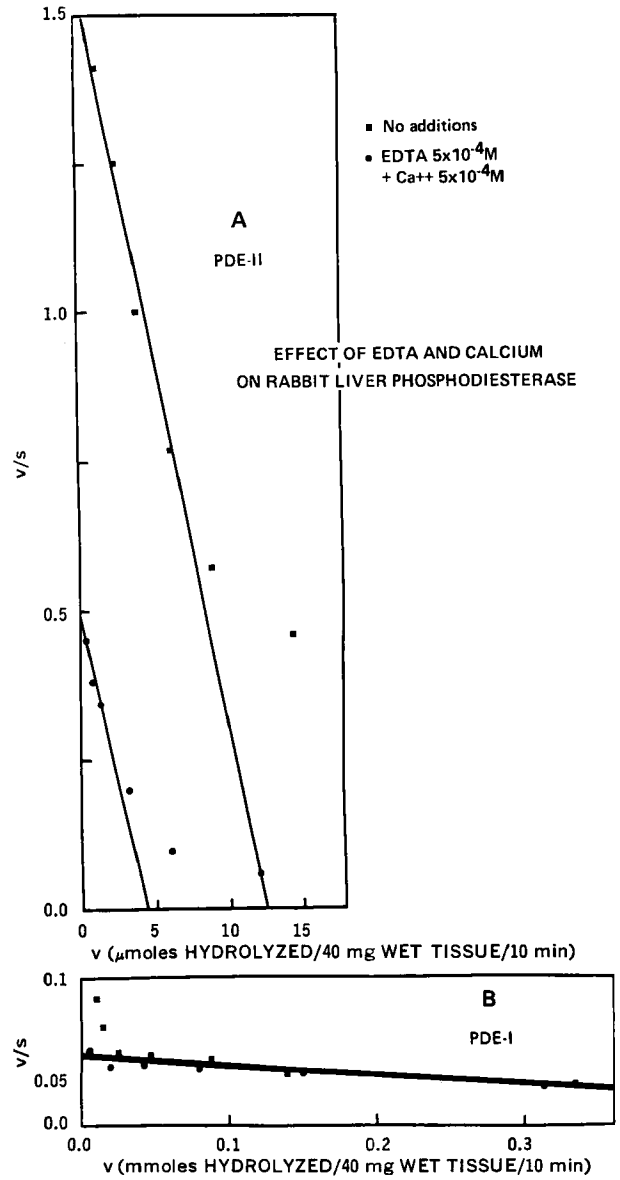


Figure 3—*Eadie plots for the effects of Ca^{+2} on the activity of cyclic AMP phosphodiesterase in rabbit liver. A represents the results obtained at low substrate concentrations (Form II) and B represents the results obtained at high substrate levels (Form I).*

ity for the low K_m phosphodiesterase is that of adenosine on the enzymes from human blood platelets. As can be seen in Fig. 4, adenosine inhibited the Form II activity with no effect on the Form I activity present in the platelet homogenates (174).

Different Forms in Different Cell Lines—One prime requirement in drug development is the tissue specificity of the drug in question. The differences between the phosphodiesterases from different tissues appear to be large enough to support the use of this enzyme as a target for the development of new drugs (55, 122, 175–180). There appear to be both structural and kinetic differences between the phosphodiesterases isolated from different tissues (121). These differences are reflected in the differential sensitivities to drugs of the enzymes from different sources (122, 157, 172, 174, 175, 177, 178, 181–193)

Table II—Inhibitor Constants (K_i) of Theophylline on Form II Activity from Guinea Pig Lung and Stomach Using Cyclic AMP or Cyclic GMP as Substrates[a]

Substrate	K_i[b]	
	Lung	Stomach
Cyclic AMP	4×10^{-4}	4×10^{-4}
Cyclic GMP	2×10^{-3}	1.3×10^{-4}

[a] Assayed according to Thompson and Appleman (102) at substrate concentrations of 1 and 2 μM and six drug concentrations. [b] Molar, determined as described in Ref. 211.

and even from neighboring areas of the brain (194). Therefore, testing the responses of phosphodiesterases from different tissues may provide preliminary clues to the possible tissue selectivity of the drug in question.

Cyclic GMP versus *Cyclic AMP Phosphodiesterases*—Although several pyrimidine and purine cyclic nucleotides are hydrolyzed by different tissue preparations (3, 55, 72, 117, 125, 195, 196), the significance of this observation is unclear since only two cyclic nucleotides appear to occur in nature, cyclic AMP and cyclic GMP, both of which are purines. The hydrolysis of the two naturally occurring cyclic nucleotides was thought at one time to be catalyzed by the same set of enzymes (197). Present evidence, however, points to the possible existence of two different sets of enzymes with widely different affinities for the two naturally occurring cyclic nucleotides (85, 126, 130, 160, 198, 199).

Recent studies seem to emphasize the differences between cyclic AMP and cyclic GMP phosphodiesterases in subcellular localization, cation requirements (166), and response to drugs (177, 181, 200–202). Since the two naturally occurring cyclic nucleotides appear to mediate different, if not antagonistic, events in many systems (203–210), it is of paramount importance to recognize the functionally different natures of cyclic AMP and cyclic GMP phosphodiesterases. The selective effects of drugs on these enzymes will greatly determine their biological effects on the tissues examined. This is exemplified by the effects of theophylline on phosphodiesterase preparations from the guinea pig stomach and lung, using each cyclic nucleodtide as a substrate (Table II). Theophylline shows greater selectivity for cyclic AMP phosphodiesterase than for cyclic GMP phosphodiesterase in the lung than in the stomach. This may at least partially explain the effects of theophylline in the two tissues.

Not only do the selective effects of a drug on cyclic AMP and cyclic GMP phosphodiesterases vary from tissue to tissue but also the effects in the same tissue vary with different drugs (177, 181, 212–215). The effects of the three phosphodiesterase inhibitors, theophylline, papaverine, and glycyrrhetinic acid, on the enzymes from the fundic mucosa of three species (Table III) serve to illustrate the latter point. Greater selectivity for cyclic AMP phosphodiesterase is correlated with greater inhibitory effect on gastric acid secretion. The three compounds have profoundly different selectivities for the specific phosphodiesteras-

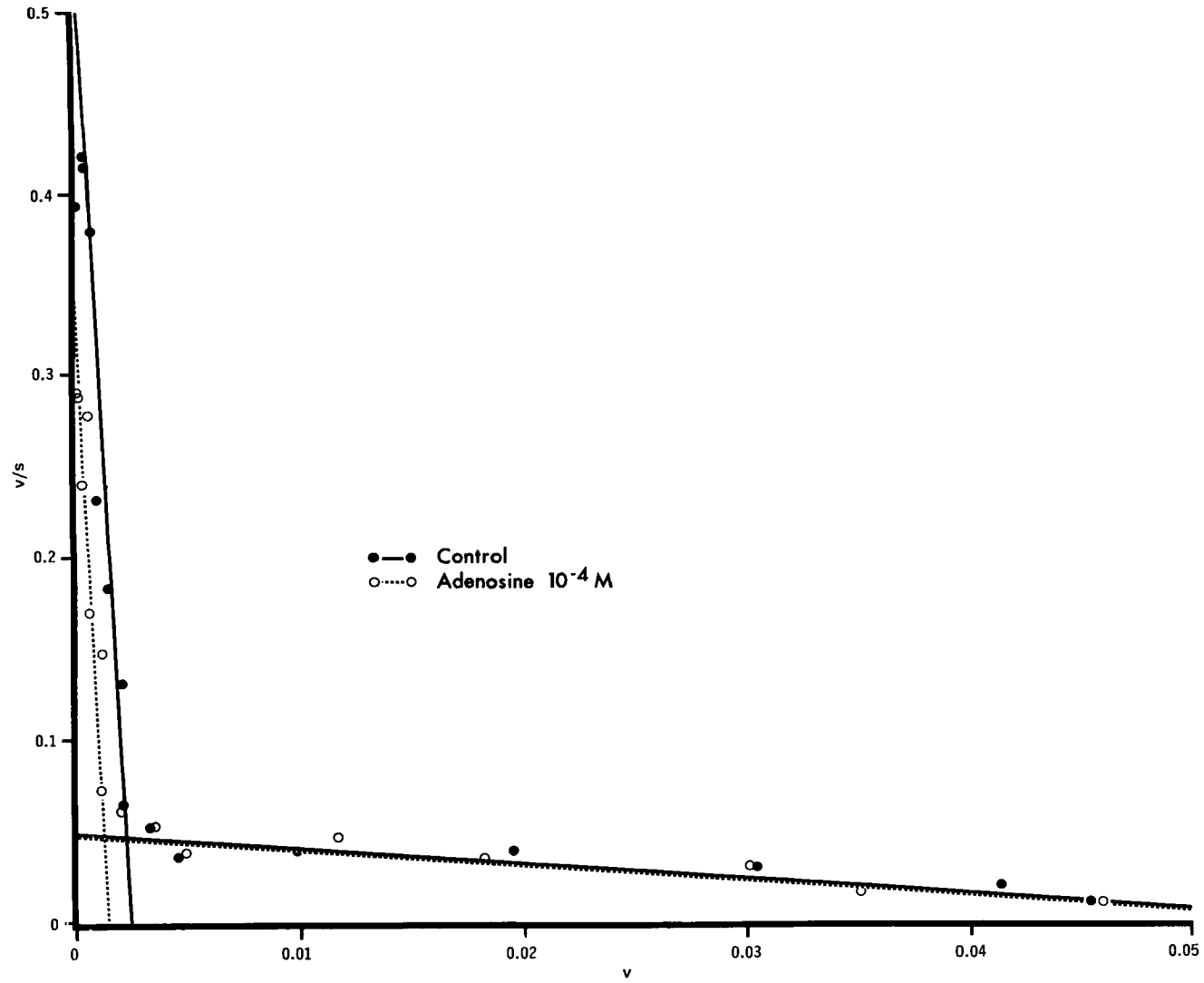


Figure 4—*Eadie plots for the effects of adenosine (10^{-4} M) on phosphodiesterase activity from human blood platelet membrane-supplemented whole homogenates. Note the selective inhibition by adenosine on Form II while no effect is evident on Form I (174).*

es and produce different effects on the output of the system (204, 212).

The realization of the possible importance of substrate selectivity in the use of phosphodiesterases as targets for the development of new drugs is quite recent. Little screening work has been reported where different substrates for the enzymes were used. This matter will probably attract more and more attention.

One important point to stress in this respect is the ability of each naturally occurring cyclic nucleotide to influence the rate of hydrolysis of the other (112, 130, 140, 167, 186, 188, 214–218). Thus, compounds selectively affecting the specific phosphodiesterase for either nucleotide will have indirect effects on the activity of the other. This is complicated by the ability of either cyclic nucleotide to both stimulate and inhibit the rate of hydrolysis of the other, depending on its concentration. This may provide an explanation for the nonconforming effects of exogenously applied cyclic nucleotides in some systems, as in the stimulatory effects of cyclic AMP on the contraction of some smooth muscles (219). It also may explain the antagonistic effects of some drugs on the levels of the two cyclic nucleotides (218).

A model enzyme system composed of specific subunits with affinity for cyclic AMP and others for cyclic GMP has been proposed (4, 215). It is quite apparent that cyclic GMP may be involved as an *in vivo* regulator of phosphodiesterase activity (130, 216). The greater sensitivity of the cyclic GMP phosphodiesterase to calcium ions (101) strongly emphasizes this possibly important role.

In general, there is greater affinity of phosphodiesterases for cyclic GMP (136, 141, 214, 215) than for cyclic AMP. This may be related to the generally lower *in vivo* concentrations of cyclic GMP.

The previously unsuspected selectivity of phosphodiesterase inhibitors for either cyclic AMP or cyclic GMP enzymes may provide an explanation for the anomalous behavior of some of these compounds *in vivo*. For example, papaverine, although far more potent than theophylline as an inhibitor of cyclic AMP phosphodiesterase, is less potent than expected in elevating plasma free fatty acids and blood glucose (220).

Table III—Selectivity of Theophylline, Papaverine Hydrochloride, and Glycyrrhetinic Acid for Low K_m Cyclic AMP and Cyclic GMP Phosphodiesterase from the Fundic Mucosa of Three Species (210) and Their Effects on Acid Secretion[a]

Substrate	Dog			Rat			Rabbit			Effect on Gastric Acid Secretion
	Substrate			Substrate			Substrate			
	Cyclic AMP	Cyclic GMP	G/A[b]	Cyclic AMP	Cyclic GMP	G/A[b]	Cyclic AMP	Cyclic GMP	G/A[b]	
	Inhibitor Constants (K_i), Molar[c]									
Theophylline	50	26	0.5	38	17	0.5	44	17	0.4	Potentiates
Papaverine	2	2.6	1.3	2.5	2.8	1.1	2.5	3	1.2	No effect
Glycyrrhetinic acid	7	13	1.9	4.0	10	2.5	7.5	15	2.0	Inhibits

[a] Enzyme activity was determined by a radioactive assay technique (102). [b] G/A = K_i(cyclic GMP as substrate)M/K_i(cyclic AMP as substrate)M; higher numbers indicate greater selectivity for cyclic AMP phosphodiesterase. [c] $\times 10^{-5}$. Determined by the method in Ref. 211 at two substrate (0.8 and 1×10^{-6} M cyclic AMP and 1 and 2×10^{-6} M cyclic GMP) and six drug concentrations bracketing the K_i value.

Interconvertibility of Phosphodiesterases—As mentioned earlier, there is some support for the possible interconvertibility of the two main forms of phosphodiesterase (79, 129, 198, 221). A measure of the change in the proportion of either form of the enzyme present in a particular experimental situation can provide an estimate of the effects of different agents on the interconvertibility of the two forms. The results with some prostaglandins, epinephrine, and aspirin are shown in Table IV. Although the effects are small, they point to a possible mechanism for the control of phosphodiesterase activity. Final confirmation of this mechanism must await further purification of the enzymes.

Two apparently interconvertible forms of the enzyme were isolated from cellular slime molds (157). The importance of sulfhydryl groups for the interconversion is apparent in this system, as evidenced by the effects of dithiothreitol. However, the latter compound has little effect in mammalian systems (222).

Natural Intracellular Activators and Inhibitors—Protein activators for phosphodiesterase were isolated from a number of tissues (84, 107, 159, 161, 222–229). These activators appear to mediate the stimulatory effects of calcium ions on the enzyme activity from the brain and may prove quite important as natural regulators of the enzyme activity in that tissue. Natural protein inhibitors for phosphodiesterase have also been described (228, 230, 231) and in some systems may have other functions (232).

A heat-stable, nondialyzable phosphodiesterase inhibitor was also isolated from soybeans (73) and slime mold (233), and an activator was isolated from the latter (100). The possible physiological role, if any, of these natural activators or inhibitors in the control of the enzyme activity has not been established. Whether interference with the effects of these activators or inhibitors can present a mechanism for possible drug effects on phosphodiesterase remains to be seen. Also it is not clear whether these activators or inhibitors are collectively similar or different (234).

ATP, pyrophosphate (235), adenosine (71, 174), citrate, isocitrate, pyruvate, oxalate, malate, and tartrate (67) were reported to inhibit the enzyme activity. The possible role of any or all of these intermediates in the control of phosphodiesterase activity *in vivo* remains to be determined.

Cyclic nucleotides appear to activate (236) and to control the rate of synthesis of phosphodiesterase (168, 237–239). Evidence primarily from tissue cultures and bacteria strongly support a role for cyclic AMP in the control of phosphodiesterase synthesis, probably *via* cyclic nucleotide-dependent protein kinase (240).

Effects of Hormones on Phosphodiesterase—Several hormones, including insulin (79, 151, 154, 156, 164, 165, 241, 242) (for review, see 3 and 4), ACTH (243), growth hormone (79), cholecystokinin and gastrin (129, 244–246), aldosterone (96), histamine (32, 247–249), epinephrine (133, 250–253), prostaglandins (110), corticoids (117, 154, 254–256), thyroxine (117, 254, 257), and plant cytokinesins (258) have been reported to affect phosphodiesterase activities in their target tissues. In most of these situations, the forms of the enzyme affected consistently appear to be the low K_m, high affinity forms. These effects tend to bolster the possible hormonal receptor function of phosphodiesterase, particularly its low K_m forms.

In none of these situations, however, has the importance of phosphodiesterase effects been unequivocally established in the mechanisms of action of these hormones. It is not clear whether the effects observed on phosphodiesterase are primary or secondary effects of the hormones studied, since similar effects are produced by cyclic GMP (112, 259) or its hydrolysis product (222), high salt solutions (260), and, possibly, lipids (261). It is not clear whether these hor-

Table IV—Effects of Compounds on the Percent Form II Activity in Membrane-Supplemented Whole Homogenate of Human Blood Platelets[a]

Drug	Drug Concentration	Form II, %
Control	—	7.1
Epinephrine	10^{-5} M	14.3
Aspirin	10^{-5} M	3.8
PGE_1	1.4×10^{-6} M	2.0
$PGF_{2\alpha}$	2×10^{-4} M	5.5
PGE_2	2×10^{-4} M	7.0

[a] Phosphodiesterase activity was determined as described for Table I. The percent Form II was calculated *via* the use of a computer program developed after the treatments of Cleland (138). The assays were done as described for Table I.

monal effects could lend themselves to modulation *via* the use of drugs. However, these effects remain potentially important.

Diseases Associated with Altered Phosphodiesterase Levels—In some situations, as in hypertension (141–143), hepatomas (262), diabetes (263), and obesity (263), increased activity of the low K_m, high affinity phosphodiesterase may be an important part of the etiology of the disease state, although this may not always be the case (20, 264). In these conditions, the use of appropriate phosphodiesterase inhibitors could alleviate the deficiency in intracellular cyclic AMP by the most desired mechanism for any drug, namely, by correcting the basic biological defect. On the other hand, there is also evidence for decreased phosphodiesterase activity in diabetes (137, 165, 265, 266) and hepatomas (267).

In some diseases, the lowered cyclic AMP levels are associated with decreased sensitivity of adenylyl cyclase to stimulation. The most feasible approach to correct the cyclic AMP deficiency under these circumstances is the inhibition of phosphodiesterase. Examples of these conditions are hypertension (141–143, 268), asthma (269), psoriasis (270), and, possibly, cancer (271, 272).

Phosphodiesterase Inhibition, Cyclic Nucleotide Accumulation, and Pharmacological Effects—Many compounds thought to act *via* phosphodiesterase inhibition and the consequent cyclic nucleotide accumulation are "uncomfortably" weak inhibitors of the enzyme, with K_i values often in the millimolar range. In most of these situations, it is doubtful that the compound could attain the high concentrations needed *in vivo*. Often, insignificant or only marginal *in vivo* phosphodiesterase inhibition can be demonstrated; yet some accumulation of the cyclic nucleotides may occur (93, 273, 274). The latter may become evident only if accompanied with cyclase stimulation.

A popular example of this is theophylline, a weak phosphodiesterase inhibitor with a K_i value of about 1 mmole or greater in most systems. It is doubtful that it reaches this high concentration *in vivo* when administered in the usual doses. No *in vivo* phosphodiesterase inhibition by theophylline can be demonstrated in most systems, yet theophylline by itself sometimes produces small elevations of cyclic nucleotide tissue levels when administered *in vivo*. It is widely believed, however, that phosphodiesterase inhibition and the consequent cyclic nucleotide elevations represent the main mechanism for its effects. The potentiation of hormonal effects by theophylline is one main criterion required to determine the possible mediator role of cyclic nucleotides in the mechanism of action of the hormones (275, 276).

Most phosphodiesterase inhibitors, including the more active ones, cause only small accumulations of cyclic nucleotides when compared with the amounts generally produced *via* adenylyl or guanylyl cyclase stimulation. In many systems, a small cyclic nucleotide elevation is all that is needed for maximal stimulation of the physiological response (274). It is not clear what role, if any, the extremely high cyclic nucleotide levels, which are sometimes produced *via* cyclase stimulation, play in the physiological effect, since, in most instances, the effect is usually essentially complete at much lower intracellular levels of the cyclic nucleotides (277).

It should always be remembered that phosphodiesterase inhibition may represent only one of several effects that phosphodiesterase inhibitors can produce. These other effects may be totally independent of the cyclic nucleotide system and may, in certain instances, be responsible for the major portion of the activity of these agents. The other effects of the methylxanthines are clearly summarized (213, 274, 278) and could serve as an example of other responses that can be produced by phosphodiesterase inhibitors, apparently unrelated to their effects on cyclic nucleotide metabolism.

Other properties of drugs may play a role in determining their effects on cellular biochemical events, *e.g.*, the ability of the drug to reach the enzyme. Membrane permeability may have been important in the case of a xanthine derivative, where striking differences between its *ex vivo* and *in vitro* potency on phosphodiesterase were demonstrated (191).

ACTIVATORS AND INHIBITORS: STRUCTURE–ACTIVITY RELATIONSHIPS

In this section, the structure–activity relationships of compounds that have been reported to affect phosphodiesterase activity will be discussed. Since most of the reported studies utilized cyclic AMP as the substrate, the activities measured were those of cyclic AMP phosphodiesterases. Not every reported compound is listed, but most entities that fall into common structural (preferably) or utility classes are included. Several miscellaneous structures may be found in the references cited. The two main objectives were: *(a)* to determine the structural features or groups that are common among compounds showing activity on phosphodiesterases, and *(b)* to determine the extent to which variation of structural moieties has allowed improved potency and/or selectivity.

Discussion of this section is complicated by the heterogeneity of the enzyme preparations used, the wide variations in the assay conditions, the different levels of substrate employed, making it difficult to determine what form of the enzyme was assayed, and the different methods used in determining the potency of the agents employed.

To present a uniform discussion of the results, it was essential to unify the method of presentation. Those studies employing a substrate concentration of 10^{-5} M or less were assumed to measure Form II (low K_m) activity, while those at higher substrate levels were assumed to estimate Form I (high K_m) activity. The enzyme source was mentioned whenever practical.

For quantification of the results, five terms were employed in the following order of preference whenever possible:

a. Inhibitor constant (K_i), in micromoles per liter.

b. Inhibitor concentration$_{50}$ (I_{50}), in micromoles per liter.

c. Percent inhibition (100 minus percent of control).

d. Potency relative to theophylline (based on K_i or I_{50} values) if the latter was employed as a standard in the same system.

e. Potency relative to papaverine (based on K_i or I_{50} values) if the latter was employed as a standard in the same system.

In the absence of either K_i or I_{50} values, no accurate relative potency could be determined. Therefore, the following four additional terms were used to give a rough estimate of potency whenever possible:

f. The compound produced greater percent inhibition than theophylline in the same system.

g. The compound produced less percent inhibition than theophylline in the same system.

h. The compound produced greater percent inhibition than papaverine in the same system.

i. The compound produced less percent inhibition than papaverine in the same system.

These letters were used as superscripts throughout the tables to qualify the potency values reported. In some instances, more than one superscript was used to provide more adequate information on the activity of the compound in question. Although I_{50} and K_i values are often similar, they are not necessarily identical, particularly with competitive inhibitors (279, 280), and were listed separately.

Activators—Compounds reported to stimulate phosphodiesterase are not as numerous as those that appeared to inhibit it. Since the most common abnormalities in cyclic nucleotide metabolism usually result in lower levels of these important mediators, inhibition of their degradation is the more desirable therapeutic target. Nevertheless, activators are of interest in diseases associated with elevated cyclic nucleotide levels, *e.g.*, cyclic AMP in diabetes (263, 281, 282), cholera (283), alcoholism (284), and mania (285) and cyclic GMP in psoriasis (286) and hypertension (287).

Several compounds of widely different structures were reported to activate phosphodiesterase from a variety of tissues (Table V). These activators include nitroglycerin and papaverine, which also inhibit the insoluble enzyme in rabbit colon homogenate, and hydralazine, which inhibits the enzyme activity in bovine mesenteric artery (289). However, the effects of these compounds on phosphodiesterase could be indirect. In most systems, crude preparations of the enzyme were used and effects on cyclic GMP synthesis and/or metabolism cannot be ruled out. Cyclic GMP has repeatedly been shown to have significant effects on cyclic AMP phosphodiesterase activity (66, 85, 111, 112, 188, 214, 222, 259).

Imidazole (70, 111, 129, 230, 288, 290, 291) is most frequently quoted as a stimulator of phosphodiesterase, although high concentrations are generally needed. It has been shown to have no effect on some phosphodiesterase systems (74, 215), to have negligible effects on rat brain phosphodiesterase (288), and possibly to even inhibit at concentrations of 10–50 m*M* (215). Its stimulating effects appear to depend on the presence of Mn^{+2} (70).

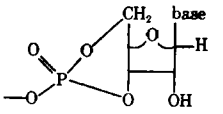
Table V—Activators of Phosphodiesterase

Name	Enzyme Source	Reference
Acetamide	*D. discoideum*	10
L-Arginine	Mouse brain	186
L-Aspartic acid	Mouse brain	186
Catecholamines	Rat adrenal	251
Cyclic GMP	Rat thymic lymphocytes	112
	Rat liver	85
Cysteine	Alaska pea seedlings	74
Dithiothreitol	Alaska pea seedlings	74
Gastrin and related peptides	Rabbit tissues (misc.)	129
Glutathione	*D. discoideum*	12
Glycine	Mouse brain	186
5′-GMP	Beef heart	222
Histamine analogs and metabolites	Rat brain	288
	Bullfrog gastric mucosa	217
Hydralazine	Bovine artery	289
Imidazole	Mouse pancreatic islet	111
	Guinea pig heart	290
	Alaska pea seedlings	74
	Rat brain	288
	Bovine brain	70
	Rat heart	291
Insulin	Rat liver	151
Lipids	Rat brain	261
Nicotine	Bovine tongue epithelium	17
Nicotinic acid	Adipose tissue	292
Nitroglycerin	Rabbit colon cytoplasm	289
Papaverine	Rabbit colon cytoplasm	289
	Cat cerebral cortex	191
Phenylalanine	Bovine tongue epithelium	17
Propylthiouracil	*D. discoideum*	10
Protein activators	Rat brain	227
	Bovine brain	234
	Bovine heart	84
	Porcine brain	84
	Human brain	84
	Several rat tissues	234
	Bovine brain cerebra	223
	Rat cerebellum	106
	Hamster islet cell tumor	107
Quinine	Bovine tongue epithelium	17
Strychnine	Bovine tongue epithelium	17
Thioacetamide	*D. discoideum*	10
Thiourea	*D. discoideum*	10
Urea	Bovine tongue epithelium	17
Others	Adipose tissue	292
	Rat mast cells	293

A number of sulfhydryl compounds significantly stimulate phosphodiesterase from a variety of sources. This fact led to the speculation that free sulfhydryl groups are essential for phosphodiesterase activity (3, 189).

In view of the possible interconvertibility of the two forms of phosphodiesterase, as discussed previously, the substrate concentrations used could significantly influence the effect produced by the compound studied. A compound that stimulates the hydrolysis of low concentrations of cyclic AMP could act *via* a shift of activity from Form I to Form II. Decreased phosphodiesterase activity at high substrate concentrations would be expected; thus, the same compound could be described as both a stimulant and an inhibitor, depending on the substrate concentration used. Examples of this duality include cholecystokinin, epinephrine, and cyclic GMP on cyclic AMP hydrolysis. This shift may underlie the obvious controversy concerning the effects of bitter taste

Table VI—Cyclic Nucleotides of Natural Bases



Name	Base	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phosphodiesterase Form	Reference
Cyclic GMP	Guanine	47[c]	Mouse pancreatic islets	7.9×10^{-6}	II	111
		22[c]	Pea seedlings	1×10^{-4}	II	74
		100[a]	Rat liver	—	II	85
		18[a]	Frog erythrocytes	—	I	214
		40[a]	Rat thymic lymphocytes	1×10^{-4}	II	112
		1.0[b]	Cat heart	—	II	188
		700[b]	Rat brain	—	II	188
		120[a]	Beef heart	1×10^{-3}	I	222
Cyclic IMP	Hypoxanthine	1.8[a], 3.8[b], 34.2[d]	Beef heart	—	II	185
		24[b], 9.6[d]	Beef brain	—	II	185
		60[a], 80[b], 3.1[d]	Rabbit lung	—	II	185
		22[b], 7.3[d]	Rabbit kidney	—	II	185
		24[a]	Frog erythrocytes	5×10^{-5}	I	66
		2.0[b]	Cat heart	—	II	188
		940[b]	Rat brain	—	II	188
		210[a]	Beef heart	1×10^{-3}	I	222
Cyclic CMP	Cytosine	19[c]	Pea seedlings	1×10^{-4}	II	74
		Inactive	Cat heart	—	II	188
		Inactive	Rat brain	—	II	188
Cyclic UMP	Uracil	Inactive	Rat brain	—	II	188
		1600[b]	Cat heart	—	II	188
Cyclic TMP	Thymine	2000[b]	Cat heart	—	II	188
		Inactive	Rat brain	—	II	188

* Superscript italic letters refer to the terms discussed under *Activators and Inhibitors: Structure–Activity Relationships.*

principles on phosphodiesterase activity in the tongue epithelium (17, 18).

Inhibitors—*Cyclic Nucleotide Derivatives*—Although only two cyclic nucleotides are known to exist in nature, their universal and important effects have stimulated the synthesis of a large variety of cyclic phosphate derivatives of both natural and synthetic bases. Two basic biological targets are easily discernible behind this tremendous synthetic effort: *(a)* to produce compounds that could mimic the effects of the naturally occurring messengers on their target enzymes (cyclic nucleotide-dependent protein kinases) while resisting phosphodiesterase hydrolysis, and *(b)* to inhibit phosphodiesterase activity and thus raise the intracellular concentrations of the two naturally occurring cyclic nucleotides. Although other biological targets should also be considered (1), only these two have been consistently pursued.

Due to the extremely large number of compounds that have been studied for their effects on phosphodiesterase, only selected examples of inhibitor action will be presented here. Detailed lists of these compounds and their activities can be found elsewhere (177, 183, 294–299).

From a theoretical standpoint, the ability of cyclic nucleotide derivatives to act as substrates for phosphodiesterase should reflect their ability to act as inhibitors of the enzyme, since they will compete for the same site on the enzyme (60). However, this relationship does not seem to hold true in a number of situations. On the other hand, structural requirements for good phosphodiesterase inhibition in cyclic nucleotide derivatives appear to be generally similar to those needed to produce good activation of the cyclic nucleotide protein kinases (183). The latter enzymes are generally believed to translate changes in cyclic nucleotide levels into biochemical events by phosphorylating key enzymes and thus activating or inhibiting them (300).

1. Cyclic nucleotides of natural bases: Perhaps the simplest group of cyclic AMP analogs includes those compounds in which the base moiety is not adenine but another biologically occurring purine or pyrimidine derivative (Table VI). The less familiar cyclic nucleotides and their respective bases are the following: cyclic GMP, guanine; cyclic inosine monophosphate (cyclic IMP), hypoxanthine; cyclic cytidine monophosphate (cyclic CMP), cytosine; cyclic uridine monophosphate (cyclic UMP), uracil; and cyclic thymidine monophosphate (cyclic TMP), thymine. The last two are often inactive or only weakly active as phosphodiesterase inhibitors compared to their biocongeners (66, 188). The potency of cyclic IMP is often found to be of the same magnitude as that of cyclic GMP (66, 188, 222); cyclic CMP is sometimes ineffective as an inhibitor (66, 188) but is nearly equal in potency to cyclic GMP as an inhibitor of Form I from pea seedlings (74).

2. Cyclic nucleotides of synthetic bases: Several types of cyclic AMP analogs have been studied on phosphodiesterases from a variety of tissues (Table VII). Compounds of two series (Groups A and B) were generally more potent than theophylline on rabbit lung and bovine heart Form II (184). Three other groups, including imidazole nucleotides (Group I), N^1,N^6-ethenonucleotides (Group H), and substituted inosine nucleotides (Group E), were more variable in their inhibitory potency (184). Data from a similar study (302) indicated relatively little variation in I_{50} values within a series of 8-substituted analogs of cy-

Table VII—Cyclic Nucleotides of Synthetic Bases

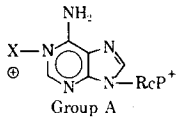
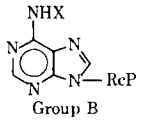
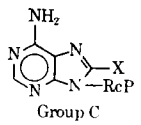
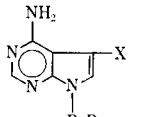
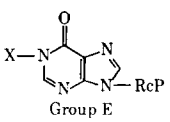
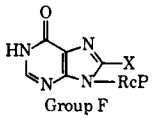
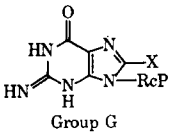
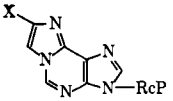
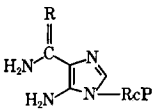
Substitution	Base	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phospho-diesterase Form	Reference
X = O	 Group A	35[b], 7.1[d]	Rabbit lung	—	II	184
		50[b], 2.6[d]	Bovine heart	—	II	184
X = OCH_3		150[b], 1.7[d]	Rabbit lung	—	II	184
		90[b], 1.4[d]	Bovine heart	—	II	184
X = OC_2H_5		45[b], 5.5[d]	Rabbit lung	—	II	184
		120[b], 1.1[d]	Bovine heart	—	II	184
X = $OCH_2C_6H_5$		35[b], 7.1[d]	Rabbit lung	—	II	184
		80[b], 1.6[d]	Bovine heart	—	II	184
X = CH_3		65[b], 3.8[d]	Rabbit lung	—	II	184
		80[b], 1.6[d]	Bovine heart	—	II	184
X = $CH_2C_6H_5$	 Group B	67[c]	*D. discoideum*	2×10^{-4}	I	10
X = COC_6H_5		72[c]	*D. discoideum*	2×10^{-4}	I	10
X = COC_3H_7-*n*		46[b], 5.4[d]	Rabbit lung	—	II	172
		110[a]	Rabbit lung	—	II	301
X = OH		13[b], 19.2[d]	Rabbit lung	—	II	184
		150[b], 0.87[d]	Bovine heart	—	II	184
X = OCH_3		100[b], 2.5[d]	Rabbit lung	—	II	184
		80[b], 1.6[d]	Bovine heart	—	II	184
X = OC_2H_5		42[b], 6.0[d]	Rabbit lung	—	II	184
		68[b], 1.9[d]	Bovine heart	—	II	184
X = $OCH_2C_6H_5$		190[b], 1.3[d]	Rabbit lung	—	II	184
		240[b], 0.54[d]	Bovine heart	—	II	184
X = $CONHCH_3$		260[b], 0.9[d]	Rabbit lung	—	II	183
X = $CONHC_3H_7$-*n*		330[b], 0.7[d]	Rabbit lung	—	II	183
X = $CONHC_6H_5$		160[b], 1.4[d]	Rabbit lung	—	II	183
X = $COOC_2H_5$		210[b], 1.1[d]	Rabbit lung	—	II	183
X = SH	 Group C	540[b]	Rat brain	—	II	188
		300[b]	Cat heart	—	II	188
		20[b]	Rabbit lung	—	I	302
X = SCH_3		125[b]	Rat brain	—	II	188
		39[b]	Cat heart	—	II	188
		24[b]	Beef heart	—	I	302
X = SC_2H_5		52[b]	Rat brain	—	II	188
		41[b]	Cat heart	—	II	188
X = SCH_2CH_2OH		200[b]	Rat brain	—	II	188
		44[b]	Cat heart	—	II	188
X = $SCH_2C_6H_5$		40[b]	Rat brain	—	II	188
		24[b]	Cat heart	—	II	188
		22[b], 5.9[d]	Beef heart	—	I	183
		60[b], 3.8[d]	Rabbit lung	—	I	183
X = NH_2		400[b]	Rat brain	—	II	188
		23[b]	Cat heart	—	II	188
X = N_3		150[b]	Rat brain	—	II	188
		90[b]	Cat heart	—	II	188
X = $NHCH_3$		700[b]	Rat brain	—	II	188
		160[b]	Cat heart	—	II	188
X = $N(CH_3)_2$		2300[b]	Rat brain	—	II	188
		3300[b]	Cat heart	—	II	188
X = $NHCH_2CH_2OH$		4400[b]	Rat brain	—	II	188
		270[b]	Cat heart	—	II	188
X = OH		Inactive	Rat brain	—	II	188
		Inactive	Cat heart	—	II	188
X = OCH_3		600[b]	Rat brain	—	II	188
		130[b]	Cat heart	—	II	188
X = Br		67[b]	Rat brain	—	II	188
		16[b]	Cat heart	—	II	188
X = Br		52[c]	*D. discoideum*	2×10^{-4}	I	10
X =		20[c]	*D. discoideum*	2×10^{-4}	I	10
X = S—C_6H_4—*p*-Cl		200[b]	Rabbit lung	—	I	302
		22[b]	Beef heart	—	I	302
X = H	 Group D	20[b]	Beef heart	—	I	303
		12[b]	Rabbit lung	—	I	303
X = CN		2.0[b]	Beef heart	—	I	303
		2.4[b]	Rabbit lung	—	I	303
X = $CONH_2$		5.0[b]	Beef heart	—	I	303
		6.7[b]	Rabbit lung	—	I	303
X = H	 Group E	80[b], 3.1[d]	Rabbit lung	—	II	184
		3.8[b], 34.2[d]	Bovine heart	—	II	184
X = OH		330[b], 0.76[d]	Rabbit lung	—	II	184
		830[b], 0.16[d]	Bovine heart	—	II	184

Table VII—(*Continued*)

Substitution	Base	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phospho-diesterase Form	Reference
X = $NHCH_2C_6H_5$	 Group F	11[c]	*D. discoideum*	2×10^{-4}	I	10
X = Br		28[c]	*D. discoideum*	2×10^{-4}	I	10
X = $SCH_2C_6H_5$		67[b]	Rabbit lung	—	I	302
		13[b]	Beef heart	—	I	302
X = $NHCH_2C_6H_5$	 Group G	19[c]	*D. discoideum*	2×10^{-4}	I	10
X = Br		32[c]	*D. discoideum*	2×10^{-4}	I	10
		150[b]	Rabbit lung	—	I	302
		48[b]	Beef heart	—	I	302
X = H	 Group H	280[b], 0.9[d]	Rabbit lung	—	II	184
		590[b], 0.22[d]	Bovine heart	—	II	184
X = C_6H_5		160[b], 1.6[d]	Rabbit lung	—	II	184
		73[b], 0.18[d]	Bovine heart	—	II	184
R = O	 Group I	100[b], 2.5[d]	Rabbit lung	—	II	184
		170[b], 0.76[d]	Bovine heart	—	II	184
R = S		80[b], 3.1[d]	Rabbit lung	—	II	184
		60[b], 2.2[d]	Bovine heart	—	II	184

* See footnote to Table VI. † RcP = β-D-ribofuranosyl cyclic 3,5-phosphate.

clic AMP (Group C), with the possible exception of 8-*p*-chlorophenylthio cyclic AMP which showed considerable selectivity for beef heart over rabbit lung enzyme.

Other workers (188), examining a series of cyclic AMP derivatives with substituents in the 8-position (Group C), showed that 8-bromo and 8-benzylthio cyclic AMP were the most potent inhibitors of the series on cat heart and rat brain Form II, respectively. The former enzyme appeared more sensitive to inhibition.

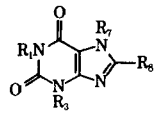
In a study on *Dictyostelium discoideum* Form I (10) (Groups B and C), the 8-substituted cyclic AMP derivatives were somewhat less potent inhibitors than the 6-substituted amino congeners. This effect was attributed to a greater steric hindrance for binding of the 8-substituted compounds, although neither N^6- nor 8-substitution profoundly affected the rate

Table VIII—Variations in the Sugar-Phosphate Moiety of Cyclic Nucleotides

Name	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phospho-diesterase Form	Reference
2′,3′-Cyclic AMP	1700[b]	Rat brain	—	II	188
	600[b]	Cat heart	—	II	188
	5[c]	Pea seedlings	1×10^{-4}	II	74
2′,3′-Cyclic GMP	210[b]	Cat heart	—	II	188
	Inactive	Rat brain	—	II	188
	15[c]	Rat thymic erythrocyte	1×10^{-4}	II	112
2′,3′-Cyclic IMP	2000[b]	Cat heart	—	II	188
2′,3′-Cyclic UMP	Inactive	Cat heart	—	II	188
	Inactive	Rat brain	—	II	188
	Inactive	Pea seedlings	1×10^{-4}	II	74
2′,3′-Cyclic CMP	Inactive	Rat brain	—	II	188
2′-*O*-Acetyl cyclic AMP	5[b], 5.0[d]	Rabbit lung	—	II	172
2′-*O*-Methyl cyclic AMP	4.8[b], 52.1[d]	Rabbit lung	—	II	172
2′-*O*-(2,4-DNP) cyclic AMP	25[b], 10.0[d]	Rabbit lung	—	II	172
2′-*O*-Butyryl cyclic AMP	5.6[b], 44.6[d]	Rabbit lung	—	II	172
	26[a]	Bovine thyroid	—	II	301
2′-Deoxy cyclic AMP	11[b], 22.7[d]	Rabbit lung	—	II	172
N^6,2′-*O*-Dibutyryl cyclic AMP	230[b], 1.1[d]	Rabbit lung	—	II	172
	160[a]	Bovine thyroid	—	II	301
	650[b]	Rat brain	—	II	188
	100[b]	Cat heart	—	II	188
5′-Methylene-5′-deoxy cyclic AMP	Inactive	Rabbit brain	1×10^{-4}	II	305
5′-Thio-5′-deoxy cyclic AMP	15[a], 12[b], 10.8[d]	Beef heart	—	II	185
	3.5[a], 2.4[b], 104[d]	Rabbit lung	—	II	185
5′-*N*-*n*-Octylamino-5′-deoxy cyclic AMP	200[a]	Beef heart	—	I	306
$S^{4'}$-Cyclic AMP	56[c]	Bovine heart	1×10^{-3}	II	307

* See footnote to Table VI.

Table IX—Xanthine Derivatives



Name	R_1	R_3	R_7	R_8	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phosphodiesterase Form	Reference
Caffeine	CH_3	CH_3	CH_3	H	27[c,f]	Cat cerebral cortex	2.5×10^{-3}	I	191
					38[c,g]	Rabbit skeletal muscle	1×10^{-3}	II	199
					3000[a], 0.6[d]	Rat adrenals	—	I	312
					31[c,g]	Beef brain	1.2×10^{-2}	I	313
					150[b], 0.8[d]	Rat brain	—	II	297
					<1.0[d]	Rat fat cell	5×10^{-4}	II	93
					48[c,g]	Bovine taste papillae	3×10^{-3}	I	18
					1000[a]	Mouse pancreatic islet	5.3×10^{-3}	II	111
					18[c,g]	Cockroach brain	1×10^{-4}	II	35
					2400[a]	Beef heart	—	II	213
					Inactive	Pea seedlings	1×10^{-4}	II	74
					930[b], 0.62[d]	Rat cerebral cortex	—	II	19
					69[c,g]	Mouse brain	1×10^{-2}	I	186
Theobromine	H	CH_3	CH_3	H	28[c]	Rabbit skeletal muscle	1×10^{-3}	II	199
					4500[a], 0.4[d]	Rat adrenals	—	I	312
					41[c,g]	Bovine taste papillae	3×10^{-3}	I	18
					150[b], 0.8[d]	Rat brain	—	II	297
					<1.0[d]	Rat fat cell	5×10^{-4}	II	93
					38[c,g]	Mouse brain	1×10^{-2}	I	186
	C_2H_5	C_2H_5	H	H	200[b], 0.13[e]	Hamster islet cell tumor	—	II	107
					>1.0[d]	Rat fat cell	4×10^{-4}	II	93
SC 2964	CH_3	C_4H_9-*i*	H	H	~15.0[d]	Rat fat cell	5×10^{-5}	II	93
					89[c]	Mouse pancreatic islet	8×10^{-4}	II	111
	C_5H_{11}-*n*	C_2H_5	H	H	~1.0[d]	Rat fat cell	4×10^{-4}	II	93
	CH_3	CH_3	H	SH	<1.0[d]	Rat fat cell	5×10^{-4}	II	93
	CH_3	CH_3	H	CH_3	160[a], 2.6[d]	Beef heart	5×10^{-4}	I	314
	CH_3	CH_3	H	C_2H_5	100[a], 4.2[d]	Beef heart	5×10^{-4}	I	314
	CH_3	CH_3	H	C_3H_7-*n*	210[a], 2.0[d]	Beef heart	5×10^{-4}	I	314
	CH_3	CH_3	H	C_4H_9-*n*	70[a], 6.0[d]	Beef heart	5×10^{-4}	I	314
	CH_3	CH_3	H	C_5H_{11}-*n*	40[a], 10.5[d]	Beef heart	5×10^{-4}	I	314
	CH_3	CH_3	H	C_6H_{13}-*n*	20[a], 21.0[d]	Beef heart	5×10^{-4}	I	314
	CH_3	CH_3	H	C_7H_{15}-*n*	Inactive	Beef heart	5×10^{-4}	I	314
	CH_3	CH_3	H	$CH_2CH_2CH_2CH_2OH$	Inactive	Beef heart	5×10^{-4}	I	314
	CH_3	CH_3	H	C_3H_7-*i*	50[a], 8.4[d]	Beef heart	5×10^{-4}	I	314
	CH_3	CH_3	H	C_4H_9-*i*	150[a], 2.8[d]	Beef heart	5×10^{-4}	I	314
	CH_3	CH_3	H	C_3H_5-*c*	60[a], 7.0[d]	Beef heart	5×10^{-4}	I	314
	CH_3	CH_3	H	C_4H_7-*c*	90[a], 4.7[d]	Beef heart	5×10^{-4}	I	314
	CH_3	CH_3	H	C_5H_9-*c*	210[a], 2.0[d]	Beef heart	5×10^{-4}	I	314
Etamiphylline	CH_3	CH_3	$CH_2CH_2N(C_2H_5)_2$	H	10.5[c,g]	Beef brain	6.25×10^{-3}	I	200
Oxyphylline	CH_3	CH_3	CH_2CH_2OH	H	22.5[c,g]	Beef brain	6.25×10^{-3}	I	200
Diprophylline	CH_3	CH_3	$CH_2CHOHCH_2OH$	H	22.8[c,g]	Beef brain	6.25×10^{-3}	I	200
Etamiphylline iodomethylate	CH_3	CH_3	$CH_2CH_2\overset{+}{N}CH_3(C_2H_5)_2I^-$	H	Inactive	Beef brain	6.25×10^{-3}	I	200
	CH_3	CH_3	$CH_2CH_2NH_2$	H	12.1[c,g]	Beef brain	6.25×10^{-3}	I	200
	CH_3	CH_3	$CH_2CH_2NHCH_2C_5H_5$	H	30.8[c,g]	Beef brain	6.25×10^{-3}	I	200
	CH_3	CH_3	$CH_2CH_2\overset{+}{N}(CH_3)_2CH_2C_6H_5I^-$	H	Inactive	Beef brain	6.25×10^{-3}	I	200

	CH_3	CH_3	CH_2CH_2OH		53[c,g]	Beef brain	1.2×10^{-2}	I	313
	CH_3	CH_3	$CH_2CHOHCH_2OH$		50[c,g]	Beef brain	1.2×10^{-2}	I	313
	CH_3	CH_3	CH_2COOH		Inactive	Beef brain	1.2×10^{-2}	I	313
	CH_3	CH_3	$CH_2CH_2N(CH_3)_2$		45[c,g]	Beef brain	1.2×10^{-2}	I	313
	CH_3	CH_3	$CH_2CH_2CH_2NH_2$		48[c,g]	Beef brain	1.2×10^{-2}	I	313
				H	25[c]	Human umbilical artery	1.8×10^{-4}	I	192
Pentoxifylline	$CH_3CO(CH_2)_4$	CH_3	CH_3		50[c]	Human umbilical vein	1.8×10^{-4}	I	192
				H	36[c]	Human umbilical artery	1.8×10^{-4}	I	192
SK 7	$CH_3(CH_2)_5$	CH_3	CH_3		56[c]	Human umbilical vein	1.8×10^{-4}	I	192

* See footnote to Table VI.

of hydrolysis of these derivatives by phosphodiesterase. 8-Bromo and 8-benzylamino derivatives of cyclic GMP and cyclic IMP (Groups G and F, respectively) were also examined on *D. discoideum* phosphodiesterase and found to be generally less effective than the cyclic AMP derivatives, although only the 8-bromo substituent was common to all three types of cyclic nucleotides.

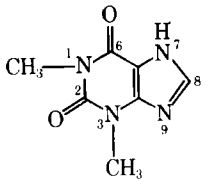
A series of cyclic AMP derivatives examined on beef heart and rabbit lung phosphodiesterase included an N^6-ethoxycarbonyl and several N^6-carbamoyl derivatives (Group B). The N^6-(N-methylcarbamoyl) and the N^6-ethoxycarbonyl derivatives were similar to theophylline in potency and selectivity, while the propylcarbamoyl and phenylcarbamoyl analogs selectively inhibited the rabbit lung and beef heart enzymes, respectively (183).

Tubercidin cyclic phosphate (Group D, X = H) is a 7-position carbon isostere of cyclic AMP; it and two closely related compounds, toyocomycin (X = CN) and sangivomycin (X = $CONH_2$), were all very good inhibitors of beef heart and rabbit lung Form I. The corresponding heterocyclic bases were also good inhibitors, but the analogous nucleosides and nucleoside 5′-phosphates were generally much weaker (303).

As might be anticipated, the most potent cyclic nucleotide inhibitors of phosphodiesterase generally have intact purine rings which resemble adenine, guanine, or hypoxanthine rather closely. Analogs of cyclic AMP having substituents in the 1- or 8-position or attached to the 6-amino group may be quite potent, depending on the nature of the substituent. Compounds with substituents bonded to the 8-position through a nitrogen or oxygen atom are often less potent than those having an 8-sulfur attachment. Isosteric substitutions (304) in the heterocyclic skeletons of cyclic nucleotides also seem to offer considerable promise for the development of potent phosphodiesterase inhibitors.

3. Variation of sugar phosphate moiety of cyclic nucleotides: Many cyclic nucleotides have been prepared that incorporate variations of the normal β-D-ribofuranosyl cyclic 3′,5′-phosphate moiety; representative examples are included in Table VIII. One such class of compounds is the 2′,3′-cyclic nucleoside phosphates, some of which were 0.001–0.005 as potent as the corresponding 3′,5′-congeners on Form II from cat heart and rat brain (188). In other systems, they were either weak or noninhibitors (66, 74).

An examination of several 2′-*O*-substituted cyclic AMP derivatives on Form II led to the conclusion that the beef heart and rabbit lung enzymes were more sensitive than similar beef brain and rabbit kidney systems to inhibition by compounds having relatively small groups in the 2′-*O*-position. Beef brain enzyme was found to be more sensitive to inhibitors bearing the bulkier 2′-*O*-(2,4-dinitrophenyl) and 2′-*O*-butyryl groups (172). The 2′-*O*-butyryl derivative is generally more potent than the N^6-butyryl (see also Table VII, Group B), which is more potent than the familiar N^6,2′-*O*-dibutyryl derivative (172, 301). The usefulness, however, of dibutyryl cyclic AMP as a tool for studying biochemical mechanisms far sur-



III

passes its utility as an inhibitor of phosphodiesterase. Its better cell penetration and slower metabolism compared to the parent cyclic AMP make it an excellent example of drug latentiation (308); nevertheless, its effects on intact biological systems must be viewed with caution (309, 310).

A number of excellent reports dealing with sugar phosphate variations in cyclic nucleotides are not discussed here (10, 183, 185, 311).

Xanthine Derivatives—Theophylline (III) is the most common standard reference agent used in studies of phosphodiesterase inhibitors. In a variety of guinea pig and rat tissues, it shows a moderate amount of tissue selectivity which appears to be species dependent (175, 176).

Caffeine is not consistently more or less active than theophylline, but commonly varies from 0.5 to 2 times as potent (Table IX). Theobromine, however, is less potent than theophylline in a variety of tissues.

Numerous xanthine derivatives have been synthesized, many of which are substituted at the 8-position as well as on the three commonly substituted secondary nitrogens. Several theophylline derivatives with substituted ethyl groups in the 7-position were less inhibitory than the parent structure on beef brain Form I, although etamiphylline is described as a better respiratory stimulant than theophylline (200). Etamiphylline iodomethylate, which has no inhibitory properties on phosphodiesterase, also has interesting *in vivo* properties. Highest potency was observed with an *N*-benzylaminoethyl function in the 7-position, while a quaternary nitrogen on the 7-substituent was detrimental (200). Another group of 7-substituted xanthines having a 3-pyridylmethyl function in the 8-position was also less potent than theophylline on beef brain Form I (313).

Two vasoactive substances are 1-substituted theobromine derivatives. SK 7 was more potent than pentoxifylline[2] as an inhibitor of umbilical phosphodiesterases, but their potencies relative to theophylline were not reported (192).

A series of 8-alkyl theophyllines showed interesting properties on beef heart Form I. 8-*n*-Hexyltheophylline was the most potent member, being 21 times as active as theophylline. Surprisingly, the 8-*n*-heptyl homolog was not effective. Equally unexpected was the fact that the 8-ω-hydroxybutyl derivative was ineffective, although the 8-butyl congener was 6 times as potent as theophylline. Compounds having four- and five-carbon branched and cycloalkyl groups in the 8-position were less effective than their straight chain counterparts; however, the C_3 groupings of isopropyl and cyclopropyl yielded analogs that were several times more potent than the *n*-propyl analog (314).

[2] BL 191.

Table X—Inhibitor Constants of Papaverine on Form II Activity from Selected Guinea Pig and Rat Tissue (175)

Tissue		K_i Value, μM[a]
Guinea Pig:	Brain	100
	Heart	180
	Kidneys	150
	Liver	30
	Lungs	50
Rat:	Brain	50
	Heart	40
	Kidneys	50
	Liver	100
	Lungs	25

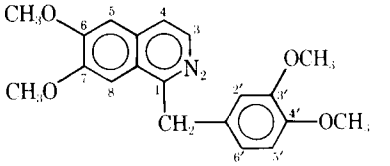
[a] Determined as described for Table II.

The 3-isobutyl analog of theophylline, SC 2964, was 15 times as potent as theophylline on rat fat cell Form II, a system in which substantial increases in lipolysis were associated with only partial phosphodiesterase inhibition. Close agreement was obtained between potencies as lipolytic agents and phosphodiesterase inhibitors. Substitution of a sulfhydryl group in the 8-position of theophylline did not improve its potency (93).

Replacing the 3-methyl of theophylline with more bulky, lipophilic groups yields compounds with improved potency, while increasing alkyl chain length up to hexyl at the 8-position is quite advantageous. Compounds with substituted alkyl substituents in the 7-position offer no improvement over theophylline, although an additional 8-substituent may be helpful.

Papaverine and Related Isoquinolines—The utility of the opium alkaloid papaverine as a standard reference for phosphodiesterase inhibition is well established. It effectively inhibits both Form I (18, 312, 315–321) and Form II (19, 107, 121, 171, 175, 181, 273, 322) from a variety of tissues. However, papaverine appears to activate phosphodiesterase from the cytoplasmic fraction of rabbit colon (289) and the cat cerebral cortex (191). While commonly exhibiting a potency from 10 to 1000 times that of theophylline, papaverine rarely shows appreciable tissue selectivity in its actions (Table X).

Replacing the 7-methoxy of papaverine with a hydroxy reduced its potency substantially on beef heart phosphodiesterase (Table XI). A similar replacement in the 3′-position (Ro 20-0518) did not affect its potency significantly; but at the 4′-position, the introduction of a hydroxy group resulted in a compound 2–3 times as potent as papaverine (315). When all four methoxys of papaverine were replaced with ethoxys, potency was not appreciably altered (317, 318,



papaverine

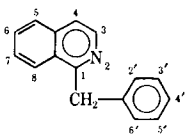
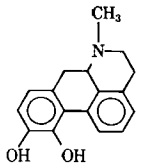
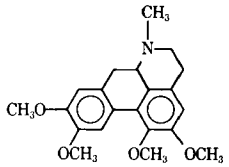
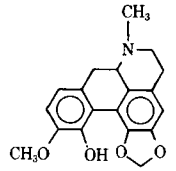


Table XI—Benzylisoquinoline Derivatives

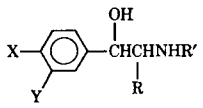
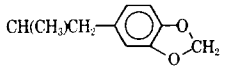
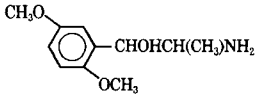
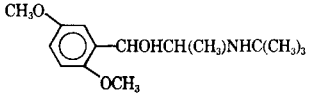
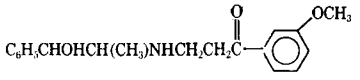
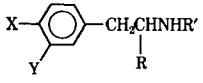
Name	Substituents at Position 3	6	7	3′	4′	6′	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phosphodiesterase Form	Reference
Ro 20-0518	H	CH_3O	HO	CH_3O	CH_3O	H	9[c,i]	Beef heart	1×10^{-5}	I	315
	H	CH_3O	CH_3O	HO	CH_3O	H	32[c,h]	Beef heart	1×10^{-5}	I	315
	H	CH_3O	CH_3O	CH_3O	HO	H	66[c,h]	Beef heart	1×10^{-5}	I	315
Ethaverine	H	C_2H_5O	C_2H_5O	C_2H_5O	C_2H_5O	H	5.6[a], 0.7[e]	Beef heart	—	?	323
							40[c]	Human platelets	5×10^{-5}	I	317
							15[a], 0.3[e]	Human platelets	—	I	318
Papaveroline	H	HO	HO	HO	HO	H	22[a], 0.2[e]	Beef heart	—	?	323
							Negligible	Guinea pig brain	1×10^{-4}	I	319
	C_2H_5	CH_3O	CH_3O	CH_3O	CH_3O	H	0.8[a], 2.5[e]	Rabbit ileum	—	II	324
6-Bromopapaverine	H	CH_3O	CH_3O	CH_3O	CH_3O	Br	61[c,i]	Guinea pig brain	2×10^{-4}	I	319
PV_2	H	CH_3O	CH_3O	H	Cl	H	12[a], 2.8[e]	Beef heart	—	I	320
Dioxyline	CH_3	CH_3O	CH_3O	CH_3O	C_2H_5O	H	59[a], 0.08[e]	Human platelets	—	I	318
	H	CH_3O	CH_3O	H	H	H	6.5[a], 0.6[e]	Beef heart	—	?	323
	H	H	H	CH_3O	CH_3O	H	22[a], 0.2[e]	Beef heart	—	?	323
	C_2H_5	CH_3O	CH_3O	H	H	H	48[e,f,h]	Human platelets	5×10^{-5}	I	317
Eupaverine	CH_3	OCH_2O		OCH_2O		H	25[a], 0.9[e]	Beef heart	—	I	316
							34[a], 1.0[e]	Beef heart	—	I	320
							0.6[a], 3.3[e]	Rabbit ileum	—	II	324
							7[a], 0.8[e]	Beef coronary artery	—	I	325, 94
							30[a], 1.3[e]	Rat adrenal	—	I	312
	H	H	H	H	H	H	Negligible	Beef heart	1×10^{-5}	?	323
Related Alkaloids											
Apomorphine							15[b], 0.8[e]	Dog cerebral cortex	—	II	121, 122
							15[b], 0.67[e]	Rat cerebral cortex	—	II	19
							220[b], 0.1[e]	Dog red blood cell	—	II	122
							170[b], 0.07[e]	Rat red blood cell	—	II	121, 122
(−)-Glaucine							36[a], 0.1[e]	Beef heart	—	?	323
(+)-Glaucine							39[a], 0.1[e]	Beef heart	—	?	323
Bulbocapnine							46[b], 0.22[e]	Rat cerebral cortex	—	II	19

* See footnote to Table VI.

323) but four hydroxys were clearly detrimental (319, 323). Replacement of both the 3′- and 4′-methoxys with hydrogen retained activity but potency decreased when the 6- and 7-methoxys were removed. Deletion of the entire 1-veratryl group of papaverine affords 6,7-dimethoxyisoquinoline, which retained only about 3% of the potency of papaverine on beef heart phosphodiesterase (323). Removal of the 3′- and 4′-methoxy groups and addition of a 3-ethyl function did not change the inhibitory potency of papaverine on human platelet Form I (317). Addition of a bromine atom at the 6′-position of either papaverine or papaverinol resulted in a more potent inhibitor of both the soluble and particulate forms of guinea pig brain Form I (319).

Replacement of both pairs of methoxys with methylenedioxy functions and addition of a 3-methyl group produces a compound (eupaverine) that was

Table XII—Adrenergic Amines and Related Compounds

Name	X	Y	R	R′	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phosphodiesterase Form	Reference
A. Phenethanolamines 									
l-Norepinephrine	OH	OH	H	H	48[c]	Beef heart	5×10^{-5}	I	252
					~17[c]	Guinea pig lung	1×10^{-4}	I	250
					~5[c]	Guinea pig lung	1×10^{-4}	II	250
					>2000[b]	Rat cerebral cortex	—	II	19
l-Epinephrine	OH	OH	H	CH_3	7[b], 42[c,f]	Beef heart	5×10^{-5}	I	252
					~30[c]	Guinea pig lung	1×10^{-4}	I	250
					Inactive	Guinea pig lung	1×10^{-4}	II	250
					12[c,g,i]	Beef heart	5×10^{-5}	II	252
					>2000[b]	Rat cerebral cortex	—	II	19
l-Isoproterenol	OH	OH	H	$CH(CH_3)_2$	46[c]	Beef heart	5×10^{-5}	I	252
					~37[c]	Guinea pig lung	1×10^{-4}	I	250
					Inactive	Guinea pig lung	1×10^{-4}	II	250
d,l-Isoproterenol	OH	OH	H	$CH(CH_3)_2$	45[c]	Beef heart	5×10^{-5}	II	252
Albuterol	OH	CH_2OH	H	$C(CH_3)_3$	7[c]	Beef heart	5×10^{-5}	I	252
Protokylol	OH	OH	H		47[c]	Beef heart	5×10^{-5}	I	252
Nylidrin	OH	H	CH_3	$CH(CH_3)C_2H_4C_6H_5$	10,000[a], 0.08[d]	Human platelets	—	I	318
Isoetharine	OH	OH	C_2H_5	$CH(CH_3)_2$	10,000[a], 0.08[d]	Human platelets	—	I	318
l-Phenylephrine	H	OH	H	CH_3	Inactive	Beef heart	5×10^{-5}	I	252
l-Dichloroisoproterenol	Cl	Cl	H	$CH(CH_3)_2$	Inactive	Beef heart	5×10^{-5}	I	252
Methoxamine					Inactive	Guinea pig lung	1×10^{-4}	II	250
					~16[c]	Guinea pig lung	1×10^{-4}	I	250
Butoxamine					Inactive	Guinea pig lung	1×10^{-4}	II	250
					Inactive	Guinea pig lung	1×10^{-4}	I	250
Oxyfedrine					1800[a], 0.07[d]	Beef coronary artery	—	I	94
B. Phenethylamines 									
Dopamine	OH	OH	H	H	46[c]	Beef heart	5×10^{-5}	I	252
					750[b], 0.01[e]	Rat cerebral cortex	—	II	19
Tyramine	OH	H	H	H	Inactive	Beef heart	5×10^{-5}	I	252
3-Methoxytyramine	OH	OCH_3	H	H	Inactive	Beef heart	5×10^{-5}	I	252
					>2000[b]	Rat cerebral cortex	—	II	19
l-Dopa	OH	OH	COOH	H	53[c]	Beef heart	5×10^{-5}	I	252

l-Tyrosine	OH	H	COOH	H	Inactive	Beef heart	5×10^{-5}	I	252
Amphetamine	H	H	CH_3	H	>1000[b]	Rat brain	—	II	287
					32[c]	Mouse brain	1×10^{-2}	I	186
p-Chlorophenylalanine	Cl	H	COOH	H	>1000[b]	Rat brain	—	II	287
Prenylamine	H	H	CH_3	$CH_2CH_2CH(C_6H_5)_2$	330[e], 0.7[d]	Beef heart	—	I	326
					80[a], 0.6[d]	Beef coronary artery	—	I	94
Verapamil (iproveratril)					1000–3000[b]	Rat brain, heart; rabbit heart, artery	—	?	327
					3300[a], 0.24[d]	Human platelets	—	I	318
					530[a], 0.43[d]	Beef heart	—	I	94
3,4-Dimethoxyphenethylamine	OCH_3	OCH_3	H	H	>2000[b]	Rat cerebral cortex	—	II	19

C. Miscellaneous Catechol Derivatives

R				
3,4-Dihydroxy-				
—H	Inactive	Beef heart	5×10^{-5}	I	252
—CHO	5[c]	Beef heart	5×10^{-5}	I	252
$—CH_2COOH$	28[c]	Beef heart	5×10^{-5}	I	252
	>10,000[b], <0.82[d]	Rat erythrocyte	—	II	121
	630[b], 0.95[d]	Canine cerebral cortex	—	II	121
—CHOHCOOH	34[c]	Beef heart	5×10^{-5}	I	252
$—CH_2CH_2COOH$	28[c]	Beef heart	5×10^{-5}	I	252

* See footnote to Table VI.

equipotent to papaverine on purified Form I from beef heart (316, 320). Replacing the 1-veratryl group of papaverine with a *p*-chlorobenzyl function increased the inhibitory effects on Form I of beef heart (320). The 3-methyl-4′-ethoxy analog of papaverine (dioxyline) was considerably less potent than papaverine on human platelet Form I (318).

3,4-Dihydropapaverine had negligible activity on both the soluble and particulate forms of guinea pig brain Form I, while 3,4-dihydroethaverine retained some of the activity of papaverine in both platelet (317) and purified beef heart Form I (316). Tetrahydropapaveroline was not effective on guinea pig brain Form I (319) or beef heart Form I (325) and was a weak inhibitor on rabbit ileum Form II (although it retained good spasmolytic action in the last system) (324).

A variety of isoquinolines with groups other than benzyl (or substituted benzyl) in the 1-position was ineffective on guinea pig brain Form I, as were those bearing representative pyridylmethyl functions in the same position (319); nevertheless, quinoparine, the desmethylene analog of papaverine, was more potent than papaverine on beef heart Form I (320).

The quaternary salt, papaverine methiodide, was not effective on beef heart phosphodiesterase (323).

These data support the conclusion (323) that the 6,7-dimethoxy substituents contribute more to the effectiveness of papaverine as a phosphodiesterase inhibitor than do the methoxy functions in the benzyl group. Furthermore, alkyl substitution in the 3-position of the heterocyclic ring retains activity, as does hydroxylation of the methylene group, but substitution of hydroxy for methoxy groups on the isoquinoline rings is generally undesirable. Partial or complete saturation of the *N*-containing ring of papaverine analogs does not provide a clear trend in phosphodiesterase inhibition, but quaternization of the nitrogen is clearly detrimental.

Although a direct comparison is lacking, morphine and codeine appear much less effective than apomorphine as inhibitors of Form II (122, 297). The apomorphine congeners, glaucine (323) and bulbocapnine (19), are effective inhibitors but both are less potent than papaverine.

Adrenergic Amines and Related Compounds—The catechol system is important to phosphodiesterase inhibition in both phenethylamines and phenethanolamines. Although catechol, resorcinol, and hydroquinone (50 μM) do not themselves inhibit phosphodiesterase activity (252), significant potency in phenethylamines favors the 3,4-dihydroxy configuration (Table XII). Replacement of the 3-hydroxy group on *l*-dopa with hydrogen (*l*-tyrosine) destroyed all activity; but if the carboxy function was changed to hydrogen (dopamine), little change was observed. Methylation of the 3-hydroxy group of dopamine destroyed activity (252).

Although they are not as potent as their amine-containing congeners, several nitrogen-free catechol derivatives also showed considerable activity on beef heart Form I (252). Those derivatives showing activi-

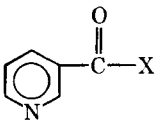
ty contained either an aldehyde or a carboxylic acid function, although benzaldehyde was remarkably weak.

The presence of the β-OH group is clearly not essential for phosphodiesterase inhibition in catecholamines (*e.g.*, dopamine *versus* norepinephrine); furthermore, the optical configuration about the β-carbon of isoproterenol is not critical, nor is the size of the amine substituent (252). As in the case of phenethylamines, anything other than 3,4-dihydroxy substitution on phenethanolamines resulted in drastically reduced potency in the system studied. Slight activity in the case of albuterol[3] may be attributed to the "homo-catechol" system (252).

Inhibition of phosphodiesterase by the adrenergic hormones occurs preferentially at high substrate concentrations. Two studies referred to in Table XII as Form II studies (250, 252) used a substrate concentration of 10^{-5} *M*, which is marginal for testing Form II activity. When phosphodiesterase studies using lower concentrations of cyclic AMP were carried out (253), a stimulant effect of these hormones on phosphodiesterase activity from rat liver was observed. This phosphodiesterase stimulant action was suggested as the intracellular mechanism for the α-adrenergic effects of these catechols, which were repeatedly shown to be associated with decreased intracellular levels of cyclic AMP (139, 276, 328, 329). This strongly reemphasizes the importance of the selection of the substrate concentration used for the type of effects observed.

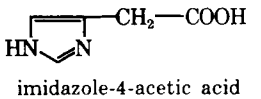
Pyridine and Quinoline Derivatives—Of several pyridine dicarboxylic acids tested on Form I from *Brevibacterium liquefaciens*, dipicolinic acid was clearly superior at very high inhibitor concentrations (71) (Table XIII). Nicotinic acid is included here as an inhibitor, although it also stimulated phosphodiesterase from adipose tissue (292). As an inhibitor of rat liver Form I (330), nicotinic acid was considerably inferior in potency to nicotinamide, *N,N*-diethylnicotinamide, and 3-acetylpyridine. Analogs of nicotinic acid having longer side chains were weak or inactive on rat fat cell Form II (331).

Other nicotinamide analogs (IV), retaining the 3-pyridylcarbonyl moiety and varying the other residue (X), were described as potent inhibitors of the rat liver soluble enzyme. Ethyl nicotinate (X = OC_2H_5), the strongest inhibitor among the agents investigated, was also able to increase hepatic cyclic AMP levels following intraperitoneal injection (332).

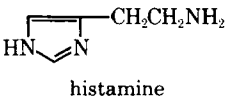


IV: X = CH_3, OCH_3, OC_2H_5, NH_2, NHC_2H_5, and $N(C_2H_5)_2$

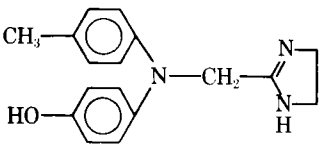
Although only a few quinoline derivatives have been investigated, their potencies as inhibitors of phosphodiesterase appear to be minimal, at least on



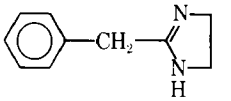
imidazole-4-acetic acid
V



histamine
VI



phentolamine
VII



tolazoline
VIII

Form I. 8-Hydroxyquinoline, as well as dipicolinic acid, probably does not actually inhibit phosphodiesterase but rather inactivates it by metal-ion chelation (71). In fact, a variety of metal-chelating carboxylic acids has been reported to inhibit phosphodiesterase activity from a number of sources (3, 67, 296).

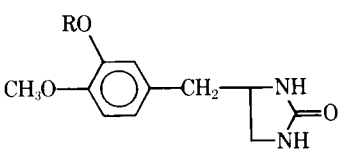
Indole Derivatives—Serotonin weakly inhibited phosphodiesterase activity in beef heart (252) (Table XIV) while reserpine more substantially inhibited the enzyme from the brains of trout (44), rat (297), and cockroach (35). A synthetic derivative of reserpine, 1-diethylaminoethylreserpine, was 200 times more potent than theophylline as an inhibitor of rabbit brain cortex Form I (333).

Dihydroergotamine was about twice as potent as theophylline on cat cerebral cortex Form I (191) but showed a uniform, pronounced inhibition of phosphodiesterase in other organs only at high, unphysiological concentrations (10^{-3} *M*) (190). Only in cat brain phosphodiesterase did both dihydroergotamine and an hydrogenated ergot alkaloid mixture[4] induce an accumulation of cyclic AMP at physiological dose levels (190). It has been reported that dihydroergotamine inhibits both adenyl cyclase and phosphodiesterase; thus it antagonizes catecholamine-stimulated lipolysis by adenyl cyclase inhibition and potentiates the effects of other lipolytic agents *via* phosphodiesterase inhibition (336).

Nine dihydroergotamine derivatives were approximately equal in potency to caffeine and theophylline on cat brain gray matter Form I (334). Brucine and strychnine were weak inhibitors (18) of Form I while the latter was also reported as an activator (17) of the enzyme.

2-Bromolysergic acid diethylamide was 5 times as potent as theophylline *in vitro* (290) and 15–40 times as potent as theophylline on guinea pig heart phosphodiesterase *ex vivo* (335).

Imidazole Derivatives—Imidazole itself is most often reported as an activator of both Forms I and II

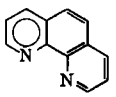


IX: R = CH_3, Ro 7-2956
X: R = n-C_4H_9, Ro 20-1724

[3] AH 3365.

[4] Hydergine.

Table XIII—Pyridine and Quinoline Derivatives

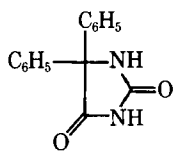
Name	Substituent(s)	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phosphodiesterase Form	Reference
		Pyridines				
	3-Acetyl	~70[c]	Rat liver	?	I	330
	3-*N*,*N*-Diethylcarboxamido	~70[c]	Rat liver	?	I	330
	3—CH=CH—COOH	Weak	Rat fat cells	1×10^{-4}	II	331
	3-$(CH_2)_n$COOH (n = 0,1,2,3)	Inactive	Rat fat cells	1×10^{-4}	II	331
	3-$(CH_2)_4$COOH	Weak	Rat fat cells	1×10^{-4}	II	331
	2,3-Dicarboxyl	16.0[c]	*B. liquefaciens*	1×10^{-2}	I	71
	2,4-Dicarboxyl	38.5[c]	*B. liquefaciens*	1×10^{-2}	I	71
	2,5-Dicarboxyl	21.5[c]	*B. liquefaciens*	1×10^{-2}	I	71
Dipicolinic acid	2,6-Dicarboxyl	94.8[c]	*B. liquefaciens*	1×10^{-2}	I	71
α,α'-Dipyridyl	2-(2-Pyridyl)	8.6[c]	*B. liquefaciens*	1×10^{-2}	I	71
Nicotinic acid	3-Carboxyl	~20[c]	Rat liver	4×10^{-2}	I	330
Nicotinamide	3-Carboxamido	~70[c]	Rat liver	4×10^{-2}	I	330
NADP	—	Weak	Rat liver	2×10^{-2}	I	330
		Quinolines				
8-Hydroxyquinoline		60[c]	*B. liquefaciens*	1×10^{-2}	I	71
ortho-Phenanthroline		25[c]	Bovine brain	1×10^{-4}	I	70
Quinoline		17[c]	Bovine brain	1×10^{-4}	I	70

* See footnote to Table VI.

but occasionally does not significantly affect the enzyme (74, 215). Imidazole-4-acetic acid (V) (217) and histamine (VI) (10^{-2} *M*) (248, 281, 337) also showed stimulant effects on the enzyme (see *Activators*), although the latter did not affect the activity from beef heart (252), bullfrog gastric mucosa (217), or cyclic GMP phosphodiesterase from rat brain (53).

The α-blocking agent phentolamine (VII) effectively inhibited Form I from mouse brain (186) and rat brain (288) at high concentrations, although the structurally related α-blocker, tolazoline (VIII), was considerably weaker in one system (288) and possibly stimulatory in the other. Low concentrations of phentolamine did not inhibit beef heart Form I (252). While evidence does not support the conclusion that α-adrenergic blocking agents as a class inhibit phosphodiesterase, it has been suggested (288) that phosphodiesterase inhibition as well as α-adrenergic blockade may be involved in the actions of phentolamine.

Several members (IX and X) of a class of 4-(3,4-dialkoxybenzyl)-2-imidazolidinones were reported to be surprisingly potent inhibitors of phosphodiesterase. The prototype of the series, Ro 7-2956, was 42 times as potent as theophylline in this rat red blood cell system. Within this family of compounds, activity was greatly dependent upon the nature and position of the aromatic ring substituents. Inhibition increased with the length of the *meta*-alkoxy group and decreased with the length of the *para*-alkoxy function (338).



XI: diphenylhydantoin

Addition of a carboxy group to the 4-position of the heterocyclic ring in Ro 7-2956 decreased activity markedly, as did changing the aromatic group to naphthyl. Opening the heterocyclic ring to form a diamine reduced activity to nearly that of theophylline. Resolution of Ro 7-2956 showed that the *l*-enantiomer had more than 3 times the potency of the *d*-form and nearly twice that of the racemic compound. Both theophylline and Ro 20-1724 inhibited rat erythrocyte phosphodiesterase in a mixed manner, with both competitive and noncompetitive aspects (177). Subsequent work (121, 122) pointed out pronounced differences in the sensitivity of phosphodiesterases from a variety of tissues to Ro 20-1724 when compared with papaverine and theophylline. Ro 20-1724 also selectively inhibited hydrolysis of cyclic AMP compared to cyclic GMP (177).

The imidazolidinedione diphenylhydantoin (XI) was reported to be one-fourth as potent as the theophylline on rat brain Form II (297) and less than 2% as active as papaverine on the enzyme from the hamster pancreatic islet cell tumor (107).

Table XIV—Indole Derivatives

Name	Substituent	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phosphodiesterase Form	Reference
Serotonin		Weak	Beef heart	5×10^{-5}	I	252
Reserpine		170[b], 0.7[d]	Rat brain	—	II	297
		39[c,f]	Cockroach brain	1×10^{-4}	II	35
		23[c]	Trout brain	5×10^{-4}	I	44
	1-Diethylaminoethyl	0.55[a], 200[d]	Rabbit brain cortex	—	I	333
Dihydroergotamine		29[c,f]	Cat cerebral cortex	2.5×10^{-5}	I	191
Dihydroergotamine analogs (a total of nine)		~1.0[d]	Cat brain gray matter	2.5×10^{-5}	I	334
Lysergide (lysergic acid diethylamide)	2-Bromo	100[a], 5.0[d]	Guinea pig heart	—	?	290
		15–40[d]	Guinea pig heart (*ex vivo*)	3×10^{-4}	?	335
		68[a], 2.6[d]	Beef heart	—	I	326
Strychnine nitrate		40[c,g]	Bovine taste papillae	6×10^{-4}	I	18
Brucine		39.2[c,g]	Bovine taste papillae	6×10^{-4}	I	18

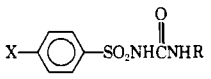
* See footnote to Table VI.

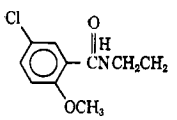
Sulfonylureas—When comparisons are made to caffeine or theophylline, the sulfonylureas are invariably less potent, even in tissues other than pancreas. Chlorpropamide and tolbutamide are most often reported to inhibit phosphodiesterase but neither is consistently the more potent. It would appear from the data in Table XV that the nature of the alkyl and ring substituents is not very important in structure–activity relationships, but an insufficient number of derivatives has been reported for any clear relationships to be outlined.

One attempt was made to show that the phosphodiesterase inhibitory action of the sulfonylureas depends on the intact structure since a mixture of toluenesulfonic acid and butylurea had very little inhibitory activity (341). This should not imply that dividing the molecule at some other point (*e.g.*, *p*-toluenesulfonamide and *N*-butylformamide) would necessarily produce the same result.

Benzothiadiazines and Related Compounds—All benzothiadiazine derivatives cited (Table XVI) as phosphodiesterase inhibitors are of the 1,1-dioxide group and only diazoxide is not of the diuretic class, which almost by definition bears a sulfamoyl group in the 7-position. Diazoxide and six thiazide diuretics were compared with caffeine and theophylline on beef heart Form I, and all compounds tested inhibited the enzyme but were less potent than caffeine and less than one-half as potent as theophylline (342). Most of the potency differences between the ben-

Table XV—Sulfonylureas



Name	X	R	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phosphodiesterase Form	Reference
Tolbutamide	CH_3	C_4H_9-*n*	23.0[c]	Rat kidney	5×10^{-3}	II	339
			59[c]	Rat adrenals	1×10^{-2}	?	340
			28[c,g], 3000[a]	Bovine thyroid	5×10^{-3}	I	301
			53[c]	Hamster islet cell tumor	1×10^{-2}	II	341
			3000[b]	Hamster islet cell tumor	—	II	107
Toluenesulfonic acid + butylurea			15[c,g]	Hamster islet cell tumor	1×10^{-2}	II	341
Chlorpropamide	Cl	C_3H_7-*n*	25.7[c]	Rat kidney	5×10^{-3}	II	339
			47[c]	Rat adrenals	1×10^{-2}	?	340
			27[c,g]	Bovine thyroid	5×10^{-3}	I	301
Acetohexamide	CH_3CO		46[c]	Rat adrenals	1×10^{-2}	?	340
Tolazamide	CH_3		57[c]	Rat adrenals	1×10^{-2}	?	340
Glyburide (glibenclamide)		H	20[c]	Mouse pancreatic islet	—†	II	111

* See footnote to Table VI. † Inhibitor concentration, 8.7 μg/ml.

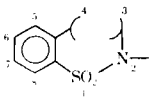
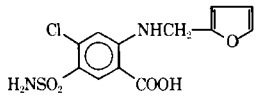
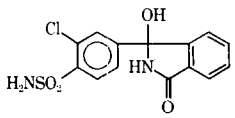


Table XVI—Benzothiadiazine Derivatives and Related Compounds

(4-3) Moiety	Substituent at Position 2	6	7	Name	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phosphodiesterase Form	Reference
—N=C(CH$_3$)—	H	H	Cl	Diazoxide	0.28[d]	Beef heart	$\sim10^{-3}$	I	342
					11[c]	Bovine artery	10^{-3}	I	289
					450[a]	Beef heart	—	I	343
					1200[b]	Hamster islet cell tumor	—	II	107
—N=CH—	H	Cl	SO_2NH_2	Chlorothiazide	0.31[d]	Beef heart	$\sim10^{-3}$	I	342
					5200[a], 0.54[d]	Guinea pig kidney	—	II	175
					2900[a], 0.45[d]	Rat kidney	—	II	175
—NHCH$_2$—	H	Cl	SO_2NH_2	Hydrochlorothiazide	0.02[d]	Beef heart	$\sim10^{-3}$	I	342
					1500[a]	Beef heart	—	I	343
—NH—CH(CHCl$_2$)—	H	Cl	SO_2NH_2	Trichlormethiazide	0.11[d]	Beef heart	$\sim10^{-3}$	I	342
—NH—CH$_2$—	H	CF_3	SO_2NH_2	Hydroflumethiazide	0.08[d]	Beef heart	$\sim10^{-3}$	I	342
—NH—CH(CH$_2$C$_6$H$_5$)—	H	CF_3	SO_2NH_2	Bendroflumethiazide	0.05[d]	Beef heart	$\sim10^{-3}$	I	342
—NH—CH(CH$_2$SCH$_2$CF$_3$)—	CH_3	F	SO_2NH_2	Polythiazide	0.41[d]	Beef heart	$\sim10^{-3}$	I	342
				Furosemide	500[a]	Beef heart	—	I	343
				Chlorthalidone	2500[a], 0.06[d]	Beef heart	—	I	344

* See footnote to Table VI.

zothiadiazine derivatives are not great, making it hazardous to extrapolate structure–activity relationships in too much detail; however, a few points seem worth mentioning.

Saturation of the 3,4-double bond of chlorothiazide decreased potency by a factor of 15 on beef heart Form I; other 3,4-saturated derivatives, except polythiazide, were less potent than chlorothiazide. The effects of alkyl or substituted alkyl groups in the 3-position appear to be minimal. Obviously, the 7-sulfamoyl function with the accompanying diuretic activity is not essential for phosphodiesterase inhibition, as shown by the case of diazoxide. Substitution in the 6-position does not appear to be of special significance where the substituent is H, Cl, or CF_3. The influence on phosphodiesterase activity of the 6-fluoro and 2-alkyl substituents of polythiazide, the most potent member of the series on beef heart Form I, remains to be elucidated.

Table XVII—Inhibitor Constants of Chlorothiazide on Phosphodiesterase Activity from Selected Guinea Pig and Rat Tissues (175)

Tissue	K_i Form II, mM[a]
Guinea Pig:	
Kidneys	5.2
Lungs	13.0
Brain, heart, liver	>85.0
Rat:	
Kidneys	2.9
Liver	85.0
Brain, heart, lungs	>85.0

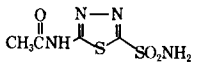
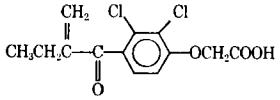
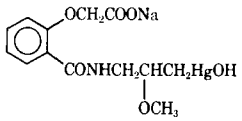
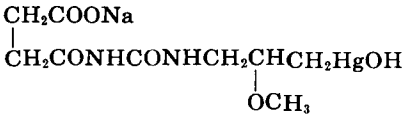
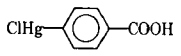
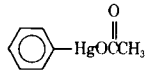
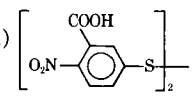
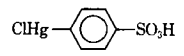
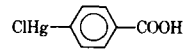
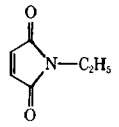
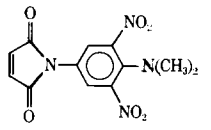
[a] Determined as described for Table II.

Although the thiazide diuretics are not potent phosphodiesterase inhibitors, the inhibitor constants (K_i) for one derivative, chlorothiazide, are lower for Form II from kidney tissue (guinea pig and rat) than for any of the other tissues studied (Table XVII), indicating marked selectivity for the kidney enzyme.

Two other diuretics, structurally related to the benzothiadiazines, were also reported to inhibit phosphodiesterase. Chlorthalidone (344) had about one-fifteenth the potency of theophylline, while furosemide (343) was three times as potent as hydrochlorothiazide on beef heart Form I.

Miscellaneous Diuretics and Sulfhydryl-Binding Agents—The importance of sulfhydryl groups for phosphodiesterase activity, as discussed earlier, is

Table XVIII—Miscellaneous Diuretics and Sulfhydryl-Binding Agents

Name	Structure	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phosphodiesterase Form	Reference
Acetazolamide		6000[a], 0.027[d]	Beef heart	—	I	343, 344
Ethacrynic acid		400[a], 0.4[d] 35[c]	Beef heart Guinea pig heart	— 2×10^{-3}	I ?	343, 344 189
Mersalyl		72[c]	Guinea pig heart	2×10^{-3}	?	189
Meralluride		67[c]	Guinea pig heart	2×10^{-3}	?	189
p-Chloromercuribenzoic acid		65[c]	Guinea pig heart	2×10^{-3}	?	189
Phenylmercuric acetate		96[c]	Guinea pig heart	2×10^{-3}	?	189
Methylmercuric chloride	CH_3HgCl	76[c]	Guinea pig heart	2×10^{-3}	?	189
Mercuric chloride	$HgCl_2$	92[c]	Guinea pig heart	2×10^{-3}	?	189
Iodoacetic acid	ICH_2COOH	12.4[c]	Bullfrog gastric mucosa	1×10^{-3}	I	217
		5[c]	Guinea pig heart	2×10^{-3}	?	189
5,5′-Dithiobis(2-nitrobenzoic acid)		27.0[c]	Bullfrog gastric mucosa	1×10^{-3}	I	217
p-Chloromercuribenzenesulfonic acid		58.0[c]	Bullfrog gastric mucosa	1×10^{-3}	I	217
p-Chloromercuribenzoic acid		61.0[c]	Bullfrog gastric mucosa	1×10^{-3}	I	217
N-Ethylmaleimide		5[c]	Guinea pig heart	2×10^{-3}	?	189
N-(4-Dimethylamino-3,5-dinitrophenyl)-maleimide		100[c]	Bullfrog gastric mucosa	1×10^{-3}	I	217

* See footnote to Table VI.

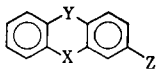
reemphasized by the results shown in Table XVIII. Several mercurial diuretics and other sulfhydryl-binding agents appear to inhibit strongly the enzyme from a number of sources (189). However, the incomplete reversal of inhibition by dithiothreitol throws some doubt on sulfhydryl binding as the only mechanism by which these compounds inhibit phosphodiesterase (189). Furthermore, acetazolamide and ethacrynic acid, with no apparent sulfhydryl-binding ability, still inhibit the enzyme (343, 344).

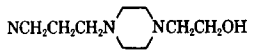
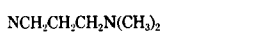
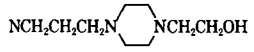
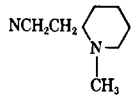
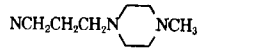
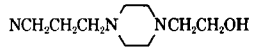
Psychotropic Agents—The reported ability of the vast majority of psychotropic agents to inhibit phosphodiesterase from a variety of tissues must be tempered by some additional observations:

1. Phenothiazine derivatives have also been reported to inhibit adenyl cyclase in brain (345) as well as other tissues (346).

2. Three tricyclic antidepressants, imipramine, desipramine, and amitriptyline, have been found to be potent stimulators of cyclic AMP formation in

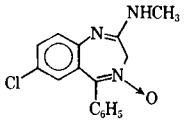
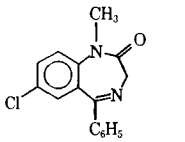
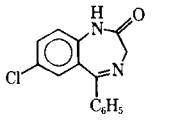
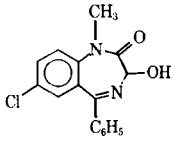
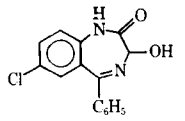
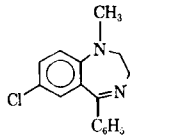
Table XIX—Psychotropic Agents: Tricyclic Derivatives



Name	X	Y	Z	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phosphodiesterase Form	Reference
Chlorpromazine	$NCH_2CH_2CH_2N(CH_3)_2$	S	Cl	170[b], 0.7[d]	Rat brain	—	II	297
				14[c]	Beef heart	5×10^{-5}	I	333
				50[c]	Rabbit brain	5×10^{-5}	I	333
				85[c]	Ox heart	1×10^{-3}	I	357
				80[a]	Rat brain	—	II	358, 359
				69[c]	Mouse brain	1×10^{-3}	I	186
Promazine	$NCH_2CH_2CH_2N(CH_3)_2$	S	H	2[c]	Beef heart	5×10^{-5}	I	333
				24[c]	Rabbit brain	5×10^{-5}	I	333
Fluphenazine		S	CF_3	48[b], 2.5[d]	Rat brain	—	II	297
				18[c]	Beef heart	5×10^{-5}	I	333
				64[c]	Rabbit brain	5×10^{-5}	I	333
Trifluprom azine		S	CF_3	300[b], 0.4[d]	Rat brain	—	II	297
				130[a]	Rat brain	—	II	358, 359
Perphenazine		S	Cl	14[c]	Beef heart	5×10^{-5}	I	333
				60[c]	Rabbit brain	5×10^{-5}	I	333
Thioridazine		S	SCH_3	180[b]	Rat brain	—	II	297
Prochlorperazine		S	Cl	14[c]	Beef heart	5×10^{-5}	I	333
				64[c]	Rabbit brain	5×10^{-5}	I	333
Chlorprothixene	$C{=}CHCH_2CH_2N(CH_3)_2$	S	Cl	170[a]	Rat brain	—	II	358, 359
Imipramine	$NCH_2CH_2CH_2N(CH_3)_2$	CH_2CH_2	H	222[a], 0.4[d]	Rat adipose	—	II	171
				765[a], 1.0[d]	Rat adipose	—	I	171
				430[d]	Rat brain	—	II	358, 359
				40[c]	Ox heart	1×10^{-3}	I	357
				700[b], 0.2[d]	Rat brain	—	II	297
				90[c,f]	Human brain	1×10^{-3}	I	178
				58[c]	Human platelets	1×10^{-3}	I	178
				36[c]	Mouse brain	5×10^{-3}	I	186
Desipramine	$NCH_2CH_2CH_2NHCH_3$	CH_2CH_2	H	45[c]	Ox heart	1×10^{-3}	I	357
				480[a]	Rat brain	—	II	358, 359
				36[c]	Mouse brain	5×10^{-3}	I	186
Trimepramine	$NCH_2CH(CH_3)CH_2N(CH_3)_2$	CH_2CH_2	H	55[c]	Ox heart	1×10^{-3}	I	357
Nortriptyline	$C{=}CHCH_2CH_2NHCH_3$	CH_2CH_2	H	70[c]	Ox heart	1×10^{-3}	I	357
				200[b], 0.6[d]	Rat brain	—	II	297
				86[c,f]	Human brain	1×10^{-3}	I	178
				34[c,g]	Human platelets	1×10^{-3}	I	178
				66[c]	Mouse brain	1×10^{-3}	I	186
Amitriptyline	$C{=}CHCH_2CH_2N(CH_3)_2$	CH_2CH_2	H	70[c]	Ox heart	1×10^{-3}	I	357
				460[b], 0.3[d]	Rat brain	—	II	297
				450[a]	Rat brain	—	II	358, 359
Opipramol		CH=CH	H	86[c,f]	Human brain	1×10^{-6}	I	178
				25[c,g]	Human platelets	1×10^{-6}	I	178
				80[c]	Ox heart	1×10^{-3}	I	357
Protriptyline	$CHCH_2CH_2CH_2NHCH_3$	CH=CH	H	65[c]	Ox heart	1×10^{-3}	I	357
Doxepin	$C{=}CHCH_2CH_2N(CH_3)_2$	O—CH_2	H	>1000[b]	Rat brain	—	II	297

* See footnote to Table VI.

Table XX—Psychotropic Agents: Benzodiazepine Derivatives (181)

Name	Structure	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phosphodiesterase Form
Chlordiazepoxide		29[c] 15[c] 18[c]	Cat temporal cortex Cat hippocampus Cat cerebellum	5×10^{-5} 5×10^{-5} 5×10^{-5}	II II II
Diazepam		55[c] 44[c] 42[c]	Cat temporal cortex Cat hippocampus Cat cerebellum	5×10^{-5} 5×10^{-5} 5×10^{-5}	II II II
N-Demethyldiazepam		34[c] 22[c] 15[c]	Cat temporal cortex Cat hippocampus Cat cerebellum	5×10^{-5} 5×10^{-5} 5×10^{-5}	II II II
N-Methyloxazepam		33[c] 19[c] 27[c]	Cat temporal cortex Cat hippocampus Cat cerebellum	5×10^{-5} 5×10^{-5} 5×10^{-5}	II II II
Oxazepam		15[c] 11[c] 11[c]	Cat temporal cortex Cat hippocampus Cat cerebellum	5×10^{-5} 5×10^{-5} 5×10^{-5}	II II II
Medazepam		18[b], 11.1[d] 25[b], 32[d] 15[b], 67[d]	Cat cortex Rat brain Neuroblastoma	— — —	II II II

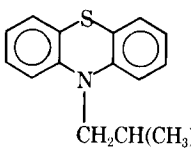
* See footnote to Table VI.

guinea pig cerebral slices (347) at doses where their phosphodiesterase inhibition is marginal; chlorpromazine and perphenazine had no significant effect on cyclic AMP levels. Theophylline at 10^{-3} *M* inhibited the effect of imipramine by >90% on cerebral slices (347). A similar situation was observed with adenosine (348–354). This antagonism may involve a competition between theophylline on the one hand and antidepressants or adenosine on the other for the receptor sites on phosphodiesterase, allowing the intracellular levels of cyclic AMP to rise through other mechanisms. Other workers reported that of nortriptyline, anitriptyline, desipramine, and imipramine, only the last had significant activity on ox heart Form II and it was less potent than caffeine (355). It is also possible that these compounds are more active on the activated enzyme, *i.e.*, in presence of the protein activator shown to be present in the brain and discussed previously.

3. Regarding a causal relationship between phosphodiesterase inhibition and the action of tricyclic antidepressants, it has been cautioned that, while levels of cyclic AMP in brain tissue may be of importance in regulating mental states, inhibition of brain phosphodiesterase by a drug does not necessarily confer antidepressant activity. A case in point is that of promethazine (XII), which is as potent an inhibitor of rat brain phosphodiesterase as certain tricyclic antidepressants but has no antidepressant effect *in vivo* (356).

A point-by-point interpretation of structure *versus* activity for the psychotropic agents would probably be fruitless as well as confusing. However, nearly all tricyclic psychotropic agents (Table XIX) have shown respectable potency as inhibitors of phosphodiesterase in most systems, depending on the concentration used.

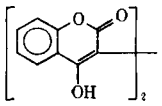
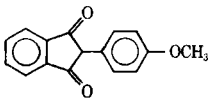
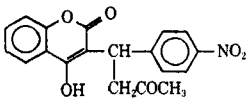
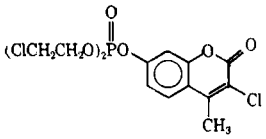
The minor tranquilizers of the chlordiazepoxide type (Table XX) have not been studied extensively as phosphodiesterase inhibitors but show considerable inhibition at low concentrations on the enzymes from the cat CNS (181). Haloperidol, a substituted butyrophenone, has invariably shown little potency on phosphodiesterases (297, 333, 358, 359).



$CH_2CH(CH_3)N(CH_3)_2$

XII: promethazine

Table XXI—Anticoagulants (363)

Name	Structure	Potency*	Enzyme Source	Phosphodiesterase Form
Dicumarol		35[b]	Human platelets	II
Anisindione		240[b]	Human platelets	II
Acenocoumarol		270[b]	Human platelets	II
Haloxan		10[b]	Human platelets	II

* See footnote to Table VI.

The relationship of cyclic AMP levels to brain function has been extensively reviewed (54, 177, 360–362).

Anticoagulants—Anticoagulants of the type shown in Table XXI may inhibit phosphodiesterase and raise platelet cyclic AMP levels (363); this has been shown to mediate platelet-aggregation inhibition (318, 364). Thus, these compounds may inhibit the formation of fibrin clots by their effects on prothrombin metabolism, and they may inhibit platelet aggregation by virtue of their phosphodiesterase inhibitory action.

Miscellaneous Heterocyclic Compounds of Synthetic Origin—A number of heterocyclic compounds with important pharmacological profiles have been found to be strong inhibitors of phosphodiesterase (Table XXII). Dipyridamole, a derivative of pyrimido[5,4-*d*]pyrimidine which was introduced several years ago as a coronary vasodilator, is a potent inhibitor of platelet aggregation and a very effective phosphodiesterase inhibitor. Nevertheless, it is generally less potent than papaverine (94, 181, 316, 318, 325). Although it showed considerable selectivity for human platelet over human brain phosphodiesterase (178), dipyridamole was less potent than its congener RA 233 on the human platelet enzyme (365). RA 233, which lacks one of the two equivalent piperidino moieties found in dipyridamole, was particularly effective against the human platelet enzyme (322, 365).

A thieno[3,2-*d*]pyrimidine derivative[5] was equipotent to dipyridamole on human platelet Form I but was less potent than the dipyridamole relative, RA 233. There was no correlation between the action of any one of these three pyrimidine derivatives and the inhibitory effect on ADP-induced platelet aggregation (365).

[b] VK 744.

SQ 20,009, a substituted pyrazolo[3,4-*b*]pyridine, is a potent inhibitor of rat brain Form II (213). Being considerably more potent on brain phosphodiesterase than on the heart, lipocyte, or adrenal enzymes (213), it is perhaps not surprising that it possesses marked antianxiety activity (297).

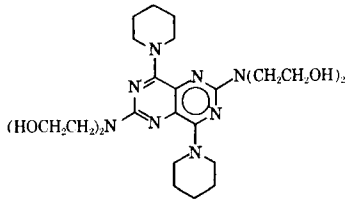
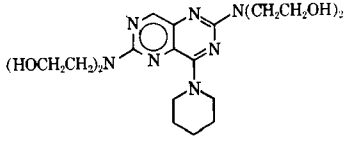
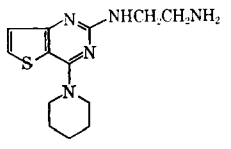
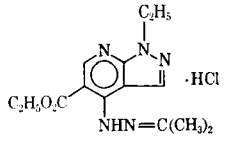
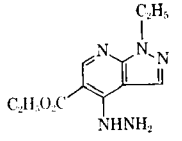
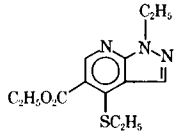
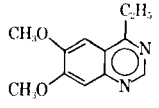
Two close relatives of SQ 20,009 also possess the pyrazolopyridine skeleton. SK 20,006, which lacks only the 4-isopropylidine moiety, was less potent than SQ 20,009 on rat brain and rat adrenal phosphodiesterase but more potent on the rat lipocyte enzyme (213). The 4-ethylthio analog, SQ 65,442, was more potent than theophylline on cockroach brain Form II (35).

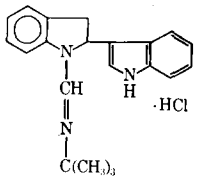
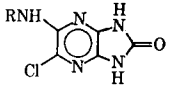
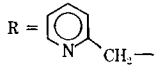
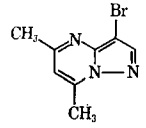
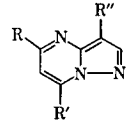
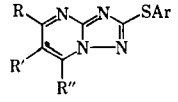
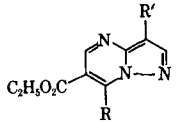
The bronchodilator–vasodilator compound known generically as quazodine is an effective inhibitor of phosphodiesterase from a number of tissues. It has a pharmacological profile similar to that of theophylline and was approximately twice as potent as the latter on phosphodiesterase from bovine thyroid (301) and a variety of rabbit tissues (176); it was more than 6 times as potent as theophylline on the enzyme from bovine brain (176) and 16 times more potent on human platelet Form I (318).

The interesting diuretic properties of MJ 8592-1, an indolylindoline (376), prompted a study of its activity on phosphodiesterase. Although it was not especially potent on phosphodiesterase from a variety of guinea pig and rat tissues, it preferentially inhibited the kidney enzyme in both species. This selective inhibition of phosphodiesterase was proposed as a mechanism for the diuretic effects of MJ 8592-1 (175).

CEIP, CDIP, and a pyridylmethyl analog are imidazo[4,5-*b*]pyrazine derivatives which were studied as hypotensives and inhibitors of phosphodiesterase (366). CEIP, which also has bronchodilator, cardiac stimulant, and peripheral vasodilator properties, is

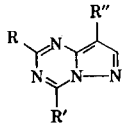
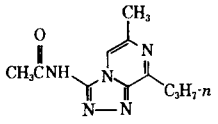
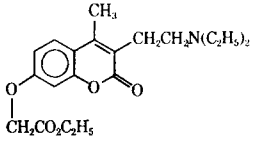
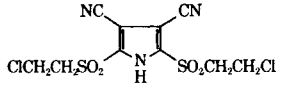
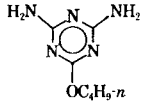
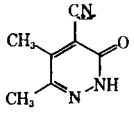
Table XXII—Miscellaneous Heterocyclic Compounds of Synthetic Origin

Name	Structure	Potency	Enzyme Source	Inhibitor Concentration, moles/liter	Phosphodiesterase Form	Reference
Dipyridamole		22[c,g]	Human brain	1×10^{-5}	I	178
		85[c,f]	Human platelets	1×10^{-5}	I	178
		>1.0[d]	Ox heart, rabbit brain, human platelets	—	II	322
		45[c]	Human platelets	1.2×10^{-4}	I	365
		62[a], 0.4[e]	Beef heart	—	I	316
		17[a], 0.3[e]	Human platelets	—	I	318
		15[b], 0.8[e]	Cat cortex	—	II	181
		12[b], 0.3[e]	Rat brain	—	II	181
		1.5[b], 3.7[e]	Neuroblastoma cell	—	II	181
		16[a], 0.35[e]	Beef heart	—	I	94, 325
RA 233		>1.0[d]	Ox heart, rabbit brain, human platelets	—	II	322
		55[c]	Human platelets	1.2×10^{-4}	I	365
VK 744		45[c]	Human platelets	1.2×10^{-4}	I	365
SQ 20,009		2[a]	Rat brain	—	II	213
		2[b]	Rat brain	—	II	297
		4.8[b]	Rabbit brain	—	II	213
		20[b]	Rat adrenal	—	II	213
		21[b]	Rat lipocytes	—	II	213
		64[a]	Cat heart	—	II	213
SQ 20,006		~10[b]	Rat lipocytes	—	II	213
		100[b], 0.1[e]	Cat cortex	—	II	181
		30[b], 0.2[e]	Neuroblastoma cell	—	II	181
		53[b], 0.07[e]	Rat brain	—	II	181
		50[b]	Cat heart	—	II	213
SQ 65,442		80[c,f]	Cockroach brain	1×10^{-4}	II	35
Quazodine		361[a], 2.5[d]	Rabbit lung	—	I	176
		1331[a], 1.7[d]	Rabbit heart	—	I	176
		181[a], 1.8[d]	Rabbit liver	—	I	176
		1540[a], 1.2[d]	Rabbit spleen	—	I	176
		800[a], 1.9[d]	Rabbit kidney	—	I	176
		1290[a], 1.7[d]	Rabbit pyloric mucosa	—	I	176
		773[a], 1.3[d]	Rabbit fundic mucosa	—	I	176

		116[a], 6.5[d]	Bovine brain	—	I	176
		59[c,f]	Bovine thyroid	1×10^{-4}	II	301
		50[a], 0.1[e]	Human platelets	—	I	318
MJ 8592-1		2700[a], 0.5[d]	Guinea pig brain	—	II	175
		Inactive	Guinea pig heart	—	II	175
		2000[a], 1.4[d]	Guinea pig kidney	—	II	175
		Inactive	Guinea pig liver	—	II	175
		11,000[a], 0.9[d]	Guinea pig lung	—	II	175
		60,000[a], 0.03[d]	Rat brain	—	II	175
		Inactive	Rat heart	—	II	175
		3400[a], 0.38[d]	Rat kidney	—	II	175
		10,000[a], 0.1[d]	Rat liver	—	II	175
		Inactive	Rat lung	—	II	175
						
CEIP	R = C_2H_5—	70[c,f]	Bovine heart	1×10^{-3}	I	366
		60[c,f]	Bovine heart	1×10^{-3}	I	182
		52[c,f]	Rabbit uterus	1×10^{-3}	I	182
		62[c,f]	Bovine pancreas	1×10^{-3}	I	182
		52[c,f]	Rat lung	1×10^{-3}	I	182
		54[c,f]	Rat heart	1×10^{-3}	I	182
CDIP	R = $(CH_3)_2NCH_2CH_2$	19[c,g]	Bovine heart	1×10^{-3}	I	366
		7[c,g]	Rabbit uterus	1×10^{-3}	I	182
		Inactive	Rat lung	1×10^{-3}	I	182
		8[c,g]	Rat heart	1×10^{-3}	I	182
		28[c,f]	Bovine heart	1×10^{-3}	I	366
ICN 3009		123[b]	Rabbit kidney	—	II	367
		140[b]	Rabbit lung	—	II	367
		60[b]	Bovine brain	—	II	367
		210[b]	Beef heart	—	II	367
3,5,7-Trisubstituted pyrazolo[1,5-*a*]-pyrimidines		≫1.0[d]	Rabbit lung, beef heart	—	II	368
2-Aralkylthio-*s*-triazolo-[1,5-*a*]pyrimidines		>1.0[d]	Rabbit lung, rabbit kidney	—	II	369
6-Carbethoxy-3,7-di-substituted pyrazolo-[1,5-*a*]pyrimidines		≫1.0[d]	Rabbit lung, rabbit kidney	—	II	370

(*continued*)

Table XXII—(*Continued*)

Name	Structure	Potency	Enzyme Source	Inhibitor Concentration, moles/liter	Phospho-diesterase Form	Reference
3,5,7-Trisubstituted pyrazolo[1,5-*a*]1,3,5-triazines		≫1.0[d]	Rabbit lung	—	II	371
ICI 58,301		62[a], 4.2[d]	Bovine heart	—	I	372, 373
Chromonar (Carbocromene)		71[c,f] 1400[a], 0.004[e]	Rat heart Beef coronary artery	1×10^{-3} —	I I	291, 324 94, 325
A		75[c,f]	Rat adipose tissue	1×10^{-3}	I	375
B		24[c,g]	Rat adipose tissue	5×10^{-3}	I	375
C		21[c,g]	Rat adipose tissue	5×10^{-3}	I	375

* See footnote to Table VI.

Table XXIII—Miscellaneous Purine and Pyrimidine Bases, Nucleosides, and Nucleotides

Name	Potency*	Enzyme Source	Inhibitor Concentration, moles/liter	Phosphodiesterase Form	Reference
		Bases and Derivatives			
Purine	12[c]	Toad bladder epithelium	5×10^{-3}	I	379
Adenine	200[b]	Rat skeletal muscle	—	I	380
	44[c,g]	Rat adipose tissue	1×10^{-2}	I	381
	~10[c,g]	Beef heart	1×10^{-3}	I	222
	35[c]	Toad bladder epithelium	2.25×10^{-3}	I	379
	112[a]	Rat skeletal muscle	—	I	380
	29[c]	Isolated rat fat cell	1×10^{-3}	II	383
	21[c,g]	Rabbit skeletal muscle	1×10^{-3}	II	199
6-Hydroxypurine	14[c,g]	Rabbit skeletal muscle	1×10^{-3}	II	199
6-Dimethylaminopurine	49[c,g]	Rabbit skeletal muscle	1×10^{-3}	II	199
	~800[a]	Rat diaphragm	—	I	382
Puromycin	800[a]	Rat diaphragm	—	I	382
Mercaptopurine	76[c,f]	Human platelets	1×10^{-3}	I	178
	76[c,f]	Human brain	1×10^{-3}	I	178
Guanine	10[c]	Beef heart	1×10^{-3}	I	222
Hypoxanthine	210[b]	Rat skeletal muscle	—	I	380
Xanthine	142[b]	Rat skeletal muscle	—	I	380
		Nucleosides			
Purine riboside	Inactive	Rat skeletal muscle	—	I	380
Adenosine	30[c]	Rat fat cells	1×10^{-3}	II	383
	52[c]	Toad bladder epithelium	5×10^{-3}	I	379
	46[c,g]	Rat adipose tissue	1×10^{-2}	I	381
	10[c]	Beef heart	1×10^{-3}	I	222
	200[b]	Rat skeletal muscle	—	I	380
	25[c,g]	Rabbit skeletal muscle	1×10^{-3}	II	199
	Inactive	Pea seedlings	1×10^{-4}	II	74
	19[c]	Mouse brain	1×10^{-3}	I	185
	21[c,h]	Rat fat cells	7×10^{-5}	II	384
Deoxyadenosine	55[c]	Rat fat cells	1×10^{-3}	II	383
	59[c,f]	Rat adipose tissue	1×10^{-2}	I	381
	24[c]	Mouse brain	1×10^{-3}	I	186
	31[c,h]	Rat fat cells	7×10^{-5}	II	384
2-Fluoroadenosine	26[c,h]	Rat fat cells	7×10^{-5}	II	384
Guanosine	24[c]	Rat fat cells	5×10^{-4}	II	383
	33[c,g]	Rat adipose tissue	2×10^{-3}	I	381
	10[c]	Beef heart	1×10^{-3}	I	222
	43[c]	Mouse brain	1×10^{-3}	I	186
Desoxyguanosine	73[c]	Mouse brain	1×10^{-3}	I	186
Inosine	342[b]	Rat skeletal muscle	—	I	380
	23[c,g]	Rat adipose tissue	2×10^{-3}	I	381
	10[c]	Toad bladder epithelium	5×10^{-3}	I	379
	24[c]	Mouse brain	1×10^{-3}	I	185
Xanthosine	162[b]	Rat skeletal muscle	—	I	380
	14[c,g]	Rat adipose tissue	2×10^{-3}	I	381
Thymidine	22[c,g]	Rat adipose tissue	1×10^{-2}	I	381
Uridine	15[c,g]	Rat adipose tissue	1×10^{-2}	I	381
Cytidine	8[c,g]	Rat adipose tissue	1×10^{-2}	I	381
		Nucleotides			
5′-GMP	Activates	Beef heart	1×10^{-3}	I	222
5′-AMP	Inactive	Beef heart	1×10^{-3}	I	222
5′-IMP	Inactive	Beef heart	1×10^{-3}	I	222
ATP	18[c]	Toad bladder epithelium	5×10^{-3}	I	379
ADP	18[c]	Toad bladder epithelium	5×10^{-3}	I	379

* See footnote to Table VI.

reportedly 2–5 times more potent than theophylline as an inhibitor of phosphodiesterase from several sources; *ex vivo* it produced up to a 10-fold increase in the cyclic AMP content of rat heart. Analogs of CEIP with more basic substituents were generally less potent (182). Although CDIP is less active than theophylline as a phosphodiesterase inhibitor *in vitro*, its *in vivo* vasodilator and bronchodilator activities are of interest (182).

Several structurally related series of fused-ring heterocycles were recently reported as inhibitors of phosphodiesterase (368–371, 377). A number of 3,5,7-trisubstituted pyrazolo[1,5-*a*]pyrimidines were considerably more potent than theophylline as inhibitors of Form II from rabbit lung and beef heart (368). One member of this series, ICN 3009, was reported to have interesting cardiotropic properties (267). Members of a series of 6-carbethoxy-3,7-disubstituted pyrazolo[1,5-*a*]pyrimidines were also considerably more potent than theophylline on Form II from rabbit lung and rabbit kidney (370).

Many members of a series of 2-aralkylthio-*s*-triazolo[1,5-*a*]pyrimidines showed significant activity on Form II of rabbit lung and kidney (369). Various 3,5,7-trisubstituted pyrazolo[1,5-*a*]-1,3,5-triazines were found to be 50–150 times more active than theophylline on Form II from rabbit lung (371); some exhibited a specificity toward lung enzyme and several

were more potent as inhibitors of cyclic AMP phosphodiesterase than of cyclic GMP phosphodiesterase (377).

ICI 58,301 is a *sym*-triazolo[4,3-*a*]pyrazine derivative and a potent inhibitor of bovine heart Form I *in vitro* (372, 373); preliminary *ex vivo* experiments with orally dosed guinea pigs indicated greater selectivity for the lung than for the heart enzyme. The effects of ICI 58,301 in reducing the degree of bronchospasm in guinea pigs could be attributed to increased levels of cyclic AMP due to phosphodiesterase inhibition (373).

Chromonar, a coronary vasodilator, is a coumarin derivative with inhibitory properties on Form I from rat heart (291, 374) and beef coronary artery (94, 325). A metabolite[6] of chromonar is only weakly active on the rat heart enzyme (291, 374).

Three lipolytic agents of widely differing structure (Compounds A, B, and C, Table XXII) have shown activity on Form I from rat adipose tissue (375).

Two benzo[*b*]thiophene derivatives, 5-chloro-3-(2-dimethylaminoethyl)benzo[*b*]thiophene hydrochloride and *N*-(4-bromobenzo[*b*]thien-2-yl)guanidinium *p*-toluenesulfonate (not tabulated), are representatives of a number of benzo[*b*]thiophene derivatives with secondary or tertiary amine side chains which reportedly show marked noncompetitive inhibition of ox heart Form I (378).

Miscellaneous Purine and Pyrimidine Bases, Nucleosides, and Nucleotides—Table XXIII includes a number of miscellaneous nucleoside derivatives that are frequently present or added to incubation mixtures where phosphodiesterase activity is measured. Their effects on phosphodiesterase assays are often disregarded, in spite of their sometimes strong inhibitory actions on the enzyme.

CONCLUSIONS

A brief exposure to the structure–activity relationships of phosphodiesterase inhibitors might tempt one to conclude that all useful drugs inhibit phosphodiesterases. While this is certainly not the case (296–298), there appears to be a correlation, if not a causal relationship, between the *in vitro* phosphodiesterase activities of a great many drugs and their *in vivo* effects. It is obvious, after scanning the tabulated material, that no single structural skeleton or moiety is sufficient to confer the action of phosphodiesterase inhibition on a substance, Rather, it is clear that compounds of widely differing structures may show phosphodiesterase inhibition. It is, however, also readily apparent that certain structural moieties, particularly amidine (N—C=N), guanidine [N—C(=N)—N], amidrazone (N—N—C=N), and 3,4-disubstituted phenethylamine, which appear so frequently in useful drug entities, are also common to a great many inhibitors of phosphodiesterases.

This preponderant phosphodiesterase inhibition among various drug classes is perhaps not unexpected since cyclic nucleotides appear to be involved in the control of nearly all facets of cellular activity and metabolism in both healthy and disease states. The cyclic nucleotides, therefore, understandably present a worthy target for the action of drugs. The association between drugs and cyclic nucleotides is strong and vital to the successful mission of both in maintaining health.

[6] Intensainsäure.

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RESEARCH ARTICLES

Decomposition of *p*-Aminosalicylic Acid in the Solid State

J. T. CARSTENSEN [x] and PAKDEE POTHISIRI

Abstract □ Aminosalicylic acid was used as a test substance to show that a solid-state decomposition governed by nucleation kinetics can give rise to first-order kinetics. The effect of sorbed moisture at a low degree of coverage was tested. Sorbed moisture theories proposed previously apply when it is realized that the rate constants in the presence of the sorbed layer may differ from the values of these parameters under anhydrous conditions.

Keyphrases □ Decomposition—aminosalicylic acid in solid state giving rise to first-order kinetics, sorbed moisture theories discussed □ Aminosalicylic acid—decomposition in the solid state, first-order decomposition patterns □ Stability—decomposition of aminosalicylic acid in solid state, sorbed moisture theories discussed, first-order decomposition patterns □ Moisture—decomposition of aminosalicylic acid in solid state

The problems of stability of solid dosage forms have been discussed, although not with great frequency, in the pharmaceutical literature. Reference is made in this report to two basic articles (1, 2). For more complete references, the reader is referred to reviews on the subject (3, 4).

The problem of how to extrapolate pharmaceutical stability data is of importance in excess determination and in label statements on expiration dating; to this end, it is necessary to know prior to extrapolation [or to determine from the data (5)] if the decomposition is zero or first order.

The stability of the drug without excipients and the stability of the drug in the presence of moisture (6) (but no other excipients) throw some light on the theoretical order of reaction and the actual mode of decomposition. In the absence of moisture, topochemical- and nucleation-governed reactions occur (3, 7, 8); of these, *some* topochemical patterns may be approximated by first-order decay (3, 9). In the absence of moisture, the more common decomposition

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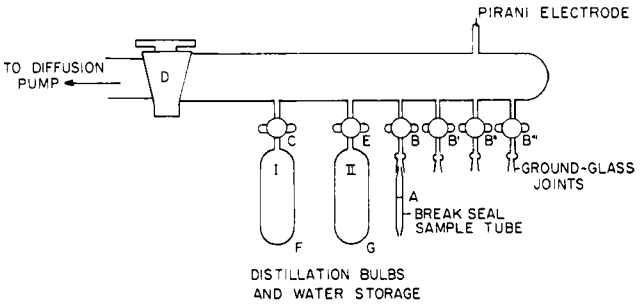

Figure 1—*Vacuum manifold used in the study.*

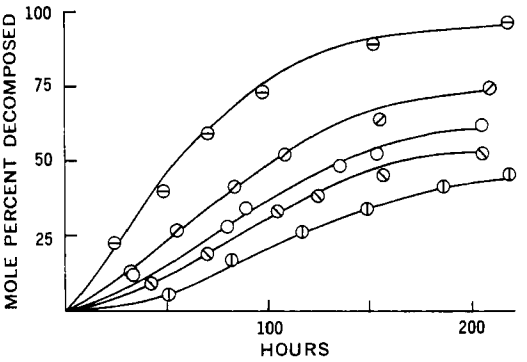

Figure 2—*Solid-state decomposition of aminosalicylic acid at 65° in the presence of moisture. Key: ◐, 5.9 torr; ⊘, 7.4 torr; ○, 11.4 torr; ⊘, 17.6 torr; and ⊖, 23.8 torr.*

pattern is governed by nucleation (7, 8), giving rise to sigmoid curves which should not be simple first or zero order.

In the presence of moisture, a simple, sorbed moisture theory (1, 2) would view the moisture present as a "bulk" saturated solution of the drug in the sorbed water; therefore, the decomposition kinetics should be dictated by the rates in saturated solution and be zero order. In the decomposition of aspirin, Leeson and Mattocks (1) found this to be the case (when pH effects were accounted for). Kornblum and Sciarrone (2) found that when moisture is present in the solid-state decomposition of aminosalicylic acid, decomposition rates are increased; in this case, however, the decomposition rates differed from those predicted by solubility.

A recent publication (3) gave tentative explanations why, theoretically, a solid could (in the absence of other ingredients) decompose in a pseudo-first-order fashion. With the presence of moisture, reaction orders should tend toward zero order if the system is considered to consist of solid plus bulk sorbed saturated moisture layer (after pH effects are accounted for).

The intent of this article is to show that first-order decomposition patterns may be tenable both *(a)* in the absence of moisture and *(b)* when moisture coverage is low. This latter feature, of course, brings the model system (aminosalicylic acid) close to the situation encountered in dosage forms. In achieving this objective, the findings of previous investigators (1, 2) will be confirmed.

EXPERIMENTAL AND RESULTS

p-Aminosalicylic acid was recrystallized from ethanol, dried *in vacuo*, ground in a mortar, and redried. The specific surface area of the powder was 0.36 m^2/g, as determined by Brunauer, Emmett, and Teller (BET) nitrogen adsorption (10).

Samples were prepared in breakseal tubes *in vacuo* on a high vacuum rack as described previously (11). A schematic drawing of the high vacuum manifold is shown in Fig. 1. The aminosalicylic acid was placed in the breakseal tube at point A. Water was placed in the distillation bulb I at point F. All stopcocks except for D were then closed, and the system was evacuated thoroughly (<0.5 mtorr). Stopcocks C and E were then opened and D was closed; liquid nitrogen was placed in a Dewar flask around G, and the water was distilled into distillation bulb II. Stopcock D was opened, the system was evacuated, D was closed, and the process was repeated with liquid nitrogen at point F. This distillation and evacuation process was carried out (four times) until lack of measurable pressure after a distillation on the Pirani gauge at H showed the water to be completely deaerated.

A constant-temperature bath was then placed at F and (with D and E closed) B was opened. In this manner, the aminosalicylic acid is exposed to a water vapor pressure dictated by the temperature of the constant-temperature bath at F. The types of constant-temperature baths used are listed in Table I. Stopcock C was occasionally closed; if the pressure measured on the Pirani gauge dropped, then equilibrium had not yet been achieved. At the point in time when the pressure no longer dropped, B and C were both closed, and the breakseal tube was removed by sealing it off by flame. Four samples could be prepared at a time (Fig. 1). A series was also prepared without moisture present.

The sealed tubes were stored at 60 ± 0.5° for the periods of time indicated in Fig. 2. After storage, the carbon dioxide evolved was determined manometrically as described by Musa (12), except a cold finger in the system was cooled to −5° to allow condensation of water without affecting the carbon dioxide pressure. Each sample was checked titrimetrically, and the decomposition was shown to be the straightforward, stoichiometric decarboxylation shown in Scheme I.

$$NH_2C_6H_3(OH)COOH \longrightarrow NH_2C_6H_4OH + CO_2$$

Scheme I

The results are shown in Fig. 2 and are tabulated in Table II.

The results from the series performed without the presence of moisture are shown in Fig. 3. Several temperatures were used.

Knowledge of the solubility of aminosalicylic acid is needed for

Table I—Constant-Temperature Baths for Controlled Water Vapor Pressure

Substance in Dewar Jar	Temperature	Water Vapor Pressure, torr
Water[a]	3.5°	5.9
Solid–liquid Benzene[b]	6.8°	7.4
Solid–liquid *p*-xylene[b]	13.2°	11.38
Water[a]	20°	17.56
Water[a]	25°	23.76

[a] This temperature is equal to the controlled air temperature. [b] This is a "slush" produced by adding liquid nitrogen to the benzene (xylene) in a Dewar jar; it produces a temperature of the melting point of benzene (xylene) as long as both solid and liquid benzene (xylene) are present.

Table II—Decomposition Rate Constants of Aminosalicylic Acid in Presence of Moisture

Vapor Pressure, *P*, torr	Log *P*	Apparent Decomposition Rate Constant, ζ hr^{-1} (Eq. 18)
5.9	0.77	0.0031
7.4	0.87	0.0035
11.4	1.06	0.0045
17.6	1.25	0.0068
23.8	1.38	0.0088

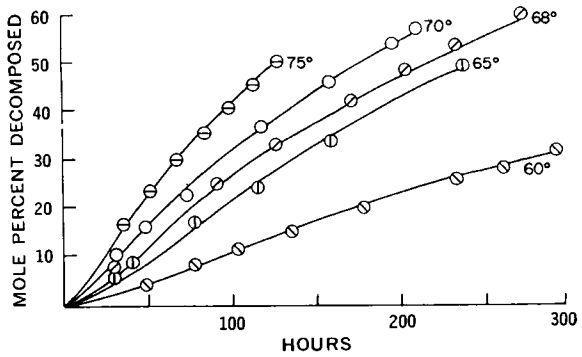

Figure 3—*Solid-state decomposition of aminosalicylic acid at various temperatures. Key:* ⊖, *75°;* O, *70°;* ⊘, *68°;* ⊕, *65°; and* ⊗, *60°.*

the analysis of the solid kinetic data where moisture is present. Forty grams of aminosalicylic acid was stored with 50 g of water in an erlenmeyer flask closed with a U-tube water trap at 65°. Samples of supernate were assayed spectrophotometrically at 300 and 230 nm (2) at various times. The contents of aminosalicylic acid and of *m*-aminophenol as a function of time are shown in Fig. 4.

The final composition approaches 2.6 moles (278 g) of *m*-aminophenol and 0.52 mole (80 g) of aminosalicylic acid per kilogram sample, *i.e.*, the solubility of aminosalicylic acid at 65° is 0.52 mole/642 g of water or 0.81 mole/kg of water. An order of magnitude estimation of the decomposition rate constant of aminosalicylic acid in solution can be made *via* $dC/dt = -k_2C$, *i.e.*, $k_2 = -(1/C)\, dC/dt$ in the following fashion. The first point on the aminosalicylic acid curve in Fig. 4 is 0.27 at 4 hr; so in the first 4 hr, C is 0.27 (or less). The first point on the *m*-aminophenol curve is 1.23, so that dC/dt is at least 1.23/4 = 0.308, so that k_2 is at least $k_2 = (1/0.27)\ 0.308 = 1.14\ \text{hr}^{-1}$. This compares fairly favorably with the value of 0.95 hr^{-1}, which will be referred to later.

To estimate the amount of moisture present as sorbed moisture on the solid itself, it is necessary to determine the moisture isotherm of the solid. This was done using the apparatus arrangement shown in Fig. 5 for determination of the isotherm at room temperature (27 ± 0.2°). All parts of the apparatus are Pyrex glass and all stopcocks are either vacuum stopcocks or hollow capillary stopcocks. The system was fused (by oxygen–gas flame) and all volumes were determined by helium gas expansion (10, 15).

Water was placed in the distillation bulb H and purified by repeated distillation between H′ and H as described earlier. Aminosalicylic acid was placed in the sample tube at J; all stopcocks were opened, and the system was thoroughly evacuated and the stopcocks were then closed. The mercury level in the McLeod gauge was then raised to the highest calibration mark (L).

Stopcocks C and F were then opened and the capillary space was

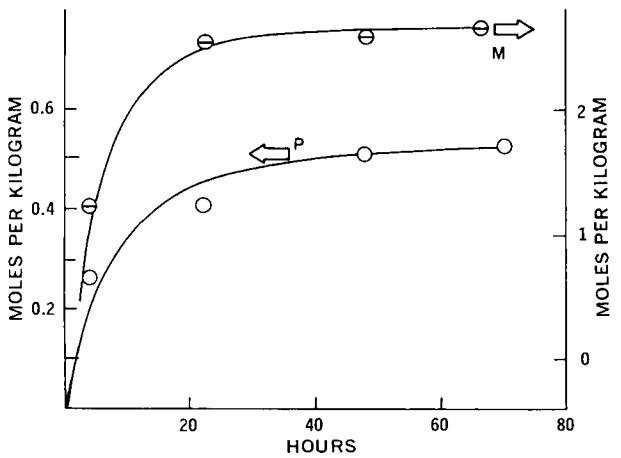

Figure 4—*Contents of aminosalicylic acid (P) and* m*-aminophenol (M) of aminosalicylic acid stored at 65° as a function of time. Key:* O, *aminosalicylic acid; and* ⊖, m*-aminophenol.*

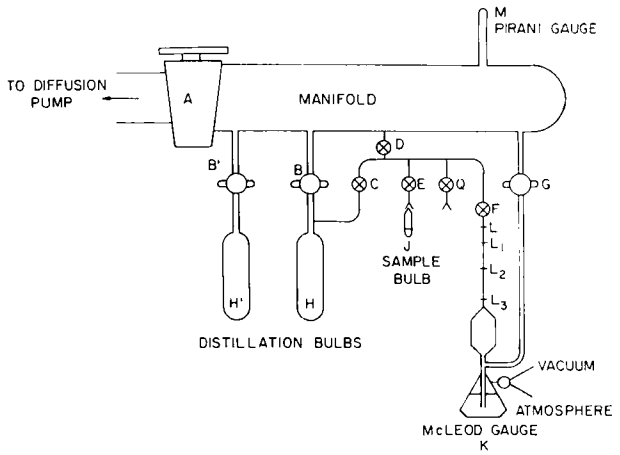

Figure 5—*Vacuum setup for determination of isotherms at room temperature.*

saturated with water vapor (4 hr), so the space between C and L would exhibit a water vapor pressure of 26.7 torr (saturation pressure of water at 27°) as registered on the McLeod gauge. Stopcock C was then closed and stopcock E was opened, and the vapor expanded into the head space over the sample.

Knowledge of the volume before and after the expansion permits calculation of the theoretical pressure (P_t) after opening stopcock E. The actual pressure (P) was measured on the McLeod gauge and was smaller than the theoretical. The difference ($\Delta P = P_t - P$) was converted into the number of moles of water, and this figure was divided by the number of moles of aminosalicylic acid in the sample to give the adsorbed amount (x) in moles per mole at equilibrium pressure P. The mercury level was then lowered to L_2, giving a new set of values for P_t, P, and x; a series of such values (giving the isotherm at 27°) is shown graphically in Fig. 6. A similar isotherm was performed for *m*-aminophenol.

The moisture isotherm at 60° presented a greater problem experimentally. The apparatus and approach outlined in Fig. 7 were used. A closed-end manometer with a side arm (EF) was made and fused onto ground-glass joints (A and B), and the manometer was loaded with mercury to the level shown. A sample of aminosalicylic acid was then placed in the noncapillary end of a breakseal tube;

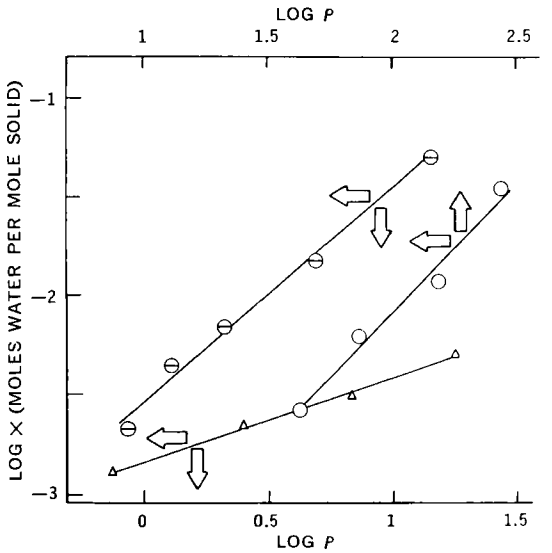

Figure 6—*Langmuir plots of moisture isotherms of aminosalicylic acid at 25°* (⊖) *and 65°* (O) *and of* m*-aminophenol at 25°* (△). *The least-squares fit equations are: aminosalicylic acid at 25°, log* x = *1.10 log* P − *2.05; aminosalicylic acid at 65°, log* x = *1.31 log* P − *3.90; and* m*-aminophenol at 25°, log* x = *0.33 log* P − *2.77.*

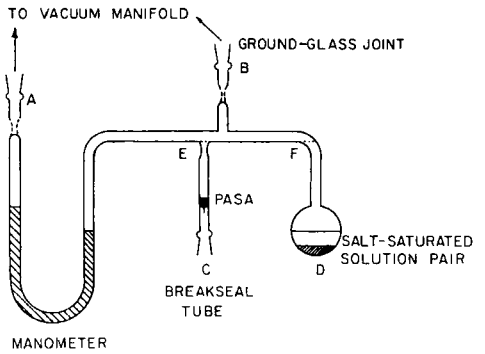

Figure 7—*Apparatus for determination of isotherms at 65°.*

this tube was fused onto the side arm at E and a bulb of water (D) was fused onto the side arm at F.

The assembly was then attached to a high vacuum rack at A and B (and checked for tightness with a Tesla coil). After evacuation the apparatus was sealed with an oxygen–gas flame at A and B, and the breakseal tube was narrowed down at E. The entire apparatus was then placed in a 60° oven[1] and, after equilibrium had been obtained (as ascertained by the mercury level being constant), the breakseal tube was sealed off with an oxygen–gas flame while the apparatus was still in the constant-temperature oven.

The breakseal tube was then inverted and attached to the vacuum rack at ground-glass joint Q (Fig. 5), and the total amount of water in the sample was determined on the McLeod gauge. Four salt-saturated solution pairs were used, producing the pressures shown in Table III.

For reasons that shall become apparent in the *Discussion* section, the effect of the size of the reaction vessel was investigated. The sample holder in most of this study had a volume of 7 ml. A kinetic study was performed at 65° with sample holders of 20 and 40 ml. The results are shown in Fig. 8.

The effect of oxygen on the decomposition was studied by exposing the aminosalicylic acid sample to 50 torr pressure of oxygen prior to sealing off the sample holder and storing it at 65°. The oxygen was prepared from potassium permanganate and manganese dioxide by the method of Sanderson (16). The results are shown in Fig. 8.

The effect of saturating the atmosphere above the aminosalicylic acid sample with mercury vapor was studied by blowing a side arm onto the sample holder and depositing a drop of mercury in the side arm. The holder was kept in an upright position during the decomposition so that there would be no contact between liquid mercury and the solid sample during this period. The results are shown in Fig. 8.

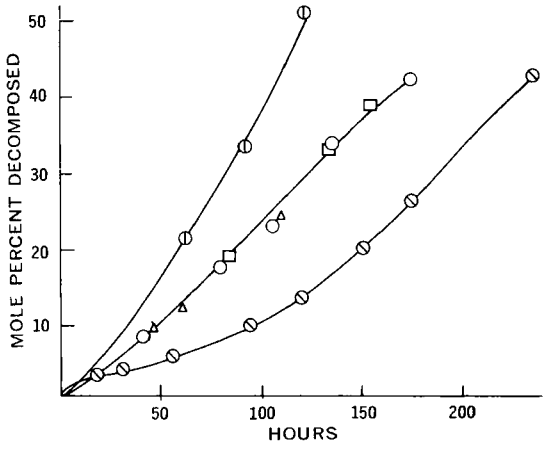

Figure 8—*Decomposition of aminosalicylic acid at 65°. Key:* ⊕, *in an atmosphere of saturated mercury vapor;* ⊘, *in an atmosphere of 50 torr pressure of oxygen;* ○, in vacuo *with a 7-ml sample holder;* △, in vacuo *with a 20-ml sample holder; and* □, in vacuo *with a 40-ml sample holder.*

[1] Model 1302, Hotpack Corp., Philadelphia, PA 19135

Table III—Salts Used in Contact with Their Saturated Solutions at 65° for Moisture Isotherms

Equilibrium Water Vapor Pressure at 65°, P, torr	Salt	Log P
148	$MgSO_4$	2.17
104	LiCl	2.02
44	$MgCl_2 \cdot 6H_2O$	1.64
26	$CaCl_2 \cdot 2H_2O$	1.41

The surface area of a series of samples was determined by BET nitrogen adsorption as described previously (10). The samples were stored for various times at 65°, and the surface area then was determined again. The results are shown in Table IV.

DISCUSSION

The decomposition of aminosalicylic acid in the solid state is a so-called Type I solid reaction—*viz.*, one where 1 mole of solid decomposes into 1 (or more) mole of a solid decomposition product and 1 (or more) mole of a gas (17). In Type I reactions, the decompositions are governed by strains in the crystal, induced by the formation of product molecules; this is referred to as nucleation (18, 19). As the strain increases, the original crystal may crack and form new surfaces upon which further decomposition can occur. Formation of the product molecules appears, at first, in the form of nuclei which are mainly located on the surface (18) or (rarely) inside the crystal (19).

Occurrence of the nuclei in rows suggests that they are confined to lines of strain in the crystal. Initiation of decomposition appears at the surface, but the decomposition progresses by two routes: *(a)* along the surface itself, and *(b)* by penetration into the crystal. An accepted and frequently substantiated theoretical model for such a decomposition mechanism is the model of Prout and Tompkins (7, 8). They assumed that there initially are N^* potential (energetic) sites for nucleation (dislocations, vacancies, surface steps, *etc.*) and that the nucleation rate is proportional to N, the number of nuclei present at time t; *i.e.*, $dN/dt = (\alpha)(N)$. The term α is a propagation probability; but at a certain point in the decomposition, the rows of nuclei start merging, so that there also is a termination probability, β, associated with the decomposition. This is expressed in the form:

$$dN/dt = [\alpha - \beta](N) \qquad \text{(Eq. 1)}$$

This cannot be integrated directly, since α and β are functions of time.

Most decompositions of Type I are sigmoid-shaped curves when decomposition is plotted *versus* time. Prout and Tompkins (7) noted that the inflection point usually occurs at $x = 0.5$ and reasoned that α must equal β at this point; they further assumed that $\beta = 0$ when $t = 0$ ($x = 0$) so that α and β could be interrelated by a function such as:

$$\beta = 2x\alpha \qquad \text{(Eq. 2)}$$

This makes β zero at time zero and $\alpha = \beta$ at the inflection point.

Table IV—Surface Area of Aminosalicylic Acid Samples after Storage at 65°

Time, t, hr	Fraction, x, Decomposed	Surface Area, A, m^2/g	Increase in Area, ΔA	$0.003t + \ln(\Delta A)$	$t-40$	$\ln(t-40)$
0	—	0.36	—	—	—	—
45	0.07	1.73	1.37	0.45	5	1.61
65	0.11	3.79	3.43	1.43	25	3.22
100	0.18	6.45	6.09	2.11	60	4.09
138	0.24	9.20	8.84	2.59	98	4.58

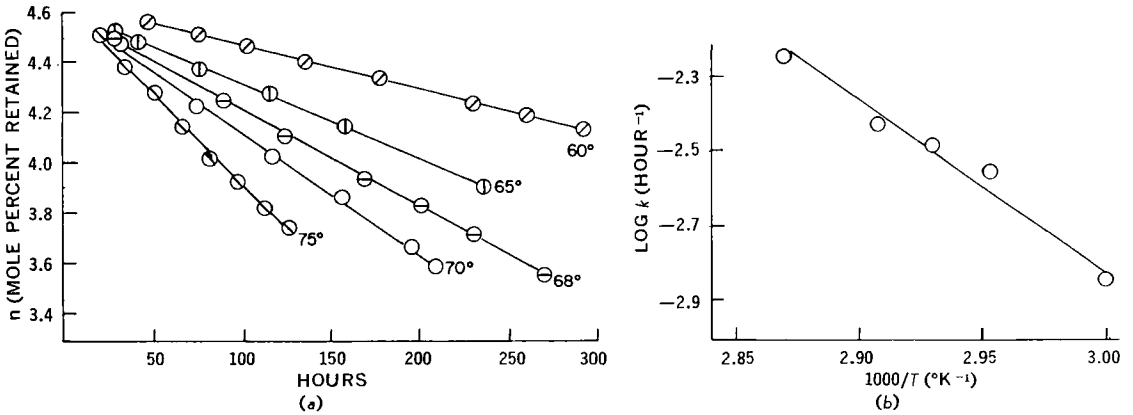

Figure 9—(a) *Data from Fig. 3 plotted according to Eq. 12.* (b) *Arrhenius plot of data from Fig. 9a.*

This reasoning is, of course, based on boundary conditions, and many functions [*e.g.*, $\beta = (2x)^2(\alpha)$] would satisfy the stated requirements as well.

Implementation of Eq. 2 and the assumption that:

$$dx/dt = k'N \qquad \text{(Eq. 3)}$$

has been shown (7) to lead to the sigmoid-shaped curve:

$$x/(1 - x) = \exp[k''(t - t_i)] \qquad \text{(Eq. 4)}$$

where t_i is the time at which the inflection point ($x = 0.5$) occurs.

It is noted from Fig. 3 that it is not justified, in the case reported here, to assume that the inflection point occurs at $x = 0.5$. An approach similar to the one used by Prout and Tompkins is therefore developed in the following section.

If a method existed for measuring N, it would be possible to test Eq. 1 directly. If one assumes that N is proportional to the increase in BET surface area, then such a method exists and this, indeed, is the purpose of following the BET surface area experimentally as a function of time (Table IV). In place of the assumptions made by Prout and Tompkins for α and β, it is assumed here that nucleation starts at some time t_0 (which is assumed to be larger than zero) with a propagation probability α_0 (<1) and that (as in the Prout–Tompkins treatment) $\alpha = \beta$ at $t = t_i$. At infinite time, the termination probability is $\beta = \beta_0$. A function that meets these requirements is:

$$\alpha - \beta = \frac{(\alpha_0 + \beta_0)(t_0 - q)}{t - q} - \beta_0 \qquad \text{(Eq. 5)}$$

where:

$$q = \frac{(\alpha_0 + \beta_0)t_0 - \beta_0 t_i}{\alpha_0} \qquad \text{(Eq. 6)}$$

Equation 6 may be rearranged to $\alpha_0(q - t_0) = \beta_0(t_0 - t_i)$; and since $t_0 - t_i < 0$, it follows that $q - t_0$ also is smaller than zero, *i.e.*, $t_0 > q > 0$.

Equation 6 inserted into Eq. 1 yields:

$$\frac{dN}{N} = \left(\frac{(\alpha_0 + \beta_0)(t_0 - q)}{t - q} - \beta_0\right)dt \qquad \text{(Eq. 7)}$$

which is integrated to:

$$\ln N = (\alpha_0 + \beta_0)(t_0 - q)\ln(t - q) - \beta_0 t + \ln Q \qquad \text{(Eq. 8)}$$

where $\ln Q$ is an integration constant. The nonlogarithmic form of this equation is:

$$N = Q(t - q)^{(\alpha_0 + \beta_0)(t_0 - q)}e^{-\beta_0 t} \qquad \text{(Eq. 9)}$$

which, inserted into Eq. 3, yields:

$$\frac{dx}{dt} = k'Q(t - q)^{(\alpha_0 + \beta_0)(t_0 - q)}e^{-\beta_0 t} \qquad \text{(Eq. 10)}$$

If $t_0 - q$ is small, then Eq. 10 takes the form:

$$\frac{dx}{dt} = k'Qe^{-\beta_0 t} \qquad \text{(Eq. 11)}$$

This is integrated to:

$$\begin{aligned} x &= 1 - e^{-\beta_0(t - t_0)} && t \geq t_0 \\ x &= 0 && t < t_0 \end{aligned} \qquad \text{(Eq. 12)}$$

The initial condition that $x = 0$ for $t < t_0$ has been invoked in going from Eq. 11 to Eq. 12 as has the boundary condition that $x = 1$ for $t \to \infty$; this latter implies that $k'(Q/\beta_0) = 1$. Equation 12 is a *first-order expression.* When t_0 is not close to q, then the expressions become more complicated. The treatment for this case is detailed in the *Appendix.* Data treated according to first-order presentation are graphed in Fig. 9*a*, and the slopes (β_0) are treated by Arrhenius plotting in Fig. 9*b*. The fits are good; β_0 at 65° is 0.003 hr^{-1}.

If the increase in surface area during decomposition is propor-

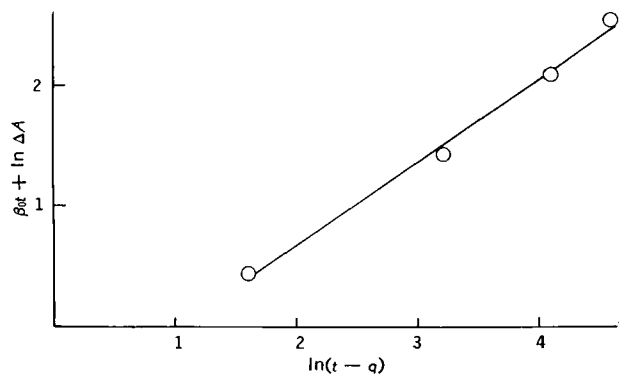

Figure 10—*Data from Table IV treated by Eq. 13 with* q = *40.*

Table V—Amounts of Moisture Adsorbed on the Samples Studied for Decomposition

P, torr	*x* (Fig. 6), moles Water/mole Amino-salicylic Acid	$V \times 10^3$, liters Water/mole Amino-salicylic Acid
5.9	0.063	1.14
7.4	0.081	1.46
11.4	0.131	2.36
17.6	0.211	3.80
23.8	0.294	5.30

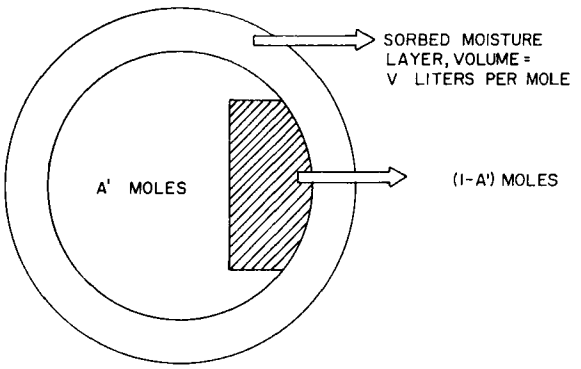

Figure 11—*Schematic drawing of the proposed model.*

tional to N, *i.e.*, $\Delta A = \lambda N$, then Eq. 8 becomes:

$$\ln(\Delta A) + \beta_0 t = (\alpha_0 + \beta_0)(t_0 - q)\ln(t - q) + \ln(Q/\lambda) \quad \text{(Eq. 13)}$$

The data in Table III were plotted in Fig. 10, using $\beta_0 = 0.003\ \text{hr}^{-1}$ and various values of q. The value $q = 40$ was found to linearize the surface area data best according to Eq. 13.

Although Prout and Tompkins (7) and Prout and Herley (8) found Eq. 4 to hold up to $x = 0.1$, they had to assume that the propagation constant had a form of k/t in the accelerative period; this then is an assumption over and above the ones stated. The amount and severity of assumptions made in the development of Eq. 12 are less. Once the order of reaction is established, the data reported here can be compared with data reported by other investigators (2, 20). The data are compared in the form $k = k_0 \exp(-E_a/RT)$, where E_a is the activation energy, R is the gas constant, and T is the absolute temperature.

From Ref. 20.

$$k = 4.3 \times 10^{14} \exp(-20{,}500/RT) \quad \text{(Eq. 14}a\text{)}$$

From Ref. 2:

$$k = 6.3 \times 10^{25} \exp(-41{,}000/RT) \quad \text{(Eq. 14}b\text{)}$$

And from this study:

$$\beta_0 = k = 3.2 \times 10^{10} \exp(-30{,}300/RT) \quad \text{(Eq. 14}c\text{)}$$

Although these expressions seem to differ to a substantial degree, the differences are not as great as the numbers in the equations would imply at first glance. For instance, 1 mole stored for 1 hr at 80° would show 0.986, 0.995, and 0.992 mole remaining by Eqs. 14*a*, 14*b*, and 14*c*, respectively.

It is instructive, however, to seek the cause of the differences. The data by Sheinker and Persiyanova (20) were generated in an atmosphere saturated with mercury vapor. Mercury has a vapor pressure of 0.07 and 0.2 torr at 75 and 90°, respectively; these are the principal temperatures of their study. The results obtained in the study reported here (Fig. 8) show that mercury vapor accelerates the decomposition of aminosalicylic acid and this constitutes one explanation why the rate constants of Ref. 20 in general are higher than the rate constants reported here. Their reaction vessel

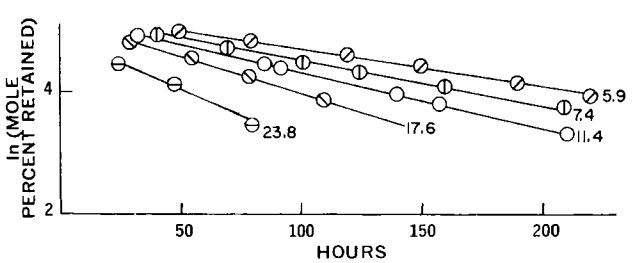

Figure 12—*Data from Fig. 2 plotted by Eq. 18.*

was (presumably) considerably larger than the one used here but, as shown in Fig. 8, this should not matter.

To find an explanation for the difference between the data of Kornblum and Sciarrone (2) and the results from this study, it should be noted that the former was not carried out *in vacuo, i.e.*, atmospheric oxygen and nitrogen were present in the headspace. For this reason, a study was carried out here with oxygen in the sample holder headspace.

Ermer (21) and Thoma (22) studied the decomposition of aminosalicylic acid in solution in the presence of oxygen and found a definite effect as compared to studies in the absence of oxygen. If there, indeed, is an oxidative effect, then the reaction rate would be expected to increase. The decomposition of solid aminosalicylic acid in the presence of oxygen (Fig. 8) does, surprisingly, not imply a straightforward increase in reaction rate but rather a rapid initial rate followed by a somewhat decreased rate in the accelerative period. The reason for this is not quite clear, but the following explanation may be feasible, although no attempts to substantiate the individual points are made here.

The rapid initial rate may be a result of an oxidative reaction of the molecules on the surface; eventually the particles are completely covered by the oxidation product, and this may retard the propagation rate and cause the subsequent decrease in reaction rate. The point is that the oxidative interference with the actual decomposition explains the small difference between the data by Kornblum and Sciarrone (2) and the data in this study. By virtue of some air being present, the experimental setup by Kornblum and Sciarrone is closer to real pharmaceutical systems than the model study reported here.

Leeson and Mattocks (1), in their work on aspirin decomposition, showed that in the presence of moisture the decomposition of the solid can be accounted for by considering the adsorbed moisture as a saturated aspirin solution. The aspirin decomposes in solution with a first-order rate constant, k'', and has a solubility of S. Knowing the volume, V, of moisture adsorbed allows calculation of a loss figure, and this value was found to be very close to the experimentally determined value.

Contrary to this, Kornblum and Sciarrone (2) found that for aminosalicylic acid, using a decomposition rate constant in solution of $k_2 = 1.986\ \text{hr}^{-1}$ (21), a sorbed moisture theory would lead to the conclusion that 80% of the solid would be in solution, a fact not substantiated by visual observation. The conclusion is based on the rate constants determined in solution by Ermer (21); even though better values for k_2 (differing from Ermer's by a factor of two) have been reported (23) subsequent to the appearance of the study by Kornblum and Sciarrone, their conclusion still remains valid. Kornblum and Sciarrone did not quantitate the amounts of moisture adsorbed and hence had to refrain from quantitating their observation. The work reported here attempts to accomplish this.

If the volume of moisture adsorbed on the solid follows a Freundlich moisture isotherm, as implied by Fig. 6, then:

$$x = K(P^{1/n}) \quad \text{(Eq. 15)}$$

or:

$$V = x/55.5 = K(P^{1/n}) \quad \text{(Eq. 16)}$$

where K and n are Freundlich parameters, x is moles of water adsorbed per mole of aminosalicylic acid, and V is kilograms of water adsorbed per mole of aminosalicylic acid.

The vapor pressures used in Table I (Fig. 2) can be converted to amount of moisture adsorbed by the equations shown in Fig. 6, and these values are shown in Table V. The samples used in the kinetic study at 65° are prepared at 25° and therefore are heated from 298 to 338°K. The samples are 0.3-g samples in a 7-ml tube. It can be shown by gas law application that, at the lowest pressure, at most 4.6×10^{-7} mole out of 1.32×10^{-4} mole of water adsorbed is lost to the vapor phase by the heating of the closed tube. At the highest pressure, at most 1.87×10^{-6} mole is lost to the vapor phase out of a total of 6.17×10^{-4} adsorbed. It is therefore assumed, in the following, that the volumes listed in Table VI are the volumes of water adsorbed onto the solid in the kinetic study.

The overall decomposition of aminosalicylic acid would be that

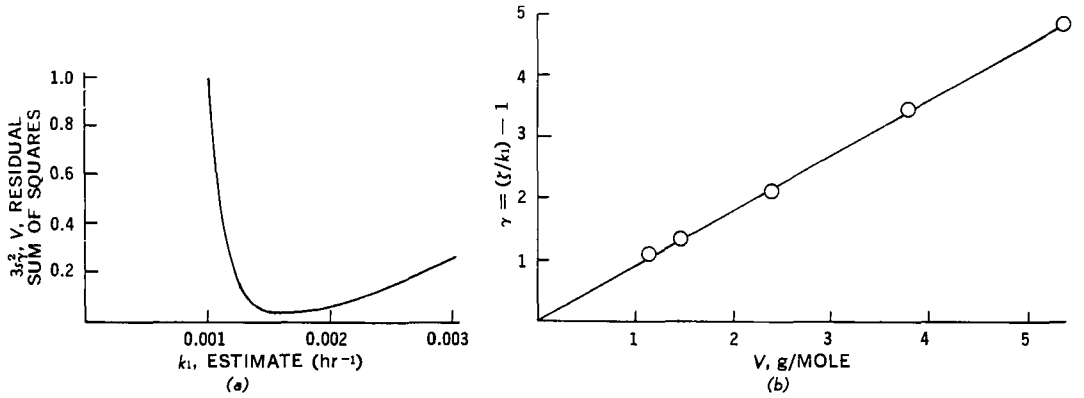

Figure 13—(a) *Sum of squares of residuals for data in Tables II and V plotted* versus *estimate of* k_1 *used in Eq. 19.* (b) *Data from Tables II and V plotted according to Eq. 19 with an estimate of* $k_1 = 0.0015\ hr^{-1}$.

of the solid and of the saturated solution if the mechanism is not diffusion controlled (24). The overall stoichiometry is the same in the solid state as in solution (Scheme II).

$$HOC_6H_3(NH_2)COOH \longrightarrow HOC_6H_4NH_2 + CO_2$$

Scheme II

The decomposition rate constant is k_1 in the solid and k_2 in solution in the sorbed moisture layer (Fig. 11). Starting with 1 mole at time zero, there will be A' moles of aminosalicylic acid left at time t; assuming that the moisture adsorbed to the *m*-aminophenol is *not* part of the "available" sorbed moisture layer[2] means that the amount in solution is $A'VS$, where V is kilograms of water adsorbed per mole of solid, and S is solubility of aminosalicylic acid in the liquid layers in moles per kilogram of water. The amount of solid aminosalicylic acid present is $A' - A'VS$. The decomposition rate then is given by:

$$-\frac{dA'}{dt} = k_1(A' - A'VS) + k_2A'VS \qquad \text{(Eq. 17)}$$

The solution to this equation is:

$$\ln A' = -k_1\{1 + [(k_2/k_1) - 1]VS\}t \qquad \text{(Eq. 18)}$$

Denoting the slope of a plot of ln A' *versus* t by $-\zeta$, *i.e.*:

$$\gamma = (\zeta/k_1) - 1 = [(k_2/k_1) - 1]VS \qquad \text{(Eq. 19)}$$

it is clear that a plot of γ *versus* V should give a straight line through the origin. The data from Fig. 2 are plotted according to Eq. 18 in Fig. 12, and linearity is good in all cases.

The value of k_1 is not necessarily identical to β_0; the moisture present on the surface may well dissolve the active sites. This is akin to the etch test for quantitative determination of numbers of dislocations on the surfaces of metals. These dissolve more rapidly and, therefore, by short exposure to acid, leave an etch mark. If the active sites are dissolved, the nucleation would be interfered with, leading to a smaller value for k_1 than for β_0 (although, in the presence of moisture, the overall decomposition rate is increased).

By denoting the quantity $[(k_2/k_1)-1]S$ by a, Eq. 19 has the form $\gamma = aV$. By using different values of k_1 to evaluate γ, different values of a will result. The least-squares fit formula $a = \Sigma\gamma V/\Sigma V^2$ was used; the residual sum of squares was computed for each estimate of k_1 and is shown graphically in Fig. 13*a*. It is obvious that the minimum in the sum of squares plot is for $k_1 = 0.0015$ hr^{-1}, *i.e.*, about one-half the value of β_0. The data plotted according to Eq. 19 by this estimate of k_1 are shown in Fig. 13*b*.

[2] If all the moisture is assumed to be available, then the expression:

$$\ln[(A' + \gamma)/(1 + \gamma)] = -k_1t$$

is arrived at. This equation gives a poorer fit than Eqs. 18 and 19.

The slope of this best least-squares fit is 0.9×10^3 moles/liter, *i.e.*:

$$[(k_2/0.0015) - 1]S = 0.9 \times 10^3 \qquad \text{(Eq. 20)}$$

It is, therefore, possible to calculate the solubility of aminosalicylic acid in water at 65° from a known value of k_2 (Table VI). The calculated values bracket the value found from the solid kinetic data; the data, hence, are not incompatible with the sorbed moisture theory of Leeson and Mattocks (1).

The average surface area per mole during a decomposition is $6.141 \times 10^4 = 8.5 \times 10^6$ cm^2. Noting that the largest moisture volume adsorbed is 5.3 ml and that, therefore, the moisture layer is at most $5.3/(8.5 \times 10^6)$ cm = 62 Å thick, it follows that there is at most 15 "layers" of moisture involved. This degree of moisture coverage is considerably smaller than that in previous investigations (1, 2); this has been done deliberately to achieve a moisture coverage more in line with what might be expected in a dosage form.

APPENDIX

In Eq. 10 the following substitutions are made:

$$(\alpha_0 + \beta_0)(t_0 - q) = m$$
$$u = t - q (du = dt,\ t = u + q)$$

followed by:

$$z = \beta_0 u (dz = \beta_0 du)$$

This gives:

$$dx = \{(k')(Q)(e^{-\beta_0 q})[\beta_0^{-(m+1)}]\}(z^m)(e^{-z})dz = M(z^m)(e^{-z})dz \qquad \text{(Eq. A1)}$$

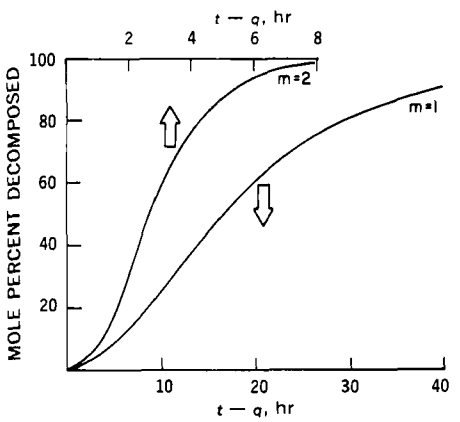

Figure 14—*Theoretical decomposition curves according to Eq. A4 with* m = *1 and* m = *2 and* $\beta_0 = 0.1\ hr^{-1}$.

Table VI—Solubilities of Aminosalicylic Acid (molal) at 65° Calculated from Eq. 20 Using Reported Values of Solution Decomposition Rate Constant (k_2, hr^{-1})

k_2, hr^{-1}	Reference	Solubility (Molal) from Eq. 20
1.99	21	0.68
0.95	23	1.42
>1.14	This study	<1.19

where $M = (k')(Q)(e^{-\beta_0 q})[\beta_0^{-(m+1)}]$ is used for convenience. If Eq. A1 is integrated from $t = t_0$ (or prior times) to infinity, $x = 1$ (total decomposition); $t_0 > q$, so the integration yields unity if carried from q to infinity; if $t = q$, $u = z = 0$, so integrating Eq. A1 from $z = 0$ to infinity gives:

$$1 = M\int_0^\infty (z^m)(e^{-z})dz = \Gamma(m+1) \qquad \text{(Eq. A2)}$$

so:

$$M = \Gamma^{-1}(m+1) \qquad \text{(Eq. A3)}$$

where $\Gamma(m+1)$ is the γ-function evaluated at $m+1$. Equation A1, by partial integration, can be shown to have the general solution:

$$x = 1 - \left(e^{-\beta_0(t-q)}\sum_0^m [\beta_0(t-q)]^{m-i}[m!/(m-i)!]\right) \qquad \text{(Eq. A4)}$$

when m is an integer. Examples of these types of curves, showing the typical S-shape, are shown for $m = 1$ and $m = 2$ in Fig. 14.

From Eq. 2 it is seen that the inflection point of these curves occurs when:

$$d^2x/dz^2 = 0 = M(e^{-z})[z^{(m-1)}](m-z) \qquad \text{(Eq. A5)}$$

i.e., when $z = \beta_0(t-q) = m$.

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Kinetics of Sulfite-Induced Anaerobic Degradation of Epinephrine

B. R. HAJRATWALA

Abstract □ The rate constant–pH profile of the rate of sulfite-induced disappearance of epinephrine from an aqueous solution under anaerobic conditions was determined at 81° in the 3.63–5.00 pH range at the ionic strength of 0.2. The profile shows a linear relationship with a positive slope. The anaerobic rate shows buffer catalysis above pH 4.4. Metabisulfite was found to be more catalytic than either bisulfite or acetone bisulfite. The concentration of epinephrine used was 5.5×10^{-5} mole/liter.

Keyphrases □ Epinephrine—kinetics of sulfite-induced anaerobic degradation □ Antioxidants—effect on epinephrine degradation, kinetics of sulfite-induced anaerobic degradation □ Degradation—epinephrine, sulfite induced, kinetics □ Stability—epinephrine, kinetics of sulfite-induced degradation □ Sulfites—role in anaerobic degradation of epinephrine, kinetics

Epinephrine degradation in aqueous solutions can occur *via* racemization or oxidation, either in the presence or absence of oxygen. Of various studies (1–5) on epinephrine degradation by racemization, only one (5) was a kinetic study and it was limited to the 0.05–1.40 pH range. The epinephrine concentration ranged from 0.05 to 0.3 mole/liter. The study shows that the racemization of epinephrine in acidic solutions is specific hydrogen-ion catalyzed.

The kinetics of bisulfite-induced degradation of epinephrine were studied (6–10), and the bimolecular kinetics of epinephrine with sulfite ion were established. The study was done in the 4–7 pH range. The kinetics of epinephrine degradation in solution by molecular oxygen are a complex function of epinephrine concentration and oxygen (11).

The degradation kinetics of epinephrine when present in other products, *e.g.*, lidocaine hydrochloride injection (12), have not been published. The purpose of this investigation was to study the anaerobic degradation of epinephrine in aqueous solution over the 3.63–5.00 pH range (this generally covers the range of USP XVIII products containing epinephrine) as a preliminary to stability studies on products containing epinephrine. The concentration of epinephrine used was 0.001% (5.5×10^{-5} mole/liter), the maximum allowed in lidocaine hydrochloride injection. The degradation of epinephrine was induced by sodium metabisulfite.

EXPERIMENTAL

Materials—All materials used were of analytically pure grade. These included epinephrine (the *l*-form), anhydrous sodium acetate, acetic acid, sodium chloride, sodium bisulfite, sodium metabisulfite, and sodium acetone bisulfite.

Standardization of Epinephrine—The epinephrine was standardized against primary standard epinephrine bitartrate USP using a spectrofluorometric method (12). It was found to contain 99.82% epinephrine.

Kinetic Studies—An accurately weighed quantity (approximately 10 mg) of epinephrine was added to a 1-liter amber-colored volumetric flask and dissolved in an appropriate buffer solution containing 0.05% sodium metabisulfite (sodium chloride added to desired ionic strength), which had been preheated to 81°. Type I glass vials (30 ml) were preheated to 81° and filled with approximately 30 ml each of epinephrine solution under anaerobic conditions (using nitrogen). The vials were tightly sealed with rubber stoppers[1] and aluminum seals and were then placed in a constant-temperature circulator bath previously adjusted to 81 ± 0.1°.

The vials were allowed to equilibrate thermally. At appropriate time intervals, vials were removed from the bath and chilled, and their contents were analyzed for epinephrine using the USP XVIII spectrofluorometric method (12). The pH of each sample was measured to ensure the constancy of pH during the entire procedure. The degradation followed first-order kinetics (Fig. 1). The apparent first-order rate constants (Table I) were calculated using re-

Table I—Effect of Acetate Buffer Concentration on Degradation of Epinephrine at 81° and Ionic Strength 0.2

pH	Buffer Concentration, mole/liter	$k \times 10^3$, hr^{-1}	$t_{1/2}$, hr
3.63	0.02	2.98	233
	0.03	2.78	250
	0.07	2.89	241
4.15	0.02	3.96	175
	0.03	4.13	168
	0.07	3.86	179
4.62	0.01	4.83	144
	0.03	5.01	138
	0.07	4.94	140
	0.10	4.90	141
5.00	0.03	6.08	114
	0.07	6.35	109
	0.10	6.70	103

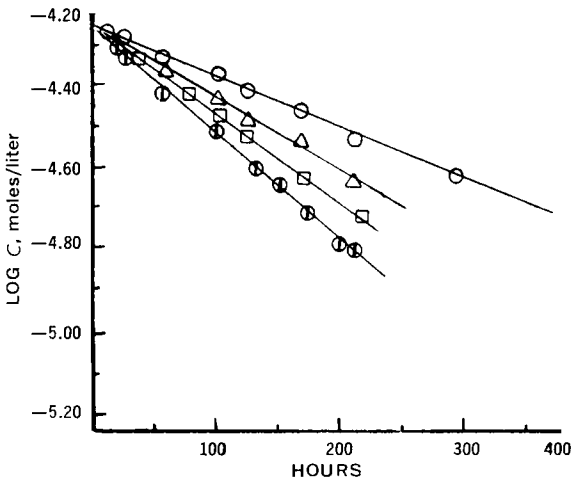

Figure 1—*Plot showing the overall first-order character of anaerobic degradation of epinephrine at various pH values in 0.03* M *acetate buffer at 81°. Key:* ○, *pH 3.63;* △, *pH 4.15;* □, *pH 4.62; and* ◐, *pH 5.00.*

[1] 1109 compound rubber stoppers, West Co., Phoenixville, PA 19460

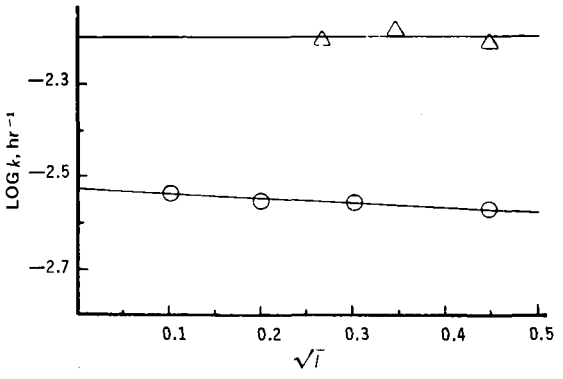

Figure 2—*Effect of ionic strength* (I) *on the degradation of epinephrine at 81° in 0.1* M *acetate buffers at various pH values. Key:* ○, *pH 3.63; and* △, *pH 5.00.*

gression analysis and 5–10 points. Correlation coefficients were between −0.918 and −0.999.

Effect of Ionic Strength—The effect of ionic strength was investigated at pH 3.63 and 5.00, using 0.1 *M* acetate buffers adjusted to ionic strengths of 0.01, 0.04, 0.07, 0.09, 0.12, and 0.20 by addition of sodium chloride (Fig. 2). The concentration of metabisulfite used was 0.05%.

Effect of Various Antioxidants—The antioxidants used were sodium bisulfite, sodium metabisulfite, and sodium acetone bisulfite in concentrations of 2.63×10^{-3} mole/liter in 0.03 *M* acetate buffer at pH 4.63 and ionic strength of 0.02 (Table II).

Degradation in Acetate Buffers—The degradation of epinephrine was studied in buffer containing varying concentrations of acetate at pH 3.63, 4.15, 4.62, and 5.00 at constant ionic strength of 0.2. Figure 3 shows the relationship between buffer concentration and the rate of degradation of epinephrine. The concentration of metabisulfite used was 0.05%.

pH–Rate Constant Profile—Table III shows the effect of hydrogen-ion concentration. The first-order rate constants (k_0) were obtained by extrapolation from Fig. 3 at zero buffer concentration. The pH–rate constant profile is shown in Fig. 4.

RESULTS AND DISCUSSION

At pH 5.00, the rate constant is independent of ionic strength. This indicates either the reaction of positive or negative ions with a neutral molecule or a unimolecular reaction of a positive, negative, or neutral molecule. At pH 3.63, the rate constant decreases with increasing ionic strength with a slope much less than 1 (approximately 0.1). A negative slope of less than 1 suggests that the reaction of ions of unlike sign is not dominating and that other reactions are occurring at this pH.

Table II shows that metabisulfite-induced degradation is greater than that for bisulfite or acetone bisulfite at pH 4.63. Riegelman and Fischer (13) showed that boric acid, which chelates with the catechol nucleus of epinephrine, protects it from inactivation by sulfite (6). Unfortunately, boric acid is toxic and cannot be used in injectables. Hence, sulfites continue to be used as antioxidants in marketed epinephrine-containing products (14), although they are far from ideal.

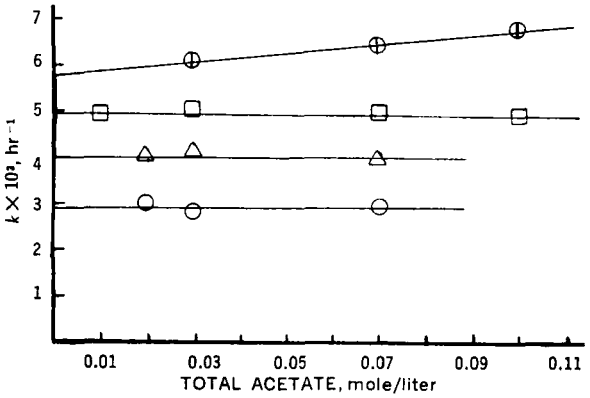

Figure 3—*Effect of acetate concentration on the rate of degradation of epinephrine at 81° at various pH values and ionic strength of 0.2. Key:* ○, *pH 3.63;* △, *pH 4.15;* □, *pH 4.62; and* ⊕, *pH 5.00.*

Table II—Effect of Various Antioxidants (2.63×10^{-3} mole/liter) on Anaerobic Degradation of Epinephrine in 0.03 *M* Acetate Buffer, pH 4.73, at 81° and Ionic Strength 0.02

Antioxidant	$k \times 10^3$, hr^{-1}	$t_{1/2}$, hr
Sodium bisulfite	3.81	182
Sodium acetone bisulfite	3.97	175
Sodium metabisulfite	4.92	141

The effect of total acetate concentration on the rate of degradation of epinephrine is shown in Fig. 3. The total acetate concentration is:

$$[Ac]_T = [HOAc] + [OAc^-] \qquad \text{(Eq. 1)}$$

where [HOAc] is undissociated acetic acid and [OAc^-] is dissociated acetic acid. The rate constants shown in Table I were obtained from log concentration *versus* time plots at each buffer concentration.

Only at pH 5.00 does increasing total acetate concentration increase the rate of degradation of epinephrine. Using the Harned and Owen equation (15), the pKa for acetic acid was calculated as 4.88 at 81°. The epinephrine pKa is 8.55 (16). Since this value is far removed from the pH range used in this study, the temperature effect on pKa of epinephrine was ignored.

In the pH range studied, epinephrine exists almost entirely as the positively charged (EpH^+) species. At pH 4.62 and below, neither the HOAc nor the OAc^- species is catalytic to the EpH^+ species. At pH 5.00, acetate (probably OAc^-) appears to be catalytic to the EpH^+ species.

The pH–rate constant profile is a valuable way of presenting kinetic data for reactions that are pH sensitive, *e.g.*, degradation of epinephrine. The pH–rate constant relationship of Table III can be expressed by the linear equation:

$$k_0(hr^{-1}) = 2.10 \times 10^{-3}pH - 4.76 \times 10^{-3} \qquad \text{(Eq. 2)}$$

with a standard error of 3.7×10^{-5}.

In considering the effect of pH on the degradation of epinephrine, the following reactions are taken into account:

$$Ep \xrightarrow{k_1} \text{products} \qquad \text{(Reaction 1)}$$

$$Ep + H^+ \xrightarrow{k_2} \text{products} \qquad \text{(Reaction 2)}$$

$$Ep + OH^- \xrightarrow{k_3} \text{products} \qquad \text{(Reaction 3)}$$

$$EpH^+ \xrightarrow{k_4} \text{products} \qquad \text{(Reaction 4)}$$

$$EpH^+ + H^+ \xrightarrow{k_5} \text{products} \qquad \text{(Reaction 5)}$$

$$EpH^+ + OH^- \xrightarrow{k_6} \text{products} \qquad \text{(Reaction 6)}$$

where Ep is unprotonated base and EpH^+ is protonated base, and:

$$Ep + H_2A \xrightarrow{k_7} \text{products} \qquad \text{(Reaction 7)}$$

$$Ep + HA^- \xrightarrow{k_8} \text{products} \qquad \text{(Reaction 8)}$$

$$Ep + A^{-2} \xrightarrow{k_9} \text{products} \qquad \text{(Reaction 9)}$$

$$EpH^+ + H_2A \xrightarrow{k_{10}} \text{products} \qquad \text{(Reaction 10)}$$

$$EpH^+ + HA^- \xrightarrow{k_{11}} \text{products} \qquad \text{(Reaction 11)}$$

$$EpH^+ + A^{-2} \xrightarrow{k_{12}} \text{products} \qquad \text{(Reaction 12)}$$

Table III—Catalytic Effect of Hydrogen Ion on Degradation of Epinephrine at 81° and Ionic Strength 0.2[a]

pH	$k_0 \times 10^3$, hr^{-1}	$k_{calc} \times 10^3$, hr^{-1}
3.63	2.85	2.867
4.15	4.00	3.960
4.62	4.92	4.947
5.00	5.75	5.746

[a] The k_0 values were obtained by extrapolation from Fig. 3; the k_{calc} values were obtained using Eq. 2.

where H_2A, HA, and A^{-2} are undissociated, monodissociated, and didissociated species of sulfurous acid, respectively. Kinetically, Reactions 1 and 6, 2 and 4, 7 and 11, and 8 and 12 are equivalent and cannot be distinguished from each other. Thus, Reactions 1, 2, 3, 5, 7, 8, 9, and 10 are considered. The overall velocity of the reaction should be equal to the sum of the rates of these reactions:

$$-\frac{d(Ep)_T}{dt} = k_1(Ep) + k_2(Ep)(H^+) + k_3(Ep)(OH^-) + k_5(EpH^+)(H^+) + k_7(Ep)(H_2A) + k_8(Ep)(HA^-) + k_9(Ep)(A^{-2}) + k_{10}(EpH^+)(H_2A) \quad \text{(Eq. 3)}$$

Because of the overall first-order character of the reaction:

$$-\frac{d(Ep)_T}{dt} = k_{obs}(Ep)_T \quad \text{(Eq. 4)}$$

combining Eq. 4 and:

$$K_a^e = \frac{(H^+)(Ep)}{(EpH^+)} \quad \text{(Eq. 5)}$$

$$K_{a_1}^s = \frac{(H^+)(HA^-)}{(H_2A)} \quad \text{(Eq. 6)}$$

$$K_{a_2}^s = \frac{(H^+)(A^{-2})}{(HA^-)} \quad \text{(Eq. 7)}$$

and substituting in Eq. 3 gives:

$$k_{obs} = \frac{k_1K_a^e}{(K_a^e + H^+)} + \frac{k_2K_a^e(H^+)}{(K_a^e + H^+)} + \frac{k_3K_a^eK_w/(H^+)}{(K_a^e + H^+)} + \frac{k_5(H^+)^2}{(K_a^e + H^+)} + \frac{k_7K_a^eA_T(H^+)^2}{(K_a^e + H^+)[(H^+)^2 + K_{a_1}^sH^+ + K_{a_1}^sK_{a_2}^s]} + \frac{k_8K_a^eK_{a_1}^sA_T(H^+)}{(K_a^e + H^+)[(H^+)^2 + K_{a_1}^sH^+ + K_{a_1}^sK_{a_2}^s]} + \frac{k_9K_a^eK_{a_1}^sK_{a_2}^sA_T}{(K_a^e + H^+)[(H^+)^2 + K_{a_1}^sH^+ + K_{a_1}^sK_{a_2}^s]} + \frac{k_{10}(H^+)^3A_T}{(K_a^e + H^+)[(H^+)^2 + K_{a_1}^sH^+ + K_{a_1}^sK_{a_2}^s]} \quad \text{(Eq. 8)}$$

where $A_T = (H_2A) + (HA^-) + (A^{-2})$, $(Ep)_T = (EpH^+) + (Ep)$, and K_w = autoprotolysis constant.

For the pH range studied, $H^+ \gg K_a^e$ and $K_{a_2}^s \ll H^+ \ll K_{a_1}^s$ [$K_{a_1}^s = 1.54 \times 10^{-2}$ and $K_{a_2}^s = 1.02 \times 10^{-7}$ (19)]. Thus, Eq. 8 reduces to:

$$k_{obs} = a + b(H^+) + c/(H^+) + d/(H^+)^2 \quad \text{(Eq. 9)}$$

where $a = k_2K_a^e + (k_7K_aA_T/K_{a_1}^s)$, $b = k_5 + (k_{10}A_T/K_{a_1}^s)$, $c = k_1K_a^e + k_8K_a^eA_T$, and $d = k_3K_a^eK_w + k_9K_a^eK_{a_1}^sA_T$. However, Eq. 9 does not fit the experimental data.

There are three possible reasons for the apparent failure of the proposed mechanism to fit the experimental results:

1. The mechanism is more complex than the one proposed, probably involving free radicals.

2. The proposed mechanism is incorrect and the various assumptions made in proposing the set of 12 reactions are incorrect. However, this is not very likely; the complex degradation of morphine and ascorbic acid was successfully described on the basis of similar principles (18, 19).

3. The mechanism may hold good, but over the narrow pH range the polynomial (Eq. 9) has an approximately linear segment.

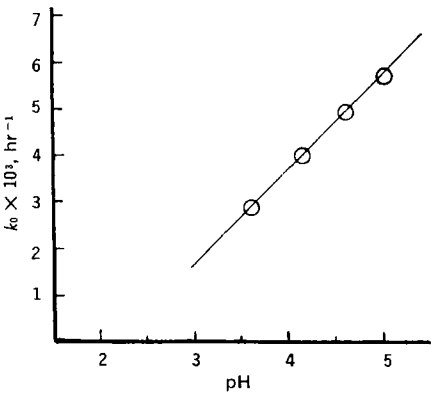

Figure 4—*The pH–rate constant profile for sulfite-induced epinephrine degradation in aqueous solution at 81° and ionic strength of 0.2.*

SUMMARY AND CONCLUSION

The pH dependence of sulfite-induced anaerobic degradation of epinephrine was determined at 81° over the 3.63–5.00 pH range at the ionic strength of 0.2. The rate of disappearance of epinephrine was obtained by determining the concentration of unreacted epinephrine *versus* time using the USP XVIII spectrofluorometric method. An apparent first-order rate of degradation was observed.

The primary salt effect was almost zero at pH 3.63 and 5.00. Acetate ions, undissociated and monodissociated, are not catalytic to protonated epinephrine at pH 4.62 and below. At pH 5.00, monodissociated acetate ions appear to be catalytic to protonated epinephrine species. The pH–rate constant profile shows a linear relationship in the pH regions studied (Eq. 2).

The pH–rate constant profile is not readily explained on a mechanism based on a set of 12 reactions (among species present).

The effects of various antioxidants show that metabisulfite is more catalytic to epinephrine degradation than bisulfite or acetone bisulfite.

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Solubility of Nonelectrolytes in Polar Solvents III: Alkyl *p*-Aminobenzoates in Polar and Mixed Solvents

S. H. YALKOWSKY ˣ, G. L. AMIDON *, G. ZOGRAFI *, and G. L. FLYNN ‡

Abstract □ The relative solubilities of *n*-alkyl *p*-aminobenzoates in water, propylene glycol–water mixtures, propylene glycol, and several other pharmaceutically important solvents can be predicted on the basis of a theoretical equation. This equation relates the activity coefficient of the hydrophobic portion of the molecule to the product of its surface area and its interfacial tension [free energy per unit area of a hydrocarbon (tetradecane) against the polar or semipolar solvent of interest]. The assumptions, conclusions, and applicability of the theoretical relationship are compared to those of the Scatchard–Hildebrand approach.

Keyphrases □ *p*-Aminobenzoates, alkyl—solubility in polar and mixed solvents, equation developed for predicting solubility □ Alkyl *p*-aminobenzoates—solubility in polar and mixed solvents, equation developed for predicting solubility □ Solubility—alkyl *p*-aminobenzoates in polar and mixed solvents, equation developed for predicting solubility, compared to Scatchard–Hildebrand approach □ Solvents, polar—solubility of nonelectrolytes (alkyl *p*-aminobenzoates)

From a pharmaceutical point of view, the most important physical-chemical property of a substance is its aqueous solubility. In addition to designating the maximum concentration (blood level) attainable for a drug, aqueous solubility is a dominant factor in partitioning and adsorption onto biological surfaces. Solubility in water-miscible polar solvents and in mixed aqueous solvents is also of great potential utility in the design of parenteral, topical, and liquid vehicles for drugs.

The ability to predict the effects of even simple structural modifications or vehicle modifications on solubility can be of great value in the design of improved drugs and drug delivery systems. Theoretical descriptions of solubility have mainly been restricted to either nonpolar solutes in nonpolar solvents (1–4) or to salts and other highly polar solutes in water (5) and are thus not directly applicable to either aqueous (or polar) solvents of pharmaceutical interest. Several empirical correlations between structure and aqueous solubility have been published (6–8) but have not received wide acceptance.

Recently, the authors (9, 10) applied an "interfacial" model to the solubilities of aliphatic alcohols and hydrocarbons in water. This model equates the combined attractive and repulsive forces between the hydrocarbon portion of the molecule and water with the product of the molecular surface area and the free energy per unit area (the latter being related to the curvature corrected hydrocarbon–water interfacial tension). It has been used successfully for primary, secondary, tertiary, linear, branched, and cyclic alcohols and hydrocarbons (10) and also for other liquid series[1]. It is also applicable to series whose members are crystalline provided that the ideal solubility (determined from thermal data) is taken into account.

THEORETICAL

In an ideal solution, the solute–solute and solvent–solvent interactions are equivalent to the solute–solvent interactions, and there is no change in heat or volume on mixing. Thus, the only thermodynamic factor affecting solubility is the entropy of mixing, which results in infinite miscibility or a mole fractional solute solubility (X_2) of unity. This is frequently written as:

$$-\log (X_2)^{\text{ideal}} = 0 \qquad \text{(Eq. 1)}$$

If the solute is a solid, the crystal lattice energy opposes the solution process. The magnitude of this effect on solubility is approximately:

$$-\log (X_2)^{\text{ideal}} = \frac{\Delta H_f}{2.303R} \frac{(T_f - T)}{T_f T} \qquad \text{(Eq. 2)}$$

where ΔH_f is the molar heat of fusion of the crystal having an absolute melting point of T_f, R is the gas constant, and T is the absolute temperature. At the melting point, where the solute becomes a liquid, $(T_f - T)$ vanishes and Eq. 2 becomes Eq. 1.

Virtually all pharmaceutically important solutes have aqueous and polar solvent solubilities well below their ideal values. For these solutes, the deviation from ideality is described by an activity coefficient (ac) defined so that:

$$-\log X_2^{\text{exp}} = \log X_2^{\text{ideal}} + \log (ac) \qquad \text{(Eq. 3)}$$

The activity coefficient reflects the sum of: *(a)* the work required to remove a solute molecule from its surrounding of other solute molecules, W_{22}; *(b)* the work required to create a cavity in the sol-

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vent large enough to contain a solute molecule, W_{11}; and *(c)* the work gained on the insertion of the solute molecule into the cavity, W_{12}. Mathematically, it can be shown that (1, 2):

$$-\log(ac) = (W_{22} + W_{11} - 2W_{12})\frac{V_2\phi_1^2}{2.303RT} \qquad \text{(Eq. 4)}$$

where V_2 is the solute partial molal volume, and ϕ_1 is the solvent volume fraction. For dilute solutions, ϕ_1 and ϕ_1^2 can be approximated by unity.

Systems for which the size and polarity of the solute and solvent do not differ greatly are termed regular solutions. For these regular solutions, the term W_{12} (which is difficult to evaluate) can be accurately approximated by the geometric mean of W_{11} and W_{22} (which are easily measured):

$$W_{12} = \sqrt{W_{11}W_{22}} \qquad \text{(Eq. 5)}$$

and Eq. 4 becomes:

$$\log(ac) = -(W_{11}^{1/2} - W_{22}^{1/2})^2\frac{V_2}{2.303RT} \qquad \text{(Eq. 6)}$$

for dilute solutions. The square roots of the work terms are frequently designated as solubility parameters, δ_1 and δ_2. Thus:

$$\log(ac) = -(\delta_1 - \delta_2)^2\frac{V_2}{2.303RT} \qquad \text{(Eq. 7)}$$

For aqueous or polar solvent solutions of most drugs, the geometric mean approximation (Eq. 5) is not valid and Eqs. 6 and 7 are thus not applicable. However, Eq. 4 is still valid and would be useful if it were possible to measure W_{12} (or δ_{12}) conveniently.

In a previous publication (10), the authors discussed a two-dimensional analogy to Eq. 4, where W_{11}, W_{12}, and W_{22} are replaced by the surface and interfacial tensions γ_1, γ_2, and γ_{12} and the partial molal volume is replaced by molar surface area A_2.

Mathematically, the work required to remove a solute molecule from a bulk phase is equal to the surface area created times the surface tension of the liquid. (Curvature corrections will be discussed later.) Since removal of a single molecule from a bulk liquid phase does not measurably change the bulk phase surface area, it is only necessary to consider half of the work of cohesion:

$$\tfrac{1}{2}WC = \gamma_2 A_2 \qquad \text{(Eq. 8)}$$

Likewise, the work required to create a cavity in the solute phase capable of accommodating the solute is equal to half the solvent work of cohesion:

$$\tfrac{1}{2}WC = \gamma_1 A_2 \qquad \text{(Eq. 9)}$$

Finally, the work involved in the insertion of the solute molecule into the solvent cavity is given by the work of adhesion between the two species:

$$WA = -(\gamma_1 + \gamma_2 - \gamma_{12})A_2 \qquad \text{(Eq. 10)}$$

These three steps, which are strictly analogous to those used in deriving Eq. 4, can be summed and used to give:

$$\log(ac) = -\frac{\gamma_{12}A_2}{2.303RT} \qquad \text{(Eq. 11)}$$

The main advantage of using the two-dimensional model over the more commonly used three-dimensional one is that, for the systems of interest, the term γ_{12} is experimentally measurable whereas δ_{12} is not. Interestingly, the interfacial tension is difficult to measure for substances of similar polarity where the geometric mean rule is valid; conversely, Eq. 11 is useful for situations where Eq. 7 is inapplicable so that the two equations are complimentary rather than competitive.

Equation 11 can be obtained more intuitively by a modification of standard cavity theories of solubility. In general, these theories consider the energy required to create a cavity or hole in the solvent and then add a solute–solvent interaction term. The use of the interfacial rather than the surface tension eliminates the need to use a specific interaction term because γ_{12} is already dependent upon solute–solvent interactions.

Another means of obtaining Eq. 11 utilizes the relationship (1) that $\gamma A = \delta^2 V$ which, when substituted into Eq. 4, gives:

$$-\log(ac) = \frac{(\gamma_1 + \gamma_2 - 2\gamma_{12})A}{2.303RT} \qquad \text{(Eq. 12)}$$

for dilute solutions. With Antanow's rule, which states that $\gamma_1 + \gamma_2 - \gamma_{12} = 0$, Eq. 11 derives directly from Eq. 4. This derivation is not to be preferred because it relies upon the geometric mean rule and Antanow's rule, both of which are not generally valid. It is presented only to illustrate the parallelism between the two approaches.

To make Eq. 11 applicable to solutions of nonelectrolytes in polar solvents, consider how both γ_{12} and A_2 vary with structure. It is convenient to consider, as did Langmuir (11), the aliphatic and nonaliphatic portions of the solute separately. The total surface area A_2 of each solute is then equal to the area of the polar (*p*-aminobenzoate) moiety A_p plus the area of the aliphatic hydrocarbon chain A_h:

$$A_2 = A_p + A_h \qquad \text{(Eq. 13)}$$

When using this relationship, Eq. 11 becomes:

$$\log(ac) = \frac{\gamma_{1p}A_p + \gamma_{1h}A_h}{2.303RT} \qquad \text{(Eq. 14)}$$

where γ_{1h} is the microscopic aliphatic hydrocarbon–solvent interfacial tension, and γ_{1p} is an analogous two-dimensional term dependent upon the interactions between the solvent and the polar portion of the solute. These terms are similar to those first proposed by Langmuir (11) in his principle of independent surface activity.

If γ_{1p} and A_p are both independent of the size of the alkyl group, the change in activity coefficient with homologation would be expected to be directly proportional to the change in the hydrocarbon surface area A_h. The quantity $\gamma_{1p}A_p$ could then be determined from the intercept at A_p. These values are presently under study for a large number of polar groups. However, the present paper considers only changes in the hydrophobic portion of semipolar solutes with particular emphasis on normal aliphatic chains [branched chains were considered previously (10)].

The method of calculating the surface area of a molecule has been described in detail (10, 12). Briefly, the calculation involves determining the most likely molecular conformation of the atomic centers and then determining the areas from the van der Waals radii of the atoms and the solvent. Overlapping areas and areas not accessible to solvent are not included.

In the calculation of the interfacial tension, two factors must be considered: *(a)* the dependence of γ_{12} on the hydrocarbon moiety, and *(b)* its dependence on both the high curvature that exists at the microscopic interface surrounding a single molecule and the density difference associated with the change from a macroscopic to microscopic interface. Studies (13–15) have shown that hydrocarbon–water interfacial tension, although definitely dependent on hydrocarbon structure, falls within a narrow range and is not greatly affected by branching. Some selected values are listed in

Table I—Selected Hydrocarbon–Water Interfacial Tensions

Hydrocarbon	Interfacial Tension Reported		
	Ref. 14	Ref. 15	Ref. 13
n-Pentane	49.0[a]	—	—
	50.2[a]		
2-Methylbutane	49.64[a]	—	—
n-Hexane	50.8[a]	51.1	49.5
	51.25[a]		
Cyclohexane	—	51.2	—
n-Octane	51.7[a]	50.8	—
	50.8[a]		
	51.68[a]		
2,2,4-Trimethylpentane	50.1[a]	—	—
n-Decane	—	51.2	—
Decalin	—	51.4	—
Tetradecane	—	52.2	51.9
White mineral oil	—	51.3	—

[a] These are literature values gathered by Pomerantz *et al.* (14).

Table II—Logarithms of Mole Functional Solubilities of Alkyl *p*-Aminobenzoates in Various Solvents

Solvent[a]	Ester (Surface Area)				
	Ethyl (391.5 Å²)	Butyl (455.2 Å²)	Hexyl (518.8 Å²)	Octyl (582.4 Å²)	Dodecyl (709.6 Å²)
Ideal: 25°	−0.619	−0.406	−0.669	−0.933	−1.731
37°	−0.490	−0.239	−0.426	−0.680	−1.438
Water: 25°	−4.060	−4.378	−5.252	−7.647	
37°	−3.734	−4.508	−5.715	−8.124	−9.544
Methanol	−0.950	−0.519	−1.039	−1.729	−2.798
Ethanol	−1.080	−0.615	−1.027	−1.509	−2.370
Ethylene glycol	−1.588	−1.576	−2.374	−3.139	−4.750
Propylene glycol, 37°	−1.161	−0.710	−1.153	−1.688	−2.968
Glycerin	−1.952	−2.660	−3.494	−4.520	−6.733
Formamide	−1.671	−1.826	−2.656	−3.466	−5.063
N-Methylformamide	−0.695	−0.542	−0.678	−1.168	−2.388
N,*N*-Dimethylformamide	−0.371	−0.242	−0.359	−0.530	−2.113
Hexane, 37°	−2.778	−2.222	−2.285	−2.495	−3.015
Silicone oil, 37°	−1.005	−0.721	−0.890	−1.138	−1.771

[a] All data for 25° unless otherwise indicated.

Table I, from which it is seen that 50 dynes/in. is a good representative value. Zografi and Yalkowsky (13) made similar observations for all other solvents used in this study.

A water molecule at a highly convex surface is in contact with more water molecules than if it were at a planar surface. Since some of these neighboring molecules are on the hydrocarbon side of a plane tangent to the interface, there are stronger inward (toward the hydrocarbon) forces than there are at a flat surface. These inward forces counterbalance some outward attractive forces and thus reduce the interfacial tension. Also, a bulk interfacial tension involves void spaces in the hydrocarbon phase, which cannot be present at the microscopic interface as it has been defined here.

On this basis, Eq. 11 is modified to give:

$$\log(ac) = \frac{-C\gamma_{1h}A_h}{2.303RT} \qquad \text{(Eq. 15)}$$

where C is a curvature correction factor [due cognizance is given to the complex nature of the molecular interface in defining C (10)].

The important feature of Eq. 15 is that if C is a constant, the bulk hydrocarbon–solvent interfacial tension is a meaningful parameter with which to correlate solubility. The exact value of C may be dependent upon the size of the solvent molecule and the degree of curvature (16, 17). However, since the solvents being considered do not vary greatly in size, this variation will be ignored in the present paper.

From Eq. 15, one would expect the ability of each member of a group of solvents to solubilize a methylene group, *i.e.*, the activity coefficient of a methylene group, to be directly proportional to the hydrocarbon–solvent interfacial tension. This could be extended to mixed solvents, provided that there is no preferential localization of either component at the bulk or molecular interface.

EXPERIMENTAL

Materials—The *p*-aminobenzoate esters studied were selected from those used in Ref. 18. All solvents were of reagent grade and were used as received. The water was deionized.

Solubility Measurements—Solutions containing excess solid were allowed to equilibrate for 3 days with mild agitation. They were then filtered through a thermally equilibrated filter, diluted with methanol or hexane, and read spectrophotometrically.

Table III—Logarithms of Mole Fractional Activity Coefficients of Alkyl *p*-Aminobenzoates in Various Solvents

Solvent[a]	Ester					Slope, $\Delta \log(ac)$ / Δn
	Ethyl	Butyl	Hexyl	Octyl	Dodecyl	
Water	−3.441	−4.472	−5.583	−6.654	—	−0.537
Water, 37°	−3.245	−4.269	−5.299	−6.444	−8.106[b]	−0.525
Methanol	−0.331	−0.114	−0.370	−0.737	−1.068	−0.089
Ethanol	−0.461	−0.209	−0.359	−0.516	−0.639	−0.029
Ethylene glycol	−0.969	−1.170	−1.706	−2.146	−3.019	−0.212
Propylene glycol, 37°	−0.372	−0.472	−0.728	−1.009	−1.530	−0.120
Glycerin	−1.334	−2.254	−2.825	−3.509	−5.002	−0.358
Formamide	−1.053	−1.421	−1.987	−2.474	−3.332	−0.233
N-Methylformamide	−0.077	−0.046	−0.010	−0.175	−0.658	−0.059
N,*N*-Dimethylformamide	+0.248	+0.164	+0.309	+0.463	−0.383	−0.050
Hexane, 37°	−2.288	−1.983	−1.859	−1.815	−1.668	+0.056
Silicone oil, 37°	−0.515	−0.482	−0.465	−0.458	−0.333	+0.017

[a] All data for 25° unless otherwise noted. [b] Not used in calculation of slope because of uncertainty of experimental measurement.

Table IV—Logarithms of Mole Fractional Solubilities of Alkyl *p*-Aminobenzoates in Propylene Glycol–Water Mixtures

Solvent[a]		Ester				
Water, %	Propylene Glycol, %	Ethyl	Butyl	Hexyl	Octyl	Dodecyl
100	0	−3.734	−4.500	−5.715	−7.142	−9.540
80	20	−3.329	−3.909	−5.086	−6.168	−8.433
60	40	−2.720	−3.108	−4.234	−5.245	−7.347
40	60	−2.337	−2.250	−3.115	−3.987	−5.470
20	80	−1.828	−1.345	−2.321	−2.961	−4.386
0	100	−1.161	−0.710	−1.153	−1.688	−2.968

[a] All data for 37°.

Table V—Logarithms of Mole Fractional Activity Coefficients of Alkyl *p*-Aminobenzoates in Propylene Glycol–Water Mixtures

Solvent[a] Composition	Ester					Slope, $\Delta \log (ac)$ / Δn
	Ethyl	Butyl	Hexyl	Octyl	Dodecyl	
Water	−3.245	−4.268	−5.289	−6.444	−8.106[b]	−0.525
20% Propylene glycol	−2.840	−3.671	−4.661	−5.488	−6.995	−0.419
40% Propylene glycol	−2.230	−2.870	−3.809	−4.566	−5.910	−0.375
60% Propylene glycol	−1.848	−2.012	−2.690	−3.307	−4.032	−0.234
80% Propylene glycol	−1.339	−1.107	−1.895	−2.281	−2.948	−0.183
Propylene glycol	−0.372	−0.472	−0.728	−1.009	−1.530	−0.120

[a] All data for 37°. [b] Not used in calculation of slope because of uncertainty of experimental measurement.

Ideal Solubility Determinations—These values were calculated using Eq. 2, the heat of fusion, and the melting temperature. The heats of fusion were determined by a differential scanning calorimeter. The melting points were determined by hot-stage microscopy and differential scanning calorimetry, both as described previously (18).

Interfacial Tensions—The interfacial tensions were determined against tetradecane using the Wilhelmy plate method. The experimental details along with the dependence of interfacial tension on hydrocarbon density were described in a separate publication (13).

RESULTS

The ideal mole fractional solubilities of ethyl, butyl, hexyl, octyl, and dodecyl *p*-aminobenzoates at 25 and 37° are given in Table II. These values were calculated from the experimentally measured heats and temperatures of fusion as described. Their relationship to changes in crystal structure with chain length was discussed previously (18). The experimental mole fractional solubilities of the five esters in each of the 11 pure polar solvents considered are also given in Table II.

The experimental and ideal mole fractional solubilities of Table II and Eq. 3 were used to calculate the activity coefficients of each ester in each solvent. The logarithms of these values are listed in Table III. The expected linear decline in log *(ac)* with increasing chain length was observed in nearly all solvents. The sensitivity, σ, of the solvent to an additional methylene unit can be defined as:

$$\sigma = \frac{\Delta \log (ac)}{\Delta n} \qquad \text{(Eq. 16)}$$

(This relationship is generally not valid for $n = 0$ because the free acid can undergo donor hydrogen bonding and cannot be considered part of the series.) The value of σ for each solvent is given in the last column of Table III. The values of σ for water at 25 and 37° are close to each other and to the value determined for the alcohols and hydrocarbons (10, 11) and other series (6–8).

The logarithms of the solubilities and activity coefficients of the esters in mixed propylene glycol–water solutions are given in Tables IV and V. Each mixed solvent behaves as a pure solvent having a value of σ which is directly dependent upon its composition.

The molecular surface areas of each normal alkyl ester of *p*-aminobenzoic acid were calculated by Hermann's (12) method using an effective solvent radius of 1.5 Å as described previously (10). From these values, it is found that the surface area of the esters above propyl, like the alcohols and hydrocarbons, increases by 31.8 Å^2/methylene unit (see Table II). The interfacial tensions of each solvent against tetradecane (a representative hydrocarbon) are listed in Table VI. For mixed solvent systems the interfacial tensions were also calculated as the composition weighted average of the pure solvent values:

$$\gamma_{1h} = \gamma_{wh} f_w + \gamma_{ch} f_c \qquad \text{(Eq. 17)}$$

where w and c represent water and cosolvent, respectively. The corresponding surface tensions, solubility parameters, and dielectric constants are also listed in Table VI for comparative purposes.

DISCUSSION

Semilogarithmic plots of mole fractional solubility *versus* chain length for the alkyl *p*-aminobenzoates in any of the solvents studied (mixed or pure) show a break in linearity at $n = 4$ (see Fig. 2 of Ref. 9 and Figs. 2–4 of Ref. 18). This break has been shown to be due to the fact that the butyl ester has the weakest crystal lattice energy of the series and thus has the greatest ideal solubility. When the ideal solubility is divided into the observed solubilities, good linearity between log *(ac)* and chain length is found.

The slopes of these lines (Tables III and V) can be seen to be roughly dependent upon solvent polarity: $\sigma_{hexane} = +0.06$, $\sigma_{glycerin} = -0.36$, and $\sigma_{water} = -0.53$. Comparisons of σ with each parameter in Table VI were considered, but the best correlation was obtained with interfacial tension; therefore, the discussion will be confined to interfacial tension for which linearity may be expected.

Since the increase in surface area per methylene unit is a constant (31.8 Å^2 above the propyl ester), Eqs. 15 and 16 can be combined to give:

$$\sigma = \frac{-C\gamma_{1h}}{2.303RT} \qquad \text{(Eq. 18)}$$

Table VI—Solvent Properties

Solvent	Solubility Parameter	Dielectric Constant	Surface Tension	Interfacial Tension against Tetradecane
Water	23.4	78.5	72.0	51.9
Methanol	14.5	33	23.0	4.2
Ethanol	12.8	24.3	22.2	—
Ethylene glycol	17.1	37.7	48.8	19.3
Propylene glycol	15.0	32.1	37.1	12.5
Glycerin	17.7	42.5	64.9	35.2
Formamide	19.4	109.5	58.7	31.2
N-Methylformamide	16.1	190	40.1	12.3
N,*N*-Dimethylformamide	12.2	36.7	36.6	4.6
Hexane	7.3		18.5	0.0
Silicone oil	—	—	—	0.0
20% Propylene glycol	21.4[a]	77.6[a]	65.2[a]–53.3[b]	44.1[a]–34.2[b]
40% Propylene glycol	19.8[a]	59.9[a]	58.4[a]–47.0[b]	36.2[a]–28.9[b]
60% Propylene glycol	18.2[a]	48.7[a]	51.6[a]–42.9[b]	28.3[a]–22.9[b]
80% Propylene glycol	16.5[a]	41.4[b]	44.8[a]–39.1[b]	20.4[a]–17.1[b]

[a] Estimated as the sum of the properties of the pure solvent times their volume fraction. [b] Experimentally measured.

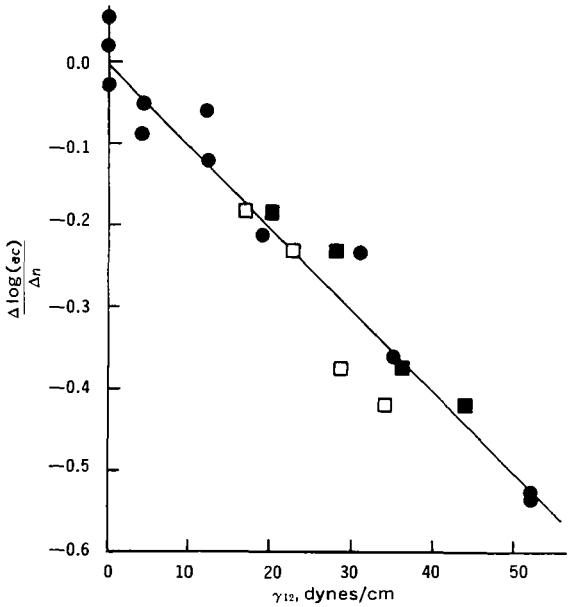

Figure 1—*Dependence of methylene group activity coefficient in a solvent upon the solvent–tetradecane interfacial tension. Key: ●, pure solvents; □, experimental interfacial tension of mixtures; and ■, calculated interfacial tension of mixtures.*

The slope of σ *versus* γ_{1h} (Fig. 1) is equal to $-C/2.303RT$ (or $-0.033C$ at 25°). The observed slope is 0.01, which corresponds to a value of about one-third for C. This value is in good agreement with theoretical estimates (16, 17) and with the value obtained (10) for alcohols and hydrocarbons in water. Furthermore, the good linearity of Fig. 1 lends support to the assumption of a constant curvature correction factor and thus to the use of interfacial tension as a parameter with which to correlate solubility. The effects of chain branching and positional isomerism in each solvent having positive hydrocarbon–water interfacial tensions should be the same; reducing the surface area reduces the interfacial area and, by Eq. 11, increases solubility.

In Fig. 1, both the experimentally measured and the calculated interfacial tensions are shown for the propylene glycol–water mixtures against tetradecane. The difference between the two is not unexpected. It is well known that semipolar components of aqueous solutions tend to be absorbed onto nonpolar interfaces. This accumulation of a surface excess of the less polar component causes a greater lowering of the surface or interfacial tension than would be observed for a completely homogeneous mixture. Since the calculated values are based upon complete homogeneity, they are, of necessity, higher than the measured values. At a microscopic interface within the bulk of the solution, it seems likely that there will be little or no accumulation of a surface excess of the cosolvents being considered. (Such an accumulation would effectively amount to complexation if it did occur to an appreciable extent.)

While this postulate cannot be proved, it is interesting to consider the following possible reasons for its justification:

1. The development of a surface excess is a slow diffusion-dependent process. The thermal motion of the particles (molecules) would tend to hinder the build-up of an absorbed layer.

2. At the highly curved molecular interface, the interfacial tension is much less than at a microscopic hydrocarbon or air interface. Thus, there would be a much smaller change in free energy per unit area when a third component (cosolvent) goes to the interface.

The theory of regular solutions (Eqs. 2 and 7) has been used successfully to describe the solubilities of methyl through nonyl and dodecyl *p*-aminobenzoates in hexane (18) but was unable to handle the aqueous solubilities of these same compounds. The present approach (Eqs. 2 and 11), while giving satisfactory results for hexane, is not sensitive to changes in nonpolar solvents. This is because it is mechanically impossible to obtain accurate interfacial tension of such solvents against pure hydrocarbon surfaces.

Furthermore, since γ_{12} cannot be less than zero, it is not possible to predict the slight increase in log *(ac)* with chain length in some solvents, nor can the curvature of such data be predicted. Fortunately, as mentioned previously, these properties are handled well by regular solution theory. On the other hand, the use of Eq. 7 cannot predict the linearity of the log *(ac) versus n* data observed in the polar solvents and the mixed solvents in this study.

The limiting factor to the validity of the theory presented and of regular solution theory is the evaluation of the solute–solvent term, γ_{12} or δ_{12}. The former can be measured for substances whose polarity is significantly different from one another while the latter is calculable for substances similar in polarity. Together, the two equations cover a wide range of solvents and solutes and bring one a step closer to being able to predict solubility *a priori*. Investigations are already underway to determine the effects of common organic substituents on activity coefficients. However, for the present, γ_{12} applies only to saturated hydrocarbons which represent the alkyl portion of the solute.

CONCLUSION

A derivation is presented which equates RT log *(ac)* to $\gamma_{1h}A_h$, the hydrocarbon–solvent interfacial tension times the molecular surface area of the hydrocarbon portion of a molecule. This equation, which is a two-dimensional analogy to the Scatchard–Hildebrand equation, avoids the need to rely upon the geometric mean rule and overcomes certain limitations on the application of the latter. It has been shown to be useful for predicting the effects of hydrophobic substituents on the activity coefficients of various semipolar substances in water, mixed aqueous solvents, and pure nonaqueous polar and semipolar solvents.

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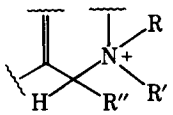
Stereochemical Preferences for Curarimimetic Neuromuscular Junction Blockade I: Enantiomeric Monoquaternary Amines as Probes

PAUL W. ERHARDT and TAITO O. SOINE ˣ

Abstract □ Seven pairs of monoquaternary enantiomeric neuromuscular junction blocking agents were prepared in which the carbon asymmetry is adjacent to the quaternized nitrogen moiety. The tertiary amines from which the blocking species were obtained are carnegine, laudanosine, *N*-methylpavine, corydine, isocorydine, glaucine, and boldine. Curarimimetic potencies, obtained with an *in vivo* cat tongue–hypoglossal nerve preparation, were obtained for the enantiomeric methiodides of each of these amines. Possible contributions to activity by preferential binding to blood components or by selective inhibition of acetylcholinesterase also were studied. The combined studies indicate that there is a modest preference by the neuromuscular junction of the cat for monoquaternary blockers with the *(S)*-configuration.

Keyphrases □ Curarimimetic activity—stereochemical preferences for neuromuscular junction blockade, seven pairs of enantiomeric monoquaternary amines (carnegine, laudanosine, *N*-methylpavine, corydine, isocorydine, glaucine, and boldine as methiodides) as probes □ Neuromuscular junction blockade (curarimimetic)—stereochemical preferences, seven pairs of enantiomeric monoquaternary amines as methiodides as probes □ Amines, enantiomeric monoquaternary—as probes for determination of stereochemical preferences for curarimimetic neuromuscular junction blockade □ Blocking agents, neuromuscular—carnegine, laudanosine, *N*-methylpavine, corydine, isocorydine, glaucine, and boldine (as methiodides), stereochemical preferences for curarimimetic neuromuscular junction blockade, enantiomeric monoquaternary amines as probes

The structural requirements for neuromuscular junction blockade have been a heavily researched area in the study of chemical activity on physiological function (1). Most applicable parameters have received intensive study, and it is surprising that little has been done regarding enantiomeric comparisons (2). The following structure illustrates the nature of the asymmetry commonly possessed by curarimimetic neuromuscular junction blockers:



The possibility that carbon asymmetry adjacent to the quaternary nitrogen may cause potency differences among the stereochemical isomers was indicated by the work of King (3), who found that (+)-tubocurarine was 20–60 times more potent than (−)-tubocurarine. Similarly, earlier work (4) indicated that nitrogen asymmetry, obtained by methyl quaternization of canadine, can also lead to differing neuromuscular junction blocking potencies among the various stereochemical isomers.

Since these initial findings, support for such a premise has been rather sparse since a systematic investigation of the effects of asymmetry on curarimimetic activity is nonexistent. However, in cases where configurational isomerism was encountered, different potencies among the isomers also were observed (2, 5–9).

This series of studies addresses itself to the elucidation of stereochemical parameters, resulting from configurational isomerism, that may affect neuromuscular junction blocking potency. Asymmetry *per se* is not necessary for blocking activity, and these studies are not presupposed on finding such differences. Instead, they are based on establishing the extent, if any, of such differences in the hope of further defining the characteristics of the binding site for these types of blocking agents.

In beginning such a study, it seemed reasonable to start at the simplest level, that is to employ as probes molecules possessing only a single asymmetric atom. In terms of pharmacological design and for synthetic simplicity, it was decided to pattern the probes after a *hemi*-curare type of structure. The resulting tetrahydroisoquinoline types, after quaternization, would then bear an onium head in a "pachy-curare" arrangement typical of curarimimetic agents. This approach was taken by earlier workers (10, 11), who found that such structural types do indeed show weak curare-like activity. The synthesized probes (I–XIV) were subjected to bioassay in this initial work.

Further studies will employ more complex probes and will consider the stereochemical preferences of bisquaternaries with asymmetric carbon atoms, monoquaternaries with carbon–nitrogen asymmetry and simple nitrogen asymmetry, and bisquaternaries with carbon–nitrogen asymmetry and uncomplicated nitrogen asymmetry.

CHEMISTRY

The laudanosinium (I and II) and *N,N*-dimethylpavinium (III and IV) probes were synthesized from papaverine. Treatment of papaverine methiodide with methanolic sodium borohydride (12) produced (±)-laudanosine. Its resolution was achieved *via* the *O,O*-di-*p*-toluoyl bitartrate salts (13). Deracemization with quinic acid (14, 15), tartaric acid, and *O,O*-dibenzoyltartaric acid (16) was unsuccessful. Treatment of papaverine methiodide with sodium borohydride in pyridine (17) yielded the partially reduced 1,2-dihydro compound, which was cyclized to (±)-*N*-methylpavine by the usual acid treatment (18). Resolution was accomplished *via* the bitartrate salts (19). Methyl iodide quaternization of each enantiomeric pair produced the desired probe compounds.

The carneginium probes (V and VI) were synthesized from *N*-acetylhomoveratrylamine which, under Bischler–Napieralski reaction conditions (20) and subsequent reduction with sodium borohydride in methanol (21), yielded (±)-salsolidine. This compound was deracemized *via* the *O,O*-dibenzoyl bitartrate salts (21).

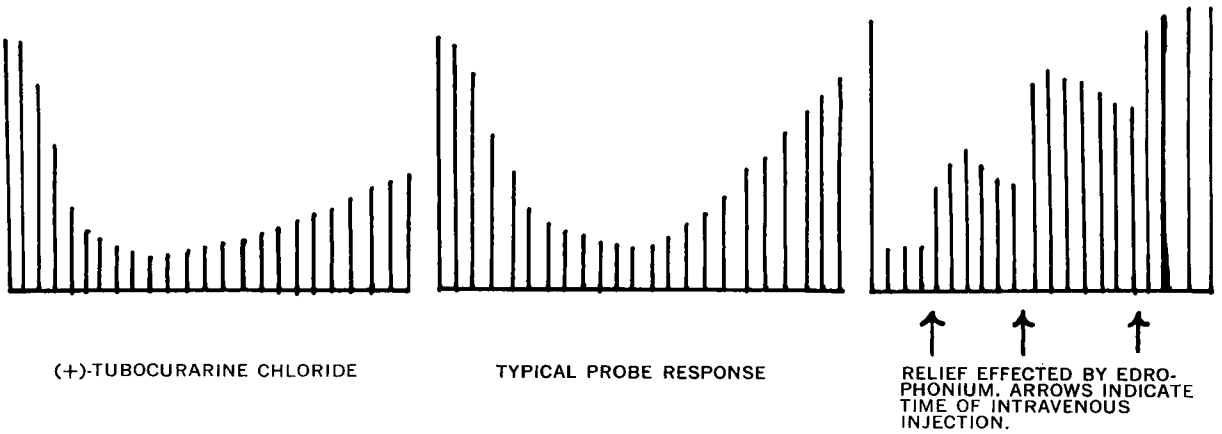
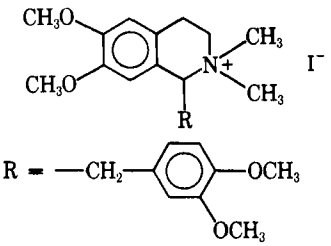


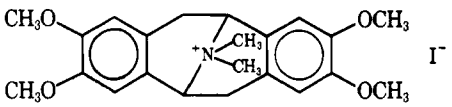
Figure 1—*Pattern of neuromuscular junction block onset, duration, and relief.*

Methyl iodide treatment of each enantiomer under buffered conditions provided the desired probe pair.

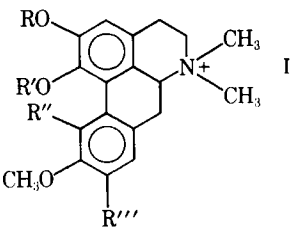
The aporphinium-type probes were prepared from the commercially available, naturally occurring tertiary bases, all of which possessed the *(S)*-configuration. Methyl iodide quaternization of these [(+)-glaucine, (+)-corydine, (+)-isocorydine, and (+)-boldine] gave the *(S)*-component of the desired enantiomeric probe pairs (VII, IX, XI, and XIII). Racemization of the optically active



I: (*S*)-*N*-methyllaudanosinium iodide
II: (*R*)-*N*-methyllaudanosinium iodide
R = CH_3
V: (*S*)-*N*-methylcarneginium iodide
VI: (*R*)-*N*-methylcarneginium iodide



III: (*S,S*)-*N,N*-dimethylpavinium iodide
IV: (*R,R*)-*N,N*-dimethylpavinium iodide



R = R′ = CH_3, R″ = H, R‴ = OCH_3
VII: (*S*)-*N*-methylglaucinium iodide
VIII: (*R*)-*N*-methylglaucinium iodide
R = CH_3, R′ = R‴ = H, R″ = OCH_3
IX: (*S*)-*N*-methylcorydinium iodide
X: (*R*)-*N*-methylcorydinium iodide
R = R′ = CH_3, R″ = OH, R‴ = H
XI: (*S*)-*N*-methylisocorydinium iodide
XII: (*R*)-*N*-methylisocorydinium iodide
R = R″ = H, R′ = CH_3, R‴ = OH
XIII: (*S*)-*N*-methylboldinium iodide
XIV: (*R*)-*N*-methylboldinium iodide

bases was effected by the catalytic hydrogenation (22) in acetic acid (21). Deracemization of the racemates of glaucine, corydine, and isocorydine was accomplished *via* the bitartrate route suggested (23) for glaucine.

Although many attempts were made to resolve the boldine racemate, it was only possible through the *O,O*-di-*p*-toluoyl bitartrate salts. Again, methyl iodide quaternization of the resultant *(R)*-enantiomers was successful and provided the other half of the required enantiomeric probe pairs (VIII, X, XII, and XIV). All of the compounds prepared, other than known compounds, were characterized by melting point, optical rotation, TLC, and spectral methods (IR, NMR, UV, and mass spectroscopy). Final compounds were also subjected to elemental analysis. All analytical and spectral data were in accord with known values. Melting point, optical rotation, and TLC data were obtained again, after physiological testing, to ensure that the quality of the test probes had not changed.

BIOLOGY

In the selection of an assay for neuromuscular junction blocking abilities, two considerations led to the decision that an *in vivo* cat preparation would be the most appropriate:

1. It is well established (24–26) that among various species the cat most closely resembles humans in its response to neuromuscular junction blocking agents.
2. Maclogan (27) pointed out that isolated nerve–muscle preparations do not simulate the intact animal in overall response, duration of action, and recovery patterns.

Although several *in vivo* cat preparations have been described, they all employ a muscle removed from its natural surroundings and necessitate precautionary measures such as the maintenance of a controlled muscle temperature (28–31). However, the hypoglossal nerve–tongue muscle preparation allows the muscle to be maintained in its natural surroundings, and previous experience in these laboratories (21) established its suitability for this type of study[1].

In assessing the nature of the blockade produced by the monoquaternary probes, several observations support the conclusion that the block is of a curarimimetic type:

1. No initial contraction or muscular fasciculation, characteristic of depolarizing-type blockers in their action on cat muscle (1), was observed.
2. The blocking patterns (Fig. 1) of the probes were identical to those of (+)-tubocurarine in their onset of action and decay to maximal effect, although (+)-tubocurarine had a much longer duration of action which manifested itself in a recovery phase with a more gentle slope.
3. The blocks established by the probes and by (+)-tubocurarine were shown to be cumulative at low doses.
4. The blocks established by (+)-tubocurarine and by the

[1] The method has been used as a teaching tool in the pharmacology laboratories of the University of Minnesota and Michigan State University, but it apparently has not been described in the literature. Therefore, a detailed description of this method appears in the *Experimental* section.

Table I—Neuromuscular Junction Blocking Potencies

Drug	r^{2a}	ED_{50}, $\times 10^{-4}$ mmole/kg	Duration, min	ED_{50} Potency Ratio to (+)-Tubocurarine ($1/N$)	Slope Comparison[b] [$F_S(dF', dF)$]	Previous Work[c]
(+)-Tubocurarine	0.73	1.32	35	1		
XIII	0.85	17.06	20	13	0.018 (2, 27) +	
XIV	0.80	27.72	20	20		
IX	0.51	16.98	11	13	0.571 (2, 30) +	
X	0.66	38.10	9	29		
XI	0.94	41.41	15	31	0.295 (2, 27) +	(32–34) − (35) +
XII	0.80	47.67	15	36		
III	0.69	49.09	30	37	1.511 (2, 30) +	
IV	0.78	85.92	30	65		
VII	0.85	79.48	8	60	9.515 (2, 27) −	(36) − (37) +
VIII	0.75	128.97	7	97		
I	0.72	96.99	12	73	1.602 (3, 46) +	(9, 10, 38, 39) +
II	0.79	167.94	15	127		
V	0.92	260.88	10	197	3.073 (2, 27) +	
VI	0.86	488.98	9	370		

[a] When the raw data for a single animal were treated statistically, r^2 was considerably higher (>0.90) in all cases. The lower r^2 value obtained when the data for three cats were pooled can then be taken as an expression of individual variation. [b] Comparisons were made between the regression line slopes of the probes and (+)-tubocurarine (+ indicates that the slopes are not statistically dissimilar; — indicates that the slopes are statistically dissimilar). [c] Reference to prior studies on the racemic modifications (+ indicates potency agreement; — indicates potency disagreement).

Table II—Enantiomeric Neuromuscular Junction Blocking Potency Comparisons

Drug	Enantiomer	ED_{50} with 95% Confidence Limits, mg/kg			ED_{50} Potency Ratio of Enantiomers	Elevation[a] Comparison [$F_{el}(dF', dF)$]
		Low	ED_{50}	Up		
(+)-Tubocurarine		0.08	0.09	0.16		
I	(*S*)	3.29	4.84	11.68	1.8:1	3.198 (1, 25) −
II	(*R*)	5.24	8.38	20.56		
III	(*S,S*)	1.64	2.44	4.28	1.7:1	6.939 (1, 19) +
IV	(*R,R*)	3.43	4.27	5.86		
V	(*S*)	8.39	9.47	11.07	1.9:1	18.267 (1, 16) +
VI	(*R*)	13.39	17.75	24.70		
VII	(*S*)	3.58	3.95	4.36	1.7:1	17.240 (1, 16) +
VIII	(*R*)	5.07	6.41	8.00		
IX	(*S*)	0.29	0.82	1.64	2.2:1	7.992 (1, 19) +
X	(*R*)	1.30	1.84	3.33		
XI	(*S*)	1.70	2.00	2.40	1.2:1	−
XII	(*R*)	1.70	2.32	4.35		
XIII	(*S*)	0.61	0.80	1.22	1.5:1	4.563 (1, 16) +
XIV	(*R*)	0.90	1.30	1.50		

[a] Statistical comparisons were made between the line elevations of enantiomeric pairs to evaluate the significance of the difference in ED_{50} values (+ indicates a statistically significant difference; — indicates that the difference is not statistically significant).

probes showed identical, characteristic stepwise patterns of recovery when edrophonium, an acetylcholinesterase inhibitor, was administered (Fig. 1).

5. A statistical comparison of the slopes of log dose *versus* probit response linear regression lines (Fig. 2) for (+)-tubocurarine and the probes revealed that, in all cases except for the glauciniums, their slopes were not statistically different. This finding in itself is taken to mean a similar mechanism of action.

Table I presents the neuromuscular junction blocking potency data and is arranged so that the more potent inhibitors are at the top of the table. The phenolic aporphiniums are more potent than the aporphiniums which possess only aryl ether type of functional-

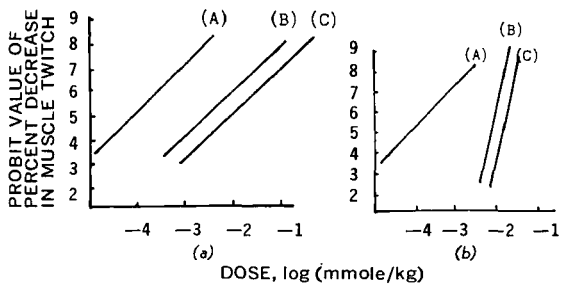


Figure 2—*Computer program-derived linear regression plots of neuromuscular junction blockage data.* (a) *Typical data represented by actual data for the* N-*methylcorydinium iodides. Key: A,* (+)-*tubocurarine; B, IX; and C, X.* (b) *Aberrant data obtained for the* N-*methylglaucinium iodides. Key: A,* (+)-*tubocurarine; B, VII; and C, VIII.*

ity, although it is usually expected that alkylation of phenolic groups leads to an enhancement in neuromuscular junction blocking potency (5, 40). However, the fully aryl ether type of aporphiniums are represented only by the glauciniums and, as already stated, these probes are unique in that their slopes show a statistically significant deviation from the slope of (+)-tubocurarine.

In addition to the neuromuscular junction activity, an initial fall in blood pressure was observed intermittently for the probes and for (+)-tubocurarine. The effect is common (41) for quaternary-type compounds and may be attributed to either a weak muscarinic-type activity or, more likely, to an initial histamine release effected by these agents since the effect was of a transient nature.

Another observation was that during the initial dosing phase with (+)-tubocurarine, equal subsequent doses effected a greater blockade. This phenomenon is presumed to be due to the filling of other more generalized receptors for quaternary-type compounds. The possibility that these receptors may not be located at the neuromuscular junction is exemplified by the red blood cells in which the membrane contains cholinesterase and bears a net negative charge (42, 43). After saturation of these depots with either (+)-tubocurarine or the probes, the phenomenon was no longer observed and linear relationships between dose and response could be obtained through the remainder of the experiment.

Because of this phenomenon, it became important to establish that these probes had an activity of their own and that they were not acting by the mere displacement of (+)-tubocurarine from the nonspecific receptors. Therefore, the Latin-square method was adopted into the drug administration protocol such that each new animal preparation was titrated and initially tested with a different drug. Three probes were tested in three different animal preparations, and each probe was tested with at least three different concentrations in each animal. Potency ratios were found to be the same in all preparations and were independent of the order of testing.

Table II presents the neuromuscular junction blocking potency

Table III—Enantiomeric Probe Binding to Blood Components

Drug	Test Concentration, mg/ml	Percent Bound to Plasma Proteins	Percent Bound to Red Blood Cells
I	0.085	9 ± 2	22 ± 2
II	0.085	8 ± 2	10 ± 2
III	0.040	9 ± 3	7 ± 2
IV	0.040	7 ± 3	10 ± 2
V	0.170	2 ± 3	—
VI	0.170	4 ± 3	—
VII	0.065	33 ± 8	12 ± 2
VIII	0.065	20 ± 6	13 ± 2
IX	0.016	50 ± 6	—
X	0.016	52 ± 4	—
XI	0.028	69 ± 7	22 ± 2
XII	0.028	59 ± 7	16 ± 2
XIII	0.013	68 ± 7	—
XIV	0.013	56 ± 6	—

relationships between the *(S)*- and *(R)*-enantiomers. That the *(S)*-enantiomers have a subtle, statistically significant potency magnitude difference from the *(R)*-enantiomers is clearly demonstrated. However, this difference reflects the gross response observations from an *in vivo* bioassay and cannot, at this point, be attributed to a stereochemical preference exhibited by certain neuromuscular junction receptors. Other physiological parameters that could cause such differences are: different absorption rates between enantiomers, unequal distributions due to either a preferential access to certain compartments or a preferential binding in certain compartments having equal accessibility, different metabolic rates, differing rates of excretion, and, most important, different inhibitory interactions with acetylcholinesterase. Since acetylcholinesterase is so intimately associated with the neuromuscular junction, its consideration is essential for studies of this type, even by those workers who employ isolated nerve–muscle preparations as their bioassay.

The time course of less than 5 sec to obtain maximal blocking effect after intravenous injection with all compounds tested represents very nearly the bolus head of the injection (44); on this basis, differential absorption, excretion, and metabolism rates are unlikely factors to be considered in seeking causes for differences in enantiomeric potency. Similarly, preferential access to certain compartments can probably be disregarded, although preferential binding (*e.g.*, to plasma protein) in certain compartments having equal accessibility cannot be dismissed as a possibility. Finally, preferential inhibition of acetylcholinesterase by one of the enantiomers certainly cannot be disregarded on this basis and, therefore, requires further investigation.

That the blood compartment may have binding preferences for one of the enantiomeric species was a primary consideration, al-

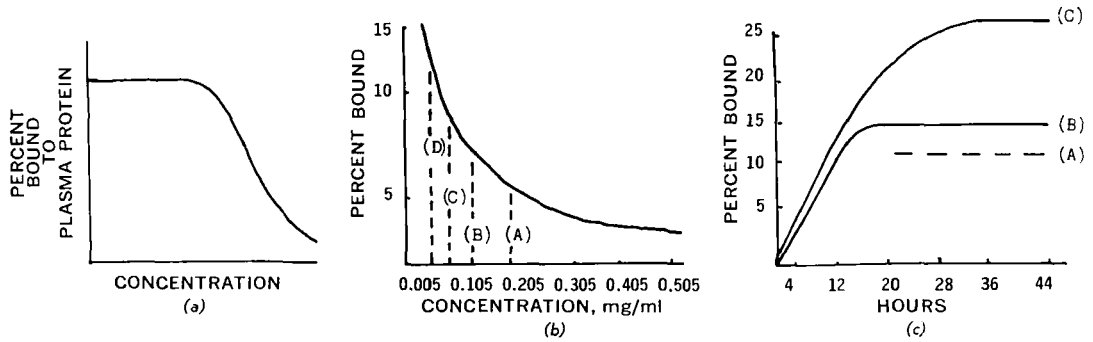


Figure 3—*Plasma protein binding patterns and* in vivo *drug distribution compartment concentrations.* (a) *Theoretical pattern of binding to plasma proteins as a function of increasing drug concentration.* (b) *Binding pattern observed within the measurable concentration range for the probes (actual data for I). Also illustrated are* in vivo *distribution compartment concentrations using the plasma volume (A), the total blood volume (B), the total extracellular water (C), and the total body water (D).* (c) *Typical dialysis rate data (actual data for I). Key: A, calculated loss due to dilution; B, control dialysis with membrane containing buffer solution only; and C, normal dialysis.*

Table IV—Acetylcholinesterase Inhibition

Drug	Enantiomer	*In Vivo* Concentration Range, $\times 10^{-5}$ M[a]	Wilkinson K_i, $\times 10^{-4}$ M[b]	Wilkinson V_{max}, μmoles/unit/min	Inhibition[c]
Acetylcholine (natural substrate, K_m)			2.72 ± 0.06	1.07 ± 0.01	
Edrophonium			0.033 ± 0.001	0.7 ± 0.2	
Neostigmine			0.0011 ± 0.0003	1.1 ± 0.4	
I	(*S*)	2–6	13 ± 0.2	1.1 ± 0.1	C
II	(*R*)	4–10	6.5 ± 0.8	1.0 ± 0.1	C
III	(*S,S*)	2–6	7.1 ± 0.4	0.8 ± 0.2	C
IV	(*R,R*)	4–10	6.1 ± 0.5	0.7 ± 0.3	C
V	(*S*)	13–32	8.2 ± 0.4	1.4 ± 0.3	C
VI	(*R*)	24–60	3.4 ± 0.3	1.19 ± 0.08	C
VII	(*S*)	4–10	1.7 ± 0.3	0.5 ± 0.1	I
VIII	(*R*)	6–16	2.3 ± 0.4	0.5 ± 0.04	I
IX	(*S*)	0.8–2	3.4 ± 0.2	1.4 ± 0.3	C
X	(*R*)	2–5	6.7 ± 0.3	1.1 ± 0.2	C
XI	(*S*)	2–5	3.9 ± 0.8	1.1 ± 0.3	C
XII	(*R*)	2–6	2.8 ± 0.5	1.6 ± 0.5	C
XIII	(*S*)	1–2	1.8 ± 0.4	0.3 ± 0.2	I
XIV	(*R*)	1–3	1.0 ± 0.3	1.0 ± 0.3	I

[a] Lower concentrations were calculated using the total body water as a distribution compartment, and upper concentrations were calculated using the total extracellular water as a distribution compartment. [b] Determined by calculation from computer program-(Wilkinson) generated, altered K_m values. [c] Determined graphically by Lineweaver–Burk plot (C = competitive and I = inconclusive).

though differences in enantiomeric activities on this basis are not common; a rather high degree of binding to the blood components is required before significant alterations in drug distribution patterns are observed (45). Nevertheless, two blood components may be entertained as potential binding sites, namely, plasma proteins and the red blood cells, since both have been implicated to some degree in previous studies (43, 46).

Plasma protein binding studies, utilizing a membrane equilibrium dialysis technique (47) and UV spectrophotometric measurement for concentration assay, were undertaken first. Preliminary studies had established that a UV assay was appropriate in terms of sensitivity in the concentration ranges expected and because the probes showed a conformity to the Beer–Lambert law. Figure 3 illustrates the nature of the results obtained. Failure to obtain the percent bound plateau at low concentrations as predicted by theory (48) is readily explained by the inability of UV spectrophotometry to assay at appropriately low concentrations for the agents employed as probes in this work. However, the concentration range that has been successfully studied is well below the range (Fig. 3) regarded as a minimum for an *in vivo* ED_{50}-based concentration range (44).

Furthermore, since the objective of the study was to compare the differences in binding between *(S)*- and *(R)*-enantiomers under identical, simulated *in vivo* conditions, whether or not measurements were made at the theoretical plateau levels became an academic question. Therefore, the concentrations employed were calculated from the average of the ED_{50} doses of the enantiomeric pair using the whole blood volume (44) as an appropriate *in vivo* distribution compartment (45). The results of the plasma protein binding study are recorded in Table III and clearly indicate that there are no significant differences in binding of the enantiomers; therefore, this type of interaction cannot account for the differences in neuromuscular junction blocking potency.

Aside from the question of enantiomeric binding comparisons, it was observed that there is a significantly greater binding propensity among the phenolic probes than among the nonphenolic ones. Since binding is a function of concentration in the range studied, Table III cannot be used for such a comparison. However, Fig. 4 shows this difference clearly. This behavior is in accord with observations of earlier workers (46) who found similar binding enhancement in the presence of hydrogen-bonding phenolic or hydroxyl groups.

Binding to red blood cells was studied next. Four of the seven pairs of enantiomers under consideration were selected for the study and represent all of the structural types present among the probes. Thus, I and II were representative of the tetrahydroisoquinoliniums, III and IV were representative of the *N*-methylpaviniums, and VII and VIII and XI and XII were representative of the nonphenolic and phenolic aporphiniums, respectively. Dialysis, in this case, was not necessary since centrifugation could remove the red blood cells from the sample and produce a clear solution suitable for UV analysis.

The same concentrations used in the plasma protein binding study were employed in determining the extent of binding to the red blood cells (Table III). While statistically different binding is observed for the enantiomeric cases of I and II and XI and XII, it would be unreasonable to consider these to have a causal relationship with the observed *(S)*- greater than *(R)*-enantiomeric neuromuscular junction potency differences because the result [*(S)* bound greater than *(R)*] is opposite to what would be required.

Preferential inhibition of acetylcholinesterase by one of the enantiomers, which would presumably tend to offset the neuromuscular junction blocking activity by that enantiomer and thereby result in a greater apparent activity for the other enantiomer, was the final possibility studied. The pH–stat method (49–52) was chosen. In this method, the acetic acid produced by enzymatic hydrolysis of acetylcholine is automatically titrated with base so that the pH is maintained at 7.5.

The enzymatic velocity so obtained was treated by a computer analysis[2], using the method of Wilkinson (53), to generate K_m and V_{max} data having standard deviation error limits associated with each value. From these data, the K_i values could be calculated by employing simple Michaelis relationships[3]. Lineweaver–Burk plots (54) were also performed to aid in the assessment of the type of inhibition exhibited by these agents.

The results of these studies are recorded in Table IV. The K_m and V_{max} data for acetylcholine indicate that the method is both valid [the K_m being in general agreement with literature values (49–51, 55) and the V_{max} being in exact agreement[4]] and highly accurate (the K_m error limit being only ±2% and that for V_{max} being only ±1%). The expansion of these error limits when the inhibitors are present implies that the interaction of these agents with the enzyme is more complex than that dictated for narrow error range limits by the simple mathematical model used in the data regression technique. However, the values are accurate enough in that they distinguished enantiomeric potency differences in five of the seven probe pairs. Also, the agreement between the Wilkinson V_{max} data and the Lineweaver–Burk data strengthens the validity of the generated data.

As expected (56–59), the results indicate that the probes are,

[2] CDC-6400 computer program.

[3]
$$\frac{X \text{ intercept}}{\text{altered } X \text{ intercept}} = 1 + \frac{[I]}{K_i}$$

[4] One unit of acetylcholinesterase hydrolyzes 1 μmole of acetylcholine/min at 25° (Worthington Biochemical Corp.).

Table V—Racemic Bases

Compound	Preparation Reference	Aporphine Racemization Time, hr	Overall Yield, %	Melting Point	Literature Melting Point
(±)-Laudanosine	12		83	114–116°	115–116° (60)
[(±)-1-(3′,4′-dimethoxybenzyl)-2-methyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline]				172–174° (picrate) 214–216° (methiodide)	174–175° 215–217°
(±)-Salsolidine	20		35	201–202° (picrate)	201–201.5° (61)
[(±)-1,2-dimethyl-6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline]					
(±)-*N*-Methylpavine	17		20	134–138°	139–140° (18)
[(±)-2,3,8,9-tetramethoxy-*N*-methylpavinane]	18				
(±)-Glaucine	21	36	51	134–137°	137–139° (60)
[(±)-1,2,9,10-tetramethoxyaporphine]	22				
(±)-Corydine	22	72	75	164–166° 226° (·HCl)	165–167° (62) 228° (63)
[(±)-1-hydroxy-2,10,11-trimethoxyaporphine]					
(±)-Isocorydine	22	125	56	120° (amorphous solid)	
[(±)-1,2,10-trimethoxy-11-hydroxyaporphine]					
(±)-Boldine[a]	22	125	78	86° (amorphous solid)	
[(±)-1,10-dimethoxy-2,9-dihydroxyaporphine]					

[a] If the boldine or boldinium-type compounds were left standing in the open for several days, decomposition would occur. Aqueous solutions were even less stable and darkened after a single day. This behavior is in accord with the literature (60).

mainly only very weak inhibitors of the enzyme, since they show K_i values larger than the K_m value for acetylcholine. The only two exceptions are the glauciniums (VII and VIII) and the boldiniums (XIII and XIV), whose K_i values are slightly less than the K_m value for acetylcholine, but they are still regarded as only weak inhibitors. In general, the nature of this inhibition would appear to be largely competitive, as shown by the insignificant alteration of the Wilkinson V_{max} data and by Lineweaver–Burk plot assessment. Again, the glauciniums and boldiniums are exceptions and probably have a different or mixed mechanism of action.

The K_i values are generally larger than the appropriate *in vivo* neuromuscular junction ED_{50} blocking concentrations by a factor of nearly 10. This weak inhibitory activity, when coupled with *in vivo* concentration considerations, indicates that the action of the probes on acetylcholinesterase can contribute only insignificantly to the overall neuromuscular junction blocking activity observed as the gross response to these agents. Nevertheless, such small enzyme interactions possibly could be enough to distort the gross neuromuscular junction responses between the *(S)*- and *(R)*-enantiomers if the *(S)*- and *(R)*-enantiomer interactions with the enzyme show substantial differences.

An assessment of this possibility requires a specific examination of Table IV in terms of *(S)*- and *(R)*-enantiomeric differences. Statistically, an *(R)* > *(S)* potency relationship exists in four cases, *(S)* > *(R)* in one case, and *(S)* = *(R)* in the remaining two cases. Clearly, the definite trend in gross neuromuscular junction blocking potency, where the *(S)*-enantiomers are always more potent than the *(R)*-enantiomers, is not so unequivocally approached in the anticholinesterase activity series. Therefore, it does not seem reasonable to equate the gross enantiomeric differences with some causative relationship involving antiacetylcholinesterase enantiomeric potency differences.

Furthermore, for one of the enantiomeric pairs (I and II) showing the most substantial difference in inhibitory potency, the difference in gross neuromuscular junction blocking potency is not even statistically significant. Similarly, for the enantiomeric pair (IX and X) showing the largest gross neuromuscular junction blocking potency difference, the inhibitory difference is in the opposite direction [*(S)* > *(R)*] to that required for the production of the gross neuromuscular junction response difference if the difference is to be attributed to the difference in enzyme interactions. Both of these findings serve to negate further the notion that there is a causal relationship between antiacetylcholinesterase enantiomeric potency differences and gross neuromuscular junction blocking enantiomeric potency differences.

All of the evidence, therefore, implies that the inhibitory activity is of too low a potency and is too random in nature to result in the observed unequivocal relationship between the *(S)*- and *(R)*-enantiomers in gross neuromuscular junction blocking potency.

EXPERIMENTAL[5]

Racemic Bases—The preparation of the individual racemic laudanosine, salsolidine, and *N*-methylpavine bases was performed according to conventional published methods, although (+)-glaucine[6], (+)-corydine[7], (+)-isocorydine[7], and (+)-boldine[8] were obtained from commercial sources. The latter four compounds were racemized by the method of Kametani *et al.* (22), using Adam's catalyst under catalytic hydrogenation conditions.

Table V summarizes the overall yields, physical constants, and references for the preparative procedures employed. Trivial names, historically assigned, are used for convenience, together with a statement of the more complex systematic name.

Resolution of Racemic Bases—Deracemization was accomplished through the formation of diastereomeric bitartrate salts obtained through interaction of the racemic base with the appropriate (+)- or (−)-tartaric or substituted tartaric acid. With racemic laudanosine, salsolidine, and *N*-methylpavine, it was necessary to use both the (+)- and (−)-forms of the tartaric acids; with the commercially obtainable, optically active bases, it was necessary to use only one of them.

The purity of the resolved bases was maintained at a high level. Table VI summarizes the yields and physical constants for the enantiomeric pairs and indicates the deracemization techniques employed.

[5] Melting points were determined on a Thomas-Hoover melting-point apparatus and are uncorrected. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. Analyses were performed by M-H-W Laboratories, Garden City, Mich. TLC was conducted on Eastman Chromagram (No. 6060) sheets in a minimum of three different solvent systems, and visualization was done with both UV lamp and iodine vapor. IR spectra were obtained as KBr samples with either a Perkin-Elmer 237 B grating IR spectrometer or a Beckman IR 9 spectrometer. UV spectra were determined on a Cary 14 recording spectrophotometer. NMR spectra were measured with a Varian Associates model A-60D or T-60 NMR spectrometer, using tetramethylsilane as an internal standard. Mass spectral determinations were performed by Mass Spectrometry Laboratory Services, Department of Chemistry, University of Minnesota, Minneapolis, Minn., using a AEI-MS 30 mass spectrometer or a Hitachi Perkin-Elmer RMU-6D instrument.

[6] Pierce Chemical Co., Rockford, Ill.

[7] Obtained many years ago from Eastman Chemical Co., Rochester, N.Y.

[8] Nutritional Biochemicals Corp., Cleveland, Ohio.

Table VI—Enantiomeric Bases

Compound	Resolution Reference	Resolving Acid (Solvent)	Yield, %	Melting Point	$[\alpha]_D^{28}$	Literature Melting Point	$[\alpha]_D$
(+)-(*S*)-Laudanosine	13	*O,O*-Di-*p*-toluoyltartaric [ethyl acetate–acetone (7:3)]	82	109–110°	55.4 (c 1, $CHCl_3$)	109° (13)	56.8 ($CHCl_3$)
(−)-(*R*)-Laudanosine		—	54	110°	−55.2 (c 1, $CHCl_3$)	109° (13)	−56.8 ($CHCl_3$)
(+)-(*R*)-Salsolidine	21	*O,O*-Dibenzoyltartaric (ethanol)	35	Liquid	60 (c 1, C_2H_5OH)	—	60.3 (C_2H_5OH) (21)
(−)-(*S*)-Salsolidine		—	40	Liquid	−59 (c 0.5, C_2H_5OH)	—	−59.9 (C_2H_5OH) (61)
(+)-(*R,R*)-*N*-Methylpavine	19	Tartaric acid (ethanol)	64	151–153°	209 (c 1, C_2H_5OH)	151–153° (19)	207.8 (C_2H_5OH)
(−)-(*S,S*)-*N*-Methylpavine		—	66	151–152°	−209 (c 1, C_2H_5OH)	152–153° (19)	−209 (C_2H_5OH)
(+)-(*S*)-Glaucine[a]	23	None[b]	—	118–120°	115 (c 0.5, C_2H_5OH)	119–120° (63)	115 (C_2H_5OH)
(−)-(*R*)-Glaucine		(+)-Tartaric (ethanol)	56	Oil[c]	−115 (c 0.5, C_2H_5OH)	—	−115 (C_2H_5OH) (23)
(+)-(*S*)-Corydine[a]	62	None[b]	—	147–149°	204 (c 2, $CHCl_3$)	149° (62)	204 ($CHCl_3$) (63)
(−)-(*R*)-Corydine		(+)-Tartaric (water)	45	146–148°	−205 (c 2, $CHCl_3$)	149° (62)	−206 ($CHCl_3$)
(+)-(*S*)-Isocorydine[a]	—	None[b]	—	182–184°	194 (c 0.5, $CHCl_3$)	185° (63)	195 ($CHCl_3$)
(−)-(*R*)-Isocorydine		(+)-Tartaric (50% aqueous ethanol)	15	Oil	−194 (c 0.5, $CHCl_3$)	—	—
(+)-(*S*)-Boldine[a,d]	—	None[b]	—	160–163°	112 (c 0.5, CH_3OH)	161–163° (63)	114 (C_2H_5OH)
(−)-(*R*)-Boldine[d]		(+)-*O,O*-Di-*p*-toluoyl-(−)-tartaric (ethyl acetate–acetone)	24	160–163°	−111 (c 0.5, CH_3OH)	—	

[a] Physical data and literature values were obtained and are reported for these commercially available, naturally occurring (*S*)-enantiomers for the purpose of comparison with the unnatural (*R*)-enantiomers obtained by deracemization. [b] Naturally occurring alkaloids not requiring deracemization and obtained from commercial sources. [c] All other physical and spectral data were in accord with the literature values. Since the next preparative step involved simple nitrogen quaternization, the pursuit of crystalline material seemed more reasonable after this next reaction step; extraordinary attempts to produce crystalline material were not pursued at this stage. [d] Literature data and the values obtained in this work are for boldine obtained from chloroform solutions.

Table VII—Probe Compounds

Compound	Yield, %	Melting Point	$[\alpha]_D^{28}$	Literature Melting Point	$[\alpha]_D$
I	90	218–220° dec.	118 (c 1, C_2H_5OH)	218–221° dec. (64)	120 (C_2H_5OH)
II	90	216–219° dec.	−117 (c 1, C_2H_5OH)	218–221° dec.	−120 (C_2H_5OH)
III	91	273–276°	−204 (c 0.5, 50% aq. CH_3OH)	270–280° (60)	—[a]
IV	91	272–276°	204 (c 0.5, 50% aq. CH_3OH)	270–280°	—[a]
V	68	198–202° dec.	10.6 (c 1, C_2H_5OH)	211° dec. (in a sealed tube) (63)	—[a]
VI	52	196–200° dec.	−10.7 (c 1, C_2H_5OH)	211° dec. (in a sealed tube)	—[a]
VII	69	220–222° dec.	78 (c 0.5, C_2H_5OH)	224–225° (60)	72.5
VIII	65	223–224° dec.	−77 (c 0.5, C_2H_5OH)	224–225°	−72.5
IX	64	212–214° dec.	136 (c 0.8, $CHCl_3$)	228–230° dec. (62)	—[a]
X	43	214–216° dec.	−138 (c 0.8, $CHCl_3$)		—[a]
XI	75	239° dec.	148 (c 0.3, H_2O)	231–232° (rapid heating) (60)	139.9 (62) 143.3
XII	70	236° dec.	−146 (c 0.5, H_2O)		—[a]
XIII	31	178–182°	5.4 (c 0.5, CH_3OH)	"...boldine...does not yield crystalline salts" (62)	—[a]
XIV	28	180–182°	−5.2 (c 0.5, CH_3OH)		—[a]

[a] Since optical rotation data were not found in the literature for these compounds, their analyses are reported in Table VIII.

Table VIII—Elemental Analyses

Probe	Formula	Analysis, % Calculated C	H	I	N	Found C	H	I	N
III	$C_{22}H_{28}INO_4 \cdot 2H_2O$	49.54	6.02	23.84	2.63	49.70	5.88	23.64	2.50
IV						49.67	5.84	23.96	2.55
V	$C_{14}H_{22}INO_2$	46.28	6.06	34.99	3.86	46.38	5.82	34.82	3.66
VI						46.16	5.85	35.16	3.69
IX	$C_{21}H_{26}INO_4 \cdot 2H_2O$	48.55	5.78	24.47	2.69	48.10	5.43	23.88	2.50
X						48.65	5.77	24.67	2.48
XII	$C_{21}H_{26}INO_4$	52.17	5.38	26.29	2.90	52.07	5.13	26.10	2.79
XIII	$C_{20}H_{24}INO_4$	51.13	5.11	27.06	2.98	51.09	4.99	26.89	2.77
XIV						51.10	4.85	27.33	2.76

Quaternization of Enantiomeric Bases—Simple treatment of a methanolic solution of the base with excess methyl iodide was the general procedure. In the case of the salsolidines (secondary amines), it was necessary to buffer the reaction carefully with sodium bicarbonate to take up the elaborated hydrogen iodide to permit complete quaternization.

Table VII records the yields and physical constants for these final compounds. Elemental analyses for previously unreported compounds are recorded in Table VIII.

Neuromuscular Junction Blocking Bioassay—Male cats, 2.25–4 kg, were anesthetized by intraperitoneal injection of either sodium pentobarbital or allobarbituric acid. The femoral artery and vein were exposed by surgery and cannulated. The arterial cannula was connected to a force transducer[9], which was coupled[10] to a recording polygraph[11]. An accurate tracing of the animal's blood pressure was thus obtained and used to ascertain the general condition of the specimen and the depth of anesthesia. Finally, it was used for verification of death which was effected with intravenous administration of a saturated potassium chloride solution at the completion of the experiment.

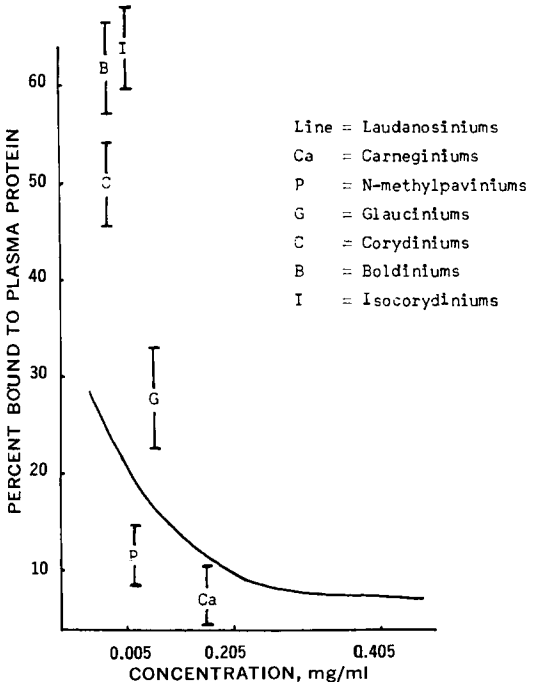


Figure 4—*Relationship of protein binding between different drug families.*

The venous cannula, intended for drug administration, led to a three-way stopcock, which allowed very small volumes of probe solutions to be flushed into the animal *via* another larger syringe containing normal saline. A tracheotomy was performed and the animal respired comfortably with the assistance of a respirator[12] (16–20 strokes/min; 20–30 ml/stroke). The hypoglossal nerve was surgically exposed, and an electrode was placed under the nerve so that a supramaximal (1–5 v) square (1-msec duration, 4.5-sec delay) pulse was given by an electrical stimulator[13]. The tongue muscle was tied with surgical thread leading to a strain-gauge transducer[14] and, *via* a coupler[10], allowed for muscle-twitch–pen-flick recording on the polygraph (Fig. 1). All drug solutions were prepared in normal saline, and the administration protocol followed the Latin-square method described previously.

Data Regression—Raw data, as percent decrease from control response, were converted to probit values. These values *versus* log dose values were used in a linear regression program to generate computer-derived lines having 95% confidence limits associated with their error parameters. From the line and error parameters, the following computer programmed calculations were performed: an ED_{50} with 95% confidence limits, a statistical comparison of the slopes between the different drugs, and a comparison between the ED_{50} values in an indirect manner *via* a statistical comparison of the line elevations between different drugs.

Plasma Protein Binding Assay—The equilibrium dialysis setup was identical to that described by Abdel-Monem *et al.* (47). Plasma proteins were obtained from cat whole blood by using the supernate after centrifugation (2000×*g* for 20 min at 25°). All solutions were prepared from isotonic pH 7.4 phosphate buffer solution; the drug concentrations are specified in Table III.

UV methods were used for the assay of probe concentration in the external medium. Appropriate control runs were performed to account for drug losses from the external medium due to simple dilution and dialysis membrane binding (Fig. 3). Dialyses were run for 48 hr (double the time required for equilibrium). The concentration assay was performed before and after dialysis and the difference, after accounting for dilution and membrane binding losses, was used to calculate the percent bound to plasma proteins. Each drug was studied in triplicate, and the data were treated as an average value plus or minus an error limit, which would account for 100% of the data.

Red Blood Cell Binding Assay—Red blood cells were obtained from cat whole blood by four repetitions of centrifugation (2000 × *g* for 20 min at 25°), washing with isotonic pH 7.4 phosphate buffer solution, and, finally, dilution with buffer solution to approximately the same volume of whole blood from which they were obtained. The red blood cell solutions and drug solutions were equilibrated for 8 hr (double the time required for equilibration); then the red blood cells, with bound drug, were removed by centrifugation. The probe concentrations employed are specified in Table III.

UV assays were used to determine probe concentration before

[9] Beckman 215071.
[10] Beckman type 9853 coupler.
[11] Beckman type R-411 dynograph.
[12] Harvard apparaus respirator for small animals.
[13] Grass S44.
[14] Statham model UC-3.

and after equilibration. The difference, after accounting for dilution effects, was used to calculate the percent of probe bound to the red blood cells. Each probe was studied in triplicate, and the data were treated in exactly the same manner as for the plasma protein binding study.

Acetylcholinesterase Inhibition Studies—The pH–stat method was previously described (49–52). Experiments were performed with an automatic titrator[15] with recording features. Each run employed 0.35 unit[4] of eel acetylcholinesterase[16]. Five concentrations of acetylcholine chloride[17], varying from 1.0 to 5.0 × 10^{-4} *M*, were used as substrate. The titrant solution was 0.0005 *N* NaOH solution.

Three inhibitor concentrations were used with at least four substrate concentrations so that a minimum of 12 runs was performed for each probe. Inhibitor concentrations were in the range of 2–15 × 10^{-4} *M* and were such that an observable decrease in enzymatic velocity was obtained. All solutions were prepared from doubly distilled deionized water and contained 0.02 *M* $MgCl_2$ and 0.1 *M* NaCl. The enzyme solution also contained 0.005% bovine serum albumin.

All runs were performed at 28°. Aqueous hydrolysis of acetylcholine (51) was found to be insignificant, as was the need for a stream of nitrogen (50) in the reaction vessel. The data regression is described under *Biology*.

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[15] Copenhagen Radiometer automatic titrator setup employing a type SBR 2C titrigraph, a type TTT2 titrator, and a type ABU 12 autoburet.

[16] Product No. 5310, code ECHP, Worthington Biochemical Corp., Freehold, N.J.

[17] Sigma Chemical Co., St. Louis, Mo.

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Stereochemical Preferences for Curarimimetic Neuromuscular Junction Blockade II: Enantiomeric Bisquaternary Amines as Probes

ALMOURSI A. GENENAH [*], TAITO O. SOINE [x], and NADIM A. SHAATH

Abstract □ Two pairs of bisquaternary enantiomeric neuromuscular junction blocking agents as well as their diastereomeric *meso*-forms were prepared in which the carbon asymmetry is adjacent to the quaternary center. The tertiary amines from which the blocking species were obtained by methyl iodide treatment were *N*-methylpavine and 1,1′-dodecamethylenebis(6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline). Blocking potencies were determined by the mouse inclined screen assay and by the cat tongue–hypoglossal nerve technique. The mouse assay showed no statistical difference between the enantiomeric probes derived from *N*-methylpavine and only a modest superiority of the *(R–R)* isomer over the *(S–S)* isomer in the case of the tetrahydroisoquinoline compounds. The cat assay showed a modest statistically significant *(R–R)* > *(S–S)* difference in potencies in both kinds of probes. The diastereomeric *meso*-compounds were less active than the enantiomers in mice but were of intermediate activity in the cat determination. Acetylcholinesterase-inhibiting activity was determined for each probe to discount potency differences from this source, and no significant differences in blocking potency attributable to preferential enzyme inhibition by the probes were noted.

Keyphrases □ Curarimimetic neuromuscular junction blockade—stereochemical preferences, enantiomeric bisquaternary amines as probes □ Neuromuscular junction blockade, curarimimetic—stereochemical preferences, enantiomeric bisquaternary amines as probes □ *N*-Methylpavine (enantiomeric bisquaternary amines)—probes for stereochemical preferences for curarimimetic neuromuscular junction blockade □ 1,1′-Dodecamethylenebis(6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline (enantiomeric bisquaternary amines)—probes for stereochemical preferences for curarimimetic neuromuscular junction blockade

The initial report (1) from these laboratories concerning the possibility of stereochemical preferences being exhibited at the neuromuscular junction toward nondepolarizing blocking agents of the curare type was concerned with several monoquaternary enantiomeric probes derived from alkaloids related to tetrahydroisoquinoline. These studies examined the possibility that the exclusive, but modest, *(S)* > *(R)* (about 1.8:1) blocking potency difference shown by the cat assay could have been due to stereopreferential binding of the probes by blood components and/or by stereoselective acetylcholinesterase inhibition since other factors such as absorption, excretion, and metabolism differences were unlikely due to the rapid onset of block. The blood binding studies on both plasma protein and red blood cells indicated a low order of binding and a reversed order from that which might account for potency differences. The stereoselective inhibition of acetylcholinesterase, while it had the correct orientation in some cases, was of such a low order of activity and so random in its focus that it could not be seriously considered as causal for the observed potency differences.

DISCUSSION

Since neuromuscular junction blockers have traditionally been thought of as bisquaternaries, in spite of the recent disclosure that (+)-tubocurarine is actually a monoquaternary (2), it seemed appropriate to test neuromuscular junction stereochemical preferences on these types to see whether the preferences determined for monoquaternaries extended to the bisquaternaries. Unpublished observations in these laboratories had shown that quaternization of (±)-*N*-methylpavine (I) with 1,10-diiododecane produced a potent neuromuscular junction blocking agent (II) comparable in activity to (+)-tubocurarine. Therefore, the enantiomeric forms (II*a* and II*b*) of this quaternary blocker as well as the *meso*-form (II*c*) were considered to be suitable probes in the determination of stereochemical preferences at the neuromuscular junction for blocking agents.

Stereochemical preferences can only be considered valid when made between enantiomeric forms since diastereomers have different physical and chemical properties whereas enantiomers only differ in rotatory effect on polarized light. On the other hand, the fact that enantiomers may be operating in an asymmetric biological environment necessitates giving attention to preferential plasma protein and/or red blood cell binding as well as to stereopreferential acetylcholinesterase inhibition. Previous studies (1) demonstrated that blood components probably need not be considered as causal for differences in activity. However, acetylcholinesterase inhibition cannot be ruled out as a possible factor, even though it was inoperative in the case of the monoquaternaries, because it is

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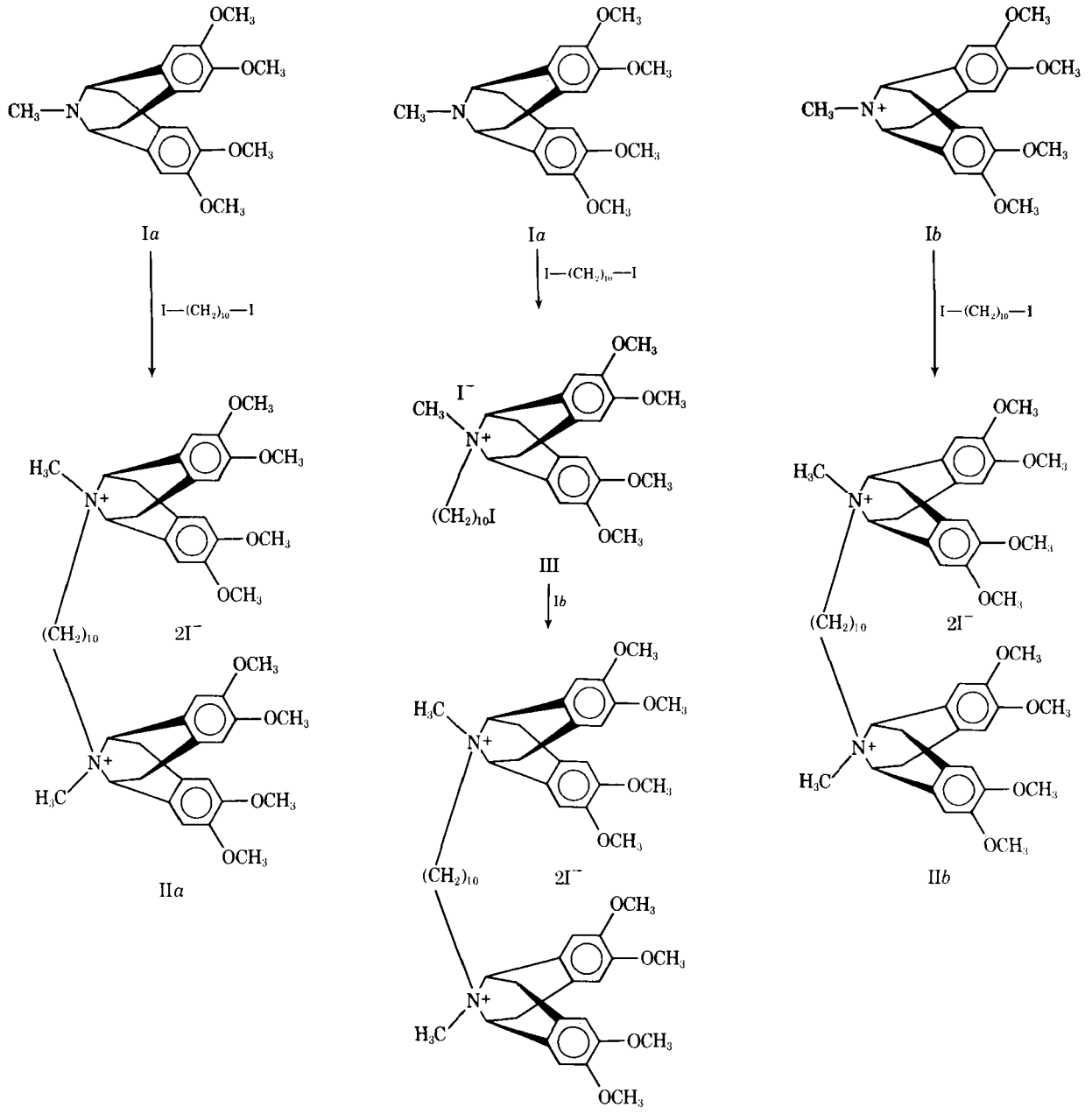


Scheme I

well known that benzoquinonium[1], a bisquaternary, has marked enzyme inhibitory properties (3).

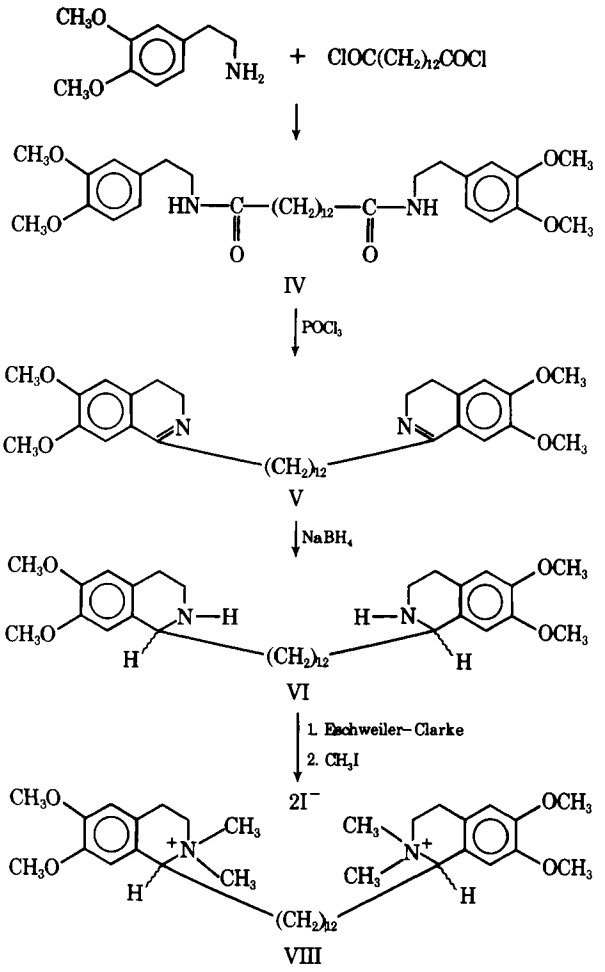
The *N*-methylpavine enantiomers were prepared according to the procedures elaborated in the literature (4, 5). The enantiomeric quaternary probes (II*a* and II*b*) were prepared by treatment of the appropriate enantiomer with 1,10-diiododecane; the preparation of the *meso*-form (II*c*) required the intermediate preparation of (+)-*(R)*-*N*-methylpavine-*N*-(10-iododecane) iodide (III), which was then reacted with (−)-*(S)*-*N*-methylpavine to achieve the desired *meso*-product (II*c*). The reactions are summarized in Scheme I.

The selection of the quaternary derivative derived from 1,1′-dodecamethylenebis(6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline) (VIII) as a useful probe was based on the report of its activity by Smith *et al.* (6) and the obvious opportunity to use it as an enantiomeric probe. Smith *et al.* indicated only that activity differences existed between two fractions from the synthetic process which had differing solubilities, obviously the racemic pair and the *meso*-form. The possibility for exploring enantiomeric differences of the racemate as well as the fact that this paper has been cited as an example of such differences (7) suggested that it be an appropriate candidate.

The chemical preparation of VIII (said to be in press) has not appeared to date, although it is apparent that these kinds of compounds are readily prepared through the reactions shown in Scheme II with, initially, formation of an appropriate diamide (IV) by condensation of 1,12-dodecanedicarboxylic acid chloride with homoveratrylamine. Under Bischler–Napieralski ring closure conditions (8), the bis ring closure occurs to yield the appropriate 3,4-dihydroisoquinoline (V). Reduction of V with methanolic sodium borohydride and conversion to the hydrochloride salt led to a mixture of racemic and *meso*-1,1′-dodecamethylenebis(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline) dihydrochlorides (VI). The separation of the two forms was not as easily accomplished by simple water recrystallization to effect separation on a solubility basis as was suggested by Smith *et al.* (6). The experience of the present authors was that absolute ethanol was a far better differentiating agent in a solubility sense, as indicated by the experimental results.

To distinguish between the *meso* and racemic fractions, the free

[1] Mytolon.



Scheme II

base of each was regenerated and converted to the bi[(−)-*O,O*-di-*p*-toluoyltartrate] salt and then subjected to recrystallization from different solvents and solvent combinations. Only the salts from the racemic form (arbitrarily designated as the A fraction) showed marked changes in both melting point and optical rotation when recrystallized from a specific solvent mixture [ethanol–ethyl acetate–acetone (1:2:2)], and it was concluded that this form was the one that could be resolved. Indeed, continued recrystallization of this salt from the same solvent system resulted in a product that had no change in melting point or rotation and which, when treated appropriately with base, yielded the (+)-isomer (VI*a*) as an oil. Treatment of the mother liquors by formation of the bitartrate of the isomeric (+)-*O,O*-di-*p*-toluoyltartaric acid, using the same recrystallization methods, resulted in the enantiomeric salt being obtained, which yielded the corresponding (−)-isomer (VI*b*) as an oil in the same manner.

At this point, the observations of Battersby and Edwards (9) were pertinent to the problem of determining the absolute configurations of the two enantiomers. The experience of these authors indicated rather convincingly that a correlation exists between optical rotation and absolute configuration if it can be shown that a specific shift in rotation can be correlated with a change in solvent polarity. In short, if a positive shift in rotation under the specified conditions can be identified, the compound has an *(S)*-configuration and vice versa. Table I shows the results obtained with the two isomers under consideration and certainly indicates that the (+)-isomer possesses the *(R)*-configuration and that the (−)-isomer possesses the *(S)*-configuration. Establishment of the absolute configurations led to the *N*-methylation of the corresponding secondary amines (VI*a* and VI*b*) by the Eschweiler–Clarke method to the corresponding oily enantiomers, which were then converted to the desired probes (VIII*a* and VIII*b*) by treatment with methyl iodide. Similar treatment of the *meso*-form (Fraction B) yielded the necessary quaternary probe (VIII*c*).

Table I—Effect of Solvent Polarity on Optical Rotation of Enantiomeric 1,1′-Dodecamethylenebis(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinolines)

	Molecular Rotation in			
Enantiomer	C_6H_6	$CHCl_3$	CH_3OH	Amine·2HCl CH_3OH
VI*a*	+16.6	+15.5	+2.8	0.0
VI*b*	−16.0	−12.1	−2.5	0.0

The pharmacological results encompass both a mouse inclined screen test and the cat tongue–hypoglossal nerve assay method. The mouse test (10) was initially preferred because it was simple, relatively inexpensive, reasonably fast, and adaptable to statistical analysis. However, cat data may be more meaningful (1). The relative potency ratios were calculated using 100 as the standard potency of (+)-tubocurarine. Analyses of the dose–response data for each individual compound showed no significant deviation from parallelism of the dose–response curves observed among the three compounds in each series or between each compound and (+)-tubocurarine. The test for parallelism was important since nonparallelism would imply a different mechanism of action which would, of course, negate any finding of potency differences.

Tables II and III give the ED_{50} values and potency ratios (based on ED_{50}) for (+)-tubocurarine and the compounds synthesized for this study. The results show that, in mice, no significant difference for the *N*-methylpavine-derived probes could be detected between the enantiomers, although both were significantly more potent than the *meso*-isomer. With the 1,1′-dodecamethylenebis(6,7-dimethoxy-2,2-dimethyl-1,2,3,4-tetrahydroisoquinolinium) iodides, the *(R–R)*-isomer was approximately twice as potent as its enantiomer and both were more potent than the *meso*-form [*i.e.*, the *(R–S)* form]. In the cat assay (Table III), a more definitive predominance of the *(R–R)*-enantiomer was evident although, again, the ratio approximates 2:1. It is interesting that, in the cat assay, the diastereomeric *(R–S)* form adopts a potency position intermediate between the enantiomers that is significantly different than the mouse assay data, which show the *(R–S)* forms being less active in both cases.

Assessing all data presented in these experiments leads to the inescapable conclusions that the *(R–R)* bisquaternaries tend to have a greater neuromuscular junction blocking potency than the *(S–S)* forms and that the potencies of the diastereomeric *(R–S)* forms are dependent on the animal used for assay.

The differences in cited activity were also examined in the light of the ability of these compounds to inhibit acetylcholinesterase which, undoubtedly, could influence the gross measurement of neuromuscular junction blocking activity. The methods were described previously (1), and it was determined that there were no significant differences in blocking ability by the various isomers (Table IV). On this basis, it must be concluded that there is a modest but significant predominance in blocking activity by the *(R–R)* absolute configuration in these types of bisquaternaries. This finding is exactly the reverse of the findings with monoquaternary enantiomeric probes (1). The significance of this difference is not apparent at this time.

EXPERIMENTAL[2]

Preparation of Enantiomeric *N*-Methylpavines—The *N*-methylpavines (I) were prepared according to the published procedure (3, 4). The racemic base was resolved by (+)-L-tartaric acid

[2] Melting points were determined on a Thomas-Hoover melting-point apparatus and are uncorrected. TLC was conducted on Eastman chromagram sheet 6060 silica gel, and visualization was done with both UV lamp and iodine vapor. Elemental analyses were performed by M-H-W Laboratories, Garden City, Mich. Optical rotations were measured on a Perkin-Elmer 141 polarimeter. IR spectra were determined in mineral oil or KBr with a Perkin-Elmer 237B grating IR spectrophotometer. NMR spectra were measured with a Varian Associates model A-60D NMR spectrometer. Mass spectral determinations were performed by Mass Spectrometry Laboratory Services, Department of Chemistry, University of Minnesota, Minneapolis, MN 55455, using an AEI-MS30 mass spectrometer or a Hitachi Perkin-Elmer RMU-6D mass spectrometer.

Table II—ED_{50} and Potency Ratios in the Mouse Assay for Neuromuscular Junction Blocking Potency

Compound	ED_{50}, mg/kg	Potency Ratios [(+)-Tubocurarine = 100]
(+)-Tubocurarine	0.34	100
II*a*	0.45	76
II*b*	0.42	81
II*c*	0.72	47
VIII*a*	2.00	17
VIII*b*	3.80	9
VIII*c*	5.60	6

to afford (+)-*(R)*-*N*-methylpavine (I*a*), mp 150–151°, $[\alpha]_D^{25}$ +211° (c 1.0, C_2H_5OH), and by (−)-D-tartaric acid to afford (−)-*(S)*-*N*-methylpavine (I*b*), mp 151–153°, $[\alpha]_D^{25}$ −210° (c 1.0, C_2H_5OH). These values were in excellent agreement with literature values (4).

***N,N'*-Decamethylenebis[(+)-*(R)*-*N*-methylpavinium Iodide] (II*a*)**—1,10-Diiododecane (1 g, 0.0025 mole) and (+)-*N*-methylpavine (3.5 g, 0.01 mole) were dissolved in dry benzene (30 ml) and then refluxed in an oil bath at 95° for 5 days. Ethanol was occasionally added dropwise to keep the reaction mixture homogeneous, and then the mixture was cooled in a refrigerator for 2 days. The solid cake which separated was obtained by decantation, crushed, and washed twice with cold benzene. Attempted crystallization by dissolving in methanol and adding ether failed to give a crystalline product; but the resulting gummy precipitate, when rubbed with a glass rod, consistently gave a white solid, mp 178–181° dec., $[\alpha]_D^{25}$ +186° (c 1.0, CH_3OH).

Anal.—Calc. for $C_{52}H_{70}I_2N_2O_8$: C, 56.52; H, 6.34; N, 2.54. Found: C, 56.25; H, 6.41; N, 2.42.

***N,N'*-Decamethylenebis[(−)-*(S)*-*N*-methylpavinium Iodide] (II*b*)**—This compound was prepared in the same manner as the (+)-isomer by refluxing 1,10-diiododecane (1.0 g, 0.0025 mole) and the (−)-*N*-methylpavine (3.5 g, 0.01 mole) in dry benzene for 5 days to give 2.0 g of product, mp 181–182° dec., $[\alpha]_D^{25}$ −190° (c 1.0, CH_3OH).

Anal.—Calc. for $C_{52}H_{70}I_2N_2O_8$: C, 56.52; H, 6.34; N, 2.54. Found: C, 56.26; H, 6.20; N, 2.36.

(+)-*(R)*-*N*-Methylpavine-*N*-(10-iododecane) Iodide (III)—(+)-*N*-Methylpavine (1.0 g) was dissolved in anhydrous benzene (10 ml) and added dropwise to a refluxing solution of 1,10-diiododecane (5 g) in dry benzene (30 ml) in an oil bath at 95° with vigorous stirring and refluxing. The addition was completed in 6 hr; the reaction mixture was allowed to reflux for 2 days, cooled to room temperature, and stored in a refrigerator for 2 days. The solid layer was separated by decantation, crushed, washed twice with cold benzene, filtered, and dried. The solid residue was lixiviated with methanol–ether (1:1), and the insoluble residue was removed by filtration. Evaporation of the filtrate and recrystallization of the residue by dissolving in methanol and dropping into a large excess of ether yielded 0.6 g of a white crystalline powder, mp 129–132° (prior softening at 115°), $[\alpha]_D^{25}$ +150° (c 1.0, CH_3OH).

Anal.—Calc. for $C_{31}H_{45}I_2NO_4$: C, 49.66; H, 6.01; I, 33.9; N, 1.87. Found: C, 50.34; H, 6.21; I, 33.1; N, 2.13.

This was the best analysis achieved and was not improved by additional attempts at purification.

***meso*-*N,N'*-Decamethylenebis(*N*-methylpavinium Iodide) (II*c*)**—Compound III (0.4 g, 0.00053 mole) was dissolved in ethanol together with (−)-*N*-methylpavine (1.0 g, 0.0028 mole), refluxed in an oil bath for 4 days at 95°, cooled, and added dropwise to a large excess of ether. The precipitate was collected, dissolved in methanol, and added dropwise to a large excess of ether. The process was repeated three times to yield 0.3 g of a white powder, mp 179–181° (with decomposition and prior softening at 165°) $[\alpha]_D^{25}$ +26.7° (c 1.0, CH_3OH).

Anal.—Calc. for $C_{52}H_{70}I_2N_2O_8$: C, 56.52; H, 6.34; I, 23.0; N, 2.54. Found: C, 56.41; H, 6.28; I, 23.2; N, 2.47.

1,12-Dodecamethylenebis[2-(3',4'-dimethoxyphenyl)ethylamide] (IV)—2-(3',4'-Dimethoxyphenyl)ethylamine (36 g, 0.2 mole) was dissolved in benzene (500 ml) in a 2-liter flask fitted with a condenser, a mechanical stirrer, and an addition funnel containing 1,12-dodecanedicarboxylic acid chloride [prepared from 1,12-dodecanedicarboxylic acid (26 g) and thionyl chloride (70 ml) in benzene]. The acid chloride solution was added dropwise to the amine solution with vigorous stirring and, after the addition was completed, the reaction mixture was allowed to cool to room temperature and filtered. The residue was recrystallized twice from ethanol to yield 54.6 g of white crystals (93%), mp 152–153°; IR (cm^{-1}): 1642 (C=O) and 3310 (N—H).

Table III—ED_{50} and Potency Ratios in Cat Assay for Neuromuscular Junction Blocking Potency

Compound	ED_{50}[a], mg/kg	Potency Ratios [(+)-Tubocurarine = 100]	ED_{50} Potency Ratio of Isomers[b] (Most Potent = 1)
(+)-Tubocurarine	0.15	100	—
II*a*	0.010	1500	1.0
II*b*	0.021	714	2.1
II*c*	0.019	790	1.9
VIII*a*	0.320	47	1.0
VIII*b*	0.780	19	2.4
VIII*c*	0.530	28	1.7

[a] These values are with a 95% confidence limit. [b] A statistical comparison was made between the line elevations of the isomers to evaluate the significance of the difference in ED_{50} values. For the series II*a*, II*b*, and II*c*, the values of F_{el} (*dF'*, *dF*) were 1.95 (2, 36); for the series VIII*a*, VIII*b*, and VIII*c*, the values were 0.83 (2, 32). In both cases the slopes between the isomers were not statistically different and, thus, the observed potency ratios are real and statistically different.

Anal.—Calc. for $C_{34}H_{52}N_2O_6$: C, 69.86; H, 8.90; N, 4.79. Found: C, 70.08; H, 8.75; N, 4.82.

1,1'-Dodecamethylenebis(6,7-dimethoxy-3,4-dihydroisoquinoline) (V)—The preceding diamide (IV) (50 g, 0.086 mole) was dissolved in dry chloroform (300 ml), and phosphorus oxychloride (50 ml) was added. The mixture then was refluxed in an oil bath at 75° for 3 hr and poured into ice water. The mixture was made alkaline with 10% aqueous sodium hydroxide and then extracted with chloroform (5 × 250 ml). The chloroform extract was washed with water, dried, and evaporated to dryness. The residue was recrystallized from benzene to yield 40 g (85%) of white crystals, mp 104–105°. The IR and NMR data were compatible with the expected structure.

Anal.—Calc. for $C_{34}H_{48}N_2O_4$: C, 74.45; H, 8.76; N, 5.11. Found: C, 74.51; H, 8.69; N, 5.21.

1,1'-Dodecamethylenebis(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline) Dihydrochlorides (VI)—Compound V (35 g) was dissolved in methanol containing 1% water (300 ml), and the resulting solution was then cooled in an ice bath. Sodium borohydride (30 g) was added to this solution in small portions with stirring while not allowing the temperature to rise above 5°. Upon completion of the addition, the continuously stirred solution was allowed to stand at room temperature for 30 min and then refluxed on a steam bath for 2 hr. It was then cooled to room temperature

Table IV—Acetylcholinesterase Inhibition by Enantiomeric Neuromuscular Junction Blockers

Compound	Wilkinson K_i[a], $\times 10^{-4}$ *M*	Wilkinson V_{max}, mole/unit/min	K_i Ratio between Enantiomers
Acetylcholine	2.56 ± 0.02 (K_m)	1.008 ± 0.05	
(+)-Tubocurarine	4.29 ± 0.40	1.55 ± 0.70	
II*a*	0.75 ± 0.10	0.53 ± 0.10	0.914:1.00
II*b*	0.82 ± 0.08	0.55 ± 0.05	
VIII*a*	8.27 ± 0.04	0.26 ± 0.01	0.779:1.00
VIII*b*	10.62 ± 0.06	0.31 ± 0.03	

[a] Determined by calculation from computer program (Wilkinson) generated, altered K_m values.

and the solvent was removed under reduced pressure. The residue was suspended in water (100 ml), made alkaline with 10% aqueous sodium hydroxide, and extracted with chloroform (5 × 150 ml). The chloroform extract was washed with water, dried, and stripped of solvent under reduced pressure to leave an oily residue (33.7 g, 96%) which, when stored in a refrigerator overnight, was converted into a soft solid. This soft solid, dissolved in 200 ml of anhydrous ether, was treated with dry hydrogen chloride gas with continuous stirring until precipitation ceased. The precipitate was removed by filtration and recrystallized from ethanol to yield 33.0 g of white crystals, mp 142–144°.

Anal.—Calc. for $C_{34}H_{54}Cl_2N_2O_4$: C, 65.28; H, 8.64; Cl, 11.36; N, 4.48. Found: C, 65.40; H, 8.59; Cl, 11.51; N, 4.39.

Separation of *meso* and Racemic 1,1′-Dodecamethylenebis(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline) Dihydrochlorides (VI)—The separation was carried out be repeated recrystallizations of the VI dihydrochloride salt from absolute ethanol until no further change in the melting points of the differently soluble fractions was observed. The fraction least soluble in absolute ethanol (A), mp 199–201°, provided a yield of 6.0 g; the more soluble fraction (B), mp 138–141°, provided a yield of 5.4 g.

Identification of the Racemic and *meso* Species—The free bases of Fractions A and B were obtained by dissolving 5 g of each salt separately in 50 ml of water and then alkalinizing with 10% aqueous sodium hydroxide followed by ether extraction (4 × 50 ml) to give an ethereal solution which was dried and stripped of solvent. Each oily residue (2.0 g, 0.0036 mole) was dissolved in ethanol (15 ml) and added to 15 ml of a hot alcoholic solution of (−)-*O,O*-di-*p*-toluoyltartaric acid (2.8 g, 0.0072 mole), and the resulting mixture was allowed to cool overnight in a refrigerator. The solid cake in each case was then separated and dried. The salt from Fraction A was a white solid, mp 133–135°, $[\alpha]_D^{25}$ −79.5° (c 1.0, CH_3OH); the salt of Fraction B was a semisolid and solidified only after several washings with ether to give a product, mp 130–132°, $[\alpha]_D^{25}$ −77.9° (c 1.0, CH_3OH).

Recrystallization of both salts from ethanol–ethyl acetate–acetone (1:2:2) separated a nicely crystalline solid only from the salt of Fraction A, whereas the salt from Fraction B always formed a soft semisolid cake. Repeated recrystallization of the salt of Fraction A finally resulted in 0.6 g of a white crystalline solid (VI-A), mp 155–157°, $[\alpha]_D^{25}$ −65.8° (c 1.0, CH_3OH).

Anal.—Calc. for $C_{74}H_{88}N_2O_{20}$: C, 67.07; H, 6.65; N, 2.11. Found: C, 66.96; H, 6.61; N, 2.02.

(+)-1,1′-Dodecamethylenebis(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline) (VI*a*)—Compound VI-A (0.5 g) was suspended in water, alkalinized with 10% aqueous sodium hydroxide, and extracted with ether. The ethereal extract was then washed with water, dried, and evaporated to dryness, leaving 0.2 g of an oily residue, $[\alpha]_D^{25}$ +2.5 ± 0.1° (c 1.0, $CHCl_3$). Its dihydrochloride showed a melting point of 199–201° and $[\alpha]_{365}^{25}$ of −421 ± 0.5° (c 1.0, CH_3OH).

(−)-1,1′-Dodecamethylenebis(6,7-dimethoxy-1,2,3,4-tetrahydroisoquinoline) (VI*b*)—The mother liquors left from the recrystallization of VI-A were collected and stripped of solvent; the residue was dissolved in water, alkalinized with 10% aqueous sodium hydroxide, and extracted with ether. The ethereal extract was washed and dried, and the solvent was evaporated to leave an oily residue (1.5 g). This oily residue was dissolved in 15 ml of ethanol, added to 15 ml of a hot alcoholic solution of (+)-*O,O*-di-*p*-toluoyltartaric acid (2 g), and then left overnight in a refrigerator. The solid cake which formed was separated and recrystallized extensively in the same way as for VI-A to give 0.7 g of white crystalline solid, mp 154–156°, $[\alpha]_D^{25}$ +66.1° (c 1.0, CH_3OH).

Anal.—Calc. for $C_{74}H_{88}N_2O_{20}$: C, 67.07; H, 6.65; N, 2.11. Found: C, 67.10; H, 6.70; N, 2.21.

The free base was obtained in exactly the same way as for VI*a* to give 0.3 g as an oily residue, $[\alpha]_D^{25}$ −2.3 ± 0.1° (c 1.0, $CHCl_3$). Its dihydrochloride showed a melting point of 198–200° and a $[\alpha]_{365}^{25}$ of +395 ± 0.5° (c 1.0, CH_3OH).

(+)-1,1′-Dodecamethylenebis(6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline) (VII*a*)—Compound VI*a* (0.2 g) was dissolved in a cold mixture of 5 ml of 90% formic acid and 4 ml of 40% aqueous formaldehyde, and the solution was then heated on a steam bath for 16 hr. The reaction mixture was cooled to room temperature, treated with 5 ml of 4 *N* HCl, and evaporated to dryness under reduced pressure. The residue was then dissolved in water, made alkaline with 10% aqueous potassium hydroxide, and extracted with ether. The ethereal extract was washed, dried, and evaporated to leave an oily residue (0.18 g), $[\alpha]_D^{25}$ +3.2 ± 0.1° (c 1.0, CH_3OH).

Quaternization of VII*a* with methyl iodide in methanol afforded VIII*a* as a pale-yellow powder, which started decomposing at 220° and charred at about 350°, $[\alpha]_D^{25}$ +2.8° (c 1.0, CH_3OH).

Anal.—Calc. for $C_{38}H_{62}I_2N_2O_4$: C, 52.78; H, 7.17; I, 29.38; N, 3.24. Found: C, 52.53; H, 7.3; I, 29.2; N, 3.25.

(−)-1,1′-Dodecamethylenebis(6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline) (VII*b*)—Compound VI*b* (0.3 g) was methylated with formic acid and formaldehyde in the same manner as was VI*a* to yield finally 0.25 g of an oily product, $[\alpha]_D^{25}$ −3.1 ± 0.1° (c 1.0, CH_3OH).

Quaternization of VII*b* with methyl iodide gave VIII*b* as a pale-yellow powder, which started decomposing at 220° and charred at about 360°, $[\alpha]_D^{25}$ −2.8° (c 1.0, CH_3OH).

Anal.—Calc. for $C_{38}H_{62}I_2N_2O_4$: C, 52.78; H, 7.17; I, 29.38; N, 3.24. Found: C, 52.91; H, 7.05; I, 29.1; N, 3.29.

***meso*-1,1′-Dodecamethylenebis(6,7-dimethoxy-2-methyl-1,2,3,4-tetrahydroisoquinoline) (VII*c*)**—The *meso*-base was methylated in the same manner as VI*a* to yield an oily residue.

Quaternization with methyl iodide gave VIII*c* as a white powder, which started decomposing at about 210° and charred at about 340°.

Anal.—Calc. for $C_{38}H_{62}I_2N_2O_4$: C, 52.78; H, 7.17; I, 29.38; N, 3.24. Found: C, 52.99; H, 7.30; I, 29.20; N, 3.11.

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Stereochemical Preferences for Curarimimetic Neuromuscular Junction Blockade III: Enantiomeric Bisquaternary Amines Related to Benzoquinonium as Probes

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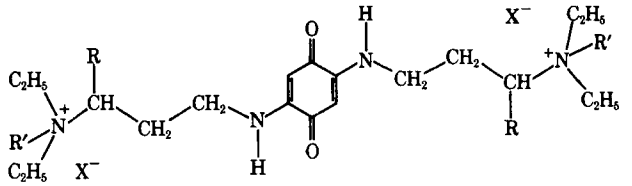
Abstract □ Enantiomeric neuromuscular junction blocking agents, which are of the benzoquinonium type but which have a methyl group introduced adjacent to the quaternary moieties to provide an asymmetric center, were synthesized and tested to determine whether the neuromuscular junction exhibits the relatively modest *(R) > (S)* superiority shown toward previously tested bisquaternaries. Testing included a mouse inclined screen assay and an *in vivo* cat hypoglossal nerve–tongue preparation, as well as standard estimations of anticholinesterase activity since the candidate compounds are known to have such a component in their activity spectrum. The observed 2:1 difference in blocking activity favoring the compound with an *(R)*-configuration is the same as that for previously tested bisquaternaries, both in direction and magnitude. Furthermore, it cannot be accounted for by preferential inhibition of acetylcholinesterase by the *(S)*-enantiomer. Absolute configurations of the enantiomers were assigned on the basis of comparisons with compounds of known configuration.

Keyphrases □ Curarimimetic activity—stereochemical preferences for neuromuscular junction blockade, enantiomeric bisquaternary amines related to benzoquinonium as probes □ Neuromuscular junction blockade (curarimimetic)—stereochemical preferences, enantiomeric bisquaternary amines related to benzoquinonium as probes □ Benzoquinonium—related enantiomeric bisquaternary amines as probes for stereochemical preferences for curarimimetic neuromuscular junction blockade □ Amines, enantiomeric bisquaternary, related to benzoquinonium—probes for determining stereochemical preferences for curarimimetic neuromuscular junction blockade

Previous studies (1, 2) were concerned with stereochemical preferences exhibited by the neuromuscular junction toward nondepolarizing blocking agents. It is, of course, recognized that asymmetry is not requisite to activity, but it is possible that differences in blocking activity by enantiomeric species could be of use in defining the characteristics of the anionic site of the hypothetical neuromuscular junction receptor.

The previously examined bisquaternaries (2) could be assumed to have answered the question of stereochemical preference, since both enantiomeric pairs showed a statistically significant *(R) > (S)* superiority in blocking potency. However, both of the previously reported probes were formally related to tetrahydroisoquinoline and, even though they were somewhat related to (+)-tubocurarine in this respect, it seemed desirable to test the generality of the *(R) > (S)* superiority in a nontetrahydroisoquinoline system.

An obvious candidate was benzoquinonium (I), since it has been recognized as a nondepolarizing blocker, presumably of the curare type, although it has no asymmetric centers. Nevertheless, synthetic introduction of a methyl group adjacent to the quaternary moiety was considered possible and would lead to a desired asymmetric probe without, hopefully, eliminating neuromuscular junction blocking activity. Although the introduction of the methyl group adjacent to the quaternary moiety was accomplished without difficulty, it proved to be impossible to quaternize the tertiary amine with the required benzyl halide, presumably because of the steric hindrance introduced by the adjacent methyl group.



I: R = H, R′ = $CH_2C_6H_5$, X = Cl
II: R = R′ = CH_3, X = I

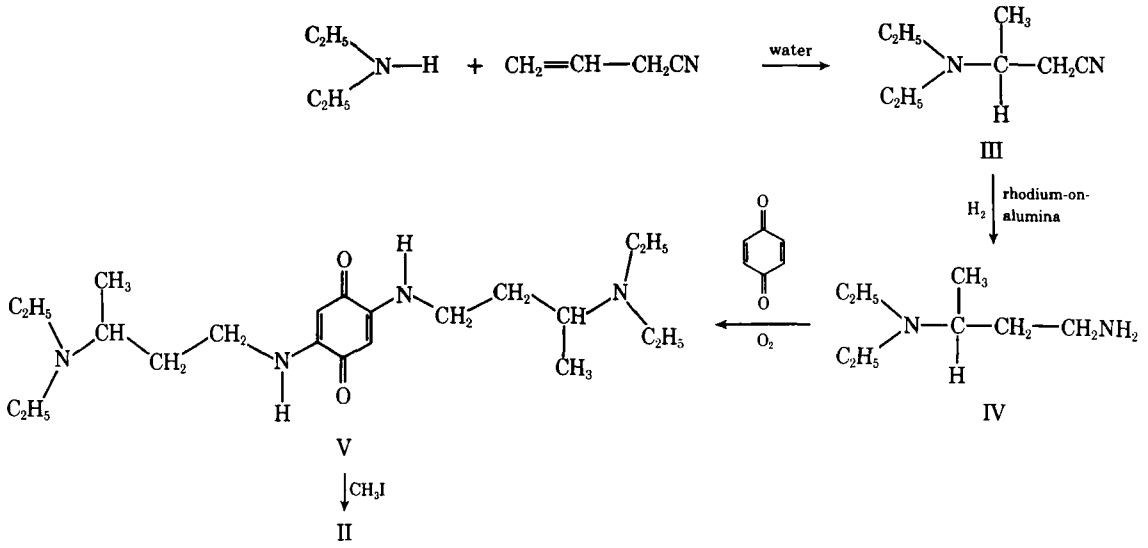
Therefore, methyl iodide as a quaternizing agent was employed, primarily because the original paper (3) describing the synthesis of benzoquinonium indicated that methyl quaternization provided blocking agents with activity comparable to compounds having benzyl as the quaternizing moiety. The present study bears out the earlier findings, since the substitution of a methyl for benzyl still provides compounds with activity comparable to the parent compound.

DISCUSSION

The synthetic route to 2,5-bis[(3-diethylamino)butylamino]-1,4-benzoquinone dimethiodide (II)[1] is shown in Scheme I. Attempted preparation of 3-diethylaminobutyronitrile (III) utilizing crotononitrile was unsuccessful. Apparently, a combination of steric and electronic factors exerted by the methyl group make the double bond resistant to nucleophilic addition, since diethylamine has been reported to add readily to acrylonitrile (4). However, the amine adds readily to allyl cyanide to give III, according to the method of Dahlbom (5), in good yield. In this case, the reaction mechanism is probably electrophilic in nature.

Compound IV was prepared in good yield by hydrogenation of III over a rhodium-on-alumina catalyst at moderate pressures. Purification of IV by the usual methods, *e.g.*, fractional distillation, could not be effected because of excessive foaming which was not alleviated by the usual methods. However, the structural identity of the compound was substantiated by IR (loss of CN stretching frequency and the appearance of NH stretching frequencies) and

[1] Because of the several nomenclature changes reflected in *Chemical Abstracts* for I, the present authors sought the advice of Dr. Kurt L. Loening, Director of Nomenclature at the Chemical Abstracts Service, who kindly furnished the most recent systematic nomenclature for the new compound: 4,4′-[(3,6-dioxo-1,4-cyclohexadiene-1,4-diyl)diimino]bis[*N*,*N*-diethyl-*N*-methyl-2-butanaminium] diiodide. Since this nomenclature is not suitable for the primary literature, the use of the alternative nomenclature as given in the text is suggested.



Scheme I—Synthetic route for preparation of II

NMR (appearance of new protons in the methylene region and complication of the methylene splitting patterns).

The reduction product (IV) was used without further purification to prepare V by reaction with either hydroquinone or benzoquinone in dioxane in the presence of oxygen. The product, obtained in rather poor yield, was in the form of bright-orange crystals, with the major part of the reaction being in the form of intractable tars. The original intention was to convert V to the benzyl quaternary salt since this would have mimicked most closely the parent compound, benzoquinonium (I). However, all attempts to prepare the benzyl quaternary derivative were unsuccessful, probably due to the steric influence of the methyl group adjacent to the tertiary nitrogens.

Since the original studies (3) had indicated that methyl quaternization, as previously mentioned, was not inimical to activity, it was attempted by treating V with a 10-fold excess of methyl iodide in dioxane–ethanol (2:1) with heating at moderate temperatures for several hours. The desired product (II) was obtained as an orange solid, which appeared to decompose on attempted crystallization. Therefore, it was best treated by careful washing of the obtained precipitate using dioxane–ethanol (2:1).

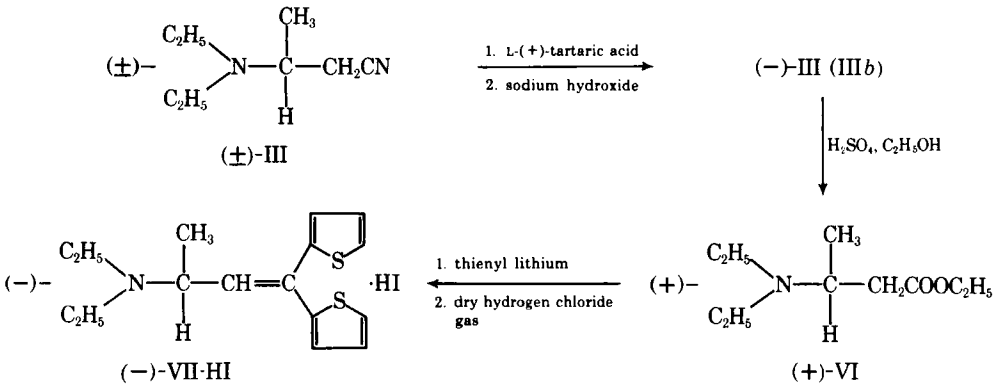
Since there are three possible isomers (a racemic pair and a *meso*-form) when II is prepared from racemic materials, it was necessary to deracemize at a stage prior to the final condensation. The deracemization was most conveniently carried out through the diastereomeric bitartrates of III; the rotatory data are given in Table I. The enantiomeric forms, *IIIa and IIIb, allowed the preparation* of the enantiomeric forms of IV, V, and II according to the methods developed on the racemic materials. The corresponding rotatory data are recorded in Table I together with the absolute configurations as determined here.

Because the objective of this study was to determine stereochemical relevancy to neuromuscular junction blocking ability, it was necessary to determine the absolute configuration of the blocking agents which was most readily attainable by determining this parameter for the enantiomeric forms of III. Scheme II depicts the transformations used to relate III*b* of unknown configuration to (+)-VII-HI of known configuration.

Table I—Physical Data on Compounds Prepared

Compound	Prepared from	Yield, %	Boiling Point (mm) or Melting Point	n_D^{23}	$[\alpha]^{20}$ 589	578	546	436	365	Solvent, mg/ml	Absolute Configuration
II	(±)-V	61	234–235°	—	—	—	—	—	—	—	—
II*a*	(+)-V	87	233.5–234.5°	—	—	34[a]	−74[a]	−446[a]	—	Water (0.6)	(*R*)
II*b*	(−)-V	87	233–234°	—	—	−33[a]	73[a]	452[a]	—	Water (0.6)	(*S*)
III	—	86	72–73° (10)	1.4362	—	—	—	—	—	—	—
III*a*	(±)-III	41[b]	—	1.4353	17.8	18.4	—	37.2	61.0	Absolute ethanol (20)	(*R*)
III*b*	(±)-III	40[b]	—	1.4350	−18.7	−19.5	—	−38.9	−63.8	Absolute ethanol (20)	(*S*)
IV	(±)-III	86	—	1.4420	—	—	—	—	—	—	—
IV*a*	(+)-III	80	—	1.4419	44.7	45.7	—	91.1	146.0	Absolute ethanol (20)	(*R*)
IV*b*	(−)-III	81	—	1.4418	−45.2	−47.2	—	−94.4	−147.5	Absolute ethanol (20)	(*S*)
V	(±)-IV	35	131–132°	—	—	—	—	—	—	—	—
V*a*	(+)-IV	38	124–125°	—	—	131[a]	—	—	—	Absolute ethanol (0.6)	(*R*)
V*b*	(−)-IV	44	124–125°	—	—	−135[a]	—	—	—	Absolute ethanol (0.6)	(*S*)
VI	(−)-III	72	96–97° (15)	1.4298	15.5	—	—	—	—	Absolute ethanol (1.0)	(*S*)
VII	(+)-VI	44	142–144°	—	−101.0	—	—	—	—	Absolute ethanol (0.9)	(*S*)

[a] Because of their orange color, the optical rotations were difficult to obtain. Concentrations were very dilute; consequently, the error in the smaller rotations (less than 100°) is probably larger than normal due to instrument noise and variability. [b] A 100% yield is equivalent to 50% of the starting racemic form.



Scheme II—Reactions involved in absolute configuration studies

The absolute configuration of (+)-VII-HI rests on the work of Beckett and Casy (6), who showed that D-(−)-alanine and (+)-VII-HI possess identical configurations through an independent synthetic conversion. Since VII-HI derived from III*b* (see Scheme II) had a negative optical rotation, it follows that III*b* has a configuration opposite to *(R)*- or D-(−)-alanine and, therefore, must have a configuration identical with *(S)*- or L-(+)-alanine. It may be added, parenthetically, that the present paper is the first to establish the absolute configuration of (+)-VI to be of the *(S)* variety, although the fact that an optically active form of it was used in the preparation (6) of (+)-VII-HI is obvious.

As a result of the failure to find a solid configurational statement for VI, the anticipated simple conversion of III to VI to determine configuration ended up in the necessity of converting III to VII. The present study provides a firm absolute configurational assignment for the enantiomers of III and VI and obviates the necessity of preparing (+)- or (−)-VII-HI to reach a decision. Finally, because III*b* has been shown to be of the *(S)*-configuration, it follows that II*b* derived from it has the same configuration and that II*a*, of necessity, is *(R)*.

The pharmacological testing employed the mouse inclined screen assay and the cat tongue–hypoglossal nerve assay, both of which were described previously (1, 2). In the mouse assay (Table II), the *(S)*-isomer was found to be about one-half as toxic as its enantiomer and about three-fourths as effective as a blocking agent. The cat assay (Table III) was more informative for reasons discussed previously (1) and showed an approximate 2:1 ratio of superiority for the *(R)*-configuration over the *(S)* in a preparation that is probably more meaningful than the mouse assay. This finding is in agreement with previous findings (2) with bisquaternaries obtained from tetrahydroisoquinoline compounds.

The examination of preferential inhibition of acetylcholinesterase (Table IV) was of interest. Although the compounds under study, as well as the parent compound (II), are strong inhibitors of acetylcholinesterase, it has been shown rather convincingly that the differences in enzyme-blocking ability cannot account for potency differences.

EXPERIMENTAL[2]

3-Diethylaminobutyronitrile (III)—This compound was prepared, according to the method of Dahlbom (5), from allyl cyanide treated with dimethylamine to give a product agreeing with the literature values, bp 83–85°/11 mm, n_D^{20} 1.4362.

3-Diethylaminobutylamine (IV)—This compound was prepared, according to the method of Freifelder (7), utilizing a rhodium-on-alumina catalyst. Evaporation of the solvent (CH_3OH) afforded 86% of a light-yellow liquid, which could not be distilled because of its foaming properties. The spectral characteristics of the yellow oil were consistent with those expected for IV. Therefore, the product was used as such without further purification, since all attempts to purify it seemed to cause further degradation.

2,5-Bis[(3-diethylamino)butylamino]-1,4-benzoquinone (V)—The preparation of this compound was carried out essentially according to the procedure of Cavallito *et al.* (3). The amine (IV) (5.0 g, 37 mmoles) in 10 ml of dry dioxane was added (10 min) to a cooled and stirred solution of benzoquinone (1.9 g, 18 mmoles) in 30 ml of dioxane. After addition was complete, the reaction mixture was allowed to come to room temperature and a slow stream of oxygen was passed through the solution with stirring for about 15 hr.

The reaction mixture was then concentrated to leave a tarry residue, which resisted recrystallization attempts after providing the product (V) as a dark-red semicrystalline solid. When recrystallized from ethanol–water, this product gave bright-orange-colored crystals, mp 131–132°. IR and NMR spectral characteristics were in accord with the expected structure.

Anal.—Calc. for $C_{22}H_{40}N_4O_2$: C, 67.32; H, 10.27; N, 14.27. Found: C, 67.48; H, 10.25; N, 14.22.

2,5-Bis[(3-diethylamino)butylamino]-1,4-benzoquinone Dimethiodide (II)—The preparation of this compound was carried out as described previously (3). This procedure involved the quaternization of V with methyl iodide (1.1 g, 8.0 mmoles) in absolute ethanol–dioxane (1:2), with 3–5 hr of heating at 40–50°. The precipitate that formed was separated by filtration, washed thoroughly with ethanol–dioxane (1:2), and dried to leave II as a brick-red solid, mp 234–235°. IR and NMR data were in accord with the expected structure.

Resolution of 3-Diethylaminobutyronitrile (III)—Both of the tartaric acids available [(+) and (−)] for use as deracemizing agents were used in the separation of the enantiomeric forms. Thus, L-(+)-tartaric acid (17.3 g, 115 mmoles) dissolved in the minimum volume of hot absolute ethanol was reacted with III (15 g, 115 mmoles) in about 20 ml of absolute ethanol. The solution was allowed to cool to room temperature and then was cooled overnight at 0°. The solvent was decanted and the gummy precipitate was triturated with acetone to give the product as a white solid.

Three recrystallizations from absolute ethanol provided 9.3 g (58%) of the bitartrate salt having a constant rotation of $[\alpha]_D^{20}$ +

Table II—ED_{50}, LD_{50}, and Potency Ratios in the Mouse Assay for Neuromuscular Junction Blocking Potency

Compound	ED_{50}, mg/kg	LD_{50}, mg/kg	Potency Ratios Based on ED_{50} [(+)-Tubocurarine = 100]
(+)-Tubocurarine	0.34	—	100
II*a* (+)	0.35	0.45	97
II*b* (−)	0.50	1.00	68
II*c* (±)	0.45	0.60	75

[2] Melting points were determined on a Thomas-Hoover melting-point apparatus and are uncorrected. Boiling points also are uncorrected. Elemental analyses were performed by M-H-W Microanalytical Laboratories, Garden City, Mich. Moist extracts were dried with anhydrous calcium or sodium sulfate or as otherwise specified. Solvents were routinely removed at about 20 mm pressure, using a rotating-flask evaporator. TLC was routinely carried out on Eastman Chromagram (No. 6060) sheets, and visualization was done with both UV lamps and iodine vapor. Optical rotations were obtained in either absolute ethanol or water, using a Perkin-Elmer 141 polarimeter. NMR spectra were obtained as 10–20% solutions in $CDCl_3$, using tetramethylsilane as an internal standard, on a Varian A-60D or T-60 spectrometer. Physical data on prepared compounds are in Table I.

Table III—ED_{50} and Potency Ratios in the Cat Assay for Neuromuscular Junction Blocking Potency

Compound	ED_{50}, mg/kg	ED_{50} Potency Ratio and Elevation Comparison	ED_{50} Potency Ratio of Enantiomers and Elevation Comparison (Most Potent = 1)
(+)-Tubocurarine	0.09	100	—
II*a* (+)	0.155	90 $F_{el}(dF', dF)^a$ =	1 $F_{el}(dF', dF)^a$ =
II*b* (−)	0.330	45 0.199 (2,24)	2 0.569 (1,12)

[a] Statistical comparisons were made between the line elevations of the isomers to evaluate the significance of the difference in ED_{50} values. Both values indicate that the slopes are not statistically different and that the potency differences are significant.

Table IV—Inhibition of Acetylcholinesterase by II*a* and II*b*

Compound	Wilkinson K_i[a]	Wilkinson V_{max}, moles/unit/min
Acetylcholine	$2.56 \pm 0.02 \times 10^{-4}$ (K_m)	1.008 ± 0.05
(+)-Tubocurarine	$4.29 \pm 0.4 \times 10^{-4}$	1.55 ± 0.7
II*a* (+)	$4.17 \pm 0.16 \times 10^{-6}$	0.385 ± 0.16
II*b* (−)	$4.01 \pm 0.06 \times 10^{-6}$	0.373 ± 0.09

[a] Determined by calculation from computer program (Wilkinson) generated, altered K_m values.

22.9° (c 1.0, H_2O). This material was combined with final fractions from two other resolutions (total 26.0 g) and dissolved in 50 ml of water. The aqueous solution was alkalinized with sodium hydroxide and extracted with ether, and the ethereal extract was dried over anhydrous calcium sulfate. Evaporation of the ether left 11.8 g of III*b*.

The same procedure using D-(−)-tartaric acid and III, recovered from the mother liquors of the L-(+)-tartaric acid treatment, afforded 10.1 g of III*a*.

(+)-Ethyl-3-diethylaminobutyrate (VI)—Compound III*b* (4.9 g, 70 mmoles) was dissolved in 15 ml of absolute ethanol and treated with 5.5 ml of concentrated sulfuric acid (8), and the resultant mixture was maintained at 130° for 16 hr in a stainless steel bomb. After cooling, the reaction mixture was poured into 70 g of cracked ice and the alcohol was removed under vacuum. The reaction product was washed with aqueous sodium bicarbonate solution and then distilled under reduced pressure to give 3.4 g of an oily VI. Since the IR and NMR spectral characteristics of this compound were in accord with the expected structure, it was used without further purification for the preparation of VII.

(−)-3-Diethylamino-1,1-di(2′-thienyl)but-1-ene [(−)-VII]—Adamson's (9) procedure was used, in which the initial step was the reaction of VI with thienyl lithium[3] to provide 3-diethylamino-1,1-di(2′-thienyl)butan-1-ol, mp 73–74° [lit. (9) mp 75–76°]. The aminoalcohol (0.23 g) in chloroform (3 ml) was converted to the aminobutene (VII) by passing dry hydrogen chloride into the solution for 10 min. The solvent was removed under reduced pressure, and the residue in water was stirred with charcoal for a few minutes at 60° and then filtered.

Alkalinization of the filtrate with dilute aqueous ammonia, ether extraction of the liberated base, and removal of solvent from the dried (anhydrous sodium sulfate) extract provided 120 mg of (−)-aminobutene [(−)-VII] as an oil. It gave a hydriodide, buff plates (from ether–ethanol), with a melting point and optical rotation substantially in agreement with the literature values (6), mp 139–140° and $[\alpha]_D^{20}$ +109 ± 2°, for its enantiomer.

Neuromuscular Junction Blocking Bioassay—Two different assays were used; the first was an all-or-none-type response and the second was a graded response.

Mouse Assay—Tests for the activity of II, II*a*, and II*b* were carried out on white male Swiss-Webster mice following essentially the procedure of Hoppe (10), which is much the same as that of Cavallito *et al.* (3) except for the time of observation. Groups of six mice were injected intraperitoneally with doses given in a volume of 0.01 ml/g of body weight. The mice were then placed on an inclined screen at a 45° angle. Mice falling off the screen within 15 min were considered to give a positive reaction. The results are summarized in Table II.

Cat Assay—The hypoglossal nerve–cat tongue muscle preparation employed was described previously (1). Table III summarizes the neuromuscular junction blocking potencies obtained by this assay in comparison with (+)-tubocurarine.

Acetylcholinesterase Inhibition—Inhibitory potency against acetylcholinesterase[4] was determined for the enantiomeric II*a* and II*b* as well as for (+)-tubocurarine in essentially the same way as described previously (1, 2). The method employs the pH-stat method of Cocolas *et al.* (11) and provided enzymatic velocity, which was computer analyzed using the Wilkinson method (12) to generate K_m and V_{max} data from which K_i values could be calculated by employing simple Michaelis relationships. The results are recorded in Table IV.

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Systems Approach to Vaginal Delivery of Drugs I: Development of *In Situ* Vaginal Drug Absorption Procedure

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Abstract □ In the framework of the development of drug delivery systems for locally administered contraceptive drugs, a reliable method that can afford quantitative evaluation of drug absorption behavior was explored using the rabbit doe. A system was constructed based upon perfusing the drug solution in the vaginal tract. For this purpose, a "rib-cage"-type cell was constructed and surgically implanted in the rabbit prior to an experiment. The primary purpose of the present paper is to evaluate the method, including the surgical operation and the perfusion system. The absorption experiments were carried out using *n*-butanol-1-^{14}C as the model solute to survey the reproducibility of the absorption behavior. Experiments were conducted with a number of rabbits on several successive days to determine the day-to-day and animal-to-animal variations. The drug disappearance in the reservoir followed first-order kinetics from which the apparent permeability coefficient was calculated. The results indicated that a set of experiments may be carried out on a single animal and that the method generally affords rather high precision.

Keyphrases □ Vaginal drug delivery system—development of *in situ* vaginal drug absorption procedure, equipment and equations, rabbits □ Drug delivery systems—contraceptive drugs, development of *in situ* vaginal drug absorption procedure, equipment and equations, rabbits □ Contraceptive drugs—procedure to evaluate drug absorption from vagina, equipment and equations, rabbits □ Absorption, drug—*in situ* vaginal procedure, equipment and equations, rabbits

The development of reliable contraceptive systems intended for local vaginal administration will require in large measure the understanding of the complex processes of drug absorption and interactions between the drug delivery device (formulation) and the vaginal tract. For the drug delivery process, the following may be considered as critical: (*a*) the release of drug, (*b*) the permeability of the vaginal membrane, (*c*) drug transport to the site of action, and (*d*) drug reaction with the "bioreceptor."

From current studies of drug transport across the buccal membrane (1, 2), the intestinal mucosa (3, 4), and skin (5), which appears to be morphologically similar to the vaginal membrane, it may be inferred that either the release or the absorption steps can be, and likely are, the determinants of the relative biological activities within a given class of compounds. For certain drug delivery systems (*e.g.*, the silicone matrixes), the mechanisms for drug release are now reasonably well understood (6, 7). In contrast, little, except for some grossly qualitative information (8–10), is available on the rates and mechanisms of mass transport in the vagina. Without a more rigorous definition of this step in the total drug release and absorption picture, the rational drug delivery in the vaginal tract is not possible. In other words, an understanding of the mechanism of drug transport and interactions subsequent to the release from the vaginal device should make possible the *a priori* development of maximally effective contraceptive drug delivery systems.

The purposes of the present report are to describe and assess a method for the determination of vaginal permeabilities, using the rabbit doe as the prototype animal and *n*-butanol as a test compound.

EXPERIMENTAL

Animals—Mature female New Zealand White rabbits (7–9 months old, 4.5–5.0 kg), which were parous, were used. With this weight range, the vaginal tract was about 2 cm in diameter and 8 cm in length, with relatively uniform dimensions. The rabbits were maintained in a temperature-controlled room (25°) with free access to water and normal pelleted food diet.

Perfusion System—The perfusion system consists of two sections (Fig. 1). The first includes the "rib-cage" cell, surgically implanted in the vaginal tract, and the fenestra rotunda (Figs. 2 and 3). The second section is maintained on the laboratory bench and involves a rotating and reciprocating pump[1] and a constant-temperature solution reservoir. The entire perfusion path in the system is of glass tubing and stainless steel material, except for the portions between the cage and the fenestra rotunda, which was polyethylene tubing[2]. The washing device and drain bottles are connected through two three-way stopcocks to the reservoir. During the experiments the two sections are securely attached to provide a closed system for perfusion.

Rib-Cage Cell—The rib-cage cell was designed to fit the rabbit's vaginal dimensions in a way that provides a constant volume and surface area in the absorption compartment (Fig. 3). The cell was constructed with two smoothened Teflon endings connected by eight stainless steel rods. The geometrical volume and surface area enclosed by this cell and surrounding vaginal wall are 8.1 cm^3 and 22.5 cm^2, respectively. The stainless steel inlet and outlet for the perfused solution were installed through one Teflon ending, from which polyethylene tubings were led to the fenestra rotunda. Because of anatomical obstruction, the rib-cage cell could not be inserted intravaginally. Thus, the cell had to be implanted surgically into the vaginal tract.

Fenestra Rotunda—The fenestra rotunda, made of a medical grade silicone rubber sheet[3], was intended to provide a connection shut-off route between the two sections of the perfusion system (Fig. 3). It also assists in positioning the rib-cage cell in the vaginal tract. The solution is perfused through the rib-cage cell through the stainless steel inlet and outlet of the fenestra rotunda, which is permanently fixed on the abdomen of the animal.

Procedure for Implantation of Rib-Cage Cell—The procedure for the implantation of the cell involved a surgical operation near the cervical end of the vaginal tract (Fig. 2). For the operation the rabbit was anesthetized with pentobarbital, and the abdomen was clipped and sterilized with iodine tincture. A central line incision of 6–7 cm in length was made in the lower portion of the abdo-

[1] FMI lab pump, model RRP 2G 150, Fluid Metering Inc., Oyster Bay, N.Y.
[2] INTRAMEDIC PE 205, Clay Adams, Division of Becton, Dickinson & Co., Parsippany, NJ 07054
[3] Silastic, Dow Corning Corp., Midland, Mich.

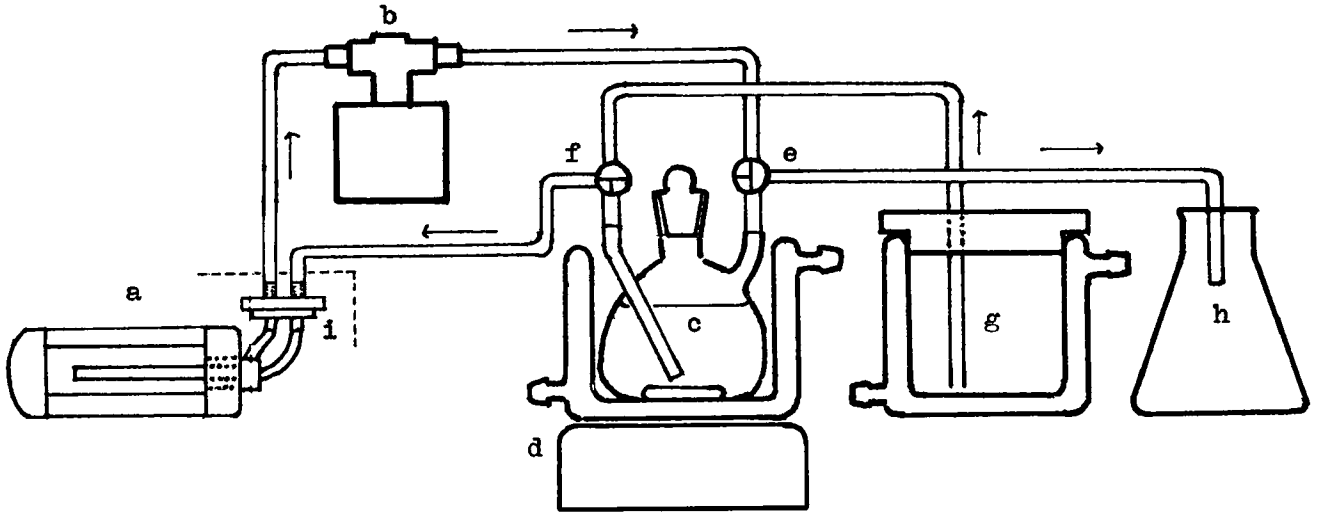


Figure 1—*Diagram of perfusion system. Key: a, rib-cage cell; b, perfusion pump; c, reservoir of solution; d, magnetic stirrer; e, three-way stopcock to returning and drain; f, three-way stopcock to circulating and washing; g, reservoir of washing solution; h, drain beaker; and i, fenestra rotunda.*

men, exposing the vaginal tract. Then the wall of the upper part of the vagina (about 0.5–~1 cm from cervixes) was opened to an appropriate length to insert the rib-cage cell, which was soaked and sterilized in advance in a surgical anti-infection solution. The incised surface of the vaginal wall was then closed by stitching with surgical gut suture (0-0). The polyethylene tubes passing through the vaginal wall were properly adjusted in length and led to the fenestra which, in turn, was positioned and stitched onto the abdominal surface. A silk string was led from the rib-cage cell along the polyethylene tubes and tied to the fenestra rotunda to prevent the cell from slipping when the rabbit assumes the normal position after the operation. The string also serves to minimize the tension on the polyethylene tubings. After the operation, an oxytetracycline hydrochloride solution was injected (10 mg/kg im) to prevent systemic infections. The whole perfusion system was then irrigated by a normal saline solution to remove blood left from the operation. This also served to confirm that the perfusing solution was able to circulate freely throughout the system.

Theoretical Analysis of Circulation Dynamics for Perfusion System—The perfusion system basically consists of a rib-cage cell, a reservoir of solution, and a pump (Scheme I). Compartment I in Scheme I is the reservoir of external solution where drug samples are taken, and Compartment II is the solution of the rib-cage cell in the vagina.

The purposes of the following mathematical description and analysis of the perfusion system are to determine: (*a*) the lag time after which the circulation dynamics of the system are at steady-state, (*b*) the steady-state relationship of the drug solution in the

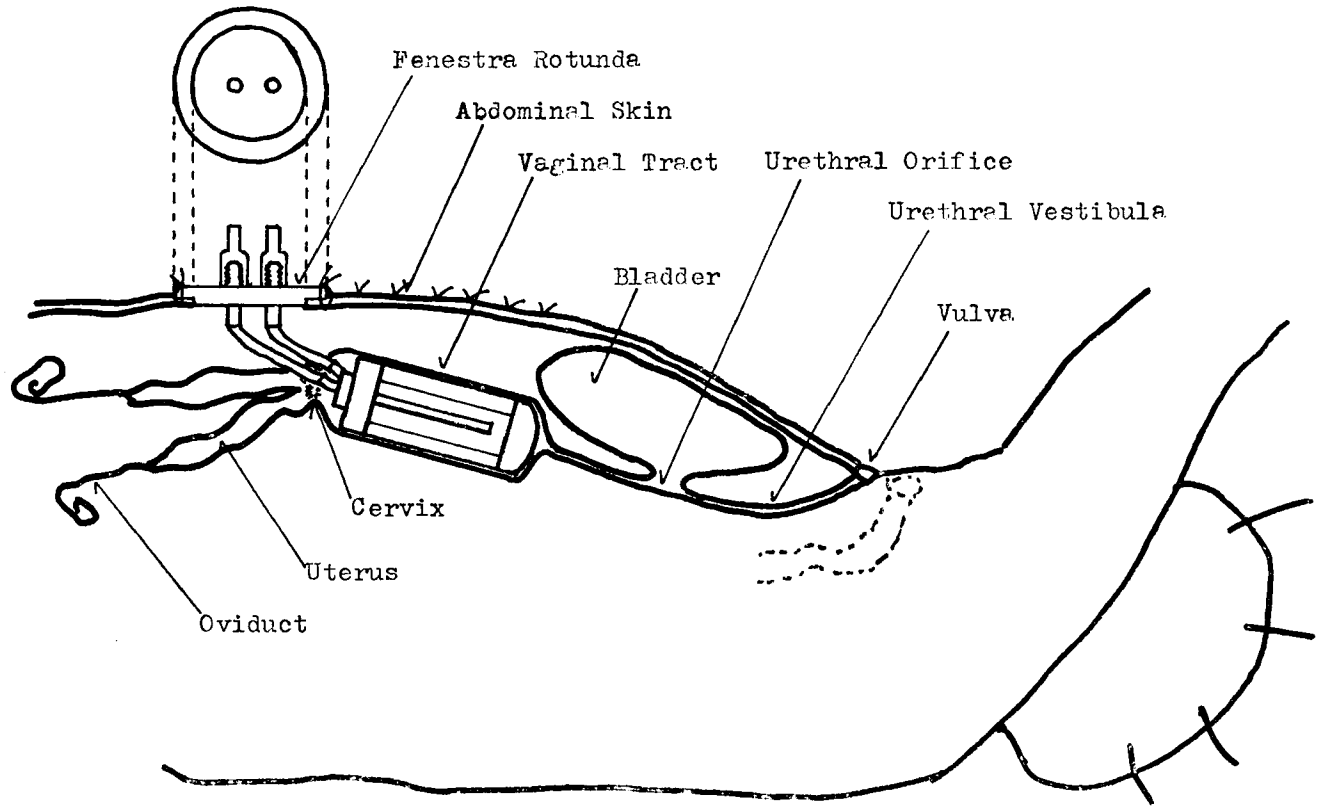


Figure 2—*Position of implanted rib-cage cell in vaginal tract.*

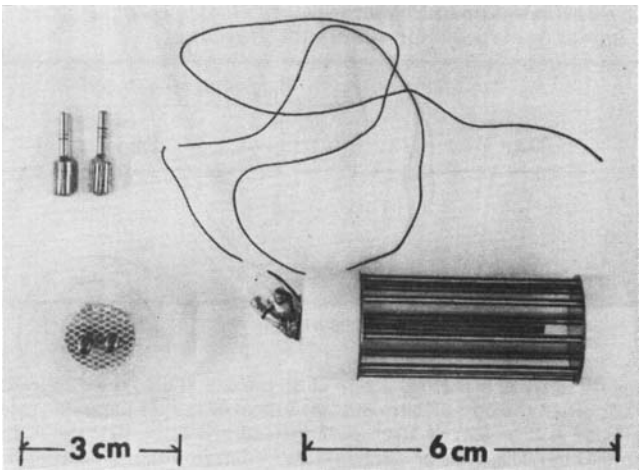


Figure 3—*Surgically implanted parts. Left: fenestra rotunda with the stainless steel inlet and outlet. Right: rib-cage cell with polyethylene tubings and silk string attachments.*

external sampling compartment to that in the vaginal compartment at any time, and, consequently, (c) the steady-state vaginal absorption rate constant. Equations 1 and 2 give the rates of change in the concentrations of Compartments I and II, respectively:

$$\frac{dC_I}{dt} = -\frac{R_f}{V_I}(C_I - C_{II}) \qquad \text{(Eq. 1)}$$

$$\frac{dC_{II}}{dt} = \frac{R_f}{V_{II}}(C_I - C_{II}) - \frac{P_f}{V_{II}}C_{II} \qquad \text{(Eq. 2)}$$

where C_I and C_{II} are the concentrations of Compartments I and II, respectively; R_f (cubic centimeters per second) is the circulating rate of the solution; V_I and V_{II} are the volumes of Compartments I and II, respectively; and P_f (cubic centimeters per second) is the absorption rate coefficient which corresponds eventually to the apparent permeability coefficient of the vaginal mucosal membrane. The total concentration change of drug in the perfusing solution can be given by:

$$\frac{dC_t}{dt} = \frac{V_I}{V_t}\frac{dC_I}{dt} + \frac{V_{II}}{V_t}\frac{dC_{II}}{dt} \qquad \text{(Eq. 3)}$$

where C_t is the total concentration in the perfusing solution, and V_t is the total volume of the perfusing solution ($V_I + V_{II}$).

For a two-compartment linear system, the equations of the system are two simultaneous first-order linear differential equations in two variables. The solution for these equations is given by:

$$C_I = \frac{K_1}{V_I}e^{-m_1 t} + \frac{K_2}{V_I}e^{-m_2 t} \qquad \text{(Eq. 4)}$$

$$C_{II} = K_1\left(\frac{1}{V_I} - \frac{m_1}{R_f}\right)e^{-m_1 t} + K_2\left(\frac{1}{V_I} - \frac{m_2}{R_f}\right)e^{-m_2 t} \qquad \text{(Eq. 5)}$$

where:

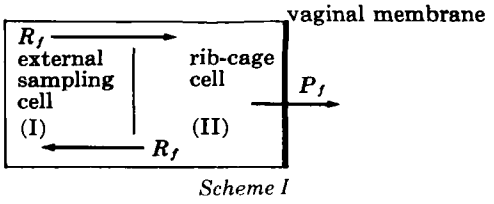
$$m_1 = \frac{R_f V_t + P_f V_I + \sqrt{(R_f V_t + P_f V_I)^2 - 4R_f P_f V_{II} V_I}}{2V_I V_{II}} \qquad \text{(Eq. 6)}$$

$$m_2 = \frac{R_f V_t + P_f V_I - \sqrt{(R_f V_t + P_f V_I)^2 - 4R_f P_f V_{II} V_I}}{2V_I V_{II}} \qquad \text{(Eq. 7)}$$

and K_1 and K_2 are determined by the initial conditions. From the initial conditions, $C_I = C_0$ and $C_{II} = 0$ at $t = 0$. Therefore:

$$K_1 = \frac{(V_I m_2 - R_f)C_0}{m_2 - m_1} \qquad \text{(Eq. 8)}$$

$$K_2 = \frac{(R_f - V_I m_1)C_0}{m_2 - m_1} \qquad \text{(Eq. 9)}$$



Scheme I

where C_0 is the initial concentration.

After substituting Eqs. 8 and 9 into Eqs. 4 and 5 and then taking their ratios:

$$\frac{p_2 C_{II} - q_2 C_I}{q_1 C_I - p_1 C_{II}} = \frac{e^{-m_1 t}}{e^{-m_2 t}} \qquad \text{(Eq. 10)}$$

where:

$$p_1 = R_f(V_I m_2 - R_f) \qquad \text{(Eq. 10a)}$$

$$p_2 = R_f(R_f - V_I m_1) \qquad \text{(Eq. 10b)}$$

$$q_1 = (V_I m_2 - R_f)(R_f - V_I m_1) \qquad \text{(Eq. 10c)}$$

$$q_2 = (R_f - V_I m_2)(R_f - V_I m_1) \qquad \text{(Eq. 10d)}$$

Accordingly, the relationship between C_I and C_{II} can be expressed as:

$$C_{II} = \frac{q_2 e^{-m_2 t} + q_1 e^{-m_1 t}}{p_2 e^{-m_2 t} + p_1 e^{-m_1 t}} C_I \qquad \text{(Eq. 11)}$$

Substituting Eq. 11 into Eq. 3:

$$\frac{dC_I}{dt} = -\frac{P_f(q_2 e^{-m_2 t} + q_1 e^{-m_1 t})}{V_I(p_2 e^{-m_2 t} + p_1 e^{-m_1 t}) + V_{II}(q_2 e^{-m_2 t} + q_1 e^{-m_1 t})} C_I \qquad \text{(Eq. 12)}$$

After a lag time, the circulation dynamics of the perfusion system will be at steady state so the ratio C_{II}/C_I in Eq. 11 converges to q_2/p_2. Also, at steady state Eq. 12 reduces to:

$$\frac{dC_I}{dt} = -kC_I \qquad \text{(Eq. 13)}$$

$$k = \frac{P_f q_2}{V_I p_2 + V_{II} q_2} \qquad \text{(Eq. 14)}$$

where k is the first-order rate constant.

Under the condition where the perfusion rate, R_f, is much greater than the membrane absorption rate, P_f, it may be assumed that the rate change of Compartment I equals that of Compartment II; that is:

$$-\frac{R_f}{V_I}(C_I - C_{II}) = \frac{R_f}{V_{II}}(C_I - C_{II}) - \frac{P_f}{V_I}C_{II} \qquad \text{(Eq. 15)}$$

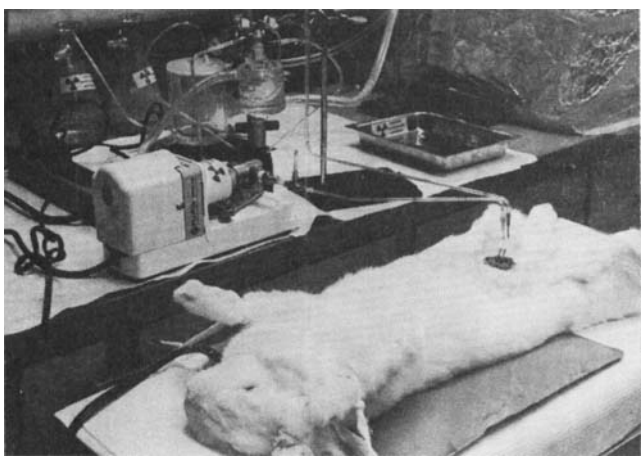


Figure 4—*Layout of perfusion system during an absorption experiment.*

Table I—Influence of Perfusion Rate to Concentration Difference between Compartments I and II and to the First-Order Rate Constant[a]

R_f, ml/min	P_f/R_f	Lag Time, sec	Upper Limit of C_I/C_{II}	C_I/C_{II}	k, sec^{-1}
35	0.38	75	1.38	1.31	5.34×10^{-3}
	0.038	105	1.038	1.03	6.47×10^{-4}
25	0.54	105	1.54	1.45	4.94×10^{-3}
	0.054	165	1.056	1.04	6.41×10^{-4}
15	0.90	150	1.90	1.77	4.17×10^{-3}
	0.090	180	1.090	1.07	6.28×10^{-4}

[a] For these calculations, these dimensional constants were used: $k_{max} = (P_f/V_I) = (AD/V_I h) = 6.62 \times 10^{-3}$ sec^{-1}, A = surface area (22.5 cm^2), D = diffusion coefficient in aqueous phase (1×10^{-5} cm^2/sec), h = thickness of aqueous diffusion layer (10^{-3} cm), V_I = volume of Compartment I (26 cm^3), and V_{II} = volume of Compartment II (8 cm^3).

or:

$$C_{II} = \frac{1}{1 + \frac{V_I P_f}{V_I R_f}} C_I \qquad \text{(Eq. 16)}$$

When substituting Eq. 13 into Eq. 1:

$$\frac{dC_I}{dt} = - \frac{1}{\frac{V_I}{P_f} + \frac{V_{II}}{R_f}} C_I \qquad \text{(Eq. 17)}$$

According to the assumption, the second term of the denominator of the first-order rate constant would be negligible so that the rate of change in drug concentration in the external sampling compartment would be principally due to absorption by the vaginal membrane.

From Eq. 2, the rate change of the concentration of Compartment II should be negative for all time and therefore $1 \leq (C_I/C_{II}) < 1 + (P_f/R_f)$. The upper limit of the concentration ratio between Compartments I and II could be estimated from the introduction of reasonable values for the exposed area, the diffusion coefficient in the aqueous phase, and the thickness of the aqueous diffusion layer (unstirred aqueous layer) into P_f according to the physical model study of membrane transport (11).

Table I shows the influence of the perfusion rate to the first-order rate constant and to the concentration difference between the external sampling compartment and the rib-cage cell compartment. The ratio between C_I and C_{II} becomes constant after a certain lag time and its magnitude is dependent upon the perfusion rate constant, R_f, relative to the absorption rate constant as described by P_f.

Under the maximum absorption rate situation where the maximum rate constant $k_{max} = 6.62 \times 10^{-3}$ sec^{-1}, the ratio $C_I/C_{II} = 1.31$ when $R_f = 35$ ml/min and $C_I/C_{II} = 1.45$ when $R_f = 25$ ml/min. This maximum absorption rate would correspond to the situation in which the rate is governed by the unstirred aqueous layer in front of the vaginal membrane and not by the vaginal membrane itself. A conservative value of 10 μm thickness for the unstirred layer is employed in the calculations. On the other hand, when the absorption rate constant is indicative of a more membrane-controlled case, *i.e.*, $k = 6.62 \times 10^{-4}$ sec^{-1}, the ratio C_I/C_{II} is more nearly unity at a perfusion rate of 35 ml/min. Thus, the *apparent* first-order absorption rate constant (uncorrected for perfusion rate effects) will be 6.47×10^{-4} sec^{-1} instead of 6.62×10^{-4} sec^{-1}. In conclusion, this type of analysis aids in determining the appropriate perfusion rates for the different situations in the experiment to follow.

Table II—Apparent Permeability Coefficients[a], P_{app}, of Vaginal Absorption for *n*-Butanol (Rabbit 1)

Day	P_{app} (cm/sec) $\times 10^4$ First Experiment	Second Experiment
1	1.91	1.65
2	1.95	2.21
3	2.09	1.98
4	2.18	2.69
5	2.18	2.50

[a] Arithmetic mean = 2.13 ± 0.28 *SD*.

Table III—Apparent Permeability Coefficients[a], P_{app}, of Vaginal Absorption for *n*-Butanol (Rabbit 2)

Day	P_{app} (cm/sec) $\times 10^4$ First Experiment	Second Experiment
1	2.07	1.67
2	2.07	2.28
3	—	—
4	—	—
5	2.04	1.55

[a] Arithmetic mean = 1.94 ± 0.26 *SD*.

Procedure for Absorption Studies—The absorption experiments were carried out on 5 successive days starting 3 days after surgery. On each day, two successive experiments were conducted with each rabbit. This procedure allows more experiments to be conducted within a short period without exhausting the animal.

The rabbit was anesthetized with sodium pentobarbital, 25–50 mg/kg, and connected to the perfusion system through the stainless steel inlet and outlet of the fenestra rotunda (Fig. 4). A heating pad was kept under the rabbit to maintain body temperature during the experiments. The vagina was then washed by perfusion with a normal saline solution for 10 min at a flow rate of 35 ml/min. Then the saline was replaced by the buffer solution to be used in the experiment and the washing was continued for an additional 5 min. The buffer was 0.115 *M* phosphate buffer (pH 6.0) made isotonic with sodium chloride.

With the system filled with buffer solution, the stopcocks were switched to introduce the reservoir and its contents (20 ml of buffer) into the system and the perfusion was started. All perfusion experiments were carried out under the flow rate of 35 ml/min at 37°. Two minutes later, an aliquot of a saline solution of *n*-butanol-1-^{14}C was rapidly injected into the reservoir and the first sample (zero time) was then taken after 2 min more. The losses in the butanol concentration were followed by taking 0.05-ml samples from the reservoir every 10 min for about 1 hr. After the first experiment on each day, the system was emptied of the drug solution and washed with saline for 15 min and then with the buffer solution for an additional 5 min. This washing procedure was sufficient to remove all drug from the system. A sample taken from the reservoir before starting the second absorption experiment showed that no drug was present. The butanol activity in the samples was determined by means of a liquid scintillation counter[4] using dioxane solution[5].

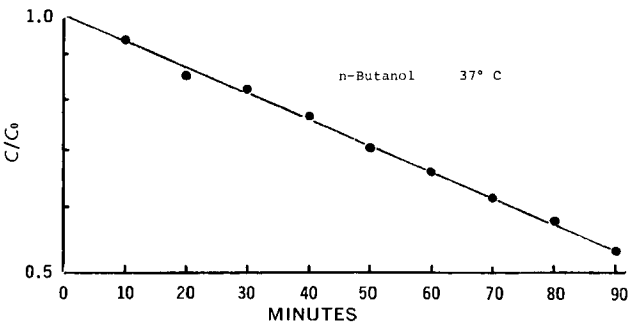


Figure 5—*Typical plot of the concentration change in the sampling reservoir as a function of time showing the first-order kinetics.*

[4] Beckman LS 200.

[5] The solution consisted of: 2,5-diphenyloxazole, 8 g; naphthalene, 100 g; and dioxane, *q.s.*, 1000 ml.

Table IV—Apparent Permeability Coefficients[a], P_{app}, of Vaginal Absorption for *n*-Butanol (Rabbit 3)

Day	P_{app} (cm/sec) × 10^4 First Experiment	P_{app} (cm/sec) × 10^4 Second Experiment
1	2.11	2.16
2	1.71	2.10
3	2.17	1.80
4	2.38	2.30
5	2.37	2.25

[a] Arithmetic mean = 2.13 ± 0.21 *SD*.

RESULTS AND DISCUSSION

General Observations—Some general observations were made on the state of the animals after surgery and during the experimental period. The rabbits behaved normally in their actions and discharged urine and stool as usual. Losses in body weight of up to 10% were generally observed for the first 2–5 days after surgery. However, after this period, most animals showed an improvement in appetite and some began to gain weight. The abdominal incision wound showed considerable healing during this period. After completion of the perfusion experiments, the animals were sacrificed and the site of the surgery was examined. Conglutination was found in the area, indicating normal recovery after surgery and the regaining of normal blood circulation in the area. The vagina itself appeared normal except for what appeared to be a slight thickening of the wall.

In the initial design, the supporting stainless steel ribs of the rib-cage cell were covered with medical grade polyethylene tubing[2]. However, in these instances, inflammation (congestion and bleeding) of the mucosal surface of the vaginal tract was observed. In all subsequent perfusion experiments, the polyethylene sleeve was not used and no abnormalities were observed.

One important assumption in the design of the rib-cage cell was that the cell would form a leaktight chamber with the vaginal walls. This appeared to always hold true, since the volume of the perfusion solution remained constant with time. The success achieved may be attributed to the combination of having the cell dimensions matched to those of the vagina and of having negative pressure in the absorption chamber during the perfusion runs.

Although isotonic solutions were used in the absorption experiments, the possible net transfer of water with time was also determined with ^{14}C-polyethylene glycol 4000. Using isotonic buffered solution at pH 6.0, no loss of water was observed in 1 hr. Accordingly, the influence of the water flow on drug movement was assumed to be negligible.

In separate experiments involving no vaginal membrane, it was also established that there was no detectable absorption of *n*-butanol to the materials of the perfusion setup.

Absorption Experiments with *n*-Butanol—Figure 5 represents the results of a typical experiment conducted for 90 min. The linear relationship obtained on a semilogarithmic scale indicates that the drug disappearance follows a first-order pattern. Thus, the rate of disappearance could be expressed by:

$$\frac{dC_b}{dt} = -K_u C_b \qquad \text{(Eq. 18)}$$

$$K_u = \frac{A}{V} P_{app} \qquad \text{(Eq. 19)}$$

where C_b is the concentration of the drug in the bulk, K_u is the first-order rate constant, A is the effective surface area of vaginal tract, V is the volume of the perfusion solution, and P_{app} is the ap-

Table V—Buffer Systems[a] Employed in Assessing the Effect of pH and Buffer Species on Membrane Permeability

pH 3.0

Citrate Phosphate Buffer:		Biphthalate Buffer:	
Citric acid monohydrate	16.81 g	Potassium biphthalate	10.21 g
Na_2HPO_4	7.12 g	1.0 *N* HCl	21.8 ml
10 *N* NaOH to adjust			

pH 6.0

Citrate Buffer:		Phosphate Buffer:	
Citric acid monohydrate	19.75 g	NaH_2PO_4	11.4 g
10 *N* NaOH to adjust		10 *N* NaOH to adjust	

pH 8.0

Borate Buffer:		Phosphate Buffer:	
Boric acid	12.37 g	Na_2HPO_4	7.1 g
10 *N* NaOH to adjust		1.0 *N* HCl	0.4 ml
Tromethamine Buffer:			
Tromethamine	0.608 g		
1.0 *N* HCl	27.9 ml		

pH 9.8

Borate Buffer:	
Boric acid	12.37 g
10 *N* NaOH to adjust	

[a] All buffer solutions were made up to 1000 ml and adjusted to isoosmotic conditions (300 mosmoles/kg) with sodium chloride.

Table VI—Effect of pH and Buffer on the Apparent Permeability Coefficient of *n*-Butanol in Vaginal Tract[a]

pH	Apparent Permeability Coefficient P_{app} × 10^4 cm/sec		
3.0	Citrate–Phosphate Buffer	Biphthalate Buffer	
	2.24	1.67	
	2.34	2.04	
	2.76	1.48	
	2.08	1.82	
	Av. 2.35	Av. 1.75	
6.0	Citrate Buffer	Phosphate Buffer	
	2.30	1.73	
	1.85	1.73	
	1.48	1.56	
	Av. 1.87	1.62	
		1.82	
		1.65	
		Av. 1.68	
8.0	Borate Buffer	Phosphate Buffer	Tromethamine Buffer
	0.83	1.64	2.35
	0.89	1.74	2.17
	Av. 0.86	Av. 1.69	1.43
			1.65
			1.81
			Av. 1.88
9.8	Borate		
	0.69		
	0.75		
	0.79		
	0.89		
	Av. 0.78		

[a] Each experiment was carried out for 1 hr.

Table VII—Crossover Experiments Involving Various pH and Buffer Systems[a] (P_{app} × 10^4 cm/sec)

Day	First Experiment pH	First Experiment P_{app}	Second Experiment pH	Second Experiment P_{app}
1	3.0 (C)	2.24	9.8 (B)	0.79
2	9.8 (B)	0.89	6.0 (P)	1.82
3	6.0 (P)	1.65	3.0 (C)	2.34
4	3.0 (C)	2.76	9.8 (B)	0.75
5	9.8 (B)	0.69	6.0 (P)	1.73

[a] C = citrate buffer, P = phosphate buffer, and B = borate buffer.

parent permeability coefficient. The ratio of the surface area to the volume of solution is 0.66.

Absorption experiments were carried out with three rabbits (Tables II–IV). For Rabbits 1 and 3, the experiments were carried out and continued up to the 5th day with two successive runs each day. For Rabbit 2, there were some difficulties with the anesthesia on the 3rd and the 4th days so that experiments on these days were not performed. The data show overall good reproducibility of the method. Both the animal-to-animal and the day-to-day variations were statistically insignificant. Consequently, the method should be useful in carrying out quantitative experiments on a single rabbit as well as with a set of animals.

Effects of pH and Buffer Species on Permeability of Vaginal Membrane—For studies on the vaginal absorption of weak electrolytes, knowledge of the possible adverse effects of pH and buffer species on the integrity and permeability characteristics of the membrane is required. These effects were quantitatively assessed by absorption experiments utilizing *n*-butanol as the reference. The buffer systems employed are listed in Table V.

As can be seen in Table VI, the apparent permeability coefficient of *n*-butanol was the same at pH 3.0 with the phthalate buffer and at pH 6.0 and 8.0 with the phosphate buffers. In comparison, the average P_{app} values for the citrate buffer systems tended to be greater and those for the borate buffers were consistently lower. Although there were wider variations in the experimental results with the tromethamine buffer as compared to the variations with the other buffers at various pH, the average P_{app} was higher than the P_{app} for the phthalate and phosphate buffers. The pH of all buffer solutions changed no more than 0.1 unit in 1 hr.

Although it is not explicit in Table VI, the tabulations of the permeability coefficients are the overall results of crossover experiments involving pH and buffer systems. The crossover experiments gave reproducible and consistent results. As one example, an absorption experiment carried out at pH 6.0 with the phosphate buffer gave a higher P_{app} than that of an immediate followup experiment at pH 9.8 with the borate buffer on the same rabbit (Table VII). The result was the same when the order of the experiments was reversed.

These results show that the membrane is affected by both pH and buffer as adjudged by its permeability to *n*-butanol. There appears to be little effect on the membrane when phthalate and phosphate buffers are used. The citrate and borate buffers and, perhaps, the tromethamine buffer do affect membrane permeability. However, these effects upon the membrane appear to be reversible and not related to the integrity of the membrane.

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Timed Release from Polymeric Films Containing Drugs and Kinetics of Drug Release

M. DONBROW [x] and M. FRIEDMAN

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Keyphrases □ Ethylcellulose films containing caffeine or salicylic acid—timed release, first-order and diffusion-controlled models discussed □ Timed release of caffeine or salicylic acid from ethylcellulose films—first-order and diffusion-controlled models discussed □ Drug release from polymeric films—first-order and diffusion-controlled mechanisms discussed □ Polymer films containing drugs—kinetics of drug release, first-order and diffusion-controlled models discussed

The methods previously used in achieving timed-release formulations of drugs may be related to four types of processes: *(a)* coating the drug or preparation, *(b)* embedding the drug in fatty, plastic, or hydrophilic matrixes, *(c)* binding the drug to an ion-exchange resin, or *(d)* forming a complex or other chemical derivative of the drug (1). The possibility exists that incorporation of the drug into a polymer

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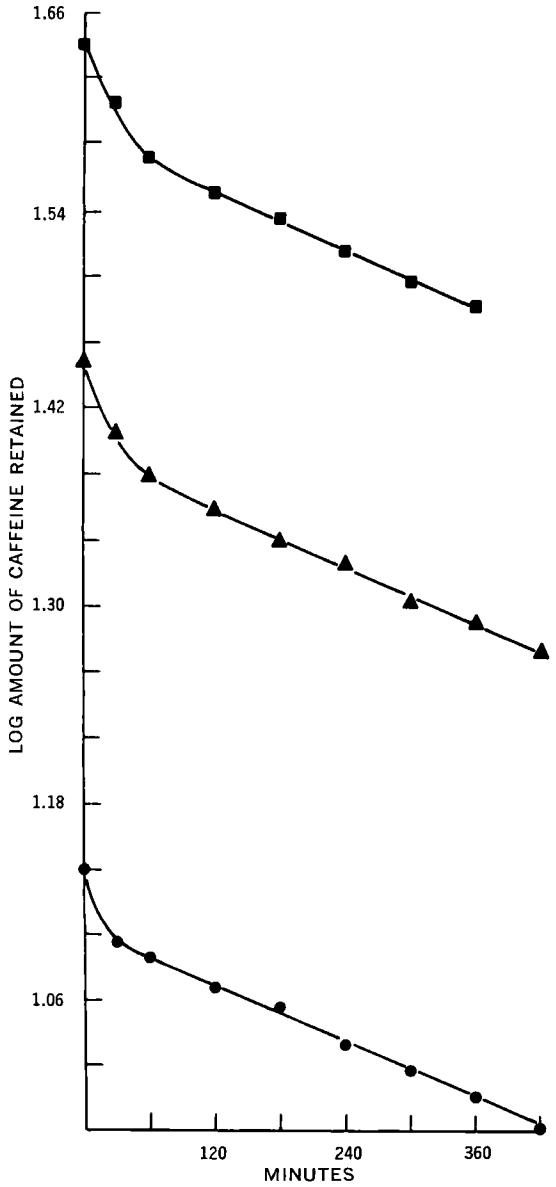

Figure 1—*Apparent first-order release profile for caffeine. Key (caffeine content of film): ●, 5%; ▲, 10%; and ■, 15%.*

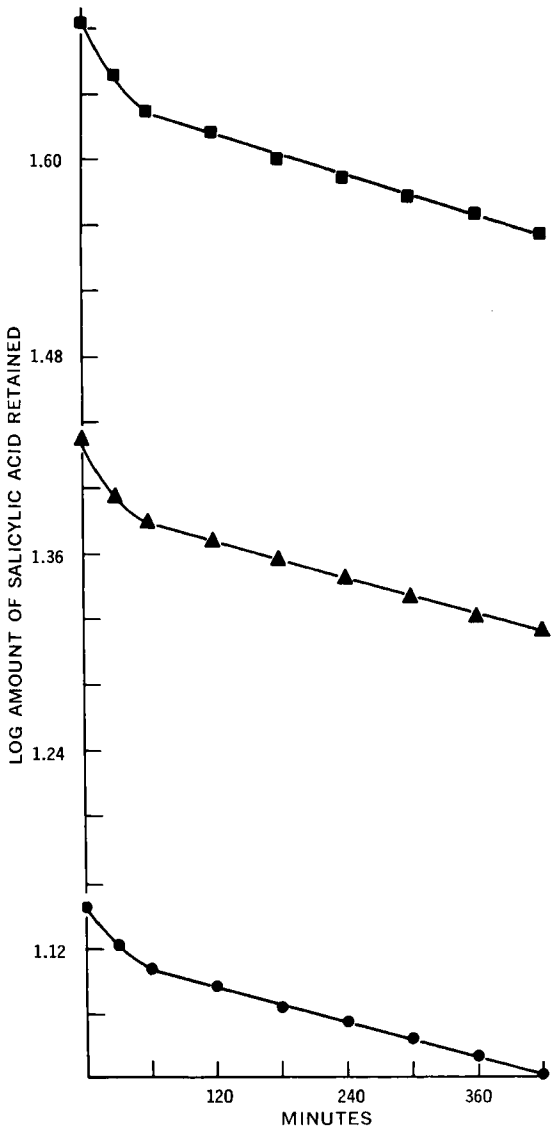

Figure 2—*Apparent first-order release profile for salicylic acid. Key (salicylic acid content of film): ●, 5%; ▲, 10%; and ■, 15%.*

film during its production might yield a useful new class of timed-release preparation. Such films have not been described for peroral use, but Sciarra and Gidwani (2, 3) investigated the preparation of such films for aerosol application to the skin and proposed kinetics for the release of gentian violet, cetylpyridinium chloride, and benzalkonium chloride, which were used as model drugs.

The present authors investigated the permeability of mixed films of ethylcellulose to caffeine (4) and salicylic acid. This report describes the preparation of cast films containing these drugs and the release patterns of these drugs from the films investigated. The applicability of the models of Higuchi (5) and Sciarra and Gidwani (2, 3) to the kinetics of release is examined.

THEORETICAL

The factors determining drug release rates are of particular importance in the formulation and production of timed-release preparations. To control these factors, it is necessary to determine the correct equation for the release patterns observed and to investigate the effects of changing the parameters in the equations upon the kinetics of release.

For film-incorporated drug, classical diffusion models would seem to be applicable (6). Higuchi (5) developed equations for linear and spherical diffusion-controlled release of drugs from insoluble solid wax or plastic matrixes. Where the drug is removed from a slab of matrix by a leaching process, the amount of drug, Q, liberated per unit surface area of matrix into the external medium in time t is given by the equation:

$$Q = \sqrt{\frac{\epsilon}{\tau} D(2A - \epsilon C_s) C_s t} \qquad \text{(Eq. 1)}$$

where A = initial drug concentration in the matrix; C_s = the solubility and D = the diffusion coefficient of the drug in the leaching medium, respectively; ϵ = porosity; and τ = tortuosity of the matrix. Higuchi's equation was found to be applicable in several studies (7–10) on wax-embedded and similar products and would appear to be a possible model for the behavior of film-incorporated drugs.

However, Sciarra and Gidwani (2, 3) based their treatment on the Noyes–Whitney law and reported that the release pattern from

Table I—Film Preparation and Composition

Composition of Film-Forming Solution[a]		Drug Concentration in Films, % w/w	Method of Film Preparation (12)
Ethylcellulose, % w/v	Drug[b], % w/v		
9.5	0.5	5	Glass substrate
9.0	1.0	10	Glass substrate
8.5	1.5	15	Glass substrate

[a] The solvent was chloroform. [b] Salicylic acid or caffeine.

their polymer films agreed with the now classical, first-order release equation:

$$\log A = \log A_0 - \frac{kt}{2.303} \qquad \text{(Eq. 2)}$$

in which A = quantity of drug remaining in the film at time t, and A_0 = initial quantity of the drug in the film. This form of release has been found applicable to many other kinds of timed-release preparations (11).

Although it is conceivable that release may be diffusion controlled in some systems and dissolution controlled in others, depending on parameters such as the permeability of the polymer to water, the solubility of the drug in the polymer and in water, and the particle size of the drug, dissolution control would not be expected to be a normal feature of timed release at low fluxes of drug and water. In this study, the applicability of the two models is investigated for two drugs having similar molecular weights and solubilities in water but vastly different affinities toward organic substances and solvents.

EXPERIMENTAL

Preparation of Films—The films were prepared by the techniques of Kanig and Goodman (12). Their composition and the method of preparation are given in Table I; 10% solutions of mixed solids were used.

Determination of Drug Concentration in Films—The initial drug concentrations in the films were determined spectrophotometrically using samples of the films dissolved in chloroform. Reference standard solutions of the drugs were prepared using the same solvent. No interferences due to the ethylcellulose occurred at the wavelengths of measurement: 273 nm for caffeine and 296 nm for salicylic acid.

Determination of Release Rate—Membranes were attached using an adhesive[1] to the rim of a cylindrical sintered-glass funnel (diameter 3.29 cm), which gave a film area of 33.98 cm^2. The inverted funnel was immersed in water at 37°, the water being mixed and circulated through a 1-cm flowcell in the spectrophotometer[2] using a polystaltic pump[3] operated at a flow rate of 20 ml/min. The film thickness was within 72–76 μm and film weight was 276–296 mg. The total volume of water used was 400 ml. Each experiment was performed in triplicate, and the mean calculated results were reproducible to within 1.5% of the mean.

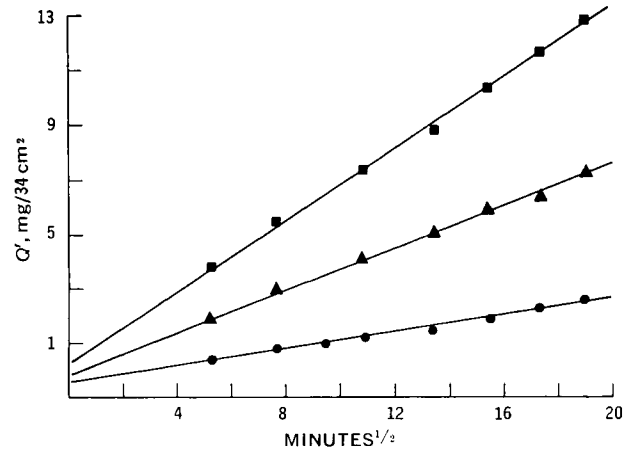

Figure 3—*Apparent diffusion-controlled release profiles, according to Higuchi's model (Eq. 1), for caffeine. Key (caffeine in film): ●, 5%; ▲, 10%; and ■, 15%.*

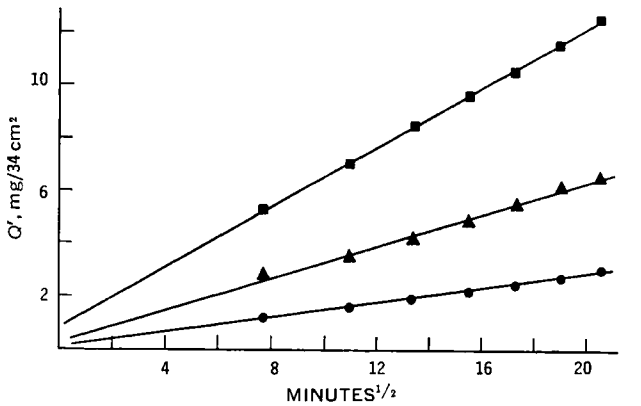

Figure 4—*Apparent diffusion-controlled release profiles, according to Higuchi's model (Eq. 1), for salicylic acid. Key (salicylic acid in film): ●, 5%; ▲, 10%; and ■, 15%.*

RESULTS AND DISCUSSION

Figures 1 and 2 show that both caffeine and salicylic acid appear to give first-order release patterns. The initial curvature can be attributed to the presence of surface drug and can be ignored.

From these figures it is also seen that the rate constant remains relatively constant with change in concentration of the drug in the film; this fact is in agreement with a first-order release model. However, when the amounts of drug released were plotted against the square root of time, straight lines were also obtained (Figs. 3 and 4). These linear plots appear to indicate that drug release in these systems is diffusion controlled, in accordance with Higuchi's equation. Furthermore, the increase in the release rate as the quantity of drug in the film is increased also agrees with this model.

Since both first-order and square root of time plots are acceptably linear, a more stringent test was needed to distinguish be-

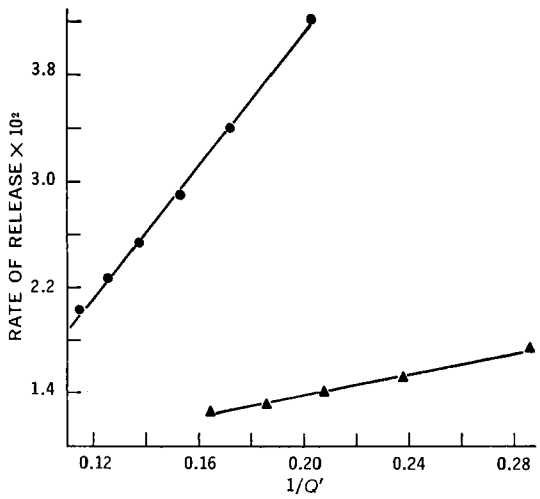

Figure 5—*Plot of release rate against reciprocal of amount of drug released, Q'. Key: ●, 10% caffeine in film; and ▲, 10% salicylic acid in film.*

[1] Silicone pressure-sensitive adhesive, Dow Corning Corp., Midland, Mich.

[2] Unicam SP 1800, Pye Unicam Ltd., Cambridge, England.

[3] Buchler Instruments, Inc., Fort Lee, N.J.

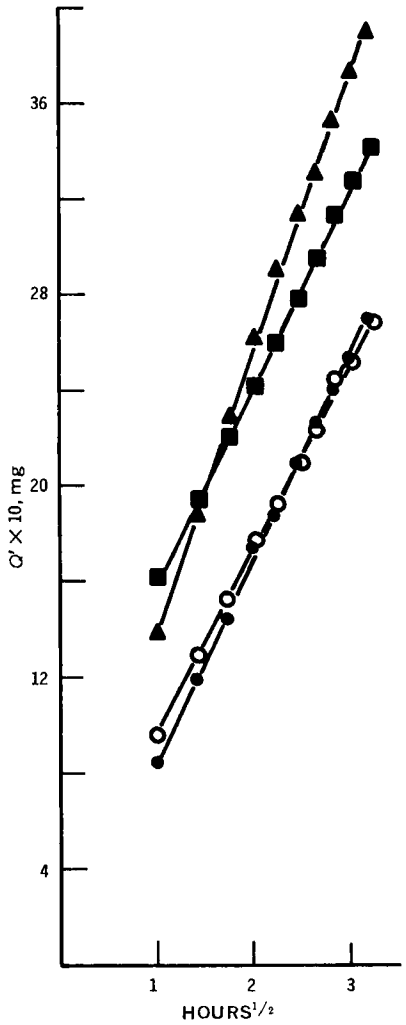

Figure 6—*Apparent diffusion-controlled release profiles, according to Higuchi's model (Eq. 1). Key (for cetylpyridinium chloride): ■, Fig. 7C of Ref. 3; and ○, Fig. 7B of Ref. 3. Key (for benzalkonium chloride): ●, Fig. 9B of Ref. 3; and ▲, Fig. 9D of Ref. 3.*

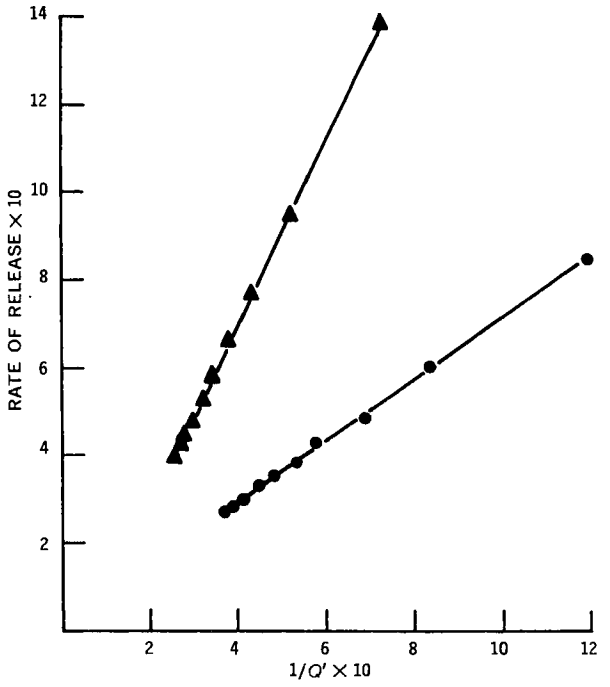

Figure 7—*Plot of release rate of benzalkonium chloride from polyamide films against reciprocals of amount released, Q'. Key (for benzalkonium chloride): ▲, Fig. 9D of Ref. 3; and ●, Fig. 9B of Ref. 3.*

tween the mechanisms. A suitable treatment was developed based upon use of predicted rate equations corresponding to Eqs. 1 and 2 (13). For the diffusion-controlled mechanism, the rate will be inversely proportional to the total amount of drug released, Q', in accordance with Eq. 3:

$$\frac{dQ'}{dt} = \frac{K^2S^2}{2Q'} \qquad \text{(Eq. 3)}$$

where $Q' = QS$ (S = surface area of film). Then:

$$K = \left[\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s\right]^{1/2} \qquad \text{(Eq. 4)}$$

The rate predicted by first-order kinetics, however, is given by:

$$\frac{dQ'}{dt} = kA_0 - kQ' \qquad \text{(Eq. 5)}$$

where $A = A_0 - Q'$. This indicates that the rate is proportional to Q' rather than inversely proportional as predicted by the diffusion model.

The rates of release were determined by measurement of the slopes of Q' *versus* time curves. The two mechanisms were clearly differentiated by plots of rates as functions of Q' and of $1/Q'$, because the plots of rate *versus* $1/Q'$ proved to be linear (Fig. 5) and those of rate *versus* Q' curved throughout the whole of the release period, indicating that the process is diffusion controlled. This conclusion was substantiated by the use of Eq. 6, the logarithmic form of Eq. 1:

$$\log Q' = \log K' + 1/2 \log t \qquad \text{(Eq. 6)}$$

where $K' = (K)(S)$.

Plots of log Q' against log t were linear and the slope was 0.5 for both drugs, confirming the diffusion model.

It is a matter of some interest to know whether the mechanism of release from polymer films varies according to the type of drug incorporated. Since Sciarra and Gidwani's (2, 3) results were obtained using the salts cetylpyridinium chloride and benzalkonium chloride, which are considerably more water soluble than caffeine and salicylic acid and form micelles, it seemed possible that a different mechanism might indeed be operative.

However, a replot of their results (Ref. 3, Figs. 7B, 7C, 9B, and 9D) showed linearity in the relationship between the quantity of drug released from the film and the square root of time (Fig. 6), so that the diffusion model also describes their results. That this model is the correct one for the release of cetylpyridinium chloride and benzalkonium chloride from the films was demonstrated by rate plots, which showed unequivocally that the rate was inversely and not directly proportional to Q' (Fig. 7), and by log–log plots, which gave slopes of 0.5, showing that the release mechanism was the same as for caffeine and salicylic acid.

The apparent first-order pattern observed probably reflects the use of a medicinally reasonable period for the study rather than a period sufficiently long to produce significant exhaustion of the film contents, which would be necessary for testing the physical mechanism of release. It is recommended that in all partial drug release studies, the rate plots against Q' and $1/Q'$ and the log–log plots used in the present work should be applied routinely where the mechanism of release is of interest. Moreover, Q *versus* t or $t^{1/2}$ plots or data should always be given to indicate the actual release pattern, since first-order plots showing log of drug retained in the product, even when linear, do not give the requisite information for predicting accurately the drug release over the full range of the dosage present.

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Chemical Constituents of Gentianaceae XIV: Tetraoxygenated and Pentaoxygenated Xanthones of *Swertia purpurascens* Wall.

S. GHOSAL [x], P. V. SHARMA, R. K. CHAUDHURI, and S. K. BHATTACHARYA *

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Keyphrases □ *Swertia purpurascens* Wall. (Gentianaceae)—five tetraoxygenated and three pentaoxygenated xanthones isolated and identified, screened for pharmacological activity □ Gentianaceae—chemical constituents, five tetraoxygenated and three pentaoxygenated xanthones isolated and identified from *S. purpurascens*, screened for pharmacological activity □ Xanthones, tetraoxygenated and pentaoxygenated—isolated and identified from *S. purpurascens*, screened for pharmacological activity □ Medicinal plants—isolation and identification of tetraoxygenated and pentaoxygenated xanthones from *S. purpurascens*, screened for pharmacological activity

Swertia purpurascens Wall. (Gentianaceae) is widely distributed in India in the temperate North Western Himalayas, 1524–3658 m (5000–12,000 ft), from Kashmir to Kumaon. The plant is used in the Indian system of medicine as a substitute for *Swertia chirata* Buch.-Ham. (1).

In a recent paper (2), isolation and characterization of three tetraoxygenated xanthone *O*-glucosides from the water-soluble xanthone fraction of this plant were reported. Since xanthone-bearing plants generally elaborate multiple xanthones (3), the earlier investigation has now been complemented by examination of the less polar xanthone fractions of this plant. This study has resulted in the isolation and identification of seven tetra- and pentaoxygenated xanthones, three of which are new naturally occurring compounds.

Another reason for the present investigation was to determine the pharmacological profile of activity of the free xanthones on the central nervous system (CNS) of laboratory animals. It was earlier demonstrated (2–6) that, while polyoxygenated xanthones and a xanthone C-glucoside, mangiferin, produced central stimulant action (mediated *via* monoamine oxidase inhibition) (4–6), the corresponding xanthone *O*-glucosides and related compounds showed definite signs of CNS depressant action (antipsycho-

Table I—Glucoxanthone and Glycoflavones Occurring in the Genera *Swertia* and *Gentiana*[a]

Species	Compound	Reference
S. swertopsis	Mangiferin (VIII), isovitexin (IX), homoorientin (X)	15
S. japonica	Orientin (XI), swertiajaponin (XII), swertisin (XIII)	16
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S. chirata	Mangiferin (VIII)	3
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[a] *Note added in proof: G. verna* was recently shown to contain mangiferin and isoorientin. [K. Hostettmann and A. Jacotguillarmod, *Helv. Chim. Acta*, **57**, 1155(1974).]

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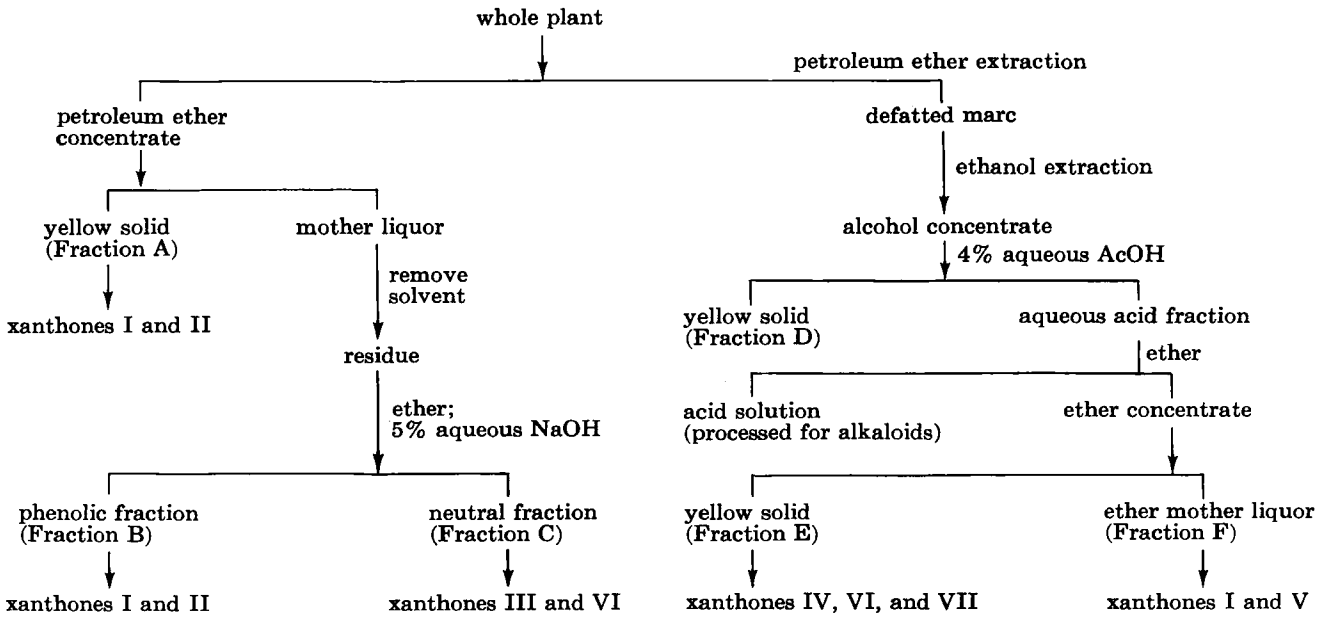


Scheme I—Isolation of tetra- and pentaoxygenated xanthones of S. purpurascens

tic action) (2–4, 6) in albino mice and rats. It was therefore thought worthwhile to study the pharmacological profile of activity of the total free xanthones of *S. purpurascens*.

The details of the chemical and pharmacological evaluations are incorporated in this paper. Additionally, the chemotaxonomic significance of the cooccurrence of several polyoxygenated xanthones, xanthone *O*-glucosides (2), and a glucoflavone (swertisin) (7) in a single plant species is appraised.

EXPERIMENTAL[1]

Extraction of *S. purpurascens*[2]—The isolation of polyoxygenated xanthones[3] from the whole plant of *S. purpurascens* (1 kg) was accomplished following the procedure shown in Scheme I. Dried and milled plant material was continuously extracted in a soxhlet apparatus with petroleum ether (bp 60–80°) and then with ethanol (28 hr each). The two extracts were separately processed.

Isolation of Xanthones from Petroleum Ether Extract—The petroleum ether extract was concentrated (about 150 ml) and the concentrate was kept at room temperature overnight when a yellow solid (7.4 g, Fraction A) separated. The solid was collected by filtration and the mother liquor was evaporated to dryness (13.5 g). A portion of the residue (2.5 g), from the mother liquor was dissolved in ether, and the phenolic and nonphenolic constituents were separated by extraction with aqueous sodium hydroxide (5%, 3 × 30 ml). The alkaline solution, after the usual workup, afforded a brown solid (78 mg, Fraction B). The neutral fraction was obtained as a pale-yellow solid (1.54 g, Fraction C).

Separation of Xanthones from Fraction A—A portion of the solid (1.4 g) was intimately mixed with silica gel (about 10 g) and placed over a silica gel column (20 × 3 cm). The column was eluted with benzene (5 liters), and fractions were collected (100 ml). The middle benzene fractions, on concentration, gave a yellow solid (0.488 g), which was partly soluble in aqueous sodium carbonate (5%). The alkali-insoluble solid was filtered, washed with water, and dried.

Xanthone I (1,5,8-Trihydroxy-3-methoxyxanthone)—The alkali-insoluble solid crystallized from ethanol as shining yellow needles (0.312 g), mp 270–271°. The melting point, mixed melting point, R_f value, and UV, PMR, and mass spectra of the compound were identical with those of authentic 1,5,8-trihydroxy-3-methoxyxanthone (3, 8). The 5,8-dimethyl ether of the xanthone, prepared with ethereal diazomethane, crystallized from ethanol as pale-yellow needles, mp and mixed mp 205°.

Xanthone II (1,3,8-Trihydroxy-5-methoxyxanthone)—The xanthone from the aqueous sodium carbonate-soluble fraction was liberated with hydrochloric acid and extracted with chloroform. The residue (0.102 g) from the chloroform extract crystallized from ethanol as yellow needles, mp 263–265°. The melting point, mixed melting point, R_f value, and UV, PMR, and mass spectra of the compound were identical with those of authentic 1,3,8-trihydroxy-5-methoxyxanthone (3, 8). The 1,3,8-trimethyl ether, prepared with dimethyl sulfate and potassium carbonate in anhydrous acetone (46 hr), crystallized from ethanol as colorless needles, mp 209–210°. The melting point, mixed melting point, and R_f value were identical with those of 1,3,5,8-tetramethoxyxanthone.

Separation of Xanthones from Fraction B—The phenolic constituents (78 mg) showed six major spots with analytical TLC. Preparative layer chromatography of this fraction afforded further crops of xanthones I (41 mg) and II (8 mg). The remaining components of the mixture could not be determined because of their similar R_f values.

Separation of Xanthones from Fraction C—The solid (1.54 g) from Fraction C was dissolved in chloroform and chromatographed over silica gel. Elution was carried out with petroleum ether, benzene, benzene–chloroform (1:1), and chloroform, 3 liters each. Fractions (100 ml) were collected. The later benzene and early benzene–chloroform eluates afforded a mixture of two xanthones, which were separated by preparative layer chromatography using chloroform–benzene–acetic acid (100:40:2) as the solvent.

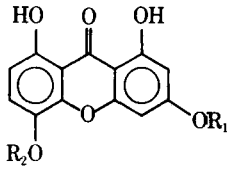
1-Hydroxy-3,7,8-trimethoxyxanthone (Decussatin) (III)—The upper preparative layer zone (R_f 0.6) was eluted with chloroform, and the residue (68 mg) was crystallized from ethanol as yellow needles, mp 148–149°. The melting point, mixed melting point, R_f value, and UV, PMR, and mass spectra of the compound were identical with those of authentic decussatin (3, 8).

1-Hydroxy-3,4,7,8-tetramethoxyxanthone (VI)—The lower preparative layer zone (R_f 0.2), after the usual workup, yielded 1-hydroxy-3,4,7,8-tetramethoxyxanthone as yellow needles (12 mg), mp 188–190°. The melting point, mixed melting point, R_f value, and UV absorption spectrum of the compound were identical with those of an authentic synthetic sample (prepared from decussatin by persulfate oxidation followed by methylation with ethereal diazomethane) of 1-hydroxy-3,4,7,8-tetramethoxyxanthone.

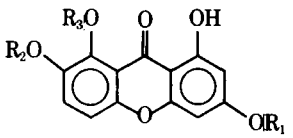
[1] The general directions were reported previously (2).

[2] The plant materials were collected in two lots, in June 1972 and June 1973, by Mr. M. R. Uniyal, Survey of Indian Medicinal Plants Unit, Central Council of Research in Indian Medicine and Homoeopathy, Jogindernagar, Himachal Pradesh. Voucher specimens have been preserved at the Pharmaceutical Chemistry Research Laboratory, Department of Pharmaceutics, Banaras Hindu University.

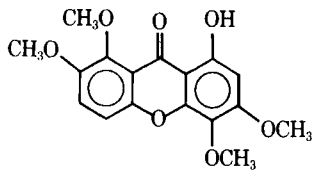
[3] All reported compounds showed satisfactory combustion analyses.



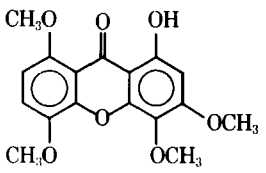
I: $R_1 = CH_3$, $R_2 = H$
II: $R_1 = H$, $R_2 = CH_3$
V: $R_1 = R_2 = H$



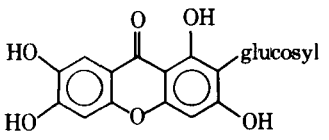
III: $R_1 = R_2 = R_3 = CH_3$
IV: $R_1 = R_2 = R_3 = H$



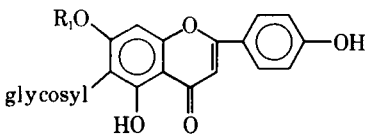
VI



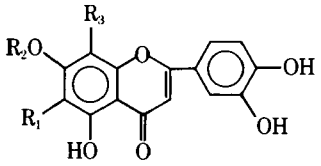
VII



VIII



IX: $R_1 = H$
XIII: $R_1 = CH_3$



X: $R_1 = H$, $R_2 = CH_3$, $R_3 =$ glycosyl
XI: $R_1 = R_2 = H$, $R_3 =$ glycosyl
XII: $R_1 =$ glycosyl, $R_2 = CH_3$, $R_3 = H$
XIV: $R_1 =$ glycosyl, $R_2 = R_3 = H$

In addition to these two xanthones, preparative layer chromatography furnished two minor xanthones from the middle zones. The identity of these two xanthones is currently being investigated.

Isolation of Xanthones from Alcoholic Extract—The ethanol extract of the defatted plant material was concentrated, and the concentrate was allowed to stand at room temperature overnight to detect the presence of mangiferin (8). Since no precipitate appeared, the extract was further concentrated to a syrupy liquid and was poured into aqueous acetic acid (4%, 200 ml). The mixture was kept at room temperature overnight. The precipitated pale-yellow solid was collected by filtration (Fraction D, xanthone *O*-glucosides) (2). The clarified acidic aqueous solution was extracted with ether (5 × 100 ml). The combined ether extracts were processed in the usual way and a dull-yellow solid (0.52 g) was obtained (Fraction E). The ether mother liquor (Fraction F) afforded two xanthones by column chromatography.

Separation of Xanthones from Fraction E—The solid (0.52 g) from Fraction E was extracted several times with hot chloroform to separate the chloroform-soluble fraction from the insoluble one.

1,3,7,8-Tetrahydroxyxanthone (IV)—The chloroform-insoluble solid (0.12 g) crystallized from methanol as yellow needles, mp 333–335°. The melting point, mixed melting point, R_f value, and UV and PMR spectra of the compound were identical with those of authentic 1,3,7,8-tetrahydroxyxanthone (3). The 3,7,8-trimethyl ether, prepared with ethereal diazomethane, crystallized from ethanol as yellow needles, mp and mixed mp (with decussatin) 148–149°.

1-Hydroxy-3,4,5,8-tetramethoxyxanthone (VII) and 1-Hydroxy-3,4,7,8-tetramethoxyxanthone (VI)—The methanol mother liquor, after separation of IV, was treated several times with ethereal diazomethane. The methylated product was subjected to preparative layer chromatography. The upper preparative layer zone afforded a further crop of decussatin (17 mg). The middle zone afforded VII (11 mg) as yellow needles, mp 186–188°. The melting point, mixed melting point, R_f value, and UV and mass spectra were identical with those of authentic, synthetic 1-hydroxy-3,4,5,8-tetramethoxyxanthone (9) (prepared from 1-hydroxy-3,5,8-trimethoxyxanthone by persulfate oxidation followed by methylation with diazomethane). The lower preparative layer zone afforded VI (15 mg) as yellow needles, mp 188–190°.

Separation of Xanthones from Fraction F—The ether mother liquor, after separation of Fraction E, was chromatographed over silica gel (20 × 1.8 cm). The column was eluted with benzene, benzene–chloroform (1:1), and chloroform, 2 liters each. The benzene and benzene–chloroform eluates afforded a further crop of xanthone I (92 mg).

1,3,5,8-Tetrahydroxyxanthone (V)—The chloroform eluates gave a brown solid (82 mg), which crystallized from ethanol as needles, mp 293–295° (M^+, 260, 100%). The trimethyl ether, prepared with ethereal diazomethane, was found to be identical with 1-hydroxy-3,5,8-trimethoxyxanthone (3) in all respects.

Persulfate Oxidation of 1-Hydroxy-3,5,8-trimethoxyxanthone—To a stirred solution of 1-hydroxy-3,5,8-trimethoxyxanthone (0.112 g) in aqueous potassium hydroxide (1.8 g in 2 ml of water) and pyridine (2 ml), an aqueous solution of potassium persulfate (0.23 g in 5 ml of water) was added over 3 hr. After another hour, the solution was neutralized to Congo red and the turbidity was removed by centrifugation. Further acidification of the centrifugate and treatment at 50° with sodium sulfite gave a precipitate which was extracted with ether.

The ether extract was worked up in the usual way. The residue (28 mg) was dissolved in methanol (5 ml) and treated with an excess of ethereal diazomethane. The methyl ether, 1-hydroxy-3,4,5,8-tetramethoxyxanthone, crystallized from ethanol as yellow needles, mp 186–188° [lit. (9) mp 187–188°]; *m/e* 332 (M^+, 100%). The UV absorption spectrum was identical to that reported in the literature (9).

The persulfate oxidation of decussatin and subsequent methylation of the product to 1-hydroxy-3,4,7,8-tetramethoxyxanthone were accomplished following similar procedures as described earlier.

RESULTS AND DISCUSSION

The entire plants were milled and extracted for polyoxygenated xanthones. The isolation and purification of the individual compounds (Scheme I) were accomplished by solvent extraction, column chromatography, preparative layer chromatography, and derivatization. The identity of the known compounds was established by UV, PMR, and mass spectra evidence and by direct comparison with reference samples (3, 8). The characterization of only the new xanthones is described here.

1-Hydroxy-3,4,7,8-tetramethoxyxanthone (VI)—The compound, $C_{17}H_{16}O_7$ (M^+, 332, 100%), mp 188–190°, is a monohydroxytetramethoxyxanthone in which the hydroxyl group is strongly chelated, since it remained unaffected with ethereal diazomethane but formed the permethyl ether with dimethyl sulfate and potassium carbonate in acetone. This finding was also supported by PMR data. The UV absorption spectrum of the compound is characteristic of a 1,3,4,7,8-pentaoxygenated xanthone (9).

The 60-MHz PMR spectrum of the compound, in deuterochloroform, showed one strongly chelated proton at δ 13.04 (C_1—OH), four methoxyl groups at δ 3.94–4.02, and three aromatic protons at δ 6.40 (1H, s, C_2—H), 7.05 (1H, d, J = 9 Hz, C_5—H), and 7.28 (1H, d, J = 9 Hz, C_6—H). In the mass spectrum of the xanthone, aside from the molecular ion peak, intense fragment ion peaks appeared at *m/e* 317 (35%), 302 (48), 289 (20), and 259 (10), associated with the loss of CH_3, CH_2O, C_2H_3O complex, and CH_2O + C_2H_3O groups, respectively, as supported by substantial abundances of metastable ions (3, 8, 10, 11).

On the basis of these observations and conclusions, the pentaox-

ygenated xanthone of *S. purpurascens* is assigned the 1-hydroxy-3,4,7,8-tetramethoxyxanthone structure (VI). Supporting evidence in favor of this conclusion was gained from its synthesis from decussatin. Persulfate oxidation of decussatin, followed by methylation with ethereal diazomethane, gave VI identical with the naturally occurring compound in all respects.

Partially Methylated Pentaoxygenated Xanthones Characterized as 1-Hydroxytetramethoxyxanthones (VI and VII)—TLC of the mixture of xanthones in Fraction E (Scheme I) showed the presence of two strongly polar components. These compounds could not be isolated directly by preparative layer chromatography. Methylation of the mixture with ethereal diazomethane, followed by preparative layer chromatography, afforded the two pentaoxygenated xanthones as homogeneous entities.

The identity of the two compounds was established by direct comparison with authentic synthetic 1-hydroxy-3,4,5,8-tetramethoxyxanthone (VII) and VI. Complete demethylation of the two monohydroxytetramethoxyxanthones with hydrobromic acid afforded the pentahydroxyxanthones, which were more polar than their naturally occurring counterparts. From this observation, it is reasonable to expect that the two pentaoxygenated xanthones (1,3,4,5,8- and 1,3,4,7,8-) are partially methylated. Because of the small quantities of these compounds, further identification was not possible.

Members of the genus *Swertia* are known to elaborate liberally the tetraoxygenated (1,3,5,8- and 1,3,7,8-) xanthones (3, 12); 1,3,4,5,8- and 1,3,4,7,8-pentaoxygenated xanthones are the first two pentaoxygenated xanthone patterns isolated from a member of this genus. The two oxygenation patterns were previously encountered only in the genus *Gentiana* (9). The resemblance in chemical characters of the two genera, *Swertia* and *Gentiana*, is striking. Thus, another significant similarity between the two genera was observed in the flavonoids cooccurring with the polyoxygenated xanthones.

The general pattern of flavonoid distribution in these two genera is the common one of glycoflavones (Table I). Exceptions were noted only in *G. septemfida* and *G. makinoi* (13), where glycoflavones were replaced by flavones. In *S. chirata, S. swertopsis,* and *G. lutea,* in addition to the glycoflavones, a glucoxanthone, mangiferin, was found to cooccur with glycoflavones. Mangiferin is a unique chemical character; both in its distribution and biogenesis, it seems to be more closely related to flavonoids than to xanthone derivatives (3, 8, 12, 14).

These observations and the assumption that the free flavones are vicariously represented by the glycoflavones in the mentioned *Swertia* and *Gentiana* species (Table I) give rise to a number of interesting speculations in plant biochemistry about the route by which synthesis of the flavones could be switched to glycoflavones; the reason for the sparse distribution of mangiferin in xanthone-bearing plants; and phytochemical relationship between taxa on the basis of oxygenation patterns of xanthones. Further studies are clearly necessary to draw any conclusion in this direction.

PHARMACOLOGY

Investigation of the pharmacological activity of the total xanthones (I–VII plus unidentified minor entities) of *S. purpurascens* was conducted on: *(a)* gross general behavior of albino mice and rats, *(b)* hexobarbital narcosis in mice, *(c)* reserpine-induced ptosis and sedation in mice, *(d)* amphetamine toxicity in aggregated mice, *(e)* subanalgesic doses of morphine in albino rats, *(f)* norepinephrine pressor response in pentobarbital-anesthetized dog, and *(g)* toxicity in albino rats.

Albino mice and rats, bred from the CDRI strains, and healthy mongrel dogs were used. The total xanthones were dissolved in 2% aqueous sodium carbonate solution and injected in a dose of 50 mg/kg ip (unless stated otherwise), keeping the volume of the injection at 1 ml/kg. The same volume of the vehicle was injected intraperitoneally to control animals. All experiments were conducted at ambient temperature of 24 ± 2°, according to previously described procedures (5, 6).

The total xanthones produced definite signs of CNS stimulation in albino mice and rats, as evidenced by the increased spontaneous motility, hyperactivity, fine tremors, and piloerection in their gross general behavior; significant potentiation (110% greater than the control) of hexobarbital narcosis; reversal of ptosis and sedation induced by reserpine; potentiation of amphetamine toxicity in aggregated mice (the mortality in the drug-treated group was 6/10 compared to 1/10 in the control group); and potentiation of analgesic effect of subanalgesic doses of morphine in albino rats (but had no analgesic effect *per se*). The total xanthones did not produce any significant potentiation (up to a dose of 100 mg/kg) of norepinephrine pressor response in dogs. The total xanthones were nontoxic up to a dose of 200 mg/kg ip in albino rats.

All methods used are accepted techniques for determining monoamine oxidase inhibiting activity of a compound *in vivo*. The central stimulant actions produced by the total xanthones of *S. purpurascens* are comparable to those of mangiferin (5, 6). Moreover, *in vitro* experiments with mangiferin definitely established that the central stimulant action of this glucoxanthone was mediated *via* monoamine oxidase inhibition. The details of this latter finding will be reported soon. Although the monoamine oxidase inhibiting activity of the total xanthones was not very pronounced, it had the advantage of being devoid of any adverse side effects, *e.g.*, high toxicity and potentiation of pressor effects of tyramine (and equivalents), commonly associated with well-known monoamine oxidase inhibitors.

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Antitumor Agents II: Nitrogen Analogs of Mycophenolic Acid

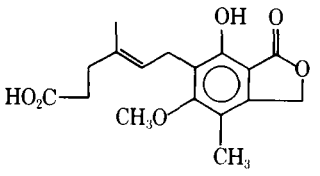
J. A. BEISLER ˣ and SHARON S. HILLERY

Abstract □ Mycophenolic acid, a novel antibiotic of low toxicity containing no nitrogen atoms in its structure, induces tumor regression in several murine solid tumor assays. It has been reported in extensive structure–activity studies that chemical modifications on the antibiotic itself reduce or eliminate antitumor activity. With the objective of antitumor activity enhancement, nitrogen-containing analogs of mycophenolic acid were synthesized according to a program directed toward the ultimate synthesis of close bioisosteres of the antibiotic. Initial efforts reported here describe the terpenoid side-chain degradation of *N*-geranyl-2(1*H*)-pyridones and *N*-geranylglutarimides, where the terminal isopropylidene is replaced with a carboxyl group as it occurs in mycophenolic acid. The resulting nitrogen-containing analogs of the antitumor antibiotic were inactive in the L-1210 and Walker 256 tumor systems.

Keyphrases □ Mycophenolic acid—synthesis and antitumor evaluation of nitrogen analogs □ Antitumor agents—synthesis and antitumor evaluation of nitrogen analogs of mycophenolic acid

Mycophenolic acid (I) was first isolated more than 75 years ago (1) from *Penicillium* culture filtrates and was subsequently shown to possess weak antifungal and antibacterial properties (2–4). Renewed interest in the metabolite was created by communications from three laboratories (5–7) describing its antiviral and antitumor properties.

Virus-induced Rous sarcoma and Friend leukemia were found to be inhibited by I. Although I does not inhibit the growth of L-1210, P-388, and other murine leukemias, it does cause tumor regression in the Walker 256, Sarcoma 180, Lewis lung, and Mecca



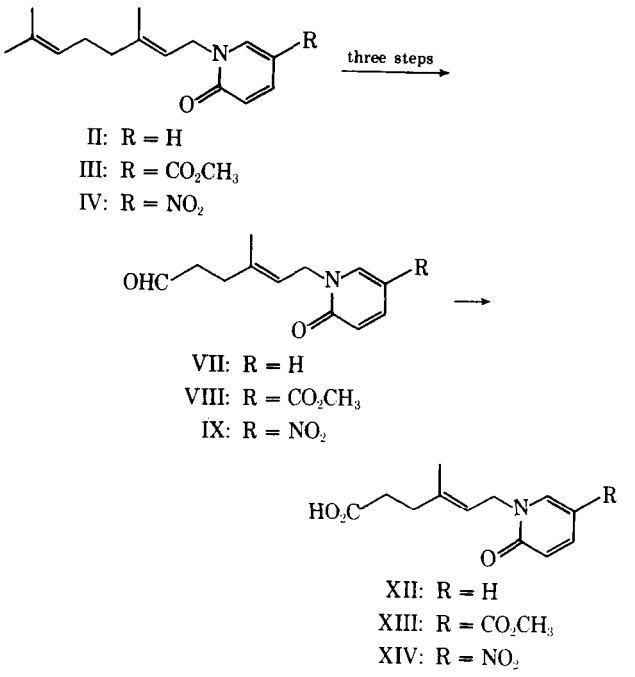
I

lymphosarcoma solid tumor systems. The solid metastasizing tumors can be considered good experimental models of the clinical situation wherein chemotherapy finds its greatest challenge.

Extensive structure–activity relationship studies (8, 9) revealed that even slight structural modifications of I reduced or eliminated antitumor activity. None of the synthetic analogs of I reported to date has shown an activity greater than the parent.

Because of its interesting spectrum of antitumor activity, its unique structure with respect to other antitumor agents, and its low toxicity in rodents (9) and as observed in clinical trials (10, 11), a synthetic study was initiated to develop novel analogs of I as part of a continuing interest in antitumor agents[1].

[1] Part I of series: J. A. Beisler, *J. Med. Chem.*, 14, 1116(1971).

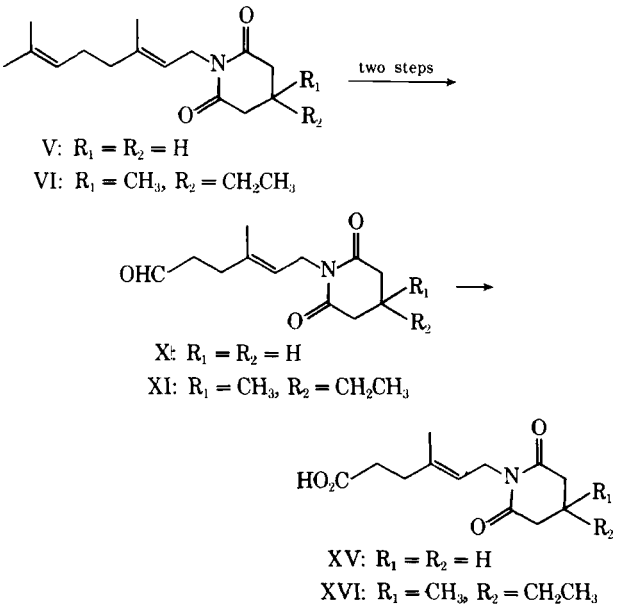


Scheme I

Accordingly, this report describes current efforts to prepare nitrogen analogs of mycophenolic acid.

RESULTS AND DISCUSSION

Tracer experiments have shown that the isoprenoid side chain of mycophenolic acid arises biosynthetically by a selective cleavage of the central double bond in a farnesyl phthalide precursor (12). The



Scheme II

Table I—Experimental Data

Compound	Boiling Point (mm Hg) Melting Point (Solvent)	Procedure	Yield, %	Formula	Analysis, % Calc.	Found
II	126–130°(0.2)	A	73	$C_{15}H_{21}NO$	C 77.88 H 9.15 N 6.05	78.15 9.40 5.89
III	169–171°(0.15)	A	70	$C_{17}H_{23}NO_3$	C 70.56 H 8.01 N 4.84	70.36 8.03 5.01
IV	38–40° (hexane)	A	76	$C_{15}H_{20}N_2O_3$	C 65.19 H 7.30 N 10.14	64.97 7.50 9.93
V	144–148°(0.3)	B	68	$C_{15}H_{23}NO_2$	C 72.25 H 9.30 N 5.62	72.29 9.44 5.41
VI	140–144°(0.15)	B	79	$C_{18}H_{29}NO_2$	C 74.18 H 10.03 N 4.81	74.11 10.11 4.69
VII[a]	140–141° (ethanol)	A	43[b]	$C_{18}H_{19}N_5O_5$	C 56.10 H 4.98 N 18.17	55.97 4.91 17.96
VIII[c]	135–136° (water)	A	62[b]	$C_{15}H_{20}N_4O_4$	C 56.24 H 6.29 N 17.49	56.15 6.21 17.32
IX[a]	185–186° (dimethylformamide–water)	A	35[b]	$C_{18}H_{18}N_6O_7$	C 50.23 H 4.22 N 19.53	50.39 4.28 19.57
X[a]	119–120° (ethanol)	B	56[d]	$C_{18}H_{21}N_5O_6$	C 53.59 H 5.25 N 17.36	53.54 5.07 17.30
XI[a]	107–109° (ethanol)	B	93[d]	$C_{21}H_{27}N_5O_6$	C 56.62 H 6.11 N 15.72	56.46 6.03 15.60
XII	Oil	—	60	$C_{12}H_{15}NO_3$	C 65.14 H 6.83 N 6.33	65.12 6.84 6.24
XII[e]	112–113° (2-propanol–ether)	—	—	$C_{12}H_{14}NO_3Tl$	C 33.94 H 3.32 N 3.30	34.08 3.38 3.28
XIII	90–91° (ethyl acetate–hexane)	—	52	$C_{14}H_{17}NO_5$	C 60.20 H 6.14 N 5.02	60.34 6.17 4.96
XIV	87–88° (ether–cyclohexane)	—	76	$C_{12}H_{14}N_2O_3$	C 54.13 H 5.30 N 10.52	54.27 5.35 10.48
XV	95–96° (ether)	—	63	$C_{12}H_{17}NO_4$	C 60.24 H 7.16 N 5.85	60.45 6.99 5.78
XVI	Oil	—	74	$C_{15}H_{23}NO_4$	C 64.03 H 8.24 N 4.98	63.90 8.36 5.07

[a] Characterized in melting point and analyzed as the 2,4-dinitrophenylhydrazone. [b] Percentage yield over three synthetic steps. [c] Characterized in melting point and analyzed as the semicarbazone. [d] Percentage yield over two synthetic steps. [e] The thallium salt of the carboxylic acid.

cleavage of the 15-carbon atom farnesyl side chain results in the loss of eight atoms while forming the carboxyl function characteristic of the antibiotic.

It was thought that a similar procedure could be adopted from the biosynthesis to develop analogs of I in the necessary multigram amounts required for antitumor testing. However, the C_{10} geranyl group (*i.e.*, having one less isoprene unit than the farnesyl group) seemed more practical for laboratory synthesis and would also afford a double bond in the side chain in the desired position and with a *trans*-disposition as required by I.

It has been demonstrated that one may selectively epoxidize the terminal isopropylidene of the tetrahydropyranyl and acetyl derivatives of geraniol (13). Therefore, it was hoped that the geranyl group bonded to a heterocyclic moiety could also be selectively epoxidized, leading ultimately to the ejection of a three-carbon fragment and formation of a terminal carboxyl group. To that end, geranyl chloride (14) was condensed with 2(1*H*)-pyridone, 5-carbomethoxy-2(1*H*)-pyridone, and 5-nitro-2(1*H*)-pyridone in dimethylformamide solution in the presence of sodium hydride to give the *N*-alkylated products II, III, and IV, respectively.

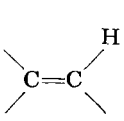
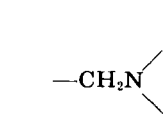
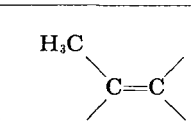
The geranyl pyridones were epoxidized in moderate yield with *m*-chloroperbenzoic acid at the terminal double bond in each instance to the exclusion of any other ether-soluble epoxide products[2]. Following conversion of the epoxides to glycol derivatives with dilute sulfuric acid, the respective aldehydes (VII, VIII, and IX) were formed *via* sodium metaperiodate oxidation (Scheme I).

Glutarimide and bemegride (4-ethyl-4-methylglutarimide) were converted into salts with lithium hydroxide in dimethylformamide solution and condensed with geranyl chloride to provide V and VI, respectively. Epoxidation of V and VI proceeded selectively as already described for the pyridone derivatives (Scheme II). However, through the agency of periodic acid in ether solution, it was possible to cleave the epoxides of V and VI directly into the respective aldehydes (X and XI) without a glycol intermediate.

The aldehydes derived from both the pyridones and the glutarimides were oxidized to their respective carboxylic acids (XII–XVI) with freshly precipitated silver oxide.

[2] In spite of a 50% excess of peracid, it was never quite possible to bring the reaction to completion. TLC always indicated a small amount of starting material.

Table II—Spectral Data

Compound	NMR, ppm, δ					IR, cm^{-1}			
	CHO or COOH								
II		5.26 (m,2H)	4.64 (d,2H)	1.82 (s,3H)	1.62 (s,3H) 1.69 (s,3H)		1653	1582	1529
III		5.17 (m,2H)	4.59 (d,2H)	1.81 (s,3H)	1.61 (s,3H) 1.66 (s,3H)	1720	1660	1610	1540
IV		5.19 (m,2H)	4.57 (d,2H)	1.83 (s,3H)	1.59 (s,3H) 1.67 (s,3H)		1670	1606	1555
V		5.05 (m,2H)	4.23 (d,2H)	1.75 (s,3H)	1.58 (s,3H) 1.66 (s,3H)	1714	1668		
VI		5.18 (m,2H)	4.43 (d,2H)	1.80 (s,3H)	1.60 (s,3H) 1.68 (s,3H)				
VII	9.80 (t,1H)	5.40 (t,1H)	4.60 (d,2H)	1.83 (s,3H)		1712	1647	1572	
VIII	9.80 (t,1H)	5.36 (t,1H)	4.60 (d,2H)	1.83 (s,3H)		1710	1650	1600	1553
IX	9.84 (t,1H)	5.41 (t,1H)	4.66 (d,2H)	1.87 (s,3H)		1716	1669	1604	
X	9.66 (t,1H)	5.21 (t,1H)	4.42 (d,2H)	1.82 (s,3H)		1711	1658		
XI	9.75 (t,1H)	5.18 (t,1H)	4.47 (d,2H)	1.81 (s,3H)					
XII	9.49 (s,1H)	5.38 (t,1H)	4.62 (d,2H)	1.81 (s,3H)		1700	1646	1573	
XIII	9.81 (s,1H)	5.40 (t,1H)	4.62 (d,2H)	1.84 (s,3H)		1705	1660	1600	
XIV	9.72 (s,1H)	5.43 (t,1H)	4.66 (d,2H)	1.86 (s,3H)					
XV	10.03 (s,1H)	5.22 (t,1H)	4.41 (d,2H)	1.82 (s,3H)		1715	1705	1665	
XVI	9.33 (s,1H)	5.18 (t,1H)	4.38 (d,2H)	1.80 (s,3H)		1710	1660		

Tables I and II provide analytical and spectral data in support of the assigned chemical structures.

PHARMACOLOGY

The geranyl derivatives (II–VI) and the carboxylic acids (XII–XVI) were both tested in mice for antitumor activity in the L-1210 leukemia system; the former were suspended in saline with polysorbate 80[3], and the latter carboxylic acids were dissolved in 0.2 *N* NaOH and the pH was adjusted to 8 or 9 prior to administration. In addition, the carboxylic acids were tested in rats using the Walker 256 system.

L-1210 Leukemia System—In separate evaluations, according to the standard protocol (15) of the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, the compounds were administered on Days 1, 5, and 9 and then daily beginning on Day 1 and continuing to Day 9.

Compounds II–VI and XII–XVI showed no significant activity (Table III), as indicated by the ratio of the mean survival time of the test animals over that of the control animals expressed as a percentage (% T/C). A % T/C value greater than 125 is considered indicative of activity in this test system.

Walker 256 System—Since it has been shown (5) that the Walker 256 assay in rats is sensitive to I[4], it was essential that Compounds XII–XVI, within which the exact mycophenolic acid side chain is incorporated, be evaluated in the Walker system. The test was conducted according to the standard Walker 256 protocol (16), where an inoculum of tumor homogenate containing 10^6 viable cells was implanted intramuscularly in random-bred albino rats. Treatment was begun on Day 3 and continued daily through Day 6.

The ratio of the mean weight of excised tumors of the test animals over that of the control animals was the evaluation parameter expressed as % T/C; a value of 42% or less is considered necessary to demonstrate activity. By that criterion, the I analogs (XII–XVI) showed no activity at the dose levels included.

Conclusion—The inactivity of the I analogs (XII–XVI) is probably due to the incomplete functionalization of the heterocyclic portion of the molecules, especially the absence of the lactone group, which perhaps imparts I with an alkylating capacity. Future synthetic goals in this area will concentrate on nitrogen-containing structures which are more rigorously bioisosteric with I.

[3] Tween 80, Sigma Chemical Co.

[4] In this screening laboratory, as a control, mycophenolic acid at 100 mg/kg caused a 90% inhibition of the Walker tumor (*i.e.*, % T/C = 10).

EXPERIMENTAL[5]

***N*-Alkylation with Geranyl Chloride**—*Procedure A, Pyridones II–IV*—To a suspension of 3.15 g (123 mmoles) of sodium hydride in 300 ml of dry dimethylformamide was added, in small portions, the requisite 2(1*H*)-pyridone (120 mmoles). After stirring the reaction solution, which was protected from moisture with an anhydrous calcium sulfate drying tube, for 1 hr at room temperature, a solution of 20.7 g (120 mmoles) of geranyl chloride (14) in 50 ml of dry dimethylformamide was added, and stirring was continued at room temperature for 24 hr.

The reaction solution was poured into 3 liters of water, and the product was collected by multiple ether extraction. After washing once with water, the extracts were dried and concentrated to an oil under water-pump vacuum. The products were purified by vacuum distillation or, in the instance of IV, by crystallization.

Procedure B, Glutarimides V and VI—The appropriate glutarimide (50 mmoles) and lithium hydroxide monohydrate (2.10 g, 50 mmoles) in 100 ml of dimethylformamide were stirred for 1 hr at room temperature while the reaction solution was protected from moisture with a drying tube. Geranyl chloride (8.63 g, 50 mmoles) in 30 ml of dimethylformamide was then added, and the reaction mixture was stirred for 24 hr. The product isolation was identical to that described in Procedure A.

Side-Chain Degradation—*Procedure A, Aldehydes VII–IX*—A cold solution of the *N*-geranyl-2(1*H*)-pyridone (35 mmoles) in 180 ml of anhydrous ether was poured into a cold solution of 85% *m*-chloroperbenzoic acid (10.75 g, 53 mmoles) in 180 ml of anhydrous ether. The resulting reaction solution was stored at about −12° for 24 hr. After shaking the ether solution twice with 1 *N* NaOH solution, it was dried and evaporated to give the epoxide in moderate yield as a clear oil. TLC (silica G, chloroform) analysis invariably indicated a small amount of unreacted starting material[2], which was carried through the next two synthetic steps.

The crude epoxide (13 mmoles) was stirred 30–60 min with 100 ml of 10% H_2SO_4 solution while cooling the reaction flask in an ice bath. After extracting once with a small portion of ether, which

[5] NMR spectra were determined with a Varian A-60 spectrometer, and chemical shifts are expressed as delta (δ) values downfield from tetramethylsilane as the internal standard. IR spectra were recorded with a Perkin-Elmer model 521 spectrophotometer. Combustion analyses were performed by Galbraith Laboratories, Inc., Knoxville, Tenn. Dimethylformamide was dried over a 3A molecular sieve prior to use. Extracts were dried with anhydrous sodium sulfate. Pure geraniol (98%) for the preparation of geranyl chloride was obtained from Bush Boake Allen, Inc., Norwood, N.J. Capillary melting points and boiling points are uncorrected.

Table III—Antitumor Evaluation

Compound	L-1210, % T/C (Dose)[a]	Walker 256, % T/C (Dose)[a]	NSC Number
II	93(50)[b]		160,456
III	101(400)		163,543
IV	97(400)		160,454
V	93(400)		160,455
VI	97(400)		169,795
XII	96(400)	106(200)	173,105
XIII	98(400)	102(200)	173,104
XIV	97(400)	71(400)	173,106
XV	93(400)	106(400)	173,108
XVI	93(400)	104(400)	173,107

[a] The specified dose (milligrams per kilogram) was the highest dose level given, producing the indicated % T/C in a dose–response assay. [b] Toxic at 100 mg/kg.

was discarded, the reaction solution was saturated with sodium chloride and the product was collected by exhaustive methylene chloride extraction. After drying the extracts, solvent removal *in vacuo* gave essentially a quantitative yield of the glycol. For solubility reasons, it was necessary to add 20 ml of 1,2-dimethoxyethane to the dilute sulfuric acid in reaction with the epoxide of IV; the ether extraction was then omitted.

In a typical run, the glycol (5 mmoles) was oxidatively cleaved in 50 ml of methanol–water (1:1) by a dropwise addition of sodium metaperiodate (2.14 g, 10 mmoles) in 50 ml of methanol–water (1:1). After the addition, the mixture was stirred in the dark at room temperature for 1.0–1.5 hr. The inorganic precipitate was removed from the reaction mixture by filtration, and the filtrate was concentrated to half under vacuum.

Following saturation of the concentrate with sodium chloride, the solution was thoroughly extracted with methylene chloride. The dried extracts were evaporated to afford the aldehydes in good yield, which were characterized as 2,4-dinitrophenylhydrazone derivatives except for II. Compound II was characterized as a semicarbazone.

Procedure B, Aldehydes X and XI—The epoxidation of the *N*-geranylglutarimides (V and VI) was carried out in the same way as in Procedure A. The aldehydes X and XI were formed in good yield directly by treating the corresponding epoxides with ethereal periodic acid. Thus, 10 mmoles of epoxide in 200 ml of ether was treated, while stirring, with 11 mmoles (2.51 g) of periodic acid in 200 ml of ether by dropwise addition at room temperature.

The reaction mixture was stirred for 30 min after the addition and then filtered. The filtrate was washed once with cold, dilute sodium bicarbonate solution and dried. Evaporation of the ether *in vacuo* gave the aldehydes as colorless oils, which were characterized *via* the 2,4-dinitrophenylhydrazones.

Preparation of the Carboxylic Acids (XII–XVI)—Silver oxide (Ag_2O) was freshly prepared by adding a solution of 5.25 g of sodium hydroxide in 75 ml of water to 10.5 g of silver nitrate in 75 ml of water. The oxide was collected by vacuum filtration, washed several times with water, and suspended in 100 ml of water. The suspension was added to 20 mmoles of aldehyde (VII–XI) slurried in water and stirred at room temperature for 2–6 hr. Aldehydes IX and XI were dissolved in dimethoxyethane and dioxane, respectively, prior to the addition of the silver oxide suspension.

At the end of the reaction time, the reaction mixture was made basic with saturated sodium bicarbonate solution, stirred an additional 30 min, and then vacuum filtered through a pad of diatomaceous silica[6]. After removing neutral materials from the filtrate by ether extraction, the filtrate was acidified with 10% nitric acid solution, saturated with potassium nitrate, and extracted exhaustively with methylene chloride. Evaporation of the extracts gave the carboxylic acids.

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[6] Celite, Johns-Manville Co.

Hydrodynamic Analog Model for Pharmacokinetics I: Model and Its Usefulness

V. S. VAIDHYANATHAN

Abstract □ A novel hydrodynamic model for drug distribution, when the drug is administered intravenously in physiological systems, is presented. In addition to obtaining results of familiar multicompartment models, the theory presents fresh insights into pharmacokinetic problems. A detailed procedure is included for evaluating drug–physiological system parameters such as the elimination rate constant, volumes of distribution, and permeability properties from the experimental time course of concentration of the central compartment.

Keyphrases □ Model, hydrodynamic analog—proposed for pharmacokinetics, applications, equations □ *Diffusion model*, hydrodynamic, analog—proposed for pharmacokinetics, applications, equations □ Pharmacokinetics—hydrodynamic analog model proposed, evaluation of elimination rate constant, volume of distribution, and permeability properties

Several examples of experimental studies of drug elimination from plasma following absorption and initial distribution are available (1–4). Intravenous administration of a drug assures that the administered substance is initially distributed in plasma and then to other tissues including the kidneys, and finally it is eliminated from the body. The salient feature of these studies is that variations of drug concentration in plasma with time can be adequately represented as the sum of two or three exponential functions, the characteristic parameters of which are evaluated experimentally.

The theoretical foundations of such pharmacokinetic experiments are based on the two- and three-compartment models (1, 2). This report presents a novel hydrodynamic picture of what is probably happening to substances administered intravenously (5). The model presented is simplified for clarity and can possibly account for many experimental observations, including the kink observed in the plot of plasma concentration of dicumarol (4) *versus* time.

The model presented enables one to relate the kinetic parameters of familiar compartment models with physical parameters such as the permeability of barriers separating the compartments. The model can be improved by inclusion of appropriate chemical reactions with which the drug can participate in certain compartments and may help in quantitizing phenomena such as bone growth in physiology. Of immediate interest is that the model sheds new light on certain aspects of existing compartment model theories.

HYDRODYNAMIC ANALOG OF THREE-COMPARTMENT MODEL ANALYSIS

Consider the flow of a liquid into Compartments 2 and 3 from Compartment 1 and to the outside through a tap connected to Compartment 2 (Fig. 1). Assume that at time $t = 0$, the liquid is poured into Compartment 1, with no liquid present in Compartments 2 and 3. The partition between Compartments 1 and 3 is porous and has an effective pore area, A_{13}, and thickness, h_{13}. Similarly, the partition between Compartments 1 and 2 is also porous, with an effective pore area, A_{12}, and thickness, h_{12}.

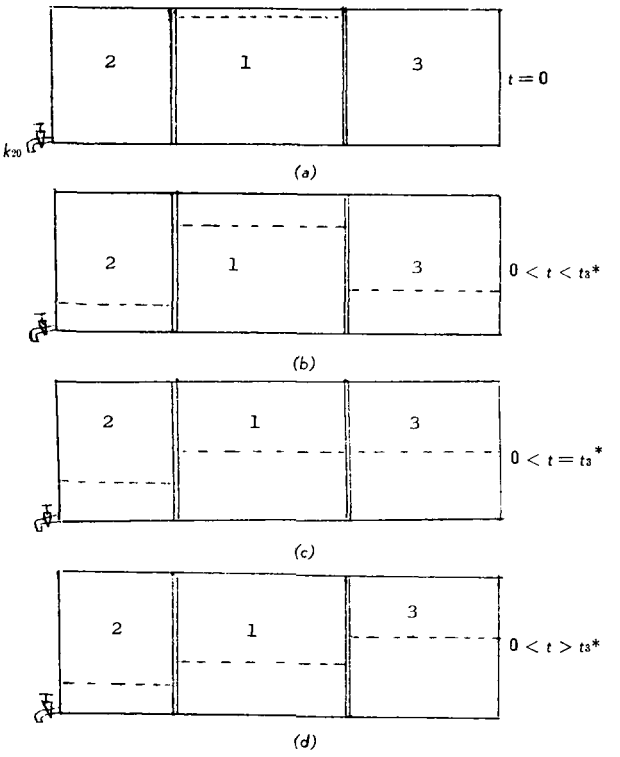

Figure 1—*Hydrodynamic analogy of the three-compartment system.*

Then study the levels of liquid in the three compartments at four arbitrary times (Fig. 1). Initially the liquid flows from Compartment 1 to Compartments 2 and 3 at different rates, depending on the porosity of partitions and the volumes of compartments. This results in a rise of the levels of liquid in Compartments 2 and 3 and a corresponding decrease in the level of liquid in Compartment 1. As time progresses, the difference in levels between Compartments 1 and 3 decreases and the levels in Compartments 1 and 3 become equal at a certain time, $t = t_3^*$. At this time, the net flow of liquid between Compartments 1 and 3 vanishes.

If the tap connected to Compartment 2 has been closed all of the time, there will come a time, $t = t^0$, when levels in all three compartments become equal and the net flow between compartments vanishes. (Discussion about whether t^0 is finite or infinite is subtle and not very relevant to the present discussion.) This represents the most stable equilibrium state of the system. Since, however, the tap connected to Compartment 2 is always (slightly) open, this equilibrium state is never reached in the system depicted in Fig. 1 for reasonable times. The stable state is when all of the liquid in the three compartments has drained to the outside. Thus, for times greater than t_3^*, the direction of flow of liquid between Compartments 1 and 3 reverses and the levels of liquids in the three compartments attain the configuration depicted in Fig. 1*d*. Flow to the outside through the tap continues until all of the liquid is depleted.

The multicompartment models of pharmacokinetics can now be studied with the help of this hydrodynamic analogy, where levels

are to be replaced by concentrations. Certain conclusions of a general nature can be obtained from this extremely simple example.

First, net loss of material from the system occurs through the elimination process (*i.e.*, leak through the tap ignoring any metabolic process loss in compartments), which may be adequately represented by a first-order rate constant, k_{20}. Thus, one has the mass conservation equation for the three-compartment model:

$$(d/dt)\{X_1(t)V_1 + X_2(t)V_2 + X_3(t)V_3\} = -k_{20}V_2X_2(t) \quad \text{(Eq. 1)}$$

where $X_1(t)$, $X_2(t)$, and $X_3(t)$ are concentrations in Compartments 1, 2, and 3, respectively, at time t; and V_1, V_2, and V_3 are the volumes of distribution of the material in Compartments 1, 2, and 3, respectively. The rate of loss of material from the system at time t is assumed to be proportional to the concentration in Compartment 2 at time t, the negative sign in Eq. 1 denoting the loss.

If $Q_{12}(t)$ and $Q_{13}(t)$ represent the net (total) amount of substance transferred from Compartment 1 to Compartments 2 and 3, respectively, from time zero to time t, the mass balance equation may be written as (6):

$$\dot{X}_1(t) + \dot{X}_2(t) + \dot{X}_3(t) = +\dot{Q}_{12}\{(V_1 - V_2)/V_1V_2\} + \dot{Q}_{13}\{(V_1 - V_3)/V_1V_3\} - k_{20}X_2(t) \quad \text{(Eq. 2)}$$

where the super dots indicate first time derivatives of appropriate quantities.

Only when the volumes of distribution of the three compartments equal one another does one get a relation of the type (7):

$$\dot{X}_1(t) + \dot{X}_2(t) + \dot{X}_3(t) = -k_{20}X_2(t) \quad \text{(Eq. 3)}$$

Second, the appropriate initial conditions are that at time $t = 0$, one has:

$$X_1(0) = \text{dose}/V_1 \quad \text{(Eq. 4}a\text{)}$$
$$X_2(0) = X_3(0) = 0 \quad \text{(Eq. 4}b\text{)}$$

The third conclusion that one arrives at is that the transport from Compartment 1 to 3 occurs only in one direction at any time t; the direction of transport is determined by the difference in concentrations between Compartments 1 and 3. Fourth, there exists a positive definite critical time t_3^*, at which the flow between Compartments 1 and 3 vanishes. For times greater than t_3^*, the loss in concentration in Compartment 1 due to the flow to Compartment 2 and the outside is partly offset by the flow of material from Compartment 3 to 1.

The fifth conclusion is that if the leak to the outside is eliminated, *i.e.*, the tap is closed at all times, it takes a minimum finite time, t^0, for the system to reach equilibrium when concentrations in the three compartments will be equal. When k_{20} is equal to zero, only for times equal to and greater than t^0, the right-hand side of Eq. 3 vanishes and Eq. 3 is valid with unequal volumes of distribution of compartments.

Finally, the sixth conclusion that one arrives at from the simple hydrodynamic model is that the variation in concentrations of compartments with respect to time for the system is determined by the set of differential equations:

$$\dot{X}_1(t) = -\{(\delta_{12} + \delta_{13})/V_1\}X_1 + (\delta_{12}/V_1)X_2 + (\delta_{13}/V_1)X_3 \quad \text{(Eq. 5}a\text{)}$$
$$\dot{X}_2(t) = -\{(\delta_{12}/V_2) + k_{20}\}X_2 + (\delta_{12}/V_2)X_1 \quad \text{(Eq. 5}b\text{)}$$
$$\dot{X}_3(t) = -(\delta_{13}/V_3)X_3 + (\delta_{13}/V_3)X_1 \quad \text{(Eq. 5}c\text{)}$$

The constant parameters δ_{12} and δ_{13} can be identified with the use of Fick's law (8) as:

$$\delta_{12} = (D_{12}A_{12}/h_{12}) \quad \text{(Eq. 6}a\text{)}$$
$$\delta_{13} = (D_{13}A_{13}/h_{13}) \quad \text{(Eq. 6}b\text{)}$$

where D_{12} and D_{13} are the diffusion coefficients of administered substance in the barrier between Compartments 1 and 2 and Compartments 1 and 3, respectively; A_{12} and h_{12} are the effective pore area and thickness, respectively, of the barrier separating Compartments 1 and 2; and A_{13} and h_{13} are the effective pore area and thickness, respectively, of the barrier separating Compartments 1 and 3.

The differential equations of conventional pharmacokinetics deal in amounts rather than in concentrations. With the identifications:

$$k_{12} = (\delta_{12}/V_1) \quad k_{21} = (\delta_{12}/V_2) \quad \text{(Eq. 7}a\text{)}$$
$$k_{13} = (\delta_{13}/V_1) \quad k_{31} = (\delta_{13}/V_3) \quad \text{(Eq. 7}b\text{)}$$

where k_{12}, k_{21}, k_{13}, and k_{31} are the first-order rate constants of the familiar three-compartment model, one obtains a one-to-one correspondence between the hydrodynamic analog and the familiar pharmacokinetic expressions.

It is a necessary consequence of the hydrodynamic analogy model and principles of irreversible thermodynamics that:

$$k_{12}^*V_1 = k_{21}^*V_2 \quad \text{(Eq. 8}a\text{)}$$
$$k_{13}^*V_1 = k_{31}^*V_3 \quad \text{(Eq. 8}b\text{)}$$

Thus, with the hydrodynamic model, one is able to relate diffusion coefficients in the barrier, effective pore area, and permeability of the substances across the barrier with the kinetic first-order rate coefficients of the familiar compartmental model equations.

THEORETICAL

Based on the assumption that the rate of transfer of solute material between adjacent compartments at any time t is proportional to the difference in concentrations between the compartments at time t, one has the set of coupled differential equations:

$$\dot{Q}_{12}(t) = \delta_{12}[X_1(t) - X_2(t)] \quad \text{(Eq. 9}a\text{)}$$
$$\dot{Q}_{13}(t) = \delta_{13}[X_1(t) - X_3(t)] \quad \text{(Eq. 9}b\text{)}$$

where $\dot{Q}_{12}(t)$ is the rate of transfer of solute from Compartment 1 to 2 at time t. The concentrations of drug substance in Compartments 1 and 3 at any time t are related to $Q_{12}(t)$ and $Q_{13}(t)$ by the relations (6, 8):

$$X_1(t) = (\text{dose}/V_1) - (1/V_1)\{Q_{12}(t) + Q_{13}(t)\} \quad \text{(Eq. 10}a\text{)}$$
$$X_3(t) = Q_{13}(t)/V_3 \quad \text{(Eq. 10}b\text{)}$$

With k_{20} nonvanishing, the concentration in Compartment 2 at time t is related to $Q_{12}(t)$ by the differential equation:

$$\dot{X}_2(t) + k_{20}X_2(t) = \dot{Q}_{12}(t)/V_2 \quad \text{(Eq. 11)}$$

To obtain the solutions of Eqs. 9*a* and 9*b* subject to the constraints of Eqs. 10*a*, 10*b*, and 11, it is convenient to differentiate once more with respect to time:

$$\ddot{Q}_{12}(t) = \delta_{12}[\dot{X}_1 - \dot{X}_2] \quad \text{(Eq. 12}a\text{)}$$
$$\ddot{Q}_{13}(t) = \delta_{13}[\dot{X}_1 - \dot{X}_3] \quad \text{(Eq. 12}b\text{)}$$

Since the solution of Eq. 11 is evident with vanishing $\dot{Q}_{12}$, assume that the solution of Eqs. 12*a* and 12*b* is given by:

$$X_2(t) = A(t)\exp(-k_{20}t) \quad \text{(Eq. 13)}$$

where $A(t)$ is an unknown function of t to be determined. Use of Eqs. 10*a*, 10*b*, 11, and 13 in Eqs. 12*a* and 12*b* yields, upon rearrangement:

$$\dot{Q}_{12}(t) = \dot{A}(t)V_2\exp(-k_{20}t) \quad \text{(Eq. 14}a\text{)}$$
$$\dot{Q}_{13}(t) = -(V_1V_2/\delta_{12})\{\exp(-k_{20}t)[\ddot{A} + \dot{A}(\alpha_{12} - k_{20}) - A(k_{20}\delta_{12}/V_2)] \quad \text{(Eq. 14}b\text{)}$$
$$\alpha_{12} = \delta_{12}(V_1 + V_2)/V_1V_2 \quad \text{(Eq. 14}c\text{)}$$

Substitution of the results of Eqs. 14*a*–14*c* in Eqs. 15*a* and 15*b* obtainable from Eq. 12*b*—*viz.*:

$$\dddot{Q}_{13} + \alpha_{13}\ddot{Q}_{13} = -(\delta_{13}/V_1)\dot{Q}_{12}(t) \quad \text{(Eq. 15}a\text{)}$$
$$\alpha_{13} = \delta_{13}(V_1 + V_3)/V_1V_3 \quad \text{(Eq. 15}b\text{)}$$

yields a third-order differential equation for the function $A(t)$ with constant coefficients as:

$$\dddot{A} + \ddot{A}P + \dot{A}Q + AR = 0 \qquad \text{(Eq. 16)}$$

where P, Q, and R are time-independent parameters related to the permeabilities, volumes of compartments, and k_{20} by:

$$P = \alpha_{12} + \alpha_{13} - 2k_{20} \qquad \text{(Eq. 17a)}$$

$$Q = [k_{20}\{k_{20} - (\delta_{12}/V_2) - \alpha_{12} - \alpha_{13}\} + \alpha_{12}\alpha_{13} - (\delta_{12}\delta_{13}/V_1^2)] \qquad \text{(Eq. 17b)}$$

$$R = k_{20}(\delta_{12}/V_2)[k_{20} - \alpha_{13}] \qquad \text{(Eq. 17c)}$$

Evidently, the solution of the differential Eq. 16 is:

$$A(t) = \sum_{\sigma=1}^{3} C_\sigma \exp(r_\sigma t) \qquad \text{(Eq. 18)}$$

where C_σ (σ = 1, 2, 3) are constants of integration to be determined from boundary conditions, and r_σ (σ = 1, 2, 3) are the three roots of the cubic equation:

$$r^3 + r^2P + rQ + R = 0 \qquad \text{(Eq. 19)}$$

TIME DEPENDENCE OF CONCENTRATIONS IN COMPARTMENTS

From the results of the previous section, one is now in a position to write down the expressions for time dependence of concentrations in the three compartments:

$$X_1(t) = \sum_\sigma C_\sigma\{1 + mr_\sigma\} \exp(\mu_\sigma t) \qquad \text{(Eq. 20a)}$$

$$X_2(t) = \sum_\sigma C_\sigma \exp(\mu_\sigma t) \qquad \text{(Eq. 20b)}$$

$$X_3(t) = \sum_\sigma C_\sigma \exp(\mu_\sigma t)[1 + r_\sigma m + (V_1V_2/\delta_{12}\delta_{13})M_\sigma] \qquad \text{(Eq. 20c)}$$

$$M_\sigma = r_\sigma^2 + r_\sigma(\alpha_{12} - k_{20}) - k_{20}(\delta_{12}/V_2) \qquad \text{(Eq. 20d)}$$

$$\mu_\sigma = r_\sigma - k_{20} \text{ and } m = (V_2/\delta_{12}) \qquad \text{(Eq. 20e)}$$

Utilizing the initial conditions that at time $t = 0$ the concentrations in Compartments 2 and 3 are null and that the concentration in Compartment 1 equals (dose/V_1), one has from Eqs. 20a–20e:

$$\sum_{\sigma=1}^{3} C_\sigma r_\sigma = \eta \qquad \text{(Eq. 21a)}$$

$$\sum_{\sigma=1}^{3} C_\sigma = 0 \qquad \text{(Eq. 21b)}$$

$$\sum_{\sigma=1}^{3} C_\sigma r_\sigma^2 = \eta\omega \qquad \text{(Eq. 21c)}$$

$$\omega = k_{20} - \alpha_{12} - (\delta_{13}/V_1) \qquad \text{(Eq. 21d)}$$

$$\eta = \text{dose}(\delta_{12}/V_1V_2) \qquad \text{(Eq. 21e)}$$

Solving the set of simultaneous Eqs. 21a–21c, one has the expressions for the three integration constants for the three-compartment model:

$$C_1 = \eta(\omega - r_2 - r_3)/\{(r_1 - r_3)(r_1 - r_2)\} \qquad \text{(Eq. 22a)}$$

$$C_2 = \eta(r_1 + r_3 - \omega)/\{(r_1 - r_2)(r_2 - r_3)\} \qquad \text{(Eq. 22b)}$$

$$C_3 = \eta(\omega - r_1 - r_2)/\{(r_1 - r_3)(r_2 - r_3)\} \qquad \text{(Eq. 22c)}$$

Thus, one has the result that all integration constants are proportional to dose and one can define a parameter p such that:

$$C_2 = C_1p \qquad \text{(Eq. 23a)}$$

$$C_3 = -(1 + p)C_1 \qquad \text{(Eq. 23b)}$$

$$p = [(r_1 + r_3 - \omega) \times (r_1 - r_3)]/[(\omega - r_2 - r_3)(r_2 - r_3)] \qquad \text{(Eq. 23c)}$$

The parameter p is characteristic of the three-compartment pharmacokinetic system. The salient point is that even though the results of this analysis in Eqs. 20a–20e resemble the results of the familiar three-compartment model, as the sum of three exponential terms, one of the integration constants must have a sign opposite to the signs of other two integration constants. This result has the implication (which is not generally recognized) that a *function of the type* $\sum_\sigma A_\sigma \exp(\mu_\sigma t)$, *where* A_σ*'s are constants and* μ_σ*'s are negative definite time-independent quantities, can have extremum values.* This aspect is utilized to explain (9) the observed kink in the plot of plasma concentration *versus* time[1].

EVALUATION OF SYSTEM PARAMETERS FROM EXPERIMENTAL DATA

Aside from the profferred explanation for the possible existence and observation of kinks in the plot of the concentration of the central compartment *versus* time (9), the main results of the current approach presented in Eqs. 20a–20e for the time dependence of concentrations of the three compartments are in agreement with the familiar compartment model analysis. However, the significant feature of the hydrodynamic analog is that the physical meaning of the observed slopes, intercepts, and rate coefficients can be presented.

The parameters characteristic of the system are the permeabilities of the barrier between Compartments 1 and 2, δ_{12}, the barrier between Compartments 1 and 3, δ_{13}, the volumes of distribution of drug in the three compartments, V_σ, and the elimination rate constant, k_{20}. One now evidently needs a detailed procedure for evaluating the parameters characteristic of the physiological system and the drug from experimental data. Due to limitations of the experimental procedure in the physiological system, such experimental information is (unfortunately) usually restricted to the time course of the concentration of drug in Compartment 1.

This analysis categorically points out that the usual identification of the asymptotic slope for large times of the plot of ln $X_1(t)$ with the elimination rate constant k_{20} is untenable. This asymptotic slope equals the smallest of the three decay constants, say $(-\mu_3)$ equal to $(k_{20} - r_3)$.

The familiar resolution of the plot of ln X_1 *versus* t, into sum of three linear plots, yields the values of μ_1, μ_2, and μ_3 for the slopes. Corresponding intercepts at time $t = 0$ yield the values of, for example, i_1, i_2, and i_3. These are the known quantities at present from experimental data. Defining the computable quantities j_σ by:

$$\ln j_\sigma = i_\sigma \qquad \text{(Eq. 24a)}$$

$$j_\sigma = C_\sigma(1 + r_\sigma m);\ \sigma = 1, 2, 3 \qquad \text{(Eq. 24b)}$$

one has the values of $C_\sigma(1 + r_\sigma m)$, where m equals (V_2/δ_{12}).

Additional information available from experimental data is the concentration of drug in Compartment 1 at time $t = 0$—*viz.:*

$$X_1(0) = j_1 + j_2 + j_3 = \text{dose}/V_1 \qquad \text{(Eq. 25)}$$

From a knowledge of the initial dose and $X_1(0)$, the volume of distribution of Compartment 1, V_1, is known.

Since μ_1, μ_2, and μ_3 are known, one can compute the quantities A and C:

$$A = k_{20} + \alpha_{12} + \alpha_{13} \qquad \text{(Eq. 26a)}$$

$$C = k_{20}(\delta_{13}/V_3)(\delta_{12}/V_1) \qquad \text{(Eq. 26b)}$$

from the relations:

$$A = -(\mu_1 + \mu_2 + \mu_3) = P + 3k_{20} \qquad \text{(Eq. 27a)}$$

$$C = -(\mu_1\mu_2\mu_3) = k_{20}^3 + k_{20}^2P + k_{20}Q + R \qquad \text{(Eq. 27b)}$$

Equations 26a, 26b, 27a, and 27b follow from recognition of the fact that the decay constants μ_σ's are the three distinct roots of the cubic equation:

$$\mu_\sigma^3 + \mu_\sigma A + \mu_\sigma B + C = 0 \qquad \text{(Eq. 28a)}$$

$$B = \mu_1\mu_2 + \mu_1\mu_3 + \mu_2\mu_3 = Q + 2k_{20}P + 3k_{20}^2 \qquad \text{(Eq. 28b)}$$

[1] Since a critical examination of the physical and mathematical results of the multicompartment hydrodynamic diffusion analog may not be of much interest to the general reader, these aspects are presented in the next paper.

From Eqs. 20*a*–20*e*, 21*a*–21*e*, and 25, one has:

$$X_1(0) = (C_1m)[(\mu_1 - \mu_3) + p(\mu_2 - \mu_3)] \quad \text{(Eq. 29}a\text{)}$$

$$p\{j_1 - (C_1m)(\mu_1 - \mu_2)\} = j_2 \quad \text{(Eq. 29}b\text{)}$$

Elimination of p from Eqs. 29*a* and 29*b* yields a quadratic expression for the unknown (C_1m):

$$(C_1m)^2(\mu_1 - \mu_2)(\mu_1 - \mu_3) - (C_1m)\{j_1(\mu_1 - \mu_3) + j_2(\mu_2 - \mu_3) + X_1(0)(\mu_1 - \mu_2)\} + j_1X_1(0) = 0 \quad \text{(Eq. 30)}$$

Since all quantities except (C_1m) of Eq. 30 are known from experimental information, one can solve for the value of (C_1m). Then p can be solved using:

$$p = (\mu_2 - \mu_3)^{-1}[\{X_1(0)/(C_1m)\} - (\mu_1 - \mu_3)] \quad \text{(Eq. 31)}$$

One has from Eqs. 21*c* and 23*c* two relations relating ω and k_{20}:

$$\omega(\text{dose}/V_1) = (C_1m)\{\mu_1^2 - \mu_3^2) + p(\mu_2^2 - \mu_3^2) + 2k_{20}[(\mu_1 - \mu_3) + p(\mu_2 - \mu_3)]\} \quad \text{(Eq. 32)}$$

$$\omega = 2k_{20} + [\{(\mu_1^2 - \mu_3^2) + p(\mu_2^2 - \mu_3^2)\}/\{(\mu_1 - \mu_3) + p(\mu_2 - \mu_3)\}] \quad \text{(Eq. 33)}$$

Since p and (C_1m) are now known, Eqs. 32 and 33 should be solved simultaneously for ω and k_{20}. From the knowledge of k_{20}, μ_σ, and j_σ, the values of C_σ, r_σ, and m are computed. From the values of V_1 and V_2 thus obtained, δ_{12} can be computed. Since ω is defined in Eq. 21*d*, one can now compute (δ_{13}/V_1). The value of α_{13} is computed using:

$$\alpha_{13} = A - k_{20} - \alpha_{12} \quad \text{(Eq. 34)}$$

The value of (δ_{13}/V_3) is obtained using:

$$(\delta_{13}/V_3) = \alpha_{13} - (\delta_{13}/V_1) = A + \omega - 2k_{20} \quad \text{(Eq. 35)}$$

From these procedures, all parameters characteristic of the three-compartment pharmacokinetic system and drug are thus computed. Since the quadratic Eq. 30 may yield two distinct values of (C_1m), one of these values may be discarded in certain cases as physically meaningless, using the physical realistic condition that the value of α_{13} should be greater than (δ_{13}/V_1).

The physically meaningful condition that α_{13} is greater than (δ_{13}/V_1) leads to the condition that when the two roots of Eq. 30 are distinct, one of them is meaningless. This conclusion is arrived at by the arguments presented in the *Appendix*.

COMPLETE SPECIFICATION OF PARAMETERS IN THREE-COMPARTMENT SYSTEMS

The prescription presented can be systematically characterized as follows. Compute V_1, the volume of distribution of the central compartment, using Eq. 25. Compute the product (C_1m) using Eq. 30, and compute p using Eq. 31. Then compute ω and k_{20} using Eqs. 32 and 33 simultaneously, and compute r_1, r_2, and r_3 using the relations $r_\sigma = \mu_\sigma + k_{20}$.

Compute C_1 using the relation $C_1 = j_1 - r_1(C_1m)$; compute C_2 and C_3 using Eqs. 23*a* and 23*b*, respectively. The sum of $(\alpha_{12} + \alpha_{13})$ and $\{\alpha_{12} + (\delta_{13}/V_1)\}$ are known from Eqs. 34 and 35. Since (δ_{13}/V_3) is also known from Eq. 35, Eq. 26*b* yields the value of (δ_{12}/V_1). Thus, δ_{12} is now known. Since m is known from values of C_1 and (C_1m), V_2 is computable. Thus, calculation of individual values of α_{12} and α_{13} is now feasible with Eq. 26*a*. Compute (δ_{13}/V_1) from knowledge of α_{13} and (δ_{13}/V_3).

CONCLUSIONS

The motivation behind the development of the hydrodynamic diffusion model as an alternative to more familiar pharmacokinetic multicompartment models has been many fold. The present work derived inspiration from the classic work of Northrop and Anson (6).

The similarity of the method of resolution of compound radioactivity into component contributions and familiar multicompartment models of pharmacokinetics is at best only superficial. Radioactive decay processes have the unique advantages that decay constants are independent of environment and that the integration constants encountered are just the concentrations at initial time of independently decaying individual components. In two- or three-compartment models, the integration constants, although proportional to dose, are functions of decay constants.

The decay constants of familiar pharmacokinetics are determined by system parameters such as volumes of distribution of various compartments, permeability properties of barriers partitioning the system into compartments, and the behavior of the drug itself. In familiar compartment models, much of this information is latent in the kinetic rate coefficients, and extraction of physical properties of the system from such rate coefficients is messy. It is also maintained that these decay constants are independent of volumes of distribution in familiar pharmacokinetics. Introduction of five rate constants, while three are sufficient, to describe the flow processes in three-compartment models and balancing gain *versus* loss by forward flow minus backward flow is unnecessary and aesthetically unappealing.

Other motivations behind the present work are to explain possible observations of kinks in the concentration time plot of central compartments (4) and to analyze the theoretical possibility of observation of aperiodic oscillatory phenomena with certain drugs in certain physiological systems. Some of these considerations are presented in the following paper (9). It should be emphasized, however, that observation of even one such kink requires that the drug and the physiological systems simulate a multicompartment system with the number of compartments greater than three.

A reliable method of computation of characteristic parameters of the system from experimental data in preference to the familiar procedure is presented. One can incorporate improvements such as multiple doses given at different times through the central compartment as well as metabolic reactions in various compartments and barriers with which the administered drug can participate. Extension of the method to the multicompartment model is straightforward and naturally tedious. The presented model can be elaborated further to include real situations.

APPENDIX

It is shown here that the physical condition that α_{13} is greater than (δ_{13}/V_1) results always in only one physically meaningful root of Eq. 30. Defining $a = (\mu_1 - \mu_2)$, $b = (\mu_1 - \mu_3)$, $c = (\mu_2 - \mu_3)$, $Y = (C_1m)$, and $d = (\delta_{13}/V_3)$, one has Eqs. 22*a*–22*c* written as:

$$Yab = X_1(0)(d + \mu_1) \quad \text{(Eq. A1}a\text{)}$$

$$Ypac = -X_1(0)(d + \mu_2) \quad \text{(Eq. A1}b\text{)}$$

$$Y(1 + p)bc = -X_1(0)(d + \mu_3) \quad \text{(Eq. A1}c\text{)}$$

The physical condition that α_{13} is greater than (δ_{13}/V_1) demands that d is positive definite. Equations 29*a* and 29*b* become:

$$X_1(0) = Y(b + pc) \quad \text{(Eq. A2}a\text{)}$$

$$p = j_2/(j_1 - Ya) \quad \text{(Eq. A2}b\text{)}$$

Replacing Y in Eqs. A2*a* and A2*b* by the result of Eq. A1*a* yields:

$$j_2c = X_1(0)(d + \mu_1 - a) - j_1b + j_1\{ab/(d + \mu_1)\} \quad \text{(Eq. A3)}$$

The coefficient of (C_1m) of Eq. 30 becomes:

$$X_1(0)(d + \mu_1) + j_1\{ab/(d + \mu_1)\} \quad \text{(Eq. A4)}$$

and the two roots of Eq. 30 are:

$$Y^+ = X_1(0)(d + \mu_1)/ab \quad \text{(Eq. A5}a\text{)}$$

$$Y^- = j_1/(d + \mu_1) \quad \text{(Eq. A5}b\text{)}$$

Thus, solution for (C_1m) expressed by Eq. A5*a* is in agreement with Eq. A1*a*. Since $j_1 = C_1 + C_1m(\mu_1 + k_{20})$, one has from Eq. A5*b* that:

$$1 = m(d - k_{20}) \quad \text{(Eq. A6)}$$

If d is zero or negative, or if d is positive and has a magnitude less than k_{20}, one has the nonphysical result that m should be negative. Thus, the second root of Eq. 30 becomes meaningless

when d is zero or negative. Unless the value of j_1 equals $X_1(0)\{(d + \mu_1)^2/(ab)\}$, the two roots of Eq. 30 will be distinct.

DEFINITION OF SYMBOLS

μ_σ = decay constants of exponentials
r_σ = roots of cubic equation (Eq. 19)
V_σ = volume of distribution of the compartments
C_σ = integration constants proportional to dose
t_1^* = time at which minimum occurs in $X_1(t)$
t_1^{**} = time at which maximum may occur in $X_1(t)$
t_2^* = time at which maximum occurs in $X_2(t)$
t_3^* = time at which maximum occurs in $X_3(t)$; $X_3(t_3^*) = X_1(t_3^*)$
$t(0)$ = time at which extremum occurs in $F(t)$
X_σ = concentration in Compartment σ
i_σ = intercepts of three resolved plots whose sum depicts $\ln X(t)$
δ_{12} = permeability of drug across the barrier between Compartments 1 and 2
$\alpha_{12} = (\delta_{12}/V_1) + (\delta_{12}/V_2)$

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Hydrodynamic Analog Model for Pharmacokinetics II: Critical Examination of Model and Its Contribution to Pharmacokinetics

V. S. VAIDHYANATHAN

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Keyphrases □ Models, hydrodynamic diffusion analog—proposed for pharmacokinetics, critical examination, equations □ Diffusion model, hydrodynamic, analog—proposed for pharmacokinetics, critical examination, equations □ Pharmacokinetics—hydrodynamic diffusion analog model proposed and critically examined, equations

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ANALYSIS

Mathematically, both the hydrodynamic analog and familiar multicompartment models essentially involve solutions of coupled first-order linear differential equations of the kind (2):

$$\dot{X}(t) = AX(t) \qquad \text{(Eq. 1)}$$

where X is an n-component vector whose elements represent concentrations at time t of the n-compartment diffusion model (1).

In the familiar pharmacokinetic model, these vector elements represent the amount of substance present in each compartment. The super dot in Eq. 1 denotes the first time derivative. The time-independent elements of matrix A of Eq. 1 represent the permeabilities of the drug in barriers between connected compartments in the hydrodynamic diffusion model. Thus, when there is no connection between Compartments i and j, the corresponding matrix element A_{ij} is zero.

In the familiar pharmacokinetic model, the elements of the corresponding matrix are linear combinations of assumed first-order rate constants. For example, the partial contribution (due to the existence of connectivity with Compartment j) to the rate of decrease in the amount of material in Compartment i is assumed to be given by:

$$\dot{Y}_i = -k_{ij}Y_i + k_{ji}Y_j \qquad \text{(Eq. 2)}$$

where Y_j and Y_i are the amounts in Compartments j and i, respectively, at time t; and the k_{ij}'s are the assumed first-order rate constants.

Thus, volumes of distribution of various compartments are not introduced in the formulation of the familiar pharmacokinetic model and these need to be extracted from experimental data on the basis of *ad hoc* assumptions. The decay constants of conventional pharmacokinetics are considered not as functions of volumes of distribution. Since volumes of distribution of various compartments in an n-compartment model definitely play a role in the material distribution in various compartments at arbitrary finite

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where Y_j and Y_i are the amounts in Compartments j and i, respectively, at time t; and the k_{ij}'s are the assumed first-order rate constants.

Thus, volumes of distribution of various compartments are not introduced in the formulation of the familiar pharmacokinetic model and these need to be extracted from experimental data on the basis of *ad hoc* assumptions. The decay constants of conventional pharmacokinetics are considered not as functions of volumes of distribution. Since volumes of distribution of various compartments in an n-compartment model definitely play a role in the material distribution in various compartments at arbitrary finite

time, t, such volumes of distribution lie buried in the expression of familiar pharmacokinetics, which is a solution of Eq. 1, namely that the amount of material of Compartment j is given by:

$$Y_j(t) = \sum_{i=1}^{n} A_{ji} \exp(\lambda_i t) \qquad \text{(Eq. 3)}$$

In the diffusion model (1), one defines the partial contribution to the rate of decrease in concentration of Compartment i, due to connectivity with Compartment j, by the relation (see Eqs. 9 and 10 of Ref. 1):

$$V_i \dot{X}_i(t) = \delta_{ij}[X_i - X_j] \qquad \text{(Eq. 4)}$$

In this manner, the influence of volumes of distribution of various compartments on the time course of concentrations in various connected compartments are explicitly introduced in the formulation of the differential equations in the hydrodynamic (diffusion) analog (1). This approach has the advantage of precisely incorporating the influence of the volumes of distribution, the permeability properties of barriers between connected compartments to drug, and the elimination rate constants with the decay constants experimentally measurable and the coefficients of the solution of Eq. 1—*viz.* (see Eqs. 17, 22, and 26 of Ref. 1):

$$X_j(t) = \sum_{\sigma=1}^{n} S_{j\sigma} \exp(\mu_\sigma t) \qquad \text{(Eq. 5)}$$

Thus, there are no uncertainties about the role played by characteristic parameters of the physiological system in the determination of the time course of concentrations of drug in any compartment. This aspect may be considered the salient feature of the diffusion analog. In addition, the exact relationships existing between coefficients $S_{j\sigma}$ and $S_{i\sigma}$ can be expressed in terms of eigenvalues.

Returning now to another aspect of the pharmacokinetic problem, one recognizes that concentrations in compartments other than the central one (Compartments 2 and 3 of the three-compartment model of Ref. 1) are zero at initial time and at $t = \infty$. Since concentrations are positive definite quantities for intermediate finite times, there must exist a finite time t_σ^* when $\dot{X}_\sigma = 0$. Thus, the concentration–time plot of the $(n - 1)$ compartments of n-compartment systems should all exhibit maxima. If the nth central compartment also exhibits extremum times at which $\dot{X}_1(t)$ vanishes, then the observation of kinks and shoulders becomes amenable to explanation within the context of the diffusion model of pharmacokinetics.

DESCRIPTIVE ANALYSIS OF BEHAVIOR OF $X_1(t)$

The time dependence of concentrations in the three compartments as depicted by the theory (1) are schematically presented in Fig. 1. Curves II and III represent the time dependence of concentrations of Compartments 2 and 3, respectively, exhibiting extrema at times t_2^* and t_3^*. These are the shapes of concentration–time curves that will be observed for a three-compartment system with no chemical reactions.

Figure 1 also shows three alternative curves, I*a*, I*b*, and I*c*, which are mutually exclusive for the time dependence of concentration of drug in Compartment 1. Plot I*a* is the familiar monotonic curve experimentally observed for $X_1(t)$ in many systems. This can be resolved into three distinct exponential functions by the well-known procedure of resolution of the plot of ln $X_1(t)$ into three linear plots with positive intercepts i_1, i_2, and i_3 on the concentration axis. The procedures of obtaining the permeability coefficients, volumes of distribution, and elimination rate constants from such experimental data were presented previously (1).

Plots I*b* and I*c* of Fig. 1 are schematic examples of $X_1(t)$, which exhibit one extremum and two extremum times, respectively. Plot I*c* is similar to the case of dicumarol behavior upon intravenous administration, exhibiting the "kink." Plot I*b* may be considered as a special case of plot I*c*, where the two extremum points t_1^* and t_1^{**} as well as the inflection time coincide such that the time derivative of $X_1(t)$, while remaining negative most of the time, momentarily approaches zero. Such a behavior occurs when the rate of gain of material from Compartment 3 by Compartment 1 equals the rate of loss to Compartment 2 from Compartment 1.

For the three-compartment system, the time t_2^* at which concentration in Compartment 2 attains maximum value is given by:

$$p = (C_2/C_1) = (\mu_1 - \mu_3 F)/(\mu_3 F - \mu_2 G) \qquad \text{(Eq. 6a)}$$

$$F = \exp(\mu_3 - \mu_1)t_2^* \qquad \text{(Eq. 6b)}$$

$$G = \exp(\mu_2 - \mu_1)t_2^* \qquad \text{(Eq. 6c)}$$

Similarly, the time t_3^* at which concentration in Compartment 3 reaches a maximum value is given by:

$$p = (\mu_1 R_1 - \mu_3 R_3 H)/(\mu_3 R_3 H - \mu_2 R_2 I) \qquad \text{(Eq. 7a)}$$

$$H = \exp(\mu_3 - \mu_1)t_3^* \qquad \text{(Eq. 7b)}$$

$$I = \exp(\mu_2 - \mu_1)t_3^* \qquad \text{(Eq. 7c)}$$

$$R_\sigma(\sigma = 1, 2, 3) = [1 + m\{r_\sigma + (V_1/\delta_{13})M_\sigma\}] \qquad \text{(Eq. 7d)}$$

Thus, the characteristic times t_2^* and t_3^* are independent of dose and are related to each other and constant parameters of the system by:

$$\mu_1\mu_3(R_3H - R_1F) + \mu_1\mu_2(R_1G - R_2I) + \mu_2\mu_3(R_2IF - R_3GH) = 0 \qquad \text{(Eq. 8)}$$

CONDITIONS FOR OBSERVATION OF KINK

Assume that a system obeying the three-compartment hydrodynamic model exhibits a kink similar to I*c* of Fig. 1 in the concentration–time plot of the central compartment concentration. Thus, both t_1^* and t_1^{**} exist for this system and are observable. When two such extremum times exist, evidently an inflection point $t_1^\dagger$ should also exist. When these three real times exist, one has from Eqs. 20*a*–20*e* of Ref. 1:

$$\mu_1 S_1 + p\mu_2 S_2 Y_2^* = (1 + p)\mu_3 S_3 Y_3^* \qquad \text{(Eq. 9a)}$$

$$\mu_1^2 S_1 + p\mu_2^2 S_2 Z_2 = (1 + p)\mu_3^2 S_3 Z_3 \qquad \text{(Eq. 9b)}$$

$$\mu_1 S_1 + p\mu_2 S_2 Y_2^{**} = (1 + p)\mu_3 S_3 Y_3^{**} \qquad \text{(Eq. 9c)}$$

where:

$$S_\sigma = 1 + r_\sigma m \qquad \text{(Eq. 10a)}$$

$$Y_2^* = \exp(\mu_2 - \mu_1)t_1^* \qquad \text{(Eq. 10b)}$$

$$Y_3^* = \exp(\mu_3 - \mu_1)t_1^* \qquad \text{(Eq. 10c)}$$

$$Z_2 = \exp(\mu_2 - \mu_1)t_1^\dagger \qquad \text{(Eq. 10d)}$$

$$Z_3 = \exp(\mu_3 - \mu_1)t_1^\dagger \qquad \text{(Eq. 10e)}$$

$$Y_2^{**} = \exp(\mu_2 - \mu_1)t_1^{**} \qquad \text{(Eq. 10f)}$$

$$Y_3^{**} = \exp(\mu_3 - \mu_1)t_1^{**} \qquad \text{(Eq. 10g)}$$

Similar to the existence of the critical point in van der Waals' isotherm, when the three times t_1^*, $t_1^\dagger$, and t_1^{**} equal each other, one has the expression:

$$\exp\{(\mu_2 - \mu_3)t_1^*\} = [(1 + p)S_3\mu_3(\mu_1 - \mu_3)]/[pS_2\mu_2(\mu_1 - \mu_2)] \qquad \text{(Eq. 11)}$$

The conclusion that one arrives at from Eqs. 9 and 11 is that these critical times are independent of dose. This conclusion is substantiated by the case of dicumarol where the time at which kink occurs is essentially independent of the dose of drug.

Since the left-hand side of Eq. 11 is positive definite, in order that t_1^* exists and is real and observable, the necessary condition is:

$$(1 + p)S_3/(pS_2) = -C_3(1 + r_3 m)/[C_2(1 + r_2 m)] > 0 \qquad \text{(Eq. 12)}$$

When t_1^*, $t_1^\dagger$, and t_1^{**} exist and are distinct, one has:

$$-j_3/j_2 = (\mu_1 Y_2^* - \mu_2 Z_2)/(\mu_1 Y_3^* - \mu_3 Z_3) \qquad \text{(Eq. 13a)}$$

$$= (Y_2^* - Y_2^{**})/(Y_3^* - Y_3^{**}) \qquad \text{(Eq. 13b)}$$

For the sake of brevity, the following discussion to explain the possibility of experimentally observing curves of types I*b* and I*c* of Fig. 1 is limited to systems in which α_{13} is greater than k_{20}. It can be shown (see *Appendix*) that C_1 and C_3 are negative definite for

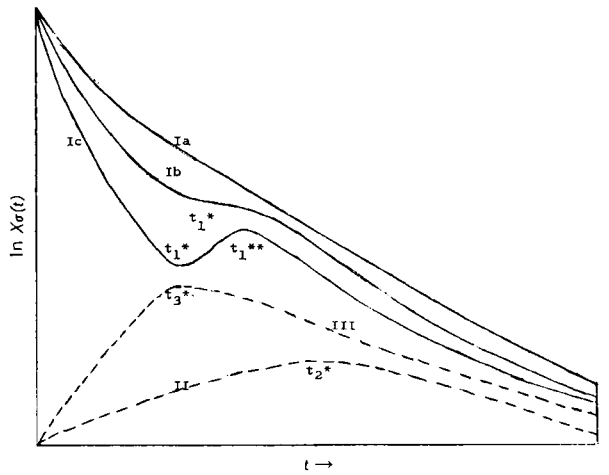

Figure 1—*Curves II and III represent time dependence of concentrations in Compartments 2 and 3, respectively, of the three-compartment hydrodynamic model. The time dependence of concentrations of Compartment 1 is represented schematically by plot Ia when there is no extremum time, by plot Ib when there is one extremum time at* t_1^**, and by plot Ic when there are two extremum times.*

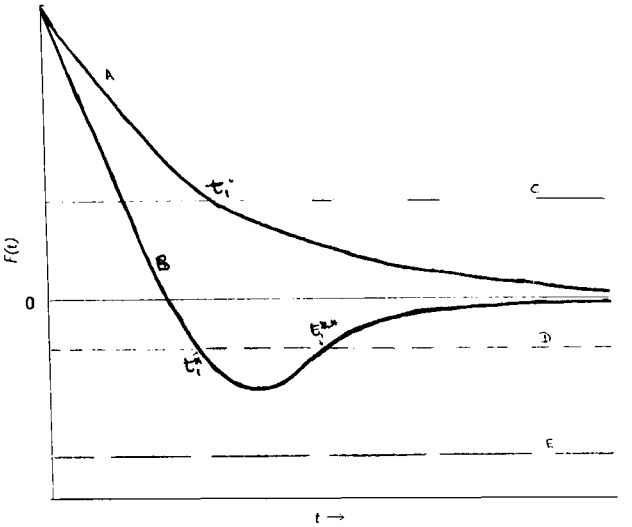

Figure 2—*Time dependence of the function* F(t) *is shown schematically by curve A when there is no extremum and by curve B when there is an extremum. Curves C, D, and E represent three possible values of* $(-C_3S_3)$.

this system. Recall from Eqs. 17*a* and 20*e* of Ref. 1 that one has:

$$\alpha_{12} + \alpha_{13} + k_{20} = -(\mu_1 + \mu_2 + \mu_3) > 0 \quad \text{(Eq. 14)}$$

The parameters α_{12}, α_{13}, μ_σ's, k_{20}, and r_σ's have dimensions of reciprocal seconds. Thus, all of these parameters can be expressed as proportional to k_{20}, the proportionality constants being simple numbers.

When specifically considering the case of r_1 and r_2 being negative and r_3 positive, both S_1 and S_2 are negative provided that the magnitudes of r_1m and r_2m are greater than unity; S_3 is positive definite. The requirement that both S_1 and S_2 are negative is assured when $(\delta_{12}/V_2) < \alpha_{12} < k_{20}$.

Under these conditions, both C_1S_1 and C_3S_3 are positive, while C_2S_2 is negative. With these preliminaries, one is now in a position to discuss the existence or nonexistence of t_1^*, the time at which time derivative of $X_1(t)$ vanishes.

A function $F(t)$, defined by:

$$F(t) = C_1S_1(\mu_1/\mu_3)\exp[(\mu_1 - \mu_3)t] + C_2S_2(\mu_2/\mu_3)\exp[(\mu_2 - \mu_3)t] \quad \text{(Eq. 15a)}$$

$$F(t_1^*) = -C_3S_3 \quad \text{(Eq. 15b)}$$

has the behavior of either plot A or B of Fig. 2. Note that $F(0) = \dot{X}_1(0)/\mu_3$ is positive. It can be shown that $F(t)$ can be either monotonic with time or at best can have one and only one extremum point. When no extremum point exists, the behavior of $F(t)$ is schematically represented by plot A of Fig. 2.

When an extremum point exists in $F(t)$, the behavior of $F(t)$ is represented by plot B of Fig. 2. The extremum point of $F(t)$, t_0, is given by:

$$t_0 = (\mu_2 - \mu_1)^{-1}\ln|[-C_1S_1\mu_1(\mu_1 - \mu_3)]/[C_2S_2\mu_2(\mu_2 - \mu_3)]| \quad \text{(Eq. 16)}$$

In order that t_0 exists, the quantity within the brackets of Eq. 16 should be positive and greater than unity. Figure 2 also shows plots C, D, and E, drawn parallel to the time axis and representing three possible magnitudes of C_3S_3; C_3S_3 is negative in curve C and positive in plots D and E.

The condition that $X_1(t)$ is positive definite for all finite times requires that C_3S_3 is positive definite. The intersection point between $F(t)$ and the line drawn parallel to the time axis with magnitude C_3S_3 evidently denotes the time t_1^*, at which time $\dot{X}_1(t) = 0$. Thus, when $F(t)$ for a system is monotonic with time, no extremum point in $X_1(t)$ occurs. Also, when for a system $F(t)$ has an extremum and has the shape of curve B, if the magnitude of C_3S_3 is large so that no intersection occurs, then no extremum point occurs in $X_1(t)$. Thus, experimental exhibition of the kink in the plot of $X_1(t)$ of a drug in a physiological system, simulating a three-compartment model, is critically dependent on the magnitude of C_3S_3 (hence on i_3) and on the existence of t_0 of $F(t)$.

Observation of kinks in the plot of $X_1(t)$, as in the case of dicumarol, is not excluded by the hydrodynamic diffusion analog of compartment models in pharmacokinetics. Since extremely stringent conditions of the relations among physiological parameters need to be satisfied for the observation of such kinks, it is no wonder that most systems exhibit only plot Ia of Fig. 1.

AN ILLUSTRATIVE CALCULATION

One may compute that $k_{20}m = 2.50$ for a system having the following magnitudes for the various parameters: $(\delta_{13}/V_1) = 0.1733\ k_{20}$, $(\delta_{12}/V_1) = 0.36\ k_{20}$, $\alpha_{12} = 0.76\ k_{20}$, and $\alpha_{13} = 1.24\ k_{20}$. One may also compute by the methods described that, for this system, $\mu_1 = -1.6\ k_{20}$, $\mu_2 = -1.2\ k_{20}$, and $\mu_3 = -0.2\ k_{20}$; $p = -1.1577$ for this system. One may verify that for this system the plot of $X_1(t)$/dose resembles plot I*b* of Fig. 1, with one extremum point occurring slightly above $2.3\ (k_{20})^{-1}$.

DISCUSSION

The presented analysis can be extended to n-compartment systems, with arbitrary choice of connectives. The main results of such deliberations are that the time dependence of concentrations of any compartment can be expressed in the form of Eq. 5 with n exponential terms. The decay constants are related to the volumes of distribution and permeabilities of barriers between compartments for the drug, by relations analogous to Eqs. 17 and 27 of Ref. 1, except that one has to find the roots of an nth order polynomial.

However, with appropriate values for the physiological parameters of the system to the drug, corresponding functions $F_n(t)$ can have more than one extremum time. Thus, with appropriate value of C_nS_n, which should be positive definite, the plot of the concentration of the central compartment in which drug is introduced initially can, in principle, exhibit $(n - 1)$ extremum times (3–5) at which $\dot{X}_1(t) = 0$. However, since $\dot{X}_1(t)$ is negative definite for very small times and for very large times, the number of physically realizable extremum points should be an even number. Therefore, observation of two such kinks for a specified drug in the plot of ln $X_1(t)$ requires that the physiological system behave for this drug as a five-compartment system.

If the drug participates in a chemical reaction in the barriers connecting compartments, then these have the effect of altering the corresponding matrix element of matrix A of Eq. 1. Thus, active transport of a drug across a specified barrier has the effect of changing the magnitude and sign of the corresponding matrix element.

If the drug participates in a chemical reaction in the compartment fluid phase of the physiological system, such effects may contribute to either alteration of corresponding element of vector X of Eq. 1 or, in principle, change an n-compartment model to behave as an $(n + 1)$-compartment model. These considerations will be discussed in future publications; for the present, it is concluded that such a phenomenon is capable of possibly explaining certain types of "aperiodic biological oscillatory" after effects observed with certain drugs in physiological systems.

APPENDIX

Defining the quantities ϵ and ζ by:

$$\epsilon = \alpha_{12} + (\delta_{13}/V_1) \quad \text{(Eq. A1}a\text{)}$$

$$\zeta = \epsilon + k_{20} \quad \text{(Eq. A1}b\text{)}$$

one has:

$$\omega = k_{20} - \epsilon \quad \text{(Eq. A2)}$$

Equations 22a–22c of Ref. 1 can now be expressed as:

$$C_1(J_1/\eta) = -(\mu_3 + \mu_2) - \zeta \quad \text{(Eq. A3}a\text{)}$$

$$C_2(J_2/\eta) = (\mu_1 + \mu_3) + \zeta \quad \text{(Eq. A3}b\text{)}$$

$$C_3(J_3/\eta) = -(\mu_1 + \mu_2) - \zeta \quad \text{(Eq. A3}c\text{)}$$

$$J_1 = (\mu_1 - \mu_2)(\mu_1 - \mu_3) \quad \text{(Eq. A3}d\text{)}$$

$$J_2 = (\mu_1 - \mu_2)(\mu_2 - \mu_3) \quad \text{(Eq. A3}e\text{)}$$

$$J_3 = (\mu_1 - \mu_3)(\mu_2 - \mu_3) \quad \text{(Eq. A3}f\text{)}$$

The J_σ's, η, and ζ are positive definite. The decay constants are distinct and are negative definite. They may be ordered such that their magnitudes satisfy the inequalities of $|\mu_1| > |\mu_2| > |\mu_3|$.

It is evident that both C_1 and C_3 are negative when the magnitude of ζ is greater than $-(\mu_1 + \mu_2)$ [greater than $-(\mu_2 + \mu_3)$]. Because of the constraint expressed by Eq. 21b of Ref. 1, C_2 should be positive definite, which is assured by Eq. A3b.

The condition imposed by Eq. 12 for the existence of t_1^* is that the ratio $(1 + r_3m)/(1 + r_2m)$ should be positive. This condition is evidently satisfied if the roots of cubic Eq. 19 of Ref. 1, r_2 and r_3, are both positive and have a magnitude less than k_{20}. This condition is also satisfied if both r_2 and r_3 are negative, provided the magnitudes of r_2m and r_3m are greater than unity. It is also satisfied if either r_2 or r_3 is negative, provided the product of negative root and m has a magnitude less than unity.

When α_{13} is greater than k_{20}, from Eq. 17c of Ref. 1 it follows that $R = -(r_1r_2r_3)$ is negative. This requires that, when r_1 is negative, r_2 and r_3 must have opposite signs.

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Potential Anticancer Agents II: Antitumor and Cytotoxic Lignans from *Linum album* (Linaceae)

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Keyphrases □ *Linum album* (Linaceae)—isolation and identification of antitumor and cytotoxic lignans □ Lignans—isolation and identification from *L. album*, screened for anticancer and cytotoxic properties □ Anticancer agents, potential—isolation and identification of antitumor and cytotoxic lignans from *L. album*, screened for activity

Linum album was found to be active against the P-388 leukemia and 9KB cell assay system in a random collection screening program for new anticancer agents. The chloroform extract of *L. album* yielded podophyllotoxin, α- and β-peltatins, and a new lignan, 3′-demethylpodophyllotoxin. Based upon spectroscopic data, a structure was proposed for 3′-demethylpodophyllotoxin, which was verified by preparation of a derivative of known structure. The lignans podophyllotoxin, α- and β-peltatins, and 3′-demethylpodophyllotoxin were shown to be at least partially responsible for the antitumor and cytotoxic activity of *L. album* extracts.

EXPERIMENTAL[1]

Biological Activity—An ethanolic extract of *L. album* was evaluated for cytotoxicity; it was found active against Eagle's 9KB carcinoma of the nasopharynx in cell culture (ED_{50} = 2.3, 2.5, and 1.4 μg/ml)[2] and showed *in vivo* activity against the P-388 lymphocytic leukemia in mice (T/C 136, 126, and 108 at 133, 88, and 88 mg/kg, respectively)[3]. Test methods employed were those of the Drug Research and Development Branch of the National Cancer Institute (1).

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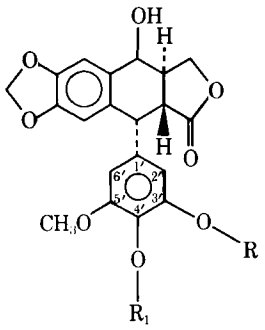


lignan	R	R_1
I	CH_3	CH_3
II	CH_3	OH
III	OH	CH_3

Fractionation of the initial plant material into Fractions A, C, and D (Scheme I) showed that the activity was mainly concentrated in Fraction A (1.8 μg/ml in the 9KB system and T/C values in mice infected with the P-388 leukemia of 130, 125, and 125 at doses of 400, 200, and 100 mg/kg, respectively). Fraction C was cytotoxic (ED_{50} = 1.4 μg/ml) as well as toxic in mice infected with the P-388 leukemia at doses of 100–400 mg/kg. Fraction D was inactive in the 9KB and P-388 systems. These data are presented in Table I.

Fractionation of Plant Material—A sample of whole plant material (6.6 kg) was continuously extracted in a Lloyd extractor with petroleum ether (bp 30–60°) until the extract was colorless. This extract was then concentrated to a viscous, light-brown syrup weighing 82 g (Fraction A). The defatted plant material was air dried and continuously extracted with methanol (3 liters), and the methanol solution was concentrated *in vacuo* to yield 390 g of a thick, dark-brown syrup (Fraction B).

Fraction B was partitioned between chloroform (6 liters) and water (3 liters). The chloroform fraction (Fraction C) was separated, dried over anhydrous sodium sulfate, and concentrated to dryness *in vacuo* to give a residue of 107 g. The aqueous phase was frozen and lyophilized to yield a residue weighing 280 g (Fraction D).

Fractionation of Fraction C—The chloroform-soluble fraction (C, 100 g) was dissolved in methanol and intimately mixed with 250 g of silica gel PF_{254}. Following evaporation of the methanol *in vacuo*, the powdered mixture was slowly added to the top of a chloroform slurry-packed column containing 3.0 kg of silica gel PF_{254}. Elution was initiated with chloroform, and 4.0-liter fractions were collected.

The eluent was changed to solvents of increasing polarity as the fractions were monitored by spraying the developed plates with sulfuric acid and heating at 120° for 10 min. The fractions were combined into five groups having different TLC patterns and were subsequently assayed for 9KB and P-388 leukemia activity (Table II).

Table I—Antitumor and Cytotoxic Screening Data on *L. album*

Extract	Fraction	Tumor Systems: 9KB ED_{50}, μg/ml[a]	Tumor Systems: P-388 Dose/Percent[b], (mg/kg)/(T/C)
50% ethanol	B001	2.3	133/136
		2.5	88/126
		1.4	
50% ethanol	B002	1.4	88/108
	A	1.8	400/130
			200/125
			100/125
	C	1.4	400/TT[c]
			200/TT
			100/TT
	D	100.0	400/95
			200/110
			100/110

[a] An active fraction is one that exhibits an $ED_{50} \leq 20.0$ μg/ml. [b] An active fraction is one that exhibits an increase in survival time ≥125%. [c] TT = too toxic.

Table II—Chromatographic Separation of Fraction C

Fractions[a]	Eluent	Weight, g	9KB[b] ED_{50}, μg/ml	P-388[c], Dose/Percent, (mg/kg)/(T/C)
1–4	Chloroform	1.79	100.0	130/90
5	Chloroform	16.2	12.0	100/135
6	Chloroform	20.8	6.9	100/135
7	Chloroform	2.4	6.0	140/140
8–13	Chloroform–methanol (1:1)	13.0	79.0	400/135

[a] Each fraction was 4.0 liters. [b] An active fraction is one that exhibits an $ED_{50} \leq 20$ μg/ml. [c] An active fraction is one that exhibits an increase in survival time ≥125%.

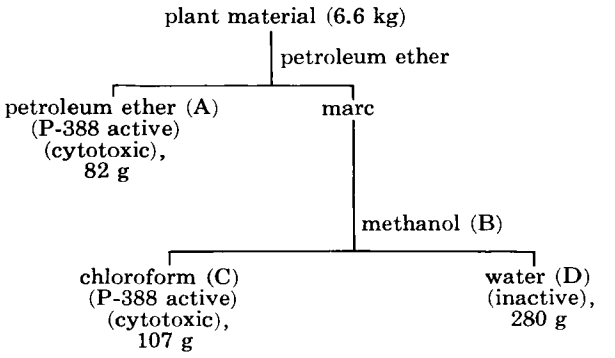
Detection of α-Peltatin and β-Peltatin—A 55-mg sample derived from fraction 6 (Table II) gave a strong positive phenol test with diazotized *p*-nitroaniline spray reagent (2). To remove the phenolic constituents from this mixture, the sample was dissolved in ether (10 ml) and extracted with 10 ml of 10% sodium carbonate solution. The sodium carbonate solution was then acidified with concentrated hydrochloric acid, and the acidic solution was extracted with ethyl acetate.

After drying the organic phase over anhydrous sodium sulfate, it was filtered and evaporated to dryness *in vacuo* to yield a residue (31 mg). TLC analysis of this sample indicated that it was a complex mixture of phenolic compounds. Co-TLC with reference α-peltatin and β-peltatin revealed the presence of these two compounds in the sample[4]. Both peltatins gave a reddish color when sprayed with diazotized *p*-nitroaniline.

α-Peltatin gave an R_f value of 0.30 in benzene–95% ethanol (9:1), 0.20 in benzene–chloroform–methanol (10:5:0.5), and 0.10 in methylene chloride–ethyl acetate (5:1); β-peltatin gave R_f values of 0.40, 0.25, and 0.15, respectively, in these three solvent systems. The complexity of the sample and the small quantity precluded further purification.

Isolation of Podophyllotoxin—Fraction 6 (Table II) was further chromatographed to yield several subfractions, which contained podophyllotoxin (I) as indicated by co-TLC with reference podophyllotoxin[4]. However, these combined samples (278 mg) proved complex and exhibited a green color. These subfractions were partitioned between petroleum ether and 10% aqueous methanol in an attempt to simplify the mixture. Only the aqueous methanol fraction showed the presence of podophyllotoxin.

The residue (220 mg), after evaporation of methanol, was dissolved in benzene (2 ml) and chromatographed[5]. Elution with 1



Scheme I

[4] Supplied by Dr. J. L. Hartwell, Drug Research and Development, National Cancer Institute, Bethesda, Md.

[5] Twenty grams of Florisil F-101 packed in benzene, Fisher Scientific Co., Fair Lawn, N.J.

Table III—NMR Spectra of 3′-Demethylpodophyllotoxin[a]

Proton Assignment	Chemical Shift, δ, ppm	Multiplicity, J, Hz (in $CDCl_3$)	Integration	Chemical Shift, δ, ppm	Multiplicity, J, Hz (in Benzene-d_6)	Integration
Aliphatic	2.75–3.55	Multiplet	2	—	—	—
Ar-OCH_3	3.82	Singlet	6	3.55	Singlet	3
				3.58	Singlet	3
Aliphatic	4.55	Multiplet	2	—	—	—
Methylenedioxy	5.99	Singlet	2	—	—	—
Aromatic	6.01	Doublet (J = 2 Hz)	1	6.28	Doublet (J = 2 Hz)	1
Aromatic	6.45	Singlet	1	6.40	Singlet	1
Aromatic	6.78	Doublet (J = 2 Hz)	1	6.97	Doublet (J = 2 Hz)	1
Aromatic	7.18	Singlet	1	7.10	Singlet	1

[a] The spectra were recorded at 60 MHz.

liter of benzene removed the green color. Subsequent elution with chloroform (1 liter) and evaporation of the eluant *in vacuo* afforded a pale-yellow residue (154 mg). Crystallization and recrystallization of this material, using benzene–petroleum ether, yielded white crystals (30 mg), mp 115–120°.

A UV[6] spectrum exhibited absorption with λ_{max} at 292 nm (log ε 3.29), and bands in the IR spectrum[7] (KBr) at ν_{max} 3400 (OH) and 1750 (lactone) cm^{-1} were observed. The mass spectrum[8] showed a molecular ion at *m/e* 414 (100%). The IR, mass spectral, and TLC properties were identical with those of podophyllotoxin (3–5), and there was no depression of the melting point following a mixed melting-point determination. The absolute configuration of I has recently been determined by X-ray analysis (6).

Isolation and Characterization of 3′-Demethylpodophyllotoxin—Fraction 6 from the first silica gel column (Table II) gave a positive test with diazotized *p*-nitroaniline spray reagent (2). Subsequent purification of this fraction, using another silica gel PF_{254} column, afforded a semipurified material (99 mg) containing a phenolic component. The sample was dissolved in chloroform (1 ml), applied to a 2.0-mm thick silica gel PF_{254} plate, and developed with ethyl acetate–petroleum ether (9:1).

The zone corresponding to R_f 0.70 was removed, eluted with methanol, and filtered, and the filtrate was taken to dryness *in vacuo*. The resulting pale-yellow amorphous solid (30 mg), mp 85–105°, gave a UV absorption with λ_{max} at 289 nm (log ε 3.30), indicative of a podophyllin derivative (5); important bands in the IR (KBr) spectrum were at ν_{max} 3400 (OH), 1760 (lactone), and 1590 (aromatic) cm^{-1}. TLC of the isolate on silica gel G gave R_f 0.65 using 1-butanol–acetic acid–water (4:1:1) and R_f 0.25 using ethyl acetate–methanol (95:5). The color produced with diazotized *p*-nitroaniline spray reagent was bright orange.

The mass spectrum of this isolate showed a molecular ion at *m/e* 400 (100%), and the fragmentation pattern was characteristic of that produced by lignan derivatives (3, 4). The loss of water (M^+ − 18) (5.0%) indicated that an alcohol group was present, and fragments at *m/e* 154 (23%) and *m/e* 167 (16%) indicated the presence of a phenolic group in ring D in addition to two methoxyl groups (Scheme II). This suggested that the isolate might be identical with the known lignan 4′-demethylpodophyllotoxin (II). However, co-TLC and mixed melting-point determination with an authentic sample of 4′-demethylpodophyllotoxin eliminated this possibility[9].

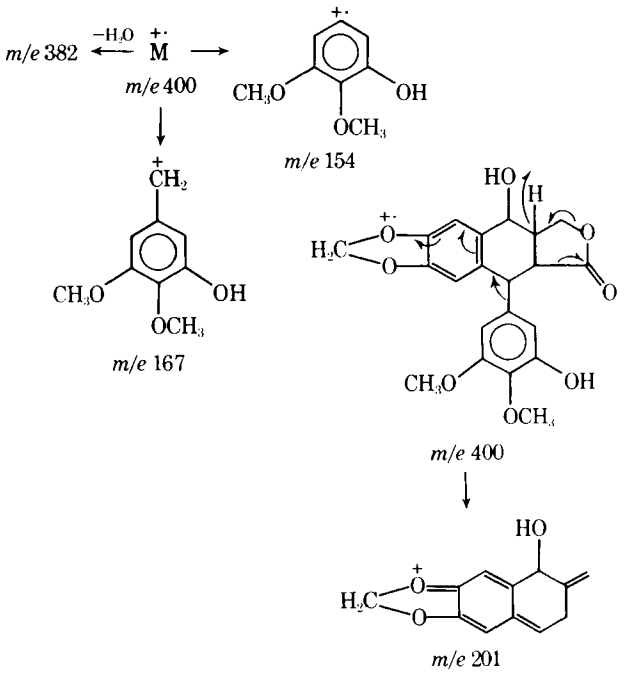
The NMR spectrum[10] in $CDCl_3$ (Table III) confirmed the presence of two methoxyl groups, exhibiting a singlet integrating for six protons at δ 3.82. Another singlet was also observed, and the chemical shift of δ 5.99, together with the integration for two protons, suggested a methylenedioxy group. Comparison of this spectrum with that of II[9] indicated that only the aromatic region was different.

In the NMR spectrum of II, the presence of a singlet at δ 6.42, integrating for two protons, was representative of the equivalent 2′ and 6′ aromatic protons. No such absorption was observed for the isolate, but a pair of doublets (J = 2 Hz), each integrating for one proton, was seen at δ 6.78 and 6.01. The absorption at δ 6.01 was somewhat obscured by the methylenedioxy peak at δ 5.99. Irradiation at δ 6.01 collapsed the doublet at δ 6.78 to a singlet. Back-irradiation produced a similar result for the doublet at δ 6.01.

An NMR spectrum was taken using benzene-d_6 (Table II) as the solvent, with the hope of observing solvent shifts of the aromatic and the methoxyl protons. Indeed, the methoxyl protons were shifted and split into two singlets at δ 3.55 and 3.58, indicating that the methoxyl groups were nonequivalent; the fact that they appeared as a singlet at δ 3.82 in the spectrum taken in $CDCl_3$ could now be attributed to its characteristic in the solvent. Furthermore, the aromatic protons were shifted so that the two doublets were observed at δ 6.28 (J = 2 Hz) and 6.97 (J = 2 Hz). A double-resonance experiment again indicated that these two protons were coupled to each other.

These data indicated that the two aromatic protons in ring D were nonequivalent and *meta* (J = 2 Hz) to each other and also that the two methoxyl groups were nonequivalent. At this point, only five possible arrangements of the three substituents (*i.e.*, two methoxyl and one OH) on ring D could be considered.

Only one of these possible compounds would give rise to podophyllotoxin. Methylation of the isolate with diazomethane did in-



Scheme II—Mass spectral fragmentation of 3′-demethylpodophyllotoxin

[6] Recorded using a Beckman model DB-G grating UV spectrophotometer.
[7] Recorded using a Beckman model IR-18A IR spectrophotometer.
[8] Recorded using a Hitachi Perkin-Elmer model RMU-6D mass spectrometer.
[9] Supplied by Sandoz Inc., Basel, Switzerland.
[10] Recorded using a Varian T-60A instrument at 60 MHz.

deed yield podophyllotoxin, hence proving that the isolate was 3′-demethylpodophyllotoxin (III).

No differences were observed in the NMR spectra of the aliphatic regions of I, II, or III, indicating that III has the same stereochemistry at the corresponding positions as I. This is the first report of the isolation of III from nature.

DISCUSSION

Podophyllotoxin (I) was reported previously to be cytotoxic in the 9KB system, with an ED_{50} of <0.01 μg/ml (7); it is active in the Lewis lung (LL) carcinoma system (mice) at a minimum effective dose (MED) of 2 mg/kg, giving a tumor weight inhibition of 64% (7). Furthermore, it is active in the P-388 leukemia system with an MED of ~2 mg/kg, producing a 70% increase in lifespan (7). This cytotoxic lignan is also active in the Walker 256 (IM) carcinosarcoma system; tumor weight is inhibited 73% at an MED of 6 mg/kg.

However, podophyllotoxin has been reported to be inactive in the following tumor systems: sarcoma 180, adenocarcinoma 755, L-1210 leukemia (intraperitoneal), Friend virus leukemia, melanotic melanoma, plasmacytoma No. 1, HS1 human sarcoma, L-1210 leukemia (subcutaneous, delayed treatment), and P-1798 lymphosarcoma (7).

Other workers (8) stated that podophyllotoxin is effective against the L-1210 leukemia, lymphosarcoma, lymphocarcoma 2, mammary adenocarcinoma C3HBA, melanoma S-91, and rat carcinoma 1643, but experimental data were not presented. Extensive studies with podophyllotoxin have also been published regarding its effect on the sarcoma 37 in mice (9).

Clinical experience with podophyllotoxin has been disappointing. In the one available published study, Savel (10) reported that 25 patients with neoplastic disease were studied, of whom 17 were treated to the point of bone marrow or GI toxicity with doses of 0.5–1.0 mg/kg iv. Side effects (nausea, vomiting, diarrhea, oral ulcers, and fever) were frequent. Transient, clinically insignificant regression of tumor masses and lymph nodes occurred in one-third of the patients with reticulum cell sarcoma, two-thirds of the patients with Hodgkin's disease, and one-seventh of the patients with adenocarcinoma. One patient with chronic granulocytic leukemia and rapidly enlarging adenopathy had a marked regression of lymph nodes and spleen size.

α-Peltatin and β-peltatin have been found effective in treating the following types of tumors in laboratory animals: L-1210 leukemia, lymphosarcoma 1, lymphosarcoma 2, mammary adenocarcinoma C3HBA, melanoma S-91, and rat carcinoma 1643 (8), as well as the sarcoma 37 (9), but neither lignan was effective against the Caspari mammary adenocarcinoma (11). However, in clinical trials, α-peltatin by intravenous administration was of no significant therapeutic value in human neoplastic disease (12). β-Peltatin was clinically evaluated in children with dediastinal neuroblastoma with undramatic results (13).

The new lignan, 3′-demethylpodophyllotoxin (III), exhibited an ED_{50} of 1.0 μg/ml against Eagle's 9KB carcinoma and a prolongation of life of 130% T/C at a dose of 1 mg/kg against the P-388 lymphocytic leukemia.

Whereas it does not appear that podophyllotoxin-type lignan aglycones will prove to be effective chemotherapeutic agents for the treatment of human neoplastic diseases, certain derivatives of the water-soluble glycoside forms of these lignans, *i.e.*, 4′-demethylepipodophyllotoxin-9-(4,6-*O*-2-thenylidene)-β-D-glucopyranoside (14–19), have been used clinically with some success.

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Nonlinear Tissue Disposition: Salicylic Acid in Rat Brain

M. A. GONZALEZ *, T. N. TOZER ˣ, and D. T. T. CHANG

Abstract □ A model was developed to detect nonlinear disposition of a drug in a tissue. The model was experimentally tested relating to salicylic acid disposition in the brain. Experimental data obtained in rats are reported for doses of 25 and 400 mg/kg ip. The parameters measured for each dose were the ratio of the area under the brain concentration–time curve to the area under the plasma concentration–time curve and the ratio of the maximum brain concentration of salicylic acid to the plasma concentration at that point in time. The ratios increased with dose; furthermore, ratios calculated using plasma concentrations corrected for plasma protein binding were dose dependent. Calculations performed on literature data for salicylic acid disposition in mouse brain corroborated the results of this study. The existence of a saturable transport system for the elimination of salicylic acid from the brain is supported by the data presented. The rationale necessary to apply the model to any tissue is discussed.

Keyphrases □ Transport, drug—nonlinear tissue disposition, salicylic acid in rat brain, model proposed, equations □ Drug disposition—nonlinear tissue disposition, salicylic acid in rat brain, model proposed, equations □ Tissue disposition, nonlinear—model proposed, equations derived, salicylic acid in rat brain as an example □ Salicylic acid—nonlinear tissue disposition in rat brain, model proposed, equations

A number of saturable processes may produce disproportionate changes in the tissue distribution of a drug when the dose of the drug is increased. Facilitated transport is one such process; in its presence the result is nonlinear tissue distribution. The existence of transport systems for the elimination of organic acids from the brain and cerebrospinal fluid has been documented (1–5). Steinwall (2, 3) postulated that such transport systems could be contributing to the hindrance of blood-to-brain transfer; that is, these systems could be a functional component of the blood–brain barrier.

Confirmation of an acid transport system in the brain requires knowledge of factors governing the distribution and disposition of drugs in the brain such as: (*a*) binding to blood proteins and tissue components, (*b*) metabolism of the drug, (*c*) diffusion in and out of the tissue, and (*d*) bulk flow of the cerebrospinal fluid. Similar processes are functional in other tissues as well as the brain.

This paper proposes a model to detect the presence of saturable processes that would influence the disposition of a drug in a tissue. The model is experimentally tested as it relates to the transport of salicylic acid from the brain; however, the model should be applicable to drug disposition in other tissues.

THEORETICAL

Consider the simplest model for the distribution of drugs into tissues, the case in which a drug enters and leaves a tissue only by passive diffusion. For such a system the change in the amount of drug in the tissue with time, dT/dt, may be expressed by an equation based on Fick's first law of diffusion and the assumption that only unbound drug is diffusible:

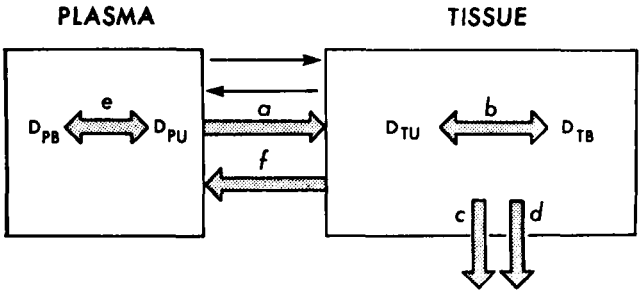

Figure 1—*Schematic diagram of the processes of disposition of a drug in a tissue. Key: thin arrows, passive diffusion; heavy arrows, saturable processes;* a, f, *unidirectional transport to and from tissue;* c, *metabolism;* d, *excretion; and* b, e, *binding to tissue components and plasma proteins. Symbols* D_{PB}, D_{PU}, D_{TB}, *and* D_{TU} *are the amounts of bound and unbound drug in plasma and tissue, respectively.*

$$\frac{dT}{dt} = k(C_1 - C_2) \qquad \text{(Eq. 1)}$$

where C_1 is the concentration of drug in the plasma free from protein binding, C_2 is the unbound concentration of drug in the tissue, and k represents a hybrid constant (units of clearance) for passage of the drug between plasma and tissue.

The rate of entry of the drug into the tissue is kC_1, while kC_2 is the rate of exit of the drug from the tissue. The integral of the rate of entry with time is the amount entering the tissue, $\int_0^\infty kC_1\,dt$, while the integral of the rate of exit with time equals the amount leaving the tissue, $\int_0^\infty kC_2\,dt$. The total amount that enters the tissue must equal the total amount that leaves the tissue; therefore:

$$\int_0^\infty C_1\,dt = \int_0^\infty C_2\,dt \qquad \text{(Eq. 2)}$$

Passive diffusion requires that the area under the unbound plasma concentration–time curve be equal to the area under the unbound tissue concentration–time curve.

In an experiment in which steady-state plasma and tissue levels are attained, that is $dT/dt = 0$, from Eq. 1:

$$C_1^{ss} = C_2^{ss} \qquad \text{(Eq. 3)}$$

where C_1^{ss} is the unbound plasma concentration at steady state, and C_2^{ss} is the unbound tissue concentration at steady state. The same relationship would be applicable for an experiment in which a single dose is administered. At the point in time, t_{max}, when the rates into and out of the tissue are equal, the unbound tissue level is maximal, C_2^{max}, and equal to the unbound plasma concentration, $C_1^{t\,max}$:

$$C_1^{t\,max} = C_2^{max} \qquad \text{(Eq. 4)}$$

Plasma and Tissue Binding—Unbound concentrations of a drug are not readily determined, particularly within a tissue. Usually, the total plasma concentration, C_P, and the total tissue concentration, C_T, are measured. The total plasma and tissue concentrations are complex functions of their respective unbound concentrations. The functions depend on the affinity of a drug for the plasma proteins and tissue components and on the total number of binding sites. The unbound concentration of the drug in the plasma is a fraction, α_1, of the total plasma concentration:

$$C_1 = \alpha_1 C_P \qquad \text{(Eq. 5)}$$

The fraction unbound varies with the total plasma concentration and, thus, with the dose. Consequently, the relationship between the area under the unbound concentration–time curve to the area under the total concentration–time curve becomes a function of the dose. This ratio can be thought of as the ratio of the mean value of C_1 with time, $\bar{C}_1$, to the mean value of C_P with time, $\bar{C}_P$, that is, the ratio of the time average values:

$$\frac{\int_0^\infty C_1\,dt}{\int_0^\infty C_P\,dt} = \frac{\int_0^\infty C_1\,dt \Big/ \int_0^\infty dt}{\int_0^\infty C_P\,dt \Big/ \int_0^\infty dt} = \frac{\bar{C}_1}{\bar{C}_P} \qquad \text{(Eq. 6)}$$

Similarly, for the tissue:

$$C_2 = \alpha_2 C_T \qquad \text{(Eq. 7)}$$

where α_2 is the fraction of drug in the tissue that is not bound to tissue components and:

$$\frac{\int_0^\infty C_2\,dt}{\int_0^\infty C_T\,dt} = \frac{\bar{C}_2}{\bar{C}_T} \qquad \text{(Eq. 8)}$$

Since the areas under the unbound plasma concentration–time curve and the unbound tissue concentration–time curve must be equal (Eq. 2), $\bar{C}_1$ equals $\bar{C}_2$. Dividing Eq. 6 by Eq. 8 yields:

$$R = \frac{\int_0^\infty C_T\,dt}{\int_0^\infty C_P\,dt} = \frac{\bar{C}_T}{\bar{C}_P} \qquad \text{(Eq. 9)}$$

The ratio, R, referred to as the area distribution ratio, is a measure of the concentration of drug in the tissue with time relative to that in the plasma with time. For both plasma and tissue, the fraction of the drug unbound normally increases with concentration; as the dose is increased, therefore, $\bar{C}_1/\bar{C}_P$ and $\bar{C}_2/\bar{C}_T$ increase. An increase or decrease of R with increasing dose depends on whether the plasma protein binding or tissue binding is the more readily saturable. The former results in an increase in R, the latter in a decrease.

In steady-state experiments, the fractions unbound in plasma, α_1^{ss}, and in tissue, α_2^{ss}, are:

$$\alpha_1^{ss} = \frac{C_1^{ss}}{C_P^{ss}} \qquad \text{(Eq. 10)}$$

$$\alpha_2^{ss} = \frac{C_2^{ss}}{C_T^{ss}} \qquad \text{(Eq. 11)}$$

The total plasma and tissue concentrations at steady state are represented by C_P^{ss} and C_T^{ss}, respectively. As given in Eq. 3, the steady-state unbound concentrations in plasma and tissue are equal; therefore, dividing Eq. 10 by Eq. 11 yields a steady-state distribution ratio, R^{ss}:

$$R^{ss} = \frac{C_T^{ss}}{C_P^{ss}} = \frac{\alpha_1^{ss}}{\alpha_2^{ss}} \qquad \text{(Eq. 12)}$$

Similarly, in an experiment in which a single dose is given, there is a time, t_{max}, when the unbound tissue concentration equals the unbound plasma concentration as given in Eq. 4. The total concentration in the tissue, C_T^{max}, is at a maximum at the same point in time when C_2^{max} is achieved, assuming tissue binding is instantaneous. A t_{max} distribution ratio, R^{max}, may then be defined as:

$$R^{max} = \frac{C_T^{max}}{C_P^{t_{max}}} = \frac{\alpha_1^{max}}{\alpha_2^{max}} \qquad \text{(Eq. 13)}$$

The plasma concentration at the same time point when the tissue is at the maximum concentration is defined as $C_P^{t_{max}}$. The values of R^{ss} and R^{max} vary with dose in a similar fashion to R due to the decreased binding at higher concentrations.

Saturable Transport Systems—The presence of transport, metabolic, or excretory mechanisms also give rise to a change in R, R^{ss}, and R^{max} with dose, as determined by Eqs. 9, 12, and 13, respectively. As an example, consider the case in which the rate of entry into a tissue may be kinetically expressed as a combination of passive diffusion and a saturable transport. For such a case:

$$\text{rate in} = kC_1 + \frac{aC_1}{b + C_1} \qquad \text{(Eq. 14)}$$

where the maximum rate of transport is represented by a, b is the dissociation constant for the "drug–carrier" complex, and k is as defined in Eq. 1. The amount that enters the tissue is obtained by integration of Eq. 14:

$$\text{amount entering tissue} = k\int_0^\infty C_1\,dt + a\int_0^\infty \frac{C_1}{b + C_1}\,dt \qquad \text{(Eq. 15)}$$

When assuming that a drug leaves the tissue only by passive diffusion, the total amount leaving the tissue is $k\int_0^\infty C_2\,dt$, as shown in the derivation of Eq. 2. Furthermore, if the assumption is made that no protein binding occurs in the plasma or tissue, substituting C_P for C_1 and C_T for C_2 leads to the following:

$$k\int_0^\infty C_T\,dt = k\int_0^\infty C_P\,dt + a\int_0^\infty \frac{C_P}{b + C_P}\,dt \qquad \text{(Eq. 16)}$$

that is, the total amount leaving the tissue must equal that entering the tissue. The area distribution ratio as expressed in Eq. 9 becomes:

$$R = 1 + \frac{\frac{a}{k}\int_0^\infty \frac{C_P}{b + C_P}\,dt}{\int_0^\infty C_P\,dt} \qquad \text{(Eq. 17)}$$

The transport in gives rise to values of R greater than 1; however, as the dose is increased to the point that C_P exceeds b for prolonged periods, the value of R decreases toward a value of unity.

Conversely, transport out of the tissue with passive diffusion in and out of the tissue and no plasma or tissue binding results in the following:

$$\text{rate in} = kC_P \qquad \text{(Eq. 18)}$$

$$\text{rate out} = kC_T + \frac{a'C_T}{b' + C_T} \qquad \text{(Eq. 19)}$$

where a' is the maximum rate of transport out, and b' is the dissociation constant for the drug–carrier complex. Since the total amount entering the tissue equals the total amount leaving the tissue:

$$k\int_0^\infty C_P\,dt = k\int_0^\infty C_T\,dt + a'\int_0^\infty \frac{C_T}{b' + C_T}\,dt \qquad \text{(Eq. 20)}$$

the area distribution ratio then becomes:

$$R = 1 - \frac{\frac{a'}{k}\int_0^\infty \frac{C_T}{b' + C_T}\,dt}{\int_0^\infty C_P\,dt} \qquad \text{(Eq. 21)}$$

In this case, R is less than unity and an increase in dose gives rise to an increase in its value toward unity. The same argument holds for saturable metabolism or excretory mechanisms in the tissue.

For the steady-state condition, if transport into the tissue is present as expressed in Eq. 14 and only passive diffusion accounts for the exit of the drug, then by assuming no binding effects the rate out of the tissue, kC_T, must equal $kC_P + [aC_P/(b + C_P)]$, the rate into the tissue. A steady-state distribution ratio is then defined by the following:

$$R^{ss} = 1 + \frac{a}{k(b + C_P^{ss})} \qquad \text{(Eq. 22)}$$

For transport out of the tissue:

$$R^{ss} = 1 - \frac{\frac{a'}{k}C_T^{ss}}{C_P^{ss}(b' + C_T^{ss})} \qquad \text{(Eq. 23)}$$

Following a bolus dose, the t_{max} distribution ratios are identical to Eqs. 22 and 23; C_T^{max} and $C_P^{t_{max}}$ replace C_T^{ss} and C_P^{ss}, respectively, in Eqs. 22 and 23.

A change in a distribution ratio with dose indicates the existence of nonlinear disposition of a drug in a tissue. As previously discussed, the direction of change in the distribution ratio with dose

Table I—Salicylic Acid Disposition in Rat Brain and Plasma

Hours after Treatment	Total Plasma Concentration[a], μg/ml	Assayed Brain Concentration[a], μg/ml
	25-mg/kg Dose	
0.5	105.8 ± 1.9	3.98 ± 0.45
1.0	96.5 ± 2.3	4.16 ± 0.30
5.0	65.2 ± 4.3	2.89 ± 0.16
10.0	15.2 ± 2.8	0.60 ± 0.08
15.0	8.5 ± 1.2	0.26 ± 0.03
20.0	4.2 ± 1.2	0.12 ± 0.04
	400-mg/kg Dose	
1.0	561.2 ± 41.7	119.7 ± 15.4
5.0	380.4 ± 14.4	71.9 ± 3.0
10.0	357.6 ± 22.7	62.4 ± 4.5
20.0	187.7 ± 14.7	21.3 ± 2.5
30.0	153.0 ± 5.1	12.1 ± 1.0
40.0	62.3 ± 12.8	3.4 ± 0.8
50.0	26.2 ± 7.2	1.2 ± 0.4

[a] The mean of five animals and standard error.

indicates which nonlinear process might exist. In reality, however, combinations of saturable processes are likely to be present.

Figure 1 schematically shows the mechanisms that would result in a dose-dependent distribution ratio. For combinations of saturable processes, the net change in the distribution ratio is a result of the dominant process if the effects are opposite. If the effects reinforce each other, the change is exaggerated. An increase or decrease in a distribution ratio with dose could thus help discern which saturable process is involved. For example, *if a distribution ratio decreases with dose*:

1. A unidirectional transport into the tissue may exist. Such a transport is described by process *a* in Fig. 1. As the dose increases, the transport system approaches saturation, resulting in tissue concentrations of the drug that are proportionately lower at the higher plasma concentrations.

2. Tissue binding of the drug may be occurring, process *b*. The possibility in this case is one of saturation of binding sites within the tissue as the dose increases. The apparent effect would be that the tissue loses the ability to accumulate the drug.

3. Metabolism of the drug in the tissue may be increasing, process *c*. This is an unlikely possibility unless the drug activates its own metabolism or the experiment involves chronic dosing with a drug which induces its own metabolizing enzymes.

If a distribution ratio increases with dose:

1. The degree of plasma protein binding may not be constant as the dose is increased, process *e*. This is to be expected, since the fraction of drug unbound normally increases with concentration.

2. A metabolic or an excretory system capable of being saturated may exist in the tissue, processes *c* and *d*, respectively. The administration of increasing doses results in a lower rate of elimination relative to the amount in the tissue. The net result would be an increase in tissue levels larger than the corresponding increase in plasma levels.

3. Elimination from the tissue may involve a saturable transport system, process *f*.

Correction for Plasma Protein Binding—An increase in a distribution ratio with dose due to the saturation of plasma protein binding can be readily ascertained. When assuming that the entry of a drug into a tissue depends upon the unbound drug in plasma, the ratio of the area under the tissue concentration–time curve to the area under the unbound plasma concentration–time curve would be independent of plasma protein binding. This ratio may be determined by dividing Eq. 9 by Eq. 6:

$$R_{cor} = \frac{\int_0^\infty C_T\,dt}{\int_0^\infty C_1\,dt} \qquad \text{(Eq. 24)}$$

The ratio, R_{cor}, is referred to as the area distribution ratio corrected for plasma protein binding.

A steady-state distribution ratio corrected for plasma protein binding, R_{cor}^{ss}, may be determined from steady-state tissue and unbound plasma concentrations:

$$R_{cor}^{ss} = \frac{C_T^{ss}}{C_1^{ss}} \qquad \text{(Eq. 25)}$$

Similarly, following a single dose, the t_{max} distribution ratio corrected for plasma protein binding is:

$$R_{cor}^{max} = \frac{C_T^{max}}{C_1^{t_{max}}} \qquad \text{(Eq. 26)}$$

The values of R_{cor}, R_{cor}^{ss}, and R_{cor}^{max} should be unity unless tissue binding or other saturable processes occur in the tissue. If the distribution ratios corrected for protein binding do not change over the dosage range tested, saturation of plasma protein binding is the mechanism by which R, R^{ss}, and R^{max} vary with dose. Any change in the corrected distribution ratios indicates the existence of other saturable processes. The direction of the change gives a further indication of the probable mechanisms.

EXPERIMENTAL

Materials—All chemicals used were of analytical grade; the sodium salicylate[1] was of certified grade.

Apparatus—A spectrophotofluorometer[2] was utilized in the salicylic acid assays. Protein binding studies were carried out with an equilibrium dialysis apparatus[3].

Animal Procedures—Male Sprague–Dawley rats[4], 190–210 g, were used. Two major studies were carried out: in one, the rats received a dose of sodium salicylate corresponding to 25 mg/kg; in the other, the dose was 400 mg/kg.

Several groups with five rats per group were randomly selected for each study. Except for the control group, each rat received an intraperitoneal injection of 1 ml of a sodium salicylate solution. After treatment the animals were sacrificed at different times by decapitation.

Upon decapitation the blood from each rat was collected in a 50-ml beaker containing 200 μl of a 0.05 *M* solution of edetic acid [(ethylenedinitrilo)tetraacetic acid disodium salt]. After centrifugation of blood, 1 ml of plasma was transferred to a centrifuge tube. The brain from each rat was removed after decapitation and quickly frozen in dry ice after blotting with a tissue to remove excess surface blood. Brain and plasma samples were stored frozen until the time of analysis.

Assay Procedures—Plasma samples were assayed using the procedure of Rowland and Riegelman (6). Standard curves were prepared by extracting control plasma samples containing known amounts of salicylic acid.

To assay the brain samples for salicylic acid, each brain was homogenized in a tissue grinder[5] using two volumes of distilled water for every gram of brain. Three milliliters of homogenate was transferred into a 20-ml culture tube containing approximately 2 g of sodium chloride and 0.3 ml of 6 *N* HCl. After mixing the samples, 5 ml of a solution of equal volumes of ether and *n*-heptane was added to the samples, and the samples were gently shaken mechanically for 30 min.

After centrifugation to separate the phases, an aliquot of the organic phase was extracted with 2 ml of a 0.5 *M* phosphate buffer, pH 7. The phosphate buffer extracts were read on the spectrophotofluorometer at the wavelengths for maximum activation and fluorescence, as determined using a standard sample of sodium salicylate in phosphate buffer. By using the described extraction procedure, known amounts of salicylic acid were added to homogenates of control brains to prepare the standard curves.

The value obtained for the concentration of salicylic acid in the brain homogenate had to be corrected for the contribution of drug from the residual blood. By using a value of 11 μl as the residual blood per gram of brain (7) and calculating the concentration of salicylic acid in the blood from the concentrations in plasma[6], it was possible to determine the amount of salicylic acid that had to be subtracted from the amount assayed in the brain.

[1] Fisher Scientific Co.

[2] Aminco-Bowman spectrophotofluorometer model 8210.

[3] Dianorm equilibrium dialysis system, supplied by Innovativ-Medizin AG., P.O. Box 31, CH-Esslingen, Switzerland.

[4] Horton Laboratories, Inc.

[5] Tenbroeck.

[6] M. A. Gonzalez and T. N. Tozer, unpublished data.

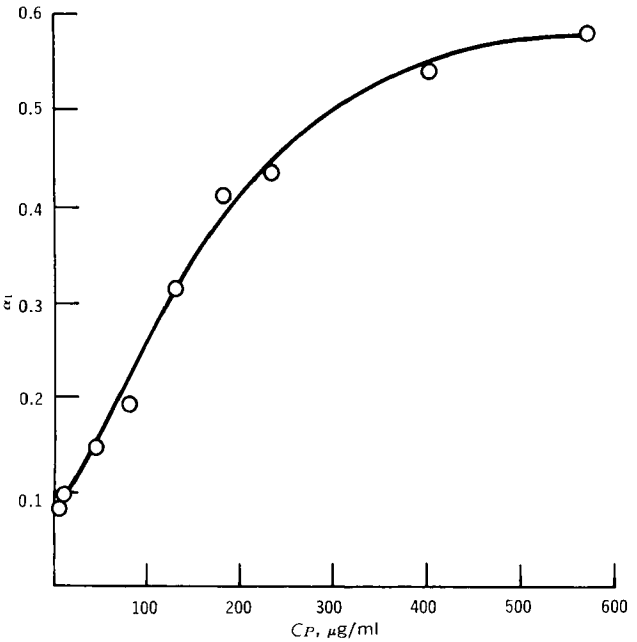

Figure 2—*Unbound fraction of total plasma concentration, α_1, as a function of total plasma concentration, C_P.*

Protein Binding Study—The degree of plasma protein binding for salicylic acid was determined using the equilibrium dialysis system. The apparatus consists of 20 cells (Teflon), each of which is divided into two compartments or half-cells with a 1-ml volume by a section of tubing[7] with a thickness of 0.025 mm. The cells are mounted on a rotor so that agitation is accomplished by rotation of the cells. The entire apparatus is watertight, so that it may be immersed in a constant-temperature water bath.

In the present study, 1 ml of a solution of sodium salicylate in Krebs–Ringer bicarbonate buffer was introduced with a tuberculin syringe into one half-cell, and 1 ml of fresh rat plasma was simultaneously injected into the other half-cell. Since the apparatus has 20 cells, it was possible to dialyze different concentrations of salicylic acid at one time. Preliminary experimentation showed equilibrium to be reached within 1–2 hr; therefore, the dialysis was carried out for 3 hr at 37°.

After equilibration, the solutions in the buffer and plasma half-cells were analyzed using the plasma assay previously mentioned. The concentration of drug in the buffer half-cell was taken to represent the concentration of the free ligand on both sides of the membrane. The total concentration of the bound and free ligand was obtained from the analysis of the plasma half-cell. The fraction of salicylate unbound was thus calculated by dividing the concentration in the buffer half-cell by the concentration in the plasma half-cell.

RESULTS AND DISCUSSION

The salicylate concentrations assayed in plasma and brain are summarized in Table I. The samples were analyzed separately; the values reported are the arithmetic means of five separate plasma or brain samples.

The results of the plasma protein binding study are depicted in Fig. 2. By using this graph, the values for total salicylate in plasma, C_P in Table I, were converted to the concentrations of unbound salicylic acid, C_1. The brain concentrations reported in Table I were corrected for the salicylic acid contributed by the residual blood as discussed previously. The corrected tissue concentrations, C_T, together with the unbound concentrations of salicylic acid in plasma are plotted in Fig. 3.

The disposition of salicylic acid in rats is dose dependent, as evidenced by the difference in the apparent half-lives of elimination for the 25- and 400-mg/kg doses. A comparison of the half-lives calculated from the slopes of the plasma curves in Fig. 3 within the first 20–30 hr accentuates the dose dependency. The half-life of salicylic acid in the plasma after a 25-mg/kg dose was 2.8 hr, while a half-life of 11 hr resulted from a 400-mg/kg dose.

Dose-dependent elimination of salicylic acid in humans was reported previously by Levy *et al.* (8). These authors pointed out that monoexponential fits of plasma data are incorrect and that nonlinear kinetics best describe salicylic acid disposition; however, these conclusions are based on studies in individuals. Nonlinear kinetic parameters can be obtained by curve fitting the data from each individual. The present investigation involved a population study; therefore, calculations of nonlinear parameters are not as valid as when an individual is the test subject.

Dose dependency in the time course of the unbound plasma concentration will give rise to disproportionately larger areas under the C_1–time curve; since the amount entering a tissue must equal the amount leaving a tissue, the ratio of the areas under the C_T–time curve to the area under the C_1–time curve should not change. That is, the distribution ratio is independent of the time course of a drug in the body; saturable processes must be present to account for changes in this ratio with dose.

As presented in the *Theoretical* section, the calculation of the distribution ratios, R and R^{max}, after different doses of a drug serves as a test of the model described by Eqs. 9 and 13. To determine R, the areas under the cartesian plots of C_T–time and C_P–time were estimated using the trapezoidal rule. To determine R^{max}, C_T^{max} was approximated by selecting the highest mean value of C_T achieved. The plasma concentration obtained at the same time as C_T^{max} was used for C_P^{max}. The results are reported in Table II under the headings R and R^{max}. The large increase in R and R^{max} with increasing dose indicates nonlinear disposition of salicylic acid in the brain.

The increase in the distribution ratios could be attributed to a decrease in the fraction of salicylic acid bound in the plasma as the plasma concentration increases. To test this possibility, the distribution ratios corrected for plasma protein binding, R_{cor} and R_{cor}^{max}, were calculated from the data in Fig. 3. The corrected distribution ratios calculated for the two doses are listed in Table II. Inasmuch as these distribution ratios increase with dose, metabolism, excretion, or transport out of the brain must be involved in the salicylic acid elimination. Transport involvement in the entry of salicylic acid or tissue binding of the drug in the brain would cause a decrease in the distribution ratios if these processes were solely present.

As previously discussed, a combination of processes could be involved, but the change in the distribution ratios would be dependent on the dominant mechanisms. For example, entry and exit of salicylic acid in the brain could both be carrier mediated; however, if the transport out were more readily saturated than the transport in, the distribution ratios would increase with dose.

As further verification of the dose dependency of the distribution ratios for salicylic acid, R^{max} and R_{cor}^{max} were calculated using literature data for salicylic acid distribution in mice (9, 10). These studies included data for brain, unbound blood, and total blood concentration of salicylic acid at different times following administration of 50–800 mg/kg ip. The results of these calculations are

Table II—Experimentally Determined Distribution Ratios

Dose, mg/kg	R	R^{max}	R_{cor}	R_{cor}^{max}
25	0.035	0.035	0.180	0.146
400	0.139	0.205	0.302	0.353

Table III—Distribution Ratios Calculated from the Data of Sturman *et al.* (9) and McArthur *et al.* (10) in Mice

Dose, mg/kg	R^{max}	R_{cor}^{max}
50	0.153	0.305
100	0.213	0.386
200	0.278	0.487
400	0.294	0.506
800	0.459	0.766

[7] Visking.

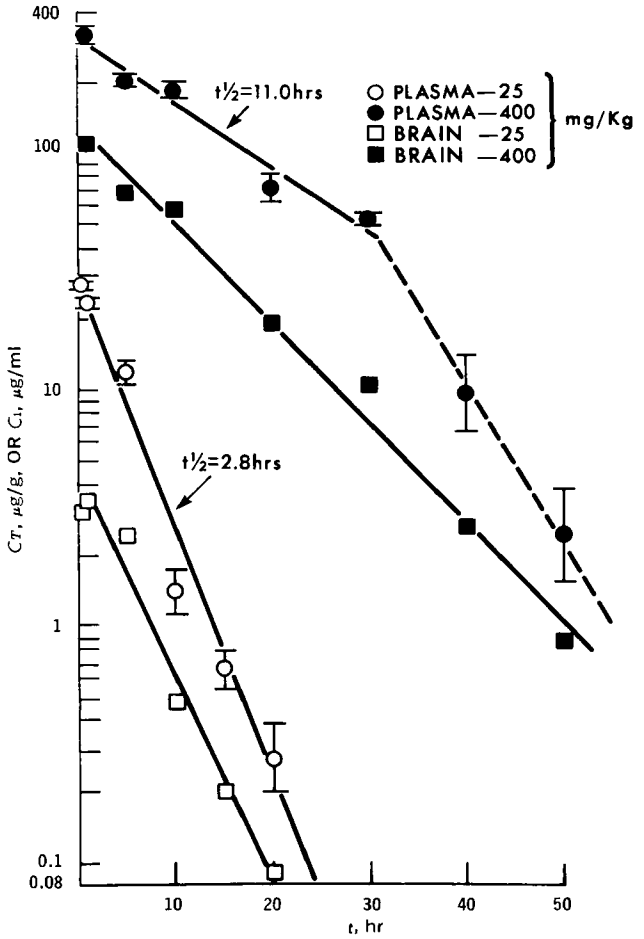

Figure 3—*Semilogarithmic plots of salicylic acid concentrations in rat brain and plasma* versus *time after administration of 25 and 400 mg/kg ip.* C_1 *is the unbound concentration of salicylic acid in the plasma;* C_T *is the concentration of salicylic acid in the brain corrected for residual blood.*

summarized in Table III; the results agree with those of the present study; that is, the distribution ratios increase with dose.

One interpretation of the changes in the values of the distribution ratios could be the existence of a saturable metabolic pathway in the brain, as discussed in the *Theoretical* section. This explanation is unlikely since Wolff and Austen (11) reported no salicylate metabolites in rat brains. Sturman *et al* (9) reached the same conclusion in their studies with mice.

An alternative explanation is the existence of a saturable transport system which functions to rid the brain of unwanted acidic compounds, in this case salicylic acid. As previously discussed, if the rate of elimination relative to the amount in the tissue becomes lower due to saturation of the transport system, the tissue levels increase more than the corresponding increase in plasma levels. This would result in a distribution ratio that increases with dose. The hypothesis that a transport system is responsible for the dose dependency of the distribution ratios presupposes that there are no major physiological changes produced by the drug under study. Especially critical is that the cerebral blood flow remain unchanged throughout the study.

The fact that the values for the distribution ratios corrected for plasma protein binding are always less than one also points to a transport system that prevents salicylic acid in the brain from achieving equilibrium with the plasma. Referring to Eqs. 24 and 26, if there were tissue binding, R_{cor} and R_{max} should be greater than one. McArthur *et al.* (10) reported that binding does not occur in brain tissue. The values of R_{cor} and R_{cor}^{max}, therefore, should be equal to one if there is no metabolism in the tissue, no transport system, and no other means of drug elimination. Cerebrospinal fluid bulk flow could be invoked as responsible for the elimination of acids from the brain, but this process is not saturable and does not explain the dose dependency of the corrected distribution ratios. Since the metabolism is considered unlikely, the existence of a saturable transport system is postulated.

If it were possible to saturate completely a transport system in the brain, the corrected distribution ratios should approach unity. This is observed with the R_{cor}^{max} values in Table III.

The dose dependency of the distribution ratios does not conclusively prove the existence of a transport system for the elimination of salicylic acid from the brain. Additional proof could be obtained by the administration of known inhibitors of acid transport systems if the distribution values should increase in the presence of the inhibitor.

The model and techniques presented should be applicable to any tissue. Further studies are planned to test the general usefulness of the model as a means of screening drugs for saturable metabolism, binding, and transport within specific tissues.

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Isolation of 19α-H-Lupeol from *Maclura pomifera*

J. E. GEARIEN ˣ **and MICHAEL KLEIN**

Abstract □ A reinvestigation of the constituents of the Osage orange (*Maclura pomifera*) yielded, in addition to the previously reported triterpenes (lupeol, butyrospermol, and lupane-3β,20-diol), the pigments osajin and pomiferin, and a previously unreported constituent. The structure of this new compound was investigated. On the basis of spectroscopic and chemical data, it appeared to be an epimer of lupeol and is referred to as 19α-H-lupeol.

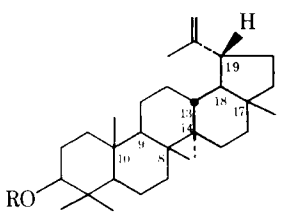
Keyphrases □ Osage orange *(Maclura pomifera)*—reinvestigation of constituents, isolation and identification of 19α-H-lupeol □ *Maclura pomifera* (Osage orange)—reinvestigation of constituents, isolation and identification of 19α-H-lupeol □ 19α-H-Lupeol—isolation, identification from Osage orange *(Maclura pomifera)*

The resistance of the Osage orange to insect attack led to a reexamination of the constituents of its fruit. Along with previously reported constituents (1–5), an unreported epimer of lupeol was also isolated.

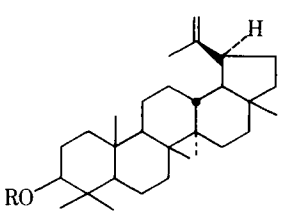
The dried fruit of the Osage orange yielded a mixture of triterpenes and pigments when extracted with petroleum ether. After separation of the pigments by chromatography, the triterpene constituents were obtained as a viscous oil. After refluxing the oil with alcoholic potassium hydroxide and extraction with ether, a solid containing the triterpene alcohols was obtained. When chromatographed on basic alumina with various solvents (Scheme I), a partial separation of the constituents was obtained.

Each fraction was treated with a mixture of acetic anhydride–pyridine. Butyrospermyl acetate and lupenyl acetate (I*b*) were isolated from the petroleum ether fraction; lupane-3β,20-diol 3-monoacetate was isolated from the alcohol fraction when eluted with benzene–ethanol (3:1). In addition, elution of a fraction with petroleum ether–benzene (4:1) from basic alumina gave a compound later shown to be 19α-H-lupenyl acetate (II*b*), a previously unreported constituent whose IR spectrum was similar to that of I*b*.

Their NMR spectra appeared identical except in the methine region at δ 1.59–1.65. Mass spectrum fragmentation patterns, of which several peaks were assigned previously (4) as being characteristic of the lupane ring system, were identical except for the percent relative abundance for certain peaks and therefore did not provide conclusive data in distinguishing between the two. Parent ions were *m/e* 468 for I*b* and II*b* and *m/e* 426 for the alcohols I*a* and II*a*. Specific rotations differed, and mixed melting points were depressed.



I*a*: R = H
I*b*: R = —$COCH_3$



II*a*: R = H
II*b*: R = —$COCH_3$

EXPERIMENTAL[1]

Materials—The ripe fruit[2] of the Osage orange tree was gathered in a grove in Chicago, Ill., in fall 1967. The alumina[3] was obtained commercially. Chromatographic solvents were distilled prior to use. Petroleum ether used for chromatography had a boiling range of 40–60°.

Chromatographic columns were prepared by mixing the adsorbent with petroleum ether into a slurry, pouring into a column, and allowing to settle. Excess petroleum ether was recycled through the column. Compounds were then adsorbed onto the column and eluted with the indicated solvents.

Extraction of Triterpenes and Pigments—The ripe fruit of the Osage orange tree was dried in an oven at 60°, and it was then broken with a hammer into fine pieces and crushed to a powder in a mortar and pestle. It (74 g) was extracted with petroleum ether in a soxhlet extraction apparatus for 48 hr. After this time, the extraction material was replaced with a fresh portion.

This procedure was repeated 50 times until a total of 3.5 kg of dried fruit was extracted. Evaporation of the extract on the flash evaporator yielded a mixture of a solid yellow powder in a brown-colored oil (600 g, which amounted to 17.1% of the dried fruit).

Separation of Triterpenes and Pigments—The petroleum ether extract (70 g) was placed on a basic alumina column (1.5 kg) and eluted with 500 ml each of petroleum ether, carbon tetrachloride–petroleum ether (1:1), and finally carbon tetrachloride. The remaining yellow material on the column was eluted exhaustively with 500 ml of methanol. Evaporation of the solvent yielded a dark-brown oil (15 g), from which the pigments osajin and pomiferin were obtained, following the procedure of Wolfram *et al.* (5).

Hydrolysis of Osage Orange Extract—The fractions of Osage orange extract that were eluted with petroleum ether, petroleum ether–carbon tetrachloride (1:1), and carbon tetrachloride were combined. The solvent was evaporated on the flash evaporator, yielding a thick brown oil (50 g). To this oil was added 5 liters of 95% ethanol and 20.0 g of potassium hydroxide. The resulting mixture was heated at reflux for 72 hr.

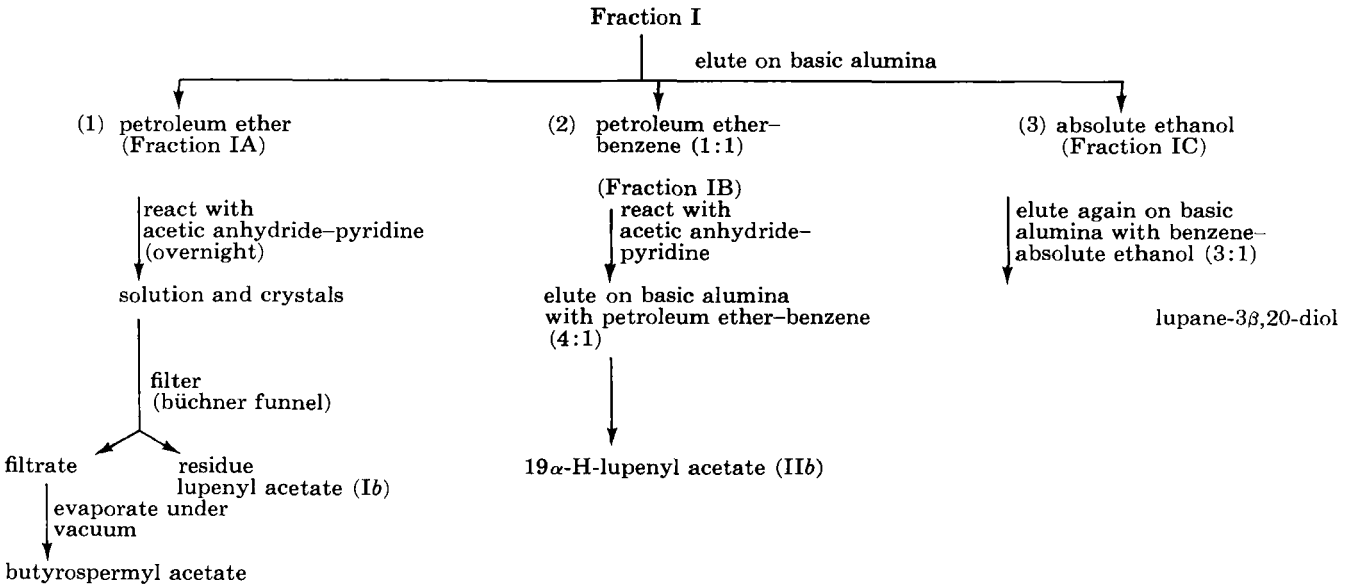
The alcohol was removed on a flash evaporator, leaving a thick yellow oil. Water (1 liter) was added, and the mixture was extracted with three 1-liter portions of methylene chloride. The methylene chloride portion was evaporated, yielding 25 g of light-brown solid material.

Separation of Triterpene Alcohols—The mixture of triterpene alcohols (25 g) was chromatographed on 200 g of basic alumina. Exhaustive elution with petroleum ether (1.5 liters) yielded a

[1] Melting points were determined on a Thomas-Hoover capillary melting-point apparatus and are uncorrected. Elemental analyses for carbon and hydrogen were performed by Dr. Kurt Eder, Geneva, Switzerland, and by Micro-Tech Laboratories, Inc., Skokie, Ill. The NMR spectra were determined using a Bruker model HFXO5 90-MHz spectrometer and were recorded downfield from tetramethylsilane as an internal reference. IR spectra were obtained with a Perkin-Elmer recording spectrophotometer, model 337, using KBr pellets. IR spectra with microquantities were determined with the aid of a Wilks scientific model 9 single-beam internal reflection attachment on a KRS-5 reflector plate (50 × 20 mm). Mass spectral data were obtained by means of the Hitachi Perkin-Elmer model RMU-6D spectrometer. UV spectra were obtained using a Perkin-Elmer model 202 double-beam recording spectrophotometer. Optical rotation were performed on a polarometer (No. 283) by O. C. Rudolph & Sons, Caldwell, N.J., in chloroform. Authentic samples of lupeol acetate, butyrospermol, and lupane-3β,20-diol 3-monoacetate were obtained through the courtesy of Professor K. G. Lewis, University of Australia, Armisdale, WSW.

[2] The fruit was identified by Professor Frank A. Crane, Department of Pharmacognosy and Pharmacology, University of Illinois at the Medical Center, Chicago. A sample is kept at that institution.

[3] Chromatographic Alumina F-20 (Activity I, basic), Alcoa Chemicals.



Scheme I—Separation of constituents

light-brown solution which, after removal of the solvent, gave a solid (Fraction I, 15 g). Further purification of Fraction I (2.5 g) by chromatography was required, eluting on basic alumina (100 g). Three solvent systems were applied. The fraction eluted with petroleum ether was labeled IA (1.1 g). Elution with petroleum ether–benzene (1:1) yielded Fraction IB (0.25 g), and final elution with absolute ethanol yielded Fraction IC (0.1 g).

Fraction IA: A Mixture of Butyrospermol and Lupeol—Removal of the solvent of Fraction IA yielded a brown solid. Fraction IA was recrystallized from acetone, collected on a büchner funnel, and washed with acetone to remove the adhering tar-like material. The fine white crystals remaining were allowed to air dry and then were recrystallized from 95% ethanol to give a white solid (1.0 g), which was treated overnight at room temperature with pyridine (10 ml) and acetic anhydride (15 ml) to give a solid monoacetate. The reaction mixture was filtered and the solid was recrystallized three times from 95% ethanol to give 0.48 g of lupenyl acetate, mp 213–214°, $[\alpha]_D^{25}$ +42° [lit. (6) mp 214–215°, $[\alpha]_D$ +41°].

Anal.—Calc. for $C_{32}H_{52}O_2$: C, 82.02; H, 11.11. Found; C, 81.96; H, 11.25.

Formation of 29-norlupan-20-one-3β-yl acetate (VIII), by reaction of lupenyl acetate with ozone, showed the position of a C-19 methine proton in the NMR spectrum at δ 3.70 ppm as a quartet (J = 4.6 Hz).

To a solution of 0.1 g (0.02 mole) of lupenyl acetate in 10 ml of absolute ethanol was added a solution containing 0.02 g (0.036 mmole) of potassium hydroxide in 10 ml of 70% ethanol. The mixture was heated at reflux for 4 hr. The solvent was removed on the flash evaporator, and 20 ml of water was added. Lupeol was extracted with methylene chloride.

The methylene chloride was evaporated on the flash evaporator, and the remaining white solid was recrystallized from acetone. The white crystals were dissolved in petroleum ether and chromatographed on 50 g of basic alumina. Elution with benzene–absolute ethanol (5:1) gave lupeol. Lupeol was recrystallized from acetone to give fine white needles, mp 210–211°, $[\alpha]_D^{25}$ +30° [lit. (16) mp 210–212°, $[\alpha]_D^{20}$ +26.4°]. Lupeol (0.08 g, 0.019 mmole) was obtained in 94% yield.

The filtrate from the acetylation that led to the formation of Fraction IA was evaporated by removal of the solvent on the flash evaporator. The yellow flaky residue was recrystallized from 95% ethanol using decolorizing activated charcoal. Further recrystallizations from acetone yielded butyrospermyl acetate (0.41 g), mp 139–140°, $[\alpha]_D^{25}$ +12° [lit. (6) mp 142.5–144°, $[\alpha]_D$ +10°].

The IR spectrum was identical with that of the product of acetylation of an authentic sample of butyrospermol, and there was no depression of melting point upon admixture of that acetylation product. The mass spectrum indicated m/e 468.

To a solution of 20 mg (0.04 mmole) of butyrospermyl acetate in 10 ml of 95% ethanol was added 10 mg (0.25 mmole) of sodium hydroxide in 10 ml of 70% ethanol. The resulting solution was heated to reflux for 2 hr. The solvent was evaporated, water was added, and the butyrospermol was extracted with ether several times. The ether fraction was evaporated to dryness, and the resulting solid was recrystallized several times from methanol, mp 112–113°, $[\alpha]_D^{25}$ −12° [lit. (6) mp 111–112°, $[\alpha]_D^{25}$ −13°].

It was identical with authentic butyrospermol and showed no depression of melting point upon admixture of an authentic sample. The IR spectra were identical.

Anal.—Calc. for $C_{30}H_{50}O$: C, 84.50; H, 11.74. Found: C, 84.30; H, 11.58.

Fraction IB—Removal of petroleum ether and benzene from Fraction IB yielded a brown-colored tar. Recrystallization of the substance from acetone yielded a tan-colored solid, which was collected on a büchner funnel, washed with acetone–water, and then allowed to dry. This procedure gave 0.2 g of Fraction IB.

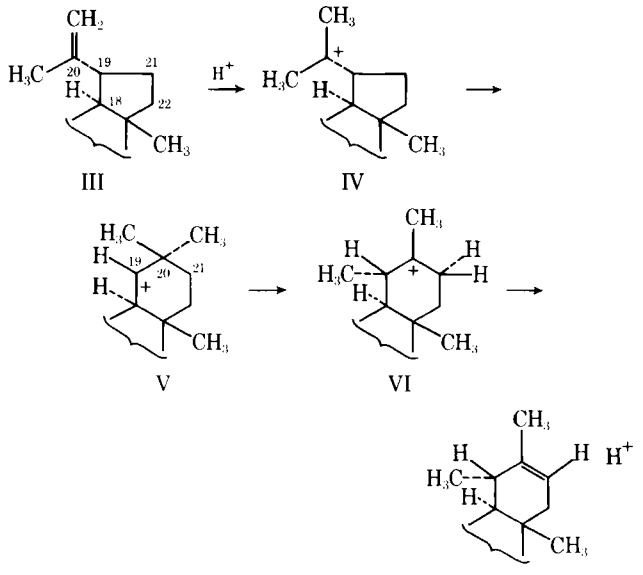
Fraction IB (2.0 g) was chromatographed on 100 g of basic alumina. Elution with petroleum ether–benzene (1:1) gave a fraction, which was recrystallized from acetone and then 95% ethanol to give a solid, mp 180–182°. Treatment with 5 ml of pyridine and 10 ml of acetic anhydride, heating at reflux for 10 min, gave a product which crystallized upon standing at room temperature for 1 hr. The product was recrystallized from 95% ethanol, mp 193–195°, in a yield of 1.5 g (corresponding to 0.38% of the dried fruit).

The IR spectra of the products from Fractions IA and IB showed the presence of a vinylidene group. Both spectra were similar with lupeol (Ia) and lupenyl acetate (Ib), respectively.

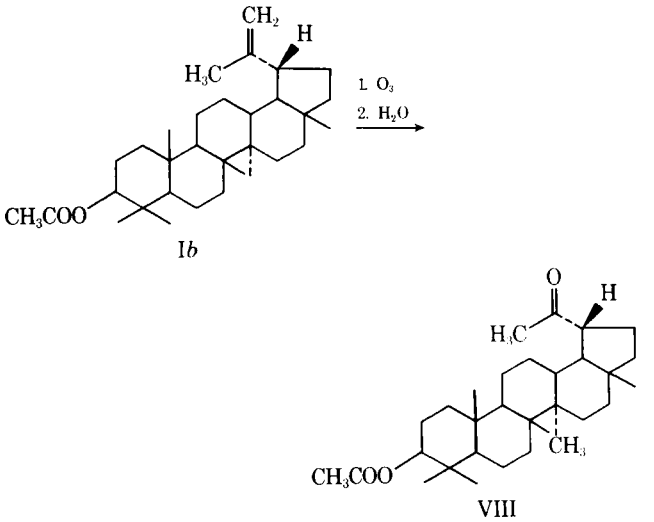
*Separation of 19α-H-Lupenyl Acetate (II*b)—The acetylated product (1.2 g) was chromatographed on 200 g of basic alumina. It was eluted with petroleum ether–benzene (4:1), and 15-ml fractions were collected. The last two fractions (14 and 15) (87 mg, 0.021% of the dried fruit) were combined and recrystallized from 95% ethanol to yield a solid, mp 210–211.5°, $[\alpha]_D^{25}$ +15° (dioxane). The mixed melting point with an authentic sample of lupenyl acetate was depressed to 193°.

There were absorption bands in the IR that indicated the presence of the vinylidene group (3100, 1650, and 880 cm^{-1}); NMR ($CDCl_3$): δ 0.84 (s, 9, 4α, 4β, 10β-CH_3), 1.027 (s, 3, 8β-CH_3), 0.929 (s, 3, 14α-CH_3), 0.780 (s, 3, 17β-CH_3), 2.03 (s, 3, acetoxyl-CH_3), 1.681 (s, 3, 30-CH_3), 4.60 (d, 2, J = 0.1 Hz, vinyl protons on C-29), and AB pattern at δ 4.46 (q, 1, J = 6 Hz, 3α-H); mass spectrum m/e (relative intensity) of major peaks (70 ev): 468 (100) M^+, 453 (28.6) (M − CH_3), 408 (18.5) (M − CH_3COOH), 393 (19.0) [M − (CH_3COOH + CH_3)]: 468 (100), 453 (27.6), 408 (14.9), 393 (8.6); (13 ev): 468 (100), 453 (26.0), 408 (10.4), 393 (7.8); and (10 ev): 468 (100) and 453 (29.2). Formation of the methyl ketone (IX) by reaction with ozone indicated the C-19 methine proton in the NMR spectrum as a quartet at δ 4.46.

Anal.—Calc. for $C_{32}H_{52}O_2$: C, 82.05; H, 11.11. Found: C, 81.67; H, 11.37.



Scheme II—Mechanism of formation of Ψ-taraxasterol (VII) from lupeol (III)



Scheme III—Formation of 29-norlupan-20-one-3β-yl acetate (VIII) by reaction of lupenyl acetate with ozone

Fractions 9 through 13, thought to be a mixture, were combined and recrystallized from 95% ethanol, mp 193–197°. Combined fractions 14 and 15 were again eluted on basic alumina with petroleum ether–benzene (4:1) and gave white crystals, eluting identically.

To a solution of 10 mg (0.02 mmole) of 19α-H-lupenyl acetate in 10 ml of 95% ethanol was added 10 mg (0.18 mmole) of potassium hydroxide in 10 ml of 70% ethanol. The resulting mixture was heated to reflux for 3 hr. The solvent was removed on the flash evaporator, 10 ml of water was added, and 19α-H-lupeol was extracted with ether several times. The ether fraction was evaporated to dryness, and the white solid remaining was recrystallized from acetone and then 95% ethanol, mp 204–206°, $[\alpha]_D^{25}$ +10°.

Anal.—Calc. for $C_{30}H_{50}O$: C, 84.50; H, 11.11. Found: C, 84.42; H, 11.39.

The mass spectrum indicated *m/e* 426; NMR ($CDCl_3$): δ 0.76 (s, 3, 4, CH_3), 0.828 (s, 3, 4β-CH_3), 0.960 (s, 3, 10β-CH_3), and 3.15 (q, 1, J = 6.0 Hz, 3α-H).

Fraction IC—Removal of the solvent from Fraction IC yielded a tar-like substance, which was washed with acetone and then recrystallized from 95% ethanol using activated charcoal. The product (0.08 g) was chromatographed on 100 g of basic alumina with benzene–absolute ethanol (3:1) to give an orange-colored viscous oil (15 mg). Treatment with pyridine (3 ml) and acetic anhydride (10 ml) overnight gave a monoacetate which, after recrystallization with acetone and then 95% ethanol, gave needles, mp 253–255°, $[\alpha]_D^{25}$ +16° [lit. (6) mp 248.5–250°, $[\alpha]_D^{25}$ +13.5°].

There was no depression of melting point upon admixture of an authentic sample of lupane-3β,20-diol 3-monoacetate. The IR spectrum was identical with that of an authentic sample. There was a sharp OH stretch at 3520 cm^{-1}, C=O stretch at 1720 cm^{-1}, and C—O stretch at 1250 cm^{-1}. The mass spectrum gave *m/e* 486.

To a solution of 0.3 g (0.06 mmole) of lupane-3β,20-diol 3-monoacetate in 10 ml of 95% ethanol was added 0.1 g (0.18 mmole) of potassium hydroxide in 20 ml of 70% ethanol. The solution was heated in reflux for 30 min, and the solvent was distilled off. Water (10 ml) was added to the white-colored residue and the product was extracted with ether. After distillation of the ether, the product was chromatographed on basic alumina (100 g) with benzene–absolute ethanol (1:1) to yield lupane-3,20-diol, mp 238–240°, $[\alpha]_D^{25}$ +3.9° [lit. (6) mp 238–241°, $[\alpha]_D$ +4°].

Reaction of Ib with Ozone—Compound Ib (1.0 g, 0.21 mmole) in 20 ml of chloroform was treated with a slow stream of ozone at 10° for 1 hr. Completion of the formation of the ozonide was ensured by inserting the outlet tube into a 5% potassium iodide solution. The solvent was removed *in vacuo* at room temperature, leaving the transparent glass-like ozonide to which 100 ml of water was added. The mixture was heated for 1 hr and then steam distilled. To the distillate was added an alcoholic solution of 2,4-dinitrophenylhydrazine. A yellow solid formed, which was collected on a büchner funnel and allowed to dry. The yellow solid was recrystallized from 95% ethanol to give needles, mp 163° [lit. (7) mp 166°]. There was no depression of the melting point upon admixture of an authentic sample of formaldehyde 2,4-dinitrophenylhydrazone. The hydrazone derivative corresponded to a 30% yield of formaldehyde.

The residue from the steam distillation was evaporated to yield a white solid, which was extracted with ether and washed several times with 10% $NaHCO_3$. The ether was removed and the product was recrystallized three times from acetone and then twice from 95% ethanol to yield white needles, mp 255–257°, $[\alpha]_D^{25}$ +9.8° [lit. (9) mp 258–260°, $[\alpha]_D^{20}$ +8.3°]. 29-Norlupan-20-one-3β-yl acetate (0.3 g, 0.063 mmole) was obtained in a 30% yield.

The IR spectrum indicated loss of the peaks corresponding to the vinylidene group in lupenyl acetate at 3100, 1650, and 880 cm^{-1}. The carbonyl ketone group showed a peak at 1695 cm^{-1}. NMR ($CDCl_3$): δ 0.84 (s, 9, 4α, 4β, 10β-CH_3), 1.02 (s, 3, 8β-CH_3), 0.960 (s, 3, 14α-CH_3), 0.764 (s, 3, 17β-CH_3), 2.143 (s, 30-CH_3), 2.03 (s, 3, acetoxyl-CH_3), *AB* pattern at δ 4.46 (q, 1, J = 6.0 Hz, 3α-H), and 3.70 (q, 1, J = 4.6, Hz, C-29 H); mass spectrum *m/e* (relative intensity) of major peaks (70) ev): 470 (24.1) (M^+), 455 (1.9) (M − CH_3), 440 (2.8) (M − $2CH_3$), 425 (1.9) (M − $3CH_3$), 410 (100) (M − CH_3COOH), 395 (29.6) [M − (CH_3COOH + CH_3)], 377 (3.7), 367 (44.6), 354 (6.5), 341 (13.9), and 328 (13.9).

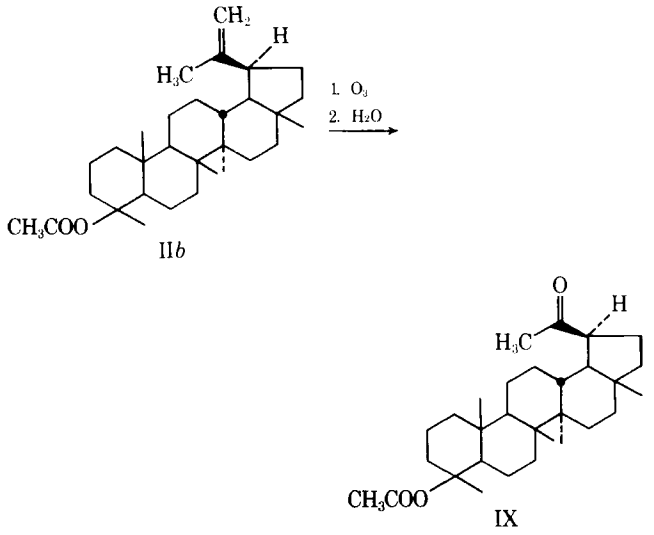
Anal.—Calc. for $C_{31}H_{50}O_3$: C, 79.15; H, 10.64. Found: C, 78.97; H, 10.50.

Reaction of 19α-H-Lupenyl Acetate with Ozone—Following the procedure for the ozonolysis of lupenyl acetate, 29-nor-19α-H-lupan-20-one-3β-yl acetate was formed from the reaction of ozone with 19α-H-lupenyl acetate. The product was recrystallized three times with acetone and then two times with methanol, mp 249–251°, $[\alpha]_D^{25}$ +43°.

IR spectra showed the loss of peaks, indicating the presence of the vinylidene group at 3100, 1650, and 880 cm^{-1}. The ketone carbonyl group appeared at 1690 cm^{-1}. NMR ($CDCl_3$): δ 0.84 (s, 9, 4α, 4β, 10β-CH_3), 1.02 (s, 3, 8β-CH_3), 0.949 (s, 3, 14α-CH_3), 0.749 (s, 3, 17β-CH_3), 2.143 (s, 3, 30-CH_3), 2.03 (s, 3, acetoxyl-CH_3), *AB* pattern at δ 4.46 (q, 1, J = 6 Hz, 3α-H), and 4.10 (q, 1, J = 4.8 Hz, C-29 H); mass spectrum *m/e* (relative intensity) of major peaks (70 ev): 470 (24.0) (M^+), 455 (2.6) (M − CH_3), 440 (1717) (M − $2CH_3$), 425 (5.3) (M − $3CH_3$), 410 (100) (M − CH_3COOH), 395 (30.3) (M − CH_3COOH), 377 (4.4), 367 (49.3), 354 (6.6), 341 (13.9), and 328 (13.9).

Anal.—Calc. for $C_{31}H_{50}O_3$: C, 79.15; H, 10.64. Found: C, 78.90; H, 10.57.

Equilibration of 29-Norlupan-20-one-3β-yl Acetate (VIII) with Sodium Ethoxide—A solution of 65 mg (0.14 mmole) of VIII in 10 ml of absolute ethanol was added to a solution of 26 mg (0.4 mmole) of sodium ethoxide in 10 ml of absolute ethanol. The solution was stirred at room temperature for 24 hr. The solvent was re-



Scheme IV—Formation of 29-nor-19α-H-lupan-20-3β-yl acetate (IX) by reaction of 19α-H-lupenyl acetate with ozone

moved on the flash evaporator. To the resulting brown solid, 40 ml of water was added. The mixture was stirred at room temperature for 2 hr and then extracted with ether.

The ether was evaporated on the flash evaporator, and to the resulting product was added 10 ml of a solution of acetic anhydride–pyridine (5:1). The mixture was heated for 10 min and then allowed to stand at room temperature for several days, during which time crystals formed. The crystals were collected and recrystallized three times from acetone and then twice from methanol to give 50 mg (76%) of a solid, mp 250–251°, $[\alpha]_D^{25}$ +43°.

Anal.—Calc. for $C_{31}H_{48}O_3$: C, 79.15; H, 10.64. Found: C, 78.90; H, 10.57.

The product was identical with the product of reaction of 19α-H-lupenyl acetate with ozone and showed no depression of melting point upon admixture of that product.

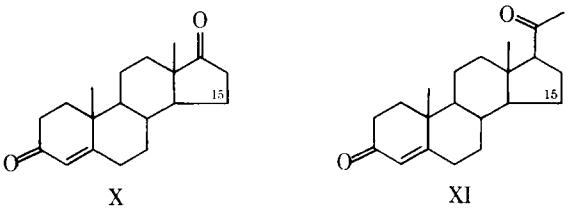
Conversion of Lupeol (Ia) to Ψ-Taraxasteryl Acetate (VII)—Following the procedure of Halsall *et al.* (13), lupeol (3.0 g, 0.7 mmole) was converted to Ψ-taraxasterol, which was reacted with acetic anhydride (10 ml) and pyridine (5 ml). The product was recrystallized from 95% ethanol several times and eluted over 100 g of basic alumina with petroleum ether–benzene (1:1) to give Ψ-taraxasteryl acetate (0.1 g, 20% yield), which was isolated as long white needles, mp 243–244°, $[\alpha]_D^{25}$ +62° [lit. (9) mp 238–240°, $[\alpha]_D^{25}$ +56°].

Conversion of 19α-H-Lupeol (IIa) to Ψ-Taraxasteryl Acetate (VII)—Following the procedure used in the conversion of lupeol to Ψ-taraxasteryl acetate, 19α-H-lupeol (40 g, 0.09 mmole) was converted to the identical product, mp 241–242°. There was no depression of melting point upon admixture of Ψ-taraxasteryl acetate; IR and mass spectra were identical.

DISCUSSION

Structural Characterization of Epimeric 19α-H-Lupenyl Acetate (IIb)—The lupane carbon skeleton contains 10 areas of potential dissymmetry including C-3; the carbon atoms comprising ring junctures C-5 and C-10, C-8 and C-9, C-13 and C-14, and C-17 and C-18; and the carbon atom at which the isopropenyl group is located, C-19. Acidic isomerization of 19α-H-lupeol (IIa), under the conditions specified by Halsall *et al.* (13), produced the identical pentacyclic triterpene as produced by lupeol (Ia), Ψ-taraxasterol (see partial structure VII, Scheme II), which was isolated as the 3-acetate.

From the mechanism proposed by Halsall *et al.* (13) (Scheme II), the only asymmetric site disturbed by these conditions is C-19. The first step predicts protonation of C-19 of partial structure III, leading to the formation of carbonium ion IV with the positive center located at C-20. Ring enlargement follows to form carbonium ion V, which places the positive charge at C-19. Migration of the α-axial methyl group on C-20 leads to carbonium ion VI which, after loss of a proton, gives Ψ-taraxasterol (VII). Not precluding the possibility of another isomer or mechanism leading to formation of VII, identical structures for the two epimers I and II in rings A, B, C, and D were indicated.



Prominent peaks for lupenyl acetate (Ib) and 19α-H-lupenyl acetate (IIb) in their NMR spectra were similar. Major differences in the spectra were uninterpretable occurring in the methine area at δ 1.59–1.65 ppm. To shift C-19 protons of Ib and IIb irrespectively away from interference, both Ib and IIb were treated with ozone to yield the corresponding ketones, 29-norlupan-20-one-3β-yl acetate (VIII) and 29-nor-19α-H-lupan-20-one-3β-yl acetate (IX) (Schemes III and IV).

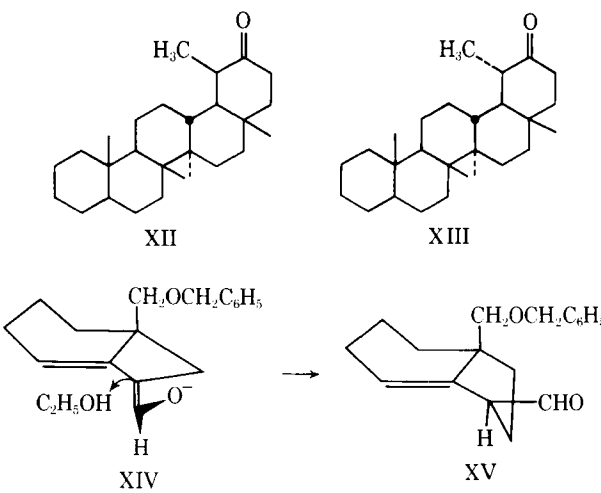
Ketone formation with ozone was used to avoid possible disturbance of the asymmetry of C-19, such as in oxidation in acidic media with chromic anhydride (8). However, acidic by-products are formed by ozone reaction (13). Previously, the ketone purification was performed by removal of acids by extraction with 10% potassium hydroxide (8). To prevent epimerization at C-19, this was avoided by use of sodium bicarbonate.

The NMR spectrum of VIII indicated a methine proton at δ 3.70 ppm, J = 4.6 Hz, attributed to the C-19 methine proton. It is adjacent to the carbonyl and would be expected to shift downfield. The methine proton appeared as a quartet (δ 3.82, 3.74, 3.67, and 3.59), in which first-order rules could be applied for its interpretation. The proton is in the environment of three nonequivalent protons, so four peaks would be expected.

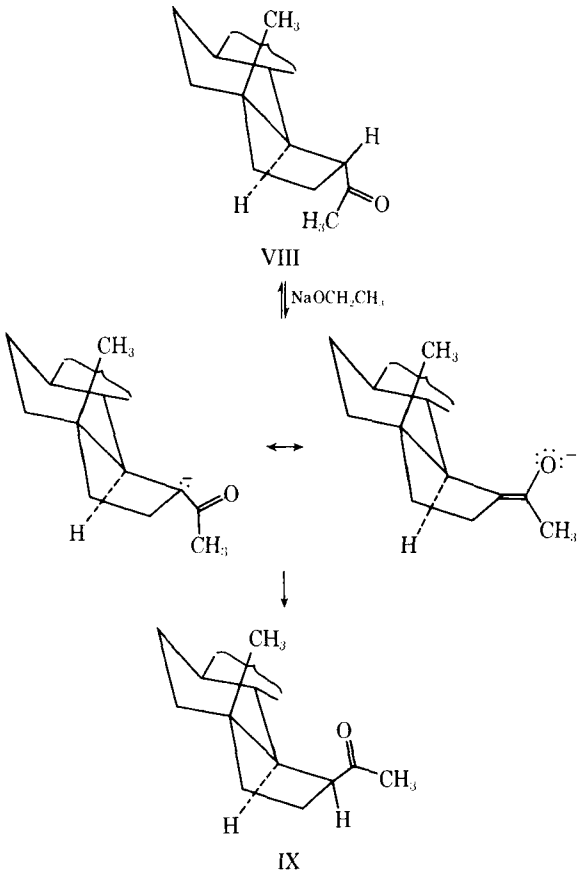
If Ib and IIb were epimeric at C-19, ozonolysis of the latter should yield Compound IX. The NMR spectrum of its product (IX) indicated the C-19 methine proton at δ 4.10, J = 4.8 Hz, as a quartet (δ 4.22, 4.14, 4.06, and 3.98), in which first-order rules were applied for its interpretation. No other proton in Ib could show a quartet in which the coupling constant is about 4.8 Hz unless that proton were at C-19.

The chemical shift difference between the two C-19 quartets of VIII and IX is δ 0.40. The difference in chemical shift compared to measurements obtained from 15-substituted hydroxy and acetoxy derivatives of Δ^4-androstene-3,17-dione (X) and Δ^4-pregnene-3,20-dione (XI) (13). The C-15 position of the five-membered rings in X and XI can correspond to substituents of C-19 in I or II. In both sets of molecules, each site in the five-membered ring is the third carbon atom from the β-methyl at the ring junction and the first carbon atom from the α-hydrogen at the ring juncture; they are, therefore, models for comparison.

The chemical shifts of the protons attached to the same carbon



Scheme V



Scheme VI—Formation of 29-nor-19H-lupan-20-3-yl acetate (VIII) from 29-norlupan-20-one-3-yl acetate (IX) with sodium ethoxide, followed by dilution with water and reesterification of the C-3 hydroxy group

atom within the skeletons of X and XI are characteristic of the position and configuration in which these protons are situated (13). There is a high field shift of an axial proton relative to its equatorial counterpart when these compounds are substituted alcohols. The chemical shift difference for the C-15 methine protons is δ 0.20 + 0.03; for the corresponding 15-substituted acetates, it is δ 0.20 (13).

Epimerization of ketone VIII to IX occurred with sodium ethoxide. The strong base affected only the asymmetry of one carbon atom in VIII, C-19. The epimerization of VIII to IX is similar to the epimerization of 30-nortaraxastan-20-one (XII) to 30-nor-19α-H-taraxastan-20-one (XIII), when absorbed from benzene onto neutral alumina [Activity I, pH 7–8 (14)]. In addition, Marshall and Greene (14) indicated the stereochemical outcome of the reaction of the enolate of 7a-benzyloxymethyl-2,3,4,5,6,7,7a-hexahydroindene-3-carboxaldehyde (XIV) with ethanol. Protonation of the enolate by ethanol occurred from the less hindered side to give the β,γ-unsaturated aldehyde (XV), in which the carboxaldehyde group is in the β-axial orientation (Scheme V).

Upon equilibrating VIII with sodium ethoxide, followed by dilution with water, extraction with ether, and reesterification with acetic anhydride–pyridine, only one pure ketone (IX) was isolated (Scheme VI). Where a ring is rigid, such as in steroidal ketones, addition reactions to the carbonyl function proceed from the equatorial side (15). As illustrated in Scheme VI, after removal of the C-19 proton, the asymmetry at that site is disturbed. The configuration at C-19 of the final product IX is determined in the addition step, in which the proton adds from the sterically more accessible side equatorially. Therefore, the acetyl group of the final product IX is exclusively in the β-axial orientation.

Since the product of equilibrium of VIII in base gave IX and this was identical with the product of ozonation of II*b* to IX (Scheme IV), it then follows that the difference between I*b* and II*b* is solely at C-19.

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Bioavailability and Pharmacokinetics of Prednisone in Humans

A. R. DiSANTO ˣ and K. A. DeSANTE

Abstract □ In a clinical study involving 22 normal adult volunteers, the bioavailability and pharmacokinetics of prednisone tablets with varying dissolution rates were evaluated. Serum levels were measured by a radioimmunoassay for prednisolone. Absorption rate constants and serum half-lives are presented. Substantial serum levels of prednisolone were attained quite rapidly (within 0.5 hr). The observed serum levels were statistically analyzed and fitted to the one-compartment open model with first-order absorption and elimination. A qualitative correlation between the *in vitro* dissolution rate and the calculated initial absorption rate constants was found. However, the *in vitro* dissolution rates were not predictive of the overall bioavailability of the prednisone tablets tested in terms of peak concentration and area under the serum concentration–time curve.

Keyphrases □ Prednisone—bioavailability and pharmacokinetics in humans, absorption rate constants and serum half-lives, 50-mg tablet compared to 10 5-mg tablets, *in vitro–in vivo* correlations discussed □ Bioavailability—prednisone in humans, 50-mg tablet compared to 10 5-mg tablets, *in vitro–in vivo* correlations discussed □ Pharmacokinetics—prednisone in humans, absorption rate constants and serum half-lives, *in vitro–in vivo* implications

With the advances in analytical technology, it is now possible to measure circulating levels of exogenous steroids (1–5). Applying the principles of pharmacokinetics to these serum levels enables one to define and quantitate the processes of absorption, distribution, metabolism, and excretion. Through the understanding of these processes, the clinician can more rationally assign optimal dosage regimens. However, this use assumes that the available drug products are reliable in terms of delivering the prescribed dose of the drug to the general circulation at essentially the same rate and extent; *i.e.*, release of the drug from the drug delivery system (capsule, tablet, *etc.*) is not variable from manufacturer to manufacturer nor from lot to lot.

Among the more important aspects associated with the rate and extent of absorption of an orally administered solid dosage form are: *(a)* the dissolution characteristics of the drug, and *(b)* the ability of the drug to permeate the GI membrane. The absorption of any given therapeutic agent could potentially be rate limited by either process. However, when several different formulations of the same drug are compared, the intrinsic permeability of the drug is usually constant for the formulations tested and the rate of solution of the solid drug may then become the major variable among the formulations.

In recent years, much emphasis has been placed on the *in vitro* screening of a drug's dissolution properties as an index of *in vivo* absorption characteristics for a given formulation. In several instances, such *in vivo–in vitro* correlations have been found (6, 7).

The purposes of this study were to: *(a)* define and quantitate the processes of absorption and elimination of prednisone after oral administration of a single 50-mg dose, and *(b)* establish the bioequivalence of a slowly dissolving 50-mg prednisone tablet to an equivalent dose of a rapidly dissolving 5-mg prednisone tablet.

Table I—Physicochemical Characteristics of Prednisone Products Evaluated

	Treatment A, Prednisone, 50-mg Tablet	Treatment B, Prednisone, 10 5-mg Tablets
Assay	48.6 mg/tablet	5.0 mg/tablet
Content uniformity:		
High	113.2% of 50 mg	99.2% of 5 mg
Low	108.6% of 50 mg	97.6% of 5 mg
Average	110.7% of 50 mg	98.2% of 5 mg
Dissolution rates:		
USP test, $T_{60\%}$, not more than 20 min at 100 rpm:		
Average, min	60.4	2.3[a]
Range, min	50.0–80.0	1.7–2.8
Percent in 20 min		
Average	34.8	100
Range	26.5–42.0	—
Disintegration time, min:		
High	1.5	2.0
Low	1.3	0.8
Average	1.5	1.0

[a] For 10 5-mg tablets.

EXPERIMENTAL

Subjects—Twenty-two healthy volunteers (15 females and seven males) averaging 25 years of age, range 19–44, with normal screening vital signs and laboratory parameters were selected. No volunteers with active peptic ulcers, tuberculosis, or psychosis were accepted. The subjects did not receive any steroid medication for 30 days and any other medication for 7 days preceding the start of the study, and they received only the medication prescribed in the protocol. The average weight of the subjects was 64.5 kg (142 lb), range 47.7–88.5 kg (range 105–195 lb).

Medication and Dosage—At zero time of each treatment period, each subject received medication as follows: Treatment A, one 50-mg tablet of prednisone[1] given as a single oral dose; and Treatment B, 10 5-mg tablets of prednisone[2] given as a single oral dose of 50 mg. Individual subjects were assigned treatments according to a randomized two-way crossover design. Seven days separated the medication day of Period I from the medication day of Period II.

Study Conditions—Subjects were required to fast overnight and for 2 hr immediately after the medication was administered. The medication was taken at zero time with 180 ml (6 fl oz) of water. No beverages (except water) were permitted during the fasting period. The subjects were not permitted to engage in any strenuous or athletic activities during the days of administration.

Sampling and Collection of Blood for Steroid Assay—Blood was taken from each subject at zero time, *i.e.*, just before dosing with each medication, and then again at 0.5, 1.0, 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, and 24 hr after zero time. Ten milliliters of blood was

[1] Deltasone, Upjohn Lot 16,970.
[2] Deltasone, Upjohn Lot 131AJ.

Table II—Average Serum Prednisolone Concentrations and Related Parameters Obtained from 22 Normal Adults Each Receiving Single Oral Doses of 50 mg of Prednisone

	Treatment A, Prednisone, 50-mg Tablet	Treatment B, Prednisone, 10 5-mg Tablets	Significance Level of Differences between Treatment Averages A and B
Average serum concentration, μg/100 ml, at:			
0.5 hr	40.76	57.29	$0.01 < p < 0.025$
1.0 hr	70.00	77.07	N.S.
2.0 hr	79.50	82.34	N.S.
3.0 hr	80.70	69.42	$0.01 < p < 0.025$
4.0 hr	68.63	60.60	N.S.
6.0 hr	49.35	48.01	N.S.
8.0 hr	36.04	33.07	N.S.
12.0 hr	15.30	17.41	N.S.
24.0 hr	2.06	5.21	N.S.
Peak of average serum concentration curve, μg/100 ml	80.7	82.3	N.S.
Average of individual peak serum concentration, μg/100 ml	92.6	89.4	N.S.
Average area under serum concentration curve, 0 → 12 hr, μg/100 ml × hours	573.5	559.4	N.S.
Average area under serum concentration curve, 0 → 24 hr, μg/100 ml × hours	677.6	695.1	N.S.
Average area under serum concentration curve, 0 → ∞ hr, μg/100 × hours	686.2	732.7	N.S.
Average peak time, hr	2.07	1.73	N.S.
FD/V	126.8	112.15	N.S.
Biological half-life ($t_{1/2}$), hr:			
Arithmetic mean	2.95	3.57	N.S.
Harmonic mean	2.57	2.92	N.S.
Elimination rate constant (K), hr^{-1}	0.27	0.25	N.S.
Absorption half-life ($A_{1/2}$), hr:			
Arithmetic mean	0.99	0.66	$0.025 < p < 0.05$
Harmonic mean	0.61	0.35	$0.01 < p < 0.025$
Absorption rate constant (k), hr^{-1}	1.15	1.97	$0.01 < p < 0.025$

drawn at each sampling time. Serum was harvested from all samples as soon after drawing as possible and immediately frozen.

Physical and Chemical Characteristics of Medication—The physical and chemical characteristics of the two formulations are presented in Table I. Both formulations have similar potency, content uniformity, and disintegration times, but the dissolution rates are vastly different. These dissolution rates were determined by the rotating-basket assembly of NF XIII. The USP XVII specification for dissolution of 5-mg tablets of prednisone is that 60% of the drug should dissolve in not more than 20 min.

Assay Methodology—Recent developments in radioimmunoassay procedures have produced an assay that measures circulating levels of prednisolone in serum. Since prednisone is metabolized rapidly *in vivo* to prednisolone (8, 9), the development of this assay procedure allows for the determination of the bioavailability of prednisone. The specificity and sensitivity of the prednisolone assay used in this study were reported previously (1).

RESULTS

The average serum prednisolone concentration–time relationships obtained for 22 normal adults after administration of single oral doses of 50 mg of prednisone given as either one 50-mg tablet or 10 5-mg tablets of prednisone are shown in Fig. 1. The average serum prednisolone concentrations and the related parameters of peak height concentration and area under the serum concentration–time curve, as well as a summary of the statistical analysis, are presented in Table II.

With the exception of the 0.5- and the 3.0-hr sampling times, there were no statistically significant differences between the average prednisolone concentrations attained with each treatment. The mean peak serum concentration was 89 μg/100 ml (range 45.17–175.42) for the 5-mg tablet and 93 μg/100 ml (range 50.81–157.9) for the 50-mg tablet. The 0–24-hr areas under the serum concentration–time curve were 678 and 695 μg/100 ml × hours for the 50-mg and 10 × 5-mg tablets, respectively.

Preliminary estimates of the parameters were graphically obtained by the feathering (method of residuals) technique, using the serum data plotted on semilogarithmic graph paper. Each individual subject's data were then fitted by the method of least squares, using an iterative digital computer program (NONLIN) and a digital computer[3], to conform to Scheme I (10):

$$\frac{(F)(D)}{t=0} \xrightarrow{k} \boxed{\begin{matrix} V \\ C \end{matrix}} \xrightarrow{K}$$

$$C = \frac{(F)(D)}{V}\left(\frac{k}{k-K}\right)[e^{-Kt} - e^{-kt}]$$

Scheme I

where F = fraction of dose absorbed, D = dose, k = first-order rate constant for absorption, K = first-order rate constant for elimination, C = concentration of drug in serum, and V = volume of distribution.

In the computer fitting, the graphical estimates of the parameters were used as initial estimates, and the serum concentrations were assigned equal weights. The least-squares estimates of the individual rate constants (k and K) for absorption and elimination are listed in Table III.

Both the individual and average serum concentration–time data were fitted to the one-compartment model with first-order absorption (Scheme I). In Fig. 1, the average serum prednisolone levels are plotted *versus* time, as are those predicted by Scheme I. The average rate constants used in generating these lines are listed in Table III. The r^2 and correlation coefficients between observed and predicted values for these computer-fitted data points were 0.99, indicating an excellent fit[4].

The serum half-lives calculated for prednisolone were not significantly different for either treatment, and the overall mean serum half-life from the 44 observations was approximately 3.25 hr (2.95 hr for the 50-mg tablet and 3.57 hr for the 5-mg tablets). The mean of the individuals' rate constants of elimination was 0.27 hr^{-1} for the 50-mg tablet, and it was 0.25 hr^{-1} for the 5-mg tablets. The respective mean rate constants of absorption for the 50-mg tablet and the 10 5-mg tablets were 1.15 and 1.97 hr^{-1}; their corresponding absorption half-lives were 0.99 and 0.66 hr.

[3] IBM 360/50.

[4] r^2 = (Σ squared observations − Σ squared deviations)/Σ squared observations.

Table III—Individual Pharmacokinetic Parameters Obtained from 22 Normal Adults Each Receiving Single Oral Doses of 50 mg of Prednisone (Data Fitted to One-Compartment Open Model Using a Digital Computer Programmed with NONLIN)

	Treatment A, Prednisone, 50-mg Tablet						Treatment B, Prednisone, 10 5-mg Tablets					
Subject	k, hr^{-1}	$A_{1/2}$, hr	K, hr^{-1}	$t_{1/2}$, hr	r^2	cor	k, hr^{-1}	$A_{1/2}$, hr	K, hr^{-1}	$t_{1/2}$, hr	r^2	cor
1	0.41	1.68	0.29	2.36	0.972	0.963	3.98	0.17	0.16	4.32	0.997	0.996
2	1.46	0.48	0.28	2.46	0.997	0.996	2.38	0.30	0.21	3.30	0.995	0.993
3	1.52	0.46	0.08	3.79	0.991	0.986	0.60	1.15	0.18	3.77	0.986	0.975
4	0.81	0.86	0.55	1.27	0.995	0.995	4.98	0.14	0.23	2.97	0.999	0.999
5	1.35	0.51	0.23	3.03	0.913	0.878	1.45	0.48	0.24	2.87	0.990	0.986
6	0.68	1.02	0.20	3.55	0.998	0.995	2.86	0.24	0.14	4.81	0.996	0.991
7	0.28	2.52	0.23	3.02	0.984	0.982	0.72	0.96	0.04[a]	17.58[a]	0.971	0.928
8	1.90	0.37	0.10	7.17	0.995	0.988	1.41	0.49	0.10	6.76	0.995	0.989
9	1.91	0.36	0.12	5.90	0.997	0.994	3.35	0.21	0.11	6.45	0.978	0.958
10	1.44	0.48	0.32	2.16	0.997	0.995	2.05	0.34	0.24	2.86	0.998	0.996
11	0.46	1.50	0.35	1.96	0.985	0.978	1.35	0.51	0.07	4.05	0.985	0.969
12	1.26	0.55	0.27	2.56	0.974	0.957	1.43	0.48	0.30	2.29	0.996	0.994
13	0.91	0.77	0.25	2.76	0.974	0.956	1.12	0.62	0.27	2.61	0.996	0.993
14	0.81	0.85	0.35	2.00	0.999	0.998	0.20	3.46	0.85	0.81	0.944	0.912
15	0.51	1.35	0.23	3.07	0.990	0.983	0.84	0.83	0.14	4.80	0.986	0.975
16	0.26	2.70	0.19	3.67	0.992	0.993	3.07	0.23	0.14	4.85	0.998	0.996
17	0.53	1.30	0.35	2.00	0.997	0.994	1.48	0.47	0.24	2.92	0.993	0.986
18	1.57	0.44	0.23	3.01	0.977	0.953	1.33	0.52	0.31	2.21	0.993	0.988
19	0.42	1.64	0.31	2.25	0.990	0.984	2.43	0.29	0.12	5.67	0.994	0.989
20	0.58	1.19	0.39	1.81	0.997	0.996	0.65	1.07	0.40	1.75	0.963	0.950
21	1.20	0.58	0.26	2.62	0.997	0.996	0.47	1.49	0.41	1.70	0.992	0.986
22	5.00	0.14	0.28	2.45	0.991	0.989	5.00	0.14	0.22	3.19	0.993	0.985
Mean	1.15	0.99	0.27	2.95	0.986	0.979	1.97	0.66	0.25	3.57	0.988	0.979
SD	1.01	0.47	0.10	1.77			1.40	0.52	0.17	2.14		
Average data	0.92	0.76	0.19	3.72	0.999	0.998	1.74	0.40	1.15	4.57	0.999	0.999

[a] Data not amenable to kinetic analysis.

The absorption half-life was statistically ($p < 0.05$) lower for the 5-mg tablets than for the 50-mg tablet, indicating a qualitative correlation between the *in vitro* dissolution rates and the calculated initial absorption rate constants. However, since the peak serum concentrations, the time of peak serum concentration, and the area under the serum concentration–time curve did not differ statistically, no true correlation between the bioavailability of two formulations and the *in vitro* dissolution was obtained. Hence, from a clinical view, the differences suggested by the dissolution rates were meaningless.

The rate and degree of absorption are dependent upon both the dissolution and the permeability characteristics of a drug. The prednisone from both preparations has the same permeability characteristics, and any discrepancies in bioavailability could not be dependent upon this parameter. Since the peak heights, the areas, and the peak times all indicate equivalent bioavailability (both in extent and rate of availability), it can be concluded that the presently used dissolution rate technique for prednisone is not sufficiently predictive of the commonly utilized *in vivo* bioavailability parameters. Although the pharmacokinetic analysis indicated a slower rate of absorption *in vivo* with an increase in $T_{60\%}$ *in vitro*, the peak concentration and the time of the peak serum levels of prednisolone (2.07 and 1.73 hr) were not significantly different.

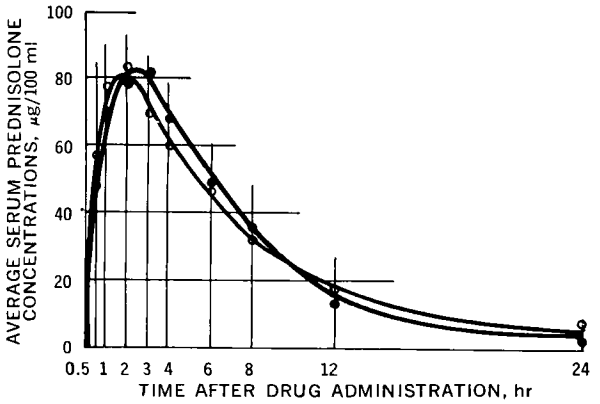

Figure 1—*Average serum prednisolone concentrations (μg/100 ml) obtained for 22 normal adults after receiving 50 mg of prednisone given as: ●, Treatment A, single oral dose of 50-mg tablet of prednisone; or ○, Treatment B, single oral dose of 10 5-mg tablets of prednisone. Lines were generated by digital computer with average parameters of Table III.*

DISCUSSION

The intent of the authors was not to take issue with the dissolution rate specifications for prednisone but rather to caution the reader in the interpretation of dissolution data. As expressed by Barr (11), *in vitro* dissolution rate measurements have been used in various ways. The two most important are: *(a)* as a sensitive quality control procedure to detect changes in the release characteristics due to lot-to-lot variation, formulation changes, or storage conditions; and *(b)* to provide an indication of differences in *in vivo* absorption characteristics of the drug and serve as a secondary standard to detect dosage forms with a potential for poor bioavailability.

Important requirements for the use of an *in vitro* dissolution method as a quality control procedure are its sensitivity, reproducibility, and ability to detect small differences in dissolution rate. These differences may or may not be indicative of differences in *in vivo* bioavailability. When *in vitro* dissolution rate methods are used to assess possible differences in the bioavailability of different formulations of the same drug, the principal requirements are a high degree of correlation between the *in vivo* and *in vitro* methods and the selectivity of the methods to reject poor products.

The fact that a tablet that did not meet compendial dissolution rate specifications was bioequivalent to a tablet with almost a 25-fold faster *in vitro* dissolution rate should be a caution against accepting the results of a battery of *in vitro* tests as criteria for *in vivo* bioequivalence of two formulations. Much of the differences observed in the dissolution rates between these tablets could possibly be attributed to the dissolution rate apparatus employed. By using a different dissolution rate apparatus or different conditions with the same apparatus, an *in vitro–in vivo* correlation that can predict prednisone bioavailability from dissolution rate data may yet be determined.

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Simple and Reliable Method for Serial Sampling of Blood from Rats

R. A. UPTON

Abstract □ A technique for serial sampling of whole blood, plasma, or serum from unanesthetized, unrestrained rats is described. This technique is sufficiently rapid, reliable, and independent of the need for elaborate preparation, specialized equipment, or practiced skills to appeal to a wide range of experimental and teaching situations requiring multiple sampling from many animals. A simple surgical procedure implants a one-piece jugular cannula cut from a commercial coil of silicone polymer tubing. Multiple blood samples are almost immediately available for 5 weeks or more. Plasma or serum samples are readily obtainable from each blood sample without transfer from the syringe in which it is collected. Intravenous injection through the cannula does not prejudice later sampling protocol.

Keyphrases □ Blood sampling, serial—simple and reliable method, technique and equipment described, rats □ Serial sampling, blood—simple and reliable method, technique and equipment described, rats

The size of the rat makes it a convenient laboratory animal for instructional classes and limited facility situations or where statistical treatment of data necessitates a number of subjects. For pharmacokinetic studies in particular, a simple technique for serial sampling of blood from these animals would be most useful.

The methods available, however, are unreliable, traumatic for the subject, difficult for the operator, or require elaborate preparation or specialized equipment. The following modification of the Popovic and Popovic (1) and Weeks and Davis (2) methods involves a simple, rapid surgical procedure, requiring neither elaborate preparation nor equipment more specialized than medical-grade tubing. Cardiac blood samples from the unanesthetized, unrestrained rat can then be taken repetitively and almost instantaneously. Plasma or serum samples are available for very little extra outlay of time or effort.

The modifications described thus eliminate problems counter to the demands of those very situations that favor choice of the rat as a laboratory subject. The technique is well suited to a wide range of experimental and teaching situations requiring multiple samples from large numbers of animals.

EXPERIMENTAL

Surgical Procedure—The rat is lightly anesthetized with ether, and the hair is removed from about a 2-cm square of the skin on the back of the neck. The animal is then held on its back by taping the forelegs to a board with adhesive tape. Light anesthesia is maintained for the rest of the surgical operation by applying a small wad of tissue paper over both the rat's nose and mouth and moistening the wad with ether occasionally.

Hair is clipped from above the right jugular vein, and a 2–3-cm skin incision is made in an anterior direction from above the midpoint of the right collar bone. By blunt dissection, about 1 cm of the external jugular vein is exposed forward from its emergence from below the collar bone. A 14-gauge hypodermic needle, from which the hub has been removed, is inserted under the skin at a point in line with, and about 1 cm anterior to, the exposed section of the jugular vein. It is passed subcutaneously but directly (*i.e.*, over the right shoulder but under the ear) to pierce the skin in the center of the clipped area on the back of the neck and just above the shoulder blades.

A 150-mm length of silicone polymer tubing is threaded through the needle, and the latter is removed through the back of the neck to leave the tubing lying subcutaneously. A syringe charged with heparinized saline is attached to the dorsal end of the tubing, and the tubing is rinsed with a little of the solution.

The exposed section of the vein is ligated anteriorly, and the ends of the tie are left uncut. By taping these ends to the operating board beside the subject's head, slight traction can be applied to the vein. A short oblique cut is made in the vein with a pair of fine scissors just below the ligature. By lifting the resultant flap with a pair of fine forceps and rotating the tubing, the ventral end of the tubing can easily be inserted into the vein. Thirty millimeters of tubing is passed toward the heart.

The tubing is secured by tying firmly around the vein just above the collar bone and again closer to the point of insertion of the can-

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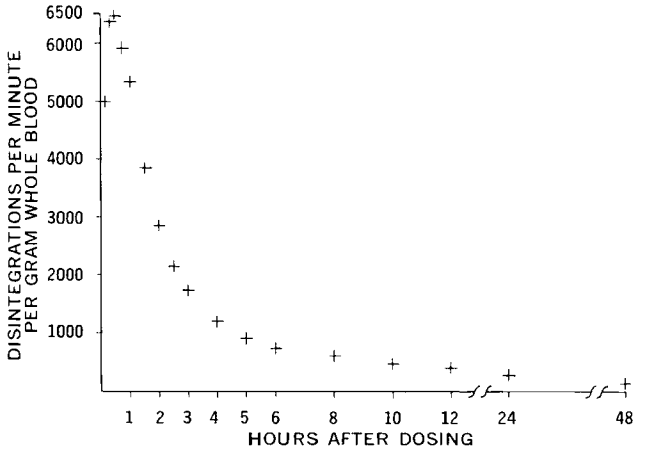

Figure 1—*Pharmacokinetic study. A 282-g rat was dosed intraperitoneally with 7.5 × 10⁶ dpm ¹⁴C-antifolate Compound 1, 12 hr after cannula implantation.*

nula. The ends of the anterior ligature are firmly tied around the exposed tube; another tie is made below the ligature but above the point of insertion, thus securing the exposed cannula to the depleted section of the vein. Possible occlusion of the cannula can be monitored after each tie by movement of the plunger of the syringe still attached.

Parted subcutaneous tissue and musculature are reunited by discontinuous sutures. Where possible, the sutures are passed around the tubing. Three or four usually suffice. The ventral skin incision can then be closed, also with discontinuous sutures (six to eight). A firm tie is made around the tubing and skin just before the tubing emerges at the back of the neck; the ends of the tie are left long and are used to form a small loop above the skin. After injection of 0.2 ml heparinized saline, the cannula can be cut with a bevel to a length of 10–20 mm and stoppered with a light plug fashioned from 24-gauge stainless steel wire.

A translucent, seamless silicone tubing[1] (0.51 mm i.d., 0.94 mm o.d.) was used. Sterilization was found to be unnecessary. The tubing was wiped free of visible lint prior to insertion into the vein. Vein wall obstruction of the cannula was never a problem if the tubing was beveled by trimming to an arrowhead and the tip of the arrowhead was removed by a transverse cut. Correct location of the cannula in the vein was assisted by marking the tubing, *e.g.*, 40 mm from the bevel. The heparinized saline used was normal saline containing heparin, 10 units/ml.

While not expensive, the cannula could be cleaned without difficulty and reused indefinitely.

Cannulated subjects were individually housed. All animals survived surgery, regained consciousness within minutes after the operations described, and moved apparently unencumbered after about 0.5 hr. Presence of the cannula caused no obvious discomfort. Where possible after effects of light ether anesthesia presented no objections, animals could be used for sampling procedures almost immediately.

Sampling Procedure—The following technique was employed for obtaining blood samples for pharmacokinetic studies from cannulated male Wistar rats, 250–300 g, approximately 4 months old. Each was housed individually in a urine–feces separating apparatus, having a cage of 25-cm diameter horizontal cross section. (Any similarly sized, shallow roofless cage would be suitable.) Handling of the animal was found to be unnecessary. Rather, movement of the animal (rare) during the withdrawal procedure was followed by a complementary movement of the hands manipulating the cannula or attached equipment.

A small clip was positioned at the back of the neck, both to clamp the suture loop and occlude the exposed tubing. The plug was removed from the cannula. A syringe fitted with a 23-gauge needle and containing 0.2 ml heparinized saline was inserted into the cannula. The cannula was released from the clip, which was allowed to hang from the suture loop. Then 0.2 ml of blood was withdrawn into the syringe, held vertically to minimize mixing of blood and saline.

[1] Silastic 602-135, Dow Corning International Ltd.

Table I—Short Sampling Interval

Sampling Time, min after Dosing[a,b]	Weight of Blood Samples, g Whole Blood	Content, dpm ¹⁴C/g Blood
60.0	0.2068	5851
61.0	0.1988	5864
62.0	0.2006	5728
63.0	0.1962	5461
64.0	0.1930	5231
64.5	0.1953	4957
65.0	0.2017	4754
65.5	0.2043	4571
66.0	0.2100	4527
66.5	0.2058	4323

[a] A 282-g rat was dosed intraperitoneally with 7.5 × 10⁶ dpm ¹⁴C-antifolate Compound 1, 10 days after cannula implantation. [b] Standard procedure (withdrawal of 0.2 ml of blood prior to each sample and return with 0.2 ml of heparinized saline after each sample) was omitted after 64.0 min for 30-sec sampling.

The syringe was removed from the needle and stored vertically in a needle scabbard taped to a wall, and a fresh syringe was fixed to the needle still attached to the cannula. The sample of blood was withdrawn and the syringe was removed. The first syringe was then returned to the needle, and the blood followed by the upper layer of heparinized saline was injected into the animal. The cannula was then reguided between the jaws of the clip, and the needle was removed.

Use of the clip enabled rapid access to samples without excessive blood loss or introduction of air. Entry of air could be further minimized by additional application of the clip between syringe manipulations. Use of the loop of suture material prevented drag on the cannula. Subjects wore the clip arrangement for up to 12 hr when sampling was frequent. When sampling intervals were longer, *e.g.*, overnight, the lighter plug replaced the clip and the loop was not necessary. Hold-up volume of the cannula and attached needle plus dead space between the needle and syringe was 20–25 μl. Removal of the needle bevel aided preservation of the tubing material.

During one pharmacokinetic study, each rat provided 15 samples of 0.2 ml blood at 10, 20, 30, 45, and 60 min and thereafter every 30 min up to and including 6 hr after dosing. In another such study, 17 samples were removed from each subject with the same regimen to 1 hr but with the remainder of the samples spaced to 48 hr[2] (Fig. 1). In all cases, samples could be taken within seconds of the nominated times.

Sampling could be comfortably achieved every minute. For more frequent sampling, the advantages of rinsing the cannula disappeared. By omission of rinsing and blood volume replacement, samples could be withdrawn every 30 sec without rush (Table I). These samples contained the contents of the dead space, giving for each 0.2 ml sample about 10% contamination from the previous sample. This contamination could be reduced by skillful use of the air introduced when syringes were changed without application of the clip.

Two rats were retained for 5 weeks, after which time blood samples could still be freely removed. Both subjects continued to show no patent ill effects. For these longer term animals, a loose rather than firm tie was made at the back of the neck with a fairly coarse sewing silk and the loop was constructed as before. A small piece of adhesive tape was folded over the cannula and loop such that both were fixed between the two adhesive surfaces. The resulting protective sheath was trimmed.

Where the cannula was used as frequently as daily, no special rinse-out precautions were necessary. For the first use of the cannula after more than 24 hr, initial withdrawal of a small blood sample was advisable. Cannulas not undergoing use were rinsed weekly, at which time clots may have formed in the line. These clots

[2] Whole blood samples were digested with Soluene (Packard Instrument Co.) and isopropanol in preweighed vials, bleached with hydrogen peroxide, and counted in acidified Instagel (Packard Instrument Co.). Correction for counting efficiency was made by external standard.

could often be withdrawn before injection of 0.2 ml heparinized saline. Occasionally they had to be reinjected into the animal with the saline.

Preparation of Plasma or Serum Samples—Whole blood samples collected in the described manner were nonhemolyzed. Plasma or serum could be obtained from each blood sample without transfer from the syringe in which it was collected. The syringe itself was centrifuged in an inverted position after provision was made to prevent depression of the plunger by centrifugal force. This was very simply achieved by slipping over the plunger a short piece of beverage tubing whose lumen diameter did not allow passage of the body of the syringe. It was even more simply achieved with syringes of the plastic disposable type by shortening the plunger with a large pair of scissors.

After centrifugation, hematocrit values could be conveniently obtained from the syringe scale before removal of each plasma sample. Plasma or serum was removed either by aspiration from the top or by depression of the plunger (reinsertion of the severed end of the plunger).

Intravenous Injection—The cannula described also was used successfully as a site for intravenous injection, where the injection was followed by 0.2 ml of heparinized saline. This procedure did not influence blood samples later obtained from the same site. Injection of liquids as viscous as dimethyl sulfoxide presented no difficulty, and even pure glycerin could be injected (0.5 ml within 1 min).

DISCUSSION

Cannula implantation techniques appear to have been largely avoided for pharmacokinetic studies in the rat, probably because of elaborate preparation, requisite surgical skill, or unreliability of repeated blood sampling associated with this approach. Because alternative techniques do not offer great control or facility for blood sampling, use of the rat as a pharmacokinetic subject has been more limited than its use elsewhere.

The techniques described here are designed for pharmacokinetic studies and are based on the properties (3, 4) of a silicone polymer cannula rather than polyethylene, which has been used in almost all reported instances of implantation of blood-collecting cannulas in rats. The installation of the silicone cannula is simple and rapid, and its use is convenient and reliable.

The advantages of a one-piece cannula are achieved by utilizing the greater softness and flexibility of silicone polymer tubing to fulfill both internal and external requirements. Laborious cannula manufacture is avoided, since the tubing is used virtually as supplied commercially.

Exteriorization of the cannula by previously described circuitous and difficult routes and the provision of elaborate anchors for the cannula are bypassed since the cannula is sufficiently secured by the firmly tied sutures, which partially embed into the outer wall of the soft tubing. Furthermore, without the rigid curves present in previously described cannulas it is possible to rotate the tubing after it is placed subcutaneously, thereby facilitating its insertion into the vein. Since exposition of the jugular vein and suture after insertion of the cannula are not complex tasks, the simplifications described in both inserting and exteriorizing the tubing render the surgical procedure sufficiently undemanding for untrained personnel to perform.

Use of a clip to regulate flow during frequent sampling is far more convenient and effective than use of plugs and is without the expense, unavailability, and dead volume problems of taps. These advantages, significant for pharmacokinetic work, could not have been realized with polyethylene tubing, which does not recover clamping.

Failure of previous cannulas to allow repeated sampling has arisen from three main areas:

1. Tendency for removal by the subject or operator. Attempted removal of the silicone tubing by the subject has rarely been observed. Less discomfort was a probable consequence of its greater softness and flexibility and also of the direct route of exteriorization. The elasticity of the tubing also allowed a certain degree of tension to be applied to the cannula without detrimental effects.

2. Obstruction of the orifice by intravascular structures (*viz.*, vein wall) under negative pressure. This has not been a problem since use of the bevel design described. With the ability to rotate the tubing, even this more complex bevel could be inserted into the vein without difficulty.

3. Blockage within the cannula. The use of a one-piece cannula has eliminated connecting sites with their tendency to promote blockage and resist flow of viscous fluids. Furthermore, permanent surfaces in contact with blood were solely of silicone polymer, which has been reported to be free of the tendency to effect thrombus formation. Blockage has not been a problem with frequently used cannulas nor with properly maintained, longer term cannulas.

The sampling techniques described have been combined with urine collection procedures (5) and the technique of Light *et al.* (6) for bile collection in the unanesthetized rat. These combinations have enabled the establishment of statistically well-defined pharmacokinetic models for certain antifolate drugs, including a close investigation into the contribution made by enterohepatic cycling.

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Timed-Release Depot for Anticancer Agents

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Abstract □ The timed-release of anticancer agents from composites with poly(lactic acid) was studied in rats. In the case of cyclophosphamide–poly(lactic acid) composites, 67% of the administered dose was released within 34 days. With *cis*-dichlorodiammineplatinum (II), the amount of drug released was only 9.3% within the same period. This difference might be attributed to the different solubilities of these two drugs in the polymer. Electron spectroscopy chemical analysis, a new tool, was used to investigate the extent of diffusion of drugs in polymer films.

Keyphrases □ Cyclophosphamide—timed-release formulation with poly(lactic acid), release characteristics, electron spectra □ *cis*-Dichlorodiammineplatinum (II)—timed-release formulation with poly(lactic acid), release characteristics, electron spectra □ Anticancer agents—poly(lactic acid) timed-release formulations of cyclophosphamide and *cis*-dichlorodiammineplatinum (II) □ Timed-release formulations—cyclophosphamide and *cis*-dichlorodiammineplatinum (II) with poly(lactic acid)

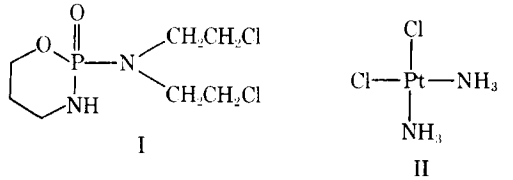
The controlled release of narcotic antagonists from composites with poly(lactic acid) in film and in particle form was reported previously (1, 2). No data have been found on the release of anticancer agents from polymeric matrixes. The present investigation was undertaken to determine in experiments *in vivo* the amounts of two potent anticancer agents, cyclophosphamide[1], 2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (Compound I), and *cis*-dichlorodiammineplatinum[2] (II) (Compound II) (3–5), released from poly(lactic acid) composites.

EXPERIMENTAL

The electron spectra were recorded on an electron spectrometer for chemical analysis[3]. The scan rate was 0.05 ev/sec. The recorder used the following parameters: integral *X–Y*; *X* axis scale, 2 ev/cm; *Y* sensitivities compared on the same basis.

Preparation of Cyclophosphamide (I)–Poly(lactic acid) Composites—A mixture of 0.3680 g of I, 1.3996 g of poly(lactic acid) (1), and 0.0975 g of tributyl citrate was dissolved in 100 ml of methylene chloride. The solution was evaporated to dryness; the residue, wrapped in aluminum foil, was melt-pressed[4] at 170° under a total load of 3 metric tons for 30 sec (shims 0.91 mm thick were used) to produce translucent films of uniform thickness in which no imperfection due to air or gas was observed.

Preparation of *cis*-Dichlorodiammineplatinum (II)–Poly(lactic acid) Composites—Compound II (0.5078 g) was added to a solution of poly(lactic acid) (1) (1.8675 g) and tributyl citrate (0.1257 g) in methylene chloride. The fine suspension was evaporated to dryness, and the residue was pressed as in the preparation of I composites. The films obtained were opaque and of uniform thickness, in which no imperfection due to air or gas was observed.



I

II

[1] Cytoxan, Mead Johnson Laboratories, Evansville, Ind.

[2] Colonial Metal Inc., Elkton, Md.

[3] DuPont 650 electron spectrometer. The authors thank Mr. John Flynn of E. I. DuPont Co. for assistance with and use of the DuPont 650 electron spectrometer.

[4] Carver laboratory press model C.

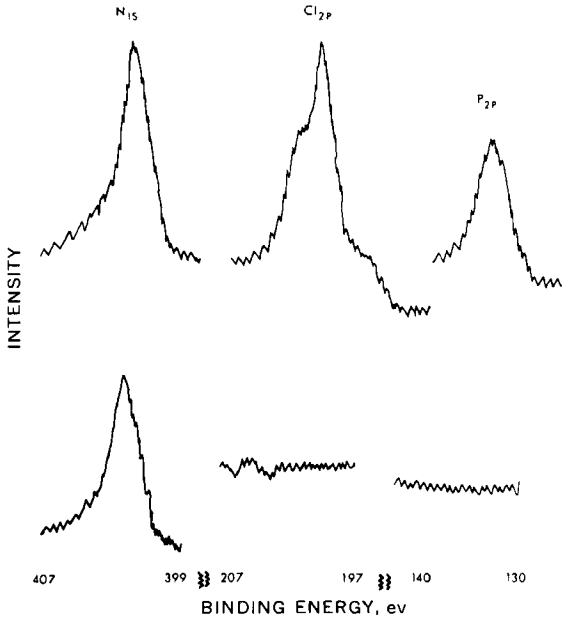


Figure 1—*Electron spectra of a I–poly(lactic acid) composite before implantation (top) and at the end of the experiment (bottom).*

Experiments *In Vivo* with I Composites—These experiments were performed on groups of three male Sprague–Dawley rats following the implantation method previously described (1), using films of 2-cm² size and 120-mg weight (average of three tests) and containing 20% of drug. The amount of I released during the test was determined from the percentages of nitrogen and phosphorous present in the film at the end of the test (N, 0.77%; P, 0.70%) compared with those present in the film before implantation (N, 2.14%; P, 2.37%).

Experiments *In Vivo* with II Composites—These experiments were performed on groups of three male Sprague–Dawley rats by the implantation method previously described (1), using films of 2 cm² and 360-mg weight (average of three experiments) and containing 20% of drug or 47 mg of platinum per rat. The release rate of drug as a function of time was determined by measuring periodically the amounts of platinum excreted in urine during the test. The total amount of drug released from the composite was determined from the percent of platinum found in the film at the end of the test (Pt, 11.8%) compared with that present in the film before implantation (Pt, 13.01%).

RESULTS AND DISCUSSION

As shown by the amounts of nitrogen and phosphorus present in the I–poly(lactic acid) composites before and after the *in vivo* test, an average of 67% of the administered dose is released over 34 days. This result demonstrates the feasibility of a sustained delivery of I from the poly(lactic acid) composite. This feasibility is further substantiated by the electron spectroscopy chemical analysis spectra of the composite recorded before implantation and at the end of the test (Fig. 1). The peaks of chlorine and phosphorus atoms show a considerable decrease in intensity at the end of the test.

Determination of the release rate of I by measuring the amounts

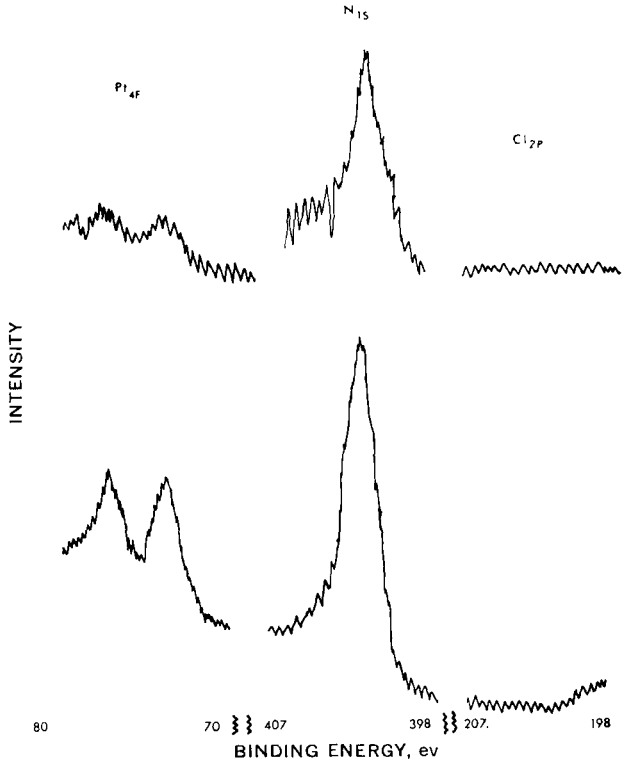


Figure 2—*Electron spectra of II–poly(lactic acid) composite before implantation (top) and at the end of the experiment (bottom).*

of nitrogen and phosphorus excreted in urine was not performed since nitrogen and phosphorus compounds are normally present in urine.

The release rate of II from poly(lactic acid) composites, determined from the amount of platinum in urine, is slow and decreases to a small amount after 18 days from implantation (Table I). The cumulative amount of drug in the urine, expressed as percent of the administered dose, is 3.85; the amount of drug released from the composite during the test, calculated from the amount of drug remaining in the film at the end of the test, is 9.3%. These results indicate that a significant amount of drug unaccounted for either remains sequestered within the animal or is eliminated through ways other than the urine. The presence of large amounts of drug left in the composite at the end of the experiment is also shown by the electron spectroscopy chemical analysis spectra (Fig. 2).

The relatively low amount of platinum compound in the surface

Table I—Amounts of Platinum and Cumulative Percent of Dose Excreted in Urine

Period, days	Milligrams of Platinum in 4 Days	Cumulative Milligrams of Platinum	Cumulative Percent of Dose
0–4	0.88	0.88	1.87
4–8	0.52	1.40	2.97
8–12	0.24	1.65	3.21
12–18	0.06	1.71	3.63
18–26	0.05	1.76	3.74
26–34	0.05	1.81	3.85

of the original film, as shown by the electron energy spectra, is attributed to its very low solubility in the polymer and its low rate of diffusion. But I, being more soluble in the polymer, appeared to a considerable extent in the surface of the original film. These solubility observations are further borne out by the physical appearance of the respective films, that of I being translucent and that of platinum compound being opaque. The electron spectroscopic technique is being studied further for measuring the diffusion rate of drugs in polymer films.

In addition, differences in the release of these two drugs can be attributed to differences in solubilities in water. The solubility of I in water is 40 g/liter (6) and that of II is 2.2 g/liter (7).

These results prove the feasibility of a timed-release system for delivering anticancer agents and suggest that this concept of timed release is of great potential for use in cancer therapy.

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Hydrolysis of Fatty Acid Esters of Acetaminophen in Buffered Pancreatic Lipase Systems I

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Abstract □ A series of fatty acid esters of acetaminophen were prepared beginning with the acetate, the propionate, and all even-numbered fatty acids and going through the octadecanoate. The enzymatic hydrolysis of all derivatives was studied *in vitro* with varying amounts of lipase added to the hydrolysis mixtures. Under the conditions of the *in vitro* hydrolysis, it was observed that all derivatives were hydrolyzed more readily in an aqueous medium at pH 7.8. A positive relationship was seen between the hydrolysis rates and the concentration of lipase at this pH. There was a negative relationship between the chain length of the acyl moiety and the corresponding hydrolysis rates. The short chain esters were hydrolyzed at rates many times more rapid than the long chain esters. The intermediate chain-length esters, *p*-acetamidophenyl decanoate, *p*-acetamidophenyl laurate, and *p*-acetamidophenyl myristate, were hydrolyzed at intermediate time periods extending over 12 hr, approaching completion at 97.5, 87.5, and 80.5%, respectively, when 18 Wilson units of lipase was used in each milliliter of hydrolysis mixture. The longer chain esters, *p*-acetamidophenyl palmitate and *p*-acetamidophenyl stearate, were hydrolyzed to the extent of 16 and 8%, respectively, over 12 hr under the same *in vitro* conditions.

Keyphrases □ Acetaminophen—fatty acid esters, hydrolysis in buffered pancreatic lipase systems □ Esters, acetaminophen, fatty acid—hydrolysis in buffered pancreatic lipase systems □ Hydrolysis—fatty acid esters of acetaminophen, buffered pancreatic lipase systems □ Lipase—enzymatic hydrolysis of fatty acid esters of acetaminophen

The objective of this work was to investigate the feasibility of employing fatty acid ester derivatives of a drug for use as a substrate for GI enzymes to control the release of the free drug from its dosage form. A second objective was to determine if a combination of the esters and appropriate enzymes in a dosage form could be utilized to achieve a uniform and prolonged therapeutic effect from a single dosage unit of the drug without producing an excessive initial plasma concentration. Acetaminophen (*N*-acetyl-*p*-aminophenol, 4-hydroxyacetanilid) was selected as a model drug for this study.

The synthesis of *p*-acetamidophenol acetate, butyrate, hexanoate, and stearate and enzymatic hydrolysis in serum lipase as reported previously (1) provided the basis for the investigation. In the previous study, it was noted that the short chain esters of acetaminophen were hydrolyzed very rapidly while the stearate was hydrolyzed so slowly that no attempt was made to quantitate the results. In view of these findings, it was felt that a complete series of saturated fatty acid esters should be prepared and tested in pancreatic lipase for comparative purposes. Intermediate to the hexanoate and the stearate should be one or more esters that could be hydrolyzed slowly over 10–12-hr in the presence of GI enzymes.

Fletcher *et al.* (2) investigated the absorption of lincomycin and esters of lincomycin from rat intestines. They reported that the 7-butyrate and 7-propionate esters of lincomycin were absorbed more rapidly than the corresponding 2-esters. This difference was attributed to intestinal hydrolysis rates for the two positions on lincomycin in which the 7-esters were hydrolyzed more slowly than the 2-esters. Since both types of esters were absorbed to some extent intact, increased absorption of these soluble intact esters over the lincomycin base was explained on the basis of an increase in lipid solubility.

Glazko *et al.* (3, 4) found that the intact esters of chloramphenicol are poorly absorbed and gave indirect evidence that the rate of absorption of chloramphenicol depends upon the rate of enzymatic hydrolysis. The hydrolysis rates for these esters appeared to depend on particle size, chemical nature of the esters, and the ability of the compound to be wetted.

Contrary to previous reports, they found that some of the more water-soluble esters were hydrolyzed more slowly than water-insoluble esters such as the palmitate if the latter had a sufficiently large surface exposed to enzymatic attack. Suspensions containing very small particles of chloramphenicol palmitate produced excellent blood levels in dogs, while dry crystalline preparations were poorly absorbed. When the esters of chloramphenicol were not readily hydrolyzed *in vitro* by enzymes under optimum conditions, poor absorption resulted regardless of the physical form of the esters.

GI absorption of drugs in particulate form usually results after the drug is in the dissolved state (5). When dissolution is rate limiting, particle size is of great importance to the rate of transfer from the GI tract to ultimate sites of action. In a recent review article about particle size, it was reported that when the solubility of a drug is less than 0.1 mg/ml, the effect of particle size could be an important factor in consideration of physiological availability, while other workers contend a solubility up to 1 mg/ml may be a factor in drug availability (5). Fatty acid esters of acetaminophen are poorly soluble in water, and the GI absorption of acetaminophen should be limited by the amount of enzymes facilitating ester hydrolysis in the intestines and the available surface area in the dosage unit.

EXPERIMENTAL

Reagents—The following were used: acetaminophen[1], acetic anhydride[2], propionic anhydride[3], butyric anhydride[3], hexanoyl

[1] Eastman Organic Chemicals.
[2] J. T. Baker Chemical Co.
[3] Aldrich Chemical Co.

Table I—Acetaminophen Derivatives, Melting Points, Solubilities, and Average Particle Size

Derivative	Melting Point	Solubility, mg/ml	Particle Size, Average Diameter in Microns[a]
Acetaminophen	168–172°	20.00	—
Acetate	153–156°	0.32	30.0
Propionate	130–133°	0.32	11.5
Butyrate	140–143°	0.33	11.0
Hexanoate	107–109°	0.018	32.0
Octanoate	103–105°	0.010	8.7
Decanoate	107–109°	0.009	7.3
Laurate (dodecanoate)	111–113°	0.006	4.3
Myristate (tetradecanoate)	114–116°	0.006	2.7
Palmitate (hexadecanoate)	117–118°	0.005	2.7
Stearate (octadecanoate)	117–118°	0.005	2.1

[a] As determined by the Fisher subsieve sizer.

chloride[1], octanoyl chloride[1], decanoyl chloride[4], dodecanoyl chloride[1], tetradecanoyl chloride[1], hexadecanoyl chloride[1], octadecanoyl chloride[1], and lipase[5].

Instruments—The necessary equipment included a submersion rotator[6], a subsieve sizer[7], and a spectrophotometer[8].

Preparation of Acetaminophen Esters—A general synthetic procedure was followed in the preparation of the derivatives of acetaminophen (5) and involved the heating with stirring for 24 hr of the required acid anhydride or acyl halide with acetaminophen in anhydrous pyridine. The derivatives were precipitated from cold water, washed in a 5% solution of sodium carbonate, and recrystallized from *n*-propyl alcohol and water except in the case of *p*-acetamidophenyl propionate where methanol and water were used. The preparation of the esters of acetaminophen from the acid anhydrides using excess amounts of acetyl, propionyl, and butyryl anhydrides presented no problem in the isolation of the products.

Fatty acid chlorides and acetaminophen were used in approximately equimolar quantities in the synthesis of the larger analogs of this series of derivatives. The products were all white, crystalline powders except the acetate and stearate, which were off-white powders. The acetaminophen derivatives along with their melting points, solubilities, and average particle sizes are shown in Table I.

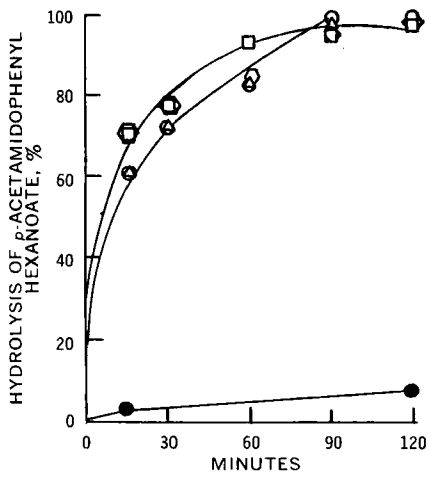

Figure 1—*Percent hydrolysis of* p-*acetamidophenyl hexanoate with varied amounts of lipase and calcium chloride in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities): ○, 18 units/ml; □, 9 units/ml; △, 4.5 units/ml; ○, 2.25 units/ml; and ●, control, 0 units/ml.*

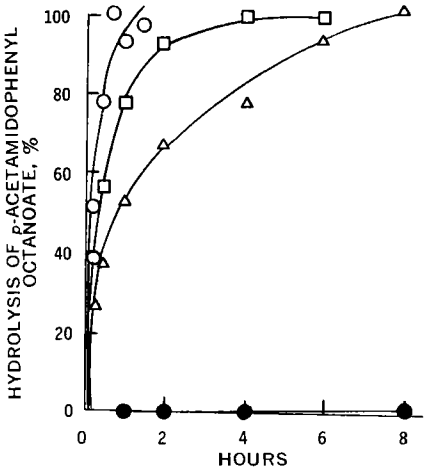

Figure 2—*Percent hydrolysis of* p-*acetamidophenyl octanoate with varied amounts of lipase and calcium chloride in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities): ○, 9 units/ml; □, 4.5 units/ml; △, 2.25 units/ml; and ●, control, 0 units/ml.*

Solubilities—The solubilities of the acetaminophen derivatives were determined by placing an excess of each derivative in water at 25°. Test tubes containing the derivative and distilled water were agitated with the submersion rotator for 6 hr. The clear filtrates obtained by filtration through filter paper[9] were diluted successively until absorption could be determined spectrophotometrically. Dilutions were unnecessary with the larger esters because the saturated solutions beginning with the octanoate show very low absorbance.

The results of these determinations are recorded in Table I. The acetate, propionate, and butyrate derivatives, according to official description, were classified as "very slightly soluble," while the remaining derivatives were "insoluble" (7).

Particle Size—Prior to *in vitro* and *in vivo* testing, all derivatives were passed through a 120-mesh wire cloth sieve. Further classification of particle size was accomplished by the determination[7] of average particle diameters for each derivative.

Lipase Activity—Commercial preparations of pancreatic lipase were assayed for the hydrolytic activity with olive oil as the substrate by the method of Lazo-Wasem (8). All references to lipase activity in this study were based on the Wilson unit per milligram. The lipase used in the *in vitro* hydrolysis studies had an activity of 2.96 Wilson units (±0.069 *SE*).

Stability of Esters in Simulated Gastric Fluid—To establish the stability of the derivatives under simulated gastric conditions, two representative esters were selected: one short chain fatty acid derivative and one long chain fatty acid derivative. Separately, 100 μmoles of *p*-acetamidophenyl acetate and *p*-acetamidophenyl decanoate was digested in 10 ml of simulated gastric fluid USP (pH 1.35), maintained at 37°, agitated, and titrated with 0.0499 *N* sodium hydroxide solution on a recording pH meter for 4 hr. No appreciable hydrolysis in either sample (less than 5% hydrolysis) was noted.

Preparation of Hydrolysis Formulation—An accurately weighed sample of ester and enzyme was triturated in a cold mortar. Individual weighings were made from each batch to contain 100 μmoles of the ester, which was equivalent to 15.1 mg of acetaminophen. The required ester and enzyme were transferred to dry Pyrex test tubes (15-ml capacity). A 0.5-ml aliquot of a 0.2 *M* solution of calcium chloride was added to, and mixed with, the powder prior to the addition of 9.5 ml of a 0.1 *M* phosphate buffer solution, also containing 0.1 *M* sodium chloride. The pH of the buffer

[4] Matheson, Coleman & Bell Co.
[5] Reheis Chemical Co.
[6] Scientific Industries, Inc.
[7] Model 95, Fisher Scientific Co.
[8] Beckman DU model 2400, Beckman Instruments, Inc.
[9] Whatman No. 1.

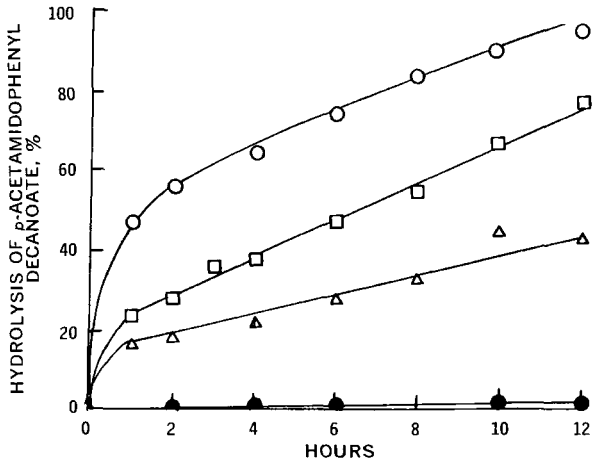

Figure 3—*Percent hydrolysis of* p-*acetamidophenyl decanoate with calcium chloride and varied amounts of lipase in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities):* ○, *18 units/ml;* □, *9 units/ml;* △, *4.5 units/ml; and* ●, *control, 0 units/ml.*

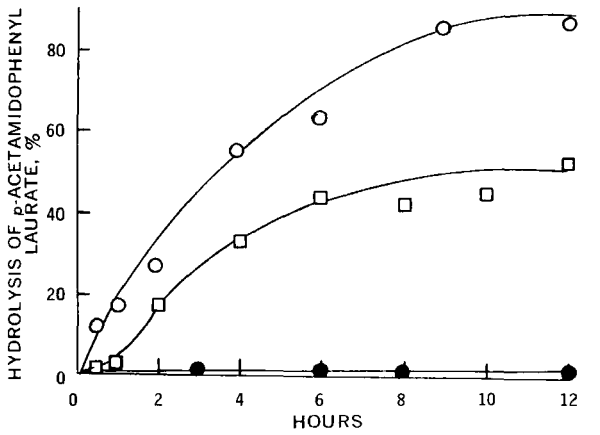

Figure 4—*Percent hydrolysis of* p-*acetamidophenyl laurate with calcium chloride and varied amounts of lipase in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities):* ○, *18 units/ml;* □, *9 units/ml; and* ●, *control, 0 units/ml.*

solution was adjusted to accommodate the addition of calcium chloride with a resultant pH of 7.8.

Each tube was stoppered and placed on a submersion rotator. The submersion rotator was placed in a constant-temperature bath maintained at 37° and rotated at a maximum speed of 30 cpm. At timed intervals, one or more of the tubes were removed from the bath and filtered through filter paper[9]. A 0.1-ml sample of the clear filtrate was diluted to 10 ml with distilled water, and the absorbance was determined against a reagent blank at 245 nm.

At 100% hydrolysis, each dilution contained 15.1 μg/ml of acetaminophen. The derivatives beginning with the hexanoate through the stearate were assayed by Procedure B (see *Analytical*), which takes advantage of a great difference in the solubility of acetaminophen as compared with the ester solubility and the dilution factor of 1:100 employed.

Analytical—The acetate, propionate, and butyrate derivatives are sufficiently soluble to require a different assay approach from the longer carbon derivatives. Acetaminophen absorbs more strongly at 280 nm than do the ester derivatives. The assay was accomplished by using an equimolar quantity of each derivative in the reagent blank containing an inactive enzyme, and the extent of hydrolysis was determined by the increase in absorbance as compared with a standard mixture of the ester and acetaminophen.

Procedure A: Determination of Acetaminophen in Presence of Acetaminophen Derivatives—Standard solutions containing 75.5 μg/ml (0.5 μmole/ml) of acetaminophen and 0.5 μmole/ml of the short chain esters (*p*-acetamidophenyl acetate, propionate, and butyrate equivalent to 75.5 μg/ml of acetaminophen) were prepared in the *in vitro* reagents. The esters of acetaminophen do not absorb strongly at 280 nm, but acetaminophen absorbs strongly at this wavelength.

In the determination of percent hydrolysis in the *in vitro* experiments, Procedure B was followed except that a 0.50-ml sample was used instead of a 0.10-ml sample and the dilutions were read at 280 nm against a reagent blank containing the ester.

Procedure B: Determination of Acetaminophen in In Vitro *Hydrolysis of Long Chain Acetaminophen Derivatives*—A 30.2-mg sample of acetaminophen, accurately weighed, was transferred to a 100-ml volumetric flask, and the flask was diluted to volume with 0.1 *M* phosphate buffer, pH 7.8. Aliquots of 1.0, 0.5, 0.4, 0.3, 0.2, 0.1, and 0.05 ml of the standard solution were diluted to 1 ml with the phosphate buffer and diluted to 10.0 ml with distilled water. The absorbance of each sample against a reagent blank was measured at 245 nm.

RESULTS AND DISCUSSION

All melting points are consistent with those reported for *p*-acetamidophenyl acetate, butyrate, hexanoate, and stearate (1). The melting point for *p*-acetamidophenyl propionate prepared in this laboratory was slightly higher (about 4°) than that previously reported (9). The melting points for this series decrease from about 154° for the acetate to a minimum of 104° for the octanoate derivative and from the octanoate increase with fatty acid chain length to 118° for the stearate.

The melting point observed for the propionate derivative (132°) suggests an oscillating series similar to that reported for the fatty acid ester derivatives of salicylic acid, in which the fatty acid moieties with the even number of carbon atoms exhibited higher melting points in a series of compounds than the odd numbered in the same series (10).

Hofstee (10) recognized that an assay for crude lipase, using olive oil as the substrate, would not serve as a comparative standard for all substrates. The purpose of the assay here was to establish the quality of the various preparations and to standardize the activity in terms of Wilson units in the *in vitro* studies. The assay was especially useful in following the stability of the enzyme preparations during the investigation.

The release of acetaminophen in this study depends solely upon the hydrolysis of each derivative. The rate of hydrolysis of all derivatives was enhanced by the presence of pancreatic lipase as seen in the *in vitro* hydrolysis studies. Any loss of enzyme activity would seriously alter the outcome of an experiment, and esters of acetaminophen are hydrolyzed very slowly under gastric conditions.

The results obtained by Procedures A and B are illustrated in Figs. 1–7 except for *p*-acetamidophenyl acetate, *p*-acetamidophenyl propionate, and *p*-acetamidophenyl butyrate, which were hydrolyzed so rapidly as to be almost complete in 15 min or less.

p-Acetamidophenyl hexanoate was hydrolyzed more rapidly than the corresponding octanoate ester. Both esters were hydrolyzed less rapidly than the short chain esters under the same hy-

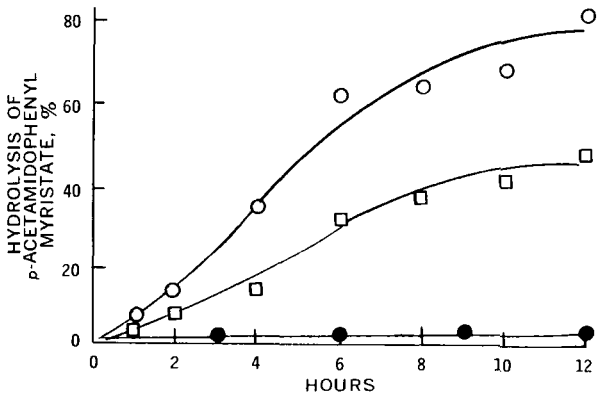

Figure 5—*Percent hydrolysis of* p-*acetamidophenyl myristate with calcium chloride and varied amounts of lipase in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities):* ○, *18 units/ml;* □, *9 units/ml; and* ●, *control, 0 units/ml.*

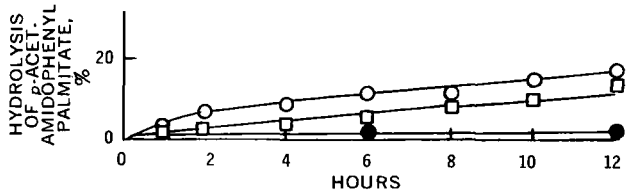

Figure 6—*Percent hydrolysis of* p-*acetamidophenyl palmitate with calcium chloride and varied amounts of lipase in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities): ○, 18 units/ml; □, 9 units/ml; and ●, control, 0 units/ml.*

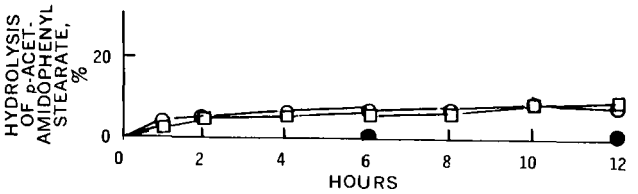

Figure 7—*Percent hydrolysis of* p-*acetamidophenyl stearate with calcium chloride and varied amounts of lipase in a phosphate buffer, pH 7.8, at 37°. Key (lipase activities): ○, 18 units/ml; □, 9 units/ml; and ●, control, 0 units/ml.*

drolysis conditions. None of the short chain esters through the octanoate ester appears to saturate the enzymes available in the hydrolysis system for any of the hydrolysis formulas shown in Figs. 1 and 2.

When the substrate concentration is in excess of the amount required to attain maximal velocity, the reaction proceeds at a zero-order rate; however, if the concentration is less than a saturation concentration, the rate of reaction becomes dependent upon the concentration of substrate and the reaction proceeds at a first-order rate (11). This appears to be the case with the short chain derivatives under these conditions.

In general, as the chain length in the fatty acid moiety was increased, a decrease in the hydrolysis rate was noted. The effects of varied lipase concentration are demonstrated in Figs. 3–5, showing the percent hydrolysis of *p*-acetamidophenyl decanoate, laurate, and myristate, respectively. A marked decrease in the rate of hydrolysis was noted for all three derivatives as the concentration of enzyme was decreased. The short chain esters were hydrolyzed rapidly at all enzyme concentrations. The long chain esters, *p*-acetamidophenyl palmitate and stearate, were hydrolyzed very slowly at the two enzyme concentrations studied.

Some difference was noted in Fig. 6 for *p*-acetamidophenyl palmitate but not to the same extent as observed for the derivatives of intermediate chain length. An insignificant difference was seen for *p*-acetamidophenyl stearate (Fig. 7). Both were hydrolyzed less than 10% in 12 hr. Since one purpose of the study was to find a derivative that would be hydrolyzed slowly and completely over a 12-hr period, it was apparent that the derivatives and some of the *in vitro* conditions illustrated in Figs. 3–5 for *p*-acetamidophenyl decanoate, laurate, and myristate met this specification.

Formulas of *p*-acetamidophenyl palmitate and stearate in Figs. 6 and 7 released acetaminophen at a rate lower than the therapeutic level. The short chain derivatives, *p*-acetamidophenyl acetate, propionate, and butyrate were hydrolyzed so rapidly that it was doubtful if the rate of hydrolysis would be a limiting step in their absorption from the GI tract if compared with acetaminophen.

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Fluorometric Assay of Thioguanine

JOE M. FINKEL

Abstract □ A spectrophotofluorometric method is described for the assay of thioguanine. The assay involves the oxidation of thioguanine with potassium permanganate to a fluorescent product. The method is suitable for routine quality control in laboratory preparations of thioguanine.

Keyphrases □ Thioguanine—spectrophotofluorometric assay □ Spectrophotofluorometry—determination, thioguanine

Basic quantitative data concerning drug dosage are required to obtain effective chemotherapy against tumor cells. Very sensitive and specific assays are needed to determine the anticipated low concentrations of anticancer drugs and their metabolites in various biological systems. Fluorometry, which affords the required sensitivity and specificity, has been used in this laboratory for such analytical determinations (1–5).

As part of an integrated program to investigate the pharmacokinetics and physiological disposition of antileukemic drugs, this laboratory became concerned with thioguanine (2-aminopurine-6-thiol), which inhibits a wide spectrum of neoplasms (6–9). In the initial studies, a quality control method was required for thioguanine injection solutions. Also, the method had to be adaptable for the assay of thioguanine in serum.

A literature survey revealed a limited number of applicable assays for thioguanine. One method employed thioguanine labeled with ^{35}S (10), whereas other assays used ^{14}C (7, 9). Although tracer methods generally extend the limits of sensitivity of an assay, they often lack specificity without prior separation of the drug from labeled metabolites. Pittillo and Woolley (11) reported a microbiological assay for thioguanine that had a lower limit of sensitivity of 1.0 μg/ml and required 24-hr preincubation and 16-hr incubation periods.

This report describes a simple and sensitive fluorometric assay for thioguanine standards which are oxidized with alkaline potassium permanganate solution to a fluorescent product. Fluorescence emission is measured at 410 nm with an excitation wavelength of 330 nm.

EXPERIMENTAL

Instrumentation—Fluorescence measurements were made with a spectrophotofluorometer[1] and an x–y recorder. The following instrument settings were used for all measurements: slit arrangement, No. 4; photomultiplier shutter, 4 mm; and sensitivity control, 40. Stainless steel mirrors were inserted in the cell compartment to increase the amount of radiation reaching the photomultiplier tube.

UV absorption measurements were made with a spectrophotometer[2].

Reagents—Combustion analyses for thioguanine[3] showed 35.9% carbon, 3.0% hydrogen, and 42.2% nitrogen. Calculated values were 35.9% carbon, 3.0% hydrogen, and 41.9% nitrogen. The UV absorption spectrum at pH 1 gave λ_{max} 257 (ϵ_m 8120) and 347 (ϵ_m 20,425) nm. These data agreed with previously reported values (12).

Thioguanine standards in water were prepared in a concentration range of 0.003–1.0 μg/ml from a 10-μg/ml stock solution. A drop of 6 N NaOH was added to 100 ml of the stock solution to dissolve the thioguanine.

Carbonate–bicarbonate buffer (pH 10.1) was prepared by the addition of 30.0 ml of 0.2 M sodium carbonate solution to 20.0 ml of 0.2 M sodium bicarbonate solution.

Potassium permanganate, 0.24% (w/v), and 30% hydrogen peroxide were also used.

Analytical Procedures—To 0.5 ml of pH 10.1 carbonate–bicarbonate buffer in a centrifuge tube was added 0.5 ml of thioguanine standard solution. After the buffered solution was stirred, 0.5 ml of 0.24% potassium permanganate was added to initiate oxidation of the drug. After 10 min of oxidation, the excess permanganate was reduced by the addition of 1 drop of 30% hydrogen peroxide. The resulting mixture was stirred thoroughly and centrifuged at 1640×g for 10 min. The clear, colorless supernate was measured fluorometrically at 410 nm when excited at 330 nm.

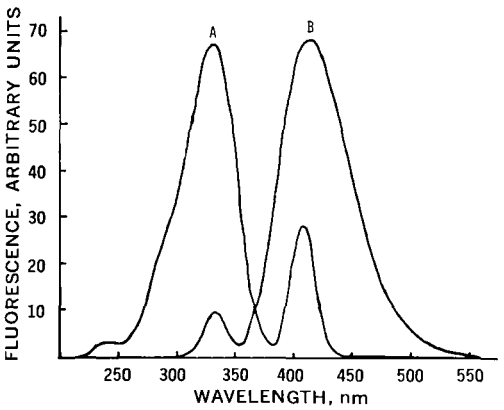

Figure 1—*Excitation spectrum (A) and fluorescence spectrum (B) of oxidized thioguanine (1.0 μg/ml).*

RESULTS AND DISCUSSION

Fluorescence Studies—The assay for thioguanine reported here is a modification of a fluorometric assay for mercaptopurine (1). Both compounds are susceptible to similar chemical changes. Since thioguanine does not have a high quantum yield of fluorescence, the drug was oxidized with alkaline potassium permanganate to a fluorescent product. The oxidation product was identified as 2-aminopurine-6-sulfonate.

[1] Aminco-Bowman.

[2] Cary model 17.

[3] Supplied by Dr. J. A. Montgomery, Organic Department, Southern Research Institute. All other purine derivatives used in this work were supplied by Dr. R. F. Pittillo, Microbiology Division, Southern Research Institute.

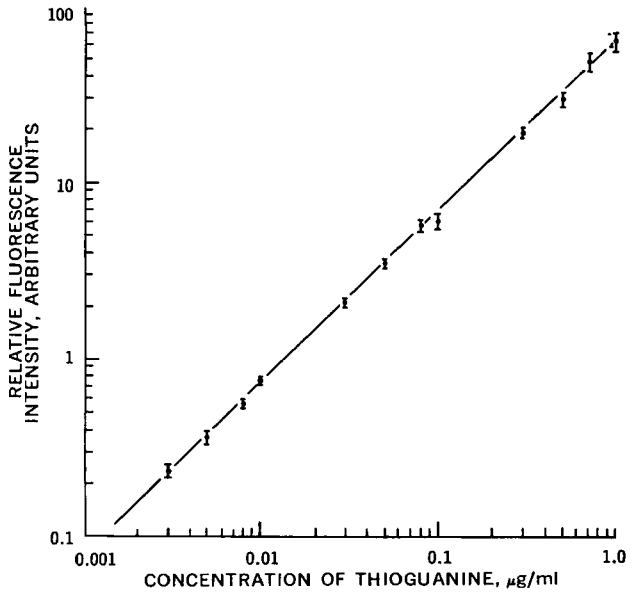

Figure 2—*Relative fluorescence intensities of thioguanine standards after potassium permanganate oxidation.*

The oxidations of thioguanine and mercaptopurine and their corresponding sulfinates with alkaline potassium permanganate to sulfonates were reported elsewhere (1, 13). Figure 1 shows the excitation and fluorescence spectra of thioguanine after oxidation. The maximum excitation occurs at 330 nm, and with that excitation wavelength the maximum fluorescence occurs at 410 nm.

The relative fluorescence intensity is linear with the concentration of thioguanine in water for the three concentration ranges examined (Fig. 2). The values plotted on the abscissa are concentrations of the thioguanine standards submitted to oxidation and do not include the 1 to 3 dilution of the 0.5-ml standard solution with buffer and permanganate.

Sensitivity—The lower limit of detection of thioguanine with the fluorometric assay is 0.003 μg/ml, whereas the upper limit is 1.0 μg/ml. Samples expected to have high drug concentration must be diluted. The average background relative fluorescence intensity of a water blank was 0.13 ± 0.02 arbitrary unit.

Reproducibility—Twenty samples containing 0.8 μg/ml of thioguanine were assayed by the fluorometric method. A set of 10 samples was measured in the morning, and a second set of 10 samples was determined in the afternoon. The mean value and standard deviation of the combined sets were 0.87 ± 0.07 μg/ml. The value of the afternoon samples was 0.84 ± 0.01 μg/ml, which indicates a better reproducibility as familiarity with the method and skills improved.

Interferences—To determine the specificity of the fluorometric assay for thioguanine, the fluorescence spectra of some related purine derivatives were examined. Table I presents the relative fluorescence intensity at 410 nm for each derivative oxidized and excited at 330 nm. Because of high fluorescence emission, two of the compounds were diluted to 1.0 μg/ml but all other purine derivatives were measured at a concentration of 10 μg/ml. Only one sample listed in Table I was not oxidized so as to observe differences of fluorescence intensities between unoxidized and oxidized thioguanine.

Table I—Relative Fluorescence Intensities of Purine Derivatives after Oxidation with Potassium Permanganate

Compound	Concentration, μg/ml	Relative Fluorescence Intensity
Thioguanine (unoxidized)	10	0.53
Thioguanine (oxidized)	1	62.49
6-Thioguanosine	1	49.20
Guanine	10	0.03
Xanthine	10	0.52
6-Thioxanthine	10	72.40
6-Thiouric acid	10	0.34

Although thioguanine did not strongly fluoresce, oxidation of the drug gave a product with over a 1000-fold increase in fluorescence intensity. Guanine, which serves as a starting material in the synthesis of thioguanine, produced the lowest relative fluorescence intensity and, thus, cannot interfere with this fluorometric assay. Only thioguanosine and 6-thioxanthine showed enough fluorescence to interfere; however, the presence of these two compounds in a thioguanine standard preparation is unlikely.

Although this fluorometric assay is presented for monitoring laboratory preparations of thioguanine, the assay with modifications is applicable to biological samples. Studies concerned with the measurement of thioguanine serum levels in mice, dogs, and monkeys were conducted in these laboratories. The *in vivo* studies will be reported.

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High-Pressure Liquid Chromatographic Determination of Chlorpropamide in Tablet Formulations

D. MOLINS, C. K. WONG, D. M. COHEN, and K. P. MUNNELLY ˣ

Abstract □ A method was developed for the quantitative determination of chlorpropamide in tablet formulations by high-pressure liquid chromatography after homogenization of the sample with methanol.

Keyphrases □ Chlorpropamide tablets—high-pressure liquid chromatographic analysis after homogenization with methanol □ High-pressure liquid chromatography—analysis, chlorpropamide tablets

Chlorpropamide is an oral hypoglycemic agent of the sulfonylurea class. It is indicated in uncomplicated diabetes mellitus of the stable, mild, or moderately severe, nonketotic, maturity-onset type that cannot be completely controlled by diet alone. Various methods for the determination of chlorpropamide have been reported, including GLC (1), TLC (2, 3), titrimetry (4, 5), and spectrophotometry (5–9). However, these methods do not have the rapidity, simplicity, and sensitivity found in high-pressure liquid chromatographic (HPLC) methodology.

Recently, a quantitative HPLC method for sulfonylureas involving reverse-phase chromatography was published (10). A simple, direct, and extremely rapid HPLC procedure for the quantitation of chlorpropamide in tablet formulations after homogenization of the sample with methanol is the subject of this report.

EXPERIMENTAL

Equipment—A liquid chromatograph[1], operated at ambient temperature and equipped with a UV detector for monitoring the column effluent at 254 nm, was used. The column was 1-m × 2.1-mm (i.d.) stainless steel tubing, dry packed with silica gel[2] (140–200 mesh) obtained by fractionation through U.S. standard sieves[3]. A variable-speed homogenizer[4] equipped with a 250-ml cup was also used.

Reagents—USP chlorpropamide reference standard was dried at 60° for 2 hr before use. Bis(dodecyl) phthalate[5], practical grade, was the internal standard. Analytical reagent grade methanol[6] was the mobile phase.

Preparation of Standard Solution—Dissolve approximately 300 mg of bis(dodecyl) phthalate in 300 ml of methanol. Accurately weigh approximately 200 mg of chlorpropamide standard, transfer quantitatively to a 100-ml volumetric flask, and dissolve in and dilute to volume with methanol. Pipet 5 ml of the bis(dodecyl) phthalate solution and 5 ml of the chlorpropamide standard solution into a 50-ml volumetric flask and dilute to volume with methanol.

Preparation of Sample Solution—Determine the average tablet weight of 20 tablets. Pulverize the tablets, weigh an amount equivalent to 250 mg of chlorpropamide, and transfer the powder to a 250-ml homogenizer cup. Pipet 100 ml of methanol into the cup and homogenize for 3 min at maximum speed in an ice bath to dissolve the chlorpropamide completely. Centrifuge the suspension, pipet 5 ml of the supernate and 5 ml of the bis(dodecyl) phthalate solution into a 50-ml volumetric flask, and dilute to volume with methanol.

Chromatography—Condition the column for 24 hr with the mobile phase at a flow rate of 0.5 ml/min. This procedure is necessary for newly packed columns; conditioning is not required for previously used columns. Inject 3 μl of the standard solution and adjust either the pressure or flow rate so that the chlorpropamide

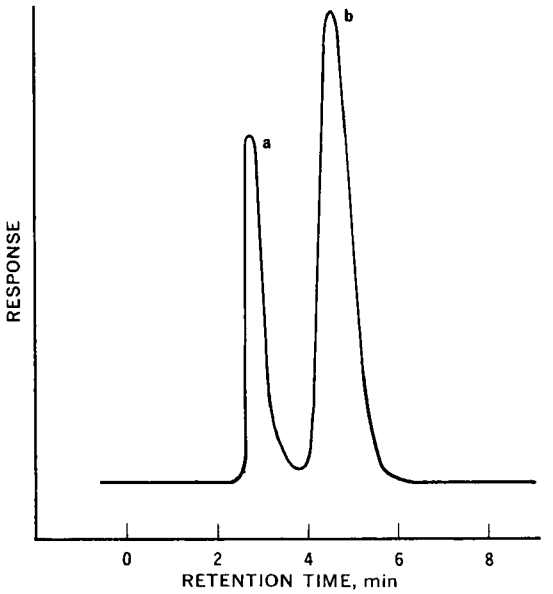

Figure 1—*Representative chromatogram. Key: a, bis(dodecyl) phthalate; and b, chlorpropamide.*

Table I—Percent Recovery[a] of Chlorpropamide from Spiked Placebos

Day	Weight Number	Injection 1	Injection 2
1	1	101.3	102.1
	2	101.1	98.9
	3	98.4	99.6
	4	98.7	101.3
2	1	100.8	98.7
	2	100.0	100.8
	3	100.4	98.4
	4	99.2	99.2
3	1	98.7	100.4
	2	101.4	102.7
	3	100.4	98.4
	4	100.4	99.2
4	1	99.6	101.3
	2	96.6	100.4
	3	100.4	98.8
	4	99.2	100.0

[a] Overall average recovery = 99.9%.

[1] DuPont 820.
[2] Silica gel 60 (0.063–0.200 mm), Brinkmann Instruments, Westbury, N.Y.
[3] Newark Wire Cloth Co., Newark, N.J.
[4] Sorvall Omni-Mixer.
[5] Eastman Kodak.
[6] Mallinckrodt.

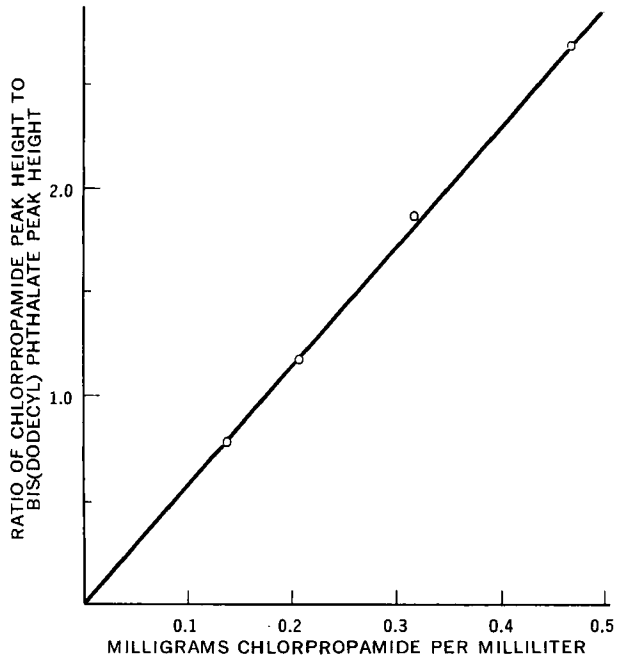

Figure 2—*Relationship of varying amounts of chlorpropamide to a constant bis(dodecyl) phthalate concentration.*

exhibits a retention time of approximately 5 min. Repeat if necessary.

Inject 3 μl of the standard solution followed by two 3-μl injections of the sample solution and one 3-μl injection of the standard solution, allowing sufficient time between injections for development of the chromatograms. The peak heights obtained are used for the calculations.

Calculations—Calculate the quantity of the chlorpropamide in milligrams per tablet according to the following formula:

$$\text{mg/tablet} = \frac{R_{spl} \times \text{standard weight (mg)} \times \text{average weight (mg)}}{R_{std} \times \text{sample weight (mg)}} \quad \text{(Eq. 1)}$$

where R_{spl} is the average ratio of the chlorpropamide peak height to the bis(dodecyl) phthalate peak height in the sample solution injection, and R_{std} is the average ratio of the chlorpropamide peak height to the bis(dodecyl) phthalate peak height in the standard solution before and after the sample injections.

RESULTS AND DISCUSSION

A typical chromatogram of chlorpropamide and bis(dodecyl) phthalate is shown in Fig. 1. Figure 2 shows the linear relationship of varying amounts of chlorpropamide to a constant amount of bis(dodecyl) phthalate.

The accuracy and precision of the method were tested by the following experiment. Four weights of a placebo blend in which known quantities of chlorpropamide had been added were assayed per day for 4 consecutive days. The average percent recovery (Table I) was 99.9 with 95% confidence limits of 99.4–100.4. The estimates of precision (Table II) were obtained using the analysis of variance statistical technique. Ninety-five percent of individual results will not vary from each other (*i.e.*, from the mean) by more than ±2.7%. The standard error for the average of two injections per sample was ±0.9%.

The proposed HPLC method was compared to a UV spectroscopic assay (6) and to a modification of a TLC method (2); the latter utilizes silica gel GF plates and chloroform–acetone–butanol–water (90:95:5:2.5) as the developing solvent. The results obtained (Table III) by the proposed method were in good agreement with the UV and TLC assays. All samples were within the requirements of USP XVIII for chlorpropamide tablets by the HPLC method.

Table II—Estimates of Precision for Determination of Chlorpropamide in Tablet Formulations

Day	Number of Weights per Day	Number of Injections per Weight	Estimates of Precision[a], %
1	1	1	±2.7
1	1	2	±1.9
1	2	2	±1.4
1	3	2	±1.1
1	4	2	±1.0
Injection to injection within a weight on a day			±2.7

[a] Ninety-five percent of the individual results or averages of 2, 3, 6, or 12 results will not vary from each other by more than the percentages quoted. These estimates include variability due to days, weights, and injections. The estimate of precision for injection to injection within a weight on a day excludes variability due to days and weights.

Table III—Comparison of HPLC, UV, and TLC Methods for Determination of Chlorpropamide in Tablet Formulations[a]

Sample	HPLC	UV	TLC
1	245	264	256
2	255	259	251
3	256	259	249
4	249	255	246
5	250	249	263
6	249	257	257
7	252	259	249
8	258	256	244
Average	252	257	252

[a] All results are reported as milligrams per tablet based on a composite sample of five tablets.

Concerning the behavior of the silica gel column, the retention times increased with use time and the column lost its efficiency after 2–3 weeks of continuous use. However, excellent reproducibility was obtained from column to column. A possible explanation of this behavior is that separation is accomplished by both adsorption and partition chromatography and is, therefore, dependent on the moisture content or activity of the silica gel.

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Colorimetric Analysis of Hexachlorophene in Topical Formulations

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Abstract □ The commonly used 4-aminoantipyrine dye formation procedure for hexachlorophene analysis in topical formulations was modified to overcome interference due to other components. Bar soaps and nonemulsion formulations are analyzed directly, employing a chloroform back-extraction stage of the dye prior to quantitation. Hexachlorophene in emulsions and liquid soaps is determined using a TLC separation prior to dye formation.

Keyphrases □ Hexachlorophene—colorimetric analysis in topical formulations, modified 4-aminoantipyrine dye formation method, elimination of interferences □ 4-Aminoantipyrine—dye formation for hexachlorophene colorimetric determination, modifications to overcome interferences □ Colorimetry—analysis, hexachlorophene in topical formulations, 4-aminoantipyrine dye formation, modified to eliminate interferences

Direct UV spectrophotometry (1, 2), absorbance measurements after sample cleanup (3, 4), NMR spectroscopy (5), GLC (6), and, more recently, high speed liquid chromatography (7) have been used to quantitate hexachlorophene (I) in topical formulations. However, the colorimetric determination of the dye formed when I reacts with 4-aminoantipyrine (II) (8–10) is the method most commonly used for analyzing such preparations (11–13).

The 4-aminoantipyrine procedure for analysis of I gives rise to difficulties due to instability of the dye formed (11, 14) and to interference from surfactants present in preparations (15). This report describes a modified II method for the analysis of I which overcomes many of these difficulties. The TLC procedures also described allow the application of the colorimetric method to emulsions.

EXPERIMENTAL[1]

Weak Ammonia Buffer (11)—Dissolve 67.5 g of ammonium chloride in 570 ml of concentrated ammonium hydroxide, dilute to 1 liter with distilled water, and mix. Dilute 2.0 ml of this solution to 1 liter with distilled water to afford the required weak ammonia buffer.

General Procedure (A)—*4-Aminoantipyrine Solution*—Prepare a 0.5% (w/v) solution of II in distilled water.

Potassium Ferricyanide Solution—Prepare a 2.0% (w/v) solution of potassium ferricyanide in distilled water.

Hexachlorophene Standards—Dissolve about 25 mg of reference standard quality I, accurately weighed, in 100.0 ml of dimethylformamide and mix. To individual 25-ml volumetric flasks, add, respectively, 2.0, 3.0, 4.0, and 5.0 ml of this solution and dilute to volume with dimethylformamide.

Sample Preparation—Transfer an accurately weighed aliquot of powdered bar soap, toothpaste, or dusting powder equivalent to about 0.55 mg of I to a centrifuge tube. Add 15.0 ml of dimethylformamide and mix thoroughly for 45 min using a mechanical shaker. Centrifuge or filter the solution to remove insoluble material.

Color Development and Hexachlorophene Determination—Transfer 1.0 ml of solution containing standard or sample to a 25-ml volumetric flask, add 20 ml of weak ammonia buffer and 1.0 ml of II reagent, and mix. Add 1.0 ml of potassium ferricyanide reagent, immediately dilute to volume with weak ammonia buffer, and again mix. Immediately transfer 15.0 ml of this solution to a 60-ml separator containing about 6 g of sodium chloride. Add 7.0 ml of chloroform and shake for 1–2 min. Allow layers to separate and collect most of the chloroform layer.

Filter the chloroform solution through a filter[2] into a 1.0-cm spectrophotometer cell. Measure the absorbance of the sample solution at 483 nm against a blank prepared in an identical manner but substitute 1.0 ml of dimethylformamide for solutions containing I.

Prepare a calibration curve plotting absorbances of standard solutions against micrograms per milliliter of I in the final chloroform solution.

Calculations—Calculate the percent of I using:

$$\% \text{ w/v I} = \frac{Cs \times 175}{Ws \times 10} \qquad \text{(Eq. 1)}$$

where Cs = micrograms per milliliter of I from the graph, and Ws = weight of sample taken in milligrams.

Chromatographic Method (B)—*Buffered 4-Aminoantipyrine Solution*—Prepare a 0.5% (w/v) solution of II in weak ammonia buffer.

Buffered Potassium Ferricyanide Solution—Prepare a 2.0% (w/v) solution of potassium ferricyanide in weak ammonia buffer.

Hexachlorophene Standards—Dissolve about 25 mg of reference standard quality I, accurately weighed, in ethanol and dilute to 25.0 ml.

Sample Preparation—Transfer to a centrifuge tube an accurately weighed aliquot of emulsion, solution, or other sample equivalent to about 10 mg of I. Add 10.0 ml of ethanol and mix thoroughly for 45 min, employing a mechanical shaker. If necessary, centrifuge or filter the resultant solution to remove insoluble material.

TLC and Isolation Procedure—Use self-prepared or precoated fluorescent layers of silica gel, 250 μm thick.

On a line 1 cm from one edge of the silica gel layer, apply duplicate spots using 10.0, 15.0, and 20.0 μl of standard solutions of I, alternating such spots with three others prepared with 15.0 μl of sample solution. Develop the plate for 15 cm in a paper-lined chamber, using ethyl acetate–methanol (9:1) (4) as the solvent.

Air dry the plate and examine it under shortwave UV light. Delineate I spots and collect silica gel from individual spot areas using a suitable apparatus (16). Isolate I by washing the silica gel with 6 ml of ethanol, collecting the solvent in a suitable tube or flask. Evaporate the ethanol to dryness.

Color Development and Hexachlorophene Determination—Carefully dissolve the residue obtained from silica gel extraction in 0.5 ml of ethanol and add 13.0 ml of weak ammonia buffer and 1.0 ml of buffered II solution. Mix, add 1.0 ml of buffered potassium ferricyanide solution, and again mix. To the resulting solution, immediately add about 6 g of sodium chloride and 7.0 ml of chloroform and shake vigorously for 1–2 min. Allow the layers to separate.

Using a 10-ml syringe and suitable needle, draw off the chloroform layer without delay. Pass this solution through a filter[2] into a 1.0-cm cell; measure the absorbance at 483 nm against a blank pre-

[1] Chemicals of analytical reagent grade or the equivalent are used. Ethanol means 95% ethanol.

[2] Filter holder SX 00013000 and filters PHWP01300, Millipore Corp., Bedford, Mass.

Table I—Analysis of Hexachlorophene in Topical Formulations by the General Colorimetric Procedure

Sample	Labeled Amount, w/w %	Found Label Claim, w/w %	Coefficient of Variation	Number of Determinations[a]
Simulated soap	2.0	97.56	1.60	8
A soap	2.0	101.05	1.93	5
B soap	2.16	101.4	0.92	3
C soap	Unlabeled	2.16[b]	1.70	3
D douche	2.0	102.05	0.51	6
E powder	0.5	100.63	2.91	6
F tooth-paste	Unlabeled	0.045[b]	3.33	4
G liquid soap	0.4	90.15	2.14	3

[a] Each determination constituted a separate weighing followed by color development in triplicate. [b] Percent on a weight basis.

Table II—Analysis of Topical Emulsions by the Specific Colorimetric Procedure

Sample	Labeled Amount, %	Percent Recovery, General Colorimetric Method	Percent Recovery, UV Absorbance	TLC/Colorimetric		
				Mean Percent Recovery	Coefficient of Variation	Number of Plates (Duplicate Spots)
G	0.4	90.15	104.4	103.17	0.43	2
H	0.7	89.47	108.7	105.2	2.74	3
I	0.5	94.80	163.1	94.5	2.08	3
J	3.0	96.89	105.5	105.7	2.08	3

pared as described but using a similar portion of the same silica gel layer *that contains no I*. Prepare a calibration curve plotting absorbances of standard solutions against micrograms per milliliter of I in the final chloroform solution and determine the content of I in the samples.

Calculations—The percent of I is calculated using Eq. 2:

$$\% \text{ w/w I} = \frac{Cs \times 525}{Ws} \qquad \text{(Eq. 2)}$$

where Cs = micrograms per milliliter of I from the graph, and Ws = weight of sample taken in milligrams.

DISCUSSION

Although phenols are commonly analyzed by the II color procedure, the dyes formed during color development are unstable in the alkaline solutions in which they must be generated (11, 14). This problem has been overcome for many phenols by extraction of the dye into nonpolar solvents such as chloroform (11, 14). This procedure was reported to be inapplicable to analysis of I because of the insolubility of the I–II dye in chloroform (11, 14). The present investigation indicated that this dye could be taken up in chloroform by "salting out" from the aqueous solution with common salt.

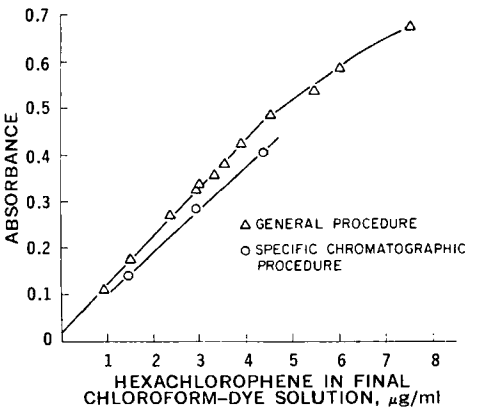

Figure 1—*Hexachlorophene calibration curves.*

The partition coefficient of the I–II dye between brine and chloroform appears to favor the latter. Solubility of the dye in chloroform was found to be in excess of 4.5 μg/ml. However, at concentrations over this level, volumes and shaking times became critical so that it was difficult to obtain reproducible results. Thus, in method development, sample sizes and solvent volumes were adjusted to keep I levels below 4.5 μg/ml.

Various aqueous phase–chloroform ratios were examined for extracting I–II dye into the chloroform layer. While the dye appeared only in the organic layer with ratios as low as 3:1, 15 ml of aqueous phase to 7 ml of chloroform was most convenient because of subsequent manipulation of the organic layer.

Using the color development and partitioning procedure, which subsequently became the proposed method, various concentrations of I were examined to determine whether dye solutions obeyed Beer's law at the absorbance maximum, 483 nm. Figure 1 demonstrates the direct relationship between concentration and absorbance (and also shows solubility characteristics of the I–II dye in chloroform).

Based on these considerations, the *General Procedure* for I analysis was developed for the examination of samples. The sample size proposed provides a I concentration in the final I–II dye solution of about 3.1 μg/ml and an absorbance near 0.33. This ensures that specimens containing overages of up to 150% will be in the linear range of the graph. Similarly samples containing as little as 50% of the label claim for I also will be in the accurate and precise range of the graph. All this, of course, assumes no interference from other components in formulations.

Since there are reports that analysis of I by II dye formation is adversely affected by soap and other surfactants (15), various organic solvents were examined to find one that would, ideally, isolate I and leave behind interfering substances from soap and other topical formulations. Chloroform did not isolate all of the I present in simulated samples while ethanol was nonselective, dissolving fatty acid salts and other materials so that lower than expected absorbances were obtained. Dimethylformamide, which has been used previously in I analysis (1), was found to be a good solvent for I in bar soap, dusting powder, and toothpaste but left some ambiguity when used with emulsion formulations.

Dimethylformamide was used in the isolation of I from a number of specimens of soap and other products (Table I). The coefficients of variation observed with some samples reflect difficulties in sampling (varying moisture levels, lack of homogeneity of specimens, *etc.*), since analysis of a solution of I in dimethylformamide

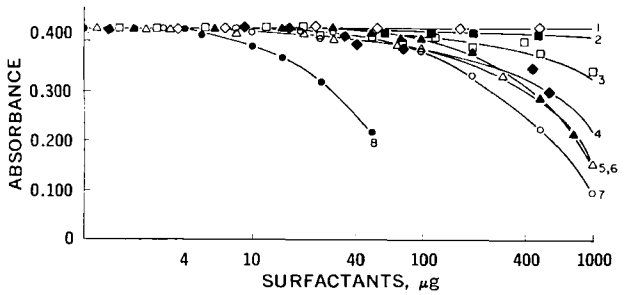

Figure 2—*Effects of surfactants on a constant concentration of 100 mg/25 ml hexachlorophene. Key: 1, ◇, sodium lauryl sulfate; 2, ■, sorbitan monooleate; 3, □, polyoxyethylene (23) lauryl ether; 4, ◆, polysorbate 80; 5, △, polyoxyl 30 stearate; 6, ▲, polyoxyl 8 stearate; 7, ○, polyoxyethylene (4) lauryl ether; and 8, ●, benzalkonium chloride.*

indicated recoveries of 97.9% with a coefficient of variation of 0.87.

Application of the proposed I method to emulsions left some doubt as to the accuracy of the procedure, since all values obtained were below labeled amounts (Table II). In addition, a liquid soap solution (Sample G) was low. To assess these results, Samples G–K inclusive were examined spectrophotometrically at 299 nm in acidified methanol. This study indicated higher I contents than were obtained by the II procedure (Table II).

These results, however, did not remove all ambiguity, since the absorbance curves for all samples showed the presence of background absorbance with maxima broader than those observed with equivalent concentrations of pure I. Thus, surfactants and other components could be interfering with both the II dye and direct UV procedures.

To determine the scope of the effects of surfactants on I analysis by II dye formation, solutions of I were treated with various levels of a number of commonly encountered surfactants, the II dye was developed, and absorbances were measured. Figure 2 demonstrates the limited tolerance of this colorimetric procedure to the presence of nonionic and cationic surfactants. In contrast, high levels of sodium lauryl sulfate did not interfere with color development.

It was apparent from this and other work (15) that it was necessary to isolate I from other emulsion components prior to color development. TLC on silica gel, with ethyl acetate–methanol (9:1) as solvent (4), was effective. In addition, this system separated I from other phenols such as dichlorophen, *o*-phenylphenol, 2,4-dichlorophenol, phenol, 2-benzyl-4-chlorophenol, 2,4,5-trichlorophenol, 4-chloro-3,5-dimethylphenol, and trichlorocarbanilide (Table III), so a high level of specificity could be obtained.

Direct scaling down of the general colorimetric procedure (A) for examining samples that had been chromatographed was not possible because of the limited buffering power of weak ammonia buffer. Where pH values of final solutions in the general procedure were always in the 9.45–9.67 range, direct scaling down afforded varying pH values with some as low as 8.35. Preparation of II and potassium ferricyanide reagents in buffer overcame this problem so that final values for the proposed specific chromatographic procedure (B) were always between 9.5 and 9.7.

TLC of standard solutions of I with subsequent isolation of I and color development indicated that 86% I was consistently recovered from chromatographic layers. Using this method, emulsion Samples H–J inclusive and the liquid soap solution G were analyzed (Table II).

Data derived from the *Chromatographic Method* were in good agreement with that obtained from UV absorbance measurements with the exception of Sample J (Table II). In this case, results obtained with Method B were very close to those obtained with the general colorimetric procedure. These data indicate that results obtained from general procedures, either colorimetric or UV, for quantitating I in emulsions and liquid soaps cannot be considered reliable. Thus, prior to utilizing a general method, the specific procedure must be applied to establish reliability. These problems do not arise with bar soap since both the general and specific colorimetric procedures afforded similar values.

Table III—Typical R_f Values of Antimicrobial Agents Examined

Compounds	R_f
Hexachlorophene	0.42
Trichlorocarbanilide	0.58
Phenol	0.62
2,4-Dichlorophenol	0.62
2,4,5-Trichlorophenol	0.62
o-Phenylphenol	0.63
4-Chloro-3,5-dimethylphenol	0.63
Dichlorophen	0.63
2-Benzyl-4-chlorophenol	0.64

The proposed modified colorimetric procedures for I analysis enables one to analyze occasional or replicate samples of bar soap and solutions containing I with minimum preparation time, while the specific procedure described enables the examination of emulsions with a high level of accuracy.

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Determination of Sorbitol and Mannitol in Pharmaceuticals by GLC of Tris-*n*-Butyldiboronate Esters

DAVID L. SONDACK

Abstract □ A rapid method for determining sorbitol or mannitol in pharmaceuticals by GLC using the tris-*n*-butyldiboronate esters is described. An internal standard unrelated to the hexitols is utilized to avoid the problems of cross-contamination introduced by the use of other hexitols.

Keyphrases □ Sorbitol—GLC determination in pharmaceuticals using tris-*n*-butyldiboronate esters □ Mannitol—GLC determination in pharmaceuticals using tris-*n*-butyldiboronate esters □ GLC—determination, sorbitol and mannitol in pharmaceuticals using tris-*n*-butyldiboronate esters

Sorbitol and mannitol have been used as artificial sweeteners in pharmaceutical preparations. The analytical procedure described in the USP (1) is tedious and cumbersome because it involves column chromatography, periodate reaction, and iodometry. Derivatization of the hexitols to their hexaacetate derivatives facilitates analysis by GLC due to increased volatility (2), but the method is still somewhat time consuming and the most suitable liquid phase found is unstable (3). The rapidity of silylation procedures does not provide a convenient assay since the common hexitols are not resolved (4).

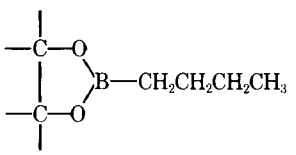
Alkane boronic acids aid in the volatilization of various organic compounds (5) and have been shown to resolve the common hexitols (6). With the method presented here, the sorbitol or mannitol content of pharmaceutical preparations may be determined by GLC utilizing the *n*-butylboronic acid derivatives (I).

EXPERIMENTAL

Equipment—A gas chromatograph[1] equipped with a flame-ionization detector was used. The detector signal was fed to a computer[2] for peak integration and to a 1-mv recorder[3] with a chart speed of 15 in./hr and a 1-sec full-scale response. Samples were injected with a 10-μl syringe[4].

Helium was used as the carrier gas, and electrolytic hydrogen and oxygen were used in the detector. The stationary phase, 2% OV-17 on 80–100-mesh Chromosorb G[5], was packed in borosilicate glass columns, 1.22 m × 0.64 cm o.d. All chemicals used were reagent grade or the best quality available.

Operating Conditions—The column was operated isothermally at 220° with the detector block and injection port at 240°. The helium flow rate was 55 ml/min with an inlet pressure of 40 psi. The



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[1] Hewlett-Packard model 402.
[2] IBM 1800.
[3] Honeywell Electronik 16.
[4] Hamilton 701.
[5] Applied Science Laboratories, State College, Pa.

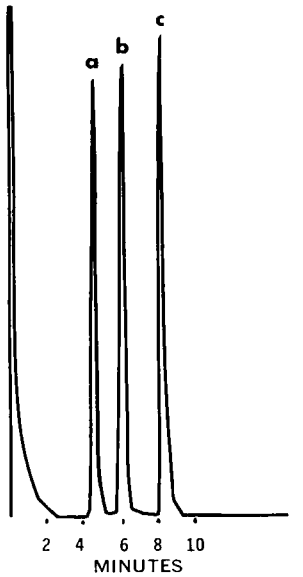

Figure 1—*Gas–liquid chromatogram of tris-*n*-butyldiboronate esters of mannitol (peak A) and sorbitol (peak B) and of* m*-diphenylbenzene, the internal standard (peak C).*

electrometer range was 100 with an attenuation of 8. Sample injections between 1 and 5 μl were made.

Internal Standard—*m*-Diphenylbenzene, 2.5 mg/ml, in pyridine was used as the internal standard.

Hexitol Analysis—Solutions (2.5 mg/ml) of reference standard sorbitol or mannitol were prepared in deionized water. Sample solutions were diluted volumetrically to provide approximately the same concentration. Aliquots of 1.00 ml were withdrawn and delivered by pipet to a screw-capped vial and placed in a vacuum desiccator over sodium hydroxide pellets or other suitable desiccant. After careful evacuation to avoid sample loss due to bubbling, the samples were allowed to stand in the desiccator overnight.

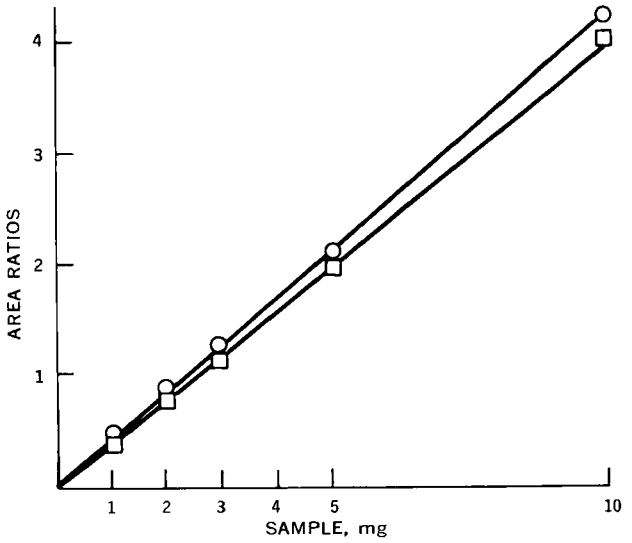

Figure 2—*Linearity of response of mannitol (○) and sorbitol (□) with concentration (conditions as in text).*

Table I—Determination of Sorbitol and Mannitol in Various Samples

Sample	Milligrams Taken	Sorbitol Found, mg	Mannitol Found, mg
Sorbitol			
1	34.2	30.6	3.7
2	33.7	30.2	3.6
3	31.6	28.3	3.3
4	29.9	26.8	3.2
5	30.8	27.6	3.4
6	30.4	27.2	3.3
Mannitol			
1	36.5	3.6	32.7
2	36.0	3.5	32.2
3	33.4	3.3	39.9
4	32.6	3.1	29.2

Approximately 10 mg of *n*-butylboronic acid[6] and exactly 1.00 ml of internal standard solution were added to the dried samples. The reaction was completed almost immediately, and 1-μl portions were injected onto the column.

RESULTS AND DISCUSSION

The chromatogram shown in Fig. 1 is of the tris-*n*-butyldiboronate esters (6) of mannitol and sorbitol under the described conditions. No mannitol or sorbitol samples obtained were free of mutual contamination. It is advisable to observe the precaution offered by Eisenberg (6), *i.e.*, to ascertain the purity of the *n*-butylboronic acid reagent by GLC of a pyridine solution on the OV-17 column at 92°. It follows, then, that excess reagent elutes with the solvent front during hexitol analysis. The reagent used here was essentially free of any impurities.

The response of the analytical system was linear to a concentration four times that described in Fig. 2. The residual standard deviations calculated for five replicate samples of mannitol and sorbitol, analyzed separately, were both ±1.2% (Table I). These values compare favorably with those using the method of Manius *et al.* (3). The advantages of this method are the ease of derivatization, the shorter analysis time, and the greater thermal stability of the liquid phase.

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Keyphrases □ Chlorpromazine hydrochloride—chemistry of nonaqueous titration, method proposed, compared to official methods □ Nonaqueous titration—chlorpromazine hydrochloride, chemistry □ Titration, nonaqueous—chlorpromazine hydrochloride, chemistry

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The pure compound is titrated potentiometrically in glacial acetic acid medium, according to the USP XVIII and the BP 1963, and in acetone using methyl orange indicator according to the BP 1968. The injection is assayed spectrophotometrically in the three compendia. In the BP 1968 and the BP 1963 methods, the injection solution is diluted with 0.1 *N* hydrochloric acid solution to a suitable concentration and then the absorbance is measured directly. In the USP XVIII method, several extraction processes are carried out before measurement.

Tablets are determined spectrophotometrically according to the USP XVIII and the BP 1968. The BP 1963, however, recommends the titration of chlorpromazine hydrochloride content of tablets against standard ceric ammonium sulfate solution.

DISCUSSION

Several methods of analysis have been proposed for chlorpromazine hydrochloride involving gravimetry, titrimetry, spectroscopy, colorimetry, and chromatography. Spectrophotometric and colorimetric methods include the estimation of chlorpromazine in biological fluids (4) by measuring the red color produced by concentrated sulfuric acid. Colorimetric determination of chlorpromazine in tablets containing methampyrone [sodium (antipyrinylmethylamino)methanesulfonate] was reported (5).

A method using acid indicator dyes was suggested (6) for the de-

Table I—Determination of Sorbitol and Mannitol in Various Samples

Sample	Milligrams Taken	Sorbitol Found, mg	Mannitol Found, mg
Sorbitol			
1	34.2	30.6	3.7
2	33.7	30.2	3.6
3	31.6	28.3	3.3
4	29.9	26.8	3.2
5	30.8	27.6	3.4
6	30.4	27.2	3.3
Mannitol			
1	36.5	3.6	32.7
2	36.0	3.5	32.2
3	33.4	3.3	39.9
4	32.6	3.1	29.2

Approximately 10 mg of *n*-butylboronic acid[6] and exactly 1.00 ml of internal standard solution were added to the dried samples. The reaction was completed almost immediately, and 1-μl portions were injected onto the column.

RESULTS AND DISCUSSION

The chromatogram shown in Fig. 1 is of the tris-*n*-butyldiboronate esters (6) of mannitol and sorbitol under the described conditions. No mannitol or sorbitol samples obtained were free of mutual contamination. It is advisable to observe the precaution offered by Eisenberg (6), *i.e.*, to ascertain the purity of the *n*-butylboronic acid reagent by GLC of a pyridine solution on the OV-17 column at 92°. It follows, then, that excess reagent elutes with the solvent front during hexitol analysis. The reagent used here was essentially free of any impurities.

The response of the analytical system was linear to a concentration four times that described in Fig. 2. The residual standard deviations calculated for five replicate samples of mannitol and sorbitol, analyzed separately, were both ±1.2% (Table I). These values compare favorably with those using the method of Manius *et al.* (3). The advantages of this method are the ease of derivatization, the shorter analysis time, and the greater thermal stability of the liquid phase.

[6] Pierce Chemical Co.

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A method using acid indicator dyes was suggested (6) for the de-

termination of chlorpromazine hydrochloride; the reaction product with bromcresol purple was isolated and its structure was elucidated with elemental, IR, and UV analyses. Chlorpromazine was determined in biological specimens by UV spectrophotometry and corroborated by TLC (7). A spectrophotometric method was developed for the routine assay of conjugated and unconjugated chlorpromazine metabolites in human urine (8).

Among the many available titrimetric and gravimetric methods for the determination of chlorpromazine hydrochloride is an attempt (9) to determine the nitrogenous base gravimetrically by precipitation with silicotungstic acid in acid solution. The oxidation of the phenothiazine nucleus with standard bromate (10) was used to determine chlorpromazine, and the excess bromate was estimated.

A thermometric titration procedure for chlorpromazine hydrochloride using standard sodium hydroxide solution as the titrant was suggested (11). The neutralization of the protonated nitrogen with base resulted in an enthalpogram with a sharp end-point. The phenothiazine nitrogen, being not sufficiently basic in aqueous solution, failed to exhibit an end-point when titrated *versus* standard hydrochloric acid solution.

Amperometric titration of chlorpromazine (12) was accomplished using a molybdenum anode and a graphite, platinum, or tungsten cathode. Chlorpromazine, in tablets, was determined by diphasic titration against sodium lauryl sulfate, using methyl yellow as the indicator (13). It was stated that this method is of particular value for determining phenothiazine derivatives in the presence of other organic bases. A photometric titration procedure was also described (14) for determining this compound in several dosage forms, using ceric sulfate as titrant.

Methods involving titration in nonaqueous solvents were reviewed (15). Chlorpromazine hydrochloride, in pure form as well as in various dosage forms, was titrated (16), using acetone as the solvent and methyl red as the indicator. The titrant was a standard perchloric acid solution in dioxane. Alternatively, the nitrogenous base was liberated by aqueous potassium hydroxide solution and extracted by hexane, acetone was added, and the solution was titrated as described.

Chlorpromazine and some other phenothiazine derivatives were titrated in a mixture of ethylene dichloride and glacial acetic acid against aceteous perchloric acid solution, using tropaeolin OO as the indicator (17). It was also shown (18) that neutral or almost neutral solvents, such as acetone and methylcyanide, were more satisfactory than acid solvents, such as glacial acetic acid, in determining chlorpromazine hydrochloride in pharmaceutical preparations.

The most widely used method (19) for the determination of the hydrochlorides of nitrogenous bases cannot be applied to the determination of chlorpromazine hydrochloride due to the development of an intense red color which interferes with the color change of crystal violet indicator. Therefore, the purpose of the present investigation was to develop a nonaqueous titrimetric procedure applicable to the determination of chlorpromazine hydrochloride, in pure form as well as in different dosage forms, in which the end-point detection can be accomplished visually with crystal violet and glacial acetic acid, the most widely used indicator and solvent in nonaqueous titration.

EXPERIMENTAL

Apparatus and Materials—The following were used: a suitable titrimeter[1] equipped with a combination electrode[2], a magnetic stirrer, and a microburet of 10-ml capacity graduated to 0.02 ml.

Solutions—The following were used: a 0.1 *N* perchloric acid solution in glacial acetic acid, 5 and 6% (w/v) solutions of mercuric acetate in glacial acetic acid, a 0.2% (w/v) solution of crystal violet indicator in glacial acetic acid, and a saturated solution of methyl orange in acetone.

Acetous perchloric acid solution was standardized against potassium acid phthalate[3], previously dried at 110° for 2 hr, using glacial acetic acid as the solvent. The end-point was determined potentiometrically.

[1] Pye model 79.
[2] Pye Catalog No. 401 E07, series No. 546022.
[3] Rhone-Poulenc, France.

Table I—Determination of Chlorpromazine Hydrochloride in Pure Form, Tablets, and Injections Using the Proposed Visual Nonaqueous Titration Method

Sample	Weight, mg	Weight Found, mg	Recovery, %
Pure compound			
1	309.7	311.3	100.52
2	296.9	298.4	100.50
3	176.8	178.1	100.73
4	167.1	166.9	99.88
5	142.1	142.6	100.35
Mean percent recovery (P' = 0.05)			100.39 ± 0.39
Tablets (25 mg)			
1	300.0	298.4	99.47
2	252.7	251.0	99.33
3	207.7	206.6	99.47
Tablets (10 mg)			
4	155.5	154.1	99.09
5	103.5	102.9	99.42
6	103.2	103.3	100.09
Mean percent recovery (P' = 0.05)			99.48 ± 0.35
Injections (50 mg/2 ml)			
1	375.0	373.0	99.47
2	250.0	246.9	98.76
Injections (25 mg/5 ml)			
3	125.0	124.5	99.60
4	100.0	99.5	99.50
Mean percent recovery (P' = 0.05)			99.33 ± 0.61

For comparative purposes, chlorpromazine hydrochloride[3] was assayed by the official procedures of the USP XVIII and the BP 1963 and 1968.

Determination of Chlorpromazine Hydrochloride by Proposed Visual Procedure—An amount of chlorpromazine hydrochloride, as specified in Table I, was accurately weighed and dissolved in 50 ml of glacial acetic acid contained in a 150-ml titration beaker. Ten milliliters of 5% (w/v) mercuric acetate solution, 1.0 g of powdered ascorbic acid, and 2 drops of crystal violet indicator solution were added.

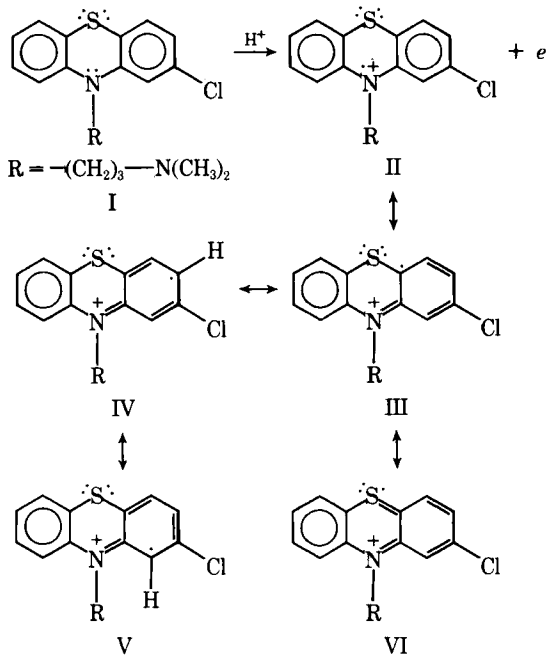
The solution, magnetically stirred, was titrated potentiometrically against 0.1 *N* acetous perchloric acid solution. The end-point in the titration was determined from the inflection in the titration curve, obtained by plotting the volume of titrant in milliliters against the millivolt readings. The proper color change of the indicator coinciding with the potentiometric end-point was noted. Such a color change was found to be from violet to blue. The titration was also performed as described but with 50 ml of a glacial acetic acid–dioxane (1:1) mixture as the solvent.

Analysis of Tablets—Twenty tablets were weighed and powdered. An amount of powdered tablets equivalent to a weight of chlorpromazine hydrochloride as shown in Table I was accurately weighed and placed in a 150-ml titration beaker containing 50 ml of glacial acetic acid. Then 10 ml of mercuric acetate solution, 1.0 g of ascorbic acid powder, and 3 drops of crystal violet indicator solution were added. The solution was stirred magnetically for several minutes and titrated to a blue color change of the indicator.

Analysis of Injections—A volume of the mixed contents of ampuls equivalent to an amount of chlorpromazine hydrochloride as indicated in Table I was pipetted, exactly, into a 150-ml titration beaker. The solution was evaporated on a water bath to a small volume (3–5 ml), and about 6.0 ml of acetic anhydride was added for each milliliter of solution remaining in the titration beaker. Then the solution was heated on a hot plate until it began to boil. The solution was cooled, and 10 ml each of glacial acetic acid and mercuric acetate solution, 1.0 g of ascorbic acid powder, and 2 drops of crystal violet indicator solution were added. It was then titrated visually with 0.1 *N* acetous perchloric acid as described.

RESULTS

Chlorpromazine hydrochloride, being a phenothiazine derivative, is sensitive to oxidation. Therefore, in glacial acetic acid medium, the oxidizing effect of perchloric acid is catalyzed by mercuric acetate (20), giving a red-colored oxidation product that makes visual end-point detection with crystal violet indicator impossible.



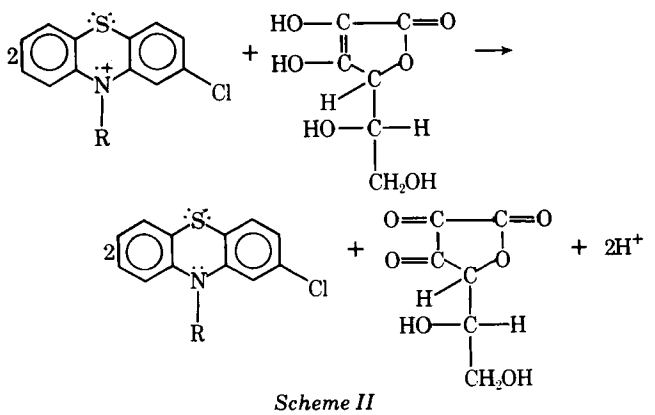
Scheme I

For this reason, the official compendia such as USP XVIII and BP 1963 employ potentiometric, rather than visual, end-point detection in the nonaqueous titration of chlorpromazine hydrochloride in glacial acetic acid solvent. BP 1968 replaces glacial acetic acid with acetone and uses a saturated solution of methyl orange in acetone as the indicator.

The formation of intensely colored compounds in acid solution of phenothiazine was explained by Michaelis *et al.* (21). Using methylene blue, a phenothazine derivative, as an example, these investigators proposed a free radical formation which depended on a state of equivalent resonance. Their explanation is satisfactory for the color developed by this compound, because it possesses two auxochrome amino groups in the 3- and 7-positions which are capable of participating in the development of a semiquinoid free radical structure and hence facilitate color production.

This explanation is not satisfactory, however, for phenothiazine derivatives stripped of all auxochrome groups. This objection was later resolved by the same authors (22) by pointing out that colored semiquinone radicals of phenothiazine as they exist in acid solution are stabilized by resonance involving the two bridge atoms, nitrogen and sulfur. They stated that such colored semiquinone free radicals can be obtained in a high concentration and in a quite stable condition in 80% acetic acid.

Chatten and Harris (23) reported the titration behavior of a number of phenothiazine derivatives, including chlorpromazine, in nonaqueous media and concluded that the reaction between chlorpromazine and perchloric acid in nitromethane solvent produced a distinctly colored compound, ranging from an initial pink to a final rose-red color. These authors attributed the formation and stabili-



Scheme II

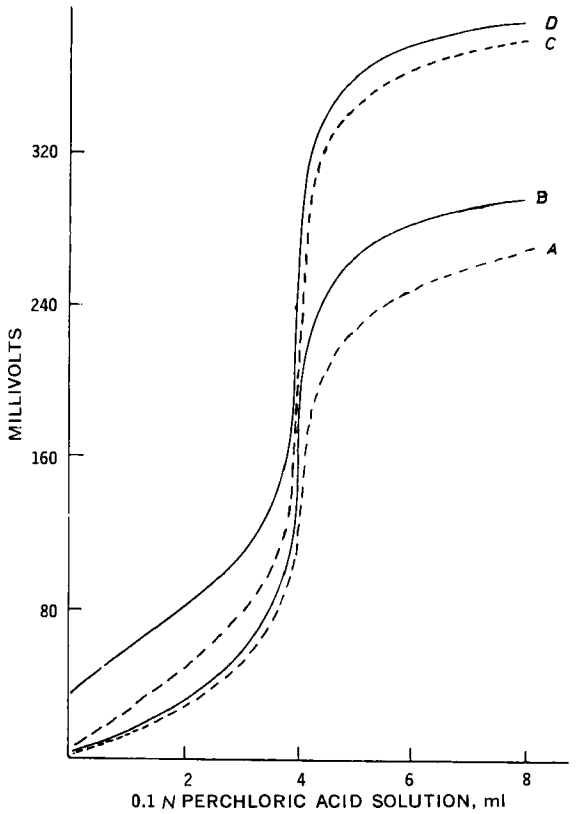


Figure 1—*Potentiometric titration of chlorpromazine hydrochloride. Key: A, USP XVIII and BP 1963 procedures in glacial acetic acid; B, proposed procedure in glacial acetic acid and using ascorbic acid; C, USP XVIII and BP 1963 procedures in glacial acetic acid–dioxane mixture; and D, proposed procedure in glacial acetic acid–dioxane mixture and using ascorbic acid.*

zation of the colored compound to complex formation between the resonating nitromethane and the titrated phenothiazine derivative, and they indicated that such a complex could result from ionic attraction of the oppositely charged centers in the phenothiazine compound and the carbanion-acinitromethane resonate.

Thus, the formation and stabilization of the semiquinone free radical, which is responsible for the red color production, may be due to the possible formation of many resonating forms. Scheme I illustrates the mechanism suggested for such a resonance. In acetic acid medium, chlorpromazine (I) is oxidized by the action of perchloric acid, whereby it loses one electron to give the semiquinone free radical (II). This structure is in resonance with Structures III, IV, and V, which arise from II by resonance involving one of the two benzene rings of the phenothiazine structure. When resonance involves the other benzene ring, resonating structures analogous to III, IV, and V would be possible from Structure II.

In addition, more resonating structures are expected to be formed *via* another route involving the two bridge atoms, nitrogen and sulfur. Such a route is represented by Structures II, III, and VI when resonance involves one of the benzene rings. However, resonance involving the other benzene ring through this route can be represented by Structure II, the structure corresponding to III, and Structure VI.

The formation of a red-colored solution during the titration of chlorpromazine hydrochloride with perchloric acid solution was prevented by the addition of finely powdered ascorbic acid to the contents of the titration beaker before beginning the titration. The absence of color from chlorpromazine solution throughout titration enabled accurate and sharp visual end-point detection, which is impossible in the USP XVIII and BP 1963 nonaqueous titration procedures.

The immediate disappearance of the red color and the subsequent prevention of such a color formation during the titration are due to reduction with ascorbic acid. The semiquinone free radical

Table II—Determination of Chlorpromazine Hydrochloride According to USP XVIII, BP 1963, and BP 1968 Methods

Sample	USP XVIII and BP 1963		BP 1968	
	Weight, mg	Recovery, %	Weight, mg	Recovery, %
1	608.1	100.10	291.0	100.15
2	299.1	101.39	172.7	99.50
3	199.9	100.00	89.7	101.65
4	142.1	101.33	79.1	101.18
5	141.6	100.69	58.2	100.39
	Mean percent recovery ($P' = 0.05$)	100.70 ± 0.81	Mean percent recovery ($P' = 0.05$)	100.57 ± 1.05

is reduced to chlorpromazine, whereby dehydroascorbic acid is formed as illustrated by Scheme II. Ascorbic acid and its oxidation product, dehydroascorbic acid, being neutral in glacial acetic acid, do not interfere with the determination of chlorpromazine hydrochloride.

Typical titration curves for the determination of chlorpromazine hydrochloride according to the proposed method and the USP XVIII and BP 1963 procedures in glacial acetic acid and in a glacial acetic acid–dioxane solvent system are illustrated in Fig. 1. Curve B, which represents the titration of the nitrogenous base hydrochloride in glacial acetic acid according to the proposed method, indicates that a sharper end-point detection is obtained when compared with the USP XVIII and BP 1963 procedures (curve A). The relative magnitudes in the breaks of the two curves are 540 and 340 mv/ml of titrant, respectively.

The visual end-point detection was also found to be sharp when the proposed method was applied. This difference in the sharpness of end-points may be attributed to the fact that in the official procedures a mixture of chlorpromazine and the semiquinone free radical, rather than a single compound, is titrated; in the proposed method, the formation of such a free radical is prevented. The semiquinone free radical is expected to be a weaker base than chlorpromazine in view of the presence of a positive charge, which decreases the electron density within the semiquinone free radical structure. Titration in the glacial acetic acid–dioxane (1:1) solvent system yielded an even sharper end-point detection.

Chlorpromazine contains two distinctly different types of nitrogen atoms, a tertiary amino and a heterocyclic nitrogen. The resonance within the heterocyclic ring system of chlorpromazine is such that the electron density around the heterocyclic nitrogen atom is incapable of reacting with protons. The tertiary nitrogen atom, being a strong base, is capable of holding a proton when titrated with perchloric acid solution. Therefore, in the solvent systems employed in this study, chlorpromazine behaves as a monoacidic base and gives only one inflection point when titrated potentiometrically.

The scope of the proposed visual titration method for control purposes was demonstrated by its application to samples of chlorpromazine hydrochloride in pure form and in synthetic dosage forms such as tablets and injections. Commonly used excipients, insoluble fillers, diluents, and lubricants such as lactose, starch, and talc do not interfere with the determination. An aliquot of powder mass of tablets is titrated directly, without preliminary extraction of the active constituent, after sufficient time is allowed for its solubility. The water content of injections is removed by evaporation to a small bulk, and the last traces are allowed to react with a sufficient amount of acetic anhydride.

The results of the titration of chlorpromazine hydrochloride, according to the proposed visual nonaqueous titration method, are shown in Table I. The mean percent recoveries ($P' = 0.05$) of the pure form, tablets, and injections are 100.39 ± 0.39, 99.48 ± 0.35, and 99.33 ± 0.61, respectively.

Table II lists the recovery data obtained when chlorpromazine hydrochloride is determined by the potentiometric nonaqueous titration method of USP XVIII and BP 1963 and by the BP 1968 procedure. The mean percent recoveries ($P' = 0.05$) are 100.70 ± 0.81 and 100.57 ± 1.05, respectively. Statistical analysis of the two sets of results given in this table indicated that the difference between the two mean percentage recoveries is statistically insignificant and that the two methods are equally precise and almost equally accurate. Statistical comparison of the data obtained using the proposed visual method and the potentiometric USP XVIII and BP 1963 procedure showed that the two methods are equally precise. However, the proposed method is more accurate.

With the proposed method, the end-point detection is accomplished visually using crystal violet indicator; such detection is not possible with the official methods. The proposed method also has the advantage of being applicable to chlorpromazine hydrochloride in the pure state as well as in dosage forms. The official compendia employ different assay methods for different dosage forms. Glacial acetic acid and crystal violet indicator, the most widely used solvent and indicator in nonaqueous titration, are also employed. The proposed method is useful and attractive to the pharmaceutical control chemist for routine analysis.

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GLC Determination of Saccharin in Pharmaceutical Products

E. M. RATCHIK and V. VISWANATHAN ˣ

Abstract □ A GLC method was developed for the assay of saccharin in pharmaceutical products. The procedure requires silylation with *N,O*-bis(trimethylsilyl)acetamide and subsequent chromatography on trifluoropropyl methyl silicone, using *n*-octacosane as an internal standard.

Keyphrases □ Saccharin—GLC determination in pharmaceutical products, silyl derivatives formed □ GLC—analysis, saccharin in pharmaceutical products, silyl derivatives formed

Subsequent to the ban on the use of cyclamates in pharmaceutical products, saccharin has been subjected to increased scrutiny and governmental regulations (1). Various methods for the quantitative and qualitative determination of saccharin have been reported, including TLC (2), IR (3), and a nonaqueous titration after separation by ion-exchange chromatography (4). One early reference to a GLC procedure involved the preparation of the *N*-methyl ester using diazomethane (5). Other investigators reported variations such as changes in the liquid phase and use of an internal standard (6, 7).

Conacher and O'Brien (8) reported a GLC method for the determination of saccharin in soft drinks, using diazomethane as the methylating agent and methyl silicone (JXR) as the liquid phase. According to their report, the methylation of saccharin consistently gave two peaks in a 17:3 ratio, which they postulated to be the *N*-methyl derivative of saccharin and the *O*-methyl derivative of pseudosaccharin, respectively. Their quantitative data were based on area measurements of the rapidly eluted, poorly shaped major peak (8).

This paper describes the determination of saccharin in pharmaceutical products utilizing silylation with *N,O*-bis(trimethylsilyl)acetamide[1]. This procedure offers several improvements upon previous methods. Diazomethane is toxic, potentially explosive, and rather difficult to prepare and use, while the silylation reaction is rapid and leads to a derivative needing no further treatment prior to chromatography.

A literature survey indicated a complete lack of assay methods for saccharin in complex pharmaceutical dosage forms. The ion-exchange chromatographic method is time consuming and suitable only for simple aqueous preparations, since any other acid or acid salt in the dosage form interferes with the determination.

The present method was found to be completely free of interferences from active and inactive ingredients in the products analyzed.

[1] Regis Chemical Co.

Table I—Saccharin Assay Results

Product	Claim	Found[a]
Tablet A	15.0 mg/tablet	Batch 1: 15.1 Batch 2: 14.0 Batch 3: 14.1 Batch 4: 14.9
Tablet B	30 mg/tablet	Batch 1: 28.0 Batch 2: 29.0
Tablet C	0.76 mg/tablet	Batch 1: 0.80 Batch 2: 0.73 Batch 3: 0.72 Batch 4: 0.74
Elixir A[b]	5.0 mg/5 ml	4.9
Elixir B[b]	10.8 mg/5 ml	11.6
Elixir C[b]	14.4 mg/5 ml	14.4
Elixir D[b]	3.6 mg/5 ml	3.8
Syrup[b]	5.5 mg/5 ml	5.5
Suspension	5.0 mg/5 ml	Batch 1: 5.1 Batch 2: 5.0 Batch 3: 5.0

[a] Average of duplicate determinations calculated as sodium salt. [b] Synthetic formulations.

EXPERIMENTAL

Equipment—A gas chromatograph[2] equipped with a single hydrogen flame-ionization detector and interfaced with a digital computer[3] was used. The column was 0.9-m × 0.3-cm (3-ft × 0.125-in.) o.d., type 304 stainless steel tubing, packed with 100–120-mesh silanized diatomaceous earth[4] and coated with 5% trifluoropropyl methyl silicone (OV-210).

The operating temperatures were: column, 160°; detector, 250°; and injector port, 240°. Nitrogen was the carrier gas with a flow rate of 30 ml/min. The hydrogen gas flow was adjusted for maximum sensitivity at 30 ml/min, and the air flow was 300 ml/min. The electrometer range was 10^{-10} amp/mv.

N,O-Bis(trimethylsilyl)acetamide was used as the silylating reagent. The isopropyl ether (bp 67–69°) used was alcohol free[5]. All other chemicals were reagent grade with the exception of the hydrocarbon *n*-octacosane[6], the internal standard, which was practical grade.

Preparation of Internal Standard Stock Solution—One hundred milligrams of *n*-octacosane was accurately weighed into a 50-ml volumetric flask and dissolved in and diluted to volume with cyclohexane.

Preparation of Reference Standard Stock Solution—Seventy-six milligrams of reference saccharin was accurately weighed into a 100-ml volumetric flask and dissolved in and diluted to volume with methanol.

Preparation of Reference Standard—A 4.0-ml aliquot of the reference standard stock solution was pipetted into a 10-ml volumetric flask and evaporated to dryness on a steam bath under nitrogen. Then 1.0 ml of silyl reagent was pipetted into the residue and mixed well. The flask was stoppered and heated on a steam bath for 30 min. After cooling to room temperature, 1.0 ml of the internal standard stock solution was pipetted into the flask and thoroughly mixed.

Preparation of Samples—A suitable quantity of the syrup, suspension, or pulverized tablet mass equivalent to approximately

[2] Varian Aerograph
[3] PACE system, Electronic Associates Inc.
[4] Varaport 30, Varian Aerograph.
[5] Matheson, Coleman & Bell.
[6] Eastman Kodak.

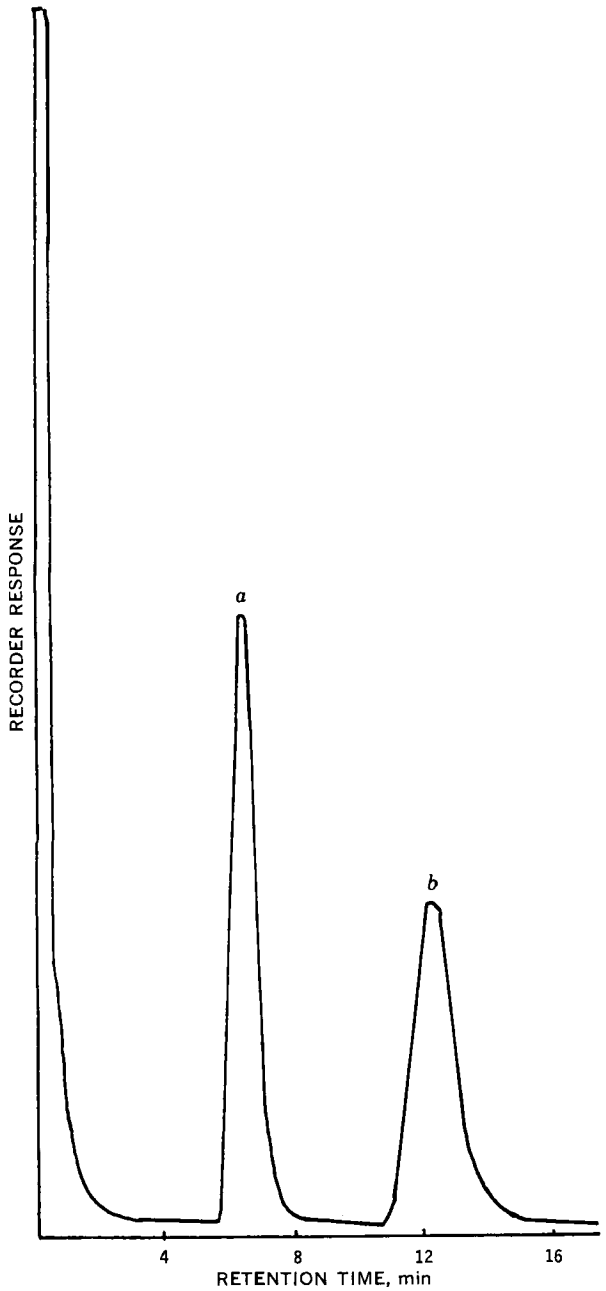

Figure 1—*Gas chromatogram of a sample preparation containing* (a) *saccharin as the trimethylsilyl derivative and* (b) n-*octacosane.*

6.0 mg of saccharin was accurately transferred into a 50-ml centrifuge tube. Twenty-five milliliters of 10% aqueous hydrochloric acid was added, mixed well, and allowed to stand for 15 min. This mixture was extracted with five 20-ml portions of isopropyl ether and shaken mechanically for 15 min each time. All extracts were passed through anhydrous sodium sulfate and collected in a 125-ml erlenmeyer flask.

The extract was evaporated to dryness on a steam bath under nitrogen, and the residue was dissolved in 4.0 ml of methanol. A 2.0-ml portion of this solution was transferred into a 10-ml volumetric flask and evaporated to dryness. Then 1.0 ml of silyl reagent was added to the residue and mixed well. The flask was stoppered and heated on a steam bath for 30 min. After cooling to room temperature, 1.0 ml of the internal standard stock solution was pipetted into the flask and thoroughly mixed.

Procedure—The chromatographic column was conditioned by making a few 10-μl injections of the working standard solution. Several 5-μl injections of the standard and sample solutions were made to establish the response factor and to calculate the saccharin content of the samples.

Table II—Statistical Evaluation

Determinations	Saccharin, mg/Tablet
1	0.85
2	0.78
3	0.80
4	0.82
5	0.83
6	0.80
Mean	0.81
SD	±0.025
95% Confidence limit	±0.0262

Calculations—The response factor for saccharin is determined as follows:

$$RF_S = \frac{A_S(\text{std}) \times C_{IS}}{A_{IS}(\text{std}) \times C_S} \qquad \text{(Eq. 1)}$$

where:

A_S(std) = peak area of saccharin in the working standard solution
A_{IS}(std) = peak area of internal standard in the working standard solution
C_{IS} = concentration of internal standard (milligrams per milliliter) in the working standard solution
C_S = concentration of saccharin (milligrams per milliliter) in the working standard solution

The saccharin content in pharmaceutical products can be calculated as follows:

$$\text{mg saccharin/dosage unit} = \frac{A_S(\text{spl}) \times C_{IS} \times \text{dilution factor}}{A_{IS}(\text{spl}) \times RF_S \times \text{sample weight (g or ml)}} \qquad \text{(Eq. 2)}$$

where A is the area, C is the concentration, and RF is the response factor (Eq. 1). The conversion factor is milligrams saccharin × 1.317 = sodium saccharin.

RESULTS AND DISCUSSION

Peak Characterization—GLC–mass spectrometry data indicated the molecular ion of the derivative to be at m/e 255, which corresponds to the monotrimethylsilyl derivative of saccharin. IR and NMR studies of the silylated product showed the loss of the imino proton, indicating that the reaction probably produced *N*-trimethylsilyl derivative.

An IR scan of the peak eluting from the GLC column, using an interfaced IR spectrophotometer, indicated the presence of the expected carbonyl band at 1700 cm^{-1} in addition to a strong band at 1250 cm^{-1} due to the substitution of a trimethylsilyl group. A more conclusive characterization of the derivative or the effects of enolization of saccharin, if any, would be beyond the scope of this paper.

Linearity—The detector response for saccharin was found to be linear in the range of 0.24–2.33 mg/ml at a constant internal standard concentration. The slope of the linearity curve was calculated to be 0.646 with a Y intercept of −0.006.

A study of silylation time was carried out using intervals of 10–60 min on the steam bath. The results indicated that the reaction is essentially complete in 20 min.

Precision and Accuracy—Analytical data obtained for several pharmaceutical products indicate that the procedure is applicable to a wide range of dosage forms without any modifications in analytical techniques (Fig. 1 and Table I). A statistical evaluation of the precision of the method was carried out for an antacid tablet formulation (Table II).

Recovery—A study was also undertaken to estimate the recovery of saccharin from the dosage forms. Formulations to which known amounts of sodium saccharin were added prior to acidification and extraction were analyzed and indicated essentially complete recovery (Table III).

Table III—Recovery Data

Product	Initial Assay	Saccharin Added, mg	Final Assay	Recovery, %
Tablet	16.9 mg/tablet	4.8	21.7 mg/tablet	100
Tablet	32.6 mg/tablet	9.3	42.0 mg/tablet	100
Suspension	5.1 mg/tablet	5.1	10.5 mg/5 ml	103
Suspension	5.1 mg/5 ml	10.5	16.3 mg/5 ml	105
Elixir	3.8 mg/5 ml	5.5	9.1 mg/5 ml	98
Elixir	3.8 mg/5 ml	10.2	13.6 mg/5 ml	97

Interferences—To establish the applicability of this procedure to various pharmaceutical dosage forms, synthetic formulations were made by adding sodium saccharin and were analyzed in addition to commercially available products. By the use of saccharin-free placebo formulations, it was found that pharmaceutically active ingredients such as ascorbic acid, nicotinyl alcohol, aprobarbital, dextromethorphan hydrobromide, chlorpheniramine maleate, acetaminophen, and sulfamethoxazole did not interfere with the analysis.

One pharmaceutical product contained a vitamin B-complex mixture made up of thiamine mononitrate, riboflavin phosphate, pyridoxine hydrochloride, niacinamide, and calcium pantothenate. The ingredients in the mixture did not cause any interference. Commonly used pharmaceutical excipients such as corn syrup, sucrose, citric acid, sodium citrate, sodium benzoate, propylene glycol, stearic acid, magnesium stearate, and mannitol also did not affect the utility of this method.

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PHARMACEUTICAL TECHNOLOGY

Drying Rates of Tablet Granulations I: Effect of Certain Granulating Adjuvants on Drying Rates

BALDEV R. BHUTANI [x] and VISHNU N. BHATIA

Abstract □ The design and construction of a laboratory size dryer and other accessories suitable for investigating the drying rate kinetics of granules under controlled external conditions are described. Granulations of lactose and sulfathiazole, representing water-soluble and insoluble materials, were prepared using various commonly used binders, and their drying rates were determined. The binders and diluents affected the drying rate curves for these granulations both qualitatively and quantitatively. Granules made with starch paste and gelatin solution required maximum time and energy for drying and those made with simple syrup USP required the least among the binders studied. Generally, three linear slopes were observed when the drying rate was plotted against the moisture remaining, indicating that granulation drying may be considered as occurring through three distinct phases.

Keyphrases □ Tablet granulations—effect of granulating adjuvants on drying rates □ Granulating adjuvants—effect on drying rates of tablet granulations □ Drying rates, tablet granulations—effect of granulating adjuvants □ Excipients—effect of granulating adjuvants on drying rates of tablet granulations

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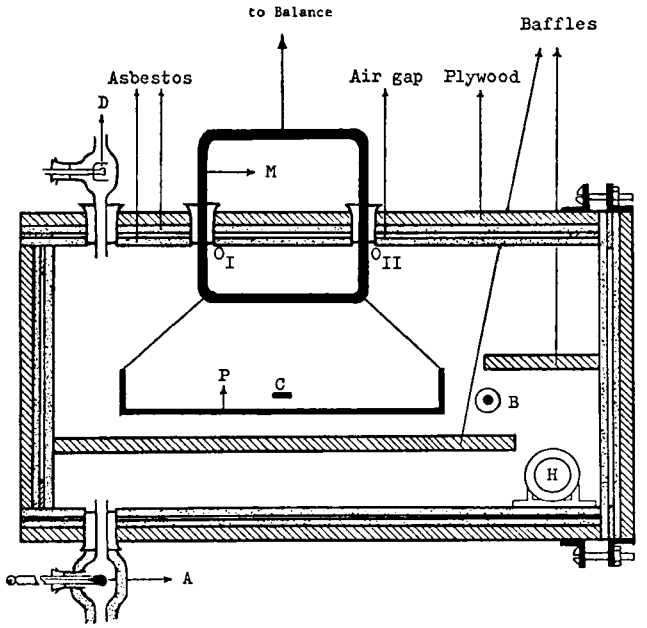

Figure 1—*Cross section of the dryer designed for this study. Key: H, heating coil; B and C, thermocouples; A and D, thermometers; M, metal loop; O_I and O_{II}, openings through the dryer; and P, drying pan.*

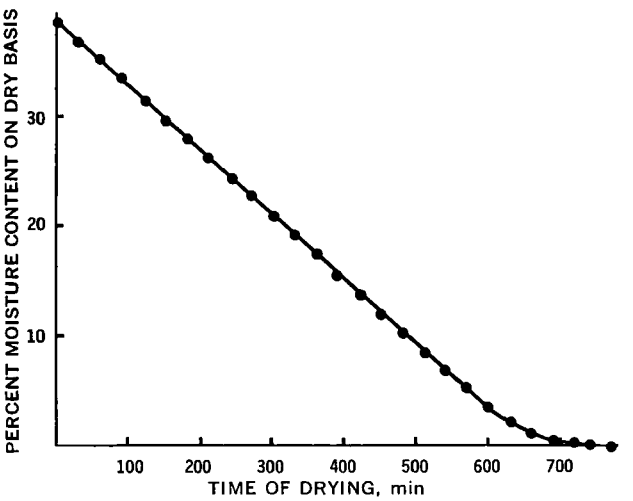

Figure 2—*Drying rate curve for sulfathiazole granulation prepared using distilled water as the binder and plotted as moisture content on dry basis* versus *time of drying.*

the tableting machines, little attention has been paid to the efficiency of the drying operation following the wet granulation. The high heat energy consumption involved in the drying of tablet granulations makes it a very expensive operation. This is particularly true for the last phases of the drying operation aimed at removing moisture in the range of 10.0–0.5% (w/w).

Even though the drying rates of various materials have been studied in the chemical industry (1–5), little effort has been made to understand the kinetics of drying of porous materials such as tablet granules. Pharmaceutical granulations can vary widely, based upon differences in porosity and the mechanism by which the moisture is held within them. Therefore, the drying rate kinetics of these pharmaceutical granulations must depend upon the physicochemical nature of the ingredients as well as the solutions of the binding agents used to form them.

Most binding agents used for wet granulations, such as starch paste, acacia mucilage, gelatin solution, simple syrup, methylcellulose solution, and corn syrup, are hydrophilic in nature. These binders increase the bulk density and reduce the porosity of the powder, thereby diminishing the effective surface area for evaporation. Hydrophilic binders also retard the rate of evaporation of moisture by lowering the vapor pressure of liquid moisture. In addition, the amount of moisture introduced into the granules and retained by them after drying varies greatly with different binding agents.

The purpose of the present investigation was to design experimental conditions to study the drying rate of the granules under controlled external conditions. Using such drying conditions, attempts were made to elucidate the comparative effect of some commonly recommended binding agents. These include starch (7, 8), gum acacia (7), corn syrup (7), gelatin (9), simple syrup (9), carboxymethylcellulose (10), and povidone (11, 12). Additionally, the effects of binder concentrations and diluents on the drying rates of the granulations were studied.

EXPERIMENTAL

Equipment Design—To study the rate of drying of granules based upon the internal mechanism of liquid flow and independent of external conditions of drying such as temperature, humidity, air flow rate, and state of subdivision, a special setup was required. An experimental apparatus was designed and fabricated, consisting of three sections: *(a)* dryer, *(b)* drying air humidity and flow rate control line, and *(c)* air heating chamber.

The dryer was a 54 × 46 × 30-cm well-insulated box (Fig. 1). The sides of the dryer were constructed from four layers consisting of (from outside to inside) 1.27 cm plywood, 0.64 cm asbestos, 0.32 cm air gap, and 0.64 cm asbestos. An additional insulation layer was added while studying the effect of physical properties of the granulations upon the rate of the first falling rate period.

The inner side of the dryer was sprayed with aluminum paint to cut down heat loss by radiation. The dryer was fitted with a reasonably quick opening door at one end. The rubber gasket was placed between the dryer and the door to make an airtight closure. To improve air circulation, the dryer contained horizontal baffles made of 1.27 cm plywood.

A square sample pan, P (30 × 30 × 3.8 cm), was suspended by its corners with a thick metal wire from two corners of a square-shaped metal loop, M (15 × 15 cm), made from a 0.64-cm diameter iron rod. The other two corners of the metal loop extended outside the dryer through the openings, O_I and O_{II}, in the upper side of the dryer. These two corners were connected to the weighing pan of the balance as shown in Fig. 1.

The two openings through which the loop M extended outside were fitted with rubber stoppers, each having a central hole equal to the diameter of the iron rod of the loop. These openings were always kept closed except when the weight was being recorded. A heating coil, H, connected in series to a variable resistor was placed inside to preheat the dryer to achieve the experimental conditions faster.

Thermocouples were placed at points B, C, and D in Fig. 1 and the temperature was read[1]. Thermocouple C was placed over the surface of the sample granules. The temperature at point A was measured using a mercury thermometer. Temperatures at points

[1] On a Tele-Thermometer, model 44, 12 channel, Yellow Springs Instrument Co., Inc., Yellow Springs, Ohio. It is capable of accurately measuring temperature differences of up to 1° F.

Table I—Initial and Final Moisture Contents, Critical Points, Slopes of Falling Rate Periods, and Drying Rates during Constant Rate Period of Sulfathiazole Granules Made with Various Binding Agents

Binding Agent	Initial Moisture Content, % w/w	Final Moisture Content, % w/w	Total Drying Time, min	Drying Rate during CRP[a]	Critical Points, % Moisture on Dry Basis		Slopes of Falling Rate Periods[b]	
					First Point	Second Point	First Slope	Second Slope
Distilled water	38.9	0.0	810	34.5	15.00	2.80	0.55	11.00
Starch paste, 10% (w/w)	37.5	0.3	875	31.7	20.05	2.50	0.45	11.00
Starch paste, 15% (w/w)	37.9	0.8	925	31.0	22.00	3.35	0.56	8.75
Acacia mucilage, 10% (w/w)	30.4	0.3	705	34.5	15.00	3.00	1.00	11.30
Acacia mucilage, 15% (w/w)	29.4	0.3	755	30.0	16.00	3.00	0.69	8.34
Gelatin solution, 10% (w/w)	28.7	0.8	840	30.5	22.00	2.50	0.63	10.61
Gelatin solution, 15% (w/w)	28.2	0.9	825	—	—[c]	3.00	0.52	9.30
Povidone NF solution, 10% (w/w)	29.0	0.2	755	28.0	12.00	2.00	0.67	11.00
Methylcellulose solution, 3% (w/w)	33.0	0.6	955	28.0	20.00	2.70	0.50	8.00
Simple syrup USP	11.2	0.1	525	—	—[d]	1.65	0.50	6.45
Corn sugar solution, 50% (w/w)	17.6	1.0	730	—	—[d]	2.80	0.88	6.50

[a] CRP = constant rate period [grams/(hour) (kilogram of dry solid)]. [b] Units are grams per hour kilogram. [c] Above 25.00 (projected). [d] Above initial moisture content.

A, B, C, and D will be referred to as heating chamber, inlet air, granules surface bed, and outlet air temperatures, respectively.

To obtain drying air at constant flow rate and humidity, incoming air from a blower was cooled in the range of from −10 to −15°, using a salt–ice bath to condense most moisture. Final traces of moisture were removed by bubbling the air through two successive containers of concentrated sulfuric acid. The air was then passed through two empty conical flasks plugged with glass wool to retain any suspended acid droplets, followed by passage through solid sodium hydroxide to remove the last traces of sulfuric acid. The air thus treated was considered perfectly dry[2].

This air was passed through a Rotameter[3] to measure the air velocity which could be adjusted by a Hoffman clamp. The resulting air was forced through an air heating chamber, which consisted of a metallic box, 15 × 15 × 18 cm, well insulated from the environment by a 1.90-cm layer of foam rubber. A heating coil controlled by a variable resistor was fitted inside the heating chamber to heat the air to a required temperature.

Sulfathiazole Granulation Studies—The granules were prepared by adding the binding agent to sulfathiazole powder in a blender mixer until a suitable wet mass was obtained. This mass was then forced through a 10-mesh standard screen. The initial moisture content of these granulations was determined using a moisture determination balance[4]. Enough wet granules were spread uniformly to provide a 1.27-cm bed over the sample pan P of the dryer. The pan was suspended inside the dryer, and a thermocouple was placed over the surface of the granules. The door of the dryer was closed and the dryer was heated to approximately 70° for 30 min, employing the heating coil H and the heated inlet air at a rate of 3.40×10^6 cm³/hr.

The electric current passing through the coil was then gradually reduced to zero, such that the inlet air temperature was stabilized at 50° and the weight of the entire suspended system (weighing assembly plus granules) was noted. From this point on, readings for the inlet air, granules surface bed, outlet air temperatures, and weight of the suspended system were recorded periodically until the weight of the suspended system was constant. The dryer was then opened and the final moisture content of the dried granules was determined using the moisture balance.

The data collected were used to calculate the drying rate [grams of moisture evaporated/(hour)(kilograms of dry solid)] and the amounts of moisture lost every 30 min during the drying operation for every batch of granulation. Each experiment was carried out at least twice. In most cases there was no statistically significant difference in the results, the range of variation being within ±1% (w/w) moisture content for the critical points. However, if the two experimental results differed significantly, the experiment was repeated until two consecutive experimental results fell within required limits.

Lactose Granulation Studies—The procedure employed to study lactose granulations was identical to that used for sulfathiazole granulations except for the drying conditions. In this case, the dryer was heated to approximately 60° for 30 min, employing the heating coil H and the heated inlet air at a rate of 2.83×10^6 cm³/hr after placing the granulation in the sample pan. The electric current passing through the coil H was then gradually reduced to zero, such that the inlet drying air was stabilized at 40°. The drying rate of lactose granulation was not studied at 50° because at this temperature lactose turned yellow, indicating a chemical change.

RESULTS AND DISCUSSION

When a solid is dried experimentally, the data usually obtained relating moisture content to time of drying can be plotted in various forms:

1. Moisture content (dry basis) *versus* time of drying
2. Drying rate [grams of moisture evaporated/(hour) (centimeters² of drying area)] *versus* moisture content [grams of free moisture/gram of dry solid]

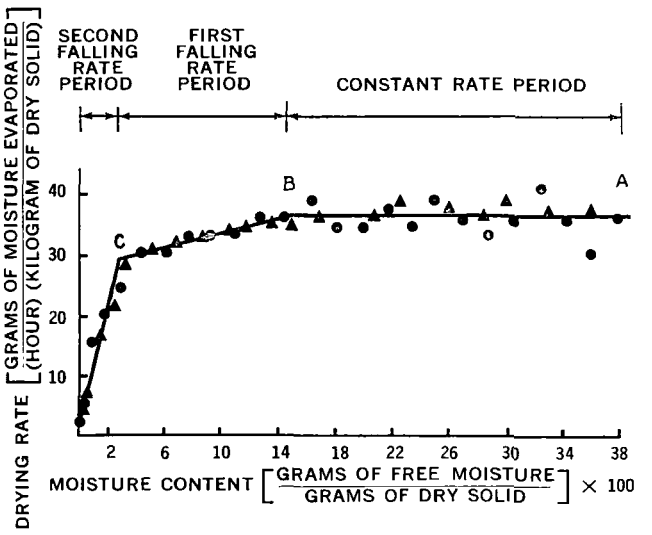

Figure 3—*Drying rate curve for sulfathiazole granulations prepared using distilled water as the binder and plotted as drying rate* [*grams of moisture evaporated/(hour)* (*kilogram of dry solid*)] versus *moisture content of granulation. Key:* ●, *points from Experiment 1; and* ▲, *points from Experiment 2.*

[2] Twenty cubic feet of the resulting air was passed through a drying tube containing Drierite and no increase in the weight of the drying tube was observed.

[3] Tube size R-6-25-B, Brooks Instrument Division, Hatfield, Pa.

[4] Model 6000, Ohaus Scale Corp., Union, N.J.

Table II—Initial and Final Moisture Contents, Critical Points, Slopes of Falling Rate Periods, and Drying Rates during Constant Rate Period of Lactose Granules Made with Various Binding Agents

Binding Agent	Initial Moisture Content, % w/w	Final Moisture Content, % w/w	Total Drying Time, min	Drying Rate during CRP[a]	Critical Points, % Moisture on Dry Basis		Slopes of Falling Rate Periods[b]	
					First Point	Second Point	First Slope	Second Slope
Distilled water	14.8	0.6	700	21.5	6.60	2.00	1.47	10.10
Starch paste, 10% (w/w)	19.5	1.5	780	20.7	11.50	3.00	0.95	8.70
Starch paste, 15% (w/w)	28.2	1.8	840	20.2	13.80	4.35	0.70	10.30
Acacia mucilage, 10% (w/w)	19.4	3.5	625	21.3	11.0	5.30	1.56	8.25
Acacia mucilage, 15% (w/w)	17.4	4.3	685	19.3	11.2	5.40	1.35	10.90
Gelatin solution, 10% (w/w)	19.2	3.0	840	17.8	10.8	4.50	0.91	7.50
Gelatin solution, 15% (w/w)	18.6	4.2	780	17.7	12.40	5.80	0.55	6.94
Povidone NF solution, 10% (w/w)	21.6	4.5	775	18.0	11.30	6.20	1.21	7.69
Povidone NF solution, 15% (w/w)	22.0	4.8	780	19.5	11.40	6.20	1.67	9.25
Methylcellulose, 30% (w/w)	17.2	3.7	600	17.3	9.6	4.45	1.00	7.08
Simple syrup USP	8.2	2.6	415	—	—	3.16	1.61	7.40
Corn sugar solution, 50% (w/w)	16.4	3.7	675	5.0	8.05	4.5	2.50	7.80

[a] CRP = constant rate period [grams/(hour)(kilogram of dry solid)]. [b] Units are grams per hour kilogram.

3. Drying rate [grams of moisture evaporated/(hour) (centimeters^2 of drying area)] *versus* time of drying

4. Drying rate [grams of moisture evaporated/(hour) (gram of dry solid)] *versus* moisture content [grams of free moisture/gram of dry solid]

The first method of plotting normally gives a smooth curve and shows no sharp breaks. The fact that the drying rate is subject to variation with time of drying and moisture content of solid can be better illustrated by the latter three methods. For example, when experimental data for sulfathiazole granulations, made using distilled water as the binder, were plotted using Method 1, a smooth curve was obtained (Fig. 2). However, when the same data[5] were plotted following Method 4, the curve showed three distinct phases of drying (Fig. 3).

The slopes of the straight lines in Fig. 3 were obtained using linear regression analysis. The drying rate curve showed a constant rate period segment, which is a straight line parallel to the X axis. This constant rate period was followed by two additional straight lines, representing the first and the second falling rate periods. The drying rate curve for lactose granulations was similar in nature (Fig. 4). The presence of three phases of drying could be explained based upon the mechanism of moisture movement within the solid phase during drying (1–4, 6).

All drying rate curves exhibited this typical drying pattern as described here. However, in some cases, the constant rate period was not observed since the initial moisture content of the granules was below the first critical moisture content. The critical points, the slopes of the falling rate periods, and the drying rates during constant rate periods obtained for each binder are presented in Tables I and II.

During the constant rate period, the rate of drying is constant since the surface conditions remain unchanged. The temperature of the bed, the humidity, the velocity, and the temperature of the drying air are constant. Furthermore, the fraction of wetted surface of the drying particles remains the same. Moisture movement within the solid is rapid enough to maintain a steady state such that the surface is always saturated with liquid moisture. The moisture diffusing to the drying air from the surface of the solids is immediately replaced by the moisture from the interior. The end of the constant rate period is reached when the concentration of the moisture in the solid corresponds to point B in Fig. 3. This point, termed the first critical point, initiates the first falling rate period.

After the first critical moisture content point has been reached, the rate of drying begins to fall off linearly with the moisture content of the solid. This falling rate period corresponds to the phase of the drying cycle when only part of the solid surface is wet because the internal moisture migration is inadequate to replace the moisture evaporated from the surface of the solids. As the liquid moisture is progressively removed from the solid, the fraction of the pore volume occupied by air increases and there is insufficient moisture left to maintain a continuous film across the pores. This initiates the second falling rate period (point C in Fig. 3). After this state is reached, evaporation of liquid moisture takes place inside the pores and then mass transfer occurs by a vapor diffusion process.

Since binding agents change the physical characteristics of the granulations, the curves were plotted as drying rate: grams of moisture evaporated/(hour) (kilogram of dry powder) *versus* moisture content (grams of free moisture/gram of dry solid). The percentage of moisture needed to prepare a suitable wet mass for granulation of lactose and sulfathiazole ranged between 8.3 and 28.2% (w/w) for lactose and 11.2 and 38.9% (w/w) for sulfathiazole. One commonly used binder, starch paste, introduced the maximum amount of moisture into the granules. More time and energy were required to dry the granules made with starch paste than the granules made with other commonly used binders such as acacia mucilage, solutions of povidone NF, and gelatin solution.

Of all the binders studied, simple syrup USP introduced the minimum amounts of moisture [8.2% (w/w) for lactose and 11.2% (w/w) for sulfathiazole]. These amounts were approximately one-

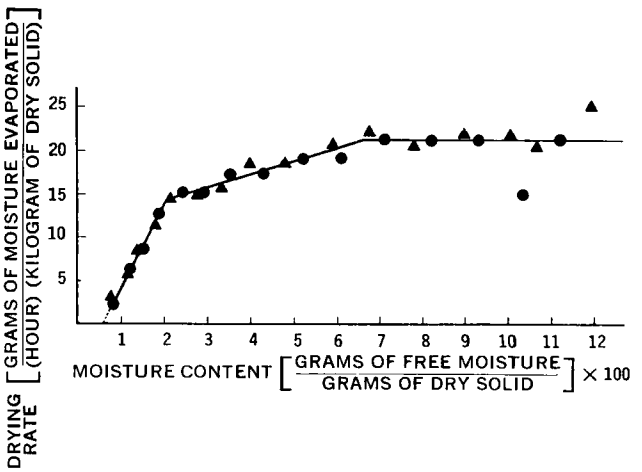

Figure 4—*Drying rate curve for lactose granulations prepared using distilled water as the binder and plotted as drying rate [grams of moisture evaporated/(hour) (kilogram of dry solid)] versus moisture content of granulation. Key: ●, points from Experiment 1; and ▲, points from Experiment 2.*

[5] Experimental data for two separate experiments for the same granulations were plotted on the same graph. This technique was used to minimize experimental errors.

third of those used for 15% (w/w) starch paste. Correspondingly, the time needed to remove the moisture from granules made with simple syrup was observed to be only about 40% of that needed for the granules made with 15% (w/w) starch paste. All of the other binders studied introduced moisture ranging between these two extreme values of 15% (w/w) starch paste and simple syrup USP.

The amount of moisture introduced into the granules during the granulation process also varied with the concentration of the solution or the suspension of the binding agent used. These amounts increased with increasing concentrations of binders in the case of starch paste, decreased with mucilage of acacia and gelatin solution, and remained almost constant for solutions of povidone NF. The increase in the initial moisture content of the granules in the case of starch paste is related to the considerable thickening that occurs with increasing concentrations of the paste. This thickening or reduced fluidity decreases the wetting ability of the paste.

The amount of moisture retained appears related to the affinity of granulation for water. With both lactose and sulfathiazole granulations, the binding agents increased the moisture-retaining capacity of the granules. The quantity of moisture retained by the granules increased with the increasing concentration of the binding agents used. Since moisture is often a major cause of deterioration of medicaments, the final moisture content of the granulations is usually a major concern for the industrial pharmacist.

The drying rates during the constant rate period were highest for granules prepared with distilled water as a binding agent, because it had the least effect on the bulk density and porosity of the chemical. The drying rate was the least for corn sugar solution. The rate of drying dropped by about 25% for granules made with corn sugar solution; 15% for granules made with 3% (w/w) methylcellulose, 10% (w/w) gelatin solution, 15% (w/w) gelatin solution, and 10% (w/w) povidone NF solution; and 5–10% for granules made with 15% (w/w) starch paste, 15% (w/w) acacia mucilage, and 15% (w/w) povidone NF solution.

In some cases (lactose granules made with simple syrup, gelatin solution, and corn sugar solution) the constant drying rate period was not observed since the first critical point apparently existed above the initial moisture content of the granules. In the case of sulfathiazole granulations prepared using gelatin solution, the economy of the drying process was greatly affected, since all moisture must be evaporated during the falling rate periods, which constitute more expensive phases of the drying process. When more concentrated solutions or suspensions of the binders were employed, most of the drying operation took place in the falling rate periods. This results due to a decrease in the granulations porosity whereby the moisture migration from the interior of the granules to the surface becomes more difficult so unsaturation of the surface occurs at earlier stages of drying.

The slopes of the first and second falling rate periods for lactose and sulfathiazole granules made with different binding agents represent the rates of decrease of drying rates in relation to decreasing moisture content of the granules. Higher values for these slopes represent a faster change in the lowering of the drying rate or a longer drying period. A comparative evaluation of binding agents for their effect on the slopes of falling rate periods as well as the effect of various physical parameters of the granulations upon this phase of drying will be the subject of future studies.

CONCLUSIONS

A laboratory size dryer as well as other accessories, designed and constructed to study the drying rate kinetics of porous granulations, was used to evaluate the effect of binders on the drying rates for lactose and sulfathiazole granules. The drying rate data obtained showed three distinct phases of drying, which were both qualitatively and quantitatively different for different binders and diluents.

Future uses of this setup will include studying the effect of physical properties of the granulations upon the drying rates and the mechanism of drying.

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Stability Study of Nitroglycerin Sublingual Tablets

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Abstract □ Nitroglycerin sublingual tablets were studied over a 1-year period to determine tablet stability in terms of loss of strength, uniformity of tablets, and degradation of the drug itself. Tablets from six different firms were analyzed by a semiautomated procedure. The samples included two molded tablets and four compressed tablets, ranging in age at the time of initial assay from 40 days to over 1 year. The results indicated that there is a loss of strength of nitroglycerin tablets and that refrigeration slows down this loss. The study also indicated that these tablets were stable during the year of testing in terms of tablet uniformity and degradation of nitroglycerin.

Keyphrases □ Nitroglycerin sublingual tablets—stability study, loss of strength, uniformity of tablets, and degradation of drug □ Tablets, sublingual nitroglycerin—stability □ Content uniformity—nitroglycerin sublingual tablets □ Stability—nitroglycerin sublingual tablets, loss of strength, uniformity of tablets, and degradation of drug

Much recent research has been devoted to the tableting, packaging, storage, and analysis of sublingual nitroglycerin tablets. In 1973, Fusari (1) reported that tablets that were uniform when manufactured developed increased individual tablet variation upon storage because of the volatilization and subsequent intertablet migration of nitroglycerin. It has been reported (1–3) that packing materials, such as cotton and rayon, in contact with nitroglycerin tablets absorb varying amounts of nitroglycerin.

In light of all that has been reported about the problems of packaging and storage of sublingual nitroglycerin, a study was carried out to determine the stability of sublingual nitroglycerin in tablets in terms of loss of strength, uniformity of tablets, and degradation of the drug itself.

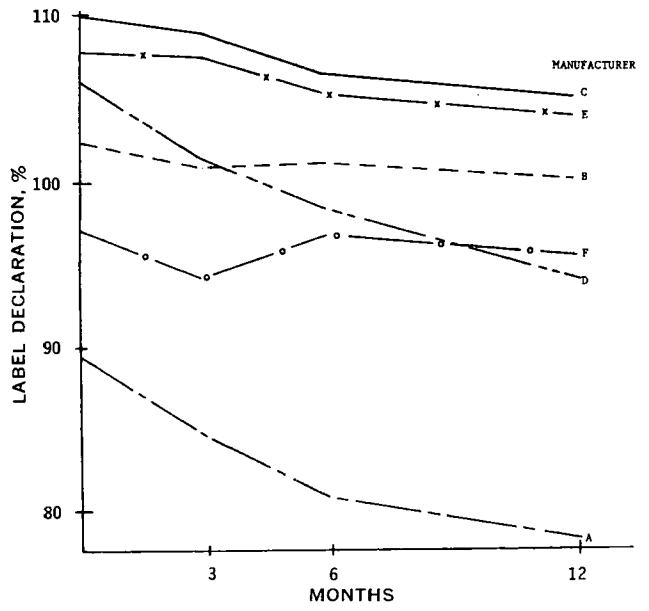

Figure 1—*Average of the 180 individual tablet assays (in percent of label declaration)* versus *time of storage after receipt.*

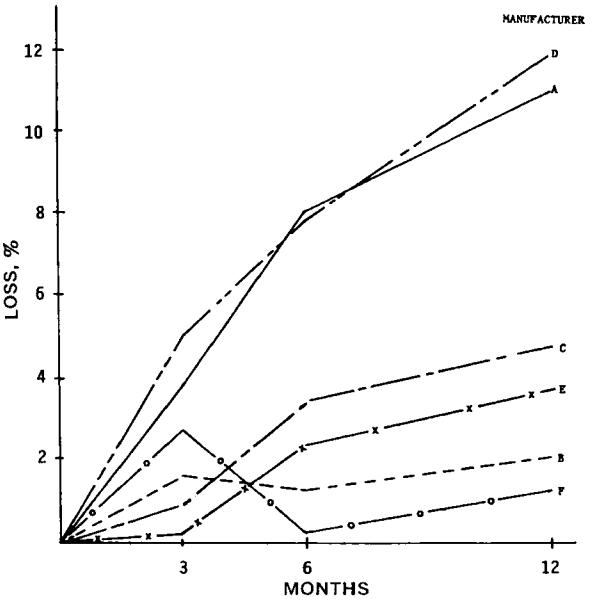

Figure 2—*Loss in percent of label declaration* versus *time of storage in this laboratory.*

One batch was sampled from each manufacturer of sublingual nitroglycerin. This sample consisted of 24 bottles from the batch most recently released by the firm's quality control department. The samples used were as follows.

Manufacturer A: 0.65-mg ($\frac{1}{100}$-gr) compressed tablets, manufactured December 24, 1971; 1000-tablet amber glass bottle, metal cap with paper liner either waxed or plastic covered, shrink-fit seal over bottle mouth, and cotton packing in bottle.

Manufacturer B: 0.6-mg molded tablets, manufactured November 16 to December 10, 1971; 100-tablet amber glass bottle, metal cap, and rayon packing.

Manufacturer C: 0.6-mg molded tablets, manufactured November 22 to December 7, 1971; 100-tablet linear polyethylene bottle, tin-plated screw cap with linear paraffin wax on liner, glassine paper seal on bottle, and rayon packing.

Manufacturer D: 0.4-mg compressed tablets, manufactured February 22 to February 26, 1971; 100-tablet amber plastic bottle, metal screw cap with liner, and cotton packing.

Manufacturer E: 0.43-mg ($\frac{1}{150}$-gr) compressed tablets, manufactured July 19 to August 9, 1971; 1000-tablet bottle; nine amber plastic with metal cap and 15 amber glass with plastic cap, both with shrink-fit seal and all with cotton packing.

Manufacturer F: 0.6-mg compressed tablets, released by quality control October 11, 1971; 1000-tab-

Table I—Average of 180 Tablets[a]

Manufacturer	Dosage	Approximate Age of Sample at Assay, days	Percent of Label Declaration		
			Average of 180 Tablets	Coefficient of Variation, %	Range
A	0.65 mg ($^1/_{100}$ gr)	40	89.4	5.0	77.3–109.2
			89.5 (T)	4.6	
			88.9 (M)	4.2	
			89.6 (B)	6.2	
		130	84.5	5.0	71.8–96.4
			84.1 (T)	5.7	
			84.8 (M)	5.3	
			84.5 (B)	4.1	
		220	81.4	5.9	70.8–97.3
			80.9 (T)	5.9	
			83.3 (M)	5.6	
			80.1 (B)	5.7	
		405	78.4	5.2	70.4–94.1
			79.0 (T)	5.3	
			78.4 (M)	4.9	
			77.7 (B)	5.5	
B	0.6 mg	72	102.4	4.9	89.2–115
			101.8 (T)	4.6	
			102.1 (M)	5.4	
			103.4 (B)	4.6	
		162	100.8	4.9	87.3–114.8
			101.9 (T)	4.6	
			100.1 (M)	5.2	
			100.6 (B)	5.2	
		252	101.2	5.2	86.1–117.1
			101.8 (T)	6.1	
			101.4 (M)	4.8	
			100.5 (B)	4.8	
		435	100.4	4.6	88.5–112.4
			100.8 (T)	4.7	
			100.9 (M)	4.5	
			99.4 (B)	4.7	
C	0.6 mg	80	110.0	7.7	74.5–134.0
			111.7 (T)	8.4	
			108.8 (M)	8.7	
			109.5 (B)	6.0	
		170	109.1	7.3	86.6–129.2
			108.8 (T)	8.0	
			110.3 (M)	5.8	
			108.1 (B)	8.0	
		260	106.5	8.0	78.7–134.8
			108.7 (T)	7.3	
			106.1 (M)	7.7	
			104.6 (B)	9.1	
		448	105.2	7.5	82.6–121.9
			104.9 (T)	8.1	
			104.5 (M)	6.9	
			106.0 (B)	7.5	
D	0.4 mg	387	106.5	6.1	89.3–129.7
			107.0 (T)	5.9	
			105.3 (M)	5.9	
			107.3 (B)	6.4	
		473	101.4	5.5	90.0–120.5
			101.7 (T)	6.1	
			100.9 (M)	5.2	
			101.5 (B)	5.1	
		568	98.6	5.2	88.9–117.4
			97.7 (T)	5.2	
			99.1 (M)	5.3	
			99.1 (B)	5.1	
		746	94.6	5.3	79.6–111.6
			94.8 (T)	5.5	
			93.6 (M)	5.4	
			95.4 (B)	5.0	
E[b]	0.43 mg ($^1/_{150}$ gr)	243	107.8	4.0	94.5–117.1
			107.4 (T)	4.1	
			107.8 (M)	3.8	
			108.3 (B)	4.0	
		334	107.6	3.5	103.2–127.4
			107.7 (T)	4.0	

(*continued*)

Table I—(*Continued*)

Manufacturer	Dosage	Approximate Age of Sample at Assay, days	Percent of Label Declaration		
			Average of 180 Tablets	Coefficient of Variation, %	Range
			108.0 (M)	4.7	
			107.1 (B)	3.7	
		426	105.4	4.2	87.0–117.2
			104.2 (T)	4.4	
			105.7 (M)	4.3	
			106.3 (B)	4.0	
		608	104.1	5.0	91.6–127.7
			101.6 (T)	4.5	
			104.5 (M)	5.1	
			106.2 (B)	5.4	
F[c]	0.6 mg	209	97.0	21.8	50.5–200.4
			96.7 (T)	17.7	
			94.9 (M)	23.2	
			99.4 (B)	24.6	
		302	94.2	18.8	54.5–145.7
			87.8 (T)	16.8	
			97.6 (M)	18.4	
			97.1 (B)	21.1	
		393	96.8	21.0	51.8–167.8
			97.1 (T)	22.6	
			92.6 (M)	19.7	
			100.8 (B)	21.1	
		577	95.8	22.9	47.3–167.6
			92.2 (T)	21.8	
			96.6 (M)	23.3	
			98.6 (B)	23.3	

[a] Sixty each from top (T), middle (M), and bottom (B) of the bottle at 0, 3, 6, and 12 months after receipt in this laboratory. [b] Batch failed USP XVIII tablet disintegration test. [c] Batch failed USP XVIII content uniformity requirement.

let amber glass bottle, metal screw cap with shrink-fit seal-closures, and cotton packing.

EXPERIMENTAL

Analytical Protocol—Six of the 24 bottles in each batch were analyzed within 1 month of receipt. From each of the six freshly opened containers, 10 tablets were sampled from the top layer, 10 from the middle layer, and 10 from the bottom layer (180 tablets total). These 18 groups of tablets were kept discrete and traceable to the point of sampling (bottle and layer).

The remaining 18 bottles from the batch were divided into three groups of six each for subsequent analyses. The subsequent analyses were performed 3, 6, and 12 months after the date of the original analysis and utilized the identical sampling technique.

Table I shows the average of these 180 assay results together with the averages of the 60 tablets from the top, middle, and bottom of the six bottles. Figure 1 is a graphic representation of the average assay of the 180 tablets in relation to the time of storage of the samples after receipt. Figure 2 shows the percent loss of the six products based on the average of the 180 individual tablet assays.

The six containers opened for the original analyses (zero time) *only* were treated additionally as follows. After initial analysis, the bottles were tightly reclosed. Three bottles were stored at 5°, and the remaining three bottles were stored at ambient temperatures ranging from 15.5 to 33°. After 3, 6, and 12 months, 11 tablets were sampled at random from each of six containers, and the containers were tightly reclosed after each withdrawal.

Ten of the 11 tablets from each container were individually analyzed by the same procedure used for the original analysis; the remaining tablet from each bottle was analyzed by TLC. Table II shows the average assays for tablets from the three bottles stored at 5° (10 from each bottle) and the three bottles (10 from each bottle) stored at 12.8–29.4°. These analyses were made on the same bottle of tablets each of the assay times of 0, 3, 6, and 12 months. Figure 3 shows the difference in percent of label claim between the averages of those stored at 5° and those stored at room temperature.

Bottle packing material and cap liners were analyzed for nitroglycerin by quantitative TLC to see if the loss in strength could be attributed to adsorption onto the packing material. Table III shows the results of the TLC analysis performed on freshly opened bottles.

Principles—The nitroglycerin tablet is disintegrated with a small amount of water and then extracted with a suitable aliquot of isooctane. A portion of the isooctane extract is sampled by an automated analyzer system, which hydrolyzes the nitroglycerin with tetramethylammonium hydroxide in 1-propanol, yielding 2 moles of nitrite ion from each mole of nitroglycerin (4).

p-Chloroaniline in propanol is diazotized by the nitrite formed; the diazotized product is then coupled with *N*-(1-naphthyl)ethylenediamine in 1-propanol. The absorbance of the resultant color is determined at 550 nm.

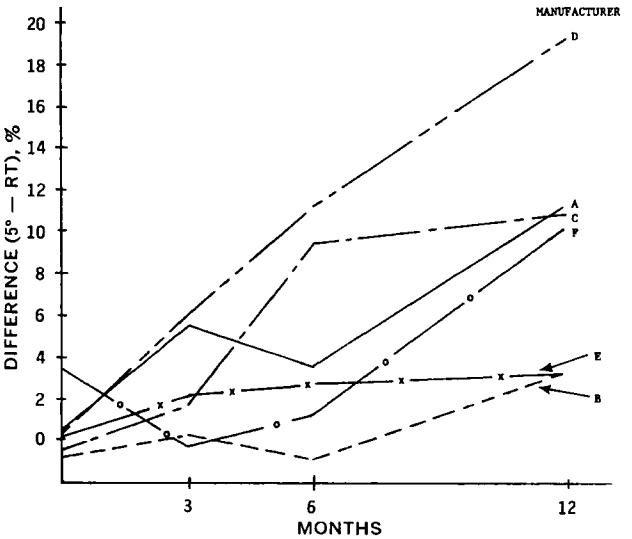

Figure 3—*Difference in percent of label declaration between tablets stored at 5° and room temperature (RT) versus time of storage in this laboratory.*

Table II—Summary of Analysis of Subsamples 1–3 (Refrigerated) and 4–6 (Room Temperature)

Manufacturer	Dosage	Approximate Age of Sample at Assay, days	Subsample	Percent of Label Declaration: Average (Number of Tablets)	Percent of Label Declaration: Coefficient of Variation, %
A	0.65 mg ($^1/_{100}$ gr)	40	1–3	89.6 (90)	5.3
			4–6	89.1 (90)	4.8
		130	1–3	90.6 (30)	6.5
			4–6	85.1 (30)	5.3
		220	1–3	87.8 (30)	6.3
			4–6	84.3 (30)	5.5
		405	1–3	87.5 (30)	4.3
			4–6	76.3 (30)	5.1
B	0.6 mg	72	1–3	102.0 (90)	4.8
			4–6	102.8 (90)	4.6
		162	1–3	101.8 (30)	6.5
			4–6	101.6 (30)	3.6
		252	1–3	99.3 (30)	4.3
			4–6	100.2 (30)	5.3
		435	1–3	101.0 (30)	6.6
			4–6	97.9 (30)	4.6
C	0.6 mg	80	1–3	109.7 (90)	7.6
			4–6	110.2 (90)	8.1
		170	1–3	107.3 (30)	7.7
			4–6	105.6 (30)	10.2
		260	1–3	113.7 (30)	7.9
			4–6	104.3 (30)	10.7
		435	1–3	103.3 (30)	10.0
			4–6	92.3 (30)	13.5
D	0.4 mg	22	1–3	106.7 (90)	6.1
			4–6	106.3 (90)	6.0
		108	1–3	105.3 (30)	5.2
			4–6	99.4 (30)	5.0
		203	1–3	103.1 (30)	5.9
			4–6	92.0 (30)	5.0
		381	1–3	101.7 (30)	5.5
			4–6	82.7 (30)	5.7
E	0.43 mg ($^1/_{150}$ gr)	243	1–3	107.9 (90)	3.5
			4–6	107.7 (90)	4.4
		334	1–3	108.9 (30)	3.5
			4–6	106.8 (20)	4.0
		426	1–3	109.3 (30)	4.0
			4–6	106.6 (20)	4.3
		608	1–3	107.8 (30)	3.2
			4–6	104.6 (20)	2.9
F	0.6 mg	209	1–3	99.7 (90)	22.1
			4–6	94.3 (90)	21.5
		302	1–3	96.5 (30)	22.8
			4–6	96.8 (30)	24.7
		393	1–3	97.0 (30)	20.7
			4–6	95.8 (30)	17.1
		577	1–3	101.7 (30)	25.7
			4–6	91.7 (30)	17.9

Apparatus—The automated analyzer[1] system consisted of a liquid sampler II equipped with stainless steel cover plate[2], a proportioning pump I, a colorimeter equipped with either an 8- or a 15-mm tubular flow cell (depending on drug dosage level) and 550-nm filters, and a single-pen recorder[3], linear in transmittance and provided with paper printed in absorbance units, moving at 46 cm (18 in.)/hr. Figure 4 is a manifold diagram showing the coils, tubing, and fittings.

Reagents—The following were used:

Nitroglycerin Adsorbate—About 10% nitroglycerin adsorbed on lactose, standardized against potassium nitrate using the AOAC total nitrate method (5).

Isooctane—ACS reagent grade 2,2,4-trimethylpentane.

1-Propanol—ACS reagent grade.

Tetramethylammonium Hydroxide Solution—Prepared by diluting 25 ml of aqueous 10% tetramethylammonium hydroxide to 1000 ml with 1-propanol.

p-*Chloroaniline Solution*—Prepared by adding 500 mg to 100 ml of hydrochloric acid, shaking the mixture to disperse, diluting to 1000 ml with 1-propanol, and shaking to dissolve. A fresh solution should be prepared weekly.

N-*(1-Naphthyl)ethylenediamine Dihydrochloride Solution*—Prepared by adding 1 g to 5 ml of water, shaking the mixture to disperse, adding 5 ml of hydrochloric acid, diluting to 1000 ml with 1-propanol, and shaking to dissolve. A fresh solution should be prepared weekly.

Nitroglycerin Standard Solution—(a) For analysis of 0.3 and 0.6 mg/tablet: An amount of adsorbate equivalent to approximately 12 mg of nitroglycerin is accurately weighed, transferred to a 1000-ml volumetric flask containing 400 ml of isooctane, shaken intermittently for 15 min, diluted to volume with isooctane, and

[1] AutoAnalyzer, Technicon Corp., Tarrytown, NY 10591
[2] Technicon No. 127-B029.
[3] Bristol.

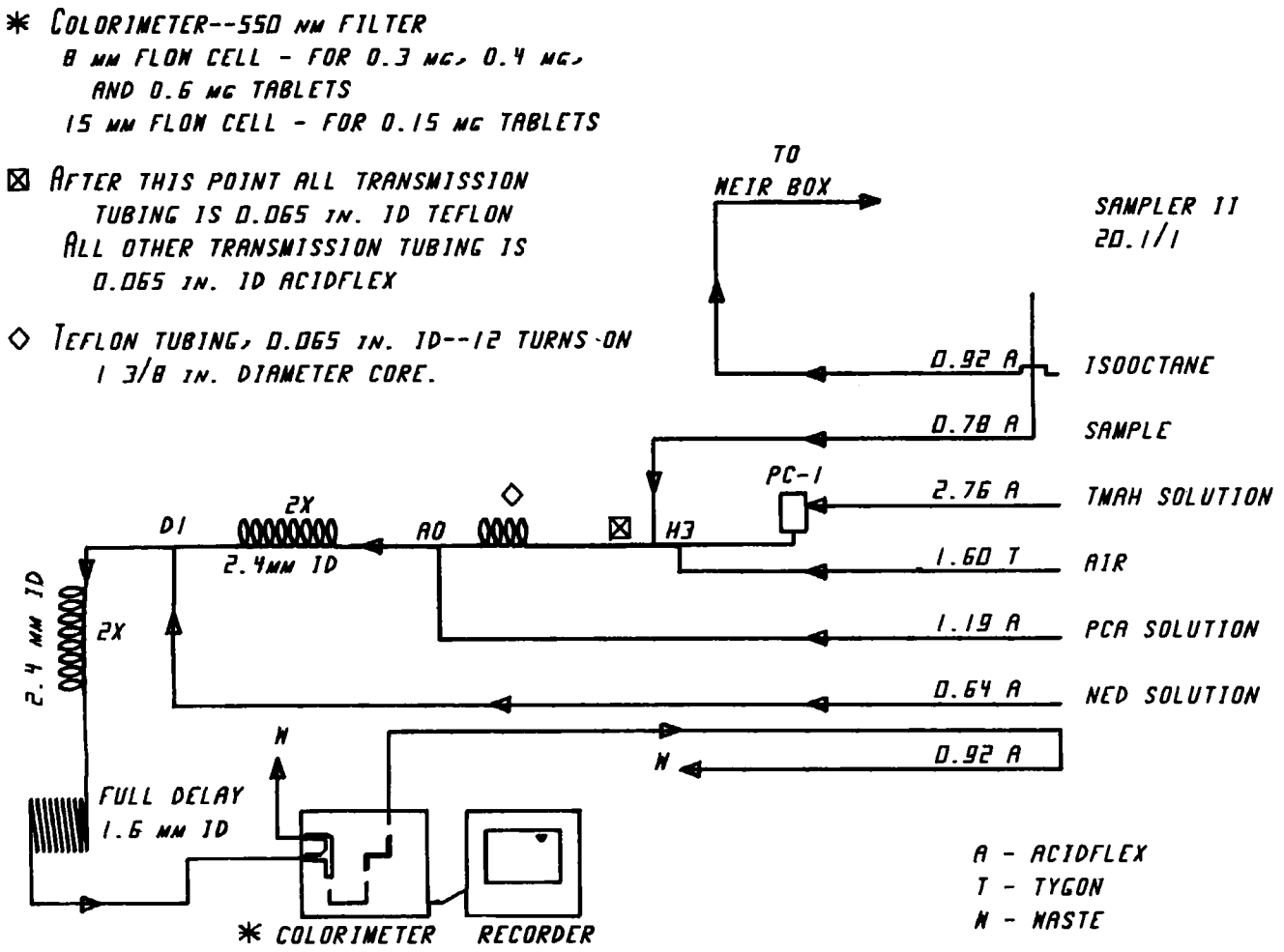

Figure 4—*Manifold diagram for semiautomated method of analysis for nitroglycerin tablets.*

mixed. Just before use, a 50-ml aliquot is shaken with 1 ml of water for 1 min, and the clear isooctane layer is decanted into the automated analyzer cup.

(b) For analysis of 0.4 mg/tablet: An amount of adsorbate equivalent to 16 mg of nitroglycerin is accurately weighed and treated as in *(a)*.

(c) For analysis of 0.15 mg/tablet: Solution *a* (25.0 ml) is transferred to a 125-ml glass-stoppered erlenmeyer flask; then 25.0 ml of isooctane is added, and the solution is mixed. Just before use, it is shaken for 1 min with 1 ml of water, and the clear isooctane layer is decanted into the automated analyzer cup. The dry isooctane solutions are stable for at least 2 months if kept tightly stoppered.

Sample Preparation—Single tablets are disintegrated with 0.5 or 1.0 ml of water in a glass-stoppered flask, 25 or 50 ml of isooctane is added, and the solution is shaken mechanically for 15 min. The amounts of solvents necessary per dosage level are:

dosage, mg	water, ml	isooctane, ml
0.6	1.0	50
0.4	0.5	25
0.3	0.5	25
0.15	0.5	25

The decanted isooctane extract is sampled from 8.5-ml polyethylene cups by the sampler at a rate of 20 samples/hr with a sample-to-wash ratio of 1:1. The sample stream is mixed with a stream of tetramethylammonium hydroxide solution and air in a Teflon coil; *p*-chloroaniline solution is added and mixed into the resulting stream, followed by *N*-(1-naphthyl)ethylenediamine dihydrochloride solution. The stream is delayed with a full delay coil and then passed through a colorimeter having 550-nm filters.

An 8-mm flow cell is used for dosage levels of 0.3, 0.4, and 0.6 mg; a 15-mm flow cell is used for a dosage level of 0.15 mg. The sampling pattern is two standards, five samples, one standard, five samples, one standard, five samples, *etc.* The first standard on each sample wheel is ignored in calculations because some absorption takes place in the tubing. A stainless steel sample cover plate must be used, and the sample solutions should not stand in the cups any longer than necessary before sampling because of evaporation of solvent.

TLC—*TLC Plates*—Silica gel G[4] (20 × 20 cm) plates were prepared (for the quantitative visual estimation method) by scooping out nine channels (2 mm wide) to give 10 bands of thin layer 18 mm wide.

Silica gel GF[4] (20 × 20 cm) prescored plates were prepared (for the quantitative colorimetric estimation by TLC) by scooping out three channels (2 mm wide, each channel at score mark on glass plate) to give four bands of thin layer 38 mm wide.

Developing Solution—Benzene–ethyl acetate–acetic acid (16:4:1) (6) was used.

Spray Reagent—A solution of 1% *N*-(1-naphthyl)ethylenediamine dihydrochloride in methanol was prepared. Visualization was achieved by exposing the plates to UV light after spraying.

Standard—Potassium nitrate standard solution and standard preparation were prepared according to USP XVIII.

Plate Standard[5]—A mixture of nitroglycerin, 1,3-dinitroglycerin, 1,2-dinitroglycerin, and mononitroglycerin in alcohol was prepared and applied to the TLC plates using disposable pipets.

Packing Material—The plugs of packing material from the bot-

[4] Supplied by Analtech, Brinkmann Instruments, Inc., Westbury, NJ 11590

[5] Supplied by Dr. Philip Needleman, Washington University School of Medicine.

Table III—Results of TLC Analysis of Packing Material and Cap Liners

Manufacturer	Approximate Age of Material at Assay, days	Material	Found, mg		
			Nitroglycerin	1,3-Dinitroglycerin	1,2-Dinitroglycerin
A	220	Cotton packing	1.03	0.07	0.05
		Liner	—	—	—
	405	Cotton packing	1.1	0.17	0.06
		Liner	0.01	—	—
B	252	Rayon packing	0.03	—	—
		Liner	0.003	—	—
	435	Rayon packing	0.05	—	—
		Liner	0.01	—	—
C	260	Rayon packing	0.26	0.05	0.01
		Liner	0.01	—	—
	448	Rayon packing	0.31	0.098	0.02
		Liner	0.004	—	—
D	203	Cotton packing	1.4	0.01	0.01
		Liner	0.27	—	—
	381	Cotton packing	2.0	0.04	0.03
		Liner	0.22	—	—
E	243	Cotton packing	0.274	0.01	0.01
		Liner	0.014	—	—
F	302	Cotton packing	2.8	0.08	0.63
		Liner	0.05	—	—
	393	Cotton packing	7.1	0.12	0.1
		Liner	0.02	—	—
	577	Cotton packing	8.8	0.22	0.2
		Liner	Trace	Trace	Trace

tles were placed in a 5-ml hypodermic syringe with about 3 ml of methanol and agitated by rotating between the fingertips. They were then injected into disposable pipets containing a small amount of powder[6] to control the flow of liquid to the TLC plate. Pipets were standing at right angles to the plate with their tips touching the plate. Four replacement washes with methanol were applied.

Wax Paper Liners—The wax paper liners from the bottles in the sample were attached to wires (by rolling them around the wire) and churned up and down in the disposable pipets containing about 0.5 ml of methanol (pipet tips were plugged with small amounts of powder[6] to control the flow of liquid to the TLC plate). Pipets were standing at right angles to the TLC plate with their tips touching the plate. Four replacement washes were applied.

Plate Development—The plate was developed to about 4 cm in methanol, air dried, developed an additional 10 cm with developing solution, and air dried again.

Quantitative Visual Estimation—The developed unscored TLC plates were sprayed and visualized after exposure to UV light for several minutes and by visual comparison with standards on the same plate.

Colorimetric Estimation by Quantitative TLC—Each of the developed, scored TLC plates was snapped along score marks into four sections. Sections containing standards were sprayed and visualized under UV light. The broken plates were reassembled, and the thin-layer areas on sample and blank sections corresponding to the visualized standard nitroglycerin spot were removed and placed in 100-ml volumetric flasks with 1 ml of acetic acid and allowed to stand for 30 min. Two milliliters of phenoldisulfonic acid was added and the flask was allowed to stand another 15 min; then 50 ml of water and 10 ml of ammonium hydroxide were added.

After cooling to room temperature, the solution was diluted to 100 ml with water and scanned in a spectrophotometer from 600 or 550 nm to 350 nm against a reagent blank. Blank solutions of adsorbent were carried through the analysis and subtracted.

Quality Control of Analytical System—It was imperative that some quality assurance checks be made on the automated method during the year of testing. The 10% nitroglycerin adsorbate was used throughout the study and was assayed periodically to ensure that it had not lost nitroglycerin. It first assayed (January 1972) at 10.1%. Subsequent assays in June 1972 and February 1973 were also 10.1%. In each case the total nitrate method was used with potassium nitrate as the standard.

To ensure that the system was performing satisfactorily, a composite of 0.6-mg nitroglycerin tablets consisting of ground tablet material was analyzed throughout the year. This composite was kept in a refrigerator at 5°. It was analyzed 10 times before the start of the study, and the percent coefficient of variation of the system was 0.50. As can be seen from the data in Table IV, the system had good precision and gave essentially the same assay results over the year.

Table IV—Analysis of 0.6-mg Quality Control Composite

Date	Percent Found[a]	*SD*	Coefficient of Variation, %
January 1972	106.4 (10)[b]	0.53	0.50
February 4, 1972	106.1	—	—
February 22, 1972	108.2	—	—
February 23, 1972	108.6 (2)	—	—
May 3, 1972	105.0 (3)	—	—
May 9, 1972	106.1	—	—
August 10, 1972	106.7 (3)	—	—
November 27, 1972	107.1 (8)	0.78	0.73
December 11, 1972	106.2 (8)	0.82	0.77
February 1, 1973	104.7 (4)	—	—
February 7, 1973	107.0 (5)	—	—
February 14, 1973	106.1	—	—
February 20, 1973	104.9 (7)	—	—
May 8, 1973	106.0 (7)	—	—
Average of assays during study of February 4 to May 8	106.2 (51)	1.26	1.19

[a] Based upon label declaration. [b] Values in parentheses are numbers of assays performed on that date.

[6] SilicAR TLC 4 GF, Mallinckrodt Chemical Works, St. Louis, MO 63160

Table V—Analysis of Variance on Averages of 180 Assays

Months	Manufacturer A	B	C	D	E	F
0	89.4	102.4	110.0	106.5	107.8	97.0
3	84.5	100.8	109.1	101.3	107.6	94.2
6	81.4	101.2	106.5	98.6	105.4	96.8
12	78.4	100.4	105.1	94.6	104.1	95.8
Observed F ratio	200.54	5.12	13.15	144.42	26.70	0.74
Significant	Yes	Yes	Yes	Yes	Yes	No
Critical value $F_{0.95}$ (3, ∞) = 2.60						

Table VI—Analysis of Variance on Room Temperature Average Assay (RTA) *versus* Refrigerated Average Assay (RFA)

	Manufacturer A	B	C	D	E	F
3 Months						
RTA	85.1	101.6	105.6	99.4	106.8	96.8
RFA	90.6	101.8	107.3	105.3	108.9	96.5
Observed F	16.51	0.0102	0.452	19.9	3.153	0.0025
Difference significant	Yes	No	No	Yes	No	No
6 Months						
RTA	84.3	100.2	104.3	92.0	106.6	95.8
RFA	87.8	99.3	113.7	103.1	109.3	97.0
Observed F	6.83	0.618	12.830	62.94	4.513	0.0562
Difference significant	Yes	No	Yes	Yes	Yes	No[a]
12 Months						
RTA	76.3	97.9	92.3	82.7	104.6	91.7
RFA	87.5	101.0	103.3	101.7	107.8	101.7
Observed F	127.91	4.481	16.511	204.6	11.379	2.5996
Difference significant	Yes	Yes	Yes	Yes	Yes	No[a]
Critical value $F_{0.95}$ (1, 60) = 4.00						

[a] Note large coefficient of variation percent in Table II for this batch.

RESULTS AND DISCUSSION

The results shown in Table I indicate a loss in strength of the nitroglycerin with age, with Manufacturers A and D's products showing substantial losses (Figs. 1 and 2). A one-way analysis of variance (one variable) (7) was performed to test if the difference in the results between the time periods was significant. Table V shows the averages for the 180 assays for that time period and the observed F ratio calculated using one-way analysis of variance. The only sample that did not show a statistical difference in the means was Manufacturer F's product.

The data in Tables I and II also indicate that there was no increase in intertablet variation throughout the year in samples stored in the refrigerator and at room temperature, as evidenced by the coefficient of variation of the tablets analyzed for the test period. The range of the assays remained about the same for each assay period, with the highs being no higher or the lows lower. These results are not in direct conflict with the findings in the recent publications of Fusari (1, 8). Fusari's data indicate that the increase in variability develops within 1 month of manufacture. The batches used in this study were more than 1 month old before the authors' initial assay. In addition, four of the six batches under study here were compressed tablets. Fusari referred only to molded tal ets.

The results shown in Table II indicate a difference in analysis of tablets from bottles stored at 5° and at room temperature (Fig. 3). An analysis of variance was performed to test if the difference between the average assays at each time was statistically significant. Table VI shows the averages of the 30 assays from the three bottles stored in the refrigerator and the three bottles stored at room temperature, as well as the observed F ratio. Only Manufacturer F's product failed to show a significant difference at 12 months. Manufacturers A and D showed a statistically significant difference as early as 3 months, Manufacturers C and E showed a statistically significant difference at 6 months, and Manufacturer B did not show a difference until 12 months.

An additional statistical analysis of variance was performed on the data in Table I to determine whether the drug stratified between different levels in the bottles. No stratification was found. No degradation of nitroglycerin to 1,3-dinitroglycerin, 1,2-dinitroglycerin, or the mononitroglycerins was observed in the tablets by TLC.

Table III shows the results of the analysis for the nitroglycerin in the cap liners and packing materials. As reported earlier, the bottle packing absorbed varying amounts of nitroglycerin; cap liners absorbed a lesser amount.

In summary, the data presented here show:

1. There is a loss of strength of nitroglycerin tablets from previously unopened bottles upon storage.
2. Refrigeration of nitroglycerin tablets slows down the loss of nitroglycerin.
3. The nitroglycerin tablets used in this study, four compressed tablets and two molded tablets, were stable during the year of testing in terms of uniformity of tablets and degradation of the drug.

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Automated Conductimetric Titrimeter: Use in Studying Ionic Solute–Solute Interactions

G. I. MUKHAYER, S. S. DAVIS [x], and ERIC TOMLINSON

Abstract □ An automatic conductimetric titration apparatus is described in which the delivery of titrant is effected using a motorized automatic syringe having a wide range of injection rates. Two applications of the apparatus are described: *(a)* determination of the CMC of cationic and anionic surfactants, and *(b)* study of the interaction of an organic anion (sodium lauryl sulfate) and organic cations (phosphonium salts). The accuracy and precision of results using this apparatus are superior to results obtained by the tedious manual method.

Keyphrases □ Titrimeter, automated conductimetric—design, application to studying solute–solute interactions □ Syringe, motorized automatic—component of automatic conductimetric titration apparatus, application to solute–solute interactions □ Conductimetric titrations—automatic apparatus described, applied to solute–solute interactions

The physical property of solution conductance is well established, and conductimetric titration measurements can be used for various purposes. In pharmacy such measurements find application in studies on association and micellization, interaction (*i.e.*, complexation) between species of opposite charge, and analytical quality control. However, the usual method of addition of titrant or complexing agent to the titration vessel is tedious, and the results are often subject to experimental inaccuracies.

A conductimetric titration of one ion against another of opposite charge will provide information on ion-pair formation, complexation (as described by the solubility product), and, if one ion is surface active, the effect of the added species (counterion) on micelle formation. In addition, a study of the interaction of a large organic ion with congeneric members of a series of ions of opposite charge can lead to the calculation of thermodynamic quantities and, subsequently, the derivation of extrathermodynamic parameters (1) for use in linear free energy relationships to examine structure–activity correlations by regression analysis.

Studies on the variation of the free energy term with temperature should lead to an insight into enthalpy and entropy contributions to the interaction and, in consequence, the processes responsible for such complex formation. Because small errors in measurement of the solubility product (and any association constant) will lead to greater errors when the free energy–temperature relation is differentiated (2), an essential requirement of measurement is that the experimental data are of high accuracy. Such accuracy cannot be obtained with manual or semiautomatic methods.

Thus, an accurate automatic conductimetric titration apparatus was developed, using commercially available components, which is suitable for pharmaceutical systems. Semiautomatic conductimetric titrimeters for use in analytical measurements were described previously (3, 4).

EXPERIMENTAL

Apparatus (Fig. 1)—A conductivity bridge[1], which is continuously and automatically balanced, is connected to a dip-type conductivity cell contained in a thermostated beaker. The temperature is maintained constant by water pumped from a water bath and cooler unit. The titration mixture is agitated constantly by a magnetic stirrer. The measured conductivity, which can be read directly from the bridge, is normally recorded in the form of a continuous trace on a chart recorder.

In titrations where an external standard is used, a similar thermostated beaker is connected to the water bath; a dip cell, which has a comparable cell constant (±0.01) to the other cell, is connected to the external standard terminal of the bridge. The stirring conditions in the external standard are maintained the same as in the unknown during the titration.

The delivery of the titrant is maintained constant using a motorized automatic syringe[2], which has a wide range of delivery rates according to the capacity of the special syringes used. Where an external standard is used, two syringes of the same capacity are employed and driven simultaneously at the same rate.

Calibration—The conductivity bridge was trimmed and calibrated as described in the instrument manual.

The automatic motorized syringe equipment is supplied with special syringes, consisting of a calibrated glass barrel and a steel plunger fitted with a rubber gasket. The accuracy and reproducibility of the volume delivered from the syringes were tested by two techniques: *(a)* the syringe was allowed to drain into a dry, previously calibrated volumetric flask of a suitable volume; and *(b)* the syringe was filled with a potassium chloride solution of known conductivity and this was titrated into a blank of double-distilled water of known volume. The conductivity was recorded as a func-

[1] Wayne-Kerr B642 universal bridge, Wayne-Kerr, Surrey, England.
[2] R. Braun, Melsungen, West Germany.

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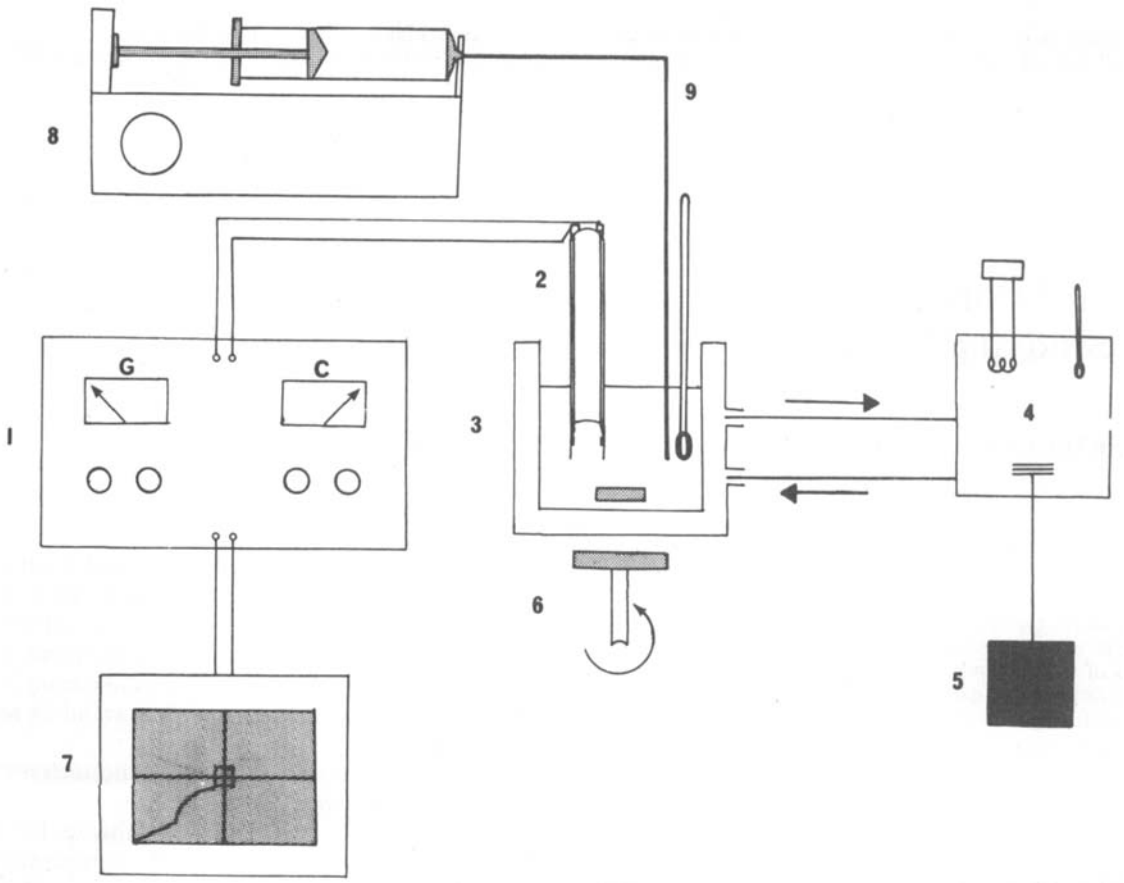

Figure 1—*Schematic diagram of automatic conductimetric titrimeter. Key: 1, conductivity bridge; 2, dip-type conductivity cell; 3, thermostated beaker; 4, water bath; 5, cooler unit; 6, magnetic stirrer; 7, chart recorder; 8, motorized automatic syringe; and 9, stainless steel cannula.*

tion of the added volume. The variation as compared to the figures supplied by the manufacturer was ±0.03%.

The specific conductance at any instant of the titration can be calculated from the following equation:

$$K_{spt} = (K_i + K_t)F_tC_{cell} \qquad \text{(Eq. 1)}$$

where K_{spt} = specific conductance at time t of the titration; K_i = initial conductance in the titration vessel at zero concentration of titrant; K_t = recorded conductance at time t; C_{cell} = cell constant; and F_t = dilution factor at time t where $F_t = [(V + V_t)/V]$, where V = initial volume, and V_t = volume delivered by the syringe.

Average variations of the specific conductivity obtained from the automatic procedure as compared with a calibration curve made for standard solutions of the electrolyte were ±0.1%.

In titrations using the external standard technique, the syringes and the dip cells were matched by the electrolyte method alone. The two beakers utilized were filled with equal volumes of double-distilled, deionized water, and the two syringes were filled with equal volumes of the same electrolyte solution. The magnetic stirrers were adjusted to give the same rate of stirring; the conductivity bridge was trimmed, set, and calibrated as before. When properly balanced, there was no change in the recorded conductivity when the same electrolyte solution was titrated into two different beakers.

Applications—*Determination of Critical Micelle Concentration (CMC)*—A plot of specific conductivity against concentration for a detergent solution leads to a change in the slope obtained in the region of the CMC. Experimental points may be considered to lie along two straight lines, the extrapolated intersection of which can be taken as the CMC (5). For these studies, the conductivity was recorded as a function of volume when surfactant solutions (benzalkonium chloride[3] and sodium lauryl sulfate[4]) were titrated against 50.0 ml of double-distilled, deionized water ($K_i < 1.0 \times 10^{-6}$ mho/cm at 25°).

Interaction between Large Organic Ions of Opposite Charge—The interaction between sodium lauryl sulfate and various phosphonium salts[5] in an aqueous medium was studied in a range of concentrations. The phosphonium compounds were recrystallized from ethanol before use.

A phosphonium salt solution (50.0 ml) was contained in the titration beaker, and a 1×10^{-1} M solution of sodium lauryl sulfate was added slowly from the syringe.

External Standard Technique for Following Complexation—The interaction between two ions and the subsequent solubilization of the complex can be studied to an even higher degree of precision using an external standard technique. An extra thermostated beaker containing 50.0 ml of conductivity water was fitted with a dip-type conductivity cell that had a similar cell constant to the other cell (1.40 *versus* 1.39). This was connected to the external standard side of the conductivity bridge. The other thermostated beaker contained the solution of phosphonium compound. The conductivity bridge was then balanced to give a null point reading. The sodium lauryl sulfate solution was then titrated into the two beakers simultaneously.

RESULTS AND DISCUSSION

CMC—It has long been established that the antibacterial action of the quaternary ammonium salts is related to their physical

[3] Sterling-Winthrop Research Laboratories, Rensselaer, N.Y.
[4] Specially purified grade, British Drug Houses, Poole, England.
[5] Maybridge Chemical Co., Cornwall, England.

properties and, in particular, their relative surface activity to their relative lethal capacity (6). Commercially available benzalkonium chloride products are a mixture of alkyl benzyldimethylammonium salts comprised of the C_8–C_{16} homologs. Work reported here is part of a study undertaken to examine relationships between the surface activity of pure alkyl benzyldimethylammonium chlorides and their antibacterial activity.

Published literature values for the CMC's of the examined series are at variance (7, 8), and the conductimetric titrimeter provides a rapid and extremely accurate method of establishing the CMC's of benzalkonium chloride.

Figure 2 shows the plot of log CMC *versus* alkyl chain length for data obtained in the present study and previously reported values. The results (7) obtained using a lowering of the surface tension method, although smaller than those in this study at all chain lengths, are in reasonably close agreement. The difference is possibly due to the fact that the surface tension method requires a high degree of purity of the surfactant solution.

The results (8) determined with a dye titration method are far lower and show an anomalous break in the curve at $n = 12$. This is probably due to the dye molecules promoting micelle formation. There is no evidence of "odd–even" effects where values for even-numbered homologs fall on a different line than odd-numbered homologs (9).

The relationship between CMC and chain length *(n)* in Fig. 2 is given by:

$$\log \text{CMC} = a - bn \qquad \text{(Eq. 2)}$$

where a and b are constants. The gradient b is 0.36, indicating that the addition of each methylene group reduces the CMC by a factor of 2.3.

If micelle formation is considered as a phase separation process, a free energy value of −507 cal/mole can be calculated for the transfer of a methylene group from an aqueous environment to the micelle. [The nonideality of monomeric species *in solution* and the small correction for the entropy of mixing of the micellar phase with the solvent were ignored. These factors will have a small influence on the calculated result (10).]

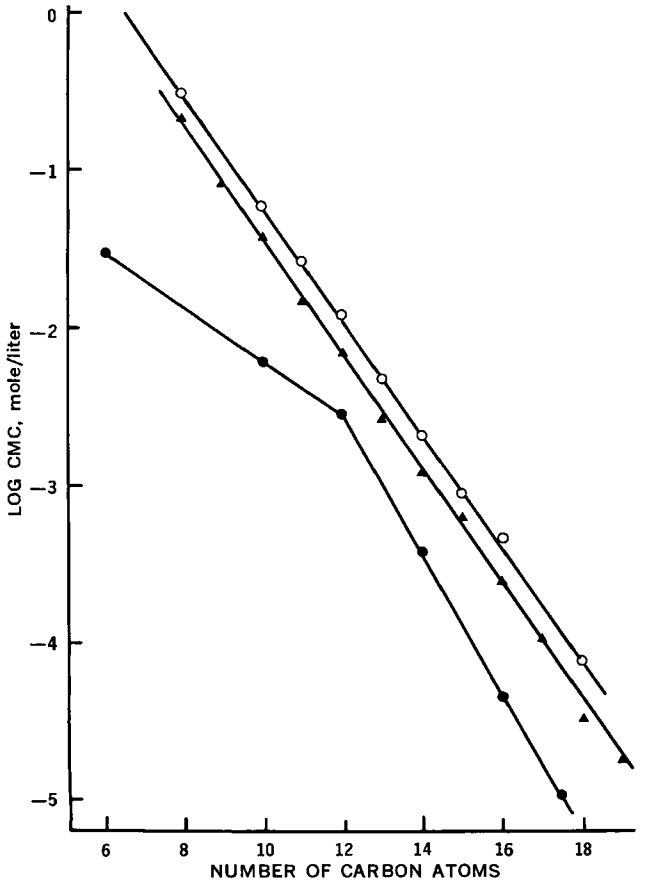

Figure 2—*Log CMC* versus *alkyl chain length for alkyl benzyldimethylammonium chlorides. Key:* ○, *this work;* ▲, *Ref. 7; and* ●, *Ref. 8.*

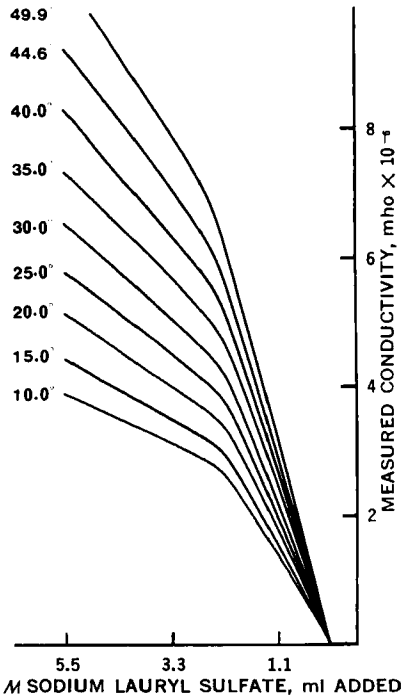

Figure 3—*Experimental curves from chart recorder showing change in conductivity with added sodium lauryl sulfate.*

Figure 3 shows the experimental curves of conductivity *versus* volume of added sodium lauryl sulfate solution (0.2 *M*) for the 10–50° range. The calculated CMC values at different temperatures were compared to values reported by Goddard and Benson (11) in Fig. 4. At each temperature, slightly higher values for the CMC were recorded.

The conductivity of solutions of sodium lauryl sulfate below the CMC was also monitored using this titrator, and the equivalent conductivities were calculated and compared to the Onsager limiting slope. Deviations from the slope could be attributed to premicellar aggregation of surfactant molecules (12).

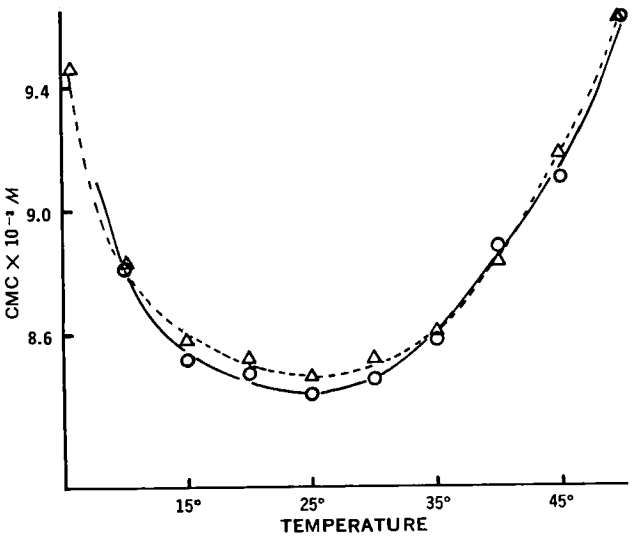

Figure 4—*Change of CMC with temperature for sodium lauryl sulfate. Key:* △, *this work; and* ○, *Ref. 11.*

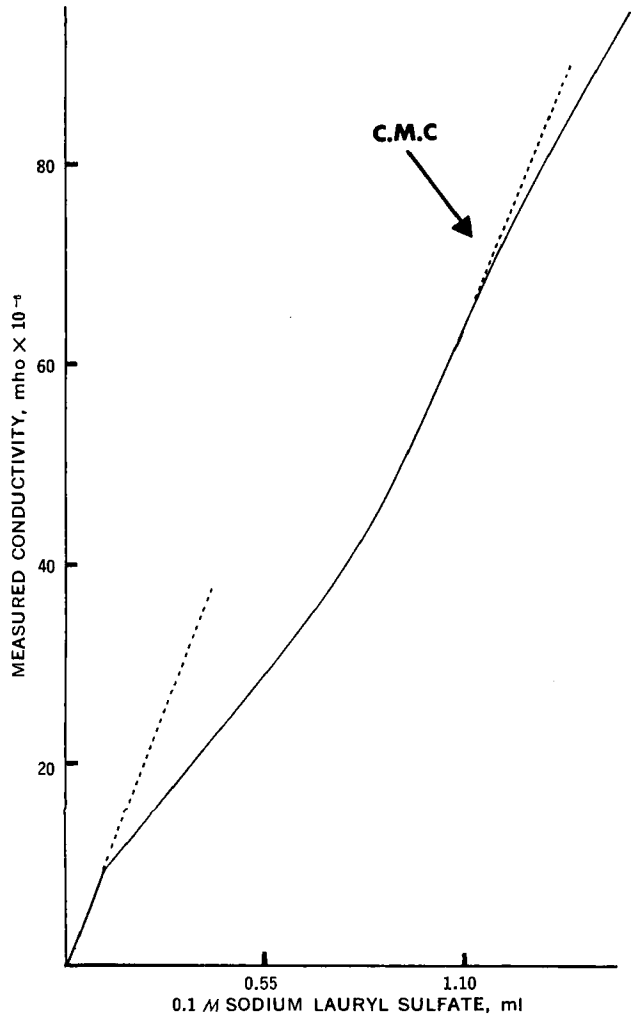

Figure 5—*Conductimetric titration of sodium lauryl sulfate (0.1 M solution) against butyltriphenylphosphonium bromide (1.4×10^{-3} M solution).*

Interaction between Large Ions—A typical conductivity curve for the titration of sodium lauryl sulfate against a solution of phosphonium compound is shown in Fig. 5. It consists of three separate regions. At a low concentration of added sodium lauryl sulfate, there is a linear relationship between specific conductivity and sodium lauryl sulfate concentration. In this region the components of the solution are in an almost fully ionized state. However, there is the possibility of aggregation and limited ion-pair formation.

A second region is heralded by a break in the curve (the solution normally goes turbid at this point), followed by a curvilinear region. The sudden change in the gradient of the conductivity–volume of titrant plot is due to the separation of a complex between sodium lauryl sulfate and the phosphonium salt.

The change in this end-point with different phosphonium-ion concentrations is shown in Fig. 6 as a double logarithmic plot. The gradient of the straight line is 1.0, indicating that a 1:1 complex has been formed. The solubility product is:

$$K_s = [\text{sodium lauryl sulfate}] \times [\text{phosphonium}] \quad \text{(Eq. 3)}$$

Finally, a third region occurs when the CMC of sodium lauryl sulfate is reached. This produces another change in the slope of the conductivity relationship, but it is less distinct than the complexation point. At the CMC, the complex is solubilized and the turbid solution begins to clear.

The coefficient of variation of these titrations in reproducing the same titration curve and the same end-point was ±0.1%.

The change in solubility product with alkyl sulfate chain length is shown in Fig. 7. It is apparent that each methylene group added to the alkyl chain changes the solubility product by a constant factor of 3.6. The corresponding change in free energy is −766 cal/mole. This value is much larger than the free energy increment for the methylene group for micelle formation (Fig. 2). However, it is somewhat smaller than the increment for transfer of the methylene group from an aqueous environment to a nonpolar solvent (13). Such values suggest that the turbid phase separating at the first end-point is a liquid-like coacervate system rather than a solid precipitate (1).

A detailed treatment of the thermodynamics of complexation between alkyl sulfates and phosphonium compounds and the effects of different experimental conditions and additives will be published later.

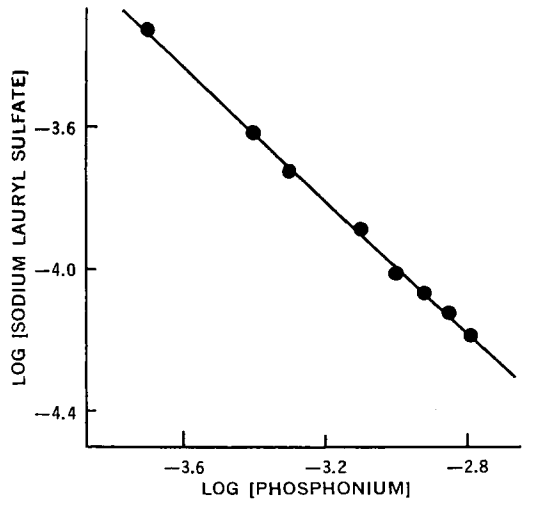

Figure 6—*Titration of sodium lauryl sulfate solutions against different concentrations of phosphonium salt (benzyltriphenylphosphonium chloride) (double log plot).*

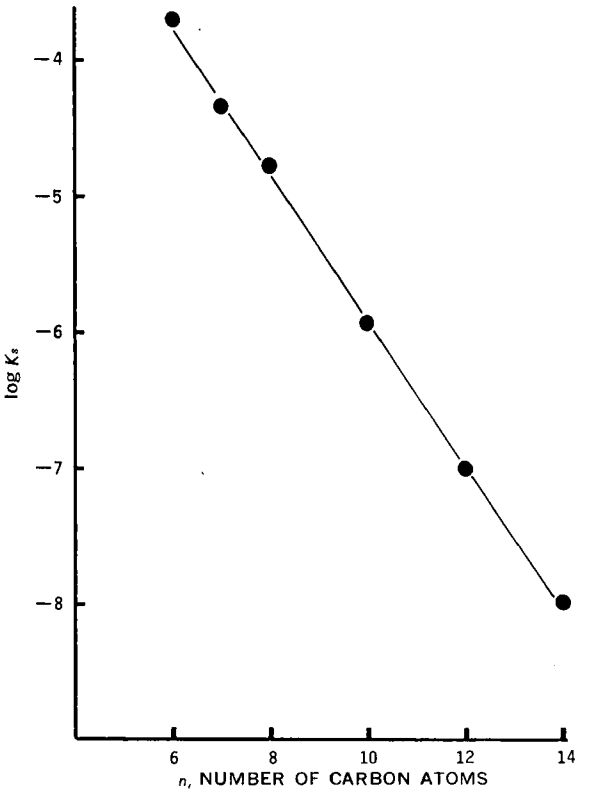

Figure 7—*Log* K_s versus *chain length for the interaction of sodium lauryl sulfate with benzyltriphenylphosphonium chloride.*

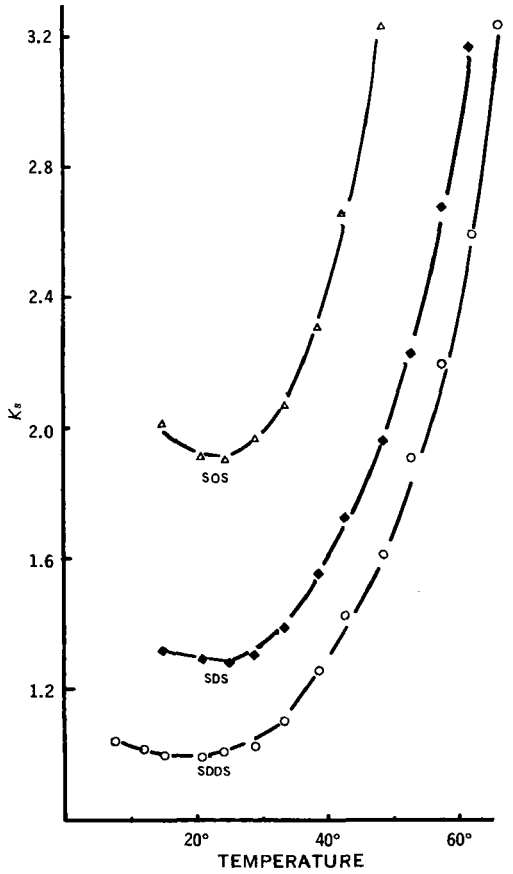

Figure 8—*Change of* K_s *with temperature for the interaction of alkyl sulfates with benzyltriphenylphosphonium chloride. [SOS = sodium octyl sulfate,* $K_s \times 10^{-5}$*; SDS = sodium decyl sulfate,* $K_s \times 10^{-6}$*; and SDDS = sodium lauryl sulfate,* $K_s \times 10^{-7}$*.]*

The effect of temperature on the solubility product was studied using a given phosphonium compound and three different alkyl sulfate homologs (Fig. 8). The solubility product passes through a minimum in the region of 25° in a similar manner to the CMC of the alkyl sulfates alone (Fig. 4 and Ref. 11).

Where the external standard technique is used, the conductivity *versus* volume titrant added plot should be a horizontal line in the absence of complexation. However, for the anion–cation system studied, there is a difference in conductivity reading between the two beakers due to interaction and complexation (Fig. 9). Three distinct regions are evident:

1. An initial region where there is only a small conductivity difference. This is considered to be due to the formation of ion-pairs in water (14) and a possible premicellar aggregation (dimer formation) of the anion (sodium lauryl sulfate) (12).
2. The complexation region.
3. The micellization region.

Therefore, the external standard method provides a rapid and accurate method for the detection of the solubility product and the CMC. An association constant for ion-pair formation can be calculated from the initial region.

The theoretical change in conductivity due to complex formation can be calculated from experimentally determined values of equivalent conductance of the alkyl sulfate, phosphonium compound, and sodium and chloride ions. Above the complexation point, Eq. 3 becomes:

$$K_s = [\text{sodium lauryl sulfate} - x] \times [\text{phosphonium} - x] \quad \text{(Eq. 4)}$$

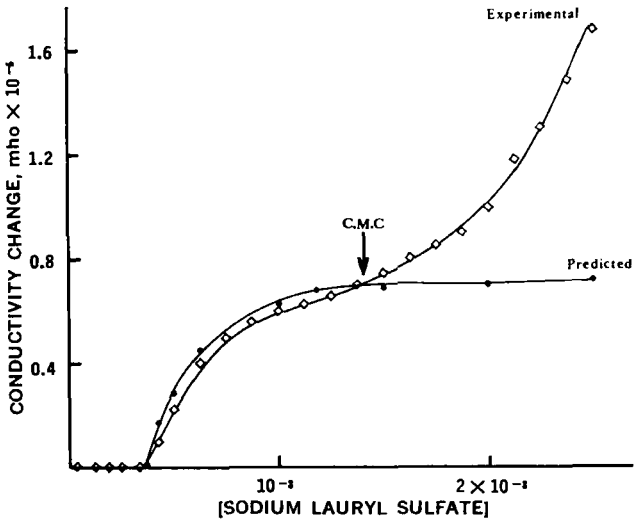

Figure 9—*Interaction between sodium lauryl sulfate and benzyltriphenylphosphonium chloride (*3×10^{-4} M*) using the external standard technique. Key: □, experimental; and ●, predicted.*

where x is the concentration of complex formed. The value of x can be found by solving the quadratic equation resulting from Eq. 4, and from this the theoretical fall in conductivity can be calculated. The agreement between theoretical and experimental data is good up until micelle formation. Furthermore, the comparison of theoretical and experimental data allows the CMC for the mixed system to be determined accurately.

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[x] To whom inquiries should be directed.

Structure of Lupeol and Its 19α-H-Isomer

J. E. GEARIEN ˣ, L. BAUER, M. KLEIN, and B. LEVINE

Abstract □ The silver-ion-assisted dehydrochlorination rearrangement of 19α-chloro-18α-oleanane-3β-ol (lupeol hydrochloride) was reinvestigated. The alkene so obtained was ozonized to yield an epimeric mixture (at C-19) of two methyl ketones. The mechanism of the rearrangement of the chloro derivative to lupeol and its 19α-H-isomer is reinterpreted in the light of these new findings.

Keyphrases □ Lupeol and 19α-H-isomer—structure determination □ 19α-Chloro-18α-oleanane-3β-ol (lupeol hydrochloride)—structure determination

The isolation of 19α-H-lupeol (Ia) from the fruit of the Osage orange *(Maclura pomifera)* (1) prompted a reexamination of the method exployed by Halsall *et al.* (2) in assigning the configuration of lupeol (IIa) at C-19. These investigators based their conclusion on the dehydrochlorination of 19α-chloro-18α-oleanene-3β-ol (IIIa, lupeol hydrochloride) to furnish lupeol. This reaction involved the elimination of hydrogen chloride with the simultaneous ring contraction from a six- to a five-membered ring. The conclusions reached by Halsall *et al.* were predicated on the assumption that a set of stereospecific reactions was involved in the overall transformation.

DISCUSSION

Halsall *et al.* set out to determine the configuration at C-19 of lupeol in the following manner. First, lupeol was treated with ethanolic hydrogen chloride solution at room temperature to produce IIIa. They determined that the chloro group in this new product is in the equatorial position and that H-19 is then axial (2). Since the product of their dehydrohalogenation was not the alkene germanicol but rather lupeol, they reasoned that the normal type E_2 *trans*-elimination of hydrogen chloride was not possible.

Instead, Halsall *et al.* (2) suggested a kind of stereospecific displacement of the chloro group by a neighboring methylene group, which would be equivalent to a stereospecific ring contraction, to lead to an isopropyl carbonium ion. This is reproduced, as they conceived it in Scheme I. This overall conversion is analogous to a number of similar ring contractions of ring A in various triterpenes as, for example, the reaction of the methanesulfonate of cholestanol to give 3β-isopropyl derivatives (3). Since Halsall *et al.* (2) isolated only one isomer from IIIa and confirmed it to be lupeol, they used the mechanism in their Scheme I as evidence for assigning the configuration of the proton at C-19 in IIa[1].

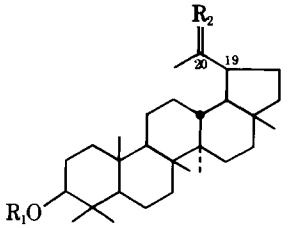
In repeating the work of Halsall *et al.*, IIa was converted first to IIIa. Dehydrohalogenation and subsequent acetylation, using their procedure, gave a mixture which consisted of Ib and IIb. The prime data used by Halsall *et al.* to identify IIa were the melting point and optical rotation values. In this study, PMR spectroscopy was used to examine the product further.

The C-19 methine protons in this mixture were obscured by the envelope of PMR signals due to the other methylene and methine protons. To analyze the mixture more meaningfully, the structure would have to be modified to shift H-19 downfield [from $(CH_3)_4Si$]. Such an anisotropic shift was realized when Ib and IIb were converted to the corresponding ketones, V and IV, respectively. To accomplish this, the reaction mixture from the rearrangement was treated with ozone. A mixture of ketones, 3β-acetoxy-30-norlupan-20-one (IV) and its α-H, at C-19, isomer (V) was obtained.

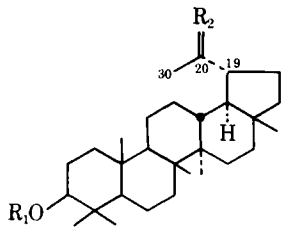
Extreme care was exercised to avoid strong base during the work-up to prevent epimerization. It was then possible to examine the PMR spectrum for the presence of these isomers. The PMR spectrum of the product of the ozonolysis indicated that a mixture contained the epimeric ketones at C-19 in the ratio of approximately 1:1. Two quartets were observed in this PMR spectrum: δ 3.71 (J = 4.20 Hz) and 4.10 (J = 4.80 Hz). These quartets and the rest of the spectrum paralleled the chemical shifts and coupling constants reported previously for IV and V, respectively (1).

It appears that the rearrangement of IIIa yields lupeol and its 19α-epimer. A synchronous process could be accommodated if ring E in IIIa is more in a skewed conformation than was assumed by Halsall *et al.* (2). The bulky substituents surrounding the chloro group might force considerable distortion of ring E and, in effect, force the ring to be more planar. Djerassi *et al.* (4) noted that some compounds that contain a *gem*-dimethyl grouping, as at C-20 in III, in the vicinity of the chromophore showed inverted rotatory dispersion curves, attributed to conformational distortion of the system. Thus, the attacking methylene carbon could displace the chloro group either from above or below the plane of that ring.

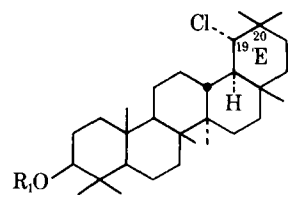
An alternative explanation is offered. Abstraction of chloride ion by silver ion usually creates an incipient carbonium ion, which



Ia: R_1 = H, R_2 = CH_2
Ib: R_1 = $COCH_3$, R_2 = CH_2
V: R_1 = $COCH_3$, R_2 = O

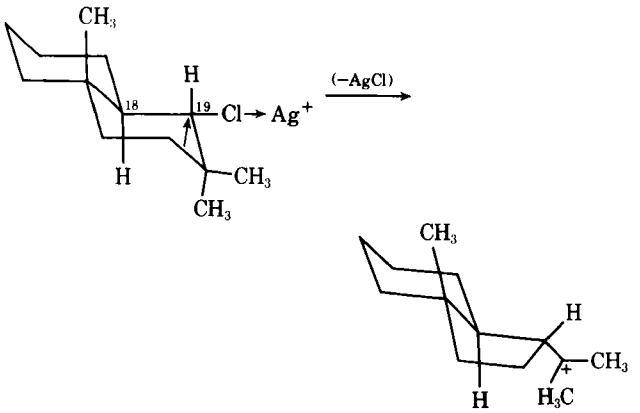


IIa: R_1 = H, R_2 = CH_2
IIb: R_1 = $COCH_3$, R_2 = CH_2
IV: R_1 = $COCH_3$, R_2 = O

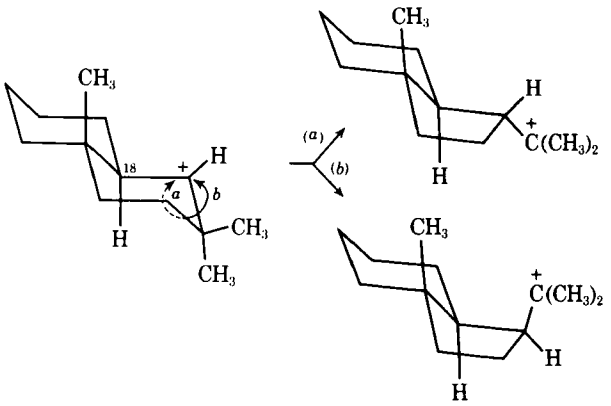


IIIa: R_1 = H
IIIb: R_1 = $COCH_3$

[1] While this work was in progress, the configuration at C-19 was settled by the total synthesis of lupeol [G. Stork, S. Uyeo, T. Wakamatsu, P. Grieco, and J. Labovits, *J. Amer. Chem. Soc.*, **93**, 4945(1971)]. Its stereochemistry was important in assignment of related triterpenoids. See A. S. Samson and R. Stevenson, *Org. Prep. Proced.*, **5**, 59(1973); and E. Klinotova, N. Hovorkova, J. Klinot, and A. Vystrcil, *Collect. Czech. Chem. Commun.*, **38**, 1179(1973).



Scheme I—E_2 trans-elimination of 19α-chloro-18α-oleanane-3β-ol when ring E is represented in the chair conformation



Scheme II—Ring contraction via carbonium ion at C-19

then would be expected to produce two plausible rearrangement products (Scheme II). Loss of a proton from the carbonium ion would then furnish IIa (from path *a*) and IIb (from path *b*).

EXPERIMENTAL[2]

Materials—The silica gel powder[3] used was 60–200 mesh in size.

Chromatographic solvents were distilled prior to use. The petroleum ether used for chromatography, as well as in the extraction procedure, had a boiling range of 40–60°.

Preparation of Chromatographic Columns—The column[4] used was 500 × 50 mm. The chromatographic columns were prepared by the slurry method. Benzene and silica gel powder were mixed into a slurry, and the slurry was then poured into the column and allowed to settle. Petroleum ether was then passed through the column until all of the benzene was removed.

Extraction of Lupeol (Ia)—Dry lupini beans[5] (the seed of *Lupinus* spp.) (500 g) were soaked overnight in water. On the following day the testae from the seeds were removed and placed in an oven for 12 hr at 100°. The dried testae were then ground in an electric blender until a fine powder was obtained. This procedure was repeated 25 times, until a total of 12.5 kg of lupini beans was used. From this amount, 2000 g of dried testa powder was collected.

The dried testa powder (1000 g) was extracted with petroleum ether for 72 hr in a soxhlet extraction apparatus. The defatted powdered material was then replaced with the remaining 1000 g, and the extraction process was repeated.

Evaporation of the extract yielded a light-yellow solid (14.0 g).

The solid (1.0 g) was dissolved in a minimum amount of chloroform. This solution was mixed with dry silica gel powder (2.0 g) and placed in an oven for 12 hr at 100°. The resulting mixture was then layered at the top of a packed chromatographic column.

The column was eluted with: *(a)* petroleum ether (1000 ml), *(b)* petroleum ether–benzene (4:1, 1000 ml), *(c)* petroleum ether–benzene (2:1, 500 ml), and *(d)* petroleum ether–benzene (1:1, 500 ml). The column was then eluted exhaustively with benzene.

Evaporation of the benzene fraction yielded a white powder, which was recrystallized twice from acetone and once from 95% ethanol to yield lupeol as white needles, mp 210–212° [lit. (5) mp 211–212°]. Lupeol (9.0 g) was obtained in 0.45% yield based on the dry lupini bean.

The IR spectrum was identical with that of an authentic sample. The melting point was undepressed upon admixture of an authentic sample.

19α-Chloro-18β-oleanane-3β-ol (IIIa)—Dry ethanol (800 ml) was saturated with hydrogen chloride gas at 0° and gradually added, with periodic cooling, to a solution of lupeol (9.0 g, 0.021 mole) in dry ethanol (500 ml). The mixture was allowed to stand for 5 days at 20° and then diluted with water (700 ml), and extraction with chloroform yielded the product. Two recrystallizations from 95% ethanol yielded needles (3.80 g), mp 206–208°.

Further purification was effected by chromatographing the product (0.76 g) on a silica gel column, prepared in benzene–petroleum ether (1:4). Elution with a mixture of benzene–ether (1:1, 500 ml) yielded a fraction, which was recrystallized twice from 95% ethanol to give needles (0.58 g), mp 210–212° [lit. (2) mp 211–212°]. This chromatographic procedure was repeated five times, and 19α-chloro-18α-oleanane-3β-ol (2.90 g, 0.0063 mole) was obtained in a 32.2% yield. The IR spectrum indicated the loss of the characteristic vinylidene bands at 3100, 1650, and 880 cm^{-1}.

Anal.—Calc. for $C_{30}H_{51}ClO$: C, 77.84; H, 11.03; Cl, 7.68. Found: C, 77.76; H, 10.99; Cl, 7.75.

Dehydrochlorination and Acetylation of IIIa—19α-Chloro-18α-oleanane-3β-ol (2.90 g, 0.0063 mole) was dissolved in hot 95% ethanol (300 ml) and heated at reflux for 20 hr with silver acetate (4.0 g). During this time the suspended solid darkened and a silver mirror formed on the surface of the flask. The mixture was then diluted with water (500 ml) and extracted with ether. Ether was evaporated, and the remaining solid was refluxed for 2 hr with acetic anhydride (35 ml).

This solution was then poured into water (250 ml), and the aqueous solution was extracted with ether. After removal of ether, the residue was recrystallized twice from 95% ethanol to yield white needles, mp 206–208° [lit. (2) mp 215–216° for lupenyl acetate, lit. (1) mp 210–211.5° for 19α-H-lupenyl acetate]. The product (2.10 g, 0.0043 mole) was obtained in a 72.4% yield.

The IR spectrum indicated the presence of a vinylidene group by the presence of peaks at 3100, 1650, and 880 cm^{-1}. The broad O—H stretching band in the starting alcohol had disappeared, with the appearance of a C—O stretching band at 1240 cm^{-1}, and a C=O stretching band appeared as a sharp peak at 1750 cm^{-1}.

Anal.—Calc. for $C_{32}H_{52}O_2$: C, 82.02; H, 11.11. Found: C, 81.97; H, 11.19.

Ozonolysis of Dehydrochlorinated and Acetylated Products (IV and V) from IIIa—A solution of lupenyl acetate and/or its epimer (2.10 g, 0.0043 mole) in chloroform (40 ml) was treated at 10° with a slow stream of ozone for 1 hr. Completion of formation of the ozonide was ensured by inserting the outlet tube into a 5% potassium iodide solution. Immediate oxidation of the iodide indicated that the reaction was complete.

The solvent was evaporated, leaving the ozonide as an oil. Water (200 ml) was added, and the mixture was heated for 1 hr and then steam distilled. To the distillate was added an alcoholic solution of 2,4-dinitrophenylhydrazone.

After the steam distillation, the aqueous phase was extracted with ether. Removal of ether furnished a product, which was re-

[2] Melting points were determined on a Thomas-Hoover capillary melting-point apparatus and are uncorrected. Elemental analyses for carbon, hydrogen, and chlorine were performed by Micro-Tech Laboratories, Inc., Skokie, Ill. The NMR spectrum was determined using a Bruker, model HFX05, 90-MHz spectrometer and was recorded downfield from tetramethylsilane as an internal reference. The solvent used was deuterated chloroform. IR spectra were obtained with a Perkin-Elmer model 700 recording spectrophotometer. Nujol mulls were used for all samples run. Ozone was generated by passing oxygen through a Welsbach Corp. model T23 ozonizer.

[3] J. T. Baker Chemical Co., Phillipsburg, N.J.

[4] Kontes, Vineland, N.J.

[5] Purchased from Conti-Di-Savoia European Specialties, Chicago, Ill.

crystallized twice from aetone and then twice from 95% ethanol to yield white needles, mp 244–246° [lit. (5) mp 260–262° for 29-norlupan-20-one-3β-yl acetate [lit. (5) mp 249–251° for 29-nor-19α-H-lupan-20-one-3β-yl acetate]. The product (0.57 g, 0.0012 mole) was obtained in a 27.0% yield.

The IR spectrum indicated loss of the peaks corresponding to the vinylidene group at 3100, 1650, and 880 cm^{-1}. Carbonyl absorption at 1700 cm^{-1} was observed.

Anal.—Calc. for $C_{31}H_{50}O_3$: C, 79.15; H, 10.64. Found: C, 79.01; H, 10.50.

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[x] To whom inquiries should be directed.

Synthesis of Substituted Benzylidinohydrazines and Their Monoamine Oxidase Inhibitory and Anticonvulsant Properties

SURENDRA S. PARMAR [*‡x], A. K. GUPTA [‡], T. K. GUPTA [‡], and VIRGIL I. STENBERG [*]

Abstract □ Some N^1-(4-acetamidobenzoyl)-N^2-(substituted phenyl carboxylate)benzylidinohydrazines were synthesized, characterized, and evaluated for their ability to inhibit monoamine oxidase *in vitro*. All substituted benzylidinohydrazines inhibited monoamine oxidase activity of rat brain homogenates. These compounds possessed anticonvulsant activity, which was reflected by the protection afforded against pentylenetetrazol-induced convulsions; they also potentiated pentobarbital-induced hypnosis in mice. Monoamine oxidase inhibitory effectiveness of these substituted benzylidinohydrazines was unrelated to their anticonvulsant activity and their ability to potentiate pentobarbital-induced hypnosis.

Keyphrases □ Benzylidinohydrazines—synthesis, characterization, and *in vitro* inhibition of monoamine oxidase □ Structure–activity relationships—benzylidinohydrazines, anticonvulsant activity and inhibition of monoamine oxidase activity of rat brain homogenates □ Monoamine oxidase inhibitors—synthesis of benzylidinohydrazines □ Anticonvulsant activity—benzylidinohydrazines

Many hydrazine derivatives are monoamine oxidase inhibitors (1). Such enzyme inhibitors have been shown to possess anticonvulsant properties (2). Furthermore, psychotropic (3) and anticonvulsant (4) properties exhibited by benzylidine derivatives led to the synthesis of N^1-(4-acetamidobenzoyl)-N^2-(substituted phenyl carboxylate)benzylidinohydrazines. The ability of these benzylidinohydrazines to inhibit monoamine oxidase activity of rat brain homogenates was investigated in an attempt to correlate enzyme inhibitory effectiveness with anticonvulsant activity and ability to potentiate pentobarbital-induced hypnosis.

EXPERIMENTAL

Chemistry—*Ethyl 4-Aminobenzoate*—Ethyl 4-aminobenzoate was prepared by the esterification of 4-aminobenzoic acid by the method reported earlier, mp 91° (5).

Ethyl 4-Acetamidobenzoate—In a conical flask containing water, 18.3 ml of concentrated hydrochloric acid and 35 g of ethyl 4-aminobenzoate (0.22 mole) were introduced with stirring. To this solution, 256 ml of distilled acetic anhydride was added. The reaction mixture was added to a solution of 33 g of sodium acetate in 100 ml of water and stirred vigorously. On cooling, the solid that separated was filtered, washed, dried, and recrystallized from ethanol, mp 117°.

(4-Acetamidobenzoyl)hydrazine—A mixture of ethyl 4-acetamidobenzoate (0.4 mole) and 0.4 mole of hydrazine hydrate (99–100%) in absolute ethanol was refluxed on a steam bath for 15 hr. Excess ethanol was distilled, and the hydrazine that separated on cooling was filtered and recrystallized from ethanol, mp 277°.

N^1-*(4-Acetamidobenzoyl)*-N^2-*substituted Benzylidinohydrazines*—A mixture of 4-acetamidobenzoylhydrazine (0.1 mole) and a suitable salicylaldehyde (0.1 mole) in ethanol with a few drops of acetic acid was refluxed on a steam bath for 4–5 hr. Excess ethanol was removed by distillation. The solid mass that separated was collected by filtration, washed with water, dried, and recrystallized from ethanol. These compounds were characterized by their sharp melting points and elemental analyses (Table I).

N^1-*(4-Acetamidobenzoyl)*-N^2-*(aryl-substituted Phenyl Carboxylate)benzylidinohydrazines*—N^1-(4-Acetamidobenzoyl)-N^2-substituted benzylidinohydrazine (0.025 mole) in dry benzene (20 ml) was mixed with the appropriate benzoyl chloride, and the resulting mixture was refluxed on a steam bath for 5–6 hr. Excess benzene was removed by distillation, and the crude product that separated on cooling was filtered, washed first with sodium bicarbonate and then with water, and recrystallized from ethanol. These substituted benzylidinohydrazines, characterized by their sharp melting points and elemental analyses, are recorded in Table II.

Determination of Monoamine Oxidase Activity—Male rats weighing 150–200 g were killed by decapitation. Brains were quick-

crystallized twice from aetone and then twice from 95% ethanol to yield white needles, mp 244–246° [lit. (5) mp 260–262° for 29-norlupan-20-one-3β-yl acetate [lit. (5) mp 249–251° for 29-nor-19α-H-lupan-20-one-3β-yl acetate]. The product (0.57 g, 0.0012 mole) was obtained in a 27.0% yield.

The IR spectrum indicated loss of the peaks corresponding to the vinylidene group at 3100, 1650, and 880 cm^{-1}. Carbonyl absorption at 1700 cm^{-1} was observed.

Anal.—Calc. for $C_{31}H_{50}O_3$: C, 79.15; H, 10.64. Found: C, 79.01; H, 10.50.

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Synthesis of Substituted Benzylidinohydrazines and Their Monoamine Oxidase Inhibitory and Anticonvulsant Properties

SURENDRA S. PARMAR [*‡x], A. K. GUPTA [‡], T. K. GUPTA [‡], and VIRGIL I. STENBERG [*]

Abstract □ Some N^1-(4-acetamidobenzoyl)-N^2-(substituted phenyl carboxylate)benzylidinohydrazines were synthesized, characterized, and evaluated for their ability to inhibit monoamine oxidase *in vitro*. All substituted benzylidinohydrazines inhibited monoamine oxidase activity of rat brain homogenates. These compounds possessed anticonvulsant activity, which was reflected by the protection afforded against pentylenetetrazol-induced convulsions; they also potentiated pentobarbital-induced hypnosis in mice. Monoamine oxidase inhibitory effectiveness of these substituted benzylidinohydrazines was unrelated to their anticonvulsant activity and their ability to potentiate pentobarbital-induced hypnosis.

Keyphrases □ Benzylidinohydrazines—synthesis, characterization, and *in vitro* inhibition of monoamine oxidase □ Structure–activity relationships—benzylidinohydrazines, anticonvulsant activity and inhibition of monoamine oxidase activity of rat brain homogenates □ Monoamine oxidase inhibitors—synthesis of benzylidinohydrazines □ Anticonvulsant activity—benzylidinohydrazines

Many hydrazine derivatives are monoamine oxidase inhibitors (1). Such enzyme inhibitors have been shown to possess anticonvulsant properties (2). Furthermore, psychotropic (3) and anticonvulsant (4) properties exhibited by benzylidine derivatives led to the synthesis of N^1-(4-acetamidobenzoyl)-N^2-(substituted phenyl carboxylate)benzylidinohydrazines. The ability of these benzylidinohydrazines to inhibit monoamine oxidase activity of rat brain homogenates was investigated in an attempt to correlate enzyme inhibitory effectiveness with anticonvulsant activity and ability to potentiate pentobarbital-induced hypnosis.

EXPERIMENTAL

Chemistry—*Ethyl 4-Aminobenzoate*—Ethyl 4-aminobenzoate was prepared by the esterification of 4-aminobenzoic acid by the method reported earlier, mp 91° (5).

Ethyl 4-Acetamidobenzoate—In a conical flask containing water, 18.3 ml of concentrated hydrochloric acid and 35 g of ethyl 4-aminobenzoate (0.22 mole) were introduced with stirring. To this solution, 256 ml of distilled acetic anhydride was added. The reaction mixture was added to a solution of 33 g of sodium acetate in 100 ml of water and stirred vigorously. On cooling, the solid that separated was filtered, washed, dried, and recrystallized from ethanol, mp 117°.

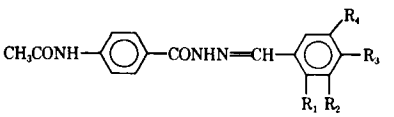
(4-Acetamidobenzoyl)hydrazine—A mixture of ethyl 4-acetamidobenzoate (0.4 mole) and 0.4 mole of hydrazine hydrate (99–100%) in absolute ethanol was refluxed on a steam bath for 15 hr. Excess ethanol was distilled, and the hydrazine that separated on cooling was filtered and recrystallized from ethanol, mp 277°.

N^1-*(4-Acetamidobenzoyl)*-N^2-*substituted Benzylidinohydrazines*—A mixture of 4-acetamidobenzoylhydrazine (0.1 mole) and a suitable salicylaldehyde (0.1 mole) in ethanol with a few drops of acetic acid was refluxed on a steam bath for 4–5 hr. Excess ethanol was removed by distillation. The solid mass that separated was collected by filtration, washed with water, dried, and recrystallized from ethanol. These compounds were characterized by their sharp melting points and elemental analyses (Table I).

N^1-*(4-Acetamidobenzoyl)*-N^2-*(aryl-substituted Phenyl Carboxylate)benzylidinohydrazines*—N^1-(4-Acetamidobenzoyl)-N^2-substituted benzylidinohydrazine (0.025 mole) in dry benzene (20 ml) was mixed with the appropriate benzoyl chloride, and the resulting mixture was refluxed on a steam bath for 5–6 hr. Excess benzene was removed by distillation, and the crude product that separated on cooling was filtered, washed first with sodium bicarbonate and then with water, and recrystallized from ethanol. These substituted benzylidinohydrazines, characterized by their sharp melting points and elemental analyses, are recorded in Table II.

Determination of Monoamine Oxidase Activity—Male rats weighing 150–200 g were killed by decapitation. Brains were quick-

Table I—Physical Constants of N^1-(4-Acetamidobenzoyl)-N^2-substituted Benzylidinohydrazines



Compound	R_1	R_2	R_3	R_4	Melting Point[a]	Yield, %	Molecular Formula[b]	Analysis, % Calc.	Found
I	H	H	OH	OCH_3	255°	80	$C_{17}H_{17}N_3O_4$	C 62.38	62.36
								H 5.20	5.22
								N 12.84	12.88
II	OH	H	H	H	275°	65	$C_{16}H_{15}N_3O_3$	C 64.64	64.53
								H 5.05	5.00
								N 14.14	14.20
III	H	OH	H	H	285°	70	$C_{16}H_{15}N_3O_3$	C 64.64	64.49
								H 5.05	5.20
								N 14.14	14.18
IV	H	H	OH	H	300°	85	$C_{16}H_{15}N_3O_3$	C 64.64	64.70
								H 5.05	5.10
								N 14.14	14.10

[a] Melting points were taken in open capillary tubes and are uncorrected. [b] All compounds were recrystallized from ethanol.

ly removed and homogenized[1] in ice-cold 0.25 *M* sucrose (2%, w/v). The reaction mixture (final concentration) consisted of 0.5 ml of phosphate buffer (0.2 *M*, pH 7.5), 1×10^{-4} *M* kynuramine, 0.5 ml of brain homogenate (equivalent to 10 mg of wet weight of the tissue), and water to a total volume of 3 ml. The monoamine oxidase activity of brain homogenates was determined after incubation at 37° in air for 30 min (6).

The various substituted benzylidinohydrazines were added to the brain homogenate to produce a final concentration of 5×10^{-5} and 1×10^{-4} *M* and incubated for 10 min before adding kynuramine. The mixture was then incubated for an additional 30 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (w/v), and the precipitated proteins were removed by centrifugation.

Suitable 1-ml aliquots of the supernatant solution were taken in 2 ml of 1 *N* NaOH and were assayed spectrophotofluorometrically[2] for 4-hydroxyquinoline content. An increase in absorbance provided a direct measurement of 4-hydroxyquinoline formation, which was taken as an index of the enzyme activity. The percent inhibition was calculated from the decrease observed in absorbance, and this value provided an index of the inhibitory property of these substituted benzylidinohydrazines.

Determination of Anticonvulsant Activity—Anticonvulsant activity was determined in mice of either sex weighing 25–30 g. The mice were divided into groups of 10, keeping the group weights as nearly the same as possible. All benzylidinohydrazines were suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v).

The test compounds were injected in a group of 10 animals at a dose of 100 mg/kg ip. Four hours later, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose of pentylenetetrazol has been shown to produce convulsions in most untreated mice and was also found to produce 100% mortality during a 24-hr period.

The mice were observed for 60 min for the occurrence of seizures. An episode of clonic spasm that persisted for a minimum of 5 sec was considered a threshold convulsion (7). Transient intermittent jerks and tremulousness were not counted. Animals devoid of threshold convulsions during the 60-min period were considered protected. The number of animals protected in each group was recorded, and the anticonvulsant activity of the benzylidinohydrazines was represented as percent protection. The mice were then observed for 24 hr and mortality was recorded.

Potentiation of Pentobarbital (Sodium) Sleeping Time—The method of Winter (8) was followed to investigate the ability of benzylidinohydrazines to potentiate pentobarbital-induced hypnosis. Mice weighing 20–25 g were divided into groups of six animals. One group of six animals was used for each compound, while another group of six mice served as the control. Pentobarbital, when administered in a dose of 40 mg/kg ip to the control group, was found to produce sleep. All benzylidinohydrazines were suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v) and were injected at a dose of 100 mg/kg ip 30 min prior to the administration of pentobarbital.

The animals were observed for sleep as evidenced by loss of the righting reflex. The degree of potentiation produced by these benzylidinohydrazines was calculated by the total average time of sleep observed in experimental animals divided by the total average time of sleep observed in control animals.

Toxicity—The approximate LD_{50} value for each benzylidinohydrazine was determined in albino mice by the method reported by Smith (9). The test compounds, suspended in 5% aqueous gum acacia, were administered intraperitoneally to groups of 10 mice.

RESULTS AND DISCUSSION

Monoamine oxidase inhibitory effectiveness of benzylidinohydrazines at the final concentrations of 1×10^{-4} and 5×10^{-5} *M*, using kynuramine as the substrate and rat brain homogenates as the source of the enzyme, are recorded in Table III. All evaluated compounds possessed monoamine oxidase inhibitory activity. Maximum inhibition was observed with the compound possessing a 3-nitrophenyl carboxylate group at position R_1 of the benzylidine moiety (Compound X). The presence of a 4-acetamidophenyl carboxylate group at R_1, R_2, or R_3 of the benzylidine moiety was found to result in a relatively lower degree of monoamine oxidase inhibition when compared to other substituted phenyl carboxylates.

As is evident from Table III, benzylidinohydrazines at a dose of 100 mg/kg possessed anticonvulsant activity against pentylenetetrazol-induced convulsions; the degree of protection ranged from 10 to 50%. Maximum protection was observed with the compounds having a 3-nitrophenyl carboxylate group (Compund VI) or an acetyl salicylate group (Compound XVIII) at R_3 of the benzylidine moiety of these hydrazines. Benzylidinohydrazines having a 4-acetamidophenyl carboxylate group at R_2 or R_3 (Compounds XV and XIX) possessed weak anticonvulsant activity.

The degree of protection afforded by these compounds against pentylenetetrazol-induced death during 24 hr in mice was unrelated to their anticonvulsant activity (Table III). The low toxicity of these compounds in albino mice was reflected by their high approximate LD_{50} values, which were 1000 mg/kg or greater on intraperitoneal administration.

All evaluated benzylidinohydrazines potentiated sleeping time induced by pentobarbital in mice at a dose of 100 mg/kg (Table III). Benzylidinohydrazines having a phenylcarboxyl, 4-acetamidophenyl carboxylate, or acetyl salicylate group at R_3 with a methoxy substituent at R_4 and a 3-nitrophenyl carboxylate group at R_3 of the benzylidine moiety prolonged the sleeping time twice that observed with the administration of pentobarbital alone.

[1] Potter–Elvehjem.
[2] Aminco-Bowman spectrophotofluorometer.

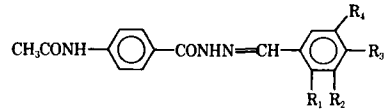


Table II—Physical Constants of N^2-(4-Acetamidobenzoyl)-N^2-(aryl-substituted Phenyl Carboxylate)benzylidinohydrazines

Compound	R_1	R_2	R_3	R_4	Melting Point[a]	Yield, %	Molecular Formula[b]	Analysis, %	
								Calc.	Found
V	H	H	$OOCC_6H_5$	OCH_3	240°	80	$C_{24}H_{21}N_3O_5$	C 66.82	66.78
								H 4.87	4.82
								N 9.74	9.62
VI	H	H	3-$(NO_2)C_6H_4OCO$	OCH_3	243°	60	$C_{24}H_{20}N_4O_7$	C 60.50	60.47
								H 4.20	4.18
								N 11.76	11.82
VII	H	H	4-$(NHCOCH_3)C_6H_4OCO$	OCH_3	125°	85	$C_{26}H_{24}N_4O_6$	C 63.93	63.78
								H 4.92	4.88
								N 11.47	11.52
VIII	H	H	2-$(OCOCH_3)C_6H_4OCO$	OCH_3	215°	60	$C_{26}H_{23}N_3O_7$	C 63.80	63.77
								H 4.70	4.73
								N 8.59	8.61
IX	$OOCC_6H_5$	H	H	H	263°	65	$C_{23}H_{19}N_3O_4$	C 68.83	68.82
								H 4.74	4.73
								N 10.47	10.51
X	3-$(NO_2)C_6H_4OCO$	H	H	H	218°	68	$C_{23}H_{18}N_4O_6$	C 61.88	61.92
								H 4.04	4.21
								N 12.56	12.40
XI	4-$(NHCOCH_3)C_6H_4OCO$	H	H	H	260°	72	$C_{25}H_{22}N_4O_5$	C 65.50	65.70
								H 4.80	4.77
								N 12.23	12.20
XII	2-$(OCOCH_3)C_6H_4OCO$	H	H	H	250°	68	$C_{25}H_{21}N_3O_6$	C 65.36	65.32
								H 4.58	4.47
								N 9.15	9.10
XIII	H	$OOCC_6H_5$	H	H	275°	75	$C_{23}H_{19}N_3O_4$	C 68.83	68.88
								H 4.74	4.80
								N 10.47	10.52
XIV	H	3-$(NO_2)C_6H_4OCO$	H	H	268°	59	$C_{23}H_{18}N_4O_6$	C 61.88	61.73
								H 4.04	4.00
								N 12.56	12.62
XV	H	4-$(NHCOCH_3)C_6H_4OCO$	H	H	265°	78	$C_{25}H_{22}N_4O_5$	C 65.50	65.30
								H 4.80	4.72
								N 12.23	12.42
XVI	H	2-$(OCOCH_3)C_6H_4OCO$	H	H	266°	65	$C_{25}H_{21}N_3O_6$	C 65.36	65.33
								H 4.58	4.56
								N 9.15	9.20
XVII	H	H	$OOCC_6H_5$	H	275°	75	$C_{23}H_{19}N_3O_4$	C 68.83	68.66
								H 4.74	4.71
								N 10.47	10.58
XVIII	H	H	3-$(NO_2)C_6H_4OCO$	H	280°	70	$C_{23}H_{18}N_4O_6$	C 61.88	61.90
								H 4.04	3.98
								N 12.56	12.50
XIX	H	H	4-$(NHCOCH_3)C_6H_4OCO$	H	270°	80	$C_{25}H_{22}N_4O_5$	C 65.50	65.38
								H 4.80	4.76
								N 12.23	12.30
XX	H	H	2-$(OCOCH_3)C_6H_4OCO$	H	276°	63	$C_{25}H_{21}N_3O_6$	C 65.36	65.25
								H 4.58	4.62
								N 9.16	9.12

[a] Melting points were taken in open capillary tubes and are uncorrected. [b] All compounds were recrystallized from ethanol.

Table III—Monoamine Oxidase Inhibitory and Pharmacological Properties of N^1-(4-Acetylaminobenzoyl)-N^2-(substituted Phenyl Carboxylate)benzylidinohydrazines

Compound	Monoamine Oxidase Inhibition[a], %		Approximate LD_{50}, mg/kg	Anticonvulsant Activity[b], % Protection	Pentylenetetrazol Mortality[c], %	Potentiation of Pentobarbital Sleeping Time, × Control[d]
	5×10^{-5} *M*	1×10^{-4} *M*				
V	16.7 ± 0.8	36.7 ± 0.5	>1000	20	50	2.2
VI	31.7 ± 0.9	57.9 ± 0.8	>1000	50	30	1.7
VII	15.4 ± 0.7	36.7 ± 0.6	>1000	30	40	2.1
VIII	19.2 ± 0.6	52.9 ± 1.1	>1000	30	30	2.1
IX	67.9 ± 1.2	81.7 ± 2.1	>1000	30	50	1.5
X	81.7 ± 1.1	92.9 ± 2.0	1000	30	50	2.3
XI	45.4 ± 1.0	65.4 ± 1.5	1000	30	50	1.6
XII	77.9 ± 1.0	84.2 ± 2.2	1000	30	50	1.9
XIII	19.2 ± 0.7	55.4 ± 2.4	>1000	40	40	1.7
XIV	30.4 ± 0.7	52.9 ± 1.9	>1000	40	50	2.0
XV	22.9 ± 0.6	26.7 ± 0.7	>1000	10	60	1.6
XVI	20.4 ± 0.8	30.4 ± 0.5	>1000	30	50	1.8
XVII	32.9 ± 0.9	50.4 ± 1.1	>1000	30	50	1.7
XVIII	33.7 ± 0.7	57.9 ± 1.3	>1000	50	20	2.1
XIX	27.9 ± 0.6	50.2 ± 0.9	>1000	10	90	2.0
XX	54.2 ± 0.8	71.7 ± 2.0	>1000	50	50	1.4

[a] Each experiment was done in duplicate. All values represent mean values of percent inhibition with ± standard error of the mean calculated from three separate experiments. Assay procedure and the contents of the reaction mixture are as described in the text. All compounds were dissolved in propylene glycol (100%), and an equivalent amount of propylene glycol was added to the control tubes containing kynuramine alone in the absence of the test compounds. [b] Anticonvulsant activity was determined at a dose of 100 mg/kg as described in the *Experimental* section. [c] Represents mortality during 24 hr in each group of animals administered pentylenetetrazol. [d] Control value for pentobarbital (40 mg/kg) sleeping time was 29 min, which was taken as 1 for evaluation of the ability of substituted benzylidinohydrazines to potentiate pentobarbital sleeping time.

These observations did not provide a correlation between the monoamine oxidase inhibitory effectiveness of these benzylidinohydrazines and their anticonvulsant activity or their ability to potentiate pentobarbital-induced hypnosis. Detailed pharmacological studies of the effects of these benzylidinohydrazines may possibly reflect a basis for their ability to inhibit monoamine oxidase.

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Azulene Analogs of Pharmacological Agents III: Acute Toxicity and Local Anesthetic Activity of Azulylamides and Azulenecarboxamides

P. H. DOUKAS ˣ, T. J. SPEAKER, and R. S. THOMPSON

Abstract □ This paper describes the acute toxicity of two azulylamides and six azulenecarboxamides and the nerve conduction-inhibiting properties of some of these compounds. The azulene derivatives are compared to their benzenoid prototypes, lidocaine and procainamide, as part of a continuing investigation of the biological properties of nonbenzenoid aromatic compounds.

Keyphrases □ Azulene analogs (azulylamides and azulenecarboxamides)—acute toxicity and local anesthetic activity □ Azulylamides and azulenecarboxamides—acute toxicity and local anesthetic activity □ Structure–activity relationships—azulene analogs–local anesthetic activity

Previous papers (1, 2) reported the synthesis of azulene analogs of procainamide and lidocaine. The acute toxicity and nerve conduction-inhibiting properties are now reported for several of these analogs.

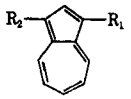
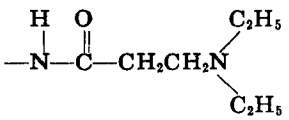
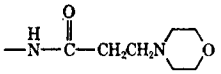
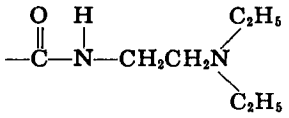
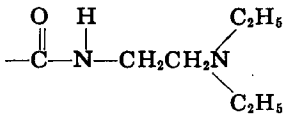
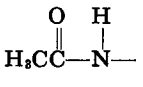
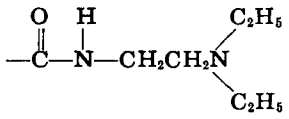
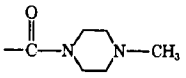
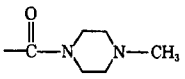
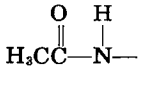
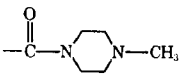


Table I—LD_{50} Values of Some Azulylamides and Azulenecarboxamides[a,b]

Compound	R_2	R_1	LD_{50} (95% Confidence Limits) mg/kg	LD_{50} (95% Confidence Limits) mmoles/kg	LD_{50} Ratios[c] Azulenoid Lidocaine	LD_{50} Ratios[c] Azulenoid Procainamide
I-1	H—		229 (212–252)	0.749 (0.69–0.82)	1.1	0.45
I-2	H—		210 (171–264)	0.737 (0.60–0.93)	1.1	0.46
II-1	H—		178 (150–220)	0.582 (0.49–0.66)	0.85	0.35
II-2	O_2N—		221 (148–338)	0.629 (0.41–0.96)	0.92	0.38
II-3			367 (212–575)	1.00 (0.58–1.57)	1.46	0.61
III-1	H—		268 (243–289)	0.926 (0.84–1.02)	1.36	0.57
III-2	O_2N—		188 (131–275)	0.561 (0.39–0.82)	0.82	0.34
III-3			>485[d]	>1.5[d]	>1.4	>0.9
Lidocaine			160 (135–198)	0.682 (0.57–0.77)		
Procainamide			447 (407–486)	1.64 (1.49–1.81)		

[a] All values are for the hydrochloride salts. [b] The chemical names of the compounds are as follows: I-1, 1-(3-diethylaminopropionamido)azulene; I-2, 1-(3-morpholinopropionamido)azulene; II-1, 1-(2-diethylaminoethyl)azulenecarboxamide; II-2, 1-(2-diethylaminoethyl)-3-nitroazulenecarboxamide; II-3, 1-(2-diethylaminoethyl)-3-acetamidoazulenecarboxamide, III-1, 1-(*N*-methylpiperazine)azulenecarboxamide; III-2, 1-(*N*-methylpiperazine)-3-nitroazulenecarboxamide; and III-3, 1-(*N*-methylpiperazine)-3-acetamidoazulenecarboxamide. [c] Calculated on a millimoles per kilogram basis. [d] Insufficient compound was available to allow close estimation of the LD_{50} values or of the 95% confidence limits.

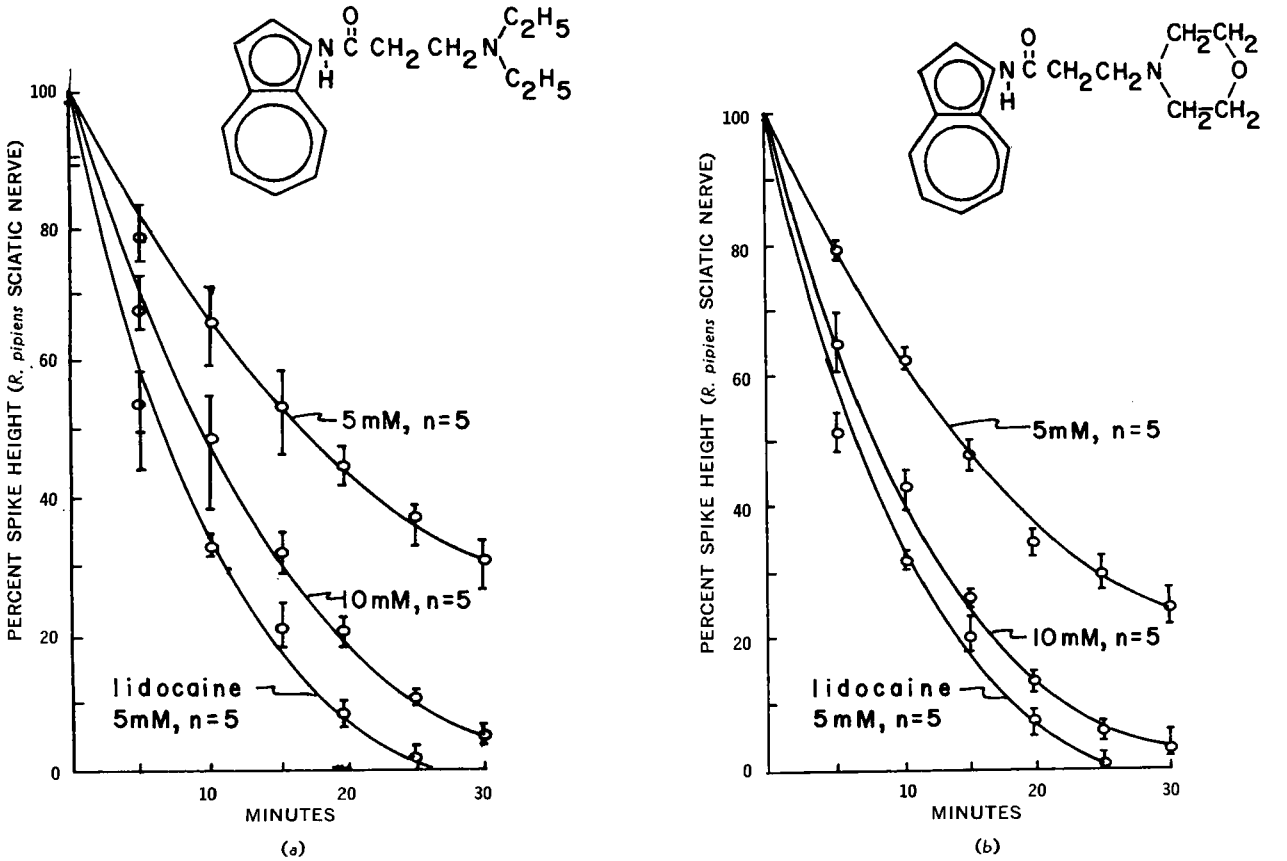


Figure 1—*Inhibition of sciatic nerve action potential* (R. pipiens) *with time at 5- and 10-mmole concentrations of:* (a) *1-(3-diethylaminopropionamido)azulene (I-1), and* (b) *1-(3-morpholinopropionamido)azulene (I-2). Both compounds are compared to 5 mmoles of lidocaine. Each experimental point represents the mean of five experiments; the standard deviation for each point is also shown.*

The compounds included in this study were of three classes: azulylamides (Type I), secondary azulenecarboxamides (Type II), and tertiary azulenecarboxamides (Type III). Intraperitoneal LD_{50} values determined in male Swiss–Webster mice, according to the method of Weil (3), are given in Table I. On the basis of the LD_{50} values, candidate compounds from each series were selected for evaluation as nerve blocking agents. Isolated frog sciatic nerve preparations were employed to determine the degree of inhibition of nerve conduction relative to the pattern compounds. The changes in percentage inhibition as a function of time and of drug concentration are presented in Figs. 1 and 2.

EXPERIMENTAL

LD_{50} Determinations—Naive male Swiss–Webster mice[1], 25 ± 9 g, were randomly divided into five or six groups of four mice each. The groups were housed separately in metabolism cages at 25 ± 2° and provided with food[2] and tap water *ad libitum*, except during the first 4 hr of each experiment when food was withheld. Mice in each group were identified by tail marking. All experimental and control injections were made intraperitoneally.

All drugs for LD_{50} studies were dissolved in enough 0.9% sodium chloride solution that the administered dose was contained in 0.1 ml/10 g of body weight. All solutions were prepared fresh on the day of use, except those of sodium chloride which were obtained from commercial 30-ml multiple-dose vials[3].

All surviving animals were observed over 72 hr; all deaths occurred within 24 hr of dosing.

Nerve Conduction Measurements—Freshly removed sciatic nerves of male frogs (*Rana pipiens*) were used in all measurements of inhibition of nerve conduction. Nerves were kept bathed in oxygenated (95% O_2–5% CO_2) frog Ringer's solution and were maintained at 21 ± 0.5°. All drugs for nerve conduction studies were dissolved in frog Ringer's solution, and all solutions were prepared fresh on the day of use according to the method of Condouris (4).

For measurement of nerve conduction, nerves were suspended across pairs of stimulating and recording platinum electrodes in an oxygenated, humidified nerve chamber. Drug was applied to each nerve by allowing it to dip into a small drug-containing bath positioned between the stimulating and recording electrodes.

Supramaximal stimulation[4] with trains (140 msec; delay = 1 sec) of square wave pulses at a frequency of 89 cycles/sec and a duration of 0.07 msec was utilized. The nerve action potential arising from the recording electrodes was displayed oscillographically[5]. Nerves were permitted to equilibrate in drug-free frog Ringer's solution for 30 min prior to the initiation of an experiment. Measurements of the spike height just prior to drug exposure were taken as individual control values for each nerve.

At the end of the equilibration period, the drug-free Ringer's solution was replaced with drug-containing Ringer's solution and the spike height was measured at 5-min intervals. The nerve remained

[1] West Jersey Biological Supply, Wenonah, N.J.
[2] Purina laboratory chow.
[3] Baxter Laboratories, Division of Travenol Laboratories, Inc., Morton Grove, Ill.
[4] Laboratory stimulator model 104A, American Electronic Laboratories Inc., Colmar, Pa.
[5] Tektronix oscilloscope model 502A, Tektronix, Inc., Beavorton, Ore.

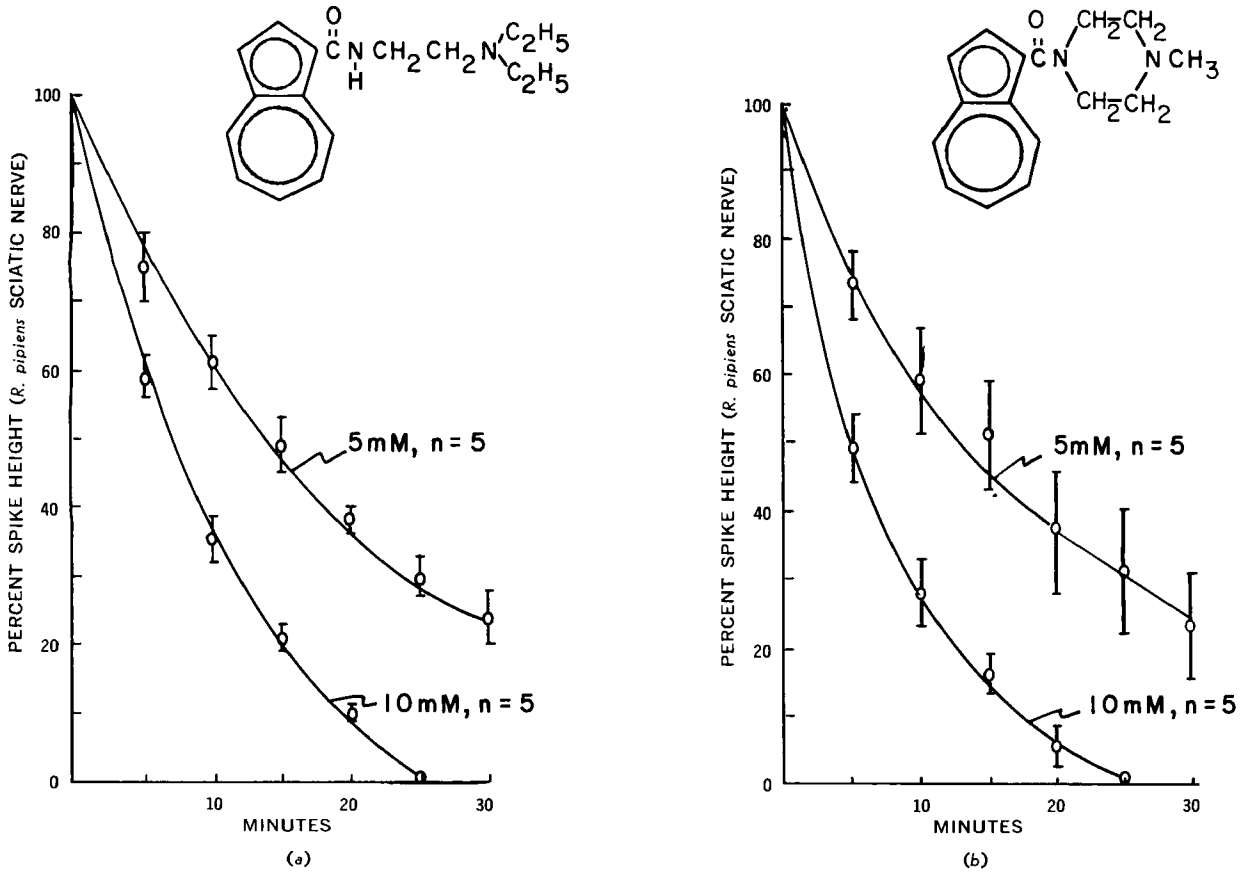


Figure 2—*Inhibition of sciatic nerve action potential* (R. pipiens) *with time at 5- and 10-mmole concentrations of:* (a) *1-(2-diethylaminoethyl)azulenecarboxamide (II-1), and* (b) *1-(N-methylpiperazine)azulenecarboxamide (III-1). Each experimental point represents the mean of five experiments; the standard deviation for each point is also shown.*

in contact with the drug solution for the duration of the experiment. A fresh nerve was used for each experiment, and no nerve was exposed to a second drug-containing solution.

The reversibility of drug effects was assessed by repeated washing of the nerve preparation with drug-free Ringer's solution until the action potential was restored.

Preliminary range-finding experiments established the concentrations at which 50% inhibition of conduction could be established within 30 min. These experimentally determined concentrations for each azulenoid were then used in subsequent replicate experiments. Experiments were replicated five or more times.

All azulenoid compounds tested were synthesized in these laboratories (1, 2). Lidocaine hydrochloride[6] and procainamide hydrochloride[7] were used as comparative controls and 0.9% sodium chloride solution and frog Ringer's solution were used as vehicle controls in LD_{50} and nerve conduction studies, respectively.

RESULTS AND DISCUSSION

The LD_{50} doses and 95% confidence limits of the several compounds are presented in Table I, both as milligrams per kilogram and as millimoles per kilogram, the latter to provide a rapid method of comparison between the compounds. The ratios of the LD_{50} values, in millimoles per kilogram, of the azulene derivatives and the benzenoid prototypes are also listed in the table.

The two azulylamides have approximately the same LD_{50} values as lidocaine and have about twice the toxicity of procainamide. The azulenecarboxamides, by contrast, have LD_{50} values extending over a threefold range, depending on substitution. The parent and 3-nitro-substituted carboxamides are substantially more toxic than are the corresponding 3-acetamido derivatives; similarly, substitution of an acetamido group on the annular system reduces the toxicity of the parent compound by at least half. In the case of Compound III-3, the LD_{50} value is of the same order as that of procainamide on a millimoles per kilogram basis.

All carboxamides except the 3-acetamidopiperazineamido analog are more toxic than procainamide, but three of these (Compounds II-3, III-1, and III-3) are of lesser toxicity than lidocaine.

Lethal doses of both experimental and reference compounds appeared to produce death by respiratory collapse. The sequence of toxic effects was as follows. In all instances, the mice were initially depressed following administration of the compounds. Subsequently, they suffered prolonged mixed clonic and tonic convulsions. Death, when it occurred, was a result of respiratory failure. The intensity and duration of convulsions were roughly proportional to the administered dose.

The intensity of the convulsions was greatest in the 3-nitro-substituted compounds, intermediate in the 3-unsubstituted compounds, and least in the 3-acetamido compounds. These highly colored compounds and their metabolites were detected in urine soon after administration. The metabolic transformation of selected azulylcarboxamides is currently under investigation. Gross observation upon necropsy showed the large and small intestines, mesentery, and bladder to be deeply stained by the azulenoid compounds.

All 3-unsubstituted azulene derivatives produced reversible graded dose–effect relationships in isolated nerve preparations. Two- to threefold changes in concentration of azulenoid produced about twofold changes in the time required for a 50% inhibition of conduction, with higher concentrations producing more rapid effects.

Figures 1*a* and 1*b* show time–effect relationships on frog nerve conduction produced by the azulylamides I-1 and I-2. Neither of these compounds is as potent on a molar basis as is the prototype compound lidocaine.

Figures 2*a* and 2*b* show the time–effect relationship on frog

[6] Supplied by Dr. Bertil Takman, Astra Laboratories, Worcester, Mass.
[7] K and K Laboratories, Jamaica, N.Y.

nerve conduction produced by the azulenecarboxamides II-1 and III-1. Although the prototype compound for these carboxamides, procainamide, is without effect on frog sciatic nerve, both azulenoid compounds inhibited conduction. Compared with lidocaine, these compounds are about half as active.

The 3-nitro compounds (II-2 and III-2) consistently produced an irreversible inhibition of conduction graded with concentration. However, in contrast with the other compounds, the dose–effect relationships produced by the nitro derivatives were extremely variable from nerve to nerve.

The 3-acetamidocarboxamides are devoid of nerve conduction-inhibiting activity at concentrations up to 50 mmoles.

REFERENCES

(1) P. H. Doukas and T. J. Speaker, *J. Pharm. Sci.*, **60**, 184(1971).

(2) *Ibid.*, **60**, 479(1971).

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[x] To whom inquiries should be directed.

Chromatography on Lipophilic Dextran Gels for Fractionation of Low Molecular Weight Compounds I: Steroid Digitonides

M. M. EL-OLEMY [*] and S. J. STOHS [x]

Abstract □ A method for the separation of 3β-hydroxysterols from other sterols is presented. The method involves precipitating 3β-hydroxysterols as the digitonides. The digitonide is then decomposed and separated into components using chromatography on highly cross-linked lipophilic polysaccharide gels. The digitonin in the mother liquor is separated from other sterols using the same chromatographic procedure.

Keyphrases □ Dextran gel chromatography—decomposition of steroid digitonides □ 3β-Hydroxysterols—separation from other sterols by dextran gel chromatography □ Chromatography—separation of 3β-hydroxysterols from other sterols, dextran gel columns

The ability of digitonin to form a sparingly soluble complex with an equivalent amount of 3β-hydroxysteroids was first noted in 1910 (1). The manipulation of concentration (2) as well as the use of gathering agents such as aluminum chloride and aluminum hydroxide was later introduced to permit the rapid and quantitative precipitation of steroid digitonides (3–5). Specificity of digitonide formation to 3β-hydroxysterols (6) and 3β-hydroxysteroidal sapogenins (7) has been shown. Haslam and Klyne (6) showed that 3β-hydroxysterols of the 5α series (A/B *trans*) and Δ^5-3β-hydroxysterols are precipitated at a higher dilution than 3β-hydroxysterols of the 5β series (A/B *cis*). This property can be used to fractionate the 3β-hydroxysterols further (6).

DISCUSSION

Digitonide formation has been used in the quantitative estimation of 3β-hydroxysteroids. A gravimetric assay was proposed (1) while more recently a photometric assay was used for this purpose (2, 4, 8, 9). A radioisotopic assay was also proposed (10).

Decomposition of steroid digitonides has afforded a means for the isolation of 3β-hydroxysteroids. Windaus (1) proposed prolonged boiling with xylene. Schöenheimer and Dam (11) decomposed the digitonides in pyridine. Ether was then added to precipitate digitonin and the steroids were obtained by evaporation of the filtrate.

Mikhelovich (12) showed that the Schöenheimer and Dam (11) method results in less decomposition of either the steroid or digitonin than the Windaus (1) method. Bergmann (13) modified the Schöenheimer and Dam procedure by heating the digitonide with anhydrous pyridine at 70–100°, evaporating pyridine, and extracting the steroid with ether in a soxhlet apparatus. The digitonin was then recovered by extraction with hot 90% alcohol.

Sobel *et al.* (9) extracted the steroids with petroleum ether after digitonide decomposition and demonstrated that pyridine was superior to acetic acid for digitonide decomposition. Issidorides *et al.* (14) used dimethyl sulfoxide to decompose the digitonide. Digitonin remained in solution, while the precipitated steroids were extracted with hexane. The digitonin was subsequently obtained by evaporation of dimethyl sulfoxide.

Digitonide formation has been used to isolate digitonin-like compounds by precipitation with cholesterol. Chanley *et al.* (15) used this procedure in the isolation of holothurin A from the sea cucumber, *Actinopygo agassiza*, while Issidorides *et al.* (14) used this procedure for the recovery of tomatine and honothurin.

^{3}H-Digitonin has been employed to determine the subcellular location of sterols by autoradiography (16). Digitonin has also been used to fragment chloroplasts. The enzymes associated with fragments of various particle sizes can then be examined (17).

Several sources of errors may be encountered when using digitonin to precipitate sterols quantitatively (18). Terpenoids and other products are also precipitated, while excess digitonin cannot be readily removed from the precipitate (18).

Occasionally, the separation of 3β-hydroxysterols from 3α-, 3-dehydro-, or other sterols is required. This report describes a method for effecting such fractionation using digitonide preparations and lipophilic, highly cross-linked dextran gel[1] chromatography. The procedure involves precipitation of 3β-hydroxysterols as the digitonides. The digitonides, in solution, are applied to this column, using benzene–methanol (1:1) as the eluting solvent,

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whereby the digitonides decompose and the components become separated at the same time. The mother liquor from the digitonide preparation, containing excess digitonin and other sterols, can also be separated into its components using the same type of column.

The procedure is especially suitable for heat-labile sterols, since a minimum of heat is utilized. This method is particularly suited for small amounts of materials but can be easily adapted to a larger scale. It can also be adapted to the isolation of the digitonin-like compounds by precipitation with cholesterol and subsequent fractionation.

The procedure was first successfully tested on an artificial mixture of the cholesterol and progesterone. It was then applied with equal success to an experimental mixture of stigmasterol and $\Delta^{4,22}$-stigmastadien-3-one obtained from the oxidation of stigmasterol. The oxidation of stigmasterol does not readily go to completion, and the mixture cannot be completely separated by recrystallization or column chromatography on silica gel.

EXPERIMENTAL

Δ^{4-22}-Stigmastadien-3-one from Stigmasterol—$\Delta^{4,22}$-Stigmastadien-3-one was synthesized by the Oppenauer (19) oxidation process. Stigmasterol (2 g) was dissolved in a dry mixture of 30 ml benzene and 15 ml acetone with 8 g aluminum *tert*-butoxide, and the mixture was refluxed for 8 hr. Upon cooling, 4 ml of water was added, followed by 10 ml of 10% H_2SO_4 with vigorous shaking and an additional 30 ml of water. The organic phase was removed, and the aqueous phase was reextracted with benzene. The benzene fractions were pooled, dried over anhydrous sodium sulfate, and concentrated *in vacuo*.

The reaction products were tested on silica gel H plates using isopropyl ether–petroleum ether–acetic acid (70:30:1) (20). The spots, when visualized by spraying with sulfuric acid and heating at 110° for 10 min, indicated a $\Delta^{4,22}$-stigmastadien-3-one to stigmasterol ratio of about 3:1. The mixture was recrystallized once from ethanol before being used for digitonide preparation.

Preparation of Digitonide—The oxidation product (20 mg) was dissolved in 1 ml of hot 90% ethanol. To this solution was added 4 ml of 1% digitonin in 90% ethanol. The solution was concentrated under nitrogen to 3 ml and cooled, and the crystals that formed (22 mg) were filtered. The mother liquor was then concentrated to 1 ml and cooled to obtain a second crop of the digitonide. The combined stigmasterol digitonide obtained was recrystallized once from ethanol to obtain 16 mg of the product, which was used in the chromatography.

The mother liquor was tested in TLC, using the system silica gel H plates with isopropyl ether–petroleum ether–acetic acid (70:30:1). $\Delta^{4,22}$-Stigmastadien-3-one and digitonin were detected with no trace of stigmasterol.

Dextran Gel Chromatography—A column (130 × 2 cm) of lipophilic dextran gel[1] was used for the fractionation of both the steroid digitonide and its mother liquor. A column was prepared using a slurry of the gel in benzene–methanol (1:1), eluting with the same solvent under a head of 150 cm at a flow rate of 1.0 ml/min, and collecting 5-ml fractions. Variation of flow rate between 0.5 ml and 1.3 ml/min did not hinder the separation. The relative concentration of each fraction was determined by means of TLC on silica gel H plates with isopropyl ether–petroleum ether–acetic acid (70:30:1). The relative concentrations were determined according to the size of each spot and density of the color obtained by spraying with 20% H_2SO_4 and heating the plates at 110° for 10 min.

Stigmasterol Digitonide—The digitonide, 10 mg, was dissolved in 1 ml of methanol–benzene (1:1) and applied to the described column. The digitonide simply decomposes on the column without the use of xylene, pyridine, acetic acid, or heat. In these investigations, digitonin was eluted in fractions 57–63 with the peak in fraction 60, while stigmasterol was obtained in fractions 65–74 with the peak in fraction 70.

The fractions containing stigmasterol were combined and evaporated to obtain stigmasterol, which was purified by recrystallization. Greater than 90% of the initially chromatographed amounts of both products were routinely recovered by this procedure. Larger amounts of materials can be separated effectively using larger columns.

Mother Liquor—The mother liquor was evaporated to dryness and dissolved in 1 ml of benzene–methanol (1:1). A 0.5-ml aliquot of this solution was applied to a dextran gel column. Digitonin was eluted in fractions 56–63 with the peak fraction at 61, while $\Delta^{4,22}$-stigmastadien-3-one was obtained in fractions 63–78 with the peak fraction at 68.

The fractions containing only $\Delta^{4,22}$-stigmastadien-3-one were combined and evaporated *in vacuo*. The residue was recrystallized from ethanol to obtain $\Delta^{4,22}$-stigmastadien-3-one in a pure form (R_f in TLC and mass spectroscopy).

[1] Sephadex LH-20, Pharmacia Fine Chemicals, Inc., Piscataway, NJ 08854

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Chromatography on Lipophilic Dextran Gels for Fractionation of Low Molecular Weight Compounds II: Separation of Cardiac Glycosides from Cardiac Aglycones

M. M. EL-OLEMY * and S. J. STOHS ˣ

Abstract □ A simple method is presented for the separation of cardiac glycosides from cardiac aglycones using highly cross-linked lipophilic dextran gel chromatography with 95% ethanol as the solvent.

Keyphrases □ Dextran gel chromatography—separation of cardiac glycosides from cardiac aglycones □ Cardiac glycosides—separation from cardiac aglycones using dextran gel chromatography □ Chromatography—separation of cardiac glycosides from cardiac aglycones

The successful use of lipophilic dextran gel chromatography for the separation of cardiac glycosides from cardiac genins in a simple and reproducible manner is reported. The force responsible for the separation appears primarily to be a molecular sieving effect.

DISCUSSION

Several chromatographic techniques for the separation and purification of various cardiac glycosides have been extensively used. The techniques most often employed include paper chromatography (1) and TLC (2). Column chromatography (3–5) and countercurrent separation (6, 7) have also been used.

The use of dextran gel chromatography for the separation of compounds of varying molecular weight has been well documented. The separation of various classes of steroids and lipids was attempted using the lipophilic dextran gel[1] (8–11) or partition chromatography on hydrophilic dextran gels of the G type (9, 12, 13). Recently, a hydroxycyclohexyl derivative (14) and four hydroxyalicyclic derivatives of the lipophilic dextran gel[1] (15) were prepared, and standard elution volumes for a model set of steroidal compounds were reported for columns prepared for these modified materials (14, 15). Lipophilic dextran gels, however, have not been used for the separation of cardenolides, although column chromatography on another dextran gel[2] was used in the purification of cardiac glycosides and cardiac genins extracted from cardiac tissues of experimental animals (16).

The chromatographic method reported here was initially applied to artificial mixtures of two or more of the following compounds: digitoxin (mol. wt. 764.92), gitoxin (mol. wt. 780.92), digitoxigenin (mol. wt. 374.50), and gitoxigenin (mol. wt. 390.50). The procedure was then successfully used for the separation of a mixture of ^{3}H-digitoxigenin and ^{3}H-digitoxin prepared by the incomplete acid hydrolysis of ^{3}H-digitoxin. Since radioactive digitoxigenin is not commercially available, while ^{3}H-(G)-digitoxin can be purchased, it was desired to prepare ^{3}H-digitoxigenin from ^{3}H-(G)-digitoxin by acid hydrolysis and subsequent purification. The isolated ^{3}H-digitoxigenin is being used in a series of experiments designed to determine the metabolism of digitoxigenin by tissue homogenates from various experimental animals.

Several solvent systems of 95% ethanol, 70% ethanol, and methanol–chloroform (1:1) were used for eluting the columns[1]. The best separation was obtained using 95% ethanol.

The method provides an effective separation of the glycosides digitoxin and gitoxin from the aglycones digitoxigenin and gitoxigenin. However, there was no separation of the two aglycones from one another, or of the two glycosides from each other under the conditions employed. Apparently, the difference of only 16 molecular weight units is not sufficient to make these separations possible.

This method can be utilized for the preparative separation of the cardiac glycosides from their aglycones.

EXPERIMENTAL

Hydrolysis of ^{3}H-Digitoxin—To 10 μCi of uniformly labeled ^{3}H-digitoxin[3] (specific activity 1 mCi/μmole) was added 5 mg of nonradioactive digitoxin. The sample was dissolved in 10 ml of methanol–water (7:3), 60 μl of concentrated sulfuric acid was added, and the mixture was refluxed for 4 min. The reaction mixture was subsequently extracted with chloroform. The chloroform was then evaporated to dryness, and the residue was dissolved in dry benzene until ready for chromatography.

The residue was then evaporated to dryness and dissolved in 2 ml of 95% ethanol, an aliquot of which was applied to the column. Acid decomposition of the liberated digitoxigenin began to occur before complete hydrolysis of the sugar moiety of digitoxin was achieved, so a method for separating the digitoxigenin from the incompletely hydrolyzed digitoxin was needed.

Separation of Hydrolysis Mixture on Lipophilic Dextran Gels—An aliquot (1 ml) of the ethanolic solution obtained was applied to a column, 2.5 cm in diameter and containing 60 g of the gel in 95% ethanol. Fractions (1 ml) were collected at a flow rate of 0.5 ml/min under a pressure head of 50 cm. Separation of cardenolides from the glycosides was possible with flow rates from 0.3 to 0.7 ml/min.

Fractions were tested by TLC on silica gel H[4] plates with either methylene chloride–methanol (85:15) (17) or cyclohexane–acetone–acetic acid (65:33:2) (18) as the developing solvent. After development, the plates were sprayed with ferric chloride in acetic acid (2 ml of 5% $FeCl_3$ in 20 ml of acetic acid), heated at 100° for 5 min, and cooled. The plates were then sprayed with 50% H_2SO_4 and heated again for 10 min.

The cardiac glycosides and aglycones give yellow to green or blue colors. The relative concentrations of the fractions were determined according to the size and density of the color of the spot of TLC and/or by liquid scintillation counting for the tritium-labeled samples (19). Both digitoxin and gitoxin were eluted in fractions 81–90 with a peak at fraction 84, while digitoxigenin and gitoxigenin were eluted in fractions 94–107 with a peak at fraction 102.

The fractions containing each compound were combined and evaporated to dryness *in vacuo* to obtain chromatographically pure cardiac glycosides and cardenolides as determined by TLC. Approximately 50% of the radioactivity was recovered as digitoxigenin following the hydrolysis of ^{3}H-(G)-digitoxin.

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Antidermatophytic Activity of 2-Thiotetrahydro-1,3,5-thiadiazines and Isothiocyanates

VIJAYA MANOHAR *, S. V. K. NARASIMHA MURTHY ‡§, M. SIRSI *, G. RAMANANDA RAO *x, and P. L. NARASIMHA RAO ‡

Abstract □ Benzyl, furfuryl, tetrahydronaphthyl, α-picolyl, and α-homopicolyl derivatives of 2-thiotetrahydro-1,3,5-thiadiazines were studied and found to be more active than β-picolyl and pyridyl analogs on dermatophytes, including the more resistant *Microsporum canis*.

Keyphrases □ 2-Thiotetrahydro-1,3,5-thiadiazines—antidermatophytic activity □ Isothiocyanates—antidermatophytic activity □ Antidermatophytic activity—2-thiotetrahydro-1,3,5-thiadiazines and isothiocyanates

It has been demonstrated that pterigospermin and its fission product benzyl isothiocyanate are potent antifungal agents (1–7). Phytopathogenic and saprophytic fungi, especially the yeasts, show sensitivity to isothiocyanates and their producers (8). However, inadequate dermal absorption, low solubility, and vesication are but a few of the factors that limit their topical application in the treatment of dermoid infections.

It was thought that substitution of the benzyl moiety by other isosteric groups, particularly those with basic residues, would make the compounds more water soluble to overcome the limitations in their use. This report presents the results of studies to test this hypothesis.

EXPERIMENTAL

Organisms—*Trichophyton mentagrophytes*[1] (HM115), *T. rubrum*[1] (HM186), *T. violaceum*[1] (HM164), *Epidermophyton floccosum*[1] (HM78), *Microsporum canis*[1] (I73), and *Candida albicans*[2] (Z248) were used. All dermatophytes and yeasts were maintained on Sabouraud's glucose agar slants.

Inocula—Spore suspensions from 15-day agar slant cultures in sterile saline were prepared, and 0.1 ml of the suspension or of an 18-hr broth culture of *C. albicans* was used as the inoculum in the serial dilution method.

Compounds—Solutions (1 mg/ml) were made in slightly acidified 50% ethanol (basic compounds), 20% dimethylformamide (griseofulvin), or acetone.

Antifungal Activity—Tubes in duplicate containing log concentrations, 0, 0.1, 1.0, 10.0, and 100 μg/ml, of the test compounds in 5.0 ml of Sabouraud's glucose broth were inoculated and incubated at 30° for 14 days (dermatophytes) or for 2 days (yeast). Tubes containing solvents at corresponding concentration levels were also included as blanks. The minimum inhibitory concentration (MIC) required for complete inhibition of growth was scored based on at least two independent experiments.

RESULTS AND DISCUSSION

The results of initial screening of antifungal activity of a few isothiocyanates and their producers, substituted 2-thiotetrahydro-1,3,5-thiadiazines, and other substances are reported in Table I. Benzyl isothiocyanate (XXI) inhibited the dermatophytes and *C. albicans* in the concentration range of 0.1–5.0 μg/ml, while the corresponding thiadiazine (X) did so at a level of 1.0–10.0 μg/ml. Similar levels of activity were displayed by furfuryl and tetrahydronaphthyl analogs (XIII and XIV, respectively). α-Picolyl and α-homopicolyl derivatives (III and VI, respectively) were distinctly more active then the β- and γ-isomers (IV and V, respectively) and far more potent than the pyridyl compounds (I and II, respectively).

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Table I—Antifungal Activity of Thiadiazines and Isothiocyanates on Dermatophytes

	Compound	MIC, μg/ml					
		T. mentagrophytes	*T. rubrum*	*T. violaceum*	*E. floccosum*	*M. canis*	*C. albicans*
	3,5-Disubstituted 2-Thiotetrahydro-1,3,5-thiadiazines						
I	α-Pyridyl	100	100	100	100	100	50
II	β-Pyridyl	100	10	100	100	100	50
III	α-Picolyl	10	10	10	10	100	4
IV	β-Picolyl[a]	100	100	10	100	100	10
V	γ-Picolyl[a]	100	100	10	100	100	15
VI	α-Homopicolyl	10	1.0	10	0.1	0.1	10
VII	*o*-Methylphenyl	100	100	100	100	n.a.[a]	—[b]
VIII	*m*-Methylphenyl	100	100	100	100	n.a.	—
IX	*p*-Methylphenyl	100	100	100	100	n.a.	—
X	Benzyl	10	10	1.0	10	10	6
XI	*o*-Methylbenzyl	10	10	0.1	10	10	—
XII	*p*-Methylbenzyl	100	0.1	0.1	10	10	—
XIII	Furfuryl	1.0	0.1	0.1	10	10	—
XIV	Tetrahydronaphthyl	0.1	0.1	0.1	0.1	10	5
XV	Isobutyl	10	100	100	100	100	—
XVI	*o*-Chlorobenzyl[a]	n.a.	n.a.	n.a.	n.a.	n.a.	2
XVII	*o,p*-Dichlorobenzyl[c]	n.a.	n.a.	n.a.	n.a.	n.a.	5
XVIII	*m,p*-Dichlorobenzyl[c]	n.a.	n.a.	n.a.	n.a.	n.a.	5
XIX	*p*-Bromobenzyl[c]	n.a.	n.a.	n.a.	n.a.	n.a.	4
XX	*o,p*-Dibromobenzyl	n.a.	n.a.	n.a.	n.a.	n.a.	5
	Other Compounds						
XXI	Benzyl isothiocyanate	1.0	0.1	0.1	0.1	1.0	5
XXII	Allyl isothiocyanate	100	100	100	100	100	—
XXIII	*ac*-Tetrahydro-β-naphthyl isothiocyanate	1.0	1.0	0.1	0.1	10	5
XXIV	Griseofulvin	10	10	1.0	1.0	20	—

[a] Reference 11. [b] n.a. = not active up to 1000 μg/ml. — = not tested. [c] Reference 6.

While the homologous phenyl and other compounds were less active, halogenated derivatives (XVI–XX), which markedly inhibited yeasts and other phytopathogens (6), failed to show any antidermatophytic activity. Higher levels of thiadiazines, which expectedly produced active isothiocyanates *in vivo* (9, 10), may be needed to bring about complete inhibitory activity.

A few compounds seemed comparable to, or better than, griseofulvin in their antidermatophytic activity. *M. canis*, which was relatively more resistant to griseofulvin, was equally susceptible as the other dermatophytes to the action of furfuryl and tetrahydronaphthyl compounds (XIII and XIV, respectively) and even more susceptible to VI. These compounds are definitely more potent antidermatophytic agents than the benzyl analogs.

The solvents used, ethanol, dimethylformamide, and acetone, did not inhibit the growth of dermatophytes and yeasts.

In exploratory clinical trials, treatment with IV and X appears quite effective in superficial dermatomycoses.

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Disintegration Properties of Calcium Phosphate Dibasic Dihydrate Tablets

K. A. KHAN * and C. T. RHODES ‡×

Abstract □ The effect of compressional force on the disintegration time of tablets prepared from calcium phosphate dibasic dihydrate containing various tablet disintegrants was examined. The results show that effects of compressional force on disintegration time are of two types. The first type is that of insoluble disintegrants, *e.g.*, starch and a cation-exchange resin, where the disintegration time initially shows a dramatic decrease. After this decrease, a further increase in compressional force appears to have no effect on the disintegration time. The second type is that of soluble disintegrants, *e.g.*, calcium sodium alginate, sodium carboxymethylcellulose, and sodium starch glycolate, where variation in compressional force has very little effect on the disintegration time. These results are discussed in terms of the differing mechanism whereby these substances act as disintegrants.

Keyphrases □ Calcium phosphate dibasic dihydrate—disintegration time □ Disintegrants—effect of compressional force on disintegration of starch, cation-exchange resin, calcium sodium alginate, sodium carboxymethylcellulose, sodium starch glycolate

Several investigators have studied the effect of compressional force on the disintegration time of tablets. Most published reports have shown that an increase in compressional force increases the disintegration time. The disintegration time for tablets was shown (1, 2) to continue to increase as the compressional force increased, even though the compression ratio of the tablets had reached a maximum.

Similarly, a semilogarithmic relationship was found (3, 4) between the disintegration time and the compressional force for certain tablets made by static methods, despite the fact that their porosities reached constant values. A linear relationship between the compression pressure and disintegration time of tablets containing lactose and potato starch was found (5). From studies (6) of starch and aspirin tablets, a two-phase increase was reported when the results were plotted on a linear scale. When using various celluloses in different organic and inorganic bases, there was a two-stage linear increase in the logarithm of the disintegration time with increasing compression pressure (7).

It was found that although the disintegration time increases with increasing compressional force, the relationship differs with each formulation (8). An entirely different relationship was recently shown (9) for dicalcium phosphate dihydrate tablets containing a cation-exchange resin; an increase in compressional force enhanced the dissolution efficiency and reduced the disintegration time. Following this work, it was considered important to examine the effect of the presence of different disintegrants on the relationship between compressional force and disintegration time of tablets prepared from an insoluble direct-compression system, since these areas have not been investigated previously.

EXPERIMENTAL

Materials—Starch (BP grade), calcium sodium alginate[1], cation-exchange resin[2], sodium starch glycolate[3], sodium carboxymethylcellulose[4], calcium phosphate dibasic dihydrate[5] (unmilled), and magnesium stearate (BPC grade) were used.

Methods—The excipients were mixed with the lubricant and 10% (w/w) of disintegrant by quartering and shaking vigorously in a plastic bag. The lubricant used was 1% magnesium stearate, which was passed through a No. 60 hand screen before blending with the other powders. The tablets were made on an instrumented single-punch machine[6] using 1.02-cm (0.4-in.) flat punches.

The upper punch forces were measured by using Kistler load cells in a similar manner as described by Marshall (10). The particle density of the mixtures was determined using a specific gravity bottle, with benzene as the supernatant liquid. Apparent tablet densities were obtained from thickness and diameter measurements of at least 10 tablets.

Tablet porosity was calculated from particle density and apparent tablet density values. The mean hardness[7] of at least five tablets was obtained. Disintegration times were determined on a disintegration apparatus[8] using the BP method. The mean of at least five determinations was obtained.

RESULTS AND DISCUSSION

Figure 1 shows the effect of compressional force on the porosities of the calcium phosphate tablets containing different disinte-

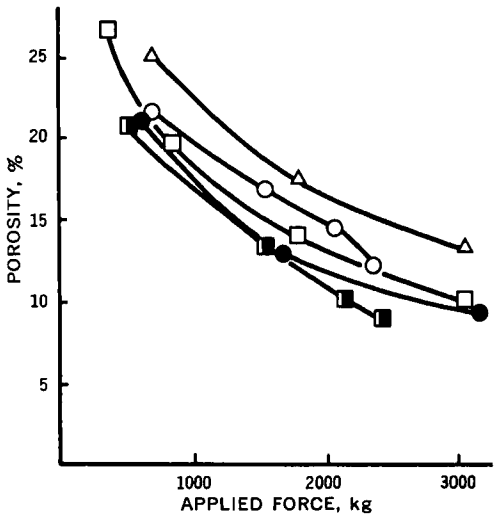

Figure 1—*Effect of applied force on the porosity of tablets prepared from calcium phosphate dibasic dihydrate containing different disintegrants. Key:* △, *cation-exchange resin;* ○, *sodium carboxymethylcellulose;* □, *sodium starch glycolate;* ●, *calcium alginate; and* ■, *starch.*

[1] Alginate F417, supplied by Alginate Industries, London, England.
[2] Amberlite 1RP88, supplied by Lemming Chemical Ltd., London, England.
[3] Primojel, supplied by Gordon Slater Ltd., Cheshire, England.
[4] Courlose P20, British Celanese Ltd., Coventry, England.
[5] Supplied by Albright and Wilson, Idbury, Worcestershire, England.
[6] Manesty type F3.
[7] Erweka hardness tester.
[8] Manesty.

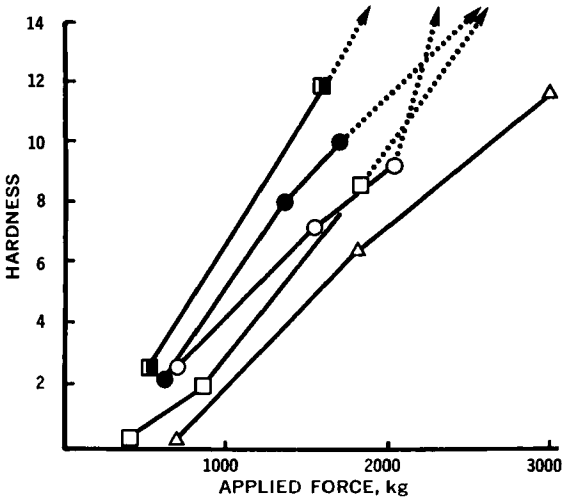

Figure 2—*Effect of applied force on the hardness of tablets prepared from calcium phosphate dibasic dihydrate containing different disintegrants. Key: △, cation-exchange resin; ○, sodium carboxymethylcellulose; □, sodium starch glycolate; ●, calcium sodium alginate; and ■, starch.*

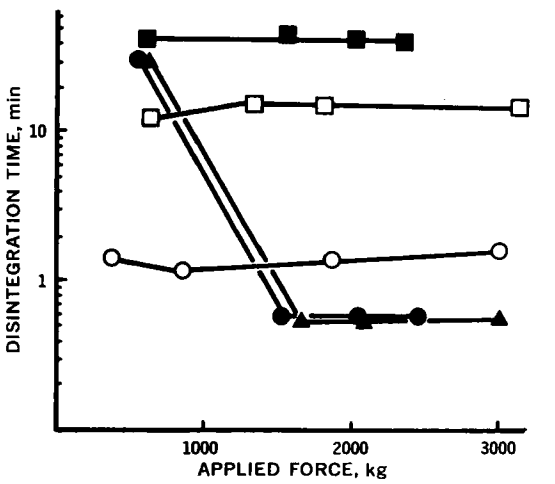

Figure 3—*Effect of applied force on the disintegration time of tablets prepared from calcium phosphate dibasic dihydrate containing different disintegrants. Key: ▲, cation-exchange resin; ■, sodium carboxymethylcellulose; ○, sodium starch glycolate; □, calcium sodium alginate; and ●, starch.*

grants. It is apparent that the porosity of all tablets decreased with increasing compressional force. The rate of decrease in porosities with increasing pressure appears to be the same with all disintegrants. The cation-exchange resin produced the most porous tablets and starch the least porous.

It is well known that starch undergoes plastic deformation under compression (11); its glidant action may have also contributed to the closer packing. The higher porosity of tablets containing the cation-exchange resin probably resulted mainly from the poor compression characteristics of this disintegrant (12). The poor adhesion between the cation-exchange resin and calcium phosphate particles is further confirmed by the effect of compressional force on tablet hardness (Fig. 2). Here again, the tablets containing cation-exchange resin were softer.

The effect of compressional force on tablet disintegration time reveals that there are two types of disintegrant behavior (Fig. 3). In the first type (starch and the cation-exchange resin), the disintegration time initially shows a dramatic decrease, after which a further increase in compressional force appears to have no effect on the disintegration time. In the second type (calcium sodium alginate, sodium carboxymethylcellulose, and sodium starch glycolate), variation of compressional force has very little effect on the disintegration time.

The literature generally reports that harder tablets take longer to disintegrate, but Fig. 3 shows that in the systems investigated the reverse appears to be the case. Although the finding of Higuchi *et al.* (4) cannot be compared with those presented in this paper because of great differences in the formulations used, there is one important similarity. These workers found an initial decrease in the disintegration time for tablets containing starch. A similar behavior was found in this study for tablets containing starch and the cation-exchange resin (Fig. 3).

This behavior might be explained by the hypothesis proposed (1) for potato starch and phenacetin tablets. As the compression force increases, the tablet porosity decreases, and the pore diameter may also decrease, as noted previously (13, 14) using tablets of aspirin and starch. At low compression forces, often as much as 20% void space exists within the tablet, and the pore diameter is large. Penetration by the disintegration fluid is, therefore, very rapid, and the disintegrant particles begin to swell and fill the void space without immediately affecting tablet disintegration.

As the compression force increases, the time taken for penetration of fluid and the swelling of disintegrant particles with subsequent tablet disintegration is increased by the reduction in pore size. There is, however, a decrease in porosity (Fig. 1), which means that the swelling particles have a more immediate effect on the tablet structure. The effectiveness of starch and the cation-exchange resin, when used at 10% concentration, perhaps counteracts the effect of reduced penetration. Thus, further increase in compressional force does not affect the disintegration time.

Other reasons for the reduction of disintegration time with increased compressional force (for tablets prepared from calcium phosphate dibasic dihydrate) have been described (9). The phenomenon of initial decrease in disintegration time with increasing compression force is more pronounced for insoluble disintegrants because the rate-determining step becomes that of water penetration with subsequent swelling of disintegrant particles.

The second group of disintegrants (sodium starch glycolate, sodium carboxymethylcellulose, and calcium sodium alginate) is somewhat soluble. The disintegration of tablets containing these disintegrants is brought about by a number of factors, including dissolution of disintegrant particles. After the penetration of water, the disintegrant particles absorb water and begin to swell and also to dissolve. All these factors are additive in their effect and counteract the effect of reduced penetration due to reduction in pore size. The result (Fig. 3) is that disintegration time is largely unaffected by the increase in compressional force.

In conclusion, it is suggested that the effect of compressional force on tablet disintegration may depend upon the disintegrant and the diluent used. Generalizations such as the harder the tablet, the slower the disintegration rate are not applicable to all systems.

This study also reveals an extremely important property of calcium phosphate dibasic dihydrate. The disintegration time of tablets prepared from this material, contrary to most published reports, does not increase with increasing compressional force.

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Improved Synthesis of 18-Hydroxydeoxycorticosterone

W. DAVID SLAUNWHITE and A. J. SOLO ˣ

Abstract □ Hypoiodite photolysis of 3β-acetoxy-5-pregnen-20β-ol gave 3β-acetoxy-5-pregnene-18,20-lactone in 46% yield. Lithium aluminum hydride reduction of the latter afforded 3β,18,20β-trihydroxy-5-pregnene (91% yield) which, on Oppenauer oxidation, was converted to 18-hydroxyprogesterone (66%). Lead tetraacetate oxidation followed by mild saponification gave 18-hydroxydeoxycorticosterone (58% yield).

Keyphrases □ 18-Hydroxydeoxycorticosterone—synthesis □ 18,-20-Oxido-20-hydroxy-4-pregnen-3-one—synthesis □ 18-Hydroxyprogesterone—synthesis

Because recent evidence suggests that 18-hydroxydeoxycorticosterone (I) may be an important causative agent in hypertension (1–6), considerable interest recently has been shown in the synthesis of this compound. Syntheses reported to date have either been overly long (7), low in yield (7, 8), or lacking in needed experimental detail (9). The details of a short, reproducible, high yield synthesis of I from 3β-acetoxy-5-pregnen-20-one (II) are now reported (Scheme I).

DISCUSSION

3β-Acetoxy-5-pregnen-20β-ol (III), prepared in 69% yield by borohydride reduction of pregnenolone acetate (II), was subjected to photolysis in the presence of lead tetraacetate and iodine (10) to afford 3β-acetoxy-5-pregnene-18,20-lactone (IV) in a yield of 46%. Lithium aluminum hydride reduced IV to 3β,18,20β-trihydroxy-5-pregnene (V) in a yield of 91%. Oppenauer oxidation converted V to 18,20-oxido-20-hydroxy-4-pregnen-3-one (VI) in 66% yield.

The latter hemiketal (VI) was oxidized by lead tetraacetate in acetic acid (9) and then hydrolyzed with dilute hydroxide to form I. Reaction for 18 hr with excess tetraacetate caused the intermediate 21-acetoxy Compound VII to cleave to form the lactone VIII. However, by limiting the reaction time to 6 hr, VI could be transformed to I in 58% yield. An attempt to improve this yield by limiting the amount of tetraacetate proved futile.

In summary, the reported procedure has permitted the reproducible preparation of I from a cheap and readily available starting material in an overall yield of 11%.

EXPERIMENTAL[1]

3β-Acetoxy-5-pregnen-20β-ol (III)—Pregnenolone acetate (20 g) in tetrahydrofuran (100 ml) was added to sodium borohydride (3 g) in water (10 ml) and stirred at room temperature for 72 hr. Acetic acid was added and then the mixture was diluted with water. The solvent was concentrated under reduced pressure.

The solid was filtered, dried, and then chromatographed on a column of 200 g of acidic alumina. Elution with benzene–ethyl acetate (100:20) afforded III in a yield of 14 g (69%); mp 167° [lit. (11) mp 165°]; IR ($CHCl_3$): ν 3610 and 1720 cm^{-1}; NMR: δ 0.78 (s, C-18 H), 1.04 (s, C-19 H), 2.03 [s, $O(O{=})CCH_3$], and 5.39 (d, $J \approx 4$ Hz, C-5 H).

3β-Acetoxy-5-pregnene-18,20-lactone (IV)—To a stirred suspension of lead tetraacetate (15 g) and calcium carbonate (5 g) in cyclohexane (500 ml) at 80° were added iodine (4 g) and III (5 g). The stirred mixture was refluxed and irradiated with a 250-w tungsten lamp for 150 min and then was cooled and filtered. The filter cake was washed with cyclohexane, and the filtrate was washed with aqueous sodium thiosulfate (5%) and water. After pyridine (1.25 ml) was added, the filtrate was concentrated under reduced pressure.

The residue was dissolved in acetone (100 ml), and silver chromate (2.5 g) was added. The mixture was cooled to 0°, and 5.7 ml of a solution prepared by dissolving chromium trioxide (13.3 g) in sulfuric acid (11.5 ml) and diluting to 50 ml with water was added slowly. After 60 min a solution of sodium acetate (60 g) in water (100 ml) was added. The mixture was extracted two times with benzene.

The organic layer was washed (water), dried (magnesium sulfate), and concentrated under reduced pressure. Crystallization from benzene–ether gave IV in a yield of 2.4 g (46%), mp 208–210°; IR ($CHCl_3$): ν 1745 and 1720 cm^{-1}; NMR: δ 1.13 (s, C-19 H), 2.02 [s, $O(O{=})CCH_3$], and 5.40 (s, C-5 H).

Anal.—Calc. for $C_{23}H_{32}O_4$: C, 74.16; H, 8.66. Found: C, 74.23; H, 8.66.

3β,18,20β-Trihydroxy-5-pregnene (V)—A mixture of IV (1 g) and lithium aluminum hydride (1 g) in tetrahydrofuran (60 ml)

[1] Melting points were determined in an open capillary tube on a Mel-temp apparatus and are uncorrected. IR spectra were recorded on a Beckman IR-8. NMR spectra were determined on a Varian A-60 spectrometer in deuterochloroform solution and are reported in parts per million downfield from a tetramethylsilane internal standard. Elemental analyses were determined by Atlantic Microlab, Inc.

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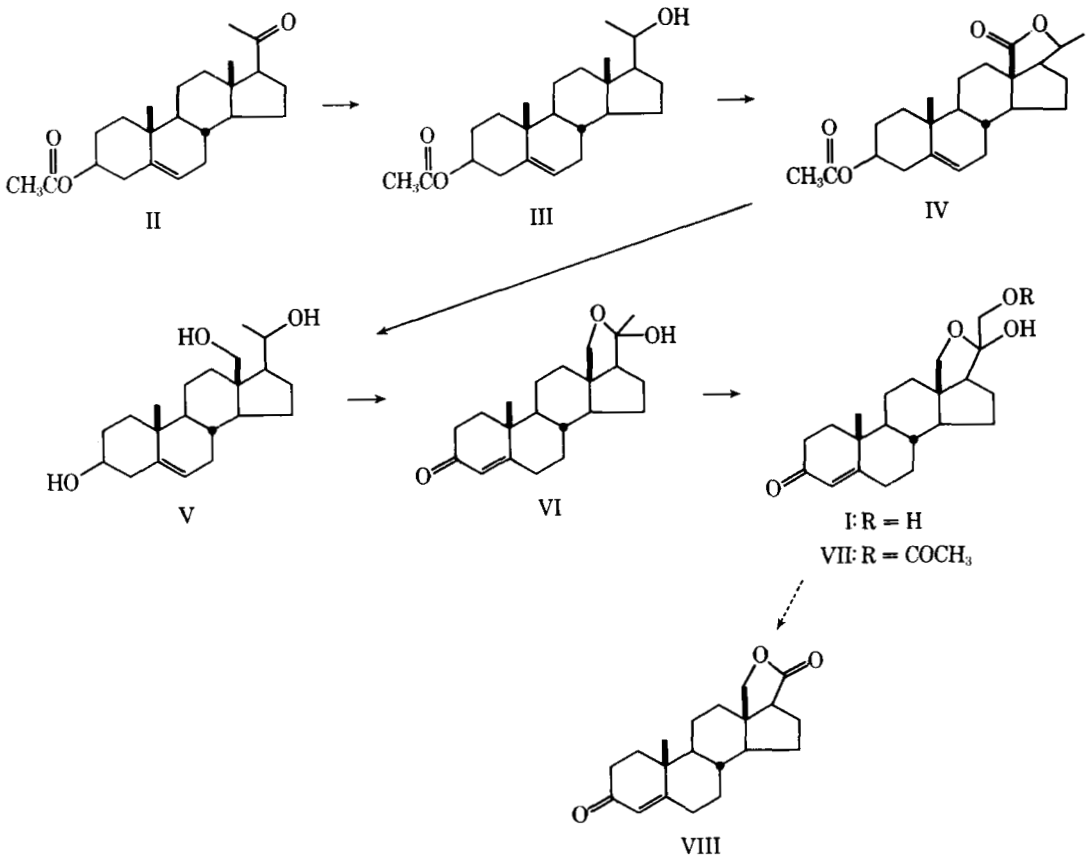

Scheme I

was stirred overnight at room temperature. Standard work-up afforded V which, when recrystallized from acetone, afforded an analytical sample, 0.82 g (91%); IR (mineral oil): ν 3370 cm^{-1}.

Anal.—Calc. for $C_{21}H_{34}O_3$: C, 75.41; H, 10.25. Found: C, 75.18; H, 10.08.

18,20-Oxido-20-hydroxy-4-pregnen-3-one (VI)—Ten milliliters was distilled from a mixture of toluene (80 ml), cyclohexanone (25 ml), and V (0.80 g). Then aluminum isopropoxide (0.80 g) was added, and the resulting mixture was heated under reflux for 5 hr. Standard work-up afforded VI, which crystallized from methylene chloride–ether in a yield of 0.50 g (66%), mp 172–175° [lit. (7) mp 173–182°]; IR ($CHCl_3$): ν 3440, 1660, and 1610 cm^{-1}; NMR: δ 1.11 (s, C-19 H), 1.47 (s, C-21 H), 3.71 (s, C-18 H), and 4.67 (s, C-4 H).

18,20-Oxido-20,21-dihydroxy-4-pregnen-3-one (I)—A mixture of VI (100 mg), lead tetraacetate (100 mg), and acetic acid (1.5 ml) was stirred at room temperature for 6 hr. The solution was poured on ice, extracted with ethyl acetate, washed with water, dried over magnesium sulfate, and concentrated under reduced pressure. The residue, consisting of a mixture of VI, VII, and VIII, was dissolved in methanol (10 ml). To the solution was added 1 ml of 0.1 *N* potassium hydroxide. The mixture was stirred for 2 hr at room temperature and then was neutralized with acetic acid and concentrated under reduced pressure.

The residue was dissolved in ethyl acetate, washed with water, dried over magnesium sulfate, concentrated, and purified by thick-layer chromatography on silica gel. Acetone–benzene (18:82) was used to develop the plate. The most polar band afforded I, in a yield of 60 mg (58%), mp 191–193° [lit. (7) mp 191–195°]; IR ($CHCl_3$): ν 3415, 1660, and 1610 cm^{-1}.

A slightly less polar band contained VI and an appreciably less polar band contained the lactone VIII, IR ($CHCl_3$): ν 1760 cm^{-1}. The NMR spectrum showed loss of acetate CH_3 and the C-18 protons appeared as an *AB* pattern centered at δ 4.05.

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Simple Isolated Perfused Artery Preparation: Vasoconstrictor Evaluation

CHESTER M. DAVIS and THOMAS F. BURKS ˣ

Abstract □ Small mesenteric arteries free of all extraarterial tissues were obtained from anesthetized dogs and perfused *in vitro* with Krebs solution. Vasoconstrictor responses of these arteries to intraarterial levarterenol and epinephrine were dose related and equivalent to those of arteries surrounded by fat and other tissues. Responsiveness was stable for at least 60 min. This simple preparation is useful for the study of vasoconstrictor phenomena in uncomplicated arterial tissue.

Keyphrases □ Vasoconstrictor evaluation—simple isolated perfused artery preparation described, response to catecholamines determined □ Artery preparation—preparation of simple isolated perfused artery described, vasoconstrictor evaluation □ Vascular smooth muscle—description of simple isolated perfused artery preparation, vasoconstrictor evaluation

It is often desirable in pharmacological analysis to examine drug effects on arterial smooth muscle. Also, an uncomplicated technique for measurement of resistance in small isolated arteries can be advantageous in physiological studies of vascular smooth muscle.

Two methods are generally used in investigations of isolated vascular muscle reactivity: measurement of flow or pressure in perfused vessels, and direct recording of tension or length in vascular muscle strips (1). Smaller vessels that participate in regulation of peripheral resistance have usually not been studied by either method because of difficulty in preparation. The isolated artery preparation described here is relatively simple to prepare and should be easily adaptable to various test procedures.

EXPERIMENTAL

Artery Preparation—Mongrel dogs of either sex, 8–20 kg, were anesthetized with sodium pentobarbital (30 mg/kg) or sodium thiopental (15 mg/kg) and sodium barbital (250 mg/kg) administered intravenously. The anesthetic was supplemented as required during the experiment by administration into a cannulated femoral vein.

The small intestine was exposed by a midline abdominal incision, and an arcade of mesenteric artery supplying a small segment of intestine was chosen for isolation. The mesentery on either side of the arcade was cut with scissors, and umbilical tape ligatures were tied around the intestine on either side of the arterial fan. The mesenteric artery in the arcade chosen was then cannulated with a 4–6-cm length of polyethylene catheter (1.27 mm o.d.; PE 90), usually drawn down to slightly smaller dimensions (1.0 mm o.d.) at its tip and attached to a 10-ml syringe filled with Krebs solution.

After the catheter was tied into the artery, 5 ml of Krebs solution was gently flushed through the vasculature of the gut section to remove any blood. Another ligature was then tied centrally to the cannula, and the artery and periarterial tissue were clipped loose above the cannula and from the serosal surface of the intestine. Each dog was capable of supplying at least 10 such arteries. This procedure provides an "undissected" mesenteric artery, which could be perfused with a physiological salt solution (2).

The mesentery over the artery was then gently split with the tip of an iris forceps along the length of the artery and over the visible branches. With only slight traction on the artery, and with the aid of the iris forceps, the artery was gently dissected free of the periarterial tissues and its small terminals were cut wherever desired. It was generally convenient to prepare an artery with terminals of 0.2–0.5 mm o.d. or smaller. One isolated artery is illustrated in Fig. 1. Since the dissection of the artery required no longer than 1 min and small amounts of Krebs solution could be forced through the lumen every few seconds, it was not necessary to moisten the artery with an externally applied saline solution.

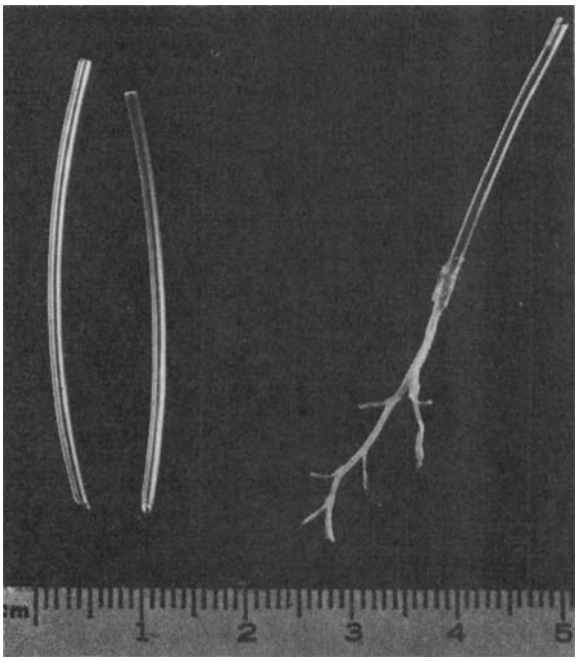

Figure 1—*Photograph of polyethylene cannulas and isolated mesenteric artery. The cannula on the left is the original tubing (PE 90); the one on the right was drawn down to a tip opening of approximately 1 mm o.d. The artery illustrated is typical of the ones employed in this study with terminal vessels less than 0.5 mm in diameter.*

Perfusion—The cannula of the artery was attached to Tygon and polyethylene tubing leading from a constant-flow pulsatile pump[1], and the artery was perfused with Krebs bicarbonate solution warmed to 37° and bubbled with 95% O_2–5% CO_2. The rate of flow varied from 10 to 20 ml/min, but a flow rate of 13 ml/min produced a perfusion pressure in most arteries of 25–30 mm Hg above the resistance offered by the cannula alone. Since flow was held constant, the perfusion pressure was directly proportional to the arterial resistance. Perfusion pressure was measured from a T-connection between the pump and the artery by a pressure transducer[2] and was recorded on an oscillographic recorder[3].

Intraarterial injections were made with a 100-μl syringe in volumes of 0.01–0.1 ml into injectable tubing immediately above the arterial cannula. A 2–3-cm section of injectable tubing was prepared by wrapping one or two thicknesses of adhesive tape around ordinary rubber tubing.

The artery was usually suspended from the cannula into the

[1] Sigmamotor type T-8.
[2] Statham P23Db.
[3] Beckman type RM dynograph.

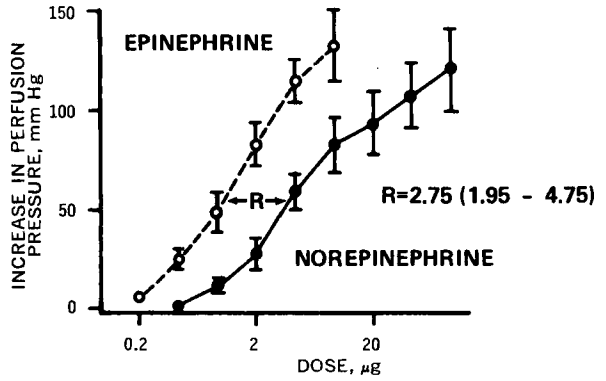

Figure 2—*Dose–response relationships of pressor responses (changes in perfusion pressure) to epinephrine and levarterenol (norepinephrine) administered intraarterially in isolated mesenteric arteries. R indicates that 1 dose unit of epinephrine produced an increase in perfusion pressure equivalent to 2.75 units of levarterenol. Each point is the mean (±SEM) of responses in preparations from six animals.*

warm, moist air of the muscle chamber of a conventional isolated muscle bath, and the perfusate was allowed to drain from the bottom of the bath. In some experiments, the artery was exposed to room air without observable changes in responsiveness.

Drugs and Chemicals—The vasoconstrictor agents employed were levarterenol (norepinephrine) bitartrate[4] and epinephrine bitartrate[5]. Doses are expressed as the free bases. Krebs bicarbonate solution of the following composition was employed as the perfusion medium (in millimoles): NaCl, 118.0; KCl, 4.7; $CaCl_2$, 2.5; KH_2PO_4, 1.1; $MgSO_4$, 0.5; $NaHCO_3$, 25.0; and glucose, 10.0.

Experimental Design—Two groups of six dogs were used. In one group, two dissected arteries were removed from each dog and each artery was injected with all dose levels of either levarterenol or epinephrine. These responses were employed in construction of dose–response curves. In the other groups of animals, six undissected arteries were removed from each dog and were attached to the perfusion apparatus. After their responsiveness to levarterenol and epinephrine stabilized, extraarterial tissues were removed and responses of the dissected arteries to the adrenergic amines were determined.

Effects of removing extraarterial tissues on responsiveness were compared by Student's *t* test, paired comparisons (3). Dose–response curves were compared by parallel line bioassay (4). Relative differences in arterial reactivity among dogs and by position removed (whether first or sixth) were determined by a Latin-square design analysis of variance (5).

RESULTS

The arteries were removed quickly and without difficulty from the donor animals. Once perfusion was begun, the baseline perfusion pressure usually stabilized within 5–10 min and remained relatively constant for at least 60 min. Test doses of catecholamine were administered at 2-min intervals beginning about 5 min after perfusion was begun. Vasoconstrictor responses to the intraarterial catecholamines became maximal within 10–20 min and remained stable for 30–60 min.

A uniform time interval (1.5–4 min) between doses improved reproducibility of the responses. In most arteries, perfusion pressure after a response returned to baseline within 2 min of the catecholamine injection. Within the limits of 0.01–0.1 ml, the volume of the injection did not affect the magnitude of the responses. There was also no difference in responsiveness whether the solvent for the catecholamines was purified water, 0.9% sodium chloride, or Krebs solution.

There was a significant variation between animals in responsiveness of their isolated arteries to levarterenol and epinephrine. In a series of six dogs, two of the animals provided arteries significantly more reactive than those from the remaining four dogs. Different arteries from the same dog produced comparable responses. The position of the artery removed (whether first or third) did not affect responsiveness to the catecholamines.

[4] Levophed, Winthrop.
[5] Suprarenin, Winthrop.

Table I—Comparative Responsiveness of Isolated Mesenteric Arteries before and after Removal of Extraarterial Tissues (n = 36)

	Before	After	p
Baseline perfusion pressure, mm Hg[a]	26 ± 1	41 ± 3	<0.01
Responses, mm Hg, to:			
Levarterenol (1.6–4.0 μg)	93 ± 4	100 ± 5	NS
Epinephrine (0.8–2.0 μg)	87 ± 5	96 ± 6	NS

[a] Each artery was perfused at identical flow rates before and after clearing extraarterial tissues.

The magnitude of vasoconstrictor responses to intraarterial levarterenol and epinephrine was proportional to the dose levels administered (Fig. 2). Dose–response curves to the two catecholamines were parallel, but epinephrine was 2.7 times more potent than levarterenol as a vasoconstrictor.

To determine whether removal of periarterial tissues from the "undissected" arteries resulted in loss of fine juxtaintestinal arterial branches and whether the imposition of the surgical trauma affected responses, the reactivity of the isolated arteries was compared before and after dissection from periarterial tissue (Table I). Baseline perfusion pressures at identical rates of flow before and after dissection were slightly increased after the dissection procedure. There was no significant change in the magnitude of responses to levarterenol and epinephrine due to dissection. An example of the responses of an artery before and after removal of periarterial tissues is illustrated in Fig. 3.

DISCUSSION

The isolated artery preparation described here provides a measure of arterial resistance, is easily prepared, and is quite reactive to catecholamines. Two further features are reproducibility of responses and absence of fat and other periarterial tissues. The isolated artery preparation introduced by Rogers *et al.* (2) has the advantage of associated periarterial nerves but the disadvantage of a large mass of extraarterial fat and connective tissue which can serve as a reservoir of ions and as a depot for lipid-soluble drugs and hormones. Isolated resistance vessels from the rabbit brain or mesentery, described by Uchida *et al.* (6), provide small (50–250 μm o.d.) terminal vessels but lack the advantage of ease of preparation since a dissection microscope and a small (400 μm o.d.) cannula tip are required.

There was concern that dissection of the artery from surrounding tissues would result in excess surgical trauma or loss of smaller terminal vessels near the intestine. Therefore, responses before and after clearing the arteries of extraarterial tissues were compared. The results showed no decreases in baseline perfusion pressure that would have resulted if fine resistance vessels were removed in the dissection process (6). The pressure changes induced by the catecholamines also were not decreased after dissection.

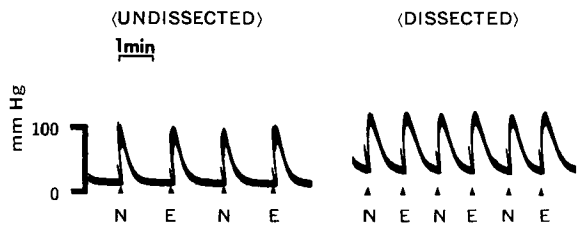

Figure 3—*Responses of an isolated artery to levarterenol (norepinephrine) (N, 3 μg) and to epinephrine (E, 1 μg) before (undissected) and after (dissected) removal of extraarterial fat and other tissues. The artery was perfused at constant flow of 11 ml/min. Baseline perfusion pressure was increased after dissection, but the changes in perfusion pressure produced by the catecholamines were not changed.*

These observations indicate that the major portion of the resistance segments were retained after the dissection process.

Responsiveness to intraarterially injected catecholamines was in the same general range as reported for similar preparations (2, 6). The potency ratio between epinephrine and levarterenol of 2.75 (1.95–4.75) agrees closely with that reported by Rogers *et al.* (2) of 2.61 (1.56–3.66).

This isolated artery preparation can provide a useful method of testing vasoconstrictor responses to catecholamines. The arteries may be perfused while hanging in air or submerged in bath fluid in recirculating or nonrecirculating systems. They also may be stored at least overnight in refrigerated Krebs solution without loss of responsiveness to levarterenol, epinephrine, or potassium ion (7). Therefore, it seems to provide one simple alternative to vascular strips or conduit vessels in studies of vascular smooth muscle.

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N-Substituted Indanamines as Potential Hypoglycemic Agents

SAMIR Ch. LAHIRI [x], JAYANTA K. GUPTA, and ANINDYA K. MONDAL

Abstract □ A number of indanamines substituted at the terminal amino nitrogen with various aliphatic, alicyclic, heterocyclic, and aromatic ring systems were synthesized and screened for hypoglycemic activity. None was found to possess significant activity compared to tolbutamide.

Keyphrases □ Indanamines, *N*-substituted—synthesized and screened as hypoglycemic agents □ Hypoglycemic agents, potential—synthesis and screening of *N*-substituted indanamines

Hypoglycemic activity was reported among different types of indanamines (1–4). It was also observed that *n*-butylpyrrolidine alone possesses appreciable hypoglycemic activity but was extremely toxic (1). Furthermore, it is well known that among the sulfonylureas, various alicyclic, heterocyclic, and aromatic ring substitutions at the terminal urea nitrogen atom had a beneficial effect (5–8).

Therefore, attempts were made to incorporate various such ring systems at the terminal amino nitrogen atom of the indanamine ring moiety and to observe the effect on hypoglycemic activity. All of these compounds were prepared by following a reported procedure (1–4). Unlike the previous work, both the amides and the corresponding amine hydrochloride salts were used for the pharmacological evaluation of hypoglycemic activity.

EXPERIMENTAL[1]

Indan-*N*-substituted Carboxamides (Ia and IIa)—These compounds were prepared from indan-1-carboxylic acid (9, 10) (1 mole) and indan-1-acetic acid (11) (1 mole) *via* the acid chloride intermediate (3) by reaction with the corresponding primary or secondary bases (1.5 moles) in the presence of 10% NaOH solution. The amides were either extracted with a suitable solvent or obtained as a crystalline solid, which was subsequently purified by crystallization from suitable solvents. The physical properties, analyses, and hypoglycemic activity of all amides synthesized are given in Table I.

***N*-Substituted Indanamines (I and II)**—The amides (1 mole) so prepared were reduced by lithium aluminum hydride reduction (1.5 moles) in absolute ether. The excess lithium aluminum hydride was decomposed with calculated amounts of 3% NaOH solution and filtered. The filtrate was extracted with cold 2 *N* HCl until it was free from amine. The aqueous layer was basified and extracted with ether, washed, dried, and distilled under reduced pressure.

The amines were characterized as their hydrochloride salts, and these hydrochloride salts were used for hypoglycemic screening. The analyses, physical properties, and hypoglycemic activity of the amines are given in Table I.

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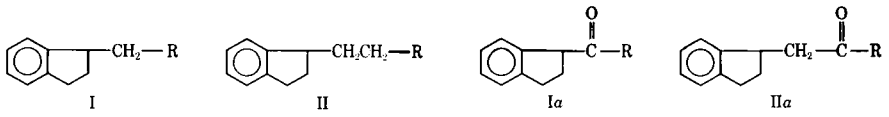

I II Ia IIa

Table I—Physical Data and Hypoglycemic Activity for the *N*-Substituted Indanamines

Compound		R	Boiling Point/mm	Formula	Analysis, % Calc.	Analysis, % Found	n_D^{30}	Melting Point	Average Percent Fall of Blood Sugar: In Normal Intact Rabbits	Average Percent Fall of Blood Sugar: In Alloxan Diabetic Rabbits
1.	Ia	Morpholino	165–170°/0.6–0.65	$C_{14}H_{17}NO_2$	C 72.7 H 7.4 N 6.1	7.25 7.3 5.8	—	92–94°[a]	11.15 ± 2.31	—
2.	Ia	Piperidino	165–168°/2–3	$C_{15}H_{19}NO$	C 78.6 H 8.3 N 6.1	78.4 8.0 6.3	1.5639	—	7.47 ± 1.12	—
3.	Ia	Pyrrolidino	170–176°/0.8–1.0	$C_{14}H_{17}NO$	C 78.1 H 7.9 N 6.5	77.8 7.7 6.4	1.5640	—	17.41 ± 2.40	22.4 ± 4.55
4.	Ia	Cyclohexyl	Solid	$C_{16}H_{21}NO$	C 79.0 H 8.6 N 5.8	79.3 8.3 5.6	—	158.5–161°[a]	14.40 ± 2.32	—
5.	Ia	Benzyl	Solid	$C_{17}H_{17}NO$	C 81.3 H 6.8 N 5.6	81.0 6.7 5.4	—	118.5–120.5°[a]	6.34 ± 1.12	—
6.	Ia	*n*-Hexyl	178–182°/0.65–0.7	$C_{16}H_{23}NO$	C 78.4 H 9.4 N 5.7	78.2 9.1 5.5	—	36–38°[a]	8.80 ± 2.55	—
7.	IIa	Morpholino	160–165°/0.6–0.7	$C_{15}H_{19}NO_2$	C 73.4 H 7.7 N 5.7	73.1 7.5 5.9	—	67–69°[a]	14.26 ± 2.78	—
8.	IIa	Piperidino	175–180°/0.6–0.7	$C_{16}H_{21}NO$	C 79.0 H 8.6 N 5.7	79.2 8.3 5.6	1.5525	—	5.74 ± 1.37	—
9.	IIa	Pyrrolidino	165–168°/0.3–0.35	$C_{15}H_{19}NO$	C 78.6 H 8.3 N 6.1	78.8 8.2 6.3	1.5532	—	4.62 ± 1.24	—
10.	IIa	Cyclohexyl	Solid	$C_{17}H_{23}NO$	C 79.4 H 8.9 N 5.4	79.1 8.6 5.2	—	126–128°[a]	7.95 ± 1.58	—
11.	IIa	Benzyl	Solid	$C_{18}H_{19}NO$	C 81.5 H 7.2 N 5.3	81.1 7.4 5.2	—	85–88°[a]	5.99 ± 1.04	—
12.	IIa	*n*-Hexyl	145–150°/0.45–0.5	$C_{17}H_{25}NO$	C 78.7 H 9.7 N 5.4	78.5 9.6 5.7	1.5341		6.95 ± 1.07	—
13.	I	Morpholino	114–116°/1–2	$C_{14}H_{19}NO$	C 77.4 H 8.8 N 6.5	77.1 8.5 6.2	1.5389	224.5–226.5°[bc]	14.51 ± 3.5	—
14.	I	Piperidino	115–120°/2–3	$C_{15}H_{21}N$	C 83.7 H 9.8 N 6.5	83.5 9.5 6.4	1.5343	220.5–222.5°[bc]	10.76 ± 1.02	–
15.	I	Pyrrolidino	110–115°/0.6–0.7	$C_{14}H_{19}N$	C 83.6 H 9.5 N 7.0	83.3 9.4 6.8	1.5383	217.5–218.5°[bc]	16.62 ± 3.01	21.15 ± 4.21
16.	I	Cyclohexyl	135–137°/0.3–0.35	$C_{16}H_{23}N$	C 83.9 H 10.0 N 6.1	84.2 10.2 5.8	1.5358	214–215°[bc]	5.39 ± 1.73	–

(*continued*)

Table I—*(Continued)*

17.	I	Benzyl	130–135°/0.8–0.9	$C_{17}H_{19}N$	C	86.1	86.4	1.5667	179–181°[b,c]	7.18 ± 2.30	—
					H	8.0	7.9				
					N	5.9	5.5				
18.	I	*n*-Hexyl	122–132°/0.5–0.6	$C_{16}H_{25}N$	C	83.1	82.8	1.5089	224–227°[b,c]	17.70 ± 4.18	20.32 ± 4.50
					H	10.8	10.5				
					N	6.1	6.3				
19.	II	Morpholino	126–130°/0.6–0.7	$C_{15}H_{21}NO$	C	77.9	77.5	1.5301	199–203°[b,c]	18.36 ± 3.85	22.35 ± 4.22
					H	9.1	9.2				
					N	6.1	5.7				
20.	II	Piperidino	130–135°/0.5–0.6	$C_{16}H_{23}N$	C	83.9	83.8	1.5310	214–216°[b,c]	12.20 ± 1.71	—
					H	10.0	9.9				
					N	6.1	6.4				
21.	II	Pyrrolidino	110–115°/0.9–1.0	$C_{15}H_{21}N$	C	83.7	83.9	1.5335	180–182°[b,c]	11.17 ± 2.64	—
					H	9.8	9.6				
					N	6.5	6.1				
22.	II	Cyclohexyl	115–120°/0.2–0.25	$C_{17}H_{25}N$	C	84.0	83.6	1.5317	178–180°[b,d]	6.11 ± 1.53	—
					H	10.3	10.4				
					N	5.8	5.5				
23.	II	Benzyl	130–135°/0.3–0.35	$C_{13}H_{21}N$	C	86.1	86.2	1.5638	235–237°[b,c]	13.46 ± 1.96	—
					H	8.4	8.1				
					N	5.6	5.1				
24.	II	*n*-Hexyl	118–120°/0.8–0.85	$C_{17}H_{27}N$	C	83.3	83.1	1.5117	202–204°[b,c]	10.98 ± 1.65	—
					H	11.0	11.3				
					N	5.7	5.6				

[a] Alcohol + water. [b] Melting point of amine hydrochloride. [c] Ethyl acetate + absolute alcohol. [d] Ethyl acetate.

Pharmacology—Eight normal healthy male rabbits, 1.5–2.0 kg, were used for hypoglycemic screening. The animals were fasted for 18 hr; after the fasting venus blood was taken, the compounds were given at a dose of 100 mg/kg either in aqueous solution or as a 0.5% carboxymethylcellulose suspension through a stomach tube.

Blood glucose concentration was followed after dosing at 3-hr intervals up to 24 hr. The blood glucose estimation was carried out by the method of Hagedorn and Jensen (12). The peak fall of blood glucose concentration was observed between the 9th and 12th hr.

DISCUSSION AND CONCLUSION

The authors previously reported hypoglycemic activity among indanamines (1–4) and since then have searched for a more potent compound in this series by incorporating various substituents at the terminal amino nitrogen of the indanamine ring moiety. Among the compounds shown in Table I, Compounds 3, 15, 18, and 19 possessed appreciable activity in normal intact rabbits. These compounds were further screened on alloxan-induced (13) diabetic rabbits (Table I).

From the results of screening, it is evident that any substitution at the amino nitrogen is not beneficial for activity. Heterocyclic ring substitution is slightly better than alicyclic and aromatic ring substitution, but the activity is not of the order of tolbutamide to warrant further investigation in the hypoglycemic field. Effect of pyrrolidine ring substitution is not as spectacular as in the case of *n*-butylpyrrolidine itself. The activity pattern and duration of activity of all these compounds are very similar. They all showed peak hypoglycemic activity between 9 and 12 hr, indicating that they probably first undergo some biotransformation to an active metabolite whose elimination pattern is also very similar.

Some compounds were screened for *in vitro* epinephrine biosynthesis inhibitory activity and antianginal and antiviral activity, but none was found to possess significant activity in any of these areas except Compound 18. Compound 18 was reported to possess some bradycardic activity and marked attenuation of peak after isoproterenol administration and hence was designated as a β-blocker.

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[x] To whom inquiries should be directed.

Dose Adjustment in Renal Failure

Keyphrases □ Renal failure—problems of dose adjustment, rate constants □ Dosage regimens—dose adjustment in renal failure □ Pharmacokinetics—dose adjustment in renal failure, rate constants

To the Editor:

In 1972, Gibaldi and Perrier (1) discussed the problems associated with dose adjustment in patients with renal insufficiency for drugs that obey two-compartment model kinetics. These authors suggested that, due to the curvilinear relationship between the hybrid rate constant β and the elimination rate constant k_{el}, assumption of a linear relationship between endogenous creatinine clearance (C_{cr}) and β may result in underestimation of β, leading to undermedication in some circumstances. They pointed out, however, that due to normal scatter generally observed in clinical data, the assumption of a linear relationship between ln $2/t_{1/2}$ and C_{cr} is still valid for practical purposes.

Added support for the latter comment may be found if one considers the relative magnitude of the distribution and elimination rate constants appropriate to the two-compartment model along with the constraints imposed on β and the associated biological half-life by the values of k_{21} and k_{el}. If one assumes that k_{el} is linearly related to C_{cr} but that k_{12} and k_{21} are invariant, then, as indicated by Gibaldi and Perrier (1), β will approach zero as k_{el} approaches zero but will approach k_{21} as k_{el} increases to large values.

To examine the relationship between curve nonlinearity and rate constant values, curves were constructed based on the parameter values given in Table I.

Plots of β *versus* k_{el} are given in Fig. 1. In each case, the plot is essentially linear at low values of β and k_{el}. When β values fall within this low range, one observes a linear relationship between β and C_{cr}. As the value of k_{el} increases, β approaches k_{21}. The maximum values for β in the four cases are 1.5, 0.1, 1.0, and 0.15 hr^{-1}, equivalent to biological half-lives of 0.46, 6.93, 0.69, and 4.62 hr, respectively.

From Fig. 1, the appropriate β/k_{el} relationships are essentially linear for values of β less than 0.5, 0.03, 0.7, and 0.1 hr^{-1}, equivalent to biological half-lives greater than 1.4, 23.1, 1.0, and 6.9 hr, respectively. In these four cases, a pseudolinear relationship is obtained between β and k_{el}, and hence β and C_{cr}, after

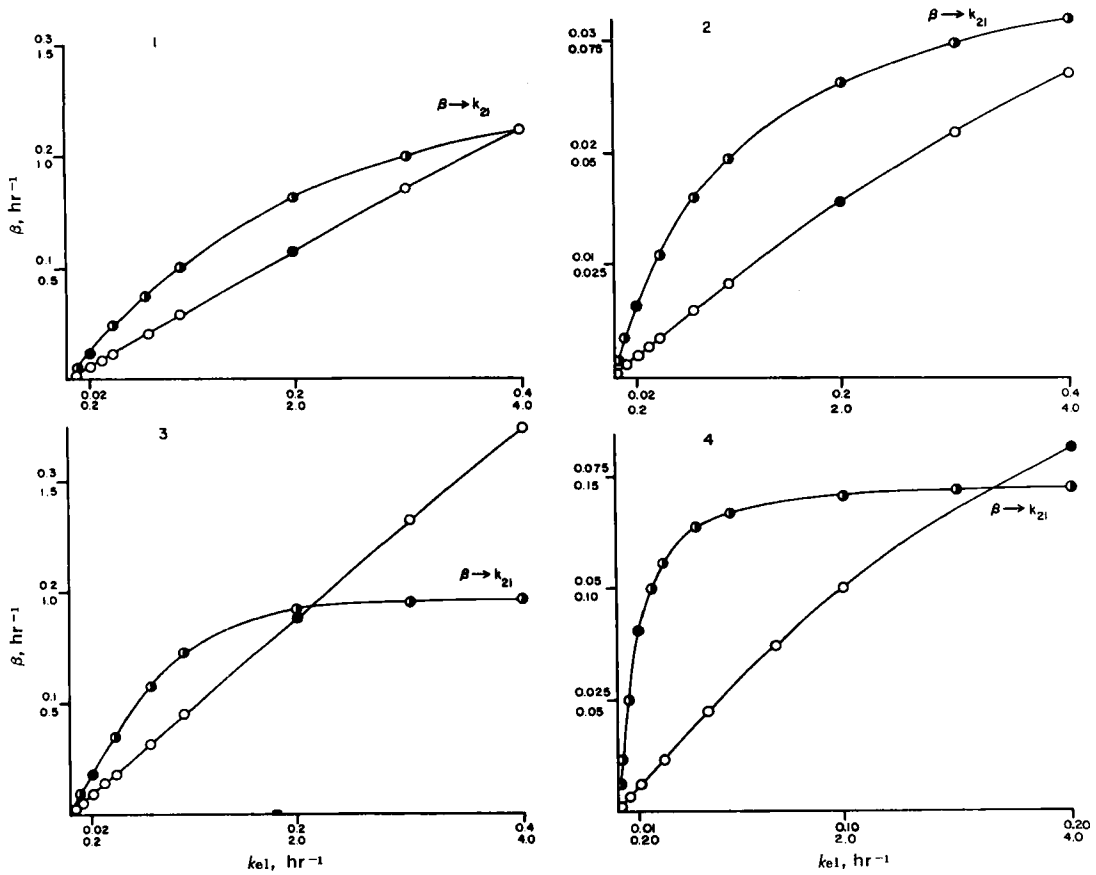

Figure 1—*Relationships between* β *and* k_{el} *in the four situations described in Table I. Key:* ◑, *bottom scale;* ○, *top scale; and* ●, *common point on both scales.*

Table I—Distribution and Elimination Rate Constants

Case	k_{12}	k_{21}	k_{el}
1	1.0	1.5	0.01 → 4.0
2	1.0	0.1	0.01 → 4.0
3	0.1	1.0	0.01 → 4.0
4	0.1	0.15	0.01 → 4.0

half-lives are increased between 1.4 and 3.3 times the minimum possible value. For most drugs, however, biological half-lives in conditions of normal renal function may be considerably longer than the minimum value and a linear relationship might be expected after a much smaller increase in half-life.

Even when the extreme case applies, it is evident from these data that dose adjustment assuming a linear relationship between ln $2/t_{1/2}$ and C_{cr} is a realistic procedure regardless of the pharmacokinetic model. The errors in the assumption would be greatest in conditions of relatively mild renal insufficiency, where dose adjustment might not be considered necessary.

(1) M. Gibaldi and D. Perrier, *J. Pharm. Sci.*, **61**, 952(1972).

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Free Amino Acids in Higher Marine Fungi

Keyphrases □ Marine fungi (higher)—determination of free amino acids from Ascomycetes and Fungi Imperfecti □ Fungi, marine—determination of free amino acids □ Amino acids, free—content of Ascomycetes and Fungi Imperfecti determined

To the Editor:

Attention has been directed to the potential importance of higher marine fungi as contributors of metabolites to marine ecosystems and producers of bioactive compounds (1). Continuing our investigations on the overall metabolic capabilities of these organisms, we now report on the free amino acids of selected marine Ascomycetes and Fungi Imperfecti. Related published reports are limited to that by Schafer and Lane (2), who identified 12 amino acids from hydrolyzed peptides of the Ascomycete *Lulworthia floridana* Meyers, and Kirk's (3) cytochemical investigations of marine pyrenomycete ascospores.

Shake cultures of 10 isolates (Table I) were grown on a yeast extract–glucose–inorganic salts[1] medium and harvested after 7 days as described previously (4). Mycelia were washed thoroughly in cold water,

[1] Rila Marine Mix, Rila Products, Teaneck, N.J.

Table I—Organisms Investigated

Ascomycetes	Isolate[a]
Lignincola laevis Höhnk	R-2
Nais inornata Kohlm.	R-4
Leptosphaeria oraemaris Linder	R-13
Corollospora maritima Werdermann	R-19
Halosphaeria mediosetigera Cribb *et* Cribb	R-524
Halosphaeria hamata (Höhnk) Kohlm.	R-577
Haligena elaterophora Kohlm.	R-601
Halosphaeria appendiculata Linder	R-605
Fungi Imperfecti	
Culcitalna achraspora Meyers *et* Moore	F-1
Zalerion maritimum (Linder) Anastasiou	R-6

[a] Refers to isolate number of particular species as cataloged in the mycological collection of P. W. Kirk, Jr., Old Dominion University, Norfolk, VA 23508

dried in forced air at 40° for 48 hr, and defatted with petroleum ether (bp 30–60°) for 12 hr in a soxhlet apparatus. Extracts of free amino acids were then prepared according to the method of Heathcote *et al.* (5), modified by extending the aqueous extraction phase to 48 hr. These extracts were desalted and purified according to Pocklington (6). The extracts, free from interfering ions and peptides, were analyzed by TLC and GLC.

Aqueous extracts were spotted on thin-layer cellulose plates and developed in a two-dimensional system: isopropanol–butanone–1 *N* HCl (12:3:5) in the first direction and butanol–acetic acid–water (2:1:1) in the second. Identification of the amino acids was achieved by comparing chromatograms of reference amino acids with those of extracts and mixtures of the two. The amino acid spots were revealed by heating plates sprayed with ninhydrin solution.

GLC analyses were based on the method described by Zumwalt *et al.* (7). The instrument[2] used was equipped with a flame-ionization detector. Instrument conditions included: silanized[3] borosilicate glass column, 1.8-m × 0.3-cm (6-ft × 0.125-in.) o.d., packed with 3% methyl silicone on calcined diatomaceous earth[4]; carrier gas, nitrogen at 45 ml/min; oven, 75° increased by 7.5°/min to 300°; detector, 250°; flash heater, 200°.

Reference trimethylsilyl amino acid derivatives were prepared as follows (8). One milligram of amino acid was dissolved in 50 ml of methanol. One milliliter of this stock solution was placed in a 0.6 × 5.1-cm (0.25 × 2-in.) screw-capped vial containing 1 ml of anhydrous methylene chloride. This was heated to 70° in a sand bath to remove the solvent and azeotropically remove all moisture. While the vial remained in the sand bath, its contents were further dried under a stream of nitrogen for 30 min.

To the dried residue was added 0.25 ml of bis(trimethylsilyl)trifluoroacetamide, and this mixture was heated for 1 hr in a sand bath at 135°. After refluxing, the vial was allowed to cool spontaneously. Derivatives of the dried free amino acid extracts were prepared in an identical manner. Freshly prepared

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To what extent these compounds were synthesized by the fungi or merely taken up from the culture medium remains to be determined because the medium, which contained yeast extract, cannot be considered as being totally chemically defined. However, an assay of yeast extract[5] indicated that alanine, proline, serine, ornithine, tryptophan, cystine, cysteine, and hydroxyproline are absent from this product. Also, arginine, a component of yeast extract, was not detected by us in any of the fungal mycelium examined.

Consequently, we feel that the array of amino acids detected in the mycelium represents metabolites synthesized by the organisms, at least in part. In addition, because natural sea water contains numerous amino acids, the cultivation procedures are not unrealistically artificial and this investigation allows better understanding of the chemical–ecological role of fungi in the marine biosphere as well as the overall metabolic activities of these organisms.

The occurrence of hydroxyproline in all species examined is noteworthy, because this amino acid is believed to occur only rarely in fungi (9). Whether hydroxyproline actually occurs less commonly in terrestrial than in marine fungi or has been overlooked in analyses of the former requires further clarification. The compound is a prime component of collagen in animals including marine species (10). Degens *et al.* (11) reported that hydroxyproline is not ubiquitous in sea water, whereas Pocklington (6) found it readily detectable. Owing to their abundance in salt marshes (12), the higher marine fungi could be an important source of this and other amino acids in detritus feeding animals of coastal and estuarine environments.

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5-[3-(2-Chloroethyl)-1-triazenyl]imidazole-4-carboxamide and a Possible Mechanism of Action of 5-[3,3-Bis(2-chloroethyl)-1-triazenyl]imidazole-4-carboxamide

Keyphrases □ Chloroethyltriazene—synthesis, antileukemic activity □ Imidazoles, triazenyl—preparation, antileukemic activity □ (Chloroethyltriazenyl)imidazole—explosive decomposition, hydrolysis to aminoimidazolecarboxamide □ Antileukemic activity—chloroethyltriazene □ Bis(2-chloroethyl)triazenes—possible mechanism of action

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[1] The following abbreviations of Compounds I–III and VII have been employed in the literature: Compound I (NSC-82196), BIC; Compound II (NSC-45388), DIC and DTIC; Compound III (NSC-407347), MIC; and Compound VII, AIC.

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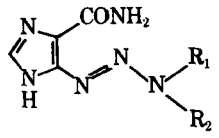
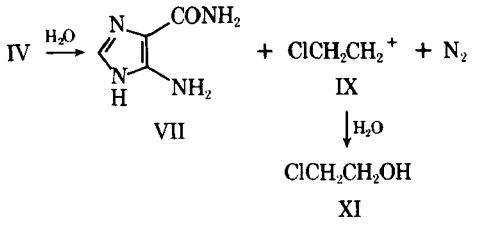


I: $R_1 = R_2 = CH_2CH_2Cl$
II: $R_1 = R_2 = CH_3$
III: $R_1 = H$, $R_2 = CH_3$
IV: $R_1 = H$, $R_2 = CH_2CH_2Cl$

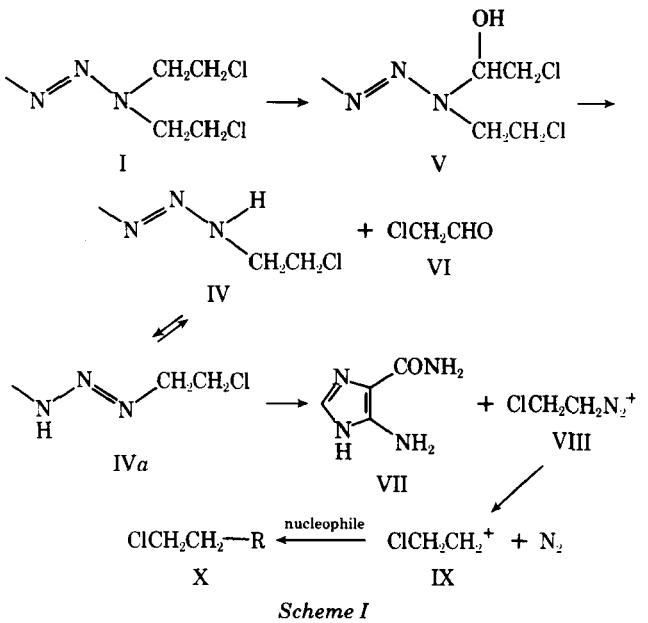


Scheme II

acids containing radioactive 7-methylguanine. Analogous results were obtained by incubating labeled II with microsomal preparations from mouse tumor tissues (7). These studies showed that II is demethylated by microsomal oxidases and that a methylating agent is generated by this process.

Similarly, studies of the metabolism of ^{14}C-labeled II (labeled at the methyl groups or at position 2 of the imidazole ring) in rats (4, 5), dogs (8, 9), mice (9), and patients (4, 5, 8–10) identified the aminoimidazole (VII) and expired carbon dioxide as metabolic products *in vivo*. Utilization of VII and of formate (both derived from labeled II) in the biosynthesis *de novo* of purine nucleotides may explain the observed labeling of nonmethylated purines (6, 9) and may account in part for the radioactivity of nucleic acids (6, 11); however, the identification of 7-methylguanine as a constituent of nucleic acids and as a urinary excretion product after administration of II confirms the metabolic generation of a methylating agent *in vivo* (6).

5-(3-Methyl-1-triazenyl)imidazole-4-carboxamide[1] (III) was shown earlier to have activity against L-1210 leukemia and to dissociate spontaneously in solution to the aminoimidazole (VII) and, presumably, to the methyldiazonium ion and then to the methylcarbonium ion (12). Taken together, the enzymatic, metabolic, biological, and chemical studies indicated that the dimethyltriazene (II) is activated by dealkylation to the monomethyltriazene (III) and that the methylcarbonium ion is the reactive species responsible for activity. Presumably, other active dialkyltriazenes are metabolized similarly, and III or an analogous monoalkyltriazene is the immediate progenitor of a methyl or other alkyl carbonium ion.



Scheme I

It has been suggested (13, 14) that I may act by dissociation to bis(2-chloroethyl)amine (nor-nitrogen mustard) and 5-diazoimidazole-4-carboxamide. However, if I is metabolized by microsomal oxidases in a manner similar to II and to phenyltriazenes (15), two highly reactive species should be formed (Scheme I). The presumed intermediate (V) should decompose spontaneously to 5-[3-(2-chloroethyl)-1-triazenyl]imidazole-4-carboxamide (IV) and to chloroacetaldehyde (VI).

By analogy to other monoalkyltriazenes (12, 16), IV should dissociate to VII and to the chloroethyldiazonium ion (VIII), which should, in turn, decompose to nitrogen and a chloroethylcarbonium ion (IX). Either the chloroethylcarbonium ion or chloroacetaldehyde (or both) may be responsible for the activity of I. One of the presumed intermediates, IV, has been prepared, studied for stability, and evaluated against leukemia L-1210.

The mono(chloroethyl)triazene (IV) was prepared by treating 2-chloroethylamine free base in dry ethyl acetate solution with 5-diazoimidazole-4-carboxamide (added in one portion) by a procedure similar to previously described procedures (12). A white precipitate was washed thoroughly with ethyl acetate, slurried with absolute ethanol in the dark, and dried *in vacuo* at room temperature[2], yielding 83%; violent explosive decomposition[3] at 114° (inserted at 80°, 3°/min); strong IR bands at 3475, 3250, 3070, 1635, 1585, 1420, and 1380 cm^{-1}.

Anal.—Calc. for $C_6H_9ClN_6O$: C, 33.26; H, 4.19; N, 38.79. Found: C, 33.38; H, 4.33; N, 38.62.

The thermal instability[2] of IV precluded mass spectral analysis, and the sparing solubility and solvolytic instability prevented proton magnetic resonance analysis. The IR spectrum of a specimen of IV kept at −15° for 1 year was unchanged.

In water or in 50% ethanol, IV decomposed in the dark to 5-aminoimidazole-4-carboxamide (VII). For example, a suspension of IV (200 mg) in water (50 ml) was stirred in the dark until essentially all IV had dissolved (about 8 hr). Evolution of a gas (presumably nitrogen) was observed. After filtration of the mixture to remove undissolved IV (2 mg), GLC of an

[2] Compound IV is very explosive; it is sensitive to heat and to shock. A specimen (prepared prior to the synthesis of I) decomposed explosively while being dried at 78°.

[3] The decomposition temperature is reproducible when it is determined as stated.

Table I—5-[3-(2-Chloroethyl)-1-triazenyl]imidazole-4-carboxamide[a] (IV) *versus* L-1210 Leukemia[b]

Dose, mg/kg	Mortality by Day 5	Average Weight Change Difference, g[c]	Number of 30-Day Survivors/Total	Nonsurvivors	
				Average Survival Time, days	T/C Ratio, %
Day 1 Schedule:					
100	2/6	−6.2t[d]	0/6	6.3/9.4	67t
50	0/6	−4.6t	2/6	14.0/10.0	140
37	0/6	−1.7	4/6	14.5/9.9	146
25	0/6	−2.2	6/6		
25	0/6	−2.0	5/6	15.0/9.9	151
17	0/6	−1.1	3/6	14.3/9.9	144
12.5	0/6	−1.3	3/6	18.0/10.0	180
6.25	0/6	−1.7	0/6	14.8/10.0	148
qd, Days 1–9:					
25	0/6	−4.2t	0/6	9.2/10.0	92
12.5	0/6	−3.3	0/6	16.0/10.0	160
9	0/6	−2.4	2/6	16.5/9.9	166
6.25	0/6	−2.4	3/6	20.3/10.0	203
6	0/6	−1.6	1/6	22.4/9.9	226
4	0/6	−1.0	0/6	15.5/9.9	156
3.12	0/6	−1.9	1/6	14.4/10.0	172

[a] Suspensions of this compound in saline + Tween 80 were administered intraperitoneally within 5 min of the preparation of the suspensions. [b] L-1210 cells (10^5) were implanted intraperitoneally in mice on Day 0. [c] Average weight change of treated mice minus average weight change of control mice by Day 5. [d] t = toxic.

aliquot of the reaction solution showed that it contained 2-chloroethanol (XI), which was identified by comparing its retention time with that of an authentic commercial sample. The concentration of XI in the aliquot corresponded to a yield of 70% of XI from the chloroethyltriazene (IV).

Lyophilization of the remaining reaction solution left a solid (123 mg, 93% yield as VII monohydrate) that was shown by TLC to be almost pure VII. (Trace amounts of three impurities were detected.) The identity of the residue was confirmed by its mass spectrum, which was compared with that of an authentic specimen of pure VII free base: *m/e* 126 (molecular ion, base peak), 109 (126 — NH_3), and 94.3 (metastable ion, 126 → 109). Evidence of slight alkylation of VII by the 2-chloroethylcarbonium ion (IX) during this experiment or in similar experiments was found in the mass spectra of the isolated products (all of which were predominantly VII). The following weak peaks in some spectra support this conclusion: *m/e* 188 (M^+ of product of alkyation of VII by IX), 170 (M^+ of product of alkylation of VII by IX and replacement of the chloro group by a hydroxy group), 152 (M^+ of product of alkylation of VII by IX followed by formation of a vinyl group or of a cyclic derivative of VII *via* loss of HCl), and 139 (loss of CH_2Cl or CH_2OH from an alkylated VII derivative).

As shown in Scheme II, the mode of decomposition is analogous, therefore, to that of the monomethyltriazene (III) and similar monoalkyltriazenes (12) and is in contrast to the intramolecular cyclization reaction of I (1, 17, 18).

In standard L-1210 leukemia tests, IV was administered intraperitoneally as a suspension on Day 1 and on Days 1–9 (qd). The data in Table I indicate that there were some "cures," as evidenced by 30-day survivors. The most effective dose was 25 mg/kg on the Day 1 schedule; in two tests, 11 of 12 treated mice survived until the end of the 30-day test period. In comparable tests (1, 2) of I against L-1210 leukemia, the optimal single and daily doses were about 200–500 mg/kg and 50–75 mg/kg/day (qd 1–30 or death), respectively. These doses are about 10-fold those of the approximate optimal doses (Table I) of IV.

The results of tests *in vivo* show that IV, if it is formed as outlined in Scheme I, could account for the observed activity of I. Therefore, the results are consistent with the activation of I by microsomal oxidases. The demonstrated mode of dissociation of IV provides evidence that the chloroethylcarbonium ion is the reactive species responsible for the activity of IV (IX → X) and, after activation, of I[4]. Because of the potent activity of IV, a corollary is that the chloroethylcarbonium ion is exceptionally effective in manifesting antineoplastic activity[5].

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[4] After the initial reaction of IX with a nucleophilic center *in vivo*, the chloroethyl derivative (X) may undergo further (intermolecular or intramolecular) reaction.

[5] Although the activity of triazenes and certain other anticancer agents may be due to the generation of methyl, chloroethyl, or other carbonium ions, it does not necessarily follow that alkylation of guanine or other nucleic acid moieties is responsible for the observed activity.

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REFLECTIONS ON A BOOK

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But the book in point is one that warrants some attention and, even more important, some thoughtful reflection.

As books go—especially compared with the scholarly tomes familiar to most of our readers—this volume does not present a particularly impressive appearance. If anything, its appearance belies its content, since it is little more than a pocket volume of 116 pages, with large size type and lots of white space on the relatively small pages.

What sets it apart, however, is the message it delivers—a message that is virtually a call to revolution in pharmacy. The volume is titled "*The Four Walls of Pharmacy*," and it is subtitled "*Professional Power with and for the People.*"

Moreover, if its title and message set it apart, what stamps it with the seal of credibility is the identity of its authors. No wild-eyed, long-haired disciple from some radical, left-wing organization was responsible for its preparation. To the contrary, the senior author is none other than Henry M. Burlage, Ph.D., pharmaceutical scientist, long-time dean of the College of Pharmacy at the University of Texas, and pharmacy teacher for over 50 years. The coauthor is his son, Robb K. Burlage, a Harvard-trained authority in economics, who has spent over a decade in specialized activities as a health economist.

The cover flap, in somewhat of an understatement, asserts: "*This book will surely provoke those in pharmacy and related fields, for no point of debate is left untouched. . . . Challenging old-fashioned thinking, the authors categorically state: 'Pharmacy needs lobbying power . . . .'*"

The "four walls" alluded to in the title refer to the Drug Industry (Wall I), Education (Wall II), the "Medical Team" (Wall III), and Government (Wall IV).

The authors waste no time in exposing their assessment of the drug industry, subtitling this chapter "The Noble Exploiters" and claiming in their opening paragraph that "ethical" is a nice word to go with drugs, but in the corporate or drug industry realm "it is a farce." Finally, they conclude this chapter by explaining that the industry is discussed as their first "wall of the pharmacists' captivity" because it dominates directly as well as indirectly through each of the other three "walls" mentioned above.

In turn, attention is then focused on each of these other realms and the role they play and have played in constraining, restricting, and thwarting pharmacy in its effort to attain its potential as a profession and in its ability to serve the best interests of the public. Moreover, other related activities in the pharmacy sphere come in for their lumps as well; regarding state boards of pharmacy—"In all too many cases, the appointments are political in payment for support"; regarding the "captive pharmacy press"—because of the dependency upon advertising income, "it is questionable whether the press of the state associations and some representing the national associations is a *free* press"; and the hypocrisy of pharmacy organizations which take in members who engage in the very activities that the organization was established to oppose.

Therefore, while no facet of the pharmacy universe escapes either caustic comment or at least a critical eye, it is the assessment of pharmacy education and pharmaceutical research that is most pertinent to the majority of our readers. And again, our traditional systems and accepted approaches are questioned and challenged. For example: "*The senior author, for fifty years a pharmaceutical educator, placed his early faith, along with many of his colleagues, in the potentiality of scientific education to reconstruct professional practice. And yet pharmacy as a public interest profession, despite vital scientific and organizational progress, is today more trapped by the 'Four Walls' than before.*"

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Lest our comments be misinterpreted, we are not necessarily approving or endorsing the content of this volume. We do, however, feel that the authors are correct that major and fundamental changes are necessary throughout pharmacy—including education, research, and drug manufacturing—if pharmacy is to fulfill its proper role in society. And it just may be that some, if not most, of the authors' comments are right on target!

—EGF

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REVIEW ARTICLE

Rationale for Design of Biologically Reversible Drug Derivatives: Prodrugs

A. A. SINKULA [x] **and S. H. YALKOWSKY**

Keyphrases □ Drug derivatives, biologically reversible—rationale for design of prodrugs, physicochemical and biological considerations, applications, review □ Prodrugs—rationale for design, physicochemical and biological considerations, applications, modifications affecting absorption, site direction, depot, taste and odor, and irritation, review □ Reversible drug derivatives—rationale for design for prodrugs, physicochemical and biological considerations, applications, review □ Chemical modification—prodrugs, physicochemical and biological considerations, applications, review □ Latentiated drugs—design of prodrugs, review

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Nearly all therapeutic agents possess various physicochemical and biological properties, some desirable and others undesirable. In general, the pharmaceutical world is concerned with minimizing the number and magnitude of undesirable properties of a drug while retaining the desirable therapeutic activity.

Improvement of drug efficacy can be accomplished by biological, physical, or chemical means. The biological approach entails varying the route of administration. Examples include the injectable route to optimize onset of action, maximize bioavailability (enhanced blood levels), and eliminate gastric irritation and acid-catalyzed drug degradation. Versatility is severely limited when utilizing the biological approach, because alternative routes of administration are frequently unavailable and are always less convenient than oral administration.

A greater degree of flexibility of drug modification is offered by the physical approach, commonly referred to as dosage form design. The elements and philosophy of this approach were discussed by Schroeter (1) and others (2–8). The highest degree of flexibility in altering drug efficacy, however, is offered by the chemical approach.

Drug derivatization has been long recognized as an important means of producing better pharmaceuti-

cals. Bayer, as far back as 1899, synthesized the drug aspirin in an attempt to improve the therapeutic activity of salicylic acid. Since that time, literally thousands of drug derivatives have been synthesized and tested. These drug derivatives can be broadly classified into two categories: irreversible or reversible. Irreversible derivatives or analogs are usually synthesized for the purpose of finding a similar, new, biologically active entity possessing increased potency, a broader spectrum of activity, or some other desirable property not possessed by the parent compound. A reversible drug derivative utilizes a chemical moiety of proven biological activity (the parent molecule) and seeks to deliver it to the site of action while overcoming some inherent drawback to the use of the parent compound.

In the case of the analog, precautions must be taken as to what functional group can be modifed since indiscriminate modification may destroy all bioactivity. The reversible derivative can be modified at any functionality without undue concern for its involvement at the receptor level since it is reversible by definition. These facts eliminate the need to determine the bioactive center(s) in the molecule and offer the chemist a greater number of chemical sites at which to modify.

In general, three approaches are followed in the search for new drug agents: *(a)* the general screening approach in which chemical substances from any source are tested for their effect against a predetermined disease or disease state, *(b)* the chemical modification of existing drug substances whose biological effects are known, and *(c)* mimicking nature by biochemical design, where a compound is made to exert an action in a manner similar to a known biochemical substance (9). Any lead compounds obtained from these three approaches are usually further modified chemically to gain the biologically most potent representatives of the series.

This review will focus on approach *(b)*, the chemical modification of existing drug substances whose biological effects are known. It will be limited solely to biologically reversible derivatives, *i.e.*, those compounds that, upon introduction to the appropriate biological system, revert back to the parent molecule by virtue of enzymatic and/or chemical lability. To provide an interpretive review of the area, references from the journal and patent literature will be selected to illustrate the various principles discussed. The discussion will of necessity not be comprehensive but will nonetheless cover the significant aspects of the discipline.

Reversible derivatives (10–14) have also been termed prodrugs (15–22) and latentiated drugs (23–25) and have been designed to eliminate a variety of undesirable properties such as bitterness, odor, gastric upset, and poor absorption. Many comprehensive reviews of both reversible and irreversible drug derivatives (23–32) have appeared recently.

Harper (25) must be credited with coining the term "drug latentiation," and he defined it as "the chemical modification of a biologically active compound to form a new compound, which upon *in vivo* enzymatic attack will liberate the parent compound." Kupchan *et al.* (33) extended this definition operationally by including nonenzymatic processes as well for regeneration of the parent compound. By inference, latentiation implies a time lag element or time component involved in regenerating the bioactive parent molecule *in vivo*. Since most latentiated drug substances *per se* are biologically inactive, this concept is important for those drugs that are metabolized or excreted too rapidly to provide adequate clinical efficacy.

Albert (34, 35), in discussing the selective toxicity of drug molecules, elucidated the proagent or prodrug concept. The term prodrug is general in that it includes latentiated drug derivatives as well as substances that are converted after administration to the actual substance that combines with receptors. These actual substances may be active metabolites of the parent molecule. Harper (24), on the other hand, discussed the concept of structural formulation and defined it as "the modification of a biologically active compound at a point not essential for binding to an active site in the biological receptor, so that although the desired biological effect is retained, the resulting changes in physicochemical properties cause alteration in the absorption, distribution or metabolism of the drug; the parent compound, however, is not liberated in the body." Structural formulation differs from irreversible derivative formation in that the former retains bioactivity *in vivo* whereas the latter may or may not do so. The distinction is subtle but may have profound ramifications in the rational approach to the synthesis of bioactive agents.

Any number of inherent disadvantages may preclude the use of the parent drug molecule in clinical practice. Among those properties considered disadvantageous in a drug molecule are bitterness or tartness, offensive odor, gastric or intestinal upset and irritation, pain on injection, lack of absorption, slow or rapid metabolism, and lack of stability in the bulk state, the dosage form, or *in vivo* (*i.e.*, gastric instability).

In many cases, undesirable properties in a drug molecule cannot be overcome by conventional pharmaceutical formulation or route of administration changes, so the method of choice becomes reversible derivative formation. In the intelligent design of reversible drug derivatives, it is necessary to consider two questions:

1. What structural modification(s) of the parent molecule are necessary to reduce or eliminate the particular undesirable effect?
2. What conditions are available *in vivo* (enzymes, pH, *etc.*) to regenerate the parent molecule from the derivative?

The first question requires an extensive knowledge of structure–activity relationships as they apply to elimination of these undesirable properties. The second question is dependent on a rather sophisticated knowledge of biology. Complete answers to these two questions are obviously not yet available to the medicinal chemist. A limited body of knowledge is available, however, and this knowledge, if used judiciously, can form a basis for the rational design of revers-

ible drug derivatives. The remainder of this review will consider specific undesirable drug properties and possible means of eliminating these properties by using reversible drug derivatives. Examples of drugs whose properties have been successfully modified are provided in the tables.

GENERAL CONSIDERATIONS

Physicochemical—*Absorption*—Probably the most fruitful area of reversible derivatization is the improvement of passive drug absorption through epithelial tissue. Many studies involving *in vivo, in situ,* and *in vitro* systems have been conducted to elucidate the role of chemical structure in drug absorption. The similarities and differences among some more widely accepted theories also will be discussed here.

Nearly all of the empiricisms and theories in current use agree that the addition of a hydrophobic group to a compound usually increases its absorption. It is also agreed that this increase in absorption is a direct consequence of the increase in the biological lipid–water partition coefficient resulting from the added hydrophobic moiety. Although there is a lack of agreement as to what, if any, *in vitro* partitioning system best mimics the biological situation, octanol–water apparently is the most useful for correlative purposes.

From both the physicochemical and the biological point of view, octanol–water is the most extensively studied system. Leo *et al.* (36) tabulated and critically evaluated thousands of octanol–water partition coefficients and developed a system of rules for estimating values for compounds that have not yet been studied.

It is important to realize that the theoretical and empirical relationships to be discussed are equally applicable to reversible derivatives as well as nonreversible derivatives (analogs), because all of these relationships rely upon partitioning and because the presence of a reversible linkage does not normally alter the relative partition coefficients of the members of a series. For example, in the octanol–water system, the ratio of the partition coefficients of ethyl and butyl benzoates is the same as the ratio for *p*-ethyl- and *p*-butylbenzoic acids, even though the esters are reversible while the acids are not. Another consequence of the dependence of absorbability on partitioning is the fact that the unionized form of a dissociable molecule is absorbed more efficiently than its ionic species. The quantitative relationships between pH, pK, and partition coefficient are well known (37, 38) and will not be discussed here.

To get a clear picture of the role of hydrophobicity in drug transport, it is necessary to cover a wide range of partition coefficients. The most convenient and economical way of accomplishing this task is to construct a homologous series. The partition coefficient of the *n*th number of any homologous series, PC_n, in any solvent system can be described by:

$$\log(PC_n) = \log(PC_0) + \pi^s n \qquad \text{(Eq. 1)}$$

Table I[a]—Values of π_{CH_2} for Some Common Solvents and for Red Blood Cell Ghosts

Solvent	π_{CH_2}
Ether	0.573
Ether	0.612
Octanol	0.500
Chloroform	0.609
Olive oil	0.525
Castor oil	0.545
Red blood cell ghosts	0.526

[a] Adapted, with permission, from Ref. 46.

where PC_0 is a constant dependent on both the series and the solvent system, and π^s is a constant dependent only on the solvent system. According to the notation of Leo *et al.* (36), π can be an incremental constant for any substituent; but when dealing primarily with homologous series, π will designate π_{CH_2} unless otherwise specified.

The values of π (π_{CH_2}) for several organic solvents and red blood cell ghosts against water are listed in Table I. From the octanol–water π value of 0.5, it can be seen that only seven consecutive homologs are needed to cover a 1000-fold range of partition coefficients in half-log increments. Spanning this broad range of values minimizes the effects of random error and normal biological variation. Homologous series are particularly well suited for studying transport because the alkyl group usually does not interfere with the interaction of the active portion of the molecule and the receptor site. Therefore, it is frequently possible to measure the relative biological activities of homologs and then equate these values to relative transport rates.

The earliest structure–activity or structure–transport workers all recognized the parallelism between the logarithms of the biological response *(BR)* and the lipid–water partition coefficient *(PC)*. Equations of the form:

$$\log(BR) = a + b\log(PC) \qquad \text{(Eq. 2)}$$

were proposed (39–44). For the simple case of a homologous series, Eqs. 1 and 2 are combined giving:

$$\log(BR) = b\pi^s + b\log(PC_0) + a \qquad \text{(Eq. 3)}$$

which describes the so-called linear structure–activity relationship.

The value of a in Eq. 2 or 3 is a measure of the degree of similarity of the *in vitro* and the true *in vivo* partitioning systems. If a is equal to unity, the systems are equivalent in their relative affinities for a methylene group. (It is possible for π_{CH_2} to be the same for a pair of systems and, at the same time, for other substituent constants such as π_{OH} to be quite different. For specific examples, see Ref. 36.) When considering a homologous series, it is not necessary to use any reference *in vitro* partitioning system. Flynn and Yalkowsky (45) showed that $b\pi^s$ is equal to π^B, the π value in the biological system. Thus, Eq. 3 becomes:

$$\log(BR) = A + \pi^B n \qquad \text{(Eq. 4)}$$

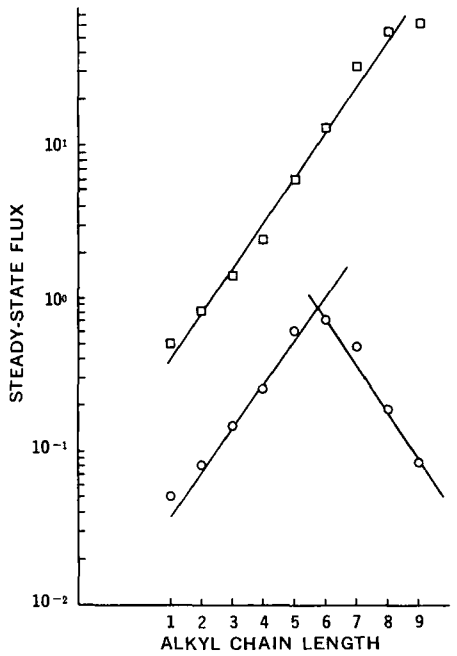


Figure 1—*Data of Scheuplein and Blank (48) for permeation of normal aliphatic alcohols across human stratum corneum* in vitro. *Key:* □, *concentration-normalized flux; and* ○, *flux from 0.10* M *solution (last four points represent saturated solutions). (Adapted, with permission, from Ref. 46.)*

where A is equal to the constant portion of Eq. 3 and is, therefore, dependent upon both the biological system and the homologous series.

Yalkowsky and Flynn (46) recently evaluated a large number of linear chain-length–activity relationships which are dependent upon passive transport. They observed, as expected, that π^B for a particular biological system is essentially independent of the series under study. They found that the value of π^B for most simple organisms (bacteria, fungus, erythrocytes, *etc.*) is 0.46 ± 0.03; *i.e.*, on the average, there is a 2.9-fold increase in activity for each additional methylene group. It was also observed that the value of π^B for epithelial and GI tissue of higher animals is around 0.25, which corresponds to an incremental constant for each methylene group of about 1.8 for the change in activity (or transport) with chain length. This low value is in agreement with the fact that these tissues contain a significant fraction of polar components.

The linear increase in the logarithm of the transport rate with chain length is expected from basic permeability theory. The flux, F, or transport rate of a substrate across a rate-determining lipid phase or membrane separating two aqueous phases at a concentration differential of ΔC is:

$$F = \frac{PC}{R}\Delta C \qquad \text{(Eq. 5)}$$

where PC is the lipid–water partition coefficient of the substrate, and R is the resistance of the membrane to its diffusion. Collander (47), using chara cells, was among the first to show the relationship between permeability and partitioning in a biological system. More recently, excellent correlation was shown (48) between the permeability of nine aliphatic alcohols across excised human stratum corneum and their stratum corneum–water partition coefficients. From these data (Fig. 1), it can be seen that there is a great increase in permeability between methanol and nonanol.

The exponential increase in transport rate and thus activity with extention of the alkyl group cannot go on indefinitely. If experiments are carried to high enough chain lengths, there is a leveling off or plateauing of the curve and ultimately a decrease in activity with increasing hydrophobicity. While there is reasonably good agreement in the literature about the ascending portion of the structure–activity curve, there is a great deal of controversy about the reason for and even the shape of the apical or descending portions of the curve.

Hansch (49) described the overall shape of the structure–activity curve by a parabolic equation of the form:

$$\log(BR) = a + b\log(PC) + c\log^2(PC) \qquad \text{(Eq. 6)}$$

He showed that Eq. 6 gives better statistical fit to many sets of data than the linear Eq. 2. The improved correlation is undoubtedly at least partially due to the greater degree of flexibility produced by the additional variable c; but since c is always negative, it cannot be regarded as simply another adjustable parameter. One difficulty that arises with the parabolic equation is that the values of the coefficients, a and b in Eqs. 2 and 6, have no relationship to one another and parabolic equations having the same ascending slopes can appear quite different.

Since Hansch's parabolic relationship is based upon a countercurrent distribution-type model (49), the curve is nowhere linear and is symmetrical about an optimum value of log (PC). The nonlinearity of the parabola makes it difficult to reconcile with the linear case described by Eq. 2. Furthermore, the symmetry is not consistent with the many reasons given by Hansch and Clayton (50) to explain the decrease in activity with chain length. Nevertheless, in spite of the theoretical shortcomings, the Hansch parabolic relationship can be extremely useful in the empirical analysis of structure–activity data and in the prediction of optimum partition coefficients for biological activity. From a practical standpoint, it is frequently more useful than the more theoretically valid relationships that will be discussed.

Wagner and Sedman (51) recently analyzed much of Hansch's data and found that a statistically better fit is obtained with an equation of the form:

$$(BR) = \frac{1}{a + (b/PC)} \qquad \text{(Eq. 7)}$$

Others also showed good fit of biological activity data and transport data to equations similar to Eq. 7 (46, 52, 53). One of the earliest uses of this equation was by Zwolinski *et al.* (54) who studied the permeability of various plant cells to the members of several homologous series. They also showed that double-recip-

rocal plots [$(BR)^{-1}$ *versus* $(PC)^{-1}$] are linear and can be used to evaluate the constants a and b conveniently. Another important feature of Eq. 7 is that it is applicable to both linear and nonlinear data.

The mathematical form of Eq. 7 can be obtained directly from Eq. 5 by the incorporation of an additional resistance in series with that of the membrane. Equation 5 then becomes:

$$BR \propto F = \frac{\Delta C}{(R_m/PC) + R_{aq}} \qquad \text{(Eq. 8)}$$

This added resistance, R_{aq}, results from the unstirred aqueous layers adjacent to the membrane which must be traversed by any solute passing through the membrane. For a more complete description of the derivation of Eq. 8 and the importance of unstirred layers (or diffusion layers as they are often called), the reader is referred to Refs. 45 and 46. For an alternative treatment based on extraction theory leading to another equation of the same form as Eq. 7, see Ref. 51.

For the study of a homologous series, it is convenient to combine Eq. 1 with the logarithmic form of Eq. 8 (or 7) to get:

$$\log(BR) = \log(\Delta C) - \log(PC_0) - \pi n - \log(R_m + R_{aq}PC_0 10^{\pi n}) \qquad \text{(Eq. 9)}$$

which is shown schematically in Fig. 2. It can be seen that, according to Eq. 9, there is a linear increase in log BR with chain length but that at some point [when $R_{aq} \geq (R_m/PC)$] the curve levels off and approaches a limiting value. These equations can satisfactorily describe most structure–activity data, but they do not explain the descending portion of the curve.

Because of the wide variety of reasons for a decline in activity with increasing chain length, no single equation can explain all available data. Hansch and Clayton (50) and Yalkowsky and Flynn (46) listed about a dozen possible reasons for this decline. These reasons can be broadly classified into those that are dependent upon a biological parameter (*e.g.*, enzyme specificity, conformational distortion of the active site, metabolism, and poisoning of enzymes) and those that are related to some physical property (*e.g.*, solubility, complex formation, micelle formation, partitioning into inert phases, and binding to inert surfaces). The former are the most difficult to correlate by simple theories but can be handled quite satisfactorily by equations such as Eq. 6. The latter all have one important feature in common; they can generally be described mathematically by:

$$\log(P_n) = \log(P_0) + \alpha n \qquad \text{(Eq. 10)}$$

where P_n is the value of any property of the nth homolog of the series, P_0 is a reference value, and α is a well-defined constant[1].

[1] Occasionally, it is necessary to use a higher order polynomial of n to describe P_n. This would alter the subsequent mathematical treatment slightly but not the general conclusions (see Ref. 52).

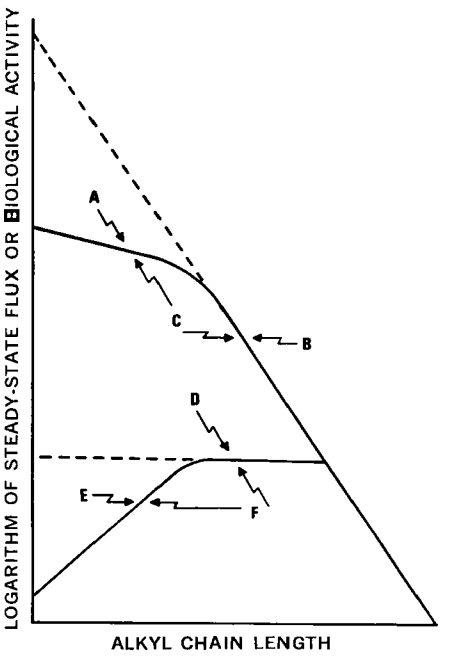


Figure 2—*Hypothetical chain-length activity relationships. (Adapted, with permission, from Ref. 52.)*

Yalkowsky *et al.* (46) theoretically characterized the effects of behavior on the transport rate by using solubility as an example. Based on literature data for nearly 20 homologous series in water, they found[1]:

$$\log(S_n) = \log(S_0) - \delta n \qquad \text{(Eq. 11)}$$

where S_n is the solubility of the nth member of the series, and S_0 is a constant.

The effect of solubility on transport rate is to limit the attainable concentration differential so that the maximum value of Eq. 8 becomes:

$$F = \frac{S}{(R_m/PC) + R_{aq}} = \frac{(S_0 10^{-\delta n}/R_m)}{PC_0 10^{\pi n} + R_{aq}} \qquad \text{(Eq. 12)}$$

which, in logarithmic form, is:

$$\log(F) = \log(S_0 PC_0) + (\pi - \delta)n - \log(R_m + R_{aq}PC_0 10^{\pi n}) \qquad \text{(Eq. 13)}$$

These equations can now describe a "parabolic" structure–activity curve on the basis of transport-limited activity and basic physical–chemical relationships. Figure 2 shows the expected dependence of transport across a biological barrier (and activity dependent thereupon) for the members of a homologous series predicted by Eqs. 9 and 13. The scales are arbitrary but show that the break occurs at the same chain length for the equimolar and saturated cases. Figure 3 shows an experimental verification of Eqs. 9 and 13. These data were obtained from turnover time experiments with goldfish (52). The agreement between experimental and theoretical data, while not necessarily proving the theory, gives a positive indication of its utility.

Solubility—The primary role of solubility in determining drug absorption is obvious since only the

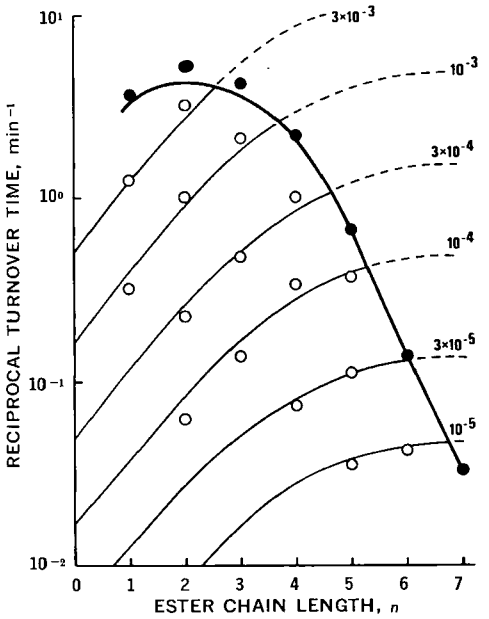


Figure 3—*Turnover times produced by various concentrations and saturated solutions of* n-*alkyl* p-*aminobenzoates. Points are experimental:* ○, *unsaturated; and* ●, *saturated. Lines are theoretical:* ——, *unsaturated; and* ——, *saturated. (Reprinted, with permission, from Ref. 52.)*

drug that is in solution is available for absorption. Since virtually any structural modification that alters solubility will also alter ionization and partitioning, it is somewhat difficult to provide clearcut examples of the relationship between solubility and absorption. Some effects of structure on aqueous solubility will be discussed later. The importance of solubility was apparent in the previous discussion of homologous series. It frequently is the factor responsible for the parabolic shape of many structure–activity curves.

For homologous series, solubility in aqueous media generally decreases by a factor of 4.0 for each methylene unit. Branched alkyl moieties decrease aqueous solubility to a lesser extent than linear chains. It was shown that this results because the former have a lower surface area than the latter and because the solubility of over 70 aliphatic alcohols (linear, cyclic, and branched) is directly proportional to the hydrocarbon surface area (55). An earlier study (56) showed a similar correlation for hydrocarbons. Many empirical correlations have appeared which are useful for estimating the aqueous solubility of organic liquids (57–59). These correlations, while far from complete, can be of value in evaluating the effect of a structural modification on solubility.

Since many drugs are either weak acids or weak bases or their salts, dissociation must be regarded as an important factor in determining absorbability. It is generally known (37, 38) that the unionized form of a drug is absorbed far more efficiently than the ionic species, even though the latter is more soluble. The explanation for this observation lies in the fact that the increase in the partition coefficient in going from a salt to free acid (or free base) usually exceeds the corresponding decrease in solubility by several orders of magnitude. This is analogous to π being greater than δ in Eq. 13. The greater absorbability of the unionized species over the salt is further amplified by the fact that it is rarely practical to give saturated solutions of the salt.

Many workers (37, 38) regard the absorption of ionic species as nonexistent and treat the undissociated species in a manner similar to the treatment of nonelectrolytes discussed. Others (60, 61) have shown that certain ionic drugs are absorbed in their undissociated state, either directly or by ion-pair or complex formation. While these mechanisms are certainly operative in specific instances, they are not of sufficient importance to be of concern here. Consequently, the term ΔC in Eqs. 5, 8, and 9 must refer only to the concentration differential of the undissociated form of the drug. In each aqueous phase, the concentration of unionized species, C_u, can be related to the total concentration, C_t, the pH, and the pK of the weak acidic drug by:

$$C_u = \frac{C_t}{1 + 10^{(\mathrm{pH}-\mathrm{pK})}} \qquad \text{(Eq. 14)}$$

A similar equation can be written for basic drugs. The combination of Eqs. 14 and 5 is known as pH–partition theory. The direct application of this theory to gastric and intestinal absorption has been only partially successful. The data seem to indicate that the intestinal pH is closer to 5.5 than it is to the accepted value of 7.4. This observation led to the postulation of a region of the intestinal lumen adjacent to its surface which has a pH of 5.5 and which is in equilibrium with the bulk of the lumen (37). This virtual pH hypothesis has been criticized (62) because its existence would have no effect on the amount of unionized drug at the luminal surface.

Dissociation behavior, however, is important in designing reversible derivatives when the linkage involves the ionizable group. Thus, a pH-sensitive drug such as 15-methylprostaglandin $F_{2\alpha}$ becomes unionizable when converted to its methyl ester.

If absorption is to be increased by adjusting hydrophobicity, a linkage must be selected that will remain intact until absorption is complete, with subsequent release of the parent molecule into the bloodstream or at some specified tissue. To choose such a linkage rationally, an awareness of what linkage-cleaving enzymes are present in the GI tract, the liver, the blood, and the various body tissues is necessary.

Biological—*Enzymes*—In a broad sense, the basis for the rational design of biologically reversible drug derivatives is predicated on the ability of the host tissue or organism to regenerate the drug derivative to the bioactive parent molecular species. The manner in which this is frequently accomplished is through the mediation of an enzyme or enzyme system within the host. In this respect, Bender (63) and Jencks (64) reviewed the mechanisms of enzyme catalysis. The enzyme(s) may be widely distributed throughout the host tissue (*e.g.*, esterase) or localized in site-specific tissue (*e.g.*, amidase). Enzymes *per se* have been characterized chemically, but their efficiency and

Table II—Reversible Derivative and Prodrug Linkages and Enzymes Responsible for Their *In Vivo* Hydrolysis

Linkage	Hydrolyzing Enzyme	Tissue	Reference
Ester			
Short–medium chain aliphatic	Cholinesterase	Liver, kidney, gut	149–160, 161, 162
	Ester hydrolase	Blood, intestinal mucosa	
	Lipase		
	Cholesterol esterase		
	Acetylcholinesterase		
	Acetyl esterase		163
	Aldehyde oxidase	General distribution	
	Lipase	Liver	
	Carboxypeptidase	Gut	105, 107
Long chain aliphatic	Pancreatic lipase		103, 116
Carbonate	Pancreatin	Intestine	
	Lipase		164
	Aliesterase		165–168
	Carboxypeptidase		
	Cholinesterase	Blood	
Hemiester	Esterases	Blood	169
Phosphate, organic	Acid phosphatase	Blood	170–173
	Alkaline phosphatase	Blood	
	Acid phosphatase III	Blood	174, 175
Pyrophosphate	Pyrophosphatase I	Liver, gut, blood	176
Sulfate, organic	Steroid sulfatase	Liver, blood	177
	Arylsulfohydrolase A and B (phenolsulfatase, arylsulfatase)	Liver	178, 179
	Arylsulfohydrolase C	Liver	180
	Estrogen sulfohydrolase	Liver, gut	181, 182
	Steroid-3β-sulfohydrolase	Liver	183–185
	Steroid-21-sulfatase	Placenta	186
Amide	Amidase	Liver	156, 187–193
		Neoplastic tissue	
		Walker carcinosarcoma 256	194
		Dunning rat leukemia	195
		Neoplastic tissue	191
Amino acid	Proteolytic enzymes	Gut	105, 107, 196
		Neoplastic tissue	110
	Chymotrypsins A and B		106
	Trypsin		105, 107
	Carboxypeptidase A		105, 107
	Carboxypeptidase B		108, 109, 197, 198
Azo	Azoreductase	Liver	199
		Walker rat carcinoma	200
		Sarcoma 180	
		Adenocarcinoma 755	201
		Lymphoid leukemia L-1210	
		Liver	202–205[a]
		Gut microflora	66, 68
Carbamate	Carbamidase	Liver	206
		Walker carcinosarcoma 256 (rats)	115
		Adenocarcinoma 755 (mice)	115
		Liver	207
Phosphamide	Phosphoramidases	Neoplastic tissue (liver)	208–216
		Liver	
Glucosiduronate (β-glucuronide)	β-Glucuronidase	Liver	217–220
		Gut	221
		Blood	
		Gut microflora	66, 68
N-Acetylglucosaminide	α-*N*-Acetylglucosaminidase	Liver, gut, blood	222, 223
	β-*N*-Acetylglucosaminidase		224
β-Glucoside	β-Glucosidase	Gut, liver	176
		Gut microflora	66, 68

[a] This excellent review covers a wide variety of bioreversible nitrogen mustard drug derivatives.

specificity have generally not been quantitatively determined *in vivo*.

Quantitative predictions of the effect of drug derivative enzymatic catalysis *in vivo* are difficult due to the variety and complexity of the enzyme systems involved. The liver, for example, considered the most important organ for drug degradation, contains a complex, nonspecific variety of enzyme systems. The reactivity of these systems varies widely in individuals and largely depends on genetic and hormonal factors, sex, age, *etc.*, making absolute predictions impossible. Furthermore, *in vitro* enzyme hydrolysis studies can be misleading if not conducted with several animal species. For example, a species specificity was demonstrated when hydrolysis rates of the reversible derivative 4-acetamidophenyl-2,2,2-trichloroethyl carbonate were compared using human and rat plasma (65). Hydrolysis proceeded about four times faster in rat plasma than in human plasma, probably due to a greater abundance of specific and/or nonspecific esterases present in rat plasma. This same phenomenon has been demonstrated for a se-

ries of lincomycin 2,7-dialkylcarbonate esters (12).

A number of chemical linkages covalently bonded to a drug substrate that are known to be hydrolyzed by certain common enzymes are listed in Table II. The host tissues in which these enzymatically catalyzed reactions occur are also shown. Although qualitative in nature, this outline provides the medicinal chemist with a powerful tool for designing bioreversible drug derivatives tailored to meet a specific therapeutic need, route of administration, and dosage regimen.

Enzymes considered important to orally administered reversible derivatives are found in the gut, gut wall, blood, and liver. The gut of several animal species, including humans, contains a variety of GI microflora whose enzyme systems are capable of hydrolyzing a number of reversible drug linkages. Thus, the naturally occurring glycosides amygdalin and cycasin are hydrolyzed by gut microfloral enzymes to their aglycones methylazoxymethanol and mandelonitrile, respectively (66). β-Glucuronidases of similar microflora are capable of hydrolyzing stilbestrol and indomethacin glucuronides excreted in the bile. The parent drug is then reabsorbed from the intestine. This hydrolytic activity thus serves as an important event in the enterohepatic circulation of these drug entities (66, 67). Azo linkages are hydrolytically reduced by azoreductase enzymes present in gut microflora. The reversible drug derivative sulfachrysoidine[2] is converted to the parent bioactive species sulfanilamide by this mechanism (66, 67).

Experiments in humans and germ-free rats have shown that sulfasalazine (salicylazosulfapyridine), a drug used in the treatment of ulcerative colitis, is enzymatically reduced to 5-aminosalicylate and sulfapyridine in the GI tract. The former has been implicated for its direct effect on the colon (68, 69). Other chemical linkages known to be hydrolyzed by GI microorganisms are carboxylic esters, sulfate and nitrate esters, amides, and sulfamates (70).

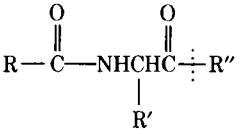
Esterases—The distribution of esterases is ubiquitous and includes a wide assortment of vertebrate tissues and blood serum (71–75). Nonspecific esterases, as the name implies, hydrolyze a variety of ester types including aliphatic, aromatic, and thiol esters (76, 77) and amino acid esters (78, 79), as well as a number of aromatic amides (76) such as acetanilid, phenacetin, and lidocaine. Specific esterases include acetylcholinesterase (80), cholesterol esterase (81), and possibly vitamin A esterase (82). For a comprehensive review of carboxylic ester hydrolases, see Ref. 76. Many bioreversible drug derivatives have been designed to take advantage of the catalytic activity of these esterases.

Lipases, another subclass of hydrolases, can be considered as carboxylesterases with one difference—they are unable to hydrolyze substrate ester derivatives that are fully dispersed (soluble) in water. There appears to be a minimum degree of molecular aggregation necessary for their hydrolytic activity (83). Lipases have recently been shown to hydrolyze molecules in a micelle (84, 85). Two classes of lipases are considered important for hydrolysis of reversible drug derivatives: tissue lipases and lipases found in the digestive tract due to discharge by specialized organs. The latter type is exemplified by pancreatic lipase. Good correlation was established between the hydrolysis rate of a micellar solution of tripropionin and lipase esterolytic activity on these solutions containing approximately 13 monomers per micelle (84).

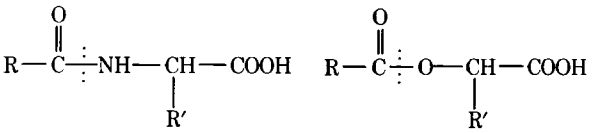
The rapid hydrolysis of clindamycin palmitate given orally may be due to such hydrolytic activity since this drug exists as a micellar solution (86). A similar lipase is thought to hydrolyze micellar solutions of cholesterol and vitamin A esters (87–90). The fact that short chain fatty acid esters are not hydrolyzed as readily as longer chain (C_{10}–C_{18}) esters may be due to the fact that short chain esters are, in general, water soluble whereas the longer chain esters tend to form micellar solutions.

Other lipases that may affect bioreversible derivatives have been found in the microsomal fraction of rat intestinal mucosa (91) and postheparin plasma (92). They hydrolyze short chain monoglycerides and simple esters but not cholesterol esters or long chain di- and triglycerides. A gastric lipase has been found in the microsomal fraction of gastric mucosa (93, 94) which hydrolyzes a number of variable chain-length triglycerides (95). Another lipase of interest is the so-called lipoprotein lipase. It can be detected in plasma within 20 sec after an injection of heparin, suggesting that it may be attached to blood capillary walls (96, 97). The liver contains at least three known lipases: one acidic lipase (98–100) and two alkaline lipases (99, 101). These and other lipases have been reviewed (102, 103).

Another class of hydrolases that may be important in the hydrolysis of bioreversible drug derivatives is the proteolytic enzymes (peptidases) found in the duodenum and intestinal epithelium. The amino acid derivatives hydrolyzed by the endopeptidases can be represented by the general formula:



Those hydrolyzed by the carboxypeptidases can be represented by the general formulas:



peptide substrates ester substrates

The dotted lines represent the point of hydrolysis by the peptidase. Studies with glycylglycine indicate that hydrolysis occurs within the intestinal epithelial cell rather than at the microvilli surface (104). Some of the better known endopeptidases include the chy-

[2] Prontosil.

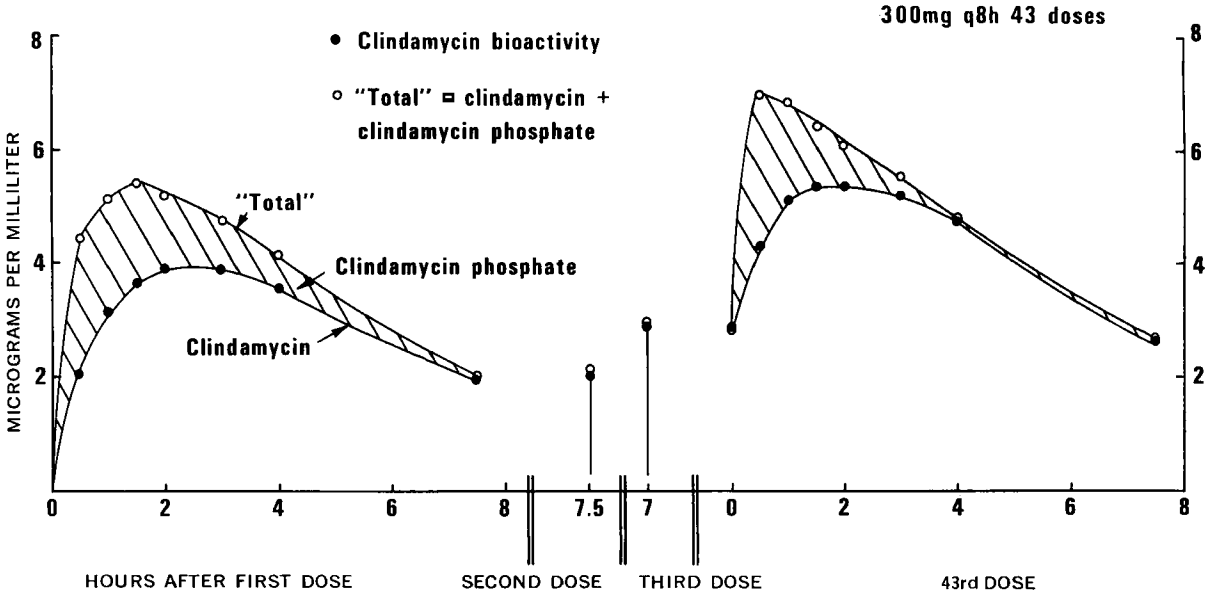


Figure 4—*Mean serum concentrations (micrograms per milliliter) of clindamycin and clindamycin phosphate after intramuscular injections of clindamycin phosphate. (Adapted, with permission, from Ref. 173.)*

motrypsins. These enzymes catalyze the hydrolysis of amide substrates containing the aromatic amino acids phenylalanine, tryptophan, and tyrosine. Moreover, esters of these derivatives are cleaved more rapidly than the amides (105). Chymotrypsin C differs from chymotrypsins A and B in its ability to hydrolyze more rapidly the ester, amide, and peptide bonds involving leucine (106). Trypsin, another peptidase found in the intestine, acts exclusively on peptide, amide, and ester bonds formed with arginine and lysine (105, 107).

Carboxypeptidase A and B, exopeptidases found in the intestine, catalyze the hydrolysis of ester and peptide bonds of derivatives containing aliphatic side chains such as alanine (105), leucine (105), isoleucine (105), and hippuryl-L-lysine (108, 109).

The abundance of proteolytic enzymes found in cancer cells led to the synthesis of glycine bis(2-chloroethyl)amide as a bioreversible form of nitrogen mustard which might selectively be hydrolyzed in such cancer cells (110).

Other investigators have reported on the effect of amidases on the hydrolysis of aminoacylanilides (111–113) and penicillin derivatives (114).

Carbamidases present in Walker carcinosarcoma 256 were deemed responsible for hydrolysis of various bis(1-aziridinyl)phosphinyl carbamates to the bioactive alkylating bis(1-aziridinyl)phosphinyl radical (115).

Keller (116) and Rhodes (117) have reviewed the properties and functions of a wide variety of proteolytic enzymes, and other comprehensive reviews have appeared elsewhere (118).

Alkaline Phosphatase—Alkaline phosphatases (orthophosphoric monoester phosphohydrolases) catalyze the hydrolysis of many alkyl and aryl monophosphate esters. This family of enzymes is localized mainly in kidney and intestinal mucosa (119). Ossifying cartilage is also a rich source of such phosphatase (120) and has an optimum activity at pH 8.4–9.4 (121), similar to that found in blood plasma (122). Isotope labeling studies support the belief that alkaline phosphatase acts *via* a transferase mechanism whereby a phosphoryl moiety is directly transferred from the phosphate ester to an acceptor alcohol (123). It was also shown that Zn^{+2} (2–3 g-atoms/mole of enzyme) was essential for catalytic activity (124, 125). These enzymes catalyze the hydrolysis of a wide variety of ester substrates, including phosphate monoesters, diesters, and phosphoroamidate (126–128).

Acid Phosphatase—Like alkaline phosphatase, the acid phosphatases or orthoester phosphohydrolases are widely distributed throughout nature. Their distribution in mammalian tissue differs somewhat from alkaline phosphatase, being found predominantly in red blood cells (129, 130), plasma (131), prostatic tissue (132–134), spleen (135, 136), and liver (137).

The availability of acid phosphatases in red blood cells and plasma may account for the rapid hydrolysis of clindamycin phosphate to clindamycin when clindamycin phosphate is administered intramuscularly (Fig. 4). Peak serum concentrations of clindamycin appear in less than 0.5 hr, reflecting rapid hydrolysis of the phosphate ester in the systemic circulation. Intramuscular administration of this derivative results in delayed absorption of clindamycin, due in part to rate-limiting diffusion from the injection site as the phosphate ester, with subsequent serum hydrolysis.

The biologically reversible antineoplastic drug, diethylstilbestrol diphosphate, was designed to take advantage of the high phosphatase activity in prostatic carcinoma tissue. Given as an intramuscular injection, the derivative was thought to diffuse from the injection site as the ester and circulate to the prostatic tissue where hydrolysis occurred, thus accu-

Table III—Reversible Drug Derivatives Utilized as Modifiers of Absorption (Oral, Percutaneous, and Parenteral)

Parent Molecule	Reversible Modification or Linkage	Route of Administration	Property Modified	Reference
Convallatoxin	Ketal	Oral	Resorption	228
Hydantoin	Alkyl ester	Oral	Absorption	229
	Amino ester			
Chlorphenesin	Glycine ester	Oral	Absorption	230
	Alanine ester			
Acetaminophen	Caffeine complex	Oral	Absorption	231
Acetylsalicylic acid (aspirin)	THAM salt	Oral	Absorption	232
Acetylsalicylic acid (aspirin)	Acetamidophenyl ester	Oral	Absorption	233
N-Allylnoroxymorphone (naloxone)	Acetate ester	Oral	Absorption	148
	Sulfate ester			
Nicotinic acid	Mesoinositol penta-nicotinate	Oral	Absorption	234
15-Methylprostaglandin $F_{2\alpha}$	Methyl ester	Oral	Absorption	227
Procaine	Polyethylene glycol (carbamate)	Percutaneous Conjunctival	Absorption	235
α-Amino-*p*-toluenesulfonamide (4-homosulfanilamide)	Acetate salt	Percutaneous	Absorption	30, 236
Hexachlorophene	Ester	Percutaneous	Absorption	21
	Ether			
	Hemiester			
Oleandomycin	Acetate ester	Oral	Absorption	237
Erythromycin	Ester, alkyl	Oral	Absorption	14, 238, 239
Clindamycin	Ester, alkyl	Oral	Absorption	13, 240
	Ester, phosphate	Intramuscular		241
α-Carboxybenzylpenicillin	Ester, mono- and dialkyl	Oral	Absorption	242
α-Aryl-β-aminoethyl penicillin	Alkoxymethyl esters	Oral	Absorption	243
Penicillin, general structure	Diethylaminoethyl ester, alkoxymethyl esters, ether	Oral	Absorption	244
α-Aminobenzylpenicillin (ampicillin)	Azide	Intravenous, oral	Absorption	245, 246
6-(D-α-Sulfoaminophenylacetamido)penicillin	Pivaloyloxymethyl ester	Oral	Absorption	247
Carbenicillin	Indanyl ester	Oral	Absorption	248, 249
α-Aminobenzylpenicillin (ampicillin)	Acyloxymethyl ester	Oral	Absorption	225, 226, 250–253
Penicillin G	Amide with 1,2-benziso-thiazol-3(2*H*)-one 1,1-dioxide	Intramuscular	Absorption	254
Penicillin V				
α-Aminobenzylpenicillin (ampicillin)	*N*,*N*-Isopropylidene adduct	Oral	Absorption	255, 256
Hetacillin	Pivaloyloxymethyl ester	Oral	Absorption	257
Doxycycline	Polymetaphosphate complex	Oral	Absorption	258
Colistin	Lower alkyl esters	Oral	Absorption	259
Tetracycline	Betaine salt	Oral	Gastric absorption	260
7-Acylaminocephalosporins	Ring-substituted acyloxybenzyl esters	Oral	Absorption	261
	Acyloxyalkyl esters	Oral	Absorption	262
α-Amino (or ureido) cyclohexadienylalkyl penicillins and cephalosporins	Acyloxymethyl esters	Oral	Absorption	263
7-Acylaminocephalosporins	Imino ether	Oral	Absorption	264
Nandrolone	Phenylpropionate, decanoate esters	Intramuscular	Absorption	266
9α-Fluorohydrocortisone (fludrocortisone)	Acetate ester	Subcutaneous	Absorption	266
Estradiol	Enol ether	Oral, parenteral	Absorption	267
	Acetal			
Oxymetholone	Ethoxycarbonate ester	Oral, subcutaneous	Absorption	268
Methylprednisolone	Acetate ester	Intramuscular	Absorption	269
Testosterone	*N*-Acetylglucosaminide	Oral	Absorption	270, 271
	Glucosiduronate (trimethylsilyl) ether	Oral		
19-Nortestosterone	17-β-Adamantoate	Intramuscular	Absorption	272
9-(β-D-Arabinofuranosyl) adenine	Acetate, formate, propionate, esters	Oral	Absorption	273
Cortisol, prednisolone, dexamethasone	21-Phosphate ester	Intramuscular, intravenous	Absorption	274
	21-Hemisuccinate ester	Subcutaneous		
Prostaglandin	Alkylsilyl ether	Percutaneous, oral, intrauterine	Absorption	275
Salicylic acid	Carbonate ester	Oral	Absorption	276, 277

mulating as diethylstilbestrol in this localized site (23). Good antitumor activity was similarly demonstrated in mouse plasma cell tumor ADJ/PC6A with a series of phosphate esters of *p*-hydroxy-*N,N*-bis(2-chloroethyl)aniline. The activity was attributed to hydrolysis of the phosphate esters by acid and alkaline phosphatases present in the tumor (138, 139).

Comprehensive reviews on these important classes of enzymes have been written (140, 141), and Schapira reviewed the properties of various enzymes present in neoplastic tissue (142).

Sulfatases—The sulfate ester-hydrolyzing enzymes are not necessarily limited to the catalytic hydrolysis of sulfate ester anions but exert their effects on sulfatophosphates (sulfatophosphate sulfohydrolases) and sulfamates (sulfamatases). The sulfatases can be subdivided into aryl, alkyl, and steroid sulfatases. The function of the aryl and alkyl sulfatases is obscure, although they are found in liver (143, 144) and brain (145, 146) tissue. The steroid sulfatases, however, catalyze the hydrolysis of several types of steroid sulfate esters including estrone sulfate, androstenolone sulfate, etiocholanolone sulfate, and cortisone-21-sulfate.

A series of estrogen sulfates was synthesized (147), but no biological testing of these esters was reported. Linder and Fishman (148) found low narcotic antagonist activity upon intravenous administration of an isomeric monosulfate and a disulfate ester of naloxone.

Although virtually no drug derivatives containing a sulfate ester moiety have been explored, their use could be rationalized in therapeutic indications analogous to those of drug phosphate esters.

For obvious reasons, drugs possessing low aqueous solubility would be excluded from the intravenous route of administration. Reversible derivatives designed to enhance aqueous solubility of such drugs *in vivo* include hemiesters, phosphate, and, possibly, sulfate esters.

APPLICATIONS

Absorption—Table III lists some presently marketed drugs that contain reversible linkages designed to enhance absorption of the parent molecule. This list of examples is designed to highlight the variety and scope of chemical linkages utilized to modify a wide selection of parent drug molecules. Parent molecules include representatives from many diverse classes of drugs, and chemical linkages include complexes and salts as well as covalently bound moieties. Since absorption is a general phenomenon, several routes of administration are included to illustrate the versatility of this form of drug modification.

One fine example illustrating the importance of reversible derivative formation and its effect on absorption is found in studies with ampicillin (D-α-aminobenzylpenicillin) (225, 226). This drug is relatively stable in an acidic medium but poorly absorbed orally. A series of acyloxymethyl esters (I*a*–I*h*) was synthesized to provide a hydrophobic form of ampicillin that would be rapidly hydrolyzed *in vivo* after absorption to enhance serum levels of this antibiotic.

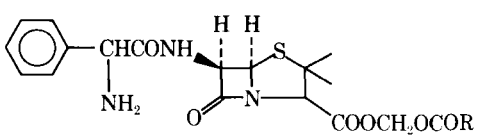
Table IV[a]—Hydrolysis of Acyloxymethyl Esters of Ampicillin at pH 7.4 and 37° in the Presence of 10% Human Serum[b]

Ester	Percent Hydrolyzed[c] after 30 min
Acetoxymethyl (I*a*)	89
Propionyloxymethyl (I*b*)	80
κ-Butyryloxymethyl (I*c*)	85
Isobutyryloxymethyl (I*d*)	89
Pivaloyloxymethyl (I*f*)	22
α-Ethyl-κ-butyryloxymethyl (I*g*)	23
Benzoyloxymethyl (I*h*)	67

[a] Adapted, with permission, from Ref. 225. [b] The starting concentration of the esters was 1/35 mmole ~ 10 μg/ml of free ampicillin. [c] The figures do not indicate the exact degree of hydrolysis since, under the applied conditions, ampicillin as well as its esters undergoes transformations (probably polymerization) resulting in loss of antibacterial activity.

In vitro hydrolysis studies were carried out on several ampicillin esters (Table IV). It was apparent that the straight chain esters were more rapidly hydrolyzed than those with branched chains. This *in vitro* study, while indicative of the relative degree of enzymatic *in vitro* lability of these esters, was not conclusive in determining which ester might suitably be used orally *in vivo*. It would appear that the pivaloyloxymethyl ester (I*f*) is hydrolyzed too slowly to be of use clinically. In point of fact, absorption studies in humans (226) indicated that this ester was absorbed almost quantitatively and enzymatically hydrolyzed rapidly after absorption (Fig. 5). This ester hydrochloride of ampicillin represents the right degree of hydrophobicity for absorption coupled with the required amount of enzymatic lability necessary for hydrolysis to produce extremely high plasma levels of ampicillin. Peak serum levels of ampicillin (administered as pivampicillin) were more than three times greater than ampicillin administered above. This ratio of peak serum levels was invariant in both fasted and fed subjects. Since this study, acyloxymethyl esters of several antibiotics have been made in an attempt to duplicate the success achieved with ampicillin (Table III).

15-Methylprostaglandin $F_{2\alpha}$ and its methyl ester provide yet another example of how a bioreversible derivative can enhance absorption of a parent drug molecule. Magee *et al.* (227) determined the rate of absorption of 15-methylprostaglandin $F_{2\alpha}$ and its methyl ester in rats by using an *in situ* method of sequential sampling of the duodenal contents. They found that absorption of the ester was biphasic with



I*a*: R = CH_3
I*b*: R = CH_2CH_3
I*c*: R = $(CH_2)_2CH_3$
I*d*: R = $CH(CH_3)_2$
I*f*: R = $C(CH_3)_3$
I*g*: R = $CH(CH_2CH_3)_2$
I*h*: R = C_6H_5

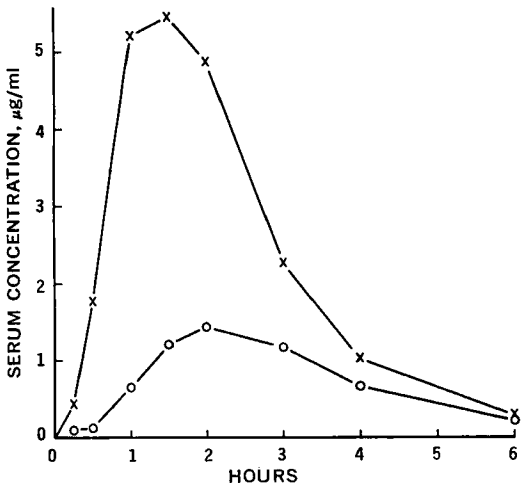


Figure 5—*Mean serum levels of ampicillin in normal volunteers following oral administration of 250 mg of amoicillin (O) and 358 mg of pivaloyloxymethylampicillin hydrochloride (≈250 mg of ampicillin) (X) immediately after breakfast. (Adapted, with permission, from Ref. 225.)*

initial rapid uptake followed by slow continuous absorption (Fig. 6).

The rapid phase of absorption declined as the ester was cleaved to the parent free acid. Higher doses of ester resulted in more drug being absorbed, with no ester being detectable in the serum (Fig. 7). These researchers concluded that the methyl ester was absorbed very rapidly, with hydrolysis by esterases occurring in the intestinal lumen and serum. Serum

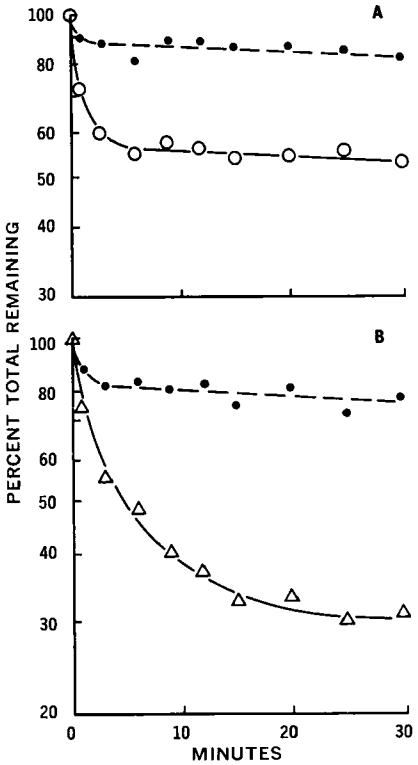


Figure 6—*Absorption of a low dose (A) (0.05 mg/ml) and of a high dose (B) (0.5 mg/ml) of 15-methyl-^{14}C-prostaglandin $F_{2\alpha}$ methyl ester. The dashed lines show changes in fluid volume. (Adapted, with permission, from Ref. 227.)*

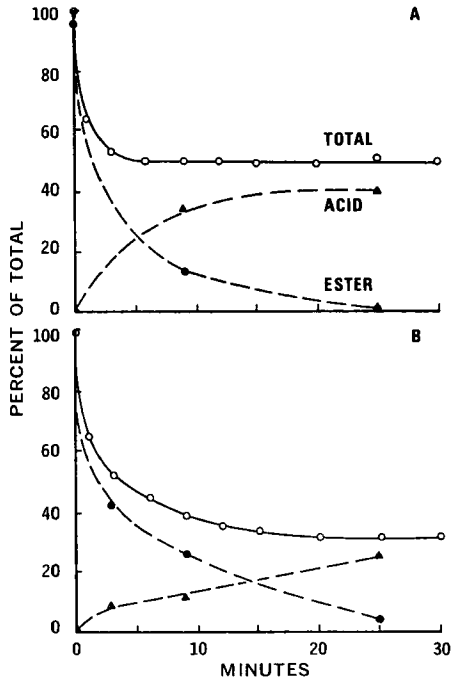


Figure 7—*Hydrolysis of 15-methyl-^{14}C-prostaglandin $F_{2\alpha}$ methyl ester to the free acid in the lumen of the small intestine. Same experiment as shown in Fig. 6. Key: A, low dose; B, high dose; ▲, 15-methyl-^{14}C-prostaglandin $F_{2\alpha}$; ●, 15-methyl-^{14}C-prostaglandin $F_{2\alpha}$ methyl ester; and ○, ● + ▲. (Adapted, with permission, from Ref. 227.)*

levels of parent prostaglandin 10–20 times greater were observed when the methyl ester was administered.

Site Direction—Historically, research in the area of site-directed, reversible derivative chemotherapy has been generated mainly by empirical data and by intuitive notions and correlations. The fault lies not with the chemist and biologist but rather with the paucity of definitive data that might be used for planning and execution of meaningful studies in this area.

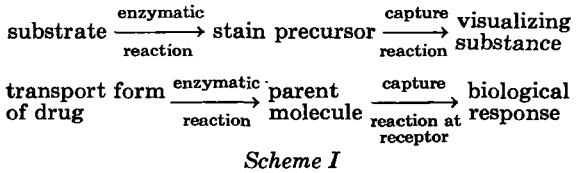
The concepts of reversible drug derivative chemotherapy to specific tissue can be generally divided into two classes:

Site directed, in which the reversible derivative is transported and distributed preferentially to site-specific tissue according to the physicochemical properties of the derivative (*e.g.*, hydrophobicity–hydrophilicity relationships, pKa, molecular size and geometry, and solubility). This subject was discussed in the *Absorption* section.

Site activated, which relates to the regeneration of the parent molecule either by enzymatic or chemical hydrolysis in the target organ or site-specific tissue.

The rationale for the synthesis of site-directed drugs evolved primarily in the area of cancer chemotherapy but can be summarized by the following basic premises that apply to chemotherapy of any localized disease state:

1. Certain disease conditions are localized in specific tissue.
2. The drugs utilized in these disease states affect



Scheme I

normal as well as diseased tissue and may be metabolized and excreted prior to reaching the diseased tissue.

3. Diseased tissue possesses enzymes or enzyme systems differing in their nature and abundance from normal tissue.

4. These enzymes are capable of regenerating the bioactive species of a reversible derivative.

Several excellent papers and reviews are based on these principles (160, 163, 278–283).

Harper (23), in discussing drug localization in specific tissue, drew analogies to enzyme histochemistry in which the histochemical staining process was likened to site-specific drug localization (Scheme I). By drawing inferences from Scheme I and observing the relationship of the low solubility of certain dyes and their high association by hydrogen bonding, substituent effects and steric properties, and subsequent binding to protein, it was concluded that optimal conditions for drug transport to site-specific tissue involved steric interactions (fit) of the drug with the receptor and also the influence of distribution, transport, excretion, and duration of action to these drug–protein interactions.

The ideal reversible drug derivative might possess the following characteristics:

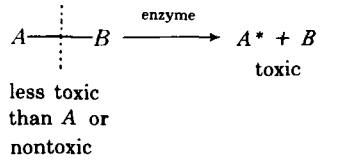
1. Exclusive and complete transport to the diseased tissue or target organ, including affinity and interaction with these cell systems and tissues.

2. Absence of binding of the derivative to protein or tissue not specifically diseased without degradation (hydrolysis) or metabolism of the derivative prior to contact with the diseased bioenvironment; lack of toxicity for normal tissue within the diseased environment and other normal tissue in the body.

3. Complete elimination from the body once the disease state is eliminated or once complete remission is effected.

The ideal derivative consists of a bioactive parent molecule containing a suitable transport group sufficiently stable to deliver the molecule to the site of activity (site direction) containing the necessary enzyme systems and/or chemical bioenvironment sufficient to regenerate the parent molecule at the diseased site (site activation). While the ideal derivative is yet to be made, several approaches have been utilized in a variety of attempts at providing optimal therapeutic response and site specificity. They can be categorized as: *(a)* utilization of the enhanced enzymatic activity present in diseased or dividing cells, especially neoplastic cells, but not present in normal, nondividing cells; *(b)* utilization of the lower pH of neoplastic tissue relative to that of most normal cells; and *(c)* utilization of lipophilic and/or hydrophilic derivatives to enhance transport and subsequent bioactivity to site-specific tissue.

Following the first approach, Tsou *et al.* (191) synthesized a series of nitrogen mustard reversible amide derivatives designed to regenerate the parent molecule in neoplastic tissue by taking advantage of the difference in the level of enzymatic amidase activity between normal and neoplastic cells. Assuming amidase activity to be localized in the tumor cell, they schematically represented what might occur at the site (Scheme II):



Scheme II

where *A—B* represents a nontoxic bioreversible derivative of *A* (toxic to neoplastic tissue), and the dotted line represents the site of hydrolysis of the derivative by the enzyme.

Of several derivatives synthesized, *N,N*-diallyl-β-aziridinopropionamide (DAAP) was found to be active intraperitoneally against the Dunning leukemia system in both ascites and solid form, and data were obtained at doses between 20 and 40 mg/kg. Leucopenia, a common manifestation of toxicity with other bisfunctional alkylating agents, was not apparent with *N,N*-diallyl-β-aziridinopropionamide, indicating that the derivative was selective in its action against dividing (neoplastic) tissue.

Other examples of this approach can be found in Table V.

Another property, namely lower relative pH of certain neoplastic cells (284, 285), has been exploited in the design of bioreversible cancer chemotherapy drug derivatives. The fact that pH is lower in tumor cells may be due to the high rate of aerobic and anaerobic glycolysis, the end result being the production of lactic acid. This acid may accumulate in tumor cells if its production is greater than an offsetting neutralization due to diffusion of buffering substances into the cells from the arterial circulation and by acid diffusing from the cell into venous blood (286, 287). If the preceding elimination mechanisms were not operative, the pH of the tumor cells would probably decrease to a limiting value of ~6 (288).

Papanastassiou *et al.* (287) investigated the *in vivo* acidic lability of a series of alkylaryldiazoamino compounds known to undergo facile acid hydrolysis *in vitro* (Scheme III). Of those compounds tested, *p*-[*N,N*-bis(2-methanesulfonoxyethyl)amino]benzenediazonium fluoroborate (II), while somewhat toxic, prolonged the lifespan of L-1210 leukemia-implanted mice to 157% of controls at a dose of 1.25 mg/kg. It produced cures in five out of six mice with ascites Dunning leukemia. Several other compounds tested were inactive when R = CH_2CH_2F and Y = NO_2 or

$$YArN{=}NNR_2 \xrightarrow{H_3O^+} YArN_2^+ + HNR_2$$

Scheme III

$COOC_2H_5$. The choice of R and Y substituents appears to be critical to activity.

Table V—Reversible Drug Derivatives Utilized as Modifiers and Enhancers of Drug Transport to Site-Specific Tissue

Parent Molecule	Reversible Modification or Linkage	Route of Administration	Target Tissue or Organ	Reference
Isocyanate, R—N=C=O	Haloalkyl nitrosourea	Intraperitoneal Intravenous Intracerebral Subcutaneous Oral	L-1210 leukemia	301–303
Testosterone	Propionate ester	Intramuscular	Breast carcinoma	304
Dromostanolone	Propionate ester	Intramuscular	Breast carcinoma	305
Urea	Hydroxyl		Myelogenous leukemia	306
Normeperidine	Amide	Subcutaneous	CNS	307
Morphine, phenazocine	Ether	Subcutaneous	CNS	308
Tetrahydrocannabinol	Ester	Oral, intramuscular	CNS	152
Diethylstilbestrol	4,4′-Diphosphate ester	Intramuscular	Prostatic carcinoma	23
	4,4′-Dicarbamate	Intramuscular	Prostatic carcinoma	309
Methotrexate, 3′,5′-dichloromethotrexate	Dialkyl esters		L-1210 leukemia	154
Phenethylamine, *dl*- and *d*-amphetamine, *l*-ephedrine, *dl*-*p*-hydroxyamphetamine	Carbonate	Intraperitoneal	Brain	207, 310
Dopamine, 3,4-dihydroxyphenethylamine	Diacyl esters		Brain	311
Norepinephrine	Silyl ether Acetate ester	Intravenous	Brain Heart	312
Cytosine arabinoside (cytarabine)	5′-Acylate 5′-Sulfonate	Intraperitoneal Subcutaneous	L-1210 leukemia	151
Hydroxyurea	Alkyl ether	Intravaginal	Vaginal mucosa	206
Dichloroisocyanatophosphine oxide	Carbamate		Walker carcinosarcoma 256	115
N,*N*-Bis(2-chloroethyl)-phosphorodiamidic acid	Cyclic phosphoramide	Intraperitoneal Intravenous	Malignant lymphomas Neuroblastoma Ovarian adenocarcinoma Breast carcinoma Wilms tumor	210, 214 215 297, 299
Nitrogen mustard (2,2′-dichloro-*N*-methyldiethylamine)	Phosphorodiamidic acid	Intraperitoneal	Dunning ascites tumor L-1210 leukemia	216
Nitrogen mustard	Aminomethyl tetracycline	Intraperitoneal	Adenocarcinoma 755 L-1210 leukemia	192
Allylamine	Amide	Intravenous	Adenocarcinoma 755	191
Nitrogen mustard	Cholesterol, estradiol, dehydroepiandrosterone esters	Oral	Mammary tumor Leukemia	155, 313
Nitrogen mustard	Peptide (glycine, phenylalanine)	Intraperitoneal	Hepatoma Walker carcinosarcoma 256	314 315, 316
5-Diazoimidazole-4-carboxamide	Dialkyl triazene	Intraperitoneal	Sarcoma 180 Adenocarcinoma 755 L-1210 lymphoid leukemia	201
Nitrogen mustard	Azobenzene	Intraperitoneal	Liver	200, 205, 317
Dichlorodiamine, dichlorodialkylamine	Platinum coordination complex	Intraperitoneal Intravenous	Solid sarcoma 180 Walker 256 carcinosarcoma Dunning leukemia Primary Lewis lung carcinoma	318, 319

In other studies utilizing the neoplastic cell pH hypothesis, Shealy *et al.* (201) found that the lifespan of L-1210-implanted mice increased 57% over controls when dosed with 5(or 4)-(dimethyltriazeno)imidazole-4(or 5)-carboxamide (III). These compounds are acid labile (300) and were thought to regenerate the parent bioactive 5-diazoimidazole-4-carboxamide *in vivo* as the internal salt (IV). A pH difference between neoplastic and normal tissue and its effect on bioactivity of antineoplastic agents were also studied (289).

A third approach, currently receiving increased attention, concerns the correlation of structure–activity relationships with lipophilic character of the drug derivative. A study (290) of some quantitative structure–activity relationships of a series of nitrosoureas and triazinoimidazoles found that for each series the lipophilic character (a crude measure of absorption and transport) of each type of drug was parabolically related to its activity against L-1210 leukemia in mice. A parabolic relationship was found for 23 nitrosoureas when log P was plotted against log $(1/c)$ (Fig. 8).

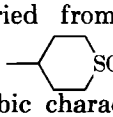
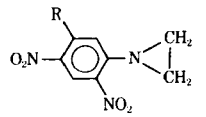
Log P was taken as a measure of hydrophobic character of the derivative molecule where P was its octanol–water partition coefficient. Log $1/c$ is a measure of the bioactivity of the drug. When X = Cl or F and R substituents varied from 4-*tert*-butylcyclohexyl (log $P = 4.51$) to  (log $P = -0.41$), the optimum hydrophobic character occurred at log P from about -0.4 to -0.6. Steric and electronic effects on bioactivity were not considered in this theoretical study (290). Based primarily on hydrophobic consid-

Table VI[a]—Solubility, Partition Coefficients, Substituent Constants, and Biological Activity of *N*-Substituted 1-Aziridino-2,4-dinitrobenzamides[b]



Compound	R	*S*, μmoles/ml	P^E	P^B	Log P^E	π	π'	ED_{90}, μmoles/kg	LD_{50}, μmoles/kg
1	H	1.01	30	>100	1.48	—	—	100	1100
	CONHR′								
16	R′ = H	5.04	0.50	0.14	−0.28	−1.76	—	1.6	112
41	CH_3	1.41	0.87	1	−0.06	−1.54	0.22	<9	113
42	C_2H_5	0.75	2	3.1	0.30	−1.18	0.58	3.6	100
43	*n*-Propyl	0.13	5.4	12	0.73	−0.75	1.01	34	95
44	Isopropyl	0.32	4.5	9	0.65	−0.83	0.93	5.1	54
45	Cyclopropyl	0.41	1.7	3.3	0.23	−1.25	0.51	3.4	110
46	Isobutyl	0.14	14	30	1.15	−0.33	1.43	29	146
47	*tert*-Butyl	0.68	30	55	1.48	0	1.76	65	227
	$CONHR_2$								
52	$R_2 = (CH_3)_2$	3.3	1.1	15	0.04	−1.44	0.32	−ve	65
53	$(C_2H_5)_2$	2.6	7.5	>100	0.86	−0.62	1.14	−ve	81
54	$—(CH_2)_5—$	0.68	9.2	>100	0.95	−0.53	1.23	109	281
55	$—(CH_2)_2O(CH_2)_2—$	1.3	1.2	15	0.08	−1.40	0.36	186	560

[a] Adapted, with permission, from Ref. 289. [b] *S* = solubility in water containing 2% dimethyl sulfoxide at 25°, P^E = ether–water partition coefficient, P^B = benzene–water partition coefficient, $\pi = \log P^x - \log P^H$ (for ether–water coefficients), and $\pi' = \log P^x - \log P^{CONH_2}$ (for ether–water coefficients).

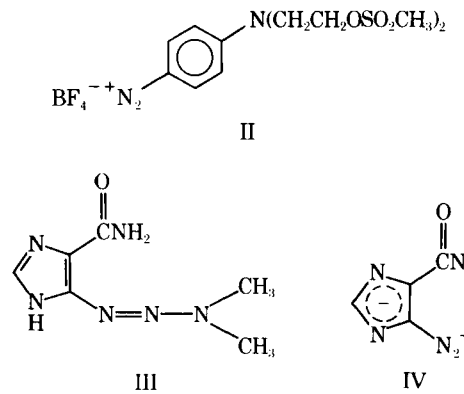
erations and the parabolic relationship, however, it was suggested that nitrosourea derivatives containing more hydrophilic substituents might yield more potent and less toxic drugs, although there are not enough data in Fig. 8 to substantiate the maximum value accurately. The trend, however, is apparent.

It was also shown that an approximate measure of the derivatives' LD_{10} (dose required to kill 10% of test animals) was related parabolically to log *P*. Maximum toxicity was found at log $P_0 = 0.4$. Log P_0 for toxicity was determined by taking the derivative *d* log 1/*c*/*d* log *P* and was found to be approximately 1 log unit higher than for potency, implying that derivatives containing more hydrophilic substituents, while increasing potency, simultaneously decrease toxicity. Wheeler *et al.* (291) confirmed these observations.

In contrast to the finding of Hansch *et al.* (290) of a parabolic relationship correlating log *P* with activity in a series of nitrosourea derivatives, Khan and Ross (289) found a linear relationship between log *P* and activity in a series of *N*-substituted 1-aziridino-2,4-dinitrobenzamides. Thus, they demonstrated a correlation between the water–lipid partition coefficients of a number of substituted amides of 5-aziridino-2,4-dinitrobenzamide (CB 1954) and the expected amide linkage sensitivity toward enzymatic hydrolysis in one instance and the chemotherapeutic index in the Walker tumor assay on the other. Not surprisingly, the highest antitumor activity was demonstrated by the more hydrophilic and easily hydrolyzed substituted amide derivatives. Table VI summarizes the results of solubility, partition coefficient, substituent constants, and bioactivity data for this series of amide derivatives.

The derivatives were tested as inhibitors of growth in transplanted Walker rat carcinoma 256; the LD_{50}, or median lethal dose for tumor-bearing animals, and the ED_{90}, or the dose that produced a 90% inhibition of tumor growth, were reported. The LD_{50}/ED_{90} ratio was taken as the chemotherapeutic index (C.I.). A plot of log $1/ED_{90}$ or log $1/LD_{50}$ *versus* π results in a linear relationship for a series of monosubstituted (but not disubstituted) amide derivatives (Fig. 9).

It can thus be seen that the presence of a monosubstituted amide group in 5-aziridino-2,4-dinitrobenzamide confers favorable water–lipid partitioning prop-



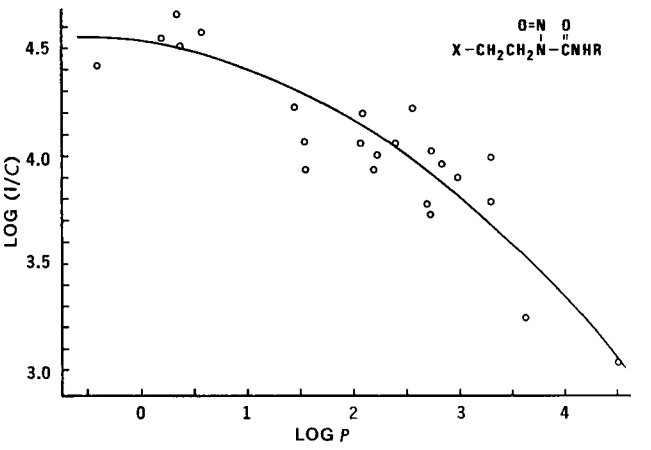


Figure 8—*Experimental data (log 1/C) for 23 nitrosourea derivatives plotted against log P. (Adapted, with permission, from Ref. 290.)*

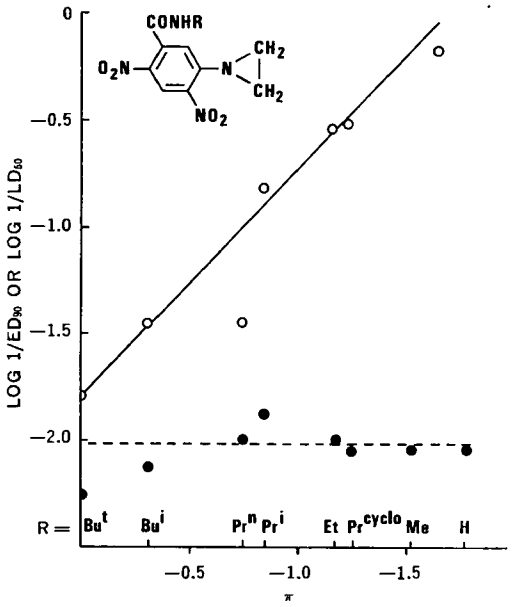


Figure 9—*Plot of substrate constant, π, against log 1/ED_{90} (○) and log 1/LD_{50} (●) for 5-aziridino-2,4-dinitrobenzamides. (Adapted, with permission, from Ref. 289.)*

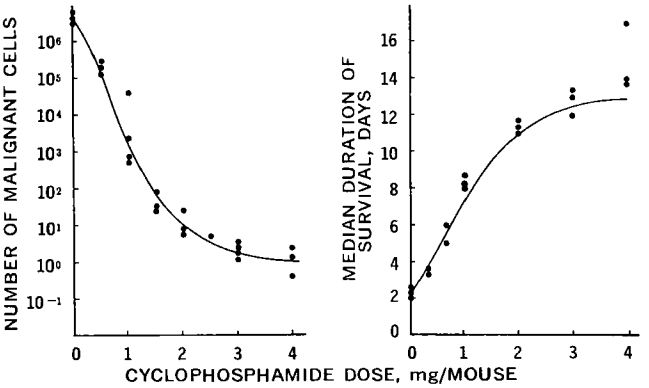
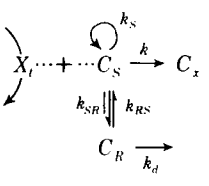


Figure 10—*Dose–cell survival and dose–animal survival curves for the effect of cyclophosphamide on lymphoma cells in the mouse femur. The cell survival line was obtained by least-squares fit of the data to the model of Scheme IV. The theoretical duration of animal survival curve was generated with the values* $C_T^0 = 2.2 \times 10^7$ *cells,* $k_s = 0.0603\ hr^{-1}$, *and cell number causing animal death* $= 8.6 \times 10^8$ *cells. (Adapted, with permission, from Ref. 293.)*

erties yet maintains sufficient *in vivo* stability *via* the amide linkage such that hydrolysis is selective within sensitive tumor cells. These two factors, coupled with the fact that host toxicity of the derivatives is not highly dependent on π or ease of amide hydrolysis, suggest that membrane permeabilities of Walker tumor cells differ from normal cells. Possibly the tumor intercellular enzymatic activity is sufficient to hydrolyze the amide to a nondiffusing anion, thus destroying toxicity as well as bioactivity.

Similar studies were performed on busulfan[3] (1,4-dimethanesulfonoxybutane, 1,4-butanediol dimethanesulfonate) and related sulfonic acid esters (292). Good correlations between water–lipid ratios and bioactivity (neutrophil-depressing activity) were found.

In a study aimed at providing a rational approach to the development of improved site-specific chemotherapeutic agents, Jusko (293) developed a pharmacodynamic model utilizing dose–time–cell survival curves generated by the administration of cell cycle specific agents. The model assumes an irreversible, bimolecular mechanism of drug–receptor interaction that serves as the interface between the pharmacokinetic parameters of the drug and the cell cycle–cell proliferation kinetics of both normal and neoplastic cells. Dose–time–cell survival curves were analyzed using the model for several drugs including the reversible derivative cyclophosphamide. The effects of this derivative on hematopoietic and lymphoma cells were examined, and it was found that the primary *in vivo* determinant of selective chemotherapy was operative during the proliferative state of the cells.

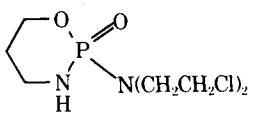


Scheme IV

Scheme IV illustrates the model utilized by Jusko to characterize the effects of these chemotherapeutic agents, where C_S represents the concentration of cells (proliferative) sensitive to the drug, C_R is the number of insensitive (resting) cells, k_{RS} and k_{SR} are interconversion rate constants between the two cell populations, X_t represents the transient amount of drug to which the cells are exposed, and k_S and k_d are rate constants acting on sensitive *(C_S)* and insensitive *(C_R)* cells, respectively. The concentration of sterilized or killed cells is represented by C_x, and k is the association rate constant for bimolecular, irreversible drug–receptor interaction.

Perhaps the most important parameter of the model is k_S, the "chemotherapy constant," which is determined by the assumptions that: *(a)* k_{RS} and k_{SR} are relatively constant, and *(b)* the rate constant for cell kill $(k \times X_t)$ can be related to the dose (D_0) and the number of doses *(N)* of drug by $k \int_0^\infty X_t \times dt = Nk_SD_0$.

This parameter implicitly includes the distribution, elimination, and drug–receptor association rate constants. Figure 10 illustrates dose–cell survival and dose–animal survival curves for cyclophosphamide on lymphoma cells.



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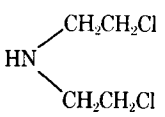

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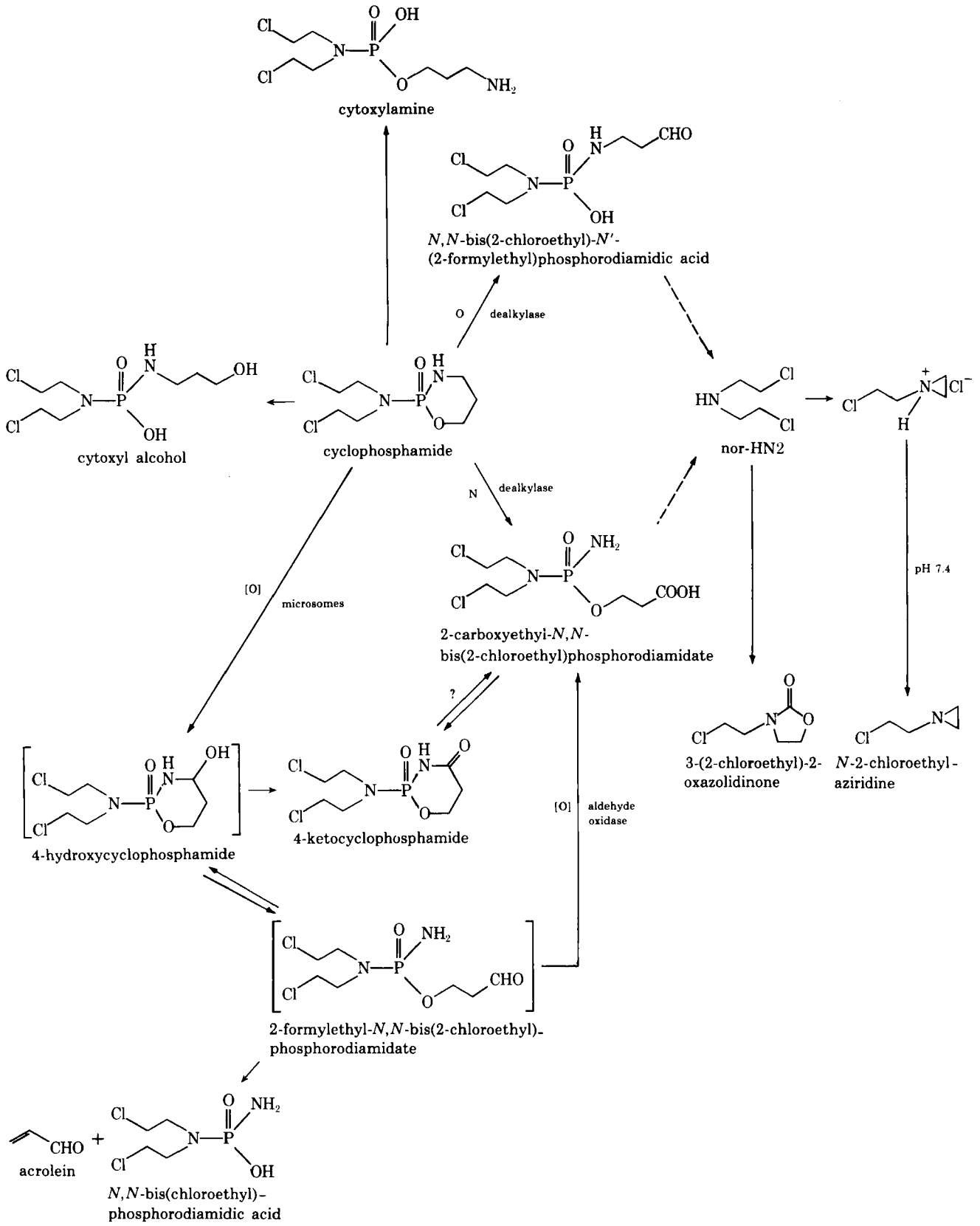


Scheme V

Pharmacokinetic studies such as this allow estimates of biological effects (cell kill) to be made at the receptor level as a function of both dose and time. Obviously, absorption and distribution of derivative to site-specific tissue must also be considered as important variables (see discussion on absorption). This type of modeling allows the medicinal chemist to evaluate grossly the effects of a parent molecule at

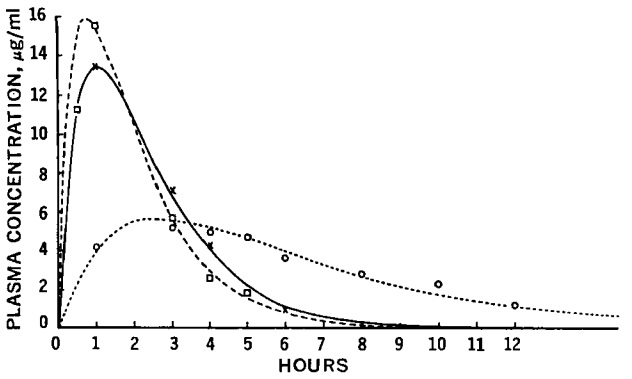


Figure 11—*Concentration of dihydrostreptomycin in the plasma of dogs as a function of time after intramuscular administration of 4 mg/kg of dihydrostreptomycin (as sulfate) in water (□) and in cottonseed oil (×) and of 4 mg/kg of dihydrostreptomycin (as pamoate) in cottonseed oil (○). Dotted lines give one-compartment open model fits. (Adapted, with permission, from Ref. 321.)*

the receptor level and to provide an approximation of the magnitude of the effect of the reversible linkage on transport and distribution to such site-specific tissue.

The interesting bioreversible cytotoxic derivative whose *in vivo* biodegradation has remained obscure for many years is cyclophosphamide [2-[bis(2-chloroethyl)amino]tetrahydro-2*H*-1,3,2-oxazaphosphorine 2-oxide (V)]. This agent differs from other alkylating agents in that it shows a significantly greater selectivity against a variety of tumor cells (211, 294, 295) as judged by its relatively high therapeutic index *in vivo* (215). The greater safety margin of this drug is due to its reduced general and organotropic toxicity. Furthermore, leucotoxicity is less severe and more readily reversible.

Cyclophosphamide was originally synthesized as a reversible derivative of 2,2′-dichlorodiethylamine (nor-HN2, VI). Hindsight proved this assumption to be false based on subsequent work on this derivative.

On administration of cyclophosphamide to animals containing a variety of tumor systems, however, the cytotoxicity was significantly greater than with nor-HN2 (211, 296). A concerted effort (209, 210, 214, 215, 297–299) was undertaken to characterize the bioreversibility and metabolism pattern of this drug (Scheme V). Several of the products and metabolites possess cytotoxic activity comparable to the activity of administered cyclophosphamide. Recent studies with ^{14}C-labeled cyclophosphamide in mouse liver microsomal systems showed *N,N*-bis(2-chloroethyl)-phosphorodiamidic acid to be an active cytotoxic agent generated in this system (297, 298). The detection of acrolein as a product of microsomal oxidation suggested that metabolism of V proceeded *via* an open chain aldehyde to *N,N*-bis(2-chloroethyl)phosphorodiamidic acid (Scheme V). Montgomery *et al.* (160) treated in detail the metabolism of this drug and its various forms.

The example of cyclophosphamide and the implications apparent from a reversible derivative standpoint are profound. The relatively simple modification of a drug molecule (nor-HN2?) has gradually developed into an exceedingly complex and as yet unresolved problem in the determination of the mechanism of action of this important drug.

Table V illustrates selected examples of several classes of drugs that have been reversibly modified for utilization as site-directed and site-activated chemotherapeutic agents.

Depot—Both the physical and chemical approaches have proved useful in imparting depot activity to a drug. Ritschel (320) recently published a thorough review of the various synthetic and mechanical techniques for producing depot activity in parenterals.

The basic premise for depot activity is the localization of the drug derivative in some biological depot or site within the organism, with subsequent slow release from that site at a sufficient rate to provide the active form of the drug in therapeutically efficacious quantities. The duration of activity of such a drug depends upon its physical and chemical properties.

Two important considerations in rational design are: *(a)* the solubility of the derivative in the vehicle (*e.g.*, oleaginous) and the bulk aqueous phase (extracellular water at the site of administration) where the release rate is solubility controlled, and *(b)* diffusion of drug derivative from the vehicle where release is diffusion controlled.

Once the derivative is released (solubilized) at the site of administration, two factors become important: *(a)* regeneration of parent molecule at or near the site by slow, nonenzymatic hydrolysis on contact with body or tissue fluids, *e.g.*, repository sulfones; and *(b)* regeneration of the parent bioactive substance by enzymatic hydrolysis either at the site or in a central compartment (*e.g.*, blood). This sequence of events can be described by a consecutive first-order model (Scheme VI):

$$A \xrightarrow{k_a} B \xrightarrow{k_r} C \xrightarrow{k_e} D$$

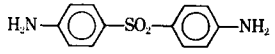
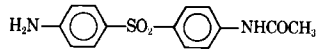
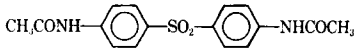
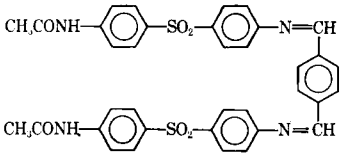
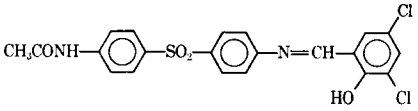
Scheme VI

where A represents the amount of drug at the injection site, B is the amount of derivative in the central body compartment (blood and rapidly equilibrating tissue), C is the amount of regenerated active species, and D is the amount of drug eliminated from the central compartment at any time. The terms k_a, the absorption rate constant, k_r, the derivative's regeneration rate constant, and k_e, the elimination rate constant, govern the drug transfer rate from the depot and central compartment.

Caldwell *et al.* (321) utilized this model in studying the depot properties of dihydrostreptomycin pamoate and in estimating k_a (absorption rate constant) for this salt. The plasma concentration–time plots for the sulfate ($t_{1/2}$ = 0.33 and 0.52 hr) and pamoate ($t_{1/2}$ = 3.46 hr) salts of dihydrostreptomycin are shown in Fig. 11.

The bioavailability of the parent molecule depends on the type and rate of hydrolysis of the reversible derivative. Elslager and coworkers (322–325) synthesized several bioreversible dapsone (diaminodiphenyl sulfone, 4,4′-sulfonyldianiline, DDS) derivatives designed to hydrolyze either enzymatically or nonenzy-

Table VII[a]—Comparative Antimalarial, Antileprotic, and Metabolic Data on Dapsone and Derivatives

Compound	Structure	Weeks Mice Protected		Rats[d]			
				Urinary Excretion			
		Plasmodium berghei[b]	*Mycobacterium leprae*[c]	% Excreted in 30 days	Estimated Half-Life, days	Peak Blood Level, μg/ml	Peak Methemoglobin Level, g/100 ml
Dapsone (DDS)		<1	2	57	9	13.8	3.9
4′-Sulfanilylacetamide (MADDS)		3.5	—	50	32	1.3	1.2
Acedapsone (DADDS)		12	>8	7	>200	0.2	0
4′,4‴-[*p*-Phenylene-bis(methylidyneimino-*p*-phenylenesulfonyl)]-bisacetanilid (PSBA)		5–7	>8	40	55	0.4	0.2
4′-[*N*-(3,5-Dichloro-salicylidene)sulfanilyl]-acetanilide (DSA)[e]		9	>8	31	35	0.7	1.0

[a] Adapted, with permission, from Ref. 324. [b] Estimated number of weeks 50% of mice were protected following a single subcutaneous 400-mg/kg dose of drug suspended in benzyl benzoate–castor oil (40:60). [c] First drug injection was 400 mg/kg given 58 days after infection with *M. leprae;* subsequent injections were 200 mg/kg at intervals of 0.5, 1, or 2 months. [d] Drugs given as single subcutaneous doses of 400 mg/kg in a volume of 5 ml/kg of 1.5% pectin and 0.1% Tween 60 in distilled water. [e] Reviewers' abbreviation.

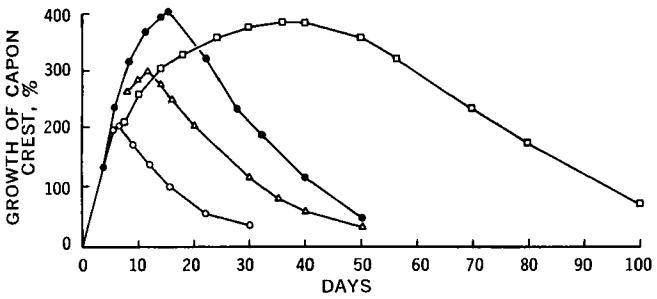


Figure 12—*Influence of ester type of testosterone on growth of capon crest. Key: ○, propionate ester; △, isobutyrate ester; ●, n-valerate ester; and □, undecylenate ester. (Adapted, with permission, from Ref. 327.)*

matically *in vivo*. A series of 4′-(*N*-acylsulfanilyl)anilides (322) was made to take advantage of their antimalarial and antileprotic activities in a repository form. The acyl group varied from hydrogen to palmityl, with a range of solubilities in pH 7 buffer solutions from 0.013 (hydrogen) to 0.0002 (palmityl) mg/ml. Optimum repository activity occurred with acedapsone (4′,4‴-sulfonylbisacetanilide, DADDS) at a solubility of 0.003 mg/ml. This derivative was slowly hydrolyzed by deacetylases to regenerate dapsone *in vivo*.

Other derivatives were designed to take advantage of nonenzymatic lability in body tissue and fluid and included a variety of 4′,4‴-[bis(amino-*p*-phenylenesulfonyl)]bisanilides (323) and 4′-[(*N*-aralkylidene-, benzylidene-, and naphthylidene)sulfanilyl]anilides (324). Of these latter compounds, 4′,4‴ - [*p* - phenylenebis (methylidyneimino-*p*-phenylenesulfonyl)] bisacetanilide (PSBA) and 4′-[*N*-(3,5-dichlorosalicylidene)sulfanilyl]acetanilide (DSA) proved noteworthy. Antimalarial and antileprotic activity data as well as approximate pharmacokinetic data for these derivatives are summarized in Table VII.

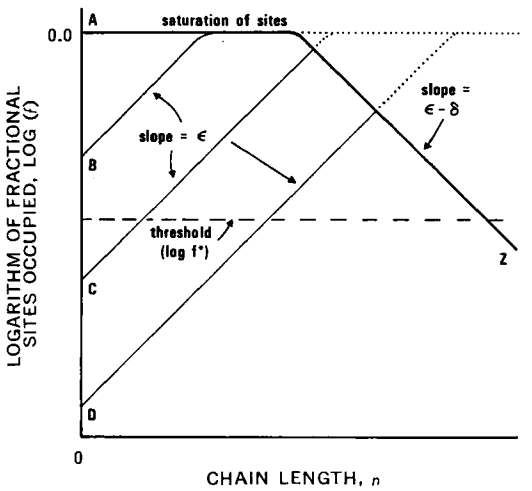


Figure 13—*Theoretical curves for the fractional occupancy of a perfect Langmuirian receptor phase as a function of alkyl chain length under the condition that* $S_0(k_1/k_{-1})_0 \gg 1$. *The heavy line, line A–Z, is the expected trend for saturated solutions. Profiles initiating at points B, C, and D are idealized profiles obtained using fixed concentrations* C_1, C_2, *and* C_3, *respectively, where* $S_0 > C_1 > C_2 > C_3$. *In such cases, log (f) increases linearly with chain length until the saturation limitation is obtained. Thereafter, the profiles are superimposed on the saturation profile. The dotted lines represent the continuing trends expected, neglecting the solubility restriction. "Parabolic" curves can be generated at a fixed concentration. The apex of the "parabola," the chain length of maximal activity, can be flat or broad, depending on concentration. Its position on the chain-length axis is also concentration dependent. (Reprinted, with permission, from Ref. 367.)*

Pinder (326) recently reviewed antimalarial drugs possessing depot and repository properties.

Steroids have been the subject of intense study as clinically acceptable forms of depot drugs. Steroidal bioactivity has been prolonged by forming esters of various lengths and various degrees of steric hindrance. Figure 12 illustrates the effect of chain length on duration of activity (the bioactivity of the parent steroid manifested as capon crest growth) for a series of testosterone esters (327). Winter and Porter (266) similarly correlated rates of absorption of several esters of hydrocortisone and 9α-fluorocortisone as a function of duration of activity.

Miescher *et al.* (328), in a classic study, demonstrated extended duration and bioavailability in a series of testosterone aliphatic esters. With the long chain esters (palmitate and stearate), the increased duration was probably due to delayed absorption (solubility rate controlled) and steric hindrance (slowed hydrolysis of ester). Rapala *et al.* (272) demonstrated a prolonged activity due to steric hindrance with substituted and nonsubstituted adamantoate esters of testosterone.

Various drug classes designed specifically as agents for depot therapeutic efficacy by use of the reversible derivative are listed in Table VIII.

Organoleptic Properties—*Taste*—Taste is a complex combination of sensations including gustation, olfaction, tactility, and responses to heat and cold. In humans, the sensation of taste is confined primarily to the dorsal surface of the tongue, the soft palate, the epiglottis, and parts of the gullet. In children, however, the taste receptors are distributed over larger areas of the mouth. Basically, there are four so-called primary tastes: sour, bitter, salty, and sweet. Others include astringent, metallic, and alkaline (soapy). Pfaffman (362) estimated that a human has 10^4 taste buds including taste nerves and other sensory nerves stimulated by foods. Each taste bud is innervated by from two to 10 nerve fibers with another 10^4 sensory fibers that serve as monitors for pain, temperature, tactility, and other stimuli.

Mechanism of Taste Receptor Stimulation—Electrophysiological measurements have been made by inserting microelectrodes into single taste cells and measuring the electric potential change between the inside and outside of the cell after stimulation with a chemical substance (363, 364). It was found that the molecular geography of the receptor surface changes after such stimulation, thus initiating a chain of events that ultimately manifests itself as a taste response.

Potassium ions, present in abundance within the cell, are released due to increased cell permeability near the associated nerve axon, and this phenomenon accounts for the electric potential change. This change initiates electrical nerve impulses of a few

Table VIII—Reversible Drug Derivatives Utilized as Modifiers of Duration of Activity (Prolonged, Depot Action)

Parent Molecule	Reversible Modification or Linkage	Route of Administration	Reference
Nicotinic acid	Dextran and carboxymethyldextran esters	Oral	329
Streptomycin	Alginic acid salt	Intramuscular	330
Dihydrostreptomycin	Pamoate salt	Intramuscular	321
Cytarabine (ara-C)	5′-Adamantoate ester	Intraperitoneal	331
Ara-adenosine (ara-A)	5′-Palmitate and 5′-benzoate esters	Intraperitoneal	332
Deoxyribonucleosides, ribonucleosides	5′-Adamantoate ester	Intraperitoneal	333
3-*o*-Toloxy-1,2-propanediol	Phenoxyacetate ester	Intraperitoneal	334
	Hemisuccinate ester	Intraperitoneal	335
Ampicillin	Dibenzylethylenediamine salt	Intramuscular	336
Substituted acetamidopenicillins	Carboxamido ester	Intramuscular	337
Amphotericin B	Methyl ester	Intraperitoneal	338
Neomycin	Dextran sulfate salt	Intramuscular	339
Fluphenazine	Heptanoate and decanoate esters	Intramuscular	340
Pipothiazine	Palmitate ester	Intramuscular	341–343
	Undecylenate ester	Oral	
Nandrolone	Phenylpropionate and decanoate esters	Intramuscular	265
Hydrocortisone	21-Alkyl esters	Local (topical)	266
Testosterone	17-β-Alkyl esters	Intramuscular	33, 272, 328, 344
6α-9α-Difluorocorticosteroid	Alkyl ortho esters	Intramuscular	345
Estradiol	17-Cycloalkenyl esters	Oral	346
	Oligomeric esters	Subcutaneous	347
Estradiol	Enol ethers and acetals	Oral, parenteral	267
Estradiol	Formate ester	Intramuscular	348
Nortestosterone	Terpenoate ester	Subcutaneous	349
Aldotestosterone, 1-dehydro-aldosterone	21-Benzoate ester	Oral, parenteral	350
Estrogen	3-Cyclopentyl ether	Parenteral	351
Betamethasone	21-Adamantoate ester	Oral, rectal, parenteral	352
Phendimetrazine	Pamoate salt	Oral	353[a]
Imipramine	Pamoate salt	Oral	354
Oleandomycin	Acetate, propionate ester	Oral	355
Aspirin (acetylsalicylic acid)	Alkyl carbonate ester	Oral	356
2-Phenylacetamido-, 2-amino-2-phenylacetamido-, phenylimidazolidinylpenicillins	*N*-Acyl-6-acylamino amide	Oral	337
Prostaglandin	2-(4-Imidazolyl)ethylamine salt	Oral	357
Dopamine	Amino acid amide	Oral	193
Chloramphenicol	Mono- and bis(trimethylsilyl) ethers	Parenteral	358
Dapsone (diaminodiphenylsulfone)	Acetamide	Intramuscular	359
	Formamide	Oral	360
Cycloguanil	Pamoate salt	Intramuscular	361

[a] This article tabulates a variety of pamoate salts for several classes of drugs.

milliseconds duration, with 100–200 impulses/sec. The intensity of the signal to the brain is a function of the number of nerve fibers activated and the impulse frequency.

Beidler (365) found that the magnitude of a taste response increases at a declining rate with concentration until further concentration increases fail to elicit a response. The relationship between concentration and the magnitude of a steady-state response is:

$$R = \frac{CKR_m}{CK + 1} \qquad \text{(Eq. 15)}$$

where C is the concentration of the stimulant, R is the electrical response magnitude, R_m is the maximal response magnitude at saturation, and K is the reaction equilibrium constant of stimulus–receptor. Plotting R *versus* C affords a sigmoidal curve, the middle portion of which can be utilized for comparisons between concentration and response. The utility of the comparison is invalid at low and high concentrations. Other studies provided evidence of weak substrate adsorption to the taste receptor surface (366).

When suspensions are considered, simply replace C by S for drug solubility. The effects of a homologous series on adsorption onto receptors was studied recently (367). In an analysis similar to that presented for absorption (see section on absorption), it was shown that for subsaturated solutions the response increases with alkyl chain length until R reaches R_m. It was also shown that for suspensions there can be an increased response with chain length for the lower homologs. This results from the fact that the value of K, which is essentially a Langmuirian adsorption constant, increases as a homologous series is ascended. However, as the series becomes highly hydrophobic, the decrease in solubility that accompanies chain extention overshadows the increase in the adsorption constant and the response begins to diminish with chain length until it drops below the threshold value. These relationships are summarized in Fig. 13.

Duncan (368) postulated that taste stimulation may be due to enzymatic mediation. This contention was disputed by Beidler (369) who cited lack of experimental evidence for this thesis plus the fact that such interactions are highly pH dependent whereas a taste response elicited by sodium chloride, for in-

Table IX—Reversible Drug Derivatives Utilized as Modifiers of Taste and Odor Properties

Parent Molecule	Reversible Modification or Linkage	Property Modified	Reference
Pivampicillin	Probenecid salt	Bitterness	378
Tetracycline	3,4,5-Trimethoxybenzoate salt	Bitterness	379
Propoxyphene	2-Naphthalenesulfonic acid salt	Bitterness	380
	4-Chloro-*m*-toluenesulfonic acid salt and 3,4-dichlorobenzenesulfonic acid salt	Bitterness	381
Oleandomycin	Acyl ester/*N*-oxide	Bitterness	355
Chloramphenicol	Palmitate ester	Bitterness	221
	α-Haloalkyl ester, phenyl ester	Bitterness	382
	Phosphite ester	Bitterness	383
	(Trimethylsilyl) ether		358
Lincomycin	Alkyl ester	Bitterness	10–12
	Phosphate ester	Bitterness	175
Clindamycin	Alkyl ester	Bitterness	13
Erythromycin	Alkyl ester/salt	Bitterness	238
	Alkyl ester	Bitterness	14, 384, 385
Ethyl mercaptan	Phthalate ester	Odor	386, 387
Trichloroethanol	Carbonate ester	Odor	16
N-Arylanthranilic acid	Glyceryl ester	Bitterness	388

stance, can occur between pH 3 and 11. He felt that the initial response was electrochemical in nature and due to creation of a disturbance in the surface cell geography with subsequent depolarization of the receptor cell.

Molecular configuration of the drug molecule plays an important role in adsorption efficiency at the receptor level. Saccharin has a taste threshold approximately $\frac{1}{500}$ that of sugar, whereas *N*-methyl-, ethyl-, or bromosaccharin is tasteless (369). Analogously, the magnitude of a bitter taste response may be modified by changing the molecular configuration of the parent molecule. Such is the case for clindamycin (parent molecule) and a series of clindamycin 2-esters (reversible derivative) (13). Solubility, as already mentioned, may play an important role in decreasing the intensity of a bitter response.

A combination of factors is perhaps operative in the demonstration of a taste response. Bitterness of a molecule, for example, may be due to the efficiency of the taste receptor–substrate adsorption reaction, which is related to the molecular geometry of the substrate. If alteration of the parent molecule occurs by derivative formation, the geometry is altered, affecting the adsorption constant. This effect, in turn, acts in concert with the aqueous solubility (or lack of such) of the derivative to eliminate the bitter taste response. Beidler (369) and Moncrieff (370) published reviews on taste theory.

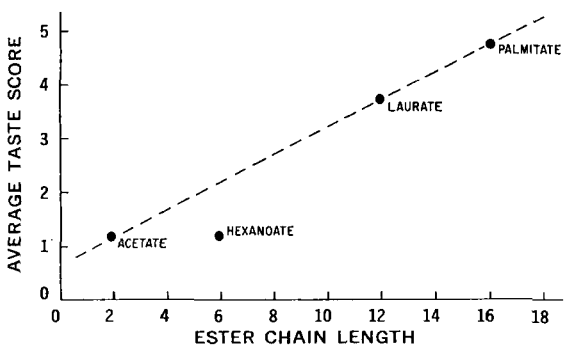


Figure 14—*Effect of clindamycin ester chain length on taste of ester in syrup. (Reprinted, with permission, from Ref. 13.)*

Odor—Odor, another organoleptic property amenable to reversible derivatization, plays an important role in the quality and intensity of taste perception. The magnitude of an olfactory response is related to concentration (371) and can be measured by recording the electrical nerve impulses generated by the olfactory receptors when they are stimulated by an odor (372). Olfactory receptor sensitivity is well known and probably depends on the adsorption of the stimulus molecule to the surface of the receptor. Certain insects can respond to concentrations of less than 10^{-17} *M* (373).

The nature of the functional group and the shape of the molecule, in conjunction with volatility, determine the intensity and duration of such adsorption. Studies (365) have been successfully applied to olfaction using the amyl acetate olfactory response (372). Several reviews and papers have been written correlating odor and molecular constitution (374–376), stereochemistry (377), and physical variables (372).

Taste Studies—The extremely bitter antibiotics have been the focus of much work in reversible drug modification. A study of clindamycin was undertaken to provide derivatives that could suitably be utilized as a tasteless pediatric form of this antibiotic (13). A series of 2- and 3-monoesters and several 2,3-biscarbonate esters was made. Four clindamycin 2-acyl esters of varying chain length (palmitate, laurate, hexanoate, and acetate) were dissolved in 30% sucrose solutions. The clindamycin ester solutions were coded A, B, C, and D, and samples of each were given to a taste panel of 26 people, with 1 hr allowed between each sample. The taste of each was rated on a scale of 1–9 (1 = poor, 9 = excellent). The raw score data were analyzed by computer to show any significant differences between samples.

The average scores (Fig. 14) indicate a linear trend of taste improvement with increasing chain length. The palmitate ester was significantly better than the laurate at the 5% level of confidence. The laurate, in turn, was significantly better than both the hexanoate and the acetate. The latter two compounds

Table X—Reversible Derivatives Utilized as Modifiers of Irritation on Injection

Parent Molecule	Reversible Modification or Linkage	Route of Administration	Reference
Chloramphenicol	1-Hemisuccinate sodium salt	Intramuscular, intravenous	169, 400
	1,3-Bishemisuccinate disodium salt or piperazine salt	Intramuscular	
Oleandomycin	Acetate, propionate esters, *N*-oxide	Intravenous, intramuscular	355
Clindamycin	Phosphate ester	Intramuscular	391, 398, 399

were ranked equally low, being very bitter tasting.

Clindamycin 2-palmitate was found to be devoid of the characteristic bitter taste of clindamycin.

Table IX illustrates several types of drug molecules that have been reversibly modified to eliminate offensive taste and odor properties inherent in the parent drug molecule.

Irritation—*Intramuscular or Intravenous Injection*—Perhaps the most effective yet intimidating mode of drug administration is *via* the parenteral route. It is efficacious in the sense that it provides an immediate therapeutic dose of medicament to those patients not able to self-administer medication such as infants and hospitalized and comatose patients. Furthermore, the frequency of dosing may be reduced by utilizing an injectable depot or repository form of the drug (see *Depot* discussion).

Patient reluctance develops, however, when an initially painful intravenous or intramuscular injection must be administered in a multiple-dose regimen. Such pain is usually accompanied by hemorrhage, edema, inflammation, and tissue necrosis (389). The intensity of pain due to injectable substances has been evaluated by use of tenderness profiles (390) and elevation of enzyme levels such as serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), and creatine phosphokinase (CPK)(390–392). Ballard (393) tabulated a variety of local reactions caused by the injection of several drug solutions and suspensions.

Several factors apparently influence the occurrence, intensity, and duration of pain generated by an injection. Among these, the most important are considered to be the solubility of drug in an aqueous medium, viscosity, pH, tonicity of the injected drug solution, volume injected, site of injection, pain tolerance of the patient, and technique of administration (394). Other factors include precipitation of drug at the injection site (391, 393, 395–397) and localized cell lysis (391).

The approach usually taken by the product formulation specialist is to add a local anesthetic to the product to reduce pain by blocking the sensory nerves around the injection site. A more rational approach involves the reversible modification of certain physicochemical properties of the offending parent drug to eliminate such pain and irritation. A number of drug molecules have been reversibly modified in such a manner to alleviate these frequently bothersome side effects (Table X).

A good example of this type of modification involves clindamycin hydrochloride, a semisynthetic antibiotic used in the treatment of infections due to sensitive Gram-positive organisms. This drug is irritating when administered by intramuscular or intravenous injection. It was postulated that this irritation might result from precipitation of the clindamycin free base at the injection site or from rapid partitioning of drug into tissue cells surrounding the injection site, resulting in cell lysis (391). Both mechanisms may be important since the aqueous solubility of the hydrochloride salt is more than 100 mg/ml at pH 6 but is only 3 mg/ml at physiological pH (~7.4), thus supporting the precipitation hypothesis.

Additionally, clindamycin free base has a partition coefficient of 1.85, which should favor passage of this species through a lipoidal barrier or membrane. At physiological pH, the free base form (pKa = 7.6) would be present at about one-half of the injected dose, lending credibility to the cell partitioning hypothesis.

The synthesis (398) and subsequent intramuscular testing (399) of clindamycin phosphate indicated that this reversible derivative of clindamycin was virtually devoid of the irritation exhibited by the parent antibiotic. There were no signs of local intolerance, and intravenous infusions of the ester produced no superficial thrombophlebitis or irritation. The phosphate ester is intrinsically inactive against bacteria but is rapidly hydrolyzed to clindamycin *in vivo*. Other studies designed to characterize the intramuscular absorption, distribution, and excretion of this derivative indicated excellent bioavailability of the derivative from the injection site as the parent antibiotic (173).

Figure 4 illustrates the findings of a multiple-dose study utilizing clindamycin and the 2-phosphate ester. The lower curve represents clindamycin, and the upper curve represents both clindamycin and clindamycin 2-phosphate. It can be seen that ester

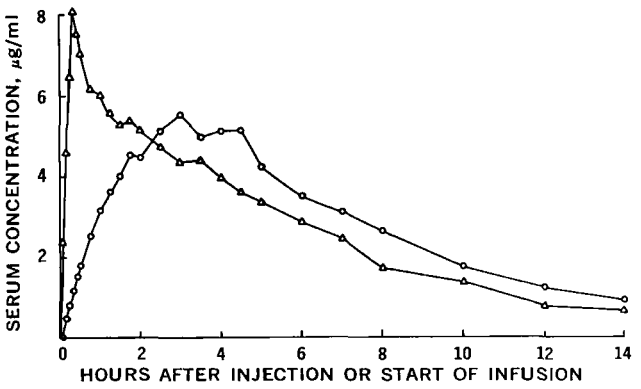


Figure 15—*Serum concentration (micrograms per milliliter) of clindamycin after 600 mg iv (△) and 600 mg im (○) clindamycin phosphate in the same subject. (Adapted, with permission, from Ref. 173.)*

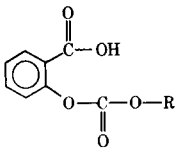


Table XI[a]—Aspirin and Carbonate Esters of Salicylic Acid: Solubilities, Partition Coefficients, and Hydrolysis Rates

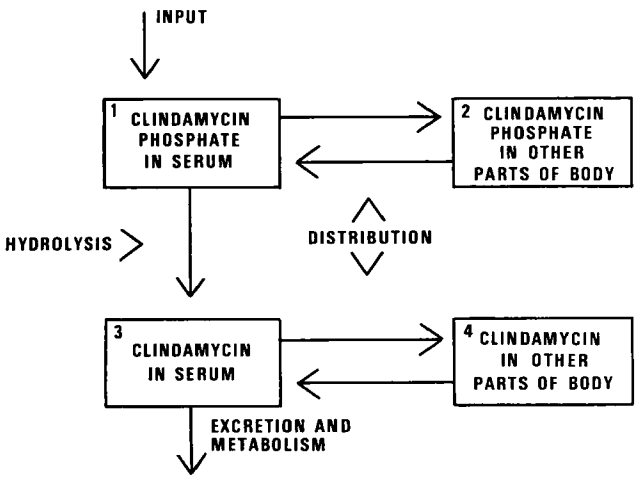
R	Solubilities, mg/ml, 37°		Cyclohexane–0.1 *N* HCl Partition Coefficient, 25° [b]	Half-Lives for Hydrolysis, 37°				
				Phosphate Buffer (0.1 *M*)		Enzyme in Phosphate Buffer (pH 7.4, 0.1 *M*)[c]		
	0.1 *N* HCl[b]	Cyclohexane		pH 7.4, hr	pH 12, min	2% Human Plasma, hr	0.05% Human Pseudocholinesterase, hr	0.05% α-Chymotrypsin, hr
Ethyl	6.7	1.1	0.17	41	3.8	13.3 (3.1)	7.4 (5.5)	53 (—)
n-Butyl	2.8	2.7	1.0	29	4.5	11.7 (2.5)	1.23 (24)	1.38 (21)
n-Hexyl	0.28	5.6	20	15	4.8	8.2 (1.8)	1.33 (11)	0.25 (58)
Trichloroethyl	1.1	0.24	0.22	0.53	1.1	0.35 (1.5)	—	—
Aspirin	5.3	0.06	0.05	10	1.5	21 (—)	3.3 (3)	6.3 (1.6)

[a] Adapted, with permission, from Ref. 276. [b] The 0.1 *N* HCl was used in these experiments to assure that the compounds were completely unionized in the aqueous phases and to reduce the degree of hydrolysis in the aqueous phase during equilibration. [c] The numbers in parentheses are $t_{1/2}$ buffer (pH 7.4)/$t_{1/2}$ enzyme ratios, which represent the degree of enzymatic catalysis.

hydrolysis was virtually complete at the 4-hr sampling time.

A more precise characterization of serum concentration–time profiles of the ester was determined by dosing intramuscularly and intravenously (Fig. 15). Ester hydrolysis occurred more rapidly with intravenous administration, and intramuscular administration showed both slower absorption and decreased peak concentration of the parent antibiotic. The implications concerning the route of administration for such a reversible derivative are important in life-threatening infections, the intravenous route being indicated.

Few studies are available in which pharmacokinetic principles have been applied to reversible drug derivatives designed for injectable purposes. Again, the studies on clindamycin phosphate are illustrative (173). A four-compartment model was used to explain much of the data generated in the study (Scheme VII). The data show that clindamycin phosphate is rapidly hydrolyzed in serum and that the parent drug is widely distributed after both intravenous and intramuscular administration. Equilibrium is reached after the third dose in a multiple-dose regimen, and serum levels of clindamycin are readily predictible.



Scheme VII—Four-compartment pharmacokinetic model for clindamycin phosphate. (Adapted, with permission, from Ref. 173.)

GI Disturbances—It has long been known that a variety of drugs can cause a wide spectrum of GI disturbances, ranging from mild gastritis to severe ulceration (401–404). How these phenomena occur is still a matter of speculation, but several modes of action have been postulated:

1. Injury to gastric mucosa through direct contact of drug to produce a localized irritating necrotizing effect.
2. Damage due to circulating drug that stimulates increased gastric secretion. [It has been shown, however, that this mechanism is nonoperative for aspirin in human subjects and animals and, in fact, may lower gastric secretory activity (405, 406), but it may be operative for other gastroirritant drugs.]
3. Interference with the mechanism(s) responsible for maintaining the integrity of the GI mucous or "mucous barrier."

This barrier, consisting of glycoprotein, is relatively resistant to proteolytic enzyme activity and acts as a protective boundary to underlying epithelial tissue.

Table XII[a]—Toxicity, Gastric Irritability, and Potency of Salicylic Acid Hexylcarbonate and Acetylsalicylic Acid (Aspirin)

	Salicylic Acid Hexylcarbonate	Acetylsalicylic Acid
LD_{50} in mice[b]	10.1	7.0
LD_{50} in rats[b]	13.0	7.6
Incidence of gastric erosions in rats with equimolar doses, %	0	90
Incidence of gastric irritation in dogs with equimolar doses, %	0	100
Antipyretic activity	Equal potency	
Analgesic activity	Equal potency	
Anti-inflammatory activity	Equal potency	

[a] Data from Ref. 277. [b] Average acute oral toxicity in moles/kg × 10^{-3}.

Table XIII—Reversible Drug Derivatives Utilized as Modifiers of GI Irritability

Parent Molecule	Reversible Modification or Linkage	Property Modified	Reference
Salicylic acid	Carbonate ester	Gastric irritation and hemorrhage	276, 277
Aspirin (acetylsalicylic acid)	4-Acetamidophenyl ester	GI blood loss	233
Aspirin (acetylsalicylic acid)	Alkyl ester	GI irritation	417
	Cyano ester		
Aspirin (acetylsalicylic acid)	Carbonate anhydride ester	GI bleeding	356
Nicotinic acid	Hydrazide	GI irritation	409
	Hydroxamic acid		
N-Arylanthranilic acid	Glyceryl ester	GI ulceration	388
21-Hydroxysteroid	Phosphate and sulfate ester	Gastric hemorrhage	418
Oleandrin	Acetate ester	GI irritation	415, 416
Gitoxin	Pentaacetate ester	GI irritation	414
Prostaglandin	Ethyl ester	Diarrhea	413
4,4′-Dihydroxydiphenyl-(2-pyridyl)methane	Alkyl ester	Gastric irritation	411, 412
	Sulfate ester		
Trichloroethanol	Carbonate ester	Stomach irritation, nausea, vomiting	16
	Phosphate ester		410
	4-Acetamidophenyl ester		17

The systemic administration of cortisone and aspirin alters the composition of this barrier in a way that decreases its protective ability (407, 408). This may be due to cellular injury resulting in decreased mucous production and increased ionic flux.

A study of the absorptive properties of a series of organic acids, including acetylsalicylic acid (aspirin), through the mucosal barrier found increased fluxes of hydrogen, chloride, sodium, and potassium ions after administration of these acids; these increases persisted after removal of the compounds (402). Evidence of bleeding was also apparent. Diffusion of acetic acid, which is unionized at gastric pH, through the gastric barrier proceeded more rapidly than diffusion of hydrochloric acid (ionized), implying that unionized hydrophobic molecules are more readily and efficiently absorbed.

In an effort to eliminate gastric irritation produced by salicylic acid, a series of hydrophobic carbonate esters of this important analgesic was prepared (276). The rationale for utilizing carbonate esters of this drug lies in the fact that they are readily hydrolyzed *in vivo* and might possess hydrophobic properties adequate to allow absorption and distribution over a greater area of the GI tract, thus reducing localized irritation. Several esters were synthesized and characterized according to their physicochemical properties (Table XI).

Based on partitioning studies and relative ease of hydrolysis (half-life) in pH 7.4 buffer and enzyme media, the hexylcarbonate ester of salicylic acid was chosen for further study. Blood level studies in dogs indicated that this ester was absorbed more slowly than acetylsalicylic acid and the other carbonate esters. After 2 hr, however, the plasma concentration–time profiles did not differ for any of the esters. A comparison of the LD_{50} values, gastric irritability, and bioactivity of salicylic acid hexylcarbonate *versus* acetylsalicylic acid (aspirin) in animals indicated significant differences in toxicity between these drug derivatives (277) (Table XII).

This study (276) stressed to the medicinal chemist the importance of performing selected physicochemical studies (*e.g.*, solubility and partitioning) and *in vitro* tests (half-lives in a variety of buffers and enzyme-rich milieu) to evaluate initially a series of reversible derivatives based on a preliminary rationale for making such derivatives. It is obvious that when such studies are performed, the choice of primary and backup candidates for further testing becomes less difficult.

Other experiments have been designed and carried out with the foregoing rationale as their basis, and the results of several of these studies are summarized in Table XIII.

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Synthesis and Antimalarial Activity of Heterocyclic Alkyl Disulfides, Thiosulfates, and Dithio Acid Derivatives

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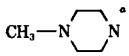
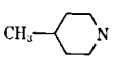
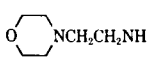
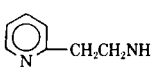
Abstract □ Based on the antimalarial activity in mice of bis(4-*p*-acetamidobenzenesulfonamidophenyl) disulfide, a series of *N*-heterocyclic alkyl disulfides and thiosulfates was synthesized and screened for antimalarial activity. Several related dithio acid dianions and *S*-blocked derivatives were also screened to provide an indication of the possible role that thiol anions might play in malaria chemotherapy. Activity was limited by toxicity with these compounds, and none of those tested, with the exception of bis(4-*p*-acetamidobenzenesulfonamidophenyl) disulfide, showed curative activity in either a mouse or chick test.

Keyphrases □ Heterocyclic alkyl disulfides, thiosulfates, and dithio acid derivatives—synthesis and antimalarial activity □ Disulfides, heterocyclic alkyl—synthesis and antimalarial activity □ Thiosulfates, heterocyclic alkyl—synthesis and antimalarial activity □ Dithio acid derivatives—synthesis and antimalarial activity □ Antimalarial agents, potential—synthesis, screening of heterocyclic alkyl disulfides, thiosulfates, dithio acid derivatives

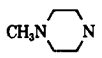
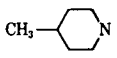
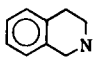
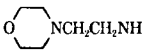
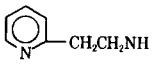
The ability of the disulfides of aminoalkylthiols to bind reversibly to DNA, RNA, and other nucleoproteins has been known for some years (1). The discovery of this ability led to a postulation (2), in regard to radiation protection, that many radioprotective bis(aminoalkyl) disulfides would fit those areas of the DNA helix uncovered by histones and undergo complex formation. The resulting complexes were believed to aid in restorative effects, one being a decrease in rate or temporary cessation of DNA replica-

Table I—*N*-Heterocyclylethanethiols $R-CH_2-CH_2-SH$

R	Boiling Point (mm)	n_D^{25}	Yield, %	Formula	Analysis, % Calc.	Found
	59–60° (1)	1.5061	78	$C_7H_{16}N_2S$	—	—
	45–46° (1)	1.5015	83	$C_7H_{15}NS$	—	—
	73–75° (4)	1.5053	82	$C_6H_{13}NOS$	—	—
	127–129° (1.5)	1.5783	73	$C_{11}H_{15}NS$	C 68.29 H 7.76 N 7.25 S 16.59	68.62 7.51 7.40 16.32
	35–36° (1.5)	1.5033	90	$C_6H_{13}NS$	C 54.89 H 10.00 N 10.67 S 24.43	55.05 9.97 10.16 24.16
	73° (3.5)	1.5010	78	$C_8H_{17}NS$	C 60.31 H 10.79 N 8.79	60.00 10.50 8.35
	58–59° (1.5)	1.4938	80	$C_8H_{17}NS$	C 60.31 H 10.79 N 8.79	59.90 10.60 9.20
	67–71° (3.5)	1.4957	50	$C_8H_{17}NS$	C 60.31 H 10.79 N 8.79	60.50 10.90 8.46
	122–124° (1.5)	1.5130	71	$C_8H_{18}N_2OS$	C 50.48 H 9.55 N 14.72	50.37 9.91 14.71
	130–132° (0.3)	1.5676	63	$C_9H_{14}N_2S$	C 59.89 H 7.76 N 15.37	60.14 7.85 15.59

[a] Reference 5.

Table II—*N*-Heterocyclylethyl Disulfides R–CH_2–CH_2–S–S–CH_2–CH_2–R

R	Salt	Melting Point	Yield, %	Formula	Analysis, % Calc.	Found
	4HCl	254–255°	24	$C_{14}H_{30}N_4S_2 \cdot 4HCl$	C 36.21 H 7.41 N 12.06 S 13.79	35.99 7.46 12.22 14.14
	2HCl	270–271°	54	$C_{14}H_{28}N_2S_2 \cdot 2HCl$	C 46.53 H 8.37 N 7.75 S 17.73	46.53 8.18 7.92 17.42
	2HCl	229–230°	60	$C_{12}H_{24}N_2O_2S_2 \cdot 2HCl$	C 39.44 H 7.19 N 7.66 S 17.55	39.54 7.17 7.42 17.48
	2HCl	227–228°	56	$C_{12}H_{24}N_2S_2 \cdot 2HCl$	C 43.22 H 7.88 N 8.40 S 19.23	42.88 7.62 8.20 19.45
	2HCl	235–237°	59	$C_{16}H_{32}N_2S_2 \cdot 2HCl$	C 49.31 H 8.81 N 7.19	49.58 8.71 7.46
	2HCl	239–240°	73	$C_{16}H_{32}N_2S_2 \cdot 2HCl$	C 49.31 H 8.81 N 7.19	49.66 8.59 7.34
		n_D^{25} 1.6064	92	$C_{22}H_{28}N_2S_2$	C 68.59 H 7.54 N 7.54 S 16.67	68.26 7.39 7.49 16.88
		1.5365	64	$C_{16}H_{32}N_2S_2$	C 60.69 H 10.21 N 8.85 S 20.25	60.4 10.5 9.05 19.59
		1.5376	62	$C_{16}H_{34}N_4O_2S_2$	C 50.75 H 9.07 N 14.80 S 16.93	50.3 9.3 14.7 16.5
		1.5918	43	$C_{18}H_{26}N_4S_2$	C 59.62 H 7.24 N 15.46 S 17.68	59.5 7.02 15.09 17.99

tion, allowing repair to be effected before alterations were replicated. It is possible that this postulation may be of value in designing agents effective against malaria parasites, particularly where such compounds may bind more strongly to DNA and halt the replication process.

DISCUSSION

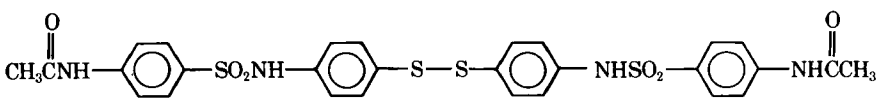
One compound, bis(4-*p*-acetamidobenzenesulfonamidophenyl) disulfide (I), has already been reported (3) that might act through such a mechanism. This compound effected a cure in one of five mice infected with *Plasmodium berghei* when administered at a toxic level (640 mg/kg); subsequent testing data are reported here. To test this idea more thoroughly, a series of *N*-substituted bis(aminoalkyl) disulfides was synthesized. These compounds carry heterocyclic rings that should increase binding ability to nucleic acids.

In addition, to determine whether compounds of this type act through the sulfur function as a thiolating agent, similar structures having thiosulfate groups in place of disulfide, as well as a few dithio acid dianions, were prepared for comparison of antimalarial activity. A thiosulfuric acid derivative of quinine, in which the thiosulfate group is located a distance of two carbons from the basic nitrogen, was recently synthesized and found to have greater activity against *P. berghei* KBG 173 in mice than did quinine (4).

Compounds of the type Het—CH_2CH_2SX were synthesized, where Het refers to a reduced nitrogen-containing heterocyclic ring and X to either hydrogen, a disulfide, or thiosulfate function. The method employed for the mercaptans was mercaptoethylation of the heterocyclic nitrogen using ethylene monothiocarbonate (5). Use of an excess of amine minimized polymerization of ethylene sulfide. All mercaptans obtained showed the loss of NH-stretching peaks and the presence of the weak SH-stretching peak at 2550–2600 cm^{-1}. Yields ranged from 70 to 90%. Physical data are listed in Table I.

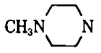
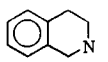
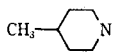
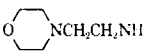
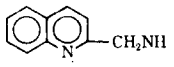
Disulfides were prepared by oxidation of the mercaptans with dimethyl sulfoxide and were generally isolated as the dihydrochlorides. The disulfide dihydrochlorides were obtained as oils, which were crystallized by trituration under anhydrous ether or ethanol. In a few cases the oils could not be crystallized, and the compounds were isolated as the free amines. All disulfides showed loss of the SH-stretching peak in the IR and gave positive nitroprusside tests for a disulfide. Yields ranged from 24 to 92%. Physical data are listed in Table II.

Synthesis of the thiosulfuric acids was accomplished by reaction of the heterocyclic alkyl halides with either sodium or thallous



I

Table III—*N*-Heterocyclylethanethiosulfuric Acids $R-CH_2-CH_2-S_2O_3H$

R	Melting Point	Yield, %	Formula	Analysis, % Calc.	Found
	186–187.5°	24	$C_7H_{16}N_2O_3S_2$	C 34.98 H 6.68 N 11.66 S 26.68	34.51 7.44 11.16 26.25
	180–182°	40	$C_7H_{15}NO_3S_2$	C 37.31 H 6.72 N 6.21 S 28.41	37.32 6.70 6.29 28.20
	181–183°	61	$C_6H_{13}NO_4S_2$	C 31.70 H 5.78 N 6.16 S 28.21	32.01 5.70 6.18 28.42
	165–167°	28	$C_6H_{13}NO_3S_2$	C 34.10 H 6.21 N 6.63 S 30.34	34.24 6.19 6.60 30.40
	154–155°	54	$C_{11}H_{15}NO_3S_2$	C 48.32 H 5.54 N 5.12	48.05 5.53 4.92
	162.5–164°	35	$C_8H_{17}NO_3S_2$	C 40.14 H 7.17 N 5.81 S 26.79	40.50 7.31 6.06 26.49
	173–174.5°	62	$C_8H_{17}NO_3S_2$	C 40.14 H 7.17 N 5.81 S 26.79	40.42 7.20 6.04 26.58
	185–185.5°	31	$C_8H_{17}NO_3S_2$	C 40.14 H 7.17 N 5.81 S 26.79	39.89 7.43 5.65 26.82
	158–160°	30	$C_8H_{18}N_2O_4S_2$	C 35.52 H 6.71 N 10.36 S 23.71	35.4 6.8 10.2 23.87
	187–188°	57	$C_{12}H_{14}N_2O_3S_2$	C 48.30 H 4.73 N 9.39	48.3 4.8 9.3

thiosulfate or by reaction of either the mercaptan or disulfide with potassium metabisulfite. 2-(2-Quinolylmethylamino)ethanethiosulfuric acid and 2-(2-*N*-morpholinylethylamino)ethanethiosulfuric acid were prepared by treating 2-chloromethylquinoline and *N*-(2-chloroethyl)morpholine, respectively, with 2-aminoethanethiosulfuric acid. The morpholine derivative was also prepared from the thiol by reaction with potassium metabisulfite. The thiosulfates were generally water soluble and could be recrystallized. Characteristic peaks near 1020, 1170, and 1230 cm^{-1} (6) were exhibited by all thiosulfates; yields ranged from 24 to 63%. Physical data for the thiosulfuric acids are listed in Table III.

The dithio acid dianions were prepared as the dipotassium salts using the procedure of Foye and Kauffman (7), which employed the condensation of carbon disulfide with substituted acetonitriles with *tert*-butoxide as the condensing agent. None of these compounds could be recrystallized or even reprecipitated. One of the more stable of these dimercaptoacrylonitriles, the 2-phenyl derivative, was converted to the 4-phenyl-4-hydroxydithiolane-2-ylidene on condensation with α-bromoacetophenone, using the procedure of Campaigne and Haaf (8). This compound was dehydrated to the corresponding 1,3-dithiole in sulfuric acid at 0°, and the product provided an *S*-blocked derivative of a dithio acid dianion. Other derivatives of 2-cyano-3,3-dimercaptoacrylonitrile in which the sulfur function was blocked, *e.g.*, with methyls or a 2,3-pyrazinyl group, were prepared for comparison of antimalarial activities. The corresponding quinoxaline derivative was also prepared using 2,3-dichloroquinoxaline.

Antimalarial testing was carried out against *P. berghei* in mice according to the method of Osdene *et al.* (9). Results were supplied by the Walter Reed Army Institute of Research. The lead compound for this series, bis(4-*p*-acetamidobenzenesulfonamidophenyl) disulfide (I), was described as curative in this test. A sulfone which was submitted, 2-amino-5-thiazolyl-4-nitrophenylsulfone (II), was found to be active.

Among the disulfides tested, the *N*-pyrrolidylethyl (V) and *N*-3-methylpiperidylethyl (VII) disulfides showed differences in mean survival times from those of the controls (which survived 6.1 days) of 5.3 and 5.8 days, respectively. The corresponding thiosulfuric acids showed mean survival time differences of only 1.3 and 0.2 day, respectively. The other disulfides and thiosulfuric acids, as well as the dithio acid dianions and their *S*-blocked derivatives, showed very little difference in mean survival time over that of the controls (Table IV).

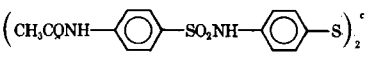
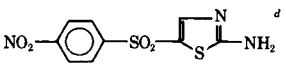
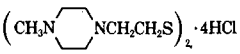
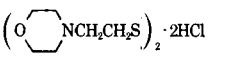
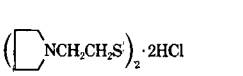
Several compounds were also evaluated for prophylactic action in chicks against *P. gallinaceum* according to the procedure of Rane and Rane (10). This test was uniformly fatal to untreated controls in 72–96 hr. In this test the disulfide (I), curative in the mouse test, showed a difference in mean survival time over controls of only 0.5 day, and the active sulfone (II) gave a mean survival time difference of only 0.4 day (Table V).

EXPERIMENTAL[1]

***N*-Heterocyclylethanethiols**—The cyclic amines were dried over calcium hydride and filtered prior to use. To a refluxing solution of the amine (0.3 mole) in 100 ml of anhydrous toluene was

[1] Melting points were determined with a Mel-Temp capillary melting-point block and are uncorrected. IR data were obtained using a Perkin-Elmer model 137B spectrometer with sodium chloride optics. NMR spectra were obtained with a Varian A-60 spectrometer using tetramethylsilane as the external or internal standard. Elemental analyses were done by F. B. Strauss, Oxford, England; Carol K. Fitz, Carlisle, Mass.; or Russell J. Werby, Boston, Mass. TLC was carried out using silica gel, and products were detected by exposure to iodine vapor. The heterocyclic amines were obtained from Aldrich Chemical Co., Eastman Organic Chemicals, or Fisher Scientific Co.

Table IV—Antimalarial Activities in Mice[a]

Number	Compound	Dose, mg/kg	Difference in Mean Survival Time, days (Test Minus Control)[b]
I		40	0.5
		80	4.1
		160	8.4 (a) (t,1)
		320	9.9 (a) (t,1)
		640	20.9 (c,1) (t,3)
II		40	2.3
		80	4.9
		160	6.1 (a)
		320	9.5 (a)
		640	12.9 (a)
III		640	0.4
IV		160	0.8 (t,3)
V		20	0.6
		40	4.0
		80	4.4
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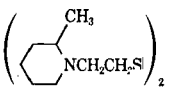
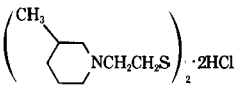
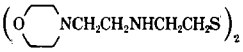
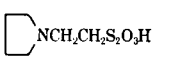
VI		160	0.0
VII		40	0.6
		80	1.2
		160	4.0
		320	5.8
VIII		160	0.0
IX		320	0.0
X		20	0.4
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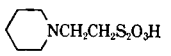
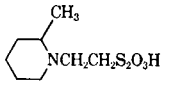
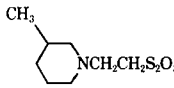
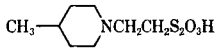
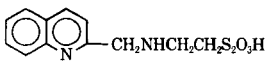
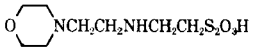
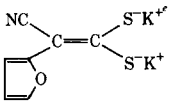
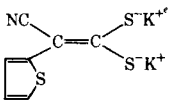
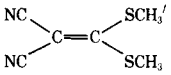
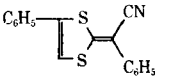
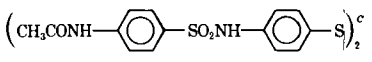
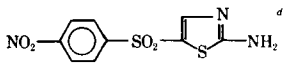
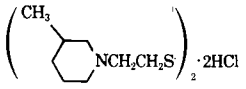
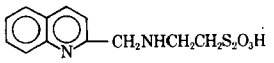
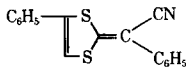
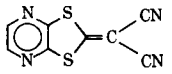
XI		160	0.8 (t,3)
XII		160	0.2
XIII		160	0.2
XIV		160	0.4
XV		160	0.4
		320	0.9 (t,3)
XVI		40	0.3
		80	0.9 (t,3)
XVII		320	0.1
XVIII		320	0.5
XIX		40	0.0 (t,5)
XX		160	0.3
		320	1.1

[a] The compounds were tested against *P. berghei* KBG 173 by administration to five male mice at a given concentration in a single subcutaneous dose 72 hr after infection. [b] The mean survival time of the controls was 6.1 days. Deaths during Days 2–5 after drug administration were attributed to toxicity (t, number that died). Compounds were classified as active (a) when the mean survival time of the treated mice was twice that of the controls, *i.e.*, test − control > 6.1 days, and curative (c, number of surviving mice) when test animals lived 60 days postinfection. [c] Reference 3. [d] Source: Aldrich Chemical Co. [e] Reference 7. [f] Reference 12.

Table V—Antimalarial Activities in Chicks[a]

Number	Compound	Dose, mg/kg	Difference in Mean Survival Time, days (Test Minus Control)[b]
I		100	0.5
II		30 60 120	0.4 0.4 0.4
VII		160	0.0
XV		240	0.0
XX		160	0.0
XXI		120	0.4

[a] The compounds were tested against *P. gallinaceum* Brumpt 8A injected into five chicks from heart blood of infected chicks. The compounds, suspended in peanut oil, were administered subcutaneously in a single dose immediately after infection. [b] The mean survival time of the control chicks was 72–96 hr. Deaths that occurred within 48 hr after infection were considered due to toxicity of the compound (t, number that died). An increase of 100% in survival time was recognized as the minimum effective response. Chicks with survival times of 30 days were recorded as cured (c, number of survivors). [c] Reference 3. [d] Source: Aldrich Chemical Co.

added 0.1 mole of ethylene monothiocarbonate dropwise over 20 min. The resulting solution was allowed to reflux overnight, and it was distilled *in vacuo* through a 17.7-cm (7-in.) vigreux column to yield a colorless oil. Physical constants of the mercaptans are listed in Table I.

***N*-Heterocyclylethyl Disulfide Dihydrochlorides**—In a representative procedure, 0.07 mole of mercaptan was dissolved in 25 ml of dimethyl sulfoxide, and the colorless solution was heated at 80–90° for 18–20 hr. The resulting solution was poured into 300 ml of ice water, and the mixture was stirred for 4 hr. The oily disulfide was extracted twice with ether, and the combined extracts were washed with water.

The ether layer was dried over magnesium sulfate, and the ether was removed by flash evaporation to give a clear oil, which was triturated with absolute ethanol to yield a partially solidified oil. To this was added concentrated hydrochloric acid in 95% ethanol, the solvent was removed by flash evaporation, and the resulting hydrochloride was recrystallized twice from 95% ethanol to yield colorless crystals. Physical constants of the disulfide dihydrochlorides are listed in Table II.

***N*-Heterocyclylethyl Disulfides**—In a representative procedure, 0.06 mole of mercaptan was dissolved in 25 ml of dimethyl sulfoxide, and the solution was heated at 85–90° overnight. The resulting solution was poured into 300 ml of ice water, the oily mixture was extracted twice with methylene chloride, and the combined extracts were washed twice with water. The methylene chloride layer was treated with charcoal, dried over magnesium sulfate, and filtered. The solution was flash evaporated to yield a yellow or brown oil. Physical constants of the disulfides are listed in Table II.

2-(*N*-4-Methylpiperazinyl)ethanethiosulfuric Acid—This procedure is representative of the conversion of mercaptans to thiosulfuric acids. 1-(2-Mercaptoethyl)-4-methylpiperazine (7.35 g, 0.046 mole) was dissolved in 60 ml of methanol, and potassium metabisulfite (11.1 g, 0.050 mole) was added with 10 ml of water. The mixture was refluxed overnight and was filtered while hot to remove potassium thiosulfate. The filtrate was flash evaporated to a solid, and the solid was taken up in boiling methanol and filtered. A precipitate of 3.1 g (24% yield) of colorless crystals was obtained on cooling, mp 186–187.5°.

2-(*N*-Piperidyl)ethanethiosulfuric Acid—This procedure is representative of the conversion of halides to thiosulfuric acids. To a solution of *N*-(2-chloroethyl)piperidine hydrochloride (18.4 g, 0.1 mole) in 50 ml of water were added sodium thiosulfate pentahydrate (24.8 g, 0.1 mole) and an additional 50 ml of water. The solution was refluxed for 1 hr and was flash evaporated to give an oil; this oil was taken up in methanol and filtered. The methanol was removed by flash evaporation to give an oil, which was crystallized from 95% ethanol to yield 8.9 g (39.5%) of colorless crystals, mp 180–182°.

2-(2-Quinolylmethylamino)ethanethiosulfuric Acid—To a warm solution of sodium hydroxide in 95% ethanol (2 g in 70 ml) was added rapidly, with stirring, a hot solution of 2-aminoethanethiosulfuric acid (11) (7.85 g, 0.05 mole) in 5 ml of water. To this mixture under reflux was added dropwise 2-chloromethylquinoline (4.44 g, 0.025 mole) in 95% ethanol (50 ml). Heating and stirring were continued for 17 hr, and the mixture was concentrated to about 20 ml and was washed with ether to remove unreacted 2-chloromethylquinoline.

The solution was neutralized with acetic acid and allowed to stand at 0° for 2 days. The resulting semisolid was filtered to give 4.2 g (57%) of product, mp 183–185° (after recrystallization from ethanol–water, mp 187–188°); R_f 0.6 [silica gel plates with *n*-butanol–ethanol–water (3:1:1)]; IR (KBr): 1020, 1185, and 1240 ($S_2O_3^-$) and 3500 (NH) cm^{-1}.

2-(1-Cyanobenzylidene)-4-phenyl-1,3-dithiole—A mixture of 2-phenyl-3,3-dimercaptoacrylonitrile dipotassium salt (7) (23.7 g, 0.082 mole), sodium bicarbonate (16.4 g), and bromoacetophenone (19.9 g, 0.10 mole) was stirred at room temperature for 24 hr. The resulting solid was filtered, and concentrated sulfuric acid (50 ml) was added to the cooled filtrate. This mixture was cooled at 0° for 5 hr, and dilution with water precipitated a yellow solid. The product was filtered, washed with water, and recrystallized from chloroform, giving 5.3 g (22% yield), mp 162–163°; NMR (trifluoroacetic acid): δ 7.38 (5H).

Anal.—Calc. for $C_{17}H_{11}NS_2$: C, 69.60; H, 3.78; N, 4.77. Found: C, 69.85; H, 4.20; N, 4.57.

2-Dicyanomethylenepyrazino[2,3-*d*]dithiole—The dipotassium salt of 2-cyano-3,3-dimercaptoacrylonitrile (7) (7.8 g, 0.033 mole) was dissolved in 50 ml of dimethylformamide, and 2,3-dichloropyrazine (4.44 g, 0.03 mole) was added. The solution was heated at 85° for 16 hr, becoming black. It was diluted with 100 ml of water, cooled to 5°, and filtered. The dried brown powder (2.0 g) was sublimed at 0.5 mm with a free flame, giving 1.1 g (17% yield) of yellow powder, mp 175–205°, and 0.25 g (4%) of yellow needles, mp 212–213°; IR: 2210 (C≡N), 1490, 1342, 1148, 1075 (pyrazine ring), and 850 (CH) cm^{-1}.

Anal.—Calc. for $C_8H_2N_4S_2$: C, 44.02; H, 0 92; N, 25.70; S, 29.38. Found: C, 43.68; H, 0.96; N, 25.41; S, 28.98.

2-Dicyanomethylenequinoxalino[2,3-*d*]dithiole—The dipotassium salt of 2-cyano-3,3-dimercaptoacrylonitrile (7) (7.8 g,

0.033 mole), 2,3-dichloroquinoxaline (6.0 g, 0.03 mole), methanol (200 ml), and water (20 ml) were heated under reflux for 2 hr, diluted with 150 ml of water, and filtered. The dried solid was recrystallized from 250 ml of dimethylformamide and dried at 140° (0.2 mm) for 2 hr to give 3.2 g (25%) of bright-yellow crystals, mp 298–320° dec.; IR: 2210 (C≡N), 1505, 1180, and 1120 (quinoxaline ring) cm^{-1}.

Anal.—Calc. for $C_{12}H_4N_4S_2$: C, 53.71; H, 1.50; N, 20.88; S, 23.90. Found: C, 54.07; H, 1.74; N, 20.5; S, 23.6.

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[x] To whom inquiries should be directed.

Bisulfite-Ion-Catalyzed Degradation of Fluorouracil

GERALD S. RORK and IAN H. PITMAN [x]

Abstract □ 5-Fluorouracil (I) reversibly adds bisulfite ion across its $>C_5{=}C_6<$ bond to form 5-fluoro-5,6-dihydrouracil-6-sulfonate (II). The pH-independent equilibrium constant for this reaction was calculated to be 560 M^{-1} at ionic strength 1.00 M at 25°. Compound II was observed to be unstable in alkaline solution and reacted to yield uracil-6-sulfonic acid, fluoride ion, and α-fluoro-β-ureidopropionic acid-β-sulfonate (III), along with I (*via* a loss of HSO_3^-). In strongly alkaline conditions, *i.e.*, 1 M NaOH, III was observed to undergo a subsequent reaction to produce a compound believed to be α-fluoro-β-ureidoacrylic acid (V) or fluoromalonaldehydic acid (VII). These irreversible degradation reactions of II lead to a complete degradation of I in sodium bisulfite solutions. The similarity of this bisulfite-ion-catalyzed degradation of I to its hydrolytic degradation is discussed.

Keyphrases □ Fluorouracil—bisulfite-ion-catalyzed degradation □ Sodium bisulfite—role in degradation of 5-fluorouracil, nucleophilic addition □ Equilibrium—bisulfite ion addition to fluorouracil

It has been reported (1) that the antimetabolite 5-fluorouracil (I) undergoes a reversible, covalent addition of bisulfite ion (HSO_3^-) across its 5,6-carbon–carbon double bond to yield 5-fluoro-5,6-dihydrouracil-6-sulfonate (II). Additional findings that II undergoes irreversible reactions in sodium bisulfite solutions leading to complete degradation of I (Scheme I) are now reported.

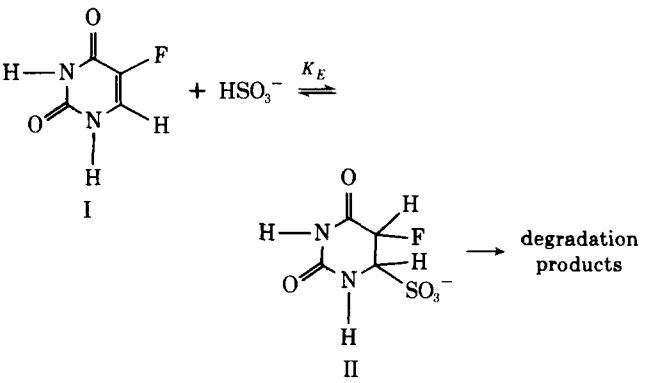
Although I is comparatively stable at room temperature in aqueous buffers that do not contain sodium bisulfite[1], it has been observed to degrade at higher temperatures and in alkaline conditions (2). It has been suggested that the first step in these reactions is covalent hydration of the 5,6-double bond of I to produce 5- or 6-hydroxy-5-fluoro-5,6-dihydrouracil, which then degrades to nonchromophoric products (*via* opening of the pyrimidine ring) (2).

The results of the present study are discussed in terms of similarities between the water-catalyzed and bisulfite-ion-catalyzed degradations of I.

EXPERIMENTAL

Materials and Equipment—Fluorouracil[2] was used as received. All water was redistilled from a Pyrex apparatus before use. All other chemicals were a reagent grade and were used without additional purification.

Potassium 5-fluoro-5,6-dihydrouracil-6-sulfonate monohydrate



Scheme I

[1] Fluorouracil is formulated for injection as a pH 9 solution (adjusted with sodium hydroxide) at a concentration of 50 mg/ml. Fluorouracil package insert, Roche Laboratories, Dec. 1972.

[2] Sigma Chemical Co.

0.033 mole), 2,3-dichloroquinoxaline (6.0 g, 0.03 mole), methanol (200 ml), and water (20 ml) were heated under reflux for 2 hr, diluted with 150 ml of water, and filtered. The dried solid was recrystallized from 250 ml of dimethylformamide and dried at 140° (0.2 mm) for 2 hr to give 3.2 g (25%) of bright-yellow crystals, mp 298–320° dec.; IR: 2210 (C≡N), 1505, 1180, and 1120 (quinoxaline ring) cm^{-1}.

Anal.—Calc. for $C_{12}H_4N_4S_2$: C, 53.71; H, 1.50; N, 20.88; S, 23.90. Found: C, 54.07; H, 1.74; N, 20.5; S, 23.6.

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Bisulfite-Ion-Catalyzed Degradation of Fluorouracil

GERALD S. RORK and IAN H. PITMAN [x]

Abstract □ 5-Fluorouracil (I) reversibly adds bisulfite ion across its $>C_5{=}C_6<$ bond to form 5-fluoro-5,6-dihydrouracil-6-sulfonate (II). The pH-independent equilibrium constant for this reaction was calculated to be 560 M^{-1} at ionic strength 1.00 M at 25°. Compound II was observed to be unstable in alkaline solution and reacted to yield uracil-6-sulfonic acid, fluoride ion, and α-fluoro-β-ureidopropionic acid-β-sulfonate (III), along with I (*via* a loss of HSO_3^-). In strongly alkaline conditions, *i.e.*, 1 M NaOH, III was observed to undergo a subsequent reaction to produce a compound believed to be α-fluoro-β-ureidoacrylic acid (V) or fluoromalonaldehydic acid (VII). These irreversible degradation reactions of II lead to a complete degradation of I in sodium bisulfite solutions. The similarity of this bisulfite-ion-catalyzed degradation of I to its hydrolytic degradation is discussed.

Keyphrases □ Fluorouracil—bisulfite-ion-catalyzed degradation □ Sodium bisulfite—role in degradation of 5-fluorouracil, nucleophilic addition □ Equilibrium—bisulfite ion addition to fluorouracil

It has been reported (1) that the antimetabolite 5-fluorouracil (I) undergoes a reversible, covalent addition of bisulfite ion (HSO_3^-) across its 5,6-carbon–carbon double bond to yield 5-fluoro-5,6-dihydrouracil-6-sulfonate (II). Additional findings that II undergoes irreversible reactions in sodium bisulfite solutions leading to complete degradation of I (Scheme I) are now reported.

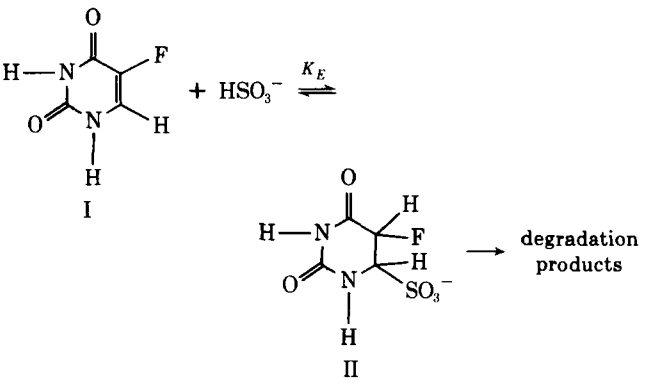
Although I is comparatively stable at room temperature in aqueous buffers that do not contain sodium bisulfite[1], it has been observed to degrade at higher temperatures and in alkaline conditions (2). It has been suggested that the first step in these reactions is covalent hydration of the 5,6-double bond of I to produce 5- or 6-hydroxy-5-fluoro-5,6-dihydrouracil, which then degrades to nonchromophoric products (*via* opening of the pyrimidine ring) (2).

The results of the present study are discussed in terms of similarities between the water-catalyzed and bisulfite-ion-catalyzed degradations of I.

EXPERIMENTAL

Materials and Equipment—Fluorouracil[2] was used as received. All water was redistilled from a Pyrex apparatus before use. All other chemicals were a reagent grade and were used without additional purification.

Potassium 5-fluoro-5,6-dihydrouracil-6-sulfonate monohydrate



Scheme I

[1] Fluorouracil is formulated for injection as a pH 9 solution (adjusted with sodium hydroxide) at a concentration of 50 mg/ml. Fluorouracil package insert, Roche Laboratories, Dec. 1972.

[2] Sigma Chemical Co.

was isolated from the reaction of I in a potassium sulfite solution at pH 5. Approximately 0.3 g of I was added to 4 ml of a saturated solution of potassium sulfite, which had been adjusted to pH 5 with concentrated hydrochloride acid. After stirring at room temperature for 1 hr, the mixture was heated to 95° and a clear solution was obtained.

White crystals formed on cooling and were collected by filtration and then washed with cold water and anhydrous ether. The NMR and UV spectra of this material are consistent with that previously reported (1) for the sodium salt of II, which was isolated in a different procedure.

Anal.—Calc. for $C_4H_6FKN_2O_6S$: C, 17.90; H, 2.24; N, 10.44. Found: C, 17.65; H, 2.26; N, 10.16.

Similarly, potassium 5-fluoro-1,3,5-trideutero-5,6-dihydrouracil-6-sulfonate with one molecule of D_2O of crystallization was isolated when the this procedure was followed using D_2O and DCl in place of H_2O and HCl.

Anal.—Calc. for $C_4HD_5FKN_2O_6S$: C, 17.58; D and H (as H), 2.20; N, 10.25. Found: C, 17.41; D and H, 2.17; N, 9.86.

Kinetic and Equilibria Measurements—The kinetics and equilibria of addition of HSO_3^- to I were measured by following the loss of absorbance of I in the 270–290-nm region that occurred after mixing solutions of I with aqueous buffer solutions containing sodium bisulfite. Unless otherwise stated, all solutions were measured at ionic strength 1.00 *M* (adjusted with potassium chloride) and at 25°.

Typically, 0.005–0.010 ml of a 0.1 *M* solution of I at pH 10 was added to a 1-cm cell. The cell contained 3.0 ml of an aqueous buffer containing sodium bisulfite and had been equilibrated at 25° in the cell compartment of a spectrophotometer. (The reference cell contained an identical solution and the instrument had been balanced at zero absorbance.) The cell was then inverted several times to ensure complete mixing, and the resultant loss in absorbance was followed as a function of time.

Observed first-order rate constants (k_{obs} values) were calculated from the slopes of semilogarithmic plots of the absorbance changes, $(A - A_\infty)$, *versus* time. Since II did not have any significant absorbance in the 270–290 region, the absorbance value at the start of the reaction A_0, and the absorbance value at equilibrium, A_∞, could be used to calculate an observed equilibrium constant for the addition of HSO_3^- to I. The equilibrium constants were calculated as described under *Results and Discussion*.

The kinetics of reactions of II in aqueous solutions were followed by mixing 0.005–0.010 ml of a 0.10 *M* solution of II in water with 3.0 ml of a particular buffer and observing the resultant absorbance changes that occurred in the UV spectrum of the solution.

A value of 6.50 ± 0.03 was calculated for the pKa of HSO_3^- from a potentiometric titration of sodium sulfite with standard hydrochloric acid solution at ionic strength 1.00 *M* (adjusted with potassium chloride) at 25°. The pKa of I was 7.71 ± 0.05 from spectrophotometric measurements at ionic strength 1.00 *M* at 25°.

Paper Chromatography—The paper chromatographic system used to separate the degradation products of the reaction of II in alkaline solution utilized 95% ethanol–acetic acid–water (80:10:10) as the developing solvent (ascending) on filter paper[3]. Strips 4 × 18 cm were cut, and duplicate spottings of the solution to be analyzed were made 1 cm from each edge of the strip and 1 cm from the bottom.

The developing solvent was allowed to ascend 10–12 cm and the strip was then allowed to dry. Spots were detected by three different methods: 1, illuminating by a 254-nm lamp; 2, spraying with a 5% methanolic potassium hydroxide solution, drying, and illuminating with a 254-nm lamp; and 3, spraying with a 3% solution of *p*-dimethylaminobenzaldehyde which was also 5% in hydrochloric acid.

Since duplicate spottings were made, the developed chromatogram was cut in half from top to bottom. Methods 1 and 2 were applied to one half, while Method 3 was applied to the other.

RESULTS AND DISCUSSION

Kinetics and Thermodynamics of Addition of HSO_3^- to I—The reversible addition of HSO_3^- to I is believed[4] to involve attack by SO_3^{-2} at C-6, followed by protonation at C-5, in the forward direction (addition) and general and specific base-catalyzed removal of the C-5 proton, followed by elimination of SO_3^{-2}, in the reverse direction (desulfonation). A similar two-step process has been suggested to occur in the reversible addition of HSO_3^- to other uracil derivatives (3). The kinetic results of the present study closely parallel those obtained by Sedor *et al.*[4] and thus have not been included in detail.

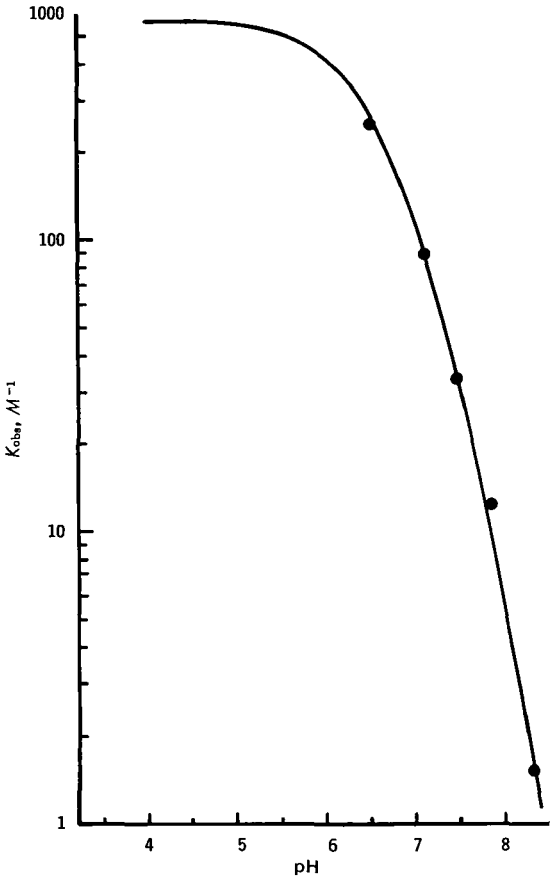


Figure 1—*Semilogarithmic plot of the observed equilibrium constants for addition of HSO_3^- to 5-fluorouracil, K_{obs} values versus pH. The solid line was calculated from Eq. 2 with K_E = 560 M^{-1}. All values were measured at ionic strength at 1.00 M and at 25°.*

Values of the observed equilibrium constant for addition of HSO_3^- to I (Scheme I) were calculated from the slopes of plots of $(A_0 - A_\infty)/A_\infty$ values *versus* the total sulfites concentration in solution, $[S_T] = [HSO_3^-] + [SO_3^{-2}]$, for a series of solutions at constant pH:

$$K_E = \frac{[II]}{[I][HSO_3^-]} \qquad \text{(Eq. 1}a\text{)}$$

$$K_{obs} = \frac{[II]}{[I_T][S_T]} = \frac{(A_0 - A_\infty)}{A_\infty[S_T]} \qquad \text{(Eq. 1}b\text{)}$$

where the term $[I_T]$ represents the concentration of 5-fluorouracil neutral molecule plus the concentration of 5-fluorouracil monoanion ($[I_T] = [I] + [I^-]$).

It is expected that II exists predominantly as a monoanion in the pH range investigated (6.5–8.3) since its pKa for ionization of an imino proton is approximately 10.0. The pKa of 5,6-dihydrouracil-6-sulfonate was previously observed to be 10.65 (3). The K_{obs} values can thus be expressed in terms of the pH-independent equilibrium constant, K_E, and the acid dissociation constants for HSO_3^-, $K_a^{HSO_3^-}$ and I, K_a^I, as shown in Eq. 2:

$$K_{obs} = K_E\left(\frac{[H^+]}{[H^+] + K_a^I}\right)\left(\frac{[H^+]}{[H^+] + K_a^{HSO_3^-}}\right) \qquad \text{(Eq. 2)}$$

[3] Whatman No. 2.
[4] F. A. Sedor, D. G. Jacobson, and E. G. Sander, to be published.

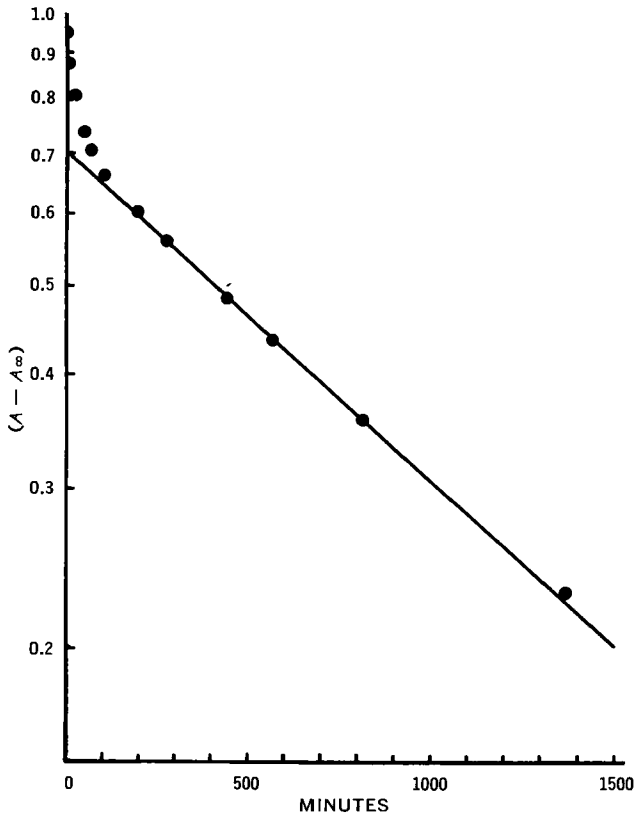


Figure 2—*Semilogarithmic plot* versus *time of the absorbance changes that occurred at 285 nm upon addition of I to a solution that was 0.233* M *in sodium sulfite and 0.3* M *in tromethamine buffer at pH 8.31, ionic strength 1.00* M, *25°.*

Figure 1 shows the equilibrium pH profile of K_{obs} values calculated from Eq. 1*b*. The values are plotted semilogarithmically *versus* pH, and the solid line was calculated from Eq. 2 using a value of $K_E = 560\ M^{-1}$, with $pKa^I = 7.71$ and $pKa^{HSO_3^-} = 6.50$. At constant total sulfite concentration, the equilibrium formation of II becomes less favorable as the pH is increased above 5. Equation 2 appears to describe this dependency adequately.

Degradation Products of II—At pH values below 8, only a first-order approach to equilibrium was observed when solutions of I were mixed with sodium bisulfite solutions. However, an additional reaction was observed in solutions containing tromethamine buffer and sodium sulfite at pH 8.31.

Figure 2 shows a semilogarithmic plot of the absorbance changes, $(A-{}^{I}A_{\infty})$, observed at 285 nm after a solution of I was mixed with a solution at pH 8.31, which was 0.233 *M* in sodium sulfite and 0.30 *M* in tromethamine buffer. These data suggested that II slowly degraded under these conditions. An observed first-order rate constant for this reaction was calculated to be 1.6×10^{-5} sec^{-1} from the terminal slope of Fig. 2. The intercept at zero time of this line was taken as the A_∞ value for the first reaction in calculating the observed equilibrium constant, K_{obs}, for the addition of HSO_3^- to I at this pH.

Further evidence that II underwent degradation reactions in alkaline solutions came from the observation that II and 5-deutero-II (5-*d*-II) did not yield quantitative amounts of I *via* a base-catalyzed desulfonation reaction. Figure 3 shows the UV spectrum of a 2.00×10^{-4} *M* solution of I in 0.01 *N* NaOH solution along with the UV spectra of 2.00×10^{-4} *M* solutions of II and 5-*d*-II in 0.01 *N* NaOH solution, which had been allowed to stand for 40 min at 25°. A first-order appearance of the absorbance due to I was observed for each of these two solutions, and the observed rate constants were calculated to be 5.5×10^{-3} sec^{-1} for II and 2.8×10^{-3} sec^{-1} for 5-*d*-II.

From the spectral data shown in Fig. 3, it was calculated that the yield of I was 45% from II and 17.5% from 5-*d*-II. The lower percentage yield of I from 5-*d*-II is believed to result from a decrease in the rate of the desulfonation reaction relative to a competing side reaction, which occurred when a deuterium was incorporated at C-5 of II. Primary kinetic deuterium isotope effects in the range of 4–5 have been observed in the desulfonation reaction of similar HSO_3^- adducts of uracils (3). Any deuterium isotope effect on the side reaction was apparently smaller than that on the desulfonation reaction. Thus, these data suggest that a side reaction competes with the desulfonation of II and that it results in the formation of a compound with virtually no absorbance in the 240–310-nm region.

Figure 4 illustrates the changes in the UV spectrum that occurred with time in a solution that was 1.67×10^{-4} *M* in II and 0.01 *N* in sodium hydroxide. An isosbestic point was observed at 256 nm during the reaction of II to I for at least seven half-lives (15 min). These spectral data are also consistent with a reaction scheme in which a side reaction occurs parallel to the desulfonation reaction and leads to the formation of a nonchromophoric product. The slow increase in absorbance in the 260-nm region, resulting in loss of the isosbestic point, will be discussed below.

NMR spectroscopy and paper chromatography were utilized to investigate the nature of the degradation products formed from 5-*d*-II in alkaline solutions. The deuterated derivative was studied since it gave a higher percentage of products other than I, as mentioned previously.

The NMR spectra of Compounds II (spectrum A) and 5-*d*-II (spectrum B) in D_2O are shown in Fig. 5. After the pD of the solution of 5-*d*-II in D_2O was increased to approximately 12 with a NaOD solution, the NMR spectrum of the resultant solution, spectrum C, indicated that the singlet at 4.9 ppm (due to 5-*d*-II) had disappeared and that signals due to I (doublet centered at 7.6 ppm, $J = 5$ Hz) and an unidentified compound (doublet centered at 5.25 ppm, $J = 29$ Hz) had developed.

The formation of a bright-yellow color when an acidic solution of *p*-dimethylaminobenzaldehyde was added to a portion of this solution suggested that some opening of the pyrimidine ring had occurred. Neither 5-*d*-II nor I gave a positive test with this reagent, which is specific for primary (*versus* secondary) amines, amides, and ureido functions (4).

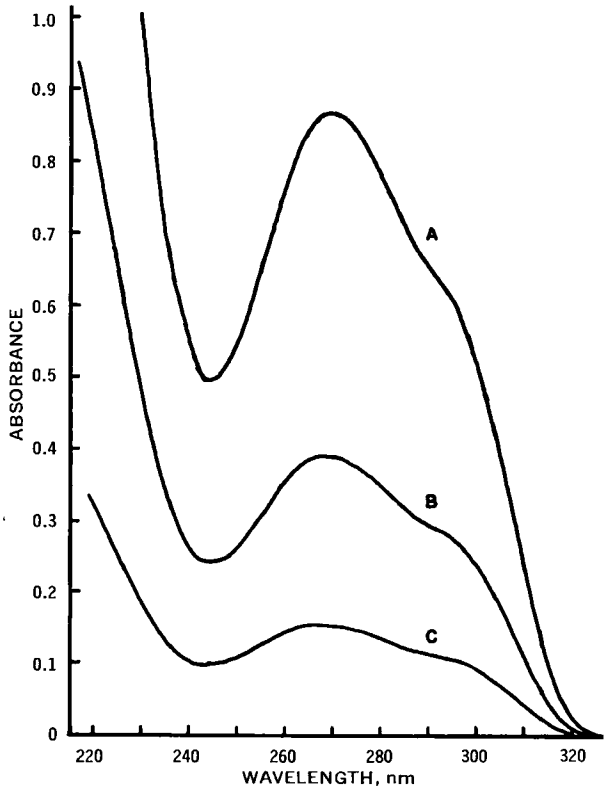


Figure 3—*UV spectra of 2.00 × 10⁻⁴* M *solutions of II (B) and 5-d-II (C) in a 0.01* N *NaOH solution measured 40 min after the solutions were made. The spectrum of a 2.00 × 10⁻⁴* M *solution of I in 0.01* N *NaOH solution is shown for comparison (A).*

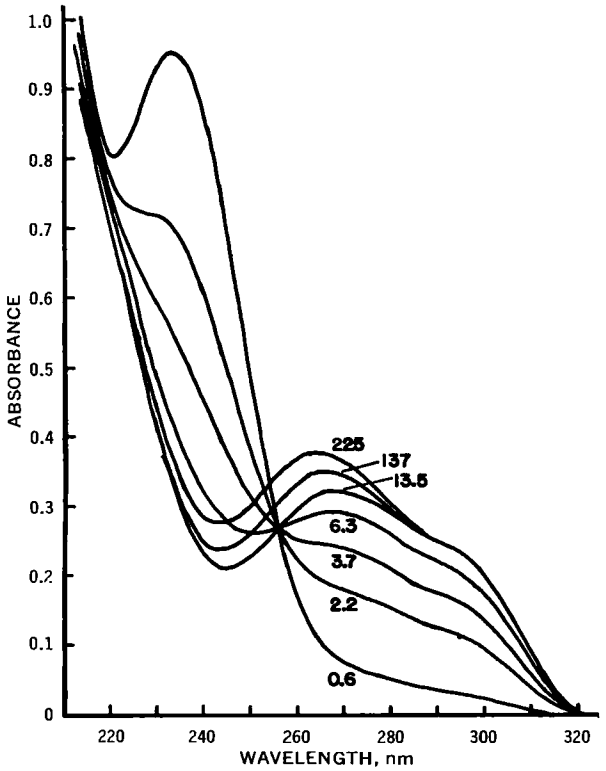
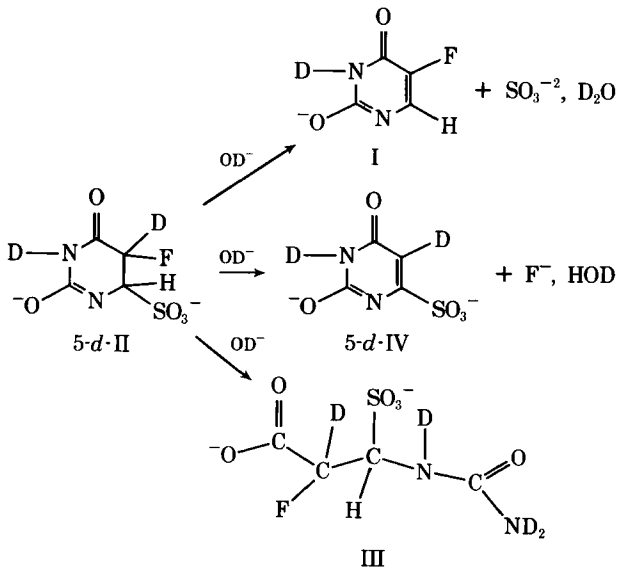


Figure 4—*UV spectral changes that occurred upon addition of II to a 0.01* N *NaOH solution to yield a 1.67* $\times$ 10^{-4} M *solution. The time, in minutes, after the addition of II is appropriately labeled for each scan.*

It is known (5, 6) that 5,6-dihydrouracils hydrolyze at the 3,4-linkage in alkaline solution to yield β-ureidopropionic acids, which readily develop a similar yellow color with this reagent. It is expected that 5-*d*-II would undergo a similar 3,4-ring opening since the fluorine at C-5 would activate the C-4 carbonyl group toward attack by OH^-. In the paper chromatographic analysis of this solution, as described under *Experimental*, only one spot (R_f 0.07) was observed to develop a yellow color when sprayed with an acidic solution of *p*-dimethylaminobenzaldehyde. This spot was detectable with a 254-nm UV lamp only after spraying with a 5% methanolic potassium hydroxide solution.

Apparently, the component exhibited in this low R_f spot and the doublet at 5.25 ppm in spectrum C of Fig. 5 is α-deutero-α-fluoro-β-ureidopropionic acid-β-sulfonate (III), which was formed *via* base-catalyzed 3,4-ring opening of 5-*d*-II (Scheme II). Compounds II and 5-*d*-II were not observed to have measurable vicinal H—F couplings, apparently because of the specific orientation of the C_5—F and C_6—H bonds.



Scheme II

The large coupling of 29 Hz, assigned to III, is typical of vicinal H—F couplings on saturated carbon (7). Opening of the ring would allow rotation about the carbon–carbon bond, providing a different orientation of H and F. Thus, 3,4-ring opening could drastically change the coupling constant of the C_6—H and not markedly change its chemical shift.

Two other spots were observed in the paper chromatographic analysis: one at R_f 0.50 and one at R_f 0.16. The spot at R_f 0.50 was detected with a 254-nm UV lamp and the R_f value was identical to that of authentic 5-fluorouracil (I). The spot at R_f 0.16 was also detectable with a 254-nm UV lamp but was less intense. This spot was cut from the chromatogram and eluted with a small volume of water.

The UV spectrum of this solution strongly suggested that the compound was uracil-6-sulfonic acid (IV) (present as a sulfonate salt). This identification was based on the reported (8) spectral data of authentic IV [pKa_2 = 7.2; λ_{max} 264 nm (ϵ 8470 cm^{-1} M^{-1}) in 0.1 *N* HCl and λ_{max} 293 nm (ϵ 9190 cm^{-1} M^{-1}) in 0.1 *N* NaOH]. The spectrum showed λ_{max} 264 nm at pH <5, which then shifted to longer wavelengths with an isosbestic point at 277 nm as the pH of the solution was increased. At pH 11–12, λ_{max} 293 nm was observed; the ratio of the 293-nm peak to the 264-nm peak (at pH 5) was 1.06. This value is in good agreement with the literature value of 1.09.

A strong, positive test for the presence of fluoride ion was obtained for this solution, whose NMR spectrum and paper chroma-

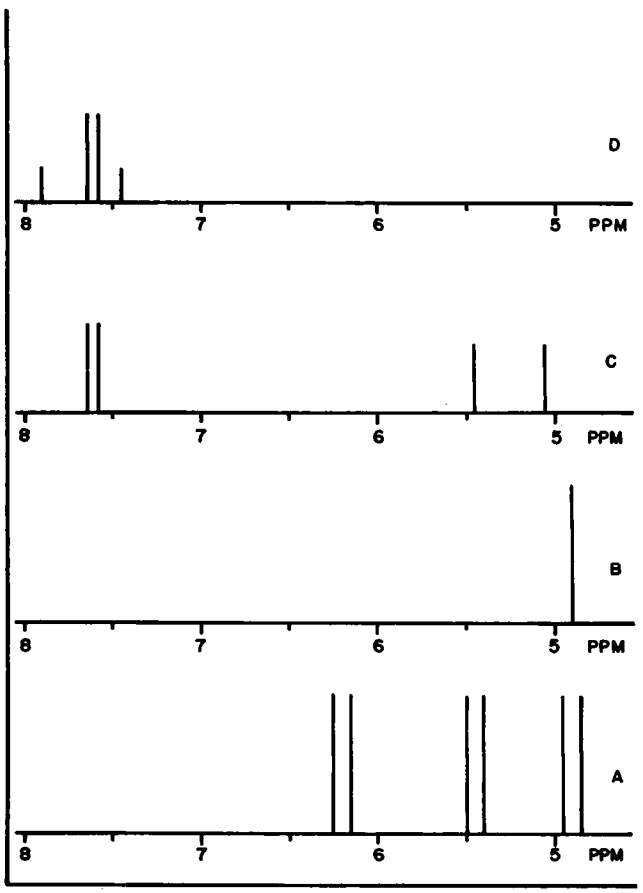
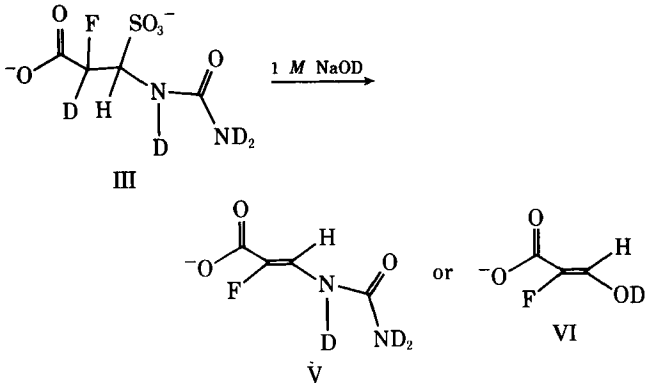


Figure 5—*NMR spectral data for a solution of II in D_2O (A) and a solution of 5-d-II in D_2O (B). Spectrum C was obtained several minutes after addition of sufficient NaOD solution in D_2O to bring the pD of Solution B to approximately 12–13. Spectrum D was measured several minutes after the addition of additional NaOD solution (40% NaOD in D_2O) to Solution B.*



Scheme III

tographic behavior has just been discussed, when it was treated with a zirconium–alizarin solution (9). Neither 5-*d*-II, I, nor any of the solution components gave a positive test with this reagent. Thus, it appears that the spot at R_f 0.16 is due to 5-*d*-IV, formed *via* HF elimination (Scheme II). This compound would obviously not have appeared in spectrum C of Fig. 5 since it contains no hydrogen substituents. It apparently is a relatively minor product in these reactions.

UV spectral data (Fig. 4) indicated that the nonchromophoric product of the side reaction mentioned, presumably Compound III, slowly underwent a subsequent reaction in alkaline conditions to produce an unknown compound with a strong chromophore in the 240–280-nm region. When the R_f 0.07 spot was eluted with water and the resultant solution was made 1 *M* in sodium hydroxide, a peak with λ_{max} 260 nm slowly developed over 1 hr.

Similarly, when the solution whose NMR spectrum is shown as spectrum C in Fig. 5 was made strongly alkaline (by addition of several drops of 40% NaOD in D_2O to the sample tube), the doublet at 5.25 ppm was lost and a doublet centered at 7.67 ppm, with a coupling constant of 27 Hz, was observed as shown in spectrum D.

These UV and NMR spectral changes that occurred in strongly alkaline conditions are apparently due to a degradation of III to either α-fluoro-β-ureidoacrylic acid (V) or fluoromalonaldehydic acid (VI) (Scheme III). Compound VI has been reported (10) as a degradation product of 5-fluoro-6-hydroxy-5,6-dihydrouracil (VII) in 1 *M* NaOH solution, along with urea and a small amount (6%) of I.

In a 1 *M* NaOH solution, VII was reported to have a strong chromophore (13,300 cm^{-1} M^{-1}) with a λ_{max} of 260 nm. This UV behavior seems very similar to that observed for the alkaline degradation product of the R_f 0.07 spot. The fact that the doublet shown in spectrum D of Fig. 5 is very similar in chemical shift to the C_6—H of I suggests a structure such as that of V or VI. The large observed coupling constant of 27 Hz is consistent with reported *trans*-H—F vicinal couplings on carbon–carbon double bonds (7).

A comparison of the reaction sequences in Schemes I and II with the postulated (2) mechanism of degradation of I in aqueous buffers that did not contain sodium bisulfite indicates that the irreversible degradation of I in both cases is most likely initiated by covalent addition of a nucleophilic reagent to I to yield a 5,6-dihydrouracil. In the former case, HSO_3^- is the nucleophilic reagent; in the latter case, it is water.

Because of the structural similarities of 5-fluoro-6-hydroxy-5,6-dihydrouracil and II, it seems likely that the magnitude of their rate constants for irreversible degradation would be similar. The irreversible degradation reactions postulated for both compounds involve attack of OH^- at their C-4 carbonyl groups and elimination of HF across their 5,6-bonds. Hence, the rate at which I undergoes irreversible degradation would be determined largely by the magnitudes of the rate and equilibrium constants for addition of HSO_3^- or H_2O to I.

Previous studies (11, 12) have indicated that HSO_3^- adds much more rapidly than H_2O to >C=N— and >C=O groups and that the magnitudes of the equilibrium constants for these reactions are much larger for HSO_3^- addition than for H_2O addition. A similar relationship is expected to apply to additions of these species to >C=C< bonds. Hence, it is not surprising that I is irreversibly degraded much more rapidly in solutions that contain sodium bisulfite than it is in water.

Consequently, it is expected that whenever the 5,6-double bond of 5-fluorouracil is saturated, the resultant compound will be unstable with respect to the degradation reactions discussed. The antimetabolic properties of 5-fluorouracil are believed to be due to an inhibition of the enzyme thymidylate synthetase. In a recent study of the binding of 5-fluoro-2-deoxyuridylate to this enzyme in the presence of 5,10-methylenetetrahydrofolate, the formation of a 5,6-substituted-5-fluoro-5,6-dihydrouracil structure in the enzyme–substrate complex was proposed (13). Based on the results of the present study, a binding of this type may also lead to increased rates of degradation of the pyrimidine ring structure or possibly to loss of HF across the 5,6-bond.

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Crystal Structures of Azathioprine Dihydrate and 6-Methylmercaptopurine Trihydrate

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Abstract □ The crystal and molecular structures of 6-methylmercaptopurine trihydrate and of azathioprine dihydrate were determined by the use of three-dimensional, X-ray diffractometer data and were refined by least squares. Both molecules crystallize in the N(9)-H tautomer form, in contrast to the N(7)-H tautomer form found in crystals of 6-mercaptopurine. Unlike 6-mercaptopurine, or other thiopurines that have unsubstituted thio groups, the sulfur atoms of 6-methylmercaptopurine and azathioprine do not act as hydrogen-bond acceptors in the crystal structures. These two derivatives of 6-mercaptopurine assume a conformation in which the substituents on the sulfur atom are directed away from the imidazole moiety of the purine.

Keyphrases □ Azathioprine dihydrate—crystal and molecular structures □ 6-Methylmercaptopurine trihydrate—crystal and molecular structures □ Mercaptopurine derivatives—crystal and molecular structures of azathioprine dihydrate and 6-methylmercaptopurine trihydrate □ Crystallography—determination of structures of azathioprine dihydrate and 6-methylmercaptopurine trihydrate

A number of synthetic purine and pyrimidine analogs are useful chemotherapeutic agents (1–3). Of the purine analogs that are effective in cancer chemotherapy, mercaptopurine is one of the most widely used (3–5). However, its effectiveness is limited by its rapid degradation *in vivo* (6), and derivatives have been synthesized with the intent of preventing this *in vivo* degradation. A particularly effective derivative is azathioprine[1], 6-[(1-methyl-4-nitroimidazol-5-yl)mercapto]purine, in which the protective group is a nitroimidazolyl moiety (7). Azathioprine reacts with sulfhydryl groups, resulting in the slow liberation of free 6-mercaptopurine.

Acquired resistance to mercaptopurine represents the second major obstacle to its successful use (2, 8). For *in vivo* activation, mercaptopurine must be first converted to its ribonucleotide (by the enzyme inosinic-guanylic pyrophosphorylase) and then to 6-methylmercaptopurine ribonucleotide (1, 2). When administered as the ribonucleoside, 6-methylmercaptopurine is capable of inhibiting the growth of cells that have acquired resistance to mercaptopurine by loss of inosinic-guanylic pyrophosphorylase (1). This analog ribonucleoside, mistaken for adenosine by the enzyme adenosine kinase, is converted directly to the ribonucleotide, thus bypassing the pathway responsible for resistance to mercaptopurine (1).

This paper describes the crystal structures of azathioprine dihydrate and 6-methylmercaptopurine trihydrate and discusses some apparent structural effects produced by the addition of substituents to the sulfur atom of mercaptopurine.

[1] Imuran, Burroughs Wellcome Co.; preferred chemical name is 6-[(1-methyl-4-nitroimidazol-5-yl)thio]purine.

Table I—Crystal Data[a]

	Azathioprine Dihydrate	6-Methylmercaptopurine Trihydrate
Stoichiometry	$C_9H_7N_7O_2S \cdot 2H_2O$	$C_6H_6N_4S \cdot 3H_2O$
Z	4	4
Space Group	$P2_1/c$	$P2_1/c$
a	4.560 (1) Å	10.721 (1) Å
b	15.491 (1)	6.976 (2)
c	19.447 (3)	15.027 (2)
β	93.61 (1)°	115.04 (1)°
ρ (calculated)	1.518 g cm^{-3}	1.437 g cm^{-3}
ρ (observed)	1.51 g cm^{-3}	1.43 g cm^{-3}
μ (CuK$\bar{\alpha}$)	22.8 cm^{-1}	27.0 cm^{-1}

[a] The density was measured by flotation in a mixture of benzene and carbon tetrachloride.

EXPERIMENTAL

Yellow crystals of azathioprine were obtained by slowly evaporating a water–ethanol solution; colorless crystals of 6-methylmercaptopurine were grown from a solution of water and dimethylamine. Weissenberg and oscillation photographs showed the crystals to be monoclinic, space group $P2_1/c$, as indicated by the systematic absence of reflections h0l with l odd and 0k0 with k odd.

Data for azathioprine were obtained from a crystal fragment with approximate dimensions of 0.30, 0.20, and 0.03 mm. Data for 6-methylmercaptopurine were obtained from a crystal fragment with approximate dimensions of 0.30, 0.20, and 0.10 mm. All angular and intensity data were collected with an X-ray diffractometer[2], by use of a scintillation counter and nickel-filtered copper radiation.

Cell parameters were measured before and after intensity data were collected. Approximate cell parameters for use in collection of intensity data were calculated by a least-squares analysis of the angular settings for several medium-angle reflections (CuK$\bar{\alpha}$, λ = 1.5418 Å). Accurate values for the cell parameters were determined immediately after data collection by a least-squares analysis of 2θ values for high angle reflections (CuKα_1, λ = 1.54051 Å). The final cell parameters were based on nine reflections from each compound; these parameters were not significantly different from those obtained prior to data collection. Crystal data, including the final cell parameters, are given in Table I.

Intensity data were measured by use of a 2θ scanning technique. The scanning speed was 1°/min, and a 20-sec background measurement was performed at each terminus of the scans. All unique reflections with $2\theta < 128°$ were measured, including 2279 reflections for azathioprine and 1697 reflections for 6-methylmercaptopurine. Those reflections with scan counts below background levels were given their calculated negative intensity values and were retained in all subsequent calculations.

The intensities were assigned variances, $\sigma^2(I)$, according to counting statistics plus a correctional term $(0.03S)^2$, S being the scan count. The intensities and their variances were corrected for Lorentz and polarization effects, and absorption corrections were applied by using the program ORABS (9). The data sets were scaled using Wilson (10) plots.

The trial structure of azathioprine was obtained by the heavy atom method, using sulfur as the heavy atom, and the trial structure for 6-methylmercaptopurine was obtained by direct methods, with the use of the computer program MULTAN (11). A modified version of the full-matrix least-squares program ORFLS was used

[2] Picker FACS-1.

Table II—Heavy Atom Parameters and Their Standard Deviations[a]

Atom	x	y	z	β_{11}	β_{22}	β_{33}	β_{12}	β_{13}	β_{23}
Azathioprine Dihydrate									
S	7467 (2)	2359 (1)	2230 (1)	684 (7)	41 (1)	24 (1)	49 (2)	41 (1)	7 (1)
N(1)	4199 (7)	3781 (2)	1949 (1)	439 (18)	38 (2)	20 (1)	16 (4)	16 (3)	5 (1)
C(2)	3293 (9)	4427 (2)	1533 (2)	407 (23)	40 (2)	23 (1)	4 (6)	17 (4)	1 (1)
N(3)	4123 (6)	4608 (2)	904 (1)	444 (18)	38 (2)	19 (1)	−7 (4)	4 (3)	3 (1)
C(4)	6072 (8)	4037 (2)	693 (2)	393 (21)	38 (2)	18 (1)	−26 (5)	8 (4)	0 (1)
C(5)	7167 (8)	3330 (2)	1072 (2)	402 (21)	35 (2)	20 (1)	−10 (5)	6 (4)	1 (1)
C(6)	6144 (8)	3236 (2)	1719 (2)	388 (21)	35 (2)	20 (1)	−10 (5)	−2 (4)	3 (1)
N(7)	9171 (7)	2874 (2)	705 (2)	500 (19)	45 (2)	21 (1)	14 (5)	22 (4)	−1 (1)
C(8)	9212 (9)	3308 (3)	126 (2)	468 (25)	49 (2)	22 (1)	−1 (6)	24 (4)	−5 (1)
N(9)	7442 (7)	4018 (2)	87 (2)	514 (21)	44 (2)	18 (1)	−3 (5)	10 (4)	4 (1)
C(10)	6080 (8)	2590 (2)	3021 (2)	454 (22)	31 (2)	20 (1)	18 (5)	10 (4)	4 (1)
C(11)	4253 (8)	2138 (2)	3424 (2)	467 (23)	32 (2)	20 (1)	7 (5)	2 (4)	5 (1)
N(12)	3919 (7)	2517 (2)	4041 (2)	603 (22)	47 (2)	23 (1)	4 (5)	22 (4)	3 (1)
C(13)	5568 (10)	3204 (3)	4023 (2)	660 (29)	42 (2)	22 (1)	1 (7)	9 (5)	−1 (1)
N(14)	6918 (7)	3282 (2)	3422 (2)	452 (19)	38 (2)	26 (1)	−6 (5)	12 (4)	4 (1)
C(15)	8877 (13)	3997 (3)	3249 (3)	598 (33)	46 (2)	38 (2)	−26 (8)	7 (7)	7 (2)
N(16)	2772 (8)	1344 (2)	3248 (2)	602 (23)	36 (2)	32 (1)	5 (5)	−1 (4)	7 (1)
O(17)	3382 (8)	959 (2)	2731 (2)	1056 (27)	41 (2)	34 (1)	−24 (5)	26 (4)	−4 (1)
O(18)	914 (7)	1093 (2)	3639 (2)	778 (23)	58 (2)	51 (1)	−73 (5)	70 (4)	6 (1)
O(W1)	1203 (14)	1165 (3)	822 (4)	2018 (65)	94 (4)	241 (6)	153 (12)	236 (16)	64 (4)
O(W2)	6145 (17)	340 (5)	685 (8)	1899 (77)	130 (6)	546 (16)	−23 (16)	−86 (25)	137 (8)
6-Methylmercaptopurine Trihydrate									
S	7926 (1)	2332 (1)	741 (1)	49 (1)	215 (1)	49 (1)	−5 (1)	22 (1)	−23 (1)
N(1)	5284 (1)	1946 (2)	−577 (1)	64 (1)	137 (2)	35 (1)	−4 (1)	19 (1)	0 (1)
C(2)	3928 (1)	2075 (2)	−823 (1)	64 (2)	140 (3)	34 (1)	−4 (2)	13 (1)	4 (1)
N(3)	3293 (1)	2636 (2)	−278 (1)	52 (1)	136 (2)	40 (1)	−2 (1)	14 (1)	5 (1)
C(4)	4189 (1)	3137 (2)	631 (1)	56 (1)	102 (3)	38 (1)	1 (2)	21 (1)	9 (1)
C(5)	5611 (1)	3100 (2)	983 (1)	52 (1)	114 (3)	34 (1)	−3 (2)	17 (1)	4 (1)
C(6)	6152 (1)	2462 (2)	338 (1)	54 (1)	102 (3)	38 (1)	−1 (1)	21 (1)	5 (1)
N(7)	6214 (1)	3711 (2)	1953 (1)	63 (1)	178 (3)	35 (1)	−6 (2)	20 (1)	−4 (1)
C(8)	5159 (1)	4084 (2)	2152 (1)	78 (2)	184 (3)	37 (1)	−8 (2)	29 (1)	−4 (1)
N(9)	3917 (1)	3765 (2)	1390 (1)	59 (1)	164 (3)	44 (1)	2 (2)	29 (1)	5 (1)
C(10)	8109 (2)	1489 (3)	−325 (1)	85 (2)	242 (5)	57 (1)	0 (2)	44 (1)	−10 (2)
O(W1)	375 (1)	3198 (2)	−1444 (1)	60 (1)	184 (3)	57 (1)	−6 (1)	16 (1)	2 (1)
O(W2)	1432 (1)	3908 (2)	1426 (1)	115 (2)	222 (3)	154 (2)	38 (2)	102 (1)	54 (2)
O(W3)	8956 (1)	5272 (2)	2916 (1)	67 (1)	197 (2)	46 (1)	−13 (1)	20 (1)	−2 (1)

[a] Values were multiplied by 10^4. Temperature factors are coefficients in the expression $T = \exp(-\beta_{11}h^2 - \beta_{22}k^2 - \beta_{33}l^2 - 2\beta_{12}hk - 2\beta_{13}hl - 2\beta_{23}kl)$. The values of the isotropic extinction parameter are $g = 0.040$ (6) for azathioprine dihydrate and $g = 0.079$ (3) for 6-methylmercaptopurine trihydrate.

to refine the trial structures (12, 13). The quantity minimized was $\Sigma w(F_0^2 - F_c^2/k^2)^2$, with k as a scale factor and the weight w equal to $1/\sigma^2(F_0^2)$. Scattering factors for the nonhydrogen atoms were from the "International Tables for X-ray Crystallography" (14), anomalous dispersion correction factors for these atoms were from Cromer and Liberman (15), and hydrogen-atom scattering factors were from Stewart *et al.* (16).

All hydrogen atoms except those bonded to the two water molecules in azathioprine dihydrate, which exhibited excessive thermal motion, were located in difference Fourier maps, which were calculated during the latter stages of refinement. Final cycles of refinement included all positional parameters, anisotropic temperature factors for the nonhydrogen atoms, isotropic temperature factors for the hydrogen atoms, and Zachariasen's (17) isotropic extinction parameter g [as formulated by Coppens and Hamilton (18)]. The final R index ($\Sigma||F_0| - |F_c||/\Sigma|F_0|$) is 0.079 for azathioprine dihydrate and 0.029 for 6-methylmercaptopurine trihydrate.

During the last cycle of refinement, no parameter shifted more than one-fifth of its estimated standard deviation. Final three-dimensional electron-density difference maps showed no peaks or troughs that exceeded 0.53 e/Å^3 for azathioprine dihydrate and 0.19 e/Å^3 for 6-methylmercaptopurine trihydrate.

RESULTS[3]

Table II lists the final heavy atom parameters and their estimated standard deviations. Table III gives the hydrogen atom parameters and their estimated standard deviations. For nonhydrogen atoms of azathioprine and 6-methylmercaptopurine, the estimated errors in positional coordinates are about 0.004 and 0.002 Å, respectively; for hydrogen atoms of the two purines, these errors are 0.05 and 0.03 Å, respectively.

Figure 1 shows the conformations, thermal ellipsoids, and bond lengths for azathioprine and 6-methylmercaptopurine. Bond angles are listed in Table IV. Both molecules crystallize in the N(9)-

Table III—Hydrogen Atom Parameters and Their Standard Deviations[a]

Atom	x	y	z	B, Å^2
Azathioprine Dihydrate				
H(C2)	200 (7)	481 (2)	171 (1)	2.6 (0.7)
H(C8)	1032 (8)	318 (2)	−22 (2)	4.3 (0.9)
H(N9)	710 (9)	441 (2)	−26 (2)	6.0 (1.1)
H(C13)	577 (8)	364 (2)	436 (2)	4.3 (0.9)
H(C15)	1047 (10)	382 (3)	293 (2)	6.6 (1.2)
H′(C15)	959 (10)	421 (3)	366 (2)	7.1 (1.5)
H″(C15)	770 (10)	441 (3)	301 (2)	7.4 (1.4)
6-Methylmercaptopurine Trihydrate				
H(C2)	335 (2)	167 (2)	−148 (1)	3.5 (0.3)
H(C8)	524 (1)	454 (2)	278 (1)	3.2 (0.3)
H(N9)	310 (2)	391 (3)	139 (1)	4.6 (0.4)
H(C10)	906 (2)	156 (4)	−17 (2)	7.0 (0.5)
H′(C10)	768 (2)	228 (3)	−89 (2)	6.7 (0.6)
H″(C10)	777 (2)	23 (4)	−49 (2)	7.2 (0.6)
H(W1)	3 (2)	210 (3)	−159 (1)	4.8 (0.5)
H′(W1)	110 (2)	303 (3)	−106 (2)	5.3 (0.5)
H(W2)	123 (2)	290 (3)	154 (2)	6.3 (0.6)
H′(W2)	91 (2)	469 (3)	138 (2)	6.6 (0.6)
H(W3)	818 (2)	484 (3)	264 (1)	5.0 (0.5)
H′(W3)	914 (2)	569 (3)	247 (1)	5.0 (0.5)

[a] Positional parameters were multiplied by 10^3.

[3] Tables of observed and calculated structure factors will be furnished by the authors upon request.

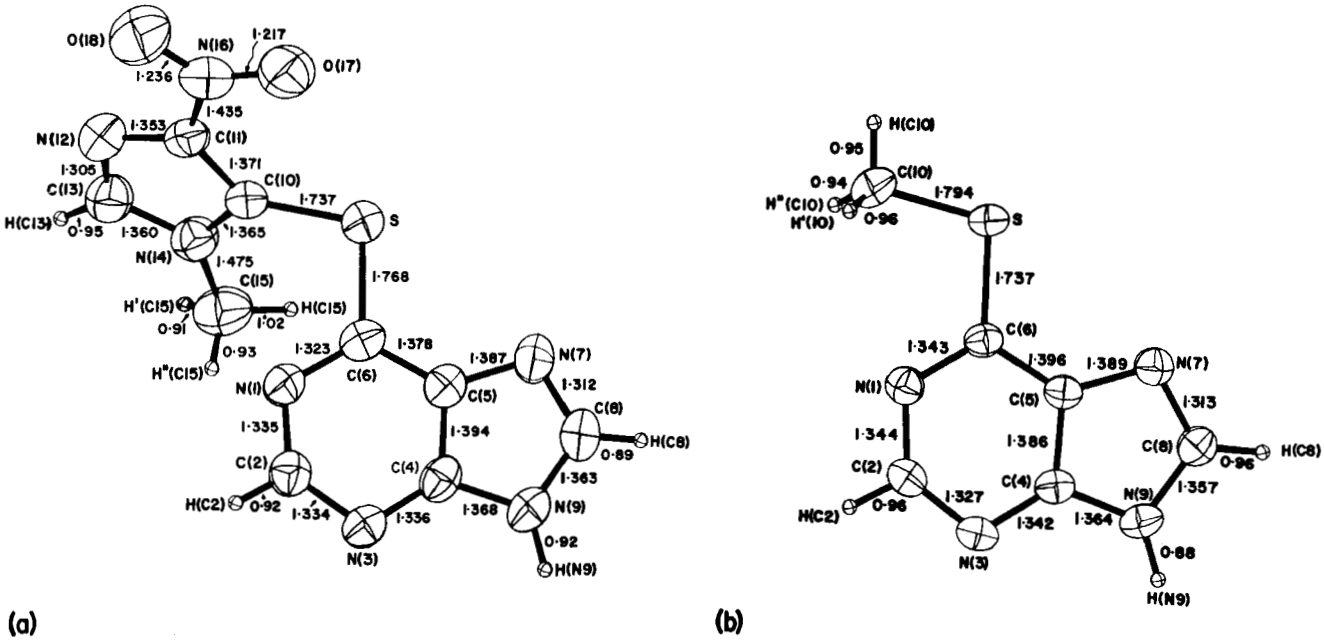


Figure 1—*Perspective view of the molecules, including bond lengths: (a) azathioprine, and (b) 6-methylmercaptopurine. Estimated standard deviations in bond lengths between nonhydrogen atoms are about 0.006 and 0.003 Å for the azathioprine and 6-methylmercaptopurine structures, respectively. Estimated standard deviations in bond lengths involving hydrogen atoms are about 0.06 and 0.03 Å in the azathioprine and 6-methylmercaptopurine structures, respectively. Nonhydrogen atoms are represented by thermal ellipsoids defined by the principal axes of thermal vibration and scaled to include 50% probability. Hydrogen atoms are represented by spheres of 0.07 Å radius. This drawing, as well as Figs. 2 and 4, was prepared by using the computer program ORTEP (32).*

H tautomer form and display a conformation in which the substituents bonded to the sulfur atom (nitroimidazolyl and methyl groups) are directed away from the imidazole moiety of the base [the S-C(10) bond is *trans* to the C(5)-C(6) bond]. It appears that nitroimidazolyl and methyl substituents exert different influences on the geometry of the purine moieties. The most significant differences between corresponding bond lengths and angles in the two structures involve the sulfur atom and/or atom C(6), and it is likely that they are due to inductive effects of the substituents.

Table IV—Bond Angles Involving Nonhydrogen Atoms[a]

	Azathioprine Dihydrate	6-Methylmercaptopurine Trihydrate
C(6)-N(1)-C(2)	117.6°	117.6°
N(1)-C(2)-N(3)	128.2°	129.0°
C(2)-N(3)-C(4)	112.0°	111.8°
N(3)-C(4)-C(5)	125.5°	125.6°
N(3)-C(4)-N(9)	128.8°	128.3°
C(5)-C(4)-N(9)	105.7°	106.1°
C(4)-C(5)-C(6)	115.8°	117.0°
C(4)-C(5)-N(7)	110.8°	110.1°
C(6)-C(5)-N(7)	133.4°	132.9°
C(5)-C(6)-N(1)	120.9°	119.0°
C(5)-C(6)-S	118.2°	119.3°
N(1)-C(6)-S	120.9°	121.6°
C(5)-N(7)-C(8)	103.0°	103.7°
N(7)-C(8)-N(9)	114.9°	114.0°
C(4)-N(9)-C(8)	105.6°	106.1°
C(6)-S-C(10)	102.1°	102.9°
C(11)-C(10)-S	132.0°	—
C(11)-C(10)-N(14)	103.5°	—
N(14)-C(10)-S	124.2°	—
C(10)-C(11)-N(12)	113.0°	—
C(10)-C(11)-N(16)	126.6°	—
N(12)-C(11)-N(16)	120.3°	—
C(11)-N(12)-C(13)	103.4°	—
N(12)-C(13)-N(14)	112.8°	—
C(13)-N(14)-C(10)	107.2°	—
C(13)-N(14)-C(15)	125.1°	—
C(10)-N(14)-C(15)	127.7°	—
C(11)-N(16)-O(17)	119.2°	—
C(11)-N(16)-O(18)	117.0°	—
O(17)-N(16)-O(18)	123.8°	—

[a] Estimated standard deviations are about 0.3° for azathioprine and 0.2° for 6-methylmercaptopurine.

The purine rings are almost planar, and none of the atoms deviates from the least-squares purine planes by more than 0.015 Å in azathioprine and 0.008 Å in 6-methylmercaptopurine. In azathioprine, atoms S and C(10) are displaced from the purine plane by 0.021 and 0.307 Å, respectively; in 6-methylmercaptopurine, these two atoms are displaced by 0.036 and 0.023 Å, respectively. The large displacement of C(10) of azathioprine is probably due to steric hindrance between the bulky nitroimidazolyl moiety and the purine ring.

The crystal-packing scheme for azathioprine is depicted in Fig. 2. Hydrogen-bond distances and angles are listed in Table V. Azathioprine molecules are joined by pairs of N(9)-H···N(3) hydrogen bonds across crystallographic inversion centers. The purine moieties are stacked in the *a* direction, with adjacent bases along *a* being separated by an interplanar spacing of 3.29 Å; the stacking pattern, as viewed perpendicular to the plane of the purine moiety, is shown in Fig. 3*a*. The water molecules, which display extremely large apparent thermal motion, are hydrogen bonded together, resulting in channels that run parallel to the *a* axis.

Figure 4 depicts the crystal-packing scheme for 6-methylmercaptopurine. Hydrogen-bond dimensions are included in Table V. The water molecules are hydrogen bonded together and to the

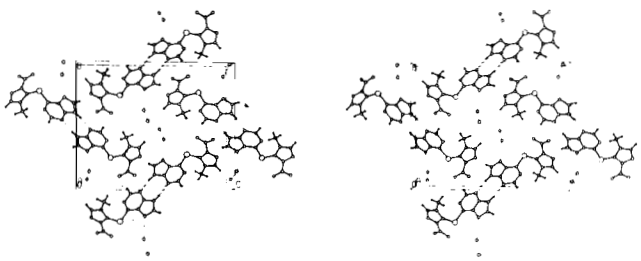


Figure 2—*Stereo drawing of the crystal packing of azathioprine dihydrate, as viewed down the* a *axis. The water molecules are represented by circles. Hydrogen bonds are depicted as fine lines.*

Table V—Hydrogen Bond Distances and Angles[a]

Donor Atom	Hydrogen Atom	Acceptor Atom	Distances, Å		Donor–Hydrogen–Acceptor Angle
			Donor–Acceptor	Hydrogen–Acceptor	
Azathioprine Dihydrate					
N(9)	H(N9)	N(3) e	2.929	2.02	169°
O(W1)[b]		N(7) b	2.810		
O(W1)[b]		O(W2) a	2.618		
O(W1)[b]		O(W2) b	2.635		
O(W2)[b]		O(W1) a	2.618		
O(W2)[b]		O(W1) c	2.635		
O(W2)[b]		O(W2) d	2.992		
6-Methylmercaptopurine Trihydrate					
N(9)	H(N9)	O(W2) a	2.689	1.81	173°
O(W1)	H(W1)	O(W3) f	2.804	1.98	171°
O(W1)	H′(W1)	N(3) a	2.893	2.15	166°
O(W2)	H(W2)	O(W3) g	2.817	2.05	169°
O(W2)	H′(W2)	O(W1) h	2.807	2.04	173°
O(W3)	H(W3)	N(7) a	2.884	2.07	179°
O(W3)	H′(W3)	O(W1) e	2.808	1.98	177°

[a] Symmetry codes—a: x, y, z; b: x − 1, y, z; c: x + 1, y, z; d: −x + 1, −y, −z; e: −x + 1, −y + 1, −z; f: x − 1, −y + ½, z − ½; g: −x + 1, y − ½, −z + ½; and h: −x, −y + 1, −z. [b] Possible hydrogen-bonded contacts. Positions of these hydrogen atoms could not be determined.

N(3), N(7), and N(9) sites. The channels of hydrogen-bonded water molecules run in the *b* direction. There are no hydrogen bonds between molecules of 6-methylmercaptopurine. The bases form planar ribbons running in the *c* direction and lying nearly parallel to the *ac* plane. The ribbons of bases are stacked in the *b* direction, with interpurine stacking distances of 3.33 and 3.24 Å. The stacking pattern is shown in Fig. 3*b*.

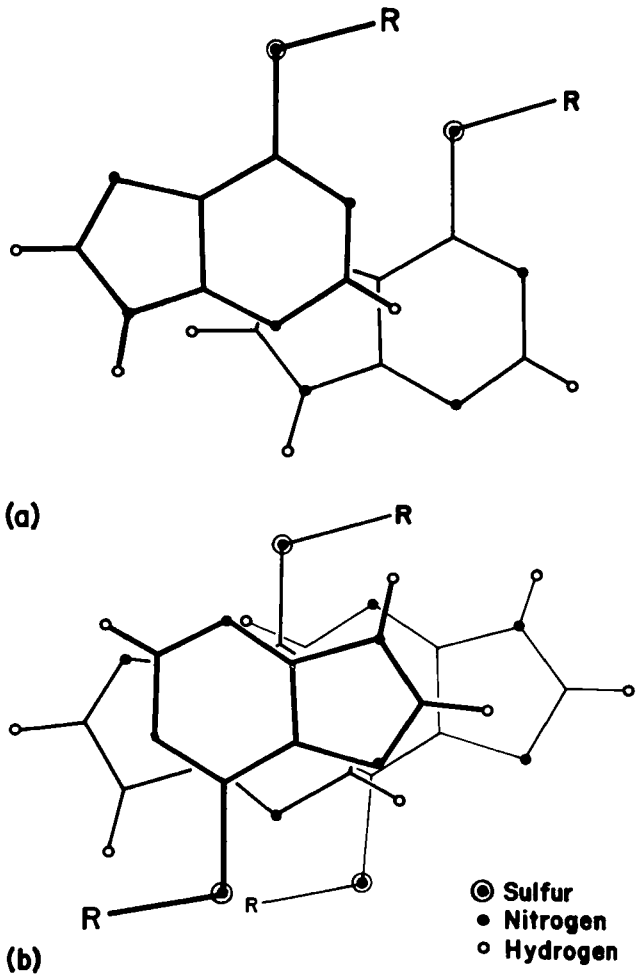


Figure 3—*Base stacking patterns, as viewed perpendicular to the planes of the purine rings. Key: (a), azathioprine; and (b), 6-methylmercaptopurine.*

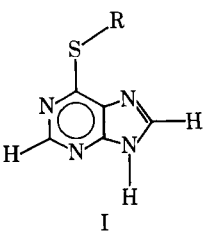
DISCUSSION

Mercaptopurine derivatives with a substituent bonded to the sulfur atom would be expected to assume one of two conformations that are related to each other by a rotation of 180° around the C(6)-S bond. To maintain partial double-bond character in the C(6)-S bond, the mercapto group must be approximately coplanar with the purine moiety; therefore, the substituent must point either toward (Structure I) or away from (Structure II) the imidazole ring of the base.

Examination of N^6-methyladenine (19) and other N^6-monosubstituted adenine derivatives (20–22) revealed that even relatively small aliphatic groups at the N(6) position tend to point away from the imidazole ring [*trans* to the C(5)-C(6) bond]. Molecular orbital calculations for N^6-monosubstituted adenine derivatives indicate that this *trans*-conformation is the more stable (23), and examination of space-filling molecular models suggests that the alternate conformation (in which the substituent is positioned *cis* to the imidazole moiety) is destabilized by interference between the substituent and atom N(7). Molecular models of azathioprine and 6-methylmercaptopurine show that similar interactions occur between atom N(7) and the substituents of these compounds when the substituents are *cis* to the imidazole moiety, so it is not surprising to find that both azathioprine and 6-methylmercaptopurine assume the *trans*-conformation depicted in Structure II.

Azathioprine and 6-methylmercaptopurine both crystallize in the N(9)-H tautomer form, the same tautomer found in crystals of N^6-(Δ^2-isopentenyl)-2-methylmercaptoadenine (24), another derivative that contains a substituent bonded to the sulfur atom. In contrast, 6-mercaptopurine (25, 26), 6-thioguanine (27), and 2-thio-6-methylpurine (28, 29), all of which possess unsubstituted thio groups, crystallize as N(7)-H tautomers.

It appears that the sulfur atoms of 6-methylmercaptopurine and azathioprine may be poor hydrogen bond acceptors, since neither of these atoms participates in hydrogen bonding in the crystal structures. Similarly, in the crystal structure of N^6-(Δ^2-isopentenyl)-2-methylmercaptoadenine (24), the sulfur atom is not in-



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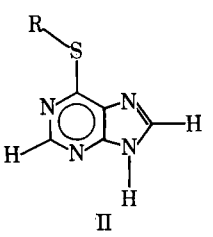

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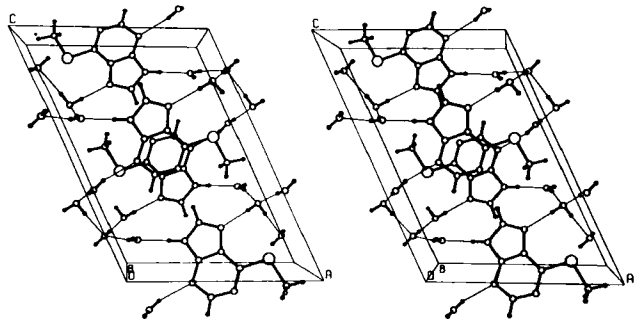

Figure 4—*Stereo drawing of the crystal packing of 6-methylmercaptopurine trihydrate, as viewed down the* b *axis. Hydrogen bonds are depicted as fine lines.*

volved in hydrogen bonding. In contrast, the unsubstituted thio groups are hydrogen bond acceptors in the crystal structures of 6-mercaptopurine (25, 26), 6-thiopurine riboside (30), 6-thioguanine (27), 6-thioguanosine (31), and 2-thio-6-methylpurine (28, 29). These crystallographic results suggest that the addition of substituents to the sulfur atom of 6-mercaptopurine may have an appreciable effect on the hydrogen-bonding properties of the base.

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Reduction of Blue Tetrazolium by Corticosteroids

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Abstract □ Pseudo-first-order rate constants were observed for the reaction of corticosteroid or corticosteroid esters with blue tetrazolium. The data indicate that the reactivity of corticosteroids is, in part, a function of their geometry in that corticosteroid reactivity toward blue tetrazolium increases with increasing planarity of the steroid molecule and that corticosteroid esters must be hydrolyzed as a necessary prerequisite to reaction with blue tetrazolium. Evidence is presented indicating that free radicals are not involved in the blue tetrazolium reaction with corticosteroids. Certain pharmaceutically important compounds such as pyrocatechol derivatives and hydroquinone appear to reduce blue tetrazolium by the anion free radical mechanism proposed previously. A spectrophotometric method for determining the number of reduction units transferred to blue tetrazolium per molecule is described.

Keyphrases □ Blue tetrazolium—reduction by corticosteroids, pseudo-first-order rate constants, mechanism discussed □ Corticosteroids—reduction of blue tetrazolium, pseudo-first-order rate constants, mechanism discussed □ Steroids—reaction of corticosteroid and corticosteroid esters with blue tetrazolium, mechanism

The blue tetrazolium reaction (1) is widely used for the analysis of corticosteroids and is, with slight modification, the official procedure [USP XVIII (2) and NF XIII(3)]. In very basic alcoholic solution, the C_{17} side chain of the corticosteroid is oxidized and blue tetrazolium (I) is reduced to a colored formazan whose concentration is measured spectrophotometrically. Extensive investigations (4–16) of the reaction conditions established that the analytical procedure is subject to many variables, which are minimized by analyzing blank, standard, and sample solutions concurrently.

Kinetic and mechanistic studies, although not extensively investigated, revealed certain important aspects of the blue tetrazolium reaction. For example, the rate of the reaction of cortisone and hydrocortisone with I was found to be first order in the corticosteroid (17). Of these two corticosteroids, cortisone was observed to be the more reactive toward I.

In that study, it was also established that ester hydrolysis of cortisone acetate was a prerequisite to reaction with I. Significant variations in the reaction rates of corticosteroids of closely related structures with I have been noted (6, 13–15, 18).

Meyer and Lindberg (18) established that the α-ketol moiety in corticosteroids and other α-ketol-containing compounds is responsible for the reduction of I and that the electron-donating ability of substituent groups may be correlated with the rate of formazan development.

Sinsheimer and Salim (19) showed that some nonketol compounds such as polyhydric phenols, quinones, and certain active hydrogen compounds are sufficiently reactive to permit quantitative analysis *via* formazan formation. They proposed a free radical mechanism based on similar structural reactivity in the blue tetrazolium reaction to the reaction of the same compounds toward oxygen.

This paper shows that the reduction of blue tetrazolium by corticosteroids and several other compounds occurs as a unit function. The difference in reaction rates of several corticosteroids is related to their differences in structure. The results are consistent for the 25 steroids studied.

EXPERIMENTAL

Apparatus—The following were used: a UV–visible ratio recording spectrophotometer[1] with 1-cm quartz cells, an electrobalance[2], a computer[3], an electron-spin resonance instrument[4], and a TLC apparatus[5].

Reagents—Alcohol USP, absolute methanol, acetic acid, and 50% hydrogen peroxide were all analyzed reagent grade. The 1% tetramethylammonium hydroxide (II) was prepared by diluting 5.00 ml of 10% aqueous[6] II to 50.0 ml with alcohol USP (95% ethanol). Solutions of iodine, 0.010 mg/ml in alcohol USP, and blue tetrazolium[7], 5.0 mg/ml in absolute methanol, were prepared. Corticosteroid standard solutions contained 0.010 mg/ml in alcohol USP unless otherwise indicated.

General Procedure—The procedure followed, unless otherwise specified, is the official procedure given in USP XVIII (2), in which a 20.00-ml volume of standard or sample corticosteroid in alcohol USP is treated with 2.00 ml of I reagent (5 mg/ml), followed by 2.00 ml of 1% II. The absorbance is measured against a reagent blank 90 min after the addition of II.

Corticosteroid Decomposition by II—Duplicate 20.0-ml aliquots of betamethasone, betamethasone 17-benzoate, betamethasone 21-benzoate, and dichlorisone acetate were pipetted into glass-stoppered flasks, and 2.00 ml of 1% II was added to one set. Both sets were then stoppered and placed in the dark for 24 hr. Two milliliters of I (5 mg/ml) were added to the set to which II had been added, and the solution was scanned for 90 min after the addition of I. The general procedure was followed in the other set.

Effect of Water on Blue Tetrazolium Reagent-.Five-milliliter aliquots of I were diluted 1:1 with water or with alcohol USP and allowed to stand for 16 hr. A blank and triplicate aliquots of hydrocortisone were run in accordance with the general procedure except that the diluted I was used.

Oxygen Effect—Six 20.0-ml aliquots of alcohol USP were pipetted into 25-ml glass-stoppered flasks. Oxygen was bubbled through two aliquots and simultaneously through glass-stoppered cylinders containing I and II for 20 min. This procedure was repeated for two other aliquots using nitrogen instead of oxygen. The remaining two air-saturated aliquots were used as controls.

All six aliquots were run by the general procedure, except that the saturated samples of I and II were used with the respective saturated aliquots and all samples were scanned against alcohol USP instead of a reagent blank.

Electron-Spin Resonance Study—Cortisone (0.100 mg/ml in alcohol USP) was treated by the general procedure. Immediately after the addition of 1% II, the sample was scanned in an electron-spin resonance spectrometer. After the scan, the solution was

[1] Cary model 15.
[2] Cahn model G-2.
[3] Digital Equipment Corp. PDP/8e and IBM 360 model 25.
[4] Varian model 4502-13.
[5] Eastman 6060 silica gel with fluorescent indicator.
[6] Eastman Organic Chemicals.
[7] Dajac Laboratories.

Table I—Decomposition of Selected Corticosteroids by Tetramethylammonium Hydroxide

Corticosteroid	Source[a]	Amount, mg	Absorbance: Decomposed Steroid[b]	Absorbance: Control	Decomposition[c], %
Betamethasone	1	0.202	0.195	0.479	59
Betamethasone 17-benzoate	2	0.241	0.193	0.422	54
Betamethasone 21-benzoate	2	0.243	0.187	0.414	55
Dichlorisone acetate	3	0.203	0.078	0.453	83

[a] 1 = NF Reference Standard; 2 = Warner Lambert Co., Morris Plains, N.J.; 3 = Division of Pharmaceutical Sciences, Food and Drug Administration, Washington, D.C. [b] Steroid plus 1% II was mixed for 24 hr before quantitation. [c] Calculated as:

$$100 - \frac{\text{absorbance of decomposed steroid}}{\text{absorbance of control}} \times 100$$

swept with nitrogen gas for 20 min and rescanned for 40 min from the time of addition of 1% II. Cortisone (1.00 mg/ml in alcohol USP) was treated similarly, except that the second scan occurred 90 min after the addition of 1% II. Scans were also made of alcohol USP, alcohol USP plus I, and alcohol USP plus I and 1% II.

Rate Studies—A 20.0-ml aliquot of a standard and a 20.0-ml blank of alcohol were treated by the general procedure, with zero time taken as the time of the addition of 1% II to the standard solution. Both solutions were transferred to cells as rapidly as possible and placed in the spectrophotometer, and readings were made each minute at 525 nm until the reaction reached essential completion.

The procedure was repeated for a total of 25 different corticosteroids. Rate constants were calculated by a computer program. This procedure was repeated for several noncorticosteroid steroids and other selected pharmaceutical compounds.

Hydrolysis of Betamethasone 17-Benzoate—Duplicate 20.0-ml aliquots of the betamethasone and betamethasone 17-benzoate standards were treated with 2.00 ml of 1% II and allowed to stand for 75 and 135 min, respectively; the reaction was terminated by the addition of 1.00 ml of acetic acid. The solutions were washed into a separator with 75 ml of water and extracted five times with 20.0-ml portions of chloroform.

Each extract was washed through a second separator containing 15 ml of water and filtered through chloroform-washed cotton, and the combined extract was taken to dryness carefully under air on the steam bath. The residues were dissolved in 0.2 ml of methanol, and 1 μl was spotted with standards at the same concentrations on a TLC plate and developed with chloroform–methanol (36:1).

Effect of Free Radical Reaction Inhibitor—One set of 10.0-ml aliquots of hydrocortisone (0.020 mg/ml in alcohol USP), fluoxymesterone (0.020 mg/ml in alcohol USP), hydroquinone (0.010 mg/ml in alcohol USP), and isoproterenol sulfate (0.006 mg/ml in alcohol USP) was mixed with 10.0 ml of iodine reagent, and another set was mixed with 10.0 ml of alcohol USP. Both sets were run *versus* a blank by the procedure discussed under *Rate Studies*.

RESULTS AND DISCUSSION

Experiments designed to determine the extent of reactions between corticosteroids and/or formazan with the ingredients of the reaction medium were carried out with typical compounds before rate studies were attempted.

Table I shows the results of the study of the interaction of a typical corticosteroid and corticosteroid esters with II. Since both the corticosteroid and its esters were partially decomposed after 24 hr of contact with II, the reaction of II evidently occurs also at the C_{17} side chain and competes with I during the usual 90-min reaction time. For the longer periods needed in some of the rate studies, increased base degradation and less formazan formation are expect-

Table II—Rate Constants and Absorbance per Micromole for Selected Corticosteroids

	Source[a]	T_i[b], min	A'[c]	k[d]	Relative Rate[e]	Time to A_{max}, min	Maximum Absorbance	Absorbance per Micromole
Betamethasone	1[f]	71	0.300	0.024	3.2	370	0.460	0.894
Flurandrenolide	4	40	0.454	0.056	7.4	90	0.597	1.161
19-Norhydrocortisone	6	40	0.515	0.072	9.5	90	0.597	1.012
Hydrocortisone	5	34	0.545	0.090	12	60	0.596	1.076
Prednisolone	5	34	0.548	0.092	12	130	0.610	1.092
Triamcinolone	5	38	0.999	0.093	12	90	1.112	2.193
11-Desoxycortisone	7	36	0.548	0.094	12	90	0.572	0.990
Methylprednisolone	1[f]	28	0.502	0.096	13	70	0.549	1.007
5-Pregnen-3β,21-diol-20-one	7	33	0.479	0.096	13	90	0.516	0.857
Corticosterone	7	32	0.531	0.098	13	90	0.579	1.010
Dexamethasone	1[f]	34	0.519	0.098	13	50	0.544	1.069
11-Desoxycorticosterone	7	31	0.575	0.109	14	90	0.616	1.011
Fluprednisolone	1[f]	25	0.497	0.121	16	61	0.547	1.034
Cortisone	8	16	0.557	0.243	32	90	0.583	1.045
Dihydrocortisone	7	18	0.560	0.257	34	60	0.566	1.017
Prednisone	5	13	0.556	0.305	40	31	0.568	1.012
Betamethasone 17-benzoate	2[f]	90	0.130	0.0076	1	394	0.441	0.913
Betamethasone 21-benzoate	2[f]	90	0.133	0.017	2.2	470	0.438	0.878
Hydrocortisone acetate	5	36	0.479	0.081	11	60	0.520	1.046
Prednisolone acetate	5	33	0.473	0.086	11	90	0.517	1.030
11-Desoxycorticosterone acetate	5	33	0.499	0.090	12	60	0.563	1.048
Dichlorisone acetate	3[f]	29	0.451	0.102	13	90	0.477	1.070
Dihydrocortisone acetate	7	20	0.490	0.200	26	40	0.503	1.009
Cortisone acetate	5	18	0.501	0.230	30	90	0.528	1.056
Prednisone acetate	7	15	0.490	0.307	40	90	0.497	0.998

[a] 4 = Eli Lilly and Co., Indianapolis, Ind.; 5 = USP Reference Standard; 6 = Syntex Research, Palo Alto, Calif.; 7 = Schwarz/Mann, Orangeburg, N.Y.; 8 = K and K Laboratories, Inc., Plainview, N.Y. [b] T_i is time in minutes of observation for calculation of rate constant. [c] A' is optimum absorbance for calculation of rate constant. [d] k is rate constant. [e] Compared to betamethasone 17-benzoate, which has the slowest rate. [f] See Table I for source.

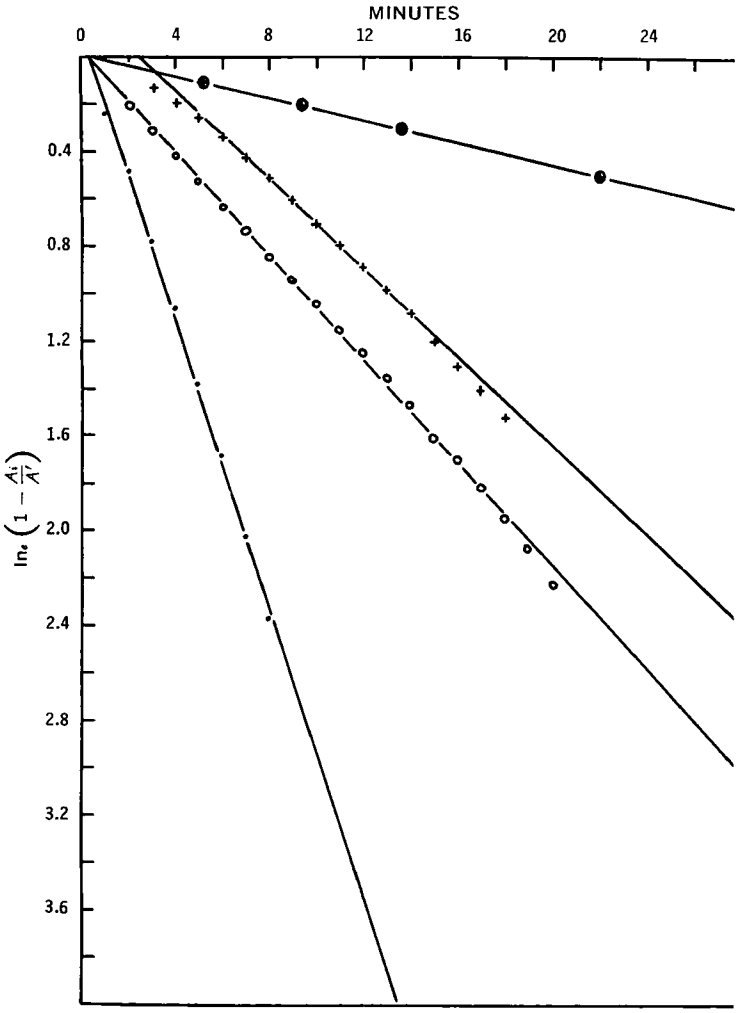

Figure 1—*Reaction rates of typical corticosteroids. Key: ⊕, betamethasone; +, 11-desoxycorticosterone acetate; ○, 11-desoxycorticosterone; and ●, prednisone.*

ed. As an example, the time necessary to attain a maximum absorbance of 0.460 for betamethasone is 370 min, whereas an equimolar amount of flurandrenolide reaches a maximum absorbance of 0.597 in 90 min.

Triplicate aliquots of hydrocortisone run by the general procedure, using a 1:1 dilution of the solution of I prepared for the study with water, gave absorbances of 0.561, 0.561, and 0.562, while the control group with a 1:1 dilution of the same solution of I with alcohol USP gave absorbances of 0.577, 0.577, and 0.576. The results indicate that water does not cause decomposition of I; the 3% decrease in sensitivity probably is due to the apparent pH effect of water (16).

The results of the study of the effect of oxygen on the blue tetrazolium blank showed that the average net absorbance of duplicates run *versus* alcohol USP as the blank was 0.070 for the air-saturated control, 0.078 for the oxygen-saturated solutions, and 0.084 for the nitrogen-saturated solutions. Under the conditions studied, oxygen apparently does not contribute to the blank in the blue tetrazolium reaction.

In addition, the blue tetrazolium–corticosteroid reaction was monitored by electron-spin resonance. The electron-spin spectrum showed the presence of an unpaired electron, which was a function of the concentration of the cortisone used and which did not change during the 90 min required for the reaction of cortisone with I to go to completion. This indicates that the unpaired electron was not involved in the production of formazan.

The results of the rate study of the blue tetrazolium reaction with corticosteroids are shown in Table II. Pseudo-first-order rate constants were observed for all 25 corticosteroids. The rate constants were obtained by performing a least-squares regression on the following equation:

$$\ln_e\left(1-\frac{A_i}{A'}\right) = kT_i + E \qquad \text{(Eq. 1)}$$

where A_i is the absorbance at any time (T_i), A' is the optimum absorbance, k is the rate constant, and E is the intercept. The intercept for a first-order equation should be zero, but the intercept of the regression line is shifted due to the time lag caused by the addition of the last reagent. The index of determination (R^2) for the regressions was greater than 0.99 in most cases.

The rate constants listed in Table II range from 7.6×10^{-3} for betamethasone 17-benzoate to 3.07×10^{-1} for prednisone acetate, which indicates that prednisone acetate reacts 40 times faster than betamethasone 17-benzoate.

The reaction of I with 0.55 μmole of hydrocortisone in the presence of 0.41 μmole of iodine showed an absorbance of 0.412 after 22

Table III—Reduction Units Furnished by Noncorticosteroid Pharmaceuticals

Compound	Source[a]	Sample, μmoles	Reaction Time	Maximum Absorbance	Absorbance Per Micromole	Number of Reduction Units[b]
Hydroquinone	9	0.1101	5 hr	1.107	1.179	1.14
Epinephrine bitartrate	8[c]	0.2934	10 min	0.931	3.173	3.08
Isoproterenol sulfate	3[c]	0.0692	30 min	0.281	4.061	3.94
Isoproterenol hydrochloride	5[c]	0.2077	40 min	0.784	3.774	3.66

[a] 9 = Fisher Scientific Co., Fairlawn, N.J. [b] Calculated as 1.031 absorbance units equals 1 reduction unit. [c] See previous tables for source.

Table IV—Molar Absorptivity of Selected Compounds

Compound	Source[a]	Absorbance	Concentration, g/liter	Molecular Weight	Molar Absorptivity[b]	Number of Reduction Units
Hydrocortisone	5	0.596	0.01004	362.47	21,500	1
Hydroquinone	9	1.070	0.004995	110.11	23,600	1
Triamcinolone	5	1.112	0.0100	394.45	43,900	2
Epinephrine bitartrate	8	0.931	0.004890	333.29	63,500	3
Isoproterenol hydrochloride	5	0.784	0.002573	247.70	75,600	4
Isoproterenol sulfate	3	0.281	0.001008	291.31	81,200	4

[a] See previous tables for source. [b] Maximum error is 1.75% as determined by National Bureau of Standard Calibration.

min, while the absorbance of a duplicate without iodine was 0.408 for the same time. Evidently, the reaction rate of hydrocortisone with I is not inhibited by iodine, even though iodine is a radical scavenger which inhibits radical reactions. This failure to inhibit suggests that the oxidation of corticosteroids by I does not proceed by a free radical mechanism.

The reactivity of the corticosteroids toward I appears to be, at least in part, a function of their molecular shape. Steroids with C_{16} substitution in either the α or β position react the slowest, steroids that possess a planar A ring and have sp^3 C_{11} substitution show intermediate reactivity, and steroids possessing a C_{11} carbonyl react the fastest with I. Thus the more planar the steroid molecule, the faster is the reaction with I.

This trend, coupled with the electron-spin resonance experiment, is evidence that a bimolecular reaction between I and the corticosteroid occurs in which a reduction unit from the ketol group is transferred from the steroid to I to form the formazan. In addition, the approach of the blue tetrazolium molecule occurs presumably at the β face of the steroid since greatest rate retardations are observed for steroids having β substitution near the ketol group (*e.g.*, C_{16}).

The rates of reaction of several corticosteroid esters with I were also studied (Table II). Graphical representation of typical kinetic data (Fig. 1) indicates that ester hydrolysis occurs prior to and during the early stages of formazan development, thus substantiating Guttman's (17) similar observations in the case of cortisone acetate. In addition, TLC analysis of a partially completed (25%) reaction mixture of betamethasone 17-benzoate and I revealed that the ester had been completely converted to betamethasone and its base decomposition products.

The order of reactivity of the esters listed in Table II parallels that of the corresponding corticosteroids toward I. Overall reaction rate constants are listed in Table II. The deviation between the rate constant for corticosteroids and their corresponding esters other than betamethasone reflects the generally small contribution of the hydrolysis rate to the overall reaction rate constant, k.

Calculation of the absorbance per micromole of most of the corticosteroids listed in Table II yields an average value of 1.031 ± 0.056, which corresponds to the transfer of one reduction unit. The three betamethasones were not included in the average since the long period required for their reactions with I resulted in low absorbance values due to extensive basic decomposition of the steroid.

The absorbance per micromole value (2.193) for triamcinolone suggests that two reduction units are transferred per molecule of triamcinolone. Transfer of the second reduction unit probably arises from the formation of a second α-ketol group during the base degradation of the initially formed glyoxal derivative (20, 21), which contains both C_{16} hydroxyl and C_{17} carbonyl groups.

Several pharmaceutically important compounds known to reduce I (22) were tested (Table III). These results indicate that hydroquinone, epinephrine, and isoproterenol sulfate reduce 1, 3, and 4 equivalents of I, respectively. The high reduction value for hydroquinone (1.14 units) may be due to oxidative cleavage of the quinone ring after the reduction is complete, as suggested by Sinsheimer and Salim (19).

The reaction with epinephrine bitartrate was complete in 10 min and thus was not subject to basic degradation by II under the conditions of the general procedure. Also, an aliquot of alcoholic epinephrine bitartrate produced no adrenochrome when treated with II for 24 hr, but addition of I to this solution after 24 hr produced a formazan with typical spectrum. This finding suggests that the absorbance value of 1.058/reduction unit is close to the true value. The reaction of I with hydroquinone and with isoproterenol sulfate was greatly inhibited by the presence of iodine, which indicates that the anion intermediate radical mechanism proposed (19) takes place in these systems.

The results of the study of the effect of 0.41 μmole of iodine on the reduction of I by 0.59 μmole of fluoxymesterone by the general procedure showed no inhibition of the rate of reaction during the first 30 min or after 20 hr when compared with an equimolar solution of fluoxymesterone containing no iodine. Androst-4-ene-3,17-dione-type steroids have been shown (18) to reduce I. In that study, androst-4-ene-3,17-dione was observed to be oxidized by I to a C_6 carbonyl derivative *via* the corresponding C_6 alcohol intermediate.

Table IV shows molar absorptivity values calculated by the method given in USP XVIII (2, p. 825). It appears that there is a linear relationship between the molar absorptivity and the number of reduction units involved in the reaction, with the molar absorptivity being approximately 20,000 for each unit.

The nature of the formazan produced in these reactions and the effect of solvent on the rate of reaction of I with various steroids and pharmaceutically important compounds are currently being investigated.

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Reactions of 2,2-Dimethylaziridine-Type Alkylating Agents in Biological Systems II: Comparative Pharmacokinetics in Dogs

DAVID LALKA x, W. J. JUSKO, and T. J. BARDOS

Abstract □ Pharmacokinetic studies in dogs were performed with a series of three 2,2-dimethylaziridine antineoplastic agents. These alkylating agents were relatively unstable in all aqueous media, with half-lives of 9–15 min in whole canine blood. This finding indicates that the aziridine compounds are capable of undergoing destruction in all biological tissues. A pharmacokinetic analysis of the plasma concentration *versus* time data was performed using a two-compartment open model, which includes drug elimination from both compartments. The three aziridine compounds showed similar pharmacokinetic behavior with respect to their loss from plasma, but they exhibited differences in their *in vivo* release of a potent alkylating intermediate (2,2-dimethylaziridine) and a second product, which was a strong inhibitor of pseudocholinesterase.

Keyphrases □ 2,2-Dimethylaziridines—reactions in biological systems, comparative pharmacokinetics in dogs □ Aziridines—2,2-dimethylaziridine-type alkylating agents, reactions in biological systems, comparative pharmacokinetics in dogs □ Antineoplastic agents—reactions of 2,2-dimethylaziridine-type alkylating agents in biological systems, comparative pharmacokinetics in dogs □ Pharmacokinetics—2,2-dimethylaziridine-type alkylating agents in biological systems, dogs

The 2,2-dimethylaziridine-type alkylating agents ethyl bis(2,2-dimethylaziridinyl)phosphinate (I), bis(2,2 - dimethylaziridinyl)phosphinylurethan (II), and tris(2,2-dimethyl-1-aziridinyl)phosphine oxide (III) have been shown to possess significant antitumor activity in a number of animal systems (1–3) and in humans (4–6). One characteristic chemical property is their unusually rapid hydrolysis with concomitant loss of their alkylating activity (7).

Since the effectiveness of an alkylating agent is probably a function of its reactivity toward various nucleophilic targets and of its local concentration and contact time with these targets, the *in vivo* activity of these particularly unstable alkylating agents is an interesting phenomenon and suggested that a study be made of the pharmacokinetic behavior of these drugs. The plasma concentration–time curves for such agents represent useful data for the scientific application of these drugs to the treatment of neoplastic diseases. Furthermore, a pharmacokinetic study of a series of agents with related structures could provide useful information for the design of new antineoplastic drugs.

This report describes the pharmacokinetic behavior of I–III in the dog following various routes of administration and the identification of 2,2-dimethylaziridine (IV) as part of the metabolic pathway of I and III in the plasma of dogs.

EXPERIMENTAL

Materials—The reagents used in the stability studies, pharmacokinetic studies, and analytical procedures were purchased and/or purified as described previously (7).

Analytical Procedures—The measurement of alkylating activity and the quantitative analysis of samples were performed with 4-(*p*-nitrobenzyl)pyridine, using the previously reported procedures (7).

Stability Studies—The rate of loss of I–III in various buffers, urine, plasma, and whole blood was studied. The methodology used was described previously (7).

Pharmacokinetic Studies—Male mongrel dogs, 20–28 kg, were fasted for approximately 18 hr before drug administration. The animals were lightly anesthetized with pentobarbital sodium, and one cephalic vein was catheterized for the collection of blood. Preliminary studies showed that the 2,2-dimethylaziridine compounds were stable in alkaline solution. Therefore, they were prepared for injection by dissolution in 5 ml of 0.05 *M* Na_2CO_3 (pH adjusted to 11.0) or 10^{-3} *M* KOH.

All three compounds were administered by intravenous injection, while II was also given by intramuscular injection into the gluteal muscle and by injection into the peritoneal cavity. Pretreatment with proadifen hydrochloride[1] [2-(diethylamino)-ethyl-2,2-diphenylvalerate hydrochloride] involved a dose of 20 mg/kg sc 1 hr before the administration of II.

Sample collection (sufficient blood to perform two to five determinations) was accomplished quickly (in about 20 sec) because of the rapid rate of change of plasma drug concentration. The time of sampling was assumed to be the midpoint of this brief collection interval. The catheter was maintained open by filling it with a heparin solution (100 units/ml in 0.9% NaCl). The contents of the catheter were discarded before each sample was drawn. The other mechanical features of sample handling were performed as described previously (7).

RESULTS

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RESULTS

Stability Studies—The *in vitro* stability of I–III was measured in various buffers and biological media, using a broader varia-

Table I—Rate of Destruction of Compounds I–III in Various Media

Medium, Temperature, pH	Half-Life, min I	II	III
Heparinized human blood, 36°, 7.36[a]	17	11	7
Heparinized human blood, 25°, 7.36	—	—	39
Heparinized human plasma, 37°, 7.36	—	—	26
Citrated human plasma, 36°, 7.38	32	32	25
Citrated human plasma, 25°, 7.40	—	92	—
Heparinized canine blood, 36°, 7.48[a]	15	12	8
Heparinized canine plasma, 36°, 7.48	48	42	28
Human urine, 37°, 7.80[b]	44	31	22
Sodium phosphate, 0.066 *M*, 36°, 7.50	42	23	—
Sodium bicarbonate, 0.60 *M*, 25°, 7.80[a]	136	103	60
Sodium hydroxide, 0.001 *M*, 25°, 10.9	>153	>170	>60
Potassium hydrogen phthalate, 0.01 *M*, 25°, 5.0[b]	2.0	1.0	<0.5

[a] TLC examination of samples drawn at various times indicated that at least 95% of the alkylating species was parent compound. The trace alkylating substance occasionally observed after incubation had the chromatographic characteristics of 2,2-dimethylaziridine. [b] A few percent of alkylating activity remained after more than 10 half-lives of parent compound. In this case, TLC studies confirmed that 2,2-dimethylaziridine was the residual alkylating species.

tion of experimental conditions than in previous studies (7) to assess factors that might effect their *in vivo* destruction. As shown in Table I, they react rapidly in plasma, urine, and whole blood. The shorter half-time in whole blood as compared to plasma suggests that cells can provide nucleophilic targets for reaction with these agents.

The data from the experiments in buffer solutions show that compounds of this series behave similarly in that they are very unstable in acidic media (pH 5) but do not undergo measurable hydrolysis in 3 hr at pH 10.8. For this reason, these drugs were prepared for injection in alkaline vehicles and the blood samples were alkalinized quickly after collection.

Pharmacokinetic Studies—The time course of plasma concentrations of I following intravenous administration to three dogs is shown in Fig. 1. The results obtained in the individual animals are nearly identical and show rapid, biphasic disposition with a β-phase half-life of about 7.3 min. Compounds II and III behaved in a similar manner (Figs. 2 and 3).

The metabolic inhibitor[1] was also administered to a dog receiving II. As shown in Fig. 2, there was essentially no difference in plasma levels of II in pretreated *versus* unpretreated animals. This suggests that microsomal oxidative enzymes do not play a major role in the biotransformation of II. In fact, the stability data in various biological media (Table I) suggest that these aziridine compounds are eliminated primarily by hydrolysis and chemical reaction with cellular constituents.

One dog was given half of the usual dose of II (Fig. 2). The plasma concentrations of the drug were almost exactly 50% of those observed following the normal dose (12 mg/kg). This suggests that, over the clinically useful range (4–6), the pharmacokinetic behavior of this compound is not dose dependent in the mongrel dog.

Pharmacokinetic Analysis—The plasma concentration, C_p, *versus* time, t, data observed following intravenous administration of the drugs were computer fitted to the biexponential equation:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

by nonlinear least-squares regression analysis (8). The parameters

[1] SK&F 525-A.

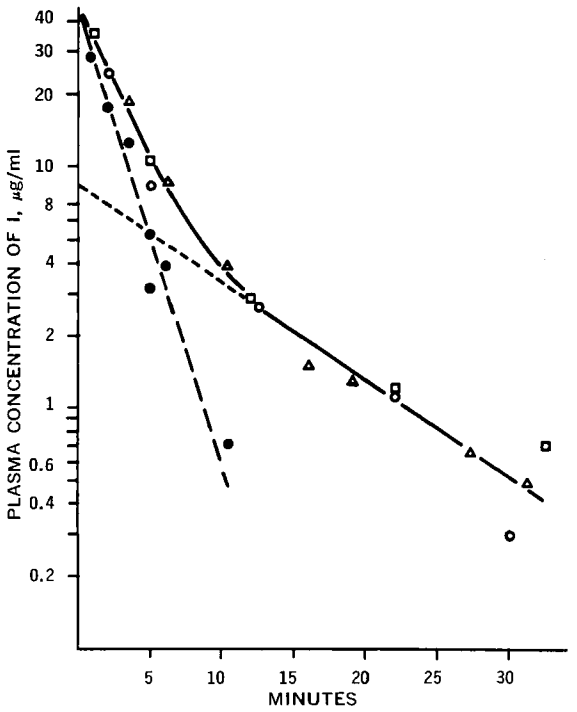


Figure 1—*Time course of I in plasma following rapid intravenous administration of 12 mg/kg to three dogs. Key: □, Dog 1; ○, Dog 2; △, Dog 3; and ●, points obtained by the method of residuals. The curves were obtained by computer least-squares regression of all data simultaneously.*

A and B are zero-time intercepts and α and β are disposition slope constants. The solid lines in Figs. 1–3 show the least-squares computer fit to the experimental data, and the calculated parameters are listed in Table II. The 95% confidence limits for the plasma half-life of I ($t_{1/2\beta}$ = 7.3 min) in the β-phase are 4.2 and 10.4 min.

The pharmacokinetic model chosen for further analysis of these data is depicted in Scheme I. A two-compartment system was selected because the plasma concentration data were biexponential, showing both distribution and elimination phases. Drug loss from both the peripheral (rate constant k_r) and central (rate constant K_{el}) compartments was incorporated into this model because, as

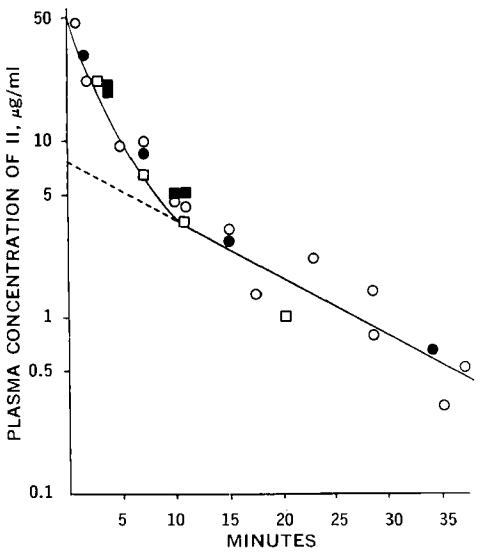


Figure 2—*Time course of II in plasma following intravenous administration of 12 mg/kg (○), 12 mg/kg following pretreatment with proadifen hydrochloride (□), 6 mg/kg normalized to 12 mg/kg (●), and multiple doses of 12 mg/kg (■) treated by the method of superposition.*

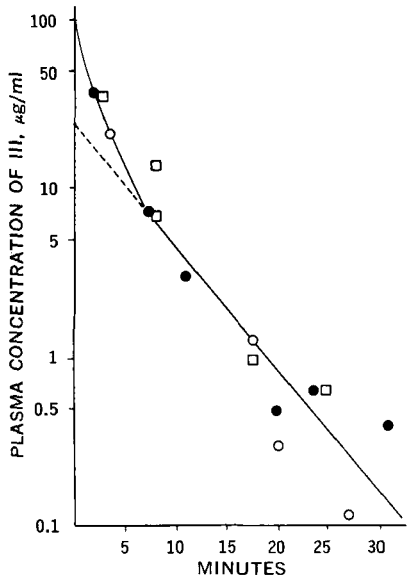


Figure 3—*Time course of plasma levels of III following intravenous administration of 24 mg/kg to three dogs. Key: □, Dog 1; ●, Dog 2; and ○, Dog 3. The curve represents the nonlinear least-squares computer fit of the data.*

shown in Table I, these aziridine compounds are destroyed in all aqueous media under physiological conditions.

A detailed solution to this type of model was evolved recently (9). Therefore, the *Appendix* contains only the equations that provide numerical parameters of interest with regard to the pharmacokinetics of I–III and of other alkylating agents (10–12).

The least-squares regression parameters, as well as some calculated pharmacokinetic constants, for I–III are listed in Table II. From these data, it can be seen that the volume of the central compartment, V_c, for all of these agents exceeds the plasma volume of the dog (about 0.05 liter/kg). This indicates that these compounds distribute rapidly out of the plasma into tissues. The total body clearance, Cl_B, of these agents is extremely rapid; for example, Compound III is cleared at a rate of about 2200 ml/min.

Further direct pharmacokinetic analysis of the data is not realistic, because it is not possible to quantitate the rates of loss of these compounds in the plasma and tissue compartments of the model separately. However, since a nonspecific mechanism of elimination appears to account for loss of I–III from the body, some relationship probably exists between k_r and K_{el}. The relative $t_{1/2}$ values for I–III in the media listed in Table I provide some indication of the rate of reaction of this agent with cellular components, but the *in vivo* situation is complicated by the possibility that hepatic and renal mechanisms are also involved in the removal of these drugs. Therefore, various k_r–K_{el} ratios were assumed in seeking an array of possible analytical solutions to the two-compartment model and to demonstrate the magnitude of error in such estimations.

The calculated transfer (k_{12} and k_{21}) and elimination (K_{el} and k_r) rate constants for I are listed in Table III. When k_r is allowed

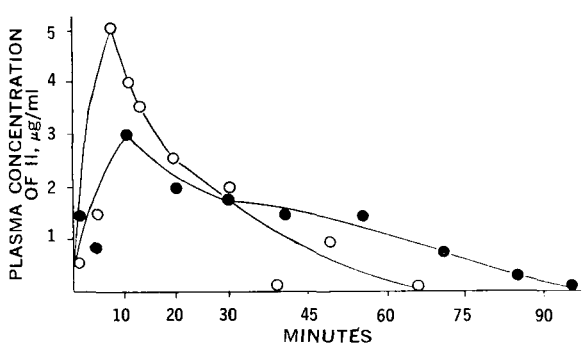
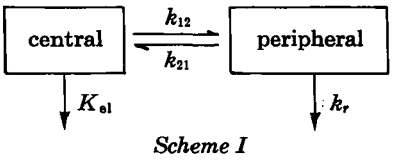


Figure 4—*Plasma concentrations of II at various times following intraperitoneal injection of 12 mg/kg to two dogs.*



Scheme I

to vary in the range $0.25\ K_{el} \leq k_r \leq 2K_{el}$ (a realistic range), the transfer rate constants only change by a factor of about two. However, it is not possible to obtain unique numerical values for these parameters for any of the agents because of the uncertainty of the rate of loss of compound in extravascular fluids.

Effect of Administration Route—The intramuscular injection of II has been used clinically (4), and the intraperitoneal route has been used during animal experimentation (3). Therefore, blood level data following administration of II by these routes were obtained and are shown in Figs. 4 and 5.

The area under the plasma concentration–time curves *(AUC)* following intraperitoneal or intramuscular administration was substantially less than that observed following intravenous dosing. This incomplete drug availability is probably caused by destruction of the drug at the site of injection. The AUC_{ip}'s for two dogs were 58 and 62% of the AUC_{iv}, while the intramuscular route yielded 58 and 69% in two other dogs. The limited availability of this compound *via* the intramuscular route suggests that the dose of drug should probably be increased when this route is used clinically.

Decomposition Studies—Thin-layer chromatograms of plasma extracts from dogs treated intravenously with I–III were examined for the parent compound and other 4-(*p*-nitrobenzyl)pyridine-reactive substances, using a previously described spray reagent (7). 2,2-Dimethylaziridine (IV), a minor hydrolysis product of I–III (see Table I and Ref. 7), was observed in extracts from dogs treated with I and III, but it could not be identified unequivocally in the extracts from dogs treated with II.

The quantity of IV found after injection of III was more than could be expected on the basis of simple hydrolysis (estimated from the size and intensity of the spots on at least three TLC plates from each dog and compared with cochromatographed standards). Therefore, it is suspected that some III is metabolized to IV. The quantity of IV generated from I probably could have arisen *via* simple hydrolysis.

The extraction step of the TLC procedure for the identification of alkylating compounds recovers about 40% of IV added to a sample of canine whole blood and about 80% of I–III. The procedure for the quantitative estimation of alkylating agents in whole blood recovers about 95% of I and III and about 60% of II. However, only about 10% of IV is recovered by this method. Therefore, the curves presented in Figs. 1–3 may be viewed as representing plasma concentration of the parent compound with little or no interference from IV.

Since an appreciable amount of IV is formed from III and some is formed from I, it seemed worthwhile to obtain plasma concentration–time data for IV. The only animals that received IV had received an intravenous dose of another alkylating agent prior to the experiment. A dose of 16 mg/kg was used. At least 100 min was allowed to elapse between the doses of the two drugs. Blood sampling was confined to about the same postinjection period when the 2,2-dimethylaziridine phosphoramides were studied.

Because the extraction procedure recovers so little compound

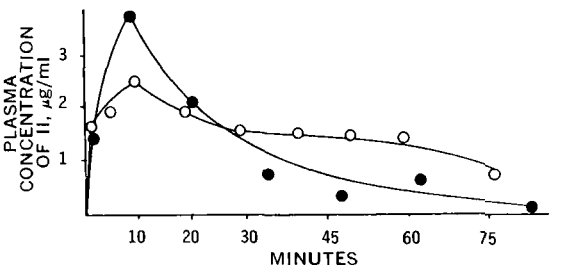


Figure 5—*Plasma concentrations of II at various times following intramuscular injection of a dose of 12 mg/kg to two dogs.*

Table II—Least-Squares Regression Parameters and Pharmacokinetic Constants for Compounds I–III

Parameter[a], units	I	II	III
A, $\mu g\ ml^{-1}$	42	46	41
α, min^{-1}	0.409	0.413	0.68
B, $\mu g\ ml^{-1}$	8.5	7.6	12
β, min^{-1}	0.095	0.074	0.14
V_c, liters kg^{-1}	0.24	0.225	0.25
Area, min $\mu g\ ml^{-1}$	191	214	142
C_p^0, $\mu g\ ml^{-1}$	50.5	53.6	53
Cl_B, ml min^{-1}	1530	1375	2200
$t_{1/2}\beta$, min	7.3	9.7	5.0

[a] Data normalized to a dose of 12 mg/kg.

(10%), it is necessary to interpret the results of these experiments conservatively. However, these experiments seemed worthwhile because they might help to show that IV does not cause interference in the blood level measurements following the administration of III. Comparison of Figs. 6 and 3 supports the view that the log-linear phase observed following the administration of III represents the plasma concentrations of III with little interference from IV, because the rates of decline of plasma alkylating activity between 15 and 40 min are very different following administration of III and IV.

Furthermore, IV appears to possess pharmacokinetic characteristics that are quite different from those of I–III. This may have some importance in the future design of 2,2-dimethylaziridines. That is, given the bis(2,2-dimethylaziridinyl)phosphine oxide structure, the nature of the group attached to the R position seems to have very little influence upon the pharmacokinetic characteristics of the compound. Since dimethylaziridine seems to act quite differently, it might be worthwhile to examine the effect of linking 2,2-dimethylaziridine to groups other than the phosphoryloxy moiety.

Preliminary data concerning the generation of a potent cholinesterase inhibitory intermediate from II were reported (13), and a comparative study of I–III was performed previously (14). These data show that the hydrolysis products of I cause substantially more cholinesterase inhibition (measured as inhibition of the procaine esterase activity of horse serum cholinesterase) than do the hydrolysis products of II and that III produces very little inhibitory intermediate(s). Since some clinical toxicity caused by II (5, 15) has been attributed to cholinesterase inhibition, and since it has been suggested that this intermediate may be pharmacologically important, it seems essential to consider the effects of the parent compound, IV, and the cholinesterase inhibitor intermediate(s) in the pharmacology and toxicology of I–III.

DISCUSSION

Chemotherapeutic Implications—A tumor could behave as a portion of the highly perfused central compartment (possible examples are renal and bronchogenic carcinomas) or as a portion of the lesser perfused peripheral compartment (perhaps carcinoma of the breast), or it might not be visualized at all by compartment modeling (possibly tumors of the central nervous system). Thus, it is of interest to examine the time course of drug levels outside of the central compartment. Because the three agents studied have similar pharmacokinetic properties, only the data for I (Fig. 1) will be analyzed.

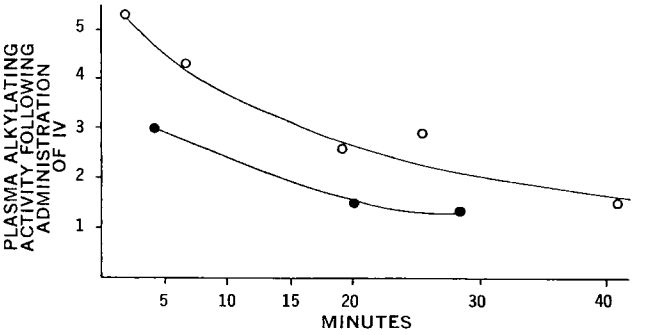


Figure 6—*Plasma levels of IV at various times following intravenous administration of a dose of 16 mg/kg to two dogs. The plasma concentrations of dimethylaziridine have been converted to alkylating activity corresponding to micrograms of III per milliliter.*

By using Eqs. A9 and A10 *(Appendix)*, the time course of drug in the central and peripheral compartments was calculated, using the various possible pharmacokinetic rate constants in Table III. As shown in Fig. 7, the quantity of drug in the peripheral compartment reaches a maximum (X_t^{max}) in about 5 min. The time required to reach this maximum is independent of k_r. However, an interesting phenomenon occurs with respect to the total amount of drug passing through the peripheral compartment. As the value of k_r increases, the value of X_t increases while the amount–time profile of drug in the central compartment remains constant.

Thus, as the ability for destruction of the drug increases in the peripheral compartment, it appears that more drug is shunted into this site in order for an identical plasma drug concentration curve to be seen. This is evidenced by the concomitant increase in k_{12}, and the decrease in k_{21}, and also by the increase in V_d^{ss} and tissue clearance, Cl_T, as k_r increases (Table III). In view of the unusually rapid hydrolysis of I–III at pH below 7.5 (Table I), it seems reasonable to assume that the magnitude of k_r largely reflects the rate of hydrolysis of the drug. This should be particularly the case if tumor tissues, which are known to be relatively acidic (pH ~6), form part of the peripheral compartment.

These pharmacokinetic considerations (Table III and Fig. 7) suggest that, given two drugs producing identical plasma concentrations at equal doses or infusion rates, the agent that hydrolyzes more rapidly may be more effective against tumors of the peripheral compartment because more of the drug will be located in the tissues at a given time. Although only limited data are available (1, 2), III does seem to be more effective than I in this type of tumor.

There is another interesting aspect of this interpretation. It was reported previously from this laboratory (16) that the ED_{90} values for a large series of alkylating agents correlate well with the rates of hydrolysis of these agents when the tumor studied was probably acting as a portion of the peripheral compartment (Walker carcinoma 256, transplanted subcutaneously in the flank region of male rats). Thus, the rate of hydrolysis of these compounds may influence both their chemotherapeutic activity and their pharmacokinetic behavior and could be a major correlative parameter in structure–activity studies.

Table III—Effect of Extravascular Elimination on the Calculated Pharmacokinetic Constants for Compound I

z, Ratio of $k_r:K_{el}$	k_{12}, min^{-1}	k_{21}, min^{-1}	K_{el}, min^{-1}	k_r, min^{-1}	V_d^{ss}, liters[a]	X_t^{max}, mg[a]	C_t^{max}, mg/liter[b]	Cl_p, ml/min	Cl_T, ml/min
0	0.093	0.148	0.263	0	9.52	44	11.9	1531	0
0.1	0.108	0.123	0.249	0.0249	10.05	52	11.9	1449	82
0.2	0.133	0.103	0.223	0.0446	11.05	63	11.9	1298	233
0.25	0.145	0.095	0.211	0.0527	11.52	68	11.9	1228	303
0.5	0.199	0.069	0.157	0.0786	13.63	93	11.9	913	618
1.0	0.261	0.053	0.0951	0.0951	16.08	122	11.9	553	978
2.0	0.304	0.045	0.0515	0.103	17.75	142	11.9	300	1231

[a] Calculated using Eq. A10. [b] Calculated from: $C_t^{max} = X_t^{max}/(V_d^{ss} - V_c)$.

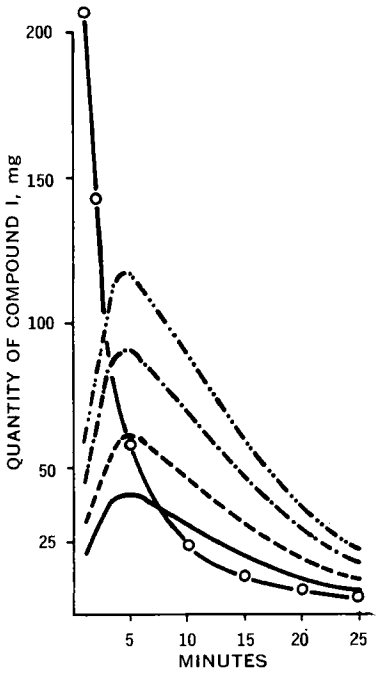


Figure 7—*Amount of I in the central compartment* (O——O) *and in the peripheral compartment as a function of time assuming* $k_r = 0$ (——), $k_r = 0.2\ K_{el}$ (– – – –), $k_r = 0.5\ K_{el}$ (–·–·–·), *and* $k_r = K_{el}$ (–· · –· · –).

APPENDIX

The differential equations that describe the two-compartment model shown in Scheme I are:

$$\frac{dX_c}{dt} = k_{21}X_t - k_{12}X_c - K_{el}X_c \qquad \text{(Eq. A1)}$$

and:

$$\frac{dX_t}{dt} = k_{12}X_c - k_{21}X_t - k_rX_t \qquad \text{(Eq. A2)}$$

where the initial conditions are X_c^0 = dose and $X_t^0 = 0$.

These equations can be solved using LaPlace transforms and matrix algebra. Equations for the various rate constants were provided by Suzuki and Saitoh (9) as follows:

$$k_{21} = \frac{A(\beta - \alpha)}{A + B} + \alpha - k_r \qquad \text{(Eq. A3)}$$

$$K_{el} = \frac{\alpha\beta - k_{12}k_r}{k_{21} + k_r} \qquad \text{(Eq. A4)}$$

$$k_{12} = \alpha + \beta - k_{21} - k_r - K_{el} \qquad \text{(Eq. A5)}$$

$$k_r = \frac{B(\alpha - \beta)}{(A + B)} + \beta - k_{21} \qquad \text{(Eq. A6)}$$

Values for the biexponential parameters A, α, B, and β are obtained by fitting the experimental data to Eq. 1. In addition, the volume of the central compartment, V_c, and the steady-state volume of distribution can be calculated as follows:

$$V_c = \text{dose}/C_p^0 = \text{dose}/(A + B) \qquad \text{(Eq. A7)}$$

$$V_d^{ss} = V_c\left(1 + \frac{k_{12}}{k_{12} + k_r}\right) \qquad \text{(Eq. A8)}$$

The amounts of drug in the central, X_c, and peripheral, X_t, compartments as a function of time are:

$$X_c = \frac{X_c^0(k_{21} + k_r - \alpha)}{\beta - \alpha}e^{-\alpha t} + \frac{X_c^0(k_{21} + k_r - \beta)}{\alpha - \beta}e^{-\beta t} \qquad \text{(Eq. A9)}$$

$$X_t = \frac{-k_{12}X_c^0}{\alpha - \beta}e^{-\alpha t} + \frac{k_{12}X_c^0}{\alpha - \beta}e^{-\beta t} \qquad \text{(Eq. A10)}$$

Numerical Evaluation of Rate Constants—Equations A3–A6 allow the calculation of k_{12}, k_{21}, and K_{el} when given known values of k_r. This is not feasible in the present study; thus the values of the various rate constants must be approximated by relating the value of k_r to that of K_{el}. In the present situation, it is assumed that k_r is proportional to K_{el} by the relationship:

$$k_r = zK_{el} \qquad \text{(Eq. A11)}$$

where z is a nonnegative real number. Solution of Eq. A4 by substituting Eqs. A11, A3, and A5 yields:

$$K_{el} = \frac{\alpha\beta - (zK_{el})\left[\alpha + \beta - K_{el} - \dfrac{A\beta + B\alpha}{A + B}\right]}{\dfrac{A\beta + B\alpha}{A + B}} \qquad \text{(Eq. A12)}$$

Crossmultiplying and equating the entire expression to zero yield:

$$0 = \alpha\beta + \left[-z\alpha - z\beta + (z - 1)\frac{A\beta + B\alpha}{A + B}K_{el} + zK_{el}^2\right] \qquad \text{(Eq. A13)}$$

Solution of this quadratic equation for any z yields one positive root for K_{el}. The values of k_r, k_{21}, and k_{12} can then be obtained from Eqs. A11, A3, and A5, respectively.

Clearance Parameters—The body clearance, Cl_B, is a model-independent parameter which can be calculated from:

$$Cl_B = \text{dose/area} \qquad \text{(Eq. A14)}$$

where area is the total area under the plasma concentration *versus* time curve obtained either by trapezoidal approximation or by integration of Eq. 1. The plasma clearance, Cl_p, is usually considered to reflect the sum of processes such as renal and hepatic clearances of drug from the plasma or central compartment and is obtained from:

$$Cl_p = (K_{el})(V_c) \qquad \text{(Eq. A15)}$$

The body and plasma clearance terms are often used interchangeably, but they are actually identical only when elimination does not occur outside of the central compartment. When elimination takes place in the peripheral compartment, the body clearance should obviously exceed the plasma clearance. It is, therefore, useful and appropriate to define the difference between the two values as the tissue clearance. The calculation involves:

$$Cl_T = Cl_B - Cl_p \qquad \text{(Eq. A16)}$$

Comparison of such clearance values provides an indication of the relative contribution of central and peripheral compartment clearance mechanisms to overall elimination of the drug from the body.

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Cycloalkanones V: Synthesis, Distribution, and Effects on Triglyceride Metabolism

IRIS H. HALL [x], ROBERT G. LAMB [*], MEI-HENG H. MAR, GERALD L. CARLSON, and CLAUDE PIANTADOSI

Abstract □ The ^{14}C-labeled 2,8-dibenzylcyclooctanone was synthesized to study its absorption, distribution, and excretion in rats. Maximum drug absorption from the GI tract occurred between 12 and 14 hr after administration. The major organs possessed maximum amounts of the drug in 1 hr, with the liver concentrating the most with 6.56% ^{14}C and the muscle mass reaching a maximum of 41% ^{14}C after 14 hr. The drug remained in the GI tract over the first 6 hr and was associated with the lipid and glycogen fractions. Eighty-seven percent was eliminated in the feces after 72 hr. 2,8-Dibenzylcyclooctanone caused a significant reduction *in vitro* of dihydroxyacetone phosphatase acyltransferase and *sn*-glycerol-3-phosphate acyltransferase, which is the proposed mechanism for the observed *in vivo* reduction of hepatic, intestinal, and serum triglycerides and total glycerolipids. *In vivo* administration of the drug resulted in a depression of liver acid phosphatidyl phosphatase, acid phosphatase and lipase, and adipose lipase. The drug increased the rates of excretion of exogenous cholesterol, palmitic acid, and progesterone.

Keyphrases □ Cycloalkanones—synthesis of radiolabeled 2,8-dibenzylcyclooctanone, tissue distribution, effects on triglyceride metabolism, rats □ 2,8-Dibenzylcyclooctanone, radiolabeled—synthesis, distribution to tissues, effects on triglyceride metabolism, rats □ Triglyceride metabolism—effects of 2,8-dibenzylcyclooctanone □ Hypolipidemic activity—2,8-dibenzylcyclooctanone

Previously, the synthesis and hypolipidemic activity of cyclooctanone derivatives were reported (1). In these studies, 2,8-dibenzylcyclooctanone (I) lowered serum cholesterol by 50%, triglyceride by 42%, and glycerol by 31% in Sprague–Dawley rats. The structure–activity relationship of some derivatives was reported (2), delineating the minimum requirement for hypocholesterolemic activity in this series of compounds.

In a continuing effort to study the mechanism of action, I was labeled with ^{14}C and studies were conducted on absorption, distribution, and excretion. Studies also were performed on the effects of I on triglyceride metabolism.

EXPERIMENTAL

Organic Synthesis—The chemical method used for isotopic labeling of I was derived from that of Piantadosi *et al.* (1). Sodium (0.5 g) was dissolved in 12.5 ml of absolute ethanol, and the solution was cooled to room temperature. Four milliliters of this solution was added to a mixture of benzaldehyde (650 mg, 6.13 mmoles) and cyclooctanone (386.8 mg, 3.065 mmoles) in a 10-ml round-bottom flask equipped with a condenser protected by a drying tube. The mixture was stirred at room temperature for 4 hr. At this time, 5 ml of water was added and the mixture was extracted three times with 10 ml of benzene.

The combined extracts were evaporated to yield a viscous residue, and the residue was purified by silica gel column chromatography (35 g, 2.4 × 30 cm). Benzene was used as an eluent, and the first 130 ml of eluent contained no product. The desired 2,8-dibenzylidenecyclooctanone was obtained in the next 60 ml, and this eluate was evaporated to yield 320 mg (35%) of pure 2,8-dibenzylidenecyclooctanone as a pale-yellow solid, mp 108–110° [lit. mp 108–110° (1) and mp 111° (3)].

Ten percent palladium-on-carbon (50 mg) and sodium methylate (30 mg) were added to a solution of 2,8-dibenzylidenecyclooctanone (320 mg, 1.06 mmoles) in 25 ml of ethyl acetate. The mixture was hydrogenated until exactly 2.12 mmoles of hydrogen was absorbed. The catalyst was filtered and washed with 5 ml of hot ethyl acetate. The filtrate was evaporated to yield a viscous residue, which was purified by silica gel column chromatography (12 g, 1.2 × 40 cm), using benzene as the elution solvent. Fractions of 2 ml were collected. After a forerun of 56 ml, 32 ml was collected and evaporated to dryness. All fractions were shown to contain only I by TLC [R_f 0.72 in benzene–chloroform (95:5)]. The yield was 190 mg (62%), mp 82–83° [lit. (1) mp 82–83°].

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For the second run, 31.3 mg (2 mCi) of benzaldehyde-(carbonyl-

[1] Amersham Searle.

Table I—Percent Recovery (Mean ± Standard Deviation) of Radioactivity in the GI Tract of Male Sprague–Dawley Rats following Oral Administration of ^{14}C-2,8-Dibenzylcyclooctanone (20 mg/kg)

Sample (n = 6)	0.083 hr	0.25 hr	0.50 hr	1 hr	2 hr	6 hr
Esophagus content	0.501 ± 0.159	0.194	0.086 ± 0.032	0.152 ± 0.102	0.107 ± 0.064	0.123 ± 0.066
Stomach content	85.17 ± 2.65	53.41 ± 10.32	52.97 ± 6.25	31.54 ± 9.91	21.64 ± 5.43	7.21 ± 0.81
Stomach	2.66 ± 0.53	13.7 ± 2.61	5.70 ± 4.03	7.80 ± 5.13	4.72 ± 1.53	6.99 ± 3.56
Small intestine content	0.615 ± 0.171	4.91 ± 1.87	9.72 ± 7.18	20.2 ± 5.22	32.7 ± 9.14	29.5 ± 2.42
Small intestine	0.358 ± 0.130	8.70 ± 2.10	8.92 ± 7.35	14.45 ± 2.25	19.6 ± 2.65	7.81 ± 2.79
Cecum content	0.020 ± 0.024	0.160 ± 0.060	0.042 ± 0.048	0.103 ± 0.063	0.216 ± 0.116	22.7 ± 3.67
Cecum	0.025 ± 0.023	0.160 ± 0.081	0.048 ± 0.024	0.035 ± 0.027	0.069 ± 0.051	1.03 ± 0.241
Large intestine content	0.017 ± 0.016	0.205 ± 0.049	0.032 ± 0.0236	0.042 ± 0.010	0.0947 ± 0.0118	7.03 ± 1.50
Large intestine	0.078 ± 0.041	0.057 ± 0.013	0.044 ± 0.036	0.241 ± 0.194	0.179 ± 0.103	0.512 ± 0.356
Head (site of administration)	8.31 ± 0.65	3.66 ± 1.52	7.04 ± 2.10	5.06 ± 3.66	4.89 ± 0.33	2.50 ± 0.42
Feces	—	—	0.182 ± 0.147	0.730 ± 0.589	0.762 ± 0.681	1.63 ± 0.652
	94.754	85.156	84.734	80.363	84.977	87.035

Sample (n = 6)	14 hr	18 hr	24 hr	48 hr	72 hr
Esophagus content	0.005 ± 0.001	0.089 ± 0.021	—	0.001 ± 0	0.003 ± 0.001
Stomach content	2.86 ± 1.35	4.42 ± 2.76	0.530 ± 0.014	0.014 ± 0.012	0.061 ± 0.031
Stomach	0.615 ± 0.133	0.596 ± 0.451		0.021 ± 0.025	0.144 ± 0.097
Small intestine content	10.0 ± 3.01	26.6 ± 9.78	5.74 ± 0.63	2.40 ± 1.40	1.73 ± 2.02
Small intestine	1.31 ± 0.81	7.81 ± 3.72	0.400 ± 0.156	0.556 ± 0.406	0.625 ± 0.32
Cecum content	11.8 ± 2.03	15.4 ± 3.78	1.96 ± 0.36	0.356 ± 0.047	0.625 ± 0.605
Cecum	0.326 ± 0.263	3.68 ± 1.72	0.020 ± 0.023	0.055 ± 0.008	0.053 ± 0.056
Large intestine content	4.03 ± 2.04	0.032 ± 0.026	0.115 ± 0.010	0.271 ± 0.098	0.487 ± 0.337
Large intestine	0.201 ± 0.205	0.131 ± 0.117	0.130 ± 0.127	0.032 ± 0.028	0.127 ± 0.119
Head (site of administration)	1.79 ± 0.88	2.91 ± 0.24	3.89 ± 0.82	1.69 ± 0.49	2.07 ± 0.95
Feces	18.8 ± 11.7	21.79 ± 2.97	57.6 ± 9.62	76.1 ± 10.6	86.7 ± 20.2
	51.737	83.458	70.385	81.496	92.653

^{14}C)[2] (6.8 mCi/mmole) and 619 mg of unlabeled benzaldehyde were used. The yield of product, Compound I, was 270 mg (31% based on the starting material) with a specific activity of 450 μCi/mmole (1.47 μCi/mg).

Radioactivity—The radioactivity was measured by placing a known volume of material in a scintillation vial; then 10 ml of the following scintillation fluid was added: 2,5-diphenyloxazole[3] and 1,4-bis[2-(5-phenyloxazolyl)]benzene[4] in toluene. These vials were counted in a scintillation counter[5], using the channel ratio technique to correct for quenching.

Animals—Male Sprague–Dawley rats[6], 120 g, were used. Prior to the experiment, the animals were maintained on lab chow[7] and water *ad libitum*.

Compound I was suspended in 1% carboxymethylcellulose solution. Each animal was administered 20 mg/kg of ^{14}C-2,8-dibenzylcyclooctanone (specific activity 450–460 μCi/mmole) orally by an esophageal intubation needle in 0.2 ml of 1% carboxymethylcellulose.

Collection of Samples—At appointed time periods (0.083, 0.25, 0.5, 1, 2, 6, 12 or 14, 18, 24, and 48 or 72 hr), the rats were killed by decapitation; the blood was drained from the carotid artery and immediately heparinized (0.2 ml heparin sodium[8]). The liver, kidneys, brain, heart, testes, adrenal glands, spleen, and each section of the GI tract with its contents were excised. The tissues were weighed and homogenized with 10 volumes of distilled water (w/v). The urine and feces were collected at 0.25, 0.5, 1, 2, 6, 12, 18, 24, 48, and 72 hr.

Radioactivity of Freeze-Dried Tissue Samples—These samples were lyophilized using a freeze dryer[9]. Dried samples (~50 mg wet weight) were combusted[10], or dried samples were weighed into scintillation vials (~10 mg) and 1 ml of tissue solubilizer[11] was added to each vial. These vials were closed tightly and heated to 55° for 20 hr. Ten milliliters of the following scintillation fluid was added: 10 g of 2,5-bis[2-(5-*tert*-butylbenzoxazolyl)]thiophene (scintillation grade), 200 g of naphthalene, 1500 ml of toluene, 1000 ml of methylcellulose, and 89 ml of water[12].

Radioactivity of Wet Homogenate—Aliquots (1 ml) of the wet homogenate were placed in scintillation vials, and 2 ml of tissue solubilizer was added to each vial; the vials were closed tightly and heated to 55° for 20 hr. After cooling to room temperature, 10 ml of scintillation fluid was added containing 1,4-bis[2-(5-phenyloxazolyl)]benzene[4] and 10.8 g of 2,5-diphenyloxazole[3] in 2 liters of toluene and 1.2 liters of octoxynol[13]. Aliquots (0.1 ml) of the tissue and feces homogenates were placed on a filter disk[14] and dried. The head and body were solubilized separately by heating in 2 volumes of 30% potassium hydroxide at 80° overnight (4). Aliquots were taken to determine the radioactivity in scintillation fluid.

***In Vitro* Absorption**—The stomach and the first half of the small intestine were removed from male rats. The contents of each portion of the gut were washed out with pH 7.2 Hanke's buffer. The radiolabeled drug was dissolved in 1% carboxymethylcellulose and placed in the lumen of the gut with an intubation needle. The gut was tied at both ends with No. 40 cotton thread so that no leakage of materials occurred. Then the gut was placed in 20 ml of pH 7.2 Hanke's buffer reinforced with 1 g/liter of dextrose and placed in a shaker[15] at 37°.

At intervals of 1 hr, aliquots were taken from the medium bathing the tissue to determine radioactivity. At the end of 6 hr, the radioactivity of the contents and a 10% homogenate in water of each segment of gut was determined.

***In Vivo* Absorption**—Male rats were administered 20 mg/kg of ^{14}C-2,8-dibenzylcyclooctanone orally. The animals were sacrificed after 2 hr, and the stomach and duodenum were removed with

[2] ICN, Pharmaceutical, Inc.
[3] PPO, New England Nuclear.
[4] POPOP, Packard.
[5] Packard Tri-Carb model 3320.
[6] Zivic Miller, Allison Park, Pa.
[7] Purina rodent lab chow.
[8] Riker Lab Inc., 5000 USP units/ml.
[9] Virtis automatic model 10-010.
[10] Packard Tri-Carb model 305 sample oxidizer.
[11] Protosol, 0.5 *M*, New England Nuclear.
[12] BBT.
[13] Triton X-100, Rohm and Haas Co.
[14] Whatman No. 1.
[15] Dubnoff.

Table II—Percent Recovery (Mean ± Standard Deviation) of Radioactivity in the Tissue of Male Sprague–Dawley Rats following Oral Administration of ^{14}C-2,8-Dibenzylcyclooctanone (20 mg/kg)

Sample (n = 6)	0.083 hr	0.25 hr	0.50 hr	1 hr	2 hr	6 hr
Liver	0.120 ± 0.009	0.722 ± 0.172	1.580 ± 0.248	6.563 ± 3.63	3.565 ± 2.02	2.221 ± 0.958
Kidneys	0.015 ± 0.002	0.202	0.225 ± 0.192	0.359 ± 0.081	0.147 ± 0.131	0.112 ± 0.075
Brain	0.026 ± 0.024	0.097 ± 0.014	0.047 ± 0.041	0.371 ± 0.331	0.071 ± 0.065	0.013 ± 0.010
Lungs	0.073 ± 0.056	0.268 ± 1.05	0.048 ± 0.030	0.214 ± 0.195	0.027 ± 0.022	0.151 ± 0.064
Heart	0.015 ± 0.014	0.253 ± 0.248	0.028 ± 0.029	0.080 ± 0.100	0.035 ± 0.034	0.009 ± 0.014
Spleen	0.004 ± 0	0.327 ± 0.227	0.021 ± 0.020	0.068 ± 0.050	0.009 ± 0.010	0.006 ± 0.010
Testes	0.003 ± 0	0.493 ± 0.329	0.049 ± 0.009	0.362 ± 0.051	0.047 ± 0.023	0.027 ± 0.030
Blood	0.001 ± 0	0.167 ± 0.158	0.015 ± 0.003	0.160 ± 0.032	0.045 ± 0.041	0.018 ± 0.004
Adrenals	0.011 ± 0.014	0.395 ± 0.231	0.027 ± 0.022	0.130 ± 0.150	0.033 ± 0.030	0.008 ± 0.001
Adipose tissue	0.377 ± 0.230	0.473 ± 0.300	0.356 ± 0.129	0.881 ± 0.797	0.767 ± 0.365	0.708 ± 0.631
Muscle mass	0.897 ± 0.085	5.05 ± 0.037	12.298 ± 1.632	12.504 ± 6.761	7.63 ± 5.43	5.943 ± 1.832
	1.54	8.447	14.694	21.692	12.376	9.215
Total recovery	96.296	93.603	99.428	102.05	97.353	96.251

Sample (n = 6)	14 hr	18 hr	24 hr	48 hr	72 hr
Liver	0.247 ± 0.246	2.58 ± 1.12	0.165 ± 0.233	0.242 ± 0.046	0.007 ± 0.033
Kidneys	0.009 ± 0.001	0.111 ± 0.103	0	0.022 ± 0.003	0.058 ± 0.009
Brain	0	0.071 ± 0.069	0.055 ± 0.028	0.014 ± 0.017	0.041 ± 0.044
Lungs	0.070 ± 0.072	0.040 ± 0.042	0	0	0.066 ±
Heart	0	0.060 ± 0.010	0	0.006 ± 0.001	0.012 ± 0.003
Spleen	0	0.042 ± 0.006	0	0.002 ± 0	0.051 ± 0.007
Testes	0.016 ± 0.003	0.069 ± 0.066	0	0.021 ± 0.031	0.128 ± 0.114
Blood	0	0.026 ± 0.005	0	0.021 ± 0.024	0
Adrenals	0	0.031 ± 0.011	0	0.006 ± 0	0.050 ± 0.008
Adipose tissue	0.434 ± 0.213	0.432 ± 0.329	0	0.238 ± 0.117	0.018 ± 0.016
Muscle mass	40.91 ± 1.29	7.98 ± 1.64	21.107 ± 9.072	7.566 ± 3.814	10.788 ± 9.532
	41.686	11.242	21.327	8.131	11.319
Total recovery	93.423	94.692	91.712	89.627	102.972

their contents. The chyme was removed from the GI tract, and a 10% homogenate of the tissue or food in 0.25 *M* sucrose plus 0.001 *M* ethylenedinitrilotetraacetic acid was prepared. Aliquots of the whole homogenates were extracted three times with chloroform and once with 10% methanol–90% chloroform.

Aliquots were taken from the organic and aqueous extract to determine radioactivity. Forty milliliters of the aqueous extract was treated with 10 ml of 20% trichloroacetic acid and centrifuged to obtain a supernate and pellet. Ten milliliters of the whole homogenates was extracted by the method of Shibko *et al.* (5) to obtain the ribonucleic acid, deoxyribonucleic acid, lipid, glycogen, and protein fractions.

***In Vivo* Hepatic and Intestinal Glycerolipid Formation from 1,3-^{14}C-Glycerol**—Male Sprague–Dawley rats, 300–350 g, were divided into two groups which received either 0.5 ml of 1% carboxymethylcellulose or 10 mg/kg of I suspended in 1% carboxymethylcellulose daily for 5 days by an oral intubation needle. After 5 days, both control and experimental animals were injected intraperitoneally with 1 ml of isotonic saline containing 5 μCi of 1,3-^{14}C-glycerol (6.5 μCi/μmole). Seventeen minutes later, the animals were anesthetized with ether, the abdomen was opened, and blood was removed from the abdominal aorta. The liver and small intestine were removed, washed with 0.05 *M* tromethamine–hydrochloric acid (pH 7.5), quickly frozen in dry ice–acetone, and stored at −40°.

Lipids from duplicate 1-g samples of tissue were extracted by the Bligh and Dyer procedure (6). Serum, hepatic and intestinal triglyceride, and serum glycerol levels and the rate of glycerolipid formation from 1,3-^{14}C-glycerol were determined by techniques described previously (7). The time course of 1,3-^{14}C-glycerol incorporation into intestinal triglycerides and total hepatic and intestinal glycerolipids was similar to that described for hepatic triglycerides (7). Therefore, the 17-min time point was used to estimate *in vivo* hepatic and intestinal triglyceride and total glycerolipid formation.

***sn*-Glycerol-3-phosphate Acyltransferase**—Rat liver microsomes were prepared as previously described (8) and incubated with 30 m*M* tromethamine–hydrochloric acid (pH 8.0), 3.3 m*M* magnesium chloride, 0.7 m*M* dithiothreitol, 50 μ*M* coenzyme A, 0.5 m*M* *sn*-1,3-^{14}C-glycerol-3-phosphate, 3.3 m*M* adenosine triphosphate, 0.60 m*M* ammonium palmitate, and 1.25 mg of albumin in a total volume of 0.40 ml. These conditions were optimum for total glycerolipid formation (8). After a 30-min incubation, reactions were stopped by the addition of 3 ml of chloroform–methanol (1:2).

The lipids were extracted by the Bligh and Dyer procedure (6) and separated into phosphatidic acid and neutral lipid by TLC in a solvent system containing chloroform–methanol–3.5 *N* ammonium hydroxide (65:35:8) (8). Areas of the TLC plates which corresponded to known lipid standards were scraped into scintillation vials, and the radioactivity was determined with a scintillation counter.

Dihydroxyacetone Phosphate Acyltransferase—The incubation mixture optimum for dihydroxyacetone phosphate esterification contained 1.5 m*M* dihydroxyacetone phosphate, 1.3 m*M* reduced nicotinamide adenosine dinucleotide, 25 m*M* tromethamine–hydrochloric acid (pH 7.5), 1.5 m*M* magnesium chloride, 40 μ*M* coenzyme A, 2.8 m*M* adenosine triphosphate, 0.3 m*M* dithiothreitol, 0.7 m*M* 1-^{14}C-palmitate (0.1 μCi), 1.25 mg of fatty acid-poor albumin, and 0.3 mg of rat liver microsomal protein in a total volume of 0.5 ml. Reactions were started by the addition of microsomal protein and stopped after a 30-min incubation by the addition of 3 ml of chloroform–methanol (1:2). Lipids were extracted and identified as described for *sn*-glycerol-3-phosphate acyltransferase.

Lipase, Acid Phosphatase, and Phosphatidyl Phosphatase Activity—Rats, 180 g, were treated in the identical manner as outlined in the *in vivo* studies. After 5 days, the liver and adipose tissue were excised and blood was collected so that the following enzymatic assays could be performed: liver phosphatidyl phosphatase at pH 7.0 (control value = 0.152 mg phosphate released in 30 min/g wet liver) and at pH 5.0 (0.448 mg phosphate released in 30 min/g wet liver) (9), free acid phosphatase (0.598 mg phosphate released in 10 min/g wet tissue), total acid phosphatase (11) (1.798 mg phosphate released in 10 min/g wet liver), hormone sensitive lipase (10) (6.4 ml of 0.025 *N* sodium hydroxide/ml of serum) and lipolysis (12) (1.7 ml of 0.05 *N* sodium hydroxide/ml of serum), and adipose hormone sensitive lipase (10) (22.5 ml of 0.025 *N* sodium hydroxide/g wet tissue). Inorganic phosphate was determined by the procedure of Chen *et al.* (11).

Table III—Effect of 2,8-Dibenzylcyclooctanone on Serum, Hepatic, and Intestinal Triglycerides, Serum Glycerol, and Liver Weight

Experiment (n)	Triglycerides				
	Serum, mg % ± *SEM*	Hepatic, mg/g ± *SEM*	Intestinal, mg/g ± *SEM*	Serum Glycerol, nmoles/ml ± *SEM*	Liver Weight, g ± *SEM*
Control (5)	128.0 ± 22.9	6.48 ± 0.63	14.8 ± 1.9	154.6 ± 24.7	13.1 ± 0.6
2,8-Dibenzylcyclooctanone	65.5 ± 11.1[a]	5.02 ± 0.73	10.9 ± 2.3	38.2 ± 4.2[b]	12.7 ± 1.2

[a] $p \leq 0.05$ (level of significance from control group). [b] $p \leq 0.001$ (level of significance from control group).

Table IV—Effect of 2,8-Dibenzylcyclooctanone on *In Vivo* Hepatic, Intestinal, and Serum Triglycerides and Total Glycerolipid Formation from 1,3-^{14}C-Glycerol

Experiment (n)	Intestine, nmoles/17 min/g ± *SEM*		Liver, nmoles/17 min/g ± *SEM*		Serum, nmoles/17 min/ml ± *SEM*
	Triglycerides	Total	Triglycerides	Total	Triglycerides
Control (5)	21.4 ± 3.3	33.1 ± 2.5	95.9 ± 10.8	131.5 ± 14.7	21.3 ± 4.9
2,8-Dibenzylcyclooctanone	7.5 ± 1.2[a]	13.3 ± 3.8[b]	30.9 ± 3.5[a]	45.3 ± 4.7[b]	5.8 ± 0.8[a]

[a] $p \leq 0.01$ (level of significance from control group). [b] $p \leq 0.001$ (level of significance from control group).

Lipid Excretion—Other rats, treated identically, were given ^{14}C-cholesterol (1 μCi), ^{14}C-palmitic acid (2 μCi), or ^{3}H-7-progesterone (60 μCi) orally on the 5th day; the feces were collected after 24 hr. Homogenates of the feces in water were prepared, and aliquots were taken for radioactive determination. The homogenates were extracted three times with chloroform and once with chloroform–methanol (90:10) to obtain the lipid fraction; this fraction was dried and weighed.

RESULTS

Distribution of 2,8-Dibenzylcyclooctanone (I)— The freeze-dried sample and wet homogenate sample had no significant accumulation of ^{14}C-2,8-dibenzylcyclooctanone or its metabolites[16] in the kidneys, heart, brain, testes, or adrenal glands. Since there was such a low recovery of ^{14}C in the freeze-dried samples, studies were performed on the total animals to determine the percent recovery of the administered radioactive dose.

Table I notes the percent recovery of radioactivity in the GI tract after oral administration of ^{14}C-2,8-dibenzylcyclooctanone. Very high concentrations of ^{14}C were found in the GI tract and its contents, representing 75–89% of the ^{14}C administered over the first 6 hr, with the largest percentage present in the stomach and small intestine. After 6 hr, the ^{14}C-2,8-dibenzylcyclooctanone began to decrease in the upper GI tract and gradually built up in the excreted feces. Table I shows that about 58, 76, and 87% of the ^{14}C was excreted in the feces 24, 48, and 87 hr, respectively, after an oral dosing.

The total carcass (muscle mass) of the rat reached 41% ^{14}C-2,8-dibenzylcyclooctanone after 14 hr (Table II). The major organs of the body reached a maximum absorption of I at 1 hr. A second peak was reached at 18 hr, especially in the liver. Fourteen hours probably represents the maximum absorption of the drug from the GI tract, since only 31% of the ^{14}C-2,8-dibenzylcyclooctanone remained in the GI tract at this time.

Organ distribution of ^{14}C-2,8-dibenzylcyclooctanone after oral administration is shown in Table II. These data on whole homogenates reflect the same conclusions as with the freeze-dried samples, whether combusted or not. Only a small percentage (9.2% at 1 hr) of ^{14}C-2,8-dibenzylcyclooctanone was found in the tissues, and the concentration found was in the following order: liver ≫ adipose tissue > testes = kidneys = brain > lungs > blood > adrenal > heart > spleen. The blood was corrected for a total blood volume of 17.5 ml for rats.

Absorption—Since a large amount of ^{14}C-2,8-dibenzylcyclooctanone was observed in the upper GI tract during the first 6 hr of the percent recovery studies, absorption studies were undertaken to determine why the drug remained there. It had been expected that the drug would be lipophilic and be absorbed rapidly. However, *in vitro* incubation studies indicated that after 6 hr 83% of ^{14}C-2,8-dibenzylcyclooctanone remained in the stomach in an isolated preparation and 84% remained in the small intestine in an isolated preparation, indicating that the drug was poorly absorbed from either site, which was congruent with the *in vivo* distribution data.

When homogenates were extracted, it was observed that of the total ^{14}C-2,8-dibenzylcyclooctanone present in the GI contents, 5.6% was in the deoxyribonucleic acid fraction, 10.9% in the ribonucleic acid, 4.1% in the protein, 38.2% in the lipid, 40.6% in the glycogen, and 0.5% in the water. Of the total ^{14}C-2,8-dibenzylcyclooctanone present in the GI tissue homogenate, 5.0% was in the deoxyribonucleic acid, 18.0% in the ribonucleic acid, 1.5% in the protein, 33.8% in the lipid, 38.8% in the glycogen, and 2.9% in the water. The ^{14}C-labeled drug or its metabolite appears to be isolated predominantly with the glycogen and lipid fractions in both the GI contents (79%) and the GI tissue (73%).

When whole homogenates were extracted (chloroform–methanol), it was observed that 65% of the ^{14}C-2,8-dibenzylcyclooctanone was in the aqueous fraction of the GI contents and 86% was in the aqueous fraction of the GI tissue. When the aqueous fraction was treated with 20% trichloroacetic acid, the ^{14}C-2,8-dibenzylcyclooctanone remained in the supernate rather than the pellet. These results indicate that the ^{14}C-drug is binding to a component in the glycogen and lipid fractions.

Tissue Triglyceride, Serum Glycerol, and Liver Weight—The administration of 10 mg/kg of I to rats for 5 days reduced mean serum, liver, and intestinal triglyceride concentrations to 49, 22.5, and 26.5%, respectively (Table III). Serum glycerol concentrations were reduced 75.3%, and there was no change in mean liver weight in rats receiving I for 5 days. During the 5-day period, rats receiving I gained 22.0 ± 3.6 g/kg and those receiving 1% car-

Table V—*In Vitro* Effect of 2,8-Dibenzylcyclooctanone on Hepatic *sn*-Glycerol-3-phosphate Acyltransferase and Dihydroxyacetone Phosphate Acyltransferase Activity

2,8-Dibenzylcyclooctanone, m*M*	*sn*-Glycerol-3-phosphatase Acyltransferase Relative Rate	Dihydroxyacetone Phosphate Acyltransferase Relative Rate
0	1.00	1.00
0.75	0.44	0.43
1.5	0.37	0.34
2.3	0.33	0.28
3.0	0.32	0.22

[16] Although no metabolites have been identified to date, the ^{14}C-isotope would also be distributed in any metabolites.

Table VI—Effects of 2,8-Dibenzylcyclooctanone on Enzyme Activities of Sprague–Dawley Rats *In Vivo*[a]

	1% Carboxymethylcellulose (n = 8)	2,8-Dibenzylcyclooctanone, 10 mg/kg/day for 5 days	p
Liver:			
Phosphatidyl phosphatase			
pH 7.0	100 ± 12	104 ± 11	—
pH 5.0	100 ± 6	87 ± 7	0.050
Lipase	100 ± 11	64 ± 14	0.001
Acid phosphatase			
Free	100 ± 15	65 ± 16	0.001
Total	100 ± 13	80 ± 14	0.050
Serum:			
Lipase	100 ± 29	94 ± 33	—
Lipolysis test[b]	100 ± 25	41 ± 21	0.001
Adipose tissue:			
Lipase	100 ± 17	64 ± 13	0.001

[a] The number of animals in the group is expressed as n. All values are expressed as percent of control noted as mean and standard deviation, $\bar{x} \pm SD$. The probable significant level (p) between each test group and the control group was determined by the Student t test. [b] Reference 12.

boxymethylcellulose gained 68.3 ± 8.2 g/kg. The difference in weight gain may be partially explained by a decrease in food intake for animals receiving I (1).

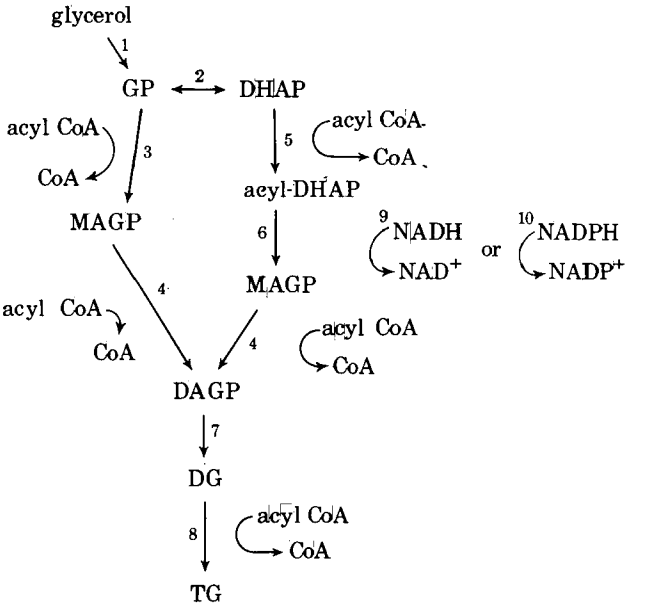
***In Vivo* Glycerolipid Synthesis**—Table IV shows the effect of I on the *in vivo* incorporation of 1,3-^{14}C-glycerol into hepatic, intestinal, and serum triglycerides and total glycerolipids. These calculations are based on the specific activity of serum glycerol 17 min after intraperitoneal injection of 1,3-^{14}C-glycerol. The methodology used for these calculations was presented previously (7).

The administration of I produced marked decreases in hepatic and intestinal triglyceride and total glycerolipid synthesis from 1,3-^{14}C-glycerol. The amount of labeled triglyceride found in the serum was also reduced 73% in animals treated with I. These results suggest that I may lower serum triglyceride levels by inhibition of hepatic and intestinal triglyceride synthesis. Since both total glycerolipid formation and triglyceride formation from 1,3-^{14}C-glycerol are reduced to the same degree by administration of I, this agent may reduce triglyceride formation by inhibiting an early step of glycerolipid metabolism (Scheme I).

Both the liver and intestine produce serum triglycerides. However, in the postabsorptive state, the liver is the major source of serum triglycerides. Therefore, the effect of I on individual reactions of hepatic glycerolipid metabolism was studied in more detail.

***In Vitro* Hepatic Glycerolipid Metabolism**—The effect of increasing concentrations of I on dihydroxyacetone phosphate acyltransferase and *sn*-glycerol-3-phosphate acyltransferase is shown in Table V. Compound I produced greater than 50% inhibition of both reactions at 0.75 m*M*. Since both reactions govern initial steps of glycerolipid metabolism (Scheme I) (13), I may exert its lipid-lowering action by inhibition of these steps *in vivo*.

***In Vivo* Lipase and Phosphatase Activity**—Compound I had



Scheme I—Pathways for Synthesis of Triglycerides: Step 1, glycerol kinase; Step 2, sn-*glycerol-3-phosphate (GP) dehydrogenase; Step 3,* sn-*glycerol-3-phosphate (GP) acyltransferase; Step 4, monoacylglycerophosphate (MAGP) acyltransferase; Step 5, dihydroxyacetone phosphate (DHAP) acyltransferase; Step 6, acyldihydroxyacetone phosphate reductase; Step 7, phosphatidate (DAGP) phosphatase; Step 8, diglyceride (DG) acyltransferase; Step 9, nicotinamide adenine dinucleotide; and Step 10, nicotinamide adenine dinucleotide phosphate.*

no effect on neutral phosphatidyl phosphatase in the liver but caused a reduction in the activity of liver acid phosphatidyl phosphatase, free and total acid phosphatase, and lipase (Table VI). Serum lipase was unaltered, but adipose lipase was significantly reduced. The lipolysis test was drastically reduced by I, indicating low levels of triglycerides and fatty acids in the plasma. The drug increased the rates of excretion of orally administered, labeled, exogenous cholesterol, progesterone, and palmitic acid (Table VII).

DISCUSSION

The results presented here and in a previous report (1) demonstrate that I lowers serum triglyceride and glycerol levels in rats. This agent may reduce serum triglycerides by inhibiting hepatic and intestinal triglyceride formation *in vivo*, since the incorporation of 1,3-^{14}C-glycerol into hepatic, intestinal, and serum triglycerides was markedly reduced by I. The fall in serum triglyceride levels is accompanied by a decrease in hepatic and intestinal triglyceride concentrations, which also may indicate a reduction in triglyceride formation by these tissues.

Scheme I outlines the basic biosynthetic pathways of hepatic glycerolipid metabolism. In this study, 1,3-^{14}C-glycerol was used to estimate *in vivo* glycerolipid metabolism. Labeled glycerol is taken up by tissues and converted to *sn*-glycerol-3-phosphate (GP) by the action of glycerokinase (Step 1, Scheme I). *sn*-Glycerol-3-phosphate may be converted to dihydroxyacetone phosphate

Table VII—Percent Recovery (Mean ± Standard Deviation) of Radioactivity in Fecal Lipid Excretion after Treatment with 2,8-Dibenzylcyclooctanone

	Control		Treated	
n = 6	6 hr	24 hr	6 hr	24 hr
Lipid extracted, mg	—	100		129
Exogenous				
^{14}C-Palmitic acid	100 ± 2	100 ± 6	190 ± 1[a]	297 ± 9[a]
^{14}C-Cholesterol	100 ± 15	100 ± 9	225 ± 14[a]	124 ± 12
^{3}H-Progesterone	100 ± 11	100 ± 9	178 ± 11[a]	237 ± 21[a]

[a] $p \geq 0.001$.

(DHAP) by *sn*-glycerol-3-phosphate dehydrogenase (Step 2, Scheme I). Both *sn*-glycerol-3-phosphate and dihydroxyacetone phosphate are converted to triglyceride (TG) by the sequential esterification steps.

Since hepatic total glycerolipid and triglyceride formation from 1,3-^{14}C-glycerol were reduced to the same degree by I (Table IV), this agent may inhibit an early step of glycerolipid metabolism *in vivo*. *In vitro* studies demonstrated that I inhibits *sn*-glycerol-3-phosphate acyltransferase (Step 3, Scheme I) and dihydroxyacetone phosphate acyltransferase (Step 5, Scheme I). An *in vivo* study also demonstrated that acid phosphatidate (DAGP) phosphatase (Step 7, Scheme I) was slightly reduced by I.

Other effects of I were decreased liver and adipose tissue lipase activity, reduced free and total acid phosphatase activity, and increased excretion of cholesterol, palmitic acid (triglyceride), and steroid hormone. Reduced lipase activity may explain the marked reduction in serum glycerol levels (Table III).

A number of agents effectively reduce serum triglycerides in humans and experimental animals (7, 14–26). However, the mode of action of these agents has not been elucidated. The hypolipidemic effect of I may partially be explained by inhibition of triglyceride formation, reduced lipolysis, and increased excretion of lipids. The effect of this agent on other factors that would lower serum lipids such as fatty acid biosynthesis, triglyceride clearance and release, and lipoprotein synthesis still remains to be studied.

The absorption and distribution data demonstrated that only minute amounts of I were being absorbed from the GI tract. The drug or its metabolites remained associated with the glycogen and lipid component(s) of the digestive tract. Only minimal concentrations of the drug were found in the blood. The muscle mass possessed more drug than the major organs of the body. The liver concentrated 6.5% of the drug in 1 hr, and a second peak (2.6%) in the liver was reached at 18 hr. This second peak may represent recirculation of the drug since the muscle mass decreased from this point in time and the fecal content increased. The drug and its metabolites were essentially eliminated from the body *via* the feces after 72 hr. Thus, it can be concluded that only very small quantities of the drug actually reached the cellular level.

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Evaluation of Nuclear-Substituted Styryl Ketones and Related Compounds for Antitumor and Cytotoxic Properties

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Abstract □ A number of nuclear-substituted styryl ketones, the related Mannich bases, and allyl alcohols were synthesized and evaluated for antitumor activity, principally in the L-1210 lymphoid leukemia and P-388 lymphocytic leukemia screening tests. The cytotoxicity of some of these compounds assessed in Eagle's 9KB carcinoma cell culture system was also recorded. Two of the Mannich bases showed promising levels of activity in the P-388 screening; of the results obtained to date, over one-third of the derivatives showed cytotoxicity at dose levels of 1–3 ppm. Other pharmacological results of these compounds are briefly reported.

Keyphrases □ Styryl ketones, nuclear substituted—synthesis, spectroscopy, and antitumor evaluation □ Mannich bases (related to styryl ketones)—antitumor evaluation □ Allyl alcohols from styryl ketones and related Mannich bases—antitumor evaluation □ Antitumor activity—nuclear-substituted styryl ketones and related Mannich bases and allyl alcohols

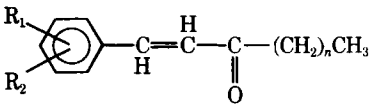
Approximately 70 years ago, it was shown that conjugated olefinic ketones undergo nucleophilic attack with thiols (1, 2) which, along with amines and carboxylate and phosphate anions, constituted the primary target sites for biological alkylating agents *in vivo* (3). Recent studies have demonstrated the reactivity of α,β-unsaturated ketones with thiols (4, 5) and amines (6), and enzyme-catalyzed alkylation of the thiol group of cysteine with α,β-unsaturated carbonyl compounds has been observed (7, 8).

It is known that Mannich bases have antitumor properties (9, 10) and the formation of the water-soluble Mannich bases derived from α,β-unsaturated ketones seems of interest. The higher chemical and biological reactivity of allyl alcohol compared to the saturated analog (11, 12) suggested the synthesis of substituted allyl alcohols as potential alkylating agents. Furthermore, it has been shown that the pyrrolizidine alkaloids with tumor-inhibiting properties possess an allylic ester function (13), which has been shown to react with thiols to give the corresponding S-alkylated product (14). The carbonyl reduction of both α,β-unsaturated ketones and the corresponding Mannich bases followed by esterification would lead to substituted allyl alcohols and esters that may have antitumor properties. The aim of the present investigation, therefore, was to synthesize a number of substituted styryl ketones (I), the related Mannich bases (II), and finally the related allyl alcohols and esters (III) for evaluation in various tumor systems, principally the L-1210 and P-388 leukemias *in vivo* and the KB cell culture system *in vitro*.

RESULTS AND DISCUSSION

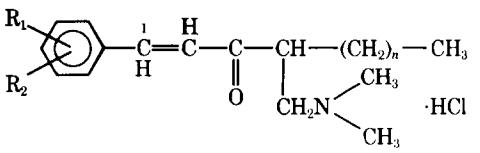
With the exception of I*i*, the styryl ketones were synthesized by the Claisen–Schmidt condensation between the appropriate benzaldehyde and either 2-octanone or 2-butanone. The possibility exists of condensation of the aromatic aldehyde with either the methyl or methylene group of the 2-alkanone. Base-catalyzed condensation between benzaldehyde and 2-octanone gave I*a*, whereas acid-catalyzed condensation gave an oil which was shown to consist mainly of 3-benzylidene-2-octanone (I*m*).

GLC analysis of the crude reaction products indicated that the base-catalyzed reaction did not give the methylene condensation product (I*m*) and that the acid-catalyzed reaction did not furnish the methyl condensation product (I*a*). However, the reaction product between benzaldehyde and 2-butanone was shown to consist of 84% I*j* and 16% I*n*, which was unexpected since earlier work had



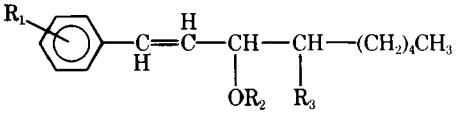
I

I*a*: $R_1 = R_2 = H$, $n = 5$
I*b*: $R_1 = 2\text{-Cl}$, $R_2 = H$, $n = 5$
I*c*: $R_1 = 3\text{-Cl}$, $R_2 = H$, $n = 5$
I*d*: $R_1 = 4\text{-Cl}$, $R_2 = H$, $n = 5$
I*e*: $R_1 = 2\text{-Cl}$, $R_2 = 4\text{-Cl}$, $n = 5$
I*f*: $R_1 = 2\text{-Cl}$, $R_2 = 6\text{-Cl}$, $n = 5$
I*g*: $R_1 = 3\text{-Cl}$, $R_2 = 4\text{-Cl}$, $n = 5$
I*h*: $R_1 = 4\text{-N(CH}_3)_2$, $R_2 = H$, $n = 5$
I*i*: $R_1 = 4\text{-}\overset{+}{\text{N}}(\text{CH}_3)_3\overset{-}{\text{I}}$, $R_2 = H$, $n = 5$
I*j*: $R_1 = R_2 = H$, $n = 1$
I*k*: $R_1 = 4\text{-Cl}$, $R_2 = H$, $n = 1$
I*l*: $R_1 = 4\text{-NO}_2$, $R_2 = H$, $n = 1$



II

II*a*: $R_1 = R_2 = H$, $n = 4$
II*b*: $R_1 = 2\text{-Cl}$, $R_2 = H$, $n = 4$
II*c*: $R_1 = 4\text{-Cl}$, $R_2 = H$, $n = 4$
II*d*: $R_1 = 2\text{-Cl}$, $R_2 = 4\text{-Cl}$, $n = 4$
II*e*: $R_1 = 2\text{-Cl}$, $R_2 = 6\text{-Cl}$, $n = 4$
II*f*: $R_1 = 3\text{-Cl}$, $R_2 = 4\text{-Cl}$, $n = 4$
II*g*: $R_1 = R_2 = H$, $n = 0$



III

III*a*: $R_1 = 4\text{-Cl}$, $R_2 = R_3 = H$
III*b*: $R_1 = R_2 = H$, $R_3 = CH_2N(CH_3)_2 \cdot HCl$
III*c*: $R_1 = R_2 = H$, $R_3 = CH_2\overset{+}{\text{N}}(CH_3)_3\overset{-}{\text{I}}$
III*d*: $R_1 = 2\text{-Cl}$, $R_2 = H$, $R_3 = CH_2N(CH_3)_2 \cdot HCl$
III*e*: $R_1 = 2\text{-Cl}$, $R_2 = CO$–C$_6$H$_4$–NO_2, $R_3 = CH_2N(CH_3)_2 \cdot HCl$
III*f*: $R_1 = H$, $R_2 = CO$–C$_6$H$_4$–NO_2, $R_3 = CH_2N(CH_3)_2 \cdot HCl$

Table I—Evaluation of Some (*E*)-1-(Substituted phenyl)-1-alken-3-ones (Series I) in the L-1210 and KB Test System

Compound	Vehicle[a]	Route of Injection	Interval[b] of Injection, days	Number of Injections	Dose per Injection, mg/kg	Survivors[c] (out of 6)	Animal[d] Weight Difference, g	T/C, %[e]	KB[f]
I*a*	7	Subcutaneous	1	1	400	6	−1.8	100	>100
					200	6	−0.5	91	
					100	6	+0.5	94	
	4	Subcutaneous	4	3	400	0	—	—	
					200	0	—	—	
					100	0	—	—	
I*b*	7	Intraperitoneal	1	9	400	6	−1.6	93	23
					200	6	−1.7	92	
					100	6	+0.5	98	
I*c*	M	Intraperitoneal	4	3	400[g]	6	−0.5	91	>10
					200[g]	6	+0.2	102	
					100[g]	6	−1.0	104	
	T	Intraperitoneal	1	9	400	6	−3.3	95	
					200	6	−0.7	88	
					100	6	−1.0	88	
I*d*	T	Intraperitoneal	4	3	400	6	−1.4	93	30
					200	6	+0.4	98	
					100	6	−0.3	96	
	C	Intraperitoneal	1	9	400	6	−2.5	92	
					200	5	−2.1	90	
					100	6	−0.3	93	
I*e*	C	Intraperitoneal	4	3	400	6	−2.3	93	28
					200	6	−2.1	91	
					100	6	−1.4	86	
	C	Intraperitoneal	1	9	400	6	−3.1	95	
					200	6	−1.0	90	
					100	6	−0.7	95	
I*f*	7	Subcutaneous	1	1	350	6	−0.4	108	N.A.
I*g*	C	Intraperitoneal	4	3	400	6	−1.1	98	27
					200	6	−0.3	93	
					100	6	−1.0	101	
	C	Intraperitoneal	1	9	400	2	−3.6	—	
					200	5	−3.1	92	
					100	6	−1.6	92	
I*h*	4	Subcutaneous	1	1	400	6	−0.7	97	>100
					200	6	+0.1	105	
					100	6	+0.1	97	
	4	Subcutaneous	4	3	400	6	−1.6	102	
					200	6	−0.6	103	
					100	6	−1.6	105	
I*i*	4	Subcutaneous	1	1	400	0	—	—	24
					200	2	+1.3	—	
					100	6	−1.4	86	
	4	Subcutaneous	4	3	100	6	−1.0	106	
					50	6	−0.5	92	
					25	6	+0.9	94	
	4	Subcutaneous	1	9	100	0	—	—	
					50	6	−0.9	97	
					25	6	−1.3	93	
I*j*	M	Intraperitoneal	4	3	400	6	−0.4	91	N.A.
					200	6	−0.3	107	
					100	6	+0.2	96	
I*k*	M	Intraperitoneal	4	3	400	6	+0.5	102	25
					200	6	−0.2	104	
					100	6	−0.1	98	
	M	Intraperitoneal	1	9	400	6	−1.1	97	
					200	6	+1.2	97	
					100	6	+0.8	98	
I*l*	7	Intraperitoneal	4	3	400	6	−0.4	93	N.A.
					200	6	−0.8	93	
					100	6	−1.0	101	

[a] C = acetone, D = alcohol, M = hydroxypropylcellulose, S = saline sonified, T = saline with Tween 80, 2 = saline, 4 = steroid suspending solution, and 7 = unspecified vehicle. [b] 1 = injections given daily, and 4 = injections made every 4 days. [c] Number of survivors on 5th day after the first injection. [d] Average weight of test group minus average weight of control animals. [e] Ratio of survival time of treated animals to control animals expressed as a percentage. [f] The figures (in micrograms per milliliter) in the KB cell culture screen indicate the dose inhibiting 50% growth of human epidermoid carcinoma of the nasopharynx in Eagle's medium. N.A. = result not available. [g] The CDF_1 strain of mouse used.

shown that in dilute alkali the reaction product consisted of I*j* (15). The compounds in Series I submitted for screening were shown by spectroscopic means to be formed by condensation between the methyl portion of the appropriate 2-alkanone and the aromatic aldehyde.

The quaternary ammonium compound (I*i*) was prepared by quaternization of I*h* with methyl iodide. Attempts to condense *p*-nitrobenzaldehyde with 2-octanone to give 1-(*p*-nitrophenyl)-1-nonen-3-one using aqueous sodium hydroxide solution were unsuccessful. Other workers have also noted the failure of nitrobenzaldehydes to react with both γ-butyrolactone and 2-hexanone in the presence of aqueous alkali (16, 17). Piperidine acetate has

Table II—Evaluation of Some (*E*)-4-Dimethylaminomethyl-1-(substituted phenyl)-1-nonen-3-one Hydrochlorides (Series II) in the L-1210 and KB Test Systems

Compound	Vehicle[a]	Route of Injection	Interval[b] of Injection, days	Number of Injections	Dose per Injection, mg/kg	Survivors[c] (out of 6)	Animal[d] Weight Difference, g	T/C, %[e]	KB[f]
II*a*	4	Subcutaneous	1	1	400	5	−4.3	100	1.5
					200	5	+0.2	93	
					100	5	−2.9	93	
	7	Subcutaneous	4	3	400	1	−1.1	—	
					200	6	−1.1	90	
					100	6	−0.9	101	
	7	Subcutaneous	1	9	200	0	—	—	
					100	6	−1.4	102	
					50	6	−0.8	95	
II*b*	7	Intraperitoneal	1	9	400	0	—	—	2.4
					200	0	—	—	
					100	5	−3.8	72	
II*c*	4	Subcutaneous	1	1	400	0	—	—	1.7
					200	5	−2.9	116	
					100	6	−0.3	91	
	4	Subcutaneous	4	3	200	4	−1.3	79	
					100	6	−0.4	78	
					50	6	+0.3	95	
	7	Subcutaneous	1	9	50	6	−1.9	93	
					25	6	+0.2	100	
					12.5	6	−0.4	100	
	7	Subcutaneous	1	9	170	0	—	—	
					115	4	+0.2	100	
					75	6	+0.3	98	
II*d*	D	Intraperitoneal	4	3	400	0	—	—	1.0
					200	0	—	—	
					100	2	—	—	
	D	Intraperitoneal	4	3	50	6	−0.9	97	
					25	6	+0.5	97	
					12.5	6	−0.4	87	
	D	Intraperitoneal	1	9	50[g]	5	−3.6	82	
					25[g]	5	−2.2	102	
					12.5[g]	6	−0.7	102	
II*e*	M	Intraperitoneal	1	1	400	0	—	—	2.8
					200	1	—	—	
					100	5	−2.8	94	
	M	Intraperitoneal	1	9	100	2	—	—	
					50	6	−4.7	75	
					25	6	−3.6	106	
	7	Intraperitoneal	1	9	25	6	−1.4	104	
					12.5	6	−0.3	101	
					6.25	6	−0.1	101	
II*f*	C	Intraperitoneal	4	3	400	0	—	—	1.2
					200	0	—	—	
					100	0	—	—	
	C	Intraperitoneal	4	3	50	3	—	—	
					25	6	−1.4	92	
					12.5	6	+1.1	95	
	C	Intraperitoneal	1	9	25[g]	6	−3.9	97	
					12.5[g]	6	−1.0	94	
					6.25[g]	6	−0.5	92	
	2	Intraperitoneal	1	9	80[g]	0	—	—	
					55[g]	0	—	—	
					35[g]	1	—	—	
II*g*	S	Intraperitoneal	4	3	400	0	—	—	1.2
					200	0	—	—	
					100	6	−0.7	86	
	S	Intraperitoneal	4	3	50[g]	6	−0.6	102	
					25[g]	6	+0.4	106	
					12.5[g]	6	−0.5	102	
	S	Intraperitoneal	1	9	50	6	−2.1	107	
					25	6	−0.6	104	
					12.5	6	−0.8	103	
	S	Intraperitoneal	1	9	170	0	—	—	
					115	3	—	—	
					75	6	0	84	

[a–g] See Table I.

been employed successfully as the catalyst in the synthesis of 3-(*p*-nitrobenzylidene)-2,4-pentanedione from 2,4-pentanedione and *p*-nitrobenzaldehyde (18), but use of this catalyst in the present investigation led only to the isolation of unreacted starting materials and a low yield of a multicomponent oil. The literature describes the preparation of an analog of 1-(*p*-nitrophenyl)-1-nonen-

Table III—Evaluation of Some Substituted (*E*)-1-Phenyl-1-nonen-3-ols and Related Compounds (Series III) against L-1210 and KB Test Systems

Compound	Vehicle[a]	Route of Injection	Interval[b] of Injection, days	Number of Injections	Dose per Injection, mg/kg	Survivors[c] (out of 6)	Animal[d] Weight Difference, g	T/C, %[e]	KB[f]
III*a*	T	Intraperitoneal	4	3	400[g]	6	−0.2	111	N.A.
					200[g]	6	−0.5	102	
					100[g]	6	−0.1	110	
	T	Intraperitoneal	1	9	400	6	−0.6	—	
					200	6	−3.8	91	
					100	6	−2.1	94	
III*b*	4	Subcutaneous	4	3	400	4	+0.8	61	6.4
					200	6	−0.6	75	
					100	6	−0.6	75	
	7	Intraperitoneal	4	3	50	6	+0.8	103	
					25	6	+0.1	106	
					12.5	6	−0.5	119	
	4	Subcutaneous	1	9	50	6	−0.9	97	
					25	6	−0.7	98	
					12.5	6	−0.9	97	
III*c*	T	Intraperitoneal	4	3	400	0	—	—	42
					200	0	—	—	
					100	0	—	—	
	2	Intraperitoneal	4	3	20	0	—	—	
					10	6	+0.6	98	
					5.0	6	+0.3	103	
	2	Intraperitoneal	1	9	10	6	−0.6	99	
					5.0	6	−0.7	97	
					2.5	6	+0.1	97	
III*d*	2	Intraperitoneal	4	3	400	1	—	—	3.2
					200	0	—	—	
					100	5	−0.8	88	
	2	Intraperitoneal	4	3	100	5	−1.6	95	
					50	6	−0.2	97	
					25	6	−0.6	96	
	2	Intraperitoneal	1	9	100	3	—	—	
					50	6	−0.5	94	
					25	6	+0.3	102	
III*e*	T	Intraperitoneal	4	3	400	6	+0.1	92	N.A.
					200	6	+0.1	92	
					100	6	+0.1	95	
III*f*	T	Intraperitoneal	4	3	400	5	−0.2	106	>10
					200	6	−0.6	105	
					100	6	−0.4	90	
	T	Intraperitoneal	1	9	400	3	—	—	
					200	6	−4.9	100	
					100	5	−3.4	97	

[a–g] See Table I.

3-one, namely I*l*, which was synthesized by an aldol condensation between 2-butanone and *p*-nitrobenzaldehyde followed by dehydration of one of the intermediate hydroxyketones (19). Two short-chain analogs of I*l*, namely I*j* and I*k*, were prepared by Claisen–Schmidt condensation of the appropriate aldehyde and 2-butanone.

The styryl ketones I were shown by GLC to be homogeneous. Examination of the PMR spectra of the styryl ketones in Series I showed that the olefinic protons at C-1 and C-2 resonated in the range of δ 7.32–7.80 and 6.37–6.85, respectively. An olefinic coupling constant of 16 Hz was observed, indicating the *(E)*-configuration of the double bond (17, 20). A strong IR band at 980 cm^{-1}, due to the carbon–hydrogen out-of-plane vibration characteristic of ethylenic compounds possessing the *(E)*-configuration (21), was noted in most compounds. Two carbonyl absorptions at 1695–1675 and 1690–1640 cm^{-1} due to the presence of s-*cis*- and s-*trans*-rotational isomers (17, 22, 23) were also observed.

In an attempt to prepare I*h*, using the conditions of the normal Claisen–Schmidt condensation, only unreacted starting materials were obtained, even when the time of heating under reflux was extended from 24 to 58 hr. However, Neilsen and Dubin (24) prepared I*h* by employing sodium hydroxide in a mixture of ethanol (92%) and water (8%), a solvent known to enhance dimer formation of styryl ketones (25, 26). In the present study, I*h* (48% yield) was obtained along with a colorless solid (IV) (~2% yield), which was shown to be a dimer of I*h*.

The preparation and properties of the Mannich bases II*a*–II*f* and the related compounds V*a* and V*b* were reported recently (27). The syntheses and spectral characteristics of the substituted allyl alcohols and esters (III) and the related compound (VI) are described in the literature (28) or under *Experimental*. In an attempt to prepare *(E)*-1-(*p*-dimethylaminophenyl)-1-nonen-3-ol by sodium borohydride reduction of I*h*, a mixture of the desired alcohol (70%) and unreacted I*h* (30%) was obtained, which was not separated by fractional crystallization or by preparative GLC.

The antineoplastic activity of a variety of epoxides (29, 30) suggested the preparation of 1,2-epoxy-1-(*p*-chlorophenyl)-3-nonanone by reaction of I*d* with hydrogen peroxide. The derivative obtained gave the predicted spectral characteristics for the epoxide and was homogeneous by TLC, but its rapid decomposition precluded its submission for screening.

The evaluation of the styryl ketones (I) against L-1210 lymphoid leukemia in mice is shown in Table I. None of the compounds showed activity. In the P-388 lymphocytic leukemia screen, I*c* showed no activity (Table V). In the KB test system *in vitro*, which is a measure of cytotoxicity and may indicate tumor-inhibiting properties (31), the chlorophenyl derivatives (I*b*, I*d*, I*e*, I*g*, and I*k*) and the quaternary ammonium iodide (I*i*) had a low level of activity, namely 23–30 ppm (Table I). At 400 mg/kg, no toxicity appeared in the mice, with the exception of Compounds I*g* and I*i*.

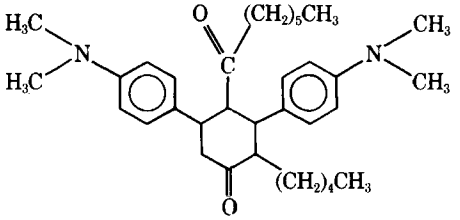
Table IV—Evaluation of IV, V*a*, V*b*, and VI in the L-1210 and KB Test Systems

Compound	Vehicle[a]	Route of Injection	Interval[b] of Injection, days	Number of Injections	Dose per Injection, mg/kg	Survivors[c] (out of 6)	Animal[d] Weight Difference, g	T/C, %[e]	KB[f]
IV	T	Intraperitoneal	4	3	400	6	−1.0	92	N.A.
					200	6	+1.1	90	
					100	6	−0.4	89	
	T	Intraperitoneal	1	9	400	6	−1.8	95	
					200	6	−0.5	95	
					100	6	+1.2	86	
V*a*	M	Intraperitoneal	4	3	400	2	—	—	2.1
					200	4	−1.3	106	
					100	6	−1.8	96	
	T	Intraperitoneal	1	9	400	0	—	—	
					200	0	—	—	
					100	4	−2.1	—	
V*b*	M	Intraperitoneal	4	3	400	6	−2.4	103	2.3
					200	5	−1.6	100	
					100	6	−1.0	102	
	M	Intraperitoneal	1	9	200	6	−0.1	95	
					100	4	+5.2	97	
					50	5	+0.2	103	
VI	M	Intraperitoneal	1	9	400	6	−2.6	113	N.A.
					200	5	−1.2	102	
					100	6	−1.3	103	

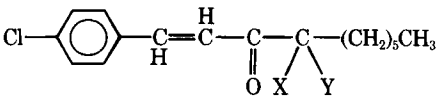
[a-f] See Table I.

The Mannich bases (II) showed no appreciable activity against the L-1210 lymphoid leukemia (Table II), although a high level of cytotoxicity (KB test) may be seen in this series for the results obtained to date. In the P-388 leukemia screen (Table V), II*d* and II*f* showed promising levels of activity, with increases in mean survival time of 30 and 42% at doses of 18 and 6.25 mg/kg, respectively. If the rate of reactivity of styryl ketones with biologically important nucleophiles is considered to be dependent on the fractional positive charge on C-1, then maximum activity would be expected to occur when the nuclear substituents are two chlorine atoms as in II*d*, II*e*, and II*f*. In the case of compounds containing one or two *ortho*-chloro atoms, II*d* and II*e*, steric impedence of an approaching nucleophile would occur, reducing reactivity; the effect would be less in the case of II*d*, which contains only one chlorine atom. In the case of II*f*, neither of the two chlorine atoms occupies the *ortho*-position on the aromatic ring so reaction with nucleophiles should be increased.

Activity against P-388 leukemia decreases in the order II*f*, II*d*, and II*e*, following a pattern of probable decreased chemical reactivity with nucleophiles at the cellular level. The toxicity in mice

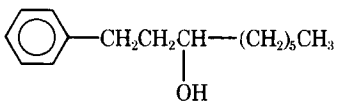


IV



V

V*a*: X = $CH_2N(CH_3)_3I$, Y = H
V*b*: X, Y = CH_2



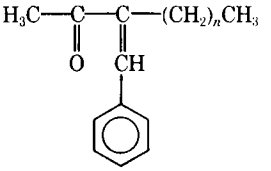
VI

for the chlorinated compounds II*b*–II*f* is higher than for the unsubstituted compound, II*a*. In addition, II*g*, possessing a shorter alkyl chain than II*a*, had greater toxicity. The mammalian toxicity increased in the dichloro derivatives II*d*–II*f* in the same sequence as the increases in activity in the P-388 screen. Compounds II*a*–II*c* showed no appreciable activity against the Ehrlich ascites tumor in mice (Table VI).

The allyl alcohol (III*a*) formed by reduction of I*d* showed only minor improvements in the L-1210 screen when the injections were given every 4 days. Both compounds were nontoxic at 400 mg/kg. Reduction of the Mannich bases II*a* and II*b* gave III*b* and III*d*, respectively. Both of these compounds had similar levels of toxicity and inactivity in the L-1210 screen as their precursors, although III*b* had a marginal level of activity (T/C 119%) when the injections were made every 4 days. While esterification of III*b* to give III*f* did not alter mammalian toxicity, quaternization of III*b* as the free base gave III*c*, with sharply increased mammalian toxicity. Esterification of III*d* gave III*e*, which showed reduced mammalian toxicity, but neither of the esters III*e* and III*f* had activity in the L-1210 screen. In the Ehrlich ascites screen (Table VI), III*b* and III*c* showed no activity but marginal activity (T/C 118%) was found for III*d*.

The possible metabolite of II*c*, namely V*b*, showed decreased toxicity in mice but no improvement in activity in the L-1210 screen (Table IV). The saturated alcohol (VI) was inactive and nontoxic at the dose levels examined.

Compounds I*a*, I*b*, I*d*, I*f*, I*h*, and I*i* were screened against *Streptococcus faecalis*, *Escherichia coli*, and *Candida albicans* at concentrations of 200 and 10 μg; the Mannich bases II*a*–II*c*, II*e*, and III*e* were screened against *S. faecalis* and *E. coli* at the same concentrations. Compound I*a* inhibited the growth of *S. faecalis* at 10 μg, and there was only partial growth of this organism when 10 μg of I*b*, I*d*, or I*f* was added to the medium. The remaining compounds were inactive against the organisms at a concentration of 200 μg.



I*m*: *n* = 4
I*n*: *n* = 0

In view of the fact that the potent diuretic ethacrynic acid possesses an α,β-unsaturated keto group attached to an aromatic ring containing two chlorine atoms, it was decided to examine II*d* and II*f* for possible diuretic activity. At 15 mg/kg, II*d* and II*f* showed marked antidiuretic activity with approximately 37 and 39%, respectively, of the urine output of the control rats. At 2 mg/kg, II*d* had 72% of the urine output of the control rats while II*f* had neither antidiuretic nor diuretic activity.

EXPERIMENTAL[1]

Substituted Styryl Ketones (I*a*–I*l*)—The preparation of I*a*, I*b*, I*d*–I*h*, I*j*, and I*k* was described previously (32). Utilization of the general method used earlier (32) gave *(E)*-1-(*m*-chlorophenyl)-1-nonen-3-one (I*c*) as colorless crystals (33%), mp 34°; IR (KBr): 1695 (s) (C=O), 1665 (s) (C=O), 1615 (s) (C=C), and 980 (s) (*trans*-CH=CH) cm^{-1}; NMR (CCl_4, 0.5 *M*): δ 7.33 (m, 5, C_1H and aromatic H), 6.55 (d, 1, $J_{2,1}$ = 16 Hz, C_2H), 2.53 (t, 2, $J_{4,5}$ = 6.5 Hz, C_4H_2), 1.87–1.10 (m, 8, CH_2), and 0.90 (m, 3, C_9H_3) ppm; mass spectrum: 250 (M^+, relative intensity 15%).

Anal.—Calc. for $C_{15}H_{19}ClO$: C, 71.84; H, 7.64. Found: C, 71.99; H, 7.51.

(E)-1-(*p*-Dimethylaminophenyl)-1-nonen-3-one methiodide (I*i*) was prepared by heating under reflux a mixture of I*h* (20.00 g, 0.077 mole), methyl iodide (21.86 g, 0.154 mole), and ethanol (20 ml) as brown platelets (19.69 g, 64%) from methanol, mp 142° dec; IR (KBr): 1695 (s) (C=O), 1665 (s) (C=O), and 1620 (C=C) cm^{-1}; NMR ($CDCl_3$): δ 7.80 (m, 5, C_1H and aromatic H), 6.75 (d, 1, $J_{2,1}$ = 16 Hz, C_2H), 4.03 [s, 9, $^+N(CH_3)_3$], 2.67 (t, 2, $J_{4,5}$ = 6.5 Hz, C_4H_2), 2.00–1.10 [m, 8, $(CH_2)_4$], and 0.90 (m, 3, C_9H_3) ppm.

Anal.—Calc. for $C_{18}H_{28}INO$: C, 53.87; H, 7.03. Found: C, 54.05; H, 7.22.

(E)-1-(*p*-Nitrophenyl)-1-penten-3-one (I*l*) was prepared in 7% yield from 2-butanone and *p*-nitrobenzaldehyde, according to a literature method (19), as orange crystals from ethanol, mp 108.5° [lit. (19) mp 108–110°]; IR (KBr): 1695 (s) (C=O), 1670 (s) (C=O), and 1620 (s) (C=C) cm^{-1}; NMR ($CDCl_3$, 0.5 *M*): δ 8.23 (m, 2, aromatic H), 7.63 (m, 3, C_1H and aromatic H), 6.85 (d, 1, $J_{2,1}$ = 16 Hz, C_2H), 2.75 (q, 2, $J_{4,5}$ = 7.5 Hz, C_4H_2), and 1.18 (t, 3, $J_{5,4}$ = 7.5 Hz, C_5H_3) ppm.

Anal.—Calc. for $C_{11}H_{11}NO_3$: C, 64.38; H, 5.40; N, 6.83. Found: C, 64.55; H, 5.45; N, 6.87.

In an attempt to prepare *(E)*-1-(*p*-nitrophenyl)-1-nonen-3-one, 2-octanone (16.03 g, 0.125 mole) and *p*-nitrobenzaldehyde (3.78 g, 0.025 mole) were added to piperidine acetate (from 2.1 g from piperidine and 2.0 g acetic acid). The mixture was stirred at room temperature for 2 hr and then poured onto ice; the precipitate was collected and recrystallized from ethanol to give unreacted *p*-nitrobenzaldehyde (0.445 g) (melting point and NMR evidence). Extraction of the aqueous phase with ether (3 × 40 ml) gave a brown oil (0.63 g), shown by GLC analysis to consist of at least seven components.

Preparation of 3-Benzylidene-2-alkanones (I*m* and I*n*)—A stirred mixture of 2-octanone (12.82 g, 0.10 mole) and benzaldehyde (10.61 g, 0.10 mole) was cooled (−5°), and hydrogen chloride gas (1.82 g, 0.05 mole) was bubbled through the reactants. The mixture was stirred at room temperature for 24 hr and heated under reflux for 2.5 hr to give a dark-brown oil, which was distilled to give a yellow oil, 6.60 g, bp 130°/9.0 mm [lit. (33) bp 161–162°/14 mm]. GLC analysis showed that two products were present, the major one (I*m*) to the extent of 95%; IR (neat): 1660 (s) (C=O) and 1620 (m) (C=C) cm^{-1}; NMR (CCl_4): δ 7.29 (m, 6, phenyl H and benzylidene H), 2.31 (broad s, 5, C_1H_3 and C_4H_2), 1.60–1.10 [m, 6, $(CH_2)_3$], and 0.90 (m, 3, C_8H_3) ppm.

GLC analysis of the crude reaction product between benzaldehyde and 2-butanone indicated a mixture of I*j* (84%) with a longer retention time than I*n* (16%). The mixture was separated using preparative GLC (10% Carbowax 20M, 215°). The characteristics of I*n* are as follows: IR (neat): 1665 (s) (C=O) and 1625 (s) (C=C) cm^{-1}; NMR (CCl_4): δ 7.30 (m, 6), 2.40 (s, 3), and 2.00 (d, 3, J = 2Hz) ppm; mass spectrum: 160 (M^+, relative intensity 100%).

3,5-Bis(*p*-dimethylaminophenyl)-4-heptanoyl-2-pentylcyclohexanone (IV)—This compound was obtained from the reaction between *p*-dimethylaminobenzaldehyde (74.58 g, 0.50 mole) and 2-octanone (64.10 g, 0.50 mole) in ethanol (340 ml) and 25% aqueous sodium hydroxide solution (30 ml). After the reaction mixture had been stirred at room temperature for 1 hr, it was set aside for 22 hr at room temperature; then I*h* (61.98 g, 48%) was removed by filtration. The mother liquor was concentrated *in vacuo* to give a semisolid. TLC on silica gel, using benzene–acetic acid–95% ethanol (10:1:1 v/v), showed that I*h* and a less mobile component were present. The semisolid was washed with the minimum quantity of ethanol to give a colorless powder (3.97 g), which was removed by filtration. Recrystallization from ethanol–ethyl acetate (1:1 v/v) gave IV (2.293 g, 1.8%), with an R_f value corresponding to the less mobile compound on TLC, mp 176–177° [lit. (24) mp 184–186°]; IR (KBr): 1720 (s) (C=O) and 1695 (s) (C=O) cm^{-1}; mass spectrum: 518 (M^+, relative intensity 100%).

Anal.—Calc. for $C_{35}H_{50}N_2O_2$: C, 78.71; H, 9.72; N, 5.40. Found: C, 78.90; H, 9.74; N, 5.18.

Substituted 4-Dimethylaminomethyl-1-phenyl-1-nonen-3-one Hydrochlorides (II*a*–II*g*) and V*a* and V*b*—The preparation of II*a*–II*f* and V*a* and V*b* was described previously (27). *(E)*-5-Dimethylamino-4-methyl-1-phenyl-1-penten-3-one hydrochloride (II*g*) was prepared by the general method (27), crystallizing as a colorless hygroscopic powder (43%) from acetone, mp 114–115° [lit. (34) mp 115–117° for 1:1 maleate salt]; IR (KBr): 1685 (s) (C=O), 1660 (w) (C=O), 1615 (s) (C=C), and 975 (s) (*trans*-CH=CH) cm^{-1}; NMR ($CDCl_3$, 0.5 *M*): δ 12.5–11.7 (broad s, 1, ^+NH, exchanged with D_2O), 7.75 (d, 1, $J_{1,2}$ = 16 Hz, C_1H), 7.47 (m, 5, phenyl H), 6.82 (d, 1, $J_{2,1}$ = 16 Hz, C_2H), 3.73 (m, 2, C_5H_2), 3.33–2.50 [m, 7, C_4H and $^+N(CH_3)_2$], and 1.33 (d, 3, J = 6.5 Hz, C_4CH_3) ppm.

Anal.—Calc. for $C_{14}H_{20}ClNO$: C, 66.26; H, 7.94; N, 5.52. Found: C, 65.75; H, 7.90; N, 5.48.

Substituted 1-Phenyl-1-nonen-3-ols (III*a*–III*d*) and Related Compounds (III*e*, III*f*, and VI)—The preparation of III*a*, VI, and diastereoisomeric modifications of III*b*–III*d* and III*f* was reported previously (28). The samples evaluated in the L-1210 screens (Table III) were the major diastereoisomers in the cases of III*d* and III*f* and a preponderance of the major isomers from the mixture in the cases of III*b*, mp 122–122.5° [lit. (28) mp 126°] and III*c*, mp 110–112° [lit. (28) mp 112.5–113.5°]. The alcohols III*b* (mp 120–121.5°) and III*c* (mp 110–112°) were screened against the Ehrlich ascites tumor (Table VI). The III*d* alcohol was a pure sample of the major isomer (NMR evidence).

The synthesis of III*e* was accomplished by the esterification of a diastereoisomeric mixture of *(E)*-1-(*o*-chlorophenyl)-4-dimethylaminomethyl-1-nonen-3-ols (5.10 g, 0.0164 mole) (28) with *p*-nitrobenzoyl chloride (3.70 g, 0.020 mole) in ether, using the method previously described (28), except that the time of stirring at room temperature was 24 hr. The colorless crystals deposited (6.73 g), mp 140–144°, were recrystallized from ethanol to give III*e* (1.33 g, 16%), mp 177° dec. The compound was homogeneous on silica gel thin-layer plates [R_f 0.67; *n*-butanol–acetic acid–water (12:3:5 v/v)]; IR (KBr): 1715 (s) (C=O), 1530 (s) (NO_2), and 965 (s) (*trans*-CH=CH) cm^{-1}; NMR ($CDCl_3$): δ 12.93–12.27 (broad s, 1, ^+NH, exchanged with D_2O), 8.28 (s, 4, *p*-$NO_2C_6H_4$), 7.37 (m, 5, C_1H and

[1] Melting points were determined on a Gallenkamp MF-370 apparatus. Boiling points and melting points are uncorrected. The NMR spectra were determined using a Varian T-60 spectrometer, with tetramethylsilane as the internal standard. IR absorption spectra were recorded on a Unicam SP-200G spectrophotometer previously calibrated with polystyrene. Band intensities are denoted as s (strong), m (medium), and w (weak). Mass spectra were determined at 70 ev on an AE1 MS-12 single-focusing mass spectrometer, operated by Mr. D. Bain of the Department of Chemistry and Chemical Engineering, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. The instrument used a heated inlet system operating near the melting point of the compound, and samples were introduced by direct probe technique. Elemental analyses were performed by Dr. F. B. Strauss, Microanalytical Laboratories, Oxford, England, and by Mr. R. M. Smith of the College of Pharmacy, University of Saskatchewan, Saskatoon, who used a Coleman model 33 carbon–hydrogen analyzer.

TLC plates, 0.5 mm thick, were prepared using silica gel G (E. Merck and Co.) and subsequently heated at 120° for 2 hr prior to use. The chromatograms were developed (20–120 min), and the compounds were detected with a spray composed of a 1% (w/v) aqueous solution of potassium permanganate containing 1% sulfuric acid. GLC was undertaken on a Pye 104 model 64 instrument using the conditions previously described (27). For preparative GLC, a Hewlett-Packard 5750B research instrument was employed. The aluminum columns [1.8 m × 0.952 cm (6 ft × 0.375 in.) o.d.] were packed with 10% Carbowax on Chromosorb G, 60–80 mesh, acid washed and silanized. Organic extracts were washed with water and dried over anhydrous magnesium sulfate.

Table V—Activity of Some Styryl Derivatives against P-388 Lymphocytic Leukemia

Compound	Vehicle[a]	Route of Injection	Interval[b] of Injection, days	Number of Injections	Dose per Injection, mg/kg	Survivors[c] (out of 6)	Animal[d] Weight Difference, g	T/C, %[e]
Ic	2	Intraperitoneal	4	3	400	6	+1.2	100
					200	6	+0.5	0
					100	6	+0.7	88
	T	Intraperitoneal	1	9	400[g]	6	−0.7	104
					200[g]	6	−0.8	104
					100[g]	6	−0.5	108
IId	2	Intraperitoneal	1	9	25[g]	6	−2.1	115
					12.5[g]	6	−1.9	125
					6.25[g]	6	−0.6	90
	2	Intraperitoneal	1	9	18[g]	6	−3.3	130
					12.5[g]	6	−2.2	120
					8.3[g]	6	−0.2	110
	D	Intraperitoneal	1	9	35	6	−3.5	110
	2	Intraperitoneal	1	9	18	5	−2.8	122
					12	6	−1.9	122
					8.0	6	−2.3	122
IIe	M	Intraperitoneal	1	9	25	4	−5.4	60
					6.25	6	−5.8	110
					12.5	6	−0.7	100
	M	Intraperitoneal	4	3	12.5	6	−0.6	104
					6.25	6	+0.2	109
					3.12	6	+0.5	104
					40	6	−4.0	90
					28	6	−2.5	115
					18	6	−4.9	105
	2	Intraperitoneal	1	1	28	6	−1.7	100
					14	6	−1.0	110
					7.0	6	+0.3	110
					95	3	—	—
					65	6	−1.8	104
					40	6	−1.7	109
IIf	2	Intraperitoneal	1	9	80	0	—	—
					55	0	—	—
					35	0	—	—
					25	5	−4.1	136
					12.5	6	−3.7	126
					6.25	6	−2.2	142
					4.12	6	−1.7	105
					2.75	5	+1.1	110
	2	Intraperitoneal	1	1	400	0	—	—
					200	0	—	—
					100	2	—	—
					50	6	−1.6	100
					25	6	0	106
					12.5	6	+0.3	106
					6.25	6	+0.1	106
IIg	2	Intraperitoneal	1	9	110	2	—	—
					75	6	−0.8	109
					37.5	6	0	109
					18.7	6	−0.2	100
	2	Intraperitoneal	4	3	225	0	—	—

[a–e,g] See Table I.

o-ClC_6H_4), 7.27 (m, 2, C_2H and C_3H), 3.53–2.37 [m, 9, C_4H and $CH_2N(CH_3)_2$], 2.07–1.10 [m, 8, $(CH_2)_4$], and 0.90 (m, 3, C_9H_3) ppm.

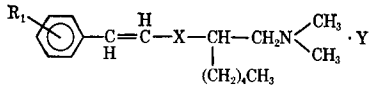
Anal.—Calc. for $C_{25}H_{32}Cl_2N_2O_4$: C, 60.60; H, 6.51; N, 5.67. Found: C, 60.64; H, 6.55; N, 5.75.

Attempted Preparation of *(E)*-1-(*p*-Dimethylaminophenyl)-1-nonen-3-ol—A solution of sodium borohydride (1.135 g, 0.03 mole) in water (18 ml, adjusted to pH 8.5 with aqueous sodium hydroxide solution) was added dropwise to a solution of Ih (7.780 g, 0.03 mole) in methanol (45 ml) at 0° under nitrogen. The reaction mixture was stirred at 0° for 1 hr and then at room temperature for 19 hr, after which the solvents were removed *in vacuo* to give a yellow residue; this residue was added to water (150 ml) at pH 4. The pH of the reaction mixture was adjusted to pH 7.5 and extracted with ether to give a yellow solid (7.30 g). TLC of the solid on silica gel, using *n*-butanol–acetic acid–water (12:3:5 v/v), showed the presence of at least two poorly resolved components. NMR spectroscopy indicated a mixture of Ih (~30%) and the desired alcohol (~70%); NMR ($CDCl_3$): δ 6.62 (dd, $J_{2,1}$ = 16 Hz, $J_{2,3}$ = 6.5 Hz, C_2H), 4.20 (q, J = 6 Hz, C_3H), and 1.90 (s, C_3OH, exchanged with D_2O) ppm. Fractional crystallization from petroleum ether, hexane, methylene chloride, and ethanol was unsuccessful. The crude solid changed to a red oil upon storage in a vacuum desiccator.

1,2-Epoxy-1-(*p*-chlorophenyl)-3-nonanone—A solution of hydrogen peroxide (4.25 g, 0.125 mole, 14.2 ml of 30% solution) in aqueous sodium hydroxide solution (5% w/v, 15 ml) was added dropwise to a solution of Id (3.135 g, 0.0125 mole) in dioxane (30 ml) at 10°. The mixture was stirred at 10–12° for 0.5 hr and, after standing at room temperature for 16 hr, water (350 ml) was added and the resultant precipitate was removed by filtration and dried overnight in a vacuum desiccator protected from light. The colorless precipitate (2.505 g, 75%), mp 61–62°, was recrystallized from petroleum ether (bp 60–80°), which did not alter the melting point. The title epoxide migrated as a single spot on silica gel thin-layer plates, using benzene–acetic acid–water (10:1:1 v/v) as the developing solvent; IR (KBr): 1710 (s) (C=O) cm^{-1}; NMR (CCl_4, 0.5 *M*): δ 7.20 (m, 4, aromatic H), 3.87 (d, 1, $J_{1,2}$ = 2.0 Hz, C_1H), 3.25 (d, 1, $J_{2,1}$ = 2.0 Hz, C_2H), 2.40 (m, 2, C_4H_2), 1.73–1.07 [m, 8, $(CH_2)_4$], and 1.07–0.67 (m, 3, C_9H_3) ppm.

A sample of the epoxide became yellow, mp 48–49°, after stand-

Table VI—Activity of Some Styryl Derivatives against Ehrlich Ascites Tumor in Mice



Compound	R_1	X	Y	Dose, mg/kg[a]	T/C, %
II*a*	H	C=O	HCl	10	103
II*b*	2-Cl	C=O	HCl	25	111
				10	108
II*c*	4-Cl	C=O	HCl	25	110
				10	111
III*b*	H	CH(OH)	HCl	25	101
III*c*	H	CH(OH)	CH_3I	25	104
III*d*	2-Cl	CH(OH)	HCl	50	118
				25	117

[a] The same dose was given for 7 consecutive days. The control animals received injections of physiological saline.

ing at room temperature for 3 days; after 5 days, it became a yellow oil. Another sample of the title compound was stored at 0°; after 10 and 30 days, it melted at 59–60 and 51–54°, respectively, after which it became a yellow oil [IR (neat) showed absorptions at 3350 and 1630 cm^{-1}, which were absent in the pure epoxide].

Screening of Compounds—The screening results listed in Tables I–V were carried out by the Drug Research and Development Division of the National Cancer Institute, Bethesda, Md., using the N.C.I. protocols (35). Unless otherwise stated, the BDF_1 strain of mouse was used as the host. In the evaluation of the compounds against the Ehrlich ascites tumor[2] (Table VI), the compounds were dissolved in physiological saline and injected intraperitoneally 24 hr after inoculation with 10^6 tumor cells. Ten tumor-bearing female Swiss mice were used in each experiment, with the 10 control animals receiving physiological saline intraperitoneally.

The antimicrobial evaluations were carried out using several strains of microorganisms[3]. The compounds were dissolved in methanol and diluted with sterile broth. *S. faecalis* (stock number 40) and *E. coli* (stock number 7) were added to the culture and incubated at 37°; growth was checked at 18 and 24 hr. The culture media containing the compounds and *C. albicans* (stock number 96) were incubated at 25°.

In the screen to assess the potential of II*d* and II*f* as diuretic agents, adult male albino rats of the Wistar strain, weighing between 225 and 275 g, were fasted overnight (18 hr) with water *ad libitum*. The rats were divided into groups of eight, one control group and one group for each dose level of 2 and 15 mg/kg. Each rat received an oral load of 2.4 ml of 0.9% NaCl/100 g of body weight, administered by means of a graduated syringe fitted with a stomach tube. The saline–drug solution or suspension (1.0 ml) was administered to each rat intraperitoneally and, in addition, the control group received 1 ml/kg of 0.9% NaCl intraperitoneally.

The rats were placed in metabolism cages and urine was collected and recorded for a 6-hr period. It was found that 25 control groups of eight rats excreted 52.58% of the volume of the saline load with a standard deviation of 11.04%. At 15 mg/kg, II*d*-, II*f*-, and hydrochlorothiazide-treated rats excreted 19.37[4], 20.50, and 137.38% of the volume of the saline load, respectively. At 2 mg/kg, II*d*-, II*f*-, and hydrochlorothiazide-treated rats excreted 37.65, 52.79, and 113.52% of the volume of the saline load, respectively.

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[2] Carried out by the Department of Cancer and Medical Research, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

[3] From the Department of Veterinary Physiology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

[4] Figure is the mean percentage excretion from five animals.

Research, University of Saskatchewan, Saskatoon, Saskatchewan, undertook the testing against the Ehrlich ascites carcinoma. The antimicrobial evaluation of certain of the compounds described was supervised by Dr. V. S. Gupta of the Department of Veterinary Physiology, University of Saskatchewan. Thanks are extended to Mrs. J. L. Brandvold who prepared several of the compounds and to Dr. D. G. K. Gorecki of the College of Pharmacy, University of Saskatchewan, who undertook the screening of IId and IIf as potential diuretic agents.

[x] To whom inquiries should be directed.

Indolizines II: Search for Potential Oral Hypoglycemic Agents

A. U. DE [x] and B. P. SAHA

Abstract □ A few 1,2-bis(*N*-alkylaminomethyl)indolizines, simple indolizinecarboxylic acids, and several 6-alkoxyindolizine-2-carboxylic acids were synthesized and screened as possible oral hypoglycemic agents. The absence of any significant hypoglycemic activity excludes these compounds from the predicted structural lead provided by some hypoglycemic *Vinca* alkaloids, such as vincamine, vindoline, and vindolinine, having the indolizine ring as one structural component. But an extension of the rationale that indolizines are also the structural components of some carcinolytic *Vinca* alkaloids, such as vincristine and vinblastine, used in cancer chemotherapy provided encouraging results. One indolizine derivative showed significant antineoplastic activity in Ehrlich ascites carcinoma.

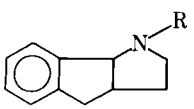
Keyphrases □ Indolizine derivatives—synthesized and screened as possible oral hypoglycemic agents □ Hypoglycemic agents, potential—synthesis and screening of indolizine derivatives □ Antineoplastic agents—screening of indolizine derivatives

The rationale for undertaking the synthesis of some *N*-alkyl 1,2,3,3a,4,8b-hexahydroindeno[1,2-*b*]pyrroles (I) and 2-(*N*-alkylaminomethyl)indolizines (II) as possible oral hypoglycemic agents was discussed previously (1, 2). Three additional compounds (I, R = *n*-pentyl, and II, R = *n*-pentyl or *n*-hexyl) were prepared according to the reported methods (1, 2) and biologically evaluated (Table I).

The failure of the compounds (II) to show any significant activity might be due to some undesirable biotransformation taking place through the very active 1- and 3-positions of II in any of the four intermediate steps of absorption, transport, barrier passage, and metabolism. Therefore, indolizine derivatives of type III, having only one active position free, were synthesized (Scheme I).

EXPERIMENTAL

Chemistry—Ethyl 2-pyridyl acetate (III*b*), prepared from 2-picolyllithium (III*a*) and ethyl carbonate according to the modification proposed by Goldberg *et al.* (3), was condensed with ethyl bromopyruvate to furnish indolizine 1,2-dicarboxylate (III*c*) (4). The diester was treated with various alkylamines to give 1,2-bis(*N*-alkylcarboxamido)indolizines (III*d*) and subsequently reduced with lithium aluminum hydride to 1,2-bis(*N*-alkylaminomethyl)-indolizines (III). The reaction between III*c* and an amine might also lead to an imide (III*e*) under the conditions followed, but the usual elemental analysis and IR and NMR spectra confirmed the diamide structure (III*d*).



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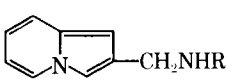

II

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Compound	Compound Type	R	Maximum Blood Sugar Lowering, %
1	I	H	5
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4	II	*n*-C_6H_{13}	10
5	III	CH_3	5
6	III	C_2H_5	Nil
7	III	*n*-C_3H_7	Nil
8	III	*n*-C_4H_9	Nil
9	III	*n*-C_5H_{11}	5
10	IV	H	5
11	IV	COOH	4
12	V	CH_3	3
13	V	C_2H_5	11
14	Tolbutamide		40

[a] Hypoglycemic tests were carried out by Central Drug Research Institute, Lucknow, India.

The synthesized compounds (III) were inactive. The introduction of two basic side chains with one active position free in III abolished the activity in comparison to II. It appeared that introducing an activating group and an acid function in the indolizine moiety while keeping positions 1 and 3 free might lead to better biological response. Moreover, various carboxylic acids and their derivatives, such as 5-methylpyrazole-3-carboxylic acid, 5-methylisoxazole-3-carboxylic acid (5), salicylic acid, and mesoxalic acid (6), have significant activity.

The simple indolizinecarboxylic acids (IV: R = H or COOH) were already known (4, 7) and prepared accordingly. 2-Methylpyridine-5-sulfonic acid (V*b*), obtained from 2-picoline (V*a*) by sulfonation with fuming sulfuric acid (8), was fused with potassium hydroxide to 5-hydroxy-2-methylpyridine (V*c*) and subsequently methylated with diazomethane to 5-methoxy-2-methylpyridine (V*d*: R = methyl) according to a modification of the method of Marion and Cockburn (9).

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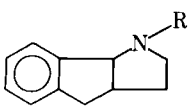

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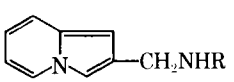

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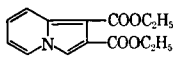
Condensation of 5-alkoxy-2-methylpyridines (V*d*) with ethyl bromopyruvate and their subsequent cyclization with sodium bi-

Table II—Antineoplastic Activity[a] of Diethylindolizine-1,2-dicarboxylate (IIIc) in Ehrlich Ascites Carcinoma



Cells Injected into Each Animal	Average Number of Cells per Milliliter in Control (C)	Average Number of Cells per Milliliter in Test (T)	T/C of Cells	Percent Inhibition of Ascitic Cells, (1 − T/C) × 100	Average Weight of Ascitic Fluid in Control (C), g	Average Weight of Ascitic Fluid in Test (T), g	T/C Ascitic Fluid	Percent Inhibition of Ascitic Fluid, (1 − T/C) × 100
18.5×10^6	450.0×10^6	80.5×10^6	0.1788	82.12	0.8	0.2	0.25	75.00
Mitomycin (as standard), 18.5×10^6	450.0×10^6	0	0	100.00	0.8	0	0	100.00

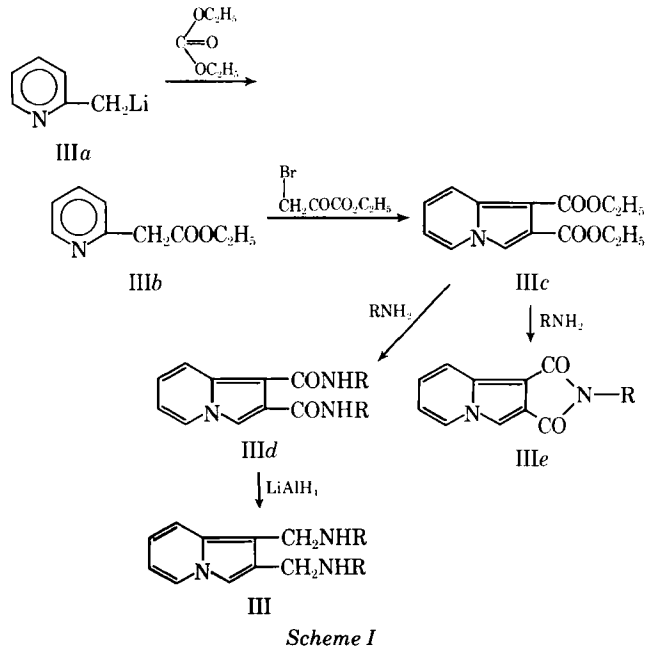
[a] Antineoplastic activity test was carried out by B.C. Panda (14).

carbonate (7) gave 6-alkoxyindolizine-2-carboxylic acids (V: R = methyl or ethyl), confirmed by the usual elemental analysis and IR and NMR spectra (Scheme II). Attempts to prepare 6-hydroxyindolizine-2-carboxylic acid (V: R = H) from Vc and ethyl bromopyruvate according to various methods (4, 7, 10) failed in the cyclization step, although the quaternary salt formation in the intermediate step took place. This failure in cyclization of the quaternary salt might be due to some deactivating influence of the hydroxy group in contrast to an alkoxy substituent at the same position.

Biological Activity—Hypoglycemic activity of the synthesized compounds are recorded in Table I. The absence of any significant activity in the indolizine derivatives (II–V) excludes at least this class of compounds (11) from the purview of Svoboda's (12) prediction on the structural lead provided by some hypoglycemic *Vinca* alkaloids. However, three types of structural variations on a prototype molecule were examined. A comparison of the relative activities of the compounds (II–V) indicates that the introduction of two basic side chains and the freeing of one active position as in III substantially decrease the activity. The other two variations, made by including an activating group and/or an acid function, retain less (type IV compounds) or about the same degree of activity (type V compounds) as the prototypes.

Hypoglycemic activity was determined orally at a dose level of 250 mg/kg body weight in each group of three overnight fasted albino rats, using tolbutamide as the standard. Blood sugar was estimated at 0, 2, and 4 hr according to the method of Nelson and Somogyi (13), and the maximum blood sugar lowering values were recorded.

The rationale for the synthesis of some indolizine derivatives,



Scheme I

being the constituents of some carcinolytic *Vinca* alkaloids such as vincristine and vinblastine, has been extended in the field of cancer chemotherapy also. With an exploratory objective, several indolizine derivatives were tested in Ehrlich ascites carcinoma, with ascitic live cell count and tumor weight being the activity parameters (14). Diethyl indolizine-1,2-dicarboxylate showed significant activity (Table II). Both the control and test groups contained five healthy Swiss albino mice of the same sex and approximately the same age and body weight (18–20 g).

The compounds were administered as suspensions in sterile buffered isotonic saline (pH 7.2) at a dose level of 50 mg/kg ip. Mitomycin, also in the same vehicle, was used as a standard at a dose level of 1 mg/kg. A fixed number of Ehrlich ascites carcinoma cells, collected from the donor mice, was transplanted into the intraperitoneal cavities of the control and test groups.

A day of incubation was allowed; then from the 2nd day seven consecutive doses were administered. Evaluation was made on the 9th day on the basis of the live cell counts and weight of the ascitic fluids collected from the intraperitoneal cavities of the control and test groups. Food and water were withheld 6 hr before the testing operation started.

Systematic investigations are being conducted with a wide variety of indolizines, and the results of investigations will be reported later.

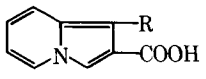
Syntheses[1]—N-n-*Pentyl-1,2,3,3a,4,8b-Hexahydroindeno[1,2-b]-pyrrole (I)*—Compound I was prepared according to the method described earlier (1) from 1,2,3,3a,4,8b-hexahydroindeno[1,2-*b*]pyrrole (2 g, 12.58 mmoles) and *n*-pentyl bromide (2.09 g, 13.84 mmoles). The product was distilled at 120°/0.6 mm to provide a colorless liquid. The yield was 0.7 g (24%), mp (picrate) 118–119°.

Anal.—Calc. for $C_{16}H_{23}N$: C, 83.85; H, 10.04; N, 6.11. Found: C, 83.90; H, 9.71; N, 5.93.

1,2-Bis(N-alkylcarboxamido)indolizines (IIId)—A mixture of diethyl indolizine-1,2-dicarboxylate (IIIc, 1 mole) and the appropriate alkylamine (2.2 moles) was heated in a sealed tube for 8 hr in a water bath. The diamide was purified by crystallization from dilute ethanol (Table III).

IIId (R = methyl): IR ν_{max}^{KBr} 3201 (NH) and 1652 (C=O) cm^{-1}. IIId (R = *n*- butyl): IR ν_{max}^{KBr} 3020 (NH) and 1650 (C=O) cm^{-1}. IIId (R = methyl): NMR ($CDCl_3$) τ 1.93–2.26 (3 H, 3-H, 5-H, and 8-H), 2.83–3.57 (2 H, 6-H and 7-H), 5.5–5.9 (2 H, 1-CON*H*CH_3 and 2-CON*H*CH_3), 7–7.16 (3 H, 2-CONHC*H*$_3$), and 8.53–8.77 (3 H, 1-CONHC*H*$_3$), a total of 13 protons. The ratio of aliphatic protons to aromatic protons was: calc., 1.6; found, 1.576.

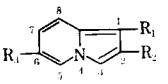
1,2-Bis(N-alkylaminomethyl)indolizines (III)—The appropriate diamide (IIId, 1 mole) was reduced with lithium aluminum hy-



IV

[1] All melting points are uncorrected. IR spectra were determined in a Perkin-Elmer spectrophotometer, and NMR spectra were determined in a Varian A60 spectrometer calibrated against tetramethylsilane at 60 MHz, 540-cps sweep width, and 300-cps sweep offset.

Table III—Characteristics of Substituted Indolizines



Compound	R_1	R_2	R_3	Melting Point	Boiling Point	Formula	Analysis, %	Calc.	Found
II[a]	H	—CONH-*n*-C_5H_{11}	H	158–160° dec.	120–125°/ 0.8 mm	$C_{14}H_{18}N_2O$	C	73.05	73.13
							H	7.83	7.90
							N	12.17	12.21
II[a]	H	—CONH-*n*-C_6H_{13}	H	—	120–125°/ 0.5–0.6 mm	$C_{15}H_{20}N_2O$	C	73.77	73.82
							H	8.20	8.12
							N	11.48	11.53
II[a]	H	—CH_2NH-*n*-C_5H_{11}	H	—	110–115°/ 0.9 mm	$C_{14}H_{20}N_2$	C	77.77	77.41
							H	9.26	9.62
							N	12.96	12.54
II[a]	H	—CH_2NH-*n*-C_6H_{13}	H	—	115–120°/ 0.5–0.7 mm	$C_{15}H_{22}N_2$	C	78.27	78.44
							H	9.57	9.41
							N	12.17	12.20
III*d*[b]	$R_1 = R_2$	—$CONHCH_3$	H	168–170°	—	$C_{12}H_{13}N_3O_2$	C	62.34	62.40
							H	5.63	5.61
							N	18.18	18.18
III*d*	$R_1 = R_2$	—$CONHC_2H_5$	H	184–186°	—	$C_{14}H_{17}N_3O_2$	C	64.86	65.00
							H	6.56	6.52
							N	16.21	16.11
III*d*	$R_1 = R_2$	—CONH-*n*-C_3H_7	H	110–112°	—	$C_{16}H_{21}N_3O_2$	C	66.89	66.81
							H	7.32	7.36
							N	14.64	14.90
III*d*	$R_1 = R_2$	—CONH-*n*-C_4H_9	H	108–110°	—	$C_{18}H_{25}N_3O_2$	C	68.58	68.50
							H	7.93	8.01
							N	13.33	13.42
III*d*	$R_1 = R_2$	—CONH-*n*-C_5H_{11}	H	113–115°	—	$C_{20}H_{29}N_3O_2$	C	69.97	70.11
							H	8.45	8.41
							N	12.25	12.30
III[c,d]	$R_1 = R_2$	—CH_2NHCH_3	H	103–104°	—	$C_{12}H_{17}N_3$	C	70.94	71.10
							H	8.37	8.29
							N	20.68	20.60
III[d]	$R_1 = R_2$	—$CH_2NHC_2H_5$	H	—	72–74°/ 0.8 mm	$C_{14}H_{21}N_3$	C	72.73	72.75
							H	9.09	9.10
							N	18.18	18.20
III[e]	$R_1 = R_2$	—CH_2NH-*n*-C_3H_7	H	—	105°/0.6 mm	$C_{16}H_{25}N_3$	C	74.13	74.32
							H	9.65	9.51
							N	16.21	16.15
III[e]	$R_1 = R_2$	—CH_2NH-*n*-C_4H_9	H	—	60–65°/1 mm	$C_{18}H_{29}N_3$	C	75.26	74.98
							H	10.10	10.31
							N	14.63	14.51
III[d]	$R_1 = R_2$	—CH_2NH-*n*-C_5H_{11}	H	—	65–67°/0.65 mm	$C_{20}H_{33}N_3$	C	76.19	76.10
							H	10.47	10.51
							N	13.33	13.41
V	H	COOH	OCH_3	170–172° dec.	—	$C_{10}H_9NO_3$	C	62.84	63.00
							H	4.71	4.52
							N	7.33	7.50
V	H	COOH	OC_2H_5	193–194° dec.	—	$C_{11}H_{11}NO_3$	C	64.39	64.50
							H	5.37	5.41
							N	6.82	6.72
V*e*	H	$COOCH_3$	OCH_3	96–97°	—	$C_{11}H_{11}NO_3$	C	64.39	64.51
							H	5.37	5.21
							N	6.82	6.84

[a] Methods described in Ref. 2. [b] Prepared from dry methylamine gas. [c] Crystallized from petroleum ether (bp 40–60°). [d] Reduction was done in tetrahydrofuran. [e] Reduction was done in ether.

dride (2 moles) in sodium-dried tetrahydrofuran or ether by refluxing for 16 hr. Excess lithium aluminum hydride was decomposed with water and filtered, and the filtrate was dried over anhydrous sodium sulfate. The solvent was removed *in vacuo*, and the residual crude amine was purified by crystallization from petroleum ether (bp 40–60°) or by distillation under reduced pressure to yield a colorless to pale-yellow product (Table III).

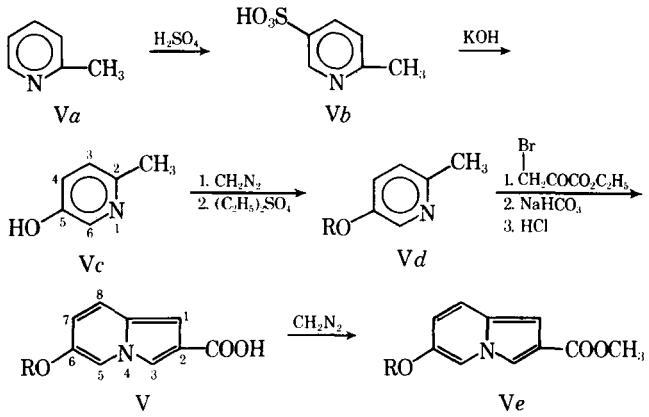
5-Hydroxy-2-methylpyridine (Vc)—2-Methylpyridine-5-sulfonic acid (V*b*, 28 g, 0.162 mole), potassium hydroxide (102 g, 1.82 moles), and water (5 ml) were thoroughly mixed in a wrought-iron pan. The mass was slowly heated to 200° when it melted and was maintained at this temperature for 10 min. The melt was cooled, dissolved in water (100 ml), partly neutralized with about 100 ml of concentrated hydrochloric acid, treated with charcoal, and filtered.

The filtrate was finally neutralized with carbon dioxide under cooling in an ice bath. A pale-yellow phenolic compound precipitated, and it was filtered, washed with two 5-ml portions of ice water, dried at 100°, and extracted with dry chloroform in a soxhlet apparatus. The solvent was removed to yield white and extremely light needles (12 g), mp 167–169°. A second crop of phenol (4 g) was obtained by evaporating the mother liquor in an enamel tray on a steam bath to dryness and then in a vacuum desiccator and finally extracting with chloroform as already described. The total yield was 16 g (90.9%).

5-Alkoxy-2-methylpyridines (Vd)—5-Methoxy-2-methylpyridine (V*d*:R = methyl) was prepared from 5-hydroxy-2-methylpyridine (Vc:0.10 mole) and diazomethane (0.26 mole) in the usual way (9), mp (picrate) 133–134°.

5-Ethoxy-2-methylpyridine (V*d*: R = ethyl) was prepared as follows. 5-Hydroxy-2-methylpyridine (Vc: 12 g) was dissolved in sodium hydroxide (10%, 44 ml), and diethyl sulfate (20.4 g) was added dropwise with stirring and heating on a water bath. The reaction mixture was kept distinctly alkaline to phenolphthalein by the simultaneous dropwise addition of sodium hydroxide (10%). After the addition was over, the reaction mixture was refluxed for 8 hr, cooled, and extracted with ether.

The ether layer was dried over anhydrous sodium sulfate. Ether was removed and the residual liquid was distilled at 50–55°/1 mm to yield 5-ethoxy-2-methylpyridine as a colorless liquid (4 g,



Scheme II

26.33%); the picrate crystallized from diluted ethanol, mp 142–144°. The alkaline mother liquor was neutralized with carbon dioxide, and 5-hydroxy-2-methylpyridine (1 g) was recovered.

Anal. —Calc. for $C_8H_{11}NO$: C, 70.08; H, 8.03; N, 10.22. Found: C, 70.1; H, 8.20; N, 9.91.

6-Alkoxyindolizine-2-carboxylic Acids (V) —A solution of 5-alkoxy-2-methylpyridine (V*d*: R = ethyl, 4 g, 19.5 mmoles) and ethyl bromopyruvate (5.7 g, 29.2 mmoles) in chilled absolute ethanol (22.8 ml) was heated under anhydrous conditions on a steam bath for 4 hr and kept at room temperature for 3 days. To reduce the reaction time, the method was modified by using cold, dry acetone (15 ml) in place of ethanol, and the mixture was heated on a steam bath for 1.5 hr only. The solvent was removed by distillation under reduced pressure, the cold residue was diluted with water (20 ml), and the solution was extracted with chloroform to remove the colored impurities.

Sodium bicarbonate was added until effervescence ceased, and the liberated 5-alkoxy-2-methylpyridine (1.8 g) was extracted with ether. Sodium bicarbonate (2.3 g) was added to the aqueous solution and the mixture was heated on a steam bath for 4 hr. While still warm the solution was acidified with 2 *N* hydrochloric acid to furnish a buff to chocolate-colored precipitate and was then recrystallized from ethanol (1 g). It gave intense blue fluorescence in ethanol and blue spots with Ehrlich's reagent (Table III).

6-Methoxyindolizine-2-carboxylic acid (V: R = methyl): IR ν_{max}^{KBr} 2950 (OH), 1670 (C=O), and 1252 (C—O) cm^{-1}. 6-Ethoxyindolizine-2-carboxylic acid (V: R = ethyl): IR ν_{max}^{KBr} 2980 (OH), 1671 (C=O), and 1255 (C—O) cm^{-1}.

Methyl-6-methoxyindolizine-2-carboxylate (Ve: R = Methyl)— To a suspension of 6-methoxyindolizine-2-carboxylic acid (V: R = methyl, 0.7 g) in dry ether (5 ml), a solution of diazomethane (0.3 g) in ether (15 ml) was added slowly to maintain a gentle evolution of nitrogen gas. The reaction was allowed to continue overnight and the resulting solution was filtered. The solvent was removed to furnish a crude product, which was crystallized from diluted ethanol as yellow crystals (0.1 g), mp 96–97° (Table III); IR: ν_{max}^{KBr} 1713 (C=O) and 1220 (C—O) cm^{-1}; NMR ($CDCl_3$): τ 6.23–6.33 (6 H, OCH_3 and $COOCH_3$) and 2.396–3.698 (5 H, 1-H, 3-H, 5-H, 7-H, and 8-H), a total of 11 protons. The ratio of aliphatic protons to aromatic protons was: calc., 1.2; found, 1.18.

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Stability, Absorption, Excretion, and Distribution of Nafiverine

SHUN-ICHI NAITO [x] **and TADASHI YAMAMOTO** *

Abstract □ The stability of a new antispasmodic, nafiverine, in an aqueous solution and in rabbit plasma was investigated. Metabolites of nafiverine in blood, bile, and urine were determined quantitatively. The effectiveness of an intramuscular administration of nafiverine was comparable to that of an intravenous administration. One metabolite of nafiverine, *N*-(2-hydroxyethyl)-*N'*-[α-(naphthyl)propionyloxy-2-ethyl]piperazine, is a new compound and its synthesis is described.

Keyphrases □ Nafiverine—stability, absorption, excretion, and distribution, intramuscular, intravenous, and oral administrations, rats, rabbits, and mice □ Stability—nafiverine, aqueous solution and rabbit plasma □ Absorption—nafiverine, intramuscular, intravenous, and oral administrations, rats, rabbits, and mice □ Distribution—nafiverine, intramuscular, intravenous, and oral administrations, rats, rabbits, and mice □ Metabolism—nafiverine, rats, rabbits, and mice

Nafiverine[1], *N,N'*-di[α-(1-naphthyl)propionyloxy-2-ethyl]piperazine (I), is a new synthetic antispasmodic which shows low toxicity and a high myolytic activity on the smooth muscles of the ileum, uterus, and coronary vessels (1, 2). The present work investigated its stability in aqueous solution, absorption, excretion, and distribution.

EXPERIMENTAL[2]

α-(1-Naphthyl)propionic Acid (III)—A mixture of 80 g of sodium hydroxide and 800 ml of 50% ethanol was added to 61.2 g of dihydrochloride of I, and this mixture was stirred for 30 min at room temperature. After the addition of 250 g of ice to the reaction mixture, 190 ml of concentrated hydrochloric acid was added to make the mixture acidic. The mixture was diluted to 2 liters with water and was stored in a freezer. Crystals separated and were collected by filtration and recrystallized from ethanol as colorless scales (40 g), mp 147.5–148.5°. The melting point of III agreed with that reported by Blicke and Feldkamp (3).

Anal.—Calc. for $C_{11}H_{11}O_2$: C, 77.98; H, 6.04. Found: C, 77.98; H, 6.34.

***N,N'*-Di(2-hydroxyethyl)piperazine (IV)**—After filtration of III, the filtrate was made alkaline (pH about 10.0) by the addition of 5% sodium hydroxide solution and evaporated to dryness. The residue was extracted with 300 ml of chloroform, and the chloroform extract was evaporated to obtain about 9.3 g of crude IV. This residue was recrystallized from ethanol to colorless prisms, mp 134.0–135.5°; the melting point agreed with that reported by Pyman (4).

Anal.—Calc. for $C_8H_{18}N_2O_2$: C, 55.14; H, 10.41; N, 16.08. Found: C, 55.42; H, 10.61; N, 16.32.

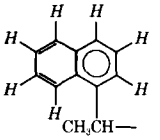
***N*-(2-Hydroxyethyl)-*N'*-[α-(1-naphthyl)propionyloxy-2-ethyl]piperazine (V)**—A solution of 17.6 g (0.08 mole) of α-(1-naphthyl)propionyl chloride (obtained by the treatment of III with thionyl chloride) in 100 ml of chloroform was gradually added dropwise during 2.5 hr to a refluxing mixture of 41.8 g (0.24 mole) of IV and 450 ml of chloroform. After evaporation of chloroform, the residue was mixed with dilute sodium bicarbonate solution to pH 9–10. This mixture was extracted with ether, and the ethereal solution was dried over anhydrous sodium sulfate after washing with a small amount of water. A yellowish oily substance (34.4 g) was obtained by evaporation of ether.

The oily residue was reacted with methanesulfonic acid in cold methanol, and the methanol was removed *in vacuo*. The residue thus obtained was mixed with 140 ml of water and stored in a refrigerator. A white precipitate (dimethanesulfonate of I) separated and was filtered. The filtrate was washed with ether and then extracted with ether after the medium was made alkaline with 8% sodium bicarbonate solution. From this ethereal solution, 12.4 g of crude V was obtained after evaporation of ether. The crude V was purified by TLC and identified as its methanesulfonate.

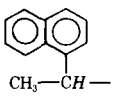
The TLC conditions were: adsorbent, Diatomite (Kieselgel G) in thickness of 1 mm; solvent, benzene–ethyl acetate–diethylamine (7:2:1); and R_f values (20°), 0 (III), 0.25–0.46 (V), and 0.53–0.57 (I).

Pure V (1.1 g) was obtained from 2.5 g of crude V by extraction of the corresponding spots with methanol.

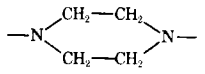
Dimethanesulfonate (VI) of V—Fifty milliliters of methanol containing 6.73 g of methanesulfonic acid (0.07 mole) was added gradually under ice cooling and stirring to a mixture of 12.4 g of V (0.035 mole) and 100 ml of methanol. Ethyl acetate was added to the reaction mixture until formation of turbidity and then methanol was added until the turbidity vanished. When the mixture was stored overnight in a refrigerator, colorless crystals of VI separated (14 g), mp 114–115°; NMR ($CDCl_3$)[3] of V: τ 1.78–2.63 (7H, m)



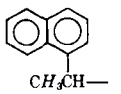
5.50 (1H, q, J = 7.0 Hz)



5.81 (2H, t, J = 5.5 Hz, —COOCH_2CH$_2$N<), 6.43 (2H, t, J = 5.3 Hz, >N—CH$_2$CH_2OH), 6.47 (1H, s, >N—CH$_2$CH$_2$OH), 7.54 (2H, t, J = 5.5 Hz, —COOCH$_2$CH_2N<), 7.57 (2H, t, J = 5.3 Hz, >NCH_2CH$_2$OH), 7.72 (8H, s)



and 8.33 (3H, d, J = 7.0 Hz)



IR (liquid film): ν_{COO} 1735 and ν_{OH} 3400 cm^{-1}.

Anal.—Calc. for $C_{23}H_{36}N_2O_9S_2$: C, 50.35; H, 6.61; N, 5.11. Found: C, 50.13; H, 6.63; N, 5.21.

Determination of I, II, V, and VI (Methyl Orange Method)—A mixture of 3 ml of water, 1.5 g of sodium chloride, 8 ml of chloroform, and 1 ml of plasma or aqueous solution containing I, II, V, or VI was shaken vigorously for 1 hr. Four milliliters of the chloroform layer after centrifugation was subjected to the methyl orange method (5). The chloroform layer was mixed with 3 ml of an equivolume mixture of 0.35% methyl orange solution and 1.75% boric acid solution, and the mixture was shaken for 10 min. Three milliliters of the chloroform layer after centrifugation was mixed with 0.5 ml of ethanol containing 2% sulfuric acid, and the absorbance of this mixture was determined at 520 nm. A mixture of 0.9 ml of plasma and 0.1 ml of a compound solution of known concentration was treated as described to prepare a calibration curve for plasma concentration.

Determination of IV (Folin Method)—The modified Folin method (6) was used to quantitate IV.

Determination of III (Formalin Method)—A mixture of 1 ml of plasma, 3 ml of 0.033 *N* HCl, 1.5 g of sodium chloride, and 8 ml

[1] Instituto De Angeli Co., Ltd., Italy.

[2] Nafiverine dimethanesulfonate (II) was obtained from the Instituto De Angeli Co., Ltd., Italy.

[3] The internal standard was tetramethylsilane.

Table I—Tabulation of Slope (k_1)[a], Heat of Activation, and Other Data Derived from Arrhenius Plots of Pseudo-First-Order Rates for Thermal Degradation of II and VI in Aqueous Solutions

	II	VI
Rate constant (k_1) at 40°	27.8×10^{-3}	17.1×10^{-3}
Rate constant (k_1) at 50°	99.4×10^{-3}	31.9×10^{-3}
Rate constant (k_1) at 60°	330.5×10^{-3}	57.6×10^{-3}
Heat of activation, ΔHa in kcal/mole	25.7	12.6
P[b]	16.4	7.1
Predicted rate constant k_1 at 25°	3.5×10^{-3}	6.2×10^{-3}

[a] The rate constant k_1 is in reciprocal days. [b] Log (frequency factor).

of ethyl acetate was shaken for 1 hr. After centrifugation, the evaporated residue from 4 ml of the ethyl acetate layer was reacted with 1 ml of 3% paraformaldehyde in concentrated sulfuric acid for 1 hr at room temperature. Absorbance of this mixture after the addition of 3 ml of concentrated sulfuric acid was measured at 640 nm. A mixture of 0.9 ml of plasma and 0.1 ml of III solution of known concentration was treated as described to prepare a calibration curve.

Stability Test of II and VI in Aqueous Solution—A 1% solution of II or a 0.3% solution of VI stored at 40, 50, or 60° was used for the stability tests.

To assay II or VI, one spot, having the same R_f value as the authentic sample of pure II or VI after development of 10 μl of the test solution by TLC, was shaken with a mixture of 3 ml of water, 1.5 g of sodium chloride, and 8 ml of chloroform for 1 hr. Four milliliters of the chloroform layer after centrifugation was treated by the methyl orange method to quantitate II or VI. The TLC solvent was ethyl acetate–benzene–ethylamine (7:5:1), the color developer was Folin reagent, and the adsorbent was Diatomite (Kieselgel G), 0.25 mm in thickness. The R_f values of I, III, IV, and V at 20° were 0.90, 0.03, 0.03, and 0.35, respectively.

Stability Test of II in Rabbit Plasma *In Vitro*—A mixture of 0.9 ml of rabbit plasma and 0.1 ml of 0.1% II solution was stored for 2 hr at 0 and 20°. One spot, having the same R_f value as the authentic sample of pure II after development of 0.1 ml of the mixture by TLC, was shaken with a mixture of 3 ml of water, 1.5 g of sodium chloride, and 8 ml of chloroform for 1 hr. After centrifugation, 4 ml of the chloroform layer was treated by the methyl orange method or Folin method to quantitate II, IV, and VI. The TLC solvent was ethyl acetate–benzene–diethylamine (2:7:1), the color developer was Folin reagent, and the adsorbent was Diatomite (Kieselgel G), 0.5 mm in thickness. The R_f values of the authentic samples at 20° were 0.01 (IV), 0.35 (VI), and 0.85 (II).

Distribution of II and Its Metabolites in Animal Tissues—

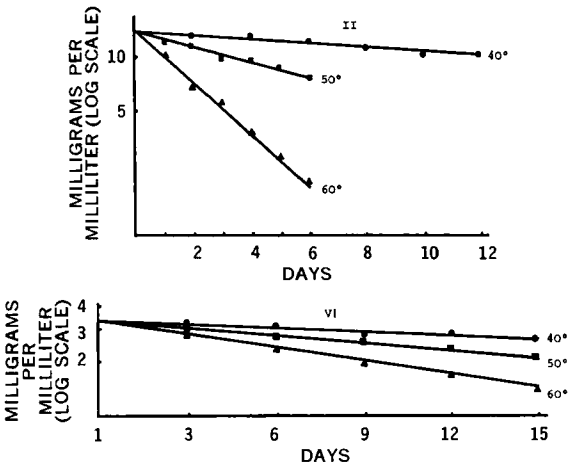


Figure 1—*Content of II or VI in aqueous solution. Initial concentration was: II, 14 mg/ml; and VI, 3.3 mg/ml. All data are the mean values of three experiments under the same conditions.*

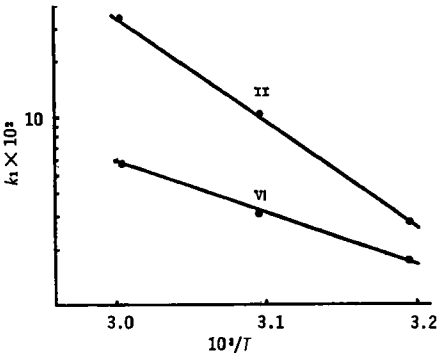


Figure 2—*Arrhenius plots of II and VI in aqueous solution. T = absolute temperature; and* k_1 *= decomposition rate constant in reciprocal days.*

Each group consisted of three male and three female rabbits (average weight 2.3 kg) or of five male and five female rats (Wistar strain, average weight 140 g). To rabbits and rats, 87 and 100 mg/kg of II were administered intramuscularly, respectively.

Eight organs (brain, lungs, heart, liver, kidneys, spleen, uterus, and testicle) were used for the determination of II–V. The sample sizes were: 4 g, brain and kidneys; 3 g, lungs and testicle; 2 g, heart;

Table II—Distribution of Metabolites I, III, IV, and V[a] of Nafiverine after Intramuscular Administration of Nafiverine Dimethanesulfonate to Rabbits

Organ	Hour	I		III		IV		V	
		mmoles/g	μg/g	mmoles/g	μg/g	mmoles/g	μg/g	mmoles/g	μg/g
Brain	2	0.12 ± 0.02	86 ± 14	0.19 ± 0.05	38 ± 9	1.14 ± 0.05	199 ± 9	0.05 ± 0.01	29 ± 8
	4	0.17 ± 0.01	120 ± 7	0.06 ± 0.01	11 ± 2	1.30 ± 0.16	226 ± 28	0.08 ± 0.01	46 ± 4
Lungs	2	0.10 ± 0.01	75 ± 6	0.54 ± 0.13	108 ± 26	1.41 ± 0.18	246 ± 31	0.06 ± 0.01	32 ± 7
	4	0.07 ± 0.02	51 ± 14	0.06 ± 0.01	11 ± 2	0.32 ± 0.20	56 ± 35	0.07 ± 0.00	39 ± 1
Heart	2	0.16 ± 0.00	117 ± 3	0.16 ± 0.01	31 ± 2	0.33 ± 0.11	57 ± 19	0.08 ± 0.01	42 ± 3
	4	0.17 ± 0.02	126 ± 19	0.05 ± 0.01	9 ± 2	0.22 ± 0.09	38 ± 16	0.08 ± 0.01	42 ± 6
Liver	2	—	—	0.10 ± 0.02	19 ± 4	0.58 ± 0.34	101 ± 58	—	—
	4	—	—	0.32 ± 0.26	62 ± 51	0.70 ± 0.14	121 ± 24	—	—
Spleen	2	0.23 ± 0.00	163 ± 2	0.65 ± 0.31	129 ± 62	1.29 ± 0.05	225 ± 9	0.10 ± 0.02	56 ± 12
	4	0.18 ± 0.02	128 ± 15	0.38 ± 0.10	76 ± 19	1.19 ± 0.22	206 ± 37	0.11 ± 0.05	61 ± 26
Kidneys	2	0.09 ± 0.02	65 ± 12	0.69 ± 0.08	137 ± 20	1.23 ± 0.25	214 ± 44	0.07 ± 0.01	36 ± 5
	4	0.10 ± 0.01	72 ± 9	0.71 ± 0.04	142 ± 9	0.93 ± 0.32	163 ± 56	0.06 ± 0.00	32 ± 3
Uterus	2	0.24 ± 0.05	174 ± 37	0.69 ± 0.14	138 ± 29	1.38 ± 0.14	240 ± 25	0.22 ± 0.06	118 ± 32
	4	0.14 ± 0.05	102 ± 41	0.43 ± 0.12	84 ± 23	1.12 ± 0.52	195 ± 91	0.08 ± 0.01	46 ± 6
Testicle	2	0.13 ± 0.04	94 ± 29	0.08 ± 0.06	15 ± 11	0.16 ± 0.05	28 ± 8	0.05 ± 0.01	28 ± 8
	4	—	—	0.01 ± 0.00	2 ± 0	0.04 ± 0.01	7 ± 2	—	—

[a] See text.

Table III—Distribution of Metabolites I, III, IV and V[a] of Nafiverine after Intramuscular Administration of Nafiverine Dimethanesulfonate to Rats

Organ	Hour	I		III		IV		V	
		mmoles/g	μg/g	mmoles/g	μg/g	mmoles/g	μg/g	mmoles/g	μg/g
Brain	1	—	...	0.12	23	0.03	5	—	...
	2	—	...	0.11	22	—	...	—	...
	6	—	...	0.03	6	—	...	—	...
Lungs	1	0.12	91	0.03	5	0.03	6	0.08	44
	2	—	...	0.13	25	0.06	11	—	...
	6	—	...	0.11	22	—	...	—	...
Heart	1	0.13	93	0.13	25	0.05	9	0.03	17
	2	0.09	65	1.32	263	0.21	37	0.03	16
	6	—	...	0.03	6	0.10	18	—	...
Liver	1	—	...	—	...	0.02	3	—	...
	2	—	...	—	...	0.08	14	—	...
	6	—	...	—	...	—	...	—	...
Spleen	1	0.08	58	0.06	11	0.04	7	0.05	25
	2	0.07	48	0.15	29	0.38	66	0.06	34
	6	—	...	—	...	—	...	—	...
Kidneys	1	0.11	79	0.11	22	0.25	44	0.08	42
	2	0.09	64	—	...	0.22	39	0.05	24
	6	—	...	—	...	0.10	17	—	...
Uterus	1	0.19	139	0.02	3	0.06	11	0.07	39
	2	—	...	—	...	0.32	55	—	...
	6	—	...	—	...	—	...	—	...
Testicle	1	—	...	0.06	11	0.02	3	—	...
	2	—	...	—	...	—	...	—	...
	6	—	...	—	...	—	...	—	...

[a] See text.

15 g, liver; and 1 g, spleen and uterus. These samples were homogenized with water, using 7 ml for brain, spleen, and kidney; 6 ml for lung, uterus, and testicle; and 8 ml for heart and liver.

The homogenate was centrifuged and 1 ml of the supernate was treated by the same method as used for the determination of III and IV.

For the determination of II and V, 4 ml of the supernate from the centrifuged homogenate was mixed with 1.5 g of sodium chloride and 8 ml of chloroform; this mixture was shaken vigorously for 1 hr. After centrifugation, 4 ml of the chloroform layer was evaporated, the residue was dissolved in 100 μl of ethanol, and 50 μl of the ethanol solution was submitted to TLC. The TLC and following procedures used were the same as described in *Stability Test of II in Rabbit Plasma* In Vitro. Organs of rabbits and rats not receiving any drug were treated in the same manner to serve as controls. Mixtures of control homogenate spiked with II, III, IV, or V solution of known concentration were treated as already described to prepare a calibration curve.

Collection of Rabbit Bile—Three female rabbits, 2.0–2.5 kg each, were anesthetized with 1 g/kg of urethan subcutaneously following a fasting period of approximately 24 hr. A polyethylene tube was inserted into the bile duct, and the bile was collected for 7.5 hr after administration of II under continuous anesthesia with urethan. At the end of the 1st (control) hr, the compound was given intravenously, intramuscularly, or orally.

Determination of Metabolites of II in Rabbit Bile—To 1 ml of rabbit bile in an ice-cold centrifuge tube, 2 ml of ethanol was added and the mixture was stirred thoroughly. After standing for 30 min under cooling with ice water, 0.5 ml of the supernate from centrifugation was mixed with 0.5 ml of water. This mixture was used for the determination of III or IV by using the formalin method and Folin method.

Detection of Urinary Metabolites of II in Rabbits—Urine was collected for 24 hr from four female rabbits (average weight 3.2 kg) receiving 1000 mg of II orally. After freeze evaporation of urine, 500 mg of the residue was mixed with 2 ml of water, and 0.1 ml of the supernate after centrifugation of this mixture was submitted to TLC.

The TLC conditions were: adsorbent, Diatomite (Kieselgel G), 0.5 mm in thickness; distance developed, 15 cm; Solvent A, acetone–ethyl acetate–chloroform (3:2:1); Solvent B, benzene–ethyl acetate–diethylamine (7:2:1); Solvent C, chloroform–methanol–28% ammonia (10:4:1); Solvent D, benzene–acetone–methanol (7:2:1); and color developer, Folin reagent or saturated solution of potassium dichromate in concentrated sulfuric acid. The R_f values were: Solvent A, 0.93 (I), 0.71 (III), 0.01 (IV), and 0.07 (V); Solvent B, 0.85 (I), 0.01 (III), 0.07 (IV), and 0.46 (V); Solvent C, 0.98 (I),

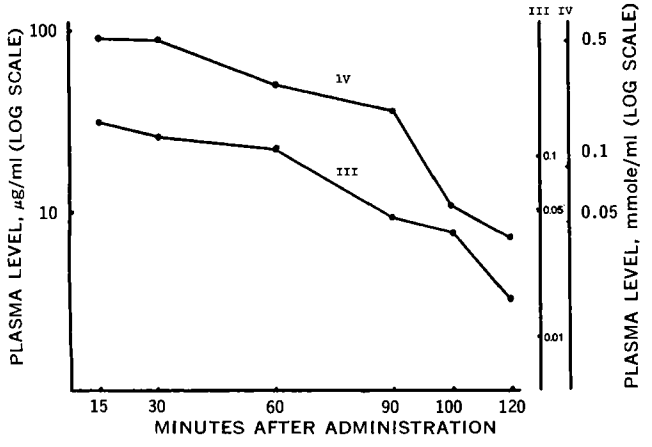


Figure 3—*Plasma level of metabolites of nafiverine dimethanesulfonate following intravenous administration of 50 mg to five female rabbits weighing about 2.4 kg each.*

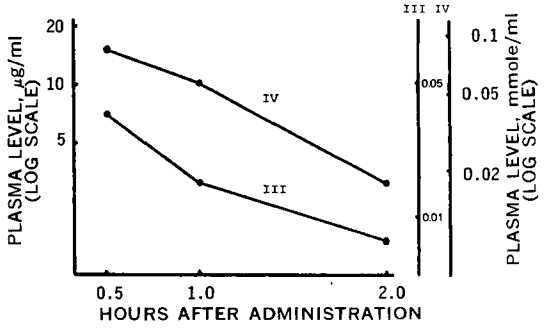


Figure 4—*Plasma level of metabolites of nafiverine dimethanesulfonate following intramuscular administration of 50 mg to five female rabbits weighing about 2.8 kg each.*

Table IV—Amount[a] of Metabolites III and IV[b] of Nafiverine Excreted in Female Rabbit Bile for 7.5 hr after Administration of Nafiverine Dimethanesulfonate

Route	Mean Body Weight, kg	Dose, mg	Metabolite	μg	% [c]	Bile Volume, ml/kg
Intravenous	2.3	50	III:	406 ± 131[d]	1.5 ± 0.5	16.1 ± 2.7[d]
			IV:	1790 ± 686	15.0 ± 5.8	
Intramuscular	2.3	50	III:	92 ± 50	0.3 ± 0.2	14.2 ± 6.5
			IV:	1211 ± 484	10.2 ± 4.1	
Oral	2.1	100	III:	—	—	14.9 ± 2.5
			IV:	—	—	
Control	2.3					19.8 ± 2.9

[a] Each group consisted of three rabbits. [b] See text. [c] Percent of III or IV was calculated from a ratio of 2 moles of excreted III or 1 mole of excreted IV to 1 mole of nafiverine dimethanesulfonate. [d] Mean value ± *SE*.

0.85 (III), 0.83 (IV), and 0.98 (V); and Solvent D, 0.96 (I), 0.52 (III), 0.02 (IV), and 0.39 (V).

Assay of Metabolites of II in Rabbit Urine—A mixture of 1 ml of water and 500 mg of the lyophilized residue of urine of rabbits receiving II in different doses and by different routes of administration was centrifuged, and 10 μl of the supernate was submitted to TLC. The two spots corresponding to III and IV were each scraped off from the TLC plate developed with a mixed solvent of chloroform–methanol–28% ammonia (10:4:1) and extracted with 2 ml of ethanol by standing for 30 min at room temperature.

After centrifugation, 1 ml of the supernate of the ethanolic extract was used for determination of III and IV. To assay III, the evaporated residue of the supernate was treated by the formalin method. In the determination of IV, the supernate was treated by the Folin method (6). The calibration curves of the two compounds were prepared separately by TLC as described by spotting the solution of the pure compound of a known concentration.

RESULTS AND DISCUSSION

The assay procedures used in the present work (*i.e.*, methyl orange method for I, II, V, and VI; Folin method for IV; and formalin method for III) are all very sensitive, and it was ascertained that 1 μg of any of the six compounds in the experimental conditions used is assayable by the respective assay method. Furthermore, III and IV do not show color reaction by the Folin method and the formalin method, respectively.

Since there are two ester bonds in the chemical structure of II, the stability of II in aqueous solution was examined to find the reason for its stability in animal blood. In the stability tests of II and VI in aqueous solutions, II and VI are not detected and assayed as themselves (dimethanesulfonate) but as I and V (free forms), respectively, by the TLC determination. From TLC of the test solutions with which the stability tests were performed, I, III, IV, and V were detected as the decomposition products of II; and III, IV, and V were detected as the decomposition products of VI.

From the assay results (Fig. 1) of unchanged II or VI, the kinetics of degradation of II and VI at 40, 50, and 60 ± 1° were interpreted on the basis of the pseudo-first-order reactions. Several constants (7) concerning the stabilities of II and VI (Table I) were derived from the results shown in Fig. 1; the Arrhenius plot is shown in Fig. 2.

As shown by TLC, I and V did not appear in animal blood following administration of II. Plasma levels of Metabolites III and IV after administration of II are shown in Figs. 3–7. In Fig. 3, blood levels of the metabolites were determined until 2 hr after administration of II. Figure 4 shows that both III and IV disappeared 3 hr after their administration; in Fig. 5, IV vanished 7 hr after administration, although zero values could not be plotted on logarithmic paper. In rats (Fig. 6), both III and IV disappeared 4 hr after administration. In mice (Fig. 7), III and IV vanished 8 and 4 hr, respectively, after administration.

To see some of the reasons for the absence of I and V in animal blood, the stability of II in rabbit plasma was investigated *in vitro*. The methyl orange method can be used to quantitate both I and V; therefore, the remaining percent of I is the amount of a mixture of I and V in plasma. The remaining percentages of I at 0.5, 1, and 2 hr after storage of a mixture of rabbit plasma and II at 20 ± 1° were 45.2, 12.4, and 1.1%, respectively. On the other hand, the remaining percentage of I at 2 hr after storage of the same mixture at 0° was 97.7%. In addition, V was detected by TLC in a mixture of plasma and II stored at 20° for 0.5 and 1 hr, but V could not be

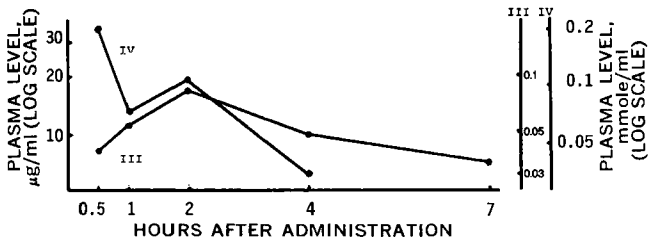


Figure 5—*Plasma level of metabolites of nafiverine dimethanesulfonate following oral administration of 100 mg to three female rabbits weighing about 2.3 kg each.*

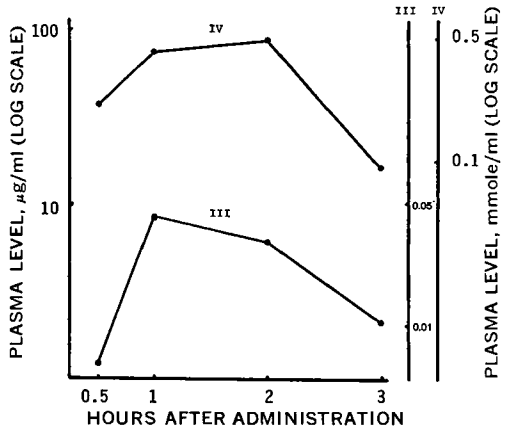


Figure 6—*Plasma level of metabolites of nafiverine dimethanesulfonate following intramuscular administration of 100 mg to five female rats (Wistar strain, average weight 135 g).*

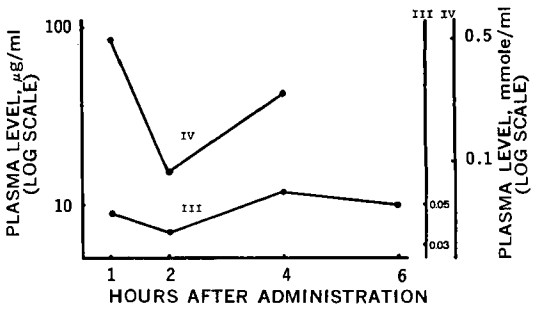


Figure 7—*Plasma level of metabolites of nafiverine dimethanesulfonate following intramuscular administration of 100 mg to 10 male mice (dd strain, average weight 20 g).*

Table V—Excretion of Metabolites III and IV[a] of Nafiverine after Administration of Nafiverine Dimethanesulfonate to Rabbits

Rabbits Used	Mean Body Weight, kg	Sex	Route	Dose, mg	Metabolite	Excreted Amount, mg 0–24 hr	24–48 hr	48–72 hr	72–96 hr	Total	Excretion[b], %
4	3.2	Male	Oral	1000	III	75.2	18.9	1.8	—	95.9	17.6
					IV	16.5	7.2	3.2	2.7	29.6	12.4
3	2.2	Female	Oral	100	III	5.8	7.7	0.4	0.2	14.1	25.8
					IV	2.3	2.7	0.4	—	5.4	22.7
4	3.0	Male	Intramuscular	900[c]	III	14.0	58.6	34.5	3.9	111.0	22.6
					IV	10.1	19.7	13.1	5.1	48.0	22.4
3	3.3	Female	Intramuscular	50	III	5.1	1.1	0.3	—	6.5	23.8
					IV	4.1	1.5	1.1	1.3	8.0	67.2
3	2.8	Female	Intravenous	50	III	5.6	1.2	0.5	—	7.3	26.7
					IV	5.2	1.6	1.3	—	8.1	68.0

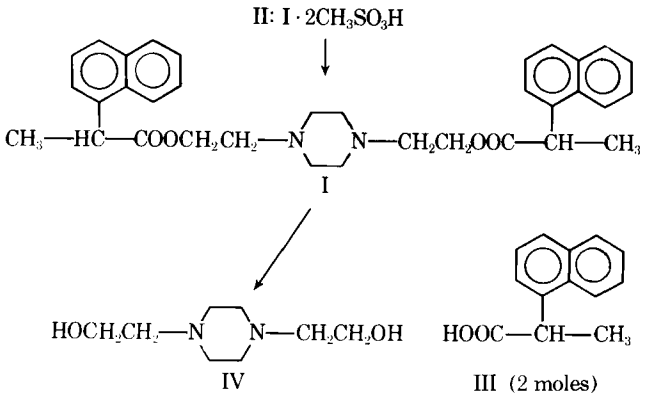
[a] See text. [b] Percent of III or IV was calculated from a ratio of 2 moles of excreted III or 1 mole of excreted IV to 1 mole of nafiverine dimethanesulfonate. [c] Nafiverine dimethanesulfonate (300 mg) was given three times every 2 hr to a rabbit.

found in the mixture stored at 0 or 20° for 2 hr. These facts revealed that I is unstable and rapidly hydrolyzed in rabbit plasma at 20°. As shown in Tables II and III, Metabolites I, III, IV, and V are distributed in animal organs but none of these metabolites is found 24 hr after intramuscular administration of II to rabbits and rats. It is interesting that a relatively large amount of metabolites of II was distributed in the uterus (rabbits and rats) but not in testicles.

The amount of metabolites of I excreted in rabbit bile during 7.5 hr after administration by different routes is shown in Table IV. No metabolites of I were found using the methyl orange method, and this fact supports the absence of I and V. Metabolites III and IV were found as the metabolites of I in rabbit bile. About 17% of the administered II was excreted in rabbit bile after its intravenous administration, while about 11% of the administered II was excreted in bile after its intramuscular administration. On the other hand, no metabolites were found after oral administration. It is desirable to examine the reasons for the considerable differences in the amount of metabolites excreted by different routes of administration. Compound II showed no effect on the volume of rabbit bile excreted during 7.5 hr.

Urine collected from rabbits receiving II was lyophilized and the residue was examined by TLC for the presence of metabolites. Urine was collected for 4 days after administration of II, since urine collected from 96 to 120 hr after the administration contained no chemical or its metabolites, as evidenced by TLC. Two metabolites, III and IV, were detected in rabbit urine by TLC. Two spots representing III and IV were separated and identified by using Solvent C for TLC. A micromelting-point determination of the mixture of evaporated residue of ethanolic extract (obtained from 5–10 spots having the same R_f value) and an authentic sample was used for identification.

To detect some metabolites present in conjugated form in rabbit urine, the lyophilized residue of urine was hydrolyzed with 5 *N* hydrochloric acid under the same conditions as reported for aminoethanesulfonylpiperidine (8). After neutralization with 5 *N* sodium carbonate solution, the mixture was submitted to TLC, using Solvents A, B, C, and D. No metabolites except III and IV were found, and no increase was found in the amounts of III and IV excreted. Therefore, III and IV in rabbit urine were directly determined by using the formalin method and the Folin method, respectively, as described under *Experimental*.



Scheme I

As shown in Table V, the fact that about 68% of IV was excreted after intravenous administration compared to about 67% after intramuscular administration shows that the availability of II after intramuscular administration is not different from that after intravenous administration. The ratio of the amount of IV to that of III in rabbit urine was near 1 after oral administration of II and intramuscular administration of 900 mg of II, while the ratio was above 2.5 after intramuscular administration of 50 mg of II and intravenous administration of II. The reason for these changes in the ratio according to the dose or the route of administration should be investigated.

In the present work, the main fate of II in animals should be as shown in Scheme I.

SUMMARY

1. Stabilities of nafiverine dimethanesulfonate and *N*-(2-hydroxyethyl) - *N'*-[α-(1-naphthyl)propionyloxy-2-ethyl]piperazine dimethanesulfonate were measured. The latter is a new compound and its synthesis was described.

2. Nafiverine could not be detected in animal blood but was present in animal tissues after the administration of its dimethanesulfonate.

3. In animal blood, bile, and urine, two metabolites, α-(1-naphthyl)propionic acid and *N,N'*-di(2-hydroxyethyl)piperazine, were detected and assayed.

4. Absorption of nafiverine dimethanesulfonate was almost the same by intramuscular administration as by intravenous administration on the basis of its excretion in rabbit urine.

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Spasmolytic Constituents of *Cedrus deodara* (Roxb.) Loud: Pharmacological Evaluation of Himachalol

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Abstract □ Himachalol has been identified as the major antispasmodic constituent in the wood of *Cedrus deodara*. The pharmacological studies of himachalol on various isolated smooth muscles (guinea pig ileum, rabbit jejunum, rat uterus, and guinea pig seminal vesicle) and against different agonists (acetylcholine, histamine, serotonin, nicotine, and barium chloride) indicated spasmolytic activity similar to that of papaverine. It was a more potent antagonist of barium chloride-induced spasm of guinea pig ileum than papaverine but less effective in reverting a similar spasm of rabbit jejunum and had no relaxing effect alone. In the conscious immobilized cat, intragastric administration of himachalol or papaverine (100 mg/kg) produced equal inhibition of carbachol-induced spasm of the intestine, lasting about 2 hr, but himachalol had a faster onset of action. Himachalol was devoid of spasmolytic effect on the bronchial musculature of guinea pig but was 3.3 times more potent than papaverine in antagonizing epinephrine-induced contraction of the guinea pig seminal vesicle. Intravenous injection of himachalol (3–10 mg/kg) in the cat produced a dose-dependent fall in blood pressure and an increased femoral blood flow.

Keyphrases □ *Cedrus deodara*—spasmolytic constituents, pharmacological evaluation of himachalol □ Himachalol—spasmolytic constituent of *C. deodara*, pharmacological evaluation □ Spasmogens—pharmacological evaluation of himachalol □ Medicinal plants—pharmacological evaluation of himachalol from *C. deodara*

During the biological screening of Indian plants for the presence of active substances, it was observed that a 50% ethanol extract from the wood of *Cedrus deodara* (Roxb.) Loud (N.O. Pinaceae)[1] possessed significant antispasmodic activity (1). Detailed studies were undertaken, and the present article describes the identification of the major spasmolytic constituent as the known sesquiterpene himachalol (I) (2, 3) and its pharmacological evaluation.

EXPERIMENTAL

Extraction and Identification of Himachalol—The alcoholic extract of the plant wood (10 kg) was separated into petroleum ether-soluble, chloroform-soluble, water-soluble, and water-insoluble fractions. The petroleum ether-soluble fraction (510 g) showed an enhancement of antispasmodic activity, and it was subjected to chromatography over alumina (10 kg) in hexane solution.

The progressive elution of the column by solvents with increasing polarity and the biological evaluation of the resultant eluates led to the isolation of a fraction (65.3 g), which was eluted with hexane–benzene (1:1). This fraction was dissolved in acetonitrile (80 ml) and allowed to stand in a deep freeze when a crystalline deposit was obtained. It was filtered and recrystallized as colorless rhombuses (23 g), mp 67°. It was found to be homogeneous by TLC and GLC and exhibited antispasmodic activity.

This crystalline substance, $C_{15}H_{26}O$ (M^+ 222), was an unsaturated sesquiterpene alcohol; IR (KBr): 3320, 1130, 1025, 1650, and 862 cm^{-1}; NMR ($CDCl_3$): 0.86, 1.0 (3H, each s, two quaternary CH_3), 1.23 (3H, s, one CH_3 attached to a carbon linked to an oxygen), 1.65 (3H, one vinylic CH_3), and 5.55 (1H, d, J = 5 Hz, one olefinic proton) ppm. On the basis of physical and spectral data, it was identified as himachalol (2, 3).

Acute Toxicity—Mice of either sex, 15–25 g, were divided into groups of 10 each. They were deprived of food for 16 hr and administered graded doses of himachalol and papaverine orally or intraperitoneally. Himachalol was used as a suspension in gum acacia, and papaverine hydrochloride was used as an aqueous solution.

The volume of the oral and intraperitoneal injections never exceeded 0.2 and 0.1 ml/10 g of body weight, respectively. The mortality over the next 72 hr was recorded, and the LD_{50} value was calculated by the probit analysis method (4).

***In Vitro* Spasmolytic Activity**—*Guinea Pig Ileum*—Sections of ileum (4–5 cm long) were suspended in an organ bath of 16-ml capacity, containing aerated Tyrode solution at 35–36°. Acetylcholine chloride (1×10^{-8} g/ml), histamine acid phosphate (2.5×10^{-8} g/ml), serotonin (5×10^{-7} g/ml), nicotine sulfate (5×10^{-7} g/ml), and barium chloride (2×10^{-5} g/ml) were used as spasmogens and left in contact with the tissue for 15–20 sec. Contractions (1:6 magnification; 1 g tension) in response to spasmogen alone and in the presence of various concentrations of himachalol or papaverine were recorded using a frontal writing lever on a smoked drum.

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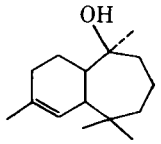

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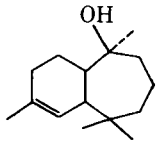

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[1] The plant sample was identified by Dr. B. Gupta and Mr. B. N. Mehrotra of the Botany Unit, Central Drug Research Institute, Lucknow, India. A voucher specimen (No. 27) has been preserved in the institute herbarium.

mg/kg sc) on 2 successive days and sacrificed 18 hr after the second injection. One uterine horn was mounted in an organ bath containing 16 ml of aerated de Jalon solution at 31°. The contractions were recorded with a frontal writing lever as in the case of gut preparations. The spasmogen oxytocin[2] (0.06 mU/ml) or serotonin (2.5×10^{-7} g/ml) was added every 5 min, and himachalol was added 1 min before the spasmogen.

Guinea Pig Seminal Vesicle—The preparation was set up in oxygenated Locke solution at 37° as described by Stone and Loew (6). Epinephrine (5×10^{-6} g/ml) was used as the agonist. Himachalol or papaverine was added to the bath 3 min before the addition of epinephrine and washed out 3 min later with two changes of bath solution. The relative potency of himachalol with respect to papaverine was calculated from log dose–response curves for the two agents.

Guinea Pig Tracheal Chain—The tracheal chain, containing 10–14 rings, was set up as described by Castillo and De Beer (7). The effect of himachalol or papaverine on the responses of the tissue to histamine (2.5×10^{-6} g/ml) and acetylcholine (7.5×10^{-6} g/ml) was tested after contact for 1 min with the tissue.

***In Vivo* Spasmolytic Activity**—*GI Propulsion of Charcoal Suspension in Rats*—Experiments were performed in rats, 100–225 g, fasted for 18–24 hr. A standardized charcoal meal (10% charcoal in 2% gum acacia in water) was administered orally to groups of five to six rats each. Himachalol or papaverine (20–100 mg/kg) was administered orally or intraperitoneally before, after, or along with the charcoal meal in a volume maintained at 1 ml/100 g of body weight. Control animals were given normal saline.

The time for which himachalol or papaverine was allowed to act varied from 15 to 60 min, depending on the time of administration in relation to the charcoal meal. Animals were sacrificed by decapitation 30 min after the test meal. The small intestine from the pylorus to the cecum was quickly removed, and the total intestinal length as well as the length through which the charcoal suspension progressed was measured. These measurements were used for calculating the percentage of the total intestinal length through which the charcoal meal traveled.

Effect on Intestinal Movements in Cats—In the first series of experiments, the effect of himachalol on normal intestinal motility was studied in anesthetized (pentobarbital sodium, 35 mg/kg iv) cats. The right femoral vein, left common carotid artery, and trachea were routinely cannulated. The abdomen was opened by a midline incision. One end of an actively motile loop of the small intestine was attached to Jackson's enterograph, and the other end was connected by a thread to an isotonic frontal writing lever. The intestinal movements as well as the carotid blood pressure were recorded on a smoked kymograph. Himachalol or papaverine was administered intravenously in doses ranging from 1 to 10 mg/kg.

The second series of experiments was performed with 12 cats, 3–4 kg. The surgery was performed under ether anesthesia, and the animal was then immobilized with hayatin methiodide (0.3 mg/kg iv) (8). All cut surfaces were infiltrated with 2% procaine to avoid pain. Positive pressure artificial ventilation was given through a tracheal cannula. A small rubber catheter was introduced through the esophagus into the stomach for administration of himachalol or papaverine.

The intestinal movements were recorded as already described. The intestine was brought into a state of contraction by injecting carbachol (1×10^{-5} g/kg) through the cannulated femoral vein. The inhibitory effect of himachalol or papaverine on the carbachol-induced spasm was measured after intragastric administration of 10, 30, and 100 mg/kg of himachalol or papaverine.

Guinea Pig Bronchial Muscle—The method used was based on that of Konzett and Rossler (9). Guinea pigs, 400–650 g, were anesthetized with urethan (1.2 g/kg). The trachea and the jugular vein were cannulated, and artificial ventilation was given at the rate of 72 strokes/min by means of a constant-volume pump delivering 10–15 ml of air/stroke. The air escaping through the side arm of the tracheal cannula operated the float in a manometer (Condon) to record its volume on the kymograph.

Histamine administration (5 μg/kg iv) was spaced at intervals of 15 min before and after himachalol or papaverine administration (1–10 mg/kg). The percent increase or decrease in the height of the excursion of the float indicated the intensity of bronchoconstriction or bronchodilatation, respectively.

[2] Syntocinon.

Table I—Comparison of Spasmolytic Activity of Himachalol with Papaverine on Various Isolated Smooth Muscle Preparations

Tissue	Species	Spasmogen	ED_{50} of Himachalol, μM	Relative Potency (Papaverine = 1)
Ileum	Guinea pig	Acetylcholine	8.1	1.47
		Histamine	3.8	1.74
		Serotonin	3.1	2.30
		Nicotine	2.3	3.60
		Barium	1.1	4.12
Jejunum	Rabbit	Barium	11.7	0.38
Uterus	Rat	Serotonin	52.6	0.12
		Oxytocin	14.8	0.32
Seminal vesicle	Guinea pig	Epinephrine	12.6	3.3

Effect on Cardiovascular System—*Isolated Guinea Pig Auricle*—The preparation was set up according to the method described earlier (10). Himachalol was dissolved in 0.05 ml of alcohol and added to the bath solution to study its effect on the rate, amplitude, and maximal frequency response to electrical stimulation.

Blood Pressure and Blood Flow Studies in Cats—Animals of both sexes, 3–4.5 kg, were anesthetized with chloralose (80 mg/kg iv) or pentobarbital sodium (35 mg/kg iv) and tracheotomized. The right femoral vein was cannulated for injecting drugs. Carotid blood pressure was monitored with a pressure transducer[3]. The left femoral artery was exposed, and a flow transducer with a lumen of 2-mm diameter attached to electromagnetic blood flowmeter[4] was placed around the artery. Both arterial blood flow and blood pressure were recorded[5].

In some cats, blood pressure and contraction of the nictitating membrane elicited by electrical stimulation of the preganglionic cervical sympathetic nerve were also recorded on a kymograph. Responses to epinephrine, acetylcholine, and histamine were recorded before and after himachalol.

RESULTS

Acute Toxicity—The LD_{50} value of himachalol (with 95% confidence limits) in mice by the oral route was 265 mg/kg (182–394), and it was 247 mg/kg (191–323) by the intraperitoneal route. The LD_{50} values for papaverine (with 95% confidence limits) were 129 mg/kg (111–149) and 116 mg/kg (107–126) by the oral and intraperitoneal routes, respectively.

***In Vitro* Spasmolytic Activity**—Himachalol was dissolved in alcohol and added to the bath in a volume of 0.04 ml to make a final solvent concentration of 0.25% in the bath. This amount of alcohol alone had no effect on isolated smooth muscle preparations. Himachalol, like papaverine, in increasing concentrations (4.5×10^{-7}–2.25×10^{-5} *M*) produced a graded antagonism to acetylcholine, histamine, serotonin, nicotine, and barium in the guinea pig ileum preparation. Himachalol was 4.1 times more potent than papaverine in antagonizing the action of barium and 1.47 times more potent than papaverine against acetylcholine. The relative potencies against other spasmogens were between these extremes (Table I).

In one experiment the ED_{50} concentration of himachalol and 2.5×10^{-7} g/ml histamine were added to 20 ml of Tyrode solution and allowed to stand for 10 min. When the organ bath containing the ileum was filled with this mixture, the contraction was only 50% of that obtained with the same concentration of histamine alone. Since a prolonged contact failed to increase antagonism by himachalol, chemical antagonism was considered unlikely.

In rabbit jejunum preparations, however, himachalol (4.5×10^{-6}–1.35×10^{-4} *M*) was only one-third as active as papaverine in antagonizing the spasm induced by barium chloride. Unlike pa-

[3] Statham P23 DC.
[4] Biotronex Laboratory model 610.
[5] On a Grass model P-7 polygraph.

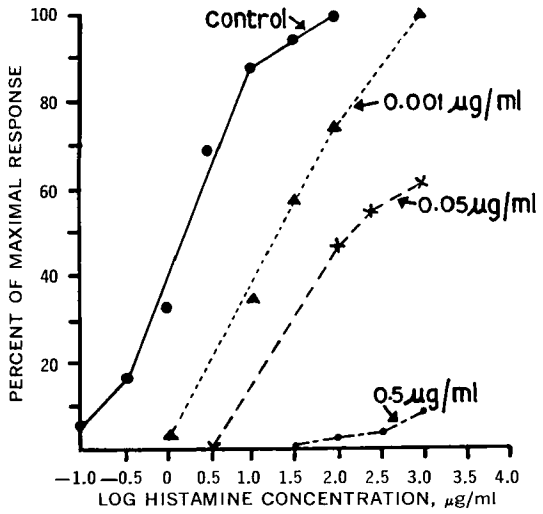

Figure 1—*Cumulative dose–response curve of ileum to histamine in the absence and presence of three concentrations of himachalol.*

paverine, it did not have any direct effect on tone and motility of the jejunum. Similarly, himachalol had a weaker spasmolytic activity against oxytocin and serotonin in the rat uterus. Himachalol (4.5×10^{-6}–4.5×10^{-5} *M*), however, strongly inhibited epinephrine-induced spasm of the guinea pig seminal vesicle, and the antagonism lasted for 6–9 min.

The relative potencies of himachalol and papaverine, assessed by calculating the ED_{50} values in various isolated smooth muscle preparations, are shown in Table I. Himachalol (4.5×10^{-6}–1.35×10^{-4} *M*) tended to cause contraction of the tracheal chain, whereas papaverine in similar concentrations relaxed the tissue. Himachalol at the same time caused a 30–50% reduction in contractions produced by acetylcholine and histamine, whereas papaverine completely abolished the effect of these spasmogens.

Cumulative dose–response studies in the guinea pig ileum showed that, in lower concentrations, both himachalol and papaverine could shift the curve for histamine to the right and that the maximal response to histamine could be obtained by increasing its concentration. In the presence of higher concentrations of either, however, histamine failed to achieve its maximum height of contraction (Figs. 1 and 2).

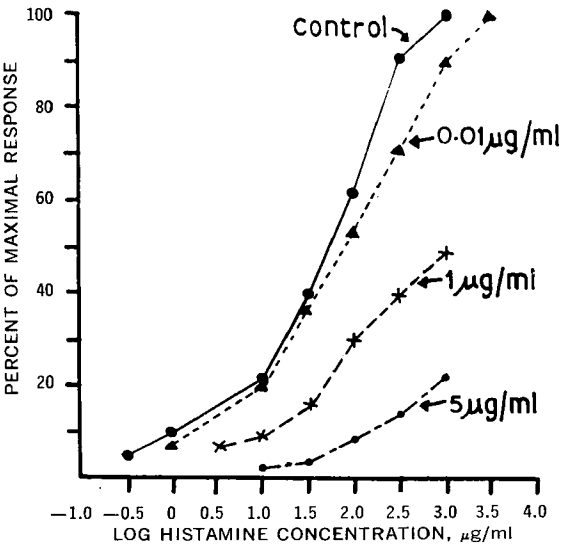

Figure 2—*Cumulative dose–response curve of ileum to histamine and the curves obtained in the presence of three concentrations of papaverine.*

Table II—Effects of Himachalol and Papaverine on Blood Pressure and Intestinal Movements in Cats under Pentobarbital Anesthesia

Compound	Dose, mg/kg iv	Fall in Blood Pressure, mm Hg ± *SE*	Duration of Fall, min ± *SE*	Average Intestinal Relaxation, mm
Himachalol	1	0 (4)[a]	—	9.5 (2)
	3	48 ± 7 (10)	5 ± 0.5	11.7 (4)
	10	65 ± 8 (5)	16 ± 6	27.0 (2)
Papaverine	1	33 ± 4 (4)	5 ± 2	7.0 (2)
	3	50 ± 4 (8)	17 ± 5	11.5 (4)
	10	81 ± 5 (4)	40 ± 5	19.15 (2)

[a] Figures in parentheses indicate number of experiments.

***In Vivo* Spasmolytic Activity**—Oral administration of up to 100 mg/kg himachalol or papaverine had no significant effect on the peristaltic movements in rats. Papaverine, however, significantly inhibited the propulsion of a charcoal meal in rats when given intraperitoneally 30 min before the test meal; himachalol had no such effect.

In anesthetized cats the effect of intravenously administered himachalol and papaverine was compared at doses ranging from 1 to 10 mg/kg on the normal tone and movements of the intestine. Both himachalol and papaverine decreased the tone of the GI musculature and, to a smaller extent, the amplitude of rhythmic contractions. The relaxation lasted for 1–5 min and was generally more marked with himachalol (Table II).

In conscious immobilized cats, intragastric administration of 10 mg/kg himachalol or papaverine had no effect. Higher doses (30–100 mg/kg) reduced the spontaneous motility as well as the spasm induced by carbachol (10 µg/kg). The reduction in both cases was proportional to the dose. Himachalol and papaverine were almost equiactive in inhibiting spontaneous activity, but papaverine caused greater reduction of carbachol-induced spasm (Fig. 3).

Both himachalol and papaverine seemed to have poor absorption from the intestine, since the fall in blood pressure was not evident even at a dose of 100 mg/kg by the intragastric route. There was an appreciable fall in blood pressure in the same preparation when himachalol or papaverine was given intravenously at a dose of 3 mg/kg.

The duration of the spasmolytic action of himachalol and papaverine was also compared. In these animals, carbachol injections were spaced at intervals of 15 min to produce spasm of the intestine. The reduction in the height of contraction was observed for 2 hr following a single intragastric administration of himachalol or papaverine. The results obtained with 100 mg/kg of both agents were plotted in Fig. 4. Himachalol had a faster but somewhat less sustained action than papaverine.

In the Konzett–Rossler preparation, himachalol caused bronchoconstriction in guinea pigs in doses of 1–10 mg/kg iv in 0.05 ml of ethanol. This amount of ethanol alone had no effect on the bronchial musculature. The spasm produced by histamine was not blocked by himachalol in a dose of 3 mg/kg. Similar doses of papaverine caused pronounced relaxation and partially inhibited the effect of histamine.

Both himachalol and papaverine occasionally potentiated the response to histamine. Figure 5 illustrates an experiment in which himachalol-induced bronchoconstriction remained unaffected by prior administration of a dose of pyrilamine maleate (5 mg/kg iv) that completely abolished the histamine effect.

Cardiovascular Effects of Himachalol—In anesthetized cats, himachalol (1–10 mg/kg iv) caused a dose-dependent fall in blood pressure, which slowly returned to its original level in 5–15 min. The hypotensive effect was less than that of an equal dose of papaverine (Table II).

There was a partial antagonism to the pressor effect of epinephrine and to the depressor response to histamine and acetylcholine. The depressor action of himachalol was not modified by pretreatment with pyrilamine maleate (5 mg/kg iv) or atropine sulfate (2 mg/kg iv).

The respiration and contraction of the nictitating membrane elicited by electrical stimulation of the preganglionic sympathetic

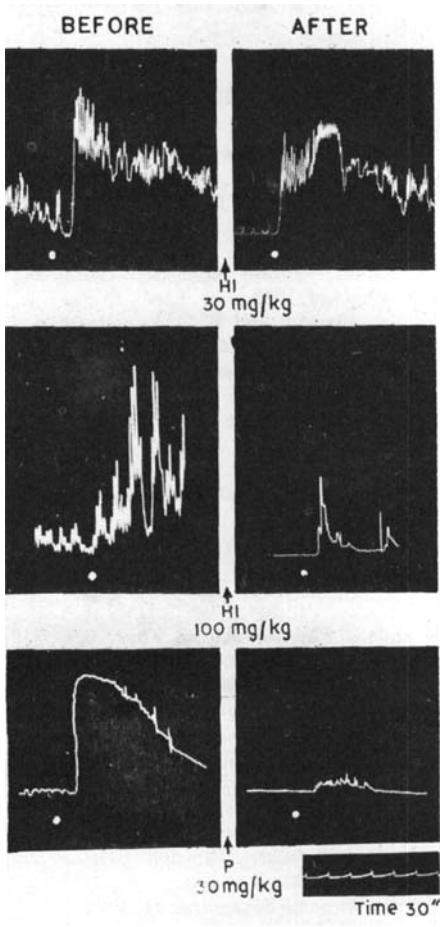

Figure 3—*Kymograph tracing showing the effect of carbachol (10 μg/kg iv) on intestinal movements of an immobilized cat before and after intragastric administration of himachalol (30 mg/kg, upper panel; 100 mg/kg, middle panel) and papaverine (30 mg/kg, lower panel).*

nerve were not affected up to a dose of 10 mg/kg iv. At a dose of 5 mg/kg, himachalol increased the femoral blood flow by 7.7% while lowering the blood pressure by 12%. Papaverine in a similar dose produced hypotension (28.5%) and enhanced the flow by 8.7%. The records from a typical experiment are shown in Fig. 6.

Cardiac rate was not affected by himachalol in these animals. The rate and amplitude of a spontaneously beating auricle were depressed by himachalol at concentrations of 4.5×10^{-6}–4.5×10^{-5} *M*. The maximal frequency response was also inhibited by 9–21%, depending on concentration. Papaverine in equal concentrations depressed the contractility and maximal frequency response to the same extent.

DISCUSSION

In gut preparations, there are abundant distinct receptors with which different agonists like acetylcholine, histamine, serotonin, nicotine, and barium chloride react to elicit a contractile response. While histamine seems to act directly on the muscle cells, others are known to act both directly through specific receptors and indirectly through nerve terminals (11–15).

Since himachalol has been found to inhibit the effect of all of the spasmogens, it appears to exert its action in the sequence of events following an interaction between the spasmogen and the receptor on the muscle cell. Papaverine also shows the same phenomenon. These observations establish the fact that, like papaverine, himachalol has nonspecific spasmolytic activity not only on the smooth muscle preparations of the gut but also in the guinea pig seminal vesicle and rat uterus.

Cumulative dose–response studies (Figs. 1 and 2) seem to indicate that himachalol, like papaverine, is a noncompetitive antagonist at high concentrations and a competitive antagonist at low concentrations. Himachalol is more potent than papaverine in guinea pig ileal and seminal vesicle preparations, whereas it is less effective than papaverine in the rat uterus, rabbit jejunum, and guinea pig tracheal chain. The species and organ variations seem to account for the decrease in potency in the latter preparations.

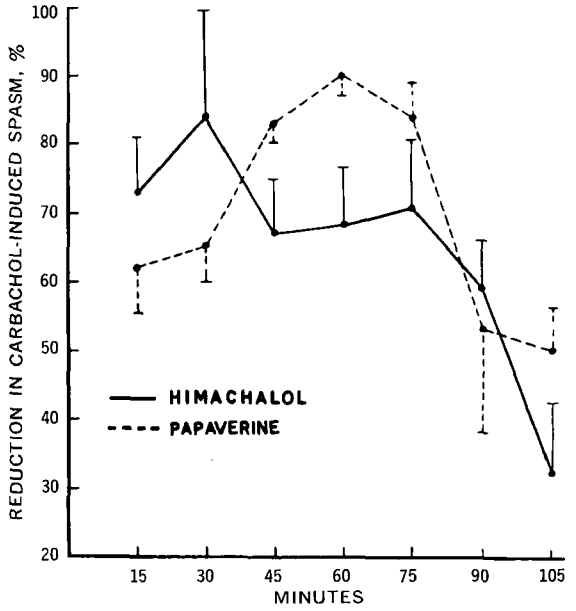

Figure 4—*Effect of himachalol and papaverine (100 mg/kg) on carbachol-induced spasm of the intestine in immobilized conscious cats. Mean values with standard errors obtained from five experiments each have been plotted. Himachalol has a faster but less sustained action.*

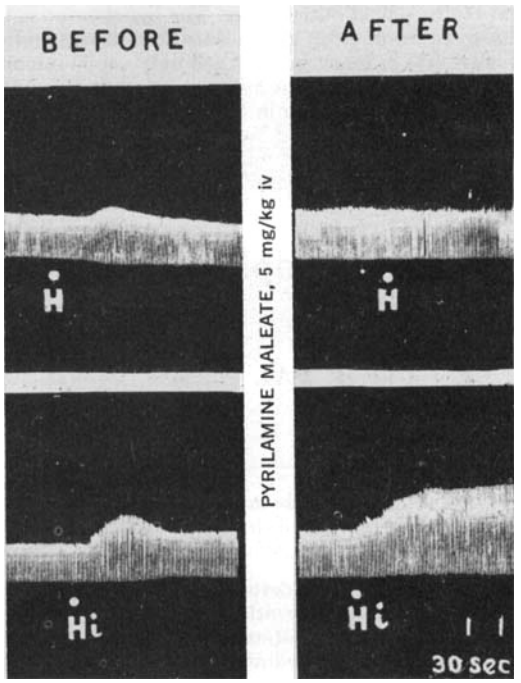

Figure 5—*Kymograph record of the effect of histamine and himachalol on the bronchial resistance in anesthetized guinea pigs. The left-hand panel shows bronchoconstriction caused by histamine (H, 5 μg/kg) and himachalol (Hi, 3 mg/kg). The right-hand panel shows antagonism of histamine (H) by pretreatment with pyrilamine maleate (5 mg/kg iv) and no effect on himachalol (Hi)-induced constriction.*

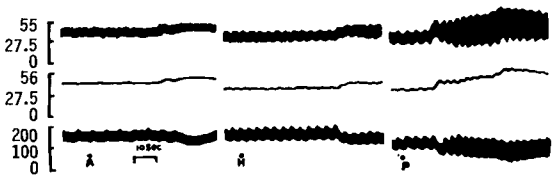

Figure 6—*Polygraph records showing, from below upward, the effects of himachalol and papaverine on blood pressure, mean femoral blood flow, and pulsatile femoral blood flow in an anesthetized cat. Key: A, control response to 0.25 ml of alcohol alone 30 sec after intravenous injection; H, hypotension and increase in the mean and pulsatile femoral blood flow 30 sec after intravenous injection of 5 mg/kg of himachalol dissolved in 0.25 ml of alcohol; and P, marked hypotension and increase in the mean and pulsatile femoral blood flow following injection of papaverine (2 mg/kg iv).*

There is a qualitative difference in the effect of the two compounds on guinea pig bronchial muscle. Himachalol causes constriction of bronchial muscle and papaverine causes relaxation. The constriction caused by himachalol cannot be blocked by pyrilamine, an antihistaminic agent, and, therefore, it seems unlikely that the constriction is due to release of histamine (Fig. 5). It is also unlikely that pulmonary congestion caused by himachalol would bring about bronchoconstriction by diminishing air inflow through bronchial tubes since papaverine causes greater vasodilation but does not cause any increase in bronchial resistance. Bronchoconstriction probably is the result of a directly stimulating action of himachalol in some unknown way.

Himachalol, like papaverine, produces systemic hypotension and peripheral vasodilation. The hypotensive effect is not mediated by cholinergic or histaminic receptor sites since the hypotension remains unaffected following pretreatment with atropine and pyrilamine. A stimulation of β-adrenergic receptors by himachalol can also be ruled out due to the absence of any tachycardia or relaxant effect on the bronchial musculature. The hypotension is unlikely to be due to a cardiac effect either, since there is no significant effect on heart rate in intact animals and only a mild inhibitory effect on the isolated auricle. The hypotension, therefore, appears to result mainly from a decrease in the peripheral resistance due to vasodilation caused by a direct relaxant action on the vascular smooth muscle.

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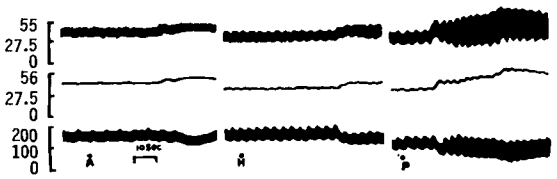
Possible Antineoplastic Agents I

A. U. DE ˣ and D. PAL

Abstract □ A few thalidomide and glutarimide derivatives were synthesized. Several compounds possessed significant antineoplastic activity against Ehrlich ascites carcinoma in Swiss albino mice.

Keyphrases □ Thalidomide derivatives—6-alkyl-2-[3′- or 4′-nitrophthalimido]glutarimides synthesized and screened as possible antineoplastic agents □ Glutarimide derivatives—6-alkyl-3-phenylglutarimides synthesized and screened as possible antineoplastic agents □ Antineoplastic agents, potential—synthesis and screening of thalidomide and glutarimide derivatives

The teratogenic effect of thalidomide (2-phthalimidoglutarimide, I) has been well established in humans and animals (1). To account for this manifestation, Faigle *et al.* (2) isolated a number of suspected metabolites of thalidomide and showed them to be derived from D-glutamic acid, an unnatural amino acid, in place of L-glutamic acid, the natural amino acid. They also pointed out the similarity between *N*-(*O*-carbobenzoxyl)glutamic acid, a metabolite of thalidomide, and folic acid. All of these observations led them to conclude that the metabolites of thalidomide might act as vitamin antagonists or antimetabolites. Since then, a number of claims and counterclaims on the antiglutamine, antifolic, and antivitamin activities of thalidomide have been made, the objective being to utilize thalidomide as a possible antineoplastic agent.



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Abstract □ A few thalidomide and glutarimide derivatives were synthesized. Several compounds possessed significant antineoplastic activity against Ehrlich ascites carcinoma in Swiss albino mice.

Keyphrases □ Thalidomide derivatives—6-alkyl-2-[3′- or 4′-nitrophthalimido]glutarimides synthesized and screened as possible antineoplastic agents □ Glutarimide derivatives—6-alkyl-3-phenylglutarimides synthesized and screened as possible antineoplastic agents □ Antineoplastic agents, potential—synthesis and screening of thalidomide and glutarimide derivatives

The teratogenic effect of thalidomide (2-phthalimidoglutarimide, I) has been well established in humans and animals (1). To account for this manifestation, Faigle *et al.* (2) isolated a number of suspected metabolites of thalidomide and showed them to be derived from D-glutamic acid, an unnatural amino acid, in place of L-glutamic acid, the natural amino acid. They also pointed out the similarity between *N*-(*O*-carbobenzoxyl)glutamic acid, a metabolite of thalidomide, and folic acid. All of these observations led them to conclude that the metabolites of thalidomide might act as vitamin antagonists or antimetabolites. Since then, a number of claims and counterclaims on the antiglutamine, antifolic, and antivitamin activities of thalidomide have been made, the objective being to utilize thalidomide as a possible antineoplastic agent.

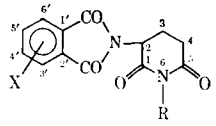


Table I—Antineoplastic Activity of 6-Alkyl-2-[3′- or 4′-nitrophthalimido]glutarimides (II) in Ehrlich Ascites Carcinoma

Compound	R	X	Cells Injected into Each Animal	Average Number of Cells per Milliliter in Control (C)	Average Number of Cells per Milliliter in Test (T)	T/C of Cells	Percent Inhibition of Ascitic Cells, (1 − T/C) × 100	Average Weight of Ascitic Fluid in Control (C), g	Average Weight of Ascitic Fluid in Test (T), g	T/C of Ascitic Fluid	Percent Inhibition of Ascitic Fluid, (1 − T/C) × 100
II*a*	H	H	2.65×10^6	366.3×10^6	200.8×10^6	0.5480	45.20	0.8	0.32	0.40	60.00
II*b*	Methyl	H	2.65×10^6	366.3×10^6	25.45×10^6	0.06947	93.053	0.8	0.5	0.625	37.50
II*c*	Ethyl	H	2.65×10^6	366.3×10^6	6.6×10^6	0.01801	98.199	0.8	0.4	0.50	50.00
II*d*	*n*-Propyl	H	2.65×10^6	366.3×10^6	48.0×10^6	0.1310	86.90	0.8	0.26	0.325	67.50
II*e*	*n*-Butyl	H	2.65×10^6	366.3×10^6	126.0×10^6	0.3440	65.60	0.8	0.2	0.25	75.00
II*f*	*n*-Pentyl	H	2.3625×10^6	282.32×10^6	205.4×10^6	0.7276	27.24	1.225	0.68	0.555	44.50
II*g*	*n*-Hexyl	H	2.3625×10^6	282.32×10^6	4.7×10^6	0.01665	98.335	1.225	0.06	0.04898	95.102
II*h*	H	3′-Nitro	2.3625×10^6	282.32×10^6	9.28×10^6	0.03287	96.713	1.225	0.1	0.08162	91.838
II*i*	H	4′-Nitro	2.3625×10^6	282.32×10^6	8.55×10^6	0.03029	96.971	1.225	0.08	0.0653	93.47
Mitomycin (as standard)			2.3625×10^6	282.32×10^6	0	0	100.00	1.225	0	0	100.00

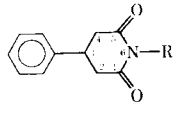
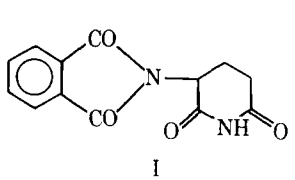


Table II—Antineoplastic Activity of 6-Alkyl-3-phenylglutarimides (V) in Ehrlich Ascites Carcinoma

Compound	R	Cells Injected into Each Animal	Average Number of Cells per Milliliter in Control (C)	Average Number of Cells per Milliliter in Test (T)	T/C of Cells	Percent Inhibition of Ascitic Cells, (1 − T/C) × 100	Average Weight of Ascitic Fluid in Control (C), g	Average Weight of Ascitic Fluid in Test (T), g	T/C of Ascitic Fluid	Percent Inhibition of Ascitic Fluid, (1 − T/C) × 100
V*a*	H	1.715×10^6	212.0×10^6	4.4×10^6	0.02076	97.924	0.667	0.1	0.1409	85.91
V*b*	Methyl	1.715×10^6	212.0×10^6	10.0×10^6	0.04718	95.282	0.667	0.09	0.1349	86.51
V*c*	Ethyl	1.715×10^6	212.0×10^6	13.34×10^6	0.06294	93.706	0.667	0.12	0.1799	82.01
V*d*	*n*-Propyl	1.56×10^6	565.2×10^6	17.38×10^6	0.03075	96.925	0.734	0.12	0.01635	98.365
V*e*	*n*-Butyl	1.56×10^6	565.2×10^6	21.95×10^6	0.03884	96.116	0.734	0.1	0.01362	98.638
V*f*	*n*-Pentyl	1.5×10^6	564.0×10^6	91.2×10^6	0.1617	83.83	0.7	0.2	0.2857	71.43
V*g*	*n*-Hexyl	1.5×10^6	564.0×10^6	216.75×10^6	0.3844	61.56	0.7	0.2	0.2857	71.43
V*h*	Phenyl	1.5×10^6	564.0×10^6	54.35×10^6	0.09636	90.364	0.7	0.6	0.8572	14.28
Mitomycin (as standard)		1.56×10^6	565.2×10^6	0	0	100.00	0.734	0	0	100.00



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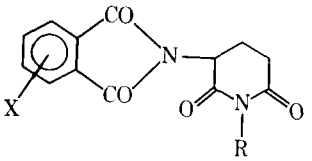

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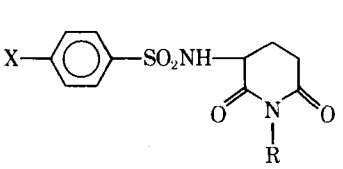

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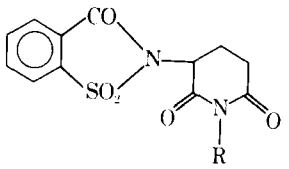

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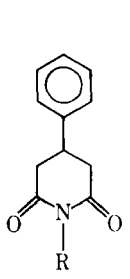

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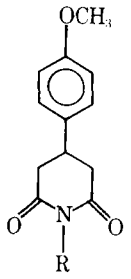

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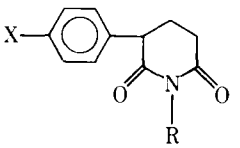


VII

DISCUSSION

The teratogenic activity of thalidomide has been viewed by many as the manifestation of its cytostatic activity and has been tested against a wide variety of experimental tumors including Ehrlich ascites, carcinoma, sarcoma, HeLa cell cancer, myeloma, and lymphoma (3–13). Although the results have not been very encouraging, thalidomide has been reported to limit the manifestation and growth of certain tumors. It has also been shown to possess a specific antiproliferative effect (4) necessary for their arrest.

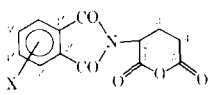
These observations are not unexpected, because Pastac (14) pointed out the analogy between the growth regulators of plant tissues and those of animals. In the former case, the growth regulators are derived from phthalic acid and naphthylamine, while in the latter thalidomide is derived from phthalic acid and glutamic acid. It was also observed (15) that folic acid-dependent embryological tumors might be susceptible to thalidomide. Besides this, there are two significant similarities between thalidomide and several alkylating agents. Thalidomide and these alkylating agents cause teratogenesis (16); furthermore, these alkylating agents, in general, alkylate DNA (17), whereas thalidomide acylates DNA and RNA in the fetus (18).

Although work with thalidomide as a possible antineoplastic agent is abundant, its derivatives have not been investigated extensively. This situation prompted the synthesis and biological evaluation of a few derivatives (II–IV). Derivative II was chosen to bring about changes both in the phthalimide and glutarimide portions of the original molecule, while derivatives of types III and IV include a sulfonamide group either in the straight chain or cyclic part of the molecule so as to result in appreciable antineoplastic activity.

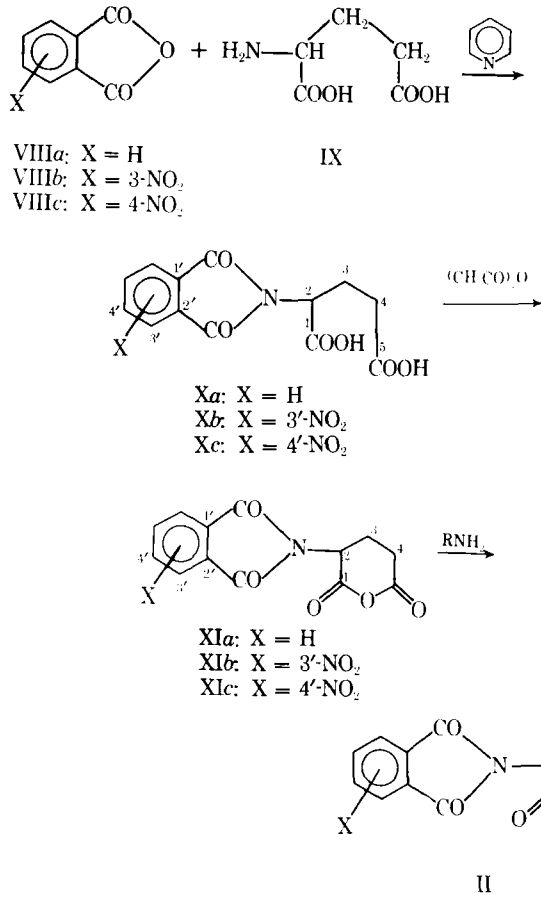
The antineoplastic antibiotics, cycloheximide and streptovitacin A, like thalidomide, are characterized by the presence of a glutarimide moiety in their structures. Therefore, the synthesis and biological evaluation of a few derivatives of the types V–VII were also undertaken.

This article describes the synthesis and activity of II and V; the synthesis and activity of other derivatives will be communicated subsequently with molecular orbital indexes calculated and a regression model built on the glutarimide moiety for the quantitative structure–activity relationships in this class of compounds.

Table III—2-[3′- or 4′-Nitrophthalimido]glutaric Anhydrides (XI)



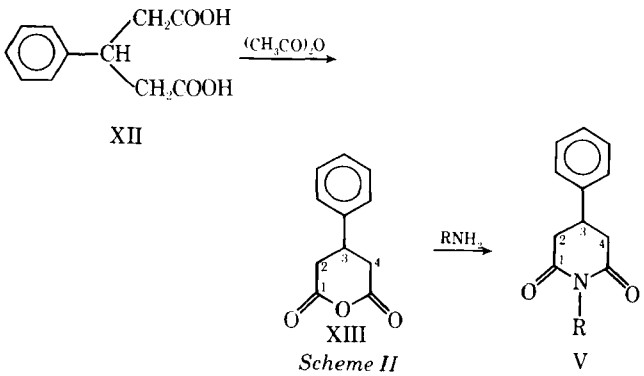
Compound	X	Melting Point	Yield, %
XI*a*	H	195–197°	76
XI*b*	3′-Nitro	213–215°	64
XI*c*	4′-Nitro	221–223°	60



Scheme I

EXPERIMENTAL

Chemistry—Phthalic anhydride and its 3- and 4-nitro derivatives (VIII) (19) were condensed with glutamic acid (IX) in the presence of pyridine to the diacids (X) and subsequently treated with acetic anhydride to furnish the anhydrides (XI) (20). Conversion of the diacids (X*b* and X*c*) to the corresponding anhydrides (XI*b* and XI*c*) was accomplished under mild conditions to avoid unnecessary decomposition. Attempts to remove any excess pyridine and acetic anhydride under reduced pressure with heating re-



Scheme II

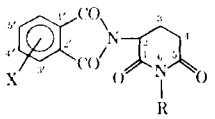


Table IV—6-Alkyl-2-[3′- or 4′-nitrophthalimido]glutarimides (II)

Compound	R	X	Yield, %	Melting Point	Boiling Point	Formula	Analysis, % Calc.	Found
II*a*	H[a]	H	70	258–260°	—	$C_{13}H_{10}N_2O_4$	C 60.47 H 3.88 N 10.85	60.81 4.32 10.57
II*b*	Methyl[b]	H	42	131–133°	—	$C_{14}H_{12}N_2O_4$	C 61.76 H 4.41 N 10.29	62.12 4.88 10.70
II*c*	Ethyl[b]	H	46	75–77°	—	$C_{15}H_{14}N_2O_4$	C 62.94 H 4.90 N 9.79	62.57 5.27 9.43
II*d*	*n*-Propyl	H	60	66–68°	—	$C_{16}H_{16}N_2O_4$	C 64.00 H 5.33 N 9.33	63.82 5.27 9.32
II*e*	*n*-Butyl	H	64	29–31°	135–137°/ 2 mm	$C_{17}H_{18}N_2O_4$	C 64.97 H 5.73 N 8.92	65.01 6.22 8.56
II*f*	*n*-Pentyl	H	60	19–21°	110–112°/ 1 mm	$C_{18}H_{20}N_2O_4$	C 65.85 H 6.10 N 8.54	66.28 6.45 8.72
II*g*	*n*-Hexyl	H	65	34–36°	100–102°/ 0.9 mm	$C_{19}H_{22}N_2O_4$	C 66.67 H 6.43 N 8.19	66.62 6.91 8.31
II*h*	H	3′-Nitro	30	262–264° dec.	—	$C_{13}H_9N_3O_6$	C 51.49 H 2.97 N 13.86	51.13 3.41 14.18
II*i*	H	4′-Nitro	27	278–280° dec.	—	$C_{13}H_9N_3O_6$	C 51.49 H 2.97 N 13.86	51.24 3.35 13.84

[a] Lit. (24) mp 271° and (25) mp 244°. [b] Purified by sublimation.

sulted in considerable decomposition and, consequently, were avoided.

The anhydrides (XI) were treated with ammonia (21) or the respective amines to give the desired imides (II) (Scheme I). Conversion of the anhydrides (XI*b* and XI*c*) to the corresponding imides (II*h* and II*i*) with ammonia was accomplished with considerable decomposition, lowering the yield appreciably. 3-Phenylglutaric acid (XII) (22) was converted to 3-phenylglutaric anhydride (XIII) with acetic anhydride (19) and subsequently treated with ammonia (21) and various amines to furnish the desired imides (V) (23) (Scheme II).

Biological Evaluation—Several groups of Swiss albino mice, each containing five healthy animals of the same sex and approximately the same age and body weight (18–20 g), were selected at random and kept in different cages under identical conditions. One group served as the control; the others were the test groups.

The mice were marked by cutting the fur at different parts of the body, with individual weights being recorded at the same time. Ehrlich ascites carcinoma cells, collected from the donor mice, were suspended in sterile isotonic saline, and the number of living cells per milliliter of this suspension was counted. A fixed number of the viable cells was implanted into the intraperitoneal cavity of each mouse. In this instance, the tumor cells multiplied relatively freely and ascites developed. A day of incubation was allowed to establish the disease before drug administration.

From the 2nd day of transplantation up to the 8th day, a suitable dose of the drug suspension, usually 50 mg/kg ip in buffered isotonic saline (pH 7.2), was injected to the test groups. The weight of each mouse was recorded every alternate day to assess the toxicity, activity, and other specificity of the screening program. Thus, seven consecutive doses were administered. On the 9th day, food and water were withheld for 6 hr before the testing operation started.

Fluid from the peritoneal cavity of each mouse of both the control and test groups was drawn by a sterile syringe, diluted to a suitable volume with sterile ice-cold isotonic saline, and preserved in an ice bath. The total number of living cells per milliliter of fluid of each mouse was separately counted with trypan blue indicator; only the dead cells took the stain. The individual weight of each animal was recorded before sacrificing. The peritoneal cavity was dissected and the fluid was sucked by cotton or filter papers; the weight of each mouse after sacrifice was recorded.

The evaluation of test compounds was made by comparing the cell counts and tumor weights of the test groups with those of the control. Mitomycin was used as a standard in these tests at a dose of 1 mg/kg in buffered isotonic saline (Tables I and II).

Activity—Antineoplastic potency of the synthesized compounds (II and V) is recorded in Tables I and II. Significant activity was observed. In the thalidomide series, appreciable activity was observed in the *n*-hexyl (II*g*) and nitro (II*h* and II*i*) derivatives based on live cell counts and tumor weights as the activity parameters. In the glutarimide series, maximum activity was observed in a simple and known compound (V*a*). The other significantly active compounds in this series were the methyl, ethyl, *n*-propyl, and *n*-butyl derivatives (V*b*–V*e*).

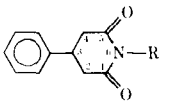
Syntheses[1]—*2-[3′- or 4′-Nitrophthalimido]glutaric Anhydrides (XIb and XIc)*—A suspension of the anhydrides (VIII*b* and VIII*c*, 20 mmoles) and L-glutamic acid (IX, 20 mmoles) in dry pyridine (12 ml) was refluxed on an oil bath for about 6 hr. Only the clear solution was transferred, by decantation, to a conical flask. Acetic anhydride (9 ml) was added to this clear solution. The solution was refluxed on a very low flame for 10 min or heated on a water bath for 1 hr and kept in the cold overnight. The anhydride crystals were filtered and washed with ether to give the product. The anhydrides were crystallized from dry ethyl acetate (Table III).

2-[3′- or 4′-Nitrophthalimido]glutarimides (II h and II i)—The appropriate anhydride (XI*b* or XI*c*) was heated at about 220–240° in an oil bath until it melted. Then dry ammonia gas was bubbled through the molten mass for about 15 min and the whole mass became tarry. The mass was cooled and crystallized from 90% ethanol with charcoal treatment, yielding 27–30% (Table IV).

6-Alkyl-2-phthalimidoglutarimides (II b–II g)—A mixture of 2-phthalimidoglutaric anhydride (XI*a*, 1 mole) and the corresponding amine (1.2 moles) was heated in a sealed tube for 8 hr in an oil bath at 210–220°. The imides were purified by sublimation

[1] All melting points are uncorrected.

Table V—6-Alkyl-3-phenylglutarimides (V)



Compound	R	Yield, %	Melting Point	Formula	Analysis, % Calc.	Found
V*a*	H	85	173–174°	$C_{11}H_{11}NO_2$	C 69.84 H 5.82 N 7.41	70.12 6.23 7.29
V*b*	Methyl	64	138–140°	$C_{12}H_{13}NO_2$	C 70.94 H 6.40 N 6.90	71.32 6.71 7.25
V*c*	Ethyl	62	86–88°	$C_{13}H_{15}NO_2$	C 71.89 H 6.91 N 6.45	71.58 7.30 6.76
V*d*	*n*-Propyl	72	104–106°	$C_{14}H_{17}NO_2$	C 72.73 H 7.36 N 6.06	73.02 7.48 6.17
V*e*	*n*-Butyl	76	112–114°	$C_{15}H_{19}NO_2$	C 73.47 H 7.76 N 5.71	73.81 7.45 5.38
V*f*	*n*-Pentyl	72	79–81°	$C_{16}H_{21}NO_2$	C 74.13 H 8.11 N 5.41	74.49 8.43 5.42
V*g*	*n*-Hexyl	68	88–90°	$C_{17}H_{23}NO_2$	C 74.71 H 8.42 N 5.13	74.89 8.26 4.87
V*h*	Phenyl	70	188–190°	$C_{17}H_{15}NO_2$	C 76.98 H 5.66 N 5.28	76.63 6.02 5.33

or by crystallization from water or dilute ethanol or were distilled *in vacuo* (Table IV).

6-Alkyl-3-phenylglutarimides (*V*)—Dry ammonia gas was bubbled through the molten 3-phenylglutaric anhydride (XIII) to furnish the imide (V*a*). A mixture of the anhydride (XIII, 1 mole) and the respective amine (1.2 moles) was heated in a sealed tube for 8 hr in an oil bath at 150–160° to furnish the crude imides (V*b*–V*h*), which were purified by crystallization from water or dilute ethanol with charcoal treatment (Table V).

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Influence of Topical Anesthesia on Tear Dynamics and Ocular Drug Bioavailability in Albino Rabbits

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Abstract □ The bioavailability of topically applied ocular drugs is very poor, due largely to drug loss through drainage and tear turnover. The use of high viscosity solutions or solid matrixes to delay or eliminate drainage is the usual approach for decreasing drug loss but the alternative approach of chemically reducing tear turnover and/or solution drainage has not been investigated. By means of a simple isotopic dilution technique, using radioactive technetium sulfur colloid, the quantitative influence of topical anesthetics on tear production and instilled solution drainage was determined. The reduction in the rate of tear turnover and solution drainage varies for different anesthetics and is dose dependent. The implication of these results for some long accepted clinical procedures is discussed, and questions are raised regarding the present understanding of the mechanisms of tear production. Quantitation of precorneal drug loss through instilled solution drainage and tear turnover permits the establishment of a baseline for ocular drug bioavailability. Aqueous humor drug concentration *versus* time profiles of radioactive pilocarpine nitrate were obtained, both in the presence and absence of topical anesthesia. The results verify the importance of tear turnover and instilled solution drainage as a major route of drug loss in the eye. Moreover, the success of the present study in improving ocular drug bioavailability by the chemical approach of repressing solution drainage and tear turnover suggests that this approach is viable for improving drug bioavailability.

Keyphrases □ Ophthalmic drug bioavailability—effect of topical anesthetics on solution drainage and tear turnover, rabbits □ Anesthetics, topical—effect on tear production and solution drainage, ophthalmic drug bioavailability evaluated, rabbits □ Tear dynamics and ophthalmic drug bioavailability—effect of topical anesthetics, rabbits □ Bioavailability, ophthalmic—effect of topical anesthetics on solution drainage and tear turnover, rabbits

Ophthalmic drug bioavailability is generally conceded to be very poor from topically applied solutions. For most drugs, less than 1% of an instilled dose crosses the cornea to the anterior chamber. Several routes of loss account for this poor drug availability, but the two major mechanisms appear to be drainage loss of an instilled solution and the continuous replenishment of tears, *i.e.*, tear turnover. A recent publication from this laboratory (1) considered the influence of solution drainage and tear turnover on drug bioavailability and reported rate constants for drainage and tear turnover, but a quantitative model of drug availability was not developed. Clearly, to maximize drug activity through improved availability in the eye, a complete mechanistic understanding of drug movement and loss is needed.

Any attempt to minimize drug loss by controlling instilled solution drainage and/or tear turnover should prove useful in improving ocular drug bioavailability. The principal approach has been to use viscous solutions (2–6) which delay instilled solution drainage but are presumed not to influence tear turnover. These attempts, however, have not been based on fundamental information related to tear production and movement or instilled fluid dynamics but rather on clinical observations to improve a particular biological response.

Since it is clear that tear dynamics are an important factor in ophthalmic drug bioavailability, an alternative approach to using formulation factors to increase corneal contact time would be to decrease the extent of instilled solution drainage and tear production through the use of drugs or to utilize the normal circadian rhythm, *i.e.*, apply a drug at bedtime since tears are not formed during sleep. Neither approach has been previously explored, but the use of drugs to decrease or inhibit selectively tear turnover and/or solution drainage to improve drug bioavailability seems particularly attractive. It has been shown (7) that rabbits under systemic anesthesia, whose tear production is inhibited (1, 8), achieve significantly higher aqueous humor levels of an instilled drug.

In the present work, the feasibility of controlling tear turnover and/or instilled solution drainage through chemical means is examined. In addition, the extent of improvement in ocular drug bioavailability, as a function of tear turnover and solution drainage, is discussed.

EXPERIMENTAL

Materials—Water was doubly distilled from alkaline permanganate in an all-glass distillation apparatus.

Technetium solutions were prepared using a package[1] of solutions and equipment used to prepare technetium, ^{99m}Tc, suspensions. The preparative procedure was described previously (1). Local anesthetics were obtained from commercial sources[2–4]. All other chemicals were either reagent or analytical grade.

Standardized sterile Schirmer tear strips were obtained commercially[5].

Male, albino rabbits, 1.8–2.4 kg, were used throughout this study[6]. The rabbits were fed a regular diet with no restrictions on the amount of food or water consumed.

Tritiated pilocarpine was obtained commercially[7] and was purified immediately before use. The reasons for the purification and the methods used were reported earlier (9). The specific activity of the pilocarpine used throughout these studies was 18.8 Ci/mmole.

Methods—*Determination of Onset and Duration of Action of Local Anesthetics*—For each local anesthetic tested, three rabbits were positioned in restraining boxes so that they remained approximately in their normal posture. A drop of the appropriate

[1] Collokit, Abbott Radio-Pharmaceuticals, North Chicago, Ill.
[2] Tetracaine hydrochloride, 0.5%, Cooper Laboratories, Wayne, N.J.
[3] Proparacaine hydrochloride, 0.5% (Ophthetic), Allergan Pharmaceuticals, Irvine, Calif.
[4] Lidocaine hydrochloride, 1% (Seracaine), Rachelle Laboratories, Long Beach, Calif.
[5] Cooper Labs, Wayne, N.J.
[6] Klubertanz, Edgerton, Wis.
[7] New England Nuclear, Boston, Mass.

Table I—Determination of Onset and Duration of Action of Topically Applied Proparacaine Hydrochloride, 0.5%, by the Corneal Reflex Technique

Time after Instillation, min	Rabbit 1		Rabbit 2		Rabbit 3	
	Anesthetized	Control	Anesthetized	Control	Anesthetized	Control
5	−[a]	+[b]	+	+	−	++
10	=[c]	++[d]	−	+	=	+
15	=	+	−	+	=	++
20	=	++	+	+	+	++
25	+	++	+	++	+	++
30	+	+	+	+	++	++
35	+	+	+	+	++	++

[a] A (—) response indicates that there was no corneal reflex produced by a single touch of the probe in the center of the cornea. [b] A (+) response indicates that a single touch of the probe to the cornea evoked a corneal reflex response, but the response was somewhat delayed and not severe. [c] A (=) response indicates that further and more severe probing of the corneal still did not evoke a response. [d] A (++) response indcates that a single touch with the probe produced an immediate and severe response.

type and concentration of local anesthetic was instilled onto the cornea of one eye, using commercial plastic dropper bottles that deliver a drop of 50–65 μl when held completely inverted. A drop of normal saline was instilled into the second eye of each rabbit as a control.

At 5-min intervals, the effectiveness of the local anesthetic was measured by means of a corneal reflex test. A thin smooth glass rod was touched to the center of the cornea, and the blink response was observed. In this way, a qualitative measure of the onset and duration of action of the local anesthetics was obtained. This procedure was helpful in gaining an idea of which drugs and what dosing regimens might be useful in the isotope studies, and it provided a means of observing the general response of the eye under the influence of local anesthetics.

Determination of Lacrimal Turnover Rate and Instilled Solution Drainage Rate—The general procedure for the determination of drainage rate and lacrimal fluid turnover rate by the nonsampling method was reported previously (1). In experiments where a local anesthetic was used, 10 min was allowed to elapse following instillation of local anesthetic into the eye before technetium was instilled and counting commenced. The 10-min interval was selected to allow complete drainage of the anesthetic solution (10).

Schirmer Number 1 Test—Rabbits were positioned in restraining boxes in the normal manner. The test was administered in a dimly lit, indirectly illuminated room. Care was exercised so that the strips did not touch the cornea because of the pain and reflex lacrimation produced, which invalidates the test. The rounded bent end of the sterile strips were hooked over the eyelid border at the junction of the middle and nasal one-third of the lower eyelid margin. After 5 min, the strips were removed and the length of the moistened area of the strips was measured.

Basic Secretion Test—The test procedure is the same as for the Schirmer Number 1 test, except that 2 drops of 0.5% proparacaine hydrochloride were instilled into each eye and a cotton swab moistened with 4 drops of 4% cocaine hydrochloride was held on the lower lid for 15 sec prior to inserting the Schirmer strips. After 2 min, the lower cul-de-sac was dried with a cotton swab and the strips were inserted. A similar test was also performed after instilling 5 drops of 0.5% tetracaine hydrochloride at 1-min intervals. Ten minutes after the last drop of tetracaine hydrochloride was instilled, the strips were inserted and the test begun.

Aqueous Humor Concentration versus *Time Profiles for Pilocarpine Nitrate in Presence and Absence of Topical Anesthesia*—All test animals were held in wooden restraining boxes in the normal upright position during the experiments to minimize movement. The animals were acclimated to these test conditions by being placed in the restraining boxes for 2–3 hr on several successive days before the experiment (1).

In the case of animals in the absence of topical anesthesia, 25 μl of 1×10^{-2} *M* tritiated pilocarpine nitrate was instilled onto the cornea, collecting in the lower cul-de-sac. During instillation, the lower lid was pulled slightly away from the globe but was immediately returned to its normal position after instillation. Where topical anesthesia was employed, 1 drop of 0.5% tetracaine hydrochloride was instilled per minute for 5 min; 10 min was allowed to elapse, and then 25 μl of tritiated pilocarpine nitrate solution was instilled.

At 5, 10, 15, 20, 30, 60, and 90 min postinstillation, rabbits were sacrificed by rapid injection of an overdose of pentobarbital sodium into a marginal ear vein. Eyes were rinsed with distilled water and wiped dry with tissues, after which 100–150 μl of aqueous humor was withdrawn from the anterior chamber.

One hundred microliters of aqueous humor was transferred[8] to scintillation counting vials containing 20 ml of liquid scintillation solution[9]. The vials had been refrigerated for 24 hr prior to addition of the samples and were stored in the dark at room temperature for at least 48 hr before counting to minimize chemiluminescence. Two consecutive 1-min counts of each sample were made using a liquid scintillation spectrometer[10]. Quenching of radioactivity by the aqueous humor was found to be relatively constant from animal to animal; therefore, blank corrections were made on the basis of aqueous humor from a single animal in each experiment.

RESULTS

Determination of Onset of Action, Duration of Action, and Depth of Anesthesia of Various Topically Applied Local Anesthetics—The onset and duration of anesthesia from the various local anesthetics are functions of the intrinsic activity of the local anesthetic, the concentration of the agent used, and the dosing regimen.

Table I indicates the results obtained with 0.5% proparacaine hydrochloride. From the data, it was determined that the onset of action of 0.5% proparacaine hydrochloride was from 0 to 10 min, with a duration of action of 10–20 min. At the 10-min point, all three anesthetized eyes began to appear dry, in noticeable contrast to the control eyes. At the 25-min point, all anesthetized eyes appeared to be regaining their moisture; and by 35 min, the anesthetized eyes could not be distinguished from the control eyes on the basis of visual appearance of the tear film.

Table II reports the data obtained from identical experiments using 0.5% tetracaine hydrochloride. The table indicates an onset of action of 0–25 min and a duration of action of 30–55 min. As was the case with proparacaine hydrochloride, drying of the eye and return to normal appearance seemed to approximate closely the loss and resumption of the corneal reflex. In comparison with proparacaine hydrochloride, tetracaine hydrochloride apparently acted somewhat slower but had a much longer duration of action. In addition, the incidence of the deeper (=) anesthesia appeared to be sustained for longer periods with tetracaine. The implications of these observations will become clearer on examination of the results of the isotope studies to follow.

Similar experiments were performed using lidocaine hydrochloride. In the three rabbits tested, 1% lidocaine hydrochloride appeared to have little or no effect on the corneal reflex. In addition, there apparently was little, if any, inhibition of lacrimation; *i.e.*, the anesthetized eyes of all rabbits displayed no observable differences in appearance from the control eyes.

These experiments were in no way intended to be definitive

[8] Biopette, Schwarz/Mann, Orangeburg, N.Y.
[9] Aquasol, New England Nuclear, Boston, Mass.
[10] Packard 2002, Packard Instrument Co., Downers Grove, Ill.

Table II—Determination of Onset and Duration of Action of Topically Applied Tetracaine Hydrochloride by the Corneal Reflex Technique

Time after Instillation, min	Rabbit 1		Rabbit 2		Rabbit 3	
	Anesthetized	Control	Anesthetized	Control	Anesthetized	Control
5	−[a]	++[d]	++	++	++	++
10	=[c]	+[b]	−	++	+	++
15	=	+	=	++	+	++
20	=	+	−	+	+	++
25	=	+	=	+	−	++
30	=	+	=	+	−	++
35	=	+	=	+	=	++
40	=	+	=	++	−	++
45	−	+	−	+	=	++
50	−	+	=	+	−	+
55	+	+	+	++	+	++
60	+	+	−	+	+	++
65	+	+	+	+	+	++
70	+	+	+	+	+	++
75	+	+	+	+	+	++

[a,b,c,d] See footnotes to Table I.

quantitative studies; in fact, with somewhat more sophisticated equipment, more quantitative methods for measuring corneal sensitivity are available (11). However, the experiments performed were reproducible when repeated on the same rabbits and do provide a basis for judging the effectiveness of various local anesthetics as well as a means of making some overall observations as to changes in general characteristics of the precorneal portion of the eye while under the influence of these drugs.

Determination of Lacrimal Fluid Turnover Rate and Instilled Volume Drainage Rate in Rabbits under Topical Local Anesthesia—Varying numbers of drops of 0.5% tetracaine hydrochloride were instilled into the eyes of rabbits at 1-min intervals. Following a lapse of 10 min after the last drop of local anesthetic, 25 μl of radioactive technetium suspension was instilled. By monitoring the decline in technetium in the tear film, it is possible to determine tear turnover and drainage rates as previously described (1, 12). Table III shows the data obtained for lacrimal turnover rates following various numbers of drops of 0.5% tetracaine hydrochloride as well as after 1 drop of 0.5% proparacaine hydrochloride.

Analysis of the data reveals that both tetracaine hydrochloride and proparacaine hydrochloride caused a significant decrease in the normal turnover rate of the lacrimal fluid, indicating decreased tear production. Moreover, the tetracaine data reveal a dose dependency in that the larger number of drops resulted in a greater decrease in tear production than the smaller number of drops. In addition, tetracaine hydrochloride had a more pronounced effect in reducing the turnover rate than proparacaine hydrochloride at a given dose level. This observation is consistent with the results of the previous section that the duration of action and the duration of deep anesthesia were greater with tetracaine hydrochloride than with proparacaine hydrochloride.

As stated in Table III, the turnover rates are calculated by multiplying the observed first-order rate constants by the lacrimal volume of 7.5 μl. Rabbits under general anesthesia were found to accommodate a larger lacrimal volume of about 12 μl (1). It is not unreasonable to assume that this may also be the case with rabbits under topical local anesthesia. If so, the drop in turnover rate in Table III would not be as large in magnitude as reported, but it would still be a significant decrease.

Table IV shows the data obtained for the drainage rate constants following a 25-μl instilled dose of technetium. As was the case with turnover rates, tetracaine hydrochloride was able to reduce substantially the drainage rate of an instilled solution. The trend is that the greater the number of drops, the more pronounced is the effect. Also, 1 drop of tetracaine hydrochloride had a significantly greater effect in decreasing drainage than did a similar dose of proparacaine hydrochloride. In fact, a single drop of proparacaine hydrochloride had no effect in decreasing drainage of a 25-μl instilled dose of technetium suspension when compared with the data for fully awake unanesthetized rabbits (1).

Table III—Turnover Rate of Lacrimal Fluid in Rabbits following Various Doses of Local Anesthetics

Number of Drops of Local Anesthetic Instilled	Turnover Rate[a], μl/min	Standard Error of Mean	Number of Determinations
Tetracaine hydrochloride, 0.5%			
0[b]	0.66	0.03	—
1	0.20[c]	0.02	7
2	0.19[c]	0.04	11
3	0.13[c]	0.04	5
4	0.11[c,d]	0.02	4
5	0.10[c,d]	0.02	7
Proparacaine hydrochloride, 0.5%			
1	0.27[c]	0.04	4

[a] Obtained by multiplying the observed first-order rate constant times the lacrimal volume, found to be 7.5 μl in fully awake unanesthetized rabbits (1). [b] Refers to data obtained in fully awake, unanesthetized animals (1). [c] Statistical analysis of these values using the Student *t* distribution (95% level) shows them to be significantly different from the value for unanesthetized animals. [d] Statistically different (95% level) from the values for 1 and 2 drops.

Table IV—First-Order Drainage Rate Constants for a 25-μl Instilled Volume of Technetium Suspension following Various Doses of Local Anesthetics Using the Nonsampling Technique

Number of Drops of Local Anesthetic Instilled	First-Order Drainage Rate Constant, min^{-1}	Standard Error of Mean	Number of Determinations
Tetracaine hydrochloride, 0.5%			
0[a]	0.54	—[b]	3
1	0.39[c]	0.04	7
2	0.35[c]	0.06	9
3	0.42[c]	0.12	4
4	0.12[d,e]	0.05	3
5	0.06[d,e]	0.02	4
Proparacaine hydrochloride, 0.5%			
1	0.73[c]	0.10	3

[a] Refers to data obtained in fully awake, unanesthetized animals (1). [b] Reported to be within ±5% of the mean (1). [c] Statistical analysis of these values using the Student *t* distribution (95% level) shows no significant difference from the value for unanesthetized animals; however, on the 90% level, the value for 1 and 2 drops of 0.5% tetracaine is significantly different from the value for unanesthetized animals. [d] Statistical analysis of these values using the Student *t* distribution (99% level) shows them to be significantly different from the value for unanesthetized animals. [e] Statistically different (90% level) from the values for 1, 2, and 3 drops.

Table V—Schirmer Test in Rabbits

	Wetting in 5 min, mm	Standard Error of Mean	Number of Eyes Tested
Schirmer Number 1 test	14	1.10	14
Basic secretion test	5.6[a]	0.89	13
5 Drops of 0.5% tetracaine hydrochloride	3.9[a]	0.75	12

[a] No significant difference between these values (Student t distribution, 95% level).

The standard errors for some values in Table IV appear to be quite large; however, this is not unexpected due to the nature of the experiments; *e.g.*, time taken to position the probe (1) is critical, and there is a large potential for individual variations in the drainage apparatus among different rabbits.

Schirmer Number 1 and Basic Secretion Tests—The Schirmer Number 1 test and the basic secretion test were performed in rabbits as described in the *Experimental* section. In addition, the Schirmer test was performed following 5 drops of 0.5% tetracaine hydrochloride, the strips being inserted 10 min after the last drop of tetracaine (Table V).

In humans, a measurement of 15 mm of moistened area in 5 min in the Schirmer Number 1 test is regarded as a standard for normal tear production. The value of 14 mm in rabbits reported here is quite comparable to humans. Both the basic secretion test and the test performed following 5 drops of 0.5% tetracaine hydrochloride showed significant dropoffs in the tear production from the Schirmer Number 1 test; however, neither test showed as large a percentage decrease in tear production as was obtained in the technetium studies.

Effect of Topical Anesthesia on Aqueous Humor Levels of Pilocarpine Nitrate—Table VI shows data obtained when 25 μl of a 10^{-2} M solution of tritiated pilocarpine nitrate was instilled into the eyes of rabbits, both in the presence and absence of 0.5% tetracaine hydrochloride. Significantly greater aqueous humor levels of pilocarpine nitrate were obtained in the presence of topical anesthesia. In fact, computer analysis of these aqueous humor concentration–time profiles reveals the area under the curve for the experiments in the presence of tetracaine hydrochloride to be 2.5 times greater than in the absence of tetracaine hydrochloride, indicating that topically applied local anesthetics can indeed affect the bioavailability of an instilled drug solution. The pharmacokinetic analysis further revealed that the peak aqueous humor concentration in both cases occurred at 25 min and that the absorption and elimination rate constants were essentially unchanged by the use of the local anesthetic.

Table VI—Aqueous Humor Concentration of Pilocarpine Nitrate at Various Times Postinstillation from 25 μl of a 1×10^{-2} M Topically Applied Solution in the Presence and Absence of Topical Anesthesia

Minutes	Pilocarpine Nitrate in Aqueous Humor, μg/ml	Instilled Pilocarpine Nitrate Present, %	Number of Eyes
No Topical Anesthetic			
5	0.36 ± 0.03[a]	0.11	10
10	0.85 ± 0.13	0.25	8
15	0.68 ± 0.06	0.20	9
20	1.20 ± 0.10	0.36	12
30	0.66 ± 0.06	0.20	12
60	0.67 ± 0.05	0.20	7
90	0.24 ± 0.02	0.07	7
5 Drops of 0.5% Tetracaine Hydrochloride			
5	0.52 ± 0.03	0.16	8
10	1.89 ± 0.08	0.56	6
15	2.29 ± 0.22	0.69	9
20	2.64 ± 0.20	0.78	10
30	2.73 ± 0.15	0.81	10
60	1.44 ± 0.11	0.43	6
90	0.62 ± 0.10	0.18	8

[a] Refers to standard error of the mean.

DISCUSSION

Physiological and Clinical Implications of Influence of Local Anesthesia on Tear Dynamics—Boberg-Ans (11) examined the influence of retrobulbar injection of local anesthetics in humans on corneal sensitivity and tear production. His findings appear to substantiate the results reported here with regard to the dose dependency of the effects of local anesthetics on tear production. While the results cannot be compared quantitatively due to the differences in experimental procedure, the qualitative similarity of the effect lends validity to the use of rabbits in such studies. Boberg-Ans made no attempt to correlate the effects of local anesthesia on tear production with any possible influence on topical drug bioavailability in the eye.

Topical anesthetics used chronically may inhibit regeneration of the corneal epithelium (13, 14). In severe cases, such use can even lead to permanent reduction in visual acuity (15). Moreover, it seems that clinically the most effective topical anesthetics are those that are also the most toxic systemically (16). Computer analysis of the pilocarpine aqueous humor concentration *versus* time profiles reported here indicate that peak aqueous humor concentrations occur at about 25 min postinstillation both in the presence and absence of topical anesthesia. In addition, both the absorption and elimination rate constants are essentially the same in both the unanesthetized and anesthetized cases. The constancy of both the peak times and the absorption rate constants suggests that corneal damage is not responsible for the increased aqueous humor levels of drug, supporting the proposed mechanism that the increased drug levels are due to decreased tear production and/or instilled solution drainage.

When treating various eye disorders with some topically applied drugs, particularly antibiotics, many ophthalmologists commence therapy with a very large loading dose followed by smaller doses at regular intervals (17). The use of topical anesthetics prior to the initial dose would enable this initial dose to be reduced significantly with no resultant loss of drug at the active site. In this way, the benefit of a high drug titer at the target area can be achieved while minimizing the possibility of toxic side effects due to drug loss to the drainage apparatus.

The basic secretion test is used clinically to differentiate the function of the "basic secretors" from the "reflex secretors." The results reported here with regard to the Schirmer Number 1 test and the basic secretion test in rabbits seem to parallel the results in humans. This finding tends to support the use of rabbits as a model in studies to determine the effects of tears on drug movement in the eye.

However, the findings raise some serious problems with regard to the validity of these tests. First is the obvious need to standardize the concentration, type, and dosage of local anesthetic used since the degree of inhibition of tear production is a function of these variables. Second is the question of the mechanism of tear production itself. The major premise of the basic secretion test is that the local anesthetic will inhibit only the reflex secretors, thereby permitting a measurement of the function of the basic secretors. The basic secretors are thought to contribute approximately 50% to the normal tear output (18). Within the quantitative limits of this test, the basic secretion test in rabbits roughly supports this assumption since a 60% reduction was obtained. However, in rabbits, with 5 drops of tetracaine hydrochloride, using the more quantitative technetium dilution technique, an 85% reduction in tear production was obtained. There are several possible explanations for this finding:

1. The Schirmer method, being rather qualitative in nature, is not precise enough to enable one to make definitive statements about the degree of tear production.
2. The basic secretion test does not employ a high enough level of anesthesia to inhibit all reflex secretors completely. Therefore, the test does not give a true measure of basic secretion.
3. Local anesthetics do not selectively decrease only the reflex secretors but decrease all tear-producing glands to a very low level.
4. Local anesthetics shut off reflex secretors at a low level due to their effect on the efferent, parasympathetic nerve supply; then at higher levels they affect the basic secretors by exerting a local ef-

fect on the various glands. The reason for not being able to shut off the secretions completely is the difficulty of reaching all of the glands involved in basic secretion. This could explain the rapid dropoff in tear turnover seen with low levels of topical anesthetic, *i.e.*, inhibition of the reflex secretors, followed by a very slow decline with higher anesthetic levels due to a local effect on the basic secretors.

All of these points raise questions with regard to the understanding of the mechanism of tear production.

Finally, an important point should be made concerning the use of topical anesthetics as a tool to increase ocular drug bioavailability. The finding is that it is possible to improve drug bioavailability in the eye by chemically reducing tear turnover and instilled solution drainage. Thus, while local anesthetics are probably not the optimum agents to use, they do support the concept.

Effects of Tear Turnover and Instilled Solution Drainage on Amount of Drug Available for Absorption from an Instilled Solution—To illustrate the enormous effect that tear turnover and drainage can have on the amount of an instilled drug solution available for absorption, some simple calculations were made using some modest approximations. For the first 5 min after instillation of a drug solution, drainage is the primary factor responsible for drug loss from the precorneal pocket (1). In rabbits, at approximately 5 min postinstillation, the total precorneal volume has returned to the normal lacrimal volume; at this point, tear turnover accounts for loss of drug from the lacrimal fluid. The lacrimal fluid turnover rate of 0.66 μl/min divided by the normal lacrimal fluid volume of 7.5 μl gives the first-order turnover rate constant of 0.088 min^{-1}.

After 5 drops of 0.5% tetracaine hydrochloride, the corresponding apparent turnover rate constant is reduced to 0.013 min^{-1}. This is referred to as an apparent rate constant since if under topical anesthesia the eye can hold a larger than normal volume, as is the case in general anesthesia (1), the rate constant would be even smaller than reported here. The drainage rate constants in the absence and presence of tetracaine hydrochloride are 0.54 and 0.06 min^{-1}, respectively.

Using these rate constants, the amount of drug remaining in the lacrimal fluid at any time can be calculated. For example, suppose 25 μl of a 1×10^{-2} *M* pilocarpine nitrate solution was instilled into the eyes and, for purposes of simplicity, it is assumed that no drug absorption occurs. This corresponds to instilling 67.82 μg of pilocarpine nitrate. After 5 min in the absence of topical anesthesia, 4.56 μg of drug remains; after 5 drops of tetracaine hydrochloride, 50.24 μg remains. Similarly, after 20 min, 1.22 and 41.13 μg remain, respectively. From such calculations, it is obvious that the use of topical anesthesia results in a tremendously greater amount of drug potentially available for absorption into the aqueous humor.

The aqueous humor concentration *versus* time profiles for tritiated pilocarpine nitrate in the presence and absence of topical anesthesia confirm the influence of tear turnover and instilled solution drainage on aqueous humor levels of instilled drug solutions. The increase is not as large as might be expected from the differences of drug remaining in the tear film, but this can possibly be explained due to nonproductive scleral absorption and possible adsorption of drug on the lids and other eye tissues. This aspect of drug loss from the eye is currently being investigated.

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Practical Kinetics I: Quantitation of Barbiturate Stability by TLC

H. V. MAULDING ˣ, J. POLESUK, and D. ROSENBAUM

Abstract □ The purpose of this work was the application of established techniques to the quantitation of stability investigations of liquid formulations as well as in solution kinetic studies. The process consists of streaking aliquots of reaction solution on TLC plates, development, elution, and analysis of the substances in the eluate. Several barbiturates were investigated regarding their decomposition at elevated temperatures. Reasonable correlation is noted between obtained rate constants and literature data. This technique represents a utilitarian approach to stability screening of compounds in solution, aqueous or otherwise, where chromatographic separation and analytical methodology for the pure compound are available.

Keyphrases □ Barbiturates—stability, quantitation by TLC □ Stability—barbiturates, quantitation by TLC □ TLC—monitoring and quantitating barbiturate stability

This report outlines a rather general method for studying the kinetics of a wide variety of chemical compounds. It obviates the necessity for time-consuming development of analytical methodology where complications arise such as product interference with the analysis of intact substances. Although the basic method is well known, this report shows its general applicability to the formulating pharmacist and routine stability studies.

The two prerequisites for implementation of the method are: *(a)* an available assay for a solution of the starting material, and *(b)* a TLC separation of reaction product or products from the parent compound. These kinetic studies, utilizing quantitative TLC, have been carried out on diverse chemical classes with success in all of the cases considered. No particular expertise appears necessary beyond the normal requirements of technical work.

This method is well suited for evaluation of the stability parameters of solution and parenteral formulations, thus making it of particular value to the developmental pharmacist. It offers the advantage of being relatively inexpensive and within the price range of most laboratories, since the only equipment required is a plate streaker and syringe for spotting. Techniques such as spectrophotodensitometry and high-pressure liquid chromatography might serve the same purpose but both entail a much larger financial expenditure.

The technique is not a panacea and suffers from some inherent limitations. Compounds such as erythromycin, which defy chemical analysis in solution, are not readily examined by this method, nor are substances that are not easily separated by TLC from degradation products such as tetracycline. The method is somewhat tedious in that care and precautions must be taken to prevent loss in scraping, eluting, *etc.*

Several barbiturates were scrutinized in this work since this group of medicinal compounds is of considerable importance. Additionally, this class has sufficient published data available for comparisons regarding their degradation rate constants at various temperatures and pH values (1–4).

EXPERIMENTAL[1]

TLC—The general procedure utilizing thiamylal is given, but other barbiturates were separated by the same system. Solutions were made of the barbiturates in various buffers and sodium hydroxide solutions. A solution of 2 mg/ml thiamylal sodium was heated at 80.4 ± 0.1° in a constant-temperature oil bath. Samples were withdrawn periodically from the reaction solution, and 0.25 ml was streaked[2] on silica gel fluorescent plates[3] with occasional air drying to confine the bandwidth. Full plates were streaked[4] with a sample on each half of the normally scored plate. The development solution was isopropyl alcohol–chloroform–ammonium hydroxide (9:9:2), with a running distance of 15 cm (5).

The barbiturate, R_f 0.8, was visualized by short wavelength UV light (255 nm). Products could be observed on treatment with iodine vapor, followed by heating to remove the vapor. The band of silica gel containing the barbiturate was marked under the UV light, scraped by microspatula, and collected. The silica gel containing the barbiturate was washed with portions of 0.1 *N* NH_4OH to 50 ml in a volumetric flask, followed by filtration through a sintered-glass filter. In lieu of filtration, the silica gel was centrifuged with 50 ml of 0.1 *N* NH_4OH.

Spectrophotometric Analysis—The clear supernate from the centrifuged silica gel, or the solution following filtration, was read in the UV against a blank prepared from a corresponding blank plate treated in the same manner as the chromatograph containing the barbiturate. Solvent blanks could also be used with little discrepancy in results. Samples were read[5] in standard 1-cm cells with scans from 350 to 220 nm.

pH Measurements—The pH values of the sodium hydroxide solutions used were calculated from activity coefficient data available in the literature (6); other pH values were determined on a pH meter[6] standardized at the specified temperatures, utilizing phthalate and borate buffer solutions (7).

RESULTS AND DISCUSSION

A readily applicable stability screening method is of considerable value to the solution or parenteral formulator. Utilization of the presented technique, which represents a synthesis of well-established procedures, may save the time required for development of analytical methodology in situations where interactions or interferences exist or where the molecules do not lend themselves to routine techniques. Previously, the tendency with these substances has been to follow the rate of decomposition visually on TLC plates as a function of time at several pH values and temperatures. This qualitative type of analysis, while giving an estimation, does not allow arrival at legitimate quantitative data.

[1] The barbiturates were supplied by Ganes Chemical Co., Carlstadt, N.J., with the exception of thiamylal sodium, which was obtained from Parke, Davis and Co., Detroit, Mich. The substances were used "as is" with no further purification. All other materials employed were of analytical reagent grade.

[2] Streaked by means of a 0.5-ml syringe, Hamilton Co., Whittier, Calif.

[3] Silica Gel 60 F-254, 0.25-mm plates, 20 × 20 cm, EM Labs. Inc., Elmsford, N.Y.

[4] Plate holder for quantitative TLC made by Applied Science Laboratories Inc., State College, Pa.

[5] Cary 14 or 11 recording spectrophotometer.

[6] Metrohm.

Table I—Observed or Apparent First-Order Rate Constants (k in seconds^{-1} × 10^6) for Decomposition of Some Barbiturates (2 mg/ml) in Aqueous Solution at 80.4°

Barbiturate	Buffer	pH[a]	λ_{max}[b], nm	Rate Constant from TLC Plate Elutions[c]	Rate Constants from the Literature[d]
Thiamylal	Phosphate[e]	7.5	255	11.9	11.2
Thiamylal	Borate[e]	10.1	305	54.8	47.2
Thiamylal	0.1 *N* NaOH	11.5	305	276	257
Phenobarbital	0.033 *N* NaOH	10.99	240	152	171
Phenobarbital	0.1 *N* NaOH	11.5	240	302	363
Phenobarbital	0.1 *N* NaOH	11.5	240	311[f]	363
Butalbital	Phosphate	8.5	240	3.9	4.1
Butalbital	0.05 *N* NaOH	11.2	240	49	52
Butalbital	0.1 *N* NaOH	11.5	240	91	87
Amobarbital	Borate	8.0	240	6.5	6.7
Amobarbital	0.1 *N* NaOH	11.5	240	83.7	90.5

[a] Description of methods of pH measurement are given under *Experimental*. [b] Measured in 0.1 *N* NH_4OH. [c] Plates were run after spotting with 0.25 ml of solution and were eluted with 50 ml of 0.1 *N* NH_4OH. See *Experimental*. Runs were carried out in duplicate at least by two different operators on different days. Results are averages of the kinetic runs done in each case. [d] References 1 and 3. Temperature = 80°. [e] See Refs. 1 and 3 for preparation of buffers. [f] Run was carried out on 0.50 g phenobarbital/liter or one-fourth of normally used concentration.

Analytical problems may be circumvented with subsequent timesaving by elution of compounds from properly spotted thin-layer plates followed by classical analysis. This procedure has been employed successfully in this laboratory, although little specific documentation is found in the literature. However, it has been applied only to compounds of molecular weight up to 520. Undoubtedly, difficulties may be expected with higher molecular weight, more complex organic molecules.

Since first-order processes are approximated due to control of reaction parameters, absolute values regarding concentration terms are not necessary. As long as the same volumes of solution are spotted each time, apparent first-order kinetics will hold. The syringe used fulfilled this condition by allowing streaking of the plate with equivalent volumes of each sample.

Table I lists some barbiturates and the conditions under which they were studied. The preponderance of the work was carried out at ~80° due to the reaction velocities and general convenience of this temperature with these compounds. The correlation may be noted between the rate constants found experimentally and those previously derived by classical solution kinetics.

A statistical comparison of the two sets of data in Table I is negated because the temperature reported was 80° (2, 3) while these investigations were carried out at 80.4°.

Table II gives a typical set of runs with thiamylal carried out by three different operators. These runs were done on different solutions on different days. The data gave a standard error of 7 with a 95% confidence interval of 262–290 sec^{-1} × 10^6 for the rate constant k.

Figure 1 illustrates a typical absorbance loss of thiamylal in 0.1 *N* NaOH as a function of time. Experiments were generally carried out for two or more half-lives.

Figure 2 gives some first-order plots for thiamylal at pH 10.1 and in 0.1 *N* NaOH at 80.4° at both 255 and 305 nm. The same slope is observed for both wavelengths under each condition.

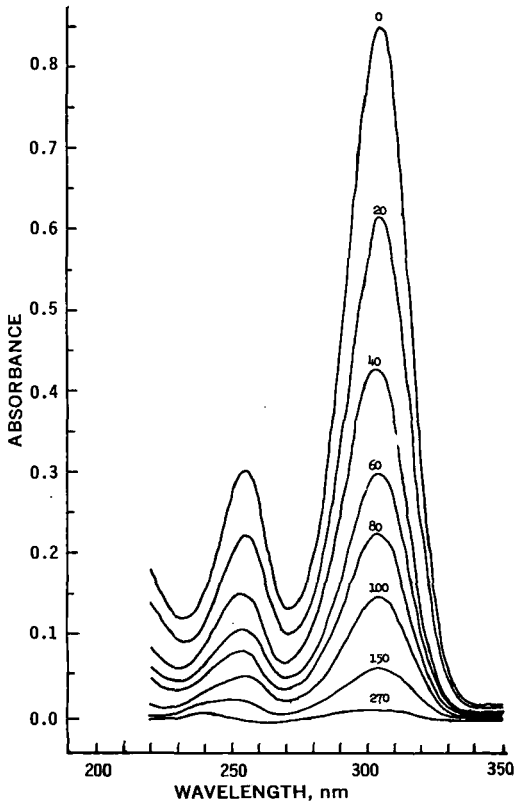

Figure 1—*Spectral change for hydrolysis of thiamylal sodium (2 mg/ml) in 0.1 N NaOH at 80.4° with time in minutes (listed on graph). Intact thiamylal was eluted from plates and read in 0.1 N NH_4OH.*

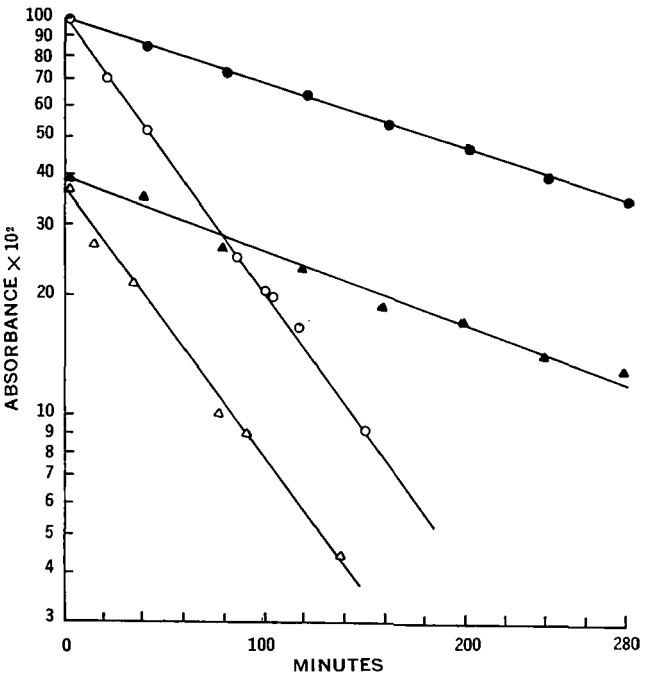

Figure 2—*Apparent first-order plots for hydrolysis of thiamylal sodium (2 mg/ml) in 0.1 N NaOH and at pH 10.1 and 80.4°. Rate constants were determined from absorbance values obtained at 255 and 305 nm. Thiamylal was extracted from silica gel thin-layer plates and read in 0.1 N NH_4OH. Key: ●, pH 10.1, 305 nm; ▲, pH 10.1, 255 nm; ○, 0.1 N NaOH, 305 nm; and △, 0.1 M NaOH, 255 nm.*

Table II—Rate Constants for Hydrolysis of Thiamylal[a] in 0.1 *N* NaOH at 80.4°

Run	k, $sec^{-1} \times 10^{6}$ [b]
1	291
2	288
3	284
4	263
5	256
Average	276[c,d]

[a] Thiamylal was read in 0.1 *N* NH_4OH at 305 nm (255 nm gives similar values). Initial concentration was 2 g/liter. [b] Results were determined by three operators at different times. [c] $SD = \sqrt{(X_i - \bar{X})^2/(n-1)} = 16$, $SE = SD/\sqrt{n} = 7$. [d] The 95% confidence interval = 262–290 $sec^{-1} \times 10^6$.

Barbiturates function as excellent models because they break down into several compounds—malonuric acids, acetic acids, urea, acylureides, diamides, *etc.* (2–4, 8–11). These products are easily separated from the parent molecule by the thin-layer system used (5). With some barbiturates, only the parent compound may be seen on fluorescent plates under short wavelength UV light. Other compounds may be observed using iodine vapor, followed by removal of the color on heating the TLC plates (2). Urea must be visualized in another manner (2). The anionic form of the pyrimidine ring system is easily extractable from the TLC plates into 0.1 *N* NH_4OH, which serves as a suitable solvent for many anionic substances. Methanol, methanolic hydrochloric acid, and 0.1 *N* HCl serve as eluants for amines and neutral molecules adsorbed onto silica gel.

Figure 3 shows absorbance diminution as a function of time for a 2-mg/ml solution of phenobarbital in 0.033 *N* NaOH at 80.4°. The spectra represented are typical, and no attempts were made to select the best curves for representation.

Several plots of log absorbance *versus* time are given in Fig. 4. This figure is a good illustration of the results obtained from the thin-layer plates for the various barbiturates. The four barbiturates examined exemplify most types currently used in therapy with regard to the nature of substituents at positions 2 and 5. There is no reason to expect that all barbiturates normally utilized medicinally cannot be analyzed in this manner.

Problems can arise in these studies when the substrate will not dissolve in amounts of 1–2 mg/ml, as occurs with barbiturates as the pH approaches pKa (pH ≈ pKa). In such cases, cosolvents may be used. Pseudo- or apparent first-order kinetics hold well in these concentrations (2 mg/ml, about 8×10^{-3} *M*) in the cases investigated. Phenobarbital was run in 10^{-4} *M* solution (Table I) with success, although working at this concentration was tedious as well as somewhat inconvenient.

When syrups containing relatively large quantities of sugars and/or polyhydric alcohols are analyzed, it may be best to extract the active compound from the aqueous solution into a suitable solvent for spotting (*i.e.*, chloroform, ether, *etc.*) due to difficulties encountered in spotting and poor running characteristics of chromatograms of syrups spotted directly without prior extraction.

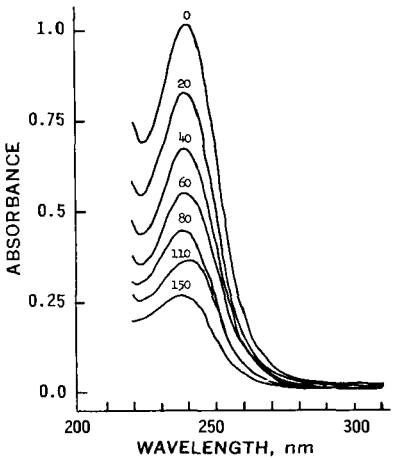

Figure 3—*Absorbance diminution of phenobarbital sodium (2 mg/ml) in 0.033* N *NaOH at 80° with time in minutes (listed on graph). Intact phenobarbital was extracted from silica gel thin-layer plates and read in 0.1* N *NH_4OH. See* Experimental.

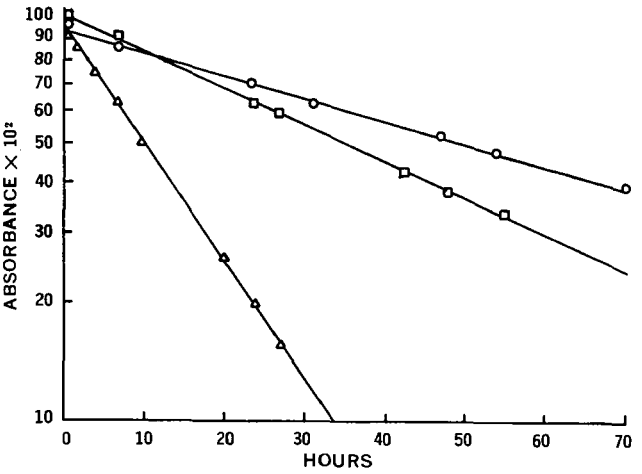

Figure 4—*Apparent first-order plots for hydrolysis of barbiturate (2 mg/ml) as a function of time. Reaction was followed by loss of absorbance at specified wavelengths (Table I). Samples were eluted from silica gel thin-layer plates as discussed in* Experimental. *Temperature was 80.4°. Key:* ○, *butalbital sodium, pH 8.5;* □, *amobarbital sodium, pH 8.5; and* △, *thiamylal sodium, pH 10.1.*

The quality of TLC plates used is critical. The plates specified under *Experimental* work satisfactorily. Problems can arise in the 230–260-nm range, because many plates tend to contain impurities that absorb in this spectral region upon elution. At higher wavelengths, most commercial plates are acceptable. When absorbance occurs, it may be circumvented by predeveloping the plates prior to spotting. Caution must be taken when streaking the plate to prevent scoring which interferes with proper development. The barbiturates generally ran to R_f 0.7–0.8; but when lower amounts were streaked (0.134 mg relative to the normally used 0.5 mg), the barbiturate phenobarbital ran considerably higher than normal, R_f 0.9+.

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Practical Kinetics II: Quantitation of Procaine Stability by TLC

H. V. MAULDING ˣ and A. F. MICHAELIS

Abstract □ This work illustrates further application of TLC as a tool in kinetic investigations of organic molecules. The procedure consists of: (*a*) streaking or spotting a known volume of reaction solution on thin-layer plates, (*b*) developing the plates, (*c*) eluting the compound under study from the adsorbent, and (*d*) assaying the material in solution. Procaine was examined regarding its hydrolysis at above ambient temperatures. The reaction was monitored spectrally and by the TLC technique. Velocity constants derived by the two methods were similar in value. Rate constants obtained from procaine samples eluted from silica gel plates following development were in good agreement with those previously reported in the literature.

Keyphrases □ Procaine—quantitation of stability by TLC, rate constants □ Hydrolysis—quantitation of procaine stability by TLC, rate constants □ Stability, procaine—TLC determination □ TLC—determination, procaine stability

Further utilization of quantitative TLC as a technique for rapid and accurate kinetic screening of solutions is reported. The method is broadened to include basic and cationic compounds whereas previously acidic or anionic substances, the barbiturates, were surveyed.

The same general approach is extended to a basic compound, procaine, by means of alterations of the elution solvents. Procaine was used as a prototype due to its medicinal importance as well as the relatively large amount of data regarding the rate constants of its decomposition available (1–3).

This amino ester, pKa_1 8.95, is hydrolyzed in solution, with consequent production of *p*-aminobenzoic acid and diethylaminoethanol. The examined solutions were an order of magnitude below those normally employed medicinally. The commercial article could be analyzed following a dilution.

EXPERIMENTAL

TLC—Procaine hydrochloride (mp 152–155°) was found chromatographically [methanol–ammonia (99:1)] to contain only traces of *p*-aminobenzoic acid. This substance was used without further purification. The methanol–ammonia solvent system was used to develop kinetic samples with a running distance of 15 cm on silica gel fluorescent plates[1]. Procaine, R_f 0.7–0.8, and *p*-aminobenzoic acid, R_f 1.0, were visualized under short wavelength UV light (255 nm). This system is an adaptation of a previously reported (4) solvent system.

Kinetic Studies—A solution of procaine hydrochloride (2 mg/ml) was prepared in the appropriate buffer solution. The samples were placed in constant-temperature baths (±0.1°), and aliquots were periodically withdrawn by pipet as the reaction progressed. A 0.25-ml[2] portion of the reaction solution was streaked[3] on full-sized (20 × 20 cm) silica gel fluorescent plates[1], with two samples placed one on each half of a manually scored plate. Forced air drying was used to confine the bandwidth as the plate was streaked.

The plate was developed in methanol–ammonia (99:1), and the band of silica gel containing the starting material was marked under UV light. The silica gel containing procaine was removed by scraping with a microspatula and collected. This material was extracted with 10 ml of methanol followed by pH 6 phosphate buffer to make 50 ml. The solid particles of silica gel were removed by filtration through a sintered-glass filter followed by analysis of the solution.

Analysis of Solutions of Procaine from TLC Plates—The methanol–pH 6 buffer from the preceding elution of the thin-layer plates was read on a UV recording spectrophotometer[4]. The absorbance was read at 289 nm (±1) against the appropriate blank.

UV Analysis—Samples were taken concomitantly with those used for the TLC assay. The aliquots were diluted to volume with the appropriate buffer, with the absorbance values taken as a function of time at 289 nm (±1). The UV analyses of intact procaine were carried out directly, without need for separation, at 289 nm following pH adjustment to 9.5. This was possible due to the disparity in molar absorptivities of procaine and *p*-aminobenzoate anion, with values of 7.5 and 17.5 × 10^3 liters/mole cm, respectively (1).

The equation employed was:

$$\log (A_t - A_\infty) = \log (A_0 - A_\infty) - kt/2.303 \quad \text{(Eq. 1)}$$

where A_t is absorbance at time t, A_∞ is the absorbance at infinite time (*i.e.*, *p*-aminobenzoate ion), and A_0 is the initial absorbance or that of procaine at time = 0.

Buffer Solutions and pH—Ammonia buffers were prepared following the general directions of Higuchi *et al.* (1), and borate buffers were made as previously specified (5) (Table I).

The pH measurements were carried out on a pH meter[5] standardized at the specified temperatures by borate and phthalate solutions (6). The pH of the reaction solutions was determined before, during, and following each kinetic run.

RESULTS AND DISCUSSION

This work comprises an expansion of the TLC method as a means for rapidly determining the kinetic parameters of solutions. The technique has been found suitable for formulation screening as well as for classical kinetic investigations. The process depends upon separation of the starting material (usually 0.1–0.5 mg) from products on thin-layer plates, which is possible in theory and generally in practice.

Barbituric acids and their anionic forms were previously shown to be fitted to analysis in this manner. Ramification of the technique is presented to include basic compounds and their cationic forms. The amino ester, procaine, was readily amenable to this treatment. This compound was selected due to the large amount of data regarding the velocity constants and other parameters of its hydrolysis already available (1, 2).

The cleavage of the ester linkage was monitored spectrophotometrically. Procaine was separated from *p*-aminobenzoic acid by TLC with the starting material being eluted from the plates and then read in the UV at 289 nm (±1).

The expression:

$$\log A_t = \log A_0 - kt/2.303 \quad \text{(Eq. 2)}$$

[1] Silica gel 60 F-254, 0.25-mm plates, EM Labs. Inc., Elmsford, N.Y.
[2] Using a 0.5-ml syringe fitted for sieve collar attachment, Hamilton Co., Inc., Whittier, Calif.; Applied Science Catalog No. 17710.
[3] Streaker apparatus made by Applied Science Labs. Inc., State College, Pa.; Applied Science Catalog No. 17700.
[4] Perkin-Elmer 202.
[5] Metrohm.

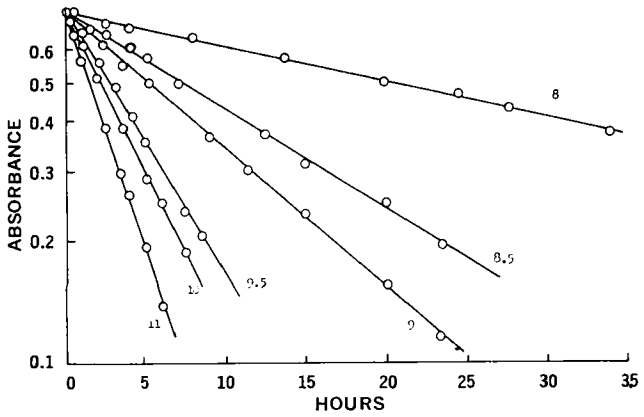

Figure 1—*Decomposition of procaine (concentration of 2 mg/ml at 40°) in aqueous solution as a function of time. Various pH values are indicated on the graph. Analyses were by quantitative TLC.*

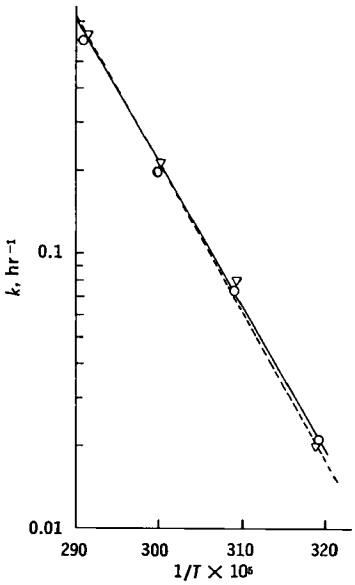

Figure 3—*Temperature dependency for hydrolysis of cationic procaine in pH 8 buffer at 40°. Key: ---▽---, rate constants evaluated by TLC procedure; and —○—, rate constants determined by spectrophotometric analysis.*

was utilized for this pure material, where A_t and A_0 represent absorbances at times t and zero. The term k is the observed or pseudo-first-order rate constant, with t being the time.

The procaine in solution was followed using the equation:

$$\log (A_t - A_\infty) = \log (A_0 - A_\infty) - kt/2.303 \quad \text{(Eq. 3)}$$

where the terms are the same as in Eq. 2 except A_∞, which is the absorbance at infinite time.

Table I lists the observed first-order velocity constants derived from the thin-layer method (Eq. 2) along with those obtained simultaneously by the spectrophotometric procedure (Eq. 3). These are compared to those previously reported by other workers (1, 2).

Kinetic runs were carried out in duplicate for each pH and temperature investigated. The method is quick and applicable from operator to operator, usually in ±10% limits. Once, unacceptable results were found by both the thin-layer and spectroscopic methods. The plots of log concentration, *i.e.*, absorbance, *versus* time resulted in a curved line in both cases. Something apparently was wrong with the solution employed. This was shown to be a loss of ammonia from unsealed flasks containing ammonia–hydrochloric acid buffer with subsequent change (lowering) of pH.

At least seven data points were taken for each run listed in Table I. Table II illustrates five typical runs by the same operator at pH 9.5 and 40° and gives an idea of the reproducibility of the technique.

Problems were encountered with the ammonia buffers. The pH values often changed by as much as one unit at higher pH's during the run. The phenomenon is well known and was previously reported (1). For this reason, borate buffers were found to be much more acceptable in the pH 8–11 region (5). None of the borate buffers exhibited this tendency to change pH during the reaction.

Most runs were carried out for a minimum of two half-lives. Below this point the analytical points tended to show more scatter as assayed by both methods.

The thin-layer separation of reactant and products is a prime

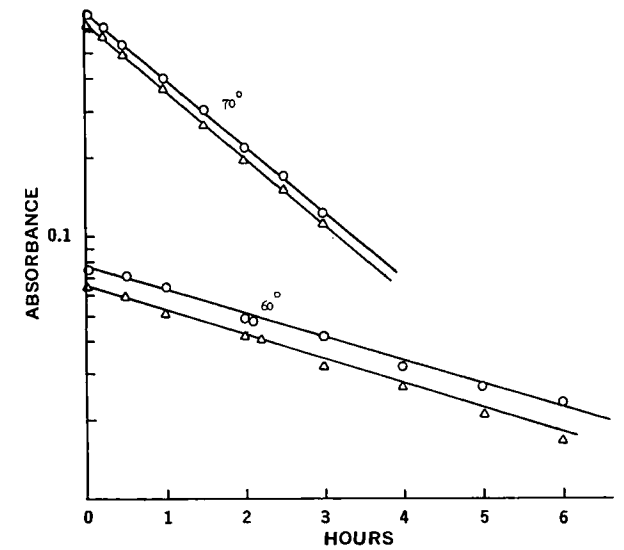

Figure 2—*Comparison of TLC and spectrophotometric results of hydrolysis of 2 mg/ml procaine at pH 8 at 60 and 70°. Absorbance is plotted* versus *time. Key: ○, spectrophotometric absorbances; and △, TLC absorbances. Two "semilog" scales were utilized for convenience in visualization.*

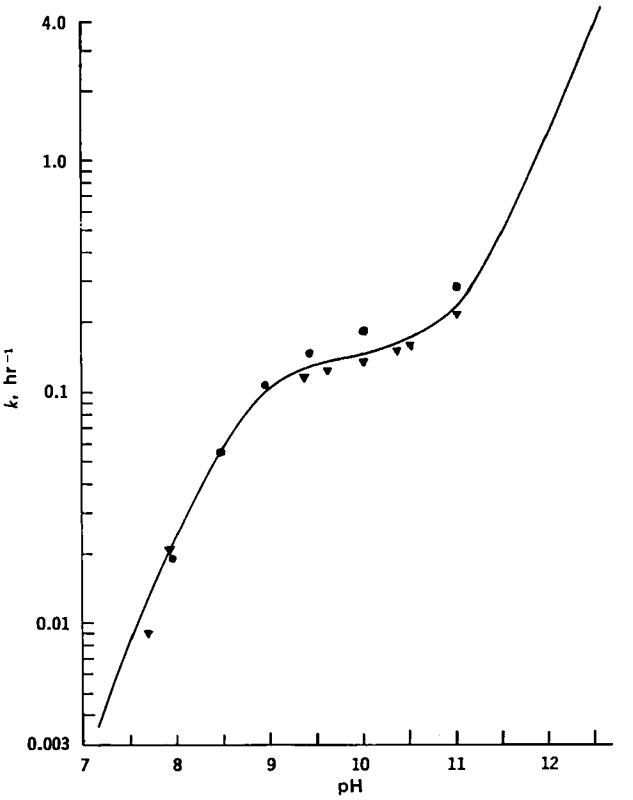

Figure 4—*Log k–pH profile of procaine at 40°. Solid curve represents a plot of the results of Higuchi* et al. *(1). Key: ●, data obtained by Siegel* et al. *(2); and ▼, results obtained from TLC (Table I). Values were determined in this work in borate buffer (Table I).*

Table I—Observed First-Order Rate Constants[a] (k, hr^{-1}) for Hydrolysis of Procaine (2 mg/ml) in Aqueous Solution at Various Hydrogen-Ion Concentrations and Temperatures

Buffer[b]	pH[c]	Temperature	Velocity Constants[d] TLC	Velocity Constants[d] Spectrophotometric	Ref. 1	Ref. 2
Ammonia–hydrochloric acid	7.6	40°	0.007	0.006	0.009	—
Ammonia–hydrochloric acid	8.0	40°	0.018	0.017	0.021	0.018
Borate	8.0	40°	0.021	0.024	(0.021)[e]	—
Borate	8.0	50°	0.079	0.075	(0.073)[e]	—
Borate	8.0	60°	0.21	0.22	(0.21)[e]	—
Borate	8.0	70°	0.63	0.61	(0.64)[e]	—
Borate	8.0	40°	0.020	0.019	(0.021)[e]	—
Ammonia–hydrochloric acid	8.5	40°	0.042[f]	0.046[f]	0.058	0.053
Ammonia–hydrochloric acid	9.0	40°	0.080[f]	0.084[f]	0.105	0.116
Borate	9.0	40°	0.101	0.099	(0.105)[e]	—
Ammonia–hydrochloric acid	9.5	40°	0.09[f]	0.10[f]	0.13	0.15
Borate	9.5	40°	0.12	0.12	(0.13)[e]	—
Ammonia–hydrochloric acid	10.0	40°	0.12[f]	0.12[f]	0.15	0.40
Borate	10.0	40°	0.14	0.15	(0.14)[e]	—
Borate	10.5	40°	0.16	0.17	(0.17)[e]	—
Ammonia–hydrochloric acid	11.0	40°	0.15[f]	0.16[f]	0.21	0.31
Borate	11.0	40°	0.19	0.18	(0.21)[e]	—

[a] Determined following elution of TLC plates with methanol–pH 6 phosphate buffer read at 289 (±1) nm. [b] Borate buffer prepared by adding boric acid to 8 g of sodium chloride in 500 ml of water. When proper pH is attained, add water to 1 liter. Ammonia buffers prepared as in Ref. 1. [c] The pH was measured before, during, and after the kinetic run. [d] Velocity constants taken from same aliquot for TLC and spectrophotometric analysis. [e] Carried out in ammonia–hydrochloric acid buffer in Ref. 1. In this work it was done as indicated in borate. [f] Values low, probably due to pH drop during the reaction of unsealed reaction vessels.

requisite of the procedure. It was effected using the system of methanol–ammonia (99:1) in a modification of a previously reported development solution (4). This separation eliminates the need for development of a differential analytical method, which can pose problems when products interfere, *etc.* The spectra obtained from the eluate should be that of the pure compound, procaine, as was found to be the case.

Procaine was readily removed from the adsorbent, fluorescent silica gel, by means of methanol followed by pH 6 buffer. Methanol and methanol–0.1 *N* HCl or acidic buffers have been generally acceptable for the several amines studied to date.

Care and precaution must be taken when deciding on the solvent for elution of the adsorbent because first trials may not lead to proper extraction. It is best if one spends a day or two removing known amounts of material from the plates and thereby selects the best solvent or solvent system prior to initiation of kinetic studies. Choice of a suitable extraction solvent is sometimes tricky but is possible with all compounds studied to date.

Figure 1 gives typical plots of log absorbance *versus* time derived from various pH values at 40° as done by quantitative TLC.

Figure 2 shows a comparison of data derived from spectrophotometric and thin-layer procedures at different hydrogen-ion concentrations. Close correlation between the rate constants from the two methods is obvious.

An estimate of the apparent energy of activation for the reaction of protonated procaine and hydroxide ion, pH 8, is given in Fig. 3. Utilizing the Arrhenius relationship:

$$\log k = \log P - Ea/2.303RT \qquad \text{(Eq. 4)}$$

where k is the observed first-order rate constant. The plot of log k against $1/T°$ (absolute) gives a straight line, the slope of which is equal to $Ea/2.303R$. The apparent energy of activation, Ea, was 25.6 kcal/mole for the TLC procedure compared to 23.8 kcal/mole from the solution directly (Fig. 3). Higuchi *et al.* (1) derived 24.0 kcal/mole for the same reaction.

Table II—Reaction Velocity Constants for Decomposition of Procaine in pH 9.5 Borate Buffer[a] at 40°

Run	k, hr^{-1}
1	0.11
2	0.13
3	0.12
4	0.12
5	0.13
Average[b]	0.12[c]

[a] Ammonia–hydrochloric acid buffers give somewhat lower values due to change of pH with time. [b] Average of spectrophotometric runs was 0.12. Value from Ref. 1 was 0.13 using ammonia–hydrochloric acid buffer. [c] Rate constants were measured following elution of plates and read at 289 (±1) nm in methanol–pH 6 phosphate buffer. See *Experimental*.

Figure 4 represents a comparison of data obtained from the literature with those in Table I. The continuous solid line records a replot of the results of Higuchi *et al.* (1). This is a graph of the relationship:

$$\log k_{obs} = k_1[OH^-]_{fBH^+} + k_2[OH^-]_{fB} \qquad \text{(Eq. 5)}$$

where f_{BH+} and f_B are fractions ionized and nonionized, respectively; and k_1 and k_2 are rate constants with $pKa_1 \approx 9$.

Various data points for the log k–pH profile are seen along with those obtained in this study. Reasonable correlation may be seen between the three sets of results, with a slight possible error resultant from estimations necessarily made in replotting.

No series defects in the total system were noted during the investigation. One spurious set of data was obtained by both methods examined. One unanswered question was the fact that initial assays by both methods were not always the same absolute values as they should have been assuming homogeneous solution and negligible breakdown. Figure 2 illustrates this matter. No effect on the latter assays was noted; the phenomenon is unexplained, with no aberration in the velocity constants being brought about by this incongruity. The reason for this is that as long as a constant value is streaked, the relative results are the same if the solution is homogeneous.

The TLC procedure is an inexpensive and simple method. No elegant equipment is needed as may be the case with gas or liquid chromatography. The total cost including the plate streaker is less than $500.00. It also possesses the potentiality of being applicable to most compounds.

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Practical Kinetics III: Benzodiazepine Hydrolysis

H. V. MAULDING [x], J. P. NAZARENO, J. E. PEARSON, and A. F. MICHAELIS

Abstract □ The velocity constants for chlordiazepoxide hydrolysis were measured by independent techniques. A quantitative TLC kinetic procedure is compared with an extractive method. The data derived from both processes are in approximate agreement, further exemplifying the feasibility of TLC for rapid stability evaluation of liquid formulations as well as solution kinetic studies. In the extractive procedure, benzodiazepine–substrate was separated from the lactam product by methylene chloride extraction of acidic aqueous solution. The TLC procedure consisted of separation on silica gel plates followed by elution and subsequent analysis. The log k–pH relationship for the hydrolysis representing water addition coupled with expulsion of methylamine is presented. This function is characterized by water and hydroxide-ion attack on monoprotic species along with specific hydrogen-ion catalysis at higher hydronium-ion concentrations, and the rate law for the decomposition of chlordiazepoxide is given. Through several half-times (pH 0.15–11.5, 79.5°), this hydrolytic reaction generating lactam predominated; however, more benzophenone was formed as the pH decreased. Velocity constants were invariant over a 200-fold concentration range. The subsequent acid-facilitated cleavage of lactam to benzophenone was not further investigated. Both general acid catalysis and general base catalysis were evidenced, with borate, acetate, formate, and phosphate buffers accelerating the conversion of chlordiazepoxide to lactam. At pH values below neutrality, nonlinear dependency of the rate constant on buffer concentration was observed. This finding may be explained by a change in the rate-determining step as buffer concentration varied.

Keyphrases □ Benzodiazepines—hydrolysis of chlordiazepoxide, velocity constants, conversion to lactam, TLC and extraction procedures compared □ Chlordiazepoxide—hydrolysis, velocity constants, conversion to lactam, TLC and extraction procedures compared □ Hydrolysis—benzodiazepines, velocity constants for chlordiazepoxide hydrolysis, TLC and extraction procedures compared □ Kinetics—benzodiazepine hydrolysis

This investigation was undertaken to study the kinetics of chlordiazepoxide hydrolysis as well as to compare pertinent rate data concerning breakdown of the compound using both classical and quantitative TLC analytical techniques.

TLC techniques have been utilized in these laboratories for rapid determination of solution stability of diverse classes of medicinal agents. TLC is applicable when separations of reactant–product(s) are available, coupled with satisfactory analytical procedures for eluted compounds.

The benzodiazepines were of interest because they represent prototypes giving mutual substrate–product interference in the UV region, limiting straight UV spectroscopy in their study (1). Therefore, a kinetic study of the hydrolysis was instituted using both quantitative TLC and spectrophotometric (following separation of reactants from products) methods. The aim, besides delineation of the kinetic profile of chlordiazepoxide hydrolysis, was to compare the agreement between techniques.

In the spectrophotometric procedure, the lactam formed by hydrolysis was readily removed from acidic solutions of the parent, chlordiazepoxide, by partitioning into methylene chloride, allowing a comparative means for determination of velocity constants.

EXPERIMENTAL

Materials—Glass-distilled water was used in all experiments. Reagent grade inorganic salts were employed without further purification.

Chlordiazepoxide reference standard[1], 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide reference standard[1], and 2-amino-5-chlorobenzophenone[1,2] were used.

Kinetic Measurements—Stock Solution 1A was prepared containing 0.90 mg/ml chlordiazepoxide. Two milliliters of this solution was pipetted into 200-ml flasks containing 198 ml of appropriate buffer solution previously equilibrated at the specified temperature, giving a final concentration of 2.98×10^{-5} M. Ten-milliliter samples were periodically withdrawn by pipet and then acidified below pH 1.1 by dropwise addition of concentrated hydrochloric acid.

The aqueous acidic solution was extracted with 3 × 10-ml portions of reagent methylene chloride (methylene chloride was discarded) in a separator, and the aqueous layer was read in the UV at 244 (or 305) nm. Residual absorbances were generally less than 5% of the initial readings and were subtracted for calculation of velocity constants. First-order rate constants were readily evaluated from:

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The observed rate constants derived in this manner were generally reproducible within ±10%.

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Table I—Observed First-Order Rate Constants[a,b] (k_{obs} in hr^{-1}) for Hydrolysis of Chlordiazepoxide (2.98×10^{-5} *M* at 79.5 ± 0.1°, Ionic Strength 0.5)[c]

pH[d]	Buffer, Molarity		k_{obs}, hr^{-1}[e]			
	[HCl]					
0.14	1.0			1.51	—	
0.24	0.8			1.14	—	
0.38	0.6			0.91	—	
0.46	0.5			0.82	—	
0.55	0.4			0.64	—	
0.84	0.2			0.36	0.31[f]	
1.12	0.1			0.26	0.20[f]	
1.40	0.05			0.18	0.16[f]	
1.76	0.02			0.14	0.12[f]	
2.05	0.01			0.12	0.11[f]	
	[H_3PO_4]	[$H_2PO_4^-$]	0.25[g]	0.5[g]	0.75[g]	1.0[g]
1.70	0.3	0.2	0.34	0.49	0.61	0.72
1.76	0.25	0.25	0.36	0.48	0.60	0.70
2.0	0.2	0.3	0.31	0.39	0.47	0.57
2.5	0.1	0.4	0.27	0.36	0.43	0.52
	[HCOOH]	[$HCOO^-$][h]	0.1[i]	0.2[i]	0.3[i]	0.5[i]
3.0	—	—	0.28	0.38	0.46	0.65
3.5	—	—	0.25	0.32	0.39	0.53
3.75	—	—	0.25	0.33	0.40	0.55
4.0	—	—	0.23	0.28	0.33	0.38
	[CH_3COOH]	[CH_3COO^-][h]	0.1[i]	0.2[i]	0.3[i]	0.5[i]
4.0	—	—	0.37	0.52	0.67	0.94
4.5	—	—	0.29	0.39	0.47	0.63
5.0	—	—	0.25	0.28	0.32	0.36
5.4	—	—	0.24	0.26	0.29	0.35
	[$H_2PO_4^-$]	[HPO_4^{-2}]				
6.0	0.2875	0.0605	0.29[j]	0.36[j]	0.44[j]	0.53[j]
7.1	0.020	0.0579	0.23[k]	0.24[l]	—	—
7.9	0.006	0.06	0.23[k]	0.22[l]	—	—
	[H_3BO_3]	[NaOH]		0.1[m]	0.5[m]	
8.9	1.0	0.5		0.24	0.27	
10.9	—	0.02		0.23	0.22[f]	
11.5	—	0.10		0.22	0.23[f]	
—	—	Water —		0.18[n]	0.23[o]	

[a] Analyses consisted of acidification with concentrated hydrochloric acid (dropwise) to pH about 1.1 of a 10-ml aliquot of chlordiazepoxide (2.98×10^{-5} *M*) in appropriate buffer solution followed by extraction with 3 × 10 ml of methylene chloride. The aqueous solution was monitored at either 244 or 307 nm. Acidic solutions were extracted directly with methylene chloride and read. [b] The k_{obs} value was reproducible ±10% from run to run. [c] Ionic strength was made to 0.5 *M* with sodium chloride except with 0.5 *M* HCl or where otherwise specified. [d] The pH values of buffers were measured at 79–80° on a Metrohm pH meter standardized at this temperature. The pH of hydrochloric acid and sodium hydroxide solutions were calculated from pH = $-\log r\pm$ [HCl] and pKw = pH + pOH_s where pOH = $-\log r\pm$ [NaOH]. Activity coefficient, $r\pm$, and pKw data were available in the literature (2). [e] Velocity constants were determined at least in duplicate. Values given are averaged. [f] These constants were determined without addition of sodium chloride to 0.5 *M*. [g] Four columns represent $[H_3PO_4]/[H_2PO_4^-]$ at 1/4, 1/2, 3/4, and 1 × molar concentrations of that stated under buffer heading. Buffer was prepared by addition of 1 *N* HCl to 0.5 *M* $NaH_2PO_4 \cdot H_2O$. [h] Formate and acetate buffers were made by dissolving 0.5 *M* NaOH in water followed by addition of formic or acetic acid to the desired pH and then distilled water to 1 liter. [i] Runs made in 0.1, 0.2, 0.3, and 0.5 *M* acetate and formate, respectively. [j] Carried out at 0.35, 0.21, 0.14, and 0.07 *M* with regard to total buffer. [k] Ionic strength = 0.2. [l] Run at 0.5 *M* total buffer. [m] Run in 0.1 and 0.5 *M* with regard to each buffer component. [n] Chlordiazepoxide hydrochloride in distilled water. [o] Chlordiazepoxide base in distilled water.

starting material was marked under UV light.

The silica gel containing the chlordiazepoxide was removed by scraping and extracted with 25 ml of methanol, followed by filtration through a sintered-glass filter. The filtrate was made up to 50 ml and read[6] at 305 or 244 nm. Log absorbance was plotted *versus* time, and k_{obs} values were obtained using Eq. 1. Rate constants were reproducible within ±10%. Residual absorbance was nil at both wavelengths.

The pH determinations were made[7] at specified temperatures standardized with phthalate and borate buffers (pH 4 and 9.2, respectively) (2). The pH value of hydrochloric acid and sodium hydroxide solutions was determined from pH = $-\log a_{HCl}$ and pKw + pOH where pOH = $-\log a_{NaOH}$. The values for the activity coefficients are available from the literature (3).

Buffers were prepared as stated in Table I. Sodium chloride was added to ionic strength 0.5 unless otherwise specified.

Product Analysis—The products of the hydrolysis of chlordiazepoxide are the corresponding lactam and benzophenone and, occasionally, a spot of unknown origin. These are separated from one another readily by TLC, employing *fresh* solutions of benzene–dioxane–ethanol–ammonia (50:44:5:1 or 60:30:5:1). The following R_f values were obtained from two systems:

	50:44:5:1	60:30:5:1
chlordiazepoxide	0.5	0.65
lactam	0.4	0.55
benzophenone	0.95	0.95
unknown	0.7	0.8

These R_f determinations were made by spotting 0.25 ml of a 2-mg/ml solution on silica gel fluorescent plates[5]. The R_f values may change slightly from day to day and run to run but their relationship to each other remains relatively constant.

RESULTS AND DISCUSSION

The chemistry and kinetics of hydrolysis of several benzodiazepines were reported previously (1). The reaction under consideration is the transformation (Scheme I) of neutral or cationic chlordiazepoxide (I or IH^+) to the lactam (II). Further decomposition to

[6] Cary 14 recording spectrophotometer.
[7] Metrohm pH meter.

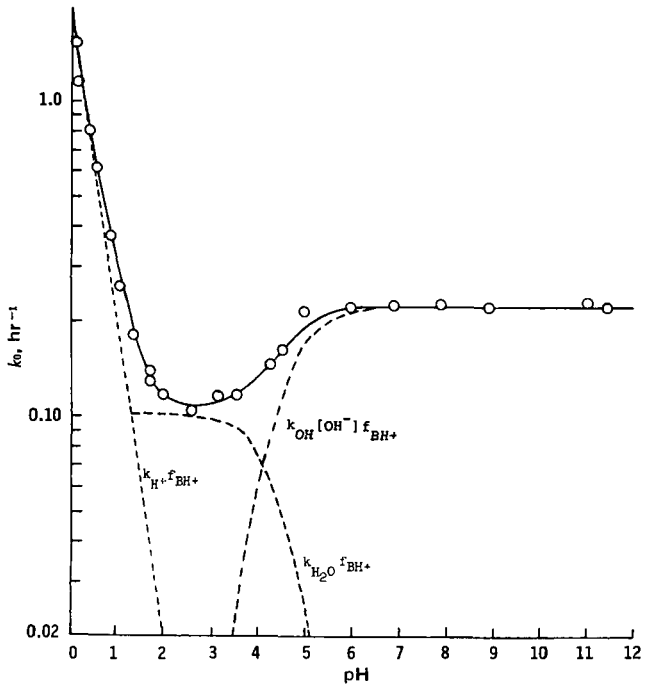


Figure 1—*Rate–pH profile for the hydrolysis of chlordiazepoxide at 79.5° and ionic strength 0.5* M. *Values of* k_0, *except those in hydrochloric acid and sodium hydroxide solution, were obtained from extrapolation of plots of* k_{obs} *against buffer concentration. The circles indicate actual data points or the extrapolated value. The three dashed-line segments are indicative of* k_{H^+}, k_{H_2O}, *and* k_{OH^-}, *with the solid line illustrating a summation of the three.*

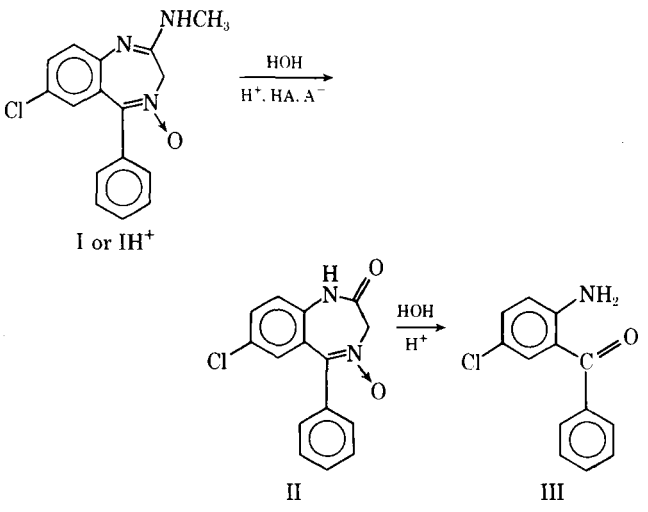
the yellow benzophenone (III) occurred in all of the more acidic reaction mixtures, as previously reported (1).

Intermediates with the diazepine ring opened were reported but were apparently not present in appreciable amounts under the conditions of this study (1).

The benzophenone was not observed to cause interference with the extractive analytical method, because it was separable along with lactam from aqueous acidic solutions containing the starting material.

The hydrolysis of chlordiazepoxide followed strict first-order kinetics in each run. The observed first-order rate constants, k_{obs}, were evaluated utilizing Eq. 1 or:

$$\log(A_t - A_\infty) = \log(A_0 - A_\infty) - k_{obs}t/2.303 \qquad \text{(Eq. 2)}$$



Scheme I

Table II—Effect of Ionic Strength on Hydrolysis of Chlordiazepoxide in Acidic Solution at 79.5°

[HCl]	pH[a]	k_{obs}, hr^{-1} Additional Ionic Strength from Sodium Chloride, M[b] 0.0	0.17	0.43	0.96
0.01 *N*	2.05	0.110	0.113	0.119	0.097
0.02 *N*	1.76	0.120	0.125	0.131	0.134
0.04 *N*	1.49	0.136	0.148	0.158	0.167
0.06 *N*	1.32	0.154	0.173	0.205	0.198
0.10 *N*	1.12	0.207	0.253	0.284	0.312

[a] Measured pH values with hydrochloric acid added where necessary to bring solutions in each set to constant pH value. [b] Total ionic strength for each experiment is total of the concentration of hydrochloric acid in the left column plus additional ionic strength by sodium chloride addition. The exact amounts added were 2, 5, and 11 g of NaCl/200 ml of reaction solution.

where A_t, A_0, and A_∞ represent absorbances at times t, 0, and infinity, respectively.

With the TLC procedure, A_∞ is 0, thus simplifying Eq. 2. Plots derived from Eq. 2 exhibited linearity over three or more half-times, with residual absorbances in the vicinity of 3% of the initial value for the extractive procedure.

The λ_{max} 245 nm was employed for spectrophotometric readings in this work, although the maximum at 305 nm is equally suitable from a theoretical as well as practical point of view.

Rate constants were invariant over a 200-fold chlordiazepoxide concentration range.

Catalysis by Hydrogen Ions and Solvent—The pH dependencies of the observed first-order rate constants, k_{obs}, in the absence of general acids and bases, k_0, is shown in Fig. 1. The k_0 plotted in Fig. 1 was derived from reaction mixtures containing only hydrochloric acid (Tables I and II) or from the intercepts of graphs of k_{obs} *versus* total buffer concentrations of 0.02–0.1 *M* between pH 2.5 and 5.0 (Fig. 2 and Table III). The estimation of k_0 between pH 6 and 11.5 was obtained from sodium hydroxide solutions or by extrapolation of k_{obs} to zero buffer concentration (Table I).

Chlordiazepoxide normally exists in two ionic forms under the conditions studied (4). The log k_0–pH profile (Fig. 1) consists of three portions. These segments are hydrogen-ion-catalyzed hydrolysis of protonated substrate and the interaction of water and hydroxide with the same substrate (5). A second alternative for hydroxide reacting with protonated substrate is the kinetically equivalent interactions of water with neutral chlordiazepoxide, thus producing a curve of the shape previously reported (6). The expression:

$$k_0 = (k_{H^+}a_{H^+} + k_{H_2O} + k_{OH^-}[OH^-])f_{BH^+} \qquad \text{(Eq. 3)}$$

where $k_{OH^-}[OH^-]f_{BH^+}$ is kinetically equivalent to $k'_{H_2O}f_B$, is characterized in Fig. 1. The terms f_{BH^+} and f_B are the fractions of chlordiazepoxide present in the protonated and neutral forms, with a_{H^+} being the hydrogen-ion activity. The second-order veloci-

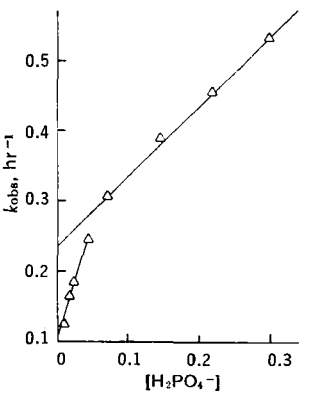


Figure 2—*Influence of pH 1.9 phosphate buffer on hydrolysis of chlordiazepoxide at 79.5° and ionic strength 0.5* M. *The ratio of* H_3PO_4–$H_2PO_4^-$ *is 2:3 in all solutions. Extrapolation of results below and above 0.1* M [$H_2PO_4^-$] *obviously leads to two different* k_0 *values. If one line were drawn connecting all points, it would be curved. This nonlinear dependency is illustrative of a change in the rate-determining step or the presence of at least two rate constants over the range of buffer concentrations represented.*

Table III—Rate Constants (k_{obs} in hr^{-1}) for Hydrolysis at Low Total Buffer Concentrations (0.02–0.10 *M*) at 79.5° and Ionic Strength 0.2

pH	Buffer	k_{obs}, hr^{-1} M^a 0.01	0.02	0.03	0.05	k_{app}[b]
1.90	$[H_3PO_4][H_2PO_4^-]$	0.121	0.158	0.180	0.222	1.2
2.62	$[ClCH_2COOH][ClCH_2COO^-]$	0.115	0.149	0.166	0.230	1.3
4.55	$[CH_3COOH][CH_3COO^-]$	0.169	0.195	0.244	0.312	1.7
		M^c 0.005	0.0075	0.015	0.02	
4.25	$[HCOOH][HCOO^-]$	0.169	0.204	0.241	0.249	1.3
3.1	$[CH_3CH_2OCH_2COOH][CH_3CH_2OCH_2COO^-]$	0.152	0.181	0.186	0.194	1.3

[a] Represents equimolar quantities of each buffer component. [b] Calculated from slope of plots of k_{obs} *versus* total buffer concentrations; measured in M^{-1} hr^{-1}. [c] Represents molarity of acidic component. Molarity of basic component = 3 × molarity of acidic component in each case.

ty constants are $k_{H^+} = 2.05\ M^{-1}\ hr^{-1}$ and $k_{OH^-} = 2.3 \times 10^7\ M^{-1}\ hr^{-1}$, with the first-order constants being $k_{H_2O} = 0.10$ and $k'_{H_2O} = 0.223\ hr^{-1}$ at 79.5°, where 55.5 *M* water is incorporated into the constant.

Since f_{BH^+} and f_B may be written as $[H^+]/([H^+] + K_a)$ and $K_a/(K_a + [H^+])$, respectively, Eq. 3 can be stated as:

$$k_0 = (k_{H^+}a_{H^+} + k_{H_2O} + k_{OH^-}[OH^-])([H^+]/([H^+] + K_a)) \quad \text{(Eq. 4)}$$

The individual constants comprising the rate–pH profile (Fig. 1) were dissected in the following manner. The k_{H^+} was evaluated at high acidities (pH 0.15–1.0), while the k_{OH^-} was established from the results over the 6–11.5 pH region. The k_{H_2O} could be obtained from dilute acidic solutions (about 0.005 *N* HCl) at pH ≈ 1.5–3.0, where neither k_{H^+} nor k_{OH^-} was appreciably operative, or by the difference: $k_0 - (k_{H^+}a_{H^+} + k_{OH^-}[OH^-]) = k_{H_2O}$ in the 1.5–3.0 pH region. The pKa estimated at half-neutralization of a solution of chlordiazepoxide in 50% polyethylene glycol 300–water was 4.6–4.8 at 80°; Fig. 1 illustrates the amount contributed by the individual constants k_{H^+}, k_{H_2O}, and k_{OH^-}.

A surprising, although not unprecedented, feature of the rate–pH profile is the apparently lower rate constant for the spontaneous rate of hydrolysis of protonated species relative to the neutral molecule[8]. This phenomenon appears to argue against a predominantly nucleophilic role for the water reaction with chlordiazepoxide. Were nucleophilic attack the case, one would anticipate a higher velocity constant for the protonated base as compared to the neutral molecule.

This more rapid reaction of "water" with neutral relative to protonated species may represent a reaction of hydroxide ion with the conjugate acid as $k_{OH^-}[OH^-]\ f_{BH^+}$ and not the kinetically equivalent k_{H_2O}/f_B. Alternatively, it may exemplify rate-determining breakdown of the tetrahedral addition intermediate through a neutral transition state (*i.e.*, a transition state with no net charge). Examples of this phenomenon have been reported in the hydrolysis of imidate and thioimidate esters (7, 8).

A second point refuting solvent catalysis of the neutral substrate is the absence of observable buffer attack in the 7–11 pH region. Where water serves as the catalytic species, one might expect catalytic effect by HPO_4^{-2}, borate, *etc.* (9).

Catalysis by General Acids and Bases—General acids and bases are effective catalysts for the conversion of protonated chlordiazepoxide to the lactam (Scheme I) while only slight, almost negligible, catalysis of the neutral molecule is observed. Figures 3 and 4 illustrate plots of k_{obs} and k_{app} against buffer component (9) and percent acid (10), respectively, at total buffer concentrations greater than 0.1 *M*. Both methods are feasible for estimation of the specific catalytic constants (second-order rate constants) for catalysis by acidic and basic species comprising the buffer, k_{HA} and k_{A^-}. Table I lists k_{obs} values for different conditions; Table IV summarizes the second-order rate constants, k_{app}, for buffer catalysis at total buffer concentration >0.1 *M*.

Table III shows the k_{app} for lower concentrations of buffer which produce rate constants inconsistent with those found at higher buffer concentrations.

Figure 2 exemplifies catalysis over the entire realm of buffer concentrations employed for phosphate buffer at pH 1.9 and 79.5°. The overall slope is comprised of at least two separate rate constants, as previously reported (11–14). This nonlinear dependency

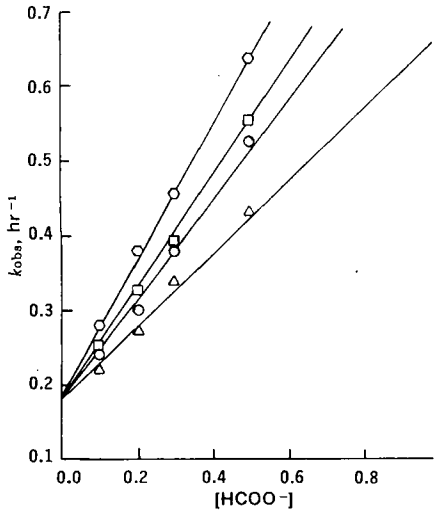


Figure 3—*Dependency of the observed first-order rate constant on the concentration of formate ion in the hydrolysis of chlordiazepoxide at 79.5° and ionic strength 0.5* M. *The extrapolation to zero buffer concentration leads to erroneous results* (k_0 *is apparently 0.18* hr^{-1}) *due to nonlinear dependency at formate concentrations of less than 0.1* M. *Key:* ○, *pH 3.0;* □, *pH 3.25;* ○, *pH 3.5; and* △, *pH 4.0.*

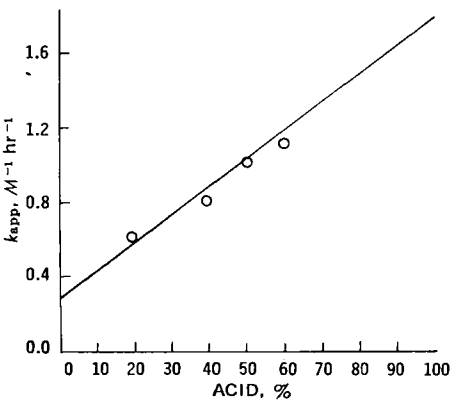


Figure 4—*Apparent second-order rate constants for catalysis of chlordiazepoxide hydrolysis by phosphoric acid–dihydrogen phosphate buffers at 79.5° as a function of the percentage of the acidic component in the buffer. The values* $k_{H_3PO_4} = 1.7\ M^{-1}\ hr^{-1}$ *and* $k_{H_2PO_4^-} = 0.3\ M^{-1}\ hr^{-1}$ *are obtained by extrapolation to 0 and 100% acid, respectively.*

[8] Dr. W. P. Jencks, personal communication.

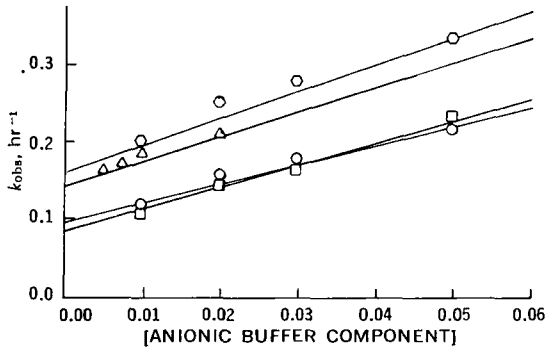


Figure 5—*Dependency of the velocity constants on buffer concentration at total buffer = 0.10* M *and below. Intercepts of plots with ordinate represent* k_0 *values utilized for rate–pH profile (Fig. 1). Key: ○, acetate, pH 4.55; △, ethoxyacetate, pH 3.1; ○, chloroacetate, pH 2.6; and □,* $H_2PO_4^-$*, pH 1.9.*

of the data on buffer concentration is probably brought about by more than one velocity constant being operative over the 0.01–1.0 *M* buffer range. This leads to erroneous k_0 values upon extrapolation (Figs. 3 and 4), unless results of 0.01–0.10 *M* buffers are included. The phenomenon is indicative of a change in the rate-limiting step as the buffer components in solution increase (11–13). This nonlinear relationship is particularly influential in the pH range where appreciable protonated chlordiazepoxide, BH^+, is present (pH 1.8–5.0). At pH values below 1.8, the phenomenon may hold, but it was not investigated with acid–anionic buffers because hydrochloric acid sufficed.

An example of this fallacious extrapolation is given in Fig. 3, where a value of $k_0 = 0.18\ M^{-1}$ is indicated for several pH values (acetate buffer) when the intercepts are actually considerably lower.

It is evident from the latter data in Table I that the degree of buffer catalysis is decreased or almost eliminated at pH >7, indicating an absence of buffer effect on neutral chlordiazepoxide.

The contribution of each buffer constituent of acid and acid anion to hydrolysis of protonated chlordiazepoxide, BH^+, may be estimated by plots of k_{obs} *versus* anion as shown in Fig. 3, utilizing the acidic dissociation constant of the buffer being investigated:

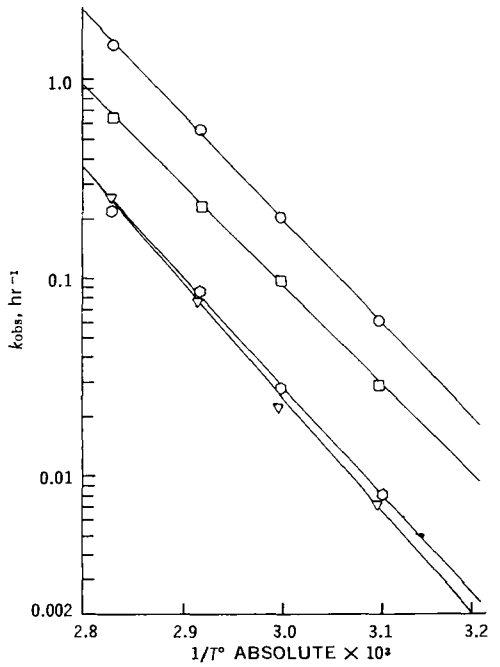


Figure 6—*Arrhenius parameters for hydrolysis of chlordiazepoxide. Key: ○, 1.0* N *HCl; □, pH 4.5 acetate buffer, ionic strength 0.5; △, pH 7 phosphate buffer, ionic strength 0.5; and ○, 0.1* N *NaOH.*

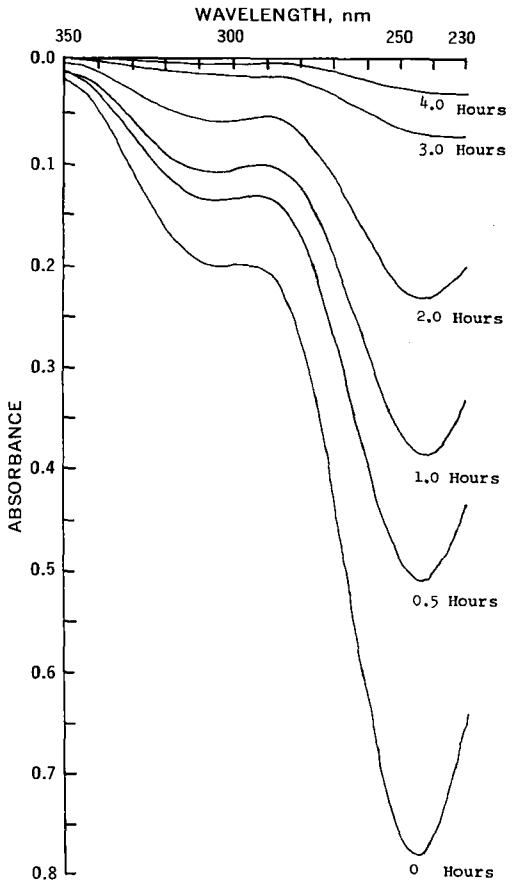


Figure 7—*Disappearance of absorbance as a function of time. Chlordiazepoxide was extracted from TLC plates at the hourly intervals listed on the spectrophotometric curves. The reaction conditions were 0.3* M *acetate, pH 4.0, ionic strength 0.5* M*, 79.5°. Peaks exhibit corresponding diminution.*

($K_a = [H^+][A^-]/[HA]$). The results presented in Figs. 3 and 4 are for total acidic and anionic concentrations of 0.1–1.0 *M*.

Equation 4 may then be written with the introduction of terms for the buffer where total buffer is less than 0.1 *M*:

$$k_{obs} = k_0 + (k_{HA}[HA] + k_{A^-}[A^-])(f_{BH^+}) \qquad \text{(Eq. 5)}$$

or:

$$k_{obs} = k_0 + \{k_{HA}([H^+]/K_a) + k_{A^-}\}[A^-] \qquad \text{(Eq. 6)}$$

where $f_{BH^+} \approx 1$; *i.e.*, pH < 3.5; pKa ≈ 4.6–4.8 at 80°.

Therefore, a plot of the $k_{obs} - k_0$ against [A−] where buffer concentration is >0.1 *M* gives a straight line with:

$$\text{slope} = k_{HA}([H^+]/K_a) + k_{A^-} \qquad \text{(Eq. 7)}$$

From a graph of Eq. 6 with slopes of a given buffer combination (*e.g.*, acetate–acetic acid) at several pH values, the slope obtained equals k_{HA} with an intercept of k_{A^-} (9). When the data of Fig. 3 are plotted utilizing Eq. 6, the values are: $k_{HCOOH} = 0.92\ M^{-1}\ hr^{-1}$ and $k_{HCOO^-} = 0.48\ M^{-1}\ hr^{-1}$ at 79.5°.

The k_{HA} and k_{A^-} values may be determined utilizing plots of k_{app} against the percentage acid in the buffers, as given by Sander and Jencks (10) and exemplified in Fig. 4.

The following results were obtained from Fig. 4: $k_{H_3PO_4} = 1.7\ M^{-1}\ hr^{-1}$ and $k_{H_2PO_4} = 0.3\ M^{-1}\ hr^{-1}$ at 79.5°. These are in close proximity to the values obtained from Eqs. 6 and 7.

Table III summarizes some k_{obs} and k_{app} values for lower buffer concentrations (0.02–0.1 *M*). Figure 2 also depicts the influence of buffer on the observed first-order rate constant, with at least two slopes being obvious. Extrapolations of results in Table III were utilized in the determination of the k_0 shown in Fig. 1. Figure 5 illustrates the dependency of velocity constants (Table III) on the anionic component at lower buffer concentrations.

Table IV—Values of Apparent Second-Order Rate Constants[a] (k_{app} in M^{-1} hr^{-1}) for Buffer-Catalyzed Hydrolysis of Chlordiazepoxide at 79.5° and Ionic Strength 0.5 M

Acid	pH[b]	Acid[c], %	Concentration Range[d], M	k_{app}, M^{-1} hr^{-1}[e]
H_3PO_4	1.70	60	0.125–0.50	0.89
	1.76	50	0.125–0.50	0.90
	2.0	40	0.125–0.50	0.49
	2.5	20	0.125–0.50	0.36
HCOOH	3.0	—	0.1–0.5	0.92
	3.5	—	0.1–0.5	0.71
	3.75	—	0.1–0.5	0.68
	4.0	—	0.1–0.5	0.49
CH_3COOH[f]	4.0	—	0.1–0.5	1.92
	4.5	—	0.1–0.5	0.90
	5.0	—	0.1–0.5	0.38
	5.5	—	0.1–0.5	0.30
$H_2PO_4^-$	6.0	80	0.085–0.34	1.03

[a] Measured as stated under *Experimental* utilizing 2.98×10^{-5} M chlordiazepoxide. [b] The pH values were measured at 79° on a pH meter standardized at that temperature. [c] Percentage acid is not listed for acetate and formate solutions become they were prepared by addition of the appropriate acid to 0.5 M NaOH with dilution to 1 liter. [d] Concentration refers to total buffer, except for formate and acetate where acetate and formate ions were used in the calculation because total buffer was not known from the preparation method except by calculation from pH and pKa of the appropriate solutions. [e] Obtained from slopes of plots of k_{obs} against total buffer concentration, except in cases of acetate and formate buffers where anionic component was utilized for abscissa. [f] Chlordiazepoxide has pKa of about 4.6–4.9 at 79°; therefore, both protonated and nonprotonated species are present in the acetate buffer region.

At pH 4 and above, both appreciable cationic and neutral substrates are present so a mixture of rate constants is actually given in which the effects of various acids and their anions, namely acetate buffer, on both protonated and nonprotonated chlordiazepoxide should be considered for rigorous analysis.

Arrhenius Parameters—Estimates of the apparent activation energies were made from the Arrhenius equation:

$$\log k_{obs} = \log P - Ea/2.303RT \qquad \text{(Eq. 8)}$$

Values derived from the preceding expression vary between 23.0 and 25.2 kcal/mole under the condition shown in Fig. 6. This information corroborates the analytical method employed as well as giving the *Ea* determinations.

Ionic Strength Effects—In the acidic region where hydronium-ion catalysis on cationic substrate is evident, it may be expected that the Bronsted–Bjerrum equation (15) might hold in some modified form (16) or at least that a qualitative agreement might be seen. This was seemingly the case as may be seen from the results in Table II, which shows a slight increase in the velocity constants.

Reaction Products—Over the entire pH range investigated, the reaction sequence given in Scheme I predominated. Other products of the hydrolysis at higher temperatures were reported (1, 17) but only a fourth trace spot was evidenced with the TLC systems employed. Formation of the benzophenone was increased as the pH scale was descended.

Quantitative TLC and Comparisons—An original aim of the work was to investigate the rate constant discrepancies taking place when chlordiazepoxide was subjected to quantitative thin-layer kinetic analysis. For this reason, a second analytical method (extraction of acidified aqueous substrate with methylene chloride) was applied as a check. It is now evident that buffer catalysis along with the previously mentioned nonlinear dependency of rate constants on buffer concentrations contributed to the difficulties encountered.

Figure 7 illustrates a typical absorbance loss for chlordiazepoxide (80°, pH 4, Ac^- = 0.3 M) when subjected to quantitative TLC. The k_{obs} is 0.77 hr^{-1}, in comparison to 0.80 hr^{-1} determined by the extractive procedure. Figure 8 shows the reaction rate constant at pH 2 (phosphate buffer) and 80° plotted at both wavelengths (244 and 305 nm), with linearity at both wavelengths as determined by quantitative TLC. Table V lists data derived from both techniques and illustrates a reasonable degree of correlation between the two

Table V—Comparative Results of k_{obs} for Chlordiazepoxide Hydrolysis of Quantitative TLC Method Relative to the Extractive Procedure at 79.5°

pH	Buffer	k_{obs}, hr^{-1} Quantitative TLC	k_{obs}, hr^{-1} Extraction[a]
0.46	0.5 N HCl	0.74	0.82
1.12	0.1 N HCl	0.23	0.20
1.40	0.05 N HCl	0.17	0.16
2.0	Phosphate[b]	0.41	0.36
3.0	Formate[c]	0.61	0.65
4.0	Acetate[c]	0.91	0.74
4.5	Acetate[c]	0.58	0.63
5.0	Acetate[c]	0.41	0.36
6.0	Phosphate[d]	0.29	0.29
7.1	Phosphate	0.20	0.23
11.5	0.1 N NaOH	0.21	0.22

[a] Taken from Table I. [b] Buffer contains 0.125 M of each component at ionic strength 0.5. [c] 0.5 M acetate and formate ion. [d] See Table I, pH 6.0 and 7.1 for buffers.

sets. It was concluded that the quantitative thin-layer method works well for this benzodiazepine as well as certain congeners.

Problems initially arising were attributed to:

1. Influence of buffer catalysis when precautions were not taken to hold buffer concentration and anionic and basic components of the buffers constant at the given pH values.
2. Insolubility of the substrate in aqueous solutions as the pH increased. Concentrations of 1–2 mg/ml were used at pH 5. Whereas the solutions were homogeneous at 60–80°, they were prone to precipitate upon cooling to room temperature during the streaking process (see *Experimental*).
3. The nonlinear dependency of rate constants and buffer concentrations.

CONCLUSIONS

The hydrolytic degradation of chlordiazepoxide in aqueous solution *via* the lactam was studied over the 0.15–11.5 pH range at 79.5°. The lactam and benzophenone were the principal products formed under these conditions, so the reaction investigated was conversion of chlordiazepoxide to the corresponding lactam. Intermediates previously reported between the lactam and benzophenone (1) were not observed, with the exception of one of unknown origin seen by TLC. These intermediates may be transient in nature under the conditions employed.

This reaction is catalyzed by hydrogen and hydroxide ions, general acids, and general bases and undergoes a solvent reaction with

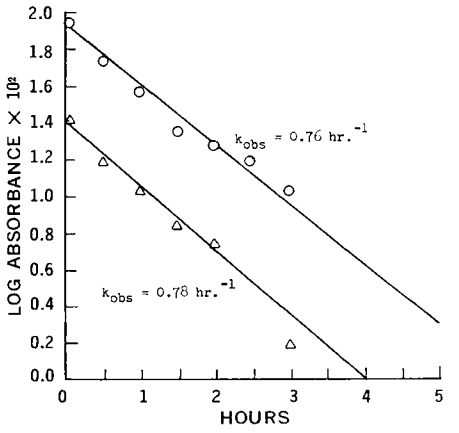


Figure 8—*Plots of results obtained from pH 2 phosphate buffer, H_3PO_4–$H_2PO_4^-$ = 0.3 M/0.45 M, ionic strength 0.5, temperature 80°. Data were obtained from samples periodically streaked and then separated by TLC, followed by elution from adsorbent and analysis at the pertinent wavelengths. Key:* O, *245 nm; and* △, *305 nm. See Fig. 7 for a typical plot of the absorbance diminution as function of time.*

protonated and neutral substrates. The following rate law may be written:

$$k_{obs} = (k_{H^+}a_{H^+} + k_{H_2O} + k_{OH^-}[OH^-] + k_{HA}[HA] + k_A[A^-])\, f_{BH^+} \quad \text{(Eq. 9)}$$

where f_{BH^+} and f_B represent the fractions of protonated and neutral chlordiazepoxide at a given hydrogen-ion concentration; pKa = 4.6–4.8 measured in 50% polyethylene glycol 300 at 80°.

The term $k_{OH^-}[OH^-]f_{BH^+}$, Eqs. 4 and 9, may be expressed as the kinetically equivalent $k_{H_2O}f_B$, as pointed out previously (8, 9).

Nonlinear dependency of the rate constant with buffer concentration in the 1.7–4.5 pH range was seen, which is likely the result of a change in the rate-limiting step with an increase of buffer in solution (11–14).

An extraction procedure was developed as a comparative method for the quantitative TLC process. The technique consisted of extraction of the decomposition products into methylene chloride from acidified chlordiazepoxide followed by UV analysis of the acidic aqueous layer.

The quantitative TLC method worked well for chlordiazepoxide, with initially discovered discrepancies being the consequence of a lack of control of the buffer concentration and occasional precipitation of the substance from solution upon cooling during the streaking procedure. When these factors were controlled, results were obtained in accord with those from the extraction procedure.

The work presented here corroborates applicability of the TLC technique to molecules that do not lend themselves to kinetic analysis by standard differential UV techniques. Thus, the delays occasionally encountered in assay development may be circumvented. Although this procedure is not the ultimate in simplicity, it affords ready availability of stability results to the formulator as well as to the kineticist.

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Simultaneous Determination of Phenylbutazone and Oxyphenbutazone in Plasma by High-Speed Liquid Chromatography

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Abstract □ A sensitive, specific, high-speed liquid chromatographic procedure is described for the simultaneous determination of phenylbutazone and its metabolite, oxyphenbutazone, in plasma. Acidified plasma is partitioned with cyclohexane–ether (1:1) containing the 2,4-dinitrophenylhydrazone of 3,4-dimethoxybenzaldehyde as an internal standard. The organic extract is reduced to dryness, the resulting residue is redissolved in chloroform, and aliquots of this solution are chromatographed on an adsorption column, using a mobile phase of 0.002% acetic acid and 23.0% tetrahydrofuran in *n*-hexane at 35°. Use of a UV detector permits quantitative analysis of samples containing less than 0.25 μg/ml of phenylbutazone or oxyphenbutazone.

Keyphrases □ Phenylbutazone–oxyphenbutazone—simultaneous determination in plasma, high-speed liquid chromatography □ Oxyphenbutazone–phenylbutazone—simultaneous determination in plasma, high-speed liquid chromatography □ Liquid chromatography, high speed—simultaneous determination in plasma of phenylbutazone and oxyphenbutazone

Several spectrophotometric procedures (1–6) and a GLC method (7) have been reported for the determination of phenylbutazone in biological fluids; however, fewer methods have been described for the estimation of two known metabolites (1) of this drug, oxyphenbutazone (1-phenyl-2-*p*-hydroxyphenyl-3,5-dioxo-4-butylpyrazolidine) and hydroxyphenylbutazone [1,2-diphenyl-3,5-dioxo-4-(3-hydroxybutyl)pyrazolidine].

The classical method of Burns *et al.* (1) is not of sufficient sensitivity to estimate phenylbutazone and oxyphenbutazone in biological fluids following single

protonated and neutral substrates. The following rate law may be written:

$$k_{obs} = (k_{H^+}a_{H^+} + k_{H_2O} + k_{OH^-}[OH^-] + k_{HA}[HA] + k_A[A^-])\, f_{BH^+} \quad \text{(Eq. 9)}$$

where f_{BH^+} and f_B represent the fractions of protonated and neutral chlordiazepoxide at a given hydrogen-ion concentration; pKa = 4.6–4.8 measured in 50% polyethylene glycol 300 at 80°.

The term $k_{OH^-}[OH^-]f_{BH^+}$, Eqs. 4 and 9, may be expressed as the kinetically equivalent $k_{H_2O}f_B$, as pointed out previously (8, 9).

Nonlinear dependency of the rate constant with buffer concentration in the 1.7–4.5 pH range was seen, which is likely the result of a change in the rate-limiting step with an increase of buffer in solution (11–14).

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The work presented here corroborates applicability of the TLC technique to molecules that do not lend themselves to kinetic analysis by standard differential UV techniques. Thus, the delays occasionally encountered in assay development may be circumvented. Although this procedure is not the ultimate in simplicity, it affords ready availability of stability results to the formulator as well as to the kineticist.

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Simultaneous Determination of Phenylbutazone and Oxyphenbutazone in Plasma by High-Speed Liquid Chromatography

N. J. POUND * and R. W. SEARS

Abstract □ A sensitive, specific, high-speed liquid chromatographic procedure is described for the simultaneous determination of phenylbutazone and its metabolite, oxyphenbutazone, in plasma. Acidified plasma is partitioned with cyclohexane–ether (1:1) containing the 2,4-dinitrophenylhydrazone of 3,4-dimethoxybenzaldehyde as an internal standard. The organic extract is reduced to dryness, the resulting residue is redissolved in chloroform, and aliquots of this solution are chromatographed on an adsorption column, using a mobile phase of 0.002% acetic acid and 23.0% tetrahydrofuran in *n*-hexane at 35°. Use of a UV detector permits quantitative analysis of samples containing less than 0.25 μg/ml of phenylbutazone or oxyphenbutazone.

Keyphrases □ Phenylbutazone–oxyphenbutazone—simultaneous determination in plasma, high-speed liquid chromatography □ Oxyphenbutazone–phenylbutazone—simultaneous determination in plasma, high-speed liquid chromatography □ Liquid chromatography, high speed—simultaneous determination in plasma of phenylbutazone and oxyphenbutazone

Several spectrophotometric procedures (1–6) and a GLC method (7) have been reported for the determination of phenylbutazone in biological fluids; however, fewer methods have been described for the estimation of two known metabolites (1) of this drug, oxyphenbutazone (1-phenyl-2-*p*-hydroxyphenyl-3,5-dioxo-4-butylpyrazolidine) and hydroxyphenylbutazone [1,2-diphenyl-3,5-dioxo-4-(3-hydroxybutyl)pyrazolidine].

The classical method of Burns *et al.* (1) is not of sufficient sensitivity to estimate phenylbutazone and oxyphenbutazone in biological fluids following single

doses of phenylbutazone. Although the need for a sensitive and specific method for estimating phenylbutazone in plasma has been met by recently reported high-speed liquid chromatographic (HSLC) (8) and GLC procedures (9), the development of HSLC and GLC methods for measuring oxyphenbutazone in plasma, following single doses of phenylbutazone, was undertaken for pharmacokinetic studies under investigation in this laboratory (10).

The HSLC procedure described compares favorably with the recently reported GLC method (11). It possesses adequate sensitivity for single-dose studies, is somewhat less complex than the GLC procedure, and permits the simultaneous determination of both phenylbutazone and oxyphenbutazone from a single plasma extract.

EXPERIMENTAL

Materials—Complimentary samples of phenylbutazone[1], oxyphenbutazone[2], and hydroxyphenylbutazone[2] were used as received. Tetrahydrofuran[3], freshly distilled and dried over molecular sieves[3], and spectrograde *n*-hexane[4] were used as solvents. The 2,4-dinitrophenylhydrazone of 3,4-dimethoxybenzaldehyde, used as the internal standard, was prepared by the reported procedure (12), mp 264–266° [lit. (12) mp 264°].

Chromatographic Procedure—Details of the modified constant-pressure high-speed liquid chromatograph[5] used in this study were previously reported (13). The chromatograph was equipped with a stop-flow injection port[5], a constant-temperature water bath[6], and a fixed-wavelength (254 nm) UV absorbance detector[5].

The adsorption column[7] (100 cm × 1.8 mm i.d., 304 stainless steel) was dry packed by a procedure similar to the one described by Kirkland (14), then coiled (radius 15 cm), and fitted horizontally into the water bath of the instrument. Analyses were performed using a mobile phase of 0.002% acetic acid and 23.0% tetrahydrofuran in *n*-hexane at a flow rate of 60 ml/hr (475 psi) and a temperature of 35°. The mobile phase was "degassed" by applying vacuum (about 100 mm) to the solvent reservoir for approximately 1 min before use. Extraction residues were redissolved in chloroform, and 10-μl aliquots of this solution were injected directly on-column with a 25-μl syringe[8] using a stop-flow injection technique.

Preparation of Standard Solutions—Standard solutions of phenylbutazone and oxyphenbutazone were prepared by dissolving a sample of the drug substance, accurately weighed, in 95% ethanol (1 ml) in a 100-ml volumetric flask and then bringing the solution to volume with phosphate buffer (pH 7.2). Aliquots of each solution were mixed and further diluted with water to produce a final solution containing the desired concentration of phenylbutazone and oxyphenbutazone.

Spiked plasma solutions were prepared by transferring an aliquot (1.0 ml) of the aqueous phenylbutazone–oxyphenbutazone solution to a 5-ml volumetric flask, bringing the solution to volume with citrated human plasma, and mixing thoroughly.

Analytical Procedure—An aliquot (1.0 ml) of the plasma sample was transferred to a 10-ml screw-capped centrifuge tube, 1 *M* hydrochloric acid (1 ml) and a solution of the internal standard (15 – 75 μg/ml, accurately weighed) in cyclohexane–ether (1:1) (6.0 ml) were added, and the tube was tumbled[9] for 15 min at 30 rpm. The tube was then centrifuged for 2–3 min at 3000 rpm, and the upper organic layer was transferred, with a Pasteur pipet, to a 15-ml conical screw-capped centrifuge tube.

The solvent was evaporated under nitrogen in a constant-temperature (60°) water bath. Chloroform (1 ml), which was used to rinse the inner walls of the tubes, was also evaporated. Aliquots (10 μl) of the solution, prepared by promptly redissolving the resulting residue in 50–200 μl of chloroform to give an anticipated drug concentration of 35–200 ng/μl, were chromatographed.

Peak height ratios were calculated by dividing the peak height of phenylbutazone or oxyphenbutazone by the peak height of the internal standard. The amount of each drug substance was then calculated from the respective calibration curve.

[1] Mount Royal Chemicals, Montreal, Quebec, Canada.
[2] Ciba-Geigy Pharmaceuticals, Canada.
[3] Fisher Scientific, Montreal, Quebec, Canada.
[4] Caledon Laboratories Ltd., Georgetown, Ontario, Canada.
[5] Model 4000, Varian Aerograph, Palo Alto, Calif.
[6] Model 3084, Labline Industries, Melrose Park, Ill.
[7] Sil-X adsorbent, Perkin-Elmer Corp., Norwalk, Conn.
[8] Model 702N, Hamilton Co., Reno, Nev.
[9] Multi-Rotator, Scientific Industries Ltd., Springfield, Mass.

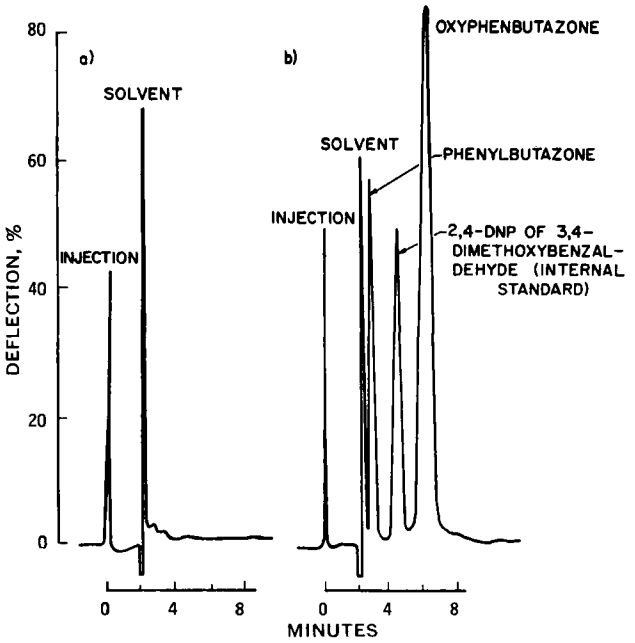

Figure 1—*High-speed chromatograms of human plasma extracts. Key: a, control plasma; and b, plasma containing 2.0 μg/ml phenylbutazone, 2.1 μg/ml internal standard, and 7.7 μg/ml oxyphenbutazone. The conditions were: 1 m × 1.8 mm i.d. column using a mobile phase of 0.002% acetic acid, 23.0% tetrahydrofuran in* n*-hexane at a flow rate of 60 ml/hr at 35°, and a detector attenuation of 0.04 absorbance unit full scale.*

RESULTS AND DISCUSSION

Figure 1*b* illustrates the chromatogram obtained from the analysis of a plasma sample containing 2.0 μg/ml of phenylbutazone, 2.1 μg/ml of internal standard, and 7.7 μg/ml of oxyphenbutazone, using the described chromatographic system. This figure repre-

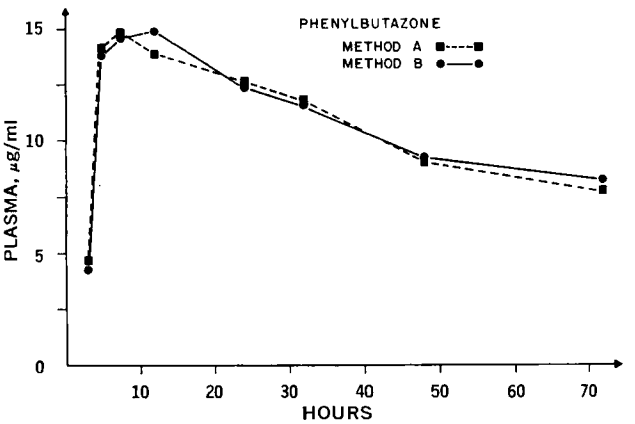

Figure 2—*Phenylbutazone concentration in plasma of human volunteer following single oral dose of 2 × 100-mg phenylbutazone tablets.*

Table I—Analysis of Spiked Phenylbutazone–Oxyphenbutazone Plasma Samples[a]

Sample	Phenylbutazone, μg/ml			Oxyphenbutazone, μg/ml		
	Calc.	Found	Recovery, %	Calc.	Found	Recovery, %
1	6.53	6.49	99.39	6.46	6.38	98.76
2	4.13	4.11	99.52	4.30	4.31	100.23
3	8.49	8.61	101.41	8.88	8.99	101.24
4	7.78	7.61	97.81	7.74	7.57	97.80
5	1.58	1.57	99.37	1.86	1.84	98.92
6	0.23	0.22	99.13	0.31	0.31	98.73
Mean			99.44			99.28
RSD, %			±1.17			±1.26

[a] Average analysis of duplicate aliquots.

sents an on-column injection of 0.4 μg of phenylbutazone and 1.5 μg of oxyphenbutazone. Analysis is achieved in less than 8 min. Although a small amount of interference does occur with the phenylbutazone peak, no compounds that interfere with oxyphenbutazone are extracted from plasma (Fig. 1*a*). The chromatogram shown in Fig. 1*a* is typical of the blank obtained from plasma samples from six volunteers.

Adsorption chromatography was chosen because it offers the advantage of rapid analyses and excellent column stability. A single column was used throughout this study. A simple solvent system of *n*-hexane modified with 23.0% tetrahydrofuran was used as a mobile phase. The addition of a small amount (0.002%) of acetic acid to the mobile phase reduced the slight tendency of the peaks to tail. Operation at 35° reduced column back pressure (475 psi) and improved column efficiency.

Although hydroxyphenylbutazone, the other known metabolite of phenylbutazone (1), is not completely resolved from oxyphenbutazone, this compound could be detected readily if it were present in a sample. Under these chromatographic conditions, retention times of 2.73, 4.33, 5.36, and 6.12 min are obtained for phenylbutazone, the internal standard, hydroxyphenylbutazone, and oxyphenbutazone, respectively. No hydroxyphenylbutazone has been detected in any of the single-dose studies performed in this laboratory (10).

Analyses were performed as described. To avoid degradation of the sample extracts (8), the residues were promptly redissolved in chloroform (50–200 μl) and chromatographed. No decomposition of the drug substances was detected. Chloroform was chosen as the solvent for redissolving the sample residues since the internal standard, phenylbutazone, and oxyphenbutazone are all readily soluble in this solvent, the injection of 10 μl of chloroform does not affect the resolution of the separation, and the vapor pressure of chloroform is high enough to prevent excessive loss due to evaporation between injections.

Quantitation was performed by peak height measurement to avoid the problems associated with the integration of the small broad peaks frequently encountered in HSLC (8). Previous work in this laboratory (8, 15) has indicated that peak height data compare favorably with those obtained by digital integration.

The 2,4-dinitrophenylhydrazone of 3,4-dimethoxybenzaldehyde was used as an internal standard. It was added directly with the extracting solvent before partitioning the samples, thereby reducing errors caused during transfer and evaporation procedures. The use of 2,4-dinitrophenylhydrazine derivatives as internal standards shows particular promise due to their ease of synthesis, stability, and intense UV absorbance at 254 nm. Judicious selection of the ketone or aldehyde reagent makes it possible to prepare internal standards with the required retention time for a particular separation.

Comparable values for linearity and sample recoveries (>98%) were obtained for both phenylbutazone and oxyphenbutazone from spiked solutions prepared with either plasma or water. The use of water instead of plasma facilitated the preparation of synthetic solutions used to prepare and check the calibration curves. Calibration curves for phenylbutazone and oxyphenbutazone were obtained by plotting the response *versus* weight ratio of drug–internal standard for a series of synthetic aqueous mixtures of both drugs. Both plots were straight lines ($y = mx$) over the concentration range of from 0.230 to 37.64 μg/ml for phenylbutazone or from 0.315 to 43.37 μg/ml for oxyphenbutazone. Mean slope values of 1.146 and 0.460 were obtained for phenylbutazone and oxyphenbutazone, respectively. These curves were checked daily, and the difference between the calculated and found response ratios of the standard solutions was never more than 2%.

The precision of this procedure is reflected by the data obtained from the analysis of six aliquots of each of two spiked plasma samples. Recovery values of 0.228 ± 0.004 and 0.311 ± 0.004 μg/ml were obtained from the analysis of samples containing 0.230 and 0.315 μg/ml of phenylbutazone and oxyphenbutazone, respectively. Similarly, values of 7.61 ± 0.12 and 7.57 ± 0.09 μg/ml were obtained for samples containing 7.78 μg/ml of phenylbutazone and 7.61 μg/ml of oxyphenbutazone. These results were obtained using a detector attenuation of 0.04 absorbance unit full scale (aufs). At maximum detector sensitivity (0.005 aufs), the on-column injection of approximately 15 ng (0.075 μg/ml of plasma) of phenylbutazone would produce a readily detectable (10% of full scale) recorder response. The minimum detectable amount of oxyphenbutazone would be somewhat higher (20 ng/injection).

The results from the analysis of six spiked plasma samples reflect the accuracy of the procedure (Table I). Mean recovery values of 99.44 ± 1.17 and 99.28 ± 1.26% were obtained for phenylbutazone and oxyphenbutazone, respectively. All six plasma blanks used contained a small, relatively constant amount of extraneous material which produced some interference with the phenylbutazone peak. The chromatogram shown in Fig. 1*a* is typical. The phenylbutazone data shown in Table I were corrected by subtracting the value of the plasma blank from the height of the phenylbutazone peak before calculations were performed.

The accuracy and precision of this method are further substantiated by the results presented in Figs. 2 and 3. Figure 2 illustrates the 72-hr plasma phenylbutazone profiles of a human subject [male, 90.8 kg (200 lb)] who had been administered 2 × 100-mg tablets of phenylbutazone. Samples of blood (10 ml) were withdrawn from the cubital vein by means of heparinized containers[10]. Blood samples were centrifuged, and the plasma was transferred and stored at −10°. Samples were stable for at least 2 weeks under these conditions (8, 9). The profiles shown in Fig. 2 were obtained using the procedure described in this article (Method B), which permits the simultaneous determination of both phenylbutazone and oxyphenbutazone, and a previously reported HSLC method (Method A) (8), which provided only for the determination of phenylbutazone. The two profiles compare favorably.

Phenylbutazone and oxyphenbutazone plasma levels of a male volunteer [90.8 kg (200 lb)] who had been given a 400-mg dose of phenylbutazone in solution were also monitored over 388 hr. Profiles obtained by both this HSLC procedure and a recently developed GLC (11) method are shown in Fig. 3. The two techniques compare favorably. Overall differences between the two procedures were 8.1 and 7.7% for phenylbutazone and oxyphenbutazone, respectively.

In conclusion, the HSLC procedure described here provides a rapid, sensitive, and precise method for the simultaneous determination of plasma levels of phenylbutazone and oxyphenbutazone. It offers the added advantage that since the second metabolite, hydroxyphenylbutazone, can be clearly distinguished, it can be em-

[10] Vacutainers, Beckton Dickinson & Co., Mississauga, Ontario, Canada.

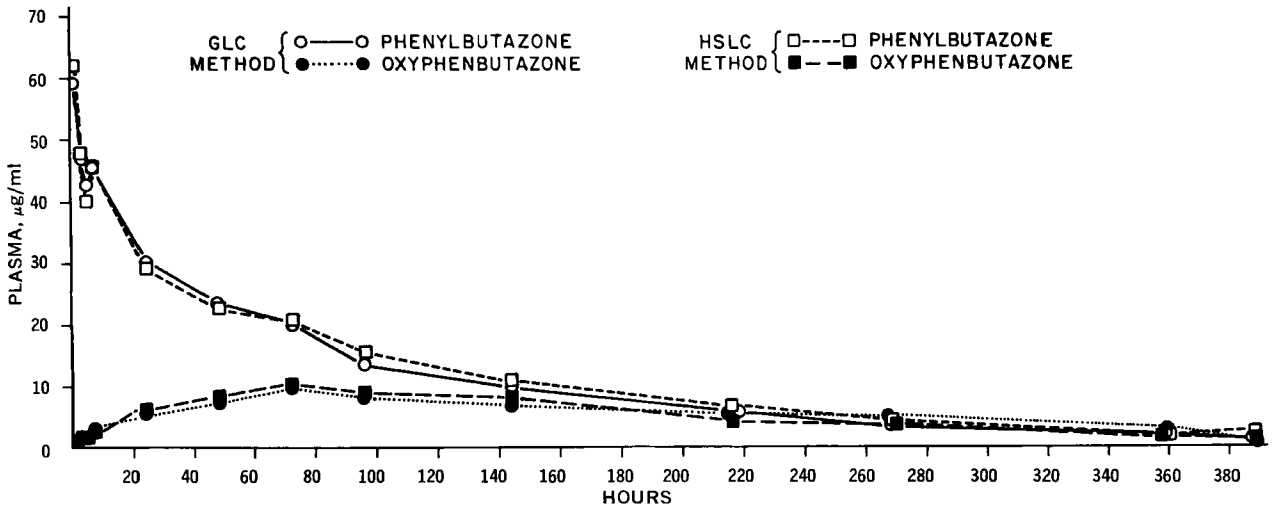

Figure 3—*Comparison of plasma levels determined by HSLC and GLC procedures (11) following a single oral dose of 400-mg solution of phenylbutazone to a volunteer.*

ployed for the analysis of phenylbutazone and oxyphenbutazone in multiple-dose as well as single-dose pharmacokinetic studies.

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Submicrogram Assay for Scopolamine in Plasma and Urine

W. F. BAYNE ˣ, F. T. TAO, and N. CRISOLOGO

Abstract □ A GLC–mass spectrometric method for scopolamine, sensitive to 50 pg/ml for a 4-ml plasma or urine sample, was developed. The method used a deuterated internal standard to minimize variability in absolute recovery in the extraction procedure. Scopoline and deuterated scopoline were formed from the base-catalyzed hydrolysis of scopolamine and the internal standard and were analyzed as the heptafluorobutyrates, using a GLC–mass spectrometric system by monitoring the *m/e* 138 and 141 fragments, respectively.

Keyphrases □ Scopolamine—submicrogram assay in plasma and urine, heptafluorobutyryl derivatives □ GLC–mass spectrometry—analysis, scopolamine in plasma and urine

Scopolamine, an anticholinergic drug, is a tropane alkaloid. The existing methods of assaying scopolamine in biological fluids, such as GLC (1) and acid-dye techniques (2, 3), lack sensitivity when therapeutic doses are administered. The most definitive study on scopolamine and its metabolites in biological fluids and tissues involved the use of radioactively labeled scopolamine in mice, rats, guinea pigs, and marmosets (4). This method was unsuitable for humans.

It was decided to develop a sensitive assay, using a stable isotope-substituted, internal standard in conjunction with GLC–mass spectrometry, that could be used to assay scopolamine in biological fluids after administration of an antinauseant dose. The method involved monitoring a fragment, produced under electron-impact ionization, of the heptafluorobutyryl derivative of scopoline (I). Scopoline was formed from the base-catalyzed hydrolysis of scopolamine.

Walle and Ehrsson (5) previously made the heptafluorobutyryl derivative of tropine, a hydrolysis product of atropine, in a study of the electron-capture responses of amino and alcoholic compounds. Blake *et al.* (6) recently used the diheptafluorobutyryl and dipentafluoropropionyl derivatives of 2-hydroxymethyltropine, formed through lithium aluminum hydride reduction of cocaine, to monitor cocaine indirectly in horse urine by electron-capture detection.

The pharmacokinetics of amphetamine and phentermine have been investigated by GLC–mass spectrometry, using trifluoroacetyl derivatives and deuterium-substituted analogs as internal standards (7). A similar approach was developed for homovanillic acid in cerebrospinal fluid, using the pentafluoropropionyl derivatives of the methyl ester of homovanillic acid (8). Homovanillic acid was assayed both by GLC–mass spectrometry, with the trideuterated methyl ester as an internal standard, and by electron-capture detection, with a homolog of homovanillic acid as the internal standard. As has been pointed out previously, some fluorinated acyl derivatives are readily adapted to both GLC–mass spectrometry and electron-capture detection studies because of favorable chromatographic properties in the subnanogram range (9).

The heptafluorobutyryl derivative of scopoline was used not for its sensitivity toward electron-capture detection but because it chromatographed well at the picogram level and showed a favorable fragmentation pattern under electron-impact ionization. The use of the deuterated internal standard for selective ion monitoring negated the necessity of finding an internal standard with similar partitioning properties, but with different retention times, which would have been required if electron-capture detection was used. Also, the use of "windows" (selective ion monitoring) in the GLC–mass spectrometric method greatly reduced the problem of biological interferences as compared to electron-capture detection.

EXPERIMENTAL

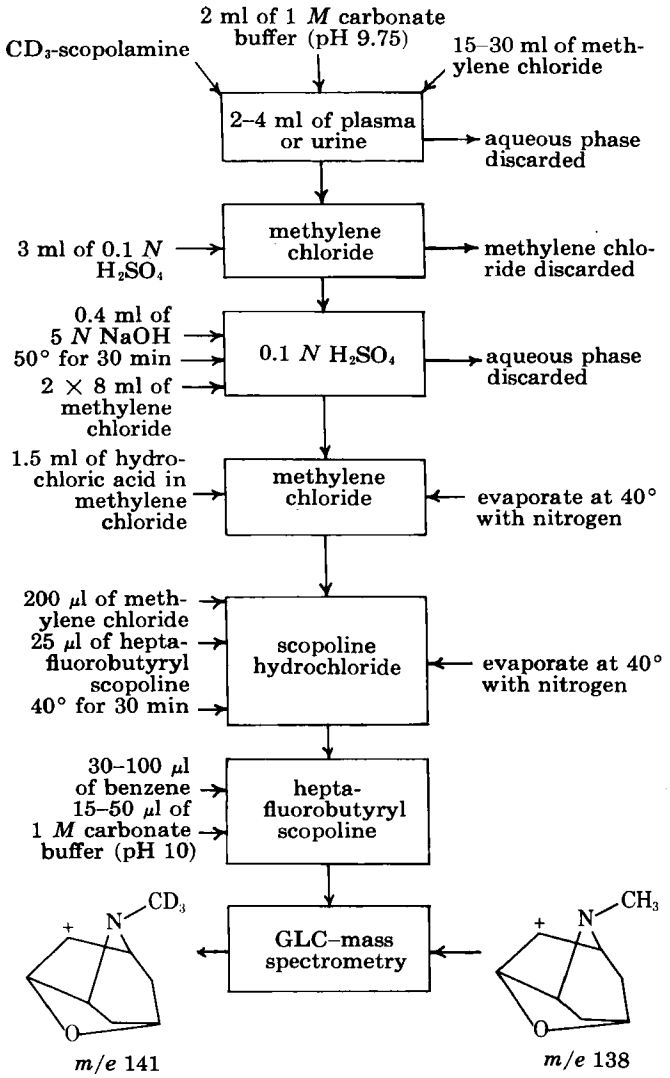
Materials—A GLC quadrupole mass filter system[1], in conjunction with an automatic peak selector[1], was used in the assay. An electron energy of 30 ev and an ionization current of 0.5–1.0 mamp were employed. The automatic peak selector was wired so that only two channels were used, maximizing the signal-to-noise ratio. The *m/e* 138 fragment of I and the *m/e* 141 fragment of the heptafluorobutyryl derivative of trideuteromethylscopoline (II) were monitored.

The sampling time was 10 msec. A preamp filter of 300 amu/sec and 30 μF capacitance for the output signal for each channel were used. It was sometimes necessary to use between 10 and 30 mv bucking for the 141 channel as compared to the 138 channel when small amounts of internal standard were used because of the inherent background within the system.

Glass columns, 1.8 m (6 ft), 2 mm i.d., were silanized and packed with either 3% OV-17 on Gas Chrom Q[2] (100–120) or 1% OV-225 on Gas Chrom Q[2] (100–120). Plasma samples were chromatographed on 3% OV-17 at 95° at a flow rate of 30 ml/min, while urine samples were chromatographed on 1% OV-225 under similar conditions. The retention time of I was 2.5 min on the former column and 2.2 min on the latter column. OV-225 invariably resolved peaks, which occasionally occurred in urine samples, from I that were not resolved with OV-17. Also, a reduction in column temperature could be used to improve resolution of I from peaks endogenous to the urine samples if blanks suggested the possibility of interferences. However, this was rarely necessary. Plasma samples were chromatographed on 3% OV-17 since there were no interfering peaks and this column bled less than did the OV-225.

Cylindrical separators (30 or 60 ml), with Teflon valves and stoppers, and 13-ml centrifuge tubes were used. The glassware was silanized with 5% dimethyldichlorosilane[2] in pyridine for 4 hr. The glassware was then washed with toluene, methanol, and methylene chloride and dried at 80°. The glassware was used up to 15 times before resilanization. After being used in an assay, the glassware was washed with laboratory detergent, methanol, and methylene chloride. The cylindrical separators and centrifuge tubes, together with the Teflon stoppers, were shaken vigorously with methanol

[1] Model 1015 Finnigan Corp., Sunnyvale, Calif.
[2] Applied Science Laboratories, Inc., State College, Pa.



Scheme I—Flow diagram of extraction procedure

and methylene chloride and dried just prior to performing an assay.

Synthesis of (−)-Scopolamine-(N-CD_3) Hydrobromide Hydrate—The deuterated compound was synthesized by the method of Schmidt *et al.* (11) who synthesized (−)-scopolamine-(N-$^{14}CH_3$) hydrobromide hydrate. Deuterated methyl iodide of 99.5 atom % isotopic purity[3] was used. The deuterated preparation gave a single peak by GLC, whose retention time was the same as scopolamine. The percent of the CH_3 moiety relative to the CD_3 was approximately 1%, as indicated by GLC–mass spectrometry.

Extraction Procedure—(−)-Scopolamine-(N-CD_3) hydrobromide hydrate, the internal standard, was added to 2–4 ml of plasma or urine. To the biological sample, contained in 30- or 60-ml cylindrical separators, were added 2 ml of 1 *M* carbonate buffer (pH 9.75) and 15–30 ml of methylene chloride[4]. If 4 ml of plasma was used, a large volume (30 ml) of methylene chloride was used to prevent emulsion formation. This was not a problem with urine. Also, the volume of organic solvent could be reduced in plasma extractions if 5% isopropanol[4] in methylene chloride was used. The inclusion of 5% isopropanol produced no extraneous peaks in the chromatograms.

The cylindrical separators were shaken vigorously in a horizontal position in a shaker[5] for 20 min and were then centrifuged at

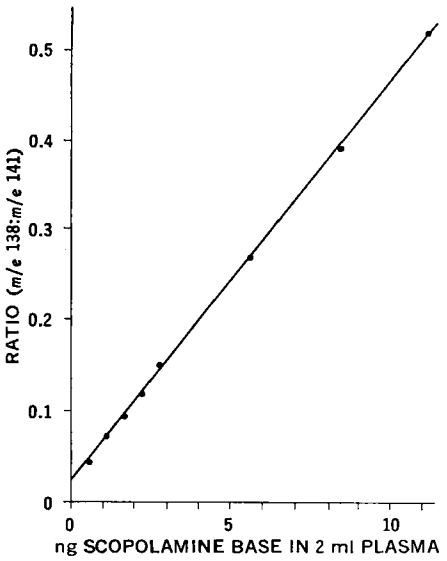


Figure 1—*Standard curve of scopolamine in plasma; 24.1 ng of internal standard, CD_3-scopolamine hydrobromide hydrate, was used.*

2000 rpm; the organic layer was removed through the Teflon valve into a 30-ml cylindrical separator containing 3 ml of 0.1 *N* H_2SO_4. These separators were then vigorously shaken for 10 min and centrifuged at 1500 rpm, and the organic layer was removed and discarded through the Teflon valve. To the 3 ml of 0.1 *N* H_2SO_4 was added 0.4 ml of 5 *N* NaOH, and the tubes were heated in a convection oven at 50–60° for 30 min.

After cooling, the alkaline aqueous phase was extracted with 8 ml of methylene chloride, the tubes were centrifuged at 1000 rpm, and the organic layer was released into a 13-ml centrifuge tube. Methylene chloride (1.5 ml), into which hydrogen chloride gas was bubbled, was added to this organic extract and the contents were vortexed. The contents were evaporated at 40° under a gentle stream of nitrogen[6] to approximately 1 ml. The aqueous alkaline phase was again extracted with 8 ml of methylene chloride and the organic phase was added to the 1 ml of the first extract in the 13-ml centrifuge tubes. After adding 1.5 ml of methylene chloride containing hydrogen chloride and vortexing, the contents were evaporated to dryness.

Care was taken to discontinue evaporation when the centrifuge tubes appeared dry. To the "invisible" residue was added 200 µl of methylene chloride plus 25 µl of heptafluorobutyric anhydride[7]. The centrifuge tubes were capped with Teflon stoppers and the contents were heated at 40° in a water bath. The centrifuge tubes were immersed to the 1-ml graduation so that the methylene chloride condensed in the upper part of the centrifuge tube and created a washing action. After 30 min the centrifuge tubes were spun briefly at 1500 rpm to concentrate the methylene chloride in the bottom of the tube. Methylene chloride and excess derivatizing reagent were removed under a gentle stream of nitrogen at 40°. The evaporation interval was 5 min.

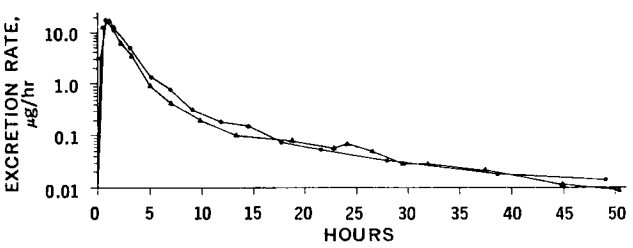


Figure 2—*Urinary excretion profiles following oral doses of scopolamine. Key: ▲, Subject 1, 906 µg (base); and ●, Subject 2, 800 µg (base).*

[3] ICN Isotope and Nuclear Division, Cleveland, Ohio.
[4] Nanograde solvents, Mallinckrodt Chemical Works, St. Louis, Mo.
[5] Eberbach Corp., Ann Arbor, Mich.
[6] N-Evap, Organomation Associates, Shrewsbury, Mass.
[7] Pierce Chemical Co., Rockford, Ill.

Table I—Parameters for Nonlinear Regression Fit of Urinary Excretion Data

	Six-Parameter Equation					
	A	*B*	*C*	*a*	*b*	*c*
Subject 1	35.5	0.29	116	0.72	0.068	4.5
Subject 2	30.1	0.28	50.7	0.58	0.066	3.1
	Four-Parameter Equation					
	A		*B*	*a*	*b*	
Subject 1	34.4		0.29	0.71	0.068	
Subject 2	26.9		0.27	0.56	0.065	

To the residue, 30–100 μl of benzene, followed by 15–50 μl of 1 *M* carbonate buffer (pH 10), was added. The contents were vortexed and then centrifuged at 2000 rpm. Up to 10 μl of the benzene layer was injected. The contents of the centrifuge tubes were maintained at 10° in a refrigerated centrifuge to minimize loss of the benzene layer due to evaporation. See Scheme I for a flow diagram of the extraction procedure.

Standard Curve and Sample Analysis—A three-point standard curve was constructed for each series of analyses because of significant slope and intercept variations of standard curves from day to day. A statistical analysis of this variation is presented in the discussion. Scopolamine hydrobromide[8] was added to either plasma or urine in amounts ranging from 2 to 30 ng when, for example, 30 ng of internal standard was employed. If smaller or larger quantities of the internal standard were employed, the quantities of scopolamine added were adjusted so that the ratios would span the range from about 0.1 to 1.0.

Quantities of CD_3-scopolamine hydrobromide hydrate between 2 and 30 ng were added to 2–4 ml of plasma or urine, depending on the level of drug expected in the biological fluids. If a ratio of less than 0.05 was expected for a given sample, the amount of internal standard was reduced to increase the ratio. However, the same standard curve, constructed with a different quantity of internal standard, could be used to obtain the amount of scopolamine in the sample. The ratio was used to obtain the quantity of scopolamine present in the biological sample from the standard curve. This ratio was multiplied by the ratio of the internal standard in the sample to the internal standard used to construct the standard curve (Fig. 1). This technique was similar to that employed by Garrett and Hunt in the analysis of tetrahydrocannabinol (10).

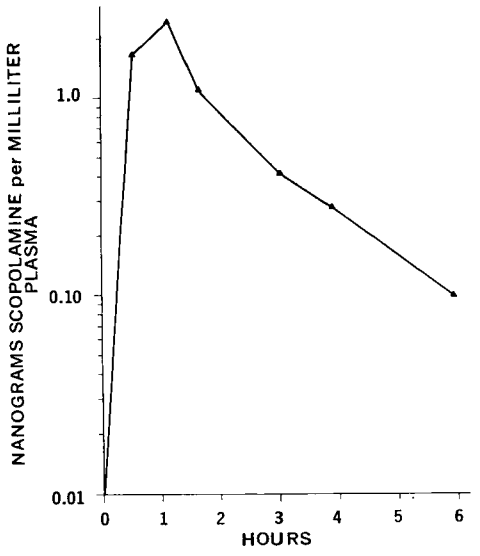


Figure 3—*Plasma levels following oral dose of scopolamine for Subject 1, 906 μg (base).*

[8] USP Reference Standard.

Table II—Major Mass Fragments of Heptafluorobutyryl CH_3-Scopoline and Heptafluorobutyryl CD_3-Scopoline

Heptafluorobutyryl CH_3-Scopoline		Heptafluorobutyryl CD_3-Scopoline	
m/e	% of *m/e* 81	*m/e*	% of *m/e* 81
81	100	81	100
138	86.1	141	81.2
94	20.8	97	22.2
96	11.3	99	10.9
108	6.3	111	4.9
110	5.4	113	5.0
154	3.1	157	3.0
169	2.5	169	1.8
351	0.4	354	0.35
---	—	138	0.82

RESULTS

The urinary excretion profiles for two subjects and the plasma levels of scopolamine for one of these subjects following oral administration of scopolamine are presented in Figs. 2 and 3, respectively. Figure 4 shows typical chromatograms obtained in the analysis of urine samples. The instantaneous clearance for scopolamine can be calculated from the curves and is close to 120 ml/min, indicating that glomerular filtration is the prime mode of clearance. The total amount of scopolamine excreted from both subjects was 4–5% of the orally administered dose.

The urinary excretion curves were fitted by a nonlinear regression program to the equation $dD_E/dt = Ae^{-at} + Be^{-bt} - Ce^{-ct}$ for each subject (Table I). Since there was a scarcity of data points prior to the maximum excretion rate, since there was the possibility of a lag time, and since *c*, the rate constant for absorption, was significantly larger than *a*, it was decided to fit the curves to equation $dD_E/dt = Ae^{-at} + Be^{-bt}$, neglecting points prior to the maximum (Table I). Thus, the parameters for the *a* and *b* phases of the

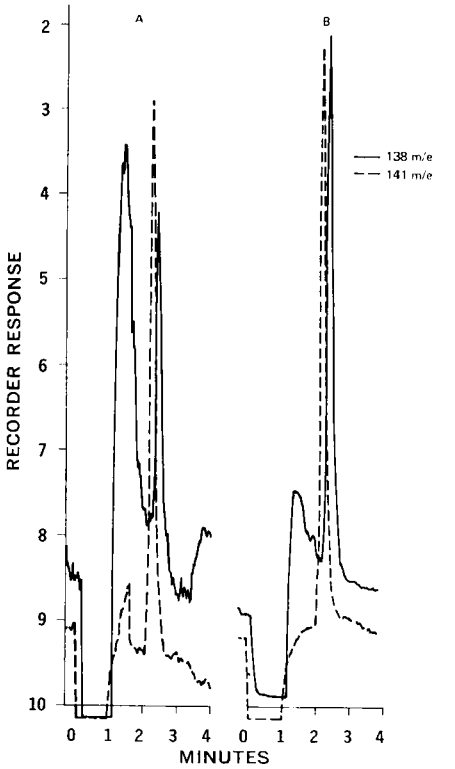


Figure 4—*Chromatograms of heptafluorobutyryl scopoline and heptafluorobutyryl CD_3-scopoline obtained from typical urine samples. Key: A, 0.192 ng/ml scopolamine (calculated) and 6.78 ng of internal standard; and B, 1.97 ng/ml scopolamine (calculated) and 13.56 ng of internal standard.*

excretion curves were not greatly affected by inclusion or neglect of the first-order absorption term. The parameters are functions of the rate constants for a two-compartment open model.

DISCUSSION

When scopolamine was derivatized with heptafluorobutyric anhydride and chromatographed, there was a tendency for the molecule to undergo pyrolytic elimination of heptafluorobutyric acid in the injection port. The presence of aposcopolamine was confirmed with GLC–mass spectrometry by a strong *m/e* 285 molecular ion. Therefore, it was decided to use a more indirect method, namely, the hydrolysis of scopolamine to scopoline and its conversion to a heptafluorobutyryl derivative (I), since it was more stable when chromatographed.

Compound I had excellent chromatographic properties in the 50–100-pg range. A quadrupole mass filter was used as a detector. The major mass fragments are listed in Table II. The most abundant fragment, retaining the three deuterium atoms at 30 ev, was *m/e* 141. Therefore, the *m/e* 138 and 141 fragments, representative of I and II, respectively, were monitored simultaneously. It was necessary to derivatize the free OH group in scopoline to prevent adsorption on the column. Furthermore, the ionization of the heptafluorobutyryl scopoline derivative (I) resulted in significantly less fragmentation than the ionization of scopoline under similar conditions.

Compound I had a tendency to undergo hydrolysis. However, 75% of the acylated derivative remained after 20 hr if benzene and carbonate buffer were added to the residue. The derivative was less stable in benzene in the absence of carbonate and markedly less stable in methylene chloride. When the hydrolysis of I was followed using a flame-ionization detector, a gradual increase in scopoline could be detected. Possibly, the carbonate liberated the free amine from a complex of I and heptafluorobutyric acid which was more susceptible to hydrolysis.

Absolute recoveries were investigated by adding 10 ng of scopolamine to 2 ml of plasma and conducting the extraction procedure. The recoveries, when measured against a standard prepared by derivatizing a known quantity of scopoline, ranged between 80 and 95%. The use of the deuterated internal standard removed this variability. However, high absolute recoveries were still desirable since the concentrations of scopolamine in the biological fluids were generally very small.

As mentioned previously, the method was somewhat indirect. The major metabolite of scopolamine was shown to be scopolamine glucuronide in animal studies (4), which was not extracted in the assay procedure. Scopine and aposcopolamine, minor metabolites, interfere with measurement of scopolamine. However, the method was developed to evaluate various dosage forms in human subjects rather than to elucidate the metabolism of the drug. A more refined method to discern the metabolites is now under investigation.

The average residual sum of squares for 13 regression lines for 13 three-point standard curves was 6.05×10^{-4}. The difference between the sum of squares of the 39 data points about a single regression line and the above-mentioned sum of squares was 6.23×10^{-3}. The ratio between the two variances was 5.58. The value from F tables yielded F (24, 13, 0.99) = 3.59. Thus, there was strong indication of significant variation in the standard curves.

The average value of the slopes, when expressed as the ratio of the *m/e* 138 to 141 responses times the ratio of the amount of deuterated scopolamine to scopolamine, was 1.03 ± 3.2% *RSD*. The average value and relative standard deviation of the intercepts were 0.0167 and ±55%, respectively. The large variation in the intercept was largely due to the degree of resolution between the *m/e* 138 and 139 peaks. The much smaller variation in the slope was probably due to the accuracy in focusing on the *m/e* 138 and 141 fragments.

Sjöguist and Änggård (8) noted similar problems in the assay for homovanillic acid. Therefore, it was decided to run a three-point standard curve each time a series of biological samples was to be analyzed. To test the precision of the assay, five aliquots of a urine sample were analyzed on different days, each with its own standard curve. The average value and relative standard deviation were 254 pg/ml and 2.5%, respectively. The variations of the slope and intercept during an analytical run were negligible.

The response ratio was determined for seven 4-ml aliquots of urine. The concentration of scopolamine in this urine, from a patient who had taken an oral dose of scopolamine, was 144 pg/ml. The average value of the response ratio was 0.105 ± 2.9% *RSD*. Thus, the consistency in the addition of the internal standard and the extraction procedure was very good.

A statistical analysis of the precision in determining the response ratios was recently presented in a manufacturer's application bulletin (12). Large amounts of internal standard relative to the endogenous compound can decrease the precision when the internal standard contributes to the *m/e* fragment monitored for the endogenous compound. For this reason, the amount of CD_3-scopolamine added to samples was chosen to yield response ratios between 0.1 and 2.0. If a particular sample had a response ratio significantly different from this range, it was usually rerun with an appropriately adjusted amount of internal standard.

CONCLUSION

The experiments demonstrate the utility of selective ion monitoring in conjunction with stable isotopes as internal standards in pharmacokinetic studies. The requirements in developing sensitive assays using this technique are that: *(a)* the compound or a derivative chromatograph well at the subnanogram level, and *(b)* the compound or derivative fragment under electron-impact ionization in a manner to produce a few fragments representing a significant portion of the total ionization. By using a GLC–mass spectrometric method for scopolamine, the urinary excretion rates for the unconjugated drug, which represented 4–5% of an oral dose of less than 1 mg, were followed over five orders of magnitude.

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Alkylation by Secondary Alcohols III: Fusion of Medicinal Sulfanilamides with Benzhydrol

R. E. MOSKALYK [x] and J. L. MALICKY

Abstract □ The fusion of certain sulfanilamides with benzhydrol in the presence of anhydrous zinc chloride affords several different products, depending primarily on the temperature at which the reaction is carried out. With sulfanilamide itself, three different products were isolated at 100, 160, and 180°. A sequence of steps is suggested to account for the three products, one of which involves an intramolecular rearrangement of a benzhydryl moiety. The fusion of benzhydrol with *p*-toluidine gives 2,6-dibenzhydrylaniline and not the *N,N*-dibenzhydryl derivative as previously reported.

Keyphrases □ Sulfanilamides—fusion with benzhydrol, effect of reaction temperature, isolation and identification of products □ Benzhydrol—fusion of medicinal sulfanilamides, alkylation by secondary alcohols, effect of temperature □ Alkylation—by secondary alcohols, fusion of medicinal sulfanilamides with benzhydrol, effect of reaction temperature

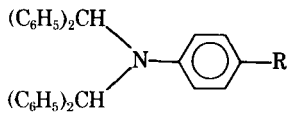
Sulfanilamides yield a number of interesting and useful substitution products when reacted with certain secondary alcohols under various reaction conditions. Initially, this reaction was studied employing xanthydrol as the alkylating agent (1). More recently, the reaction of sulfanilamides with benzhydrol in nitromethane, using perchloric acid as the catalyst, was reported (2). The products were normally the corresponding N^4-monobenzhydryl derivatives of type I and these proved to be stable crystalline compounds most suitable for the identification and differentiation of the antibacterial sulfanilamides.

The fusion of benzhydrol with a number of *para*-substituted anilines, in the presence of zinc chloride, gave the corresponding *N,N*-dibenzhydryl derivatives (II) (3, 4). Since this type of substitution had not been previously encountered in this laboratory under any reaction conditions, this investigation was initiated to prepare and study the products of the fusion reaction involving benzhydrol and the various sulfanilamides.

These compounds would be interesting not only from the standpoint of their potential use in the qualitative analysis of this class of drugs but also as model compounds for theoretical studies of both the structure–activity relationships and the mode of action of the medicinal sulfanilamides.

EXPERIMENTAL[1]

2,6-Dibenzhydryl-4-methylaniline (III)—Compound III was



II

R = $(C_6H_5)_2CH$ (3), CH_3, $CH(CH_3)_2$, Cl, Br, NO_2, $COOCH_3$, $COOCH_2CH_3$, OCH_3, and OCH_2CH_3 (4)

obtained by following the method reported by Giraud (4) for the preparation of *N,N*-dibenzhydryl-4-methylaniline (II, R = CH_3). To 2 g (0.019 mole) of *p*-toluidine and 3.5 g (0.038 mole) of benzhydrol was added 1 g of zinc chloride. The reaction mixture was heated in an open tube in a glycerin bath for 2 hr at approximately 150°.

After cooling, the solid residue was stirred with ethanol, resulting in the immediate formation of fine crystals which melted at 188° after several crystallizations from ethanol [lit. (4) mp 188° for the product of this reaction]; IR (mineral oil): 3390 and 3322 (NH_2) cm^{-1}; NMR ($CDCl_3$): τ 2.82 (s, 20H, C_6H_5), 3.55 (s, 2H, aromatic), 4.53 (s, 2H, methine), 6.74 (s, 2H, NH_2, D_2O exchangeable), and 8.03 (s, 3H, CH_3).

Anal.—Calc. for $C_{33}H_{29}N$: C, 90.16; H, 6.65; N, 3.19. Found: C, 90.14; H, 6.54; N, 3.42.

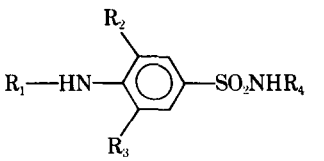
N^1-(2-Pyridyl)-N^4,3-dibenzhydrylsulfanilamide (IV)—Compound IV was obtained by fusing 1.0 g (0.004 mole) of sulfapyridine with 1.47 g (0.008 mole) of benzhydrol in the presence of 1.1 g (0.008 mole) of zinc chloride at 150° for 10 min. The same product was also obtained from a similar reaction mixture fused for 45 min at 185°. Stirring with ethanol afforded a white product which, after three crystallizations from ethanol, melted at 232.5–234.5°; IR (mineral oil): 3390 (NH) and 1147 (SO_2) cm^{-1}.

Anal.—Calc. for $C_{37}H_{31}N_3O_2S$: C, 76.39; H, 5.37; N, 7.22. Found: C,,76.26; H, 5.43; N, 7.07.

N^1-(2-Pyrimidyl)-N^4,3-dibenzhydrylsulfanilamide (V)—Compound V was prepared by fusing 2.2 g (0.005 mole) of benzhydrol and 1.8 g (0.01 mole) of sulfadiazine in the presence of 1.36 g (0.01 mole) of zinc chloride at 150° for 5 min. Treatment of the cooled reaction mixture with ethanol yielded the title compound which, when recrystallized twice from an acetone–water mixture, melted at 249–250°; IR (mineral oil): 3367 (NH) and 1163 (SO_2) cm^{-1}.

Anal.—Calc. for $C_{36}H_{30}N_4O_2S$: C, 74.20; H, 5.19; N, 9.62. Found: C, 74.24; H, 5.22; N, 9.48.

3,5-Dibenzhydrylsulfanilamide (VI)—Fusion of 2.2 g (0.012



compound	R_1	R_2	R_3	R_4
I	$(C_6H_5)_2CH$	H	H	H
IV	$(C_6H_5)_2CH$	$(C_6H_5)_2CH$	H	2-pyridyl
V	$(C_6H_5)_2CH$	$(C_6H_5)_2CH$	H	2-pyrimidyl
VI	H	$(C_6H_5)_2CH$	$(C_6H_5)_2CH$	H
VII	H	$(C_6H_5)_2CH$	$(C_6H_5)_2CH$	9-xanthenyl
VIII	$(C_6H_5)_2CH$	$(C_6H_5)_2CH$	H	$(C_6H_5)_2CH$
IX	H	$(C_6H_5)_2CH$	$(C_6H_5)_2CH$	$(C_6H_5)_2CH$
X	H	H	H	H
XI	$(C_6H_5)_2CH$	H	H	$(C_6H_5)_2CH$

[1] Melting points were determined using a Thomas-Hoover capillary melting-point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer IR spectrophotometer, model 21, as Nujol mulls. NMR spectra were taken on a Varian Associates model A-60 spectrometer. Elemental analyses were performed by Messrs. Weiler and Strauss, Oxford, England, and by the Microanalysis Laboratory, Department of Chemistry, University of Alberta. Benzhydrol was obtained as the commercial product from Eastman Kodak Co., Rochester, N.Y., and used without further purification. The sulfanilamides were obtained commercially or were gifts from the manufacturers.

mole) of benzhydrol and 1.0 g (0.006 mole) of sulfanilamide in the presence of 1.6 g (0.012 mole) of zinc chloride for 30 min at 180° yielded a pasty material. On cooling and stirring with ethanol, the title compound was isolated, mp 296–297° (ethanol); IR (mineral oil): 3378, 3300, and 3205 (NH_2 and SO_2NH_2) cm^{-1}.

Anal.—Calc. for $C_{32}H_{28}N_2O_2S$: C, 76.16; H, 5.59; N, 5.55. Found: C, 76.25; H, 5.81; N, 5.35.

N′,3,5-Tribenzhydrylsulfanilamide (IX)—Fusion of 3.0 g (0.016 mole) of benzhydrol and 1.0 g (0.006 mole) of sulfanilamide in the presence of 2.2 g (0.016 mole) of zinc chloride at 160° for 3 min, followed by stirring in ethanol, yielded a white product. After several recrystallizations from ethanol, the compound melted at 296°. A mixed melting point with VI showed no depression.

The remainder of the solution was concentrated and the gummy residue was extracted with chloroform. Upon evaporation of the chloroform, a gummy material was again isolated. However, stirring the gum in ethanol yielded a white precipitate which, after several crystallizations from ethanol, melted at 193–194.5°; IR (mineral oil): 3390 and 3300 (NH_2) cm^{-1}.

Anal.—Calc. for $C_{45}H_{38}N_2O_2S$: C, 80.56; H, 5.71; N, 4.18. Found: C, 80.69; H, 5.78; N, 4.42.

N′-Xanthenyl-3,5-dibenzhydrylsulfanilamide (VII)—Compound VII was prepared by reacting VI with xanthydrol following the procedure of Moskalyk and Chatten (1); mp 207–208° (acetone–water); IR (mineral oil): 3356, 3249, and 3289 (NH_2 and SO_2NH) cm^{-1}.

Anal.—Calc. for $C_{45}H_{36}N_2O_2S$: C, 78.92; H, 5.30; N, 4.09. Found: C, 78.38; H, 5.31; N, 4.40.

The compound could not be purified further.

RESULTS AND DISCUSSION

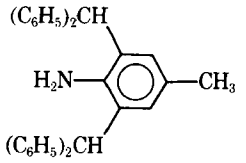
The physical properties of the first few compounds isolated from the fusion reaction with several sulfanilamides did not appear to correspond to the type of structure illustrated by II. Accordingly, the reaction of one of the compounds reported by Giraud (4), *p*-toluidine, was reexamined. Upon duplicating the procedure, a compound with an identical melting point (188°) to that reported was recovered. Elemental analysis verified that the compound was a disubstituted derivative. However, examination of the NMR and IR spectra revealed that the product was 2,6-dibenzhydryl-4-methylaniline (III).

The NMR spectrum consisted of five very well-resolved singlets. A 20-proton singlet at τ 2.82 accounted for the aromatic protons on the two benzhydryl groups. Three two-proton singlets at τ 3.55, 4.53, and 6.74 accounted for the aromatic, methine, and amino protons, respectively. The latter protons exchanged on deuteration. The last singlet, representing three protons, could be assigned to the methyl group (τ 8.03). In the IR, the spectrum still exhibited two peaks in the amino-stretching region, although these were depressed in intensity when compared to the parent amine.

In view of these results, the structure assigned to this compound by Giraud (4) (II, R = CH_3) is incorrect. Furthermore, this same structural assignment made to the eight other *para*-substituted aniline derivatives represented in II now appear to be in doubt also.

Sulfacetamide, when fused with benzhydrol at 150° for 2 hr, gave a product which did not exhibit any carbonyl or sulfonyl absorption in the IR region. It was shown to be 2,6-dibenzhydrylaniline[2], a compound that had been isolated previously from a reaction of *p*-aminobenzoic acid with benzhydrol (2). Fusion of the latter two compounds also gave 2,6-dibenzhydrylaniline.

Under similar reaction conditions, sulfapyridine failed to give a recoverable product. However, when the reaction was interrupted after 10 min at 150°, a white crystalline material was recovered which proved to be the dibenzhydryl derivative IV. The IR spectrum showed but a single band in the amino-stretching region characteristic of N^4-monosubstitution. The sulfonamide hydrogen was not substituted, as evidenced by the titratability of the compound as an acid[3]. The SO_2 symmetric stretching frequency at



III

1147 cm^{-1} suggested that the compound existed in the amido tautomeric form[4], as illustrated in IV. Treatment of N^4-monobenzhydrylsulfapyridine, prepared earlier (2), with benzhydrol under these fusion conditions gave IV, further supporting this structural assignment.

Sulfadiazine also yielded a disubstituted derivative when fused with benzhydrol in the presence of zinc chloride for 5 min. This product was likewise shown to be substituted on the amino nitrogen and in the position *ortho* to the nitrogen (V). These compounds (IV and V) represent yet another type of substitution pattern possible under fusion conditions and thus cast further doubt on the validity of Giraud's structural assignments.

Fusing sulfabenz with benzhydrol for 2 min afforded a monosubstituted product in very low yield. The compound was shown to be the N^4-monobenzhydryl derivative[5] reported previously. No disubstituted derivatives could be isolated from any of the fusion reactions carried out for longer periods.

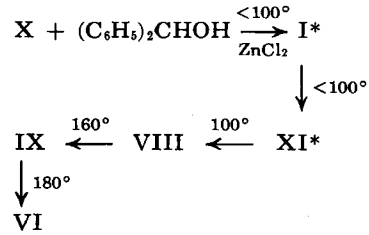
The preceding observations suggest that the first step in the alkylation reactions under fusion conditions is the substitution of a hydrogen on the N^4-nitrogen atom. This conclusion was borne out in the more detailed studies of the fusion reaction with sulfanilamide itself.

Sulfanilamide, when fused with a 2 molar ratio of benzhydrol at 180° for 30 min, afforded the dibenzhydryl derivative VI. Reaction of VI with xanthydrol gave VII. Employing a 3:1 molar ratio of benzhydrol to sulfanilamide, under identical reaction conditions, again gave VI, as did the reaction with a 5:1 molar ratio. The failure of these reactions with large excesses of benzhydrol to give derivatives alkylated also at the N'-position was puzzling in view of the ease with which this site can be alkylated with either benzhydrol (2) or xanthydrol (1) in solution.

Since alkylation at the N'-position with xanthydrol proceeds readily at room temperature and that with benzhydrol proceeds readily at about 70°, it seemed probable that N'-benzhydryl derivatives were being formed in the reaction but that they were subsequently cleaved at these higher fusion temperatures (150–180°). In support of this postulate, when a 3 molar ratio of benzhydrol was fused with sulfanilamide at 100° for 5 min, VIII[6] was isolated in high yield. To exemplify further the influence of the temperature, fusion of benzhydrol with sulfanilamide (3:1 molar ratio) at 160° afforded two compounds: VI and IX. Reaction of VI with benzhydrol in nitromethane and perchloric acid catalyst gave IX; with xanthydrol, it gave VII.

The preceding results suggest the sequence of steps for the reaction of benzhydrol with sulfanilamide at different fusion temperatures illustrated by Scheme I.

Although I and XI were not isolated under fusion conditions, I had been recovered previously from the corresponding reaction in



*Scheme I—*Not isolated under fusion conditions*

[2] Isolated from the reaction mixture after stirring with ethanol, mp 174–175.5° (acetone–water) [lit. (2) mp 174–175.5°]; IR (mineral oil): 3425 and 3356 (NH_2) cm^{-1}.

[3] Titrated in chloroform with 0.1 *N* KOH in methanol, using phenolphthalein as the indicator.

[4] The SO_2 symmetric stretching frequencies in the 1160–1145 and 1145–1125 cm^{-1} ranges have been assigned to the amido and imido forms, respectively (1, 5).

[5] Isolated by extraction of the gummy residue with chloroform, mp 215–216° (ethanol) [lit. (2) mp 216–218°].

[6] With a melting point of 206–208° (dioxane–water) [lit. (2) mp 206–208°].

solution (2) and the xanthenyl analog of XI had been reported (1). The formation of VIII from an equimolar reaction suggests that the monosubstituted derivative I is more reactive than the parent sulfanilamide X to further alkylation and that the resulting product XI is even more susceptible to further alkylation.

The conversion of VIII to IX at 160° involves the rearrangement of a benzhydryl moiety from the N^4-position to the second *ortho*-position on the phenyl ring. Some evidence was obtained to imply that this migration is an intramolecular one and could either occur by a concerted process or through an ion-pair intermediate. When VIII was heated in the presence of zinc chloride and pure sulfanilamide (X), the only product that could be isolated was IX, and there was no indication of any cross-products of an intermolecular process being formed. When this reaction was repeated using naphthalene rather than sulfanilamide, once again IX was the only product isolated. Fusion of I with sulfanilamide in the presence of zinc chloride somewhat surprisingly gave VI. Since no free benzhydrol was present in the reaction mixture, the second benzhydryl radical must have come from another molecule of I. This result tends to support an intramolecular rearrangement *via* an ion-pair mechanism.

When VIII was heated at 210° with an equal weight of sulfanilamide (X) in the absence of zinc chloride, only starting material was recovered. Therefore, these benzhydryl rearrangements are not simply thermodynamic, and the zinc chloride obviously functions as a Lewis acid in a manner analogous to that found in the benzidine rearrangement.

Rearrangements involving benzhydryl groups have been reported, but the mechanistic aspects have not been studied in detail. The rearrangement of the benzhydryl ether of *o*-cresol, in the presence of zinc chloride, to give the corresponding *p*-benzhydryl-*o*-methylphenol was reported (6), and the rearrangement of *N*-benzhydrylsalicylamide to the corresponding *para*-substituted phenol was observed (7). *N*-Benzhydryl-*o*-toluidine was found to yield two products, the *p*-benzhydryl- and the *o,p*-dibenzhydrylanilines, when heated in a hydrochloric–acetic acid medium (8). When the reaction was carried out in the presence of *o*-cresol, the cross-product, *p*-benzhydryl-*o*-methylphenol, was also recovered; accordingly, it was concluded that the rearrangement occurred through an intermolecular process. The apparent intramolecular rearrangement of the benzhydryl group observed in this study does not appear to have a precedent.

Attempted fusion reactions with sulfamerazine, sulfamethazine, sulfachlorpyridazine, sulfamethoxypyridazine, sulfaphenazole, sulfaquinoxaline, sulfamethoxazole, sulfadimethoxine, sulfaproxyline, sulfisoxazole, sulfamethizole, sulfathiazole, sulfaethidole, and sulfaguanidine afforded gums which could not be crystallized. Numerous attempts employing different reaction temperatures, different reaction times, and varying amounts of catalyst with each sulfanilamide invariably gave unresolvable gums. The only exceptions were sulfaquinoxaline[7] and sulfamethizole[8], both of which afforded the corresponding N^4-monobenzhydryl derivatives at lower fusion temperatures for shorter periods. Therefore, contrary to the report by Giraud (4), this reaction, under the conditions used in this study, did not yield *N,N*-dibenzhydryl derivatives with *para*-substituted anilines nor any other readily recoverable products with the majority of the sulfanilamides studied.

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[x] To whom inquiries should be directed.

[7] Fusion at 130° for 10 min, mp 223–225° (acetone–water) [lit. (2) mp 224–225°].

[8] Fusion at 130° for 5 min and stirring of the residue with ethanol, mp 214–215.5° (chloroform–hexane) [lit. (2) mp 212–212.5°].

Active Antitumor Components in a Decomposed Amino Sugar I: Effect of Sugar Structure on Activity

A. HAMEED KHAN and JOHN S. DRISCOLL ×

Abstract □ While pure methyl 5-(2-chloroethylamino)-5-deoxy-2,3-*O*-isopropylidene-β-D-ribofuranoside hydrochloride has no L-1210 leukemia activity, a decomposed sample was found to be very active. One of several approaches taken to determine the nature of the active component involved a study of how sugar structure affects antitumor activity. A number of aminoribose derivatives were prepared and tested against the murine L-1210 and P-388 leukemia and the B-16 melanoma tumor systems. Compounds were tested as pure materials and as synthetically degraded mixtures. Both the β-haloethyl group and a secondary amine were required for highest activity.

Keyphrases □ Amino sugar, decomposed—active antitumor components, effect of sugar structure on activity □ Sugars, amino—active antitumor components in decomposed samples, effect of structure on activity □ Antitumor activity—decomposed amino sugar □ Structure–activity relationships—decomposed amino sugar–antitumor activity

The one-arm sugar mustard, methyl 5-(2-chloroethylamino)-5-deoxy-2,3-*O*-isopropylidene-β-D-ribofuranoside hydrochloride (I), was prepared during studies of the antitumor activity of ribose alkylating agents (1). Although I was inactive in the murine L-1210 leukemia system when first tested, it was discovered later that a decomposed sample of I was highly active (T/C > 200%)[1] in that system. The entire sample was utilized, however, in the testing program. Resynthesis of I again confirmed the inactivity of the pure material.

A two-pronged approach was taken to determine the structure(s) of the active component(s) in the decomposed mixture of I. One study attempted purposely to degrade pure I and isolate and identify the active components[2]. The second approach, the subject of the study described here, was an investigation of the effect of chemical structure on the activity of purposely degraded samples. A method was devised to accelerate the production of active material from 5 years to 5 hr, and it was planned to prepare and degrade certain aminoribose derivatives to provide information about the structural characteristics required to produce active material from I.

RESULTS AND DISCUSSION

The first series of compounds prepared (Scheme I) was designed to answer the following two questions:

1. Was the nitrogen mustard group necessary for activity since it imparted no activity to I?
2. Was a secondary amine required or would a tertiary amine produce activity?

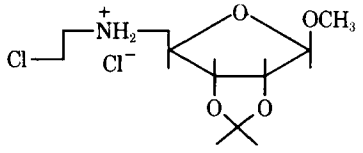
In an attempt to answer the first question, Compounds VI–IX were synthesized. The first key intermediate prepared was the tosylate (IV). It was so poorly reactive with the required amine nucleophiles, however, that the mesylate (V) had to be synthesized to obtain the desired amino sugars. The *N*-propyl derivative (VI) was synthesized, because in this compound the reactive chlorine was replaced with a completely inert methyl group while leaving the partition coefficient relatively undisturbed ($\pi_{CH_3} = 0.5$; $\pi_{Cl} = 0.39$) (3). The other three derivatives should be somewhat more hydrophilic than I. The bromo analog of I (XV) was also synthesized.

Compounds VI–IX and XV were tested in the leukemia L-1210 system. They were tested both as analytically pure materials and as the product from the synthetic degradation process shown to produce an active mixture from pure, inactive I. None of the nonhalogen-containing derivatives were L-1210 active in either form (pure or decomposed) when tested at 400, 200, and 100 mg/kg QD 1-9 or Q4D (2). The bromo analog (XV), however, was active both as a pure compound and after degradation (Table I). This activity is in contrast to that of the chloro compound (I), which was active only after degradation. Pure XV was highly active in the leukemia P-388 system (2). Since the yield of XV was low (Table II), it was not tested in the degraded form in the P-388 system and was tested only on the Day 1 schedule in L-1210.

A comparison between pure and degraded XV (Day 1 only) in L-1210 shows the degraded form to be more active. It is also less toxic based on mouse weight loss. While pure XV is L-1210 active and pure I is not, there are not enough data on degraded XV to make a definitive comparison of the two materials. It appears, however, that a halogen atom is required in the molecule to produce L-1210 activity in a degraded sample.

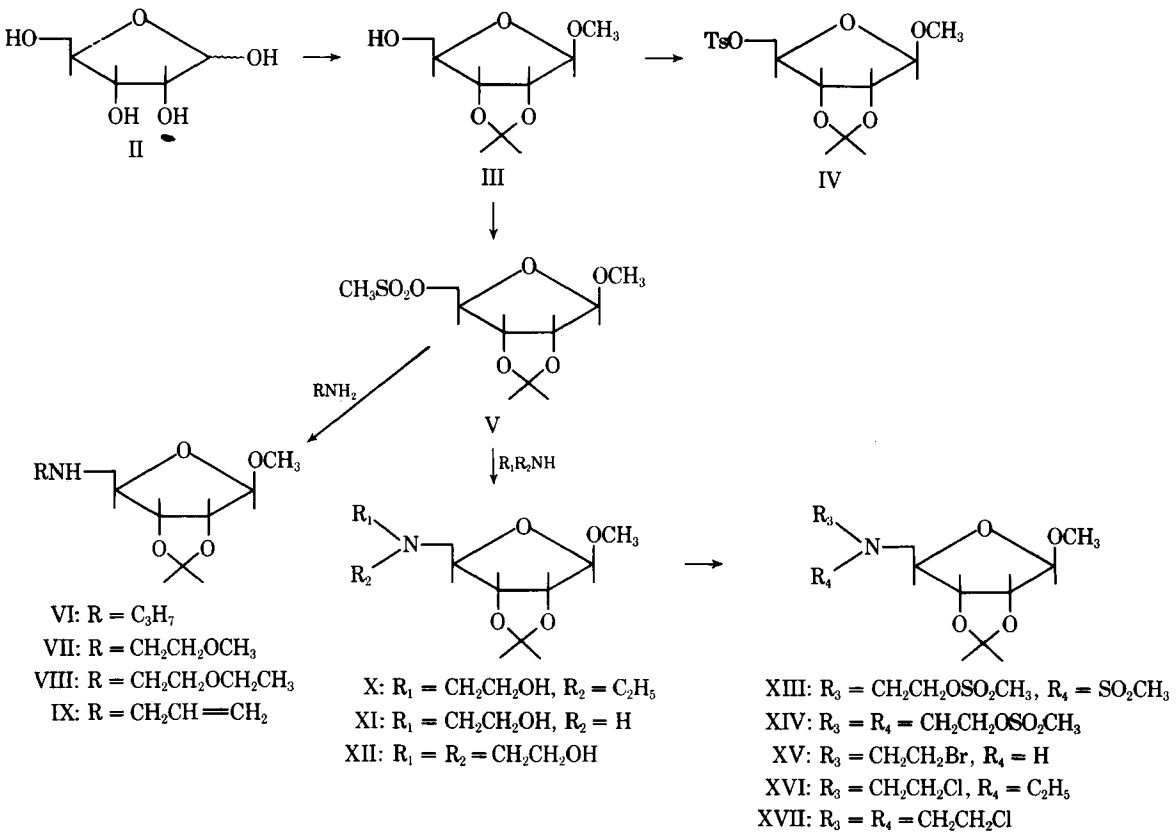
Compound XVI was prepared to explore the effect of additional substitution on the amine nitrogen. Since compounds of general type XVIII were possible degradation products based on analogy with the aminoxylose system (4), it appeared mechanistically impossible to produce XVIII from a tertiary amine such as XVI. Pure XVI had been prepared and tested (L-1210) in 1960, but that sample was inactive in limited testing. When the isopropylidene blocking group was removed from XVI, an active compound resulted (1). Compound XVI was resynthesized and a retest of the pure material showed reproducible L-1210 activity (Table I). Activity was also found in the leukemia P-388 and in the B-16 melanoma test systems. Degradation under standard conditions did produce a marginally L-1210 active mixture, but the activity was reduced, not enhanced as was found with secondary amines I and XV. Doses of degraded XVI higher than 3.1 mg/kg were toxic. A conclusion can be made that the change from a secondary to a tertiary amine in this series adversely affects the L-1210 activity of the degraded product by destroying the pure active compound, by nonproduction of an active intermediate, or both.

The two-arm mustard (XVII) also had been prepared and tested (1). Although the initial test of XVII showed L-1210 activity, confirmation testing was negative. Compound XVII was resynthesized for this study but in considerably lower yield than XVI. For this reason, only the pure, nondegraded material was tested (Table I). The bis(β-chloroethyl) compound was confirmed active in both the



I

[1] T/C = treated × 100%/control. For protocol details, see Ref. 2.
[2] This will be described in a subsequent report.



Scheme I—Synthesis of amino sugars

L-1210 and P-388 systems. This finding contrasts with the lack of L-1210 activity for the mono(β-chloroethyl) derivative, I. Compounds II–V and X–XIV (Scheme I) were evaluated both pure and as degraded materials in the L-1210 system and were inactive (T/C < 125%).

NMR evidence[2] indicates that acetone and methanol (loss of isopropylidene and methylglycoside) are liberated early in the acidic degradation procedure. The initial product of these reactions should have structure XXIV (Scheme II). This made the preparation of XXIV for testing desirable. Among all the amino sugars initially prepared, however, only I could not be hydrolyzed to a stable deblocked product (1).

Because of the suspected instability of XXIV, a reaction sequence was devised (Scheme II) that allowed the final step to proceed under mild conditions. The debenzylation of XXIII by hydrogen over palladium-on-charcoal in the aqueous acidic solution gave the reduced, hydrolyzed product, XXIV. A periodate oxidation was carried out to characterize this reaction product (11). Methyl

Table I—Murine Antitumor Activity

Compound	Form	Tumor System	Treatment Schedule[a]	Dose, mg/kg/day	Activity (T/C), %
XV	Pure	L-1210	Day 1 only	100	125
	Pure	L-1210	Days 1, 5, 9	100	157
				50	131
	Pure	L-1210	QD 1–9	36	137
	Pure	P-388	QD 1–9	36	250
				18	205
				9	188
	Degraded	L-1210	Day 1 only	400	144
				200	126
XVI	Pure	L-1210	QD 1–9	18.5	144
				12.5	126
	Pure	P-388	QD 1–9	12.5	188
				8.2	211
				4.0	195
				2.0	158
	Pure	B-16	QD 1–9	7.2	145
				4.3	129
	Degraded	L-1210	QD 1–9	3.1	125
XVII	Pure	L-1210	Days 1, 5, 9	6.2	139
				4.1	130
				2.7	138
				1.7	133
	Pure	P-388	QD 1–9	3.1	227
				1.0	241
				0.3	154

[a] Reference 2.

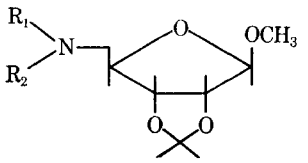


Table II—Physical Constants for Some Aminoribose Derivatives

Compound	R_1	R_2	Melting Point	Yield, %	Formula	Analysis, % Calc.	Found
VI	$CH_2CH_2CH_3$	H	145°	67	$C_{12}H_{23}NO_4 \cdot HCl$	C 51.16	51.46
						H 8.52	8.80
						Cl 12.61	12.56
						N 4.97	4.82
VII	$CH_2CH_2OCH_3$	H	104°	76	$C_{12}H_{23}NO_5 \cdot HCl$	C 48.35	48.10
						H 8.10	8.31
						Cl 11.93	11.70
						N 4.70	4.65
VIII	$CH_2CH_2OC_2H_5$	H	150°	36	$C_{13}H_{25}NO_5 \cdot HCl$	C 50.08	50.10
						H 8.35	8.20
						Cl 11.40	11.20
						N 4.49	4.55
IX	$CH_2CH{=}CH_2$	H	135°	37	$C_{12}H_{21}NO_4 \cdot HCl$	C 51.62	51.93
						H 7.88	8.03
						Cl 12.73	12.52
						N 5.02	4.93
X	CH_2CH_2OH	CH_2CH_3	135°	59	$C_{13}H_{25}NO_5 \cdot HCl$	C 50.10	49.80
						H 8.35	8.29
						Cl 11.49	11.73
						N 4.49	4.41
XI	CH_2CH_2OH	H	142°	86	$C_{11}H_{21}NO_5 \cdot HCl$	C 46.56	46.54
						H 7.76	7.57
						Cl 12.52	12.50
						N 4.94	4.90
XII	CH_2CH_2OH	CH_2CH_2OH	148°	33	$C_{13}H_{25}NO_6 \cdot HCl$	C 47.63	47.56
						H 7.93	8.06
						Cl 10.84	10.76
						N 4.27	4.21
XIII	SO_2CH_3	$CH_2CH_2OSO_2CH_3$	89°	52	$C_{13}H_{25}NO_9S_2$	C 38.70	38.69
						H 6.25	6.23
						N 3.47	3.24
						S 15.90	15.80
XIV	$CH_2CH_2OSO_2CH_3$	$CH_2CH_2OSO_2CH_3$	80°	36	$C_{15}H_{29}NO_{10}S_2$	C 40.27	40.24
						H 6.48	6.28
						N 3.13	3.40
						S 14.32	14.62
XVI	CH_2CH_2Cl	C_2H_5	117°	82	$C_{13}H_{24}ClNO_4 \cdot HCl$	C 47.28	47.13
						H 7.57	7.87
						Cl 21.52	21.98
						N 4.24	3.98
XVII	CH_2CH_2Cl	CH_2CH_2Cl	128°	32	$C_{13}H_{23}Cl_2NO_4 \cdot HCl$	C 42.80	42.78
						H 6.58	6.83
						Cl 29.22	29.24
						N 3.84	3.62

α-D-mannopyranoside was used to standardize the procedure (12). Compound XXIV consumed 2 moles of periodate with the formation of 1 mole of formic acid. This result is consistent with the structure shown for XXIV but inconsistent with the structures expected for a hydroxypiperidine rearrangement product or an open chain sugar.

Compounds XIX–XXIII were inactive in the L-1210 system. Enough XXIV was obtained to carry out an L-1210 dose–response test (800, 400, 200, and 100 mg/kg) on the Day 1 treatment schedule (2). This test was negative at all doses. Decomposed samples of I have given T/C values > 200% under the same treatment conditions. The search for the active component in decomposed I is continuing.

EXPERIMENTAL[3]

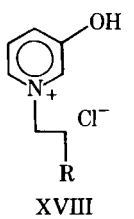
Decomposition Studies—The deionized water used was deoxygenated for 24 hr with nitrogen prior to being warmed to 60° in a three-necked, round-bottom flask equipped with a reflux condenser, magnetic stirrer, and a gas inlet tube. A slow stream of nitrogen was passed into the flask during the reaction period. The material to be degraded was added to the warm solution to give a concentration of 100 mg/ml. The stirred solution was maintained at 60° for 5.0 hr, cooled, and lyophilized to give the test mixture.

Methyl 2,3-*O*-Isopropylidene-β-D-ribofuranoside (III)—This compound was prepared as a yellow syrup [method of Leonard and Carraway (5)] in 62% yield on a 30-g scale.

Methyl 2,3-*O*-Isopropylidene-5-*O*-*p*-toluenesulfonyl-β-D-ribofuranoside (IV)—This material was prepared as white needles (14.9 g, 82%), mp 82–83° [lit. (6) mp 83.5–84.5°] by the method of Shunk *et al.* (6).

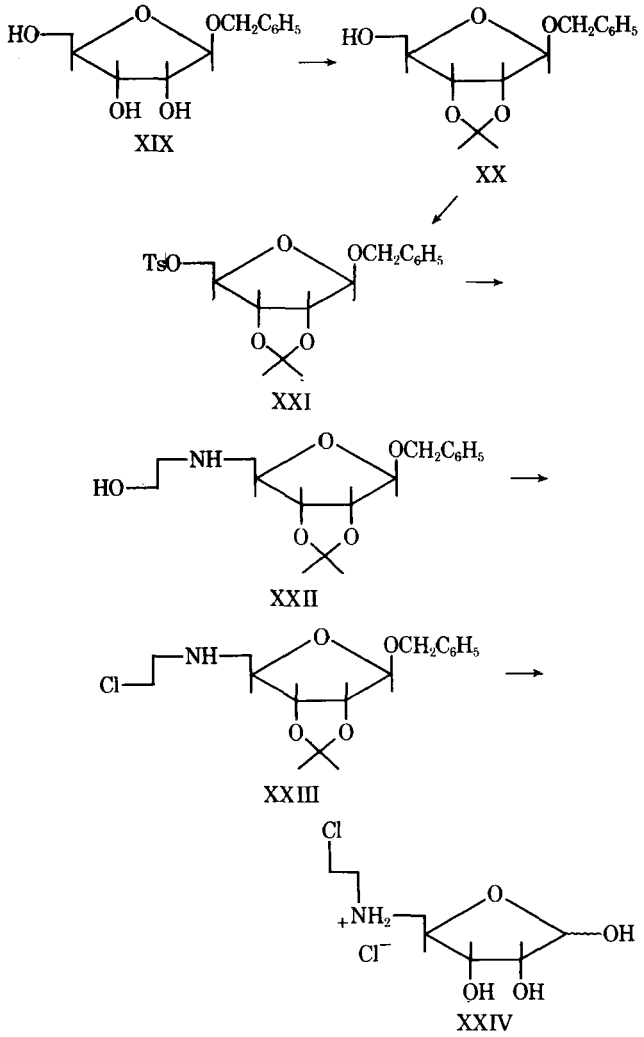
Methyl 2,3-*O*-Isopropylidene-5-*O*-methanesulfonyl-β-D-ribofuranoside (V)—This material was prepared by the procedure of Kissman and Weiss (7), as a white crystalline solid (49 g, 88%), mp 75–76° [lit. (7) mp 73–74°].

Methyl 5-Alkylamino-5-deoxy-2,3-*O*-isopropylidene-β-D-ribofuranosides (General Procedure for VI–IX)—A solution of



XVIII

[3] Melting points were determined on a Thomas-Hoover apparatus and are uncorrected. Elemental analyses were carried out by Dr. W. C. Alford, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health.



Scheme II—Synthesis of deblocked I

V (5.0 g) in 25 ml of the appropriate amine was heated in a pressure bottle at 115° for 17 hr. The solution was cooled and concentrated *in vacuo*. The residue was dissolved in water and extracted with ether (3 × 100 ml). The ether layer was washed with water and dried (sodium sulfate). Addition of dry hydrogen chloride to the ether solution caused the product to precipitate as the hydrochloride salt. The products were recrystallized from methanol–ether (Table II).

Methyl 5-(2-Hydroxyethylamino-5-deoxy-2,3-*O*-isopropylidene-β-D-ribofuranosides) (General Procedure for X–XII)—A stirred mixture of V (10 g) and the appropriate ethanolamine derivative (30 ml) was heated at 150° for 4 hr. The solution was cooled and diluted with chloroform (100 ml). The chloroform solution was washed with four 10-ml portions of water, and the chloroform solution was dried (sodium sulfate). The chloroform was removed *in vacuo*, and the residual oil was dissolved in dry ether to which dry hydrogen chloride was added. The resulting white crystalline solids were isolated (Table II).

Methyl 2,3-*O*-Isopropylidene-5-deoxy-5-[(2-hydroxyethyl)(methylsulfonyl)amino]-β-D-ribofuranoside Methanesulfonate (Ester) (General Procedure for XIII and XIV)—To a stirred, dry ice–acetone cooled solution of XI (6.7 g) in dry pyridine (30 ml) was added, dropwise, methanesulfonyl chloride (5 ml). After the addition was complete (0.5 hr), the reaction mixture was refrigerated overnight. The mixture was added slowly to a vigorously stirred ice–water mixture. The resulting solution was extracted with chloroform. The chloroform layer was washed with water, dried (sodium sulfate), and concentrated to dryness. The resulting solid was recrystallized from ethanol (Table II).

Methyl 5-(2-Bromoethylamino)-5-deoxy-2,3-*O*-isopropylidene-β-D-ribofuranoside Hydrobromide (XV)—A solution of XI (1.5 g) in dry methylene chloride (20 ml) and 2,6-lutidene (0.7 ml) was heated gently on a steam bath under anhydrous conditions. To the refluxing solution was added a solution of thionyl bromide (1.0 ml) in dry methylene chloride (10 ml). After the addition was complete (1 hr), the solvent was distilled and the residual solid was poured into a stirred ice–water mixture containing 10% sodium bicarbonate. The solution was extracted with ethyl acetate, and the extract was dried (sodium sulfate) and passed through a column of alumina. The eluate was distilled under reduced pressure to remove the solvent, and the residue was dissolved in dried ether. A white crystalline solid precipitated (0.4 g, 26%, mp 138°) upon the addition of dry hydrogen bromide gas.

Anal.—Calc. for $C_{11}H_{21}Br_2NO_4$: C, 33.70; H, 5.37; Br, 40.92; N, 3.58. Found: C, 33.30; H, 5.59; Br, 40.79; N, 3.45.

Methyl 2,3-*O*-Isopropylidene-5-[*N*-ethyl-(2-chloroethyl)-amino]-5-deoxy-β-D-ribofuranoside Hydrochloride (General Procedure for XVI and XVII)—A solution of X (9.48 g) in dry methylene chloride (25 ml) was saturated with dry hydrogen chloride under anhydrous conditions. Thionyl chloride (10 ml) was added dropwise to the refluxing solution. After the addition was complete (2 hr), the solution was refluxed for 1 hr. Excess solvent was removed *in vacuo*. The residue was dissolved in dry ether (1 liter) and saturated with dry hydrogen chloride. On standing overnight, a white crystalline solid separated from the solution (Table II).

Benzyl β-D-Ribofuranoside (XIX)—This compound was prepared by the method of Ness *et al.* (8) in 69% yield. The resulting semisolid syrup was recrystallized from ethyl acetate to give white prisms, mp 103° [lit. (8) mp 105°].

Benzyl 2,3-*O*-Isopropylidene-β-D-ribofuranoside (XX)—A mixture of XIX (6.6 g), anhydrous acetone (90 ml), 2,2-dimethoxypropane (10 ml), and anhydrous copper sulfate (5 g) was stirred at room temperature overnight. The solution was filtered through a layer of charcoal, and the solvent was removed under reduced pressure. The residual solid was crystallized from ether to give 6.9 g (88%) of white microneedles, mp 105° [lit. (9) mp 104–105°].

Benzyl 2,3-*O*-Isopropylidene-5-*p*-toluenesulfonyl-β-D-ribofuranoside (XXI)—This compound was prepared by the method of Hanessian and Haskell (10) to give 10.2 g (66%) of white needles, mp 95° [lit. (10) mp 93–95°].

Benzyl 2,3-*O*-Isopropylidene-5-[(2-hydroxyethyl)amino]-5-deoxy-β-D-ribofuranoside (XXII)—A suspension of XXI (10.0 g) in ethanolamine (40 ml) was heated at 160° for 4 hr. The cooled solution was diluted with chloroform (100 ml) and washed with three 10-ml portions of water. After drying (sodium sulfate), the solvent was removed *in vacuo* and the residual oil was distilled (175°/0.2 torr). The distillate solidified on standing overnight to give 6.2 g (86%) of white crystals, mp 45°.

Anal.—Calc. for $C_{17}H_{25}NO_5$: C, 63.14; H, 7.74; N, 4.33. Found: C, 63.26; H, 7.89; N, 4.11.

Benzyl 2,3-Isopropylidene-5-[(2-chloroethyl)amino]-β-D-ribofuranoside Hydrochloride (XXIII)—A solution of XXII (2.0 g) in dry methylene chloride (15 ml) was saturated with dry hydrogen chloride. Thionyl chloride (5 ml) in methylene chloride (20 ml) was added dropwise to the refluxing solution. After addition was complete (1 hr), the solution was heated under reflux for 3 hr. The solvent was removed *in vacuo*, and the residue was dissolved in chloroform. The solution was washed once with a 10% ice-cold solution of sodium carbonate and twice with water and was dried (sodium sulfate). The chloroform was removed *in vacuo*, and the residue was dissolved in dry ether (100 ml). An oily residue resulted upon addition of dry hydrogen chloride. This residue solidified on standing overnight. Recrystallization from dimethylformamide–ether gave 1.2 g (51%) of yellow prisms, mp 148°.

Anal.—Calc. for $C_{17}H_{25}Cl_2NO_4$: C, 53.96; H, 6.61; Cl, 18.78; N, 3.70. Found: C, 53.85; H, 6.58; Cl, 18.39; N, 3.71.

5-[(2-Chloroethyl)amino]-5-deoxy-D-ribofuranoside Hydrochloride (XXIV)—A solution of XXIII (0.3 g) in water (20 ml) containing concentrated hydrochloric acid (0.3 ml) was reacted with hydrogen over 10% palladium-on-charcoal catalyst (0.2 g) for 22 hr at 23° in a Parr apparatus. The solution was filtered and lyophilized to give 0.2 g (88%) of a highly hygroscopic white crystalline solid.

Anal.—Calc. for $C_7H_{15}Cl_2NO_4 \cdot 2H_2O$: C, 29.58; H, 6.59; Cl, 25.00; N, 4.93. Found: C, 29.70; H, 6.46; Cl, 24.60; N, 4.84.

The periodate oxidation of XXIV was carried out using the

method of Jackson and Hudson (11, 12). Titration over 5 hr showed that 2.1 moles of periodate was consumed and that 0.98 mole of formic acid had been produced by the oxidation, indicating a ribofuranose ring structure.

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Effect of Ethanol on Theophylline Absorption in Humans

RENU KOYSOOKO, ELLIOT F. ELLIS, and GERHARD LEVY [x]

Abstract □ This study was carried out to determine if ethanol, which enhances theophylline absorption from the rat small intestine, has a similar effect when administered orally to human subjects. Seven normal adults received 200 mg of theophylline/m^2 of body surface area, in 50 ml of either aqueous solution or hydroalcoholic solution containing 20% ethanol. There was no significant difference in the average plasma concentrations of theophylline produced by these two solutions, but three subjects (all female) experienced nausea after taking the aqueous solution while none became nauseous after taking theophylline in the hydroalcoholic solution.

Keyphrases □ Theophylline—effect of ethanol on absorption, humans □ Ethanol—effect on absorption of theophylline, humans □ Absorption—theophylline, effect of ethanol, humans

Theophylline is an effective bronchodilator used widely for the treatment of asthma. It is desirable to have oral dosage forms available from which this drug is rapidly absorbed so that acute attacks of asthma can be treated and relieved promptly at home. There have been claims that the absorption of theophylline is enhanced if administered in hydroalcoholic solution, but the evidence is conflicting (1–4).

Recently, an initial concentration of 5% ethanol was found to increase significantly the absorption of theophylline from a ligated segment of small intestine of anesthetized rats, and a constant concentration of 2% ethanol (and even lower concentrations in unpublished studies) increased appreciably the absorption of theophylline from the perfused small intestine of anesthetized rats (5). This absorption-enhancing effect of ethanol was associated with an increase in the net flux of water from the intestinal lumen and may be due to solvent drag (5). The study described here was initiated to determine if the rate of absorption of theophylline can be increased in humans by administering the drug in hydroalcoholic rather than in aqueous solution.

EXPERIMENTAL

Seven healthy volunteers (four females and three males), 24–33 years old and capable by education and background (registered nurses and graduate students in pharmaceutics) to give informed consent, participated in the study. They were instructed to abstain from coffee, tea, chocolate, cola drinks, and alcoholic beverages for 24 hr before and during the study and to take no drugs (except oral contraceptives if these were used regularly) for 3 days before and during the study.

Theophylline, 200 mg/m^2 body surface area, dissolved either in 50 ml of water or in 50 ml of hydroalcoholic solution containing 20% (v/v) ethanol, was administered in the morning 1 hr after a light breakfast. The two preparations were given in crossover fashion, 1 week apart. Fifteen milliliters of blood was withdrawn through an indwelling venous catheter into a heparinized syringe immediately before drug administration, and 10-ml blood samples were obtained at 10, 30, 60, 120, 240, 360, and 480 min thereafter. Plasma was separated and stored in a freezer pending assay.

At the end of the experiment, each subject was given a card with the questions: "Did you notice any pharmacologic effects during the day? If so, what and when?" There was no additional questioning, elaboration, or discussion to prevent any biased suggestive influences on the response to the question.

Theophylline concentrations in plasma were determined by the spectrophotometric method of Schack and Waxler (6), modified for smaller sample volumes and greater sensitivity (7). Blank values and recovery of theophylline were determined for each subject by assaying the zero-time plasma as such and after adding theophylline to yield a concentration of 10 μg/ml. The blank values ranged from 1.48 to 3.47 μg apparent theophylline/ml, and the recovery of added drug ranged from 85.1 to 97.7%. The analytical re-

method of Jackson and Hudson (11, 12). Titration over 5 hr showed that 2.1 moles of periodate was consumed and that 0.98 mole of formic acid had been produced by the oxidation, indicating a ribofuranose ring structure.

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Table I—Theophylline Concentration in Plasma of Healthy Adults after Oral Administration of an Aqueous Solution of Theophylline[a]

Subject	Sex	Age, years	Body Weight, kg	Height, cm	Minutes 10	30	60	120	240	360	480
					Theophylline Concentration, μg/ml						
1	F	24	49.5	160	3.59	11.91	11.80	13.35	10.05	7.43	4.83
2	F	27	63.0	171	2.99	8.62	10.71	9.82	8.30	7.25	5.78
3	F	33	63.0	162	6.75	14.88	13.64	11.78	9.37	8.07	6.75
4	F	28	55.2	160	2.02	9.69	13.14	13.74	10.51	7.82	6.47
5	M	27	71.4	169	6.96	9.95	11.41	11.16	9.37	8.07	7.65
6	M	28	66.8	173	14.34	13.98	13.52	11.37	8.58	7.68	6.15
7	M	29	75.0	188	14.08	13.46	12.70	10.94	8.13	6.15	5.02
Mean					7.25	11.78	12.42	11.74	9.19	7.50	6.09
SD					5.10	2.42	1.13	1.38	0.90	0.67	0.99

[a] Two hundred milligrams of theophylline/m² of body surface area.

sults were corrected for each individual according to these values. Ethanol concentrations in plasma were determined by the GC method of Cooper (8) with certain modifications (5).

RESULTS

The plasma theophylline concentrations after oral administration of the drug in aqueous and hydroalcoholic solution are listed in Tables I and II, respectively, and the average data are shown in Fig. 1. There was no statistically significant difference (by paired *t* test) between the theophylline concentrations produced by these two solutions at any time, but peak concentrations in four of the seven subjects appeared earlier with the aqueous solution. Theophylline concentration peaks in the other three subjects occurred at the same time with both solutions.

The concentrations of ethanol in the plasma after administration of theophylline in hydroalcoholic solution are listed in Table III. Maximum concentrations were usually achieved at 30 min; the ethanol concentrations in plasma became negligible 2 hr after administration of the hydroalcoholic solution. The apparent biological half-life of theophylline, determined from the exponential portion of the individual plasma concentration curves, averaged slightly less than 7 hr (Table IV). Three of the seven subjects (all but one of the female subjects in the study) reported nausea after administration of the aqueous solution, while there were no reports of nausea after administration of the hydroalcoholic solution (Table V).

DISCUSSION

The minimum effective plasma concentration of theophylline is generally considered to be about 5 μg/ml, and the average therapeutic concentration is about 10 μg/ml (9). The results of this study show that the minimum effective concentration could be achieved in most subjects within 10 min and maintained in the subjects for 480 min after administration of 200 mg theophylline/m² in either aqueous or hydroalcoholic solution. Therapeutically optimum concentrations occurred usually at 60 min and only occasionally as early as 10 min after drug administration.

Considering the desirability of achieving optimum plasma theophylline concentrations rapidly in case of an acute asthma attack, there is a rational basis for attempting to enhance the rate of theophylline absorption beyond that which can usually be achieved by oral administration of the drug in aqueous solution. While low concentrations of ethanol were found to enhance theophylline absorption in rats when a solution of the drug was introduced directly into the small intestine (5), a similar effect was not apparent upon oral administration to human subjects under the conditions of this study. It is possible that the absorption-enhancing effect of ethanol on theophylline may have been dampened by the process of gastric emptying in this study. The amount of ethanol taken by the subjects may even have been sufficient to inhibit gastric emptying (10, 11) and thereby to decrease actually the rate of theophylline absorption. Use of smaller amounts of ethanol or of one of the other absorption-enhancing agents recently discovered in this laboratory[1] may be more rewarding.

There have been reports (12, 13) of a feeling of mild inebriation in some subjects after oral administration of 75 ml of a hydroalcoholic solution of theophylline containing 20% ethanol. The results of this study, using similar doses of ethanol, indicate that the concentrations in plasma are well below those found to elicit a noticeable pharmacological effect (14, 15). Interestingly, none of the subjects complained of nausea after taking theophylline in hydroalcoholic solution but three subjects, all women, reported nausea after taking theophylline in aqueous solution. Since the theophylline concentration in the plasma of these three subjects was

Table II—Theophylline Concentration in Plasma of Healthy Adults after Oral Administration of a Hydroalcoholic Solution of Theophylline[a]

Subject	Minutes 10	30	60	120	240	360	480
	Theophylline Concentration in Plasma, μg/ml						
1	2.70	3.45	10.91	13.15	12.30	6.07	4.37
2	5.13	8.88	11.33	11.72	9.89	7.86	7.00
3	7.88	11.01	11.09	11.35	8.69	6.60	5.07
4	5.73	8.69	10.51	11.11	9.88	7.79	6.74
5	8.10	11.12	12.15	11.70	8.94	7.99	7.19
6	9.26	8.51	13.23	10.41	8.54	7.55	6.22
7	2.91	9.82	14.08	12.90	10.59	7.99	7.61
Mean	5.96	8.78	11.90	11.76	9.83	7.41	6.31
SD	2.58	2.58	1.32	0.97	1.32	0.76	1.19

[a] Two hundred milligrams of theophylline/m² of body surface area.

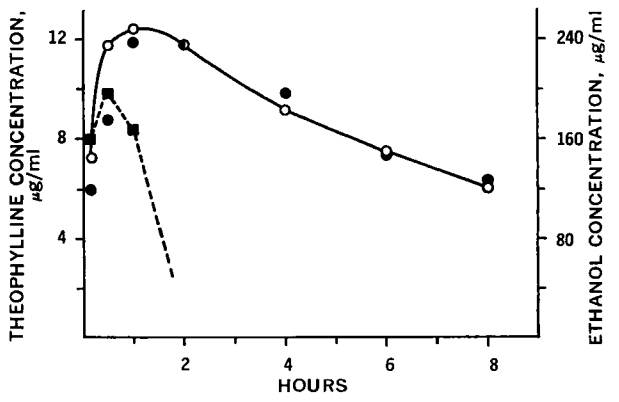

Figure 1—*Mean plasma theophylline concentrations in seven normal adult subjects after oral administration of 200 mg of theophylline/m² of body surface area in aqueous solution* (○) *and hydroalcoholic solution* (●). *The ethanol content of the latter was equivalent to 10 ml/m² of body surface area and produced the plasma concentrations represented by* ■ *and the dashed line.*

[1] J. B. Houston and G. Levy, to be published.

Table III—Ethanol Concentration in Plasma of Normal Adults after Oral Administration of a Hydroalcoholic Solution of Theophylline[a]

Subject	Minutes 10	30	60	120
Ethanol Concentration in Plasma, μg/ml				
1	193.0	204.2	165.9	—[b]
2	82.2	132.8	21.5	—[b]
3	180.3	190.7	223.0	—[b]
4	73.3	204.3	172.1	53.0
5	229.8	206.7	89.4	—[b]
6	229.5	221.3	143.2	38.7
7	140.0	222.6	342.0	69.2
Mean	161.2	197.5	166.7	
SD	64.8	30.6	102.3	

[a] Ten milliliters of ethanol/m² of body surface area. [b] Less than 20 μg/ml.

not unusually high and not higher than that of the other subjects in the study, the nausea probably was due to a local rather than central effect of the drug. The observations are at best suggestive, but they are in agreement with those of a previous investigation (2). Jørgensen and Møller reported that two subjects who took 400 mg theophylline in water suffered from severe abdominal colic and nausea while no such effects were noted in 16 patients when theophylline was administered in hydroalcoholic solution containing 20% ethanol (2).

The apparent biological half-life of theophylline found in this study ranged from about 4 to 9 hr and averaged slightly less than 7 hr. These results are well within the range of previous studies on adult subjects but somewhat longer on the average than the mean values of 4.4 and 5.2 hr observed by Mitenko and Ogilvie (16) and Jenne *et al.* (17), respectively, after intravenous injection. It is possible that protracted absorption affected the biological half-life estimates in this study and they have therefore been designated as apparent half-lives.

Table IV—Apparent Biological Half-Life of Theophylline in Normal Adults after Oral Administration of Aqueous and Hydroalcoholic Solutions

Subject	Apparent Half-Life, hr: Aqueous Solution	Hydroalcoholic Solution
1	3.78	4.19
2	8.03	7.80
3	7.06	5.15
4	5.71	7.23
5	8.56	8.81
6	7.09	8.31
7	5.30	7.43
Mean	6.50	6.98
SD	1.67	1.69

Table V—Incidence of Nausea after Administration of Theophylline in Aqueous and Hydroalcoholic Solutions

Subject	Aqueous Solution	Hydroalcoholic Solution
1	−	−
2	+	−
3	+	−
4	+	−
5	−	−
6	−	−
7	−	−

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Pharmacokinetics of Carbamazepine in Monkeys following Intravenous and Oral Administration

R. H. LEVY ˣ, J. S. LOCKARD, J. R. GREEN, P. FRIEL, and L. MARTIS

Abstract □ The pharmacokinetics of carbamazepine were evaluated in four male rhesus monkeys. A 20-mg/kg dose was administered by intravenous (5-min) infusion and orally (nasal–gastric intubation) in a propylene glycol–ethanol–water solvent. Plasma and urine determinations were performed by GLC. All semilogarithmic intravenous curves exhibited an irregular decay behavior in the first 3-hr period, followed by a linear disappearance phase ($T_{1/2}$ = 1.0–2.4 hr). Urinary excretion measurements confirmed the short elimination half-life and showed that less than 1% of the dose was excreted unchanged. Oral studies also yielded a short elimination half-life (1.0–1.60 hr), which was confirmed by urinary excretion measurements. The oral curves were analyzed pharmacokinetically. The fraction of the dose reaching the systemic circulation ranged between 58 and 87%. Measurable (but insignificant) amounts of drug were found in the feces after intravenous and oral administrations.

Keyphrases □ Carbamazepine—pharmacokinetics, intravenous and oral administrations, monkeys □ Pharmacokinetics—carbamazepine, intravenous and oral administrations, monkeys □ Disposition—carbamazepine, acute intravenous and oral doses, monkeys

Carbamazepine, an iminostilbene derivative, is presently the drug of choice for the treatment of trigeminal neuralgia (1). Similarly to diphenylhydantoin, which is occasionally used for trigeminal neuralgia, it inhibits posttetanic potentiation (2). Following its pharmacological testing, which showed marked anticonvulsant properties in animals (3), it was used in a series of clinical trials as an antiepileptic drug, mostly in Europe and Australia (4–7). Carbamazepine has drawn special attention since several investigators reported that it possesses comparatively less sedative effects than other anticonvulsants and that it produces a "positive psychotropic effect" (8). It is currently under investigation in the United States (9).

Studies on its metabolic fate in animals or humans recently appeared (10, 11), but much less information has been accumulated regarding its pharmacokinetic properties (12, 13). The present studies were undertaken to study the disposition of carbamazepine in the monkey following acute intravenous and oral doses of the drug.

EXPERIMENTAL

Materials—Carbamazepine[1] and cyheptamide[2], the internal standard in the GLC assay, were used. All solvents used were reagent grade.

Subjects—Four male adolescent rhesus monkeys *(Macaca mulatta)*, 3–5 kg, were used. The monkeys were adapted to primate restraining chairs but housed in individual cages between experimental days.

Drug Administration—*Drug Solution and Dosage*—Carbamazepine has a very low water solubility in a relatively wide pH range. Therefore, it was put into solution in a 50% propylene glycol, 20% ethanol, and 30% water mixture at a concentration of 10 mg/ml. Propylene glycol and water without ethanol did not maintain the carbamazepine in solution at room temperature; carbamazepine tended to crystalize out at concentrations less than 20% ethanol. A compatible sterile solution without the drug but with the same percentages of propylene glycol, ethanol, and water was used for flushing the infusion tube.

All experiments (intravenous and oral) were performed at one dose level, 20 mg/kg. This dose (which corresponds to the upper dosing range in patients) was well tolerated by the monkeys and yielded easily quantifiable plasma concentrations.

Procedure—A monkey was placed in a carrying holder at the start of an experimental day (8 am) before the animal's daily feeding period. (The lower extremities, dorsally from the calf to the ankle, were shaved to emphasize the small saphenous veins and branching collaterals.)

In preparation for the drawing of blood samples, a 21-gauge needle of a heparin lock (intermittent infusion set with reseal injection site) was inserted into the small saphenous vein of one leg. The needle, butterfly tab, and junction of the trailing polyethylene tubing were sutured to the skin to secure the infusion set at four points. The animal was then employed in either an intravenous or oral experiment.

Intravenous Experiment—After the heparin lock was positioned and while the monkey was still in the carrying holder, a 21-gauge butterfly needle with tubing was inserted into the small saphenous vein of the other leg for drug infusion.

Unless otherwise indicated, carbamazepine was infused at a constant rate over 5 min, followed immediately by 2 ml of flushing solution. Subsequent to the initial three blood samples, the animal was placed in a primate chair and food and water were made available.

Oral Experiment (Nasal–Gastric)—At least a week intervened between the intravenous and oral experiments. After the heparin lock was secured, the animal was placed in a primate restraining chair and intubated nasally with a polyethylene, 38-cm (15-in.) gastric tube. The drug solution was injected directly into the stomach, followed immediately by 2 ml of flushing solution. The animal was not fed until 4 hr after drug administration; water was allowed *ad libitum*.

Table I—Pharmacokinetic Parameters of Carbamazepine Obtained after Intravenous Administration

Experiment	Animal	Elimination Half-Life, hr	Volume of Distribution, V_d, liter/kg	Total Body Plasma Clearance, liter/hr/kg
P1	M1	1.20	0.883	0.51
P2	M1	1.04	0.885	0.59
P3	M2	1.00	0.722	0.50
P4	M2	1.50	0.959	0.43
P5	M3	2.38	1.580	0.46
P6	M4	1.22	0.968	0.55

[1] Geigy Pharmaceuticals, Ardsley, N.Y.
[2] Ayerst Laboratories, New York, N.Y.

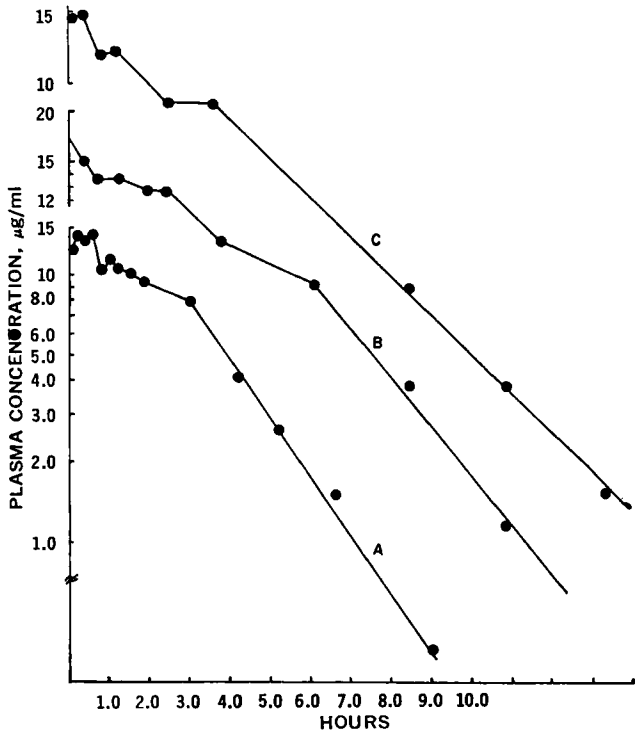


Figure 1—*Plasma concentration–time curves resulting from a 5-min intravenous infusion of carbamazepine (20 mg/kg). Key: A, Monkey M1; B, Monkey M2; and C, Monkey M4.*

Blood and Excreta Samples—Eleven to 14 blood samples were collected over 24 hr in both intravenous and oral experiments. Before obtaining each sample, a needle was inserted into the heparin lock. Then 0.2 ml of heparin was injected into the saphenous vein, and 5–10 drops of blood were allowed to drip until the viscosity and color of the blood were regular.

The blood (1.5–2 ml) was collected in a 3-ml vial containing potassium ethylene diaminetetraacetic acid (EDTA) and potassium sorbate. After each sample, the heparin lock was refilled and the blood was centrifuged (2000 rpm) for approximately 10 min. The plasma was transferred to another container, capped, coded, and frozen at −15° until assayed.

Urine was sampled from 0 to 48 hr and feces from 0 to 96 hr. Urine was collected in screened dropping pans, which prevented contamination by feces. The feces were collected in bags taped to the monkey buttocks bars. The stomach plate of the primate chair had underarm supports, which prevented a monkey from pivoting and contaminating its feces with urine.

The volume of urine at each scheduled collection was recorded. After mixing, a sample of approximately 2 ml was placed in a vial, capped, coded, and frozen at −15°. The gram weight of each scheduled feces collected was recorded, and the total amount was placed in a container, capped, coded, and frozen at −15°.

Plasma, Urine, and Feces Assays—All plasma determinations were made by the GLC procedure of Friel and Green (14) where carbamazepine is assayed directly, using cyheptamide as an internal standard. One milliliter of undiluted urine samples was extracted and chromatographed in the same manner as plasma samples.

Feces were weighed, diluted approximately 1:5 (w/v) with distilled water, and homogenized manually. Then an aliquot (1–15 ml, representing 0.2–4.0 g dry fecal weight) was transferred to a 40-ml glass-stoppered centrifuge tube, and 5 μg of cyheptamide, 2 ml of phosphate buffer (pH 7.2), and 15 ml of chloroform were added. The mixture was shaken manually for 2 min and centrifuged. The chloroform layer was removed and filtered into a centrifuge tube, and the chloroform extract was evaporated to dryness in a water bath at 50° under a gentle stream of air. The remainder of the extraction procedure was identical to that used for plasma and urine samples, *i.e.*, cyclohexane wash, reextraction into chloroform, and evaporation.

Table II—Urinary Excretion of Unchanged Carbamazepine following Intravenous and Oral Administrations

Experiment	Animal	Percent of Dose Excreted in Time Period		
		0–8 hr	0–24 hr	0–48 hr
P1	M1	0.09	0.13	0.59
P3	M2	0.38	0.39	—
P4	M2	0.24	0.45	—
P5	M3	0.17	0.19	—
P6	M4	0.31	0.46	—
P7	M3	0.073	0.083	0.102
P8	M1	Trace	Trace	—
P9	M4	0.46	0.48	0.68
P10	M2	0.12	0.25	—
O1	M1	0.37	0.47	—
O2	M2	0.17	0.17	—
O3	M3	0.06	0.11	—
O4	M4	0.15	0.15	0.15
O5	M2	0.06	0.08	0.09

Calibration curves constructed from spiked monkey plasma, urine, and feces were run with each set of unknown samples. They were linear in the range examined (2–16 μg). Recoveries (relative to aqueous standards) for plasma, urine, and feces were 97, 109, and 101%, respectively. Precision values obtained from a set of duplicate determinations were 4.9, 3.9, and 4.8% for plasma, urine, and feces, respectively.

RESULTS AND DISCUSSION

Kinetics following Intravenous Administration—The plasma concentration–time curves obtained following intravenous administration of carbamazepine to Monkeys M1, M2, and M4 (Experiments P1, P4, and P6, respectively) are shown in Fig. 1. The intravenous curves present several peculiarities. Each semilogarithmic curve is generally composed of two regions: an initial portion with atypical decay followed by a linear portion with an apparent first-order disappearance where a half-life measurement can be made.

Table I gives the pharmacokinetic parameters obtained from all intravenous experiments. The elimination half-lives were obtained from a nonlinear least-squares fit of the terminal monoexponential portion of each curve. For each curve the number of points used in the fit was maximized while the corresponding increase in sum of squares was minimized. The data in Table I show that carbamazepine is rapidly eliminated from the systemic circulation, its biological half-life ranging between 1 and 2.5 hr. These results are in agreement with the values of 55–75 min recently reported for the half-life of carbamazepine in monkeys (13).

The atypical postinjection decay of carbamazepine does not permit an unequivocal assessment of the most appropriate pharmacokinetic model. Total body clearance was computed from the ratio of dose to area under the plasma concentration–time curve (calculated by the trapezoidal rule), and the volume of distribution was derived from the total body clearance and elimination half-life (Table I).

Table II gives the results of urinary excretion measurements performed during all intravenous experiments (P1 and P3–P10). Carbamazepine is probably extensively metabolized since less than 1% of the dose can be recovered unchanged in urine after more than 10 half-lives. These findings concur with a 1% urinary recovery found previously (13).

Table II also shows that in most instances the majority of the total fraction excreted unchanged appears in the first 8 hr, substantiating the rapid elimination found from plasma data. In a few instances (Experiments P4 and P10), the fraction excreted in the first 8 hr is approximately 50% of the total fraction excreted unchanged. The irregularity in frequency of micturition (a collection time interval labeled 0–8 hr can in fact correspond to a much shorter excretion time period) may account for this observation. In Experiments P1 and P9, there appears to be appreciable excretion within the 24–48-hr interval. However, too few 24–48-hr collections were obtained to assess the significance of this finding.

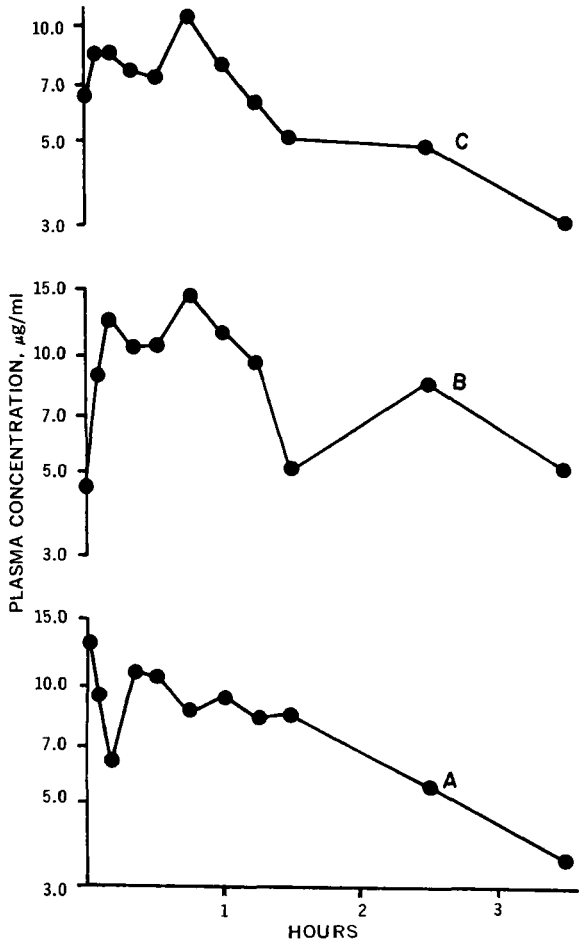


Figure 2—*Plasma levels of carbamazepine resulting from an intravenous infusion of 20 mg/kg in Monkey M2. Key: A, 20-min infusion, solvent of propylene glycol–ethanol–water; B, 5-min infusion, solvent of polyethylene glycol 400–water; and C, 20-min infusion, solvent of polyethylene glycol 400–water.*

The unusual pharmacokinetic behavior of carbamazepine in the first 2 hr after intravenous administration is not explained easily. In view of its very low water solubility, the possibility exists that the plasma level irregularities are related to a phenomenon of drug precipitation in plasma followed by redissolution. Two approaches were used to test this hypothesis. Since the drug was infused over a relatively short time (5 min) in all intravenous experiments shown in Fig. 1, it was decided to infuse the same volume of drug solution over 20 min in Monkey M2 (Experiment P10) for which two intravenous curves were already available (Experiments P3 and P4). Plasma samples as well as urine and feces were collected and assayed. The resulting plasma concentration–time curve is shown in

Table III—Excretion of Carbamazepine in Feces following Intravenous and Oral Dosing

Experiment	Animal	Amount Excreted in Time Period, μg			
		0–8 hr	8–24 hr	24–48 hr	48–96 hr
P1	M1	11.2	Trace	Trace	
P4	M2	16.2	7.8	27.6	
P7	M3	⟶	Trace[a]	4.5	
P9	M4	14.9	<7.0	<7.5	
P10	M2	8.4	8.4	—	
O1	M1	18.5	6.3	Trace	
O3	M3	1.6	1.3	Trace	
O4	M4	⟶	Trace[a]	Trace	
O5	M2	13.8	113.7	7.7	
O6	M1	⟶	⟶	3.8[b]	6.6

[a] 0–24-hr sample. [b] 0–48-hr sample.

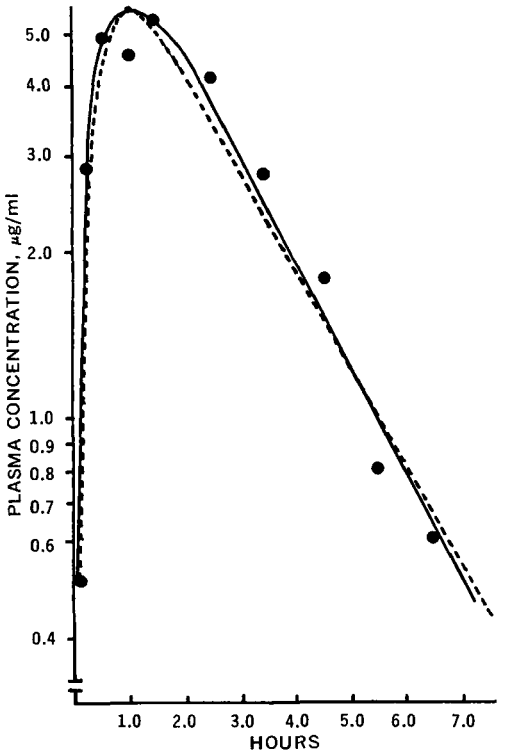


Figure 3—*Plasma concentration–time curve resulting from intragastric intubation of carbamazepine (20 mg/kg) in Monkey M1. Continuous line is the least-squares fit to a biexponential equation (Scheme I); dotted line is the least-squares fit to a triexponential equation (Scheme II).*

Fig. 2 (curve A), where the time scale has been expanded to illustrate the postinjection phenomenon. When this curve is compared to the initial portion of the curves in Fig. 1, it becomes apparent that the pattern is quite similar.

There is still another possibility within this hypothesis of drug precipitation, namely, that the observed phenomenon is related to the solvent mixture used to dissolve carbamazepine. To test this possibility, a new solution of carbamazepine was prepared (at the same concentration) in an equal weight mixture of polyethylene glycol 400 and water. The same dose and volume administered in all the previous intravenous experiments were administered to Monkey M2 over a 5- (Experiment P11) and 20- (Experiment P12) min constant infusion period (Fig. 2, curves B and C, respectively). The pattern observed here is similar to those obtained in Experiments P3, P4, and P10. Therefore, changing both the solvent mixture and the infusion time did not eliminate the postinjection plasma level fluctuations.

Another series of experiments was undertaken to investigate the nature of the plasma level fluctuations in the postinjection period. Feces were collected (three times during 48 hr) during Experiments P1 and P4 and assayed for unchanged drug (Table III). It was found that the first 8-hr samples contained 11 and 16 μg of drug for Experiments P1 and P4, respectively. In the latter experiments, 8 and 28 μg of drug were measured in the following 16- and 24-hr samples, respectively.

To confirm these findings, similar intravenous experiments were

Table IV—Pharmacokinetic Parameters of Carbamazepine Obtained after Oral Administration

Experiment	Animal	One-Compartment Model			Physiological Availability, %
		A	K_a, hr^{-1}	K_e, hr^{-1}	
O1	M1	53	0.764	0.619	58.4
O2	M2	200	0.483	0.455	86.6
O3	M3	200	0.467	0.434	75.2
O4	M4	472	0.582	0.568	65.0

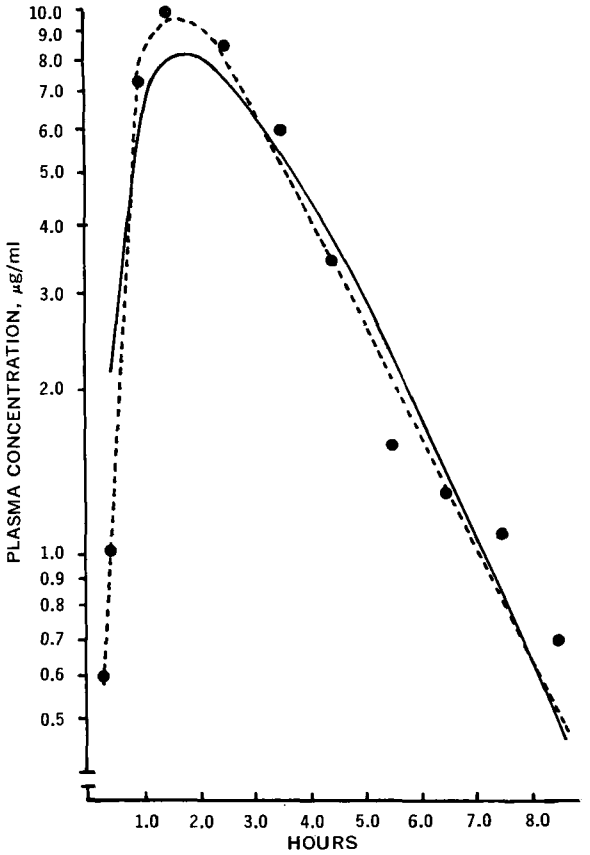
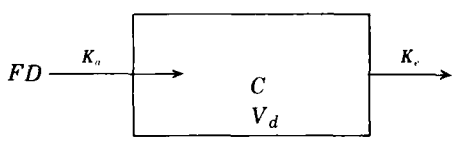


Figure 4—*Plasma concentration–time curve resulting from intragastric intubation of carbamazepine (20 mg/kg) in Monkey M2. Continuous line is the least-squares fit to a biexponential equation (Scheme II); dotted line is the least-squares fit to a triexponential equation (Scheme I).*

performed in each animal and the results show measurable quantities of drug in the feces at various times (Table III). These findings support the hypothesis that carbamazepine undergoes enterohepatic cycling in the monkey under the present experimental conditions. Hence, it is possible that the characteristic fluctuations observed in the 60–90-min period postinjection are caused by reabsorption of the drug after secretion in the bile and emptying in the duodenum. (In addition, food ingestion could influence this phenomenon.)

However, it is not possible to assess the fraction of the dose cycled from the amount of drug measured in the feces, since that amount represents only the portion of the fraction cycled that was not reabsorbed. This phenomenon will be discussed further. Enterohepatic cycling has also been suggested in humans (15).

Kinetics following Oral Administration—Since no information could be gathered from the intravenous data regarding the type of model that describes the pharmacokinetic behavior of carbamazepine in monkeys, it was decided to approach the kinetic analysis of oral data using the one-compartment open model with absorption (Scheme I).

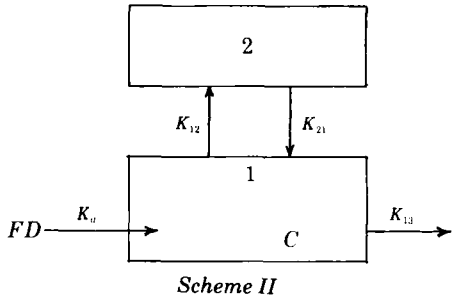


Scheme I

The integrated expression for the one-compartment model is of the form:

$$C = A(e^{-K_e t} - e^{-K_a t}) \qquad \text{(Eq. 1)}$$

where K_a and K_e are the first-order absorption and elimination rate constants, respectively, and A is a function of K_a, K_e, V_d (volume of distribution), and the fraction of the dose absorbed FD. Accordingly, the oral data were fitted to a biexponential equation *with weighting factors of 1 and 1/C,* using a nonlinear least-squares program[3] (16). (Initial estimates of the parameters were obtained using a log-peeling technique.)



Scheme II

Table IV gives the pharmacokinetic parameters obtained for weighted data (reciprocal weighting gave a lower sum of squares, with improved fit at the tail end of the curve). However, these parameters are empirical equation parameters rather than unique model parameters because of the closeness of values of K_a and K_e. Nevertheless, the elimination half-lives calculated from these parameters are in good agreement with those obtained after intravenous administration (Table I). Figures 3–6 show that an adequate fit is obtained for Monkeys M1 and M3 but not for M2 and M4. Hence, a two-compartment model with absorption was also considered (Scheme II)(17).

It was found that in all four instances the data points could be adequately fitted to a triexponential equation of the form:

$$C = \sum_{i=1}^{3} A_i e^{-m_i t} \qquad \text{(Eq. 2)}$$

where A_i is a function of the fraction of the dose absorbed, FD, the volume of distribution of Compartment 1, V_1, and the various first-order rate constants, K_a, K_{12}, K_{21}, K_{13}; and m_i is only a

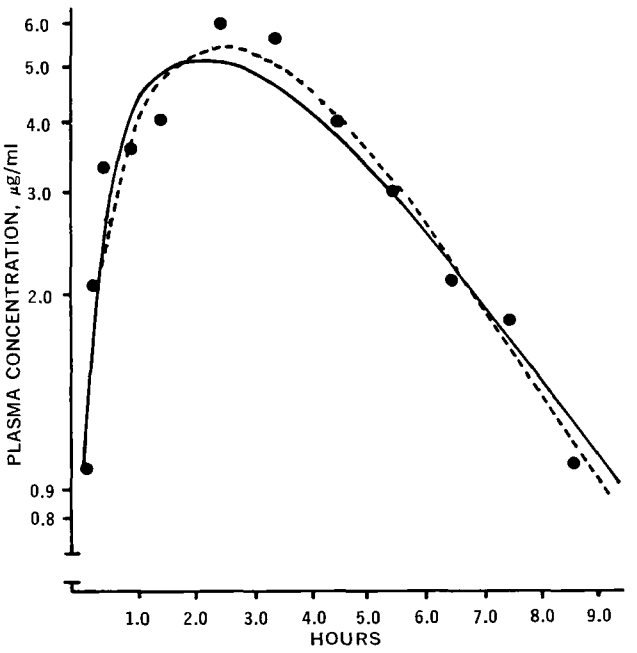


Figure 5—*Plasma concentration–time curve resulting from intragastric intubation of carbamazepine (20 mg/kg) in Monkey M3. Continuous line is the least-squares fit to a biexponential equation (Scheme I); dotted line is the least-squares fit to a triexponential equation (Scheme II).*

[3] On a CDC 6400 computer.

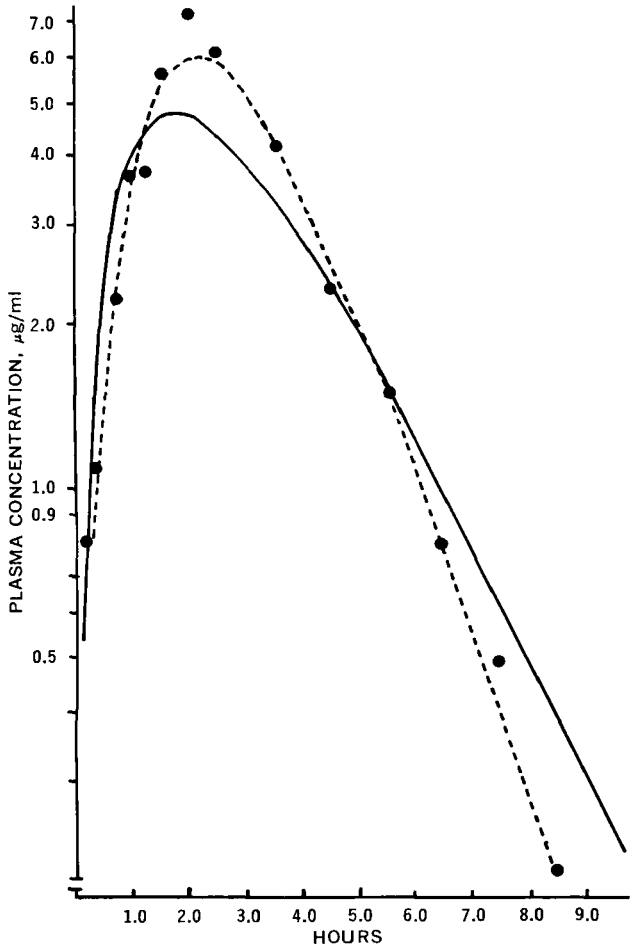


Figure 6—*Plasma concentration–time curve resulting from intragastric intubation of carbamazepine (20 mg/kg) in Monkey M4. Continuous line is the least-squares fit to a biexponential equation (Scheme I); dotted line is the least-squares fit to a triexponential equation (Scheme II).*

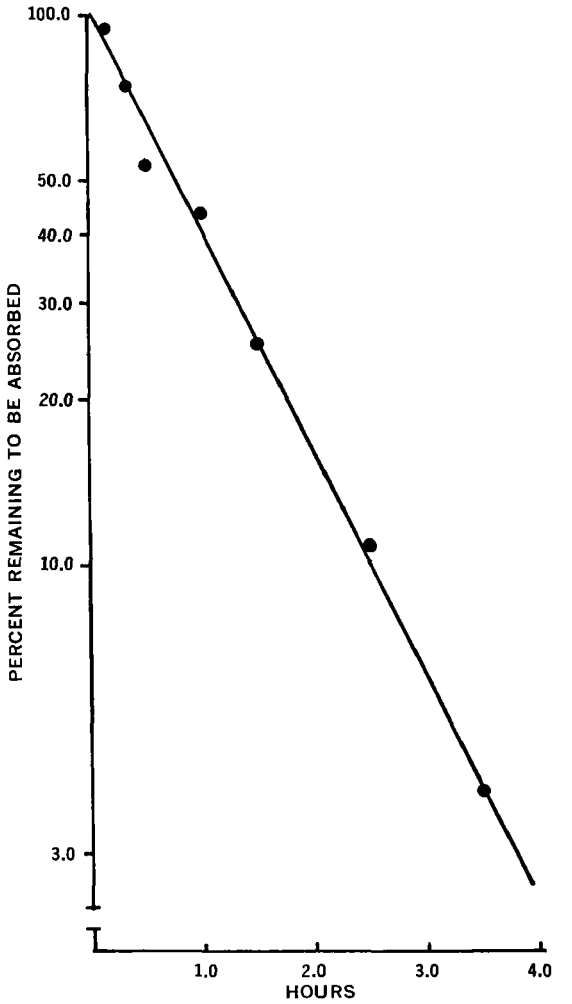


Figure 7—*Semilogarithmic plot of percent drug remaining to be absorbed* versus *time constructed according to Scheme I (Monkey M1).*

function of the first-order rate constants. For Monkeys M2 and M4, the latter fit was more adequate than that previously obtained with Eq. 1.

An attempt was made to calculate the parameters of the two-compartment model from the equation parameters, but it was not possible to obtain reliable model parameters. The parameters α and K_a could not be distinguished from each other because of the lack of prior information from intravenous data; therefore, K_{21}, K_{12}, and K_{13} could not be computed. Also, the parameters A_i and m_i had large standard deviations. Furthermore, the fact that several factors such as initial estimates, weighting function, and number of data points altered drastically the values of the parameters (and their standard deviations) further warned of their unreliability. These and other aspects of the (questionable) reliability of model parameters obtained by nonlinear least-squares fitting procedures were discussed (18), and a more recent review of this subject has also appeared (19).

Wagner–Nelson plots were constructed to gain a better understanding of the absorption process. However, the usual approach of obtaining K_e (from the terminal portion of the oral curve) could not be used in the present case because of the closeness of the absorption and elimination rate constants. Thus, for each animal the elimination rate constant obtained from intravenous data was utilized in the calculation of percent absorbed *versus* time plots. The log percent unabsorbed *versus* time plots yielded straight lines for all four animals (Fig. 7), and the corresponding absorption half-lives (0.52–1.16 hr) were somewhat shorter than those obtained from curve fitting to Eq. 1.

Physiological availability was calculated from the ratio of areas under the plasma concentration–time curves obtained after oral and intravenous administrations. The values obtained (58–87%) indicate incomplete systemic availability for this particular formulation (Table IV). The incomplete bioavailability could be attributed to one or more of several possibilities. Incomplete absorption appears unlikely since concomitant measurements of unabsorbed drug in feces revealed less than 1% of the dose (Table III). The possibility of a "first-pass" effect was considered (20). The fraction of the oral dose appearing in the systemic circulation, θ, was calculated from metabolic clearances and rhesus monkey liver blood flow data (21). The values of θ ranged between 0.87 and 0.85, indicating that this hypothesis could reasonably account for incomplete availability. Also, if non-first-order enterohepatic cycling were present, the method used to measure physiological availability would become invalid. Metabolism of carbamazepine in the gut or in the gut wall would also contribute to incomplete systemic availability. Consequently, a definitive assessment of the physiological availability of carbamazepine in monkey must await further study.

Urinary excretion measurements were also performed during the oral studies (Experiments O1–O5, Table II). The data confirm the findings of the parenteral studies that less than 1% of the dose appears unchanged in urine and that most of it appears in the first 8-hr collection.

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PHARMACEUTICAL ANALYSIS

New Color Reaction for Determination of Bacitracin in Ophthalmic Ointments

J. DOULAKAS

Abstract □ A colorimetric method was developed for the rapid analysis of bacitracin in ophthalmic ointments. The method involves the oxidation of α-aminocarboxylic acid with sodium hypobromite in an alkaline medium and condensation of the resulting aldehyde with phloroglucinol in concentrated hydrochloric acid to yield a pink color which gives an absorbance maximum at 505 nm. The relationship between absorbance and the quantity of bacitracin reacted obeyed Beer's law over the 15–100-μg/ml concentration range studied, and the produced color was stable for several hours. Furthermore, the method, which can be applied directly to the aqueous dissolution sample, gave results comparable to the official microbiological analytical procedure. The standard deviation is equal to ±1.81%.

Keyphrases □ Bacitracin—colorimetric analysis in ophthalmic ointments □ Ophthalmic ointments—colorimetric analysis of bacitracin □ Ointments, ophthalmic—colorimetric analysis of bacitracin □ Colorimetry—analysis, bacitracin in ophthalmic ointments

Although numerous microbiological methods (1–9) have been reported for the determination of bacitracin, the chemical analysis of this antibiotic in dosage forms has been a difficult task. The current widely accepted procedure for the determination of bacitracin employs a microbiological assay. In general, this procedure requires considerably more time than do chemical methods. For this reason, it was thought desirable to develop a chemical assay for the antibiotic in the hope of achieving rapid results with greater accuracy and precision. UV spectrophotometry, because of the very low absorbance of bacitracin, can be used for identification purposes rather than for a quantitative determination (10, 11).

Stretton *et al.* (12) reported the separation of zinc bacitracin, neomycin sulfate, and polymyxin B sulfate by various chromatographic procedures. They worked with the pure antibiotics and determined them colorimetrically, using a ninhydrin reagent, according to Maehr and Schaffner (13), after previous electrophoretic separation.

A simpler and shorter approach seemed to be one which might take advantage of the lability of the aminocarboxylic groups toward oxidation. Since these groups can be oxidized to the aldehyde (14) by sodium hypobromite fairly readily and further condensed with phloroglucinol (15), it was anticipated that this technique could be employed for a quantitative assay of bacitracin.

In the present investigation, it was found that bacitracin could be quantitatively determined by employing a colorimetric technique. The procedure is based upon heating the antibiotic with alkali sodium

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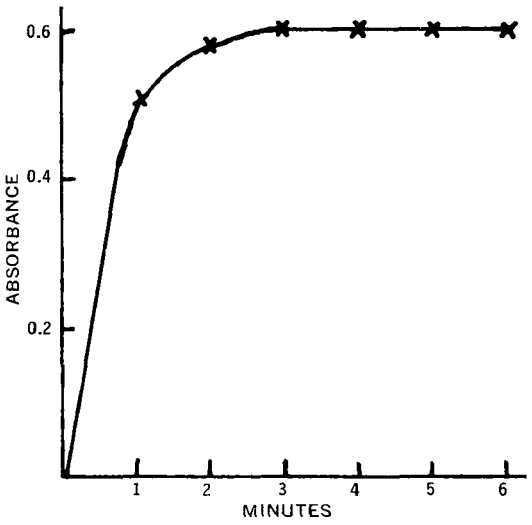


Figure 1—*Reaction time at 100°.*

hypobromite and condensing the resulting aldehyde with phloroglucinol reagent in an acid medium. The absorbance of the color produced is measured spectrophotometrically at 505 nm. The color-producing reaction with bacitracin gives excellent results and has not been previously reported.

EXPERIMENTAL

Instrumentation—Spectra and absorbance measurements were made with a double-beam spectrophotometer[1]. Matched glass-cells with a 2-cm optical path were used.

Reagents and Chemicals—Bromine and phloroglucinol, analytical reagent grade[2], were used as the chromogenic reagents. All solvents and other reagents used were also analytical reagent grade[2].

Sodium Hypobromite Solution—Dissolve 2 g of bromine into a 100-ml glass-stoppered volumetric flask, which has been previously half-filled with 1 *N* sodium hydroxide solution. Dilute to volume with the same solvent.

Phloroglucinol Reagent—Dissolve 0.5 g of phloroglucinol, in a 50-ml glass-stoppered volumetric flask, in 15 ml of distilled water by warming in hot water and shaking. Dilute to volume with concentrated hydrochloric acid (37%).

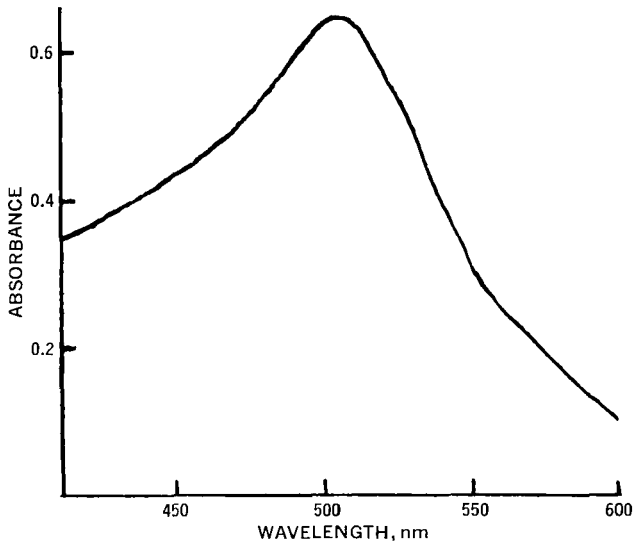


Figure 2—*Absorption spectrum of bacitracin (80 μg/ml).*

[1] Hitachi Perkin-Elmer model 124 (UV and visible).
[2] E. Merck Ltd., Darmstadt, West Germany.

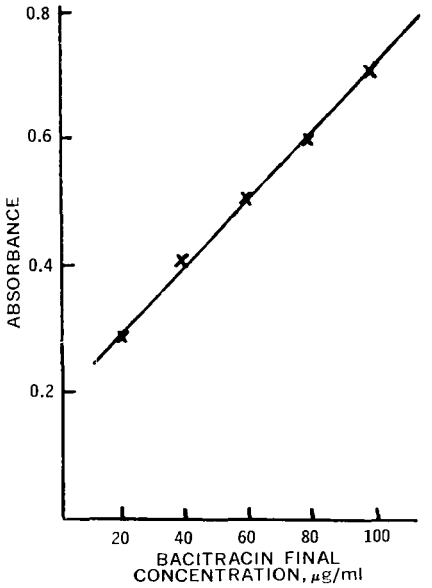


Figure 3—*Relationship between absorbance and concentration of bacitracin.*

Standard Reference Solution—Bacitracin[3], pure according to BP 1968, with a biological potency of 65 units/mg, was used. The purity of the commercially available bacitracin was checked by UV, TLC, and microbiological assay and was found acceptable. It was used as such without further purification. Bacitracin (10 mg) was dissolved in distilled water in a 50-ml glass-stoppered volumetric flask and diluted to volume with the same solvent.

Analytical Procedure—Weigh accurately a sample of the ophthalmic ointment, equivalent to approximately 10 mg (650 units) of bacitracin, into a 50-ml glass-stoppered centrifuge tube fitted with a ground-glass joint. Add 2 ml of ligroin (40–60°), warm slightly (*e.g.*, in a water bath at 40°), and shake briefly until the contents of the tube become a homogeneous viscous mass. Add 4.0 ml of distilled water and shake mechanically for 1 hr.

To assist solution of the active compounds, interrupt the shaking two or three times for 1-min intervals and place the tube briefly in the water bath each time. Finally, centrifuge for 5 min at 3000 rpm. Remove the aqueous (lower) phase with the help of a pipet, and filter it through a filter paper previously washed with distilled water and dried. Pipet 2.0 ml of the filtrate into a 25-ml glass-stoppered volumetric flask and fill to volume with distilled water.

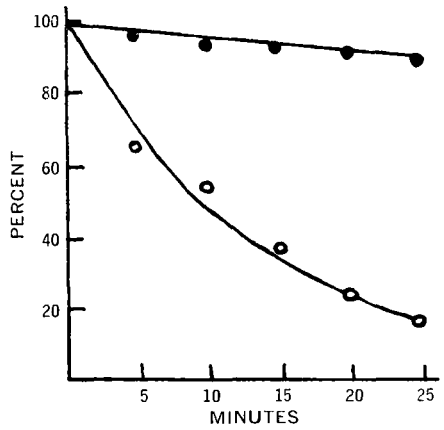


Figure 4—*Decomposition of bacitracin at 50 ± 0.1° (pH 13). Key: ●, colorimetric determination; and ○, biological determination (1 mg/ml).*

[3] Sigma Chemical Co.

Table I—Analysis of Known Bacitracin Ophthalmic Ointment for Bacitracin by Two Different Methods

Trials	Assay by the Sodium Hypobromite–Phloroglucinol Reagent, mg/g	Assay by the Ninhydrin Reagent, mg/g
1	4.00	3.98
2	3.95	3.87
3	3.89	3.90
4	4.00	4.01
5	4.00	3.88
6	3.92	4.09
7	3.80	3.91
8	4.01	4.14
9	3.96	3.93
10	3.90	3.86
	$\bar{X}$ = 3.94	3.96
	SD = ±0.07	0.10

Color Formation—Pipet 4.0 ml of an aqueous solution containing 60–200 μg bacitracin/ml into a 20-ml glass test tube, and add 1.0 ml of sodium hypobromite solution. Place the tube in a boiling water bath for 5 min and then add 3.0 ml of phloroglucinol reagent. Stir and develop the color by placing the tube in the bath again for a further 10 min. A pink stable color is developed. Cool by placing the tube in cold water (20°) for about 10 min.

Pipet 2.0 ml of 95% ethanol into the tube (occasionally a faint opalescence develops which disappears when ethanol is added) and shake well. Determine the absorbance, A_p, of the solution at 505 nm in a 2-cm cell against a reagent blank using a suitable spectrophotometer. Concomitantly carry out the reaction with the reference standard bacitracin in a 20-ml glass test tube, and follow the procedure described for color formation.

By designating the bacitracin standard weighed out in milligrams as C_s and the absorbance of the standard solution produced as A_s, the bacitracin present in the ointment can be calculated from the equation:

$$\text{bacitracin (mg/g)} = \frac{A_p \times C_s}{A_s \times \text{sample weight (g)}} \quad \text{(Eq. 1)}$$

Multiplying this equation by the factor I, where I indicates the microbiological potency of the standard bacitracin in units per milligram, one gets units of bacitracin per gram of ointment.

TLC—Ointments containing bacitracin and neomycin as active compounds were extracted as described. Bacitracin was then determined after TLC because neomycin interferes with the determination of bacitracin using this color reaction.

TLC was carried out on 20 × 20-cm glass plates coated with an approximately 300-μm layer of silica gel GF_{254}[4] activated at 110° for 45 min. The plates were treated with the developing solvent of *n*-butanol–acetic acid–water–pyridine (30:22:38:6, v/v) (12) over the whole 20-cm length and finally dried with a hair drier.

Using a microsyringe, apply 200 μl (approximately 500–800 μg of neomycin sulfate and bacitracin) of standard and test solutions as two thin uniform strips on the starting line of a prepared TLC plate. Take care not to damage the silica gel layer, which is easily flaked off the plate. Leave a 6-cm length in the middle of the plate for the blank. Develop the plate in the developing solvent for about 2.5 hr and then dry it, first by removing most of the solvent with the hair drier and then by placing the plate in a vacuum oven at 50° and 5–10 torr for 30 min. This procedure avoids decomposition of bacitracin.

Viewing the plate under a UV lamp (254 nm), mark out the standard and test zones of bacitracin situated on the upper half of the chromatogram, with R_f about 0.86, and scrape them separately off the plate using a spatula. Neomycin spots were visualized by spraying with ninhydrin reagent, R_f about 0.65. Place each zone in a 50-ml centrifuge tube fitted with a ground-glass joint, and place an equally sized area of adsorbent from the middle 6-cm section of the plate (representing the adsorbent background) into a third tube for preparing the blank.

[4] Merck.

Table II—Comparative Assay of Bacitracin in Ophthalmic Ointments Containing 4 mg/g Bacitracin

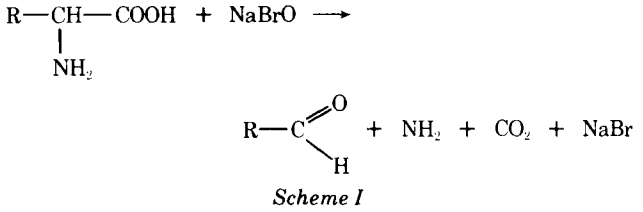
Sample	Colorimetric Method, %	Microbiological Method, %
1	102.3	113.6
2	99.1	107.2
3	97.3	104.1
4	96.8	97.8
5	100.4	96.6
6	98.2	98.8
7	97.7	92.7
8	99.4	103.2
	$\bar{X}$ = 98.9	101.75
	SD = ±1.81	±6.66
	RSD = ±1.81	±6.66

Into each tube, pipet 5.0 ml of pH 13 phosphate buffer (dissolve 100 mg of sodium phosphate dibasic dihydrate in 100 ml of 0.1 *N* sodium hydroxide, pH 12.7), and extract the active compound by placing the tubes on a mechanical shaker for 30 min. Centrifuge for 5 min at 3000 rpm.

Pipet 4.0 ml of each of the three supernatant solutions into three 20-ml test tubes, and carry out the color reaction in exactly the same manner as described under *Color Formation*, beginning with "... add 1.0 ml of sodium hypobromite solution." Bacitracin can be calculated from Eq. 1.

RESULTS AND DISCUSSION

By considering bacitracin as a compound containing aminocarboxylic acids of the type R—C(—NH_2)—H—COOH, the possible oxidation reaction is shown in Scheme I (14).

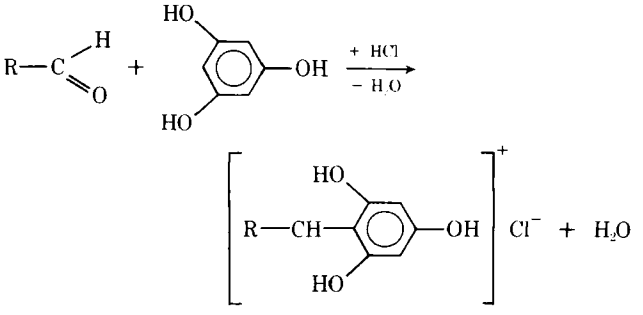


Scheme I

The α-aminocarboxylic acids are both deaminated and decarboxylated on treatment with alkali hypobromite. An aldehyde with one carbon atom less than in the original amino acid results.

The resulting aldehyde condenses with phloroglucinol in concentrated hydrochloric acid to yield pink-colored monomethine chloride (Scheme II) (15). The concentration optimum of the sodium hypobromite reagent was determined using concentrations of 1, 1.5, 2, 2.5, and 3% of active bromine. The best results were obtained with the reagent at 2%. The exact concentration of the reagent is very important, because large variations may lead to considerable changes in color intensity. Therefore, the concentration may be checked by prior iodometric titration of the bromine.

The reaction time was determined following the color development at the temperature of the boiling water bath. The results (Fig. 1) indicated that a heating time interval of only 5 min with the alkaline sodium hypobromite reagent is required to obtain



Scheme II

Table III—Assay of Bacitracin after TLC

Sample	Recovery, %
1	104.0
2	100.0
3	99.4
4	100.6
5	105.2
6	96.1

complete reaction. The optimum reaction time for the phloroglucinol reagent was 10 min, and only the 1% concentration in 31% hydrochloric acid was used.

The reaction reaches a maximum after 5 min and remains almost stable for several hours. This asset is very important for a method to be used regularly in quality control and for series determinations. Twenty-four hours after the beginning of the reaction, the maximum of the visible absorption spectrum remains constant at 505 nm while the absorbance shows a small increase of about 4% (Fig. 2).

Linearity—Concentration was found to be proportional to absorbance for the chromophore produced by reaction of bacitracin with the chromogenic reagent. A typical Beer's law plot is shown in Fig. 3. The linearity of the reaction was investigated in a concentration range of 15–100 μg/ml of bacitracin at the maximum at 505 nm. For concentrations lower than 10 μg/ml, the relationship is not linear.

Ten replicates of an eye ointment preparation, containing 4 mg (250 units) of bacitracin/g, gave an average of 3.94 mg/g with a relative standard deviation of 0.07 mg. The results were compared with those obtained by the ninhydrin method according to Langner and Heckel (16) (Table I). Data comparing the colorimetric method with the microbiological method on eight different samples of premix are presented in Table II.

The microbiological determination was carried out by the petri dish method (17, 18) using Difco Penassay seed agar (pH 6.6 after sterilization) as the culture medium and *Micrococcus flavus* (ATCC 10240) as the test microorganism.

The analytical method described here was used for the analysis of bacitracin in two commercial preparations after the TLC technique. The assay was performed on six samples, and the results obtained (Table III) show the suitability of this analytical procedure. The ninhydrin reagent gave unsatisfactory results after TLC.

Interferences—The method is relatively nonspecific and, therefore, is not stability indicating. In addition, any compound susceptible to oxidation by sodium hypobromite in an alkaline medium (such as other aminocarboxylic acid-containing compounds as well as neomycin) would probably react with phloroglucinol and, consequently, interfere with the determination of bacitracin using this procedure; suitable separation steps such as TLC or ion-exchange chromatography would be required. Figure 4 shows that the biological activity of bacitracin decreases more rapidly than is indicated by the colorimetric method.

A solution containing 1 mg/ml bacitracin in pH 13 phosphate buffer (see under *TLC*) was heated rapidly at 50° (water bath). Then an aliquot of this solution was neutralized, cooled, and diluted suitably for the colorimetric and microbiological methods. This solution was used as the standard for the further aliquots pipetted out in 5-min intervals, which were also neutralized, cooled, and diluted suitably. The colorimetric results were compared to those of the microbiological method.

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GLC Determination of Nicotinamide in Multivitamin Formulations after Conversion to Nicotinonitrile

JÖRGEN VESSMAN ˣ and SIGNHILD STRÖMBERG

Abstract □ A method was developed for the quantitative GLC determination of nicotinamide. It is based on the conversion of nicotinamide to nicotinonitrile. This dehydration reaction is mediated with trifluoroacetic anhydride and catalyzed by base. The GLC properties of this nitrile are excellent. The reaction conditions, extraction, and stability properties of the derivative as well as the choice of internal standard are discussed.

Keyphrases □ Nicotinamide—GLC determination in multivitamin formulations, conversion to nicotinonitrile □ Multivitamin formulations—GLC analysis of nicotinamide, conversion to nicotinonitrile □ GLC—analysis, nicotinamide in multivitamin formulations, nicotinonitrile measured after dehydration

GLC has been widely used in the pharmaceutical analysis for the quantitative determination of various products. In the heterogeneous group of vitamins, only a few are amenable to GLC. Some approaches have been successful, particularly among the fat-soluble ones.

Nicotinamide was gas chromatographed early (1). The adsorptive properties of the compound have made the practical use of GLC methods difficult, although Prosser and Sheppard (2) and Ashby and Deavin (3) reported reproducible results. Silylation of nicotinamide was suggested also (4–6).

The present method utilizes a rapid dehydration reaction with trifluoroacetic anhydride (I) to form the nicotinonitrile and the subsequent GLC determination of that product. The GLC properties are improved considerably, permitting quantitative determination in pharmaceutical formulations with a precision of ±2.0% or less.

EXPERIMENTAL

Apparatus—A gas chromatograph[1] with a flame-ionization detector was used with nitrogen (30 ml/min) as the carrier gas. The glass column (1.5 m × 0.18 cm) was filled with 20% Carbowax 20 M terephthalic acid on 100–120-mesh Gas Chrom P, acid washed and silanized. The column temperature was 155°.

Chemicals and Reagents—Trifluoroacetic anhydride[2] (I), pyridine, methanol, and chloroform were of analytical grade. 6-Methylnicotinamide was synthesized from 6-methylnicotinic acid[3].

The pH 1.2 buffer for extraction of tablets was prepared by mixing 2.0 g of sodium chloride, 80 ml of concentrated hydrochloric acid, and water to 1 liter.

A 0.05% solution of bromthymol blue indicator solution was prepared in water.

Procedure—*Standard Solution Preparation*—Weigh accurately about 25 mg of nicotinamide into a 25-ml volumetric flask. Dissolve in and dilute to volume with methanol.

Weigh accurately about 25 mg of 6-methylnicotinamide into a 25-ml volumetric flask. Dissolve in and dilute to volume with methanol. This solution is referred to as the internal standard.

Standard Preparation—Transfer 0.10, 0.20, and 0.30 ml of standard solution into three glass-stoppered 12-ml centrifuge tubes. Add 0.20 ml of the internal standard solution to each tube and evaporate to dryness on a boiling water bath.

Add 0.4 ml of pyridine and wait until the residue is dissolved (5–10 min). Add 0.2 ml of chloroform and 0.2 ml of trifluoroacetic anhydride. Stopper the tubes, shake gently, and let stand for 5 min in an ice bath.

Add 1 drop of bromthymol blue indicator solution and 5 *M* sodium hydroxide dropwise to a blue color. Be careful not to add an excess of the hydroxide. Cool the tubes and add 1.5 ml of chloroform. Shake for 2 min and centrifuge. Discard all of the aqueous phase, and inject about 3 μl of the chloroform layer into the gas chromatograph.

Calibration Curve—Construct the calibration curve by plotting the weight ratio of nicotinamide–6-methylnicotinamide on the *x*-axis against their peak height ratios on the *y*-axis.

Sample Preparation: Liquid Formulations (1 mg/g)—Weigh 2.0 ml of the sample in a 100-ml evaporation flask. Add 2.0 ml of the internal standard solution, and evaporate to dryness in a vacuum with a rotating evaporator on a boiling water bath.

Dissolve the residue in 1 ml of methanol. To facilitate the dissolution, warm gently and shake for 5 min. Add 9.0 ml of chloroform while shaking and then add a spoon of sodium sulfate. Filter through silanized glass wool.

Pipet 1.0 ml of the clear solution into a 12-ml glass-stoppered centrifuge tube. Proceed as described for the preparation of standards.

Sample Preparation: Tablets (20 mg/Tablet)—Place one tablet into a 25-ml erlenmeyer flask, and add 3.0 ml of pH 1.2 buffer. Warm and shake for 15 min in a mechanical shaker. Transfer quantitatively into a 25-ml volumetric flask, and dilute to volume with methanol.

Pipet 0.25 ml of the sample solution and 0.20 ml of the internal standard solution into a glass-stoppered 12-ml centrifuge tube. Proceed as described for the preparation of standards.

RESULTS AND DISCUSSION

In studies of the perfluoroacylation of primary and secondary amides, Ehrsson and Mellström (7) found that primary amides were dehydrated to nitriles. This reaction has been of great value to improve the GLC properties of some primary amides studied in this laboratory. Therefore, it was of interest to apply this reaction to the quantitative determination of nicotinamide.

Derivative Formation—The formation of the nitrile was verified with mass spectrometry. Base catalysis was necessary according to Ehrsson and Mellström (7), who used 1.0 *M* trimethylamine in ethyl acetate. With 1 *M* triethylamine in chloroform, the dehydration of nicotinamide with I was accomplished rapidly. From a GLC point of view, pyridine was the better choice because the chromatograms were cleaner and had a much narrower solvent front.

With pyridine as the catalyst, the dehydration was very rapid, with a quantitative yield in less than 10 sec. The amount of pyridine needed for pure nicotinamide extracts was around 25 μl, giving a final pyridine concentration in the reaction mixture of 0.52 *M* (final volume ~0.8 ml).

The amount of trifluoroacetic anhydride needed for quantitative reaction was found to be 100 μl when pure nicotinamide solutions were reacted. However, these amounts of reactants were ineffective when extracts from liquid multivitamin preparations were analyzed, probably because of the poor solubility of the residue from these extracts in too small volumes of reagents. Instead, 400 μl of pyridine, 200 μl of chloroform, and 200 μl of I were used, and the dried extract was dissolved in pyridine before the anhydride was added.

[1] Varian 1400.
[2] Merck.
[3] Pfaltz and Bauer, Flushing, N.Y.

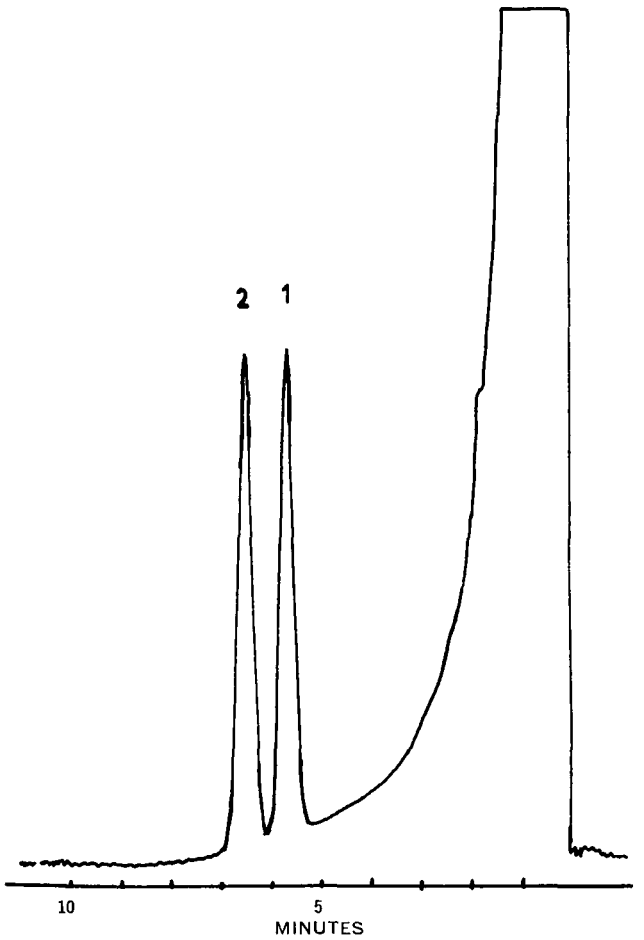

Figure 1—*Gas chromatogram of nicotinonitrile (1) and its 6-methyl analog (2) obtained from an extract of a liquid multivitamin preparation containing 1 mg nicotinamide/g.*

The reaction has to be done in the absence of water. Therefore, extracts or aliquots of aqueous samples were taken to dryness before the derivative formation step.

Extraction of Nicotinonitrile—After the dehydration reaction, it was not practical to inject the organic phase directly because the solvent front tailed immensely. Careful neutralization of the reaction mixture (indicator present), preferrably in an ice bath, and immediate separation of the phases gave an organic extract which, upon injection, gave acceptable chromatograms (Fig. 1).

Partition experiments with nicotinonitrile between chloroform and aqueous buffers showed that the extraction efficiency decreased below pH 4. Above pH 4 and with equal phase volumes, the extraction into chloroform was 94% (measured gas chromatographically against a lipophilic internal standard, decafluorobenzophenone). To obtain an extraction efficiency greater than 99%, the phase volume ratio (organic–aqueous) was adjusted to 3. To keep the volume of hydroxide needed to neutralize the trifluoroacetic acid as low as possible, 5 *M* sodium hydroxide was used. This was of particular importance in the analysis of liquid formulations. In that way the concentration of the nicotinonitrile in chloroform was kept high enough to permit the injection of reasonable volumes (≤5 μl, to keep the solvent front of reasonable size).

Stability of Nicotinonitrile—One source of variation was the hydrolysis of the nitriles when the reaction mixture was in contact with the alkaline aqueous phase. This was particularly apparent when a large number of samples were analyzed in a series.

Of the tested analogs to nicotinonitrile, isonicotinonitrile was most sensitive to hydrolysis. After 1 hr in contact with 5 *M* sodium hydroxide, about 25% had degraded. For nicotinamide and its 6-methyl analog, the figures were about 6 and 1%, respectively. These figures were measured against the lipophilic compound dodecane in the organic phase.

Table I—Relative Retention Times of Various Nitriles Derived from Pyridine Carboxylic Acids

Substance	Nitrile in Position	Relative Retention
Nicotinonitrile	3	1.00[a]
6-Methylnicotinonitrile	3	1.16
6-Chloronicotinonitrile	3	2.66
Isonicotinonitrile	4	0.75
Picolinonitrile	1	1.77

[a] Retention time of nicotinonitrile at 155° = 6.1 min.

To minimize these hydrolysis effects, the neutralization was carefully performed in an ice bath in the presence of bromthymol blue. After centrifugation the aqueous phase was completely removed.

GLC—GLC properties of the nicotinonitrile were excellent in comparison with those of nicotinamide, as was recently shown by Ponomarev *et al.* (6). No signs of memory effects, *i.e.*, desorption of compounds from previous injections, were observed. The choice of Carbowax 20 M terephthalic acid as the stationary phase was chiefly dictated by the fact that this column gave the best separation of nicotinonitrile from pyridine in the solvent front (Fig. 1). After a day's use with extracts from liquid formulations, it was advantageous to exchange the first part of the column filling (2–4 cm) to inhibit clogging.

Choice of Internal Standard—Four analogous compounds were studied as suitable internal standards. The relative retention times of the nitriles are given in Table I. The best choice as an internal standard from a retention point of view was 6-methylnicotinamide followed by isonicotinamide. The latter would be better from an extraction point of view because the extractability was equivalent to that of nicotinamide. However, due to the greater risk for hydrolytic losses with isonicotinonitrile, the 6-methyl analog was preferred.

The dehydration of the analogous compounds was found to be as rapid as for nicotinamide.

The internal standard was added at the beginning and was present in all steps. This was of particular importance in the analysis of liquid formulations.

Quantitative Applications—The method has been applied on several multivitamin formulations. Tablets were extracted with an acidic buffer, and the extract was diluted with methanol to a concentration of 1 mg/ml. An aliquot of this solution was taken to dryness, and the residue was then treated with the reagents.

Liquid formulations were mixed with methanol and the internal standard solution and then taken to dryness in a vacuum. The residue was admixed with methanol and heated on a water bath for 1 min to dissolve it. Then chloroform and dried sodium sulfate were added, whereby precipitation of some constituents occurred. The chloroform layer was filtered through silanized glass wool, and then an aliquot of the extract was evaporated to dryness and processed. Sometimes the residues obtained at this point were greasy and required a couple of minutes to dissolve in pyridine.

For liquid formulations, the relative standard deviation was ±1.0%; tablets gave a somewhat higher value, ±2%. The method has been successfully used in the quality control laboratory for 1 year. This method can be applied in stability studies because the formation of the nitrile is not very probable under normal storage conditions.

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[x] To whom inquiries should be directed.

Liquid Chromatography of Clindamycin 2-Phosphate on Triethylaminoethyl Cellulose

WALTER MOROZOWICH [x] and ROGER G. WILLIAMS

Abstract □ The separation of clindamycin 2-phosphate from clindamycin 3-phosphate, clindamycin 4-phosphate, clindamycin B 2-phosphate, and lincomycin 2-phosphate was achieved by liquid chromatography on triethylaminoethyl cellulose using a 254-nm monitor. The compounds have low molar absorptivities at 254 nm (<17), and UV detection is made possible by the high capacity support triethylaminoethyl cellulose. Linear peak height response *versus* concentration allows rapid quantitation of clindamycin 2-phosphate.

Keyphrases □ Clindamycin 2-phosphate—separation from other clindamycin compounds, triethylaminoethyl cellulose column, liquid chromatography □ Triethylaminoethyl cellulose—used for liquid chromatographic separation of clindamycin 2-phosphate from other clindamycin compounds □ Liquid chromatography—separation, clindamycin 2-phosphate from other clindamycin compounds

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A liquid chromatograph[2] with a 254-nm detector was used.

Column Packing—Triethylaminoethyl cellulose[3] was freed of fines by slurrying 1–4% suspensions in water and allowing the material to settle for 1–1.5 hr. The supernate was discarded and the slurrying–settling process was repeated six times. The purified support was isolated by filtration, washed with acetone, and air dried for 24 hr.

A funnel was attached to a 2.1-mm i.d. × 1-m stainless steel column, and 5–10 mg of triethylaminoethyl cellulose was added from an inverted container supported on a 50-mesh screen by brief mechanical vibration of the screen. The side of the column was vibrated for a few seconds, and the material within was compacted with a loosely fitting stainless steel rod (1.8-mm diameter) with an applied hand force of 0.2–0.7 kg (0.5–1.5 lb). The tamping rod was turned 120°, and three such compressions were made after each addition of support. Excessive compaction force results in columns with poor flow rates, and practice is required to produce columns with adequate flow (0.4–1.5 ml/min) using 2000 psi at room temperature.

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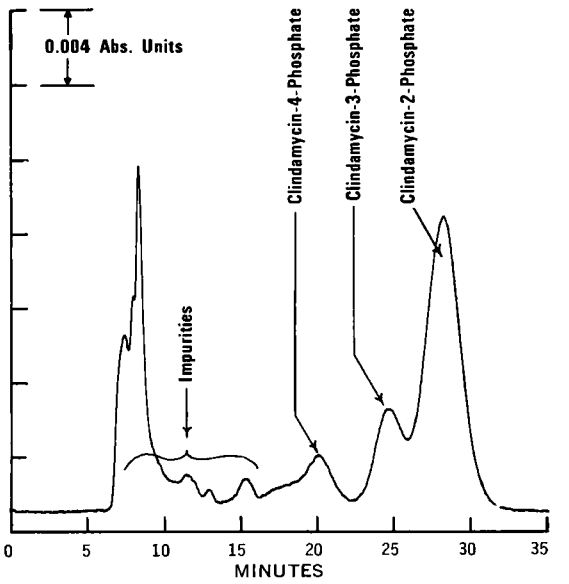

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[1] Cleocin Phosphate, The Upjohn Co.
[2] Du Pont model 820.
[3] Cellex-T, BioRad Laboratories, Richmond, Calif.
[4] Hamilton 701N, Chaney type II adaptor.

(7) H. Ehrsson and B. Mellström, *Acta Pharm. Suecica*, **9**, 107(1972).

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[x] To whom inquiries should be directed.

Liquid Chromatography of Clindamycin 2-Phosphate on Triethylaminoethyl Cellulose

WALTER MOROZOWICH [x] and ROGER G. WILLIAMS

Abstract □ The separation of clindamycin 2-phosphate from clindamycin 3-phosphate, clindamycin 4-phosphate, clindamycin B 2-phosphate, and lincomycin 2-phosphate was achieved by liquid chromatography on triethylaminoethyl cellulose using a 254-nm monitor. The compounds have low molar absorptivities at 254 nm (<17), and UV detection is made possible by the high capacity support triethylaminoethyl cellulose. Linear peak height response *versus* concentration allows rapid quantitation of clindamycin 2-phosphate.

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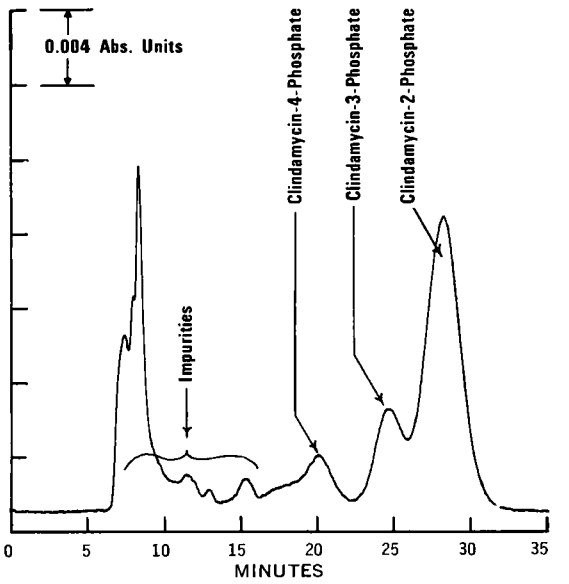

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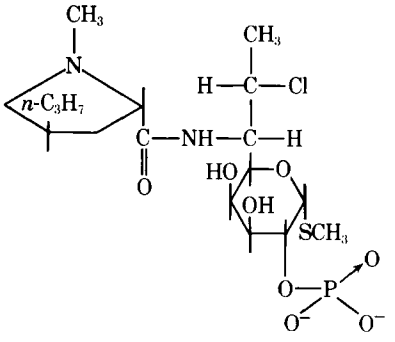
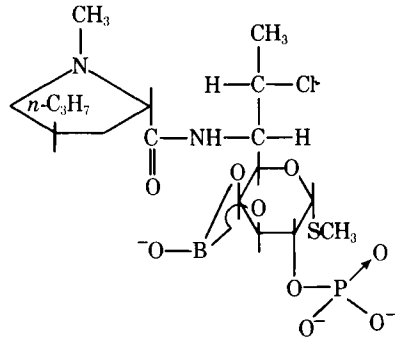
reactive *cis*-3,4-diol:

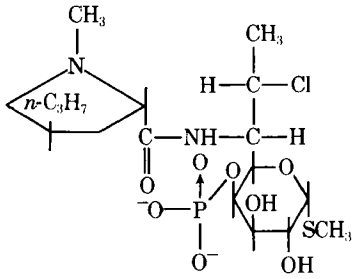


I: clindamycin 2-phosphate



II: clindamycin 2-phosphate–borate complex

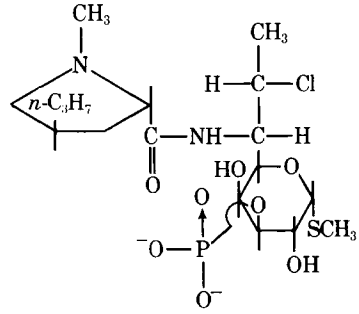
nonreactive *trans*-2,3-diol:



III: clindamycin 4-phosphate

+

nonreactive 2,4-diol:



IV: clindamycin 3-phosphate

Scheme I—Complexation of isomeric clindamycin phosphate esters with borate

clindamycin phosphate ester in 3 ml of 0.25 *M* boric acid (pH 8.8) was treated with 1 ml of 0.1 *M* magnesium chloride. Alkaline phosphatase[5], 50 mg, 1.45 units/g, was added and the mixture was vigorously shaken in a 15-ml centrifuge tube until solution was attained. After 18 hr at 37 ± 2°, the solution was shaken vigorously with 8 ml of chloroform. The mixture was centrifuged at 20,000 rpm for 30 min and the aqueous layer was removed by aspiration.

The clear chloroform layer was dried with sodium sulfate, and a 3-ml aliquot was taken to dryness under a stream of nitrogen. Trifluoroacetic anhydride (0.1 ml) was added and the vial was sealed with a Teflon-faced screw cap. The solution then was heated at 45° for 1 hr. After cooling to room temperature, about 10 mg of anhydrous sodium carbonate was added. The container was resealed immediately, and 1-μl samples were taken by syringe penetration through the Teflon-lined septum. GLC was conducted on a gas chromatograph[6] (flame-ionization detector), using a 0.6-cm × 0.6-m (0.25-in. × 2-ft) glass column containing 1% SE-30 on Gas Chrom Q (100–120 mesh) at 170°.

Crude Clindamycin 3-Phosphate and Clindamycin 4-Phosphate—A solution of 98.5 g of clindamycin 2-palmitate hydrochloride (3) in a mixture of 145 ml of pyridine and 290 ml of acetone was added dropwise to a solution of 14.3 ml of phosphorus oxychloride in a mixture of 70 ml of pyridine and 70 ml of acetone. After 0.5 hr, 600 g of ice was added and the solvent was removed *in vacuo*.

The residue was dissolved in a mixture of methanol, ether, water, and ammonium hydroxide (280 ml of each). After 20 hr at room temperature, the solution was extracted with ether and the aqueous phase was taken to dryness under vacuum. The residue was chromatographed on 190 g of silica gel[7] (0.05–0.2 mm), using chloroform–methanol–water–ammonium hydroxide (8:8:1:1). The pooled product fraction was evaporated to dryness and the residue was dissolved in methanol (100 ml). The solution was desalted by careful acidification with sulfuric acid.

After filtration, removal of the solvent gave 3.5 g of a crude mixture containing clindamycin 3-phosphate and clindamycin 4-phosphate (R_f 0.4 and 0.3, respectively, on borate–silica gel plates). The R_f values were identical with those of the authentic 3-phosphate reported previously (2) and the authentic 4-phosphate prepared by a modified route. GLC–mass spectral[8] analysis, after conversion of authentic clindamycin 4-phosphate to the tris(trimethylsilyl) derivative (1% OV-1 on Gas Chrom Q, retention time of 2.8 min at 210°), gave the following key fragmentation: *m/e* 792 (M^+), 777

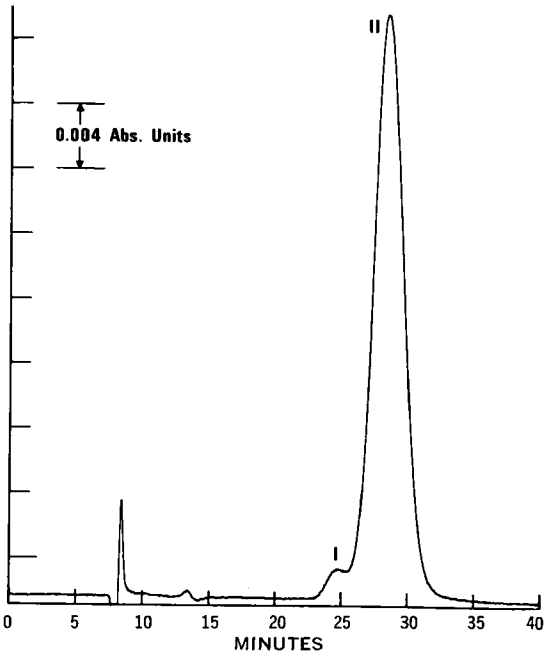


Figure 2—*Liquid chromatogram of 1.05 mg of pure clindamycin 2-phosphate (7 μl of a 15% solution, pH 8.85). Conditions were the same as in Fig. 1.*

[5] Worthington Biochemicals.
[6] Hewlett-Packard model 402-B.
[7] Merck.
[8] LKB 9000.

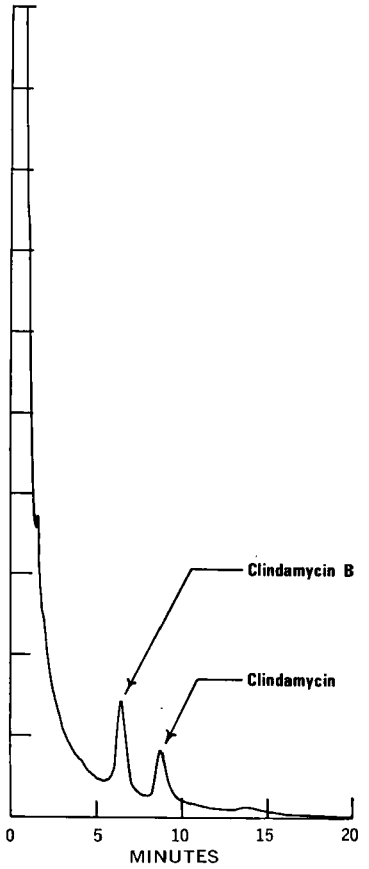


Figure 3—*Gas chromatogram of peak I from Fig. 2 after phosphatase hydrolysis and conversion to the trifluoroacetate derivative (conditions: 1% SE-30, 170°).*

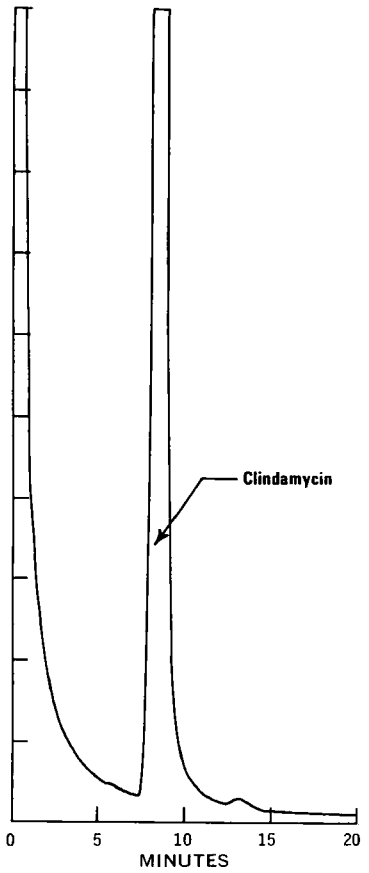


Figure 4—*GLC of peak II from Fig. 2 after phosphatase hydrolysis and conversion to the trifluoroacetate derivative. Conditions were the same as in Fig. 3.*

($M^+ - CH_3$), 756 ($M^+ - HCl$), 741 ($M^+ - HCl - CH_3$), and 713 ($M^+ - HCl - C_3H_7$).

Phosphate analysis, according to the method of Bartlett and Lewis (4), showed a phosphate content of 5.25% for authentic clindamycin 4-phosphate, which is in agreement with the theoretical value of 5.76%.

RESULTS

Borate was chosen as the mobile phase for liquid chromatographic studies since reversible interaction should occur with clindamycin 2-phosphate (I) but not with clindamycin 3-phosphate (IV) or clindamycin 4-phosphate (III) (Scheme I). Borate interaction is specific for vicinal *cis*-diols (5) and is provided only with the 2-phosphate ester.

Liquid chromatography was conducted using a 254-nm detector; the conditions for chromatography were: 0.25 *M* boric acid, pH 8.80, 410 psi, 0.23 ml/min at 60°. The isomeric phosphate esters were eluted in the order clindamycin 4-phosphate < clindamycin 3-phosphate < clindamycin 2-phosphate (Fig. 1), as verified by TLC analysis of the collected peaks.

As expected, clindamycin 2-phosphate was eluted last, presumably due to the retarding influence of the increased anionic character of the borate complex II. The separation of the 2- and 3-phosphate esters in the borate system was superior to that provided by several other electrolytes. Lincomycin 2-phosphate (6) is well separated from clindamycin 2-phosphate (retention times of 21.7 and 28.2 min, respectively).

The chromatograms of pure clindamycin 2-phosphate show the presence of a minor peak preceding the major peak (Fig. 2). Peak collection followed by phosphatase hydrolysis (2) and GLC analysis in comparison with authentic standards showed that the major liquid chromatographic peak II (Fig. 2) was clindamycin 2-phosphate and the minor liquid chromatographic peak I was clindamycin B 2-phosphate. Phosphatase hydrolysis liberated clindamycin from the former and clindamycin B from the latter, as established by GLC (Figs. 3 and 4) in comparison to retention times with authentic samples (7). Clindamycin B is the 4′-ethyl analog of clindamycin, which is produced in the synthesis of clindamycin (8) from the biosynthetically prepared lincomycin containing lincomycin B (9).

The theoretical plate number (10) for the clindamycin 2-phosphate peak (Fig. 2) showed that approximately 750 theoretical plates were attained on the 1-m triethylaminoethyl cellulose column. The column is remarkably stable, and one column was used for 3 months at 60° with little change in retention times or theo-

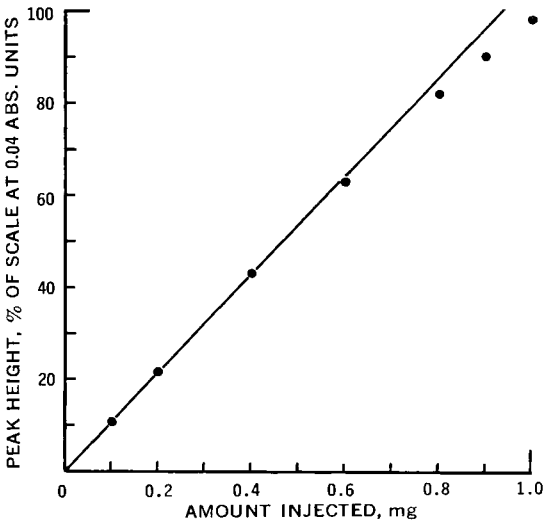


Figure 5—*Liquid chromatographic peak height response* versus *amount of clindamycin 2-phosphate injected.*

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Plots of peak height *versus* amount of clindamycin 2-phosphate injected showed slight deviation from linearity at high sample charges (0.8–1.0 mg) (Fig. 5). Below 0.6 mg, excellent linearity is observed and peak heights can be used for quantitation.

DISCUSSION

The separation of clindamycin 2-phosphate from isomeric and related phosphate esters is sufficient to allow quantitation by the peak height approach.

Detection of clindamycin 2-phosphate by UV monitoring of background absorbance is remarkable and worthy of comment. The molar absorptivity of clindamycin 2-phosphate at 254 nm is only 16.8 in water at pH 8.90. Detection of clindamycin phosphate was made possible only by use of the high capacity support triethylaminoethyl cellulose. The exchange capacity of a 2.1-mm × 1-m column is 810–1160 μEq, as calculated from the column weight and the exchange capacity of the support. This value is approximately 20 times larger than the conventional liquid chromatographic ion-exchange supports which normally contain 48–60 μEq in the same size column (11). The use of high capacity supports should allow the detection of many compounds with low background absorbance at 254 nm.

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A. G. BUTTERFIELD [x], D. W. HUGHES, W. L. WILSON, and N. J. POUND

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Rolitetracycline (I) (2-*N*-pyrrolidinomethyltetracycline) is an antibiotic formed by *N*-aminomethylation of the carboxamide function of tetracycline (II). Compound I is formulated either as the base or nitrate salt for intravenous or intramuscular use. These formulations are intended to be made at the time of use with water suitable for injection.

The official method of assay of I in Canada (1) is microbiological. This method, however, gives no information on the extent of hydrolysis of I, since the test organism is also sensitive to II, nor any estimate of epimerization or degradation products expected to be formed under the assay conditions used. One would expect I, a Mannich base, to be susceptible to hydrolysis in aqueous solution, and Brunzell (2) demonstrated that aqueous solutions of I are indeed rapidly hydrolyzed with the formation of II and 4-epitetracycline (III).

By using high-speed liquid chromatography (HSLC), the stability of I was studied and it was confirmed that aqueous solutions are rapidly hydrolyzed to II (3). In addition, formulations of I were also found to contain II. Although the hydrolysis of I to II does not involve a loss of microbiological activity, the presence of II in formulations could be an indication of poor manufacturing practice.

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By using high-speed liquid chromatography (HSLC), the stability of I was studied and it was confirmed that aqueous solutions are rapidly hydrolyzed to II (3). In addition, formulations of I were also found to contain II. Although the hydrolysis of I to II does not involve a loss of microbiological activity, the presence of II in formulations could be an indication of poor manufacturing practice.

This report describes a rapid HSLC procedure for the simultaneous assay of I and II in rolitetracycline formulations. Being precise and highly specific, the method represents a significant improvement over present analytical procedures for the quality control of rolitetracycline formulations.

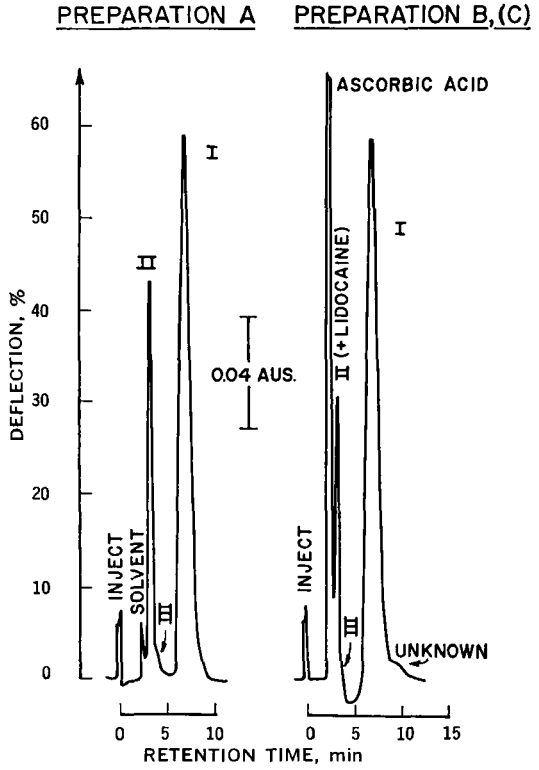

Figure 1—*High-speed liquid chromatograms of typical partially hydrolyzed rolitetracycline and rolitetracycline nitrate formulations. Key: A, rolitetracycline intravenous formulation; B, rolitetracycline nitrate intravenous formulation; and, C, rolitetracycline nitrate intramuscular formulation.*

EXPERIMENTAL

Materials—Rolitetracycline base (I)[1], rolitetracycline nitrate[2] (I-nitrate), tetracycline (II)[3], lidocaine hydrochloride[4], magnesium gluconate[5], and ascorbic acid[6] were used as obtained.

Apparatus—A liquid chromatograph[7] fitted with a constant-temperature water bath, a "septumless" injection port, and a fixed wavelength (254 nm) UV absorption detector (attenuated to 0.32 absorbance unit full scale) was used.

Chromatographic Parameters—A column (225 cm × 3.2 mm o.d. × 1.8 mm i.d., 304 stainless steel) was dry packed with a pellicular cation-exchange resin[8] by a procedure similar to that reported by Kirkland (4) and coiled (diameter, 25 cm) to fit horizontally into the water bath of the instrument. Each liter of mobile phase [0.10 *M* Na^+, 0.003 *M* ethylenediaminetetraacetate ($EDTA^{-2}$), and 40% ethanol; pH 4.35] was prepared by diluting a solution of ethanol (400 ml), 1.0 *M* sodium hydroxide solution (93.3 ml), and 0.05 *M* disodium ethylenediamine tetraacetate (66.7 ml) with water to a volume of 800 ml, adjusting to an apparent pH of 4.35 with acetic acid, and then bringing the solution to volume (1000 ml) with water.

Occasionally a small amount of flocculant precipitate formed during this procedure; however, it readily dissolved when the mobile phase was heated under reflux to remove dissolved gases and did not reprecipitate on cooling. The degassed solvent was stored in the solvent reservoirs of the instrument. A flow rate of 60 ml/hr (4100 psi) and an operating temperature of 25° were used throughout.

[1] Hoechst Pharmaceuticals, Montreal, Quebec, Canada.
[2] Bristol Laboratories of Canada, Candiac, Quebec, Canada.
[3] United States Pharmacopeia, Rockville, Md.
[4] Astra Pharmaceuticals (Canada) Ltd., Cooksville, Ontario, Canada.
[5] K&K Laboratories, Plainview, N.Y.
[6] British Drug Houses, Toronto, Ontario, Canada.
[7] Model 4100, Varian Aerograph, Walnut Creek, Calif.
[8] Pellionex CP-128, Northgate Laboratories, Hamden, Conn.

Table I—Labeled Composition of Rolitetracycline (I) and Rolitetracycline Nitrate Formulations

Formulation	I, mg/Vial	I-Nitrate, mg I/Vial	Magnesium Gluconate, mg/Vial	Ascorbic Acid, mg/Vial	Lidocaine Hydrochloride, mg/Vial
A_1[a]	275	—	434.5	—	—
A_2[a]	110	—	173.8	—	—
B[a]	—	350	273	575	—
C_1[b]	—	350	273	575	40
C_2[b]	—	150	109	246	40

[a] Intravenous formulations. [b] Intramuscular formulations.

Determination of Purity of Rolitetracycline Standard—Approximately 50 mg of the bulk drug standard of I was accurately weighed into a 2-ml volumetric flask. The sample was dissolved with shaking (10 sec), and the solution was immediately brought to volume with aqueous magnesium gluconate (43.4 mg/ml). The flask was placed in a water bath at 25°. Aliquots (0.25 μl) of the sample solution were withdrawn and injected, using a 1-μl syringe fitted with a reproducibility adaptor[9], at about 12-min intervals for a 3-hr period. The flask was shaken briefly prior to each sampling, and the time from solution makeup to elution of the peak due to I was recorded for each injection.

Determination of Synthetic Mixtures—*Rolitetracycline*—The desired amount of I was accurately weighed into a 2-ml volumetric flask. The sample was dissolved with shaking (about 10 sec), and the solution was brought to volume with aqueous magnesium gluconate (43.4 mg/ml). The flask was immediately placed in an ice bath (0°). The sample was shaken at 10-min intervals during the 30 min allowed for cooling. After 30 min, triplicate 0.25-μl aliquots were chromatographed.

Rolitetracycline Nitrate—The required amounts of I-nitrate and ascorbic acid (115 mg) were accurately weighed into a 2-ml volumetric flask. In the case of the synthetic intramuscular formulations, lidocaine hydrochloride (8.0 mg) was also added to the sample. The samples were then dissolved, brought to volume with magnesium gluconate solution, chilled, and chromatographed in the same manner as the synthetic mixture of I.

Determination of Commercial Products—*Drug Substances*—A sample of the drug substance (I or I-nitrate), equivalent to approximately 50 mg of I, was accurately weighed into a 2-ml volumetric flask, and the sample was treated by the procedure previously described for synthetic mixtures of I.

Formulations—The contents of a single vial (275 or 350 mg of I or I-nitrate) were dissolved in water and immediately transferred quantitatively to a 10-ml volumetric flask. In the case of 110- or 150-mg formulations, a 5-ml volumetric flask was used. The solution was then brought to volume with water, shaken, and placed in

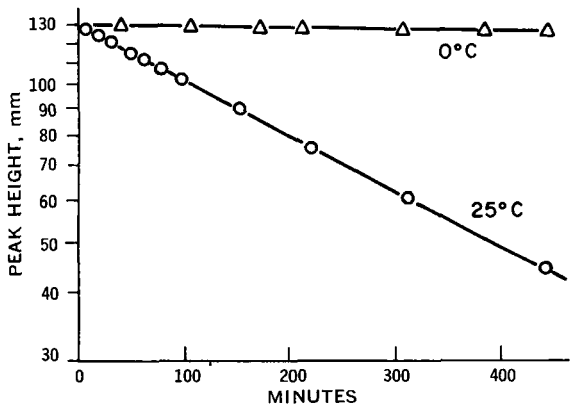

Figure 2—*Plot of log peak height* versus *time in solution from chromatograms of rolitetracycline solutions at two temperatures.*

[9] Model 7101N CH, Hamilton Co. Reno, Nev.

Table II—HSLC Analysis of Synthetic Rolitetracycline Formulations

Synthetic Formulations	Rolitetracycline (I)			Tetracycline (II)[a]			Total[a]		
	Calc., μg	Found, μg	Recovery, %	Calc., μg	Found, μg	Recovery, %	Calc., μg	Found, μg	Recovery, %
I	4.54	4.56	100.4	0.29	0.28	97	4.83	4.84	100.2
II	7.23	7.22	99.9	0.42	0.42	100	7.65	7.64	99.9
III	4.40	4.38	99.6	—	—	—	4.40	4.38	99.6
IV	5.43	5.42	99.7	—	—	—	5.43	5.42	99.7
Mean recovery, %			99.9			98			99.8
RSD, %			±0.4			±2			±0.3

[a] Expressed as equivalent amount of I.

Table III—HSLC Analysis of Synthetic Rolitetracycline Nitrate Formulations

Synthetic Formulations	Rolitetracycline (I)			Tetracycline[a] (II)			Total[a,d]		
	Calc., μg	Found, μg	Recovery, %	Calc., μg	Found, μg	Recovery, %	Calc., μg	Found, μg	Recovery, %
I[b]	3.43	3.41	98.6	0.17	0.17	100	3.60	3.58	99.5
II[b]	6.18	6.13	99.2	0.60	0.59	98	6.78	6.72	99.1
III[c]	3.68	3.66	99.5	0.93	0.95	102	4.61	4.61	100.0
IV[c]	4.23	4.15	98.1	0.80	0.81	101	5.03	4.96	98.6
Mean recovery, %			98.9			100			99.3
RSD, %			±0.6			±2			±0.6

[a] Expressed as equivalent amount of I. [b] Synthetic intramuscular formulation. [c] Synthetic intravenous formulation. [d] Total micrograms of I and II.

an ice bath. After cooling for 30 min, with shaking at 10-min intervals, triplicate 0.25-μl aliquots of the solutions were chromatographed.

Calculations—Concentrations of I and II were determined using the following expressions:

$$\text{I (micrograms per injection)} = (P_R + b)/m_R \quad \text{(Eq. 1)}$$

$$\text{II (micrograms per injection)} = [P_T - P_B - P_R(C)]/m_T \quad \text{(Eq. 2)}$$

$$\text{II (micrograms as I)} = \text{II (micrograms)} \times D \quad \text{(Eq. 3)}$$

where:

P_R = peak height for I (millimeters)
b = y intercept of calibration curve for I (− 5.7 mm)[10]
m_R = slope of calibration curve for I (24.1 mm/μg)[10]
P_T = peak height for II (millimeters)
P_B = peak height for sample blank (millimeters)
C = factor to correct for decomposition of I to II during sample preparation (0.061)[10]
m_T = slope of calibration curve for II (62.5 mm/μg)[10]
D = factor to express weight of II as an equivalent weight of I (1.187 = mol. wt. I/mol. wt. II)

RESULTS AND DISCUSSION

The high-speed cation-exchange chromatography system used in this analysis is a modification of one described in a previous study of the chromatographic behavior of a number of tetracycline derivatives (5). In the present investigation, the ethanol concentration of the mobile phase was increased to 40% and the operating temperature was reduced to 25° to minimize on-column hydrolysis of I.

Figure 1 illustrates the chromatograms obtained from the analysis of three, partially degraded, commercial formulations. Preparation A is made up of I and magnesium gluconate; while Preparation B contains I-nitrate, ascorbic acid, and magnesium gluconate. Preparation C is an intramuscular I-nitrate formulation containing lidocaine hydrochloride as well as ascorbic acid and magnesium gluconate. In addition to I and II, small amounts of III can also be detected in these degraded samples.

Under these conditions, retention times of 2.5, 3.1, 3.7, and 6.6 min were obtained for ascorbic acid, II, III, and I, respectively, while lidocaine hydrochloride and magnesium gluconate chromatographed as small peaks with retention times of 3.0 and 2.8 min, respectively. The latter two compounds are not resolved from the peak due to II; however, because of their low detector response, they are equivalent to less than 0.15 μg/injection of II in a typical formulation. A correction is made for the interference of these compounds by using a blank value (P_B) in Eq. 2.

Peaks due to two as yet unidentified substances, with retention times of 4.3 and 9.3 min, were also detected when I-nitrate preparations were chromatographed. One substance was present only in solutions containing ascorbic acid and produced a negative peak (4.3 min). A similar peak was also detected when aqueous solutions of ascorbic acid were chromatographed and is thought to be due to a displacement desorption of some other compound with a low UV extinction coefficient. The second substance (9.3 min) was present in all I-nitrate bulk drug samples and formulations examined. This peak may be due to 4-epirolitetracycline (IV). Neither of these peaks interferes with the determination of I or II in I-nitrate formulations.

Simultaneous quantitative analysis of both I and II is possible by relating peak height measurements to calibration curves for each of these two compounds. However, due to the intrinsic instability of solutions of I, it has been necessary to include correction factors in Eq. 2 to compensate for the formation of II from I during sample preparation.

The rate of degradation of I and I-nitrate is affected mainly by temperature (Fig. 2) and, to a lesser extent, by the concentrations of magnesium gluconate or of I in the sample solution[11]. Therefore, to facilitate accurate quantitative analysis and to permit the analysis of all formulations from a single calibration curve for I, samples containing a relatively high concentration of I (22–35 mg/ml) were prepared and immediately cooled to 0°. During the analysis, these samples were maintained at 0°. From Fig. 2 it can be seen that the rate of degradation is retarded to such an extent at 0° that any changes in the composition of the sample are negligible over the 1–2 hr required for an analysis. In fact, continued analysis of synthetic mixtures of I and I-nitrate over 7 hr at 0° showed less than 1.5% degradation.

The purity of the standard of I used throughout this study was determined relative to an FDA standard by UV (1) (99.4%), microbiological[12] (100%), and HSLC (100%) methods. HSLC was used to monitor the formation of II in three magnesium gluconate solutions of the standard of I at 25° over 3 hr. Extrapolation of the curve for II back to zero time indicated that no II was initially present in the sample; *i.e.*, it contained 100% I.

[10] Factors obtained under experimental conditions described here.

[11] Unpublished data.

[12] Reported by supplier.

Table IV—Analysis of Rolitetracycline (I) and Rolitetracycline Nitrate Formulations[a]

Sample	HSLC				Microbiological[e]	UV
	I	II[b]	IV[b,c]	Total[d]		
Rolitetracycline:						
110 mg, intravenous	105.6	1.3	—	106.9	111	106.3
275 mg, intravenous	110.9	0.7	—	111.6	114	110.3
Rolitetracycline nitrate:						
150 mg, intramuscular	104.0	4.2	14.0	108.2	112	122.2
350 mg, intramuscular	109.5	5.5	6.9	115.0	113	121.9
350 mg, intramuscular	113.3	6.9	13.7	120.2	118	133.9
350 mg, intravenous	111.9	7.1	7.4	119.0	115	126.4

[a] Results expressed as percent of labeled claim for I. [b] Expressed as equivalent amount of I. [c] By difference, UV minus Total. [d] Total I and II, *i.e.*, microbiologically active components. [e] Average of two determinations.

An absolute injection technique was used throughout this study. A relative standard deviation of 1.4% was obtained from the peak height measurements for the injection of five 0.25-μl (4.7-μg) aliquots of a sample of I maintained at 0°. Calibration curves were prepared for both I and II. A plot of peak height for I, against the amount of I injected for eight synthetic mixtures of I, was linear over a concentration range of 8–44 mg/ml (2–11 μg/injection) (Fig. 3). A least-squares analysis of the data showed that the linear portion of the curve could be expressed by an equation for a straight line, with a slope of 24.1 mm/μg and a y-intercept of −5.7 mm. Synthetic mixtures, prepared daily, were used throughout the study to check the calibration curve. The difference from the calculated values was never more than 1%.

A similar procedure was used for the determination of II in the formulations; however, the equation for the calibration curve for II was corrected for background interference caused by the presence of lidocaine hydrochloride or magnesium gluconate in the samples and for the amount of II formed from I during sample preparation. Analysis of six samples of pure I, cooled to 0° and left to equilibrate for 30 min, showed that a peak for II was obtained which was 6.1 ± 0.5% of the measured peak for I. Therefore, a factor of 0.061 (C, Eq. 2) multiplied by the measured I peak height (P_R, Eq. 2) was included to correct the measured II peak height for the formation of II during sample preparation and equilibration at 0°. Since measured I peak heights *versus* micrograms of I injected were plotted to obtain the calibration curve for I, no correction for decomposition during sample makeup was required in Eq. 1.

The calibration curve obtained from the analysis of solutions of II, in 0.01 M HCl, over a concentration range of 2–32 mg/ml (0.5–8 μg/injection) was a straight line through the origin with a mean slope of 62.5 mm/μg.

The value for the blank (P_B, Eq. 2) is readily obtained from the analysis of a blank solution containing the approximate concentration of ascorbic acid, lidocaine hydrochloride, and magnesium gluconate found in the formulation (Table I).

To facilitate correlation of the analytical data, it was convenient to express the weight of II as that weight of I from which it could have formed. Therefore, a third factor, 1.187 (D, Eq. 3), was included to convert the weight of II to the equivalent weight of I.

The results obtained from the analysis of synthetic formulations of I containing varying amounts of I and II are listed in Table II. Excellent correlation between the theoretical and observed values for the individual components and the total concentration of tetracyclines, expressed as I, was obtained. Similar correlation was observed between the HSLC and UV analyses of commercial formulations of I (Table IV).

The situation was somewhat more complex, however, in the case of I-nitrate and its formulations. The theoretical amount of I in pure I-nitrate bulk drug is 89.5%. Microbiological[12] and UV (1) analyses of I-nitrate bulk drug showed it to be pure (microbiological, 98%; UV, 102.5%). However, HSLC analysis of three replicates of the sample indicated that it contained 88.4 ± 0.3% of the expected amount of I along with an amount of II equivalent to 4.2 ± 0.2% of the expected amount of I. Thus, it can be concluded that the bulk drug contains at least one other UV-absorbing substance (about 7.4%) in addition to I and II. This observation is further substantiated by the fact that the HSLC chromatograms obtained for all I-nitrate bulk drugs and formulations contained an extraneous peak (9.3 min).

This extraneous peak was not due to the presence of 4-epianhydrotetracycline or anhydrotetracycline in the sample, since these compounds were found to have retention times of 8.6 and 4.9 min, respectively, under these conditions. It may, however, be due to IV-nitrate since it elutes after I. Previous work (5) showed that four 4-epitetracyclines were more retentive than the corresponding epimers. Furthermore, the microbiological assay results for the I-nitrate formulations (Table IV) are significantly lower than the UV results. This finding could be explained by the fact that during such an analysis IV would be hydrolyzed to III, which has been reported to possess approximately 5% of the microbiological activity of II (6). It has been shown[11] that I is more than 80% hydrolyzed in less than 30 min under the conditions specified for the microbiological assay. In addition, the formation of appreciable amounts of IV by the treatment of I with acetic acid was reported previously (7). When a sample of I was treated under these conditions and chromatographed, the size of the peak at retention time 9.3 min was increased to approximately two-thirds the size of the peak due to I. Finally, IV would be assayed by the UV method as II, since the tetracycline moiety is measured by this method. This would explain why the HSLC and UV methods agree for formulations of I, where none of this third compound is detected, and yet disagree where the compound is present, as in the I-nitrate formulations (Table IV).

The results from the analysis of a series of synthetic I-nitrate mixtures (Table III) showed good correlation between the calculated and found values for I and II in these mixtures. Mean recovery values of 98.9 ± 0.6 and 100.2 ± 2.8% were obtained for I and II, respectively. The UV analyses shown in Table IV indicate a higher concentration of total tetracyclines than the total amount of I and II determined by HSLC. This result is as expected, since the chro-

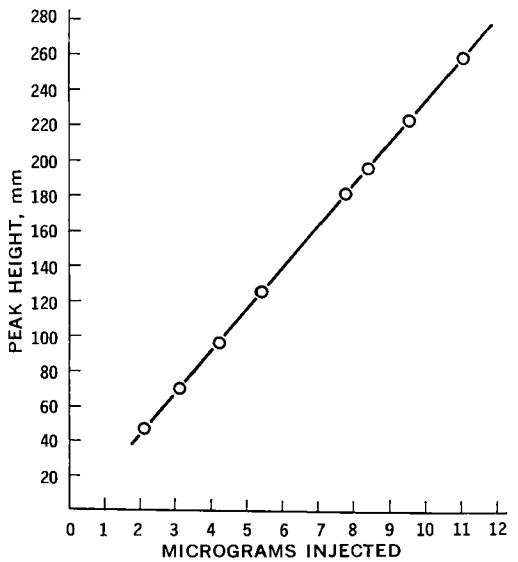

Figure 3—*Plot of peak height* versus *micrograms of rolitetracycline from chromatograms of rolitetracycline standard solutions.*

matogram of each of these mixtures possessed the small extraneous peak at 9.3 min.

The average amount of IV in the formulations (Table IV) was estimated, by difference, to be 10.5%. The UV analyses (mean = 126.1%) indicated an average "overfill" of 26.1%; therefore, the average amount of IV in the formulation is equivalent to 8.3% of the total tetracyclines, expressed as I. This value is comparable to the 7.4% found in the particular sample of I-nitrate bulk drug substance used in this study. Close correlation was also obtained between the results of the microbiological analyses and the total amount of I and II determined by the HSLC procedure. This finding is consistant with the fact that III, which would be formed by the hydrolysis of IV, is less microbiologically active than II (6).

In conclusion, the HSLC procedure provides a rapid, precise method for the simultaneous qualitative and quantitative analysis of rolitetracycline and its hydrolysis product, tetracycline, in both rolitetracycline and rolitetracycline nitrate formulations. A third component found in rolitetracycline nitrate formulations has not been identified conclusively, but UV and microbiological assays indicate that this compound is present at levels as high as 10% of total tetracyclines, contains a tetracycline moiety, and is comparatively inactive microbiologically. A tentative identification as 4-epirolitetracycline is suggested.

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PHARMACEUTICAL TECHNOLOGY

Versatile Unit for Filling Gelatin Capsules with Drugs or Chemicals

GEORGE R. THOMPSON [x] and ADAM CUNNINGHAM

Abstract □ A new, inexpensive device was developed for making individually prepared capsules that can be administered to experimental animals. The device was designed for accurate and rapid production of large numbers of various capsule sizes and drug dosages. During 12 months of usage, 21 capsules were prepared weekly for each of 44 dogs (924 total capsules) in approximately 2.5 workhours/week. Each capsule contained a precise amount of drug to administer a specific dosage to each individual dog. No difficulties were encountered in the manufacture or utilization of this device, and it can be operated by untrained personnel.

Keyphrases □ Capsules—device for making individually prepared capsules, design and application □ Gelatin capsules—rapid production of various capsule sizes and drug dosages, device described □ Equipment—rapid production of various capsules and drug dosages, design and application

Drugs and chemicals intended for human consumption or exposure must first be evaluated for toxicity in laboratory animals. The Food and Drug Administration and other governmental regulatory agencies recommend that the route of administration in animals corresponds to the route for proposed human usage (1). For subacute or chronic oral administration of food additives, pesticides, or other materials to rodents, the compounds can be incorporated into the diet. Dogs and monkeys can receive daily *per os* administration of drugs or other orally consumed chemicals by administration of the materials as tablets, in capsules, or by intubation of solutions or suspensions. Each of these three alternatives for drug administration involves significant shortcomings with large numbers of animals for long periods.

Manual production of tablets or capsules requires considerable effort and adds unduly to the cost of an experiment[1]. Preparation of tablets or capsules with commercial units decreases time and cost but sacrifices accuracy in dosage due to unit-to-unit variation (2, 3) and loss of the ability to adjust *precisely* for

[1] Manual preparation of 924 capsules/week for 44 dogs in a 1-year toxicity study could cost more than $15,800.

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Table I—Softening Times of No. 13 Gelatin Capsules

Capsule Contents	Storage Temperature	Softening Time (min)[a], $\bar{x} \pm SD$
1.0 ml of water	22.8°	12.54 ± 1.67
1.0 ml of water	9.0°	18.50 ± 2.21
1.0 ml of water	−9.0°	20.04 ± 2.25
0.82 g of gelatin powder + 1.0 ml of water	22.8°	25.20 ± 2.27
0.82 g of gelatin powder + 1.0 ml of water	9.0°	>120[b]
0.82 g of gelatin powder + 1.0 ml of water	−9.0°	>120[c]

[a] Mean values for five individual capsules per group. The softening time was defined as the elapsed time from instillation of the water until the capsules became too soft for administration to animals. [b] These five capsules were slightly soft within 20 min, but they were never judged too soft for administration; they were only "rubbery" instead of "collapsible." [c] These five capsules never became soft and they were frozen within a few hours.

short-term changes in body weights of the animals. This last deficiency can be partially rectified by preparing several units with different dosages and administering multiple units to approximate the desired dose. However, considerable accuracy in dosage can still be lost, and substantial time and effort are required to total manually the units for dosing each animal. In addition, commercial production of tablets or capsules offers cost savings only if thousands of units are prepared simultaneously; the units must, therefore, be handled and stored so that problems with physical instability are avoided (4).

Gastric intubation of solutions or suspensions to dogs and monkeys provides an accurate dosage and is relatively inexpensive. However, daily intubation for protracted periods can produce aspiration pneumonitis (5, 6) as well as a low incidence of intubation accidents.

DISCUSSION

Solutions or suspensions can be accurately formulated for administration to animals; but when these formulations are placed in gelatin capsules, dissolution of the capsule is an undesirable effect. Capsule dissolution can be significantly retarded by cooling filled capsules, adding powdered gelatin to the capsule prior to addition of a liquid formulation, or by a combination of these two techniques (Table I).

Cooled No. 13 capsules filled with 1.0 ml of water became too soft to handle within a mean time of 18.5 min when refrigerated at 9.0° and within 20.0 min when stored at −9.0°. Similar capsules stored at ambient temperature (22.8°) softened in an average of 12.5 min. Addition of an average of 0.82 g of powdered gelatin prior to 1.0 ml of water resulted in a mean softening time of 25.2 min at ambient temperature; similarly filled capsules stored at 9° were slightly soft in approximately 15–20 min, but these capsules were never too soft to handle. Capsules with powdered gelatin and water stored at −9° never became soft and were frozen within a few hours. Therefore, the device was designed to add a consistent amount of powdered gelatin to each capsule just prior to addition of the drug solution. The capsules were then quickly frozen until needed during the next week. The drug used in these studies was stable in frozen capsules for more than 6 weeks.

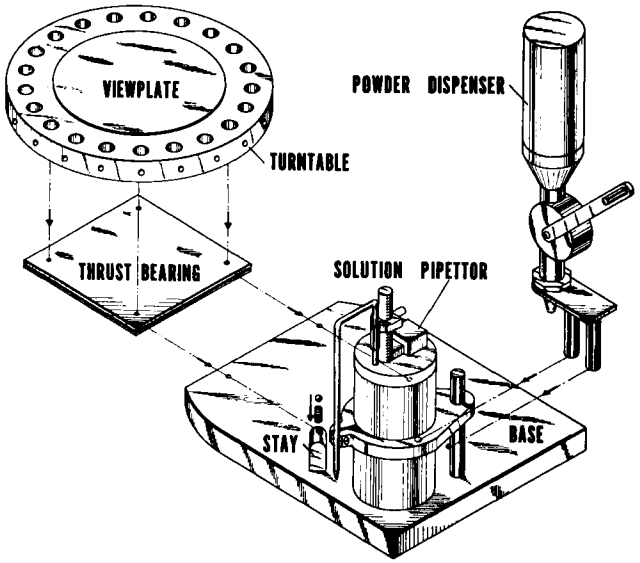

Figure 1—*Schematic presentation of the unit assemblage. The base of the unit measured 25.4 × 30.4 cm (10 × 12 in.), and the thrust bearing was 10.2 × 10.2 cm (4 × 4 in.). The diameter of the turntable was 24.76 cm (9.75 in.)*

EXPERIMENTAL

The details of the design and construction of the capsule-filling unit are illustrated in Fig. 1. The base and turntable were cut from scrap pieces of 2.54-cm (1-in.) oak and varnished to seal the wood for protection against spilled solutions and powders. Sufficient holes for a 1-week/animal supply of capsules were drilled into the turntable, and the hole diameters in various turntables were varied to accommodate different capsule sizes (Table II). The edge of the turntable was pitted perpendicular to each capsule hole, and these pits served as sites for engaging the stay. The white formica view-

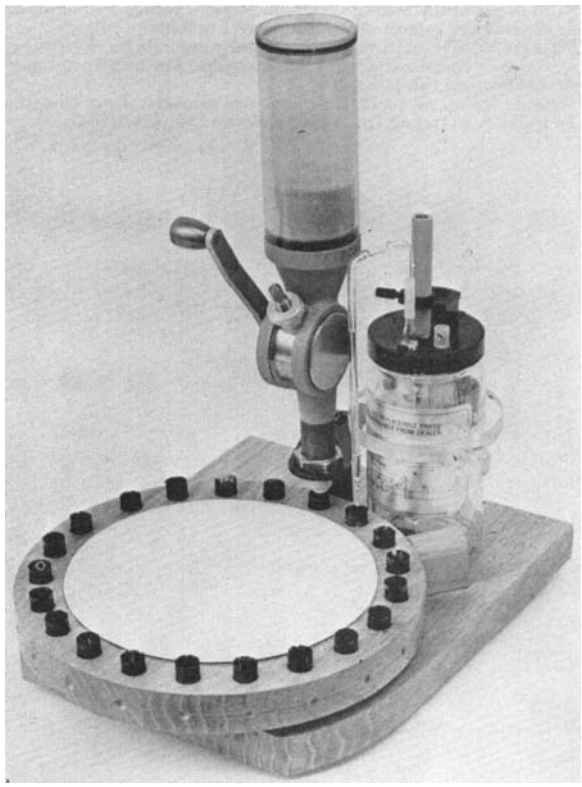

Figure 2—*View of the assembled unit.*

Table II—Physical Dimensions of Gelatin Capsules

Capsule No.	Length, cm	Diameter, cm	Maximum Volume[a], ml
13[b]	2.966	1.460	2.63
00[c]	2.324	0.840	0.93
0[d]	2.128	0.741	0.67
1[d]	1.905	0.675	0.49
2[d]	1.800	0.622	0.38
3[c]	1.617	0.571	0.28
4[d]	1.332	0.523	0.21

[a] Maximum volume of water that could be placed in the bottom half of the capsule. Capsules 00–4, containing their maximum volumes of water, were too soft to handle within 1–2 min; No. 13 capsules were too soft to handle within approximately 12.5 min. [b] Michigan Capsule Co. [c] Eli Lilly. [d] Parke-Davis.

plate offered an easily cleaned surface for checking the integrity of capsules before and after filling.

The thrust bearing and wooden stay were utilized to allow easy and reproducible alignment of each capsule under the powder dispenser[2] and/or the pipet[3]. The powder dispenser and solution pipet were positioned so that they simultaneously centered over capsules. When capsules smaller than the No. 13 were utilized, a special funnel had to be attached to the bottom of the powder dispenser. An extension of glass tubing was attached to the pipet to minimize the distance between the delivery tip and the capsule. The assembled unit is shown in Fig. 2.

Volumes of formulation to be dispensed into each capsule for an individual animal were calculated from the weekly body weight of the animal, the dose to be administered, and the solution concentration. The formulation was placed in the pipet, which had been adjusted for the desired volume for each capsule, and the gelatin powder was placed in the reservoir of the dispenser. All holes in the turntable were then filled with the bottom half of the appropriate size of capsule. The first two capsules were filled with gelatin powder; but while the second capsule was filled with the powder, the first capsule was filled with the proper formulation volume from the pipet. Therefore, as the turntable was rotated, two capsules were filled simultaneously—one with gelatin powder and one with drug solution onto gelatin powder. As soon as the turntable completed one revolution, all capsules were capped and stored in a freezer. Following this procedure, 21 capsules could be filled with gelatin powder and formulation in less than 1 min.

[2] Uniflow model II powder measure, available commercially or from RCBS Inc., Oroville, CA 95965. This particular powder dispenser has been found to be extremely accurate. Ten samples at a setting for approximately 0.82 g of powdered gelatin varied from 0.8071 to 0.8282 g with an $\bar{x} \pm SD$ of 0.8202 ± 0.0055. This unit may be sufficiently accurate for dispensing powdered drugs or chemicals directly into capsules, but no attempt has been made to investigate this possibility.

[3] Oxford Pipettor model P5058-2, available through various scientific supply houses or from Oxford Laboratories, Foster City, CA 94404

SUMMARY

This device circumvents or minimizes essentially all problems of dosage accuracy, physical instability of dosage units, labor costs, and incidental pathology. Costs for materials in this unit were less than $70.00, and only 10 hr was required for its construction. The unit can be operated efficiently by untrained personnel with little chance for technical error. Care and maintenance of this device are extremely easy, since all contaminated units or surfaces are either removable or openly exposed for cleaning.

During 12 months of usage, 924 capsules were prepared weekly for 44 dogs, and each capsule contained a precise amount of drug to administer a specific dosage (on the basis of milligrams per kilogram) to each individual animal. Dosages were easily adjusted each week for changes in body weights of the individual dogs by adjusting the volume of drug solution pipetted into each capsule.

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Measurement of Surface Color and Color Difference of Tablet Colorants by Tristimulus Colorimetry

FRANCES M. BOGDANSKY

Abstract □ The surface color of a series of color dispersions containing from one to three FD&C or D&C dyes suspended in a sucrose syrup was examined using tristimulus colorimetry. CIE (x, y) and Hunter (a, b) chromaticity data, determined directly from an integrating sphere colorimeter or from reflectance spectra and suitable calculations, were used to order materials by color on expanded chromaticity diagrams. Color difference was evaluated quantitatively from Hunter (a, b) data using the Judd color difference formula. Quality control specifications were established either by defining areas of acceptable color by applying limits to the chromaticity coordinates of color standards or by requiring the Judd color difference to be less than a specific number of National Bureau of Standards standard units. This method eliminates the subjective character of color evaluation and permits the quantitation of visual color difference. These differences can be used, in conjunction with defined color standards, to develop color specifications for tablet colorants, finished tablets, and liquid and cream pharmaceutical preparations.

Keyphrases □ Color difference and surface color—measurement of tablet colorants by tristimulus colorimetry □ Tristimulus colorimetry—measurement of surface color and color difference of tablet colorants □ Chromaticity data—surface color of series of FD&C and D&C color dispersions, tristimulus colorimetry □ Colorimetry, tristimulus—surface color and color difference of tablet colorants

Many pharmaceutical tablets are coated with sugar suspensions of FD&C and D&C dyes for purposes of aesthetics, identification, and stability. Since it is desirable that all tablets coated with a specific colorant appear to be the same color, it is important that the coating material be of uniform and consistent color. The evaluation of surface color is usually a subjective test, dependent only on the ability of an individual to discriminate color differences visually.

Because the eye is not an analytical instrument, it is not capable of precisely defining color or of consistently discriminating small differences in color between two similar substances. The eye also possesses only a limited memory for color, and the visual storage of color data is, therefore, extremely difficult. Visual color determinations are complicated further by the fact that different individuals perceive the same color differently, and even the same individual often describes a single color in a different manner at different times.

Subjective color determination usually involves a visual comparison of the sample with some arbitrary color standard. This procedure necessitates reliance on color standards that are subject to change with time and are not permanent records of color. As a result, methods often involve the frequent redefinition of color standards which can lead eventually to a gradual, but significant, change in acceptable color. There is, therefore, a necessity to define color in absolute terms and to develop a numerical method of color evaluation.

Spectrophotometric techniques for the evaluation of surface color are not new. Trichromatic color measurement (tristimulus colorimetry) has been used successfully in the paint, textile, and plastic industries for many years (1, 2). Some work also has been reported on color determination and color control in the food industry (3, 4). More recently, colorimetry has been employed in the determination of color stability of pharmaceutical tablet formulations (5–18) and in the production of color matches for solid dosage forms (19–21). This work describes the measurement of surface color by tristimulus colorimetry and the determination of color specifications for the routine control of tablet colorants.

THEORY

Any color can be matched by an appropriate mixture of three selected radiations, usually saturated red (700 nm), green (546 nm), and blue (436 nm) (19). This phenomenon of three-color mixing and matching is attributed to the existence of a triple-receptor system in the eye, where the entire range of color sensations is derived from variations in the magnitude of responses in three forms of cone receptors. The spectral components of a beam of light entering the eye are combined additively when focused on the cone receptors of the retina, so an additive color mixture system must provide the basis of color measurement (20). A system has been developed by the Commission Internationale de l'Eclairge (CIE) in terms of the amounts of three defined stimuli which, when mixed in fixed proportions, produce a complete color match with some established standard. The tristimulus values (X, Y, and Z) of any sample are defined as the magnitudes of these three standard stimuli needed to produce a color match.

When a colored surface is illuminated by a standardized illuminant and the flux incident on that reflecting surface has the spectral energy distribution, P_λ, the tristimulus values of the reflected light are given by (19):

$$X = \int_0^\infty \bar{x}_\lambda P_\lambda \rho_\lambda \, d\lambda \Big/ \int_0^\infty \bar{y}_\lambda P_\lambda \, d\lambda \simeq \sum_{380}^{770} \bar{x}_\lambda P_\lambda \rho_\lambda \Big/ \sum_{380}^{770} \bar{y}_\lambda P_\lambda \quad \text{(Eq. 1)}$$

$$Y = \int_0^\infty \bar{y}_\lambda P_\lambda \rho_\lambda \, d\lambda \Big/ \int_0^\infty \bar{y}_\lambda P_\lambda \, d\lambda \simeq \sum_{380}^{770} \bar{y}_\lambda P_\lambda \rho_\lambda \Big/ \sum_{380}^{770} \bar{y}_\lambda P_\lambda \quad \text{(Eq. 2)}$$

$$Z = \int_0^\infty \bar{z}_\lambda P_\lambda \rho_\lambda \, d\lambda \Big/ \int_0^\infty \bar{y}_\lambda P_\lambda \, d\lambda \simeq \sum_{380}^{770} \bar{z}_\lambda P_\lambda \rho_\lambda \Big/ \sum_{380}^{770} \bar{y}_\lambda P_\lambda \quad \text{(Eq. 3)}$$

where ρ_λ is the reflectance, $\rho_\lambda = 1.000$ for 100.0% reflectance, and $\bar{x}_\lambda$, $\bar{y}_\lambda$, and $\bar{z}_\lambda$ are the spectral tristimulus values (distribution coefficients) of the equal energy spectrum that have been determined from color matching experiments. Since the products $\bar{x}_\lambda P_\lambda$, $\bar{y}_\lambda P_\lambda$, and $\bar{z}_\lambda P_\lambda$ for the standard sources are used frequently, tables of these products have been prepared with normalized $\bar{y}_\lambda$ distribution coefficients so that $\int_0^\infty \bar{y}_\lambda P_\lambda \, d\lambda = 100.000$[1]. The Y tristimulus value is a direct measure of the percent luminance of the colored surface. The chromaticity coordinates of the reflected light are calculated as follows:

[1] The normalized energy-weighted distribution coefficients for numerous illuminants including the S_c and D-6500 sources and the spectral distribution coefficients have been tabulated by CIE and are found in the literature (Ref. 20, pp. 316–330).

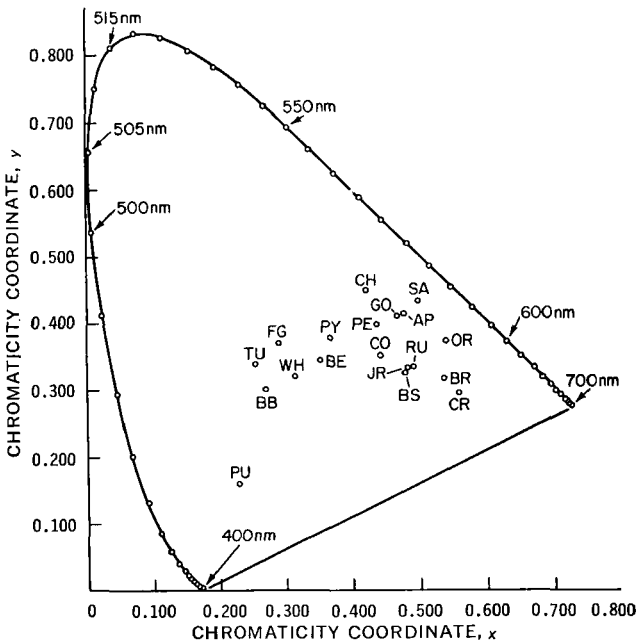

Figure 1—*An* (x, y) *chromaticity diagram prepared from the 1931 CIE chromaticity coordinates for a field subtending 2° (Ref. 19, p. 316). The colorants listed in Table I are located by their* (x, y) *coordinates to define the region of the diagram where real colors are found.*

$$x = X/(X + Y + Z) \qquad \text{(Eq. 4}a\text{)}$$

$$y = Y/(X + Y + Z) \qquad \text{(Eq. 4}b\text{)}$$

The x and y chromaticities can then be employed as coordinates to locate the position of the sample in a two-dimensional chromaticity diagram.

The separation of points on the chromaticity diagram represents real differences in color. Color difference determinations are, however, complicated because significant differences in (x, y) chromaticity values do not necessarily produce significant visual differences in color for all spectral regions (21). As a result, a nonuniform distribution of colors on the chromaticity diagram is obtained.

To produce a more uniform distribution of colors, a transformation of the chromaticity diagram is required such that the distance between any two colors is a direct measure of their visual differences. One transformation that has been widely used is the Hunter (a, b) scale. The (a, b) coordinates of a sample are derived from the (x, y) chromaticity coordinates by the following expressions (22):

$$a = [17.5Y^{1/2}][1.02(x/y) - 1.0] \qquad \text{(Eq. 5)}$$

$$b = [5.927Y^{1/2}][x + 2.181y - 1.0]/y \qquad \text{(Eq. 6)}$$

A more uniform color diagram is obtained when a and b are employed as the coordinates of the chromaticity diagram.

Several methods are available for determining color difference quantitatively. The Judd color difference formula is widely used, especially in the form shown in Eq. 7 (23):

$$\Delta E = [\Delta L^2 + \Delta a^2 + \Delta b^2]^{1/2} \qquad \text{(Eq. 7)}$$

where $\Delta L = [10Y_1^{1/2}] - [10Y_2^{1/2}]$, $\Delta a = a_1 - a_2$, $\Delta b = b_1 - b_2$, and the color difference, ΔE, is expressed in National Bureau of Standards (NBS) units. The NBS unit, the color difference seen when $\Delta E = 1$, is about three times smaller than can be perceived visually under the best conditions and corresponds to a chromaticity change of 0.0015–0.0025 in x and y for points in the center of the (x, y) diagram.

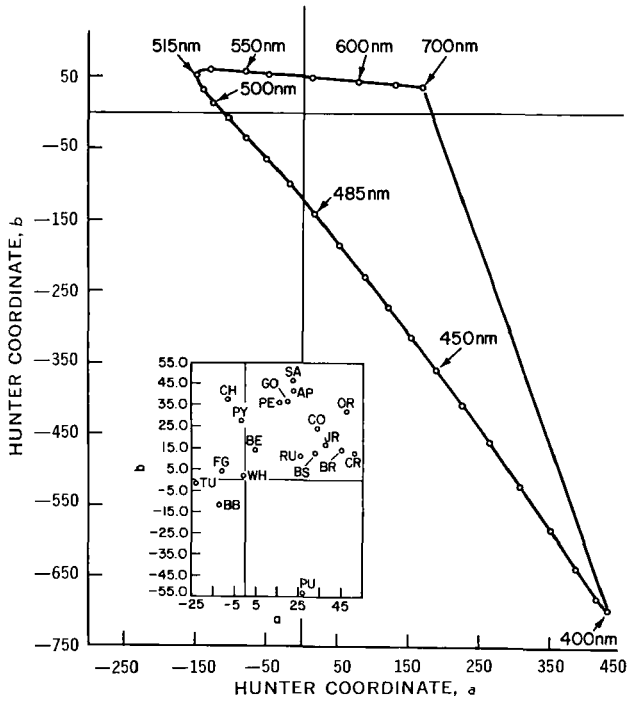

Figure 2—*Hunter* (a, b) *diagram prepared from the 1931 2° CIE chromaticity coordinates using the transformations shown in Eqs. 5 and 6. The expanded portion of the diagram shows the location of the colorants listed in Table I.*

EXPERIMENTAL

The color dispersions investigated were purchased commercially[2]. Each dispersion contains from one to three D&C or FD&C dyes adsorbed on alumina and suspended in a mixture of sucrose syrup, titanium dioxide, and sodium benzoate.

Spectral samples were prepared by spreading 5-μm layers of the suspension on chromatography paper[3] with a Bird applicator[4]. Reflectance spectra for all samples were obtained over the 380–770-nm wavelength region using a reflectance spectrophotometer[5] equipped with a tungsten lamp. Spectra were recorded against vitrolite reference plates[6]. The reflectance due to the reference was determined by running two matched vitrolite plates, and corrections for this reflectance always were made before the tristimulus values were calculated. Tristimulus and chromaticity data were calculated from reflectance values using Eqs. 1–4*b* and the CIE 1964 10° energy-weighted distribution coefficients for the D-6500[7] standard illuminant[8].

Hunter (a, b) coordinates and some tristimulus data were obtained using the CIE 1931 2° field and the standard illuminant, S_c[9], with a direct readout colorimeter[10] equipped with a tungsten-halogen lamp and novoil filters[11]. All samples were examined using the colorimeter large sample beam and 1.9-cm (0.75-in.) aperture.

[2] Colorcon, Inc., West Point, Pa.
[3] Whatman 3MM chromatography paper.
[4] Precision Gage and Tool Co., Dayton, Ohio.
[5] Beckman DB-G spectrophotometer with reflectance attachment, Beckman Instruments, Fullerton, Calif.
[6] Beckman Instruments, Fullerton, Calif.
[7] The Colorimetry Commission of CIE has defined standard spectral distributions at various color temperatures, and the source D-6500, with a color temperature of 6500°K, has been selected as typical of average daylight (Ref. 19, p. 7).
[8] Individual calculations were made at 10-nm intervals over the 770–380-nm wavelength region, and a summation of these was made to evaluate X, Y, and Z. The energy-weighted distribution coefficients used are tabulated in the literature (Ref. 19, pp. 316–330).
[9] The 1931 CIE source S_c, with a color temperature of 6700°K, approximates average daylight. The source consists of a gas-filled tungsten lamp used in conjunction with two color filters (Ref. 19, p. 309).
[10] Gardner XL-10, Gardner Laboratory, Inc., Bethesda, Md.
[11] Gardner Laboratory, Inc., Bethesda, Md. The novoil filters absorb UV light and simplify measurements on fluorescent samples.

Table I—(x, y) and (a, b) Chromaticity Data for Colorants

Sample	Color	Composition[a]	x[b]	y	$10Y^{1/2}$	a	b
AP	Apricot	Y5, Y6	0.474 ± 0.0002	0.415 ± 0.0001	77.3 ± 0.06	22.5 ± 0.10	41.7 ± 0.06
BB	Baby blue	B1, Y5	0.269 ± 0.0004	0.302 ± 0.0002	78.0 ± 0.06	72.0 ± 0.06	−11.3 ± 0.10
BE	Beige	B2, R2, Y5	0.351 ± 0.0001	0.346 ± 0.0002	79.4 ± 0.10	5.0 ± 0.06	14.2 ± 0.06
BR	Brick red	R7, R36	0.536 ± 0.0028	0.318 ± 0.0010	35.1 ± 0.06	44.7 ± 0.06	14.3 ± 0.06
BS	Burnt sienna	B2, R3, Y5	0.477 ± 0.0000	0.326 ± 0.0000	38.5 ± 0.06	32.7 ± 0.06	12.7 ± 0.06
CH	Chartreuse	G3, Y5	0.418 ± 0.0004	0.449 ± 0.0005	72.6 ± 0.07	−6.5 ± 0.05	37.9 ± 0.11
CO	Coral	R3, Y5	0.441 ± 0.0004	0.353 ± 0.0003	69.8 ± 0.06	33.7 ± 0.15	24.5 ± 0.06
CR	Cranberry	R3, Y5, Y6	0.557 ± 0.0010	0.296 ± 0.0006	32.2 ± 0.06	50.8 ± 0.25	12.5 ± 0.12
FG	Forest green	B1, V1, Y5	0.289 ± 0.0006	0.371 ± 0.0019	29.1 ± 0.12	−10.5 ± 0.12	4.0 ± 0.15
GO	Gold	R2, Y5, Y6	0.468 ± 0.0004	0.411 ± 0.0000	70.7 ± 0.10	19.7 ± 0.06	36.9 ± 0.06
JR	Jasper red	R2, R3, Y5, Y6	0.481 ± 0.0004	0.334 ± 0.0001	46.3 ± 0.06	37.4 ± 0.15	16.8 ± 0.06
OR	Orange	R3, Y5	0.538 ± 0.0005	0.375 ± 0.0006	57.9 ± 0.06	47.3 ± 0.12	32.2 ± 0.06
PE	Peach	Y5, Y6	0.434 ± 0.0001	0.398 ± 0.0002	81.1 ± 0.06	16.0 ± 0.06	36.3 ± 0.06
PU	Purple	B1, V1, Y5	0.229 ± 0.0008	0.160 ± 0.0013	34.2 ± 0.43	27.0 ± 0.32	−53.1 ± 0.65
PY	Pale yellow	Y5	0.367 ± 0.0002	0.378 ± 0.0000	93.7 ± 0.06	−1.6 ± 0.06	28.0 ± 0.06
RU	Rust	R2, R3, R36	0.489 ± 0.0018	0.336 ± 0.0020	30.8 ± 0.11	25.9 ± 0.06	11.4 ± 0.06
SA	Saffron	Y5	0.496 ± 0.0004	0.435 ± 0.0005	77.0 ± 0.20	22.2 ± 0.35	46.4 ± 0.17
TU	Turquoise	B1, V1, Y5	0.253 ± 0.0004	0.338 ± 0.0005	56.0 ± 0.17	−23.1 ± 0.17	−1.1 ± 0.10
WH	White	B2	0.314 ± 0.0001	0.322 ± 0.0000	95.4 ± 0.28	−0.6 ± 0.06	2.4 ± 0.11

[a] Samples are dispersions of FD&C or D&C lakes in sucrose suspensions containing titanium dioxide and sodium benzoate. B1 = FD&C Blue No. 1, B2 = FD&C Blue No. 2, G3 = FD&C Green No. 3, R2 = FD&C Red No. 2, R3 = FD&C Red No. 3, R7 = D&C Red No. 7, R36 = D&C Red No. 36, V1 = FD&C Violet No. 1 (delisted), Y5 = FD&C Yellow No. 5, and Y6 = FD&C Yellow No. 6. [b] The (x, y) and (a, b) data were obtained with a colorimeter having a field subtending 2° and using the standard illuminant S_c.

RESULTS AND DISCUSSION

The 1931 CIE (x, y) spectral chromaticity coordinates are plotted in Fig. 1, and the Hunter (a, b) transformation of these coordinates is shown in Fig. 2. The curved portion of both diagrams represents the loci of spectral colors. Pure white for illuminant S_c is located near the center in each diagram with coordinates x = 0.3100, y = 0.3161 and a = 0.031, b = −0.042 (24). Sample colors are represented by points lying within the spectral boundary. The effect of the Hunter transformation is to shorten the green region (550–490 nm) and to expand the blue region (490–400 nm), producing a more uniform chromaticity diagram.

Once a color is located on a chromaticity diagram by its (x, y) coordinates, an additional property of the color, dominant wavelength, can be defined (25). The dominant wavelength is determined by drawing a line from the white point through the color point to intersect the spectral locus at some specific wavelength. For colors lying below the white point, *i.e.*, in the purple and maroon regions, the dominant wavelength is the complimentary wavelength found by drawing a line from the color point through the white point to intersect the spectrum locus.

To evaluate surface colors quantitatively and to locate these colors on both the (x, y) and (a, b) chromaticity diagrams, data for a series of color dispersions were obtained (Table I). Relative standard deviations were calculated for both (x, y) and (a, b) chromaticity coordinates for all samples and were found to be less than ±1.2% for most colorants. Chromaticity data are highly reproducible and, therefore, useful for the determination of small color differences. The data from Table I are plotted on the (x, y) diagram (Fig. 1) and the expanded portion of the (a, b) chromaticity plot shown in Fig. 2. Both diagrams locate materials by color. Colors on the Hunter (a, b) diagram are noticeably more separated, especially in the red–yellow and blue–green regions. As a consequence, very similar colors, *e.g.*, BS, JR, and RU and AP and GO, are more easily distinguished on the (a, b) than on the (x, y) chromaticity plot. Since the Hunter (a, b) scale was designed to create a more uniform measurement of color difference, equal increments in either a or b produce nearly the same visual color difference in the red as in the green and blue regions of the diagram.

Small differences in color were evaluated using three color suspensions, the chartreuse material (CH), the purple suspension (PU), and the white colorant (WH). Color data were obtained either directly, using an integrating sphere colorimeter, or from reflectance spectra and suitable calculations using Eqs. 1–4*b* (Table II). A two-way analysis of variance showed the data for all samples of each of the three colorants to be significantly different from each other. In addition, the statistical analysis showed 10 replicate determinations of color data for a single sample to be not signifi-

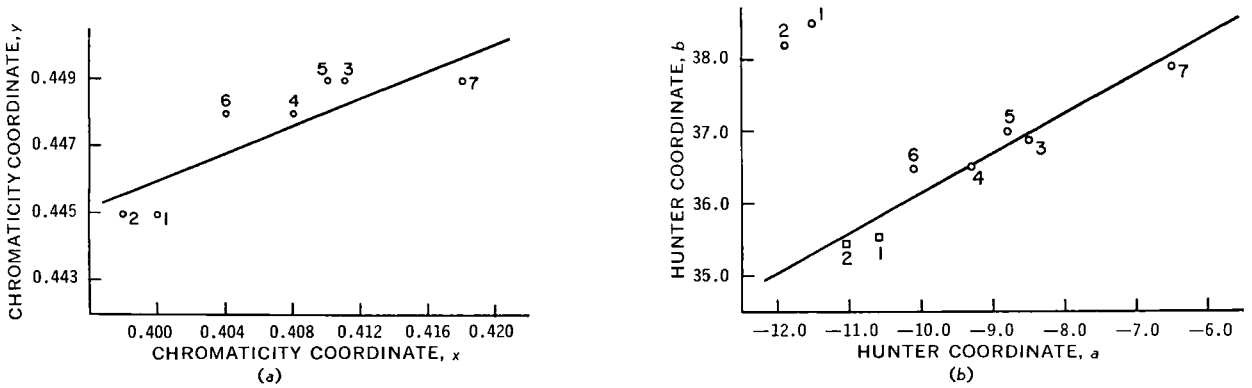

Figure 3—(a) *Expanded* (x, y) *chromaticity diagram for the chartreuse material (CH). Chromaticity coordinates were obtained using the 1931 CIE 2° energy-weighted distribution coefficients for illuminant* S_c. (b) *Expanded Hunter* (a, b) *diagram for the chartreuse colorant (CH). The* (a, b) *coordinate values were determined for the 1931 CIE 2° field data and the standard illuminant* S_c. *Key:* ○, *experimental data; and* □, *calculated data.*

Table II—(x, y) and (a, b) Chromaticity Data for Samples of Three Colorants

Colorant	Sample	x	y	$10Y^{1/2}$	a	b
CH[a]	1[b]	0.400	0.445	78.6	−11.5	38.6
	2[b]	0.398	0.445	78.0	−11.9	38.2
	3	0.411	0.449	72.2	−8.5	36.9
	4	0.408	0.448	72.0	−9.3	36.5
	5	0.410	0.449	72.5	−8.8	37.0
	6	0.404	0.448	72.9	−10.1	36.5
	7	0.418 ± 0.0004	0.449 ± 0.0005	72.6 ± 0.06	−6.5 ± 0.04	37.9 ± 0.13
PU[c]	1	0.238	0.207	43.1	16.1	−43.0
	2	0.236	0.182			
	3	0.238	0.187	38.2	24.3	−51.2
	4	0.237	0.178	36.4	26.1	−54.0
	5	0.235	0.185	37.1	24.3	−52.2
	6	0.238	0.192	39.8	21.9	−49.9
	7	0.236	0.194			
	8	0.234 ± 0.0016	0.190 ± 0.0006	40.5	22.8	−53.6
	9	0.238	0.185			
	10	0.233	0.173	34.2 ± 0.40	27.0 ± 0.29	−53.1 ± 0.61
WH[c]	1	0.318	0.336	92.6	−0.7	4.0
	2	0.325	0.340	91.1	0.0	6.0
	3	0.324	0.340	91.9	−0.1	5.8
	4	0.325	0.341	90.9	−0.6	5.5
	5	0.314 ± 0.0003	0.330 ± 0.0002	95.4 ± 0.23	−0.5 ± 0.03	2.4 ± 0.07
	6	0.311	0.328	92.9	−1.2	0.5

[a] The (x, y) and (a, b) data were obtained with a colorimeter having a field subtending 2° and using the standard illuminant S_c. [b] The (x, y) data were converted to (a, b) data using Eqs. 5 and 6 with $10Y^{1/2} = 72.4$, and the following results were obtained: Sample 1, $a = -10.6$, $b = 35.6$; and Sample 2, $a = -11.0$, $b = 35.4$. These values are used only for illustrative purposes. [c] The (x, y) data were obtained from reflectance spectra and suitable calculations using 10° energy-weighted distribution coefficients for the D-6500 standard illuminant. The (a, b) data were obtained with a colorimeter having a 2° field and using the standard illuminant S_c.

cantly different. As a result, small differences in color between different samples of a single colorant are real and not a consequence of experimental error.

The chartreuse material (CH) is a mixture of FD&C Yellow No. 5 and FD&C Green No. 3 in a sugar suspension. Seven discrete samples of this material were examined, and the expanded chromaticity diagrams, prepared by plotting the (x, y) and (a, b) data from Table II, are shown in Figs. 3*a* and 3*b*. The positions of the samples on the diagrams define their color. The line drawn on the (x, y) plot represents the direction of color shading found for these samples, from green at the lower left to yellow at the upper right. The (a, b) plot is more complex, since five of the seven points lie on a straight line which again defines color shading from green at the lower left to yellow at the upper right, while two points, representing the color positions of Samples 1 and 2, lie significantly off this line. Closer examination of the data and the expressions for the (a, b) transformations provides an adequate explanation. Equations 5 and 6, for the conversion of (x, y) to (a, b) data, include the term $Y^{1/2}$, which is a measure of the lightness of the sample. A linear (a, b) relationship is expected from a linear (x, y) relationship only when the $Y^{1/2}$ term remains constant. The data in Table II show that all samples except 1 and 2 have nearly the same $[10Y^{1/2}]$ (average $[10Y^{1/2}]$ for Samples 3–7 is 72.4). If the average value of $[10Y^{1/2}]$ found for Samples 3–7 is used to convert the (x, y) to (a, b) data for Samples 1 and 2, the resulting values lie on the line. Consequently, (a, b) data and their associated chromaticity diagrams have the advantage of discriminating not only differences in surface color but also differences in sample lightness.

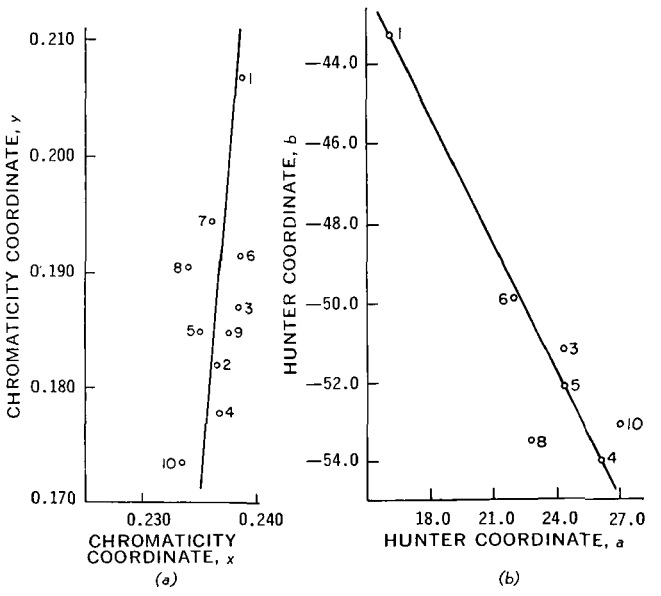

Figure 4—(a) *Expanded* (x, y) *chromaticity diagram for the purple color dispersion (PU) using the CIE 1964 10° energy-weighted distribution coefficients and the D-6500 illuminant.* (b) *The expanded Hunter* (a, b) *diagram for the purple material (PU). Coordinate values were determined for standard illuminant* S_c *and the 1931 2° field data.*

Chromaticity diagrams also can be used for the quality control evaluation of surface color and for the establishment of acceptable limits of color variation. The method can be illustrated using the data for the chartreuse material in Table II and Figs. 3*a* and 3*b*. Those samples of the chartreuse material considered to have acceptable color as judged subjectively were identified and located on the chromaticity diagrams. In this example, only Samples 3–6 were thought to be acceptable; Samples 1 and 2 were considered too green and Sample 7 too yellow. An area of acceptable color was created by placing limits on the (x, y) and (a, b) coordinates: for the (x, y) diagram, $0.402 \leq x \leq 0.414$ and $0.446 \leq y \leq 0.450$; for the Hunter (a, b) diagram, $-7.2 \leq a \leq -10.7$ and $35.5 \leq b \leq 37.7$.

If a reference standard is established for any surface color, all further subjectivity in color difference measurement can be eliminated by employing the color difference formula shown in Eq. 7. Color difference can be expressed in NBS units, and limits of acceptability for a sample when compared to a standard also can be defined in NBS units rather than by a subjective examination. For example, Sample 4 of the chartreuse material was designated as the reference standard and acceptable samples were required to have color differences less than 3 NBS units when calculated relative to the standard. Suitable calculations using Eq. 7 showed Samples 1, 2, and 7 to have color differences of 7.3, 6.8, and 3.2 NBS units, respectively, while Samples 3, 5, and 6 had differences of only 0.92, 0.87, and 1.2 NBS units, respectively. Samples 1, 2, and 7, since they differed from the standard by more than 3 NBS units, did not have acceptable surface colors.

A series of samples of the purple colorant (PU), a sugar suspension of three FD&C dyes (Blue No. 1, Yellow No. 5, and Violet No.

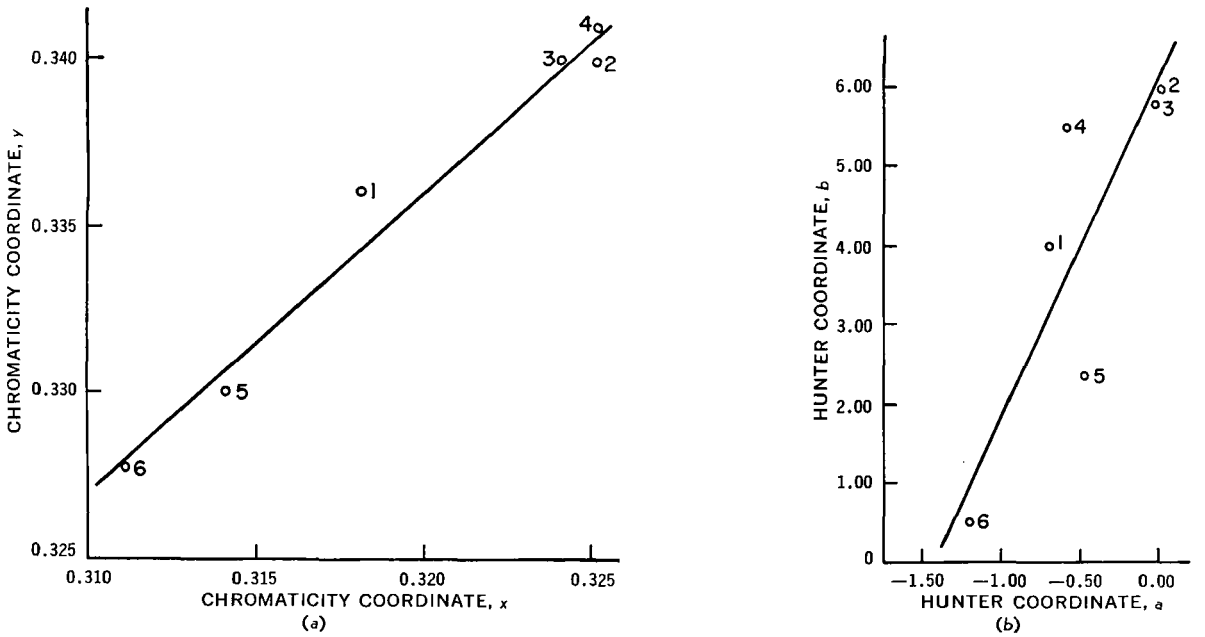

Figure 5—(a) *Expanded* (x, y) *chromaticity diagram for the white color dispersion (WH). Coordinates were evaluated using the 1964 10° energy-weighted distribution coefficients for the illuminant D-6500.* (b) *Expanded Hunter* (a, b) *diagram for colorant (WH). Coordinates were obtained for illuminant* S_c *and the 1931 2° field data.*

1[12]) also was examined, and the color data are collected in Table II. The expanded scale chromaticity diagrams (Figs. 4*a* and 4*b*) describe the gradual shading of this material from a deep, intense purple at the lower extreme to a violet color with a distinct blue cast at the upper extreme. The line drawn through the data points on both chromaticity diagrams defines the direction of color shading as described by the dominant wavelength of the samples. As a result, the differences in the slopes of the (x, y) and (a, b) plots are directly related to the transformation of the (x, y) spectral boundary (Figs. 1 and 2). In both chromaticity diagrams, Sample 1 is significantly different from all other samples, having far more blue color. The desirable color for this purple material was selected subjectively as the color of Samples 4 and 10. Samples 1, 6, 7, and 8 were considered too blue to be acceptable.

A region of acceptable color was created by placing arbitrary limitations on the color coordinates: $0.230 \leq x \leq 0.243$ and $0.167 \leq y \leq 0.188$ and $23.3 \leq a \leq 29.3$ and $-50.5 \leq b \leq -56.5$. Under these conditions, Samples 3 and 8 became borderline cases. If Sample 10 is defined as the reference standard for the purple colorant, color difference can be evaluated in terms of NBS units using the Judd formula. The following values were obtained for Samples 1, 3, 4, 5, 6, and 8, respectively: 17.3, 5.2, 1.8, 4.1, 8.2, and 7.6 NBS units. For control purposes, a ΔE value greater than 5 will cause a sample to be rejected because of unsuitable surface color. For the data presented, only Samples 4 and 5 are unquestionably acceptable while Sample 3 is borderline.

Color data were obtained for a third dispersion, the white colorant (WH), which is a suspension of FD&C Blue No. 2 in a sucrose solution. These data are shown graphically in Figs. 5*a* and 5*b*. The linear relationships found for both the (x, y) and (a, b) chromaticity plots represent the shading of samples from the blue region at the lower left to the yellow region in the upper right. A subjective examination of the white color samples revealed that Samples 2–4 were all yellowish and were, therefore, unacceptable. Sample 5 was considered most desirable while Samples 1 and 6, although having slight yellow and blue casts, respectively, represented the limits of colorant acceptability. On this basis, the following limits on the chromaticity coordinates were established: $0.310 \leq x \leq 0.319$ and $0.327 \leq y \leq 0.337$, $-1.25 \leq a \leq -0.25$ and $0.50 \leq b \leq 4.50$. In terms of NBS units, these limits represent ±3 units around the coordinates for Sample 5.

[12] FD&C Violet No. 1 has been delisted by the Food and Drug Administration. The samples examined were several years old and did contain FD&C Violet No. 1.

CONCLUSIONS

Surface color and small differences in color are evaluated quantitatively for a series of tablet colorants from tristimulus and (x, y) chromaticity values, determined either directly from integrating sphere colorimeters or from reflectance spectra and suitable calculations. Color data are plotted to give chromaticity diagrams which function to order materials by color. These diagrams eliminate subjectivity from color evaluation and from subsequent sample acceptance or rejection by permitting the selection of a color standard and applying limits to the chromaticity coordinates to define an area of acceptable color. Since the (x, y) scale is not uniform, limits of acceptability are established independently for both the x and y coordinates and for every distinct color.

In an effort to simplify the measurement of color differences, the more uniform Hunter scale is used. Hunter (a, b) coordinates are obtained either directly from an integrating sphere colorimeter or from a transformation of the (x, y) coordinates. Expanded chromaticity diagrams prepared from the Hunter data also order materials by color and have the added advantage that the distance between any two colors can be regarded as nearly a direct measure of visual difference. The use of these uniform scales permits the definition of color difference in terms of the NBS unit, and limits of acceptability are established in terms of these units.

The compilation of tristimulus and chromaticity information provides a permanent detailed description of a colored sample and defines quantitatively the color difference between the sample and its established standard. The subjectivity of an individual in discriminating color difference is, therefore, replaced by an absolute numerical method that permits the establishment of discrete control specifications for surface colors.

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This work documents a comparison of starch USP and a modified cornstarch incorporated in the granules of several drug formulations. Their processing properties and their relative pharmaceutical availabilities, as indicated by dissolution measurements, were studied.

EXPERIMENTAL

Materials—Excipients used in preparing the tablets included starch USP, a modified cornstarch[1], microcrystalline cellulose[2], spray-dried lactose[3], and magnesium stearate USP. Active ingredients included acetaminophen[4], ascorbic acid[5], chlorothiazide[5], levodopa[6], methyldopa[5], and probenecid[5].

Tablet Preparation—The active ingredients selected were all at a dosage of 500 mg, and tablet composition was identical for all drugs. The excipients were maintained constant in the experimental plan, although the formulation may not have been ideal for any one of the drugs. Batch sizes remained constant at 1000 tablets.

Granulations of 500 g of each active ingredient and 60 g of either starch USP or the modified cornstarch with a 7.0% starch paste (65°) were processed in a planetary mixer[7]. In all cases, the starch paste was prepared with starch USP. The wet granulations were manually screened through a No. 6 screen and oven dried overnight at 45°. After dry milling[8], the granulations were blended[9]

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Table I—Granulation Properties

Drug	A, Methyldopa		B, Acetaminophen		C, Ascorbic Acid		D, Chlorothiazide		E, Levodopa		F, Probenecid	
Starch	Modified	USP	Modified	USP	Modified	USP	Modified	USP	Modified	USP	Modified	USP
Loss on drying, %	10.2	10.0	0.8	1.0	1.4	1.7	1.0	1.4	1.0	1.4	0.6	1.4
Sieve analysis, % on screen												
No. 30	12.0	11.8	5.5	1.6	16.2	4.8	4.2	5.6	2.6	3.8	2.2	1.1
No. 50	32.8	37.2	36.2	24.7	37.2	23.3	23.9	10.4	23.7	8.8	15.9	10.7
No. 100	22.8	20.1	35.6	40.3	26.0	35.5	35.5	81.3	28.4	17.1	57.3	14.2
No. 200	15.7	16.4	22.6	32.8	13.5	29.5	30.3	2.7	23.5	27.7	21.5	59.5
No. 325	13.7	10.3	0.1	0.6	5.2	6.4	5.2	—	19.2	30.2	2.9	13.7
Base	3.0	4.2	—	—	1.8	0.5	0.8	—	2.7	12.5	0.1	0.8
Mean granule diameter, μm[a]	385	430	380	300	480	290	290	340	250	135	320	170
Density, g/ml												
Bulk	0.578	0.633	0.459	0.449	0.733	0.695	0.569	0.466	0.614	0.594	0.343	0.325
Tapped	0.770	0.879	0.695	0.736	0.991	1.007	0.769	0.790	0.787	0.848	0.528	0.551
Compressibility, %	24.94	27.99	33.46	38.99	37.92	30.98	26.01	41.01	21.98	29.95	35.04	41.02
Angle of repose	49°38′	53°55′	55°25′	55°25′	48°55′	51°27′	52°18′	58°44′	51°27′	53°08′	56°49′	58°44′
Flow, sec	4	14	14	17	5	16	7	49	8	26	59	68
Flow properties on rotary press	Good	Fair	Good	Poor	Good	Good	Good	No run	Good	Poor	No run	No run

[a] Obtained from graphs of the sieve analysis data.

with 130 g of microcrystalline cellulose and lubricated with 0.6% magnesium stearate.

Tablets were compressed on a rotary machine[10] operating at 20 rpm and equipped with only two sets of capsule-shaped punches, 0.71 × 1.82 cm (9/32 × 23/32 in.), stationed on opposite sides of the die table.

Testing Procedures—Sieve analyses were performed on samples of the milled granulations, using a nest of sieves and an electromagnetic sieving machine[11]. The compressibility factor was calculated using:

$$\text{percent compressibility} = \frac{P - L}{P} \times 100 \quad \text{(Eq. 1)}$$

where P is packed density, and L is loose density. Densities were measured using a graduated cylinder and a motorized tapping device[12] set to operate for 2000 cycles. Loss-on-drying moisture determinations were obtained with a moisture balance[13].

The angle of repose was measured for each lubricated mixture using the method of Nelson (3), where the repose angle ϕ is defined by:

$$\phi = \tan^{-1}\frac{h}{r} \quad \text{(Eq. 2)}$$

in which h is the height and r is the radius[14] of the base of the cone formed by the powder. Flow time was determined by the flow of 60 g of material through a stainless steel funnel with a 1.5-cm opening.

The tablet properties measured included 20 individual weights determined on a semimicro analytical balance[15], 20 individual thickness measurements made on a dial comparator[16], and breaking strength measurements determined on a motorized tester[17]. The disintegration time of six tablets was measured by the method described in USP XVIII.

Dissolution measurements were carried out by the method specified in USP XVIII with a 50-rpm rate of rotation. The dissolution medium was 0.1 N HCl unless noted otherwise.

RESULTS AND DISCUSSION

The various drugs were all treated in the same manner, and comparisons were made between the two formulations of each active ingredient, *i.e.*, one containing starch USP and one containing the modified starch, and not between active ingredients.

The physical properties of the granulations (Table I) showed some differences. In most cases, the formulation containing the modified starch appeared to yield a granulation with a slightly larger mean diameter, indicating more efficient granulation. Correspondingly, the granulations containing starch USP were generally more dense. There was very little difference in the moisture levels between the two granulations of a given drug.

The three factors of compressibility, angle of repose, and time of flow, which should give some indication of flow properties, generally predict that the granulations containing the modified starch will perform better. The lower the compressibility (4) and the lower the response angle (3), the better is the predicted flow.

This was shown to be true in every case where the material flowed sufficiently to produce tablets. With probenecid (Table I), neither granulation would flow well enough to compress a tablet on the rotary machine, even when operated manually. With ascorbic acid, the two formulations seemed to perform equally well. In the other four cases, the formulation containing the modified starch performed better on the rotary machine than its counterpart containing starch.

Although this evaluation is subjective in the case of methyldopa, it is less so with the others. Two of the formulations containing starch USP (Table I, B and E) required that the machine be turned slowly by hand to obtain tablets that did not cap at the take-off bar. The starch USP counterpart of a third (Table I, D) would not flow sufficiently to run at all.

The three formulations that would not flow sufficiently to fill the die cavity yielded repose angles >56° whereas all others were below 56°. Time of flow through a funnel also appeared to give correlation with nonflowability, since the same three formulations gave values of 49 sec or greater.

The physical properties of the resulting tablets (Table II) support this evaluation. The weight control, as indicated by the rela-

[10] Stokes model BB2, modified by removal of the front ejection cam so only one compressing station was used.
[11] Geoscience Instrument Corp., Mt. Vernon, N.Y.
[12] Model JEL-ST2, Numec Instrument & Control Corp., Monroeville, Pa.
[13] Central Scientific Co., Chicago, Ill.
[14] The value of r in this set of experiments was 2.55 cm.
[15] Model H20T, Mettler Instrument Corp., Princeton, N.J.
[16] Model 282M, B. C. Ames Co., Waltham, Mass.
[17] Heberlein hardness tester, Cherry Burrell, Park Ridge, Ill.

Table II—Tablet Properties

Drug	Methyldopa			Acetaminophen			Ascorbic Acid		Chlorothiazide		Levodopa		Probenecid	
Starch	Modified		USP	Modified		USP	Modified	USP	Modified	USP	Modified	USP	Modified	USP
Weight, mg	—[a]	—[b]	—	—[a]	—[b]	—	—	—	—	—	—	—	—	—
Mean[c]	722	710	710	547	550	547	673	711	678	—	681	697	—	—
RSD, %	0.57	2.28	2.38	1.56	1.76	3.55	1.09	1.91	0.97	—	1.13	23.14	—	—
Thickness, mm														
Mean	673	733	659	528	578	525	555	567	583	—	618	597	—	—
RSD, %	0.25	0.22	0.64	0.80	0.34	2.52	0.28	0.58	0.18	—	0.34	0.79	—	—
Hardness, kg														
Mean	13.02	6.32	8.3	6.15	3.45	2.12	12.62	12.72	11.08	—	8.90	8.88	—	—
RSD, %	7.37	15.02	11.69	13.16	16.30	17.83	8.86	10.45	7.96	—	9.56	14.66	—	—
Disintegration time, sec	58	37	58	100	40	148	142	65	35	—	72	62	—	—

[a] Tablets prepared under pressure equivalent to starch USP formulation. [b] Decreased hardness to match starch USP formulation. [c] Although the formulations are identical for all drugs studied, the mean tablet weights differ due to the density differences exhibited by the granulations. The tablet machine fill weight adjustment was not changed during the experiment.

Table III—Tablet Hardness at Equivalent Pressures

Drug	Hardness at Equivalent Pressures, kg	
	Tablet with Starch USP	Tablet with Modified Starch
Methyldopa	7.5	10.5
Acetaminophen	5.0	6.5
Ascorbic acid	5.0	6.5
Chlorothiazide	11.0	15.5
Levoaopa	5.0	8.0
Probenecid	9.5	13.0

tive standard deviation, was always higher for the granulation containing starch USP than for its modified starch counterpart. The other physical properties were adequate in all cases, with the exception of the hardness of the starch USP formulation of acetaminophen which could not be increased.

The ability to lessen the tendency for capping and splitting of the tablet, as mentioned previously, represents a significant advantage of the modified starch. This material apparently acts as a stronger internal binder than starch USP; but if too much binding power is contributed, disintegration and dissolution could be adversely affected. What is required is an internal binder that also has disintegrant properties[18].

The disintegration values (Table II) are all satisfactory, indicating that the additional binding capacity of the modified cornstarch is not detrimental. Moreover, the dissolution profiles show a difference between the two granulations when a comparison was available. As shown in Fig. 1, the release profile of the modified starch formula is consistently higher than that of the corresponding starch USP formula for the five drugs that did yield tablets.

These comparisons were made on tablets of approximately the same hardness values for a given drug. In general, the hardness value for the tablet containing starch USP was the maximum possible; *i.e.*, no additional compression pressure could be applied. For the drugs in Figs. 1*a* and 1*b*, harder tablets were made from the modified starch formulation (13 kg for methyldopa and 6 kg for acetaminophen); the release profiles, although lower with increased pressure, were still higher than those for the corresponding starch USP tablets. In one case (chlorothiazide), the tablet containing starch USP was prepared manually with a hydraulic press[19] to obtain a dissolution comparison.

To illustrate the differences in internal binding power at equivalent pressures, tablets were compressed from each mixture on the manual hydraulic press. The image in this case was a round, flat beveled edge tablet, 1.27 cm (0.5 in.) in diameter. Since the compressional force remained constant at 2000 lb, the comparisons in Table III show that the formulation containing the modified starch for any drug yielded a harder tablet than did the formulation containing starch USP.

As noted, each drug selected for the study was treated in the same manner with regard to formulation, granulation, *etc.* For the one soluble drug in the group, ascorbic acid, the mixture was overwet during granulation as expected. For this reason, the experiment was repeated with a 37% decrease in the quantity of the water used for the starch paste.

Although both formulations performed adequately on the rotary tablet press, the one containing the modified starch appeared superior again. However, no significant difference was observed in the two dissolution profiles (Fig. 2). The formulation containing the modified starch showed no difference from the previous experiment (Fig. 1*c*), and that containing starch USP improved to an equivalent level. This finding appears to indicate that the modified starch formulation is relatively insensitive to this change in processing but that the formulation containing starch USP can be adversely affected by overgranulation.

Direct Compression—The ingredients selected for the experi-

[18] According to the manufacturer's literature, Sta Rx 1500 starch differs from starch USP only from water solubility and particle-size standpoints. It contains a 20% maximum cold water-soluble fraction.

[19] Carver laboratory press, Fred S. Carver, Inc., Summit, N.J.

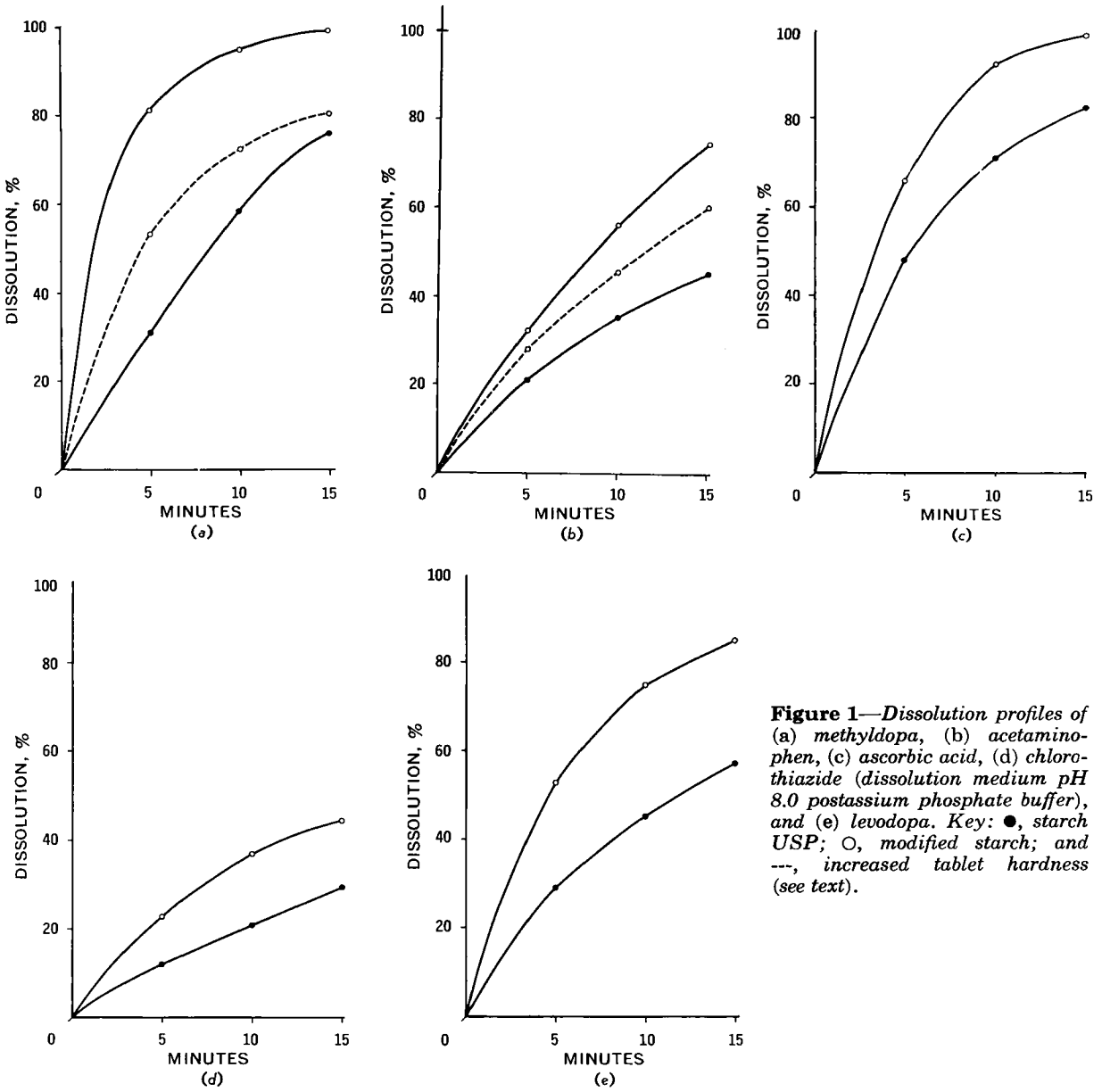

Figure 1—*Dissolution profiles of* (a) *methyldopa*, (b) *acetaminophen*, (c) *ascorbic acid*, (d) *chlorothiazide* (*dissolution medium pH 8.0 postassium phosphate buffer*), *and* (e) *levodopa. Key:* ●, *starch USP;* ○, *modified starch; and* ---, *increased tablet hardness* (*see text*).

mental formulation in this work, as mentioned previously, are marketed mainly as direct compression excipients. It has been proposed that a direct compression formulation is preferable for several reasons, one being that the drug is not bound by granulation and, therefore, its availability should be superior. This formulation was suitable for such an evaluation since the same ingredients could be used both ways. In addition, the percentage of the drug in the formulation was so high that the primary drawback of direct compression formulations, possible lack of content uniformity, was not a concern.

One soluble and one insoluble drug (ascorbic acid and chlorothiazide, respectively) were selected, and the identical formulations were prepared as in the previous section but this time by the appropriate simple mixing techniques in a twin-shell blender[9].

With respect to processing, these direct compression mixtures were inferior in all cases. None would flow well enough on the rotary tablet machine to fill the die even when the machine was operated manually. This result was not completely unexpected when so great a percentage of the formulation was the active ingredient which then controlled the physical properties. The experiment was continued, however, by compressing manually on the hydraulic press.

The dissolution profiles on the resulting tablets (Fig. 3) were not those expected. Although the formulation containing the modified starch for each drug did exhibit a faster release than the starch USP counterpart, each direct compression mixture gave a slower release than its granulated counterpart (Figs. 1*c* and 1*d*). The values for tablet hardness were held constant for the two experiments. Thus, it is not always true that the granulation of an active ingredient is detrimental to its availability.

Soluble Excipient—The effect of including a soluble component in the formulation under study was of interest. For this reason, the two chlorothiazide granulations were prepared as before but were then mixed with a directly compressible lactose rather than the microcrystalline cellulose.

Again, the processing characteristics of the formulation containing the modified starch were superior to the starch USP counterpart. While tablets of 15–16 kg in hardness were prepared from the former, the latter produced only tablets of 4–6 kg on the rotary tablet press.

For dissolution testing, tablets of equivalent hardnesses were prepared by hydraulic press as before. In the case of these lactose formulations (Fig. 4), the one containing starch USP gave a higher release rate than did its modified starch counterpart. The release of the starch USP–lactose formulation was essentially equivalent to that of the starch USP–microcrystalline cellulose formulation, and both were slower than the modified starch–microcrystalline cellulose formulation (Fig. 1*d*).

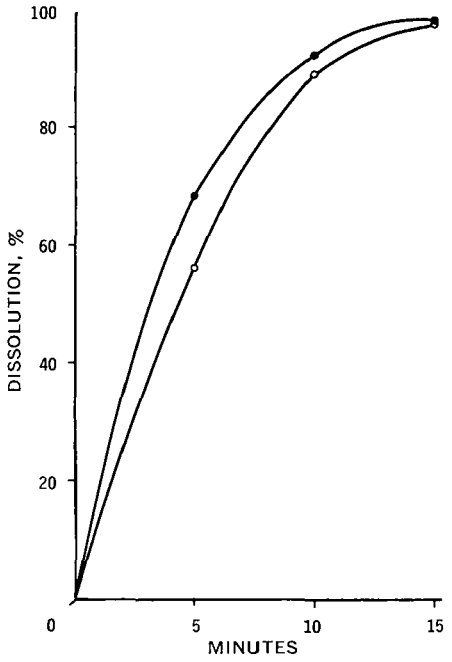

Figure 2—*Release profile for ascorbic acid tablets. Key:* ●, *starch USP; and* ○, *modified starch (see text).*

A possible explanation for the release of the modified starch–lactose formulation being lower than its microcrystalline cellulose counterpart may be that the wicking action of the microcrystalline cellulose allows the dissolution medium to act on the granules quickly, thus bringing into play the properties of the modified starch; in the other formulation, the activity of the solvent is divided between the dissolution of the lactose and the action on the granules. Thus, in the case of a specialized ingredient such as modified starch, it is important to consider the effect of each ingredient in the formulation on the specialized property one is trying to utilize.

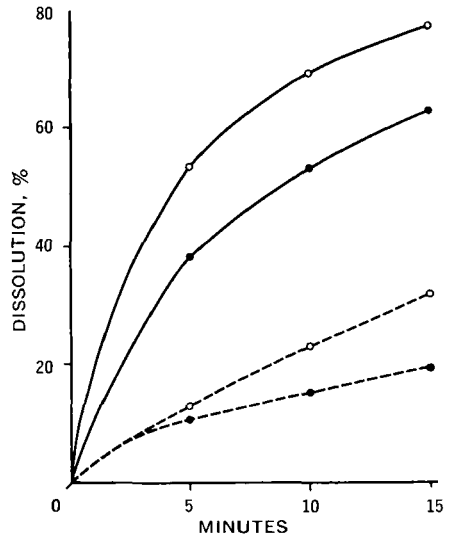

Figure 3—*Release profiles for direct compression formulations. Key:* ●, *starch USP;* ○, *modified starch;* ——; *ascorbic acid; and* ---, *chlorothiazide.*

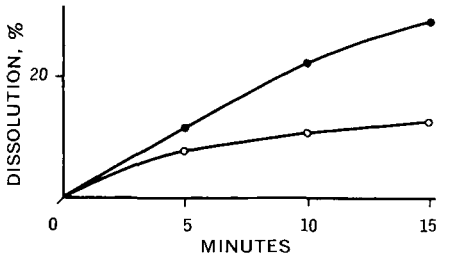

Figure 4—*Dissolution profiles for chlorothiazide formulations containing lactose. Key:* ●, *starch USP; and* ○, *modified starch.*

SUMMARY

Formulations of several drugs were prepared by incorporating either starch USP or a modified starch within the granule and subsequently adding microcrystalline cellulose. In general, for a given drug, the formulation containing the modified starch performed better during processing (flow and weight uniformity) and exhibited a slightly faster release rate (under the conditions of the test) than did its starch USP counterpart. The modified starch mixture produced a harder tablet than the starch USP mixture when compressional force was held constant. The data also indicate that the mixture containing modified starch may be less sensitive to changes in processing, *i.e.*, overgranulating.

When granulated and direct compression mixtures of the same ingredients were prepared, the granulated mixtures performed better with respect to both processing and dissolution testing. Substitution of a soluble component, lactose, for the microcrystalline cellulose did not significantly change the properties of the starch USP formulation but did lower the release rate of the modified starch formulation.

The results of this study show that although modified starch is marketed as a direct compression excipient, it may also be used in the granulation technique with possible improvement in processing properties and pharmaceutical availability as measured by dissolution.

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Pressure Changes in Bottles during Sterilization by Autoclaving

M. C. ALLWOOD [x], R. HAMBLETON, and S. BEVERLEY *

Abstract □ A method is described to measure changes in pressure inside a bottle during autoclaving without disturbing the integrity of the seal. Experiments showed that, in a perfectly sealed bottle, the pressure rose to a maximum of 58.2 psi absolute. If the rubber liner was unable to maintain the seal, air leaked slowly from the bottle both during the heating-up and the early sterilization period.

Keyphrases □ Sterilization, autoclaving—pressure changes in bottles, method for measurement □ Bottles, sealed—pressure changes during sterilization by autoclaving, method for measurement □ Seals, bottles—effects of sterilization by autoclaving, pressure changes in bottles measured

The use of screw-capped bottles as containers for parenteral fluids and noninjectable water has recently been criticized (1). The protection against microbial contamination offered by such systems to sterile fluids has been shown to be inadequate (2). One serious fault with many rubber seals is their inability to prevent air from escaping from inside the bottles during autoclaving (3, 4). The presence of air above the liquid contents of the bottle ensures that the pressure within that bottle will be greater than the pressure in the autoclave during sterilization.

The objective of this study was to measure changes in pressure inside a typical screw-capped bottle during sterilization when the container remained hermetically sealed and also when an imperfect bottle seal allowed air to escape. The standard 1-liter noninjectable water bottle[1] was used as the test system. A bottle was modified so that pressure changes inside such a container could be measured during autoclaving. The highest pressure readings obtained were compared with calculated maximum values.

THEORETICAL

The theoretical maximum bottle pressure during autoclaving at 121° is calculated as follows. The pressure within a bottle is the sum of the partial pressures of air and steam. The pressure of steam at elevated temperatures is known, and the component due to expansion of the air in the bottle may be calculated according to the ideal gas equation where (PV/T) = a constant. Then V (the head-space volume of the bottle) is reduced at elevated temperatures by the expansion of water in the bottle. By comparing the density of water at 20° (0.9983 g/cm^3) and 121° (0.9421 g/cm^3) (5), the increase in volume over this range is:

$$\left(\frac{0.9981}{0.9421} - 1\right)10^2 = 5.97\% \qquad \text{(Eq. 1)}$$

Therefore, the expansion of 1 liter of water will reduce the head-space volume in a bottle at 121° by 59.7 ml. Conversely, the expansion of the glass will result in a potential increase in volume, estimated to be about 1.0 ml, for the 1-liter soda-lime glass bottle[1] (linear coefficient of expansion of glass = 9.8×10^{-6}).

Other factors to be considered include the air content of the water placed in the bottle. If water at 20° is air saturated at a pressure of 1 atm, it contains 18.7 ml air/liter (water for injection rapidly becomes resaturated after distillation). At 121°, virtually all of this air would be expelled, adding to the pressure produced in the head space. Also, a small volume of the air in the bottle head space will be displaced by water vapor, equal to 4.4 ml at 20° (saturation vapor pressure of water at 20° is 175 mm Hg). Therefore, under these conditions, in a perfectly sealed noninjectable water bottle[1], the internal pressure (IP) at 121° will be (assuming standard atmospheric pressure conditions):

$$IP = \left[\frac{14.7(220.0 + 18.7) - 4.4(394}{293(221.0 - 59.7)}\right] + 14.7 + 15.0 =$$

58.4 psi (absolute) (Eq. 2)

EXPERIMENTAL

A hole, 1 cm in diameter, was drilled into the base of a 1-liter noninjectable water bottle. Into this was fixed a rubber tire valve casing, which was connected *via* a length of stainless steel reinforced polytetrafluoroethylene flexible tubing to an instrumentation port in the roof of the autoclave chamber. A pressure transducer[2] was sealed into the same instrument port on the outside of the autoclave chamber. Within the bottle, a length of glass tubing extended from the tire valve to the air space (Fig. 1).

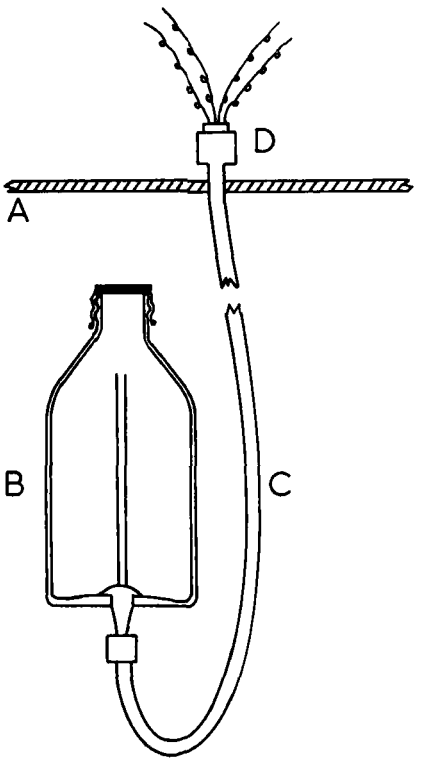

Figure 1—*Apparatus for measuring pressure changes inside the noninjectable water bottle during autoclaving. Key: A, roof of autoclave chamber; B, noninjectable water bottle; C, pressure measurement pipe; and D, pressure transducer.*

[1] Supplied by the U.K. Department of Health and Social Security.

[2] Type ITQS, Kulite Semiconductor Ltd., Basingstoke, United Kingdom.

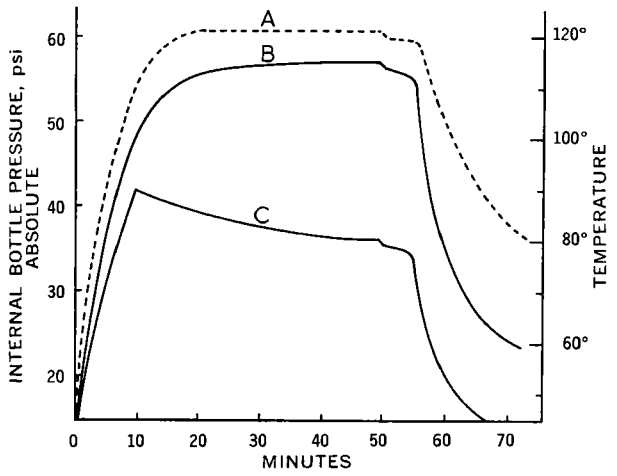

Figure 2—*Temperature and head-space pressure changes in noninjectable water bottles containing 1 liter of distilled water, sealed with flat rubber liners and autoclaved at 121°. Key: A, changes in temperature; B, changes in pressure, perfectly sealed bottle; and C, changes in pressure, leaking bottle seal.*

Pressure changes were recorded on a potentiometric recorder; temperature measurements, also recorded, were made with a copper–constantan thermocouple in a second bottle placed alongside that adapted for pressure measurements in the autoclave chamber. The bottles were filled with 1 liter of distilled water at 20°. They were then autoclaved and spray cooled[3].

RESULTS AND DISCUSSION

Figure 2 shows temperature and pressure changes in a noninjectable water bottle, sealed with flat rubber liner and aluminum screw cap, autoclaved at 121°. Curve A shows the temperature profile during autoclaving within the bottle head space. Curve B shows the concomitant pressure changes in a well-sealed bottle. Allowing for the additional volume of air within the pressure measurement tubing and the volume of glass tubing in the bottle, the maximum theoretical pressure in the experimental system described in Fig. 1 would be 57.2 psi (absolute).

The pressure inside the bottle rose to a level close to the maximum theoretical value, attaining a pressure of 57.0 psi (absolute) at equilibrium (adjustments were made for day-to-day variations in atmospheric pressure). On cooling the bottle to 20° after autoclaving, the pressure was found to return to atmospheric pressure, indicating that no air had been leaked from the bottle head space during the sterilization procedure.

These findings suggest that the apparatus described is suitable for detecting pressure changes within glass containers during autoclaving. It is essential that the tubing employed to connect the bottle to the pressure detection device is temperature and pressure resistant. It is also important to ensure that the pressure transducer is resistant to steam and responds linearly over the range of temperature up to 121°.

Clearly, the closure of a bottle is placed under considerable strain during autoclaving. Where the seal was not maintained, the pressure increased until a point was reached beyond which it gradually fell again due to leakage of air from the bottle (curve C). On cooling to 20°, such bottles were always observed to contain a vacuum. Results, therefore, show that air may escape from the bottle during the heating-up phase of the sterilization cycle.

The air space increases rapidly in temperature, resulting in an initial rapid increase in pressure, followed by expansion and evaporation of water. If the seal is imperfect, then at some stage during the heating process, the rubber liner is lifted sufficiently from its seating on the rim of the bottle to allow air to escape. This leakage then continues slowly as the temperature of the bottle and its contents rise. This is probably aided by the expansion of the aluminum cap. As soon as the spray cooling commences, steam in the head space condenses and the rubber liner is sucked back onto the rim of the bottle to reform the seal. When cool, the bottle will consequently contain a vacuum. Results reported elsewhere (3) have shown that, in the majority of cases of the seal leaking, up to 90% of the air originally present is lost from the bottle head space.

[3] Getinge model SAR 450, Getinge A.G., Uppsala, Sweden.

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Effect of Aging on Physical Properties of Phenylbutazone Tablets

D. BARRETT * and J. T. FELL ‡ x

Abstract □ A study was made of the influence of age on the physical properties of phenylbutazone tablets BP, with special reference to *in vitro* dissolution rates. Past batches of tablets showed a progressive decrease in dissolution rate with age. This effect could be simulated in short periods by using elevated temperatures. The effect appears to be connected with the subcoat layer of the sugar coating of the tablet which adheres more strongly to the tablet core and slows down its disintegration.

Keyphrases □ Phenylbutazone tablets—effect of aging on physical properties ■ Aging—effect on physical properties of phenylbutazone tablets □ Stability—effect of aging on physical properties of phenylbutazone tablets ■ Dissolution rate—effect of aging, phenylbutazone tablets

The effects of storage time and temperature on such tablet properties as hardness and disintegration time have been examined (1–5). The results were highly dependent on the formulation under study.

Alam and Parrott (6) recognized the importance of dissolution rate and the possible effect that changes in this property with time may have on bioavailability. In a study using hydrochlorothiazide tablets, they showed a close correspondence between changes in dissolution rate occurring at elevated temperatures and those occurring after prolonged storage at room temperature. Retardation of dissolution rate was also reported (7) for sodium salicylate tablets, and a possible effect with acetaminophen tablets was mentioned (8).

The present study was undertaken to examine the effect of age on the physical properties of phenylbutazone tablets BP. These tablets are sugar coated, and both the cores and the sugar-coated tablets have been examined. Several studies examined the *in vitro* dissolution behavior and *in vivo* availability of various brands of phenylbutazone tablets (9–13). Wide variations between brands were noted, but the influence of the age of the product does not appear to have been taken into account.

EXPERIMENTAL

Tablets—All tablets examined were 100-mg phenylbutazone tablets prepared to BP standards. A series of both cores and coated tablets with varying manufacturing dates were obtained which had been prepared according to the same formulation and standards. Relevant information is given in Table I.

The most recently manufactured batch was subjected to controlled temperature studies. Tablets were stored in tightly sealed, well-filled bottles and were exposed to temperatures of 20 (representing an even room temperature), 37, and 50°. Tablets stored at elevated temperatures were allowed to attain room temperature before testing and were stored at 20° until all tests were completed. Testing was carried out at suitable time intervals dependent on storage temperature.

Dissolution Studies—Tablet dissolution was studied by the beaker method of Levy and Hayes (14). The dissolution fluid, contained in a 2-liter beaker (13-cm diameter, 19 cm high) was 1 liter of simulated intestinal fluid USP without pancreatin, maintained at 37 ± 0.5°. The fluid was stirred with a Perspex paddle consisting of three blades (2 cm long, 1.3 cm high) set at an angle of 30° to the vertical on a 1.5-cm diameter shaft. This paddle was immersed centrally in the dissolution fluid to a depth of 2.7 cm below the surface of the fluid and 4.8 cm from the bottom of the beaker. The paddle was driven at a constant speed of 75 rpm.

The colored outermost layers of the sugar coat were removed by washing prior to dissolution to prevent any possible interference with the assay. The tablet was placed centrally in the fluid, and 2-ml samples were withdrawn at suitable time intervals and filtered[1]. After suitable dilution, the samples were assayed spectrophotometrically for phenylbutazone at 264 nm. Each tablet was allowed to dissolve in its entirety. The excipients present in the tablet, other than the color, did not interfere with the assay.

Disintegration Time—This was measured using the disintegration apparatus of BP 1973, using 250 ml of simulated intestinal fluid without pancreatin at 37°. The disintegration times of the tablets were determined individually.

Tablet Strength—The strength of the tablet cores was measured by diametral compression using a standard testing instrument[2]. Tensile failure was not observed in all cases, and the results are expressed as the mean breaking load of five individual determinations.

RESULTS AND DISCUSSION

The results from the dissolution studies were interpreted by apparent first-order kinetics as derived by Wagner (15). Plots of log (percent undissolved) against time gave straight lines, the slopes of which were taken as a measure of the dissolution rate. The data were plotted down to 10% undissolved; lines were fitted to these plots by the method of least-squares linear regression using a desk computer[3], programmed accordingly. Correlation coefficients indicated that a significant linear correlation existed ($p = 0.05$) in all cases.

Experiments with tablets in which interference by the color was not a problem indicated that the preliminary washing did not affect the slopes of the dissolution plots. Parameters such as a $t_{50\%}$ value, which would include a lag time with the sugar-coated tablets, were not used because these would be affected slightly by the washing procedure.

The slopes of these plots for tablets manufactured at different times are given in Table I. There was a continuing decrease in the mean regression line slope with age for both the cores and the sugar-coated tablets. The most marked change, however, occurred with the sugar-coated tablets. These results, of course, must be treated with caution because initial dissolution rates are unknown and the differences may merely be due to interbatch variation. However, the progressive decrease in dissolution rate strongly suggests an aging effect.

The vastly greater change in dissolution rate for the sugar-coated tablets than for the cores indicates that if an aging effect is present, it is in some way connected with a change in the sugar coat. Visual observation of the tablets in the dissolution apparatus showed different "break-up" patterns for tablets of different ages. All of the cores and the more recently manufactured sugar-coated tablets disintegrated readily.

With Batch 02/70, there was a tendency for fragments of the core to adhere to the subcoat. With Batches 05/69 and 04/69, the

[1] Millipore filters (0.45 μm), Millipore U.K. Ltd., London NW10 7SP, United Kingdom.

[2] Floor model, Instron Ltd., High Wycombe, United Kingdom.

[3] Model 9810 A, Hewlett-Packard, Loveland, CO 80537

Table I—*Results from Past Batches of Tablets*

Batch Number[a]	Tablet Cores: Mean (*SD*) of Regression Line Slopes	Tablet Cores: Mean (*SD*) of Disintegration Time, min	Strength, kg	Sugar-Coated Tablets: Mean (*SD*) of Regression Line Slopes	Sugar-Coated Tablets: Mean (*SD*) of Disintegration Time, min
07/72	−0.0319 (0.00427)	5.8 (1.76)	3.13	−0.0250 (0.00217)	9.7 (1.93)
03/71	−0.0220 (0.00214)	11.6 (1.89)	1.97	−0.0221 (0.00207)	19.4 (1.78)
02/70	−0.0206 (0.00099)	1.5 (0.40)	1.24	−0.0194 (0.00195)	9.5 (0.80)
05/69	−0.0193 (0.00171)	1.2 (0.19)	1.76	−0.0059 (0.00326)	~15[b]
04/69	−0.0183 (0.00187)	4.7 (1.69)	2.05	−0.0022 (0.00091)	~40[b]

[a] Dates of manufacture. Cores: 07/72, Dec. 1972; 03/71, Mar. 1971; 02/70, Mar. 1970; 05/69, May 1969; and 04/69, Apr. 1969. Sugar coated: 07/72 Jan. 1973; 03/71, Apr. 1971; 02/70, Apr. 1970; 05/69, May 1969; and 04/69, May 1969. [b] Disintegration times could not be determined more precisely due to the tendency of fragments of the tablet core to adhere to fragments of the subcoat, making it difficult to detect the end-point of the test.

Table II—Results from Tablets Stored at Controlled Temperatures

Storage Conditions	Tablet Cores: Mean (*SD*) of Regression Line Slope	Tablet Cores: Mean (*SD*) of Disintegration Time, min	Strength, kg	Sugar-Coated Tablets: Mean (*SD*) of Regression Line Slope	Sugar-Coated Tablets: Mean (*SD*) of Disintegration Time, min
Initially	−0.0235 (0.00292)	13.8 (5.13)	2.06	−0.0274 (0.00346)	7.6 (1.86)
20°, 6 weeks	−0.0254 (0.00211)	14.4 (4.39)	2.33	−0.0272 (0.00179)	8.1 (4.10)
20°, 14 weeks	−0.0248 (0.00271)	14.1 (4.61)	2.41	−0.0283 (0.00369)	7.9 (1.67)
37°, 3 weeks	−0.0259 (0.00265)	7.7 (2.03)	2.61	−0.0260 (0.00153)	6.9 (1.66)
37°, 6 weeks	−0.0234 (0.00144)	9.9 (2.12)	2.96	−0.0272 (0.00215)	6.9 (2.09)
37°, 14 weeks	−0.0241 (0.00261)	10.6 (2.27)	2.75	−0.0240 (0.00258)	8.7 (2.17)
50°, 2 weeks	−0.0223 (0.00123)	12.7 (5.82)	3.13	−0.0252 (0.00276)	7.5 (2.51)
50°, 4 weeks	−0.0237 (0.00133)	15.1 (4.67)	3.06	−0.0288 (0.00228)	16.4 (7.84)
50°, 6 weeks	−0.0223 (0.00333)	13.5 (6.46)	3.00	−0.0231 (0.00665)	20.8 (8.67)
50°, 9 weeks	−0.0240 (0.00241)	14.1 (4.76)	3.01	−0.0151 (0.00373)	~25[a]
50°, 14 weeks	−0.0231 (0.00203)	13.9 (5.01)	2.98	−0.0115 (0.00510)	~40[a]

[a] See Table I.

tablet split into two halves, and these persisted throughout the test. The subcoat appeared to adhere to the core and prevent it from breaking up completely, thus impeding dissolution. Differences in the extent of this adherence would account for the greater spread of results noted with these tablets.

In the case of Batch 04/69, the core showed continued resistance to break up even after the tablet coat had broken away. This could have been due to the presence of a film of adhesive material from the coating process on its surface.

The strength and disintegration times of these tablets are given in Table I. A progressive change in disintegration time with age of manufacture was not apparent with the tablet cores. Of the sugar-coated tablets, Batch 03/71 had a high disintegration time, not in keeping with its dissolution rate, but Batches 05/69 and 04/69 both had disintegration times that may be correlated with their extended dissolution times. The values of the breaking loads of the tablets did not show any trend with age.

The results from the controlled temperature studies are given in Table II. In the 14-week storage period, the dissolution rate from the tablet cores remained essentially unchanged. Sugar-coated tablets stored at 20 and 37° were similar, but those stored at 50° showed an aging effect. Results after 14 weeks of storage at 50° show an increasing reduction in dissolution rate with a wider spread in the values. These results agree qualitatively with those from the tablets manufactured during the past 5 years.

The break-up pattern of the tablets stored at 50° was similar to that observed with the older tablets. Again it appeared that the innermost subcoat was in some way impeding tablet break up and dissolution. Tablet subcoats are often a gelatin–acacia mixture, the materials that both Alam and Parrott (6) and Kristofferson and Keto (7) found were most susceptible to aging effects when used as granulating agents.

No appreciable change in disintegration times for cores stored at 20 or 50° was noted, although there appeared to be a decrease in those stored at 37°. For the sugar-coated tablets, storage at 20 or 37° did not affect the disintegration time, except for 14 weeks at 37°, but an increase was observed at 50° which may be related to the decrease in dissolution rate. The strength of the tablet cores rose with storage at all temperatures to a constant value. This increase does not appear to be related to dissolution rate or disintegration time, as was also observed previously (6).

The results presented indicate an aging effect with phenylbutazone tablets that appears to be connected with the subcoating of the sugar coat. This raises the possibility of the effect being present in other sugar-coated tablets. The use of elevated temperatures enables qualitative simulation of the aging in short time periods.

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x To whom inquiries should be directed.

NOTES

5-Chloro-2-pyrimidinyl Analog of Dantrolene

THOMAS J. SCHWAN x and K. O. ELLIS

Abstract □ 1-{[5-(5-Chloro-2-pyrimidinyl)furfurylidene]amino}-hydantoin, a structural analog of the skeletal muscle contraction antagonist dantrolene, was synthesized and found to have no skeletal muscle relaxant activity.

Keyphrases □ Dantrolene—synthesis and screening for muscle relaxant activity of 5-chloro-2-pyrimidinyl analog □ 1-{[5-(5-Chloro-2-pyrimidinyl)furfurylidene]amino}hydantoin—structural analog of dantrolene, synthesized and screened for muscle relaxant activity □ Muscle relaxant activity—5-chloro-2-pyrimidinyl analog of dantrolene

The unique skeletal muscle relaxant activity of dantrolene, 1-{[5-(*p*-nitrophenyl)furfurylidene]amino}hydantoin (I), has been demonstrated (1, 2). It has been pharmacologically categorized as a skeletal muscle contraction antagonist (3).

The 5-chloro-2-pyrimidinyl group was shown to replace effectively the *p*-nitrophenyl moiety in a series of nonsteroidal hypocholesterimic agents (II) (4). Thus, the 5-chloro-2-pyrimidinyl analog of the prototype was desired for evaluation for skeletal muscle relaxant activity.

DISCUSSION

Although the requisite 5-(substituted phenyl)-2-furaldehydes were prepared previously by coupling of the appropriate diazonium salt with furfural (5), reaction of diazotized 5-chloro-2-aminopyrimidine with furfural failed to give the desired aldehyde (III). Accordingly, an alternate route to III was devised.

Condensation of furamidine hydrochloride with mucochloric acid, using the general procedure of Budesinsky (6), gave 5-chloro-2-(2-furyl)-4-pyrimidinecarboxylic acid (IV), which was smoothly decarboxylated to 5-chloro-2-(2-furyl)pyrimidine (V) (Scheme I).

Formylation of V gave the aldehyde III, the structure of which is supported by NMR data. Although the NMR spectrum does not completely rule out a 2,3-orientation of substituents in the aldehyde, previous studies in these laboratories showed that 2-phenylfuran is formylated at the 5-position of the furan ring (7). Thus, a 2,5-disubstituted structure was assigned to III.

Treatment of III with 1-aminohydantoin hydrochloride gave VI, the 5-chloro-2-pyrimidinyl analog of I.

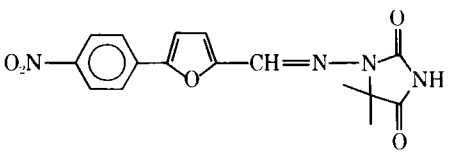
In gross observational testing in mice similar to that described by Irwin (8), VI caused no measurable skeletal muscle relaxation in oral doses up to 1600 mg/kg po. Furthermore, there was no indication of CNS activity or acute toxicity.

Direct skeletal muscle contraction antagonism was tested in the curarized pithed rat gastrocnemius muscle preparation (9). Compound VI did not affect the twitch response of the gastrocnemius muscle over a dose range of 1.0–25 mg/kg iv, indicating that it had no skeletal muscle contraction antagonism activity.

EXPERIMENTAL[1]

5-Chloro-2-(2-furyl)-4-pyrimidinecarboxylic Acid (IV)—Two solutions were prepared: Solution A contained 852 g (15.78 moles) of sodium methoxide in 6360 ml of methanol, and Solution B contained 903 g (5.34 moles) of mucochloric acid in 2285 ml of methanol.

To a solution of 1150 g (7.83 moles) of furamidine hydrochloride in 2610 ml of methanol stirred at 55° was added rapidly 3800 ml of Solution A. The reaction was endothermic. The mixture was reheated to 55°, and 1625 ml of Solution B was added while the mix-



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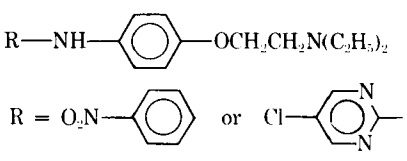

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[1] Melting points were determined on a Mel-Temp apparatus and those below 230° are corrected. IR spectra were determined as mineral oil mulls using a Perkin-Elmer 137B spectrophotometer. The NMR spectrum was obtained on a Varian A-60A instrument and was compared with tetramethylsilane as an internal standard.

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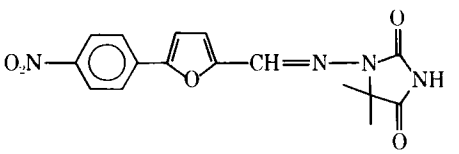

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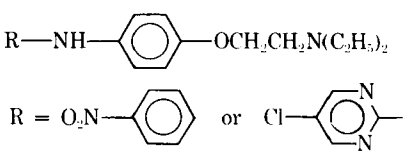

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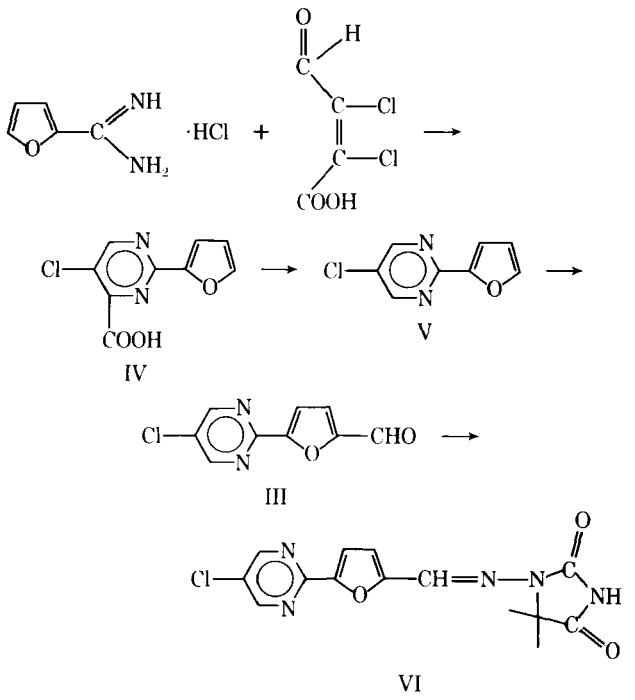

Scheme I

ture was maintained at 55–60° with an ice bath. After the mixture was stirred at 55–60° for 15 min, the remainder of Solution A was added. The mixture was reheated to 55°, and the remainder of Solution B was added as already described. The mixture was stirred at 55–60° for 2.5 hr and cooled overnight, and the solid was filtered.

The filter cake was washed with cold methanol, air dried, and taken up in 10 liters of cold water. Acidification with dilute hydrochloric acid gave 473 g (39%) of the product, mp 153–154°. The analytical sample, mp 160–161°, was obtained by recrystallization from water; IR (μm): 5.84 (C=O, acid), 6.32, and 6.41 (C=C, C=N).

Anal.—Calc. for $C_9H_5ClN_2O_3$: C, 48.13; H, 2.24; Cl, 15.79; N, 12.47. Found: C, 48.17; H, 2.34; Cl, 15.86; N, 12.07.

5-Chloro-2-(2-furyl)pyrimidine (V)—The carboxylic acid IV (300 g, 1.33 moles) was heated at 160–170° for 5 min. The cooled mixture was refluxed with 1 liter of chloroform for 1 hr and the suspension was decolorized and filtered. Upon removal of the solvent *in vacuo*, there was obtained 238 g (99%) of the product, mp 106–109°. The analytical sample, mp 108–110°, was obtained by recrystallization from heptane; IR (μm): 6.30 (C=C, C=N) and no C=O absorption at 5.80–6.10.

Anal.—Calc. for $C_8H_5ClN_2O$: C, 53.20; H, 2.79; Cl, 19.63; N, 15.51. Found: C, 53.05; H, 3.08; Cl, 19.63; N, 15.00.

5-(5-Chloro-2-pyrimidinyl)-2-furfural (III)—To 1000 ml of dimethylformamide, stirred and cooled at 0–10°, was added 183 ml (307 g, 2.0 moles) of phosphorus oxychloride. To the cooled solution was added a solution of 181 g (1.0 mole) of V in 600 ml of dimethylformamide. The mixture was stirred at 90–100° for 8 hr, cooled, and poured cautiously into 5 liters of 2% sodium hydroxide. An additional 450 ml of 20% sodium hydroxide was added, and the mixture was stirred at room temperature for 30 min.

The mixture was acidified with concentrated hydrochloric acid and the solid was filtered, washed with water, and dried to give 143 g (68%) of the crude aldehyde, mp 179–182°. The analytical sample, mp 209–210°, was obtained by recrystallization from benzene; IR (μm): 6.00 (C=O, aldehyde); NMR (dimethyl sulfoxide-d_6): δ 7.55, 7.70 (doublets, J = 4 Hz, 2, furan C—H), 9.80 (s, 1, aldehyde C—H), and 9.06 (s, 2, pyrimidine C_4-H and C_6-H).

Anal.—Calc. for $C_9H_5ClN_2O_2$: C, 51.82; H, 2.42; N, 13.43. Found: C, 51.79; H, 2.40; N, 13.35.

1-{[5-(5-Chloro-2-pyrimidinyl)furfurylidene]amino}hydantoin (VI)—To a stirred solution of 41.6 g (0.20 mole) of III in 800 ml of dimethylformamide at 50° was added slowly a solution of 60.8 g (0.40 mole) of 1-aminohydantoin hydrochloride in 300 ml of water. The mixture was immediately poured into 3 liters of ice water and the solid was filtered, washed thoroughly with water, and dried. Recrystallization from dimethylformamide–water gave 35 g (57%) of the product, mp 318–320°. The analytical sample, mp 323–325°, was obtained by recrystallization from dimethylformamide–water; IR (μm): 5.63 and 5.79 (C=O, hydantoin).

Anal.—Calc. for $C_{12}H_8ClN_5O_3$: C, 47.15; H, 2.64; N, 22.91. Found: C, 47.20; H, 2.64; N, 22.60.

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Inhibition of *Pseudomonas* Burn Wound Infection by Mafenide Dry Foam

PATRICK N. CATANIA ˣ and JAMES C. KING

Abstract □ The results of an *in vivo* evaluation of 8.5% mafenide dry foam are described. Using burned guinea pigs infected with *Pseudomonas aeruginosa*, mafenide was applied every 12 hr as the dry foam or as the commercially available ointment. After 156 hr of therapy with the medicated dosage forms, the previously infected areas did not demonstrate the presence of *Pseudomonas*. However, all nonmedicated, infected controls produced positive cultures. Both medicated dosage forms demonstrated equivalent efficacy in the inhibition of *Pseudomonas* on burn wounds.

Keyphrases □ Mafenide—dry foam formulation, inhibition of *Pseudomonas* burn wound infection □ Burns—inhibition of *Pseudomonas* burn wound infection by mafenide dry foam □ *Pseudomonas* infections in burns—inhibition by mafenide dry foam

Earlier (1), the formulation and *in vitro* evaluation of medicated dry foams were reported. The dry foam was described as a light, flexible, aerated film, with hydrophilic properties that permit ease of application and removal from denuded surfaces. Furthermore, it was postulated that the local use of medicated dry foams may obviate some disadvantages associated with currently available dosage forms. This report describes the results of the *in vivo* evaluation of mafenide dry foam.

EXPERIMENTAL

***In Vivo* Evaluation**—The burned, infected guinea pig was selected as the animal model. Fifteen male guinea pigs, approximately 300 g, were randomly selected. Approximately 24 hr after shaving the dorsum of the animals, four burns were inflicted upon the back of each animal under ether anesthesia.

A burning procedure utilized steam projected through an orifice 8 mm in diameter. In this manner, steam, cooled to 80–90°, was directed onto the backs of the animals for 10 sec to obtain four full thickness thermal injuries. The animals were then returned to individual cages with ample food and water and observed for 24 hr.

After this period, three burns on each animal were inoculated by swabbing with an overnight broth culture of *Pseudomonas aeruginosa* (ATCC 9721); the fourth burn on the animal served as the burned, uninfected control. Of the three infected areas, one was medicated with 0.1 g of 8.5% mafenide ointment[1], applied by means of a sterile blade. The second infected area was medicated with a 2.5-cm square of 8.5% mafenide dry foam. The third area served as the burned, infected control.

Swab cultures were obtained every 12 hr, immediately prior to the application of the dosage form. Repeat applications were continued at these same time intervals for 156 hr.

To determine the effect of the unmedicated vehicles upon infected burns, 15 infected burns were treated with nonmedicated dry foam and 15 infected burns were treated with a nonmedicated ointment[2]. These burns were treated and cultured as described every 12 hr for 72 hr (Table I).

Identification of *Pseudomonas*—Since mixed colonies were cultured from burn areas, appropriate identification tests were performed to distinguish the test bacteria from other microorganisms. Initial differentiation was made with the Gram stain (2). The ability of *Pseudomonas* species to produce a diffusible, blue-green pigment, pyocyanin (3), also aided in the identification. In addition, most strains elaborate another pigment known as pyoverdin (fluorescein) that fluoresces upon exposure to UV light (4, 5). For this reason, a short wavelength UV lamp[3] was used as an aid in identifying and quantifying the *Pseudomonas* isolated from the guinea pig burn areas.

Visual examination of the Gram-negative bacteria permitted the differentiation of three types of bacteria: fluorescent, diffusible pigment-producing colonies; ivory-colored colonies; and yellow-orange colonies. Test for oxidase and indole were performed on representative samples of these three Gram-negative bacteria to substantiate the preliminary identification (Table II).

Table I—Results of Mafenide Therapy on Infected Burns

Hours[a]	Number of Animals[b] with Positive *Pseudomonas* Cultures[c] after Treatment[d] with: Mafenide Dry Foam	Mafenide Ointment	Uninfected Control	Infected Control[e]
12	14	4	2	15
24	1	1	5	15
36	0	0	6	15
48	0	0	5	15
60	0	0	8	15
72	0	0	8	15
84	0	0	8	15
96	0	0	10	15
108	0	0	5	15
120	0	0	8	15
132	0	0	8	15
144	0	0	8	15
156	0	0	6	15

[a] Times listed are the hours after inoculation with an overnight broth culture of *Ps. aeruginosa*. [b] Each of 15 animals received four full thickness thermal injuries. [c] A positive culture was considered to be one in which five or more *Ps. aeruginosa* colonies were isolated and identified from a burn. [d] Treatment consisted of 8.5% mafenide ointment or dry foam, applied to the involved site every 12 hr for 156 hr. In addition, one burn per animal served as the uninfected untreated control, and one burn per animal served as the infected untreated control. Student *t* computations indicate $p < 0.005$ after 24 hr of therapy. [e] Other controls: All cultures from 15 burns treated with nonmedicated ointment every 12 hr for 72 hr were positive for *Pseudomonas*, and 89 of 90 cultures obtained from 15 burns similarly treated with nonmedicated dry foam were positive.

RESULTS AND DISCUSSION

With initial surface growth of *Pseudomonas* being of primary concern in *Pseudomonas* burn wound infection (6, 7), the burned, infected guinea pig was selected as the *in vivo* model for comparing the effectiveness of mafenide dry foam with that of the corresponding ointment. Since supra-eschar bacterial colonization is usually considered to be the first stage of local sepsis, it was assumed that eradication of surface colonies would indicate the effectiveness of the mafenide-containing dosage forms. Therefore, a negative swab culture was selected as the parameter for evaluating the medicated preparations.

By developing suitable burning techniques, four thermal injuries of uniform size were inflicted onto the dorsum of each animal. In this manner, the two medicated dosage forms could be evaluated on each of 15 animals. Furthermore, the effect of diffusion from

[1] Sulfamylon Cream, Winthrop Laboratories, Inc., New York, N.Y.
[2] Neobase Ointment, Burroughs Wellcome & Co., Research Triangle Park, N.C.
[3] Mineralight, Ultra-Violet Products, Inc., San Gabriel, Calif.

Table II—Results of Microbiological Identification Tests

Organism[a]	Identification Test				
	Gram Stain	Indole	Oxidase	Fluorescence[b]	Pigment[c]
Ps. aeruginosa (ATCC 9721)	−	−	+	+	+
Unknown (fluorescent)	−	−	+	+	+
Unknown (ivory colored)	−	+	−	−	−
Unknown (yellow colored)	−	−	−	−	−

[a] Unknown organisms were isolated from burn wounds during *in vivo* studies. Representative samples of these were subjected to the tests indicated to identify *Pseudomonas*. [b] Fluorescence was observed with the naked eye using UV light. [c] Production of a diffusible pigment indicates a positive result.

one area to another was determined by utilizing one burn per animal as the infected, untreated control. It was assumed that if diffusion of medication from a treated area to an untreated area were to occur, the untreated, infected burns would not demonstrate the presence of *Pseudomonas*. However, the untreated, infected burns produced positive cultures for the test organism throughout the study. For this reason, it was assumed that diffusion of the active ingredient from one burn to another did not occur and, therefore, did not have an inhibitory effect upon the test organism in untreated areas. Therefore, the use of four burns per animal, each treated with a different dosage form, appeared to be a reasonable study method.

To determine if cross-contamination might occur from an infected burn to an adjacent area of no colonization, the fourth burn on each animal was not seeded. This burned, uninfected, untreated control served to demonstrate that recontamination was occurring throughout the study, since six of these areas produced positive cultures at the end of the study. From this observation, it seemed probable that cross-contamination of the other burns was occurring at a similar rate.

However, since the burns treated with the medicated dry foam or medicated ointment produced no positive cultures, it is suggested that both medicated dosage forms were equally effective in inhibiting the growth of *Pseudomonas in vivo*. That is, both preparations eradicated supra-eschar colonization after 36 hr of therapy and subsequently prevented the expected cross-contamination due to the presence of *Pseudomonas* on the other burn areas. Both the *in vitro* and *in vivo* methods of evaluation suggest that the dry foam and ointment are equally effective dosage forms for mafenide.

To demonstrate visually the presence of *Ps. aeruginosa* on the burns, the methods of Ward *et al.* (5) and Caplan (8) were used. At the conclusion of the study, UV light was directed onto the burns to detect the characteristic fluorescence of the test bacteria. Unfortunately, mafenide fluoresced with a similar intensity and color to that of fluorescein; therefore, no differentiation could be made between the presence of *Pseudomonas* and the presence of mafenide.

The untreated, infected areas produced fluorescence characteristic of *Pseudomonas*. Of the 15 infected, untreated burns, all produced fluorescence on exposure to UV light. This indirect visual demonstration of the presence of the test organism tends to validate the swabbing technique used, because all infected, untreated burns produced positive cultures for *Pseudomonas*.

In addition, macroscopic examination of the burned, uninfected areas under UV light produced fluorescence with five burns. In contrast, six of these areas produced cultures positive for *Pseudomonas*. This discrepancy between the number of fluorescent areas and the number of areas with positive cultures may be explained by the limitation of the UV technique. According to Polk *et al.* (9), the fluorescence of *Pseudomonas* may be observed with the naked eye as the concentration approaches and exceeds 10^5 organisms/cm^2. Evidently, the contamination and proliferation of *Pseudomonas* on the initially uninfected areas were not of sufficient magnitude, so the fluorescence was not visible on the sixth area.

SUMMARY AND CONCLUSIONS

This report provides additional data regarding a medicated dry foam for local therapy. Using the burned, infected guinea pig, the efficacy of 8.5% mafenide dry foam was compared with that of the corresponding commercial ointment. Since both medicated dosage forms demonstrated equivalent efficacy in inhibiting *Ps. aeruginosa* on burn wounds, further study is indicated to assess the utility of a medicated dry foam as a useful approach to local therapy in human subjects.

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Alkaloids of *Vinca* Species V: Structure Elucidation of Herbadine, an Alkaloid Isolated from *Vinca libanotica*

G. H. AYNILIAN, C. L. BELL, and N. R. FARNSWORTH *

Abstract □ Herbadine, a novel dihydroindole alkaloid, was isolated from the aerial parts of *Vinca libanotica* Zucc. (Apocynaceae). The physical and spectral data (UV, IR, NMR, and mass spectroscopy) indicated the alkaloid to be a derivative of ajmaline. Comparative high-resolution NMR studies with quebrachidine and high-resolution mass spectral studies established the structure of the alkaloid to be 2-epi-3-hydroxyquebrachidine. Herbadine was previously isolated from another species by Russian workers and a structure was postulated. The current paper gives evidence for a corrected structure of herbadine.

Keyphrases □ Herbadine—structure elucidation, *V. libanotica* alkaloid □ *Vinca libanotica* alkaloids—structure elucidation of herbadine □ Alkaloids—*Vinca* species, structure elucidation of herbadine

In a previous article (1) the structures and NMR spectra of vincamajine (I) and herbamine (III), both isolated from *Vinca libanotica* Zucc. (Apocynaceae) (2), were reported. The structure of another dihydroindole alkaloid is now elucidated. This alkaloid, also isolated from *V. libanotica* (2), has a molecular formula of $C_{21}H_{24}N_2O_4$ and possesses physical and spectral properties very similar to herbadine (V), recently reported to be isolated from *V. herbacea* (3). However, in accordance with the revised structure for herbamine (1) and based on high-resolution mass spectral studies of herbadine together with high resolution NMR studies at 220 MHz of both herbadine and the structurally related quebrachidine (II), a new structure (IV) is postulated for herbadine.

EXPERIMENTAL[1]

Previously, the isolation and characterization of several alkaloids including herbadine from *V. libanotica* were reported (2). Herbadine exhibited a melting point of 202–205° dec. and a UV spectrum in methanol having maxima at λ 292 (log ε 3.48), 242 (3.98), and 212 (4.57) nm, which are typical of alkaloids having the dihydroindole moiety. An IR spectrum (KBr) gave absorption bands at ν_{max} 3450 (s) (NH and/or OH), 2950 (s), 2875 (w), 2400 (w), 1725 (s) ($COOCH_3$), 1615 (s) (indoline), 1560 (w), 1540 (w), 1460 (w), 1380 (w), 1310 (w), 1290 (w), 1240 (s) ($COOCH_3$), 1200 (w), 1140 (s), 1100 (w), 1060 (s), 1040 (w), 980 (w), 950 (w), 900 (m), 860 (m), 810 (w), 780 (w), 760 (w), and 735 (1,2-disubstituted benzene) cm^{-1}.

The mass spectrum gave a molecular ion at M^+ 368 (32%), followed by ions at *m/e* 337 (2), 252 (2), 251 (2), 178 (3), 168 (1), 167 (1), 166 (1), 158 (1), 157 (1), 150 (3), 147 (3), 144 (5), 143 (20), 135 (5), 130 (10), 121 (2), and 117 (100).

The high-resolution mass spectrum revealed that the fragments at *m/e* 130 and 143 corresponded to the N_a-indole moiety with one and two additional carbon atoms. The peak at *m/e* 144 was a doublet consisting not only of $C_{10}H_{10}N$ but also of C_9H_6NO; the latter requires retention of the oxygen at C_{17} and is found in all ajmaline derivatives bearing a 17-hydroxy group (4). This is not the case in the structure (V) proposed for herbadine by the Russian workers, who did not determine the elemental composition of the mass spectral fragments. The base peak at *m/e* 117 (C_8H_7N) could be best represented by the N_a-indole skeleton.

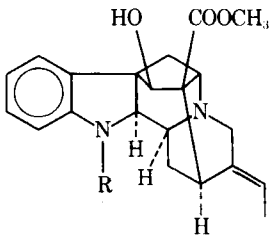
The NMR spectrum of the alkaloid, determined in CD_3OD at 90 MHz, exhibited some similarities with the spectrum of quebrachidine. Thus, it was assumed initially that herbadine (M^+ 368) was an hydroxyl derivative of quebrachidine (M^+ 352), the structure of which was established based on mass spectral studies (5).

Since NMR data can be used as a simple means of differentiating between alkaloids of the ajmaline and 2-epiajmaline series (1) and since no comprehensive NMR assignments have been reported for quebrachidine, the high-resolution NMR spectra of both herbadine and quebrachidine were determined in pyridine-d_5 at 220 MHz (Table I).

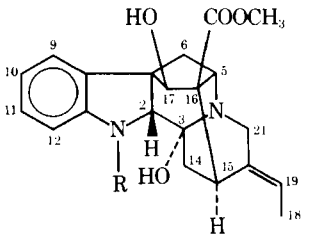
DISCUSSION

The basic differences observed between the NMR spectra of herbadine and quebrachidine were the same as the ones observed between herbamine and vincamajine (1), thus proving that herbadine belongs to the ajmaline series. In addition, in the high-resolution mass spectrum, the elemental composition of the prominent ions (M^+ 368, *m/e* 144, 143, 130, and 117) was 14 units ($—CH_2—$) lower than the ions of herbamine, suggesting that herbadine was the N_a-demethyl derivative of herbamine.

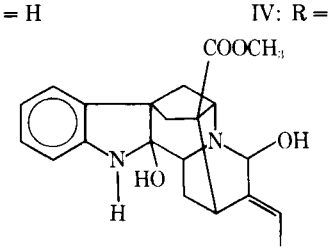
In the NMR spectrum of herbadine, two doublets at δ 4.50 and 3.40 (J = 17 Hz) were attributed to the two C_{21} protons; in quebra-



I: R = CH_3
II: R = H



III: R = CH_3
IV: R = H



V

[1] The UV spectrum was determined using a Beckman model DB-G spectrophotometer, and the IR spectrum was determined using a Beckman model IR 18A spectrophotometer in a KBr pellet. NMR spectra of herbadine and quebrachidine were recorded in pyridine-d_5 solutions on a Varian HR-220 instrument operating at 220 MHz, with tetramethylsilane as an internal standard and with chemical shifts reported in δ (parts per million) units. The high-resolution mass spectrum (70 ev) of herbadine was recorded using a C.E.C. model 110 double-focusing spectrometer at Eli Lilly and Co.

The plant material was authenticated as *V. libanotica* Zucc. (Apocynaceae) by Dr. W. T. Stearn, British Museum of Natural History, London, England. A voucher specimen (SP-2315) representing the collection has been placed in the Herbarium of the Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center.

Table I—NMR Spectral Data of Herbadine and Quebrachidine Determined at 220 MHz in Pyridine-d_5

Assignments	Herbadine		Quebrachidine	
	Chemical Shift, δ[a]	Coupling Constants, Hz	Chemical Shift, δ[a]	Coupling Constants, Hz
C_{9}—H	7.75–7.30 (m)		7.55–6.95 (m)	
C_{11}—H	7.75–7.30 (m)		7.55–6.95 (m)	
C_3—OH	7.75–7.30 (m)		—	
C_{17}—OH	7.75–7.30 (m)		7.55–6.95 (m)	
C_{12}—H	7.17 (d)	$J_{11,12} = 6.5$	7.55–6.95 (m)	
C_{10}—H	7.00 (t)	$J_{9,10} = J_{10,11} = 6.5$	7.55–6.95 (m)	
$-N_bH$	6.65 (d)	$J_{2,NH} = 3$	6.10 (d)	$J_{2,NH} = 4$
C_{19}—H	5.32 (q)	$J_{18,19} = 6$	5.32 (q)	$J_{18,19} = 7$
C_{17}—H	4.85 (d)	$J_{17,OH} = 5$	4.84 (d)	$J_{17,OH} = 6$
$C_{21\beta}$—H	4.50 (d)	$J_{21\alpha,21\beta} = 17$	3.62 (m)	
C_2—H	4.24 (d)	$J_{2,NH} = 3$	4.17 (d × d)	$J_{2,3} = 3.5$, $J_{2,NH} = 4$
C_5—H	4.16 (d)	$J_{5,6\beta} = 4.5$	3.99 (d)	$J_{5,6\beta} = 6$
$-COOCH_3$	3.90 (s)		3.88 (s)	
C_{15}—H	3.86 (d)	$J_{14\beta,15} = 4$	3.80 (d)	$J_{14\beta,15} = 5$
$C_{21\alpha}$—H	3.42 (d)	$J_{21\alpha,21\beta} = 17$	3.62 (m)	
$C_{14\beta}$—H	3.40 (d × d)	$J_{14\beta,15} = 4$	2.91 (d × d)	$J_{14\beta,15} = 5$
		$J_{14\alpha,14\beta} = 15$	3.62 (m)	$J_{14\alpha,14\beta} = 14$
$C_{6\beta}$—H	3.23 (d × d)	$J_{6\alpha,6\beta} = 12$	3.25 (d × d)	$J_{5,6\beta} = 6$
		$J_{5,6\beta} = 4.5$		$J_{6\alpha,6\beta} = 12$
$C_{6\alpha}$—H	2.12 (d)	$J_{6\alpha,6\beta} = 12$	2.02 (d)	$J_{6\alpha,6\beta} = 12$
$C_{14\alpha}$—H	1.84 (d)	$J_{14\alpha,14\beta} = 15$	1.50 (d × d)	$J_{3,14\alpha} = 10$
				$J_{14\alpha,14\beta} = 14$
C_{18}—CH_3	1.71 (d)	$J_{18,19} = 6$	1.72 (d × t)	$J_{18,19} = 7$
				$J_{18,21} = 2$

[a] Chemical shifts measured in parts per million downfield from internal tetramethylsilane reference.

chidine, the signals of both C_{21} protons were in the form of a multiplet at δ 3.62. This finding was in accordance with the fact that the C_{21} protons in the ajmaline series are in nonequivalent environments due to their orientation with respect to the highly anisotropic ethylidine side chain and the unshared pair of electrons of N_b, while in the 2-epiajmaline series, the C_{21} protons are symmetrically disposed with respect to these two groups. Thus, in herbadine the C_{21} does not bear an alcohol group and this makes Structure V not feasible.

In the spectrum of quebrachidine, the signal of C_2—H is made up of a doublet of doublets at δ 4.17 due to the coupling to both C_3—H and NH protons. By contrast, in herbadine, the C_2—H signal is only a doublet at δ 4.24, showing only coupling to the NH proton. A Dreiding model of herbadine revealed that the signal of C_2—H should not show appreciable coupling to the C_3 proton, regardless of the presence of a C_3 proton, since the dihedral angle between these two protons is about 85° ($J_{2,3} \cong 0$). A similar phenomenon was observed in sandwicine and its derivatives (6).

A doublet at δ 4.85 and 4.84 in the spectra of herbadine and quebrachidine, respectively, represents the C_{17} protons of the two alkaloids. The presence of this signal in herbadine is an additional proof that an OH group should be present at C_{17} and not as shown in V.

A doublet at δ 1.84 ($J_{14\alpha,14\beta} = 15$ Hz) represents $C_{14\alpha}$—H, showing that only geminal coupling exists. However, in quebrachidine, the signal of $C_{14\alpha}$—H is made up of a doublet of doublets at δ 1.50 ($J_{3,14\alpha} = 10$ Hz, $J_{14\alpha,14\beta} = 14$ Hz), showing that, in addition to the geminal coupling, this proton is coupled to C_3—H. Since in both the ajmaline and 2-epiajmaline series the dihedral angle between $C_{14\alpha}$—H and C_3—H is about 10° and a large coupling constant is expected, then in herbadine the C_3—H must be substituted with an OH group since the signal of $C_{14\alpha}$—H is a simple doublet.

The remaining protons in both compounds give signals that are well separated from signals of adjacent protons, and the resultant spectrum is easily interpreted on the basis of first-order analysis. Furthermore, the spectra of herbadine and quebrachidine agree closely with those of herbamine and vincamajine, respectively, as reported previously (1), and assignments of signals were based on this prior analysis.

The NMR data, together with the other physical constants, establishes herbadine to be 2-epi-3-hydroxyquebrachidine.

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[x] To whom inquiries should be directed.

Conversion of Agroclavine to Setoclavine and Isosetoclavine in Cell-Free Extracts from *Claviceps* sp. SD 58 and in a Thioglycolate–Iron (II) System

RAGHBIR S. BAJWA and JOHN A. ANDERSON ˣ

Abstract □ Agroclavine was converted to setoclavine and isosetoclavine in crude extracts from *Claviceps* sp. SD 58. The ratio of setoclavine to isosetoclavine was 0.95. The conversion with boiled crude extract was 68% of the conversion with unboiled extract. In a thioglycolate–iron (II) system at 40° for 5 hr, 45.5% of agroclavine was converted to setoclavine and isosetoclavine. At 70° for 4 hr in the thioglycolate–iron (II) system, 4% of 4-dimethylallyltryptophan was converted to clavicipitic acid.

Keyphrases □ Agroclavine—conversion to setoclavine and isosetoclavine, cell-free extracts from *Claviceps* sp. SD 58 and thioglycolate–iron (II) □ Setoclavine and isosetoclavine—converted from agroclavine, cell-free extracts from *Claviceps* sp. SD 58 and thioglycolate–iron (II) □ Thioglycolate–iron (II)—conversion of agroclavine to setoclavine and isosetoclavine □ *Claviceps* sp. SD 58—conversion of agroclavine to setoclavine and isosetoclavine

Clavine alkaloid-producing strains of ergot fungus produce setoclavine, isosetoclavine, penniclavine, and isopenniclavine. These alkaloids are derived from agroclavine and elymoclavine (1). Beliveau and Ramstad (2) proposed that the hydroxylation was nonenzymatic, since both isomers, *i.e.*, setoclavine–isosetoclavine and penniclavine–isopenniclavine, were formed in most fungal species that carried out the conversion.

Shough and Taylor (3) showed that horseradish peroxidase in the presence of hydrogen peroxide could catalyze the 8-hydroxylation of agroclavine and elymoclavine. However, Jindra *et al.* (4) reported that *Claviceps* sp. SD 58 had high catalase activity but contained very little peroxidase. Wilson *et al.* (5) observed that mammalian cytochrome P-450 converted agroclavine to setoclavine and isosetoclavine in addition to other products, and NADPH was required as a cofactor.

This article reports the 8-hydroxylation of agroclavine and elymoclavine in a thioglycolate–iron (II) system, which may resemble the system for nonenzymatic formation of setoclavine, isosetoclavine, penniclavine, and isopenniclavine, in cultures of *Claviceps* sp. SD 58.

EXPERIMENTAL[1]

Materials—Radioactive agroclavine and elymoclavine were prepared by feeding ^{14}C-methylene-tryptophan to *Claviceps purpurea* PRL 1980. After 5 days of growth in mannitol medium (6) supplemented with 400 mg/liter L-tryptophan and 2.0 m*M* niacinamide, 100 μCi of ^{14}C-tryptophan was added to 25 ml of culture. After 15 days, the culture was homogenized and filtered. The residue was washed with water, and washings and the filtrate were extracted with ammonia–ether.

The ether extract was extracted with 0.1 *N* sulfuric acid, and the aqueous extract was made basic with sodium carbonate and then extracted with ether. The ether extract was evaporated to dryness, and the residue was dissolved in ethanol. The extract was fractionated on TLC with ethyl acetate–acetone–dimethylformamide (5:5:1).

Agroclavine and elymoclavine were further purified by preparative TLC, using chloroform–methanol (4:1) and chloroform–diethylamine (9:1) solvents. Setoclavine and isosetoclavine were also separated from the alkaloid extract by preparative TLC as described for the incubated systems.

4-Dimethylallyl-[3-^{14}C]-tryptophan was synthesized according to procedures of Plieninger and coworkers (7–9). The decarboxylation of 4-bromoindole-2-carboxylic acid was carried out as described by Kohler and Anderson (10). ^{14}C-Formaldehyde was used in the conversion of 4-dimethylallylindole to the gramine. The specific activity of the final product was 4.9 μCi/μmole.

Procedures—*Claviceps* sp. SD 58 was grown in yeast extract–mannitol medium NL-406 (11) for 10–16 days, and the culture was filtered on a buchner funnel. The cells were washed with 0.02 *M* phosphate buffer (pH 7.0), suspended in the same buffer, and homogenized[2] for 3–6 min at maximum speed. The mixture was centrifuged at 10,000×*g* for 20 min, and the supernate (crude extract) was collected. All operations for preparation of the crude extract were carried out at 0–5°.

The incubation mixture consisted of crude extract (7.0 mg/ml), 1.9×10^{-4} *M* agroclavine (8.4 μCi), and 1.0 mg streptomycin sulfate in a final volume of 2.2 ml. Crude extract and chemically defined mixtures were incubated on a shaker[3] at 25°, unless another temperature is indicated.

After incubation, alkaloids were extracted as described for the preparation of labeled alkaloids, and preparative TLC was carried out with chloroform–triethylamine (9:1). After elution of setoclavine and isosetoclavine with 1.0% ammonium hydroxide in ethanol, the samples were dried, dissolved in 0.5% 2,5-diphenyloxazole in toluene, and counted[4]. The percent conversion was the percent of radioactivity applied to the TLC plate recovered from the region of the plate containing setoclavine or isosetoclavine. Radioactive penniclavine and isopenniclavine were fractionated by preparative TLC with chloroform–methanol (4:1).

Conversion of agroclavine to setoclavine and isosetoclavine in the chemically defined systems was also measured fluorometrically. After incubation, 8 ml of methanol was added to 2 ml of incubation mixture. The fluorescence was then measured[5] with excitation at 325 nm and emission at 425 nm. These wavelengths corresponded to the excitation and emission maxima. Reference setoclavine and isosetoclavine had excitation and emission maxima at the same wavelengths. The intensity of fluorescence correlated well with the amounts of setoclavine and isosetoclavine estimated visually by TLC.

After reaction of dimethylallyl-[3-^{14}C]-tryptophan in the thioglycolate–iron (II) system, the incubation mixture was adjusted to

[1] *Claviceps* sp. SD 58 was provided by Dr. J. E. Robbers and reference clavicipitic acid was provided by Dr. H. G. Floss of Purdue University, La-Fayette, Ind. Reference setoclavine, isosetoclavine, and penniclavine were gifts from Dr. Von Wartburg and Dr. Stadler, Sandoz Ltd., Basle, Switzerland.

[2] Virtis "45" homogenizer.

[3] *Dubnoff.*

[4] Beckman model L 200 liquid scintillation counter.

[5] Aminco-Bowman spectrophotofluorometer.

Table I—Effect of pH on Conversion of Agroclavine to Setoclavine and Isosetoclavine in Thioglycolate–Iron (II) System[a]

pH	Conversion, %	
	Setoclavine	Isosetoclavine
4.0	20.7	19.7
4.5	22.8	22.7
5.0	18.9	18.3
5.5	15.1	13.9
6.0	14.5	14.6
7.0	9.1	8.3
8.0	7.6	6.6
9.0	7.2	7.6
5.5[b]	1.7	3.6

[a] The incubation mixture contained 3.5×10^{-4} *M* ^{14}C-agroclavine (0.064 μCi), 4.0×10^{-4} *M* iron (II) sulfate, 0.02 *M* sodium thioglycolate, and buffer in final volume of 2.4 ml. Sodium acetate buffer (0.5 *M*) was used for pH 4.0, 4.5, 5.0, and 5.5, 0.2 *M* sodium phosphate buffer was used for pH 6.0, 7.0, and 8.0, and 0.2 *M* tromethamine hydrochloride buffer was used for pH 9.0. The reaction was carried out for 5 hr at 40°. [b] Buffer only.

pH 5.0 with dilute hydrochloric acid and passed through a column of cation-exchange resin[6]. Alkaloids and amino acids were eluted from the resin with 5% ammonium hydroxide solution. The eluate was dried under vacuum on a rotary evaporator, and the residue was dissolved in 0.5 ml of 95% ethanol containing 1.0% ammonium hydroxide for TLC.

RESULTS AND DISCUSSION

The percent of setoclavine and isosetoclavine of the total alkaloid extract from cultures of *Claviceps* sp. SD 58 was 1.42 and 1.79%, respectively. The percent conversion of agroclavine to setoclavine was 1.87% with crude extract and 1.12% with crude extract that had been boiled for 10 min. The percent conversion of agroclavine to isosetoclavine was 1.97% with crude extract and 1.48% with boiled crude extract.

Several systems were tested for 8-hydroxylation of agroclavine, and the most effective system was thioglycolate[7]–iron (II). Under optimum conditions, conversion to setoclavine and isosetoclavine combined was 45.5% (Table I). The conversion was favored at low pH. The ratio of setoclavine–isosetoclavine was 1 throughout the pH range of 4.0–9.0. ^{14}C-Elymoclavine was converted to penniclavine (5.8%) and isopenniclavine (6.0%) in this system.

The efficient conversion of agroclavine to setoclavine and isosetoclavine in a nonenzymatic system, the ratio of setoclavine–isosetoclavine near 1 in the cultures as well as in crude extracts, and the stability of most activity to boiling indicate that the majority of the formation of setoclavine and isosetoclavine in cultures of *Claviceps* sp. SD 58 is nonenzymatic.

Dimethylallyl-[3-^{14}C]-tryptophan was also incubated in the thioglycolate–iron (II) system. At 40°, there was little conversion of dimethylallyltryptophan to Van Urk's positive products. The concentrations of thioglycolate and iron (II) were varied, and higher temperatures were used to increase conversion. Conditions that appeared to give the best conversion to Van Urk's positive products were 4.0×10^{-3} *M* sodium thioglycolate, 2.0×10^{-4} *M* iron (II) sulfate, 3.8×10^{-4} *M* dimethylallyl-[3-^{14}C]-tryptophan (0.2 μCi), and 0.5 *M* sodium acetate (pH 4.0) in a final volume of 2.2 ml incubated for 4 hr at 70°.

The products were fractionated on cation-exchange resin and on TLC with developing solvents of chloroform–methanol–acetic acid (70:20:5 and 8:4:1) and methyl acetate–isopropanol–ammonia (45:35:20). A radioactive spot corresponded to reference clavicipitic acid in these three solvents. The percent conversion to clavicipitic acid was 4.0%. Fifty percent of the radioactivity was recovered in a number of spots below dimethylallyltryptophan on TLC.

The 8-hydroxylation of agroclavine and elymoclavine and the conversion of dimethylallyltryptophan to clavicipitic acid in the thioglycolate–iron (II) system probably proceed by similar mechanisms. A reasonable initial step would be radical formation through removal of the allylic hydrogen. Activation at the allylic carbon of dimethylallyltryptophan is consistent with the recently revised structure of clavicipitic acid, in which a C—N bond connects the α-amino nitrogen and the allylic carbon (12). Further studies are necessary to determine the reactions involved in formation of the products in this system.

In contrast to the failure to obtain significant enzymatic 8-hydroxylation of agroclavine and elymoclavine, dimethylallyltryptophan is actively converted to clavicipitic acid in extracts from *Claviceps* sp. SD 58[8]. The thioglycolate–iron (II) and related systems should be useful models for the enzymatic reaction.

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[x] To whom inquiries should be directed.

[6] Dowex 50(H$^+$) (100–200 mesh), Dow Chemical Co.

[7] Sodium thioglycolate, Sigma Chemical Co.

[8] Unpublished results.

Fluorometric and GLC Analyses of Aristolochic Acid

KOPPAKA V. RAO ˣ, YASAR TANRIKUT, and KARON KILLION

Abstract □ Aristolochic acid is a natural product with possible implication in Balkan endemic nephropathy. A convenient fluorometric assay for the compound is described, based on reduction to the lactam and measurement of the intensity of fluorescence. The limit of sensitivity was 0.05 μg/ml. A GLC assay is also described, based on flash methylation of aristolochic acid and its lactam using trimethylanilinium hydroxide. Conditions for optimum performance with a sensitivity limit of 1–5 μg/ml are described.

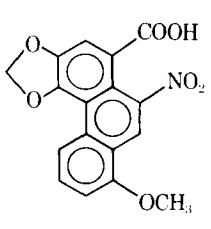
Keyphrases □ Aristolochic acid—fluorometric and GLC analyses □ Fluorometry—analysis, aristolochic acid □ GLC—analysis, aristolochic acid

Aristolochic acid is a natural product of unusual interest. It is one of the few naturally occurring organic compounds to contain a nitro group. The compound has significant activity against certain experimental neoplasms such as adenocarcinoma 755 (1). After a brief study, clinical trials in human patients were abandoned because of its nephrotoxic effects (2). More recently, the compound has been implicated as a probable causative agent in Balkan endemic nephropathy (3, 4).

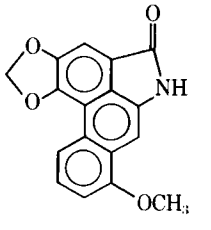
The plant, *Aristolochia clematitis*, grows as a weed in certain areas of the Balkan region. It has been suggested that the aristolochic acid from this weed might find itself either in the diet (through contamination of its seeds in the wheat) or the water supplies (through seepage from the roots). To assess the validity of this hypothesis, it is necessary to examine large numbers of grain and water samples from the region as well as samples of biological fluids from patients affected. A convenient and sensitive test is therefore essential.

Paper chromatographic analysis was used to detect and separate aristolochic acid in body fluids (5), and it was also separated by chromatography on a mixture of silica gel and cellulose (6). Spectral methods were used in both procedures for the identification. Polarography was employed for the quantitative analysis of the acid (7).

Aristolochic acid (I) is a phenanthrene derivative (8-methoxy-6-nitrophenanthro-[3,4-*d*]-1,3-dioxole-5-carboxylic acid). It has a characteristic UV absorption spectrum with maxima at 390, 318, and 250 nm (log ε 3.81, 4.08, and 4.34, respectively). Although the intensity at 250 nm is high, serious interference from other compounds absorbing in this region makes it useless for an analytical method. The compound exhibits no characteristic fluorescence. However, on reduction, it forms a lactam (II) which does show intense fluorescence (8). This property, therefore, offers a possibility for a fluorometric assay for aristolochic acid. This paper describes a convenient and sensitive assay based on reduction to the lactam and measurement of the intensity of fluorescence.



I



II

A second possible assay method based on GLC was studied. It is common practice to convert carboxylic acids to their esters to increase their volatility in this system. One convenient reagent is diazomethane, which gives quantitative conversion to the methyl ester. Recently, a methylation method using 0.1 *M* trimethylanilinium hydroxide was described, which has the advantage that the reaction can be carried out in the gas chromatograph itself (9). Application of this method to the analysis of aristolochic acid was studied, and the results are presented here.

EXPERIMENTAL

The sample of aristolochic acid was obtained by extraction from the seeds of *A. clematitis*[1] (6, 10). Finely ground seeds (100 g) were stirred with ethanol for 24 hr and filtered. After three such extractions, the combined extract was concentrated to a syrup and partitioned between ethyl acetate and 0.5 *N* HCl. The solvent layer was washed twice with 5% aqueous sodium bicarbonate. The yellow precipitate obtained on acidification of the bicarbonate layers was filtered and purified by chromatography on silicic acid[2] (100 mesh), with chloroform as the solvent. The major yellow band on concentration gave aristolochic acid, which was further purified by preparative TLC using a silica gel plate[3] developed with chloroform–methanol (9:1).

The aristolochic acid recovered from the bright-yellow band was crystallized twice from methanol–chloroform (1:1), mp 283–285°. It behaved as a single component in TLC in two different systems, and its spectral properties were identical with those described in the literature. GLC analysis (described below) also indicated its homogeneity.

To make the assays more representative of *A. clematitis* and to have a more readily available standard, the yellow precipitate (which was a mixture of two major and two or three minor members of the aristolochic acid group) was crystallized twice from methanol and chloroform. The bright-yellow crystalline solid thus obtained consisted of aristolochic acid (75%) and aristolochic acid-II (25%)[4]. It was used for the routine GLC assays.

Fluorometric Assay—A fluorescence spectrophotometer[5] with a 150-w xenon lamp was used. The monochromator was calibrated against the line emission spectrum of xenon. The spectra were uncorrected for wavelength response of the lamp, monochromator,

[1] A sample of dried fruits from Yugoslavia was received from Morton Robins, Director, Regional Medical Program Service, Department of Health, Education, and Welfare. A voucher sample was preserved at the herbarium in the Department of Botany, University of Florida.

[2] Mallinckrodt.

[3] E. Merck, PF254+360.

[4] This refers to a compound with the same structure as that of aristolochic acid but without the methoxyl group. The nomenclature is that used in *Chemical Abstracts*.

[5] Perkin-Elmer model MPF-2A.

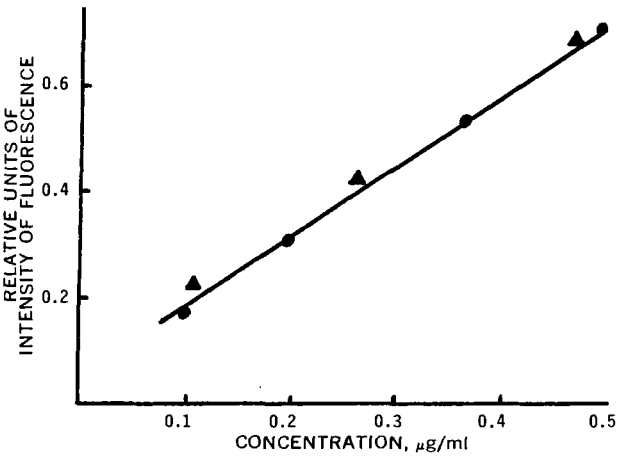


Figure 1—*Standard curve for fluorometric assay of aristolochic acid* (●). *Key:* ▲, *recovery from urine.*

and phototube. The solutions were not freed from dissolved air. Measurements were made in 1-cm silica cells.

The following procedure was used. To 5 ml of a solution of aristolochic acid in 1% aqueous sodium bicarbonate (concentration range 0.05–0.5 μg/ml) in a 30-ml separator was added solid sodium hydrosulfite (10–20 mg). After 5 min, the solution was diluted with 5 ml of 1 *M* phosphate buffer (pH 7) and extracted with chloroform (5 ml). The extract was filtered through a small piece of cotton into a 10-ml volumetric flask. A second extraction was carried out with chloroform, and the two were combined in the volumetric flask and diluted to volume.

The reduction and extraction were found to be quantitative, as shown by UV spectrophotometry. The fluorescence spectrum of the extract was obtained with the excitation wavelength set at 340 nm. Depending on the settings for the emission slit, excitation slit, and reference and sample sensitivities, the overall sensitivity of the assay can vary. With median settings, linearity of response was observed in the concentration range of 0.05–0.5 μg/ml. It is necessary to set up a "standard curve" for each set of assays performed.

GLC—The assays were performed on a gas chromatograph[6] equipped with a flame-ionization detector. A glass U-shaped column (183 cm long and 0.63 cm o.d.) was packed with 3% SE 30 on *100–200-mesh column packing*[7], *conditioned at 300° for 18 hr.* Helium, the carrier gas, was maintained at 55 ml/min; the flow rate for hydrogen was 45 ml/min and that for air was 300 ml/min. The injector and detector were kept at 300°. The column oven temperature was maintained at 260°.

Procedure for Biological Samples—Urine samples (1–5 ml) containing aristolochic acid were extracted at pH 2–3 with chloroform (10 ml) in a 30-ml separator. The solvent layer was placed in a centrifuge tube and evaporated to dryness in a water bath (50°) under a gentle stream of air filtered through glass wool.

Derivatization—Trimethylanilinium hydroxide (9) (100 μl of 0.1 *M*) was added to the evaporated sample, and the volume was made up to 2 ml with methanol. Then 5 μl of this solution was injected into the gas chromatograph. Two injections were made for each sample. Under the conditions described, aristolochic acid emerged as a sharp peak at 9.7 min.

Table I—Recovery of Aristolochic Acid Added to Urine

Added, mg/ml	Recovered, mg/ml	Recovery, %
0.15	0.15	100
0.30	0.30	100
0.30	0.29	97
0.45	0.44	98

RESULTS AND DISCUSSION

When the chloroform solutions of the aristololactam were excited at 340 nm, a characteristic emission spectrum with a maximum at 425 nm was obtained. Using this spectrum as the standard, quantitative recoveries of aristolochic acid were obtained at various concentrations (Fig. 1). Recovery of added aristolochic acid to urine is also shown in Fig. 1. Depending on the sensitivity settings used, the baseline of the fluorescence spectrum may vary and the standard curve may not pass through the origin. The deviation is usually slight, and the use of a standard curve with each batch of assays will compensate for it.

Three reducing agents were studied: stannous chloride, ammonium sulfide, and sodium hydrosulfite. Of these, stannous chloride requires acid conditions at which aristolochic acid has rather low solubility. Ammonium sulfide gave slow reduction. Sodium hydrosulfite gave the fastest reaction in a neutral medium.

The lactam showed much lower intensity of fluorescence in an aqueous medium, and interference from other biological constituents could cause problems. Hence, extraction with a solvent was considered necessary. Among the three solvents studied (ether, ethyl acetate, and chloroform), chloroform was the most convenient. Two extractions at pH 7 gave complete recovery of the lactam in the solvent layer.

The fluorometric assay is convenient and sensitive. With proper controls and higher sensitivity settings, concentrations of 0.02–0.05 μg/ml of aristolochic acid can be determined after reduction to the lactam.

For the GLC assay, a plot of the peak height *versus* the concentration is shown in Fig. 2. The minimum detectable concentration was 0.01 mg/ml (or 0.025 μg/5 μl injected.) By using a 2- or 5-ml sample, the limit can be lowered further.

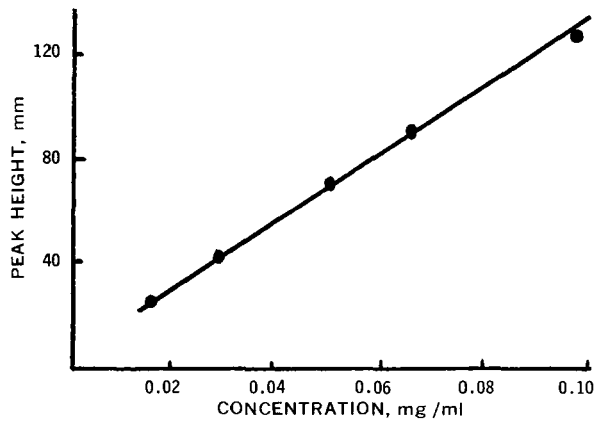


Figure 2—*Standard curve for aristolochic acid.*

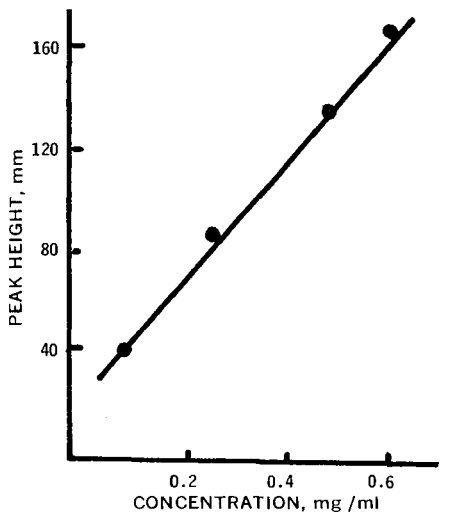


Figure 3—*Recovery of aristolochic acid from urine.*

[6] Varian model 2100 (Varian Aerograph, Walnut Creek, Calif.) equipped with a Varian Model A-25 strip-chart recorder.
[7] Varaport 30 from Varian Aerograph, Walnut Creek, Calif.

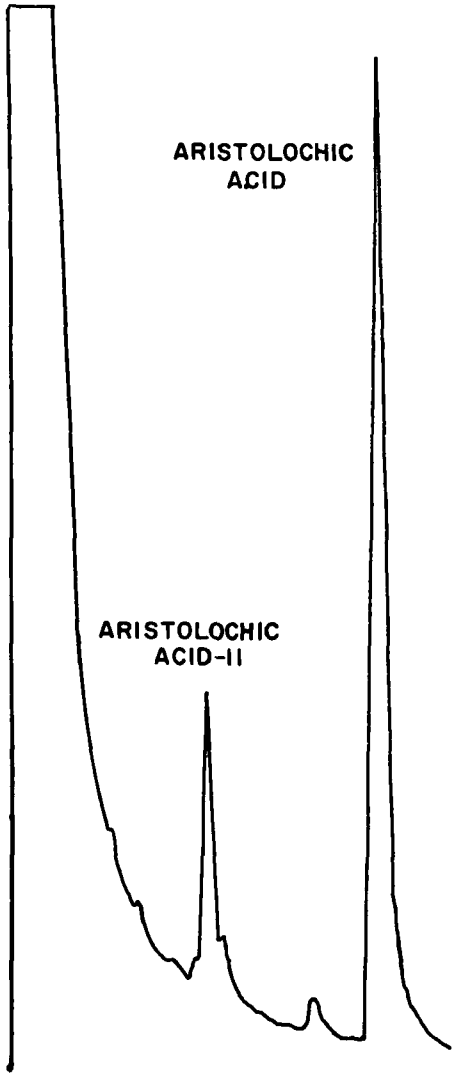


Figure 4—*Gas chromatogram of aristolochic acid with the addition of trimethylanilinium hydroxide. (Aristolochic acid concentration = 0.4 mg/ml; attenuation, 8×10^{-11}. For other details, see text.)*

Recovery of aristolochic acid added to urine is shown in Table I and Fig. 3. A blank was set up and processed the same way, and it gave no interfering peaks in the region of interest.

To confirm that the peak observed was due to the methyl ester, aristolochic acid was esterified with diazomethane and the authentic sample of the methyl ester was injected into the gas chromatograph. A peak with the same retention time (9.7 min) was observed. Figure 4 shows a typical chromatogram of aristolochic acid together with the minor component isolated from *A. clematitis*.

Since there is a possibility that aristolochic acid might undergo metabolic reduction and be excreted as the lactam, it was decided to study the GLC characteristics of this compound. The lactam was prepared by the reduction of aristolochic acid with sodium hydrosulfite, and a standard curve was obtained in the usual manner (Fig. 5). The compound emerged as a sharp peak with a retention time of 3.0 min.

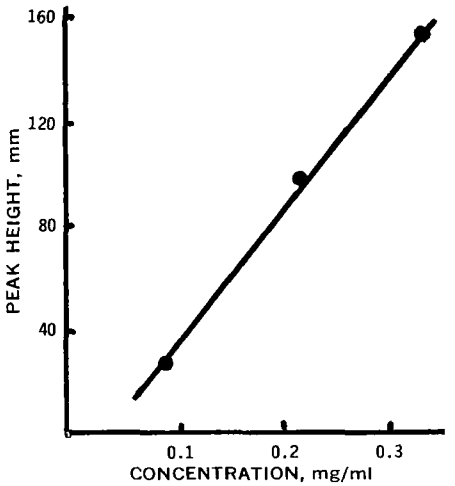


Figure 5—*Standard curve for reduced aristolochic acid (lactam).*

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[x] To whom inquiries should be directed.

Long Acting Delivery Systems for Narcotic Antagonists II: Release Rates of Naltrexone from Poly(lactic Acid) Composites

SEYMOUR YOLLES *x, THOMAS D. LEAFE *, JAMES H. R. WOODLAND *, and FRANCIS J. MEYER ‡

Abstract □ Parallel *in vitro* and *in vivo* release rates of tritiated naltrexone from poly(lactic acid) composites were studied. The *in vitro* release of naltrexone was 67% of the dose over a 35-day test period, while the *in vivo* release was only 24% within 70 days. Apparently, an exchange of the tritium for the hydrogen of the body water takes place, indicating that urinary excretion radioactivity is not a reliable measure for estimating the naltrexone released. Naltrexone–poly(lactic acid) composites showed effective blocking action to morphine in rats (24 days), dogs (29 days), monkeys (20 days), and mice (21 days).

Keyphrases □ Naltrexone—release rates from poly(lactic acid) composites, long acting delivery systems for narcotic antagonists □ Narcotic antagonists—long acting delivery systems, naltrexone–poly(lactic acid) formulation, release rates □ Dosage forms—long acting delivery systems for narcotic antagonists, release rates of naltrexone from poly(lactic acid) composites □ Formulations—naltrexone–poly(lactic acid) composites, release rates

The development of systems for controlled release of narcotic antagonists from polymeric matrixes, such as polyethylene and poly(lactic acid), has been the objective of research by this laboratory since 1970. The results of the behavior of cyclazocine[1] (3-cyclopropylmethyl - 1,2,3,4,5,6-hexahydro-6,11-dimethyl-2,6-methano-3-benzazocin-8-ol)–poly(lactic acid) composites were published recently (1). The present paper reports on the release rates of naltrexone[2] (17-cyclopropylmethyl - 4,5α - epoxy - 3,14 - dihydroxymorphinan-6-one), which was described (2, 3) to be a useful narcotic antagonist with less agonistic activity than cyclazocine, from poly(lactic acid) composites.

EXPERIMENTAL

All countings were performed with liquid scintillation spectrometers[3]. The counting solution consisted of a mixture of 2,5-diphenyloxazole (22.0 g), 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (0.4 g), and a nonionic wetting agent[4] (1000 ml) diluted to 4000 ml with toluene in the *in vivo* experiments. A scintillation liquid[5] was used in the *in vitro* experiments.

Preparation of Naltrexone–Poly(lactic Acid) Composites—A chloroform solution of tritiated naltrexone[6] [specific activity 83.9×10^9 dpm/ml (1.0 ml)] was diluted to 1000 ml with methylene chloride. To this solution (30 ml) were added, for the preparation of composites containing 20% naltrexone, unlabeled naltrexone base[2] (4.0 g) and tributyl citrate (1.0 g) in methylene chloride (200 ml) and then 15 g of poly(lactic acid) (1). For the preparation of composites containing 35% naltrexone, unlabeled naltrexone (10.5 g) and tributyl citrate (1.5 g) in methylene chloride (200 ml) and then 18.0 g of poly(lactic acid) (1) were added to the original solution of labeled naltrexone (45 ml).

In the preparation of both types of composites, the solvent was flashed off under reduced pressure and the residue, wrapped in aluminum foil, was melt-pressed[7] at 170° under a total load of 3 metric tons for 30 sec (shims 0.91 mm thick were used) to produce films of uniform thickness in which no imperfection due to air or gas was observed. The films were ground in a blender[8].

The particles obtained were screened, and fractions of an average size of 500–710 μm were collected. The specific radioactivity, determined by combustion of the polymer matrixes and measurement of the radioactivity in the water trapped in a scintillation spectrometer[9], was 107×10^6 dpm/g for the sample containing 20% naltrexone and 61.4×10^6 dpm/g for the sample containing 35% naltrexone.

***In Vitro* Tests**—A sample of the composite containing 20% naltrexone (650 mg) was sewn into a cheesecloth sack and anchored under the water level of the sample holder of the modified extractor (Raab) described in a previous paper (1). The drug was extracted with tepid (29 ± 3°) water as the solvent. Samples of the aqueous extract were collected periodically, and the radioactivity was measured. The values of naltrexone extracted in each interval of time are reported as percent of dose initially present in the sample, calculated on the basis of disintegrations per minute.

At the end of the experiment, the sample of composite left in the extractor was dissolved in methylene chloride and the radioactivity of the obtained solution was measured. The total radioactivity of the extracted aqueous solution plus the radioactivity found in the sample after extraction checked with that present in the sample before extraction.

***In Vivo* Tests**—These tests were performed on groups of three male Sprague–Dawley rats, 550–600 g, using the following injection method. Into a 5-ml plastic syringe, whose opening at the tip was first enlarged with a 0.21 cm (0.081-in.) drill and then plugged, were added, while stirring, carboxymethylcellulose[10] (210 mg), the composite containing 20% naltrexone (400 mg), and normal saline (portionwise, 0.5-ml portions) until the total volume was 3 ml. The time of mixing and stirring was about 1 min.

An intimate mixture of particles in nearly clear, viscous carboxymethylcellulose resulted. The plug at the tip of the barrel was then replaced with a 12-gauge, thin wall needle. The injection site on the rat was shaved, the area having been selected by measuring the length of the needle from the nape of the neck to a lower section of the back. The animal was lightly anesthetized with ether to

Table I—Blocking Action of Naltrexone–Poly(lactic Acid) Composites

Animal	Number of Animals	Dose, mg/kg
Rats[a]	70	240
Dogs[b]	5	17
Monkeys[a]	4	30
Mice[c]	10	39

[a] D. A. McCarthy, Parke, Davis and Co., Ann Arbor, Mich., personal communication. [b] W. R. Martin, Addiction Research Center, Lexington, Ky., personal communication. [c] R. H. Reuning, College of Pharmacy, Ohio State University, personal communication.

[1] Sterling-Winthrop, New York, N.Y.
[2] Endo Laboratories, Garden City, Long Island, N.Y.
[3] Packard Tri-Carb model 3003 (Packard Instruments, Downers Grove, Ill.) was used in the *in vivo* experiments, and a Beckman LS-100 (Beckman Instruments, Fullerton, Calif.) was used in the *in vitro* experiments.
[4] Triton X-100, Rohm and Haas, Philadelphia, Pa.
[5] Aquasol, New England Nuclear Corp., Boston, Mass.
[6] Tritiated by the catalytic exchange method by New England Nuclear Corp., Boston, Mass.
[7] Carver laboratory press model D.
[8] Waring.
[9] Packard Tri-Carb model 3003.
[10] 7LF, Hercules, Inc., Wilmington, Del.

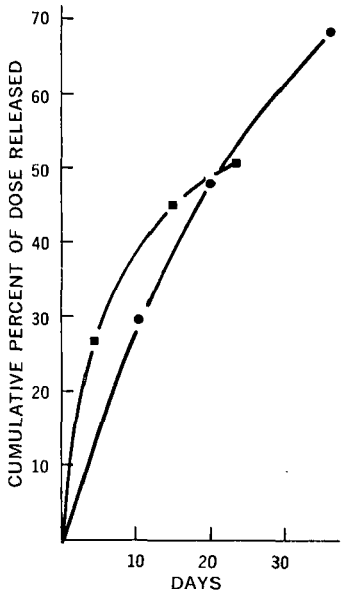

Figure 1—*Cumulative amounts of naltrexone and cyclazocine released* in vitro *from composites in particle form. Key:* ■, *cyclazocine (Ref. 1, Sample F); and* ●, *naltrexone.*

avoid excessive movement. The time required for injection was approximately 15 sec. On removing the needle, the opening was pressed and supported with the finger, painted with antiseptic, and immediately closed with a clip or adhesive tape. The animals were immediately placed into individual metabolism cages designed for the collection of urine.

The collected urine and cage washings were combined and counted on a daily basis over the first 10 days and then every 4 days for the duration of the experiment. The urine samples were diluted to 100 ml with water, and samples of 1 ml were pipetted into 15 ml of scintillation solution and radioassayed by liquid scintillation counting techniques. Internal standardization was used for the calculation of counting efficiency. The values of naltrexone delivered in each interval of time are reported as percent of dose released calculated on the basis of disintegrations per minute.

Determination of Tritiated Naltrexone Remaining in Composites at End of *In Vivo* Tests—A sample of the composite was collected from the sacrificed animal and extracted with chloroform. The radioactivity of the extract was 0.43×10^6 dpm (1% of the original radioactivity).

Exchange of Tritium from Tritiated Naltrexone for Hydrogen of Water—A solution of radioactive naltrexone in methylene chloride (0.1 ml) (83.1×10^6 dpm/ml, experimentally determined) was evaporated to dryness in a 250-ml beaker. To the residue was added 50 ml of distilled water. The obtained solution (specific radioactivity 2.6×10^4 dpm/ml) was frozen on the inside wall of a 1000-ml, round-bottom, single-necked flask connected to a dry ice-cooled moisture trap and kept under vacuum (<0.01 torr) overnight. The water (49 ml) condensed in the trap showed a radioactivity of 4400 dpm/ml (17% of the radioactive tag was lyophilized).

Blocking Action to Morphine—The blocking action of naltrexone composites to morphine was determined in rats (Sprague–Dawley), dogs, monkeys (*Macaca mulatta*), and mice by injecting suspensions of 35% naltrexone–poly(lactic acid) composites in 7% carboxymethylcellulose gel (Table I). The duration of morphine antagonistic activity was determined in rats and in mice by using the tail pinch test; in dogs by measuring the flexor reflex, the skin twitch reflex, the pulse rate, and the pupillary diameter; and in monkeys by measuring the changes in morphine-induced prolongation of interblinking time.

RESULTS AND DISCUSSION

The results of the *in vitro* tests are shown in Fig. 1 in comparison with those obtained with cyclazocine composites under the same conditions. The total amount of naltrexone release within 35 days was 67% of the dose originally present in the composite. The time at which one-half of the dose had been delivered, $T_{1/2}$, was 22 days, and the average daily delivery of naltrexone was 1.53 mg. This delivery is comparable to that found for cyclazocine.

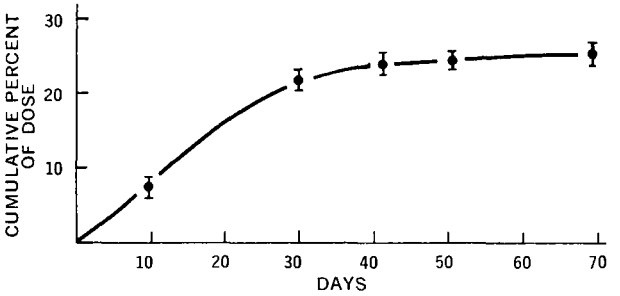

Figure 2—*Cumulative amounts of naltrexone excreted in urine, expressed as percent of dose. Each point represents the mean observation on three animals* ± SE. *In this work, the standard error is equal to the standard deviation divided by the square root of the number of animals tested:* ($SE = \sigma/\sqrt{N}$).

The *in vivo* test results (Fig. 2) show that only 24% of the administered dose was excreted in urine over a 70-day period. However, the determination of the drug in the composite at the end of the test showed that only 1% of the original dose remained in the composite.

The considerable difference in the amounts of naltrexone released in the *in vivo* and *in vitro* tests and the discrepancy between the amount of naltrexone excreted and that left in the composite led to the investigation of the stability of the tritium label on the naltrexone molecule. This investigation was performed by freeze-drying samples of tritiated naltrexone in aqueous solutions and determining the radioactivity of the condensed water. An amount of 17% of the radioactivity originally present in naltrexone was found in the condensed water, indicating exchange of the tritium for the hydrogen of water.

These results show that urinary excretion radioactivity is not a reliable measure for estimating the naltrexone released in *in vivo* tests. At present, other methods are being investigated that are not based on radioactivity, such as chromatography.

Significant blocking action against the effect of morphine was observed in rats throughout the 24-day test period[11]. The tests on dogs showed a highly significant level of blockage for all measures, except lowering of pulse rate, through the 21st day and a significant level of blockage for the flexor reflex through the 29th day (3). Complete antagonism to the depressant effect of morphine on the blinking rate occurred 24 hr after antagonist injection in monkeys. The estimated duration of statistically significant activity was approximately 20 days[11]. In the tests on mice, a 21-day average duration of blocking action was observed.

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[11] D. A. McCarthy, Parke, Davis and Co., 1973, personal communication.

Determination of Stability Constants of Stannous Fluoride Complexes by Potentiostatic Titration

K. G. NELSON [x] **and K. N. AMIN** *

Abstract □ The stability constants for stannous fluoride complexes were determined by potentiostatic titration. The method involves incremental additions of fluoride wherein each addition is followed by titration with stannous such that there is no change in the electromotive force developed between the fluoride ion and the reference electrodes. The values obtained were $\beta_1 = 4 \times 10^3$, $\beta_2 = 1.1 \times 10^7$, and $\beta_3 = 1 \times 10^9$. The results of this work suggest that the potentiostatic method would be useful for determining stability constants in complexation systems involving an ion for which a specific ion electrode is available.

Keyphrases □ Stannous fluoride complexes—determination of stability constants, potentiostatic titration □ Titration, potentiostatic—determination of stability constants of stannous fluoride □ Stability constants—stannous fluoride complexes, potentiostatic titration

Stannous fluoride is well recognized as a topically applied anticaries agent. Past work (1–3) has demonstrated that the various products formed from the reaction of stannous fluoride and hydroxyapatite are $Sn_3F_3PO_4$, $Sn_4(PO_4)_2(OH)_2 \cdot H_2O$, $SnHPO_4$, and CaF_2, depending on the experimental conditions. A thorough description of the solution chemistry of stannous fluoride is needed for an understanding of the reactions and for the construction of stability fields for this system.

DISCUSSION

Stannous ion forms three soluble complexes with fluoride in aqueous solution. The stoichiometry of these complexes is SnF^+, SnF_2, and SnF_3^-. Their stability constants have been determined by potentiometric titration (4) and by polarography (5), but there are wide discrepancies in the reported values.

Kankare (6) recently reported a novel potentiostatic technique for determining dissociation constants of weak acids (or bases). Briefly, this method involves the titration of incremental increases of the weak acid with a strong base such that the electromotive force of the glass–reference electrode system remains constant. This procedure permits certain mathematical simplifications in data analysis and circumvents problems associated with the liquid junction potential and non-Nernstian electrode response. The potentiostatic technique could be useful for studying equilibria in systems containing an ion for which there exists a specific ion electrode.

The purposes of this study were: *(a)* to provide a set of stability constants for the stannous fluoride complexes to help clarify the discrepancy in reported values, and *(b)* to demonstrate the applicability of the potentiostatic technique to the study of a metal-ion complex system.

THEORETICAL

In an aqueous solution of pH 4 or below containing stannous and fluoride ions, the equilibria shown in Scheme I prevail.

$$Sn^{+2} + iF^- \rightleftharpoons SnF_i^{(2-i)} \qquad i = 1,2,3$$

Scheme I

The corresponding complexation stability constants are:

$$\beta_i = \frac{[SnF_i^{(2-i)}]}{[Sn^{+2}][F^-]^i} \qquad \text{(Eq. 1)}$$

There are two additional equilibria involving fluoride ions (Scheme II).

$$HF \rightleftharpoons H^+ + F^- \qquad K_{HF} = \frac{[H^+][F^-]}{[HF]}$$

$$HF_2^- \rightleftharpoons HF + F^- \qquad K_{HF_2} = \frac{[HF][F^-]}{[HF_2^-]}$$

Scheme II

Let the total amount (in moles) of stannous be s, the total amount of the fluoride be f, and the volume be V. Then the mass balance equations are as follows:

$$\frac{f}{V} = \sum_{i=1}^{3} i\beta_i[Sn^{+2}][F^-]^i + [F^-] + [HF] + 2[HF_2^-] \qquad \text{(Eq. 2)}$$

$$\frac{s}{V} = \sum_{i=1}^{3} \beta_i[Sn^{+2}][F^-]^i + [Sn^{+2}] \qquad \text{(Eq. 3)}$$

Rearranging Eq. 2 and dividing by Eq. 3 give:

$$Z = \frac{f - V\left[[F^-] + \frac{[F^-][H^+]}{K_{HF}} + \frac{2[F^-]^2[H^+]}{K_{HF}K_{HF_2}}\right]}{s} = \frac{\sum_{i=1}^{3} i\beta_i[F^-]^i}{1 + \sum_{i=1}^{3} \beta_i[F^-]^i} \qquad \text{(Eq. 4)}$$

where z may be considered the degree of complexation of fluoride with stannous.

Rearrangement of Eq. 4 gives:

$$f = sZ + V\left[[F^-] + \frac{[H^+][F^-]}{K_{HF}} + \frac{2[H^+][F^-]^2}{K_{HF}K_{HF_2}}\right] \qquad \text{(Eq. 5)}$$

At constant fluoride-ion and hydrogen-ion concentrations, the unique condition for a potentiostatic titration, Eq. 5 predicts a linear relationship between f and s. A modification of this expression is necessary, however, due to the increase in V during the titration. At any time during the titration:

$$V = V_0 + \frac{s}{C_s} + \frac{f}{C_f} \qquad \text{(Eq. 6)}$$

where V_0 is the initial volume, and C_s and C_f are the concentrations of the stannous and fluoride titrant solutions, respectively. By substituting Eq. 6 into Eq. 5 and letting:

$$X = [F^-] + \frac{[F^-][H^+]}{K_{HF}} + \frac{2[F^-]^2[H^+]}{K_{HF}K_{HF_2}} \qquad \text{(Eq. 7)}$$

the following equation can be obtained:

$$f = s\frac{Z + X/C_s}{1 - (X/C_f)} + \frac{V_0X}{1 - (X/C_f)} \qquad \text{(Eq. 8)}$$

Table I—Potentiostatic Data

Experiment	pH	Fluoride, moles × 10^5				Stannous, moles × 10^5			m	r^a
		f_0	f_1	f_2	f_3	s_1	s_2	s_3		
1	3.22	1.0	11.04	5.0	8.97	12.103	6.0611	10.9	0.8261	0.99999
2	3.24	1.75	10.0	5.21	—	9.0976	4.3604	—	1.1255	0.99980
3	3.26	2.5	10.01	5.01	7.41	6.98	3.58	5.405	1.4058	0.99993
4	3.30	4.0	10.0	5.0	6.07	6.275	2.991	3.624	1.6335	0.99990
5	3.38	6.0	10.0	5.13	3.89	5.411	2.818	2.006	1.8527	0.99996
6	3.46	8.0	10.0	7.2	—	4.73	3.50	—	2.0912	0.99997
7	3.53	10.0	10.0	5.0	—	4.734	2.266	—	2.1382	0.99994
8	3.62	14.0	6.13	—	—	2.723	—	—	2.251	—

[a] Correlation coefficient.

Table II—Stability Constants for Stannous Fluoride Complexes at 25°

Sources	Methods	Ionic Strength	β_1	β_2	β_3
Hall–Slater (4)	Potentiometric	0.85[a]	1.80×10^6	5.79×10^8	1.77×10^9
Bond–Taylor (5)	Polarographic	1.00[a]	1.0×10^4	7.0×10^6	2.70×10^9
This work	Potentiostatic	0.10	4.0×10^3	1.1×10^7	1.0×10^9

[a] Sodium perchlorate.

which is of the linear form:

$$f = sm + b \qquad \text{(Eq. 9)}$$

Plotting fluoride consumption *versus* stannous consumption for the potentiostatic titration directly gives m = slope and b = intercept, from which X and Z can be easily calculated. Since pH can be obtained experimentally and K_{HF} and K_{HF_2} are reported constants, fluoride concentration, $[F^-]$, can be calculated from X by Eq. 7. Sets of Z and $[F^-]$ are thus obtained from potentiostatic titrations and are used to calculate the stability constants through a rearrangement of Eq. 4:

$$Z = \sum_{i=1}^{3} (i - Z)[F^-]^i \beta_i \qquad \text{(Eq. 10)}$$

EXPERIMENTAL

Materials and Apparatus—The source of fluoride was a standard solution[1] containing 0.1 ± 0.0005 mole of sodium fluoride/liter. Stannous sulfate was prepared by the method described by Donaldson and Moser (7). The remaining chemicals were reagent grade and were used without further purification except potassium dichromate, which was recrystallized twice from water. All solutions were prepared using boiled, double-distilled water stored under a nitrogen atmosphere.

A potentiometric titrator[2] was used as a null-point detector for the potentiostatic titration, as a pH meter, and as a potentiometer for the stannous analysis. For these applications a fluoride-ion electrode[3], a glass electrode[4], and a platinum electrode[5], respectively, were used in conjunction with a silver–silver chloride low leak reference electrode[6]. The titration vessel consisted of a 50-ml capacity plastic beaker[7], which was maintained at 25° with a constant-temperature water bath[8].

The stannous sulfate solution was delivered with a micrometer syringe[9] or an ultraprecision micrometer buret[10]. An automatic buret[11] was used for dispensing the fluoride and potassium dichromate solutions and was capable of delivering 1-μl volumes. The micrometer buret and syringe volumes could be read with an accuracy of 0.1 μl. A Teflon-coated stirring bar and a magnetic stirrer[12] were used to agitate the solution.

Method—A 25-ml volume of the titration medium, consisting of 0.03333 M sodium sulfate and 0.005% concentrated sulfuric acid, was pipetted into the titration vessel. A continuous flow of nitrogen was maintained over the solution. A small, accurately known amount of fluoride, f_0, was added and the potential was allowed to stabilize. The end-point for the titration was set at this potential on the titrator.

Aliquots of the fluoride solution were then added corresponding to amounts f_1, f_2, and f_3. Each addition of fluoride was followed by a titration back to the adjusted end-point with corresponding amounts of stannous s_1, s_2, and s_3. The stannous sulfate solution was freshly prepared for each experiment by dissolving 400 mg in 2 ml of water and filtering through a membrane[13] filter of 0.22-μm pore size. After each experiment the titration mixture was subjected to stannous analysis by the method of Collins and Nebergall (8) to ensure that the amount of stannous present would be accurately known.

The pH was monitored by means of a duplicate experiment. It was determined after the initial fluoride addition and at the completion of the titration. There was no noticeable or measurable change in pH.

RESULTS

The data obtained from the potentiostatic titrations (Table I) were processed by performing a least-squares calculation on the cumulative additions of fluoride and stannous. The linearity predicted by theory (Eq. 8) is quite satisfactory as displayed by the correlation coefficients. The slopes and intercepts (f_0) shown were used to determine sets of Z and $[F^-]$ by means of Eqs. 7–9 and using $K_{HF_2} = 2.59 \times 10^{-1}$ (9) and $K_{HF} = 9.312 \times 10^{-4}$ (10).

To determine the concentration stability constants from the sets of Z and $[F^-]$, it was first assumed for experiments 1–3 that $[SnF_3^-]$ was sufficiently small that it could be ignored. Values for β_1 and β_2 were then calculated by solving simultaneous equations (i = 1, 2 in Eq. 10). This value of β_1 was used in a modified Leden's method (11) to get a better estimate for β_2. The procedure involved plotting $\log [Z - (1 - Z)\beta_1]/(2 - Z)$ *versus* $\log [F^-]$ for the data of experiments 1–4. If the $[SnF_3^-]$ is sufficiently low, this plot should have a slope of two. Such was the case so that the intercept was used to obtain the better estimate of β_2. By using β_1 and β_2, the method of Leden was used to determine β_3. The stability constants

[1] Orion Catalog No. 94-09-06, Orion Research Inc., Cambridge, Mass.
[2] Radiometer TTTlc, Radiometer A/S, Copenhagen, Denmark.
[3] Model 94-09A, Orion Research Inc., Cambridge, Mass.
[4] pH electrode No. 39301, Beckman Instruments, Inc., Fullerton, Calif.
[5] Beckman Instruments, Inc., Fullerton, Calif.
[6] Perma probe solid-state reference electrode No. 39406, Beckman Instruments, Inc., Fullerton, Calif.
[7] TPX, No. 1203, Nalgene Labware, Rochester, N.Y.
[8] Model FK, Haake Instruments, Inc., Rochelle Park, N.J.
[9] Agla, Burroughs Wellcome & Co., London, England.
[10] Catalog No. 7876, Roger Gilmont Instruments, Inc., Great Neck, N.Y.
[11] ABU 1B, B150, Radiometer A/S, Copenhagen, Denmark.
[12] Sargent-Welch Scientific Co., Skokie, Ill.
[13] GSWP 013, Millipore Corp., Bedford, Mass.

Table III—Comparison of Calculated and Experimental Values of Z

Experimental	Calculated
0.822	0.826
1.117	1.213
1.391	1.426
1.607	1.684
1.808	1.872
2.025	1.988
2.055	2.069
2.132	2.184

obtained along with those reported in the literature are given in Table II.

It can be seen that there is wide discrepancy in the reported stability constant for SnF^+. The reported value (4) was determined by regression analysis. In a review article, Bond (12) cautioned against applying statistical analysis of complexation data without appropriate weighing of the data relative to the known chemistry of the system. Using Leden's method, Bond calculated the following stability constants from the data of Hall and Slater (4): $\beta_1 = 3 \times 10^4$, $\beta_2 = 1.5 \times 10^8$, and $\beta_3 = 2 \times 10^{10}$. Thus, the new β_1 is in closer agreement with the value reported by Bond and Taylor (5), but they expressed uncertainty in their reported value because of curvature in their Leden plot.

The agreement in reported values of β_3 is satisfactory considering the differences in experimental methods, computational analyses, and ionic strengths. The β_2 values reported range over two orders of magnitude, with the one determined in this work occupying an intermediate position. If the values are transformed to association constants for the sequential reaction shown in Scheme III, then the respective constants for the current work would be 4×10^3, 2.75×10^3, and 9.09×10^1.

$$SnF_{i-1}^{(3-i)} + F^- \rightleftharpoons SnF_i^{(2-i)} \qquad i = 1, 2, 3$$

Scheme III

The respective constants of Bond and Taylor (5) would be 1.2×10^4, 4×10^2, and 6.02×10^2. It can be seen that the latter values are not sequentially in order, which is physically unrealistic.

To assess whether stability constants are satisfactory, it has been suggested that a point-by-point comparison be made between experimental and calculated values (12). Such a comparison is shown in Table III. The stability constants reported in this work are quite satisfactory by this criterion and give a much better fit than if other reported sets of β are used.

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Dehydration of Tetracycline

K. D. SCHLECHT *[x] **and C. W. FRANK** [‡]

Abstract □ The dehydration of tetracycline at the C-5a–C-6 position as a function of acidity was investigated at various temperatures. The rate was first order with respect to tetracycline and with respect to $[H^+]$. Rate constants and an activation energy are reported. Tetracycline was unstable in dilute acid.

Keyphrases □ Tetracycline—dehydration kinetics, effect of acidity at various temperatures □ Dehydration kinetics—tetracycline, effects of acidity at various temperatures □ Stability—tetracycline, dehydration, effects of acidity at various temperatures

Tetracyclines have been subjected to numerous reactions to aid in elucidating their structures; one reaction involves dehydration at the C-5a–C-6 position in the presence of warm mineral acids (1–4). When tetracycline [4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-2-naphthacenecarboxamide] (I) is dehydrated, anhydrotetracycline [4-(dimethylamino)-1,4,4a,5,12,12a-hexahydro-3,10,11,12a-tetrahydroxy-6-methyl-1,12-dioxo-2-naphthacenecarboxamide] (II) is formed; II has aromatic character in both the C- and D-rings.

The benzylic C-6 position of I has a tertiary hydroxyl, which is *trans* to the adjacent C-5aH, thereby making the hydroxyl very acid labile and anhydro formation an easy process. Recently, the ease of anhydro formation has been used in the analysis of tetracyclines. Generally, I and its main degradation products [II, 4-epitetracycline (III), and 4-epianhydrotetracycline (IV)] can be separated by TLC, treat-

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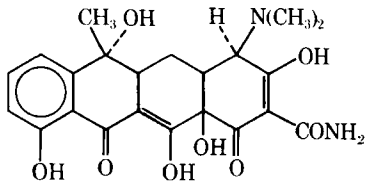

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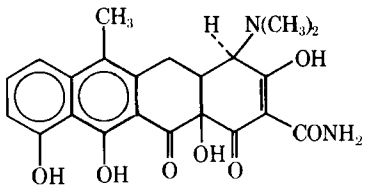

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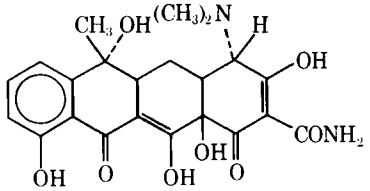

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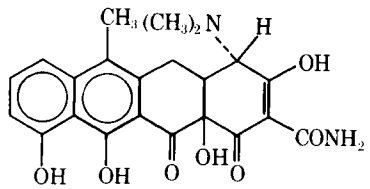

IV

ed with hydrochloric acid to ensure that each constituent exists as the corresponding anhydro compound, and determined spectrophotometrically (5–8).

Reports of the time and conditions required for quantitative conversion to the anhydro compound seem to vary: 2 *N* HCl and heating at 100° for 5 min (5), 5 *N* HCl (time and temperature were not mentioned) (8), and 2 *N* HCl and heating "over hot water" for 5 min (6).

In 1968, Clive (9) compiled some very approximate half-lives (determined by changes in bioactivity) of various tetracyclines degrading to the anhydro species at various temperatures and acid strengths. Aside from the original reports of anhydro formation, little has been reported characterizing the reaction. Further characterization of anhydro formation would be useful for quantitative conversion and pharmaceutical studies since IV is generally believed to be responsible for a "reversible Fanconi-type syndrome," which has been observed in patients who have taken degraded tetracyclines, resulting in lengthy syndrome treatment (sometimes in excess of 1 year) (6, 10).

EXPERIMENTAL

I-HCl was obtained from a commercial source. Compound II was prepared by a method similar to that reported by McCormick *et al.* (11); 4.0 g of I-HCl was dissolved in 2-propanol–methanol–hydrochloric acid (4:1:2), heated to 70° for at least 0.5 hr, cooled, and filtered. Recrystallization from methanol–hydrochloric acid (30:1) yielded yellow needle-like crystals, which were dried under vacuum for 2 days, yielding 1.91 g of crystals, mp 219–222° dec. The product was found to be pure by TLC. The TLC method of analysis used acid-washed Kieselguhr MN slurried with 5% edetic acid (EDTA) at pH 7.5, as reported by Fernandez *et al.* (6).

Spectra and absorbances of kinetic reactions were obtained on a

Table I—Observed Rate Constants for II Formation at Various [H$^+$] Values and Temperatures and 1.00 *M* Ionic Strength[a]

Temperature	[H$^+$]	$10^4\ k_{obs}$, sec^{-1}
30°	0.250	0.212
	0.375	0.322
	0.500	0.452
	0.625	0.565
	0.750	0.675
	0.825	0.790
	1.00	0.903
35°	0.125	0.214
	0.250	0.430
	0.375	0.622
	0.500	0.862
	0.625	1.07
	0.750	1.30
	0.875	1.52
	1.00	1.72
40°	0.125	0.418
	0.375	1.20
	0.500	1.61
	0.875	2.76
	1.00	3.14
45°	0.125	0.862
	0.250	1.60
	0.375	2.40
	0.500	3.21
	0.625	4.11
	0.750	4.77
	0.875	5.69
	1.00	6.84
50°	0.125	1.57
	0.375	4.65
	0.500	6.38
	0.625	7.61
	0.750	9.43
	0.875	10.8
	1.00	12.0

[a] Monitored at 432 nm, $[I]_0 = 1 \times 10^{-4}$ *M*.

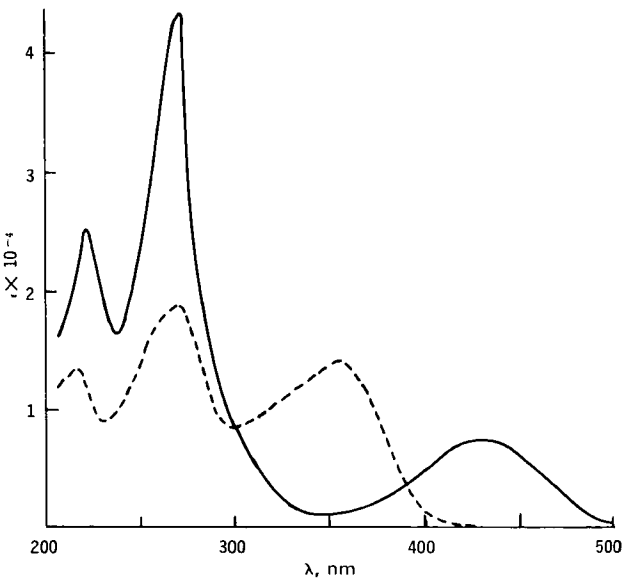

Figure 1—*UV–visible spectra of tetracycline* (- - -) *and anhydrotetracycline* (——) *(both in 0.03* N *HCl).*

Table II—k Values for II at Various Temperatures

Temperature	10^4 k, M^{-1} sec^{-1}, $\pm SD$ (Estimated)
30°	0.924 ± 0.008
35°	1.74 ± 0.014
37°	2.28 (calculated value)
40°	3.12 ± 0.02
45°	6.43 ± 0.09
50°	12.0 ± 0.25

spectrophotometer[1] equipped with a thermostated cell holder and using matched silica cells (10.0 mm). Aliquots of 1.25 M HCl were used to acidify the tetracycline solutions, and 1.00 M ionic strength was maintained using aliquots of 1.25 M KCl.

RESULTS

The UV–visible spectra of I and II in 0.03 N HCl are shown in Fig. 1. It is apparent that the kinetics of anhydro formation may be followed at either 360 or 432 nm. The agreement of the observed rate constants, k_{obs}, obtained at both wavelengths was well within experimental error. Apparent first-order kinetics fit the data with respect to I over more than 80% of the reaction:

$$\text{rate} = k_{obs}[\text{I}] \qquad \text{(Eq. 1)}$$

for a specific $[H^+]$.

When the initial concentration of I was increased by a factor of 10, the fit was still excellent and agreed, within experimental error, with the data at the lower concentration. The k_{obs} values obtained from 30 to 50° at various $[H^+]$ values are given in Table I. The k_{obs} of Eq. 1 contains a dependence on $[H^+]$. A plot of k_{obs} *versus* $[H^+]$ yielded a straight line and zero intercept at each temperature studied. This finding is consistent with the following overall rate law:

$$\text{rate} = k[\text{I}][H^+] \qquad \text{(Eq. 2)}$$

Table II lists the overall rate constants, k, obtained between 30 and 50°, as well as the estimated rate constant at 37°.

An Arrhenius plot of the data yielded an activation energy, Ea, for anhydro formation of 25.1 kcal/mole (SD 0.6).

DISCUSSION

The reported data at constant ionic strength suggest that the reaction is fairly simple: attack of H^+ on the tetracycline species in solution. Under the conditions reported here, the tetracycline is protonated at the C-4 nitrogen. The dehydrated reaction mixtures were analyzed for evidence of epimerization at the C-4 position, which is a significant degradation reaction in less acidic solutions (12). TLC was used, and no epimerization products were detected.

The data also suggest that I is not as stable in slightly acidic solutions as might be desired. Calculations with the results reported here indicate that 10% of a I solution in 0.1 M HCl (1 M ionic strength) would degrade to II in a little more than 3 hr.

Extrapolation of the results to stronger hydrochloric acid concentrations indicates that conversion to II is not as rapid as was previously assumed for quantitative determinations of I (7, 8, 10). The use of both 2 N HCl at elevated temperature and 5 N HCl near room temperature has been reported. The data presented here suggest that heating is required to attain quantitative conversions to II even in strong acid solutions.

[1] Coleman 124.

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x To whom inquiries should be directed.

New Compounds: Addition Products of Heterocyclic Chalcones

GIANLUIGI SOLDATI

Abstract □ Michael-type adducts, 1,3-heterocyclic-3-mercaptopropan-1-ones, were prepared by the base-catalyzed reaction of heterocyclic chalcones with thiols. These new compounds were found to be active as fungicides.

Keyphrases □ Chalcones, heterocyclic—addition products, 1,3-heterocyclic-3-mercaptopropan-1-ones synthesized as potential fungicides □ 1,3-Heterocyclic-3-mercaptopropan-1-ones—synthesized as potential fungicides □ Fungicides, potential—synthesis of Michael-type adducts, 1,3-heterocyclic-3-mercaptopropan-1-ones

Some addition products of heterocyclic chalcones with thiols were prepared in view of potential biological properties. Bronchodilatory (1), coronary spasmolytic (2–5), bacteriostatic (6), tuberculostatic (7), staphylostatic (8), and anthelmintic (9) activities have been reported for heterocyclic chalcones; recently, these compounds also showed an inhibitory effect on the suprarenal gland cortex of the rat (10).

The intermediate chalcones (1,3-heterocyclic-2-propen-1-ones) were prepared by the conventional (11) aldehyde–ketone base-catalyzed condensation. In turn, these chalcones were used for the Michael-type adducts, 1,3-heterocyclic-3-mercaptopropan-1-ones (Table I). A base-catalyzed ethanolic solution refluxed for 2–3 hr gave good yields of the adducts.

Several of the compounds showed fungicidal properties.

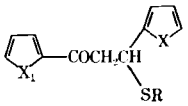

Table I—1,3-Heterocyclic-3-mercaptopropan-1-ones

Compound	X	X_1	R	Melting Point	Formula	Analysis, %	
						Calc.	Found
I	O	O	Phenyl	63–64°	$C_{17}H_{14}O_3S$	C 68.45	68.45
						H 4.73	4.71
						S 10.73	11.05
II	O	O	*p*-Tolyl	95–96°	$C_{18}H_{16}O_3S$	C 69.22	69.40
						H 5.16	5.26
						S —	—
III	O	S	*p*-Chlorophenyl	77–78°	$C_{17}H_{13}ClO_2S_2$	C 58.56	58.91
						H 3.76	3.86
						S 18.35	18.64
IV	O	S	*p*-Tolyl	75–76°	$C_{18}H_{16}O_2S_2$	C 65.85	65.82
						H 4.91	5.12
						S —	—
V	O	S	*o*-Aminophenyl (12)	74°	—		
VI	S	S	3,4-Dichlorophenyl	96–97°	$C_{17}H_{12}Cl_2OS_3$	C 51.31	51.02
						H 3.24	3.62
						S 24.17	24.23
VII	S	S	*p*-Bromophenyl	91–92°	$C_{17}H_{13}BrOS_3$	C 49.87	50.07
						H 3.20	3.16
						S 23.51	23.69
VIII	O	S	*p*-Bromophenyl	76–78°	$C_{17}H_{13}BrO_2S_2$	C 51.92	52.25
						H 3.33	3.23
						S 16.27	16.54
IX	S	O	*p*-Chlorobenzyl	61–62°	$C_{18}H_{15}ClO_2S_2$	C 59.59	59.42
						H 4.17	4.14
						S —	—
X	O	O	3,4-Dichlorophenyl	73–74°	$C_{17}H_{12}Cl_2O_3S$	C 55.78	55.44
						H 3.53	3.42
						S 8.74	8.93
XI	O	O	*p*-Bromophenyl	90–91°	$C_{17}H_{13}BrO_3S$	C 54.12	53.81
						H 3.47	3.42
						S 8.48	8.61
XII	O	O	*p*-Methoxyphenyl	66–67°	$C_{18}H_{16}O_4S$	C 65.85	66.18
						H 4.91	4.92
						S 9.75	10.00
XIII	S	O	1-Octyl	44–45°	$C_{19}H_{26}O_2S_2$	C 65.10	65.13
						H 7.48	7.42
						S —	—
XIV	S	O	1-Dodecyl	55–57°	$C_{23}H_{34}O_2S_2$	C 67.96	67.94
						H 8.43	8.52
						S —	—
XV	S	O	*p*-Bromophenyl	93–95°	$C_{17}H_{13}BrO_2S$	C 51.92	51.95
						H 3.33	3.23
						S —	—

EXPERIMENTAL

All adducts listed in Table I were prepared by the following procedure as exemplified by 1,3-di-(2-furyl)-3-(*p*-bromomercapto)-propan-1-one.

p-Bromothiophenol (5.0 g, 0.0264 mole) was added to 5.0 g (0.0265 mole) of 1,3-di-(2-furyl)-2-propen-1-one in 100 ml of ethanol, followed immediately by 5 drops of the catalyst triethylamine. After refluxing for 2 hr on a steam bath, the solution was cooled. The precipitate that formed was collected and recrystallized from ethanol–water to give a white crystalline material, mp 90–91°, in almost quantitative yield.

Anal.—Calc. for $C_{17}H_{13}BrO_3S$: C, 54.12; H, 3.47; Br, 21.18; S, 8.48. Found: C, 53.81; H, 3.42; Br, 21.02; S, 8.61.

Other intermediates used were 1-(2-furyl)-3-(2-thienyl)-2-propen-1-one, 1-(2-thienyl)-3-(2-furyl)-2-propen-1-one, and 1,3-di-(2-thienyl)-2-propen-1-one.

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COMMUNICATIONS

Influence of Peroxide Impurities in Polyethylene Glycols on Drug Stability

Keyphrases □ Polyethylene glycols—peroxide impurity, corticosteroid stability □ Antioxidants—butylated hydroxytoluene, propyl gallate □ Colorimetric assay—peroxide concentrations

To the Editor:

The poor stability of an experimental topical corticosteroid formulated in polyethylene glycol 300 was found to be due to the high concentration of peroxides in the vehicle. Removal of the impurity from the vehicle led to an increase in stability of the steroid at elevated temperatures. Boon and Mace (1) reported that degradation of tripelennamine hydrochloride in polyethylene glycol 300 was dependent on the concentration of ethylene oxide in polyethylene glycol; when the concentration of ethylene oxide exceeded 0.1%, there was measurable loss of the active constituent.

Higher molecular weight polyethylene glycols and polyethylene glycol esters, *i.e.*, polyethylene glycol 400, polyethylene glycol 1500, and polyethylene glycol 6000 distearate, used to solubilize the steroid before its incorporation into an ointment formulation (petrolatum base), all contained peroxides as impurities and the steroid showed poor stability. Except for one sample of polyethylene glycol 1500, samples of polyethylene glycols from different manufacturers all contained peroxides. In one instance, a small quantity of hydrogen peroxide had been added by the manufacturer to maintain a water-clear product.

Although the identities of the peroxides present as impurities were not determined, they are believed to consist of various organic peroxides rather than hydrogen peroxide *per se*. The concentrations of peroxide in polyethylene glycols of different molecular weights and polyethylene glycol 6000 distearate are reported in Table I. In the colorimetric assay used, the glycol sample was added to an acidified potassium iodide solution and the iodine liberated was titrated against standard thiosulfate solution.

The level of peroxide in polyethylene glycols increased with aging. Studies in these laboratories showed that the presence of selected antioxidants and water (5–10%) in the vehicle helped to decrease the concentration of peroxides. Thus, of eight antioxidants tested, butylated hydroxytoluene and propyl gallate were the most successful in this respect, and pretreatment of the vehicle with 0.005–0.05% of either agent was effective. The decomposition of peroxides under the influence of water or antioxidants was slow at room temperature but was accelerated by heating. Both the concentration of antioxidant and the duration of heating (60–80°) required for the removal of peroxide were dependent on the initial con-

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Table I—Peroxide Concentrations in Polyethylene Glycols

Manufacturer	Polyethylene Glycol 300[a]	Polyethylene Glycol 400[a]	Polyethylene Glycol 1500[b]	Polyethylene Glycol 6000 Distearate[b]
I	1.4	3.24	<0.01	1.97
II	4.86	3.95	4.26	N.T.[c]
III	9.3	5.7	N.T.	1.92[d]

[a] Microequivalents of thiosulfate per milliliter of glycol. [b] Microequivalents of thiosulfate per gram of glycol. [c] N.T. = not tested. [d] Additional vendor.

centration of peroxide. The presence of water in the formulation helped prevent any further production of peroxide.

Some of these observations were substantiated by McKenzie (2), who studied peroxide formation and decomposition in triethylene glycol. Azaz *et al.* (3) employed antioxidants to stabilize benzocaine hydrochloride in cetomacrogol solutions containing peroxide impurities.

The reaction of trace metals in the polyethylene glycols with propyl gallate to form colored reactants was prevented by the inclusion of a small quantity of a chelating agent, *e.g.*, 20 ppm of ethylenediaminetetraacetic acid. Since all polyethylene glycols used in these studies contained less than 0.01% ethylene oxide, it is suggested that the reported (1) instability of tripelennamine hydrochloride in polyethylene glycol 300 may have been due not only to the presence of ethylene oxide in the formulation but also the presence of peroxides.

We recommend the use of only the highest quality polyethylene glycols in formulations, a determination of the stability of the active constituent in glycols under elevated temperature conditions (to assess the effect of pretreating the vehicle with antioxidants at elevated temperatures), and, finally, the incorporation of a small percent of water in the formulation.

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Constituents of *Cannabis sativa* L. IX: Stability of Synthetic and Naturally Occurring Cannabinoids in Chloroform

Keyphrases □ *Cannabis sativa* L.—stability of synthetic and naturally occurring cannabinoids in chloroform □ Cannabinoids—stability in chloroform □ Marijuana—stability of synthetic and naturally occurring cannabinoids in chloroform □ Stability—synthetic and naturally occurring cannabinoids in chloroform

To the Editor:

A report from these laboratories demonstrated that chloroform was a more efficient solvent for extracting cannabinoids from *Cannabis sativa* L. than benzene, pentane, hexane, petroleum ether, ethanol, acetone, and ether. Moreover, cannabinoids[1] extracted with chloroform were stable at ambient temperature for 144 hr (1).

Recently, Parker *et al.* (2) reported that synthetic cannabidiol was unstable in spectrograde chloroform over an 8-day period. The authors stated that: "caution should be exercised in the use of chloroform as a solvent for prolonged extraction and storage of cannabidiol." Since members of this research group (3–6) and others (7) have employed chloroform as an extracting solvent and since a working group, sponsored by the United Nations[2], on the chemistry of *Cannabis* and its components recently recommended that the procedure developed in these laboratories be used worldwide, it seemed imperative that additional data be presented in support of chloroform as the solvent of choice for extracting cannabinoids from *Cannabis*.

The basic procedure utilized in these laboratories is as follows. Samples of *Cannabis* are extracted at ambient temperature with nanograde chloroform[3] for 1 hr[4]. After that time, the chloroform is removed *in vacuo* and an ethanolic solution containing a known amount of the internal standard, androst-4-ene-3,17-dione, is added. Therefore, by allowing 15 min for workup, each sample is exposed to chloroform for a maximum of 75 min. Thus, if synthetic and naturally occurring cannabinoids are stable in chloroform for 75 min, chloroform, as previously recommended (1, 7), would be the solvent of choice for extraction of naturally occurring cannabinoids found in crude drug preparations from *C. sativa* L.

We wish to report results of a 3-month stability study using chloroform as the solvent for the following: (*a*) synthetic cannabidiol, (−)-Δ^8- and Δ^9-*trans*-tetrahydrocannabinols, $\Delta^{9,11}$-tetrahydrocannabinol (exocyclic), and cannabinol; (*b*) an extract of female Mexican *Cannabis* grown in Mississippi (coded CMEF-71, ME-A); and (*c*) a synthetic mix-

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[2] United Nations Document MNAR/9/1974.

[3] Mallinckrodt Chemical. One-gallon amber bottles are kept at ambient temperature, opened one at a time as needed, and used with minimum exposure to air, *etc.*

[4] For details, see Refs. 1 and 4–6. Androst-4-ene-3,17-dione was first used as an internal standard by Davis *et al.*, *Lloydia*, **33**, 453(1970).

Table I—Peroxide Concentrations in Polyethylene Glycols

Manufacturer	Polyethylene Glycol 300[a]	Polyethylene Glycol 400[a]	Polyethylene Glycol 1500[b]	Polyethylene Glycol 6000 Distearate[b]
I	1.4	3.24	<0.01	1.97
II	4.86	3.95	4.26	N.T.[c]
III	9.3	5.7	N.T.	1.92[d]

[a] Microequivalents of thiosulfate per milliliter of glycol. [b] Microequivalents of thiosulfate per gram of glycol. [c] N.T. = not tested. [d] Additional vendor.

centration of peroxide. The presence of water in the formulation helped prevent any further production of peroxide.

Some of these observations were substantiated by McKenzie (2), who studied peroxide formation and decomposition in triethylene glycol. Azaz *et al.* (3) employed antioxidants to stabilize benzocaine hydrochloride in cetomacrogol solutions containing peroxide impurities.

The reaction of trace metals in the polyethylene glycols with propyl gallate to form colored reactants was prevented by the inclusion of a small quantity of a chelating agent, *e.g.*, 20 ppm of ethylenediaminetetraacetic acid. Since all polyethylene glycols used in these studies contained less than 0.01% ethylene oxide, it is suggested that the reported (1) instability of tripelennamine hydrochloride in polyethylene glycol 300 may have been due not only to the presence of ethylene oxide in the formulation but also the presence of peroxides.

We recommend the use of only the highest quality polyethylene glycols in formulations, a determination of the stability of the active constituent in glycols under elevated temperature conditions (to assess the effect of pretreating the vehicle with antioxidants at elevated temperatures), and, finally, the incorporation of a small percent of water in the formulation.

(1) P. F. G. Boon and A. W. Mace, *J. Pharm. Pharmacol., Suppl.*, **20**, 32S(1968).

(2) D. A. McKenzie, "A Study of Peroxide Formation and Decomposition in Certain Glycol Systems," Technical Service Report, Union Carbide Corp., Tarrytown, N.Y., Feb. 1970.

(3) E. Azaz, M. Donbrow, and R. Hamburger, *Pharm. J.*, **211**, 15(1973).

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Constituents of *Cannabis sativa* L. IX: Stability of Synthetic and Naturally Occurring Cannabinoids in Chloroform

Keyphrases □ *Cannabis sativa* L.—stability of synthetic and naturally occurring cannabinoids in chloroform □ Cannabinoids—stability in chloroform □ Marijuana—stability of synthetic and naturally occurring cannabinoids in chloroform □ Stability—synthetic and naturally occurring cannabinoids in chloroform

To the Editor:

A report from these laboratories demonstrated that chloroform was a more efficient solvent for extracting cannabinoids from *Cannabis sativa* L. than benzene, pentane, hexane, petroleum ether, ethanol, acetone, and ether. Moreover, cannabinoids[1] extracted with chloroform were stable at ambient temperature for 144 hr (1).

Recently, Parker *et al.* (2) reported that synthetic cannabidiol was unstable in spectrograde chloroform over an 8-day period. The authors stated that: "caution should be exercised in the use of chloroform as a solvent for prolonged extraction and storage of cannabidiol." Since members of this research group (3–6) and others (7) have employed chloroform as an extracting solvent and since a working group, sponsored by the United Nations[2], on the chemistry of *Cannabis* and its components recently recommended that the procedure developed in these laboratories be used worldwide, it seemed imperative that additional data be presented in support of chloroform as the solvent of choice for extracting cannabinoids from *Cannabis*.

The basic procedure utilized in these laboratories is as follows. Samples of *Cannabis* are extracted at ambient temperature with nanograde chloroform[3] for 1 hr[4]. After that time, the chloroform is removed *in vacuo* and an ethanolic solution containing a known amount of the internal standard, androst-4-ene-3,17-dione, is added. Therefore, by allowing 15 min for workup, each sample is exposed to chloroform for a maximum of 75 min. Thus, if synthetic and naturally occurring cannabinoids are stable in chloroform for 75 min, chloroform, as previously recommended (1, 7), would be the solvent of choice for extraction of naturally occurring cannabinoids found in crude drug preparations from *C. sativa* L.

We wish to report results of a 3-month stability study using chloroform as the solvent for the following: (*a*) synthetic cannabidiol, (−)-Δ^8- and Δ^9-*trans*-tetrahydrocannabinols, $\Delta^{9,11}$-tetrahydrocannabinol (exocyclic), and cannabinol; (*b*) an extract of female Mexican *Cannabis* grown in Mississippi (coded CMEF-71, ME-A); and (*c*) a synthetic mix-

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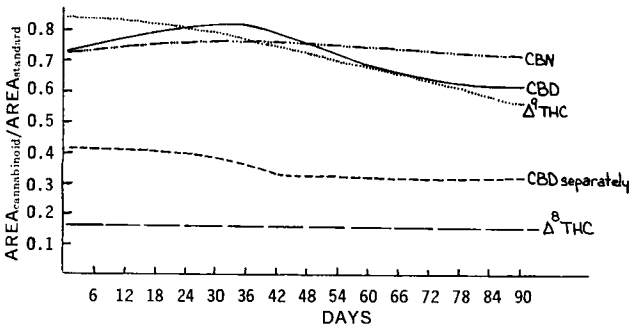

Figure 1—*Graph showing the stability of a mixture of the synthetic cannabinoids [cannabinol, (−)-Δ⁸- and (−)-Δ⁹-trans-tetrahydrocannabinols, and cannabidiol] stored in chloroform over 90 days. Cannabidiol stored separately in chloroform is also presented. The results reported are the area of the cannabinoid divided by the area of the internal standard* versus *the time in days. (THC = tetrahydrocannabinol, CBD = cannabidiol, and CBN = cannabinol.)*

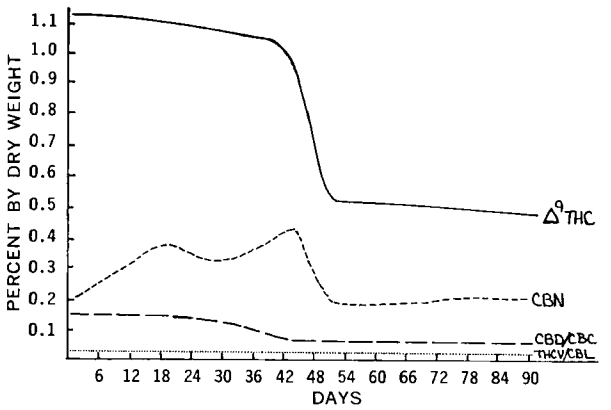

Figure 2—*Graph showing the concentrations of an extract of female Mexican* Cannabis *grown in Mississippi and stored in chloroform for 90 days. The concentrations (percent by dry weight) are plotted against the time in days. [THCV/CBL = a mixture of (−)-Δ⁹-trans-tetrahydrocannabidivarin and cannabicyclol, CBD/CBC = a mixture of cannabidiol and cannabichromene, CBN = cannabinol, and Δ⁹THC = tetrahydrocannabinol.]*

ture of cannabidiol, (−)-Δ⁸- and Δ⁹-*trans*-tetrahydrocannabinols, and cannabinol.

Each chloroform solution was maintained at ambient temperature for 21 days. During this time, each solution was exposed to natural and artificial light. Due to consistent solvent evaporation, each solution was subsequently stored at 6° for the remainder of the experiment. Each sample contained a known amount of the internal standard, and results are reported as the area of the cannabinoid peak divided by the area of the internal standard peak *versus* time in days.

The area under the curve was calculated originally by peak height times width at half-height and was replicated with a GLC–computer system as previously reported (8). GC conditions also have been reported (1, 3–6, 8).

Figure 1 depicts graphically the stability of synthetic cannabidiol as well as a synthetic mixture of cannabidiol, (−)-Δ⁸- and Δ⁹-*trans*-tetrahydrocannabinols, and cannabinol stored in chloroform. Data from these laboratories show that synthetic cannabidiol in chloroform is stable within experimental error for 21 days. The stability diminishes slightly from Day 21 to Day 41 and thereafter remains constant for a 90-day period. With the synthetic mixture, the peak of cannabidiol showed an increase from Day 1 to Day 34, followed by a gradual decline. The slight increase observed for cannabidiol in the synthetic mixture may have been due to an artifact. When synthetic cannabidiol, cannabinol, and (−)-Δ⁸- and Δ⁹-*trans*-tetrahydrocannabinols are mixed and subsequently subjected to GLC analyses, a peak not observed in chromatograms from their respective individual analyses is consistently observed.

The stability of cannabinol and (−)-Δ⁸-*trans*-tetrahydrocannabinol was constant within experimental error in the synthetic mixture over 90 days. However, some diminution in the area under the (−)-Δ⁹-*trans*-tetrahydrocannabinol peak was observed and may have been responsible for the very slight increase in the amount of cannabinol. However, this increase in cannabinol does not account for the total loss of (−)-Δ⁹-*trans*-tetrahydrocannabinol. These data support the Turner *et al.* (6) statement that (−)-Δ⁹-*trans*-tetrahydrocannabinol does not decompose quantitatively to cannabinol.

Figure 2 concerns an extract from female Mexican *C. sativa* grown in Mississippi (coded CMEF-71), which contains a mixture of cannabidiol and cannabichromene, and a mixture of (−)-Δ⁹-*trans*-tetrahydrocannabidivarin and cannabicyclol, as well as cannabinol and (−)-Δ⁹-tetrahydrocannabinol. In these laboratories, (−)-Δ⁸-*trans*-tetrahydrocannabinol was not observed in fresh plant samples when careful analytical parameters were used. (−)-Δ⁹-*trans*-Tetrahydrocannabinol showed a very slight decrease from Day 1 through Day 38 and then underwent a precipitous decrease from Day 44 to Day 50. From Day 50 to Day 90, it once again was relatively stable.

As shown in Fig. 2, cannabinol stability is erratic. There is no lucid interpretation of this behavior. Cannabinol increased from a concentration of 0.2% at Day 1 to a concentration of approximately 0.35% at Day 19. The cannabinoid then began a decline to Day 26, where it again ascended to a high of approximately 0.4% concentration on Day 44. From Day 44 to Day 50, cannabinol decreased once again to approximately 0.2% and then remained fairly constant from that time on.

Turner *et al.* (6) reported a similar decomposition pattern for (−)-Δ⁹-*trans*-tetrahydrocannabinol in dry female Mexican *Cannabis* after storage at 37° for 60 weeks. It was postulated that a free radical moiety might be responsible for the precipitous decomposition.

The combined peak for cannabichromene and cannabidiol was stable from Day 1 through Day 19. Slight decomposition then began until Day 44, after which time it remained stable. This decrease was probably due to the decomposition of cannabichromene. Mass spectral data and data from the 6% OV-1 column have shown that in this particular variant the area under the peak classically labeled cannabidiol is approximately 85% cannabichromene and only 15%

cannabidiol. Cannabichromene is a relatively unstable molecule and decomposes quite readily when exposed to heat, light, and acidic and basic conditions (9). Combined (−)-Δ^9-*trans*-tetrahydrocannabidivarin and cannabicyclol was constant until Day 20. It then declined very little and very slowly. Cannabigerol, although not shown in Fig. 2 due to its small concentration, was very stable.

The data in Fig. 2 cannot be easily explained. Certain phenomena have been reported (9) regarding the ambiguity of cannabichromene decomposing to cannabicyclol. Other than a solvent effect and a "biophysical" parameter not yet defined, this group can offer no explanation of most intrinsic data in Fig. 2.

Individual synthetic cannabinoids and a mixture of synthetic cannabinoids are stable in chloroform and do not decompose in these laboratories, as Parker *et al.* (2) reported for cannabidiol. The instability reported by Parker could be due to impure solvents. We have observed unusual stability problems with certain batches of chloroform. These problems were circumvented by using nanograde and certain brands of spectrograde chloroform[5]. Thus, our data indicate cannabinoids, synthetic or naturally occurring, to be stable in chloroform within experimental error for much longer time periods than are required in a routine analysis. We do, however, agree with Parker *et al.* that cannabinoids should not be stored in chloroform for a prolonged period. Therefore, from published data (1) and data contained within, chloroform remains the solvent of choice for extracting cannabinoids from *Cannabis* preparations.

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(4) P. S. Fetterman and C. E. Turner, *ibid.*, **61**, 1476(1972).

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(7) P. Lerner, *Bull. Narcotics*, **21**, 39(1969).

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Disposition of *N,N*-Bis(phenylcarbamoylmethyl)dimethyl Ammonium Chloride in the Rat: An Interesting Example of First-Pass Metabolism

Keyphrases □ *N,N*-Bis(phenylcarbamoylmethyl)dimethyl ammonium chloride—disposition in rat, first-pass metabolism □ Metabolism, first pass—*N,N*-bis(phenylcarbamoylmethyl)dimethyl ammonium chloride in rat □ Lidocaine derivatives—disposition of *N,N*-bis(phenylcarbamoylmethyl)dimethyl ammonium chloride in rat, first-pass metabolism

To the Editor:

It has recently been demonstrated that the disposition of *N,N*-bis(phenylcarbamoylmethyl)dimethyl ammonium chloride (I), a quaternized lidocaine derivative with antiarrhythmic activity, is dependent on the route of administration in the rat (1). The present report is concerned with a quantitative estimate of the degree to which I is subjected to a first-pass effect in the rat and with a quantitative comparison of the predicted *versus* the observed disposition of the drug after intraperitoneal administration.

After an intravenous (tail vein) dose of 2.5 mg of ^{3}H-I to Sprague–Dawley rats, 35% of the administered radioactivity was ultimately excreted in the urine in the form of I and a carboxylic acid metabolite (II). Radiochromatogram scans of urine samples subjected to high voltage electrophoresis, in addition to reverse isotope dilution data, indicated that about 70% of the total radioactivity in the urine could be accounted for by I.

With intravenous administration of an equal dose to rats with ligated bile ducts, 80% of the radioactive dose was found in the urine. Since the observed increase in urinary excretion of tritium in bile duct-ligated rats may be attributed to "spill over" into the urine of drug and/or metabolite normally excreted in bile, it was estimated that in normal rats 45% of the administered radioactivity is excreted in the bile. Reverse isotope dilution of bile samples revealed less than 5% intact drug (I), while radiochromatogram scans of electrophoregrams of bile samples indicated that a single compound (II) accounted for about 80% of the total radioactivity in the bile. The data are reasonably consistent with Scheme I, where F_s indicates the fraction of administered radioactivity reaching the systemic circulation; f_m, f_u, and f_y represent the fractions of intact drug reaching the systemic circulation that are converted to metabolite, excreted as such, and unaccounted for, respectively; and f_b' and f_u' denote the fractions of the total amount of formed metabolite that undergo biliary and urinary excretion, respectively. The parenthetical values represent percent of administered radioactivity.

Upon intraperitoneal injection of a 2.5-mg dose of ^{3}H-I to normal Sprague–Dawley rats, one finds significantly less tritium ultimately excreted in the urine than is observed after tail vein injection (22%

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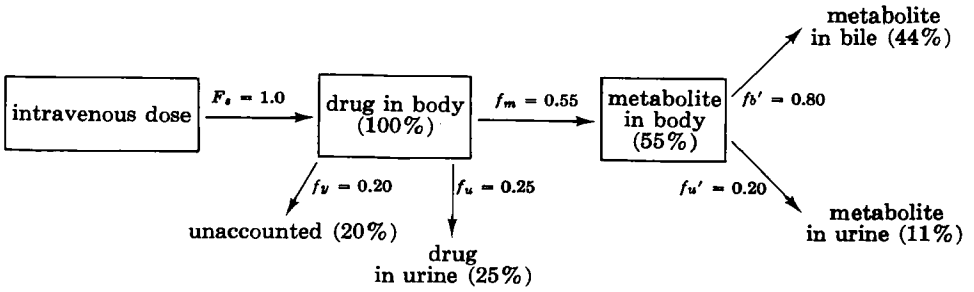
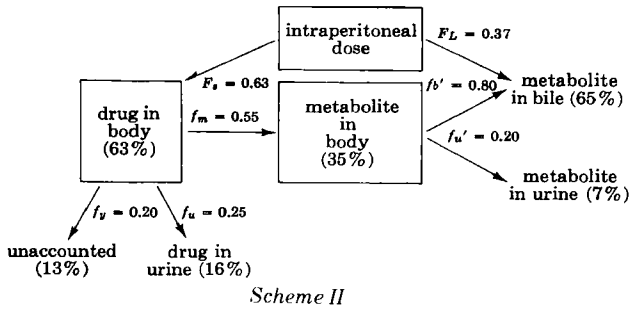


Scheme I

versus 35%, $p < 0.01$). This observation raised the possibility that after intraperitoneal administration the drug undergoes a first-pass phenomenon, leading to a biliary shunt. Confirmation of this hypothesis was obtained by finding comparable total urinary recovery after either intravenous or intraperitoneal administration to rats with ligated bile ducts. A number of previous studies (2–5) with drugs manifesting first-pass metabolism consistently showed that the ratio of metabolite to unchanged drug in the urine is significantly greater after "hepatic" (intraperitoneal, oral, *etc.*) administration than after "systemic" (intravenous, intramuscular, *etc.*) administration. Surprisingly, this was not the case with I. Irrespective of the route of administration, about 70% of the total radioactivity in the urine represented intact drug while the metabolite (II) accounted for the balance; essentially no intact drug was found in the bile.

The data suggest that, during passage of drug through the liver after intraperitoneal administration, a portion of the dose is irreversibly lost by metabolic conversion and subsequent biliary excretion while the balance of the dose reaches the systemic circulation. The fraction of the dose reaching the systemic circulation after intraperitoneal administration is calculable directly from the ratio of total radioactivity excreted in the urine after intraperitoneal and intravenous dosing, *i.e.*, 22/35 = 0.63. Accordingly, Scheme I was modified to take into consideration the first-pass effect and a quantitative metabolic scheme for drug disposition after intraperitoneal administration was devised (Scheme II), where F_L is the fraction of the dose undergoing first-pass metabolism. It is assumed that all of the metabolite formed during the first pass is quantitatively excreted in the bile. All other symbols and notations are as defined in Scheme I.



Scheme II

Scheme II is in remarkably good agreement with experimental observations in the rat after intraperitoneal administration. As noted, 22% of the intraperitoneal dose of ^{3}H-I was excreted in the urine, whereas Scheme II predicts 23% (16% I and 7% II). After intraperitoneal administration of I to rats with ligated bile ducts, 14% of the total radioactivity could not be accounted for in the urine. Scheme II predicts that 13% of the dose would be unaccountable. The difference between the urinary excretion of total tritium after intraperitoneal administration to rats with ligated and patent bile ducts indicates that, in normal rats, 64% of the radioactive dose is ultimately excreted in the bile. Biliary excretion of total radioactivity 24 hr after intraperitoneal administration of ^{3}H-I to bile duct-cannulated rats accounted for 48% of the dose. Extrapolation of these data to 72 hr after drug administration to estimate total biliary excretion yields a value of 59%. Both estimates of total biliary excretion of tritium are in excellent agreement with the value of 65% calculated in Scheme II.

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Reduced Binding of Sulfaethidole to Bovine Serum Albumin in Presence of Benzalkonium Chloride

Keyphrases □ Sulfaethidole—reduced binding to bovine serum albumin in presence of benzalkonium chloride □ Benzalkonium chloride—effect on binding of sulfaethidole to bovine serum albumin □ Binding—sulfaethidole to bovine serum albumin, effect of benzalkonium chloride

To the Editor:

In an earlier publication (1) we showed that a range of acidic drugs was capable of displacing the long acting sulfonamide, sulfaethidole, from its primary binding site whereas some quaternary ammonium compounds were not. We have continued these displacement experiments with a broader range of basic drugs, using the same experimental conditions, namely 1.45×10^{-5} *M* bovine serum albumin and 2.52×10^{-5} *M* sulfaethidole in a pH 7.4 physiological phosphate buffer, for the circular dichroic technique.

The following basic drugs showed less than 2% displacement of sulfaethidole: phenylpropanolamine (7.04×10^{-4} *M*), caffeine (1.03×10^{-4} *M*), tripelennamine (3.43×10^{-5} *M*), atropine (2.88×10^{-4} *M*), propranolol (2.03×10^{-4} *M*), imipramine (2.42×10^{-5} *M*), and benzocaine (6.32×10^{-5} *M*). However, the germicide, benzalkonium[1], greatly reduced the induced ellipticity of sulfaethidole and hence its albumin binding (Fig. 1).

Benzalkonium chloride is a mixture of alkyl dimethylbenzylammonium chlorides where the alkyl chain predominantly contains 12 or 14 carbon atoms. By using an average molecular weight of 372 and assuming that one molecule of benzalkonium displaces one molecule of sulfaethidole, a binding constant of $1.4 \times 10^4\ M^{-1}$ can be calculated by the method previously described. This value of 10^4 is in good agreement with the binding constants quoted for the binding of other long chain quaternary ammonium compounds to bovine serum albumin (2–4) and suggests that benzalkonium shares at least some areas of a binding site with the anionic sulfaethidole.

The sulfaethidole curves shown in Fig. 1 do not cross over at exactly the same point and the shape of the smaller curves, those with the highest benzalkonium concentration, are somewhat distorted because the benzalkonium slightly modifies the circular dichroism of bovine serum albumin in the 250–300-nm region. This region is a fingerprint region for the aromatic residues of the protein, and the changes in the spectrum suggest a change in the polarity of the immediate environment of these residues, possibly as a result of conformational changes in the albumin.

The circular dichroism spectrum of proteins below 250 nm is primarily due to the secondary structure of the albumin; when using a benzalkonium concentration of 2.28×10^{-4} *M* and a bovine serum albumin concentration of 1.45×10^{-6} *M*, a reduction in ellipticity of 4.5% was observed in the 205–225-nm region. This finding suggests that some conformational change is produced by benzalkonium; however, the spectrum in this region is complicated by the contributions from the aromatic residues of the albumin.

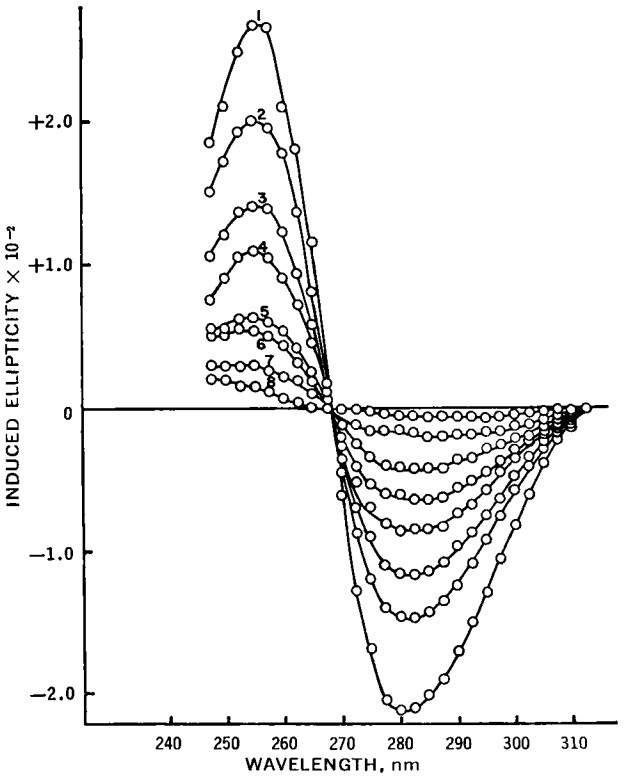

Figure 1—*Induced ellipticity of the sulfaethidole–bovine serum albumin complex in the presence of benzalkonium. Measurements were made in 10-mm cells at pH 7.4. The concentration of bovine serum albumin was 1.45 × 10⁻⁵ M, and that of sulfaethidole was 2.52 × 10⁻⁵ M. The concentrations of benzalkonium were: 1, 0; 2, 0.91 × 10⁻⁴ M; 3, 1.46 × 10⁻⁴ M; 4, 1.82 × 10⁻⁴ M; 5, 2.28 × 10⁻⁴ M; 6, 2.74 × 10⁻⁴ M; 7, 3.66 × 10⁻⁴ M; and 8, 4.57 × 10⁻⁴ M.*

If this change in ellipticity is due to conformational changes, then the changes are smaller than those found with anionic detergents under similar conditions.

Benzalkonium is usually used in a concentration of 1 in 10,000 as a preservative; even if injected, the large dilution factor would make the conformational changes in the albumin insignificant. However, the direct application of this concentration to mucous membranes may cause some denaturation of the membrane protein.

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Cholelithiasis Chemotherapy: An *In Vitro* Approach

Keyphrases □ Cholelithiasis chemotherapy—identification of cholesterol gallstone-dissolving agents, *in vitro* screening system □ Gallstones, cholesterol—dissolution, identification of drugs, *in vitro* screening system □ Cholesterol gallstones—dissolution, *in vitro* screening system to identify potential chemotherapeutic agents

To the Editor:

Cholelithiasis, a disease state resulting from deficiencies in cholesterol transport within the biliary system, is known to have afflicted humankind for centuries (1). Today, surgical removal of gallbladders and their stones is one of the most commonly performed operations with a third of a million cholecystectomies yearly in the United States alone (2, 3). Many treatments have been recommended including herbs, mineral waters, olive oil, and turpentine (4). More recent approaches to chemotherapy have generally been based on the feeding of endogenous components of bile, bile salts (5–8), or phospholipids (9). Clinical success with chenodeoxycholic acid administration was recently reported (8). Other studies have assessed the role of steroids (10), cod liver oil (11), and some drugs (12) on gallstone dissolution in rabbits fed a lithogenic diet.

The purpose of this research was to identify agents capable of bringing about the dissolution of cholesterol gallstones following oral administration. An *in vitro* screening system was developed which can monitor changes in the cholesterol-holding capacity of bile upon the addition of a test compound. The rationale for such a screening system is based on the physical–chemical properties of bile.

Bile salts will spontaneously aggregate into micelles above a critical temperature and concentration. Alone, these micelles have little ability to solubilize cholesterol; but in the presence of lecithin, the formation of mixed micelles enables the transport of large quantities of cholesterol (14). Small *et al.* (15) described this mixed micelle as a cylinder of bile salts, with hydrophilic sides facing the aqueous phase, surrounding an inner core of lecithin. The hydrophilic ends of the lecithin comprise the top and bottom of the cylindrical mixed micelle. Lecithin can interact with cholesterol to form liquid crystalline aggregates that are not soluble in aqueous media. Bile salts can engulf such aggregates to form mixed micelles and, in so doing, solubilize larger quantities of cholesterol. A complete description of these relationships may be found elsewhere (16).

The solubility of cholesterol in these four-component systems (bile salt, lecithin, cholesterol, and water) has been extensively studied. However, it has been difficult to find agreement on the same solubility values. Recently, the factors responsible for the discrepancies were identified (17) and the basal solubility of cholesterol was found to be in the order of 5 mole % (17–19). With this equilibrium solubility value defined, it has become possible to design a screening system capable of identifying compounds that can modify the equilibrium solubility of cholesterol in bile; such compounds would be potentially useful as cholelitholytic agents (20).

The cholesterol-solubilizing potential of a homologous series of quaternary nicotinic acids was studied in an aqueous *in vitro* system devoid of biliary components (13). While such a procedure measures the inherent solubilizing characteristics of a compound, it fails to identify compounds that cannot form micelles or water-soluble complexes with cholesterol but can otherwise interact with the biliary transport system to increase its carrying capacity (lecithin, for example). There is also the possibility of a compound interfering with the structure and function of the biliary micelles. Thus, it was considered worthwhile to employ a primary *in vitro* screening procedure based upon the bile salt–lecithin system. Secondary tests could then consider the inherent solubilizing ability of active compounds to gain information on their mechanism of action. A secondary test evaluating the effect of an active compound on the kinetics of gallstone dissolution is warranted; recent studies have discovered barriers to cholesterol dissolution in undersaturated bile systems (21). The development of a primary screen and some preliminary solubility enhancement data are the subjects of this report.

Excess hydrated crystalline cholesterol spiked with cholesterol-4-^{14}C was added to a lecithin–bile salt solution containing 38 m*M* egg lecithin and 135 m*M* conjugated bovine bile salts in a 0.05 *M* phosphate buffer (pH 7.4), as previously described (22). The solubility of cholesterol at 37° in this milieu was followed until equilibrium was reached. Because the long incubation times and numerous samplings of the bulk cholesterol–lecithin–bile salt suspension permitted microbial alterations, it was necessary to develop methods for sterilization and storage of the system. An incubating–stirring apparatus was designed that allowed for storage and aseptic sampling of this suspension (23).

When the cholesterol reached its equilibrium solubility, homogeneous aliquots of the suspension were placed into prewarmed vials containing the carefully weighed drug candidates. The systems were incubated with shaking at 37° for 24 hr. The solubility of cholesterol was then measured by liquid scintillation counting of an aliquot of the filtrate obtained by membrane filtration. Efficient determination of cholesterol solubility and recording of data were abetted through the use of automated liquid scintillation counting instruments equipped with punch-tape outputs. The tape was fed into a time-share terminal and, with the use of appropriate programs, back-

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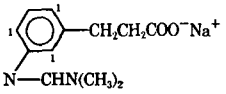
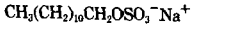
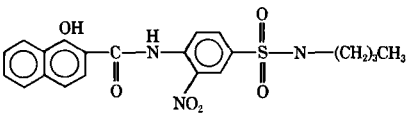
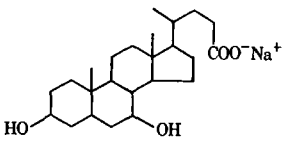
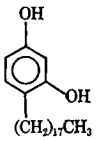
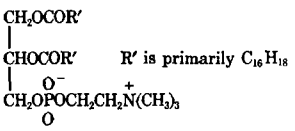
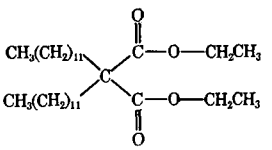
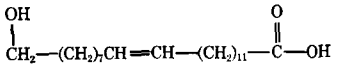
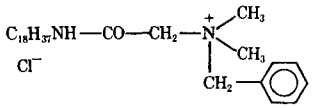
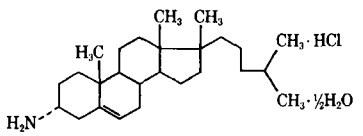
Table I—Cholesterol Solubilizing Evaluation of Selected Compounds

Compound	Structure	Cholesterol Solubility Enhancement as Percent of Control
Sodium ipodate[a]		95
Sodium lauryl sulfate[b]		97
1-Hydroxy-2′-nitro-4′-(butylsulfamoyl)-2-naphthanilide[c]		99
Chenodeoxycholic acid sodium salt[d]		100
4-*n*-Octadecylresorcinol[e]		115
Egg lecithin		116
Bis(dodecyl) malonic acid diethyl ester[e]		117
Erucyl alcohol[f]		119
Benzyldimethyl[(octadecylcarbamoyl) methyl]ammonium chloride[g]		121
Cholesteryl-3-α-amine hydrochloride hydrate[h]		122

[a] Courtesy of E. R. Squibb & Sons, New Brunswick, N.J. [b] Fisher Scientific Co., Fairlawn, N.J. [c] Aldrich Chemical Co., Milwaukee, Wis. [d] Courtesy of A. F Hofmann, M.D., Rochester, Minn. [e] Alfred Bader Chemical Co., Milwaukee, Wis. [f] Koch-Light Laboratories Ltd., Colnbrook Buckinghamshire, England. [g] Smith Kline & French Laboratories, Philadelphia, Pa.

ground and quench effects were eliminated and the solubility of cholesterol in test vials was compared to that of the controls. The results were recorded as solubility enhancement as a percent of control.

For a compound to be active *in vivo* by physical enhancement, it would have to be excreted in the bile. Smith (24) discussed the physical–chemical factors responsible for directing a compound toward biliary excretion. Where feasible, these principles (polarity, molecular weight, and stereochemistry) were used to guide selection of screening candidates. Compounds from diverse chemical families were selected for initial screening to maximize the possibilities of identifying active moieties. Candidates for screening

were obtained from the indicated sources and used as received. Sufficient compound was carefully weighed into a glass vial so that a 10 m*M* concentration of drug resulted upon addition of 2 ml of stock suspension.

Some representative data appear in Table I. With the introduction of a drug candidate, lipid interactions in the lecithin–bile salt micelle become more difficult to explain than in the quaternary system. The purpose of the drug is to mimic the action of either bile salt or lecithin, with the result of an increased cholesterol-carrying capacity. A compound may interact with the micelle in such a way that the holding capacity is not increased or decreased. This would be dependent on the nature of the drug–micelle interaction: position of drug in micelle, nature of charge on drug, charge density, branching of drug, *etc.* The *in vitro* screen is designed to measure the result of the five-component interaction, *i.e.*, the solubility of cholesterol in the presence of a drug–lecithin–bile salt system.

A major class of drugs designed to be passed through the biliary tract is the cholecystographic agents. Archer *et al.* (25) demonstrated the importance of chain length in determining whether a potential cholecystographic agent would be excreted *via* the urinary or biliary system. It was shown that if the chain was too small, the compound was eliminated by the kidneys; if the chain was too large, the drug was not intestinally absorbed. Thus, a critical length for biliary excretion existed. A cholecystographic agent, sodium ipodate, was not found to influence the solubility of cholesterol in this screening system. The most active compounds appear to contain an alkyl chain and thus show more of a structural resemblance to lecithin than to bile salts.

A bile salt, chenodeoxycholic acid, has little activity in this system, which was not unexpected since bile salts alone are not known to be good cholesterol solubilizers. The addition of lecithin or other insoluble swelling amphiphiles (16) can swell the bile salt micelle and provide an environment capable of dissolving large quantities of cholesterol. Oral administration of chenodeoxycholic acid has been found to bring about remission of cholesterol gallstones in humans by making the bile undersaturated with respect to cholesterol (8); the biliary secretion of cholesterol is lowered in relation to the bile salt and lecithin output. The chenodeoxycholic acid would seem to be acting *more* by virtue of its physiological than physical characteristics.

A viable *in vitro* screening procedure capable of identifying compounds that significantly enhance the biliary holding capacity for cholesterol has been developed. Data are presented for some typical compounds, selected because of a particular discrete characteristic. Moreover, the application of physical–chemical principles to devise and evaluate a particular disease state has been accomplished. As we learn more about the complexities of the human body, certainly the utilization of *in vitro* model systems to screen potential compounds preliminarily should become commonplace in the search for potential drug products.

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REVIEWS

Assay of Vitamins in Pharmaceutical Preparations. By MANZUR-UL-HAQUE HASHMI. Wiley, New York, NY 10016, 1973. 512 pp. 24 × 16 cm. Price $32.00.

This up-to-date (through 1971) compilation of methods will be of great benefit to anyone interested in the analysis of vitamins in pharmaceutical preparations. For the first time, all published methods are gathered into one volume with sufficient detail to allow analysis of vitamin products by the method deemed most suitable for the preparation being analyzed. A sufficient number of references are given for each type of determination in the event the analyst desires further information.

Each chapter deals with an individual vitamin with separate chapters for multivitamin preparations and for automated analysis. Critical evaluations of the methods are included together with brief discussions of the detail procedures. A separate chapter concerning the stability of vitamins in multivitamin preparations contains a very comprehensive bibliography on the subject. In all, the book covers 215 individual analytical methods for 18 different vitamins documented by more than 700 literature references, 44 figures, and 15 tables.

All USP XVIII methods for the analysis of vitamins have been included with appropriate references. Necessary modifications by different authors and critical opinion expressed in the literature about the USP procedures have been mentioned to inform readers about the latest developments. The British Pharmacopoeia and the National Formulary methods which differ from the USP procedures are also described.

This compilation of methods is recommended for all analysts interested in or involved with the analysis of vitamin preparations.

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Drug Design, Volume IV. Edited by E. J. ARIENS. Academic, 111 5th Ave., New York, NY 10003, 1973. 489 pp. 16 × 24 cm. Price $35.00.

Those lured by the title into the hope that with this text on the drafting table they can proceed to design model compounds guaranteed to become drugs of choice should first turn to page 290. Here, in this excellent chapter on "The Design of Biologically Active Nucleosides," Alexander Bloch lists some two dozen parameters that would have to be known for each species in which the compound is intended for use "in order to proceed rationally" to design drugs, and warns that "even then we would probably have missed a variety of more complex parameters, and would still not be able to predict its (the drug's) most sensitive site of action." Bloch goes on, however, to demonstrate the versatility of the nucleosides, from the viewpoint of both structure and application.

In the next chapter, appropriately the last, the patient is the victim, the therapeutic aim is death, the undesirable side effect development of resistance to toxicity. Perverse as inclusion of this article on "The Design of Insecticidal Chlorohydrocarbon Derivatives," by G. T. Brooks might seem, it nevertheless is an interesting addition to the text, including theoretical considerations, detailed structure–activity data, and modifications to counteract detoxification.

Unfortunately, equal value cannot be claimed for the opening three chapters. The first, by Leslie Benet on "Biopharmaceutics as a Basis for the Design of Drug Products" elaborates the obvious. One can almost visualize the good professor wagging his finger as he patiently explains (page 4) that "quickly" means "rapidly." He also seems to suffer from the illusion that there is for most drugs a 1:1 correspondence between blood levels and clinical effect.

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Advances in Biochemical Engineering. Edited by T. K. GHOSE, A. FIECHTER, and N. BLAKEBROUGH. Springer-Verlag New York Inc., 175 Fifth Ave., New York, NY 10011, 1974. 290 pp. 16 × 24 cm. Price $29.60.

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Aromatic Amino Acids in the Brain. Ciba Foundation Symposium 22. Edited by G. E. W. WOLSTENHOLME and D. W. FITZSIMONS. Associated Scientific Publishers, P.O. Box 211, Amsterdam, The Netherlands, 1974. 396 pp. 17 × 24.5 cm.

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Research Methods in Neurochemistry, Vol. 2. Edited by N. MARKS and R. RODNIGHT. Plenum, 227 W. 17th St., New York, NY 10011, 1974. 414 pp. 15.5 × 23 cm. Price $24.50.

Atlas of Protein Spectra in the Ultraviolet and Visible Regions. Edited by D. M. KIRSCHENBAUM. Plenum, 227 W. 17th St., New York, NY 10011, 1974. 623 pp. 21.5 × 28.5 cm. Price $39.50.

Obstetric Therapeutics. Edited by D. F. HAWKINS. Williams & Wilkins, Baltimore, MD 21202, 1974. 594 pp. 15.5 × 24 cm. Price $29.25.

Cyclic AMP, Cell Growth, and the Immune Response. Edited by W. BRAUN, L. M. LICHTENSTEIN, and C. W. PARKER. Springer-Verlag New York, Inc., 175 Fifth Ave., New York, NY 10011, 1974. 416 pp. 16 × 24 cm. Price $23.80.

Assessment of the Carcinogenicity and Mutagenicity of Chemicals. Report of a WHO Scientific Group. World Health Organization, Geneva, Switzerland, 1974. 19 pp. 14 × 20 cm. Price Sw.fr. 4.

Metal Ions in Biological Systems. Simple Complexes, Vol. 1. Edited by H. SIGEL. Dekker, 305 E. 45th St., New York, N.Y., 1974. 267 pp. 15.5 × 23.5 cm. Price $21.75.

Homogeneous Hydrogenation. By B. R. JAMES. Wiley, 605 Third Ave., New York, NY 10016, 1973. 525 pp. 24 × 15.5 cm. Price $24.95.

Journal of Pharmaceutical Sciences

MARCH 1975

VOLUME 64 NUMBER 3

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Assistant Editor

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All expressions of opinion and statements of supposed fact appearing in articles or editorials carried in this journal are published on the authority of the writer over whose name they appear and are not to be regarded as necessarily expressing the policies or views of the American Pharmaceutical Association.

Offices—Editorial, Advertising, and Subscription Offices: 2215 Constitution Ave., N.W., Washington, DC 20037. Printing Offices: 20th & Northampton Streets, Easton, PA 18042

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THE ECONOMICS OF PUBLICATION

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Well, as bad as the situation may be in the case of food prices and other commodities sold in the supermarket, printers and printing companies have been increasing prices to their clients at an even faster pace. This is the result of all the customary factors, including labor, taxes, equipment, utilities, and so on, but it is especially due to one other; namely, the cost of paper stock.

Anyone even remotely connected with the production of printed material has probably had the shocking experience during the past year or so of being told that the price of paper was increasing on a monthly or even a weekly basis. The overall result was that comparable paper stock ended up costing anywhere from double to four times as much as it did about a year earlier.

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All of this is rather evident from an inspection of the APhA financial statements. Although these statements are released and distributed each year—as in the case of most financial reports—they make awfully dull reading, and few people give them even a cursory examination.

But if they did read these reports, they would note that *J. Pharm. Sci.* income has been growing modestly from $244,000 in 1972, to $260,000 in 1973, to $290,000 in 1974, and to $288,000 in 1975 (all figures rounded and latter figures estimated).

Simultaneously, however, during this same period of modest income growth, direct expenses attributable to production of *J. Pharm. Sci.* have grown by leaps and bounds from $265,000 in 1972, to $293,000 in 1973, to $322,000 in 1974, and to $370,000 in 1975.

Without going into any great detail, two things are evident from these figures; namely:

(a) As the first observation, the APhA member subscriber pays only a small additional premium to receive this Journal, but a substantial proportion of his or her membership dues is allocated to support the Journal. At the current rate of total income resulting from subscription charges, page charges, advertising revenue, and reprint sales, APhA is providing a very substantial subsidy from membership dues—for 1975 this will amount to approximately $82,000—in order to make J. Pharm. Sci. *available.*

(b) As the second observation, we may look at how this "cost/benefit ratio" translates to each individual Journal recipient, whether the recipient is a member subscriber, nonmember subscriber, or institutional subscriber. With an overall circulation of approximately 12,000 and an anticipated 1975 production cost of $370,000, it is evident that the proportionate cost of simply providing the average subscriber with the Journal for a period of one year is running just under $31—and this figure of $31 is extremely conservative since it does not include any overhead and similar types of occupancy expenses.

Every clergyman tells his congregation something to the effect that: "I do not wish to talk about money, but . . . .".Similarly, we would also prefer to avoid doing so. However, we feel that these facts and figures will be revealing to our readers, and especially to our APhA member subscribers by acquainting them more fully with one of their membership benefits.

We also feel that these facts and figures will provide a better understanding of why all associations, societies, and commercial publishers are seriously reexamining their publication programs and in many cases are instituting or at least considering radical changes in them. If the publishers of *Life, Look,* and the *Saturday Evening Post* had discerned the winds of change early enough and taken action in time to adjust to them, their demise may have been avoided, and these once-popular mass-circulation weekly magazines would probably have survived. By the same token, current trends and changing circumstances make it appear that technical and scientific periodicals will need to undergo some major changes in coming years if they are to survive and prosper.

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Chemical Classification of Plants

Recently, a commentary appeared in the "Open Forum"[1] discussing a book review published in *J. Pharm. Sci.* [**63**, 1344(1974)]. As author of the review, I wish to deny the two implications stated in the commentary: ". . . the reviewer implies that review articles on plant antitumor agents may be confusing to the chemically (and not botanically) oriented reader and that chemically oriented reviews in this area are not available."

As to the first, the intended implication, which is apparent to me after reading the review, is that of all the articles published on naturally occurring substances (not only reviews and not just reviews on plant antitumor agents), most of them present data within a taxonomic framework and this may (perhaps not) be difficult reading for those whose training and experience are not botanically oriented. I readily acknowledge that the botanical classification approach well serves its purpose, and I made no suggestion to the contrary. However, I pointed out that the approach in this publication more closely represents a chemical classification than a taxonomic approach. I believe this is in keeping with the primary purpose of a book review, which is to describe the contents and methods of data presentation.

Second, in my judgment, there are no statements in the reviewer's comments that would in any way imply that chemically oriented reviews in this area are not available. Whether the reviewer was aware of chemically oriented reviews in this area is not a pertinent point, but can anyone with even a peripheral interest in plant antitumor agents not be aware of the work of Dr. S. M. Kupchan and some of the other authors listed by the commentary writers?

Charles M. Darling
School of Pharmacy
Auburn University
Auburn, AL 36830

Received December 6, 1974.

[1] N. R. Farnsworth and G. A. Cordell, *J. Pharm. Sci.*, **63** (11), vi(1974).

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Some time ago the Editor of *J. Pharm. Sci.* asked for opinions on current review practices for manuscripts published in this Journal. Only a few individuals responded, and the opinions did not appear to be strong one way or another. Because our opinions were split on the issue, we did not feel that our remarks would contribute to the question.

However, a unique way of handling this problem is evidenced in the journal *Current Anthropology*. This process has been described in detail[1], but it would seem of interest to describe briefly the method used by this journal, and perhaps by others, for handling the review process of manuscripts.

Copies of the manuscript are sent to about *20* scholars whose data cards indicate their competence in the subject matter. They are asked to evaluate the manuscript in terms of accuracy, importance, and interest to the profession and to make suggestions for improvement. Their replies, analyzed by the editorial staff, provide a firm basis for a decision on the manuscript—whether it be accepted as it is or returned to the author so that he or she might take account of the suggestions from the referees or seek a more appropriate journal for the manuscript. These reviews are published immediately following the article in the journal and often amount to the length of a full manuscript themselves. The author then has the option to reply to these reviews, and such replies are published immediately following the reviews.

It seems to us that this is an amazingly simple and fair way of treating the "peer review" problem in which everyone has an opportunity to express his or her opinion. The major disadvantages are: *(a)* there would be an additional burden placed on the editorial staff of the journal, *(b)* a larger number of competent reviewers would be needed, who would be willing to respond rapidly so as to avoid delays in publication, and *(c)* publication of most articles would be somewhat delayed. The obvious advantages to this system are: *(a)* even though an article was judged inappropriate for publication, the author could still request publication knowing that the comments and analysis of the referees would be made known to the readership, and *(b)* the author would have an opportunity to rebut, in writing, the criticisms of the referees (if needed).

Obviously, the referees would have to be instructed to prepare reviews that would reflect a manuscript after being corrected for trivial errors. This type of a review system would perhaps markedly change the type of review received by the editor and would force some authors to be more careful in the preparation of their manuscripts.

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March 20–22
American Society for Clinical Pharmacology and Therapeutics, Annual Meeting, Washington Hilton Hotel, Washington, D.C.

March 22
USP Convention, Mayflower Hotel, Washington, D.C.

April 6–11
American Chemical Society, National Meeting, Philadelphia, Pa. (Contact Meetings Department, American Chemical Society, 1155 16th St., N.W., Washington, DC 20036)

April 19–24
APhA Annual Meeting, San Francisco, Calif.

April 27–May 2
American Society for Microbiology, New York Hilton Hotel, New York, N.Y. (Contact R. W. Sarber, 1913 Eye St., N.W., Washington, DC 20006)

April 28–30
Pharmaceutical Manufacturers Association, Annual Meeting, Boca Raton, Fla.

May 1–4
American Society of Internal Medicine, Mayflower Hotel, Washington, D.C. (Contact William Ramsey, 535 Central Tower Building, 703 Market St., San Francisco, CA 94103)

May 2–3
American College of Clinical Pharmacology, Chalfonte-Haddon Hall, Atlantic City, N.J. (Contact Ms. Barbara Renaud, 2 E. 103rd St., New York, NY 10029)

May 2–4
Association of Clinical Scientists, Governors Inn, Research Triangle Park, N.C. (Contact F. W. Sunderman, Jr., M.D., University of Connecticut School of Medicine, P.O. Box G, Farmington, CT 06032)

May 4–5
American Federation for Clinical Research, Chalfonte-Haddon Hall, Atlantic City, N.J. (Contact C. B. Slack, 6900 Grove Road, Thorofare, NJ 08086)

May 5
American Society for Clinical Investigation, Chalfonte-Haddon Hall, Atlantic City, N.J. (Contact H. E. Williams, M.D., Room 4401, San Francisco General Hospital, San Francisco, CA 94110)

May 11–14
The Proprietary Association, Annual Meeting, The Greenbrier, White Sulphur Springs, W.Va.

May 19–21
National Research Council, Annual Scientific Meeting, National Academy of Sciences Auditorium, Washington, D.C.

May 22–24
International Congress on Patient Counseling, Amsterdam, The Netherlands (Contact K. Ellison Davis, Excerpta Medica Foundation, P.O. Box 1126, Jan van Gaelnstr., 335, Amsterdam, The Netherlands)

May 26–30
International Conference on Prostaglandins, Florence, Italy (Contact Dr. G. C. Folco, Scientific Secretary, Institute of Pharmacology and Pharmacognosy, University of Milan, via Andrea Del Sarto, 21-20129, Milan, Italy)

June 15–18
Society for Economic Botany, Annual Meeting, University of Maryland, Baltimore, Md. (Contact Dr. Ralph N. Blomster, School of Pharmacy, University of Maryland, 636 W. Lombard St., Baltimore, MD 21201)

June 15–19
American Medical Association, Annual Meeting, Atlantic City, N.J. (Contact Dr. E. B. Howard, 535 N. Dearborn St., Chicago, IL 60610)

June 16–18
International Symposium on Mass Spectrometry in Biochemistry and Medicine, Alghero, Sardinia, Italy (Contact A. Frigerio, Istituto di Ricerche Farmacologiche, Mario Negri, Via Evitrea 62, 20157, Milan, Italy)

June 18–20
Drug Information Association, Annual Meeting, Sheraton Boston Hotel, Boston, Mass.

July 13–18
International Congress on Clinical Chemistry, Toronto, Ontario, Canada (Contact Dr. F. H. Sims, Women's College Hospital, Toronto, Ontario, Canada)

August 11–15
Gordon Research Conference, Medicinal Chemistry, Colby Women's College, New London, N.H. (Contact Dr. Alexander M. Cruickshank, Gordon Research Conferences, University of Rhode Island, Kingston, RI 02881)

August 24–29
American Chemical Society, National Meeting, Chicago, Ill. (Contact A. T. Winstead, American Chemical Society, 1155 16th St., N.W., Washington, DC 20036)

September 1–5
International Congress of Pharmaceutical Sciences, Dublin, Ireland (Contact Congress Secretary, 35th International Congress of Pharmaceutical Sciences, 44 Northumberland Road, Ballsbridge, Dublin 4, Ireland)

October 1–5
American College of Apothecaries, Annual Meeting, Four Seasons Sheraton, Toronto, Ontario, Canada

October 6–8
ASTM Committee E-19 on Chromatography, Annual Meeting, Sheraton-Houston, Houston, Tex. (Contact Mr. Jack Stamm, Instrument Products Division, Photo Products Department, Du Pont Co., Wilmington, DE 19898)

October 13–16
Association of Official Analytical Chemists, Annual Meeting, Marriott Hotel, Twin Bridges, Washington, D.C. (Contact L. G. Ensminger, Association of Official Analytical Chemists, Box 540 Benjamin Franklin Station, Washington, DC 20044)

October 17
APhA Academy of Pharmaceutical Sciences, Annual Eastern Regional Meeting, Governor Morris Inn, Morristown, N.J. (Contact Dr. Daniel Mufson, USV Pharmaceutical Corp., 1 Scarsdale Road, Tuckahoe, NY 10707)

November 2–6
Society for Neuroscience, Annual Meeting, New York, N.Y. (Contact Neuroscience Meeting Headquarters, Room 200 A, Lee Building, 9650 Rockville Pike, Bethesda, MD 20014)

November 16–20
American Public Health Association, Annual Meeting, Chicago, Ill.

November 16–20
APhA Academy of Pharmaceutical Sciences, National Meeting, Marriott Motor Hotel, Atlanta, Ga.

November 29–December 4
American Medical Association, Clinical Meeting, Honolulu, Hawaii

November 30–December 5
American Chemical Society, National Meeting, Mexico City, Mexico (Contact Meetings Department, 1155 16th St., N.W., Washington, DC 20036)

1976

April 26–May 1
Third International Symposium on Detection and Prevention of Cancer, Americana Hotel, New York, N.Y. (Contact Dr. Herbert E. Nieburgs, Mount Sinai School of Medicine, City University of New York, Fifth Ave. and 100th St., New York, NY 10029)

Items to be considered for inclusion or corrections should be addressed to:

J. Pharm. Sci. Calendar
2215 Constitution Avenue, N.W.
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Journal of Pharmaceutical Sciences

MARCH 1975
VOLUME 64 NUMBER 3

REVIEW ARTICLE

Studies with Deuterated Drugs

MARTIN I. BLAKE *x, HENRY L. CRESPI ‡, and JOSEPH J. KATZ ‡

Keyphrases □ Drugs, deuterated—deuterium biological and isotope effects, toxicity and pharmacological and biological effects of deuterium oxide, heavy water as a drug, various deuterated drugs, applications, review □ Deuterated drugs—barbiturates, hormones, anesthetics, antibiotics, alkaloids, amino acids, sympathomimetic amines, applications, review □ Heavy water—carcinolytic agent, measure of total body water, and neutron radiography, review □ Deuterium oxide—toxicity and pharmacological and biological effects, review

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INTRODUCTION

A drug may be defined as an agent used in the diagnosis, cure, mitigation, treatment, or prevention of disease in humans or animals. Included in this category are articles (other than food) used to affect the structure or any function of the body of humans or animals. This definition distinguishes a drug from a chemical. A drug that contains a radioactive nuclide in the molecule is known as a radiopharmaceutical, and several dozens of these are useful clinical agents.

At the present time, there are no drugs on the market that contain deuterium in the molecule or, for that matter, are enriched in any other stable isotope. Numerous deuterated drugs have been obtained by organic synthesis or by biosynthesis. Several of these substances are completely deuterated, some are highly deuterated, and others are partially deuterated, but the majority are deuterated only in specific molecular positions.

Deuterated drugs have proved useful in studying isotope effects, in permitting a better understanding of the mechanism of drug action, and in elucidating metabolic and biosynthetic pathways. Some are simply laboratory curiosities awaiting future study. Heavy water (^2H_2O) itself has been examined for therapeutic applications. This review is concerned with the laboratory application of both heavy water and deuterated drugs.

Table I—Comparison of Some Physical Properties of Water and Deuterium Oxide[a]

Property	Water	Deuterium Oxide
Melting point	0.00°	3.81°
Boiling point	100.00°	101.72°
Density, d_4^{20}, g/ml	0.9982	1.1056
Temperature of maximum density	3.98°	11.23°
Viscosity, centipoise, 20°	1.005	1.25
Surface tension, dynes/cm, 25°	71.97	71.93
Dielectric constant, 25°	78.54	78.93
Heat of formation, cal/mole, liquid	−68,320	−70,410
Free energy of formation, cal/mole, liquid	−56,690	−58,200
Entropy, eu/mole, liquid	16.75	18.19
Heat of fusion, cal/mole	1,436	1,515
Heat of vaporization, cal/mole, 25°	10,515	10,846
Zero-point energy, cal/mole	13,219	9,664

[a] See Ref. 15.

Biological Effects of Deuterium—Deuterium (^{2}H, D) is the rare, stable heavy isotope of hydrogen (^{1}H). It was discovered (1, 2) in 1932 and was isolated in a high state of purity (3) shortly thereafter. Deuterium occurs in nature to the extent of about 1 part in 6400 and is obtained primarily by isolation of deuterium oxide from ordinary water; its concentration ranges from 0.0156% in sea water to 0.0139% in fresh water. Heavy water is the simplest deuterium-containing compound and differs from ordinary water in many of its properties (Table I). It can be reasonably expected that the mass differences associated with the replacement of deuterium by hydrogen in a molecule will distinctly affect its physical and chemical properties. Such mass differences can also be expected to produce changes in the biological behavior of deuterium compounds.

Extensive deuterium isotope studies were precluded in the 1930's and early 1940's because of the great scarcity of heavy water. Since heavy water is very suitable as a moderator in certain types of nuclear reactors, efficient methods have been developed to isolate heavy water from natural sources. Hence, deuterium (as heavy water) has become a tonnage, high purity, industrial chemical available at low to moderate cost. The ready availability of heavy water to the scientific community as a result of advances in nuclear technology has resulted in a burgeoning interest in the biological effects of deuterium.

The biological implications of deuterium substitution were recognized almost immediately after its discovery. It soon became apparent that extensive replacement of hydrogen by deuterium in biological systems could produce deleterious effects. The early literature for 1932–1950 has been reviewed thoroughly (4). Much of the early work, however, is contradictory and of questionable value, primarily because of poor experimental design forced by inadequate and impure supplies of heavy water and because of the absence of suitable analytical techniques for measuring accurately the deuterium content of biological systems.

A conference under the auspices of the New York Academy of Sciences in 1960 (5) did much to revive and stimulate interest in studies on the biological effects of deuterium. Many of the relevant papers in that monograph will be referred to later in this review. In the same year, Katz published reports (6, 7) that summarized both the early studies in this field and also included new research undertaken during the 1950's, primarily at Argonne National Laboratory.

The physiological effects of deuterium were the subject of a comprehensive monograph (8) in 1963. Flaumenhaft *et al.* (9) reviewed the biological effects of deuterium and focused their comments on isotope effects on the cellular level. They noted specifically the isotope effects on the growth of microorganisms and higher plants in deuterated media and the cytology of deuterated cells.

Katz, Crespi, Blake, and coworkers (10–15) published a series of articles covering various aspects of deuterium isotope effect studies, with special emphasis on applications related to problems of a chemical and biochemical nature. The culturing of various microorganisms in deuterated media, the effect of deuteration on the stability of certain proteins and nucleic acids, and isotope effects in the metabolism of deuterated glucose and mannose by ascites tumor cells were reviewed (10).

Another review (11) covered the effect of deuterium substitution on the conformation of certain biopolymers and deuterium isotope effects in carbohydrate metabolism and in the potentiation of tumor chemotherapy. A third paper (12) described the cultivation of deuterated organisms and their utilization as a practical source of ^{2}H and ^{1}H–^{2}H compounds that are very useful for PMR spectroscopy of biopolymers; it also described the utility of deuterated organisms for following the path of hydrogen in living organisms.

In 1965, Katz (13) presented a comprehensive review of deuterium isotope effects on living organisms and biopolymers and illustrated the power of these methods by a discussion of biogenesis of chlorophyll. In a more recent review, the application of NMR studies to the biosynthesis of the important plant constituents bacteriochlorophyll, chlorophylls *a* and *b*, and the clavine alkaloids was discussed (14). The most recent and perhaps the most comprehensive review of deuterium isotope effects on biological systems was published in 1971 (15). In the present review, principal emphasis will be directed to deuterated drugs and their application.

Deuterium Isotope Effects—The basis for the frequently profound chemical and biological consequences of deuteration merits some discussion. The kinetic isotope effect on the rates of chemical reaction resulting from substitution of deuterium for hydrogen, which must certainly be implicated in the biological effects of deuterium, has received thorough theoretical treatment (16–20). The difference in mass between deuterium and hydrogen causes the vibrational frequencies of carbon, oxygen, and nitrogen bonds to deuterium to have lower frequencies than corresponding bonds to hydrogen. As a result, the

chemical bonds involving ^2H will generally be more stable than those of ^1H.

It has been calculated that the zero-point energy (lowest ground-state vibrational level) for many bonds to deuterium is about 1.2–1.5 kcal/mole smaller than for bonds to hydrogen. The zero-point energy of a bond undergoing chemical reaction reflects the ease with which the molecule is activated from a ground state to the transition state required for bond scission to occur. The more stable deuterium bond requires a greater energy of activation to achieve the transition state; as a consequence, the rate of reaction involving a bond to deuterium is generally slower than that involving a bond to hydrogen. Thus, substitution of deuterium for hydrogen in a chemical bond can affect significantly the rate of bond cleavage and exert marked effects on the relative rates of chemical reactions.

Large isotope effects on reaction rates are apparent where cleavage involves a bond to deuterium at the reaction site. In such instances the effect is referred to as a primary isotope effect and is usually expressed in terms of the ratio of the specific rate constants k_H/k_D. Bigeleisen (21) calculated the maximum possible ratios in specific rate constants for a number of stable and radioactive isotopes (Table II). Wiberg (17) calculated that at 25° the maximum positive primary kinetic isotope effect that can be expected in a chemical reaction involving the breaking of bonds to hydrogen is in the range of 7–10 for C—H, N—H, and O—H *versus* C—D, N—D, and O—D, respectively. For a number of reasons, these maximum ratios are not realized; more generally, values of k_H/k_D are in the range of 2–5.

An observable isotope effect will only be apparent, of course, where the breaking of a C—H or C—D bond is involved in the rate-determining step. In certain acid–base-catalyzed reactions, depending on the mechanism, an inverse isotope effect may take place (22). The pronounced effects on rates of chemical reactions make deuterium isotope studies a particularly useful tool for elucidating the mechanisms of many reactions of biochemical significance (8).

Deuterium atoms in nonexchangeable positions located near, but not at, the reaction center can give rise to secondary isotope effects. While deuterium secondary isotope effects are real and measurable, they are usually much smaller than primary isotope effects. Belleau (23) indicated that the k_H/k_D for secondary isotope effects falls in the range of 1.05–1.25. Secondary isotope effects, although small, may be important in biological systems sensitive to kinetic effects. The physical organic chemist also makes a distinction between solvent isotope effects, where only the isotopic composition of the medium has been altered, and primary and secondary isotope effects resulting from the replacement of hydrogen by deuterium in carbon to hydrogen bonds of organic compounds.

A recent review article (15) pointed out that in living organisms and in many biochemical and physiological systems the distinction between solvent and primary and secondary isotope effects is not always clear. Isotope effects produced by hydrogen atoms that exchange very slowly (if, in fact, they exchange at all) with water, as is the case for most carbon–hydrogen bonds, must be distinguished from those in which exchange occurs very rapidly, as is generally the case for hydrogen atoms bonded to oxygen, sulfur, or nitrogen. Compounds occurring in biological systems will generally have both types of exchangeable hydrogen present. If the isotopic composition of the solvent is altered, this will effect a corresponding change in the isotopic composition of the exchangeable hydrogen in the solute molecules. In living systems, therefore, solvent isotope effects include contributions from both primary and secondary isotope effects.

Table II—Estimated Maximum Possible Rate Constant Ratios at 25° for Various Stable Isotopes

Natural Abundance Isotope	Heavy Isotope	Rate Ratio[a], k_l/k_2
^1H	^2H	18
^1H	^3H	60
^{12}C	^{13}C	1.25
^{12}C	^{14}C	1.5
^{14}N	^{15}N	1.14
^{16}O	^{18}O	1.19
^{32}S	^{35}S	1.05

[a] Rate constant for the light isotope is the numerator.

The compositional or constitutional isotope effects have been defined (15) as those arising from alterations in the isotopic composition of the nonexchangeable hydrogens in the molecule in which all hydrogen sites in nonexchangeable positions are occupied by deuterium. Hybrid constitutional deuterium isotope effects occur when both hydrogen and deuterium are present in nonexchangeable positions. When the isotopic composition of living organisms is changed, as when animals are administered deuterium oxide in the drinking water, the initial effects of deuteration are attributable primarily to a solvent isotope effect. When the deuterium oxide enters the metabolic processes of the organism and is used for the biosynthesis of compounds containing both hydrogen and deuterium, then, in addition to a solvent isotope effect, hybrid constitutional isotope effects are induced.

Finally, in an organism grown in an environment of deuterium free of any hydrogen, constitutional isotope effects become predominant. In the deuteration of living organisms, both water and deuterium oxide are usually present and hybrid isotope effects are most prominent. These hybrid isotope effects are most commonly encountered in living organisms subjected to partial deuteration and are the most difficult isotope effects to interpret at the molecular level.

General Toxicity of Deuterium Oxide—Since deuterium oxide resembles ordinary water so closely, it is natural to speculate on the effects of this apparently small difference in chemical composition on the toxicological properties of the molecule. Concern over the toxicity of heavy water to living organisms was first expressed (24) soon after its discovery. A mouse

fed the equivalent of about 1 g of pure heavy water over a 3-hr period survived the ordeal but showed definite signs of intoxication. Lewis (24) noted that: "The more he [the mouse] drank of the heavy water the thirstier he became." Unfortunately, the experiment had to be terminated because the limited supply of heavy water was exhausted.

An extensive series of studies on the physiological effects of deuterium oxide in mice was next conducted over a 5-year period starting in 1934 (25). The acute lethal dose for deuterium oxide in mice was 5–7 ml/10 g of body weight. This amount was observed to be the lethal dose even when the deuterium oxide was administered as a 50% solution in water. When "concentrated" heavy water was administered orally, a crisis was reached on about the 5th day, at which time the body water became about one-third replaced by deuterium oxide and death generally occurred around the 7th day. However, a mouse ingesting 40% D_2O in its drinking water for a 2-month period showed no apparent ill effects other than retarded weight gain; mice whose body fluids reached 20% deuteration showed no noticeable harmful effects when the heavy water was removed from the diet.

Barbour and Trace (26) fed mice pure (99.5%) deuterium oxide at a dosage rate of 1 ml/10 g of body weight/day. This intake generally proved fatal in 7 days, at which time the mice were deuterated to the extent of 40–50%[1]. Death in these mice was preceded by a characteristic sequence of toxic manifestations.

More recently, Thomson (8) thoroughly reviewed the literature dealing with the physiological and toxicological effects of deuterium oxide in mammals. Katz (7) reported that mice and rats cannot long survive replacement of more than one-third of their body water by deuterium oxide. At the 20% replacement level, rats become hyperexcitable and more aggressive than normal; when the plasma levels approach 30% D_2O, rats frequently convulse when handled. When the body fluids are at the 35% deuteration level, death generally results. Thomson (27) also observed that rats drinking deuterium oxide died when about one-third of their body water was replaced by deuterium oxide. He concluded that the toxic effects were attributable to the summation of a multitude of small changes affecting the rates of enzymatic reactions in the body.

Czajka and Finkel (28) maintained mice on 25% D_2O in the drinking water for up to 280 days without adverse effect on body weight or longevity. In these mice, ingestion of 25% D_2O caused incorporation in the body fluids of about 18–20% deuterium. When the deuterium content of the drinking water was raised to the 25–30% level, fetal viability ceased. Katz *et al.* (29) found that the median survival times for mice deuterated to different deuterium levels ranged from 60 days for mice drinking 40% D_2O to 12 days for mice drinking 75% D_2O. Differences in survival time were related to the rates at which deuteration reached toxic levels and not to differences in deuterium levels *per se*. Most animals died when the body tissue fluid concentration rose to between 30 and 40% D_2O.

Czajka *et al.* (30) studied the toxic manifestations of deuterium in two dogs. One beagle was maintained at 20% D_2O in the body fluids for 50 days, and the other was maintained at the toxic range of 33–35% for a brief period. Deuteration was effected by replacement of ordinary water with deuterium oxide in the food and drink. These dogs (approximately 10 kg) appear to be the largest animals subjected to extensive deuteration. A 30% concentration in the blood plasma appears to be close to the acute danger level for dogs, as it is for mice and rats. Thus, in intact mammals the magnitude of deuterium effect appears on the whole to be quite independent of the size of the organism.

In one report (31) on humans, no ill effects were noted over 4 months when the body water was replaced with deuterium oxide to the extent of 0.5%. In mammals it appears that up to 15% deuterium can be tolerated, but severe toxic effects and possibly death may result at the 30% level and higher. Although toxic effects appear to correlate with the overall level of deuteration, deuterium probably elicits harmful effects in subtle ways even in low concentration.

Incorporation *in vivo* of small amounts of deuterium into sensitive molecules such as enzymes, nucleic acids, and similar important substances possibly could have serious consequences. Knapp and Gaffney (32) pointed out that the small amounts of stable isotopes involved in labeling experiments probably would not cause harmful effects. Nevertheless, they cautioned that it would be wise to label the molecules in molecular positions that are metabolically stable to minimize the extent of distribution throughout the body. The possible toxicity of stable isotopes at low concentrations clearly merits further study.

The ultimate cause of death from deuteration is not clear. Numerous disturbances are observed including renal function impairment, central nervous system (CNS) disturbances, cardiac involvement, enzymic interferences, hormonal imbalances, and glucose metabolism disturbances. All of these factors seem to be involved in the death of the animal, and no single factor appears to be the principal cause of death. Only a few studies have been directed toward finding ways to increase the tolerance of animals to the ravages of high concentrations of deuterium oxide in the body fluids. An extensive study (33) was conducted in which a number of hormones, vitamins, and vitamin mixtures were examined for possible beneficial effects on the ability of mice to survive toxic levels of deuteration. The mice were administered daily injections of hormones, vitamins, or vitamin mixtures while being maintained on 50 or 75% D_2O in the drinking water, but the improvement in survival was marginal at best.

Notwithstanding the damaging effects imposed on animals from high levels of deuteration, deuterium oxide must nevertheless be considered a remarkably nontoxic substance. Few, if any, components of the

[1] Percent deuterium oxide throughout this paper refers to atom percent D in the aqueous fluid. A mixture of water and deuterium oxide is related by a mobile equilibrium: $H_2O + D_2O \rightleftharpoons 2\,HOD$.

body can be replaced to anywhere the same extent as can water by deuterium oxide without the gravest consequences.

Pharmacological and Biological Effects of Deuterium Oxide—Morowitz and Brown (4) reviewed the early physiological studies in this area, and Thomson (8) prepared a comprehensive summary of the literature up to the year 1963.

Heavy water is readily absorbed from the GI tract and rapidly equilibrates with the body fluids. There appears to be no selective excretion of heavy water by the kidney. Pure (99.8 atom % D) heavy water has a taste not too different from distilled water or deaerated water. It is readily administered to animals in their drinking water, and its palatability may be enhanced by addition of traces of sodium chloride. Smith (34) suggested that mice are capable of discriminating between deuterium oxide and water, and he attributed this ability to the formation of the more stable deuterium—oxygen bonds which may be involved in the interaction between the water molecule and "a gustatory receptor site." This study was later challenged (35), since the data were based on only two mice (litter mates)[2].

By the selection of the appropriate deuterium oxide concentration in the drinking water, the equilibrium deuterium oxide concentration in the body fluids can be readily controlled. The course of deuteration has been intensively studied in mice (26, 29, 36) and rats (27). Figure 1 illustrates the rate of deuteration (as reflected by the deuterium content of the urine) as a function of time when varying concentrations of heavy water were administered to mice in their drinking water. An equilibrium state in the body fluids was reached by about the 10th day. With low concentrations of heavy water in the drinking water, the deuterium level in urine reached about 90% that of the ingested water. Where the deuterium oxide content of the drinking water was in the 20–50% range, equilibrium urine values were approached more slowly and were only 70–80% that of the drinking water. Dilution was due primarily to the fact that the food administered to the animal was of ordinary isotopic composition. Similar results are obtained when the course of deuteration is assessed in terms of the deuterium content of the blood serum.

Both mice and rats react similarly to the effects of deuterium ingestion. When the deuterium content of blood serum approaches 20%, signs of hyperirritability are observed and the mice show a tendency to convulse. Their coats become rough and they tend to lose weight rapidly. If the deuterium level is raised to still higher levels, the animals show a pilomotor response and become stuporous, the body temperature drops markedly, and death may occur in a short time. Thomson (27) noted that replacement of the body water by 15% D_2O resulted in signs of hyperactivity in rats. At the 20–25% replacement level, they became hyperexcitable and tended to convulse when stimulated. The animals ceased to groom themselves,

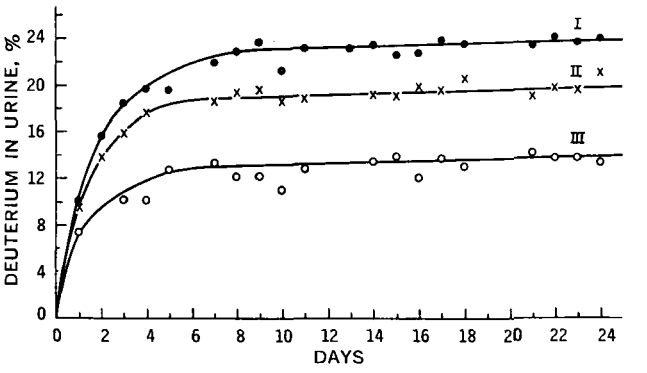


Figure 1—*Course of body fluid deuteration of mice drinking deuterium oxide of various concentrations. Key: I, 30% D_2O; II, 25% D_2O; and III, 15% D_2O. Each point represents a mean of from five to 22 determinations. (Reprinted, with permission, from Ref. 36.)*

ulcers were noted on their paws, and their tails showed signs of necrosis. At the 30% deuterium level, the rats became comatose and refused food; there was a precipitous loss in weight, followed by death due to respiratory depression.

Katz *et al.* (29) found that in mice the brain incorporates deuterium at a slower rate than do the kidneys, liver, and spleen. Lower deuterium incorporation into the brain is probably due to the high lipid content of the brain with its large amount of nonexchangeable hydrogen. Incorporation of deuterium proceeds principally by biosynthesis. The rate at which deuterium is eliminated from the body fluids when deuterium oxide is withdrawn from the body was also studied. The deuterium level of the urine fell rapidly, although appreciable amounts were still detectable after 7 days of withdrawal.

The effects of extensive deuteration on the internal organs of mice and rats were detailed (29, 36), and the effects of deuteration on liver enzyme activity, kidney function, blood constituents, and enzyme systems in rats were discussed (27). Rabinowitz *et al.* (37) studied the effects of heavy water ingestion on hepatic lipogenesis. The physiological effect of extensive deuteration on specific organs and body functions in mice and rats was considered (26, 38).

The physiological consequences of deuteration in two dogs were described (30). (This study was referred to earlier.) Particular attention was paid to alterations in the blood picture and the ECG. Although the two dogs were maintained in a highly deuterated state under different conditions, the changes produced were the same in both dogs. The effects on the blood picture were consistent with those reported in mice (37, 39).

Bachner *et al.* (40) studied the pathological effects of deuteration in mice drinking 75% D_2O. Renal tubular damage was observed as well as destructive changes in the salivary gland ducts. Zunker and McKay (41) also reported kidney damage in mice drinking 75% D_2O. Serious pathological changes were observed in the kidney structure of deuterated mice (42). Thomson (8) reviewed in detail the literature concerning the effects of deuteration on histological

[2] In our experience, mice accustomed to drinking chlorinated water tend to discriminate against deuterium oxide.

changes, on blood and plasma constituents, and on enzyme systems in mammals.

One significant effect of deuteration in mice is on reproductive capacity. Several studies indicated that drinking heavy water produces sterility in mice (43–45). A decrease in litter size and a decrease in the percent of fertile matings were noted when deuterated males were mated with untreated females. Almost complete sterility was produced in male mice who drank 30% D_2O for 6 weeks. Sterile matings resulted from male mice who were returned to ordinary water for at least 2 weeks after a period of deuteration with 30% D_2O. A high incidence of degenerating eggs was found when male mice that were on heavy water for 7 weeks and then were returned to ordinary water for 2 weeks were mated with untreated females. These researchers suggested that when deuterium is substituted for hydrogen, changes are produced in the physical forces that bind macromolecules and that these changes may produce an abnormal genetic material in developing sperm which causes fertilized eggs to degenerate.

Gross and Spindel (46, 47) described in detail the antimitotic activity of deuterium oxide. They attributed abnormalities in the cell division process to viscosity changes caused by changes in the hydrogen bonding properties of deuterated biopolymers. Because heavy water has a pronounced effect on cell division in most organisms, it is not unexpected for rapidly dividing tissues in animals, particularly the reproductive tissues, to be adversely affected by deuterium oxide.

Czajka and Finkel (28) investigated the effect of administration of 25% D_2O in drinking water on the reproductive potential of female mice. Deuteration of the mice was instituted at various times before and after mating to obtain data on the effects on pregnancy and viability of the newborn. Fewer pregnancies were carried to term in deuterated animals, and there was a marked increase in the incidence of wholly nonviable litters along with a decrease in the survival of newborn mice. The ability of female mice to carry through a normal pregnancy and bear viable offsprings was definitely impaired by deuterium. The greatest reduction in fetal viability occurred when the dams were substantially deuterated at the time of fetus implantation (about the 5th day after mating). Fetal viability ceased when the deuterium oxide content of the drinking water exceeded 30%. These untoward effects on reproductive potential were reversed when the female mice were returned to ordinary drinking water.

It was noted that spermatogenesis was essentially normal in deuterated male mice, suggesting that the genetic material in the partially deuterated sperm may be abnormal (48). In another study, prolonged deuteration of mice led to total aspermia, accompanied by the formation of multinucleate giant cells in the testes (49). Hughes and Glass (50) pointed out that the inability of deuterated male mice to produce viable offsprings was due to the failure of the sperm to fertilize the egg and not to the failure of the fertilized egg to develop. The fetuses of deuterated mice were studied, and it was observed that major disturbances occurred in the development of the nervous system (51). The extreme complexity of the effects of deuteration on reproduction was emphasized.

Deuteration in mice at a gradual rate of increase up to one-fifth saturation of the body fluids produced an elevation in body temperature of about 1°, concomitant with an increase in the metabolic rate of about 20% above normal (52). In an earlier study, Barbour (53) noted a lowering of the basal metabolism rate in mice injected subcutaneously with 99% D_2O. Increased levels of deuterium oxide in the body fluids of mice were shown to produce a decrease in metabolic rate and body temperature (26). With a dose of pure deuterium oxide (1.5 ml/10 g of mouse body weight), the temperature of the animals began to fall on the 1st day. With all doses of 50% D_2O, the temperature fall was delayed; with 40% D_2O, the temperature did not begin to fall until the 13th day. A fall in metabolism rate also occurred simultaneously with the fall in temperature.

The effect of heavy water on the heart rate was studied (54). The frequency of pulsation of the excised heart of the frog was slowed in 20% heavy water. The effect of heavy water was similar to that produced by lowering the temperature. This effect was confirmed in a study of ECG changes (55). Barnes and Warren (56) also studied the effects of high deuterium oxide concentrations on the auricles of the turtle heart. They found that 75% heavy water lessened the amplitude of the auricular beat. According to these investigators, the effect of heavy water was equivalent to lowering the body temperature about 5°.

Szczesniak *et al.* (57) determined whether externally applied water can penetrate mammalian skin. Young male rats were immersed for 6–7 hr in 40% heavy water maintained at a temperature of 35°. Blood samples drawn from the pumping heart were analyzed for deuterium content. The analytical data provided evidence that heavy water did penetrate the skin and entered systemic circulation.

The effect of deuterium oxide on wound healing in rats drinking 25% heavy water was studied (58). In these rats the equilibrium deuterium content in the body fluids was 15% deuterium. Skin incisions were made in the abdominal wall, and the effect of deuteration on the healing process was studied. The breaking strength of the wounds after 27 and 47 days of healing was about 40% less in the deuterated animals than in the controls. Cellular population and ground substance were similar in the incisions of both groups. The wounds in the deuterated rats healed abnormally in comparison to the control animals. It was concluded that the untoward effects of deuterium oxide on wound healing were due primarily to the solvation effects of deuterium oxide on developing collagen and that direct incorporation of deuterium into the collagen macromolecule is of minor importance.

Classification of Deuterated Drugs—The number of drugs that have been deuterated has been increasing rapidly. These include compounds contain-

ing a single deuterium atom in a specific molecular position and drugs that are fully deuterated, prepared either by biosynthesis or organic synthesis. Attention has generally been directed to the preparation of the deuterated drug molecule, but the synthesis of intermediate compounds utilized ultimately in the synthesis of a drug has been the object of some research.

The drugs covered in this review are categorized for convenience and the discussion is not based on a strict pharmacological or chemical system. We have made a serious attempt to include all relevant material and apologize for any inadvertent omissions.

HEAVY WATER AS A DRUG

Heavy Water as a Carcinolytic Agent—Deuterium oxide as an agent for controlling tumor growth was one of the earliest therapeutic applications of heavy water to be explored. Because deuteration affects the kinetics of cellular reactions, the consequences of deuteration are more severe the higher the level of metabolic activity. Embryonic tissue and neoplastic tissue are mainly rapidly proliferating tissues and thus should be particularly susceptible to the inhibitory action of deuterium oxide on cell division (36). The hope is that the detrimental effects of deuterium to tumor growth are more severe than the other consequences to the host.

In 1936, Fischer (59) noted that mouse carcinoma cells and fowl sarcoma cells in tissue cultures were unable to grow when the deuterium oxide content of the medium was 50–70%. Early studies (60) indicated that the rate of growth of transplanted lymphosarcoma and mammary carcinoma in mice was reduced to one-half when the animals drank 40% D_2O as compared to mice drinking ordinary water. However, the mice on deuterium oxide died faster because of the toxic side effects of deuterium oxide. Mice with carcinoma drinking 60% D_2O died even sooner than mice on 40% D_2O. Several investigations (61–63) found that mouse and rat tumors were unaffected by heavy water in concentrations as high as 94%.

In more recent studies (36), it was reported that the growth rate of Krebs-2 ascites tumors and P-1534 lymphatic leukemia was reduced in host mice deuterated to a 25% level, which is below the toxic limit for deuteration in mice. The glutamic–oxalacetic transaminase (GOT) determination in the blood serum and in the ascites plasma indicated that there was tumor cell destruction at this level of deuteration.

In a study of Ehrlich ascites tumor in mice, deuterating the mice to 30% increased survival time by about 6 days while deuterating to 40% had no sparing effect, with the toxic effects apparently nullifying any therapeutic advantage (64). It appears to be established that deuteration inhibits tumor growth but does not arrest it at deuterium concentrations tolerated by the host.

Finkel and coworkers (65, 66) observed that the growth of Krebs-2A ascites tumors in mice was depressed when the deuterium oxide levels in the body fluids ranged from 13 to 32%. With increasing deuteration, there was a progressive elevation in serum GOT in tumor-bearing mice and an even greater elevation in the ascites plasma value. These findings suggest that deuteration to high levels leads to tumor cell injury with a release of GOT into the ascites fluid, from where it is subsequently carried into the general circulation and yields elevated serum GOT values.

A combination of deuteration with chemotherapeutic agents may offer beneficial effects in tumor therapy (36), and this possibility was explored (65, 67). A potentiating effect for certain antitumor agents was reported in mice inoculated with solid and ascites Krebs-2 tumors. Fluorouracil was more effective in controlling tumors in mice drinking 30% D_2O than in nondeuterated control mice. Similar results were obtained with mercaptopurine, colchicine, and cyclophosphamide. Additive effects were noted with deuterium oxide and fluorouracil, while a distinct potentiation resulted when deuterium oxide was combined with colchicine or cyclophosphamide. Aminopterin and cyclophosphamide showed an enhanced activity against mouse ascites leukemia in deuterated mice. However, combination therapy was generally accompanied by a shortened survival time; in addition, deuteration may cause an increase in the toxicity of some chemotherapeutic agents.

Katz *et al.* (36) also examined the effect of deuteration on the radiation sensitivity of mice. In some experiments, deuterium oxide produced a slight prolongation of survival time in X-irradiated mice. The effects of deuteration and X-ray treatments on myelogenous leukemia in mice were studied (68). Oral administration of 25% deuterium oxide in drinking water produced a significant prolongation in survival time. X-radiation, in addition to deuteration, gave additive results, but a synergistic effect was not detected. Mice deuterated prior to irradiation were provided some protection against the lethal effects of X-rays.

French *et al.* (69) studied the effect of deuteration in mice infected with ectromelia virus, and they compared the effect on survival time and histopathological characteristics in two groups of infected mice. Mice receiving 30% D_2O in drinking water showed a slight prolongation in survival time and a slight delay in onset of necrosis in the internal organs examined.

The action of nitrogen mustard and deuterium oxide on mitosis and on the nucleic acid content of Ehrlich ascites tumor cells was compared (70). Deuterium oxide only blocks the ability of cells to divide, while nitrogen mustard prevents mitosis irreversibly. The presence of deuterium oxide caused a decrease of desoxyribose bound to purine.

Patients with uterine cervical cancer were treated by local administration of pure heavy water into the tumor and beneficial effects were reported (71). Heavy water also showed a potentiating effect when combined with a chemotherapeutic agent in these studies.

The effects of 30% D_2O administered in the drinking water on a virus-induced mouse leukemia were noted (72). Deuterium was administered in the

drinking water at different time periods after inoculation of the tumor virus. The earlier the initiation of deuterium oxide administration, the higher was the fraction of survivors, suggesting that deuterium oxide may have antileukemia virus activity. Furthermore, 30% D_2O was more beneficial than 15%.

In summary, numerous studies have shown that deuterium does inhibit the growth of tumors but, unfortunately, the concentrations of deuterium required cannot be tolerated by the host.

Heavy Water as Measure of Total Body Water—Heavy water is used for the measurement of total body water in humans by isotope dilution analysis. At the Mayo Clinic (73), this routine diagnostic procedure involves the following basic steps:

1. The patient receives 25 ml of pure deuterium oxide orally.
2. Urine collections are obtained at 2, 3, and 4 hr after administration of the deuterium oxide dose or 2, 4, and 6 hr in patients suspect of fluid retention.
3. Urine is reduced by passage over heated zinc (74).
4. The products of reduction (HD and H_2 gases) are analyzed by a mass spectrometer[3] for ratios of masses 3 and 2. By calibration of the mass spectrometer with known concentrations of HD:H_2, the sample moles percent deuterium oxide "excess" is calculated from the HD:H_2 ratio.
5. The calculation of total body water is then computed from the expression $V_2 = (C_1V_1)/C_2$, where V_2 is the total body water; C_1 and V_1 are the concentration and volume of deuterium oxide administered, respectively; and C_2 is the deuterium content of the urine at equilibrium.
6. Total body water calculated from the last two urine samples are averaged and taken to be the observed total body water. If the values of the last two samples disagree by more than 5%, equilibration is assumed incomplete and an additional specimen is obtained.

The procedure utilized at the Mayo Clinic is based on literature methods (74, 75).

Heavy Water in Neutron Radiography—In recent years, neutron radiography has made possible a number of interesting and potentially useful applications in biomedical research and clinical diagnosis. In certain respects, neutron radiography is similar to X-radiography. However, the two techniques are complementary rather than overlapping; neutron radiographs often reveal information not readily apparent from conventional X-rays.

The subject of neutron radiography was comprehensively treated (76), and various biomedical applications of the procedure were discussed recently (77, 78). The utility of heavy water as a biological contrast agent in slow neutron radiography also was explored (79).

Replacement of a substantial amount of hydrogen with deuterium by exchange with deuterium oxide (the process referred to as deuteration) reduces the attenuation of slow neutrons traversing tissue because the absorption and scattering probabilities of neutrons are much greater for hydrogen than for deuterium. Deuteration is effected by simply contacting the tissue with heavy water, whereby exchange takes place rapidly. The extent of deuteration depends on the chemical nature of the tissue and the number of ionizable or exchangeable hydrogens it possesses. The reader is reminded that hydrogen atoms bonded directly to carbon atoms are not exchangeable, while hydrogen atoms bonded to nitrogen, oxygen, or sulfur are readily exchangeable.

Fatty tissues consist largely of hydrocarbon chains, which contain essentially only nonexchangeable hydrogen. After deuteration, fatty components therefore form an image with high contrast against surrounding muscle, bone, and most body fluids. Fats retain their ^1H content and remain opaque to neutrons. A high level of exchange takes place in muscle tissue, because proteins and carbohydrates have large numbers of exchangeable hydrogen. Since bones are largely inorganic in nature, they are generally radiolucent to neutrons, even without deuteration. The bone marrow does contain variable amounts of connective tissue, proteins, and fats. Extensive deuteration does occur in viable bones, resulting in high contrasts between bones and fatty tissues in neutron radiographs of deuterated sections.

A significant contrast between tumor tissue and surrounding tissue in specimen radiographs was noted (77). Although the specimen was not deuterated in this case, it was suggested that the difference in the neutron absorption pattern may have been due to a higher hydrogen content in the tumor than in normal tissue. In a more recent study (79), tissue slices from an adenocarcinoma of the colon were soaked in heavy water for 24 hr, and comparisons were made among the X-ray, neutron radiograph, and microscopic examinations of the histological section. The neutron radiograph revealed greater contrast between the fat and other tissues. Neutron radiography has been demonstrated to be a unique method for imaging bone marrow directly. The radiograph of a deuterated specimen (a finger soaked in heavy water for 24 hr) showed strong contrast between the radiopaque fatty bone marrow and the radiolucent bone cortex and the connective tissue in the joints.

Heavy water has been employed in a selective imaging technique whereby contrasts were produced by controlled deuteration in localized areas of an animal's body. A heavy water solution containing dextran, glucose, and sodium chloride was infused into both carotid arteries of a living rat over 4 hr. All organs except the brain and aqueous humor of the eye were extensively deuterated. A neutron radiograph of the rat's head revealed high contrast between these organs and the surrounding tissues which became radiolucent to neutrons. When dextran was deleted from the perfusion liquid, the contrast was not observed. Apparently, dextran interferes with the exchange process across the blood–brain and blood–aqueous humor barriers, and this technique may be useful in studying the effect of drugs and other factors on transport across these barriers.

[3] AEI MS 20.

Heavy water as a contrast medium in neutron radiography has limited clinical application at present. It is readily applied to isolated tissue sections by simply soaking the specimen in heavy water. Heavy water may be administered by injection to live research animals within the constraints, of course, of the neutron radiation dose and the toxicity of the heavy water. The thickness of the tissue or organ that may be effectively imaged by neutron radiography increases as the extent of deuteration becomes greater.

Although heavy water in small amounts is generally not toxic to humans or animals, the concentrations required for effective neutron radiography *in vivo* are large and probably beyond the dosage that can be readily tolerated by humans.

Parks *et al.* (79) considered the possibility of effecting deuteration in rats by a single large dose of deuterium oxide administered by intraperitoneal or intravenous injection. They found that rats survived injections of heavy water that did not exceed 40% of the total body fluid weight. This dose resulted in less than 30% deuterium replacement in the body fluids. According to these investigators, this level of deuteration is probably not sufficient for clinical applications of neutron radiography. Even at this level of deuteration, the thickness of the specimen that may be radiographed is only 5 cm.

The extent of deuteration in rats may be increased to 50% by an isolated segment infusion technique. This level of deuteration in the muscles of the hindlimbs did not prove fatal. Similar levels were achieved in the brain of rats by injection of heavy water into the carotid arteries. Parks *et al.* (79) speculated on the possible application of this technique in humans. Deuteration at the 50% level would permit radiography of a limb 7 cm thick while imposing a high, although clinically acceptable, neutron radiation dose. It is questionable whether the risks imposed by extensive deuteration of a human limb would be outweighed by the advantages derived from such a drastic procedure. A review of clinical applications of neutron radiography was presented recently (80).

BARBITURATES

The pharmacological effect of deuterium substitution in butethal[4] (5-*n*-butyl-5-ethylbarbituric acid) was studied (81, 82). 3′-Dideuteriobutethal, which contains two deuterium atoms on carbon 3 of the butyl side chain in the 5-position, was synthesized. The deuterated derivative was compared with the nondeuterated form by intraperitoneal injection in mice and measurement of the duration of induced sleeping time, based on the time from loss to return of the righting reflex. The deuterated barbiturate increased sleeping time better than twofold in comparison with the nondeuterated controls. The biological half-life of the former was about 2.5 times longer than the latter. *In vitro* studies were conducted by incubation with mouse liver microsomes, and an isotope effect of about 1.6 was observed for the *in vitro* rate of hydroxylation of the deuterated compound compared to the nondeuterated molecule.

The major metabolite of butethal is 5-(3-hydroxy-*n*-butyl)-5-ethylbarbituric acid. If the oxidative process involving the penultimate carbon of the side chain of the molecule is the rate-limiting step, replacement of hydrogen with deuterium at the site of oxidation should decrease the rate of oxidation, increase the biological half-life, and prolong the pharmacological activity. Not excluded in these studies is the possibility that deuterated butethal may be less readily metabolized because of a weaker affinity for hepatic microsomal enzymes. Studies with 4′-deuteriobutethal seemed to indicate that the terminal carbon of the side chain may also be involved in the metabolism of butethal.

The effects of deuteration on the activity of 5-(1-methylbutyl)-5-ethylbarbituric acid were investigated (83). The 3′-dideuterio derivative was synthesized, and its anesthetic potency was compared with the ordinary drug in mice. While the onset of sedation was similar in both compounds, the time to peak depression was delayed and the total sleeping time was extended significantly by the deuterated derivative. The biological half-life, determined in dogs, was 1.7–2.0 times greater for the deuterated compound than for the drug of ordinary isotopic composition. The metabolic process and mechanism of action appeared to be the same as those noted for butethal.

The effect of deuterium substitution on the metabolism of phenobarbital in dogs and rats was reported (84). Two deuterated derivatives were studied: one containing a deuterium atom in the *para*-position of the phenyl group at position 5 and the other containing five deuterium atoms in the ethyl group in position 5 in addition to a deuterium atom in the *para*-position of the phenyl group. There was no apparent difference in the rates of disappearance of these compounds and the control from the plasma, which indicated that there was no significant isotope effect. Although the major route of metabolism of phenobarbital in humans, dogs, and rats is through hydroxylation of the *para*-position of the phenyl group, it does not appear that this process is rate limiting. Apparently, biological aromatic hydroxylation lacks a deuterium isotope effect.

Sleep time studies with mice indicated that there were no significant differences in sedative potency among the compounds studied, and there appeared to be no significant difference in the degree of stimulation of drug-metabolizing enzymes. It was also indicated that the 5-ethyl substituent contributes only in a minor way to the sedative activity of barbiturates, perhaps by increasing the lipid solubility of the molecule. There was no apparent alteration in the metabolic pathway of the deuterated drug. Alterations in the pharmacological activity of barbiturates are often dependent on structural changes involving longer and more highly branched alkyl substituents in position 5. Further work based on kinetic isotope studies should be of considerable value in determining the precise effect of molecular changes on the pharmacological function of barbiturates.

[4] Neonal.

The estrogenic activity of α-bromo-α,β-diphenyl-β-pentadeuteriophenylethylene was compared to its nondeuterated analog and found to be considerably less active (85). The pharmacological activity apparently depends on the oxidation to the corresponding bis-*p*-hydroxy derivative, which has a structure similar to diethylstilbestrol. The decrease in activity of the deuterated form is the result of a primary kinetic isotope effect on the oxidation of a C—D bond. The assay procedure (Allen–Doisy test) was based on the induction of estrus in ovariectomized rats. No significant difference was noted between the two compounds in their preoestrus-inducing activity.

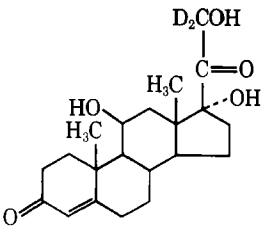
11α-Deutero-17α-hydroxycorticosterone was synthesized, and its biological activity was compared to hydrocortisone (86). In the oral glycogen deposition test in mice and in the oral systemic granuloma assay in rats, the approximate relative potencies of the deuterated derivative compared to hydrocortisone were 0.7 to 1.0 and 1.5, respectively.

The androgenic activity of 3α-deutero-17α-methyl-5α-androstane-3β,17β-diol was compared to the corresponding ^{1}H derivative (87). The 3-hydroxy form is probably androgenically inactive as such and must be oxidized *in vivo* to the keto derivative, which is a potent androgen. If the oxidation to the 3-ketone with the loss of the 3α-hydrogen or 3α-deuterium is the rate-determining step, then the 3α-deutero compound should be oxidized more slowly and therefore exhibit lower androgenic activity than the 3α-hydrogen compound.

This possibility was tested in castrated male rats, and it was found that the ^{1}H compound was 3.36 times more active than the deuterated compound on the basis of the ventral prostate response and 4.65 times more active in the response of the seminal vesicles, demonstrating that oxidation to the keto form is a necessary step for androgenic activity in the rat. This result is interpreted to be due to an *in vivo* isotope effect on the oxidation of the 3-hydroxy group, which involves the cleavage of the 3α-deuterium—carbon bond. No difference was noted in androgenic activity between testosterone and 17α-deuterotestosterone in the castrated rat when administered orally or subcutaneously.

A number of deuterated deaminooxytocin derivatives, variously substituted in the 1β-mercaptopropionic acid position or in the 5-asparagine position were synthesized (88, 89). The biological activity of the deuterated derivatives was compared with deaminooxytocin. Statistical analysis of the data revealed that, within experimental error, all compounds elicited the same vasodepressor and oxytocic activities. It was also shown that deuterated amino acids can be incorporated into peptides, using ordinary synthetic procedures, without significant loss of deuterium.

Pitman *et al.* (90) synthesized hydrocortisone (I) in which two deuterium atoms were incorporated in the C-21 position. Its stability against degradation in aqueous solution under both aerobic and anaerobic conditions was compared with that of hydrocortisone. Under oxidative conditions, the deuterated compound was more stable than the protio form of hydrocortisone. The explanation may be that the reaction mechanism for the degradation involves a rate-determining enolization in the C-17 position of the dihydroxyacetone side chain.



I: hydrocortisone-d_2

Lemieux *et al.* (91) studied the *in vivo* oxidation of the antidiabetic drug tolbutamide (*N*-*p*-toluenesulfonyl-*N'*-*n*-butylurea) to *N*-*p*-carboxybenzenesulfonyl-*N'*-*n*-butylurea. The rate of oxidation in adult human subjects was essentially unaffected by complete deuteration of the methyl group of the toluene residue, as measured by the rate of the excretion of the oxidation product. Tagg *et al.* (92) identified hydroxymethyltolbutamide as the major oxidation product in the urine of rats and rabbits administered tolbutamide, a finding confirmed by Thomas and Ikeda (93). The tolbutamide was shown to be metabolized by liver microsomes in the rat. Only a small isotope effect (H/D = 1.14) was noted in comparing the oxidation rates of tolbutamide containing three deuterium atoms in the methyl group with nondeuterated tolbutamide. The small isotope effect indicates that the breaking of carbon—hydrogen bonds is not the rate-determining step in the oxidative reaction.

The specificity of the pharmacological action of dopamine was examined by substituting deuterium for hydrogen in the alkyl side chain (94). Both α,α'-dideuteriodopamine and β,β'-dideuteriodopamine were synthesized chemically and tested for activity in the dog. No difference in pharmacological activity was noted between the deuterated dopamines and the protio analog, suggesting that this agent acts directly on a dopaminergic receptor without involving the formation of intermediate compounds. If hydroxylation were the rate-determining step involved in the pharmacological activity, then the rate of *in vivo* conversion to norepinephrine would have been reduced due to a primary deuterium isotope effect involving the β-carbon position. If dopamine activity were effected through the monoamine oxidase enzyme, then a comparable effect would have been noted as a result of substitution in the α-carbon position. The absence of these effects supports the concept that dopamine acts directly on a receptor.

Barbour and Dickerson (95) observed the effect of deuterium oxide at 20, 50, and 75% on the hydrolysis of acetylcholine bromide both in the presence and in the absence of cholinesterase. Dog blood serum served as the source of cholinesterase. Deuterium oxide slowed the hydrolysis of acetylcholine to the same extent whether or not enzyme was present. The

rate of hydrolysis was decreased 10.6% in 20% D_2O, 23% in 50% D_2O, and about 33% in 75% D_2O. Apparently, the inhibition of hydrolysis cannot be attributed to poisoning of the enzyme.

Acetylcholine was synthesized with three hydrogen atoms of the acetyl group replaced by deuterium atoms; about a 30% reduction in potency was reported as a result of the deuterium substitution (96).

Belleau (23) synthesized acetylcholine bromide variously substituted with deuterium: Compound A, three deuterium atoms in the acetyl group as synthesized earlier (96); Compound B, two deuterium atoms in the choline part of the molecule alpha to the ester linkage; Compound C, two deuteriums in the choline part beta to the ester linkage; and Compound D, full deuteration of the three methyl groups attached to the quaternary nitrogen. Michaelis constants were obtained for the isomeric deuterated acetylcholine bromides. The respective constants were identical, $4.5 \pm 0.3 \times 10^{-4}$ *M*. Each compound was further tested for cholinergic activity, using the hypotensive response of the dog to intravenous administration before and after treatment with physostigmine. The absence of deuterium isotope effects on the binding of acetylcholine with the esterase enzyme or the cholinergic receptor is at variance with previous observations (96) or a 30% reduction in potency for Compound A.

ANESTHETICS

The anesthetic properties of deuterated chloroform in dogs and mice were studied (97). The exhaled deuterated chloroform had the same isotopic composition as the inhaled chloroform, which indicated that no exchange had taken place. In mice, the response to the anesthetic action of both forms of chloroform was similar. In dogs, the anesthetic syndrome was the same for both compounds. But, on the basis of limited studies, it appeared that deuterated chloroform was somewhat more potent; 20% more ordinary chloroform was required to achieve the same stage of anesthesia as the deuterated form when compared in the same five dogs. It did not appear that chloroform was metabolized to any significant extent.

The anesthetic properties of fully deuterated ethyl ether were also studied (98). The anesthetic syndrome in mice was identical to that for the protio analog. On the basis of mass spectrometric analysis, it was determined that no deuterium exchange had taken place, suggesting that the anesthetic action of ether does not depend on chemical interaction with the biological systems of the body and that it is evoked by the intact molecule involving weak interactions rather than chemical reactions. In an earlier study (99), it was shown that fully deuterated ethylene and acetylene elicited anesthesia in dogs and cats similar to that of ordinary ethylene and acetylene.

The effect of deuterium oxide on the local anesthetic activity of procaine was studied, using the cornea of the guinea pig as the test site (100). The ED_{50} of procaine in deuterium oxide was found to be about one-half of the ED_{50} for procaine in water. No significant difference in the toxicity of procaine was found with either solvent. Stability studies indicated that procaine is more stable in deuterium oxide solutions than in water solutions at comparable pH values. Greater activity of procaine in deuterium oxide was accounted for by the fact that procaine free base is more stable in deuterium oxide solutions and also that a deuterium oxide solution of procaine contains more free base than the corresponding aqueous solution at comparable pH values[5].

The effect of α-deuteration on the metabolism and depressant action of ethanol was studied, using the mouse as the test animal (101). Ethanol and α-deuterioethanol were administered intraperitoneally (4 g/kg). The deuterated compound slowed the rate of metabolism from 794 to 692 mg/kg/hr. Sleeping time was increased from 110 to 170 min for the deuterio compound after the injection of 5 g/kg for both compounds.

ANTIBIOTICS

Shaffer *et al.* (106) examined the effects of deuteration on the growth rate and morphology of the fungi *Penicillium notatum* and *Aspergillus fonsecaeus*. These organisms were grown over 3 months in media containing 10–99% D_2O and protio substrates. Severe growth inhibition was noted at the 50% D_2O level in the *P. notatum* cultures and at the 66% level in the *A. fonsecaeus* cultures. In both fungi, the normal floating-mat type of growth was replaced at the highest deuterium oxide levels by a submerged flocculated type of growth. Pigment production decreased as the deuterium oxide level increased, and sporulation was inhibited in both fungi. *P. notatum* sporulated in media containing 0, 10, and 25% D_2O, while *A. fonsecaeus* sporulated in all but the 99% D_2O medium. Combustion analysis data indicated that the extent of deuterium incorporation into fixed positions in the mycelia was only about 50% that of the deuterium level of the medium, indicative of the ^1H contribution from the ^1H- glucose used in the culture medium. The effect of deuteration on antibiotic production was not investigated in these studies.

Nona *et al.* (107) investigated the effect of deuteration on the growth of *Penicillium janczewskii*, a mold that produces the antifungal antibiotic griseofulvin. The mold was cultured by the shake culture technique and by surface culture in media containing 50, 75, and 99.6% D_2O with protio or deuterio sugars as the carbon source. Griseofulvin titers, pigmentation, and mycelial dry weights were all markedly depressed as the deuterium oxide content of the medium was increased. Corn steep solids and vitamin B complex appeared to enhance both mycelial growth and griseofulvin production in highly deuterated media. In a highly deuterated environment, the mold apparently uses nutrients to support vital metabolic activities and meets only those needs required for

[5] Some literature reports (102–105) have dealt with relating the pD scale and the operational pH in deuterium oxide solutions. Generally, a correction of about 0.40 pH unit is added to the "apparent" pH of a heavy water solution as measured by a conventional glass electrode.

survival. Griseofulvin biosynthesis appears to be nonessential for mold survival and can be depressed without damage to the organism.

A subsequent study (108) found a "replacement culture" technique to be useful for obtaining nonessential metabolites. The mycelial contents of shake cultures grown in a water medium were used as the starting tissue for preparative replacement cultures. After about 1 week's growth, the vegetative tissue was removed from the culture, washed free of water with deuterium oxide, and redispersed in a deuterium oxide medium. When the replacement deuterium oxide medium contained deuterio sugars, fully deuterated griseofulvin was produced; when protio sugars were included in the deuterium oxide medium, the griseofulvin was partially deuterated. In the ^{2}H-cultures, griseofulvin did not accumulate to any appreciable extent before the 15th day of incubation. Therefore, the ^{1}H-mycelium younger than 15 days could be used in a replacement culture without fear of product contamination. This procedure reduced the 50–60 days generally required for maximum antibiotic production by the direct fermentation method to about 30–35 days. No significant increase in antibiotic production was noted by the latter method. The antibiotic was isolated and purified by TLC. PMR (^{1}HMR) spectral analysis indicated complete deuteration in the sample obtained from a fully deuterated replacement medium.

The antifungal activity of the isolated deuterated antibiotic was compared (109) with that of ordinary griseofulvin by the plate assay method, with *Microsporum gypseum* as the test organism, and the deuterated antibiotic was found to be about 10% more active than the protio form. The increased activity of the deuterated griseofulvin may be due to an increased efficiency of action resulting from a primary isotope effect at the target site where the rupture of C—D bonds may be directly involved, or the increased stability of the deuterated molecule may play a role in that the fungus may not be able to metabolize deuterated griseofulvin as readily as it does the protio form. However, a better understanding of the mechanism of antifungal activity of ordinary griseofulvin is needed before the effect of deuterium replacement is more completely understood.

Mohammed *et al.* (110, 111) reported the effects of deuterium oxide on the growth and antibiotic production of 13 low-producing strains of two species of *Penicillium* and three high-producing strains of *P. chrysogenum*. However, the deuterated antibiotic was not isolated in these investigations.

Deuterophenylacetyl-^{15}N-DL-valine was synthesized where 41% of the hydrogen in the benzene ring was replaced with deuterium (112). This compound was used to study the incorporation of phenylacetic acid and valine into the benzylpenicillin molecule during fermentation. It was learned from deuterium analyses that the phenylacetyl moiety was incorporated directly into benzylpenicillin. Very little ^{15}N was found in the molecule, from which it was concluded that the nitrogen of the amide group did not originate from the added labeled valine.

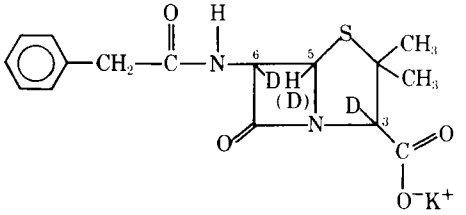
Deuteriobenzylpenicillin, in which the benzene ring contained five deuterium atoms, was utilized as a label for monitoring net penicillin production during fermentation (113).

Deuteriobenzyl-d_7-penicillin was synthesized chemically and used to study the deuterium isotope effect on the biological activity of penicillin (114). The biological activity of the selectively deuterated penicillin was compared to that of the protio form by a turbidimetric assay procedure, using *Staphylococcus aureus* as the test organism. The relative potency (penicillin to deuteriopenicillin) was reported to be 1.25. It was suggested that since the binding of the benzylpenicillin molecule to proteins is apparently mediated by the benzyl moiety and not by the β-lactam-thiazolidine nucleus (115), alteration in the rate and strength of binding may account for decreased bioactivity of the deuterated molecule.

P. chrysogenum (Wisconsin strain 53-414) was cultured in a defined medium containing glucose, acetate, lactate, and phenylacetate as carbon sources and pure deuterium oxide as the solvent (116). Partially deuterated benzylpenicillin was isolated from the culture containing protio substrates and deuterium oxide. The extent of solvent participation in the biosynthesis of penicillin was determined by analysis of the ^{1}HMR spectrum. The culturing procedure involved a modification of the replacement technique, whereby a thick seed culture was produced in a protio medium. The mycelial pad was separated, rinsed with deuterium oxide, and used as an inoculum for the deuterated culture.

Incorporation of deuterium appears almost complete at the C-3 position of the thiazolidine ring and the C-6 position of the β-lactam ring (II). Only partial incorporation of deuterium (60–70%) at the C-5 position was noted. The deuterium atoms in the C-5 and C-6 positions probably arise during the biosynthesis of cysteine. The deuterium at the C-3 position may arise from the precursor valine or in the final step of biosynthesis involving the thiazolidine ring closure, whereby a deuterium may be incorporated through solvent participation.

A highly deuterated penicillin was obtained (117) by using deuterated substrates in the replacement culture medium. Analysis of the ^{1}HMR spectrum revealed an average deuterium replacement of 89%. Complete deuteration was noted in the phenylacetyl group, at the C-3 position of the thiazolidine ring, and at the C-6 position of the β-lactam ring. Partial ^{2}H incorporation (64%) was noted at the C-5 position of the β-lactam ring and in the methyl groups (77%) at the C-2 position of the thiazolidine ring. When deuterated pyruvate (the precursor for the two meth-



II: partially deuterated benzylpenicillin

yl groups attached to C-2) was included in the medium, the methyl group contained only ^1H. However, when deuterated pyruvate was incorporated in the seed culture medium, deuterium was present in the methyl groups. The methyl groups are apparently preformed in the seed inoculum with little or no additional biosynthesis of methyl groups during fermentation.

The antibiotic potency of highly deuterated benzylpenicillin was compared (118) with the compound of normal isotopic composition by the USP cup-plate assay, with *Sarcina lutea* as the test organism. A relative potency (H/D) of 1.23 was obtained. Thus, as also noted by Laskar and Mrtek (114), deuterium in the penicillin molecule affected the potency adversely. Penicillin acts by inhibiting cell wall formation, possibly at the cross-linking level in mucopeptide synthesis. According to Wise and Park (119), the penicillin molecule fits the active site of a hypothetical transpeptidase enzyme. The β-lactam ring of the penicillin molecule apparently reacts with the enzyme and inactivation results. Penicillin has been shown (120) to be bound irreversibly to a "penicillin-binding component" of bacterial cell walls. The component is presumably the transpeptidase enzyme. The binding strength of deuterated penicillin to the enzyme would be expected to be less than the protio form of penicillin, thus accounting for a decrease in potency.

The deuterium isotope effect on the antibiotic activity of chloramphenicol was studied (121). This antibiotic apparently interferes with the synthesis of proteins essential in cell division (122). The antibiotic may bind irreversibly to an enzyme active center or covalently to an enzyme product used in a subsequent biosynthetic step. Chloramphenicol may act through a hydrogen radical transfer mechanism (123). The benzylic C—H bond is proposed as the point of attack at the receptor site. The antibiotic apparently forms a free radical, which binds covalently to the enzyme. The α-deuterio derivative should possess a lower activity if, in fact, the rate-determining step is a deuterium radical transfer.

α-Deuteriochloramphenicol was synthesized and its antibacterial activity was compared with the protio analog using *Escherichia coli* as the test organism. An isotope effect of 1.27 was obtained based on inhibitory rate constant measurements; *i.e.*, the deuterio form had only 80% of the activity of the protio species. This result is consistent with the hypothesis that the benzylic C—H bond is broken in the rate-determining step and with the concept that the antibiotic blocks an enzyme covalently.

ALKALOIDS

Beckett *et al.* (124) postulated that *N*-dealkylation at the central receptor site is the initial process responsible for morphine analgesia. Since this is an enzymatic oxidative reaction, involving the breaking of C—N and C—H bonds, the rate of demethylation and the pharmacological activity depend on the ease with which these bonds are oxidized.

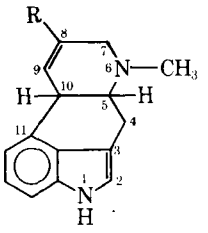
Morphine, in which the *N*-methyl group was completely deuterated, was prepared and then the effect of deuteration on the analgesic potency and enzymatic *N*-demethylation of the molecule was studied (125, 126). A significant lowering in potency in mice (tail-flick method), as well as the LD_{50}, irrespective of the mode of administration was found; no differences in time of onset, maximal effect, or duration of action were detected. The potency ratio (morphine to deuteriomorphine) ranged from 1.56 to 3.00, depending on the mode of administration. Absorption effects were ruled out since similar results were obtained when the drugs were administered intracerebrally and intravenously.

The rate of morphine demethylation was studied *in vitro* by measuring the formaldehyde released when morphine was incubated with rat liver microsomes. An isotope effect (k_H/k_D) of 1.4 was reported. Based on the Michaelis and velocity constants, the observed isotope effect apparently results from a weaker bonding of the morphine molecule to the *N*-demethylating enzyme. The difference in potencies may thus be due to a decreased affinity of the deuterated morphine for the receptor site, assisted by a diminished reaction rate of the bound material.

A series of studies (127–129) examined the biosynthesis of a water-soluble group of clavine alkaloids elaborated by certain strains of *Claviceps purpurea*. Conditions were established for the optimal growth of the *Claviceps* strain 47 A-Tyler in a fully deuterated form (127). At high concentrations of deuterium oxide, saprophytic growth was highly dependent on the nature of the carbon source. This strain of *Claviceps* was well maintained and produced high yields of alkaloids in water on a medium containing a mixture of 5–15% protiomannitol and 0.5% protiosuccinic acid.

At deuterium oxide levels up to 75%, growth and alkaloid production were realized in submerged and mat cultures with 7% protiosuccinic acid as the only carbon source. Reduction of the phosphate content in the nutrient medium stimulated alkaloid production in water cultures but only slightly in deuterium oxide cultures. Complete deuteration of the organism and successful maintenance of the culture were achieved on a low phosphate nutrient medium containing 2% fully deuterated monosaccharides and 0.5% deuteriosuccinic acid plus traces of vitamins. However, at high levels of deuterium oxide, alkaloid production was severely impaired, although the organism grew well.

Further studies (128) used a replacement culture technique for the biosynthesis of highly deuterated clavine alkaloids. Cultures grown in an ordinary hydrogen environment (water and protio substrates) were transferred to a deuterium oxide medium containing only phosphate buffer salts and tryptophan, phenylalanine, succinic acid, and sugars, either in ^1H or ^2H forms. Multimilligram amounts of deuterated alkaloids were obtained from preparative replacement cultures containing phenylalanine and deuteriosuccinic acid as substrates in pure deuterium oxide. The alkaloid fractions consisted of elymoclavine (III)



III: elymoclavine: R = CH_2OH
IV: agroclavine: R = CH_3

with minor amounts of agroclavine (IV) and were isolated by solvent extraction.

No alkaloids were elaborated when deuterated tissue was used in a replacement culture experiment independent of whether the replacement medium was made with water or deuterium oxide. Alkaloid production is apparently a nonessential activity, and organisms that produce this class of compounds do so only under quite specific and probably nonobligatory conditions. As indicated by Mrtek *et al.* (128), a primary effect of deuteration in biological systems appears to be the suppression of nonessential metabolism, which in a way represents an afterthought for the organism. High deuterium oxide concentrations make *Claviceps* use available metabolites for essential nutrition at the expense of alkaloid synthesis, a result similar to that observed by others (107, 116). The replacement culture technique provides a mechanism for circumventing at least some problems encountered in the adaptation of the fungus to a deuterated environment.

The third study of this series (129) provided a detailed magnetic resonance spectral analysis of a number of the clavine alkaloids. The spectra were used to establish the isotopic composition of elymoclavine biosynthesized in deuterium oxide with selected protio substrates. The results indicated a large degree of solvent participation during alkaloid biosynthesis. Alkaloids produced in this way contain both hydrogen and deuterium and are referred to as isotope hybrid compounds. The hydrogen-to-deuterium ratios at the different hydrogen positions in the molecule can be determined from ^{1}HMR data. Elymoclavine obtained from replacement cultures in 99% D_2O with protiosuccinate and protiophenylalanine as substrates was highly deuterated at all positions. The hydrogen atoms at positions 10 and 17 (III) were completely deuterated, and the hydrogens at positions 4, 5, 6, 7, and 9 contained 68–86% deuterium, indicating extensive labilization of carbon-bound hydrogen during biosynthesis.

Blake *et al.* (130) studied the effects of deuteration on peppermint (*Mentha piperita*). This appears to be the first detailed study of the effects of extensive replacement of hydrogen by deuterium on the growth of a higher plant. Cuttings were grown hydroponically in nutrient solutions containing varying concentrations of deuterium oxide. There was a progressive inhibitory effect on growth with an increase in the deuterium oxide content of the nutrient. The effect became particularly pronounced at the 50% level, and growth essentially stopped in 70% D_2O. There was impairment in the ability to flower at all levels of deuterium oxide, which seems to parallel fertility effects in mammals as noted earlier. This probably results because of the high metabolic activity associated with reproduction and the large deuterium kinetic isotope effect that might be expected in such a situation.

A comparison was made between the deuterium content of the water distilled from the deep-frozen plant (labile water), which represents the isotopic composition of the exchangeable hydrogen in the plant, and the deuterium content of the water obtained from the combustion of dried plants, which represents the organically bound exchangeable and nonexchangeable deuterium. The deuterium content of the former was found to be approximately 75% that of the nutrient solution in which the plant was grown, while the latter was about 50% of the deuterium content of the nutrient solution. Thus, the ratio of organically bound to labile deuterium was about 0.66, somewhat higher than that reported for animals (29).

A subsequent study (131) described the histological effects of deuterium. The major effect of deuterium on the growth of peppermint appeared to be inhibition of cell division; in general, the effects were more pronounced in actively growing tissues. The third study of this series (132) reported the effects of certain growth regulators on the growth of mint at different concentrations of deuterium oxide in the nutrient solution. The inhibitory effects of deuterium on the cellular level were not reversed by the plant growth hormones used, but maleic hydrazide, usually considered to be a plant growth inhibitor, stimulated the growth of partially deuterated peppermint plants. The metabolic effects of maleic hydrazide are apparently converted into stimulation because of the contrasted metabolic effects of deuteration.

Although peppermint is not an alkaloid-producing plant, the studies described with mint did suggest the possibility of utilizing an alkaloid-producing plant for the preparation of deuterated or partially deuterated alkaloids by biosynthesis. A series of studies was undertaken involving the growth of the plant *Atropa belladonna* in heavy water. This plant produces atropine and related alkaloids. The general morphological effects of deuteration on belladonna plants grown in nutrient solution containing 30, 50, and 60% D_2O were described (133). The extent of deuterium uptake by the plant, the effect of deuteration on seed germination, and the effect on alkaloid content were examined.

The growth of belladonna was strongly inhibited by deuterium oxide concentrations greater than 50%, and the morphological effects were qualitatively similar to those reported earlier for peppermint plants. In 60% D_2O, the effects were so drastic that the plants were not recognizable as belladonna; plant elongation was slight, only a few pale poorly expanded leaves developed, and there was no indication of inflorescence. In general, with increasing concentrations of deuterium oxide, there was a marked reduction of normal growth patterns; above 50% D_2O in the

medium, there was a loss of reproductive capacity.

Most disappointing, however, was the progressive inhibitory effect on alkaloid production as the deuterium content of the plant increased, a situation not at all unlike that described for the clavine alkaloids (128). In fact, plants grown in 60% D_2O medium did not produce sufficient plant material to permit an analysis for alkaloid content.

A subsequent study in this series described the effects of deuteration on flower, fruit, and seed development in *A. belladonna* grown in high concentrations of deuterium oxide (134). Intercomparison of plants grown through three generations of exposure to heavy water indicated that the most drastic growth inhibition took place during first generation growth in a deuterium environment. After three generations, some adjustment to the deuterium in the environment appeared to occur as the plants showed better growth in 50% D_2O. Blake *et al.* (135) reported that in 50% D_2O, only 75% of belladonna seeds germinated; in concentrations above 50% D_2O, the germination rate was reduced to zero.

A replacement culture technique (128) was used to study the effect of deuteration on alkaloid production in *A. belladonna* (136). Belladonna plants grown to maturity in aqueous (water) nutrient solution were transplanted to nutrient solutions containing 50, 60, 75, and 100% D_2O. The plants placed into a 99.7% D_2O medium showed drastic effects of deuteration within hours, and all plants died in several days. The plants transplanted into 75% D_2O survived about 3 weeks. The 50 and 60% D_2O plants survived the stresses imposed by deuterium and were harvested after a growth period of 7.5 months. Alkaloid production was reduced drastically, ranging from about one-third to one-tenth that of the control plants. The alkaloid content was too small and the amount of plant material collected too little to permit isolation of alkaloid for further study. Although total alkaloid analysis and analysis for atropine and scopolamine content were performed, the isotopic composition of the alkaloid fraction was not determined. Although the time for transplantation was selected when the rate of alkaloid synthesis in the plant was greatest, extremely low alkaloid analyses were obtained for the deuterated plants at harvest. It seems likely that alkaloid production was completely inhibited upon transfer of the plants from normal growth in water to the deuterated nutrient.

All studies reported in this section, much of the work summarized in the section on antibiotics, and some of the investigations covered in the amino acid section were conducted in a series of collaborative efforts between the Chemistry Division of Argonne National Laboratory and the College of Pharmacy of the University of Illinois. These collaborative programs were the subject of a recent article (137).

AMINO ACIDS

The successful culturing of several species of algae in 99.6% D_2O was reported about 15 years ago (138–140), and the details of mass culturing on a continuous basis were published (141) shortly thereafter. The algae are grown autotrophically in deuterium oxide containing inorganic salts, with carbon dioxide as the source of carbon. Under these conditions, all compounds synthesized by the algae are fully deuterated[6].

The identification and isolation of fully deuterated amino acids and monosaccharides in alga hydrolysate were described (142). The isolated amino acids included deuterioalanine, deuterioglutamic acid, deuterioaspartic acid, and deuterioglycine. Deuteriomannose and deuterioglucose were also isolated in greater than gram amounts by chromatography on cellulose, and the amino acids were obtained by passage of the ionic fraction of the hydrolysate through an ion-exchange[7] column. Cohen and Putter (143) reported the preparative separation of most deuterated amino acids present in the protein hydrolysate prepared from the algae *Scenedesmus obliquus* grown in heavy water.

The chemical synthesis of a series of fully deuterated amino acids was described including deuterated glycine (144); deuterated aspartic acid, glutamic acid, asparagine, and glutamine (145, 146); and deuterated phenylalanine (147). One objective of these studies was to prepare highly deuterated derivatives for use in the synthesis of deuterated analogs of oxytocin and vasopressin.

A nonenzymatic transamination method was employed for the preparation of "amino acids" deuterated in the α-position (148). The protio amino acid was heated for 70 hr at 120° with salicylaldehyde in the presence of copper ion in deuterium oxide. The α-substituted deuterio amino acids prepared in this way included glycine, alanine, glutamic acid, valine, isoleucine, aspartic acid, and phenylalanine. α-Deuterioglutamic acid and α,β,β-trideuterioglutamic acid were prepared by enzymatic transamination of glutamic acid with pig heart glutamate–pyruvate aminotransferase and pig heart glutamate–oxaloacetate aminotransferase, respectively (149). α-Deuterioglutamic acid was synthesized by heating the acetylamino acid at 100° in deuterium oxide in the presence of acetic acid-d_1 and acetic anhydride (150).

Guroff *et al.* (151, 152) synthesized phenylalanine with a deuterium atom exclusively in the *para*-position of the phenyl ring by the catalytic reduction of the *para*-bromo derivative of phenylalanine. The *p*-deuteriophenylalanine was used as a substrate for *Pseudomonas* phenylalanine hydroxylase. Hydroxylation of the phenylalanine resulted in the formation of *p*-tyrosine, with the concomitant migration of the deuterium atom to the *meta*-position. Although a significant isotope effect was observed in this study, Guroff and Daly (153), in a subsequent report dealing with the migration of a deuterium atom during phenylalanine hydroxylation, concluded that no isotope effect was observed. The same substrate and enzyme

[6] The term "fully deuterated" and the prefix "deuterio" are used here to indicate that nonexchangeable hydrogen atoms have been replaced with deuterium atoms.

[7] Dowex-50.

system were used in both studies. The authors explained that the discrepancy was apparently caused by the presence of impurities in the deuterated substrate used in the earlier study, which resulted in an overestimation of the actual amount of phenylalanine present. This hydroxylation-induced migration was named the "NIH shift" and was the subject of an extensive review (154). Frequent reference is made to the utilization of deuterated substrates in studies concerned with the elucidation of the mechanism involved in this important phenomenon.

The preparation of specifically labeled or fully deuterated amino acids has provided useful compounds for the synthesis of deuterated drugs, for studying the mechanism of biochemical reactions, and for kinetic isotope studies.

The deuterium kinetic isotope effect in nonenzymatic transamination of alanine was studied with pyridoxal (155). L-Deuterioalanine was isolated from the hydrolysate of the algae *Scenedesmus obliquus* grown in deuterium oxide (142). In the solvent water, the reaction of L-protioalanine with pyridoxal was 2.4 times faster than for L-deuterioalanine, and in deuterium oxide the reaction with L-protioalanine was 2.9 times faster than with L-deuterioalanine. Lin *et al.* (156) determined the isotope effect in the nonenzymatic transamination of L-glutamic acid. L-Deuterioglutamic acid was isolated from deuterated algae as described earlier (142). The reaction with protioglutamic acid was 2.1 times faster than with deuterioglutamic acid. The reaction was found to be general acid–base catalyzed.

Fang *et al.* (157) investigated the kinetic isotope effects in the enzymatic transamination of fully deuterated glutamic acid (isolated from deuterated algae hydrolysate), α-deuterioglutamic acid (synthesized from protioglutamic acid), and 3,3,4,4-d_4-glutamic acid (synthesized from fully deuterated glutamic acid). The glutamate–oxaloacetate aminotransferase enzyme system was employed in the transamination reactions. The two compounds with deuterium in the α-position showed an isotope effect (V_H/V_D) of 1.85, while the compound having a hydrogen in the α-position and deuterium atoms in the β- and γ-positions showed an isotope effect (V_H/V_D) of 1.26. The latter isotope effect was explained in terms of a secondary isotope effect where the deuterium atoms of the molecule decrease the rate of α-hydrogen elimination, the rate-limiting step of the reaction. The data indicated that the bond to the α-carbon and not the bond to the β-carbon is broken during transamination.

L-Deuteriophenylalanine was isolated from the ionic fraction of the cell wall hydrolysate of algae grown in a medium containing 99.6% D_2O (158). The transamination rate of the protio and deuterio analogs to the corresponding analogs of phenylpyruvic acid was compared by using rat brain extract as the enzyme system. The deuterium isotope effect (V_H/V_D) was found to be 1.64.

Partially (37%) deuterated DL-phenylalanine was used to study the metabolic pathways by which phenylalanine is converted to tyrosine (159). A normal patient, a patient with phenylketonuria, and a patient with hyperphenylalaninemia were administered doses of 200 mg/kg of deuterated phenylalanine, and the deuterium content of the excreted aromatic acids was determined. In the healthy subject, the *m*-hydroxyphenylacetic acid produced by metabolism was markedly deuterated, but this was not the case in the patient suffering from phenylketonuria. Thus, *m*-hydroxylation of phenylacetic acid in this disease appears to be blocked. This was also true for the hyperphenylalaninemic patient. No deuterium was incorporated into the metabolites of tyrosine by patients with phenylketonuria and hyperphenylalaninemia. That mandelic acid is a product of phenylalanine metabolism was confirmed in both the healthy as well as the phenylketonuric patient. It was suggested that another metabolic pathway for phenylalanine degradation may exist in patients with hyperphenylalaninemia. Some reservation was expressed about the conclusions arrived at since DL-phenylalanine was used in these studies rather than the naturally occurring L-form.

Methionine containing a deuteriomethyl group was used to study the mechanism of transmethylation in biological systems (160, 161). It was shown clearly that the methyl group is transferred from methionine to choline and creatine in animals. It was further established that the hydrogen atoms of the methyl groups do not exchange with the hydrogen atoms of the body water and that the methyl group is transferred as a unit in the transmethylation process.

Triggle and Moran (162) investigated the kinetic isotope effect involved in the rate of oxidation of α-deuteriophenylglycine and α-deuteriotyrosine by L-aminooxidase. The synthetic procedures yielded 95% deuterium labeling in the α-position. An isotope effect (k_H/k_D) of 1.41 was reported. On the basis of the isotope effect, it was clearly established that removal of an α-hydrogen is rate determining in the enzymatic oxidation of tyrosine and phenylglycine.

Rhodes *et al.* (163) used L-deuterioglutamic acid and L-deuterioaspartic acid, isolated from the ionic fraction of the hydrolysate of deuterated algae, as the starting compounds in the synthesis of 2,6-dioxo-3-phthalimidopiperidine-3,4,4,5,5-d_5 and 2,5-dioxo-3-phthalimidopyrrolidine-3,4,4-d_3. Inasmuch as these compounds are partially deuterated forms of thalidomide and *N*-phthalylaspartimide, respectively, and because both substances elicit sedative and teratogenic activity, the effect of deuterium substitution on these activities can be evaluated. Such studies may provide information about the mechanism by which these agents produce their teratogenic effects.

SYMPATHOMIMETIC AMINES

Deuterium oxide had sympathomimetic activity when substantial amounts of it replaced the body water of mice (164, 165). At the 20% saturation level, the animals showed definite signs of sympathomimetic stimulation including exophthalmos and the pilomotor reaction. It was suggested that deuterium oxide protected the natural sympathetic hormones

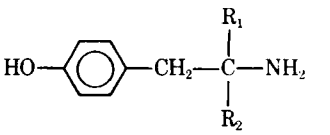
by delaying their disappearance from the body.

Fully deuterated *dl*-epinephrine was synthesized in which 90% of the hydrogen atoms were substituted with deuterium atoms (166). Injection into a cat produced an elevation in blood pressure indistinguishable from that resulting from ordinary epinephrine. It was concluded that deuteration caused little, if any, change in the physiological activity of epinephrine.

The effect of deuterium substitution in certain sympathomimetic amines on their interaction with the enzyme monoamine oxidase was studied (167–169). (The catecholamines, such as epinephrine and norepinephrine, are apparently inactivated primarily through *O*-methylation rather than through attack by monoamine oxidase.) Tyramine was synthesized in which the α-hydrogens were replaced with deuterium atoms. Since tyramine is a good substrate for monoamine oxidase, the rate of oxidation by the enzyme should be decreased in view of the fact that C—D bond cleavage is involved in the reaction. An isotope effect (k_H/k_D) of 2.3 for the oxidation was observed. The magnitude of the pressor response, as noted in cats given barbiturates, was about the same for both compounds, but the duration of effect (pressor area) was twice as prolonged for the dideuterio compound. The magnitude and duration of nictitating membrane contractions were likewise twice as large for the deuterated derivative. No difference in pressor response was noted when *l*-α-dideuteriorepinephrine was compared with *l*-norepinephrine, indicating that at the effector cell level norepinephrine is probably not attacked by monoamine oxidase.

The stereospecific nature of the observed isotope effects was also studied. On the basis of a three-point contact between substrate and enzyme or receptor site, only one enantiomeric form of α-monodeuteriotyramine (V) should be responsible for the isotope effect noted with dideuteriotyramine. Both optical isomers were prepared by enzymic decarboxylation of tyrosine. *In vitro* studies with liver monoamine oxidase showed one optical isomer to be 2.3 times as reactive as the other, a ratio similar to that of the isotope effect noted when the dideuterio compound was compared to the protio form. This clearly demonstrates that the two α-hydrogens in tyramine are not equivalent for the enzyme, and that the observed results are explainable in terms of a three-point contact between substrate and enzyme. The stereospecificity of the isotope effect was also demonstrated from the effects on arterial pressure and nictitating membrane contraction. Belleau *et al.* (167–169) claimed these studies to be the first pharmacological ones in which conclusions about mechanism were based on kinetic isotope effects. These studies have indeed provided significant information on certain aspects of the mechanism of adrenergic activity not readily attainable through more conventional methods.

The effect of deuterium substitution at the carbon alpha to the amine group in the substrate kynuramine on the kinetics of oxidation by monoamine oxidase was examined (169, 170). A primary isotope effect of 2.1 (k_H/k_D) was obtained, which is comparable to the isotope effect reported for the corresponding



V: $R_1 = H$, $R_2 = D$; $R_1 = D$, $R_2 = H$

tyramine compounds as previously noted. Also studied was the effect of deuterium atoms at the carbon beta to the amine group in kynuramine. In this case there was a reduction in the rate of oxidation by monoamine oxidase, with a secondary deuterium isotope effect of 1.18. The ratio of the Michaelis constants for α-d_2-kynuramine to kynuramine (K_m^D/K_m^H) was 3.0, while the Michaelis constant for the β-d_2-kynuramine was identical to that for kynuramine. The α-d_2 compound apparently forms a weaker complex with monoamine oxidase, whereas the β-d_2 compound is bound by the enzyme to about the same extent as kynuramine.

The deuterium isotope effect on the enzymatic deamination of *l*-amphetamine was studied (171). This process appears to be the major metabolic pathway for amphetamine in rabbits, dogs, and humans. Oxidative deamination studies were performed *in vitro* on *l*-amphetamine containing a deuterium on the α-carbon atom by following the substrate concentration in the enzymatic reaction with microsomal enzymes in a rabbit liver homogenate. Relative rates of metabolism of the protio and deuterio forms of amphetamine were determined by simultaneous incubations under identical conditions. The ratio of apparent rate constants (k_H/k_D) based on initial velocities was found to be 2.0 ± 0.3. Since cleavage of a carbon—hydrogen bond is involved in the deamination process and since this step of the reaction is rate limiting, a significant deuterium isotope effect was not unexpected.

Deuterium isotope effects on the metabolism of amphetamine and *N*-substituted amphetamines were studied in humans (172, 173). When the excretion rate of α-deuterio-*d*-amphetamine was compared with that of its protio analog, a small, but not quantitatively determined, deuterium isotope effect was observed. Larger isotope effects were noted in the *dextro*-isomers of the *N*-alkyl-substituted amphetamines than in the *levo*-forms.

Deuterium-labeled amphetamine, *p*-methoxyamphetamine, and α-methyltyramine were synthesized primarily for use as internal standards in GC–mass spectrophotometric procedures for the analysis of amphetamine and its metabolites (174). These compounds were variously labeled with deuterium in the alkyl side chain attached to the phenyl group. Deuterium-labeled phentermine (2-methyl-1-phenylisopropylamine) was synthesized in which the 2-methyl group was fully deuterated (175). The labeled compound was prepared specifically for use in comparative metabolic studies with amphetamine, as suggested in the previous paper. Further reference to the metabolic studies will be made in the section on *Bioanalytical Applications*.

MISCELLANEOUS DEUTERATED COMPOUNDS OF BIOLOGICAL IMPORTANCE

A number of deuterated drugs and related products will be considered here since they do not fit appropriately into any of the categories of drugs already covered.

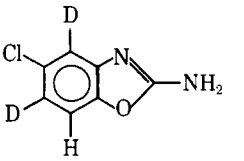
In the section on amino acids, it was noted that some amino acids and monosaccharides were isolated from the hydrolysate of the algae *Scenedesmus obliquus* grown in a medium containing 99.6% D_2O (142). The sugars were fractionated from the nonionic fraction of the hydrolysate on a cellulose chromatographic column. Fully deuterated mannose and glucose were isolated and purified in greater than gram amounts, and some of their properties were studied. These sugars have been incorporated into deuterated nutrient media used for the culturing of organisms that elaborate natural products of therapeutic value. The sugars have been used in mechanistic studies of the biosynthesis of highly deuterated drugs including penicillin (117), griseofulvin (107), clavine alkaloids (127), and riboflavin (176).

Li *et al.* (177) studied the primary and secondary deuterium isotope effects on the rate of mutarotation of fully deuterated glucose and mannose. The decrease in the rate of mutarotation observed in deuterium oxide was attributed to a primary isotope effect arising from the breaking and reforming of O—H and O—D bonds, and a secondary isotope effect was attributed to the C—D bonds in the molecule that were responsible for a further decrease in the rate of mutarotation.

GLC was used to separate fully deuterated from protio carbohydrates (178). Complete separation of glucose and glucose-d_7 was reported. Other carbohydrate mixtures were only partially separated.

A number of fully deuterated fatty acids from *S. obliquus* cultured in heavy water were isolated and characterized (179). The fatty acid composition of the total lipids and of individual lipid classes was determined. The fatty acids, as their methyl esters, were characterized by GLC and mass, IR, and ^{1}HMR spectroscopy. Rohwedder *et al.* (180) determined the composition of the fatty acid fraction obtained from the deuterated algae *S. obliquus* and assigned the IR spectra of the fatty acids. McCloskey *et al.* (181) studied the GC and mass spectrometric properties of fully deuterated fatty acids obtained from the same deuterated algae source.

Zoxazolamine, 2-amino-5-chlorobenzoxazole, is a muscle relaxant metabolized by ring hydroxylation at C-6 to yield 2-amino-5-chloro-6-hydroxybenzoxazole. Dideuteriozoxazolamine-4,6-d_2 (VI) was synthesized, and the ^{1}HMR spectral data were used to establish the exact structure of the compound (182). From *in vivo* studies, it was concluded that there was no isotope effect on the duration of paralysis in rats after drug administration, since the behavior of ordinary and dideuteriozoxazolamine was not significantly different. *In vitro* metabolic studies were conducted with liver microsomes. The deuterated compound was metabolized more slowly than the protio compound (k_H/k_D = 1.4). The isotope effect may be due to a lower affinity of the deuterated compound for the degradative enzyme or to a slower cleavage rate of the C—D bond compared to the C—H bond. No explanation for the absence of a difference in the duration of paralysis between the two forms of zoxazolamine was advanced despite a difference in the *in vitro* degradation rates by the liver microsomes.

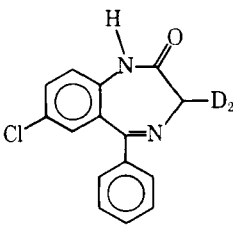


VI: zoxazolamine-4,6-d_2

O-Demethylation is an oxidative reaction catalyzed by the liver microsomes in drug detoxification. The effect produced on the enzymic demethylation of *o*-nitroanisole when the hydrogens of the O—CH_3 group were replaced with deuterium atoms was studied (183). Because the rupture of carbon—hydrogen bonds is involved in the demethylation process, a deuterium isotope effect should be evident if this step in the reaction is rate limiting. On the basis of the rate of formation of the reaction products, a deuterium isotope effect (k_H/k_D) of about 2 was noted. This primary isotope effect could also account for a lowered Michaelis–Menten constant, and a $K_m{}^D/K_m{}^H$ of 0.4 was indeed observed. However, considering the complexity of the enzyme system (liver microsomes), a straightforward interpretation of the change in K_m is not possible.

Marcucci *et al.* (184) investigated the effect of deuteration on the anticonvulsant activity of *N*-demethyldiazepam (7-chloro-1*H*-5-phenyl-1,4-benzodiazepin-2-one), a major metabolite of diazepam with pharmacological properties similar to the parent compound. *In vivo*, *N*-demethyldiazepam is hydroxylated in the C-3 position to form oxazepam, which accumulates in the brain and is responsible for the anticonvulsant activity of the parent compound. *In vitro*, oxidation to the hydroxyl derivative is effected by the liver microsomal system.

Deuterated *N*-demethyldiazepam (VII) was synthesized in which two deuterium atoms were introduced into the C-3 position. If cleavage of the carbon—hydrogen bond in the oxidative process is rate limiting, then deuteration would be expected to affect the metabolism and pharmacological activity in mice. *In vitro* studies revealed that the deuterated form oxidized at a slower rate to form oxazepam than did the protio form of *N*-demethyldiazepam. *In vivo* studies in mice indicated that the anticonvulsant ac-



VII: *N*-demethyldiazepam-d_2

tivity was reduced from 20 to 5 hr for the deuterated compound and that the ED_{50} was increased from 198 μg/kg for the protio compound to 288 μg/kg for the deuterated analog. The decreased activity of the deuterated compound is apparently due to a decreased accumulation of the hydroxylated derivative, oxazepam, in the brain of the mice because of the reduced rate of oxidation of the parent deuterated compound. It was concluded that decreased oxazepam formation in the case of deuterated *N*-demethyldiazepam may arise from one of the following causes: (*a*) a reduced rate of cleavage of the carbon—deuterium bond, (*b*) a lower affinity for the active enzyme site, or (*c*) the formation of a more stable complex with the enzyme.

Hydroxylating enzymes present in liver microsomes play an important role in the detoxification of foreign organic compounds. In the conversion of acetanilide to *p*-hydroxyacetanilide by rabbit liver microsomes, molecular oxygen was incorporated by the microsomes into the hydroxyl group of *p*-hydroxyacetanilide (185). The rate of hydroxylation of pentadeuterioacetanilide was compared to that of ordinary acetanilide to determine whether the breaking of a carbon—hydrogen bond was rate limiting in the microsomal hydroxylation of acetanilide (186). The ratio, k_H/k_D, turned out to be unity, indicating the absence of any deuterium isotope effect and suggesting that cleavage of the carbon—hydrogen bond is not rate limiting. The slow step in the reaction may instead be the incorporation of molecular oxygen in the *para*-position or possibly the fixation of oxygen by the enzyme.

Hydroxylation of *p*-deuterioacetanilide by the hydrolase enzymes in rabbit liver microsomes formed *m*-deuterio-*p*-hydroxyacetanilide (187). The enzyme-catalyzed aromatic hydroxylation appears to be accompanied by a stepwise migration of the group (deuterium in this instance) displaced by the hydroxyl. While a mechanism for the reaction was postulated, possible kinetic isotope effects were not considered.

BIOANALYTICAL APPLICATIONS

Methods—Stable isotopes, particularly deuterium, are proving useful as tracers for drug distribution and metabolism studies. The many advantages of stable isotopes for these purposes include the absence of possible radiation hazards, especially important where children and pregnant women are concerned. Simpler procedures and precautions are required during the synthesis and the general handling of compounds enriched with stable isotopes. In addition to deuterium, ^{13}C, ^{15}N, and ^{18}O are useful stable isotopes for such studies. Deuterium and ^{13}C offer the additional advantage over radioactive tritium and ^{14}C of smaller mass differences as compared to the more abundant natural isotopic form. A smaller mass difference leads in general to a smaller kinetic isotope effect and therefore to a smaller perturbation in the data obtained from tracer experiments. In the case of nitrogen and oxygen, of course, there are no radioactive species of sufficiently long half-life for use as tracers or labels.

A major deterrent to more extensive use of stable isotopes for tracer studies has been the lack of suitable instrumentation that provides for simple, sensitive, specific, and reasonably inexpensive methods of quantitation. These considerations are particularly important where low concentrations of labeled molecules are involved. For radioactive labels, of course, there are convenient, inexpensive, and sensitive methods for detection of emitted radiation; these methods permit detection of labeled drugs or metabolites at the picogram concentration level. Because of concern over possible radiation hazards, however, radioactive isotopes are generally not used for *in vivo* metabolic studies. Radiometric procedures also lack specificity, because the isotope content in a sample is usually measured without regard to the way in which it is incorporated.

The use of stable isotopes for the quantitative determination of compounds can be based on an isotope dilution analysis technique, which is more commonly used with radioactive isotopes. Stable isotopes were first used in this manner by Rittenberg and Foster (188) who employed ^{15}N-labeled amino acids for the determination of glycine, glutamic acid, and aspartic acid in fibrin hydrolysates and deuterium-labeled fatty acid for the determination of palmitic acid in rat fat. Isotope ratios of the isolated and purified compounds were determined by mass spectrographic analysis after combustion.

The development of GC–mass spectrometry–alternating voltage acceleration instrumentation has greatly simplified this procedure and has made possible the application of stable isotope dilution analysis to intact molecules. The need for isolation, purification, and combustion has been largely eliminated. Numerous applications of this technique to the determination of drugs and their metabolites have appeared in the literature in recent years. Some of these applications have already been mentioned, and additional ones will be noted briefly here.

The mass spectrometer is perhaps the most suitable instrument for the detection of stable isotopes. However, complicated separation and purification procedures are usually required for mass spectrometric analysis, and poor yields frequently result. A technique has been developed which combines the high resolving power of GC and the sensitivity and specific ion-detecting ability of the mass spectrometer, thus simplifying considerably the problems encountered previously with mass spectral analysis. GC–mass spectrometry has found wide application in the qualitative identification of drugs (189), especially where small quantities of drugs and metabolites may be involved. The use of stable isotopes in clinical pharmacology, particularly as tracers for drug distribution and metabolism studies, were reviewed (32).

A modification of the GC–mass spectrometry technique, referred to as "mass fragmentography," was introduced in 1968 (190) for the identification of chlorpromazine and some of its metabolites in human blood. The use of a multiple-ion detector accessory

with a gas chromatograph–mass spectrometer permits the mass spectrometer to serve as a sensitive, selective, and molecule-specific GC detector. Molecular species in the picogram range have been detected with this procedure. Specific ions and fragments formed by the compound of interest that elute from the gas chromatograph and enter the mass spectrometer can be conveniently monitored. The mass spectrometer detector selectively monitors only compounds yielding fragments at preselected mass numbers, so the complete mass spectrum need not be scanned.

The use of an alternating voltage accelerator was introduced into the mass spectrometer system, permitting continuous monitoring of the intensity of several ions or fragments (191). Thus, the composition of certain unresolved or partially resolved mixtures in GC effluents can be determined. Two mass numbers were used in determining the composition of a mixture of glucose and glucose-d_7 that was rendered volatile by formation of the penta-*O*-trimethylsilyl derivatives. Samples as small as 0.1 μg could be conveniently analyzed. Mixtures of epiandrosterone and dehydroepiandrosterone were determined with as little as 20 ng of the compounds.

Hammar *et al.* (190) were apparently the first to apply mass fragmentography to the characterization of drugs and their metabolites. They pointed out the extensive possibilities offered, particularly since modern drugs generally have known structures and synthetic analogs containing stable isotope labels are readily available. When mass numbers are employed for continuous monitoring, the peak areas obtained are proportional to the abundance of the fragment in question and are therefore applicable for quantitation. This system of analysis is generally referred to as GC–mass spectrometry–alternating voltage acceleration, and instrumentation with this capability is commercially available.

Several variations in the labeling techniques are utilized. In drug metabolism studies, labeled drug may be added prior to extraction, derivatization, and analysis. This technique of adding a "carrier" compound prior to GC corrects for error introduced as a result of processing tissue samples. This procedure requires the addition of a relatively large quantity of a carrier compound that possesses chemical and physical properties similar to the compound of interest.

Deuterated analogs are ideal carriers. Deuterium is the least expensive of the commonly used stable isotopes and is available in the widest variety of compounds; it is readily introduced into compounds by the usual synthetic methods. Gaffney *et al.* (192) described the use of "internal standards" labeled with stable isotopes that correct for losses of the compound under study during the GLC treatment of the sample. With the mass spectrometer as the detector for the GLC effluent, chemical differences between the compound being determined and the internal standard are not necessary, because mass differences alone are sufficient to discriminate between the compound and the standard.

The ideal internal standard is the actual compound under investigation modified to contain an increased mass by the introduction of a stable isotope in appropriate molecular positions. Quantitation depends on a comparison of the response of the mass spectrometer at two masses, one mass characteristic of the internal standard and the other characteristic of the substance being analyzed. Cho *et al.* (193) noted that since internal standards are isotopic variants of the compound being studied, fragmentation patterns and retention times will be essentially identical.

It is desirable for the standard to have more than one labeled atom. The masses of the fragment ions selected for measurements could therefore differ by perhaps 2 or 3 mass units. Knapp *et al.* (194) developed the technique of using stable isotopes for introducing visually conspicuous mass spectral "isotope clusters" into drug molecules as labels or markers. They prepared trideuterium-labeled nortriptyline, in which the three hydrogen atoms attached to the *N*-methyl group were replaced with deuterium atoms. An M, M + 3 doublet was created by an equimolar mixture of the labeled and nonlabeled drug, and this mixture was used for following the fate of the drug and its metabolic products. The drug and any of its metabolites retaining the labeled moiety showed an M, M + 3 doublet in the mass spectrum for the molecular ion and any fragment ions containing the labeled moiety. The concept of multiple deuterium labeling has been applied in sequence analysis of proteins by mass spectrometry (195, 196).

Applications Involving Deuterated Drugs—Chlorpromazine and several of its metabolites have been identified in human blood (190). Desmethylchlorpromazine and didesmethylchlorpromazine have been identified in plasma and in red blood cells after treatment with β-glucuronidase. 2-Chlorophenothiazinylpropionic acid was identified in plasma after treatment with β-glucuronidase.

The metabolic pathways of deuterated tyrosine and leucine were studied in human subjects (197). Healthy controls and patients suffering from certain metabolic disorders were administered oral doses of 3,5-dideuterio-*l*-tyrosine and *dl*-deuterioleucine (apparently a trideuterio-derivative). The urine was collected and examined for amino acid metabolites using GC–mass spectrometry. In three mentally retarded patients, high concentrations of deuterated hippuric acid and benzoic acid were detected after orally loading the patients with deuterated tyrosine. No isotopic labeling of either acid was found in normal subjects after loading with deuteriotyrosine.

Deuterated 3-hydroxyisovaleric acid was detected in a patient with suspected "sweaty feet syndrome" after oral administration of deuterated leucine. This compound is excreted in increased amounts in patients with the metabolic defect involving leucine degradation (β-hydroxyisovalericacidemia). The significance of stable isotope labeling and GC–mass spectrometry detection technique in studying metabolic pathways *in vivo* was emphasized, particularly in patients with metabolic defects.

Three variously deuterium-substituted amphetamines were synthesized and evaluated for use as internal standards in the GC–mass spectrometry assay for the quantitative determination of amphetamine in biological fluids (193). Because of their desirable GLC and mass spectral properties, the trifluoroacetic acid amide derivatives were used.

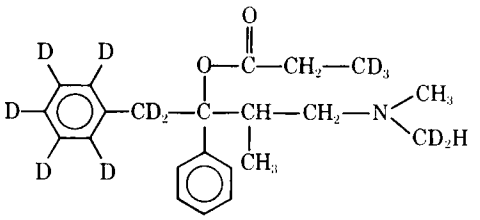
A procedure based on GC–mass spectrometry was developed for the quantitative determination of prostaglandin E_1 (PGE_1) as the methyl ester and *O*-methyl oxime (methoxime) derivative (198). The same derivative was used as a deuterated carrier, except that the methyl of the methoxime contained three deuterium atoms. The ions at *m/e* 470 and 473, produced by electron impact on the effluent, were monitored. The method was sensitive at the nanogram level.

Axen *et al.* (199) reported the GC–mass spectrometry determination of prostaglandins E_2 and $F_{2\alpha}$, which permitted the determination of picomole amounts. The corresponding tetradeuterio (3,3,4,5-d_4) derivatives were synthesized and served as deuterium-labeled carriers. This method is sufficiently sensitive and specific to be useful for the analysis of plasma prostaglandins and for studies involving enzymatic synthesis of prostaglandins *in vitro* from endogenous precursors.

The simultaneous microestimation of choline and acetylcholine was reported using GC–mass spectrometry (200, 201). The corresponding compounds containing nine deuterium atoms in the *N*-methyl groups served as internal standards and carriers. This procedure permitted the quantitative determination of 1 pmole or less of compound.

A very sensitive and specific assay for 5-hydroxyindole-3-acetic acid in human cerebrospinal fluid was reported based on GC–mass spectrometry analysis of the diheptafluorobutyryl methyl ester derivative (202). The two major ions (*m/e* 538 and 597) of the mass spectrum were monitored after elution from the chromatographic column. The dideuterium-labeled 5-hydroxyindole-3-acetic acid was synthesized and served as an internal standard for the quantitation of 5-hydroxyindole-3-acetic acid in cerebrospinal fluid. An analysis required less than 2 min and permitted an accurate determination of 5-hydroxyindole-3-acetic acid in the range of 2–50 ng/ml of cerebrospinal fluid when 2 ml of cerebrospinal fluid was taken for analysis. In the 8–20-ng/ml range, the standard deviation for the method was less than 7%; it was 1–2% at higher levels of 5-hydroxyindole-3-acetic acid.

Deuterated nortriptyline was used as an internal standard for unlabeled nortriptyline (192, 194). In the synthesis of the labeled molecule, two deuterium atoms were introduced into the side chain as $-CHCH_2CD_2NHCH_3$ (the M + 2 amu compound). The trifluoroacetate derivative was prepared by treatment with trifluoroacetic anhydride. Equimolar mixtures of the nortriptylines at the 50–100-pg concentration level were used for producing mass fragmentograms of nortriptyline trifluoroacetate (M = 232) and nortriptyline trifluoroacetate-d_2 (M = 234). Mass fragmentograms of extracts of human plasma samples containing nortriptyline and its deuterated



VIII: propoxyphene (variously labeled)

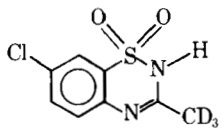
analog were also obtained. Elution and resolution of the two compounds were quite satisfactory.

The stable isotope labeling technique was used to identify the metabolites of nortriptyline in human urine and bile (203). The trideuterium derivative was prepared in which the three deuterium atoms were located in the *N*-methyl position. A single 25-mg oral dose containing an equimolar mixture of the deuterated and unlabeled nortriptylines was administered to adult humans. By mixing stable isotope-labeled and unlabeled drugs, an artificially created isotopic doublet (M, M + 3) was produced, which served as a conspicuous spectral marker for identifying drug metabolites in urine and bile. Urine extracts treated with trifluoroacetic anhydride were examined by GC–mass spectrometry, and the parent drug and any metabolites retaining the labeled moiety were readily identified by the presence of the doublet. Mass fragmentography was successfully employed where metabolites were present in quantities insufficient for ordinary mass spectral scanning.

On the basis of the essentially equal intensity of the artificially created doublet, the following were identified in urine: unchanged nortriptyline and the desmethyl, 10-hydroxy, and 10-hydroxydesmethyl derivatives of nortriptyline. The same compounds were also identified in human bile in the same relative amounts as found in urine. The advantages and further possibilities of the mass fragmentography technique in drug metabolism studies were emphasized. The small dose requirement and the absence of radiation hazards associated with the use of radioactive isotopes make stable isotope labeling particularly attractive for studies involving children and pregnant women.

Clarke and Foltz (204) used morphine, in which the *N*-methyl group contained three deuterium atoms, as a carrier and as an internal standard for determining low morphine levels in human urine by the reverse isotope dilution technique. The deuterated morphine exhibits mass spectral fragments 3 amu higher than those for morphine. The labeled morphine was added to the urine prior to the extraction process, and the morphine content of the urine sample was determined from the ratio of ion current peaks corresponding to the nonlabeled morphine and the added carrier-labeled morphine. The method was sensitive to the nanogram concentration level.

The biotransformation of propoxyphene in humans was studied (205, 206) using variously deuterium labeled forms (VIII) of the drug: (*a*) propoxyphene-d_2, in which one *N*-methyl group was replaced by the $N—CD_2H$ group; (*b*) propoxyphene-d_3, in which the methyl group of the propionyl func-



IX: diazoxide-d_3

tion was replaced by CD_3; and (*c*) propoxyphene-d_7, in which all the hydrogens of the benzyl group were replaced with deuterium. In this study, mixtures of the unlabeled drug and the deuterium-labeled form were used to create isotope clusters in the mass fragmentation patterns. These clusters served as tags for identifying the parent drug and drug metabolites in complex mixtures containing endogenous components such as tissues and body fluids.

The labeled drug was administered orally to human subjects as a 1:1 mixture with unlabeled drug. Urine samples were extracted and the extracts were analyzed by GC–mass spectrometry. Drug metabolites in the mixture were detected by analyzing the fragmentation pattern of each component for the presence of M, M + 2; M, M + 3; and M, M + 7 doublets. Analysis was also performed on the fragmentation patterns obtained from urine extracts of subjects administered only unlabeled or labeled drug. In addition to unmetabolized drug, the following metabolites were identified: norpropoxyphene, dinorpropoxyphene, propoxyphene carbinol, norpropoxyphene carbinol, dinorpropoxyphene carbinol, hydroxypropoxyphene, hydroxynorpropoxyphene, and 6-phenyl-6-benzyl-2-ethyl-5,6-dihydro-5-methyl-4*H*-1,3-oxazine.

The concentration of *N,N*-dimethyltryptamine in human plasma was estimated by stable isotope dilution analysis (207). *N,N*-Dimethyltryptamine is a psychotomimetic agent which, along with related methylated indoleamines, has been identified in the blood and urine of schizophrenics. However, a causative role in mental disease has not been established. Dideuterio-*N,N*-dimethyltryptamine was synthesized in which the two deuterium atoms were located on the carbon alpha to the indole ring. Dideuterio-*N,N*-dimethyltryptamine served as a carrier and was added to the plasma at the beginning of the isolation procedure. It also served as an internal standard in the quantitation step of the GC–mass spectrometry analysis for *N,N*-dimethyltryptamine in the isolate. The sensitivity limit was 0.5 ± 0.2 ng/ml of plasma.

A stable isotope dilution and mass fragmentography assay was used in pharmacokinetic studies with diazoxide, an antihypertensive and hyperglycemic agent (208). Diazoxide-d_3 (IX) was synthesized, in which the three deuterium atoms were located in the methyl group attached to the ring system. This compound served as a carrier and internal standard for determining plasma concentrations of the drug. The sensitivity of the procedure was reported to be about 1 ng/sample. The plasma half-life of the drug ranged between 20 and 53 hr in four adult patients.

Hofmann *et al.* (209) synthesized deuterium-substituted bile acids. A stable isotope dilution technique was developed for determining the bile acid pool size which required only 10 mg of deuterium-labeled bile acid. In patients with cholesterol gallstones, the gallbladder was found to be supersaturated with cholesterol while the amount of circulating bile acids was generally lower than normal. This finding was confirmed in 60 patients with gallstones.

CONCLUSION

An impressive number of drugs have been synthesized or biosynthesized that are variously substituted with deuterium; while they have not found application in drug therapy, they are being used to study the mechanism of drug action, to elucidate metabolic and biosynthetic pathways, and to study kinetic isotopic effects. Deuterated drugs labeled in specific molecular positions are proving invaluable in drug distribution and metabolism studies. Mass fragmentography makes good use of deuterated drugs for the detection of specific ions and molecular fragments representative of metabolic products present in the body fluids at extremely low concentrations.

Heavy water is employed as a routine diagnostic agent for measuring total body water and shows promise as a contrast medium in certain biomedical applications of neutron radiography. Some studies have indicated that heavy water inhibits tumor growth but, unfortunately, the concentrations required are not well tolerated by the host.

The toxicological effects resulting from extensive deuteration in mice, rats, and dogs have been described. In intact mammals the magnitude of deuterium toxicity appears mainly to be independent of the size of the animal. It appears that up to 15% deuterium replacement can be tolerated by mammals, but severe toxic effects and even death result when the level exceeds 30%. The physiological effects of deuteration on numerous specific organs and body functions have been thoroughly studied, including the effects on liver activity, kidney function, blood constituents, enzyme systems, reproductive capacity and cell division, the heart, the nervous system, metabolism, and even the wound-healing process.

A variety of unicellular microorganisms has been cultured in a fully deuterated environment. However, higher plants show a graded response to ^2H_2O, with 60–70% the maximum level tolerated in the nutrient medium. A primary effect of deuteration appears to be suppression of the production of such important natural products as alkaloids and antibiotics. However, the problem has been circumvented to some extent by using replacement culture techniques.

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Rational Development of a Soluble Prodrug of a Cytotoxic Nucleoside: Preparation and Properties of Arabinosyladenine 5′-Formate

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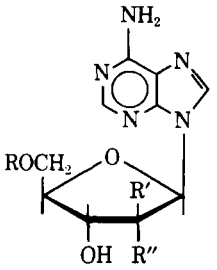
Abstract □ The 5′-*O*-formate ester of arabinosyladenine was synthesized and shown to be suitable as a water-soluble prodrug, being at least 60 times more soluble than the parent cytotoxic nucleoside. This increased solubility was ascribed to a decrease in intermolecular interaction in the crystalline state, as evidenced by an ~90° lower melting point for the ester relative to the parent compound. The prodrug reverted to the parent compound in aqueous solution, its pH–rate profile being V-shaped with maximum stability at a pH ~4.2, corresponding to a half-life of about 10 days. The rate of hydrolysis of the prodrug at 37° in 91% human serum and 91% whole blood was studied. At an initial concentration of 0.4 mg of prodrug/ml of 91% whole blood, reversion to arabinosyladenine appeared to be essentially complete in about 15 min. The prodrug did not appear to be subject to enzymatic deamination. This feature, together with the good solubility of the prodrug, makes possible the effective formulation of arabinosyladenine for intravenous purposes. The rationale involved in the general design of a prodrug and the specific considerations necessitated in the case of adenine arabinoside are discussed.

Keyphrases □ Arabinosyladenine 5′-formate—synthesized as highly water-soluble prodrug, hydrolysis rates in biological fluids □ Prodrugs—synthesis of highly water-soluble arabinosyladenine 5′-formate, hydrolysis rates in biological fluids □ Cytotoxic nucleosides—synthesis of arabinosyladenine 5′-formate as highly water-soluble prodrug, hydrolysis rates in biological fluids

The nucleoside arabinosyladenine[1] (I) is an epimer of adenosine (II) and has been shown to be an effective agent in the inhibition of the growth of viruses and tumors in various *in vitro* and *in vivo* tests (1–6). The low water solubility[2] (0.0018 *M*, 0.5 mg/ml) of this drug and the large (2.5 g) human doses suggested have hampered its clinical testing[3]. Infusions of several liters of intravenous vehicle containing the dissolved drug have been employed in some cases[3]. The present studies were undertaken to develop a more soluble prodrug form of I and thus reduce the minimum volume in which this dose (2.5 g) could be dissolved.



I: R = R″ = H, R′ = OH
II: R = R′ = H, R″ = OH
III: R = HCO, R′ = OH, R″ = H

DISCUSSION

A comparison of the chemical structures of I and II shows that they differ only in their stereochemical configuration at the 2′-position of the sugar group. Both substances appear to be quite polar, and they might be expected to be similarly solvated in aqueous solution. The lower solubility of I (1.8×10^{-3} *M*) relative to II (~0.02 *M*)[2] may be explained by stronger intermolecular hydrogen bonding in the crystal lattice of I due to the different geometry. This rationalization appears to be supported by the observed differences in melting points (I, mp 258–260°; II, mp 235°), where the higher melting material is less soluble.

One method of achieving an apparent increase in the solubility of I is through the use of a water-soluble prodrug form (7, 8) of I. This method involves the identification of a suitable chemical derivative of I that reverts to I under *in vivo* conditions. Two different approaches for increasing solubility through prodrug formation are immediately apparent. The first is to prepare a derivative considerably more hydrophilic than I, in which case the increase in solubility would be achieved through increased solvent–solute interactions. The 5′-phosphate of I (9), which is simply an epimer of adenosine monophosphate, is an example of this type of prodrug and has already been reported.

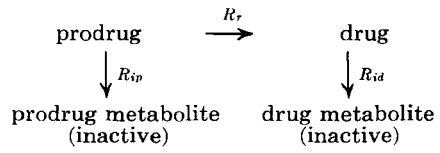
The second type of prodrug with increased solubility relies on the perturbation of the geometry of the crystal and/or a decrease in the capacity for intermolecular interaction to achieve the increase in solubility. Such changes would be expected to increase the free energy of the crystalline solute and thereby would tend to increase solubility. An example of this approach is the N^6-(dimethylamino)methylene derivative of I (10).

Although the specific prodrug examples cited successfully achieved significant increases in solubility, other criteria, such as the rate of release of the drug from the prodrug form, also should be considered in the design of a prodrug.

If the purpose of utilizing the prodrug approach is to attain improved drug delivery as evidenced by greater concentrations of the drug in the body fluids, then it is the position of these investigators that the prodrug should not persist appreciably once the delivery function has been achieved. Therefore, unless designed specifically for other purposes such as timed release, prodrugs should be rapidly converted to the drug itself in the relevant biological medium. If such is not the case and the drug is released over a period of hours, some doubt will exist as to whether the nature of the observed pharmacological response is due to the drug or to the prodrug. This is particularly important in cases where the prodrug itself, because of inherent chemical or structural features, may be likely to exert a biological response.

The rate of regeneration of a drug from its prodrug form may also be important from a kinetic standpoint when attempting to achieve increased blood levels of drug. A simplified representation

[1] Arabinosyladenine is referred to in a number of ways in the literature including 9-β-D-arabinofuranosyladenine, ara-A, NSC-404241, and adenine arabinoside.
[2] Determined in these laboratories.
[3] J. P. Davignon, National Cancer Institute, personal communication.



Scheme I

of potential problems of the kinetic type are presented in Scheme I, where R_{ip} and R_{id} represent the rates of inactivation of the prodrug and the drug, respectively, and R_r represents the rate of release of the drug from the prodrug. From an inspection of Scheme I, it is obvious that the concentration of the drug that can be attained is dependent on the magnitude of R_r relative to R_{ip} and R_{id}. As the magnitude of the ratio $R_r/[R_{ip} + R_{id}]$ increases, the concentration of the drug that can be achieved is increased.

When one considers that arabinosyladenine undergoes enzymatic deamination (11, 12) to the corresponding biologically inactive hypoxanthine (6) rather rapidly[4], it is obvious that a slow rate of reversion, such as that exhibited by the previously reported prodrugs of I (9, 10), will result in extremely low concentrations of I *in vivo*. Hence, an important consideration in this work was the identification of a prodrug that, *in vivo*, would rapidly revert to I as well as provide the desired solubility properties.

The chosen approach for increasing the solubility of I involved the formation of a prodrug exhibiting decreased intermolecular hydrogen bonding in the crystalline state and thereby increasing the activity of the solid. It was anticipated that this objective could be achieved by reducing the hydrogen donor capacity of the sugar portion of the molecule. The replacement of the hydrogen atom of a hydroxy group by acylation or alkylation was expected to accomplish this goal. Again, any such derivative should rapidly revert to I under *in vivo* conditions. The well-known facts that esters are normally more rapidly cleaved than are ethers and that blood contains esterases suggested that an ester would be preferred over an ether as a prodrug.

Esterification of the 5′-hydroxy group was decided upon, since it was expected to offer some advantages. First, the 5′-hydroxy is a primary alcohol, while the 2′- and 3′-hydroxy groups are secondary alcohols. Since it was shown (13) with a series of butyl and propyl esters that the esters of primary alcohols are normally cleaved more readily than the corresponding esters of secondary alcohols, an ester at the 5′-position would be expected to be the most rapidly hydrolyzed of the possible esters of I.

Second, earlier work (6, 11) on adenosine and related compounds strongly suggested that a free hydroxy group at the 5′-position was required to serve as a good substrate for adenosine deaminase. Therefore, derivatization at the 5′-hydroxy would be expected to prevent or retard the enzymatic deamination of III as discussed previously. Such protection from deamination, if realized, would then assure that reversion of the prodrug would occur prior to any deamination.

The specific choice of the formyl group as the acyl group of choice for esterification of the 5′-position was based on the greater facility with which formate esters are hydrolyzed in comparison to more bulky homologs (14, 15) such as acetate and propionate. Another consideration in this choice was that the formyl group, being extremely compact, would not be expected to introduce any appreciable lipophilic character into the otherwise polar nature of I. On the basis of these considerations, arabinosyladenine 5′-formate[5] (III) appeared to be an attractive candidate as a prodrug of I.

EXPERIMENTAL

Anhydrous I[6], mp 258–260°, was used as supplied. All other chemicals used were of the highest commercial grade available. The water was triply distilled, with the final distillation being from acid permanganate solution in an all-glass still.

Liquid Chromatography—High-pressure liquid chromatography (HPLC) was used for monitoring and optimizing the conditions for the synthesis of III and for following the kinetics of hydrolysis of III. Except for the eluants, the chromatographic systems used were identical for both purposes. The liquid chromatograph[7] was equipped with a UV (254 nm) detector and a 0.5-m stainless steel column having an internal diameter of 2 mm. The stationary phase was a strong cation-exchange resin[8]. Samples were eluted at 600 psig, which resulted in a flow rate of about 1 ml/min.

Since the chromatograph used was not insulated or thermostated, column temperatures during operation were subject to fluctuations in room temperature. The temperature varied from day to day but was in the range of 23–27°; fluctuations within the time period of a given run or series of runs were normally <1.5°. Such temperature variations caused retention times to vary by as much as 20–30%; peak heights were also found to vary. Because of such variation in peak heights, integration of the area of the peaks was performed using a planimeter. This approach yielded results that, when normalized as described later, were not noticeably affected by the temperature variation. Detailed differences between the HPLC systems used will be discussed.

System Used in Following the Synthesis of III—The eluant system used for monitoring and optimizing the synthesis of III was an aqueous solution containing 0.04 *M* NaH_2PO_4 and 0.05 *M* $NaNO_3$, pH 4.5. This system resulted in the separation of several components. Typical retention times were as follows: I = 1 min, III = 2 min, II (adenine)[9] = 2.5 min, arabinosyladenine 3′-formate[10] (IV) = 4 min, and arabinosyladenine 3′,5′-diformate[11] (V) = 12.5 min.

System Used in Following Hydrolysis of III—In studies on the hydrolysis of III, the eluant used was aqueous 0.04 *M* NaH_2PO_4, which had been adjusted to pH 4.2 with 10% H_3PO_4 in water. Although retention times varied somewhat with temperature fluctuations, typical values for the retention times of I and III were 1.5 and 4.5 min, respectively.

Preparation, Isolation, and Characterization of III—The synthetic method and conditions reported here were arrived at after monitoring the reaction by liquid chromatography and attempting to optimize the conditions with respect to the yield of III.

Compound I (10.0 g) was dissolved in anhydrous formic acid (16.0 ml) and maintained at 5° for 96 hr. The isolation of III was accomplished by the use of conventional column chromatography according to the following procedure.

The reaction mixture (after 96 hr) was diluted to 2 liters with ether while being stirred vigorously with a magnetic stirrer. The crystalline solid that separated was allowed to settle, and the supernate was decanted. The precipitate was washed with an additional 500 ml of ether, which was decanted. The ether solutions were combined (2.5 liters) and passed through a chromatographic column (7 cm i.d.) previously packed with an ether slurry of silica gel[12] (450 g). The solid residue remaining after the washing procedure was dissolved in acetone–ether (4:1, 3 liters) and passed through the column. Further elution with 2 liters of acetone–ether (9:1) followed.

These eluate fractions were found by HPLC analysis to contain little of the desired product and were discarded. Further elution with acetone (8 liters) was carried out and the eluate was concentrated *in vacuo* at room temperature. The gummy residue obtained was transferred to a crystallizing dish, using a minimal amount of acetone. A crystalline solid formed spontaneously on standing. The filtered and dried product (5.5 g, 50% yield) was found by HPLC analysis to contain >96% of III with the remaining 4% consisting of unreacted I and some II, IV, and V. Recrystallization from acetone resulted in a solid, mp 168–170°, which appeared to be essentially homogeneous by HPLC and TLC [silica gel, chloroform–methanol (2:1), R_f 0.4]. This material was charac-

[4] Dr. Florence White of the National Cancer Institute stated, in a personal communication, that the deamination resulted in loss of antitumor activity and that the biological half-life for the enzymatic deamination of I was ~30 min.

[5] The prodrug, arabinosyladenine 5-formate, was recently assigned the identification number NSC 171240 by the National Cancer Institute.

[6] Furnished by the National Cancer Institute.

[7] DuPont model 840.

[8] SCX on Zipax, DuPont.

[9] Compounds II and III were not completely resolved, but the presence of small amounts of II was apparent as a small shoulder or peak on the much larger III peak. The retention time was confirmed with authentic II.

[10] Compound IV was not completely characterized but was tentatively identified as the 3′-formate ester of I.

[11] Compound V was isolated, characterized, and definitely established to be the 3′,5′-diformate ester of I (mp 153–155°).

[12] Silicar CC-7, Mallinckrodt.

terized as arabinosyladenine 5′-formate (III); IR (KBr): 1730 (s, C=O) cm^{-1}; NMR (dimethyl sulfoxide-d_6): δ 8.25 (s, 1, 5′-O_2CH), 8.16 (s, 2, 2- and 8-H), 7.35–7.05 (m, 2, N—H), 6.36 (d, J = 4 Hz, 1, 1′-H), 5.95–5.55 (m, 2, 3′- and 2′-O—H), 4.55–4.35 (m, 2, 5′-H), and 4.35–3.85 (m, 3, 4′-, 3′-, and 2′-H) ppm.

Elemental analysis for carbon, hydrogen, and nitrogen was within 0.3% of theory for $C_{11}H_{13}N_5O_5$.

Preparation of Whole Blood and Serum Samples—Serum was prepared by inducing rapid and complete clotting of the blood samples through agitation with glass rods. The resulting sample was centrifuged and the supernatant serum was collected. When whole blood was desired, potassium oxalate (~2 mg/ml of blood) was added to the freshly drawn sample to prevent coagulation.

Kinetic Studies—*Hydrolysis in Aqueous Solutions*—Reactions were initiated by adding freshly prepared aqueous solutions of III to the buffered media in small screw-capped vials. For studies carried out at 25°, the solutions were preequilibrated in a water bath[13]; sampling was begun immediately after mixing. For studies at elevated temperatures, samples were mixed at room temperature and subsequently placed in the thermostated bath; no sampling was done for at least 15 min. Initial concentrations of III were approximately 1 mg/ml (~0.003 M).

The reaction was monitored by HPLC analysis of aliquots of the sample solution removed at various times. In runs at elevated temperatures, aliquots of the sample solutions were taken, the reactions were stopped by chilling in an ice bath, and the aliquots were subsequently chromatographed. In the case of reactions at 25°, aliquots were removed and chromatographed directly. Sample volumes used for HPLC were kept small (1–1.5 μl), since in large volumes the buffers in the reaction mixture were sometimes capable of altering the pH of the mobile phase sufficiently to disturb the chromatographic separation.

To minimize errors due to the possibility of slight variations in the volume of injected samples and the slight changes in instrument sensitivity that appeared to accompany the temperature-dependent fluctuations, the peak areas for III were normalized. The normalization procedure took into account the facts that the reaction involves the hydrolysis of III to yield only I and that the UV detector response to III was only 97% of the response to I on a molar basis. The normalization equation is given in Eq. 1:

$$A_{III,N} = \frac{A_{III,E}}{A_{III,E} + 0.97\,A_{I,E}} \qquad \textbf{(Eq. 1)}$$

where $A_{III,N}$ = normalized area of the peak corresponding to III, $A_{III,E}$ = area of the peak corresponding to III as determined experimentally, and $A_{I,E}$ = area of the peak corresponding to I as determined experimentally. Plots of log $A_{III,N}$ *versus* time were used to calculate the apparent first-order rate constants of the hydrolysis reaction.

Hydrolysis in Serum and Blood—Reactions in serum and blood were initiated by adding one volume of a freshly prepared stock solution of III to 10 volumes of either serum or whole blood, both having been previously equilibrated at 37°. After a predetermined time, the protein was precipitated using tungstate-sulfuric acid method of Folin and Wu (16). The precipitant was prechilled to 0° to quench the reaction rapidly. The sample was allowed to stand until coagulation and hemolysis were complete. The samples were then centrifuged, and the supernatant liquid was used for chromatographic analysis.

Such samples did not appear to undergo further reaction to any appreciable extent during the time necessary to complete the separation and analysis. In this case, because of the enzymatic deamination of I in blood (11, 12), normalization as described above was impossible.

Lyophilization—An aqueous solution containing ~30 mg of III/ml of water was prepared. Aliquots were placed in suitable screw-capped vials, and these vials were immediately frozen in a freezing mixture of solid carbon dioxide and acetone. The sample vials were then placed in the lyophilizer[14] and freeze dried at a pressure of about 0.2 mm Hg and an initial shelf temperature of from −20 to −25°. The shelf temperature rose to near 0° over 24 hr, during which time most water had been removed. The shelf temperature was then increased to 25°, and the samples were maintained for about 8 more hours at a pressure of about 6.1 mm Hg. The water content of the freeze-dried samples as determined by loss in weight was ~1–2%.

RESULTS

Preparation and Characterization—Compound III was synthesized[15] by dissolving I in anhydrous formic acid (18)[16] and allowing the resulting solution to stand at 5° for 4 days. Isolated yields of III in excess of 50% were obtained. The yield was decreased and the problem of greater quantities of undesired side products was worsened when higher temperatures were used. In addition to III, the 3′-formoyl and the 3′,5′-diformoyl derivatives of I (see *Experimental*) were produced along with a small percentage of adenine (20). The various products were separated by column chromatography and analytically pure III was obtained after recrystallization from acetone.

The characterization of III was based on satisfactory analytical results and NMR spectra. It has been established from studies of model nucleosides and derived esters of known structure that the methylene and methine protons experience deshielding only when attached to the carbon involved in the ester bond. The two methylene (5′) and the three methine (2′, 3′, 4′) protons of arabinosyladenine are represented by two multiplets in the δ 4.35–3.55 region. In the spectrum of the monoformate (by analysis, $C_{11}H_{13}N_5O_5$), two of those five protons are deshielded; this result is compatible only with Structure III.

Physical Properties—The white crystalline prodrug melted at 166–168°, about 90° lower than I. Due to the decomposition of III in water, as discussed later, it was not possible to determine the equilibrium aqueous solubility of the prodrug. However, a concentration of about 34 mg of III/ml of solution was achieved within 1 hr when an amount of the solid in excess of the solubility was agitated with water at 25°. On the basis of these data, it appears that the solubility of III in water is at least 34 mg/ml or nearly 0.12 M. This solubility value for III corresponds to the equivalent of ~30.5 mg of I/ml of water, which represents something more than a 60-fold increase in the aqueous molar solubility of III relative to that found for I. This 60-fold increase in the apparent solubility of the drug means that the minimum volume of solution necessary to deliver the equivalent of 2.5 g of I would be reduced from 5 or 6 liters with I itself to about 80 ml with the prodrug.

A comparison of the melting points and the aqueous solubility behavior of I and III clearly demonstrates that formylation of the 5′-position resulted in a significant decrease in intermolecular interaction in the crystalline state. As stated earlier, the qualitative results observed were anticipated on the basis of the hypothesis that acylation of the 5′-hydroxy group would eliminate the ability of that group to act as a hydrogen donor and thereby decrease the extent of intermolecular hydrogen bonding in the solid state. The observed effects on physical properties may arise simply as a direct result of the inability of the 5′-position to act as a proton donor, or they may be due to decreased hydrogen bonding caused by more gross changes in intermolecular and intramolecular geometry in the solid state due to formylation at the 5′-position.

Hydrolysis in Blood and Serum—As stated earlier, it was anticipated that the esterases in body fluids would enzymatically catalyze the hydrolysis of the ester prodrug and thereby facilitate rapid and complete reversion to I. The hydrolysis of III at 37° in 91% serum and in 91% whole blood was studied; the data obtained showed that, at an initial concentration of about 2 mg of III/ml of fluid, 50% of the prodrug was lost in about 15–18 min in 91% plasma. However, in 91% whole blood, 50% disappearance of III occurred in about 6–8 min. The observed difference in the time required for disappearance of 50% of the prodrug is probably due to the loss of certain enzymes or enzyme activities, which may occur during the separation of the serum from the whole blood. Observations of this kind have been reported previously for the hydrolysis of aspirin in blood and plasma (21).

More extensive studies in 91% whole blood were performed, and

[13] The thermostated water baths used maintained temperatures at the stated value ±0.2°.

[14] Virtis model 10-800 attached to a Cenco Hyrac 14 vacuum pump.

[15] The synthesis of III was first accomplished in these laboratories utilizing acetic-formic anhydride (17) in formic acid. This method suffered from low overall yields and is, therefore, not presented in detail.

[16] Acylation under acidic conditions has been shown to yield selectively 5′-esters in the case of certain nucleosides (19).

Table I—Comparison of the Biological Activity of I and III against Two Experimental Tumors in Mice

Treatment[a]	Survival Time[b] for Mice Inoculated with Tumor Cells, days	
	Ehrlich Tumor[c]	Hepatoma 134[d] Tumor
Control	11.6 ± 2.2	21.5 ± 1.3
I	21.0 ± 1.3	25.3 ± 2.0
III	21.0 ± 2.0	25.5 ± 1.0

[a] I was given at 25 mg/kg ip twice daily on Days 1–4 after inoculation with tumor cells. Compound III was given in equimolar doses according to the same schedule. [b] The values given represent the average survival and the average mean deviation for groups consisting of six mice. [c] ICR female mice were inoculated intraperitoneally with 10^6 Ehrlich cells. [d] C3H female mice were inoculated intraperitoneally with 5×10^6 Hepatoma 134 cells.

some typical data are presented in Fig. 1. It was apparent from data of this type that the disappearance of III corresponded quite well to the appearance of I in the initial part of the reaction. However, after about 25% of III had been converted to I, the rates of appearance of I and disappearance of III began to differ markedly and the concentration of I actually reached a maximum and began to decrease prior to the complete disappearance of III. This observation may be explained on the basis of the occurrence of the enzymatic deamination of I to yield the arabinosylhypoxanthine, as previously reported (9, 11) and discussed.

Figure 1 also shows that the apparent rate of hydrolysis of III, when expressed as a percent of the initial concentration, is dependent upon the initial concentration of III. The apparent rate of reversion of III was observed to be more rapid at the lower initial concentration of III, suggesting that the reaction order was less than unity. This observation is probably due to the relatively high concentration of III used, which caused saturation (21) of the esterases involved in the hydrolysis. The initial concentration of 0.4 mg of III/ml of blood represents the range of concentration that would be encountered if a dose of III, which is the equivalent of a 2.0-g dose of I, were administered to an average 70-kg person.

From Fig. 1, it is apparent that even at the lower initial concentration of III, the peak concentration of I approached 50% of the theoretical value. Furthermore, the peak concentration of I expressed as a fraction of the initial dose of III increased as the initial concentration of III was increased; even at the higher initial concentration of III, nearly complete hydrolysis of the prodrug occurred in about 20–25 min.

The positive intercepts of the curves corresponding to the concentration of I at each initial concentration of III are due at least in part to the presence of some I in the aqueous stock solutions prior to the addition of blood.

The data obtained from the blood studies clearly demonstrate that the prodrug rapidly reverts to I and results in a concentration of I that is not currently attainable with I or either of the previously reported prodrug forms (9, 10) in biological fluids.

***In Vivo* Activity**—Subsequent to the *in vitro* studies in blood and serum, the prodrug was compared *in vivo* in both the Ehrlich ascites and Hepatoma 134 rodent tumor systems (Table I)[17]. In both systems, III appeared to have activity equivalent to I when administered intraperitoneally. To obtain meaningful results, evaluation of the prodrug as a method of delivering I should be carried out in systems that possess esterase activity.

***In Vitro* Chemical Stability**—Although the aqueous solubility of III and the *in vitro* and *in vivo* data obtained are such that III would appear to be a prodrug form of I satisfactory for use in intravenous solutions, it was necessary to investigate various other properties and behavior related to stability and formulation before fully evaluating the suitability of III as a prodrug.

As expected, in aqueous buffered solution, III underwent a simple hydrolysis at the 5′-position to yield quantitatively I and formic acid. The reaction was studied at pH ≃ 1–8, and the kinetics of the process appeared to be first order throughout this pH range. The rate of reaction in this region was virtually independent of ionic strength but extremely pH dependent. The log rate–pH profile for hydrolysis of III at 25° is V-shaped (Fig. 2) and is qualitatively similar to that commonly observed for ester hydrolyses, with slopes approaching an absolute value of unity at pH ≤ 2.5 and ≥ 7.0.

[17] These studies were conducted by G. A. LePage, McEachern Laboratory, University of Alberta, Edmonton, Alberta, Canada, who provided the data to the authors.

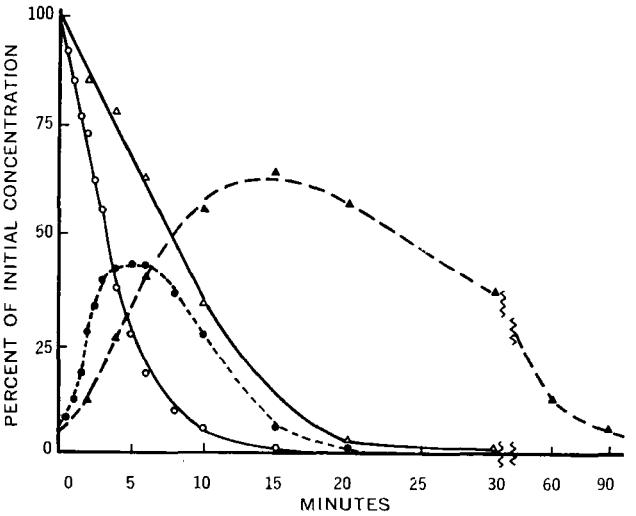


Figure 1—*Plot of concentrations of III and I in 91% whole human blood at 37° as a function of time and the initial concentration of III. The triangles represent the concentrations of III (△) and I (▲) at an initial concentration of 2 mg of III/ml of blood solution. The circles represent the concentrations of III (○) and I (●) where the initial concentration of III was 0.4 mg/ml.*

The buffer systems used in the pH range studied were phosphate, chloroacetate, and acetate. All of these buffers were observed to catalyze the ester hydrolyses to a limited extent. Because of such catalysis, the values for the rate constants at the various pH values, as shown in Fig. 2, were obtained by extrapolation to zero buffer concentration of the linear relationship between the observed rate constant and buffer concentration for a given buffer system at that particular pH.

As can be seen from Fig. 2, the minimum spontaneous hydrolyt-

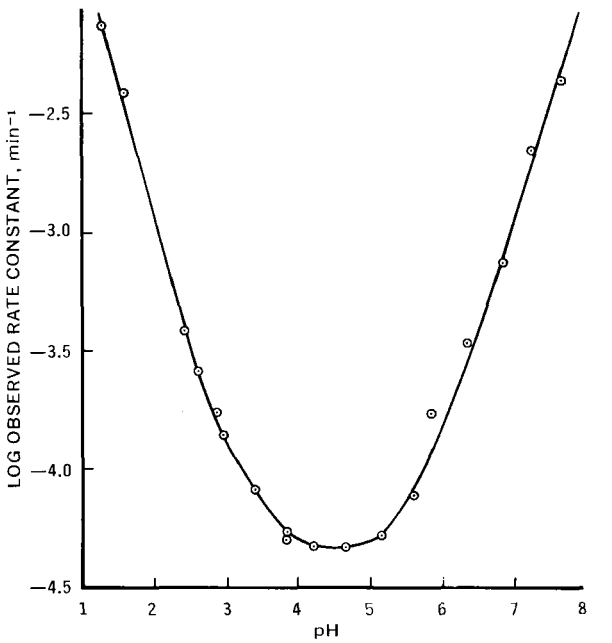


Figure 2—*Log rate–pH profile for the spontaneous hydrolysis of III at 25°, ionic strength 0.05. The data points for the various pH values were obtained by extrapolation of plots of observed rates* versus *buffer concentration at each pH.*

Table II—Temperature Effect on the Rate of Hydrolysis of III at pH 4.2, Ionic Strength 0.05

T	k_0, $\times 10^4$ min^{-1a}	$t_{1/2}$, days
25°	0.47	10.0
36.8°	1.4	3.4
60°	82	0.68

[a] These values are for the spontaneous hydrolysis of III and were obtained by extrapolation to zero buffer concentration.

ic rate, or the maximum stability of III, is found at pH ~4.2. At this pH the observed first-order rate constant was about 4.7×10^{-5} min^{-1} which corresponds to a half-life of about 10 days. At pH 7.4, which approximates physiological pH, the hydrolytic rate constant as shown in Fig. 2 is about 2.5×10^{-2} min^{-1} which corresponds to a half-life of about 4.5 hr.

The effects of temperature on the rate of hydrolysis of III at pH 4.2 were determined and these data are presented in Table II. The rate constants were obtained by extrapolation to zero buffer concentration from a series of buffered solutions. The energy of activation for the hydrolytic reaction was calculated from the data in Table I and was found to be about 16 kcal/mole.

Formulation Considerations—On the basis of the kinetic data, it was apparent that III would not be suitably stable for formulation as ready-to-use solution even at refrigerated temperatures. However, the 10-day half-life for hydrolysis at pH 4.2 and room temperature appeared to be compatible with its use as a formulation to be reconstituted as a solution, at pH 4.2., within several hours prior to use.

The suitability of the prodrug for use in a lyophilized dosage form was examined in a limited study. An unbuffered aqueous solution containing ~30 mg of III/ml, which is equivalent to ~27 mg of I/ml, was prepared and 1-ml aliquots were quickly frozen and lyophilized. The freeze-drying process resulted in a white porous cake which was readily reconstituted as a solution upon the addition of 1 ml of water. The lyophilization process and subsequent storage of the freeze-dried product in sealed vials for 3 months at 4° resulted in less than 1% degradation of III. These data seem to suggest strongly that formulation of III as a lyophilized dosage form would present no major problems.

CONCLUSION

On the basis of these results, it appears that III meets virtually all the important criteria for a prodrug form of arabinosyladenine which would be suitable for formulation in parenteral solutions.

More importantly, this work has demonstrated that the design of a prodrug can be accomplished through use of a rational approach. Such an approach requires that the underlying causes which necessitate the use of the prodrug approach be defined and understood. It may then be possible to identify several alternatives which may overcome these basic difficulties. However, only those specific approaches which are compatible with the overall requirements and objectives of the chosen or required pharmaceutical system can be utilized effectively.

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Percutaneous Absorption of Drugs IV: Percutaneous Absorption of Drugs from Oily Vehicles

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Abstract □ The percutaneous absorption and retention of salicylic acid and carbinoxamine from four oily vehicles (liquid paraffin, oleic acid, hexadecyl alcohol, and isopropyl myristate) were studied by employing a recirculation apparatus. The absorption followed first-order kinetics, with the exception of the initial period. The vehicle that had a strong affinity to the drug showed a poor drug-releasing effect, and poor absorption and retention of drugs by the skin were observed. Higher absorption rate constants were observed for damaged skin than intact skin. The acceleration of absorption because of skin damage, however, was not so great from liquid paraffin as from the aqueous solution. The amount of drugs retained in the damaged skin declined following the initial increase.

Keyphrases □ Salicylic acid in oily vehicles—correlations between *in vitro* adsorption and *in vivo* percutaneous absorption, physicochemical parameters □ Carbinoxamine in oily vehicles—correlations between *in vitro* adsorption and *in vivo* percutaneous absorption, physicochemical parameters □ Vehicles, oily—percutaneous absorption of salicylic acid and carbinoxamine from liquid paraffin, hexadecyl alcohol, oleic acid, and isopropyl myristate □ Absorption, percutaneous—salicylic acid and carbinoxamine from oily vehicles, intact and damaged skin

In previous studies (1–3), the percutaneous absorption of salicylic acid and carbinoxamine was investigated using the intact and damaged skin of male guinea pigs. The factors involved in the percutaneous absorption from aqueous solutions were examined by measuring the amount of the drugs retained in the skin and the amount lost from the recirculating solutions. Ointment and cream bases are generally used in topical dosage forms. It is, therefore, possible that these vehicles interact with drugs and modify their release rate from the bases. Moreover, vehicles may have effects on the permeability of drugs through the cutaneous tissue barrier and on the barrier itself.

The percutaneous absorption of drugs from vegetable oils and animal fats that tend to penetrate through the skin was reported to be superior to that from mineral oils (4–6). The effects of various oily vehicles on the percutaneous absorption of salicylic acid was studied by measuring the amount of the drug excreted in the urine; the drug was substantially absorbed from lard and lanolin but only slightly absorbed from petrolatum[1] (7, 8). Strakosch (9) reported that the extent of absorption of salicylic acid was not affected by the type of emulsion, but Stolar *et al.* (10) pointed out that the extent of absorption of drugs varies with the type of emulsion.

The absorption of salicylic acid from hydrophilic ointment was less than that from yellow petrolatum, simple ointment, and absorption ointment, although the influence of the ointment base was not great (11).

[1] Vaseline.

Studies on the absorption of salicylic acid from polyethylene glycol 400 and four other hydrophilic vehicles showed that the partition coefficient of the drug between the vehicle and benzene was well correlated with the degree of drug absorption from the vehicle (12). An experimental model for the thermodynamic consideration of the relation between the vehicle and percutaneous absorption was presented (13, 14), but many problems remain to be solved.

In the present study, the authors used oily vehicles to examine the influence of four different types of bases, *i.e.*, liquid paraffin, oleic acid, hexadecyl alcohol, and isopropyl myristate, on the percutaneous absorption of drugs and compared the absorption from the oily vehicles with that from aqueous solutions.

EXPERIMENTAL

Drugs—Salicylic acid (pKa = 3.0) was employed as an acidic drug, and carbinoxamine (pKa = 8.9) was used as a basic drug. These drugs were dissolved in hexadecyl alcohol, oleic acid, or isopropyl myristate to give a concentration of 500 μg/ml. Carbinoxamine was dissolved in liquid paraffin to give concentrations of 250, 500, and 1000 μg/ml; the concentrations of salicylic acid were 75, 150, and 300 μg/ml because of its poor solubility in liquid paraffin.

Oily Vehicles—Light liquid paraffin (specific gravity 0.855, bp 180–190°/5 mm Hg), hexadecyl alcohol (2-hexyl-1-decanol, specific gravity 0.842, bp 195–205°/50 mm Hg), oleic acid (specific gravity 0.895, bp 230–235°/15 mm Hg), and isopropyl myristate (specific gravity 0.850, bp 158–160°/5 mm Hg) were used immediately after distillation of the reagent grade oils.

Absorption Experiments—A recirculation apparatus, described previously (1), was fixed to the abdominal skin of a male guinea pig, 300–400 g, after removal of the abdominal hair with an electric hair clipper. The abdominal skin from which the stratum corneum was removed by stripping 20 times with cellophane adhesive tape was used as a damaged skin. During the recirculation of test solutions at a constant flow rate, an aliquot of the test solution was pipetted at fixed time intervals and the amount of the drug absorbed was calculated from the decrease in the concentration of the drug in the recirculating solution. Experimental conditions were the same as reported previously (1–3): area of skin, 2.25 π cm^2; temperature of solutions, 31 ± 1°; recirculating flow rate, 15 ± 5 ml/min; solution volume, 25 ml; and room temperature, 23 ± 2°.

Measurement of Cutaneous Retention of Drugs—Male guinea pigs were killed immediately after recirculation of a drug solution for a given time, and the area of the abdominal skin to which the apparatus was fixed was wiped with absorbent cotton to remove the drug solution. The skin of the area was then excised to the corium, and the drug in the skin was determined by the analytical methods described previously (2, 3).

Determination of Partition Coefficients—Ten milliliters of an isotonic solution of sodium chloride was added to 10 ml of the oily vehicles containing the drug at the same concentrations as the test solutions in the absorption experiment. The mixtures were immersed in a thermostated water bath at 31°, and the container was shaken in the bath for 30 sec every 10 min. When equilibrium was attained (approximately 4 hr), the oily phase was separated by centrifuging and the drug concentration was determined spectro-

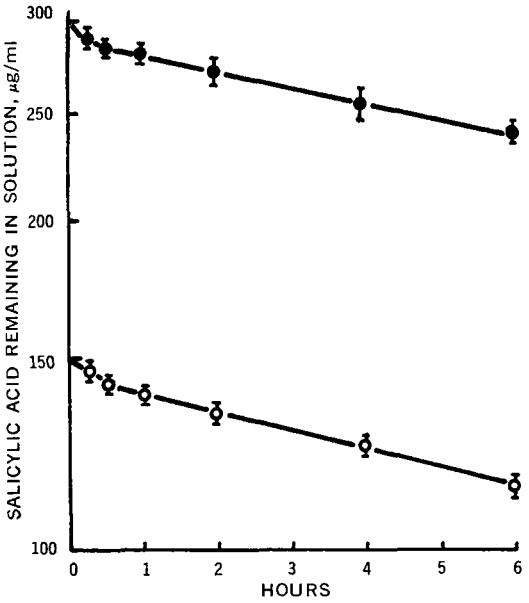

Figure 1—*Logarithmic plots of salicylic acid remaining in the recirculating liquid paraffin solution against time. Initial concentrations of salicylic acid were 300 (●) and 150 (○) μg/ml (mean ± standard error of five experiments).*

photometrically. The partition coefficient was calculated by comparison of the equilibrium concentration with the initial concentration of the drug in the oily vehicle.

Determination of Solubility of Salicylic Acid—Each oily vehicle was placed in a glass-stoppered test tube, and excess salicylic acid was added. The tubes were then shaken in the thermostated water bath. An aliquot of the supernate was pipetted at fixed time intervals until the equilibrium concentration was obtained. The equilibrium value was taken as the solubility of drug in the oily vehicles.

Experiment on Adsorption of Drugs to Excised Skin *In Vitro*—Twenty milliliters of each oily vehicle containing a known amount of drug was placed in a glass-stoppered 50-ml test tube, and a section of abdominal skin (2.25 π cm^2 in size) of the guinea

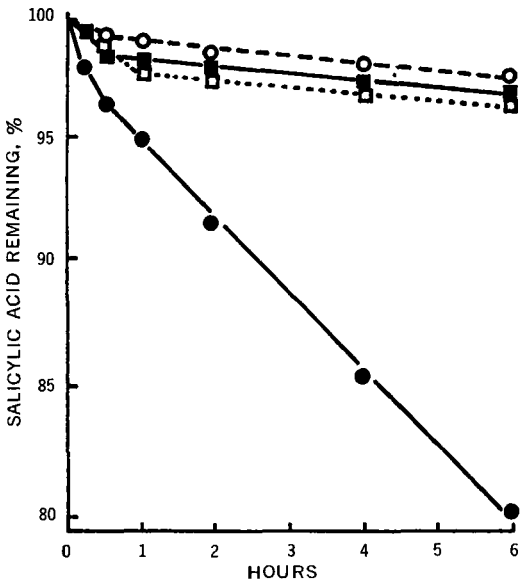

Figure 2—*Logarithmic plots of salicylic acid remaining in various oily vehicles against time. Key: ●, liquid paraffin; ○, oleic acid; ■, hexadecyl alcohol; and □, isopropyl myristate. Initial concentrations of salicylic acid were 300 μg/ml in liquid paraffin and 500 μg/ml in the rest. Each plot represents the mean of three to six experiments.*

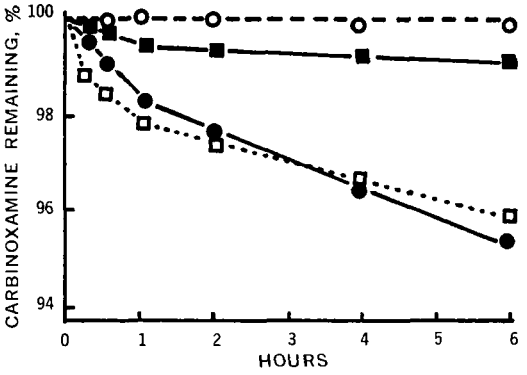

Figure 3—*Logarithmic plots of carbinoxamine remaining in various oily vehicles against time. Key: ●, liquid paraffin; ○, oleic acid; ■, hexadecyl alcohol; and □, isopropyl myristate. Initial concentrations of carbinoxamine were 500 μg/ml.*

pig was added. The tube was placed in the thermostated water bath at 31° and shaken occasionally. An aliquot of the solution was withdrawn at suitable time intervals over 96 hr to determine the equilibrium amount of drug adsorbed by the excised skin.

Change in Solution Volume—The change in the solution volume during the experimental period was determined because absorption of the oily vehicle through the skin was expected. The volume of the test solution at the starting point of this experiment was compared with that after recirculation for 6 hr. The absorption of the oily vehicles through both the intact skin and the damaged skin during the experimental period was negligible.

Determination of Salicylic Acid in Oily Vehicles—Salicylic acid in oleic acid was determined by the colorimetric method using ferric nitrate (15). Determination of salicylic acid in the other oily vehicles was carried out by partly modifying the method used for determination of the drug in an aqueous solution (1). One milliliter of the test solution was placed in a glass-stoppered test tube, and 2 ml of chloroform and 8 ml of 0.2 *N* NaOH were added. After shaking for 20 min, the tube was centrifuged and 2 ml of the aqueous layer was transferred to another test tube. To this tube was added 5 ml of chloroform. After shaking for 20 min and centrifuging, sali-

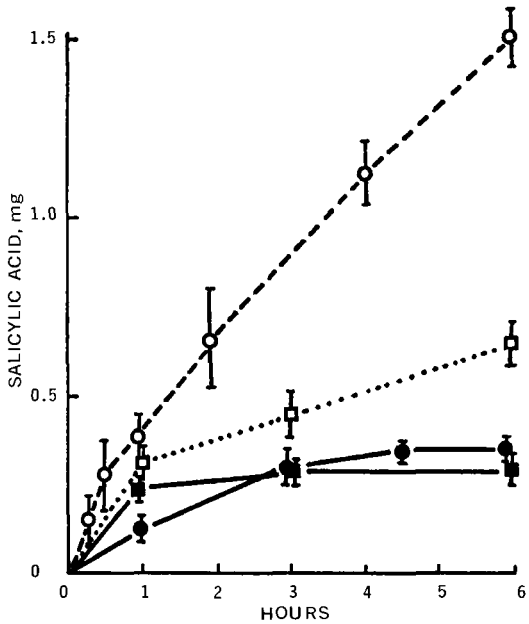

Figure 4—*Percutaneous absorption and retention patterns of salicylic acid. Key: ●, amount retained in the skin from liquid paraffin; ○, amount absorbed from liquid paraffin; ■, amount retained in the skin from buffer solution, pH 3.0; and □, amount absorbed from buffer solution, pH 3.0. Initial concentrations of salicylic acid were 300 μg/ml in liquid paraffin and 500 μg/ml in the buffer solution.*

Table I—Relationship between Percutaneous Absorption and Physical Properties of Drugs

Drugs	Oily Vehicles	Amount Absorbed during 1–6 hr, %	Solubility at 31°, mg/ml	Water–Oil Partition Coefficient at 31°	Amount Adsorbed by Excised Skin *In Vitro*, mg
Salicylic acid	Liquid paraffin	14.6	0.495	41.5	3.56
	Isopropyl myristate	1.7	43.1	0.17	2.26
	Hexadecyl alcohol	1.6	79.2	0.17	1.57
	Oleic acid	1.5	45.2	0.24	0.73
Carbinoxamine	Liquid paraffin	2.8	Very soluble	0.49	0.92
	Isopropyl myristate	1.7		1.09	1.18
	Hexadecyl alcohol	0.0		0.51	0.16
	Oleic acid	0.0		1.02	0.001

cylic acid in the chloroform layer was determined spectrophotometrically at 310 nm.

Determination of Carbinoxamine in Oily Vehicles—Carbinoxamine in oily vehicles was determined by partly modifying the previously reported method (1) for the determination of the drug in an aqueous solution. One milliliter of test solution was placed in a glass-stoppered test tube, and 2 ml of chloroform and 8 ml of 0.2 *N* HCl were added. After shaking for 20 min, the tube was centrifuged, 3 ml of the aqueous layer was transferred to another test tube, and 1 ml of 2 *N* NaOH and 6 ml of chloroform were added. After shaking the tube for 20 min, it was centrifuged and carbinoxamine in the chloroform layer was determined spectrophotometrically at 262.5 nm.

The calibration curves of both salicylic acid and carbinoxamine in oily vehicles were obtained in the 75–1000-μg/ml concentration range prior to the determination of both drugs in oily vehicles. The standard deviation of these data was 1.3%, and these methods were able to determine the drugs with sufficient precision.

Determination of Drug Contents in Skin—Salicylic acid and carbinoxamine in the skin were determined by the method described previously (2).

RESULTS AND DISCUSSION

Absorption of Drugs through Intact Skin—*Percutaneous Absorption of Drugs from Oily Vehicles*—When the logarithmic concentrations of salicylic acid in liquid paraffin were plotted against time, straight lines were obtained except for the initial period (Fig. 1). The slopes of the lines were independent of the initial concentration of drug. Similar straight lines also were obtained with carbinoxamine. Therefore, the disappearance of the drugs from liquid paraffin followed first-order kinetics as was the case from the aqueous solution (1).

From the measurement of salicylic acid concentrations in the recirculating medium at 1 and 6 hr of perfusion for three initial concentrations of 75, 150, and 300 μg/ml, the percentage of salicylic acid absorbed during the 5-hr period was calculated for each initial concentration. The values were around 15% irrespective of the initial concentration. Similarly, the percentage of carbinoxamine absorbed during the same period was around 2.8 for three initial concentrations of 250, 500, and 1000 μg/ml. These observations indicate no saturation phenomenon in the concentration range and thus tend to suggest that the percutaneous absorption of both drugs from liquid paraffin is a simple passive transport.

Figure 2 shows the time course of absorption of salicylic acid from hexadecyl alcohol, oleic acid, and isopropyl myristate together with that from liquid paraffin. The corresponding plots for carbinoxamine are presented in Fig. 3. Little carbinoxamine was absorbed from oleic acid, whereas some carbinoxamine disappeared from hexadecyl alcohol only during the 1st hr. For all salicylic acid systems and carbinoxamine in liquid paraffin and isopropyl myristate, the drug concentration of the recirculating solutions de-

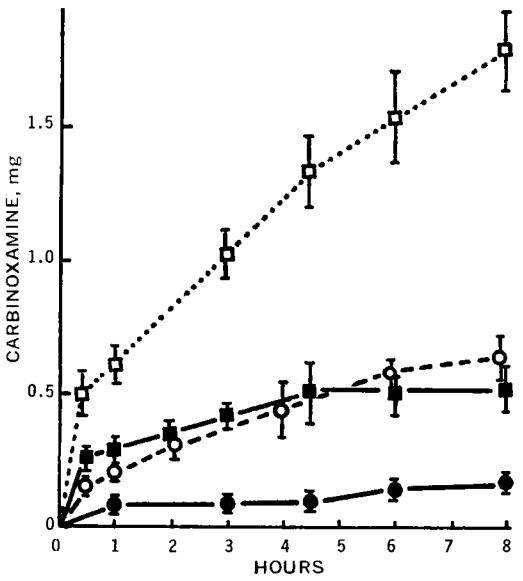

Figure 5—*Percutaneous absorption and retention patterns of carbinoxamine. Key: ●, amount retained in the skin from liquid paraffin; ○, amount absorbed from liquid paraffin; ■, amount retained in the skin from buffer solution, pH 9.0; and □, amount absorbed from buffer solution, pH 9.0. Initial concentrations of carbinoxamine were 500 μg/ml.*

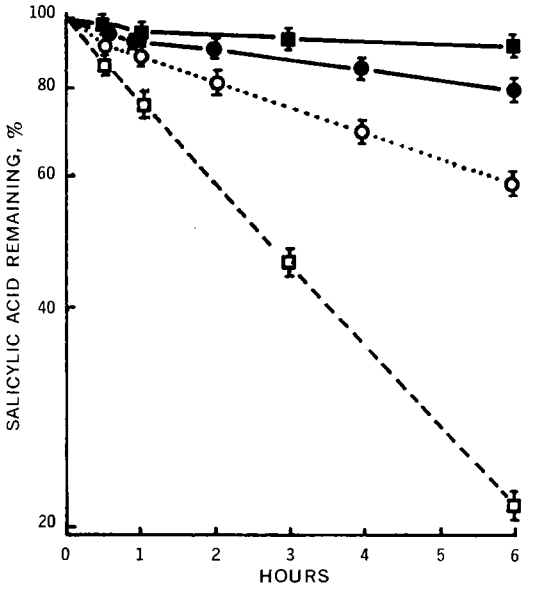

Figure 6—*Logarithmic plots of salicylic acid remaining in the recirculating solution against time. Key: ●, liquid paraffin on intact skin; ○, liquid paraffin on damaged skin; ■, buffer solution, pH 3.0, on intact skin; and □, buffer solution, pH 3.0, on damaged skin. Initial concentrations of salicylic acid were 500 μg/ml in the buffer solution and 300 μg/ml in liquid paraffin.*

Table II—Relationship between Percutaneous Absorption of Salicylic Acid and Composition of Vehicle

Composition of Vehicle, %		Amount Absorbed during 1–6 hr, %	Solubility at 31°, mg/ml	Water–Oil Partition Coefficient at 31°
Liquid Paraffin	Isopropyl Myristate			
100	0	14.6	0.495	41.5
90	10	5.7	3.21	1.73
75	25	3.9	7.97	0.75
50	50	2.8	17.6	0.39
25	75	2.6	29.5	0.27
0	100	1.7	43.1	0.17

creased, following first-order kinetics after a lapse of the initial period.

The solubilities of salicylic acid in these vehicles and the water–oil partition coefficients of both drugs were investigated to examine the cause of the difference in the percutaneous absorption among these vehicles (Table I). The solubility of salicylic acid in liquid paraffin was lower than that in other solvents, whereas the water–oil partition coefficient was higher. These physical properties of salicylic acid may account for its greater absorption from liquid paraffin than from other oils. For carbinoxamine, however, the difference in the amount absorbed among these vehicles was not so great because its partition coefficients were all small and of similar magnitude.

The amount of drug adsorbed by the excised skin *in vitro* from the four different vehicles (Table I) seems to be related to absorption in the initial period from the recirculating solution (Fig. 2). Only a trace of carbinoxamine was adsorbed from oleic acid, while the amounts absorbed from liquid paraffin and isopropyl myristate were as large as about 1 mg. This finding is also reflected by absorption of carbinoxamine in the initial period (Fig. 3).

To examine the correlation between the affinity of a drug to vehicles and the amount absorbed, liquid paraffin and isopropyl myristate, which differ widely from each other in solubility and absorption characteristics of salicylic acid, were mixed in different proportions, and the variation of percutaneous absorption of salicylic acid with solvent composition was investigated (Table II). The solubility of salicylic acid in the vehicles increased and the water–oil partition coefficient decreased as the proportion of isopropyl myristate was increased. Thus, it became evident that the amount of the drug absorbed tends to decrease as the affinity of drug to the vehicle is increased.

Kakemi *et al.* (16) postulated two mechanisms for rectal absorption of sulfonamides from oils: the drug dissolved in the oil is absorbed into the rectal mucosa either directly or through the mucosal secretion. When the latter mechanism prevails over the former, the water–oil partition coefficient of the drug between the aqueous

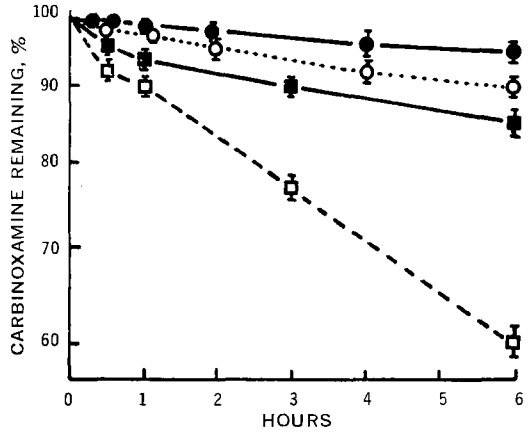

Figure 7—*Logarithmic plots of carbinoxamine remaining in the recirculating solution against time. Key:* ●, *liquid paraffin on intact skin;* ○, *liquid paraffin on damaged skin;* ■, *buffer solution, pH 9.0, on intact skin; and* □, *buffer solution, pH 9.0, on damaged skin. Initial concentrations of carbinoxamine were 500 μg/ml.*

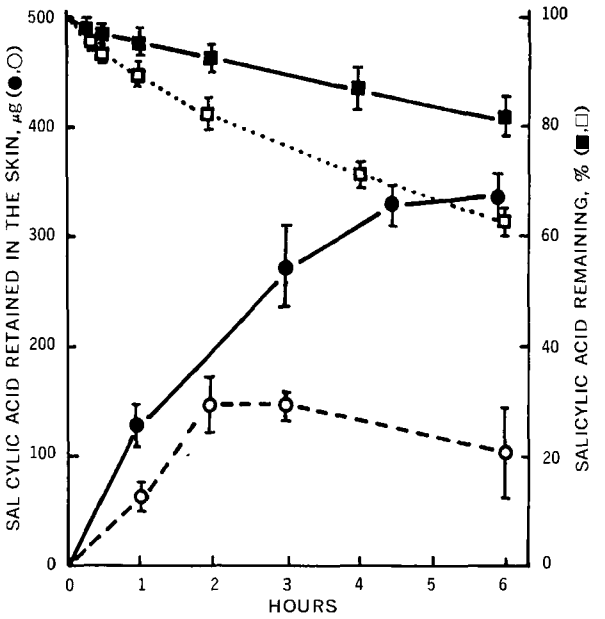

Figure 8—*Percutaneous absorption pattern of salicylic acid from liquid paraffin. Key:* ●, *amount retained in intact skin;* ○, *amount retained in damaged skin;* ■, *percent remaining in liquid paraffin applied on intact skin; and* □, *percent remaining in liquid paraffin applied on damaged skin. Initial concentrations of salicylic acid were 300 μg/ml.*

and oily phases is expected to influence greatly the absorption rate of drugs. In the reverse situation, this would not be true.

As in the case of rectal absorption of drugs from oils, these two mechanisms may be considered for percutaneous absorption, *i.e.*, direct absorption and absorption *via* the aqueous secretion (secreted by the skin). The second mechanism is more likely to be predominating in the percutaneous absorption of salicylic acid from oils. This proposition is based on the fact that the amount of salicylic acid absorbed from liquid paraffin, which has a large drug-releasing ability (high partition coefficient), was much larger than that from other oils having a strong affinity to salicylic acid and very poor drug-releasing power (low partition coefficient). The absorption of carbinoxamine, on the other hand, cannot be interpreted similarly and requires further consideration.

Time Course of Cutaneous Retention of Drugs from Oily Vehicles—Figure 4 shows the comparison of the amount of salicylic acid absorbed and that retained in the skin when two different vehicles (*i.e.*, liquid paraffin and an aqueous solution) were used. A similar comparison is presented for carbinoxamine in Fig. 5. The amount retained in the skin and that absorbed from the aqueous vehicle increased with time in a similar manner during the initial stage of the experiment with salicylic acid as described previously (2). But the former quickly approached a plateau value.

From the liquid paraffin, however, the amount absorbed during the initial period was much larger than the amount retained. After 6 hr of recirculation, the ratio of the amount of the drugs retained to that absorbed from liquid paraffin was lower than that from the aqueous vehicle, *i.e.*, 0.20 and 0.25 from liquid paraffin for salicylic acid and carbinoxamine, respectively, and 0.45 and 0.35 from the aqueous solution for the same drugs, respectively. A similar trend was observed for the other three oily vehicles.

These variations in cutaneous retention phenomenon with the nature of the vehicle may be attributed to the fact that the stratum corneum is highly swollen with water when the vehicle is an aqueous solution, whereas no swelling takes place when oily vehicles such as liquid paraffin are employed. The swelling may enhance the retention of drugs because it increases the volume of the stratum corneum.

Absorption of Drugs through Damaged Skin—*Percutaneous Absorption of Drugs from Oily Vehicles*—In Figs. 6 and 7 the percentage of salicylic acid or carbinoxamine remaining in the recirculating solution is plotted against time for the aqueous solution and liquid paraffin. Absorption kinetics are also compared between the

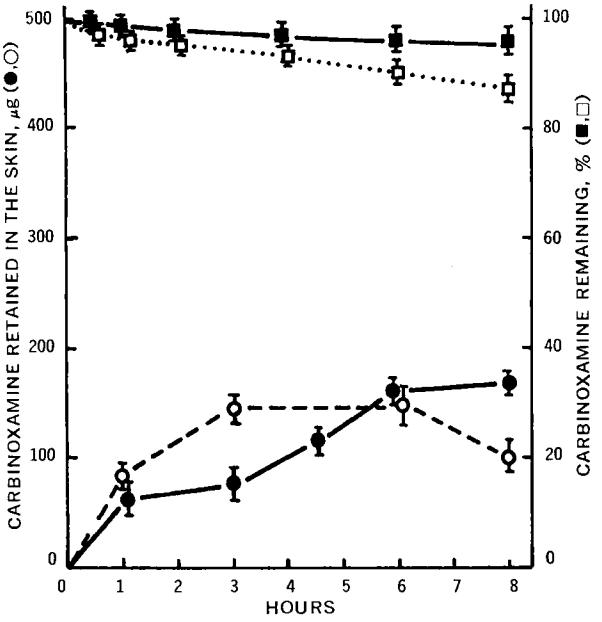

Figure 9—*Percutaneous absorption pattern of carbinoxamine from liquid paraffin. Symbols are the same as in Fig. 8. Initial concentrations of carbinoxamine were 500 μg/ml.*

intact and damaged skins. From the aqueous solution, the absorption rate constant through the damaged skin during the 1–6-hr recirculation period was about 10 times greater for salicylic acid and 3 times greater for carbinoxamine than the corresponding rate constant through the intact skin. However, the absorption rate constant of the drugs through the damaged skin from liquid paraffin was only about twice as much as that through the intact skin.

These results indicated that the damaged skin is particularly permeable to drugs dissolved in water. Drugs dissolved in liquid paraffin may also be absorbed through the damaged skin after being released into an aqueous secretion. Thus, the transfer of the drug into the secretion from oil may be considered the rate-determining step.

Time Course of Cutaneous Retention of Drugs—Figures 8 and 9 show the percentage of the drug absorbed from the recirculating liquid paraffin solution and the corresponding amount retained in the skin for the damaged and intact skins plotted against time. As for salicylic acid (Fig. 8), the amount retained in the damaged skin was less than that in the intact skin and tended to decrease after 2 hr. The absorption of the drug through the damaged skin from the recirculating solution was so rapid that the concentration of the drug remaining in the recirculating solution was decreased to 80% of the original concentration after 2 hr of recirculation and to 60% after 6 hr. Thus, the decrease in salicylic acid concentration in the solution as perfusion continues is most likely to be responsible for the decrease in the amount retained in the skin. Such a decrease was not observed in the intact skin.

As for carbinoxamine, on the other hand, the amount retained in the dámaged skin was larger than that in the intact skin during the 6 hr of recirculation, but the situation was reversed after 6 hr (Fig. 9). The carbinoxamine concentration in the recirculating solution remained at 90% of the initial concentration even after 6 hr of recirculation. The amount of carbinoxamine retained in the damaged skin showed a tendency to decrease only after 6 hr.

These results suggest that the observed difference in cutaneous retention behavior of drug between the intact and damaged skins is attributed to the better absorption of drug through the damaged skin than through the intact skin, whereby the concentration of drug in the recirculating solution falls during the experiment.

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Assay for Acetazolamide in Plasma

W. F. BAYNE ˣ, G. ROGERS, and N. CRISOLOGO

Abstract □ A method for the analysis of acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, sensitive to 25 ng/ml in plasma, was developed. After extraction of acetazolamide and its propionyl analog, 5-propionamido-1,3,4-thiadiazole-2-sulfonamide, the internal standard, from plasma with ethyl acetate and removal of lipids from the residue of the ethyl acetate extract with methylene chloride, the sulfonamides were chromatographed on an octadecyl trichlorosilane bonded phase using high-pressure liquid chromatography. The method was developed to study plasma level profiles of different dosage forms of acetazolamide.

Keyphrases □ Acetazolamide—high-pressure liquid chromatographic analysis in plasma □ High-pressure liquid chromatography—analysis, acetazolamide in plasma

Acetazolamide, 5-acetamido-1,3,4-thiadiazole-2-sulfonamide, is a carbonic anhydrase inhibitor used to lower intraocular pressure through a reduced production of aqueous humor. Its inhibitory effect on carbonic anhydrase was used to develop an assay for acetazolamide in biological fluids (1). The drug was reported to be 90% excreted, on the average, in humans after oral administration (2). The enzymatic method appears to be fairly specific; only metabolites that inhibit carbonic anhydrase should interfere with the specificity of the assay.

A number of unsuccessful attempts to develop chemical methods for acetazolamide in biological fluids were described (1). However, a modification of the Bratton and Marshall procedure (3), a colorimetric method sensitive to 5 μg/ml, was reported for acetazolamide (4). Attempts to prepare acetazolamide derivatives suitable for GLC analysis by the authors were generally unsuccessful. Secondary sulfonamides were methylated with diazomethane (5), but a number of products resulted when acetazolamide, a primary sulfonamide, was treated with diazomethane in this laboratory.

Blessington (6) successfully methylated primary and secondary sulfonamides using a method employed by Pettitt and Stouffer (7) for alkylation of amino acids. Application of this method, utilizing the sulfinyl carbanion of dimethyl sulfoxide and methyl iodide, to acetazolamide yielded one product which was not successfully characterized by GC–mass spectrometry.

Table I—Absolute Recoveries of Acetazolamide from Plasma

Acetazolamide, μg	Recovery, % (Number of Determinations)
0.1	68.0 (3)
0.25	74.9 (2)
0.5	77.3 (3)
0.75	79.3 (2)
1.0	87.0 (3)
2.5	84.0 (2)
5.0	94.2 (3)
10.0	87.0 (1)

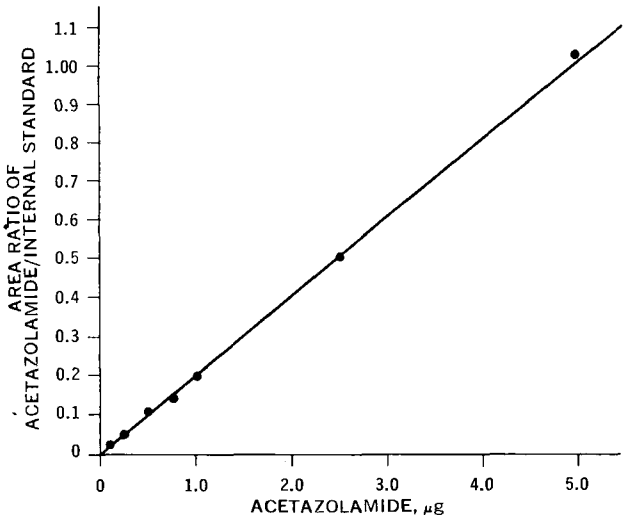

Figure 1—*Standard curve for acetazolamide (5.32 μg of internal standard).*

An extractive alkylation technique recently used to methylate chlorthalidone (8) was investigated by the authors for its potential use in preparing a volatile methylated acetazolamide derivative. Although a single peak resulted under the chromatographic conditions, the peak actually consisted of two compounds, the trimethylated derivative of acetazolamide and the tetramethylated derivative of 5-amino-1,3,4-thiadiazole-2-sulfonamide (I). If the propionyl analog of acetazolamide was methylated, the trimethylated derivative of the analog and I resulted. In this case, the two compounds were resolved, the former having the longer retention time.

Kram (9) separated a number of sulfonamides using high-pressure liquid chromatography with pellicular anion-exchange resins. A recent publication (10) indicated the feasibility of separating sulfonamides by reverse phase chromatography. Therefore, a method for acetazolamide, sensitive to 25 ng/ml plasma, was developed using high-pressure liquid chromatography that requires no prior derivatization.

EXPERIMENTAL

Materials—A piston-driven, constant-flow, pulse-free pump[1], with a 2000-psi limit, in conjunction with a septumless injector and 254-nm UV detector[2] (8-μl flow cell), was used for analyzing samples. The output was recorded on a variable span, potentiometric recorder. Most separations were performed on a 1-m, 6.35-mm o.d., 2-mm i.d., precision-bored stainless steel column packed with an octadecyl trichlorosilane bonded phase[3] with a particle size of 30–44 μm. Sodium acetate buffer (0.05 *M*, pH 4.5) with 2% (v/v)

[1] Instrumentation Specialties Co., Lincoln, Neb.
[2] Varian Instrument Division, Palo Alto, Calif.
[3] Vydac Reverse Phase, Applied Science Laboratories, State College, Pa.

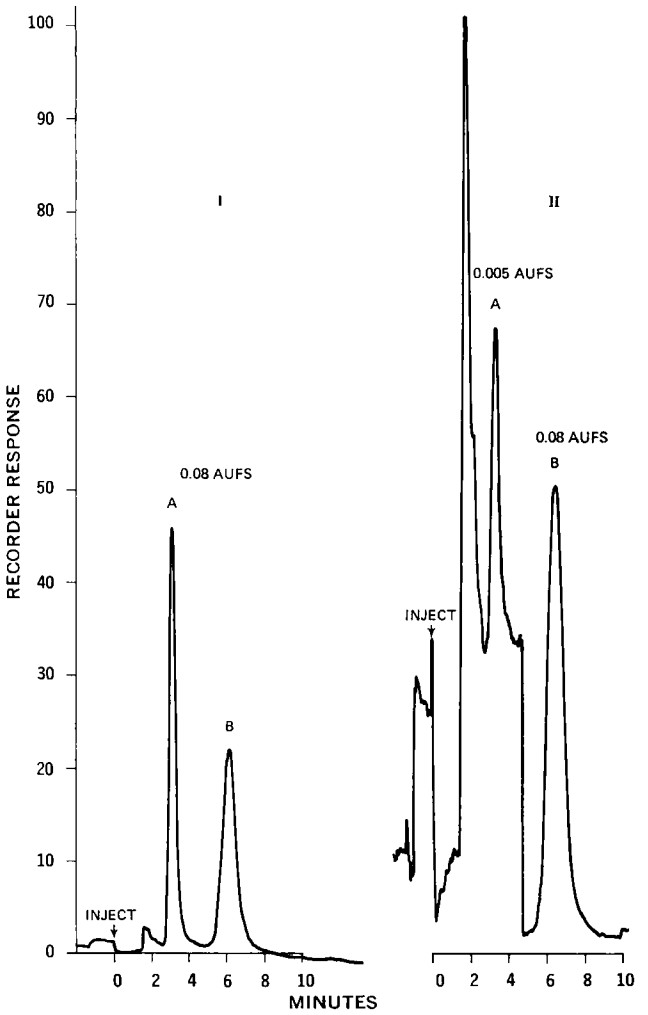

Figure 2—*Chromatograms of acetazolamide (A) and internal standard (B) in 1 ml of plasma. Key: I, 0.1 μg of acetazolamide and 5.32 μg of internal standard; and II, 5.0 μg of acetazolamide and 5.32 μg of internal standard.*

methanol was employed as the mobile phase. The flow rate was 48 ml/hr with pressures in the neighborhood of 1050 psi.

Some separations were performed on a 30-cm, 4-mm i.d. column packed with octadecyl trichlorosilane bonded to less than 10 μm diameter packing material which recently became available[4]. In this case, the same buffer was used but contained 15% (v/v) methanol. The flow rate was 120 ml/hr with pressures of about 1500 psi.

Reagents—Glycine buffer was prepared by addition of sulfuric acid to an aqueous glycine solution to obtain a pH 2 buffer. Sodium acetate and ammonium acetate buffers were prepared by titrating acetic acid with sodium hydroxide or ammonium hydroxide, respectively, to obtain pH 4.5 buffers.

Extraction Procedure and Standard Curve—Solutions of acetazolamide[5] and internal standard, 5-propionamido-1,3,4-thiadiazole-2-sulfonamide[6], were prepared daily in 0.01 *N* NaOH at concentrations of 1 mg/ml. The acetazolamide solution was diluted 1:100 and 1:20 with 0.01 *N* NaOH prior to addition to plasma. To 1-ml quantities of plasma, contained in 40-ml Teflon-stoppered[7] centrifuge tubes, 0.1, 0.25, 0.5, 0.75, 1.0, 2.5, and 5.0 μg of acetazolamide were added. The 1:100 dilution was used for the first four samples, while the 1:20 dilution was used for the last three samples.

From a 1:10 dilution of the internal standard solution, a constant amount of internal standard, 5.32 μg, was added to the solutions containing the different quantities of acetazolamide. Three milliliters of 0.5 *M* ammonium acetate buffer (pH 4.5) was added to the plasma. The aqueous phase was extracted twice with 10-ml portions of ethyl acetate, the contents being shaken for 5 min and centrifuged at 2500 rpm after each extraction. The combined organic phase was transferred to another 40-ml centrifuge tube and evaporated at 40° with a stream of nitrogen.

To the yellow residue, 4 ml of 0.25 *M* glycine buffer (pH 2) was added. The aqueous phase was extracted twice with 5-ml portions of methylene chloride and the organic phase, which retained the yellow pigments, was discarded. During this extraction, the contents were shaken gently to prevent emulsion formation. The aqueous phase was then extracted twice with 6-ml portions of ethyl acetate, and the combined organic extracts were evaporated to dryness. The residue was dissolved in 100 μl of 0.01 *N* NaOH, and the contents were sonified to ensure dissolution. Then 10–25 μl of the 0.01 *N* NaOH solution was injected onto the chromatographic column by the stop-flow technique. The peak areas of acetazolamide and the internal standard at 254 nm were determined by multiplying the peak height times the width at half-height.

The standard curve and typical chromatograms obtained in its preparation are presented in Figs. 1 and 2, respectively. Figure 3 illustrates the chromatogram obtained from a sample containing 25 ng/ml acetazolamide, together with a blank, using the highly efficient, small-diameter packing material.

Analysis of Samples—Approximately 5 μg of the internal stan-

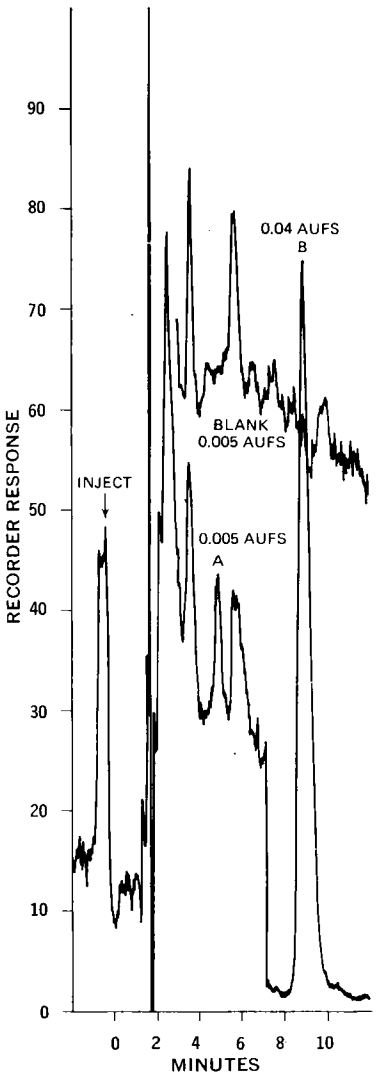

Figure 3—*Chromatograms of acetazolamide (A: 25.2 ng/ml plasma), internal standard (B: 2.58 μg/ml plasma), and blank.*

[4] μBondapak C18, Waters Associates, Milford, Mass.
[5] Sigma, St. Louis, Mo.
[6] Gift of Lederle Laboratories, Pearl River, N.Y.
[7] Kontes, Vineland, N.J.

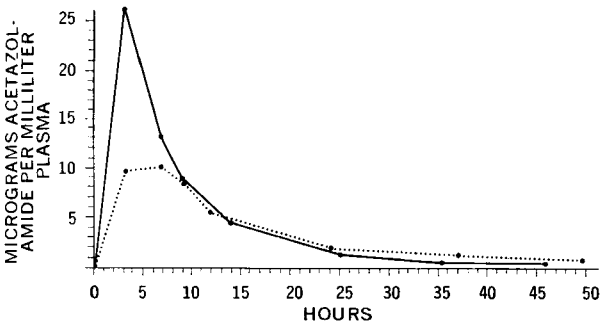

Figure 4—*Plasma level profiles for different dosage forms of acetazolamide. Key: —, tablet, 500 mg; and . . . ., timed-release formulation, 500 mg.*

dard, 5-propionamido-1,3,4-thiadiazole-2-sulfonamide, accurately known through weighing and dilution, was added to the plasma samples to be analyzed. The extraction procedure was then performed as described. The standard curve was used to determine the quantity of acetazolamide present in a known volume of sample. To reduce build-up of highly retained compounds, which reduced the efficiency of the column after repeated injections, it was convenient to inject 50 μl of methanol or dimethylformamide after each sample injection.

RESULTS

When the slope (0.205) for the regression line of the data for the standard curve was multiplied by the amount of internal standard employed (5.32 μg), a value of 1.09 was obtained. This value was very close to the molecular weight ratio (1.06) of the acetamido and propionamido analogs. The molar extinction coefficient for the two compounds was 8.52×10^3 at 254 nm.

The plasma level–time curves for a 500-mg oral tablet and a 500-mg timed-release formulation[8] are presented in Fig. 4. Significant plasma concentrations exist for both dosage forms 45 hr after administration. This finding can be explained by the strong affinity of carbonic anhydrase in the red blood cells and other tissues for acetazolamide. This affinity results in extremely small concentrations of free drug within these tissues and hence small gradients for the transport of drug from these tissues (11). A full analysis of the pharmacokinetics will be presented in the future.

DISCUSSION

Acetazolamide was initially chromatographed on pellicular anion-exchange column. However, the efficiency of the column rapidly declined with repeated injections of biological samples. Acetazolamide was reported to have two pKa's of 7.4 and 9.1 (12). The absorption maximum shifts from 265 nm for the nonionized form to 290 nm for the totally ionized form. Since a 254-nm UV detector was employed, sensitivity was progressively lost as the pH of the buffers was increased from 7 to 9 to increase the retention of acetazolamide. Therefore, reverse phase chromatography was employed. There was no evidence of column degradation with continuous use.

[8] Sequel, Lederle Laboratories, Pearl River, N.Y.

Table II—Area Ratios for Timed-Release Formulation

Hours	Area Ratio, Acetazolamide/ Internal Standard
3.3	1.96; 1.96
6.25	2.02; 2.11
9.25	1.76; 1.73
12.25	1.11; 1.12
24.25	0.409; 0.422
37.25	0.224; 0.234
49.25	0.182; 0.174

The absolute recoveries of acetazolamide from 1 ml of plasma (Table I) ranged from 68 to 94% for concentrations of 0.1–10.0 μg/ml. These recoveries were determined by adding known quantities of acetazolamide to plasma and comparing the amounts recovered, using the previously discussed extraction scheme, to known amounts of acetazolamide injected directly into the liquid chromatograph from the solution used to spike the plasma samples. The recoveries showed the generally expected trend of higher percent recoveries with higher concentrations. This variability was overcome by using an appropriate internal standard with similar partitioning properties.

To check the precision in performing the assay procedure with an internal standard, plasma concentrations from the orally administered timed-release formulation were determined in duplicate. The measured ratios, corrected to the 5.32 μg of internal standard used in the construction of the standard curve, are presented in Table II.

The described analysis, with some modification, can obviously be extended to other sulfonamides and their metabolites.

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Absorption of Metrizamide from Cerebrospinal Fluid to Blood: Pharmacokinetics in Humans

KLAES GOLMAN

Abstract □ The rate of transfer of contrast agents from cerebrospinal fluid to blood is of clinical importance in radiological examinations of the subarachnoid space. Metrizamide, a potential contrast medium, was injected intrathecally to humans and serum levels at different times after injection were measured. A one-compartment open model was found to apply to the data. Considerable individual variations were found, but the mean absorption rate constant indicated that more than 50% of the absorbed dose had disappeared from the cerebrospinal fluid 0.75 hr after injection. TLC of the fraction excreted in the urine showed that metrizamide was not metabolized in the body.

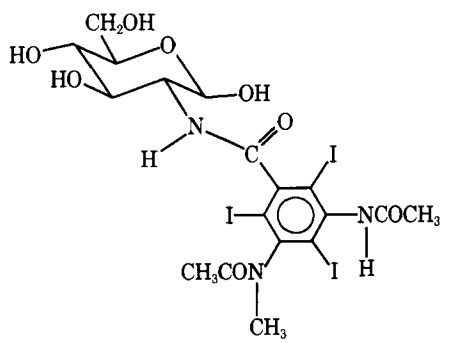
Keyphrases □ Metrizamide—pharmacokinetics after intrathecal administration, concentration in cerebrospinal fluid, humans □ Pharmacokinetics—metrizamide absorption from subarachnoid space after intrathecal administration, humans □ Radiology—pharmacokinetics of metrizamide absorption from subarachnoid space after intrathecal administration, humans □ Absorption kinetics—metrizamide from cerebrospinal fluid, humans

Metrizamide (I) is a contrast agent (1) developed for use in the subarachnoid space. The absorption of metrizamide and other water-soluble contrast agents from cerebrospinal fluid to blood was previously followed by evaluation of X-ray films taken at different times after intrathecal injection of the contrast agents (2–6). However, it is difficult from subjective evaluation of X-ray films to estimate the biological half-life of the contrast agent in the subarachnoid space. Therefore, the absorption process was examined further by determining pharmacokinetic parameters describing the absorption, distribution, and elimination of metrizamide after intrathecal injection in humans.

EXPERIMENTAL

Subjects—The subjects were patients who volunteered to participate after the test was discussed with them. The patients were part of a previously reported study (2), and each patient was examined and had a medical evaluation as described in that study.

Methods—For the present study the following sampling was relevant. Venous blood samples were taken at 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, and 23 hr after intrathecal injection of 1700 mg of I (170 mg I/ml) in the lumbar region (L1–L4). The weight of the patients was in the 59–94-kg range. Cerebrospinal fluid samples were obtained by a second spinal puncture taken at 6 or 24 hr after the injection.



I

Quantitative urine collection was performed during the first 24 hr after injection. Serum and cerebrospinal fluid samples were analyzed for their iodine content by a modification of the alkaline ashing method[1] (7) and urine samples were analyzed by the oxygen flask combustion method[2] (8).

Pharmacokinetic Analysis—To determine the kinetic constants related to the absorption of metrizamide into the blood, a one-compartment open model was used. In this model the serum concentration follows the equation of Teorell (9):

$$C = \frac{FDk}{V(k - K_E)}[e^{-K_E(T-T_0)} - e^{-k(T-T_0)}] \qquad \text{(Eq. 1)}$$

where:

C = serum concentration
T = time after injection
T_0 = lag time
F = fraction of dose absorbed
D = dose
k = first-order rate constant for absorption
K_E = first-order rate constant for excretion
V = apparent volume of distribution

Initial estimates for the parameters k, K_E, and V/F were obtained from observed serum levels using the feathering technique described by Wagner (10). Final estimates were found by fitting the theoretical curve (Eq. 1) to the observed values. The curve fitting was done by computer[3]. From the final estimates for the absorption rate constant, the percentage absorbed from the subarachnoid space was calculated as a function of time by the equation:

$$\left(\frac{A_T}{FD}\right)100 = (1 - e^{-kT})100 \qquad \text{(Eq. 2)}$$

where A_T = amount of substance that has entered the volume of distribution at time T. The derivation of this equation was described previously (11). All calculations using Eq. 2 have been done

Table I—Serum Values and Percent Absorbed from the Subarachnoid Space after Intrathecal Injection

Time after Injection, hr	Observed I Serum Concentration, μg/ml		Percent of Dose Absorbed[a]
	Mean ± SD	Range	
0.5	6.0 ± 5.1	1.2–7.8	30.2
0.75	8.6 ± 6.9	1.6–22.4	51.7
1	11.4 ± 8.3	3.2–28.4	66.6
1.5	15.4 ± 8.6	5.2–28.0	84.0
2	15.6 ± 8.2	5.2–29.2	92.4
3	14.9 ± 5.8	5.6–24.0	98.2
4	13.9 ± 4.6	7.2–21.2	99.6
6	11.7 ± 3.9	7.2–18.4	99.9
23	3.0 ± 1.7	1.0–4.4	100.0

[a] Calculated from Eq. 2.

[1] At the Central Laboratory, Ullevål Hospital, Oslo, Norway.
[2] By the Analytical Department of Nyegaard & Co, Oslo, Norway.
[3] A nonlinear iterative program written in BASIC for the Honeywell-Bull time-sharing system.

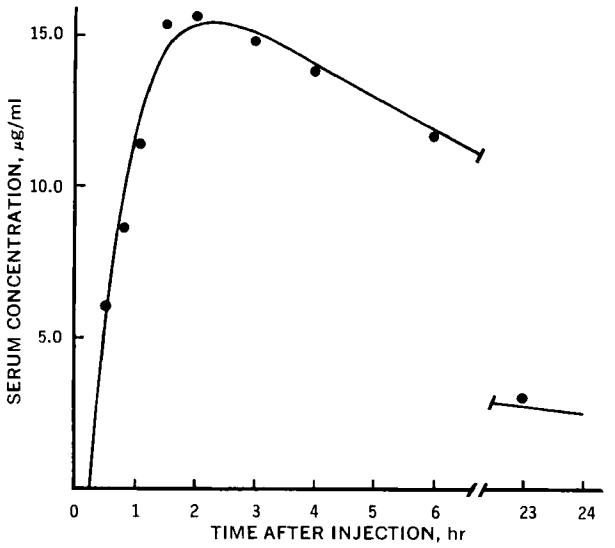


Figure 1—*Serum iodine concentrations. Mean values of observed concentrations after intrathecal injection of metrizamide in eight patients* (●). *Theoretical curve of best fit was calculated from Eq. 1* (—).

with the value of $F = 1$, assuming all metrizamide to be absorbed from the subarachnoid space.

The concentrations of metrizamide in the cerebrospinal fluid at different times after injection were also used for calculation of the biological half-life of metrizamide in the subarachnoid space. When assuming first-order kinetics, the biological half-life ($T_{1/2}$) was calculated by the equation:

$$T_{1/2} = \frac{\ln 2(T_2 - T_1)}{\ln C_1 - \ln C_2} \qquad \text{(Eq. 3)}$$

where C_1 = concentration at time T_1 (hours) after injection, and C_2 = concentration at time T_2 (hours) after injection.

Metabolism—All urine samples were examined for metabolites by TLC. The urine was applied to silica gel plates, which were developed in two systems as described by Frey (12). After development the plates were examined under UV light.

RESULTS

Serum Concentration—Eight patients were injected with metrizamide intrathecally. Metrizamide could be detected in the blood after about 15 min, and the maximum serum concentration appeared about 2 hr after injection (Table I). Relatively large individual variations were found. Estimates for the pharmacokinetic parameters were determined for each subject from the observed serum concentrations and are presented in Table II, where the parameters calculated from the mean serum values are shown also. The fit of the theoretical curve to the observed data is shown in Fig. 1.

Biological Half-Life—From the estimated mean rate constant for absorption, the percentage absorbed from the subarachnoid space to blood at different times after injection was calculated using Eq. 2. The values (Table I) show that about half of the dose disappeared from the cerebrospinal fluid in 0.75 hr.

In three patients the concentration of metrizamide in the cerebrospinal fluid was measured 6 hr after injection, and in three other patients the concentration was measured 24 hr after injection (Table II). When the mean values of these measurements were used for calculation, the biological half-life of metrizamide in the subarachnoid space was found to be 11 hr.

Excretion in Urine—In the six patients from which urine was sampled, 55–86% of the administered I was recovered within 23 hr.

Metabolism—The metabolic studies showed that no metabolites of metrizamide were found in the part of the dose excreted in the urine (Table II). The detection limit was estimated to be less than 5% of the recovered dose.

DISCUSSION

The results support the preliminary assumption that the one-compartment open model is valid for describing the absorption of metrizamide from the subarachnoid space to blood, as was found in a previous study (11) where metrizamide was injected intracisternally to cats. In this study, the first-order constant for absorption was also 1.5 hr^{-1}. However, in neither of the two studies was an attempt made to incorporate the amount of metrizamide that penetrates into the extracellular space of the nervous tissues into the pharmacokinetic model because of the acceptable fit to the blood data obtained by the one-compartment open model.

From the structure of the ependyma (13), it is probable that some injected metrizamide enters the extracellular space of the nervous tissue relatively fast. This migration implies a longer biological half-life of metrizamide in the subarachnoid space than is found by the one-compartment open model and may partly explain the discrepancy found between the two different methods of measuring the biological half-lives used in the present study.

Another source of error is that when the biological half-life of metrizamide in the subarachnoid space was determined from measurements of the cerebrospinal fluid concentration, the same group of patients was not examined 6 and 24 hr after injection. Furthermore, the calculation used in Eq. 3 is based on the assumption that metrizamide has the same distribution at 6 and at 24 hr after injection. This may not be the case, but the results of Prockop and Fishman (14) indicate that the assumption is valid. They found that the concentration of inulin followed a straight line in a semilogarithmic plot of cerebrospinal fluid concentration *versus* time. The biological half-life of inulin was 68.8 min^{-1} during the observation period of 30–150 min after percutaneous injection in cisterna magna of dogs.

In the three patients examined 6 hr after the injection, a mean concentration of 3600 μg of I/ml was found. This value is only one-fourth of that which would have been found (Table II) if the volume of the subarachnoid space in humans is 118 ml (13), if no disappearance had taken place, and if the contrast agent had distributed uniformly in the cerebrospinal fluid. A uniform distribution in the cerebrospinal fluid within the first few hours after the injection is not likely, and the value of 3600 μg of I/ml thus shows that the biological half-life of metrizamide in the cerebrospinal fluid is far less than 11 hr during the first 6 hr after injection.

In the previous study (11) as well as in this study, considerable variation in the serum levels was found between subjects. This finding should be related to the observations by Skalpe *et al.* (2) who, by examining the radiographs, found that no contrast could be seen in some patients 3–5 hr after injection while traces could still be seen in other patients after 8 hr. The disappearance of other contrast agents from the subarachnoid space after intrathecal injection in humans also seems to show similar individual variations. Thus, Schmerwitz and Rösch (5) found meglumine iocarmate to be completely "radiologically" absorbed within 2–8 hr after injection. For meglumine iothalamate, the corresponding time was 4–8 hr (6).

One reason for the individual variation in the data in the present study is the variation in dose per unit body weight. Another, and perhaps the main, reason may be that the patients made different movements after the injection, thus provoking differences in the circulation of the cerebrospinal fluid. It has been shown that movements of the body are very important for the circulation in spinal cerebrospinal fluid of cats. Grundy (15) found virtually no movement of dye from 7 to 85 min after sacral injection when the cats were kept immobile. Since the importance of the movements for the circulation was not recognized in the previous study in cats (11) and in this study, special attention was not paid to this factor so the rate constants for absorption found cannot be correlated with the movements of the patients and the handling of the cats.

Inflammatory involvements of the meninges may also change the absorption rate from cerebrospinal fluid (14, 16, 17). If, however, inflammatory involvements of the meninges are present and have any effect on absorption, they must mainly be located in the lumbar area of the subarachnoid space, because radiographs showed that no visible contrast passed above the 12th thoracic vertebra. Although the mean value of T_0 shows that 0.25 hr passes after the injection before metrizamide enters the blood, the fact that the blood level reaches its peak within 2 hr after injection correlated with the assumed slow circulation in the cerebrospinal

Table II—Pharmacokinetic Constants, Cerebrospinal Fluid Concentrations, and Urinary Recovery after Intrathecal Injection of 1700 mg of I

Patient	k, Rate Constant for Absorption, hr^{-1}	K_E Rate Constant for Excretion, hr^{-1}	T_0, Lag Time, hr	$\frac{V}{F}$, ml/kg	Cerebrospinal Fluid Concentration[a,b]		Percent Recovered in Urine 23 hr after Injection
					6 hr after Injection, μg I/ml	24 hr after Injection, μg I/ml	
1	2.1	0.08	0.54	96	2200	—	86
2	1.4	0.07	0.16	130	—	1510	63
3	1.3	0.20	0.41	141	—	—	60
4	0.4	0.04	0.13	210	—	1540	55
5	0.7	0.14	0.29	117	—	460	79
6	1.0	0.23	0.16	53	200	—	82
7	0.3	0.13	0.25	80	8500	—	—
8	5.7	0.12	0.38	80	—	—	—
Mean ± SD[c]	1.5 ± 0.2	0.09 ± 0.01	0.25 ± 0.05	121 ± 5			

[a] The cerebrospinal fluid was obtained by a second spinal puncture. [b] When assuming uniform distribution in the subarachnoid space and no disappearance from the cerebrospinal fluid, the concentration would have been 14,400 μg of I/ml. [c] Calculated from the observed mean serum values in Table I.

fluid (15) and makes it probable that, with the doses used in this study, most of the absorption takes place in the lower part of the spinal canal.

CONCLUSION

Absorption and excretion of metrizamide after intrathecal injection in the lumbar area of humans seem to follow first-order kinetics. The one-compartment open model is valid for calculations of the blood level data obtained after intrathecal injection of metrizamide. After intrathecal injection of 10 ml of metrizamide (170 mg of I/ml) in the lumbar area, most absorption probably takes place from the lower part of the spinal canal. About 70% of the injected dose is excreted in the urine within 24 hr after the injection.

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Development and Evaluation of a Dexamethasone Timed-Release Aerosol Formulation

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Abstract □ Dexamethasone microcapsules were prepared and *in vitro* parameters were determined by sieve analysis which yielded materials retained on Nos. 80-, 100-, 200-, and 270-mesh sieves. Release rate and direct total determinations indicated that some measure of timed release appeared in all four sieve samples. The pH–release rate profiles of the microcapsules indicated that dissolution was pH independent. Colloidal silica and isopropyl myristate formulations provided the best suspending characteristics of all aerosol formulations evaluated. Delivery rate, pressure, and evacuation testing demonstrated the usability and functionality of the aerosol. An evaluation was made on the effect on urinary excretion levels of 17-hydroxycorticosteroid when a physical admixture of the drug and timed-release microcapsules were sprayed on rabbits. The significantly higher 17-hydroxycorticosteroid levels obtained in urine of animals treated with the admixture after 1 day may have been attributed to both an immediate suppression of the endogenous steroid and the "spilling over" of excess dexamethasone. A significant difference also was found at the 90% confidence level among the 24-, 48-, and 72-hr 17-hydroxycorticosteroid levels in the admixture and the microcapsules, which substantiated the delayed effect of the latter.

Keyphrases □ Dexamethasone—development and evaluation of timed-release aerosol formulation, preparation of microcapsules □ Aerosols—development and evaluation of dexamethasone timed-release formulation □ Timed-release formulations—dexamethasone aerosol, development and evaluation □ Topical preparations—dexamethasone timed-release aerosol formulation, development and evaluation

Sciarra and Gidwani (1) discussed the kinetics that influence the rate of release of substances from various polymeric films and found that several polymer films inhibited the release of the drug and may be suitable for producing a sustained-action aerosol spray. The film-forming agents, ethylcellulose and polyamide, are compatible with aerosol propellants and are suitable for spraying (2). The kinetics of drug release were studied from polymeric films formulated as an aerosol dressing (3).

The methods for delaying the activity of medicaments to produce orally and parenterally administered timed-release pharmaceuticals have been reviewed (4). The preparation of nylon microcapsules and their application to pharmaceutical dosage forms were reported (5), as well as a listing of the possible methods of microencapsulation (6). An *in vitro* test method for the characterization of oral timed-release materials appears in the National Formulary (7). The influence of particle size in an aerosol dosage form and the various factors influencing the particle size of a substance dispensed from an aerosol were reported (8).

Scoggins (9), using fluocinolone acetonide, reported depression of urinary steroid excretion caused by suppression of endogenous adrenocorticosteroid production, indicating absorption of the drug. The decrease in concentration of 17-hydroxycorticosteroids in the urine was suggested to reflect a partial shutdown of adrenal gland activity because of the absorbed steroid (10).

This study concerns the microencapsulation of dexamethasone by the phase separation method of Rowe (11). Various polymers were investigated to determine the most suitable one for coating the active drug. Since the particle size of the microencapsulated material is critical when used in an aerosol formulation, attention was given to methods for the separation of the microencapsulated substance into various particle-size ranges. The suspension, resuspension, and prevention of agglomeration and caking of the particles were considered using several dispersing agents. Finally, the 17-hydroxycorticosteroid urine level was determined in rabbits both before and after application of the spray to determine the extent of the release of dexamethasone from the film.

Dexamethasone was chosen for this study because it is a commonly used medicinal in topical products and can be easily detected. It has been shown that topical applications of steroids, when absorbed, bring about various detectable *in vivo* responses (12, 13).

EXPERIMENTAL

Preparation of Microcapsules—Methylcellulose, ethylcellulose, polyvinyl acetate, and polyvinyl alcohol were used. One major problem that occurred during the preparation of the microcapsules involved the separation of the mass into discrete particles. This problem was overcome by using ethylcellulose as the polymeric material, varying the order of mixing, and controlling the rate of addition of the solvents.

Fifty grams of ethylcellulose NF was added to 450 g of toluene by sprinkling it into the vortex of the toluene, using a laboratory mixer[1] at a speed of approximately 100 rpm. The solution was placed into a sealed container and allowed to stand overnight. This procedure produced a clear solution. Approximately 70–90 ml of petroleum ether[2] was added dropwise to the ethylcellulose solution contained in a 1000-ml beaker, using a 100-ml pipet, until a persistent turbidity appeared. Five grams of dexamethasone[3] was added and mixed well. The addition of the remainder of the petroleum ether was alternated between the addition of talc USP and ether in approximately three equal applications (a total of 400 ml of petroleum ether and 110 g of talc was used).

The coated particles were filtered in several small portions and washed several times with petroleum ether. The particles were separated and rack dried at room temperature. Microcrystals produced by this method were calculated to contain between 3.0 and 4.0% dexamethasone. Actually, 137 g of solids containing approximately 3.7% dexamethasone was obtained by this method. Figure 1 illustrates the type of particle obtained.

Particle-Size Analysis—The particle-size distribution of the dried particles was determined[4] with Nos. 60-, 80-, 100-, 200-, and

[1] Lightnin.
[2] Boiling range of 30–60°, Specialty Chemicals Division, Phillips Petroleum Co., Bartlesville, Okla.
[3] Micromilled, S. B. Penick and Co., New York, N.Y.
[4] Pulverit Classification Equipment type RB3020, Geoscience Instruments Corp., Mount Vernon, N.Y.

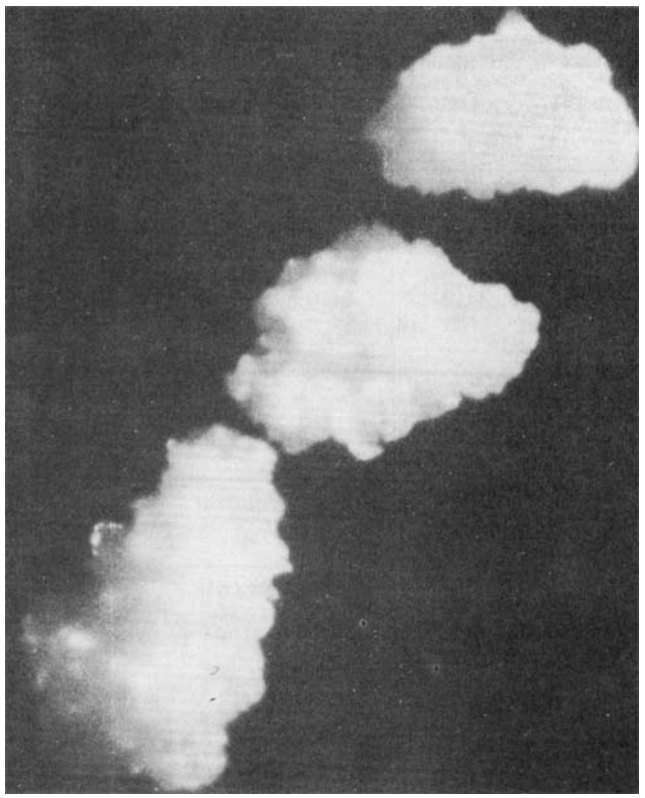

Figure 1—*Dexamethasone microcapsules (40×).*

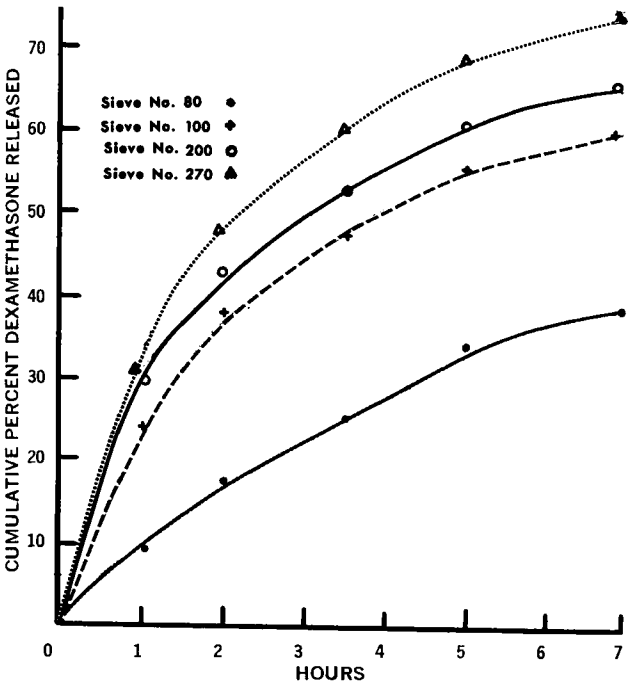

Figure 2—*Release rate from dexamethasone microcapsules of varying sieve sizes.*

270-mesh sieves. The portions collected on each screen were retained for further study. Additional microcapsules were prepared and the particles were classified as indicated so that a sufficient amount of material was available for the studies. Approximately 120 g of microcapsules was collected per batch and five batches were prepared.

Determination of Dexamethasone in Dexamethasone Microcapsules—The amount of dexamethasone in the microcapsules was determined by exposing the microcapsules to extracting fluids of varying pH. Simulated gastric fluid and intestinal fluid (7) were prepared without the inclusion of enzyme so as to obtain extracting fluids with pH values of 1.2, 2.5, 4.5, 7.0, and 7.5. Other fluids of varying pH could also have been used.

Two hundred and fifty milligrams of dexamethasone microcapsules was placed into a 200-ml bottle, and 150 ml of pH 1.2 fluid was added to the bottle, previously warmed to 37°. The bottle was tightly capped and rotated in a rotating-bottle apparatus at 28–30 rpm for 1 hr at 37 ± 1.0°. The pH 1.2 extracting fluid was decanted from the bottle through a 325-mesh stainless steel screen while the residue was retained in the bottle.

Residue collected on the screen was quantitatively returned to the original bottle with 150 ml of pH 2.5 extracting fluid, previously warmed to 37°. The bottle was tightly capped, returned to the apparatus, and rotated. This procedure was repeated at the end of 2 hr using pH 4.5 fluid, at the end of 3.5 hr using pH 7.0 fluid, and at the end of 5 hr using pH 7.5 fluid. After 7 hr of total rotation time, the pH 7.5 fluid was decanted from the bottle and the residue was retained for assay.

Release Rate Procedure—About 50 mg of dexamethasone standard was accurately weighed and quantitatively transferred to a 500-ml separator, and about 160 ml of water was added. The sample and the residue were transferred to individual 500-ml separators. These three samples were extracted with five 50-ml portions of chloroform. The chloroform layers were placed into 250-ml volumetric flasks by being passed dropwise through chloroform-wetted anhydrous sodium sulfate into a filtering funnel and were brought to volume with chloroform. An aliquot of each solution was pipetted into a 50-ml centrifuge tube and evaporated to dryness.

Assay of Samples—Twenty milliliters of ethanol was added to each tube, the solution was stirred for 5 min, and 2 ml each of blue tetrazolium solution (14) and tetramethylammonium hydroxide solution (15) was added to each tube. The tubes were then allowed to stand for 90 min. A blank was prepared in a similar manner. The absorbances of the sample and standard were read against the blank at 525 nm. The amount of dexamethasone was calculated as follows:

$$\frac{S}{Sa} \times \frac{C}{1000} \times \text{D.F.} = \text{mg dexamethasone released/g} \quad \text{(Eq. 1)}$$

where S = absorbance of sample at 525 nm, Sa = absorbance of standard at 525 nm, C = concentration of standard in micrograms per milliliter, and D.F. = dilution factors. Furthermore:

$$\frac{Bi}{\Sigma Bi} \times 100 = \text{\% dexamethasone released per time period} \quad \text{(Eq. 2)}$$

where Bi = mg dexamethasone/g released during the ith time period, and ΣBi = cumulative amount released in milligrams per gram. These results are shown in Table I and Fig. 2.

Direct Total Determination—Approximately 1 g of dexamethasone microcapsules was ground and finely powdered. An amount equivalent to 4 mg of dexamethasone was accurately weighed and transferred to a 100-ml volumetric flask, and 70 ml of ethanol was added. The solution was shaken for 30 min and brought to volume with ethanol. A 15-ml aliquot from each flask was transferred to a 100-ml volumetric flask and brought to volume with ethanol. The sample containing the No. 270 microcapsules was not diluted.

The standard was prepared by accurately weighing 50 mg of dexamethasone standard and dissolving it in enough ethanol to yield a total volume of 250 ml. A 3-ml aliquot was placed into a 100-ml volumetric flask and brought to volume with ethanol. Five-milliliter aliquots of each sample and 20 ml of the standard solution were pipetted into 50-ml centrifuge tubes. Each sample was then treated with the same reagents as previously indicated and the absorbance was determined as described under *Assay of Samples*. The direct total of dexamethasone was determined as follows:

$$\frac{S}{Sa} \times \frac{C}{1000} \times \frac{100}{W} \times \text{D.F.} = \text{mg dexamethasone/g} \quad \text{(Eq. 3)}$$

where S = absorbance of sample at 525 nm, Sa = absorbance of standard at 525 nm, C = concentration of standard in micro-

Table I—*In Vitro* Release Rate[a] and Direct Total Determinations of Dexamethasone Microcapsules

Hours	Sieve No. 80		Sieve No. 100		Sieve No. 200		Sieve No. 270	
	Determination Number		Determination Number		Determination Number		Determination Number	
	1	2	1	2	1	2	1	2
1	8.0	10.0	24.6	25.0	30.8	29.9	25.4	34.5
2	16.4	18.3	36.0	39.8	40.5	45.5	44.2	50.9
3.5	24.4	25.6	46.2	49.1	51.7	53.9	59.1	61.2
5	32.6	34.8	55.1	56.6	60.5	61.6	70.1	68.4
7	37.0	39.2	59.0	61.3	65.2	66.0	74.9	73.7
Residue	63.1	60.8	40.9	38.7	34.9	34.0	25.0	26.2
Direct total[b], mg/g	45.3		45.4		46.7		41.3	

[a] Cumulative weight percent dexamethasone released. [b] Average of two determinations.

Table II—Formulations Prepared

Ingredients	Formula Number					
	1	2	3	4	5	6
	Percent (w/w)					
Dexamethasone microcapsules	12.5	12.5	12.5	12.5	12.5	12.5
Fumed silica[a]	—	0.5	0.5	0.5	0.5	0.5
Oleyl alcohol	—	—	5.0	—	—	—
Isopropyl myristate	—	—	—	5.0	—	—
Talc USP	—	—	—	—	5.0	—
Mineral oil USP	—	—	—	—	—	5.0
Dichlorodifluoromethane NF–dichlorotetrafluoroethane NF (40:60)[b]	87.5	87.0	82.0	82.0	82.0	82.0

[a] Cab-O-Sil M-5, Cabot Corp., Boston, Mass. [b] Propellant 12/114.

grams per milliliter, W = sample weight in grams, and D.F. = dilution factors. These results are included in Table I.

Determination of pH Profile of Dissolution Rate of No. 100 Sieve Dexamethasone Microcapsules—The dissolution rate of the dexamethasone microcapsules, No. 100 sieve (149 μm), was determined as a function of pH. Two hundred and fifty milligrams of the dexamethasone microcapsules was exposed to pH 1.2, 2.5, 4.5, 7.0, and 7.5 fluids. The fluid and the method used were as previously described. Determinations were made at 1.0, 3.5, and 7.0 hr. The decanted media, as well as the residues, were treated, diluted, and assayed according to the procedures previously outlined. The results are reported as cumulative percent dexamethasone released at varying times and pH values (Fig. 3).

Formulation of Aerosol Containing Dexamethasone Microcapsules—Several formulations (Table II) were prepared in aerosol compatibility tubes calibrated in centimeters. The propellants were added by the pressure fill method, and the tube was inverted three times to disperse fully the suspended material. The dexamethasone microcapsules had an approximate concentration of 40 mg/g. The incorporation of 7.5 g/formulation was, therefore, equivalent to approximately 0.5% dexamethasone/tube.

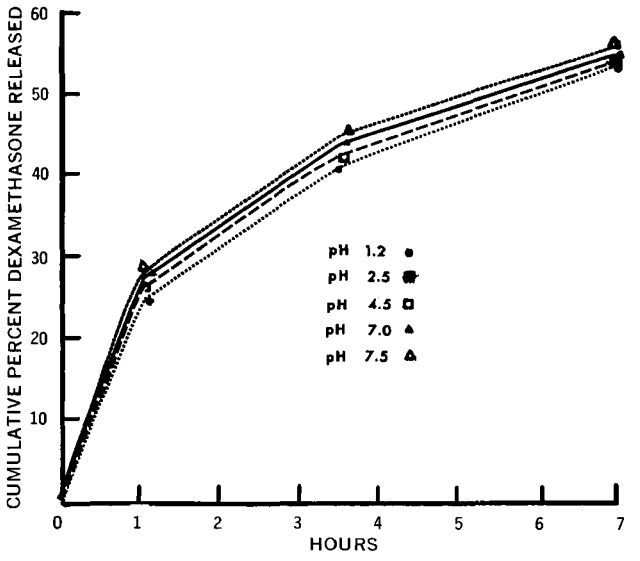

Figure 3—*Dissolution of dexamethasone microcapsules, sieve No. 100 material, at different pH values.*

The aerosols were prepared by mixing the dexamethasone microcapsules with the other solids present in the formulation. The dispersing agent was then blended with the solids and added to the compatibility tube, which was sealed with an aerosol valve[5]. Dichlorodifluoromethane–dichlorotetrafluoroethane (40:60) was added by the pressure process, and the tubes were allowed to stand 2–5 days and then evaluated. The sedimentation rate of each preparation was determined (Table III).

Evaluation of Aerosol Formulation—Based upon the results shown in Table III, Formulation 4 was selected for further study. Six aerosol containers were prepared as previously outlined, except that the samples were prepared in tin-plated aerosol cans. The valve previously described was also used for this portion of the study.

Approximately 60 g of product was placed into each container. The dip tubes were removed from the valves used on the samples for the assays to facilitate the removal of the propellant without discharging the microcapsules from the container. The samples were then evaluated for delivery rate, pressure, evacuation, and release of active ingredient.

Delivery Rate—The delivery rate was determined on two cans 5 days after filling. The aerosol product was sprayed, weighed, and equilibrated to 25 ± 1°. The can was set into position under the delivery apparatus (16). The actuator was set to deliver product for 10 sec, and then the can was cleaned and reweighed. Three determinations per container were made. The average of these readings produced a delivery rate of 1.75 g/sec.

Pressure Testing—The vapor pressure was determined on the two cans used for the delivery rate. The aerosol can was placed into a water bath at 21 ± 1°, and a gauge was prepressurized with

[5] Precision Valve Co., Yonkers, N.Y.; 5.08 × 0.05-cm (2 × 0.020-in.) orifice, radius core (nylon) stem, Buna N (regular durometer) gasket, type 302 stainless steel spring, 0.2-cm (0.08-in.) nylon body, flat dimpled tin plate, epon-coated mounting cup, standard dip tube, and 0.05-cm (0.02-in.) actuator.

Table III—Sedimentation Rate of Aerosol Formulations

Formula Number	Height of Supernate at $T_{30\ sec}$, cm	Height of Sediment at $T_{2\ hr}$, cm	Total Height at $T_{2\ hr}$, cm	$\frac{A}{C-B} \times 100$[a], %
1	1.4	1.6	3.1	93.3
2	0.5	2.0	3.1	45.5
4	0.45	2.0	3.5	30.0
5	0.5	2.0	2.9	55.6
6	1.1	2.0	3.5	73.3

[a] Ratio, in terms of percentage, of supernatant height at T = 30 sec to supernatant height at T = 2 hr. [b] Coagulation occurred with Formulation 3 and it was deleted from the study.

Table IV—Assay of Dexamethasone Microcapsules from an Aerosol

Hours	Cumulative Percent		
	Determination Number 1	2	Control
1	36.4	31.5	34.5
2	56.2	49.1	50.9
3.5	71.4	65.6	61.2
5	78.7	76.1	68.4
7	84.1	84.0	73.7
Residue	15.8	15.9	26.2
Direct total, mg/g	39.6[a]		41.3

[a] Average of two determinations.

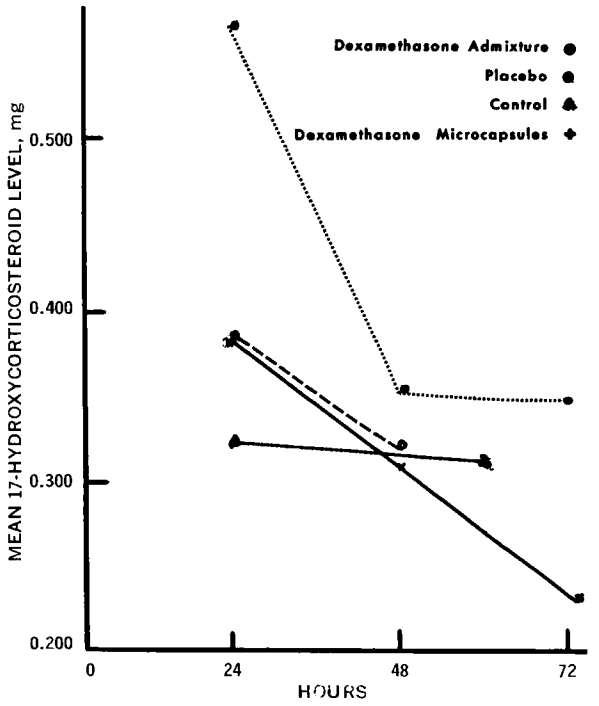

Figure 4—*Mean 17-hydroxycorticosteroid levels in rabbit urine.*

nitrogen. The pressure was then obtained with the use of the prepressurized gauge (17), and the average of two determinations on each can was 49 psig.

Evacuation Testing—Two containers, 5 days after preparation, were weighed and completely evacuated over a 24-hr period. Each can was reweighed, and the loss was determined by difference. This value was 57.7 g, representing the average for the two cans.

Analytical Testing—An assay was performed on two containers, 48 hr after preparation, and the result was compared to a control representing the analysis of the initial dexamethasone microcapsules, No. 270 sieve material, from which the aerosol products were originally prepared (Table IV).

***In Vivo* Evaluation of Aerosol**—To ascertain the release of the active ingredient from the encapsulated material sprayed from the aerosol container, five male New Zealand rabbits, approximately 4 months old, were fed rabbit pellets[6] and were allowed water *ad libitum*. They were placed in separate cages placed at an angle to allow the urine to flow into vessels suspended at the lower end of the cages. The urine passed through a fiberglass filter before flowing into the vessels.

The backs of the animals were shaved and the necks were collared[7]. The aerosol product was sprayed onto their backs at a distance of approximately 20.3 cm (8 in.), and the test areas were then occluded by wrapping[8]. The urine volume was recorded, and the samples were frozen for subsequent assay.

Three aerosol preparations were used: *(a)* a placebo container in which the inactive ingredients were mixed together and then pressurized, *(b)* a container in which the drug and the inactive ingredients were admixed, and *(c)* the delayed drug release microcapsules, No. 270 sieve, in an aerosol can. This phase of the study was carried out as follows:

1. A control urine sample was collected from each of the five rabbits after 24 and 48 hr. The placebo container was then sprayed for 2 sec. Urine samples were collected after 24 and 48 hr and assayed (Table V).

2. Two days after the last collection, the container with the nondelayed preparation was sprayed for 2 sec onto each of the five rabbits. Urine samples were collected after 24, 48, and 72 hr and assayed (Table VI).

3. Seven days after the last collection of the nondelayed preparation, urine samples were collected from each rabbit to determine if a return to the mean placebo value had occurred (Table VI).

4. After the urine collection in Step 3, the timed-release product was sprayed for 2 sec onto each of the five rabbits. Urine samples were collected after 24, 48, and 72 hr and assayed as previously described (Table VII). The data were then treated statistically (Table VIII).

DISCUSSION

A photograph, at 40× magnification, taken with reflected light (Fig. 1), identifies the microcapsules formed in this process as irregular-shaped particles containing a wall of polymer film. This shape is typical of microcapsules produced by this method. While it is desirable to have uniform particles, the results shown in Table I indicate the range of particles produced by this method. Usable microcapsules chosen for this study were those retained on Nos. 80-, 100-, 200-, and 270-mesh sieves, although the larger sized particles would tend to settle faster and may cause valve clogging. In most cases, particles 60 μm and less in diameter tend to present a minimum amount of valve clogging problems when formulated as an aerosol.

The selection of the most suitable method for the preparation of the dexamethasone microcapsules was performed experimentally. The major problem encountered was related to capsule wall agglomeration. Ethylcellulose, when wet, produced a sticky material which appeared to enhance agglomeration. Once the solvent evaporated from the agglomerated material, it was impossible to bring about separation. The use of a mineral silicate, talc (11), minimized but did not eliminate this problem. However, by repeated washings with petroleum ether, this problem was reduced to a minimum.

The values for the *in vitro* release rate and direct total determinations are shown in Table I. The results indicate that some measure of timed release appeared in all four sieve samples tested according to the methods noted. As can be seen from Fig. 2, as the particle size of the microcapsule was decreased, the release rate increased; after 7 hr, approximately 74% of the dexamethasone contained in the material on the No. 270-mesh screen was released while only about 38% of dexamethasone was released from the No.

[6] Ralston Purina Co., St. Louis, Mo.
[7] Medicollar, EVSCO Pharmaceutical Corp., Oceanside, N.Y.
[8] Saran Wrap, Dow Chemical Co., Midland, Mich.

Table V—17-Hydroxycorticosteroid Levels in Rabbit Urine (Control and Placebo)

	Control						Placebo					
	24-hr Sample			48-hr Sample			24-hr Sample			48-hr Sample		
Rabbit Number	Urine Volume/ 24 hr, ml	Concentration/ ml, μg	Concentration/ 24 hr, mg	Urine Volume/ 24 hr, ml	Concentration/ ml, μg	Concentration/ 24 hr, mg	Urine Volume/ 24 hr, ml	Concentration/ ml, μg	Concentration/ 24 hr, mg	Urine Volume/ 24 hr, ml	Concentration/ ml, μg	Concentration/ 24 hr, mg
1	192	1.94	0.373	162	2.01	0.326	274	1.66	0.455	105	3.28	0.344
2	222	1.44	0.320	240	2.07	0.497	242	2.04	0.494	252	1.32	0.333
3	65	2.99	0.194	110	2.19	0.241	82	4.35	0.357	76	4.44	0.337
4	122	3.06	0.373	85	3.85	0.327	75	4.29	0.322	60	4.13	0.248
5	130	2.70	0.351	42	4.19	0.176	70	4.14	0.290	94	3.49	0.328

Table VI—17-Hydroxycorticosteroid Levels in Rabbit Urine after Treatment with Dexamethasone Aerosol Admixture

	24-hr Sample			48-hr Sample			72-hr Sample			24-hr Control (7 Days after 72-hr Sample)		
Rabbit Number	Urine Volume/ 24 hr, ml	Concentration/ ml, μg	Concentration/ 24 hr, mg	Urine Volume/ 24 hr, ml	Concentration/ ml, μg	Concentration/ 24 hr, mg	Urine Volume/ 24 hr, ml	Concentration/ ml, μg	Concentration/ 24 hr, mg	Urine Volume/ 24 hr, ml	Concentration/ ml, μg	Concentration/ 24 hr, mg
1	216	1.71	0.369	133	2.70	0.359	200	1.44	0.288	240	1.39	0.334
2	418	1.88	0.786	285	1.30	0.371	340	1.16	0.394	266	1.80	0.479
3	150	4.52	0.678	93	3.73	0.347	104	3.11	0.323	142	2.08	0.295
4	126	4.16	0.524	142	2.67	0.379	158	2.21	0.349	174	2.11	0.367
5	156	3.02	0.471	87	3.48	0.303	82	4.57	0.375	80	4.18	0.334

80-mesh sieve. The fact that this pattern of release of steroid was obtained is not surprising, since the release would be expected to be dependent upon particle size. It is also possible that some polymer wall material may have been ruptured during the separation process, resulting in a premature release.

Assays were performed on the four sieve sizes selected using the rotating-bottle apparatus and method desribed. There appears to be good reproducibility between the assays performed, although variations in the *in vitro* release rate between the different particle-size fractions were noted. The proximity of values in the direct total determinations for all four sieve sizes indicates a measure of uniformity in the different samples. Therefore, since the amount of drug content is approximately the same, the variation in release rate appears to be related to variations in the polymer wall deposition.

The data shown in Fig. 3 indicate that there is essentially no difference in the dissolution rate of dexamethasone microcapsules at various pH values. Dexamethasone dissolution from these microcapsules apparently is pH independent under the conditions studied. In light of the apparent pH independency of the microcapsules, there should be essentially no difficulty in using the NF (7) oral release rate method to characterize microcapsules for topical use from a pH point of view. Since the pH of skin is approximately 4–6 and the study was carried out in this and other pH ranges, these results should be applicable for a topical preparation.

Of the various dispersing agents studied, a combination of fumed silica and isopropyl myristate seemed to be the most useful (Table III). It was necessary to provide a formulation that would have good suspending characteristics to obtain a uniform delivery of the drug from the system. In addition, no evidence of valve clogging or leakage was noted.

To determine whether there would be any adverse effect on the *in vitro* delayed release of the drug from the microcapsules when packaged in the aerosol containers, a sample of the microcapsules from the aerosol was assayed. These results (Table IV), when compared against the initial results for the No. 270-mesh material, indicated that there was relatively little, if any, effect upon the delayed release of the drug. Evidently the coating produced by this technique is not destroyed or damaged in the presence of the propellant and the dispersing agents studied.

Seven days was considered an adequate interval to allow for full excretion of the drug from the time the admixture and microcapsule preparations were applied. As can be seen from Tables V, VI, and VIII, the concentrations of 17-hydroxycorticosteroid in 24 hr for the control and the control taken 7 days after the 72-hr admixture was applied were not significantly different ($p > 0.3$).

Tables VI and VII provide the values for the urine volume, 17-hydroxycorticosteroid concentration per milliliter of urine, and total 17-hydroxycorticosteroid levels found in the 24-hr periods when spraying immediate release and timed-release material. Figure 4 shows the difference obtained when plotting mean value *versus* time. The high 17-hydroxycorticosteroid level in animals treated with the admixture may be attributed to both an immediate suppression of the endogenous steroid and the "spilling over" of

Table VII—17-Hydroxycorticosteroid Levels in Rabbit Urine after Treatment with Dexamethasone Aerosol Microcapsules

	24-hr Sample			48-hr Sample			72-hr Sample		
Rabbit Number	Urine Volume/ 24 hr, ml	Concentration/ml, μg	Concentration/24 hr, ml	Urine Volume/ 24 hr, ml	Concentration/ ml, μg	Concentration/24 hr, mg	Urine Volume/ 24 hr, ml	Concentration/ml, μg	Concentration/24 hr, mg
1	222	1.48	0.329	182	1.98	0.360	202	0.92	0.186
2	370	1.69	0.625	300	1.03	0.309	312	0.51	0.159
3	158	2.06	0.326	160	2.05	0.328	120	3.04	0.365
4	159	1.85	0.294	126	2.81	0.354	103	1.37	0.141
5	60	4.98	0.299	56	3.39	0.190	90	3.42	0.308

Table VIII—Levels of Significance of 17-Hydroxycorticosteroid in Rabbit Urine Samples

Samples Compared (Paired Data)	Mean ± Standard Error of Mean	17-Hydroxycorticosteroid Levels	
		t	p
24-hr Control and 24-hr Placebo	0.322 ± 0.035 0.384 ± 0.039	1.212	>0.25
48-hr Control and 48-hr Placebo	0.313 ± 0.054 0.318 ± 0.018	0.081	>0.9
24-hr Placebo and 24-hr Admixture	0.384 ± 0.039 0.566 ± 0.074	2.536	>0.05
48-hr Placebo and 48-hr Admixture	0.318 ± 0.018 0.352 ± 0.013	1.280	>0.25
24-hr Placebo and 24-hr Dexamethasone microcapsules	0.384 ± 0.039 0.375 ± 0.063	0.213	>0.8
48-hr Placebo and 48-hr Dexamethasone microcapsules	0.318 ± 0.018 0.308 ± 0.031	0.248	>0.8
24-hr Dexamethasone admixture and 24-hr Dexamethasone microcapsules	0.566 ± 0.074 0.375 ± 0.063	3.772	>0.02
48-hr Dexamethasone admixture and 48-hr Dexamethasone microcapsules	0.352 ± 0.013 0.308 ± 0.031	2.171	>0.1
72-hr Dexamethasone admixture and 72-hr Dexamethasone microcapsules	0.346 ± 0.019 0.232 ± 0.044	2.284	>0.1
24-hr Control and 24-hr Control (7 days after 72-hr admixture collection)	0.322 ± 0.035 0.362 ± 0.031	1.035	>0.3

excess dexamethasone, with a subsequent rapid increase in the 17-hydroxycorticosteroid content in the urine, hence the significant increase from the placebo as noted in Fig. 4.

However, a difference is seen in release and absorption between the dexamethasone admixture and microcapsules (Fig. 4). The values for the microcapsules for Days 1 and 2 are approximately equal to the control and placebo values, indicating that there is a delay in the release of dexamethasone. Not until Day 3 is a difference noted as indicated by the suppression of the 17-hydroxycorticosteroid content.

To treat the results statistically, the results were evaluated as paired data (Table VIII). While the general level of biological significance is 0.05 (95% confidence level) or less, it is believed that, with the number of experiments conducted in this evaluaton, any value less than 0.1 (90% confidence level) could be of possible significance.

From Table VIII the p values for the 24-hr control and placebo are not significantly different, indicating that the handling and treatment of the animals with the placebo had no effect upon the 17-hydroxycorticosteroid content of the urine.

The p values for the 24-hr placebo and dexamethasone admixture show significant variation, attributed to the effect of the immediate release material. The values for the 48-hr placebo and admixture are not significant, indicating the return to the norm. The p values for the 24-hr placebo and dexamethasone microcapsules show no significant variation which can be attributed to the prevention of the absorption of the dexamethasone from the microcapsules. The p values for the 24-, 48-, and 72-hr dexamethasone admixture and microcapsules show significant differences at the 90% confidence levels.

CONCLUSIONS

On the basis of the obtained results, the following may be concluded:

1. An aerosol preparation containing a microencapsulated drug can be prepared. Dexamethasone microcapsules, prepared by the phase separation technique using an ethylcellulose film, exhibit timed release in a pH range of 1.2–7.5 and the release appears to be pH independent.

2. The phase separation technique produces microcapsules with a suitable size distribution for dispensing as an aerosol dosage form. Those particles in the range of about 50 μm in diameter are most desirable for this application.

3. A formulation consisting of dexamethasone microcapsules (53 μm in diameter) together with suitable dispersing agents and propellants can be dispensed without rapid sedimentation and valve clogging.

4. A statistically significant reduction in the urinary level of 17-hydroxycorticosteroids in rabbits can be obtained from a timed-release dexamethasone aerosol when sprayed topically and compared to a dexamethasone preparation in which the ingredients are simply admixed.

5. No release of dexamethasone from the microcapsules was obtained over 24–48 hr, but reduction in the 17-hydroxycorticosteroid level (indicating absorption of dexamethasone) was noted over 72 hr. The use of higher concentrations of dexamethasone initially in the microcapsules could possibly have shown a more significant effect.

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Effect of Flavoring Oils on Preservative Concentrations in Oral Liquid Dosage Forms

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Abstract □ The partitioning of methyl-, ethyl-, propyl-, and butylparabens into flavoring oils from aqueous systems was studied. The partitioning is dependent on the concentration of the flavoring oil, the pH of the aqueous medium, and the nature and concentration of additives to the aqueous medium.

Keyphrases □ Flavoring oils—effect on preservative concentrations in oral liquid dosage forms, partitioning of parabens □ Preservatives—effect of flavoring oils on concentrations in oral liquid dosage forms, partitioning of parabens □ Parabens—partitioning from aqueous systems, effect of flavoring oils on concentrations in oral liquid dosage forms □ Interactions—parabens–flavoring agents in aqueous medium, effect on preservative concentrations, oral liquid dosage forms

Although the search for effective preservatives continues unabated, esters of *p*-hydroxybenzoic acid are still the most frequently used preservatives in oral pharmaceutical preparations. Several recent articles discussed the conditions in which various preservatives are effective and various conditions or agents that reduced or destroyed their effectiveness (1–3).

The effect of partitioning on the antimicrobial activity of preservatives was discussed (4). In a mixed system containing an oil phase, emulsified or as a separate layer, the concentration of antimicrobial agent required was higher than that in a completely aqueous system (5). Higher concentrations of preservatives are required to compensate for the quantities made unavailable for antimicrobial action due to adsorption, binding, or solubilization by nonionic surfactants or partitioning into a nonaqueous phase (6–11).

The theory behind partitioning of a preservative in an oil–water system was discussed (12). Several reports (7, 8, 10, 13) also described the partitioning of a preservative between two phases and the equilibria involved in partitioning and binding of a preservative to surfactants. Patel and Romanowski (12) further showed microbiologically the partitioning and binding effect of the oils and the surfactants on the preservatives. It has been noted that creams and emulsions are more difficult to preserve than aqueous solutions (14–18). The significant part played by partitioning of the preservatives between aqueous and nonaqueous phases, allowing availability of only a portion of the total quantity of the preservatives in the aqueous phase where contaminants normally multiply, has been stressed (19–22).

Flavoring agents are used in most oral liquid pharmaceutical preparations. These flavoring agents are composed of aromatic oils, natural or synthetic, and other ingredients to make them compatible with aqueous systems.

Preliminary observations in this laboratory showed that the solubility of parabens in these flavoring agents was extremely high. Since parabens are used in low concentrations, their high solubility in flavoring agents may cause depletion of the preservatives from the aqueous phase, with a consequent reduction in overall preservative activity. The purpose of this paper is to report the interaction between parabens and flavoring agents in an aqueous medium.

EXPERIMENTAL

General Partitioning Study—A 0.15% solution of methylparaben was prepared in a buffer solution of specified pH. Fifty-milliliter aliquots of the solution were transferred to 125-ml erlenmeyer flasks, and 0.5 ml of flavoring oil (1% of the total volume) was added to each flask. Respective blanks were also prepared containing flavoring oil but no methylparaben in the buffer solutions. The flasks were mounted on a wrist shaker[1] and shaken for 12 hr. The insoluble oily component in the mixture was then separated by a combination of centrifugation and column filtration technique.

The mixture was centrifuged at 10,000 rpm, corresponding to approximately 12,000 rcf. in a high-speed analytical centrifuge[2] for about 30 min. After centrifugation, the aqueous layer was separated from the floating oily layer using a Pasteur pipet. Aqueous layers from different tubes corresponding to one sample were pooled. This aqueous portion was then passed through an acid-washed kieselguhr column. The columns were prepared as follows.

A 10-ml disposable plastic syringe was plugged with a wad of glass wool of about 10-mm constant thickness. Two grams of acid-washed dry kieselguhr was weighed into the syringe. The column was lightly tapped down to a 35-mm column height, corresponding to the 6-ml mark on the plastic syringe, and the mixture was passed through the column. The first 5 ml of the effluent was re-

[1] Burrell.

[2] I.E.C. international centrifuge model H-T; rcf. = reciprocal centrifugal force.

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Effect of Flavoring Oils on Preservative Concentrations in Oral Liquid Dosage Forms

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Abstract □ The partitioning of methyl-, ethyl-, propyl-, and butylparabens into flavoring oils from aqueous systems was studied. The partitioning is dependent on the concentration of the flavoring oil, the pH of the aqueous medium, and the nature and concentration of additives to the aqueous medium.

Keyphrases □ Flavoring oils—effect on preservative concentrations in oral liquid dosage forms, partitioning of parabens □ Preservatives—effect of flavoring oils on concentrations in oral liquid dosage forms, partitioning of parabens □ Parabens—partitioning from aqueous systems, effect of flavoring oils on concentrations in oral liquid dosage forms □ Interactions—parabens–flavoring agents in aqueous medium, effect on preservative concentrations, oral liquid dosage forms

Although the search for effective preservatives continues unabated, esters of *p*-hydroxybenzoic acid are still the most frequently used preservatives in oral pharmaceutical preparations. Several recent articles discussed the conditions in which various preservatives are effective and various conditions or agents that reduced or destroyed their effectiveness (1–3).

The effect of partitioning on the antimicrobial activity of preservatives was discussed (4). In a mixed system containing an oil phase, emulsified or as a separate layer, the concentration of antimicrobial agent required was higher than that in a completely aqueous system (5). Higher concentrations of preservatives are required to compensate for the quantities made unavailable for antimicrobial action due to adsorption, binding, or solubilization by nonionic surfactants or partitioning into a nonaqueous phase (6–11).

The theory behind partitioning of a preservative in an oil–water system was discussed (12). Several reports (7, 8, 10, 13) also described the partitioning of a preservative between two phases and the equilibria involved in partitioning and binding of a preservative to surfactants. Patel and Romanowski (12) further showed microbiologically the partitioning and binding effect of the oils and the surfactants on the preservatives. It has been noted that creams and emulsions are more difficult to preserve than aqueous solutions (14–18). The significant part played by partitioning of the preservatives between aqueous and nonaqueous phases, allowing availability of only a portion of the total quantity of the preservatives in the aqueous phase where contaminants normally multiply, has been stressed (19–22).

Flavoring agents are used in most oral liquid pharmaceutical preparations. These flavoring agents are composed of aromatic oils, natural or synthetic, and other ingredients to make them compatible with aqueous systems.

Preliminary observations in this laboratory showed that the solubility of parabens in these flavoring agents was extremely high. Since parabens are used in low concentrations, their high solubility in flavoring agents may cause depletion of the preservatives from the aqueous phase, with a consequent reduction in overall preservative activity. The purpose of this paper is to report the interaction between parabens and flavoring agents in an aqueous medium.

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Table I—Partitioning of Propylparaben from Acetate Buffer into Oils (1.0%)

	Concentration of Propylparaben in Aqueous Phase, mg/100 ml	
	Before Partitioning	After Partitioning
Orange oil	73.3	66.0
Lemon oil	73.3	55.6
Anise oil	73.3	48.9
Peppermint oil	73.3	12.6
Spearmint oil	73.3	0.2

Table II—Solubility (in g/100 ml) of Parabens in Distilled Water, Peppermint Oil, and Spearmint Oil

Paraben	Distilled Water	Peppermint	Spearmint
Methyl	0.25	15.68	20.54
Ethyl	0.17	19.97	23.93
Propyl	0.05	25.56	32.51
Butyl	0.02	44.17	45.59

jected, and the next 25–30 ml was collected for further work. The solutions serving as blanks contained parabens but no flavoring oil or contained flavoring oil but no parabens, and they were treated the same way.

Paraben Assay—The solutions containing parabens were assayed spectrophotometrically. The filtrates or the standard aqueous solutions were diluted first with methanol containing 20% 0.1 *N* HCl. These solutions were then further diluted with pure methanol. The absorbance of the final solution was measured[3] at 256 nm, the λ_{max} for undissociated parabens, against a corresponding blank solution. Intermittently, UV spectra of these solutions were obtained[4] to check the absence of any interference from extraneous matter as well as to be sure that parabens were present in the undissociated form. Interfering species capable of absorbing in the UV region were acetate ion (λ_{max} 205 nm), spearmint oil (λ_{max} 245 nm), and peppermint oil (λ_{max} 252 nm).

The sample solutions needed a minimum of a 3:100 dilution with acidic methanol. At this dilution the contribution to absorbance from the interfering moieties was minimal. The absorbance measurements of the sample solutions also were made against blank solutions containing the same ingredients except flavoring oil or paraben, as the case may be, in exactly the same concentrations and treated identically to sample solutions. This procedure compensated for the possible contribution in absorbance measurements from interfering moieties.

Solubility of Paraben in Flavoring Oils—Ten milliliters of spearmint oil and peppermint oil was saturated individually with methylparaben, ethylparaben, propylparaben, and butylparaben at room temperature. These solutions were shaken in the presence of an excess of solute for 24 hr on a wrist shaker. The flasks were then transferred to a 25° thermostated shaker bath for further shaking for 24 hr. The solutions were filtered through a fine-porosity sintered-glass funnel, and the filtrates were adequately diluted with pure methanol. The spectrophotometric absorbances of the solutions were then measured at 256 nm against the proper blanks.

In another experiment, the solubility of propylparaben in lemon, orange, spearmint, and peppermint oils was compared in a similar way. The solubilities of all four esters in water also were determined in a similar way except for cutting the shaking time to 8 hr to avoid possible degradation of the compounds in water. Degradation was not controlled, nor was it accounted for in the final results.

pH–Partition Studies—Acetate, phosphate, and borate buffers, pH 4–9, were prepared. Solutions of propylparaben in a concentration of approximately 20 mg/100 ml were prepared in buffer solution. Spearmint oil and peppermint oil were added to these solutions in quantities to obtain concentrations of 0.25 and 0.4%, respectively. The mixtures were equilibrated by shaking on the wrist shaker for 16 hr at room temperature, and the aqueous layer was then separated and assayed for propylparaben. Buffer solutions containing propylparaben but no oil were shaken for the same length of time to account for any possible pH-dependent degradation of propylparaben in aqueous solutions.

Effect of Salt on Partitioning—Sodium chloride or sugar was added to solutions of 0.02% propylparaben in pH 4.7 acetate buffer in concentrations ranging from 0.25 to 3.0 *M* in salt or sugar. These solutions were then equilibrated with 0.15% spearmint oil for 16 hr at room temperature. The separation of the aqueous layer and the assay of propylparaben in it followed.

[3] Beckman DU spectrophotometer.
[4] Cary model 15 recording spectrophotometer.

Table III—Effect of Concentration of Peppermint Oil on Partitioning of Propylparaben from 0.025% Solution in Acetate Buffer

Peppermint Oil, %	Concentration of Paraben, mg/100 ml
0.00	73.6
0.10	49.4
0.20	27.0
0.40	13.6
0.60	8.5
0.80	6.7

Table IV—Effect of pH on Partitioning of Propylparaben from 0.025% Solution in Buffer into 0.25% Spearmint Oil

pH	Concentration of Paraben in Aqueous Phase, mg/100 ml		C_{oil}/C_{aq}[a]
	Before Partitioning (B.P.)	After Partitioning (A.P.)	$\frac{\text{B.P.} - \text{A.P.}}{\text{A.P.}}$
5.60	79.8	9.3	7.61
6.74	79.8	11.1	6.18
7.35	79.6	13.6	4.84
7.45	79.6	14.7	4.41
8.56	79.1	39.8	0.99
9.01	79.7	55.1	0.45

[a] No volume adjustments were made.

RESULTS AND DISCUSSION

The partitioning of propylparaben from a pH 4.7 acetate buffer into flavoring oils added in a concentration of 1.0% is shown in Table I. All of the oils showed some partitioning effect. However,

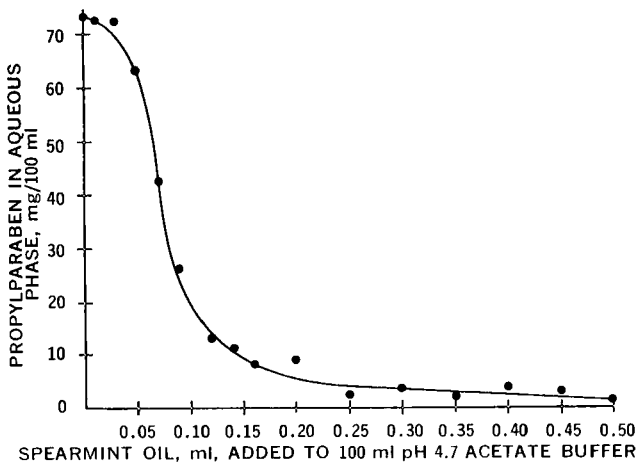

Figure 1—*Partitioning of propylparaben from solution in pH 4.7 acetate buffer into spearmint oil as a function of concentration of spearmint oil.*

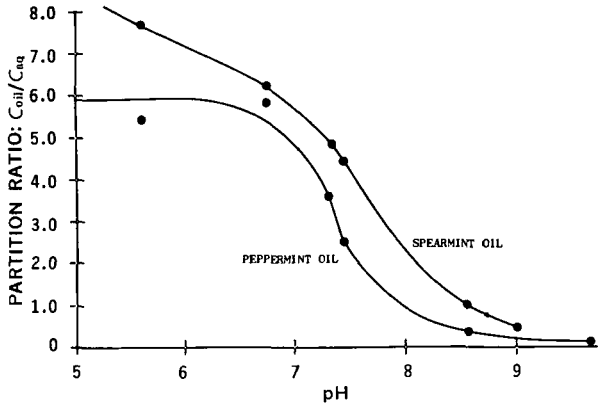

Figure 2—*Partitioning of propylparaben from solution in aqueous buffer into 0.4% peppermint oil and 0.25% spearmint oil as a function of pH of aqueous buffer solution.*

the effect was observed best with peppermint oil and spearmint oil, so further work was confined to them.

The solubilities of four esters of *p*-hydroxybenzoic acid in distilled water, peppermint oil, and spearmint oil are given in Table II. As reported in the literature, the solubility in water decreases and the solubility in oil increases with the increase in the chain length of the ester.

The effect of the concentration of the flavoring oil on the partitioning of propylparaben from its solution in acetate buffer is shown in Table III. Even at 0.1% oil concentration, almost 30% of the propylparaben partitioned into the oil. The same effect of the concentration of oil on partitioning was observed with spearmint oil (Fig. 1).

The effect of pH on the partitioning of propylparaben from a 0.025% solution in buffers into 0.25% spearmint oil is shown in Table IV. No attempt was made to calculate the true partition coefficients since the volume adjustments would give tremendously high numbers. Only ratios of concentrations of paraben in oil and aqueous phase are given in Table IV. It can be observed from the data that as the pH of the propylparaben solution is raised, the concentration of ionized species in relation to unionized species increases. Since the solubility of ionized species in the nonaqueous solvents is negligible, the amount partitioning into the oil layer decreases steadily with the increase in pH. The same observation was made with peppermint oil. The results for the effect of pH on the partitioning are shown graphically in Fig. 2 for both peppermint oil and spearmint oil. The sigmoidal curve seen for peppermint oil with its inflection point at pH 7.4 corresponds to the titration curve of a buffer species with a pKa of 7.4.

The effect of the length of the side chain in the ester linkage was investigated by determining the ratio of the concentration of the four esters in an aqueous acetate buffer and a nonaqueous dispersed phase of mixtures containing a fixed amount of added flavoring oil—0.2% spearmint oil or 0.2% peppermint oil (Tables V and VI). A plot of the partition ratio against the molecular weight of the esters in the spearmint and peppermint oils is shown in Fig. 3. From the plot it can be observed that a longer chain length causes higher partitioning into the oil. Thus, butylparaben partitions the most into the oil followed by propyl-, ethyl-, and methylparaben, in that order. This order follows the order of solubility of the

Table V—Partitioning of Parabens Using a Constant Quantity of Spearmint Oil (0.2%) in Acetate Buffer

Paraben	Concentration of Parabens in Aqueous Phase, mg/100 ml: Before Partitioning (B.P.)	After Partitioning (A.P.)	$\frac{C_{oil}/C_{aq}\ \text{B.P.} - \text{A.P.}}{\text{A.P.}}$
Methyl	59.2	36.6	0.62
Ethyl	69.1	19.2	2.60
Propyl	68.7	1.4	48.1
Butyl	47.1	0.0	—

Table VI—Partitioning of Parabens Using a Constant Quantity of Peppermint Oil (0.2%) in Acetate Buffer

Paraben	Concentration of Parabens in Aqueous Phase, mg/100 ml: Before Partitioning (B.P.)	After Partitioning (A.P.)	$\frac{C_{oil}/C_{aq}\ \text{B.P.} - \text{A.P.}}{\text{A.P.}}$
Methyl	58.8	55.7	0.06
Ethyl	74.0	51.4	0.44
Propyl	64.9	26.0	1.50
Butyl	60.1	9.1	5.77

esters in oil themselves. The longer chain length of the ester linkage imparts the characteristic of higher solubility in the nonaqueous phase (Fig. 3).

Since any solution in need of preservation would contain ionic and nonionic materials, the effect of their presence on partitioning of propylparaben from a 0.05% solution into a 0.15% spearmint oil was studied. Sodium chloride was added as an ionic material, and sucrose was added as a nonionic material (Tables VII and VIII). As the concentration of sodium chloride in solution increased, the amount of propylparaben partitioning into oil increased. This effect was conspicuously absent with sugar.

The increased partitioning of parabens in the presence of sodium chloride may be explained on the basis of the salting out of oil; the amount of oil separating increased with an increased concentration of salt in the solution. The inherent solubility of these flavoring oils is fairly low. In the presence of a strong electrolyte, some of the dissolved oil or its components—terpenes—would come out of solution. This oil would then disperse throughout the aqueous phase as a nonaqueous organic phase, exerting its full partitioning effect and causing depletion of parabens from the aqueous phase.

In the foregoing experiments, the concentration of oils used was 4–10 times the normal concentrations used as flavoring agents. The exaggerated conditions were necessary to discover the possible interaction of the flavoring oils at their normally lower levels. The solubility of the flavoring oils is dependent on the concentration of other additives, particularly ionic compounds. As noticed in the effect of concentration of sodium chloride on the partitioning of propylparaben, the ionic compound would increase the partitioning of propylparaben into oil or cause salting out of the oil from the solution which, in turn, would be available for partitioning of more

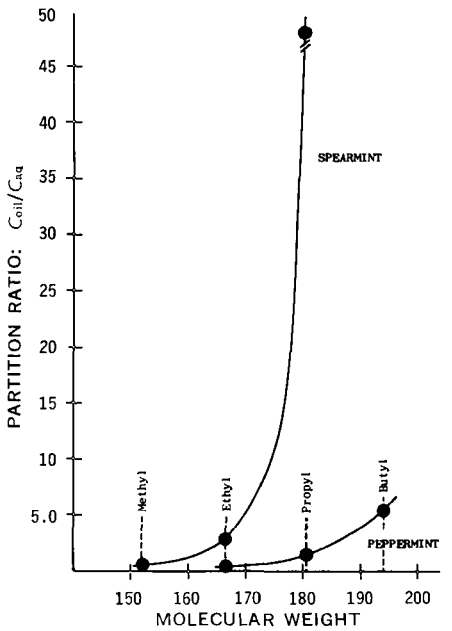

Figure 3—*Partitioning of parabens from solution in pH 4.7 acetate buffer solution into peppermint oil and spearmint oil as a function of molecular weight of parabens.*

Table VII—Effect of Sodium Chloride Concentration on Partitioning of Propylparaben from 0.05% Solution into 0.15% Spearmint Oil

Molarity of Salt in Acetate Buffer	pH	Concentration of Paraben in Aqueous Phase, mg/100 ml		$\frac{C_{oil}/C_{aq}}{\frac{B.P. - A.P.}{A.P.}}$
		Before Partitioning (B.P.)	After Partitioning (A.P.)	
0.00	4.60	77.2	52.1	0.48
0.25	4.50	77.2	46.9	0.65
0.50	4.45	77.2	38.4	1.01
0.75	4.40	77.2	33.3	1.32
1.00	4.40	77.2	23.1	2.34
2.00	4.30	77.2	9.8	6.87
3.00	4.28	77.2	2.4	31.18

paraben. The overall effect is depletion of parabens from the aqueous phase.

A higher pH would decrease the amount of parabens partitioned into the oils because of the higher concentration of dissociated paraben molecules in the aqueous medium. The dissociated molecule does not have the preservative action, so adjustment of pH to reduce partitioning would not be helpful.

An experimental antacid formulation was prepared containing a combination of methylparaben (1.0 mg/ml) and propylparaben (0.5 mg/ml) as a preservative. This formulation was flavored with anise oil, and another similar formulation was flavored with peppermint oil. The concentration of anise oil was 5.5 times that of peppermint oil.

Both formulations were challenged with *Pseudomonas aeruginosa* and *Paracolobactrum coliforme* separately with 10,000,000 and 500,000 organisms/ml. A mixed culture of the two was also used as a challenge inoculum at two levels. Over a 4-week testing period during which seeded bottles were incubated at 25 and 37° and plated on growth media, the formulation containing peppermint oil did not show growth, indicating adequacy of the preservative system. However, the preparation containing anise oil as a flavoring agent showed growth of *Pseudomonas* in the 2nd week. When the concentration of propylparaben in the anise oil formulation was raised to 0.7 mg/ml, the formulation withstood the microbial challenge test and was comparable to a control formulation. Therefore, it was concluded that in the presence of a small concentration of flavoring oils, the partitioning of parabens into flavoring oil does occur, causing a decrease in the preservative action. The antimicrobial action of the flavoring oils makes quantitative microbial evaluation of this partitioning effect in pharmaceutical formulations very difficult. A method for such evaluation is under consideration.

Table VIII—Effect of Sucrose Concentration on Partitioning of Propylparaben from 0.025% Solution into 0.1% Spearmint Oil

Molarity of Sucrose in Acetate Buffer	Concentration of Paraben in Aqueous Phase, mg/100 ml	
	Before Partitioning	After Partitioning
0.00	48.5	48.5
0.25	48.5	49.5
0.50	48.5	49.6
0.75	48.5	53.2
1.00	48.5	49.2
2.00	48.5	45.8

CONCLUSION

This study demonstrates that parabens used as preservatives in oral liquid pharmaceuticals partition into flavoring oils. The partitioning effect depends upon the concentration of the flavoring oils, the pH of the aqueous medium, and the nature of other additives. The depletion of parabens from the aqueous phase may lower the concentration of the preservatives below the critical required level for preservative action.

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Nature of Anionic or α-Site of Cholinesterase

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Abstract □ The nature of the so-called anionic binding site of acetylcholinesterase was investigated using a technique called receptor mapping using model interaction calculations. The results support the suggestion that coulombic forces play only a minor role in the binding event at this enzyme site.

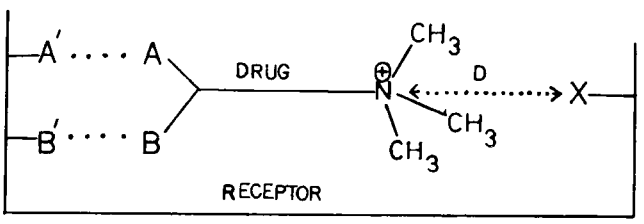
Keyphrases □ Acetylcholinesterase—anionic binding studied, receptor mapping using model interaction calculations, role of coulombic forces □ Binding sites, acetylcholinesterase—anionic binding site, receptor mapping using model interaction calculations, coulombic forces □ Enzymes, acetylcholinesterase—theoretical studies on nature of anionic binding site □ Receptor mapping using model interaction calculations—theoretical studies on nature of anionic binding site of acetylcholinesterase □ Coulombic forces—role in binding of acetylcholinesterase, anionic site

The widely held view (1) concerning the active features of cholinesterase is that two principal regions exist, a catalytic or esteratic site and a binding or anionic site. The esteratic site is thought to be a serine residue, engaging in acylation with acetylcholine or a similar ester. This site can be phosphorylated or carbamylated, leading to a slowly dissociable ester and, hence, to effective inhibition of the enzyme. The binding site is that region of the enzyme near the esteratic site capable of interacting or binding to the onium groups of acetylcholine or similar compounds. It has been postulated that this feature is anionic, since the complimentary onium group of the substrate bears a net positive charge. This interaction was presumed to involve an ion-pair. In addition to an ionic component, Wilson (1) postulated a significant role for dispersion forces.

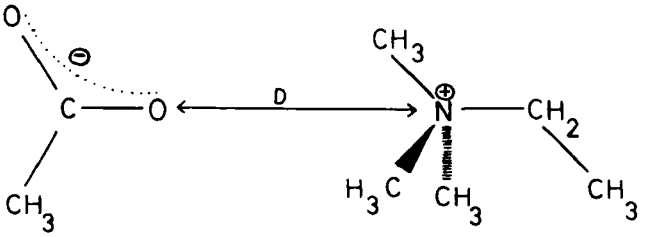
DISCUSSION

In a recent treatise, O'Brien (2) discussed work leading to some new views on the nature of the binding site and proposed that binding sites exist on cholinesterase, each responsive to different types of substrates or inhibitors. O'Brien renamed the classical anionic site the α-site. This enzyme feature must bind to acetylcholine for hydrolysis to occur. Other sites, β and γ, are engaged by different classes of inhibitors.

From accumulated evidence, O'Brien made a case for the view that the α-site is not ionic but is a region of the enzyme (side chain) essentially nonpolar in character and engaging in a noncoulombic interaction with the onium group of acetylcholine. Specifically, O'Brien proposed two alternatives to the general view of an anionic α-site: *(a)* an α-site composed of an inducible dipole which forms an ion-induced dipole with the acetylcholine onium group, or *(b)* a completely nonpolar α-site engaging in van der Waals interaction with the acetylcholine onium residue.



Scheme I—Drug–receptor model showing binding features A and B locked at A' and B', respectively, and distance D between receptor feature X and onium group



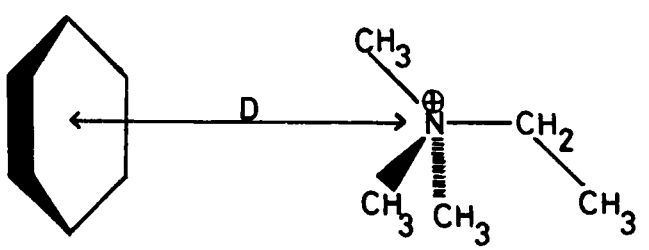
Scheme II—Mode of interaction between acetic acid-ion and acetylcholine model

Some insight into these three possibilities may be gained using a theoretical approach recently employed by Kier and Aldrich (3) and Höltje and Kier (4, 5). This approach, referred to as receptor mapping using model interactions calculations, involves the calculation of interaction energies (electrostatic, polarization, dispersion, and repulsion) between a series of reactive or biologically active molecules and a group of model compounds simulating a variety of likely receptor or enzyme active site moieties. These moieties are models derived from amino acid side chains.

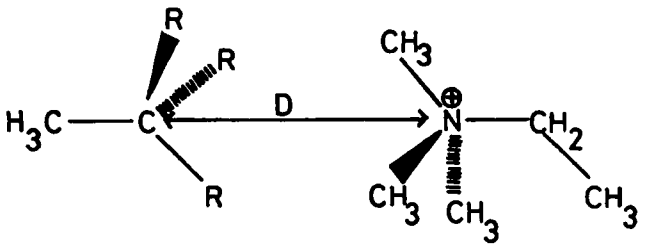
The assumption has been made that the receptor or enzyme active site is composed of a constellation of amino acid side chains. It is also assumed that one amino acid side chain is the dominant structural feature engaging a particular part of the substrate molecule or drug pharmacophore.

The problem is then to calculate the interaction energy between a structural feature under study in the drug or substrate molecule and a model compound simulating a possible receptor or enzyme active site. By performing these calculations on a series of drugs or substrates in which a variation in structure has been introduced at the moiety under study, a pattern of interaction energies can be obtained for each of several candidates for the receptor or active site. An attempt is made to correlate the trend in calculated interaction energies with a catalytic reactivity index or a biological property. The better the correlation, the more reasonable is this candidate for the receptor or active site.

Two concepts of enzymatic rate enhancement lead to the belief that the binding of a structural feature, remote from the catalyzed structure of the substrate, can have a direct bearing on the rate of reaction. The concept of catalysis by induction of strain, developed by Jencks (6), views the substrate as going through significant structural alteration as it is transformed through a transition state while bound at the enzyme active site. The binding forces probably vary as a function of these structural changes. At some point in the conversion of substrate to product, these binding forces are maximal. If the binding is maximal when the substrate reaches the transition state for the rate-determining step, a powerful driving force for the reaction could be provided. In effect, the enzyme is using a potential binding energy to help force the substrate toward the critical transition state structure. It is felt that these strain



Scheme III—Mode of interaction between benzene and acetylcholine model



Scheme IV—Mode of interaction between ethane (R = H) and tert-butane (R = CH_3) and acetylcholine model

forces involve noncovalent forces between enzyme and substrate. Such could be the role of structural features on a substrate, remote from the region of catalytic change.

A second concept of enzymatic rate enhancement is called substrate anchoring (7). Based upon nuclear magnetic evidence, substrates have a relatively long residence time at the active sites of enzymes. This binding as a complex is believed to be capable of accelerating the reaction rate by factors between 10^6 and 10^9. The concept presumes that the anchoring of a substrate to the enzyme greatly increases the probability of the formation of an activated complex, thereby increasing the reaction rate by large factors. The role of substituent groups on a substrate, remote from the region of catalytic change, could provide this anchoring.

Further significance can be attached to a correlation if the assumption is made that, in a series of drugs or substrates, the ratio, ρ, of any two activities in a series is related to the difference in strength of binding of the feature under study by the expression $\Delta E = -RT \ln \rho$. The better the calculated ΔE values in a series fit to the experimental ρ values, the more realistic is the model of the receptor or active site.

It is assumed that the mode of approach of the structural feature under study is constrained to a single mode of approach to the receptor model because of other points of binding between drug or substrate and receptor or enzyme (Scheme I). The calculations of interaction energies are thus made for a single mode of approach, varying the distance between the two molecules.

It is clearly recognized that simplifying assumptions and idealizing conditions are made. In reality, the receptor may be a region rather than a single moiety. The significance of these results may lie in the creation of a reasonable model which, although not proven to be reality, is isomorphic with reality. The value of such an isomorphic model lies in its abilities to explain and to predict.

EXPERIMENTAL

The calculations used were developed (8) and described previously (3–5). This approach was utilized in the first example of the prediction of a molecular conformation, specifically prostaglandin E_1 (9).

In this study, a series of acetylcholine derivatives in which the onium group was successively replaced by *tert*-butyl, isopropyl, ethyl, and methyl groups was selected. The reactivity against cholinesterase in terms of percentage of acetylcholine hydrolysis rates was measured in two separate studies (10).

The models simulating the α-site were chosen to reproduce the three proposed modes of interaction, *i.e.*, a carboxylate group (acetic acid) participating in an ionic interaction, a benzene ring participating in an ion-induced dipole interaction, and a *tert*-butyl (2,2-dimethylbutane) and methyl (ethane) group participating in a completely noncoulombic van der Waals interaction.

The modes of approach of the parent acetylcholine molecule and each model are shown in Schemes II–IV. The optimum distance separating the substrate model and the receptor model was chosen so that the entire series presented the best correlation with the hydrolysis rates. The distance separating the α-carbon atom of each member of the substrate series and the active site model was assumed to be constant for each test case of intermolecular distance. This is based on the assumption that the distal part of the molecule binds significantly to its complimentary feature (Scheme I), so that no matter what is appended on to the ethyl chain, the distance separating the active site from the α-methylene group will be constant.

Table I—Interaction Energies (Kilocalories per Mole) Calculated between Acetic Acid-Ion and Acetylcholinesterase Substrate Models[a]

R	Distance, Å (Scheme II)			
	3.5[b]	3.75	4.0	4.25
$\overset{+}{N}(CH_3)_3$	12.50	11.49	9.99	8.51
$C(CH_3)_3$	3.48	4.26	4.04	3.49
$CH(CH_3)_2$	3.09	3.45	3.18	2.74
CH_2CH_3	2.68	2.64	2.32	1.94
CH_3	2.49	2.01	1.61	1.31

[a] Scheme II shows one typical mode of interaction listed in the table: R = $^+N(CH_3)_3$. [b] Set of values at this distance used to calculate the percentage of hydrolysis (Table V).

Table II—Interaction Energies (Kilocalories per Mole) Calculated between Benzene and Acetylcholinesterase Substrate Models[a]

R	Distance, Å (Scheme III)			
	5.0	5.25	5.5	5.75[b]
$\overset{+}{N}(CH_3)_3$	3.05	2.55	2.08	1.68
$C(CH_3)_3$	1.32	1.21	1.01	0.82
$CH(CH_3)_2$	1.05	0.94	0.78	0.63
CH_2CH_3	0.77	0.66	0.54	0.43
CH_3	0.48	0.39	0.30	0.24

[a] Scheme III shows one typical mode of interaction listed in the table: R = $^+N(CH_3)_3$. [b] Set of values at this distance used to calculate the percentage of hydrolysis (Table V).

Table III—Interaction Energies (Kilocalories per Mole) Calculated between *tert*-Butane and Acetylcholinesterase Substrate Models[a]

R	Distance, Å (Scheme IV)			
	5.75	6.0	6.25	6.5[b]
$\overset{+}{N}(CH_3)_3$	3.27	2.75	2.27	1.87
$C(CH_3)_3$	1.20	1.07	0.89	0.71
$CH(CH_3)_2$	0.95	0.83	0.69	0.55
CH_2CH_3	0.70	0.60	0.48	0.39
CH_3	0.44	0.35	0.27	0.22

[a] Scheme IV shows one typical mode of interaction listed in the table: R = $^+N(CH_3)_3$. [b] Set of values at this distance used to calculate the percentage of hydrolysis (Table V).

RESULTS

The interaction energies of the model systems are given in Tables I–IV.

Using the thermodynamic formula $\Delta E = -RT \ln \rho$, the percentage of hydrolysis for the series of noncationic congeners compared

Table IV—Interaction Energies (Kilocalories per Mole) Calculated between Ethane and Acetylcholinesterase Substrate Models[a]

R	Distance, Å (Scheme IV)			
	5.0[b]	4.75	4.5	4.25
$\overset{+}{N}(CH_3)_3$	2.02	2.51	2.98	3.16
$C(CH_3)_3$	0.86	1.01	0.98	0.32
$CH(CH_3)_2$	0.67	0.80	0.82	0.45
CH_2CH_3	0.48	0.59	0.67	0.56
CH_3	0.28	0.37	0.49	0.66

[a] Scheme IV shows one typical mode of interaction listed in the table: R = $^+N(CH_3)_3$. [b] Set of values at this distance used to calculate the percentage of hydrolysis (Table V).

Table V—Experimental and Calculated Hydrolysis Rates (Percent) Relative to Acetylcholine

R	Experimental		Calculated from Model			
			Acetate	Benzene	*tert*-Butane	Ethane
$\overset{+}{N}(CH_3)_3$	100[a]	100[b]	100	100	100	100
$C(CH_3)_3$	24	60	4.37×10^{-5}	25	15	15
$CH(CH_3)_2$	14	24	2.4×10^{-5}	18	12	11
CH_2CH_3	7	16	1.23×10^{-5}	13	9	8
CH_3	3	10	0.91×10^{-5}	10	7	6

[a] Horse plasma cholinesterase. [b] Human erythrocytes cholinesterase.

to a 100% hydrolysis rate for the acetylcholine model was calculated. For this purpose, the set of data that fits best in view of the biological data was chosen for each model approach. Table V shows the values obtained together with the experimental hydrolysis rates of human erythrocytes and horse plasma cholinesterase.

Table V shows clearly that the anionic α-site model cannot explain the rather moderate differences in activity between the cationic and the noncationic acetylcholinesterase substrates, whereas the three uncharged α-binding site models behave much more realistically in terms of the hydrolysis rates. These results support the suggestion of O'Brien (2) that an anionic binding site is not a good assumption. The benzene model provides the best fitting set of values in this series, but the superiority over the two nonaromatic α-site models is not significant enough to decide clearly between an ion-induced dipole or a van der Waals interaction binding mechanism. However, the results of this study make an ion-induced dipole interaction at the α-binding site of the acetylcholinesterase, typified by a benzene ring, most likely.

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[x] To whom inquiries should be directed.

Microbiological Determination of Drug Partitioning IV: Drug–Protein Interactions

S. JAVIDAN * and ROBERT G. MRTEK ‡x

Abstract □ The protein binding characteristics of chloramphenicol, furazolium chloride, benzalkonium chloride, and phenylmercuric nitrate were described from their partitioning behavior in gelatin–acacia complex coacervate systems. Although the partitioning was determined by two different methods (microbiological and chemical), the microbiological method was more reliable for this type of investigation. Drug–protein parameters were calculated for the four antimicrobials. The advantages of the coacervate systems over other models for protein binding studies of drugs are discussed.

Keyphrases □ Protein binding characteristics—chloramphenicol, furazolium chloride, benzalkonium chloride, and phenylmercuric nitrate, partitioning behavior in gelatin–acacia complex coacervate systems, microbiological determination □ Drug–protein interactions—microbiological determination of drug partitioning, gelatin–acacia complex coacervate systems, four antimicrobial agents □ Drug partitioning—microbiological determination, drug–protein interactions, gelatin–acacia complex coacervate systems □ Microbiology—determination of drug partitioning, drug–protein interactions, four antimicrobials, gelatin–acacia complex coacervates

Binding of drugs to the protein constituents in biological systems has been demonstrated to have a tremendous effect on the ultimate action of drugs in these systems (1). Different model systems, including dialysis membranes (2–5), lipid-like organic solvents (6–10), more polar liquids (11–18), and other types of systems (19–33), have been used in the study of protein binding drugs. The use of organic solvent systems for the study of protein binding involves the tacit assumption that the organic solvents closely

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simulate the functioning biological system. Such an assumption has always been questioned and has been rejected outright by several investigators. Additionally, the study of drug–protein interactions by the use of dialysis membrane systems requires corrections due to the adsorption of drugs to the membranes and the charge deposited on such membranes.

The advantages of complex coacervate systems as appropriate biomodels have been emphasized by several investigators (34–58); several of these systems have already been used in drug partitioning studies (34, 59–62). Therefore, it seems that complex coacervate systems offer more dependable models for studying drug–protein interactions. Javidan and Mrtek (62) studied the partitioning of chloramphenicol, phenylmercuric nitrate, benzalkonium chloride, and furazolium chloride in gelatin–acacia complex coacervate systems using chemical and biological methods. In this work, β-values for the protein binding of these four antimicrobials are calculated from the partition coefficients of these substances in the gelatin–acacia complex coacervate systems.

EXPERIMENTAL

The organisms, culture media, preparation of coacervate systems, and methods of determination of partition coefficients were exactly the same as reported previously (60–62). *Escherichia coli* B/r (ATCC 23227), *Pseudomonas aeruginosa*[1], and *Staphylococcus aureus*[1] were used as test organisms. Difco E.C., BHI media, and a defined medium (63) were used for the growth of microorganisms.

During their exponential growth, organisms were introduced to a sample of the equilibrium liquid layer of a coacervate system containing drug. From the rate of the growth of the microorganism, as determined from particle counter measurements[2], the concentration of drug in the coacervate layer was determined. By means of suitable mathematic relationships, the partition coefficient was calculated. In the chemical determination of partitioning, the amount of drug was analyzed in a sample taken directly from the equilibrium layer of the system, and partition coefficients were calculated in a similar way as for the microbiological system.

RESULTS AND DISCUSSION

The partition coefficients of chloramphenicol, benzalkonium chloride, furazolium chloride, and phenylmercuric nitrate were determined as described previously (62). β-Values were calculated using the following equation (3):

$$\beta = \frac{PD}{D_t} \qquad \text{(Eq. 1)}$$

where PD shows the moles of the protein-bound drug and D_t is the total amount of drug in the system. Figures 1–4 show the results.

From Fig. 1, it is apparent that β is at a maximum for chloramphenicol when the partition coefficient approaches a constant value of 21.9. This value indicates that, of a total 6750 μg of chloramphenicol in the coacervate system, 61% is retained in the coacervate layer. Chloramphenicol is reported to be bound to plasma proteins to an extent of 45–60% (1, 64–67). Figure 1 shows that, with increasing amounts of chloramphenicol in the system, the partition coefficient of chloramphenicol and, correspondingly, its β-values increased to a maximum. When 1800 μg of chloramphenicol was present in the system, the derivative of the curve in Fig. 1 was a maximum, thereby indicating the drug concentration where

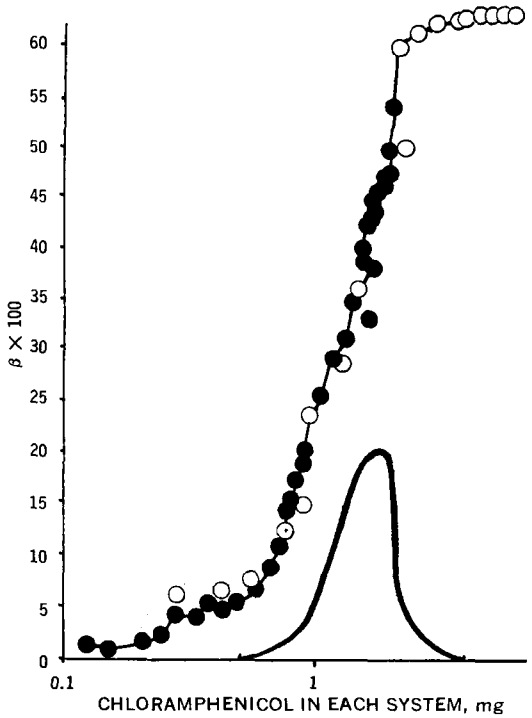

Figure 1—*Protein binding of chloramphenicol in gelatin–acacia coacervate system. Key:* ○, *determined by* S. aureus; ●, *determined by* E. coli *B/r; and lower curve, derivative function* D′ β.

binding seemed to be most complete. When the amount of drug in the coacervate system exceeded 1800 μg, the differential curve fell off.

Similarly with benzalkonium chloride, the partition coefficient in the coacervate system was quite high with relatively small amounts of drug in the system (<10,000 μg). Above this amount of drug, the partition coefficient decreased as the amount of benzalkonium chloride was increased. When the partition coefficient values plateaued at high amounts of drug (40,000 μg), 22.6% of the drug in the system was bound in the coacervate phase. The apparent decrease in partition coefficient is attributed to the inordinately larger amounts of benzalkonium chloride accumulated in the equilibrium phase, which may have been due to the micellization of benzalkonium chloride. Free monomers partition in favor of micelles above the critical micelle concentration (CMC). It is suggested that above 30,000 μg of drug in the system, the micelles were large so that any added monomer to the system could not participate in the aggregation process; therefore, the drug once again partitioned in favor of the coacervate phase. At about 15,000 μg of drug in the system [the minimum point on the derivative curve *D′ (PC)* in Fig. 2], it is postulated that the rates of protein binding in the coacervate phase and micellization are equal.

Autian (68) studied the binding effects of benzalkonium chloride in plastics and concluded that the concentration of the bound drug increased up to the vicinity of the CMC and then decreased. Although Autian's studies were made in different model systems, his results (69) followed the same pattern as described in this study. When the partition coefficient approached a constant value, 22.66% of the benzalkonium chloride was retained in the coacervate phase.

The protein binding of benzalkonium chloride already has been reported by several investigators (70–81). A number of workers have noted that some quaternary ammonium compounds were more active than many other germicides when tested in the presence of serum proteins (82, 83). In 1945, Tice and Pressman (70) investigated the effect of benzalkonium chloride on anionic and cationic gelatin and reported that anionic gelatin forms a coacervate with benzalkonium chloride, while cationic gelatin does not. These investigators commented that cationic gelatin not only does not inactivate benzalkonium chloride but augments its antibacterial activity.

[1] Culture Collection, Department of Microbiology, College of Medicine, University of Illinois at the Medical Center, Chicago, IL 60680
[2] Coulter.

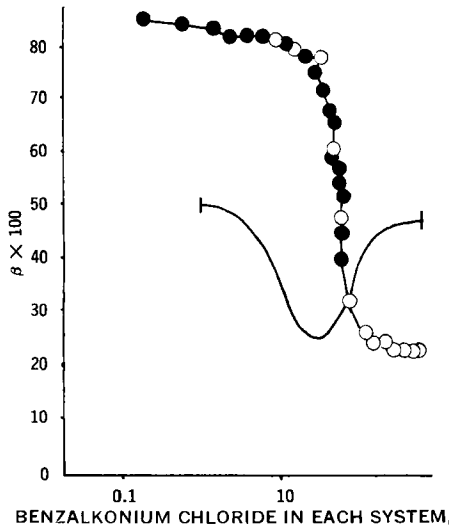

Figure 2—*Protein binding of benzalkonium chloride in gelatin–acacia coacervate system. Key:* ○, *determined by* P. aeruginosa; ●, *determined by* S. aureus; *and lower curve, derivative function* D′ β.

Protein binding of cationic nitrofurans like furazolium chloride was reported to be about 10% (81). From Fig. 3, it can be seen that when the partition coefficient approached a constant value of 2.28, β-values became 13.9%; *i.e.*, the amount of drug in the coacervate phase was 13.9% of the total amount of drug in the system. This value is acceptably close to a reported value (81). A closer examination of Fig. 3 reveals that when less than 480 μg of drug was present in the system, the increase in the partition coefficient was attributed to the protein binding of furazolium chloride in the coacervate phase. It could be postulated that micellization occurred when the amount of furazolium chloride in the system exceeded 480 μg. Since the molecules of furazolium chloride participated in the micellization process above the CMC, the partition coefficient and, consequently, the β-values decreased.

Above 6000 μg of drug in the system, the curves in Fig. 3 are analogous to those determined with benzalkonium chloride. As a matter of fact, Fig. 3 can be considered to represent a combination of Figs. 1 and 2; *i.e.*, two ongoing processes—binding and micelle formation—are involved. Furazolium chloride did not show any measurable aggregation below 480 μg, making that portion of the curve similar to Fig. 1, and it underwent micellization above 480 μg, making the curve similar to Fig. 2. Paul and Paul (84) showed from *in vivo* studies that the amount of nitrofuran bound to plasma protein in rats did not appear to be affected by the plasma concentration of the drug. The drug is apparently distributed very rapidly and the increase in plasma concentration of the drug is not as great as would be expected for a proportionate increase in dosage.

It would appear from Fig. 4 that the molecules of phenylmercuric nitrate underwent some type of aggregation in the equilibrium phase. The partition coefficient of phenylmercuric nitrate approached a constant value of 5.0, and the β-value was 26%.

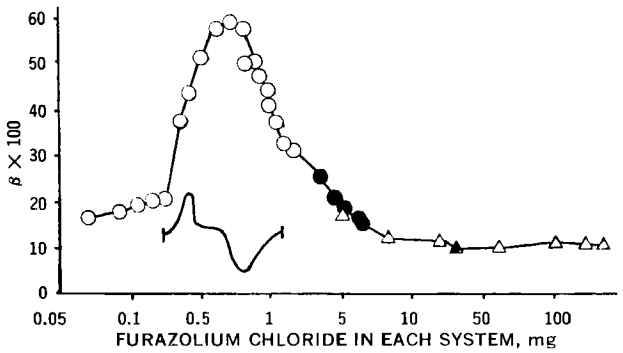

Figure 3—*Protein binding of furazolium chloride in gelatin–acacia coacervate system. Key:* ○, *determined by* E. coli *B/r;* ●, *determined by* S. aureus; △, *determined by* P. aeruginosa; *and lower curve, derivative function* D′ β.

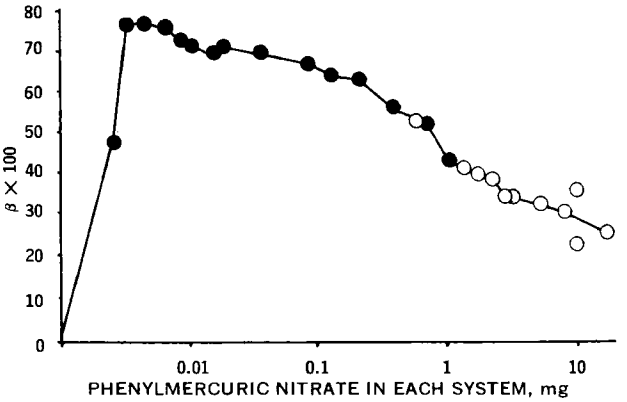

Figure 4—*Protein binding of phenylmercuric nitrate in gelatin–acacia coacervate system. Key:* ○, *determined by* E. coli *B/r; and* ●, *determined by* S. aureus.

The partitioning (as well as protein binding) of four antimicrobials was measured microbiologically in this study; however, microbiological measurement of drug partitioning and protein binding need not be confined only to antimicrobials (85–88). As long as a drug substance is capable of exhibiting any type of biological activity, it is a candidate for this type of study. There are always organisms that are responsive to the pharmacological effect that a compound exerts. In this manner, vitamins, toxic materials, nutrients, *etc.*, can be partitioned in coacervate systems and measured microbiologically. Complex coacervates can be formed from almost any protein below its isoelectric point. For this reason, the coacervate model system described here for gelatin should be extended to a variety of more important proteins in an attempt to study the protein binding of biologically significant substances.

Although the β-value is the most reliable parameter for the study of protein binding, parameters such as r and K are calculated in some instances. Calculation of r, the average moles of bound drug per mole of protein, and K, the intrinsic association constant of drug–protein interaction, could be accomplished by employing the following equations:

$$r = \frac{1000M(T - aV_2)}{198m \times 10^6} \qquad \text{(Eq. 2)}$$

$$K = \frac{M(T - aV_2)}{aV_2 198{,}000m - M(T - aV_2)} \qquad \text{(Eq. 3)}$$

where M and m are the molecular weights of the protein and the drug, respectively; a is the concentration of drug in the equilibrium liquid; V_2 is the volume of the equilibrium liquid; and T is the total amount of drug in the system.

The calculation of protein binding constants from partition coefficients obtained with heptane–water systems has been performed by several investigators (11–17, 84). But studies utilizing systems such as chloroform–water, octanol–water, or heptane–water suffer from the lack of similarity with biological systems. Coacervate systems should offer more dependable models for the study of drug–protein interactions. The similarities between protoplasm and aqueous complex coacervates have been emphasized. Takruri (34) suggested that complex coacervates might serve as biological models, although the difference between biological systems (protoplasm) and coacervates is admittedly substantial. Naturally one shortcoming of the model is that biological systems are dynamic, but coacervate systems are essentially static. These differences between protoplasm and coacervates are, however, qualitatively and quantitatively much less than those between protoplasm and chloroform, heptane, olive oil, or other pure solvents.

The advantage of complex coacervate systems over other methods for studying protein binding must include several important considerations:

1. In the method of equilibrium dialysis, the protein molecule is

required to be sufficiently large to be selectively retained by the dialysis membrane. The limits of this method preclude studies involving the binding properties of protein fragments and polypeptides, while in coacervate systems no such molecule or configurational constraints are imposed.

2. In binding studies with drugs using dialysis membranes, a significant correction usually must be made for drug adsorption by the membrane (11). Under certain circumstances the variability of this correction leads to a lower precision in the determination of the amount of drug actually bound to the protein than the usual analytical method permits.

3. Whenever the protein carries a net electrostatic charge, it is the usual practice to add dialyzable electrolytes to minimize the so-called Donnan effect. In the case of serum albumins, at least, this procedure is particularly undesirable because these proteins bind even the simplest anion such as chloride. As a result, the protein charge is altered, usually to an unknown extent.

4. By varying the protein components and other physical characteristics, coacervate systems can be specially designed for a variety of proteins and small molecules.

5. The fact that the equilibration can be established in coacervate systems by centrifugation for 10 min suggests relative speed as another advantage of coacervate systems over dialysis membranes in the study of drug–protein binding.

6. Measurement of protein binding in organic systems such as heptane–water (11) and octanol–water (14–16) does not detect the difference in ionic properties of drugs, while coacervate systems seem to serve these subtle yet important purposes. In this study the relationship between the coacervate–equilibrium liquid partition coefficient of a compound and its protein binding parameter r could be obtained from the following equation:

$$PC = \frac{198{,}000rm}{aV_1M} \qquad \text{(Eq. 4)}$$

By assuming that all drug molecules in the coacervate phase are bound to protein, the only requirement for determination of r is a knowledge of the approximate or average molecular weight of drug compounds and the protein molecules.

The usefulness of coacervates as drug–protein binding models should become a subject for further study. To this end, one could replace, entirely or in part, the equilibrium liquid from drug-bound systems with equilibrium liquids from similar systems containing no drug. The subsequent determination of drug concentration in the new equilibrium liquid may provide useful information concerning the amount of free (or reversibly bound) drug in the coacervate phase.

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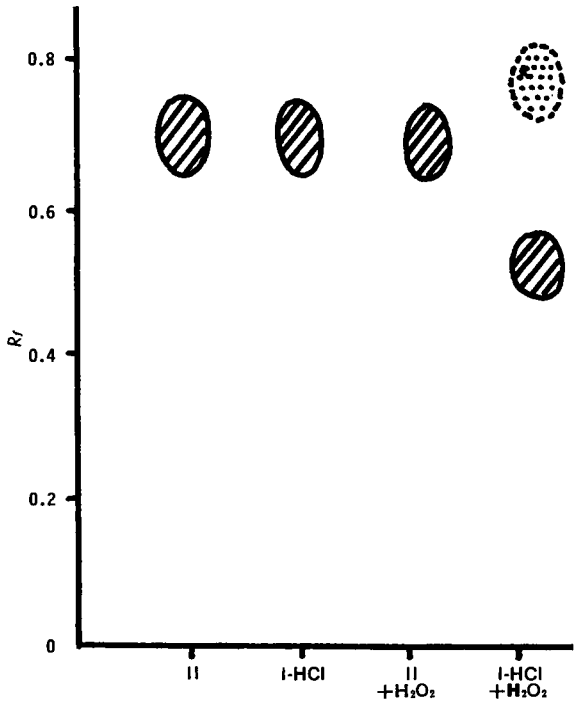


Figure 1—*Paper chromatography of I-HCl and II with 3% hydrogen peroxide. Solvent system was isopropanol–concentrated ammonia–water (7:1:2). Spots were detected by UV radiation and Ehrlich's reagent. Key: ⊘, UV absorbing spot; and ⊛, Ehrlich's reagent positive spot.*

tions of the anhydronucleoside when treated with hydrogen peroxide, since its ubiquitous occurrence in aerobic biological systems makes the reaction of considerable interest.

EXPERIMENTAL[3]

2,2′-Anhydro-1-β-D-arabinofuranosylcytosine (I) Hydrochloride—Compound I-HCl was prepared according to the modified method reported previously (13). Phosphorus oxychloride (33 ml, 0.36 mole) was placed in 200 ml of anhydrous dimethylformamide, and the mixture was set aside at room temperature for 30 min. To the solution was added 10.0 g (41 mmoles) of cytidine, and the mixture was stirred at room temperature for 3 hr and then poured into 150 ml of water to destroy the reagent. The aqueous reaction mixture was allowed to stand at room temperature for 2 hr and then was evaporated *in vacuo* to about 200 ml.

The solution was seeded with an authentic sample of the hydrochloride of I and stored in a refrigerator overnight. The white needles were separated by filtration, washed with ethanol, and dried. The hydrochloride of I was obtained in a yield of 49% (5.23 g), mp 257–259° dec. Physicochemical properties of this sample were identical with those of the authentic sample purified through ion-exchange columns described previously (13).

Paper Chromatography of I-HCl in Presence of Hydrogen Peroxide—Compound I-HCl (100 mg, 0.38 mmole) was dissolved in 1.0 ml of water, and 0.10 ml (1.30 mmole) of 30% hydrogen peroxide was added. The solution was submitted to paper chromatography and was developed with Solvent 1 for 14 hr. A single spot (R_f 0.47) was detected by UV radiation, and the aqueous extract of the spot showed UV absorption maxima at 286 (pH 1) and 258 and 316 (pH 7 and pH 13) nm. The spot consumed metaperiodate in the sodium metaperiodate–potassium iodide spray test. Another spot (R_f 0.74), colored violet with Ehrlich's reagent, was also detected. When I in the absence of hydrogen peroxide or II in the presence of hydrogen peroxide was submitted to paper chromatography and developed as described, only one spot (R_f 0.61) corresponding to II was detected (Fig. 1). The paper chromatograms of I in the presence of the reagent developed with Solvent System 2 or 4 revealed no other spots than that corresponding to I.

Treatment of Pyrimidine Nucleoside with Hydrogen Peroxide in Buffer Systems—Pyrimidine nucleosides such as I, II, 2,2′-anhydro-1-β-D-arabinofuranosyluracil, 2,2′-anhydro-5-hydroxy-1-β-D-arabinofuranosylcytosine (III), cytidine, and uridine were treated with 0.025 *M* hydrogen peroxide in buffer systems at room temperature. For example, a solution of the I-HCl (50 mg, 0.190 mmole) or II (50 mg, 0.205 mmole), 50 μl (0.65 mmole) of 30% hydrogen peroxide, and 450 μl of water was added to 25.0 ml of each of the following buffers, and the mixture was set aside at room temperature. The buffers used were 1 *M* sodium acetate (pH 4.0, 4.9, 5.5, and 5.9), 1 *M* phosphate (pH 5.8, 6.2, 7.0, and 7.9), and 1 *M* glycine–sodium hydroxide (pH 8.0, 9.0, 10.0, and 11.0).

An aliquot (0.10 ml) of each buffered mixture was added to 10.0 ml of 0.1 *N* NaOH; after 5 min at room temperature, the spectrum of the alkaline solution was recorded. Under the alkaline conditions, unreacted I was completely converted into II, and the product (III) was almost intact. From the spectra, absorbances of I and II treated with hydrogen peroxide were calculated (Figs. 2–4). The amount of hydrogen peroxide remaining in the buffered mixture except at pH 11 was determined by iodometry (Fig. 4).

The reaction of I with hydrogen peroxide at pH 7.9 in the presence of ferric chloride or allyl alcohol was also investigated. Thus,

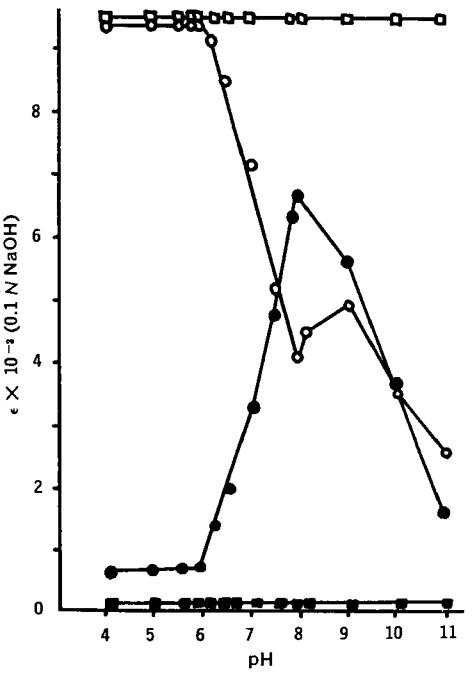


Figure 2—*Changes of UV absorption of I and II treated with hydrogen peroxide under different pH conditions. Compounds I-HCl (7.6×10^{-3} M) and II (8.2×10^{-3} M) were treated with 0.025 M hydrogen peroxide in 1 M buffer systems at room temperature for 18 hr. Absorbance at 275 and 315 nm of the mixture diluted with alkali was measured. Key: ○, I at 275 nm; ●, I at 315 nm; □, II at 275 nm; and ■, II at 315 nm.*

[3] Melting points were determined on a Buchi melting-point apparatus and are uncorrected. UV absorption was measured with a Hitachi recording spectrophotometer, EPS-3T. Optical rotations were obtained with a JASCO polarimeter, DIP-SL. NMR spectra were taken in deuteroxide (internal standard 2,2-dimethylsilapentane-5-sulfonic acid sodium salt) with a Varian-T60 spectrometer. High-resolution mass spectroscopy was measured with a CEC 110B double-focus mass spectrometer.

Paper chromatograms were run on Toyo Roshi No. 51A, using the following solvent systems: 1, isopropanol–concentrated ammonia–water (7:1:2); 2, *n*-butanol–water (84:16); 3, 5 *M* ammonium acetate–0.5 *M* tetrasodium ethylenediaminetetraacetate–saturated sodium borate–ethanol (12:0.3:48:132) (9); and 4, *n*-butanol–acetic acid–water (2:1:1). Paper electrophoresis was carried out in the following buffer systems: A, borate buffer (pH 6.0) (10); and B, 0.05 *M* phosphate buffer (pH 7.5) at 1000 v/35 cm for 1 hr. The spots were detected by UV radiation (253.6 nm) unless otherwise mentioned. The metaperiodate consumption test was done according to the usual method (11). Commercially available 30% hydrogen peroxide was used, and the content of the reagent in the solution was 440 mg (13 mmoles)/1.0 ml, estimated by iodometry. Cytarabine (II) was prepared by the authors (12, 13).

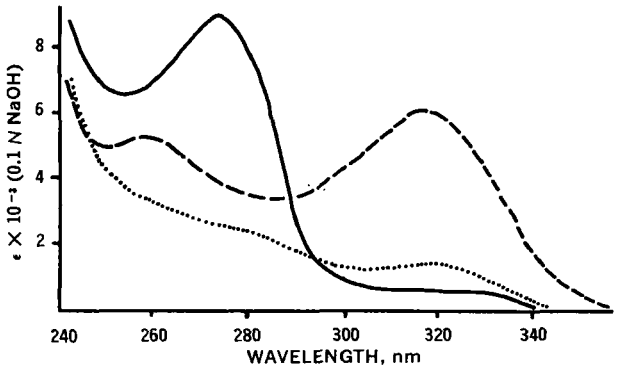


Figure 3—*Full spectra of the reaction mixture of I with hydrogen peroxide diluted with alkali. Conditions were the same described for Fig. 2. Key: —, pH 4.9; — —, pH 7.9; and ....., pH 11.0.*

a solution of the I-HCl (5.0 mg, 0.019 mmole) and 50 μl (0.65 mmole) of 30% hydrogen peroxide in 25.0 ml of 0.1 *M* phosphate buffer (pH 7.9) containing 5×10^{-5} *M* ferric chloride or 5×10^{-2} *M* allyl alcohol was allowed to stand at room temperature. An aliquot (2.0 ml) was diluted with 23 ml of 0.1 *N* NaOH or 0.1 *N* HCl, and the spectrum of the solution was recorded. The effect of ferric chloride and allyl alcohol on the reaction of I with hydrogen peroxide is presented in Fig. 6.

2,2′ - Anhydro-5-hydroxy-1-β-D-arabinofuranosylcytosine (III)—Compound I-HCl (2.00 g, 7.65 mmoles) was added to a mixture of 2.0 ml (26.0 mmoles) of hydrogen peroxide and 1.0 liter of 1 *M* phosphate buffer (pH 7.9) with stirring. The solution was kept at 30° for 18 hr. It was then chilled in an ice water bath, and the crystalline salt was removed by filtration and washed with cold water. The filtrate and washings were combined and treated with 1.0 g of 5% palladium-on-charcoal overnight until free of hydrogen peroxide as detected by potassium iodide-starch paper.

The charcoal was removed by filtration, and the filtrate was acidified to pH 1.0 with concentrated hydrochloric acid and added to a cation-exchange column[4] (250 ml). The column, which had previously been washed with water, was eluted with 250 ml of 3 *N* NH_4OH. The effluent was evaporated at 20° *in vacuo* to dryness, and the residue was triturated with ethanol to afford 1.60 g of a crude crystalline sample of III. Recrystallization from ethanol–water gave 1.20 g of white needles (65.0% yield), mp 275–281° dec., $[\alpha]_D^{25}$ −98.3° (c 0.10, water). The pKa, determined spectrophotometrically, was 5.2. NMR: δ 3.53 (2H, doublet, $H_{5'}$), 4.40 (1H, multiplet, $H_{4'}$), 5.46 (1H, doublet, $H_{2'}$, $J_{1',2'}$ = 6 Hz), 6.54 (1H, doublet, $H_{1'}$, $J_{1',2'}$ = 6 Hz), and 7.00 (1H, singlet, H_6) ppm. High-resolution mass spectroscopy showed a molecular ion peak at *m/e* 241 and an elemental composition corresponding to $C_9H_{11}N_3O_5$.

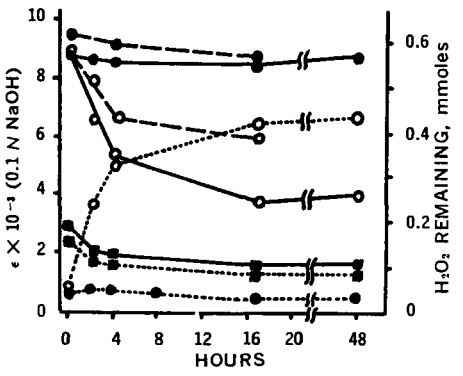


Figure 4—*Time course of the reaction of I with hydrogen peroxide. Compound I-HCl (7.6×10^{-3} M) was treated with 0.025 M hydrogen peroxide at pH 5.0, 7.9, or 11.0 and at room temperature. Absorbance at 275 and 315 nm of the mixture diluted with alkali was measured. The amount of hydrogen peroxide remaining in the mixture was also determined. Key: ●, pH 5.0; ○, pH 7.9; ■, pH 11.0; ——, absorbance at 275 nm; -----, absorbance at 315 nm; and — —, amount of hydrogen peroxide remaining.*

[4] Dowex 50 × 4, free form.

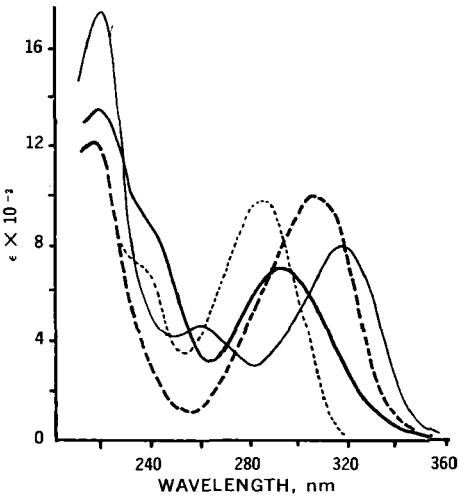


Figure 5—*UV absorption spectra of III treated with 0.1 N NaOH at 50°. Spectra were taken after the mixture was diluted with 0.1 M phosphate buffer (pH 7.0) or 0.1 N HCl. Key: —, for 0 min and then at pH 7.0; ----, for 0 min and then in 0.1 N HCl; ——, for 30 min and then at pH 7.0; and — —, for 30 min and then in 0.1 N HCl.*

Compound III consumed 2 moles of metaperiodate within 30 min and an additional 2 moles over 40 hr. A violet color was produced when III was treated with a 2% ferric chloride solution in water. Data from paper chromatographic, paper electrophoretic, and UV absorption analyses are presented in Table I.

Anal.—Calc. for $C_9H_{11}N_3O_5$: C, 44.81; H, 4.59: N, 17.42. Found: C, 44.68; H, 4.58; N, 17.46.

Alkaline Hydrolysis of III: Spectrophotometric Estimation—Compound III (10.0 mg) was dissolved in 2.0 ml of 0.1 *N* NaOH and incubated at 22 or 50° for 6 hr. An aliquot (50 μl) was diluted with 25 ml of 0.1 *N* HCl or 0.1 *M* phosphate buffer (pH 7.0), and the spectra were recorded. The spectra indicated that III was completely degraded to 5-hydroxy-1-β-D-arabinofuranosylcytosine (IV) at 22° in 6 hr and at 50° in 30 min. Treatment of III in 0.1 *N* NaOH at 50° for an additional 150 min did not show any changes in the spectrum. About 50% of III was transformed into IV at 22° in 65 min, estimated by the absorbance at 320 nm in 0.1 *N* HCl. Representative profiles are given in Fig. 5.

Attempts to Isolate the Hydrolyzed Product (IV)—Compound III (700 mg, 2.9 mmoles) was dissolved in 30 ml of concentrated ammonia and heated at 100° for 5 hr in a sealed tube. Paper chromatography (Solvents 1, 2, and 4) of the mixture revealed a new fluorescent spot detected by UV radiation. The aqueous extract of the spot showed absorption maxima at 304 and 215 (pH 1), 292 and 218 (pH 7), and 321 (pH 13) nm, very close to the maximum (292 nm at pH 7) of the known 5-hydroxycytidine (14). The spot gave a violet color with a 2% ferric chloride solution in water and was not colored by Ehrlich's reagent. The reaction mixture was evaporated *in vacuo* to dryness, and the residue was purified by passage through a cellulose column (1.7 × 50 cm) with Solvent 2 to afford a colorless gum of IV (Table I). All attempts to obtain a crystalline sample of IV failed because it gradually decomposed and darkened on treatment with solvents at room temperature.

RESULTS AND DISCUSSION

During the studies on the chemical transformations of I, it was found that paper chromatography of a solution of I-HCl in hydrogen peroxide with an alkaline solvent system quantitatively converted I into compounds other than its hydrolyzed derivative, II. Thus, paper chromatography of a mixture of I-HCl in hydrogen peroxide developed with an alkaline solvent system revealed one UV-absorbing spot and another non-UV-absorbing spot (Fig. 1). The product (III) corresponding to the UV-absorbing spot differed

Table I—Paper Chromatography, Paper Electrophoresis, and UV Absorption Data of 2,2′-Anhydro-1-β-D-arabinofuranosylcytosine Derivatives

Compound	Paper chromatography (R_f) Solvent System 1	2	3	4	Paper Electrophoresis (Mobility), cm Buffer System A[a]	B	pH	UV Absorption λ_{max}, nm ($\epsilon \times 10^{-3}$)
I·HCl	—	0.08	0.67	0.30	−6.2	−13.5	1–7	263 (10.6), 231(9.5)
II	0.61	0.14	0.66	0.44	−6.2	−7.0	1	282(13.4)[b]
							7	271(9.7)
III	0.47	0.06	0.52	0.44	−6.2	−8.5	1	284 (9.98), 215 (12.2)
							7	316 (8.2), 258 (5.0), 218.5 (17.7)
							13	316 (8.2), 257 (5.0)[d]
IV[c]	0.38	0.15	—	0.37	—	−7.0	1	304 (10.2), 215 (12.2)
							7	292 (7.3), 218 (13.8)
							13	321 (8.0)

[a] Compounds such as cytidine having a *cis*-glycol function at the sugar moiety ran −3.0 cm to cathode. [b] Reference 13. [c] Data of paper chromatography and paper electrophoresis were obtained with the purified gum of IV, and the UV absorption spectrum was obtained by the quantitative transformation of III with 0.1 *N* NaOH at 50°. [d] Measured as soon as possible.

from I or II and had the absorption maxima at 258 and 316 nm (pH 7 and 13). The reaction on the paper chromatogram using the alkaline solvent system was not observed when a mixture of I in water in the absence of hydrogen peroxide or a mixture of II and the reagent was developed. Therefore, I was converted into III and other non-UV-absorbing substance(s) by hydrogen peroxide without prior hydrolysis to II.

To characterize the pH dependence of the reaction of I with hydrogen peroxide, I and II were treated with a 3.4 molar excess of 0.025 *M* hydrogen peroxide in buffer systems (pH 4–11) at room temperature for 18 hr (Fig. 2). Although the absorbances of the mixtures of II and hydrogen peroxide were not altered over the 4–11 pH range, those of the mixtures of I and hydrogen peroxide were altered extensively, depending on the pH of the buffer systems. Thus, the absorbances at 275 nm of the mixtures of I and the reagent were not altered below pH 6 but extensively decreased above pH 6. The absorbances at 315 nm of the mixtures increased over the 6–8 pH range and decreased at more alkaline pH values. Some of the full spectra of the reaction mixtures are shown in Fig. 3. Figures 2 and 3 indicate that I reacted with hydrogen peroxide in two fashions without prior hydrolysis to II. At around pH 8, I was mainly converted into III, having an absorption maximum at 316 nm (pH 13). At around pH 11, I was mainly converted into non-UV-absorbing substance(s).

A time course study of the reaction of I with hydrogen peroxide is presented in Fig. 4. At pH 5.0, little reaction occurred over 48 hr, and little hydrogen peroxide was consumed during the first 17 hr. At pH 7.9, both the decrease of the absorbance at 275 nm and the increase at 315 nm indicated that the formation of III gradually proceeded, reaching a maximum at 17 hr. About one-third of the 3.4 equimolar amount of hydrogen peroxide initially present in the mixture was consumed after 17 hr, indicating that I had reacted with about an equimolar amount of the reagent. The yield of III at pH 7.9 after 17 hr was 73%, calculated from the ε value of III. At pH 11.0, an extensive decrease in the absorbance at 275 nm occurred within 1 min, and the formation of compounds other than non-UV-absorbing substance(s) was not significant during the reaction.

Compound III was isolated in a yield of 65% from the reaction mixture of I treated with 0.025 *M* hydrogen peroxide in 1 *M* phosphate buffer (pH 7.9) overnight. The structure of III was determined by the following observations. High-resolution mass spectroscopy and elemental analysis showed the formula of III to be $C_9H_{11}N_3O_5$. In the NMR spectrum of III, the anomeric proton signal appeared at δ 6.54 ppm with a coupling constant of 6 Hz; the signal at δ 5.46 ppm with the same coupling constant can be assigned to the 2′-proton by the double resonance technique. Chemical shift of the 2′-proton signal of III appeared very close to that of the 2,2′-cyclized compound, I[5], and was downfield from that of the uncyclized compound such as II[6], indicating that the 2,2′-anhydro linkage was retained in the molecule of III. One of the two vinylic protons at the 5- and 6-positions of I had disappeared, and one proton signal appeared at δ 7.00 ppm as a singlet. Thus, III was either the 5-hydroxy or the 6-hydroxy derivative of I.

Introduction of a hydroxy function into the 5-position of pyrimidine nucleosides produced a bathochromic and hypochromic shift of UV absorption; that at the 6-position produced a hypsochromic and hyperchromic influence (14–19). Furthermore, 5-hydroxypyrimidine nucleosides give a positive reaction with a ferric chloride solution but not with an Ehrlich's reagent, whereas 6-hydroxypyrimidine nucleosides give a positive reaction with an Ehrlich's reagent but not with a ferric chloride solution (14–19). Evidence that III was the 5-hydroxy derivative of I was provided by the UV absorption spectrum of III compared with that of I (Table I) and the violet color produced with a ferric chloride solution. The structure of III was thus established as 2,2′-anhydro-5-hydroxy-1-β-D-arabinofuranosylcytosine (Scheme I).

Compound III was quite stable in 0.1 *n* HCl at 80° for 1 hr, but it was degraded in alkali. When III was treated with concentrated ammonia at 100° for 5 hr, the major product (IV) was obtained. Compound IV, not isolated in a crystalline form, may be 5-hydroxy-1-β-D-arabinofuranosylcytosine because its UV absorption spectrum closely resembles that of the known 5-hydroxycytidine (14) and gives a positive reaction with ferric chloride solution. In 0.1 *N* NaOH, complete hydrolysis of III to IV required 6 hr at 22° and 30 min at 50° (Fig. 5); 50% hydrolysis was complete after 65 min at 22°. The anhydro bond of III was more stable than that of I, which was quantitatively converted into II in 0.1 *N* NaOH at 22° within 5 min (20). While the introduction of a halogeno group into the 5-position of I destabilized the anhydro bond (20), the introduction of a hydroxyl function stabilized the bond towards alkali.

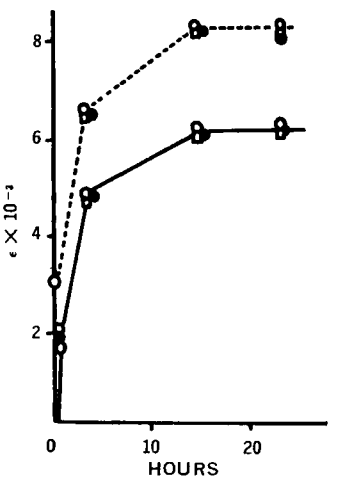
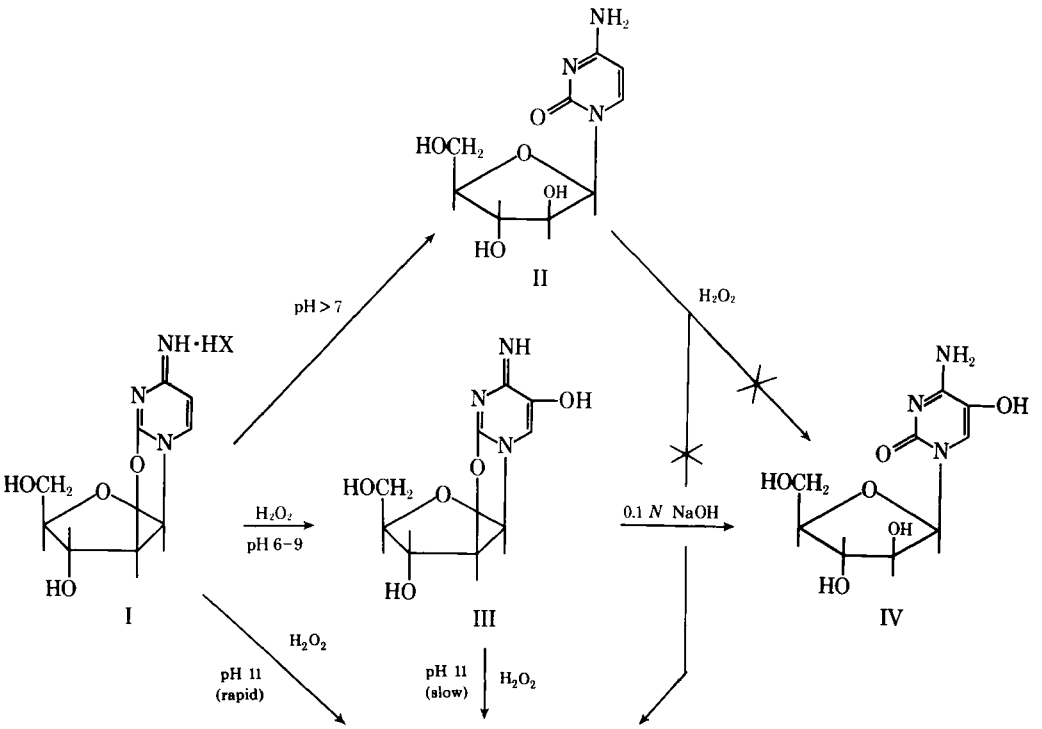


Figure 6—*Effect of ferric chloride and allyl alcohol on the reaction of I with hydrogen peroxide at pH 7.9. Compound I·HCl (7.6 × 10⁻⁴ M) was treated with 0.025 M hydrogen peroxide in 0.1 M phosphate buffer (pH 7.9) at room temperature. Absorbance at 315 nm when diluted with 0.1 N NaOH and that at 285 nm when diluted with 0.1 N HCl were measured. Key: —, 315 nm; ----, 285 nm; ○, with none; ●, with 5 × 10⁻⁵ M ferric chloride; and □, with 5 × 10⁻² M allyl alcohol.*

[5] δ 5.63 ppm (doublet, $H_{2'}$, $J_{1',2'}$ = 6 Hz).
[6] δ 4.40 ppm (triplet, $H_{2'}$, $J_{1',2'}$ = 5 Hz).



Scheme I

Formation of non-UV-absorbing substance(s) by reaction of I with hydrogen peroxide at around pH 11 was first considered to proceed *via* rapid formation and degradation of III. Thus, the reaction of III with the reagent at pH 11.0 was investigated. Although the decrease of the absorbance at 275 nm of I treated with the reagent was spontaneous and extensive (Fig. 4), that of III treated with the reagent at pH 11.0 was gradual over 20 hr, reaching about 70% of the initial one. At pH 11, the speed of transformation of III into non-UV-absorbing substance(s) was slower than that of I. Therefore, the reaction of I with hydrogen peroxide at pH 11 occurred directly without prior formation of III.

Since hydrogen peroxide is produced by redox reactions in all living cells, it may be responsible for alterations of I. The reaction of I with the reagent near neutral pH was of special interest. Reaction of I with hydrogen peroxide at pH 7.9 was not influenced by a metal ion, such as ferric chloride, or a radical trap, such as allyl alcohol, over 24 hr, indicating that the reaction of I with the reagent to form III was not due to a radical reaction (Fig. 6). In addition to II, other pyrimidine nucleosides such as cytidine, uridine, and 2,2′-anhydro-1-β-D-arabinofuranosyluracil did not suffer any significant reaction with the reagent at pH 7.9. Thus, hydroxylation of the 5-position of I with hydrogen peroxide was a reaction characteristic to the structure of I.

The effects of hydrogen peroxide on nucleic acids and their components have been investigated (21–24). The reagent with metal ions destroys the deoxyribonucleic acid molecule, causing an extensive decrease in UV absorption and melting temperature (21, 24) and liberating all four bases (21, 22). Some of the pyrimidine bases and the purine bases have been converted into non-UV-absorbing substances (22) and 2-, 7-, or 8-hydroxyadenine in the case of adenine base (23). These effects of hydrogen peroxide on nucleic acids and their components have been generally regarded as the results of radical-forming processes. The results obtained by Subbaraman *et al.* (25) on the reaction of uracil and thymine bases with alkaline hydrogen peroxide were accounted for by a mechanism in which the neutral substrate was attacked by the anion of hydrogen peroxide to form products that led to ring cleavage. The reaction of I with the reagent described here under the neutral and slightly basic conditions (pH 6–9) may be due to other mechanisms than radical-forming processes.

Compound III was studied by Hoshi *et al.* (26) as an antineoplastic agent and was found to be inactive.

SUMMARY

1. The reaction of I with a 3.4 molar excess of hydrogen peroxide (0.025 *M*) at pH 7.9 and at room temperature for 17 hr afforded III in a good yield.

2. The formation of III from I by reaction with the reagent occurred over the 6–9 pH range.

3. The reaction of I with the reagent under the neutral conditions could not be interpreted by radical-forming processes.

4. The anhydro bond of III was somewhat more stable toward alkali than that of I.

5. An extensive loss of UV absorption was observed within 1 min in the reaction of I with the reagent at pH 11, which did not proceed through II or III.

6. Compound II did not suffer significant reaction with the reagent over the 4–11 pH range.

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Quantitation of Propoxyphene and Its Major Metabolites in Heroin Addict Plasma after Large Dose Administration of Propoxyphene Napsylate

J. FRANK NASH ˣ, I. F. BENNETT, R. J. BOPP, M. K. BRUNSON, and H. R. SULLIVAN

Abstract □ A sensitive and specific GLC assay method was developed for the determination of propoxyphene, its major metabolite norpropoxyphene, and lesser known metabolites cyclic dinorpropoxyphene and/or dinorpropoxyphene in plasma of heroin addicts administered up to 800 mg of propoxyphene napsylate. The assay used a mass internal standard of pyrroliphene. The compounds were extracted from pH 9.8 carbonate-buffered plasma with butyl chloride, back-extracted into acidified water which was then washed with hexane, and reextracted with chloroform from the aqueous phase made basic. Quantitation of the drug and its metabolites was accomplished by temperature-programmed GLC. Absolute identification of the compounds chromatographed was completed by GLC–mass spectrometry.

Keyphrases □ Propoxyphene and metabolites—GLC analysis in heroin addict plasma □ Norpropoxyphene and cyclic dinorpropoxyphene—GLC analysis in heroin addict plasma after propoxyphene napsylate administration □ Dinorpropoxyphene (cyclic) and norpropoxyphene—GLC analysis in heroin addict plasma after propoxyphene napsylate administration □ GLC–mass spectrometry—analysis, propoxyphene and metabolites in heroin addict plasma

Propoxyphene hydrochloride[1] has been prescribed widely for the symptomatic relief of mild or moderate pain. Metabolic studies were conducted using radiocarbon-labeled propoxyphene (1) and with deuterium-labeled drug and GLC–mass spectrometry (2).

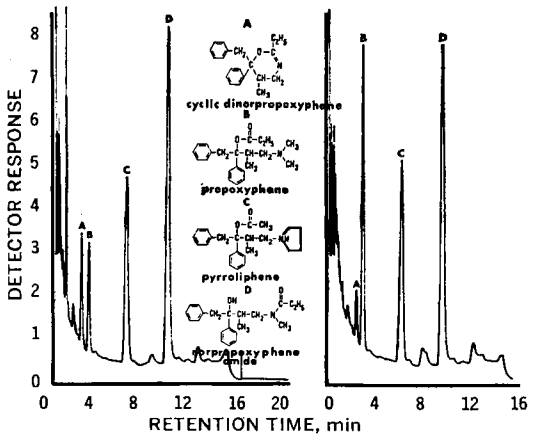

Figure 1—*Gas-liquid chromatogram (flame-ionization detector) of human plasma extracts. Left: blank plasma spiked with: Compound A, 100 ng/ml; Compound B, 100 ng/ml; Compound C, 250 ng/ml; and Compound D, 500 ng/ml. Right: patient's plasma after dose of 200 mg propoxyphene napsylate.*

The 2-naphthalenesulfonate[2], another salt of propoxyphene, has been marketed as a therapeutic agent equally as effective as the hydrochloride (3–6). The use of propoxyphene napsylate in large doses for the treatment of heroin addicts has been reported (7, 8), as has a GLC method for the determination of propoxyphene in plasma (9).

[1] α-*d*-4-(Dimethylamino)-3-methyl-1,2-diphenyl-2-propionoxybutane hydrochloride; Darvon, Eli Lilly and Co.

[2] α-*d*-4-(Dimethylamino)-3-methyl-1,2-diphenyl-2-propionoxybutane 2-naphthalenesulfonate; Darvon-N, Eli Lilly and Co.

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Quantitation of Propoxyphene and Its Major Metabolites in Heroin Addict Plasma after Large Dose Administration of Propoxyphene Napsylate

J. FRANK NASH ˣ, I. F. BENNETT, R. J. BOPP, M. K. BRUNSON, and H. R. SULLIVAN

Abstract □ A sensitive and specific GLC assay method was developed for the determination of propoxyphene, its major metabolite norpropoxyphene, and lesser known metabolites cyclic dinorpropoxyphene and/or dinorpropoxyphene in plasma of heroin addicts administered up to 800 mg of propoxyphene napsylate. The assay used a mass internal standard of pyrroliphene. The compounds were extracted from pH 9.8 carbonate-buffered plasma with butyl chloride, back-extracted into acidified water which was then washed with hexane, and reextracted with chloroform from the aqueous phase made basic. Quantitation of the drug and its metabolites was accomplished by temperature-programmed GLC. Absolute identification of the compounds chromatographed was completed by GLC–mass spectrometry.

Keyphrases □ Propoxyphene and metabolites—GLC analysis in heroin addict plasma □ Norpropoxyphene and cyclic dinorpropoxyphene—GLC analysis in heroin addict plasma after propoxyphene napsylate administration □ Dinorpropoxyphene (cyclic) and norpropoxyphene—GLC analysis in heroin addict plasma after propoxyphene napsylate administration □ GLC–mass spectrometry—analysis, propoxyphene and metabolites in heroin addict plasma

Propoxyphene hydrochloride[1] has been prescribed widely for the symptomatic relief of mild or moderate pain. Metabolic studies were conducted using radiocarbon-labeled propoxyphene (1) and with deuterium-labeled drug and GLC–mass spectrometry (2).

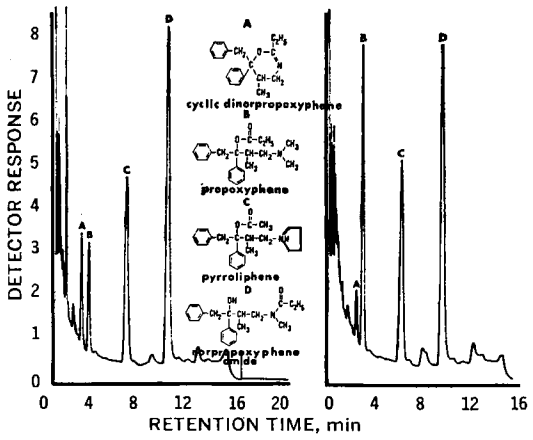


Figure 1—*Gas-liquid chromatogram (flame-ionization detector) of human plasma extracts. Left: blank plasma spiked with: Compound A, 100 ng/ml; Compound B, 100 ng/ml; Compound C, 250 ng/ml; and Compound D, 500 ng/ml. Right: patient's plasma after dose of 200 mg propoxyphene napsylate.*

The 2-naphthalenesulfonate[2], another salt of propoxyphene, has been marketed as a therapeutic agent equally as effective as the hydrochloride (3–6). The use of propoxyphene napsylate in large doses for the treatment of heroin addicts has been reported (7, 8), as has a GLC method for the determination of propoxyphene in plasma (9).

[1] α-*d*-4-(Dimethylamino)-3-methyl-1,2-diphenyl-2-propionoxybutane hydrochloride; Darvon, Eli Lilly and Co.

[2] α-*d*-4-(Dimethylamino)-3-methyl-1,2-diphenyl-2-propionoxybutane 2-naphthalenesulfonate; Darvon-N, Eli Lilly and Co.

Table I—Precision and Accuracy in Measurement of Cyclic Dinorpropoxyphene, Propoxyphene, and Norpropoxyphene added to Human Plasma

	Cyclic Dinorpropoxyphene		Propoxyphene		Norpropoxyphene	
	25 ng/ml	100 ng/ml	25 ng/ml	100 ng/ml	125 ng/ml	500 ng/ml
Mean[a]	23 (21–25)	101 (87–116)	20 (19–22)	91 (82–97)	118 (104–149)	524 (455–603)
RSD	15.9%	11.5%	6.4%	6.1%	7.7%	10.3%
RE	−8.0%	+1.0%	−20.0%	−9.0%	−6.0%	+5.0%

[a] Mean of five replicate samples (range).

Table II—Plasma Concentrations of Cyclic Dinorpropoxyphene[a], Propoxyphene, and Norpropoxyphene from Patients Administered Large Doses of Propoxyphene Napsylate

Patient	Days	Dose, Tablets of Propoxyphene Napsylate, mg	Day of Plasma Sample	Concentration of Compounds in Plasma, ng/ml		
				Cyclic Dinorpropoxyphene	Propoxyphene	Norpropoxyphene
1	1–4	800	1	0	0	0
	5–8	600	5	49	110	1194
	9–13	400	9	38	80	869
			12	28	61	627
	14–19	200	19	16	10	67
	20–24	0	24	0	11	24
2	1–4	800	1	0	0	0
	5–8	600	5	51	167	770
	9–13	400	9	45	111	503
	14–19	200	17	22	58	162
			19	21	29	96
	20–24	0	24	0	0	0
3	1–4	800	1	0	0	0
	5–8	600	5	55	228	853
	9–13	400	9	49	79	690
			13	27	33	207
	14–19	200	17	16	0	37
	20–24	0	None	—	—	—
4	1–4	800	1	0	0	0
	5–8	600	5	40	197	910
	9–13	400	9	43	161	983
			12	39	64	669
	14–19	200	17	27	12	174
	20–24	0	None	—	—	—
5	1–4	800	1	0	0	0
	5–8	600	5	107	207	1227
	9–13	400	9	61	54	550
			12	34	36	245
	14–19	200	None	—	—	—
	20–24	0	None	—	—	—

[a] The concentrations reported as cyclic dinorpropoxyphene may represent cyclic dinorpropoxyphene and/or dinorpropoxyphene.

This report presents an assay for propoxyphene and its metabolites, norpropoxyphene[3] and cyclic dinorpropoxyphene[4], in plasma from patients and gives the resultant data. With appropriate modifications, this procedure also has been used for the routine assay of propoxyphene and norpropoxyphene in plasma from subjects receiving therapeutic doses of propoxyphene hydrochloride and propoxyphene napsylate.

EXPERIMENTAL

Reagents—*α-dl*-Cyclic dinorpropoxyphene perchlorate, *α-d*-propoxyphene hydrochloride, *α-d*-norpropoxyphene citrate, and *α-d*-pyrroliphene hydrochloride[5] were synthesized[6]. The last compound was used as the mass internal standard for GLC analysis. All solvents used were analytical reagent quality except chloroform and butyl chloride, which were distilled in glass[7]. GLC packing material, 2% OV-7 on Chromosorb W (HP) (80–100 mesh)[8], and the silylating reagent dimethyldichlorosilane[9] were purchased.

Apparatus—A gas–liquid chromatograph[10] equipped with a flame-ionization detector and 1-mv recorder[11] was used. Teflon-lined screw-caps and disposable 15-ml centrifuge tubes[12] were used throughout the extraction procedure. Tapered 15-ml glass centrifuge tubes[13] previously siliconized were used for evaporation[14] of solvent.

Chromatographic Conditions—The recorder was operated at a chart speed of 0.63 cm/min. Helium carrier gas (flow rate of 60 ml/min) was filtered through 1.83-m × 1.27-cm (6-ft × 0.5-in.) copper tubing filled with pellets of molecular sieve 5A. The filter was preconditioned overnight at 200° with dry nitrogen as the conditioning gas. A glass column[8], 0.61 m (2 ft) × 3 mm i.d., was operated isothermally at 185° for 4 min followed by 4°/min programming to 230° during analyses.

[3] *α-d*-1,2-Diphenyl-3-methyl-4-methylamino-2-propionoxybutane.

[4] *α-dl*-6-Benzyl-2-ethyl-5,6-dihydro-5-methyl-6-phenyl-4*H*-1,3-oxazine. Its chemistry will be discussed in a future publication by H. R. Sullivan.

[5] *α-d*-4-Pyrrolidino-1,2-diphenyl-3-methyl-2-acetoxybutane hydrochloride.

[6] At the Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, Ind.

[7] Burdick and Jackson Laboratories, Muskegon, Mich.

[8] Ohio Valley Specialty Chemical, Inc., Marietta, Ohio.

[9] Pierce Chemical Co., Rockford, Ill.

[10] Model 5700A, Hewlett Packard, Avondale, Pa.

[11] Model 7127A, Hewlett Packard, Avondale, Pa.

[12] Corning Glass Co., Corning, N.Y.

[13] Matheson Scientific Co., Chicago, Ill.

[14] Model 106 N-Evap, Organomation Associates, Shrewsbury, Mass.

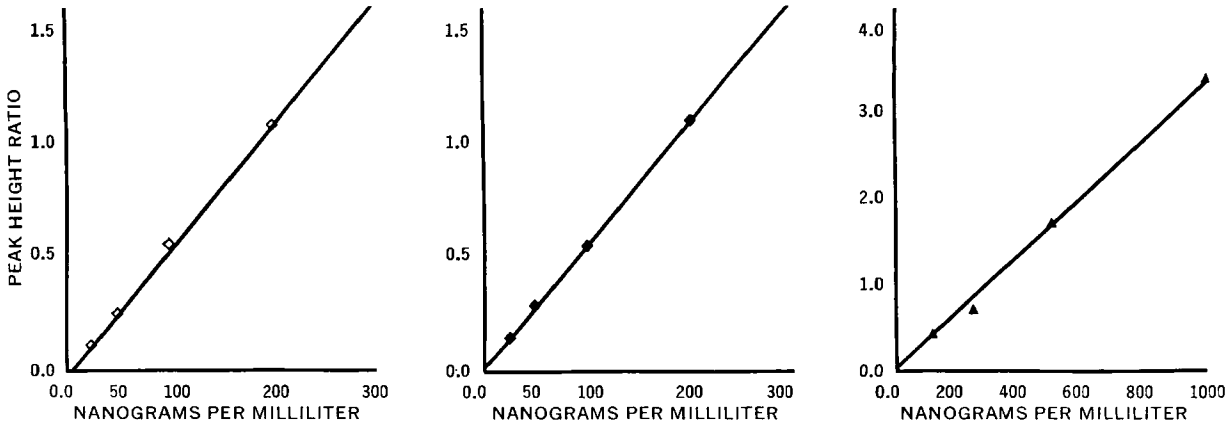


Figure 2—*Calibration curves; relationship between peak height ratio of the compound to the internal standard (pyrroliphene) and the concentration of compound in plasma. Left: cyclic dinorpropoxyphene. Center: propoxyphene. Right: norpropoxyphene.*

Newly packed columns were conditioned by turning the carrier gas on for 2–5 min, heating the column to 300°, and holding without carrier flow for 1 hr. The column was cooled to room temperature, reheated to 250°, and conditioned overnight with carrier gas flowing. The detector temperature was 250°. The oxygen and hydrogen flow rates were 240 and 60 ml/min, respectively.

Under these conditions, the following retention times were observed[15]: cyclic dinorpropoxyphene, 145 sec; propoxyphene, 175 sec; pyrroliphene, 370 sec; and norpropoxyphene amide (rearranged norpropoxyphene formed under basic conditions), 600 sec[16] (Fig. 1). At an electrometer sensitivity of 7.8×10^{-10} A, 1 μg of propoxyphene produced a full-scale deflection.

Procedure—The 15-ml tapered centrifuge tubes were washed with chromic acid cleaning solution, rinsed with water, dried, and siliconized using a 1% solution of dimethyldichlorosilane in toluene[17]. Heparinized blood was centrifuged to separate the plasma[18]. Standard solutions of cyclic dinorpropoxyphene (as the perchlorate), propoxyphene (as the hydrochloride), and norpropoxyphene (as the citrate) were added to 4 ml of blank plasma to give calibration concentrations equivalent to 0, 25, 50, 100, and 125 ng of propoxyphene and cyclic dinorpropoxyphene/ml of plasma and 0, 125, 250, 500, and 1000 ng of norpropoxyphene/ml of plasma.

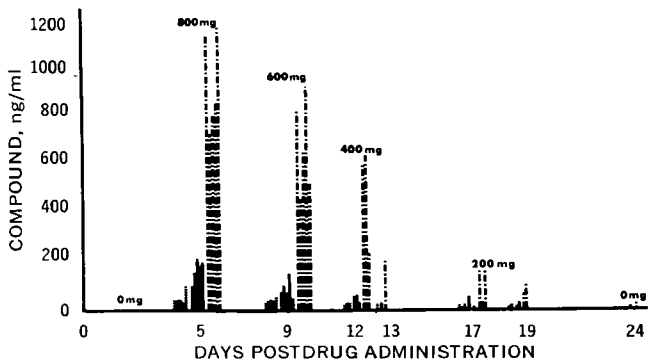


Figure 3—*Plasma concentrations of propoxyphene (—) and metabolites cyclic dinorpropoxyphene (.....) and norpropoxyphene (- - -) after administration of decreasing doses of propoxyphene napsylate to five heroin addicts.*

To each tube were added 0.5 ml of a 2-μg/ml solution of the mass internal standard and 0.5 ml of pH 9.8 carbonate buffer. The mixture was vortexed to ensure uniform distribution of the compounds in the plasma. Using a pipet[19], 10 ml of butyl chloride was added to each tube and the samples were shaken for 2 min and then centrifuged at 2000 rpm for 4 min. The upper organic phase was transferred to a second screw-capped centrifuge tube, and care was taken not to transfer any plasma. Five milliliters of 0.2 *N* hydrochloric acid was added to the samples, which were shaken for 2 min and centrifuged for 4 min. The organic phase was aspirated and discarded. To the aqueous acid was added 5 ml of hexane. The samples were shaken and centrifuged and the hexane was aspirated and discarded. Then 0.6 ml of 2 *N* sodium hydroxide was added with a 1-ml pipet[19] and the samples were vortexed.

After ensuring the pH was 10[20], 10 ml of chloroform was added and the samples were shaken and centrifuged. The upper aqueous phase was aspirated and discarded and the chloroform phase was transferred to a 15-ml siliconized, tapered centrifuge tube. Care was taken not to transfer any of the aqueous phase. The chloroform was evaporated to dryness at 37° under a gentle stream of nitrogen. To the residue was added 20 μl of carbon disulfide–chloroform (1:1) with a 20-μl pipet[21]. The samples were vortexed for 20–30 sec, and 3 μl of each sample solution was injected onto the GLC column.

Plasma from heroin addicts administered propoxyphene napsy-

[15] Methadone chromatographed at 155 sec and could be extracted and quantitated by this procedure.

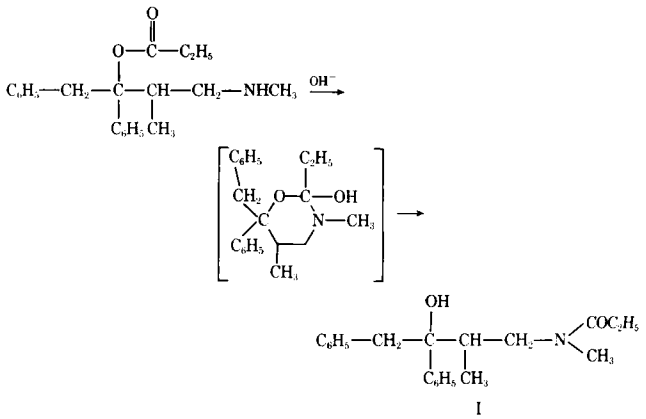
[16] Under the chromatographic conditions described, dinorpropoxyphene amide is not separated from norpropoxyphene amide and any of the former present in the plasma extracts would have been quantitated as norpropoxyphene. If dinorpropoxyphene is present, it will cyclize to an intermediate which dehydrates on column to yield cyclic dinorpropoxyphene. If the extracts are held at pH 12 for 1 hr, the dinorpropoxyphene is essentially all rearranged to dinorpropoxyphene amide. Evidence that cyclic dinorpropoxyphene is a true metabolite of propoxyphene found in plasma is supported by dog data to be presented by Mr. H. R. Sullivan in a forthcoming paper.

[17] This treatment appears to prevent adsorption of the propoxyphene compounds to the glassware.

[18] Fresh plasma or frozen plasma held at −8° for several weeks has been assayed successfully. Alternate freezing and thawing of plasma caused erratic results for propoxyphene and its metabolites.

[19] Repipet, Labindustries, Berkeley, Calif.

[20] Norpropoxyphene, as the secondary amine, could not be quantitated by the GLC procedure. Therefore, it was necessary to treat the samples with strong base, such as an alkali hydroxide, to convert the norpropoxyphene by intramolecular rearrangement through the O to N shift of the propionyl group to the very stable *N*-methyl *N*-propionyl-1,2-diphenyl-3-methyl-2-hydroxy-4-butylamine (I). The amide is readily quantitated by gas chromatography.



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[21] Precision Pipet, Medical Laboratory Automation, Inc., Mt. Vernon, N.Y.

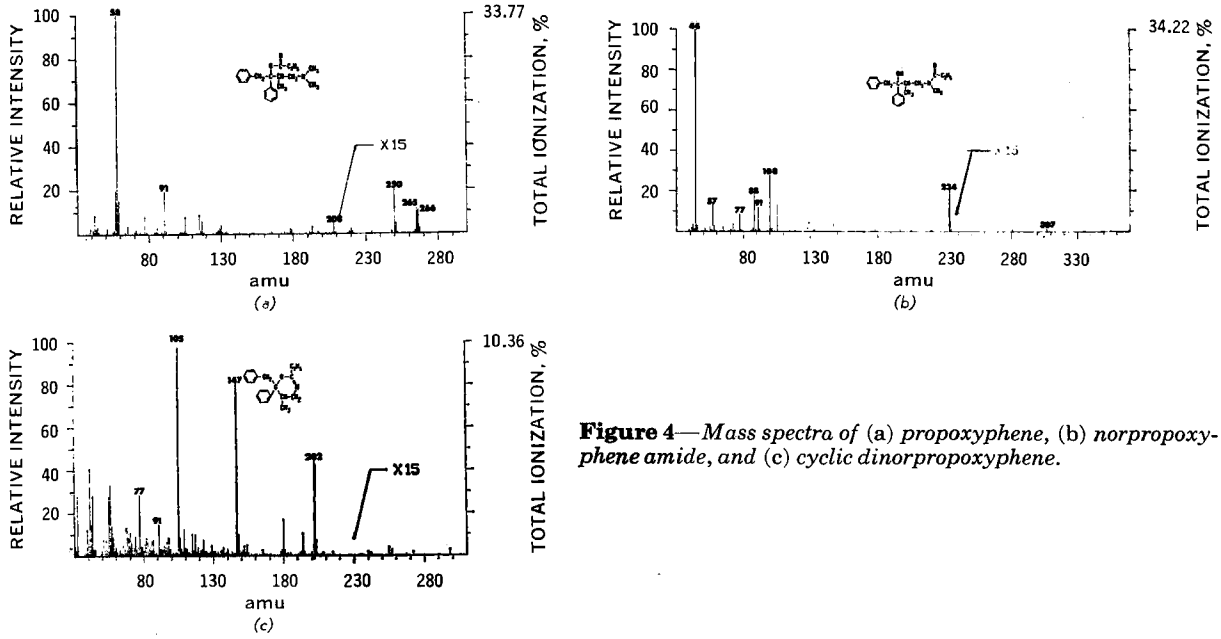

Figure 4—*Mass spectra of* (a) *propoxyphene,* (b) *norpropoxyphene amide, and* (c) *cyclic dinorpropoxyphene.*

late were analyzed similarly after addition of 0.5 ml of the mass internal standard solution.

Following chromatography of the calibration standards, peak height ratios[22] of the propoxyphene compounds to the mass internal standard were calculated. Calibration curves (Fig. 2) were prepared by plotting the peak height ratios *versus* concentrations of drug or metabolites (nanograms per milliliter). Concentrations of cyclic dinorpropoxyphene, propoxyphene, and norpropoxyphene in the sample plasma were calculated from the calibration curves.

Mass spectra of cyclic dinorpropoxyphene, propoxyphene, and norpropoxyphene amide present in the patient's plasma sample extracts were obtained using a gas chromatograph–mass spectrometer[23]. The components present in the extracts were separated using a 1.22-m (4-ft) siliconized glass column of 2.5 mm i.d. packed with 1% W-98 methylvinyl silicone gum rubber[8] on 80–100-mesh Gas Chrom Q, maintained at 160°. The carrier gas, helium, flow was 40 ml/min. The ionizing potential and trap current were 70 ev and 60 μamp, respectively. The temperature of the flash heater was 200°, and the ion source temperature was 290°. Under these conditions, the GLC retention times of cyclic dinorpropoxyphene, propoxyphene, and norpropoxyphene amide were 120, 180, and 780 sec, respectively.

RESULTS AND DISCUSSION

To determine the precision and accuracy of the assay, plasma samples were prepared containing known amounts of cyclic dinorpropoxyphene (as perchlorate), propoxyphene (as hydrochloride), and norpropoxyphene (as citrate) and were assayed by the described procedure (Table I).

Plasma samples were obtained[24] from patients undergoing heroin detoxification with propoxyphene napsylate. Table II reports the plasma concentrations found for propoxyphene and its metabolites. The blood sample was taken before each day's dose.

The histogram in Fig. 3 shows the decrease of propoxyphene and its two major metabolites in patients' plasma as the dose of propoxyphene napsylate is reduced. It is apparent that even when patients are administered large doses of propoxyphene over several weeks, neither the parent compound nor its metabolites accumulate in the blood.

Using the chromatographic conditions previously described for the analysis of plasma samples, methadone was found to elute within 10 sec of cyclic dinorpropoxyphene (see Footnote 15). Positive identification of the cyclic metabolite of propoxyphene in plasma was accomplished by GLC–mass spectrometry. Methadone was identified in a similar manner in plasma samples obtained from patients who were administered methadone. Because of these close retention times, investigators must be careful of false positives for methadone with patients being detoxified with propoxyphene. Separation of the cyclic dinorpropoxyphene and methadone by GLC is accomplished with the 1% W-98 column (120 *versus* 216 sec, respectively).

The mass spectra by which cyclic dinorpropoxyphene, propoxyphene, and norpropoxyphene amide were unambiguously identified are shown in Fig. 4. When the mass spectra of the compounds extracted from patients' plasma were compared with those of the authentic compounds, the fragmentation characteristics were identical.

The mass fragmentation pattern of propoxyphene (Fig. 4*a*) does not show an M^+ at 339 amu[25]. The M − 73 fragment seen at 266 amu represents the loss of the propionyloxy group. The fragment of 265 amu represents the loss of propionic acid (M − 74) from the molecular ion; the additional proton has been shown by deuteration to arise from the benzyl methylene group. The additional fragments observed are the loss of propionic acid plus a methyl group at 250 amu, M − (74 + 15), and the loss of the propionyloxy and dimethylaminomethyl moieties, M − (73 + 58) at 208 amu. The base peak in the spectrum is that of the dimethylaminomethyl ion of 58 amu. The ion at 91 amu represents the benzyl group and is a fragment common to propoxyphene and all of its metabolites.

The mass spectrum obtained from the alkaline rearranged norpropoxyphene (norpropoxyphene amide) is shown in Fig. 4*b*. Again no molecular ion at 325 amu is observed. A small ion at 307 amu is observed and represents the loss of water from the parent ion. The fragment at 234 amu (M − 91) arises by the loss of the benzyl group from the parent ion. The base peak in the spectrum is at 44 amu and represents the methylaminomethyl group. Other fragments observed are those of the propionyl group, 57 amu; the phenyl group, 77 amu; the benzyl group, 91 amu; the *N*-propionylmethylamino group, 100 amu; and the *N*-propionylmethylammonium ion, 88 amu.

Figure 4*c* shows the mass spectrum of cyclic dinorpropoxyphene. Again, no molecular ion is detected. The M − 91 fragment seen at 202 amu results from the loss of the benzyl group from the molecular ion. The major ion at 147 amu reflects the combined loss of the benzyl and the C_2H_5— C^+=N moieties, M − (91 + 55). The most abundant ion is at 105 amu and represents the C_6H_5—C^+=O

[22] Results were virtually unaffected when relative peak areas were used instead of relative peak heights.

[23] Model 9000, LKB-Produkter, Brommo, Sweden.

[24] Dr. F. S. Tennant, Jr.

[25] By use of chemical ionization mass spectrometry, the molecular ion was obtained by Mr. J. L. Occolowitz, The Lilly Research Laboratories.

ion. Ions at 77 and 91 amu represent the phenyl and benzyl groups, respectively.

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Mr. R. N. Booher and Mr. W. W. Turner, Chemical Research, Lilly Laboratories, synthesized the norpropoxyphene citrate and cyclic dinorpropoxyphene perchlorate. Plasma samples for this study were supplied by Dr. Forest S. Tennant, Jr., University of California at Los Angeles. The authors also thank Mr. W. E. Legan for his analysis of the plasma samples.

[x] To whom inquiries should be directed.

Intersubject Variation in Absorption of Digoxin in Normal Volunteers

DAVID H. HUFFMAN [*x], CARL V. MANION [‡§], and DANIEL L. AZARNOFF [‡]

Abstract □ The absorption of oral digoxin preparations was evaluated following single-dose administration of 0.5 mg of digoxin to 16 normal volunteers in a randomized crossover design. Absorption was estimated using the cumulative excretion of digoxin in urine for 7 days and the area under the 24-hr serum digoxin concentration curve (*AUC*). Significant intersubject variability was observed with both parameters, but this variability was greater for the *AUC*. After intravenous administration, the 7-day digoxin excretion was 68% of the dose. The elixir and a rapid dissolution tablet were significantly better absorbed (84.5 and 77.8%, respectively) than was a slow dissolution tablet (66.7%), as reflected by the fraction of the amount excreted in the urine following intravenous administration of the same dose. There was a highly significant correlation between the cumulative digoxin excretion in urine during the first 2 days compared to 7 days ($r = +0.972$, $p < 0.001$). Bioavailability of oral digoxin preparations can be reliably determined by comparison of the cumulative 2-day excretion of digoxin following a single dose.

Keyphrases □ Digoxin oral preparations—bioavailability in humans, intersubject variations □ Bioavailability of digoxin oral preparations—estimated using cumulative excretion in urine and area under serum concentration curve, intersubject variations □ Absorption of digoxin oral preparations—intersubject variations

The bioavailability of oral digoxin preparations has been studied using various protocols. Clinically important differences in bioavailability have been demonstrated among tablet preparations (1–5). Single-dose studies (1, 4–6) and steady-state studies (7) have used the peak serum concentration, the area under the serum concentration *versus* time curve (*AUC*), and the cumulative excretion of digoxin in the urine to estimate the absorption of oral digoxin preparations. Since there has been no complete agreement on the standard method for estimating digoxin bioavailability, and because a larger study was needed to determine the degree of intersubject variability, the bioavailability of digoxin from three oral preparations relative to an equivalent intravenous dose was determined in 16 normal volunteers in a randomized crossover design.

EXPERIMENTAL

Sixteen healthy hospital employees, 10 females and six males (ages 21–33), volunteered for the study. Written consent was obtained after discussing with each subject the inconveniences and hazards reasonably to be expected. All subjects had normal history and physical examinations and no evidence of cardiac, hepatic, renal, or GI disease.

After an overnight fast, 0.5 mg of digoxin was administered to each volunteer on four occasions, each 2 weeks apart. This interval allowed for the essentially complete elimination of digoxin given in the previous study. The following dosage forms were administered to each volunteer in a random sequence:

1. Two 0.25-mg digoxin tablets (I)[1,2]
2. Two 0.25-mg digoxin tablets (II)[3]
3. Ten milliliters (0.05 mg/ml) of a digoxin elixir (III)[4]
4. Two milliliters (0.25 mg/ml) of parenteral digoxin (IV)[5]

The dissolution rates of the tablets were determined by the method described by Lindenbaum *et al.* (8).

The digoxin tablets and elixir were given orally with 200 ml of water. The parenteral solution was administered intravenously over 5 min. Regardless of the route of administration, the subjects

[1] Lanoxin, Lot 474-G, Burroughs Wellcome.

[2] The dissolution of the tablets of the two Lanoxin lots resulted in 85% digoxin dissolved in 1 hr for the 474-G tablet and 65% for the 991-F tablet (R. Cresswell, personal communication). These tablets were taken from regular production runs.

[3] Lanoxin, Lot 991-F, Burroughs Wellcome.

[4] Lanoxin, Lot 816-G, Burroughs Wellcome.

[5] Lanoxin, Lot 062-F, Burroughs Wellcome.

ion. Ions at 77 and 91 amu represent the phenyl and benzyl groups, respectively.

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Intersubject Variation in Absorption of Digoxin in Normal Volunteers

DAVID H. HUFFMAN [*x], CARL V. MANION [‡§], and DANIEL L. AZARNOFF [‡]

Abstract □ The absorption of oral digoxin preparations was evaluated following single-dose administration of 0.5 mg of digoxin to 16 normal volunteers in a randomized crossover design. Absorption was estimated using the cumulative excretion of digoxin in urine for 7 days and the area under the 24-hr serum digoxin concentration curve (*AUC*). Significant intersubject variability was observed with both parameters, but this variability was greater for the *AUC*. After intravenous administration, the 7-day digoxin excretion was 68% of the dose. The elixir and a rapid dissolution tablet were significantly better absorbed (84.5 and 77.8%, respectively) than was a slow dissolution tablet (66.7%), as reflected by the fraction of the amount excreted in the urine following intravenous administration of the same dose. There was a highly significant correlation between the cumulative digoxin excretion in urine during the first 2 days compared to 7 days ($r = +0.972$, $p < 0.001$). Bioavailability of oral digoxin preparations can be reliably determined by comparison of the cumulative 2-day excretion of digoxin following a single dose.

Keyphrases □ Digoxin oral preparations—bioavailability in humans, intersubject variations □ Bioavailability of digoxin oral preparations—estimated using cumulative excretion in urine and area under serum concentration curve, intersubject variations □ Absorption of digoxin oral preparations—intersubject variations

The bioavailability of oral digoxin preparations has been studied using various protocols. Clinically important differences in bioavailability have been demonstrated among tablet preparations (1–5). Single-dose studies (1, 4–6) and steady-state studies (7) have used the peak serum concentration, the area under the serum concentration *versus* time curve (*AUC*), and the cumulative excretion of digoxin in the urine to estimate the absorption of oral digoxin preparations. Since there has been no complete agreement on the standard method for estimating digoxin bioavailability, and because a larger study was needed to determine the degree of intersubject variability, the bioavailability of digoxin from three oral preparations relative to an equivalent intravenous dose was determined in 16 normal volunteers in a randomized crossover design.

EXPERIMENTAL

Sixteen healthy hospital employees, 10 females and six males (ages 21–33), volunteered for the study. Written consent was obtained after discussing with each subject the inconveniences and hazards reasonably to be expected. All subjects had normal history and physical examinations and no evidence of cardiac, hepatic, renal, or GI disease.

After an overnight fast, 0.5 mg of digoxin was administered to each volunteer on four occasions, each 2 weeks apart. This interval allowed for the essentially complete elimination of digoxin given in the previous study. The following dosage forms were administered to each volunteer in a random sequence:

1. Two 0.25-mg digoxin tablets (I)[1,2]
2. Two 0.25-mg digoxin tablets (II)[3]
3. Ten milliliters (0.05 mg/ml) of a digoxin elixir (III)[4]
4. Two milliliters (0.25 mg/ml) of parenteral digoxin (IV)[5]

The dissolution rates of the tablets were determined by the method described by Lindenbaum *et al.* (8).

The digoxin tablets and elixir were given orally with 200 ml of water. The parenteral solution was administered intravenously over 5 min. Regardless of the route of administration, the subjects

[1] Lanoxin, Lot 474-G, Burroughs Wellcome.

[2] The dissolution of the tablets of the two Lanoxin lots resulted in 85% digoxin dissolved in 1 hr for the 474-G tablet and 65% for the 991-F tablet (R. Cresswell, personal communication). These tablets were taken from regular production runs.

[3] Lanoxin, Lot 991-F, Burroughs Wellcome.

[4] Lanoxin, Lot 816-G, Burroughs Wellcome.

[5] Lanoxin, Lot 062-F, Burroughs Wellcome.

Table I—Cumulative Excretion of Digoxin in Urine (μg/7 days)

Subject	I[a]	II[b]	III[c]	IV[d]
1	209.6	179.3	226.8	260.4
2	339.9	275.3	218.7	327.3
3	285.1	257.0	350.0	250.2
4	292.2	242.1	300.0	351.8
5	245.5	240.4	274.2	370.1
6	225.8	168.7	274.1	367.2
7	256.7	204.3	300.3	336.5
8	177.3	225.3	286.6	297.2
9	325.3	214.2	321.9	377.2
10	183.7	281.2	260.7	311.2
11	314.2	305.5	291.5	317.2
12	241.6	242.1	227.4	240.1
13	297.4	204.1	264.9	365.2
14	230.3	148.5	146.3	307.0
15	242.6	129.6	319.9	303.8
16	220.1	214.5	272.6	367.2
Mean	255.5	218.9	277.2	328.1
SE	12.3	11.1	12.0	10.1
SD	47.5	45.9	46.7	39.0
Percent of IV	77.8	66.7	84.5	100.0

Analysis of Variance (Cumulative Excretion)

Source of Variation	*df*	Sum of Squares	Mean of Squares	*F* Ratio	*p*
Between individuals	15	65712	4381	3.90	$p < 0.01$
Between formulations	3	97012	32337	28.79	$p < 0.005$
Between periods	3	17490	5830	5.19	$p < 0.01$
Error	42	47177	1123	—	—
Total	63	227391	—	—	—

Multiple Range Analysis: Test for Digoxin Excretion

Drug	I	II	III	IV
Cumulative excretion	255.5	218.9	377.2	328.1

IV *versus* III, $p < 0.005$
IV *versus* I, $p < 0.005$
IV *versus* II, $p < 0.005$
III *versus* I, $p > 0.05$, NS[e]
III *versus* II, $p < 0.005$
I *versus* II, $p < 0.05$

Multiple Range Analysis: Test for Periods

Period	1	2	3	4
Cumulative excretion	272.6	252.2	296.1	260.7

1 *versus* 2, $p > 0.05$, NS
1 *versus* 3, $p > 0.05$, NS
1 *versus* 4, $p > 0.05$, NS
2 *versus* 3, $p < 0.05$
2 *versus* 4, $p > 0.05$, NS
3 *versus* 4, $p < 0.05$

[a] Lanoxin tablet, Lot 474-G, Burroughs Wellcome. [b] Lanoxin tablet, Lot 991-F, Burroughs Wellcome. [c] Lanoxin elixir, Lot 816-G, Burroughs Wellcome. [d] Lanoxin parenteral, Lot 062-F, Burroughs Wellcome. [e] NS = not significant.

remained upright and ambulatory and were not allowed to eat for 4 hr following drug administration.

Blood for serum digoxin concentration was obtained at 0, 0.25, 0.5, 0.75, 1, 1.5, 2, 3, 4, 6, 7, 10, and 24 hr. Serum was separated and frozen at −18° until assayed. All urine excreted during the study was collected as follows: blank, 0–6, 6–12, and 12–24 hr and daily for the next 6 days.

The serum digoxin concentration was estimated by the radioimmunoassay of Smith *et al.* (9). Digoxin concentration in the urine was assayed by adding 10–50 μl of urine to blank serum. The radioimmunoassay procedure was the same as for the serum, and the concentration of digoxin in the urine was determined on the basis of the aliquot that produced values in the linear portion of the standard curve prepared in serum (5). Internal standards were used as a means of monitoring the accuracy and reproducibility of the digoxin determinations. The interassay coefficient of variation was 3.2%.

Statistical significance was determined by a two-way analysis of variance (ANOVA). When there were significant differences among the four groups by ANOVA, differences between the individual groups were determined by Duncan's multiple range test (10, 11).

RESULTS

The 7-day cumulative excretion of digoxin in the urine of the volunteers following each dosage form is given in Table I. The variability for these 16 subjects depended upon the dosage form. The coefficient of variation for the intravenous dose was 11.9%. This value is less than the coefficient of variation for the three oral dosage forms: 16.8, 18.6, and 21% for III, I, and II, respectively. This variation correlated with the percent absorbed, as reflected by the fraction of the amount excreted in urine following intravenous administration of the same dose from the three oral dosage forms, *i.e.*, the smaller the fraction absorbed, the greater the variability.

The elixir (III) was absorbed best with a mean value of 84.5%. Of the two tablets, I was 77.8% absorbed whereas II was only 66.7% absorbed. Although the mean absorption of I was less than the elixir, this difference was not statistically significant. The excretion of digoxin in the urine was significantly less for all three oral dosage forms than for the same dose given intravenously ($p < 0.005$). Sixty-six percent of the intravenous dose of digoxin was excreted during the 7-day collection period. In addition to significant differences among the various dosage forms, there were also interindividual differences (Table I).

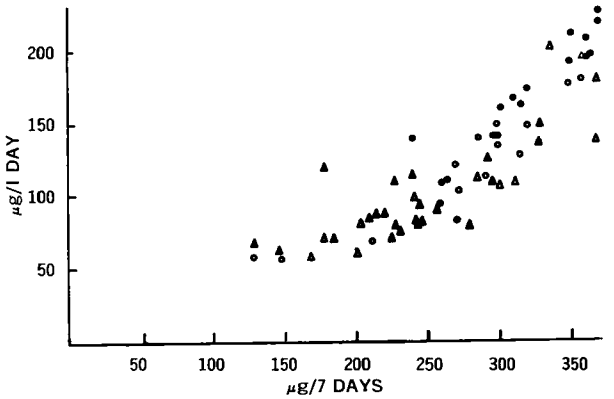

Figure 1—*Correlation between the digoxin excretion during the 1st day and the cumulative excretion of digoxin. Each value represents a separate study. Key:* ●, *intravenous;* ○, *elixir;* △, *Tablet I; and* ▲, *Tablet II* ($r = +0.84$, $p < 0.01$).

Greenblatt *et al.* (5) found a highly significant correlation between the excretion of digoxin in the urine during the 1st day following a single 0.75-mg dose and the cumulative 6-day digoxin excretion. They suggested that urine collection for 1 day was sufficient for most digoxin bioavailability studies. A significant correlation ($r = 0.84$, Fig. 1) was also found between the 1- and 7-day cumulative digoxin excretion, but the correlation ($r = 0.972$, Fig. 2) between the 2- and 7-day excretion in the urine was much better.

The mean serum digoxin concentration *versus* time following the administration of 0.5 mg of each dosage form is given in Fig. 3. The better absorbed oral preparations, I and III, had higher peak serum concentrations than II. Thereafter, the serum digoxin concentrations declined in a similar manner for the four studies. The *AUC* for the first 24 hr following drug administration was determined by the trapezoidal rule (Table II). Compared to the *AUC* for the intravenous dose, the elixir was 88% absorbed. Both tablets were significantly less well absorbed than the elixir.

Two differences were apparent between the cumulative urine excretion and the *AUC*. There was no significant difference in bioavailability between the two tablets using the *AUC*, whereas there was a significant difference between these tablets using the cumulative excretion of digoxin in the urine. Based on the *AUC*, Tablet I was significantly less well absorbed than the elixir. This finding was in contrast with the urine data in which a significant difference between Tablet I and the elixir was not demonstrated.

Two volunteers (Nos. 3 and 16) had unusual results for the *AUC* when compared to their cumulative excretion of digoxin in the urine. Unlike all other volunteers, their *AUC* was less for the intravenous dosage form than for the three oral dosage forms. The reason for this finding is unexplained. The *AUC* data were reanalyzed utilizing the mean of the 14 other volunteers for the two spurious values (Table III). Although the absorption of the oral digoxin preparations based on comparison of the *AUC*'s following intravenous administration of the same dose was now in closer agreement with the urine data, the difference in absorption of the two tablets still was not significant.

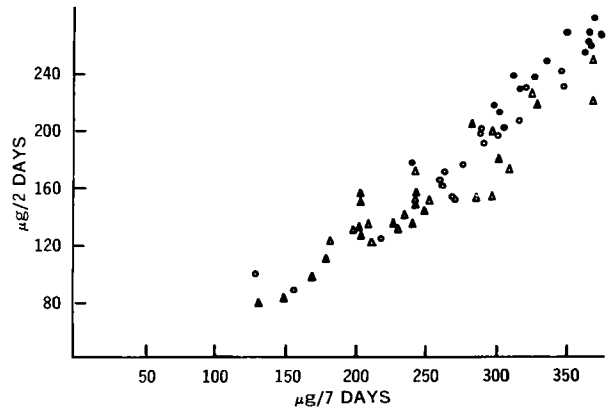

Figure 2—*Correlation between the digoxin excretion during the first 2 days and the 7-day cumulative excretion of digoxin. Each value represents a separate study. Key:* ○, *intravenous;* ○, *elixir;* △, *Tablet I; and* ▲, *Tablet II* ($r = +0.972$, $p < 0.001$).

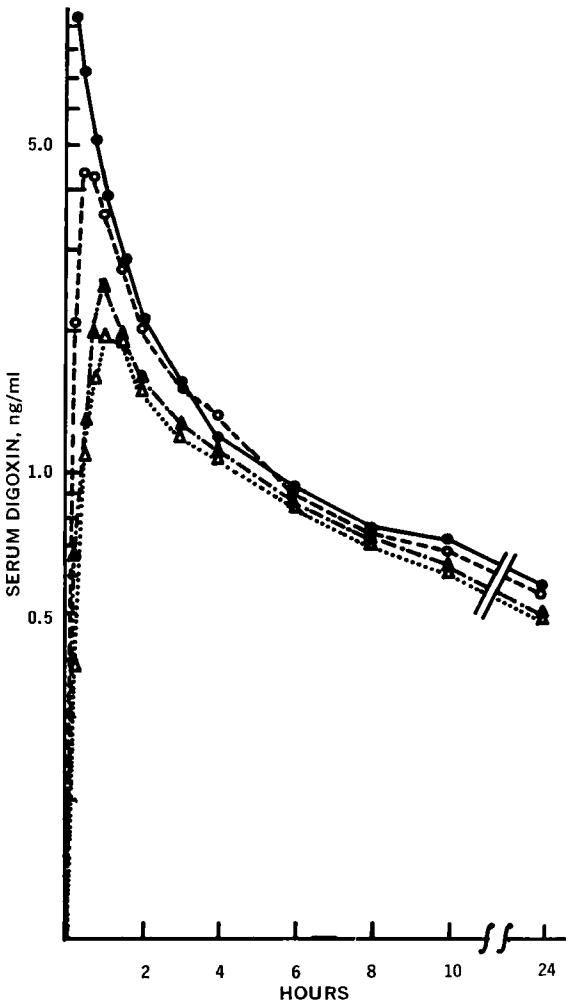

Figure 3—*Mean serum digoxin concentration during the first 24 hr following digoxin administration. Key:* ●—●, *intravenous;* ○---○, *elixer;* ▲··▲, *Tablet I; and* △···△, *Tablet II.*

DISCUSSION

These studies help to define further the variability in the cumulative excretion of digoxin and the *AUC* following a single dose administered to normal volunteers. While the results were similar using the two variables, there were important differences. Based on the cumulative excretion of digoxin in urine, the difference in the absorption of digoxin from the elixir and Tablet I was not statistically significant. Similar findings were demonstrated previously for tablets with rapid dissolution rates (12).

Both the elixir and I were better absorbed than the slow dissolution tablet. Using the *AUC*, however, the absorption of the two tablets was not significantly different despite differences in the peak serum digoxin concentrations. The discrepancy between the urine and serum data is due to a decreased absorption estimate for I using the *AUC* parameter. Since the coefficient of variation for the urine data was 16% as compared with 21% for the serum data, the cumulative excretion of digoxin is the more reliable estimate for determining the bioavailability of orally administered digoxin preparations.

Ideally, all digoxin excreted following the dose should be collected. With the assumption that the pharmacokinetics of digoxin fit a

Table II—Area under Concentration *versus* Time Curves (ng × 24 hr/ml)[a]

Subject	I	II	III	IV
1	17.2	14.0	20.6	27.5
2	15.7	14.2	29.6	32.7
3	22.8	20.0	39.1	24.3
4	23.5	19.5	22.2	33.8
5	14.3	15.4	15.7	19.6
6	20.3	18.7	19.8	21.2
7	19.4	18.9	25.9	30.7
8	23.9	16.8	21.8	30.7
9	16.7	21.6	23.0	24.0
10	20.8	20.6	19.7	30.5
11	14.7	13.4	23.0	27.0
12	21.2	21.0	21.4	27.8
13	15.8	11.9	20.7	22.6
14	17.7	21.4	20.1	21.1
15	18.2	19.2	18.5	22.1
16	11.1	28.4	26.4	19.4
Mean	18.4	18.4	22.9	25.9
SE	0.9	1.02	1.35	1.18
SD	3.5	3.96	5.25	4.58
Percent of IV	71.0	71.0	88.0	100.0

Analysis of Variance (*AUC*)

Source of Variation	*df*	Sum of Squares	Mean of Squares	*F* Ratio	*p*
Between individuals	15	529.3	35.3	2.08	$p < 0.05$
Between formulations	3	601.4	200.5	11.82	$p < 0.001$
Between periods	3	88.4	29.5	1.73	$p > 0.05$, NS[b]
Error	42	712.5	16.9	—	—
Total	63	1931.6	—	—	—

Multiple Range Analysis

Drug	I	II	III	IV
AUC	18.3	19.1	23.0	25.9

IV *versus* III, $p > 0.05$, NS
IV *versus* I, $p < 0.005$
IV *versus* II, $p < 0.005$
III *versus* I, $p < 0.05$
III *versus* II, $p < 0.05$
I *versus* II, $p > 0.05$, NS

[a] The area under the serum concentration *versus* time curve (*AUC*) was determined by the trapezoidal rule for the first 24 hr after drug administration. [b] NS = not significant.

Table III—Area under Serum Digoxin Concentration *versus* Time Curves Corrected for Subjects 3 and 16 (ng × 24 hr/ml)[a]

Drug	I	II	III	IV
Mean	18.5	17.6	21.6	26.5
Percent of IV	69.8	66.4	81.5	100.0

Analysis of Variance

Source of Variation	*df*	Sum of Squares	Mean of Squares	*F* Ratio	*p*
Between subjects	15	310.0	20.67	2.309	$p < 0.05$
Between formulations	3	767.1	255.7	28.562	$p < 0.001$
Between periods	3	11.5	3.83	0.428	$p > 0.05$, NS[b]
Error	42	376.0	8.95	—	—
Total	63	1464.00	—	—	—

Multiple Range Analysis

IV *versus* III, $p < 0.005$
IV *versus* I, $p < 0.001$
IV *versus* II, $p < 0.001$
III *versus* I, $p < 0.05$
III *versus* II, $p < 0.05$
I *versus* II, $p > 0.05$, NS

[a] The area under the concentration *versus* time curve (*AUC*) was determined by the trapezoidal rule for the first 24 hr after drug administration. [b] NS = not significant.

two-compartment open model, urine was collected for 4.2 half-lives and accounted for over 95% of the total drug eliminated. Since there was a highly significant correlation between the excretion of digoxin for the first 2 days and the 7-day cumulative excretion, a 2-day urine collection appears to be satisfactory. It is suggested that urine be collected for a minimum of 2 or preferably 3 days following the administration of digoxin. The longer collection period will be particularly important when evaluating the bioavailability of digoxin from tablets with slow absorption. In this manner, the 748 serum and 448 urine digoxin assays necessary to complete this four-way crossover study can be reduced to a mere 64.

Although bioavailability studies have not been performed in pa-

tients who are receiving digoxin, steady-state studies in normal volunteers (7) correlate with the single-dose studies, suggesting that either protocol may be used.

The dose of digoxin appears to be important with regard to its absorption. Greenblatt *et al.* (12) used a single dose of 0.75 mg and found that the absolute bioavailability relative to an intravenous infusion of digoxin was 65% for the elixir and 55% for a digoxin tablet. Both of these values are considerably lower than the findings in this study. They administered the intravenous digoxin by a 1-hr infusion and found a slightly higher percent of the intravenous dose of digoxin in the urine, which may explain these differences.

Recent studies by Greenblatt *et al.* (13) indicate that the cumulative excretion of digoxin in the urine is 7% greater following a 1-hr intravenous infusion of digoxin when compared to an intravenous injection over 3 min. This finding accounts for the difference between the 66% cumulative excretion of digoxin following the intravenous dose in this study as compared with 76% in their study (12). When this difference is considered, however, there still remains an unexplained 13% difference in the bioavailability of digoxin in the two studies. Steady-state studies suggest that the bioavailability of oral digoxin may be dose dependent (7). The differences between this study and the earlier one (5) support this possibility. Therefore, a standard dose of digoxin should be chosen for future digoxin bioavailability studies.

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Alcoholysis of Medicinally Active 5-Aminodibenzo[*a,d*]cycloheptenes

J. NALLY, J. NAZARENO, J. POLESUK, and H. V. MAULDING ˣ

Abstract □ The rate constants for alcoholic solvolysis of the hydrochloride salts of diethylaminodibenzo[*a,d*]cycloheptene and related amino congeners were determined. The objective was a study of the comparative ease of cleavage of the C—N amino linkage by various aliphatic alcohols. The interaction of protonated amines of this series with alcoholic hydroxyls presumably leads to formation of the corresponding ethers in a manner somewhat analogous to alkoxide reaction with alkyl bromides. The methyl ether produced from solvolysis of diethylaminodibenzocycloheptene hydrochloride was isolated and identified. Methanol appears to react somewhat more rapidly with the amine hydrochlorides than other aliphatic alcohols. The latter produce almost invariant velocity constants with a given amine hydrochloride. The exception was *tert*-butanol, which resulted in k_{obs} values about one-third of those given by the other alcohols. Some velocity constants in formic and acetic acids were evaluated. Generation of carbonium ions of appreciable lifetime was indicated in formic acid by the formation of a highly colored (red-violet) solution. This color may be a manifestation of the dibenzotropylium ion.

Keyphrases □ 5-Aminodibenzo[*a,d*]cycloheptenes—alcoholic solvolysis, rate constants □ Solvolysis, alcoholic—medicinally active 5-aminodibenzo[*a,d*]cycloheptenes, rate constants □ Alcohols—solvolysis of hydrochloride salts of diethylaminodibenzo[*a,d*]cycloheptene and related amino congeners, rate constants

Protonated members of the 5-aminodibenzo[*a,d*]-cycloheptane series (I) were found to be relatively unstable in aqueous solution, with rupture of the C—N linkage being the pertinent reaction (1). Insertion of a double bond between positions 10 and 11 of the cycloheptane moiety produced 5-aminodibenzo[*a,d*]cycloheptenes (II) and led to a nearly two-magnitude enhancement of the hydrolytic velocity constant. Velocity increases in each series were dependent upon the amino substituent at C-5. The rate constants for hydrolysis of these 5-amino compounds (I and II) were invariant with pH (where pH < pKa), being only a function of the nature of the various protonated amines and the specified temperature (1).

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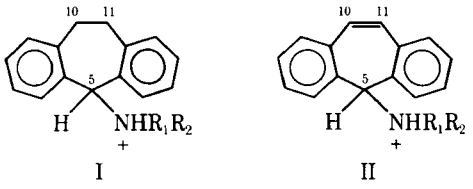
J. NALLY, J. NAZARENO, J. POLESUK, and H. V. MAULDING ˣ

Abstract □ The rate constants for alcoholic solvolysis of the hydrochloride salts of diethylaminodibenzo[*a,d*]cycloheptene and related amino congeners were determined. The objective was a study of the comparative ease of cleavage of the C—N amino linkage by various aliphatic alcohols. The interaction of protonated amines of this series with alcoholic hydroxyls presumably leads to formation of the corresponding ethers in a manner somewhat analogous to alkoxide reaction with alkyl bromides. The methyl ether produced from solvolysis of diethylaminodibenzocycloheptene hydrochloride was isolated and identified. Methanol appears to react somewhat more rapidly with the amine hydrochlorides than other aliphatic alcohols. The latter produce almost invariant velocity constants with a given amine hydrochloride. The exception was *tert*-butanol, which resulted in k_{obs} values about one-third of those given by the other alcohols. Some velocity constants in formic and acetic acids were evaluated. Generation of carbonium ions of appreciable lifetime was indicated in formic acid by the formation of a highly colored (red-violet) solution. This color may be a manifestation of the dibenzotropylium ion.

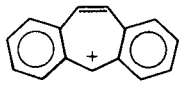
Keyphrases □ 5-Aminodibenzo[*a,d*]cycloheptenes—alcoholic solvolysis, rate constants □ Solvolysis, alcoholic—medicinally active 5-aminodibenzo[*a,d*]cycloheptenes, rate constants □ Alcohols—solvolysis of hydrochloride salts of diethylaminodibenzo[*a,d*]cycloheptene and related amino congeners, rate constants

Protonated members of the 5-aminodibenzo[*a,d*]-cycloheptane series (I) were found to be relatively unstable in aqueous solution, with rupture of the C—N linkage being the pertinent reaction (1). Insertion of a double bond between positions 10 and 11 of the cycloheptane moiety produced 5-aminodibenzo[*a,d*]cycloheptenes (II) and led to a nearly two-magnitude enhancement of the hydrolytic velocity constant. Velocity increases in each series were dependent upon the amino substituent at C-5. The rate constants for hydrolysis of these 5-amino compounds (I and II) were invariant with pH (where pH < pKa), being only a function of the nature of the various protonated amines and the specified temperature (1).



I II

R_1, R_2 = alkyl, alkyl; alkyl, hydrogen; cycloalkyl



III

Both series of compounds were previously found medicinally active as antihistaminic and anticonvulsant agents (2, 3).

The sensitivity of 5-aminodibenzocycloheptenes (II) to decomposition in aqueous solution is possibly mediated through a highly resonance-stabilized dibenzotropylium ion (III), with the positive charge being distributed over the carbon atoms of the entire molecule. However, on the basis of experimental evidence, the bimolecular attack of alcohol on the protonated amine cannot be discounted.

It became of interest to examine the reactivities of several aminodibenzocycloheptenes with ROH groups from a mechanistic viewpoint as well as from the standpoint of their tendency to function as alkylating agents. The aminodibenzocycloheptenes (II) react readily with nucleophiles such as alcoholic hydroxyls and carboxylic acid hydroxyl groups commonly found in proteins. These alkylation reactions may be related to the strong vesicatory properties of many members of this series. Sensitivity is noted with extremely dilute solutions or trace amounts of the pure material (2).

The work presented is mainly involved with the proclivity of several 5-aminodibenzocycloheptenes to interact with aliphatic alcohols, resulting in ether formation and simultaneous liberation of the primary or secondary amine during cleavage of the C—N bond (Scheme I).

EXPERIMENTAL

Preparation of Compounds—5-Aminodibenzo[*a,d*]cycloheptanes, either as the free base or hydrochloride salts, were synthesized as previously reported from 5-chlorodibenzocycloheptane[1] and the corresponding amine in benzene or toluene (1, 3).

The 5-aminodibenzo[*a,d*]cycloheptenes were isolated as the free bases or hydrochloride salts from the 5-chlorodibenzocycloheptene reaction with the corresponding liquid amine (4–6). The 5-chlorodibenzocycloheptene was prepared by treatment of the alcohol[1] with equimolar thionyl chloride in dry toluene. It was employed without further purification following removal of solvent.

Compounds utilized in this study were recrystallized and subjected to C, H, N, and Cl microanalysis. Actual values were found to be in good agreement with theoretical values (1, 6). Melting points corresponded to those previously reported for the compounds (1, 3–5) and TLC [chloroform–diethylamine (99:1)] showed only one spot.

Kinetic Procedures—Solutions of the various alcohols were equilibrated at the specified temperatures, followed by addition of 25 mg of the amine hydrochloride in solution to give a final volume of 100 ml. Four-milliliter samples were periodically withdrawn and evaporated to dryness under reduced pressure (rotary evaporator), followed by addition of 0.5 ml of 0.1 *N* HCl, 0.5 ml of 0.1 *N* NaOH, 4 ml of pH 3.44 citrate buffer[2], and 25 ml of methyl orange solution[3]. The aqueous solution was extracted three times with 30-ml portions of chloroform and made up to 100 ml in a volumetric flask. The chloroform solutions were scanned in the visible from 390 to 460 nm.

Neither the decomposition products nor their mixtures with the intact molecules in equimolar concentrations produced appreciable chloroform extractive color with methyl orange. This was noted since degraded solutions failed to interfere with the analytical procedure.

Solvent alcohols were reagent grade, anhydrous, except 95% ethanol. The other chemicals and materials used were reagent grade.

The analytical procedure was altered in the case of propylene glycol in that 2 ml of the propylene glycol containing 0.5 mg/ml amine was diluted to 50 ml, followed by adjustment of the pH to 3.44. The methyl orange was added and the method was followed as previously stated.

Degradation Products—A 250-mg sample of 5-diethylaminodibenzo[*a,d*]cycloheptene hydrochloride was heated at 50° for 18 hr in methanol solution. The resultant solution was filtered, and the filtrate was evaporated to dryness, acidified, and extracted into dry ether. The purity of the sample was checked by TLC[4] [chloroform–diethylamine (99:1)], with only one spot, R_f 0.75, being observed. The ether was evaporated, leaving an oily residue which was subjected to mass spectroscopy. The molecular ion, M^+, exhibited a value of 222, indicative of the methyl ether; NMR ($CDCl_3$): 3.3 (s, 3H, OCH_3) and 7.1 (m, 8H, aromatic protons) ppm.

Anal.—Calc. for $C_{16}H_{14}O$: C, 86.5; H, 6.3. Found: C, 86.2; H, 6.1.

RESULTS AND DISCUSSION

Rate constants were determined for the reactions depicted in Scheme I from either:

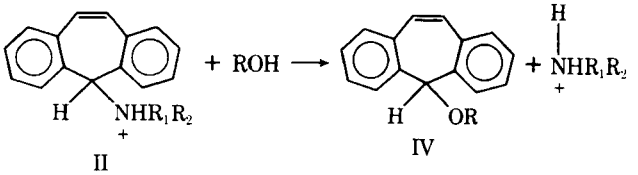
$$k_{obs} = 0.693/t_{1/2} \qquad \text{(Eq. 1)}$$

or:

$$\log (A_t - A_\infty) = \log (A_0 - A_\infty) - k_{obs}t/2.303 \qquad \text{(Eq. 2)}$$

where A_0, A_t, and A_∞ are absorbances at times = 0, t, and infinity, respectively; and k_{obs} is the observed first-order rate constant. The residual absorbance, A_∞, varied from negligible to about 2% A_0. Rate constants were evaluated in triplicate and were generally reproducible to within ±10%. Figure 1 illustrates the means of calculating k_{obs} by absorbance plots of the ion-pair complex (chloroform solution) *versus* time (Eq. 2) for several compounds in Table I.

Table I lists some alcohols examined for their effects on the reaction rate of the 5-aminodibenzocycloheptenes. Invariance of the velocity constants from alcohol to alcohol, with the exception of sterically hindered *tert*-butanol, is rather persuasive, but not conclusive, evidence of an S_N1 process; S_N processes, which for activation require only a dispersal of charge, are usually three or more times as fast in ethanol as in water (7). However, this phe-



II IV

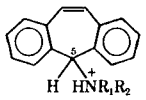
Scheme I

[1] Aldrich Chemical Co., Milwaukee, Wis.

[2] Prepared from 9.7 g of anhydrous citric acid in 900 ml of water. Thirty percent sodium hydroxide (10.5 ml) was added, and the pH was adjusted to 3.44 with concentrated hydrochloric acid, followed by dilution to 1 liter with distilled water.

[3] Two hundred and fifty milligrams of methyl orange (Eastman No. 432), 3.3 ml of 0.1 *N* NaOH, and 800 ml of water were placed in a 1-liter, volumetric flask and shaken to dissolve the contents. The pH was adjusted to 7.0 with 0.1 *N* HCl (dropwise), followed by dilution to 1 liter with distilled water.

[4] Silica gel FG, 250 μm, Analtech Inc., Newark, Del.



R_1 = alkyl, cycloalkyl, allyl
R_2 = hydrogen, alkyl

Table I—Rate Constants (k_{obs} in hours^{-1}) for Reaction of Various 5-Aminodibenzo[*a,d*]cycloheptenes in Alcohol Solution (Ether Formation) at Specified Temperatures

5-Substituent	Temperature	Water	k_{obs}[a], Alcohols: Methanol	Ethanol	Propanol	Isopropanol	Butanol	*sec*-Butanol	*tert*-Butanol	Propylene Glycol
1. Diethylamino hydrochloride	50°	0.49	1.50	1.27	1.32	2.04	1.61	1.54	0.68	1.31
2. Pyrrolidinoamino hydrochloride	60°	0.013	0.037	0.020	0.018	0.019	0.012	0.018	0.005	0.024
3. Allylamino hydrochloride	60°	0.24	0.108	0.116	0.103	0.082	0.059	0.053	0.015	0.159
4. Propylamino hydrochloride	60°	0.044	0.047	0.032	0.029	0.028	0.023	0.026	0.007	—
5. Piperidinoamino hydrochloride	60°	0.12	0.322	0.277	0.271	0.277	0.209	0.244	0.063	0.204

[a] Other conditions, listed in the order of compound, temperature, alcohol, and k_{obs}, are: diethylamine hydrochloride, 40°, ethanol, 0.26; propanol, 0.25 methanol, 0.33; and propylamino hydrochloride, 60°, neopentyl, 0.027.

nomenon also holds for reaction of neutral molecules with amine hydrochlorides. This phenomenon is manifested in the majority of the reactions of Table I.

Application of the mass law effect may be compelling evidence of an S_N1 reaction. For the sequence shown in Scheme II:

$$\overset{+}{R}NHR_1R_2 \underset{k_r}{\overset{k_f}{\rightleftharpoons}} NHR_1R_2 + R^+ \xrightarrow[k']{R'OH} ROR'$$

Scheme II

when assuming steady-state treatment, one obtains (7):

$$\text{rate} = [d\,ROR']/dt = k_f[\overset{+}{R}NHR_1R_2]\Big/\left(k_r\frac{[NHR_1R_2]}{k'} + 1\right) \text{(Eq. 3)}$$

Addition of excess amine should cause the rate of hydrolysis to slow down after some lag time. This is calculated by means of increasing the denominator in Eq. 3 through the $[NHR_1R_2]$ term. Unfortunately, the experiment cannot be carried out as with alkyl halides and halide ions because the proton exchange between added amine and protonated reactant produces precipitation of the 5-aminodibenzocycloheptene base, leaving the true mechanism to speculation. The allylic compound (Compound 3, Table I) argues against a pure S_N1 reaction due to its velocity decrease in alcoholic solution. Allylic compounds, however, are well known to undergo altered reaction mechanisms under many circumstances. This solvent effect would also be expected in the bimolecular attack of alcohol on an amine hydrochloride (7).

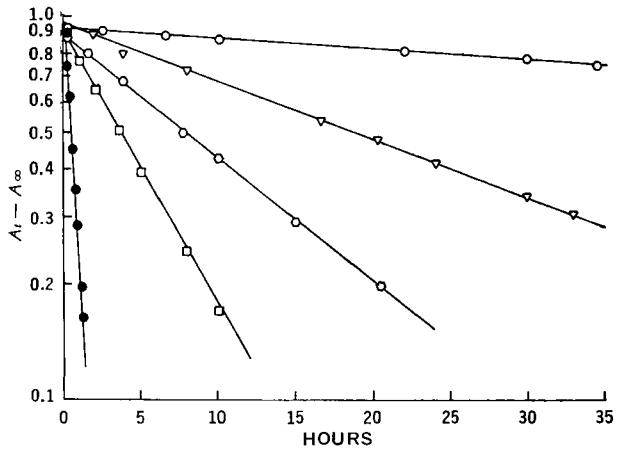


Figure 1—*Observed first-order plots for absorbance loss of various aminodibenzocycloheptenes as a function of time. The amine hydrochloride salts and alcohols utilized are listed in the key, along with the temperature for each. Key:* ●, *propylamine hydrochloride–methanol, 60°;* □, *allylamine hydrochloride–propylene glycol, 60°;* ○, *diethylamine hydrochloride–methanol, 50°;* ▽, *piperidino hydrochloride–*tert*-butanol, 60°; and* ○, *pyrrolidino hydrochloride–*tert*-butanol, 60°.*

The nonprotonated form of the compounds in Table I are unreactive, thus failing to undergo cleavage of the C—N bond which typifies this series. This could not be previously tested (1) due to the low aqueous solubility of the neutral species (free base) at pH's around and above the pKa. In alcoholic solution, this solubility problem was no longer existent and stability of the free base was observed. The addition of equimolar amounts of hydrochloric acid that protonated the amino nitrogen initiated the reaction in the usual manner.

Scheme I illustrates the expected products of the decomposition in alcoholic solution. The reaction of diethylaminodibenzocycloheptene hydrochloride in anhydrous methanol led to the anticipated methyl ether, which was isolated by preparative TLC; the structure was verified by mass spectroscopy microanalysis and NMR. In aqueous solution, three products were noted with the ether dominating (1). In the one case investigated, only the spot indicative of the methyl ether of dibenzocycloheptene was observed by TLC. No attempts were made to isolate the ethers presumably formed from the other alcohols. NMR spectroscopy shows δ 3.3 for the OCH_3 protons attached to the CH as compared to an expected value of δ 3.7 if attached to the phenyl ring. This finding corroborates a type IV structure (Scheme I).

As previously stated, the principal product in aqueous media is the bisether, which is formed both from the amine hydrochloride and from the intermediate alcohol. The ketone appears in traces and may be an artifact of oxidation of the alcohol by oxygen in the reaction solutions (1). The methyl ether appears as the only product in methanol, either through virtual absence of the alcohol in anhydrous methanol or through its low concentrations as an intermediate that prevent its observation by the methods used. The ethers, when formed, have an intrinsic stability in the reaction medium that prevents their further transformation under the reaction conditions.

Table II—Comparisons of the Observed First-Order Rate Constants (in hours^{-1}) for 5-Diethylaminodibenzocycloheptane (k_{ane}) with Those for the 5-Diethylaminodibenzocycloheptene Hydrochloride (k_{ene}) in Several Alcohols at 50°

Alcohol	k_{ane}	k_{ene}	k_{ene}/k_{ane}
Methanol	0.012	1.50	12.5
Ethanol	0.010	1.27	12.7
Isobutanol	0.0083	1.54	18.6
Isopropanol	0.0084	2.04	26.7
tert-Butanol	0.6079	0.68	8.5

Table III—Effects of Solvent Systems of Acetic Acid, Formic Acid, and Acetone on the Decomposition of 5-Diethylaminodibenzocycloheptene Hydrochloride with the k_{obs} Measured (in hours^{-1}) at 50°

Solvent[a]	k_{obs}
Acetic acid	1.46
Acetic acid + 0.5 ml of methanol	1.46
Acetic acid + 1.0 ml of methanol	1.72
Acetic acid + 5.0 ml of methanol	2.21
Formic acid, 99%	3.48
Formic acid, 99%, + 1 ml of methanol	3.52
Formic acid, 99%, + 5 ml of methanol	3.70
Reagent acetone[b]	0.41
Reagent acetone[b] + 1 ml of methanol	0.94
Reagent acetone[b] + 5 ml of methanol	1.40

[a] Run in 200-ml volumes with, for example, 5 ml of methanol added followed by formic acid addition (195 ml) to fill the volumetric flask. [b] Reagent acetone utilized from a freshly opened bottle; water content = 0.3%.

Table I shows alcohols and amines used in the investigation along with k_{obs} at specified temperatures. The following points suggest the reaction might proceed through a carbonium ion or its equivalent: *(a)* the general invariance of k_{obs} with most alcohols employed, *(b)* the slightly lower rate constant value for the hindered *tert*-butanol relative to other primary and secondary alcohols, and *(c)* the k_{ene}/k_{ane} values of greater than one magnitude. These facts, along with the comparative ease of tropylium-ion formation under the proper conditions (8), tend to support—but do not verify—a carbonium-ion hypothesis. The data for the allylic amine are disconcerting (Table I), because the observed first-order constant is smaller in alcoholic solution than in water.

Table II depicts the increase in the observed first-order rate constants with diethylaminodibenzocycloheptene hydrochloride compared with diethylaminodibenzocycloheptane hydrochloride in various alcohols at 50°. This enhancement of velocity constants in the unsaturated molecule is in agreement with the results in aqueous solution (1).

Table III shows the k_{obs} values for reaction of the diethylaminodibenzocycloheptene hydrochloride with the carboxylic hydroxyl groups of formic and acetic acids. In formic acid solution, a red-violet coloration was noted. The colored dibenzotropylium ion from the 5-chloro analog in anhydrous solvents was reported previously (9). This is circumstantial evidence for the presence of these stabilized carbonium ions under these conditions, which may be the consequence of a decreased rate of destruction of the ion rather than an increased rate of formation.

The reactivity of acetone (Table III) may be a consequence of reaction with the enolic hydroxyl as well as the water in solution.

CONCLUSIONS

The reaction of various 5-aminodibenzocycloheptenes with solvent alcohols was investigated. The k_{obs} values were found to be almost constant for a given amine in the several aliphatic alcohols studied excepting *tert*-butanol where a decrease in the observed first-order rate constant was detected.

The compounds were found to react in solutions of acetone, acetic acid, and formic acid with the latter producing a colored solution during the experiment. This may be indicative of the colored dibenzotropylium ion.

The increase in the velocity constants in alcoholic solution relative to water is characteristic of either a mechanism where only a separation of charge in the transition state is required or one representing bimolecular attack of neutral alcohol on the amine hydrochloride.

The unexpected results with the allylic compound where the k_{obs} is greater in water than in the alcohols leaves some doubts and is the principal anomaly encountered.

The magnitude of the k_{ene}/k_{ane} lends some credence to the S_N1-type mechanism, as does the general invariance of k_{obs} with alcohol employed for a given amine. Thus, the mechanism—S_N1 or S_N2—is still in doubt in this series, and a combination of the two cannot be discounted at the present time. Further experiments are in progress to elucidate the matter.

Many of these compounds are vesicant or extremely irritating. This may be a consequence of their reactivity with hydroxyls and other nucleophilic groups, such as SH, present in protein. Their relative ease of reaction may lead to alkylation of the protein materials, thus bringing about the strong response elicited upon contact with the skin.

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Combined Assay, Identification, and Foreign Related Steroids Test for Methandrostenolone by High-Speed Liquid Chromatography

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Abstract □ A high-speed liquid chromatographic system is described, which can be used for the simultaneous identification of the anabolic steroid methandrostenolone and its impurities and the quantitation of each of these compounds. Separation is effected by adsorption chromatography on a slurry-packed microparticulate silica gel column.

Keyphrases □ Methandrostenolone and related steroid impurities—simultaneous identification and quantitation by high-speed liquid chromatography □ High-speed liquid chromatography—simultaneous identification and quantitation of methandrostenolone and related steroid impurities

The synthetic steroid methandrostenolone (17β-hydroxy-17α-methylandrosta-1,4-dien-3-one) (I) is used as an anabolic agent, both medically and nonmedically (1, 2). It may be synthesized from methyltestosterone (17β-hydroxy-17α-methylandrost-4-en-3-one) (II) either microbiologically (3) or chemically (4). Steroidal impurities which may be present (5) are II, 6α,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one (III), and 6β,17β-dihydroxy-17α-methylandrosta-1,4-dien-3-one (IV). Of these, II has about twice the oral androgenic potency and about one-half of the anabolic activity of methandrostenolone (6). Therefore, its presence in any significant amount is undesirable and limited (5). The epimeric pair of 6-hydroxylated impurities would be expected to be less active than methandrostenolone, since, in general, substitution at the 6-position lowers androgenic activity (7). Nevertheless, the presence of such compounds as foreign related steroids should be controlled as much as possible within the limits of good manufacturing practice.

Present compendial assays of methandrostenolone (5, 8) are interfered with by the presence of impurities and are, therefore, less accurate then they might be. The BP (5) uses a simple UV measurement for the raw material and a condensation with dinitrophenylhydrazine, followed by colorimetric assay for the tablets, together with a TLC test for the foreign related steroids. The limits for the impurities are 0.5% for II and 2% each for III and IV. The NF (8) uses a straightforward UV measurement for both raw material and tablets and is only concerned with the presence of II as a foreign related steroid, setting a limit of 0.5% by TLC.

High-speed liquid chromatography (HSLC) is enjoying increasing usage for the analysis of steroids (9). The work described in this study is an assay method for I, together with a method for estimating the amount of each impurity. Since it is a chromatographic procedure, it is also an identification test. The method may be used for both composite and single-tablet analysis. It is fast, accurate, precise, and specific for each compound described.

EXPERIMENTAL

Reagents—Compound II[1], Compound III[2], Compound IV[2], *m*-dinitrobenzene[3], and all solvents were used as received. Compound I[4] was recrystallized from acetone and water and dried *in vacuo* before use.

HSLC Procedure—A high-speed liquid chromatograph[5] equipped with a fixed wavelength (254 nm) UV detector [attenuated 0.02 absorbance unit full-scale (aufs)], a septumless injector, and an electronic integrator[6] were used.

The silica gel[7] (4–9 μm) column (25 cm × 2.1 mm i.d. × 3.2 mm o.d.; 304 stainless steel) was prepared using a balanced-density slurry packing procedure similar to that described by Majors (10). A mobile phase of 3% (v/v) ethylene chloride and 15% (v/v) 2-propanol in *n*-hexane was used at a flow rate of 60 ml/hr (2600 psi) at ambient temperature.

Preparation of Standard Solutions—*II Standard*—Compound II, 12.5 mg, was accurately weighed into a 10-ml volumetric flask and dissolved in and diluted to volume with chloroform. Then 1.0 ml of this solution was pipetted into a 100-ml volumetric flask and diluted to volume with chloroform (Solution A, 12.5 μg/ml).

III and IV Standards—Approximately 15.6 mg each of III and IV was accurately weighed into separate 25-ml volumetric flasks and dissolved in and diluted to volume with chloroform. Then 1 ml of each solution was pipetted into separate 10-ml volumetric flasks and diluted to volume with chloroform (Solutions B and C, 62.5 μg/ml).

m-Dinitrobenzene (Internal Standard)—Approximately 9.5 mg of *m*-dinitrobenzene was accurately weighed into a 25-ml volumetric flask and dissolved in and diluted to volume with chloroform (Solution D, 380 μg/ml). Then 20 ml of Solution D was pipetted into a 100-ml volumetric flask and diluted to volume with chloroform (Solution E, 76 μg/ml).

Methandrostenolone Working Standard—Approximately 12.5 mg of recrystallized I was accurately weighed into a 5-ml volumetric flask. Aliquots (1 ml) of each of Solutions A, B, C, and D were pipetted into this flask, which was agitated gently to dissolve the

[1] National Formulary reference standard.
[2] British Pharmacopoeia, authentic substance.
[3] Fisher Scientific, Montreal, Quebec, Canada.
[4] Ciba/Geigy, Montreal, Quebec, Canada.
[5] Varian Aerograph, model 4100, Walnut Creek, Calif.
[6] Vidar Autolab, model 6300, Mountain View, Calif.
[7] LiChrosorb SI 60, Brinkmann Instruments (Canada) Ltd.; manufactured by E. Merck, Darmstadt, Germany.

Table I—Assay of Methandrostenolone[a] in Synthetic Mixtures

Sample	Amount, μg/ Injection	Recovery, %
1	1.240	99.6
2	2.480	99.8
3	2.610	101.9
4	2.750	99.6
	Mean recovery, %	= 100.2
	Coefficient of variation, %	= 1.1

[a] Containing 0.7% of III and 1.0% of IV.

methandrostenolone and diluted to volume with chloroform. This solution now contained I (2.5 mg/ml), II (2.5 μg/ml), III and IV (12.5 μg/ml), and *m*-dinitrobenzene (76 μg/ml) and corresponded to a solution of I containing 0.5% III and IV and 0.1% II.

Analysis of Methandrostenolone Tablets—*Tablet Extraction*—Single (5-mg) tablets were placed in 15 × 75-mm screw-capped test tubes and crushed with a glass rod to a fine powder. Two milliliters of Solution E was pipetted into each tube, which was closed and placed on a rotator[8] at 60 rpm for 20 min. The tube was centrifuged[9] at 3000 rpm for 5 min.

HSLC—Duplicate 1-μl aliquots[10] of the working standard solution of I were chromatographed, and the peaks corresponding to I and the internal standard were integrated. Duplicate 1-μl aliquots of each tablet extract were chromatographed, again integrating the peaks due to internal standard and I in each sample. The percent label claim of I was calculated from:

$$\% \text{ I} = \frac{N_M N_{\text{I.S.}}\ (\text{sample})}{N_M N_{\text{I.S.}}\ (\text{standard})} \times 100 \qquad \text{(Eq. 1)}$$

where N_M = integrator counts for peak from I, and $N_{\text{I.S.}}$ = integrator counts for internal standard peak.

Limit Test for II, III, and IV in Methandrostenolone Tablets—Duplicate (5-μl) aliquots[11] of the working standard solution of I were chromatographed, integrating the internal standard peak. Duplicate (5-μl) aliquots of the tablet extracts used for the assay were also chromatographed, integrating the internal standard peak. The ratio of impurities in the sample and standard was calculated from:

$$R_x = \frac{N_{\text{I.S.}} H_x\ (\text{sample})}{N_{\text{I.S.}} H_x\ (\text{standard})} \qquad \text{(Eq. 2)}$$

where R_x = ratio of compound, x, in the sample to that in the standard; $N_{\text{I.S.}}$ = integrator counts for the internal standard peak; and H_x = peak height for Compound x, where x can be II, III, or IV.

If R_x is greater than 1.0 for Compound x, the sample contains more than 0.1% (II) or 0.5% (III and IV) of that impurity.

RESULTS AND DISCUSSION

Figure 1 shows a chromatogram of a synthetic mixture of I, II, III, IV, and the internal standard, *m*-dinitrobenzene. The impurities represent 1.0, 0.7, and 1.0%, respectively.

Figure 2 shows a plot of integrator count ratio for various amounts of I and a constant amount of internal standard (76 ng/injection) *versus* the weight of I chromatographed; the response was linear to more than 3 μg of I. Subsequent work was carried out with a nominal level of 2.5 μg of I/injection.

Response curves for II, III, and IV were prepared by calculating the ratios of the peak heights to internal standard counts and plotting these values against the amount of compound injected. Compound II was studied over the 1.25–12.5-ng/μl range, while III and IV were tested over the 2.5–50-ng/μl range; 5-μl injections were used in all cases. Relative to a 1-μl injection of I at the level of 2.5 μg/μl, these ranges represent 0.05–0.5% II and 0.10–2.0% III and IV.

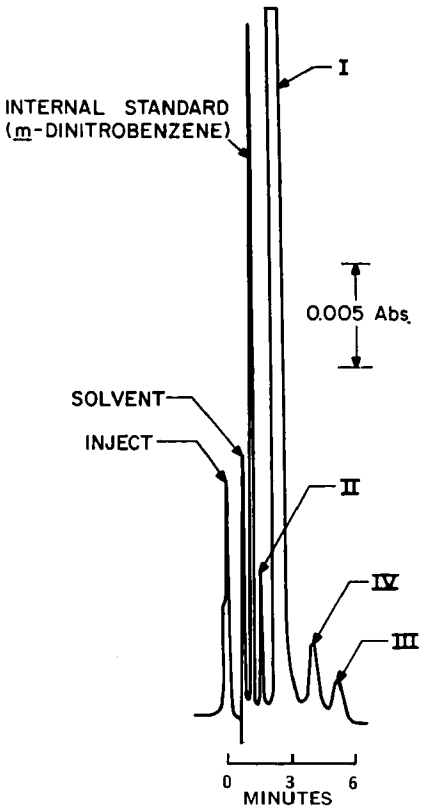

Figure 1—*High-speed liquid chromatogram of a synthetic mixture of methandrostenolone (I, 2.5 μg), methyltestosterone (II, 25 ng), 6α-hydroxymethandrostenolone (III, 17.5 ng), and 6β-hydroxymethandrostenolone (IV, 25 ng). Chromatographic conditions are given in the text.*

The calculated line of best fit in each case was a straight line through the origin ($y = mx$), with the relative standard deviations of the slopes being 20.3, 7.7, and 2.0% for II, III, and IV, respectively.

Methandrostenolone raw material and two separate reference standards (NF and BP) were chromatographed under the conditions described here and were shown to contain between 0.4 and 1.0% of each of the two impurities III and IV and negligible amounts of II. Recrystallization of the raw material from a water–acetone solution yielded I containing less than 0.03% of either impurity. This sample was used to prepare the working standard solution of I.

The chromatographic system was stable over the period required for the assay. Retention time variation on the peak from I was less than 2%. Compound I was quantitated by comparison of the ratios of integrator counts for peaks obtained for I and the internal standard when 1.0-μl aliquots of the tablet extract and working standard solution were chromatographed. Since the nominal concentration of these two compounds was the same in both solutions, direct comparison of peak height ratios multiplied by 100 gives the percent of label claim for I. Table I lists the assay results for synthetic solutions of I over a range of 1.2–2.75 μg/injection.

The limit test for the impurities in I could have been carried out with a single-injection technique, using the same chromatogram used for the assay. However, the results would have been less precise because of the small size of the impurity peaks when only 1.0 μl (2.5 μg) of solution was used (Fig. 1). For this reason, a 5-μl injection was used which gave peaks for the impurities that were large enough for quantitation but a nonlinear peak for I. Negligible interference of the parent peak with IV was observed under these conditions, and quantitation of the impurities was possible at levels as low as 0.05% for II and 0.1% for III and IV. Quantitation was effected by comparison of the ratios of impurity peak heights to in-

[8] Multi-Purpose Rotator, Scientific Industries, Springfield, Mass.
[9] HM-s centrifuge, International Equipment, Needham Heights, Mass.
[10] Hamilton 75N CH microliter syringe.
[11] Hamilton 701N CH microliter syringe.

Table II—Assay of Methandrostenolone and Impurities in Tablets

Lot		HSLC I, %	II, %	III, %	IV, %	Total, %	BP, %
A[a]	Mean =	100.0	0.06	0.35	0.76	101.2	98.8
	SD =	±2.6	±0.02	±0.04	±0.04	—	
A[b]		98.7	0.05	0.33	0.72	98.8	98.6
B[a]	Mean =	95.2	<0.05	0.51	0.67	96.4	97.7
	SD =	±3.7	—	±0.04	±0.04	—	
B[b]		96.6	<0.05	0.47	0.61	97.7	97.8

[a] Values are from analysis of 10 single tablets. [b] Values are from analysis of composite of 10 tablets, with both methods applied to same solution.

ternal standard counts from chromatograms of 5-μl aliquots of sample and working standard solutions. Peak height was used for the impurity peaks because the integrator was not always able to track peaks corresponding to small quantities of III and IV.

When six (5-μl) aliquots of the working standard solution of I were analyzed, coefficients of variation of 1.4, 21.4, 4.4, and 8.3% were obtained for I, II, IV, and III, respectively.

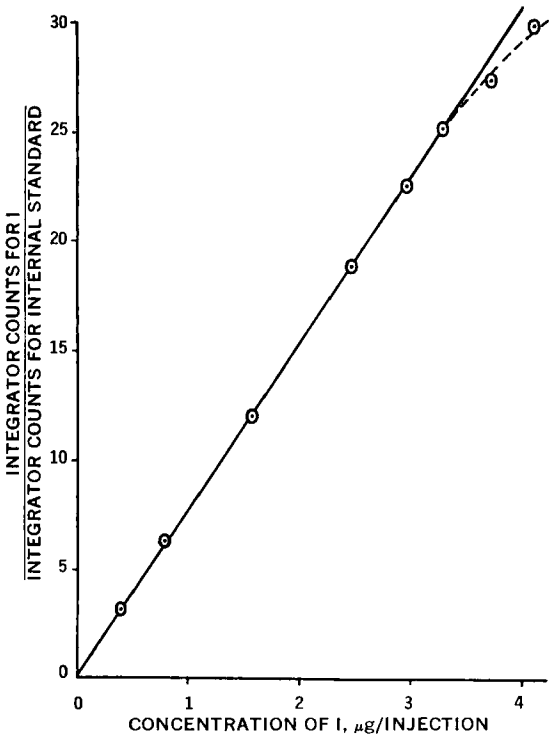

Figure 2—*Linearity curve for methandrostenolone from chromatograms of methandrostenolone standard solutions containing 76 ng/injection of* m-*dinitrobenzene as internal standard.*

Table II summarizes the results obtained when 10 single tablets (5 mg) of I, from each of two lots, were assayed by HSLC and by the BP method and when 10-tablet composites of each lot were assayed by both methods. The assay results are in excellent agreement when the total percent recovery by HSLC is compared to the BP method, especially in the case of the composite solutions assayed by both methods. The single-tablet assay results have different means, indicating a possible shortcoming in assaying only 10 tablets for content uniformity purposes. Alternatively, it may reflect real differences between two sets of tablets or differences in precision of the two methods at the single-tablet level. The coefficients of variation on the impurities were 33, 12, and 6% for II, III, and IV, respectively, at impurity levels of 0.05, 0.4, and 0.7%, respectively, and include about 3% tablet variation.

In conclusion, a precise, rapid, HSLC procedure was developed which allows either the assay of methandrostenolone and an estimate of its three impurities or a dual-injection assay of the parent compound and of these same impurities.

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Effect of Compaction on Particle Size

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Abstract □ Compressed tablets were prepared on a hydraulic press at several different compaction pressures by a standardized technique, using aspirin, dicalcium phosphate dihydrate, calcium phosphato-carbonate, alumina, and microcrystalline cellulose. All tablets except microcrystalline cellulose contained a cation-exchange resin as disintegrant. The particle-size spectra of the disintegrating compacts were evaluated using a particle-size counter or an air jet sieve. It is shown that compacts made from different materials but of the same initial particle-size spectra disintegrate to give particles of a considerably different size. Determination of the change in particle size produced by the compaction process provides useful insight into the nature of the compaction process.

Keyphrases □ Compaction pressure—effect on particle-size spectrum of disintegrating compacts, aspirin, dicalcium phosphate dihydrate, calcium phosphato-carbonate, alumina, microcrystalline cellulose □ Particle size, disintegrating compacts—determination, relationship between particle size and nature of compaction process □ Pressure, compaction—effect on particle-size spectrum of disintegrating compacts □ Compressed tablets—evaluation of particle-size spectra □ Tablets, compressed—evaluation of particle-size spectra

The relevance of particle-size control to pharmaceuticals has been recognized for many years. Most studies have been concerned with the influence of particle size on tableting characteristics (1) or on dissolution rate (2). Several studies reported the effect of compressional force on the specific surface area of tablets (3, 4) and showed that, as pressure increases, specific surface area initially increases and then decreases.

Studies of the effect of compaction on the particle size of a pharmaceutical system may be expected to provide data relevant to deciding whether the particles within the compact exist as crystal units or aggregates or have undergone extensive fragmentation. Examination of the literature reveals the comparative paucity of such work. Cid and Jaminet (5) showed that the maximum dissolution rate of the aspirin formulations they investigated was obtained from the system giving the smallest particle-size distribution when the tablet disintegrated. Smith *et al.* (6) proposed that an increase in compressional force enhances the dissolution by causing fragmentation within the compact.

Since the effect of compaction pressure on aggregation or fragmentation within a compact is related to the fundamental molecular properties of the material being compressed, it seemed likely that a study of particle-size distributions within disintegrating compacts would help elucidate both compressional properties and the effect of compressional force on dissolution behavior. Therefore, it was considered that if powders of known particle-size spectra were compressed and then allowed to disintegrate, evidence of fragmentation or interparticulate bonding might be obtained. Of course, experimental problems involved in the determination of the particle size of disintegrating compacts meant that the data obtained might represent relative rather than absolute values, even with carefully standardized techniques.

Five materials were selected for this study: alumina was chosen as a brittle material that forms unstable compacts; aspirin was chosen as a granular, directly compressible drug; and microcrystalline cellulose, dicalcium phosphate dihydrate, and calcium phosphato-carbonate complex were chosen as direct compression diluents.

EXPERIMENTAL

Materials—Aspirin[1] BP, dicalcium phosphate dihydrate[2], and calcium phosphato-carbonate complex[3] were all screened to obtain a −35 + 60 B.S.S.[4] mesh particle-size fraction (mean particle-size fraction 335 μm); fines were removed by use of an air jet sieve[5]. Because the coarse fraction of alumina[6] was not compressible, it was necessary to use a finer sieve fraction (−100 + 200 B.S.S., mean particle size 113 μm). Microcrystalline cellulose[7] was used as supplied by the manufacturer. Magnesium stearate[8] was also used in the formulations.

For the dicalcium phosphate dihydrate, calcium phosphato-carbonate complex, and alumina, 3% of the cation-exchange resin[9] was used as disintegrant. For aspirin formulations, 5% of the resin was needed.

Methods—Compacts, 13 mm in diameter, were prepared by means of a hydraulic press; the powder samples were subjected to known pressures for 30 sec. To effect disintegration of the compacts without agitation, which might cause particulate fracture, the compacts contained a particularly effective disintegrant. In the case of the compacts prepared from the insoluble materials (dicalcium phosphate dihydrate and calcium phosphato-carbonate complex), distilled water was used as the disintegration vehicle; for the other systems, appropriate saturated solutions were used.

Particle-size determinations of the disintegrated compacts were made using either a particle-size counter[10] or air jet sieve[5]. The particle-size counter, with a 400-μm orifice tube, was used where the maximum particle size was less than 160 μm. The counter was used as described previously (8), with 1% (w/v) sodium chloride as the electrolyte. For most of the particle-size determinations, the air jet sieve was used. The tablets were allowed to disintegrate and then were filtered and dried before sieve analysis. A standard procedure was adopted by which the material was sieved for 3 min on each British Standard sieve. From the amount remaining on each sieve, percentage undersize values were calculated. At least four compacts were used for each experiment; the means of the differ-

[1] Monsanto, London, England.
[2] Albright and Wilson, Oldbury, England.
[3] Calfos, London, England.
[4] British Standard sieve.
[5] Alpine.
[6] Thermo Syndicate, London, England.
[7] Avicel PH102, Honeywell and Stein, London, England.
[8] B.D.H., Poole, England.
[9] Amberlite IRP 88, Lenning, London, England.
[10] Coulter.

Table I—Effect of Applied Pressure on the Mean Particle Size of Disintegrated Compacts

Applied Pressure, MNm^{-2}	Mean Particle Size (Micrometers) of Compacts after Disintegration Containing			
	Alumina[a]	Aspirin[b]	Dicalcium Phosphate Dihydrate[b]	Calcium Phosphato-carbonate Complex[b]
74	—	277	68	208
149	82	282	100	194
298	80	272	120	183
447	66	266	149	177
597	—	263	150	180

[a] Alumina, initial mean particle size 112.5 μm. [b] Aspirin, dicalcium phosphate dihydrate, and calcium phosphato-carbonate complex, initial mean particle size 335 μm.

ent values, all of which were in good agreement with one another (±4%), were calculated.

The effect of compaction pressure on the particle-size distribution of microcrystalline cellulose, as determined by use of the particle-size counter, is shown in Fig. 1. Similar data for aspirin, dicalcium phosphate dihydrate, calcium phosphato-carbonate complex, and alumina obtained by use of the air jet sieve are shown in Figs. 2–4. The effect of compaction pressure on the mean particle size of the disintegrated compacts is shown in Table I.

RESULTS AND DISCUSSION

It can be seen in Fig. 1 that an increase in compaction pressure[11] from 74 to 298 MNm^{-2} caused some fragmentation of the microcrystalline cellulose. However, the particle-size distributions of compacts prepared at 446 and 597 MNm^{-2} were very similar to that determined at 298 MNm^{-2}. Because this material is known to undergo considerable interparticulate bonding by plastic flow (9), the results shown in Fig. 1 may initially appear to be somewhat strange. Reier and Shangraw (10), however, proposed that the most important factor contributing to the compaction of microcrystalline cellulose is hydrogen bonding. These investigators stated that crystals are compacted close enough so that hydrogen bonding between them can occur. The results presented in Fig. 1 support Reier and Shangraw's (10) hypothesis that, as may be expected, penetration of water would interrupt hydrogen bonding and break the compact into its original constituents. Thus, the curves (Fig. 1) only show fragmentation, which is caused by the effect of compression.

Figure 2 shows the effect of pressure on the particle-size distribution of compacts prepared from alumina (Fig. 2A) and aspirin (Fig. 2B). The compacts prepared from alumina were soft and very unstable. Compacts of this material apparently were formed merely by fragmentation, which increases slightly with an increase in pressure; mean particle size of the disintegrated compacts undergoes gradual reduction as the applied pressure increases. There did not appear to be any agglomeration or recombination of particles as a result of pressure. These results are similar to those obtained by Varma *et al.* (11), who studied the effect of pressure on the particle-size distribution of compacts prepared from some brittle materials.

Figure 2B shows the effect of pressure on the particle-size distribution of compacts prepared from granular aspirin. It seems that increasing pressure caused some fragmentation of aspirin crystals; the effect, however, was extremely small. The mean particle size was reduced from 277 μm when compacted at 74 MNm^{-2} to a slightly lower value of 263 μm when compacted at 597.1 MNm^{-2}. There was also very little effect on the pattern of particle-size distribution. During the initial compression, as rearrangement of these crystals takes place, the edges and corners of some crystals possibly may be knocked off, thus causing a slight decrease in particle size. After this stage, it seems that a strongly bonded system has been formed and further increases in pressure, as evident from

[11] In this paper, Standard International units of newtons per square meter are used for compaction pressures, 1 newton = kg^{-2}.

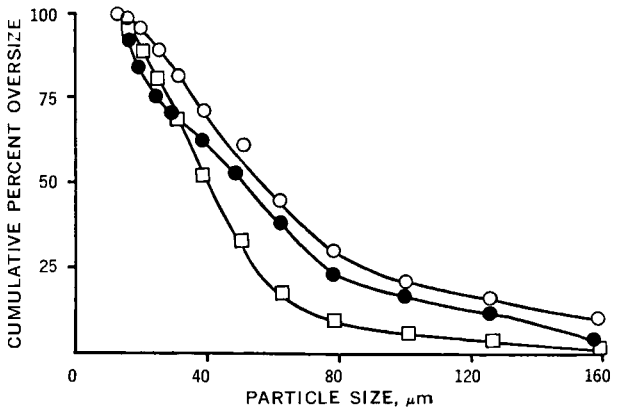

Figure 1—*Effect of pressure on the particle-size distribution of disintegrating microcrystalline cellulose compacts. Key: ○, before compaction; ●, compacted at 74 MNm^{-2}; and □, compacted at 298 MNm^{-2}.*

Fig. 2B and Table I, have very little effect on the particle-size distribution. These results are supported by the findings of Ganderton (12), who also found that aspirin crystals underwent deformation but retained their integrity at all practical pressures.

Figure 3 shows the effect of pressure on the particle-size distribution of compacts prepared from dicalcium phosphate dihydrate. The particle size of this material was reduced after compaction at 74 MNm^{-2} (mean size 68 μm), increased again when compacted at 149.3 MNm^{-2} (mean size 100 μm), and continued to increase with increasing pressure in the pressure range used in this study (Table I and Fig. 3). These results are very similar to those obtained in studies of the effect of pressure on the surface area of compacts (3, 4) in which the surface area of the compacts initially increased due to fragmentation and then decreased due to particle bonding.

Although the mean particle size of dicalcium phosphate dihydrate compacts clearly indicates the effect of pressure on the com-

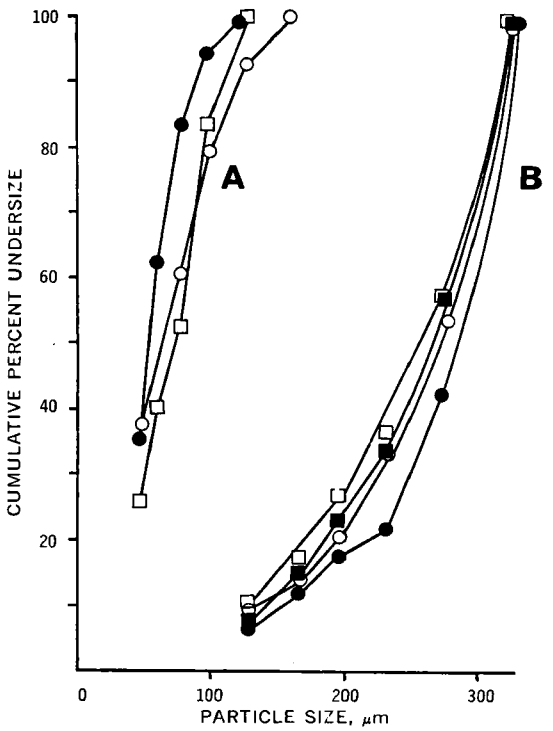

Figure 2—*Effect of pressure on the particle-size distribution of disintegrating compacts made from alumina (A) or aspirin (B). Key (A, alumina, original mean particle size 113 μm): ○, 149 MNm^{-2}; □, 298 MNm^{-2}; and ●, 446 MNm^{-2}. Key (B, aspirin, original mean particle size 336 μm): ●, 149 MNm^{-2}; ○, 298 MNm^{-2}; ■, 447 MNm^{-2}; and □, 597 MNm^{-2}.*

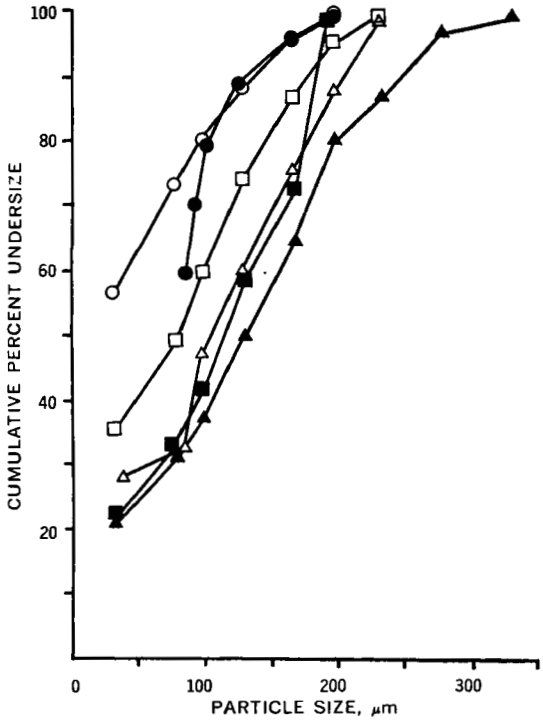

Figure 3—*Effect of pressure on the particle-size distribution of disintegrating dicalcium phosphate dihydrate compacts (original mean particle size 335 μm). Key: ○, 74 MNm⁻²; ●, 149 MNm⁻²; □, 224 MNm⁻²; △, 298 MNm⁻²; ■, 372 MNm⁻²; and ▲, 447 MNm⁻².*

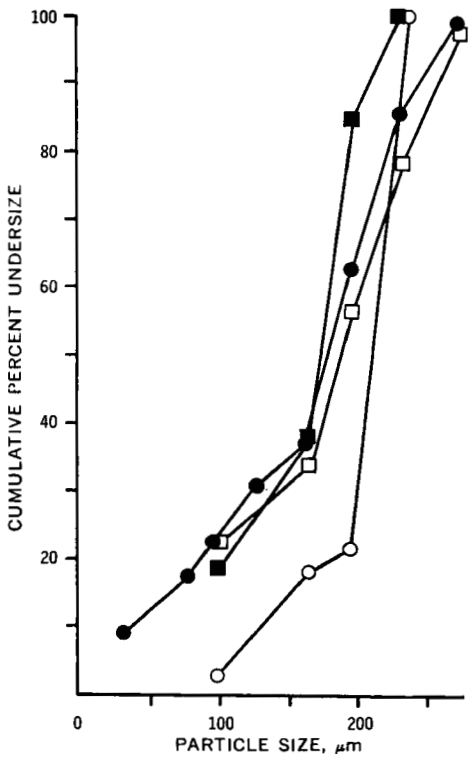

Figure 4—*Effect of pressure on the particle-size distribution of disintegrating calcium phosphato-carbonate compacts (original mean particle size 335 μm). Key: ○, 149 MNm⁻²; □, 298 MNm⁻²; ●, 447 MNm⁻²; and ■, 672 MNm⁻².*

pression of this material (Table I), the particle-size distribution curves are more informative (Fig. 3). These curves show the great amount of fragmentation that occurred during compression, with the curve changing from a very narrow size range to a much wider distribution. At low pressure, grinding is the predominant process, but as the compression increases, although grinding still continues, recombination also takes place and the distribution curve covers a greater size range and becomes flatter. These curves show that fragmentation occurs initially during compression; but as the compression is increased, bonding also takes place. Thus, the mean particle diameter and specific surface area will depend on the balance of these two processes which, in turn, depend upon the applied pressure.

Figure 4 shows the effect of pressure on the particle-size distribution of compacts prepared from calcium phosphato-carbonate complex. The effect of pressure on the particle-size distribution of this substance apparently is very similar to that of aspirin; once a strongly bonded system has been formed, a further increase in pressure causes very little fragmentation. The behavior of this material on compression differs from that of dicalcium phosphate dihydrate in that the former undergoes less fragmentation on compression and, after initial fragmentation, a further increase in pressure does not cause any apparent bonding and recombination of particles, as shown by the compression of dicalcium phosphate dihydrate (Figs. 3 and 4 and Table I).

Previously, it was shown that calcium phosphato-carbonate complex has somewhat superior compression properties to that shown by dicalcium phosphate dihydrate (7). Calcium phosphato-carbonate complex produces harder tablets at lower pressure; its compressibility also appears to be higher than that of dicalcium phosphate dihydrate. The results presented in Fig. 4 indicate that, although the increase in pressure causes some fragmentation, the curve changes from one of a narrow range to that of a much wider distribution. Mean particle size decreases only slightly with increasing pressure (Table I). From these results and those reported previously (7), it appears possible that calcium phosphato-carbonate complex is a useful direct compression diluent which forms a strongly bonded system at a low compaction pressure.

In conclusion, it seems that compacts of different materials made from the same initial particle size and compressed at the same pressures show different size distributions, which appear to be indicative of their compaction behavior. The techniques of estimating particle-size distribution of disintegrated compacts can be a useful guide to the compression properties of a formulation.

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Water-Sorption Properties of Tablet Disintegrants

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Abstract □ The water-sorption properties of four tablet disintegrants, starch, sodium carboxymethylcellulose, sodium starch glycolate, and a cation-exchange resin, were examined in the form of powders and in compressed tablets prepared from calcium phosphate dibasic dihydrate. Dissolution properties of the tablets compare well to the water-sorption properties. The effect of storage in the presence of water vapor upon tablets containing the various disintegrants was evaluated in terms of tablet hardness and disintegration time. Differences in the effects produced in the various tablet formulations can be related to the differing mechanisms whereby the disintegrants effect tablet rupture. Photomicrographic data support the conclusions drawn from the water-sorption, disintegration, and dissolution studies. Sodium starch glycolate and the cation-exchange resin merit careful consideration by formulators using calcium phosphate dibasic dihydrate or similar direct compression matrixes.

Keyphrases □ Water sorption of tablet disintegrants (starch, sodium carboxymethylcellulose, sodium starch glycolate, cation-exchange resin)—disintegration and dissolution, powders and calcium phosphate dibasic dihydrate tablets □ Tablet disintegrants (starch, sodium carboxymethylcellulose, sodium starch glycolate, cation-exchange resin)—water sorption of powders and calcium phosphate dibasic dihydrate tablets, disintegration, dissolution

Previously, the disintegration and dissolution properties of tablets containing sodium starch glycolate and a cation-exchange resin were reported (1, 2). The results of these evaluations clearly showed that both the cation-exchange resin and sodium starch glycolate are extremely efficient disintegrants in a variety of tablet systems. The objective of the present study was to examine the water-sorption properties of these disintegrants in an attempt to evaluate the basic factors controlling their disintegrant action. Starch and sodium carboxymethylcellulose were included for comparative purposes.

EXPERIMENTAL

Materials—The following were used: calcium phosphate dibasic dihydrate[1] (unmilled), corn starch[2] BP, sodium salicylate[2], amaranth[2] BPC, a cation-exchange resin[3], sodium starch glycolate[4], sodium carboxymethylcellulose[5], and magnesium stearate[6].

Dried Disintegrant Powders—The dried disintegrants were exposed to 100% relative humidity at 37°. Two replicate readings of water uptake, always in good agreement, were obtained at various times over 25 hr. Figure 1 shows the water sorption by the disintegrant as a function of time.

Table I—Effect of Humidity on Disintegration Properties of Calcium Phosphate Dibasic Dihydrate Tablets, Containing Several Disintegrants, Stored at 100% Relative Humidity at 37° [Punch Size 1.1 cm ($^7/_{16}$ in.) Flat]

Disintegrants, 10% (w/v)	Disintegration Time, min					
	Initial	2 hr	4 hr	8 hr	22 hr	30 hr
Starch	15[a]	15[a]	15[a]	15[a]	15[a]	15[a]
Sodium carboxymethylcellulose	43	50	50	55	55	60
Sodium starch glycolate	0.6	2.8	7.0	13.0	15	15
Cation-exchange resin	0.83	2.0	7.0	8.0	60	60

[a] Particles remaining.

Tablet Containing 10% Disintegrant—Calcium phosphate dibasic dihydrate was chosen as the excipient because of its very low equilibrium moisture content (2.5%). Furthermore, tablets made from the material were easier to handle after having absorbed moisture than were other tablets, such as those prepared from lactose.

The tablets containing 10% disintegrant were made using 1.1-cm ($^7/_{16}$-in.) flat punches on a single-punch tablet press[7]. All tablets were made under the same conditions of pressure and die fill settings. Fifty tablets of each formulation were used for the water-sorption study. Five lots of 10 tablets of each formulation (one lot for each time interval of 5, 10, 15, 20, and 25 hr) were weighed and labeled individually; their thickness and diameter measurements were taken, using a micrometer, before and after exposure to 100% relative humidity.

Figure 2 shows the water uptake by these tablets as a function of time. The results of expansion and thickness changes accompanying water sorption are presented in Figs. 3 and 4. (Changes in density reflect overall structural changes, whereas individual measurement of thickness and diameter changes allows observation of whether expansion occurs preferentially in, or at right angles to, the plane of compression.) These tablets were also tested for disintegration time[8] using the BP method and for hardness[9]. The effect of storage on disintegration time and hardness of tablets prepared from calcium phosphate dibasic dihydrate is shown in Tables I and II.

To express the relative affinities for water of these disintegrants, the term "absorption efficiency" was used; it is calculated in a similar way as dissolution efficiency (3). Figures 5 and 6 show the absorption efficiencies of pure disintegrants and of the disintegrants

[1] Albright and Wilson, Oldbury, England.
[2] J. M. Loveridge, Southampton, England.
[3] Amberlite IRP88, Lenning, London, England.
[4] Primojel, Slater, Winsford, England.
[5] Courlose P20, British Celanese, Coventry, England.
[6] British Drug Houses, Poole, England.
[7] Manesty F3.
[8] Manesty disintegration tester.
[9] Erweka hardness tester.

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Water-Sorption Properties of Tablet Disintegrants

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Abstract □ The water-sorption properties of four tablet disintegrants, starch, sodium carboxymethylcellulose, sodium starch glycolate, and a cation-exchange resin, were examined in the form of powders and in compressed tablets prepared from calcium phosphate dibasic dihydrate. Dissolution properties of the tablets compare well to the water-sorption properties. The effect of storage in the presence of water vapor upon tablets containing the various disintegrants was evaluated in terms of tablet hardness and disintegration time. Differences in the effects produced in the various tablet formulations can be related to the differing mechanisms whereby the disintegrants effect tablet rupture. Photomicrographic data support the conclusions drawn from the water-sorption, disintegration, and dissolution studies. Sodium starch glycolate and the cation-exchange resin merit careful consideration by formulators using calcium phosphate dibasic dihydrate or similar direct compression matrixes.

Keyphrases □ Water sorption of tablet disintegrants (starch, sodium carboxymethylcellulose, sodium starch glycolate, cation-exchange resin)—disintegration and dissolution, powders and calcium phosphate dibasic dihydrate tablets □ Tablet disintegrants (starch, sodium carboxymethylcellulose, sodium starch glycolate, cation-exchange resin)—water sorption of powders and calcium phosphate dibasic dihydrate tablets, disintegration, dissolution

Previously, the disintegration and dissolution properties of tablets containing sodium starch glycolate and a cation-exchange resin were reported (1, 2). The results of these evaluations clearly showed that both the cation-exchange resin and sodium starch glycolate are extremely efficient disintegrants in a variety of tablet systems. The objective of the present study was to examine the water-sorption properties of these disintegrants in an attempt to evaluate the basic factors controlling their disintegrant action. Starch and sodium carboxymethylcellulose were included for comparative purposes.

EXPERIMENTAL

Materials—The following were used: calcium phosphate dibasic dihydrate[1] (unmilled), corn starch[2] BP, sodium salicylate[2], amaranth[2] BPC, a cation-exchange resin[3], sodium starch glycolate[4], sodium carboxymethylcellulose[5], and magnesium stearate[6].

Dried Disintegrant Powders—The dried disintegrants were exposed to 100% relative humidity at 37°. Two replicate readings of water uptake, always in good agreement, were obtained at various times over 25 hr. Figure 1 shows the water sorption by the disintegrant as a function of time.

Table I—Effect of Humidity on Disintegration Properties of Calcium Phosphate Dibasic Dihydrate Tablets, Containing Several Disintegrants, Stored at 100% Relative Humidity at 37° [Punch Size 1.1 cm ($^7/_{16}$ in.) Flat]

Disintegrants, 10% (w/v)	Disintegration Time, min					
	Initial	2 hr	4 hr	8 hr	22 hr	30 hr
Starch	15[a]	15[a]	15[a]	15[a]	15[a]	15[a]
Sodium carboxymethylcellulose	43	50	50	55	55	60
Sodium starch glycolate	0.6	2.8	7.0	13.0	15	15
Cation-exchange resin	0.83	2.0	7.0	8.0	60	60

[a] Particles remaining.

Tablet Containing 10% Disintegrant—Calcium phosphate dibasic dihydrate was chosen as the excipient because of its very low equilibrium moisture content (2.5%). Furthermore, tablets made from the material were easier to handle after having absorbed moisture than were other tablets, such as those prepared from lactose.

The tablets containing 10% disintegrant were made using 1.1-cm ($^7/_{16}$-in.) flat punches on a single-punch tablet press[7]. All tablets were made under the same conditions of pressure and die fill settings. Fifty tablets of each formulation were used for the water-sorption study. Five lots of 10 tablets of each formulation (one lot for each time interval of 5, 10, 15, 20, and 25 hr) were weighed and labeled individually; their thickness and diameter measurements were taken, using a micrometer, before and after exposure to 100% relative humidity.

Figure 2 shows the water uptake by these tablets as a function of time. The results of expansion and thickness changes accompanying water sorption are presented in Figs. 3 and 4. (Changes in density reflect overall structural changes, whereas individual measurement of thickness and diameter changes allows observation of whether expansion occurs preferentially in, or at right angles to, the plane of compression.) These tablets were also tested for disintegration time[8] using the BP method and for hardness[9]. The effect of storage on disintegration time and hardness of tablets prepared from calcium phosphate dibasic dihydrate is shown in Tables I and II.

To express the relative affinities for water of these disintegrants, the term "absorption efficiency" was used; it is calculated in a similar way as dissolution efficiency (3). Figures 5 and 6 show the absorption efficiencies of pure disintegrants and of the disintegrants

[1] Albright and Wilson, Oldbury, England.
[2] J. M. Loveridge, Southampton, England.
[3] Amberlite IRP88, Lenning, London, England.
[4] Primojel, Slater, Winsford, England.
[5] Courlose P20, British Celanese, Coventry, England.
[6] British Drug Houses, Poole, England.
[7] Manesty F3.
[8] Manesty disintegration tester.
[9] Erweka hardness tester.

Table II—Effect of Humidity on Hardness of Calcium Phosphate Dibasic Dihydrate Tablets Stored at 100% Relative Humidity at 37°

Disintegrants, 10% (w/w)	Hardness, Erweka Units					
	Initial	2 hr	4 hr	8 hr	22 hr	30 hr
Starch	5.6	5.5	5.0	5.5	5.0	4.0
Sodium carboxymethylcellulose	5.2	3.2	2.0	2.2	2.0	2.0
Sodium starch glycolate	4.0	3.5	3.0	3.0	2.0	—
Cation-exchange resin	3.0	—	—	—	—	—

incorporated into tablets after 5 and 25 hr. Dissolution efficiencies of tablets prepared from calcium phosphate dibasic dihydrate containing 1% amaranth are shown in Fig. 7.

Surface Structure of Disintegrant Compacts after Absorbing Moisture—To obtain further information about the mechanism of action of the disintegrants evaluated in this study, compacts of disintegrants were photographed after being exposed to moisture. The disintegrant (250 mg) was compressed at 76.1 MNm^{-2}, using a hydraulic press and 13-mm flat punches. The compacts were exposed to an atmosphere of 100% relative humidity at 37°. Photomicrographs of the compacts were taken before and after 2 and 4 hr of exposure to humid conditions.

The surface structure of compacts prepared from sodium starch glycolate is shown in Figs. 8–10, and that of cation-exchange resin compacts is shown in Figs. 11–13. The structure of starch and its compacts has been extensively studied using a scanning electron microscope (4); therefore, the photomicrographs were not included in this report. Also, since the structure of sodium carboxymethylcellulose on absorbing moisture was quite similar to that of the resin compacts, only the resin compacts are shown here.

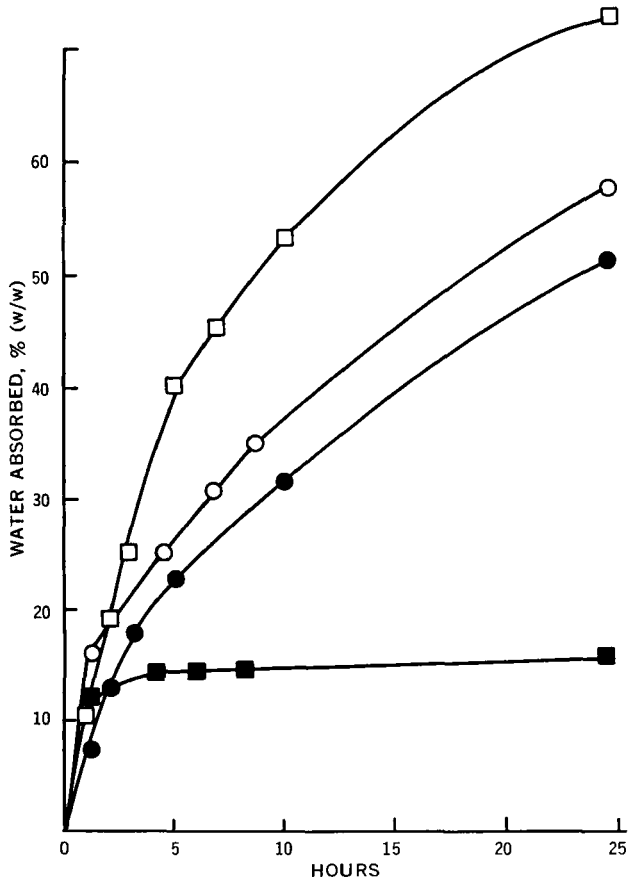

Figure 1—*Water absorption by dried disintegrant powders. Key: □, cation-exchange resin; ○, sodium starch glycolate; ●, sodium carboxymethylcellulose; and ■, starch.*

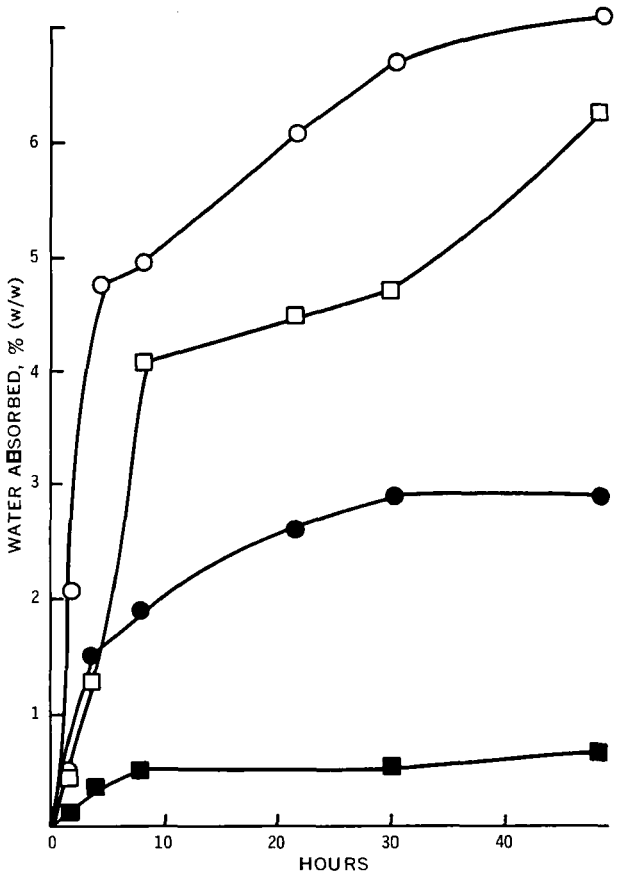

Figure 2—*Water sorption of calcium phosphate dibasic dihydrate tablets containing 10% (w/w) distintegrant. Key: ○, cation-exchange resin; □, sodium starch glycolate; ●, sodium carboxymethylcellulose; and ■, starch.*

RESULTS AND DISCUSSION

The water sorption by these disintegrants as a function of time in powder form and when incorporated in tablets is shown in Figs. 1 and 2. The cation-exchange resin exhibited the highest water uptake followed by sodium starch glycolate, sodium carboxymethylcellulose, and starch. Figure 3 shows the expansion of calcium phosphate dibasic dihydrate tablets containing 10% (w/w) disintegrant, when exposed to 100% relative humidity, as a function of time. Tablets containing 10% (w/w) cation-exchange resin showed the highest decrease in apparent density followed by tablets containing sodium starch glycolate, sodium carboxymethylcellulose, and starch. An interesting observation was made in the case of calcium phosphate dibasic dihydrate containing 10% (w/w) sodium carboxymethylcellulose; when tablets were exposed to 100% relative humidity at 37°, they showed considerably more expansion in their thickness than in their diameter (Fig. 4). It is possible that the expansion in the axis in which compression was applied is due to the elastic behavior of sodium carboxymethylcellulose after absorbing moisture. The other tablets showed more even expansion in both directions.

The results of these water-sorption studies, as evidenced by absorption efficiencies (Figs. 5 and 6), clearly demonstrate that the disintegrants with the highest water uptake are generally the most effective in most tablet systems (Fig. 7 and Refs. 1 and 2). Although no attempt is made to define fully the complete mechanism of action of these disintegrants, it appears that the main reasons for the effectiveness of sodium starch glycolate and the cation-exchange resin are their high uptake of water (Fig. 1), the relatively rapid absorption of water by tablets containing these disintegrants (Fig. 2), and their marked swelling and expansion properties (Figs. 3 and 4). It is, of course, fully expected that there may be difficulties in comparing water sorption, in hours, and tablet dissolution, in minutes.

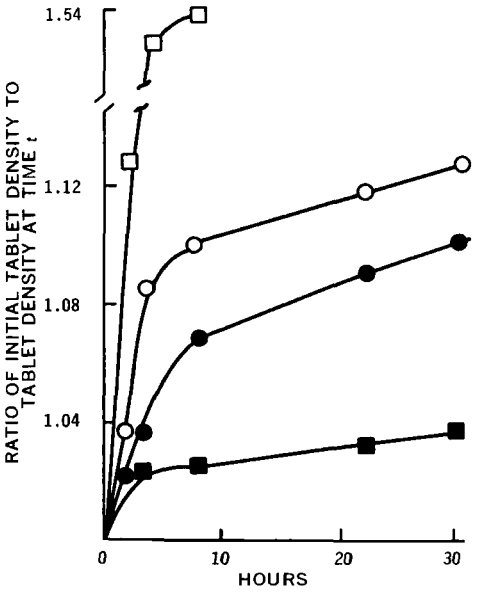

Figure 3—*Change of apparent tablet density of calcium phosphate dibasic dihydrate tablets containing 10% (w/w) disintegrant on exposure to 100% relative humidity at 37°. Key: □, cation-exchange resin; ○, sodium starch glycolate; ●, sodium carboxymethylcellulose; and ■, starch.*

The effect of storage (100% relative humidity) on disintegration time and hardness of tablets prepared from calcium phosphate dibasic dihydrate containing disintegrants is shown in Tables I and II. Generally, tablet hardness decreased and disintegration time increased under humid conditions. Although very little information is available on the effect of storage on calcium phosphate dibasic dihydrate tablets containing disintegrants, these results are in general agreement with those obtained with tablets prepared from lactose (7). The results presented in Tables I and II also show that the tablets containing effective disintegrants, which possess higher affinity for water, were most affected. The tablets containing cation-exchange resin and sodium starch glycolate showed a considerable increase in their disintegration time and a marked reduction in their strength after storage.

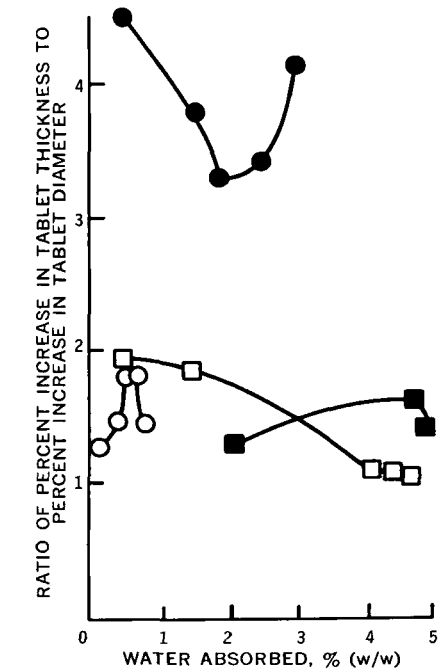

Figure 4—*Change of tablet diameter and thickness of calcium phosphate dibasic dihydrate tablets containing 10% (w/w) disintegrant when exposed to 100% relative humidity at 37°. Key: □, cation-exchange resin; ○, sodium starch glycolate; ●, sodium carboxymethylcellulose; and ■, starch.*

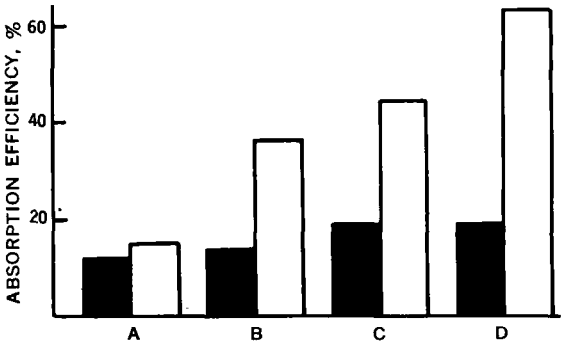

Figure 5—*Absorption efficiencies of disintegrant powders on exposure to 100% relative humidity at 37°. Dark bars represent 5 hr, and open bars represent 25 hr. Key: A, starch; B, sodium carboxymethylcellulose; C, sodium starch glycolate; and D, cation-exchange resin.*

Tablets containing a disintegrant break up rapidly in water because of the sudden and immediate application of stress. However, when a tablet containing such a disintegrant is exposed to water vapor, stress is built up slowly and the tablet absorbs some of the strain. Because the disintegrants within these tablets have lost some of their absorption and swelling ability, the disintegration time tends to increase. It can be seen that tablets containing disintegrants in which swelling is an important mechanism of action were most affected, *i.e.*, cation-exchange resin and sodium starch glycolate. Because swelling is not thought to be an important mechanism in the action of starch, the properties of tablets containing this disintegrant were not greatly affected on exposure to 100% relative humidity. The reduction in the strength of tablets can be explained by the absorption of moisture by the disintegrant, thus causing swelling and bond disruption. Because moisture has an especially deleterious effect on the disintegration time and hardness of tablets containing the most effective disintegrants (with high affinity for moisture), suitable packaging is of supreme importance when such a disintegrant is used in tablets.

A complete picture of the mechanism of action of sodium starch

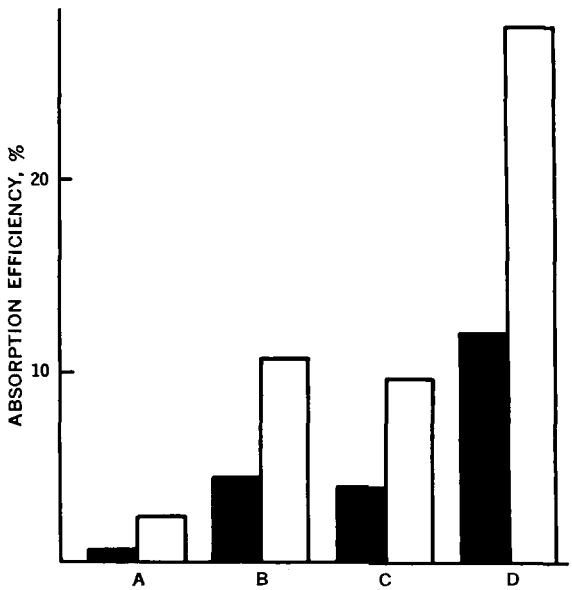

Figure 6—*Absorption efficiencies of calcium phosphate dibasic dihydrate tablets containing 10% (w/w) disintegrant on exposure to 100% relative humidity at 37°. Dark bars represent 5 hr, and open bars represent 25 hr. Key: A, starch; B, sodium carboxymethylcellulose; C, sodium starch glycolate; and D, cation-exchange resin.*

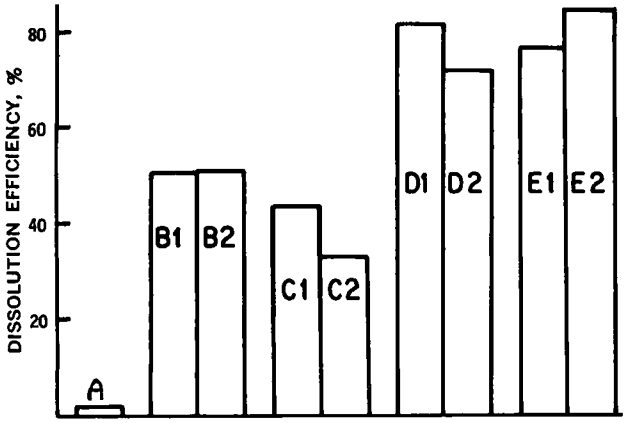

Figure 7—*Dissolution efficiencies of tablets prepared from calcium phosphate dibasic dihydrate containing 1% (w/w) amaranth as tracer. Key: A, without disintegrant; B1, starch, 5%; B2, starch, 10%; C1, sodium carboxymethylcellulose, 5%; C2, sodium carboxymethylcellulose, 10%; D1, sodium starch glycolate, 5%; D2, sodium starch glycolate, 10%; E1, cation-exchange resin, 5%; and E2, cation-exchange resin, 10%.*

glycolate emerges from the results presented in Figs. 8–10. On absorbing moisture, the sodium starch glycolate grains show considerable structural changes. Initially, the grains swell and become distorted, followed by the dissolution of disintegrant particles. The mechanism of action of sodium starch glycolate is probably similar to that of starch, as described by Lowenthal and Wood (4). Sodium starch glycolate possesses the additional advantages of being soluble and readily dispersible in water. Its spherical particles, dispersed in a tablet system, offer a larger surface, thus improving "wicking" (if this mechanism is operative) and allowing rapid penetration of water into the tablet interior (Fig. 4). The main reasons for the efficiency of this disintegrant probably are its high rate of water uptake (Fig. 1) and its marked swelling properties (Figs. 4 and 5); these factors cause pressure to be exerted within the tablet, thus breaking up interparticle bonding. This is then followed by the dissolution of sodium starch glycolate particles, which results in the crumbling and disintegration of the entire tablet structure.

Figures 11–13 show the surface structure of cation-exchange resin compacts before and after absorbing moisture (the absorbed moisture may be seen on the surface of compacts in Figs. 12 and 13). These photographs confirm previous findings that the resin possesses a very high rate of water uptake (Figs. 1–3). Kun and Kunin (5) described hydration of the cation-exchange resin as a two-step process: (*a*) the hydration of the gel phase or the microreticular phase, and (*b*) the filling of macroreticular pores with

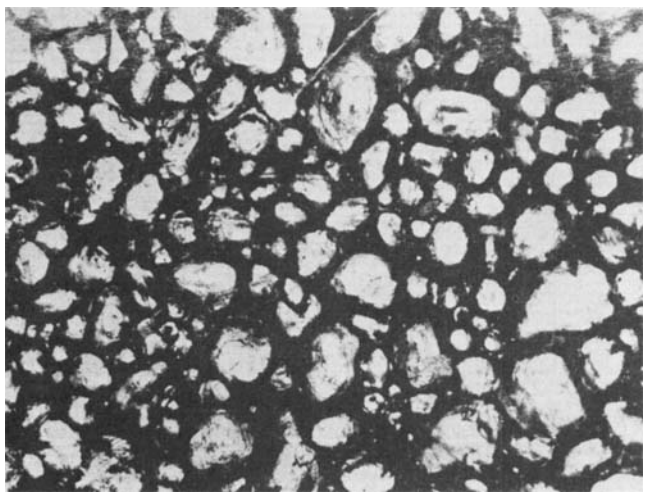

Figure 8—*Photomicrograph of sodium starch glycolate compact before exposure to humidity.*

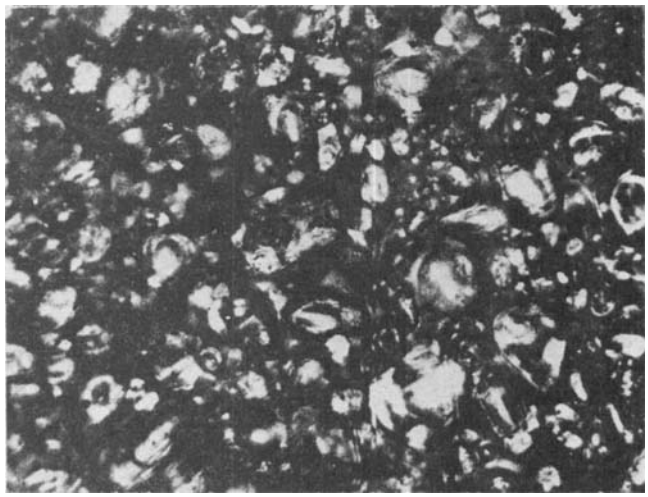

Figure 9—*Photomicrograph of sodium starch glycolate compact after 2 hr of exposure to 100% relative humidity at 37°.*

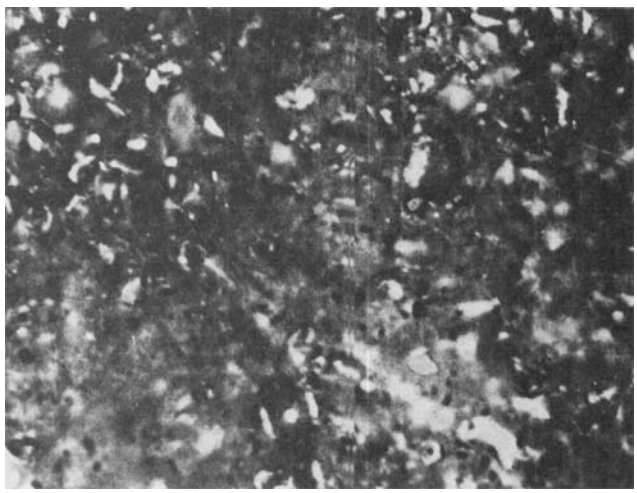

Figure 10—*Photomicrograph of sodium starch glycolate compact after 4 hr of exposure to 100% relative humidity at 37°.*

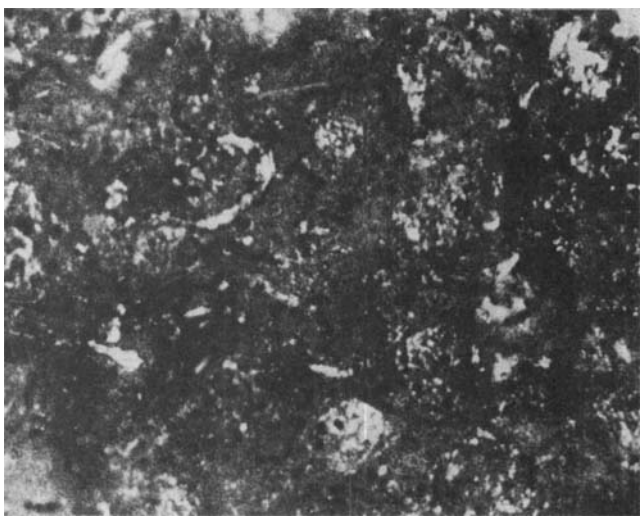

Figure 11—*Photomicrograph of cation-exchange resin compact before exposure to humidity.*

water, either by capillary condensation or hydraulically. From the results of the study of water sorption by the cation-exchange resin (Figs. 1, 2, and 11–13), it becomes clear that the disintegration of tablets containing the cation-exchange resin is probably caused by

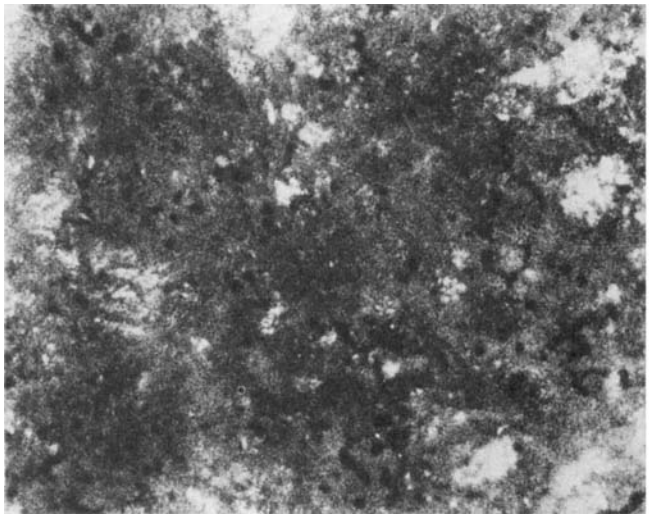

Figure 12—*Photomicrograph of cation-exchange resin compact after 2 hr of exposure to 100% relative humidity at 37°.*

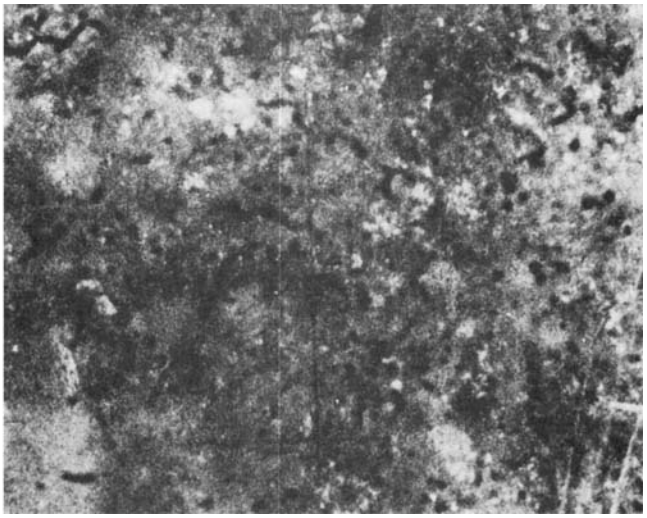

Figure 13—*Photomicrograph of cation-exchange resin compact after 4 hr of exposure to 100% relative humidity at 37°.*

the rapid penetration of water into the tablet interior and the crumbling of the tablet is brought about by the extensive swelling of resin particles. The nonadhesiveness of the cation-exchange resin in the hydrated state is also a useful property.

There is a similarity between starch and the cation-exchange resin in that both require a minimum effective concentration (1, 2). For starch it is said that a certain concentration is required to form an effective capillary system that will draw the water into the tablet interior (6, 7). Lowenthal and Wood (4), however, disagreed with this explanation and proposed that water hydrates the hydroxy group of starch molecules, causing them to move apart. The slight swelling that occurs is due to the rapid hydration step and a slower sorption of water step. These authors believed that the degree of breakup depends upon the number and size of starch grain agglomerates (total starch concentration in the tablet). The greater the number and size of agglomerate, the greater is the disruption. Similarly, a certain critical amount of cation-exchange resin would be necessary to produce sufficient swelling to rupture the tablet. Although sodium carboxymethylcellulose has a very high affinity for water, it is well known that its adhesiveness in the hydrated state is responsible for the poor disintegration properties (1, 2) of this material.

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Effects of Cyproterone Acetate on Uterine Motility in Rats

ELVERA R. ABBATIELLO ˣ and JOSEPH CATUOGNO

Abstract □ The effect of cyproterone acetate, an antiandrogen, on uterine contractility when the compound is administered to rats during midpregnancy was investigated. Pregnant Wistar rats were given cyproterone acetate subcutaneously on Days 8–11 of gestation. The results indicate that cyproterone acetate, either directly or indirectly, inhibits uterine contractility, an effect that may explain the prolongation of the gestational period observed in all treated animals.

Keyphrases □ Cyproterone acetate—effect on uterine motility, rats □ Uterine motility—effect of cyproterone acetate, rats □ Antiandrogens—effect of cyproterone acetate on uterine motility, rats

An antiandrogen is a substance that prevents the testicular hormone from exerting its normal effects on the organism without interfering with the secretion of the hormone (1). Therefore, an antiandrogen should not cause testicular atrophy. This appears to be true of the progestational steroid cyproterone acetate (6-chloro-1β,2β-dihydro-17-hydroxy-3'*H*-cyclopropa[1,2]pregna-1,4,6-triene-3,20-dione acetate). Its toxicity is low, the LD_{50} being greater than 2 g/kg orally in the mouse.

Preliminary studies have shown that cyproterone acetate is: *(a)* strongly progestational (being 250 times more active than progesterone), *(b)* weakly estrogenic (being less than $1/1000$ as active as estradiol[1]), and *(c)* weakly androgenic (being less than $1/64$ as active as testoterone propionate) (2).

When given to pregnant rats, cyproterone acetate antagonizes fetal testicular androgens, leading to feminization of the male offspring. Changes produced during sexual differentiation are always irreversible, in contrast to changes induced in the adult animal (3).

The extensive literature relative to the effects of cyproterone acetate on the developing offspring is incomplete since little, if any, work has been done to determine the effects of this compound on the pregnant mother. Recent investigations in this laboratory have shown the following:

1. The duration of gestation increases in cyproterone acetate-treated rats.
2. At the time of parturition, rats treated during gestation with cyproterone acetate cannibalized their offspring with much greater frequency than untreated rats. This effect has also been demonstrated in mice treated with cyproterone acetate (4, 5).

The present investigation was undertaken to study the effect of cyproterone acetate treatment during pregnancy on uterine contractions at the terminal stage of gestation.

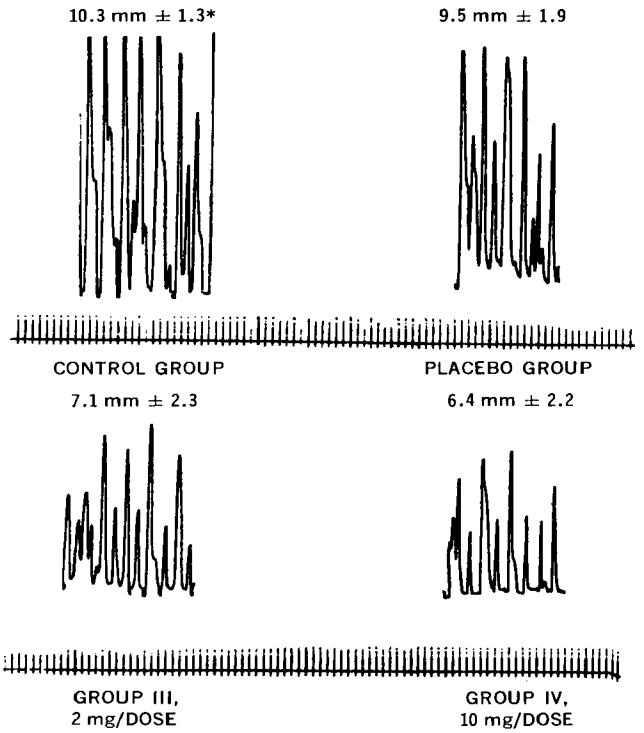

Figure 1—*Spontaneous contractions: paper speed = 0.0005 cm/sec; time marker = 30 sec; calibration = 5 mm of graph = 1 mm of contraction. *Height of contraction: mean ± SD.*

EXPERIMENTAL

Forty Wistar virgin female rats, average weight 295 g, were mated in a 2:1 ratio with 20 Wistar male rats[2]. Vaginal washings were performed to detect the presence of sperm or vaginal plugs, and positive washings were designated as Day 1 of pregnancy. The animals were then randomly divided into four groups of 10 animals.

Group I—The control group received no treatment.

Group II—The placebo group received 0.2 ml of a solution of benzyl benzoate[3] and peanut oil (50:50) subcutaneously on Days 8–11 of gestation.

Group III—This test group received 0.2 ml of a solution con-

[1] Vaginal smear method of Allen and Doisy (M. X. Zarrow *et al.*, "Experimental Endocrinology: A Sourcebook of Basic Techniques," Academic, New York, N.Y., 1964).

[2] The animals were obtained from Camm Research Institute, Wayne, N.Y.

[3] Eastman Organic Chemicals, Rochester, N.Y.

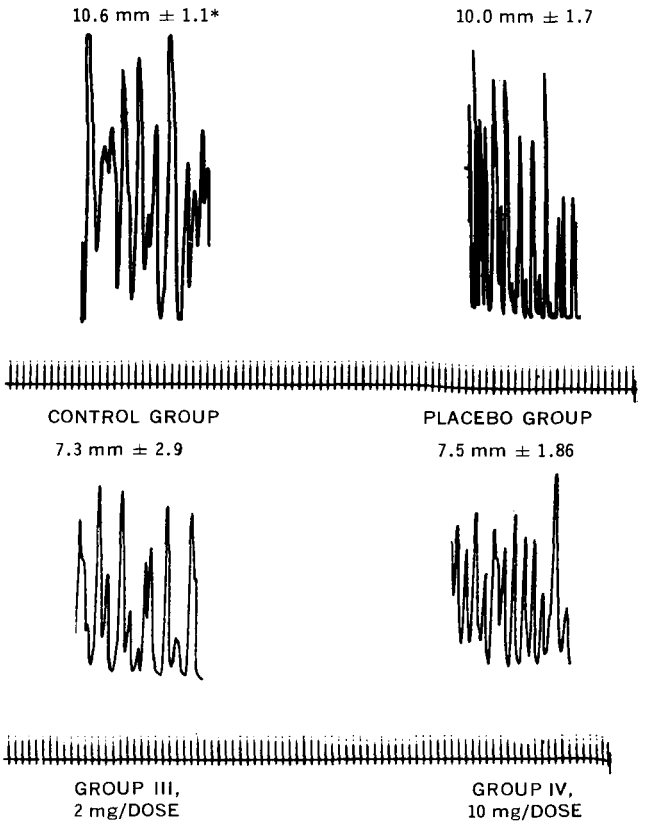

Figure 2—*The effect of 10 μunits of oxytocin on uterine contraction. Paper speed = 0.0005 cm/sec; time marker = 30 sec; calibration = 5 mm of graph = 1 mm of contraction. *Height of contraction: mean ± SD.*

taining 2 mg cyproterone acetate[4] dissolved in benzyl benzoate and 50% peanut oil subcutaneously on Days 8–11 of gestation. This dose was chosen because of its frequency of use in previous studies as the low dose producing an effect.

Group IV—This test group received 0.2 ml of a solution containing 10 mg cyproterone acetate dissolved in benzyl benzoate and 50% peanut oil subcutaneously on Days 8–11 of gestation. This dose was chosen because it was the standard dose used in several reported investigations (3, 7, 8).

On the 21st day of gestation, the animals were sacrificed. The abdominal cavity was opened, the fetuses were removed, and a 10-mm section of uterine muscle was obtained. This section was then cleaned of all fatty tissue and associated blood vessels and set up in a 10-ml isolated organ bath at 37.5°. The composition of the bathing solution was (grams per liter): NaCl, 9; KCl, 0.42; $NaHCO_3$, 0.5; $CaCl_2$, 0.24; $MgCl_2$, 0.44; and glucose, 0.5[5] (8). The bath was aerated with a gas mixture of 95% O_2 and 5% CO_2[6], and the pH was 7.

Mechanical records were obtained on the physiograph[7] through an isotonic myograph.

The spontaneous motility of the isolated preparation was studied for 45–60 min. Between 1 and 3 hr, the activity of the uterus decreases and this is the most convenient phase for the study of drug actions. The effects of 2.5 and 10 μunits of oxytocin[8] on the isolated uterine muscle were studied within this interval. Each dose of oxytocin was injected into the bath as a 0.1-ml solution.

Assay of the oxytocic effect was accomplished by a bracketing procedure (8). It consisted of four doses of oxytocin per comparison: 10 μunits followed by 2.5 μunits, then 2.5 μunits again, and, finally, 10 μunits. Each dose was allowed to act on the muscle for 10 min, followed by a flushing of the fluid and a rest period of 6 min before the next dose. The bath was also flushed 30 min after the initial setup.

[4] Lot 21236, Internal Code 11586B micronized, Schering Corp., Bloomfield, N.J.

[5] All chemicals except glucose were supplied by J. T. Baker Chemical Co., Phillipsburg, N.J. Glucose was supplied by Sigma Chemical Chemical Co., St. Louis, Mo.

[6] Matheson Co., East Rutherford, N.J.

[7] Desk model type DMP-4A, Narco-Bio-Systems, Inc., Houston, Tex.

[8] Pitocin, Parke-Davis, Detroit, Mich.

Table I—Statistical Analysis of Uterine Contractions

Spontaneous Contractions	*p*-Values[a]
Control *versus* placebo	N.S.
Control *versus* Group III	0.0025
Control *versus* Group IV	0.0005
Placebo *versus* Group III	0.0125
Placebo *versus* Group IV	0.0025
Group III *versus* Group IV	N.S.
10 μunits Oxytocin	
Control *versus* placebo	N.S.
Control *versus* Group III	0.0005
Control *versus* Group IV	0.0005
Placebo *versus* Group III	0.0005
Placebo *versus* Group IV	0.0005
Group III *versus* Group IV	N.S.
2.5 μunits Oxytocin	
Control *versus* placebo	N.S.
Control *versus* Group III	0.025
Control *versus* Group IV	N.S.
Placebo *versus* Group III	N.S.
Group III *versus* Group IV	N.S.

[a] *t*-Test of significance. Degrees of freedom = 18/test. N.S. = not significant.

RESULTS

The control group uterine sections exhibited strong, uniform, and continuous contractions, with similar results manifested by the sections obtained from placebo animals (Fig. 1). The mean strengths of contraction (*i.e.*, measurement of the strongest contraction per strip) were: control, 10.3 ± 1.3 mm; and placebo, 9.5 ± 1.9 mm.

The animals in Group III showed a variety of spontaneous activity. The range of contractions in the spontaneous activity portion was 3–10.5 mm, and the mean strength of contractions was 7.1 ± 2.3 mm. Group IV, except for three samples, exhibited weak but continuous contractions, with a mean strength of contraction of 6.4 ± 2.2 mm.

The decreased spontaneous contractions in the strips taken from the cyproterone acetate-treated animals suggests that estrogen dominance in the myometrium is not as great as in the control and placebo groups. The effects of 2.5 μunits of oxytocin on the isolated uterine sections are shown in Fig. 3. The mean strengths of contraction were: control, 7.4 ± 3.7 mm; placebo, 5.5 ± 4.0 mm; Group III, 4.7 ± 3.7 mm; and Group IV, 5.6 ± 1.8 mm.

The data obtained during this investigation indicate that no significant differences in the strengths of contractions exist between the control and placebo groups in any of the three parameters measured (Table I). Moreover, the strengths of contractions in Groups III and IV are significantly lower than those of the control and placebo groups when comparing spontaneous contractions and following the addition of 10 μunits of oxytocin to the tissue bath. Furthermore, the strengths of contractions in all groups following the addition of 2.5 μunits of oxytocin are lower but tend to return to levels comparable to those obtained spontaneously and subsequent to the addition of 10 μunits of oxytocin to the tissue bath.

DISCUSSION

In the rat, the data obtained during this investigation indicate that cyproterone acetate has an inhibiting effect on uterine contractions. This inhibitory effect is apparent in spontaneous contractions of the uterine segments as well as in oxytocin-induced contractions.

The ovulatory cycle in the rat is considered to be 5–6 days. The animal will mate only when in estrus, a period of high estrogen concentration. Once conception occurs, progesterone synthesis increases to a higher level than estrogen in an effort to maintain pregnancy. This high level of progesterone continues until approximately midpregnancy, at which time estrogen levels slowly begin to increase. Estrogen dominance occurs late in the last week

of pregnancy and is needed to initiate uterine excitability and induce contractions at a force capable of expelling the fetuses.

It appears from this study that the administration of cyproterone acetate, an antiandrogenic, progestational compound, at midpregnancy prolongs the progesterone dominance. That the action of this compound is prolonged is in agreement with the findings of Bridge and Scott (2) who observed that prostatic secretion in dogs treated with cyproterone acetate was abolished for a period greater than 47 days after the termination of drug treatment.

The duration of action of this compound would also explain the increase in the gestational period of rats treated at midpregnancy with cyproterone acetate and the inability of these animals to deliver at term. Since the undelivered fetuses continue to grow, forceful and painful expulsion might account for the high percentage of cannibalization observed in these animals (4).

Since it was determined that the force of contractions is reduced following the administration of cyproterone acetate, investigations are presently in progress to assay the levels of estrogens and progesterone in the treated animals. The results obtained from these studies are important in elucidating the mechanism of action of this compound on uterine contractility.

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Anticonvulsant Activity of *N,N'*-Bis[3-(3-substituted urea)propyl]piperazines

ARVIND K. CHATURVEDI [*], JAYANTI P. BARTHWAL [*], SURENDRA S. PARMAR [*‡x], and VIRGIL I. STENBERG [‡]

Abstract □ Several *N,N'*-bis[3-(3-substituted urea)propyl]piperazines were synthesized and characterized by their sharp melting points, elemental analyses, and IR spectra. All substituted piperazines were found to possess anticonvulsant activity, which was reflected by 20–70% protection observed against pentylenetetrazol-induced seizures in mice. Some of these compounds inhibited oxidation of pyruvic acid by rat brain homogenate. No correlation could be observed between the anticonvulsant activity possessed by these substituted piperazines and their ability to inhibit the oxidation of pyruvic acid.

Keyphrases □ *N,N'*-Bis[3-(3-substituted urea)propyl]piperazines—synthesis, anticonvulsant activity and relationship to inhibition of pyruvic acid oxidation □ Structure–activity relationships—*N,N'*-bis[3-(3-substituted urea)propyl]piperazines, anticonvulsant activity, rats, inhibition of pyruvic acid oxidation □ Oxidation, pyruvic acid—effect of piperazinoureas, relationship to anticonvulsant activity □ Piperazines, *N,N'*-bis[3-(3-substituted urea)propyl]—synthesis, anticonvulsant activity and relationship to inhibition of pyruvic acid oxidation

Anticonvulsant, antireserpine, and central nervous system (CNS) depressant properties exhibited by substituted piperazines (1, 2) and the inhibition of the oxidation of pyruvic acid by *N,N'*-bis[3-(3-substituted thiourea)propyl]piperazines (3) possessing anticonvulsant activity (4) led to the synthesis of some *N,N'*-bis[3-(3-substituted urea)propyl]piperazines (piperazinoureas). In the present study, attempts were made to correlate the anticonvulsant activity possessed by these piperazinoureas with their enzyme inhibitory effectiveness.

EXPERIMENTAL[1]

***N,N'*-Bis[3-(3-substituted urea)propyl]piperazines (I–XIII)**—Compounds I–XIII were prepared by refluxing a mixture of *N,N'*-bis(3-aminopropyl)piperazine[2] (0.01 mole) and the appropriate isocyanate (0.02 mole) in dry benzene on a steam bath for 2 hr. Excess benzene was removed by distillation under reduced pressure. The solid mass which separated out was collected by filtration and recrystallized from suitable solvents. These substituted piperazinoureas were characterized by their sharp melting points, elemental analyses[3], and IR spectra (Table I).

Determination of Anticonvulsant Activity—Anticonvulsant activity was determined in mice of either sex weighing 25–30 g. The mice were divided into groups of 10, keeping the group weights as near the same as possible. Each piperazinourea was suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compound was injected in a group of 10 animals at a dose of 100 mg/kg ip.

Four hours after the administration of the piperazinourea, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose

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Table I—Physical Constants of *N,N'*-Bis[3-(3-substituted urea)propyl]piperazines

Compound	R	Melting Point[a]	Yield, %	Recrystallization Solvent	Formula	IR Spectra[b] (CONH), cm^{-1}	Analysis, %	
							Calc.	Found
I	C_2H_5	149°	55	Dioxane	$C_{16}H_{34}N_6O_2$	1640	C 56.14	56.00
							H 9.94	10.00
							N 24.56	24.31
II	C_6H_5	202°	90	Dioxane	$C_{24}H_{34}N_6O_2$	1640	C 65.75	66.13
							H 7.76	7.85
							N 19.17	19.43
III	*o*-CH_3—C_6H_4	227°	60	Acetic acid	$C_{26}H_{38}N_6O_2$	1650	C 66.95	66.76
							H 8.15	8.42
							N 18.02	18.42
IV	*m*-CH_3—C_6H_4	195–197°	75	Nitrobenzene	$C_{26}H_{38}N_6O_2$	1650	C 66.95	67.03
							H 8.15	8.32
							N 18.02	18.13
V	*p*-CH_3—C_6H_4	225°	85	Acetic acid	$C_{26}H_{38}N_6O_2$	1660	C 66.95	67.25
							H 8.15	7.94
							N 18.02	18.20
VI	*o*-Cl—C_6H_4	235°	60	Acetic acid	$C_{24}H_{32}Cl_2N_6O_2$	1655	C 56.80	56.66
							H 6.31	5.95
							N 16.56	16.21
VII	*m*-Cl—C_6H_4	238°	77	Acetic acid	$C_{24}H_{32}Cl_2N_6O_2$	1650	C 56.80	56.96
							H 6.31	6.33
							N 16.56	16.70
VIII	*p*-Cl—C_6H_4	203° dec.	82	Acetic acid	$C_{24}H_{32}Cl_2N_6O_2$	1655	C 56.80	56.72
							H 6.31	6.55
							N 16.56	16.36
IX	*p*-Br—C_6H_4	>270°	83	Acetic acid	$C_{24}H_{32}Br_2N_6O_2$	1660	C 48.32	48.44
							H 5.37	5.57
							N 14.09	13.96
X	*o*-OCH_3—C_6H_4	175°	62	Acetonitrile	$C_{26}H_{38}N_6O_4$	1650	C 62.65	62.40
							H 7.63	7.94
							N 16.86	16.56
XI	*p*-OCH_3—C_6H_4	196°	82	Nitrobenzene	$C_{26}H_{38}N_6O_4$	1650	C 62.65	62.70
							H 7.63	7.78
							N 16.86	16.69
XII	*o*-OC_2H_5—C_6H_4	182°	60	Dioxane	$C_{28}H_{42}N_6O_4$	1655	C 63.87	64.01
							H 7.98	8.23
							N 15.96	16.08
XIII	*p*-OC_2H_5—C_6H_4	203°	80	Nitrobenzene	$C_{28}H_{42}N_6O_4$	1640	C 63.87	63.92
							H 7.98	8.15
							N 15.96	16.09

[a] All melting points were taken in open capillary tubes and are corrected. [b] All substituted piperazinoureas showed their characteristic absorptions for CONH.

of pentylenetetrazol has been shown not only to produce convulsions in almost all untreated mice but also to exhibit 100% mortality during 24 hr (5). However, no mortality was observed during 24 hr in animals treated with 100 mg/kg of the test compounds alone.

The mice were then observed for 60 min for seizures. An episode of clonic spasm that persisted at least 5 sec was considered a threshold convulsion. Transient intermittent jerks and tremulousness were not counted. Animals devoid of threshold convulsions during the 60 min were considered protected. The number of animals protected in each group was recorded, and the anticonvulsant activity of these substituted piperazinoureas was represented as percent protection. In the present study, no anticonvulsant activity was observed in animals treated with 5% aqueous gum acacia solution alone. The animals were then observed for 24 hr and their mortality was recorded.

Toxicity Studies—The approximate 50% lethal dose (approximate LD_{50} values) of the substituted piperazinoureas (Table II) was determined in albino mice of either sex, weighing 20–25 g, by following the method reported earlier (6).

Determination of Respiratory Activity of Rat Brain Homogenate[4]—Male albino rats, kept on *ad libitum* diet, were used in all experiments. Rats, 100–150 g, were sacrificed by decapitation. The brains were removed immediately and homogenized in ice-cold 0.25 *M* sucrose in a Potter–Elvehjem homogenizer in a ratio of 1:9 (w/v). All incubations were carried out at 37° in the conventional Warburg manometric apparatus, using air as the gas phase (5). The oxygen uptake was measured at 10-min intervals.

Fresh rat brain homogenate (1 ml) equivalent to 100 mg wet weight was added to chilled Warburg vessels containing 6.7 m*M* magnesium sulfate, 20 m*M* sodium hydrogen phosphate buffer solution (pH 7.4), 1 m*M* adenosine monophosphate (sodium salt), 33 m*M* potassium chloride, and 500 μg of cytochrome c in a final volume of 3 ml unless otherwise stated. The central well contained 0.2 ml of 20% KOH solution. Pyruvate was used at a final concentration of 10 m*M*. It was presumed that the endogenous NAD, present in this homogenate, was sufficient for the oxidation of sodium pyruvate.

All substituted piperazinoureas were dissolved in propylene glycol (100%) and were used at a final concentration of 1×10^{-3} *M*. An equal volume of propylene glycol was added to the control vessels.

RESULTS AND DISCUSSION

In the present study, *N,N'*-bis[3-(3-substituted urea)propyl]piperazines were synthesized and evaluated for their anticonvulsant activity. The degree of protection afforded by these piperazinoureas against pentylenetetrazol-induced seizures and the 24-hr mortalities are recorded in Table II. These results indicated that the compounds possessing 70% protection (X and XIII) also provided greater protection against pentylenetetrazol-induced mortality in the experimental animals. However, such a relationship between greater anticonvulsant activity and less mortality was not

[4] Commercial chemicals were used. Sodium pyruvate, adenosine monophosphate (sodium salt), and cytochrome c were obtained from Sigma Chemical Co., St. Louis, Mo. Other common chemicals were obtained from British Drug House, Bombay, India.

Table II—Anticonvulsant Activity of *N,N'*-Bis[3-(3-substituted urea)propyl]piperazines and Their Inhibitory Effects on the Oxidation of Pyruvic Acid by Rat Brain Homogenates

Compound	Anticonvulsant Activity[a], % Protection	Pentylenetetrazol Mortality[a] after 24 hr, %	Approximate LD_{50} Values[a], mg/kg	Inhibition[b], %
I	50	50	>3200	37.5 ± 1.44
II	50	50	>3200	Nil
III	50	40	1600	20.1 ± 1.40
IV	60	30	1600	17.4 ± 1.41
V	40	20	>1600	19.5 ± 1.95
VI	40	30	>1600	15.2 ± 1.51
VII	30	40	>1600	Nil
VIII	20	20	>1600	Nil
IX	40	30	1600	Nil
X	70	10	>1600	17.5 ± 1.20
XI	40	50	>1600	31.5 ± 0.85
XII	50	40	>1600	14.2 ± 0.92
XIII	70	20	1600	52.7 ± 2.60

[a] Screening procedures for the determination of the anticonvulsant activity and the approximate LD_{50} values are described in the text. Substituted piperazinoureas were administered (100 mg/kg ip) 4 hr before the administration of pentylenetetrazol (90 mg/kg). Mortality in pentylenetetrazol-treated animals was observed for 24 hr. [b] Vessel contents and assay procedures are described in the text. Each experiment was done in duplicate, and the values are the mean values of four separate experiments with ± standard error of the mean. The oxygen uptake was measured at 10-min intervals during a 1-hr incubation. The percentage inhibition was calculated from the decrease in the oxygen uptake per 100 mg wet brain weight. The mean oxygen uptake during oxidation of pyruvic acid, in the absence of piperazinoureas, was 150.46 ± 1.41 μl/100 mg/hr. The final concentrations of pyruvic acid and substituted piperazinoureas were 10 and 1 m*M*, respectively.

sufficiently uniform (*e.g.*, Table II: 50% protection = 50 or 40% mortality; 40% protection = 20 or 30% mortality; 20% protection = 20% mortality).

Each piperazinourea was found to be devoid not only of the sedative or CNS-depressant effect but also of 24-hr mortality in the 100-mg/kg dose used in the present study. Thus, 24-hr mortality in the experimental animals accounts for the toxic effects of pentylenetetrazol. The anticonvulsant activity of these piperazinoureas ranged from 20 to 70%. Maximum activity was observed with compounds possessing *o*-CH_3—C_6H_4 (X) and *p*-OC_2H_5—C_6H_4 (XIII) as substituents at position R of their structure. Minimum protection was observed with the piperazinourea having a *p*-Cl—C_6H_4 (VIII) substituent at position R. These piperazinoureas possessed low toxicity, which was reflected by their high approximate LD_{50} values of 1600 mg/kg or higher.

The inhibitory effects of substituted piperazinoureas on the oxidation of pyruvic acid by rat brain homogenates are presented in Table II. The degree of inhibition by these compounds, when used at a final concentration of 1×10^{-3} *M*, ranged from 14.2 to 52.7%; piperazinoureas possessing C_6H_5, *m*-Cl—C_6H_4, *p*-Cl—C_6H_4, and *m*-Br—C_6H_4 substituents at position R of their structure (II, VII, VIII, and IX, respectively) were devoid of the inhibitory property. Maximum inhibition was observed with the compound possessing the *p*-OC_2H_5—C_6H_4 substituent (XIII), while the piperazinourea possessing *o*-OC_2H_5—C_6H_4 substituent (XII) at position R was the least effective inhibitor.

Inhibition of certain metabolic processes in the brain has been proposed as a biochemical basis for the mechanism of action of various CNS depressants (7, 8). A parallelism was observed between greater *in vivo* hypnotic activity of some compounds and their ability to cause greater *in vitro* inhibition of respiration (9, 10). In spite of the maximum inhibition observed with Compound XIII, which also possessed maximum anticonvulsant activity, the present study failed to provide any definite correlation between the anticonvulsant activity possessed by these substituted piperazinoureas and their ability to inhibit the oxidation of pyruvic acid by rat brain homogenates.

Similar results were reported earlier with 3,4,5-trimethoxybenzamides (11). It is hoped that further detailed pharmacological and toxicological studies and investigations on the activity of substituted piperazinoureas on other enzyme systems will reflect a biochemical basis for their anticonvulsant activity.

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Inhibition of Supraeschar and Subeschar *Pseudomonas* Infection by Silver Sulfadiazine Dry Foam

PATRICK N. CATANIA ˣ and JAMES C. KING

Abstract □ The results of an *in vivo* evaluation of silver sulfadiazine dry foam are described. Using burned guinea pigs infected with *Pseudomonas aeruginosa*, silver sulfadiazine was applied every 12 hr as the dry foam or ointment. After 72 hr of therapy with the medicated dry foam, only one of the 15 burns remained infected while seven of the 15 burns remained positive for *Pseudomonas* after treatment with the corresponding medicated ointment ($p < 0.05 > 0.01$). It is suggested that the medicated dry foam provided significantly greater activity in treating a supraeschar burn wound infection of recent onset. In addition, a modified crossover study demonstrated that both the medicated dry foam and ointment are less effective in treating subeschar burn wound infections.

Keyphrases □ Silver sulfadiazine dry foam—inhibition of supraeschar and subeschar *Pseudomonas* infection, guinea pigs □ Burn therapy—inhibition of supraeschar and subeschar *Pseudomonas* infection by silver sulfadiazine dry foam □ *Pseudomonas* infections (burns)—inhibition by silver sulfadiazine dry foam □ Foams, dry—silver sulfadiazine, inhibition of *Pseudomonas* burn infections

Earlier, the formulation and in *vitro* evaluation of antibacterial dry foams were described (1). These data showed that the efficacy of the topical dry foams was not less than equivalent to that of corresponding medicated ointments. Furthermore, those dry foams containing silver salts appeared to possess significantly greater activity ($p < 0.001$) than that of ointments containing silver salts when therapeutic activity was monitored by *in vitro* methods. To determine further the efficacy of dry foams, the results of an *in vivo* evaluation of silver sulfadiazine dry foam are described.

EXPERIMENTAL

***In Vivo* Evaluation**—Each of 15 male guinea pigs was inflicted with four thermal injuries as previously described (2). All burns were challenged with an overnight broth culture of *Pseudomonas aeruginosa*[1], and each burn was assigned a specific therapeutic regimen. Therapy was initiated 30 min after inoculation and continued every 12 hr for 72 hr. One burn per animal was treated with 0.1 g of 2% silver sulfadiazine ointment, applied by means of a sterile blade. The second burn per animal was medicated with 2% silver sulfadiazine dry foam, applied in the form of 2.5-cm squares. An oil-in-water ointment[2] and dry foam control were applied to the third and fourth burns per animal, respectively. Swab cultures were obtained immediately prior to the application of the dosage forms. The results of this study are listed in Table I.

After 72 hr of treatment, a crossover study was initiated. The burn areas previously treated with the unmedicated dry foam were assigned therapy with the silver sulfadiazine dry foam, applied every 12 hr for 72 hr. In a similar manner, those areas previously treated with the oil-in-water ointment were assigned a treatment regimen of silver sulfadiazine ointment. Swab cultures were obtained every 12 hr for 72 hr immediately prior to the application of the dosage forms. The results are listed in Table II.

[1] ATCC 9721.

[2] Neobase Ointment, Burroughs Wellcome & Co., Research Triangle Park, N.C.

Table I—Results of Silver Sulfadiazine Therapy on Infected Burns

Hours[a]	Number of Animals[b] with Positive *Pseudomonas* Cultures[c] after Treatment[d] with: Silver Sulfadiazine Dry Foam	Silver Sulfadiazine Ointment	Dry Foam Control	Ointment Control
12	14	13	15	15
24	8	10	15	15
36	1	9	14	15
48	1	6	15	15
60	1	7	15	15
72	1[e]	7[e]	15	15

[a] Times listed are the hours after inoculation with an overnight broth culture of *Ps. aeruginosa*. [b] Each of 15 animals received four full thickness burns. [c] A positive culture was considered to be one in which five or more *Ps. aeruginosa* colonies were isolated and identified from a burn. [d] Treatment consisted of 2% silver sulfadiazine ointment, 2% silver sulfadiazine dry foam, an oil-in-water ointment control, or dry foam control applied to the involved site every 12 hr for 72 hr. [e] $p < 0.05 > 0.01$.

Identification of *Pseudomonas*—The isolation, identification, and quantification of the test bacteria were accomplished using visual, UV, and biochemical methods, as previously described (2).

RESULTS AND DISCUSSION

Earlier *in vitro* (1) and *in vivo* (2) studies demonstrated that 8.5% mafenide dry foam and 8.5% mafenide ointment were equally effective in treating *Pseudomonas* burn wound infections. However, since *in vitro* comparisons of the dry foam and ointment containing silver nitrate or silver sulfadiazine showed significant differences in therapeutic activity, *in vivo* studies were performed to document these preliminary conclusions.

Table II—Results of Crossover Silver Sulfadiazine Therapy on Infected Burns

Hours[a]	Number of Animals[b] with Positive *Pseudomonas* Cultures[c] after Treatment[d] with: Dry Foam/Silver Sulfadiazine Dry Foam	Ointment/Silver Sulfadiazine Ointment
12	15	15
24	15	15
36	14	15
48	15	15
60	15	15
72	15	15
84	13	15
96	10	12
108	7	11
120	8	8
132	6	7
144	4	5
156	4[e]	5[e]

[a] Times listed are the hours after inoculation with an overnight broth culture of *Ps. aeruginosa*. [b] Each of 15 animals received four full thickness burns. [c] A positive culture was considered to be one in which five or more *Ps. aeruginosa* colonies were isolated and identified from a burn. [d] Treatment consisted of dry foam control or an oil-in-water ointment control applied to the involved sites every 12 hr for 72 hr. This was followed by the application of 2% silver sulfadiazine dry foam or 2% silver sulfadiazine ointment to the involved sites every 12 hr for 72 hr. [e] $p = 0.1$.

After the initial 72 hr of treatment, all unmedicated control areas continued to demonstrate the presence of *Pseudomonas*. Of the 15 burns treated with silver sulfadiazine dry foam, one remained infected with the test bacteria. In contrast, seven of the 15 burns remained positive for *Pseudomonas* after treatment with the corresponding medicated ointment. Chi-square computations demonstrated the significance of these data ($p < 0.05 > 0.01$). However, at the conclusion of the *in vivo* crossover study, four burns treated with the medicated dry foam remained infected while five of the areas treated with the medicated ointment remained infected ($p = 0.1$).

In assessing the results of the initial *in vivo* study with those of the subsequent crossover study, several factors must be considered. The 72-hr delay in instituting therapy in the crossover study may have led to an infected environment not readily affected by rates of release from the dosage form. The proliferation of the test bacteria during this initial 72 hr probably resulted in widespread subeschar colonization. This movement of the bacteria into tissues below the burn produced a medium for growth not easily treated with topical agents.

In clinical use, both local and systemic therapy would be indicated to combat the advancing bacteria successfully. Furthermore, surgical excision of the devitalized eschar would be performed as an aid in the elimination of the infecting organism. The presence of eschar provides an excellent medium for bacterial growth and also provides a barrier between the applied drug product and the subeschar colonies, possibly preventing therapeutic contact and effect. Moreover, the protein binding characteristics of silver ion and the sulfonamide may prevent deep percutaneous absorption (3).

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Growth of Calcium Oxalate in Gel Systems

S. BISAILLON and R. TAWASHI [x]

Abstract □ Methods are described for growing calcium oxalate in silica and gelatin gels under different conditions. The results obtained indicate that, in silica gel, calcium oxalate grows into single individual crystals, twins, and rosettes. Bipyramidal calcium oxalate dihydrate crystals similar to those present in the urine of stone formers were prepared in the silica gel system. The gelatin gel offered a suitably structured substrate on which calcium oxalate monohydrate crystals grow into aggregates. The orientation pattern of calcium oxalate crystals suggests that the growth process is controlled by the stereospecificity of the gelatin medium supporting growth.

Keyphrases □ Calcium oxalate—growth of crystals in silica and gelatin gels, relevance to stone formation □ Crystal growth—calcium oxalate in silica and gelatin gel systems, types of growth □ Silica—substrate for growing calcium oxalate crystals □ Gelatin—substrate for growing calcium oxalate crystals

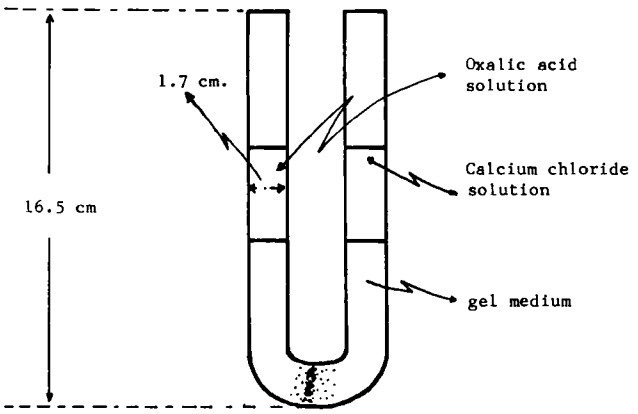

Figure 1—*Dimensions of the U-shaped tubes selected for the gel growth studies.*

Recent reports on the growth of calcium oxalate *in vitro*, either by precipitation from solution or from urine, generated valuable information on growth kinetics and the factors controlling crystal growth (1, 2). Attempts to grow crystal aggregates, similar to natural concretion, have so far met with little success (3, 4). This report describes a method for growing single crystals and artificial concretions of calcium oxalate. It is believed that this *in vitro* experimental model could be of fundamental significance in understanding the complex mechanisms of stone formation.

Studies on the crystallization of poorly soluble salts are often slowed down by the lack of methods for growing single crystals that permit close observations and understanding of the growth process and the factors controlling such phenomena. Interest in the gel medium for crystal growth has been greatly stimulated by the work of Henisch (5). Single crystals of calcium tartrate, tungstate, carbonate, and sulfate were grown using silica gel as the growth medium (6–10). This technique offered new opportunities for the production of useful single crystals and presented new observations in mechanism studies.

The growing pattern of crystals is dependent on the structure of the gel and the nature of the additives present in the gel medium. For very poorly soluble salts, the growth rate is essentially a diffusion process and the chemical reaction is not the rate-de-

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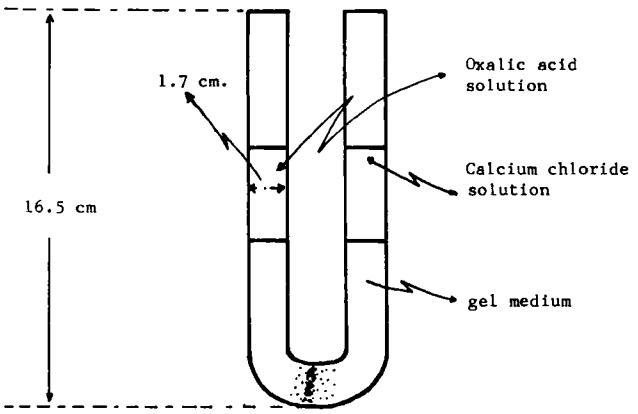

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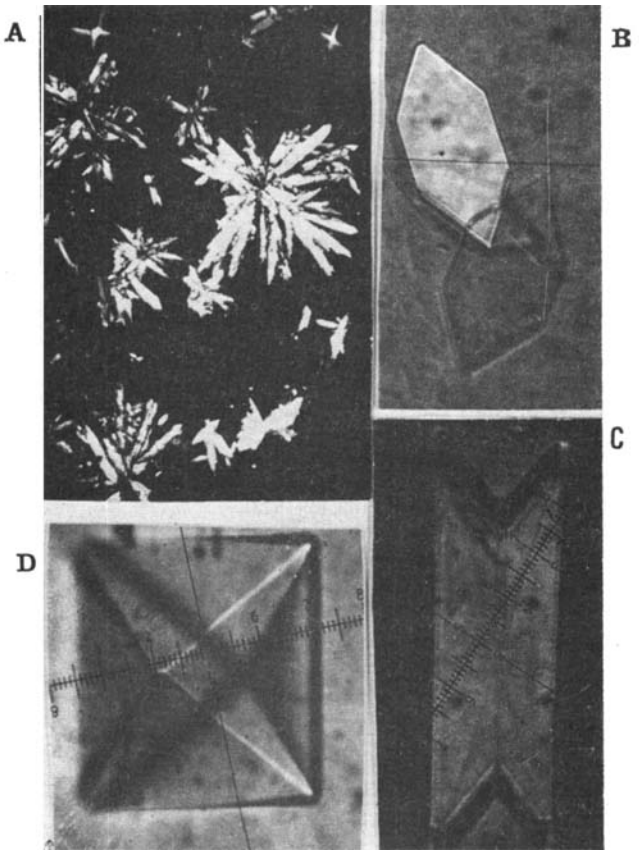

Figure 2—*Calcium oxalate crystals grown in silica gel. Key: A, calcium oxalate monohydrate (whewellite) grown as rosettes (×125); B, calcium oxalate monohydrate flat plate-like crystals (×500) grown using sodium oxatate as the source of oxalic ion instead of oxalic acid; C, twin production of whewellite (×500)—the incidence of this growth imperfection was higher when 60 ppm of magnesium was incorporated in the gel; and D, bipyramidal calcium oxalate dihydrate (weddelite) (×500) obtained when 100 ppm of pyrophosphate was added to the medium.*

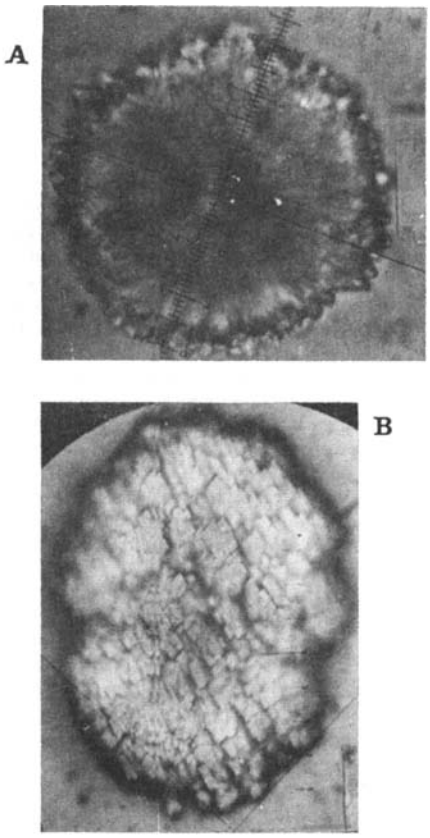

Figure 3—*Calcium oxalate crystals grown in gelatin gel. Key: A, calcium oxalate concretion (×500); and B, surface profile of the calcium oxalate crystallites in the concretions (×500).*

termining step (5). The gel acts as a support for the crystal and leads to its growth without exerting major forces upon it. The structure of the gel controls the whole process of diffusion and growth rate. The gel composition can play a role in orientation and build up of the crystalline material. Moreover, the presence of additives (any foreign substance other than the reacting species) can completely modify the crystal morphology.

In this study, calcium oxalate was selected because it represents the nucleus of two-thirds of the stones formed in the urinary tract (11) and the field of calcium oxalate crystallization is less well documented than is the crystallization of other calcium salts. From the foregoing, it is seen that the growing pattern (habit, size, quantity, and aggregation) is dependent on the structure of the gel and on the nature of the additives present in the gel medium.

EXPERIMENTAL

Two types of gels were studied: silica and gelatin. Silica was chosen because of its high inertia, and gelatin was studied because of its protein content and its structural analogy with collagen. The concentration chosen in each case to produce the gel was a compromise between the facts that a low concentration of gel will be too weak, allowing the ions to diffuse too rapidly and thus leading to numerous nuclei, and that a high concentration of gel will give poor crystals due to the very small pore size, thus interfering with the freely growing crystals.

In the case of the silica gel medium, a 10% solution of sodium metasilicate (reagent grade) was acidified to pH 6.2 with 3 *M* acetic acid. The solution was immediately poured into U-shaped tubes (Fig. 1) and stored in the dark for 24 hr to allow gelling. When additives were used to modify growth, they were incorporated in the gel medium before setting.

In the case of the gelatin (purified calfskin gelatin[1]), 55 g was dissolved in 1 liter of double-distilled water by gentle heating for 1 hr at 50°. The solution was partially cooled, and the pH was adjusted to 6.2 with tromethamine. To prevent mold growth during the incubation time, 1 ml of formaldehyde (37%) was added. Slow diffusion of the reacting ions was achieved by carefully placing 10 ml of 0.5 *M* calcium chloride on the gel surface on one side of the U-tube and 10 ml of 0.5 *M* oxalic acid on the gel surface on the other side.

The silica gel tubes were incubated at 37° and the gelatin tubes were incubated at 25° (to avoid liquefaction of the gel). After 6 days the calcium oxalate crystals were harvested and examined in polarized light under the microscope and by IR spectroscopy. The results were reproducible under each tested condition.

RESULTS AND DISCUSSION

Figure 2 shows calcium oxalate crystals grown in silica gel. In these experiments, when small concentrations (10 ppm) of magnesium, pyrophosphate, chlorophyll, and methylene blue were included as additives in silica gels, they slowed down the growth of calcium oxalate. On the other hand, the presence of pyrophosphate

[1] Eastman Kodak.

(100 ppm) favored the formation of the more energetic calcium oxalate dihydrate, apparently by lowering the energy needed for the formation of this form. It was found that calcium oxalate dihydrate formed in the presence of pyrophosphate is identical to the bipyramidal octahedral calcium oxalate dihydrate crystals obtained from the urine of chronic stone formers (12). The results obtained in silica gel are important for two reasons:

1. They emphasize the importance of environmental conditions on crystal morphology and growth rate of calcium oxalate crystals.
2. They show that it is possible to grow a single crystal of calcium oxalate dihydrate identical to those encountered *in vivo*.

In the gelatin gel system described, calcium oxalate grew in a completely different manner. Figure 3 shows the concretion found in the gelatin gel after 6 days and a surface profile illustrating the organization and orientation of the calcium oxalate crystallites in these aggregates. In this system (unlike silica gel), gelatin offered a suitably structured substrate on which calcium oxalate crystals nucleated and developed such an oriented pattern. The presence or absence of pyrophosphate or magnesium ions in the gel did not influence either the pattern of growth or the proportion of the calcium oxalate aggregates formed. This growth phenomenon indicates that the gelatin matrix and its protein moiety were capable of controlling the nucleation, growth, and orientation of calcium oxalate crystals.

Like silica gel, gelatin gel provided a favorable growth supporting medium on which the slowly diffusing calcium and oxalate ions nucleated; but by virtue of the stereospecificity of its nucleating sites, calcium oxalate crystals developed in the gelatin into such a degree of organization. The obtained results add experimental evidence for the important role of protein-structured substrate in dictating growth and specific orientation in the formation of calcium oxalate concretions. They also support Gebhardt's (13) assumption that epitaxial nucleation of stone components on suitably structured substrate (*e.g.*, collagen fibers) is the primary factor in stone formation.

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Synthesis of (4-Quinolinoamino)aminoalkyltetrahydronaphthalene Derivatives for Possible Antimalarial Activity

I. NABIH [x], S. ISMAIL, and M. NASR

Abstract □ (4-Quinolinoamino)aminoalkyltetrahydronaphthalene derivatives were synthesized in an attempt to introduce new agents with antimalarial activity.

Keyphrases □ (4-Quinolinoamino)aminoalkyltetrahydronaphthalene derivatives—synthesized and screened for antimalarial activity □ 4-Aminoquinoline compounds—synthesized and screened for antimalarial activity □ Antimalarial activity—(4-quinolinoamino)aminoalkyltetrahydronaphthalene derivatives

Compounds of the 4-aminoquinoline type still play a major role in the treatment of malaria. The development of resistance by some strains to most of the present antimalarials gave the initiative for several trials of new chemotherapeutic agents that may overcome this problem. In tropical countries where malaria eradication activities are still lacking, the provision of effective chemoprophylaxis and treatment represents a major problem.

DISCUSSION

In previous articles (1, 2), the synthesis and biological activity of a new compound 4-(7-chloro-4-quinolylamino)-2-diethylaminomethyl-5,6,7,8-tetrahydro-1-naphthol (I) were described. Structurally, the compound is related to 4-aminoquinoline and bears a substituted tetrahydronaphthalene (tetralin) system as a side chain. The rationale for including the tetralin system is that it may undergo metabolic transformation to a naphthoquinone type of structure.

Derivatives of this type have shown considerable antimalarial activity. They appear to act by inhibiting the respiration of plasmodia (3). New compounds that would structurally include both the nitrogen heterocycle and a naphthoquinone structure might be useful as antimalarials, since the resistance of the parasite to one should not imply resistance to the other because both act through different mechanisms.

Biological studies based on the response to the product by *Plasmodium berghei* in mice revealed that oral doses of I at 5–100 mg/kg are curative. In addition, the agent proved to give complete protection against later exposure to massive infection by the parasite through subcutaneous application (2), thus providing effective chemoprophylaxis beside chemotherapeutic activity.

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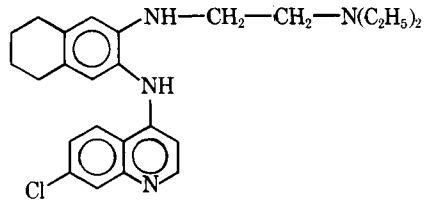
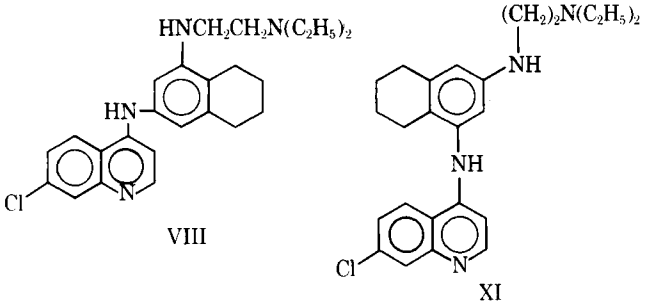


IV



VIII XI

The present work illustrates the synthesis of new compounds of the 4-aminoquinoline type, incorporating the tetrahydronaphthalene system whereby a diamino side chain is located at different positions on the aromatic part. In these structures the OH group on the aromatic part of the tetralin system was not included in order to investigate the biological significance for the presence of the phenolic OH on this part of the molecule.

Thus, it was decided to synthesize compounds structurally represented as IV, VIII, and XI. Reduction of 2-amino-3-nitro-5,6,7,8-tetrahydronaphthalene with stannous chloride and concentrated hydrochloric acid at about 150° gave 2,3-diamino-5,6,7,8-tetrahydronaphthalene (II) (4). Condensation of II with 4,7-dichloroquinoline gave 2-*N*-(7-chloro-4-quinolylamino)-3-amino-5,6,7,8-tetrahydronaphthalene (III). When III was reacted with β-diethylaminoethyl chloride, it gave 2-*N*-(7-chloro-4-quinolylamino)-3-(β-diethylaminoethylamino)-5,6,7,8-tetrahydronaphthalene (IV).

Condensation of 1-amino-3-nitro-5,6,7,8-tetrahydronaphthalene (V) with β-diethylaminoethyl chloride gave 1-(β-diethylaminoethylamino)-3-nitro-5,6,7,8-tetrahydronaphthalene (VI). Catalytic reduction of VI using 5% palladium-on-charcoal gave 1-(β-diethylaminoethylamino) - 3 - amino - 5,6,7,8-tetrahydronaphthalene (VII). On condensation with 4,7-dichloroquinoline, VII gave 1 - (β - diethylaminoethylamino) - 3-*N*-(7-chloro-4-quinolylamino)-5,6,7,8-tetrahydronaphthalene (VIII).

Condensation of V with 4,7-dichloroquinoline gave 1-*N*-(7-chloro - 4 - quinolylamino) - 3 - nitro - 5,6,7,8 - tetrahydronaphthalene (IX). Catalytic hydrogenation of IX (5% palladium-on-charcoal) gave 1-*N*-(7-chloro-4-quinolylamino)-3-amino-5,6,7,8-tetrahydronaphthalene (X). Treatment of X with diethylaminoethyl chloride yielded 1-*N*-(7-chloro-4-quinolylamino)-3-(β-diethylaminoethylamino)-5,6,7,8-tetrahydronaphthalene (XI).

EXPERIMENTAL[1]

Compound II—Compound II was prepared through reduction of 2-amino-3-nitro-5,6,7,8-tetrahydronaphthalene with stannous chloride and concentrated hydrochloric acid to give XXXVI, mp 135° [lit. (4) mp 136°].

Compound III—A mixture of 1 g (0.00617 mole) of II and 1.22 g (0.00617 mole) of 4,7-dichloroquinoline was refluxed in 10 ml of ethanol for 10 hr. Then this mixture was cooled and treated with ammonium hydroxide (25%) solution, and the precipitated product was filtered and recrystallized from ethanol to give 1.6 g (80% yield) of III, mp 300°; IR: 720 (Ar—Cl), 1590 (C=N), and 3250 (NH) cm^{-1}.

Anal.—Calc. for $C_{19}H_{17}ClN_3$: C, 70.81; H, 5.28; Cl, 10.87. Found: C, 70.53; H, 5.55; Cl, 11.01.

Compound IV—A mixture of 1 g (0.00308 mole) of III and 0.417 g (0.00308 mole) of β-diethylaminoethyl chloride was refluxed in 10 ml of ethanol for 10 hr. Then the reaction mixture was cooled and treated with 25% potassium hydroxide solution. The formed precipitate was recrystallized from ethanol to give 1.2 g of IV (91% yield), mp 180°; IR: 720 (Ar—Cl), 1590 (C=N), and 3250 (NH) cm^{-1}.

Anal.—Calc. for $C_{25}H_{31}ClN_4$: C, 71.10; H, 7.34; Cl, 8.41. Found: C, 71.03; H, 6.95; Cl, 8.81.

Compound V—Compound V was prepared by reduction of 1,3-dinitro-5,6,7,8-tetrahydronaphthalene with sodium sulfide and sodium bicarbonate, mp 77° [lit. (4) mp 78°].

Compound VI—A mixture of 1 g (0.0052 mole) of V and 0.7 g (0.0052 mole) of β-diethylaminoethyl chloride was refluxed in 10 ml of ethanol for 10 hr. The reaction was then cooled and treated with 25% ammonium hydroxide solution, the mixture was concentrated and then extracted with ether, the residual oil remaining after distillation of the ether was dissolved in absolute ethanol, and a stream of dry hydrogen chloride was allowed to pass in the solution. A hygroscopic hydrochloride was formed. The product VI was identified as the picrate, which melted after recrystallization from dilute ethanol at 128°; IR: 3340 (NH) and 3010 (cyclohexyl) cm^{-1}.

Anal.—Calc. for $C_{16}H_{25}N_3O_2 \cdot C_6H_3N_3O_7$: C, 50.58; H, 5.74; N, 16.09. Found: C, 50.75; H, 5.78; N, 16.10.

Compound VII—A mixture of 2 g (0.00686 mole) of VI (dissolved in 30 ml of absolute ethanol) and 0.3 g of 5% palladium-on-charcoal was hydrogenated. The solvent was concentrated, the charcoal was filtered, and a dry stream of hydrogen chloride was allowed to pass through the filtrate. A hygroscopic hydrochloride formed.

Product VII was identified as the picrate, which melted after recrystallization from dilute ethanol at 124–125°; IR: 3340 (NH), 3017 (cyclohexyl), and 3300 (NH_2) cm^{-1}.

Anal.—Calc. for $C_{16}H_{27}N_3 \cdot 2(C_6H_3N_3O_7)$: C, 47.63; H, 4.76; N, 17.14. Found: C, 47.31; H, 4.57; N, 17.20.

Compound VIII—A mixture of 1 g (0.00383 mole) of VII and 0.758 g (0.00383 mole) of 4,7-dichloroquinoline was refluxed in 10 ml of ethanol for 10 hr. The reaction mixture was neutralized with 25% ammonium hydroxide solution, and the formed precipitate was filtered and recrystallized from ethanol to give 1.5 g (93% yield) of VII, mp 115–117°; IR: 720 (Ar—Cl), 1590 (C=N), and 250 (NH) cm^{-1}.

Anal.—Calc. for $C_{25}H_{31}ClN_4$: C, 71.10; H, 7.34; Cl, 8.20. Found: C, 71.41; H, 7.12; Cl, 7.89.

Compound IX—A mixture of 1 g (0.00258 mole) of V and 1.02 g (0.00258 mole) of 4,7-dichloroquinoline was refluxed in 10 ml of ethanol for 10 hr. The reaction mixture was cooled and treated with 25% potassium hydroxide solution, and the formed precipitate was filtered and recrystallized from ethanol to give 1.5 g (85% yield) of IX, mp 129–130°; IR: 720 (Ar—Cl) and 250 (NH) cm^{-1}.

Anal.—Calc. for $C_{19}H_{16}ClN_3O_2$: N, 11.89; Cl, 9.91. Found: N, 11.85; Cl, 10.18.

Compound X—A mixture of 5 g (0.013 mole) of IX (dissolved in 100 ml of absolute ethanol) and 0.5 g of 5% palladium-on-charcoal was hydrogenated. The catalyst was filtered, and the solvent was concentrated. The formed precipitate was recrystallized from ethanol to give 4 g (85% yield) of X, mp 182–184°; IR: 720 (Ar—Cl) and 3250 (NH) cm^{-1}.

Anal.—Calc. for $C_{19}H_{18}ClN_3$: C, 70.58; H, 5.57; Cl, 10.83. Found: C, 70.30; H, 5.61; Cl, 10.51.

Compound XI—A mixture of 1 g (0.00308 mole) of X, 0.4 g (0.00308 mole) of XLIV, and 0.4 g (0.00308 mole) of β-diethylaminoethyl chloride was refluxed for 7 hr. The formed precipitate was isolated and recrystallized from ethanol to give 1.1 g (77% yield) of XI, mp 300° dec.; IR: 720 (Ar—Cl), 1590 (C=N), and 3250 (NH) cm^{-1}.

Anal.—Calc. for $(C_{25}H_{31}ClN_4 \cdot HCl)_2H_2O$: C, 64.34; H, 7.06; Cl, 15.20. Found: C, 64.13; H, 6.84; Cl, 14.99.

Biological Testing—Compounds IV, VII, and XI were submitted to biological testing. The test was based on the evaluation of response of *P. berghei* in mice (5). The compounds were tested by oral and subcutaneous routes, and none of the compounds showed any activity at the tolerated levels.

[1] Melting points were taken in open capillary tubes with a Gallenkamp electric melting-point apparatus and are uncorrected. The IR spectra were recorded with a Carl Zeiss Infra-cord spectrophotometer, model UR 10. Microanalyses were performed by the microanalytical laboratory, National Research Center, Cairo, U.A.R., and the Spang Microanalytical Laboratory, Ann Arbor, Mich.

These findings suggest that the presence of the naphtholic OH group, as in I, is still necessary for biological activity within this group. Apparently, due to the possibility of chelation with the cations necessary for the metabolic functions of the parasite, the oxygen of this group and the nitrogen of the neighboring side chain may contribute in the cation complex formation.

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Synthesis and Biological Properties of Alkyl Esters of Polyene Antibiotics

T. BRUZZESE [x], M. CAMBIERI, and F. RECUSANI

Abstract □ Several new alkyl esters of polyene antibiotics were prepared by an improved general procedure, and their toxicity and microbiological activity were tested. Some of these alkyl esters were more active than the nonesterified polyenes against *Candida albicans* but were less effective than the known methyl esters. Their toxicity was much less than that of the parent compounds.

Keyphrases □ Polyene antibiotics, alkyl esters—synthesis, antifungal activity □ Antifungal agents—synthesis and testing of alkyl esters of polyene antibiotics

The first attempt to reduce the toxicity of the macrolidic polyenes consisted of reacting the amino groups to give the corresponding *N*-acyl derivatives (1, 2), but these compounds were shown to be less active than the original antibiotics so chemotherapeutic employment was out of the question.

Since most natural polyenes have amphoteric characteristics, a new approach blocking the carboxyl group was developed and the first derivative, partricin methyl ester, was more active and less toxic than the parent antibiotic (3, 4). The methyl esters of other polyenes also gave good results (5, 6).

To continue this research, some homologous alkyl esters were prepared to determine whether other factors, such as the structure of the substituent group, could influence the biological activity of these polyene derivatives. The compounds were prepared by allowing the natural polyene to react with excess diazoalkane in the presence of ammonium hydroxide or other organic bases; surprisingly, the polyenes formed fewer by-products and thus biologically more active esters when in a basic medium.

The esterification of the carboxyl was confirmed by IR and NMR spectra, while TLC (Table I) on silica gel confirmed the quality of the products.

The polyene esters are pale-yellow to dark-yellow solids, which are practically insoluble in water, alkali, and the usual organic solvents and are very soluble in dimethyl sulfoxide, 2-methoxyethanol[1], and pyridine. The UV spectra show the typical absorbance patterns of the initial heptaenes and tetraenes (7).

All compounds were tested for their antifungal activity and acute toxicity in mice (LD_{50}); the results are reported in Table I in comparison with the parent substances and the known methyl esters. Since partricin is also active against protozoa, its esters were tested for their antitrichomonal activity in a preliminary manner.

EXPERIMENTAL

General Procedure of Synthesis—Concentrated ammonium hydroxide (0.5 ml) and then a 1.5% solution of diazoalkane in ether (10 ml) were added dropwise with stirring to a solution of polyene antibiotic (1 g) in dimethyl sulfoxide (20 ml). The mixture was kept at room temperature for 3 hr, and then ether (200 ml) was added to give a pasty solid. The precipitate was treated with acetone–ether, giving a high yield of the required ester.

TLC—TLC was carried out on silica gel[2] 60 F_{254}, using butanol–ethanol–acetone–32% ammonium hydroxide (2:5:1:3) as eluent and detecting the spots by exposure to UV light.

Antifungal Activity—The compounds were tested against *Candida albicans*, strain 200[3]. The test strain was cultured in Sabouraud medium[4] for 18 hr at 36° and diluted to 10% transmittance at 580 nm. Then about 0.15 ml of the diluted test strain was inoculated in 100 ml of Sabouraud medium so that the final test tubes contained 10^6 cells.

The substances were dissolved in dimethyl sulfoxide and serially diluted with sterile distilled water; 0.5 ml of each dilution was added to 4.5 ml of the inoculated broth, and all tubes were incubated for 24 hr at 36°. The results are reported as the minimum inhibitory concentration (MIC), *i.e.*, the lowest concentration of polyene antibiotic at which no visible growth was observed.

Acute Toxicity—The approximate LD_{50} was determined in groups of five female Swiss mice, 20–24 g. The compounds were

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Table I—Alkyl Esters of Polyene Antibiotics

Compounds	R_f	MIC, μg/ml[a] (*C. albicans*)	Approximate LD_{50} (Mice) mg/kg ip[b]	mg/kg iv[c]
Partricin	0.51	0.125[d]	<5[e]	—
Partricin methyl ester	0.84	0.015 (0.062)[f]	200[g]	—
Partricin ethyl ester	0.88	0.015 (0.062)	100	—
Partricin propyl ester	0.90	0.031	200	—
Partricin butyl ester	0.92	0.062	>200	—
Amphotericin B	0.41	0.125	—	5
Amphotericin B methyl ester	0.79	0.062 (0.125)	—	75
Amphotericin B ethyl ester	0.82	0.125–0.250	—	>50
Amphotericin B propyl ester	0.84	0.250	—	>50
Amphotericin B butyl ester	0.87	0.250–0.500	—	>50
Candicidin	0.55	0.062	5	—
Candicidin methyl ester	0.86	0.062	75	—
Candicidin ethyl ester	0.89	0.125 (0.500)	200	—
Candicidin propyl ester	0.92	0.125	50	—
Candicidin butyl ester	0.92	0.250	100	—
Nystatin	0.42	1	150	—
Nystatin methyl ester	0.77	1	>200	—
Nystatin ethyl ester	0.79	2–4	>200	—
Nystatin propyl ester	0.80	4 (4)	>200	—
Nystatin butyl ester	0.81	8	>200	

[a] Values in parentheses refer to samples obtained in neutral medium. [b] Suspended in 0.5% carboxymethylcellulose. [c] Solubilized with sodium deoxycholate (1:1). [d] Lit. (8) MIC 0.2 μg/ml. [e] Lit. (8) LD_{50} 0.5 mg/kg. [f] Lit. (4) MIC 0.05 μg/ml. [g] Lit. (4) LD_{50} 200 mg/kg.

administered intraperitoneally (0.5% carboxymethylcellulose suspension, 20 ml/kg), except for amphotericin and its esters which had low toxicity in suspension (LD_{50} > 1000 mg/kg) and were tested intravenously after solubilization with sodium deoxycholate (1:1). Deaths were recorded after 7 days.

RESULTS AND DISCUSSION

The results indicate that all of the esters are antifungal and that some may be more active than the parent compounds. Nevertheless, the lengthening of the aliphatic chain in the ester group (methyl to butyl) produced a gradual decrease in activity.

On the whole, the highest increases in activity were obtained by esterifying the heptaenes, while the most active derivative (methyl ester) of the tetraene nystatin was only as potent as the natural product.

The best results in the heptaenes were provided by amphotericin (the activity of its methyl ester was doubled) and particularly by partricin. The methyl and ethyl esters of partricin were the most active, their MIC value against *C. albicans* being around 0.015 μg/ml and thus eightfold more active than the nonesterified product (MIC 0.125 μg/ml). In spite of the usual decrease in activity, the butyl ester also maintained a practically doubled activity.

The acute toxicity in mice by the intraperitoneal or intravenous route appeared to be considerably reduced with all alkyl esters, and there were no marked differences within each series of esters up to the maximum doses tested.

Any antitrichomonal activity possessed by the natural antibiotic (partricin MIC 0.25 μg/ml) seemed to be maintained (although slightly reduced) after esterification. No sensitive variation was seen in the series of the homologous esters that showed MIC values around 0.5–2 μg/ml.

To conclude, the blocking of the carboxyl of the amphoteric polyene antibiotics by alkyl esterification appears to diminish the toxicity while at the same time maintaining the microbiological activity. However, lengthening the alkyl chain provides less active esters.

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Mathematical Formulation for Nonuniform Multiple Dosing

Keyphrases □ Pharmacokinetics—mathematical derivation of nonuniform multiple-dosing equation □ Multiple dosing, nonuniform—mathematical derivation of pharmacokinetic equation

To the Editor:

Consider a three-compartment model with all possible arrows between compartments and an "out" arrow from each compartment. Let k_{ij}, in inverse time units, represent a transfer rate constant from compartment i to compartment j, and let k_{i0} represent the out transfer from compartment i to the outside. Now if x_i is the amount of substance in compartment i, the law of mass action and the hypothesis of simple diffusion lead to the set of differential equations, written in matrix form, where $\dot{x} = dx/dt$:

$$\begin{pmatrix} \dot{x}_1 \\ \dot{x}_2 \\ \dot{x}_3 \end{pmatrix} = \begin{pmatrix} -k_1 & k_{21} & k_{31} \\ k_{12} & -k_2 & k_{32} \\ k_{13} & k_{23} & -k_3 \end{pmatrix} \begin{pmatrix} x_1 \\ x_2 \\ x_3 \end{pmatrix} \quad \text{(Eq. 1)}$$

Here, $k_1 = k_{10} + k_{12} + k_{13}$, $k_2 = k_{21} + k_{20} + k_{23}$, and $k_3 = k_{31} + k_{32} + k_{30}$.

Let the initial conditions be x_{10}, x_{20}, and x_{30}. Also let the 3 by 3 array of k values of Eq. 1 be represented by capital K, and let bold face denote a vector. Then Eq. 1 can be written as:

$$\mathbf{x} = K\mathbf{x} \quad \text{(Eq. 2)}$$

with initial condition vector $\mathbf{x}_0$.

The structure of the matrix K is evident. There are minus signs on the diagonal elements. The ith diagonal element is the negative sum of all the out arrows from compartment i or, in other words, the negative sum of all k values in column i and the out k from compartment i. Aside from the diagonal elements, the pattern of subscripts in the matrix K is evident. For example, for n compartments, we have:

$$K = \begin{pmatrix} -k_1 & k_{21} & \cdots & k_{n1} \\ k_{12} & -k_2 & \cdots & k_{n2} \\ \cdots & \cdots & & \cdots \\ k_{1n} & k_{2n} & \cdots & -k_n \end{pmatrix} \quad \text{(Eq. 3)}$$

where the diagonal elements are formed as described previously. In the case where some arrows in a particular model are known not to exist, the corresponding k values above are set to zero.

Now consider the use of these differential equations and the equivalent compartmental model to simulate drug transport in pharmacokinetic studies. Suppose that we want to simulate a multiple-dose regimen, where each dose is specified as to the amount of drug given and the time administered.

Niebergall *et al.* (1) stated that "no equation developed to date allows the drug to be administered at varying time intervals." The following development produces such an equation. We must first explain some of the mathematical equipment used. Let $\delta(t - a)$ denote the Dirac delta function, which can be described as a rectangle of infinite height, infinitesimal width, and area 1. More precisely, let:

$$F_w(t - a) = \begin{cases} \frac{1}{w}, a \leq t \leq a + w \\ 0 \text{ otherwise} \end{cases} \quad \text{(Eq. 4)}$$

Then:

$$\lim_{w \to 0} F_w = \delta(t - a) \quad \text{(Eq. 5)}$$

Heaviside's unit step function is defined as:

$$U(t - a) = \begin{cases} 0, t < a \\ 1, t > a \end{cases} \quad \text{(Eq. 6)}$$

which can be described as a step of height 1 located at $t = a$. Standard Laplace transform tables give:

$$L\,\delta(t) = 1 \quad \text{(Eq. 7)}$$

$$L\,\delta(t - a) = \exp(-as) \quad \text{(Eq. 8)}$$

$$LU(t) = 1/s \quad \text{(Eq. 9)}$$

$$LU(t - a) = [\exp(-as)]/s \quad \text{(Eq. 10)}$$

$$LF(t - a)U(t - a) = [\exp(-as)]f(s) \quad \text{(Eq. 11)}$$

Pipes (2) showed that ordinary Laplace transform pairs have their matrix analogs. For example, if a is a scalar:

$$L\exp(at) = (s - a)^{-1} \quad \text{(Eq. 12)}$$

but if A is a square matrix, then:

$$\exp(At) = (I + At + A^2t^2/2! + \ldots) \quad \text{(Eq. 13)}$$

and:

$$L\exp(At) = (sI - A)^{-1} \quad \text{(Eq. 14)}$$

where I is the identity matrix with ones on the diagonal and zeros elsewhere. The superscript denotes matrix inverse.

A simple example will aid in understanding the following mathematical development. Consider a one-compartment open model representing the central or "blood" compartment with an output rate constant k, with one dose injected "instantaneously" at time $t = t_0$ and another dose similarly administered at time $t = t_1$.

The differential equation for this model can be written as:

$$\dot{x} = -kx + \delta(t - t_1) \quad \text{(Eq. 15a)}$$

$$x(0) = x_0 = 1 \quad \text{(Eq. 15b)}$$

Taking the Laplace transform of both sides, we get:

$$sLx - x_0 = -kLx + \exp(-t_1 s) \quad \text{(Eq. 16)}$$

Solving for Lx, we get:

$$Lx = (s + k)^{-1}x_0 + (s + k)^{-1}\exp(-t_1 s) \quad \text{(Eq. 17)}$$

Taking the inverse Laplace transform, we get:

$$x = \exp(-kt)x_0 + \exp[-k(t - t_1)]U(t - t_1) \quad \text{(Eq. 18)}$$

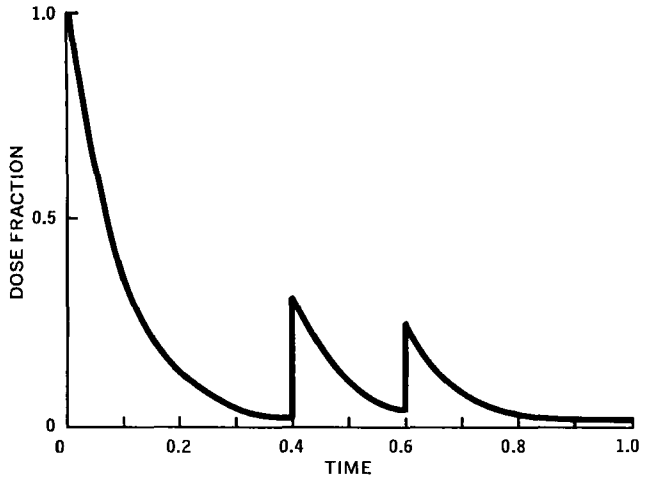


Figure 1—*Dose fraction in Compartment 1 as a function of time.*

This solution can also be written as:

$$x = \exp[-k(t - 0)]U(t - 0) + \exp[-k(t - t_1)]U(t - t_1) \quad \text{(Eq. 19)}$$

which suggests the use of a summation notation for multiple injections.

Consider a three-compartment model as above with multiple-dose amounts a_i at time t_i delivered into Compartment 1. For simplicity, let $i = 1$ and 2, so that the differential equations, in matrix notation, are:

$$\mathbf{x} = K\mathbf{x} + \begin{pmatrix} a_1\delta(t - t_1) + a_2\delta(t - t_2) \\ 0 \\ 0 \end{pmatrix} \quad \text{(Eq. 20)}$$

with a vector $\mathbf{x}_0$ of initial conditions, where δ denotes the Dirac delta function. Taking Laplace transforms, we get:

$$sL\mathbf{x} - \mathbf{x}_0 = KL\mathbf{x} + \begin{pmatrix} a_1 \exp(-t_1 s) \\ 0 \\ 0 \end{pmatrix} + \begin{pmatrix} a_2 \exp(-t_2 s) \\ 0 \\ 0 \end{pmatrix} \quad \text{(Eq. 21)}$$

Collecting terms and solving for $L\mathbf{x}$, we get:

$$L\mathbf{x} = (sI - K)^{-1}\mathbf{x}_0 + (sI - K)^{-1}\begin{pmatrix} a_1 \exp(-t_1 s) \\ 0 \\ 0 \end{pmatrix} + (sI - K)^{-1}\begin{pmatrix} a_2 \exp(-t_2 s) \\ 0 \\ 0 \end{pmatrix} \quad \text{(Eq. 22)}$$

Taking the inverse Laplace transform, we get:

$$\mathbf{x} = \exp(Kt)\mathbf{x}_0 + \exp[K(t - t_1)]\begin{pmatrix} a_1U(t - t_1) \\ 0 \\ 0 \end{pmatrix} + \exp[K(t - t_2)]\begin{pmatrix} a_2U(t - t_2) \\ 0 \\ 0 \end{pmatrix} \quad \text{(Eq. 23)}$$

where U denotes the Heaviside unit step function. When there are n compartments with m multiple doses, we have:

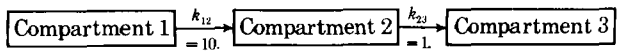
$$\mathbf{x} = \exp(Kt)\mathbf{x}_0 + \sum_{i=1}^{m} \exp[K(t - t_i)]\begin{pmatrix} a_iU(t - t_i) \\ 0 \\ \cdots \\ 0 \end{pmatrix} \quad \text{(Eq. 24)}$$

The matrix exponential function, $\exp(Kt)$, can be calculated directly from its defining expansion given in Eq. 13. Some timesaving shortcuts were given by Pipes and Hovanessian (3).

As an example, let:

$$K = \begin{pmatrix} -10. & 0. & 0. \\ 10. & -1. & 0. \\ 0. & 1. & 0. \end{pmatrix} \qquad \mathbf{x}_0 = \begin{pmatrix} 1. \\ 0. \\ 0. \end{pmatrix} \quad \text{(Eq. 25)}$$

representing Scheme I.



Scheme I

Let $a_1 = .3$, $t_1 = .4$, $a_2 = .2$, and $t_2 = .6$. Calculate $\mathbf{x}$ at $t = 1$ by substituting these values in Eq. 23 to get:

$$\mathbf{x}(1) = \begin{pmatrix} .0000 & .0000 & 0. \\ .4087 & .3679 & 0. \\ .5913 & .6321 & 1. \end{pmatrix}\begin{pmatrix} 1. \\ 0. \\ 0. \end{pmatrix} + \begin{pmatrix} .0025 & .0000 & 0. \\ .6070 & .5488 & 0. \\ .3905 & .4512 & 1. \end{pmatrix}\begin{pmatrix} .3 \\ .0 \\ .0 \end{pmatrix} + \begin{pmatrix} .0183 & .0000 & 0. \\ .7244 & .6703 & 0. \\ .2572 & .3297 & 1. \end{pmatrix}\begin{pmatrix} .2 \\ .0 \\ .0 \end{pmatrix} =$$

$$\begin{pmatrix} .0000 \\ .4087 \\ .5913 \end{pmatrix} + \begin{pmatrix} .0008 \\ .1821 \\ .1172 \end{pmatrix} + \begin{pmatrix} .0037 \\ .1449 \\ .0514 \end{pmatrix} = \begin{pmatrix} .0045 \\ .7357 \\ .7599 \end{pmatrix} \quad \text{(Eq. 26)}$$

where a computer program is used to evaluate the matrix exponential functions by Eq. 13.

To link the solution $\mathbf{x} = \exp(Kt)\mathbf{x}_0$ of Eq. 2 to its solution in terms of scalar exponentials, use known matrix theory (see, for example, Ref. 4).

Let Λ be the diagonal matrix of eigenvalues λ_1, λ_2, . . . , λ_n of K, and let D be a diagonal matrix with diagonal elements $\exp(\lambda_1 t)$, $\exp(\lambda_2 t)$, . . . , $\exp(\lambda_n t)$. Hearon (5) proved that these eigenvalues are all real and nonpositive when Eq. 2 describes a linear com-

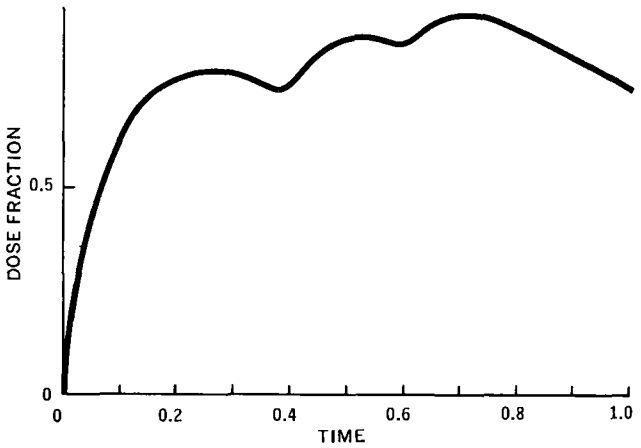


Figure 2—*Dose fraction in Compartment 2 as a function of time.*

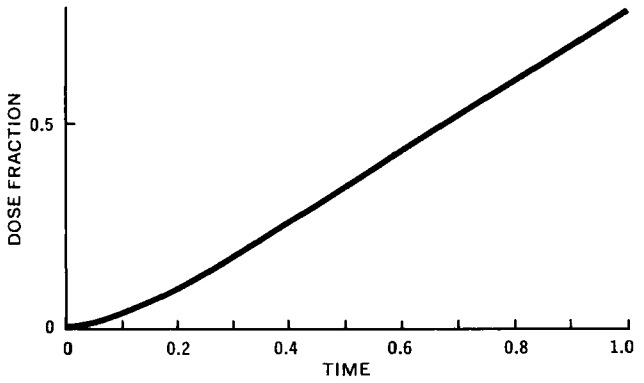


Figure 3—*Dose fraction in Compartment 3 as a function of time.*

partment model, even though K is nonsymmetric. Let $\mathbf{p}_i$ be the real eigenvector of K associated with λ_i, where $P = (\mathbf{p}_1, \mathbf{p}_2, \ldots, \mathbf{p}_n)$ and P^{-1} is the inverse of P. Then the solution of Eq. 2 can be written:

$$\mathbf{x} = PDP^{-1}\mathbf{x}_0 = \sum_{i=1}^{n} b_i \mathbf{p}_i \exp(\lambda_i t) \qquad \text{(Eq. 27)}$$

where $b = P^{-1}\mathbf{x}_0$. Thus, Eq. 24 can be written in terms of scalar exponentials, but the notation is cumbersome.

To evaluate $\mathbf{x}$ for plotting, let $G = \exp(KT)$, where T is a sufficiently small increment of time, say 0.1 in this example. Calculate G once and for all by using Eq. 13 to get:

$$G = \begin{pmatrix} .3679 & .0000 & 0. \\ .5966 & .9048 & 0. \\ .0355 & .0952 & 1. \end{pmatrix} \qquad \text{(Eq. 28)}$$

or by using the eigenvalues of K:

$$\Lambda = \begin{pmatrix} -10. & 0. & 0. \\ 0. & -1. & 0. \\ 0. & 0. & 0. \end{pmatrix} \qquad \text{(Eq. 29)}$$

and the eigenvectors of K:

$$P = \begin{pmatrix} 1.0000 & 0. & 0. \\ -1.1111 & 1. & 0. \\ 0.1111 & -1. & 1. \end{pmatrix} \qquad \text{(Eq. 30)}$$

in Eq. 27. Thus, at time $t = T$, we have:

$$\mathbf{x}(T) = G\mathbf{x}_0 \qquad \text{(Eq. 31)}$$

At $t = 2T$, we have:

$$\mathbf{x}(2T) = G\mathbf{x}(T) \qquad \text{(Eq. 32)}$$

the interpretation being that $\mathbf{x}(T)$ itself is a new set of initial conditions to use in calculating $\mathbf{x}(2T)$. Thus:

$$\mathbf{x}(3T) = G\mathbf{x}(2T) \qquad \text{(Eq. 33)}$$

which gives the values of $\mathbf{x}$ at $t = 0.3$ in the example. Now, similarly calculate $\mathbf{x}(4T)$, which gives the values of $\mathbf{x}$ at $t = 0.4$ but just before the first maintenance dose is given. Now let an asterisk denote the values of $\mathbf{x}$ immediately after the first maintenance dose is given. Then clearly:

$$\mathbf{x}^*(4T) = \mathbf{x}(4T) + (.3, .0, .0)' \qquad \text{(Eq. 34)}$$

where the prime denotes the vector transpose. Then:

$$\mathbf{x}(5T) = G\mathbf{x}^*(4T) \qquad \text{(Eq. 35)}$$

With this approach, it is clear that we can write easily the equations for nonuniform multiple dosing into each compartment, so that in general we have:

$$\mathbf{x}[(n+1)T] = G\mathbf{x}^*(nT) \qquad \text{(Eq. 36)}$$

As a check, we used the Continuous System Modeling Program (6) to integrate Eq. 20 numerically with the arguments of the example. The output plots are shown in Figs. 1–3. Plots of the solution of Eq. 20 can also be obtained by use of an analog computer, provided that one has a multiple-dose generator as described by Howell (7).

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(6) International Business Machines Corp., Manual No. GH-20-0367-4, White Plains, N.Y., Jan. 1972.

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Keyphrases □ *N*-Acetyl-*p*-aminophenol—direct colorimetric analysis, decomposition product interference examined □ Colorimetry—analysis, *N*-acetyl-*p*-aminophenol, degradation product interference examined

To the Editor:

Direct colorimetric assay of *N*-acetyl-*p*-aminophenol (I) has been effected by reaction of I with nitrous acid under mild conditions to form 2-nitro-4-acetamidophenol. This reaction was elucidated by Le Perdriel *et al.* (1), who measured the orange-red color of the phenolate ion; Inamdar and Kaji (2), working separately, assayed using the yellow of the unionized phenol.

Chafetz *et al.* (3) compared these methods with their modified technique employing an entirely aqueous system. Because these methods require only the successive addition of reagents, Daly *et al.* (4) adapted the latter technique to an automated assay apparatus, resulting in excellent recovery data with commercial formulations.

The assay of Le Perdriel *et al.* (1) resulted in good

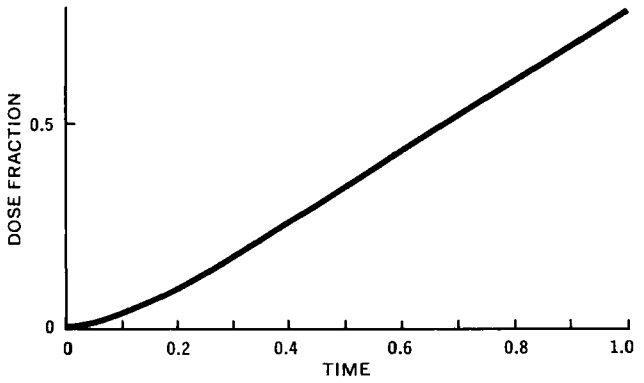

Figure 3—*Dose fraction in Compartment 3 as a function of time.*

partment model, even though K is nonsymmetric. Let $\mathbf{p}_i$ be the real eigenvector of K associated with λ_i, where $P = (\mathbf{p}_1, \mathbf{p}_2, \ldots, \mathbf{p}_n)$ and P^{-1} is the inverse of P. Then the solution of Eq. 2 can be written:

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To the Editor:

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Chafetz *et al.* (3) compared these methods with their modified technique employing an entirely aqueous system. Because these methods require only the successive addition of reagents, Daly *et al.* (4) adapted the latter technique to an automated assay apparatus, resulting in excellent recovery data with commercial formulations.

The assay of Le Perdriel *et al.* (1) resulted in good

selectivity for I; no interference was incurred by most structurally similar drugs except salicylamide (3). However, Le Perdriel *et al.* noted that when I was aged, results in excess of theory were obtained at a point where some 50% of I had degraded. This problem was strongly emphasized by Shearer *et al.* (5) who, using the Chafetz *et al.* (3) method, claimed that a minimum of 25% contribution to the absorbance was observed when an equivalent molar concentration of *p*-aminophenol was tested.

Because of the limited and confusing documentation of the true extent of interference perpetrated by the degradation products of I, the purposes of this study were to document this degree of interference and to discuss the limit of applicability of the Le Perdriel *et al.* assay *per se*.

The degree of interference or the ability of the Le Perdriel *et al.* assay method to determine I selectively in the presence of increasing percentages of the breakdown products of I is shown in Fig. 1. The assay method closely paralleled the method of Le Perdriel *et al.* An ethanolic solution of I was treated with the following reagents: 0.5 ml of 50% (v/v) hydrochloric acid and then 1 ml of a 10% (w/v) sodium nitrite solution. A reaction time of 15 min was allowed before the samples were adjusted to 10 ml with 5% (w/v) anhydrous sodium carbonate solution. The absorbance was read at 440 nm using a similarly treated 2-ml alcohol sample as the blank.

The error shown in Fig. 1 can be eliminated by using the ion-exchange separation of Koshy and Lach (6). This method involved separation of I from its degradation products using a narrow bore column[1] previously washed with 10% hydrochloric acid, distilled water, and alcohol, in that order. A flow rate of 5–10 drops/min was allowed until the sample passed to the upper level of the resin, at which time the alcohol elution flow rate was increased to approximately 20 drops/min. The eluate was collected until exactly 100 ml was obtained.

Two absorbance readings were found essential for proper determination of I after column separation. The first was measurement of the color developed, and the second was measurement of the absorbance of the small proportion of noncolumn-held yellow-brown oxidative products of *p*-aminophenol in a similar dilution with nitrite omitted. Both solutions were read using the same blank at 440 nm. Subtraction of the second from the first yielded, with 95% confidence, an assay value of 99.3 ± 0.8% of the quantity of I present up to the tried value of 67.51% simulated degradation.

The degree of interference depicted in Fig. 1, resulting from breakdown products of I in the Le Perdriel *et al.* determination of I, suggests less interference than was referred to by Shearer *et al.* (5) yet seemingly complies with the qualitative evidence of Le Perdriel *et al.* (1).

With data as to the degree of interference perpetuated by the breakdown products of I now available, quantitative determination of I by the Le Perdriel *et al.* method may be reviewed objectively.

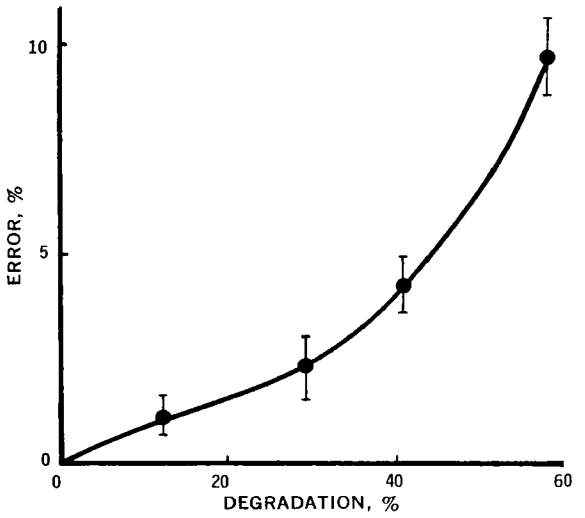

Figure 1—*Percent degradation of* N-*acetyl*-p-*aminophenol* versus *error in* N-*acetyl*-p-*aminophenol determination. The 95% confidence limits about mean values* (n = 4) *are shown.*

Consider the determination of the kinetic picture of the degradation of I. To determine effectively the order of a reaction, 90% of the reaction of the initial species should be followed. Because of the eventual growth in percentage of degradants as the reaction proceeds, the unacceptable interference (Fig. 1) of these degradation products will necessarily dictate column elution before the Le Perdriel *et al.* method *per se* may be quantitatively used.

From a practical formulation viewpoint, the concern is with 10% or less degradation. If a formulation results in a greater than 10% potency loss during a shorter period than is desirable, reformulation is necessary. The method of Le Perdriel *et al.* (1) in this instance, without column separation, would be virtually unequalled in its simplicity and quantitative potentialities, since an error determination of 1% (Fig. 1) is well within the limits of analytical error.

Thus, knowledge of the error in analysis in the presence of breakdown products (Fig. 1) is essential for the rational use of the Le Perdriel *et al.* 2-nitro-4-acetamidophenol determination of I.

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[1] Amberlite IR-120.

Role of Carbonate in Aluminum Hydroxide Gel Established by Raman and IR Analyses

Keyphrases □ Carbonate—role in aluminum hydroxide gel, Raman and IR analyses □ Aluminum hydroxide gel—role of carbonate, Raman and IR analyses □ Raman spectroscopy—association of carbonate and aluminum in aluminum hydroxide gel □ IR spectroscopy—association of carbonate and aluminum in aluminum hydroxide gel

To the Editor:

Aluminum hydroxide gel has been recognized as an effective antacid by the Food and Drug Administration (FDA) (1) and is included in USP XVIII (2). However, FDA makes no attempt to describe the structure of aluminum hydroxide gel. The USP states that aluminum hydroxide gel contains the equivalent of 3.6–4.4% of aluminum oxide in the form of aluminum hydroxide and hydrated oxide. This statement does not fully describe aluminum hydroxide gel, since a number of reports (3–6) have concluded that anions present during precipitation are incorporated in the gel structure and act to stabilize the gel. Carbonate ion is the most effective in stabilizing the gel (3). For this reason, reactive aluminum hydroxide gels usually contain carbonate. In this communication, we report that Raman and IR spectroscopy clearly demonstrate that an association exists between carbonate and aluminum in aluminum hydroxide gel. Thus, carbonate is an integral part of reactive aluminum hydroxide gel and is not simply present in solution or as a void-filling occluded ion.

The Raman spectrum of carbonate ion in solution was compared to the Raman spectrum[1] of aluminum hydroxide gel and to a crystalline sodium aluminum hydroxy carbonate gel. The aluminum hydroxide gel was prepared, following a previously described procedure (3), by the addition of a solution of aluminum chloride to a solution of sodium carbonate and sodium bicarbonate until pH 6.5 was reached. The aluminum hydroxide gel was amorphous by X-ray diffraction, contained the equivalent of 3.2% Al_2O_3, and was fully reactive as measured by the acid-consuming capacity test (2). The amount of carbonate in the gel was determined by the gasometric determination of carbon dioxide (7). The gel evolved 1.4% CO_2.

A crystalline sodium aluminum hydroxy carbonate gel containing 6.9% $NaAl(OH)_2CO_3$, which is equivalent to 2.4% Al_2O_3, was evaluated by Raman and IR spectroscopy. The crystalline gel contained the equivalent of 2.8% CO_2 and possessed X-ray diffraction bands at 5.67, 3.38, 2.784, 2.601, 2.151, 1.988, and 1.728 Å, which identify the gel as dawsonite (8).

The Raman spectrum of carbonate ion in solution (9) has a very strong band at 1063 cm^{-1}, weak bands at 1415 and 680 cm^{-1}, and an inactive band at 880 cm^{-1}. The Raman spectrum of the amorphous aluminum hydroxide gel has a broad band ranging from 1060 to 1174 cm^{-1} with the maximum at 1120 cm^{-1}. The crystalline sodium aluminum hydroxy carbonate has a sharp band at 1090 cm^{-1}.

The change in the band at 1063 cm^{-1} from a sharp peak for carbonate ion in solution to a broad band centered at 1120 cm^{-1} in the amorphous gel indicates that varying degrees of interaction are occurring between the oxygens of the carbonate ion and aluminum in the gel. These interactions destroy the symmetry possessed by the carbonate ion in solution, and the loss of symmetry causes a shift in the peak position. In aluminum hydroxide gel, numerous degrees of association are possible due to the amorphous nature of the gel. Therefore, the carbonate peak appears as a broad band reflecting the varying degrees of association of carbonate with aluminum.

In crystalline sodium aluminum hydroxy carbonate, a sharp peak appears at 1090 cm^{-1}. This shift indicates that the symmetry of carbonate ion is destroyed due to the association of carbonate with aluminum. However, the carbonate is associated with the aluminum in only one environment, as indicated by the sharp peak.

The IR spectra also indicate that definite association exists between carbonate and aluminum in aluminum hydroxide gel. Because of interference by strong water bands in the 1400–1700-cm^{-1} region, the IR spectra[2] were run as air-dried films of the gels on a zinc sulfide[3] window.

The IR spectrum of sodium carbonate has bands at 1450, 880, and 720 cm^{-1} (10). In aluminum hydroxide gel, bands associated with carbonate occur at 1525, 1415, 1100, and 850 cm^{-1}. The band at 1450 cm^{-1} for sodium carbonate has split into two bands, 1525 and 1415 cm^{-1}, in aluminum hydroxide gel. Nakamoto (10) and Healy and White (11, 12) showed that when carbonate ion coordinates to a metal, the ν_3 vibration splits into two bands. The magnitude of the splitting has been shown to be dependent on whether a uni- or a bidentate complex forms.

Carbonate bands occur at 1580, 1390, 1080, and 840 cm^{-1} in the crystalline sodium aluminum hydroxy carbonate. The carbonate band occurring at 1450 cm^{-1} for sodium carbonate has again split, but the magnitude of the split is 190 cm^{-1} for the crystalline gel in comparison to 110 cm^{-1} for the amorphous gel (Fig. 1). The magnitude of the split of this carbonate band may be useful in predicting the acid reactivity of aluminum hydroxide gel.

In addition to the splitting of the degenerate vibration, the appearance of the inactive band at 1100 cm^{-1} in the amorphous gel and 1080 cm^{-1} in the crystalline gel is also typical of a substantial lowering of symmetry due to association between carbonate and aluminum (12).

Thus, it is concluded that Raman and IR spectroscopy are useful tools for studying the role of anions in stabilizing aluminum hydroxide gel. Raman spectroscopy is especially useful because water does not interfere and the gels can be run in their natural condi-

[1] Raman spectra were run on a Spex RAMALOG 4, courtesy of Spex Industries, Metuchen, N.J.

[2] Model 421, Perkin-Elmer Corp., Norwalk, Conn.

[3] Irtran 2, Eastman Kodak, Rochester, N.Y.

Role of Carbonate in Aluminum Hydroxide Gel Established by Raman and IR Analyses

Keyphrases □ Carbonate—role in aluminum hydroxide gel, Raman and IR analyses □ Aluminum hydroxide gel—role of carbonate, Raman and IR analyses □ Raman spectroscopy—association of carbonate and aluminum in aluminum hydroxide gel □ IR spectroscopy—association of carbonate and aluminum in aluminum hydroxide gel

To the Editor:

Aluminum hydroxide gel has been recognized as an effective antacid by the Food and Drug Administration (FDA) (1) and is included in USP XVIII (2). However, FDA makes no attempt to describe the structure of aluminum hydroxide gel. The USP states that aluminum hydroxide gel contains the equivalent of 3.6–4.4% of aluminum oxide in the form of aluminum hydroxide and hydrated oxide. This statement does not fully describe aluminum hydroxide gel, since a number of reports (3–6) have concluded that anions present during precipitation are incorporated in the gel structure and act to stabilize the gel. Carbonate ion is the most effective in stabilizing the gel (3). For this reason, reactive aluminum hydroxide gels usually contain carbonate. In this communication, we report that Raman and IR spectroscopy clearly demonstrate that an association exists between carbonate and aluminum in aluminum hydroxide gel. Thus, carbonate is an integral part of reactive aluminum hydroxide gel and is not simply present in solution or as a void-filling occluded ion.

The Raman spectrum of carbonate ion in solution was compared to the Raman spectrum[1] of aluminum hydroxide gel and to a crystalline sodium aluminum hydroxy carbonate gel. The aluminum hydroxide gel was prepared, following a previously described procedure (3), by the addition of a solution of aluminum chloride to a solution of sodium carbonate and sodium bicarbonate until pH 6.5 was reached. The aluminum hydroxide gel was amorphous by X-ray diffraction, contained the equivalent of 3.2% Al_2O_3, and was fully reactive as measured by the acid-consuming capacity test (2). The amount of carbonate in the gel was determined by the gasometric determination of carbon dioxide (7). The gel evolved 1.4% CO_2.

A crystalline sodium aluminum hydroxy carbonate gel containing 6.9% $NaAl(OH)_2CO_3$, which is equivalent to 2.4% Al_2O_3, was evaluated by Raman and IR spectroscopy. The crystalline gel contained the equivalent of 2.8% CO_2 and possessed X-ray diffraction bands at 5.67, 3.38, 2.784, 2.601, 2.151, 1.988, and 1.728 Å, which identify the gel as dawsonite (8).

The Raman spectrum of carbonate ion in solution (9) has a very strong band at 1063 cm^{-1}, weak bands at 1415 and 680 cm^{-1}, and an inactive band at 880 cm^{-1}. The Raman spectrum of the amorphous aluminum hydroxide gel has a broad band ranging from 1060 to 1174 cm^{-1} with the maximum at 1120 cm^{-1}. The crystalline sodium aluminum hydroxy carbonate has a sharp band at 1090 cm^{-1}.

The change in the band at 1063 cm^{-1} from a sharp peak for carbonate ion in solution to a broad band centered at 1120 cm^{-1} in the amorphous gel indicates that varying degrees of interaction are occurring between the oxygens of the carbonate ion and aluminum in the gel. These interactions destroy the symmetry possessed by the carbonate ion in solution, and the loss of symmetry causes a shift in the peak position. In aluminum hydroxide gel, numerous degrees of association are possible due to the amorphous nature of the gel. Therefore, the carbonate peak appears as a broad band reflecting the varying degrees of association of carbonate with aluminum.

In crystalline sodium aluminum hydroxy carbonate, a sharp peak appears at 1090 cm^{-1}. This shift indicates that the symmetry of carbonate ion is destroyed due to the association of carbonate with aluminum. However, the carbonate is associated with the aluminum in only one environment, as indicated by the sharp peak.

The IR spectra also indicate that definite association exists between carbonate and aluminum in aluminum hydroxide gel. Because of interference by strong water bands in the 1400–1700-cm^{-1} region, the IR spectra[2] were run as air-dried films of the gels on a zinc sulfide[3] window.

The IR spectrum of sodium carbonate has bands at 1450, 880, and 720 cm^{-1} (10). In aluminum hydroxide gel, bands associated with carbonate occur at 1525, 1415, 1100, and 850 cm^{-1}. The band at 1450 cm^{-1} for sodium carbonate has split into two bands, 1525 and 1415 cm^{-1}, in aluminum hydroxide gel. Nakamoto (10) and Healy and White (11, 12) showed that when carbonate ion coordinates to a metal, the ν_3 vibration splits into two bands. The magnitude of the splitting has been shown to be dependent on whether a uni- or a bidentate complex forms.

Carbonate bands occur at 1580, 1390, 1080, and 840 cm^{-1} in the crystalline sodium aluminum hydroxy carbonate. The carbonate band occurring at 1450 cm^{-1} for sodium carbonate has again split, but the magnitude of the split is 190 cm^{-1} for the crystalline gel in comparison to 110 cm^{-1} for the amorphous gel (Fig. 1). The magnitude of the split of this carbonate band may be useful in predicting the acid reactivity of aluminum hydroxide gel.

In addition to the splitting of the degenerate vibration, the appearance of the inactive band at 1100 cm^{-1} in the amorphous gel and 1080 cm^{-1} in the crystalline gel is also typical of a substantial lowering of symmetry due to association between carbonate and aluminum (12).

Thus, it is concluded that Raman and IR spectroscopy are useful tools for studying the role of anions in stabilizing aluminum hydroxide gel. Raman spectroscopy is especially useful because water does not interfere and the gels can be run in their natural condi-

[1] Raman spectra were run on a Spex RAMALOG 4, courtesy of Spex Industries, Metuchen, N.J.

[2] Model 421, Perkin-Elmer Corp., Norwalk, Conn.

[3] Irtran 2, Eastman Kodak, Rochester, N.Y.

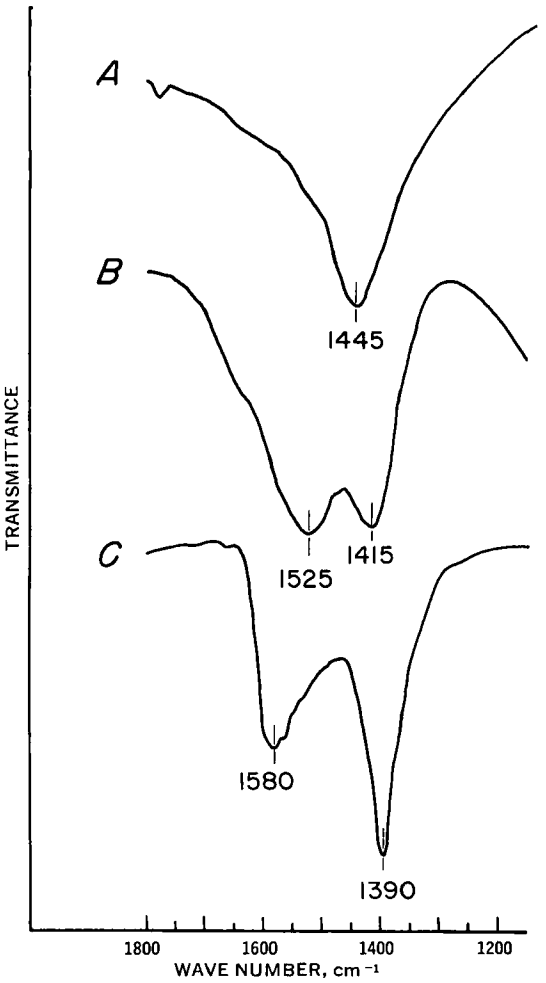

Figure 1—*IR spectra of 1200–1800-cm^{-1} region. Key: A, sodium carbonate; B, aluminum hydroxide gel; and C, sodium aluminum hydroxy carbonate.*

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A report on the role of carbonate in aluminum hydroxide-type antacids is in preparation.

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Cytolytic Effects of Bromoacetylcholine on Neuroblastoma *In Vitro*

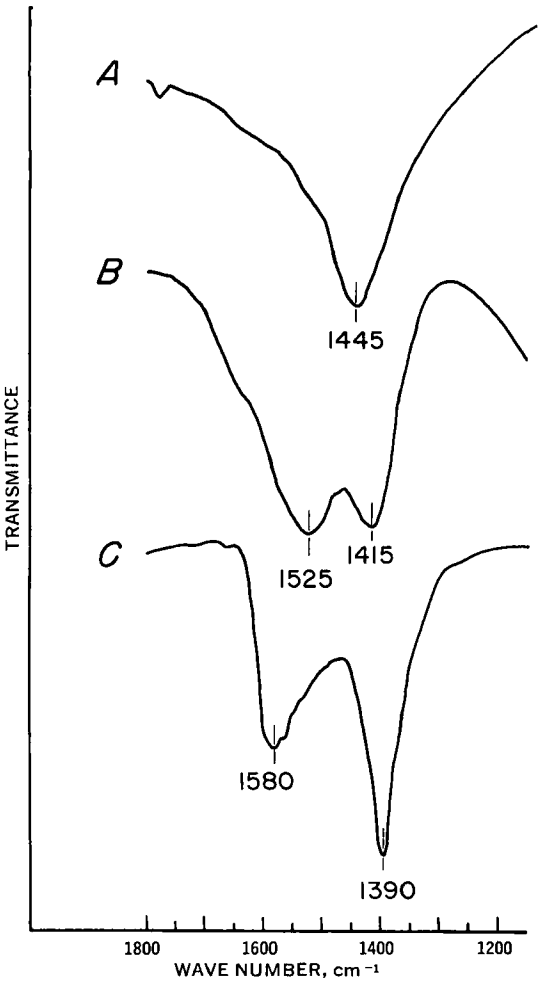
Keyphrases □ Bromoacetylcholine—cytolytic effects on neuroblastoma *in vitro* □ Neuroblastoma—cytolytic effects of bromoacetylcholine *in vitro* □ Cancer chemotherapeutic agents—cytolytic effects of bromoacetylcholine on neuroblastoma *in vitro* □ Cytolytic activity—bromoacetylcholine on neuroblastoma *in vitro*

To the Editor:

Neuroblastoma, probably the most common solid cancer in children (1), is treated with vincristine and cyclophosphamide. However, the remission rate in neuroblastoma patients is quite low and the duration of drug effectiveness is fairly short (2–4). Thus, new chemotherapeutic agents are needed to cope with this highly malignant tumor.

Recent studies revealed that the nature of neuroblastoma cells can be cholinergic, adrenergic, or inactive (5–8). It is also known that acetylcholinesterase is present in all cases and that these cells possess membranes that respond to acetylcholine and are electrically excitable, indicating the existence of cholinergic receptors at the membrane site (7, 9). Attempts made to destroy these tumor cells with an irreversible adrenolytic agent, 6-hydroxydopamine (10), have met with only partial success, possibly due to the inhibition of adrenergic components without an affect on the cholinergic parts of these tumor cells. Therefore, we proposed to kill these tumor cells with irreversible cholinolytics or with cholinergics such as bromoacetylcholine perchlorate, $BrCH_2COOCH_2CH_2{}^+N(CH_3)_3{\cdot}ClO_4{}^-$.

Bromoacetylcholine is a cholinergic agonist which stimulates both nicotinic and muscarinic receptors directly (11–13). However, when it is incubated with cell membranes for 15 min or longer, it binds irreversibly and specifically to the cholinergic receptor at the nicotinic site but not at the muscarinic site (11, 14). Therefore, it was thought that this compound might inhibit the growth of neuroblastoma cells. In this investigation, bromoacetylcholine inhibited the growth of neuroblastoma effectively at concentrations of 10^{-6}–10^{-5} *M*. Therefore, it is hoped that bromoacetylcholine can be used to treat the malignant tumor.



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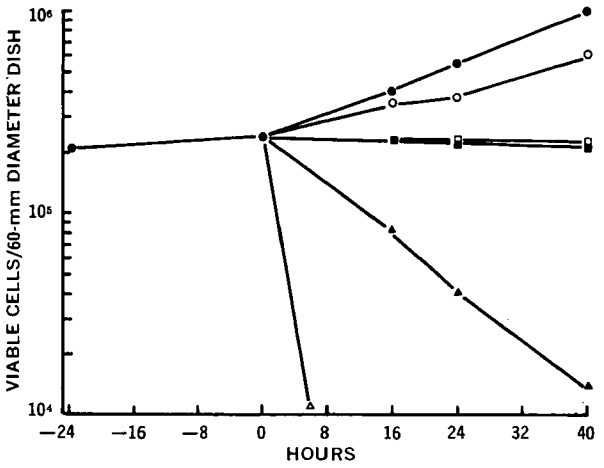

Figure 1—*Inhibition of neuroblastoma cells by bromoacetylcholine. Key:* ●, *control;* ○, *5* × 10^{-6} M *bromoacetylcholine;* □, *1* × 10^{-5} M *bromoacetylcholine for the first 16 hr and then washed away;* ■, *1* × 10^{-5} M *bromoacetylcholine;* ▲, *2* × 10^{-5} M *bromoacetylcholine; and* △, *1* × 10^{-3} M *bromoacetylcholine.*

Mouse neuroblastoma C-1300 clone N-18 was cultivated in modified Eagle medium[1] (containing, milligrams per liter: $CaCl_2$, 200; KCl, 400; $MgSO_4$, 97.72; NaCl, 6800; $NaH_2PO_4 \cdot H_2O$, 140; glucose, 1000; phenol red, 10; L-arginine hydrochloride, 126; L-cystine dihydrochloride, 31.29; L-glutamine, 292; L-histidine hydrochloride·H_2O, 42; L-isoleucine, 52.5; L-leucine, 52.4; L-lysine hydrochloride, 72.5; L-methionine, 15; L-phenylalanine, 32; L-threonine, 48; L-tryptophan, 10; L-tyrosine disodium salt, 52.1; L-valine, 46; calcium D-pantothenate, 1; choline chloride, 1; folic acid, 1; *i*-inositol, 2; nicotinamide, 1; pyridoxal, 1; riboflavin, 0.1; and thiamine hydrochloride, 1) supplemented with 10% fetal calf serum[2], 292 mg/liter L-glutamine, 2500 mg/liter $NaHCO_3$, 50 units/liter penicillin, and 50 units/liter streptomycin (7). The cells were grown at 37° under 95% air–5% CO_2 with 100% humidity.

For the culture experiments, 1 × 10^6 cells were placed in a Falcon tissue culture flask (75 cm^2) for 3–5 days until the cells were in the logarithmic phase of growth. They were then removed from the surface by a 15-min exposure to 0.05% trypsin in modified D_1 solution (7, 15). The cells were tapped off in fresh medium containing 10% fetal calf serum.

Aliquots of the cell suspension were incubated for 5 min with 0.04% trypan blue, and the titer of viable and nonviable cells was determined with a hemocytometer. Appropriate dilution was made, and the cell suspension was plated at 2 × 10^5 cells/dish (Falcon tissue culture dishes of 60 mm diameter) and grown for 24 hr. The medium was replaced with a fresh one as the control and with fresh medium plus various concentrations of bromoacetylcholine as the experimental samples. They were further incubated for 40 hr, and the cultures were removed at intervals for cell count.

[1] MEM, Earle's Powder, Catalog No. F-11, Grand Island Biological Co., Grand Island, N.Y.

[2] Catalog No. 614, Grand Island Biological Co., Grand Island, N.Y.

Figure 1 shows a complete inhibition of neuroblastoma cells by 1 × 10^{-5} *M* bromoacetylcholine with an ID_{50} of about 5 × 10^{-6} *M*. With higher concentrations of bromoacetylcholine (2 × 10^{-5}–1 × 10^{-3} *M*), neuroblastoma cells were destroyed and the cell number was reduced to below the original level. Figure 1 also shows an irreversible inhibition of neuroblastoma by bromoacetylcholine. The medium containing bromoacetylcholine was removed from dishes after 16 hr of incubation. The monolayers were rinsed and reincubated with fresh medium alone. No recovery of cell growth was observed with these treatments. These findings warrant further studies on using bromoacetylcholine to inhibit neuroblastoma *in vitro* and in experimental animals *(in vivo)*.

There are three important findings available regarding the pharmacology of bromoacetylcholine:

1. It produces cholinergic responses that can be blocked competitively by cholinergic blocking agents such as atropine (muscarinic) and tubocurarine (nicotinic) (11, 13).

2. It binds irreversibly to cholinergic receptors at nicotinic sites only but not at muscarinic sites (14).

3. It takes lower concentrations (10^{-5} *M*) to produce muscarinic responses but higher ones (10^{-3} *M*) to induce nicotinic responses (13, 14).

Based on these findings, we predict that when bromoacetylcholine is used to treat neuroblastoma patients, the muscarinic responses produced will be the major side effects. These can be eliminated by using atropine and its analogs along with bromoacetylcholine. Since the irreversible binding of bromoacetylcholine to cholinergic receptors at nicotinic sites is not affected by atropine, it will abolish side effects of bromoacetylcholine without reducing the ability of bromoacetylcholine to inhibit the neural tumor.

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Intestinal Bacterial Reduction of 4,4′-Dihydroxystilbene to 4,4′-Dihydroxybibenzyl

Keyphrases □ 4,4′-Dihydroxystilbene—intestinal bacterial reduction to 4,4′-dihydroxybibenzyl □ 4,4′-Dihydroxybibenzyl—intestinal bacterial reduction from 4,4′-dihydroxystilbene □ Metabolism—*trans*-stilbene, intestinal reduction of 4,4′-dihydroxystilbene

To the Editor:

We previously reported the metabolic reduction of the ethylenic double bond in *trans*-stilbene to yield 4,4′-dihydroxybibenzyl to the extent of 2.1% in rabbit urine and 10.6% in guinea pig urine (1, 2). Continued studies in our laboratories have shown that rats administered *trans*-α-^{14}C-stilbene intramuscularly in peanut oil excreted 4.1% of the dose as the bibenzyl compound in urine. These rat studies also have indicated that 40% of the original activity as compared to only 2.3% for rabbits is eliminated over 19 days in the feces. 4,4′-Dihydroxybibenzyl was detected by cochromatography with a reference compound in TLC Systems I and II[1]. It was the major metabolite in rat feces in a yield of 12.4% of the dose.

These species differences, which are consistent with reports (3, 4) that polarity and molecular weight determine the extent of biliary excretion in a given species, led us to postulate that biliary excretion and subsequent intestinal bacterial action are responsible for the reduction of the 4,4′-dihydroxy metabolite of *trans*-stilbene to 4,4′-dihydroxybibenzyl. That is, the increased yield of the bibenzyl compound in the rat could be due initially to an increased biliary excretion of either 4,4′-dihydroxystilbene *per se* or, more significantly, its glucuronide conjugate. It is of interest in this regard that enterohepatic circulation has been reported for the related compound, diethylstilbestrol, with extensive excretion into rat bile as the monoglucuronide followed by hydrolysis and reabsorption of diethylstilbestrol (5–7).

Recently, Scheline (8) also postulated that 4,4′-dihydroxybibenzyl is produced through reduction by intestinal microflora. His conclusion was based upon a decreased yield of the bibenzyl compound in urine of rats treated with the antibiotic neomycin sulfate or by bile duct ligation prior to the administration of *trans*-stilbene. Our studies confirm this hypothesis by direct incubation of dihydroxystilbene in an intestinal microflora extract.

Incubation studies were with 4,4′-dihydroxy-α-^{14}C-stilbene obtained by TLC (System I) of the phenolic fraction derived from an ether extract of enzyme[2]-hydrolyzed urine of rabbits administered *trans*-α-^{14}C-stilbene. The dihydroxy compound, which cochromatographed with reference material in TLC Systems I and II, was eluted from silica gel with methanol. This extract was concentrated, a few drops of polysorbate 80 were added, and the remaining methanol was removed before the sample was diluted to 1 ml with 0.1 *M* phosphate buffer (pH 7.4). An aliquot (0.1 ml = 4300 dpm) of the polysorbate 80 suspension was added to 1 ml of the incubation medium previously described (9, 10). The intestinal bacterial extract for this mixture was obtained from the intestinal and cecal contents of male Sprague–Dawley rats, 250–300 g. After incubation at 37° for 24 hr under nitrogen, the mixture was quenched with 1 *N* HCl (1 ml) and continuously extracted with ether for 48 hr. Controls were prepared in the same manner except that the intestinal bacterial extract was autoclaved for 17 min at 121° before addition to the sample tubes containing substrate.

Methanol solutions of the ether extracts of the control and test samples were investigated for transformations by TLC System I. Reference compounds cospotted with the methanol solution were visualized by quenching of the fluorescent TLC plates, while radioactive components were located using a radiochromatogram scanner[3]. Quantitation was by liquid scintillation counting of 0.64-cm (0.25-in.) strips from the TLC plates.

The TLC results from sample incubations indicated a yield of 14% of 4,4′-dihydroxybibenzyl at R_f 0.40, but there was no indication of the reduced product with TLC of the control incubation. Thus, these incubation studies confirm the reduction by intestinal microflora of 4,4′-dihydroxystilbene and, together with reports (9, 11) of the reduction of cinnamic acids to phenylpropionic acids, establish the conjugated ethylenic bond as among the moieties (12) reduced by intestinal microflora.

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[1] TLC systems employed were: I, toluene–piperidine (5:2); and II, benzene–methanol (9:1). TLC plates, 5 × 20 cm and 0.25-mm layer thickness, were precoated with silica gel F-254 (Brinkmann Instruments Co.).

[2] Hydrolysis was at 37° for 72 hr with 1000 units of β-glucuronidase–aryl sulfatase (Sigma Chemical Co.) per ml of urine.

[3] Packard radiochromatogram scanner model 7201, Packard Instrument Co.

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We previously reported the metabolic reduction of the ethylenic double bond in *trans*-stilbene to yield 4,4′-dihydroxybibenzyl to the extent of 2.1% in rabbit urine and 10.6% in guinea pig urine (1, 2). Continued studies in our laboratories have shown that rats administered *trans*-α-^{14}C-stilbene intramuscularly in peanut oil excreted 4.1% of the dose as the bibenzyl compound in urine. These rat studies also have indicated that 40% of the original activity as compared to only 2.3% for rabbits is eliminated over 19 days in the feces. 4,4′-Dihydroxybibenzyl was detected by cochromatography with a reference compound in TLC Systems I and II[1]. It was the major metabolite in rat feces in a yield of 12.4% of the dose.

These species differences, which are consistent with reports (3, 4) that polarity and molecular weight determine the extent of biliary excretion in a given species, led us to postulate that biliary excretion and subsequent intestinal bacterial action are responsible for the reduction of the 4,4′-dihydroxy metabolite of *trans*-stilbene to 4,4′-dihydroxybibenzyl. That is, the increased yield of the bibenzyl compound in the rat could be due initially to an increased biliary excretion of either 4,4′-dihydroxystilbene *per se* or, more significantly, its glucuronide conjugate. It is of interest in this regard that enterohepatic circulation has been reported for the related compound, diethylstilbestrol, with extensive excretion into rat bile as the monoglucuronide followed by hydrolysis and reabsorption of diethylstilbestrol (5–7).

Recently, Scheline (8) also postulated that 4,4′-dihydroxybibenzyl is produced through reduction by intestinal microflora. His conclusion was based upon a decreased yield of the bibenzyl compound in urine of rats treated with the antibiotic neomycin sulfate or by bile duct ligation prior to the administration of *trans*-stilbene. Our studies confirm this hypothesis by direct incubation of dihydroxystilbene in an intestinal microflora extract.

Incubation studies were with 4,4′-dihydroxy-α-^{14}C-stilbene obtained by TLC (System I) of the phenolic fraction derived from an ether extract of enzyme[2]-hydrolyzed urine of rabbits administered *trans*-α-^{14}C-stilbene. The dihydroxy compound, which cochromatographed with reference material in TLC Systems I and II, was eluted from silica gel with methanol. This extract was concentrated, a few drops of polysorbate 80 were added, and the remaining methanol was removed before the sample was diluted to 1 ml with 0.1 *M* phosphate buffer (pH 7.4). An aliquot (0.1 ml = 4300 dpm) of the polysorbate 80 suspension was added to 1 ml of the incubation medium previously described (9, 10). The intestinal bacterial extract for this mixture was obtained from the intestinal and cecal contents of male Sprague–Dawley rats, 250–300 g. After incubation at 37° for 24 hr under nitrogen, the mixture was quenched with 1 *N* HCl (1 ml) and continuously extracted with ether for 48 hr. Controls were prepared in the same manner except that the intestinal bacterial extract was autoclaved for 17 min at 121° before addition to the sample tubes containing substrate.

Methanol solutions of the ether extracts of the control and test samples were investigated for transformations by TLC System I. Reference compounds cospotted with the methanol solution were visualized by quenching of the fluorescent TLC plates, while radioactive components were located using a radiochromatogram scanner[3]. Quantitation was by liquid scintillation counting of 0.64-cm (0.25-in.) strips from the TLC plates.

The TLC results from sample incubations indicated a yield of 14% of 4,4′-dihydroxybibenzyl at R_f 0.40, but there was no indication of the reduced product with TLC of the control incubation. Thus, these incubation studies confirm the reduction by intestinal microflora of 4,4′-dihydroxystilbene and, together with reports (9, 11) of the reduction of cinnamic acids to phenylpropionic acids, establish the conjugated ethylenic bond as among the moieties (12) reduced by intestinal microflora.

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(12) R. R. Scheline, *Pharmacol. Rev.*, **25**, 451(1973).

[1] TLC systems employed were: I, toluene–piperidine (5:2); and II, benzene–methanol (9:1). TLC plates, 5 × 20 cm and 0.25-mm layer thickness, were precoated with silica gel F-254 (Brinkmann Instruments Co.).

[2] Hydrolysis was at 37° for 72 hr with 1000 units of β-glucuronidase–aryl sulfatase (Sigma Chemical Co.) per ml of urine.

[3] Packard radiochromatogram scanner model 7201, Packard Instrument Co.

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In summary, I would highly recommend this book to anyone interested in the reactivity, analysis, or aqueous stability of organic functional groups.

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Drug–Biomolecule Interactions: Interactions of Mononucleotides and Polybasic Amino Acids

JAMES C. LACEY, Jr. [x], and KENNETH M. PRUITT

Abstract □ Histones and ribosomal proteins are basic proteins that participate in gene regulation and protein synthesis, respectively. How these proteins interact with nucleic acids is not yet clear, although specificities in these interactions have been observed. Study of the interaction of mononucleotides with basic polyamino acids is one approach to understanding such interactions. The results of studies with the mononucleotides can help elucidate the normal molecular processes in biological systems and also shed light on some effects of drugs, such as puromycin and tubericidin, that are nucleotide derivatives. A review of studies on the interaction of mononucleotides and basic polyamino acids such as polylysine and polyarginine is presented. In addition, a short review of the self-associative properties of mononucleotides is given. Studies of the mononucleotide–polyamino acid interaction have involved a wide variety of techniques including equilibrium dialysis, NMR, optical rotatory dispersion, circular dichroism, and precipitate analysis.

Keyphrases □ Mononucleotides—interactions with polybasic amino acids □ Amino acids, polybasic—interactions with mononucleotides □ Drug–biomolecule interactions—mononucleotides and polybasic amino acids □ Interactions—drugs with biomolecules, symposium

Interactions of the components of nucleic acids and proteins are significant factors in the regulation of gene expression (transcription) and the control of protein synthesis (translation). These interactions are also involved in the reactions of mononucleotides and enzymes and, possibly, in the biological activity of nucleotide analog drugs such as puromycin. *In vitro* studies of the interactions of amino acids and nucleic acids and of polyamino acids and nucleic acids have shed some light on the nature of the interactions of nucleic acids and proteins *in vivo*.

There have been only a few reported studies of the interactions of amino acids and nucleic acids, but these studies indicate that there is little tendency for amino acids to bind to nucleic acids and that there is little or no specificity for the binding observed (1). However, numerous studies of polyamino acid–nucleic acid interactions revealed significant instances of specific binding. These latter studies mostly utilized polybasic amino acids such as polylysine, polyarginine, and polyornithine because of the high electrostatic affinity between these positively charged polyamino acids and the negatively charged ribose phosphate backbone of the nucleic acid.

Although charge–charge interactions are the main attractive forces between polybasic amino acids and nucleic acids, other forces are also operative, resulting in binding specificities. For example, Leng and Felsenfeld (2) found that polylysine has a preferred binding affinity for adenine–thymine-rich regions in DNA. A weaker specificity of interaction between poly-L-arginine and guanine–cytosine regions was also observed. Later, Shapiro *et al.* (3) showed that the preference of poly-L-lysine for adenine–thymine regions could be changed to guanine–cytosine regions by the addition of tetramethylammonium salts.

Furthermore, RNA polymerase has been shown to bind to a greater extent to poly dA-T than to T_4 DNA (4). The mechanism leading to such specificities is not known. Specificities in the binding of histones and nonhistone acidic proteins have also been found (5), but the mechanism behind these specific interactions is also unknown.

Studies of the binding of ribosomal proteins to ribosomal RNA recently showed that at least five ribosomal proteins of the 30S subunit of *Escherichia coli* ribosomes have specific binding sites on the 16S ribosomal RNA (6). Again, the basis for the specificity is not known.

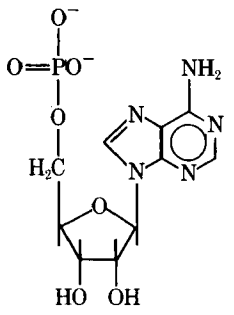
These comments are intended to place the present review of mononucleotide–polyamino acids in the proper perspective. This article is mainly a review[1] of the literature reports on mononucleotide–polybasic amino acid interactions. However, also included are some previously unreported studies from the authors' laboratory regarding the mechanism by which a lysine side chain might approach an adenine ring in a mixture of adenosine 5′-monophosphate and poly-L-lysine.

DISCUSSION

Properties of Interactants and Methods of Study—*Basic Polyamino Acids*—At pH values well below the pKa of the basic side chains, all of the side chains are positively charged and the polymers tend to assume a random coil configuration due to electrostatic repulsion. The basic polyamino acids are very soluble due to the ionic nature of the side chains. As this charge is eliminated by elevation of pH or masked by the addition of salts, the basic polyamino acid tends to go into a helical form.

The principal means for studying this transition have been optical rotatory dispersion and circular dichroism (7, 8). The random coil to helix transition normally gives rise to perturbations of the spectra in the 220–230-nm range. It has been found that poly-L-lysine and poly-L-arginine form helixes much more readily than poly-L-ornithine.

[1] Literature search was completed in August 1972.



I

Mononucleotides—The 5′-mononucleotides have several interesting properties relevant to their interaction with basic polyamino acids. In solution, the ribose-phosphate moiety is capable of freely rotating so that it might be expected to occupy randomly all possible positions relative to the plane of the organic base ring. However, NMR studies (9) indicate that the *anti*-conformation (I) is preferred over the opposite *syn*-conformation.

Another property of the mononucleotides is their propensity to self-associate so that their organic base rings are essentially parallel to each other and one above the other, forming a vertical array. This property is called base stacking and has been studied in solution using NMR. As a consequence of base stacking, signals from protons attached to the purine and pyrimidine ring systems of nucleotides are shifted upfield (to lower frequencies). The magnitude of the shift is determined by the extent of interaction of the stacking ring systems (9).

X-ray diffraction studies have confirmed the stacking tendencies of the nucleotides in the solid state (10). The tendency to self-associate by stacking is in the following order: guanosine 5′-monophosphate > adenosine 5′-monophosphate > cytidine 5′-monophosphate > uridine 5′-monophosphate.

Interaction of Mononucleotides and Basic Polyamino Acids—The work discussed here is concerned with the specificities of these interactions and with their physical–chemical basis. The effects on the interactions of changes in pH, ionic strength, length of amino acid side chain, and relative concentrations of reactants are also considered.

The history of the study of mononucleotide–polyamino acid interactions is short. The first reports in the literature came from Woese's laboratory and appeared in 1968 (11). Using equilibrium dialysis and turbidity measurements, Woese studied the interaction of the various 5′-nucleotides with poly-L-arginine. Similar experiments were already underway in the authors' laboratory. Woese found that turbidity developed when solutions of guanosine 5′-monophosphate, adenosine 5′-monophosphate, and cytidine 5′-monophosphate were mixed with solutions of poly-L-arginine. No turbidity changes were observed with uridine 5′-monophosphate. He also observed that the ease of formation of turbidity in mixtures with poly-L-arginine decreased in the order: guanosine 5′-monophosphate > adenosine 5′-monophosphate > cytidine 5′-monophosphate > uridine 5′-monophosphate. Woese pointed out that this order is the same as that observed for the base stacking tendencies of the nucleotides.

The authors found precisely the same order for the concentration dependence of turbidity changes[2] when the nucleotides were mixed with solutions of poly-L-lysine (Fig. 1) (12). These experiments were carried out in the absence of added salt to maximize the electrostatic attractions between the reactants. The addition of small amounts of sodium chloride blocked the turbidity-producing interactions. As shown in Fig. 1, the addition of nucleosides or potassium phosphate (K_2HPO_4) to solutions of poly-L-lysine did not produce significant changes in turbidity.

The changes in turbidity were due to the formation of small, translucent spheres which could be easily separated by centrifugation. Because of this property, it was possible to separate the interaction products and calculate their stoichiometry by analyzing the composition of the supernate. These data (Table I) can be interpreted in terms of a model that specifies an initial charge–charge attraction between the negative phosphate groups on the nucleotides and the positively charged ϵ-amino group of the lysine. Aggregation follows as a consequence of base stacking of bound nucleotides. This model is based on the following interpretation of the data.

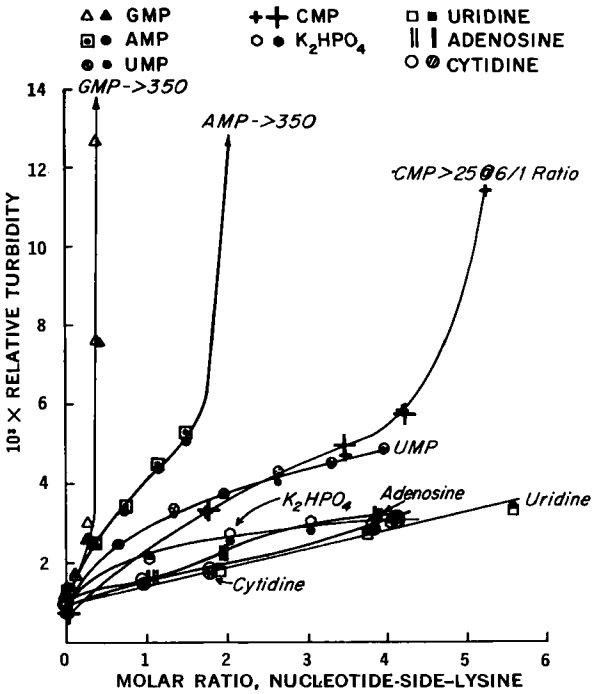


Figure 1—*Changes in relative turbidity of solutions of poly-L-lysine (100,000 mol. wt.) as solutions of 5′-mononucleotides, mononucleosides, or potassium phosphate were added at pH 8.0–9.0. The initial poly-L-lysine concentration was 1 × 10^{-3} M (lysine residue basis). Key shows symbols for duplicate samples of each additive. Numbers at top of guanosine 5′-monophosphate (GMP), adenosine 5′-monophosphate (AMP), and cytidine 5′-monophosphate (CMP) curves are extreme turbidity values reached. All turbidity values were corrected for dilution and for solvent scattering.*

At pH 8.0, all nucleotides have a net charge of −2 and the lysine residues have a charge of +1. The predicted stoichiometry for a simple charge–charge interaction would be two lysine residues for each nucleotide molecule. This is approximately the value observed for each nucleotide at pH 8.0.

At very low pH, the net negative charge on the nucleotide decreases because of protonation of both the phosphate and the purine–pyrimidine rings. At high pH, the proton is removed from the ϵ-amino group of the lysine. Thus, charge–charge interactions are different at pH extremes and the number of nucleotides bound should vary accordingly. This effect was confirmed for adenosine 5′-monophosphate. At pH 4, approximately the pKa of the nucleotide ring, adenosine 5′-monophosphate has one negative charge on the phosphate group and an average of one-half of a negative charge on the ring. The average number of bound nucleotide residues was found to increase to 1.0.

As the pH is raised, the number of bound nucleotide residues decreases. As the pH approaches the pKa of the ϵ-amino group of the lysine, the number of nucleotide residues bound decreases until no further precipitation is observed at pH 12.5.

The fact that no significant turbidity increases were found when nucleosides (lacking the phosphate group) were added to polylysine solutions further substantiates the importance of charge–charge interactions in the binding process.

The involvement of base stacking in the aggregation is suggested by the fact that the effectiveness of nucleotide precipitation of polylysine is in the order guanosine 5′-monophosphate > adenosine 5′-monophosphate > cytidine 5′-monophosphate > uridine 5′-monophosphate and that this is the same order for nucleotide base stacking tendencies.

The range of intensity of the nucleotide–polylysine aggregation reaction is very great down the series from guanosine 5′-mono-

[2] A Brice-Phoenix model 2000 light-scattering photometer was used (25°).

Table I—Stoichiometry of Binding of Nucleotides to 100,000 Molecular Weight Poly-L-lysine

Nucleotide	pH	Approximate Initial Molar Ratio Nucleotide to Lysine Residues	Molar Ratio of Nucleotide to Lysine in Residues[a]
Guanosine 5′-monophosphate	8.0	0.4	0.38
		1.0	0.40
		1.5	0.43
Adenosine 5′-monophosphate	4.0	2	No precipitate
		4	No precipitate
		8	1.0
	5.5	1	No precipitate
		2	0.60
		3	0.47
	8.0	1	No precipitate
		2	0.50
		3	0.65
		4	0.50
	11.0	1	0.16
		2	0.22
		4	0.23
	12.5	2	No precipitate
		4	No precipitate
		8	No precipitate
Cytidine 5′-monophosphate	8.0	2	No precipitate
		4	0.58
		8	0.61
Uridine 5′-monophosphate	8.0	10	0.79
		10	0.60
		10	0.89

[a] Each number is an average of duplicate analyses and was calculated by difference in concentrations before and after precipitation. Experiments were carried out at room temperature. In each experiment, the initial poly-L-lysine concentration was 1×10^{-2} *M* (lysine residue basis). The pKa of the ϵ amino group of poly-L-lysine is 10.5. The nucleotides each have a phosphate pKa at about 6.5.

phosphate to uridine 5′-monophosphate. At pH 8.0, with reactants present in equimolar amounts, guanosine 5′-monophosphate is quantitatively precipitated from polylysine solution; under the same conditions with a 10-fold molar excess of uridine 5′-monophosphate over lysine residues, only a small amount of precipitation is observed. The specificities are also clearly shown by the turbidity curves in Fig. 1.

At the same time that these experiments were being done, space-filling models were constructed to explore the stereochemical properties of the polylysine–nucleotide interactions. Around a poly-L-lysine molecule in an α-helical form, one could fit an associated helix of base-stacked mononucleotides in the *anti*-conformation (Fig. 2). This model suggested that the 2′-OH group might be an important factor in stabilizing the interaction. It also predicted a maximum binding of three nucleotides per lysine residue, a stoichiometry never observed in the laboratory.

Wagner and Arav (13) also studied the binding of mononucleotides to poly-L-lysine and poly-L-arginine, using equilibrium dialysis at pH 7.0 in the presence of 0.02 *M* acetate. Under these conditions, the declining order of association constants for nucleotide interactions with both poly-L-lysine and poly-L-arginine was guanosine 5′-monophosphate > adenosine 5′-monophosphate > cytidine 5′-monophosphate > uridine 5′-monophosphate. The same hierarchy was found for turbidity development (phase separation).

Wagner (14) was the first to point out that, at low nucleotide concentrations, binding of mononucleotides by polybasic amino acids was essentially noncooperative, followed Langmuir binding curves, and did not produce phase separation. However, as nucleotide concentration was increased at constant polyamino acid concentration, the binding became cooperative and phase separation occurred. Wagner also found that the binding affinities of nucleotides for poly-L-arginine was in the order guanosine 5′-monophosphate > adenosine 5′-monophosphate > cytidine 5′-monophosphate.

Wulff *et al.* (15) reported a continuation of this work; they studied the binding of 5′-ribonucleotides to poly-L-lysine using circular dichroism. These experiments were carried out at 1°, pH 7, in the presence of 0.02 *M* KF. Poly-L-lysine and nucleotide concentrations were 1.4 and 0.5 m*M*, respectively. Under these conditions, only guanosine 5′-monophosphate showed circular dichroic spectra that differed significantly from the sum of the spectra of the individual components. The investigators concluded that the binding of guanosine 5′-monophosphate resulted in a cooperative ordering of guanosine 5′-monophosphate molecules, probably mediated by increased base stacking. There was some indication from the data, based on circular dichroism effects at ~220 nm, that the poly-L-lysine was assuming a more helical conformation.

Rifkind and Eichorn (16) carried out an extensive study of the effect of the 5′-ribonucleotides on the helicity of poly-L-lysine, poly-L-arginine, and poly-L-ornithine. They employed optical rotatory dispersion and used equimolar amounts of monomer and polymer at either 1×10^{-3} or 5×10^{-4} *M*. Phase separation did not occur. They studied the interaction at pH 4 and 6 in both water and 60% dioxane. In water, the only nucleotides causing significant increases in helicity of any of the polyamino acids were guanosine 5′-monophosphate and inosine 5′-monophosphate (with poly-L-arginine at pH 6).

In 60% dioxane, all 5′-nucleotides induced helicity in poly-L-lysine at pH 6. At pH 4, guanosine 5′-monophosphate, inosine 5′-monophosphate, and adenosine 5′-monophosphate induced helicity in poly-L-lysine. Poly-L-ornithine became more helical only with guanosine 5′-monophosphate at both pH 6 and 4. Poly-L-arginine became more helical with all nucleotides at pH 6 (guanosine 5′-monophosphate > adenosine 5′-monophosphate > inosine 5′-monophosphate > uridine 5′-monophosphate > cytidine 5′-monophosphate) and with guanosine 5′-monophosphate > adenosine 5′-monophosphate > inosine 5′-monophosphate at pH 4. Changes in the optical rotatory dispersion spectra at ~233 nm were used to measure helicity in the polyamino acid. Greater helical ordering of the nucleotide was observed at longer wavelengths where it was found that guanosine 5′-monophosphate became more ordered than the rest of the nucleotides, probably due to its greater ability to self-associate. Rifkind and Eichorn (16) concluded that the overall ability of the 5′-ribonucleotides to induce helicity in polyamino acids was guanosine 5′-monophosphate > inosine 5′-monophosphate > adenosine 5′-monophosphate > cytidine 5′-monophosphate ≈ uridine 5′-monophosphate, which is in agreement with base-stacking tendency (17).

These results, together with those already mentioned, make it clear that the association of the nucleotides with the basic polyamino acids consists of a primary electrostatic attraction, without which the interaction does not take place, and a secondary one, the

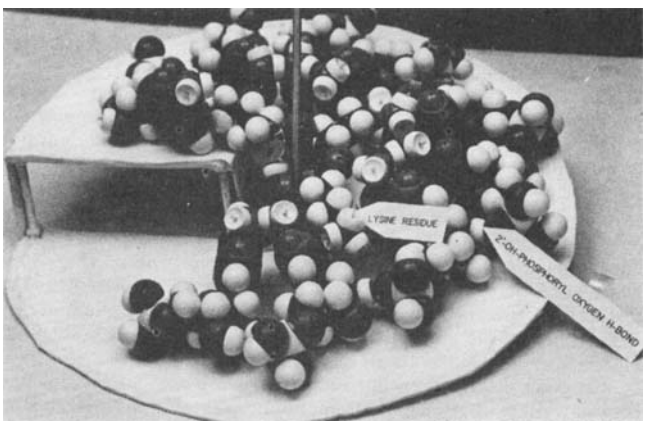


Figure 2—*Photograph of a molecular model of an α-helix of poly-L-lysine inside and complementary helix of associated adenosine 5′-monophosphate monomers outside. Note lysine side chains intercalated between adenine rings. Adenine rings are base stacked, and the nucleotides are in an approximate* anti-*conformation. The outside of the complex is a continuous sequence of ribose phosphates stabilized by hydrogen bonding of 2′-OH on one nucleotide to the phosphoryl oxygen of the next. Although the model shows hydrogen bonding of the —NH_2 of one adenine to the —NH_2 of the next, this is not a known property of adenine–adenine interactions and may not be operative in actuality.*

association of the nucleotides with themselves. The extent of cooperation of these two attractions then determines the final strength of binding.

The cooperative nature of these interactions at high nucleotide concentrations has been treated in detail (14, 18).

In model-building experiments (12), the 2′-OH groups of the nucleotides may have participated in nucleotide–nucleotide interactions through hydrogen bonding of the ribose on one nucleotide to the phosphoryl oxygen on a neighboring nucleotide. Several studies (18, 19) examined the importance of the 2′-OH group. Circular dichroic spectra showed that guanosine 5′-monophosphate and guanosine 3′-monophosphate (with 2′-OH groups) both caused changes in the spectra of poly-L-lysine at short wavelengths, indicating polyamino acid conformational change, and at high wavelengths, indicating association of the bases. However, deoxyguanosine 5′-monophosphate and deoxyguanosine 2′-monophosphate, lacking 2′-OH groups, caused only minor spectral changes.

In equilibrium dialysis experiments with the same set of nucleotides, it was found that nucleotides having the 2′-OH group possessed considerably greater nearest neighbor interaction energies than those lacking the 2′-OH group. The stacking coefficients decreased in the order guanosine 3′-monophosphate > guanosine 5′-monophosphate > deoxyguanosine 5′-monophosphate > guanosine 2′-monophosphate. The 2′-OH group is obviously an important factor in stabilizing these interactions.

Wagner (20) also explored the effect of variations in amino acid side-chain length on the interaction of polybasic amino acids with guanosine 5′-monophosphate. Polyarginine, polyhomoarginine, polyornithine, and polylysine were studied at 0°, pH 7, and in the presence of 0.02 *M* sodium acetate. The binding constants under these conditions decreased in the order polyarginine > polyhomoarginine > polyornithine > polylysine. Thus, the side chains with stronger basicity and shorter chain length had greater affinity for the nucleotide. The cooperativity of guanosine 5′-monophosphate interaction was about the same with all polymers. Circular dichroism results showed that poly-L-lysine fit best into the ordered guanosine 5′-monophosphate structure even though the other polymers had greater binding constants.

NMR Studies of Adenosine 5′-Monophosphate with Poly-L-lysine—In an NMR study of monoamino acid–mononucleotide interactions at high concentrations (21), the authors observed that adenosine 5′-monophosphate, but not the other three common mononucleotides, caused changes in the chemical shifts of amino acids. Furthermore, all three amino acids studied (lysine, arginine, and proline) showed these changes. The changes in chemical shift were downfield from the reference signal. This kind of change indicates that the amino acid side chain is oriented in the same plane as the nucleotide base ring. Since none of the other nucleotides caused such changes, it was decided that there was a generalized amino acid–adenosine 5′-monophosphate association.

The same interaction with poly-L-lysine and adenosine 5′-monophosphate was then studied. First a polymer having a molecular weight of about 100,000 was tried. After the addition of adenosine 5′-monophosphate, the lysine protons were not easily resolved. A polymer with a molecular weight of 3000 was then used. At a poly-L-lysine concentration of 0.46 *M* and pH 8.0, additions of adenosine 5′-monophosphate did not cause phase separation but did cause downfield shifts of the lysine side-chain protons (Fig. 3) as the adenosine 5′-monophosphate concentration increased. In the presence of the poly-L-lysine, the ring protons of the adenine ring showed an upfield shift with the H_8 proton shifting more than the H_2. Such upfield shifts result when base stacking occurs.

Schweizer *et al.* (9) earlier reported such a shift of the adenine proton signals of adenosine 5′-monophosphate in the presence of poly-L-lysine, but these investigators did not mention changes in the position of the lysine proton signals. These data may indicate that the lysine side chain is approaching the edge of the adenine ring in the same plane as the ring and that polylysine induces tighter stacking of the adenosine 5′-monophosphate rings. The greater shift of the H_8 proton signals would occur if the imidazole rings of the purines overlapped more than the six-membered rings. Such an offset of the rings suggests helical ordering. These data also suggest why poly-L-lysine binds preferentially to adenine–thymine-rich regions of DNA. The basis for this specificity of binding is probably the affinity of the lysine side chains for the adenine rings exposed in the grooves of the adenine–thymine regions of DNA.

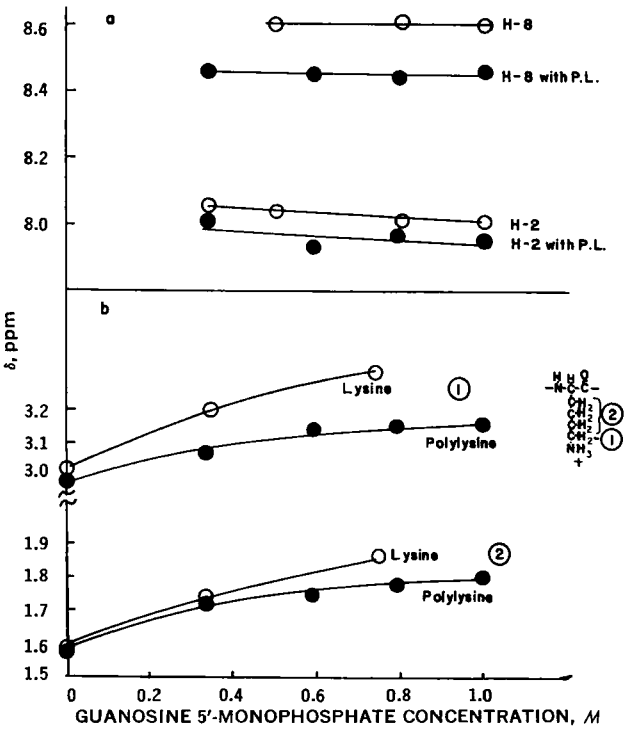


Figure 3—(*a*) *Positions of PMR signals of the protons of the adenine ring of adenosine 5′-monophosphate in aqueous (D_2O) solution at pH 8.0 and various adenosine 5′-monophosphate concentrations without* (○) *and with* (●) *poly-L-lysine (0.46* M *residue basis). An internal standard, 2,2,3,3-tetradeutero-3-(trimethylsilyl)propionic acid sodium salt, was used.* (*b*) *Positions of the amino acid side-chain protons of lysine* (○) *and poly-L-lysine* (●), *as identified in the formula, at various adenosine 5′-monophosphate concentrations at pH 8.0. Positions are relative to the internal standard. All data were collected at 25°.*

The important role played by base stacking in polyamino acid–nucleotide interactions suggests the possibility of ring–ring interactions between aromatic amino acids and DNA. There have been some reports of such interactions (22, 23). These studies have generally shown ring stacking of the aromatic amino acids with the rings of the nucleic acid bases.

SUMMARY

The results reviewed here show that the interactions of nucleotides with polybasic amino acids are strongly influenced by the nucleotide–nucleotide interactions that occur once the monomers are bound to the polyamino acid. The charge–charge interactions between negative phosphate groups and positively charged amino acid side chains are common features of all pairs of reactants. The specificity of the interaction is determined to a large extent by the variations in self-associating tendencies of the bound nucleotides.

When the monomer binding sites on the polymer are close enough to each other to allow bound monomers to interact, a cooperative binding occurs which is dependent on the specific nucleotide–nucleotide interactions. Polymer structure influences the binding process because the structure of the polymer determines the spatial relationships of the monomer binding sites. Thus, the monomer binding is influenced by such factors as the length of the amino acid side chain on the polyamino acid and helix–coil transitions in the polymer.

Both specific and nonspecific interactions can be influenced by changes in experimental conditions affecting the forces responsible for the binding. Specificity is enhanced by conditions favorable to base stacking, and binding can be prevented entirely by conditions that interfere with charge–charge interactions.

On the basis of experience with mononucleotide–polybasic amino acid interactions, it is possible to make important inferen-

ces regarding the interactions of polypeptides and nucleic acids. The coplanar interaction of lysine side chains and adenine rings suggests that exposed adenine rings in adenine–thymine-rich regions of nucleic acids may serve as important binding sites for regions of polypeptide chains having amino acid residues of appropriate structure.

Finally, these studies have important implications for the synthesis of drugs intended to interact with specific regions of nucleic acids. Such drugs should be designed to take advantage of the specificity that can be achieved by cooperative self-association once initial attachment is made to the nucleic acid.

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Drug–Biomolecule Interactions: Mechanisms and Kinetics of Interactions of Biomolecules at Interfaces

FEDERICO A. VILALLONGA

Abstract □ At the air–water interface, the area occupied by the molecules of each component of mixed monomolecular layers of cholesterol with hexadecyl alcohol, hexadecylic acid, or hexadecylamine was independent of the presence of the other component. The values of the free energy of mixing of these lipids were within the range of the entropic factor of the free energy even at high surface pressures. Mixed monolayers of cholesterol and bovine serum albumin showed a similar independence of the area per molecule and of the free energy of mixing values when the concentration of the protein was expressed in terms of amino acid residues per molecule of protein forming the mixed monolayer. Higher values of the free energy of mixing were obtained for mixed monolayers of cholesterol with dipalmitoyl lecithin and dipalmitoyl phosphatidylethanolamine than were expected from an entropic factor. The interaction between monomolecular layers of lipidic biomolecules with bulk subphase components, the energy of activation, interaction kinetics, and effects of added electrolytes were also studied. The implications of these data to a mechanism of action are discussed.

Keyphrases □ Phospholipid monolayer interactions—mechanisms and kinetics at interfaces □ Interfaces—mechanisms and kinetics of interactions, surface pressure and surface potential □ Monolayers, phospholipid—mechanisms and kinetics of interactions of biomolecules of interfaces □ Drug–biomolecule interactions—mechanisms and kinetics of interactions of biomolecules at interfaces □ Interactions—drugs with biomolecules, symposium

Drug molecules incorporated into body fluids are distributed and directed to receptors located in tissue cells. If the receptors form part of the cell membrane, as seems to be the case for some local anesthetics (1) and bactericides (2), the interaction with the receptor alters the permeability of the cell membrane and thus completely defines the pharmacological activity of the drug. If the receptors are located in subcellular

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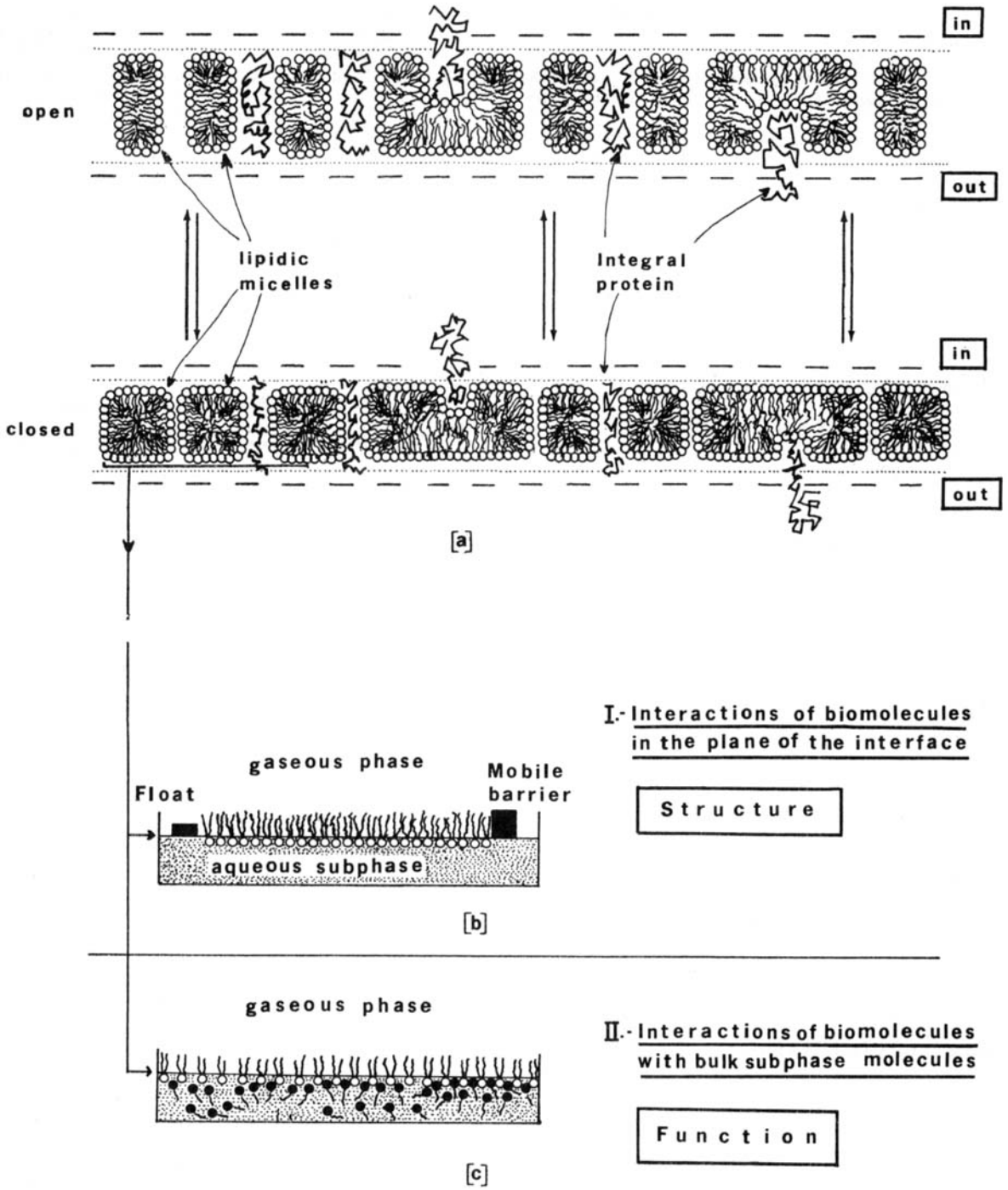

Figure 1—*Speculative diagrammatic representation of the changes of micellar form and the reversible transformation from the open to the closed configuration of biomembrane. Dashed lines represent the limits of the membrane; dotted lines represent the oriented layer of water.*

organelles, as seems to be the case for many antibiotics (2), the drug molecule must penetrate through the cell membrane to find its way to the receptor.

It follows then that, in all cases, the first stage in the exercise of the pharmacological action of a drug must be the interaction of the drug molecule with the cell membrane components.

DISCUSSION

It is not possible here to discuss the many different models of membrane structure that have been proposed since the Danielli–Dawson model (3). The reader is referred to Ref. 4 for this purpose. Biological membranes are composed mainly of lipids and proteins and maintain their integrity because of the interactions between their components. Figure 1*a* represents a speculation that brings together the "open–closed" hypothesis (5) with the existence of lipid bilayers between repeating "integral protein" units (6).

Changes in the local charge distribution in the thin crust of water that is oriented at the membrane interface provoke reversible variations of the micellar forms. In turn, they produce the reversible transformation of an "open" state to a "closed" state of biomembranes (5). Drug molecules approaching the cell interface can produce these changes.

The study of the interactions in the plane of the interface be-

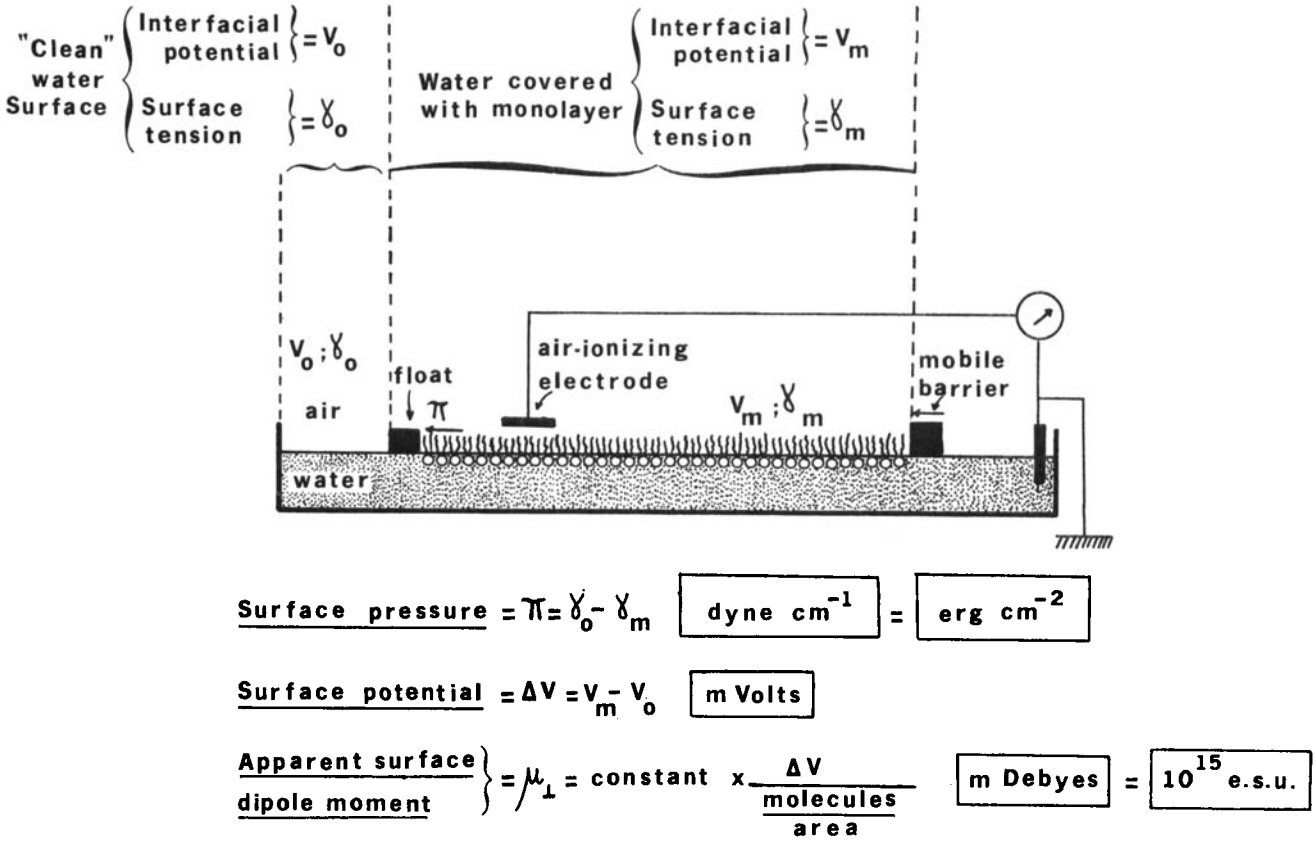

Figure 2—*Schematic representation of the surface pressure, surface potential, and apparent surface dipole moment.*

tween biomolecules of the type found in biomembranes (Fig. 1*b*) may yield useful information on the orgin and strength of the forces involved in the maintenance of the integrity of biomembranes.

The study of the interaction between monomolecular layers of biomolecules similar to those that form the cell membranes with molecules that approach from the bulk subphase (Fig. 1*c*) can further the understanding of the role of specific components of biomembranes in the interaction and of how the approaching molecule can affect the microstates of biomembranes.

To define the state of the molecules forming a monomolecular layer (7), three primary parameters are used: *(a)* the area per molecule, *(b)* the surface pressure, and *(c)* the surface potential. The area per molecule is simply obtained by dividing the total surface area of the interface by the number of molecules forming the monomolecular layer.

The surface pressure, π (Fig. 2), a force acting in a plane parallel to the interface, is defined as the difference between the surface tension of the "clean" air–water interface and the surface tension of the water covered with the monomolecular layer. The surface potential, ΔV, which corresponds to the summation of the contributions of each molecule to the interfacial potential of the monomolecular layer, is defined by the difference between the interfacial potential of the monomolecular layer and the interfacial potential of the clean air–water interface. The apparent surface dipole moment per molecule, μ, which is the contribution of each molecule to the interfacial potential of the monomolecular layer, is defined as proportional to the ratio of the surface potential to the number of molecules per unit area of the monomolecular layer.

Interactions between Biomolecules in Plane of Interface—Pure, single lipids and proteins, when spread at an interface, form monomolecular layers which can be studied and characterized by the measurement of their surface parameters. Binary mixtures of these substances spread at the air–water interface may show values of their surface parameters that adhere to or depart from those expected from the addition of the values corresponding to each single, isolated component. Analysis of the experimental data obtained can give information about interactions between the components.

Figure 3 represents the plot of the mean area per molecule, A_{ab}, obtained by dividing the total surface area by the *total* number of molecules that form the monolayer (Component *a* + Component *b*) against the molar fraction of the binary mixed monolayer (N_a or N_b) (8). The broken line (Fig. 3, line I) is obtained with the experimental points when either nonmixing or ideal mixing of the components occurs. In both cases the area occupied by each molecule of Components *a* and *b* is identical to the area that these molecules occupy when they are in a single, pure monomolecular layer of each component. The additivity rule is obeyed and for any composition of the mixed monolayer the mean surface area per molecule is given by:

$$A_{ab} = N_a A_a + N_b A_b \qquad \text{(Eq. 1)}$$

in which A_{ab} is the mean area per molecule in the binary mixed monomolecular layer; and N_a, N_b, A_a, and A_b are the molar fractions of Components *a* and *b* and the area per molecule of each of these components, respectively, when they are in a single, pure monolayer at the same conditions of surface pressure and temperature.

The enthalpy of mixing for an ideal mixing process, $\Delta H_M{}^I$, is equal to zero and from Eq. 2:

$$\Delta G_M{}^I = \Delta H_M{}^I - T\Delta S_M{}^I \qquad \text{(Eq. 2)}$$

it follows that:

$$\Delta G_M{}^I = -T\Delta S_M{}^I \qquad \text{(Eq. 3)}$$

which states that the free energy of mixing, $\Delta G_M{}^I$, or the "driving force" of the mixing process is entirely of entropic origin, $\Delta S_M{}^I$, and is a function only of the molar fraction:

$$\Delta G_M{}^I = RTN_a \ln N_a + RTN_b \ln N_b \qquad \text{(Eq. 4)}$$

in which R is the universal gas constant, T is the temperature (°K), and N_a and N_b are the molar fractions of Components *a* and *b* in the binary mixed monomolecular layer, respectively.

The full lines II, III, and IV (Fig. 3) are obtained when conden-

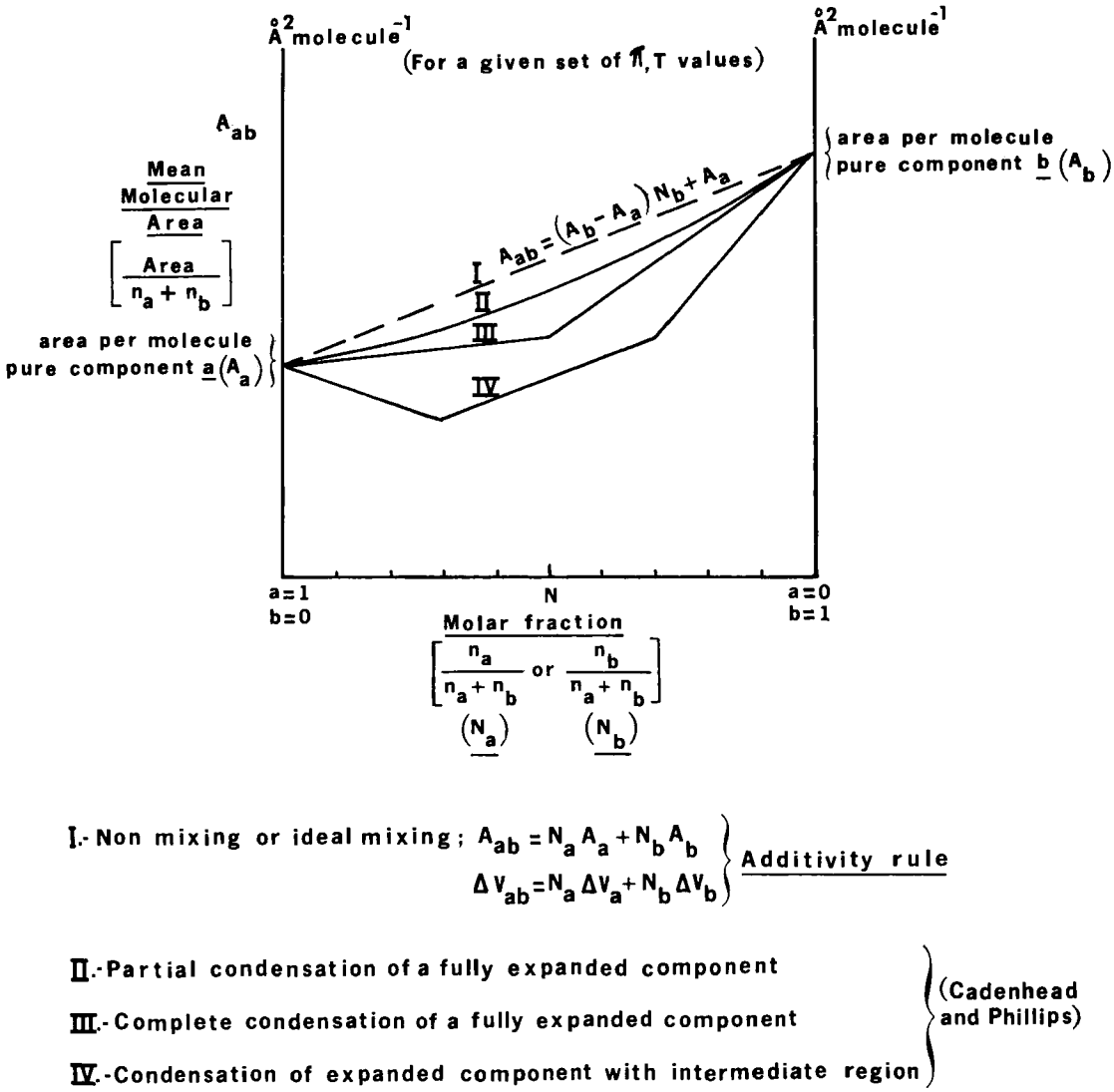

Figure 3—*Plot of the mean molecular area against molar fraction in mixed monolayers.*

sation effects occur on an expanded Component b because of the presence of Component a in the mixed monomolecular layer. Plots of the III and IV type are typically obtained at different surface pressures for mixed monomolecular layers of cholesterol and lecithin (9–13). The presence of sharp changes of slope at a given composition was taken to indicate (9) the formation of molecular complexes. This concept involves the existence of some energy of interaction between the cholesterol and lecithin molecules forming the complex.

The negative departure from the additivity rule can be explained in this particular case without referring to the postulation of complex formation (14, 15). In the expanded liquid state, adjacent lecithin molecules with their fatty acyl chains could generate an empty volume similar to an inverted cone that constitutes a "cavity" which can be "filled" with cholesterol molecules without causing an increase in the monomolecular layer.

A thermodynamic treatment (16) can be used with some restrictions (8, 12, 16–18) for the experimental evaluation of the free energy of mixing, ΔG_M, of the components of a mixed monolayer. However, evidence was presented recently (19) which indicates that the thermodynamic interpretations in the case of dipalmitoyl lecithin systems are tenuous and probably incorrect.

The excess free energy of mixing, $\Delta G_M{}^E$, is given by:

$$\Delta G_M{}^E = \int_{\pi^*}^{\pi} (\delta_{ab} - N_a\delta_a - N_b\delta_b)\, d\pi \qquad \text{(Eq. 5)}$$

where δ_{ab}, δ_a, and δ_b represent the area of the mixed monolayer and the areas of the pure, single monolayers of Components a and b, respectively; N_a and N_b represent the respective molar fractions, π is the surface pressure, and π^* is usually taken as zero.

The integrals $\int_{\pi^*}^{\pi} \delta_{ab}\, d\pi$, $\int_{\pi^*}^{\pi} \delta_a\, d\pi$, and $\int_{\pi^*}^{\pi} \delta_b\, d\pi$ can be separately evaluated by weight integration of the compression curve of the mixed monolayer, the monolayer of pure Component a, and the monolayer of pure Component b.

The free energy of mixing can be calculated (20) using:

$$AG_M = \Delta G_M{}^E + \Delta G_M{}^I \qquad \text{(Eq. 6)}$$

in which $\Delta G_M{}^I$ (see Eq. 4), being independent of the chemical nature of the components, can be numerically calculated for each value of the molar fraction.

Table I represents the results obtained by the application of this method of mixed monolayers of cholesterol and dipalmitoyl lecithin, dipalmitoyl cephalin, hexadecylamine, hexadecanol, and palmitic acid (17).

The significance of cholesterol–phospholipid interactions in the maintenance of the integrity of some biological membranes has been emphasized (21, 22). The free energy of mixing for a binary system is a measure of the tendency of its components to undergo a dissolution process. From Table I, it can be seen that the most negative value for all mixtures studied corresponds to the systems of cholesterol–dipalmitoyl lecithin and cholesterol–dipalmitoyl cephalin, which are roughly 200 cal/mole more negative than those corresponding to the ideal system or to cholesterol–hexadecanol and cholesterol–palmitic acid. The greater tendency for dissolution

Table I—Free Energy of Mixing (ΔG_M, cal/mole) at Air–Water Interface for Mixtures of Cholesterol with Phospholipid or C_{16} Hydrocarbon Chain Compounds[a]

	Molar Fraction				
	0.20	0.33	0.50	0.67	0.80
β,γ-Dipalmitoyl-DL-α-lecithin	−330	−480	−630	−510	−300
	(−210)	(−450)	(−460)	(−460)	(−300)
β,γ-Dipalmitoyl-DL-α-cephalin	−430	−440	−640	−430	−340
	(−300)	(−250)	(−430)	(−310)	(−220)
Hexadecylamine	(−240)	(−350)	(−390)	(−340)	(−280)
1-Hexadecanol	−180	−260	−370	−250	−200
	(−220)	(−290)	(−380)	(−280)	(−230)
Palmitic acid	−380	−460	−450	−350	−280
	(−330)	(−430)	(−430)	(−350)	(−280)
Ideal system, $\Delta G_M^I = RT(N_1 \ln N_1 + N_2 \ln N_2)$	−290	−370	−400	−370	−290

[a] Temperature, 20°; subphase, phosphate buffer (pH 7.2, ionic strength 0.15). Integration limits are 0 and 40 dynes/cm. Figures in parentheses correspond to the integrals between 0 and 27.5 dynes/cm.

of the phospholipids in equimolecular amounts of cholesterol at the air–water interface could originate from some interaction between cholesterol and these phospholipids.

The difference between the values of the free energy of mixing for both pairs of integration limits in the cholesterol–phospholipid systems seems to indicate that a closed, packed configuration is necessary for enhancement of mutual dissolution. Since this effect is not observed for systems containing cholesterol and hexadecanol or palmitic acid, it may be concluded that some geometrical molecular arrangement of the phospholipid molecules is necessary to increase the mutual dissolution between these molecules.

This type of deviation from the additivity rule has been observed in many mixed monomolecular layers involving lipids known to form biomembranes. From the results reported (8, 12, 13, 17, 23), it seems that specific interactions between groups cannot explain all of the experimental facts. Cavity effects and van der Waals forces between the methylene groups of the hydrocarbon chains probably are involved in this interaction. The overall picture obtained with the results points out that dispersive or van der Waals forces may constitute a main factor in the maintenance of the structure of the lipid micelles of biomembranes.

Biomembrane models suggest that proteins could be structural as well as functional components of biomembranes and that lipid–protein interactions could play an important role in the structure of biomembranes. It was suggested (24) that cholesterol monomolecular layers at the air–water interface interact with proteins injected into the subphase because of specific forces that involve the hydroxy group of cholesterol and the peptide bond of bovine serum albumin. However, the compression curves of mixed monomolecular layers of cholesterol and bovine serum albumin show no significant departure from the additivity rule in the plot of the mean molecular area as a function of the molar fraction (Fig. 4) (25).

In this case, the protein molecules were spread directly at the

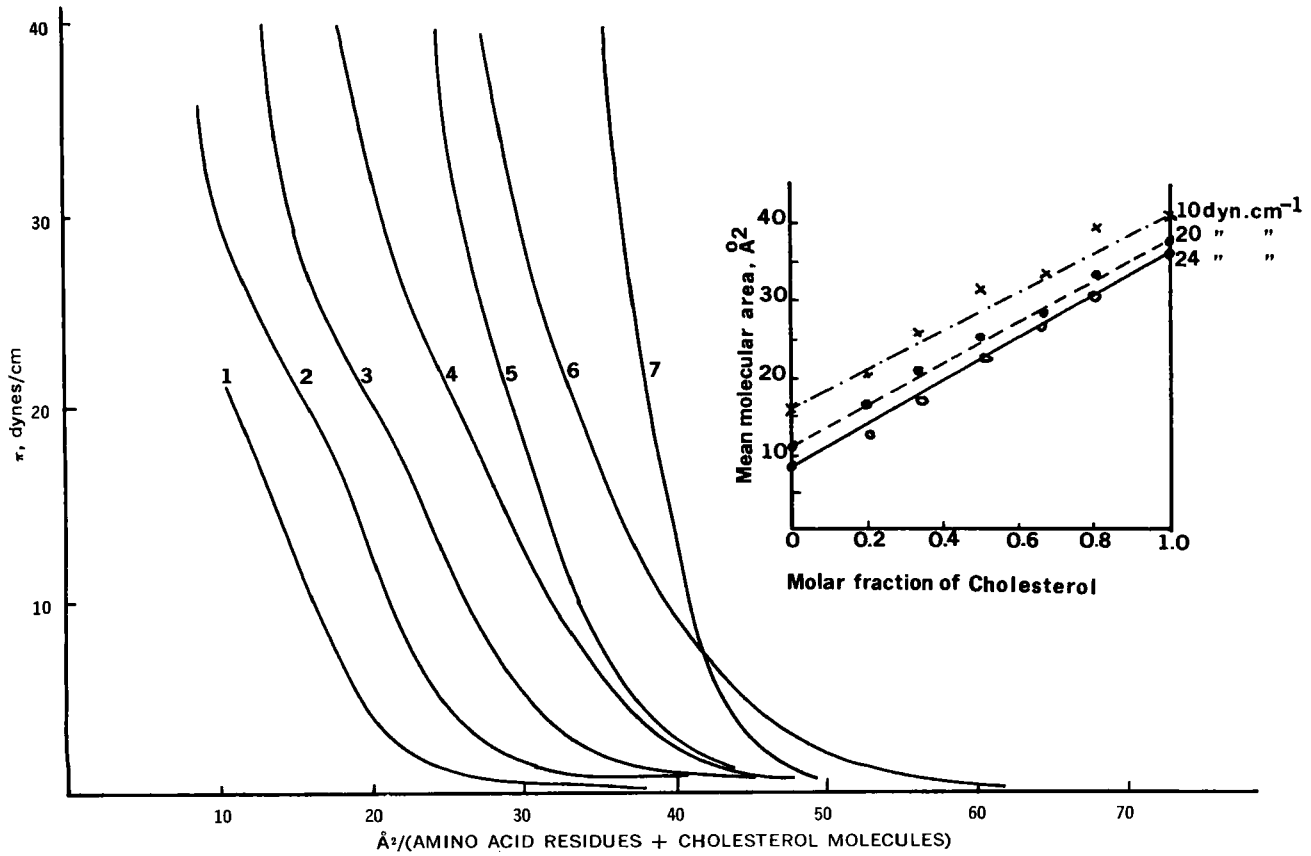

Figure 4—*Surface pressure–area curves of mixed monolayers of bovine serum albumin and cholesterol. Key: 1, 100% albumin; 2, 20% cholesterol; 3, 33% cholesterol; 4, 50% cholesterol; 5, 67% cholesterol; 6, 80% cholesterol; and 7, 100% cholesterol.*

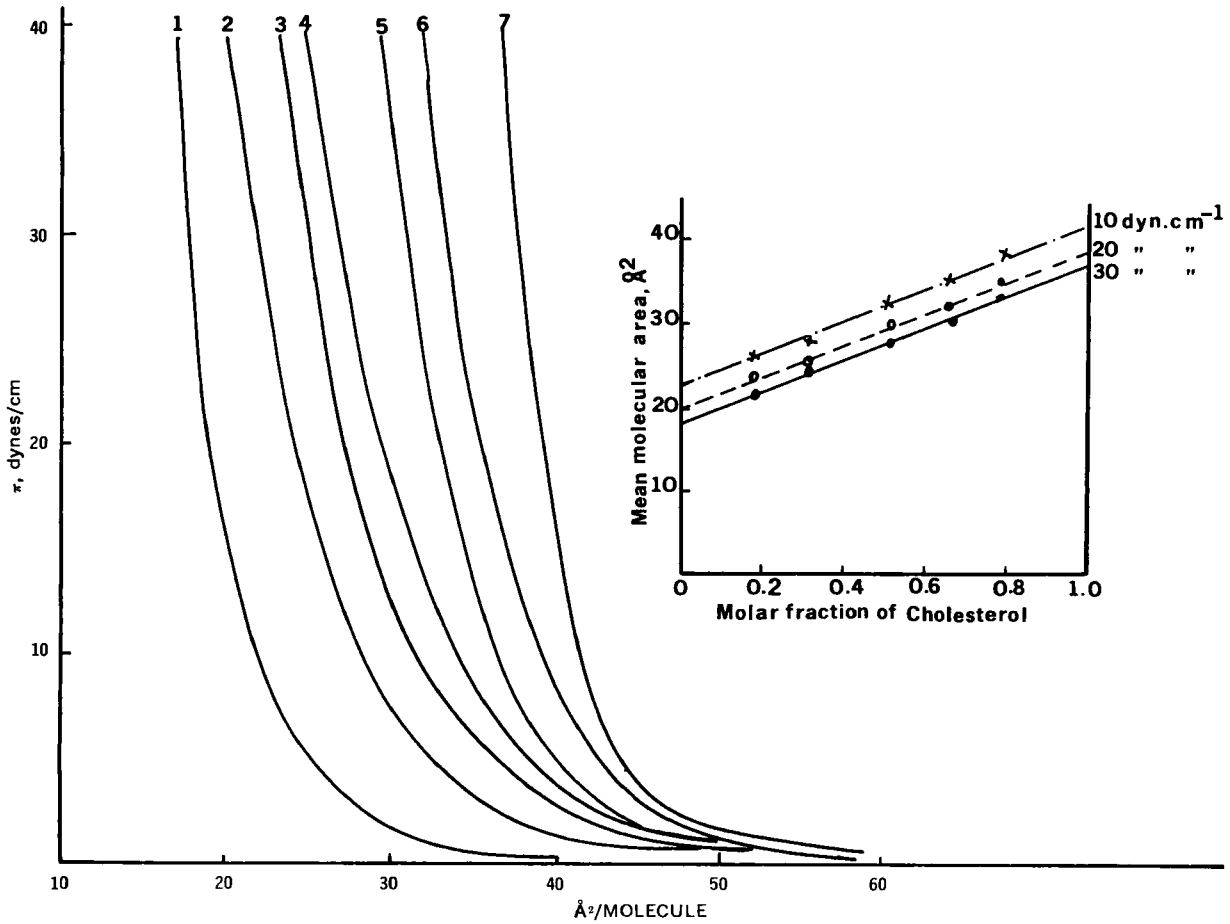

Figure 5—*Surface pressure–area curves of mixed monolayers of cetyl alcohol and cholesterol. Key: 1, 100% cetyl alcohol; 2, 20% cholesterol; 3, 33% cholesterol; 4, 50% cholesterol; 5, 67% cholesterol; 6, 80% cholesterol; and 7, 100% cholesterol*

plane of the cholesterol monomolecular layer and the mean molecular area was calculated using the number of amino acid residues per molecule of bovine serum albumin. Similar plots are obtained for mixed monomolecular layers of cholesterol and cetyl alcohol (Fig. 5). In both cases (Fig. 6), the calculation of the free energy of mixing shows that the values obtained are those to be expected from an entropic factor only, without any contribution of an excess of free energy of mixing.

Furthermore, when bovine serum albumin is added at the plane of the interface in which a cholesterol monolayer was previously spread, the plot of the increment of the surface pressure against the number of bovine serum albumin molecules is of the Langmuir type (Fig. 7). If the concentration is expressed in molar fraction calculated with the number of amino acid residues per molecule of bovine serum albumin, the plot is then a straight line (Fig. 8). The same type of linear plot of the increment of surface pressure against concentration is obtained for the addition (in the plane of the interface) of sodium hexadecyl sulfate, sodium lauryl sulfate, bovine growth hormone, insulin, or 5-valineangiotensin when (in the last three cases) the concentration is expressed as molar fractions of amino acid residues (Fig. 9).

Within the limits of the experimental error, all cases studied adhered to:

$$\pi_{tot} = \pi^{0}_{chol} + mN_R \qquad \text{(Eq. 7)}$$

in which π_{tot} represents the total surface pressure after the spreading of the protein, polypeptide, or surfactant in the plane of the cholesterol monolayer; π^{0}_{chol} represents the surface pressure of the cholesterol monomolecular layer before the spreading of the other substances; and N_R is the molar fraction in the monomolecular layer of the added substance calculated, in the case of proteins and peptides, with the number of amino acid residues.

The adherence to this equation, which represents the total vapor pressure of a tridimensional system as a function of the molar fraction, strongly suggests that the process is simply a bidimensional, nonspecific mutual dissolution between the amino acid residues (or the long hydrocarbon chains of the surfactants) and the cholesterol monomolecular layer.

Here, again, the experimental evidence seems to indicate that nonspecific forces of the van der Waals type are likely involved in the interaction of proteins with monomolecular layers of cholester-

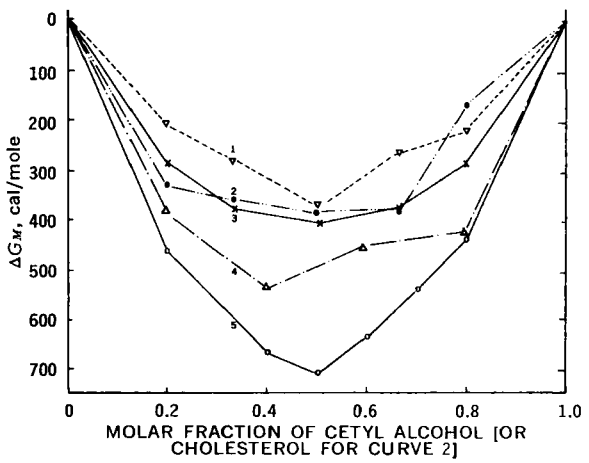

Figure 6—*Free energy of mixing, ΔG_M, as a function of molar fraction. Key: 1, △, cetyl alcohol–cholesterol; 2, ●, cholesterol–bovine serum albumin; 3, ×, ideal system, calculated from Eq. 4; 4, △, cetyl alcohol–$C_{14}H_{29}\cdot CH(CH_3)OSO_3Na$ calculated from Ref. 1; and 5, ○, cetyl alcohol–sodium hexadecyl sulfate calculated from the data of Ref. 10.*

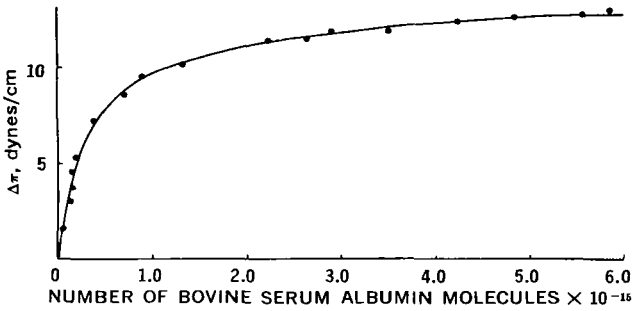

Figure 7—*Increment of surface pressure, $\Delta\pi$, in a cholesterol monolayer at 10 dynes/cm initial surface pressure with the number of bovine serum albumin molecules added at the plane of the interface at constant area.*

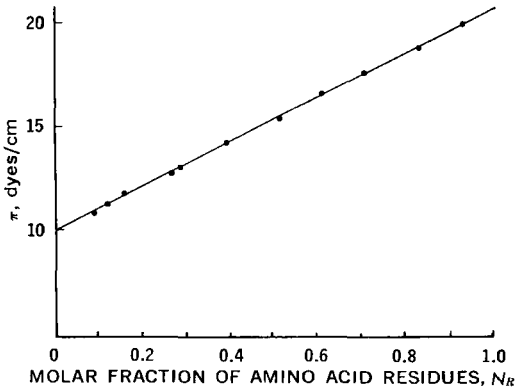

Figure 8—*Total surface pressure, π, as a function of molar fraction of amino acid residues of bovine serum albumin, N_R, at constant area. Initial surface pressure of cholesterol monolayer = 10 dynes/cm.*

ol and, consequently, that these forces could play an important role in the maintenance of the integrity of biomembranes.

Interactions of Biolipid Molecules with Subphase Components—Studies of this type have been used since the 1930's with biological processes. A review on this subject is not intended here, and the reader is referred to Refs. 26–28.

The process of the interaction of a molecule approaching to a

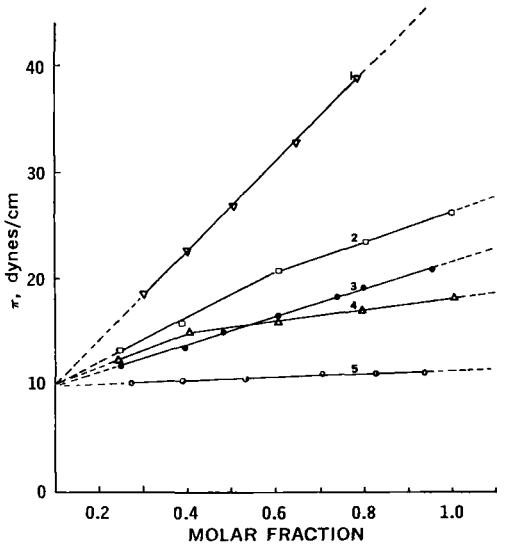

Figure 9—*Total surface pressure as a function of molar fraction for: 1, sodium hexadecyl sulfate; 2, bovine growth hormone, amino acid residues; 3, sodium lauryl sulfate; 4, insulin, amino acid residues; and 5, 5-valineangiotensin, amino acid residues. Initial surface pressure of cholesterol monolayer = 10 dynes/cm.*

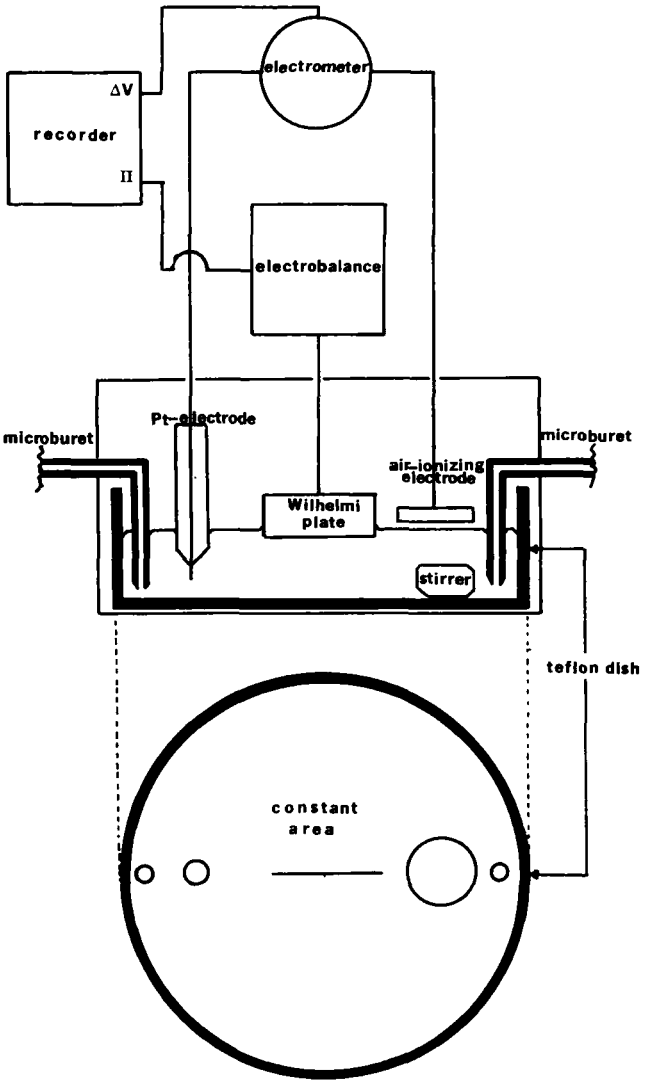

Figure 10—*Experimental assembly for the recording of changes in surface pressure and surface potential.*

monomolecular layer from the subphase can be followed by the measurement of the variations of the surface parameters as a function of time. The study can be performed at constant surface pressure or at constant surface area. When energetic values are sought, the parameter of choice is the variation of surface pressure at constant surface area after the injection of the desired molecule in the subphase beneath the monomolecular layer. This variation is a function of the excess surface concentration and the changes in the monolayer properties that could affect its surface pressure such as conformational changes and phase transitions.

Figure 10 shows a simple experimental device (29). A Teflon dish containing a final and constant volume of water provided with a Teflon-coated magnetic stirring bar is used as a trough. The tips of two identical microburets are immersed in the water. One microburet contains the solution of the substance to be injected. The other microburet is used to withdraw, prior to the injection, exactly the same volume of water to avoid effects of the variations of buoyancy on the platinum plate.

The Wilhelmy platinum plate for the measurement of the surface pressure is attached to an electrobalance. The ^{241}Am, air-ionizing electrode for the measurement of the surface potential is attached to an electrometer. The outputs of both instruments are fed into a dual-pen recorder, allowing measurement of the variation of the surface pressure and surface potential as a function of time at constant surface area and temperature.

The study of the interaction concerns two aspects: the kinetics of approach to equilibrium and the resultant equilibrium. The equilibrium has been studied by the application of a modified

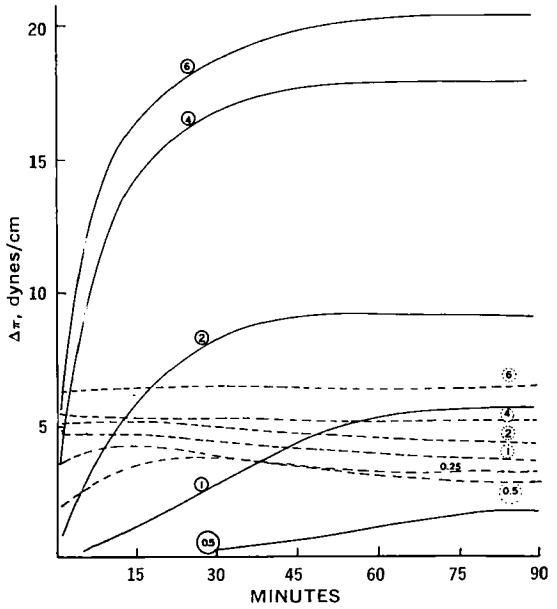

Figure 11—*Plots of the increment of surface pressure against time after the injection of sodium cetyl sulfate (——) or cetrimonium bromide (– – –) beneath a dipalmitoyl lecithin monolayer at 5 dynes/cm. Curves are labeled as to micromolar final concentrations of the injected surfactant.*

		Energy of activation, Kcal. mole^{-1}	
		Hexadecylsulfate$^-$ $CH_3(CH_2)_{15}$ SO_4^-	Hexadecyltrimethylammonium$^+$ $CH_3(CH_2)_{15}$ $N^+(CH_3)_3$
Dipalmitoyl glycerol	$2[CH_3(CH_2)_{14}]$ Esterified glycerol	10.8	10.6
Dipalmitoyl lecithin	$2[CH_3(CH_2)_{14}]$ $O\cdot P\cdot(CH_2)_2\cdot N^+(CH_3)_3$	9.4	11.8
Dipalmitoyl phosphatidyl-ethanolamine	$2[CH_3(CH_2)_{14}]$ $O\cdot P\cdot(CH_2)_2\cdot N^+H_3$	10.9	11.3
Phosphatidyl serine	$2[CH_3(CH_2)_?]$ $O\cdot P\cdot CH_2CHN^+H_3\cdot COO^-$	7.5	8.8
Phosphatidic acid	$2[CH_3(CH_2)_?]$ $O\cdot P\cdot O^-$	7.2	8.8
Behenic acid	$CH_3(CH_2)_{20}$ COO^-	9.9	12.3

Figure 12—*Energies of activation for the interaction of cetyl sulfate and cetrimonium ions with different lipids. Initial surface pressure of the monolayers = 5 dynes/cm.*

Gibbs adsorption equation (30, 31) and by the postulation of an osmotic equilibrium (32) between two presumed phases. A systematic study of the kinetics of these processes was performed only recently (29).

Figure 11 shows typical plots of the increment of surface pressure, $\Delta\pi$, against time after the injection of sodium cetyl sulfate or cetrimonium bromide beneath a dipalmitoyl lecithin monolayer at 5 dynes/cm (29). Both the kinetics and the final equilibrium vary with the nature of the polar head of the injected molecule. The interaction of cetrimonium bromide is virtually completed in the first few minutes after the injection and the effect on the surface pressure is relatively small. The interaction of sodium cetyl sulfate takes a much longer time to achieve equilibrium, and the effect on the surface pressure is more than three times greater than the effect of the cetrimonium bromide.

Energetics—The final value of the increment of the surface pressure ($\Delta\pi$, dynes/cm) as a function of the concentration in the bulk subphase of the injected molecules (n, molecules/cm^3) permits the estimation of the energy of activation of the process on the premise of a collision model and a constant entropy factor (29, 33) from:

$$1/\Delta\pi = 1/\Delta\pi_m + (B/\Delta\pi_m)(1/n) \qquad \text{(Eq. 8)}$$

$$B = (Ns/A)(2\pi_m/kT)^{1/2}(\nu)(e^{-\varphi/kT}) \qquad \text{(Eq. 9)}$$

in which (Ns/A) is the number of molecules per unit area that form the lipidic monolayer; $\pi = 3.14\ldots$; m is the mass of the approaching molecule (or ion); k and T are the Boltzman constant and the absolute temperature, respectively; and φ is the energy of activation. The frequency factor, ν, that corresponds to the vibration of an oscillator perpendicular to the plane of the interface can be assumed to be 10^{-12} sec (34).

Figure 12 shows the values of the energies of activation for the interaction of cetyl sulfate and cetyl sulfate–cetrimonium ions with several lipid and phospholipid monolayers (40).

Taking as a reference the dipalmitoyl glycerol molecule, it can be observed that the attachment of a complex phosphoryl group with a net negative charge produces a significant difference between the energies of activation of the interaction of the molecules that form the monolayer with the positive or the negative long chain hydrocarbon ion injected. The zwitterionic dipalmitoyl lecithin and dipalmitoyl phosphatidylethanolamine, with a net charge equal to zero in the conditions of the experiments, differentiate, however, through the value of the energy of activation the charge of the injected ion. This seems to indicate that even for the zwitterionic phospholipids the ionic groups of the polar hydrophilic moiety are not equivalent in the perturbation that an attached hydrocarbon chain produces in the surface pressure of the phospholipid monolayer.

The energy of activation is a function of the chain length of the injected molecule (33). Figures 13–15 show the plots of the energies of activation against the chain length for the interaction of series of alkyl sulfate and alkyl trimethylammonium ions with dipalmitoyl glycerol, dipalmitoyl phosphatidylethanolamine, and dipalmitoyl lecithin. The slope of the best straight line passing through the experimental points is related to the energy of activation per methylene group of the hydrocarbon chain of the injected ion, and the intercept could be interpreted as related to the contribution of the polar head to the total energy of interaction.

Table II shows the value of the energy of activation per methylene group and per polar group for the interaction of alkyl sulfate and alkyl trimethylammonium ions with dipalmitoyl glycerol, di-

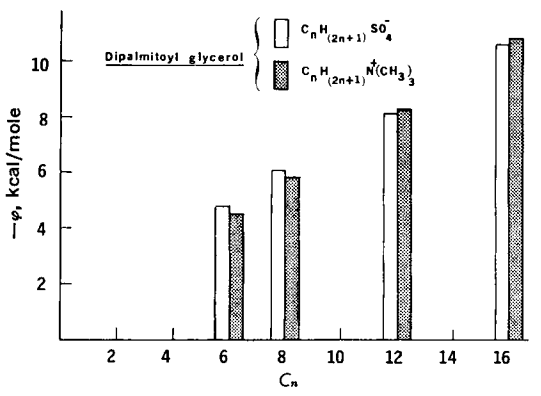

Figure 13—*Energies of activation as a function of the chain length of alkyl sulfate and alkyl trimethylammonium ions for the interaction with dipalmitoyl glycerol monolayers at 5 dynes/cm initial surface pressure.*

Table II—Energies of Activation of the Interaction of Alkyl Sulfate and Alkyl Trimethylammonium Ions with Dipalmitoyl Glycerol, Dipalmitoyl Phosphatidylethanolamine, and Dipalmitoyl Lecithin

Interaction		φ, kcal/mole				Energy of Activation, kcal/Group mole		
		n =						
Lipidic Monolayer	Surfactant Ion	6	8	12	16	SO_4^-	$N^+(CH_3)$	CH_2
Dipalmitoyl glycerol	$C_nH_{(2n+1)}\cdot SO_4^-$	−4.8	−6.0	−8.3	−10.6	−1.30	—	−0.58
	$C_nH_{(2n+1)}\cdot N^+(CH_3)_3$	−4.4	−5.8	−8.4	−10.8	—	−0.60	−0.64
Dipalmitoyl phosphatidyl-ethanolamine	$C_nH_{(2n+1)}\cdot SO_4^-$	−4.9	−6.1	−8.5	−10.8	−1.4	—	−0.62
	$C_nH_{(2n+1)}\cdot N^+(CH_3)_3$	−5.1	−6.4	−8.8	−10.3	—	−1.4	−0.62
Dipalmitoyl lecithin	$C_nH_{(2n+1)}\cdot SO_4^-$	−4.4	−5.5	−7.6	−10.0	−1.0	—	−0.56
	$C_nH_{(2n+1)}\cdot N^+(CH_3)_3$	−4.7	−6.3	−9.3	−12.4	—	−0.10	−0.77

palmitoyl phosphatidylethanolamine, and dipalmitoyl lecithin. As it can be observed, the energy of activation per methylene group is in the range of from −0.56 to −0.77 kcal/mole, and the contribution to the energy of activation of the sulfate or the trimethylammonium group always has a negative value.

The energy of adsorption at the air–clean water interface of long chain hydrocarbon ions has been measured by different investigators. Table III represents the numerical values obtained for the energy of adsorption per methylene group and per polar head calculated using literature data (36–38).

The energy of activation per methylene group in the interaction process of the long chain hydrocarbon ion with the lipidic monolayers is energetically equivalent to that of the adsorption of methylene groups at an air–clean water interface in all cases studied, with the exception perhaps of the interaction of alkyl trimethylammonium ions with dipalmitoyl lecithin monolayers.

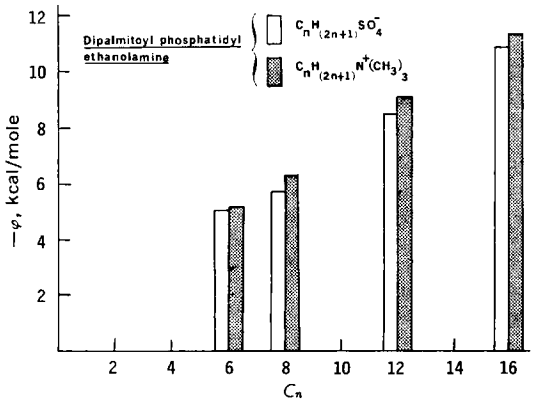

Figure 14—*Energies of activation as a function of the chain length of alkyl sulfate and alkyl trimethylammonium ions for the interaction with dipalmitoyl phosphatidylethanolamine monolayers at 5 dynes/cm initial surface pressure.*

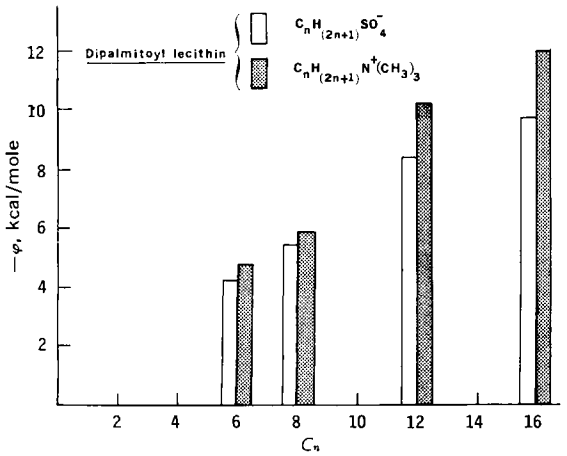

Figure 15—*Energies of activation as a function of the chain length of alkyl sulfate and alkyl trimethylammonium ions for the interaction with dipalmitoyl lecithin monolayers at 5 dynes/cm initial surface pressure.*

The energy of activation per polar head of the injected long chain hydrocarbon ion, because of its numerical value and the negative sign, is closer to the values obtained for the energy of adsorption of sulfate and trimethylammonium polar heads at a hydrocarbon–clean water interface.

The picture that emerges from these energetic findings is by far more complex than that associated with the mechanical effects suggested by the semantics of "monolayer penetration" coined by Schulman and Rideal (39). The comparison of the values obtained in the interaction processes with those obtained for the adsorption at clean interfaces (without any monolayer) suggests that the polar head group of the injected long chain hydrocarbon ion could be located near to a hydrocarbon environment with its hydrocarbon chain more likely near to an aqueous environment.

This suggestion and the differences observed in the energies of activation of the interaction of alkyl sulfate and alkyl trimethylammonium ions with the zwitterionic phospholipids point out that both the polar hydrophilic moiety of the phospholipid and the polar head of the long hydrocarbon chain determine the different perturbation that identical hydrocarbon chains can produce in the state of the monolayer. The presence of this type of phospholipids is almost constant in biomembranes and, consequently, these facts strongly suggest that the microstates of the lipid micelles of biomembranes could be differently affected according to the nature of the approaching molecule.

Kinetics—The effective change in surface pressure for the interaction of cetrimonium ions with dipalmitoyl lecithin, dipalmitoyl phosphatidylethanolamine, and dipalmitoyl glycerol monolayers is essentially an extremely fast process under the experi-

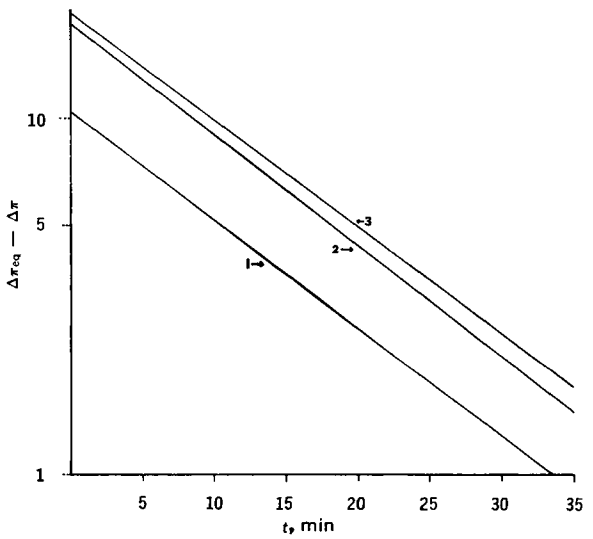

Figure 16—*Typical apparent first-order plots for the interaction of 4 × 10⁻⁶ M cetyl sulfate ion with: 1, dipalmitoyl lecithin; 2, dipalmitoyl glycerol; and 3, dipalmitoyl phosphatidylethanolamine monolayers.*

Table III—Energies of Adsorption per Group of Alkyl Sulfate and Alkyl Trimethylammonium Ion at the Hydrocarbon–Water and Air–Water Interfaces[a]

Energy of Adsorption, kcal/Group mole					
Hydrocarbon–Water			Air–Water		
SO_4^-	$N^+(CH_3)_3$	CH_2	SO_4^-	$N^+(CH_3)_3$	CH_2
−1.2	−0.70	−0.81	+2.4	+2.7	−0.63

[a] From Refs. 34 and 36–38.

mental conditions. The new steady-state values are reached within a few minutes after the injection.

The interaction of cetyl sulfate ions with these three lipids is comparatively a slower process. Figure 16 shows the plots of the logarithm of the difference between the value of the increment of the surface pressure at equilibrium, $\Delta\pi_{eq}$, and the increment at a time t, $\Delta\pi$, as a function of time for the interaction of cetyl sulfate at a final concentration of 4×10^{-6} *M* with dipalmitoyl glycerol, dipalmitoyl lecithin, and dipalmitoyl phosphatidylethanolamine (40). The linear plot suggests an apparent first-order increase of the surface pressure to a new steady-state value with almost identical values of the apparent first-order rate constant.

The presence of an inert electrolyte affects the kinetics of these processes and permits the evaluation of the apparent first-order rate constants even in the cetrimonium-ion interaction. Figure 17 shows the decrease of the apparent first-order rate constant in the presence of an increasing concentration of sodium chloride, from 0.15 to 0.45 *M* for the interaction of cetrimonium ion at a final concentration of 4×10^{-6} *M* with dipalmitoyl lecithin monolayers[1]. This same effect can be observed using potassium chloride as the added electrolyte (Fig. 18).

The plot of the logarithm of the apparent first-order constant against the square root of the ionic strength gave straight lines in both cases, with an identical negative slope of approximately unity (Fig. 18) but with a different intercept. The adherence to the straight-line equation, the negativity, and the numerical value of the slope indicate that the interaction between cetrimonium ions

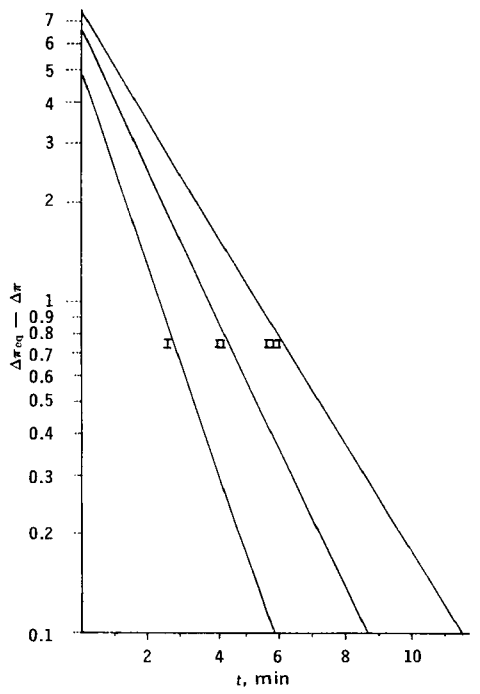

Figure 17—*Typical apparent first-order plots for the interaction of* 4×10^{-6} M *cetrimonium ions with dipalmitoyl lecithin monolayers. Key: I, 0.15* M *NaCl; II, 0.30* M *NaCl; and III, 0.45* M *NaCl.*

[1] E. R. Garrett and F. A. Vilallonga, unpublished results.

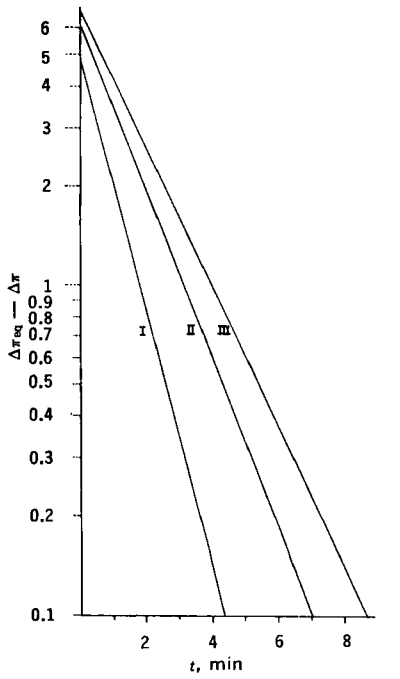

Figure 18—*Typical apparent first-order plots for the interaction of cetrimonium ions with dipalmitoyl lecithin monolayers. Key: I, 0.15* M *KCl; II, 0.30* M *KCl; and III, 0.45* M *KCl.*

with the dipalmitoyl lecithin monolayer could be described as an interaction between ions of opposite charge (40) and suggests that the first event in the process is the attachment of the polar head of the cetrimonium ion, possibly to the phosphate group of the zwitterionic polar hydrophilic moiety of dipalmitoyl lecithin.

The difference observed between the plots of the logarithm of $\Delta\pi_{eq} - \Delta\pi$ as a function of time for subphases containing sodium chloride or potassium chloride indicates that for a given ionic strength the interaction of cetrimonium ions with dipalmitoyl lecithin monolayers is a faster process in the presence of potassium ions than in the presence of sodium ions. This experimental finding could be related to selective effects observed in the compression curves of dipalmitoyl lecithin monolayers obtained on subphases containing sodium chloride, potassium chloride, or lithium chloride (41).

The selective effect of sodium ions on dipalmitoyl lecithin monolayers (42), which has been also observed on dipalmitoyl phosphatidylethanolamine monolayers (43), has been used jointly with

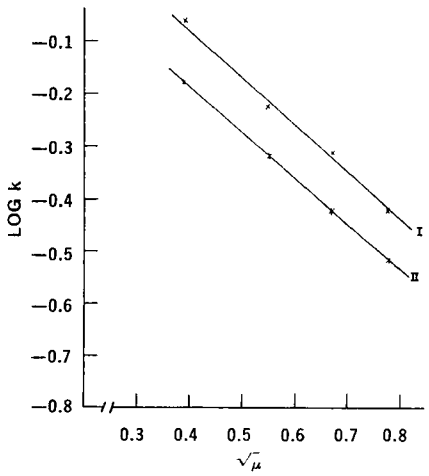

Figure 19—*Plot of the logarithm of the apparent first-order rate constant for the interaction of cetrimonium ions with dipalmitoyl lecithin monolayers against the square root of the ionic strength. Key: I, KCl; and II, NaCl.*

the measurement of unidirectional flux of sodium ions to conclude that the penetration of sodium ions through the epithelium of frog skin does not occur by simple diffusion but depends on a specific interaction with the polar group of the outer leaflet of the plasma membrane (44).

It has been suggested (45) that, if a cell membrane has a water channel whose walls are covered with phospholipids of the dipalmitoyl lecithin type, the discriminative properties of these phospholipids, when spread in monolayers (41), would cause the radius and the length of such a pore to vary according to which ion is flowing through its lumen.

It has been established that the nerve cell membrane can distinguish between sodium and potassium ions and that the permeability to these two ions is variable (46). The effect of quaternary ammonium ions injected into the axoplasm on the potassium conductance of squid axons strongly suggests that the postassium ions traverse the nerve membrane by way of pores which sodium ions cannot enter (47).

In accord with the findings on monolayer experiments, it is conceivable that the potassium ion generates *per se* the selective channel in absence of quaternary ammonium ions and that, because of its accelerating effect on the kinetics of the interaction of the quaternary ammonium ion with the phospholipids that cover the walls of the channel, it assists the closing of the activation gates when the quaternary ammonium ion is injected into the axoplasm.

CONCLUSION

A better knowledge of the interactions of biomolecules at the plane of an interface is necessary to understand the complexity of the structure of the cell interfaces and the order of magnitude of the energy required to produce nondisruptive structural changes that, in turn, can produce functional modifications of biomembranes. If these functional modifications could be produced by the approach of a selected drug molecule to the cell interface, the better knowledge of the kinetics and energetics of this type of interaction will be invaluable in the design of new and better drugs.

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Drug–Biomolecule Interactions: Binding Study of Substrate and Inhibitors to Acetylcholinesterase Using NMR

G. KATO

Abstract □ NMR was used to study the binding of acetylcholine, atropine, and physostigmine to acetylcholinesterase. Changes in the linewidth of the *N*-methyl resonance of acetylcholine, resulting from association with the enzyme during hydrolysis, were utilized to study the enzyme–substrate interaction. Physostigmine inhibited the binding of the substrate while atropine accelerated substrate hydrolysis without interfering with its binding. The dissociation constant, K_D, and the linewidth of the acetylcholinesterase–inhibitor complex, $\Delta\nu_{bound}$, for atropine and physostigmine can be estimated from the linewidth changes of the *N*-methyl and phenyl group resonances of atropine and from the *N*-methyl and C-methyl group resonances of physostigmine resulting from association with the enzyme. The results indicate that there is at least one binding site on the enzyme surface for atropine and one for physostigmine. Further evidence that the two sites are distinct is indicated by the fact that gallamine displaces atropine from its site without competing with physostigmine.

Keyphrases □ Acetylcholinesterase—binding of substrate and inhibitors, NMR □ Binding—substrate and inhibitors to acetylcholinesterase, studied by NMR □ NMR—evaluation, binding of substrate and inhibitors to acetylcholinesterase □ Drug–bimolecule interactions—binding of substrate and inhibitors to acetylcholinesterase, NMR □ Interactions—drugs with biomolecules, symposium

Since drugs and the complex biological entities on which they act are composed of molecules, their effects can accordingly be interpreted on the basis of molecular events, *i.e.*, by the interaction of drug with receptor molecules. The receptors are conceived as being special structures within the molecular framework of the cell membranes and cellular organelles. Such structures are capable of direct binding with specific, biologically active substances, and it is this binding that results in an observable physiological, pharmacological, or biochemical response.

It is widely believed that the receptors are macromolecules—probably proteins—with structures resembling those of enzymes or antibodies (1). They almost certainly include structures sensitive to acetylcholine, epinephrine, histamine, serotonin, and the peptide hormones. But while the chemical structure of most drugs is known, the structure of receptors is still unknown. The binding of a number of drugs to the enzyme acetylcholinesterase was studied with the hope of using this system as a possible model for other drug–receptor interactions (2–5).

DISCUSSION

Acetylcholinesterase catalyzes the hydrolysis of acetylcholine into choline and acetate. This reaction involves the active center of the enzyme which consists of two subsites, the anionic site and the esteratic site (6). The anionic site is responsible for binding, while the esteratic site is responsible for cleavage of the substrate molecule.

Apart from the active site, acetylcholinesterase contains noncatalytic binding sites. For example, Changeux (7) demonstrated the binding of gallamine triethiodide and tubocurarine, two receptor inhibitors that only partially inhibited the enzyme. Belleau and DiTullio (8, 9) showed that at least two anionic sites are present on acetylcholinesterase; site I is part of the active site, and site II is at the periphery and binds tubocurarine. Their results imply certain similarities between the anionic sites of acetylcholinesterase with the acetylcholine receptors of excitable membranes. Several other investigators (10–13) also showed the presence of allosteric sites on acetylcholinesterase which bind certain cholinergic ligands with more or less high affinity.

The aim of the present work was to investigate the binding of acetylcholine, atropine, and physostigmine to purified squid enzyme using a high-resolution NMR method. The binding of inhibitors to enzymes is usually studied by kinetic methods or by equilibrium dialysis. Both techniques, however, have inherent limitations. The nature of the interaction can be studied in more detail by NMR methods (14).

With NMR spectroscopy, two types of change are commonly observed in the spectrum of a small molecule when it binds to a macromolecule. The linewidths of the protons of a small molecule give information about the degree of restriction of motion of specific parts of small molecules, the degree of saturation of binding sites, and the rates of exchange. In addition, chemical shift changes for some protons of the small molecule give information on the magnetic environment of the small molecule when it is bound. Such an effect could result from nearby aromatic systems or paramagnetic ions. A recent review summarized the application of NMR spectroscopy to biochemical problems (14).

THEORETICAL

All elementary particles of the atom, such as the proton or the electron, constantly spin around their axis with a certain momentum. In NMR spectroscopy, the H atom, which has a single proton with spin $I = \frac{1}{2}$ and a magnetic moment μ is of primary interest. If the spinning proton is placed in a strong magnetic field, H_0, it will act to tilt its axis either in the direction of the field or in opposition to the field. Thus, a proton will be able to assume only one of two possible orientations, corresponding to energy levels of $-\mu H_0$ in the lower state and $+\mu H_0$ in the upper state.

The lower level corresponds to the situation in which the magnetic field, H_0, and the nuclear moment are parallel; in the upper level, they are antiparallel. To induce transitions between the two states, a discrete amount of energy, ΔE, is applied to the system. This is the energy difference between the two states and is given by $\Delta E = 2\mu H_0 = h\nu$, where h is Planck's constant and ν is the frequency of the electromagnetic radiation necessary for such a transition. For protons in a magnetic field of 14,000 gauss, the frequency of such energy is about 60 MHz, which is in the radiofrequency region.

Under ordinary conditions in a magnetic field, there is a very small difference in the population of the two levels given by a Boltzmann distribution. This small excess of protons in the lower energy state is important. The small, but finite, excess of nuclei in the lower energy state gives rise to an absorption of energy in the radiofrequency region.

The information indicates that the resonance condition can be obtained by varying either the magnetic field, H_0, or the frequency, ν. In practice, one usually works with a fixed frequency and sweeps the magnetic field through the resonance value, obtaining a spectrum of the compound. The sample is placed in a tall glass cy-

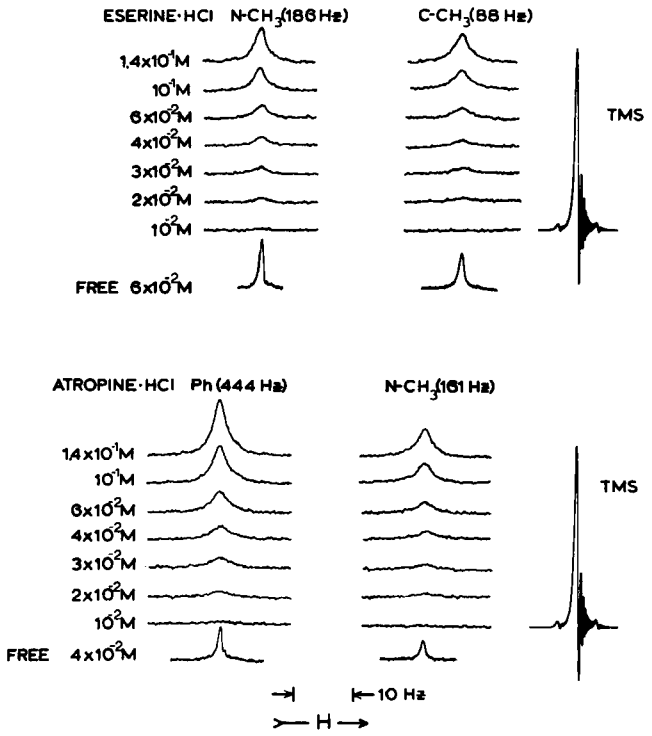

Figure 1—*(Top) The 60-MHz NMR spectra of the* N*-methyl and C-methyl resonances of physostigmine hydrochloride (eserine hydrochloride), free (lower trace) and in the presence of acetylcholinesterase (1.7 × 10⁻⁵* M*) (upper traces). (Bottom) The 60-MHz NMR spectra of the phenyl and* N*-methyl resonances of atropine hydrochloride, free (lower trace) and in the presence of acetylcholinesterase (1.7 × 10⁻⁵* M*) (upper traces). The intense resonances at right are those of protons of an external tetramethylsilane standard. Samples were dissolved in 0.1* M *phosphate buffer, pH 7.4, in D_2O, and spectra were recorded at 39°. The specific activity of squid acetylcholinesterase was 200 mmoles of acetylthiocholine/hr/mg.*

lindrical tube, which is inserted between the poles of a magnet surrounded with a coil which produces the radiofrequency field. If the field of the magnet is scanned at some fixed radiofrequency field, the protons absorb energy which can be recorded by a radio receiver. When the signal is fed directly into a recorder, a sharp peak is obtained in the spectrum.

Each different chemical group of an organic compound containing hydrogens gives rise to a separate absorption peak at different values of the applied magnetic field. Hydrogen atoms in the various chemical groups absorb at slightly different frequencies because the applied magnetic field becomes modified by the movements of the electrons and nuclei in adjacent atoms and neighboring molecules. The absolute position of the absorption cannot be obtained from the instrument. The exact position of the resonance frequencies of protons in different environments is measured from some arbitrary standard (*e.g.*, tetramethylsilane). The position of the peak is termed the chemical shift.

Upon removal of the exciting field at resonance, the Boltzmann distribution of the sample is regained by a relaxation process. This is described by two relaxation times, T_1 and T_2. T_1 is termed the spin–lattice relaxation time, and T_2 is the spin–spin relaxation time. The spin–spin relaxation rates, $1/T_2$, can be obtained from $1/T_2 = \pi \Delta\nu_{1/2}$, where $\Delta\nu_{1/2}$ is the linewidth at one-half maximum peak height. For rapid translational or rotational motion, T_2 is long and $1/T_2$ is small; hence, narrow peaks are obtained. As the molecular motions are reduced by viscosity or by interaction with a larger rigid molecule, T_2 becomes shorter and the peaks become broader.

In solution, small molecules have rapid translational and rotational molecular motion (*i.e.*, they have short correlation times) so narrow peaks are obtained. In solids, molecules occupy fixed positions and thus give rise to very broad peaks. Macromolecules such as proteins in solution give rise to complex NMR spectra due to the overlapping of resonance peaks from similar chemical groups. The longer correlation times due to slow rotational and translational motion also result in broad resonance peaks.

In certain conditions, the protein often shows no spectrum, while the interacting species shows a typical narrow line NMR spectrum. When a freely moving molecule interacts with a protein, the rotational correlation time of the small molecule is increased. As the correlation times are increased, line broadening occurs. It is the changes in the spectra of the small molecules resulting from the binding to high molecular weight macromolecules that are of interest. The width of the resonance peaks in particular are extremely sensitive to variations in the molecular environment and provide the most informative measurements for the study of molecular interaction.

For the equilibrium $E + S \rightleftharpoons ES$, describing enzyme–small molecule association:

$$K_D = \frac{[E][S]}{[ES]} \qquad \text{(Eq. 1)}$$

where $[E] = E_0 - [ES]$ and $[S] = S_0 - [ES]$. The term $[ES]$ is the concentration of the enzyme–small molecule complex, $[S_0]$ is the total concentration of the small molecule, and $[E_0]$ is the total concentration of the enzyme.

For the situation in which a small molecule binds reversibly to an enzyme, the observed linewidth of a proton on the small molecule is given by:

$$\Delta\nu_{obs} = P_{ES}\Delta\nu_{ES} + P_S\Delta\nu_S + \left(\frac{1}{\pi T_2}\right)_{ex} \qquad \text{(Eq. 2)}$$

where P_{ES} and P_S are the fractions of the small molecule bound and free in solution, respectively; $\Delta\nu_{ES}$ and $\Delta\nu_S$ are the linewidths of the bound and free species, respectively; and $(1/\pi T_2)_{ex}$ is the contribution to the observed linewidth due to exchange (15).

If the exchange lifetime is much less than $1/\Delta\nu_{ES}$, then:

$$\Delta\nu_{obs} = P_{ES}\Delta\nu_{ES} + P_S\Delta\nu_S \qquad \text{(Eq. 3)}$$

or:

$$\Delta\nu_{obs} = \left(\frac{[ES]}{[S_0]}\right)\Delta\nu_{ES} + \left(\frac{S_0 - [ES]}{S_0}\right)\Delta\nu_S \qquad \text{(Eq. 4)}$$

In all experiments, $S_0 \gg [E_0]$ so $(S_0 - [ES])/S_0 = 1$ and:

$$\Delta\nu_{obs} = \frac{[ES]}{[S_0]}\Delta\nu_{ES} + \Delta\nu_S \qquad \text{(Eq. 5)}$$

For inhibitor binding studies, the values of $\Delta\nu$ were corrected by

Table I—Linewidth Data for *N*-Methyl and Phenyl Protons of Atropine and *N*-Methyl Protons of Physostigmine Obtained from Association of Atropine and Physostigmine with Acetylcholinesterase, in Absence and Presence of Gallamine Triethiodide[a]

Inhibitor (Protons)	Concentration: Inhibitor, *M*	Concentration: Acetylcholinesterase, mg/ml	$\Delta\nu_{obs}$[b], Hz	$\Delta\Delta\nu$, Hz
Atropine (NCH_3)	0.02	0	1.5	—
Atropine (NCH_3)	0.02	4	3.9	2.4
Atropine (NCH_3) Plus gallamine	0.02 0.04	4	2.8	1.3
Atropine (phenyl)	0.02	0	1.0	
Atropine (phenyl)	0.02	4	3.1	2.1
Atropine (phenyl) Plus gallamine	0.02 0.04	4	2.2	1.2
Physostigmine (NCH_3)	0.02	0	1.2	
Physostigmine (NCH_3)	0.02	4	2.8	1.6
Physostigmine (NCH_3) Plus gallamine	0.02 0.04	4	3.0	1.8

[a] Measurements were made in 0.1 *M* phosphate buffer, pH 7.4, at 39° in a Varian A-60D spectrometer. [b] Values given are observed measured linewidths corrected for instrumental broadening (0.4 Hz).

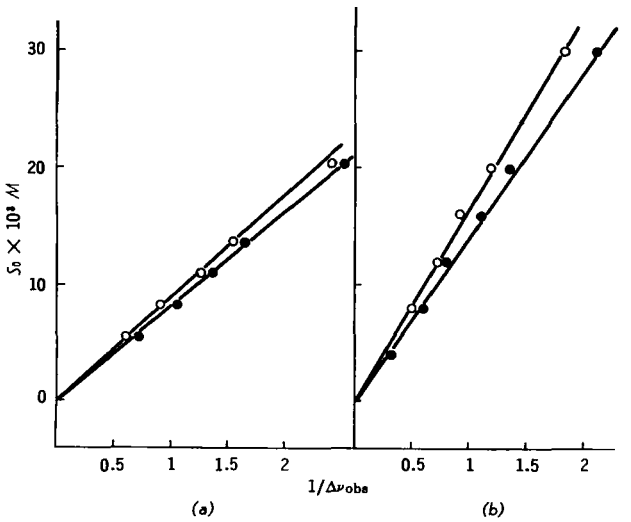

Figure 2—(a) *Plot of the reciprocal of the observed linewidth* ($\Delta\nu_{obs}$) *for the* N-*methyl* (O) *and C-methyl* (●) *group resonances of physostigmine* versus *varying concentrations of physostigmine sulfate* (S_0) *in the presence of constant concentrations of acetylcholinesterase* (1.7×10^{-5} M). (*b*) *Plot of the reciprocal of the observed linewidth* ($\Delta\nu_{obs}$) *for the phenyl* (●) *and* N-*methyl* (O) *group resonances of atropine* versus *varying concentrations of atropine sulfate* (S_0) *in the presence of a constant concentration of acetylcholinesterase* (1.7×10^{-5} M). *Samples were dissolved in 0.1* M *phosphate buffer, pH 7.4, in* D_2O, *and spectra were recorded at 39°. In these experiments the linewidth of the free inhibitor,* $\Delta\nu_S$, *was subtracted from the observed linewidth,* $\Delta\nu_{obs}$.

subtracting the values of $\Delta\nu_S$ from the measured values of $\Delta\nu$. Therefore:

$$\Delta\nu_{obs} = \frac{[ES]}{[S_0]}\Delta\nu_{ES} \qquad \text{(Eq. 6}a\text{)}$$

$$[ES] = \frac{\Delta\nu_{obs}}{\Delta\nu_{ES}}[S_0] \qquad \text{(Eq. 6}b\text{)}$$

Substitution of Eq. 1 leads to Eq. 7:

$$K_D = E_0\left[\frac{\Delta\nu_{ES} - \Delta\nu_{obs}}{\Delta\nu_{obs}}\right] - \frac{\Delta\nu_{obs}}{\Delta\nu_{ES}}S_0\left[\frac{\Delta\nu_{ES} - \Delta\nu_{obs}}{\Delta\nu_{obs}}\right] \qquad \text{(Eq. 7)}$$

If $\Delta\nu_{obs} \ll \Delta\nu_{ES}$:

$$S_0 = \frac{E_0\Delta\nu_{ES}}{\Delta\nu_{obs}} - K_D \qquad \text{(Eq. 8)}$$

A plot of S_0 *versus* $1/\Delta\nu_{obs}$ gives a line whose intercept is $-K_D$ and whose slope is used to calculate $\Delta\nu_{ES}$ (16).

EXPERIMENTAL

Binding of Atropine and Physostigmine to Acetylcholinesterase—The NMR spectrum of atropine hydrochloride in D_2O exhibits resonances at 444 and 161 Hz, which can be assigned to the phenyl and *N*-methyl group protons, respectively. The spectrum of physostigmine hydrochloride consists of a resonance at 186 Hz due to the *N*-methyl group and a resonance at 88 Hz due to the C-methyl group in the fused ring system. Because these were the most intense resonances in the spectra, they were used for the present study.

Figure 1 shows these resonances at 60 MHz, with tetramethylsilane as an external standard. The addition of highly purified acetylcholinesterase (specific activity = 200 mmoles acetylthiocholine/hr/mg; 1.7×10^{-5} *M* final concentration) to a sample of physostigmine or atropine hydrochloride at various concentrations (10^{-2}–1.4×10^{-1} *M*) resulted in the spectra shown in Fig. 1. Both resonances of atropine and physostigmine were extensively broadened without a change in their shifts.

Figure 1 illustrates that the *N*-methyl and aromatic resonances of atropine and the *N*-methyl and C-methyl resonances of physostigmine become progressively broader as the concentration of inhibitor is decreased. It is evident from these observations that the chemical exchange of both inhibitors between free and enzyme-bound species is rapid.

As described under *Theoretical*, if the enzyme–inhibitor binding is studied by varying the inhibitor concentration at a fixed concentration of enzyme, a plot of S_0 with respect to $1/\Delta\nu_{obs}$ should yield a straight line with a slope of $E_0\,\Delta\nu_{ES}$ and an intercept equal to $-K_D$, provided the described conditions are met.

The linewidth data obtained for atropine and physostigmine are plotted in this manner in Fig. 2. In these experiments, E_0 was held fixed at 17 μM. Variations of S_0 with $1/\Delta\nu_{obs}$ are linear for both peaks of atropine and physostigmine over the range of conditions used. The following linewidths for the enzyme–inhibitor complexes and dissociation constants are calculated from the least-squares lines. For physostigmine, $\Delta\nu_{ES,NCH_3} = 496 \pm 26$ Hz, $K_D = 5.9 \pm 2.4 \times 10^{-4}$ *M*, $\Delta\nu_{ES,CCH_3} = 488 \pm 16$ Hz, and $K_D = 4.3 \pm 4.0 \times 10^{-4}$ *M*. For atropine, $\Delta\nu_{ES,NCH_3} = 984 \pm 52$ Hz, $K_D = 1.2 \pm 5.8 \times 10^{-4}$ *M*, $\Delta\nu_{ES,phenyl} = 858 \pm 32$ Hz, and $K_D = 3.0 \pm 3.9 \times 10^{-4}$ *M*.

The *N*-methyl and C-methyl groups of physostigmine are immobilized to the same extent when physostigmine binds to the enzyme, as might be expected, since both groups are on the same fused ring system. With atropine the *N*-methyl group is also immobilized to approximately the same extent as the phenyl group.

Since $K_D \ll S_0$, K_D cannot be obtained with great precision. Nevertheless, it is clear that atropine and physostigmine bind with high affinity ($K_D < 1$ m*M*). The K_i values for atropine and physostigmine were then determined from kinetic studies, using acetylthiocholine as the substrate. Whereas physostigmine sulfate was an effective anticholinesterase ($K_i = 1.2$ μM), atropine sulfate inhibited poorly ($K_i = 6.0$ m*M*). If both compounds bind to the active site, both should inhibit substrate hydrolysis. Yet atropine is an ineffective anticholinesterase but binds to the enzyme with high affinity. This indicates that physostigmine may bind to the active site and atropine to another.

That atropine and physostigmine do not bind to the same sites on the enzyme surface was confirmed by adding gallamine triethiodide to a solution of acetylcholinesterase containing either atropine or physostigmine and showing that gallamine reduced the amount of bound atropine but not physostigmine. Table I gives the quantitative data obtained. Gallamine and atropine compete for

Table II—Linewidth Data for *N*-Methyl and Phenyl Protons of Atropine and *N*-Methyl and C-Methyl Protons of Physostigmine Obtained from Association of Atropine and Physostigmine with Acetylcholinesterase, in Absence and Presence of Carbamylcholine Chloride (Carbachol)[a]

Inhibitor (Protons)	Concentration: Inhibitor, *M*	Concentration: Acetylcholinesterase, mg/ml	$\Delta\nu_{obs}$[b], Hz	$\Delta\Delta\nu$, Hz
Atropine (NCH_3)	0.02	0	1.5	—
Atropine (NCH_3)	0.02	4	3.5	2.0
Atropine (NCH_3) Plus carbachol	0.02 0.10	4	2.7	1.2
Atropine (phenyl)	0.02	0	1.0	
Atropine (phenyl)	0.02	4	2.5	1.5
Atropine (phenyl) Plus carbachol	0.02 0.10	4	2.5	1.5
Physostigmine (NCH_3)	0.02	0	1.2	
Physostigmine (NCH_3)	0.02	4	3.2	2.0
Physostigmine (NCH_3) Plus carbachol	0.02 0.10	4	2.3	1.1
Physostigmine (CCH_3)	0.02	0	1.4	
Physostigmine (CCH_3)	0.02	4	3.8	2.4
Physostigmine (CCH_3) Plus carbachol	0.02 0.10	4	3.0	1.6

[a] Measurements were made in 0.1 *M* phosphate buffer, pH 7.4, at 39° in a Varian A-60D spectrometer. [b] See Table I.

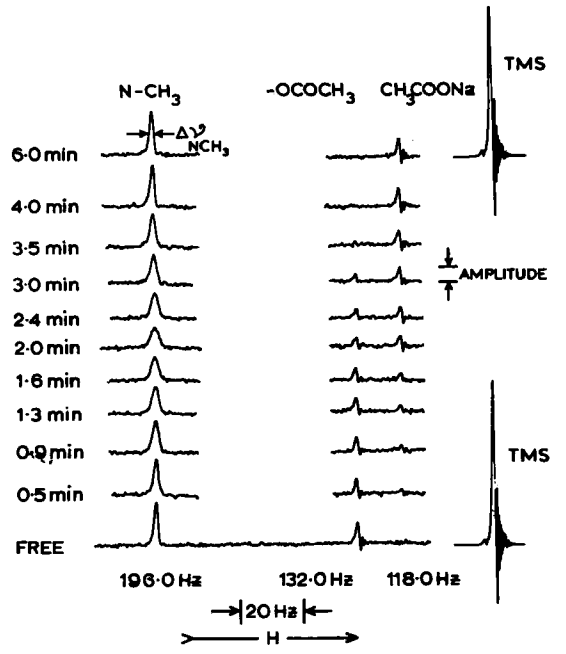

Figure 3—*Time study of the NMR spectrum of acetylcholine chloride (16.7 mM), free and after addition to acetylcholinesterase (50 mg/ml) in 0.1 M sodium phosphate buffer, pH 7.4, in D_2O at 39°. The lower trace represents a portion of the NMR spectrum of acetylcholine free in solution. The upper traces are the scans of the acetylcholine spectrum at various times after addition to acetylcholinesterase. Resonances at 196 and 132 Hz (downfield from external tetramethylsilane) are from protons in the N-methyl and acetate groups of acetylcholine. The resonance at 118 Hz is from sodium acetate protons, a product of hydrolysis of acetylcholine. The arrows on the N-methyl peak indicate the width of the resonance line at half-height ($\Delta\nu_{NCH_3}$). The arrows on the acetate peak indicate its amplitude in millimeters. A solution of tetramethylsilane was used as an external standard, and its resonance appears to the upper field of the acetylcholine resonances. Its spectrum was recorded before and after the experiment. The specific activity of squid acetylcholinesterase was 1.4 mmoles of acetylthiocholine/hr/mg.*

the same sites on the enzyme but gallamine and physostigmine do not.

Carbamylcholine chloride, which resembles the structure of acetylcholine but is not hydrolyzed by acetylcholinesterase, competes with physostigmine binding. The quantitative data are presented in Table II. Carbamylcholine also competes with the binding of atropine but, surprisingly, only competes with the N—CH_3 group of atropine while the binding of the phenyl group remains unchanged.

These results indicate that acetylcholinesterase has groups outside its active site for binding certain inhibitors such as atropine. The most decisive evidence that the atropine binding site is distinct from the active site comes from the following reasoning and observations. If it is assumed that atropine and physostigmine bind at the same site, then any compound able to compete with atropine at its binding site would be expected to compete with physostigmine. If, on the other hand, the atropine and physostigmine sites are distinct, some compounds should be able to displace atropine from its site without necessarily competing with the substrate or physostigmine. That the latter is obtained is demonstrated by the fact that gallamine inhibits atropine binding without inhibiting physostigmine binding.

Gallamine decreased the linewidth of both phenyl and *N*-methyl groups of atropine in the presence of enzyme, as expected under the present conditions, since both gallamine and atropine have a phenyl and a charged quaternary ammonium group. The phenyl group of gallamine competes with the phenyl group of atropine, and the *N*-ethyl group of gallamine displaces the *N*-methyl group of atropine.

The results with carbamylcholine are less clear. One explanation is that there are two different binding sites for atropine. In this case, the atropine molecule binds *via* the *N*-methyl group at one site and *via* the phenyl group at the other. Carbamylcholine may interfere with the binding of atropine at the first site without affecting the second. In the presence of carbamylcholine, therefore, atropine would remain bound at the second site *via* the phenyl group. This is not unlikely, since carbamylcholine does not contain an unsaturated ring.

The second possibility is that atropine binds *via* both the *N*-methyl and phenyl groups and that carbamylcholine interferes with the binding of the *N*-methyl group only, leaving the phenyl group bound. This is consistent with the idea that both groups of atropine are necessary for maximum binding to the enzyme (2, 3).

Binding of Acetylcholine to Acetylcholinesterase—The binding of acetylcholine to acetylcholinesterase was studied by measuring the change in the linewidth at half-maximum amplitude of the *N*-methyl group ($\Delta\nu_{NCH_3}$) as a function of time. The rate of hydrolysis was followed by measuring the amplitude of the acetate peak (118 Hz) as a function of time. The lower trace in Fig. 3 shows a portion of the NMR spectrum of acetylcholine chloride. The peaks are labeled N—CH_3 and —$OCOCH_3$ for the protons of the quaternary ammonium and acetate moieties, respectively.

When acetylcholine chloride was added to a solution of the enzyme and the spectrum of the solution was scanned repeatedly, the spectrum underwent several changes (upper traces of Fig. 3). The N—CH_3 group resonance became broader, reaching a maximum at 2 min, followed by a gradual sharpening of this same resonance. The amplitude of the resonance at 132 Hz (due to the acetylcholine acetate protons) decayed with time, while that at 118 Hz (due to the free acetate protons) gradually increased and reached maxi-

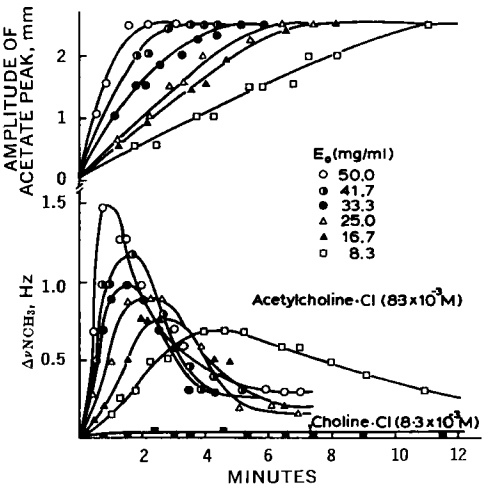

Figure 4—*Plot of the observed linewidth for the* N*-methyl group resonance of acetylcholine (lower graph) and the amplitude of the sodium acetate group resonance (upper graph)* versus *time at varying concentrations of enzyme, E_0, in the presence of the same concentration of acetylcholine chloride (8.3 mM). At zero time, a solution of acetylcholine chloride (10 μl of 1.0* M*) was rapidly mixed with a solution of acetylcholinesterase in the NMR tube. The tube was placed in the probe, and the acetylcholine spectrum was scanned repeatedly. The change in linewidth of the* N*-methyl group and the amplitude of the acetate group were measured from the same experiment. This procedure was repeated using six different concentrations of enzyme. The plot is the difference between the linewidths for acetylcholine chloride in the presence of acetylcholinesterase and in buffer only (0.8 ± 0.05 Hz). Included in the graph is the change in linewidth of the* N*-methyl group of choline chloride (8.3×10^{-3} M) when mixed with a solution of acetylcholinesterase (50 mg/ml). The solution contained 0.1* M *sodium phosphate buffer, pH 7.4, in 98% D_2O. The total volume was 0.6 ml, and the temperature was 39°. The enzyme used was identical to the one used in the experiment shown in Fig. 3.*

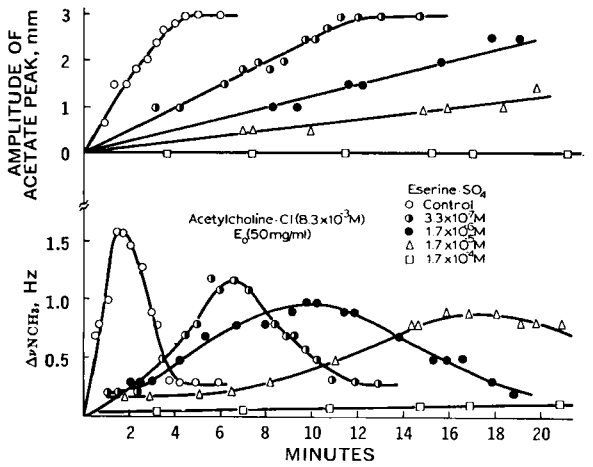

Figure 5—*Plot of the observed linewidth for the* N*-methyl group resonance of acetylcholine (lower graph) and the amplitude of the sodium acetate group resonance (upper graph) versus time at varying concentrations of physostigmine (eserine) in the presence of the same concentration of enzyme (E_0 = 50 mg/ml) and substrate (acetylcholine chloride = 8.3 mM). The experimental procedure was the same as in Figs. 2 and 3, with the exception that the enzyme was preincubated with various concentrations of physostigmine before the addition of substrate. Measurements were made in 0.1* M *phosphate buffer, pH 7.4, in D_2O at 39°.*

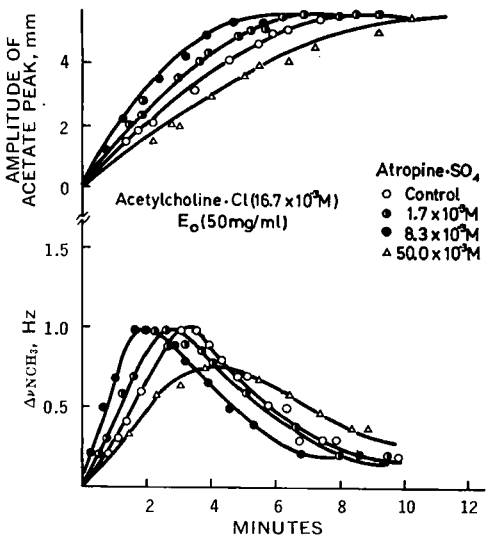

Figure 6—*Plot of the observed linewidth for the* N*-methyl group of resonance of acetylcholine (lower graph) and the amplitude of the sodium acetate group resonance (upper graph) versus time at the same concentrations of enzyme (E_0 = 50 mg/ml) and substrate (acetylcholine chloride = 16.7 mM). The experimental procedure was the same as in Figs. 2 and 3, with the exception that the enzyme was preincubated with various concentrations of atropine before the addition of substrate. Measurements were made in 0.1* M *phosphate buffer, pH 7.4, in D_2O at 39°.*

mum amplitude at 6 min. No further changes occurred after this time. While the acetylcholine acetate peak may be slightly broadened, the free acetate peak was not broadened.

The changes in $\Delta\nu_{NCH_3}$ and the amplitude of the acetate peak are shown in Fig. 4 with six different concentrations of enzyme, E_0, at the same substrate concentration. With each enzyme concentration, a characteristic change in linewidth was observed, *i.e.*, a progressive broadening followed by a decrease in linewidth after which a steady state was reached. At lower enzyme concentrations, the maximum linewidth decreased and the time of maximum broadening was delayed. When choline was added to the enzyme, its *N*-methyl resonance was not broadened. The changes in linewidth are interpreted in terms of binding of the substrate molecule to the active center of the enzyme.

Binding of Acetylcholine to Acetylcholinesterase in Presence of Physostigmine—In the presence of physostigmine sulfate, the *N*-methyl group reasonance of acetylcholine showed a decrease in the rate of line broadening, indicating a delay in the reaction as well as a decrease in maximal line broadening. These results are shown in Fig. 5 with four different concentrations of physostigmine. The upper graph shows the effect of increasing concentrations of physostigmine on the rate of acetate formation. With physostigmine sulfate = 1.7×10^{-4} *M*, line broadening and hydrolysis were completely abolished.

Binding of Acetylcholine to Acetylcholinesterase in Presence of Atropine—The effects of atropine on acetylcholine binding are uniquely different from those observed with physostigmine. In the presence of atropine, the effect on the *N*-methyl resonance was an increase in the rate of line broadening without influencing the maximum linewidth. The upper graph in Fig. 6 shows that atropine ($1.7 - 8.3 \times 10^{-3}$ *M*), under the present conditions, accelerates the rate of substrate hydrolysis. Higher concentrations of atropine (50×10^{-3} *M*) inhibit hydrolysis and substrate binding.

These findings suggest that atropine does not interfere with the binding of the substrate but instead accelerates its hydrolysis. The mechanism by which atropine or its analogs accelerate, if they are not bound at the active site, could be accounted for by allosteric interactions. That is, the binding of atropine transmits a conformational change through the structure of the enzyme which modulates its catalytic activity.

The acceleration by atropine and the inhibition by physostigmine were observed in the presence of high concentrations of substrate. Lower concentrations of substrate could not be used because they caused inaccuracies in linewidth measurements. It is possible, therefore, that these observations depend on the presence of relatively high concentrations of acetylcholine.

Some researchers (17, 18) showed that certain quaternary ammonium compounds potentiate the hydrolytic activity of acetylcholinesterase at high substrate concentrations. They suggested that these compounds accelerate the deacetylation sequence of the enzyme. If atropine accelerates deacetylation by binding to the anionic site of the active center, it must assume an orientation away from the esteratic site; otherwise it will interfere with deacetylation. This may be possible in view of the suggestion by Belleau *et al.* (19) of *exo* and *endo* binding of effectors at the esteratic site level.

Alternatively, atropine may protect the enzyme against substrate inhibition; that is, high concentrations of substrate bind to a regulatory site and exert negative cooperativity toward the active center. Atropine competes with the substrate at the regulatory site and prevents substrate inhibition. This implies that substrate inhibition may be due to an allosteric mechanism. Recent evidence (20) suggests that this may be the correct mechanism for the observed kinetic effects.

It is impossible to conclude, from the data presented here, which of the two mechanisms is correct. Nevertheless a two-site mechanism—the active site and a regulatory site—must be involved in each scheme.

The use of NMR techniques outlined in this paper overcomes some problems of other methods previously used for the study of enzyme–substrate interactions. The experimental procedure described for substrate binding is particularly useful in studying the active site region and the effects of various inhibitors on the enzyme–substrate complex. This method provides the advantage of following the rate of hydrolysis of substrate and its binding at the active site concomitantly.

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Drug–Biomolecule Interactions: Fluorescence Studies on Interaction of Aminonaphthalenesulfonic Acid Derivatives with Serum Albumins

H. W. JUN, L. A. LUZZI ˣ, and JOSEPH K. H. MA

Abstract □ The binding of the three aminonaphthalenesulfonic acid derivatives to human and bovine serum albumins was studied by measuring the fluorescence enhancement of the compounds. The number of binding sites of human and bovine serum albumins for these compounds appears to be one and two, respectively, under the experimental conditions. As the molar ratio of the fluorescent compounds to bovine serum albumin increased, the binding sites appeared to increase for the compounds. The quenching of the native fluorescence of albumin was examined by the successive addition of methanolic solutions of these compounds. 1-Anilinonaphthalene-8-sulfonate quenched the protein fluorescence to a greater extent than the other compounds studied, indicating that 1-anilinonaphthalene-8-sulfonate molecules are bound more closely to the tryptophan residues of albumin. The finding that the three compounds did not quench the fluorescence of tryptophan dissolved in water indicates no direct molecular interaction between tryptophan and the three fluorescent probes. The driving force for binding may be due to the structural characteristics of the amino acid sequence surrounding the tryptophan residues.

Keyphrases □ Aminonaphthalenesulfonic acid derivatives—binding to human and bovine serum albumins, fluorescence enhancement □ Albumin, human and bovine serum—binding of three aminonaphthalenesulfonic acid derivatives, fluorescence enhancement □ Probes—binding of three aminonaphthalenesulfonic acid derivatives to serum albumins, fluorescence enhancement □ Drug–biomolecule interactions—fluorescence studies on interaction of aminonaphthalenesulfonic acid derivatives with serum albumins □ Interactions—drugs with biomolecules, symposium

Interaction of organic molecules with body proteins has resulted in many facets of research interest in biomedical studies. When drugs are bound to plasma proteins, distribution, therapeutic efficacy, and elimination of the drugs may be altered. Biotransformation of organic compounds occurs through interactions of the compounds with metabolizing enzymes. The pharmacological effects of drugs are also thought to be mediated by interactions of the drug molecules with active site proteins.

Because of the clinical implications of such interactions, numerous studies have been carried out concerning these subjects (1, 2). Although aims and experimental techniques vary among investigators, most studies have been directed toward explaining the effects of the interactions.

In this study, fluorescence spectroscopy was employed to study the binding of three fluorescent probes to human and bovine serum albumins. A fluorescent probe is a compound with spectral properties such as optimal excitation and emission wavelengths, fluorescence intensity, and lifetime of the excited state that undergo changes reflecting its molecular environment. When probe molecules bind to protein binding sites, an enhancement of fluorescence emission and wavelength shift occur.

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Table I—Excitation and Emission Maxima (Uncorrected) of the Fluorescent Probes in pH 7.5 Phosphate Buffer and Bovine and Human Serum Albumin Solutions

	pH 7.5 Phosphate Buffer		Bovine Serum Albumin		Human Serum Albumin	
	Excitation, nm	Emission, nm	Excitation, nm	Emission, nm	Excitation, nm	Emission, nm
1-Anilinonaphthalene-8-sulfonate	360	520	376	467	380	474
N-n-Butyl-(5-dimethylamino-naphthalene)-1-sulfonamide	330	540	343	494	340	498
5-Dimethylaminonaphthalene-1-sulfonamide	328	540	347	506	345	509

The enhancement of fluorescence emission of a probe can be monitored to obtain binding information for the probe (3). The quenching of the intrinsic fluorescence of protein can also be quantitated to calculate the binding parameters of the interactant (4). The purposes of this study were to compare and to characterize the binding of three structurally similar fluorescent probes to human and bovine serum albumins by measuring the fluorescence enhancement of the probes and the fluorescence quenching of the proteins in the hope that additional information would be obtained for the binding model.

EXPERIMENTAL

Materials—The following fluorescent probes were studied: 1-anilinonaphthalene-8-sulfonate[1], *N-n*-butyl-(5-dimethylamino-naphthalene)-1-sulfonamide[2], and 5-dimethylaminonaphthalene-1-sulfonamide[3]. Crystalline human[4] and bovine[5] serum albumins were purchased commercially. Spectroscopic grade methanol was employed as a solvent for the probes. The water used was double distilled from glass. All other chemicals used were of reagent grade.

Instruments—All fluorescence measurements were made on a spectrophotofluorometer[6] equipped with a 150-w xenon lamp and 1P21 photomultiplier tube. Spectra were recorded with an *X-Y* recorder[7]. The entrance slit for the excitation light and the exit slit for the fluorescence emission were 3 and 4 mm, respectively.

Methods—Two milliliters of human and bovine serum albumin solution (1.37×10^{-6} *M*) in pH 7.5 phosphate buffer (0.05 *M*) were titrated with successive additions of 2 μl of 1×10^{-3} *M* of probe dissolved in methanol. The excitation and emission wavelengths (uncorrected) for the probes are recorded in Table I.

Titrations were carried out in fluorescence quality cells with fitted Teflon stoppers, and titrants were added manually with microsyringes[8]. After each titration, the enhancement of fluorescence intensity was recorded as a function of probe concentration. Separate titrations with successive additions of 2 μl of a 1×10^{-3} *M* probe solution were also performed at higher protein concentrations (34.2×10^{-6} *M* for bovine serum albumin and 68.5×10^{-6} *M* for human serum albumin). It was then assumed that all of the probe was bound to protein at this concentration.

Temperature was controlled at 23 ± 0.5° with thermostated cell compartments through which water from a constant-temperature bath[9] was circulated. To minimize the photodecomposition of protein, samples were exposed to the light only for the short measurement period. Blank titrations in the buffer were performed to correct the fluorescence of free probe in the absence of protein.

Treatment of Data—Enhancement of the fluorescence intensity of the probe upon addition to human and bovine serum albumins at two protein concentrations was measured, and these data were used to calculate the fractions of free and bound probe and the molar ratio of probe bound to protein (5). The binding constants and the number of binding sites of the compounds were determined by the use of the Scatchard equation (6):

$$\bar{V}/D_f = nK_a - \bar{V}K_a \qquad \text{(Eq. 1)}$$

where $\bar{V}$ is the number of moles of bound probe per mole of protein, D_f is the concentration of free probe, n is the number of binding sites on the protein molecule, and K_a is the binding constant of probe to the protein.

Measurement of Fluorescence Quenching—The quenching of the native fluorescence of human and bovine serum albumins was measured at the emission wavelength of 358 nm with the excitation at 283 nm following successive additions of probe. In titration, 2 μl of a 1×10^{-4} *M* probe solution in methanol was successively added to 2 ml of protein solution (1.37×10^{-6} *M* in pH 7.5 phosphate buffer) and the degree of quenching was recorded. The concentration of probe added to the protein solution was chosen so that it did not absorb the exciting radiation at 283 nm.

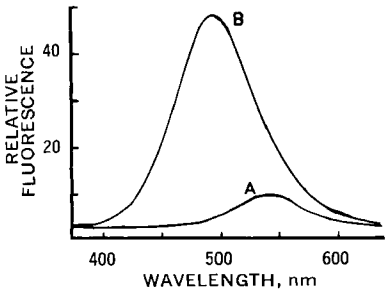

Figure 1—*Emission spectrum of* N-n-*butyl-(5-dimethylaminonaphthalene)-1-sulfonamide* (10×10^{-6} M) *in the absence (curve A) and presence (curve B) of bovine serum albumin* (1.37×10^{-6} M) *in pH 7.5 phosphate buffer. Excitation wavelengths were at 330 and 343 nm, respectively.*

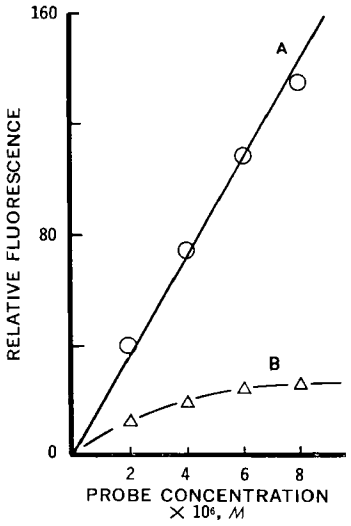

Figure 2—*Fluorescence titration curves of bovine serum albumin with* N-n-*butyl-(5-dimethylaminonaphthalene)-1-sulfonamide at higher protein concentration* (34.2×10^{-6} M) *(curve A) and lower protein concentration* (1.37×10^{-6} M) *(curve B).*

[1] Recrystallized in water, Aldrich Chemical Co., Milwaukee, Wis.
[2] Obtained from the Department of Entomology, University of Georgia, Athens, Ga.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Control No. 4619, Nutritional Biochemicals Corp.
[5] Control No. 1693, Nutritional Biochemicals Corp.
[6] Aminco-Bowman, American Instrument Co., Silver Spring, Md.
[7] Omnigraphic, Houston Instrument, Bellaire, Tex.
[8] Hamilton.
[9] Haake model FJ, Polyscience Corp., Evanston, Ill.

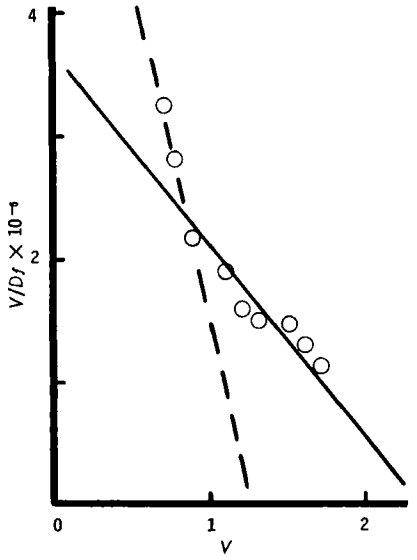

Figure 3—*Scatchard plot for 1-anilinonaphthalene-8-sulfonate–bovine serum albumin binding.*

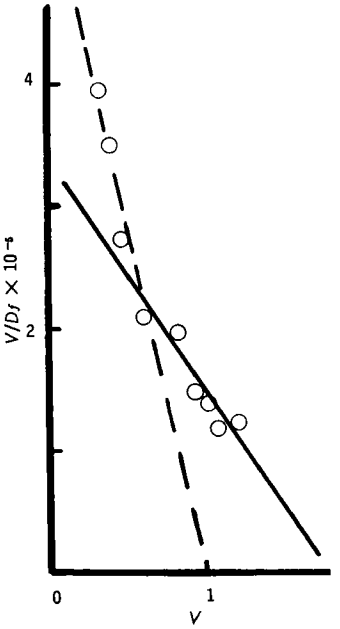

Figure 5—*Scatchard plot for 5-dimethylaminonaphthalene-1-sulfonamide–bovine serum albumin binding.*

RESULTS AND DISCUSSION

The use of fluorescence spectroscopy to study the binding of small molecules to proteins has been well established (7, 8). The aminonaphthalenesulfonic acid derivatives used in this study show an increase in fluorescence emission when bound to serum albumins. In this study the binding data were obtained by monitoring the increased fluorescence intensities of the probes (9, 10) and by measuring the quenching of the fluorescence of protein by the probes (11, 12).

Figure 1 is an emission spectrum of *N-n*-butyl-(5-dimethylaminonaphthalene)-1-sulfonamide in the absence and presence of bovine serum albumin in pH 7.5 phosphate buffer (0.05 *M*). The fluorescence emission of this compound in a buffer is minimal (curve A); but in the presence of albumin, the fluorescence intensity is greatly enhanced (curve B), with a 46-nm hypsochromic shift in the wavelength of the emission maximum. Jun *et al.* (13) reported the quantum yield of 0.059 for this compound in water and indicated that it was increased about 12 times to 0.7 when bound to bovine serum albumin.

In Fig. 2, the enhancement of fluorescence intensity is essentially dependent on the amount of the probe added at the high protein concentration (curve A). At lower protein concentration, fluorescence intensity reaches a maximum, indicating a saturation of binding sites (curve B). The large increase in fluorescence emission and hypsochromic shift in the presence of protein has been attributed to binding of the probe at hydrophobic sites (14, 15). These spectral modifications are responses to changes in the polarity of the probe environment as a result of binding.

McClure and Edelman (16) observed a significant increase in fluorescence emission of the fluorescent probe 2-*p*-toluidinylnaphthalene-6-sulfonate when placed in a solvent of lower polarity. Similar spectral shifts and fluorescence enhancement were observed for the other compounds studied. Table I summarizes the spectral shifts of the compounds in phosphate buffer and human and bovine serum albumin solutions.

Figures 3–5 are Scatchard plots obtained for probe–bovine serum albumin complexes. The plots were obtained by the method described under *Experimental* and were used to calculate the binding constant and the number of binding sites. Under the experimental conditions, the number of binding sites for the probes appears to increase from one to two as the molar ratio of probe to protein increases. The dotted line representing lower molar ratios of probe to protein indicates the number of binding sites to be close to one with possibly higher binding affinity and the solid line indicates two binding sites.

Since the quenching studies showed that the binding sites may

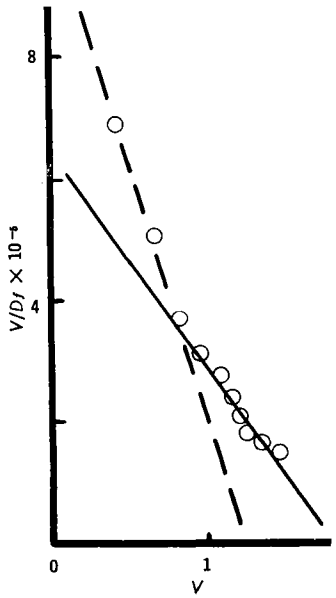

Figure 4—*Scatchard plot for* N-n-*butyl-(5-dimethylaminonaphthalene)-1-sulfonamide–bovine serum albumin binding.*

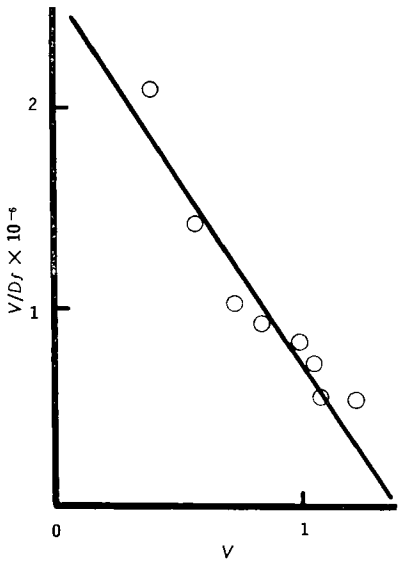

Figure 6—*Scatchard plot for 1-anilinonaphthalene-8-sulfonate–human serum albumin binding.*

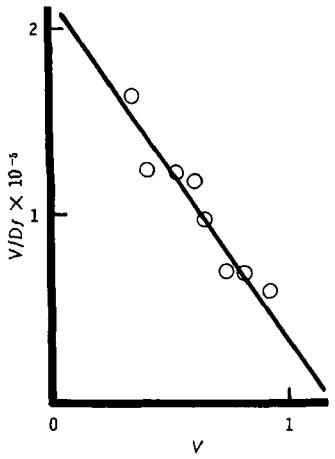

Figure 7—*Scatchard plot for* N-n-*butyl-(5-dimethylaminonaphthalene)-1-sulfonamide–human serum albumin binding.*

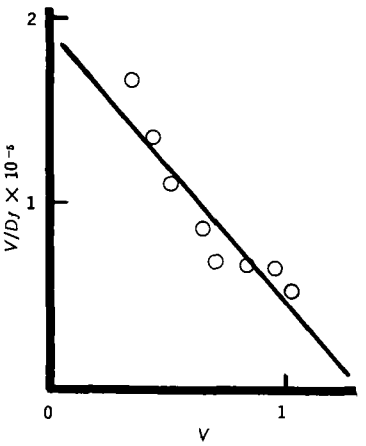

Figure 8—*Scatchard plot for 5-dimethylaminonaphthalene-1-sulfonamide–human serum albumin binding.*

be near the tryptophan residues and since bovine serum albumin is known to have more than one tryptophan binding site (17), the Scatchard plots suggest that the molecular environment of the binding sites may not be identical. At the low molar ratio of probe to protein, certain binding sites are initially available; as the probe concentration increases, more binding sites become available until all binding sites are saturated. This view may be supported by the report (18) that the total amount of salicylate bound to plasma protein was a function of drug concentration and that the capacity of binding sites increased at higher drug concentration.

The binding affinities of the probes to bovine serum albumin were in the following order: 1-anilinonaphthalene-8-sulfonate ($1.70 \times 10^6/M$), *N-n*-butyl-(5-dimethylaminonaphthalene)-1-sulfonamide ($3.66 \times 10^5/M$), and 5-dimethylaminonaphthalene-1-sulfonamide ($1.72 \times 10^5/M$). 1-Anilinonaphthalene-8-sulfonate had the highest binding constant. The difference in binding affinity between the two amide probes may be due to the involvement of the side chain in binding. Although the hydrophobic naphthalene moiety appears to be primarily responsible for the binding (19), the side chain of these compounds apparently affects binding.

Figures 6–8 are the Scatchard plots of the probes bound to human serum albumin using the same experimental conditions as with bovine serum albumin. The number of binding sites for the probes appears to be one. The orders of wavelength shift and binding affinity of the probes were similar to those for the bovine serum albumin–probe interactions. 1-Anilinonaphthalene-8-sulfonate ($1.60 \times 10^6/M$) showed the highest binding affinity followed by *N-n*-butyl-(5-dimethylaminonaphthalene)-1-sulfonamide ($1.82 \times 10^5/M$). The fluorescence enhancement was generally lower than that for the bovine serum albumin–probe complexes.

Figure 9 shows the quenching effects for the native fluorescence of albumin following the successive addition of probes. The probe, in the concentration used, did not contribute to the quenching. When 2 ml of bovine serum albumin solution (1.37×10^{-6} *M* in pH 7.5 phosphate buffer) was successively titrated with 2 μl of 1-anilinonaphthalene-8-sulfonate solution (1×10^{-4} *M* in methanol), the fluorescence emission of protein at 358 nm was diminished (curve A) when excited at 283 nm. The stoichiometry of the quenching curve shows the number of binding sites to be one and indicates that the binding sites are not saturated at this molar ratio of probe to protein.

Earlier workers (8, 11, 12) reported that the quenching of protein fluorescence in the presence of small molecules is an indication of binding. For fluorescence quenching to occur in a solution of two compounds, the quenching substance must be within a critical transfer distance (20) to the fluorophor so that the energy transfer between the two molecules takes place. Forster suggested (21) that for an energy transfer to occur the emission band of the fluorophor must overlap the absorption band of the quencher. Although the concentration of 1-anilinonaphthalene-8-sulfonate dissolved in buffer did not absorb the light at the emission wavelength of the protein (358 nm), the absorption band of the bound probe may overlap the emission band of the protein.

The finding that 1-anilinonaphthalene-8-sulfonate strongly quenches the fluorescence of albumin indicates that the binding site for this probe is in the vicinity of the tryptophan residues. Studies (22, 23) have shown that serum albumin has strong binding sites for small organic molecules located in the immediate vicinity of the tryptophan residue. Swaney and Klotz (23) reported that the environment of tryptophan residues of human serum albumin forms a cluster of apolar amino acids. This arrangement of hydrophobic clusters may be responsible for the interaction of the probes to serum albumins.

The quenching of the fluorescence of albumins by the other probes was less significant (Figs. 9B and 9C). This may be due to the molecules of 1-anilinonaphthalene-8-sulfonate being more closely bound to the tryptophan residues of the albumin. The finding that other organic compounds did not quench the fluorescence of serum albumin suggests the specificity of binding sites to the aminonaphthalenesulfonic acid derivatives. Chen and Kernohan (12) observed that 1-anilinonaphthalene-8-sulfonate did not quench the fluorescence of erythrocyte carbonic anhydrase. This observation also indicates the specificity of binding between serum

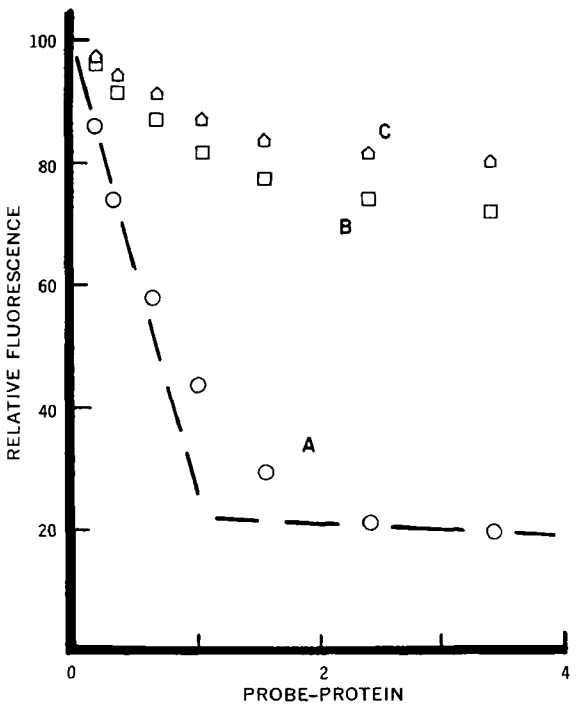

Figure 9—*Quenching curves of bovine serum albumin fluorescence by 1-anilinonaphthalene-8-sulfonate (curve A),* N-n-*butyl-(5-dimethylaminonaphthalene)-1-sulfonamide (curve B), and 5-dimethylaminonaphthalene-1-sulfonamide (curve C). The bovine serum albumin concentration in pH 7.5 phosphate buffer was 1.37×10^{-6} M. Fluorescence was measured at 358 nm (emission) and 253 nm (excitation).*

albumins and the probes used. Carbonic anhydrase may lack the structural requirement for the binding of 1-anilinonaphthalene-8-sulfonate.

In contrast to the quenching of the native fluorescence of protein by the probes, no quenching was observed upon addition of the probes to tryptophan dissolved in buffer. This indicates that the driving force for the probe–protein binding is not mediated by direct interaction between the probe and tryptophan molecules, although tryptophan has been reported to have a binding affinity to various other organic molecules (24, 25). This observation also shows that the quenching of protein fluorescence by the probes was not due to concentration or inner filter effects.

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Drug–Biomolecule Interactions: Spin-Probe Study of Effects of Anesthetics on Membrane Lipids

K. W. BUTLER

Abstract □ The electron spin resonance spectra of probes were used to study the organization and motion of molecules in hydrated stacked bilayer or liposome model membrane systems. The same steroid structures required for reduction of membrane permeability were required to produce well-ordered films of brain lipid. Alcohols and anesthetic agents influence the structure of model membranes, with their order of efficacy paralleling their pharmacological effectiveness. Spin probes were also used to demonstrate effects of calcium and local anesthetics on the rate of penetration of ascorbate into lipid bilayers.

Keyphrases □ Anesthetics—effects on membrane lipids, electron spin resonance spectra □ Lipids, membrane—effects of anesthetics, electron spin resonance spectra □ Drug–biomolecule interactions—effects of anesthetics on membrane lipids, electron spin resonance spectra □ Interactions—drugs with biomolecules, symposium

Electron spin resonance spectroscopy (ESR) examines the spectra of free radicals in an applied magnetic field. In a filled electron orbital, there are two electrons whose magnetic moments cancel; but in a free radical, there is an unpaired electron with a net magnetic moment. This electron has two different energy states, which can be qualitatively described as having the magnetic moment parallel and antiparallel to an applied magnetic field. The energy difference between these states is equal to the energy of a quantum of microwave radiation (for the usual applied magnetic fields of 3–12 kilogauss). A single absorption line is observed in the case of an isolated electron. In a free radical, the magnetic environment of the electron can split this resonance into a number of lines.

In the spin-label method, a free radical with a suitably designed functional group is reacted with the system. In the spin-probe method, selected molecules with free radical moieties are intercalated into the

albumins and the probes used. Carbonic anhydrase may lack the structural requirement for the binding of 1-anilinonaphthalene-8-sulfonate.

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In the spin-label method, a free radical with a suitably designed functional group is reacted with the system. In the spin-probe method, selected molecules with free radical moieties are intercalated into the

system. Details of the latter method, in particular with respect to the study of oriented lipid films, were discussed by Smith (1).

DISCUSSION

In this laboratory, two types of spin labels have been used primarily. One type (IV and V) is a steroid with a nitroxide group at the C_3 position. Another type (I and II) consists of a fatty acid or ester with a nitroxide group on the hydrocarbon chain. Probe III is also used occasionally as a monitor of head-group mobility.

Figure 1 describes how the spectra of a nitroxide depend upon the angle between the N—O bond and the magnetic field of the spectrometer. This bond is perpendicular to the long axis of the steroid nucleus (IV and V) and parallel to the long axis of the fatty acid (I and II). If probes IV and V orient themselves perpendicular to the plane of a lipid film and rotate rapidly about their long axes, their ESR spectra would consist of three lines separated by 6 and 19 gauss with the magnetic field perpendicular and parallel to the film, respectively.

When V is intercalated in brain lipid films, as the spectral anisotropy (difference between spectra run perpendicular and parallel to the magnetic field) decreases, the ratio of the heights of the peak 6–8 gauss downfield from the central peak and the central peak decreases when the spectra are observed with the film perpendicular to the magnetic field. This ratio, b/c, is a measure of the spectral anisotropy, varying from 1 for perfect order to 0 for a totally random array of spin probes. Under the same conditions, the separation of the three spectral lines of the spin-labeled fatty acid would be 32 and 6 gauss, respectively. For lower degrees of order, the spectral anisotropy is less.

Various theoretical models must be used to interpret these data (1–4). These spectral characteristics are observed with many lipid systems. The consistency of data obtained with both steroid and fatty acid spin probes demonstrates that the extrinsic probe is a faithful reporter of properties intrinsic to the lipid system.

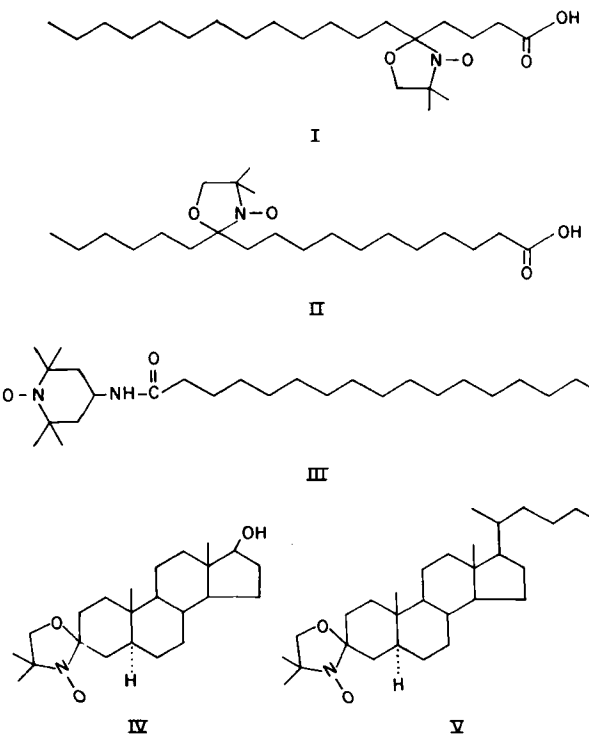
The detailed behavior of films depends upon their composition, the aqueous medium, and the temperature. The presence of ions in the aqueous medium is required for the integrity of films formed from charged lipids (2). Steroids have a marked effect; cholesterol is essential for a high degree of order in films of brain lipid (5) or egg lecithin (3) and decreases the rate and amplitude of motion and the probability of the *gauche*-conformation chains of the fatty acid in egg lecithin films (3, 4). Other steroids having a 3β-hydroxy group, a planar skeleton, and a nonpolar group at the C-17 position exert the same effect on brain lipids (5). These same factors were found by van Deenen (6) to be required for a reduction in membrane permeability.

Compounds in the aqueous phase can also influence the arrangement of molecules within the lipid phase. Proteins can be shown to exert various influences, either increasing or decreasing spectral anisotropy (1). The exact interpretation of the results is difficult, but it is certain that hydrophobic residues are involved in the disordering action. Polylysine increases order; Lys-Phe and Lys-Ala copolymers induce disorder, even in the presence of salt.

Many anesthetic agents interact with membranes. In a preliminary study, if butane or chloroform was passed in a stream of air over a hydrated lipid film, a large increase in the mobility of the spin probe occurred. This effect was dose dependent and reversible. Similar structural perturbations were observed with spin-labeled nerves and erythrocytes (7, 8).

EXPERIMENTAL

Alcohols—A dose–response study was made on the effects of a series of alcohols on human red cell lipids, lipids from the white matter of beef brain, and on egg lecithin using the cholestane spin probe (9). As the concentration of each alcohol increased, a point was reached where the separation between the lines of the spectra taken with the magnetic field of the spectrometer perpendicular to the film increased and the difference between the perpendicular and the parallel spectra decreased. At still higher alcohol concentrations, the film disintegrated. The concentration of alcohol required to cause a given change in the degree of anisotropy decreased as the chain length increased.



A plot of the concentration necessary to produce a 50% decrease in the height of the low field peak against the number of methylene groups in the alcohol is a straight line which parallels plots of the alcohol concentration necessary to inhibit tadpole reflexes, to bring about a decrease in the resistance of black lipid films, and to cause a 50% inhibition of red blood cell hemolysis. The displacement in the plots may be due to the varying amounts of disorder required to observe the various effects; more disorder is required for a 50% inhibition of red blood cell osmotic hemolysis than is required to inhibit tadpole reflexes, and even more is required to cause a resistance decrease in black lipid films. This disordering effect could cause anesthesia by directly affecting the permeability of the lipid layer, as seen with black lipid films, or by changing the lipid interaction with the membrane-bound proteins.

Local Anesthetics—Local anesthetics also increase the permeability of black lipid films (10), inhibit osmotic lysis of red cells (11, 12), and block nerve conduction (13–16). Unlike the effect of alcohols, the concentration of local anesthetic needed to cause a given response is pH dependent. This dependence has been rationalized by proposing different effects for the charged and uncharged forms of the drugs.

By using the cholestane spin probe (V), local anesthetics such as procaine, tetracaine, and butacaine affected films of brain white matter lipid in much the same manner as the alcohols. When the local anesthetic was added in a saline buffer to films that had been hydrated with the buffer alone, increasing concentrations of local anesthetic caused a decrease in the spectral anisotropy (hence order) of the films.

Unlike the effect of alcohols, the disordering effect increased with increasing pH at a fixed local anesthetic concentration. In the absence of local anesthetic, the amount of ESR spectral anisotropy was essentially constant over the pH range used. The concentrations necessary for marked disorder are high, in the toxic range according to studies performed on isolated nerves and red cells *in vitro*. It has been suggested that the uncharged form of local anesthetics penetrates membranes. The disordering effects observed may be due to disruption of the normal intermolecular forces between the phospholipids themselves or between phospholipids and cholesterol induced by high intramembrane concentrations of alcohols or local anesthetics.

As previously mentioned, films of brain lipid exhibit no ESR spectral anisotropy in the absence of cholesterol and only very slight anisotropy at 5 g % cholesterol. These films showed a very different response to the addition of local anesthetic. Whereas 1 m*M* tetracaine had no discernible effect on films containing the

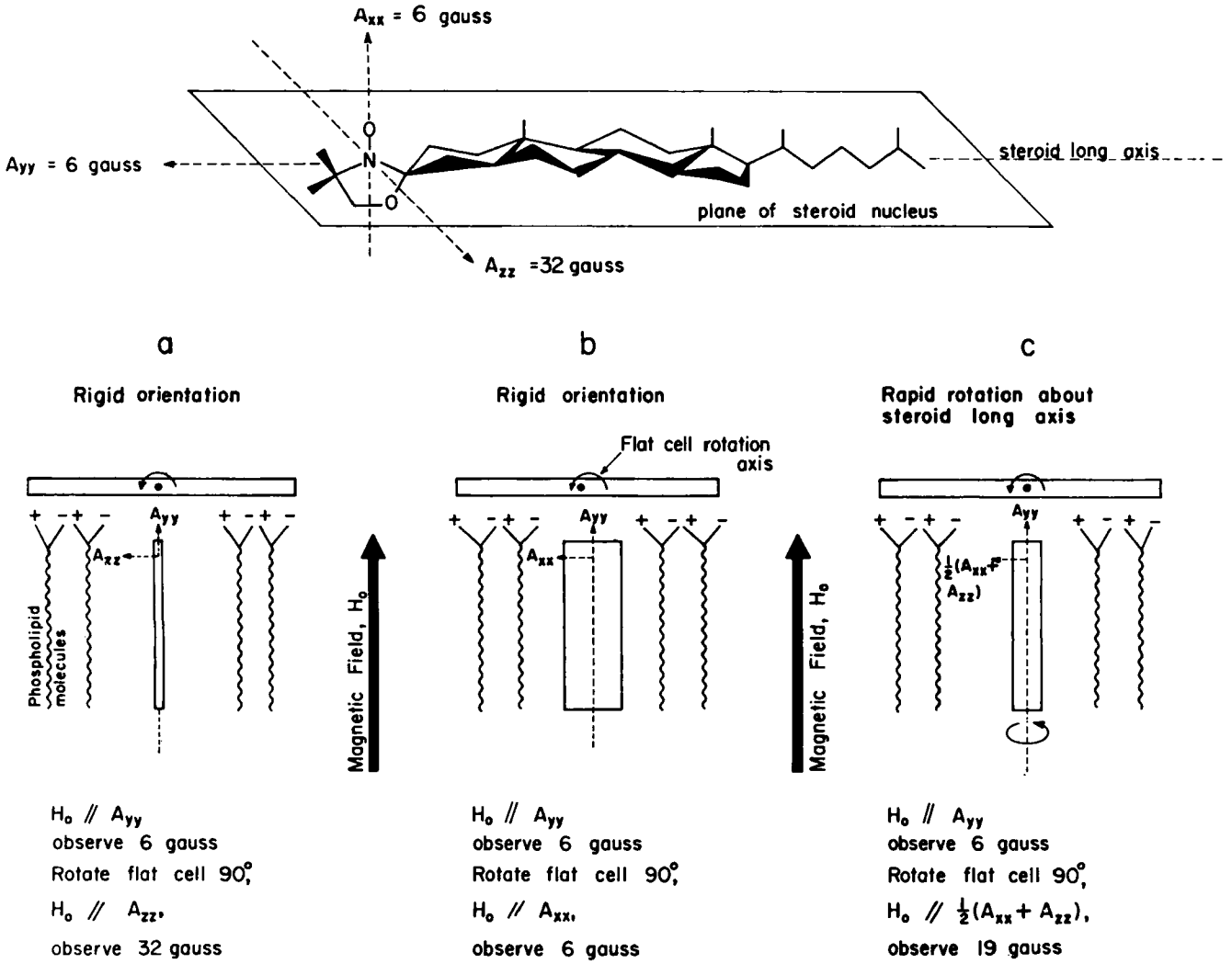


Figure 1—*Structure of V and the spectra that would be observed under various model conditions. (Reprinted, with permission, from Ref. 1.)*

normal amount of cholesterol, 1 m*M* tetracaine at pH 4.5 caused a massive increase in spectral anisotropy when applied to films containing 5% cholesterol.

Other local anesthetics also improved order when applied to films of brain lipid with 5% cholesterol at concentrations below those at which they disordered films of brain lipid with a normal cholesterol content. In addition, instead of efficacy increasing as a function of pH, each local anesthetic showed an optimal region of pH values for improving order. The optimum pH was different for each local anesthetic tested. Both uncharged and cationic forms of the local anesthetic apparently are required for this effect.

The effect of temperature on spectral anisotropy was measured with films of brain lipid. Figure 2 shows the effect of tetracaine and of cholesterol on the variation of spectral anisotropy with temperature. Both cholesterol and tetracaine caused the response to be greatly broadened.

The decrease in spectral anisotropy with a decrease in temperature is probably due to the formation of a gel phase which does not orient the spin probes; the decrease in spectral anisotropy with an increase in temperature can be ascribed to increased thermal motion of the acyl chains of the phospholipid. Cholesterol has been shown to increase the fluidity of the acyl chains below the transition temperature of the phospholipid and to decrease fluidity above the transition temperature (17). The results show that local anesthetics can exert much the same effect on brain lipid.

These results suggest two conclusions: (*a*) the response to local anesthetics of lipid films depends upon their composition, and (*b*) local anesthetics can exert a stabilizing effect on the organization of the lipids within the films. Since local anesthetics at anesthetic, nontoxic doses do not alter the resting potential of the nerve but rather block the transmission of impulses (18, 19), local anesthetics may act by stabilizing the membrane and preventing the conductance changes necessary for the propagation of action potentials.

Spin-Probe Reduction by Sodium Ascorbate—*Phosphatidyl Serine and Effect of Local Anesthetics*—The lipids used in the studies described here were extracted from biological material and consisted of a complex mixture of phospholipids, sulfatides, cerebrosides, and cholesterol. Other workers have investigated the effects of local anesthetics on the permeability, ion binding, and surface potential of pure systems or known mixtures (20). Tobias *et al.* (21) found that either cephalin (phosphatidyl serine plus phosphatidyl ethanolamine) alone or cephalin plus cholesterol showed a massive increase in resistance upon the addition of calcium. In this study, filters[1] were soaked in lipid to form the resistance barriers.

On the other hand, Papahadjopoulos (22) found that calcium increased the permeability of pure phosphatidyl serine vesicles to sodium. Local anesthetics also increased sodium permeability in the absence of calcium. At lower concentrations, these same local anesthetics opposed the effect of calcium; that is, in the presence of calcium, the addition of tetracaine or procaine decreased the sodium permeability of the liposomes.

The effects of calcium or local anesthetics on phosphatidyl serine films could not be studied since the films are unstable at pH values around neutrality, especially in the presence of calcium. Liposomes with an intercalated spin probe showed no significant spectral changes when local anesthetics, calcium, or both were

[1] Millipore.

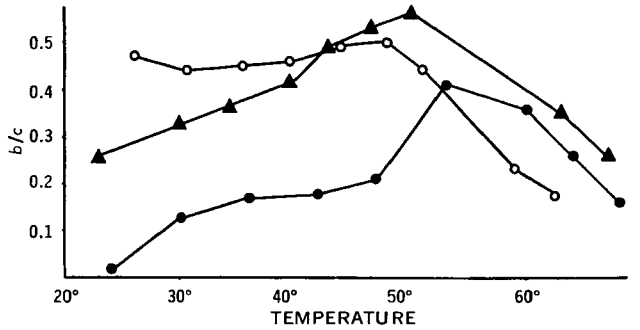


Figure 2—*Effects of tetracaine and cholesterol on the temperature response of brain lipid films. Key:* ●, *brain lipid and 5% cholesterol;* ▲, *brain lipid, 5% cholesterol, and 1 m*M *tetracaine; and* ○, *normal brain lipid.*

added. However, the permeability of these liposomes to sodium ascorbate was highly dependent upon the presence of calcium and of tetracaine. Metcalfe (23) reported that benzyl alcohol increased the rate of ascorbate penetration into liposomes. Therefore, the rate of reduction of the nitroxide moiety of the spin probe was used to monitor the local permeability of the lipid system.

With liposomes formed by water bath sonication of a mixture of phosphatidyl serine and spin probe I, the addition of 1 m*M* calcium caused a massive increase in the rate of reduction of the spin probe by ascorbate. The addition of 1 m*M* tetracaine caused a small increase in permeability, and 5 m*M* tetracaine caused a larger increase in permeability. When both 1 m*M* calcium and 1 m*M* tetracaine were present in the solution, the rate was less than with 1 m*M* calcium alone (Fig. 3).

The addition of lecithin to the system reduced the effect of calcium on the system to the point that pure lecithin liposomes showed no response to 10 m*M* calcium. As long as a calcium effect could be observed, tetracaine acted to oppose this effect. The addition of cholesterol up to a 1:1 mole ratio to the liposome did not inhibit the effect of calcium.

Other Charged Systems—Liposomes were also formed of egg lecithin and I to which either phosphatidic acid or dicetyl phosphate had been added to confer a negative charge. Both calcium and tetracaine at a concentration of 1 m*M* increased the rate of ascorbate reduction of I. Unlike the results with egg lecithin to which phosphatidyl serine had been added, the rate of reduction was greater when both calcium and tetracaine were added than with either alone (Fig. 4).

Brain Lipid Films—The results with brain lipids resembled those obtained with simple charged lipid systems. The rate of reduction of I incorporated in liposomes formed from the white matter lipid of beef brains increased upon the addition of either calcium or tetracaine at concentrations of 1 m*M*. Like the results obtained with egg lecithin plus either phosphatidic acid or dicetyl phosphate, the rate was greater with both calcium and tetracaine

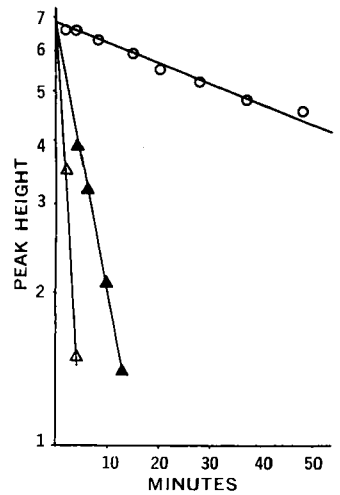


Figure 3—*Time course of the reduction of I in water bath-sonicated phosphatidyl serine vesicles by* 10^{-2} M *ascorbate at pH 7.2. Key:* ○, 7.5×10^{-3} M *ethylenediaminetetraacetate;* △, 1.0×10^{-3} M *calcium; and* ▲, 1.0×10^{-3} M *calcium* $+ 1 \times 10^{-3}$ M *tetracaine.*

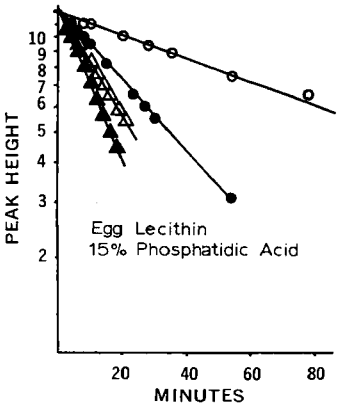


Figure 4—*Time course of the reduction of I in water bath-sonicated liposomes consisting of 85% egg lecithin and 15% phosphatidic acid by* 10^{-2} M *ascorbate at pH 7.4. Key:* ○, 7.5×10^{-3} M *ethylenediaminetetraacetate;* ●, 7.5×10^{-3} M *ethylenediaminetetraacetate* $+ 1 \times 10^{-3}$ M *tetracaine;* △, 1.0×10^{-3} M *calcium; and* ▲, 1.0×10^{-3} M *calcium* $+ 1 \times 10^{-3}$ M *tetracaine.*

than with either alone. When cholesterol was removed from the brain lipid mixture, the results, although not first order in the presence of calcium, more closely resembled the results obtained with systems containing phosphatidyl serine.

The results were qualitatively the same whether the liposomes were formed by simple hand shaking or by sonication with a probe or in a water bath. The direction of the effects of calcium and tetracaine was independent of liposome size.

Other spin probes, whose nitroxide groups lie at different depths within the lipid bilayer, were also used. The rate of reduction of the stearamide spin probe (III), in which the nitroxide group lies near the aqueous interface of the lipid bilayer, was six times as fast as the rate of reduction of I, in which the nitroxide group is buried within the lipid layer. This experiment was performed with large, hand-shaken liposomes. In water bath-sonicated (small) liposomes, V was reduced eight times as fast as was I.

Calcium and tetracaine had the same effects with these probes. Therefore, the accessibility of the interior of the lipid to the aqueous phase is concluded to be the major rate-limiting step.

CONCLUSION

It is apparent from these studies that the behavior of model systems when exposed to local anesthetics depends upon the lipid composition and upon the ions present in the aqueous environment. Phosphatidyl serine, a common constituent of nerves, responds to anesthetics in a manner very different from that of other phospholipids.

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Drug–Biomolecule Interactions: Mechanism of Ligand Interactions with Carbonic Anhydrase Studied by Magnetic Resonance Relaxation and Rapid Reaction Methods

PALMER TAYLOR

Abstract □ Kinetics of interaction between the metalloenzyme carbonic anhydrase and either monovalent anions or aromatic sulfonamides were examined by three distinct fast reaction techniques: stopped flow, equilibrium perturbation, and magnetic resonance relaxation. By correlating spectroscopic data on conformational and ionization equilibria of the complex and free species with the reaction kinetics, a relatively complete description of the mechanism can be presented. A proton-dependent equilibrium between two coordination forms of the free enzyme can be demonstrated spectroscopically. Anions selectively combine with the form predominating at low pH. For a series of carboxylate ligands, formate and substituted acetates, anion association is found to be three orders of magnitude greater than similar ligand substitution processes known in inorganic chemistry. For sulfonamide association, the attacking species are the form of carbonic anhydrase predominating at high pH and the neutral sulfonamide. Combination involving the neutral species is followed by loss of a proton to form the sulfonamido anion in the stabilized complex. This obligate proton transfer offers a probable explanation for the unique specificity of sulfonamides in inhibiting this enzyme. The anionic sulfonamido moiety can be shown to resemble closely a transition intermediate in the catalytic step of substrate hydration.

Keyphrases □ Ligand–carbonic anhydrase interactions—kinetics studied by magnetic resonance relaxation and rapid reaction methods □ Carbonic anhydrase–ligand interactions—kinetics studied by magnetic resonance relaxation and rapid reaction methods □ Drug–biomolecule interactions—mechanism of ligand interactions with carbonic anhydrase, magnetic resonance relaxation and rapid reaction methods □ Interactions—drugs with biomolecules, symposium

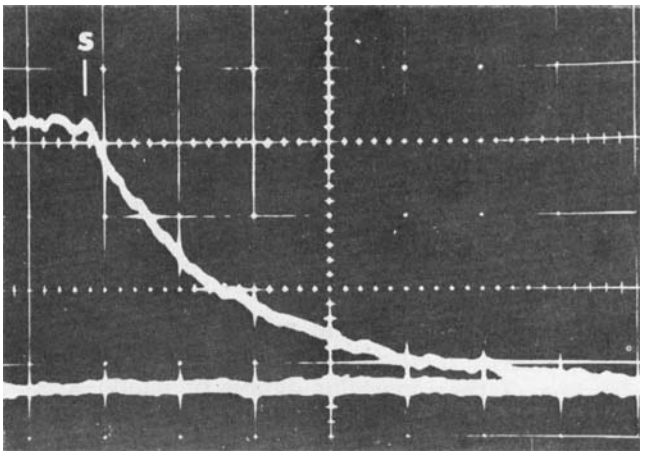

Figure 1—*Oscilloscope trace recording the change in carbonic anhydrase fluorescence for the reaction between 14 μM p-nitrobenzenesulfonamide and the human C isozyme. The oscilloscope was triggered with the initiation of flow so that the time dependence of fluorescence was measured during and after flow. Stoppage of flow occurs at s, and each large horizontal increment corresponds to 50 msec. The horizontal trace at the bottom of the screen was triggered ~10 sec later and corresponds with the fluorescence of the complex at equilibrium. Fluorescence was monitored at 345 nm; the excitation wavelength was 290 nm.*

The molecular basis of specificity in the formation of a ligand–macromolecule complex is of interest in many fields of the biological sciences, since complex formation represents the common initial event that leads to a divergence in response. The interaction of a macromolecule with a drug shows a capacity for selective recognition of ligand structure. In addition, receptors may exhibit specific capacity to translate an interaction with chemical mediators. The latter is generally considered to occur through ligand-sensitive conformational changes. Thus, in the study of specificity of drug–macromolecule interactions, both differential affinity and changes induced in the structure of the complex should be considered.

Complex formation between a drug and macromolecule usually involves an inherently rapid process(es). Examination of such interactions at a level where primary processes and transient species are detected requires methods applicable to following

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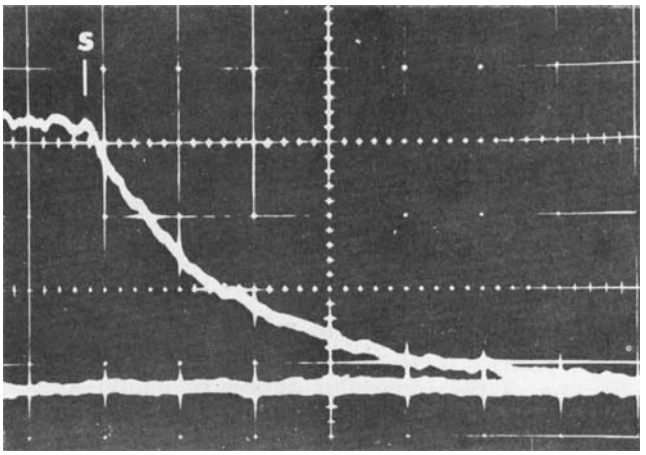

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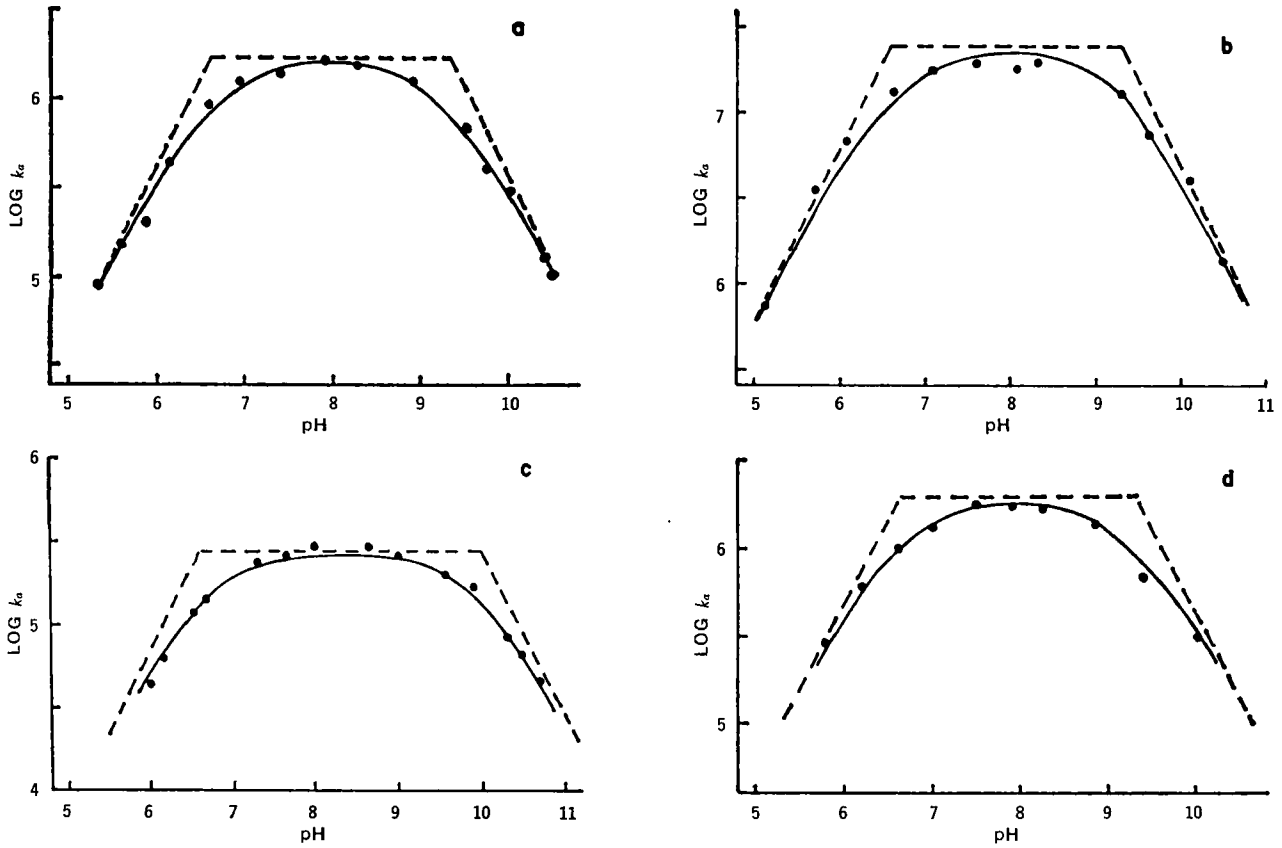


Figure 2—*The pH dependence of the bimolecular association rate constants,* k_a, *between human carbonic anhydrase C and various sulfonamides:* (a) p-*nitrobenzenesulfonamide and the Zn enzyme,* (b) *4-(salicyl-5-azo)benzenesulfonamide and the Zn enzyme,* (c) *5-dimethylaminonaphthalene-1-sulfonamide and the Zn enzyme, and* (d) p-*nitrobenzenesulfonamide and the Co (II) enzyme. The solid lines were calculated using Eq. 1, assuming* pK_E *to be 6.60 and* pK_S *to be 9.30 for* p-*nitrobenzenesulfonamide and salicylazobenzenesulfonamide and 10.00 for 5-dimethylaminonaphthalene-1-sulfonamide. The intersections of the dotted lines having slopes of 1.0 and 0.0 also yield the respective pK values. The kinetic constants were determined by stopped-flow measurements of fluorescence quenching associated with complex formation. (Reprinted, with permission, from Ref. 8.)*

reaction dynamics in the milli- and microsecond range. Kinetic methods useful in gaining an understanding of the molecular details of ligand–macromolecule interactions are discussed in this article.

Rapid mixing (stopped flow) and equilibrium perturbation techniques, when coupled to a suitable detection system, can monitor directly the course of the drug–macromolecule interaction. These two methods differ greatly in their applicable time range, the conditions under which kinetic constants are obtained, and the information that can be derived from the experiment. In addition, magnetic resonance relaxation times can be employed to estimate kinetic parameters. This situation arises when ligand exchange from two magnetically distinct environments, the binding site on the macromolecule and the bulk solvent, becomes the dominant relaxation process.

The level at which kinetic data can be interpreted is limited by the available structural information on the reacting species. Carbonic anhydrase is a metalloenzyme with a molecular weight of 29,500 and properties that are amenable to the use of spectroscopic probes. The crystal structure of this enzyme is now well characterized (1), and the active site exists at the base of a rather narrow cleft containing a single Zn atom. The Zn appears to be tetrahedrally coordinated with one H_2O and three residues from the protein core serving as ligands.

Catalysis takes place in the immediate vicinity of the Zn, and the enzyme inhibitors, monovalent anions and aromatic sulfonamides, appear to bind within the coordination sphere of the metal (1–4). The Zn may be replaced by Co (II) with the retention of very similar catalytic and inhibitor binding properties (2, 4). Substitution of this d^7-transition metal having unpaired electrons provides a valuable intrinsic probe of the enzyme coordination structure, since optical and magnetic resonance spectroscopy may be used to correlate conformational and ionization states with the reactive species.

In the selection of a system, it is also advantageous to keep ligand structure relatively simple. With the aromatic sulfonamide, only a single ionization equilibrium, the sulfonamido proton, must be considered. Conformation is also well defined with the two oxygens and the amino and aryl groups tetrahedrally disposed around the sulfur (6).

RESULTS AND DISCUSSION

Stopped-Flow Studies—The overall kinetics of association and dissociation may be followed with stopped-flow instrumentation by monitoring the quenching of the protein-tryptophan fluores-

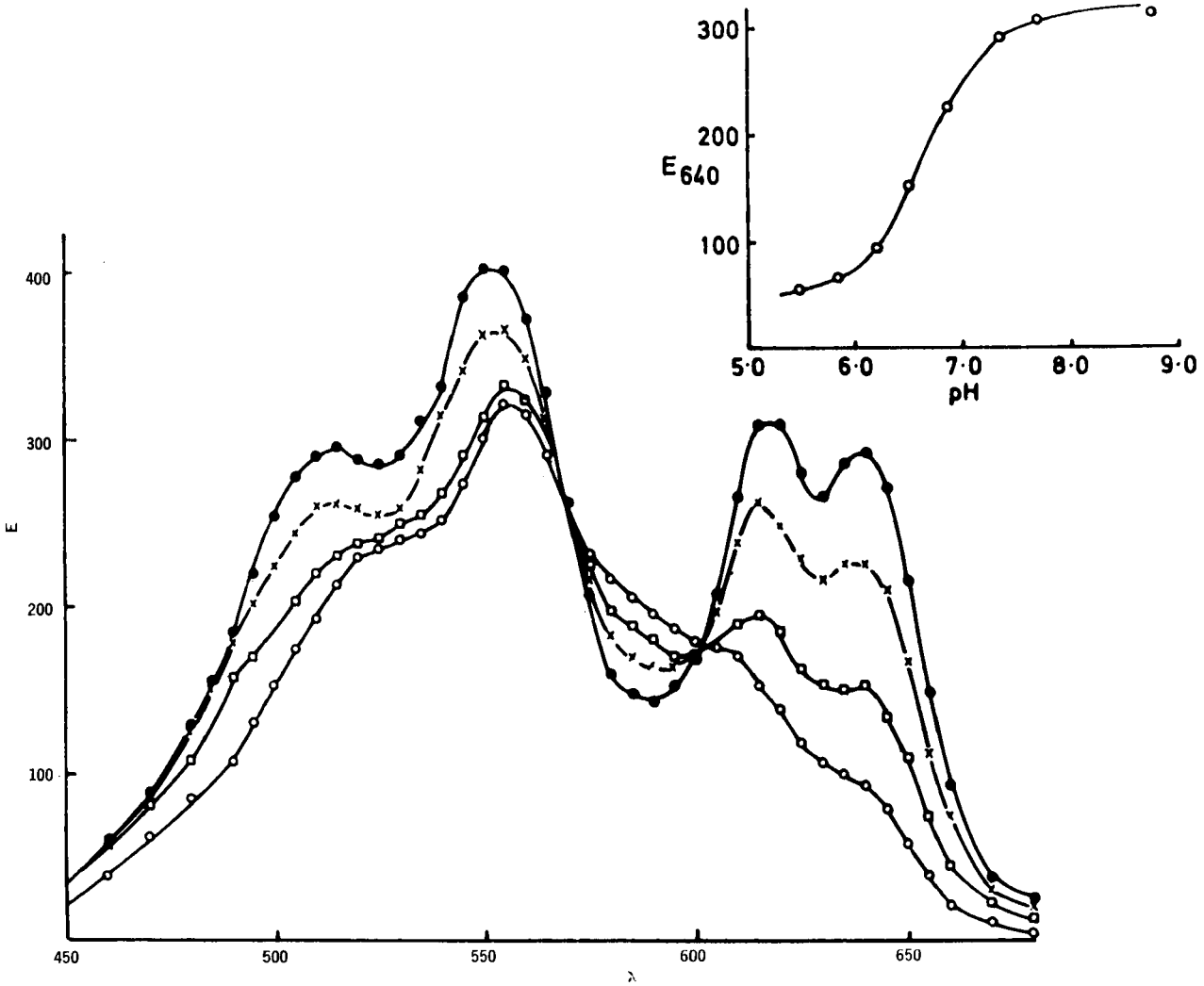


Figure 3—*The pH dependence of the Co (II) absorption spectrum of human carbonic anhydrase C; 0.32 mM enzyme in 20 mM K_2SO_4 was adjusted in pH by 0.05 N NaOH or 0.05 N H_2SO_4, and the spectra were recorded in 2.0-cm cells. Key: ○, pH 6.19; □, pH 6.50; ×, pH 6.86; and ●, pH 7.35. E is the molar absorptivity. The inset is a plot of the absorptivity for the peak at 640 nm as a function of pH. (Reprinted, with permission, from Ref. 8.)*

cence (7). If there is overlap between the tryptophan emission spectrum and the absorption spectrum of the sulfonamide, these species can serve as efficient donor–acceptor pairs of excited-state energy in the complex.

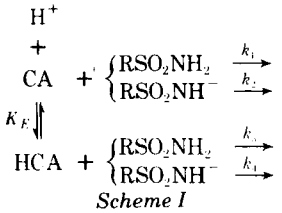
Figure 1 shows the time-dependent change in protein fluorescence associated with complexation of *p*-nitrobenzenesulfonamide and human carbonic anhydrase C. During the period where the trace is approximately horizontal, the transit time between the mixing chamber and observation tube is less than the reaction time, and fluorescence of the mixed, but unreacted, reactants is observed. At s, the flow is abruptly stopped and a first-order approach to the equilibrium fluorescence of the complex is observed.

Dissociation of the complex may also be followed by the stopped-flow procedure, which has been employed to obtain association and dissociation rate constants for a large series of sulfonamides (7). Correlation of individual kinetic parameters with structure provides an additional dimension to the usual structure–activity considerations. For example, substituent variation on the aromatic ring primarily affects the association rate process, and it is only with substitution *ortho* to the sulfonamido group that the dissociation rate becomes affected (7).

The pH dependence of the association rate between the sulfonamide and the enzyme shows two ionization equilibria which influence the association process (Fig. 2). The apparent pK at high pH corresponds with the titratable pKa of the sulfonamido proton on the various sulfonamides, while the apparent pK at 6.5 is independent of the combining ligand, suggesting that an ionization on the enzyme is responsible (8).

The Co (II) spectra of the human carbonic anhydrase C show clear isosbestic points, demonstrating a proton-dependent equilibrium between two discrete coordination forms of the enzyme (Fig. 3). The pK for this equilibrium is 6.6, which is in good accord with the pH dependence of the association rate. To confirm that the proton-dependent coordination change controls the low pH arm of the association rate, a pK of 7.3 can be assigned for both the spectral transition and the association rate in a different isozyme, carbonic anhydrase B.

Chemical modification of the β isozyme with bromoacetate forms a less active derivative in which a single histidine has been stoichiometrically carboxymethylated (9). The pH for the transition between coordination forms in the carboxymethylated enzyme shifts to 9.1, and there is a corresponding change in the ionization equilibrium affecting the sulfonamide association rate (8). Thus, Scheme I illustrates the sulfonamide association.



Scheme I

Reaction pathways represented by k_2 and k_3 may be ruled out

on the basis that they do not fit the observed pH dependence of the kinetics. Thus:

$$k_{obs} = \frac{k_1 + k_4(K_S/K_E)}{(1 + K_S/H^+)(1 + H^+/K_E)} \qquad \text{(Eq. 1)}$$

where K_E and K_S represent protonic dissociation constants on the enzyme and sulfonamide.

The pathways denoted by k_1 and k_4 exhibit identical pH dependencies, and a decision on the critical reaction pathway must be arrived at in a different way. Since k_{obs} is $2 \times 10^7\ M^{-1}\ sec^{-1}$, when $K_S/K_E = 10^{-3}$, k_4 must necessarily be greater than $10^{10}\ M^{-1}\ sec^{-1}$. A rate constant of that magnitude exceeds the diffusion limitation for a system of this nature. In other words, the low concentration product of the reactants for pathway k_4 requires that k_4 be improbably large to satisfy the observed rate. Thus, combination between the sulfonamido anion and the form of the enzyme represented by HCA can be ruled out and k_1 is left as the dominant reaction path for sulfonamide association.

Spectroscopic studies clearly show that the sulfonamido anion RSO_2NH^- is the stabilized species in the complex (10). Thus, complex formation involves initial combination between neutral species with subsequent transfer of a proton from the sulfonamide (Fig. 4). Although it is not known whether sulfonamide coordination occurs through the nitrogen or oxygen, striking similarities between sulfonamide coordination and CO_2 hydration are apparent. Both ligands combine with the coordination form of carbonic anhydrase predominating at high pH and subsequently involve a proton dissociation step.

The distances between the two oxygens in the linear CO_2 molecule and the tetrahedral sulfonamido group are similar, ~ 2.36–2.46 Å (6). As shown in Fig. 4, the sulfonamido anion in the complex rather closely resembles a transition state intermediate in CO_2 hydration. A requirement for combination by the neutral sulfonamide species followed by loss of the proton while in the bound state provides the likely explanation why aromatic sulfonic acids, although geometrically similar to the sulfonamides, exhibit a very low affinity for the enzyme.

The rate of dissociation of the complex is pH independent (7). Correspondingly, while the Co (II)·spectrum of the enzyme–sulfonamide complex differs from the free enzyme, it is unaffected by solution pH changes (7). Thus, the binding of an inhibitory ligand fixes the coordination state where no ionization equilibria affect either the coordination structure of the metal or the ligand dissociation rate.

Since the dissociation rate is pH independent, the dissociation of the sulfonamido proton does not take place in a rate-limiting step. Alternatively, an internal proton transfer between the ligand and a site on the enzyme would be consistent with the proposed mechanism.

Monovalent anions also bind to carbonic anhydrase but form

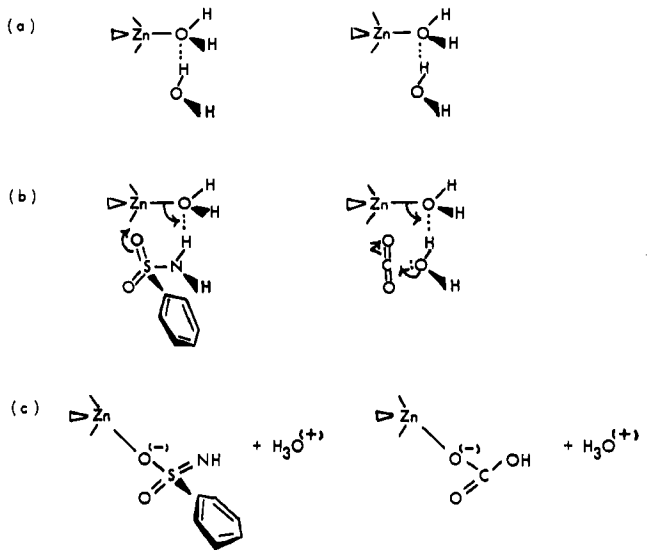


Figure 4—*Proposed scheme for sulfonamide and CO_2 interactions with the active site of carbonic anhydrase.*

Table I—Factors Controlling Relaxation of a Ligand in Association with a Paramagnetic Environment[a]

$$\frac{1}{T_2} = P\left[\frac{\frac{1}{T_{2M}^2} + \frac{1}{\tau_M T_{2M}} + \Delta\omega_M^2}{\left(\frac{1}{T_{2M}} + \frac{1}{\tau_M}\right)^2 + \Delta\omega_M^2}\right];\ P \ll 1$$

Case

a) If $\frac{1}{T_{2M}^2} > \frac{1}{\tau_M}$ or $\Delta\omega_M^2 > \frac{1}{\tau_M^2}$, then $\frac{1}{T_2} = \frac{P}{\tau_M}$

b) If $\frac{1}{\tau_M} > \Delta\omega_M^2 > \frac{1}{T_{2M}\tau_M}$, then $\frac{1}{T_2} = P\tau\Delta\omega_M^2$

c) If $\frac{1}{T_{2M}\tau_M} > \frac{1}{T_{2M}^2}, \Delta\omega_M^2$, then $\frac{1}{T_2} = \frac{P}{T_{2M}}$

Case	Relaxation Mode	Temperature Dependence	Frequency Dependence
a)	$1/\tau$	+	No change
b)	$\Delta\omega^2$	−	+ ($\alpha\omega_0^2$)
c)	$1/T_{2M}$	±	−

[a] From Swift and Connick (17). P is the ratio of ligand in the paramagnetic environment relative to the bulk solvent, τ_M is the residence time of the ligand in the paramagnetic environment, T_{2M} is the dipolar and scalar contribution to the relaxation time, and $\Delta\omega_M$ is the chemical shift difference in radians.

less affine complexes than the sulfonamides (11). The affinity of the anion–enzyme complex is pH independent and maximal at low pH, while at higher pH values the stability of the complex is inversely proportional to the hydrogen-ion concentration (Fig. 5). As in the case of the sulfonamides, ionization equilibria with pK_E's of 6.6, 7.3, and 9.1 determine the pH profile of anion binding to the C, B, and carboxymethylated B isozymes, respectively (12). Scheme II describes the specificity of ligand binding to carbonic anhydrase.

The coordination form predominating at low pH (HCA) selectively binds anions, while the high pH form (CA) is selective for sulfonamides. The pH dependence of association is dependent on the ionization equilibrium, K_E, and ionization equilibria on the ligand. Carboxymethylation of histidine$_{200}$ shifts ionization equilibrium K_E, thereby influencing the pH dependence of binding of both types of ligands.

NMR Relaxation Studies—The rapid rates of association and dissociation for the anion complex preclude measurement of the individual kinetic constants, k_a and k_{-a}, by flow methods. In an associating system, where the binding site and the solution provide magnetically distinct environments, NMR relaxation of ligand nuclei can be controlled by chemical exchange of the ligand between the free and bound states. This mode of relaxation only becomes dominant when the exchange rate is slow relative to other relaxa-

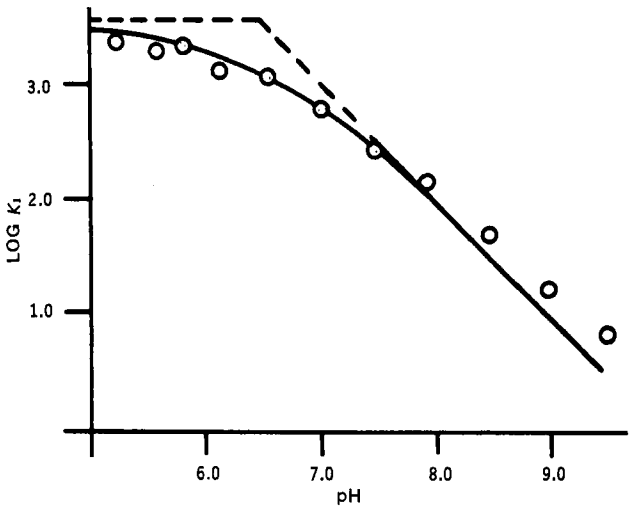


Figure 5—*The pH dependence of the equilibrium affinity of SCN^- for human carbonic anhydrase C. The solid line is calculated by assuming that $pK_E = 6.6$ and that the anions combine only with the coordination form of carbonic anhydrase predominating at low pH.*

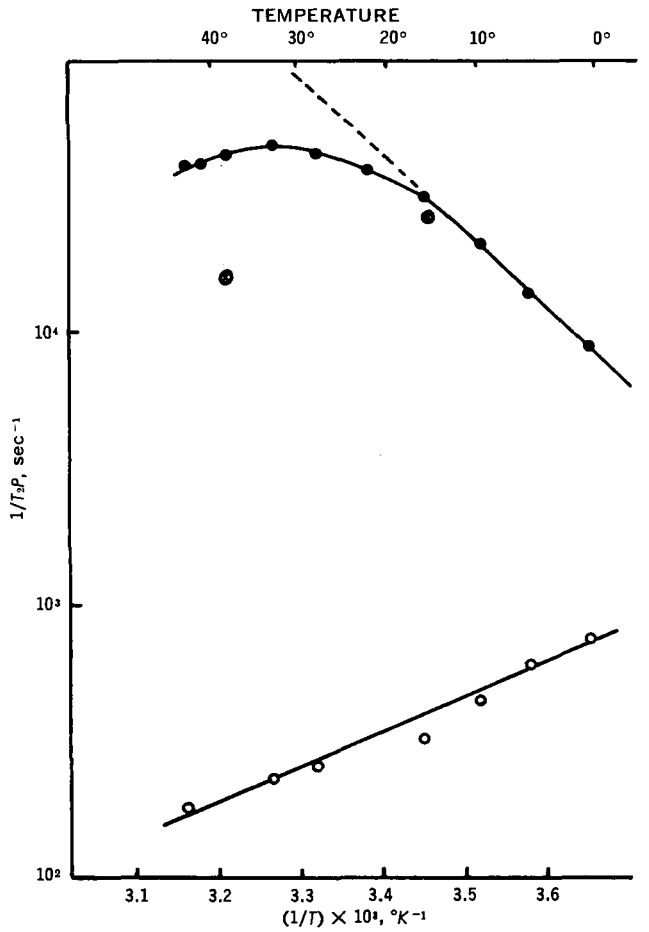
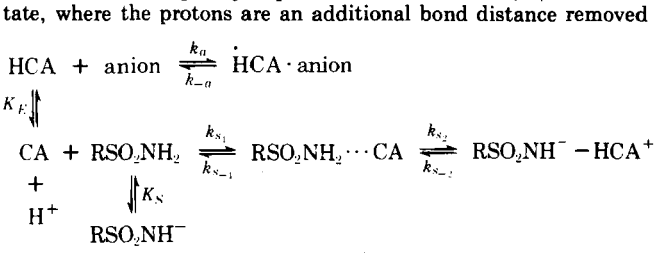


Figure 6—*Influence of temperature on the transverse relaxation rate ($1/T_2$) for formate and acetate complexes of Co (II) human carbonic anhydrase C, pH 7.6; $1/T_2P$ is plotted as a function of temperature where* P *is the ratio of bound to free ligand. Since the enzyme is at least 97% saturated at the ligand concentrations employed,* P *is equal to the ratio of added enzyme to ligand. Key: 400 mM formate in the presence of 0.1 mM enzyme at 100 MHz, ●, and at 60 MHz, ⊗; and 240 mM acetate in the presence of 1.5 mM enzyme at 100 MHz, ○. At each temperature, measurements were made in the presence and absence of* p-*carboxybenzenesulfonamide, and the plotted values represent the difference in the relaxation rates between the two conditions. The sulfonamide serves to dissociate all of the bound ligand.*

tion processes (Table I). With paramagnetic Co (II) enzymes, the contribution of electron–nuclei relaxation enhances the contribution of dipolar and scalar terms, $1/T_{2M}$, and the chemical shift difference, thereby increasing the likelihood of exchange-controlled relaxation (Case *a*).

The temperature dependence of relaxation of the single formate proton is consistent with exchange control in the low temperature range (Fig. 6) (Case *a*, Table I). Confirmation that the lifetime of the ligand in the paramagnetic environment, τ_M, controls and governs relaxation was obtained by measurements of chemical shift, $\Delta\omega_M$, and the frequency dependence of relaxation (13). For acetate, where the protons are an additional bond distance removed



Scheme II

Scheme III

from the metal, neither the magnitude of the relaxation rate nor its temperature dependence is indicative of exchange control.

Accordingly, a second nucleus must be examined to obtain additional direct kinetic information. Relaxation rate measurements with substituted fluoroacetates showed that ^{19}F relaxation at low temperatures also is governed by the exchange rate (13). On the other hand, protons on the same ligands, as would be anticipated from the acetate data, exhibit a different relaxation mode (Fig. 7). The kinetic constants, k_a and k_{-a}, may be estimated from the lifetime of the complex and equilibrium affinities for formate and the three fluoroacetates (Table II). Ligand association is a rapid process and approaches the diffusion limitation of a bimolecular reaction.

The crystal structure determination and NMR dispersion experiments provided good evidence that when inhibitory ligands bind to carbonic anhydrase a coordinated H_2O molecule is displaced (1, 14). When H_2O is the leaving group, ligand–metal association processes occur as shown in Scheme III (15).

The first step, which leads to an outersphere complex, is in rapid equilibrium with the slower ensuing step, which involves entry of the ligand into the metal coordination sphere. The second step, k_{01}, is rate limited by H_2O dissociation from the metal (15, 16); thus, ligand–metal substitution rates are sensitive to the nature of

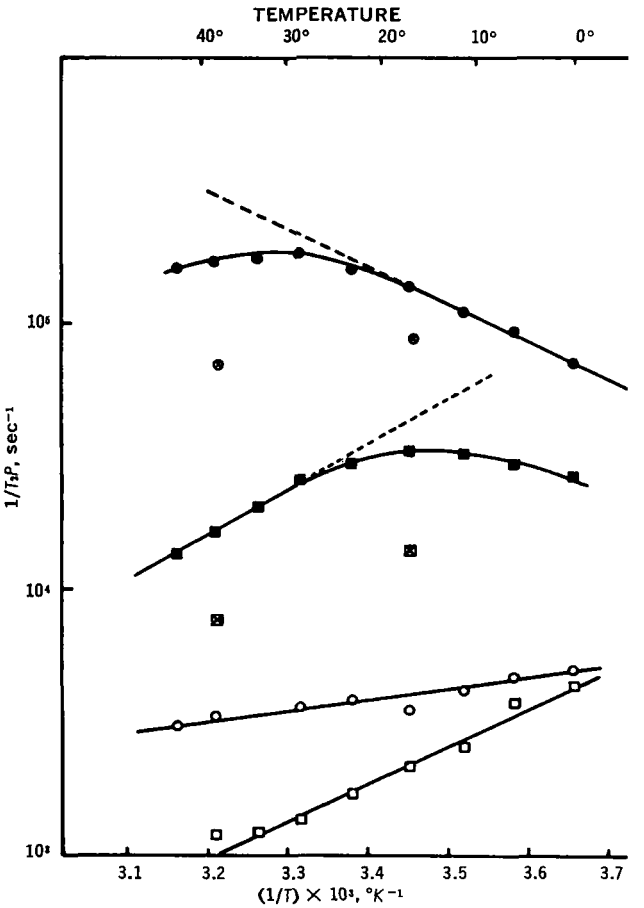


Figure 7—*Influence of temperature on the transverse relaxation rate ($1/T_2$) of ^1H and ^{19}F for mono- and difluoroacetate complexes of Co (II) carbonic anhydrase C, pH 7.6;* P *is the ratio of enzyme-to-inhibitor concentration: ●, ^{19}F in CH_2FCOO^- at 94.2 MHz; ■, ^{19}F in CHF_2COO^- at 94.2 MHz; ○, ^1H in CH_2FCOO^- at 100 MHz; □, ^1H in CHF_2COO^- at 100 MHz; ⊗, ^{19}F in CH_2FCOO^- at 56.4 MHz; and ⊠, ^{19}F in CHF_2COO^- at 56.4 MHz. The relaxation rates represent the difference in measurements made in the absence and presence of* p-*carboxybenzenesulfonamide.*

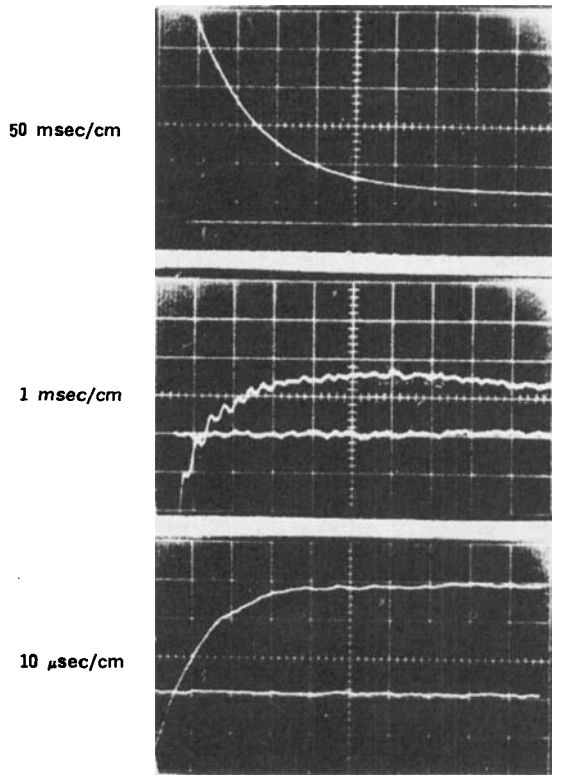


Figure 8—*Temperature-jump relaxation spectra for the association between 3-nitro-4-hydroxybenzenesulfonamide and human carbonic anhydrase B. A 30-kv pulse is discharged across a 0.7-cm path cell containing 4 × 10^{-4} M enzyme and sulfonamide in 0.2 M K_2SO_4 and 0.1 M bis(2-hydroxyethyl)-iminotris(hydroxymethyl)methane buffer, pH 6.0. Absorption at 425nm is monitored on a split-beam spectrophotometer. Key: top panel, 50-msec/cm sweeptime, τ_1 = 115 msec; middle panel, 1-msec/cm sweeptime, τ_2 = 1.1 msec; and bottom panel, 10-μsec/cm sweeptime, τ_3 = 13 μsec. The initial temperature is 18.5°.*

the metal rather than the ligand. For tetrahedral aquo–Co (II), k_{01} is estimated to be 10^5 sec^{-1}, which yields a bimolecular rate constant of the order of 10^5 M^{-1} sec^{-1} (13, 17). By contrast, the ligand association rates with the Co (II) enzyme exceed 10^8 M^{-1} sec^{-1}.

Enhanced rates of ligand substitution and turnover are obviously advantageous to the catalytic potential of an enzyme. The unusual spectra arising from *d–d** transitions, which have been observed for many metalloenzymes, indicate unique coordination geometry in the catalytically active form of the enzyme. However, it requires this kind of experiment where the dynamics of ligand exchange can be measured to establish that the metal in metalloenzymes plays a more crucial role than simply the orientation of the substrate.

Anion interactions with carbonic anhydrase can be characterized by far more rapid dissociation rates when compared with the aromatic sulfonamides. Although both monovalent anions and sulfonamides appear to coordinate with the metal, sulfonamide binding seems to involve two loci of interaction, the metal coordination sphere and a hydrophobic surface in the active site cleft to which the aromatic ring binds. The slower dissociation rate coupled with a second potential interaction site, confering stability to the complex, makes it seem probable that sulfonamide complex formation proceeds through a multistep mechanism (Scheme IV).

In a similar manner to chelation with bidentate ligands, formation of the initial complex makes the second step effectively unimolecular, imparting a statistical advantage toward complex formation. Thus, an apparent amplification of binding energy can be achieved through proper apposition of binding groups.

$$RSO_2NH_2 + CA \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} RSO_2NH_2 \cdots CA \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} RSO_2NH^- - HCA^+$$

Scheme IV

Table II—Estimated Association and Dissociation Rate Constants for Various Carboxylate Anion Complexes of Co (II) Carbonic Anhydrase C at 25°

Ligand	K_1, M [a]	k_1, M^{-1} sec^{-1} [b]	k_{-1}, sec^{-1} [c]
Formate	1.54 × 10^{-4}	3.9 × 10^8	6.0 × 10^4
Monofluoroacetate	9.1 × 10^{-4}	2.2 × 10^8	2.0 × 10^5
Difluoroacetate	5.3 × 10^{-4}	1.9 × 10^8	1.0 × 10^5
Trifluoroacetate	7.7 × 10^{-4}	2.0 × 10^8	1.5 × 10^5

[a] Intrinsic affinity: calculated from the apparent affinity at pH 7.6 and $K_1 = K_{1(app)}$ $[H^+/(H^+ + K_E)]$, where K_E is the protonic dissociation constant described in Scheme I. [b] Calculated from k_{-1} and K_1. [c] Determined from NMR measured lifetimes, τ_M, and extrapolation to 25°: $k_{-1} = 1/\tau_M$.

Equilibrium Perturbation Studies (Temperature Jump)—Low concentrations of reactants are necessary in the stopped-flow measurements; otherwise the time limit of resolution (~3 msec) is exceeded. Thus, in the stopped-flow experiment the bimolecular step, k_1, is rate limiting, and one cannot follow the reaction under conditions where a detectable concentration of intermediate would build up during the reaction. With high concentrations of reactants, a net change in reactant concentration must be achieved by methods other than mixing.

If an equilibrium between complex and free species is perturbed by the rapid adjustment of an external parameter controlling the equilibrium state, the transient approach to a new equilibrium (relaxation) can be monitored. The difference between initial and final equilibrium defines the amplitude of the relaxation step(s), and the time dependence of reequilibration can be related to the rate constants of the reaction (18). In contrast to the flow techniques, mixing is avoided and the concentration of reactants is close to the equilibrium concentration.

By discharging a high voltage pulse through a sample cell, a 5–10° temperature rise can be effected in a few microseconds. With the temperature-jump method, rate phenomena with relaxation times in the microsecond to second range are accessible to direct measurement.

The temperature-jump studies on complex formation between 3-nitro-4-hydroxybenzenesulfonamide and carbonic anhydrase B reveal as many as three relaxation processes (Fig. 8)[1], none of which is present if the reactants are examined individually. Assignment of relaxation steps to primary processes in the reaction pathway is achieved from the analysis of the concentration dependence of the relaxation rate and amplitude.

The slow step of the relaxation times, τ_1, at low concentrations is equivalent to the steady-state kinetic constants derived from the stopped-flow measurements. The relaxation step in the microsecond range, τ_2, is of low amplitude and precluded making a definitive assignment. The fast step, τ_3, shows a rate dependency on buffer concentration and appears to involve ionization in the bound state of the phenolic moiety on the sulfonamide.

A complete analysis of the relaxation profile will be presented elsewhere. It should be evident from these considerations that temperature-jump studies, with their wide temporal range of applicability, offer an essential means for examining the complete spectrum of primary processes associated with complex formation.

With ligand–macromolecule interactions where ionization and conformational changes can be anticipated to accompany complex formation, multiple kinetic and spectroscopic methods must be employed to provide a complete description of reaction mechanism. Through this approach, an understanding of the factors confering specificity in the recognition of ligand structure and the response to ligand binding can be developed.

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Drug–Biomolecule Interactions: Proton Magnetic Resonance Studies of Complex Formation between Bovine Neurophysins and Oxytocin at Molecular Level

JOHN H. GRIFFIN *§, JACK S. COHEN *x, PAUL COHEN ‡, and MARYSE CAMIER ‡

Abstract □ Proton magnetic resonance spectroscopy was used to monitor individual amino acid residues in bovine neurophysin, in the nonapeptide hormone oxytocin, and in the complex formed between them. For neurophysin I alone, a normal titration curve for the C-2 proton resonance of the lone histidine residue was obtained with an apparent ionization constant of 6.9. Addition of oxytocin to a solution of neurophysin I at pH 6.5 resulted in several changes in the spectrum. The effect on the histidine C-2 proton resonance signal indicated a slow exchange process between two states, probably representing a conformational change in the protein. The apparent pK of the histidine residue in the hormonal complex was shifted to 6.7, indicating a slightly more positive (less electron dense) environment for the histidine residue. Resonances of the single tyrosine residue of oxytocin were observed to broaden significantly, but not to shift appreciably, on the addition of neurophysin II. These observations may indicate involvement of the tyrosyl residue of oxytocin in the hormone–"carrier protein" interaction.

Keyphrases □ Neurophysins, bovine—complex formation with oxytocin, PMR □ Oxytocin—complex formation with bovine neurophysins, PMR □ Hormonal interactions—bovine neurophysins–oxytocin, PMR □ PMR—monitoring bovine neurophysins–oxytocin complex formation □ Drug–biomolecule interactions—PMR studies of complex formation between bovine neurophysins and oxytocin at the molecular level □ Interactions—drugs with biomolecules, symposium

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The neurohypophyseal nonapeptides oxytocin and 8-arginine (or 8-lysine) vasopressin are biologically important hormones known to associate noncovalently with the proteins neurophysin I and neurophysin II (11, 12). These proteins are found in the neurosecretory granules of the pituitary glands of several species associated with the hormonal peptides (13). The physical and chemical properties of the neurophysins, which have monomeric molecular weights near 10,000, have been investigated (14, 15), and some amino acid sequences for bovine neurophysins I and II have been proposed (16, 17). The study of the molecular interactions between neurophysins and peptide hormones has been approached using classical equilibrium methods (18–22) and spectroscopic techniques (23–26).

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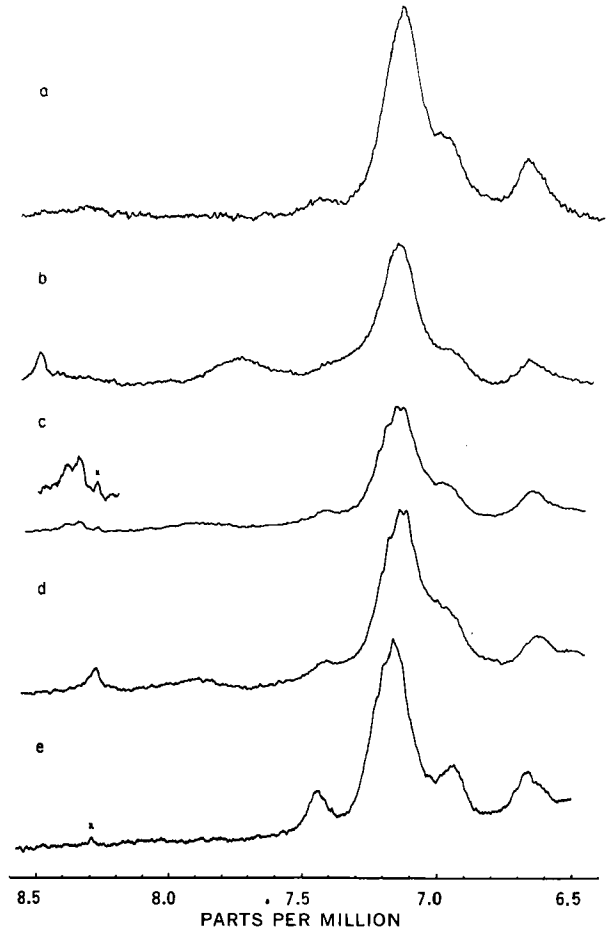

Figure 1—*Aromatic region of PMR spectra recorded in D_2O, 0.1 M NaCl, of neurophysin II at pH 6.52* (a) *and of neurophysin I at pH 2.21* (b), *5.77* (c), *6.09* (d), *and 8.85* (e). *The number of scans varied between 9 and 37. The insert in* (c) *is the same spectrum at higher gain. The peaks marked × are not due to neurophysin.*

EXPERIMENTAL[2]

Samples of neurophysins for PMR studies were lyophilized several times from D_2O and were dissolved in 0.1 *M* NaCl in D_2O[3] (100%) at a concentration of 30 mg in 0.5 ml. Measurements of pH were performed directly in the NMR sample tube as detailed previously (3), and the pH values quoted are direct meter readings[4].

Spectra were recorded on a spectrometer[5] with a probe temperature of 22 ± 1°. Time-averaging was carried out with a computer[6]. Other details of the PMR methods were described elsewhere (3). Chemical shift values are quoted in parts per million downfield from external 6% tetramethylsilane in carbon tetrachloride. Concentration measurements were checked spectrophotometrically[7]. For neurophysin, ϵ was taken as 3400 M^{-1} cm^{-1} at 260 nm (15); for oxytocin, an ϵ at 280 nm was taken as 1400 M^{-1} cm^{-1}.

As a control experiment for viscosity effects, neurohypophyseal proteins (mol. wt. ≃ 50,000) that eluted before the neurophysins during filtration[8] were used. This protein fraction was devoid of hormonal binding ability by equilibrium dialysis experiments carried out with radioactive oxytocin (22). The PMR spectrum of oxytocin was recorded in the presence of 15 mg of this protein and showed a negligible line-broadening effect (<10%).

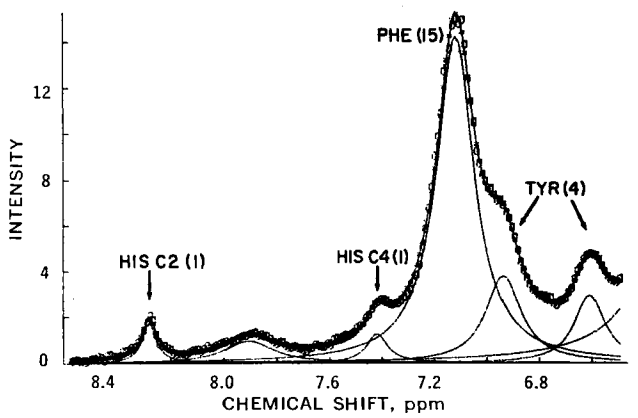

Figure 2—*PMR spectrum of neurophysin I from Fig. 1d fitted with Lorentzian curves. Key: O, observed; *, calculated; and ——, components. The assignments of the peaks are shown in the CALCOMP plot with the relative number of protons in parentheses.*

RESULTS AND DISCUSSION

Neurophysins—Aromatic protons absorb in a region of the PMR spectrum that is well resolved from the resonances of the much more numerous aliphatic protons. Imidazole C-2 ring proton resonances are even better resolved as a result of their unique chemical environment between two nitrogen atoms. The aromatic region of some PMR spectra of neurophysins I and II are shown in Fig. 1. The resonance in the low field region between 8.5 and 7.4 ppm in Figs. 1*b*–1*e* that shifts with pH may be attributed to the C-2 ring proton (8, 28, 29) of the single histidine residue of neurophysin I, which is absent from neurophysin II (30) (Fig. 1*a*).

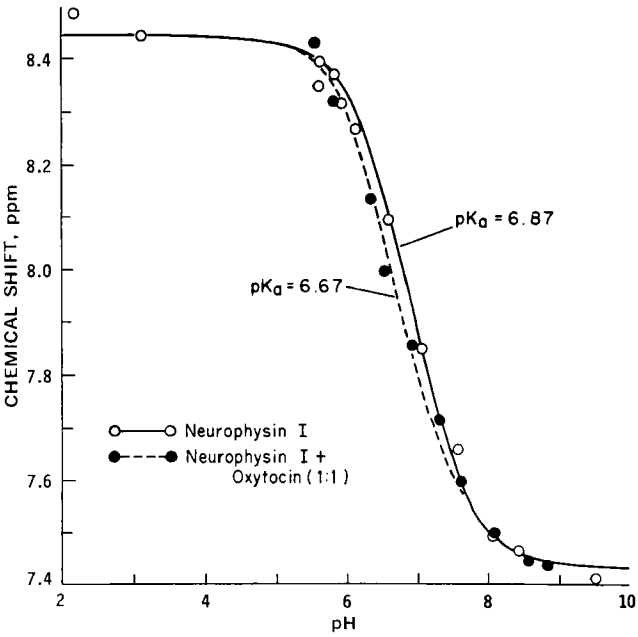

Figure 3—*Titration curves of the C-2 proton resonance of the single histidine residue of neurophysin I and of the neurophysin I–oxytocin complex. The lines are least-squares fits to the data using the theoretical expression for a simple proton association equilibrium (29).*

[2] Details of the preparation of highly purified bovine neurophysin have been described elsewhere (22). The synthetic peptide oxytocin was a gift from Dr. Guttmann, Sandoz, Basle, Switzerland.

[3] Aldrich Chemical Co.

[4] These values are not strictly pH because measurements were made in D_2O; they are not pD because measurements were made with a glass electrode containing water. It has been shown experimentally that the isotope effects on the glass electrode and on the ionization process itself almost cancel, so that no correction is made in these studies. [See D. H. Sachs, A. N. Schechter, and J. S. Cohen, *J. Biol. Chem.*, **246**, 6576(1971), and references cited therein.]

[5] Varian Associates HR 220.

[6] Varian C1024.

[7] Cary 15 spectrophotometer.

[8] Sephadex G-75.

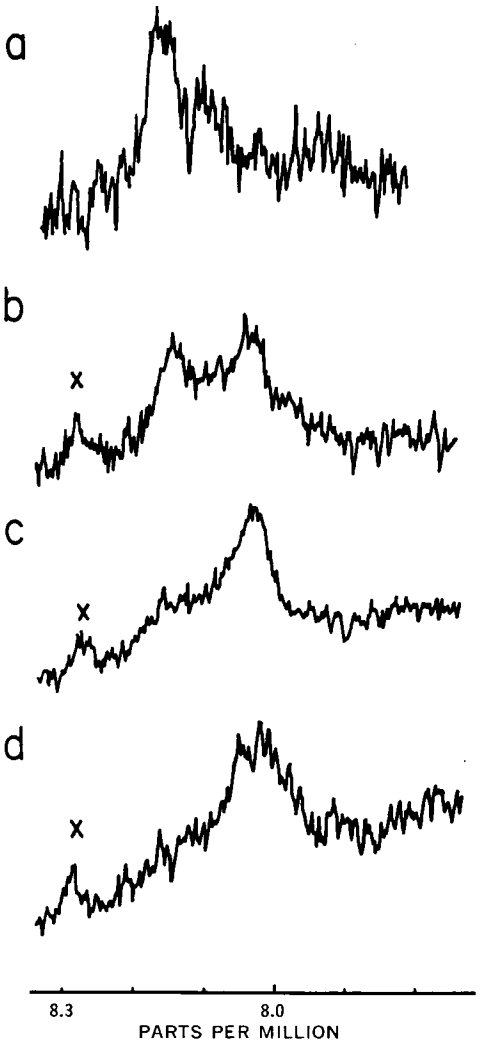

Figure 4—*PMR spectra showing the histidine C-2 proton resonance of neurophysin I at pH 6.51 in the absence of oxytocin* (a) *and at neurophysin–oxytocin molar ratios of 5:1* (b), *2.5:1* (c), *and 1:1* (d). *Solutions contained 0.1* M *NaCl in* D_2O. *The number of scans varied between 19 and 98. The peaks marked* × *are not due to neurophysin.*

Between pH 3.5 and 5.5, the resonance lines broaden due to aggregation of the protein as precipitation occurs near the isoelectric point, pH 4.4 (15, 22). Above pH 5, the protein redissolves and the lines correspondingly sharpen again. However, they remain fairly broad (5–20 Hz) considering the size of this protein compared to others studied previously (6, 31). This may result from dimer formation (15).

Another cause of line broadening may be the existence of a fairly rigid structure due to the presence of seven disulfide bonds. Reduction of internal motion causes the resonances to be broader by increasing the efficiency of dipolar relaxation between closely adjacent protons (32). The precise contribution of the two factors to the observed linewidth is difficult to estimate. A dilution by a factor of 5 to a concentration of 12 mg/ml produced no significant measurable reduction in linewidth.

The assignment of peaks in the low field region of a spectrum of neurophysin I (Fig. 1*d*) is shown in Fig. 2, in which the resonance peaks are fitted with Lorentzian functions (29). The broad resonance at chemical shift 7.75 ppm is attributed to protons that exchange slowly with water, since it disappeared entirely over 24 hr due to exchange with deuterium.

The pH titration data of the histidine C-2 proton resonance of neurophysin I are shown in Fig. 3. These data fit a theoretical curve based on a simple proton association equilibrium and give a pK value of 6.9, indicative of a normally titrating histidine residue (29). One unusual feature of the titration of the histidine C-2 proton resonance in this case was the consistent observation of a doubling of the peak at pH values between 5.6 and 5.9 (Fig. 1*c*). It was difficult to obtain spectra at slightly lower pH values due to precipitation of the protein (15); above pH 6.0, the phenomenon was no longer observable.

This doubling phenomenon could represent a slow equilibrium of the histidine residue between two environments, as previously suggested for similar observations involving histidine resonances (1, 3). Changes in the environment of the histidine residue may possibly be related to aggregation processes known to occur in this pH range (15).

Binding of Oxytocin to Neurophysins—Aliquots of oxytocin were added to a solution of neurophysin I at pH 6.5, and the effects on the PMR spectrum of the protein were monitored. A doubling of the histidine C-2 proton resonance was noted (Fig. 4). Since oxytocin does not contain a histidine residue, this phenomenon must be attributed to the effect of complex formation on the lone histidine in neurophysin I.

The presence of two separate resonances (Fig. 4*b*) at intermediate neurophysin–oxytocin molar ratios indicates a slow conformational equilibrium between two environments (1), for which the lifetime in one state must be greater than 13 msec (*i.e.*, $\gg 1/\pi\Delta\nu$, where $\Delta\nu$ is the separation between the two resonances, 25 Hz). This slow equilibrium could be the oxytocin binding process or a protein conformational change that exposes the histidine residue to two different environments. The latter possibility seems more likely in view of previous knowledge of the rates of protein conformational changes (33). No other significant changes in most regions of the spectrum were observable up to a molar ratio of 1:1.

The titration curve of the neurophysin I–oxytocin complex at a 1:1 molar ratio (>95% oxytocin binding sites filled) is shown in Fig. 3. The histidine pKa value is shifted 0.20 pH unit to a lower value due to complex formation, representing a somewhat more positive (less electron dense) environment for the histidine residue in the hormone–neurophysin I complex. Such a relatively minor pK shift suggests that the histidine residue does not play a significant role in the hormonal interaction. This conclusion might be expected

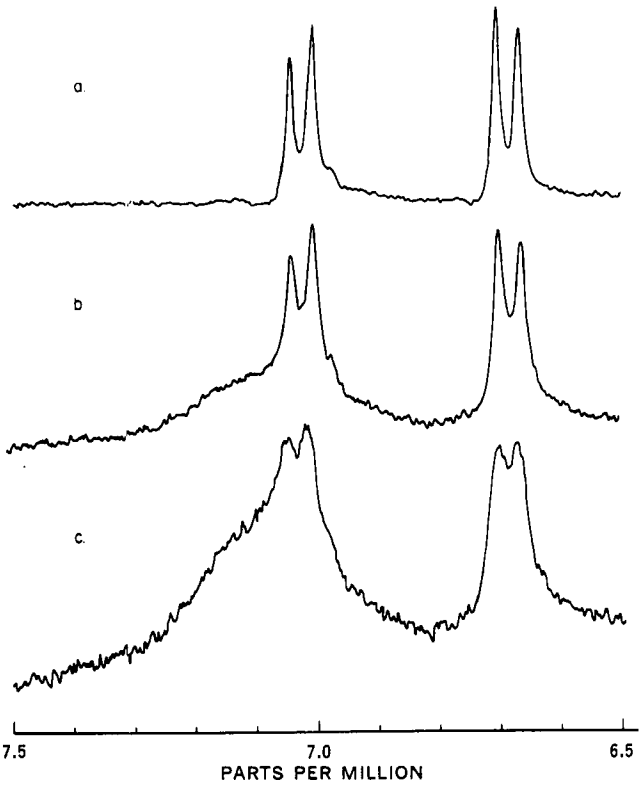

Figure 5—*Aromatic region of PMR spectra of oxytocin at pH 6.60* (a) *and after the addition of aliquots of neurophysin II to yield oxytocin–neurophysin molar ratios at pH 6.79 of 7:1* (b) *and 3:1* (c). *Solutions contained 0.1* M *NaCl in* D_2O.

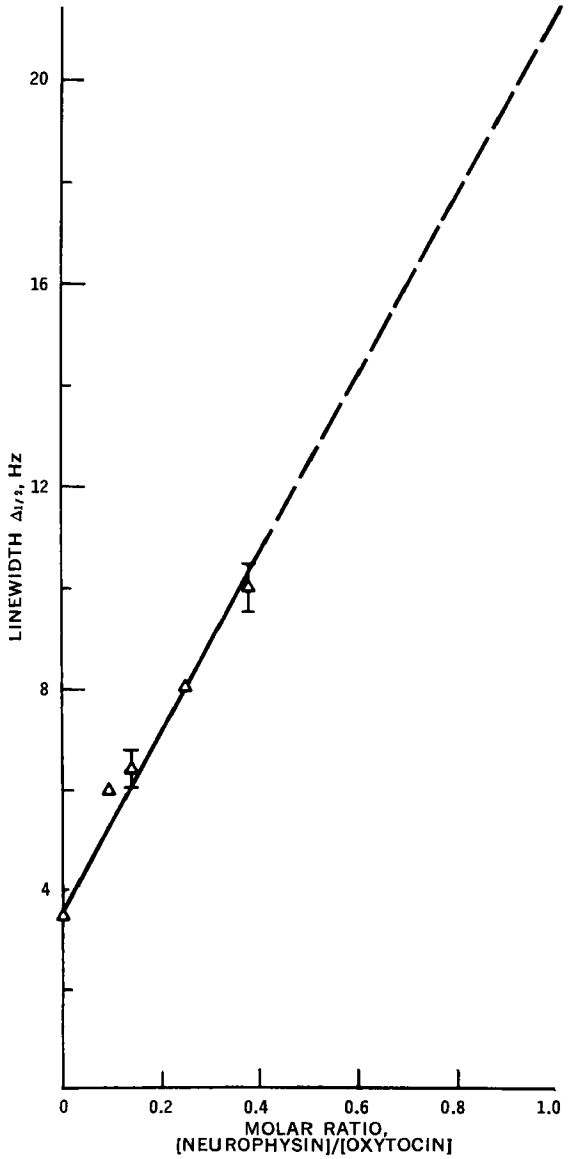

Figure 6—*Plot of the observed full linewidth at half-height of the tyrosyl resonance at 6.7 ppm of oxytocin* versus *the molar ratio of neurophysin–oxytocin.*

since neurophysin II binds oxytocin yet lacks a histidine residue.

The protein resonances obscure the oxytocin resonances in the aliphatic region of the PMR spectrum even at low protein to oxytocin molar ratios, and it is virtually impossible to distinguish the resonances of the two components. However, oxytocin contains a single tyrosine residue, and it is possible to follow the effects of the addition of neurophysin II on the tyrosyl aromatic protons. PMR spectra at several molar ratios are shown in Fig. 5. The mean value of the full width at half-height of the two tyrosine resonances in the high field doublet at 6.7 ppm is plotted in Fig. 6 as a function of the molar ratio of the total amount of each species in solution.

The apparent linear increase in linewidth of the oxytocin tyrosyl C-3 and C-5 proton resonances (Fig. 6) could arise from either a slow or a fast exchange process between two states for the tyrosyl residue. In the case of a simple bimolecular process (Scheme I):

$$\text{oxytocin} + \text{neurophysin} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \text{oxytocin–neurophysin}$$

Scheme I

the exchange of the tyrosyl residue between free and bound states can be described by equations derived for either the fast or the slow exchange limit (34). In the slow exchange limit:

$$\Delta_{1/2\,\text{obs}} = (1 - p)\Delta_{1/2\,\text{free}} + p/\tau \qquad \text{(Eq. 1)}$$

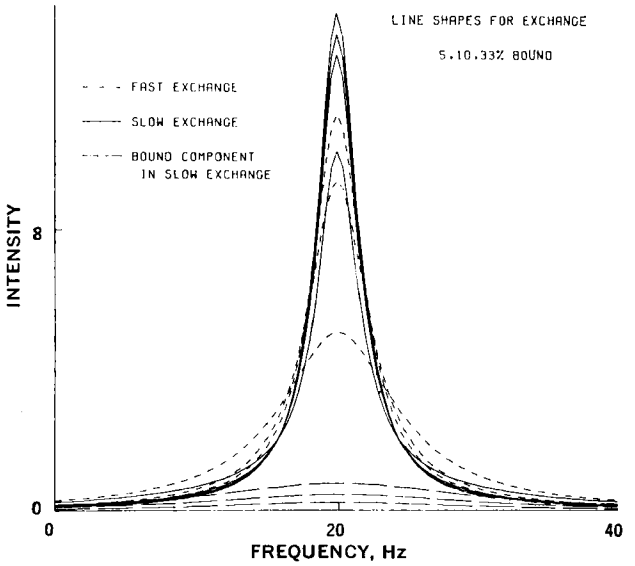

Figure 7—*Simulated peak shapes for fast and slow exchange conditions for a single Lorentzian-shaped peak. In both conditions the linewidth at half-height, $\Delta_{1/2}$, of the free component is 3.5 Hz, while that of the bound component, assumed to have the same chemical shift, is 21.5 Hz. In the slow exchange limit when $\tau \gg P$ (Eq. 1), $\Delta_{1/2obs} = \Delta_{1/2free}$, and the observed peak (solid line) is broadened by summation with the bound component. For slow exchange, the area of each of the two components is proportional to the amounts that are free and bound. For fast exchange the linewidth of the observed peak for different proportions bound was determined from Eq. 2 and is represented by the small dashed line. The actual observed values of $\Delta_{1/2}$ in each case would be:*

percent bound	*fast exchange*	*slow exchange*
0	*3.5 Hz*	*3.5 Hz*
5	*3.4 Hz*	*3.6 Hz*
10	*5.3 Hz*	*3.8 Hz*
33	*9.4 Hz*	*4.3 Hz*

while in the fast exchange limit:

$$\Delta_{1/2\,\text{obs}} = (1 - p)\Delta_{1/2\,\text{free}} + p\Delta_{1/2\,\text{bound}} \qquad \text{(Eq. 2)}$$

where $\Delta_{1/2}$ is the full linewidth at half-height, p is the fraction of oxytocin bound, and $\tau = 1/k_{-1}$, which is the mean lifetime of the complex. These equations apply when the concentration of free oxytocin is much greater than the concentration of the oxytocin–neurophysin complex and when:

$$\Delta_{1/2} = 1/\pi T_2 \qquad \text{(Eq. 3)}$$

where T_2 is the tranverse relaxation time of the observed proton.

Both Eqs. 1 and 2 can describe a linear dependence of the observed half-width on the amount of complex formed, as illustrated in Fig. 7. The possibilities of slow or fast exchange for the oxytocin–tyrosyl binding process can be distinguished. Studies at different temperatures and at different magnetic field strengths, coupled with direct measurement of the longitudinal relaxation time, T_1, should serve to distinguish these possibilities (34).

On the basis of preliminary work, Balaram *et al.*[9] concluded that the lysine vasopressin–neurophysin II complex exhibits slow exchange. However, Alazard *et al.*[10] recently found that oxytocin and lysine vasopressin show a fast exchange process with neurophysin II. This conclusion cannot necessarily be extended to all such complexes, not only because of the different hormones and conditions used but also because of recent evidence that these two peptide hormones bind differently (22).

[9] P. Balaram, A. A. Bothner-by, and E. Breslow, personal communication [see *Biochemistry*, **12**, 4695(1973)].

[10] R. Alazard, P. Cohen, J. H. Griffin, and J. S. Cohen, unpublished results.

If a slow exchange is assumed, evaluation of τ from Eq. 1 using the data shown in Fig. 6 leads to a value of 56 msec, which is consistent with the limiting value (*i.e.*, >13 msec) of the lifetime of the histidine residue in the hormonal complex. This value of τ gives 18 sec^{-1} for the dissociation rate constant, k_{-1}. Since the oxytocin–neurophysin dissociation constant, K_d, which equals k_{-1}/k_1, is approximately 2×10^{-5} M (22), an estimate of k_1, the bimolecular association rate constant, yields 9×10^5 M^{-1} sec^{-1}.

Alternatively, if a fast exchange is assumed, evaluation of $\Delta_{1/2\,bound}$ from Eq. 2 gives an extrapolated value of 22 Hz for the linewidth of the tyrosyl resonances in the complex at a 1:1 molar ratio. At this ratio, saturation of oxytocin binding sites on neurophysin occurs (19, 22). Since the observed linewidth is related through $1/T_2$ (Eq. 3) to the rotational correlation time of the tyrosyl side chain, the large increase in linewidth upon binding indicates a significant restriction in motion of the tyrosyl residue if fast exchange obtains. Involvement of the tyrosyl ring of oxytocin in the binding process, indicated here by PMR studies, is consistent with the apparent lack of binding capacity of 2-isoleucine-oxytocin (19) and of oxytocin iodinated on the tyrosyl ring (22). Experiments carried out with oligopeptides corresponding to the *N*-terminal amino acid sequence of the hormone also reinforce this idea (15, 25, 26).

Complex formation between neurophysin and oxytocin could involve hydrophobic or π–π interactions between neurophysin and the hormone's tyrosyl side chain as well as hydrogen bonding of the tyrosyl phenolic hydroxyl groups. However, π–π interactions seem unlikely in this case, since the stacking of aromatic rings produces measurable (>0.1 ppm) upfield shifts (35), and no significant change in chemical shift of the oxytocin tyrosyl resonances is observed at the highest molar ratios (Fig. 5).

Additionally, hydrogen bonding of the phenolic hydroxyl may not be essential for hormone binding since 2-phenylalanyl-oxytocin is bound almost as well as oxytocin (19). In this case, the tyrosyl residue of oxytocin seems to be bound in the hormone–neurophysin II complex through hydrophobic bonding[11].

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* To whom inquiries should be directed.

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Drug–Biomolecule Interactions: Topographical Study of Active Site of Erythrocyte Carbonic Anhydrase Using Spin-Labeled Sulfanilamide Drugs

COLIN F. CHIGNELL

Abstract □ The topography of the active sites of human erythrocyte carbonic anhydrases B and C and bovine erythrocyte carbonic anhydrase B was studied using a series of spin-labeled sulfanilamide analogs. Results show that the active site of human carbonic anhydrase C is a narrow cleft approximately 14 Å in depth. This observation is in good agreement with previously published X-ray diffraction data. While the active sites of human carbonic anhydrase B and bovine carbonic anhydrase B have the same general shape as the active site of human carbonic anhydrase C, they are slightly deeper.

Keyphrases □ Erythrocyte carbonic anhydrase—active site, topographical study, spin-labeled sulfanilamide drugs □ Carbonic anhydrase, erythrocyte—active site, topographical study, spin-labeled sulfanilamide drugs □ Sulfanilamide drugs, spin labeled—topographical study of active site of erythrocyte carbonic anhydrase □ Spin-labeled drugs—sulfanilamide, topographical study of active site of erythrocyte carbonic anhydrase □ Drug–biomolecule interactions—topographical study of active site of erythrocyte carbonic anhydrase using spin-labeled sulfanilamide drugs □ Interactions—drugs with biomolecules, symposium

Spin labels are stable free radicals that can be used as probes or reporter groups for biological macromolecules such as proteins, nucleic acids, and membranes. The first free radical to be used as a spin label was the chlorpromazine cation radical. Ohnishi and McConnell (1) studied the interaction of this spin label with DNA and found that it was bound with its aromatic plane nearly perpendicular to the helix axis of the nucleic acids. Since the chlorpromazine radical was only moderately stable in aqueous solutions, other, less reactive, spin labels were sought. The synthesis of di-*tert*-butylnitroxide (2), a free radical that was very stable in aqueous systems over wide temperature and pH ranges, had been described previously. The unique chemical and physical properties of the nitroxide group made it an ideal spin label, and various molecules containing this free radical have now been synthesized (3).

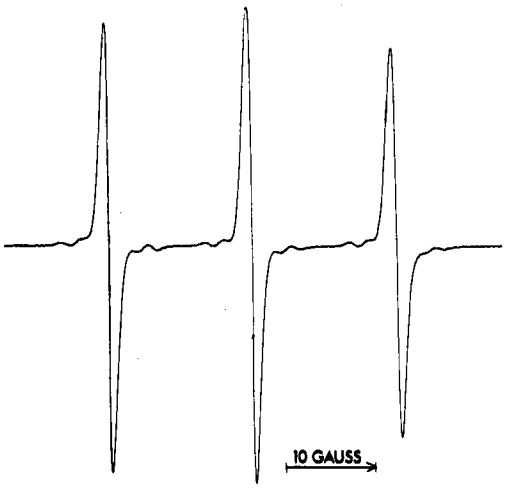


Figure 1—*Electron spin resonance spectrum of II* (2×10^{-5} M) *in 0.1* M *sodium phosphate buffer.*

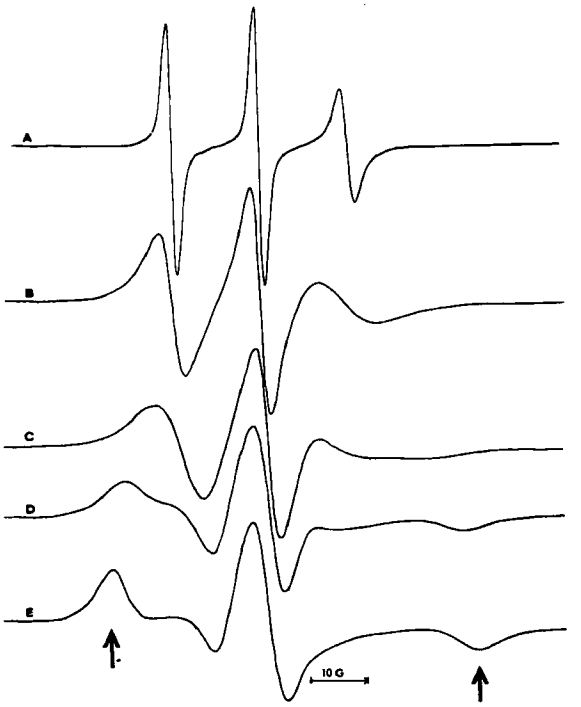
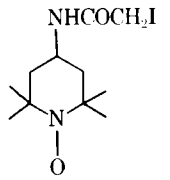


Figure 2—*Electron spin resonance spectrum of III* (1×10^{-4} M) *dissolved in glycerol. Key: A, 60°; B, 30°; C, 20°; D, 10°; and E, 0°. The two arrows indicate the positions of the low (left) and high (right) field peaks which are characteristic of a highly immobilized nitroxide radical.*

DISCUSSION

When a nitroxide spin label is dissolved in a solvent of low viscosity, such as aqueous buffer, its electron spin resonance spectrum consists of three sharp lines of nearly equal height (Fig. 1). This spectrum may be characterized by three parameters: *(a)* the position of the center line in the magnetic field (this is governed by a constant, g_0, termed the g factor); *(b)* the distance between adjacent lines, A_0, measured in gauss (G); and *(c)* the individual linewidths measured in gauss. When the environment of the nitroxide group is altered, then changes may occur in one or more of these parameters (3).

For example, di-*tert*-butylnitroxide in water yields $A_0 = 16.7$ G and $g_0 = 2.0056$, whereas di-*tert*-butylnitroxide in hexane is characterized by $A_0 = 14.8$ and $g_0 = 2.0061$. When a glycerol solution of a nitroxide spin label is cooled, the electron spin resonance spectrum of the free radical becomes broad and asymmetric (Fig. 2).



I

The limiting line shape (Fig. 2E), which is known as the rigid glass, polycrystalline, or powder spectrum of the nitroxide radical, is characterized by the appearance of distinct low and high field peaks separated by approximately 64 G (Fig. 2E).

The rigid glass spectrum is observed when the spin label is randomly oriented with respect to the laboratory magnetic field and the molecular motion of the nitroxide group is slow on the electron spin resonance time scale, *i.e.*, $\tau_c \gg 10^{-8}$ sec, where τ_c is the rotational correlation time of the spin label. Spectra approaching the rigid glass limit have been observed when nitroxide spin labels were tightly bound to macromolecules such as proteins and membranes (3).

Nitroxide spin labels can be employed in two different ways to study drug–biomolecule interactions. First, they can be attached (covalently or noncovalently) to the macromolecule of interest and function as reporter groups. This approach was used by Holmes and Piette (4) to study the interaction of chlorpromazine with erythrocyte ghost membranes. When these workers reacted an iodoacetamide spin label (I) covalently with the erythrocyte membrane, the electron spin resonance spectrum of the labeled membranes indicated that the nitroxide group had a high degree of freedom.

It would, therefore, appear that the protein sulfhydryl groups labeled by this reagent were on the outside of the membrane. When chlorpromazine was added to the spin-labeled membranes, a highly immobilized component appeared in the electron spin resonance spectrum of the nitroxide group. Holmes and Piette (4) suggested that chlorpromazine induced a conformational change in erythrocyte membranes so that some spin labels were moved into the interior. Since the highly immobilized component disappeared on removal of chlorpromazine, the conformational change was apparently reversible.

Hubbell *et al.* (5) used a similar approach to study the interaction of local anesthetic drugs, such as benzyl alcohol and lidocaine, with erythrocyte membranes. The spin labels employed included nitroxide analogs of methyl stearate and 17β-hydroxy-5α-androstane, which were incorporated noncovalently into red cell membranes. The steroid spin label rotated preferentially about its long axis, which was oriented perpendicular to the membrane surface. The addition of benzyl alcohol produced a progressive sharpening of the electron spin resonance spectrum of the spin label, which suggested that the environment of the free radical was becoming more fluid.

However, at high (lytic) concentrations of benzyl alcohol, a new spectral component appeared, which corresponded to a fraction of spin labels that were in a highly immobilized environment. This same component was also observed when the androstane spin label bound to protein was isolated from the erythrocyte membrane. Thus, the highly immobilized component that appeared in the lytic concentration range of benzyl alcohol was attributed to new protein binding sites for the spin label. These results agreed well with previous NMR studies of benzyl alcohol binding of erythrocyte membranes (6).

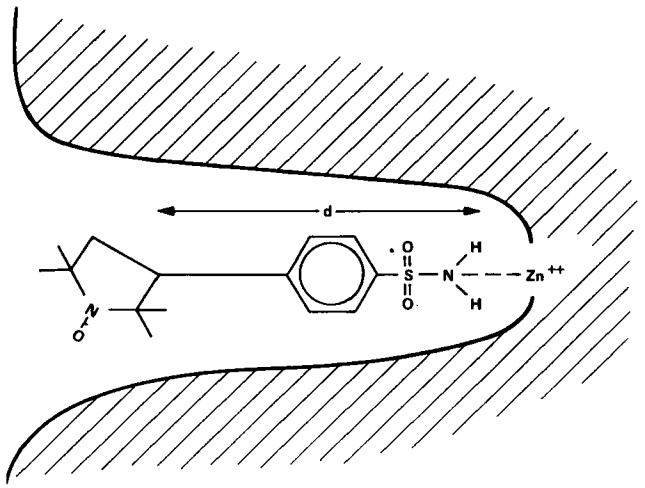


Figure 3—*Active site of human carbonic anhydrase C, showing the binding of a spin-labeled sulfanilamide analog.*

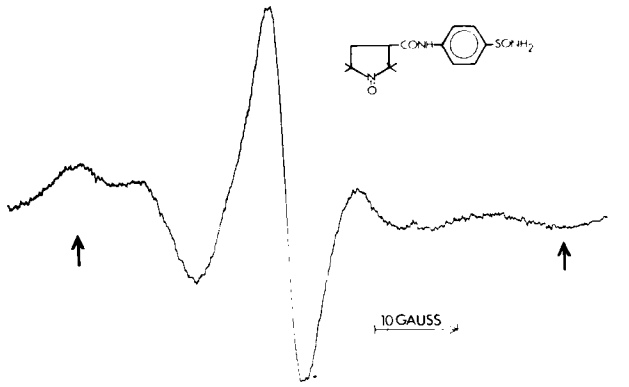


Figure 4—*Electron spin resonance spectrum of II (2×10^{-5} M) in the presence of human erythrocyte carbonic anhydrase C (1×10^{-4} M) and 0.1 M sodium phosphate buffer. The arrows indicate the positions of the low (left) and high (right) field peaks which are characteristic of a highly immobilized nitroxide radical* (cf., *Fig. 2E*).

The second way in which spin labels can be used to study drug interactions with biological systems is to incorporate the nitroxide group into the drug molecule. This approach has been used to probe the active site of erythrocyte carbonic anhydrase, a zinc-containing metalloenzyme which is inhibited by aromatic sulfonamide drugs with the general formula $ArSO_2NH_2$ (where Ar is homoaromatic or heteroaromatic).

X-ray diffraction studies of human carbonic anhydrase C have shown (7, 8) that the active site of this enzyme is a deep crevice, at the bottom of which is a single zinc atom (Fig. 3). When 3-acetoxymercuri-4-aminobenzenesulfonamide combined with human carbonic anhydrase C, the aromatic group of the inhibitor occupied a narrow cleft in the active site while the sulfonamide group was coordinated directly to the zinc through its nitrogen atom or one of the oxygen atoms (7). The topography of the active site of human carbonic anhydrase C and two other erythrocyte carbonic anhydrases was studied using a series of spin-labeled analogs of the inhibitor sulfanilamide (II–VI) in which the length of the chain separating the aromatic and pyrrolidine rings was progressively increased (Fig. 3 and Table I).

Before an electron spin resonance study of the interaction of the sulfonamide spin labels with human carbonic anhydrase C was attempted, it was necessary to establish that these compounds did indeed bind to the active site by measuring their ability to inhibit the enzymatic activity of the enzyme. Although all spin-labeled

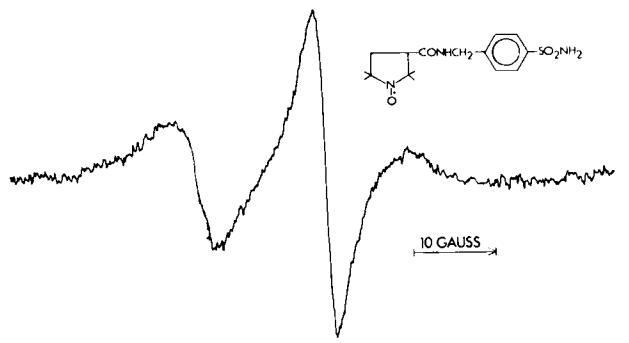
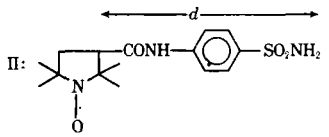
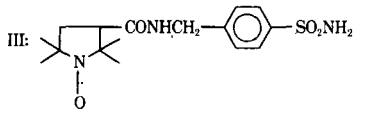
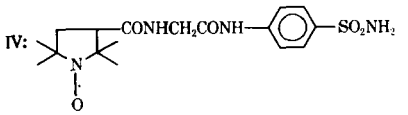
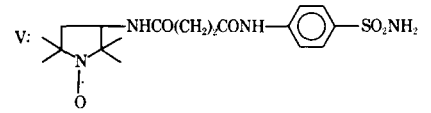
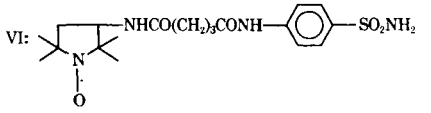


Figure 5—*Electron spin resonance spectrum of III (2×10^{-5} M) in the presence of human erythrocyte carbonic anhydrase C (1×10^{-4} M) and 0.1 M sodium phosphate buffer.*

Table I—Inhibition of the Esterase Activity of Human Erythrocyte Carbonic Anhydrases B and C by Spin-Labeled Sulfonamides

Spin Label	K_I, $M \times 10^6$			d^a, Å
	Bovine Carbonic Anhydrase B	Human Carbonic Anhydrase B	Human Carbonic Anhydrase C	
II: 	0.02	0.33	0.08	10.0
III: 	0.42	0.26	1.89	10.8
IV: 	2.71	2.78	0.58	13.6
V: 	0.78	3.94	1.11	14.5
VI: 	—	—	—	15.6

[a] Determined with Pauling–Corey–Koltun models for the fully extended conformation of each sulfonamide spin label.

sulfonamides did inhibit the ability of human carbonic anhydrase C to hydrolyze *p*-nitrophenylacetate (9), the kinetics of the interaction were noncompetitive in all cases (Table I). While this suggested that the sulfonamides did not bind to the active site of the enzyme, similar noncompetitive kinetics had already been reported for the inhibition of the esterase activity of erythrocyte carbonic anhydrase by other sulfonamides such as acetazolamide (9). Yet it is known, from X-ray diffraction studies, that these drugs bind to the active site of human carbonic anhydrase C (7, 8). Kernohan (10) suggested that the apparent noncompetitive nature of the kinetics is due to a relatively slow rate of dissociation of the sulfonamide from the active site of a carbonic anhydrase.

The electron spin resonance spectrum of II bound to human carbonic anhydrase C was broad and asymmetric, with a splitting of 59 G between the low and high field peaks (Fig. 4). Since this spectrum resembled the rigid glass spectrum of the nitroxide group (Fig. 2E), there was little doubt that the pyrrolidine ring of II was highly immobilized when this inhibitor was bound to human carbonic anhydrase C. When a single methylene group was inserted between the benzene ring and the aromatic amino group of II to give III, the nitroxide radical showed some increase in mobility at the active site of human carbonic anhydrase C (Fig. 5).

In the complex between IV and human carbonic anhydrase C, the nitroxide group exhibited considerable freedom of movement at the active site of the enzyme (Fig. 6). A further increase in the distance between the aromatic and pyrrolidine rings, to give V, did not greatly change the electron spin resonance spectrum of the spin label (Fig. 7). In their spin-label study of hapten binding to rabbit immunoglobulins, Hsia and Piette (11) obtained electron spin resonance spectra that were similar to these shown in Figs. 6 and 7. They suggested that such spectra were characteristic of spin-labeled ligands bound to rather narrow combining sites in such a way that their nitroxide groups were just outside the sites. The mobilities of the nitroxide groups shown in Figs. 5–7 can be expressed in terms of their respective rotational correlation times (12). The rotational correlation times of the nitroxide groups of III–VII reached a minimum with IV, which did not change when the chain joining the aromatic and pyrrolidine rings was further extended (Fig. 8).

Further studies showed that the pyrrolidine ring of II is also highly immobilized when this inhibitor binds to the active site of human erythrocyte carbonic anhydrase B and bovine erythrocyte carbonic anhydrase B (14). However, when the chain length separating the aromatic and pyrrolidine rings was increased, V was the first spin label of the series in which the nitroxide group showed high mobility at the active site of human carbonic anhydrase B. While similar results were obtained for bovine carbonic anhydrase B, it was also found that the nitroxide group of VI had less mobility at the active site of this enzyme than did the nitroxide group of V (Fig. 8). This finding suggested that VI may have been sufficiently flexible as to allow the pyrrolidine ring to turn back and interact with either the active site of bovine carbonic anhydrase B or an accessory binding site on the surface of the enzyme (14).

The electron spin resonance spectrum of II bound to human carbonic anhydrase C indicated quite clearly that the nitroxide group of the inhibitor was highly immobilized at the active site of the enzyme. This observation suggests that the active site of human carbonic anhydrase C is a narrow cleft and is in agreement with X-ray diffraction data of Bergstén *et al.* (8). Sulfonamide IV was the first

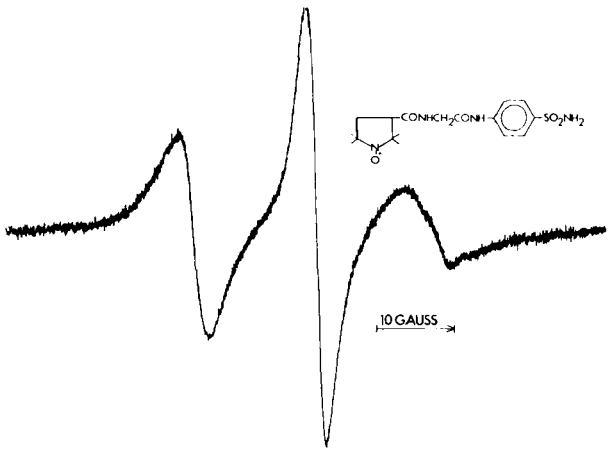


Figure 6—*Electron spin resonance spectrum of IV* (2×10^{-5} M) *in the presence of human erythrocyte carbonic anhydrase C* (1×10^{-4} M) *and 0.1 M sodium phosphate buffer.*

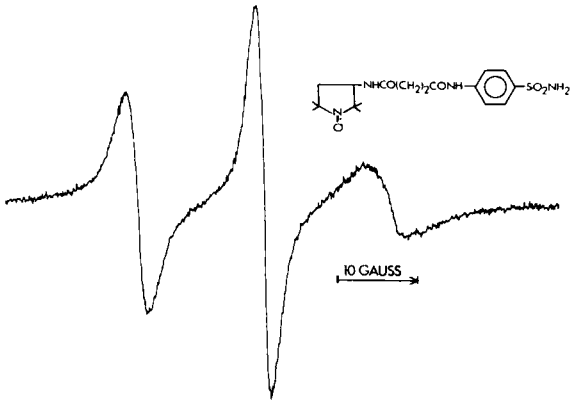


Figure 7—*Electron spin resonance spectrum of V (2×10^{-5} M) in the presence of human erythrocyte carbonic anhydrase C (1×10^{-4} M) and 0.1 M sodium phosphate buffer.*

of the spin labels in which the pyrrolidine ring appeared to be outside the active site of human carbonic anhydrase C.

The distance, *d*, between the nitrogen atom of the sulfanilamide group and the 3-position of the pyrrolidine ring can be estimated from Pauling–Corey–Koltun models to be 13.8 Å for the fully extended conformation of IV (Table I). This would set an upper limit of 14 Å for the depth of the active site of human carbonic anhydrase C. This value is in good agreement with an estimate of 15 Å obtained from X-ray diffraction studies (8). Although the active sites of human carbonic anhydrase B and bovine carbonic anhydrase B appear to have the same shape as human carbonic anhydrase C, they probably are slightly deeper.

These studies have shown that spin-labeled sulfonamides can be used to study the topography of the active site of erythrocyte carbonic anhydrase. It is hoped that spin-labeled analogs of other drug molecules, such as acetylcholine and the barbiturates (15), may make it possible to investigate their interactions with receptor biomolecules. The spin label technique also permits the rapid measurement of low concentrations of sulfonamide drugs present in biological fluids (16). A simple assay procedure for the estimation of carbonic anhydrase levels in red cell hemolysates has also been developed (15) and employs a spin-labeled sulfonamide. There now seems to be little doubt that electron spin resonance and the spin-labeling technique will not only be important tools for studying drug–biomolecule interactions at a molecular level but also may provide a versatile and sensitive assay procedure for drugs, enzymes, and other binding proteins.

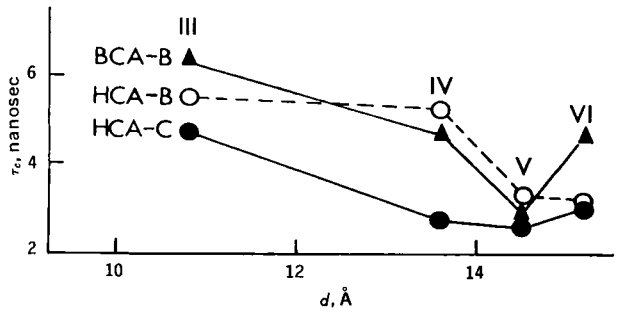


Figure 8—*Rotational correlation times, τ_c, of III–VI as a function of the distance, d, between the sulfonamide nitrogen atom and the point of attachment of the pyrrolidine ring (Fig. 3 and Table I).*

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Drug–Biomolecule Interactions: Interaction of Gentamicin with Lipid Monomolecular Films

DAVID E. AUSLANDER ˣ, ALVIN FELMEISTER †, and BARTLEY J. SCIARRONE

Abstract □ The interaction of gentamicin with monomolecular films of a series of biologically important lipids spread on an aqueous buffered subphase was studied. The surface pressure, π, of these films was determined by the Wilhelmy plate method as a function of surface area, A, and π–A curves were constructed. Changes in the π–A characteristics in the presence of gentamicin were used as a measure of antibiotic–film interaction. No interaction was observed between gentamicin and films of cholesterol, egg lecithin, dipalmitoyl lecithin, phosphatidyl ethanolamine, stearyl alcohol, and bovine ceramides at all pH values studied. Stearic acid films showed no interaction with gentamicin at pH 5. At pH 7 and 8, a small increase in pressure (~3 dynes/cm) was noted. A dramatic increase in surface pressure was observed in the presence of stearyl aldehyde films ranging from approximately 9 dynes/cm at pH 7.2 to 23 dynes/cm at pH 8.4. This effect was attributed to a Schiff-base reaction between the nonprotonated primary amino groups on the gentamicin molecule and the stearyl aldehyde. Further evidence was provided by the fact that the addition of glucose (which has been reported to participate in Schiff-base formation with amines) to the subphase inhibited the stearyl aldehyde–gentamicin interaction. Sucrose did not show a corresponding effect. The addition of sodium bisulfite, which reacts with aldehydes to form α-hydroxysulfonic acid, also inhibited the gentamicin–stearyl aldehyde interaction. It is postulated that Schiff-base formation is a step in the *in vivo* transport of gentamicin across the membrane of sensitive organisms.

Keyphrases □ Gentamicin—interaction with lipid monomolecular films, surface pressure, π–A curves □ Monomolecular films—interaction of biologically important lipids with gentamicin, surface pressure, π–A curves □ Films, monomolecular—interaction of gentamicin with lipids □ Drug–biomolecule interactions—gentamicin with monomolecular films □ Interactions—drugs with biomolecules, symposium

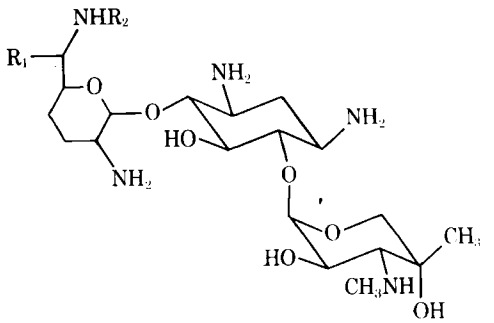
Gentamicin is a water-soluble aminoglycoside antibiotic derived from the micromonospora family and is active against both Gram-negative and Gram-positive bacteria. The gross structures of the gentamicin components (I) were recently reported (1). The biological activities of the three gentamicin components are essentially identical (2).

Gentamicin, in common with the other members of the kanamycin family, exhibits some interesting biophysical properties. It does not transport to any significant degree across the membranes of the GI tract, as evidenced by the fact that less than 2% absorption has been observed following oral administration[1]. In contrast, gentamicin does cross some specialized membranes; *e.g.*, it penetrates the aqueous humor of the eye (3), passes the placental barrier and into the amniotic fluid (4), and transports across the otic membrane (5). Furthermore, since the site of gentamicin antibacterial action is at the ribosomal level (6), it clearly must penetrate the bacterial membrane.

The mechanism by which gentamicin transports across bacterial and other membranes, however, has not been firmly established. Its solubility characteristics and its negligible oil–water partition coefficient (7) preclude dissolution or partitioning into lipoidal membranes. Its relatively large molecular size precludes transport through water-filled pores or channels, as has been postulated for some simple cations and anions. Therefore, it appears that its transport must involve, at least as a first step, some specific interaction with a cell membrane component.

Studies with other aminoglycoside antibiotics in which an interaction with bacterial cell membranes has been demonstrated (8) support this postulation. However, the specific sites on the membrane involved in the antibiotic–cell membrane interaction have not been identified, nor has the role of specific membrane lipids in this process been established.

This investigation examined the potential participation of membrane lipids in the transport of gentamicin across cell membranes. Monomolecular films were selected as the membrane model, because they provide a relatively simple, well-defined, oriented structure with some similarity to biomembranes. In addition, they allow for modification of composition of both the "membrane" (*i.e.*, the film) and the subphase on which the film is spread. Furthermore, numerous studies have demonstrated that there is a correlation between the interaction of many drugs with monomolecular films and their biological activity (9).



I

C_1: $R_1 = R_2 = CH_3$
C_{1A}: $R_1 = R_2 = H$
C_2: $R_1 = CH_3$, $R_2 = H$

[1] D. Cooper, Schering Corp., Bloomfield, N.J., personal communication, 1970.

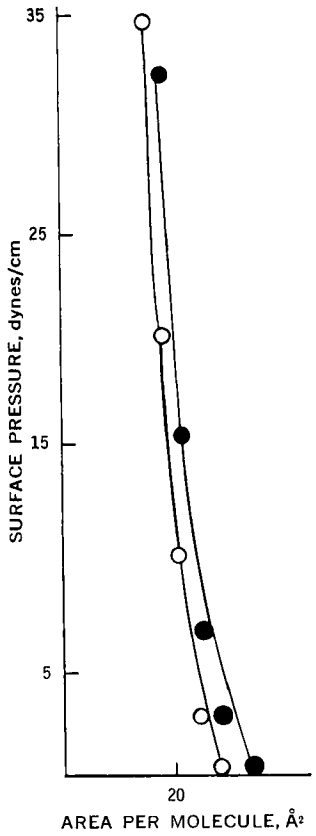


Figure 1—*Surface pressure–surface area curves of stearic acid at pH 7.0, alone* (○) *and in the presence of 1* × 10^{-3} M *gentamicin* (●).

EXPERIMENTAL

Gentamicin base[2] was obtained as a mixture of active components C_1, C_{1A}, and C_2. Stearyl aldehyde[3] (purity >99%) was obtained as a solution in carbon disulfide. The stearic acid[4], stearyl alcohol[4], cholesterol[3], L-α-dipalmitoyl lecithin[4], and egg lecithin[5] were obtained chromatographically pure. Cerebrosides[6], ceramides[6], and phosphatidyl ethanolamine[3], extracted from bovine brain, and sucrose dipalmitate[7] were used as obtained. Dextrose USP and sucrose USP were crystallized from boiling water. All inorganic chemicals used were reagent grade, and all organic solvents were of spectrophotometric grade. The water used was prepared by double distillation from an all-glass apparatus.

The film balance used to study the surface pressure–surface area (π–A) characteristics of the films was described previously (10).

The temperature of the subphase was maintained at 25 ± 0.1° by circulating water from a constant-temperature bath through a water jacket around the trough.

The lipids were placed on the surface of the subphase, using a micrometer syringe[8] capable of delivering volumes as small as 0.001 ml. The spreading solvent for the lipids was *n*-hexane, except that benzene was the spreading solvent for sucrose dipalmitate, cerebrosides, and ceramides. To allow for complete evaporation of the spreading solvent, 5 min was allowed before manual compression was initiated. After each compression, 15 sec was allowed to elapse before the next surface pressure measurement was taken.

Mixed monomolecular films of various mole fractions of egg lecithin and stearyl aldehyde were prepared by adding the appropriate quantities of each component to the surface. The number of film molecules was kept constant.

The interaction of gentamicin with the films was studied by the following procedures. Gentamicin (10^{-3} or 10^{-4} *M* concentration) was dissolved in the subphase (0.01 *M* phosphate buffer at constant ionic strength and various pH values), and the various films were spread over this subphase. Differences in the π–A characteristics of pure films and films in the presence of drug were determined and used as a measure of drug–film interactions.

[2] Schering Corp., Bloomfield, N.J.
[3] Applied Science Laboratories, State College, Pa.
[4] Mann Research Laboratories, New York, N.Y.
[5] Sylvana Laboratories, Millburn, N.J.
[6] P-L Biochemicals, Milwaukee, Wis.
[7] Pfaltz & Bauer, Flushing, N.Y.
[8] Agla.

RESULTS AND DISCUSSION

Cholesterol and the phospholipids, egg lecithin, L-α-dipalmitoyl lecithin, and phosphatidyl ethanolamine, represent components that constitute a major fraction of the lipid constituents of cell membranes. None of these materials, when spread as monomolecular films, showed any significant interaction with gentamicin (1 × 10^{-4} or 1 × 10^{-3} *M* concentration) over a pH range of 4–8.4. Bovine ceramides and stearyl alcohol also exhibited no interaction with the antibiotic under similar conditions.

These data demonstrate that gentamicin does not interact nonspecifically with lipid films and support the contention that gentamicin does not transport into bacterial membranes *via* a partitioning mechanism.

Since gentamicin is a base, the interaction with an acidic film was explored next. Stearic acid was selected as the model system. At a gentamicin concentration of 1 × 10^{-4} *M*, no interaction was observed with the stearic acid film over a pH range of 5–8. At 1 × 10^{-3} *M*, an increase of about 3 dynes/cm at all areas per molecule was observed at pH 7 (Fig. 1) and similarly at pH 8. No interaction was observed at lower pH values. Thus, it appears that, as the ionization of gentamicin is partially depressed within the physiological pH range, some film–antibiotic interaction does occur.

The natural cerebrosides, which are mixtures of galactolipids, also exhibited an interaction with 1 × 10^{-3} *M* gentamicin (Fig. 2) of about the same magnitude as was observed with the stearic acid film. Since the cerebrosides differ from the previously studied ceramides (which showed no interaction) only by the addition of a galactose sugar moiety, it appears that the observed interaction is specific and is dependent on the presence of this sugar residue.

However, when sucrose dipalmitate was spread, no interaction with gentamicin was observed. This finding indicates that the free aldehydic function of the galactose residue is essential for the interaction.

To investigate this possibility further, the interaction of gentamicin with stearyl aldehyde films was studied. The π–A curves of stearyl aldehyde films spread on a subphase in the absence of gentamicin were identical throughout the pH range of 6–8.4. In the presence of 1 × 10^{-3} *M* gentamicin, a dramatic interaction was observed, and the extent of this interaction increased regularly with increasing pH (Fig. 3). A similar, but reduced, interaction was observed at 1 × 10^{-4} *M*.

Thus, it is evident that the reaction not only is dependent on the

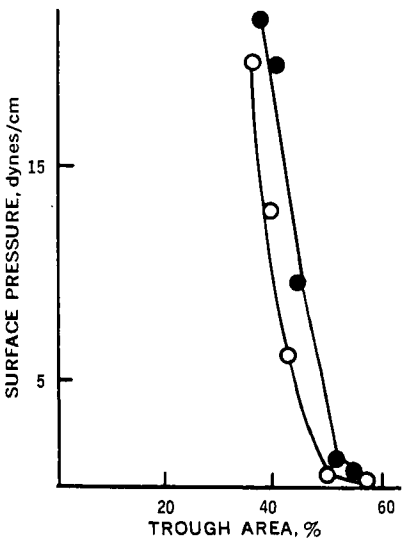


Figure 2—*Surface pressure–percent trough area curves of cerebrosides at pH 7.0, alone* (○) *and in the presence of 1* × 10^{-3} M *gentamicin* (●).

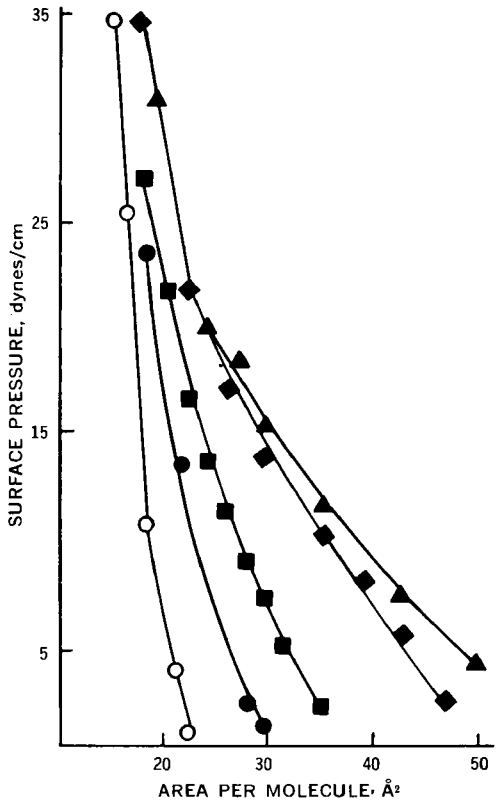


Figure 3—*Surface pressure–surface area curves of stearyl aldehyde, alone at pH values of 6.0–8.4* (○) *and in the presence of 1 × 10⁻³ M gentamicin at pH values of 7.2* (●), *7.6* (■), *8.0* (◆), *and 8.4* (▲).

presence of an aldehydic function in the film but also on the degree of protonation of the primary amino groups on the gentamicin and that the observed film–antibiotic interaction is the result of a Schiff-base interaction between these two functional groups.

The addition of glucose (1×10^{-4} *M*) into the subphase partially inhibited the film–antibiotic interaction while the addition of sucrose did not. This effect apparently was the result of a competition between the stearyl aldehyde and the aldehydic function of the glucose for the dissolved gentamicin. Sucrose, which does not have an available aldehydic function, cannot participate in this competition.

Sodium bisulfite, when added to the subphase, also inhibited the gentamicin–film interaction, apparently because of its ability to interact with the stearyl aldehyde *via* the formation of an α-hydroxysulfonic acid.

While free fatty aldehydes have been identified as natural components of various tissues (11–14), it is likely that they constitute only a small fraction of the lipid portion of cell membranes. Therefore, the effect of the addition of an "inert" lipid to the monomolecular film on the gentamicin–aldehyde interaction was determined. Egg lecithin was selected since it had shown no interaction with gentamicin and was representative of the phospholipid fraction of biomembranes. Mixed films of stearyl aldehyde and egg lecithin (mole fraction ratios of 3:1, 1:1, and 1:3) were spread, and the interaction with gentamicin was determined.

It can be seen in Fig. 4 that at a mole fraction ratio of 3:1 the interaction of gentamicin appears to be reduced over that observed with the stearyl aldehyde alone, as evidenced by the relatively small expansion of the π–A curve at low pressures. At high pressures, a condensation effect can be seen, suggesting that material is being lost from the interface. At a 1:1 mole fraction ratio, some expansion is also observed at low pressure, although less than that observed for the 3:1 system. At high pressures, however, the condensation effect is more pronounced.

At a 1:3 mole fraction ratio (Fig. 5), the condensation effect is still more pronounced and occurs at all surface pressures. Furthermore, it is most evident at the higher pH value (pH 8.0), a condition under which the gentamicin–stearyl aldehyde interaction approaches a maximum.

Thus, it appears that egg lecithin does not inhibit the gentamicin–aldehyde interaction but rather influences the ability of the resultant Schiff base to remain at the interface. This effect may be the result of the difference in the comparative strength of the interaction between neighboring stearyl aldehyde molecules and between neighboring stearyl aldehyde and egg lecithin molecules. Thus, if the latter interaction is weaker than the stearyl aldehyde–stearyl aldehyde interaction, the increase in hydrophilicity of the aldehyde as the result of its reaction with gentamicin may lead to the loss of the product (Schiff base) from an area-determining position at the interface.

CONCLUSIONS

The data demonstrate that gentamicin does not interact nonspecifically with cholesterol and phospholipid films and that it does interact with aldehydic films *via* Schiff-base formation. In a membrane composed essentially of cholesterol and phospholipids and interspersed with small amounts of fatty aldehydes, these latter compounds would serve as a site at which a gentamicin–membrane interaction might occur. Under such conditions, the increased hydrophilicity of the product (Schiff base) and the relatively weak interaction between the aldehyde and other membrane lipids would lead to desorption of the Schiff base from the membrane. This would result in the formation of pores through which intracellular material could leak out and unreacted gentamicin could enter and interact with the ribosomes within the cell.

While it is recognized that a physical model in itself cannot unequivocally elucidate the mechanism of action in a biological system, supporting data for this Schiff-base mechanism can be found in the literature. For example, alkalinization of urine promotes the activity of gentamicin (15), while *n*-alkylation of aminoglycoside

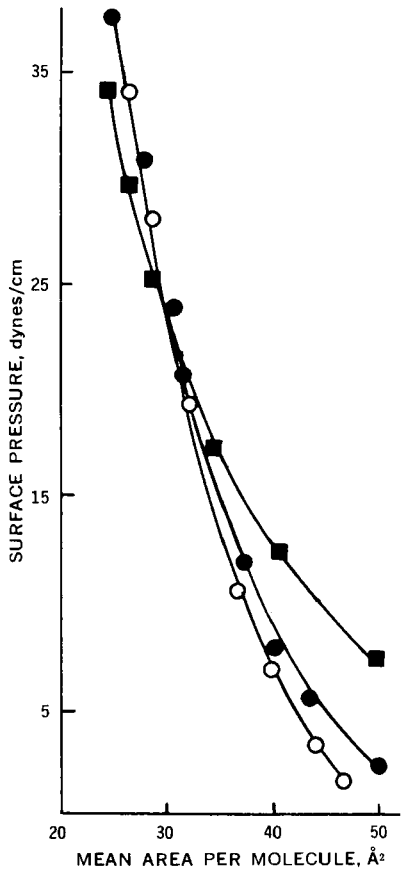


Figure 4—*Surface pressure–mean area per molecule of stearyl aldehyde plus egg lecithin at a 3:1 mole fraction ratio, alone* (○) *and in the presence of 1 × 10⁻³ M gentamicin at pH 7.0* (●) *and 8.0* (■).

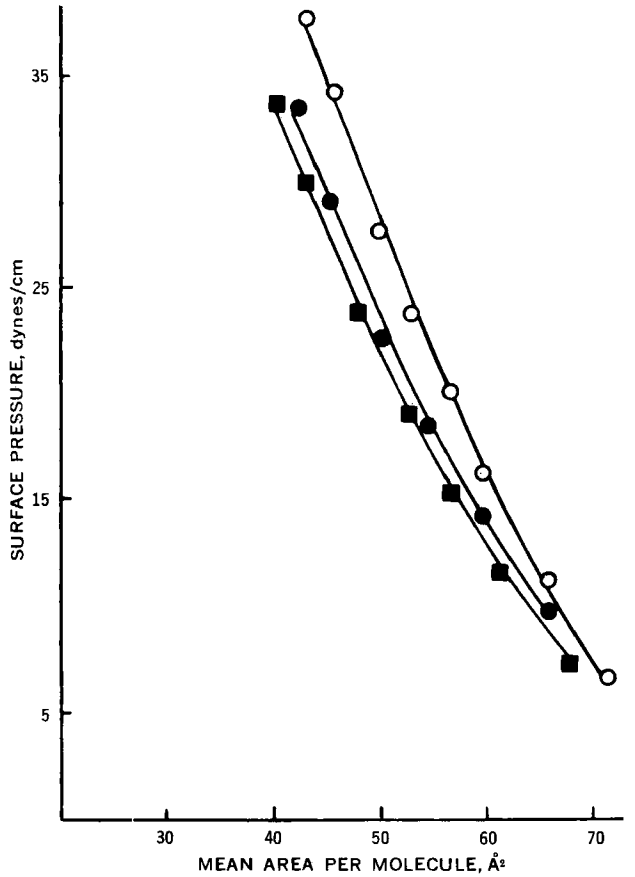


Figure 5—*Surface pressure–mean area per molecule of stearyl aldehyde plus egg lecithin at a 1:3 mole fraction ratio, alone* (○) *and in the presence of 1 × 10⁻³ M gentamicin at pH 7.0* (●) *and 8.0* (■).

antibiotics dramatically reduces their activity (16). Both of these observations correspond with the fact that only an undissociated primary amino group can participate in a Schiff-base reaction and with the postulation that such a reaction is necessary for gentamicin activity.

In addition, recent work in these laboratories (17) demonstrated that the addition of a small amount of sodium bisulfite to the growth media of *Escherichia coli* reduces the sensitivity of these organisms to gentamicin. It was postulated that this effect was the result of an interaction between the bisulfite and membrane aldehydes, which made these aldehydes less available to attack by gentamicin.

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Drug–Biomolecule Interactions: Bioelectrometric Study of the Mechanism of Carbachol Interactions with the Cornea and Its Relation to Miotic Activity

VICTOR F. SMOLEN ˣ, CHUI S. PARK, and EDWARD J. WILLIAMS

Abstract □ The augmentation of carbachol miotic activity attributable to enhanced transcorneal absorption, which results from the action of cationic adjuvants included in ophthalmic vehicles, suggested a study of carbachol–corneal tissue interaction as a further step toward understanding the phenomenon. The present study was performed *in vivo* using an innocuous electrometric technique. A fixed charge density of the corneal epithelial surface *versus* carbachol concentration profile was obtained from the electrometric results; it revealed three distinct concentration regions defined by precipitous decreases of fixed charge over extremely small concentration ranges. This anomalous behavior is attributed to cooperative alterations in the binding affinities of fixed anionic sites on the tissue surface, which result in an all-or-none release of protons and/or other microcations. The unmasked anionic sites become reoccupied with carbachol except in the last region where the reoccupation by carbachol is competitive with other cations in the solution in contact with the surface. This behavior, postulated on the basis of the construction of a carbachol–tissue binding isotherm from which thermodynamic interaction affinities were computed, was corroborated by the observed dependency of the duration of miotic activity on carbachol concentration. Allosteric interactions between anionic binding sites, which are mediated through electron inductive and electrostatic field effects and likely involve a cooperative alteration in tissue water structure, are implicated as underlying the observed phenomena.

Keyphrases □ Carbachol—interactions with cornea, relation to miotic activity, mechanism, bioelectrometry □ Ophthalmic solutions—carbachol interactions with cornea, relation to miotic activity, mechanism, bioelectrometry □ Bioavailability—ophthalmic carbachol, interactions with cornea, relation to miotic activity, mechanism, bioelectrometry □ Drug–biomolecule interactions—mechanism of carbachol interactions with cornea, relation to miotic activity, bioelectrometry □ Interactions—drugs with biomolecules, symposium

The first report (1) in the present series described the considerable augmentation of carbachol miotic activity resulting from the inclusion of benzalkonium chloride, a cationic surfactant, or a dextran (DEAE), a cationic polyelectrolyte, in an ophthalmic vehicle. This phenomenon, first reported for benzalkonium chloride (2), was discussed briefly and attributed to a modulation of carbachol–tissue interaction by the cationic adjuvants resulting in a promoted biophasic availability of the drug. The adjuvants appear to be selective in their enhancement of the transcorneal availability of the drug to its site of miotic action without concomitantly increasing systemic absorption.

The present report describes details of a bioelectrometric investigation of the interaction of carbachol with the corneal epithelial surface of rabbits. The *in vivo* bioelectrometric technique (3–8) is unique in its ability to provide data that permit characterization of drug–tissue interactions in terms of kinetic and thermodynamic parameters (7, 10–13) under physiological, product-use, conditions. The technique often provides a unique means for obtaining such information.

EXPERIMENTAL

Materials—All reagents were analytical grade except for the carbachol[1] USP (carbamylcholine chloride). Pentobarbital sodium[2] and lidocaine hydrochloride[3] were used as general and local anesthetics, respectively. Filter paper wick material[4] was used for measuring electrical potentials[5]. Fiber junction, miniature, saturated calomel electrodes[6] were attached using Tygon rubber sleeves to the filter paper wicks, which were used for corneal measurements; a glass-sleeve-type electrode[6] was used as the reference electrode immersed in the 0.15 *M* NaCl reference solution. A micromanipulator[7] was used to apply the wicks to the corneal surface. The liquid junction potential measuring apparatus was described previously (8).

The composition of the carbachol-free buffer solutions employed in the pH titration experiments was reported earlier (5). Carbachol solutions[8] were prepared by the addition of carbachol to pH 6.95 phosphate buffer solutions. Conductance measurements were performed with a conductivity bridge[9]. The animals used were approximately 2-month-old male New Zealand white rabbits, 2.0–2.5 kg.

Miotic Response Monitoring—The details of the administration of ophthalmic drug solutions into the eyes of the rabbits and the recording of drug-induced time variations in the pupillary diameter were reported previously (1, 12–14).

Bioelectrometric Methodology—The previously described dilution potential method (3–5, 15), as contrasted to an equilibrium method or the dilution-potential–time extrapolation method (11), was utilized to determine the dependency of the fixed charge density of the corneal surface on the composition of applied experimental solutions. The fixed charge density arises from the dissociation of ionogenic groups covalently bonded to or sorbed onto the biocolloids constituting the tissue surface (14).

The net density of fixed charge is generally a sensitive function of the composition of the solution contacting the tissue. The verity of observed dilution-potential measurements in reflecting the fixed charge density has been confirmed by direct experimentation (8). The often direct relationships between observed changes in fixed charge density and the extent of solute binding to tissue surfaces have also been demonstrated (6, 7, 11).

Except for some minor modifications, the employed procedure is similar to the technique described earlier for the interaction of procaine hydrochloride with the cornea of guinea pigs (3). The experimental arrangement for the present study with rabbits is illustrated in Fig. 1. The rabbits were immobilized by slowly infusing a

[1] Alcon Labs., Inc., Fort Worth, Tex.
[2] Nembutal Sodium, Abbott Laboratories.
[3] Xylocaine Hydrochloride, Astra Pharmaceutical Co.
[4] Beckman, Catalog No. 320046.
[5] Sargent SR recorder.
[6] Beckman.
[7] Model UD-1, Narishige Scientific Instrument Laboratory, Tokyo, Japan.
[8] The pH of all solutions was measured with an Orion model 801 digital pH meter.
[9] Serfass.

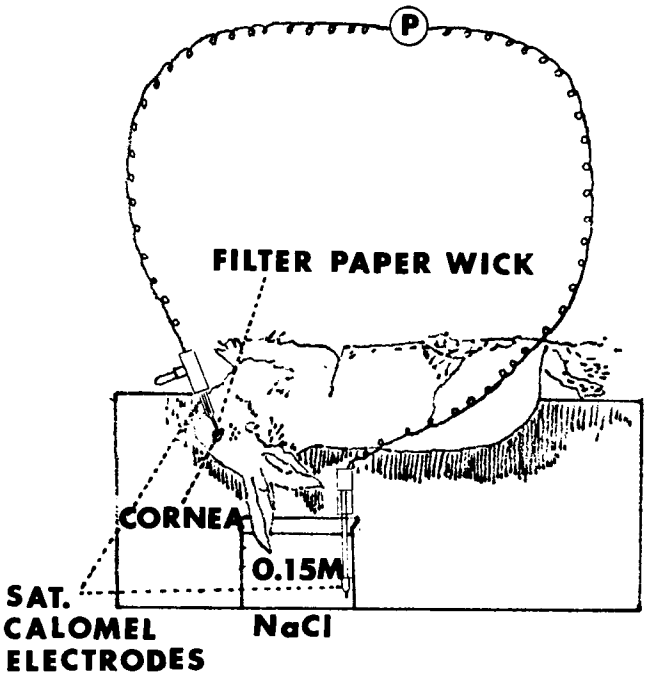

Figure 1—*Experimental arrangement for the bioelectrometric determination of the fixed charge density of the rabbit cornea.*

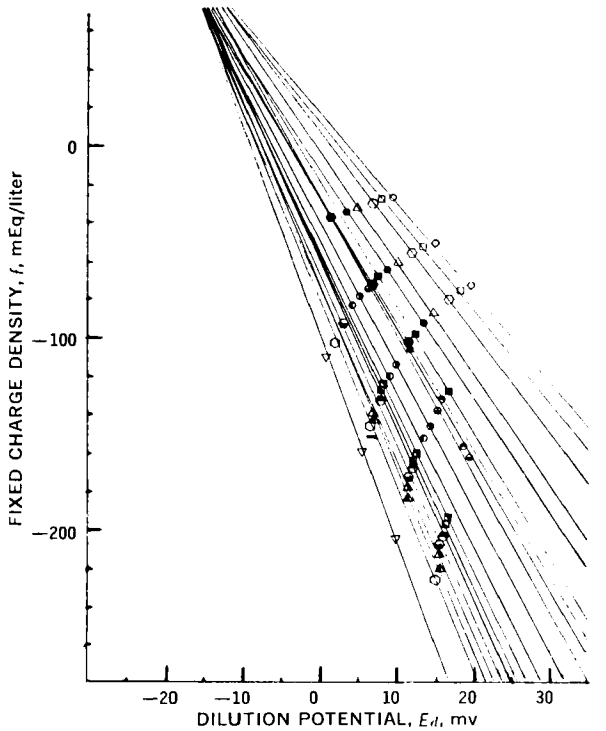

Figure 2—*Calibration plots for the transformation of experimentally observed dilution potential values into corresponding fixed charge densities. The curves relate to:* (○) *pH 6.95 buffer without carbachol and the same buffer containing varying carbachol concentrations,* i.e., (□) *0.0055*M, (○) *0.0137* M, (△) *0.0274* M, (●) *0.038* M, (■) *0.049* M, (◉) *0.055* M, (◓) *0.060* M, (◒) *0.066* M, (◑), *0.077* M, (◐) *0.088* M, (◔) *0.099* M, (◩) *0.104* M, (◓) *0.110* M, (◒) *0.115* M, (▲) *0.121* M, (◭) *0.129* M, (○) *0.137* M, *and* (▽) *0.164* M.

dilute solution (0.6% w/v) of pentobarbital sodium into a marginal ear vein. The required dose varied between 30 and 40 mg/kg.

Retrobulbar injection of 1 ml of a 1% (w/v) solution of lidocaine hydrochloride was administered to the anesthetized rabbits to diminish blinking and lacrimation and to permit freer access of the filter paper wicks to the cornea by causing the eye to protrude slightly. The retrobulbar injection of lidocaine did not alter the results of the corneal electrometric measurements. Each rabbit was allowed a minimum recovery period of 1 week between successive experiments in which the right and left eyes were used alternately.

As shown in Fig. 1, the rabbit's ear was depilated and immersed in a 0.15 *M* NaCl solution along with the saturated calomel reference electrode. This rabbit's ear served as a reference surface, and it was not disturbed during an experimental run. The dilution potential procedure for a particular solution consisted of thoroughly rinsing the cornea with buffer, blotting off the excess solution with cotton swabs and filter paper wicks, and contacting the center of the cornea with a filter paper wick which had been wetted with the experimental solution and attached to the fiber junction of an electrode.

Following contact of the solution-wetted wick with the cornea, a potential, E_1, was recorded until it became constant. For an eye thoroughly rinsed with buffer just prior to the measurement, constancy in E_1 was achieved almost immediately. Without further treatment of the eye, a second slightly smaller filter paper wick wetted with a 10-fold dilution of the experimental solution and attached to a second electrode precisely matched to the first (*i.e.*, asymmetry potentials measured between the electrodes with both immersed in 0.15 *M* NaCl solution were less than 0.1 mv) was placed within the area of the cornea covered during the measurement of E_1. A second potential, E_2, was recorded immediately upon contact of the dilute solution with the corneal surface. Each measurement of E_1 and E_2 was replicated with the same solution and its dilution; this procedure was repeated for all experimental solutions in a series.

Extreme care was exercised in the positioning of the solution-wetted wicks on the cornea and also in the swabbing of excess solution from the eye so as not to abrade the cornea or touch surrounding tissues. In each measurement, the micromanipulator was used to lower the wick slowly to the center of the cornea until electrical contact was made by pendular drops of solution adhering to the end of the wick rather than by the wick itself. Consistent adherence to this technique prevented abrasion of the cornea as well as position and movement effects on corneal potentials (16–18); such effects did not present a problem in the present study. The filter paper wick itself must not possess a fixed charge density which varies with the composition of its imbibed solutions since this can cause a considerable error in the results.

Whether a particular wick material is satisfactory or not can be determined from liquid junction potentials obtained with a strip of the wick material serving as a bridge between the experimental solutions and their 10-fold dilutions. The procedure for this determination is identical to that previously described for a strip of cation-exchange membrane material (8).

Conversion of Measured Dilution Potentials into Fixed Charge Densities—Theoretical expressions relating the difference in the two measured potentials, $(E_2 - E_1)$, termed the dilution potential, E_d, to the fixed charge density of the tissue surface for an applied experimental solution were presented previously (3, 7, 15). The equations are most conveniently solved graphically or through the construction of alignment charts.

Figure 2 presents the calibration curves used to relate experimental E_d values to corresponding values of fixed charge density for each pH 6.95 carbachol solution–10-fold dilution pair used. The curves are linear over the E_d range of interest; the slopes of the curves vary with the content of carbachol contained in their corresponding solutions. The limiting ionic conductance, λ_0, of the carbamylcholine cation required for construction of the calibration curves was determined both by conductance and liquid junction potential measurements to yield a nearly identical average value of 38.9 ohms^{-1} at 25° for carbachol concentrations ranging from 5.5 to 274 m*M* in the pH 6.95 buffer. Experimental methods for measuring λ_0 were described previously (8).

The verity of the application of the theoretical expressions for relating fixed charge density values to observed dilution potentials, *via* the calibration curves in Fig. 2, was confirmed by liquid junction potential measurements (3, 5). Fixed charge densities corresponding to dilution potentials measured with buffered solutions of varying pH, but not containing carbachol, were obtained using a previously reported alignment chart (5).

Relation of Dilution Potential Method to Other Corneal

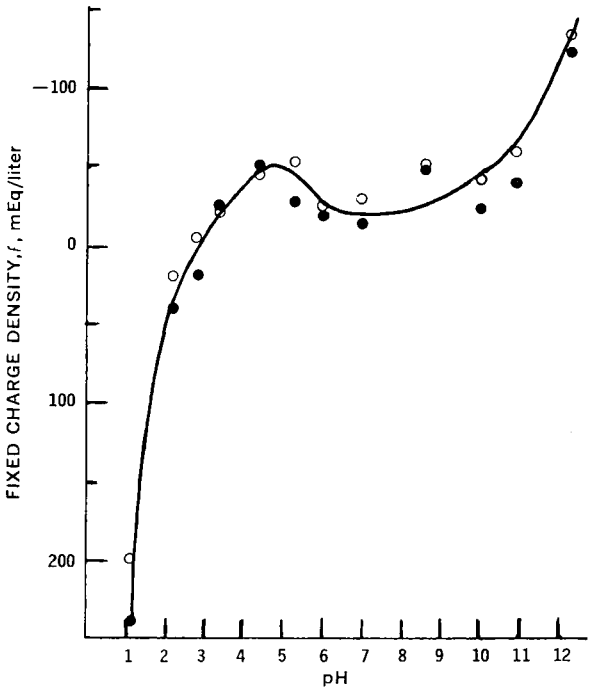

Figure 3—*Bioelectrometric pH titration curve of the corneal surface of rabbits. Key:* O, *forward titration; and* ●, *reverse titration.*

Potential Recording Techniques—In contrast to the transmembrane type of potentials recorded on the cornea by various other investigators (16–19), the dilution potentials determined in the present study are liquid junction potentials that develop across the interphase formed immediately upon contact between the experimental solution, with which the corneal surface has been allowed to equilibrate, and the 10-fold dilution of the same solution.

The E_d values depend only upon the composition of the solutions and the fixed charge characteristics of the tissue surface existing under the conditions of their application. Therefore, the E_d potentials obtained cannot be directly compared to corneal potentials reported by other investigators using other techniques which provide results that cannot be interpreted as readily (15–18). Caution is particularly required in comparing *in vivo* corneal potentials with results recorded from excised corneas. When using the dilution potential methods, differences become clearly evident. Details of the comparison of *in vivo* to *in vitro* electrometric results will be presented subsequently.

RESULTS AND DISCUSSION

pH Titration Curves of Corneal Surface—Figure 3 illustrates how the fixed charge density of the corneal surface varies with the pH of the applied solution; numerical values are presented in Table I. The plot in Fig. 3 is analogous to pH titration curves of amphoteric macromolecules in that it also describes proton ionization. It differs somewhat in that the shape of the curve also reflects the influence of other solutes in the experimental solutions applied to the tissue surface.

The variation in fixed charge density of the surface with increasing pH derives from the dissociation of protons from chemical groups covalently bonded to protein, nucleic acid, phospholipid, and mucopolysaccharide tissue constituents of the corneal surface. The variation is also affected by the binding of other ionic or uncharged solutes (present in the milieu of the tissue surface) to charged or neutral surface sites (3, 7, 15). The observed density of fixed charge at any time is a consequence of the interacting state of these biocolloids as a whole and is quite sensitive to the composition of the applied solutions.

Previous results from this laboratory describing the effects of surfactants (7, 15), hexachlorophene (5–7), and carcinogenic and inactive hydrocarbons (4) with human epidermis, as well as the interaction of procaine with the corneal surface (3), have been reported. Direct correlations between tissue binding (5, 7) or pharmacological activity (3) of the tissue-interacting solutes and observed changes in fixed charge density have been demonstrated.

Table I—Fixed Charge Density Values for the Forward (Low to High pH) and Reverse (High to Low pH) Hydrogen-Ion Titration of the Corneal Surface of Rabbits

pH	E_d, mv		Fixed Charge Density, f, ± SD, mEq/liter[a]	
	Forward	Reverse	Forward Titration	Backward Titration
1.12	−1	−8	+200 ± 46	+240 ± 30
2.20	−9	−14	+20 ± 10	+40
2.82	−9	−14	−5 ± 15	+20
3.39	−7	−6	−23 ± 3	−25
4.40	−2	−1	−44 ± 24	−51 ± 31
5.33	+6	+1	−53 ± 39	−28 ± 19
5.99	+8	+6	−24 ± 13	−19 ± 23
6.95	+9	+6	−29 ± 5	−14 ± 15
8.60	+5	+4	−51 ± 22	−49 ± 22
10.07	−3	−7	−42 ± 9	−23 ± 16
10.91	−2	−5	−60	−40
12.30	−8	−10	−135	−125

[a] The values are averages of a minimum of eight replications except for single determinations which are listed without standard deviations.

The fixed charge densities plotted as a pH titration curve in Fig. 3 appear to be independent of whether the measurements were initiated at low or high pH; a forward titration curve (pH 1.12–12.30) was not statistically different from the reverse curve (pH 12.30–1.12) at a $p \leq 0.05$ confidence level as determined using a paired t test. However, an opacity of the cornea with an accompanying red coloration and mucous lacrimation usually appeared at low (1.12–3.39) and at high (10.07–12.30) pH during the electrometric titration. It has been reported that the cornea can be severely damaged at pH 11 (21).

In view of these changes in the cornea observed at the extremes of pH, the apparent nonhysteretic nature of the forward and reverse titration curves was unexpected; the fixed charge density in the past has been shown to be a sensitive means of detecting changes in the structure and properties of tissue surfaces. The apparent failure of the bioelectrometric results to exhibit hysteresis in the acid–base binding characteristics of the cornea may be attributed to the following causes:

1. Whatever changes do occur are reversible.
2. Irreversible changes occur at depths within the cornea below those for which the electrometric results reflect the state of tissue colloids; for epidermis, the depth is approximately 3–4 μm (6, 7). Although a similar determination was not performed for the cornea, it may be assumed that the stratum corneum of the epidermis is appreciably more porous than the unkeratinized corneal epithelium and that the corneal tissue depth represented electrometrically in the present case is much less than that estimated for epidermis.
3. The damage to the corneal epithelium is the same for both acidic and alkaline pH extremes. The difference in the fixed charge density at pH 6.95, given in Table I as 29 ± 5 mEq/liter, compared to the value of 70 ± 13 mEq/liter (Table II), which was observed for corneas not previously treated with solutions other than pH 6.95, supports this conclusion.

When referring to Fig. 3, it is apparent that the isoelectric point of the corneal surface is in the vicinity of pH 3.0. At the isoelectric pH, the number of basic, fixed cationic groups exactly balances the number of acidic, fixed anionic groups titrating below the isoelectric point. The relatively low isoelectric point of the corneal surface is largely attributable to the presence of sulfate groups on its mucopolysaccharide constituents, although some carboxyl groups are also titratable below pH 3.0 (22).

Since the lowest pH at which cationic groups on the surface start to deprotonate is above pH 5 and the anionogenic groups all may be assumed to be essentially protonated at pH 1.12 (23), the observed difference in fixed charge density, ≃200 mEq/liter, which is the difference between the charge density at pH 1.12 and that at the isoelectric pH, is assumed to represent the density of fixed cationic sites on the tissue surface.

Table II—Dependence of *In Vivo* Corneal Surface Fixed Charge Density, *f*, on Carbachol Concentration in pH 6.95 Phosphate Buffer as Determined Electrometrically

Carbachol Solution Molarity	Carbachol Molarity at Corneal Surface, $r^Z M$[a]	E_d,[b] mv	$f \pm SD$, mEq/liter	Δf[c], mEq/liter
0 (buffer)	0	+19	−70 ± 13	0
0.0055	0.0069	+16	−67 ± 14	+3
0.0137	0.017	+14	−64 ± 12	+6
0.0274	0.032	+10	−58 ± 9	+12
0.038	0.044	+6	−50 ± 13	+20
0.049	0.054	+3	−37 ± 11	+30
0.055	0.058	0	−25 ± 18	+45
0.060		+5	−66 ± 17	+4
0.066	0.088	+12	−117 ± 12	−47
0.077	0.090	+4	−67 ± 19	+3
0.088	0.098	0	−50 ± 16	+20
0.099	0.108	−2	−42 ± 21	+28
0.104	0.100	−3	−34 ± 26	+36
0.110	0.111	−7	−1 ± 33	+69
0.115		−2	−54 ± 24	+16
0.121	0.125	+4	−109 ± 20	−39
0.129	0.145	−1	−65 ± 28	+5
0.137	0.153	−2	−61 ± 35	+9
0.164	0.173	−6	−37 ± 29	+33

[a] $Z = 1$ for carbamylcholine cation. [b] Average values from 28 determinations. [c] Δf = *f* (carbachol) − *f* (buffer).

It can be seen in Fig. 3 that the net fixed charge density is anionic above pH 3.0, passing through a shallow minimum at approximately pH 6.5. The apparent minimum in the curve may at first appear quite anomalous since it indicates that an increase in the cationic charge density of the surface occurs with increasing pH over the 4.5–6.5 pH range whereas deprotonation of the surface, as may ordinarily be expected to occur with increasing pH, causes the surface charge density to decrease. This same behavior has also been observed in this laboratory with titration curves obtained *in vivo* on rabbit intestinal mucosa and frog skin. Less physiologically active tissue surfaces such as epidermis and connective tissue, which do not actively transport ions across their single or multicellular barriers, exhibit this effect to a minor degree or not at all (5, 7, 15).

Presently unpublished results of additional studies with frog skin and the mucosal surface of the lower third of the rabbit duodenum have shown that the anomaly in the titration curves of these tissue surfaces is due to the binding of Na^+ ions, which occurs in increasingly larger concentrations in the higher pH buffer solutions, to the tissue surfaces at a rate exceeding the rate of deprotonation of fixed anionogenic surface sites. Experiments at fixed pH have revealed a similar U-shaped response in anionic fixed charge density to increasing concentrations of Na^+ ions, indicating that the Na^+ ions themselves can trigger their own release from tissue-binding sites.

A mechanism involving "cardinal" Na^+ binding sites, whose extent of occupation governs the affinity of "gangs" of other Na^+ binding sites by an allosteric, electron-inductive mechanism similar to that previously postulated (4–6, 15, 24–27) is hypothesized as being operative in this phenomenon and active Na^+ transport (25). A related behavior is demonstrated by the results shown here for carbachol.

Carbachol Interaction with Corneal Surface—Figure 4 contains plots of carbachol-induced changes in the fixed charge density of the rabbit cornea as a function of carbachol concentration; each point is the average of at least 28 replications performed with different rabbits. The data are summarized in Table II.

It is apparent from inspection of Fig. 4 that increases in the cationic fixed charge density of the surface with increasing carbachol concentration occur in three discrete concentration ranges. Each range is well delineated by a precipitous drop in the density of the fixed positive surface charge. Therefore, an unexpected phenomenon of a cationic sorptate interacting with an anionic sorbent to render it more anionic over a very small range of concentration is observed. In view of related phenomena reported from this laboratory, *e.g.*, hexachlorophene anion binding to a negatively charged epidermal surface which *reduces* its net anionic charge density (5, 6) and similar results from other laboratories (28), these apparently paradoxical carbachol effects on the cornea do not appear quite as unusual.

As previously reported (5), the effects of hexachlorophene anions on the fixed charge density of the epidermal colloids at pH 10 were attributed to the operation of electrostatic field effects in conjunction with inductive delocalization of electrons emanating from peptide nitrogen binding sites to increase the acid pK's of vicinal ionogenic protein side groups. The shift in pK results in an uptake of protons, as well as probably other mobile microcations, from the solution and produces a diminution in anionic fixed charge density.

A similar mechanism was postulated as being responsible for repression of the ionization of fixed anionogenic sites on skin that was observed to be induced by sodium lauryl sulfate (7). Predictive evidence for such hypotheses was presented in a later paper (20) in the form of a hexachlorophene anion-induced inversion of the specific electrical response behavior of the epidermal surface to Na^+ and K^+ ions. The present observed behavior of the fixed charge density of the corneal surface in response to carbachol interaction is most closely analogous to the influence of unionized hexachlorophene on the skin at lower pH (5) or of 3-methylcholanthrene (4); both agents manifest their effects in an increase in the net anionic fixed charge density that can be attributed to an inductive withdrawal of electrons from vicinal ionogenic sites, resulting either in their deprotonation or to their dissociation of other possible microcations with which the sites might also be occupied (6). Similar allosteric mechanisms have been postulated (24, 27, 29–31).

The ability of electron-inductive effects to be transmitted for appreciable distances through biopolymers such as proteins and nucleic acids possessing semiconductive properties has been convincingly postulated (29, 32, 33). The mechanisms postulated for the presently observed phenomena are also involved in solute-induced changes in the pK's of titratable groups of wool and soluble proteins and are manifested in lateral shifts in hydrogen bonding curves (34–36) as well as, for example, drug and calcium-ion-induced lateral shifts in the intracellular Na^+ and K^+ binding isotherms of muscle (37, 38).

With these principles in mind, the initial increases in cationic fixed charge density in each discrete carbachol concentration range (Fig. 4) can be attributed to direct electrostatic binding of carbachol cations to fixed anionic sites, similar to that demonstrated for various different cations binding to wool (39), plasma proteins (40), the epidermis (4, 41), and the cornea (3). In each

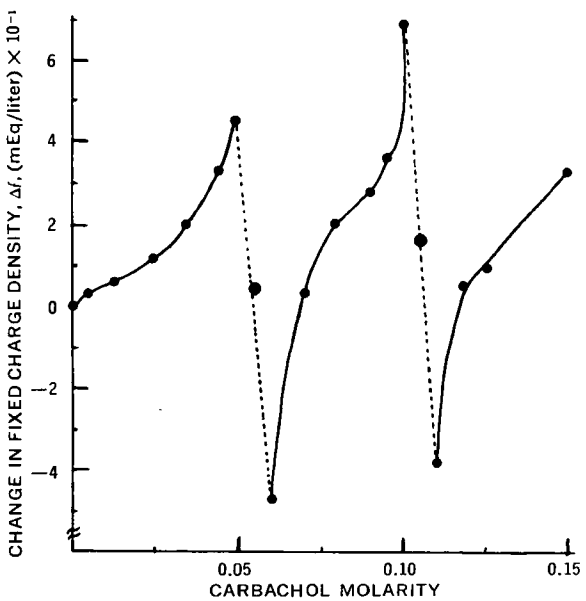

Figure 4—*Dependency of carbachol-induced changes in the fixed charge density of the corneal surface. The ordinal values are changes relative to the fixed charge density at pH 6.95 in the absence of carbachol. Each value is the average of 28 determinations performed on different rabbits.*

case, a long-range coulombic attraction has been postulated to be responsible for a sufficiently close approach of cationic solutes to anionic binding sites so as to cause electrostatic salt binding to occur and to be reinforced by secondary, short-range van der Waals forces.

The abrupt appearance of new, fixed, anionic tissue surface sites, which is observed when carbachol interacts with the cornea, may be attributed to a triggering of a cooperative (22, 29), inductively implemented, reduction in the electron charge densities of anionic sites transiently occupied by cations. These sites are vicinal to carbachol binding sites. The cooperative nature of the phenomenon may be compared to the analogous mechanism by which cationic surface-active agents denature proteins (32, 42). The carbachol-induced alterations of neighboring sites can be postulated to occur by electron withdrawal, resulting in a reduced affinity (decreased acidic pK) for binding protons and presumably other cations and depending on such factors as their hydrated diameters and relative concentrations (24, 43). This reduced binding affinity allows the release of the cations into the milieu and results in an abrupt increase in the negative fixed charge density of the tissue surface. The anionic sites vacated by the released cations become available for binding carbachol, with the process again being repeated when a sufficient density of these interaction sites becomes occupied to trigger another all-or-none type of cooperative transformation resulting in the release of additional cations.

Considering that *(a)* a carbachol-induced electron withdrawal from neighboring anionic sites causes a shift in the affinities of the sites so that they selectively prefer less hydrated, larger atomic radius ions such as K^+ or carbachol cations over Na^+ or H^+, for example, as predicted from the theory of Ling (43) and Eisenman (44) and *(b)* carbachol–salt binding can be reinforced by hydrophobic bonding and van der Waals interactions, it is reasonable to assume that carbachol cations have a decided competitive advantage over other cations in the environment with regard to reoccupying the vacated fixed anionic sites.

The proposal of Klotz (45), which implicates the presence of iceberg-like structured water on protein surfaces in the alteration of acidic pK values of ionic and ionogenic side-chain groups, lends additional insight into the mechanistic nature of carbamylcholine cation-induced cooperative changes in the fixed charge density. Structured tissue water is stabilized as water of hydrophobic hydration on nonpolar protein side-chain groups (44, 45) and peptide linkages (40). This structured water is responsible for increasing the acidic pK's of fixed ionogenic groups relative to their values in unstructured water (40); the presence of cationic quaternary ammonium groups, such as those found on carbachol, causes a disruption of the water structure (44, 47, 48) which, in turn, favors ionization of ionogenic groups.

The operation of a mechanism of this nature is possibly a contributing factor to the sudden unmasking of anionic corneal surface sites which delineate the various discrete carbachol concentration ranges in Fig. 4. The formation and disruption of structured water occur in an abrupt cooperative manner (48), which could account for the observed precipitous changes in fixed charge density which are suggestive of the involvement of a cooperative mechanism (49).

Construction of Carbachol–Tissue Sorption Isotherms from Bioelectrometric Data—Although the fixed charge density of the corneal surface at the pH of the carbachol solutions is anionic, the observable anionic fixed charge density is less than the actual density of anionic binding sites due to a partial masking of these sites by electrically compensating, fixed cationic sites. All anionic sites are postulated as being capable of participation in the drug–tissue interactions. If the binding of α cations gives rise to a change of one unit of fixed charge density, the interaction of cationic solutes with the corneal surface may be described by the equilibrium presented in Scheme I:

$$S^- + \alpha c^+ \rightleftharpoons Sc_\alpha$$

Scheme I

where α is an apparent stoichiometric coefficient. For direct electrostatic salt binding of cations to fixed anionic sites, α would have a value of unity. In cases of interactions that indirectly result in an altered fixed charge density, α may have almost any value and in fact can vary as a function of the concentration of the applied interacting solute.

Table III—Binding Data for Interaction of Carbachol in pH 6.95 Buffer with the Corneal Surface of Rabbits

Carbachol Solution Molarity	Maximum Number of Binding Sites	Stoichiometric Coefficient, α	Apparent Association Constant, K
$0–2.74 \times 10^{-2}$	275[a]	0.87	0.80
$3.8 \times 10^{-2} – 5.5 \times 10^{-2}$		3.2	5×10^2
$8.8 \times 10^{-2} – 1.11 \times 10^{-1}$	277[b]	4.7	3×10^4
$1.25 \times 10^{-1} – 1.73 \times 10^{-1}$	425[c]	1.9	36

[a] Δf_{∞_1} in milliequivalents per liter. [b] Δf_{∞_2}. [c] Δf_{∞_3}.

It can be assumed for the present that an unknown number, α, of carbamylcholine cations, c^+, reacting with each anionic binding site, S^-, to form a complex, Sc_α, is responsible for the reduction of 1 equivalent of fixed charge density; $1/\alpha$ equivalents of negative fixed charge are neutralized for each equivalent of cation bound to form the complex. In other words, an observed change in fixed charge density, Δf, in the presence and absence of the cationic sorptate, when equal to 1 equivalent, is tantamount to the interaction of α moles of the cation. The relationship between free anionic binding sites, S^-, and the fixed charge density of the surface, f, is implicit in the value of α.

The affinity of the drug–tissue interaction may be described by an apparent association constant, K, as given by Eq. 1:

$$K = \frac{Sc_\alpha}{(c^+)_c{}^\alpha (S^-)} \qquad \text{(Eq. 1)}$$

where $(c^+)_c$ is the concentration of interacting cation in the colloid surface phase.

Rossotti and Rossotti (51) indicated that any property that varies with the degree of complex formation may be used to determine its extent. In the present case, observed changes in the fixed charge density represent experimentally known quantities which can be used for this purpose. Equation 1 can be rewritten fully in terms of experimental quantities to yield:

$$K = \frac{\Delta f}{[r^z(c^+)_{aq}]^\alpha(\Delta f_\infty - \Delta f)} \qquad \text{(Eq. 2)}$$

In Eq. 2, the observed change in fixed charge density at equilibrium, Δf, represents the concentration of the complex. The interacting cations in the applied solution distribute into the colloid surface phase in accordance with a Donnan equilibrium and must, therefore, be corrected by multiplication by r^z, where r is the Donnan ratio and z is the valence of the interacting cations. In the present treatment, the value of z is 1 for the carbamylcholine cation. The number of unaffected binding sites at equilibrium is the difference between the total number of anionic sites capable of interaction, Δf_∞, and the number of affected sites, Δf.

Equation 2 may be written in the forms given by Eqs. 3 and 4 for applicability to each of the three carbachol concentration regions in Fig. 4 preceding or following precipitous changes of Δf, which, as previously discussed, are postulated as being due to a cooperative, all-or-none unmasking of anionic sites:

$$K = \frac{\Delta f + f_1}{[r^z(c^+)_{aq}]^\alpha[\Delta f_{\infty_2} - (\Delta f + f_1)]} \qquad \text{(Eq. 3)}$$

$$K = \frac{\Delta f + f_1 + f_2}{[r^z(c^+)_{aq}]^\alpha[\Delta f_{\infty_3} - (\Delta f + f_1 + f_2)]} \qquad \text{(Eq. 4)}$$

In Eqs. 2–4 Δf_∞, Δf_{∞_2}, and Δf_{∞_3} are extrapolated total numbers of anionic sites for each of the three regions; f_1 and f_2 represent the changes in Δf between regions one and two and between regions two and three, respectively. The total density of anionic fixed charge that has been influenced up to a given carbachol concentration is given by Δf, $(\Delta f + f_1)$, and $(\Delta f + f_1 + f_2)$ for the concentration regions of 0–0.055, 0.088–0.111, and 0.125–0.173 M, respectively. Equations 2–4 are, in principle, equivalent if the newly

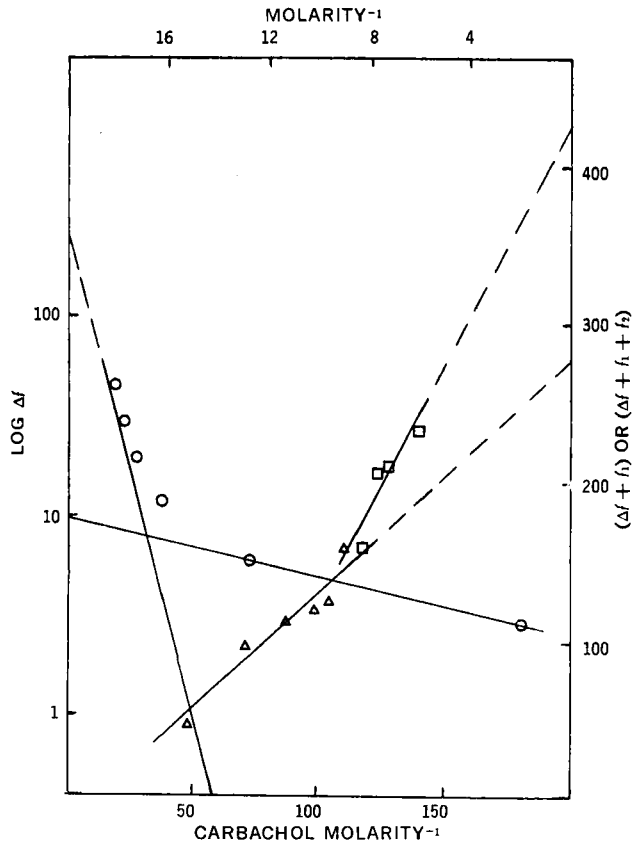

Figure 5—*Graphical determination of the maximum number of binding sites, Δf_∞, on the rabbit corneal surface which bind carbamylcholine cations at pH 6.95. The Δf_∞ values are determined by extrapolating the change in fixed charge density to infinite carbachol concentration for each of the binding regions shown corresponding to 0–5.5 × 10⁻² M (○), 8.8 × 10⁻²–1.11 × 10⁻² M (△), and 1.25 × 10⁻²–1.73 × 10⁻⁴ M (□) carbachol.*

created anionic binding sites are considered as being included in the total of cation-affected sites up to any given concentration.

Generally, some form of Eq. 2 suffices for the treatment of most solute–tissue interaction data. The observation that precipitous changes in Δf do occur attests to the verity of the expectation that heterogeneous tissue sites are characterized by differing interaction parameters. If this were not the case, the binding would be describable by a monotonic Δf *versus* carbachol concentration function indicating identical independent binding sites which, when transformed into a sorption isotherm, would be Langmuirian in appearance.

Values of Δf_∞ were obtained graphically (Fig. 5) by linear extrapolations to ordinate values corresponding to infinite concentrations of interacting carbachol. One plot of Δf *versus* the reciprocal of carbachol concentration was nonlinear, having a shape resembling an exponential decay. This appearance suggested its resolution into a sum of contributing exponentials, using the familiar peeling-off technique (52) and obtaining the sum of the least-squares values of the intercepts of semilogarithmic plots of each contributing exponential component. The results of the application of this technique are illustrated in Fig. 5, where the sum of the ordinal intercepts gives the apparent total number of available binding sites corresponding to an infinite concentration of carbachol for the 0–0.055 *M* region. Table III lists the values of total available sites appropriate for the three discrete binding regions.

Equation 5 is a linearized, logarithmic form of Eq. 2. Values of α and K may be determined from the slopes and intercepts of corresponding log–log plots of Eq. 5:

$$\log\left[\frac{\Delta f}{\Delta f_\infty - \Delta f}\right] = \log K + \alpha \log\left[r^z(c^+)_{aq}\right] \quad \text{(Eq. 5)}$$

The values of all terms appearing in Eq. 5 except α, K, and Δf_∞ are known from experimental procedures. As previously mentioned, the apparent stoichiometric coefficient, α (as well as the affinity constant, K), may be different in each concentration region as well as being concentration dependent within each region due to heterogeneity of binding sites and a varying reactivity of the sites that can be dependent upon the extent of their occupation. When this is fully the case, α and K may be approximated at each adsorptate concentration from the slope and intercept of a tangent line drawn to the curve at each abscissal concentration value. The accuracy of such a procedure could, when necessary, be enhanced by curvilinear regression fitting of the log–log plot to an empirical equation and differentiating the equation to obtain the slopes at each concentration. However, the evaluation of K and α would still remain uncertain due to the error generally characteristic of first derivative estimations.

Fortunately, it has been the experience of the authors that such log–log plots are either linear or exhibit two or more regions of linearity in which α and K are relatively constant. When this is the case, a plot of log $[\Delta f/(\Delta f_\infty - \Delta f)]$ *versus* log $[r^z(c^+)_{aq}]$ provides α as the slope of the line and log K as the intercept on the ordinate at $[r^z(c^+)_{aq}] = 1$. Equations 3 and 4 obviously can be linearized similarly to Eq. 2 and values of α and K can be evaluated.

Plots of Eqs. 2–4 for the present study are exemplified in Fig. 7. The plot for the lowest concentration region reveals two distinct straight lines, indicating a heterogeneity of binding sites within this region, with each type of site having its own α and K values. The values of α, as calculated from the least-squares slopes of the lines, are listed in Table III. The value of α in the carbachol concentration region of 0.088–0.111 *M* suggests that four to five carbamylcholine cations, when bound to the cornea, give rise to a change of approximately one unit of fixed charge density. This result is reasonable if one postulates a release of nondrug cations every time a drug cation is bound. The number of equivalents of bound cation that causes an integral value can be interpreted as representing a weighted average of integral values which must necessarily occur on a microscopic scale. The values of the association constant, K, for each concentration region are also listed in Table III. As anticipated, these values differ along with the α values in each concentration region. In the lowest concentration region,

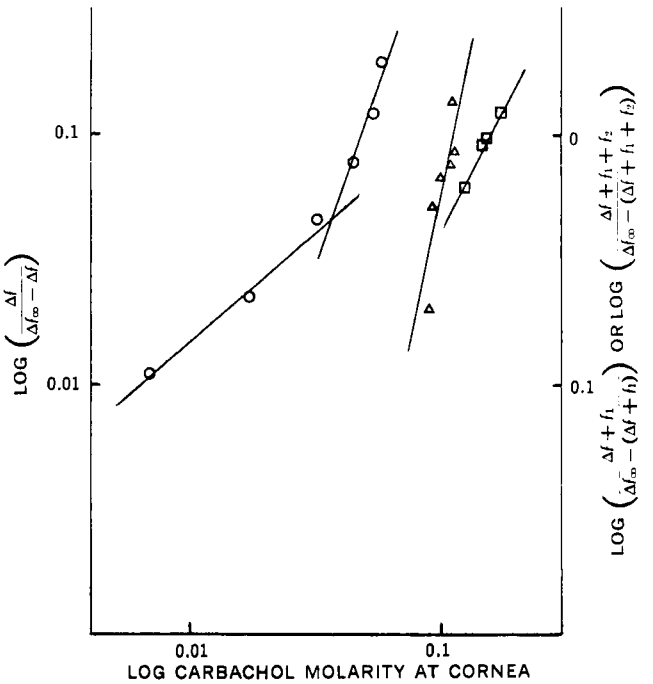

Figure 6—*Bjerrum-type plots of electrometric results of carbachol interaction with rabbit cornea. The three plots correspond to the carbachol concentration regions of 0–2.74 × 10⁻² M (○), 8.8 × 10⁻²–5.5 × 10⁻² M (△), and 1.25 × 10⁻²–1.73 × 10⁻² M (□). The ordinate scale on the right side is used for the plots corresponding to the latter two regions.*

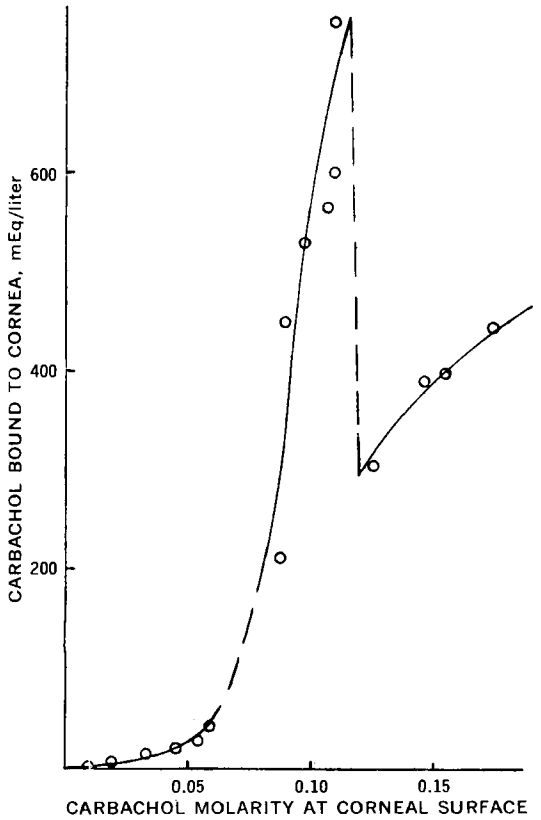

Figure 7—*Sorption isotherm describing the binding of carbamylcholine cations to the corneal surface of rabbits at pH 6.95. The dashed portions of the curve represent the intervals between the three binding regions which are defined by abrupt changes in fixed charge density.*

there are evidently two types of sites with different K values.

Having determined α values from the log–log plots, the equivalents of corneal surface-bound carbachol can be obtained by multiplying Δf [or $(\Delta f + f_1)$ or $(\Delta f + f_1 + f_2)$] by corresponding values of α. From Eqs. 2–4:

$$\left.\begin{aligned} &\alpha(Sc_a) = \alpha\Delta f \\ &\alpha(\Delta f + f_1) \\ &\alpha(\Delta f + f_1 + f_2) \end{aligned}\right\} = \text{cornea bound carbachol} \qquad \text{(Eq. 6)}$$

An adsorption isotherm may be constructed by plotting the quantity of bound carbachol as a function of the carbachol concentration in the corneal surface phase, *i.e.*, $[r^2(c^+)_{aq}]$. The cooperative nature of the drug–tissue interaction is again apparent from the shape of the isotherm in Fig. 7. Inspection of Fig. 7 reveals an unusual effect. When accepting the method by which they were attained, the results in Fig. 7 indicate that the interaction of carbachol decreased in the 0.125–0.173 M region relative to the 0.088–0.111 M region. This behavior may be rationalized by postulating that in the transition between these two concentration regions, in which new anionic sites are unmasked due to a release of cations, the released cations include carbachol; vacated sites subsequently become reoccupied by carbachol when its concentration is increased.

The decrease in the association constant for carbachol binding from a value of 3×10^4 to 36 (Table III) indicates a large decrease in affinity for carbachol interaction which underlies the desorption of carbachol from the tissue surface in the transition between the second and third concentration regions. Since there is no apparent similar discontinuity between the first and second concentration regions in Fig. 7, it can be assumed that the abrupt drop in charge density occurring between these regions (Fig. 4) reflects a release of nondrug cations, Na^+, K^+, *etc.*, from the tissue surface rather than carbamylcholine cations. Table III shows that the carbachol association constant is appreciably greater for the second region compared to the first region, indicating an enhancement of drug cation binding in the second region. The opposite occurs in the third region.

Carbachol–Corneal Surface Interaction and Drug Bioavailability Implications—Since carbachol must penetrate into, and migrate through, the cornea, it seems reasonable to expect that the size of the reservoir of drug sorbed onto the corneal surface will affect the amount of drug reaching the biophase. The amount of bound drug on the corneal surface is dependent on the availability of binding sites and the affinity of the drug for the surface; weakly bound drug is easily washed off in the lacrimal fluid. In the preceding discussion, it was postulated that drug–tissue affinity is reflected in the observed changes in the fixed charge density of the drug-treated tissue surface. A correlation may be expected between tissue-bound drug and the amounts of drug and the time they are available to exert a local pharmacological response.

The duration of the miotic response provides a measure of such drug bioavailability; the response duration is defined as the time required for the pupil diameter to return to normal. In Fig. 8, the response duration is plotted as a function of the concentration of topically administered carbachol solutions. It is evident from the figure that the duration first increases monotonically with increasing carbachol concentration; it then decreases and eventually increases again as the concentration is further raised. The sharp decrease in duration is approximately in the same concentration region where an abrupt drop in charge density (Fig. 4) and bound carbachol (Fig. 7) occurs. This abrupt change was attributed to desorption of carbamylcholine cations from tissue binding sites. It appears that the bioavailability of topically administered carbachol is dependent on the extent of interaction of carbachol with the corneal surface and can be enhanced by modifying the drug dosage form so as to increase this interaction.

In a subsequent paper of this series, it will be proposed that the adjuvant, benzalkonium chloride, enhances the bioavailability of carbachol by affecting carbamylcholine binding sites on the corneal surface. In the first paper of this series, it was proposed that this

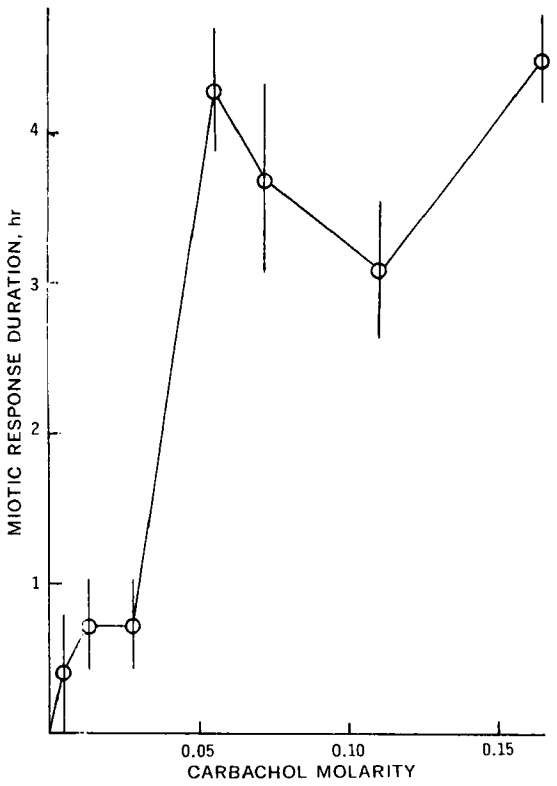

Figure 8—*Dependency of the duration of carbachol-induced miotic response activity in rabbits on the carbachol concentration in topically administered, pH 6.95, ophthalmic solutions. Each point represents the average of a minimum of four replications. The standard deviations are shown for each point.*

adjuvant also functions in facilitating the transport of carbachol through the cornea to the aqueous humor by inducing the release of bound carbamylcholine from tissue binding sites throughout the cornea (1).

As was the case in Fig. 7, no drop in response duration in the lowest carbachol concentration region is evident in Fig. 8, which supports the contention that the first abrupt drop in charge density in Fig. 4 reflects a release of nondrug cations from the corneal surface. The small initial increase in duration at very low carbachol concentrations could conceivably be a result of the low affinity of drug cations for surface sites as predicted by the small drug–tissue association constant (Table III). For these concentrations, one can speculate that the binding of carbamylcholine cations to the corneal surface also augments movement of drug cations across the cornea by creating a more favorable electrical gradient.

Iontophoresis, a procedure used to facilitate the transcorneal passage of anionic and cationic drugs (53), emphasizes, in conjunction with other evidence, the electrostatic and presumably electrochemical factors that influence passage of ophthalmic drug ions from the epithelial tear interface to the aqueous humor. Concomitant conformational changes in biocolloids resulting in morphological changes in the cells constituting the epithelial barrier must also be considered as related factors that could affect the transcorneal transport of carbachol. For example, Green and Tonjun (54) reported that an electron microscopically observed widening of intercellular spaces in the corneal epithelium results from treatment with surface-active quaternary ammonium compounds. If this occurs *in vivo, i.e.,* it is not merely a consequence of the harsh treatment of the excised corneal tissue subjected to dehydration and osmium fixation in the process of making electron micrographs, it is likely that submolecular phenomena, such as those presently described, underlie the widening process (31, 41) and, by this mechanism, could also contribute to enhancing transcorneal drug passage.

SUMMARY

The present study was directed to an elucidation of the submolecular interaction of the ophthalmic drug carbachol with the corneal surface of rabbits. An *in vivo* bioelectrometric method, which permitted determination of the fixed charge density of the corneal surface in response to varying the carbachol concentration of topically administered solutions, was implemented.

The reversible pH–titration curve of the cornea illustrates the amphoteric, predominantly acidic, nature of the tissue surface and is due to the various protein, lipid, nucleic acid, and mucopolysaccharide constituents of the corneal surface. The interaction of carbamylcholine cations with the corneal surface is a complex phenomenon which apparently involves cooperative, all-or-none changes in association affinities of fixed anionogenic groups on the surface. The mechanism of the interaction is explainable in terms of an inductive alteration of cation binding site affinities as a consequence of the binding of carbachol cations to vicinal cardinal sites. Corresponding miotic results indicate that the bioavailability of the drug is dependent on the extent of the drug–tissue interaction and reflects alterations in the values of the parameters characterizing the interaction.

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This paper is Part II in the series "Bioavailability of Ophthalmic Carbachol."

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JOHN P. BEDERKA, Jr. [*x], DANIS DAVITIYANANDA [‡], MIKEL L. MOSES [*], and NASIM AHMAD [*§]

Abstract □ Thirteen pyridine compounds, phenylbutazone, and three salicylates were studied for their effects upon the turnover of 7-^{14}C-nicotinamide dinucleotides in the mouse. The compounds were administered at equitoxic doses (LD_{25}) to 7-^{14}C-nicotinic acid- (niacin) pretreated mice, and the induced excretion of urinary-^{14}C was analyzed in terms of total ^{14}C and percentage of total ^{14}C as known metabolites of nicotinic acid. Of the 17 compounds, 12 afforded significant alterations in the total ^{14}C excreted and five of these caused alterations in the disposition of the 7-^{14}C-nicotinamide endogenously liberated from the 7-^{14}C-nicotinamide adenine dinucleotide pool. Comparative depletions of ^{14}C from brain, lungs, liver, and kidneys were studied with 10 of the pyridine compounds. Several tissues were found to be the sources of the urinary-^{14}C, with the lungs being the most accessible source. Some compounds had effects at doses less than the LD_{25}'s, as shown by increased hexobarbital sleeping time in acute experiments with rats. These pyridine compounds were initially considered to act at the level of the nicotinamide dinucleotides in the normal biosynthetic pathway (nicotinic acid site) and/or at the level of glycohydrolase (nicotinamide site). In view of the inclusion of nicotinic acid, nicotinamide, salicylic acid, and phenylbutazone in this correlation between toxicity and 7-^{14}C-nicotinamide mobilization, it is not necessary that the formation of compounds analogous to the nicotinamide dinucleotides plays a significant role in the toxic manifestations of the nicotinamide analogs. The displacement of 7-^{14}C-nicotinamide dinucleotides from their corresponding apoenzymes with subsequent metabolism of the dinucleotides could explain the noted increased 7-^{14}C-nicotinamide dinucleotide turnover and depletion which led to the toxic effects.

Keyphrases □ Coenzyme depletion—effects of 17 compounds, motor activity, sleeping time, toxicity, therapeutic effects, coenzyme binding site □ Toxicity—pyridine compounds, salicylates, phenylbutazone, nicotinic acid site, nicotinamide site, coenzyme binding site □ Metabolism—nicotinamide, coenzymes, nicotinamide dinucleotides, apoenzymes □ Drug–biomolecule interactions—drug toxicity and vitamin coenzyme depletion, symposium □ Interactions—drugs with biomolecules, symposium

Since initial toxicity studies (1, 2), McDaniel *et al.* (3) described niacin and seemingly anomalous antiniacin or toxic effects of 3-acetylpyridine and several attempts have been made to reconcile such disparities. Thus, antiniacin properties were considered in compounds related to nicotinic acid (niacin) and nicotinamide (niacinamide) (4–6). Since these studies with nicotinamide and some possible antimetabolites, attention has been focused on the nicotinamide dinucleotides[1] (NAD and NADP). The production of analogs and/or alterations in the concentrations of these dinucleotides have been considered as possible explanations of the antiniacin effects of 3-acetylpyridine (5–9) and of 6-aminonicotinamide (10).

It has been reported that nicotinamide administration results in increased levels of liver NAD, with maximal increases at 8–12 hr after the administration of 250 mg/kg ip to the mouse (11) and 1 g/kg ip to the rat (12). The latter study showed a relationship among the increased nicotinamide (23-fold), nicotinic acid (65-fold), and NAD (11-fold) levels in the liver after nicotinamide administration. This induced synthesis is in accord with the normal biosynthetic pathway (Scheme I), wherein the *in vivo* hydrolysis of nicotinamide would furnish the nicotinic acid.

Moreover, compounds other than nicotinic acid and nicotinamide have been reported to increase liver NAD levels. Kaplan *et al.* (11) showed that the administration of 3-methylpyridine (β-picoline) afforded a higher liver NAD level and more rapid rise to maximal effect than did nicotinamide. This increase could possibly be explained as a consequence of the continuous *in vivo* oxidation of 3-methylpyridine to nicotinic acid, with resultant NAD biosynthesis. Alternatively, and perhaps concomitantly, the 3-methylpyridine could cause a general tissue depletion of NAD whose mobilization could lead to the liver levels of NAD described.

Nicotinamide and related substances lend themselves readily to studies relating biological effect and biochemical change, since the normal biotransformations of nicotinamide have been intimately defined in regard to NAD biosynthesis (13) and to nicotinamide

[1] The phrase nicotinamide dinucleotides will include the oxidized [NAD (nicotinamide adenine dinucleotide) and NADP (nicotinamide adenine dinucleotide phosphate)] and corresponding reduced forms (NADH and NADPH) since quantitative assessments of the amounts of the individual substances have not been performed.

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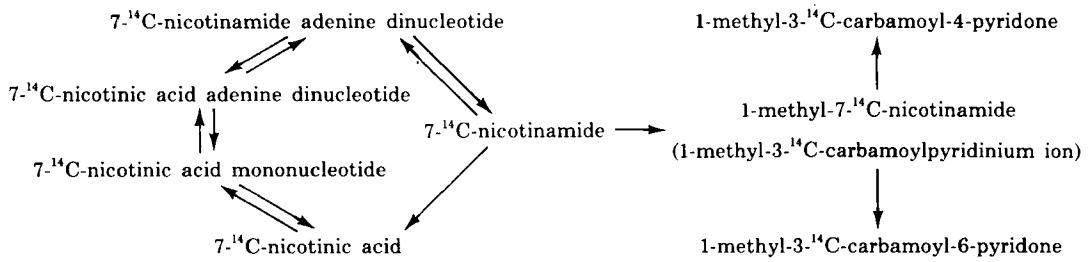


Scheme I—Biotransformation of 7-^{14}C-nicotinic acid and some urinary metabolites of 7-^{14}C-nicotinamide dinucleotide-derived 7-^{14}C-nicotinamide in the mouse

metabolism (14–17). This report describes a relationship between the acute toxicities of nicotinamide, some analogous compounds, and some highly protein bound drugs and their abilities to alter the 7-^{14}C-nicotinamide turnover from the labeled nicotinamide dinucleotides in the 7-^{14}C-nicotinic acid-pretreated mouse.

EXPERIMENTAL

Chemicals—The 7-^{14}C-nicotinic acid[2] (11 mCi/mmole) exhibited a single radioactive zone at R_f 0.2 in Solvent H. The 3-(1-hydroxyethyl)pyridine was prepared according to McKennis *et al.* (18). All other chemicals were obtained from commercial sources, and the liquids were purified by distillation prior to use.

Animals—Young adult female Swiss albino and Charles River mice, 20–32 g, and female Sprague–Dawley rats, 140–170 g, were used. The animals were housed at ambient conditions and allowed food and water *ad libitum* unless otherwise indicated.

Chromatography—Paper[3] chromatograms were developed by the descending method at ambient temperatures in Solvent H [upper phase from saturated aqueous 2-methyl-1-propanol–concentrated aqueous ammonia (50:1 v/v)] (compound and R_f): 1-methylnicotinamide, 0.07; nicotinic acid, 0.18; 1-methyl-3-carbamoyl-4-pyridone, 0.34; 1-methyl-3-carbamoyl-6-pyridone, 0.47; and nicotinamide, 0.69.

Acute Toxicity Determinations—The chemicals were administered intraperitoneally in a volume of 0.2 ml. Pyrazinamide, thionicotinamide, and phenylbutazone were administered in dimethyl sulfoxide, and isoniazid and nicotine were administered in pH 4 buffer solution[4]. Nicotinic acid and the salicylates were dissolved in aqueous sodium hydroxide, and all other chemicals were administered in aqueous solution. The LD_{25} values were calculated according to the method of Litchfield and Wilcoxon (19) after a 7-day observation period with either five or six groups of 10–30 mice per treatment.

Treatment with 7-^{14}C-Nicotinic Acid and Other Agents—Groups of five mice received 7-^{14}C-nicotinic acid (3.3 μg, 0.29 μCi) and were maintained under normal conditions for 24 hr. The mice were then divided into control and treatment groups and were manipulated before being transferred to obtain evacuation of the urinary bladder. This manipulation involved squeezing the mouse between the thumb and index finger at areas just anterior to the hindlimbs, with gentle pulling of the mouse clinging to a horizontal metal screen.

The mice were then treated with the test chemicals at a dose equal to the LD_{25} and placed into metabolism cages. Urine was collected from both the control and treated groups again, with appropriate manipulation to obtain bladder evacuation at the end of the 16-hr experiments.

Radioactivity Measurements—The scintillation solution contained 5.0 g of 2,5-diphenyloxazole and 0.3 g of *p*-bis[2-(5-phenyloxazolyl)]benzene in 1 liter of toluene. Samples were counted in scintillation solution containing 15 ml of cocktail and 3 ml of methanol. Urinary aliquots (0.1 ml) from mice pretreated with 7-^{14}C-nicotinic acid were added directly to the scintillation solution for counting.

Paper chromatograms of urinary aliquots were sectioned at 1-cm intervals perpendicular to their long axes, and each section was placed in the scintillation solution for counting. In this manner, the position (R_f) of the radioactivity on the paper could also be obtained.

Determination of Tissue-^{14}C—At the conclusion of the 16-hr experiments, the mice were sacrificed with ether. The carcasses were then maintained at about −10° until analyzed for ^{14}C. The specific tissue samples (brain, kidneys, liver, and lungs) from all animals (four or five) in each group were combined and placed in 3% sodium hydroxide solution (1 g of tissue/5 ml of solution). Dissolution was accomplished by shaking the tissue suspension contained in 20-ml glass vials overnight at ambient temperatures.

To 2 g of the tissue digest were then added a solubilizer[5] (3 ml), to obtain a pH of about 7, and 15 ml of Bray's scintillation solution (for aqueous samples: naphthalene, 60 g; 2,5-diphenyloxazole, 4 g; *p*-bis[2-(5-phenyloxazolyl)]benzene, 0.2 g; methanol, 100 ml; ethylene glycol, 20 ml; and dioxane to 1 liter).

Gross Motor Activity—The activities of the mice were quantitated in a photoactivity cage[6], and cumulative counts were obtained during 30 min after the intraperitoneal administration of each compound to duplicate groups of five mice. Control counts were obtained on different mice that received only the vehicle.

Sleeping Times—Groups of eight–12 animals were used at each dose of hexobarbital (Tables I and II). Sleeping times were recorded as the time in minutes between the loss and return of the righting reflex.

RESULTS

Toxicity—The toxicity studies afforded the LD_{25} values shown in Tables II and III. The ratios of LD_{75}/LD_{25} shown in Table III indicate a very narrow range of slopes (1.14–1.85) for the various dose–response curves, except with thionicotinamide (3.42). Although the observed range of slopes is narrow, the lines cannot be considered parallel except for the group including 3-cyanopyridine, 3-pyridinecarboxaldehyde, pyrazinamide, 3-acetylpyridine, and isoniazid according to the method of Litchfield and Wilcoxon (19).

During the 7-day observation periods, the mice died mainly between 10 and 72 hr after receiving the test chemicals at a dose equal to the LD_{25}. With nicotine and isoniazid, however, the susceptible mice died within a few minutes to less than 3 hr and no further deaths were noted in the ensuing 7 days. The general toxic signs covered the spectrum, from essentially immobile mice with thionicotinamide to opisthotonus with nicotine.

Phenylbutazone, the three amides (nicotinamide, pyrazinamide, and thionicotinamide), and the salicylates produced only sedation; at the higher doses of pyrazinamide and thionicotinamide, paralysis of the posterior extremities was noted. With 3-cyanopyridine and 3-pyridylcarbinol, a slight stimulatory effect followed by a period of brief inactivity was seen within 1 hr after administration. However, only hyperactivity and frequent convulsions were seen with isoniazid and nicotine. After the administration of doses of 3-(1-hydroxyethyl)pyridine and 3-acetylpyridine near the LD_{50}

[2] New England Nuclear Corp.
[3] Whatman No. 1.
[4] Beckman No. 3506.
[5] Bio-Solv BBS-2.
[6] Photo activity cage [61-cm (24-in.) diameter and 38-cm (15-in.) high cylinder], Lehigh Valley Electronics, Inc., Lehigh Valley, PA 18001

Table I—Sleeping Times of Female Rats Pretreated with Nicotinamide and Some Related Compounds[a]

Dose, mg/kg	Nicotinamide	3-Acetylpyridine	3-(1-Hydroxyethyl)pyridine
900	$17 \pm 2.5^{0.05}$	—[b]	—[b]
90	22 ± 3.2	—[b]	—[b]
45	29 ± 2.7	30 ± 1.8	26 ± 3.1
11	21 ± 3.1	30 ± 2.2	28 ± 0.4
45[c]	$40 \pm 5.2^{0.01}$	33 ± 3.5	$37 \pm 2.7^{0.05}$
45[c]		Nicotinic acid $34 \pm 3.2^{0.05}$	

[a] All drugs were administered intraperitoneally in aqueous solutions. The dose of hexobarbital was 50 mg/kg. Sleeping times are tabulated as mean ± *SE* in minutes. Statistical significance is indicated by superscripts, and the data were analyzed according to the *t* test. [b] Results were not obtainable at these doses due to deaths and the severe toxic signs during the 4 days of the experiments. [c] These data were obtained from acute experiments wherein the test substances were administered and 30 min later hexobarbital was also administered. Pretreatment at the other doses was carried out for 3 days prior to the administration of hexobarbital.

Table II—Relationships between Toxicity, Coenzyme Depletion, Gross Motor Activity, and Sleeping Time in the Mouse

Compound	Dose[a], mg/kg	Urinary-^{14}C[b], % Increase	Motor Activity[c], % Decrease	Sleeping Time[d], min
Methylnicotinate	450	300	42	60
3-Methylpyridine	250	290	74	139
3-Pyridylcarbinol	1500	260	93	248
Nicotinic acid	3090	190	89	203
Pyridine-3-carboxaldehyde	720	120	92	225
Methyl-3-pyridylcarbinol	240	52	68	287
Nicotinamide	1940	42	95	71
Pyrazinamide	705	40	92	86
Salicylic acid	500	27	46	32
3-Acetylpyridine	182	0	0	89
Isoniazid	103	0	16	181
Aspirin	800	0	49	34
p-Aminosalicylic acid	1800	0	28	34
Phenylbutazone	210	−25	73	79

[a] Doses are equal to the LD_{25}. [b] Shown as percentage increase above control, except for phenylbutazone, during the 16 hr after administration of the indicated compound. [c] Shown as the mean percentage decrease from control with two groups of five mice each in all comparisons. The maximal variability in the controls was ±15%. [d] Control values were equal to 32 and 34 min in the groups of 10 mice.

dose, an apparent mild sedation was noted, with return to normal activity within 20 min. During 10–24 hr after the administration of these latter two compounds, all signs discussed were seen in some mice, with deaths occurring throughout the 7-day period as described previously (20, 21).

Induced Excretion of ^{14}C—The quantitative results of the induced urinary excretion of radioactivity from the ^{14}C-labeled mice are shown in Tables II and III. The mean (429,000 dpm) of the induced ^{14}C-excretion values for the first six compounds in Table III is significantly different ($p < 0.001$, Student *t* test) from the control mean (310,000 dpm), even though one of five animals died in each of three of the four experiments. Similar data with these and some additional compounds are shown in Table II. The induced excretions of ^{14}C by nicotinic acid, 3-pyridylcarbinol, 3-methylpyridine, and methylnicotinate are much greater than the control increment. These studies indicate that 12 of the 17 compounds shown in the two tables at doses equal to their LD_{25}'s significantly altered the normal excretion of nicotinamide and/or its metabolites in the urine of the mouse.

Patterns of Urinary ^{14}C from 7-^{14}C-Nicotinic Acid-Labeled Mice—The information on the excretion of radioactive metabolites of nicotinic acid (Table IV) extends the preliminary studies of Roth *et al.* (15) and Lin and Johnson (22). The values show that less than 10% of the ^{14}C in the urine is 7-^{14}C-nicotinic acid. The control values for percent of the urinary ^{14}C excreted as 1-methylnicotinamide and pyridones show very little variation[7]. However, the values for nicotinic acid and nicotinamide in the control experiments and all four parameters in the treated groups show wide variations. Thus, comparisons between the control and treated groups can best be made in regard to percent ^{14}C-1-methylnicotinamide and percent ^{14}C-pyridones.

Five of the 11 compounds show percentages significantly different from the control means, thus indicating an altered disposition of endogenously generated free nicotinamide in the mouse. Furthermore, these effects upon 7-^{14}C-nicotinamide disposition are limited to compounds having significant effects upon total urinary-^{14}C except with the pH 4 buffer.

Tissue Distribution of ^{14}C—The relative ratios of the tissue specific activities for kidneys, lungs, brain, and liver are 1.0, 1.4, 3.1, and 4.2, respectively, and can be calculated from the values in Table V. Of the four tissue sites, pyridine-3-carboxaldehyde alone

Table III—Toxicity and ^{14}C-Nicotinamide Depletion after the Intraperitoneal Administration of Nicotinamide and Some Analogous Compounds to the Female Mouse

Compound (Molecular Weight)	LD_{25}[a], mg/kg	$\frac{LD_{75}}{LD_{25}}$	Urinary-^{14}C Increment[b], dpm
Nicotinic acid (123)	3087	1.85	578,000 (5)
Nicotinamide (122)	1940	1.14	130,000 (4)
3-Cyanopyridine (104)	810	1.43	−110,000 (5)
Pyridine-3-carboxaldehyde (107)	720	1.49	366,000 (5)
Pyrazinamide (123)	705	1.37	125,000 (4)
3-(1-Hydroxyethyl)pyridine (121)	239	1.22	160,000 (5)
3-Acetylpyridine (121)	182	1.45	19,000[c] (5)
Thionicotinamide (138)	105	3.42	80,000[c] (4)
Isoniazid (137)	103	1.38	3,000[c] (5)
Nicotine (162)	6.2	1.69	13,000[c] (5)

[a] The LD values were calculated according to the method of Litchfield and Wilcoxon with five or six groups, each containing 10–30 mice. A 7-day observation period was used for determining survival or death. [b] These values indicate the differences between the control mean ± *SD* (310,000 ± 43,000 dpm) from eight experiments and the experimentally obtained values for the number of mice (parentheses) that survived the 16-hr experiments. The control and treated groups each initially contained five mice in the ^{14}C-excretion studies. [c] These values do not differ significantly from the control mean; *i.e.*, they equal less than 2 *SD*.

[7] The urinary radioactivity corresponding chromatographically to 1-methylnicotinamide (1-methyl-3-carbamoylpyridinium ion) was shown by the isotope dilution technique in similar experiments (17) to be at least 84% accountable as 1-^{14}C-methylnicotinamide. Similarly, 1-methyl-3-carbamoyl-6-pyridone was 93% accountable as the 1-^{14}C-methyl-3-carbamoyl-6-pyridone and 1-methyl-3-carbamoyl-4-pyridone was 98% accountable *via* isolation as the 1-^{14}C-methyl-3-carbamoyl-4-pyridone *N*-oxide.

Table IV—Percentages of Radioactivity Corresponding to Known Metabolites of Nicotinamide in the Urine of ^{14}C-Labeled Mice Treated with Nicotinamide and Some Related Compounds[a]

Urinary-^{14}C Constituents	Percentage of Total Urinary Radioactivity									Mean ± *SD*
Control										
^{14}C-1-Methylnicotinamide	37	34	32	36	29	26	35	26	36	32 ± 4.6
^{14}C-Nicotinic acid	17	—	5.7	15	10	5.6	7.8	8.1	4.4	7.4 ± 5.0
^{14}C-Pyridones	38	53	53	41	40	47	55	41	46	45 ± 6.3
^{14}C-Nicotinamide	6.4	7.9	7.4	8.4	16	21	2.1	25	14	12 ± 7.5

Treated	Nicotinamide[b]	1-Methylnicotinamide	Nicotinic Acid[b]	3-Pyridylcarbinol[b]	3-Acetylpyridine	INH	Pyrazinamide[b]	Thionicotinamide[b]	3-Cyanopyridine[b]	3-PE[b]	NIC	HOH	Dimethyl Sulfoxide	pH 4	
^{14}C-1-Methylnicotinamide	11[c]	41	36	50[c]	40	27	49[c]	62[c]	75[c]	35	25	32	33	33	—
^{14}C-Nicotinic acid	10	—	3.7	7.0	<1.0	6.3	5.5	4.1	<1.0	2.6	5.5	—	9.5	18[c]	—
^{14}C-Pyridones	<1.0[c]	48	51	41	35	52	29[c]	16[c]	10[c]	40	55	57	52	46	—
^{14}C-Nicotinamide	64[c]	6.8	8.4	2.0	25	15	16	18	11	22	14	11	5.2	4.7	—

[a] The percent values are reported as 1-methylnicotinamide, 1-methyl-3-carbamoyl-4-pyridone plus 1-methyl-3-carbamoyl-6-pyridone (pyridones), and nicotinamide due to the localization of the radioactivity on paper chromatograms at R_f values coincident with those for these known metabolites of nicotinic acid. Each value represents one group of five mice. [b] The administration of these compounds was shown to have caused a significant change in the total radioactivity excreted in the urine of the 7-^{14}C-nicotinic acid-pretreated mice. [c] These values are considered to be significantly different from the corresponding mean control values; *i.e.*, they differ by more than 2 *SD* from the mean.

had significant effects at three sites. Nicotinamide and 3-(1-hydroxyethyl)pyridine had an effect at one site (lungs).

Whereas few significant decreases in the tissue specific activities are seen after treatment with the nine compounds, of the 36 values shown, only seven are above their respective control means. This indicates that a general mobilization is occurring, especially in the liver where all of the treated values are lower than the control mean. The lack of any significant tissue depletion after nicotinic acid administration was especially surprising in view of the large total urinary-^{14}C excretion shown in Table III.

Hexobarbital Sleeping Times in Rats—Chronic (3-day pretreatment with single daily intraperitoneal administrations) effects with 3-acetylpyridine and 3-(1-hydroxyethyl)pyridine were not seen due to death and severe toxic signs at the elevated doses shown in Table I. It is comparatively shown, however, that 3-day pretreatment with nicotinamide, 3-acetylpyridine, and 3-(1-hydroxyethyl)pyridine had no effects upon sleeping times. In the acute situation where hexobarbital was administered 30 min rather than 24 hr after the compound, prolonged sleeping times were seen except with 3-acetylpyridine. This lack of an effect is in agreement with the results shown in Tables II–V which also show no significant effects of 3-acetylpyridine upon processes involving the nicotinamide dinucleotides.

Motor Activity, Hexobarbital Sleeping Time, and Toxicity Correlates in Mice—As described, many compounds apparently induced a general central nervous system depression in the mice. The decreases in gross motor activities and, perhaps, the increases in hexobarbital sleeping times (Table II) support these initial observations. Thus, the altered coenzyme turnover, as shown by the tabulated changes in urinary ^{14}C, was accompanied by decreased motor activities except with aspirin (acetylsalicylic acid) and, perhaps, *p*-aminosalicylic acid since values of about 15% are within the variability exhibited in control groups.

The data in Table II also suggest a relationship between altered urinary-^{14}C (and thus nicotinamide dinucleotide turnover) and sleeping time since compounds that showed low or no alteration of urinary-^{14}C had small or no measurable effects upon the sleeping time at the indicated doses. The first eight compounds in Table II showed significant alterations in coenzyme turnover, motor activity, and sleeping time. Phenylbutazone was the only other drug that similarly altered all three parameters.

DISCUSSION

Nicotinic Acid Site—The results in Tables III and IV can be analyzed in terms of two sites of action for seven of the 10 substances. The largest increases in urinary-^{14}C excretion (Table III) were obtained with nicotinic acid and pyridine-3-carboxaldehyde. The result with nicotinic acid is interpretable as an increased synthesis and resultant turnover of NAD, with the consequent liberation of 7-^{14}C-nicotinamide as per the biosynthetic pathway (Scheme I). This normal route to NAD biosynthesis will be referred to as the nicotinic acid site of action and was recently discussed (23).

As shown in Table IV, after nicotinic acid treatment, the liberated 7-^{14}C-nicotinamide is metabolized and excreted in the normal but accelerated manner since no differences in the relative percentages between the control and treated values are apparent. The effects of pyridine-3-carboxaldehyde can be similarly explained since it may be metabolized to nicotinic acid in a manner analogous to the known oxidations of aldehydes to their corresponding acids. However, the administration of pyridine-3-carboxaldehyde results in an elevated level of ^{14}C-1-methylnicotinamide in the urine (Table IV). This indicates that pyridine-3-carboxaldehyde can alter the normal disposition of nicotinamide, perhaps competitively, in agreement with a possible action also at the nicotinamide site to be described. The lack of any obvious relationship between the toxicity and ^{14}C-depletion with 3-acetylpyridine, isoniazid, and nicotine indicates that these three substances act predominantly at other than the nicotinamide dinucleotides to exert their lethal effects.

Nicotinamide Site—Treatment with nicotinamide resulted in a significant increase in urinary-^{14}C and a considerably altered disposition of endogenously liberated 7-^{14}C-nicotinamide. Thus, the 7-^{14}C-nicotinamide entered the nicotinamide pool with the result that little methylation of the ^{14}C-nicotinamide occurred; 64%

Table V—Tissue-Specific Activities in the Mouse 40 hr after Administration of 7-^{14}C-Nicotinic Acid and the Effects of Treatment with LD_{25} Doses of Nicotinic Acid and Related Compounds

Compound	Tissue Radioactivity × 10^{-3}, dpm/g			
	Brain	Lungs	Liver	Kidneys
		Control[a]		
	6.01 ± 1.43	13.0 ± 1.99	4.46 ± 0.94	18.72 ± 6.96
		Treated		
Isoniazid	6.76	10.08	3.03	21.30
Pyridine-3-carboxaldehyde[b]	2.64[c]	4.66[c]	2.16[c]	12.56
3-Cyanopyridine[b]	4.87	12.40	2.87	11.90
Nicotinic acid[b]	5.66	12.30	2.78	12.49
Thionicotinamide[b]	7.06	14.12	3.40	18.00
Pyrazinamide[b]	5.62	15.35	2.84	20.35
3-Acetylpyridine	3.66	15.15	2.66	15.59
Nicotinamide[b]	5.25	8.53[c]	4.27	10.35
3-(1-Hydroxyethyl)pyridine[b]	3.50	7.50[c]	3.14	10.40

[a] Each control value is the mean of five control groups of five mice each ± *SD*, and each treated value is the pooled value from five mice in one treatment group. [b] These compounds caused significant alterations in 16-hr urinary excretion of ^{14}C when administered 24 hr after 7-^{14}C-nicotinic acid. [c] These values differ by more than 2 *SD* from their respective control means.

of the urinary-^{14}C was accountable as ^{14}C-nicotinamide and only 11% as ^{14}C-1-methylnicotinamide compared to the control means of 6% ^{14}C-nicotinamide and 37% ^{14}C-1-methylnicotinamide. Furthermore, essentially no ^{14}C-pyridones were found in the urine.

These relative percentage values indicate that the normal route of nicotinamide metabolism from the NAD source is from NAD to nicotinamide to 1-methylnicotinamide to pyridones with little nicotinic acid as an intermediary since the values for ^{14}C-nicotinic acid are essentially unchanged. These results with nicotinamide can be interpreted as the consequence of a competition between the administered nicotinamide and endogenous 7-^{14}C-nicotinamide at the level of glycohydrolase (EC 3.2.2.6, Scheme II), involving 7-^{14}C-nicotinamide-labeled endogenous NAD and the endogenous nicotinamide pool (7, 24, 25). This site of action at the glycohydrolase will be referred to as the nicotinamide site.

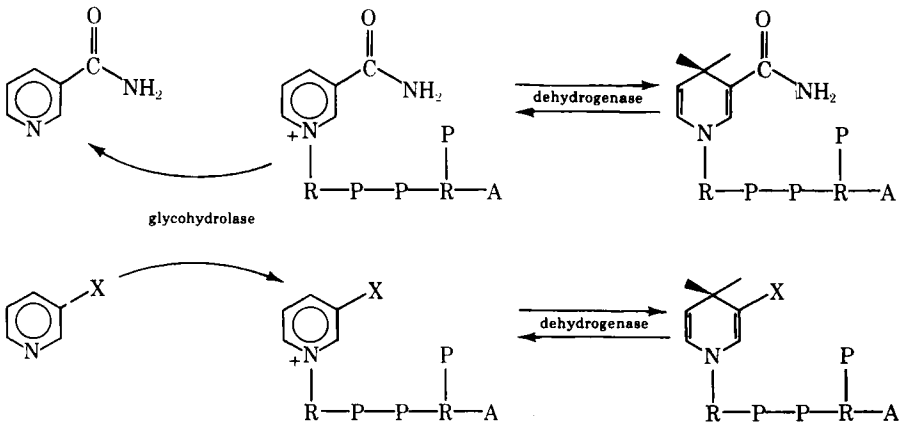
Nucleotide Source of Urinary-^{14}C—Although the effects of nicotinamide on urinary-^{14}C excretion may be interpreted as due to an action at the nicotinic acid site *via* the hydrolysis of nicotinamide to nicotinic acid, a consideration of the results with pyrazinamide, thionicotinamide, and 3-(1-hydroxyethyl)pyridine makes the nicotinamide site more probable as the major site of action of these four compounds, since the experiments afforded similar and significant effects upon urinary-^{14}C excretion (Tables III and IV). Additionally, in view of the structural differences between these compounds and nicotinic acid, it seems unlikely that either they or their metabolites could participate in the necessary enzymatic reactions in the biosynthetic pathway to NAD, thus causing a liberation of 7-^{14}C-nicotinamide.

Moreover, in view of the structural similarities especially among the three amides, it seems more plausible that a common site of action may be only at glycohydrolase. A further similarity of action among nicotinamide, pyrazinamide, and thionicotinamide is indicated by their alterations of the normal disposition of 7-^{14}C-nicotinamide (Table IV).

The increased excretion of urinary-^{14}C is perhaps the result of washing out a circulating pool of 7-^{14}C-nicotinamide and its metabolites. However, results with 1-methylnicotinamide (Table IV) argue against this possible site of action. 1-Methylnicotinamide caused neither an increased urinary-^{14}C nor an altered 7-^{14}C-nicotinamide metabolite pattern in the urine. Since the administered 1-methylnicotinamide and its pyridone metabolites would have depleted any circulating 7-^{14}C-1-methylnicotinamide and 7-^{14}C-pyridones, one can conclude that a normal turnover of the 7-^{14}C-nicotinamide dinucleotides was maintained and that the slow and normal metabolism of 7-^{14}C-nicotinamide was not measurably altered by 1-methylnicotinamide, either directly or *via* feedback inhibition of NAD metabolism.

Decreased Nucleotide Turnover—The decreased rate of NAD turnover, shown in Tables II and III as a decreased urinary-^{14}C excretion, after 3-cyanopyridine and phenylbutazone administration may have several bases. Initially, 3-cyanopyridine may inhibit the normal functioning of glycohydrolase and thus reduce the rate of NAD turnover. Additionally, the 16-hr urine volume was 2.8 ml, the lowest volume recorded in a 2.8–21-ml range with the control mean of 8.5 ml.

The decreased urinary-^{14}C after phenylbutazone administration is, however, not interpretable in terms of the two possible sites of action of the pyridine compounds since the chemical structures are apparently unrelated. However, a general action at the enzyme level *via* allosteric effects is suggested in view of the known high affinity shown by phenylbutazone for serum albumin. This allosteric effect could lead to an increased binding of the coenzyme and,



Scheme II—Illustration of the nicotinamide site of competitive antimetabolite (3-X-pyridine) incorporation into the nicotinamide dinucleotide pool

thus, an altered turnover rate. This alternative to the nicotinic acid and nicotinamide sites of action is supported by the general depleting effects (Table V) and will be discussed.

Coenzyme Binding Site—In view of the correlation between the equitoxic doses and their effects on the disposition of endogenous 7-^{14}C-nicotinamide, a cause-and-effect relationship is suggested for 12 of the 17 compounds discussed. Since nicotinic acid and nicotinamide are included in this correlation, the mediation of the toxic signs *via* abnormal dinucleotides analogous to NAD and/or NADP is seemingly obviated.

Although the dinucleotide analogs may contribute to the toxic manifestations, their formation *in vivo* would also remove the nicotinamide antimetabolites from the steady-state competition with nicotinamide at the level of glycohydrolase (26). Sund (27) reasoned that the nicotinamide dinucleotides are essentially completely bound to their respective apoenzymes. Reduction in the degree of binding could lead to their metabolism, to increased turnover, and to increased urinary-^{14}C. The loss of the apoenzyme *via* metabolism would also be a likely consequence of coenzyme depletion. Thus, the described results could be interpreted in terms of a third site of action at the level of coenzyme binding. This action allows an explanation of all observed results with the pyridine compounds, salicylic acid, and phenylbutazone.

The effects of nicotinamide, nicotinic acid, and 3-(1-hydroxyethyl)pyridine upon hexobarbital-induced sleeping times in the rat (Table I) support and extend the evidence for enzyme-related interactions of nicotinamide and other compounds that alter biological functions. An allosteric effect upon microsomal drug-metabolizing enzymes has also been reported with nicotinamide (28) and with metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) (29).

Tissue Site of Nucleotide Depletion—It has been reported (7) that the administration of 3-acetylpyridine to the mouse resulted in increased levels of liver NAD which were, however, less than those caused by nicotinamide but more than those caused by nicotinic acid. The present results show that 3-acetylpyridine was ineffective in altering the normal disposition of 7-^{14}C-nicotinamide. Since the normal pattern of metabolites of nicotinamide was obtained after 3-acetylpyridine administration, the metabolism of even these small amounts of ^{14}C-nicotinamide and ^{14}C-1-methyl nicotinamide was not altered by 3-acetylpyridine.

In contrast to the hypothesis of Herken (25), the toxicity of 3-acetylpyridine is, therefore, not likely related to interference in the normal synthesis or breakdown of NAD. Furthermore, based upon the normal urinary-^{14}C excretion after 3-acetylpyridine administration, the toxicologically significant existence of 3-acetylpyridine analogs of the nicotinamide dinucleotides seems unlikely. This failure to obtain any significant effect upon the disposition of ^{14}C-nicotinamide after 3-acetylpyridine administration argues against any overall nicotinamide-depleting effect by 3-acetylpyridine. However, a highly specific competitive interaction of 3-acetylpyridine and nicotinamide in a small vital area of the brain could have gone undetected (25, 30). An explanation of the so-called increases in liver NAD after treatment with 3-acetylpyridine would seem to require the existence of a unique substance very similar in chemical and spectral properties to NAD, *i.e.*, the "apparent liver pyridine nucleotide" suggested by Hayman *et al.* (8).

The distinct differences between the results with 3-acetylpyridine and 3-(1-hydroxyethyl)pyridine are entirely unexpected. Previous experiments (21) with mice and rats showed that the LD_{50} values for both compounds were essentially equal, and these results were confirmed by tabulated LD_{25} values in this study. Furthermore, the toxic signs and time courses of these signs were indistinguishable, whereas the onset times differed by 1 hr in previous experiments (21, 24, 31, 32). However, both 3-acetylpyridine and 3-(1-hydroxyethyl)pyridine caused similar increases in "apparent" urinary 1-methylnicotinamide and nicotinic acid in studies in the dog (33).

Again, a possible explanation of these different results may be that 3-(1-hydroxyethyl)pyridine has a more general and 3-acetylpyridine a more specific depleting effect upon the nicotinamide-containing dinucleotides. This possibility is supported by the work on depletion of tissue-^{14}C shown in Table V. The apparent site specificity shown by pyridine-3-carboxaldehyde, nicotinamide, and 3-(1-hydroxyethyl)pyridine could be extended to other levels of biological organization. Thus, depending upon the sites of depletion, either a toxic or, perhaps, therapeutic effect could be obtained.

Toxic *versus* Therapeutic Effects of Coenzyme Depletion—Nicotinic acid, 3-pyridylcarbinol, phenylbutazone, and the salicylates are therapeutically useful agents. The first two drugs are used for their vasodilator effects. The site(s) of the ^{14}C-depletion by nicotinic acid did not include the brain, kidneys, liver, and lungs (Table V). The vascular site was not investigated, but these studies are in progress with the vasodilator substances. Thus, site-specific coenzyme depletion could be a basis of toxic and/or therapeutic effects, depending upon the binding energies of the various coenzyme–apoenzyme complexes (34) and the relative affinities of the agents for the various tissues, as shown by Chang (35) with the coenzyme-depleting agent streptozotocin.

SUMMARY AND CONCLUSIONS

This investigation was initiated to ascertain a biochemical parameter that correlated with an acute toxic effect. The toxic effects of 17 compounds were studied in relation to induced changes in the turnover of coenzymes (nicotinamide adenine dinucleotides), nicotinamide metabolism, hexobarbital sleeping time, and gross motor activity in the mouse. Twelve of the compounds altered the turnover of the ^{14}C-labeled nicotinamide dinucleotide pool in the mouse, with a resultant general depression of the animals.

The increased dinucleotide turnover was discussed in terms of drug action at a nicotinic acid site, a nicotinamide site, and coenzyme binding site. The first two sites allow the possible incorporation of nicotinamide analogs into the organism and the formation of analogs of the dinucleotides, but they also serve as routes for the inactivation of these pyridine compounds. The coenzyme binding site of action allowed the best explanation of the results. It is suggested that coenzyme depletion is a more general basis of the actions of these drugs and toxicants. Thus, coenzyme depletion and concomitant metabolism of the corresponding apoenzymes may be a basic mechanism of drug action leading to toxic and/or therapeutic effects.

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A PAINFUL PROSPECT—WITH NO ANALGESIC

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In July of last year, we wrote in this column to express our concern that efforts to bring under control a serious national health problem—namely, drug abuse—had reached a level of such extremism that those efforts had come to be a health problem in themselves. Specifically, the single-minded determination to stamp out drug abuse had proceeded to the point where legitimate use of medication is seriously inhibited to the detriment of innocent persons needing such medication.

Moreover, the previous September we had devoted our editorial comments to what at that time was a current national problem; namely, shortages of various goods and commodities, such as gasoline, beef, lumber, and so on. Fortunately, at that time, there were no shortages of pharmaceutical products. Nevertheless, we used that opportunity to warn—the editorial was titled "Careless Analysis and Planning"—that a basic function of government in society is to study, analyze, predict, and anticipate various needs of the people and then, based upon this exercise, to adjust and take such actions to see that demands will be met in an orderly manner and by an efficient process.

Regrettably, our fears were subsequently realized and significant shortages of national proportions have been experienced during the past year, relating to several life-saving drugs such as ampicillin, heparin, and quinidine.

We would like to be able to say that our powers of prediction were right on target and we saw this all coming in our crystal ball. However, honesty compels us to admit that it was pure guess-work that events happened to turn out as they did. Consequently, we probably should not fault public officials for failing to have made proper plans which would have averted these shortages.

In our opinion, however, such exoneration should not extend to a situation in which knowledgeable people have widely publicized warnings of an impending shortage of critically needed medicinal agents and even have provided facts and documentation to support their cries of alarm. Given these circumstances, it seems tragic to us for responsible government officials to turn a deaf ear and take little, if any, action to avert such a crisis from developing.

Such appears to be the prospect with respect to the availability of the family of narcotic analgesics derived from opium. In our zeal to stamp out drug abuse, the United States has not only undertaken various programs and activities on the home front but has also entered into agreements abroad to induce foreign countries to curtail or eliminate the cultivation of the opium-poppy. Ignored in this strategy is the fact that codeine, morphine, and other medicinal opiate drugs are also derived from this same source, and as a concurrent effect, the available supply of these critical medicinals is rapidly diminishing.

This situation has been reported rather widely in both the professional and lay press. Perhaps the most articulate, well-documented, and compelling report on this impending catastrophe appeared as an editorial entitled, "No Opium for Pain—Threatening Medical Crisis," in the December 26, 1974 issue of the *New England Journal of Medicine*. In part, the editorial stated: *"As the matter stands today, the specter of no opium for pain in the United States is well-founded. In the event of a major epidemic or other national emergency, this critical shortage of pain-killing opiate drugs could easily cause a medical catastrophe. Under these circumstances, doctors and nurses in hospitals are about as useful as a fleet of new ambulances with little or no gasoline in their tanks."*

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Edward G. Feldmann

Antiviral Activity of Triterpenoid Saponins

Through his letter in the Open Forum section in the January 1975 issue of *J. Pharm. Sci.*, we are pleased to note that Dr. Alberto I. Calabrese had reported antiviral activity of hederin, a triterpenoid saponin, as early as 1949[1]. However, there was no way that we could have encountered this reference during our routine literature search on antiviral activity of triterpenoid saponins, the subject of our recent communication[2], since even in the *Quarterly Cumulative Index Medicus*[3], cited by Dr. Calabrese, the paper on hederin is listed *only* under the author's name. Further, this citation gives only the title, "La hederina, un nuevo antibiotico," with no indication of the antiviral activity.

We are grateful to Dr. Calabrese for bringing to the attention of *J. Pharm. Sci.* readers his original work published in 1949 and we regret not being able to locate this reference earlier.

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[1] A. I. Calabrese, *Semana Med.*, **1**, 116(1949).
[2] G. S. Rao, J. E. Sinsheimer, and K. W. Cochran, *J. Pharm. Sci.*, **63**, 471(1974).
[3] A. I. Calabrese, *Quart. Cumul. Index Medicus*, **45**, 233(1949).

Pharmacognocist versus *Pharmacognosist*

In a recent issue of this Journal (Dec. 1974, p. ix), an advertisement appeared for a "Staff Pharmacognocist." Although those of us who claim to be practitioners of pharmacognosy are always happy to see a continued need for our offspring in the industry, I would like to point out a not too uncommon error in the spelling of "pharmacognocist." The correct spelling of this unusual type of individual is, of course, pharmacognosist, being derived from the word pharmacognosy. All too often, those in the pharmaceutical sciences make the error of correlating the spelling of pharmacognosist with that of pharmacist which, of course, is derived from pharmacy.

This reminds me of an article that I once wrote for a student magazine, which was entitled "Ysongocamrahp." The point that I attempted to get across was that it matters little whether we spell pharmacognosy in the conventional manner, or backward, people still do not know what it means.

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REVIEW ARTICLE

Potential Value of Plants as Sources of New Antifertility Agents I *

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Keyphrases □ Antifertility agents—review of plant sources, classified by anatomical mechanism and folkloric route of administration □ Medicinal plants—sources of antifertility agents, classified by anatomical mechanism and folkloric route of administration, review □ Plant extracts—potential sources of antifertility agents, review □ Contraceptives—plants with active constituents, review □ *Abortifacients*—plants with active constituents, review □ Oxytocics—plants with active constituents, review □ Estrogenic plants—review of active principles

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*Editor's note: Part II of this article will appear in the May 1975 issue of the *Journal of Pharmaceutical Sciences*.

The world population explosion has pointed out the need for new and effective contraceptive agents and/or methods, having a minimum of side effects and giving a maximum protective effect. To date, the most effective and widely used contraceptives have been steroids, but these are not without side effects (1, 2).

Practically all major research to date involved with the search for new oral contraceptives has been of the synthetic type, particularly the preparation of steroid derivatives. Very little attention has been directed to the plant kingdom. There are those who continue to argue that plant products are of little importance as drugs. However, an analysis of some 1.05 billion new and refilled prescriptions dispensed from community pharmacies during 1967 showed that 25% contained one or more active principles derived from higher plants (3). Only three of the higher plant products (papaverine, ephedrine–pseudoephedrine, and caffeine) are produced commercially by synthesis, the remainder still being produced by extraction from

plants. Practically every pharmacological category of drug has as its prototype a substance of natural origin; thus it is not unreasonable to believe that the plant kingdom should yield an effective antifertility drug.

The problem underlying the search for natural antifertility drugs basically concerns deciding which of the approximately 750,000 species of higher plants should be examined in an animal system for their potential antifertility effects. There appear to be only three paths to follow, *i.e.*, *(a)* test plants that have folkloric reputation as having been used by primitive women as oral contraceptives, *(b)* test plants that are known to contain constituents that theoretically could affect the female cycle to produce antifertility effects (estrogenic sterols, coumestrols, and isoflavones) or that have a potential to contract the uterus, and *(c)* make a random collection–mass screening of all available plants for antifertility effects. The first two of these methods appear to be the most practical and realistic.

The tables in this article list those plants that have a folkloric reputation as having been used as oral contraceptives or that have been tested for antifertility effects in animals but may or may not have shown activity. We hope to point out that even though certain plants have been tested in animals for antifertility effects, the negative results presented may or may not be valid. Different species can vary in their response to different types of compounds. Furthermore, the antifertility tests used have not always been designed to test for all types of antifertility effects; that is, by using a too restricted dosing period (giving the extract only prior to mating or for only a few days after mating), for example, antifertility effects produced by some mechanisms will be identified but not those produced by others.

Because of the importance of the selection of the proper biological test to detect antifertility effects in plant extracts, the various mechanisms whereby compounds can exert an antifertility effect in various common laboratory animal species will also be reviewed.

ANTIFERTILITY MECHANISMS IN LABORATORY ANIMALS

Reproductive Cycles of Commonly Used Laboratory Species—There are a number of areas within the female mammal where substances having antifertility effects may exert their action(s); these areas are the hypothalamus, anterior pituitary, ovary, oviduct, uterus (including the endometrium, myometrium, and cervix), and vagina. A given compound may exert its antifertility effects in more than one of these areas and not necessarily by the same pharmacological mechanism. Conversely, different compounds may act in the same area to inhibit fertility but in some cases by different mechanisms.

Much information concerning the mechanisms by which chemicals may inhibit fertility has been obtained from studies carried out in commonly used laboratory species such as the rat, mouse, hamster, guinea pig, and rabbit. The reproductive cycles of these species differ among themselves as well as from those of the human and other primates. Such differences can explain, in part, the varying effects sometimes exhibited by a given antifertility substance when tested in different speeies; *i.e.*, antifertility agents interrupt reproductive function at some point, and if endogenous control at this point varies among species, different results with a given compound are possible. Consequently, and also since an understanding of antifertility mechanisms requires an understanding of reproductive function, a brief comparison of the latter in the commonly studied species will be made first. Additional comparisons among species will be made where pertinent during discussion of specific antifertility mechanisms.

Table I summarizes the major similarities and differences in the reproductive cycle function among the rat, mouse, hamster, guinea pig, and rabbit; the first four species are spontaneous ovulators, and the fifth is a coitus-induced ovulator. Schwartz (4) recently compared the ovulation-controlling systems in these two types of ovulators. In spontaneous ovulators, folliculotrophic hormone (follicle-stimulating hormone and luteinizing hormone) stimulates ovarian follicles to grow and secrete estrogen, the latter serving as a feedback signal to the hypothalamic–pituitary axis that follicles have matured. The estrogen acts with an environmental facilitating signal, *e.g.*, the time of day, to induce the ovulatory surge of luteinizing hormone. The latter also causes progesterone secretion which, in turn, acting on a background of estrogen, causes mating behavior. In induced ovulators, folliculotrophin also stimulates follicle growth and estrogen secretion. However, besides signaling the hypothalamic–pituitary axis that follicles have become ovulable, the estrogen, centrally, also induces mating behavior and hypothalamic sensitivity to feedback from coital stimulation. The latter triggers the luteinizing hormone surge which, in turn, causes progesterone secretion and ovulation.

As can be seen in Table I, complex hormonal interrelationships are involved also in the induction and maintenance of pregnancy.

Antifertility Mechanisms Classified by Anatomical Site Involved—The disruption and/or desynchronization of integrated preovulatory and preimplantational events are the means by which many kinds of compounds can impair fertility. A discussion of the mechanisms involved follows. Antifertility mechanisms involving other stages (*e.g.*, postimplantational) of the reproductive cycle also will be considered. The mechanisms will be presented according to the site in the animal at which the antifertility action is exerted.

Hypothalamus–Pituitary—These two areas are considered together for two reasons. First of all, functioning of the pituitary is under intimate control of the hypothalamus by means of hormone-specific releasing factors, *e.g.*, follicle-stimulating hormone and luteinizing hormone releasing factors (10). Secondly, the question as to whether certain substances might act on the hypothalamus and/or on the pituitary has been controversial. While steroids, for example, may exert some effect directly on the pituitary (10), evidence suggests that their major site of antifertility action is in the hypothalamus (8). Other drugs appear to act in the brain *outside* the hypothalamus; *i.e.*, certain central nervous system (CNS) depressants and autonomic blocking agents apparently block neural pathways to the hypothalamus (8, 10).

Therefore, two basic mechanisms can be considered: *(a)* disruption of the normal humoral and hormonal functions of the hypothalamus and/or pituitary, respectively, by steroids, by nonsteroidal agents (*e.g.*, methallibure[1]) having antigonadotrophic activity, and by steroid antagonists (8); and *(b)* disruption of neural input to the hypothalamus, *e.g.*, from the environment (10) and from the postulated "clock" that controls the release of gonadotrophin-releasing factor(s) in spontaneous ovulators (8).

With respect to *(a)*, antigonadotrophic activity of estrogenic steroids can be demonstrated, for example, by their ability to block compensatory hypertrophy of the remaining ovary in unilaterally ovariectomized rats (11); in women, decreased follicle-stimulating hormone excretion can be measured during usage of contraceptive doses of estrogens (12). Progestagens in women (11, 12) and presumably also in laboratory animals (13) are able to inhibit the ovulatory surge of luteinizing hormone.

Demonstrating both ovulation-inhibiting *and* antifertility effects of estrogens and progestagens in laboratory animals could present a problem, however, since spontaneous ovulators may become coitus-induced ovulators under certain circumstances (4). The occurrence of such a phenomenon could explain the less than

[1] 1-α-Methylallylthiocarbamoyl-2-methylthiocarbamoylhydrazine (ICI 33,828).

Table I—Comparison of Reproductive Cycles of Five Laboratory Species[a]

Species	Ovulation	Nonmated Cycle Length, days	Luteal Phase[b]	Luteolysis	Hormonal Requirements for Mating Behavior	Pseudopregnancy		Implantation		Pregnancy			
						Length, days	Hormonal Requirements	Day[c]	Hormonal Requirements	Endocrine Gland Requirements[d]			
										Length, days	Anterior Pituitary	Ovary	Hormonal Requirements[e]
Rat	Spontaneous	4, 5	Ps	Nonpregnant uterus[f]	Estrogen and progesterone	12–14	Estrogen, prolactin	5–6	Progesterone and estrogen	22	12	20–21	Prolactin, follicle-stimulating hormone, and luteinizing hormone
Mouse	Spontaneous	4, 5, 6	Ps	Nonpregnant uterus[f]	Estrogen and progesterone	10–12	Prolactin	4–5	Progesterone and estrogen	19–20	11–12	18–19	Prolactin; luteinizing hormone after implantation
Hamster	Spontaneous	4	Ps	Nonpregnant uterus[f]; luteinizing hormone[g]; estrogen (by inhibiting follicle-stimulating hormone)	Estrogen and progesterone	8–10	Prolactin, follicle-stimulating hormone, and luteinizing hormone	4	Progesterone	16	16	16	Prolactin, follicle-stimulating hormone, and luteinizing hormone
Guinea pig	Spontaneous	15–18	S	Nonpregnant uterus[f]; estrogen (mediated by uterus)	Estrogen and progesterone	—	—	6–7	None	67–71	3	20	None, providing viable fetuses are present *in utero*
Rabbit	Coitus induced	Relatively continuous estrus[h]	SPs	Nonpregnant uterus[f]; luteinizing hormone[g]	Estrogen	17	Estrogen	7–8	Estrogen is unnecessary	32	32	32	Estrogen, to maintain the progesterone-secreting corpora lutea

[a] Data presented taken from Refs. 4–9. [b] Ps = pseudopregnancy; functional corpora lutea found following sterile mating or cervical stimulation. S = spontaneous. SPs = in the rabbit, the luteal phase occurs spontaneously following ovulation, but ovulation must be induced, *e.g.*, by sterile mating. [c] Day 1 of pregnancy is the morning on which a copulation plug and/or sperm in the vagina are found. [d] Days of pregnancy through which endocrine glands must be present for pregnancy to be maintained; removal is compatible with pregnancy if and when the placenta can assume the role. [e] In the absence of the anterior pituitary. In the rat, the replacement therapy indicated stimulates both estrogen and progesterone secretion. In the mouse, luteinizing hormone sustains estrogen secretion. The conceptus in all five species secretes a luteotrophic hormone to varying degrees. [f] The luteolytic factor produced by the endometrium is believed to be a prostaglandin, probably $PGF_{2\alpha}$. [g] High doses of luteinizing hormone are luteolytic in these species. [h] There are seasonal variations in mating behavior and responsiveness to other ovulation-inducing procedures.

100% inhibition of ovulation produced by a norethynodrel–mestranol combination in rats (14). Nevertheless, the achievement of satisfactory contraception in women, in the absence of consistent inhibition of ovulation by contraceptive steroids (12), implies that additional antifertility mechanisms may be at work. The latter possibility is of special significance since it has been suggested that external stimuli may also be able to induce women to ovulate (8).

Nonsteroidal antigonadotrophic agents and steroid antagonists may present even further difficulties with respect to the demonstration of ovulation-inhibiting *and* antifertility (*before* fertilization) effects. As shown in Table I, estrogen and progesterone are necessary for normal mating behavior in the spontaneous ovulators, while estrogen is necessary for such behavior in coitus-induced ovulators such as the rabbit. Compounds given chronically to inhibit gonadotrophin secretion directly would indirectly inhibit ovarian secretion; if such compounds themselves had no estrogenic and/or progestagenic activity, animals treated with them possibly might not mate. Furthermore, estrogen antagonists such as 1-(*p*-diethylaminoethoxyphenyl)-1-phenyl-2-*p*-methoxyphenylethanol[2] and *trans*-1-(*p*-α-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene[3], which apparently block the estrogen feedback on the hypothalamus that is necessary for the ovulatory surge of luteinizing hormone, also can block mating behavior, at least in the rat (15, 16).

With respect to *(b)*, since the hypothalamus is subject to neural input from other areas of the brain, substances having CNS depressant activity and/or effects on neurohumoral transmission could be expected to alter gonadotrophin secretion. Pentobarbital, morphine, and atropine have been shown to block the ovulatory surge of luteinizing hormone in laboratory animals, apparently by elevating an arousal threshold in the midbrain reticular formation (17). Tranquilizers such as reserpine and chlorpromazine, anesthetics such as ether (diethyl) and halothane, and adrenergic (as well as cholinergic) blocking agents also have been shown to exert inhibitory effects (directly or indirectly) on the hypothalamus (8, 10, 18, 19). The antifertility usefulness of drugs having such effects is questionable, partly because of their other pharmacological effects but also because it has been shown that rats, at least, have sometimes become coitus-induced ovulators following blockade of their gonadotrophin surge by pentobarbital or chlorpromazine (4).

It is apparent from Table I that interference with gonadotrophin secretion may have postovulatory antifertility effects. The secretion of estrogen and/or progesterone needed for implantation in some species is under pituitary control. Luteal function also remains directly under pituitary control for half the length of pregnancy in the rat and mouse and for the entire duration of pregnancy in the hamster; it remains indirectly under pituitary control throughout pregnancy in the rabbit since *estrogen* is the ultimate luteotrophin in this species (5).

In contrast, in the guinea pig, the pituitary is unnecessary after the 3rd day of pregnancy (5); and hypophysectomized women, caused to ovulate by the sequential administration of follicle-stimulating hormone and human chorionic gonadotrophin, have become pregnant without further gonadotrophin replacement therapy (20). It is not surprising, therefore, that ergocornine, which has been shown to inhibit prolactin secretion in rats (21), has also been shown to inhibit progesterone secretion in pseudopregnant rats (22) and to interrupt pregnancy in rats (23), the latter two effects presumably mediated by the former. Ergocornine did not interrupt pregnancy in rabbits (12) or guinea pigs (13) nor interfere with luteal function in nonpregnant women (24).

Ovary—Substances having antifertility properties may exert their effects at the ovarian level by inhibiting ovulation and/or steroidogenesis. Although the actual intraovarian mechanisms causing ovulation are still unclear, it appears that protein synthesis may be involved; actinomycin D, which inhibits DNA-dependent RNA synthesis, has been shown to block pregnant mare serum gonadotrophin- and human chorionic gonadotrophin-induced ovulation in hamsters (25).

Although the mechanism(s) involved have not been elucidated, the results of work by other investigators suggest that phenoxybenzamine (26), an α-adrenergic blocking drug, and CNS depressant drugs, particularly reserpine (8), may inhibit spontaneous and pregnant mare serum gonadotrophin–human chorionic gonadotrophin-induced ovulation, respectively, by acting directly on the ovary. Experimental evidence suggests that contraceptive steroids also may act directly on the ovary to inhibit ovulation and/or some aspect of steroidogenesis (12).

Steroidogenesis, itself, may be involved in the process of ovulation. 2α-Cyano-4,4,17α-trimethylandrost-5-en-17β-ol-3-one (cyanoketone), a compound that inhibits the conversion of Δ^5-pregnenolone to progesterone, of 17α-hydroxypregnenolone to 17α-hydroxyprogesterone, and of dehydroepiandrosterone to Δ^4-androstenedione, has been shown to inhibit ovulation, possibly by interfering with steroidogenesis in the ovary (27).

As can be deduced from Table I, steroidogenesis is important for the induction and/or maintenance of pregnancy. The specific requirements vary among species, however; in the hamster and guinea pig, estrogen actually is luteolytic. The steroidogenic function of the ovaries is taken over by the placenta or the fetoplacental unit in the guinea pig by Day 20 of pregnancy (5), in the monkey by Day 25 (20), and in the human by Day 41 (28). Drugs having a direct inhibitory effect on ovarian steroidogenesis, particularly on luteal function, have the potential for interrupting pregnancy in the latter three organisms if given early enough and in the rat, mouse, hamster, and rabbit if given at any time during pregnancy. Depending on the species considered, estrogens and prostaglandins are two such classes of compounds.

As already indicated, estrogen is luteolytic in the hamster and guinea pig (Table I). In the monkey, estrogen administration early in the luteal phase has been shown to decrease progesterone secretion and to hasten the onset of menstruation (20); similar effects have been produced by estrogens in women (20, 29). The lowering of postovulatory plasma progesterone levels may be at least one mechanism by which postcoitally administered high dose estrogen exerts its antifertility effect in women (29). In contrast, in species such as the rabbit and rat in which estrogen is needed for normal luteal function, estrogen antagonists such as *trans*-1-(*p*-α-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene are likely to interfere with luteal function (30).

Prostaglandins can interrupt pregnancy in many species, but the mechanisms of action are still unclear (31); in some cases, the actions are at site(s) other than in the ovary *(vide infra)*. Administration of $PGF_{2\alpha}$ has been shown to shorten the lifespan of the corpus luteum and/or to decrease the ovarian secretion of progesterone in the rat, hamster, guinea pig, rabbit, mouse, and sheep (32); in the primate, however, effects of prostaglandins on corpus luteum function have been inconsistent (31). Even in the rat (31) and rabbit (33), susceptibility of the corpora lutea to the luteolytic effects of $PGF_{2\alpha}$ varies with the stage of pregnancy.

All species in which prostaglandins have been shown to be luteolytic are those in which the uterus influences the lifespan of the corpus luteum. The uterus in these species apparently secretes a luteolytic factor which is believed to be a prostaglandin(s) (31). In the guinea pig, hamster, and rat, at least, the endogenous luteolytic effect has been shown to be produced locally (5).

Oviduct—Since normal implantation depends on the correct timing of the arrival of the blastocyst in the uterus, disturbances of tubal transport may be accompanied by failure of implantation (8). Substances having the ability to alter oviductal motility may thereby be able to inhibit fertility. However, although ovarian hormones have been shown to influence the motility of the human oviduct *in vitro*, what effect, if any, contraceptive steroids may have on the latter in the *in vivo* situation is unknown (12). It is possible, however, that postcoitally administered, high dose estrogen may act in part by this means in the human (29, 34). The effects of some other substances on the human oviduct *in vivo* have been studied. For example, PGE_2 has been shown to inhibit tubal motility whereas $PGF_{2\alpha}$, at the same dose, has been shown to stimulate it (35).

The oviducts of rats, rabbits, and humans possess a rich sympathetic innervation, especially in the region of the isthmus, maintaining the tone of the isthmic sphincter and causing retention of fertilized ova in the oviducts. Administration of autonomic drugs, at least to these species, can influence the rate of ovum transport, although their ability to do so may be modified by estrogens and progesterone (25).

It has long been thought that luteal progestagen regulates the

[2] MER-25, ethamoxytriphetol.
[3] ICI 46,474.

relatively slow rate of passage of preimplantation ova through the oviducts (36) and that the smooth muscle of the reproductive tract exhibits maximal contractility only under the influence of estrogens (37). Recent evidence, however, suggests that smooth muscle (at least of the rabbit oviduct) is minimally active in the presence of physiological concentrations of estrogens; that its activity increases as estrogen content decreases; and that, when it is subject to the influence of estrogen, it will become increasingly active if progestagens are introduced (37). Megestrol acetate, a progestagen without estrogenic effects (38), has been shown to accelerate ova transport in the rabbit, which mechanism might contribute in part to its antifertility effect in that species (39). Nevertheless, since both estrogens and antiestrogens have been shown to alter ova transport and simultaneously to impair fertility, it would appear that a critical balance of estrogen and progesterone may be necessary for normal postovulatory events to occur (8). However, additional postovulatory antifertility mechanisms *(vide infra)* may also be playing a role in at least some cases; this possibility should not be ruled out.

Exogenous estrogen administered to many species during early pregnancy results in rapid passage of ova through the oviducts and expulsion of the ova from the uterus (36). However, at certain dosages (36) and/or when given at very specific times (40), estrogen alternatively may cause the retention of ova in the oviducts. For example, treatment of mice with 1.6 μg of estradiol on Day 1 of pregnancy, when the ova were still in the ampulla of the oviduct, resulted in retention of the ova in the oviduct (largely in the ampulla) until Day 4, presumably due to prolonged closure of the ampulla–isthmus junction. In contrast, treatment with 0.4 μg on Day 2, when the ova already were in the isthmus of the oviduct, resulted in premature entry of the ova into the uterus and a decrease in the recovery of ova by 50% (40).

A number of compounds having estrogenic activity *(vide infra)* and representing several different chemical classes have been shown to accelerate ova transport, *e.g.*, two nitriles, 2,3-bis(4-hydroxyphenyl)valeronitrile[4] and 2,3-bis(4-methoxyphenyl)pent-2-enenitrile[5], in rats (41); dienestrol [4,4′-(diethylideneethylene)diphenol] in rats (42); and nafoxidine[6] [1-[2-[*p*-(3,4-dihydro-6-methoxy-2-phenyl-1-naphthyl)phenoxy]ethyl]pyrrolidine] in mice (8, 43).

That accelerated transport of ova results in decreased fertility is, of course, partly due simply to the expulsion of the fertilized ova from the reproductive tract (36). However, it has been shown (34) that fertilized ova degenerate when transplanted into the uterus too early; an inability of the ova to survive in the uterus apparently contributes to the impaired fertility observed following accelerated ova transport from the oviducts. The decreased fertility, however, is thought to be due to the uterine environment and not to the rapid transport *per se* (8).

Nafoxidine also has been referred to as an antiestrogen *(vide infra)*, as has a structurally related compound, 1-[2-[*p*-(3-hydro-5-methoxy-2-phenylindyl)phenoxy]ethyl]diethylamine[7] (44). It is to the antiestrogenic property of these compounds and to that of *erythro*-ethyl-α′-methyl-4,4′-dihydroxybibenzyl that the investigators attribute their findings that these three compounds can cause tube locking of ova in mice.

Tube locking of ova, of course, obviously impairs fertility by preventing the fertilized ova from getting to the uterus, the normal site for implantation. The fertility of tube-locked ova *per se* may not be impaired, however, as suggested by observations made in one study in the mouse (40). Although tubal retention of ova, induced by estradiol, was associated with delayed development of the blastocele, the ova implanted normally following transfer to vehicle-treated recipients.

That the antifertility activity of a compound such as nafoxidine has been attributed to estrogenicity of the compound in some studies (8) and to its antiestrogenic activity in others (44) may seem confusing at first, but it has been difficult to determine whether many compounds have antifertility activity by virtue of their being estrogenic, their being antiestrogenic, or their having some other property (8). Certain compounds, in fact, are antiestrogenic at lower doses and estrogenic at higher doses. Furthermore, some evidence (45) suggests that there may be species differences, at least between the rat and the hamster, with respect to whether a given compound exerts antifertility activity, estrogenic activity, and/or antiestrogenic activity, differences not entirely explicable by differences in hormonal requirements between the two species (Table I).

The estrogenicity of a compound is defined most specifically as the latter's ability to produce vaginal cornification (keratinization) in ovariectomized rats or mice (the Allen–Doisy test) (11); androgens and progestagens may produce vaginal mucification, but they are incapable of producing keratinization (46). Alternatively, investigators may employ a uterotrophic bioassay in immature and/or ovariectomized rats or mice to determine estrogenicity. This test is less specific, however, since a small degree of uterine growth can be produced by androgens and progestagens (11). The latter test does have the advantage that a dose–response curve for a given compound can be obtained more objectively (47).

Antiestrogenicity similarly may be defined as the ability of a compound to inhibit the effects of standard estrogens such as estrone (11), estriol (11), and estradiol (48) when the test compound is administered with the known estrogen in one and/or both of the tests for estrogenicity. Androgens and progestagens may show antiestrogenic activity in these tests (11); the weak plant estrogens, coumestrol and genistein, also have been shown to have inhibitory effects in such tests (49). Antiestrogenicity in an antifertility test could be manifested by the blocking of a step in the reproductive cycle that requires estrogen (Table I).

Uterus—Antifertility agents that prevent ovulation and/or fertilization commonly are referred to as contraceptive agents, whereas those that act after implantation has taken place usually are called abortifacients. The term interceptive has been used by some workers (50) to refer to compounds that act after the occurrence of fertilization but prevent implantation from taking place. By this definition, then, compounds interfering with the secretion of the proper amounts of steroids needed during this time period, whether acting directly on the ovaries or indirectly by inhibiting gonadotrophin or prolactin secretion, can be referred to as interceptives, although their earlier or later administration might render some of them contraceptive or abortifacient, respectively. Drugs altering ova transport *(vide supra)* also can be considered interceptives, as can those that destroy the fertilized ova or blastocysts and those that act on the trophoblast or uterus to prevent implantation.

Experimental designs may not always be sufficient to distinguish among these specific mechanisms for given compounds. It is quite possible, furthermore, that agents exhibiting antifertility effects when given to experimental animals during the first several days postcoitum may actually be acting in more than one of these ways. Experiments in which the test compound is administered from Days 1 to 7 of pregnancy and in which only the number of implantation sites and/or litter size is determined do not elucidate which interceptive mechanisms might be involved and/or if indeed an early abortifacient action might be involved (51, 52). With additional techniques and/or more restricted dosing periods, one or more of these effects can be identified.

Although blastocyst transfer experiments (8, 53) seem to have ruled out a direct zygotoxic effect as a possible mechanism by which a number of compounds inhibiting implantation may act, observations made in a few other studies suggest the contrary. Degenerating morulae, for example, have been found in rats treated with medroxyprogesterone acetate[8] on Day 1 of pregnancy followed by estrone on Day 3 (54), and degenerating blastocysts have been found in mice treated with ethinyl estradiol on Day 1 of pregnancy (55); tubal transport appeared to be altered in the latter study as well. Inhibited growth of blastocysts, as indicated by computed blastocyst volume, together with accelerated ova transport has been demonstrated in rabbits administered the progestagen, chlormadinone acetate, for 3 days preceding the occurrence of induced ovulation (56). A mixed copolymer of phenylmethylcyclosiloxane that was administered to rabbits on Days 4 and 5 of pregnancy subsequently was found to have been taken up by the 6-day-old trophoblasts, resulting in degenerative changes in the trophoblasts (57); there was no effect on the embryonic cells. Implanta-

[4] SC-3402.
[5] SC-3296.
[6] U-11, 100A.
[7] Triethylamine, U-11, 555A.

[8] Depo-Provera.

tion did not occur; neither did changes in the uterine cells that normally accompany implantation.

That implantation *per se* can be inhibited has been shown in a number of studies. Neuraminidase in the mouse was most effective when given on Days 4 and 6 or 5 and 7 of pregnancy, times at which ova had begun implanting or had just implanted in the controls (58). The results suggested that the compound might have interfered with the development of already implanted ova as well as with the implantation of ova. *N*-(2-Chloro-1-naphthylidene)-3-amino-2,6-lutidene was more effective in preventing implantation in rats when given on Days 3–5 of pregnancy (59), while the effectiveness of 5-[[(α,α,α-trifluoro-*m*-tolyl)oxy]methyl]-2-oxazolindinethione[9] in rats required that its administration be continued beyond Day 3 of pregnancy or be begun before Day 7 (60).

Observations reported in two studies (61, 62) suggest a centrally mediated anti-implantational effect for D-6-methyl-8-cyanomethylergoline[10], a compound structurally related to the naturally occurring alkaloid agroclavine, which has been reported to inhibit implantation in mice and rats (63). D-6-Methyl-8-cyanomethylergoline was effective in rats when given orally up to Day 7 of pregnancy (61). In mice, there was failure of implantation as well as failure of occurrence of the progesterone-influenced uterine cellular changes characteristic of Day 4 of pregnancy when D-6-methyl-8-cyanomethylergoline was given on the first 2 or 3 days of pregnancy but not when given only on Day 1 (62). Treatment on Days 6 and 7 resulted in resorption of fetuses in some mice by Day 9, whereas treatment beginning on Day 7 had no effect. D-6-Methyl-8-cyanomethylergoline probably acts by suppressing prolactin release *via* stimulation of prolactin-inhibiting factor release from the hypothalamus (61); prolactin from the pituitary apparently is necessary until Day 7 of pregnancy, at which time the placenta begins secreting a luteotrophic substance for corpora lutea maintenance (62).

It has already been pointed out *(vide supra)* that a certain hormonal balance in the internal milieu of the reproductive tract appears to be important for postovulatory events to occur normally. However, although estrogen is necessary during early pregnancy in some species (Table I), it is not necessary for implantation in the rabbit, guinea pig, and hamster; furthermore, the requirements are not known for the larger domestic animals or for the primates (8), including the human (12). Nevertheless, it appears possible that nonphysiological treatment of the endometrium with hormones may impair fertility in the human.

Klopper (64), for example, pointed out that some progestagens, such as those found in oral contraceptives, produce an asynchronism between the development of the endometrial stroma and that of the endometrial glands. He suggested that an endometrium so treated might resist implantation. On an acute treatment basis, it has been suggested (60) that an excess amount of estrogen present shortly after ovulation (in mammals) might prematurely sensitize the uterus so that the latter would be in a refractory state at the time of arrival of the blastocyst.

On the other hand, it has been suggested (65) that, by virtue of their antiestrogenic properties, compounds such as *trans*-1-(*p*-α-dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene, 1-[2-[*p*-(3-hydro-5-methoxy-2-phenylindyl)phenoxy]ethyl]diethylamine, nafoxidine, and clomiphene prevent implantation, at least in the rat, by counteracting the estrogen needed for implantation; this estrogen is thought to be secreted on Day 4 of pregnancy. *trans*-1-(*p*-α-Dimethylaminoethoxyphenyl)-1,2-diphenylbut-1-ene appeared to be maximally effective when given on Day 4 and inactive when given on Day 5. Estriol, a so-called "impeding" estrogen, inhibited pregnancy *completely* when administered at a dose of 12.5 μg for the first 4 days of pregnancy but not when administered for only the first 3 days (66). Its presumably anti-implantational effect was inhibited when 0.05 μg of estradiol was administered on Day 4 together with the estriol (67).

Certain substances instilled locally into the uterus can impair fertility by affecting the endometrium in such a way that obstruction of the lumen occurs. Intrauterine instillation of a suspension of quinacrine in the rat, for example, induced a giant cell foreign body reaction in the endometrium and a consequent obstruction of the lumen, resulting in infertility for several months (68). Quinacrine instillation into the oviducts has been used as a nonsurgical means of inducing sterility in women (69).

Some compounds have postimplantational antifertility effects in nonprimate mammals but apparently not in the primate (70, 71). Demecolcine, for example, produced fetal death in rabbits and rats by means of a direct effect on the fetus; the placentae and implantation sites were not damaged. This compound had no effect on pregnancy in the macaque monkey. Clomiphene also was effective postimplantationally in the rabbit but again not in the macaque monkey. This drug, in contrast to demecolcine, affected the implantation site but exerted no effect on the fetus.

An abortifacient type of antifertility effect can be produced by compounds that stimulate uterine contractility. Sulman (72) suggested the latter as being the ultimate means by which monoamine oxidase inhibitors might induce abortion when injected into the human amnion. He reasoned that an inhibition of the amniotic monoamine oxidase enzyme system would result in serotonin's not being metabolized to 5-hydroxyindolylacetic acid. Therefore, serotonin would accumulate in the uterus and promote uterine contractility and eventually abortion. Monoamine oxidase inhibitors have been reported to interrupt pregnancy in several species, and intrauterine instillation of pargyline hydrochloride has been used successfully to induce abortion in the human (12).

The hormone, oxytocin, can be used close to term to induce labor but is ineffective as an abortifacient agent if used earlier in pregnancy (73). Prostaglandins, however, are now widely being used for the latter purpose. Observations made in humans (74), rabbits (74), and rats (75) suggest the following as the means by which prostaglandins induce abortion.

Prostaglandins appear to promote myometrial contractility which, in turn, generates internal shortening and stretch of the myometrial wall. As a result, cyclic intrauterine pressure is promoted which, together with vasoconstriction contracture, reduces uterine blood flow. The degree of this effect apparently is sufficient to compromise the endocrine function of the fetoplacental unit; as a result, estradiol and progesterone levels in the blood decrease. Following steroid withdrawal, the uterus, relieved from suppression, becomes converted into a spontaneously active, pharmacologically reactive organ, such as is normally seen only near term. The endogenous stimulatory mechanism at this time may be sufficient to complete the abortion; if not, additional prostaglandin or oxytocin may be used.

This hypothesis is supported indirectly by the results of another study (76). Three of nine women less than 4 weeks pregnant aborted completely following intravaginal administration of $PGF_{2\alpha}$. Only these three showed a significant fall in serum human chorionic gonadotrophin level, which was followed by a decrease in serum steroid level. Unfortunately, no measurements of uterine contractility were made.

Antifertility activity exerted at the level of the cervix (the neck of the uterus) or at the level of the vagina *(vide infra)* once more involves a discussion of prefertilization events. For example, the production of a cervical mucus that is "hostile" to sperm penetration is thought to be the major mechanism by which low dose progestagen-only contraceptives exert their antifertility effect in the human (77); it also has been suggested that progestagens in the human may inhibit further transport of the sperm that are able to reach the uterus (8). Testing for such activity in laboratory species, however, presents some difficulty. In the rat, mouse, hamster, and guinea pig, sperm are deposited in the uterine horns immediately after copulation (78). The rabbit, however, may be used as a model for investigating substances for contraceptive activity based on inhibition of sperm transport through the cervix; in this species, the ejaculate is deposited merely deeply within the vagina. One study did indeed show that relatively few sperm reached the anterior uterus and oviduct in rabbits pretreated for 7 days with progesterone, as compared with the number of sperm reaching these sites in the oil-treated controls (79).

Vagina—Spermicidal preparations, of course, are available for local application within the vagina. Their mechanisms of action are not well understood, and the effectiveness of many is poor (80). The five preparations that appear to have the highest rate of use effectiveness all contain polyethoxy derivatives, and four of the five contain compounds derived from phenoxypolyethoxyethanol. The results of recent experiments suggest that the effectiveness of

[9] U-11,634.
[10] 6605 VUFB, CME.

vaginal contraceptives might be able to be improved by means of the incorporation of an acrosin inhibitor into the preparation.

Acrosin is a proteolytic enzyme extractable from sperm acrosomes and is essential for penetration of the zona pellucida of the ovum by the spermatozoon during the process of fertilization (81). This trypsin-like protease has been found in sperm from a number of species: dog, rabbit, ram, bull, monkey, man, rooster (82), boar, stallion, guinea pig, hamster, and rat (83). Ejaculated sperm tend to have lower acrosin activity than do epididymal sperm, apparently due to adsorption by the sperm of an inhibitor substance present in the seminal plasma (83). This natural inhibitor is removed from the sperm during their residence in the female reproductive tract (84). Other substances that inhibit human and boar acrosin include trypsin–plasmin inhibitor bdellin B-3 from leeches, trypsin inhibitor and trypsin–plasmin inhibitor from guinea pig seminal vesicles, and the pancreatic trypsin inhibitors of pig, sheep, and dog (85). Human acrosin also is inhibited by soybean trypsin inhibitor and 1-chloro-3-tosylamido-7-amino-2-heptanone (86), a synthetic proteinase inhibitor that forms an irreversible complex with acrosin (84). Rabbit acrosin is inhibited by lima bean trypsin inhibitor, ovomucoid (87), diisopropylfluorophosphate, 1-chloro-3-tosylamido-7-amino-2-heptanone, and soybean trypsin inhibitor (81).

Of greater significance is that a number of these acrosin inhibitors have been shown to inhibit fertilization. Crystalline ovomucoid and soybean trypsin inhibitors inhibited the *in vitro* fertilization of rabbit ova by capacitated rabbit sperm (87), capacitation being defined as the physiological and biochemical change that allows for penetration and fertilization of ova (88). *In vivo* fertilization of rabbit ova was inhibited by rabbit seminal plasma trypsin inhibitor and pancreatic trypsin inhibitor, following placement into rabbits' oviducts of capacitated sperm treated with one of these inhibitors (89). *In vivo* fertilization of hen ova also was decreased in birds inseminated with rooster sperm that had been incubated with 1-chloro-3-tosylamido-7-amino-2-heptanone (82). Inhibition of fertilization following intravaginal deposition of synthetic trypsin inhibitors also has been reported (81).

This discussion has been an attempt to outline the mechanisms by which compounds having antifertility properties may exert their effects; it has been noted that different effects may be seen in different species. Only a small number of compounds showing antifertility effects have been indicated. Additional examples (predominantly synthetic compounds) can be found in Refs. 8, 10–12, 17, 34, 36–38, 44–50, 59–61, 64, 65, 68, 70, 71, and 80.

CONTRACEPTIVE AND INTERCEPTIVE PLANTS

The review of the literature in this and subsequent sections has included plants having folkloric reputations as well as those extracts shown to be active in animals or humans as antifertility agents, abortifacients, uterine stimulants, estrogenic agents, or cytotoxic agents (as applied only to antifertility effects). The search of the literature extends through 1973. Sources of references included *Chemical Abstracts, Biological Abstracts, Index Medicus,* and *Pharmacognosy Titles.*

Folkloric references can be obtained by an intensive study of ancient herbals and/or books on medical botany or by referral to review articles for which others have supposedly gleaned the older literature. Most folkloric references were obtained from the latter source, since a rather substantial number of articles and books have been acquired on medicinal folklore. No literature search of the medicinal folklore can ever be considered as complete, but we have done the best possible with available resources. Many references to books and articles on medicinal folklore could not be obtained by library personnel, even after exhaustive source searches. Such articles may or may not have contained information pertinent to the current effort.

In this section, we shall refer only to plants that have a folkloric reputation as preventing conception or that have been tested in animals or humans for the ability to prevent conception. Conception is being defined here as the successful implantation of a blastocyst in the uterine lining. Plants owing their activity to an estrogenic effect will be considered later in this review. Abortifacients and cytotoxic agents will not be considered in this section, since they do not prevent conception.

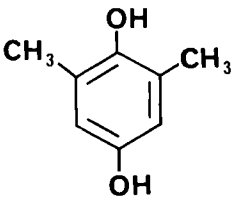
The most helpful references to plants alleged to have contraceptive (and/or interceptive) properties were the reviews of de Laszlo and Henshaw (90), Malhi and Trivedi (91), and Brondegaard (92). Many articles were encountered in which the titles implied that contraceptive plants were to be considered; however, the major thrust of such articles was an enumeration of folkloric abortifacient and/or emmenagogue plants. These will be considered in a subsequent section.

A total of 225 species of plants was found to be used as folkloric contraceptives and/or interceptives. These plants are classified into 181 genera and 76 plant families. The names of these plants, together with other pertinent data, are presented in Table II.

Another source of information on contraceptive and/or interceptive plants was a compilation of papers from the current literature in which, for one reason or another, certain plants were screened for their antifertility effects in laboratory animals. The names of these plants, together with other data such as the plant part used, the type of solvent used for preparing the extract to be tested, the test animal, and comments relative to the results, are presented in Table III. There are 145 species of plants tabulated in Table III, and these are classified into 132 genera and 57 families.

Active Contraceptive Agents—Only a few purified active antifertility principles from higher plants have been isolated. A brief summary will be made concerning each of these principles.

m-*Xylohydroquinone*—In his review of studies leading up to the isolation of *m*-xylohydroquinone (I), Sanyal (174) indicated that a study involving the vitamin contents of different cereals in India had led to the observation that male and female rats fed on a restricted diet consisting entirely of "matar" or peas *(Pisum sativum)* did not produce offspring. Sanyal (143) also cited a study in which litter production in mice was decreased when the animals were fed *P. sativum* at a level of 20% in the diet; litter production was completely abolished when the level was raised to 30%. He noted that the population of Tibet had remained stationary for the previous 200 years and, coincidentally, that the staple diet of the Tibetians consisted of barley and peas.



I: *m*-xylohydroquinone

The active principle of peas was finally isolated from pea oil and characterized as I (174). The compound produced fetal resorption in pregnant rats when given during the first 8 or 10 days of pregnancy but had no effect when given later. Further observations in rats suggested that I might be acting as a vitamin E antagonist.

Sanyal (174) cited a clinical trial of I. The pregnancy rate in Indian women was reduced by about one-half in those receiving this compound as compared with those receiving a placebo. Compound I appeared to be nontoxic in women treated twice monthly with 150–300- (147) or 300–350- (146) mg doses.

Price (175), without citing references, more recently reported that later workers had not been able to substantiate the earlier claims for the antifertility effects of I.

Lithospermic Acid—One of the most intriguing of the antifertility plants is *Lithospermum ruderale.* It was first used by the Navajo Indians as a contraceptive, as reported in 1941 (97). This plant is an herb indigenous to some of the Rocky Mountain states and is commonly known as "stoneweed." In 1945, Cranston (176) reported that when this plant was fed to adult female mice with previously regular estrous cycles, the mice developed prolonged periods of diestrus. This finding was confirmed later (177, 178). In 1949, Cranston and Robinson (179) demonstrated that extracts of *L. ruderale* decreased the gonadotrophic activity of the pituitary glands of adult female mice without producing irreversible or histological damage to the pituitary. In 1952, Kleber and Gisvold (180) confirmed that anestrus was produced in mice fed *L. ruderale,* but a phytochemical study failed to yield active principles.

Plunkett *et al.* (181) and Plunkett and Noble (182) studied the effects of *Lithospermum* extracts on rats and demonstrated that:

Table II—Folkloric Antifertility Plants

Plant Name	Part[a]	Method of Use	Country	Reference
Aizoaceae				
Trianthema pentandra[b]	—	—	India	192
Trianthema portulacastrum[b]	—	—	India	192
Amaranthaceae				
Achyranthes aspera[b]	—	—	India	192
Amaranthus spinosus[b]	—	—	India	171
Amaryllidaceae				
Stenomesson variegatum	—	—	South America	92
Anacardiaceae				
Rhus trilobata	LF	Decoction is drunk	United States	92
Semecarpus anacardium	RT	Root is cooked in sour rice water; taken for 3 days at end of menstruation; produces sterility	India	90, 192
Semecarpus stellata	RT	Decoction is drunk in sour rice mucilage	India	92
Annonaceae				
Annona squamosa[b]	—	—	India	192
Apocynaceae				
Apocynum androsaemifolium	RT	Boiled in water and drunk once weekly	North America	90
Cerbera manghas[b]	—	—	India	171
Nerium indicum[b]	—	—	India	171
Rauvolfia serpentina[b]	—	—	India	192
Thevetia peruviana[b]	—	—	India	171
Araceae				
Acorus calamus	RT	Water decoction is drunk with milk after menstruation	India	91
Anthurium tessmannii	IF	Powdered and added to food eaten by women	Columbia	92, 93
Arisaema atrorubens	RZ	Infusion is drunk with *Asclepias syriaca*	Canada	92
Arisaema triphyllum	RT	Decoction is taken to prevent conception for 1 year	United States	90, 92
Caladium seguinium	PL	Juice is used by women to achieve temporary or permanent sterility; added to diet in prisoner-of-war camps	South America, Germany	90, 94, 95, 175
Dieffenbachia seguine	LF, ST	Chewed by both sexes	Puerto Rico, Guadeloupe, Cuba, Dominican Republic, Santa Lucia	92, 94, 95
Philodendron dyscarpium	IF	Powder is added to food eaten by women	Colombia	92, 93
Urospatha antisylleptica	SP	Powder is added to food eaten by women	Colombia	92, 93
Araliaceae				
Hedera helix	FR	Decoction of dried berries is drunk after "purification"; 1 dram causes sterility	Mediterranean	90, 92
	FL	Infusion is drunk	Iran	92
Aristolochiaceae				
Aristolochia clematitis	SD	Used to prevent conception	Hungary	90, 92
Aristolochia indica[b]	—	—	India	192
Asarum canadense	RT, RZ	Boiled slowly over a long period; decoction is drunk by women	North America	90, 92
Asclepiadaceae				
Asclepias hallii	PL	Infusion is drunk after birth	United States	90, 92
Asclepias syriaca	RT, RZ	Infusion is drunk to produce temporary sterility	Canada	90, 92
Calotropis gigantea[b]	—	—	India	192
Calotropis procera	RT	Water decoction is drunk	India	91
Marsdenia tenacissima	RT	Water decoction is drunk	India	91
Basellaceae				
Basella alba	RT	Water decoction is drunk	India	91
Berberidaceae				
Berberis aristata	EX	Water decoction is drunk	India	91, 192
Epimedium alpinum	LF, RT	Taken in wine after menstruation to prevent conception for 5 days; root causes sterility	Europe	90
Podophyllum hexandrum[b]	—	—	India	171
Betulaceae				
Betula bhojpattra	SB	Water decoction is drunk	India	91
Bignoniaceae				
Dolichandrone falcata[b]	—	—	India	192
Boraginaceae				
Cordia dichotoma	FR	Water decoction is drunk	India	91

Table II—*(Continued)*

Plant Name	Part[a]	Method of Use	Country	Reference
Cordia quarensis	RT	Chewed by young women	Africa	90
Lithospermum arvense	PL	Mixed with food	Europe	90
Lithospermum officinale	RT	Infusion of root is drunk	United States	92
Lithospermum ruderale	RT	Infusion of root is taken daily for 6 months	United States	90, 92, 97
Bromeliaceae				
Ananas comosus	FJ	Fresh juice is taken raw	Malaya	90, 192
Ananas sativa	FJ	Fresh juice is taken raw	Malaya	90
Tillandsia decomposita	FS	Decoction is drunk	South America	92
Buddlejaceae				
Buddleja asiatica	—	—	India	192
Capparidaceae				
Crataeva nurvala[b]	SB	Water decoction is drunk	India	91, 92
Caprifoliaceae				
Lonicera ciliosa	LF	Infusion is drunk	United States	92
Viburnum prunifolium	—	Hot decoction is taken before menstrual period; relieves dysmenorrhea and controls fertility	Italy	90, 92
Caricaceae				
Carica papaya[b]	—	—	India	192
Celastraceae				
Celastrus paniculatus[b]	—	—	India	192
Chenopodiaceae				
Chenopodium album	PL	Mixed with diet	Hungary	90
Salsola sp.	LF	Infusion is drunk	Algiers	92
Commelinaceae				
Aneilema conspicuum[b]	—	—	India	171
Aneilema scapiflorum[b]	—	—	India	171
Compositae				
Achillea millefolium	PL	Hot infusion is drunk or mixed with food	Europe	90
Artemisia siversiana[b]	—	—	India	171
Artemisia vulgaris[b]	—	—	India	171
Atractylis gummifera	RT	Decoction is drunk by men	Arabia	92
Bahia dissecta	RT	Boiled 30 min; drunk during menstruation; used also by men	United States	90, 92
Chrysanthemum indicum[b]	—	—	India	171, 192
Cnicus benedictus	PL	Taken as a tea	North America	90
Echinops echinatus[b]	—	—	India	171
Eupatorium odoratum	RT	Taken orally	Central America	90
Franseria artemisioides	PL	Decoction is drunk	Colombia	92
Stevia rebaudiana	LF, ST	Infusion is drunk	Paraguay	92
Tanacetum umbelliferum	—	—	India	171, 192
Convolvulaceae				
Cuscuta sp.	PL	—	United States	92
Cuscuta reflexa[b]	—	—	India	171, 192
Crassulaceae				
Crassula abyssinica	PL	Used to reestablish the menstrual cycle in case of a late period	Africa	98
Cruciferae				
Anastatica hierochuntica[b]	—	—	India	171, 192
Brassica campestris[b]	—	—	India	171
Brassica nigra	SD	Water decoction is drunk	India	91, 171
Capsella bursa-pastoris	PL	Mixed with food	Europe	90
Lepidium sativum[b]	—	—	India	171, 192
Cucurbitaceae				
Citrullus colocynthis[b]	—	—	India	171, 192
Cucumis sativus[b]	—	—	India	171
Cucumis trigonus[b]	—	—	India	171, 192
Lagenaria siceraria	FR, SD	Water decoction is drunk	India	91
Luffa acutangula[b]	—	—	India	171, 192
Luffa echinata[b]	—	—	India	171
Momordica charantia[b]	—	—	India	192
Momordica tuberosa[b]	—	—	India	192
Trichosanthes bracteata[b]	—	—	India	171
Trichosanthes cucumerina	—	—	India	171
Dioscoreaceae				
Dioscorea sativa var. *rotunda*	TU	Eaten raw without water	Australia	90
Ericaceae				
Rhododendron anthopogon[b]	—	—	India	171
Euphorbiaceae				
Croton tiglium[b]	—	—	India	171, 192
Euphorbia atoto[b]	—	—	India	171, 192
Euphorbia neriifolia	RT	Water decoction is drunk	India	91
Euphorbia resinifera[b]	—	—	India	192
Euphorbia tirucalli[b]	—	—	India	171, 192

(continued)

Table II—*(Continued)*

Plant Name	Part[a]	Method of Use	Country	Reference
Excoecaria agallocha[b]	—	—	India	192
Mallotus sp.	RT	Scrapings from root are chewed with betel and swallowed	Oceania (Buka)	90, 92
Mallotus philippinensis	FR	Water decoction is drunk	India	91
Ricinus communis	SD	Eaten 1 day after delivery	India	91
	SD	Dipped in warm blood of rabbit	Algiers	92
Gentianaceae				
Frasera speciosa	PL	Decoction; one-half cupful is taken occasionally	United States	90, 92
Gramineae				
Bambusa arundinacea	RT	Water decoction is drunk	India	91
Chusquea ramosissima	YS	Chewed	Paraguay	92
Dendrocalamus strictus[b]	—	—	India	192
Echinochloa frumentacea[b]	—	—	India	171
Guttiferae				
Garcinia morella[b]	—	—	India	192
Garcinia pedunculata[b]	—	—	India	171
Juglandaceae				
Juglans regia	LF	Infusion is drunk (with saffron)	Slovakia	92
Labiatae				
Ocimum basilicum	LF	Chewed	Gunantuna	92
Ocimum sanctum	LF	Taken orally	India	99
Origanum majorana	LF	Taken orally as a tea	Germany	90
Rosmarinus officinalis	PL	Decoction with "Ocean *Artemisia*"	Central America (Opata Indians)	92
	PL	Tea with "Ocean *Artemisia*"	Central America (Opata Indians)	90
Salvia officinalis[b]	—	—	India	192
Salvia plebeia[b]	—	—	India	192
Lauraceae				
Cinnamomum cassia[b]	—	—	India	192
Leguminosae				
Abrus precatorius	SD	Powder is eaten	India	192
Butea monosperma	FL, SD	—	India	91, 192
Cassia lanceolata[b]	—	—	India	192
Cicer arietinum[b]	—	—	India	192
Desmodium retroflexum[b]	—	—	India	192
Entada scandens	SD	Eaten raw or roasted	Australia	90, 92
Erythrina variegata var. *occidentalis*[b]	—	—	India	171, 192
Piliostigma thonningii	RT	Infusion is drunk	East Africa	92
Pisum sativum	SO	—	India	171
Prosopis algarobilla	RT	Decoction is drunk	South America	92
Rhynchosia minima[b]	—	—	India	192
Sesbania aegyptica[b]	—	—	India	91, 192
Sesbania sesban[b]	—	—	India	171
Trifolium subterraneum[b]	—	—	India	171
Uraria lagopoides[b]	—	—	India	192
Vigna phaseoloides	RT	Infusion is drunk together with root of *Piliostigma thonningii*	East Africa	92
Liliaceae				
Aloe barbadensis[b]	—	—	India	192
Asparagus acutifolia	FR	Decoction is drunk	Europe	90, 92
Asparagus officinalis	FR	Decoction is drunk	Europe	90, 92
Gloriosa superba[b]	—	—	India	192
Smilacina stellata	RT, LF	Tea from leaves; one-half cupful is drunk daily for 1 week	United States	90, 92
Veratrum californicum	RT	Decoction is drunk	United States	92
Loranthaceae				
Phoradendron flavescens	LF	Taken orally as a tea	United States	90
Lycopodiaceae				
Lycopodium annotinum	PL	Decoction is drunk	Soviet Union	92
Lythraceae				
Lawsonia inermis	—	—	India	192
Magnoliaceae				
Michelia champaca[b]	—	—	India	192
Malvaceae				
Gossypium herbaceum	RT	Decoction is drunk	South America, India	90, 92, 192
Hibiscus abelmoschus	FL	Infusion is drunk	Viti Islands	92
Hibiscus manihot[b]	—	—	India	192
Hibiscus rosa-sinensis	PT	Taken orally	India	99, 192
Hibiscus tiliaceus	FL	Smoked with tobacco	Melanesia, Gunantuna	92
Sphaeralcea munroana	RT	Decoction is drunk	United States	92
Urena lobata	LF	Leaves are chewed and juice is swallowed	New Ireland, India	90, 92, 192

Table II—*(Continued)*

Plant Name	Part[a]	Method of Use	Country	Reference
Melastomataceae				
Memecylon amplexicaule[b]	—	—	India	171
Menispermaceae				
Cissampelos pareira	RT	Water decoction is drunk	India	91
Curarea tecunarum	ST	Water extract of liana is drunk by male and female in gallon quantities	Brazil	172
Moraceae				
Cannabis sativa[b]	—	—	India	192
Moringaceae				
Moringa oleifera[b]	—	—	India	192
Myristicaceae				
Myristica fragrans[b]	—	—	India	192
Virola sp.	ST	—	Brazil	101
Myrsinaceae				
Embelia ribes	RT	Water decoction is drunk	India	91
Oleaceae				
Jasminum multiflorum[b]	—	—	India	171
Palmae				
Cocos nucifera	SD	Milk is drunk	Java	92
	FJ	Juice of ripe or unripe fruit is drunk	Pacific Islands	90
Licuala sp.	RB	Chewed and swallowed by males and females	Solomon Islands (Buka)	92
Pandanaceae				
Pandanus tectorius[b]	—	—	India	171
Papaveraceae				
Argemone mexicana[b]	—	—	India	171
Chelidonium majus	PL	Juice is drunk	Soviet Union	92
Phytolaccaceae				
Phytolacca decandra[b]	—	—	India	171
Piperaceae				
Piper aurantiacum[b]	—	—	India	192
Piper leptostachyum[b]	—	—	India	171
Piper longum[b]	—	—	India	171, 192
Piper nigrum[b]	—	—	India	171, 192
Plantaginaceae				
Plantago lanceolata	PL	Powdered plant in diet	Europe	90
Plumbaginaceae				
Plumbago indica[b]	—	—	India	171
Plumbago rosea[b]	—	—	India	192
Plumbago zeylanica	RT	Water decoction is drunk	India	91, 192
Polemoniaceae				
Phlox stansburyi	LF	Decoction is drunk	United States	92
Polygonaceae				
Eriogonum jamesii	RT	Boiled 30 min; cupful is drunk during menstruation to prevent conception; also used by males	United States	90, 92
Polygonum hydropiper	PL	Infusion is drunk	Europe	90
Polypodiaceae				
Asplenium adiantum-nigrum	—	—	India	90, 92, 171, 192
Dryopteris filix-mas	—	—	India	192
	—	Infusion is drunk	Tartarean women	92
	RT, SD	Used for sterility	Europe	90
Punicaceae				
Punica granatum[b]	—	—	India	171
Ranunculaceae				
Aconitum heterophyllum	RT	Water decoction is drunk daily	India	91
Paeonia officinalis	—	Decoction is drunk	Soviet Union	92
Rosaceae				
Geum macrophyllum	LF	Decoction is drunk	United States	92
Hagenia abyssinica[b]	—	—	India	192
Prunus emarginata	WD	Water decoction is drunk	United States	92
Prunus mahaleb[b]	—	—	India	192
Sanguisorba officinalis	PL	Mixed with normal diet	Europe	90
Rubiaceae				
Anthocephalus cadamba	—	—	India	171
Anthocephalus indicus	LF, FL	—	India	91
Randia spinosa	FR	—	India	91
Rutaceae				
Citrus maxima[b]	—	—	India	171
Citrus medica	FR	Water decoction is drunk	India	91
Salicaceae				
Populus alba	SB	Decoction of bark is drunk	Mediterranean	90, 92
Santalaceae				
Santalum album[b]	—	—	India	192
Sapindaceae				
Sapindus trifoliatus[b]	—	—	India	192

(continued)

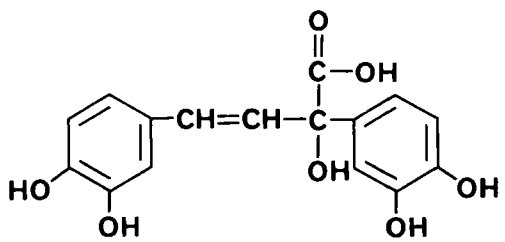
Table II—*(Continued)*

Plant Name	Part[a]	Method of Use	Country	Reference
Schizaeaceae				
Lygodium dichotomum	RT	Root is chewed with betel and some is swallowed	Solomon Islands (Buka)	90, 92
Scrophulariaceae				
Castilleja angustifolia	—	Decoction is drunk	United States	92
Solanaceae				
Datura metel	LF, FR	Water decoction is drunk	India	91
Solanum nigrum[b]	—	—	India	192
Withania somnifera[b]	—	—	India	192
Sterculiaceae				
Abroma agusta[b]	—	—	India	192
Tiliaceae				
Triumfetta bartramia[b]	—	—	India	192
Umbelliferae				
Anethum sowa	FR	Water decoction is drunk	India	91
Apium graveolens[b]	—	—	India	192
Carum carvi	FR	Water decoction is drunk	India	91
Carum roxburghianum	SD	Water decoction is drunk	India	91
Cicuta maculata	RT, RJ	Women chew and swallow roots on 4 consecutive days	United States	90, 92
Cuminum cyminum[b]	—	—	India	192
Ferula assa-foetida	—	—	India	192
Trachyspermum roxburghianum	—	—	India	171
Verbenaceae				
Callicarpa sp.	LF	Leaves are chewed and juice swallowed	Torres-Straits	90, 92
Callicarpa macrophylla[b]	—	—	India	171
Clerodendrum phlomidis[b]	—	—	India	171
Clerodendrum serratum	RT	Water decoction is drunk	India	91
Gmelina asiatica[b]	—	—	India	171
Stachytarpheta jamaicensis var. *indica*	—	—	India	171, 192
Vitex agnus-castus	PL	—	Europe	90
Vitex lagundi	RT	—	North Bougainville, Kurtachi	92
Vitex negundo	RT	Root scrapings are chewed with betel and swallowed	Solomon Islands (Buka)	90
	SD, RB	Water decoction is drunk	India	91, 192
Vitex trifolia[b]	—	—	India	192
Zingiberaceae				
Curcuma longa[b]	—	—	India	171, 192
Curcuma zedoaria[b]	—	—	India	171, 192
Globba marantia	—	—	Melanesia, Gunantuna	92

[a] EX = exudate, FJ = fruit juice, FL = flower, FR = fruit, FS = flower stems, IF = inflorescence, LF = leaf, PL = whole plant, PT = petals, RB = root bark, RJ = root juice, RT = roots, RZ = rhizome, SB = stem bark, SD = seed, SO = seed oil, SP = spadix, ST = stem, TU = tuber, WD = wood, and YS = young stems. [b] These plants have been stated to have "antifertility" properties, but it is possible that they may be abortifacients or emmenagogues.

(a) dried roots of the plant impaired development of the gonads and accessory sex organs of the immature male rat, and *(b)* injections of root extracts were at least 10 times more effective than oral administration of such extracts. Furthermore, similar effects could be produced in the mature animal. Several other groups also demonstrated antigonadotrophic activity for *L. ruderale* extracts (183–186), and Zeller *et al.* (187) showed that such extracts exert an anovulatory effect in hens.

In 1955, Graham and Noble (169) surveyed a number of plants for antigonadotrophic activity and showed this activity to be present not only in *L. ruderale* but also in *L. croccum, L. distichum, L. latifolium, L. arvense, L. officinale, Fatsia horrida, Arctostaphylos uva-ursi, Ambrosia artemisiifolia, Cnicus benedictus, Chenopodium album, Chamaelirium luteum, Amaranthus retroflexus, Borago officinalis,* and *Rubus idaeus.* The *Lithospermum* activity was found to be concentrated in the roots and increased in amounts from June to September (169). Furthermore, root extracts were highly active after a 4-year storage period, but extracts kept at 4° and pH 7.5 lost 50% of their potency after 1 week (169). Other members of the Boraginaceae, *i.e., Anchusa officinalis, Echium vulgare,* and *Symphytum officinale,* have also been shown to elicit antigonadotrophic activity (170).



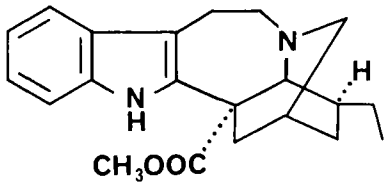
II (?): lithospermic acid

In 1958, Kemper and Loeser (188) showed that extracts of *L. officinale in vitro* and *in vivo* were capable of blocking the effect of pituitary hormones, such as hypophyseal gonadotrophins and thyrotrophin, as well as that of chorionic gonadotrophin, pregnant mare serum gonadotrophin, and prolactin.

In 1963, Johnson *et al.* (113) reported on a tentative structure for lithospermic acid (II), presumably the principal phenolic acid in members of the Boraginaceae eliciting antigonadotrophic activity. In the same year, this research group presented evidence that a very active polyphenoloxidase present in *L. ruderale* polymerizes the inactive lithospermic acid to an active polymer of unknown structure (112).

However, more recently Wagner *et al.* (115) gave evidence that "lithospermic acid" (from *S. officinale* and *Lycopus europaeus*) was a mixture of at least three different substances, based on chromatographic studies, thus refuting the structure work of Johnson *et al.* (113). They confirmed that a polyphenoloxidase preparation from the leaves of *L. europaeus* and *L. officinale* induced the lithospermic acid to acquire antigonadotrophic properties, and further showed that the addition of rutin or chlorogenic acid increased this activity.

Coronaridine—Meyer *et al.* (106) found that an aqueous ethanol extract from the roots of *Tabernaemontana heyneana* prevented pregnancy in adult female rats when the extract was administered orally. Investigation of this plant led to the isolation of a number of indole alkaloids, of which coronaridine (III) was found

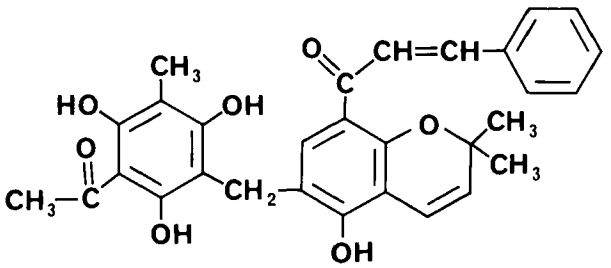


III: coronaridine

to be the active principle. Coronaridine, at a dose level of 5 mg/kg/day, prevented pregnancies and had a high degree of estrogenic activity.

Rutin—Cutting *et al.* (190) showed that a diet containing 0.1% of the flavonoid glycoside rutin impaired the fertility of female mice. But Wilson *et al.* (191) reported that a 1% rutin diet fed for 28–400 days did not modify the estrous cycle of female rats nor impair the fertility of male or female rats.

Rottlerin—The antifertility principle of *Mallotus philippinensis* has been identified as the chromene derivative rottlerin (IV) (168). At a dose level of 10 mg/kg, IV remained 100% effective for 10 days and 84% effective for 20 days; 20 mg/kg produced 100% infertility. Acetylrottlerin also was active, but isorottlerin was inactive (168).



IV: rottlerin

The isoflavones of known structure, as well as the coumestans, sterols, and cytotoxic agents, will be discussed in subsequent sections.

Antifertility Agents of Unknown Structure—Chou *et al.* (137) isolated two saponins having antifertility activity from *Gleditsia horrida*. One saponin was characterized as a triterpene attached to a single hexose sugar.

A new pyrone derivative of unknown structure, referred to as cirantin and isolated from *Citrus aurantium*, was reported to induce antifertility effects in two rabbits given oral doses of 0.75 mg/kg for 7 days (162). No further work has been found concerning this agent.

ABORTIFACIENTS, ECBOLICS, OXYTOCICS, AND EMMENAGOGUES

In this section, the literature concerning abortifacients, ecbolics, oxytocics, and emmenagogues will be reviewed relative to folkloric history, *in vitro* and *in vivo* animal studies (uterine stimulants), and human studies. When reviewing the folkloric uses of plants, it is often difficult to interpret differences between the four mentioned types of biological activities. For this review, any plant mentioned as being used to expel the fetus was considered an abortifacient. This definition groups together all ecbolics, oxytocics, and abortifacients simply as abortifacients. Also included in this category are plants said to be effective in "expelling the placenta," "useful in aiding childbirth," *etc.* Emmenagogues, on the other hand, were most often stated as such, but it is apparent that some authors consider the terms abortifacient and emmenagogue to be synonymous. We considered all plants mentioned to alter the menstrual cycle, unless specifically stated to be abortifacients, ecbolics, or oxytocics, to be emmenagogues.

There are many important reviews from which folkloric data were obtained (90, 91, 192–197), but the majority of plant names were found in minor references in the literature. The names of plants reported in the literature to have abortifacient (A) and emmenagogue (E) applications are presented in Table IV, together with the names of the plant parts used when this information was cited.

To determine whether or not plants having folkloric applications as abortifacients and/or emmenagogues might be confirmed, the literature was searched for plants whose extracts were shown to stimulate uterine tissue by means of *in vitro* or *in vivo* animal experiments. These data are presented in Table IV as (U) notations, together with the part of the plant from which the active extracts were prepared when such data were given.

Table III—Plants Evaluated in Animals or Humans for Antifertility Effects

Plant Name	Part Tested[a]	Type of Extract[b]	Dose and Route of Administration[c]	Species	Results	Reference
Acanthaceae						
Adhatoda vasica	LF	PE, ET, W	7 days before and 14 days during cohabitation in mice and 5 days after mating in rats, po	Mouse, rat	No activity	102
Amaranthaceae						
Amaranthus retroflexus	PL(?)	W	—	Rat	Antigonadotrophic activity *in vitro*	169
Anacardiaceae						
Semecarpus anacardium	SD	PE, ET, W	100 mg/kg/day on Days 1–4, po(?)	Rat	No inhibition of implantation	103
Annonaceae						
Artabotrys odoratissimus	LF	Fresh leaf	0.5–1.0 g/day for 3 days, po	Rat	Diestrus prolonged; degenerative changes in follicles; no body weight changes	104
Apocynaceae						
Apocynum androsaemifolium	PL	—	—	—	Anovulatory effect	171
Apocynum cannabinum	RT	—	—	—	Anovulatory effect	171
Catharanthus roseus	LF	Vinblastine	0.3–0.5 mg/kg, iv	Rabbit	66% reduction in normal fetuses	70
Stemmadenia galeottiana	FR	AC	Twice daily for 5 days, sc	Mouse	Number of litters reduced 44%	105
Tabernaemontana heyneana	RT	Coronaridine	5 mg/kg/day, po	Rat	Antifertility effect	106
Araceae						
Dieffenbachia seguine	SJ	Fresh stem sap, po	—	Mouse	Sterility in males 40–90 days; 30–50 days in females	95
	LF	—	—	Humans	Experimental	94

(*continued*)

Table III—*(Continued)*

Plant Name	Part Tested[a]	Type of Extract[b]	Dose and Route of Administration[c]	Species	Results	Reference
Lysichiton americanum	RT	ET/W	1–2% of diet, po	Mice	Inactive	117
Araliaceae						
Fatsia horrida	PL(?)	W	—	Rat	Antigonadotrophic effect *in vitro*	169
Asclepiadaceae						
Asclepias hallii	—	—	—	—	Anovulatory effect	171
Calotropis gigantea	RT	PE, ET, W	100 mg/kg/day on Days 1–4, po(?)	Rat	No inhibition of implantation	103
Calotropis procera	LF	PE, ET, W	100 mg/kg on Days 1–7, po	Rat	No inhibition of implantation	108
Daemia extensa	LF	In ration	po for 4 weeks; 3 g/day for rats; 9 g/day for guinea pigs	Rat, guinea pig	No effect on fertility	127
Marsdenia cundurango	BK	—	—	—	Anovulatory effect	171
Berberidaceae						
Berberis vulgaris	—	—	—	—	Anovulatory effect	171
Podophyllum peltatum	RT, RZ	Podophyllotoxin	0.25 mg, sc	Mouse	No pregnancies when treated on Day 3 or later	109
	RT, RZ	Podophyllin	5 g/ml in petroleum jelly, 0.2 ml instilled in uterus	Rat	No effect on implantation	110
Boraginaceae						
Anchusa officinalis	PL	W	—	Mouse	Antigonadotrophic activity	170
Borago officinalis	PL(?)	W	—	Rat	Antigonadotrophic activity *in vitro*	169
Echium vulgare	PL	W	—	Mouse	Antigonadotrophic activity	170
Lithospermum arvense	PX	W	—	Rat	Antigonadotrophic activity *in vitro*	169
Lithospermum croceum	PX, RT	W	—	Rat	Antigonadotrophic activity *in vitro*	169
Lithospermum distichum	PX, RT	W	—	Rat	Antigonadotrophic activity *in vitro*	169
Lithospermum latifolium	PX, RT	W	—	Rat	Antigonadotrophic activity *in vitro*	169
Lithospermum officinale	PX	W	—	Rat	Antigonadotrophic activity *in vitro*	169
	LF	W	—	Rat, guinea pig	Antigonadotrophic activity *in vitro*; diestrus in rats	188
	PL	W	1 g/day, po	Mouse	Inhibited vaginal cornification	114
Lithospermum ruderale	PX, RT	W	—	Rat	Antigonadotrophic activity *in vitro*	169
	RT	—	po	Mouse	Cessation of estrous cycles	188
	PX	—	—	Mouse	Depressed estrous cycles	180
	RT	Oxidized lithospermic acid	sc or po daily for 6 days (rat); daily injections for 7 days (hen)	Rat, hen	Antigonadotrophic activity (rat); anovulatory activity (hen)	112
	—	—	In ration, 15% of diet	Mouse	Produced anestrus; nonestrogenic and nonandrogenic	111
Pulmonaria officinalis	PL	W	—	Rat	Antigonadotrophic activity *in vitro*	115
Symphytum officinale	PL	W	—	Rat	Antigonadotrophic activity *in vitro*	115
	RT	Oxidized lithospermic acid	6–12 mg/kg, sc	Mouse	Antigonadotrophic activity increased by rutin or chlorogenic acid	115
Bromeliaceae						
Ananas comosus	RZ, FR	PE, ET, W	"Suitable amount," po	Mouse	Antifertility activity in PE and ET extracts	102
	FR	Juice	50 ml/kg on Days 1–7	Rat	60% inhibition of implantation	116
Caprifoliaceae						
Lonicera ciliosa	LF	ET/W	Dry extract as 1–2% of diet, po	Mouse	45% reduction in litters	117
Caricaceae						
Carica papaya	FP	PE, ET, W	100–500 mg/kg on Days 1–7, po	Rat	Variable inhibition of implantation	118
	SD	In ration	*Ad libitum*	Mouse	Reduced fertility with toxicity	119

Table III—*(Continued)*

Plant Name	Part Tested[a]	Type of Extract[b]	Dose and Route of Administration[c]	Species	Results	Reference
Caryophyllaceae						
Dianthus superbus	PL	W, AC	0.1 ml twice daily for 5 days, sc	Mouse	Interferes with early pregnancy; may be an estrogenic effect	120
	PL	W, AC	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters; post-copulatory effect	121
Vaccaria pyramidata	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters; no post-copulatory effect	121
Chenopodiaceae						
Chenopodium album	LF	In ration	3 g/day for 4 weeks, po	Rat	No effect on fertility	127
	LF	In ration	9 g/day for 4 weeks, po	Guinea pig	No effect on fertility	127
	PL(?)	W	—	Rat	Antigonadotrophic effect *in vitro*	169
Combretaceae						
Terminalia catappa	—	—	—	—	Antifertility effect	171
Compositae						
Ambrosia artemisifolia	PL(?)	W	—	Rat	Antigonadotrophic activity *in vitro*	169
Artemisia sp.	PL(?)	Santonin	Added to ration, po	Mouse	Delay in estrus, ovulation, and onset of mating	119
Artemisia maritima	—	—	—	—	Anti-implantation effect	171
Cnicus benedictus	PL(?)	W	—	Rat	Antigonadotrophic activity *in vitro*	169
Cnicus spicatus	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Grindelia sp.	PL	—	—	—	Anovulatory effect	171
Solidago odora	LF	—	—	—	Anovulatory effect	171
Stevia rebaudiana[d]	PL	W	10 ml of a 5% decoction for 6 days during mating	Rat	Fertility reduced 57–79%	122
Cruciferae						
Capsella bursa-pastoris	PL	In ration	20–40% of ration	Mouse	40% in ration impeded ovulation	123
Isatis oblongata	PL	W (distilled with limestone)	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Raphanus sativus	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Cucurbitaceae						
Ecballium elaterium	PL, FR	Dihydro-elatericin A	20–100 mg/kg/day, po	Mouse	Inhibition of ovulation at 100 mg/kg	124
Luffa cylindrica	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Momordica charantia	LF	W	250–500 mg/day on Days 1–7, po	Rat	No activity	125
Cupressaceae						
Thuja occidentalis	LF	—	—	—	Anovulatory effect	171
Ericaceae						
Arctostaphylos uva-ursi	PL(?)	W	—	Rat	Antigonadotrophic activity *in vitro*	169
Euphorbiaceae						
Euphorbia lathyris	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters; no post-copulatory activity	121
Jatropha curcas	FR, SD	In ration	3.3% of ration for 25 days, po	Rat	Complete inhibition of reproduction	126
Mallotus philippinensis	TR	In ration	Daily for 4 weeks, po; rat 0.75 g/day, guinea pig 3 g/day	Rat, guinea pig	Decreased mating in rats and guinea pigs	127
	TR	Rottlerin	20–840 mg/kg, po	Rat	Prevented mating	168
	—	—	—	—	Anti-implantation effect	171
	SD	PE, ET, W	7 days before and 14 days during cohabitation in mice and 5 days after mating in rats, po	Mouse, rat	No activity	102
Ricinus communis	SD	PE, ET, W	100–500 mg/kg on Days 1–7, po	Rat	No inhibition of implantation	118
Stillingia sylvatica	RT	—	—	—	Anovulatory effect	171
Gramineae						

(continued)

Table III—*(Continued)*

Plant Name	Part Tested[a]	Type of Extract[b]	Dose and Route of Administration[c]	Species	Results	Reference
Dendrocalamus strictus	LF	PE, ET, W	100 mg/kg on Days 1–7, po	Rat	No inhibition of implantation	108
Labiatae						
Hyssopus officinalis	PL	W	—	Rat	Antigonadotrophic	115
Lycopus europaeus	LF	Oxidized lithospermic acid	—	Mouse	Antigonadotrophic activity increased by rutin or chlorogenic acid	115
Lycopus lucidus	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters; postcopulatory activity	121
Majorana hortensis	PL	W	—	Rat	Antigonadotrophic	115
Marrubium officinalis	PL	W	—	Rat	Antigonadotrophic	115
Melissa officinalis	PL	W	—	Rat	Antigonadotrophic	115
Mentha piperita	PL	W	—	Rat	Antigonadotrophic	115
Ocimum sanctum	LF	PE, BZ, E, AC ET	100–200 mg/kg, po	Rat	80% antifertility for BZ extract, 60% for PE extract, others inactive	99
Orthosiphon stamineus	PL	W	—	Rat	Antigonadotrophic	115
Rosmarinus officinalis	PL	W	—	Rat	Antigonadotrophic	115
Salvia officinalis	PL	W	—	Rat	Antigonadotrophic	115
Thymus serpyllum	PL	W	—	Rat	Antigonadotrophic	115
Thymus vulgaris	PL	W	—	Rat	Antigonadotrophic	115
Lecythidaceae						
Combretodendron africanum	SB	W	Various doses, iv and sc	Rat, rabbit	Tannins and saponins inhibited estrous cycle, mating, and pregnancy	130
Leguminosae						
Abrus precatorius	LF	PE, ET, W	7 days before and 14 days during cohabitation in mice and 5 days after mating in rats, po	Mouse, rat	No antifertility activity	102
	SD	PE	150 mg/rat for 20 days before mating or on Days 1–5	Rat, mouse	Antifertility effects	131
	SD	PE, ET, W	100 mg/kg, po; PE, ET = Days 1–4; W = Days 1–7	Rat	No significant inhibition of implantation	103
	RT	PE, ET	100 mg/kg on Days 1–5, po	Rat	Prevention of nidation; ET extract has antiestrogenic activity	100
	SD	PE	7 days before and 14 days during cohabitation in mice and 5 days after mating in rats, po	Mouse, rat	Antifertility activity but toxic	102
Acacia koa	LF	ET, W	Twice daily for 5 days, sc	Mouse	Number of litters reduced 88–100%, but toxic	105
Astragalus glycyphyllus	PL	W	Continuous oral dosage	Rat	Increased duration of estrous phase of cycle	132
Butea frondosa	SD	ET, CH, W	10 mg–1 g/kg/day for 15 days, po	Rat	No change in estrous cycle; delayed mating with higher doses	133
	SD	ET, CH, W	10–1500 mg/kg/day, po; 100 mg/kg on Days 1–5, sc	Mouse, rat	Antifertility effect	133
	PT	ET	10–200 mg/kg	Mouse, rat	Antifertility effect	134
	PT, SD	ET	Various doses, parenteral	Mouse, rat	No estrogenic activity	135
Butea monosperma	SD	ET	25–200 mg/kg for 7 days, po	Rat	No inhibition of implantation	136
	FL	PE, ET, W	100 mg/kg/day on Days 1–7	Rat	No inhibition of implantation	116
Butea parviflora	SD	PE, ET, W	100 mg/kg on Days 1–7, po	Rat	No inhibition of implantation	108
Clitoria ternatea	FR	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Gleditsia horrida	FR	ET	Doses not stated, given for 5 days, sc	Mouse	Two saponins and sugars produced antifertility effect	137
Medicago sativa	IF	E, CH, acid	Not stated	Rat	E extract was estrogenic, CH extract was antiestrogenic, acid extract inter-	141

Table III—*(Continued)*

Plant Name	Part Tested[a]	Type of Extract[b]	Dose and Route of Administration[c]	Species	Results	Reference
					fered with seminal vesicle growth and potentiated action of estrogens	
	—	—	—	Rat	Antigonadotrophic	140
	PL	In ration	po	Rabbits (male)	Degenerative changes in ova after mating	142
Phaseolus aureus	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters; postcopulatory activity	121
Pisum sativum	SO	*m*-Xylohydroquinone	On Days 16 and 21 of cycle, po	Human	Reduction in relative number of pregnancies	143, 144, 146
	SD	In ration	20–30% of diet	Rat	20% decreased and 30% stopped litter formation	148
	SD	—	po	Rat	Decreased fertility	147
	SO	—	im monthly	Human	Postponed pregnancy	147
	SO	*m*-Xylohydroquinone	300–350 mg on Days 16 and 21 of cycle, monthly, po	Human	Reduction in number of pregnancies	147, 148
Psoralea corylifolia	SD	In ration	0.35 g/day for 37–77 days, po	Mouse	Fertility impaired; estrogenic effect Psoralen was not the active principle	149
Sesbania sesban	FL	W	7 days before and 14 days during cohabitation in mice and 5 days after mating in rats, po	Mouse, rat	Antifertility effect	102
	LF	W	7 days before and 14 days during cohabitation in mice and 5 days after mating in rats, po	Mouse, rat	No activity	102
Trifolium subterraneum	PL	In ration	*Ad libitum*, po	Sheep	Impaired release of luteinizing hormone by isoflavones; morphological changes in cervix	150–152
Uraria lagopoides	PL	PE, ET, W	100 mg/kg on Days 1–7, po	Rat	50% inhibition of implantation with W extract	103
Liliaceae						
Aloe barbadensis	LF	W	7 days before and 14 days during cohabitation in mice and 5 days after mating in rats, po	Mouse, rat	Antifertility activity	102
	FP	W	100–500 mg/kg on Days 1–7	Rat	No significant antiimplantation effect	116
Asagraea officinalis	SD	—	—	—	Anovulatory effect	171
Chamaelirium luteum	PL(?)	W	—	Rat	Antigonadotrophic activity *in vitro*	169
Colchicum autumnale	CO	Demecolcine	0.1–5.0 mg/kg, sc	Rabbit	Toxic to fetus when given after implantation	70
	CO	Colchicine	0.5 mg/ml saline; 0.2 ml instilled into one uterine horn	Rat	Few fetuses both horns	110
Gloriosa superba	TU	PE, ET, W	10–100 mg/kg on Days 1–7, po	Rat	No inhibition of implantation; toxic	108
Paris polyphylla	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Smilax bona-nox	RT	—	—	—	Anovulatory effect	171
Veratrum californicum	PL	In ration	*Ad libitum*, po	Sheep	Prolonged gestation associated with cyclopic fetuses	153
Magnoliaceae						
Magnolia virginiana	BK	—	—	—	Anovulatory effect	171
Michelia champaca	RT	PE, ET, W	100 mg/kg on Days 1–7, po	Rat	No inhibition of implantation	108
Malvaceae						
Gossypium herbaceum	SD, ST, SB, RB	PE, ET, W	100 mg/kg on Days 1–4 or 1–7	Rat	No significant antiimplantation effect	116
Hibiscus rosa-sinensis	PT	PE, BZ, E, ET	100–200 mg/kg, po	Rat	80% inhibition of implantation by BZ extract	99
	PT	—	—	—	Anovulatory effect	171

(continued)

Table III—*(Continued)*

Plant Name	Part Tested[a]	Type of Extract[b]	Dose and Route of Administration[c]	Species	Results	Reference
Menispermaceae						
Chondodendron tomentosum	RT	—	—	—	Anovulatory effect	171
Stephania hernandifolia	RZ	PE, ET	7 days before and 14 days during cohabitation in mice and 5 days after mating in rats; po	Mouse, rat	Antifertility effect with ET extract; increased fertility with PE extract	102
Moraceae						
Ficus pumila	LF	W	Twice daily for 5 days, sc	Mouse	61% reduction in litters	105
Moringaceae						
Moringa pterygosperma	SB	PE, ET, W	7 days before and 14 days during cohabitation in mice and 5 days after mating in rats; po	Mouse, rat	No effect by any of the extracts	102
Musaceae						
Ensete superbum	SD	AA	Various dosages for varying periods prior to and after mating, po	Mouse, rat, hamster, rabbit, guinea pig	Anti-implantational and postimplantational effects	155
Myrsinaceae						
Embelia ribes	PL	—	The following is taken in equally divided doses daily for 22 days while abstaining from intercourse: 4 drams active principle of *Embelia ribes*, 4 drams *Piper longum*, 2 drams assafoetida, and 4 drams borax	Human	Prevention of conception for 1 year	138
Myrtaceae						
Metrosideros collina	LF	W	Twice daily for 5 days, sc	Mouse	53% reduction in litters	105
Palmae						
Areca catechu	FR	PE, ET, W	100–500 mg/kg on Days 1–7, po	Rat	Inhibition of implantation with higher dose	118
	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Inactive	121
Papaveraceae						
Argemone glauca	IF	ET	Twice daily for 5 days, sc	Mouse	Reduced litters by 78%	105
	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Inactive	121
Pinaceae						
Pinus ponderosa	LF	VO, W, AC	In ration	Mouse	Complete disruption of pregnancy with W extract	156
	LF[e]	W	In ration	Mouse	See Footnote *e*	145
Piperaceae						
Peperomia sp.	LF, ST	W	Twice daily for 5 days, sc	Mouse	43–50% reduction of litters	105
Piper betle	LF, RT	PE, ET, W	7 days before and 14 days during cohabitation in mice and 5 days after mating in rats, po	Mouse, rat	Inactive	102
Piper latifolium	RT	—	—	—	Anovulatory effect	171
Piper longum	FR	—	See *Embelia ribes* (Myrsinaceae)	Human	Prevents conception for 1 year	138
	LF, RT	PE, ET, W	7 days before and 14 days during cohabitation in mice and 5 days after mating in rats, po	Mouse, rat	Inactive	102
Plumbaginaceae						
Plumbago zeylanica	RT	PE, ET, W	100 mg/kg on Days 1–7, po	Rat	No inhibition of implantation	108
Polygonaceae						
Polygonum hydropiper	PL	In ration	1 g/day	Mouse	Impaired fertility in mice	157
	PL	In ration	9 g/day	Guinea pig	Sterility in female guinea pigs	157
	RT	ET	100 mg/kg on Days 1–7	Rat	80% inhibition of implantation with PE fraction of ET extract	158

Table III—(*Continued*)

Plant Name	Part Tested[a]	Type of Extract[b]	Dose and Route of Administration[c]	Species	Results	Reference
Polygonum multiflorum	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Polypodiaceae						
Aspidium sp.	LF, ST	In ration	*Ad libitum*	Mouse	Reduced fertility with high toxicity	119
Dryopteris filix-mas	—	—	—	—	Anovulatory effect	171
Punicaceae						
Punica granatum	PC	In ration	3 g/day for 4 weeks, po	Rat	Infertile matings	127
	PC	In ration	9 g/day for 4 weeks, po	Guinea pig	Infertile matings	127
Ranunculaceae						
Aconitum napellus	RT	—	—	—	Anovulatory effect	171
Cimicifuga racemosa	RT	—	—	—	Anovulatory effect	171
Paeonia sp.	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Paeonia moutan	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Inactive	121
Rosaceae						
Hagenia abyssinica	LF, ST	In ration	*Ad libitum*	Mouse	Reduced fertility with toxicity	119
Prunus emarginata	WD	EW	1–2% of ration, po	Mouse	45% reduction of litters	117
Pyrus communis	SB	W	po and im	Mouse, rat, dog	Pregnancy interrupted at all stages	159
Quillaja saponaria	BK	—	—	—	Anovulatory effect	171
Rubus idaeus	LF	W	—	Rat	Antigonadotrophic	169
Sanguisorba officinalis	PL	In ration	20–30% of diet, po	Mouse	Prolongation of diestrus	160
	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Inactive	121
Rubiaceae						
Adina cordifolia	LF	ET	100 mg/day after copulation for 5 days, po	Rat	No effect	161
Randia dumetorum	FR	PE, ET, W	100 mg/kg on Days 1–7, po(?)	Rat	No significant inhibition of implantation	103
Rubia cordifolia	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Rutaceae						
Citrus aurantium	PC	Cirantin	0.75 mg/kg for 7 days, po	Rabbit	No pregnancies after several matings	162
Evodia rutaecarpa	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Salicaceae						
Salix sp.	BK	—	—	—	Anovulatory effect	171
Saxifragaceae						
Hydrangea arborescens	RT	—	—	—	Anovulatory effect	171
Scrophulariaceae						
Rehmannia glutinosa	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Simaroubaceae						
Brucea amarissima	SD	ET	Twice daily for 5 days, sc	Mouse	100% reduction in litters	105
Simarouba amara	BK	—	—	—	Anovulatory effect	171
Solanaceae						
Nicotiana tabacum	LF	Smoking	Smoking	Human	Alters ovarian cycle	163
	LF	Nicotine	Implant *in utero*	Rat	Increase in number of stillborn	164
Solanum dulcamara	TW	—	—	—	Anovulatory effect	171
Solanum xanthocarpum	ST, LF	In ration	Rats, 3 g/day; guinea pigs, 9 g/day for 4 weeks, po	Rat, guinea pig	No effect	127
Withania somnifera	RT	In ration	25 mg/day, po	Mouse	Reduction in fertility	165
Stemonaceae						
Stemona japonica	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Taxaceae						
Taxus baccata	LF	PE, ET, W	50–100 mg/kg on Days 1–7 with W extract and on Days 1–5 with PE and ET extracts	Rat	60% infertility with PE and W extracts	166
	ST	PE, ET, W	100 mg/kg on Days 1–4, po(?)	Rat	No significant anti-implantation effect	103

(*continued*)

Table III—(*Continued*)

Plant Name	Part Tested[a]	Type of Extract[b]	Dose and Route of Administration[c]	Species	Results	Reference
	LF	PE, ET, W	100 mg/kg on Days 1–4, po(?)	Rat	55% inhibition of implantation with W extract	103
	LF	In ration	Rat, 3 g/day; guinea pig, 9 g/day, for 4 weeks, po	Rat, guinea pig	No infertility effect	127
Thymelaeaceae						
Daphne genkwa	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Tiliaceae						
Grewia asiatica	SD	PE, ET, W	500 mg/kg on Days 1–7, po	Rat	40–66.6% inhibition of implantation	118
Umbelliferae						
Apium graveolens	SD	PE, ET, W	100 mg/kg on Days 1–7	Rat	No inhibition of implantation	116
Carum carvi	SD	In ration	3 g/day/rat; 9 g/day/guinea pig, for 4 weeks, po	Rat, guinea pig	No effect	127
Daucus carota	SD	PE, ET, W	100–500 mg/kg on Days 1–7, po	Rat	Variable inhibition of implantation	118
	SD	PE, ET, W	20–100 mg/kg on Days 1–7, po	Rat	Various results with different chromatographic fractions	52
Ferula assa-foetida	R	Resin	See *Embelia ribes* (Myrsinaceae)	Human	Prevention of conception for 1 year	138
Leptotaenia reticulata	—	—	—	—	Anovulatory effect	171
Siler divaricatum	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Decreased number of litters	121
Verbenaceae						
Clerodendrum uncinatum	PL	W	—	Rat	Antigonadotrophic	115
Verbena hastata	PL	—	—	—	Anovulatory effect	171
Verbena officinalis	PL	W	—	Rat	Antigonadotrophic	115
	PL	W	0.05–0.2 ml twice daily for 5 days, sc	Mouse	Inactive	121
Vitex agnus-castus	SD	In ration	3 g/day/rat; 9 g/day/guinea pig 4 weeks, po	Rat, guinea pig	No effect	127

[a] BK = bark, CO = corm, FL = flower, FP = fruit pulp, FR = fruit, IF = inflorescence, LF = leaf, PC = pericarp, PL = whole plant, PT = petals, PX = aerial parts, R = resin, RB = root bark, RT = root, RZ = rhizome, SB = stem bark, SD = seed, SJ = stem juice, SO = seed oil, ST = stems, TR = trichomes, TU = tuber, TW = twigs, and WD = wood. [b] Extraction solvents were: AA, absolute ethanol; AC, acetone; BZ, benzene; CH, chloroform; E, ether; ET, 95% ethanol; EW, ethanol–water (1:1); ME, methanol; PE, petroleum ether; VO, volatile oil; and W, water. [c] Routes of administration were: im, intramuscular; ip, intraperitoneal; iv, intravenous; po, oral; and sc, subcutaneous. [d] This activity could not be confirmed in at least two laboratories (128, 129). [e] Activity suspected to be due to mycotoxins produced by fungi parasitizing the pine needles (145).

Table IV—Emmenagogue, Abortifacient, and Uterine Stimulant Plants and Their Active Principles Where Known

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
Acanthaceae				
Adhatoda vasica	A, E	LF	192, 193	—
Dipteracanthus suffruticosa	A	RT	192, 193	—
Ruellia suffruticosa	See *Dipteracanthus suffruticosa*			
Strobilanthes crispus	U	LF	199	—
Agavaceae				
Agave americana	A	—	192	—
	E	LJ	193	—
	E	PL, JU	194	—
Agave lecheguilla	U	—	200	Crude saponin
Yucca gloriosa	U	—	201	—
Aizoaceae				
Carpobrotus edulis	A	FR	194	—
Trianthema decandra	A	PL	193	—
Trianthema monogyna	See *Trianthema portulacastrum*			
Trianthema pentandra	See *Trianthema decandra*			
Trianthema portulacastrum	A	—	192	—
	A	RT	193	—
Alangiaceae				
Alangium lamarckii	U	LF	202	Total alkaloids
	I	—	203	Emetine (CXXVIII)
Alismataceae				
Sagittaria sagittifolia	A	—	192	—
Amaranthaceae				
Achyranthes aspera	A	—	204	—
	A	LJ	192, 193	—
Alternanthera sessilis	A	—	194	—
Amaranthus gangeticus	E	LF	193	—
Amaranthus thunbergii	A	PL	194	—
Bragantia tomentosa	A	—	192	—

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	E	PL	193	—
Amaryllidaceae				
Clivia miniata	A	RT	194	—
	I	—	204–210	Lycorine (CXXVII)
Crinum sp.	I	—	211	Hordenine (VI)
	I	—	205–210, 218	Lycorine
Crinum asiaticum	A	—	192	—
	I	—	205–210, 218	Lycorine
Pancratium sp.	I	—	211, 310	Hordenine
	I	—	212–217, 312	Tyramine (IX)
Pancratium sickenbergi	U	BU	218	—
	I	—	205–210, 218	Lycorine
Anacardiaceae				
Anacardium occidentale	U	TE	219	—
Lannea schimperi	A	BK	220	—
Rhus coriaria	U	—	221	—
Rhus glaucescens	See *Rhus longipes*			
Rhus incana	See *Rhus longipes*			
Rhus incana var. *cuneidoliolaya*	See *Rhus longipes*			
Rhus longipes	A	PL	222	—
Rhus ruzizensis	See *Rhus longipes*			
Rhus succesanea	I	—	223	Ellagic acid (CXXXIII)
Rhus toxicodendron	U	—	224	—
Rhus villosa	See *Rhus longipes*			
Semecarpus anacardium	A	—	192	—
	A	SD	193	—
	E	SD	193	—
Spondias lutea	U	SB	219	—
Tapirira guianensis	U	SB	219	—
Annonaceae				
Annona muricata	U	LS	225	—
Annona squamosa	A	—	192	—
	A	SD	193	—
	U	—	219	—
Unona setigera	See *Uvaria rufa*			
Uvaria ridleyi	See *Uvaria rufa*			
Uvaria rufa	A	RT	195	—
Uvaria setigera	See *Uvaria rufa*			
Uvaria solanifolia	See *Uvaria rufa*			
Apocynaceae				
Aganosma marginata	A	—	192	—
	E	PL, RT	193	—
Alstonia constricta	U	BK	226, 227	Total alkaloids
	I	—	228–232	Reserpine (LXXXIX)
Alstonia scholaris	A	—	192	—
	E	BK	193	—
	I	—	228–232	Reserpine
Anodendron paniculatum	A	—	192	—
	A	RT	90	—
Carissa edulis	A	RT	233	—
Catharanthus roseus	A	RT	234	—
Cerbera odollam	A	SD, FR	193	—
	A	—	192	—
Ervatamia coronaria	A	—	192	—
	E	RT	193	—
Forsteronia floribunda	U	LS	225	—
Holarrhena antidysenterica	E	SD	91	—
Holarrhena febrifuga	A	RT, LF	194	—
	E	LF	194	—
Nerium indicum	A	RT	193	—
	E	RT	193	—
	U	—	201	—
Nerium odorum	See *Nerium indicum*			
Nerium oleander	U	—	235, 236	—
Peschiera affinis	U	RB	219	—
Pleioceras barteri	A	FR, SD, RB, LF	196	—
	E	FR, SD, RB, LF	196	—
Plumeria acuminata	A	—	193	See also *Plumeria rubra* f. *acutifolia*
Plumeria bracteata	U	RB	219	—
Plumeria rubra	A	FR	194	—
	E	FR	194	—
Plumeria rubra f. *acutifolia*	A	—	193	—
Rauvolfia serpentina	A	RT	91, 193	—
	A	—	192	—

(*continued*)

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	U	RT	237	Total alkaloids
	I	RT	228–232	Reserpine
	I	RT	238	Neoajmaline (ajmaline) (XCI)
	I	—	239	Corynanthine (LXXXVIII)
Rauvolfia vomitoria	A	LF	194	—
	E	LF	194	—
	I	—	228–232	Reserpine
Strophanthus sp.	I	—	240–244	Strophanthin (CXLIII)
Tabernanthe iboga	I	—	245, 246	Ibogaine (LXXXIII)
Thevetia nereifolia	See *Thevetia peruviana*			
Thevetia peruviana	A	SD	192, 193	—
	A	SD, PL	194	—
	E	SD	193	—
	U	—	247	—
	I	—	248	Thevetin (CXLIV)
Vallaris solanacea	U	LF	249	Crude glycosidic mixture
Vinca erecta	U	—	250	Alkaloid mixture
	H	—	257, 258	Vincamine (XC)
	I	—	251	Ervamine (LXXXV)
	I	—	252, 256	Vincamine
	I	—	251, 254, 255	Vineridine (XCV)
	I	—	251	Vinervine (LXXXVI)
Vinca major	A	—	194	—
	A	RT	259	—
	I	—	228–232	Reserpine
Araceae				
Acorus calamus	A	—	192	—
	E	RZ	193	—
Amorphophallus campanulatus	A	—	192	—
	E	RT	193	—
Cyrtosperma griffithii	See *Cyrtosperma merkusii*			
Cyrtosperma merkusii	A	SP	195	—
Dieffenbachia seguine	U	LS	225	—
Dracontium polyphyllum	A	—	192	—
	E	PL	193	—
Dracunculus vulgaris	E	PL	194	—
Lasia merkusii	See *Cyrtosperma merkusii*			
Scindapsus officinalis	E	FR	91	—
Araliaceae				
Cussonia arborea	E	RT	194	—
Hedera arborea	See *Hedera helix*			
Hedera helix	E	EX	194	—
	E	LF	197	—
Aristolochiaceae				
Aristolochia sp.	I	—	260, 261	Aristolochic acid (CL)
Aristolochia bracteata	A	—	192	—
	E	PL	193	—
Aristolochia clematitis	E	RZ	197	—
	U	—	262	—
	I	—	260, 261	Aristolochic acid
Aristolochia indica	A	—	192	—
	A	RT, PL	193	—
	A	RT	91	—
	I	—	260, 261	Aristolochic acid
Aristolochia rotunda	E	RT	197	—
	I	—	260, 261	Aristolochic acid
Asclepiadaceae				
Asclepias tuberosa	U	RT	263, 264	—
Calotropis gigantea	A	LX	265	—
	A	—	192	—
	E	LX	193	—
	U	LX	265, 266	—
	U	TW	204	—
Calotropis procera	A	—	192, 267	—
	A	PL	192, 194	—
	E	LX	193	—
	U	RT	268	—
Chlorocodon whitelii	A	PL	194	—
Daemia extensa	A	—	192	—
	E	LF	90	—
	I	—	269	Uncharacterized glycoside
Gymnema sylvestre	A	LF	194	—
	I	—	270	Betaine (CXV)
Omphalogonus nigritanus	A	LF, RT	196	—
Pergularia extensa	A	LF	196	—
	A	—	192	—
	E	LF	196	—
Tylophora asthmatica	A	—	192	—
Xysmalobium undulatum	A	RT	194	—

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	U	RT	271	Uzarine
Berberidaceae				
Berberis sp.	I	—	272, 274–280	Berberine (umbellatine) (LII)
Berberis amurensis	U	RT	277	—
	I	—	273	Jatrorrhizine (LIV)
Berberis aristata	A	—	192	—
	E	RT, WD	193	—
	I	—	274–280	Berberine
	I	—	280, 281, 597, 599	Palmatine (LV)
Berberis lycium	I	—	282	Berberine (umbellatine)
	I	—	274–280	Erysotrine (CXXIII)
Berberis thunbergii	U	—	283	—
	I	—	274, 280	Berberine
	I	—	284	Columbamine (isocorypalmine) (LIII)
	I	—	273	Jatrorrhizine
	I	—	281	Palmatine
Berberis vulgaris	U	SB	278, 285	Total alkaloids
	U	LF	286	—
	U	BK	287	—
	U	PL	288	—
	I	—	274–280	Berberine
	I	—	284	Columbamine
	I	—	273	Jatrorrhizine
	I	—	281	Palmatine
Caulophyllum thalictroides	A	RT	90	—
	E	RT	90, 289	—
Caulophyllum thalictroides var. *robustum*	U	—	283	—
Epimedium sempervirens	U	—	283	—
Leontice alberti	U	—	290	Alkaloid mixture
Nandina domestica	I	—	274–280	Berberine
	I	—	273	Jatrorrhizine
	I	—	291	Nandinine (XLIX)
Betulaceae				
Betula paltyphylla	U	—	283	—
Bignoniaceae				
Dolichandrone falcata	A	—	192	—
	A	FR, PL	193	—
Bombacaceae				
Adansonia digitata	A	—	192	—
	E	FS	193	—
Bombax malabaricum	U	SD	292, 293	—
Boraginaceae				
Cordia quarensis	A	RT	194	—
Heliotropium angiospermum	U	LS	294	—
Heliotropium indicum	A	FL	193	—
	A	—	192	—
	E	FL	193	—
	U	RT	295	—
	I	—	296	Retrornecine (LXXIII)
Tournefortia hirsutissima	U	LS	294	—
Bromeliaceae				
Ananas comosus	A	—	192	—
	A	LF, FR	193	—
	A	FR, JU	194	—
	E	LF, FR	193	—
	E	FR	194	—
Ananas sativus	A	LF, FR	193	—
	I	—	297–309	5-Hydroxytryptamine (LXXVII)
Buddlejaceae				
Buddleja asiatica	A	PL	193	—
	A	—	192	—
Burseraceae				
Balsamodendron mukul	A	—	192	See also *Commiphora mukul*
	E	—	193	—
Balsamodendron myrrha	A	—	192	See also *Commiphora myrrha*
Boswellia glabra	A	—	192	—
	E	BA, RA	193	—
Boswellia serrata	A	—	192	—
	E	VO, GU	193	—
Commiphora mukul	A	—	192	—
	E	GU, RE	193	—
Commiphora myrrha	A	—	192	—
	E	GU	193	—

(*continued*)

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
Cactaceae				
Harrisia gracillis	U	LS	294	—
Lophophora williamsii	I	PL	211, 310	Hordenine
	I	—	311	Mescaline (VII)
	I	—	212, 217, 312	Tyramine
Calycanthaceae				
Calycanthus sp.	I	—	313	Calycanthine (CXIX)
Cannabaceae				
Cannabis sativa	A	—	192	—
	A	SD	193	—
	E	PL	193	—
	U	—	219, 314–316	—
	I	—	317	Nicotine (X)
Capparidaceae				
Cadaba farinosa	A	—	192	—
	E	LF, RT	193	—
	E	LF	194	—
Cadaba indica	See *Cadaba farinosa*			
Cadaba trifoliata	A	—	192	—
	E	RT, LF	193	—
Capparis aphylla	See *Capparis decidua*			
Capparis decidua	A	—	192	—
	E	PL	193	—
Capparis spinosa	A	—	192	—
	E	RB	193	—
Crataeva religiosa	E	LF	194	—
Crataeva tapia	U	LF	219	—
Gynandropsis gynandra	A	LF	194	—
Gynandropsis pentaphylla	A	—	318	—
Caprifoliaceae				
Viburnum foetidum	A	—	192	—
	E	PL	193	—
	U	LF	267	—
Caricaceae				
Carica papaya	A	FR, LX, SD	193	—
	A	—	192	—
	E	FR, LX, SD	193	—
	I	—	297–309	5-Hydroxytryptamine
Caryophyllaceae				
Lychnis miqueliana	U	—	201	—
Silene gallica var. *quinquevulnesea*	U	—	201	—
Casuarinaceae				
Casuarina equisetifolia	A	BK	195	—
Celastraceae				
Celastrus paniculatus	A	BK, LF	193	—
	A	—	192	—
	E	LF	91, 193	—
Euonymus cannabinum	A	—	192	—
Maytenus senegalensis	A	LF, RT	220	—
Chenopodiaceae				
Beta vulgaris	A	—	192	—
	E	SD, RT, LF	193	—
	E	LF, RT	194	—
Chenopodium ambrosioides	A	PL	194	—
	A	—	192	—
	E	PL	193	—
	E	LF	194, 197	—
Chenopodium vulvaria	E	LF	194	—
Mabrina ambrosioides	See *Chenopodium ambrosioides*			
Salicornia brachiata	E	AH	193	—
Salsola arbuscula	I	—	319	Salsoline (XVII)
Chloranthaceae				
Chloranthus serratus	U	—	201	—
Cochlospermaceae				
Cochlospermum insigne	U	SB	219	—
Cochlospermum tinctorium	E	RT, ST	193	—
Cochlospermum vitifolium	U	SB	219	—
Combretaceae				
Combretum gueinzii	A	RT	194	—
Terminalia bellerica	U	FR	320	—
Terminalia catappa	U	LS	294	—
Terminalia chebula	U	—	321	—
Terminalia ivorensis	I	—	322	Ellagic acid (CXXXIII)
Commelinaceae				
Aneilema conspicuum	A	—	192	—
	E	PL	193	—
Aneilema lineolatum	A	PL	193	See also *Murdannia elata*

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
Murdannia elata	A	PL	193	—
Compositae				
Achillea millefolium	A	—	192	—
	E	FL, PL, LJ	193	—
	E	FL, LF	193	—
Anthemis nobilis	A	—	192	—
	E	VO	193	—
	E	PL	197	—
Anthemis tinctoria	E	FL, LF	197	—
Arctium lappa	U	—	201	—
Arnica sp.	U	—	324	—
Artemisia sp.	H	—	325	—
Artemisia abrotanum	E	LF, FL, SD	197	—
Artemisia absinthium	E	LF, FL	197	—
	H	—	326	—
Artemisia pontica	E	LF, FL	197	—
Artemisia siversiana	A	—	192	—
	E	PL	193	—
Artemisia vulgaris	A	PL	193	—
	A	—	192	—
	E	PL, LF, FL	193	—
	E	PL	323	—
	E	LF, RT, FL	197	—
	U	—	265	—
Atractylis lanatus	See *Carthamus lanatus*			
Atractylodes lancea	U	—	201	—
Baccharis articulata	U	—	327	—
Balsamita major	See *Tanacetum balsamita*			
Balsamita suaveolens	See *Tanacetum balsamita*			
Balsamita vulgaris	See *Tanacetum balsamita*			
Bidens pilosa	A	—	318	—
Blumea balsamifera	A	—	192	—
	E	LF	193	—
Blumea eriantha	A	—	192	—
	E	PL	193	—
Blumea lacera	A	—	192	—
	E	PL	193	—
Calcitrapa stellata	See *Centaurea calcitrapa*			
Calendula arvensis	A	—	192	—
	E	FL	193	—
	E	LF, FL	197	—
Calendula officinalis	A	—	192	—
	E	PL	197	—
	E	FL	193, 323	—
Carduus marianus	See *Silybum marianum*			
Carduus stellatus	See *Centaurea calcitrapa*			
Carthamus lanatus	E	LF, FL	197	—
Carthamus maculatus	See *Silybum marianum*			
Carthamus tinctorius	A	—	192	—
	E	FL	193	—
Centaurea calcitrapa	E	RT, FL	197	—
Centaurea centaurium	E	RT	197	—
Centaurea cyanus	A	—	192	—
	E	FL	193	—
Centaurium majus	See *Centaurea centaurium*			
Centipeda orbicularis	A	—	192	—
Chamaemelum tinctorium	See *Anthemis tinctoria*			
Chamomilla nobilis	See *Anthemis nobilis*			
Chrysanthemum indicum	A	—	192	—
	E	FL	193	—
Chrysanthemum leucanthemum	E	PL	197	—
Chrysanthemum parthenium	E	PL	197	—
Chrysanthemum sinense	E	PL	194	—
	I	—	328	Stachydrine (CXIV)
Chrysanthemum tanacetum	See *Tanacetum vulgare*			
Chrysanthemum vulgare	See *Tanacetum vulgare*			
Cichorium intybus	A	—	192	—
	E	PL	193	—
	E	SD	194	—
	E	RT	323	—
Conyza squarrosa	See *Inula conyza*			
Conyza vulgaris	See *Inula conyza*			
Costus hortensis	See *Tanacetum balsamita*			
Cota tinctoria	See *Anthemis tinctoria*			

(*continued*)

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
Cotula sp.	A	—	192	—
	E	—	193	—
Dichrocephala chrysanthemifolia	A	PL	194	—
Doronicum pardalianches	E	RZ	197	—
Echinops echinatus	A	RT	193	—
Eriocoma floribunda	U	—	329	—
Ethulia conyzoides	A	RT	222	—
Eupatorium cannabinum	A	—	192	—
	E	PL	193	—
	E	FL, RZ, LF	197	—
Eupatorium odoratum	A	—	192	—
Gazania pinnata	E	LF, FL	194	—
Glossocarida bosvallea	A	—	192	—
	E	PL	193	—
Glossocardia linearifolia	See *Glossocardia bosvallea*			
Grangea maderaspatana	A	—	192	—
	E	RT, LF	193	—
	U	FR	330	—
	U	LF	267	—
Gutierrezia microcephala	U	—	200	Crude saponin mixture
Helianthus annuus	A	—	192	—
	E	FL	193	—
Hentrophyllum lanatum	See *Carthamus lanatum*			
Inula conyza	E	LF	197	—
Inula graveolens	E	PL	194	—
Inula squarrosa	See *Inula conyza*			
Leucanthemum parthenium	See *Chrysanthemum parthenium*			
Leucanthemum vulgare	See *Chrysanthemum leucanthemum*			
Leuzea carthamoides	U	—	331	—
Matricaria chamomilla	E	PL	197	—
Matricaria parthenium	See *Chrysanthemum parthenium*			
Melanthera scandens	A	LF	194	—
Microglossa pyrifolia	A	PL	194, 233	—
Montanoa tomentosa	A	—	90, 332	—
	E	—	332	—
Ormenis nobilis	See *Anthemis nobilis*			
Petasites sp.	I	—	333	Platyphylline (LXVIII)
	I	—	333	Senecionine (LXX)
Petasites officinalis	E	RZ, LF, FL	197	—
Porophyllum lencospermum	E	RT	334	—
Pyrethrum indicum	A	RT	193	—
	A	—	192	—
Pyrethrum parthenium	See *Chrysanthemum parthenium*			
Pyrethrum tanacetum	See *Tanacetum balsamita*			
Pyrethrum umbelliferum	A	—	192	—
	A	PL	193	—
Pyrethrum vulgare	See *Tanacetum vulgare*			
Santolina chamaecyparissus	E	LF, FL, SD	197	—
Santolina incana	See *Santolina chamaecyparissus*			
Saussurea lappa	A	—	192	—
	E	RT	91, 193	—
Senecio sp.	I	—	501–503	Cytisine (XLII)
	I	—	333	Integerrimine (LXVI)
	I	—	333	Longilobine (LXX)
	I	—	333	Platyphylline (LXVIII)
	I	—	333, 335	Pterophine[e]
	I	—	333	Retrornecine (LXXIII)
	I	—	333	Riddelliine (LXIX)
	I	—	333	Senecionine (LXX)
	I	—	333	Seneciphylline (jacobine) (LXXI)
	I	—	333	Spartioidine (LXXII)
Senecio sceleratus	I	—	333	Isatidine (LXVII)
	I	—	333	Sceleratine (LXV)
Senecio spartioides	I	—	333	Spartioidine
Senecio vulgaris	E	PL	194, 197	—
	I	—	333	Jacobine (seneciphylline)
	I	—	333	Platyphylline
	I	—	336	Senecine[e]
	I	—	333	Senecionine
	I	—	333	Seneciphylline (jacobine)
Siegesbeckia orientalis	A	—	192	—
	E	PL	193	—
Silybum marianum	E	RT, SD	197	—
	I	—	212, 217, 312	Tyramine
Sonchus asper	A	—	318	—

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
Sphaeranthus sp.	A	PL	194	—
Sphaeranthus indicus	A	—	192	—
	E	PL	193	—
	U	—	337	—
Spilanthes acmella	A	—	318	—
Spina alba-hortensis	See *Silybum marianum*			
Tagetes minuta	E	LF	194	—
Tanacetum sp.	A	—	192	—
Tanacetum balsamita	E	LF, FL	197	—
Tanacetum vulgare	E	—	338	—
	E	PL	193	—
	E	FL, LF	197	—
	U	VO	339[d]	—
Tithonia diversifolia	U	LS	294	—
Tussilago farfara	A	RT, LF	193	—
	A	—	192	—
Tussilago hybrida	See *Petasites officinalis*			
Tussilago petasites	See *Petasites officinalis*			
Vernonia corymbosa	A	BU	194	—
	E	BU	194	—
Convolvulaceae				
Convolvulus bidentatus	A	PL	194	—
Convolvulus ulosepalus	A	PL	194	—
Cuscuta chinensis	A	—	204	—
Cuscuta reflexa	A	—	192	—
	A	ST, SD	193	—
	E	SD	193	—
Exogonium purga	E	TR	340	—
Ipomoea violacea	U	SD	341	Alkaloid fraction (ergonovine) (XCIII)
Operculina macrocarpa	U	TU	219	—
Rivea corymbosa	U	SD	341	Alkaloid fraction (ergonovine)
Coriariaceae				
Coriaria japonica	I	—	322	Ellagic acid
Crassulaceae				
Crassula sp.	A	RT	194	—
Cruciferae				
Anastatica hierochuntica	A	—	192	—
	A	PL	193	—
Brassica alba	A	—	192	—
Brassica cernua	U	—	201	—
Brassica juncea	E	—	193	—
Brassica nigra	A	—	192	—
Capsella bursa-pastoris	A	PL	192	—
	A	—	194	—
	E	PL	90, 194	—
	E	RT	197	—
	U	—	342	—
	I	—	212, 217, 312	Tyramine
Cheiranthus cheiri	A	—	192	—
	E	FL	193	—
Lepidium sativum	A	—	192	—
	A	SD	91, 193, 194	—
Matthiola incana	A	—	192	—
	E	LF	193	—
Matthiola tristis	A	—	192	—
	E	LF	193	—
Matthiola varia	E	LF	193	—
Nasturtium fontanum	See *Nasturtium officinale*			
Nasturtium officinale	A	—	192	—
	A	PL	90, 194	—
	E	PL	90, 194, 197	—
Raphanus sativus	A	—	192	—
	E	SD, LF	323	—
	E	SD	193, 194	—
Sisymbrium cardamime	See *Nasturtium officinale*			
Sisymbrium nasturtium	See *Nasturtium officinale*			
Cucurbitaceae				
Citrullus colocynthis	A	—	192	—
	A	RT	193	—
	E	RT	194	—
Cucumis trigonus	A	RT	193	—
	A	—	192	—
Cucurbita foetidissima	U	—	343	—
Gymnopetalum cochinchinensis	A	PL	193	—
	A	—	192	—
Luffa acutangula	A	—	192	—
Luffa acutangula var. *amara*	A	RB	193	—
Luffa cylindrica	U	LS	294	—

(*continued*)

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
Luffa echinata	A	RB	193	—
	A	—	192	—
Luffa operculata	U	FR	219	—
Momordica balsamina	A	RT, FR, SD	194	—
Momordica cabrei	U	FR, BR, RT	344	—
Momordica charantia	A	PL, RT, JU	193	—
	A	—	192	—
	A	RT	194	—
	E	PL, RT, JU	193	—
Momordica cymbalaria	See *Momordica tuberosa*			
Momordica foetida	A	—	318	—
Momordica tuberosa	A	TR	193	—
	A	—	192	—
Trichosanthes bracteata	A	FR	193	—
Trichosanthes cucumerina	A	LS	193	—
	A	—	192	—
	E	LS	193	—
Trichosanthes palmata	See *Trichosanthes bracteata*			
Wilbrandia sp.	U	—	219	—
Cupressaceae				
Juniperus sp.	U	VO	345	—
Juniperus communis	A	VO	193	—
	A	—	192	—
	E	ST, VO, FR	193	—
Juniperus sabina	E	LF	197	—
	U	—	346	—
	U	VO	347–349, 919	—
	H	—	350–353	—
Thuja occidentalis	A	—	354	—
	U	—	355, 356	—
	H	—	355	—
Cyperaceae				
Carex brevicollis	I	—	357–361	Brevicolline (LXXIX)
	I	—	362, 398	Harman
Cyperus canescens	A	—	192	—
Cyperus longus	A	—	192	—
	E	TU	193	—
Cyperus rotundus	A	—	192	—
	A	TU	193	—
	A	TU	194	—
	E	RT, TU	194	—
Cyperus scariosus	E	RT	192, 193	—
Dipsacaceae				
Scabiosa succisa	E	RZ, PX	197	—
Succisa pratensis	See *Scabiosa succisa*			
Succisa scabiosa	See *Scabiosa succisa*			
Dipterocarpaceae				
Vateria indica	A	—	192	—
Ebenaceae				
Diospyros tricolor	U	—	363	—
	I	—	363	Uncharacterized quinone[e]
Ephedraceae				
Ephedra vulgaris	U	—	364	—
	I	—	364–380	Ephedrine
	I	—	381	Pseudoephedrine (VIII)
Ericaceae				
Ledum palustre var. *nipponicum*	U	—	201	—
Erythroxylaceae				
Erythroxylum coca	A	—	192	—
	E	LF	193	—
	I	LF	382–396	Cocaine (XIII)
	I	LF	317, 798–800	Nicotine
Lyonia ovalifolia var. *elliptila*	I	—	397	Lyoniol A (lyoniatoxin) (CXL)
Euphorbiaceae				
Acalypha indica	A	—	192	—
	E	—	193	—
Acalypha petiolaris	A	RT	194	—
Antidesma ghaesaembilla	A	—	192	—
	E	WD	193	—
Croton linearis	U	LS	225	—
Croton tiglium	A	RT, SO	193	—
	A	—	192	—
Euphorbia atoto	A	JU	193	—

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	A	—	192	—
Euphorbia lathyris	I	—	398	Aesculetin (CXXXIV)
Euphorbia pilulifera	U	—	399	—
Euphorbia resinifera	A	PL	193	—
	A	—	192	—
Euphorbia restituta	A	—	194	—
Euphorbia tirucalli	A	JU	193	—
	A	—	192	—
Excoecaria agallocha	A	LX	193	—
	A	—	192	—
Jatropha glandulifera	A	—	192	—
	E	LF	193	—
Jatropha multifida	A	—	192	—
	E	SO	193	—
Mildbraedia fallax	E	PL	194	—
Phyllanthus urinaria	A	—	192	—
	A	PL	193, 194	—
	E	PL	194	—
Ricinus communis	A	—	192	—
	E	LF, SO, SD	193	—
	E	LF	194	—
	U	LS	294	—
	I	—	400	Ricin[e]
	H	SO	401–409	Castor oil
Tragia involucrata	A	—	204	—
Gentianaceae				
Gentiana kurroa	A	—	192	—
	E	PL	193	—
Geraniaceae				
Erodium sp.	I	—	212, 217, 312	Tyramine
Erodium cicutarium	A	PL	194	—
	U	—	410	—
Pelargonium fumarioides	A	PL	194	—
Pelargonium reniforme	E	PL	194	—
Sarcocaulon sp.	A	PL	194	—
Gramineae				
Andropogon iwarancusa	A	—	192	—
Andropogon monticola	A	—	192	—
Andropogon muricatus	A	—	192	See also *Vetiveria zizanioides*
Andropogon schoenanthus	E	PL	194	—
Andropogon sorghum	I	—	211, 310	Hordenine
Arundo donax	A	—	192	—
	E	RZ	193, 197, 411	—
	I	—	412, 413	Gramine (= donaxine) (LXXVI)
Bambusa arundinacea	E	LF	193	—
Bambusa bambos	A	—	192	—
Brachiaria purpurascens	U	—	414	—
Coix lacryma-jobi var. *frumentacea*	I	—	415	Palmitic acid (CLIV)
Cymbopogon citratus	U	—	201	—
Cymbopogon iwarancusa	A	—	192	—
	E	PL	193	—
Cymbopogon nardus	E	PL	194	—
Dendrocalamus strictus	A	LF	193	—
	A	—	192	—
Eleusine indica	A	LF, JU	194	—
Eriocoma floribunda	A	PL	416	—
	U	—	416	—
Lolium temulentum	E	FR	197	—
	U	—	201	—
Panicum sp.	I	—	211, 310	Hordenine
Panicum maximum	U	LS	294	—
Vetiveria zizanioides	E	RT, RZ	193	—
Guttiferae				
Garcinia morella	A	—	192	—
	A	GU, RE	193	—
Hypericum perforatum	A	—	192	—
	E	PL	193, 323	—
	U	LF	267	—
Mesua ferrea	A	—	192	—
	E	PL	193	—
Ochrocarpus longifolius	A	—	192	—
Halorrhagidaceae				
Gunnera perpensa	A	RT	194	—
Iridaceae				
Crocus sativus	A	—	192	—
	E	—	323, 417	—

(*continued*)

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	E	SG, SL	193	—
	H	—	418	—
	U	—	419	—
Iris foetidissima	E	RZ	197	—
Iris germanica	U	RT	420	—
Iris sisyrinchium	U	SD	421	—
Spatula foetida	See *Iris foetidissima*			
Labiatae				
Ajuga genevensis	I	—	422	—
Ballota foetida	See *Ballota nigra*			
Ballota nigra	E	LF, FL	197	—
Calaminta clinopodium	See *Clinopodium vulgare*			
Cataria vulgaris	See *Nepeta cataria*			
Clinopodium vulgare	E	LF, FL	197	—
Coleus sp.	A	LF	194	—
Coleus barbatus	E	PL	194	—
Coleus kilimandscharica	A	LF	194, 423	—
Fuerstia africana	E	PL	194	—
Hedemoa pulegioides	E	LF, FL.	424	—
	U	VO	425[d]	—
Hyptis pectinata	A	—	192	—
	E	PL	193	—
Hyssopus officinalis	A	—	192	—
	E	LF, PL	193	—
	E	LF	197, 323	—
Hyssopus vulgaris	See *Hyssopus officinalis*			
Iboza riparia	U	—	201	—
Lagochilus sp.	U	—	426	—
Lagochilus inebrians	I	—	328	Stachydrine (CXIV)
Lamium sp.	I	—	212, 217, 312	Tyramine
Lamium album	E	PL	197	—
Lavandula stoechas	A	—	192	—
	E	PL	193	—
Leonotis nepetaefolia	A	—	192	—
	E	PL	193	—
Leonurus cardiaca	U	—	427	K ions (?)
	I	—	428	Leonurine[e]
	I	—	328	Stachydrine
Leonurus rubiastrum	U	—	427	K ions
Leonurus sibiricus	I	LF	429	Uncharacterized alkaloid
	I	—	428	Leonurine[e]
Leucas cephalotes	A	—	192	—
	E	PL	193	—
Leucas stelligera	A	—	192	—
	E	PL	193	—
Marrubium nigrum	See *Ballota nigra*			
Marrubium vulgare	A	—	192	—
	E	PL	197	—
	U	—	430	—
Melissa graveolens	See *Melissa officinalis*			
Melissa officinalis	E	LF, FL	197	—
Mentha arvensis	A	—	192	—
	E	PL	193	—
Mentha piperita	E	LF, FL	197	—
Mentha pulegium	A	VO	194	—
	E	VO	194	—
Nepeta cataria	A	—	318	—
	E	LF, FL	192, 193, 197	—
Nepeta vulgaris	See *Nepeta cataria*			
Ocimum basilicum	A	—	204	—
	E	PL	192, 193	—
Ocimum sanctum	A	—	204	—
Origanum amaracus	E	—	197	—
Origanum dictamnus	E	LF, FL	197	—
Origanum vulgare	A	—	192	—
	E	PL	193	—
Orthosiphon pallidus	U	—	431	—
Rosmarinus officinalis	A	—	192	—
	A	FL	194	—
Salvia sp.	E	LF	323	—
Salvia officinalis	A	—	192	—
	E	LF	193	—
Salvia plebeia	A	SD	193	—
	A	—	192	—
Salvia sclarea	E	LF, FL	194	—
Stachys aquatica	See *Stachys palustris*			
Stachys betonicaefolia	U	—	432, 433	—
Stachys lanata	U	—	434	—
	I	—	328	Stachydrine
Stachys palustris	A	—	192	—

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	E	PL	193	—
	E	LF, FL	194	—
	I	—	328	Stachydrine
Stachys sylvatica	A	—	192	—
	E	PL	193	—
	U	—	435	—
	I	—	328	Stachydrine
Teucrium polium	U	—	436	—
Thymus durius	See *Thymus vulgaris*			
Thymus serpyllum	A	PL	194	—
	A	—	192	—
	E	PL	193	—
Thymus vulgaris	E	PL	194, 197	—
Ziziphora tenuior	A	—	192	—
	E	PL	193	—
Lauraceae				
Cinnamomum camphora	A	PL	194	—
	A	—	192	—
	E	—	193	—
	I	—	437	Camphor (CXXXVII)
Cinnamomum cassia	A	BK	193	—
	A	—	192	—
	E	BK	193	—
Cinnamomum tamala	A	—	192	—
Cinnamomum zeylanicum	E	VO	193	—
Laurus nobilis	A	—	192	—
	E	FR, LF	193	—
Persea sp.	I	—	212, 217, 312	Tyramine
Persea americana	E	FP	194	—
	I	—	297-309	5-Hydroxytryptamine
Persea gratissima	E	LF	197	—
	I	—	297-309	5-Hydroxytryptamine
Lecythidaceae				
Combretodendron africanum	U	—	438	—
Leguminosae				
Abrus precatorius	A	SD	193	—
	A	—	192	—
	E	SD	91	—
Acacia sp.	A	WD	194	—
	I	—	211, 310	Hordenine
	I	—	317, 798-800	Nicotine
	I	—	212, 217, 312	Tyramine
Acacia nilotica	E	LF	194	—
Acacia rugata	A	—	192	—
Aeschynomene leptophylla	A	LF	233	—
Albizia sp.	I	—	439	Albitocin[e]
Albizia adianthifolia	U	RT	440	Saponin glycoside (?)
Albizia chinensis	A	PL	194	—
	U	—	441	—
	U	BK	440	Saponin glycoside (?)
Albizia grandibracteata	U	—	441	—
	U	BK	440	Saponin glycoside (?)
Albizia gummifera	U	—	441	—
	U	BK	440	Saponin glycoside (?)
	I	—	442, 443	Albitocin[e]
Albizia lebbeck	A	BK, SD	91	—
Anagyris foetida	E	FR	197	—
	I	—	250-252, 361, 385, 444-493	Sparteine (XL)
Astragalus hamosus	A	—	192	—
	E	PL	193	—
Bauhinia gapini	U	LS	294	—
Bauhinia retusa	A	—	192	—
	E	GU	193	—
Butea frondosa	See *Butea monosperma*			
Butea monosperma	A	—	192	—
	E	FL	193	—
Caesalpinia bonduc	E	FL	194	—
Caesalpinia crista	A	—	192	—
	E	LF, BK	193	—
Caesalpinia jayabo	A	—	192	—
	E	LF	193	—
Caesalpinia nuga	See *Caesalpinia crista*			
Caesalpinia pulcherrima	A	—	192	—
	A	LF, SD	194	—
	E	LF, BK, PL	193	—
Caesalpinia sappan	A	—	192	—

(*continued*)

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	E	WD	194	—
Caesalpinia sepiaria	A	—	192	—
Cassia absus	I	—	494–498	Chaksine (CXVII)
	I	—	499	Erysotrine (CXXIII)
Cassia didymobotrya	A	—	318	—
Cassia fistula	A	FR	91	—
Cassia lanceolata	A	—	192	—
	A	LF	193	—
Cassia occidentalis	U	LS	225	—
Cassia tora	I	SD	500	Uncharacterized principle
Cicer arietinum	A	—	192	—
	A	LF	193	—
Crotalaria incana	A	—	318	—
	I	—	333	Integerrimine (LXVI)
Crotalaria juncea	A	—	192	—
	A	LF	91	—
	E	LF	91	—
	E	SD	193	—
	I	—	333	Riddelliine (LXIX)
	I	—	333	Senecionine
Crotalaria spectabilis	I	—	333	Monocrotaline (LXIV)
Cytisus sp.	I	—	501–503	Cytisine (XLII)
	I	—	504	D-Lupanine (XXXVI)
	I	—	212, 217, 312	Tyramine
Cytisus laburnum	I	—	501–503	Cytisine
	I	—	250, 252, 361, 385, 444, 493	Sparteine
Cytisus scoparius	I	—	501–503	Cytisine
	I	—	250, 252, 361, 385, 444–493	Sparteine
	I	—	212, 217, 312	Tyramine
	H	—	250–252, 361, 385, 444–493	Sparteine
Dalbergia ferruginea	A	SW, RT	90	—
	E	SW, RT	90	—
	I	—	869	Coumingidine (CVI)
	I	—	869, 870	Coumingine (CVII)
Erythrophleum guineense	I	—	869	Cassaidine (CIV)
	I	—	869, 870	Cassaine (CV)
	I	—	869–872	Erythrophleine[e]
	I	—	869, 870	Homophleine[e]
	I	—	870	Norcassaidine (CVIII)
Genista sp.	I	—	501–503	Cytisine
	I	—	867, 868	Retamine (XXXIX)
	I	—	250, 252, 361, 385, 444–493	Sparteine
Genista tinctoria	U	FL	873	—
	I	FL	873	Sugar-like principle[e]
	I	—	501–503	Cytisine
	I	—	876, 878, 879	D-Lupanine (XXXVI)
Glycyrrhiza sp.	U	—	201	—
Glycyrrhiza glabra	A	—	192	—
	E	RT	193, 323	—
Indigofera linifolia	A	—	192	—
Indigofera suffruticosa	A	—	332	—
Lespedeza bicolor var. *japonica*	U	—	201	—
	I	—	874	*N,N*-Dimethyltryptamine (LXXIV)
	I	—	874	*N,N*-Dimethyltryptamine *N*-oxide (LXXV)
	I	—	874	5-Methoxy-*N,N*-dimethyltryptamine (LXXVIII)
Leucaena glauca	A	—	192	—
	E	RT, BK	194	—
Lupinus sp.	I	—	875	17-Hydroxylupanine (XXXV)
	I	—	875	Lupamine *N*-oxide (XXXVII)
	I	—	876	17-Oxolupanine (XXXVIII)
	I	—	504	Lupinine (XLIII)
	I	—	250, 252, 361, 385, 444–493	Sparteine
	I	—	504	Trilupine (XLI)
Lupinus albus	U	—	877	—
	I	—	875	17-Hydroxylupanine
	I	—	876, 878, 879	D-Lupanine (XXXVI)
	I	—	250, 252, 361, 385, 444–493	Sparteine
Lupinus perennis	I	—	875	13-Hydroxylupanine (XXXIV)
	I	—	875	17-Hydroxylupanine
Lupinus westianus	I	—	876, 878, 879	D-Lupanine

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	I	—	250, 252, 361, 385, 444, 493	Sparteine
Maackia amurensis	U	BK	880	—
	I	—	876, 878, 879	D-Lupanine
Medicago sativa var. *italica*	U	—	201	—
	I	—	328	Stachydrine
Mucuna cylindrosperma	I	—	881, 882	Physostigmine (XCII)
Mucuna pruriens	A	—	883	See also *Mucuna prurita*
	I	LF	883	*N,N*-Dimethyltryptamine
	I	LF	883	*N,N*-Dimethyltryptamine *N*-oxide
	I	LF	883	5-Hydroxytryptamine (LXXVII)
	I	LF	883	5-Methoxy-*N,N*-dimethyltryptamine (LXXVIII)
	I	—	317, 798–800	Nicotine
Mucuna prurita	A	—	192, 883	—
	E	RT	193	—
Physostigma venenosum	I	—	881, 882	Physostigmine
	I	SD	881, 882	Physostigmine
Pisum sativum	U	SD	884	—
	I	—	212, 217, 312	Tyramine
Pithecellobium multiflorum	U	GA, SB	219	—
Poinciana pulcherrima	A	—	192	—
Rhynchosia minima	A	—	192	—
	A	LF	193	—
Saraca indica	A	—	192	—
	E	BK	193	—
	I	—	885, 886	Phenolic glycoside
Sarcococca saligna	I	—	499	Erysotrine
Sesbania aegyptiaca	A	—	192	See also *Sesbania sesban*
	E	SD	91	—
Sesbania sesban	E	SD	193	—
Smirnovia turkestana	I	—	505	Sphaerophysine
	I	—	501–503	Cytisine
	I	—	250, 252, 361, 385, 444–493	Pachycarpine (sparteine)
Spartium junceum	U	—	869	—
	I	—	501–503	Cytisine
Sphaerophysa salsula	I	—	505	Sphaerophysine
Sutherlandia microphylla	E	LF	194	—
Swartzia madagascariensis	A	SP	194	—
Tamarindus indica	A	—	192	—
Tephrosia lupinifolia	A	RT	194, 920	Rotenone
Tephrosia piscatoria	I	—	504	Rotenone
Trachylobium hornemannianum	A	—	192	—
	E	GU	193	—
Trigonella foenum-graecum	A	—	192	—
	E	PL, SD	193	—
	E	SD	91	—
	U	SD	201, 507	—
Uraria lagopoides	A	PL	193	—
	A	—	192	—
	U	—	508	—
Liliaceae				
Agapanthus africanus	A	RT	194	—
Aletris farinosa	U	RZ, RT	509	—
Allium cepa	A	—	192	—
	E	BU, JU	193	—
	U	—	510	—
Allium fistulosum	U	—	201	—
Allium kurrat	U	LF	511	—
Allium sativum	A	—	192	—
	E	BU	193	—
	U	BU	510, 512, 513	—
Allium schoenoprasum	A	—	192	—
	E	BU	193	—
Aloe sp.	H	—	514	—
Aloe barbadensis	A	—	192	—
	E	PL	193	—
Aloe capensis	U	—	515	—
Aloe ferox	A	LF	194	—
Aloe littoralis	E	PL	193	—
Aloe perryi	A	—	192	—
Aloe vera	See *Aloe barbadensis*			
Asparagus acutifolia	E	—	90	—
Asparagus officinalis	A	—	192	—
	E	FR, PL	90	—

(*continued*)

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	E	FR	194	—
Asparagus striatus	A	TU	194	—
Asphodelus sp.	U	—	516	—
Chlorophytum comosum	A	TU	194	—
Colchicum sp.	I	—	517, 518	Colchicine (CXVIII)
Dipcadi glaucum	A	PL, BU	194	—
Eriospermum latifolium	E	TU	194	—
Fritillaria roylei	I	—	519	Peimine (verticine) (C)
	I	—	519	Fritillarine (CII)
	I	—	520, 521	Fritimine[e]
Gloriosa superba	A	—	192	—
	A	RS	194, 204	—
	A	TU	91, 193	—
	E	TU	91, 193	—
Lilium candidum	E	BU, PL	197	—
Lomatophyllum reflexum	E	FL	90	—
Ornithogalum eckloni	A	PL	194	—
Urginea indica	A	—	192	—
	E	BU	193	—
Veratrum sp.	I	—	522–524	Veratrine (CI)
Linaceae				
Linum usitatissimum	A	—	192	—
	E	—	193	—
Loganiaceae				
Gelsemium sempervirens	I	—	525–527	Gelsemicine (XCVI)
	I	—	528	Gelsemine (XCVII)
Strychnos sp.	I	—	529–547	*d*-Tubocurarine (XXXIII)
	I	—	548	Macusine B (LXXXIV)
Strychnos aculeata	A	PU	549	—
Strychnos nux-vomica	A	—	192	—
	E	SD	91	—
	E	FR	193	—
	I	—	550	Brucine (XCVIII)
	I	—	551–558	Strychnine (XCIX)
Strychnos toxifera	I	—	548	Macusine B
Loranthaceae				
Oryctanthus occidentalis	U	LS	225	—
Phoradendron sp.	I	—	312	Tyramine
Phoradendron rubrum	U	LS	225	—
Viscum album	I	—	559	"Cardiac principle"
	I	—	312	Tyramine
Viscum capense	E	ST	194	—
Lycopodiaceae				
Lycopodium sp.	I	—	560	Complanatine (CXI)
	I	—	560	*Lycopodium* alkaloid L-9 (lycofawcine) (CXIII)
	I	—	560, 561	Lycopodine (CIX)
	I	—	560	Obscurine (CXII)
Lycopodium annotinum	I	—	560, 561	Annotinine (CX)
	I	—	560, 561	Lycopodine
	I	—	317, 798–800	Nicotine
	I	—	560	Obscurine
Lycopodium selago	E	PL	197	—
	I	—	560, 561	Lycopodine
Lythraceae				
Lawsonia alba	See *Lawsonia inermis*			
Lawsonia inermis	A	—	192	—
	A	RT	193	—
	E	BK, FR	193	—
Woodfordia floribunda	A	PL	194	—
	E	PL	194	—
Magnoliaceae				
Michelia champaca	A	—	192	—
	E	RT, RB	193	—
	E	RT, FL	91	—
Malpighiaceae				
Byrsonima crassifolia	A	BK, FL, FR	90	—
Byrsonima sericea	U	SB	219	—
Malvaceae				
Abelmoschus manihot	E	BK	193	—
Abutilon indicum	A	—	318	—
	A	PL	194	—
Abutilon zanzibaricum	A	—	318	—
Gossypium sp.	U	LS	225	—
Gossypium arboreum	A	—	204	—
Gossypium barbadense	A	—	562	—
	E	—	562	—
Gossypium herbaceum	A	—	192	—
	A	PL	194, 562	—
	A	RB, SD	193	—

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	A	RB	91	—
	E	—	562	—
	E	RB	193, 197	—
	E	PL	194	—
Gossypium hirsutum	A	—	192	—
	E	RT	193, 562	—
	E	—	562	—
	I	—	297–309	5-Hydroxytryptamine
Hibiscus manihot	A	—	192	See also *Abelmoschus manihot*
Hibiscus rosa-sinensis	A	—	192	—
	E	FL	91, 193	—
Malva moschata	U	—	201	—
Urena lobata	A	RT	193	—
	A	—	192	—
Melastomataceae				
Memecylon amplexicaule	A	RT	193	—
Meliaceae				
Azadirachta indica	E	LJ, FL	193	—
Cedrela toona	A	—	192	—
	E	FL	193	—
Melia azadirachta	A	—	192	See also *Azadirachta indica*
Melia azedarach	A	—	192	—
Trichilia emetica	A	—	192	—
	E	BK	193	—
Walsura piscidia	A	—	192	—
	E	BK	193	—
Menispermaceae				
Anamirta cocculus	I	—	563–566	Picrotoxin (CXXXIX)
Arcangelisia flava	A	PL	567	—
	I	—	224–280	Berberine
	I	—	284	Columbamine
	I	—	280–281	Palmatine
	I	—	273	Jatrorrhizine
Chondodendron sp.	I	—	529–547	*d*-Tubocurarine
Cissampelos mucronata	A	RT	96	—
Cissampelos pareira	A	—	192	—
	E	RT	193, 194	—
Jateorhiza columba	I	—	273	Jatrorrhizine
	I	—	280, 281, 597, 599	Palmatine
Menispermum dauricum	I	—	568	Dauricine (XXXII)
	I	—	569–574	Parasinomenine[e]
	I	—	575–582	Sinomenine (LXII)
Pycnarrhena manilliensis	A	—	195	—
Tinospora bakis	E	RT	196	—
	I	—	280, 281, 597, 599	Palmatine
	I	—	274–280	Berberine
Molluginaceae				
Mollugo pentaphylla	A	—	192	—
Moraceae				
Antiaris toxicaria	U	JU	583, 584	—
Cecropia carbonaria	U	LF	219	—
Ficus sycomorus	A	—	318	—
Moringaceae				
Moringa oleifera	A	—	192	—
	A	RT, SB	193	—
	E	RT, SB	193	—
Moringa pterygosperma	A	RB	193, 194	See also *Moringa oleifera*
	I	—	583	Uncharacterized alkaloid
Myristicaceae				
Myristica fragrans	A	—	192	—
	A	PL	193	—
Myrsinaceae				
Embelia robusta	A	—	204	—
Embelia tsjeriam-cottam	See *Embelia robusta*			
Myrtaceae				
Myrtus communis	A	—	192	—
	E	FR	193	—
Nyctaginaceae				
Boerhaavia coccinea	U	RT	219	—
Olacaceae				
Liriosma ovata	U	BK	585	—
Oleaceae				
Jasminum multifida	A	SD	193	—
Jasminum multiflorum	E	RT	193	—
Jasminum officinale	A	—	192	—

(*continued*)

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
Jasminum officinale var. *grandiflorum*	E	PL	193	—
Jasminum pubescens	See *Jasminum multiflorum*			
Jasminum sambac	A	—	192	—
	E	RT	193	—
Olea cuspidata	A	—	192	—
	E	FR	193	—
Phyllyrea latifolia	See *Phyllyrea media*			
Phyllyrea media	E	LF	197	—
Orchidaceae				
Dendrobium sp.	I	—	586	Dendrobine (CXX)
Oxalidaceae				
Oxalis acetosella	E	PL	194	—
Paeoniaceae				
Paeonia emodi	U	TU	587	—
Paeonia officinalis	E	RT	197	—
Palmae				
Areca catechu	A	—	192	—
	E	SD	193	—
	U	SD	588, 589	Tannin (?)
	I	—	590, 591	Arecoline (XI)
Cocos nucifera	A	—	192	—
Pandanaceae				
Pandanus odoratissimus	U	RT	592	—
Pandanus tectorius	A	RT	192	—
Papaveraceae				
Adlumia fungosa	I	—	593, 594	Adlumine (XIX)
	I	—	593	Bicuculline (XX)
Argemone mexicana	A	RT	195	—
	E	SO	194	—
	U	RS	595	Total alkaloids
	U	—	201	—
	I	—	274–280	Berberine
	I	—	596–598	Chelerythrine (XLIV)
	I	—	201, 597, 599	Protopine (LVIII)
Chelidonium majus	U	—	201	—
	I	—	274, 280	Berberine
	I	—	596–598	Chelerythrine
	I	—	597, 918	Chelidonine (XLV)
	I	—	201, 597, 599	Protopine (LVIII)
	I	—	250–252, 361, 385, 444–493	Sparteine
	I	—	312	Tyramine
Corydalis sp.	A	—	192	—
	E	RT	193	—
	I	—	274–280	Berberine
	I	—	593	Bicuculline
	I	—	600	Corlumine (XXII)
	I	—	597	Capauridine [(±)-capaurine] (XLVI)
	I	—	597	Capnoidine (XXI)
	I	—	601	Cularine (CXXI)
	I	—	597	Ochotensine (CXXII)
	I	—	597	Ophiocarpine (L)
	I	—	280, 281, 597, 599	Palmatine
Corydalis ambigua	I	—	602	*Corydalis alkaloid* B (tetrahydropalmatine) (LI)
	I	—	597	Adlumine
	I	—	602	*Corydalis* alkaloid J[e]
	I	—	602	*Corydalis* alkaloid L[e]
	I	—	602	*Corydalis* alkaloid M[e]
	I	—	280, 281, 597, 599	Palmatine
	I	—	597	Tetrahydropalmatine
	I	—	597	Adlumidine (XVIII)
Corydalis incisa	I	—	280, 281, 597, 599	Protopine
	I	—	597	Tetrahydropalmatine
Corydalis ochotensis	I	—	603–605	Cryptocavine (cryptopine) (LVI)
	I	—	597	Ochotensine
	I	—	201, 597, 599	Protopine
Corydalis tuberosa	I	—	597	Canadine (CXXV)
	I	—	597	Corydaline (XLVIII) (*Corydalis* alkaloid A)
	I	—	597	Tetrahydropalmatine
Fumaria micrantha	U	—	848	—
Glaucium flavum	U	—	606	—
	I	—	596, 598	Chelerythrine

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	I	—	597, 918	Chelidonine (XLV)
	I	—	201, 597, 599	Protopine
Glaucium rubrum	U	—	606	—
Hunnemannia fumariaefolia	I	—	274–280	Berberine
	I	—	596–598	Chelerythrine
	I	—	603	Hunnemannine (LVII)
Macleaya cordata	U	—	201	—
	I	—	596, 598	Chelerythrine
	I	—	201, 597, 599	Protopine
Papaver sp.	I	—	607, 849	Narcotine (XXIV)
	I	—	607, 849, 851, 852, 864, 865	Thebaine (LXIII)
Papaver somniferum	I	—	607, 865	Codeine (LX)
	I	—	608, 848–863	Morphine (LXI)
	I	—	609, 850, 852, 866	Papaverine (XVI)
Sanguinaria canadensis	A	—	603	—
	U	RT	603	Total alkaloids
	I	—	274–280	Berberine
	I	—	596–598	Chelerythrine
	I	—	201, 597, 599	Protopine
Passifloraceae				
Passiflora foetida	A	—	192	—
	E	LF	193	—
	I	—	297–309	5-Hydroxytryptamine
	U	—	610	—
Passiflora incarnata	U	—	828	Harman (LXXXI)
	U	—	823–826	Harmaline (LXXX)
Pedaliaceae				
Dicerocaryum zanguebaricum	A	LF, JU	194	—
Pedalium murex	A	FL	194	—
	A	—	192	—
	E	FJ, LF	193	—
	E	PL	194	—
Sesamum indicum	A	SD, OL	194	—
	A	SD	193	—
	A	—	192	—
	E	SD	193	—
	E	SD, OL	194	—
Phytolaccaceae				
Petiveria alliaceae	U	LS	294	—
Phytolacca americana	U	—	201	—
Phytolacca decandra	A	RT	611	—
	A	LF, SH	194	—
Pinaceae				
Pinus longifolia	A	—	192	See also *Pinus roxburghii*
Pinus ponderosa	U	LF	612	—
Pinus roxburghii	E	GU	193	—
Piperaceae				
Piper aurantiacum	A	FR	193	—
	A	—	192	—
	U	—	613	—
Piper auritum	U	LS	225	—
Piper betle	A	—	204	—
Piper longum	A	FR	91, 193	—
	A	—	192	—
	E	FR	91, 193	—
Piper nigrum	A	—	192	—
	A	FR	91, 193, 194	—
Plantaginaceae				
Plantago sp.	A	—	318	—
Plumbaginaceae				
Plumbago capensis	U	LS	294	—
Plumbago europaea	I	—	614, 615	Plumbagin (CXXXVI)
Plumbago indica	A	RT	193	—
Plumbago rosea	A	—	193	See also *Plumbago indica*
	U	RT	192, 616	—
Plumbago viscosa	See *Plumbago zeylanica*			
Plumbago zeylanica	A	RT	193	—
	A	PL	194, 195	—
	A	—	192	—
	I	—	614, 615	Plumbagin
Polygalaceae				
Polygala amara	E	RT	197	—
Polygala amarella	See *Polygala amara*			
Polygala senega	U	—	201	—
Polygala teretifolia	A	PL	194	—
Polygala uliginosa	See *Polygala amara*			
Securidaca longepedunculata	A	RB	194	—

(*continued*)

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
Polygonaceae				
Antigonon leptopus	U	LS	294	—
Fagopyrum cymosum	U	—	201	Rutin (CXLVII)
Polygonum aviculare	H	—	616	—
Polygonum bistorta	U	—	201	—
Polygonum hydropiper	A	PL	323	—
	A	—	192	—
	E	PL, LF	193	—
Polygonum senegalense	A	LF	233	—
Rheum emodi	A	—	192	—
	E	RT	193	—
Triplaris gardenriana	U	SB	219	—
Polypodiaceae				
Adiantum capillus-veneris	A	—	192	—
	E	PL	193	—
Adiantum pedatum	A	—	192	—
	E	PL	193	—
Adiantum venustum	A	—	192	—
	E	FD	193	—
Asplenium adiantum-nigrum	A	—	192	—
	E	FD	193	—
Dryopteris filix-mas	A	RT	90	—
	A	—	192	—
Pteridium aquilinum	E	RT	194	—
Portulacaceae				
Portulaca oleracea	U	LS	225	—
Talinum cunefolium	A	—	318	—
Primulaceae				
Androsale septentrionalis	A	PL	616	—
Cyclamen europaeum	E	TU	197	—
Cyclamen persicum	A	—	192	—
	E	—	193	—
Punicaceae				
Punica granatum	A	RB	194	—
	A	—	193	—
	E	RB	194	—
	U	FP	616	—
Ranunculaceae				
Anemone obtusiloba	E	LF, BK	193	—
Clematis hirsuta	A	LF	617	—
Coptis japonica	U	—	618	Crude alkaloids
	I	—	274–280	Berberine
	I	—	284	Columbamine
	I	—	273	Jateorrhizine (= jatrorrhizine)
	I	—	280, 281, 597, 599	Palmatine
Helleborus niger	A	—	192	—
	A	RT	90	—
	E	RZ	193	—
Hydrastis canadensis	U	—	619	—
	I	—	274–280	Berberine
	I	—	597	Canadine
	I	—	620–627	Hydrastine (XXIII)
	I	—	628–633	Hydrastinine (XV)
Jateorhiza palmata	U	—	618	Alkaloid extract
	I	—	280, 281, 597, 599	Palmatine
Melanthium sativum	See *Nigella sativa*			
Nigella sativa	A	—	192	—
	A	SD	91	—
	E	SD	91, 193, 197	—
Pulsatilla sp.	U	—	634	—
Ranunculus sp.	I	—	297–309	5-Hydroxytryptamine
Ranunculus sceleratus	A	—	192	—
	E	PL	193	—
Thalictrum sp.	I	—	358, 361	Thalictrimine (β-allocryptopine) (LIX)
Thalictrum revolutum	U	—	635, 636	Tertiary alkaloids
Rhamnaceae				
Zizyphus abyssinica	A	RT	220	—
Zizyphus joazeiro	U	SB	219	—
Zizyphus mauritiana	U	LS	294	—
Zizyphus undulata	U	SB	219	—
Rosaceae				
Caryophyllata urbana	See *Geum urbanum*			
Caryophyllum officinalis	See *Geum urbanum*			
Geum caryophyllum	See *Geum urbanum*			
Geum urbanum	E	RZ	197	—
Hagenia abyssinica	A	—	192	—

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	A	PL	193	—
Prunus sp.	I	—	297–309	5-Hydroxytryptamine
	I	—	212–217	Tyramine
Prunus amygdalus var. *amara*	E	SD	194	—
Prunus amygdalus var. *dulcis*	E	SD	194	—
Prunus cerasus	E	SD	194	—
Prunus mahaleb	A	—	192	—
	E	FR	193	—
Prunus persica	A	—	192	—
	E	SD	193	—
Pyracantha angustifolia	U	—	201	—
Rubus moluccanus	A	—	192	See also *Rubus rugosus*
Rubus rigidus	A	RT	194	—
Rubus rugosus	A	LF	193	—
	E	LF	193	—
Rubiaceae				
Borreria verticillata	U	RT	219	—
	I	—	637	Emetine (CXXVIII)
Cephaelis ipecachuana	I	—	637	Emetine
Cinchona calisaya	A	—	192	—
	E	—	193	—
	I	—	638	Cinchonidine (XXVII)
	I	—	638, 639	Cinchonine (XXVIII)
	I	—	638, 640	Quinidine (XXX)
	I	—	641, 682	Quinine (XXXI)
	H	—	347, 402–404, 408, 409, 683–767	Quinine
Cinchona ledgeriana	I	—	682	Hydroquinine (XXIX)
Crossopteryx africana	See *Crossopteryx febrifuga*			
Crossopteryx febrifuga	A	BK	222	—
Crossopteryx kotschyana	See *Crossopteryx febrifuga*			
Diodia barbeyana	U	RT	219	—
Guettarda angelica	U	RB	219	—
Mitragyna inermis	I	—	768, 769	Mitrinermine (XCIV) (rhynchophylline)
Mitragyna stipulosa	I	—	768, 769	Mitrinermine (rhynchophylline)
Dalbergia sisso	A	BK, WD	193	—
	A	—	192	—
Delonix regia	U	SB	219	—
Derris chinensis	I	—	194, 920	Rotenone (CXLVIII)
Derris elliptica	I	—	194, 920	Rotenone
Desmodium retroflexum	A	—	192	—
	E	RT	193	—
Desmodium tiliaefolium	A	—	192	—
Dolichos biflorus	A	—	192	—
	E	SD	193	—
Dolichos lablab	A	—	192	—
	E	LF	193	—
Elephantorrhiza sp.	A	BK	194	—
Entada africana	A	BK	194	—
Entada phaseoloides	A	SD	194	—
Eremosparton flaccidum	I	—	505	Sphaerophysine (CXVI)
Erythrina indica	A	—	192	See also *Erythrina variegata* var. *occidentalis*
Erythrina suberosa	I	—	499	Erysotrine (CXXIII)
Erythrina variegata	A	—	192	—
Erythrina variegata var. *occidentalis*	E	LF	193	—
Erythrina velutina	U	SD	219	—
Erythrophleum coumingo	I	—	869	Coumingaine[e]
Morinda citrifolia	A	—	192	—
	E	FR	193	—
Oldenlandia affinis	U	PL	770	—
	I	—	771, 772	Two polypeptides[e]
Oldenlandia herbacea	A	PL	194	—
Paederia foetida	A	—	192	—
	E	PL	193	—
Pentas purpurea	E	RT	194	—
Pseudocinchona africana	I	—	773, 774	Corynanthine (LXXXVIII)
Randia dumetorum	A	—	192	—
	A	FP	193	—
Rondeletia africana	See *Crossopteryx febrifuga*			
Rondeletia febrifuga	See *Crossopteryx febrifuga*			
Rubia cordifolia	A	—	192	—
	A	PL	194	—
	E	RT	193, 194	—
Rubia major	See *Rubia tinctorum*			
Rubia tinctorum	A	—	192	—

(*continued*)

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	E	RT	193, 197	—
Tocoyena formosa	U	RB	219	—
Uncaria sp.	I	—	775	Mitrinermine (rhynchophylline)
Uncaria gambier	I	—	775	Gambirine (LXXXVII)
Rutaceae				
Citrus sp.	I	—	776	Hesperidin (CXLV)
	I	—	212–217	Tyramine
Clausena anisata	A	PL, LF	194	—
Dictamnus albus	A	—	192	—
	E	RT	197	—
	I	—	777	Dictamnine (XXV)
	I	—	778	Skimmianine (XXVI)
Dictamnus fraxinella	See *Dictamnus albus*			
Evodia hortensis f. *hortensis*	E	BJ	779	—
	I	—	274, 280	Berberine
Evodia inu-ankend	E	FL, LF	193	—
Evodia roxburghiana	See *Evodia inu-ankend*			
Evodia rutaecarpa	U	—	201	—
Lunasia amara	I	—	780	Lunamarine (CXXIV)
Pilocarpus jaborandi	I	LF	781, 786	Pilocarpine (CXXVI)
Ruta graveolens	A	—	192	—
	A	PL	90, 194, 323	—
	A	RT	91	—
	E	PL	90, 193, 194, 323	—
	E	RT	91	—
	E	LF	197	—
	U	—	787, 789, 790	—
	H	—	326	—
	I	—	778	Skimmianine
Ruta hortensis	U	PL	791	—
Ruta montana	A	LF	197	—
Ruta sylvestris-major	See *Ruta graveolens*			
Santalaceae				
Santalum album	A	—	192, 193	—
Thesium hystrix	A	RT	194	—
Sapindaceae				
Cardiospermum halicacabum	A	—	192	—
	A	PL	194	—
	E	RT, LF	193	—
	E	PL	194	—
Sapindus emarginatus	A	FR, SD	91, 193	—
	E	SD	193	—
Sapindus trifoliatus	A	FR	91	See also *Sapindus emarginatus*
	A	—	192	—
Schmidelia affinis	See *Rhus longipes*			
Sapotaceae				
Mimusops elengi	A	FR	195	—
Saxifragaceae				
Ribes americanum	E	RT	792	—
Scrophulariaceae				
Buchnera ciliata	A	PL	793	—
Celsis caucasica	A	—	192	—
Herpestis monnieri	I	—	794	Saponin glycoside
	I	—	317, 798–800	Nicotine
Pedicularis palustris	I	—	795	Uncharacterized glycoside
Picrorhiza kurroa	A	—	192	—
	A	RZ	193	—
	E	RZ	193	—
	E	RT	91	—
Scoparia dulcis	U	RT	219	—
Sutera atropurpurea	A	FL	194	—
	E	FL	194	—
Simaroubaceae				
Picrasma ailanthoides	U	—	201	—
Simarouba versicolor	U	RB	219	—
Solanaceae				
Capsicum frutescens	U	LS	294	—
Datura tatula	U	—	201	—
	I	—	796, 797	Scopolamine (XIV)
Lycium barbatum	A	—	192	—
	E	FR	193	—
Nicotiana tabacum	A	LF	194	—
	I	—	317, 798–800	Nicotine (X)
Physalis minima	A	PL	194	—
Solanum sp.	A	—	318	—
	I	—	212–217	Tyramine

Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	I	—	297–309	5-Hydroxytryptamine
Solanum nigrum	A	—	192	—
	A	RB	193	—
Solanum paniculatum	U	LF, RT	801	—
	U	RT	219	—
Solanum variabile	U	—	801	—
Solanum verbascifolium	U	LS	294	—
Withania coagulans	A	—	192	—
	E	SD	193	—
Withania somnifera	A	—	192	—
	A	RT	193, 194	—
	I	—	317, 798–800	Nicotine
Stemonaceae				
Stemona sp.	I	—	802	Paipunine[e]
Stemona japonica	U	—	201	—
Sterculiaceae				
Abroma augusta	A	—	192	—
	A	BK	194	—
	E	RB	193	—
	E	RT, RB	194	—
	E	PL	803	—
	E	RT	91	—
	U	ST, RT	804, 805	—
Guazuma ulmifolia	U	SB	219	—
Helicteres isora	U	FR	267	—
Pentapetes phoenicea	A	—	204	—
Theobroma cacao	A	RT	195	—
Waltheria indica	A	PL	194	—
Styracaceae				
Styrax japonica	U	—	201	—
Taxaceae				
Taxus baccata	A	—	192	—
	A	LF	194	—
	E	LF	91, 197	—
	E	LF, FR	193, 194	—
	U	—	91, 791, 806	—
	H	—	807	—
	I	—	365, 380	Ephedrine (V)
Torreya nucifera	U	SD	808	—
Theophrastaceae				
Jacquinia pungens	A	—	332	—
Thymelaeaceae				
Arthrosolen polycephalus	A	PL	194	—
Daphne genkwa	U	—	201	—
Gnidia chrysantha	A	RT, FL	194	—
Lasiosiphon capitatus	A	PL	194	—
Lasiosiphon kraussianus	A	RT	220	—
Tiliaceae				
Grewia asiatica	A	—	192	—
Grewia barteri	U	—	809	—
	I	—	810	Amine-imidazole type of compound[e]
Grewia bicolor	U	—	809	—
Grewia carpinifolia	U	—	809	—
	I	—	810	Amine-imidazole type of compound[e]
Grewia cissoides	U	—	809	—
	I	—	810	Amine-imidazole type of compound[e]
Grewia cyclopetala	U	—	809, 811	—
Grewia elyseoi	U	—	809, 812	—
	I	—	810	Amine-imidazole type of compound[e]
Grewia hirsuta	A	—	192	—
	E	LF, FR	193	—
Grewia lasiodiscus	U	—	809	—
	I	—	810	Amine-imidazole type of compound[e]
Grewia malacocarpa	U	—	809	—
	I	—	810	Amine-imidazole type of compound[e]
Grewia occidentalis	A	PL	194	—
Grewia salvifolia	See *Grewia bicolor*			
Grewia venusta	U	—	809	—
	I	—	810	Amine-imidazole type of compound[e]
Grewia villosa	U	—	809	—
Triumfetta bartramia	A	—	192	—

(*continued*)

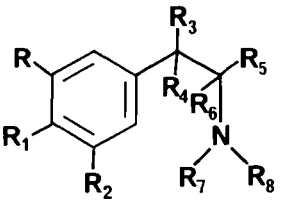
Table IV—(*Continued*)

Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
	A	RT	193	—
Triumfetta rhomboidea	A	RT	193, 194	See also *Triumfetta bartramia*
Turneraceae				
Turnera sp.	U	RT	219	—
Typhaceae				
Typha capensis	A	RT, PL	194	—
Ulmaceae				
Celtis australis	A	—	192	—
Umbelliferae				
Ammi visnaga	E	FR	197	—
	I	—	813	Khellin (visammin) (CXXXV)
Anethum foeniculum	See *Foeniculum officinale*			
Anethum graveolens	E	FR	193	—
Anethum sowa	A	—	192	—
Angelica archangelica	E	PL	323	—
Angelica sylvestris	E	RT	197	—
Apium graveolens	A	—	192	—
	A	RT, SD	193	—
	A	VO	194	—
	U	—	814	—
Athamanta cretensis	E	FR	176	—
Athamanta neumi	See *Meum athamanticum*			
Carum copticum	A	—	192	See also *Trachyspermum ammi*
	E	SD	193	—
Carum roxburghianum	A	—	192	—
	E	SD	193	—
Centella asiatica	E	PL	193	—
Chaerefolium silvestre	U	RT	814	—
Cuminum cyminum	A	FR	193	—
	A	—	192	—
	E	FR	193, 197	—
	I	—	506, 507	Coumingine (CVII)
Daucus carota	A	—	192	—
	A	SD, LF	194	—
	E	SD	193	—
	E	RT, SD	197	—
	E	RT	194	—
	U	—	814	—
Daucus creticus	See *Athamanta cretensis*			
Daucus visnaga	See *Ammi visnaga*			
Eryngium sp.	A	—	332	—
Ferula assa-foetida	A	—	192	—
Ferula caspica	U	—	815	—
Ferula foetida	E	GU, RE	193	—
Ferula nartex	E	GU	91	—
	E	ST, GU	193	—
Ferula orientalis	A	—	192	—
	E	GU, RE	193	—
Foeniculum capillaceum	See *Foeniculum officinale*			
Foeniculum officinale	E	FR, RT	197	—
Foeniculum sylvestre	See *Foeniculum officinale*			
Foeniculum vulgare	A	—	192	See also *Foeniculum officinale*
	E	SD, FR	193	—
	E	FR	194	—
Hydrocotyle asiatica	A	—	192	See also *Centella asiatica*
Imperatoria ostruthum	See *Peucedanum ostruthum*			
Imperatoria sylvestris	See *Angelica sylvestris*			
Meum athamanticum	E	RT, SD	197	—
Meum foeniculum	See *Foeniculum officinale*			
Petroselinum crispum	E	FR	194	—
Petroselinum hortense	A	—	192	—
	U	—	816	—
Petroselinum sativum	A	PL	194	—
	E	FR	194	—
	I	FR	817–822	Apiol (CXXIX)
	H	FR	823–844	Apiol
Peucedanum galbanum	A	PL	194	—
Peucedanum graveolens	A	—	192	See also *Anethum graveolens*
Peucedanum officinale	E	RT	197	—
Peucedanum ostruthum	E	RZ	197	—
Pimpinella saxifraga	E	RT	197	—
	U	—	814	—
Prangos pabularia	A	—	192	—
	E	FR, RT	193	—
Selinum sylvestre	See *Angelica sylvestris*			

Table IV—(*Continued*)

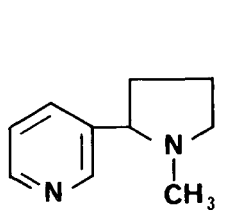
Plant Name[a]	Type of Activity[b]	Plant Part[c]	Reference	Active Constituent(s)
Steganotaenia araliacea	A	PL	194	—
Trachyspermum ammi	E	SD	193	—
Urticaceae				
Urtica sp.	I	—	270	Betaine
Urtica dioica	A	PL	194	—
	A	—	192	—
	E	PL	193	—
	I	—	297–309	5-Hydroxytryptamine
Urtica urens	A	PL	194	—
	I	—	297–309	5-Hydroxytryptamine
Valerianaceae				
Nardostachys jatamansai	A	—	192	—
	E	RT	193	—
Valeriana wallichii	A	—	192	—
	E	RT	193	—
Verbenaceae				
Clerodendrum inerme	U	LF	887	—
Clerodendrum serratum	A	—	204	—
Gmelina asiatica	A	RT	193	—
Stachytarpheta jamaicensis	A	—	192	—
	A	PL	193	—
Verbena bonaviensis	A	PL	194	—
Verbena officinalis	A	—	192	—
	I		888, 889	Verbenalin (verbenaloside) (CXLIX)
Verbena stricta	I		888, 889	Verbenalin (verbenaloside)
Vitex agnus-castus	E	FR	197	—
Vitex cannabifolia	U	—	201	—
Vitex gardneriana	U	SB	219	—
Vitex negundo	A	—	192	—
	E	FR	193	—
Vitex trifolia	A	—	192	—
	E	FR	193	—
Vitaceae				
Ampelopsis hederaceae	I	—	890–892	Catechol (CXXXI)
Cissus quadrangularis	A	—	192	—
Cissus sicyoides	U	LS	294	—
Rhoicissus cuneifolia	A	RT	194	—
Vitis quadrangularis	A	—	192	—
Vitis vinifera	E	FL	193	—
Zingiberaceae				
Alpinia speciosa	U	LF	219	—
Curcuma longa	A	—	192	—
	A	RZ, JU	194	—
	E	RZ	193	—
	U	—	201	—
Curcuma zedoaria	A	—	192	—
Elettaria cardamomum	A	—	192	—
	E	FR	193	—
Hedychium spicatum	A	—	192	—
	E	RT	193	—
Zingiber officinale	A	—	204	—
Zygophyllaceae				
Fagonia arabica	See *Fagonia cretica*			
Fagonia cretica	A	—	192	—
	E	PL	193	—
Peganum harmala	A	—	192	—
	A	PL, SD	193	—
	A	SD	91	—
	E	PL, SD	193	—
	E	—	91	—
	I	SD	893–896	Harmaline
	I	SD	893–897	Harmine (LXXXII)
Tribulus alatus	A	—	192	—
	E	FR	193	—
Tribulus terrestris	A	—	192	—
	E	RT	193	—
	I	—	898	Harman
	I	—	893–897	Harmine
Zygophyllum coccineum	U	—	899	—
Zygophyllum decumbens	U	—	900	—

[a] Plant names are as stated in the original articles except for corrections of obvious spelling errors. Arrangement is alphabetical by family, genus, and species; assignment to families is according to the system of Engler (1672). [b] A = abortifacient, ecbolic, oxytocic (folkloric); E = emmenagogue, affecting the menstrual cycle (folkloric); U = uterine stimulant as shown by *in vitro* or *in vivo* tests in animals; H = abortifacient effects as determined in humans; I = active substance isolated and shown to stimulate uterine tissue either *in vitro* or *in vivo*. [c] AH = ash, BA = balsam, BJ = bark juice, BR = branches, BK = bark, BU = bulb, EX = exudate, FL = flower, FP = fruit pulp, FR = fruit, FS = fruit shell, GU = gum, JU = juice, LF = leaf, LJ = leaf juice, LS = leaf and stem, LX = latex, OL = oil, PL = whole plant, PU = pulp, PX = aerial parts, RB = root bark, RE = resin, RS = root stalk, RT = root, RZ = rhizome, SB = stem bark, SD = seed, SG = stigma, SL = style, SO = seed oil, SP = seed pod, SW = stem wood, TE = tegumen, TR = tuberous root, TU = tuber, TW = twigs, VO = volatile oil, WD = wood, and — = plant part not stated in original article. [d] Although popularly believed to exert an oxytocic effect, the essential oil was shown to depress uterine tissue *in vitro* rather than cause a contraction. [e] Denotes that although the material was obtained as a crystalline or otherwise pure entity, the structure is not yet known.

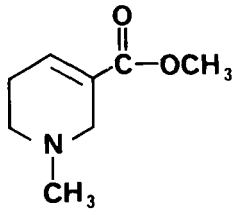


	R	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8
V: ephedrine	H	H	H	OH	H	CH_3	H	H	CH_3
VI: hordenine	H	OH	H	H	H	H	H	CH_3	CH_3
VII: mescaline	OCH_3	OCH_3	OCH_3	H	H	H	H	H	H
VIII: pseudoephedrine	H	H	H	H	OH	CH_3	H	H	CH_3
IX: tyramine	H	OH	H	H	H	H	H	H	H

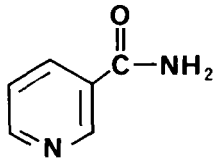
Structures of Phenethylamine Alkaloids Having Uterine Stimulant Activity



X: nicotine

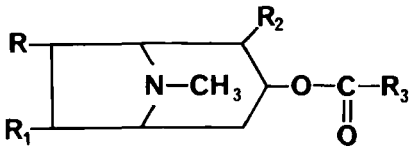


XI: arecoline



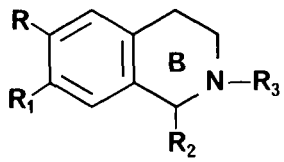
XII: nicotinamide

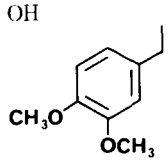
Structures of Pyridine Alkaloids Having Uterine Stimulant Activity



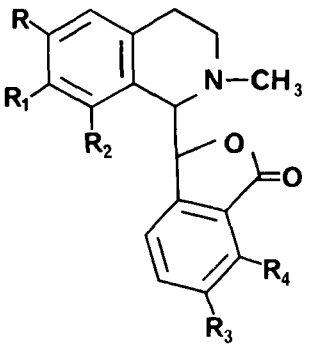
	R	R_1	R_2	R_3
XIII: cocaine	H	H	$COOCH_3$	phenyl
XIV: scopolamine	—O—		H_2	$-CH(CH_2OH)-$

Structures of Tropane Alkaloids Having Uterine Stimulant Activity



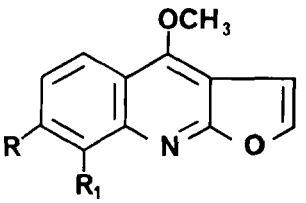
	R	R_1	R_2	R_3
XV: hydrastinine	—O—CH_2—O—		OH	CH_3
XVI: papaverine	OCH_3	OCH_3		ring B unsaturated
XVII: salsoline	OH	OCH_3	CH_3	H

Structures of Quinoline Alkaloids Having Uterine Stimulant Activity



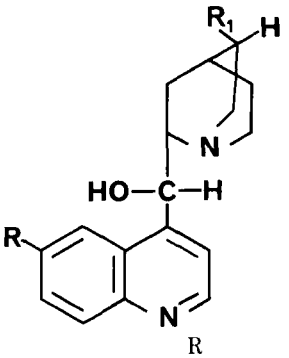
	R	R_1	R_2	R_3	R_4
XVIII: adlumidine	—O—CH_2—O—		H	—O—CH_2—O—	
XIX: adlumine	OCH_3	H	H	—O—CH_2—O—	
XX: bicuculline	—O—CH_2—O—		H	—O—CH_2—O—	
XXI: capnoidine	—O—CH_2—O—		H	—O—CH_2—O—	
XXII: corlumine	OCH_3	OCH_3	H	—O—CH_2—O—	
XXIII: hydrastine	—O—CH_2—O—		H	OCH_3	OCH_3
XXIV: narcotine	—O—CH_2—O—		OCH_3	OCH_3	OCH_3

Phthalidisoquinoline Alkaloid Structures Having Uterine Stimulant Activity



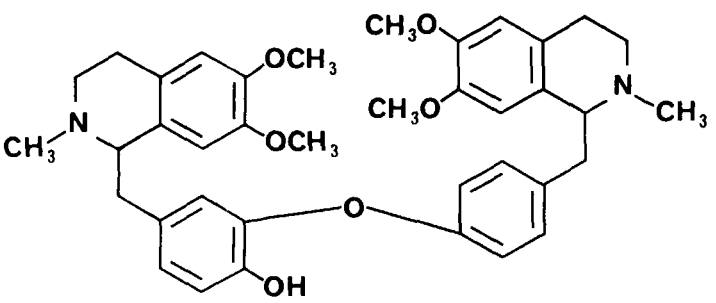
	R	R_1
XXV: dictamnine	H	H
XXVI: skimmianine	OCH_3	OCH_3

Structures of Furoquinoline Alkaloids Having Uterine Stimulant Activity

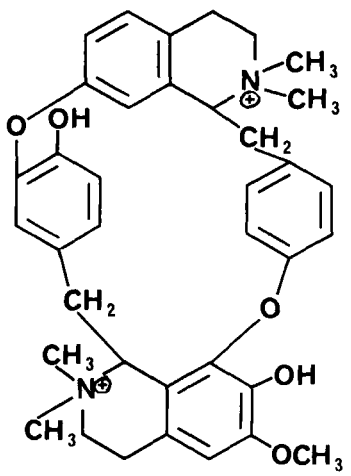


	R	R_1
XXVII: cinchonidine	H	$CH=CH_2$
XXVIII: cinchonine	H	$CH=CH_2$
XXIX: hydroquinine	OCH_3	$CH_2—CH_3$
XXX: quinidine	OCH_3	$CH=CH_2$
XXXI: quinine	OCH_3	$CH=CH_2$

Structures of Quinine Alkaloids Having Uterine Stimulant Activity

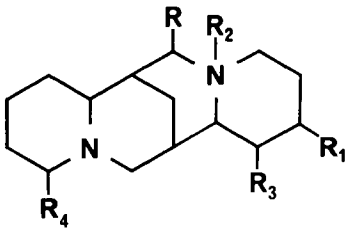


XXXII: dauricine

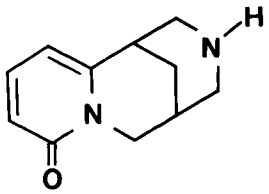


XXXIII: *d*-tubocurarine

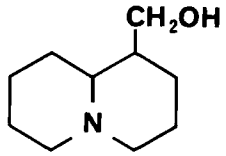
Structures of Bisbenzylisoquinoline Alkaloids Having Uterine Stimulant Activity



	R	R_1	R_2	R_3	R_4	other
XXXIV: 13-hydroxylupanine	H	OH	—	H	=O	—
XXXV: 17-hydroxylupanine	OH	H	—	H	=O	—
XXXVI: D-lupanine	H	H	—	H	=O	—
XXXVII: lupanine *N*-oxide	H	H	O	H	=O	—
XXXVIII: 17-oxolupanine	=O	H	—	H	=O	—
XXXIX: retamine	H	H	—	OH	=O	—
XL: sparteine	H	H	—	H	H	—
XLI: trilupine	H	H	—	H	=O	$\cdot HCl \cdot 2H_2O$

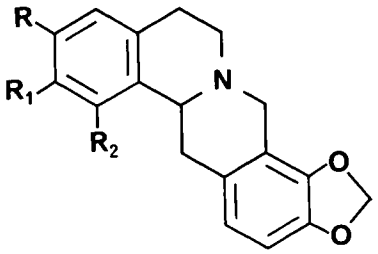


XLII: cytisine



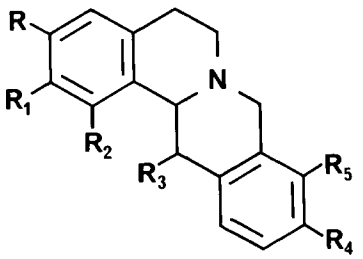
XLIII: lupinine

Structures of Quinolizidine Alkaloids Having Uterine Stimulant Activity



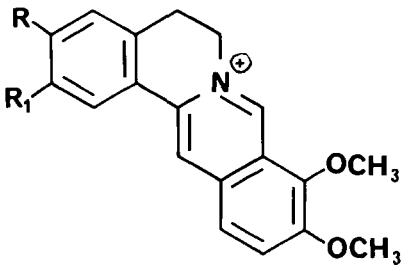
	R	R_1	R_2	other
XLIV: chelerythrine	OCH_3	OCH_3	H	unsaturated C ring
XLV: chelidonine	—O—CH_2—O—		OH	$\Delta^{1,2}$

Structures of Benzophenanthridine Alkaloids Having Uterine Stimulant Activity



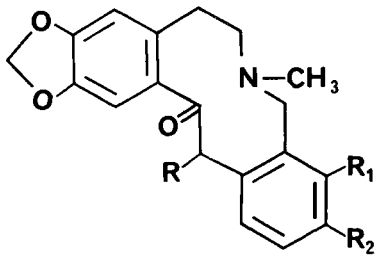
	R	R_1	R_2	R_3	R_4	R_5
XLVI: capauridine	OCH_3	OCH_3	OH	H	OCH_3	OCH_3
XLVII: capaurine	OCH_3	OCH_3	OH	H	OCH_3	OCH_3
XLVIII: corydaline	OCH_3	OCH_3	H	CH_3	OCH_3	OCH_3
XLIX: nandinine	—O—CH_2—O—		H	H	OCH_3	OH
L: ophiocarpine	—O—CH_2—O—		H	OH	OCH_3	OCH_3
LI: tetrahydropalmatine	OCH_3	OCH_3	H	H	OCH_3	OCH_3

Structures of Protoberberine Alkaloids Having Uterine Stimulant Activity



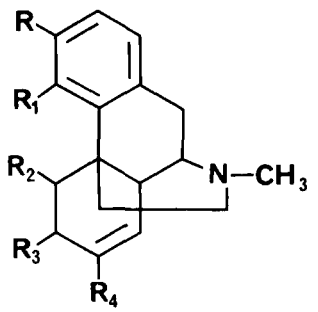
	R	R_1
LII: berberine	—O—CH_2—O—	
LIII: columbamine	OCH_3	OH
LIV: jatrorrhizine	OH	OCH_3
LV: palmatine	OCH_3	OCH_3

Structures of Protoberberine Alkaloids Having Uterine Stimulant Activity



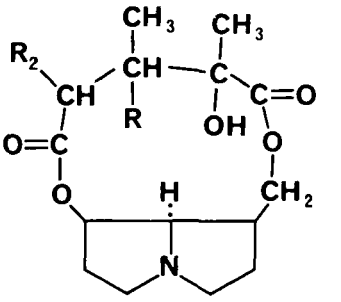
	R	R_1	R_2	other
LVI: cryptocavine	H	OCH_3	OCH_3	—
LVII: hunnemannine	CH_3	OCH_3	OCH_3	—
LVIII: protopine	H	—O—CH_2—O—		—
LIX: thalictimine	H	OCH_3	OCH_3	desoxy

Structures of Protopine Alkaloids Having Uterine Stimulant Activity



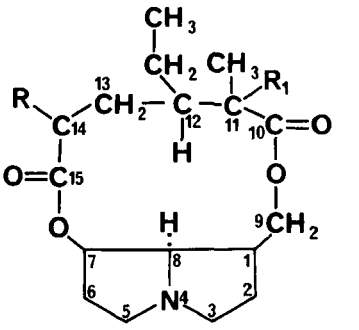
	R	R_1	R_2	R_3	R_4
LX: codeine	OCH_3	—O—		OH	H
LXI: morphine	OH	—O—		OH	H
LXII: sinomenine	OCH_3	OH	H	=O	OCH_3
LXIII: thebaine	OCH_3	—O—		OCH_3	H

Structures of Morphinan Alkaloids Having Uterine Stimulant Activity

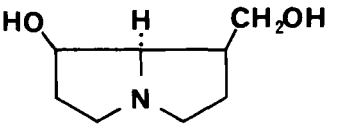


	R	R_1	R_2
LXIV: monocrotaline	OH	—CH_3	
LXV: sceleratine	H	—CH—$(CH_3)_2$	
		OH	

Structures of Pyrrolizidine Alkaloids Having Uterine Stimulant Activity

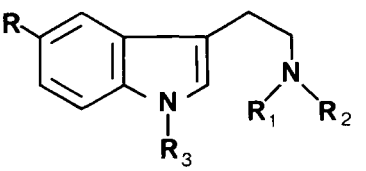


	R	R_1	other
LXVI: integerrimine	CH_3	OH	$\Delta^{1,2}$
LXVII: isatidine	CH_2OH	OH	—
LXVIII: platyphylline	CH_3	OH	—
LXIX: riddelliine	OH	CH_2OH	$\Delta^{12,13}$
LXX: senecionine	OH	CH_3	—
LXXI: seneciphylline	CH_3	OH	$\Delta^{12,13}$
LXXII: spartioidine	OH	CH_3	$\Delta^{12,13}$



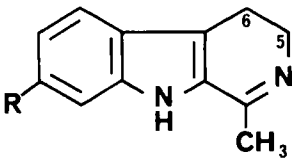
LXXIII: retrornecine

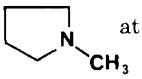
Structures of Pyrrolizidine Alkaloids Having Uterine Stimulant Activity



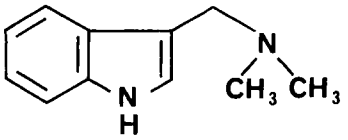
	R	R_1	R_2	R_3
LXXIV: *N,N*-dimethyltryptamine	H	CH_3	CH_3	H
LXXV: *N,N*-dimethyltryptamine *N*-oxide	H	CH_3	CH_3	H
LXXVII: 5-hydroxytryptamine	OH	H	H	O
LXXVIII: 5-methoxy-*N,N*-dimethyltryptamine	OCH_3	CH_3	CH_3	H

Structures of Tryptamine Alkaloids Having Uterine Stimulant Activity

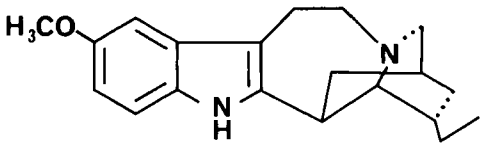


	R	other
LXXIX: brevicolline	H	 at 6-position
LXXX: harmaline	OCH_3	—
LXXXI: harman	H	—
LXXXII: harmine	OCH_3	$\Delta^{5,6}$

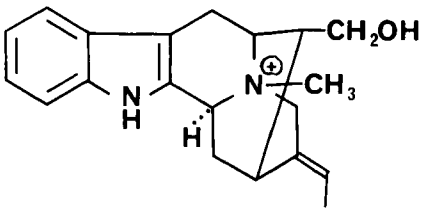
Structures of β-Carboline Alkaloids Having Uterine Stimulant Activity



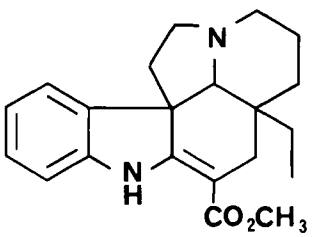
LXXVI: gramine



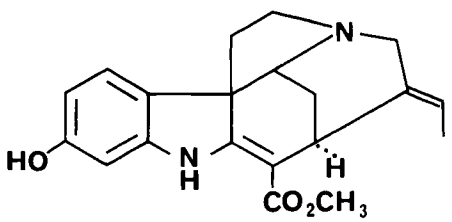
LXXXIII: ibogaine



LXXXIV: macusine B

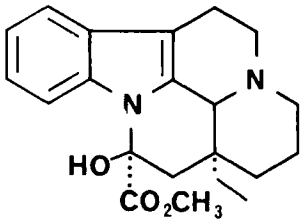


LXXXV: ervamine

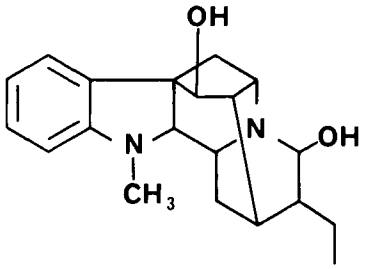


LXXXVI: vinervine

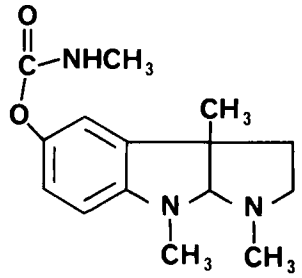
Structures of Miscellaneous Indole Alkaloids Having Uterine Stimulant Activity



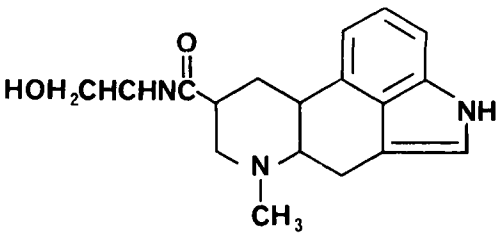
XC: vincamine



XCI: ajmaline



XCII: physostigmine



XCIII: ergonovine

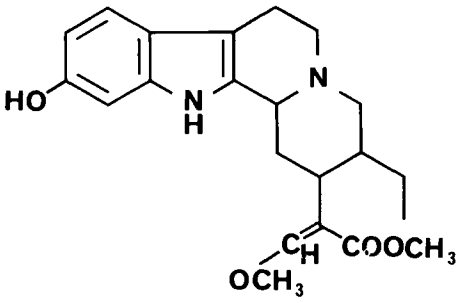
Structures of Miscellaneous Indole Alkaloids Having Uterine Stimulant Activity

During the literature search, some naturally occurring plant principles were found that were active as uterine stimulants, but the plants from which they were obtained were not noted. Whenever one of these active principles was known to occur in a plant previously noted under (A), (E), or (U), it was entered in Table IV as (I).

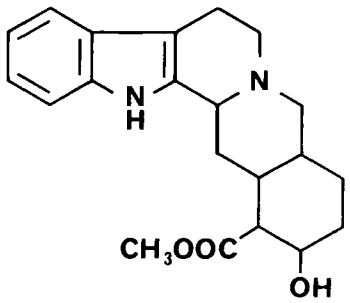
Finally, Table IV lists those plants and/or constituents that have been shown to be abortifacient or to have uterine stimulant activity as judged from tests in humans. These entries are noted as (H).

Although it is not within the scope of this presentation to give detailed information on all animal and human studies, a few remarks will be made concerning the abortifacient effects of some plant materials that have been used over the years as abortifacients.

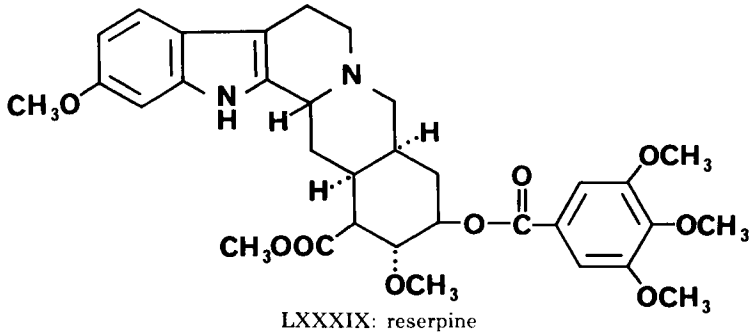
Volatile Oils—The most commonly employed volatile oils used in the past to induce abortion are those derived from *Tanacetum vulgare* (tansy), *Hedeoma pulegioides* (pennyroyal), *Ruta graveolens* (rue), *Petroselinum sativum* (apiol), and *Juniperus sabina* (savin). The literature contains virtually nothing with regard to the proven abortifacient effects of these materials. In fact, the use of these materials for abortifacient effects has been deleted from most standard pharmacology textbooks published in the last two decades. It has been shown that the excised human uterus is not stimulated by the oils of pennyroyal, savin, or tansy (198). It is



LXXXVII: gambirine



LXXXVIII: corynanthine



LXXXIX: reserpine

Structures of Miscellaneous Indole Alkaloids Having Uterine Stimulant Activity

speculated that when abortion does occur after the use of these volatile oils, it occurs only following toxic doses (198); fatal doses are not always abortifacient.

An interesting situation exists with apiol. There are two forms of this drug, "green apiol," which is the oleoresin of *P. sativum*, and "apiol" proper or "crystallized apiol," a compound of known structure. Both of these materials have a long history as abortifacients, alone or in combination with such materials as ergot extract. Numerous toxicities to apiol following its use in attempted abortion, as evidenced by a polyneuritis, were reported in the 1930's (823–832, 840, 841). It was eventually found that the toxicities were due to the presence of *ortho*-tricresyl phosphate in the apiol, presumably present as an adulterant (842, 843). It is safe to conclude that these materials were never consistently effective as abortifacients in humans and that their use will always be attendent with varying degrees of toxicity and even death (844).

Quinine and Castor Oil—These two agents have been used extensively in the past, alone or in combination with pituitary extract, to induce abortion in humans. Quinine has been reported in many studies to contract uterine tissue in both *in vitro* and *in vivo* experiments (641–682). Clinical studies are also numerous (347, 402–404, 408, 409, 683–767). It has been concluded (767) that except in cases of idiosyncrasy, quinine does not act to expel the premature fetus. There is no doubt, however, that quinine does help to promote uterine contractions after they have begun. The contractions produced by quinine in these cases are intermittent and not tetanic.

Although studies involving the use of castor oil in human abortion are numerous (401–409), one cannot find experimental studies to explain the rationale for its use. Presumably, it produces pelvic congestion as a result of intestinal irritation, as with the volatile oils (198).

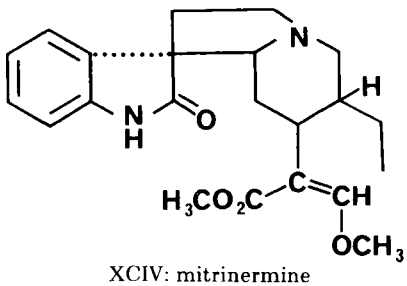
Sparteine—The only clinically useful abortifacient plant product known at present is sparteine (pachycarpine), which is an alkaloid derived from several Leguminosae species, especially those in the genera *Ammodendron, Baptisia, Cytisus, Genista, Goebelia, Lupinus, Retama, Sarothamnus, Templetonia,* and *Thermopsis* (845–847). Reports of the occurrence of sparteine in the unexpected Papaveraceae *(Chelidonium majus)* and Scrophulariaceae *(Leptorhabdos parviflora)* species should be reexamined (845–847).

Sparteine was first introduced into clinical practice as an oxytocic in 1939, and thousands of patients have been treated with it (487–489). Numerous reports confirm its *in vitro* and *in vivo* uterine stimulant activity (250, 252, 361, 385, 444–493). It has virtually all of the oxytocic properties of oxytocin and the ergot alkaloids. Untoward effects in humans almost always relate to overstimulation of the myometrium; thus, the dose of this alkaloid must be closely monitored. The latest review on the clinical use of sparteine as an oxytocic was published in 1966 (489).

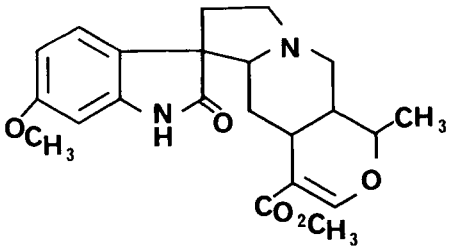
From the data presented in Tables IV and V, it can be seen that 565 species of plants are known to have a folkloric reputation for use as abortifacients, ecbolics, or emmenagogues. These 565 species are classified in 125 plant families. Of this 565 species, 225 have been shown to elicit a stimulant response when tested against uterine muscle either *in vitro* or *in vivo*, and active uterine stimulants have been isolated from 198 species. Of the isolated compounds, 148 are of known structure and 24 are of unknown structure; 122 of the isolated active compounds are alkaloids. Table V lists a number of common and widespread plant constituents that have been reported to stimulate uterine tissue either *in vivo* or *in vitro*.

Table V—Plant Principles of Widespread Occurrence Reported to Stimulate Uterine Tissue

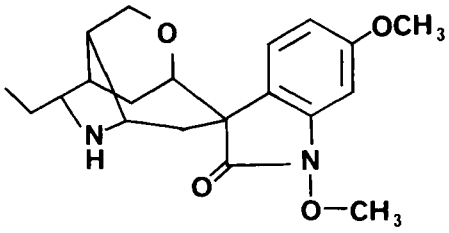
Plant Principle Type	Constituent	Reference
Simple amines	Betaine (CXV)	270
	Hordenine (VI)	310
	5-Hydroxytryptamine (LXXVII)	297–309
	Tyramine (IX)	312
Fatty acid	Arachidonic acid (CLI)	901, 902
	Linoleic acid (CLII)	901
	Linolenic acid (CLIII)	901
	Palmitic acid (CLIV)	415
Flavonoids	Hesperidin (CXLV)	776
	Quercetin (CXLVI)	903
	Rutin (CXLVII)	904
Sterols	Cholesterol (CXLI)	905
	β-Sitosterol (CXLII)	906
Polyphenols	Catechol (CXXXI)	820, 822
	Pyrogallol (CXXXII)	906
Miscellaneous	Ascorbic acid (CXXX)	907–911
	Ellagic acid (CXXXIII)	322
	Guaiazulene (CXXXVIII)	912
	Nicotinamide (XII)	913–915
	Tannin	916, 917



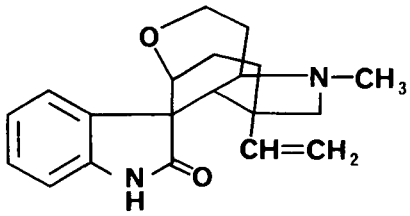
XCIV: mitrinermine



XCV: vineridine

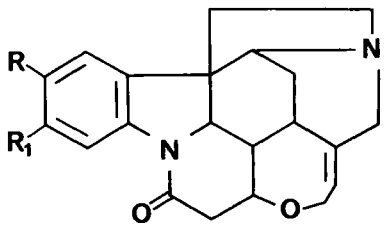


XCVI: gelsemicine



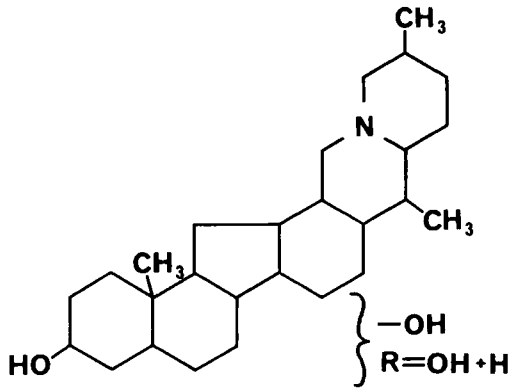
XCVII: gelsemine

Structures of Miscellaneous Indole Alkaloids Having Uterine Stimulant Activity

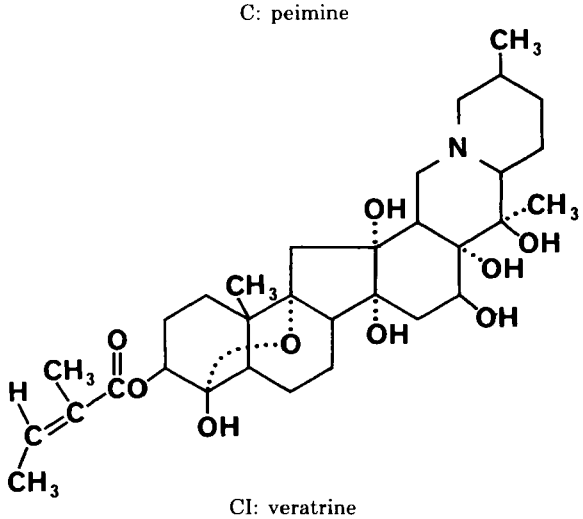


	R	R_1
XCVIII: brucine	OCH_3	OCH_3
XCIX: strychnine	H	H

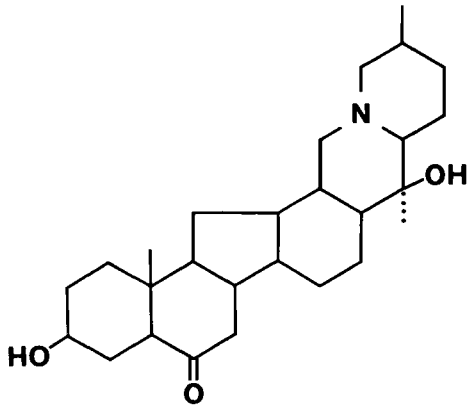
Structures of Miscellaneous Indole Alkaloids Having Uterine Stimulant Activity



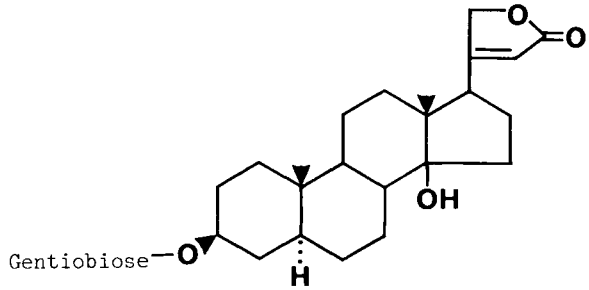
C: peimine



CI: veratrine

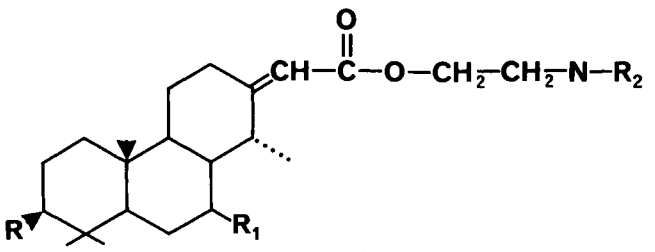


CII: fritillarine

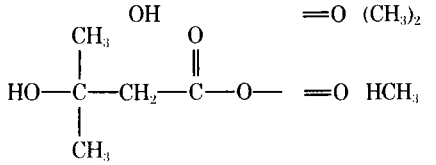


CIII: uzarine

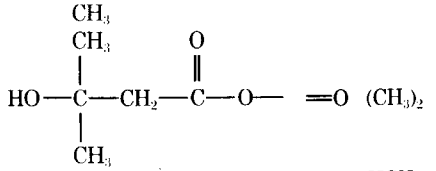
Structures of Steroid Alkaloids Having Uterine Stimulant Activity



	R	R_1	R_2
CIV: cassaidine	OH	α-OH	$(CH_3)_2$
CV: cassaine	OH	=O	$(CH_3)_2$



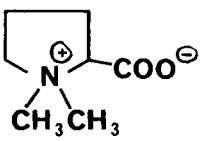
CVI: coumingidine =O HCH_3



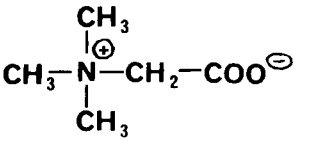
CVII: coumingine =O $(CH_3)_2$

CVIII: norcassaidine OH α-OH HCH_3

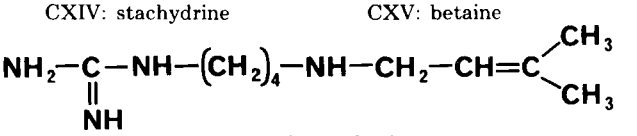
Structures of Diterpene Alkaloids Having Uterine Stimulant Activity



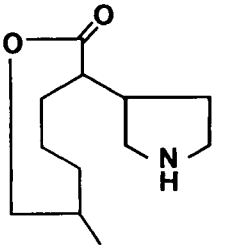
CXIV: stachydrine



CXV: betaine

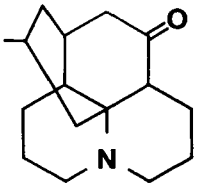


CXVI: sphaerophysine

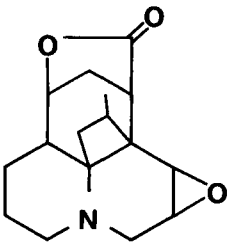


CXVII: chaksine

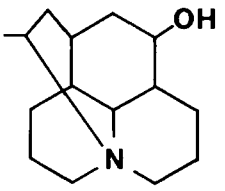
Structures of Miscellaneous Alkaloids Having Uterine Stimulant Activity



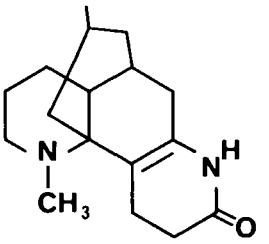
CIX: lycopodine



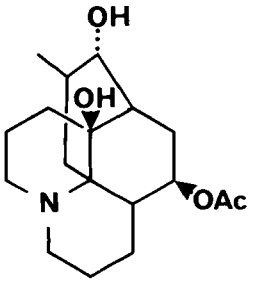
CX: annotinine



CXI: complanatine

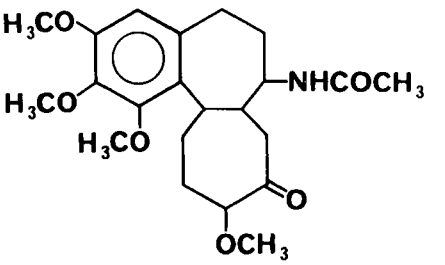


CXII: obscurine

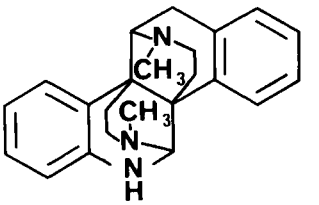


CXIII: lycofawcine

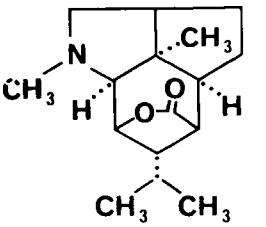
Structures of Hydrojulolidine and Related Alkaloids Having Uterine Stimulant Activity



CXVIII: colchicine

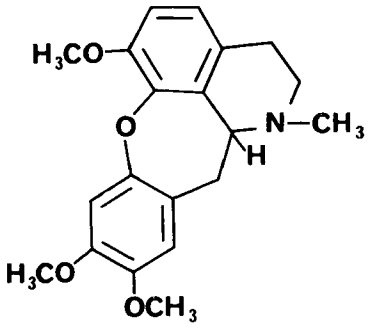


CXIX: calycanthine

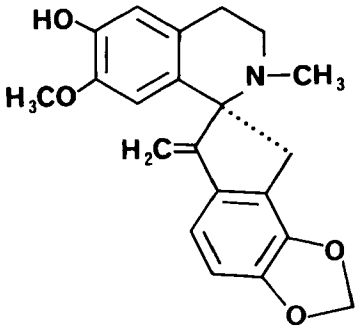


CXX: dendrobine

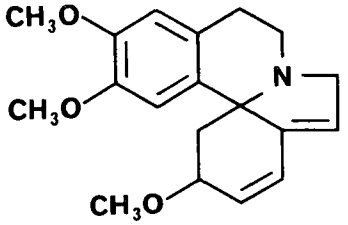
Structures of Miscellaneous Alkaloids Having Uterine Stimulant Activity



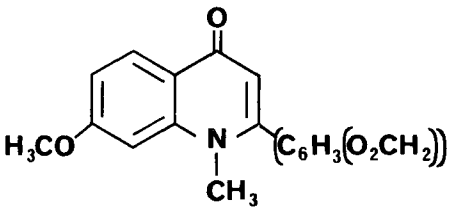
CXXI: cularine



CXXII: ochotensine

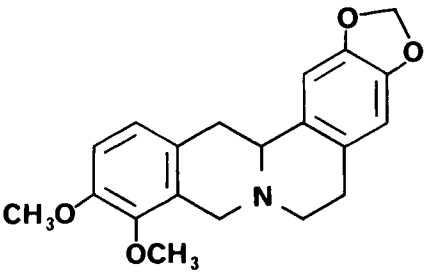


CXXIII: erysotrine

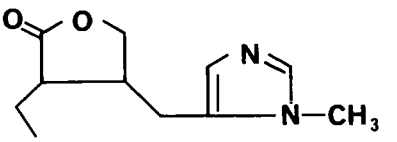


CXXIV: lunamarine

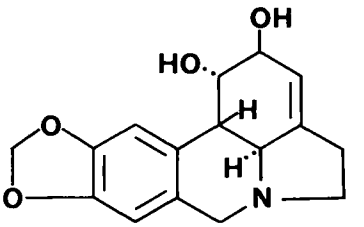
Structures of Miscellaneous Alkaloids Having Uterine Stimulant Activity



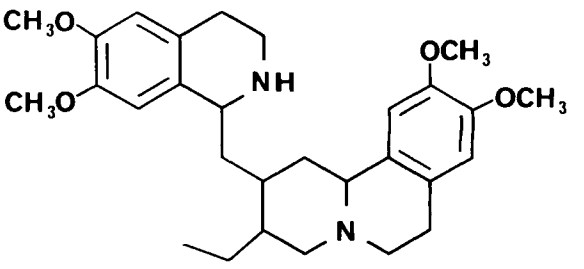
CXXV: canadine



CXXVI: pilocarpine

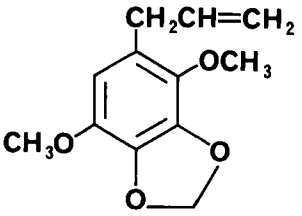


CXXVII: lycorine

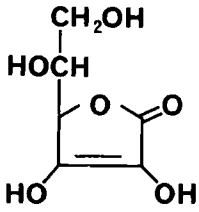


CXXVIII: emetine

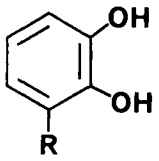
Structures of Miscellaneous Alkaloids Having Uterine Stimulant Activity



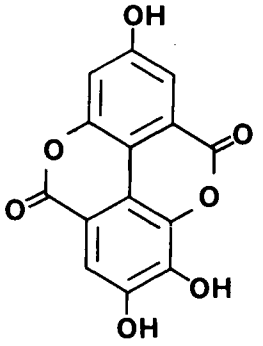
CXXIX: apiol



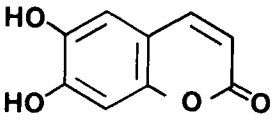
CXXX: ascorbic acid



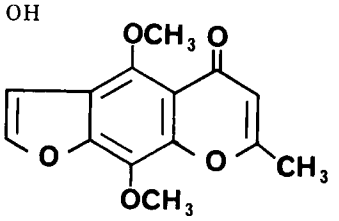
CXXXI: catechol, R = H
CXXXII: pyrogallol, R = OH



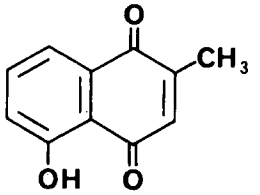
CXXXIII: ellagic acid



CXXXIV: aesculetin

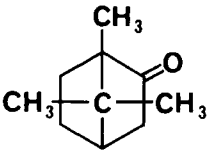


CXXXV: khellin

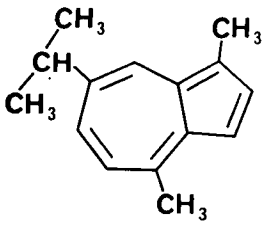


CXXXVI: plumbagin

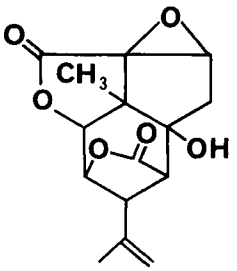
Structures of Nonalkaloid Principles Having Uterine Stimulant Activity



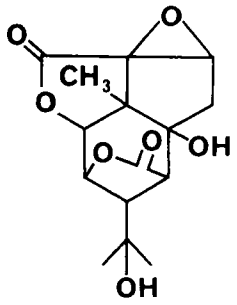
CXXXVII: camphor



CXXXVIII: guaiazulene

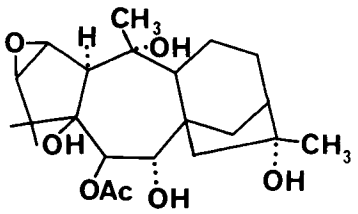


(*a*)



(*b*)

CXXXIX: picrotoxin (mixture of *a* and *b*)



CXL: lyoniatoxin

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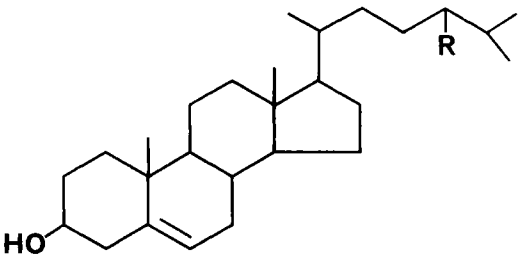
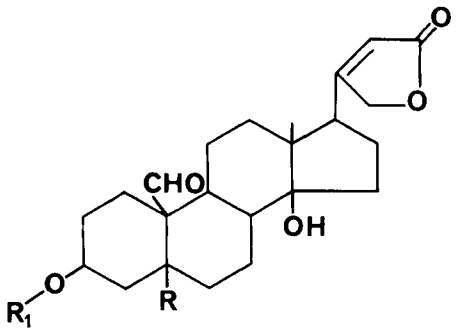


CXLI: cholesterol, R = H
CXLII: β-sitosterol, R = CH_2CH_3



	R	R_1
CXLIII: strophanthin	OH	rhamnose
CXLIV: thevetin	H	thevetose-gentiobiose

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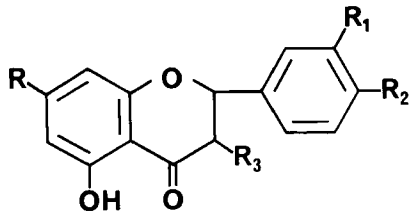
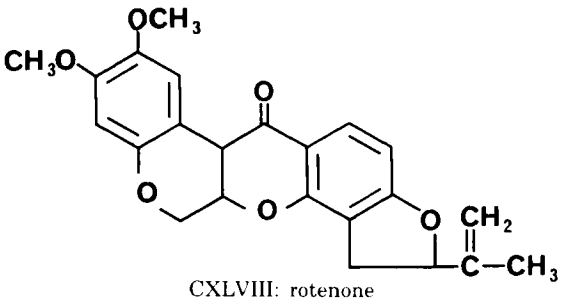
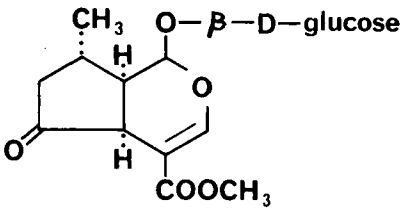


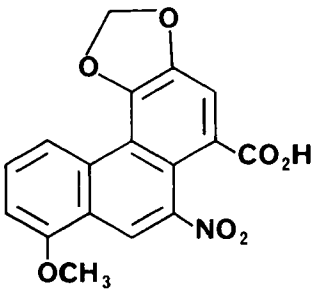
	R	R_1	R_2	R_3	other
CXLV: hesperidin	OH	OH	OH	OH	$\Delta^{2,3}$
CXLVI: quercetin	Rham·Glu	OCH_3	OCH_3	H	–
CXLVII: rutin	OH	OH	OH	*O*-rutinose	$\Delta^{2,3}$



CXLVIII: rotenone



CXLIX: verbenalin



CL: aristolochic acid

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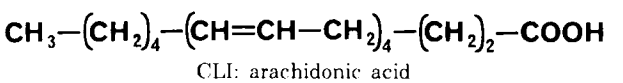
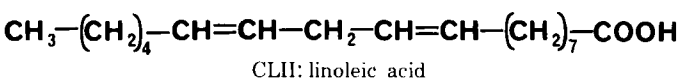
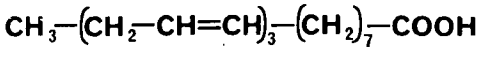


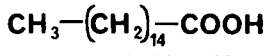
CLI: arachidonic acid



CLII: linoleic acid



CLIII: linolenic acid



CLIV: palmitic acid

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Selection of a Reference Partitioning System for Drug Design Work

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Abstract □ Consideration of the structural, polar, and transport properties of water-saturated solvent and solvent-saturated water phases of three general and eight specific binary systems leads to the conclusion that the *n*-octanol–water system is a very good all-round compromise for use as a reference system for biological partitioning in drug design work.

Keyphrases □ Partitioning systems—selection of a reference for drug design work, structural, polar, and transport properties of solvent and water phases, *n*-octanol–water system and others □ Biological partitioning—reference systems, structural, polar, and transport properties of solvent and water phases □ Transport—selection of a reference partitioning system for drug design work

Apart from its intrinsic chemical ability to act at the active site, a biologically active species can exert its influence only if it can reach the active site by a transport process that involves passage through both hydrophilic and lipophilic barriers in the biological system. As a consequence, an important aspect of drug design is the modification of the hydrophobic character of compounds possessing a desired intrinsic chemical activity so as to optimize their transport to, and their binding at, the active site. This is clearly a partitioning problem, and the need for a "synthetic" reference biological system is at once apparent if such modifications and development are to be carried on expeditiously and reproducibly. As a result, various solvent–water systems have been employed with the intent that the solvent represent the lipophilic phase of biological systems and the water represent the hydrophilic phase.

The 5806 partition coefficients listed by Leo *et al.* (1) (about 9000 values are currently in the data bank) stand as some measure of the current importance of these values. The past decade has seen a very rapid development, both in magnitude and sophistication, of the correlation of molecular structure with biological activity based on a system that considers the partition coefficient of the biologically active compound in a reference partitioning system to be a major (if not *the* major) parameter in the correlation equation. Although the original concept was put forth at the beginning of this century, the recent rapid development has been facilitated by the computerization of the techniques of linear regression analysis and the inclusion of other structural parameters; the principles are now firmly established as a major tool for drug design.

A problem associated with the current situation is that of relating the correlations based on one reference system to those based on another. This problem has been dealt with statistically (1), but it is hoped that through such papers as the present one this problem can eventually be lessened by mutual agreement on one system, or type of system, that has significant advantages over the others.

At the outset, it must be realized that all biological systems, or parts of them, are far too complicated to be really closely mimicked, even with respect only to hydrophobic characteristics, by a simple two-phase system. Furthermore, it must be realized that in biological systems the lipophilic phases are not "pure hydrocarbon" in nature but are associated with significant amounts of water held by the polar and/or ionic portions of the macromolecules of which they are composed. These polar areas and their associated water molecules have a profound effect on the lipophilicity of these phases.

There is a tendency to think of partition coefficients as coming from reference systems composed of one pure solvent phase and one pure water phase, but, in fact, the partition coefficients refer to systems composed of two binary phases, a water-saturated solvent phase and a solvent-saturated water phase. One objective of this paper is to examine the effect of saturation on the structure and properties of a given phase. The eight solvents compared here vary tremendously in their hydrophobicity and in their ability to dissolve water, but on the whole they have relatively small solubility in water.

It should also be emphasized that, to be useful, a partition coefficient *(P)* must characterize the transfer of only a simple molecular form (or forms) in which it exists in these phases. To determine *P*, the experimentally determined amounts of partitioned solute per unit volume in each phase (the ratio being referred to as the *distribution* ratio) must be corrected to take account of the various molecular species with which it is in equilibrium. For many solutes, this can be achieved rigorously only by application of coupled spectroscopic–computer techniques or inferentially from the study of *P* as a function of concentration based on the *assumed* existence of molecular species. The uncertainties in calculation of true values of *P* will vary with the partitioning systems. For routine use, it is desirable to have a reference system that minimizes these uncertainties for as wide a range of solutes as possible.

This study shows the effects of saturation on the

dielectric constant, density, viscosity, and enthalpy of activation of viscous flow, and they are interpreted along with related measurements (including IR and NMR) made by others.

EXPERIMENTAL

All experimental measurements were made at temperatures within ±0.05° of the values shown, as determined against a recently factory-calibrated piezo-electric quartz thermometer[1]. The molecular composition of the olive oil[2] was determined by GC analysis of the methyl esters of the fatty acids obtained by saponification; the molecular weight was thus calculated to be 875.6 g/mole. Oleyl alcohol was reagent grade. 1-Octanol was reagent grade subjected to the following treatment: *(a)* shaking with dilute sodium hydroxide, *(b)* shaking with dilute sulfuric acid, *(c)* drying with excess anhydrous sodium sulfate, and *(d)* fractional distillation, discarding the first fraction that contains a very small concentration of UV-absorbing contaminant. All other solvents were spectroquality.

The saturated solutions were made by a succession of vigorous shakings in glass-stoppered bottles over about a day, and they were subsequently retained in their respective baths for many days before removal of samples for the required measurements. Because of slow hydrolysis, the measurements on ethyl acetate solutions were made within an hour or so of their preparation. Density (ρ) measurements were made with standard calibrated 25-ml pycnometers, dielectric constant (ϵ) measurements were made at 5 MHz with an instrument designed for capacity substitution (2), and viscosity (η) measurements were made with viscometers[3] (using sizes appropriate for the viscosities under consideration) calibrated by suitable standards. These standards[4], conforming to ASTM oil standards, had kinematic viscosities at 25.0° of 3.702, 8.904, and 34.45 centipoise ml/g; 10-sec full sweep stopwatches were used.

Values of ΔH_{vis}, enthalpies of activation of viscous flow, were obtained from the slope of the computerized least-squares fit of the plot of log (η/ρ) *versus* $1/T$, corresponding to:

$$\log(\eta/\rho) = \frac{\Delta H_{vis}}{2.303R}\frac{1}{T} + b \qquad \text{(Eq. 1)}$$

Correlation coefficients were 0.9997 or better in all cases, except for olive oil for which the correlation coefficient was 0.990. Solubility data were taken from the literature for most cases, with consensus values being used where possible. The solubilities of oleyl alcohol and olive oil in water were determined by approximation; they are very low and the values are rough. The results are tabulated in Tables I and II.

DISCUSSION

Except for special biological situations involving "active transport" or "circulation," molecular transport is heavily dependent on diffusion processes. Self-diffusion or transport of a "marked" molecule through a liquid matrix of others identical to it is very simply related to the viscosity of that liquid. The diffusivity of a dilute solute through a liquid matrix of identical but different solvent molecules is also related to the viscosity of the solvent; but, in addition, there is a component related to a concentration gradient and thus to the entropy and enthalpy of dilution. Even in the latter case, however, the solvent usually controls the motions of the isolated solute molecules in ways that relate viscosity to the intermolecular forces and to the molar ratio of free volume to intrinsic volume (3, 4). It is not immediately obvious that viscosities expressed in the traditional "kinematic viscosity" mode (η/ρ = poise × ml/g) is related to the mass transport phenomenon of diffusion. This does become evident, however, if the poise is expressed in cgs units as η = g/sec × cm; then kinematic viscosity = η/ρ = (g/sec × cm)/(g/cm^3) = cm^2/sec, the cgs units characteristic of diffusion coefficients.

The free volume of a liquid is often considered to be the sum of a large number of empty "holes" into which diffusing molecules may slip in the transport process. As one hole is filled, another is created at the location where the diffusing molecule had just been. The enthalpy of activation of viscous flow (ΔH_{vis}) is a measure of the potential barrier that a molecule must surmount as it moves from one hole in the liquid to an adjacent hole. In this sense, it is a measure of the resistance to self-diffusion in a liquid.

Table II shows that in all of the saturated binary phases, except those involving *n*-butanol and ethyl acetate, the resistance to molecular transport was changed at the most by only 3%. Thus, for the majority of these binary phases, it can be said that internal transport characteristics are not significantly different from those of the pure major component of the phase. The binary phases involving *n*-butanol and ethyl acetate, where the changes in values of ΔH_{vis} ranged from 8 to 22%, will be dealt with later.

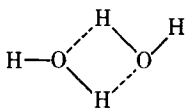
The dielectric constant (ϵ) of a liquid gives a measure of the resistance to molecular "flipping" in response to alternations of an applied electric field. For a given temperature and field frequency, the resistance to molecular rotation involves the size, polarizability, and permanent dipole (if any) of the molecules and any tendency to self-associate with other molecules to form larger aggregates. The addition of significant amounts of highly polar water to organic solvents may result in either an increase or a decrease in ϵ; as a result, molecular characteristics of liquid structure may be inferred from determination of ϵ as a function of temperature and concentration. Such inferences (based on assumed molecular models) have a greater probability of being correct if spectroscopic (usually IR and NMR) substantiation of the molecular models is possible. The following discussion, organized according to class of solvent, concerns what appears to be the appropriate aspects of the results of such measurements as they apply to the problems of determining partition coefficients.

Nonpolar and Slightly Polar Solvents—Of the solvents studied, cyclohexane is the only one that is truly nonpolar and has no capacity for hydrogen bond formation. As a result, it has no tendency for self-association into molecular aggregates, there is no liquid structure, and water is extremely insoluble in it. In a water-saturated solution, the dissolved water has no significant effect on ϵ, ρ, or η. Its solvent characteristics are typical of other saturated hydrocarbons and compounds such as carbon tetrachloride in which studies pertinent to the present discussion have been made.

Isopiestic studies (5) indicate that the very small amount of water that does dissolve in cyclohexane and carbon tetrachloride is, for all practical purposes, not associated. A recent IR study (6) of water dissolved in carbon tetrachloride indicated that about 3.6% of the dissolved water is present as a dimer, Structure I being more likely than II. The equilibrium constant for dimerization was calculated to be 2.2 M^{-1}. Since water is four times more soluble in carbon tetrachloride than in cyclohexane, it seems likely that dimerization is substantially less in cyclohexane and that, from a practical standpoint, water exists only in monomeric form in water-saturated cyclohexane.

As a partitioning liquid, this binary phase has a great affinity for nonpolar compounds and their values of P will be extremely high (the reverse is true for very polar and ionic compounds), a fact that makes the determination of P very difficult because of the very low solute solubility in one phase or the other. Partial alleviation of this difficulty is normally accomplished by selecting a suitable volume ratio of the two phases (7); but when one phase is almost totally nonpolar, this approach is quite impractical for many solutes.

This very pure nonpolar characteristic also leads to other experimental difficulties that may be impossible to overcome when measuring P values for polar solutes. To illustrate this fact, it is necessary to draw on experiments done with carbon tetrachloride, carbon disulfide, benzene, isooctane, and other nonpolar solvents. It has been found (8, 9) that when a polar solute (S) is extracted from water, there may be a many fold increase in the solubility of water in the nonpolar solvent due to the formation of hydrated solute



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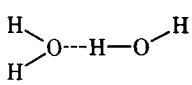

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[1] Hewlett-Packard model 2801A.
[2] Import Virgin Pompeian, Pompeian, Inc., Baltimore, MD 21224
[3] Cannon-Fenske type.
[4] Cannon Instrument Co., State College, Pa.

Table I—Densities (ρ) and Viscosities (η) of Pure and Saturated Solvents at 15–35°

Solvent	State	ρ, g/ml			η, centipoises		
		15.00°	25.00°	35.00°	15.00°	25.00°	35.00°
Cyclohexane	Pure	0.784	0.774	0.765	1.074	0.896	0.761
	Water saturated	0.784[a]	0.774[a]	0.765[a]	1.074[b]	0.896[b]	0.761[b]
Benzene	Pure	0.884	0.874	0.863	0.700	0.602	0.524
	Water saturated	0.884	0.874	0.864	0.699	0.601	0.524
Chloroform	Pure	1.493	1.475	1.453	0.595	0.538	0.489
	Water saturated	1.498	1.479	1.459	0.596	0.539	0.490
n-Butanol	Pure	0.814	0.807	0.799	3.32	2.52	1.93
	Water saturated	0.849	0.844	0.836	3.86	2.80	2.06
n-Octanol	Pure	0.828	0.822	0.815	11.0	7.61	5.43
	Water saturated	0.834	0.829	0.824	10.6	7.26	5.17
Oleyl alcohol	Pure	0.854	0.847	0.841	45.2	28.4	18.7
	Water saturated	0.855	0.850	0.840	44.1	27.9	18.4
Ethyl acetate	Pure	0.906	0.895	0.884	0.482	0.430	0.387
	Water saturated	0.911	0.900	0.899	0.520	0.460	0.410
Olive oil	Pure	0.916	0.910	0.904	101.3	63.1	41.7
	Water saturated	0.916	0.910	0.904	100.5	62.6	41.4
Water	Pure	0.999	0.997	0.994	1.14	0.894	0.720
	Cyclohexane saturated	0.999[c]	0.997[c]	0.994[c]	1.14[c]	0.894[c]	0.720[c]
	Benzene saturated	0.998	0.997	0.994	1.14	0.896	0.721
	Chloroform saturated	0.999	0.997	0.994	1.18	0.919	0.736
	n-Butanol saturated	0.989	0.987	0.983	1.57	1.16	0.895
	n-Octanol saturated	0.998	0.997	0.994	1.13	0.887	0.714
	Oleyl alcohol saturated	0.999	0.997	0.994	1.14	0.893	0.718
	Ethyl acetate saturated	0.999	0.996	0.993	1.36	1.04	0.811
	Olive oil saturated	0.999	0.997	0.994	1.15	0.895	0.720

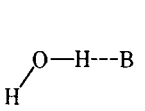
[a] Density data from Ref. 57. [b] Viscosity data from Ref. 58. [c] Density and viscosity for water-saturated cyclohexane and for cyclohexane-saturated water are assumed to be the same (to the number of significant figures given) as for the pure liquids because in each case the solubilities are less than one-fifteenth of those for the benzene–water system for which no changes in ρ and virtually no changes in η were observed.

complexes, $S(H_2O)$, $S(H_2O)_2$, *etc.*, which are in equilibrium with each other; the nonpolar character of the phase may be substantially changed by the process of partition itself. This will be especially bad with hydroxylic compounds, anilines, and other hydrogen bonding substances. This problem is in addition to the solute association (such as carboxylic acid dimerization) that often occurs and for which correction is usually made.

The extent to which the increased water content of the nonaqueous phase may also affect the solute association equilibria has not been extensively studied. However, in one study (from which it would be hazardous to generalize) it was shown (10) that in the distribution of acetamide between chloroform and water the equilibrium constant for the trimerization of acetamide in the water-saturated chloroform was not significantly different from that observed in pure chloroform. There was also no evidence for the existence of other polymers such as dimers and tetramers. This study was carried out at water phase concentrations of 0.42–9.9 *M* acetamide. However, at the solute concentrations normally used for

Table II—Dielectric Constants (ϵ) and Enthalpies of Activation of Viscous Flow (ΔH_{vis}) for Pure and Saturated Solutions at 25.0°, and the Saturation Concentrations of Water-in-Solvent and Solvent-in-Water at 25.0°

Solvent	State	Moles Water / Mole Solvent	Water Concentration, *M*	Solubility References	ϵ	ΔH_{vis}, kcal/mole
Cyclohexane	Pure				2.02	3.03
	Water saturated	0.000266	0.00245	1, 3	2.02	3.03
Benzene	Pure				2.27	2.36
	Water saturated	0.00314	0.0350	3, 45, 46	2.28	2.36
Chloroform	Pure				4.73	1.51
	Water saturated	0.00542	0.0670	1, 47	4.66	1.51
n-Butanol	Pure				17.16	4.64
	Water saturated	1.05	9.53	1, 48, 49	21.29	5.43
n-Octanol	Pure				9.78	6.07
	Water saturated	0.280	1.72	32, 34	8.58	6.23
Oleyl alcohol	Pure				3.86	7.66
	Water saturated	0.228	0.712	1	3.75	7.59
Ethyl acetate	Pure				5.98	1.73
	Water saturated	0.162	1.60	1, 50	7.41	1.87
Olive oil	Pure				3.17	5.93
	Water saturated	0.070	0.0725	1	3.17	6.07
		Moles Solvent / Mole Water	Solvent Concentration, *M*			
Water	Pure				78.54	4.03
	Cyclohexane saturated	1.18×10^{-5}	0.000651	51	76.95	4.03
	Benzene saturated	4.13×10^{-4}	0.0228	46, 51, 52, 53	76.13	4.03
	Chloroform saturated	1.16×10^{-3}	0.0637	54	75.91	4.15
	n-Butanol saturated	1.92×10^{-2}	0.977	48, 49, 55, 62	72.33	4.90
	n-Octanol saturated	7.29×10^{-5}	0.00404	49, 62, 63	76.58	4.05
	Oleyl alcohol saturated	$\sim 6.7 \times 10^{-8}$	~0.0000037		76.80	4.05
	Ethyl acetate saturated	1.64×10^{-2}	0.842	40, 50, 56	72.73	4.54
	Olive oil saturated	$\sim 2.6 \times 10^{-8}$	~0.0000014		76.18	4.07



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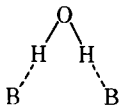

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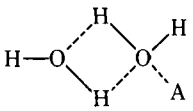

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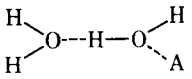

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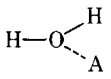


IX

drug design partition studies (10^{-2}–10^{-4} M), there would be no significant amount of trimer in the chloroform and partition would involve only the monomer.

This outlook has been refined by a detailed IR study (11) of the association of water with 17 types of Lewis bases in carbon tetrachloride (taken as typical of inert nonpolar solvents). For all of the nitrogen- and oxygen-containing Lewis bases studied (some containing two nearby basic sites), a 1:1 water–base complex was formed (III) when water was in excess and a 1:2 water–base complex (a hydrated dimer) was formed (IV) when base was in excess. Presumably an effective equilibrium between the two exists when concentrations of water and base are of comparable magnitude.

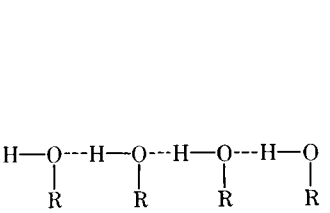
A further study of di-, tri-, and tetrabutylammonium halides also led to the conclusion that the hydrophobic cations were not hydrated at all in nonpolar solvents, but that water is hydrogen bonded to the halide ions in the same way as for the uncharged Lewis bases. Fluoride ion appears to be an exception, requiring two water molecules for hydration when water is in excess; it is suggested that this occurs through distortion of the cyclic dimer (I) and hydrogen bonding of the F^- to the two free hydrogen atoms. Normally, a water molecule is not doubly hydrogen bonded to a single base (even if there are two nearby base sites), although molecules such as 1,10-phenanthroline may represent exceptions.

NMR studies (12) have shown that when polyfunctional solutes are being partitioned, allowance must be made for intramolecular binding as well as intermolecular binding. For example, by going from 3-methyl-3-hydroxy-2-butanone to 3-methyl-4-hydroxy-2-butanone, the molecular distribution changed from 19% monomer, 30% intramolecular bound, and 51% dimer to 12% monomer, 8% intramolecular, and 80% dimer.

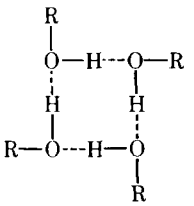
Although the arguments are by no means clearly settled (13, 14), it appears (15–17) that nonsterically hindered alcohols tend to form a fairly cleancut monomer–tetramer equilibrium in solution, with the tetramer being partly in a linear form (V) and partly in a cyclic form (VI). At 30°, for *n*-octanol in *n*-decane, the monomer–tetramer association constants are 163 M^{-3} for linear and 324 M^{-3} for cyclic to give an overall value of 490 M^{-3}. With increasing temperature, the extent of polymerization decreases and the proportion of cyclic to linear forms also decreases; at 75°, the equilibrium constants are 5.46 M^{-3} for linear and 4.89 M^{-3} for cyclic to give an overall value of 10.35 M^{-3}.

A comparison of overall association constants [490 for *n*-octanol in *n*-decane at 30°, 430 for *n*-butanol in *n*-decane at 30°, and 312 for *tert*-butanol in cyclohexane at 27° (15, 18)] shows that there is not a great difference in degree of association for nonsterically hindered alcohols in the same solvent; the significantly lower value for *tert*-butanol in cyclohexane is probably due more to some degree of steric hindrance than to the difference in solvent. Sterically hindered alcohols, such as di-*tert*-butylcarbinol (19) and 2,5-substituted phenols (20), cannot form tetramers and in this case dimers are formed in equilibrium with monomers. Partially sterically hindered alcohols will participate in a more complicated set of polymeric equilibria. For *n*-octanol in octane at 25° (14), it has been estimated that the maximum proportion of dimer is just over 5% at a concentration of 0.13 M and that this amount decreases to less than 2% at 1 M and to less than 1% at 5 M; otherwise, the only associated species is thought to be the tetramer.

To date, the structural studies of associated polar molecules in nonpolar solvents have not considered the effect of the water that



V



VI

is drawn into the nonpolar solvent by a polar solute when it is partitioned between water and the solvent.

The water concentration and all equilibria between solute hydrates, solute polymers, hydrated solute polymers, and intramolecularly bound molecules are dependent on solute concentration so that the determination of true partition coefficients corrected for these effects becomes a very formidable task. For many solutes, these effects could be minimized or eliminated by never permitting the solute concentration to exceed 10^{-3} M in the nonpolar solvent phase, but this may, in turn, introduce analytical difficulties, especially in the aqueous phase. In any case, when the biological activity for a series of compounds is compared with log P values determined in, say, an alkane–water system, the correlations are often not nearly as good as those obtained using log P values determined in the more polar *n*-octanol–water system (7, 21). The simplest binary liquids (water-saturated alkanes, cycloalkanes, carbon tetrachloride, *etc.*) thus have properties that lead to great difficulties when determining true partition coefficients and getting the best correlations with biological activity.

In many ways the properties of benzene are comparable to cyclohexane. It is a nonassociated, nonpolar liquid that has a great affinity for lipophilic hydrocarbon-like substances. However, it does differ in one important respect; its π-electron system can act as an electron pair donor for hydrogen bond formation. As a consequence, water is 15 times more soluble in benzene than in cyclohexane. The net result is that the binary phase, water-saturated benzene, is definitely more polar than cyclohexane, yet its dielectric constant is barely larger than that of pure benzene and its density and viscosity are the same as pure benzene. A variety of measurements, cryoscopic (22), isopiestic (3, 23–25), and NMR (26), leads to the conclusion that up to saturation the water dissolved in benzene is not self-associated in any polymeric form. It is believed (11, 27) to exist primarily in a hydrogen bonded solvated form such as IV, where B represents benzene, the base donor of an electron pair.

All structural and partitioning problems associated with cyclohexane also hold for benzene: the great increase in water solubility caused by partitioned polar solutes, the multiequilibria between hydrated species and multimeric species, and the effects of steric hindrance. In addition, there are the complications introduced by solutes such as alcohols competing with water for hydrogen bonding with the benzene. A thermodynamic study (28) of this competition indicated that, for the simple *n*-alkanols up through *n*-pentanol, there is a dramatic drop in the enthalpy of solution of water in alcohol–benzene solutions if the alcohol concentration exceeds a mole fraction of 0.025; larger *n*-alkanols do not show this effect. Clearly, the nature of the binary phase, water-saturated benzene, varies with the nature and concentration of partitioned alcohols and is bound to be reflected in values of partition coefficients. It has been suggested (29) that the larger alcohol molecules adopt a coiled configuration in benzene that results in a partial screening of the —OH interaction with benzene. Since more recent studies (16, 18) indicate the importance of cyclic tetramers in alcohol solutions, it may be that the proposed "shielding" comes from the long alkyl chains of these cyclic tetramers rather than from the coiling of monomers.

There can frequently be an analytical problem with the benzene–water system over and above that caused by the extreme solubility of lipophilic solutes in benzene and the insolubility in water (and the reverse for hydrophilic solutes). The most common analytical tool for determination of P is UV spectrophotometry; the very strong UV absorption by benzene renders this method entirely inapplicable, even in the benzene-saturated water phase, at wavelengths shorter than 270 nm.

As with cyclohexane, the conclusion must be reached that the benzene–water system leaves much to be desired as a reference partitioning system.

Chloroform is a slightly polar, nonassociated liquid which has a great affinity for lipophilic materials. Its lone proton is an effective electron pair acceptor in hydrogen bond formation; as a result, the

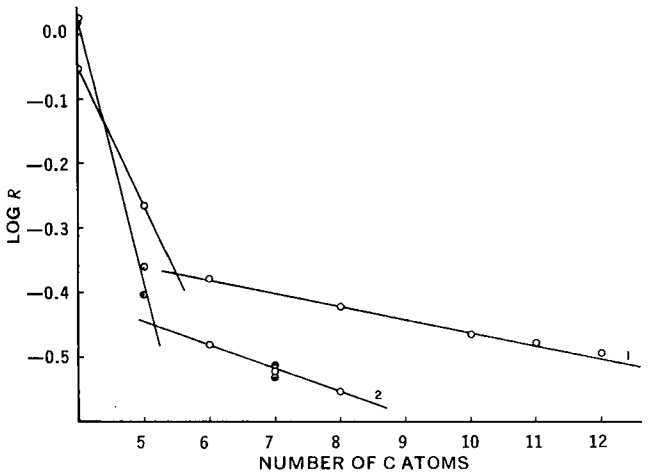


Figure 1—*Log* R versus *number of carbon atoms in* n-*alkanol chain. In curve 1,* R = *(H₂O area)/(CH₂ area), the ratio of areas under NMR peaks corresponding to dissolved water (H₂O area) and the two protons in the alcohol to which the OH is attached (CH₂ area) for alcohols saturated with water at 25°. In curve 2,* R = *moles of water/moles of alcohol in* n-*alkanols saturated with water at 25°. Key:* ○, *from Ref. 32;* ◐, *from Ref. 59;* ●, *from Refs. 48 and 49;* ◒, *from Ref. 61; and* ◓, *from Ref. 60.*

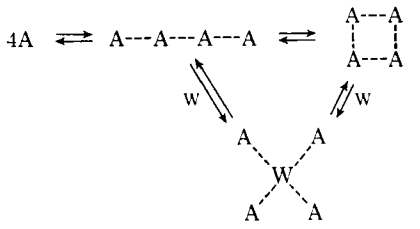
solubility of water is almost two times greater than it is in benzene. This greater solubility leads to a small but significant *decrease* in the dielectric constant of chloroform, yet it is still too small to have a significant effect on the density or viscosity of chloroform. Nevertheless, the amount of dissolved water is sufficiently large to introduce a structural complexity not observed in benzene or cyclohexane.

NMR studies (25, 29) revealed that a small amount of water dimer is hydrogen bonded to chloroform, probably as a mixture of I and II, as in VII and VIII. The majority is monomerically hydrogen bonded as in IX where A represents chloroform, the electron pair acceptor. The equilibrium dimerization constant for water in chloroform is 0.437 M^{-1} at 25° (corresponding to about 5.2% dimer at saturation), a value somewhat smaller than for other partially chlorinated hydrocarbons in which the range is usually about 8–15% dimerization (9). Since ϵ is smaller for water-saturated chloroform than for pure chloroform, the water–chloroform complexes must have a smaller μ than pure chloroform and the complexes must be sufficiently stable to behave as a polarizable species; for dimer dissociation, ΔH° = 1.8 kcal/mole.

The analytical application of UV spectrophotometry is not affected by chloroform, but all other partition problems associated with benzene apparently are also associated with chloroform, although perhaps in less extreme form. There also is the added complication of water association.

Alcohols—The problems associated with partitioning of alcohols *as solutes* between nonpolar or slightly polar solvents and water have already been discussed. Because of the highly polar nature of the hydroxy group and its great capacity for hydrogen bonding, the structure of the pure liquids and their solutions have been the object of extensive investigation. The pure alcohols are associated liquids with multiple equilibria existing between monomers and linear and cyclic multimers. For nonsterically hindered alcohols, the present IR spectral evidence heavily favors the predominance of linear and cyclic tetramers (V and VI) in equilibrium with monomers, with very little evidence for other polymeric forms (14, 16, 17).

Earlier temperature-dependent dielectric dispersion data were interpreted (30, 31) in terms of a shift in equilibria to smaller linear polymers and fewer with increasing temperature, with something of the order of four to six monomeric units per polymer at a maximum at room temperature (*n*-hexanol to *n*-decanol). However, it appears than an equally good explanation could be made in terms of decreasing the relative proportions of cyclic to linear tetramers and decreasing the proportion of tetramers to monomers with increasing temperature. Heavily substituted alcohols (especially on the α-carbon) have severe steric hindrance, which tends to reduce the concentration of monomers and to encourage the formation of cyclic polymers (32). In extreme cases, such as di-*tert*-butylcarbinol, the dimer is the largest polymer formed (17, 19).



Scheme I

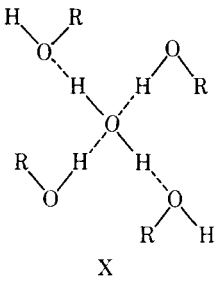
When water is added to alkanols, the simpler members of the family undergo much more profound intermolecular interactions, especially if water-saturated solutions are considered as is the case in partition measurements. In a sense, the water-saturated alkanols fall into two major categories, C_4–C_5 and C_6–C_{12}, as illustrated by water solubility in Fig. 1. C_3-Alkanols and below are miscible with water in all proportions, and C_{13}-alkanols and above are solids at room temperature. Curve 1 in Fig. 1 was determined from NMR measurements on water-saturated *n*-alkanols by taking the ratio of the area under the water peak to the area under the CH_2 peak of the carbon to which the OH is attached. Once the molar ratio of water to alcohol (W/A) exceeds ~0.40, the structure and properties of the binary phase are so greatly altered as to permit the association of a great deal more water, probably in a three-dimensional hydrogen bonded network. When the water-to-alcohol ratio in saturated solution is below ~0.40, the structure and properties of the saturated solution undergo a rather predictably slow change with increasing chain length of the alcohol.

Dielectric constant and viscosity measurements (33–35) offer some insight into the significance of this ratio. At room temperature, when water is added to the *n*-alkanols (C_1–C_5), 3-ethylpentan-3-ol, ethylene glycol, and glycerol (36), there is a steady increase in ϵ and η with increasing water concentration; but for the *n*-alkanols (C_6–C_{10}) and cyclohexanol, there is an initial decrease in ϵ and η with increasing water concentration. In the cases of 1-heptanol (at ~45°) and cyclohexanol (at ~25°), the values of ϵ go through a minimum at W/A = 0.25; in the case of 1-octanol, an apparent minimum is just reached at room temperature at W/A = 0.28 when saturation is reached. NMR data for 1-heptanol indicate (33) a relatively stable molecular aggregate at W/A = 0.25, and this finding is supported by less detailed studies with *n*-octanol (34). Added to the foregoing is the IR information about nonsterically hindered alkanols that indicates an equilibrium between monomers and linear and cyclic tetramers, an equilibrium that shifts in favor of monomers and linear tetramers with increasing temperature. The more sterically hindered an alcohol is near the α-carbon, the less able it is to form the tetramers, and cyclic dimers tend to be the preferred aggregate. The very simplest alcohols tend to form long linear multimers (37).

An oversimplified set of equilibria, which can be modified in detail for individual alcohols and which offers a general explanation for the properties of the higher alcohols and their water solutions, is shown in Scheme I, where A represents an alcohol monomer, W represents water, and the linear and cyclic tetramers are Structures V and VI. These equilibria are not meant to imply any particular kinetic mechanism of interconversion among the different forms. The water-centered complex, A_4W, is believed to have tetrahedral orientation of hydrogen bonded alcohols about the oxygen atom of water, as in X.

Qualitatively, A and cyclic A_4 have very low values of ϵ, linear A_4 has a high value of ϵ, and A_4W has a low value of ϵ that is greater than cyclic A_4. In the absence of W, only the horizontal equilibria exist; with increasing temperature, these equilibria shift to the left, causing a decrease in the amounts of both linear and cyclic A_4 and an increase in A. As a result, ϵ decreases because of the relative loss of linear A_4. If the alcohol is seriously hindered sterically, the horizontal equilibria lie far to the right and the linear and cyclic multimers are probably not tetramers but more likely dimers or trimers. In this case, an increase in temperature also shifts the equilibria to the left but ϵ increases because of the relative increase in the amount of linear multimer at the expense of cyclic multimer.

When water is added to these alcohols at room temperature,



X

there is a very strong tendency to form A_4W and all equilibria shift *in that direction; the net result is a decrease in* ϵ *for the solution* due to the loss of high ϵ linear A_4. If it is possible to add more water than corresponds to stoichiometric A_4W (*e.g.*, cyclohexanol and *n*-heptanol and smaller), ϵ will rise again due to the formation of larger aggregates of cross-linked, water-bridged A_4W units. In the case of the sterically hindered alcohols where there is a relatively small amount of linear multimer in the first place, the increase in ϵ is due to the formation of A_4W at the expense of low ϵA. The effect of adding water at each temperature must be assessed in terms of the relative amounts of A, linear A_4, and cyclic A_4 (or their counterparts in sterically hindered alcohols) that exist in the horizontal equilibria.

Although the increase in NMR line broadening for both OH and water on addition of water does indicate increased proton exchange, this exchange rate is relatively small even at the maximum corresponding to A_4W. The presence of the merest trace of many impurities will so accelerate the exchange as to cause the merging of the two peaks. The implication of this observation for partitioning is that a large fraction of solutes partitioned will probably initiate very rapid exchange, perhaps due to minor changes in pH. It is unlikely that this exchange will influence the values of *P*.

Although water–alcohol solutions are obviously complex, it would appear that a water-saturated *n*-octanol binary phase may have some advantages for partitioning. With a value of W/A = 0.28, almost all of the alcohol and water molecules are involved in the same type of A_4W complex, with but a small excess of water probably involved in cross-linking some of these complexes. Because of the large quantity of dissolved water, it is unlikely that the water concentration or the structural characteristics of this binary phase will be significantly changed as it is in the case of the nonpolar solvents. This means that low but convenient analytical concentrations of partitioned solute may be used, regardless of the polar functional groups it may possess. The balance of polarity and nonpolarity is such as to minimize the need for concern about solute self-association while simultaneously retaining the predominantly lipophilic character required for a reference biolipid system.

The complicated association equilibria that exist for alcohol and hydroxylic solutes partitioned into nonpolar or slightly polar solvents are virtually eliminated in water-saturated *n*-octanol, with each such partitioned molecule undoubtedly replacing one octanol in an A_4W complex to maintain the equivalent of a monomeric dispersion of the partitioned solute. The A_4W complex also offers, in very high concentration (~6.2 *M*), the opportunity for a highly lipophilic solute simply to "dissolve" monomerically in the matrix of highly lipophilic carbon chains of neighboring A_4W complexes. The preceding statements are supported by the long experience in this laboratory with the use of *n*-octanol, which reveals that only with certain very hydrophobic ionic species will partition measurements need to be made over a range of solute concentrations to get a true value of *P* by extrapolation to infinite dilution. This one problem may actually have as its origin the nature of the aqueous phase *(vide infra)*.

n-Heptanol and *n*-nonanol might be considered as comparable alternatives to *n*-octanol for partitioning. Water-saturated *n*-heptanol has the advantage of being somewhat less viscous but the disadvantage of having a little too much water (W/A = ~0.30, with dielectric and viscosity properties corresponding to those past the optimum A_4W). Water-saturated *n*-nonanol has the advantage of having an even more ideal water concentration (W/A = ~0.26) but the disadvantage of being more viscous.

With a molar water-to-alcohol ratio of ~1.0, it is not surprising that the structure of water-saturated *n*-butanol is extremely complex. It has been interpreted (38, 39) in terms of a whole variety of hydrogen bonded species, none of them predominant. The really serious disadvantage to the use of water-saturated butanol in a reference partitioning system is the fact that it offers so little sensitivity for discriminating between the relative hydrophobicities of solutes; the water-saturated alcohol is too similar in solvent properties to the alcohol-saturated water phase in which W/A = ~52.

Oleyl alcohol, *cis*-9-octadecen-1-ol, has been used for partition work by some investigators in the belief that it closely resembles biological phases. With an exposed double bond at the *cis*-9,10-position, it has additional hydrogen bonding potential compared to the saturated alcohols, and this, no doubt, accounts for the high water-to-alcohol ratio of 0.23 in contrast to the value of 0.17 expected from Fig. 1. At the moment, there is no experimental basis for speculating about how much of the dissolved water is associated with the double bond and how much with the hydroxy group. Also, with the bulky carbon chains, it is difficult to assess the importance of the A_4W or other complexes. However it is associated, the dissolved water has negligible effects on the dielectric and transport properties. From an experimental standpoint, water-saturated oleyl alcohol is difficult to work with because of its high viscosity (about four times that of water-saturated 1-octanol) and its tendency to form stable emulsions.

Esters—As a class, esters are polar Lewis bases whose oxygen atoms have a high potential for hydrogen bonding. As a result, they tend to dissolve more water than do the slightly polar hydrogen bonding solvents like chloroform or benzene. IR studies (11) in nonpolar solvents indicate that when water is in excess, a 1:1 ester–water complex is formed (Structure III, with B representing the ester); but when the ester is in excess as it always is in water-saturated ester, a 2:1 ester–water complex is formed (Structure IV). The increase in ϵ with an increase in water concentration also indicates an increase in concentration of bulky polar aggregates. Being more polar than water-saturated benzene or chloroform, the binary ester phase will tend to discourage the formation of self-association complexes of the partitioned solute, but these same solutes may well carry additional water into the solvent phase. It seems likely that hydroxylic solutes will participate in different varieties of association complexes with water, ester, and alcohol, depending on the degree of steric hindrance, hydrophobicity, and concentration; therefore, the determination of true partition coefficients would be very difficult.

Water-saturated ethyl acetate has been the most widely used ester for biological partitioning and is typical of the simple esters. In addition to the uncertain and variable structural problems just mentioned, it seems to be undesirably lacking in hydrophobic character for use as a reference system for a biological lipid phase. (When using values of *P* for the octanol–water system, log *P* for ethyl acetate = 0.71 compared to 3.15 for *n*-octanol.) From an analytical standpoint, it suffers from the fact that it slowly hydrolyzes, a process that may likely be catalyzed by some partitioned solutes. For example, physical measurements such as ϵ must be made within an hour to be significant (40). Therefore, immediate attention must be given to the analysis of the partitioned phases and it is not possible to keep a stock solution of water-saturated ethyl acetate for convenient use over a period of time as is customarily done in partitioning work for solvents that dissolve appreciable quantities of water. The hydrolysis products may achieve a concentration as great or greater than the solute being partitioned, the pH will be lowered by the acetic acid that is formed, and many basic solutes will form a salt which will drastically alter the apparent partition coefficient.

Being derived directly from a natural source, olive oil is in principle the best qualified of the systems considered in this paper for use as a reference biolipid phase. It is a triglyceryl ester. Other than its natural source, however, water-saturated olive oil has nothing but discouraging attributes. It does not dissolve much water. The amount that it does dissolve at 25°, 0.07 mole of water/mole of olive oil, must be considered as a third of that, on the average, per ester group. And when it is realized that a significant fraction of the long chain fatty acids esterified with the glycerol are unsaturated, then the fraction of ester groups and double bonds that are hydrogen bonded to water molecules (probably as Structure III) must be between 0.01 and 0.02. The low dielectric constant and water solubility indicate that structural problems, problems of self-association of partitioned solute, and problems of

varying degrees of hydration of polar partitioned solutes by additional water brought in by the partitioning process associated with nonpolar or slightly polar solvents will be involved here.

From a practical standpoint, there are additional difficulties. Olive oil is very difficult to work with because of its very high viscosity (about nine times that of *n*-octanol) and its discouraging tendency to form stable emulsions. Other problems also should be considered from the standpoint of its use as a reference system. Its composition varies appreciably with the source of the olive oil, as do certain of the natural contaminants which are extremely difficult to remove. It has poor storage stability, and the resulting rancidity involves changes in color, acidity, and odor, the formation of oxidation products and peroxides, and the process of slow polymerization (41–43). Other vegetable oils that have been considered for use in a reference partition system for biological lipids suffer in varying degrees from the same difficulties.

Solvent-Saturated Water—Tables I and II show the principal physical properties of the solvent-saturated water phases considered in this paper. With the exception of *n*-butanol and ethyl acetate, the solvents have very little impact. Their low water solubility causes only a small decrease in ϵ and a negligible effect on density and viscosity (diffusion and transport phenomena). Although the ratio of moles of water to moles of solvent is over 50:1 for *n*-butanol and ethyl acetate, this still corresponds to a concentration in excess of 1.1 *M*, and the physical and structural characteristics of the hydrogen bonded water molecule network are markedly affected. The unusually large increase in both η and ΔH_{vis} probably results from the water structure-forming influence of the nonpolar hydrocarbon portion of the molecules present in such a high concentration (44).

As mentioned earlier, the difference in solvent characteristics of the two phases in the *n*-butanol–water system is too small to make this system suitably sensitive as a reference system for discriminating between compounds over a wide range in hydrophobicity. Ethyl acetate has the problem of hydrolysis. Both have problems of structural interpretation in the solvent phase. The water solubility of oleyl alcohol and olive oil is so low that the effect on partitioning in the water phase can be ignored, but the usefulness of these two is limited for reasons given earlier.

The other four solvents shown in the tables have solubilities in the range of from 6×10^{-4} to 6×10^{-2} *M*. Although these concentrations have an almost negligible effect on the structure and properties as such, they may have a significant effect on partitioning. For example, hydrophobic interaction between solute and solvent in the water phase probably will result in a higher solute solubility in solvent-saturated water than in pure water. As a consequence of this enhanced water solubility, the observed values of log *P* may be lower than expected, especially for those nonpolar solutes whose water solubility is comparable to or less than the water solubility of the solvent.

Measurements carried out in this laboratory show that the increased solubility in octanol-saturated water is approximately 4.7% for naphthalene, 5.2% for phenanthrene, and 67% for anthracene. Since it has also been shown that the octanol–water partition coefficients for phenanthrene and anthracene are the same (~4.45), as expected, it is clear that solute–solvent interaction in the water phase does not necessarily lead to poor values of *P*. In the phenanthrene and anthracene cases, the great differences in effect of octanol on solubility may only reflect unusual differences existing in their *solid* states that affect the solid–dissolved solid equilibrium; even in pure water, the solubility of phenanthrene is about 20 times greater than anthracene.

Partition coefficient determinations do not (or should not) involve phases saturated with the partitioned solute or in equilibrium with its solid form. The practice of calculating values of *P* by taking the ratio of solute solubility in the separate pure phases for solutes that are fairly soluble in both (1) is a poor one from the standpoint of drug design because it ignores *(a)* the importance of activity coefficients which must be considered at high solute concentration, and *(b)* the effect that each solvent has on the solvent power of the other.

Another example of the possible importance to partitioning of the low concentration of solvent dissolved in water is the following. With increasing chain length in a homologous series of partitioned compounds, the point may be reached where the water phase solubility is so low that it is comparable to or lower than the concentration of the dissolved solvent. With still further increases in chain length, the solute solubility in the water phase may not drop off as fast as might have been expected because its solubility may be primarily determined by hydrophobic interaction with the dissolved solvent molecules. The result will be seen as a "break" in the log *P*–chain length relationship.

Such breaks have been observed with certain series of compounds, but their interpretation has not been satisfactorily resolved. Should such a break be interpreted as *(a)* hydrophobic interaction of solute with dissolved solvent as just described, *(b)* "folding" of the solute molecules with increasing hydrophobicity so as to present a smaller hydrophobic interface with water, *(c)* hydrophobic self-association equilibria between solute molecules, or *(d)* some combination of these three? Additional work is needed to clarify this point. It would seem that one way to minimize this problem in determining a true value of *P* would be to perform all partition experiments at solute concentrations smaller than the concentration of the dissolved solvent. All of the above statements presuppose the use of solute concentrations below the critical micelle concentration for those compounds that tend to form micelles.

CONCLUSIONS

Of the types of solvents considered, the best choices for a reference biolipid phase are water-saturated *n*-heptanol, *n*-octanol, and *n*-nonanol. Considering the number of compounds for which *P* values have already been determined in *n*-octanol (~3000 in the data bank of this laboratory compared to a very small number for the other two), water-saturated *n*-octanol is much the better choice. Its advantages seem to stem from having most of the *n*-octanol and water tied up in a tetrahedral hydrogen bonded complex that retains a high degree of hydrophobicity because of the four 8-carbon nonpolar chains surrounding the polar center. It discourages self-association of partitioned polar solutes by having a reasonable degree of polarity and by providing an opportunity for a solute molecule to exchange with an alcohol molecule in the tetrahedral complex. Because it contains a relatively high concentration of water, its structure, polarity, and partitioning properties will not be altered by additional water introduced to the phase by polar solutes as is the case for nonpolar and slightly polar solvents. No analytical or storage problems are associated with water-saturated *n*-octanol.

To minimize problems due to possible hydrophobic interaction of partitioned solutes with dissolved solvent in the solvent-saturated water phase, solute concentrations should be kept less than solvent concentrations in the determination of *P* values. In octanol-saturated water, solute concentrations of 10^{-3} *M* or less are normally recommended.

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[x] To whom inquiries should be directed.

Effect of Various Alcohols on Intestinal Net Water Flux and Theophylline Absorption in Rats

J. B. HOUSTON and GERHARD LEVY ˣ

Abstract □ Previous studies in this laboratory demonstrated that the rate of intestinal absorption of theophylline in rats is increased significantly by ethanol in low concentrations and that this absorption enhancing effect is associated with an increased net water flux from the intestine. It is now shown that other alcohols, namely methanol, *n*-propanol, *n*-butanol, glycerin, propylene glycol, and, to a lesser extent, mannitol and sorbitol, can also increase net water flux from the small intestine of anesthetized rats. Polyethylene glycol 200 and 400 had no such effect, suggesting that these compounds do not penetrate to a site of action that elicits the increased net water flux. At initial concentrations of 0.1 and 1.0 *M*, glycerin and propylene glycol increase significantly the intestinal absorption rate of theophylline from the small intestine of anesthetized rats. The results show that the theophylline absorption enhancing effect of ethanol is not limited to that particular alcohol.

Keyphrases □ Intestinal net water flux—effect of various alcohols, glycerin and propylene glycol effect on theophylline absorption in the rat □ Theophylline absorption—effect of glycerin and propylene glycol, rats, related to effect of various alcohols on intestinal net water flux □ Alcohols—effect on intestinal net water flux and theophylline absorption in rats

Koysooko and Levy (1) recently showed that the rate of intestinal absorption of theophylline in rats is increased significantly by ethanol in low concentrations and that there is a positive rank-order correlation between the theophylline absorption rate and the net water flux from the intestine. These observations led to three important questions: Can other alcohols also enhance theophylline absorption? Does ethanol enhance the absorption of other drugs? What is the mechanism of the absorption enhancing effect?

Reported here are results of an investigation concerned with the first of these questions. To proceed expeditiously, the effect of various concentrations of a number of alcohols on net water flux was determined first. Then, having established that most of these alcohols, in a certain concentration range, increase net water flux, the effect of two with particularly promising characteristics, glycerin and propylene glycol, on the intestinal absorption of theophylline was determined.

Table I—Effect of Polyethylene Glycol 200 and 400 on Net Water Flux from Small Intestine of Rats

Additive	Number of Animals	Rate of Net Water Flux[a], ml/cm/min × 10^4	Statistical Difference from Control
None	7	6.5 ± 0.5[b]	—
0.63% polyethylene glycol 200	4	6.6 ± 0.4	NS[c]
6.3% polyethylene glycol 200	4	7.1 ± 0.7	NS
0.63% polyethylene glycol 400	4	5.8 ± 0.8	NS
6.3% polyethylene glycol 400	4	6.0 ± 0.7	NS

[a] At steady state. [b] Mean ± *SD*. [c] NS = not significant.

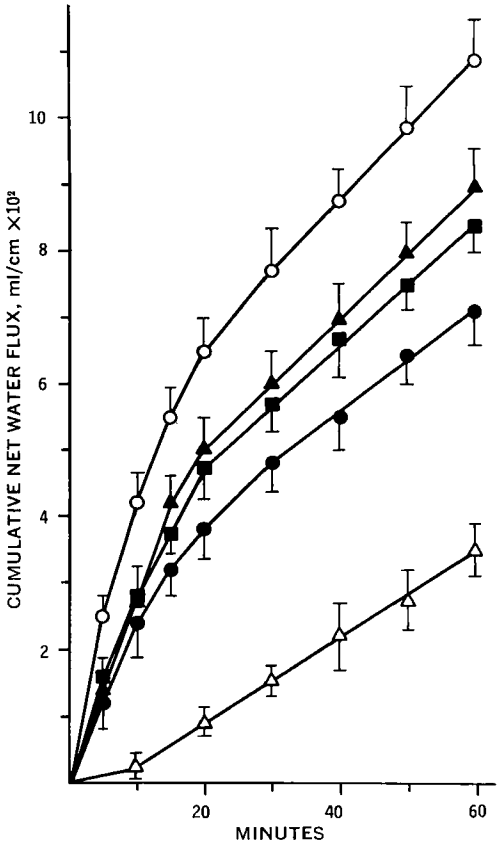

Figure 1—*Effect of alcohols in equimolar concentration on the cumulative net flux of water from the small intestine of the rat. Key:* △, *control* (n = 5); ●, n-*butanol* (n = 4); ■, *methanol* (n = 4); ▲, *ethanol* (n = 4); *and* ○, n-*propanol* (n = 4). *Vertical bars indicate 1* SD *in each direction. All alcohol concentrations were 0.25 mole/liter.*

EXPERIMENTAL

Male Sprague–Dawley rats, 280–350 g, were fasted overnight but had free access to water at all times. They were anesthetized with urethan, 1.5 g/kg ip. Net water flux from the intestine and theophylline absorption were determined as previously described (1). Briefly, a portion of small intestine was cannulated *in situ* and 7 ml of an alcohol solution was introduced into the intestinal lumen (2, 3). This solution was aspirated periodically for determination of net water flux. The solution consisted of various concentrations of methanol[1], ethanol[2], *n*-propanol[1], *n*-butanol[3], propylene glycol[1], glycerin[1], mannitol[4], sorbitol[4], polyethylene glycol 200[3], and poly-

[1] Fisher Scientific Co., Fair Lawn, N.J.
[2] Commercial Solvents Corp., Terre Haute, Ind.
[3] Baker Chemical Co., Phillipsburg, N.J.
[4] Nutritional Biochemical Corp., Cleveland, Ohio.

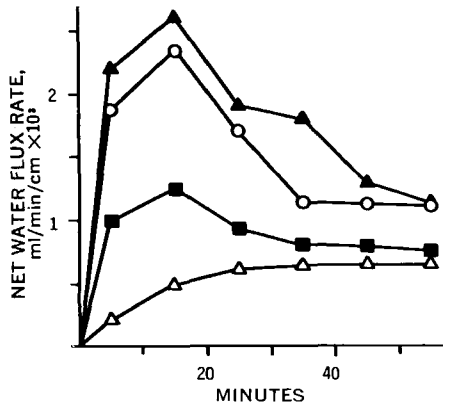

Figure 2—*Effect of various concentrations of propylene glycol on the rate of net water flux from the small intestine of the rat; mean of three experiments for propylene glycol and five experiments for control. Key:* △, *control;* ■, *0.01* M; ○, *0.1* M; *and* ▲, *1* M.

ethylene glycol 400[5] dissolved in Sørensen's phosphate buffer solution, pH 6.4. Sodium chloride was added as required to make the hypotonic solutions isotonic.

For the absorption studies, theophylline[4] was added in a concentration of 50 mg/100 ml and 0.2-ml portions were removed from the intestinal lumen periodically for determination of theophylline concentrations. The volume of drug solution in the intestine was maintained constant by addition of 0.9% sodium chloride solution from a 5-ml syringe before the removal of each sample. The difference between the total volume of saline solution added over a defined period and the volume of solution removed for assay served as a measure of net water flux. No samples were removed from solutions that did not contain theophylline. Positive and negative fluxes indicate net water flow out of and into the intestinal lumen, respectively. Theophylline concentrations were determined spectrophotometrically by the method of Schack and Waxler (4).

RESULTS AND DISCUSSION

Figure 1 shows the effect of methanol, ethanol, *n*-propanol, and

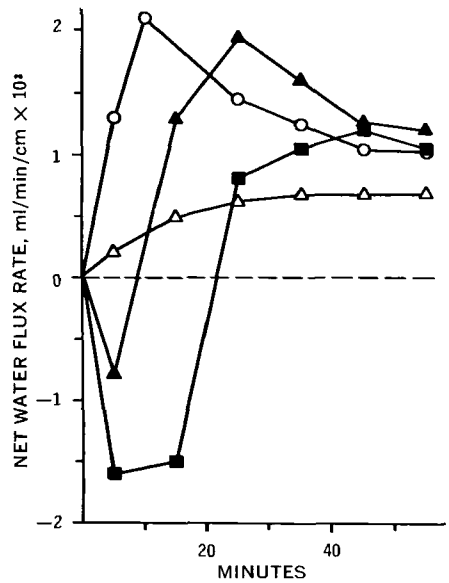

Figure 3—*Effect of various concentrations of glycerin on the rate of net water flux from the small intestine of the rat; mean of three experiments for glycerin and five experiments for control. Key:* △, *control;* ○, *0.1* M; ▲, *1* M; *and* ■, *2.5* M.

[5] Union Carbide Co., New York, N.Y.

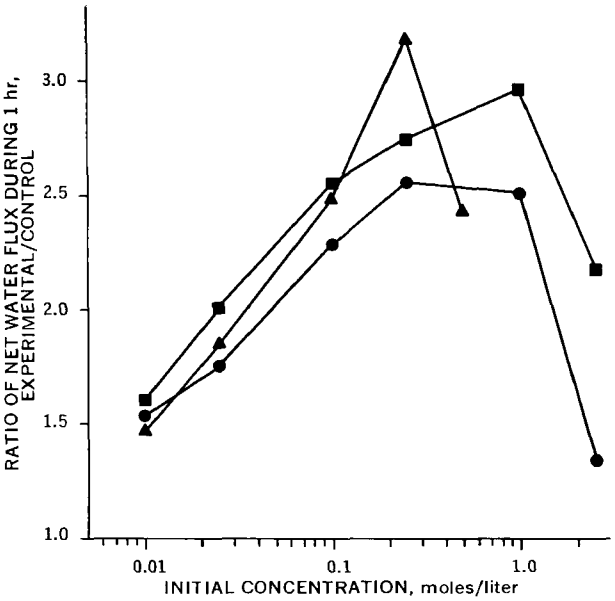

Figure 4—*Net water flux–concentration profile for ethanol* (●), *propylene glycol* (■), *and* n-*propanol* (▲) *expressed as a ratio relative to the control; mean of three experiments each. The water flux is that determined at 60 min.*

n-butanol, at an initial concentration of 0.25 *M*, on the net water flux from the rat small intestine. Following an initial apparent lag period, the net water flux from buffer solution without alcohol proceeded at an essentially constant rate of 6.5×10^{-4} ml/cm/min (Fig. 1). All four alcohols had a statistically significant net water flux enhancing effect ($p < 0.001$ for the 10- and 60-min data). The order of effectiveness was *n*-butanol < methanol ≃ ethanol < *n*-propanol. It should be recognized that the shape of the cumulative net water flux *versus* time curves is affected by the decrease of alcohol concentration with time due to absorption. Similarly, the relative order of effectiveness of the alcohols reflects not only their intrinsic activity but also their relative rate of absorption from the GI tract.

The effect on the net water flux of propylene glycol and glycerin, two compounds of particular pharmaceutical interest, was studied over a wide concentration range (from 0.01 to 2.5 *M* initial concentration). Figure 2 shows the time course of the net water flux from the intestine after instillation of 0.01, 0.1, and 1.0 *M* solutions of propylene glycol. The rate of the net water flux first increased and then slowly decreased with time.

Figure 3 represents the time course of the net water flux rates following instillation of 0.1, 1.0, and 2.5 *M* glycerin solution. The 1.0 and 2.5 *M* solutions initially caused a negative net water flux which eventually became positive and exceeded the control value. An initially negative net water flux was also produced by 2.5 *M*

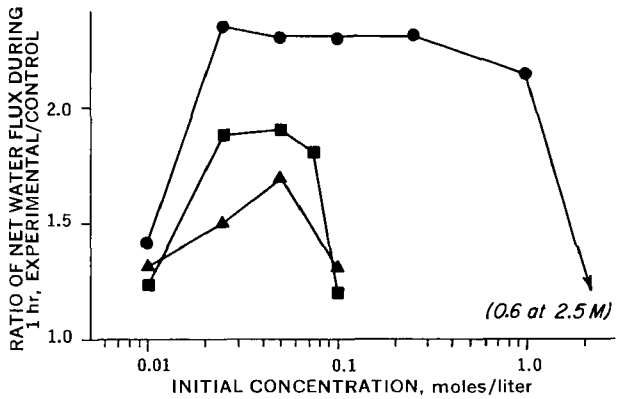

Figure 5—*Net water flux–concentration profiles for glycerin* (●), *mannitol* (■), *and sorbitol* (▲). *Details are given in previous figure.*

Table II—Effect of Propylene Glycol and Glycerin on Theophylline Absorption and Net Water Flux from Small Intestine of Rats[a]

Additive[b]	Number of Animals	Weight of Animals, g	Length of Cannulated Segment of Intestine, cm	Net Water Flux, ml/cm/25 min × 10^2	Statistical Difference from Control	Theophylline Absorption, % Unabsorbed at 25 min	Statistical Difference from Control
None	5	297 ± 34	101 ± 6	1.2 ± 0.15	—	25.3 ± 3.1	—
0.1 *M* propylene glycol	4	317 ± 17	99 ± 5	5.15 ± 0.32	$p < 0.001$	13.8 ± 3.2	$p < 0.005$
1.0 *M* propylene glycol	4	290 ± 29	102 ± 4	5.7 ± 0.72	$p < 0.001$	10.9 ± 1.5	$p < 0.005$
0.1 *M* glycerin	4	319 ± 25	102 ± 7	4.25 ± 0.33	$p < 0.001$	17.2 ± 1.9	$p < 0.01$
1.0 *M* glycerin	4	300 ± 13	101 ± 4	3.0 ± 0.40	$p < 0.001$	15.0 ± 3.7	$p < 0.005$

[a] Results are listed as mean ± *SD*. [b] All solutions contained theophylline, 50 mg/100 ml.

propylene glycol. This result was probably due to the high osmotic pressure gradient caused by these more concentrated solutions, an effect that decreases with time as the alcohol concentration is lowered by absorption.

Oral administration of a drug solution with an alcohol additive results in a gradual decrease of the alcohol concentration due to dilution with GI fluids and absorption. Therefore, it is important to determine the relationship between alcohol concentration and effect on the net water flux. Figure 4 represents such concentration–effect profiles for ethanol, *n*-propanol, and propylene glycol. The latter is more effective than ethanol and both increase the net water flux over a wide concentration range. The decrease in the effect on the net water flux at higher concentrations of the alcohols is probably due to the high osmotic pressure of the solutions which causes water flow into the intestinal lumen. In the case of *n*-propanol, this abrupt decrease in the effect on the net water flux may be due in part to the toxic effect of this alcohol. Animals that received 1 *M* solutions of *n*-propanol expired after about 30 min.

Figure 5 shows the concentration–effect profiles for glycerin, mannitol, and sorbitol. Unlike the latter two, glycerin is effective over a wide concentration range and its effect on the net water flux is almost independent of concentration over an approximately 100-fold range. Its maximum effect is, however, somewhat less than that of propylene glycol. The much less pronounced effectiveness of mannitol and sorbitol may be due to a decreased accessibility of these very polar and larger molecules to the site of action that elicits the increased net water flux. Consistent with this speculation is the lack of any effect on the net water flux of polyethylene glycol 200 and 400 (Table I). The net water flux values reported for these compounds are the steady-state values, *i.e.*, the slope of a plot of cumulative net water flux *versus* time following the initial lag phase as shown in Fig. 1 for the control (no alcohol) solution.

Propylene glycol and glycerin, in concentrations of 0.1 and 1.0 *M*, significantly increased the rate of absorption of theophylline from the rat small intestine (Figs. 6 and 7 and Table II). Thus, as previously found with ethanol (1), there is an association between enhanced absorption of theophylline and net water flux. As stated previously (1), this does not necessarily indicate a causative rela-

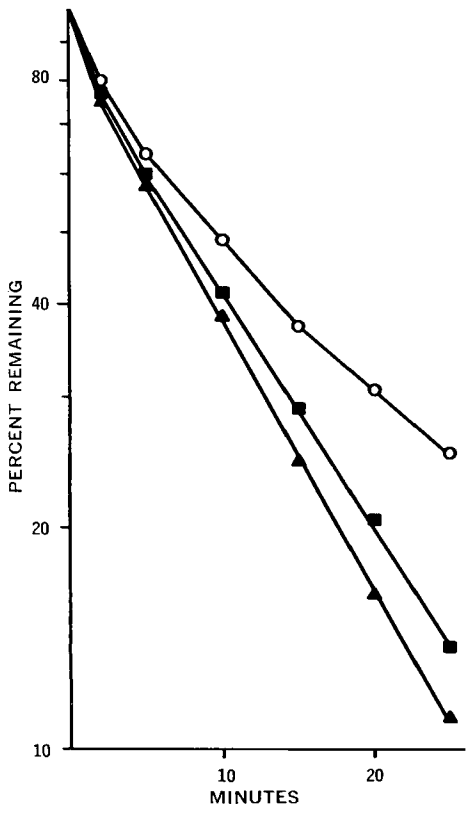

Figure 6—*Effect of propylene glycol on the time course of theophylline absorption from a 50-mg/100 ml solution instilled into a cannulated segment of small intestine of anesthetized rats. Key:* ○, *control* (n = 5); ■, *0.1* M *propylene glycol* (n = 4); *and* ▲, *1* M *propylene glycol* (n = 4).

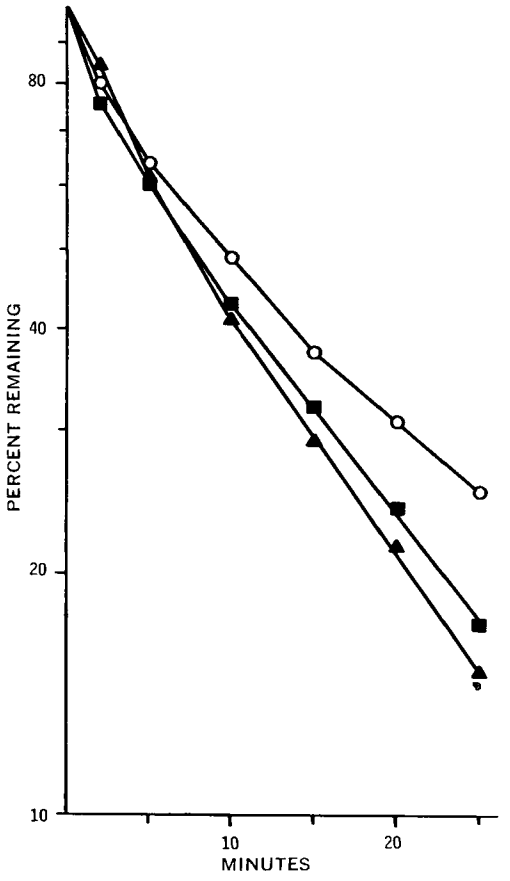

Figure 7—*Effect of glycerin on the time course of theophylline absorption from a 50-mg/100 ml solution instilled into a cannulated segment of small intestine of anesthetized rats. Key:* ○, *control;* ■, *0.1* M; *and* ▲, *1* M.

Table III—Effect of Theophylline on Net Water Flux from Small Intestine of Rats in Presence of Sørensen's Buffer, Propylene Glycol Solution, and Glycerin Solution

Additive	Net Water Flux, ml/cm/20 min × 10^2	
	Theophylline Present[a]	Theophylline Absent[b]
None	0.95 ± 0.15	0.90 ± 0.25
0.1 *M* propylene glycol	4.30 ± 0.35	4.45 ± 0.40
1 *M* propylene glycol	4.45 ± 0.70	4.55 ± 0.65
0.1 *M* glycerin	3.65 ± 0.30	3.50 ± 0.35
1 *M* glycerin	1.60 ± 0.45	1.80 ± 0.50

[a] Mean of four animals ± *SD*; initial theophylline concentration of 50 mg/100 ml. [b] Mean of three animals ± *SD*. None of these values differed significantly ($p > 0.6$) from those in the presence of theophylline.

tionship between these two effects. The mechanism of the absorption enhancing effect of the alcohols is still being investigated in this laboratory. The magnitude of the effect of propylene glycol and glycerin on the net water flux was not affected by the presence of theophylline, nor did theophylline affect the net water flux from alcohol-free solutions (Table III).

The results of the present study show that the absorption enhancing effect of ethanol on theophylline is not specific to that alcohol. It is shared by propylene glycol and glycerin and, most likely, by other alcohols that were found to increase the net water flux from the intestine. Some of the compounds studied are suitable (and are used widely) as components of pharmaceutical formulations. The fact that they act in very low concentration is particularly striking. Their potential for enhancing the absorption of certain drugs under clinical conditions remains to be explored.

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Convective Diffusion Model for a Transport-Controlled Dissolution Rate Process

KENNETH G. NELSON * **and ASHOK C. SHAH** [x]

Abstract □ A mathematical model based on convective diffusion was developed to describe the rate of dissolution from the surface of a compressed compact. Experimental studies were carried out to test the model. The basic experimental apparatus consisted of a modified rotating-filter–stationary basket dissolution test apparatus. Dissolution rates from rectangular and circular surfaces of an homologous series of *p*-aminobenzoate esters permitted testing the theory with respect to solubility, geometry, and agitation conditions. The correlation between experimental results and theory was reasonably good considering that the test conditions were somewhat less than ideal.

Keyphrases □ Diffusion model, convective—transport-controlled dissolution rate process, *p*-aminobenzoates and modified rotating-filter–stationary basket apparatus □ Dissolution rate, transport controlled—convective mathematical diffusion model tested using *p*-aminobenzoates and modified rotating-filter–stationary basket apparatus □ Transport—as controlling factor in dissolution rate process, convective mathematical diffusion model proposed and tested

Reviews of the literature concerning dissolution rates of drugs (1–3) indicate that the most widely accepted theory for dissolution rates is that proposed by Noyes and Whitney in 1897 and subsequently modified to include the stagnant or unstirred diffusion layer concept of Nernst and Brünner[1]. In this form, the rate expression is:

$$\frac{dc}{dt} = \frac{AD}{hv}(c_0 - c) \qquad \text{(Eq. 1)}$$

(The notation is defined at the end of the text.)

It can be seen that the rate is directly proportional to the area exposed, the diffusivity, and the solubility and inversely proportional to the diffusion layer thickness. The first three variables enumerated can be determined by independent measurements, whereas the effective diffusion layer thickness, h, is a model-dependent parameter. As such, its significance and utility outside the confines of the model are necessarily limited.

Transport-controlled dissolution in a stirred liquid involves two fundamental processes: molecular diffusion and forced convection as a result of fluid flow. Although hydrodynamic factors have been discussed and considered to some extent, particularly in regard to the rotating-disk technique (4, 5), a rather general

[1] See review articles for appropriate references.

Table III—Effect of Theophylline on Net Water Flux from Small Intestine of Rats in Presence of Sørensen's Buffer, Propylene Glycol Solution, and Glycerin Solution

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KENNETH G. NELSON * **and ASHOK C. SHAH** [x]

Abstract □ A mathematical model based on convective diffusion was developed to describe the rate of dissolution from the surface of a compressed compact. Experimental studies were carried out to test the model. The basic experimental apparatus consisted of a modified rotating-filter–stationary basket dissolution test apparatus. Dissolution rates from rectangular and circular surfaces of an homologous series of *p*-aminobenzoate esters permitted testing the theory with respect to solubility, geometry, and agitation conditions. The correlation between experimental results and theory was reasonably good considering that the test conditions were somewhat less than ideal.

Keyphrases □ Diffusion model, convective—transport-controlled dissolution rate process, *p*-aminobenzoates and modified rotating-filter–stationary basket apparatus □ Dissolution rate, transport controlled—convective mathematical diffusion model tested using *p*-aminobenzoates and modified rotating-filter–stationary basket apparatus □ Transport—as controlling factor in dissolution rate process, convective mathematical diffusion model proposed and tested

Reviews of the literature concerning dissolution rates of drugs (1–3) indicate that the most widely accepted theory for dissolution rates is that proposed by Noyes and Whitney in 1897 and subsequently modified to include the stagnant or unstirred diffusion layer concept of Nernst and Brünner[1]. In this form, the rate expression is:

$$\frac{dc}{dt} = \frac{AD}{hv}(c_0 - c) \qquad \text{(Eq. 1)}$$

(The notation is defined at the end of the text.)

It can be seen that the rate is directly proportional to the area exposed, the diffusivity, and the solubility and inversely proportional to the diffusion layer thickness. The first three variables enumerated can be determined by independent measurements, whereas the effective diffusion layer thickness, h, is a model-dependent parameter. As such, its significance and utility outside the confines of the model are necessarily limited.

Transport-controlled dissolution in a stirred liquid involves two fundamental processes: molecular diffusion and forced convection as a result of fluid flow. Although hydrodynamic factors have been discussed and considered to some extent, particularly in regard to the rotating-disk technique (4, 5), a rather general

[1] See review articles for appropriate references.

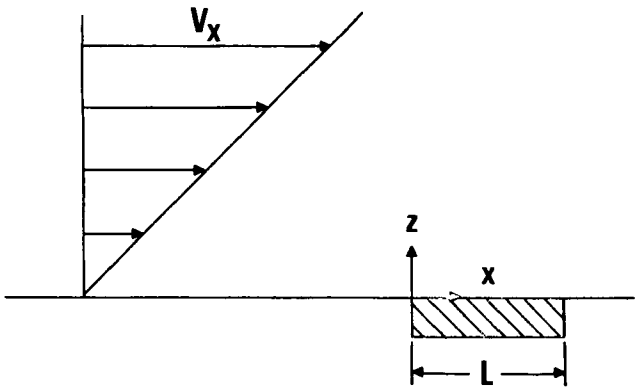

Figure 1—*Convective diffusion model.*

treatment of drug dissolution in terms of diffusion and concomitant convection has not been developed. The purposes of this study were to apply a convective diffusion model to the drug dissolution process and to test the model experimentally.

THEORETICAL

The model to be developed is based on convective diffusion theory, *i.e.*, the mathematical expressions for mass transport by diffusion and convection are combined to give a "convective diffusion" differential equation. The development of an expression for the dissolution rate, then, involves selecting appropriate boundary conditions and solving the differential equation utilizing these boundary conditions.

The convective diffusion equation was given by Levich (6) as:

$$\frac{\partial c}{\partial t} = D \text{ div grad } c - \mathbf{V} \text{ grad } c \qquad \text{(Eq. 2)}$$

The first term on the right side is the diffusion contribution, whereas the second term arises from convection. For steady state, the change in concentration with time is zero, so the steady-state convective diffusion equation is:

$$D \text{ div grad } c = \mathbf{V} \text{ grad } c \qquad \text{(Eq. 3)}$$

The physical model will be developed on the basis of the following assumptions. The liquid is flowing past the dissolving surface in the x direction, and convective flow in the y and z directions is nonexistent. The solid is dissolving by diffusion in the z direction, which is normal to the surface of the dissolving solid. With these assumptions, Eq. 3 becomes:

$$D\frac{\partial^2 c}{\partial z^2} = \mathbf{V}_x \frac{\partial c}{\partial x} \qquad \text{(Eq. 4)}$$

The liquid into which the diffusion occurs requires special consideration. In cases where a solid plane is positioned parallel to unidirectional, frictionless flow, a parabolic laminar hydrodynamic boundary layer develops on the solid (7). If the solid undergoes dissolution, the diffusion boundary layer is also well characterized (7). The flow regime in most equipment designed for drug dissolution tests, however, is turbulent to ensure efficient mixing, so the parabolic boundary layers do not develop. Under certain turbulent circumstances, however, it is customary to consider the fluid layer immediately adjacent to a solid wall as having a laminar nature (8). Thus, it will be assumed that the boundary layer into which the dissolution is occurring is laminar and, furthermore, that it may be described by a linear velocity profile. The liquid velocity term then is given by:

$$\mathbf{V}_x = \alpha z \qquad \text{(Eq. 5)}$$

The physical model is shown in Fig. 1. Substituting Eq. 5 into Eq. 4 yields:

$$D\frac{\partial^2 c}{\partial z^2} = \alpha z \frac{\partial c}{\partial x} \qquad \text{(Eq. 6)}$$

and the boundary conditions are:

$$c = c_0 \quad \text{for } 0 < x < L, \quad z = 0 \qquad \text{(Eq. 7a)}$$
$$c = 0 \quad \text{for } z = \infty \qquad \text{(Eq. 7b)}$$
$$c = 0 \quad \text{for } x = 0 \qquad \text{(Eq. 7c)}$$

The first boundary value requires that a saturated layer always exists at the solid–liquid interface and that any interfacial barrier or reaction is insignificant. The second and third boundary conditions relate to "sink" conditions.

The mathematical solution to Eq. 6 has been presented for an analogous situation (8). It is:

$$c = \frac{c_0\beta\int_{\beta}^{\infty} e^{-\beta^3}\,d\beta}{\Gamma(4/3)} \qquad \text{(Eq. 8a)}$$

$$\beta = z\left(\frac{\alpha}{9Dx}\right)^{1/3} \qquad \text{(Eq. 8b)}$$

Hence, the initial rate of dissolution is:

$$R = -D\left(\frac{\partial c}{\partial z}\right)_{z=0} \qquad \text{(Eq. 9)}$$

If Eq. 8a is substituted into Eq. 9 and the necessary differentiation is performed, Eq. 9 can be integrated over one surface of a rectangular tablet of length L (in the direction of flow) and width b to obtain:

$$R = 0.808 D^{2/3} c_0 \alpha^{1/3} b L^{2/3} \qquad \text{(Eq. 10)}$$

If Eq. 9 is integrated over one surface of a circular tablet of radius r:

$$R = 2.157 D^{2/3} c_0 \alpha^{1/3} r^{5/3} \qquad \text{(Eq. 11)}$$

Equations 10 and 11 should describe the initial dissolution rates from a surface of a rectangular tablet and a disk, respectively, provided that the boundary conditions are met and the assumptions in their derivations are valid.

EXPERIMENTAL

The dissolution rates were determined in a modified rotating-filter–stationary basket apparatus[2] which has been described previously (9, 10). In place of the stationary basket, however, a stainless steel die and holder assembly was developed that would permit the dissolution from a constant area surface of a rectangular nondisintegrating tablet (Fig. 2). The face of the die measured 4.76 × 4.76 cm and was 0.95 cm thick. The edges were beveled at 45° to promote the development of an adherent, laminar boundary layer. The opening for the tablet was 2.54 × 0.317 cm, which gives a length to width ratio of 8:1, and the die could be mounted with either the long or short dimension parallel to the direction of liquid flow. Each tablet was prepared by placing the die face down on a smooth surface, pouring the powder in the opening from the back side, and compressing with an appropriately sized rectangular punch on a hydraulic press[3].

To determine dissolution rates from circular surfaces, a clear plastic die was fabricated having the same external dimensions as the die already described. In place of the rectangular opening, a circular channel of diameter 2.22 cm was bored through the die. Therefore, a flat-faced tablet could be mounted by placing the die face down on a microscope slide and holding the tablet against the slide in the die opening with a probe while pouring molten wax in

[2] Coffman Industries, Kansas City, KS 66101
[3] Carver laboratory press, Fred S. Carver, Inc., Summit, N.J.

Table I—Effect of Die Orientation on the Dissolution Rate (R) of Ethyl p-Aminobenzoates

Revolutions per Minute	Die Orientation		Ratio
	Vertical	Horizontal	
	R, moles min^{-1}		
200	1.274×10^{-6}	8.69×10^{-7}	1.46
300	1.456×10^{-6}	9.19×10^{-7}	1.58
400	1.628×10^{-6}	1.081×10^{-7}	1.51

the opening. This arrangement would accommodate any flat-faced tablet having a diameter less than 2 cm and would ensure that the dissolving surface would be coplanar with the surface of the die.

The positioning of the die in the dissolution apparatus is a critical factor in the development of the flow pattern past the die. To ensure reproducibility and to prevent the boundary layer from separating from the die in the region of the dissolving surface, the following placement was chosen: 4.12 cm from the center of the die holder rod to the center of the rotating filter, 112° angle between the face of the die and an imaginary line connecting the midpoints of the centers of the rotating filter and the die holder and facing into the counterclockwise motion of the liquid, and 12 cm from the upper edge of the die to the lower surface of the apparatus cover. A line mark ascribed on the flask surface (Fig. 2) facilitated accurate positioning of the die. This placement was chosen by subjective evaluation of the behavior of the boundary layer at various orientations of the die in the apparatus. This behavior was observed by utilizing a tablet composed of a water-soluble dye and polyethylene glycol 6000.

The compounds selected for study were the methyl through pentyl esters of p-aminobenzoic acid, which have been well characterized (11). For a typical experiment utilizing the metal die, 200 mg of solid was compressed at 1500 lb for 1 min and the die was held above the water level in the flask until the temperature of the water (1006 ml) and the apparatus was 37°. The die was then positioned as described, and the chart recorder was started for monitoring the ester concentration with a flow cell in a spectrophotometer[4]. The molar absorptivity for these compounds is 1.652×10^4 at 285 nm.

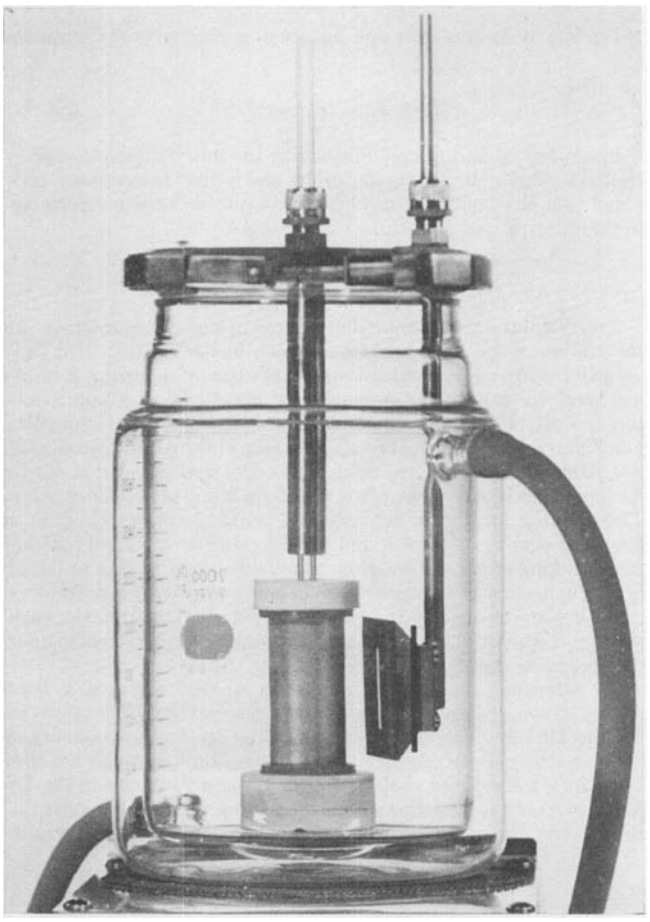

Figure 2—*Dissolution test apparatus.*

Table II—Comparison of Convective Diffusion and Unstirred Layer Theories

Parameter	Unstirred Layer	Convective Diffusion
Solubility	C_0	C_0
Surface (rectangle)	bL	$bL^{2/3}$
Surface (disk)	r^2	$r^{5/3}$
Diffusivity	D	$D^{2/3}$
Shear rate	—	$\alpha^{1/3}$

RESULTS AND DISCUSSION

The convective diffusion dissolution rate expressions (Eqs. 10 and 11) were derived assuming that the boundary layer flows past the dissolving surface in a regular manner. There may be experimental problems in meeting this requirement, however, because the dissolving surface is planar whereas the rotating-filter apparatus produces a tangential annular flow system. When using blue dye as a flow visualization technique, it was observed that the boundary layer flowed along the surface to a point about 0.5 cm forward of the trailing edge of the die, where it separated from the surface and entered the mainstream of flow. Thus, the boundary layer adhered over the dissolving surface, although it did tend to move downward from the horizontal by approximately 10°. A somewhat nonuniform blue density suggested that the boundary layer is affected by eddies entering from the main flow stream. It is expected that these phenomena will reduce the correlation between theory and experiment.

The dissolution rates for the methyl through pentyl esters of p-aminobenzoic acid were determined to test the effect of solubility. According to Eq. 10, the rate is directly proportional to solubility if all other variables remain constant. The rates were determined with the die in the vertical position and a stirring rate of 300 rpm. The solubility values were obtained from Yalkowsky *et al.* (11). Figure 3 is a plot of log R *versus* log c_0, on which a line of slope 1 was constructed to permit visual correlation with theory. The least-squares slope of this plot is 0.939 with a correlation coefficient of 0.997. The correlation between experiment and theory is

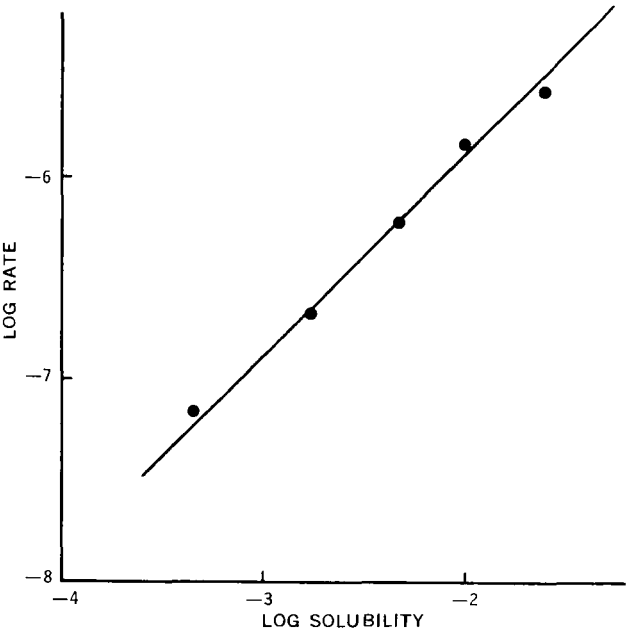

Figure 3—*Log–log plot of rate data as a function of solubility.*

[4] Beckman model DBG, Beckman Instruments, Fullerton, CA 92634

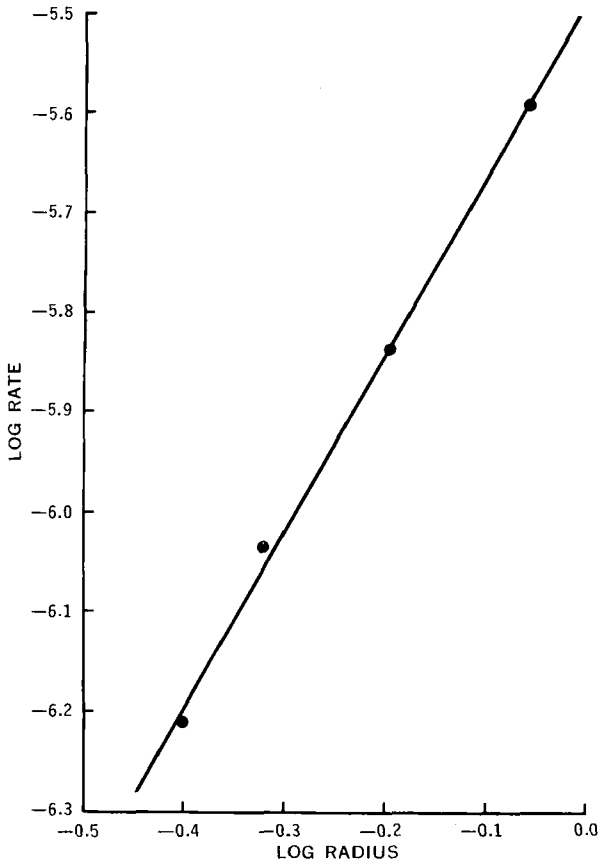

Figure 4—*Log–log plot of rate data as a function of tablet radius.*

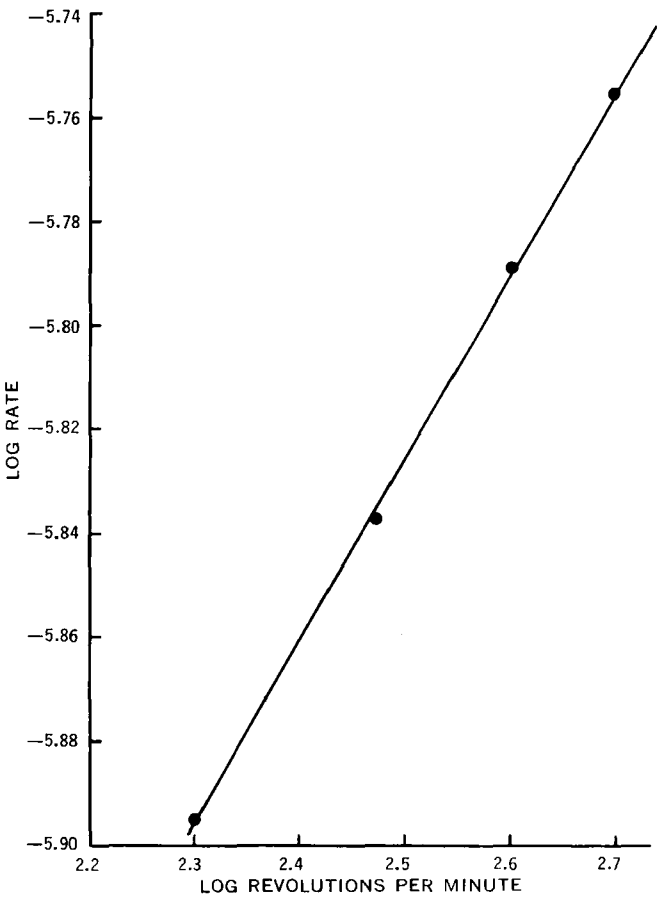

Figure 5—*Log–log plot of rate data as a function of stirring rate.*

good, considering that the diffusivity is changing by a small factor over the ester series and the presence of the more soluble species in the boundary layer may affect the value of α.

The metal die was designed to produce a rectangular tablet with a length to width ratio of 8:1 so that Eq. 10 could be tested with respect to the orientation of the tablet surface relative to the flow. According to Eq. 10, the rate of dissolution should be twice as fast with the tablet in the vertical position, *i.e.*, with the long axis perpendicular to flow, than with the tablet in the horizontal position. Table I shows data obtained using ethyl *p*-aminobenzoate with the die in the two orientations at various stirring rates. While the observed increases of about 50% in the rate in the vertical orientation relative to the horizontal do not indicate the factor of two predicted by Eq. 10, the discrepancy is probably due at least in part to the less than ideal behavior of the boundary layer.

Nevertheless, the observed differences bring out the importance of considering convection along with diffusion. The commonly employed stagnant layer model described by Eq. 1 has the rate directly proportional to area. Since the dissolving surfaces in these experiments have identical areas, Eq. 1 would require stagnant layers of different thicknesses for the two orientations. This is untenable because the flow characteristics for the two orientations are similar.

To test further the rate dependence on surface geometry, dissolution rates were determined for ethyl *p*-aminobenzoate disks mounted in the plastic die. Four tablets were used, ranging from 0.397 to 0.873 cm in radius, with a stirring rate of 200 rpm. A plot of log R *versus* log r is shown in Fig. 4. The least-squares slope of the log–log plot is 1.79, which is closer to the slope of 1.67 expected from convective diffusion theory (Eq. 11) than to 2.00 (from πr^2) in the unstirred layer theory (Eq. 1). Again, while these data are not conclusive, the influence of the convective diffusion phenomenon, *i.e.*, the build-up of solute in the hydrodynamic boundary layer, is apparent.

The one parameter in the convective diffusion theory that cannot be readily measured or calculated independently in this particular experimental system is the rate of shear. The functional dependence on the shear rate can be implicitly evaluated, however, in the following manner. If the rate of shear in the boundary layer is directly proportional to the rate of shear in the bulk liquid, *i.e.*, the stirring rate, then a plot of log R *versus* log revolutions per minute should be a straight line with a slope of 0.33, as required by Eq. 10.

Data for the dissolution rate of ethyl *p*-aminobenzoate from the rectangular die in the vertical orientation as a function of agitation conditions ranging from 200 to 500 rpm are shown as a log–log plot in Fig. 5. A line of slope one-third is drawn through the data according to theory, and the least-squares slope is 0.355 with a correlation coefficient of 0.999. It thus appears that the assumption concerning shear rate in the boundary layer is reasonable under the present conditions. Both the theory and experimental data support the fact that the dissolution rate is a relatively weak function of the stirring rate, *i.e.*, $R \propto \alpha^{1/3}$.

The convective diffusion model presented in this report predicts a dissolution rate having different functional relationships of the variables than the unstirred layer theory. Table II summarizes these dependencies for the two models. It can be seen that the differences lie in the surface area and diffusivity terms. In addition, a hydrodynamic term is present in the convective diffusion theory. Although α might be considered a model-dependent parameter not unlike h, one could design an experimental apparatus in which α would be known *a priori*. This would not be possible for h.

Convective diffusion enables one to describe drug dissolution phenomena on a more realistic basis than prevailing theories. Experimental work is being continued with an apparatus that promotes ideal flow conditions to test the model further.

NOTATIONS

A = area of dissolving surface
b = width of tablet; perpendicular to flow
c = concentration of solute
c_0 = solubility of solute
D = diffusivity of solute

h = effective diffusion layer thickness
L = length of tablet; parallel to flow
r = radius of tablet surface
R = rate of dissolution
t = time
v = volume of solution
$\mathbf{V}$ = vector describing liquid flow
V_x = x component of liquid velocity
x,y,z = cartesian coordinates
α = rate of shear in boundary layer
Γ = gamma function
β = dimensionless variable

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Substituted Thiazolidones: Selective Inhibition of Nicotinamide Adenine Dinucleotide-Dependent Oxidations and Evaluation of Their CNS Activity

SUNIL K. CHAUDHARI *, MAHIMA VERMA *, ARVIND K. CHATURVEDI *, and SURENDRA S. PARMAR *‡x

Abstract □ Eight 2-arylimino-3-(3-*N*-morpholinopropyl)thiazolid-4-ones were synthesized from the corresponding 1-aryl-3-(3-*N*-morpholinopropyl)thiocarbamides, characterized, and tested for their effects on the cellular respiratory activity of rat brain homogenates. All substituted 4-thiazolidones selectively inhibited nicotinamide adenine dinucleotide (NAD)-dependent oxidations of pyruvate, citrate, DL-isocitrate, α-ketoglutarate, malate, β-hydroxybutyrate, L-glutamate, and NADH, while the NAD-independent oxidation of succinate remained unaltered. All thiazolidones possessed some degree of anticonvulsant activity against pentylenetetrazol-induced convulsions, and the protection afforded by these compounds at a dose of 100 mg/kg ranged from 30 to 80%. The low toxicity possessed by most of these thiazolidones was reflected by their approximate LD_{50} values from 300 mg/kg to greater than 1000 mg/kg. In the present study, the anticonvulsant activity possessed by these substituted 4-thiazolidones was unrelated to their ability to inhibit selectively the NAD-dependent oxidations by rat brain homogenates. These thiazolidones exhibited depression of the CNS activity which, in some cases, was associated with the increase in respiration. All thiazolidones potentiated pentobarbital (sodium) sleeping time in mice when administered in a dose of 100 mg/kg.

Keyphrases □ Thiazolidones, 2-arylimino-3-(3-*N*-morpholinopropyl)—synthesis, inhibition of NAD-dependent oxidations and relationship to anticonvulsant activity □ Structure–activity relationships—thiazolidones, anticonvulsant activity, inhibition of NAD-dependent oxidations, rats □ Anticonvulsant activity, thiazolidones—relationship to inhibition of NAD-dependent oxidations

Thiazolidones have been shown to possess diverse biological properties including hypnotic (1), local anesthetic (2), and anticonvulsant (3, 4) activities. Recent studies indicated the anticonvulsant activity of piperazinothiocarbamides (5, 6) and their ability to inhibit nicotinamide adenine dinucleotide (NAD)-dependent oxidations. Furthermore, compounds possessing a morpholino group attached to the heterocyclic nuclei have been shown to confer greater activity and less toxicity (7). These observations led to the synthesis of some 2-arylimino-3-(3-*N*-morpholinopropyl)thiazolid-4-ones and correlation of certain pharmacological properties with their ability to inhibit NAD-dependent oxidations.

EXPERIMENTAL[1]

The various 1,3-disubstituted thiocarbamides were prepared by refluxing an equimolar quantity of 3-*N*-morpholinopropylamine and different arylisothiocyanates in dry benzene. These substituted thiocarbamides, when refluxed with chloroacetic acid and anhydrous sodium acetate in absolute ethanol, formed the desired substituted thiazolidones (8).

1 - Aryl - 3 - (3 - *N* - morpholinopropyl)thiocarbamide—3 - *N* - Morpholinopropylamine (0.01 mole) was mixed with suitable aryl isothiocyanate (0.01 mole) in 15 ml of dry benzene, and the mixture was refluxed on a steam bath for 2 hr. The reaction mixture was concentrated by removing benzene by distillation under reduced pressure. The solid that separated on cooling was filtered, washed with ether and dilute hydrochloric acid, dried, and recrystallized from ethanol. All thiocarbamides were characterized by their sharp melting points and elemental analyses (Table I).

[1] All compounds were analyzed for their carbon, hydrogen, and nitrogen contents. Melting points were taken in open capillary tubes with a partial immersion thermometer and are corrected.

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Table I—Physical Constants of Substituted Thiocarbamides

Ar—NHCSNHCH$_2$CH$_2$CH$_2$—N⟨O⟩

Compound	Ar	Melting Point[a]	Yield, %	Molecular Formula	Analysis, % Calc.	Found
I	C_6H_5	128°	80	$C_{14}H_{21}N_3OS$	C 60.21	60.40
					H 7.52	7.35
					N 15.05	15.22
II	2-CH_3—C_6H_4	188°	72	$C_{15}H_{23}N_3OS$	C 61.43	61.80
					H 7.84	7.49
					N 14.33	14.50
III	4-CH_3—C_6H_4	112°	87	$C_{15}H_{23}N_3OS$	C 61.43	61.75
					H 7.84	7.98
					N 14.33	14.20
IV	2,4-$(CH_3)_2$—C_6H_3	105°	83	$C_{16}H_{25}N_3OS$	C 62.54	62.75
					H 8.14	8.34
					N 13.67	13.59
V	2,6-$(CH_3)_2$—C_6H_3	105–107°	89	$C_{16}H_{25}N_3OS$	C 62.54	62.41
					H 8.14	8.37
					N 13.67	13.40
VI	4-OCH_3—C_6H_4	111°	82	$C_{15}H_{23}N_3O_2S$	C 58.25	58.25
					H 7.44	7.52
					N 13.59	13.69
VII	4-Cl—C_6H_4	135°	75	$C_{14}H_{20}ClN_3OS$	C 53.58	53.46
					H 6.37	6.50
					N 13.39	13.68
VIII	α-Naphthyl	115°	69	$C_{18}H_{23}N_3OS$	C 65.65	65.79
					H 6.99	7.12
					N 12.76	12.86

[a] Melting points were taken in open capillary tubes with a partial immersion thermometer and are corrected.

2-Arylimino-3-(3-*N*-morpholinopropyl)thiazolid-4-ones—A mixture of 1-aryl-3-(3-*N*-morpholinopropyl)thiocarbamide (0.01 mole), chloroacetic acid (0.01 mole), and anhydrous sodium acetate (0.015 mole) in 25 ml of absolute ethanol was refluxed on a steam bath for 4 hr. The mixture was cooled and poured into crushed ice, and the resulting solution was treated with 15% (w/v) solution of sodium carbonate until the solution was alkaline. The crude product that separated was filtered, washed several times with water, dried, and recrystallized from ethanol (Table II).

Assay of Respiratory Activity of Rat Brain Homogenate[2]—Male albino rats, 100–150 g, kept on an *ad libitum* diet, were used in all experiments. Rat brains isolated from decapitated animals were immediately homogenized in ice-cold 0.25 *M* sucrose in a Potter-Elvehjem homogenizer in a ratio of 1:9 (w/v). All incubations were carried out at 37°, and the oxygen uptake was measured by the conventional Warburg manometric technique using air as the gas phase (5). Fresh brain homogenate, equivalent to 100 mg wet brain weight, was added to the chilled Warburg vessels containing 6.7 m*M* magnesium sulfate, 20 m*M* sodium hydrogen phosphate buffer solution (pH 7.4), 1 m*M* adenosine monophosphate (sodium salt), 33 m*M* potassium chloride, and 500 μg of cytochrome c in a total volume of 3 ml unless otherwise stated.

The central well contained 0.2 ml of 20% KOH solution. Pyruvate, citrate, DL-isocitrate, α-ketoglutarate, malate, β-hydroxybutyrate, L-glutamate, NADH, and succinate were used at a final concentration of 10 m*M*, while the concentration of NADH was 0.5 m*M*. All thiazolidones were dissolved in propylene glycol (100%) and used at a final concentration of 2 m*M*. An equal volume of propylene glycol was added to the control vessels.

Determination of Anticonvulsant Activity—Anticonvulsant activity was determined against pentylenetetrazol-induced convulsions in mice of either sex weighing 25–30 g. The mice were divided into groups of 10, keeping the group weights as near the same as possible. All thiazolidones were suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compounds were administered to a group of 10 mice in a dose of 25–100 mg/kg ip. Four hours after the administration of the test compounds, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose of pentylenetetrazol was shown to induce convulsions in almost all untreated mice and produced 100% mortality during a 24-hr period. On the other hand, no mortality was observed during 24 hr in animals treated with these doses of substituted thiazolidones alone.

The mice were observed for 60 min for the occurrence of seizures. An episode of clonic convulsion persisting for at least 5 sec was considered a threshold convulsion. Transient intermittent jerks and tremulousness were disregarded. Animals devoid of threshold convulsions during the 60-min period were considered protected. The number of animals protected in each group was recorded, and the anticonvulsant activity of the substituted thiazolidones was represented as percent protection.

Potentiation of Pentobarbital Sleeping Time—The method of Winter (9) was followed to investigate the ability of substituted thiazolidones to potentiate pentobarbital-induced hypnosis. Mice, 20–25 g, were used in groups of six animals. Each compound was tested in a group of six mice while one group served as a control for pentobarbital alone. Pentobarbital (sodium), when administered in a dose of 30 mg/kg ip to the control group, produced ataxia and no sleep; an increase in the dose of pentobarbital produced sleep in normal mice.

All thiazolidones were administered in a dose of 100 mg/kg ip 1 hr prior to the administration of pentobarbital. The animals were observed regularly for sleep as evidenced by loss of the righting reflex until the animals awakened. The time of administration of pentobarbital in both the control and experimental mice treated with thiazolidones was recorded. The mean average sleeping time in each group was calculated.

Determination of Approximate LD_{50} Values—The approximate LD_{50} values of these substituted thiazolidones were determined by intraperitoneal administration in albino mice, 25–30 g, following the method reported by Smith (10).

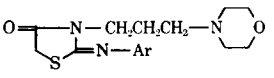
Determination of Behavioral Effects—The effects of these thiazolidones were investigated in albino mice, 25–30 g, using a dose of 100 mg/kg ip. The various effects observed were general depression, paralysis of the hindlimb associated with slight depression and an increase in the rate of respiration, and circular movements of the thiazolidone-treated mice.

RESULTS AND DISCUSSION

Inhibition of certain metabolic processes in the brain has been reported to be the mechanism of various central nervous system (CNS) depressants (11, 12). Earlier studies indicated a parallelism between *in vitro* and *in vivo* effects, since greater hypnotic activity

[2] Commercial chemicals were used in the present study. Sodium salts of pyruvic acid, citric acid, DL-isocitric acid, α-ketoglutaric acid, succinic acid, β-hydroxybutyric acid, DL-malic acid, and NADH were obtained from Sigma Chemical Co., St. Louis, Mo. Other common chemicals were obtained from the British Drug House, Bombay, India.

Table II—Physical Constants of Substituted 4-Thiazolidones



Compound	Ar	Melting Point[a]	Yield, %	Molecular Formula	Analysis, %	
					Calc.	Found
IX	C_6H_5	107°	65	$C_{16}H_{21}N_3O_2S$	C 60.19	60.47
					H 6.58	6.72
					N 13.16	13.44
X	2-CH_3—C_6H_4	73°	54	$C_{17}H_{23}N_3O_2S$	C 61.26	61.05
					H 6.90	6.72
					N 12.61	12.43
XI	4-CH_3—C_6H_4	82°	60	$C_{17}H_{23}N_3O_2S$	C 61.26	61.47
					H 6.90	7.12
					N 12.61	12.84
XII	2,4-$(CH_3)_2$—C_6H_3	53°	52	$C_{18}H_{25}N_3O_2S$	C 62.24	62.04
					H 7.20	7.10
					N 12.10	12.19
XIII	2,6-$(CH_3)_2$—C_6H_3	88°	64	$C_{18}H_{25}N_3O_2S$	C 62.24	62.16
					H 7.20	7.29
					N 12.10	11.98
XIV	4-OCH_3—C_6H_4	76°	59	$C_{17}H_{23}N_3O_3S$	C 58.45	58.60
					H 6.59	6.78
					N 12.03	12.23
XV	4-Cl—C_6H_4	55°	62	$C_{16}H_{20}ClN_3O_2S$	C 54.31	54.80
					H 5.65	6.72
					N 11.88	11.95
XVI	α-Naphthyl	112°	58	$C_{20}H_{23}N_3O_2S$	C 65.05	65.34
					H 6.23	6.19
					N 11.38	11.50

[a] Melting points were taken in open capillary tubes with a partial immersion thermometer and are corrected.

of some agents was reflected by their greater *in vitro* inhibition of respiration (13, 14). The inhibitory effects of substituted thiazolidones on the cellular respiratory activity of rat brain homogenates are presented in Table III. All thiazolidones selectively inhibited *in vitro* NAD-dependent oxidation of pyruvate, citrate, DL-isocitrate, α-ketoglutarate, malate, β-hydroxybutyrate, and L-glutamate, whereas NAD-independent oxidation of succinate remained unaltered. These results, exhibiting selective inhibition of NAD-dependent oxidations, are in agreement with earlier studies with 2-methyl-3-*o*-tolyl-4-quinazolone (15).

In the present study, *in vitro* inhibition of the oxidation of NADH was observed as was reported earlier for nitrobenzamides (16). These results have indicated possible inactivation of the transfer process of electrons in the respiratory chain by these thiazolidones by acting presumably at a site of transfer of electrons from NADH to flavine adenine dinucleotide. The enzyme inhibitory effectiveness of these thiazolidones was not uniform with regard to their ability to produce maximum inhibition during oxidation of various substrates. Compound IX, possessing an unsubstituted phenyl group attached to the thiazolidone nucleus, was the least effective compound during oxidation of all of the substrates used in the present study. The maximum inhibition of the oxidation of pyruvate and α-ketoglutarate by Compound XV, of citrate, DL-isocitrate, and malate by Compound XI, of L-glutamate and NADH by Compound X, and of β-hydroxybutyrate by Compound XII observed in these experiments failed to provide correlation between inhibitory effects and the chemical structure of these thiazolidones.

The results in Table IV compare the anticonvulsant activity possessed by these thiazolidones and their ability to potentiate pentobarbital sleeping time. All thiazolidones possessed anticonvulsant activity, which was reflected by the 30–80% protection afforded by these compounds against pentylenetetrazol-induced convulsions. Compounds IX and XV showed maximum protection, while Compound XIII afforded the least protection from pentylenetetrazol-induced convulsions. Data on anticonvulsant activity and 24-hr pentylenetetrazol-induced mortality (Table IV) indicated some association between increased protection from convulsions and decreased pentylenetetrazol mortality in experimental animals.

In the present study, intraperitoneal administration of 30 mg/kg of pentobarbital (sodium) produced ataxia and no sleep in mice. Administration of thiazolidones in a dose of 100 mg/kg ip 1 hr prior to the administration of pentobarbital produced sleep in mice. Thus, all thiazolidones potentiated pentobarbital sleeping time and the duration of sleep ranged from 21.6 to 98.6 min (Table IV). The greater potentiation of sleep observed with Compounds IX and XV, possessing maximum anticonvulsant activity, has pro-

Table III—Inhibition of the Respiratory Activity of Rat Brain Homogenate by Substituted 4-Thiazolidones

Compound	Inhibition[a], %								
	Pyruvate	Citrate	DL-Isocitrate	α-Ketoglutarate	Malate	β-Hydroxybutyrate	L-Glutamate	NADH	Succinate
IX	59.2 ± 0.7	17.4 ± 0.5	22.5 ± 0.9	35.3 ± 1.0	42.4 ± 1.4	44.0 ± 1.0	36.6 ± 1.2	30.7 ± 1.3	Nil
X	78.6 ± 0.8	43.9 ± 1.4	50.4 ± 0.7	56.9 ± 1.2	58.8 ± 0.8	60.5 ± 1.3	69.2 ± 1.5	50.1 ± 0.7	Nil
XI	72.1 ± 1.2	71.0 ± 1.5	78.1 ± 0.8	60.6 ± 1.4	59.0 ± 1.5	74.4 ± 1.5	55.0 ± 0.7	45.4 ± 0.9	Nil
XII	69.1 ± 0.9	68.0 ± 1.0	68.8 ± 1.4	40.8 ± 0.9	50.6 ± 0.9	83.7 ± 1.0	55.9 ± 0.9	45.3 ± 0.7	Nil
XIII	68.2 ± 0.8	56.7 ± 0.9	62.8 ± 0.9	44.3 ± 1.1	49.9 ± 1.0	76.3 ± 0.9	59.3 ± 0.9	40.9 ± 0.8	Nil
XIV	71.4 ± 0.9	55.4 ± 0.9	57.4 ± 1.0	57.9 ± 1.3	53.3 ± 0.9	78.3 ± 1.3	52.6 ± 1.4	40.3 ± 1.4	Nil
XV	83.3 ± 1.5	55.8 ± 1.3	60.7 ± 0.7	70.3 ± 1.5	55.4 ± 0.8	79.2 ± 1.0	65.0 ± 1.3	45.7 ± 1.3	Nil
XVI	60.4 ± 0.8	57.9 ± 1.4	60.3 ± 0.5	36.3 ± 0.9	52.6 ± 1.3	56.4 ± 1.0	53.7 ± 0.9	48.6 ± 0.9	Nil

[a] Each experiment was done in duplicate. All values represent mean values of percent inhibition with ± standard error of the mean from three separate experiments. Inhibition was determined by the decrease in the oxygen uptake/100 mg wet tissue weight/hr. Different substrates and NADH were used at a final concentration of 10 and 0.5 m*M*, respectively. All substituted thiazolidones were used at a final concentration of 2 m*M*. Vessel contents and assay procedure are as described in the text.

Table IV—Pharmacological Properties of Substituted 4-Thiazolidones

Compound	Approximate LD_{50}, mg/kg ip	Anticonvulsant Activity[a], % Protection	Pentylene-tetrazol Mortality[b], %	Potentiation of Pento-barbital Sleeping Time[c], min	Behavioral Effects[d]
IX	500	80	20	98.6 ± 10	A
X	1000	60	30	45.3 ± 9	B
XI	500	50	20	21.6 ± 5	B
XII	>1000	50	20	44.2 ± 8	C
XIII	300	30	60	77.0 ± 11	A
XIV	>1000	50	20	67.0 ± 9	A
XV	>1000	80	20	88.2 ± 7	A
XVI	>1000	50	30	51.0 ± 6	A

[a] Anticonvulsant activity was determined at a dose of 100 mg/kg ip as described in the text. [b] Represents mortality during 24 hr in each group of animals administered pentylenetetrazol. [c] Administration of pentobarbital in a dose of 30 mg/kg produced ataxia and no sleep. Sleeping time was observed in animals treated with substituted thiazolidones (100 mg/kg ip) 1 hr prior to the administration of pentobarbital. [d] Behavioral effects were observed in animals treated with substituted thiazolidones in a dose of 100 mg/kg ip, and the various effects observed are indicated as: A, slight depression; B, ataxia in hindlimbs associated with slight depression and an increase in the rate of respiration; and C, circular movements. Various screening procedures for determination of the pharmacological properties of these substituted thiazolidones are as described in the text.

vided a relationship between the anticonvulsant activity of these thiazolidones and their ability to potentiate pentobarbital-induced sleep. This relationship, however, was not sufficiently uniform to make a definite conclusion since significant potentiation of sleep was observed with Compound XIII (77.0 min) which possessed the minimum anticonvulsant activity of only 30%.

The behavioral effects observed with the administration of these thiazolidones and their approximate LD_{50} values are recorded in Table IV. The various effects observed were depression of CNS activity, which was sometimes associated with the increase in respiration, paralysis of the hindlimb, and production of circular movements. The approximate LD_{50} values of these thiazolidones were found to be from 300 to >1000 mg/kg. Most compounds possessing approximate LD_{50} values >1000 mg/kg have indicated low toxicity associated with these thiazolidones.

Results presented in the present study have failed to provide any definite correlation between selective inhibition of NAD-dependent oxidations by thiazolidones and their abilities to provide protection against pentylenetetrazol-induced convulsions and to potentiate pentobarbital-induced hypnosis. It is hoped that further detailed pharmacological and toxicological studies and investigations of the effects of substituted thiazolidones on other enzyme systems may reflect a biochemical basis for their CNS activity.

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Biosynthesis, Isolation, and Identification of 6β-Hydroxynaltrexone, a Major Human Metabolite of Naltrexone

EDWARD J. CONE ˣ, CHARLES W. GORODETZKY, and S. Y. YEH

Abstract □ Chemical reduction of naltrexone is described in an attempt to synthesize 6β-hydroxynaltrexone. Only the epimer, 6α-hydroxynaltrexone, was produced. Pilot metabolic studies on naltrexone in the dog, rat, and guinea pig were made to determine which animal produced the greatest amount of 6β-hydroxynaltrexone. The guinea pig was selected and used to produce the metabolite. Isolation and purification methods are described, and spectral data are presented for structural confirmation of the metabolite.

Keyphrases □ Naltrexone—chemical reduction to 6α-hydroxynaltrexone, metabolism in dog, rat, and guinea pig □ 6α-Hydroxynaltrexone—synthesis *via* chemical reduction of naltrexone □ 6β-Hydroxynaltrexone—isolation and identification as naltrexone metabolite in guinea pig

Naltrexone (I) is extensively metabolized in humans at the C-6 carbonyl position to 6β-hydroxynaltrexone (*N*-cyclopropylmethyl-7,8-dihydro-14-hydroxynorisomorphine) (II), with the C-6 configuration isomeric to that of morphine (1). The role of this metabolite as a possible contributor in the pharmacological response of naltrexone was of interest. A suitable quantity of II was needed for pharmacological testing and standardization of analyses of biological fluids. Several chemical reduction procedures failed to produce the metabolite with the correctly oriented β-hydroxy group. An alternative route of preparation was employed using animals as the stereospecific reducing agent. This report details the preparation of 6β-hydroxynaltrexone.

EXPERIMENTAL

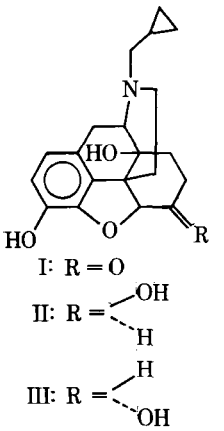
Instrumentation—*GLC*—A gas chromatograph[1] equipped with dual flame-ionization detectors and 2-mm × 1.83-m (6-ft) glass columns packed with 3% OV-17 on Gas Chrom Q, 60–80 mesh, was used. The detector and injector temperature was 280°. A temperature program was employed from 200 to 270° at a rate of 8°/min. The nitrogen carrier gas flow was 24 ml/min. The retention times of the internal standard, tetraphenylethylene, and the pentafluoropropionic anhydride derivatives of I, II, and III (two peaks) were 10.36, 5.81, 5.48, and 4.67 followed by 7.67 min.

IR—The IR spectra were obtained[2] as a KBr matrix (1%) with a 20-min scan length.

Mass Spectrometry—All mass spectra were recorded on a quadrupole GC–mass spectrometer[3]. The samples were introduced *via* the solid probe inlet, and the temperature was brought up gradually until the spectral pattern appeared. The ionizing potential was 70 ev, and the accelerating potential was 2.5 kv.

TLC Systems—*System I*—Glass fiber sheets[4] were developed with chloroform saturated with aqueous ammonia. The R_f values for I, II, and III were 0.62, 0.16, and 0.20, respectively.

System II—Silica gel G developed with benzene–diethylamine (4:1) was used. The R_f values for I, II, and III were 0.76, 0.35, and 0.35, respectively.



I: R = O

II: R = OH / H

III: R = H / OH

Attempted Chemical Synthesis of II—*Sodium Borohydride Reduction*—To 125 mg of I[5] in 25 ml of dioxane and 15 ml of methanol was added 250 mg of sodium borohydride in small portions. An immediate exothermic reaction was observed. The mixture was allowed to stir at room temperature for 2.5 hr. TLC analysis (System I visualized with potassium iodoplatinate spray) revealed that complete reduction had occurred. The solvent was evaporated under vacuum, and the residue was dissolved in 30 ml of 2 *N* NaOH and 20 ml of 40% (w/v) K_2HPO_4. The solution was extracted with chloroform, and the solvent was evaporated under vacuum. GLC analysis of the silylated residue (10% silylating reagent[6] in acetonitrile heated at 70° for 1 hr) confirmed that complete reduction to the epimer of II, *N*-cyclopropylmethyl-7,8-dihydro-14-hydroxynormorphine[5] (III) had occurred.

Sodium Amalgam Reduction—Compound I (200 mg) was reduced with 1.5 g of 5% sodium amalgam (Na-Hg) using the procedure reported by Blount *et al.* (2). The chloroform extract was analyzed by TLC (System I and visualized with potassium iodoplatinate spray) and GLC. The product of the reaction was unrelated to II or III and remained unidentified.

Iridium Tetrachloride Reduction—To a solution of iridium tetrachloride[7] (50 mg) in 1 ml of concentrated hydrochloric acid was added 40 ml of water followed by 11 ml of trimethylphosphite. Fifty milligrams of I in 40 ml of 2-propanol was added, and the mixture was refluxed overnight. After cooling, the mixture was made basic with 10 *N* NaOH and extracted with chloroform. Evaporation of solvent was followed by derivatization with pentafluoropropionic anhydride[8] (heated as a neat solution at 70° for 3 hr) in an acylation tube[9]. GLC analysis revealed that reduction to III was about 50% complete without a trace of II present.

Metabolic Studies on Animals—Pilot studies were performed on the dog, rat, and guinea pig to ascertain which species produced II as a metabolite of I. The animals were housed in stainless steel metabolic cages equipped with urine collectors. A single 50-mg dose of I was injected subcutaneously, and urine was collected periodically over 68 hr. The samples were filtered and refrigerated until analysis.

Two-milliliter aliquots of each collection were made basic (pH 9)

[1] Varian model 2700.
[2] Beckman model IR 18A.
[3] Finnigan model 1015D.
[4] Gelman I.T.L.C. type SG, Gelman Instrument Co., Ann Arbor, Mich.
[5] Provided by Endo Pharmaceutical Co., Garden City, N.Y.
[6] Regisil, Regis Chemical Co., Morton Grove, Ill.
[7] Pfaltz and Bauer, Inc., Flushing, N.Y.
[8] Pierce Chemical Co., Rockford, Ill.
[9] Regis Chemical Co., Morton Grove, Ill.

Table I—Urinary Excretion of Free and Conjugated Naltrexone and 6β-Hydroxynaltrexone in Guinea Pig and Dog

	Distribution in Urine[a]						
	Guinea Pig				Dog		
	0–2 hr	2–20 hr	20–44 hr	44–60 hr	0–5 hr	5–20 hr	20–96 hr
Naltrexone (I)	0.2	16.4	0.2	0.1	0.7	0.2	0.2
Conjugated naltrexone	1.2	58.8	1.6	0.2	161.3	24.8	0.9
6β-Hydroxynaltrexone (II)	—[b]	54.5	5.9	0.1	—[b]	—[b]	—[b]
Conjugated 6β-hydroxynaltrexone	—[b]	26.2	13.9	1.0	—[b]	—[b]	—[b]

[a] Values are reported in micrograms per milliliter for the collection period. [b] Not detectable.

with 2 *N* NaOH. One milliliter of 40% (w/v) K_2HPO_4, 0.5 g of sodium chloride, and 10 ml of chloroform were added, and the contents were shaken for 10 min. The aqueous phase was withdrawn and saved for acid hydrolysis of the conjugated drug and metabolites. The organic phase was transferred to a clean tube containing 3 ml of 2 *N* HCl and shaken for 10 min. The organic phase was discarded and the pH of the acid phase was adjusted to 9 and buffered with phosphate buffer. Ten milliliters of chloroform was added, and the contents were shaken for 10 min. The aqueous phase was aspirated and the organic phase was transferred to an acylation tube. The solvent was evaporated under nitrogen, and the residue was derivatized with pentafluoropropionic anhydride for 3 hr at 70°. Excess reagent was removed under nitrogen, and 100 μl of ethylene dichloride was added. The solution was analyzed immediately by GLC. Levels of I and II were estimated by absolute peak height based on the response of a known quantity of I.

The aqueous phase containing the conjugated drug and metabolites was hydrolyzed with excess concentrated hydrochloric acid in an autoclave for 0.5 hr at 115° and 1.27 kg/cm². After cooling, the solution was neutralized with solid potassium carbonate and extracted as described. The residue was derivatized with pentafluoropropionic anhydride and analyzed by GLC.

Isolation of II from Guinea Pig Urine—Ten white guinea pigs were injected subcutaneously twice daily with 25 mg of I in 0.5 ml of saline for 10 days. The urine was collected daily, filtered, and centrifuged, and the supernate was stored in the refrigerator.

The samples were extracted twice with chloroform (1.5 ml/ml of sample) following the adjustment of the pH to 9–9.5 with 10 *N* NaOH, buffering (40% K_2HPO_4, 1 ml for 7 ml of sample), and salting (sodium chloride, 1 g for each milliliter of sample). The combined organic extracts were centrifuged and dried over anhydrous potassium carbonate. The chloroform was removed under vacuum, and the residue was taken up in a small volume of solvent. The solution was applied to thin-layer plates and chromatographed (System II, 1000-μm layer). A 1-cm wide section of each side of the chromatogram was sprayed with potassium iodoplatinate for visualization of II. The band (R_f 0.27–0.50) corresponding to the region (R_f 0.35) where II was located from human urine was preparatively removed. The gel was washed with methanol, and the solvent was evaporated under nitrogen. The TLC isolation procedure was repeated, giving a brown oily residue. Further purification was necessary and was accomplished by solid–liquid adsorption chromatography.

The residue was fractionated into 70 10-ml fractions on a column of neutral alumina, 1.27 × 30.48 cm (0.5 × 12 in.), under gradient elution conditions using mixtures of benzene–chloroform and chloroform–methanol as the eluting solvents[10]. The fractionation procedure was monitored by TLC [System I visualized by spraying with potassium ferro-ferricyanide reagent (3) and observing under UV (254 nm)]. Fractions 54–64 [eluting solvent consisted of chloroform–methanol (4:1)] were combined and evaporated to a residue under nitrogen. The residue was extracted with chloroform, and the extract was treated with hydrogen chloride gas. A white solid precipitated immediately. The solvent was decanted, and the solid was dried under nitrogen. The solid was fractionally crystallized and recrystallized in a mixture of methanol–ether–heptane (9:1:1) to give fine, white needles of II. GLC and TLC (System I) analyses as described revealed II to contain about 3% III. The total yield of II was 187 mg (3.7% yield).

[10] The percent of chloroform in benzene was increased from 0 to 100 followed by increasing the percent of methanol in chloroform from 0 to 100 during the fractionation procedure.

RESULTS AND DISCUSSION

The complete lack of II in the chemical reduction of I prompted the consideration of a biosynthetic route as a means of preparing the metabolite. Pilot studies on the dog and guinea pig allowed estimates to be made of concentration levels of both free and conjugated I and II (Table I). Only minor amounts of free I were present in the urine of the dog, with a correspondingly high level of conjugated I in the early collections. The very active conjugation step (probably glucuronidation) along with an absence of II could account for the short biological half-life of naltrexone in the dog ($t_{1/2}$ = 2 hr) (4) as compared to humans ($t_{1/2}$ = 12 hr) (5).

The results of the study on the rat were inconclusive for the presence of II. Both free and conjugated I were detected in the

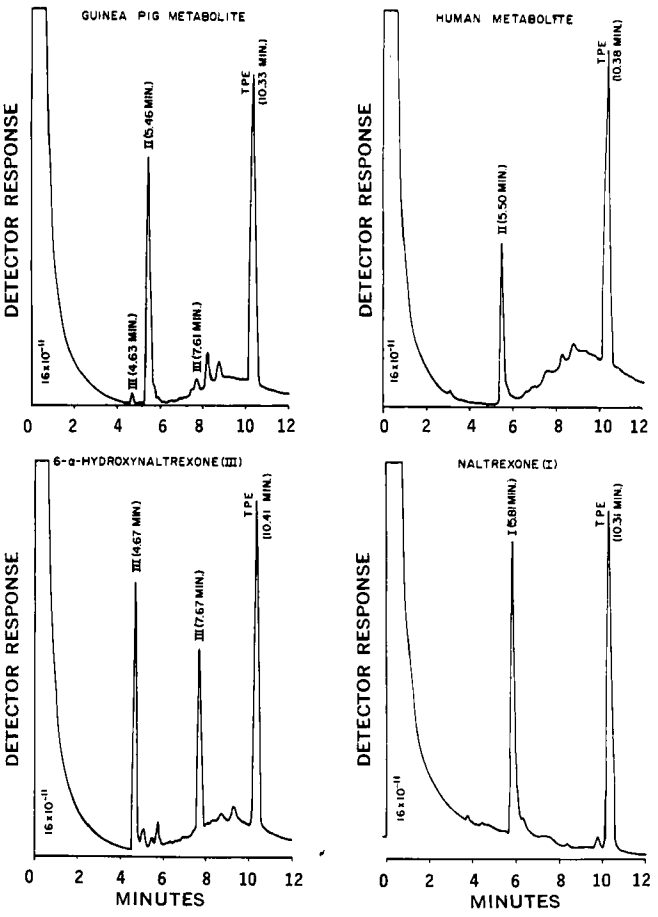


Figure 1—*Gas chromatogram of pentafluoropropionic anhydride derivatives of 6β-hydroxynaltrexone (II) isolated from the guinea pig and human (top) and 6α-hydroxynaltrexone (III) and naltrexone (I) (bottom). The internal standard was tetraphenylethylene (TPE).*

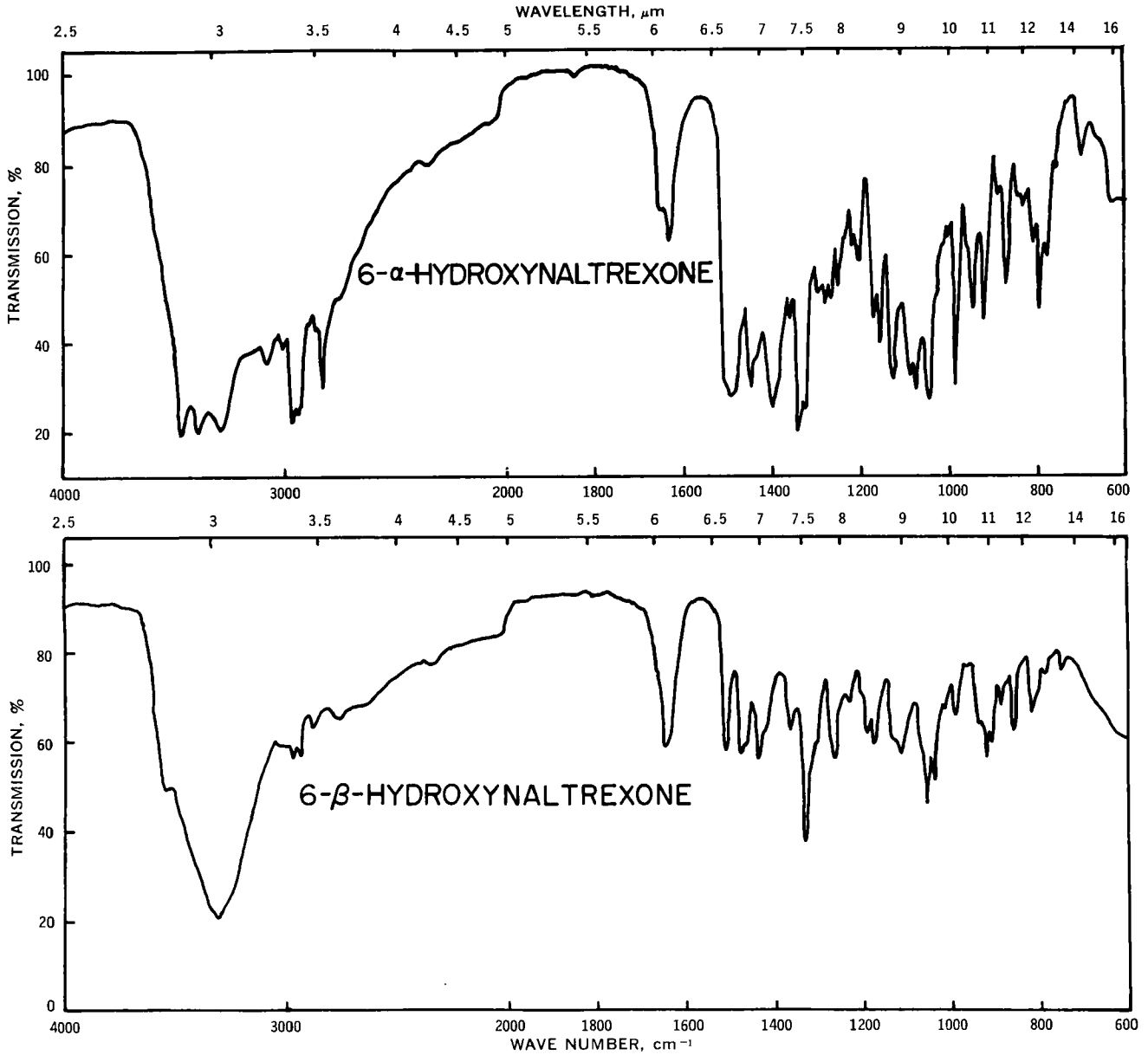


Figure 2—*IR spectra of 6α- and 6β-hydroxynaltrexone.*

urine. A small peak corresponding to the retention time of II was present in the gas chromatogram of unhydrolyzed rat urine extract. However, the relative concentration over background in the control sample made detection equivocal.

Free and conjugated I and II were present in the urine of the guinea pig. The level of free II was comparable to conjugated I while the level of free I was somewhat less than that of conjugated II. These results were encouraging, and use of the guinea pig as a preparative vehicle proved successful. Compound II was stereospecifically produced as a metabolite of I, isolated, and purified by chromatography to give pure crystalline needles of the hydrochloride.

The purity of II was evaluated by TLC (Systems I and II) and GLC (Fig. 1). The presence of about 3% III was confirmed by both techniques. A close separation of II and III can be achieved on TLC (System I) using small samples. Incomplete derivatization with pentafluoropropionic anhydride was characteristic for III and can be used beneficially for GLC identification. The lack of complete derivatization of III as opposed to II is probably explained by the lesser accessibility of the 6α-hydroxy group for derivatization. Inspection of Dreiding models of II and III indicated that the 6α-hydroxy group of III (axial position, chair form) is probably hydrogen bonded to the furan oxygen and that the 6α-hydroxy group is more sterically hindered by the surrounding ring system than that of II (equatorial position, chair form).

The origin of III in II was questioned as possibly being an artifact of the isolation procedure. To address this problem, the ratio of II to III was monitored through the purification process. Since the ratio remained essentially unchanged, it was concluded that III was a metabolite.

The GLC chromatograms of the pentafluoropropionic anhydride derivatives of I, II (obtained from human and guinea pig), and III are presented in Fig. 1. The internal standard, tetraphenylethylene, was included for comparison of relative retention times. The metabolite from humans (1) and guinea pig had the same retention time and are resolved from I and III when cochromatographed.

The IR spectra of the epimers, II and III, are shown in Fig. 2. The characteristic lack of the carbonyl band (1730 cm^{-1}) found in I is conclusive evidence for the reduction of the carbonyl bond. The fingerprint regions (910–1430 cm^{-1}) of II and III differ considerably and provide an extra means of identification of the metabolite.

The mass spectra of II and III are shown in Fig. 3. As expected, the pattern of fragmentation is quite similar. The loss of a hydroxy group ($M^+ - 17$) is typical for the reduced form, and other easily identifiable fragmentations are indicated. In addition, Fig. 3 con-

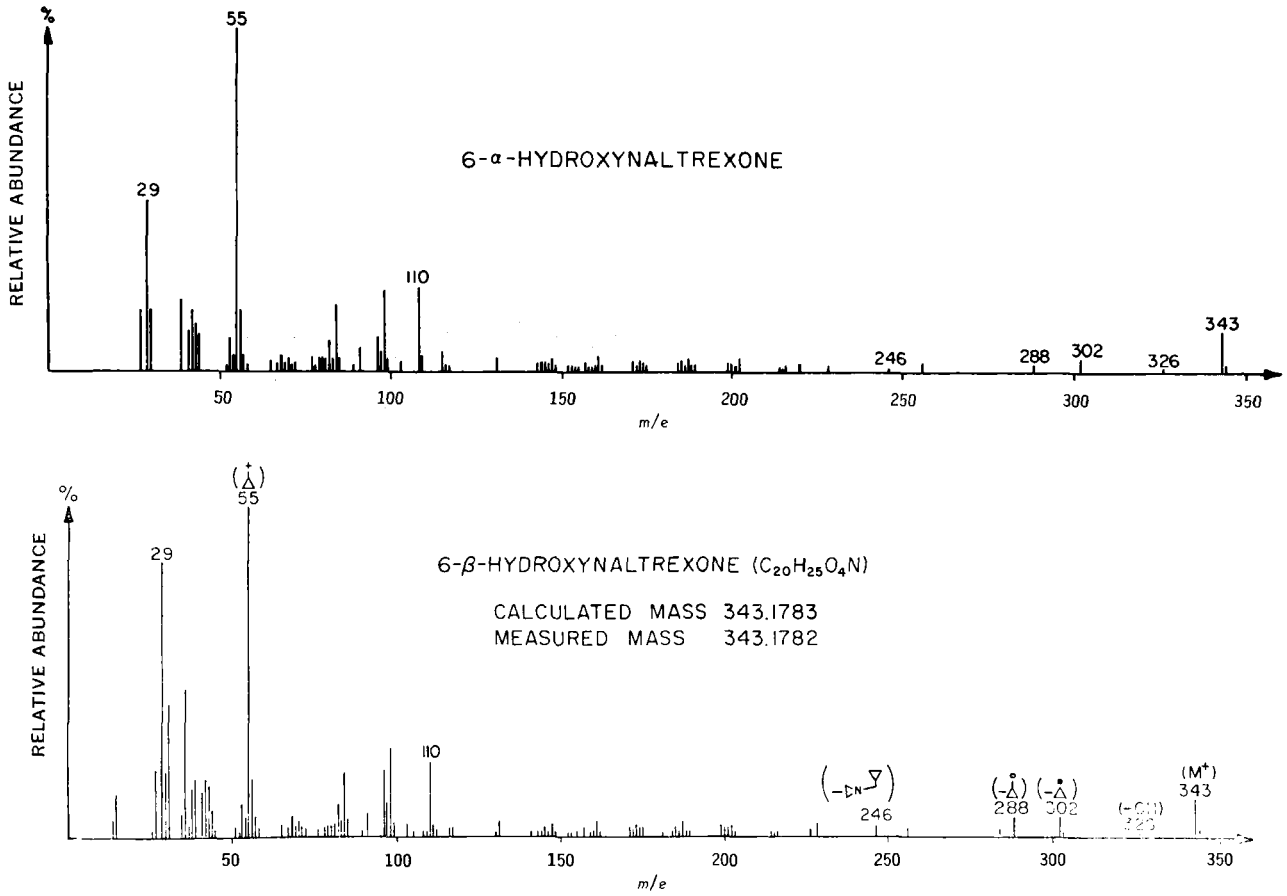


Figure 3—*Mass spectra of 6α- and 6β-hydroxynaltrexone.*

tains the high-resolution mass spectral analysis of the molecular ion of II at *m/e* 343 and confirms the structural assignment.

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Systems Approach to Study of Solute Transport across Membranes Using Suspension Cultures of Mammalian Cells III: Steady-State Diffusion Models

J. S. TURI *x, N. F. H. HO ‡, W. I. HIGUCHI ‡, and C. SHIPMAN, Jr. §

Abstract □ General physical models are derived for the diffusional transport of drugs across membranes of mammalian cells in culture suspension. These models represent different sets of possible physical processes taking place during the transport of a drug molecule. Once the diffusing species reaches the cell barrier, it may gain entrance to the cell kinetically by one of the principal quasi-steady-state mechanisms, all of which assume the cell membrane to be an integral part of the total barrier.

Keyphrases □ Transport mechanisms—eight steady-state diffusion models derived and discussed, membranes using suspension cultures of mammalian cells □ Diffusion models—eight steady-state models derived and discussed, membranes using suspension cultures of mammalian cells □ Drug transport—studied using systems approach, across membranes using suspension cultures of mammalian cells, eight steady-state diffusion models derived and discussed □ Solute binding—systems approach, drug transport across membranes using suspension cultures of mammalian cells, eight steady-state diffusion models derived and discussed

In recent years, considerable effort has been directed toward the understanding of the mechanism of drug transport across membranes. Numerous experimental systems including cell cultures have been used to study membrane transport. It is felt that these cell systems provide the means to quantify drug metabolism, distribution, and bioavailability at the site of drug action.

Requisites for quantitative, mechanistic approaches to scientific understanding are the simultaneous development of sound, theoretical physical models and the establishment of experimental systems that are accessible to rigorous analysis by physicochemical techniques.

This paper discusses some general physical models for the diffusional transport of drugs across membranes of cells in culture suspension; they represent extensions of some earlier models (1–3). These models provide the quantitative interrelationships among the variables important to the transport of drug molecules across cell membranes. They also provide the basis for the design and analysis of experiments and the foundation for development of new physical models. The use of these physical models with the experimental data is intended to describe the nature of the transport barriers, the kinds of species transported, and whether, where, and how much solute binding occurs. These models assume that the aqueous diffusion layer has a negligible effect on the transport rate.

PHYSICAL MODELS

The eight physical models, each designed to represent different sets of possible physical processes, are:

MODEL 1. Rapid equilibration in the heterogeneous cell interior in which no binding of the drug molecule to components outside the cell occurs.

MODEL 2. Rapid equilibration in the heterogeneous cell interior in which binding of the drug molecule to components outside the cell occurs.

MODEL 3. Rapid equilibration of the drug in the heterogeneous cell interior with an instantaneous, irreversible binding of the drug molecule to the cell plasma membrane at initial time.

MODEL 4. Rapid equilibration of the drug in the heterogeneous cell interior accompanied by reversible, instantaneous binding of the drug molecule to the cell plasma membrane.

MODEL 5. Rapid equilibration of the drug in the heterogeneous cell interior accompanied by reversible, instantaneous binding of the drug molecule to the cell plasma membrane followed by the permeation of both the unbound drug and the membrane-bound drug through the plasma membrane.

MODEL 6. Simultaneous transport of two drugs, each rapidly equilibrated in the heterogeneous cell interior in which neither binds to components outside the cell.

MODEL 7. Simultaneous transport of two drugs, each rapidly equilibrated in the heterogeneous cell interior in which one binds to the plasma membrane by the conditions in Model 4.

MODEL 8. Rapid equilibration in the aqueous environment in the cell, with slow simultaneous permeation of the drug into the cytoplasmic bodies and the nucleus.

A schematic diagram of the cell used for the development of these models is shown in Fig. 1.

Model 1—In Model 1 the plasma membrane is rate limiting for the diffusion of nonionic molecules.

Assuming that adsorption is negligible, the quasi-steady-state rate of uptake of a drug by a cell can be described by:

$$\frac{dC_i}{dt} = \frac{4\pi a^2 P}{V_i}\left(C_0 - \frac{C_i}{K}\right) \qquad \text{(Eq. 1)}$$

where P is the intrinsic permeability coefficient of the plasma membrane, C_i is the total drug concentration in the cell, a is the cell radius, C_0 is the drug concentration in the external medium, and K is the intrinsic partition coefficient. In reality, K is an apparent partition or distribution coefficient encompassing all interactions and may be considered equal to the total drug bound, partitioned, unbound, or otherwise distributed in the cell at equilibrium divided by $nV_iC_{0,\infty}$, where V_i is the volume of the cell. Since $V_i = (4/3)\pi a^3$, Eq. 1 becomes:

$$\frac{dC_i}{dt} = \frac{3P}{a}\left(C_0 - \frac{C_i}{K}\right) \qquad \text{(Eq. 2)}$$

Mass balance of the drug requires that:

$$T = C_0V_0 + nV_iC_i \qquad \text{(Eq. 3)}$$

where T is the total amount of drug in the system, n is the number of cells, and V_0 is the volume of the external medium. Equation 3 is only applicable to a system with a narrow cell volume distribution, as is the case with the Burkitt lymphoma cells; otherwise, the cell volume distribution should be considered.

By solving Eq. 3 for C_0, substituting into Eq. 2, and integrating between $C_i(0) = 0$ and $C_i(t)$, the solution for Model 1 is:

$$\ln\left(1 - \frac{BC_i}{A}\right) = -\frac{3BP}{a}(t) \qquad \text{(Eq. 4)}$$

where $A = (T/V_0)$, and $B = (nV_i/V_0) + (1/K)$.

The Model 1 uptake function (UF_1) is defined by rearranging Eq. 4 to give:

$$UF_1 = -\left[\frac{a}{(3)(60B)}\right]\ln\left(1 - \frac{BC_i}{A}\right) = Pt \qquad \text{(Eq. 5)}$$

where the factor 60 is necessary for determining the permeability coefficient in units of centimeters per second from the slope of the linear regression line of the UF_1 *versus* time (in minutes) plot.

For initial rates, Eq. 4 reduces to:

$$C_i = \frac{3PT}{aV_0}(t) \qquad \text{(Eq. 6)}$$

Thus, the intrinsic permeability coefficient, P, can be obtained from the initial slope of the C_i *versus* time plot or from the plot of Eq. 5 when K is known. The intrinsic partition coefficient, K, can be experimentally obtained from the equilibrium condition, *i.e.*, when $(dC_i/dt)_{t=\infty} = 0$; thus:

$$K = \frac{C_{i,\infty}}{C_{0,\infty}} \qquad \text{(Eq. 7)}$$

The intrinsic permeability coefficients may also be determined by the nonlinear regression analysis of Eq. 4.

Model 2—This model considers any drug binding to serum used in the external medium of the cell suspension system (Fig. 2). Equations 1–4 should be modified as follows.

The total concentration of drug in the aqueous medium is:

$$C_{0,T} = C_0 + C_0^* \qquad \text{(Eq. 8)}$$

where $C_{0,T}$ is the total drug concentration in the external phase, and C_0 and C_0^* are the concentrations of unbound and bound drug in the external phase, respectively. The Langmuir adsorption isotherm is assumed:

$$C_0^* = \frac{k_2C_0}{(1/k_1) + C_0} \qquad \text{(Eq. 9)}$$

where k_1 represents the fraction of sites occupied, and k_2 indicates the maximum amount that can be adsorbed. In the limit where k_1 is very small such that $1/k_1$ is much greater than C_0:

$$C_0^* = k_1k_2C_0 \qquad \text{(Eq. 10)}$$

where k_1k_2 is the linear adsorption constant of the serum-bound drug. This condition can also be expressed as shown here in Scheme I.

$$\text{drug} + \text{serum} \overset{K_b}{\rightleftharpoons} \text{drug–serum}$$

Scheme I

Therefore, the equilibrium binding constant is:

$$K_b = \frac{C_0^*}{C_0(S)} \qquad \text{(Eq. 11)}$$

whereupon, with Eq. 8:

$$C_{0,T} = C_0(1 + k_0) \qquad \text{(Eq. 12)}$$

where k_0 is equal to $K_b(S)$ by definition, K_b is the binding constant, and (S) is the serum concentration.

After substituting Eq. 12 into Eq. 2, the following rate expression for Model 2 is obtained:

$$\frac{dC_i}{dt} = \frac{3P_e}{a}\left(C_{0,T} - \frac{C_i}{K_e}\right) \qquad \text{(Eq. 13)}$$

The effective partition coefficient is:

$$K_e = \frac{C_i}{C_0 + C_0^*} = \frac{K}{1 + k_0} = \frac{K}{1 + K_b(S)} \qquad \text{(Eq. 14)}$$

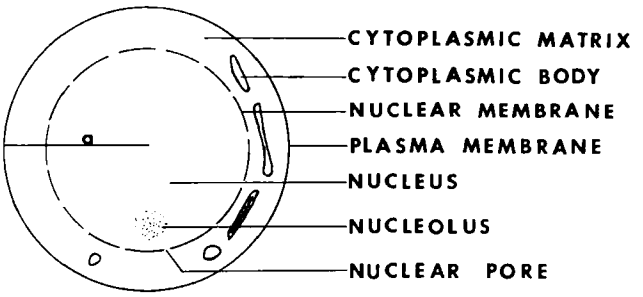

Figure 1—*Schematic description of a spherical cell of radius* a *in a viable cell culture suspension for the passive transport of nonelectrolyte drugs. In Models 1–7, the plasma membrane is the only rate-determining barrier. In Model 8, both the plasma membrane and membranes of the cytoplasmic bodies and nucleus are rate-determining barriers.*

and the effective permeability coefficient is:

$$P_e = \frac{P}{1 + k_0} = \frac{P}{1 + K_b(S)} \qquad \text{(Eq. 15)}$$

It is assumed in Eq. 15 that only the unbound drug is the membrane permeable species.

By means of Eqs. 14 and 15, the equilibrium serum–drug binding constant, K_b, can be calculated. For example, a plot of P/P_e *versus* percent of serum gives an intercept of unity and a slope equal to K_b. If the intrinsic permeability coefficient cannot be readily obtained or there is some uncertainty, then the analysis can be performed by choosing the permeability coefficient at a serum level as the reference. Thus, when choosing the permeability coefficient at 30% serum as the reference, a plot of P_{30}/P_e *versus* percent of serum gives a slope equal to $K_b/[1 + K_b(0.3)]$ and an intercept equal to $1/[1 + K_b(0.3)]$.

By using the mass balance expression:

$$T = C_{0,T}V_0 + nC_iV_i \qquad \text{(Eq. 16)}$$

the solution to Eq. 13 is:

$$\ln\left(1 - \frac{B'}{A}C_i\right) = -\frac{3P_eB'}{a}(t) \qquad \text{(Eq. 17)}$$

where $B' = (nV_i/V_0) + (1/K_e)$. The mathematical form is the same as Eq. 4 except that effective parameters are involved instead of intrinsic parameters because of the binding of the drug to the serum in the external medium.

Equations 14 and 15 are important, because they allow one to calculate the intrinsic permeability of the membrane from the effective permeability coefficients in the presence of serum, which is necessary to maintain integrity of the cells for long periods. In turn, the intrinsic permeability coefficients can be used to quantify the effect of molecular structure and functional group substituent modifications on membrane absorptivity.

Model 3—This model assumes that there is instantaneous, irreversible drug binding to the plasma membrane followed by diffu-

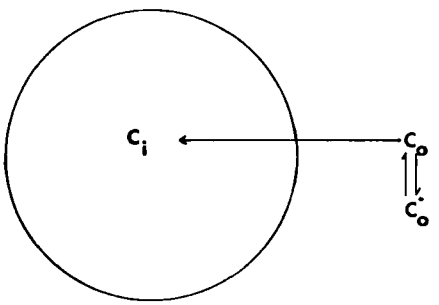

Figure 2—*Model 2: Passive transport of unbound solute across the plasma membrane with rapid distribution in the heterogeneous cell interior and reversible binding of the solute to serum and other components in the external media.*

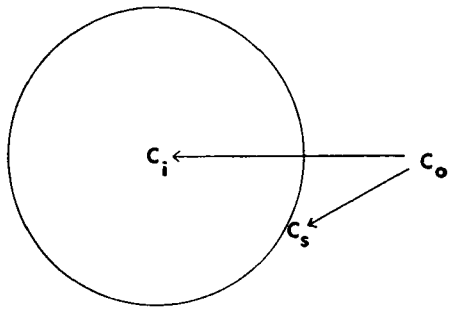

Figure 3—*Model 3: Passive transport of unbound solute across the plasma membrane with rapid distribution in the heterogeneous cell interior. There is an instantaneous, irreversible binding of the drug molecules to the external cell plasma membrane at initial time.*

sion of unbound drug molecules through the plasma membrane (Fig. 3). The following general relationships are appropriate:

$$A_c = A_i + A_s \quad \text{(Eq. 18)}$$

$$C_c = A_c/V_i \quad \text{(Eq. 19)}$$

$$C_s = A_s/V_i \quad \text{(Eq. 20)}$$

$$T = nA_c + A_0 = nC_cV_i + C_0V_0 \quad \text{(Eq. 21)}$$

where T is the total amount of drug present, A_c is the amount of drug in and on the cell, A_i is the amount inside the cell, A_s is the amount on the external surface of the cell, A_0 is the amount outside the cell, C_c is the drug concentration in and on the cell, and C_s is the drug concentration on the surface of the cell. The general mass balance relationships of Eqs. 18–21 are also applicable to Models 4 and 5.

While it is an experimental reality that C_c as a function of time is readily accessible, one encounters the problem of handling the data to assess the membrane permeability and partition coefficients clearly. This problem is resolved by the following mathematical treatment of this model.

By utilizing Eqs. 18–21:

$$C_c = C_i + C_s \quad \text{(Eq. 22)}$$

and:

$$\frac{dC_c}{dt} = \frac{dC_i}{dt} \quad \text{(Eq. 23)}$$

where $dC_s/dt = 0$ since C_s is the concentration of drug adsorbed instantaneously at initial time and irreversibly on the external surface of the plasma membrane and, consequently, is independent of the drug concentration in the external medium and time. It follows that the fundamental Eq. 2 for the quasi-steady-state rate of drug uptake by a cell can be rewritten as:

$$\frac{dC_c}{dt} = \frac{3P}{a}(X - BC_c) \quad \text{(Eq. 24)}$$

where:

$$X = \frac{T}{V_0} + \frac{C_s}{K} \quad \text{(Eq. 25)}$$

and B is as defined before. Integrating between the boundary conditions, $C_c = C_s$ at time = 0 and C_c (t):

$$\ln\left(\frac{X - BC_c}{X - BC_s}\right) = -\left(\frac{3BP}{a}\right)(t) \quad \text{(Eq. 26)}$$

which, upon rearranging, gives the Model 3 uptake function:

$$UF_3 = -\left[\frac{a}{(3)(60B)}\right]\ln\left(\frac{X - BC_c}{X - BC_s}\right) = Pt \quad \text{(Eq. 27)}$$

Before a plot of the uptake function *versus* time (in minutes)

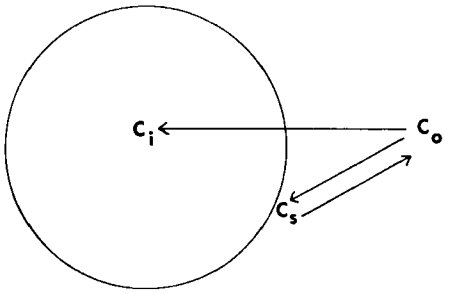

Figure 4—*Model 4: Passive transport of unbound solute across the plasma membrane with rapid distribution in the heterogeneous cell interior. There is an instantaneous, reversible binding of drug molecules to the external cell plasma membrane.*

can be made to calculate the permeability coefficient (in units of centimeters per second), the intrinsic partition coefficient must be found. Experimentally, the apparent partition coefficient, K_{ex}, is readily determined and, in turn, it is related to the intrinsic partition coefficient, K. Thus:

$$K_{ex} = \frac{C_{c,\infty}}{C_{0,\infty}} = \frac{C_{i,\infty} + C_s}{C_{0,\infty}} = K + \frac{C_s}{C_{0,\infty}} \quad \text{(Eq. 28)}$$

or:

$$K = K_{ex} - \frac{C_s}{C_{0,\infty}} \quad \text{(Eq. 29)}$$

Model 4—This model takes into account both the instantaneous and reversible drug binding to the plasma membrane and passive transport of the unbound drug molecule through the plasma membrane (Fig. 4). The concentration of drug bound to the cell surface is assumed to be proportional to the concentration of drug in the external medium:

$$C_s = K_{bm}C_0 \quad \text{(Eq. 30)}$$

where K_{bm} is the plasma membrane–drug equilibrium adsorption constant.

The uptake rate of drug into the cell interior is expressed by Eq. 2. With Eqs. 21, 22, and 30 and their derivatives, it can be shown that:

$$\frac{dC_i}{dt} = \frac{d(C_c - C_s)}{dt} = \left(1 + K_{bm}\frac{nV_i}{V_0}\right)\frac{dC_c}{dt} \quad \text{(Eq. 31)}$$

and:

$$\left(C_0 - \frac{C_i}{K}\right) = \left(1 + \frac{K_{bm}}{K}\right)\frac{T}{V_0} - \left[\left(1 + \frac{K_{bm}}{K}\right)\frac{nV_i}{V_0} + \frac{1}{K}\right]C_c \quad \text{(Eq. 32)}$$

Consequently, Eq. 2 becomes:

$$\frac{dC_c}{dt} = \frac{3P}{a(1 + K_{bm}E)}[(F - GC_c)] \quad \text{(Eq. 33)}$$

and its solution is:

$$\ln\left[\frac{F - GC_c}{F - GC_{c,0}}\right] = -\frac{3GP}{a(1 + K_{bm}E)}(t) \quad \text{(Eq. 34)}$$

where:

$$F = \left(1 + \frac{K_{bm}}{K}\right)\frac{T}{V_0} \quad \text{(Eq. 35)}$$

$$G = \left(1 + \frac{K_{bm}}{K}\right)\frac{nV_i}{V_0} + \frac{1}{K} \quad \text{(Eq. 36)}$$

$$E = \frac{nV_i}{V_0} \quad \text{(Eq. 37)}$$

and $C_{c,0}$ is the cell concentration of the drug at zero time, which is

equal to the initial surface-adsorbed drug concentration. The Model 4 uptake function is:

$$UF_4 = -\left[\frac{a(1 + K_{bm}E)}{(3)(60G)}\right]\ln\left[\frac{F - GC_c}{F - GC_{c,0}}\right] = Pt \quad \text{(Eq. 38)}$$

The drug–membrane adsorption constant may be found with the aid of Eqs. 20, 21, and 30 and from the extrapolation of a A_c *versus* t plot to $t = 0$ to get $A_{s,0}$:

$$K_{bm} = \frac{nA_{s,0}/V_i}{A_0/V_0} = \frac{nA_{s,0}}{(V_i/V_0)(T - nA_{s,0})} \quad \text{(Eq. 39)}$$

The apparent partition coefficient is determined from the equilibrium transport conditions; thus:

$$K_{ex} = \frac{C_{c,\infty}}{C_{0,\infty}} = \frac{C_{i,\infty} + K_{bm}C_{0,\infty}}{C_{0,\infty}} \quad \text{(Eq. 40)}$$

and, subsequently:

$$K = K_{ex} - K_{bm} \quad \text{(Eq. 41)}$$

Model 5—This model assumes that there is instantaneous, reversible drug binding to the plasma membrane and diffusion of both the unbound drug and the membrane-bound drug through the plasma membrane (Fig. 5). The authors cannot readily see any possible experimental means by which Model 5 can be distinguished from Model 4: however, it is presented for its academic interest. The transport of drug into the cell interior can be expressed by:

$$\frac{dC_i}{dt} = \frac{3P}{a}\left(C_0 - \frac{C_i}{K}\right) + \frac{3P_m}{a}\left(C_s - \frac{C_i}{K_m}\right) \quad \text{(Eq. 42)}$$

When employing techniques similar to those in the derivation of Model 4, the change in the cell concentration of drug with time is:

$$\frac{dC_c}{dt} = \frac{3P}{a(1 + K_{bm}E)}(F - GC_c) + \frac{3P_m}{a(1 + K_{bm}E)}(M - NC_c) \quad \text{(Eq. 43)}$$

where:

$$M = K_{bm}\left(1 + \frac{1}{K_m}\right)\frac{T}{V_0} \quad \text{(Eq. 44)}$$

$$N = K_{bm}\left(1 + \frac{1}{K_m}\right)\frac{nV_i}{V_0} + \frac{1}{K_m} \quad \text{(Eq. 45)}$$

and P and P_m are the permeability coefficients of the unbound and membrane-bound solute, respectively; K_m is the partition coefficient of the membrane-bound solute; and the terms F, G, E, and others have been previously defined. The solution is:

$$\ln\frac{(PF + P_mM) - (PG + P_mN)C_c}{(PF + P_mM) - (PG + P_mN)C_{c,0}} = -\frac{3(PG + P_mN)}{a(1 + K_{bm}E)}(t) \quad \text{(Eq. 46)}$$

The partition coefficients can be evaluated from the experimental data by the similar procedure in Model 4 and are:

$$K = C_{i,\infty}/C_{0,\infty} = K_{ex} - K_{bm} \quad \text{(Eq. 47)}$$

$$K_m = C_{i,\infty}/C_{s,\infty} = (K_{ex}/K_{bm}) - 1 \quad \text{(Eq. 48)}$$

Model 6—The next two models are useful for evaluating the simultaneous uptake of two radiolabeled solutes. Model 6 assumes that both solutes diffuse independently and that each follows the conditions of Model 1.

Then, the rate of uptake of solutes A and B by a cell can be described by:

$$\frac{dC_{i,A}}{dt} = \frac{3P_A}{a}\left(C_{0,A} - \frac{C_{i,A}}{K_A}\right) \quad \text{(Eq. 49)}$$

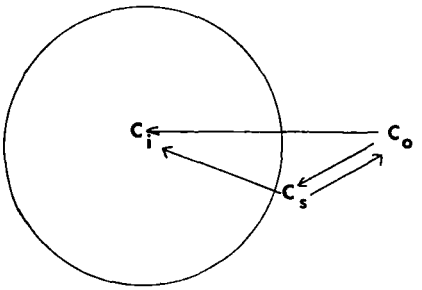

Figure 5—*Model 5: Passive transport of both the unbound solute and the external cell membrane-bound solute across the plasma membrane, with rapid distribution in the heterogeneous cell interior. The cell membrane-bound solute is also in equilibrium with the free solute concentration in the external media.*

and:

$$\frac{dC_{i,B}}{dt} = \frac{3P_B}{a}\left(C_{0,B} - \frac{C_{i,B}}{K_B}\right) \quad \text{(Eq. 50)}$$

where the definitions of the terms are the same as before and the subscripts A and B refer to the two solutes. With Eq. 2:

$$\frac{dC_{i,A}}{dt} = -\frac{3P_A}{a}\left(\frac{1}{K_A} + \frac{nV_i}{V_0}\right)C_{i,A} + \frac{3P_AT_A}{aV_0} \quad \text{(Eq. 51)}$$

and:

$$\frac{dC_{i,B}}{dt} = -\frac{3P_B}{a}\left(\frac{1}{K_B} + \frac{nV_i}{V_0}\right)C_{i,B} + \frac{3P_BT_B}{aV_0} \quad \text{(Eq. 52)}$$

and their second derivatives are:

$$\frac{d^2C_{i,A}}{dt^2} + k_A\frac{dC_{i,A}}{dt} = 0 \quad \text{(Eq. 53)}$$

$$\frac{d^2C_{i,B}}{dt^2} + k_B\frac{dC_{i,B}}{dt} = 0 \quad \text{(Eq. 54)}$$

where:

$$k_A = \frac{3P_A}{a}\left(\frac{1}{K_A} + \frac{nV_i}{V_0}\right) \quad \text{(Eq. 55)}$$

$$k_B = \frac{3P_B}{a}\left(\frac{1}{K_B} + \frac{nV_i}{V_0}\right) \quad \text{(Eq. 56)}$$

The integration of Eq. 53 and the application of the boundary conditions of $C_{i,A} = 0$ at $t = 0$ and $C_{i,A} = C_{i,A,eq}$ at $t = \infty$ yield:

$$C_{i,A} = C_{i,A,eq}(1 - e^{-k_At}) \quad \text{(Eq. 57)}$$

In a similar manner:

$$C_{i,B} = C_{i,B,eq}(1 - e^{-k_Bt}) \quad \text{(Eq. 58)}$$

where $C_{i,A,eq}$ and $C_{i,B,eq}$ are the equilibrium drug concentrations in the cell. By adding Eqs. 57 and 58:

$$C_{i,(A+B)} = C_{i,(A+B)eq} - C_{i,A,eq}e^{-k_At} - C_{i,B,eq}e^{-k_Bt} \quad \text{(Eq. 59)}$$

Experimentally, the concentration of the two radiolabeled solutes in the cell, $C_{i,(A+B)}$, as a function of time and their equilibrium concentration, $C_{i,(A+B)eq}$, are directly measurable. By utilizing the partition coefficients of solutes A and B from independent experiments and knowing the total amounts of each solute added, the equilibrium concentrations of each solute in the cells, $C_{i,A,eq}$ and $C_{i,B,eq}$, can then be calculated, *e.g.*:

$$C_{i,A,eq} = \frac{T_A}{nV_i + (V_0/K_A)} \quad \text{(Eq. 60)}$$

Finally, the respective permeability coefficients can be determined from Eq. 59.

Model 7—This model is similar to Model 6, except that solute *A* follows Model 4 and solute *B* follows Model 1. Accordingly, the solution for Model 7 is:

$$C_{c,(A+B)} = C_{c,(A+B)eq} - C_{c,B,eq}e^{-k_B t} + (C_{s,0} - C_{c,A,eq})e^{-k_A' t} \quad \text{(Eq. 61)}$$

where:

$$k_A' = \frac{3P_A G}{a(1 + K_{bm}E)} \quad \text{(Eq. 62)}$$

Model 8—This model analyzes the situation where the rate-determining barrier to equilibrium drug distribution in the monodispersed cell suspension not only includes the plasma membrane but also the membranes of the cytoplasmic bodies within the cell. The drug distributes instantaneously in the aqueous interior but permeates slowly into the cytoplasmic bodies, including the nucleus.

From Eqs. 2 and 3:

$$\frac{dC_i}{dt} = \frac{3P_e}{a}\left[\frac{T}{V_0} - \left(\frac{nV_i}{V_0} + \frac{1}{K_e}\right)C_i\right] \quad \text{(Eq. 63)}$$

The differentiation of Eq. 63 gives:

$$\frac{d^2C_i}{dt^2} = -\frac{3P_e}{a}\left(\frac{nV_i}{V_0} + \frac{1}{K_e}\right)\frac{dC_i}{dt} \quad \text{(Eq. 64)}$$

The distribution of drug within the aqueous cytoplasmic matrix and cytoplasmic bodies of each cell is:

$$C_i = (1 - \alpha_i)(C_{i,aq} + C^*_{i,aq}) + \alpha_i C_{i,cyt} \quad \text{(Eq. 65)}$$

where α_i is the fraction of cytoplasmic bodies, $(1 - \alpha_i)$ is the fraction of the cytoplasmic aqueous matrix, $C_{i,aq}$ is the unbound drug concentration in the aqueous cell interior, and $C_{i,cyt}$ is the drug concentration in the various cytoplasmic bodies. The concentration adsorbed, $C^*_{i,aq}$, in the aqueous interior is assumed to be linear with respect to the Langmuir isotherm; thus:

$$C^*_{i,aq} = K_{bi}C_{i,aq} \quad \text{(Eq. 66)}$$

where K_{bi} is the linear adsorption constant. Together with Eqs. 65 and 66, it follows that:

$$\frac{dC_i}{dt} = (1 - \alpha_i)(1 + K_{bi})\frac{dC_{i,aq}}{dt} + \alpha_i\frac{dC_{i,cyt}}{dt} \quad \text{(Eq. 67)}$$

The expression for the rate of transfer of drug from the aqueous interior of each cell into the cytoplasmic bodies is given by:

$$\left[\frac{V_0}{K'} + (1 - \alpha_i)V_i\right]\frac{dC_{i,aq}}{dt} = n_i S_i P_{e,cyt}\left(C_{i,aq} - \frac{C_{i,cyt}}{K_{e,cyt}}\right) \quad \text{(Eq. 68)}$$

and:

$$-(1 - \alpha_i)V_i\frac{dC_{i,aq}}{dt} = \alpha_i V_i\frac{dC_{i,cyt}}{dt} \quad \text{(Eq. 69)}$$

where n_i is the total number of cytoplasmic bodies with an average surface area, S_i, possessing an average effective permeability coefficient, $P_{e,cyt}$, and partition coefficient, $K_{e,cyt}$. The partition coefficient K' is the ratio of $C_{i,aq}$ to C_0. A rigorous treatment of Eqs. 68 and 69 should reflect the transport of the drug from the aqueous cytoplasmic matrix into the cytoplasmic bodies, summing over each kind of cytoplasmic bodies with each kind possessing a characteristic permeability and partition coefficient. However, since it is extremely difficult to determine experimentally the particular physical parameters within the cell with any degree of certainty, the average effect is taken as expressed here.

Differentiating Eq. 68 and then using Eq. 69 lead to the following result:

$$\frac{d^2C_{i,aq}}{dt^2} + \beta\frac{dC_{i,aq}}{dt} = 0 \quad \text{(Eq. 70)}$$

where:

$$\beta = \frac{n_i S_i P_{e,cyt}}{\left[\frac{V_0}{K'} + (1 - \alpha_i)V_i\right]}\left[1 + \frac{(1 - \alpha_i)}{\alpha_i K_{e,cyt}}\right] \quad \text{(Eq. 71)}$$

Introducing the boundary conditions of $C_{i,aq} = 0$ and $C_{i,aq(\infty)} = C_{i,aqE}$, where $C_{i,aqE}$ is the equilibrium drug concentration in the aqueous interior of the cell, the solution is:

$$C_{i,aq} = C_{i,aqE}(1 - e^{-\beta t}) \quad \text{(Eq. 72)}$$

and with Eq. 69, the derivative becomes:

$$\frac{dC_{i,aq}}{dt} = -\frac{\alpha_i}{(1 - \alpha_i)}\frac{dC_{i,cyt}}{dt} = C_{i,aqE}\beta e^{-\beta t} \quad \text{(Eq. 73)}$$

Finally, by substituting Eqs. 73 and 67 into Eq. 64, the following second-order, nonhomogeneous differential equation is obtained:

$$\frac{d^2C_i}{dt^2} + \frac{3P_e nV_i}{aV_0}\frac{dC_i}{dt} = -\frac{3P_e}{aK_e}(1 - \alpha_i)K_{bi}C_{i,aqE}\beta e^{-\beta t} \quad \text{(Eq. 74)}$$

Integration from initial to equilibrium conditions yields:

$$C_i = C_{i,E} - \left[\frac{G}{(\alpha - \beta)} + C_{i,eq}\right]e^{-\alpha t} + \frac{G}{(\alpha - \beta)}e^{-\beta t} \quad \text{(Eq. 75)}$$

where:

$$\alpha = \frac{3P_e nV_i}{aV_0} \quad \text{(Eq. 76)}$$

$$G = \frac{3P_e(1 - \alpha_i)K_{bi}C_{i,aqE}}{aK_e(\alpha - \beta)} \quad \text{(Eq. 77)}$$

CONCLUSION

Some physical models are derived for the diffusional transport of drugs across membranes of mammalian cells in culture suspension. These theoretical models represent but a few of the many ways molecules can penetrate cells. To assign mechanisms, models such as these must be used to evaluate the data and to design experiments. The experimental sequel of this theoretical presentation will demonstrate the usefulness of the physical model approach, as will be seen in the transport of various sterols and cardiac glycosides into and out of Burkitt lymphoma cells in culture suspension.

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Systems Approach to Study of Solute Transport across Membranes Using Suspension Cultures of Mammalian Cells IV: Uptake and Release Kinetics of Sterols

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Abstract □ The kinetics of uptake and release of desmosterol, cholesterol, and β-sitosterol by Burkitt lymphoma cells at 26°, pH 7.3, and isoosmotic conditions were quantitatively and mechanistically interpreted. The data are in agreement with the model involving the passive transport of the unbound sterol across the rate-determining plasma membrane, with rapid distribution within the heterogeneous cell interior. Effective permeability (P_e) and partition (K_e) coefficients of the sterols were inversely proportional to the serum concentration in the external media due to sterol–serum binding. These results are consistent with the mechanism in which only the unbound solute in the external solution participates in the membrane transport process. At all serum levels, P_e and K_e increased with increasing sterol polarity: desmosterol > cholesterol > β-sitosterol.

Keyphrases □ Membrane diffusion—systems approach to drug transport, uptake and release of desmosterol, cholesterol, and β-sitosterol by Burkitt lymphoma cells, effective permeability and partition coefficients determined and related to sterol–serum binding □ Drug transport—uptake and release kinetics, desmosterol, cholesterol, and β-sitosterol, Burkitt lymphoma cells, effective permeability and partition coefficients determined, related to sterol–serum binding □ Mammalian cells, suspension cultures—uptake and release of sterols, systems approach to study of drug transport across membranes □ Sterols—kinetics of uptake and release of desmosterol, cholesterol, and β-sitosterol by Burkitt lymphoma cells, systems approach to drug transport

This paper represents an experimental followup of the theoretical models presented earlier (1) in which the plasma membrane of the cell is the rate-determining barrier to the transport of solutes. To demonstrate the utility of the physical model approach in the design of experiments and the quantification of the relevant transport parameters and physical factors, the kinetics of the uptake and release of cholesterol, desmosterol, and β-sitosterol using Burkitt lymphoma cells in culture suspension are reported.

EXPERIMENTAL

The methods for the cultivation of Burkitt lymphoma cells were described previously (2). Included are the details of the quantitative techniques and procedures for the baseline uptake experiments of cholesterol-^{14}C by the cells in culture suspension at pH 7.3 and 26° under isoosmotic and sterile conditions in spinner flasks. The suspending media contained modified McCoys 5A, basal medium Eagle, balanced salt solution, buffers, and varying concentrations of fetal bovine serum.

To follow the changes in concentration of solute with time, both the cell and extracellular fraction were assayed by liquid scintillation counting after centrifugation. To study the release kinetics of drugs from the cell, the following two techniques were used to load the cells with solute. With the "static" equilibration technique, the cell suspension was prepared and allowed to mix with the radioactive drug at 26° for at least 48 hr. With the "prolific" equilibration technique, the cell suspension containing the radioactive drug was incubated at 37° so that the cells could grow.

After loading the cells with the solute, the cells were washed and resuspended in fresh media. Thereafter, the release studies followed the sampling procedures as outlined previously for the uptake kinetic studies (2). All uptake and release studies were performed at 26° and used identical isoosmotic media at pH 7.3. The studies were repeated at least three or four times.

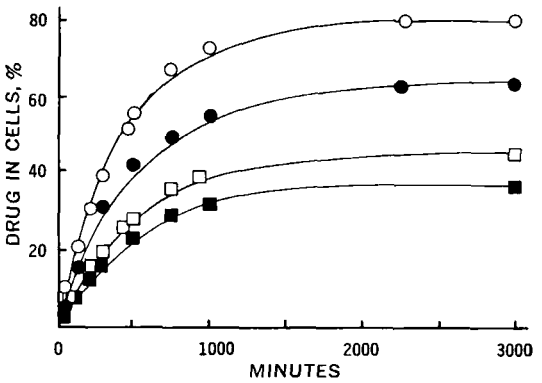

Figure 1—*Uptake of desmosterol at 26° at four fetal bovine serum concentrations. Key: ○, 0%; ●, 5%; □, 15%; and ■, 30%. Points represent the experimental data, and curves represent the best theoretical fit by nonlinear regression of Eq. 1 using the phenomenological constants in Table I.*

THEORETICAL

As will be seen, the kinetics of uptake and release of the various sterols by viable Burkitt lymphoma cells can best be explained by Model 2 (1), in which: *(a)* the interior of the cell is treated essentially as a homogeneous phase, *i.e.*, the membranes of the cytoplasmic bodies and nucleus are not the rate-determining barriers; *(b)* the plasma membrane is the rate-limiting barrier to the passive transport of the solute; and *(c)* there is binding of the solute to the serum and, perhaps, other components comprising the support media for the cell suspension. Only the essential mathematical description will be presented here.

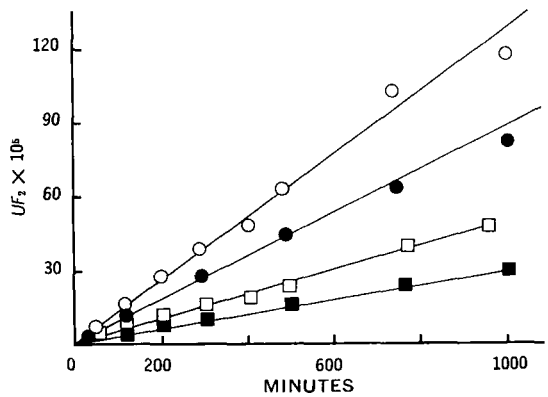

Figure 2—*Plot of uptake kinetics of desmosterol at four fetal bovine serum concentrations according to linear predictions of Eq. 1. Key: ○, 0%; ●, 5%; □, 15%; and ■, 30%. Solid curves are least-squares lines.*

Table I—Permeability Coefficients for Desmosterol Uptake and Release Kinetics at 26° from Nonlinear Regression Analysis and Partition Coefficients at Equilibrium[a]

Fetal Bovine Serum, %	Effective Partition Coefficient, Average ± *SD*		Effective Permeability Coefficient[b] × 10^7, Average ± *SD*	
	Uptake	Release	Uptake	Release
0	750 ± 44	—	13 ± 0.8	—
5	270 ± 24	288 ± 15	9.25 ± 0.5	8.80 ± 0.9
15	100 ± 10	107 ± 4	4.18 ± 0.3	3.94 ± 0.6
30	51 ± 7	56 ± 4	2.99 ± 0.4	2.64 ± 0.5

[a] Effective serum–desmosterol binding constant is K_b = 18.7. [b] Units of centimeters per second.

Table II—Permeability Coefficients for Cholesterol Uptake and Release Kinetics at 26° from Nonlinear Regression Analysis and Partition Coefficients at Equilibrium[a]

Fetal Bovine Serum, %	Effective Partition Coefficient, Average ± *SD*		Effective Permeability Coefficient[b] × 10^7, Average ± *SD*	
	Uptake	Release	Uptake	Release
0	230 ± 9	—	4.44 ± 0.6	—
5	155 ± 6	—	2.69 ± 0.4	—
15	60 ± 4	64 ± 8	1.27 ± 0.1	1.32 ± 0.2
30	34 ± 3	37 ± 2	0.88 ± 0.09	0.89 ± 0.07

[a] Effective serum–cholesterol binding constant is K_b = 15.7. [b] Units of centimeters per second.

The uptake transport function of this model, UF_2, is:

$$UF_2 = \frac{-2.303a}{180B}\log\left[1 - \frac{B}{A}C_i\right] = P_e t \qquad \text{(Eq. 1)}$$

$$A = T/V_0 \qquad \text{(Eq. 2)}$$

$$B = \frac{nV_i}{V_0} + \frac{1}{K_e} \qquad \text{(Eq. 3)}$$

where a is the radius of the cell, n is the number of cells, V_i is the volume of the cell, V_0 is the volume of the external aqueous phase, T is the total amount of drug in the system, C_i is the concentration of drug in the cell, K_e is the effective partition coefficient, P_e is the effective permeability coefficient, and t is time. Here, the initial boundary condition is $C_i(0) = 0$.

The release transport function, RF_2, is expressed by:

$$RF_2 = \frac{2.303a}{180B}\log\left[\frac{BC_i - A}{BC_i(0) - A}\right] = -P_e t \qquad \text{(Eq. 4)}$$

The initial boundary condition is $C_i = C_i(0)$. According to Eq. 1 or 4, a plot of UF_2 or RF_2 *versus* t in minutes is linear with a slope equal to P_e in units of centimeters per second.

The effective permeability coefficient is given by:

$$P_e = \frac{P}{1 + K_b(S)} \qquad \text{(Eq. 5)}$$

where K_b is the solute–serum binding constant, (S) is the serum concentration, and P is the intrinsic permeability constant of the unbound drug. It is assumed here that the serum is the only adsorbent in the external medium of the cell suspension. The effective partition coefficient is:

$$K_e = \frac{K}{1 + K_b(S)} \qquad \text{(Eq. 6)}$$

where K is the intrinsic partition coefficient.

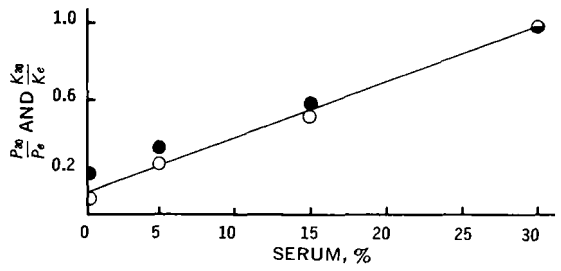

Figure 3—*Normalized plot of the effective permeability coefficients and partition coefficients of desmosterol as a function of fetal bovine serum concentration. Key:* ●, P_{30}/P_e; *and* ○, K_{30}/K_e. *The* P_{30} *and* K_{30} *values are the effective permeability and partition coefficients at the 30% fetal bovine level and are used as the references.*

RESULTS AND DISCUSSION

Uptake and Release of Desmosterol-26-^{14}C—Figure 1 shows the uptake kinetics of desmosterol at four serum concentrations. The rate of transport decreases with increasing serum concentration due to solute–serum binding. After calculating the effective partition coefficient at each serum level from the equilibrium portion of the curves and then applying the data to Eq. 1, the predicted linear relationship of the uptake function *versus* time of this

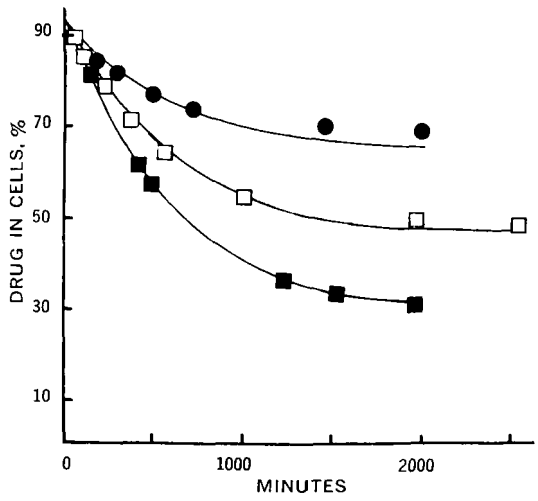

Figure 4—*Release of desmosterol at 26° at various fetal bovine serum concentrations. Key:* ●, *5% (cells equilibrated with desmosterol by the prolific method);* □, *15% (cells equilibrated by the static method); and,* ■, *30% (static equilibration method). Points represent experimental data, and solid curves are the best theoretical fit by nonlinear regression of Eq. 4 using the constants in Table I.*

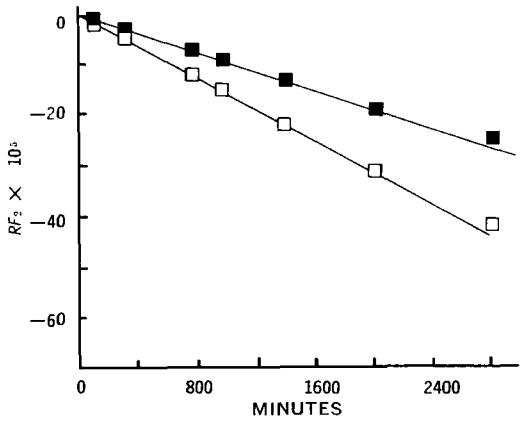

Figure 5—*Release function* versus *time of desmosterol at two fetal bovine serum concentrations. Key:* □, *15%; and* ■, *30%. The solid curve is the linear regression line.*

model is followed (Fig. 2). It is readily observed that both the effective partition and permeability coefficients decrease with increasing serum concentration.

Another indication of the fit of the theoretical model is found in Fig. 3. Here, the treatment of the experimental data followed the linear relationships of Eqs. 5 and 6, using the effective partition and permeability coefficients at the 30% serum level as reference[1]. Thus, this finding supports the theory that the unbound desmosterol is permeating through the plasma membrane.

Experiments were also done with various concentrations of desmosterol at constant serum concentration. The results showed that the desmosterol concentration did not alter the effective coefficients. This finding indicates that the concentration of serum-bound desmosterol was linearly related to the concentration of unbound drug within the experimental conditions.

Figure 4 shows the release kinetics from the cells at three fetal bovine serum levels after the cells were loaded by the static method at 26°. As expected, at higher serum levels more desmosterol diffused from the cells, reflecting a lower effective partition coefficient at higher serum levels. In Fig. 5 the profiles of the release function *versus* time show that the effective permeability coefficients decrease with increasing serum levels.

Release studies from cells loaded with desmosterol by the prolific method at 37° indicated that both radiolabeled desmosterol and cholesterol were being transported across the plasma membrane.

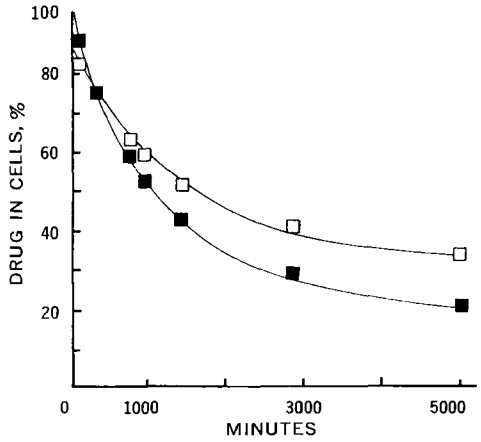

Figure 6—*Release kinetics of cholesterol at 26° and two fetal bovine serum concentrations. Cells were equilibrated with cholesterol by the static procedure. Key:* □, *15%; and* ■, *30%. Points are experimental data, and curves are the best nonlinear theoretical fit of the model using the effective permeability and partition coefficients in Table II.*

[1] In Fig. 3 the plot of P_{30}/P_e *versus* percent serum gives a slope equal to $K_b/(1 + 0.3K_b)$ and an intercept equal to $1/(1 + 0.3K_b)$.

Table III—Permeability Coefficients for β-Sitosterol Uptake Kinetics at 26° from Nonlinear Regression Analysis and Partition Coefficients at Equilibrium[a]

Fetal Bovine Serum, %	Effective Partition Coefficient, Average ± *SD*	Effective Permeability Coefficient[b] × 10^7, Average ± *SD*
0	150 ± 10.5	2.35 ± 0.55
5	45 ± 2.5	1.27 ± 0.23
15	23 ± 1.4	0.74 ± 0.06
30	12 ± 2.4	0.44 ± 0.04

[a] Effective serum–β-sitosterol binding constant is K_b = 18.8. [b] Units of centimeters per second.

These two solutes were subsequently identified by radiochromatographic analysis. The theoretical models[2] and quantitative experiments on the simultaneous transport of desmosterol and cholesterol and the bioconversion of desmosterol to cholesterol by Burkitt lymphoma cells will be reported later. No bioconversion of desmosterol was detected when the cells were loaded by the static method at 26° and when subsequent release studies were carried out at 26°.

Table I summarizes the phenomenological transport coefficients of desmosterol for Burkitt lymphoma cells. There is substantial agreement in the P_e and K_e values at the various serum levels between the uptake and release experiments. The reproducibility of the experiments is good.

The fact that the effective permeability coefficients determined either by linear or nonlinear regression analysis of the data were similar underscores the good precision of the experimental technique and design. The P_e of 1.3×10^{-6} cm/sec and K_e of 750 could be interpreted as intrinsic coefficients; however, it is not certain

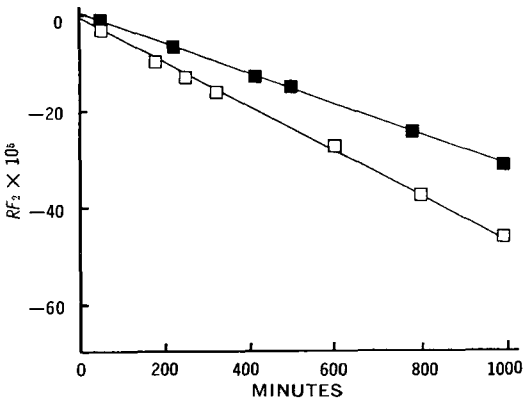

Figure 7—*Physical model treatment of release data of cholesterol in Fig. 6 by the linear Eq. 1. Key:* □, *15%; and* ■, *30%. The solid curve is the linear regression line.*

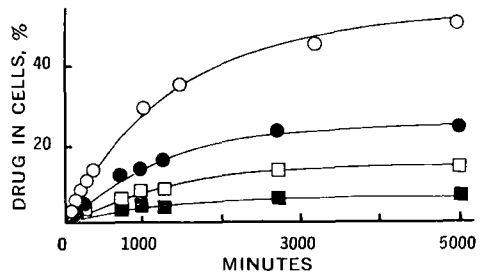

Figure 8—*Uptake of β-sitosterol at 26° and various fetal bovine serum levels. Key:* ○, *0%;* ●, *5%;* □, *15%; and* ■, *30%. The experimental data points are compared against the best theoretical fit of the model using the phenomenological constants in Table III.*

[2] H. Ando, N. F. H. Ho, W. I. Higuchi, J. S. Turi, and C. Shipman, Jr., to be published.

Table IV—Permeability Coefficients for Cholesterol, Desmosterol, and β-Sitosterol at 26° Calculated from Nonlinear Regression Analysis and Partition Coefficients at Equilibrium[a]

Fetal Bovine Serum, %	Effective Permeability Coefficient, cm/sec × 10^7			Effective Partition Coefficient		
	Desmosterol	Cholesterol	β-Sitosterol	Desmosterol	Cholesterol	β-Sitosterol
0	13.00	4.44	2.35	750	230	150
5	9.25	2.69	1.27	270	155	45
15	4.18	1.27	0.74	100	60	23
30	2.99	0.88	0.44	51	34	12

[a] Intrinsic permeability and partition coefficients can be calculated from Eqs. 5 and 6.

whether there is adsorption of desmosterol to the nonserum components of the external medium.

Uptake and Release of Cholesterol-4-^{14}C—Figure 6 shows the decrease in the percent of cholesterol in the cells with time at 26° at 15 and 30% serum concentrations. These cells were loaded by the static method. The treatment of the data by the model is shown in Fig. 7.

The results of the kinetics of cholesterol uptake were thoroughly described and reported elsewhere (2). However, the effective permeability and partition coefficients from the uptake studies at various serum levels are included in Table II with these coefficients determined from the release studies for comparison. As can be seen, the results are similar to those obtained for desmosterol in that the effective permeability and partition coefficients decreased as the serum concentration increased.

Uptake of β-Sitosterol-4-^{14}C—Figure 8 shows the β-sitosterol uptake data at four fetal bovine serum levels at a constant β-sitosterol concentration. As seen previously with cholesterol and desmosterol, β-sitosterol showed a decreased rate of uptake as the serum level increased. The uptake function *versus* time curves in Fig. 9 show very good agreement with the model, while Fig. 10 illustrates the nonlinear regression fit for the uptake of β-sitosterol at 15 and 30% fetal bovine serum. Figure 11 shows the plot of P_{30}/P_e and K_{30}/K_e *versus* percent of serum and is another indication of the fit of the theoretical model. Their absolute values are found in Table III.

General Discussion of Sterols—The experimental data and their interpretative analyses on the passive transport of the three sterols into and out of Burkitt cells in culture suspension demonstrate that the experimental methods employed are quantitative and that physically meaningful results can be obtained by the physical model approach. The evidence strongly supports that the model described in the *Theoretical* section is highly appropriate.

With regard to the other physical models reported earlier (1), Model 1 is inappropriate since it does not account for sterol–serum binding in the external media. Models 3, 4, and 5 do not apply because of the lack of evidence of plasma membrane–sterol binding. The simultaneous transport of two mutually independent solutes of Models 6 and 7 is not applicable here. Model 8 cannot satisfactorily treat the data because the uptake or release function *versus* time plots did not show a biexponential pattern, which would strongly indicate that not only the plasma membrane but also the membranes of the cytoplasmic bodies are rate-determining barriers in the cell transport of the sterols.

In summarizing this study, it is interesting to note the trends in the physical transport coefficients among the sterols (Table IV). At all serum levels, the partition and permeability coefficients increased with increasing polarity of the sterols, *i.e.*, K_e (desmosterol) > K_e (cholesterol) > K_e (β-sitosterol) and P_e (desmosterol) > P_e (cholesterol) > P_e (β-sitosterol). Similar observations were qualitatively reported (3) on uptake studies of cholesterol and β-sitosterol using L-cell mouse fibroblasts. The sterol–fetal bovine serum adsorption constant is about the same among the sterols (K_b = 15.7–18.8).

To assess the differences in the molecular structure of these sterols with regard to their transport into and out of Burkitt cells, the ratio of the permeability coefficients or the partition coefficients, using cholesterol as the reference sterol and the values at the zero percent serum level, may be a useful evaluating parameter. This technique was used in obtaining the incremental partition coefficient per CH_2 group in a *n*-alkyl chain and other chemical substituents for the buccal membrane in humans (4, 5) and the rat intestinal tract (6).

Therefore, as shown in Table V, the double bond as in desmosterol resulted in a threefold increase in the partition and permeability coefficients of cholesterol, whereas the addition of an ethyl group on the 24-position of cholesterol reduced the partition and

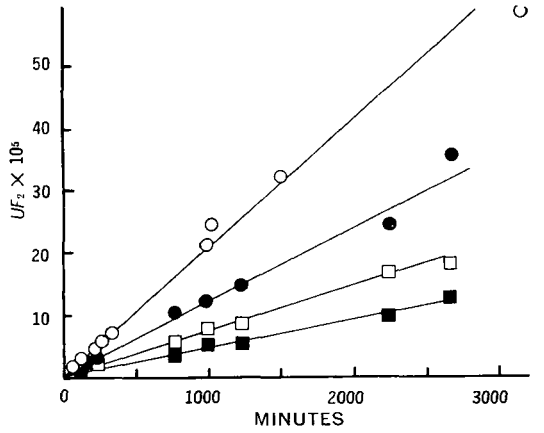

Figure 9—*Physical model treatment of uptake kinetics of β-sitosterol and various fetal bovine serum levels by the linear predictions of Eq. 1. Key: ○, 0%; ●, 5%; □, 15%; and ■, 30%.*

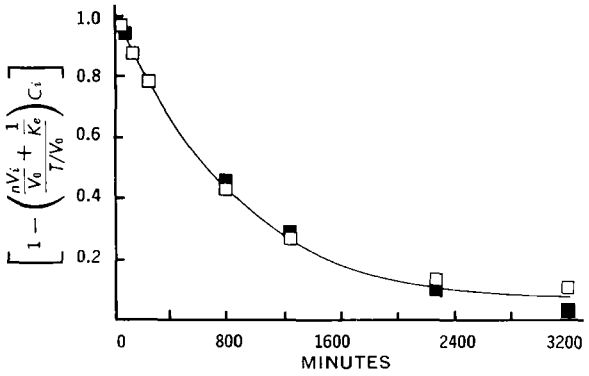

Figure 10—*Nonlinear regression analysis plot of the uptake of β-sitosterol at two fetal bovine serum levels. Key: □, 15%; and ■, 30%. Points are experimental data, and the solid curve is the theoretical fit using the constants in Table III.*

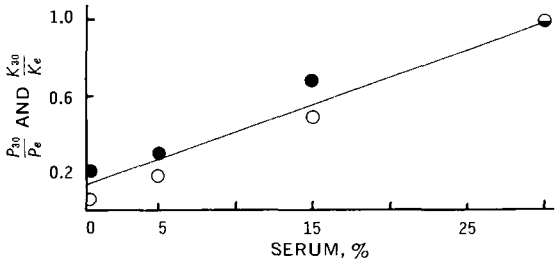

Figure 11—*Normalized plot of the effective permeability and partition coefficients of β-sitosterol as a function of fetal bovine serum concentration. Key: ●, P_{30}/P_e; and ○, K_{30}/K_e.*

Table V—Incremental Constant Evaluating Effect of Molecular Modification of Side Chain of Cholesterol on Permeability and Partition Coefficients of Desmosterol and β-Sitosterol

Parameter	Incremental Constant	
	Desmosterol	β-Sitosterol
$K_{sterol}/K_{cholesterol}$	3.26	0.65
$P_{sterol}/P_{cholesterol}$	3.0	0.53

permeability coefficients by a factor of two. The interpretation of these molecular modifications of cholesterol is unclear. It is apparent that the expectation of increased partition coefficient with decreasing polarity of the solute is not found here.

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Systems Approach to Study of Solute Transport across Membranes Using Suspension Cultures of Mammalian Cells V: Uptake and Release Kinetics of Cardiac Glycosides by Burkitt Lymphoma Cells

J. S. TURI *ˣ, N. F. H. HO ‡, W. I. HIGUCHI ‡, and C. SHIPMAN, Jr. §

Abstract □ Mass transport studies with three cardiac glycosides in the Burkitt lymphoma cell system have provided significant examples of the factorization and quantification of the influences of serum–drug binding, membrane–drug binding, cell interior binding, and intrinsic membrane permeability upon the uptake and release kinetics of drugs in living cell systems. All of the data from the glycosides are in agreement with the general physical model involving the rapid equilibration of the solute within the cell after permeation through the rate-determining plasma membrane barrier. The transport of digitoxin was influenced by membrane and serum binding and that of digoxin was influenced by membrane binding. There was no binding of ouabain to the plasma membrane and serum. The variables in the uptake and release kinetic studies at pH 7.3 included the use of viable and heat-inactivated cells, fetal bovine serum levels, and temperature.

Keyphrases □ Membrane diffusion—systems approach to drug transport, uptake and release of cardiac glycosides by Burkitt lymphoma cells, influence of serum–drug binding, membrane–drug binding, cell interior binding, and intrinsic membrane permeability □ Drug transport—uptake and release kinetics, cardiac glycosides, Burkitt lymphoma cells, influence of serum–drug binding, membrane–drug binding, cell interior binding, and intrinsic membrane permeability □ Mammalian cells, suspension culture—uptake and release of cardiac glycosides, systems approach to study of drug transport across membranes □ Cardiac glycosides—kinetics of uptake and release by Burkitt lymphoma cells, systems approach to drug transport

The lack of a mechanistic and quantitative understanding of the kinetics of drug transport to the receptor site has impeded the quantitative approach to drug design. Nearly all previous studies in the field have been of a rather descriptive nature; it has been difficult to separate, for example, the driving force factors from the kinetic factors in describing the transport and the bioavailability of drugs at the site of action from a physical–chemical standpoint.

Existing evidences indicate that one cannot always rely solely on oil–water partition coefficients, blood levels of drugs, or rates of intestinal transport as true indications of drug availability at the site of drug action. It is believed that the developed physical models and experimental techniques will give more insight into drug availability, especially at the receptor site. The concepts put forth here can be valuable in assessing molecular modifications of drugs. This study illustrates that collaborative research among pharmacokineticists, medicinal chemists, and experts in mass transport across biological membranes can be very valuable in assessing drug availability.

This paper describes the mass transport of ouabain, digoxin, and digitoxin in suspension cultures of an established cell line. This study evolved from the development of various biophysical models (1, 2) and the accompanying experimental systems and techniques. Its significance lies not only in the important implication it may have on the understanding of the

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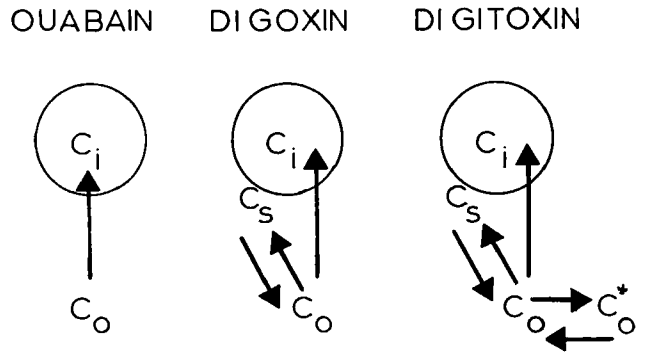

Figure 1—*Schematic physical model of the uptake transport of the cardiac glycosides. The term C_0 is the concentration of drug outside of the cell, C_i is the concentration inside of the cell, C_s is the concentration adsorbed on the plasma membrane surface, and C_0* is the concentration of serum-bound drug.*

pharmacokinetics of the cardiac glycosides in humans but also, from the standpoint of methodology, in providing examples of the factorization and quantification of the influences of serum–drug binding, membrane–drug binding, cell interior binding, and the intrinsic membrane permeability upon the uptake and release kinetics of drugs in living cell systems.

EXPERIMENTAL

Materials and Methods—The materials, methods, and procedures in the cultivation and preparation of Burkitt lymphoma cells were described previously (3). The uptake and release kinetic experiments of radiolabeled ouabain-^{3}H, digoxin-^{3}H, and digitoxin-^{3}H cardiac glycosides[1] were conducted in a manner identical to that employed previously for cholesterol, desmosterol, and β-sitosterol (3, 4).

In the uptake studies, the drug was added to 100-ml water-jacketed spinner flasks[2] containing about 4×10^8 viable cells in 100 ml of culture medium at pH 7.3, under isoosmotic and sterile conditions. The suspending medium contained modified McCoys 5A, basal medium Eagle, balanced salt solution, penicillin G (50 units/ml) and amphotericin B (1 μg/100 ml). The variables included fetal bovine serum concentrations, drug concentrations, and temperature.

To follow changes in drug concentration with time, 5-ml samples

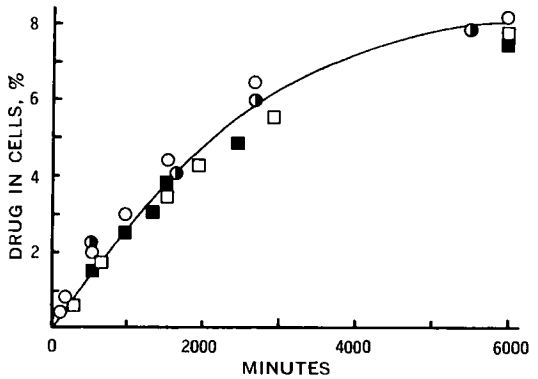

Figure 2—*Uptake of ouabain at 26° by viable and nonviable Burkitt cells at various fetal bovine serum concentrations. The solid curve represents the best theoretical fit of the model by nonlinear regression analysis. Key: ○, 0%; □, 15%; ■, 30%; and ◑, 0% (heat-inactivated cells).*

were centrifuged in thrombocytocrit tubes at 5000×g for 10 min and both the cell and extracellular fractions were assayed by liquid scintillation counting. Total cell numbers in the samples were determined[3].

To study the release kinetics, the static equilibration technique was used to load the cells with the radiolabeled drug. Viable cells in culture suspension were allowed to mix with the drug at 26° for at least 48 hr. Thereafter, the equilibrated cells were washed and resuspended in fresh medium and the release studies followed the sampling procedure for the uptake studies.

Uptake and release kinetic studies were performed at 26, 30, and 33°. Although somewhat removed from the physiological temperature of 37°, these temperatures were chosen to prevent cell division during the experiments and yet sustain a level of metabolic activity.

In some instances, nonviable cells were utilized. These were cells in which the cellular enzymes were inactivated by heat at 50° for 30 min. Viability was assessed by the erythrocin B dye exclusion method. When viable cells were used, at least 90% of the cell population remained viable after 24 hr and 50% was viable after 48 hr. As indicated by the particle counter and multichannel pulse height analyzer, the cell population and the quite narrow size distribution at the beginning and end of the experiments were essentially superimposable.

The methods and procedures of these kinds of experiments have already been shown to be precise, accurate, and reproducible. There was less than 2% drug entrapment in the centrifuged cell plug, well within the experimental error. These experimental techniques are highly quantitative when the transport rate is essentially membrane controlled (*i.e.*, low permeability coefficient), as was shown in the sterol studies and in this present study. Refined techniques are being developed for situations when the transport rate becomes less membrane controlled and more controlled by the stagnant aqueous layer about the cell.

To evaluate drug–serum binding, the experimental dialysis setup used two spinner flasks of 150-ml capacity each and a dialysis membrane placed at the flanged connection. The surface area of the exposed membrane was 5.07 cm^2 and had a pore size of 24 Å.

Uptake and Release Kinetics of Ouabain—*Theoretical*—The kinetics of cell uptake and release of ouabain can be explained by Model 1, the simplest of the various models previously derived (2). Figure 1 is a schematic illustration of the passive transport of unbound drug molecules across the rate-determining plasma membrane with rapid distribution of the drug in the heterogeneous cell interior. There is no binding of the drug to the surface of the cell nor to the serum and other components comprising the external medium of the culture suspension.

The uptake transport function of this model, UF_1, is:

$$UF_1 = -\frac{a}{180B} \ln\left[1 - \frac{B}{A}C_i\right] = Pt \qquad \text{(Eq. 1)}$$

$$A = T/V_0 \qquad \text{(Eq. 2)}$$

$$B = \frac{nV_i}{V_0} + \frac{1}{K} \qquad \text{(Eq. 3)}$$

where a is the radius of the cell, n is the number of cells, V_i is the volume of a cell, V_0 is the volume of the external aqueous phase, T is the total amount of drug in the system, C_i is the concentration of drug in a cell, K is the intrinsic partition coefficient, P is the intrinsic permeability coefficient of the plasma membrane, and t is time. The initial boundary condition is $C_i(0) = 0$.

A plot of UF_1 *versus* t in minutes is linear with a slope equal to P in units of centimeters per second. The permeability coefficient can also be found by nonlinear regression analysis of the nonlinear form of Eq. 1. In both cases, the intrinsic partition coefficient must be first determined.

For initial rates, Eq. 1 reduces to:

$$C_i = \frac{3PT}{aV_0}t \qquad \text{(Eq. 4)}$$

[1] New England Nuclear Corp., Boston, Mass.
[2] Bellco Glass, Inc., Vineland, N.J.
[3] Coulter counter model A.

Table I—Permeability and Partition Coefficients of Ouabain at Various Temperatures and Fetal Bovine Serum Concentrations from Uptake Kinetics[a]

Fetal Bovine Serum, %	Intrinsic Partition Coefficient K			Intrinsic Permeability Coefficient P, cm/sec $\times 10^8$		
	26°	30°	33°	26°	30°	33°
0	12.2 ± 0.8	16.0 ± 1.0	20.7 ± 3.1	2.08 ± 0.43	4.18 ± 0.1	7.69 ± 0.84
0[b]	14.0	—	—	2.38	—	—
5	—	—	16.5 ± 0.7	—	—	4.78 ± 0.03
15	13.0 ± 1.4	14.0 ± 1.7	15.0 ± 2.8	1.73 ± 0.11	2.76 ± 0.12	4.38 ± 0.04
15[c]	—	16.0 ± 1.4	—	—	2.49 ± 0.1	—
30	11.3 ± 1.5	—	16.0 ± 1.0	1.58 ± 1.53	—	4.35 ± 0.14

[a] The values of K were found from the equilibrium data according to Eq. 5, and the values of P were found from nonlinear regression analysis of the model. [b] Heat-inactivated Burkitt lymphoma cells used here; viable cells in culture suspension were utilized in all other studies. [c] Partition and permeability coefficients were calculated from release kinetics from viable cells at 30° using 15% fetal bovine serum.

Thus, the intrinsic permeability coefficient can be readily obtained from the initial slope of a C_i *versus* t plot.

The intrinsic partition coefficient can be experimentally found from the equilibrium condition, *i.e.*, when $(dC_i/dt)_{t=\infty} = 0$; thus:

$$K = \frac{C_{i,\infty}}{C_{0,\infty}} \qquad \text{(Eq. 5)}$$

where $C_{0,\infty}$ and $C_{i,\infty}$ are the concentrations of drug in the external medium and cell at equilibrium, respectively.

The release transport function, RF_1, is expressed by:

$$RF_1 = \frac{a}{180B} \ln\left[\frac{BC_i - A}{BC_i(0) - A}\right] = -Pt \qquad \text{(Eq. 6)}$$

The initial boundary condition is $C_i = C_i(0)$.

Analysis of Results—Figure 2 shows the uptake kinetics of ouabain by viable cells at three fetal bovine serum concentrations at 26° and by heat-inactivated cells with no fetal bovine serum. The partition coefficients are found from the plateau region of these plots. As illustrated in Fig. 3, the application of theory (Eq. 1) to the data yields the predicted linear relationship of the uptake transport function, UF_1, *versus* time.

The permeability coefficients, found by the best theoretical fit of the data in Fig. 2 and linear regression of the plot in Fig. 3, were not significantly different from each other. Table I summarizes the phenomenological transport constants of ouabain with respect to the Burkitt cell under varying conditions of temperature, cell viability, and fetal bovine serum concentration.

The following observations and comments can be made:

1. At any specific temperature, there are no significant differences between the permeability coefficient and partition coefficient themselves at the 15 and 30% fetal bovine serum levels. This is an indication that there is no occurrence of ouabain–serum binding, which will be further corroborated by dialysis experiments in the next section. However, these coefficients appear to be larger at 0% fetal bovine serum with respect to those at 15 and 30% fetal bovine serum, and the deviation tends to increase with increasing temperature. A similar observation was noted with the transport of cholesterol, desmosterol, and β-sitosterol at 26° with the Burkitt cells (4) and unpublished findings of digitoxin with red blood cells at 0% fetal bovine serum in these laboratories. There apparently is some degree of alteration of the plasma membrane in the absence of serum and the change is more apparent at higher temperatures.

2. There is a temperature dependence in the permeability coefficients. After applying the Arrhenius-type relationship of the permeability coefficients to temperature, the heat of activation is found to be about 24 kcal/mole. The temperature dependence of the partition coefficients is very small.

Evaluation of Serum Binding by Dialysis—According to the setup of the dialysis experiments, the rate of dialysis is:

$$\frac{dC_r}{dt} = \frac{A_m P_{em}}{V_r}(C_d - C_r) \qquad \text{(Eq. 7)}$$

and, with $C_r(0) = 0$, the solution is described in the following manner:

$$DF = -\frac{V_r}{120A_m} \ln\left(1 - \frac{2C_r}{T}\right) = P_{em}t \qquad \text{(Eq. 8)}$$

where C_d and C_r are the total concentrations of the drug in the donor and receiving compartments of equal volume, respectively; V_r is the volume of solution of the receiving compartment; A_m is the effective surface area of the dialysis membrane; P_{em} is the effective permeability coefficient of the membrane; t is the time; and DF is the dialysis function.

A plot of DF *versus* t in minutes gives a straight line with a slope of P_{em} in centimeters per second. The long times required to reach equilibrium are due to both the small effective surface area-to-volume ratio of 1:40 and also the pore size of the dialysis membrane of 24 Å diameter as compared to the molecular size of the cardiac glycosides.

The results in Fig. 4 clearly show that there is no serum binding with ouabain as well as digoxin. The dialysis profiles are identical between both of these cardiac glycosides at zero and 30% fetal bovine serum at 26° and pH 7.3. However, digitoxin does adsorb to

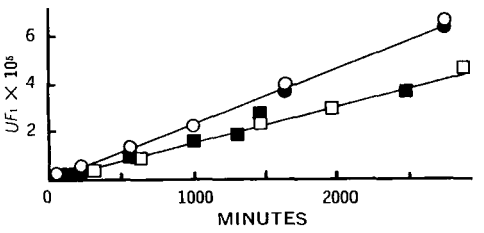

Figure 3—*Profiles of uptake kinetics of ouabain at various fetal bovine serum concentrations at 26° according to the linear predictions of Eq. 1. Solid lines are least-squares lines. Key: ○, 0%; □, 15%; ■, 30%; and ●, 0% (heat-inactivated cells). Faster uptake kinetics at 0% fetal bovine serum are attributed to altered integrity of the plasma membrane when no serum is present.*

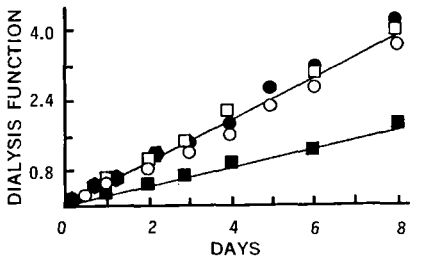

Figure 4—*Kinetics of dialysis of ouabain, digoxin, and digitoxin in the presence of fetal bovine serum at 26°. The dialysis function is defined by Eq. 8. Key: □, digitoxin–0%; ■, digitoxin–30%; ○, digoxin–0%; ●, digoxin–30%; and ●, ouabain–30%. Ouabain with no fetal bovine serum was similar to ouabain–30% fetal bovine serum. Taken together, the data show that ouabain and digoxin do not bind to fetal bovine serum while digitoxin does.*

Table II—Evaluation of Plasma Membrane–Digoxin Equilibrium Binding Constant, K_{bm}, under Varying Conditions of Initial Drug Concentrations, Serum Levels, and Temperature from Uptake Kinetic Studies[a]

Temperature	Fetal Bovine Serum, %	Initial Amount Adsorbed per 10^6 Cells, dpm	Total Amount of Drug in 5 ml Suspension, dpm	K_{bm}
26°	0	210	3.28×10^5	1.82
	15	228	3.22×10^5	1.96
33°	0	295	6.98×10^5	1.20
	5	290	6.57×10^5	1.24
	15	285	6.25×10^5	1.28
	30	294	7.05×10^5	1.17

[a] The K_{bm} is 1.89 at 26° and 1.22 at 33°.

fetal bovine serum. Similar findings were reported previously (1).

Uptake and Release Kinetics of Digoxin—*Theoretical*—The kinetics of cell uptake and release of digoxin can be appropriately described by Model 4 (2), in which the unbound drug permeates passively across the plasma membrane and distributes rapidly in the heterogeneous interior of the cell (Fig. 1). However, there is an instantaneous, reversible binding of the drug molecules to the external side of the plasma membrane. The membrane-bound drug is in equilibrium with the free drug in the external medium; there is no significant binding of the drug to the serum in the medium.

According to the model, the uptake transport function is given by:

$$UF_4 = -\frac{a(1 + K_{bm}E)}{180G} \ln\left[\frac{F - GC_c}{F - GC_c(0)}\right] = Pt \quad \text{(Eq. 9)}$$

$$E = \frac{nV_i}{V_0} \quad \text{(Eq. 10)}$$

$$F = \left(1 + \frac{K_{bm}}{K}\right)\frac{T}{V_0} \quad \text{(Eq. 11)}$$

$$G = \left(1 + \frac{K_{bm}}{K}\right)\frac{nV_i}{V_0} + \frac{1}{K} \quad \text{(Eq. 12)}$$

where K_{bm} is the plasma membrane–drug equilibrium adsorption constant; K and P are the intrinsic partition and permeability coefficients, respectively; C_c is the concentration of drug in the cell, which includes the drug within the cell and the drug adsorbed to the external surface of the plasma membrane; and $C_c(0)$ is the cell concentration at zero time and is equal to the initial surface-adsorbed drug concentration. It follows that the intrinsic permeability coefficient can be obtained from the application of the linear or nonlinear form of Eq. 9.

The membrane–drug equilibrium adsorption constant may be found by:

$$K_{bm} = \frac{nA_{s,0}}{(V_i/V_0)(T - nA_{s,0})} \quad \text{(Eq. 13)}$$

The $A_{s,0}$ is the amount of drug bound to the plasma membrane of a cell at zero time and is determined by extrapolation of a A_c (amount of drug per cell) *versus* t plot to $t = 0$.

The experimental partition coefficient, K_{ex}, is determined from the equilibrium transport conditions; thus:

$$K_{ex} = \frac{C_{c,\infty}}{C_{0,\infty}} = \frac{C_{i,\infty} + K_{bm}C_{0,\infty}}{C_{0,\infty}} \quad \text{(Eq. 14)}$$

whereupon, with Eq. 5:

$$K_{ex} = K + K_{bm} \quad \text{(Eq. 15)}$$

As one observes, the intrinsic partition coefficient K is readily calculated once K_{ex} and K_{bm} are found from the experimental data.

The release transport function, RF_4, is described by:

$$RF_4 = \frac{a(1 + K_{bm}E)}{180G} \ln\left[\frac{GC_c - F}{GC_c(0) - F}\right] = -Pt \quad \text{(Eq. 16)}$$

The model described here for digoxin is not apparently distinguishable from another model (Model 5 in Ref. 2), in which not only the unbound drug but also the external membrane-bound drug permeates across the plasma membrane into the cell. The mathematical expressions, such as Eqs. 9 and 16, are identical in form for both models. Since the authors cannot readily see any accessible experimental means to distinguish Model 5 from this Model 4 and, moreover, believe that the permeability coefficient of the unbound drug is much greater than the permeability coefficient of the membrane-bound drug, Model 4 is considered appropriate for describing the cell transport of digoxin.

Analysis of Results—The profiles of the percent of digoxin taken up by the cells with time in Fig. 5 illustrates several interesting points. The identical uptake profiles of the 5, 15, and 30% fetal bovine serum cases at 33° demonstrate that digoxin does not appreciably bind to the serum. This finding is in agreement with the conclusions of the dialysis experiments. Consistent with the results of the ouabain studies, the uptake rate is faster when there is

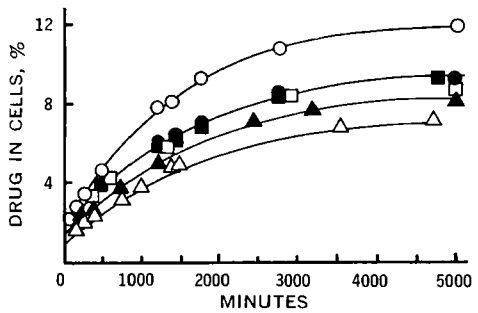

Figure 5—*Uptake rate of digoxin at 26 and 33° by viable Burkitt cells at various fetal bovine serum levels. Solid curves represent the best theoretical fit of the model by nonlinear regression analysis. Key: △, 0% (26°); ▲, 15% (26°); ○, 0% (33°); ●, 5% (33°); □, 15% (33°); and ■, 30% (33°).*

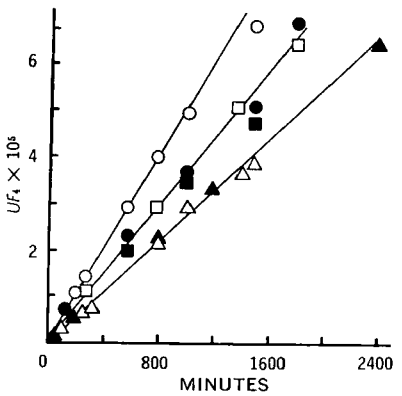

Figure 6—*Profiles of uptake kinetics of digoxin at 26 and 33° and various fetal bovine serum concentrations according to the linear predictions of Eq. 9. Key: △, 0% (26°); ▲, 15% (26°); ○, 0% (33°); ●, 5% (33°); □, 15% (33°); and ■, 30% (33°).*

Table III—Permeability and Partition Coefficients of Digoxin at Various Temperatures and Fetal Bovine Serum Concentrations from Uptake and Release Kinetic Studies[a]

Temperature	Fetal Bovine Serum, %	Intrinsic Partition Coefficient K		Intrinsic Permeability Coefficient P, cm/sec $\times 10^8$	
		Uptake	Release	Uptake	Release
26°	0	11.2 ± 2.1	—	2.93 ± 0.67	—
	15	13.0 ± 2.6	—	2.80 ± 0.56	—
33°	0	17.8 ± 2.7	18.8 ± 2.3	5.01 ± 1.1	3.52 ± 1.8
	5	13.8 ± 2.2	—	3.51 ± 0.71	—
	15	14.7 ± 1.9	17.8 ± 1.7	3.30 ± 0.62	3.75 ± 1.1
	30	12.8 ± 1.3	16.8 ± 1.5	3.21 ± 0.51	3.74 ± 0.8

[a] Values of K and P are average ± SD. The intrinsic partition coefficients were calculated from the equilibrium data according to Eqs. 14 and 15. The intrinsic permeability coefficients were found by nonlinear regression analysis of the model.

no serum present. The uptake rates increase with increasing temperature.

In Fig. 5 there are significant intercepts at zero time, indicative of an initial rapid adsorption of digoxin to the external surface of the plasma membrane. The results of further treatment of these data by the physical model are given in Table II. The plasma membrane–digoxin equilibrium adsorption constant, K_{bm}, is 1.87 at 26° and 1.22 at 33°. These constants are independent of the initial concentration of digoxin used as well as the concentration of fetal bovine serum in the external medium.

In Figs. 6 and 7, the linearity of the curves derived from the treatment of the data for uptake by Eq. 9 and for release by Eq. 16 supports the physical model described in the *Theoretical* section. Examples of the fit of the model to the data by nonlinear regression analysis are shown in Fig. 5.

The intrinsic permeability and partition coefficients from uptake and release studies at 26 and 33° and various fetal bovine serum concentrations are found in Table III. There is a tendency for the intrinsic partition coefficients at 33° to be slightly higher from the release experiments as compared to those from the uptake studies. The reason for this may be attributed to cellular changes occurring over prolonged periods in the release kinetic studies (the total time in loading the cells with digoxin and in the release kinetic portion). However, even when a lower partition coefficient is used along with a permeability coefficient calculated from an uptake experiment, the deviation of the theoretical calculations from the experimental points does not appear to be significant.

Uptake and Release Kinetics of Digitoxin—*Theoretical*—Figure 1 shows the model for the passive transport of unbound digitoxin across the plasma membrane, with rapid distribution within the heterogeneous cell interior as well as the instantaneous reversible linear adsorption of the drug to the external cell surface and the reversible linear binding with the serum in the external media. According to Ref. 2, this system corresponds to the combination of Models 2 and 4.

The mathematical description of this physical model is expressed by the following uptake transport function:

$$UF_{2,4} = -\frac{a[1 + (K_{bm}K_eE/K)]}{180G'} \ln\left[\frac{F - G'C_c}{F - G'C_c(0)}\right] = P_e t \quad \text{(Eq. 17)}$$

$$P_e = \frac{P}{1 + K_b(S)} \quad \text{(Eq. 18)}$$

$$K_e = \frac{K}{1 + K_b(S)} \quad \text{(Eq. 19)}$$

$$E = \frac{nV_i}{V_0} \quad \text{(Eq. 20)}$$

$$F = \left(1 + \frac{K_{bm}}{K}\right)\frac{T}{V_0} \quad \text{(Eq. 21)}$$

$$G' = \left(1 + \frac{K_{bm}}{K}\right)\frac{nV_i}{V_0} + \frac{1}{K_e} \quad \text{(Eq. 22)}$$

where P_e and K_e are the effective permeability and partition coefficients relating to the intrinsic permeability, P, and partition, K, coefficients and serum–drug binding; K_b is the equilibrium serum–drug binding constant; and (S) is the serum concentration in terms of the fraction of the volume of serum added in the total volume of the suspension system.

Before a plot of the uptake function *versus* time in minutes can be made to calculate the intrinsic permeability coefficient in units of centimeters per second from Eq. 17, the constants K, K_b, and K_{bm} must be found. This is readily accomplished by utilizing the data from a family of C_c *versus* t plots at different serum concentrations. The experimental partition coefficient, K_{ex}, is found from the equilibrium situation:

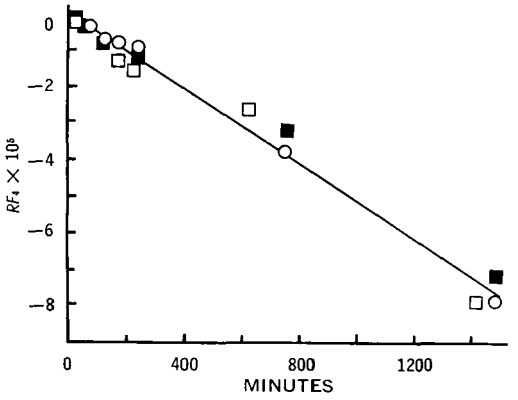

Figure 7—*Profiles of release kinetics of digoxin at 33° and various fetal bovine serum levels according to the linear predictions of Eq. 16. The solid line is derived from least-squares analysis. Key: ○, 0%; □, 15%; and ■, 30%.*

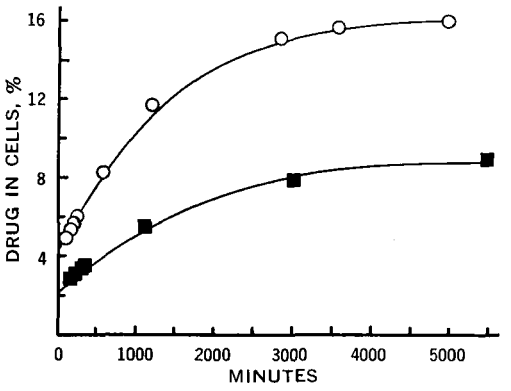

Figure 8—*Uptake rate of digitoxin by viable cells at various fetal bovine serum concentrations at 30°. Solid curves represent the best theoretical fit of the model by nonlinear regression analysis. Key: ○, 0%; and ■, 30%. The positive intercept at initial time is evidence of membrane binding. The lower intercept for the 30% fetal bovine serum case is due to the influence of serum binding and is consistent with the theory (Eqs. 23 and 24).*

Table IV—Effective and Intrinsic Plasma Membrane–Digitoxin Binding Constants at Various Serum Concentrations and Temperatures

Fetal Bovine Serum, %	Effective Membrane Binding Constant[a]			Intrinsic Membrane Binding Constant K_{bm}			Range of Initial Concentration of Digitoxin per 5 ml, dpm
	26°	30°	33°	26°	30°	33°	
0	5.94 ± 0.47	5.81 ± 0.65	5.22 ± 0.67	5.94	5.81	5.22	2×10^5–8.1×10^5
5	—	3.86 ± 0.06	3.90 ± 0.3	—	6.27	6.49	6×10^4–3.5×10^5
15	—	2.76 ± 0.27	2.39 ± 0.09	—	7.94	7.17	8.4×10^4–1.6×10^6
30	2.49 ± 0.31	1.69 ± 0.13	1.96 ± 0.24	—	8.02	9.80	2.5×10^5–8.1×10^5

[a] Values represent the averages ± *SD*. The effective plasma membrane binding constant is defined as $K_{bm}/[1 + K_b(S)]$, where K_{bm} is the intrinsic membrane–drug binding constant, K_b is the serum–drug binding constant, and (S) is the fetal bovine serum concentration. The K_b is 12.5 at 30° and 13.3 at 33°.

Table V—Effective Partition and Permeability Coefficients of Digitoxin at Various Fetal Bovine Serum Concentrations and Temperatures from Uptake Kinetic Studies[a]

Fetal Bovine Serum, %	Effective Partition Coefficients K_e			Effective Permeability Coefficients P_e, cm/sec × 10^8		
	26°	30°	33°	26°	30°	33°
0	28.6 ± 0.9	43.7 ± 3.2	52.0 ± 3.5	10.07 ± 2.48	19.8 ± 1.2	23.0 ± 1.7
5	—	25.0 ± 2.8	35.3 ± 4.9	—	13.0 ± 2.1	15.3 ± 1.1
15	—	14.3 ± 3.2	22.5 ± 2.1	—	6.99 ± 0.88	12.2 ± 3.0
30	10.7 ± 2.1	8.0 ± 1.4	12.0 ± 1.4	2.27 ± 0.33	4.05 ± 0.21	4.91 ± 0.14

[a] All values represent averages ± *SD*. The K_e is obtained from the equilibrium data and the P_e from nonlinear regression analysis of the model; K_e and P_e are defined by Eqs. 18 and 19.

$$K_{ex} = \frac{C_{c,\infty}}{C_{0,\infty}} = \frac{K + K_{bm}}{1 + K_b(S)} \qquad \text{(Eq. 23)}$$

In turn, the membrane–drug binding constant is obtained from the initial condition:

$$K_{bm} = \frac{nA_{s,0}V_0[1 + K_b(S)]}{V_r(T - nA_{s,0})} \qquad \text{(Eq. 24)}$$

The release transport function, $RF_{2,4}$, according to the model is:

$$RF_{2,4} = \frac{a[1 + (K_{bm}K_eE/K)]}{180G'} \ln\left[\frac{G'C_c - F}{G'C_c(0) - F}\right] = -P_e t \qquad \text{(Eq. 25)}$$

Analysis of Results—In Fig. 8, the decrease in the rate of uptake of digitoxin at 30° with increasing concentrations of fetal bovine serum and the percent of the drug associated with the cells at zero time found by extrapolation give evidence that there is binding of the digitoxin not only to the components of the serum but also to the external surface of the plasma membrane[4]. Similar plots are also observed with the uptake experiments at 26 and 33°. The interpretation of serum–digitoxin binding is corroborated by the results of the dialysis experiments described earlier.

The values of the effective equilibrium plasma membrane–digitoxin binding constant at various temperatures and initial concentrations of digitoxin are found in Table IV. The effective membrane–digitoxin binding constant decreases with increasing fetal bovine serum concentrations. The effective membrane binding constant is equal to $K_{bm}/[1 + K_b(S)]$. In Fig. 9, the serum binding constant, K_b, is equal to 12.5 at 30° and 13.3 at 33° and, in turn, the intrinsic membrane binding constant, K_{bm}, can be determined.

It is observed in Table IV that there is no discernible temperature dependence on the K_{bm} at 30 and 33°. Because the calculated K_{bm} is independent of the range of initial concentrations of digitoxin used with respect to a constant concentration of cells, the assumption that the concentration of digitoxin adsorbed is linearly related to the concentration of unbound species in the external fluid is justified. Upon comparing digitoxin with digoxin, the former being more nonpolar than the latter, the propensity of digitoxin to adsorb onto the plasma membrane is about three times greater.

Figure 10 shows the linear relationships of the uptake function of the experimental data at 30° *versus* time at different fetal bovine serum concentrations as predicted by the model. The linear plots of P_{30}/P_e and K_{30}/K_e *versus* percent of fetal bovine serum and the superposition of the points are other indications in support of the model (Fig. 9). The slope is equal to $K_b/(1 + 0.3K_b)$, and the intercept equals $1/(1 + 0.3K_b)$. Accordingly, the K_b is cal-

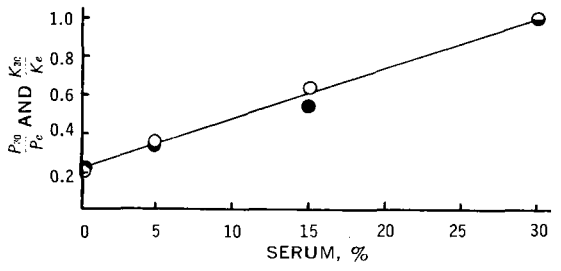

Figure 9—*Normalized plot of the effective permeability and effective partition coefficients of digitoxin as a function of fetal bovine serum concentration. The solid curve represents the least-squares lines. Key:* ●, P_{30}/P_e; *and* ○, K_{30}/K_e.

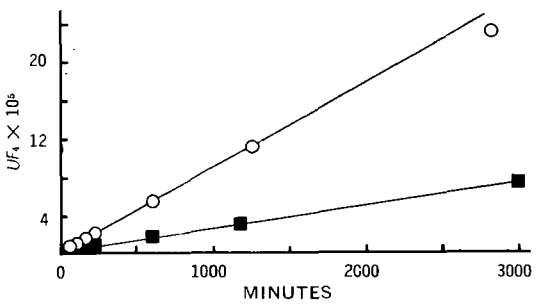

Figure 10—*Profiles of uptake kinetics of digitoxin at 30° according to the linear predictions of Eq. 17. Solid lines are least-squares lines. Key:* ○, *0% fetal bovine serum; and* ■, *30% fetal bovine serum.*

[4] From recent unpublished findings, there appears to be a rapid uptake of digitoxin within the 1st min for which a mechanistic interpretation cannot be made from the kinetic data alone. Taking the uptake and release studies together, it is deemed unlikely that the plasma membrane is not the rate-determining barrier. Autoradiographic studies within this 1-min period are being pursued to examine the distribution of digitoxin on the cell periphery and inside the cell.

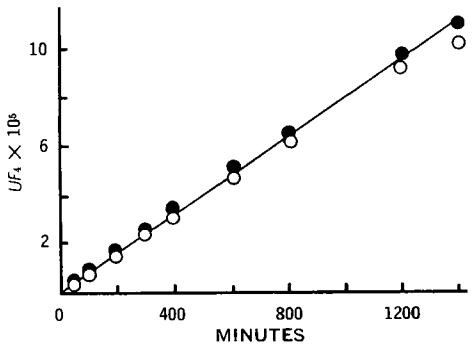

Figure 11—*Uptake kinetics of digitoxin at 30°, showing no effect of antibiotic preservatives in the medium on cell membrane permeability. Key: ●, 15% fetal bovine serum; and ○, 15% fetal bovine serum with penicillin G (50 units/ml) and amphotericin B sulfate (1 μg/100 ml).*

culated to be 12.5, which is equivalent to 92% of the digitoxin being bound reversibly to the fetal bovine serum.

The summary of the phenomenological constants (permeability and partition coefficients) and their relationships to serum levels and temperature are found in Table V.

The addition of penicillin G (50 units/ml) and amphotericin B sulfate (1 μg/100 ml) to the cell suspension to maintain sterility during the transport studies does not seem to have a measurable effect on the uptake kinetics of digitoxin at 30° and 15% fetal bovine serum (Fig. 11). The identical uptake rates by viable and heat-inactivated Burkitt lymphoma cells (Fig. 12) indicate that the transport of digitoxin does not depend upon the viability of the cell and, therefore, is passive.

The change in the concentration of digitoxin in the cell with time in the release kinetic studies and the subsequent data analysis by the release function are shown in Figs. 13 and 14, respectively.

The experimental results of the uptake and release kinetics of digitoxin agree well with the model that accounted for the simultaneous reversible membrane–drug and serum–drug binding across the plasma membrane. This was demonstrated by the linear and nonlinear regression analyses of the data with respect to the physical model. However, additional comments should be made to describe attempts to investigate whether other model variations are also applicable.

The likelihood of irreversible membrane–drug binding, as described by Model 3 (2), is not consistent with the findings that the initial concentration of digitoxin bound to the outer surface of the plasma membrane is proportional to the initial concentration in the external medium (see Table IV). Although the simultaneous passive transport of both the membrane-bound and unbound drug species across the plasma membrane is a possibility, as described by Model 5 (2), this mechanism is deemed less likely on the evidences presented here. Furthermore, if the permeability of the membrane-bound species is smaller than that of the unbound

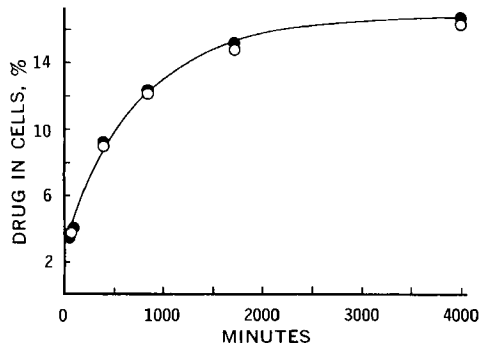

Figure 12—*Uptake of digitoxin with time at 33° and 15% fetal bovine serum. Key: ○, uptake by viable cells; and ●, uptake by heat-inactivated cells. The solid curve is the best-fit curve by nonlinear regression analysis of the model.*

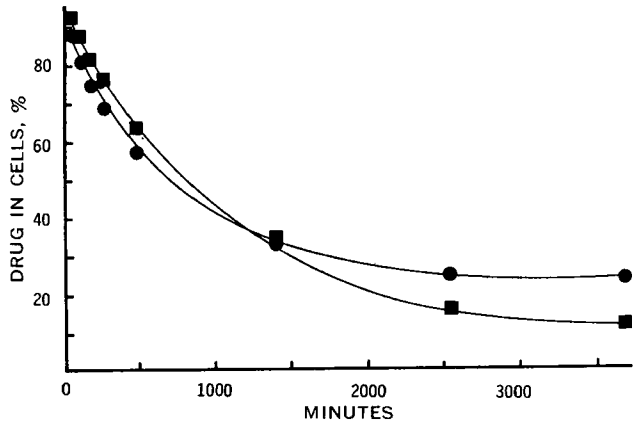

Figure 13—*Release rates of digitoxin from viable cells at 33° into the surrounding medium containing 5% (●) and 30% (■) fetal bovine serum. The cells were initially equilibrated with digitoxin using the static procedure. Solid curves represent the theoretical fit of the model by nonlinear regression analysis.*

species, this model effectively reduces to the model involving the transport of unbound species.

Simultaneous Uptake Kinetics of Ouabain and Digitoxin—*Theoretical*—This model considers the simultaneous uptake of two radiolabeled solutes, such as digitoxin-^{3}H and ouabain-^{3}H, by cells in suspension. It is assumed that both solutes diffuse independently of each other. Let digitoxin be identified as solute *A* and ouabain as solute *B*. As shown previously, digitoxin binds to the plasma membrane and fetal bovine serum while ouabain does not. The cell concentration of both solutes as a function of time is described by the following expression (2):

$$C_{c(A+B)} = (C_{c(A+B),eq} - C_{c,B,eq})e^{-k_B t} + (C_{s,0} - C_{c,A,eq})e^{-k_A t} \quad \text{(Eq. 26)}$$

where:

$$k_A = \frac{3P_{e,A}G'}{a[1 + (K_{bm}K_e E/K)]} \quad \text{(Eq. 27)}$$

$$k_B = \frac{3P_B}{a}\left(\frac{1}{K_B} + \frac{nV_i}{V_0}\right) \quad \text{(Eq. 28)}$$

Analysis of Results—The simultaneous uptake of digitoxin and ouabain at 26° without fetal bovine serum is shown in Fig. 15. Equal concentrations of each cardiac glycoside were added to the cell suspension. The membrane–digitoxin equilibrium constant, K_{bm}, was calculated using the intercept of Fig. 15, the total

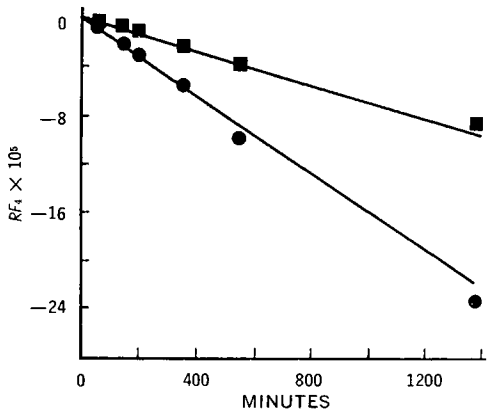

Figure 14—*Profiles of release kinetics of digitoxin at 33° according to the linear predictions of Eq. 25. Solid lines are least-squares lines. Key: ●, 5% fetal bovine serum; and ■, 30% fetal bovine serum.*

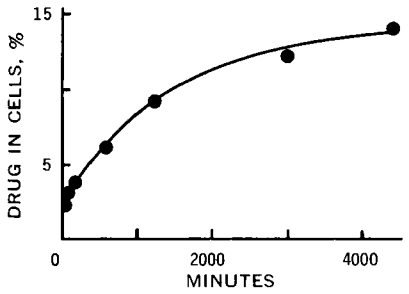

Figure 15—*Simultaneous uptake of digitoxin and ouabain with time by viable cells at 26° in the absence of fetal bovine serum. Experimental points are the sums of both drugs in the cells. The solid curve represents the theoretical predicted curve from the model using the values of the permeability and partition coefficients and other parameters of both drugs from independent experiments. The interpretation is that the transport of digitoxin is independent of that of ouabain and vice versa.*

amount of digitoxin, and the number of cells and was found to be 4.1, which is in reasonable agreement with 5.9 in Table IV. Using the intrinsic partition and permeability coefficients of the glycosides (Tables I and V) and Eq. 26, the theoretical predictions of the $C_{c(A+B)}$ *versus* time plot is in good agreement with the experimental findings. Thus, the passive uptake of digitoxin is not affected by ouabain and vice versa.

DISCUSSION

The kinetic transport data of the cardiac glycosides are consistent with the general physical model involving the rapid equilibration of these solutes within the cell after permeation through the rate-determining plasma membrane barrier. The passive transport of digitoxin is influenced by membrane and serum binding and that of digoxin by membrane binding. There is no binding of ouabain to the plasma membrane and serum.

As reported earlier (3, 4), the sterols (cholesterol, desmosterol, and β-sitosterol) provide complementary examples of the occurrence of only serum binding. In all of these cases the unbound drug molecules appear to be the only kind involved in the penetration of the plasma membrane. Significantly, these studies with the cardiac glycosides and sterols not only provide excellent examples of how each kind of physical interaction, alone and in combination, affect the total transport rate but also demonstrate the method of quantitative factorization of each physical phenomenon and combined interaction from the intrinsic transport rate.

Table VI summarizes the intrinsic phenomenological constants factored out by the analysis of the kinetic data. The intrinsic partition coefficients are direct indications of how much drug actually penetrates the cells. This is an important aspect in the design of drug molecules aside from the interaction of the molecule with the serum and plasma membrane. The intrinsic permeability coefficients indicate the rate at which the drug molecule penetrates the plasma membrane.

Table VI—Summary[a] of Intrinsic Constants of Cardiac Glycosides: Membrane Binding Constant (K_{bm}), Serum Binding Constant (K_b), Partition Coefficient (K), and Permeability Coefficient (P)[a]

Cardiac Glycoside	Temperature	K_{bm}	K_b	K	P, cm/sec × 10^8
Ouabain	26°	0	0	12.2	1.65
	30°	0	0	15.0	2.63
	33°	0	0	15.8	4.51
Digoxin	26°	1.89	0	13.0	2.80
	33°	1.22	0	13.8	3.34
Digitoxin	26°	5.94	—	29.0	10.1
	30°	7.41	12.5	40.0	20.1
	33°	7.82	13.3	59.5	27.4

[a] Values for digitoxin at 26° were taken from uptake studies at 0% fetal bovine serum. All other values were average values from uptake studies only in the presence of fetal bovine serum.

The intrinsic partition and permeability coefficients of ouabain are comparable to those of digoxin, while the coefficients for digitoxin, the most nonpolar among the three cardiac glycosides, are significantly higher. The lack of the hydroxyl group on the 12-position of digoxin giving rise to digitoxin results in significant binding to serum proteins and approximately to a sixfold increase in the membrane binding constant. There is also a threefold increase in the partition coefficient and a 10-fold increase in the permeability coefficient in favor of digitoxin over digoxin with respect to the Burkitt lymphoma cells. Thus, ouabain, digoxin, and digitoxin follow in ascending order of rapidity of their intrinsic transport rates. However, ouabain has the more rapid time for the onset and maximum intensity of cardiac activity *in vivo* following an intravenous digitalizing dose than does digoxin and, in turn, digoxin acts more rapidly than digitoxin (6). Also, the biological half-life is longest with digitoxin, followed by digoxin and then ouabain.

In extrapolating the results of this study with the Burkitt lymphoma cells and fetal bovine serum to the *in vivo* situation, this study supports the hypothesis that the onset and duration of cardiac activity of these glycosides depend upon their relative tendency to bind to serum proteins and nonspecifically to surfaces of cells in the circulatory system because the activity depends upon the availability of unbound drug species for transport across the membranes of capillaries and heart muscle cells. The intrinsic uptake and release kinetics and membrane binding of the glycosides by nonheart muscle cells and tissues will affect the biological half-life. The enterohepatic cycling of digoxin and digitoxin and the biotransformation of digitoxin are other influential, kinetically dimensional factors (7).

In the specific case of digoxin, the rate-determining step for the onset of cardiac activity may be the transport of the unbound digoxin across the capillary membrane and not across the membrane of the heart muscle cell, since the pharmacological action seems to be focused upon a surface interaction on the cell membrane. This is indicated by a recent study (8) correlating the inotropic and chronotropic activities of isolated beating neonatal rat heart cells in a culture system with membrane-bound digoxin[5].

In Figs. 3, 6, 10, 11, and 14, it is sometimes observed that the scatter of the data about the linear regression line increases as the transport of the drug proceeds to equilibrium. This is largely due to the logarithmic nature of the uptake concentration function; a small experimental error in the assay of the drug in the cells leads to a more pronounced deviation in these plots near equilibrium. At equilibrium the function equals zero and is, therefore, undefined. The deviation encountered when linear regression analysis is used disappears when the nonlinear regression technique is employed.

The experimental techniques used here are satisfactory for drugs with low permeability coefficients (5×10^{-5} and smaller) and with relatively high partition coefficients (at least 5) because the sampling time is of the order of 3 min and the centrifugation step takes 10 min. Another limitation is the low partition coefficient, which leads to analytical problems. Circumvention of this analytical constraint might be possible by increasing the cell volume fraction.

The combined usage of the mathematical models and experimental methodology developed along the guidelines of the models demonstrate the advantages of the physical model approach to the quantitative analysis of experimental data to yield meaningful phenomenological parameters. The far-reaching goal of this approach is to predict drug activity *in vivo*. This work should be regarded as a useful tool for the examination of many cells and tissues and their relationship to drugs that affect them. This system provides an excellent method for quantifying cellular drug availability as a function of molecular design. It is not intended to preclude other distribution and metabolic factors operative in the whole animal.

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M. J. KORNET ˣ, P. A. THIO, N. MALONE, and W. C. LUBAWY

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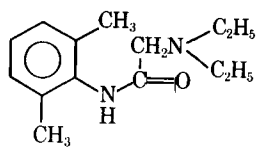

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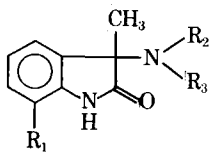

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RESULTS AND DISCUSSION

The title compounds were obtained from indoles by modification of the procedures described by Hinman and Bauman (11, 12) (Scheme I). They prepared 3-bromo-3-methyloxindole by the *N*-bromosuccinimide bromination–oxidation of skatole (11); but in repeating this work, considerable difficulty was experienced in

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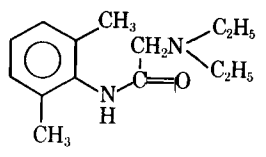

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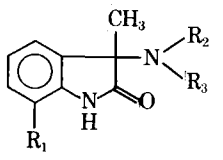

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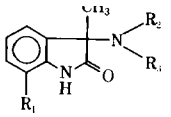
Table I—3-Amino-3-methyloxindoles



Compound	R_1	R_2	R_3	Melting Point	Mole Ratio of *N*-Bromosuccinimide to Indole	Yield[a], %	Crystallization Solvent[b]	Formula	Analysis, %	
									Calc.	Found
IIa	H	—$CH_2CH_2CH_2CH_2$—		205–206°	2:1[c]	35[d]	A	$C_{13}H_{16}N_2O\cdot(COOH)_2$	C 58.82 H 5.92 N 9.15	58.70 5.98 9.31
IIb	H	—$CH_2CH_2CH_2CH_2CH_2$—		163–164°[e]	2:1[c]	16	B	$C_{14}H_{18}N_2O$	—	—
IIc	H	—$CH_2CH_2OCH_2CH_2$—		179.5–180°	3:1	29	B	$C_{13}H_{16}N_2O_2$	C 67.22 H 6.94 N 12.06	67.36 6.99 11.88
IId	H	H	C_2H_5	192–193°	2:1[c]	24	C	$C_{11}H_{14}N_2O$	C 69.45 H 7.42 N 14.72	69.67 7.50 14.87
IIe	H	H	*n*-C_3H_7	160°	3:1	83[f]	B	$C_{12}H_{16}N_2O$	C 70.56 H 7.90 N 13.71	70.76 7.91 13.82
IIf	H	H	*n*-C_4H_9	147°	2:1[c]	28	B	$C_{13}H_{18}N_2O$	C 71.53 H 8.31 N 12.83	71.29 8.12 12.56
IIg	CH_3	H	C_2H_5	140–141°	3:1	89	D	$C_{12}H_{16}N_2O$	C 70.56 H 7.90 N 13.71	70.72 8.14 13.61
IIh	CH_3	H	*n*-C_3H_7	117–118°	3:1	76	D	$C_{13}H_{18}N_2O$	C 71.53 H 8.31 N 12.83	71.43 8.15 12.70

[a] Yield is based on starting indole. [b] A = absolute ethanol–acetone, B = ether, C = benzene, and D = hexane. [c] A 2:1 ratio was used before it was found that 3:1 gave improved yields. [d] The oxalate salt was prepared since the free base is an oil. [e] Lit. (12) mp 160–161°. [f] The yield was 17% when the mole ratio of *N*-bromosuccinimide to indole was 2:1.

separating completely the desired bromo compound from succinimide, the by-product of the reaction. Therefore, the crude 3-bromo compound was not isolated but was allowed to react directly with an amine in ether.

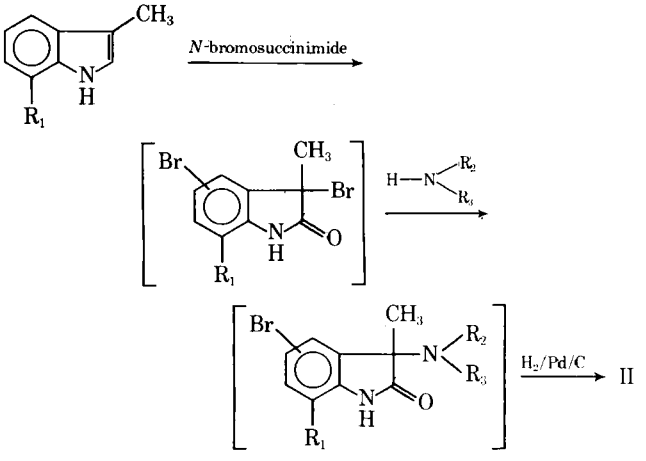
Initial experiments on the preparation of IIe [procedure of Hinman and Bauman, *N*-bromosuccinimide–indole (2:1 mole ratio)] gave a halogen-containing product presumed to be the 5-bromo derivative of IIe. Hydrogenation of this product with palladium-on-carbon catalyst in aqueous acetic acid resulted in the formation of IIe in 17% yield. Since the bromination of the aromatic ring was apparently in competition with bromination of the 3-position (11), the use of a 3:1 mole ratio of *N*-bromosuccinimide to indole followed by hydrogenolysis was tried. With such a proportion of reactants, the conversion of skatole into 3,5-dibromooxindole was expected. Under these conditions, IIe was obtained in 83% yield. These results (Table I) indicate that the optimum procedure for the preparation of II from indoles entails: *(a)* deletion of the isolation of the 3-bromo intermediate, *(b)* use of an *N*-bromosuccinimide to indole mole ratio of 3:1, and *(c)* a final hydrogenolysis step to remove aromatic bromine.

Table I records relevant data for the series of 3-amino-3-methyloxindoles. The IR spectra (KBr) exhibit a strong lactam carbonyl absorption band in the 1700–1705-cm^{-1} range. Table II contains the proton magnetic resonance (PMR) data for these compounds. All compounds shown in Table I, plus a lidocaine standard, were compared for their ability to prevent chloroform-induced arrhythmias by a modification of the procedure described by Lawson (13).

Preliminary experiments established that the chloroform-induced respiratory arrest led to cardiac arrhythmias in healthy female mice. Heart rates[1] averaged 592 ± 29 beats/min (n = 50). This value is in contrast to rates of 134 ± 19 and 137 ± 14 beats/min following ether-induced respiratory arrest at 5 and 10 min after saline injection[2], respectively. These findings are essentially in agreement with the report of Lawson (13) demonstrating the ability of chloroform to induce cardiac arrhythmias.

Subsequent studies indicated that the time of peak effect for protection from chloroform-induced arrhythmias was 10 min for lidocaine and 5 min for IIb and IIc. Therefore, lidocaine was examined 10 min following injection while the test compounds were all examined 5 min following administration.

The ability of the eight test compounds and lidocaine to counteract chloroform-induced arrhythmias is summarized in Table III. None of the test compounds was as potent as lidocaine in preventing arrhythmias, but several did display considerable activity at the 0.346-mmole/kg dose level tested. In an effort to determine more closely how compounds of this class compared to lidocaine, the median effective dose (ED_{50}) and median lethal dose (LD_{50}) of one of the more active compounds of the series, *i.e.*, 3-methyl-3-piperidinooxindole (IIb), were determined and compared to those of the standard. Graphs of the percent protection and the percent lethality *versus* dose for both compounds are shown in Figs. 1 and 2. Tests for parallelism, as determined by the method of Litchfield and Wilcoxon (14), indicated no significant difference between the curves for the two compounds either in percent protection *versus* dose or in percent lethality *versus* dose. Parallel effect curves for



Scheme I

[1] Mean ± *SE*.
[2] n = 5 in each case.

Table II—PMR Data of 3-Amino-3-methyloxindoles[a]

Compound	1-NH	3-NH	3-Methyl	ArH[b]	Other Substituents
II*a*[c]	—	—	1.93	7.35–8.18	1.82–2.32 (m) ($NCCH_2$); 3.33–3.84 (m) (NCH_2)
II*b*	9.62	—	1.61	7.04–7.81	1.32–1.90 (m) ($CH_2CH_2CH_2$); 2.58 2.98 (m) (NCH_2)
II*c*	9.68	—	1.61	7.07–7.77	2.83 (m) (NCH_2); 3.85 (m) (OCH_2)
II*d*	9.31	1.98	1.54	7.08–7.77	1.06 (t), 2.32 (q) (ethyl)
II*e*	9.40	1.94	1.47	6.86–7.53	0.82 (t), 1.08–2.67 (m) (propyl)
II*f*	9.71	1.98	1.53	7.07–7.77	0.84 (t), 0.96–2.68 (m) (butyl)
II*g*	9.96	1.96	1.50	7.04–7.51	1.03 (t), 2.29 (q) (ethyl); 2.40 (s) (benzylic CH_3)
II*h*	10.12	2.04	1.51	7.13–7.61	0.86 (t), 1.08–2.56 (m) (propyl); 2.43 (s) (benzylic CH_3)

[a] Chemical shift is given in δ units. Multiplicity is reported as: s = singlet, t = triplet, q = quartet, and m = multiplet. Spectra were determined in $CDCl_3$ with tetramethylsilane as the internal reference. [b] Multiplets. [c] Spectrum was recorded in D_2O with Tier's salt as the internal reference.

the two compounds support the probability that the aminooxindole derivative suppresses arrhythmias by a mechanism similar to that of lidocaine. The parallel toxicity curves indicate that the two compounds may also produce lethality by similar mechanisms. Since II*b* and lidocaine are related structurally, it is not surprising that they may also have a number of similarities pharmacologically.

The ED_{50}, LD_{50}, 95% confidence interval, and therapeutic indexes for II*b* and lidocaine are shown in Table IV. Although lidocaine appears to be slightly more potent in its protection from arrhythmias than II*b*, potency ratio = 1.2 (0.5–2.0)[3], this difference is not statistically significant. The marked difference in toxicity between lidocaine and II*b*, potency ratio = 3.8 (3.4–4.2)[3], is significant at the 95% level and it is this difference that accounts for the much more desirable therapeutic index seen with II*b*.

Any explanation for differences in acute toxicity between the two compounds is only speculative. Differences in ability to penetrate into the central nervous system (CNS), as well as to activate CNS receptors, may exist. Conformationally rigid molecules such as II*b* would probably be less likely to interact well with a number of different receptor groups than a compound that can assume several conformations.

Additionally, toxicity differences may be related to the eventual disposition of the compounds. Lidocaine is metabolized by oxidative deethylation of the tertiary amine to 2-ethylamino-2′,6′-acetoxylidine in rats, dogs, and humans (15). Although this metabolite is only one-third as potent as the parent compound at preventing chloroform-induced arrhythmias in mice, it is slightly more potent in producing convulsive lethality (16). If this metabolite is also formed in mice, then the parent drug and the metabolite would contribute to the toxicity demonstrated by lidocaine. Similar conversions to a metabolite that could add to the toxicity of the parent compound may not occur with II*b*.

Although these rigid compounds do not show an increase in activity, the therapeutic index for II*b* is more favorable than the corresponding value for lidocaine. This finding indicates that rigid antiarrhythmic drugs of lower toxicity and without a parallel decrease in activity can be prepared and that further efforts in this direction may result in superior drugs.

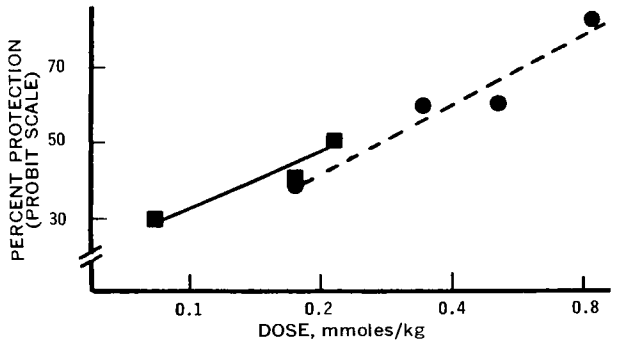


Figure 1—*Percent protection from chloroform-induced arrhythmia in female mice* versus *the administered intraperitoneal dose of II*b(●) *or lidocaine* (■).

[3] The 95% confidence interval.

EXPERIMENTAL[4]

3,7-Dimethylindole—This compound was prepared by modification of a previously reported procedure (17). A solution of 10 g (0.0764 mole) of 7-methylindole and 28 g (0.518 mole) of sodium methoxide in 150 ml of methanol was heated in a 1000-ml Parr bomb at 210–218° for approximately 12 hr and resulted in a pressure of 780 psi. The mixture was cooled to room temperature, and the contents were diluted with methanol and water. The methanol was evaporated under reduced pressure, and the residue was steam distilled to give 7.7 g (69%) of solid, mp 59°. Recrystallization from hexane (charcoal) resulted in white crystals, mp 63° [lit. (17) mp 56°]; NMR ($CDCl_3$): δ 2.33 (d, 3, CH_3 of hetero ring) and 2.43 (s, 3, benzylic H), 6.85–8.05 (m, 5, ArH, NH, and N—CH=).

3-Amino-3-methyloxindoles—The preparation of 3,7-dimethyl-3-ethylaminooxindole is described as a typical reaction. For additional data, see Table I. *N*-Bromosuccinimide (21.4 g, 0.12 mole) was added portionwise over 1 hr to a magnetically stirred solution of 5.8 g (0.04 mole) of 3,7-dimethylindole in 260 ml of purified (11) *tert*-butanol. The mixture was stirred for an additional 2 hr at room temperature. Evaporation of the solvent *in vacuo* at room temperature resulted in a syrupy residue, which was treated with 50 ml of anhydrous ether and filtered to remove the precipitated succinimide. The precipitate was washed well with ether, and the combined filtrate was diluted with additional ether to a vol-

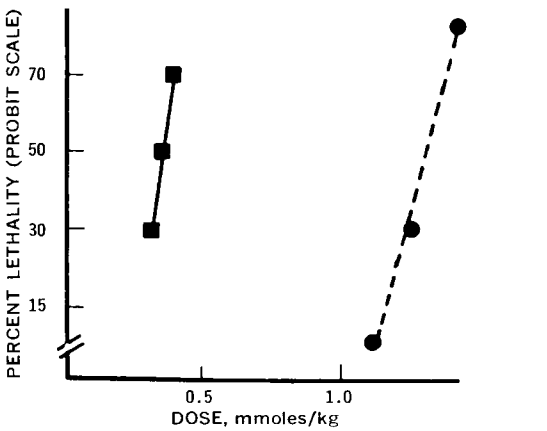


Figure 2—*Percent lethality of II*b (●) *or lidocaine* (■) *in female mice* versus *the intraperitoneal dose.*

[4] Melting points were determined with a Thomas-Hoover melting-point apparatus and are uncorrected. The structures of the compounds were confirmed by their IR and NMR spectra. IR spectra were obtained on a Beckman IR-8 spectrophotometer. NMR spectra were determined on a Varian A-60A spectrometer, using tetramethylsilane as the internal reference. Microanalyses were performed by Dr. Kurt Eder, Geneva, Switzerland, and PCR, Inc., Gainesville, Fla.

Table III—Effect of Lidocaine and Eight 3-Amino-3-methyloxindoles against Chloroform-Induced Arrhythmias in Mice

Compound	Dose, mmole/kg	Protected/Number Tested	Mean Heart Rate ± *SE*
Saline	—[a]	6/50	592 ± 29
Lidocaine hydrochloride	0.173	4/10	348 ± 44
Lidocaine hydrochloride	0.346	9/10	245 ± 22
II*a*	0.346	2/10	530 ± 62
II*b*	0.346	7/10	250 ± 64
II*c*	0.346	5/10	404 ± 80
II*d*	0.346	5/10	402 ± 99
II*e*	0.346	2/10	473 ± 60
II*f*	0.346	6/10	276 ± 66
II*g*	0.346	5/10	294 ± 58
II*h*	0.346	3/10	331 ± 40

[a] For each 10 g of body weight, 0.1 ml of saline was injected.

Table IV—ED_{50}, LD_{50}, and Therapeutic Indexes for Lidocaine and II*b* in Mice

Compound	ED_{50} (95% Confidence Interval)[a]	LD_{50} (95% Confidence Interval)[a]	Therapeutic Index
Lidocaine hydrochloride	0.22 (0.11–0.42)	0.45 (0.43–0.47)	2.0
II*b*	0.27 (0.15–0.49)	1.7 (1.5–1.9)	6.3

[a] Millimoles per kilogram.

ume of 250 ml. A solution of 33% ethylamine in absolute ethanol (20 ml) was added, and the resulting mixture was stored at room temperature for 1 week. The precipitate of ethylamine hydrobromide was filtered and washed with ether, and the ethereal solution was evaporated to a residue.

The residue was dissolved in 200 ml of 70% aqueous acetic acid and hydrogenated with 2 g of 10% Pd/C catalyst overnight on a Parr apparatus (initial hydrogen pressure = 55 psi). About 1.8 kg (4 lb) of hydrogen was absorbed. The catalyst was filtered on a sintered-glass funnel, and the solution was evaporated *in vacuo*. The semicrystalline residue was treated with 100 ml of 10% hydrochloric acid, and the mixture was diluted with water to 500 ml to dissolve all solids. This solution was then extracted twice with 100- and 75-ml portions of ether, respectively.

The aqueous phase was neutralized with solid sodium bicarbonate until the pH of the solution was 8–9. A white solid separated which was extracted once with 100 ml of chloroform and twice with 50-ml portions of chloroform. The combined chloroform extracts were dried over anhydrous magnesium sulfate. Evaporation of the dried solution gave 7.25 g (89%) of cream-colored solid, mp 138–139° with softening at 130°. Recrystallization from hexane with charcoal[5] treatment afforded 5.5 g of white product, mp 140–141°. Workup of the mother liquors resulted in a second crop of 0.77 g, mp 139.5–140.5°. The total recrystallized material amounted to 6.27 g.

Pharmacology—The antiarrhythmic screening procedure used was a modification of that described by Lawson (13). Female albino Swiss Webster mice, 20–25 g, were obtained from a local dealer[6] and given 5 days to recover from shipping stresses before use. Food and water were supplied *ad libitum*. The screening procedure consisted of injecting the animals intraperitoneally with the experimental compounds or the standard, lidocaine hydrochloride[7], dissolved in 0.1 *N* HCl. The concentration of each preparation was such that a volume of 0.1 ml was injected for each 10 g of body weight.

After injection, each mouse was placed in a chloroform-saturated atmosphere and observed. As soon as respiratory arrest occurred, usually within 1–2 min, the animal was removed from the chloroform atmosphere and an ECG tracing was obtained[8] (lead II). Ventricular rhythms above 300 beats/min were considered arrhythmic; those below 300 were considered rhythmic or protected from the chloroform-induced arrhythmia. Lethality was determined at 3 hr following intraperitoneal injection of lidocaine hydrochloride or the test compound.

The ED_{50} and LD_{50} were calculated by the method of Litchfield and Wilcoxon (14).

[5] Norit.

[6] Laboratory Supply, Indianapolis, Ind.

[7] Lidocaine hydrochloride, Pfaltz and Bauer, Flushing, N.Y.

[8] Preamplifier, MK III; amplifier, CA-200; recorder, DMP-4A; all from E & M Instrument Co., Houston, Tex.

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Determination of 17-Monochloroacetylajmaline and Its Metabolite in Plasma by TLC Fluorescence Detection

LAWRENCE J. DOMBROWSKI ˣ, ALFRED V. R. CRAIN, ROBERT S. BROWNING, and EDWARD L. PRATT

Abstract □ A sensitive and specific method is described for the determination of 17-monochloroacetylajmaline (I) and its metabolite, ajmaline (II), in plasma. Method specificity is accomplished by combining an ion-pair extraction with chromatography followed by development and utilization of reaction product fluorescence of the isolated species on silica gel. Recovery of I and II added to plasma or water averaged 70%. The major loss in the assay resulted during a solvent evaporation step and was reproducible over the concentration interval studied. The limit of detectability for I is 0.06 μg/2 ml of plasma. The method was used to determine plasma levels of I and II in the dog following a dose of 10 mg/kg iv of I.

Keyphrases □ 17-Monochloroacetylajmaline and ajmaline—TLC determination in plasma □ Ajmaline and 17-monochloroacetylajmaline—TLC determination in plasma □ TLC—determination, 17-monochloroacetylajmaline and ajmaline in plasma

Ajmaline, an alkaloid base of *Rauwolfia,* causes a direct antiarrhythmic effect in humans. Its use as a therapeutic agent in the treatment of cardiac arrhythmias is well documented (1–5). Preliminary investigations indicated that the 17-monochloroacetyl ester (I)[1] of ajmaline (II) may have advantages in controlling arrhythmias.

A sensitive and specific assay procedure was needed to study the pharmacokinetics of I in mammals. The analytical methods available for measuring the parent compound, II, include polarography, TLC, spectrofluorometry, and colorimetry (6–10). However, the cited procedures either do not possess sufficient sensitivity or are nonselective.

A procedure based upon quantitative TLC was developed in this laboratory; it permits measurement of both I and II in plasma. Detection was based upon the intense blue fluorescence produced on silica gel by I after its reaction with nitric and acetic acids. Ajmaline did not register any fluorescence with the reagents but produced a fluorescent derivative on silica gel after heating for 0.5 hr at 100°. When examined under UV light (360 nm), the intensity of the fluorescence developed for each substance was proportional to concentration. The detection limits for I and II on a silica gel plate were 0.01 and 0.10 μg, respectively.

Ion-pair extraction coupled with fluorescence developed on the TLC plate permitted measurements of I and II that were selective and sensitive.

EXPERIMENTAL

Reagents and Apparatus—A spectrodensitometer[2] equipped with a xenon mercury 200-w lamp was used. All chemicals and solvents used were analytical reagent grade. Precoated TLC plates[3] (20 × 20 cm) were used, and the solvent system was benzene–acetic acid–methanol (86:7:7).

Standards—Standard solutions of I and II were prepared in methylene chloride and diluted to obtain final drug concentrations corresponding to 20 and 120 μg/ml, respectively.

Isolation from Plasma—Two milliliters of plasma was mixed with 2 ml of 1 *M* sodium bicarbonate and extracted twice (2 min each) with 20 ml of ether. The ether extracts were pooled in a 60-ml separator and washed with 4–5 ml of water. The wash was discarded, and the ether was reextracted twice with 4 ml of 0.1 *M* hydrochloric acid. The acids were combined in a 50-ml centrifuge tube (A) and extracted with 5.0 ml methylene chloride for 1 min. After centrifuging (2000 rpm) for a few minutes, a 7.0-ml aliquot of the aqueous layer was transferred to a second 50-ml centrifuge tube (B) and reserved for the assay of II.

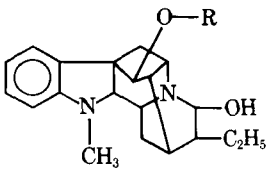
17-Monochloroacetylajmaline Assay—The methylene chloride contained in Tube A was transferred to a 15-ml glass-stoppered conical test tube and dried by mixing with 500 mg of anhydrous sodium sulfate. Depending upon the concentration of I, a suitable volume (0.25–4.0 ml) of the methylene chloride was pipetted into a conical tube and the volume was reduced to about 30 μl with a stream of dry nitrogen. The total residual volume was applied to a prescored TLC plate using a 10-μl syringe. Several 15-μl volumes of methylene chloride were used as a rinse for quantitative transfer.

Volumes of the standard solution, 2.5, 5.0, 10.0, and 20.0 μl corresponding to 0.05, 0.10, 0.20, and 0.40 μg of I, respectively, were spotted in series on the same plate containing the unknown. After development to 15 cm, the plate was air dried and lightly sprayed with 6 *M* nitric acid and then with acetic acid. The plate was lightly heated by positioning it 5–10 cm above a warm hot plate for about 60 sec. When examined under UV light (360 nm), blue fluorescent spots were observed for I at R_f 0.35. The amount of I in the unknown was then estimated by visual comparison with the standard series.

Ajmaline Assay—To Tube B were added 0.6 ml of 1 *M* sodium hydroxide and 2 ml of 1 *M* sodium bicarbonate. The mixture was extracted with 5.0 ml of methylene chloride by shaking for 2 min. After centrifuging, the aqueous phase was aspirated and discarded. The organic phase then was dried by mixing with about 500 mg of anhydrous sodium sulfate. Depending upon the concentration of II, a suitable volume (1.0–4.0 ml) of the methylene chloride was evaporated and spotted on a silica gel plate as described for the I assay.

Volumes of the standard solution, 2.5, 5.0, 10.0, and 20.0 μl corresponding to 0.3, 0.6, 1.2, and 2.4 μg of II, respectively, were also spotted on the same plate. After development to 15 cm, the plate was air dried and placed in a 100° oven for 1 hr. When examined under UV light (360 nm), fluorescent spots were observed for II at R_f 0.20. The amount of II in the unknown then was estimated by visual comparison with the standards.

Densitometry—Standards and unknowns were spotted at the



I: R = $COCH_2Cl$
II: R = H

[1] Ritmos Elle, Inverni della Beffa.
[2] Model SD 3000, Schoeffel Instrument.
[3] Silica gel 60 F-254.

Table I—Densitometric Determination of 17-Monochloroacetylajmaline (I)

Plate Number	Amount of I Spotted, μg	Number of Determinations on Each Plate	Peak Height Coefficient of Variation, %
1	0.025	5	7.0
2	0.050	5	6.3
3	0.100	5	5.2
4	0.150	5	3.8
5	0.200	5	5.0
6	0.250	5	2.4
7	0.300	5	4.8

origin at a minimum distance of 2 cm apart. No more than 2 μl was used at a time, and exactly 60-μl of total volume was used for each spot. The plate was developed, and the fluorescence was visualized as already described. The plate then was scanned using the following instrumental parameters: excitation and emission wavelengths, 365 and 465 nm; excitation and emission slitwidth, 2.0 mm each; sensitivity, 0.4; gain, 0.78; and chart speed, 10.2 cm (4 in.)/min. Calibration curves were constructed from a plot of peak height *versus* concentration.

RESULTS AND DISCUSSION

In basic methanol, I exhibits moderate fluorescence emission (excitation maximum 290 nm, emission maximum 340 nm); trace quantities of I were readily measured when present in uncomplicated systems. However, attempts to utilize this fluorescence for the measurement of I in plasma were unsuccessful because of interferences caused by components in the plasma. For example, when a volume of the body fluid (blank) was carried through various extraction procedures, a large fluorescence background was consistently produced which considerably lowered the detection sensitivity of I. This particular problem was circumvented by preparing a fluorescent derivative of I with different characteristic wavelength maxima.

A derivative was readily formed when a dilute solution containing the drug was reacted with nitric and acetic acids. The reaction was complete within a few minutes at room temperature. The unidentified product strongly fluoresced in solution at 480 nm when excited using 360-nm radiation. However, the intensity of the fluorescence was very sensitive to reaction conditions and was not reproducible. Erratic fluorescence was obtained even when variables such as time, temperature, and reagent concentrations were rigidly controlled. The nonreproducible measurement appeared to be caused by an unstable intermediate formed from the initial reaction of I with nitric acid.

Further investigation revealed that a similar fluorogen was prepared from I after the drug was adsorbed on a silica gel surface and then lightly sprayed with nitric and acetic acids. However, in this case the fluorogen prepared *in situ* was reproducible and the response was linear for quantities of the drug between 0.025 and 0.40 μg.

The development of the fluorogen was hastened when the plate was lightly heated by positioning it several inches above a hot plate for short time intervals (about 15 sec). Between each time interval, the increase in the developed fluorescence intensity was readily assessed by examining the plate under UV light. Generally, heating for about 1 min was required to attain maximum sensitivity. Longer heating times up to several minutes did not appear to alter the fluorescence signal. Heating was not essential to the development of the fluorescence, but at least 2 hr at room temperature was required to produce the maximum response.

Furthermore, it was not necessary to spray with nitric and acetic acids in the order specified. However, sensitivity for I was somewhat diminished if the order was reversed. Also, too little nitric acid or a large excess of the reagent resulted in lower sensitivity. Consistency was obtained if a plate was examined under UV light before spraying further with nitric acid. The reagent was added until the indicator fluorescence was completely quenched and produced a uniform background across the entire plate.

Determination of the nature of the fluorogen formed from I on silica gel was not attempted. The reaction appears to involve either the 17-monochloroacetyl moiety or some functional group other than hydroxy in the 17-position, since II did not produce any fluorescence when treated with nitric and acetic acids in solution or on a silica surface. An attempt was made to determine ajmaline quantitatively *via* nitric acid fluorescence by first esterifying the drug on silica gel. In this experiment, acetic anhydride rather than the toxic monochloroacetic anhydride was employed as the acylating agent. This approach apparently failed since, after treating ajmaline with the anhydride, no fluorescence response was observed on subsequent spraying with nitric acid.

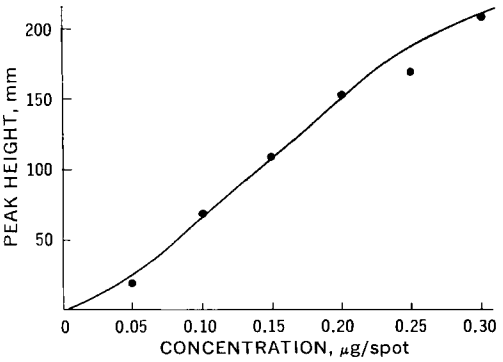


Figure 1—*Standard curve for densitometric measurement of 17-monochloroacetylajmaline.*

When adsorbed on a silica surface, ajmaline was found to produce slowly an unidentified fluorescent product on exposure to fluorescent room light at 25°. Under these conditions, several hours was required before the first visible trace of fluorescence was evident and about 8 hr was needed before the maximum sensitivity was attained. Fluorescence development was hastened when the silica plate was heated briefly at 100°. At this temperature, maximum fluorescence was attained within 0.5 hr. Extending the heating time to 2 hr did not alter the sensitivity. Therefore, a reaction time of 1 hr was chosen for the assay procedure.

An alternative method for determining ajmaline was found using the phthalaldehyde reaction previously employed by Maickel and Miller (11). The ajmaline–phthalaldehyde derivative fluoresced maximally when dissolved in concentrated sulfuric acid (excitation maximum 475 nm, emission maximum 530 nm). Because of the relatively large background produced by excess phthalaldehyde, ajmaline sensitivity was only one-half that achieved with the proposed TLC procedure.

Two amine groups are present in both I and II. Potentiometric titration of either compound with acid resulted in only one break in the titration curve. This suggested that the amine basicities are similar and are not readily resolved. Consequently, the experimentally observed pKa value is an apparent one and probably represents the mean of the two basic sites. Nevertheless, similar values were found for I and II (pKa about 7.4 each), and both compounds were extracted readily with ether from plasma. They were then back-extracted with 0.1 *M* hydrochloric acid. Methylene chloride selectively extracted I from the acid phase, the partitioning being about 97% complete. Ajmaline remained in the aqueous acid and was extracted with methylene chloride after adjusting the pH back to about 8.5.

This separation of the two alkaloids from an acid medium was surprising since both compounds have similar solubilities. The greater hydrophobic character shown by ajmaline may be due to an increase in hydrogen bonding caused by the additional hydroxy group in the 17-position. A similar observation was recently made by Eksborg *et al.* (12), who determined extraction constants for a variety of amines and various counterions and concluded that hydrophilic substituents such as hydroxy and carboxyl groups generally cause a reduction in extraction constants. Because I is predominantly protonated in 0.1 *M* hydrochloric acid, it is concluded that the extraction of I with methylene chloride from the acid medium occurs as the chloride ion-pair.

Accuracy and Precision—The recovery of added I and II from control plasma and water was determined by processing 2 ml of fluid through the complete assay procedure. The average recovery was 70% and was constant for any drug level between 0.06 and 10.0

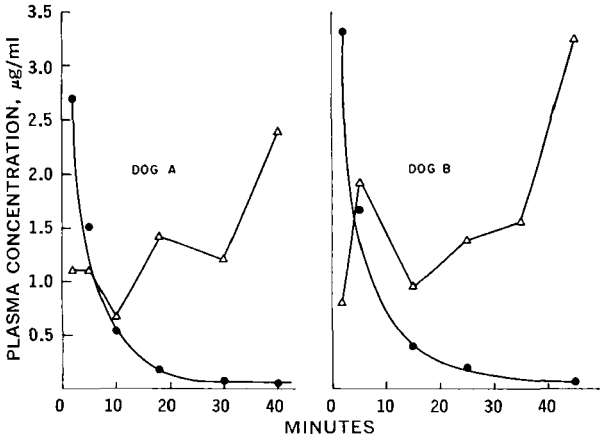


Figure 2—*Plasma profile of 17-monochloroacetylajmaline and ajmaline in Dog A (15.6 kg) and Dog B (10.5 kg), each receiving a 10-mg injection of I/kg. Key: ●, 17-monochloroacetylajmaline; and △, ajmaline.*

μg/2 ml of either fluid. The coefficient of variation also was constant for any level within the examined range. Based upon visual estimate, it is reported to be 20% (n = 3). The major recovery loss in the assay (15–20%) was due to the methylene chloride evaporation step and was determined by comparing direct standards against evaporated standards. For example, when a volume (0.25–4.0 ml) of a methylene chloride solution (0.1 μg of I/ml) was reduced to about 30 μl and transferred to a TLC plate as directed in the assay for I, only 80% of I was recovered in the process. Coating the tubes with chlorotrimethylsilane did not improve the recovery.

Using a densitometer, linear relationships between peak height and applied quantity of I were obtained up to 0.3 μg of drug/spot. A typical calibration curve is shown in Fig. 1. The small coefficients of variation reported in Table I indicate that densitometric measurements within a single plate are quite reproducible. The recovery of I from plasma (0.03–5.0 μg/ml) also averaged 70% when determined by densitometry.

Fluorescence calibration curves derived from two separate TLC plates sometimes showed sensitivity slopes with differences as great as ±30%. This large variation made it necessary to spot standards along with any unknowns on each plate. A single plate (20 × 20 cm) would conveniently accommodate four standards and five samples. The precision data reported in Table I indicate that sensitivity variations within a single plate were less than 7%. Also errors due to spot application and unevenness of spraying were satisfactorily overcome.

Plasma Levels of I and II in Dog—The proposed method was used to determine plasma levels of I and II in two beagle dogs (A and B). Each dog was medicated with I (10 mg/kg iv). Blood volumes were collected between 2 and 45 min after administration. Plasma concentrations are illustrated in Fig. 2 and show a rapid decrease in I with time. The biological half-life is approximately 3 min. Persistent and significant levels of II were obtained which increased with time. No metabolites other than ajmaline were detected in the TLC chromatogram. Further supportive evidence for the presence of I and II in the dog plasma was obtained using an alternative TLC system composed of chloroform–acetic acid (80:20) (R_f 0.49 and 0.18, respectively).

In summary, a quantitative TLC assay procedure is presented which sensitively measures 17-monochloroacetylajmaline and its metabolite, ajmaline, in plasma. Extractions prior to chromatography effectively removed interfering plasma components, and relatively simple TLC chromatograms were obtained. Method specificity results from the following: (*a*) selective extraction of I from hydrochloric acid with methylene chloride, (*b*) separation of the extractables using TLC, and (*c*) fluorescence developed by both I and II on silica gel. The proposed method was tested on dogs, and levels of both I and II were found following intravenous injection of I.

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Alkylthiocolchicines and *N*-Deacetyl-alkylthiocolchicines and Their Antileukemic Activity

GEORGE T. SHIAU, KALYAN K. DE, and ROBERT E. HARMON ˣ

Abstract □ A series of alkylthiocolchicines (methyl, ethyl, *n*-butyl, *n*-hexyl, *n*-octyl, and pinanyl) was prepared from colchicine by treatment with the appropriate alkyl mercaptan and *p*-toluenesulfonic acid. Some of these compounds (methyl-, ethyl-, and *n*-butylthiocolchicines) were deacetylated in good yields with 2 *N* hydrochloric acid in methanol. These compounds were tested for their antileukemic activity in an *in vitro* assay against L-1210 (mouse leukemia). Preliminary results showed that methylthiocolchicine is more active and the other alkylthiocolchicines are much less active than colchicine. *N*-Deacetyl-methylthiocolchicine is as active as colchicine.

Keyphrases □ Alkylthiocolchicines and *N*-deacetyl-alkylthiocolchicines—synthesized from colchicine, antileukemic activity □ Thiocolchicines, alkyl, and *N*-deacetyl-alkylthiocolchicines—synthesized from colchicine, antileukemic activity □ Colchicine derivatives—synthesis and antileukemic activity of alkylthiocolchicines and *N*-deacetyl-alkylthiocolchicines □ Antileukemic activity—synthesis and screening of alkylthiocolchicines and *N*-deacetyl-alkylthiocolchicines

The antitumor activity of colchicine, the major alkaloid of the autumn crocus, *Colchicum autumnale*, and the African climbing lily, *Gloriosa superba*, was first reported at the beginning of this century (1). The elucidation of its structure was finally completed from X-ray studies (2) and a number of total syntheses (3–8).

Colchicine is a mitotic poison, particularly in thymic, intestinal, and hematopoietic cells, which acts as a spindle poison and blocks the kinesis (9). Its effect on the mitotic spindle is thought to represent a special case of its effects on various organized, labile, fibrillar systems concerned with structure and movement (10, 11).

A study with ^{3}H-labeled colchicine showed that the drug is rapidly taken up by Ehrlich ascites and S-180 cells *in vitro* (12). The study did not establish a relationship between mitotic arrest and cytotoxicity but suggested that the latter probably arises, at least in part, from a degenerative process occurring in arrested cells. A separate investigation (13) showed that colchicine diminishes deoxycytidylate aminohydrolase activity in Ehrlich ascites cells and suggested that the drug's action on mitoses may be related to its action on this enzyme, thus regulating DNA synthesis. Numerous derivatives and analogs of colchicine have been examined for antimitotic activity, and this work has afforded a number of compounds for clinical study.

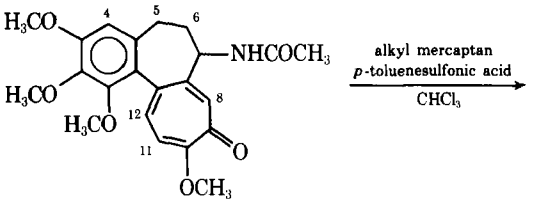
Recent reports on the muscle relaxant activity and antimitotic activity of 3-*O*-demethyl-3-*O*-β-D-glucopyranosyl-methylthiocolchicine prompted the preparation of compounds of this type for submission to the Cancer Chemotherapy National Center for evaluation in their intracranial antileukemic test system.

Table I—Antileukemic Activities of Alkylthiocolchicines and *N*-Deacetyl-alkylthiocolchicines Using the L-1210 (Mouse Leukemia) Tube Dilution *In Vitro* Assay

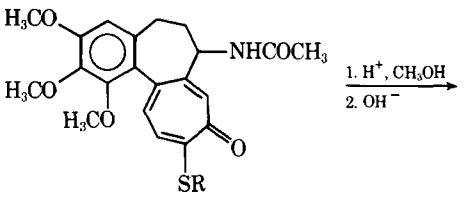
Compounds	Drug Activities, μg/ml	
	ID_{50}	ID_{90}
I (colchicine)	0.0077	0.011
II*a*	0.0018	0.0027
II*b*	0.019	0.027
II*c*	>0.1	>0.1
II*d*	>0.1	>0.1
II*e*	>0.1	>0.1
II*f*	>0.1	>0.1
III*a*	0.0070	0.011
III*b*	0.069	0.098
III*c*	>0.1	>0.1

RESULTS AND DISCUSSION

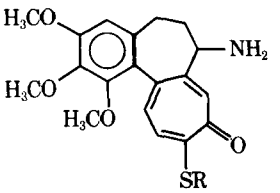
Methylthiocolchicine (II*a*) and ethylthiocolchicine (II*b*) were prepared by treating colchicine (I) with methyl and ethyl mercaptan, respectively, in the presence of *p*-toluenesulfonic acid as a cat-



I



II*a*: R = CH_3
II*b*: R = C_2H_5
II*c*: R = n-C_4H_9
II*d*: R = n-C_6H_{13}
II*e*: R = n-C_8H_{17}
II*f*: R = $C_{10}H_{17}$



III*a*: R = CH_3
III*b*: R = C_2H_5
III*c*: R = n-C_4H_9

Scheme I

alyst at room temperature for several days (14–16). However, after several preparations, it was found that the yield could be improved by using a large excess of mercaptan and by carrying out the reaction at a higher temperature in a shorter time. Under these reaction conditions, the ratio of IIa to I in the reaction mixture was as high as 20 to 1 according to UV spectroscopy.

In general, all reactions showed a gradual color change, indicating the progress of the reaction. TLC is a convenient method for following the reactions and for examining the purity of the products. The excess mercaptan can be removed by evaporation or by pouring the reaction mixture into petroleum ether (bp 30–60°). For the high boiling-point mercaptans, only the latter method affords satisfactory results.

A number of new alkylthiocolchicines, *n*-butyl-, *n*-hexyl-, *n*-octyl-, and pinanylthiocolchicines, were prepared. The yields in these cases were poor, ranging from 15 to 25% because the reactions were initially carried out at room temperature. In the case of *n*-butylthiocolchicine, the yield was improved to 45% by carrying out the reaction at 95° in a pressure tube for 4 hr. The reactions are given in Scheme I. *N*-Deacetyl-alkylthiocolchicines (III) can be obtained in good to excellent yields when alkylthiocolchicine (II) in methanol is refluxed with hydrochloric acid and basified with sodium hydroxide (16, 17).

All compounds of II and III can be purified by dry column chromatography on silica gel eluted with a solvent [ethanol–ethyl acetate (20:80, v/v)]. The R_f values in this system are 0.48, 0.75, and 0.45 for I, II, and III, respectively. However, some of the compounds could be purified satisfactorily by crystallization from suitable solvents as indicated in the *Experimental* section.

The compounds were tested[1] in an *in vitro* assay against L-1210 (mouse leukemia). The results (Table I) show that the antileukemic activity of methylthiocolchicine is greater than that of colchicine. In ethylthiocolchicine, the antileukemic activity is decreased from colchicine; in the other alkylthiocolchicines (where the alkyl group is large), the activity is decreased greatly. *N*-Deacetyl-methylthiocolchicine is as active as colchicine, while *N*-deacetyl-ethylthiocolchicine is much less active than colchicine.

EXPERIMENTAL

Commercial grade colchicine[2] was used without further purification. The melting points[3] reported are uncorrected. The IR spectra[4] of *n*-butylthiocolchicine (IIc) and *N*-deacetyl-butylthiocolchicine (IIIc) are reported here; the other spectra are practically identical with these two spectra in the region of 4000–750 cm^{-1}. The band intensities are abbreviated as follows: vs, very strong; s, strong; m, moderate; w, weak; and vw, very weak.

The UV spectra[5] were recorded using 95% ethanol as the solvent. The NMR spectra[6] were measured using tetramethylsilane as the internal standard and $CDCl_3$ as the solvent. The spectra are reported in the following order: chemical shift (δ), observed multiplicity, coupling constant, number of protons, and assignment. Signals other than for thioalkyl protons of the compounds except for IIc are omitted. They are similar in pattern as well as chemical shift to those of IIc.

Mass spectra[7] were recorded at 70 ev. Elemental analyses[8] and TLC[9] were carried out for each sample. Silica gel[10] (70–230 mesh) was used for dry column chromatography. The compounds were eluted with ethyl acetate–ethanol (80:20) and examined with UV light.

Methylthiocolchicine (IIa)—To a pressure bottle containing colchicine (20.0 g), *p*-toluenesulfonic acid monohydrate (4.0 g), chloroform (50 ml), and a magnetic bar cooled in an acetone–dry ice bath was added methyl mercaptan (110 g), also cooled previously in an acetone–dry ice bath. The pressure bottle was sealed and the reaction mixture was allowed to warm to room temperature with constant stirring. The pressure bottle was then heated in a bath at 70° for 3 hr with constant stirring. The bottle was allowed to cool to room temperature, cooled further in an acetone–dry ice bath, and then opened.

The excess methyl mercaptan was allowed to evaporate at 30–40° and was collected at acetone–dry ice temperature for reuse or was destroyed by passing through several traps filled with acidic potassium permanganate or potassium dichromate solution. The syrupy residue was dissolved in chloroform (200 ml) and washed several times with saturated sodium bicarbonate solution and finally with water. The chloroform layer was then dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. The crude residue was crystallized from hot ethyl acetate (200 ml), giving 17.5 g of shiny yellow cubes. An additional 4.9 g of crude amorphous product, that could not be crystallized from ethyl acetate, was recovered from the mother liquor. The shiny crystals were crushed and dried at 65–70° under vacuum for several hours to yield 14.3 g (69%) of methylthiocolchicine, mp 189–190° [lit. (15) mp 192–194°]; UV (95% ethanol): λ_{max} 385 (log ϵ 4.25), 290 (4.06), and 257 (4.31) nm; NMR: 2.46 (s, 3, SCH_3).

Anal.—Calc. for $C_{22}H_{25}NO_5S$: C, 63.60; H, 6.07; N, 3.37; S, 7.72. Found: C, 63.46; H, 5.92; N, 3.37; S, 7.70.

Ethylthiocolchicine (IIb)—To the solution of colchicine (16.0 g) and *p*-toluenesulfonic acid (2.4 g) in chloroform (40 ml) in a pressure bottle was introduced ethyl mercaptan (50 ml). The mixture was then sealed and heated in a bath at 90–95° for 7 hr. After the evaporation of mercaptan, the reaction mixture was neutralized with sodium bicarbonate and extracted with chloroform. The chloroform extract was washed, dried, and then evaporated to dryness under reduced pressure at 45°. The residue was dissolved in hot ethyl acetate and gave ethylthiocolchicine, 9.92 g (58% yield), of pale-yellow cubic crystals, mp 204–205° [lit. (15) mp 207–208°]; UV: λ_{max} 386 (log ϵ 4.27), 290 (4.06), and 256 (4.32) nm; NMR: 1.45 (t, J = 7.3 Hz, 3, SCH_2—CH_3) and 2.95 (q, J = 7.3 Hz, 2, SCH_2—CH_3).

Anal.—Calc. for $C_{23}H_{27}NO_5S$: C, 64.31; H, 6.34; N, 3.26; S, 7.47. Found: C, 64.15; H, 6.48; N, 3.27; S, 7.58.

***n*-Butylthiocolchicine (IIc)**—The reaction was carried out and the product was isolated as described for IIb, yielding 45%, mp 121–124°; UV: λ_{max} 387 (log ϵ 4.25), 290 (4.03), and 257 (4.29) nm; IR (intensity, assignment): 3300 (s, amide NH stretching), 3058–2840 (s, CH stretching), 1670 (s, amide carbonyl stretching), 1616 (s, tropolone carbonyl stretching), 1546 (vs), 1493 (s), 1466 (s), 1433 (s), 1408 (s), 1372 (m), 1353 (s), 1326 (s), 1287 (m), 1239 (m), 1198 (m), 1156 (m), 1139 (s), 1098 (s), 1056 (w), 1022 (s), 993 (w), 979 (m), 954 (w), 923 (m), 903 (w), 843 (w), and 826 (vw) cm^{-1}; NMR: 0.99 [t, J = 7.0 Hz, 3, S—CH_2—$(CH_2)_2$—CH_3], 1.70 [m, 4, S—$CH_2(CH_2)_2$—CH_3], 1.99 (s, 3, $COCH_3$), 2.46 (broad s, 4, CH_2 of C-5 and C-6), 2.92 (t, J = 7.0 Hz, 2, S—CH_2—), 3.69, 3.92, 3.96 (three s, 3H each, OCH_3 of C_1, C_3, and C_2)[11], 4.69 (broad s, 1, H_7), 6.56 (s, 1, H_4), 7.20 (d, *AB* pattern, $J_{11,12}$ = 10.5 Hz, 1, H_{11}), 7.32 (d, 1, H_{12}), 7.52 (s, 1, H_8), and 8.38 (broad d, J = 7 Hz, 1, NH).

Anal.—Calc. for $C_{25}H_{31}NO_5S$: C, 65.62; H, 6.83; N, 3.06; S, 7.01. Found: C, 65.33; H, 7.09; N, 2.93; S, 6.89.

***n*-Hexylthiocolchicine (IId)**—A mixture of colchicine (1.0 g), *p*-toluenesulfonic acid (0.20 g), chloroform (4 ml), and *n*-hexyl mercaptan (2 ml) was stirred in a closed container at room temperature for several weeks. The reaction mixture was poured slowly into petroleum ether (200 ml) with vigorous stirring, and a pale-yellow precipitate formed. The crude precipitate was dissolved in chloroform and purified by column chromatography. The product was crystallized from ethyl acetate–*n*-hexane, yielding 25%, mp 228–230°; UV: λ_{max} 387 (log ϵ 4.28), 290 (4.04), and 257 (4.31) nm; NMR: 0.91 [t, J = 6.5 Hz, 3, SCH_2—$(CH_2)_4$—CH_3], 1.20–1.78 [m, 8, SCH_2—$(CH_2)_4$—], and 2.90 (t, J = 6.5 Hz, 2, SCH_2—).

Anal.—Calc. for $C_{27}H_{35}NO_5S$: C, 66.78; H, 7.27; N, 2.88; S, 6.60. Found: C, 67.05; H, 7.53; N, 2.82; S, 6.71.

***n*-Octylthiocolchicine (IIe)**—The reaction was carried out and the product was isolated as described for IId. The product was obtained as amorphous powder in 20% yield; UV: λ_{max} 387 (log ϵ

[1] The Upjohn Co., Kalamazoo, Mich.
[2] S. B. Penick and Co., New York, N.Y.
[3] Thomas-Hoover Unimelt apparatus.
[4] Beckman IR-8 spectrophotometer.
[5] Cary-14 spectrophotometer.
[6] Varian A-60 spectrometer.
[7] LKB 9000 SN mass spectrometer.
[8] Midwest Microlab, Inc., Indianapolis, Ind.
[9] Eastman Chromagraph Sheet 6061 silica gel.
[10] EM Laboratories, Inc., Elmsford, N.Y.

[11] The examination of the NMR data on all alkylthiocolchicines confirmed that the peak at 4.06 (ppm, δ) is attributed to the methoxy group on tropolone ring of colchicine; the earlier assignment (Varian NMR No. 689) at 3.67 is wrong since this peak does not disappear when the tropolone methoxy group is replaced by an alkylthio group.

4.24), 290 (4.01), and 257 (4.28) nm; NMR: 0.89 [t, J = 6.5 Hz, 3, SCH_2—$(CH_2)_6$—CH_3], 1.34 [broad distorted singlet, 12, SCH_2—$(CH_2)_6$—], and 2.90 (t, J = 6.5 Hz, 2, SCH_2—).

Anal.—Calc. for $C_{29}H_{39}NO_5S$: C, 67.81; H, 7.65; N, 2.72; S, 6.24. Found: C, 67.61; H, 7.91; N, 2.52; S, 6.02.

Pinanylthiocolchicine (IIf)—The reaction was carried out and the product was isolated by the procedure described for II*d*, yielding 20%; NMR: 0.86 (s, 1), 1.10 (s, 3, CH_3), 1.25 (s, 3, CH_3), 1.47–2.76 (m, 15 but only 8 for pinanyl part, overlapped with $COCH_3$ and CH_2 of C-5 and C-6), and 2.95 (d, 2, SCH_2—).

Anal.—Calc. for $C_{31}H_{39}NO_5S$: C, 69.24; H, 7.31; N, 2.60; S, 5.96. Found: C, 69.05; H, 7.38; N, 2.58; S, 5.94.

***N*-Deacetyl-methylthiocolchicine (IIIa)**—To the clear solution of methylthiocolchicine (8.3 g) in methanol (120 ml) in a round-bottom flask was added 2 *N* hydrochloric acid (120 ml). The solution was refluxed for 40 hr, concentrated to 140 ml at 45° on a rotary evaporator, and then extracted with chloroform (5 × 120 ml). The water layer was stored, and the chloroform layer was washed with water (4 × 100 ml). The second portion of the water washings was concentrated to 30 ml and then allowed to evaporate slowly at room temperature to give yellowish green needles (2.54 g) of *N*-deacetyl-methylthiocolchicine hydrochloride (III*a*-HCl), mp 218–220°. The other three portions of the water washings were combined with the original water layer. The chloroform layer was evaporated to dryness and redissolved in hot ethyl acetate to give a mixture of III*a* and II*a* (0.64 g) in about a 3 to 2 ratio as determined by TLC.

The combined water layer was basified to pH 13 with 10 *N* sodium hydroxide and extracted with chloroform (5 × 120 ml). The combined chloroform extracts were washed with water and dried over anhydrous sodium sulfate. The clear solution was concentrated (40 ml), and then ether (240 ml) was added to give *N*-deacetyl-methylthiocolchicine (4.32 g) in crystalline form as pale-yellow shiny rectangular plates, mp 194–195° [lit. (17) mp 195°]. The combined yield of III*a* and III*a*-HCl was 89%.

For III*a*, the mass spectrum showed m/e (%) M^+ 373 (100), 345 (22), 328 (36), and 298 (42).

Anal.—Calc. for $C_{20}H_{23}NO_4S$: C, 64.32; H, 6.21; N, 3.75; S, 8.56. Found: C, 64.68; H, 6.53; N, 3.67; S, 8.86.

For III*a*-HCl, the mass spectrum showed m/e (%) M^+ 373 (355) and $H^{35}Cl$ 36 (100).

Anal.—Calc. for $C_{20}H_{23}NO_4S \cdot HCl$: C, 58.58; H, 5.90; Cl, 8.65. Found: C, 58.31; H, 5.90; Cl, 8.69.

***N*-Deacetyl-ethylthiocolchicine (III*b*)**—The reaction was carried out and the product was isolated by the procedure described for III*a*. The products III*b* and III*b*-HCl were crystallized from ethyl acetate and water, respectively, yielding 80%.

For III*b*, the melting point was 158–160° [lit. (17) mp 163°].

Anal.—Calc. for $C_{21}H_{25}NO_4S$: C, 65.09; H, 6.50; N, 3.62; S, 8.28. Found: C, 65.34; H, 6.21; N, 3.44; S, 8.14.

For III*b*-HCl, the melting point was 206–208°.

Anal.—Calc. for $C_{21}H_{25}NO_4 \cdot HCl$: Cl, 8.36. Found: Cl, 8.25.

***N*-Deacetyl-*n*-butylthiocolchicine (III*c*)**—The reaction was carried out and the product was isolated as described previously. The product was crystallized from ethyl acetate as yellow rectangular prisms, yielding 75%, mp 140–141°; IR: 3380 (m, NH_2 *anti-sym* stretching), 3320 (m, NH_2 *sym* stretching), 3050–2840 (s, CH stretching), 1615 (s, tropolone carbonyl stretching), 1560 (vs), 1493 (s), 1464 (s), 1435 (m), 1406 (s), 1376 (w), 1351 (s), 1321 (s), 1287 (w), 1235 (m), 1200 (s), 1147 (s), 1120 (w), 1092 (s), 1037 (w), 1017 (s), 1000 (m), 984 (m), 966 (m), 922 (s), 842 (w), 822 (vw), 796 (vw), and 755 (vw) cm^{-1}.

Anal.—Calc. for $C_{23}H_{29}NO_4S$: C, 66.47; H, 7.03; N, 3.37; S, 7.71. Found: C, 66.58; H, 7.12; N, 3.24; S, 7.52.

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Biochemorphology of Cyclobutanecarbonylureas

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Abstract □ Ten urea derivatives of cyclobutanecarboxylic acid were synthesized and examined for general CNS depressant properties, barbiturate potentiation, and myorelaxant, antitremorine, and anticonvulsant potencies. Although water solubility plays an important role in the activity of these compounds, other factors also appear to be involved. 1-Cyclobutanecarbonyl-3,3-dimethylurea appears to be the most active CNS depressant, whereas 1-cyclobutanecarbonyl-3-(α-naphthyl)thiourea is the most active barbiturate potentiator. 1-Cyclobutanecarbonyl-3,3-dimethylurea, 1-cyclobutanecarbonyl-3-phenylurea, and 1-cyclobutanecarbonyl-3-(2,6-xylyl)urea appear to be the most active myorelaxants, while 1-cyclobutanecarbonyl-3-propylurea, 1-cyclobutanecarbonyl-3-*tert*-butylurea, 1-cyclobutanecarbonyl-3-allylurea, and 1-cyclobutanecarbonyl-3-phenylurea apparently are the most active against pentylenetetrazol-induced convulsions. 1-Cyclobutanecarbonyl-3-*tert*-butylurea, 1-cyclobutanecarbonyl-3,3-dimethylurea, and 1-cyclobutanecarbonyl-3-phenylurea are also slightly active tremorine antagonists.

Keyphrases □ Cyclobutanecarbonylureas—synthesis, CNS activity, correlated with molecular structure and aqueous solubility □ CNS activity—synthesis and screening of cyclobutanecarbonylureas, biological activity correlated with molecular structure and aqueous solubility

In continuing research on the synthesis of new derivatives of certain cyclobutanecarboxylic acids (1–6) with central nervous system (CNS) depressant activity, 10 urea derivatives of cyclobutanecarboxylic acid were prepared by interaction of cyclobutanecarbonyl chloride with the corresponding ureas and pharmacological data were compiled. The compounds were tested for general CNS depressant properties, barbiturate potentiation, and myorelaxant, antitremorine, and anticonvulsant potencies. Furthermore, the biological activity of the compounds was correlated with molecular structure and aqueous solubility.

EXPERIMENTAL

The compounds were prepared by conventional routes involving acylation of a urea with cyclobutanecarbonyl chloride in either pyridine or dimethylformamide (3) (Table I).

White Swiss, Charles River strain mice were used once in these studies. They were previously untreated with any drug and were permitted to feed *ad libitum*.

Bioassay for Sedative and Hypnotic Properties—The depressant activity of the compounds was determined by observing their effects on the spontaneous activity and righting reflex of virgin female mice weighing 20–25 g. The drugs were given orally either as a solution or a suspension in 0.2–0.4 ml of 10% gum acacia, using a blunted and bent 18-gauge hypodermic needle feeding tube. A reduction in spontaneous activity was subjectively graded and compared with that of the control animals given the drug vehicle only.

The effect of the test drug was described in the following terms: when 100% of the animals equaled the control animals in spontaneous activity, the effect was considered as nil. When 50% or more of the test population showed a slight decrease in activity, the effect was considered slight. When 50% or more of the test population showed an intermediate decrease, the effect was considered intermediate. When 50% or more of the test animals showed a loss of righting reflex, the loss of spontaneous activity was considered marked. For each dose, five control and five test mice were used.

Barbiturate Potentiation—Female mice, 20–30 g, were used, and the test substances were administered orally as in the previous experiment. Pentobarbital sodium (50 mg/kg) was administered 30 min after the test drug. For each test and each control experiment, five mice were used. Generally, all animals lost the righting reflex within 10 min after pentobarbital injection. The animals were placed on their backs until spontaneous righting occurred. They were again placed on their backs until righting was effected within 5 sec, at which time the animals were judged to have regained the righting reflex. The measure of potentiation used was the ratio $(\text{drug} + \text{barbiturate}_{\text{sleep time}})/(\text{barbiturate}_{\text{sleep time}} + \text{drug}_{\text{sleep time}})$.

Myorelaxant Activity—Male mice, 20–22 g, were used. The test compounds were given orally as in the prior experiments, and strychnine nitrate (1.6 mg/kg ip) in distilled water was administered 30 min after the test substance. The strychnine dosage was 100% lethal in controls which died within 10–12 min. The ability of a drug to protect against strychnine-induced lethality was assessed by survival of the animals after 30 min and 24 hr. Survival for 30 min was judged as partial protection; survival for 24 hr was judged as complete protection.

Pentylenetetrazol Antagonism—Male mice, 20–25 g, were used. The test compounds were given orally as before, and pentylenetetrazol (100 mg/kg) in a 0.25% methylcellulose sterile vehicle was injected subcutaneously 30 min after the test compound. The dose was convulsive to 100% within 30 min and 99% lethal in the control animals. Drug-treated animals were observed individually for complete protection in the 30 min following injection of pentylenetetrazol. For each drug and each control, five mice were used.

Antitremorine Activity—Male mice, 20–30 g, were used. The test compounds were administered orally as before and at a dose of 1000 mg/kg. Tremorine (20 mg/kg ip) in a 0.25% methylcellulose sterile vehicle was given 30 min after the test substance. In controls, this dose produced centrally mediated tremors plus signs of parasympathetic stimulation including salivation, lacrimation, diarrhea, and urination. Subjective grading was used to establish the degree of protection against tremorine.

To judge protection, animals were suspended by the tail. When the tremor was the same as for the controls, protection was rated as nil. For a slight reduction in tremor intensity, the degree of protection was judged slight; for a slight tremor, protection was moderate; no tremor was rated as complete protection. For each drug and each control, five mice were used.

RESULTS AND DISCUSSION

To elucidate the biochemorphology of the cyclobutanecarbonylureas, the compounds in Table II were arranged according to their structural and physicochemical properties. When tested for general depressant activity, it was observed that the straight chain derivatives, Compounds 1–4, although insoluble in water, showed slight CNS depressant activity. Furthermore, 1-cyclobutanecarbonyl-3,3-dimethylurea, Compound 5, in contrast to 1-cyclobutanecarbonyl-3-methylurea, Compound 1, was highly water soluble and highly active in that long-term loss of the righting reflex was observed. This finding suggested that water solubility plays an important role in the activity of these compounds.

Introduction of a double bond into the aliphatic chain of 1-cyclobutanecarbonyl-3-propylurea, Compound 3, gave 1-cyclobutanecarbonyl-3-allylurea, Compound 6, which was identical to the parent compound in water solubility and activity. However, the allylthiourea derivative, although insoluble in water, was more active than the allylurea derivative. It caused intermediate loss of spontaneous motor activity. Similarly, the α-naphthylthiourea derivative, although insoluble in water, produced marked reduction in spontaneous motor activity, whereas the other two aromatic derivatives, Compounds 8 and 9, were insoluble in water and inactive as CNS depressants. The data suggest that, although water solubility

Table I—Cyclobutanecarbonylureas

Compound Number	Compound Name	Yield, %	Melting[a] Point	Recrystallization Solvent[b]	Molecular Formula	Analysis, % Calc.	Analysis, % Found
1	1-Cyclobutanecarbonyl-3-methylurea	80	155–158°	P/MC	$C_7H_{12}N_2O_2$	C 53.84 H 7.69 N 17.94	C 53.49 H 7.87 N 18.01
2	1-Cyclobutanecarbonyl-3-ethylurea	45	110°	A	$C_8H_{14}N_2O_2$	N 16.49	N 16.47
3	1-Cyclobutanecarbonyl-3-propylurea	70	86–88°	W/A	$C_9H_{16}N_2O_2$	C 58.69 H 8.69 N 15.21	C 58.75 H 8.90 N 15.13
4	1-Cyclobutanecarbonyl-3-*tert*-butylurea	75	137–139°	W/A	$C_{10}H_{18}N_2O_2$	C 60.60 H 9.09 N 14.14	C 60.62 H 9.17 N 14.14
5	1-Cyclobutanecarbonyl-3,3-dimethylurea	35	180°	P/MC	$C_8H_{14}N_2O_2$	N 16.47	N 17.01
6	1-Cyclobutanecarbonyl-3-allylurea	60	82°	P	$C_9H_{14}N_2O_2$	N 15.38	N 15.34
7	1-Cyclobutanecarbonyl-3-allylthiourea	80	50°	P	$C_9H_{14}N_2OS$	C 54.54 H 7.07 N 14.14	C 54.44 H 7.16 N 13.92
8	1-Cyclobutanecarbonyl-3-phenylurea	75	145°	P/MC	$C_{12}H_{14}N_2O_2$	C 66.05 H 6.42 N 12.84	C 66.35 H 6.53 N 13.00
9	1-Cyclobutanecarbonyl-3-(2,6-xylyl)urea	80	180–182°	P/MC	$C_{14}H_{18}N_2O_2$	C 68.29 H 7.23 N 11.38	C 68.28 H 7.46 N 11.33
10	1-Cyclobutanecarbonyl-3-(α-naphthyl)thiourea	85	165°	A/M	$C_{16}H_{16}N_2O$	C 67.60 H 5.63 N 9.85	C 67.49 H 5.93 N 9.58

[a] Uncorrected. [b] A = acetone, MC = methylene chloride, M = methanol, P = petroleum ether, and W = water.

plays an important role in the activity of these compounds, other factors also play a part as activity determinants.

To test the previously reported phenomenon (3) that the locus for barbiturate potentiation appeared to be functionally different from the sites involved in the directly measured depressant effect, a comparison was made of the barbiturate potentiation and general depressant effect of these compounds. It was presumed that if there were two functionally different loci for these activities, it should be possible to observe three different modes of CNS action, *i.e.*, CNS depression only (Category A), potentiation only (Category B), and both CNS depression and potentiation (Category C).

When analyzed for pentobarbital potentiation activity at a dose of 500 mg/kg, a sleep prolongation factor, R (Table II), was used as the criterion for effect. For compounds not causing loss of the righting reflex, R is a measure of true potentiation and becomes significant when greater than 1.4. As apparent from Table II, most compounds with hypnotic and sedative activity also prolonged pentobarbital sleeping time significantly and thus appear to belong to Category C. However, Compounds 1 and 2, active as hypnotics and sedatives, were found to be inactive as potentiators and belong to Category A. On the other hand, Compounds 8 and 9, found to be inactive during gross screening, potentiated the sleep-

Table II—Biological Data for Cyclobutanecarbonylureas[a]

Compound Number	Compound Name	Gross[b] Effects	Barbiturate[c] Potentiation, R	CNS Activity[d] (Category)	Strychnine Lethality Test[e] Partial	Strychnine Lethality Test[e] Complete	Pentylenetetrazol Test[f]
1	1-Cyclobutanecarbonyl-3-methylurea	S	1.4	A	3/5	1/5	0/5
2	1-Cyclobutanecarbonyl-3-ethylurea	S	0.9	A	3/5	3/5	1/5
3	1-Cyclobutanecarbonyl-3-propylurea	S	1.6	C	3/5	1/5	5/5
4	1-Cyclobutanecarbonyl-3-*tert*-butylurea	S	3.5	C	5/5	1/5	5/5
5	1-Cyclobutanecarbonyl-3,3-dimethylurea	M[g]	1.9	C	5/5	5/5	0/5
6	1-Cyclobutanecarbonyl-3-allylurea	S	1.8	C	3/5	1/5	2/5
7	1-Cyclobutanecarbonyl-3-allylthiourea	I	1.5	C	5/5	1/5	5/5
8	1-Cyclobutanecarbonyl-3-phenylurea	N	4.1	B	5/5	5/5	5/5
9	1-Cyclobutanecarbonyl-3-(2,6-xylyl)urea	N	1.8	B	5/5	5/5	3/5
10	1-Cyclobutanecarbonyl-3-(α-naphthyl)thiourea	M	5.2	C	5/5	2/5	0/5

[a] In each test the dose used was 1000 mg/kg po except where indicated. [b] M, I, S, and N represent marked, intermediate, slight, and no reduction in the spontaneous motor activity, respectively. [c] Pentobarbital sodium given intraperitoneally at a dose of 50 mg/kg; R is the ratio (drug + barbiturate$_{\text{sleep time}}$)/(drug$_{\text{sleep time}}$ + barbiturate$_{\text{sleep time}}$); the drugs were given orally at a dose of 500 mg/kg. [d] A = CNS depressant ability only, B = barbiturate potentiation only, and C = both CNS depressant and barbiturate potentiation ability. [e] Strychnine nitrate, 1.6 mg/kg ip. This dose is 100% lethal. The mice die within 10–12 min. Partial = animals protected longer than 30 min from death/animals tested. Complete = animals completely protected from death/animals tested. [f] Pentylenetetrazol, 100 mg/kg sc; animals protected/animals tested. [g] Dose of 500 mg/kg po.

ing time significantly and can be associated with Category B. With Compound 8 the potentiation was extraordinary; it potentiated the sleeping time four times.

Moreover, during gross screening, Compound 5 appeared to be the most active CNS depressant. However, in the pentobarbital potentiation analysis, Compounds 4, 8, and 10 seemed to be more active than 5; of these, Compound 10 was the most active. It prolonged the sleeping time about three times more than 1-cyclobutanecarbonyl-3,3-dimethylurea. These observations lend further support to the hypothesis that two mechanistically separate modes of action exist for these compounds: one for barbiturate potentiation and the other for depressant ability. However, the data are not sufficient to allow a definite conclusion regarding the mechanism of barbiturate potentiation. The possibility that the compounds potentiate pentobarbital sleeping time *via* the inhibition of the liver microsomal enzymatic system cannot be ruled out.

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DALE D. MANESS [x] and GERALD J. YAKATAN

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Keyphrases □ Benzodiazepines—fluorescence characteristics in strong acid □ Fluorescence characteristics—10 substituted 1,4-benzodiazepines in strong acid, fluorometric and absorptiometric titrations

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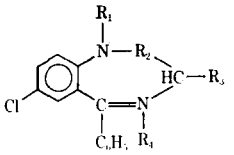


Table I—Fluorescence Characteristics and pKa Values of Substituted 1,4-Benzodiazepines Studied

Compound	R_1	R_2	R_3	R_4	pKa Fluorescence	pKa Absorbance	Fluorescence Spectral Characteristics, λ_{max} $H_0 = -10$	$H_0 = -5$
I	—	$=C(NHCH_3)-C(=O)-$	H	O	−3.5, ∼−9	−2.5, −8.7	497	525
II	H	$-C(=O)-$	H	O	−1.2, ∼−9	−1.3, −8.6	500	530
III	CH_3	$-C(=O)-$	H	O	—	−1.5, −8.0	No fluorescence	No fluorescence
IV	CH_3	$-CH_2-$	H	O	—	−2.1, −7.2	No fluorescence	No fluorescence
V	H	$-C(=O)-$	OH	—	−6.6	−2.4, −8.8	482	495
VI	H	$-C(=O)-$	H	—	−2.4, −8.2	−2.1, −8.0	482	490
VII	CH_3	$-C(=O)-$	H	—	−2.5, ∼−9	−3.0, −7.8	465	480
VIII	H	$-CH_2-$	H	—	−5.7	−1.9, −7.8	457	457
IX	CH_3	$-CH_2-$	H	—	−5.6	−1.5, −7.3	460	460
X[a]	CH_3	$-CH_2-$	H	CH_3	—	−1.5	No fluorescence	No fluorescence

[a] The azomethine group is saturated in X.

says with poor reproducibility. These problems are often attributed to the well-known dependencies of fluorescence characteristics on the solvent, solvent acidity, and charge of the emitting species. A thorough understanding of the fluorescence characteristics of drug molecules, however, can obviate many apparently undesirable aspects of fluorescence techniques, permitting the analyst to take advantage of the high sensitivity that can be realized from the successful use of fluorometry.

For example, many coumarin derivatives do not fluoresce in the 0–11 pH region but do fluoresce intensely in the Hammett acidity range (1). The analyst who does not realize that the acidity dependence of fluorescence frequently depends on the dissociation constant of the molecule in the excited state rather than (or as well as) in the ground state may well be creating a reproducibility problem where none exists. A knowledge of both pKa and pKa* values is critical to the selection of optimal conditions for fluorometric assays. An excellent review of the theory and problems involved in the choice of such conditions has been presented (2). Systematic studies of the fluorescence characteristics of drug molecules as a function of acidity are needed to provide information useful for prediction of fluorescence behavior of newly synthesized compounds.

The 1,4-benzodiazepines are widely used as tranquilizers. The ground-state acid–base equilibria of a number of 1,4-benzodiazepines have been investigated by absorption spectroscopy in the 1–13 pH region (3). Several reports have appeared concerning the fluorescence properties of 1,4-benzodiazepines (4–8). The fluorescing species in these studies were produced photochemically (4, 5), thermally in acidic solvents (6), and synthetically by derivatization (7). In only one case was the emitting species the parent compound (8), and even here the fluorescence characteristics are difficult to evaluate since the studies were run in alcoholic sulfuric, perchloric, and phosphoric acid solutions.

The physical–chemical characteristics of nitrogen heterocycles is of current interest in this laboratory. This interest resulted in the initiation of the present study—a systematic investigation of the fluorescence behavior of 1,4-benzodiazepines in strong acid solution. A series of benzodiazepines with different structures was chosen to gain insight into the structural requirements needed for fluorescence of these interesting nitrogen heterocycles. Additionally, the study encompassed the prototropic equilibria occurring in the Hammett acidity region.

EXPERIMENTAL

Apparatus—Fluorescence measurements were obtained on a recording spectrofluorometer[1] equipped with a xenon light source. Absorption spectra were recorded using a recording spectrophotometer[2].

Reagents—The 1,4-benzodiazepines[3] (Compounds I–X) were used without further purification. Analytical reagent grade sulfuric acid[4] was diluted with distilled deionized water to prepare solu-

[1] Turner model 430, G. K. Turner Associates, Palo Alto, Calif.
[2] Coleman 124, Perkin-Elmer Corp., Maywood, Ill.
[3] Compounds I–IV and VI–X were obtained from Hoffmann-La Roche, Nutley, N.J. Compound V was obtained from Wyeth Laboratories, Philadelphia, Pa.
[4] Mallinckrodt Chemical Works, St. Louis, Mo.

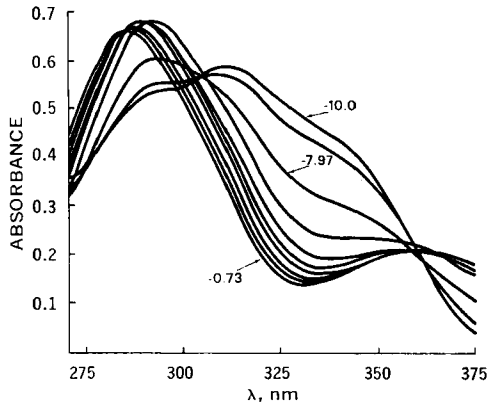


Figure 1—*Typical UV spectral changes for VII in sulfuric acid solutions. The curves are labeled as to the Hammett acidity value at which the spectrum was obtained.*

tions of varying acidity for fluorometric and absorptiometric titrations. The corrected Hammett acidity scale of Jorgenson and Hartter (9) was employed for the concentrated sulfuric acid solutions.

Solutions—All benzodiazepine solutions were prepared immediately prior to the titration and protected from light. Stock solutions approximately 10^{-2} *M* in benzodiazepine were prepared in 95% ethanol. Where the solubility of the compound in ethanol was limited, the stock solution was prepared using 10% sulfuric acid, the lowest acidity solvent used for the titration. Solutions having a final benzodiazepine concentration of approximately 10^{-4} *M* were prepared by dilution of the stock solution with the appropriate sulfuric acid solution immediately prior to obtaining the spectrum.

Stability Studies—Representative benzodiazepines were checked for stability in the strong acid solutions by the following procedure. A 10^{-3} *M* solution of the benzodiazepine was prepared in concentrated sulfuric acid ($H_0 = -10$). After 5 min at room temperature, this solution was diluted 10-fold with water to give a 10% sulfuric acid solution (10^{-4} *M* in benzodiazepine). The UV spectrum was obtained and compared to that for a 10^{-4} *M* solution prepared directly in 10% sulfuric acid. The 5-min timespan was several minutes longer than the time usually required to prepare the sample and obtain the spectrum during the titrations. Additionally, the 10^{-3} *M* solution in 100% sulfuric acid was heated for 5 min at 80° in an oil bath prior to dilution to a 10% solution and obtainment of the absorption spectrum.

6-Chloro-4-phenylquinazoline-2-carboxaldehyde, a reported acid decomposition product of oxazepam, was prepared according to the method of Bell and Childress (10). The UV absorption spectrum of this compound was also obtained in 10% sulfuric acid solution.

RESULTS AND DISCUSSION

The basic 1,4-benzodiazepine structure is shown in Table I along with the structural analogs utilized. The compounds studied were chosen to elucidate the structural requirements, if any, necessary to provide fluorescence emission. Of the 10 1,4-benzodiazepines studied, all but three fluoresced to some degree in the strongly acidic sulfuric acid media. The pertinent fluorescence characteristics of the compounds as well as the apparent pKa values obtained from both absorptiometric and fluorometric titration in strong acid are shown in Table I.

Compounds I–IV all contain the *N*-oxide group in the 4-position. Two of the four *N*-oxide benzodiazepines, I and II, exhibit fluorescence in strong acid, although of low intensity relative to other benzodiazepines studied. Compounds III and IV, which do not fluoresce in strong acid, differ significantly in structure from I and II. Compound I possesses a double bond in the 1,2-position. Compound II, through enolization, has the capability of producing the same azomethine fluorophore. Compound III, which has the carbonyl function in the 2-position like II, cannot exist in an uncharged enol form because of the methyl substituent in the 1-position. Compound IV does not possess the carbonyl function.

The remaining benzodiazepines studied reinforce the necessity for the presence of the azomethine group to provide fluorescence

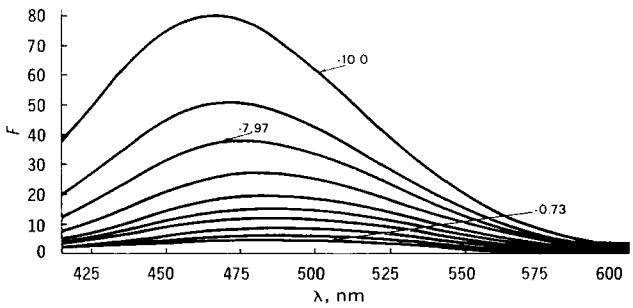


Figure 2—*Typical fluorescence spectral changes for VII in sulfuric acid solutions. The curves are labeled as to the Hammett acidity value at which the spectrum was obtained.*

in acid solution. Compounds V–IX fluoresce, whereas X, possessing neither enolization capability nor the double bond in conjunction with the aryl system, shows no fluorescence. These studies indicate that the aryl azomethine function is a requisite for fluorescence of the 1,4-benzodiazepines. The structure of the molecule being excited will also depend on the acid–base equilibria occurring in the ground state. For this reason, the prototropic reactions involved were also studied.

Figures 1 and 2 show representative spectra for the absorptiometric and fluorometric titration of VII in the Hammett acidity region. The spectral changes exhibited are representative of many of the benzodiazepines studied. As demonstrated in Fig. 1, the absorption spectra of the benzodiazepines generally show changes throughout the Hammett acidity region. Changes in the emission spectra as a function of acidity were also noted throughout the same region (Fig. 2). All of the benzodiazepines that fluoresce in strong acid show increases in fluorescence intensity with corresponding blue shifts as the acidity increases.

A typical absorptiometric titration curve for a benzodiazepine exhibiting two apparent pKa's in the Hammett acidity region is shown in Fig. 3. The fluorometric titration curves generally produced the same results as the absorbance data. Several benzodiazepines, however, did not exhibit two clearly distinct pKa's in the fluorometric titration curve when they were observed in the absorptiometric titration (Compounds III–V and VIII–X). An example of this phenomenon is shown in Fig. 4 where the fluorometric titration curve is depicted for the same compound whose absorptiometric titration appears in Fig. 3. Although two pKa's are not distinctly observable, the fluorescence change occurs over a much wider range of Hammett acidity values than would be expected for a single prototropic reaction.

The apparent pKa values determined from the titrimetric graph of the absorption and emission data (11) are given in Table I. No evidence of excited state prototropism was observed since the pKa values determined by fluorescence measurements corresponded to the pKa values obtained in the absorptiometric titrations. Spectral intensity changes were much greater for the prototropic reaction occurring in the lower Hammett acidity region than in the more

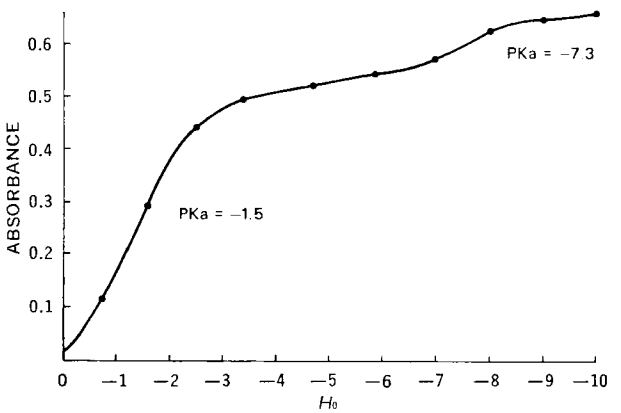
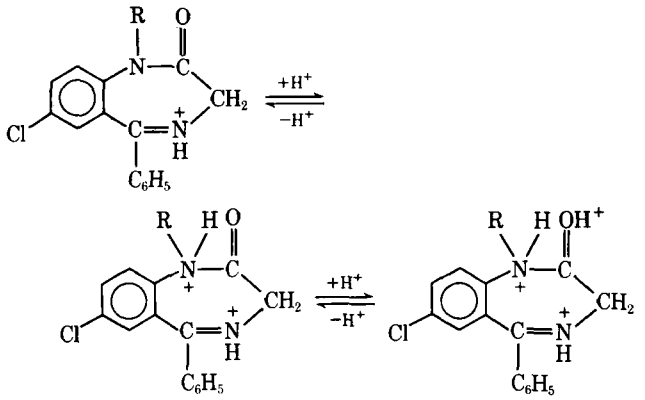


Figure 3—*Absorptiometric titration of IX in the Hammett acidity region.*



Scheme I

acidic region for I, IV, VIII, and IX (Fig. 3). Conversely, when the benzodiazepine possessed a carbonyl in the 2-position, the pKa in the strongly acidic region was very distinct and intensity changes were more pronounced than for the pKa observed in the lower Hammett acidity region. These observations may be attributed to a two-step protonation reaction for benzodiazepines having the 2-carbonyl. The first involves protonation of the 1-nitrogen—the 4-nitrogen being protonated in the pH region (3)—and the second step is carbonyl protonation. The suggested prototropic reactions are shown in Scheme I.

The benzodiazepines without proton-accepting capability in the 2-position would not be expected to show a pKa in the higher acidity region. Where a pKa is observed, the inflection in the titration curve is not very pronounced. The spectral changes observed in these higher acidities may be due to solvation effects as the medium approaches 100% sulfuric acid. If the observed spectral changes are not solvation artifacts but due to protonation, then the aromatic rings must be involved as the base moiety. This possibility

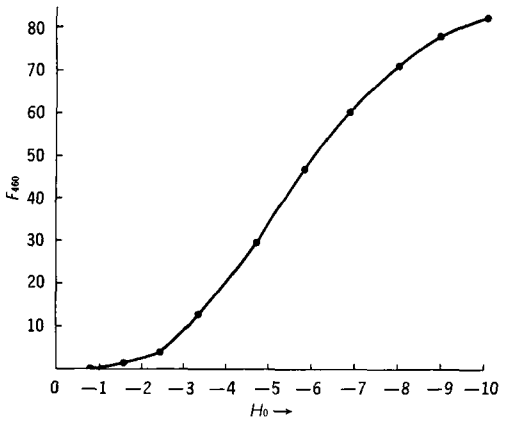


Figure 4—*Fluorometric titration of IX in the Hammett acidity region.*

seems unlikely since the positive charges residing in the portion of the molecule in conjugation with these rings should strongly decrease their basic properties. Whether protonation reaction or solvation artifact, the spectral changes are real and would be important for analytical fluorescence or absorption measurements made in the higher Hammett acidity region.

The possibility also existed that the spectral changes in the higher acidity regions were due to chemical instability of the benzodiazepines. Three compounds, V, VI, and IX, representative of the benzodiazepine structures studied were subjected to stability studies described in the *Experimental* section. The spectra obtained in 10% sulfuric acid directly as well as after treatment in 100% sulfuric acid at room temperature and at 80° were identical, indicating no apparent degradation during the timespan studied. In addition, the spectra of V and 6-chloro-4-phenylquinazoline-2-carboxaldehyde, a reported product of the acid-catalyzed isomerization of V (10), in 10% sulfuric acid are distinctly different and the formation of the degradation product would have been observed if present to any significant extent.

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Biopharmaceutic Factors in Parenteral Administration of Vitamin E

HAROLD L. NEWMARK, WILLIAM POOL, JACOB C. BAUERNFEIND, and ELMER De RITTER ˣ

Abstract □ When properly formulated, micellar-type aqueous dispersions of tocopheryl acetate are administered intravenously or intramuscularly to dogs, the rate-limiting step in the bioavailability of the physiologically active free tocopherol is the rate of hydrolysis of the acetate ester. A similar dispersion of free tocopherol yields blood levels of tocopherol many fold higher than those obtained with the acetate ester after intravenous injection and also yields much greater increases in blood levels of free tocopherol after intramuscular injection than the acetate ester formulation, particularly in the early period after the dose.

Keyphrases □ Vitamin E—biopharmaceutic factors, parenteral administration, rate-limiting step □ Tocopheryl acetate—biopharmaceutic factors, parenteral administration, rate-limiting step □ Bioavailability—tocopheryl acetate from parenteral formulations

α-Tocopherol (vitamin E) exists naturally in foods primarily in the free, unesterified form. For food fortification, pharmaceutical dosage forms, and animal supplementation, the esters, principally the acetate and succinate, are used because of their greater stability to oxidation. When administered orally to animals and humans, both esters and free α-tocopherol are fully active biologically as nutritional sources of vitamin E, indicating ready hydrolysis of the esters in the GI tract (1, 2).

Parenteral preparations containing α-tocopheryl acetate have been available commercially and for investigational use for some time, but little or no information is available concerning the kinetics of the hydrolysis needed to provide the biologically active free tocopherol from these dosage forms. A recent request from a medical research group concerned with the problems of open heart surgery prompted an investigation of this problem. During such surgery, the blood is oxygenated under high oxygen tension in an extracorporeal apparatus. This stresses the lipid antioxidant (*e.g.*, free α-tocopherol) reserves of the erythrocyte membranes, resulting in excessive, undesirable oxidative hemolysis of red cells.

The tocopherol levels in the blood simultaneously drop to very low levels, well below the acceptable minimum of 0.5 mg/100 ml. Apparently the large pools of tocopherol stored elsewhere in the body are not mobilized rapidly enough into the blood to correct this stress. Parenteral administration of *dl*-α-tocopheryl acetate in micellar aqueous dispersions surprisingly did not correct the rapid decline of the free tocopherol levels in the blood in this stress situation[1].

Rindi and Perri (3) administered a 5% aqueous emulsion of *dl*-α-tocopheryl acetate intramuscularly to humans and demonstrated a rapid increase in plasma tocopheryl acetate content, reaching a maximum at 8 hr after injection. The free tocopherol level, however, increased only slowly, reaching a maximum at about 30 hr after injection.

Table I—Formulas of Parenteral Vitamin E Products

Component	*dl*-α-Tocopheryl Acetate, 50 mg/ml	*dl*-α-Tocopherol, 50 mg/ml
dl-α-Tocopheryl acetate[a]	50 mg	—
dl-α-Tocopherol[a]	—	50 mg
Polyoxyethylated fatty acid derivative[b]	4% (w/v)	10% (v/v)
Glycerin	5% (w/v)	—
Ethyl alcohol	—	10% (v/v)
Propylene glycol	—	10% (v/v)
Benzyl alcohol	—	1% (v/v)
Thimerosal	0.1 mg	—
Disodium edetate	0.1 mg	0.1 mg
Sodium chloride	—	9 mg
Sodium acetate	0.4 mg	0.3 mg
Acetic acid	0.1 mg	2.5 mg
Water for injection, *q.s.* to	1.0 ml	1.0 ml
pH	6.0–6.6	About 4
Packaging	10-ml multiple-dose vials	2-ml single-dose ampuls

[a] Hoffmann-La Roche Inc., Nutley, N.J. [b] Emulphor EL 620, GAF Corp., New York, N.Y.

Table II—Increases in Blood Plasma Levels of Tocopherol and Tocopheryl Acetate in Dogs after Intramuscular Injection of an Aqeuous Emulsion of *dl*-α-Tocopheryl Acetate (5 mg/kg)

Blood Sample, Time after Injection	Dog Number 1106	1412	1556	Mean
Predose Level of Tocopherol, mg %				
	0.99	1.03	0.93	0.98
Increase in Level of Tocopherol, mg %				
7.5 min	0.00	0.00	0.00	0.00
15 min	0.00	0.00	0.01	0.003
30 min	0.09	0.01	0.01	0.04
60 min	0.17	0.09	0.05	0.10
2 hr	0.17	0.18	0.06	0.14
4 hr	0.31	0.26	0.15	0.21
8 hr	0.31	0.61	0.61	0.51
24 hr	0.31	0.57	0.61	0.50
Increase in Level of Tocopheryl Acetate, mg %				
7.5 min	0.3	0.5	0.1	0.3
15 min	0.3	2.0	0.9	1.1
30 min	0.6	2.9	1.2	1.6
60 min	0.7	5.0	1.6	2.4
2 hr	1.0	4.2	1.7	2.3
4 hr	1.1	4.1	1.9	2.4
8 hr	0.9	1.8	0.9	1.2
24 hr	0.2	0.1	0.4	0.2

[1] M. K. Horwitt, St. Louis University College of Medicine, personal communication.

Table III—Increases in Blood Levels of Tocopherol and Tocopheryl Acetate in a Dog after Intravenous Injection of an Aqueous Emulsion of *dl*-α-Tocopheryl Acetate (5 mg/kg)

Blood Sample, Time after Injection	Free Tocopherol, mg %	Tocopheryl Acetate, mg %
Predose	0.81	0.00
	Increase after Injection	
7.5 min	0.18	4.1
15 min	0.18	2.8
30 min	0.19	2.4
60 min	0.17	2.0
2 hr	0.21	1.7
4 hr	0.45	1.0
8 hr	0.45	1.0
24 hr	0.16	0.9

The present study was designed to obtain biopharmaceutical information for a more effective formulation of parenteral vitamin E.

EXPERIMENTAL

Materials—Aqueous dispersions of 5% *dl*-α-tocopherol NF and *dl*-α-tocopheryl acetate NF were prepared with the compositions shown in Table I. These dispersions represent modifications of formulas of injectable aqueous emulsions of fat-soluble vitamins described by Aiello and Bauernfeind (4).

These preparations are almost optically clear, have no droplets visible in a light microscope, and can be considered colloidal or micellar dispersions. The free tocopherol, being more difficult to emulsify, required more of the surfactant, a polyoxyethylated fatty acid derivative. The preparations were sterilized by bacteriological filtration through Selas candles and were aseptically subdivided into sterile ampuls and vials.

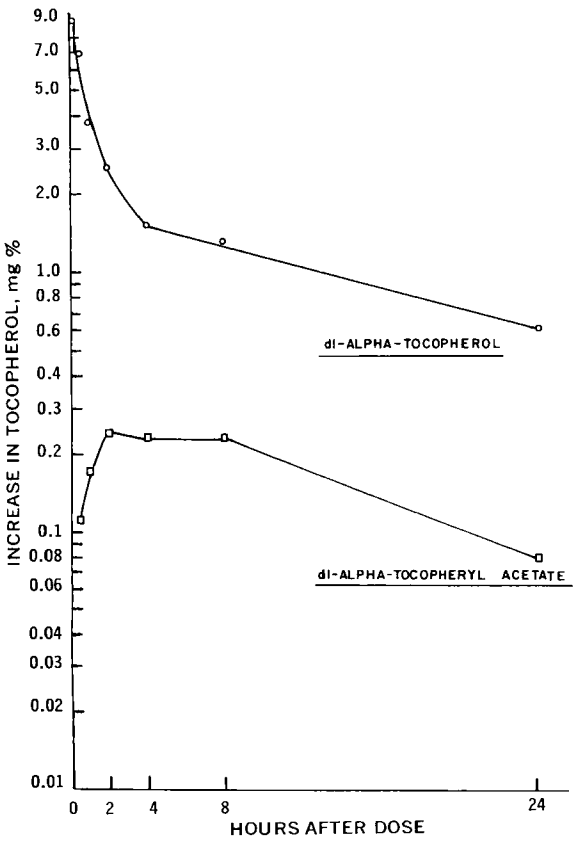

Figure 1—*Increase in blood plasma levels of tocopherol in the dog after intravenous injection of* dl-α-*tocopherol and* dl-α-*tocopheryl acetate at 5 mg/kg.*

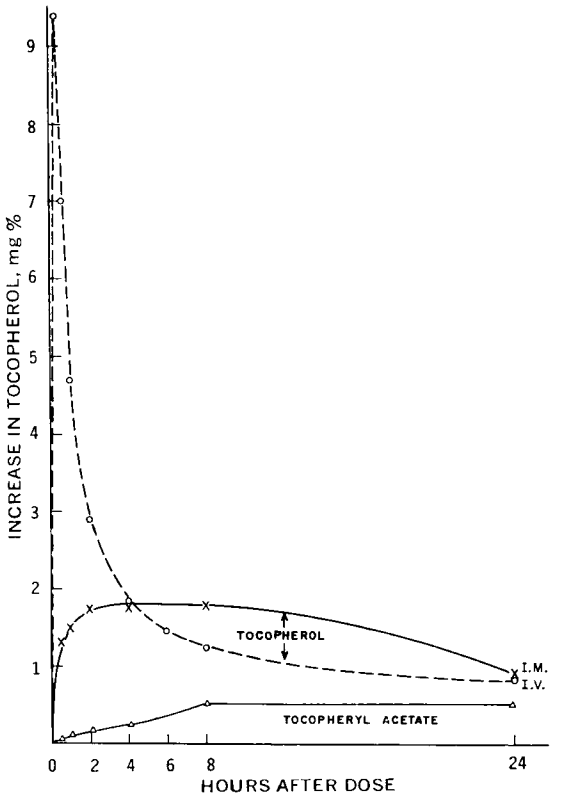

Figure 2—*Increase in tocopherol levels in dog blood plasma after injection with 5 mg/kg of* dl-α-*tocopherol (intravenous and intramuscular) or* dl-α-*tocopheryl acetate (intramuscular). Predose control levels were subtracted in each case to obtain the value shown; each curve represents the average for three dogs.*

Methods—Dogs, ~ 10 kg, were injected with 5 mg/kg of either *dl*-α-tocopherol or *dl*-α-tocopheryl acetate in the aqueous parenteral formulations. Each dog received a single injection, either intramuscularly in the gluteal muscle area or intravenously in the cephalic vein. Blood samples were drawn from each dog before drug administration and at various times after injection.

The heparinized blood samples were centrifuged and the plasma was analyzed for free tocopherol by the Emmerie–Engel color reaction utilized in the method of Quaife *et al.* (5). Plasma samples obtained from dogs that received *dl*-α-tocopheryl acetate were analyzed for free tocopherol content both before and after alkaline hydrolysis in the presence of an antioxidant, 4′-hydroxyacetanilide. The difference between these analyses represents tocopherol equivalent to the tocopheryl acetate present.

In a separate series of tests, samples of freshly drawn dog and human blood were incubated at 37° with *dl*-α-tocopheryl acetate, using the described parenteral formulation diluted 1 to 50 in 0.9% saline with 0.2 ml added to 4 ml of blood to provide a level of 5 mg/100 ml of blood. Aliquots were withdrawn at intervals and assayed for free α-tocopherol to determine the effect of whole blood in effecting hydrolysis of the acetate ester.

RESULTS AND DISCUSSION

Intramuscular injection of 5 mg/kg of *dl*-α-tocopheryl acetate in three dogs gave increases in plasma levels of free and esterified tocopherol (Table II). These data are similar to those reported for single intramuscular injections of 300 mg of an aqueous dispersion of *dl*-α-tocopheryl acetate into humans (about 5 mg/kg in 20 humans) (3). The results again demonstrate that the ester moves fairly rapidly into the bloodstream from the intramuscular site for this aqueous dispersion but that the hydrolysis to the physiologically active free tocopherol is quite slow. Apparently, little hydrolysis occurs at the injection site in the muscle or elsewhere. The similarity of these dog tests with the data of the previously mentioned human tests confirmed the suitability of the dog as an experimen-

Table IV—*In Vitro* Hydrolysis Tests of *dl*-α-Tocopheryl Acetate[a] Added to Dog or Human Blood

Sample	Hours at 37°	Dog No. 322 (Heparinized)	Dog No. 372 (Untreated)	Human Blood Sample	
				Subject 1	Subject 2
		Free Tocopherol, mg %			
Control (no tocopheryl acetate)	0	1.30	1.20	1.48	1.68
	2.0	—	—	1.43	1.64
Test (added tocopheryl acetate)	0.5	1.33	1.16	1.47	1.72
	1.0	1.36	1.07	1.43	1.66
	2.0	1.23	1.22	1.43	1.66
	3.0	1.26	1.25	—	—

[a] Aqueous emulsion (Table I) diluted 1 to 50 in 0.9% saline and 0.2 ml added to 4 ml of whole blood to provide a level of 5 mg of *dl*-α-tocopheryl acetate/100 ml.

tal animal for design of an improved parenteral formulation of vitamin E.

Intravenous administration of *dl*-α-tocopheryl acetate in a single dog at 5 mg/kg showed a similar slow hydrolysis of the ester (Table III). Although the acetate persists in the blood for many hours, only a small increase in free tocopherol levels is observed.

The increases in levels of free tocopherol in blood plasma obtained after a single intravenous administration of 5 mg/kg of *dl*-α-tocopheryl acetate (average of two dogs) or 5 mg/kg of *dl*-α-tocopherol (average of two dogs) are shown in Fig. 1. The high peak tocopherol level in the plasma of dogs receiving free tocopherol intravenously is consistent with the value expected based on the dog's blood volume. There was a many fold greater elevation of plasma levels of free tocopherol over pretreatment levels after intravenous administration of free tocopherol than with esterified tocopherol. This fact indicates again that the hydrolysis of tocopheryl acetate is the major rate-limiting step in bioavailability.

Samples of human and dog whole blood showed no significant increase in free tocopherol when inoculated with *dl*-α-tocopheryl acetate and incubated at 37° (Table IV). These results suggest that the blood itself does not have enzymes capable of rapid hydrolysis of the acetate ester.

Table V—Increase in Blood Plasma Levels of Tocopherol after Intramuscular Injection of an Aqueous Emulsion of *dl*-α-Tocopherol (5 mg/kg)

Time after Injection	Dog Number			Mean
	1103	1112	1118	
7.5 min	0.8	1.0	0.0	0.6
15 min	0.8	1.7	0.3	0.9
30 min	0.9	1.9	1.0	1.3
60 min	1.5	1.7	1.4	1.5
2 hr	1.7	2.1	1.4	1.7
4 hr	1.7	2.1	1.4	1.7
8 hr	1.7	1.9	1.9	1.8
24 hr	0.4	1.4	0.8	0.9

The data in Table V demonstrate that the emulsion of free tocopherol produces a fairly rapid and substantial rise in blood levels of tocopherol in the dog after intramuscular injection. Although the levels in the first 4 hr were lower than those obtained after intravenous administration, the area under the 24-hr curves (Fig. 2) was practically the same for both routes, indicating complete absorption of the free tocopherol from the injection site in the muscle.

Figure 2 also illustrates the much more rapid increases in blood levels of the physiologically active free tocopherol after intramuscular injection of the aqueous dispersion of free tocopherol as compared to that of tocopheryl acetate. Hydrolysis of the ester *in vivo* proceeds rather slowly, with the peak level of free tocopherol occurring 8 hr after intramuscular administration of the acetate ester and being maintained almost constant up to at least 24 hr.

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Effect of Inorganic Additives on Solutions of Nonionic Surfactants II

HANS SCHOTT ˣ and SUK KYU HAN *

Abstract □ The effect of electrolytes and urea on the cloud points of the following three nonionic polyoxyethylated surfactants was studied: cetyl, stearyl, and oleyl alcohol adducts containing 10 ethylene oxide units. The results were similar to those obtained previously with polyethylene oxide and with a polyoxyethylated alkylphenol. Nitrates of cations capable of forming stable solid complexes with model ethers like dioxane raised the cloud points in proportion to their concentrations by up to 35°. They salted the surfactants in through complexation with the ether oxygens. The order of effectiveness in raising the cloud points was lead nitrate > hydrochloric acid > cadmium nitrate > sulfuric acid ≃ magnesium nitrate > aluminum nitrate > nickel nitrate > lithium nitrate > calcium nitrate. Only the nitrates of sodium, potassium, ammonium, and cesium lowered the cloud points, salting the surfactants out. These cations do not form complexes with ether oxygens. Urea, sodium perchlorate, and sodium iodide, which break the structure of water, raised the cloud points. This salting-in process is ascribed to increased hydration of the ether groups of the polyoxyethylated surfactants due to depolymerization of water by urea and the perchlorate and iodide anions. The bromide, chloride, and sulfate of sodium lowered the cloud points. The surfactant with the alkylaryl moiety was salted in more extensively but salted out only slightly more than the three surfactants with the linear hydrocarbon chain. The data indicate the need for revising the theories of the effects of salts on the solubility of nonelectrolytes in water. None of the theories takes into account the interaction between electrolytes and nonelectrolytes, even though many nonelectrolytes compete with water as ligands for the cations.

Keyphrases □ Surfactants, nonionic—effect of inorganic additives, cloud points, salting-in and salting-out process □ Electrolytes—effect on cloud points of nonionic polyoxyethylated surfactants □ Urea—effect on cloud points of nonionic polyoxyethylated surfactants □ Inorganic additives—effect on solutions of nonionic surfactants □ Cloud points—effect of inorganic additives

Aqueous solutions of polyoxyethylated nonionic surfactants, and of polyethylene glycols which constitute their hydrophilic moiety, have an upper consolute temperature called the cloud point. Being more soluble in cold than in hot water leads to reversible phase separation on heating, and the solutions turn quite cloudy at that temperature (1–5).

Cloud points are of practical importance. Suspensions or emulsions stabilized by adsorbed nonionic surfactants tend to coagulate or coalesce, respectively, when the systems are heated to temperatures in the vicinity of the cloud point (5). This may occur, for instance, during sterilization by autoclaving. Subsequent cooling frequently fails to regenerate stable suspensions or emulsions.

Cloud points are independent of surfactant concentration between relatively wide limits (5, 6) but are markedly affected by some additives (7–10). Additives that raise cloud points salt in the polyoxyethylated compounds. By increasing the upper consolute temperature, they extend the temperature range over which homogeneous undersaturated single-phase solutions prevail.

DISCUSSION

The following equilibria are involved in the interactions that occur in the ternary system of water–electrolyte–polyoxyethylated compound, where the latter is represented by the ether linkage $R_1—O—R_2$:

$$(H_2O)_n \rightleftharpoons nH_2O$$

Scheme I

$$R_1\!\!\diagdown\!\!\diagup\!\!R_2\!\!-\!\!O + 2H_2O \rightleftharpoons R_1\!\!\diagdown\!\!\diagup\!\!R_2\!\!-\!\!O\cdot 2H_2O$$

Scheme II

$$Me^{+2} + nH_2O \rightleftharpoons [Me(OH_2)_n]^{+2}$$

Scheme III

$$XO_m^- + nH_2O \rightleftharpoons [XO_m(H_2O)_n]^-$$

Scheme IV

$$Me^{+2} + n\left(\begin{matrix}R_1\\ \diagdown\\ O\\ \diagup\\ R_2\end{matrix}\right) \rightleftharpoons \left[Me\left(\begin{matrix}O\\ \diagup\ \diagdown\\ R_1\quad R_2\end{matrix}\right)_n\right]^{+2}$$

Scheme V

Most publications on the interaction of inorganic electrolytes with aqueous solutions of polyethylene glycols (2) and polyoxyethylated nonionic surfactants (7–10) report salting out or cloud point lowering *via* dehydration, as predicted by theory (11, 12). The competition for water of hydration between an electrolyte (Scheme III for a divalent cation and Scheme IV for a monovalent, oxygenated anion) and the polyoxyethylated compound (Scheme V) leads to precipitation of the latter. An ether linkage binds up to two water molecules (13).

The following few instances of salting in or cloud point increases by electrolytes were reported for nonionic polyoxyethylated surfactants prior to publication of Part I of the present series (14). Large anions such as thiocyanate and iodide (9, 15–17), which are polarizable and constitute soft bases in Pearson's nomenclature (18), as well as perchlorate (17), which constitutes a hard base, are effective in destructuring water. They increase the hydration of polyoxyethylated compounds (Scheme II) by promoting the depolymerization of water (Scheme I). Acids (7, 9) and the nitrates of beryllium, magnesium, and calcium (15) also raised the cloud points of a nonionic surfactant, but the mechanism was disputed (15, 19).

A systematic investigation of the effect of cations on the cloud points of aqueous solutions of a branched octylphenol with 10 ethylene oxide units (Surfactant I)[1] and of an ethylene oxide polymer with an average degree of polymerization of 340–455 was made, using mostly nitrates. The nitrate ion has little or no tendency to act as a ligand for the cation, as opposed to the chloride ion. The results showed the importance of complexation of the cations by the ether linkages as represented by Scheme V. All cations that form complexes with model ethers such as dioxane, ethyl ether, and cineole (20–23) salted the two polyoxyethylated compounds in, increasing their cloud points. Included among the complexing

[1] Triton X-100, Rohm and Haas Co.

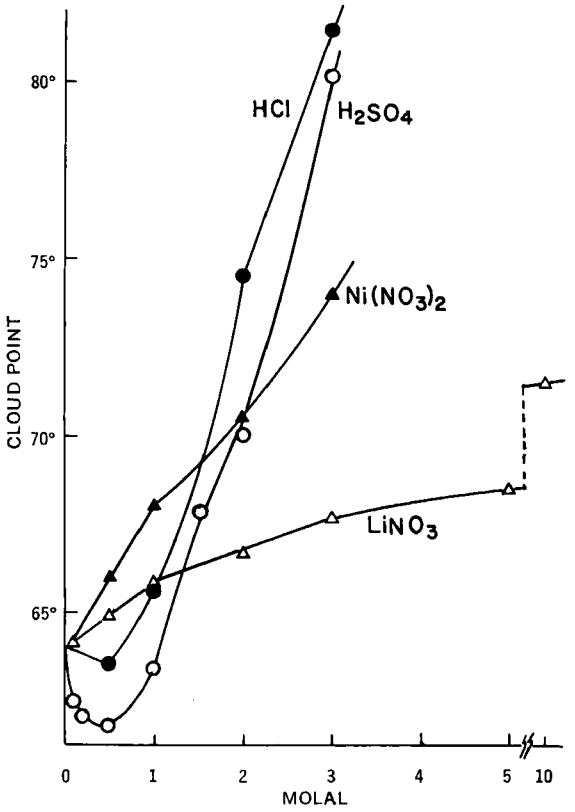

Figure 1—*Cloud point of a 1.00% solution of Surfactant II as a function of the molal concentrations of added electrolytes.*

cations were hydrogen, lithium, and all di- and trivalent cations tested. Only the monovalent cations sodium, potassium, and ammonium, whose salts do not form stable complexes with dioxane and other model ethers, lowered the cloud points of the two polyoxyethylated compounds because the cations could not form complexes with the ether oxygens of the latter. Salting out by electrolytes was found to be the exception rather than the rule, as had previously been thought (1-5, 8-12). Since dioxane can act as a bidentate ligand for cations (23), the flexible polyoxyethylene chains with their many ether linkages probably act as polydentate ligands in the complexation of the cations according to Scheme V (14).

The purpose of the present work was to extend the study of the effect of electrolytes on the cloud points of polyoxyethylated nonionic surfactants to surfactants whose hydrophobic moiety is a normal alkyl chain rather than a branched alkylaryl group as in Surfactant I. By comparing the behavior of purely aliphatic derivatives with that of an aliphatic–aromatic compound, it is possible to determine whether the aromatic ring produces specific effects in the interaction between electrolytes and nonionic surfactants. Alkali salts of anions capable of breaking and of enhancing the iceberg structure of water were also included. The results for the various compounds can be used to evaluate current theories of salt effects on nonelectrolyte solutions (11, 12).

EXPERIMENTAL

The aliphatic surfactants were ethylene oxide adducts of cetyl, stearyl, and oleyl alcohols containing an average of 10 ethylene oxide units[2]. The stearyl derivative was a waxy solid, and the other two compounds were viscous liquids or semisolids, depending on the room temperature. The properties of the four nonionic surfactants are summarized in Table I. Water was double distilled. Urea was NF grade, and all other chemicals were ACS reagent grade.

The concentrations of the ternary mixtures are expressed as molality or as percent (w/w) based on the amount of water present.

[2] Supplied by I.C.I. America (formerly Atlas Chemical Co.) as Brij 56, 76, and 96, respectively.

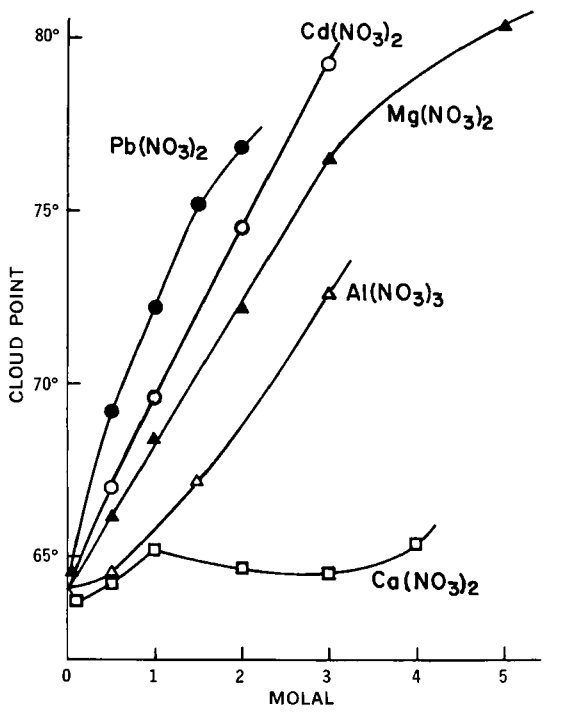

Figure 2—*Cloud point of a 1.00% solution of Surfactant II as a function of the molal concentrations of added electrolytes.*

The surfactant level was 1%. The concentration used for the alkylaryl surfactant in the previous study was 2% (14), but this difference caused only small differences in the cloud point (5, 6).

The methods for assaying the electrolyte content of the concentrated stock solutions, for preparing the ternary mixtures, and for measuring the cloud points are described in Ref. 14 with the following exception. The concentrated stock solutions of sodium bromide, iodide, and sulfate and of potassium and cesium nitrate were analyzed by drying aliquots to constant weight.

RESULTS

The effect of electrolytes on the cloud points of the polyoxyeth-

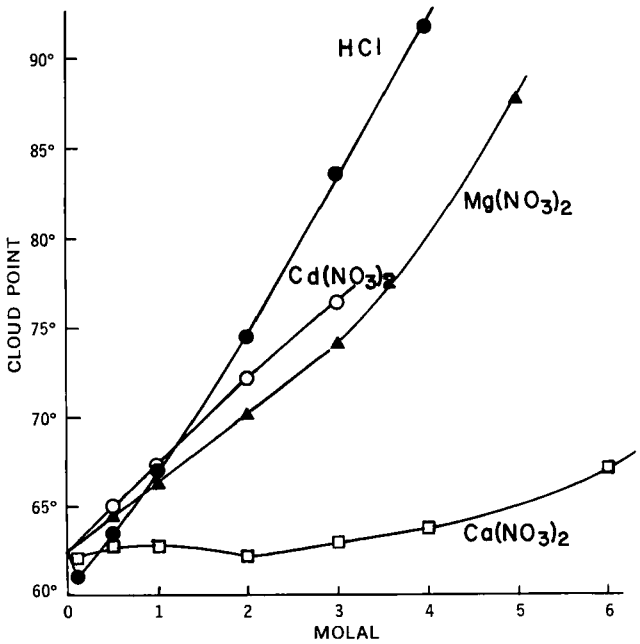

Figure 3—*Cloud point of a 1.00% solution of Surfactant III as a function of the molal concentrations of added electrolytes.*

Table I—Properties of Nonionic Surfactants Employed in the Study of Electrolyte Effects

Surfactant Number (and Formula)	Hydrophobic Moiety	Number of Ethylene Oxide Units	HLB	Cloud Point[a]	Critical Micelle Concentration at 25°	
					g/liter	moles/liter × 10^6
I [C_8H_{17}—$C_6H_4O(CH_2CH_2O)_{10}H$]	Branched octylphenol	9–10	13.4	65°	0.16	256
II [$C_{16}H_{33}O(CH_2CH_2O)_{10}H$]	Cetyl alcohol	10	12.9	64°	—	—
III [$C_{18}H_{37}O(CH_2CH_2O)_{10}H$]	Stearyl alcohol	10	12.4	62.5°	—	—
IV [$C_{18}H_{35}O(CH_2CH_2O)_{10}H$]	Oleyl alcohol	10	12.4	54°	0.065	92

[a] At 1% (w/w).

Table II—Electrolyte Concentrations Required to Raise the Cloud Points of Surfactant Solutions by 10°, in Order of Decreasing Effectiveness for Salting In

Surfactant I[a]		Surfactant II[b]		Surfactant III[b]		Surfactant IV[b]	
Electrolyte	Molality	Electrolyte	Molality	Electrolyte	Molality	Electrolyte	Molality
$Pb(NO_3)_2$	0.68	$Pb(NO_3)_2$	1.30	HCl	1.75	$Pb(NO_3)_2$	1.72
$Cd(NO_3)_2$	1.31	$Cd(NO_3)_2$	1.90	$Pb(NO_3)_2$	1.78	$Cd(NO_3)_2$	1.72
HCl	1.47	HCl	1.95	$Cd(NO_3)_2$	2.09	HCl	1.89
$Mg(NO_3)_2$	1.55	$Mg(NO_3)_2$	2.38	$Al(NO_3)_3$	2.19	H_2SO_4	2.07
H_2SO_4	1.70	H_2SO_4	2.40	H_2SO_4	2.24	$Mg(NO_3)_2$	2.07
$Al(NO_3)_3$	1.86	$Ni(NO_3)_2$	3.00	$Mg(NO_3)_2$	2.63	$Al(NO_3)_3$	3.00
$Ni(NO_3)_2$	2.0	$Al(NO_3)_3$	3.35	$Ni(NO_3)_2$	3.06	$Ni(NO_3)_2$	3.06
$LiNO_3$	4.6	$LiNO_3$	>10	$LiNO_3$	—	$LiNO_3$	10.7
$Ca(NO_3)_2$	4.7	$Ca(NO_3)_2$	—	$Ca(NO_3)_2$	—	$Ca(NO_3)_2$	—

[a] At 2% (w/w). [b] At 1% (w/w).

ylated surfactants is presented in two parts. Salts of cations that form coordination compounds with ethers are discussed first, followed by salts of cations that do not form such complexes. Urea is included in the second category.

Salting In by Complexation—Table I of Ref. 14 lists the salts of all polyvalent and of most monovalent cations used in the present study, together with their stable solid complexes in which dioxane or dioxane plus water are the ligands. Dioxane was chosen as the model ether because its adducts are more stable than those of ethyl and other monofunctional ethers (20, 21). Dioxane can act as a bidentate ligand. Hydrogen, lithium, magnesium, calcium, nickel, cadmium, aluminum, and lead form complexes with ethers. The effect of their nitrates on the cloud points of Surfactants II–IV is shown in Figs. 1–6. Hydrochloric and sulfuric acids were chosen over nitric acid to avoid oxidative degradation of the surfactants.

The effectiveness of the various electrolytes in salting in the four nonionic surfactants is rated according to two criteria. In Table II, the interpolated electrolyte concentrations required to boost the cloud point of the nonionic surfactant solutions by 10° are listed in increasing order. In Table III, the interpolated increases in cloud point achieved by the additives at the 2-molal level are listed in decreasing order. Cloud points in the presence (14) and absence (5, 6, 8) of additives were insensitive to surfactant concentrations between 1 and 4% (w/w). Since some of the cloud point–electrolyte concentration curves cross over one another, the two criteria do

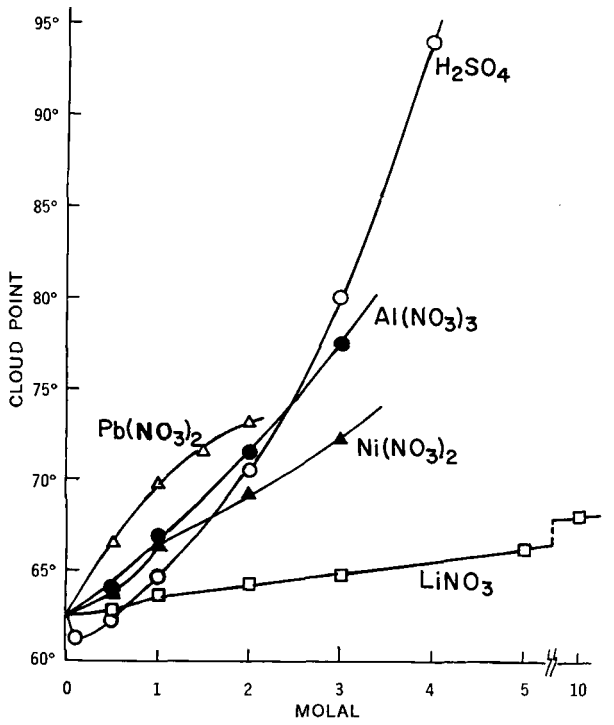

Figure 4—*Cloud point of a 1.00% solution of Surfactant III as a function of the molal concentrations of added electrolytes.*

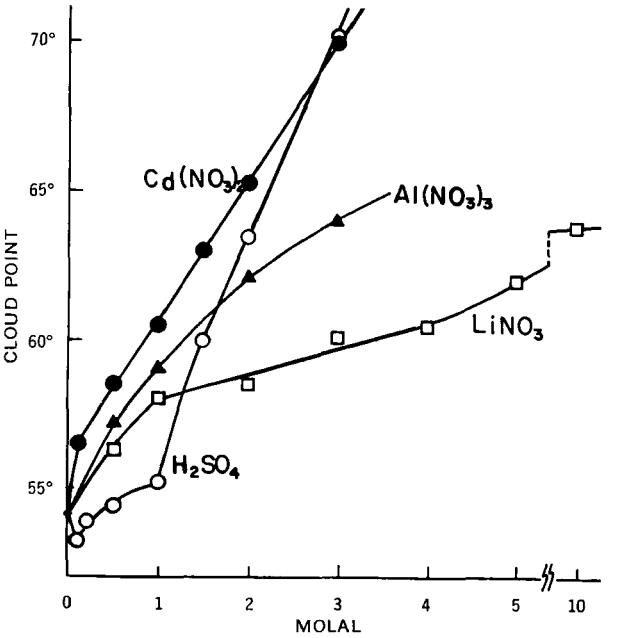

Figure 5—*Cloud point of a 1.00% solution of Surfactant IV as a function of the molal concentrations of added electrolytes.*

Table III—Cloud Point Increases Produced by Salting-In Electrolytes at 2-Molal Concentrations, in Order of Decreasing Effectiveness

Surfactant I[a]		Surfactant II[b]		Surfactant III[b]		Surfactant IV[b]	
Electrolyte	ΔC.P.[c]	Electrolyte	ΔC.P.[c]	Electrolyte	ΔC.P.[c]	Electrolyte	ΔC.P.[c]
HCl	15.0°	$Pb(NO_3)_2$	12.9°	HCl	12.0°	HCl	11.4°
$Pb(NO_3)_2$	14.6°	$Cd(NO_3)_2$	10.5°	$Pb(NO_3)_2$	10.7°	$Cd(NO_3)_2$	11.3°
H_2SO_4	14.5°	HCl	10.5°	$Cd(NO_3)_2$	9.6°	$Pb(NO_3)_2$	11.2°
$Cd(NO_3)_2$	14.3°	$Mg(NO_3)_2$	8.2°	$Al(NO_3)_3$	9.0°	$Mg(NO_3)_2$	9.7°
$Mg(NO_3)_2$	12.5°	$Ni(NO_3)_2$	6.5°	H_2SO_4	8.0°	H_2SO_4	9.5°
$Al(NO_3)_3$	10.4°	H_2SO_4	6.0°	$Mg(NO_3)_2$	8.0°	$Al(NO_3)_3$	8.1°
$Ni(NO_3)_2$	9.9°	$Al(NO_3)_3$	4.8°	$Ni(NO_3)_2$	6.7°	$Ni(NO_3)_2$	7.1°
$LiNO_3$	6.1°	$LiNO_3$	2.7°	$LiNO_3$	1.7°	$LiNO_3$	4.5°
$Ca(NO_3)_2$	2.0°	$Ca(NO_3)_2$	0.7°	$Ca(NO_3)_2$	−0.3°	$Ca(NO_3)_2$	−0.3°

[a] At 2% (w/w). [b] At 1% (w/w). [c] ΔC.P. = cloud point in 2-molal electrolyte solution minus cloud point in water.

not rank all electrolytes in the same order of salting-in efficacy even for a single surfactant. The overall order is lead nitrate > hydrochloric acid > cadmium nitrate > sulfuric acid ≅ magnesium nitrate > aluminum nitrate > nickel nitrate > lithium nitrate > calcium nitrate.

Of the four nonionic surfactants, I was salted in the most extensively and II the least. Surfactant I required less electrolyte for a 10° boost in cloud point than II, and 2-molal electrolytes raised its cloud point more than that of II. Surfactant IV was somewhat more extensively salted in than III. This difference between the four surfactants is not due to differences in the hydrophilic-lipophilic balance (HLB) since Surfactant I has the highest value and III and IV have the lowest. Moreover, the four HLB values differ by one unit only.

Apparently, the presence of the benzene ring enhanced the ability of Surfactant I to be salted in by the electrolytes. This conclusion is tentative because the cloud point of surfactants with a given hydrophobic moiety depends not only on the HLB but on the chain-length distribution of the ethylene oxide moiety as well (6). Since Surfactants I and II–IV were supplied by two different manufacturers, their polyoxyethylene chain-length distribution may well differ due to different ethoxylation conditions. Another difference is that the alkyl group of Surfactant I is branched while those of Surfactants II–IV are normal. Branching affects the cloud point (6) and may also affect the increase in cloud point by complexation with cations.

Salting In and Out by Noncomplexing Additives—Of the cations studied in Part I (14) and in the present work, only sodium, potassium, ammonium, and cesium do not form stable solid adducts with ethers (20–23). Salts of these cations as a rule salt out nonelectrolytes, except if the anions are effective structure breakers for water such as iodide, thiocyanate, and perchlorate (9, 15–17, 24). The influence of these salts on the cloud points of the nonionic surfactants will be described. Another compound well known for its ability to break the structure of water and to weaken hydrophobic bonds, namely, urea (25), was also included.

The data for Surfactants II–IV are plotted in Figs. 7–9. The effectiveness of the various noncomplexing additives in salting the four surfactants in or out are rated according to the same two criteria used for the salting-in effectiveness of the complexing electrolytes. Table IV lists the additive concentrations required to raise or lower the cloud points by 10°, while Table V lists the increases or decreases in cloud points achieved by the additives at the 2-molal level.

Surfactant I was only slightly more susceptible to salting out by

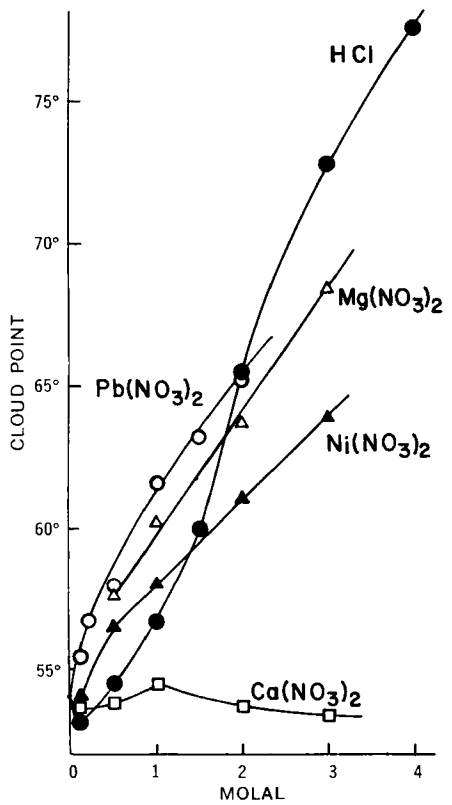

Figure 6—*Cloud point of a 1.00% solution of Surfactant IV as a function of the molal concentrations of added electrolytes.*

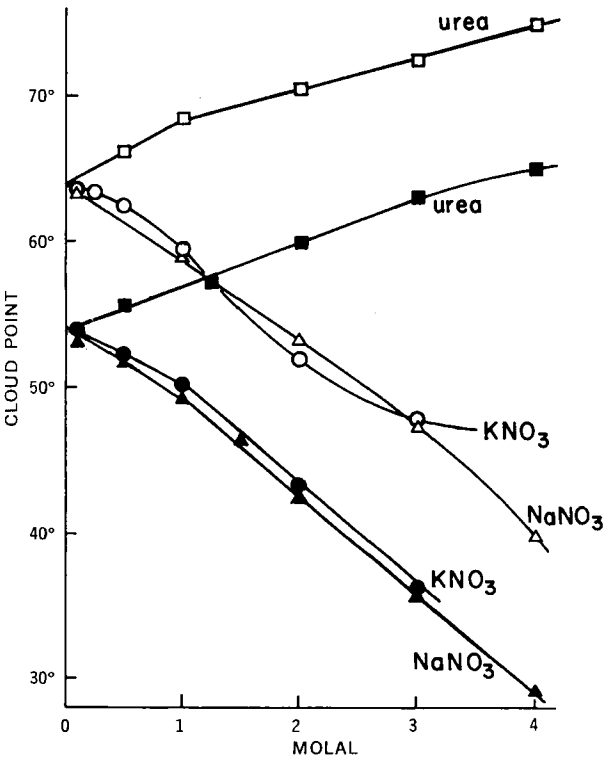

Figure 7—*Effect of the concentrations of noncomplexing additives on the cloud point of 1.00% solutions of Surfactant II (open symbols) and Surfactant IV (dark symbols).*

Table IV—Additive Concentrations Required to Raise or Lower the Cloud Points of Surfactant Solutions by 10°, in Order of Decreasing Effectiveness for Salting In or Increasing Effectiveness for Salting Out

Surfactant I[a]		Surfactant II[b]		Surfactant III[b]		Surfactant IV[b]	
Additive		Additive	Molality for 10° Increase	Additive	Molality for 10° Increase	Additive	Molality for 10° Increase
—		Urea	3.64	$NaClO_4$	0.50	Urea	3.41
				NaI	1.87		
				Urea	4.80		
	Molality for 10° Reduction		Molality for 10° Reduction		Molality for 10° Reduction		Molality for 10° Reduction
$NaNO_3$	1.58	$NaNO_3$	1.86	$CsNO_3$	2.13	KNO_3	1.91
		KNO_3	1.65	NaBr	2.00	$NaNO_3$	1.89
				$NaNO_3$	1.90		
				KNO_3	1.87		
				NaCl	0.71		
				Na_2SO_4	0.20		
				Na_2SO_4[c]	0.50		

[a] At 2% (w/w). [b] At 1% (w/w). [c] At ionic strength equivalent to 2 molal NaCl.

sodium nitrate than the other three. It required a somewhat lower salt concentration to reduce the cloud point by 10°, and 2-molal sodium nitrate lowered its cloud point by only 1–2° more than those of the other three surfactants.

The differences in the salting-out efficacy among the nitrates of the noncomplexing alkali metals are rather small. The cations are ranked in the order $Na^+ \cong K^+ > Cs^+$. The differences in the ability of the various anions to salt the nonionic surfactants in or out and the resulting spread in cloud points are far greater than the differences in the ability of the three noncomplexing cations to salt them out. This is shown by comparing the effect of the various sodium salts on the cloud points. The order of decreasing efficacy for salting in or increasing efficacy for salting out is sodium perchlorate > sodium iodide > sodium bromide ≅ sodium nitrate > sodium chloride > sodium sulfate. The spread in the cloud point of Surfactant III in the presence of these six salts exceeds 50°. The great influence of the anions on the process of salting in or out was also observed by Schick (9) and Saito (17). The order of efficacy of the anions is that of the Hofmeister or lyotropic series (26). The lower the lyotropic numbers of the anions, the greater is their ability to reduce the cloud point (9).

The three noncomplexing cations are also ranked according to the lyotropic series, but lithium and the polyvalent cations, which form complexes with the ether oxygens, are located *ipso facto* at the wrong end of that series. Urea raised the cloud points, as expected in view of its ability to reduce the structure of water.

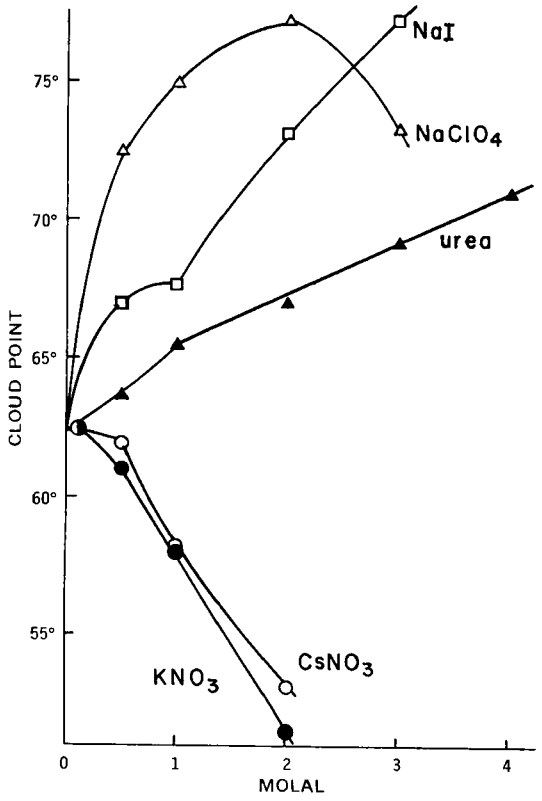

Figure 8—*Effect of the concentrations of noncomplexing additives on the cloud point of a 1.00% solution of Surfactant III.*

THEORY

The present study provides a large body of data on the salting in and salting out of four nonelectrolytes by a variety of electrolytes. Each nonelectrolyte consists of a nonpolar hydrocarbon moiety and of a polar polyoxyethylene moiety. It is of interest to use these data to evaluate current theories of the effects of electrolytes on the solubility of nonelectrolytes.

Electrostatic Theories—The electrostatic theories of Debye and McAulay and of Debye (11, 12, 27) describe salting in and out in terms of dielectric effects. They predict that a given nonelectrolyte is salted in by all electrolytes if it raises the dielectric constant of the solvent. If it lowers the dielectric constant, it is salted out by all electrolytes. The electrostatic theories do not cover the present

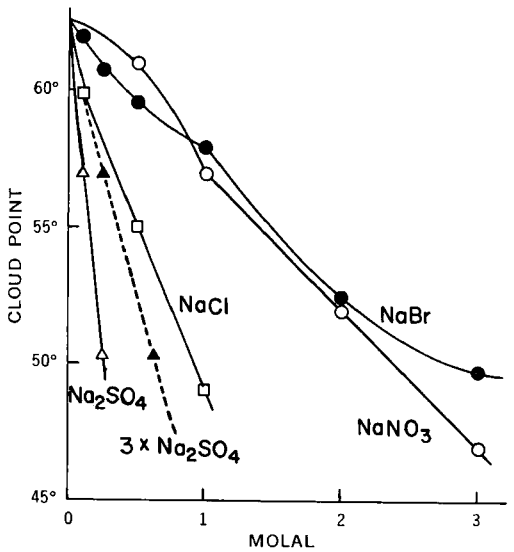

Figure 9—*Effect of the concentrations of noncomplexing electrolytes on the cloud point of a 1.00% solution of Surfactant III.*

Table V—Cloud Point Increases or Decreases Produced by Additives at 2-Molal Concentrations, in Order of Decreasing Effectiveness for Salting In or Increasing Effectivenes for Salting Out

Surfactant I[a]		Surfactant II[b]		Surfactant III[b]		Surfactant IV[b]	
Additive	ΔC.P.[c]	Additive	ΔC.P.[c]	Additive	ΔC.P.[c]	Additive	ΔC.P.[c]
		Urea	6.5°	$NaClO_4$	14.7°	Urea	6.0°
				NaI	10.7°		
				Urea	4.5°		
$NaNO_3$	−13.0°	$NaNO_3$	−10.8°	$CsNO_3$	−9.4°	KNO_3	−11.0
		KNO_3	−12.0°	NaBr	−10.0°	$NaNO_3$	−11.8°
				$NaNO_3$	−10.5°		
				KNO_3	−11.0°		
				NaCl	≈ −25.5°		
				Na_2SO_4	< −55°		
				Na_2SO_4[d]	≈ −37°		

[a] At 2% (w/w). [b] At 1% (w/w). [c] ΔC.P. = cloud point in 2-molal additive minus cloud point in water. [d] At ionic strength equivalent to 2 molal NaCl.

case where some electrolytes raised the cloud points of the polyoxyethylated surfactants, salting them in, whereas other electrolytes of the same valence lowered the cloud points, salting them out. The electrostatic theories are inadequate, because they fail to consider the electrolyte–nonelectrolyte interactions represented by Scheme V, as well as the complex structure of water, which causes certain anions to interfere with the process represented by Scheme I.

Internal Pressure Theories—The internal pressure concept was applied to salting out and in as follows (11, 28). The ion–water interaction of dissolved electrolytes causes electrostriction of water, increasing its internal pressure. When salts are added to the aqueous solution of nonelectrolytes, the increase in internal pressure tends to squeeze out the nonelectrolyte molecules from the solution, reducing their solubility or salting them out. The extent of electrostriction of water by an electrolyte can be represented by the difference between the molal volume of the pure "liquid" electrolyte, V_e, and the partial molal volume of the electrolyte at infinite dilution, $\bar{V}_e^0$.

The relationship derived by McDevit and Long (11, 28) is:

$$\ln \frac{S_n^0}{S_n} = \left[\frac{\bar{V}_n^0(V_e - \bar{V}_e^0)}{\beta RT}\right] C_e \qquad \text{(Eq. 1)}$$

where S_n^0 and S_n are the solubilities of the nonelectrolyte in water and in an electrolyte solution, respectively; β is the compressibility of water; C_e is the molar electrolyte concentration: and $\bar{V}_n^0$ is the partial molal volume of the nonelectrolyte at infinite dilution. The nonelectrolyte molecules modify the ion–water interaction merely by occupying volume, and the extent of salting in or out increases with their molecular size. Accordingly, the theory should be applied only to nonpolar solutes, because they have scant affinity toward water and electrolytes, but it was found to hold approximately for polar solutes as well (11).

According to Eq. 1, if $V_e > \bar{V}_e^0$, the solvent medium is compressed by the electrolyte and $S_n^0 > S_n$, *i.e.*, salting out occurs. If $\bar{V}_e^0 > V_e$, the ions loosen the medium and the nonelectrolyte is salted in.

By combining V_e values obtained indirectly with $\bar{V}_e^0$ values, the difference $V_e - V_e^0$ was found to be positive for most electrolytes reported, including hydrochloric acid, lithium chloride, lithium iodide, sodium perchlorate, sodium iodide, barium chloride, and cerium (III) chloride (28, 29). Since these salts either raised the cloud points of the nonionic surfactants or are expected to do so in the light of the present results, the internal pressure theory does not have the general application with which it is often credited.

In their review paper on salt effects, Long and McDevit discussed the change in the order of the cations found with basic polar nonelectrolytes like ammonia and trimethylamine as compared to the order for nonpolar nonelectrolytes. They correctly ascribed the inversion in the salting-in and salting-out order observed with the former to chemical interaction between the cations and the nonelectrolytes, specifically, to complex ion formation (11).

Hydration Theories—Hydration theories and theories based on the role of water structure in the salting in and out of nonelectrolyes by electrolytes (11, 30, 31) are either not general enough or not explicit enough to describe the present data.

Conclusions—None of the existing theories of salting in and out adequately describes the effects of electrolytes on polyoxyethylated compounds dissolved in water, mainly because they neglect electrolyte–nonelectrolyte interactions. Many nonelectrolytes are capable of acting as ligands for cations in aqueous solution, in competition with water. Thus, a comprehensive theory would have to include all five simultaneous processes represented by Schemes I–V.

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Drug Absorption VII: Influence of Mesenteric Blood Flow on Intestinal Drug Absorption in Dogs

WILLIAM G. CROUTHAMEL ˣ, LOUIS DIAMOND, LEWIS W. DITTERT, and JAMES T. DOLUISIO *

Abstract □ Intestinal absorption of sulfaethidole and haloperidol was determined using an *in situ* canine intestinal preparation. Intestinal absorption of sulfaethidole was determined at three or four mesenteric blood flow rates in each dog, ranging from unaltered flow (100%) to no flow (0%). A relatively small change in absorption rate occurred when the splanchnic blood flow rate was decreased about 35%. Further reductions in mesenteric blood flow resulted in progressive impairment of sulfaethidole absorption. The simultaneous measurement of sulfaethidole intestinal disappearance and appearance in blood indicates that sulfaethidole disappearance is equivalent to absorption. Haloperidol absorption also decreased with decreased intestinal perfusion but differed from sulfaethidole in that membrane storage of haloperidol appeared to take place during its absorption.

Keyphrases □ Absorption, drug—influence of mesenteric blood flow on intestinal absorption of sulfaethidole and haloperidol, *in situ* canine intestinal preparation □ Drug absorption—influence of mesenteric blood flow on intestinal absorption of sulfaethidole and haloperidol, *in situ* canine intestinal preparation □ Sulfaethidole—influence of mesenteric blood flow on intestinal absorption, *in situ* canine intestinal preparation □ Haloperidol—influence of mesenteric blood flow on intestinal absorption, *in situ* canine intestinal preparation □ Blood flow, mesenteric—influence on intestinal absorption of sulfaethidole and haloperidol, *in situ* canine intestinal preparation

Interest in the influence of blood flow on the GI absorption rate of drugs was stimulated by the observation (1) that periods of fasting greater than 17 hr in rats resulted in decreased rates of drug absorption. This decrease in absorption was accompanied by a concomitant blanching of the intestine, which became more pronounced with longer fasting periods. The observed changes appeared to be due to changes in intestinal blood perfusion (2).

There are many documented cases of pathological conditions leading to decreased intestinal blood perfusion. One study (3) showed that experimentally induced ventricular and supraventricular tachycardias can cause mean superior mesenteric blood flow to drop nearly 40%. Other investigators (4) reported that hemorrhagic necrosis, infarction, and gangrene of the intestinal tract secondary to decreased splanchnic perfusion may result from rapid tachycardia. A high association was reported (5) between congestive heart failure and nonobstructive intestinal ischemia. In one case, intestinal ischemia resulting from congestive heart failure produced digoxin malabsorpion severe enough to make the patient's clinical condition difficult to control (6). Intramuscular administration of digitoxin produced marked improvement in the patient. These reports suggest that the absorption of vital drugs in patients with circulatory pathology may be dangerously reduced.

BACKGROUND

It has long been known that heat and exercise produce a significant reduction in splanchnic blood flow (7–9). A study with five volunteers found that light exercise for 7–8 min decreased splanchnic blood flow by 20% (8). A fall in hepatic and splanchnic blood flow, sometimes in excess of 80%, during severe exertion was clearly demonstrated (9). Thus, it is conceivable that drug absorption following three sets of tennis or during strenuous exercise on the job could be quite different from that encountered under more restful circumstances. In this regard, exercise in a hot environment significantly decreased the absorption of 3-*O*-methylglucose but not D-xylose (10). Thus, exercise or the lack of it during bioavailability studies (studies of drug product performance) could greatly influence the apparent availability and rate of absorption of the product.

The interaction between the circulation and intestinal absorption is complex; blood flow influences absorption, but the reverse also holds true. Digitalis has been shown to be a potent splanchnic vasoconstrictor, and cardiac glycosides have precipitated intestinal necrosis (11). A reduction in splanchnic blood flow of 30–40% was found in human volunteers following a 0.25-mg dose of ouabain (12). The same effect appears to be true for digitalis (12).

The use of oral contraceptives in women also appears to affect significantly the mesenteric blood flow. Numerous reports have appeared in the last 10 years (13–15) linking the use of oral contraceptives in women to decreased intestinal blood flow. Of 24 collected cases of ischemic disease of the colon in younger persons, six were women taking oral contraceptives (13). The role of oral contraceptives in vascular diseases is unclear, and possible associated

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factors include alterations in the clotting mechanism, thickening of the vessel wall (16), and decreased blood flow (17). In some of the cases reported (14, 15), the clinical symptoms disappeared upon discontinuance of the medication but returned following its reinstitution. Therefore, changes in splanchnic blood flow produced by one drug could significantly influence the rate of absorption of a concurrently administered drug.

Despite the paucity of information available concerning the influence of intestinal blood flow on drug absorption, several recent studies have appeared concerning this topic. Most of these studies dealt with the absorption of nutrients and entailed interruption of the circulation either by collection of the mesenteric venous blood or by excorporeal perfusion of the intestine. By far the most complete study was performed by Winne and coworkers (18, 19), in which intestinal blood flow was regulated by varying the rate of blood infusion through the jugular vein of the rat, with absorption being determined by mesenteric venous collection. These investigators found that the intestinal absorption of aminopyrine, antipyrine, benzoic acid, salicylic acid, and ethanol was dependent on intestinal blood flow while ribitol was independent of blood flow (18, 19). These investigators also demonstrated the influence of blood flow on intestinal water flux (20) and on the absorption of an actively absorbed amino acid, phenylalanine (21).

A study of the absorption and metabolism of salicylamide in the rabbit found both of these processes to be profoundly influenced by intestinal blood flow (22). In an interesting study (23), partial ligation of the canine superior mesenteric artery *in vivo* resulted in the induction of a chronic malabsorption state characterized by decreased absorption of D-xylose and triolein. The intestinal absorption of the cardiac glycosides digitoxin and digoxin in the guinea pig was strongly dependent on portal blood flow (24). Williams *et al.* (10) reported that the absorption of glucose and xylose was significantly reduced when mesenteric blood perfusion *via* a pump was reduced 50%.

This report discusses in detail studies of the effects of an induced reduction in mesenteric blood flow on the rates of absorption of sulfaethidole and haloperidol from the lumen of intestinal loops, *in situ*, in the anesthetized dog.

EXPERIMENTAL

Test Animals—Mongrel dogs of either sex, 10–19 kg, were rendered free of parasites and held under the care of a veterinarian for 24 days prior to use. All animals were fasted for 16 hr prior to surgery, with water freely available.

Surgical Preparation—General anesthesia was induced 1 hr prior to surgery with 0.6 ml/kg iv allobarbital–urethan[1] (100 mg of allobarbital and 400 mg of urethan/ml). If additional anesthesia was needed during the surgical procedure, the animal received one-fourth to one-half of the initial anesthetizing dose. Following the onset of anesthesia, an endotracheal tube was inserted. Respiration was normally spontaneous and rhythmical but was supported mechanically when necessary.

A polyethylene catheter was inserted into the femoral artery to monitor arterial pressure. A venous catheter was inserted into the vena cava *via* the femoral vein for the removal of blood samples and administration of drugs or replacement solutions. Core body temperature was monitored rectally and maintained constant with a heating pad directly under the animal. Arterial blood pressure was measured using a linear core pressure transducer and recorded by a direct writing polygraph. Mean arterial blood pressure was normally maintained above 80 mm Hg. When mean arterial blood pressure dropped below this value for a significant period, the experiment was terminated and the animal was sacrificed. In cases where blood specimens were taken, blood volume was replaced with saline or dextran solution. This amount was usually 7% or less of total blood volume.

Implantation of Blood Flow Probe—Following a midline laparotomy, the entire small intestine was gently removed from the abdominal cavity, with care being taken not to disturb intestinal blood flow. The intestine was kept moist and warm during the remainder of the procedure. The superior mesenteric artery, the only source of blood in the section of the intestine used, was exposed from its origin at the aorta to the first branch, and a 3- or 4-mm noncannulating electromagnetic flow transducer[2] was then placed on the exposed artery proximal to any of its branches. The transducer was connected to a gated sign wave electromagnetic flowmeter[3] for determination of blood flow. A hydraulic blood vessel occluder[4] was placed distal to the transducer.

This arrangement allowed blood flow to the intestines to be reduced as needed and allowed constant monitoring of the flow rate. The intestines were then gently returned to the peritoneal cavity. Normally this entire isolation and implantation procedure took no more than 20 min. Following its completion, the abdomen was closed and a 1-hr stabilization period was allowed.

Preparation of Intestinal Absorption Loop—Following the 1-hr stabilization period, part of the small intestine was again exposed and the ligament of Treitz was visualized. An incision in the jejunum 30.5 cm (12 in.) distal to the ligament of Treitz was made, and a second incision was made 30.5 cm (12 in.) distal to the first. The incisions were made on the side of jejunum opposite the mesenteric attachment, and care was taken to reduce bleeding as much as possible. Two polyethylene cannulas with inflatable rubber tracheal cuffs slipped over the intestinal ends were inserted into the incisions and ligated in place. The tracheal cuffs were then inflated to prevent movement of the cannulas.

Care was taken during the entire surgical procedure to keep trauma to the intestine at a minimum. The intestine was returned to the abdominal cavity, covered with gauze, and allowed to recover for several minutes. In studies in which blood flow was altered, two or three additional intestinal segments were measured distally from the first and prepared in a similar fashion.

Determination of Drug Absorption Rates from Intestinal Loops—Following the attachment of a 60-ml syringe barrel to each cannula, perfusion fluid (25) warmed to 37° was slowly passed through the cannulated segment until the effluent was clear. Several aliquots of drug solutions (2.7 g/liter sulfaethidole or 0.7 g/liter haloperidol buffered to pH 6.0 with 0.14 *M* phosphate buffer and adjusted to isotonicity with sodium chloride) were passed through the cannulated intestinal segment and removed. Following this washing, 30 ml of fresh drug solution was introduced into the segment. Serial samples of 0.5 ml were removed as needed and assayed for sulfaethidole as described by Bratton and Marshall (26) or for haloperidol as described previously (25). Venous blood samples were obtained as needed and assayed for sulfaethidole (26).

In experiments where absorption was determined only at the control blood flow rate, a single jejunal segment was used in each dog. In experiments where blood flow was altered, a total of three or four jejunal segments was studied in each dog. At the end of a study on a given jejunal segment, the remaining drug solution was washed out of the lumen with perfusion solution, the cannulas were removed, and another jejunal segment was prepared immediately distal to the preceding segment.

Determination of Mesenteric Blood Flow Rate during Drug Absorption—To determine absolute blood flow rates, a standard curve was constructed each day by perfusing the transducer with known flow rates of saline. After implantation of the transducer, it was also necessary to close completely the hydraulic occluder on the mesenteric artery to obtain a transient recording of zero flow.

When several flow rates were to be studied in the same animal, unrestricted blood flow (100% of control) was measured and utilized for the first jejunal segment. Then flow rates of approximately 65, 35, and 0% of control were created by adjusting the hydraulic occluder, and subsequent segments were studied. Following each reduction in blood flow rate, several minutes was allowed for the animal to reequilibrate at the new condition before the absorption rate study was begun.

Although the intestinal blood flow was generally constant over the absorption period, small fluctuations did occur in a few cases. The flow rates reported are the actual observed mean flow rates rather than predetermined values.

Pharmacokinetics of Sulfaethidole in Anesthetized Dogs following Intravenous Administration—The animals were pre-

[1] Dial-Urethane, Ciba Laboratories.

[2] Medicon model Q-2030A, Medicon Instruments, Los Angeles, Calif.

[3] Medicon microflow model K 2000, Medicon Instruments, Los Angeles, Calif.

[4] Model VO-3, In Vivo Metric System, Los Angeles, Calif.

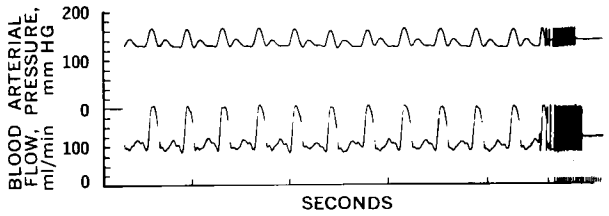
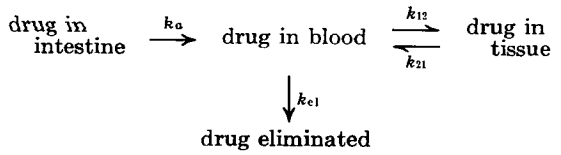


Figure 1—*Physiograph tracing of mesenteric blood flow and arterial blood pressure. Horizontal lines on right side of figure are the mean values. Tracings on left and center of figure are pulsatile values.*

pared in exactly the same way as already described except that isotonic buffer instead of drug solution was placed in the jejunal segments. An intravenous bolus injection of either 350 or 500 mg of sulfaethidole dissolved in isotonic saline was administered to each dog, and the concentration in blood at various times postadministration was determined by the method of Bratton and Marshall (26). The buffer in the jejunal lumen was also analyzed for sulfaethidole.

Pharmacokinetic Evaluation of Sulfaethidole Data—Pharmacokinetic analysis of sulfaethidole blood levels following intravenous and intestinal administration was accomplished using the SAAM25 program[5] (27). This program uses the differential equations implied by the compartmental model chosen and adjusts the model parameters until the best least-squares fit to the experimental results is obtained. In this case, computer fitting took place between the theoretical calculated levels of sulfaethidole in the blood compartment and the experimentally determined sulfaethidole blood levels using the two-compartment open model (Scheme I).



Scheme I

The rate constant k_a is the rate constant for absorption, k_{12} is the rate constant for transfer from the blood to the tissue compartment, k_{21} is the rate constant for transfer from the tissue compartment to the blood compartment, and k_{el} is the rate constant for elimination. Assumptions made in using this program include the assumption of approximate first-order kinetics for the transfer of drug between compartments and the assumption of constancy of the values of the rate constants and apparent volumes of the compartments during the experiment. The computer, in fitting the data to the theoretical curve, calculates the amount of drug in the various compartments of the model. Thus, it is possible to estimate from the blood level data the amount of sulfaethidole remaining unabsorbed from the intestinal compartment as a function of time following an intestinal dose of the drug. All data analyzed by the computer were individual data, but good agreement was found between average blood concentrations and theoretical curves predicted from the average of the individual rate constants. Initial estimates of the rate constants were determined by graphical analysis of the data.

The SAAM25 program can also be used as a digital analog computer by locking the rate constants and the volume of distribution into the program and using it to predict blood level profiles based on these values. In experiments where multiple doses of sulfaethidole were given, the rate constants and the volume of distribution for all processes except absorption were locked in. The rate constant for absorption was adjusted in conjunction with the observed absorption rate, and the calculated blood level curve was determined for each absorption period.

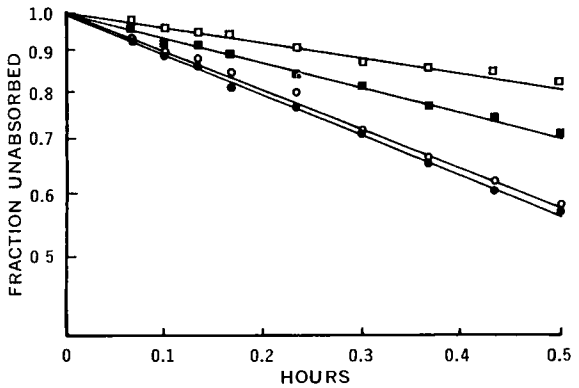


Figure 2—*Intestinal disappearance of sulfaethidole in Dog 30 at 100 (●), 64 (○), 35 (■), and 0 (□) % of control blood flow. Corresponding half-lives are 0.60, 0.626, 1.01, and 1.73 hr, respectively.*

[5] On an IBM 360-75 computer.

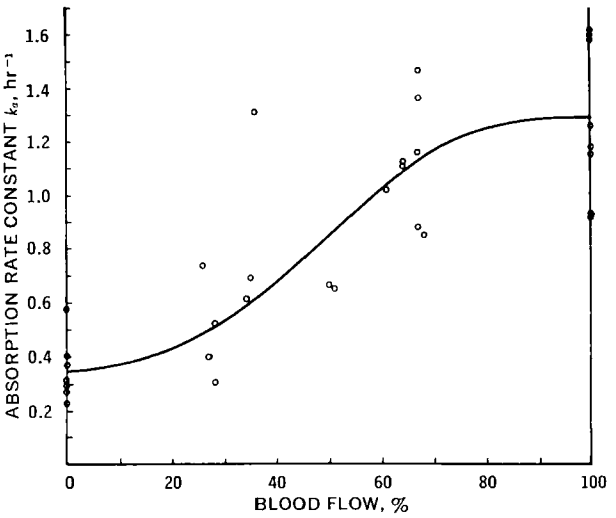


Figure 3—*Relationship between the absorption rate constant, k_a, and superior mesenteric blood flow. Each point represents one intestinal absorption experiment at one blood flow. Data are from Table I.*

RESULTS AND DISCUSSION

Intestinal Absorption of Sulfaethidole—Superior mesenteric

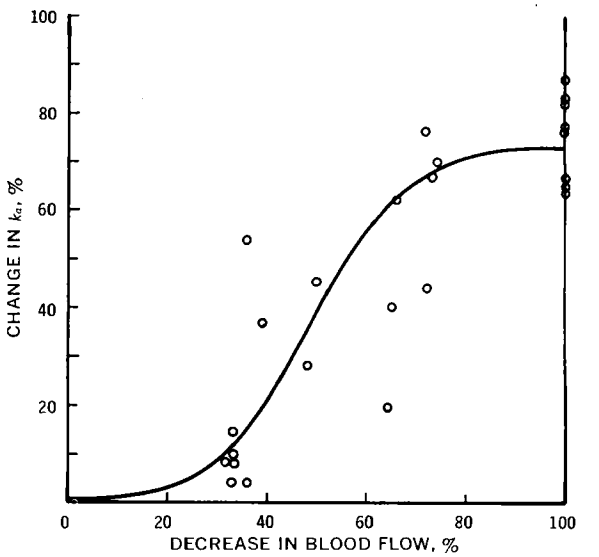


Figure 4—*Relationship between the percent change in the absorption rate constant, k_a, and the percent change in mesenteric blood flow. Data are from Table I.*

Table I—Comparison of Mesenteric Blood Flow and Sulfaethidole Absorption

Dog	Experiment	Blood Flow				Sulfaethidole Absorption		
		ml/min	ml/min/kg	% Flow	% Change in Flow	k_a[a], hr^{-1}	% Change in k_a	Half-Life, hr
13	1	165	12.0	100	0	0.924	0	0.750
	2	85	6.2	50	48	0.660	29	1.05
	3	113	8.2	68	32	0.848	8	0.817
14	1	248	20.2	100	0	1.188	0	0.583
	2	126	10.2	51	49	0.650	45	1.067
	3	68	5.5	27	73	0.392	67	1.767
	4	0	0	0	100	0.267	78	2.60
15	1	116	11.6	100	0	1.599	0	0.433
	2	71	7.1	61	39	1.014	37	0.680
	3	0	0	0	100	0.371	77	1.867
17	1	210	12.8	100	0	1.599	0	0.433
	2	141	8.6	67	33	1.363	15	0.508
	3	72	4.4	34	66	0.611	62	1.133
	4	0	0	0	100	0.291	82	2.383
20	1	112	9.8	100	0	0.924	0	0.750
	2	68	6.0	67	33	0.885	4	0.783
	3	22	1.9	28	72	0.520	44	1.333
	4	0	0	0	100	0.310	67	2.233
21	1	122	7.9	100	0	1.260	0	0.550
	2	82	5.3	67	33	1.155	8	0.600
	3	37	2.4	28	72	0.297	76	2.333
	4	0	0	0	100	0.222	82	3.116
29	1	241	15.6	100	0	1.624	0	0.427
	2	162	10.5	67	33	1.464	10	0.473
	3	87	5.6	36	64	1.308	20	0.530
	4	0	0	0	100	0.571	65	1.213
30	1	159	8.5	100	0	1.155	0	0.600
	2	107	5.8	64	36	1.106	4	0.626
	3	56	3.0	35	65	0.687	41	1.008
	4	0	0	0	100	0.400	65	1.733
34	1	160	16.0	100	0	2.445	0	0.280
	2	102	10.2	64	36	1.124	54	0.617
	3	42	4.2	26	74	0.736	70	0.942
	4	0	0	0	100	0.310	87	2.33

[a] k_a = absorption rate constant.

blood flow rates were monitored during the entire experiment; a typical recording of mesenteric blood flow is shown in Fig. 1. Although very little fluctuation generally occurred, the blood flow rates reported in Table I are the average flow rates recorded over the entire absorption experiment. The observed control blood flow rates found here compare favorably with those reported in the literature. Mean superior mesenteric blood flow rates of 12.7 ± 4.0 ml/min/kg (mean ± *SD*) (listed individually in Table I) compare closely to the 14.7 ± 4.0 ml/min/kg (mean ± *SD*) reported (28) for fasted, nonanesthetized dogs with chronically implanted flow probes.

Absorption of sulfaethidole appeared to follow first-order kinetics at all blood flow rates studied. Figure 2 shows typical intestinal disappearance curves for four different blood flow rates in a single dog (Dog 30). Absorption half-lives of 0.60, 0.626, 1.01, and 1.73 hr were found for mesenteric blood flow rates of 100, 64, 35, and 0% of control flow, respectively. Similar results for eight additional dogs are listed in Table I. The data are plotted in Fig. 3 as the absorption rate constant, k_a, *versus* the percent of control blood flow and in Fig. 4 as the percent change in the absorption rate constant *versus* the percent change in mesenteric flow. The scatter in the data reflects the animal to animal variation due to using mongrel dogs of various weights, ages, and sexes.

The results suggest that the rate of intestinal absorption of sulfaethidole is not linearly related to the rate of mesenteric blood perfusion. A decrease of about 35% in perfusion rate caused a relatively small decrease in intestinal absorption rate. Following this initial period, the absorption rate appeared to decline linearly with decreasing perfusion rate until reaching approximately 30% of control flow, where the curve again became nonlinear. There was no statistically significant difference ($p \geq 0.05$) between the absorption rate constants found at 100% blood flow and those at 61–74% blood flow. However, there was a statistically significant difference between the absorption rate constants obtained at 61–74% and 26–36% blood flow ($p \leq 0.01$) and between 26–36% and zero blood flow ($p \leq 0.05$).

The terminal nonlinearity in Figs. 3 and 4 is most likely not directly related to mesenteric blood flow since the linear portion of the curve in Fig. 3 can be extrapolated to approximately zero absorption rate at zero blood flow. Although it is generally assumed for most drugs that transfer from the intestine into the general circulation by routes other than transport by mesenteric blood is minimal (29), it is possible that alternative pathways became more

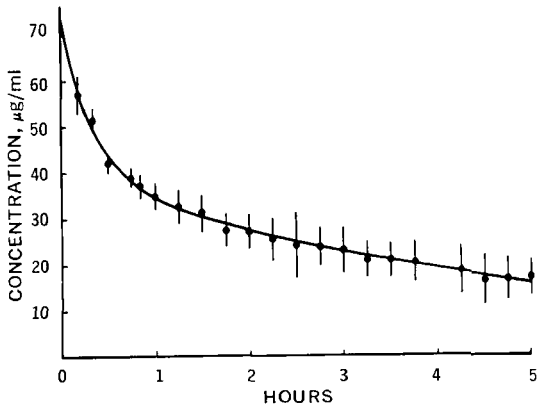


Figure 5—*Mean sulfaethidole blood levels following intravenous administration of 500 mg in three dogs. The circles (●) represent experimental data points, and the line represents the SAAM25 computer-fit curve based on the two-compartment open model. The rate constants used to draw this line are the means of individual fits found in Table II.*

Table II—Rate Constants following Intravenous Sulfaethidole Administration[a]

Dog	V_p, liters	$V_{p/kg}$, liters	k_{12}, hr^{-1}	k_{21}, hr^{-1}	k_{el}, hr^{-1}
		350-mg Dose			
23	3.23	0.222	3.32	1.41	0.209
24	5.92	0.362	2.52	1.94	0.349
25	5.62	0.413	2.32	2.67	0.329
Mean	4.93	0.332	2.72	2.01	0.296
SD	±1.49	0.099	0.53	0.63	0.076
		500-mg Dose			
40	8.93	0.633	0.706	1.281	0.249
41	6.58	0.603	0.722	1.346	0.540
48	5.92	0.290	2.64	3.06	0.217
Mean	7.14	0.509	1.36	1.89	0.335
SD	±1.58	0.190	1.11	1.01	0.178

[a] See Scheme I for explanation of terminology.

important under the conditions of low blood flow or no blood flow. The most likely explanation for this absorption at zero blood flow relates to drainage of the intestines by the lymphatic system, although other possible mechanisms have not been ruled out. In this regard, DeMarco and Levine (29) found that a significant amount (20%) of *p*-aminosalicylic acid was transported by the lymphatic system when the intestinal blood flow was reduced to zero, and Williams *et al.* (10) reported that approximately 27% of control D-xylose absorption occurred at zero blood flow. This value compares favorably with the 27% of control sulfaethidole absorption found in this study at zero blood flow.

Implantation of the flow probe appeared to have little effect on the intestinal absorption rate. Half-lives for intestinal absorption in a single dog before and after implantation of the flow probe were 0.54 and 0.58 hr, respectively. Intestinal viability at most blood flow rates did not appear to be a problem during the time period covered by the experiment. Identical absorption half-lives of 0.63 hr were found for two repetitive absorption experiments carried out in the same intestinal segment. In another experiment in which blood flow was decreased and then later increased, absorption half-lives of 0.75, 1.05, and 0.82 hr were found for blood flow rates of 100, 50, and 68%, respectively.

Little difference in absorption rate was found between the four intestinal segments used. For example, absorption half-lives of 0.45, 0.45, and 0.49 hr were found in intestinal segments 1, 3, and 4, respectively, in one dog with unaltered blood flow. These results are in agreement with other data from these studies indicating that no change occurred in the intrinsic intestinal absorption capacity or in jejunal viability with decreased blood flow.

Pharmacokinetics of Sulfaethidole after Intravenous Administration—The pharmacokinetic profile of sulfaethidole was characterized by intravenous and intestinal administrations under normal intestinal blood flow and by intestinal administration under decreased blood perfusion. Individual blood concentration–time profiles following intravenous administration of 350- and 500-mg doses of sulfaethidole were fit to the two-compartment open model (Scheme I) using the SAAM25 program (27). The rate constants k_{12}, k_{21}, and k_{el} and the volume of distribution, V_p, for the intravenous data are shown in Table II. No significant difference ($p \geq 0.05$) in the rate constants was found between the 350- and 500-mg doses.

Figure 5 shows the results for the 500-mg dose where the data points are the mean blood levels for three dogs and the line is the computer-estimated blood level based on the average of the individual rate constants for the three dogs (Table II). The data appear to be well described by the two-compartment open model. In the case of sulfaethidole, the intestine does not appear to be a preferential volume of distribution, as has been indicated for some other drugs. Prior to the intravenous injection of sulfaethidole, an intestinal segment similar to the one used in the absorption experiment was prepared but filled with buffer rather than drug solution. Less than 5% of the injected dose returned to the intestine during the experiment, with sulfaethidole intestinal levels consistently below blood levels.

Pharmacokinetics of Sulfaethidole after Intestinal Administration—*Control Blood Flow*—In the described experiments, disappearance from the intestine rather than absorption (*i.e.*, appearance in blood) was measured. To show that the disappearance rates determined in these experiments were equivalent to the absorption rates, sulfaethidole was administered to 10 dogs by a single *in situ* jejunal segment prepared as described in the *Experimental* section. Disappearance of sulfaethidole from the intestine as well as appearance in blood was determined; individual blood concentration–time data following sulfaethidole administration were analyzed by the SAAM25 program (27) using the two-compartment open model. Initial estimates of the rate constants were

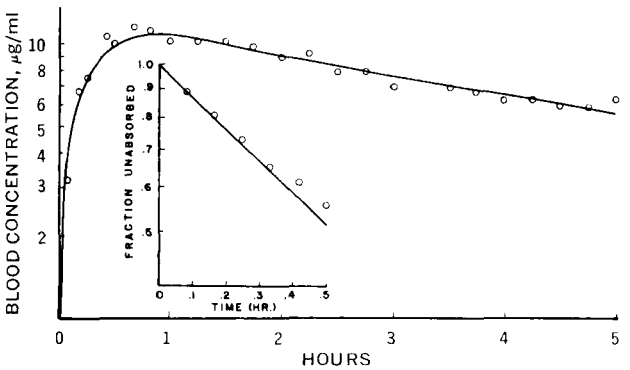


Figure 6—*Blood concentration of sulfaethidole following intestinal administration in Dog 37. The line represents the computer-calculated blood concentrations, and the circles (○) represent experimental data points. Rate constants used in generating the curve are listed in Table III. In the inset, the open circles represent the experimentally determined fraction of sulfaethidole unabsorbed from the intestine and the line represents the computer-predicted values based on the blood concentrations in this dog.*

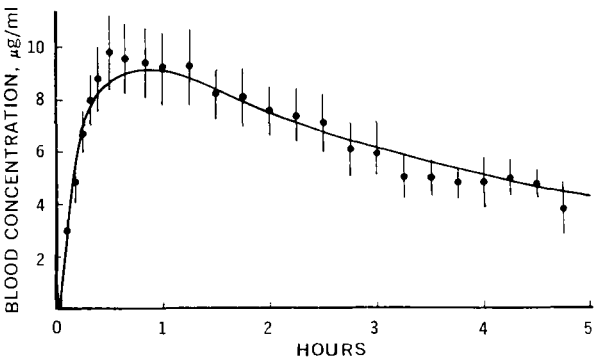


Figure 7—*Mean blood levels of sulfaethidole in 10 dogs following intestinal administration. The bars represent standard errors of the mean. The line represents the computer-calculated blood concentrations based on the mean rate constants (Table III) for these 10 dogs.*

Table III—Calculated and Observed Rate Constants for Sulfaethidole following Intestinal Administration[a]

Dog	V_p, liters	$V_{p/kg}$, liters	k_{12}, hr^{-1}	k_{21}, hr^{-1}	k_{el}, hr^{-1}	k_a (calc), hr^{-1}	k_a (obs), hr^{-1}	Sum of Squares, $\times 10^{-4}$
28	6.25	0.431	3.63	0.569	0.103	0.980	0.950	0.0017
37	1.35	0.123	2.58	0.716	0.751	0.699	0.690	0.035
39	3.55	0.300	3.20	0.893	0.570	1.280	1.07	0.014
42	0.606	0.041	1.98	0.000	0.909	0.217	0.385	0.042
43	3.33	0.272	0.543	0.222	0.373	0.853	0.790	0.480
44	2.39	0.128	1.43	3.050	0.975	2.050	1.88	0.120
45	2.30	0.120	0.949	1.220	0.900	1.000	0.885	0.062
46	2.10	0.121	1.39	0.443	0.798	1.280	1.070	0.077
47	2.53	0.164	1.78	1.030	0.379	1.350	1.150	0.220
49	1.47	0.104	2.35	0.964	0.506	0.850	0.730	0.690
Mean	2.58	0.180	1.98	0.910	0.626	1.05	0.960	
SD	± 1.56	0.117	0.973	0.841	0.286	0.482	0.394	

[a] See Scheme I for explanation of terminology.

obtained from the intravenous data. Typical data showing how the observed blood concentration–time profile is used to calculate the absorption rate in a single dog (Dog 37) are shown in Fig. 6. The rate constants calculated in this fit are found in Table III. The solid line indicates the SAAM25 estimated least-squares regression fit. The inset in this figure shows as the data points the observed intestinal disappearance rate with the solid line indicating the disappearance rate estimated by SAAM25 from the blood concentration–time data in this figure. Observed and calculated absorption half-lives of 0.99 and 1.0 hr, respectively, were found. The rate constants obtained by a similar treatment for nine additional dogs are shown in Table III.

Figures 7 and 8 show this same general relationship for all 10 dogs studied. In Fig. 7 the data points are the average blood levels for the 10 dogs and the solid line represents the blood levels generated by the SAAM25 program from the average of the individual rate constants for these dogs (Table III). Figure 8 compares the average observed fraction of the dose remaining unabsorbed in the jejunum with a line with a slope corresponding to the average absorption rate constant estimated by fitting with SAAM25 the individual blood concentration–time data in the 10 dogs. The observed and calculated absorption half-lives were 0.72 and 0.66 hr, respectively, which were not significantly different ($p \geq 0.05$). This excellent agreement between observed intestinal disappearance rates and absorption rates calculated from blood level data indicates that disappearance of sulfaethidole from the intestine is equivalent to absorption into the systemic circulation under the experimental conditions. It also indicates that the method of calculation yields results that truely reflect events in the GI lumen.

Two of the 10 dogs listed in Table III, Dogs 42 and 44, showed unusual absorption profiles. Although the reason for these unusual absorption rates was not determined, the SAAM25 program estimated the absorption rates fairly accurately from the blood level data. This further substantiates the validity of this method.

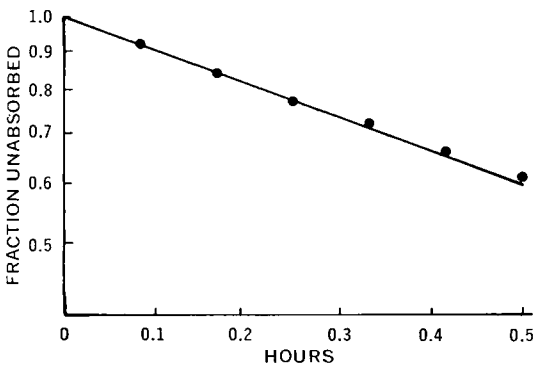


Figure 8—*Comparison of the fraction of sulfaethidole unabsorbed from the intestine and that predicted by SAAM25 from blood concentrations. The points represent the mean of 10 dogs (Table III), and the line represents the computer-calculated fraction based on the average rate constants (Table III) for the 10 dogs.*

Reduced Blood Perfusion—To verify that the kinetics of sulfaethidole described previously are valid in the experiments involving artificial reduction in blood flow and repeated administration of drug, the observed blood concentration–time profile in individual dogs was compared with that predicted by the SAAM25 program with the two-compartment open model. Therefore, the blood level data following the first administration of sulfaethidole (control blood flow) were fit by SAAM25, using the average rate constants from Table III as initial estimates. These calculated rate constants were then fixed and only the absorption rate corresponding to the disappearance of sulfaethidole from the jejunal segments at each blood flow rate was varied to generate the blood concentration–time profiles for all four absorption experiments in each dog. The results of this treatment for Dog 29 are shown in Fig. 9, where the data points are the observed blood levels and the continuous line represents the SAAM25 estimated blood levels based on the intestinal disappearance data at each blood flow. The generally good agreement here indicates that the same relationship described previously between intestinal disappearance and absorption holds even at decreased blood flow rates, and it also confirms that the pharmacokinetic model is accurately reflecting both disposition of the drug and absorption behavior from the jejunal loop.

In the last absorption experiment (IV) in Fig. 9, an increase in the blood level is apparent even though there is no mesenteric blood flow. In the SAAM25 prediction of the blood levels at zero flow, the observed intestinal disappearance rate constant (k_a = 0.571 hr^{-1}) resulted in a much better fit of the data than a rate constant of zero and is the one used in this figure. This observation is in agreement with the observed disappearance data at no flow in the previous experiments, reinforcing the concept that some sulfaethidole absorption does take place when no mesenteric blood flow is present.

Haloperidol Absorption in Reduced Intestinal Blood Perfusion—Sulfaethidole appears to be absorbed by passive diffusion of both ionized and nonionized drug species (31), but it probably has very little potential for membrane storage as described by Doluisio *et al.* (30). On the other hand, haloperidol has been shown to have a potential for membrane storage (30), and it was of interest to determine how this property might affect its absorption at decreased

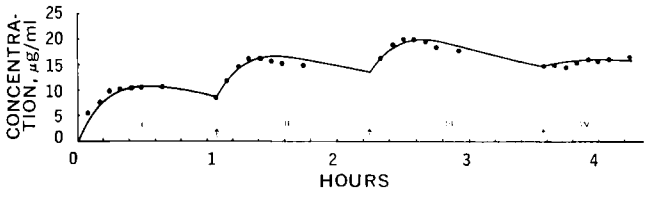


Figure 9—*Blood levels of sulfaethidole for Dog 29 under 100 (I), 65 (II), 35 (III), and 0 (IV) % flow. The circles indicate experimental data points, and the line represents the computer-estimated blood concentrations based on the intestinal disappearance rate constants for Dog 29 as described in the text. The arrows indicate when each dose was administered;* k_{12} = *1.44* hr^{-1}, k_{21} = *1.34* hr^{-1}, k_{el} = *1.26* hr^{-1}, *and* V_p = *2.30 liters.*

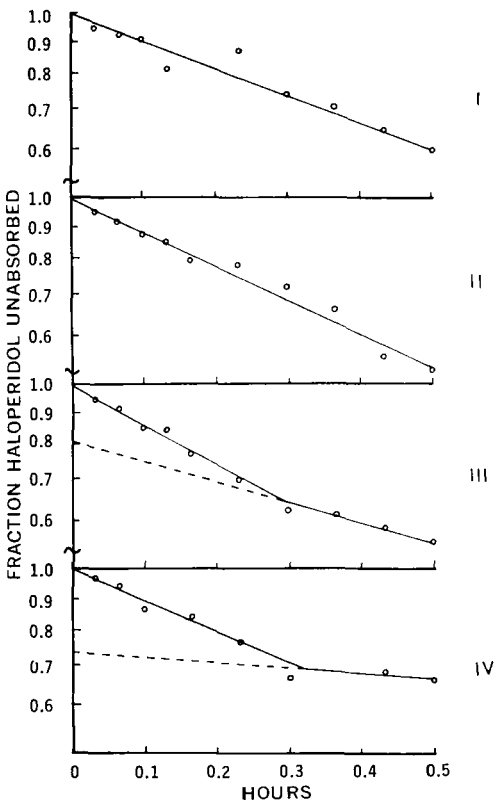


Figure 10—*Disappearance of haloperidol from the intestinal lumen at 100 (I), 70 (II), 37 (III), and 0 (IV) % flow.*

blood flow. The methodology used was similar to that of the previous studies with sulfaethidole, and the results are shown in Fig. 10. Blood levels of haloperidol were not measured because they were much too low for accurate determination. Disappearance of haloperidol from the intestine was linear at blood flow rates of 100 and 70% of control but became biphasic at lower rates of mesenteric blood flow. This finding suggests that not only the rate of intestinal disappearance of haloperidol changes with decreased blood perfusion but that a change in the absorption characteristics also occurs. The change may be due to the mechanism of absorption of haloperidol which appears to involve two steps: *(a)* transfer of haloperidol from the intestinal solution into the membrane (*i.e.*, membrane storage) and *(b)* transfer of haloperidol from the membrane into blood.

Thus, at higher rates of blood flow, *i.e.*, above 70% of control, transfer from the intestinal solution into the membrane would be the slower or rate-limiting step and would determine the overall rate of disappearance from the intestine. As blood flow decreases below 70% of control, however, the transfer of haloperidol from the membrane to blood becomes rate limiting; following the initial partitioning into the membrane, the rate of disappearance from the intestine is determined by the membrane to blood transfer rate. This hypothesis is strengthened by the observation that the initial rate of disappearance of haloperidol from the intestine remains relatively constant with decreasing blood flow while the later biphasic part of the curve appears to decrease with decreasing blood flow (Fig. 10). This was particularly apparent in the last absorption experiment where no blood flow was present, and the terminal biphasic tail of the curve is nearly flat. Membrane storage of the amino acid phenylalanine at several blood flow rates has been reported (21). In these experiments (21), disappearance from the lumen did not equal appearance in the blood and a computer program assuming a disappearance rate equal to an appearance rate did not converge.

CONCLUSION

The absorption rate of sulfaethidole is reduced by a reduction in mesenteric blood flow to the intestines. This effect does not appear to be linear. For reductions in intestinal perfusion of about 30%, a relatively small decrease in the intestinal absorption rate occurs. Changes greater than a 30% reduction in intestinal perfusion result in decreased rates of drug absorption. Measurement of sulfaethidole disappearance from the intestine and appearance in blood indicates that disappearance from the intestine is equivalent to absorption into the circulation. Haloperidol disappearance from the dog jejunal lumen occurs by a two-stage process, and a large amount of haloperidol appears to accumulate in the membrane during absorption.

Abnormal intestinal blood flow rates may be the result of pathological conditions, strenuous exercise, or the presence of other drugs. Changes in the intestinal blood flow rate during therapy could result in the increased or decreased potency of certain vital drugs. Decreased blood flow rates in certain pathological conditions could be responsible for therapeutic failures of some drugs, and differences in exercise or bedrest could be responsible for differences among subjects or poor performance of certain drug products in bioavailability studies.

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In Vivo Method for Monitoring Polysorbate 85 Effect on Epidermal Permeability

KEVIN J. RYAN and MICHAEL MEZEI ˣ

Abstract □ An *in vivo* method of monitoring the rate of water desorption from human forearms, using "dry" nitrogen gas passed over approximately 1 cm^2 of skin was investigated with the aid of a commercial electrolytic moisture analyzer. The assembled apparatus was used to evaluate the differences in water loss rates from treated and untreated (control) forearms following surfactant application. The changes in the differences were also monitored after cessation of treatment, *i.e.*, during the healing process. The apparatus provided an accurate, rapid, and painless method of monitoring relative water loss rates and, as such, could prove a useful tool in routine testing in experimental dermatology and cosmetology. The results confirm the earlier finding from an *in vitro* method with excised rabbit skin that the tested surfactant increases the permeability of the epidermis.

Keyphrases □ Permeability, epidermal—*in vivo* method for monitoring effect of polysorbate 85, relative water loss rates □ Epidermis—permeability, *in vivo* method for monitoring effect of polysorbate 85, treated and untreated human epidermis □ Polysorbate 85—effect on epidermal permeability, *in vivo* method for determining relative water loss rates □ Water loss rates—human epidermis, *in vivo* method

An *in vitro* method (1) proved satisfactory for quantitating the water content and rate of water desorption from 1.0-cm^2 samples of excised rabbit skin. The method could be applied to human skin; however, the excision of the skin sample, although virtually painless, is not readily accepted by most human subjects. In addition, an *in vitro* method can monitor the desorption of only a finite amount of water and may be, at best, unpredictably extrapolatable to the *in situ* conditions where the supply of water is essentially inexhaustible.

It is well known (2–8) that surfactants generally increase the permeability of the skin, which can easily be studied by measuring the rate of the transepidermal water loss. Accurate quantitative *in vivo* measurements from human forearms have been reported (9–13). The actual water loss under defined conditions has been determined for areas as small as 0.1 mm^2, although at least 1 mm^2 should be the minimum area considered "a representative sample for forearm skin generally" (12). These quantitative methods required considerable effort to minimize the instrumental baseline. The purpose of this study was to design a relative method that requires less stringent ambient and instrumental manipulation and, therefore, is more practical and rapid.

EXPERIMENTAL

An electrolytic moisture analyzer[1] provided the nucleus for the assembled apparatus. The "heart" of the instrument is a horizontal glass "cell" containing two platinum wires across which there is a potential difference of 75 v dc. The medium between these wires is phosphorus pentoxide, which is converted into phosphoric acid following the introduction of water into the cell by the carrier gas. Upon completion of the circuit, the water is electrolyzed and the phosphorus pentoxide is regenerated. There is a direct relationship between the amount of water in the gas and the current used. The instrument is so designed that there is a direct readout on the instrument meter in parts per million of water for a specific gas flow. The signal to the meter is connected to a recorder[2], so a continuous graph of moisture content *versus* time is obtained.

The carrier gas, ultra high purity nitrogen (containing less than 3 ppm of water), was passed *via* Teflon tubing (1 mm i.d., 4 mm o.d.) into a sampling "cup" sealed against the skin of the forearm by 400 g weight. The "dry" gas inlet was so positioned as to direct the stream of gas onto the skin surface, where it readily picked up the surface water; the "wet" gas exited from the cup *via* Teflon tubing into the instrument. The sampling cup was turned down from a 2-cm diameter brass rod, 3 cm in length. It was plastic coated to decrease porosity and heat transfer. The apparatus was in operation for several hours prior to each day's use to remove moisture that had permeated the apparatus, so that a constant baseline could be achieved.

As a test of the reproducibility of the apparatus, readings were taken on symmetrical sites on 20 subjects' left and right forearms. Prior to their participation in the study, the subjects' arms were visually inspected to ensure that no dry skin condition or other dermatological irregularities existed. The subjects were university

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[2] Coleman 165.

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Table I—Tests for the Reproducibility of Readings

Subject (Sex)	Variation[a], %
E.K. (f)	0
O.M. (f)	0
S.R. (f)	0
K.R. (m)	0
C.P. (m)	0
J.I. (f)	0
T.N. (f)	0
D.F. (m)	3.3
S.M. (f)	3.6
S.L. (m)	3.6
K.J. (m)	3.8
D.K. (m)	3.8
E.F. (f)	3.9
K.D. (f)	5.0
F.M. (m)	5.6
L.R. (m)	5.9
M.M. (m)	6.6
J.W. (f)	6.9
C.N. (f)	8.3
S.L. (f)	9.0

[a] The transepidermal water loss was measured from symmetrical sites on untreated left and right forearms of each subject, and the difference in readings is expressed as percent variation.

staff and students ranging in age from 19 to 45 years, with the majority being in the early 20's (12 females and eight males). Dense hair on the forearms of one male subject was clipped with a hair clipper[3] 1 day prior to commencement of readings.

Of the initial 20 subjects used to establish the limits of reproducibility, six (five females and one male) continued on to the evaluation of surfactant application effects. They applied the ointment base (petrolatum USP) to one forearm for the control, and the other forearm was treated with the same base containing 10% polysorbate 85[4].

The preparations were applied liberally (~0.5 g) twice daily and massaged into forearms for 10 sec. Readings were taken (prior to and during the treatment) on treated and then control forearms (order randomly varied), and the difference between the readings was expressed as a percentage of the control value. For this reason, no absolute units had to be calculated since the results were expressed as percentage difference in rates of water loss between the two sites.

One hour prior to readings, the subjects' forearms were gently washed with mild soap and warm water and patted with a tissue to remove excess water. The bare forearms were air dried to the laboratory environment while the subjects relaxed.

RESULTS AND DISCUSSION

At the commencement of the readings, the initial pen response prescribed a peak, the height of which varied as did the amount of water in the stratum corneum (which varies as does ambient temperature, humidity, physical and mental activity of the subject, blood volume, breathing rate, the ingestion of certain drugs, *etc.*). During the next 8–10 min, the peak declined to a line parallel to the baseline. This straight portion of the graph shows a constant rate of transepidermal water loss and, as such, is an indication of the "barrier function" of the epidermis.

Thirty-five percent of the readings performed on the left and right untreated forearms were identical to each other; *i.e.*, the difference in final constant rates of water loss rates was 0%. The remainder of the subjects' readings did not vary from each other by more than 9% (Table I). Thus, the limit of reproducibility of the assembled apparatus (due to environmentally, instrumentally, physiologically, and psychologically mediated variations during the time required to perform a pair of readings) was established.

When readings were performed on treated and control forearms, in every case there was a substantial increase in the rate of transepidermal water loss from the surfactant-treated arm with respect to the control arm (Table II). There was a definite correlation between the duration of treatment and an increased rate of transepidermal water loss. In half of the cases, 9 days of treatment resulted in a difference in the rate of water loss of around 100%.

Table II—Effect of Surfactant Treatment on Rate of Transepidermal Water Loss

	Subject					
	K.R.	S.R.	J.I.	K.D.	C.N.	R.S.
Day	Increase of Transepidermal Water Loss, %					
0	0	0	0	0	8.3	0
1				5.0		
2						12
3	19		10		13	30
4		66	26			
5	33					72
6				54	53	
7	52		64			
8						
9	93	100	75			100

It is readily evident that it would be impossible to reproduce consistently all of the variables present during the first hours, much less days, of readings. However, with the method's built-in controls, and by reading the control and treated forearms (order randomly varied) as close together in time as possible, the differences in readings should be due almost entirely to surfactant application.

All readings were performed in an isolated room with a relatively constant temperature of about 20° (peaks of sweat may appear on the graph above 25°). Slight fluctuations in the room's humidity and temperature during the 0.5 hr or so required for the two readings constituting a set of data did not have any noticeable effect on readings.

One subject was treated with surfactant in (ointment) base on the left arm and with base on the right arm for 9 days. The surfactant induced an increase in transepidermal water loss such that the difference in rates was 100% by the 9th day, whereupon treatment was ceased (Fig. 1). Sixteen days after cessation of treatment, while the difference was still about 12%, treatment was reinstated in a crossover fashion; *i.e.*, the control arm now became the treated arm. Since the new control arm still had a greater transepidermal water loss at the time of crossover, initially the difference in readings showed a negative value. After 3 days, the effects of the surfactant became so evident that the difference became positive and continued to increase at a greater rate than it had in phase one of the experiment (prior to crossover). Within 9 days of the crossover, the difference in rates again approached 100% and treatment was ceased. Differences again diminished, rapidly at first and then at a declining rate. By monitoring the difference in water loss rate for up to 6 weeks after cessation of treatment (and having the subject wash arms daily with mild soap and water), the declining differences in readings indicated the recovery of barrier function, which is due to the healing process.

The rate of the regeneration process is very high in the early stages. Figure 1 indicates that a 50% regain of the barrier function occurred in less than 4 days after cessation of treatment (after cessation of continuous injury), while the next 50% reparation (resulting in 0% difference in rates) required another 3–4 weeks. The time required for total regeneration of the barrier function (14) after cessation of surfactant application indicates that the full barrier function returns only with the presentation of new stratum corneum cells (unaffected by surfactant) to the surface of the epidermis.

The kinetic microhygrometric technique employed does not simulate normal environmental conditions of the epidermis. However, the method is so designed that the effectiveness of the treated forearm's epidermis in preventing excessive water loss under specific conditions can be evaluated with respect to control epidermis under the same conditions. Since the portion of the values due to sweating could be kept virtually constant by maintaining fairly constant ambient conditions (especially temperature) and psychic state, it was not deemed necessary to introduce any type of drug into the body to inhibit sweating.

To prevent the escape of carrier gas and the entrance of ambient

[3] Oster model A2-22, blade ANG-RA.
[4] Tween 85, Atlas Chemical Industries, Wilmington, Del.

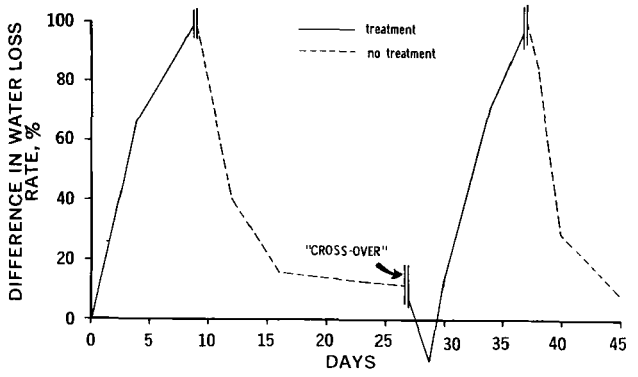

Figure 1—*Effects of surfactant treatment as measured by the percent difference in water loss rate between treated and untreated skin* versus *time. Details concerning the crossover period are given in the text.*

moisture, the cup was pressed to the skin with a weight of 400 g; this weight was determined experimentally. On untreated skin, a weight of about 200 g would be sufficient to seal the rim of the cup against normal skin. However, after several days of treatment with surfactant, the skin surface became more irregular and some 400 g was required to effect a seal, undoubtedly due to "a type of crust formation, shown microscopically to consist of necrotic and sloughing epidermis" (15).

CONCLUSION

The present study and other reports (1–8) indicate that surfactants can affect epidermal permeability. Today, with increasing exposure to epidermal insult from physical, chemical, and energy sources, the regenerating process of the epidermis not infrequently approaches overextension. Insidiously, even some of the preparations designed to improve the quality or appearance of the skin usually contain chemicals (to improve the formulation's appearance, stability, or penetration) that may actually further damage the tissue. There is, therefore, a real need for a convenient, rapid method for monitoring changes in the barrier function of the epidermis, resulting from dermatological and cosmetic preparations.

Paradoxically, damage to the barrier will cause increased water loss through the skin and the result may be "dry skin"; *i.e.*, the water content of the stratum corneum is decreased. This increased water loss through the skin will occur slightly following the application of substances that cause the removal of surface lipids (16). Substances that also have a destructive action on the barrier structure will effect a dramatic rise in the skin permeability.

Blank (17) showed that water is the only plasticizer of the stratum corneum. Accurate measurements of the actual water content of the stratum corneum, however, would provide no specific data because the water content of the stratum corneum varies (nonlinearly) with ambient humidity (11) and temperature, as well as with many other physiological and psychological conditions. A quantitative figure for the water binding capacity would be of great value in the assessment of the effects of topical applications with respect to the presence (or absence) of the yet to be determined "critical amount" of the stratum corneum's plasticizer.

The method in this paper measures the relative efficiency of the barrier function, which is dependent upon the presence of at least the critical amount of water in the stratum corneum. This method is not a quantitative measure of the *amount* of water but is a measure of its *activity*.

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Determination of Estrogens in Dosage Forms by Fluorescence Using Dansyl Chloride

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Abstract □ A sensitive, reproducible, fluorometric procedure for the determination of estrogens in pharmaceutical preparations is presented. The estrogens are determined fluorometrically following their reaction with dansyl chloride. The optimum conditions for the reaction such as pH, reaction solvent composition variations, and speed of reaction are discussed. In addition, a linearity study of the relationship between concentration and fluorescence intensity for estrone, estradiol, and ethinyl estradiol is reported. Solvent extraction procedures based on acid–base behavior or column chromatography are used when necessary to isolate the estrogen prior to reaction with dansyl chloride and fluorometric measurement. The recovery of estrogens from spiked samples indicated that the proposed method is efficient and reproducible. Comparison of the dansyl procedure with the official NF method in the analysis of estrone aqueous suspension showed the proposed method to be accurate and more precise than the NF assay. Estrone aqueous suspension, estradiol in sesame oil, estradiol valerate in castor oil and in sesame oil (in the latter case, in the presence of testosterone), estradiol benzoate in sesame oil, and ethinyl estradiol tablets (0.5 mg/tablet) from commercial sources were satisfactorily analyzed, with average results within compendial limits and no coefficient of variation greater than 2%.

Keyphrases □ Estrogens—determination in dosage forms, fluorescence using dansyl chloride, pH, reaction solvent composition, and speed of reaction, compared to compendial method □ Dansyl chloride—preparation of estrogen-dansyl derivatives, fluorometric determination of estrogens in pharmaceutical preparations, compared to compendial method □ Fluorometry—analysis, estrogens in dosage forms

Estrogens are important therapeutic agents that have been used as replacements or supplements for inadequate supplies of the natural hormone or in suppressive therapy to counter the effects of other hormones. Many analytical procedures have been described for the C_{18} estrogens, all of which are characterized by a phenolic substituent in the 3-position. Some methods are based on the modified iron–Kober colorimetric procedure (1–7), analyses that are notoriously laborious and time consuming, with the production of interfering background color as a result of the charring effect of the sulfuric acid on nonestrogenic impurities (7).

Analyses of estrogens have been developed that depend on the formation of derivatives. IR quantitative analysis has been used to measure estrone, equilin, and equilenin after formation of the benzenesulfonate phenolic esters (8). The reaction with Girard's reagent T is the basis of another IR spectrophotometric procedure that is somewhat nonspecific (9). Polarographic analysis of the Girard T (10) and nitrosophenol (11) derivatives of estrogens has been reported. Unfortunately, since the half-wave potentials for all of the different estrogens are the same, the polarographic analyses are also nonspecific.

The application of chromatographic methods to the separation and measurement of estrogens has been reported. Separations by GC have involved pure standards, pharmaceuticals, and steroids in biological media. For example, equine estrogens have been separated after silylation (12), conjugated estrogens have been analyzed after preliminary hydrolysis (13), estrone has been measured in dermatological dosage forms (14), estrogens have been monitored in human plasma (15), urinary estrogens have been determined after a preliminary TLC separation (16), and halogenated estrogens have been estimated by electron-capture behavior (17). A high-speed liquid chromatographic procedure has been described for the separation of equine estrogens (18). Conventional partition chromatography has been successful in the separation of most constituents of conjugated estrogens (19). An interesting application of the adsorptive properties of a cross-linked dextran gel[1] has been made to the separation of three common estrogenic steroids (20).

The phenomenon of fluorescence offers the clear advantage of sensitivity in estrogen analysis. Although estrogens do exhibit native fluorescence, the observed intensity is not great enough to be useful. Until this point, fluorescence procedures for estrogens were based on the Kober reaction that employs sulfuric acid to produce an analytically useful fluorophore (21–24). Simple solvent extraction could separate the estrogen from any interferences prior to the fluorogenic step (25, 26). Reaction of dansyl chloride, a substituted naphthalene sulfonyl chloride, with the phenol at the 3-position of all estrogens, is an excellent procedure that results in a fluorescent derivative. This reagent has been used in the TLC separation of estriol in pregnancy urine (27), the TLC separation of dansyl derivatives of estrone, estradiol, and estriol, and the quantitative measurement of these steroids by fluorodensitometry (28, 29). Other applications of dansyl derivative fluorescence have been made to primary and secondary amines (30–34), amino acids (30–34), and terminal amino group analysis in proteins and peptides (32–34) as well as to phenols (32–35).

This paper reports a study using the formation of the dansyl-estrogen followed by fluorescence mea-

[1] Sephadex.

surement for estrogen analysis in pharmaceutical dosage forms. Reaction conditions were investigated as to solvent composition, reagent concentration, and rate factors. The dosage form analyses were carried out under the determined conditions and yield results that were quite satisfactory.

EXPERIMENTAL[2]

Chemicals and Reagents—All reagents were of analytical reagent grade (ACS) purity and were used without further purification. Petroleum ether was ACS certified. Dansyl chloride[3] (1-dimethylaminonaphthalene-5-sulfonyl chloride) was stored in a desiccator over silica gel. Acid-washed siliceous earth[4] was used.

The following estrogen standards were used: estrone[5], 3-hydroxyestra-1,3,5(10)-trien-17-one, $C_{18}H_{22}O_2$, mp 254–256°, mol. wt. 270.4; estradiol[6] (β-estradiol), estra-1,3,5(10)-triene-3,17β-diol, $C_{18}H_{24}O_2$, mp 177–179°, mol. wt. 272.4; estradiol benzoate[6], estra-1,3,5(10)-triene-3,17β-diol 3-benzoate, $C_{25}H_{28}O_3$, mp 191–196°, mol. wt. 376.5; ethinyl estradiol[5], 17α-ethinyl-1,3,5(10)-estratriene-3,17α-diol, $C_{20}H_{24}O_2$, mp 141–143°, mol. wt. 296.4; and estradiol valerate[5], estra-1,3,5(10)-triene-3,17β-diol 17-valerate, $C_{23}H_{32}O_3$, mp 144–145°, mol. wt. 356.5. Commercial estrogen dosage forms were selected for analysis.

Preparation of Reagents—*0.01% (0.000371 M) or 0.02% (0.000742 M) Dansyl Chloride Reagents*—Dissolve 50 or 100 mg, respectively, of dansyl chloride in 500 ml of acetone. If necessary, filter the solution through fluted filter paper[7], 18.5 cm. Dansyl chloride is stable in acetone. However, the solution was prepared freshly approximately every 3 weeks.

Sodium Carbonate Aqueous Acetone Solution—Dissolve 1.00 g of sodium carbonate decahydrate in a solution of 300 ml of water and 150 ml of acetone.

Water-Washed Ether—Shake equal volumes of ether and water for 2 min in a separator. Discard the aqueous phase.

The following were all aqueous solutions: 2.5 *N* sodium hydroxide solution, 50% sulfuric acid solution (18 *N*), 1 *M* sodium bicarbonate solution, and dilute sulfuric acid (1 + 2).

Analytical Applications—Procedures are provided for the preparation of estrogen standard solutions and the treatment of the various dosage forms for the isolation of the estrogens prior to the application of the dansyl chloride reaction and fluorometric measurement.

Estrogen Standard Solutions—These solutions were prepared according to the following directions. Dissolve the appropriate quantity of estrogen, accurately weighed and dried as indicated, in about 25 ml of acetone and dilute to exactly 100 ml with acetone:

estrogen	drying conditions	quantity to be weighed, mg
estradiol	105°, 4 hr	2
estrone	105°, 4 hr	2
ethinyl estradiol	silica gel, room temperature, 4 hr	2
estradiol benzoate	105°, 3 hr	3
estradiol valerate	105°, 3 hr	2

Aqueous Suspensions for Injection—Vigorously shake the suspension to be sampled to obtain a homogeneous dispersion. Using a "to contain" pipet, transfer accurately a volume of the estradiol, estrone, or other estrogen suspension equivalent to about 4 mg of the estrogen into a 200-ml volumetric flask, wash the pipet inner bore with several increments of acetone and retain the washings in the flask, and add additional acetone in increments to the flask to a volume of about 125 ml, swirling after each addition and heating, if necessary, to aid in dissolving the estrogen. Dilute to volume, mix well, and filter the solution through filter paper[8] into a glass-stoppered conical flask, discarding the first 25 ml of filtrate. Using this sample solution, continue with the procedure *Fluorometric Determination Using Dansyl Chloride.*

Oleaginous Solutions for Injection—Using a "to contain" pipet, transfer a volume of estradiol, estrone, or other estrogen solution equivalent to about 5 mg of the estrogen into a 125-ml separator. To ensure complete transfer, wash the pipet inner bore with two 10-ml and a third 5-ml portion of petroleum ether[9], retaining the washings in the funnel. Add 15 ml of 2.5 *N* sodium hydroxide solution, shake vigorously for 2 min, and allow the phases to separate completely. Transfer the aqueous phase to a second 125-ml separator and repeat the extraction of the petroleum ether[9] with two additional 10-ml portions of 2.5 *N* sodium hydroxide solution, adding each extract to the second separator.

Wash the combined alkaline extracts with 25 ml of petroleum ether, and discard the petroleum ether phase. Add 50% sulfuric acid dropwise to acidify the combined alkaline portion to pH 1, using pH indicator paper, and cool the solution. Then add 25 ml of benzene and shake for 2 min. Transfer the acid layer to another 125-ml separator and extract with another 25-ml portion of benzene. Discard the aqueous layer. Wash the combined benzene solutions with two 5-ml portions of 1 *M* sodium bicarbonate solution and two 5-ml portions of water; discard the aqueous phases.

Pass the benzene solution through benzene-washed cotton into a beaker, and evaporate to dryness on a steam bath with the aid of a current of air. Dissolve the residue in acetone, warming if necessary to aid dissolution. Transfer the solution quantitatively to a volumetric flask of a size such that a final concentration of 0.020 mg/ml will result. Dilute to volume with acetone, and continue with the procedure *Fluorometric Determination Using Dansyl Chloride.*

Estradiol Benzoate Injectable Solution in Sesame Oil—Prepare a chromatographic column using acid-washed diatomaceous earth[4] as described by James (37).

Using a "to contain" pipet, transfer a volume of sample equivalent to 15.0 mg of estradiol benzoate in oil to a 100-ml volumetric flask. Wash the pipet inner bore with several portions of *n*-heptane, retaining the washings in the flask, and dilute to volume with *n*-heptane.

Transfer 2.0 ml of sample solution to the column, and wash the column with 75 ml of *n*-heptane, discarding the washings. Change receivers to a 250-ml glass-stoppered (TS 24/40) erlenmeyer flask, and elute the estrogen with approximately 200 ml of *n*-heptane. Evaporate the eluate to dryness, dissolve the residue in 10 ml of acetone, and add 20 ml of sodium carbonate aqueous acetone solution. Similarly, transfer 10.00 ml of the estradiol benzoate standard solution to a glass-stoppered (TS 24/40) erlenmeyer flask, and add 20 ml of sodium carbonate aqueous acetone solution. Equip both the sample and standard flask with water condensers (TS 24/40 joint), and heat at reflux for 45 min. Then quantitatively transfer the contents of the conical flasks to separate 50-ml volumetric flasks, wash each flask with two successive 3-ml portions of the sodium carbonate aqueous acetone solution, and transfer each portion quantitatively to the appropriate 50-ml volumetric flask. Add 10.00 ml of 0.02% dansyl chloride reagent, and dilute to volume with the sodium carbonate aqueous acetone solution. For a blank, add 10 ml of acetone and 10.00 ml of 0.02% dansyl chloride reagent to a 50-ml volumetric flask and dilute to volume with the sodium carbonate aqueous acetone solution. Continue with the procedure *Fluorometric Determination Using Dansyl Chloride,* beginning with "Then let the solutions stand . . . ."

Tablets—Weigh accurately 20 tablets, reduce them to a fine powder by trituration, and pass the powder through a 60-mesh sieve. Accurately weigh a portion of the sample equivalent to about 1.0 mg of ethinyl estradiol or other estrogen into a 125-ml separator. Add 10 ml of water and 0.5 ml of dilute sulfuric acid (1 + 2), extract with 25 ml of chloroform, separate the phases, and then extract with two additional 25-ml portions of chloroform.

Wash the combined chloroform solutions with two 5-ml portions of 1 *M* sodium bicarbonate solution and two 5-ml portions of water, and discard the aqueous phases. Pass the total chloroform

[2] A Perkin-Elmer model MPF-3 spectrofluorometer equipped with a xenon lamp and power source (model 150) and a Hitachi recorder (model QPD-33) were used.
[3] Aldrich Chemical Co., Cedar Knolls, NJ 07927
[4] Celite 545, Johns-Manville, Denver, CO 80217
[5] USP Reference Standard, United States Pharmacopeial Convention, Inc., Rockville, Md.
[6] NF Reference Standard, American Pharmaceutical Association, Washington, DC 20037
[7] Whatman, 2V.
[8] Whatman No. 42.
[9] For the castor oil-containing estradiol valerate dosage form, the solvent used was *n*-heptane.

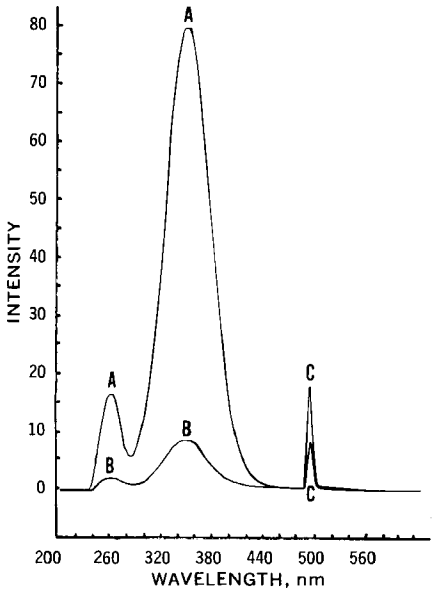


Figure 1—*Excitation spectra of dansyl-estrone* versus *the blank in chloroform; wavelengths of excitation are 264 and 355 nm (with emission at 502 nm). Key: A, dansyl-estrone; B, blank; and C, scattered radiation.*

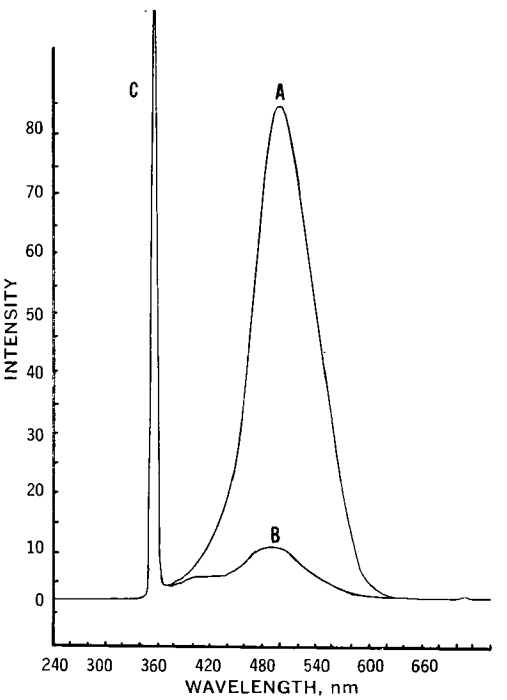


Figure 2—*Emission spectra of dansyl-estrone* versus *the blank in chloroform; wavelength of emission is 502 nm (with excitation at 355 nm). Key: A, dansyl-estrone; B, blank; and C, scattered radiation.*

extract through the chloroform-washed cotton. Evaporate the chloroform to dryness on a steam bath with the aid of a current of air. Dissolve the residue in acetone, warming if necessary to aid in dissolution, and dilute to a final concentration of 0.020 mg/ml with acetone. Continue with the procedure *Fluorometric Determination Using Dansyl Chloride.*

Fluorometric Determination Using Dansyl Chloride—Take exactly 10 ml of the sample solution, and transfer to a 50-ml volumetric flask. Add exactly 10 ml of 0.01% dansyl chloride reagent, and dilute to volume with sodium carbonate aqueous acetone solution (Solution A).

Similarly, transfer exactly 10 ml of the appropriate estrogen standard solution and exactly 10 ml of acetone to serve as a blank into separate 50-ml volumetric flasks. Add exactly 10.00 ml of 0.01% dansyl chloride reagent, and dilute to volume with the sodium carbonate aqueous acetone solution (Solution A). Then let the solutions stand in the dark for 60 min. When the time has elapsed, transfer exactly 10.00 ml from Solution A into a 125-ml separator. Extract with three 10-ml portions of water-washed ether, filter each fraction through ether-washed cotton into a 50-ml glass-stoppered conical flask, and evaporate the combined ether fractions to dryness on a steam bath under a stream of air. Dry the residue for 15 min in a vacuum desiccator containing silica gel. Transfer exactly 10.0 ml of chloroform to each flask (Solution B). Read the solutions in 1-cm quartz cells in a spectrofluorometer, setting the standard solution fluorescence to read 70% full-scale deflection; the excitation and emission wavelengths are 355 and 502 nm, respectively (Figs. 1 and 2).

Calculations—The fluorescence intensity *(F)* of the standard and sample is measured at their maximum at 502 nm. The fluorescence intensity of the blank, measured at the same wavelength, is subtracted from the readings of both the sample and standard. The concentration of estrogens *in injectables* is calculated as:

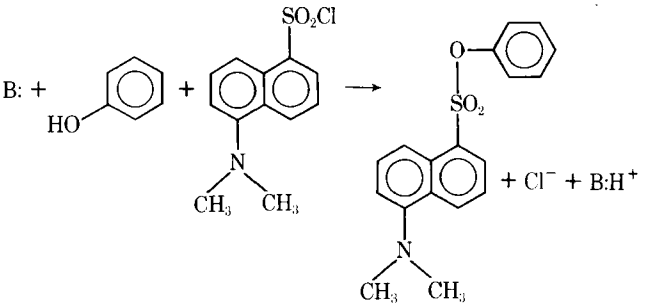
$$\frac{F_{spl} - F_{blank}}{F_{std} - F_{blank}} \times \frac{\text{weight of standard, mg}}{\text{volume of sample taken, ml}} \times \frac{\text{dilutions of standard}}{\text{dilutions of sample}} \times \frac{\text{volume of initial sample solution, ml}}{\text{volume of initial standard solution, ml}} = \frac{\text{mg of estrogen}}{\text{ml of injection taken}} \quad \text{(Eq. 1)}$$

Alternatively, the concentrations can be determined from standard curves. The concentration of estrogens *in tablets* is calculated as:

$$\frac{F_{spl} - F_{blank}}{F_{std} - F_{blank}} \times \frac{\text{weight of standard, mg}}{\text{dilutions of sample}} \times \frac{\text{dilutions of standard}}{\text{weight of sample}} \times \text{average tablet weight} = \frac{\text{mg of ethinyl estradiol}}{\text{tablet}} \quad \text{(Eq. 2)}$$

RESULTS AND DISCUSSION

Formation of Dansyl-Estrogen Derivatives—*Dansyl Chloride Reactions*—The chemical reaction of interest may be conveniently described by Scheme I.



Scheme I

The scheme is presented in a generic manner in that the base B: may be any base such as OH^- or CO_3^{-2}. It seems clear that the reaction should proceed well in an alkaline solution or in the presence of a base since the chemical change involves the displacement of the chloride ion by the phenolate species.

Solution conditions not only have an effect on the desired reaction (Scheme I) but promote a concomitant change since the base can also cause reaction of the dansyl chloride to yield an undesired product. Thus, if hydroxyl ion is the base, the reaction produces 1-dimethylaminonaphthalene-5-sulfonate. In addition, an unfortunate solvent choice could cause hydrolysis of the dansyl-estrogen derivative. The reaction conditions must permit complete formation of the dansyl-estrogen derivative and yet minimize the unde-

Table I—Recovery Study of Estrone, Estradiol, and Estradiol Benzoate in Dosage Forms

Sample	Replicates	Prepared Concentration, mg/ml	Percent Recovered (Average)	Range of Percent Recovered	*SD*	Coefficient of Variation, %
Estrone aqueous suspension	4	2[a]	100.1	99.8–100.3	±0.3	0.264
Estrone aqueous suspension	4	5[a]	102.6	101.9–103.8	±0.8	0.813
Estradiol in sesame oil solution (synthetic preparation)	7	1.018[b]	99.3	95.8–101.1	±1.9	1.92
Estradiol in sesame oil solution (synthetic preparation)	6	0.994[b]	100.6	98.6–102.9	±1.5	1.51
Estradiol benzoate in sesame oil (synthetic preparation)	4	2.99[b]	99.9	98.1–101.8	±2.0	2.03
Estradiol benzoate in sesame oil (synthetic preparation)	4	4.94[b]	99.2	97.1–101.5	±1.8	1.82

[a] Spiked with 2.892 μg of estrone. [b] 3% (v/v) benzyl alcohol.

sired reactions of the dansyl chloride and the dansyl-estrogen hydrolysis. The various reaction conditions studied are treated in the following sections.

Reaction Solvent Composition—Two factors must be considered when selecting a solvent system—*viz.*, the maintenance of a homogeneous system and the preservation at a reasonable rate of the reaction described by Scheme I. The dansyl chloride reagent is soluble in acetone, but a high proportion of acetone in the solvent reduces the velocity of the reaction (34). On the other hand, water increases the speed of the dansyl derivatization reaction but it can likewise accelerate the rate of the undesired hydrolytic conversion of the dansyl chloride to the corresponding sulfonic acid and of the desired dansyl-estrogen to the parent estrogen.

Experiments have indicated that acetone–water mixtures offer desirable advantages (27, 34). As the ratio of acetone to water is decreased from a 1:1 ratio, the solvent polarity increases and the effect is that there is a net loss of dansyl-estrogen by hydrolysis (with a resultantly diminished fluorescence) as well as an increase in the hydrolysis of dansyl chloride. The optimum solvent composition was an acetone–water system in a 2:3 volume ratio; this medium was used in the various estrogen analyses to be described.

Reagent and Base Concentrations and Reaction Rate—Study of Scheme I indicates that a 1:1 stoichiometric relationship exists between estrogen and dansyl chloride. However, to ensure the completeness of the reaction, a five- to 10-fold excess of dansyl chloride reagent was chosen and used satisfactorily.

Since the dansyl-estrogen reaction (Scheme I) proceeds by means of the estrogenic phenolate ion, the need for a base must be incorporated into the analytical solvent system. When using aqueous properties as a guide only, calculations show that there is very little phenolate species in solution at pH 8, the predominant form being the conjugate acid, phenol. At pH 9, 25% of the total phenol is present as the phenolate ion; at pH 12 the percentage rises to more than 99% phenolate ion. However, at very alkaline conditions the hydrolysis of dansyl chloride and the dansyl-estrogen derivative becomes significant, depleting the reagent and resultantly affecting the desired derivatization reaction (Scheme I).

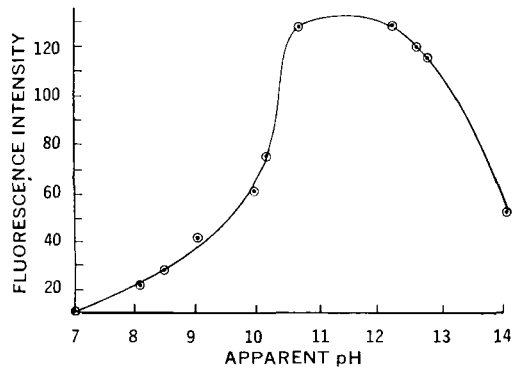


Figure 3—*Effect of apparent pH on the fluorescence intensity of dansyl-estrogen derivatives.*

The means to establish optimum alkaline conditions was taken as the production of maximum fluorescence when the dansyl-estrogen reaction was conducted under particular conditions. The results of such a study are presented in Fig. 3, and the plot indicates that an apparent pH range of 11–12 is optimum. Sodium carbonate was chosen as the base since it is convenient to use and is strong enough to achieve, but not exceed, the desired alkalinity.

To determine an effective sodium carbonate concentration, a series of studies (Fig. 4) was carried out. The concentration of sodium carbonate was varied, and the fluorescence intensity achieved by the dansyl chloride-estrogen reaction was measured as a function of time. The results indicate that in each case the maximum fluorescence is attained in about 30 min. The intensity of fluorescence is seen to be smallest when the largest quantity of 40 mg of sodium carbonate decahydrate is in solution. This phenomenon is probably ascribable to an increase in the reverse reaction (Scheme I) causing hydrolysis of the fluorescent dansyl-estrogen derivative and the dansyl chloride reagent. The intermediate concentration of sodium carbonate decahydrate results in a fluorescence that reaches a maximum within 30 min and is relatively stable for long periods. These results suggest that the best concentration of base is about 20 mg of sodium carbonate decahydrate in 30 ml of solution.

The described study that established the concentration of sodium carbonate to be used also permitted the selection of tempera-

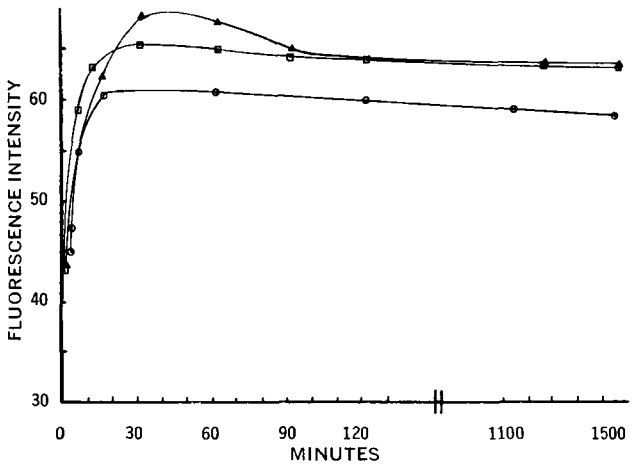


Figure 4—*Speed of reaction at various concentration levels of sodium carbonate decahydrate at 22 ± 2°. The symbols used indicate the weight of sodium carbonate decahydrate in milligrams dissolved in 30 ml of 40% H_2O in acetone for each study. Key: △, 10; □, 20; and ○, 40.*

Table II—Determination of Estrogens in Various Dosage Forms

Sample	Replicates	Declared, mg/ml or mg/Tablet	Average, %	Range	*SD*	Coefficient of Variation, %
Estrone aqueous suspension—1	8	1	1.06 (106)	1.04–1.07	±0.0119	1.12
Estrone aqueous suspension—2	8	2	1.98 (99)	1.91–2.02	±0.0389	1.97
Estrone aqueous suspension—3	5	2	2.00 (100)	1.98–2.05	±0.0306	1.53
Estrone aqueous suspension—4	6	5	4.82 (96.4)	4.80–4.85	±0.0177	0.367
Estradiol in sesame oil injection	10	1	0.955 (95.5)	0.940–0.970	±0.010	1.05
Estradiol valerate in castor oil—1	4	20	20.2 (101)	19.8–20.6	±0.370	1.83
Estradiol valerate in sesame oil—2	4	4[a]	3.91 (97.8)	3.87–3.98	±0.0497	1.27
Ethinyl estradiol tablets	8	0.5	0.487 (97.4)	0.475–0.499	±0.00998	2.04

[a] With testosterone enanthate, 90 mg/ml.

ture and time variables. From Fig. 4 it is seen that a constant fluorescence intensity is reached after 30 min at 22°. To ensure complete reaction, 60 min at room temperature was chosen as the reaction conditions for each estrogen analysis.

In this work, the manner of preparation of the reaction solution system is an important consideration, since the solvent is an acetone–water mixture with dissolved sodium carbonate. Since sodium carbonate is insoluble in acetone, aqueous solutions seem like a good way to introduce sodium carbonate. However, if an aqueous solution of sodium carbonate is added to acetone, an undesired two-phase system results. To have a homogeneous solvent, the solute sodium carbonate must be dissolved in the mixed acetone–water system.

Isolation of Dansyl-Estrogen Derivatives—Although the desirable analytical reaction may be expected to proceed to completion, the reaction solution may contain dansyl-estrogen derivative, excess dansyl chloride reagent, and dansyl hydroxide, formed by hydrolysis of the reagent. TLC monitoring of reaction solutions showed that any excess dansyl chloride is hydrolyzed to the corresponding sulfonic acid after 30 min, the analytical reaction time. Thus, to avoid interference, it becomes necessary to separate the dansyl hydroxide (a fluorescent sulfonic acid) from the dansyl-estrogen derivative.

Since the dansyl hydroxy is acidic, it forms a sodium salt and the dansyl-estrogen can be conveniently extracted from the alkaline reaction solution with a suitable organic solvent. Two of the organic solvents tried, isooctane and chloroform, were judged unsuitable because of turbidity. Ether did not give rise to turbidity and, in addition, the desired solute possessed a high partition coefficient in the ether–alkaline reaction solution. Calculations show that almost 99% of the dansyl-estrogen is extracted with the first 10-ml portion of ether. Thus, three 10-ml portions will extract virtually 100% of the dansyl-estrogen from the alkaline reaction solution.

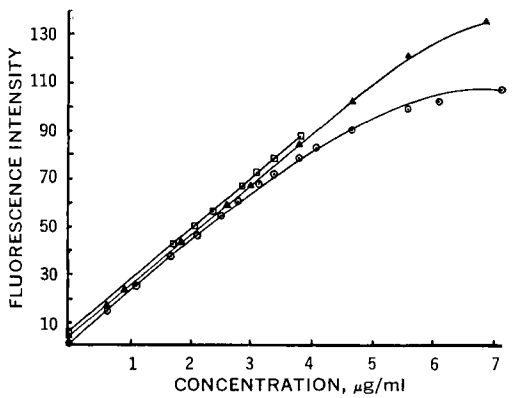


Figure 5—*Relationship between fluorescence intensity and concentration for three estrogens including a least-squares plot. Key:* □, *ethinyl estradiol;* △, *estradiol; and* ○, *estrone.*

Emission and Excitation Spectra of Dansyl-Estrogen Derivatives—The emission and excitation spectra (uncorrected) of dansyl-estrone and the blank in chloroform, the solvent to be used analytically, are shown in Figs. 1 and 2. For dansyl-estrone, two excitation maxima at 264 and 355 nm are observed. The intensity of the peak at 355 nm is approximately five times greater than the peak at 264 nm. Both excitation peaks produce an emission peak at 502 nm. Since all of the derivatives have the same fluorophore, all dansyl-estrogen derivatives have similar emission and excitation spectra.

The blank is run under identical reaction conditions utilizing the same reagents and solvents as the estrogen, except that the estrogen solution is replaced by an identical solvent volume (acetone). The blank has a different emission spectrum than dansyl-estrone, indicating that it is composed entirely of trace amounts of unextracted, intact and hydrolyzed dansyl chloride. The emission peak is at 490 nm, and the excitation peak is at 355 nm.

To minimize errors, it is highly recommended that a blank be run parallel to any estrogen determinations and that its fluorescence intensity be subtracted from that of the estrogen.

Fluorescence Intensity, Concentration, and Sensitivity—The relationship of the fluorescence intensity of the dansyl-estrogen to estrogen concentration was studied using estrone as a generic model. This type of class representation is possible since, in this instance, the fluorophore is the same for all estrogens—*viz.*, the aromatic ring A with a dansyl phenolic ester at the 3-position. Figure 5 is a least-squares plot of the fluorescence intensity of the dansyl-estrone derivative at 14 different concentrations with all solution components constant, estrone being the sole variable. The fluorescence intensity is linear with estrone concentration up to a concentration of 3 μg/ml; the correlation coefficient was calculated to be 0.996. The deviation from linearity at high concentrations is taken to be concentration quenching. Studies with estradiol and ethinyl estradiol showed linearity up to 6 and 4 μg/ml, respectively. These results indicate that the dansyl-estrogen system is amenable to analytical applications.

The sensitivity level was estimated to be 0.5 μg/ml for the final concentration of estrogens based on a minimum exhibited dansyl-estrogen fluorescence intensity of three times the blank. Below this concentration level, there are interferences due to residual and hydrolyzed dansyl chloride reagent. By using the USP XVIII procedure as a guide, it was estimated that the iron–Kober method has a sensitivity of about 1 μg/ml (36). Thus, the proposed method is about twice as sensitive.

APPLICATIONS

Isolation of Estrogens from Dosage Forms—As is usually the case, an analytical step cannot be applied until the material to be measured has been isolated from other dosage form components. The liquid–liquid extraction systems used in this work rely on the

obvious acid–base properties of estrogens. These compounds, being phenols, conveniently form water-soluble sodium salts whereas the phenol itself is soluble in a number of organic solvents.

Although the organic solvents used in the described laboratory procedures work well, some circumstances may require different solvents. This behavior is used where necessary to remove the estrogens from their dosage form matrixes. In those instances where the acid–base behavior cannot be used, chromatographic separations may be applied.

Dosage Form Analyses—To evaluate the proposed method in terms of its ability to isolate and measure estrogens, a recovery study was carried out on an aqueous suspension and an oil solution (Table I). In the case of the estrone aqueous suspension, a standard estrone was added to each sample of estrone suspension. The percentage recovery reported is based on the total estrogen present, *i.e.*, the quantity present in the original dosage form as well as the amount added. The recovery percentages and standard deviations (<1%) indicate the proposed procedure to be quite efficient and capable of measuring estrone in aqueous suspension.

The recovery and measurement of estradiol in an oleaginous medium were studied with solutions prepared in the laboratory and hence labeled "synthetic." To make the situation realistic, benzyl alcohol was included since, in some instances, it is added as a bacteriostatic agent as well as a local anesthetic. The results are excellent, with recoveries at 100.0% and standard deviations of less than 2%. These results demonstrate that the separation and measurement procedure used separates the estrogen from any interferences and that the benzyl alcohol does not interfere. Replicate analyses indicate the expected reproducibility.

It should be mentioned that phenol or phenolic compounds, *e.g.*, the parabens, used on occasion in estrogen dosage forms, would be expected to, and do in fact, interfere in the analysis. Thus, since the separation procedure presented here does not separate other phenols from the phenolic estrogens, another separation scheme would be required if nonestrogenic phenols are present or the interference is anticipated in some manner. Chlorobutanol, another preservative, did not interfere.

The use of estradiol benzoate in dosage forms in the recovery study introduces a problem heretofore not encountered since the benzoate is a phenolic ester. Since the benzoate ester does not offer a free phenolic function, the acid–base liquid extraction used for other estrogens could not be employed. A chromatographic column procedure was adopted (37, 38) to isolate the estrogen from the oil. Once the separation is complete, the ester needs to be hydrolyzed to make the phenol available for the dansyl reaction. By using the procedure described in the *Experimental* section, the analysis was carried out and the results are presented in Table I. These results are excellent (based on label statement) and are reproducible to within 2%.

The dansyl-estrogen proposed procedure was studied comparatively by using a common estrone aqueous suspension as the test sample. The latter sample was analyzed by the official NF XIII procedure, a gravimetric analysis based on reaction of the 17-keto group with a hydrazide, and the dansyl-estrogen method. The coefficient of variation for the analyses are as follows: NF XIII, 3.20%; and dansyl, 0.68%. Although the basis for each method is different, the results are comparable with a standard deviation for the NF XIII of 2.04 ± 0.065 and for dansyl of 2.06 ± 0.014, indicating that the dansyl procedure yields reliable results with acceptable reproducibility.

A number of commercial estrogen injectables and suspensions and one estrogen tablet were analyzed by this method (Table II). Inspection of these results indicates that, in each instance, the values are within acceptable limits, with the minimum percentage of 94.3%. The reproducibility is satisfactory, with only 1 *SD* slightly greater than 2%. For those products that are official compendial dosage forms, these analyses indicate them to be within rubric limits.

The analysis of estradiol valerate, although an ester, may be carried out by the general procedure since the ester is at the 17-position and does not interfere with the dansyl reaction. There is a complication, however, owing to the nature of the oil solvent used in the dosage form. In this study, the castor oil-based estradiol valerate dosage form required the use of *n*-heptane as the organic solvent. The remainder of the procedure is the same. As may be seen from Table II, one estradiol valerate sample analysis was conducted in the presence of testosterone enanthate. Since no interference is observed, as is evident from the assay values, the procedure would be expected to function satisfactorily in the presence of androgenic steroids.

One sample of ethinyl estradiol tablets (0.5 mg/tablet) was analyzed (Table II). The procedure was also applied to the analysis of single tablets satisfactorily. Although the results are more than adequate, lower potency ethinyl estradiol tablets, 0.05 mg/tablet, were difficult to analyze and results were inconsistent and nonreproducible. At this low estrogen level, the behavior may be due in part to the interference of tablet excipients and excess dansyl chloride reagent. The method of James (39) is suggested as an alternative.

At present, work is proceeding in the study of the application of the dansyl-estrogen system to the analysis of the components of conjugated estrogens.

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RALPH M. RIGGIN, ALLEN L. SCHMIDT, and PETER T. KISSINGER ˣ

Abstract □ A sensitive and very rapid assay for acetaminophen was developed based on the combination of high-performance chromatographic columns with a thin-layer electrochemical detector. Application to liquid and solid dosage forms and body fluids has been demonstrated. Great advantage derives from the detector selectivity, which permits discrimination against many potentially interfering substances without need for extensive separations or formation of derivatives. As little as 0.005% of the hydrolysis product, *p*-aminophenol, can be detected in the presence of the intact drug following cation-exchange chromatography. Acetaminophen can be quantitatively determined in serum on the 50-ng/ml level by liquid chromatography using a pellicular polyamide packing.

Keyphrases □ Acetaminophen—analysis in dosage forms, serum, and urine, high-performance liquid chromatography with electrochemical detection □ Liquid chromatography, high performance—determination of acetaminophen in pharmaceutical preparations and body fluids, electrochemical detection □ *p*-Aminophenol—determination of trace amounts in acetaminophen preparations, high-performance liquid chromatography with electrochemical detection

Fundamental advances recently have been made in liquid chromatography and analytical electrochemistry. Both fields depend on heterogeneous processes, which are now sufficiently well understood to permit optimization of the controlling parameters. This knowledge has resulted in a new enthusiasm for methods that only a few years ago were thought to be outmoded. Liquid chromatography, while easy enough to use, continues to suffer from the lack of a completely satisfactory detector, particularly one with sufficient sensitivity to compete with GC. On the other hand, modern electrochemical techniques have attained considerable sensitivity but suffer from rather poor resolution, chronic irreproducibility, and poor human engineering.

It is not surprising that great advantages accrue from combining the two technologies. Strong parallels exist with the more established association of liquid chromatography and electronic spectroscopy (both absorption and emission). In both cases, two phenomena of modest specificity are combined into a single high-resolution device. The optical instrumentation presently used in liquid chromatography is often insufficiently sensitive or selective for quantitating body fluid levels for many clinically important compounds. Although less generally applicable than optical devices, electrochemical detectors can sometimes form the basis for successful assay procedures.

Recently, a thin-layer electrochemical transducer was developed that has significant advantages over conventional electrochemical cells when coupled to high-performance liquid chromatography (1, 2). The principles of operation of this detector and other developments in hydrodynamic electrochemistry have been described (2, 3). In brief, the column effluent is passed between two plates separated by a thin spacer (typically 50 μm). Electrodes imbedded in the walls of the channel efficiently convert sample molecules into the product due to the short diffusional pathway across the moving solution film. It is possible in this manner to detect as little as 1 pg of an electroactive material while maintaining a detector dead volume of less than 1 μl.

In view of the large number of electrochemically reactive pharmaceuticals, liquid chromatography with electrochemical detection could have wide utility in quality control and drug metabolism studies. The present paper describes several procedures developed for acetaminophen (*N*-acetyl-*p*-aminophenol) in dosage forms and body fluids.

Acetaminophen is most commonly assayed in dosage forms by spectrophotometry. The drug may be monitored directly (4, 5) or following conversion to 2-nitro-4-acetamidophenol (6). Many published procedures are based on acid hydrolysis to *p*-aminophe-

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nol followed by oxidative coupling with a phenol to an indophenol dye (7). More recent approaches include stationary electrode voltammetry at a glassy carbon electrode (8) and nonaqueous potentiometric titrimetry with tetrabutylammonium hydroxide in dimethylformamide (9). None of these procedures is suitable for the determination of trace quantities of the *p*-aminophenol hydrolysis product in the presence of the intact drug.

Many papers have described the determination of total acetaminophen in body fluids by initial hydrolysis of conjugates as well as the free drug to *p*-aminophenol. This common derivative is extracted and subsequently coupled to form an azo or indophenol dye suitable for spectrophotometry (10, 11). More specific and generally more sensitive procedures are based on GLC of the free drug (12, 13) or its trimethylsilyl derivative (14). Studies of phenacetin and acetaminophen metabolism by high-resolution liquid chromatography with UV detection, whereby individual metabolites were identified and quantitated, were reported (15, 16).

EXPERIMENTAL

Reagents and Materials—Distilled water, deionized and finally distilled from alkaline potassium permanganate, was used for preparing all solutions. Acetaminophen, *p*-aminophenol, phenacetin, and salicylamide were obtained from commercial sources. *p*-Aminophenol was purified by vacuum sublimation at <1 mm Hg and 120°. Tablet and liquid dosage forms were purchased locally. All other chemicals were reagent grade and were not further purified.

Glass chromatographic columns[1], 30 cm × 2 mm i.d., were dry packed with pellicular strong cation-exchange[2] or polyamide[3] high-performance packings. Dilute sulfuric acid (0.04 *M* at 0.25 ml/min) was used as the eluent for the cation-exchange column, and phosphate buffer (0.04 *M*, pH 7.4 at 0.35 ml/min) was used for the polyamide column.

Standard solutions of acetaminophen were prepared by diluting a stock solution (1.0 mg/ml) in 0.04 *M* H_2SO_4. Instrumentation was calibrated using six solutions between 10 and 80 ng/μl. Standard solutions containing 200 ng/μl of acetaminophen and between 10 and 400 pg/μl of *p*-aminophenol (*i.e.*, 0.005–0.2%) were prepared in distilled water and used to calibrate the cation-exchange system for trace *p*-aminophenol.

Apparatus—Chromatographs were assembled from commercially available modular components, and the electrochemical detector cell was constructed as described previously (2). The instrumentation used to control the applied potential and to measure the resulting current is depicted in Fig. 1. A conventional three-electrode configuration is used with auxiliary, reference, and working electrodes of platinum, silver–silver chloride, and carbon paste, respectively. The currents encountered in detection of trace organics in the eluate from high-performance liquid chromatography are frequently below 1 namp. Therefore, it is essential to use operational amplifiers with low bias currents for OA-1 and OA-2[4] and to guard their inputs against stray leakage currents along the circuit board. The requirements for OA-3 and OA-4 are more modest; standard 741-type units will suffice.

SW1 and R14 permit selection of the appropriate applied potential. SW3 and SW4 provide a selection of the gain and filtering in the first stage of amplification. SW5, R15, OA-4, and R5 provide a balance for the standing background current encountered in the absence of an eluted zone. When SW3 is in the most sensitive position, SW6 provides a range of current sensitivities of 0.5, 1, 5, 10, 50, and 100 namp/v. The values for the passive components used are as follows: R1, 33K; R2, 100K; R3, 1M; R4, 10M; R5–R8, 1K; R9, 2K; R10, 10K; R11, 20K; R12, 100K; R13, 200K; R14, 5K; C3, 1 μF; C4, 0.47 μF; and C5, 0.01 μF.

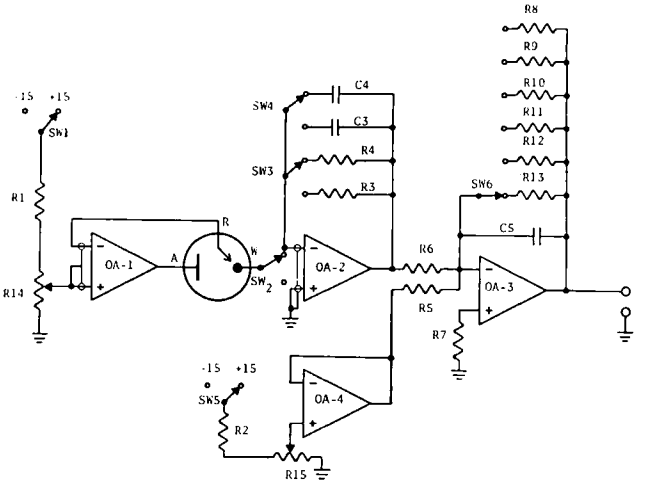

Figure 1—*Electronic instrumentation for electrochemical detection. Key: A, auxiliary electrode; R, reference electrode; and W, working electrode.*

All quantitative measurements were accomplished by analog integration of the output from OA-4 using an operational amplifier integrator with a time constant of 10 sec. The results were displayed on a digital voltmeter. The integrated response can be converted directly into the microcoulombs of charge passed for a given chromatographic peak. By using standard solutions, the amount of charge required for complete conversion can be calculated from Faraday's law. Measurements at 0.25 ml/min mobile phase velocity indicate that about 10% conversion is typical for low molecular weight phenolic compounds such as acetaminophen. Higher conversion is possible at lower flow rates or for cells with thinner channels and/or larger electrodes; however, such higher conversion does not contribute significantly to either sensitivity or precision.

The linear range of calibration plots of charge *versus* injected amount is dependent on cell geometry due to the uncompensated resistance along the thin-layer channel. For a 50-μm cell and a flow rate of 0.025 ml/min of a 0.1 *M* mobile phase electrolyte, a significant negative deviation typically occurs above several hundred nanograms for molecules with short retention times. If very large samples (*e.g.*, several micrograms) are to be injected, linearity of response can be ensured by adjusting the cell thickness up to 200 μm and/or increasing the applied potential. In general, it is more satisfactory to dilute the sample to within the established linear range.

Current–voltage curves (*i.e.*, voltammograms) for oxidation of acetaminophen and other phenolic compounds were determined using a carbon paste electrode of 3 mm diameter packed in a Teflon well with an outside diameter of 7 mm. The carbon paste was of the same composition described earlier (1). A simplified circuit using three integrated operational amplifiers was used in association with small volume (~10 ml) Pyrex cells, with the electrodes positioned in a Teflon cap[5].

Procedure—Five commercial products, as well as serum and urine samples, were analyzed for acetaminophen. Samples (2 μl) prepared according to the following procedures were injected manually onto the chromatographic columns through silicone septa using 5-μl syringes[6]. The peak areas were converted into nanograms injected by comparison with the least-squares slope determined from the six calibration standards described. The applied potential was +1.0 v *versus* a silver–silver chloride reference electrode (3 *M* NaCl) for the acetaminophen determination.

Preparation of Solid Dosage Forms—Five tablets were accurately weighed and then pulverized to a fine powder. A known per-

[1] Model MB-2-300, Chromatronix, Inc.
[2] Zipax SCX Part No. 820950002, E. I. DuPont de Nemours & Co.
[3] Pellidon, H. Reeve Angel & Co.
[4] National semiconductor LH0042CH.

[5] Photographs and construction details for locally built apparatus are available on request.
[6] Hamilton 85-N.

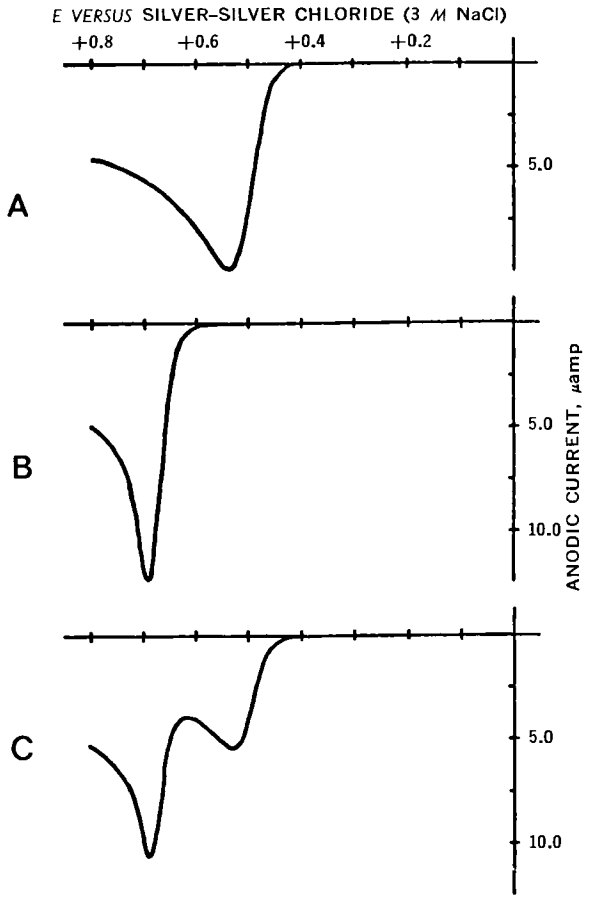

Figure 2—*Stationary electrode voltammetry (0.14 v/sec) at the carbon paste electrode* (d_w = *3 mm*) *in 0.1* M *$HClO_4$. Key: A, 1 m*M p-*aminophenol;* B, *1 m*M *acetaminophen* (N-*acetyl*-p-*aminophenol*); *and C, 0.5 m*M p-*aminophenol and 0.5 m*M *acetaminophen.*

centage of the total mass (1–4%) was then weighed and diluted to 100 ml with 0.04 *M* H_2SO_4. The solution was shaken for 5 min to ensure complete dissolution of acetaminophen. A 10-ml aliquot was transferred to a second 100-ml volumetric flask and diluted with 0.04 *M* H_2SO_4. The resulting solution, containing from 10–40 ng/μl, was used for the analysis.

Preparation of Liquid Dosage Forms—Two milliliters of the liquid product was diluted to 100 ml with 0.04 *M* H_2SO_4. A 10-ml aliquot of this initial solution was diluted again to 100 ml with 0.04 *M* H_2SO_4 to yield a final concentration in the 20–30-ng/μl range.

Determination of Trace p-*Aminophenol in Acetaminophen*—The initial solutions prepared from the dosage forms contained 100–400 ng/μl acetaminophen. These more concentrated solutions were analyzed for *p*-aminophenol. To monitor trace amounts of *p*-aminophenol successfully on the level of 0.005–0.2% of the acetaminophen present, it is necessary to decrease the response of the detector to the latter compound by lowering the applied potential. A potential of +0.65 v was satisfactory for this purpose. Therefore, it was possible to detect as little as 0.005% of *p*-aminophenol in the original dosage forms.

Determination of Acetaminophen in Serum—Three-milliliter aliquots from a normal serum pool were placed in 50-ml polyethylene centrifuge tubes containing 2 g of sodium chloride and 3 ml of saturated sodium chloride. The samples were spiked with standard acetaminophen over a 0.1–10-μg/ml range. Concentrated perchloric acid (0.5 ml) was added, and the tubes were mixed on a vortex mixer for 30 sec. Following centrifugation at 14,000 rpm for 15 min, 5 ml of the supernate was transferred to a 15-ml glass centrifuge tube. After adjustment to pH 7.0, the solution was extracted with two 5-ml portions of ethyl acetate.

The ethyl acetate layers were combined in a second 15-ml centrifuge tube and back-extracted with a single 2-ml portion of pH 7.0 McIlvaine buffer to minimize the residual uric acid. The ethyl acetate layer was then evaporated to dryness at 40° under a stream of nitrogen. The residue was brought up in 200 μl of 0.04 *M* H_2SO_4 with thorough mixing, and then 2 μl was injected onto the polyamide column and detected at a flow rate of 0.35 ml/min and a potential of +0.80 v *versus* silver–silver chloride. It is easy to make the measurement on 1 ml of serum with little sacrifice in precision by proportional adjustment of the volumes used in the extraction procedure.

The polyamide column was used to minimize interference from uric acid carried through the extraction procedure, since it is easily oxidized at a carbon electrode (17, 18). Because uric acid is not adequately resolved from acetaminophen on the cation-exchange resin, it remains a possible interference in trace determinations of the latter compound in serum and urine. Fortunately, acetaminophen is more strongly retained than uric acid on the polyamide column. The chromatographic resolution adequately compensates for the poor electrochemical selectivity for the two compounds.

Determination of Acetaminophen in Urine—The procedure was identical with that described for serum, except that 5 ml of sample was used without the addition of the sodium chloride solution. Back-extraction of the ethyl acetate with pH 7 buffer was repeated twice due to the 10-fold greater amount of uric acid present in urine. Recovery of standard additions to urine was typically 83%.

RESULTS AND DISCUSSION

The performance of an electrochemical detector can be optimized on the basis of preliminary voltammetric studies. The compounds of interest and possible interferences are examined to establish the potentials sufficient to react them at a diffusion-controlled rate. Figure 2 illustrates behavior typical of phenolic amines in acidic media. The electrochemical process involves a two-electron conversion of the compounds to intermediate species which eventually react to form benzoquinone. This chemistry has not yet been worked out for acetaminophen; however, a detailed study is in progress. Acetaminophen and *p*-aminophenol have oxidation potentials different by about 150 mv in acid solution. When the two compounds are present in a mixture at similar concentrations, they may be clearly distinguished as in Fig. 2C.

Quantitative estimation of both compounds based on the peak currents is difficult because the *p*-aminophenol wave overlaps with that due to acetaminophen in a manner not readily accounted for

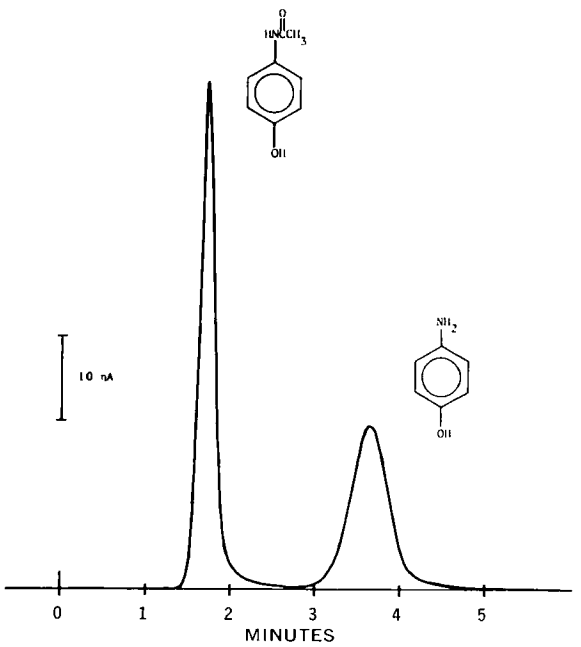

Figure 3—*Liquid chromatography of 7.5 ng acetaminophen* (N-*acetyl*-p-*aminophenol*) *and 7.5 ng* p-*aminophenol* (E = + *1.20 v* versus *silver–silver chloride 30* × *0.2-cm column, 0.25 ml/min 0.04* M *H_2SO_4*).

Table I—Analysis of Dosage Forms Containing Acetaminophen

Dosage Form	Labeled Composition, mg/Dose	Measured Composition, mg/Dose	Percent of Labeled Value Found
A (tablet)	388	373 ± 0.50%[a]	96.1
B (tablet)	194	187 ± 1.2%	96.3
C (tablet)	385	375 ± 0.41%	97.4
D (liquid)	120	121 ± 1.1%	101
E[b] (liquid)	600	589 ± 0.14%	98.2
F (liquid)	600	577 ± 0.60%	96.1
G (liquid)	600	582 ± 0.12%	97.0

[a] Relative standard deviation based on at least five determinations. [b] Liquids E, F, and G were different samples of the same dosage form purchased over a 1-year period.

mathematically. Because the heterogeneous reaction of acetaminophen is exponentially dependent on potential, at potentials near the foot of the wave in Fig. 2B, it is nearly impossible to determine trace quantities of *p*-aminophenol in the presence of a much larger amount of acetaminophen since both reactions occur to a significant extent at the peak potential of the former molecule. The problem of resolving successive electrochemical reactions is an inherent disadvantage of voltammetric methods, particularly since all compounds react at the potentials needed to quantitate the least reactive component. Since the hydrolysis of acetaminophen is a very slow reaction at ordinary temperatures (19), *p*-aminophenol is commonly present at well below 1% in dosage forms. A voltammetric approach to analysis of this undesirable decomposition product is clearly insufficient.

Physical separation of the two components is readily accomplished *via* high-performance cation-exchange chromatography (Fig. 3). From the voltammetric information, a potential of about +0.70 v or greater is sufficient to detect both compounds using electrochemical detection. Therefore, it is easily possible to make quantitative measurements on both compounds at the 100-pg level, well below the detection limit for presently available UV instruments. Nevertheless, determination of trace *p*-aminophenol is still impossible when acetaminophen is present in a 1000-fold excess. Fortunately, this problem can easily be overcome by operating the detector at lower potentials so that the response is selective to *p*-aminophenol. The same degree of selectivity is not possible with a UV detector since the absorption spectra of the two compounds are insufficiently resolved.

The results from an analysis of several popular dosage forms containing acetaminophen are given in Table I. All were found to be within the regulated specifications (20). Other components in the preparations were eluted at different retention times and/or were not electroactive. Several compounds (*e.g.*, salicylamide and phenacetin) also present as active ingredients were much more difficult to oxidize than acetaminophen and could be easily "tuned out" by adjusting the potential applied to the detecting electrode. In each sample, the *p*-aminophenol level was below 0.01% of the label value for acetaminophen, well within the regulated maximum of 0.25% for the bulk drug. It is believed that the present method for monitoring trace *p*-aminophenol is superior to the standard semiquantitative TLC assay (20).

The determination of free acetaminophen in biological fluids was easily accomplished by simple extraction following protein precipitation. In serum the response was linear over the range tested (0.1–10 μg/ml), and quantitative data (typically to about ±10% *RSD*) could be obtained on a level an order of magnitude below the capability of GC procedures (13, 14). In addition, this simple method is far less time consuming and more precise than those involving derivative formation (14).

The assay of acetaminophen in urine is more difficult than serum, primarily due to the larger concentration of uric acid. The limit for quantitative measurement is at present about 1 μg/ml. The clearance of free drug was found to be 2.1% over 24 hr for a 22-year-old male following a single 1-g dose of a commercial elixir. Conjugated metabolites (glucuronides and sulfates) can be estimated following acid or enzymatic hydrolysis in the usual manner. It is hoped that conjugated phenols derived from aromatic drugs will be able to be detected without prior hydrolysis. Recent work in this laboratory indicates that many of these are electrochemically active.

In conclusion, high-performance liquid chromatography with thin-layer electrochemical detection provides unusual sensitivity and selectivity at minimum expense. Besides acetaminophen and related molecules, many pharmaceutical products are suitable for analysis in this way (21, 22). Isoproterenol, levodopa, α-methyllevodopa, norepinephrine, epinephrine, dopamine, phenylephrine, and chloramphenicol are among the compounds that have been successfully analyzed. The applicability of the technique can be greatly expanded by nitration (23) or hydroxylation (24) of compounds containing a nonelectroactive phenyl moiety.

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Automated Procedure for Determination of Free Aromatic Amine in Water-Soluble Radiopaques

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Abstract □ The determination of free aromatic amine in water-soluble radiopaques by diazotization in 2-methoxyethanol–water, coupling with naphthylethylenediamine, and colorimetry of the azo dye has been automated. Sensitivity, precision, and accuracy are satisfactory for purity testing and stability studies.

Keyphrases □ Radiopaques (water soluble)—automated determination of free aromatic amine impurities □ Amine impurities in water-soluble radiopaques—automated determination

Many water-soluble radiopaques currently used in urography, angiography, and intravenous cholecystography are derivatives of 2,4,6-triiodobenzoic acid with at least one acylated aromatic amino group in position 3 or 5. Acylation of the amino group is normally the last step of the synthesis. Therefore, testing of nonreacted amine as a potential impurity becomes mandatory. In fact, a quantitative determination of free aromatic amine is specified in several monographs for radiopaques listed in USP XVIII and BP 1968.

The purpose of the present investigation was to develop an automated technique for the determination of free aromatic amine in radiopaques as bulk products or as injectable solutions. The application of the procedure is for routine purity testing and for extensive stability studies of the injectable radiopaque solutions, where free aromatic amine may be formed by hydrolysis of the acylamino group.

Among the methods for the determination of aromatic amine reported in the literature, the Bratton–Marshall (1) reaction was considered most suitable for automation. This reaction is used in the USP XVIII monographs for meglumine diatrizoate injection (2), sodium diatrizoate injection (3), meglumine iodipamide injection (4), sodium iodipamide injection (5), meglumine iothalamate injection (6), and sodium iothalamate injection (7); it has already been automated for assay of ethopabate (8), sulfonamides, and other drugs (9, 10).

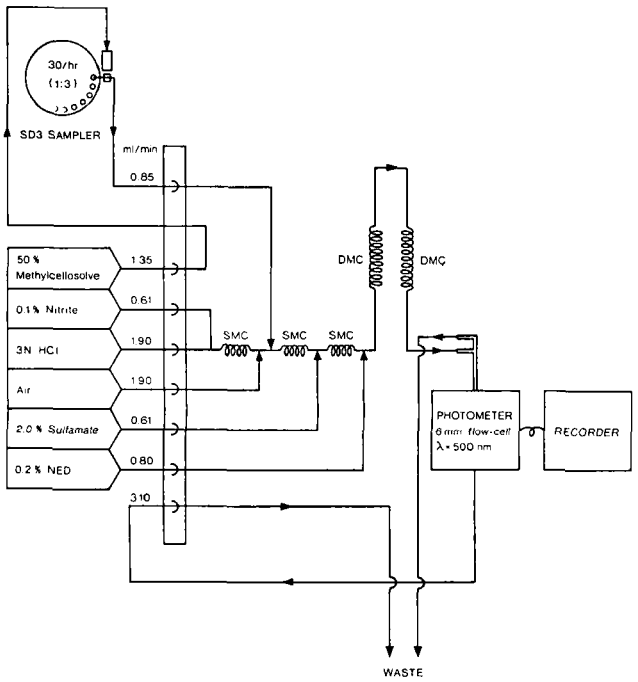

Figure 1—*Automated flow diagram (NED = naphthylethylenediamine).*

EXPERIMENTAL

Apparatus—The analytical train included the following modules: a SD3 sampler distributor[1] programmed at 30 samples/hr and set for a sample–wash ratio of 1:3, a proportioning pump[2], a flow photometer[3] equipped with a 6-mm continuous flow cell and a 500-nm interferential filter, and a recorder[4] with a chart speed of 152.4 cm/hr (60 in./hr).

Reagents[5] and Solutions—The following were used: 2-methoxyethanol[6]–distilled water (1:1, v/v), 3 *N* hydrochloric acid in 2-methoxyethanol–distilled water (1:1, v/v), 0.1% sodium nitrite in distilled water, 2% ammonium sulfamate in 2-methoxyethanol–distilled water (1:1, v/v), and 0.2% *N*-(1-naphthyl)ethylenediamine in 2 *N* hydrochloric acid–2-methoxyethanol (1:1, v/v).

Standard Solutions—The following standard solutions were prepared: 3-amino-2,4,6-triiodobenzoic acid, 3-amino-5-acetamido-2,4,6-triiodobenzoic acid, 3-amino-5-methylacetamido-2,4,6-triiodobenzoic acid, and 5-amino-2,4,6-triiodo-*N*-acetylisophthalamic acid.

Stock solutions were prepared by neutralizing 50 mg of each standard substance with 0.1 *N* NaOH and diluting to 100 ml in a volumetric flask with distilled water. These stock solutions were further diluted separately to obtain final concentrations in 2-methoxyethanol–water (1:1, v/v) ranging from 2.5 to 150 μg/ml.

The standard substances were checked for purity by carbon and iodine microanalysis, acidimetric determination of the equivalent weight, and TLC.

Sample Solution—For preparation of sample solutions from bulk substance, 5 g was neutralized with 2 *N* NaOH, diluted to 25 ml with distilled water, and then diluted to 50 ml with 2-methoxyethanol. From injectable solutions, a sample of 1–5 ml was withdrawn and diluted accurately with distilled water and then with 2-methoxyethanol to obtain a 10% solution of the radiopaque in 2-methoxyethanol–water (1:1).

Automated Procedure—The schematic diagram of Fig. 1 indi-

[1] Colorimetric laboratory analyzer model 1500, C. Erba, Milan, Italy.
[2] C. Erba, Milan, Italy.
[3] Model FF1, C. Erba, Milan, Italy.
[4] Speedomax W, Leeds & Northrup.
[5] All chemicals were E. Merck reagent grade.
[6] Methylcellosolve.

Table I—Statistical Analysis of Regression Lines[a] ($y = bx + a$)

Parameter	Standardization Number									
	1	3	5	7	9	14	15	16	19	23
b	163.6	163.2	163.0	164.1	163.5	162.1	160.1	162.6	164.4	161.7
a	−0.16	0.09	−0.27	0.00	−0.15	0.70	0.46	0.31	0.14	0.33
r	0.999986	0.999993	0.999981	0.999979	0.999988	0.999948	0.999960	0.999960	0.999967	0.999977
n	7	7	7	7	7	7	7	7	7	7
s_b	0.39	0.27	0.44	0.47	0.35	0.93	0.49	1.02	0.93	0.66
s_a	0.49	0.49	0.49	0.49	0.49	0.51	0.51	0.81	0.81	0.64
s_{xy}	±0.40	0.28	0.47	0.49	0.37	0.75	0.40	0.42	0.38	0.31

[a] y = concentration (micrograms per milliliter), x = absorbance, b = regression coefficient (slope), a = shift of regression line, s_{xy} = standard deviation of scatter around regression line, s_b = standard deviation of b, s_a = standard deviation of a, and r = correlation coefficient.

Table II—Precision and Accuracy

$R\text{-}NH_2$ Given x_i, μg/ml	$R\text{-}NH_2$ Found x_i, μg/ml										Mean Value $\bar{x}_i$, μg/ml	Precision $\left(\frac{\Sigma(x_i - \bar{x}_i)}{n-1}\right)^{1/2}$	Accuracy $x_i - \bar{x}_i$, μg/ml
	1	3	5	7	9	14	15	16	19	23			
2.41	2.29	2.54	2.17	3.11	2.47	2.97	2.72	2.70	2.12	2.57	2.57	±0.32	+0.16
4.82	4.75	4.99	4.78	5.09	4.92	5.40	5.09	5.10	4.74	4.99	4.98	0.20	+0.16
9.65	9.82	9.88	9.51	9.35	9.99	9.78	9.82	9.75	9.82	9.69	9.74	0.19	+0.09
19.30	18.98	19.18	18.96	19.20	19.14	19.02	18.97	19.03	19.48	19.08	19.10	0.16	−0.20
48.28	48.26	48.07	48.96	47.92	48.41	47.06	47.85	47.85	48.15	48.06	48.06	0.49	−0.22
96.50	97.34	96.21	97.20	96.16	95.99	96.01	95.98	96.04	97.12	96.63	96.47	0.55	−0.03
144.78	144.61	145.04	144.60	145.16	145.13	145.45	145.21	145.19	144.30	144.71	144.94	0.36	+0.16

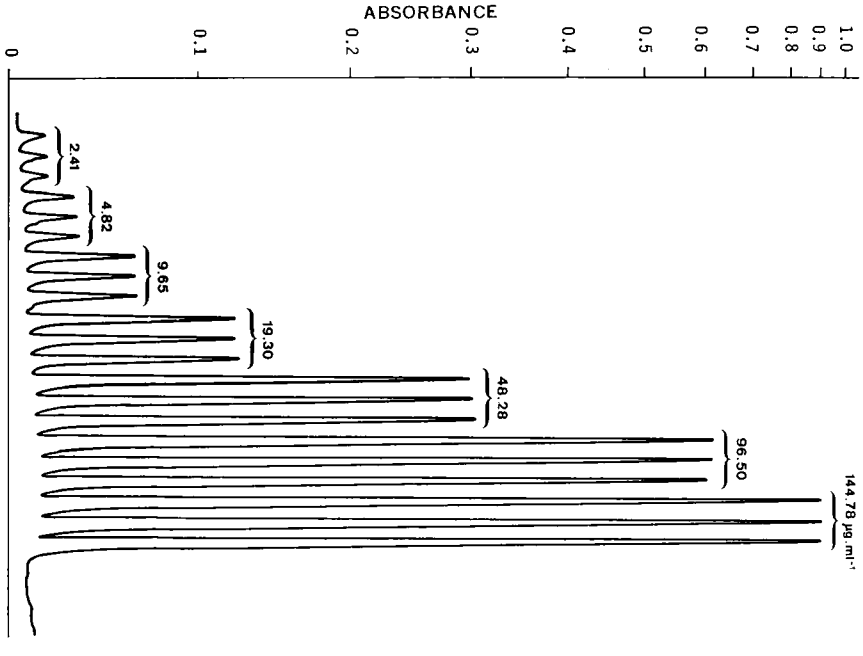

Figure 2—*Typical recording curve of 3-amino-2,4,6-triiodobenzoic acid standard solutions.*

cates the five phases of the automated procedure: (*a*) aspiration of the sample (30 sec) and washing (90 sec), (*b*) segmentation with air and combination with diazotizing reagent (reaction time of 40 sec at room temperature), (*c*) elimination of excess nitrous acid with ammonium sulfamate (40 sec), (*d*) coupling of the diazotized aromatic amine with naphthylethylenediamine (160 sec), and (*e*) continuous-flow photometry of the azo dye at 500 nm.

The flow rate of the reagents was set for obtaining the following molar ratio:

sample (200 μg/ml)	1
sodium nitrite	27
hydrochloric acid	17,000
ammonium sulfamate	325
N-(1-naphthyl)ethylenediamine	17

Prior to performing the analysis, the automated system was calibrated by triplicate assay of a series of analytical reference standards in the 2.5–150-μg/ml range. The linear regression was calculated from the recorded absorbance values (11).

Triplicate sets of five "standard" samples with a reference standard between each set were analyzed. The free amine concentration was determined from the mean absorbance measurement with the aid of the standard calibration curve[7].

RESULTS AND DISCUSSION

Linearity and Sensitivity—Figure 2 is a reproduction of a recording curve obtained by analyzing standards of sodium acetrizoate injectable solution with a free amine content in the 2.5–150-μg/ml range. The uniformity and linearity of triplicate analyses at

[7] If the mean error of the determination of one of the inserted standard samples was greater than two times the standard deviation of the calibration curves, the system was calibrated again and the new regression values were used for the following analyses. In practice, the deviation of the inserted standard sample was always ≤1.5 *SD* within the same working day.

Table III—Comparison of Automated and Manual Method

Injectable Radiopaque	Content			Free Amine Found, mg/100 ml		
	Salt, g/100 ml	Composition, %	Iodine, mg/ml	Automated Method	Manual Method	Differences
Sodium–meglumine diatrizoate	60	Meglumine 87 Sodium 13	296	3.9	3.7	+0.2
Sodium–meglumine diatrizoate	60	Meglumine 87 Sodium 13	296	4.2	3.95	$+0.2_5$
Sodium–meglumine diatrizoate	75	Meglumine 87 Sodium 13	370	5.6	5.8	−0.2
Sodium–meglumine diatrizoate	75	Meglumine 87 Sodium 13	370	6.0	6.1	−0.1
Sodium iothalamate	60	Sodium 100	282	14.4_5	15.0	-0.5_5
Sodium iothalamate	60	Sodium 100	282	12.3	12.2	+0.1
Sodium iothalamate	67	Sodium 100	400	8.1	7.9	+0.2
Sodium iothalamate	80	Sodium 100	479	23.1	23.3	−0.2
Sodium iothalamate	80	Sodium 100	479	8.6	8.6	±0.0
Meglumine iodamide	65	Meglumine 100	300	5.0	5.0	±0.0
Sodium–meglumine iodamide	62	Meglumine 77 Sodium 23	340	3.4	3.2	+0.2
Sodium–meglumine iodamide	80	Meglumine 85 Sodium 15	380	17.0	16.8	+0.2
Sodium–meglumine iodamide	80	Meglumine 45 Sodium 55	420	7.7	7.5	+0.2

the various concentrations and the return to the baseline between samples, indicating no overlap, can be noted.

Precision and Accuracy—Reproducibility of the automated technique was demonstrated by statistical analysis of 10 concentration *versus* absorbance regression lines randomly selected out of 36 calibrations performed with the same reference standards during 2 months (Tables I and II). The accuracy of the proposed procedure was evaluated by comparison of the results obtained by the automated technique with that of the USP XVIII (2, 3, 6, 7) procedure. Table III shows the excellent agreement between the two procedures for sodium and meglumine salts of diatrizoic acid and iothalamic acid.

The values for the manual method reported in Table III for iodamide salts were obtained by a manual method developed in these laboratories corresponding to the USP XVIII method for diatrizoate injectable solutions.

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Syntheses and Tissue Distribution of ^{99m}Tc-Sulfonylureas

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Abstract □ Four new tolbutamide analogs were prepared and evaluated as hypoglycemics. Hypoglycemic action was observed in two members of the class, and ^{99m}Tc-chelates were tested as potential radiopharmaceutical imaging agents.

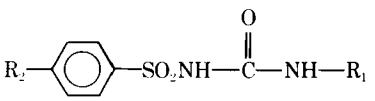
Keyphrases □ Sulfonylureas—synthesized and screened as hypoglycemics, tissue distribution of ^{99m}Tc-chelates, potential pancreatic imaging agents □ Hypoglycemic agents—synthesis and screening of sulfonylureas, tissue distribution of ^{99m}Tc-chelates □ Radiolabeled sulfonylureas—tissue distribution of ^{99m}Tc-chelates, potential pancreatic imaging agents □ Pancreatic imaging agents, potential—synthesis and tissue distribution of ^{99m}Tc-sulfonylureas

As part of a study on the development of γ-emitting radiopharmaceuticals for imaging the pancreas, several chelates of ^{99m}Tc were synthesized which were structural analogs of the pancreatic hypoglycemic, tolbutamide (I). The *para*-substituted phenylsulfonylurea moiety (I–VII) has been identified as a characteristic structural feature in numerous potent hypoglycemics such as tolbutamide (I), chlorpropamide (II), and carbutamide (III).

The structure–activity studies in this class have been reviewed (1, 2); while some active agents have been discovered in which the *para*-position (R_2) is varied, the most potent therapeutic agents appear to have a methyl at this locus. Wide variations in the nature of R_1 have been investigated, with R_2 being held constant as methyl, and agents of comparable or enhanced potency compared to tolbutamide have been uncovered (3). All of these active sulfonylureas effect a marked elevation in blood insulin levels in both nondiabetic human subjects and other individuals with noninsulin-dependent diabetes (4).

The fact that depancreatized humans are not susceptible to the hypoglycemic activity of tolbutamide is interpreted to imply a direct action on the pancreas (5). This view is further supported by the observed degranulations of the pancreatic β-cells of test animals postdosing with sulfonylureas (6), and a general pancreatotrophic behavior has been presumed for the tolylsulfonylurea moiety (1, 2, 7).

In a search for potential pancreatic delineating radiopharmaceuticals, Boyd *et al.* (8) investigated a radioiodinated analog of chlorpropamide (*i.e.*, II but with R_2 = ^{125}I). No hypoglycemic testing data were provided to indicate that the iodopropamide still retained the activity of its parent model, chlorpropamide, but β-cell degranulation was observed. The substitution of iodine (covalent radius = 1.33 Å) for chlorine (covalent radius = 0.99 Å) represents a rather significant steric modification. As more precise models of the *p*-tolylsulfonylurea type, synthetic analogs modified only at the less important locus R_1 with ligand groups capable of chelating the γ-emitting nuclide ^{99m}Tc have been prepared.

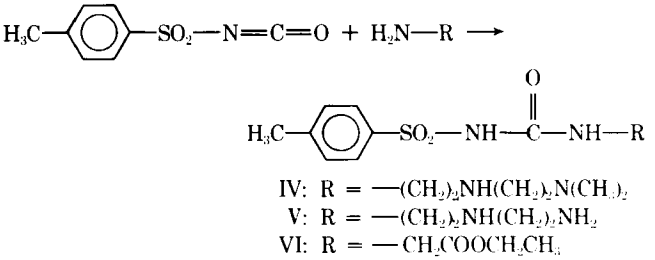


I: R_1 = *n*-butyl, R_2 = CH_3
II: R_1 = *n*-propyl, R_2 = Cl
III: R_1 = *n*-butyl, R_2 = NH_2
IV: R_1 = $—(CH_2)_2NH(CH_2)_2N(CH_3)_2$, R_2 = CH_3
V: R_1 = $—(CH_2)_2NH(CH_2)_2NH_2$, R_2 = CH_3
VI: R_1 = $—CH_2COOCH_2CH_3$, R_2 = CH_3
VII: R_1 = $—CH_2COOH$, R_2 = CH_3

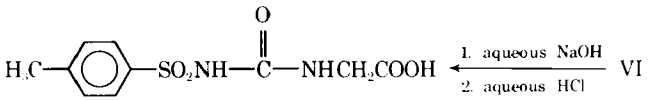
EXPERIMENTAL[1]

These candidate radiopharmaceuticals, IV–VI, were prepared by the controlled addition of the amino component to *p*-toluenesulfonyl isocyanate (Scheme I), while saponification of VI yielded VII.

1-*p*-Toluenesulfonyl-3-(*N,N*-dimethylaminoethylamino-ethyl)urea (IV)—A solution of *p*-toluenesulfonyl isocyanate (2.0 g, 0.0101 mole) in dioxane (10 ml) was added slowly (30 min) to a solution of *N,N*-dimethyldiethylenetriamine (1.32 g, 0.01 mole) in dioxane (30 ml) at room temperature. The resulting solution was stirred at room temperature for 1 hr, during which time a white precipitate formed. The precipitate was collected by filtration, washed with ether, and air dried. Recrystallization from anhydrous alcohol yielded 2.53 g (76.2%) of white hygroscopic crystals, mp 179–180°: IR (mineral oil): 3490, 3390, 3270 (NH), and 1650 (C=O) cm^{-1}; NMR (D_2O): δ 2.30 (s, 3H, $ArCH_3$), 2.52 [s, 6H, $N(CH_3)_2$], 2.86 (m, 8H, NCH_2), and 7.46 (m, 4H, ArH).



IV: R = $—(CH_2)_2NH(CH_2)_2N(CH_3)_2$
V: R = $—(CH_2)_2NH(CH_2)_2NH_2$
VI: R = $—CH_2COOCH_2CH_3$



VII

Scheme I

[1] Proton magnetic resonance (PMR) spectra were obtained on a Perkin-Elmer Hitachi R20A spectrometer, and chemical shifts are reported relative to tetramethylsilane. IR spectra were obtained on a Perkin-Elmer 257 spectrometer. Microanalyses were performed by Robertson Microanalytical Laboratory, Florham Park, N.J.

Anal.—Calc. for $C_{14}H_{24}N_4O_3S$: C, 51.19; H, 7.36; N, 17.05. Found: C, 51.18; H, 7.61; N, 16.82.

TLC on silica gel[2] 1B-F, using methanol–water (3:1), gave a single spot, R_f 0.29.

1-*p*-Toluenesulfonyl-3-(aminoethylaminoethyl)urea (V)—*p*-Toluenesulfonyl isocyanate (3.0 g, 0.0152 mole) in tetrahydrofuran (15 ml) was added slowly (30 min) to a solution of diethylenetriamine (1.58 g, 0.0156 mole) in tetrahydrofuran (70 ml) at room temperature. The resulting solution was stirred at room temperature for 30 min, during which time a white precipitate formed. The precipitate was collected by filtration, washed with ether, and dried at 65° *in vacuo*. Recrystallization from a dioxane–water mixture yielded 3.32 g (73.1%) of white hygroscopic crystals, mp 135–138°; IR (mineral oil): 3350, 3280 (NH), and 1640 (sh) (C=O) cm^{-1}; NMR (D_2O): δ 2.24 (s, 3H, $ArCH_3$), 2.88 (m, 8H, NCH_2), and 7.37 (m, 4H, ArH).

Anal.—Calc. for $C_{12}H_{20}N_4O_3S$: C, 47.97; H, 6.71. Found: C, 47.82; H, 6.86.

TLC on silica gel[2] 1B-F, using methanol–water (19:1), gave a single spot, R_f 0.86. Structure assignment was further supported by formation, in 52% yield, of a dihydro-2-imidazolone, mp 196–198°, from ethanol–water (1:1), generated by treatment of V with phosgene in dry benzene; IR (mineral oil): 1700 and 1655 (C=O) cm^{-1}.

Anal.—Calc. for $C_{13}H_{18}N_4O_4S$: C, 47.84; H, 5.56. Found: C, 47.78; H, 5.54.

1-*p*-Toluenesulfonyl-3-carbethoxymethylurea (VI)—Into 20 ml of anhydrous tetrahydrofuran was dissolved 6.3 g (0.032 mole) of *p*-toluenesulfonyl isocyanate. This mixture was added dropwise over 5 min to a solution of ethyl glycinate (3.54 g, 0.0343 mole) in 50 ml of tetrahydrofuran, during which time the temperature rose to 55°. The solution was agitated for 0.5 hr and allowed to stand at room temperature for 12 hr. Evaporation to dryness left a white powder, which was recrystallized from ethanol–water to yield 7.2 g (75%) of white needles, mp 170–171°; IR (mineral oil): 3110, 3170, 3315 (NH), 1742 (ester C=O), and 1660 (urea C=O) cm^{-1}; NMR (dimethyl sulfoxide-d_6): δ 1.15 (t, 3H, CH_3), 2.40 (s, 3H, $ArCH_3$), 3.75 (d, 2H, CH_2), 4.05 (q, 2H, CH_2), 6.80 (t, 1H, NH), and 7.61 (m, 4H, ArH).

Anal.—Calc. for $C_{12}H_{16}N_2O_5S$: C, 47.98; H, 5.37; N, 9.32. Found: C, 48.17; H, 5.62; N, 9.17.

1-*p*-Toluenesulfonyl-3-carboxymethylurea (VIII)—Compound VI (2.0 g, 6.6 mmoles) was refluxed for 1 hr in 10% aqueous sodium hydroxide solution. The solution was cooled to room temperature and acidified with concentrated hydrochloric acid, and a white solid separated from solution. This solid was redissolved by heating the mixture to 80° with sufficient 95% ethanol added to achieve homogeneity. Upon slow cooling to 0°, white needles (1.7 g or 88% of theoretical) were deposited. Upon drying *in vacuo*, these needles melted at 190° with decomposition; IR (mineral oil): 3350, 3170, 3120 (NH and OH), 1730 (acid C=O), and 1680 (urea C=O) cm^{-1}; NMR (dimethyl sulfoxide-d_6): δ 2.40 (s, 3H, $ArCH_3$), 3.68 (d, 2H, CH_2), 6.88 (t, 1H, NH), and 7.62 (m, 4H, ArH). The product was isolated as a monohydrate.

Anal.—Calc. for $C_{10}H_{12}N_2O_5S \cdot H_2O$: C, 41.37; H, 4.86; N, 9.65. Found: C, 41.40; H, 4.63; N, 9.68.

BIOLOGICAL EVALUATION[3]

For hypoglycemic testing, the candidate agents were administered as single intraperitoneal injections at 50 mg/kg in methylcellulose to three Sprague–Dawley rats; the rats were fasted for 24 hr prior to dosing. The test animals and four controls were preloaded with 1 g/kg of glucose given orally. Blood aliquots were withdrawn at 30-min intervals and analyzed for mg of glucose/100 ml of blood. Compound IV displayed hypoglycemic effects and dropped blood glucose levels by 34 ± 6 mg % at 30 min and 15 ± 1 mg % at 60 min compared to control standards. Compound V gave evidence of modest hypoglycemia at 90 min, but both VI and VII were inactive. On the basis of these results, IV was selected for technetium labeling and for detailed tissue distribution studies. Several exploratory runs were also carried out with the ^{99m}Tc-chelate of V.

[2] Baker-flex.

[3] Rat glucose tolerance studies were performed in the Biochemistry Department of McNeil Laboratories, Fort Washington, Pa., by Dr. G. F. Tutiler.

Table I—Tissue Distribution of the Sulfonylureas, Percent Dose per Gram and Standard Deviation

Tissue	15 min	30 min	1 hr
	Chelate of ^{99m}Tc and IV		
Lung	0.954 ± 0.111	0.664 ± 0.070	0.474 ± 0.009
Liver	0.915 ± 0.009	0.797 ± 0.081	0.665 ± 0.028
Spleen	0.425 ± 0.023	0.323 ± 0.023	0.237 ± 0.010
Kidney	5.402 ± 0.067	6.986 ± 0.879	6.779 ± 0.017
Pancreas	0.309 ± 0.012	0.265 ± 0.034	0.173 ± 0.007
Blood	1.165 ± 0.060	0.859 ± 0.037	0.600 ± 0.074
	Chelate of ^{99m}Tc and V		
Lung	0.759 ± 0.025	0.560 ± 0.057	0.358 ± 0.013
Liver	0.837 ± 0.003	0.690 ± 0.001	0.602 ± 0.001
Spleen	0.304 ± 0.006	0.232 ± 0.003	0.154 ± 0.001
Kidney	6.654 ± 0.202	5.907 ± 0.482	6.142 ± 0.013
Pancreas	0.312 ± 0.011	0.199 ± 0.003	0.156 ± 0.010
Blood	0.965 ± 0.002	0.636 ± 0.103	0.409 ± 0.010
	Blank Run[a]		
Lung	1.191 ± 0.142	1.072 ± 0.008	0.910 ± 0.192
Liver	6.595 ± 0.964	6.078 ± 1.600	6.114 ± 0.805
Spleen	2.438 ± 0.144	3.988 ± 0.841	2.202 ± 1.194
Kidney	6.694 ± 1.402	6.397 ± 0.815	7.179 ± 0.240
Pancreas	0.484 ± 0.368	0.497 ± 0.121	0.410 ± 0.143
Blood	2.639 ± 0.004	1.907 ± 0.073	1.569 ± 0.235

[a] Injection dose prepared by stannous chloride reduction of $Na^{99m}TcO_4$ with no sulfonylurea ligand present.

A solution of sodium pertechnetate was eluted from a technetium generator and reduced by stannous chloride to cationic technetium and a chelate prepared by the standard method published for diamine-type ligands (9). All of the chelates were purified before injection by gel filtration chromatography with saline elution. In all cases, two radioactive fractions could be eluted from the column, the first being the ^{99m}Tc-chelate of the tolbutamide analog and the second being the unreduced pertechnetate. The latter fraction was normally insignificant, reflecting nearly quantitative reduction and binding to the candidate radiopharmaceutical ligand. These observations are in accord with those reported by Eckelman *et al.* (9).

Purity of the chelate fraction was further verified by paper chromatography[4] using saline eluant (10). The R_f values obtained for the reduced hydrolyzed technetium, pertechnetate, and the ^{99m}Tc-chelate were 0.0, 0.54, and 0.77, respectively. The purity assays for the injected doses employed in the tissue distribution studies always indicated a minimum of 95% chelated material by the Eckelman–Richards method (10) of counting the separated spots on the paper chromatogram.

Tissue distribution studies were performed on pure inbred Wistar rats injected *via* the tail vein with a precalibrated dose of the labeled tolbutamide analogs IV or V. A minimum of two and a maximum of four animals were dosed and were sacrificed at 15, 30, and 60 min postinjection. Immediately after sacrificing, an autopsy was performed and the indicated tissues (Table I) were removed, dissected from fat and connective tissue, weighed, and placed in 20.3-cm (8-in.) test tubes. These tissues were counted[5], and a percent dose per gram was calculated by comparison with a standard that was counted at the same time as the tissues to correct for radioactive decay. Table I indicates the range of values obtained in each run. In an effort to optimize pancreatic uptake, several other physiologically active agents were administered immediately in advance of the labeled candidate radiopharmaceutical.

DISCUSSION

As has been observed for several other chelates, the concentration in the kidney, even at the shortest assay time, was in excess of concentrations in other target organs for ^{99m}Tc-chelates of IV and V. Some reports have suggested that chelates are not specific for any particular organ but are cleared rapidly from the blood and into the kidney by glomerular filtration (11, 12). Since ^{99m}Tc-chelates recently were developed for imaging the gallbladder (13), the

[4] Whatman No.1.

[5] Baird Atomic automatic well counter.

liver (14), amebic abscesses (15), and bone structures (16), this suggestion is obviously not a general truism because these chelates displayed the tissue specificity of the parent ligand.

Anatomical considerations dictate that for development of a satisfactory pancreatic scanning radiopharmaceutical, a suitable dose ratio of pancreas to liver be obtained. In the ^{99m}Tc-chelates of IV and V, pancreas to liver ratios of 0.35 were observed at 15 min and fell to 0.26 at the end of 1 hr. If *in vivo* dissociation of the metal ion–ligand bond, presumably by exchange of the label onto a more strongly complexing native blood protein such as transferrin *or* by exchange of the tolbutamide-like ligand onto a native blood metal ion, were occurring, tissue distribution values should be altered by preadministration of either another metal ion or another ligating group. However, predosing with ion-dextran[6] or ethylenediaminetetraacetic acid calcium salt did not appreciably alter the tissue distribution values. Further support for the conclusion that the ^{99m}Tc-chelates were remaining intact *in vivo* was obtained from a blank run (Table I) in which reduced, unchelated technetium was administered. As reported previously (17), a different distribution favoring liver uptake, by hydrolyzed, reduced technetium, was observed.

Coadministration of the injectable diuretic furosemide[7] did not alter the rapid kidney uptake nor the pancreas to liver ratio, but the anticholinergic, morphine sulfate (0.2 mg/kg iv), often employed in such studies (18, 19), did slightly enhance the ratio to 0.61 for ^{99m}Tc-V after 30 min. The low percent dose values in the pancreas and the diminished pancreas to liver ratio may reflect the fact that the β-cells, the site of action of sulfonylurea hypoglycemics (1, 2), constitute only 1–2% of the pancreas weight. In islet cell neoplasms, the β-cells represent a much higher fraction of pancreas weight (20); studies are now underway to visualize such tumors in hamsters.

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Utility of Fasting Essential Amino Acid Plasma Levels in Formulation of Nutritionally Adequate Diets III: Lowering of Rat Serum Cholesterol Levels by Lysine Supplementation

C. I. JAROWSKI ˣ and R. PYTELEWSKI

Abstract □ A lysine-supplemented ration lowered the serum cholesterol levels of rats. The level of lysine supplementation was derived from the fasting plasma essential amino acid profile.

Keyphrases □ Lysine supplementation—effect on rat serum cholesterol levels □ Amino acid supplementation—effect of added lysine on rat serum cholesterol levels □ Cholesterol levels—effect of lysine supplementation, rats □ Nutrition—effect of lysine supplementation on rat serum cholesterol levels

Earlier studies demonstrated that the molar proportions of the essential amino acids in the fasting plasma of Sprague–Dawley rats (1) and Wistar rats (2) were useful in: *(a)* determining the order of limiting essential amino acids in rations, *(b)* preparing chemically defined synthetic diets, and *(c)* ranking diets according to their biological value and net protein utilization. The average values of fasting plasma essential amino acids of human adults were also used to rank a series of protein diets fed to premature infants (2).

This paper presents an additional application of the fasting plasma profile in a nutritional study conducted with Sprague–Dawley rats. An increase in the dietary protein level resulted in a decrease in the total plasma cholesterol in chicks (3) and mice (4). The cholesterol blood levels of calves were reduced by a higher level of dietary protein (5).

It was of interest to determine how the serum cholesterol level would be influenced by feeding a lysine-supplemented ration. The level of lysine supplementation was derived from the fasting essential amino acid profile of Sprague–Dawley rats.

Table I—Calculation of the Order of Limiting Essential Amino Acids Based on the Fasting Plasma Profile of Rats[a]

Essential Amino Acid	*A*, mmoles/ liter Plasma	*B*, mmoles/ 100 g Ration	*B/A*
L-Lysine	0.389	10.80	27
L-Tryptophan	0.054	1.56	29
L-Threonine	0.199	8.22	41
L-Histidine	0.062	3.86	62
L-Arginine	0.146	9.24	63
L-Methionine + ½ cystine	0.052	3.86	74
L-Phenylalanine + tyrosine	0.115	14.86	129
L-Leucine	0.100	15.16	151
L-Valine	0.101	15.95	157
L-Isoleucine	0.060	10.51	175

[a] L-Lysine is limiting since the *B/A* value is the smallest:

$$\frac{\text{L-tryptophan } B/A \text{ value} + \text{L-lysine } B/A \text{ value}}{2} = \frac{29 + 27}{2}$$

Therefore, 2 × 0.389 = 0.778 mmole = 0.114 g of L-lysine to be added to 100 g of ration to make L-tryptophan and L-lysine equally limiting.

Table II—Serum Cholesterol Levels of Rats with and without Supplementary L-Lysine

Rat	Serum Cholesterol Levels, mg %: Ration	Ration plus Lysine (0.11%)	Ration plus Lysine for 30 Days, then Ration for 11 Days
1	128	82	113
2	120	76	117
3	134	99	131
4	130	100	119
5	122	84	109
6	126	100	124
Average	127	90	119
Average ± *SD*	127 ± 5.2	90 ± 12.4	119 ± 7.9

EXPERIMENTAL

Two groups of 20 female Sprague–Dawley rats, 216 ± 8.8 g, were fed a complete diet[1] and a lysine-supplemented ration for 30 days (0.114 g/100 g of ration). The method for calculating the L-lysine level is shown in Table I. A third group of 20 rats was fed the lysine-supplemented ration for 30 days and was then switched to the unsupplemented ration for 11 days.

After an 18-hr fast, groups of six rats were sacrificed and the collected blood was allowed to clot. Serum cholesterol was determined by the method of Levine and Zak (6) (Table II).

DISCUSSION

The data in Table II were subjected to a statistical evaluation. The serum cholesterol levels for the rats fed the lysine-supplemented ration were significantly lower than those for the control group. They also differed significantly from the group fed the control ration for 11 days. The value for the control *versus* the lysine-supplemented ration was $p < 0.001$, and the value for the rats fed the control ration for 11 days *versus* the lysine-supplemented ration was $p < 0.01$. The value for the control *versus* the group fed the control ration for 11 days was $p < 0.1$.

A suggested mechanism for these observations is that the addition of 0.114 g of lysine to 100 g of ration improves the biological value and net protein utilization. Evidence for this finding is the improved growth consistently obtained with the lysine-supplemented ration (7). Therefore, it follows that by increasing net protein utilization, less essential and nonessential amino acids will remain for intermediary metabolic conversion to cholesterol intermediates (acetate, acetoacetic acid, acetone, pyruvic acid, *etc.*).

Thus, it appears that it is possible to lower cholesterol serum levels by strategic supplementation with limiting essential amino

[1] Rockland Farms Mouse/Rat Complete Diet.

acids. The reported reversible atherosclerosis in Rhesus monkeys adds further potential significance to these observations in rats (8). This reversible atherosclerosis was achieved through dietary control. The ingestion of lysine-supplemented rations, wherein the protein component has been improved biologically by the addition of an essential amino acid, may prove to be practical in human nutrition.

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Utility of Fasting Essential Amino Acid Plasma Levels in Formulation of Nutritionally Adequate Diets IV: Lowering of Human Plasma Cholesterol and Triglyceride Levels by Lysine and Tryptophan Supplementation

P. K. RAJA and C. I. JAROWSKI [x]

Abstract □ The administration of capsules containing L-lysine monohydrochloride (0.205 g) and L-tryptophan (0.069 g) three times daily after meals resulted in a significant drop in plasma cholesterol and triglyceride levels. The proportion of amino acids in the blend was derived from the average fasting plasma essential amino acid profile.

Keyphrases □ Lysine and tryptophan supplementation—effect on human plasma cholesterol and triglyceride levels □ Tryptophan and lysine supplementation—effect on human plasma cholesterol and triglyceride levels □ Amino acid supplementation—effect of added lysine and tryptophan on human plasma cholesterol and triglyceride levels □ Cholesterol and triglyceride levels—effect of lysine and tryptophan supplementation, humans □ Nutrition—effect of lysine and tryptophan supplementation on human plasma cholesterol and triglyceride levels

Earlier studies demonstrated that the molar proportions of the essential amino acids in the fasting plasma of Sprague–Dawley rats (1–3), Wistar rats, and humans (4) were useful in *(a)* determining the order of limiting essential amino acids in rations, *(b)* preparing chemically defined synthetic diets, *(c)* ranking diets according to their biological value and net protein utilization, and *(d)* lowering serum cholesterol levels in Sprague–Dawley rats by L-lysine supplementation.

This paper presents another application of the fasting plasma profile concept in a nutritional study conducted with adult humans. Supplementation of the normally varied human dietary regimen with L-lysine and L-tryptophan was investigated since these essential amino acids are most frequently limiting. It was reasoned that provision of two essential amino acids usually found to be limiting would improve the biological value and net protein utilization of each meal. The great variety of protein combinations consumed by humans on *ad libitum* dietary regimens would be a complicating feature in a supplementation study. Lowering of serum cholesterol levels in Sprague–Dawley rats had been achieved by lysine supplementation of a standardized ration (2). Nevertheless, it was decided to determine the effect of L-lysine and L-tryptophan supplementation on the plasma levels of cholesterol and triglycerides in humans.

EXPERIMENTAL

Derivation of Capsule Formula—The average fasting human plasma concentrations of lysine and tryptophan are 0.170 and 0.052 mmole/liter, respectively (4). Thus, the ratio of lysine to tryptophan on a molar basis is 3.27 to 1. A blend of 595 g of L-lysine monohydrochloride (3.27 moles) and 204 g of L-tryptophan (1 mole) was prepared. Hard gelatin capsules (No. 1) were hand filled with 0.274 g of the blend. Each capsule contained 0.069 g of L-tryptophan and 0.205 g of L-lysine monohydrochloride. This level of supplementation was judged to be satisfactory. The minimum daily requirement of L-tryptophan and L-lysine for adult males had been established (5, 6) to be 0.25 and 0.80 g, respectively.

Human Volunteers Studied—Six persons participated in the study[1]. Phenotyping of the hyperlipidemia or hyperlipoproteinemia was established by zone electrophoresis, using agarose as the

[1] M.S.C., 48 years old, and S.C., 22 years old, mother and daughter, respectively, were members of a family with a history of hyperlipidemia. M.B. and D.G. were 56-year-old females. A.Z. was a 40-year-old male who was included in the study in spite of his being a moderate alcoholic. G.C. was a 42-year-old male who had been on clofibrate for several months.

acids. The reported reversible atherosclerosis in Rhesus monkeys adds further potential significance to these observations in rats (8). This reversible atherosclerosis was achieved through dietary control. The ingestion of lysine-supplemented rations, wherein the protein component has been improved biologically by the addition of an essential amino acid, may prove to be practical in human nutrition.

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This paper presents another application of the fasting plasma profile concept in a nutritional study conducted with adult humans. Supplementation of the normally varied human dietary regimen with L-lysine and L-tryptophan was investigated since these essential amino acids are most frequently limiting. It was reasoned that provision of two essential amino acids usually found to be limiting would improve the biological value and net protein utilization of each meal. The great variety of protein combinations consumed by humans on *ad libitum* dietary regimens would be a complicating feature in a supplementation study. Lowering of serum cholesterol levels in Sprague–Dawley rats had been achieved by lysine supplementation of a standardized ration (2). Nevertheless, it was decided to determine the effect of L-lysine and L-tryptophan supplementation on the plasma levels of cholesterol and triglycerides in humans.

EXPERIMENTAL

Derivation of Capsule Formula—The average fasting human plasma concentrations of lysine and tryptophan are 0.170 and 0.052 mmole/liter, respectively (4). Thus, the ratio of lysine to tryptophan on a molar basis is 3.27 to 1. A blend of 595 g of L-lysine monohydrochloride (3.27 moles) and 204 g of L-tryptophan (1 mole) was prepared. Hard gelatin capsules (No. 1) were hand filled with 0.274 g of the blend. Each capsule contained 0.069 g of L-tryptophan and 0.205 g of L-lysine monohydrochloride. This level of supplementation was judged to be satisfactory. The minimum daily requirement of L-tryptophan and L-lysine for adult males had been established (5, 6) to be 0.25 and 0.80 g, respectively.

Human Volunteers Studied—Six persons participated in the study[1]. Phenotyping of the hyperlipidemia or hyperlipoproteinemia was established by zone electrophoresis, using agarose as the

[1] M.S.C., 48 years old, and S.C., 22 years old, mother and daughter, respectively, were members of a family with a history of hyperlipidemia. M.B. and D.G. were 56-year-old females. A.Z. was a 40-year-old male who was included in the study in spite of his being a moderate alcoholic. G.C. was a 42-year-old male who had been on clofibrate for several months.

Table I—Effect of Lysine and Tryptophan Supplementation on Plasma Levels of Triglycerides and Cholesterol in Humans

Subject		Plasma Concentration (mg %) of Triglycerides and Cholesterol				
M.S.C.	Triglycerides	1725, 0 days	1200, 10 days	1365, 20 days	1565, 24 days	—
	Cholesterol	380, 0 days	302, 10 days	380, 20 days[a]	335, 24 days	—
S.C.	Triglycerides	215, 0 days	180, 6 days	162, 15 days	150, 30 days	102, 60 days
	Cholesterol	216, 0 days	203, 6 days	203, 15 days	210, 30 days	180, 60 days
M.B.	Triglycerides	250, 0 days	230, 7 days	200, 15 days	140, 30 days	148, 60 days
	Cholesterol	350, 0 days	310, 7 days	310, 15 days	302, 30 days	288, 60 days
A.Z.	Triglycerides	235, 0 days	195, 7 days	257, 17 days	195, 24 days	—
	Cholesterol	200, 0 days	185, 7 days	210, 17 days[b]	188, 24 days	—
D.G.	Triglycerides	288, 0 days	172, 7 days	—	—	—
	Cholesterol	240, 0 days	200, 7 days	—	—	—
G.C.	Triglycerides	255, 0 days	205, 7 days	—	—	—
	Cholesterol	252, 0 days	265, 7 days	—	—	—

[a] Subject consumed 1 dozen clams as an appetizer the night before. [b] Subject attended a cocktail party the night before.

support medium (7–9). This correlated well with the fasting plasma lipid assays as well as the appearance of the plasma samples after storage at 2–6° for 24 hr. All hyperlipoproteinemias were classified as Type IV, with the exception of Subject M.S.C. whose hyperlipoproteinemia was classified as Type V.

Protocol—All volunteers were instructed to continue their *ad libitum* dietary regimens but to take one capsule of the amino acid blend after each meal. In the case of Subject G.C., clofibrate was discontinued.

Analytical Procedure—After a 12–14-hr fast, approximately 20 ml of blood was drawn and placed in a tube containing edetic acid. After centrifuging to remove the cellular elements, the supernate was analyzed by established colorimetric procedures for cholesterol (10) and triglycerides (11). Averages for the duplicate assays performed are summarized in Table I.

DISCUSSION

The data in Table I show a downward trend in triglyceride levels in all six patients. Significant decreases were observed for S.C. (53%), M.B. (41%), and D.G. (41%). Subject G.C., formerly on clofibrate, showed a 20% decrease in plasma triglycerides after taking the amino acid pair for 7 days. The decrease in plasma cholesterol in five subjects ranged from 6 to 18%, but G.C. showed a 5% increase.

Hyperlipidemia or hyperlipoproteinemia can occur because of an increased exogenous load of cholesterol or triglyceride entering from the intestine as chylomicrons. It can also occur because of an increased release of endogenous glyceride and cholesterol entering from the liver and intestine as pre-β-lipoproteins. This increased release of lipid may be secondary to a greater flux of lipid precursors like fatty acids or carbohydrates or because of *de novo* synthesis. Hyperlipoproteinemia can occur because of a failure of the end organs, adipose tissue, and muscle sometimes to assimilate lipid due to defects in the clearing enzymes. Hyperlipoproteinemia can also occur because of abnormal solubility properties of the plasma or abnormalities in the lipoproteins themselves.

All of these factors are further modified by the general state of protein and carbohydrate metabolism and the action of a number of hormones acting predominantly by promoting lipogenesis or lipolysis in the adipose tissue, muscle, or liver. Hyperlipidemia or hyperlipoproteinemia is thus a symptom of a heterogeneous group of disorders that differ in clinical manifestations, prognosis, and responsiveness to therapy (12).

It has not been established whether lowering of serum cholesterol and triglyceride levels has a detrimental, a beneficial, or no effect on the morbidity or mortality due to atherosclerosis or coronary heart disease. Albrink (13), however, reported that the average incidence rate of coronary heart disease is doubled for individuals having cholesterol serum levels in the range of 239–261 mg % as compared with those individuals having levels in the 96–195-mg % range. In addition, the incidence of coronary heart disease was greater than fivefold for individuals having a serum triglyceride concentration in the 176–490-mg % range as compared with those with a range in concentration of 20–75 mg %. Thus, the downward trend in plasma levels of cholesterol and triglyceride achieved in this investigation by the administration of two essential amino acids given after meals is most encouraging. No toxic effects were expected and none has been encountered in any of the six subjects to date. The data obtained have led to the broadening of the scope of the investigations to attempt to establish the optimal dosage regimen, the effect of other amino acids and combinations, and the mechanism for the observed lipid lowering.

Levy and Langer (14) differentiated the hyperlipidemic drugs as follows: those that act by decreasing lipoprotein synthesis (nicotinic acid) and those that promote lipoprotein catabolism (cholestyramine). In the present investigation, the intermediary metabolic conversion of L-tryptophan to nicotinic acid was considered to be a possible mechanism. However, hypolipidemic doses of nicotinic acid range from 4.5 to 6 g/day. A mechanism proposed at this time is that, with the greater net protein utilization achieved with strategic supplementation, less essential and nonessential amino acids will be available for intermediary metabolic conversion to cholesterol and triglyceride intermediates (acetate, acetoacetic acid, acetone, pyruvic acid, *etc.*).

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Topical Mosquito Repellents VII: Alkyl Triethylene Glycol Monoethers

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Abstract □ Normal and branched-chain aliphatic monoethers of triethylene glycol are effective topical mosquito repellents. In terms of duration of protection, they are generally superior to the corresponding diethylene glycol analogs and some are superior to diethyltoluamide. The *n*-heptyl monoether of triethylene glycol affords double the protection time of diethyltoluamide under controlled laboratory conditions and appears to be a useful new mosquito repellent.

Keyphrases □ Mosquito repellents, topical—synthesis, evaluation of alkyl triethylene glycol monoethers □ Repellents, mosquito, topical—synthesis, evaluation of alkyl triethylene glycol monoethers □ Monoethers, alkyl triethylene glycol—synthesis, evaluation as mosquito repellents □ Volatility, alkyl triethylene glycol monoethers—relationship to mosquito repellency □ Diethylene glycol *versus* triethylene glycol monoethers—mosquito repellency

The quest for improved topical mosquito repellent properties was pursued among benzyl ethers in previous studies (1, 2) with positive but not dramatic results. In the present study, nonaromatic polyethers derived from ethylene glycol were examined. The compounds were prepared *via* the sodio derivatives of diethylene or triethylene glycol and the appropriate alkyl halide or tosylate and were purified by vacuum distillation.

Products[1,2] were evaluated topically on human subjects, using female *Aedes aegypti* mosquitos, as previously described (1).

EXPERIMENTAL[3]

Diethyltoluamide was obtained commercially[4]. Alkyl halides and alcohols corresponding to the R groups in Tables I and II were also obtained commercially and were used without further purification. Final products were purified and boiling points were determined by vacuum distillation using Vigreaux columns.

Preparation of Monoalkyl Triethylene Glycol Ethers (Table I) from Alkyl Halide (Method A)—An ice-cooled, mechanically stirred, three-necked, 2-liter, round-bottom flask, fitted with a powder funnel and a gas inlet allowing passage of a slow stream of nitrogen, was charged with 180 ml of triethylene glycol. Sodium hydride (57% in oil, 13.0 g, 0.13 mole) was washed of its oil by swirling with 2 × 30 ml of ether and decanting. The hydride was then added in portions. After 2 hr, the ice bath was removed and the mixture was stirred for 2 hr on a steam bath.

To this stirred dark mixture was added 0.23 mole of the alkyl halide. After 20 hr, the opaque mixture was cooled, diluted with 800 ml of water, and extracted with methylene chloride (4 × 100 ml). Sodium chloride was used to help break emulsions. The combined organic layers were washed with water (2 × 200 ml), dried (anhydrous magnesium sulfate), and rotary evaporated. The clear oil was fractionated *in vacuo* through a Vigreaux column.

Preparation of Monoalkyl Triethylene Glycol Ethers (Table I) from Tosylate (Method B)—*Preparation of Tosylate*—The alcohol (0.08 mole) was dissolved in 130 ml of dry pyridine in a 250-ml erlenmeyer flask and stirred in an ice bath while *p*-toluenesulfonyl chloride (26 g, 0.14 mole) was added in several portions. After stirring for 1 hr the solution was placed in a refrigerator (−5°) overnight.

The deep-red mixture was added to 600 ml of ice water, stirred for 5 min, and extracted with 2 × 100 ml of ligroin. The combined organic layers were washed free of pyridine with 3 *N* HCl (16 ml) and ice water (pH 3–4), then washed with 50 ml of ice water, dried with anhydrous magnesium sulfate, and evaporated *in vacuo* to yield a red oil in 99% yield. The IR spectra showed tosylate bands at 6.21 and 8.60 μm.

Alkylation of Triethylene Glycol—Triethylene glycol (180 ml) was heated on a steam bath with 1.74 g (76 mmoles, 5% excess) of sodium spheres for 1 hr in a 250-ml flask equipped with a drying tube. Gas was evolved slowly, and the sodium was dissolved to yield a clear yellow solution. The alkyl tosylate (64 mmoles) was added, and the mixture was stirred at room temperature for 3 days.

The clear, light-brown mixture was added to 1 liter of ice and water and extracted with methylene chloride or ether (3 × 200 ml). Emulsions were usually encountered and were occasionally broken by acidifying the alkaline mixture with 3 *N* HCl. The combined organic layers were washed with water (3 × 200 ml), dried (anhydrous magnesium sulfate), evaporated *in vacuo* to a yellow oil (95% crude), and distilled through a Vigreaux column *in vacuo*.

Repellency evaluations were conducted *via* both skin and cloth tests, utilizing female *A. aegypti*.

Tests on Skin—Compounds were uniformly applied in ethanol to an exposed area of the forearm of a human subject as described previously (1) (Tables I–III).

Tests on Cloth[1]—The methodology was described previously (3) except that the rate of cloth treatment was reduced by one-half until three bites per minute were received at the lowest dose. Diethyltoluamide and dimethyl phthalate were tested concurrently as standards (Table IV).

Other Tests on Skin[2]—The test methodology was similar to that used under *Tests on Skin* (Tables I and II). Results are given in Table V.

RESULTS AND DISCUSSION

A far more restricted volatility–repellency relationship was apparent in this series (Tables I and II) as compared with those previously studied (1–3). Nonetheless, maximum duration of protection at two application rates to skin was observed with Compounds I-2 and I-3 (Table I), whose volatility corresponded to boiling points in the range of 120–130°/0.5 mm Hg. They outperformed the standard, diethyltoluamide (I-14), by a substantial margin. No definite reason can be given for the comparatively poorer performance of other members of the series whose volatility would appear to be close to this optimum range, *e.g.*, Compounds I-9–I-11 (Table I); however, it is possible that the volatility of these compounds deviates from the optimum at skin temperature.

In any event, the findings suggest that maximum duration in this series is associated with C_6–C_7 alkyl ethers. Among the C_8 ethers studied, it appears that the straight-chain compound (I-4) is superior to branched isomers (I-6 and I-11) and the cyclic analog (I-12). The same generalization seems applicable to the C_7 series (I-3 and I-7) and the C_9 series (I-5, I-9, and I-10). The repellencies of the diethylene glycol analogs (Table II) were substantially infe-

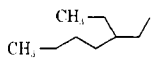
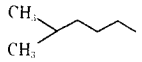
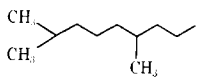
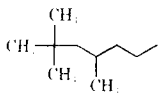
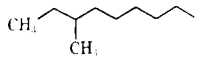
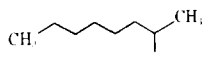
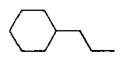
[1] Several compounds were also subjected to independent evaluation on cloth at the Gainesville laboratories of the U.S. Department of Agriculture.

[2] Two compounds were subjected to further independent evaluation at the Letterman Army Institute of Research.

[3] Elemental analyses were performed by the Microanalytical Laboratory, Department of Chemistry, Stanford University, Stanford, Calif.

[4] Eastman Organic Chemicals

Table I—Alkyl Triethylene Glycol Monoethers $R(OCH_2CH_2)_3OH$

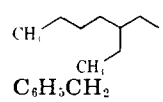
Compound	R	Yield, %	Formula	Analysis, % Calc.	Analysis, % Found	Boiling Point (mm)	Repellency, hr[a] 0.15 mg/cm²	Repellency, hr[a] 0.31 mg/cm²
I-1	n-C_5H_{11}	50[b]	$C_{11}H_{24}O_4$	C 59.97 H 10.98	59.71 11.24	111° (0.5)	5.8 (2)	9.0 (5)
I-2	n-C_6H_{13}	67[b]	$C_{12}H_{26}O_4$	C 61.50 H 11.18	61.74 11.41	122° (0.4)[c]	5.7 (30)	10.0 (30)
I-3	n-C_7H_{15}	54[b]	$C_{13}H_{28}O_4$	C 62.87 H 11.37	63.07 11.70	128° (0.4)	10.6 (4)	13.0 (4)
I-4	n-C_8H_{17}	71[b]	$C_{14}H_{30}O_4$	C 64.08 H 11.53	64.08 11.50	139° (0.45)	—	8.1 (11)
I-5	n-C_9H_{19}	60[b]	$C_{15}H_{32}O_4$	C 65.18 H 11.67	65.38 11.68	153° (0.65)	2.6 (4)	9.8 (5)
I-6		33[b]	$C_{14}H_{30}O_4$	C 64.08 H 11.53	64.04 11.63	120° (0.13)	4.7 (12)	6.4 (9)
I-7		76[b]	$C_{12}H_{26}O_4$	C 61.50 H 11.18	61.38 11.12	149° (3.25)[d]	4.3 (3)	7.0 (3)
I-8		55[b]	$C_{16}H_{34}O_4$	C 66.16 H 11.80	65.78 12.06	169° (2.0)[e]	1.3 (3)	3.3 (3)
I-9		59[b]	$C_{15}H_{32}O_4$	C 65.18 H 11.67	65.00 11.94	130° (0.35)	1.3 (4)	2.8 (3)
I-10		90[f]	$C_{15}H_{32}O_4$	C 65.18 H 11.67	64.87 11.98	140° (0.7)	1.0 (3)	1.8 (2)
I-11		50[f]	$C_{14}H_{30}O_4$	C 64.08 H 11.53	63.73 11.69	130° (0.5)	4.5 (4)	4.3 (3)
I-12		58[f]	$C_{14}H_{28}O_4$	C 64.58 H 10.84	64.54 10.77	134° (0.3)	1.1 (3)	4.8 (2)
I-13	$C_6H_5CH_2$	30[g]	$C_{13}H_{20}O_4$	C 64.98 H 8.39	64.74 8.24	148° (0.55)	—	0.25 (1)
I-14	Diethyltoluamide	—	—			100° (0.5)	3.4 (11)	6.9 (12)

[a] Number of determinations is given in parentheses. [b] Method A: prepared from bromide. [c] Lit. (4) 153–154° (5 mm). [d] About 118° /0.5 mm. [e] About 145°/0.5 mm. [f] Method B: prepared from tosylate. [g] Method A: prepared from chloride.

rior to those of the corresponding triethylene glycol ethers and, in both series, the benzyl analogs (I-13 and II-5) were inferior to the aliphatic compounds.

To obtain a more direct comparison, independent of day-to-day variations in insect avidity, topical repellency was determined simultaneously for diethylene and triethylene glycol ethers in paired tests of compounds with identical *O*-alkyl substituents (Table III). The triethylene glycol ethers were clearly superior when the R group was hexyl or heptyl. However, superiority was reversed in the *n*-octyl compounds, and the 2-ethylhexyl derivatives showed approximately equal duration. It is assumed that the increased polarity of the triethylene glycol series over that of the diethylene

Table II—Alkyl Diethylene Glycol Monoethers $R(OCH_2CH_2)_2OH$

Compound	R	Yield, %	Formula	Analysis, % Calc.	Analysis, % Found	Boiling Point (mm)	Repellency, hr[a] 0.15 mg/cm²	Repellency, hr[a] 0.31 mg/cm²
II-1	n-C_6H_{13}	~80[b]	$C_{10}H_{22}O_3$	C 63.12 H 11.65	63.21 11.57	82° (0.55)	1.8 (2)	2.8 (2)
II-2	n-C_7H_{15}	70.5[b]	$C_{11}H_{24}O_3$	C 64.66 H 11.84	64.65 11.56	93° (1.6)	—	5.9 (10)
II-3	n-C_8H_{17}	43[b]	$C_{12}H_{26}O_3$	C 66.01 H 12.00	65.78 12.03	104.5° (0.45)	—	4.5 (2)
II-4		32[b]	$C_{12}H_{26}O_3$	C 66.01 H 12.00	65.83 11.96	98° (0.5)	3.5 (3)	4.9 (7)
II-5	$C_6H_5CH_2$	27[c]	$C_{11}H_{16}O_3$	C 67.32 H 8.22	67.19 8.32	117° (0.45)	—	1.0 (1)
II-6	$(CH_3CH_2O)_2CHCH_2$	22[b]	$C_{10}H_{22}O_3$	C 54.03 H 9.98	54.02 9.92	119° (1.2)[d]	1.0 (2)	1.5 (2)

[a] Number of determinations is given in parentheses. [b] Method A: prepared from bromide and diethylene glycol. [c] Method A: prepared from chloride and diethylene glycol. [d] About 100°/0.5 mm.

Table III—Dry Protection Times[a] of Monoalkyl Ethers of Diethylene Glycol (n = 2) *versus* Triethylene Glycol (n = 3)

Compound	Mean Protection Time, hr ± *SD*			
	n-Hexyl	*n*-Heptyl	*n*-Octyl	2-Ethylhexyl
$R(OCH_2CH_2)_2OH$	3.6 ± 0.90	5.9 ± 0.95	11.0 ± 3.95	4.0 ± 0.88
$R(OCH_2CH_2)_3OH$	12.9 ± 2.77	11.6 ± 3.65	7.5 ± 5.03	5.0 ± 2.04

[a] Results are means of 10 determinations in which diethylene and triethylene glycol ethers of identical R group were paired (same test subject). Compounds were applied at 0.31 mg/cm².

Table IV—Comparative Repellent Evaluations[a]

Compound[b]	Minimum Effective Dose after Indicated Drying Time, mg/cm²	
	15 min	24 hr
I-1	0.063	0.25
I-2	0.032	0.25
I-3	0.016	0.125
I-4	0.016	0.25
I-5	0.032	0.25
I-7	0.032	0.125
I-14	0.008	0.25
Dimethyl phthalate (standard)	0.008	0.5

[a] Tests conducted by personnel at the Insects Affecting Man Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, Gainesville, Fla. [b] See Table I.

glycols of similar volatility should present durational and toxicological advantages in terms of reduced percutaneous absorption rates.

Results of minimum effective dose (MED) tests (Table IV) generally correlated with the results of topical duration tests. Both at 15 min and 24 hr after application, I-3 exhibited minimum MED values. Although these values were not as low at 15 min as those obtained for diethyltoluamide and dimethyl phthalate, I-3 was superior to both standards in the 24-hr test.

Table V—Repellent Evaluations[a]

Compound[b]	Repellency[c], hr ± *SD*	Number of Exposures
I-2	8.50 ± 4.7	12
I-3	16.75 ± 5.6	8
I-14	6.68 ± 0.81	128
2-Ethylhexanediol	3.40 ± 0.5	40

[a] Tests conducted by personnel at Letterman Army Institute of Research using 200 female *A. aegypti* in a 1.25-ft³ cage (5). [b] See Table I; compounds were applied to forearms of human volunteers at the rate of 0.31 mg/cm². [c] Mean dry protection times (prevention of biting).

The superiority of I-3 in Table I was confirmed by tests conducted independently[2] (Table V). It provided more than double the protection time afforded by diethyltoluamide to a group of volunteers under controlled laboratory conditions. Preliminary results of field tests indicate that the moderate laboratory superiority of I-2 can be nullified by conditions of heavy sweating and high insect population density. Field tests have not yet been conducted with I-3 and I-4.

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Complex Formation between Menadione and Cetylethylmorpholinium Ethosulfate: Effect on UV Photodegradation of Menadione

C. R. KOWARSKI ˣ and H. I. GHANDI

Abstract □ The process of menadione photodegradation can be enhanced or diminished by other compounds. The presence of the quaternary ammonium compound cetylethylmorpholinium ethosulfate (I) in solutions of menadione was found to slow the rate of photodegradation by UV light (253.7 nm). The mechanism of this effect may be due to complex formation between menadione and I. Complex formation was demonstrated by a shift in the absorption peaks of menadione from 245 and 260 nm to 251.5 and 261.5 nm, respectively. The equilibrium constant of this complex was calculated to be 1.647 *M*.

Keyphrases □ Menadione—effect of quaternary ammonium compound on UV photodegradation rate, complex formation □ Photodegradation of menadione by UV light—effect of quaternary ammonium compound, complex formation □ Cetylethylmorpholinium ethosulfate—effect on UV photodegradation of menadione, complex formation

Menadione (2-methyl-1,4-naphthoquinone; vitamin K_3) is degraded by exposure to light. The photolysis of menadione is diminished in the presence of anions such as chloride and bromide (1–8) but not sulfate (9). Some surfactants such as polysorbate 80 diminish the photodegradation of menadione, but polyoxyl 40 stearate enhances it (10).

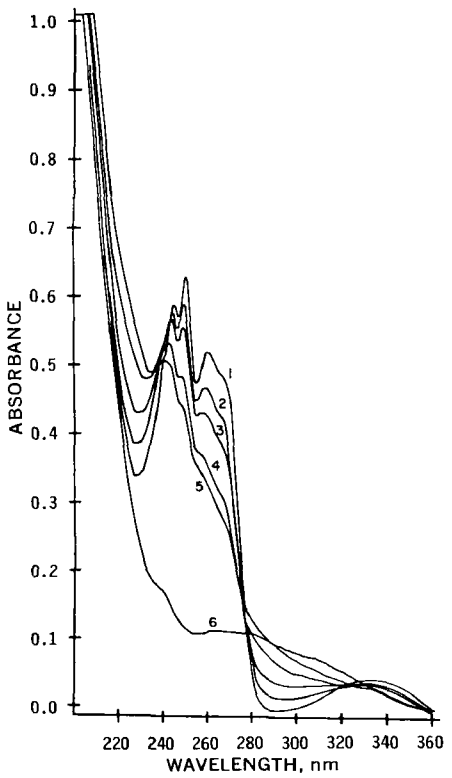

Figure 1—*Set of UV spectra obtained during photolysis of menadione. Key: 1, initial spectrum; 2, at 10 min; 3, at 20 min; 4, at 40 min; 5, at 60 min; and 6, at 3 hr from the beginning of irradiation.*

Table I—Composite Photochemical Results of Menadione in Solutions Containing Compound I

I Concentration, % (w/v)	K, $min^{-1} \times 10^{-3}$	K/K_{H_2O}, 0.2 *M* Borate Buffer
0	4.863	1.000
0.1	2.141	0.440
1.0	1.635	0.336
2.5	1.289	0.265
5.0	0.783	0.160

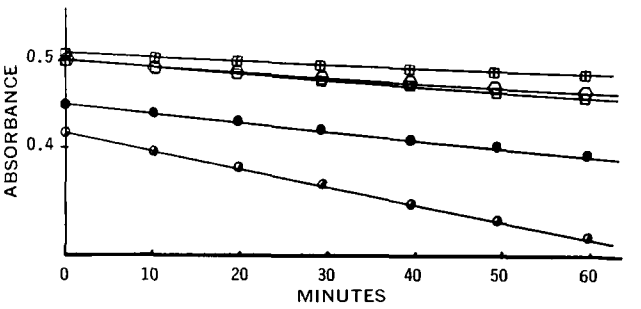

Figure 2—*Rates of photodegradation of menadione in aqueous solutions containing various concentrations of I as expressed by the change in absorbance at 250 nm with time. Key: □□, 5% I; ○, 2.5% I; □, 1% I; ●, 0.1% I; and ◑, water.*

The purpose of this study was to explore the effect of a quaternary ammonium compound on the photodegradation of menadione. Cetylethylmorpholinium ethosulfate (I) was chosen because this compound does not absorb UV light in the same region as menadione and because the sulfate ion does not have any effect on the photodegradation of menadione (9).

The naphthoquinone moiety present in menadione absorbs UV radiation over the 245–260-nm region. Therefore, photodegradation studies were carried out by irradiating with UV light at 254 nm.

EXPERIMENTAL

Materials—Menadione[1], mp 105°, and cetylethylmorpholinium ethosulfate[2] (I) were obtained commercially.

Photodegradation—The light source employed[3] was positioned at a distance of 60.0 mm from a quartz cell of 1 cm diameter. Fresh solutions of menadione in 0.2 *M* borate buffer solutions of pH 5.5 were placed in the sample cell. The absorption spectra[4] were determined during photolysis.

Interaction in Ground State—The solubility of menadione in

[1] K&K Laboratories, Plainview, N.Y.

[2] Atlas Chemical Industries, Wilmington, Del.

[3] A Pen-Ray mercury lamp, model SCT-1, black light (Eastern Corp.) with intensity of 2400 U W/cm² and 253.7 nm radiation.

[4] UV absorption was measured in a Cary model 15 double-beam scanning UV spectrophotometer and a Beckman DU spectrophotometer.

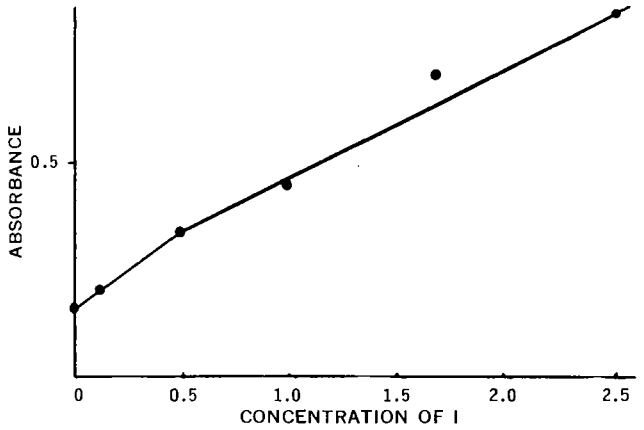

Figure 3—*Solubility of menadione in an aqueous solution as a function of the concentration of I (% w/v).*

the presence of I was studied by placing 100 mg of menadione in each of a series of 125-ml bottles containing 50 ml of 0.2 *M* borate buffer in which varying amounts of I were dissolved.

The complex determination was carried out by mixing varying concentrations of I with a constant concentration of menadione, 5.19×10^{-5} *M*, and recording the absorption spectra.

RESULTS

Kinetics of Menadione Photodegradation—The photodegradation of menadione solution was expressed as the rate of change of absorbance at 250 nm. The absorbance at this wavelength involves only the intact menadione (3, 6). A set of UV spectra obtained during photolysis is shown in Fig. 1. This figure demonstrates the gradual disappearance of the 250-nm absorption peak during photolysis. The effect of I on the rate of photodegradation was expressed by determining the apparent first-order rate constant, *K*, according to Eq. 1:

$$K = \frac{2.303}{t} \log \frac{C_0}{C} \qquad \text{(Eq. 1)}$$

For every rate study with I, a control study was run without it, and the ratio of the apparent first-order rate constants, K/K_{H_2O}, was determined (Table I and Fig. 2).

Interaction in Ground State—An increase in the solubility of menadione occurred in the presence of I, particularly when the concentration of I rose above 0.5% (w/v) (Fig. 3).

The presence of I also led to changes in the absorption spectrum of menadione. This phenomenon can be used to suggest the nature of this interaction. A maximum lowering of the 250-nm absorption peaks occurred when the concentration of I reached 2.78 *M*. At this concentration, the 245-nm absorption peak had shifted to 251.5 nm and the 260-nm absorption peak had shifted to 261.5 nm.

These shifts in the peaks, as well as the decrease in the absorbance of menadione by the addition of I, suggested a mechanism for

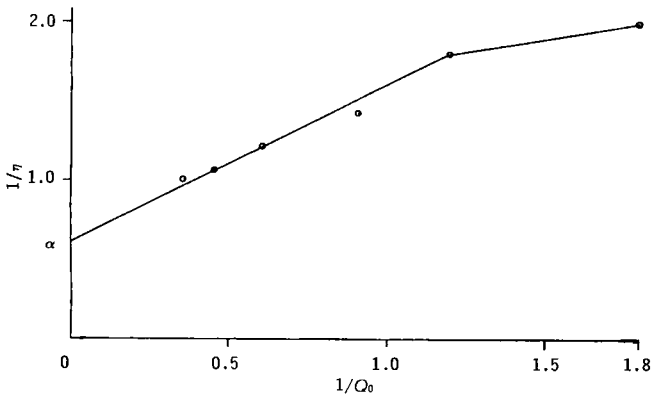

Figure 4—*Relationship between the concentration of I* [Q_0] *and the concentration of the complex* [η].

Table II—Relationship between the Concentration of Compound I [Q_0] and the Concentration of the Menadione–I Complex at 25° (Menadione Concentration = 5.19×10^{-5} *M*)

Q_0, *M*	Δi	$\frac{\Delta i}{\Delta s} = \eta$
0.5565	0.0630	2.0000
0.8347	0.0700	1.8001
1.1130	0.0880	1.4318
1.6696	0.1040	1.2116
2.2262	0.1190	1.0588
2.7825	0.1260	1.0000

the slowing down of the photodegradation of menadione. According to Kunio and Matsuoka (11), these two phenomena indicated the formation of a complex between menadione and I in the ground state.

The dissociation constant for the complex of I and menadione was calculated according to Kunio and Matsuoka (11) as follows:

$$\frac{\Delta i}{\Delta s} = \eta \qquad \text{(Eq. 2)}$$

where Δi = the difference between the absorbance of menadione in borate buffer and in various concentrations of I, and Δs = the difference between the absorbance of menadione in borate buffer and the maximal absorbance at the highest concentration of I. The absorbance was measured at 250 nm.

The quotient of the complex concentration [*MQ*] and the menadione concentration [*M*] is:

$$\beta = \frac{[MQ]}{[M]} \qquad \text{(Eq. 3)}$$

The proportionality factor is calculated with:

$$\beta = \alpha\eta \qquad \text{(Eq. 4)}$$

The equilibrium constant, *K*, is found by:

$$K = \frac{[M][Q]}{[MQ]} \qquad \text{(Eq. 5)}$$

where [*M*] and [*Q*] are the concentrations of menadione and I, respectively; and [*MQ*] is the concentration of the complex formed.

Since the concentration of the menadione is insignificant as compared to that of I, the amount of the I bound in the ground state is also insignificant as compared to the total concentration of I. Therefore, the concentration of $Q = Q_0$, $K = [(1 - \beta)/\beta]\,[Q_0]$, and Eq. 1 becomes:

$$K = \frac{1 - \alpha\eta}{\alpha\eta}\,[Q_0] \qquad \text{(Eq. 6)}$$

or:

$$\frac{1}{\eta} = \alpha K \frac{1}{[Q_0]} + \alpha \qquad \text{(Eq. 7)}$$

If $1/\eta$ *versus* $1/Q$ is plotted (as shown in Fig. 4), the intercept gives the value α. Thus, the equilibrium constant can be calculated. At a I concentration of 1.6696 *M*, the equilibrium constant was 1.647 *M* and the concentration of complex formed was 1.014×10^{-5} *M*.

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[x] To whom inquiries should be directed.

Synthesis and Evaluation of Antitumor Activity of 1-[*N*,*N*-Bis(2-chloroethyl)sulfamoylphenyl]-3,3-dialkyltriazenes

I. LALEZARI [x] and F. AFGHAHI

Abstract □ Twenty-two 1-*p*-sulfamoylphenyl-3,3-dialkyltriazenes, 1-*p*-dialkylsulfamoylphenyl-3,3-dialkyltriazenes, and 1-*p*-[*N*,*N*-bis(2-chloroethyl)sulfamoylphenyl]-3,3-dialkyltriazenes were synthesized from their corresponding amines. Six of the compounds tested were devoid of antitumor activity.

Keyphrases □ Triazene derivatives—synthesis of 22 1-*p*-sulfamoylphenyl-, 1-*p*-dialkylsulfamoylphenyl-, and 1-*p*-[*N*,*N*-bis(2-chloroethyl)sulfamoylphenyl]-substituted 3,3-dialkyltriazenes, six compounds screened for antitumor activity □ Sulfamoylphenyltriazenes—synthesis of 22 derivatives, six screened for antitumor activity □ Antitumor activity—six of 22 synthesized sulfamoylphenyltriazenes screened

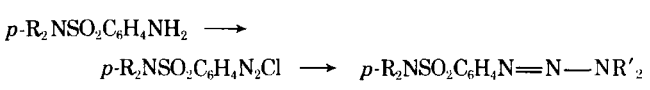
A number of 5-triazenoimidazoles with pronounced activity against experimental tumors were synthesized previously (1, 2). 5-(3,3-Dimethyl-1-triazeno)imidazole-4-carboxamide, in particular, is clinically useful for the induction of temporary remission in malignant melanoma (3). Some derivatives of phenyltriazenes have exhibited antitumor activity (4, 5), and some derivatives of N^1,N^1-bis(2-chloroethyl)-sulfanilamide were also found to be active against experimental tumors (6). Several *p*-sulfonyl- and *p*-sulfamoylcarbanilic acid esters were found to be inactive in mouse lymphoid leukemia (7, 8).

To extend these observations, it was decided to prepare compounds having *p*-sulfamoylphenyl, *p*-dialkylsulfamoylphenyl, and bis(2-chloroethyl)sulfamoylphenyl groups and the triazeno moiety in their molecules. Sulfanilamide, N^1,N^1-dialkylsulfanilamide, and N^1,N^1-bis(2-chloroethyl)sulfanilamide were readily converted to the desired triazeno derivatives by treating the appropriate diazotized amines with the secondary amines in sodium carbonate solution (Scheme I). All prepared compounds are summarized in Table I.

Compounds VI*a*, II*b*, V*b*, Ic, III*c*, and V*c* were tested *in vivo* for antitumor activity against mouse lymphoid leukemia L-1210, resulting in a T/C percent of 120 or less at 400 mg/kg (increase in survival of treated animals).

EXPERIMENTAL[1]

1 [*N*,*N*-Bis(2-chloroethyl)sulfamoylphenyl] 3,3-pentamethylenetriazene (IVc) was obtained from 2.97 g (0.01 mole) of N^1,N^1-bis(2-chloroethyl)sulfanilamide prepared according to Brintzinger *et al.* (9). The sulfanilamide derivative was added to a mixture of 25 g of crushed ice and 3.5 ml of concentrated hydrochloric acid with vigorous stirring at 0–5°. Diazotization was achieved by the slow addition, accompanied by thorough agitation, of sodium nitrite [0.7 g (0.01 mole) dissolved in 5 ml of water]. After standing at 0–5° for 20 min, the solution was filtered rapidly



Scheme I

[1] Melting points were measured on a Kofler hot-stage microscope and are uncorrected. The IR spectra were recorded on a Leitz model III spectrograph. NMR spectra were taken on a Varian A60A instrument. Mass spectra were recorded on a Varian Mat 111 spectrograph. UV spectra were obtained using a Varian-Techtron 635 recording instrument. All compounds were subjected to IR, NMR, and mass spectroscopy, and the results were as expected.

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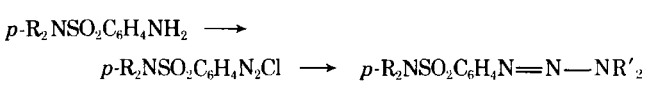

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Table I—1-*p*-Sulfamoylphenyl- and 1-*p*-Dialkylsulfamoylphenyl-3,3-dialkyltriazenes

Compound	Melting Point	Yield, %	Formula	Analysis, % Calc.	Found
I*a*	178–182°	58	$C_8H_{12}N_4O_2S$	C 42.11	42.18
				H 5.26	5.31
II*a*	128°	63	$C_{10}H_{16}N_4O_2S$	C 46.88	46.95
				H 6.25	6.17
III*a*	118–120°	49	$C_{12}H_{20}N_4O_2S$	C 50.70	50.61
				H 7.04	6.92
IV*a*	206–210°	68	$C_{10}H_{14}N_4O_2S$	C 47.24	47.14
				H 5.51	5.52
V*a*	150–154°	75	$C_{11}H_{16}N_4O_2S$	C 49.25	49.37
				H 5.97	6.06
VI*a*	169–173°	69	$C_{10}H_{14}N_4O_3S$	C 44.44	44.63
				H 5.19	5.12
VII*a*	198–200°	48	$C_{12}H_{18}N_4O_2S$	C 51.06	51.17
				H 6.38	6.20
VIII*a*	200–202°	56	$C_{11}H_{17}N_5O_2S$	C 46.64	46.60
				H 6.01	5.93
I*b*	118–120°	63	$C_{10}H_{16}N_4O_2S$	C 46.88	46.78
				H 6.25	6.28
II*b*	104–107°	66	$C_{12}H_{20}N_4O_2S$	C 50.70	50.88
				H 7.04	7.14
III*b*	167–170°	78	$C_{12}H_{18}N_4O_2S$	C 51.06	51.01
				H 6.38	6.44
IV*b*	135–138°	81	$C_{13}H_{20}N_4O_2S$	C 52.70	52.88
				H 6.76	6.80
V*b*	143–146°	73	$C_{12}H_{18}N_4O_3S$	C 48.32	48.41
				H 6.04	6.02
VI*b*	135–136°	59	$C_{14}H_{22}N_4O_2S$	C 54.19	54.17
				H 7.10	7.20
VII*b*	133–136°	48	$C_{13}H_{21}N_5O_2S$	C 50.16	50.28
				H 6.75	6.79
I*c*	100–102°	57	$C_{12}H_{18}Cl_2N_4O_2S$	C 40.79	40.78
				H 5.10	5.25
II*c*	63–64°	53	$C_{14}H_{22}Cl_2N_4O_2S$	C 44.09	44.22
				H 5.77	5.66
III*c*	150–152°	64	$C_{14}H_{20}Cl_2N_4O_2S$	C 44.33	44.50
				H 5.28	5.21
IV*c*	101–103°	72	$C_{15}H_{22}Cl_2N_4O_2S$	C 45.80	45.72
				H 5.60	5.55
V*c*	117–118°	83	$C_{14}H_{20}Cl_2N_4O_2S$	C 42.53	42.68
				H 5.06	4.98
VI*c*	108–110°	56	$C_{16}H_{24}Cl_2N_4O_2S$	C 47.17	47.26
				H 5.90	5.83
VII*c*	110–114°	63	$C_{15}H_{23}Cl_2N_5O_2S$	C 44.12	44.30
				H 5.64	5.69

and the cold solution was carefully added to a solution of 1 ml (0.012 mole) of piperidine in 25 ml of water containing 2.12 g (0.02 mole) of anhydrous sodium carbonate. The precipitate was filtered, washed with cold water, and recrystallized from aqueous ethanol to give 2.82 g (72%), mp 101–103°, mol. wt. 393 (by mass spectroscopy); UV_{max} 322 nm; IR (KBr): ν_{max} 3330, 2880, 2775, 1582, 1448, 1420, 1392, 1338, 1286, 1258, 1219, 1187, 1165, 1147, 1101, 943, 869, 851, 842, 781, 746, and 711 cm^{-1}; NMR (CCl_4): δ 1.76 (m, 6H, piperidine H), 3.51 (m, 8H, CH_2—CH_2), 3.80 (m, 4H, piperidine H), and 7.33–7.80 (q, 4H, ArH).

All other triazene derivatives reported in Table I were prepared similarly.

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In Vitro Antibacterial Activity of Drugs against Human Intestinal Anaerobic Bacteria

M. W. BROWN

Abstract □ The *in vitro* antibacterial activity, against the major groups of anaerobic fecal bacteria, of a series of drugs was examined by an agar diffusion test and the minimum inhibitory concentration (MIC) test. Only the phenothiazines and amitriptyline showed any marked antibacterial activity, the MIC's being in the 40–640-μg/ml range. It is suggested that the detergent nature of the molecules of these drugs in aqueous solution is responsible for the observed antibacterial activity.

Keyphrases □ Antibacterial activity—commonly used drugs tested against human intestinal anaerobic bacteria *in vitro* □ Drugs—commonly used drugs tested for antibacterial activity against human intestinal anaerobic bacteria *in vitro* □ Phenothiazines—antibacterial activity against human intestinal anaerobic bacteria determined *in vitro*

Many drugs introduced into the human body are excreted *via* the feces, either as the parent molecule or conjugated with glycine or glucuronic acid (1). Porter and Beresford (2) showed that chlorpromazine was excreted in the urine, while Walkenstein and Seifter (3) reported the presence of phenothiazine compounds in the feces of dogs given the drugs orally. In both cases the drug was present as the free base and as metabolites, *e.g.*, chlorpromazine sulfoxide. Porter and Beresford (2) also claimed that only 4% of an oral dose of chlorpromazine (100 mg) was excreted in the urine within 7 days. Therefore, the possibility exists that the intestinal flora will come into contact with either the drugs or their metabolites, since it has also been shown that intestinal and fecal bacteria can deconjugate glycine and glucuronic acid conjugates to release the free drug. Substances were identified in human feces that had the spectrophotometric and polarographic characteristics of chlorpromazine and chlorpromazine sulfoxide 24 hr after an oral dose of chlorpromazine (100 mg) (4, 5). These compounds were not isolated and identified, but a concomitant decrease from 6×10^{10} to 2×10^{10} viable anaerobes/g of wet feces was noted and some GI distress occurred.

EXPERIMENTAL

Anaerobic bacteria were isolated from fresh human feces using a rumen fluid roll tube (medium) (6) and the method described by Brown (5). The isolates were broadly classified into three groups (5):

Group I—Anaerobic, Gram-negative asporogenous rods exhibiting very pleomorphic forms. The organisms in this group belong to the genera *Bacteroides, Sphaerophorus, Ristella, Capsularis, Fusobacterium,* and *Eggerthella.*

Group II—Anaerobic, Gram-positive, nonmotile, asporogenous rods. These organisms belong to the genera *Bifidobacterium, Catenebacterium, Eubacterium, Propionibacterium,* and *Actinomyces* (7).

Group III—Anaerobic, Gram-positive cocci of the genera *Peptococcus* and *Peptostreptococcus* (7).

These groups included all anaerobic bacteria occurring in normal human feces at the level of 10^9–10^{10}/g of wet feces. A preliminary test for drug sensitivity was performed on the isolates, using an antibiotic disk sensitivity test method modified for the testing of obligate anaerobes (5). The isolates were streaked onto rumen fluid glucose agar slants and incubated at 37° for 48 hr to ensure that there were no contaminants. Growth was washed from the slant with dilution solution (6), and the absorbance was adjusted to 0.2 (420 nm). This suspension (0.2 ml) was used to inoculate molten agar (50 ml) in a 120-ml (4-oz.) medical flat bottle which, after mixing, was allowed to set on one large face of the bottle. Filter paper disks containing the drug (50 μg) were placed on the agar surface and left for 1 hr at room temperature to allow the drug to diffuse into the agar.

The bottles were incubated at 37° for 48 hr before the zones of inhibition were measured. To ensure that the noninhibitory drugs in the disk test were actually desorbing from the disk and diffusing through the agar, the following test was performed. Disks of the drug were placed on uncontaminated agar and the drug was allowed to diffuse into the agar for 1 hr at room temperature. The disk was removed and the residual drug on the disk was assayed polarographically. The drugs were also detected in small pieces of agar cut from the plate at different distances from the disk. The minimum inhibitory concentration (MIC) was investigated using

Table I—Inhibition of Anaerobic Fecal Bacteria by Commonly Used Drugs

		Drug[a] and Zone[b] of Inhibition										
Culture Group	Number of Isolates Tested	Chlorpromazine	Promazine	Trifluoperazine	Fluphenazine	Promethazine	Amitriptyline	Chlordiazepoxide	Diazepam	Phenobarbital Sodium	Metformin	Tolbutamide
I	40	+++	+	++	+	++	+++	−	−	−	−	−
II	10	−	−	−	−	−	−	−	−	−	−	−
III	8	−	−	−	−	−	−	−	−	−	−	−

[a] Drug concentration = 50 μg. disk. [b] Zone diameters: − = no zone, + = 10 mm, ++ = 10–20 mm, and +++ = 20–30 mm.

Table II Drugs[a] Tested for Antibacterial Activity[b]

Culture Group	Number of Isolates Tested	Chlorpromazine				Promazine				Trifluoperazine				Fluphenazine				Promethazine				Amitriptyline			
		a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
I	40	18	22	—	—	—	20	20	—	30	10	—	—	—	20	20	—	—	30	10	—	20	20	—	—
II	10	—	4	6	—	—	7	3	—	—	3	7	—	—	10	—	—	—	7	3	—	—	8	2	—
III	8	—	2	6	—	—	8	—	—	—	—	8	—	—	—	8	—	—	—	8	—	—	—	8	—

Culture Group	Number of Isolates Tested	Chlordiazepoxide				Diazepam				Phenobarbital Sodium				Metformin				Tolbutamide			
		a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d	a	b	c	d
I	40	—	—	—	40	—	—	—	40	—	—	—	40	—	—	—	40	—	—	—	40
II	10	—	—	—	10	—	—	—	10	—	—	—	10	—	—	—	10	—	—	—	10
III	8	—	—	—	8	—	—	—	8	—	—	—	8	—	—	—	8	—	—	—	8

[a] Concentrations of drugs in micrograms per milliliter: a = 40–80, b = 80–160, c = 160–320, and d = 320–640. [b] Figures indicate the number of isolates with that MIC.

rumen fluid glucose broth. Those drugs not showing any inhibition in the disk test were also examined by this method to exclude further the possibility mentioned previously.

Inocula for the broth cultures were produced as in the disk test. Sterile solutions of the drugs were added to sterile broth to give concentrations of 10, 20, 40, 80, 160, 320, and 640 μg/ml. Concentrations in excess of 640 μg/ml were found to precipitate the broth constituents.

RESULTS AND DISCUSSION

Of the drugs examined, only the phenothiazine derivatives and amitriptyline had any marked antimicrobial activity in the sensitivity disk test. This activity was limited to the Group I bacteria (Table I). However, in the MIC determinations where higher concentrations of drugs were used, these compounds were again the only active ones, but Group II and III organisms were also inhibited (Table II). The antimicrobial activity of the phenothiazines has been known for some time; Raffel *et al.* (8) reported that these compounds inhibited *Mycobacterium tuberculosum*, and Agarwal and Ignatur (9) found that chlorpromazine had a lytic effect on *Escherichia coli* G cells.

Phenothiazine was originally used as an anthelmintic (10) but was discarded because of toxic side effects. It has been shown to cause GI disturbances, *e.g.*, nausea, vomiting, and diarrhea. Haden (11) reported that patients treated with chlorpromazine complained of GI pain and nausea after 2 days. All phenothiazines tested exhibited a marked antibacterial action against the Group I (*Bacteroides*) bacteria. Since these organisms constitute the majority of flora in the latter segment of the small intestine, large intestine, and rectum (10^{10}–5×10^{11}/g of wet feces), any drug inhibiting them will permit the outgrowth of other commensals and possibly pathogens, *e.g.*, yeasts, candida, proteus, and clostridia. This alteration in the floral balance could result in large amounts of gas being produced and/or changes in pH and redox potential (Eh) of the gut contents (4). Thus, the distress suffered by persons ingesting the phenothiazines could be due to changes in gut flora.

The MIC results (Table II) indicate that the phenothiazines were all inhibitory at a similar concentration, suggesting some nonspecific activity. Several reports (12, 13) showed that the phenothiazine compounds and amitriptyline were amphipathic molecules in aqueous solution. It is possible that the inhibitory activity of these compounds was due to a surface-active effect on the bacterial cell membrane. It is unlikely, however, that the inhibitory action was solely due to a surface phenomenon since both chlordiazepoxide and diazepam, which are known to have surface-active properties, showed no antibacterial activity *in vitro* against the organisms tested (Table II). Furthermore, there is little evidence to suggest that chlordiazepoxide and diazepam cause intestinal distress.

The disk test has been criticized because it was considered that some drugs would not desorb from the disk and diffuse in the agar. Results obtained by placing disks on uncontaminated agar showed that only small amounts of drug were left on the disk after 1 hr and the drug could also be detected in sections of agar away from disk. Moreover, there was extremely good correlation between the disk tests (Table I) and MIC results (Table II).

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Excretion of the Active Principle of *Catha edulis* (Miraa) in Human Urine

C. K. MAITAI *x and G. M. MUGERA ‡

Abstract □ *d*-Norpseudoephedrine, a central stimulant present in *Catha edulis*, is excreted unchanged in human urine. The alkaloid was detected in urine 30–50 min after ingestion of synthetic *d*-norpseudoephedrine, and trace amounts of the drug could still be detected 24 hr later. Approximately 40% of ingested *d*-norpseudoephedrine was recovered in urine in the first 6 hr.

Keyphrases □ *Catha edulis*—urinary excretion of *d*-norpseudoephedrine after mastication, humans □ *d*-Norpseudoephedrine—urinary excretion after mastication of *Catha edulis*, humans □ CNS stimulants—urinary excretion of *d*-norpseudoephedrine after mastication of *Catha edulis*, humans

Catha edulis Forsk. (Celastraceae) is an evergreen shrub, which usually is about 3–7 m tall but can grow to a height of 18 m under favorable climatic and soil conditions. It is found in the Eastern part of Africa, from Ethiopia to South Africa, and in Arabia. In Kenya, the plant is cultivated on a commercial scale on the slopes of Mount Kenya in the Nyambeni Division of the Meru District. The shoots of this plant are masticated while fresh to combat mental fatigue, allay hunger, and generally induce euphoria (1). The earliest recorded direct reference to the use of *Catha* was in a 1237 (2) prescription for relief of depression. Alles *et al.* (3) showed that the central stimulant activity of *Catha* can be attributed wholly to its *d*-norpseudoephedrine content.

Information gathered from those who masticate *Catha* indicated that the effect on the central nervous system is biphasic, *i.e.* initial stimulation followed by a compensatory phase of depression (4). It was reported that it is necessary to chew the material continuously for sustained mental stimulation, an effect that could be interpreted to mean that the material is rapidly eliminated from the body. A literature survey has not revealed any study of the elimination of *d*-norpseudoephedrine from the human body and, accordingly, this aspect was investigated. Since most phenylalkylamine derivatives are eliminated through the renal route (5), the present study was restricted to investigation of the presence of *d*-norpseudoephedrine and metabolites in urine.

EXPERIMENTAL

Each of four human volunteers was requested to masticate three bundles of *Catha* material at a rate of about two bundles per hour and to submit samples of urine at predetermined intervals. The average weight of a fresh bundle of *Catha* material was 80.6 g, of which 70–80% was ingested; the rest, a fibrous residue, was discarded. The volunteers had only drunk milk in the morning and were requested to give samples of urine just before the experiment and thereafter at the following intervals: 0.5–1, 1–2, 2–4, 6–8, 8–12, 12–15, and 15–24 hr. No food or beverage was consumed in the first 6 hr of the experiment, but the volunteers were encouraged to drink water freely so as to promote frequent voiding of urine.

Qualitative examination of urine for *d*-norpseudoephedrine and metabolites was carried out as follows. To each urine sample (usually 20–50 ml) was added 1–2 ml of saturated lead acetate solution and, after thorough mixing, centrifugation was performed to remove any precipitate. The urine was acidified with 0.1 *N* sulfuric acid, and any precipitate of lead sulfate was removed by centrifugation. The acidic supernatant liquid was extracted with an equal volume of ether three times to remove organic acids and neutrals.

The aqueous phase (urine) was made alkaline with 2 *N* sodium hydroxide and then saturated with sodium chloride and extracted with an equal volume of ether for 8 hr. Preliminary work had shown that the "salting-out" process with sodium chloride improves the percentage recovery of *d*-norpseudoephedrine from urine considerably. This extraction was repeated three times, and the combined ether extract was washed twice with 2 ml of 2.5% sodium bicarbonate solution and dried with anhydrous sodium sulfate. The ether was then distilled off, and the basic residue was examined by TLC and GLC techniques.

This experiment was repeated using synthetic *d*-norpseudoephedrine. Approximately 30 mg was accurately weighed, dissolved in a small volume of water, and given to each of four human volunteers with urine samples collected as described previously.

Examination of residues for *d*-norpseudoephedrine and metabolites using TLC was carried out as follows. Residues were taken up in 2 ml of ether, and approximately 2–5 μl was spotted on a TLC plate coated with silica gel. Approximately 5 μl of the following reference compounds was also spotted on the same plate as the residue: *l*-ephedrine, *d*-pseudoephedrine, and *d*-norpseudoephedrine. Plates were developed in one of the following solvent systems: A, butanol–acetic acid–water (60:15:25); B, butanol saturated with water (upper phase); C, methanol–ammonia (100:1.5); or D, isopropanol–water–ammonia (80:15:5). The spots were revealed by ex-

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Table I—R_f Value of Basic Residue Isolated from Urine of Persons Who Had Consumed *C. edulis* Compared to that of Closely Related Compounds

Compound	Solvent			
	A	B	C	D
d-Norpseudoephedrine	0.61	0.31	0.47	0.80
Basic residue from urine	0.61	0.31	0.47	0.80
l-Ephedrine	0.53	0.26	0.39	0.75
d-Pseudoephedrine	0.53	0.26	0.39	0.75

posing the plates to iodine vapor and, after warming to remove iodine, the plates were sprayed with either ninhydrin or iodoplatinate reagents (6).

The basic residue from urine was also examined with a gas chromatograph[1] equipped with a flame-ionization detector. The GLC column used was stainless steel, 0.3 cm (0.125 in.) o.d, 1.5 m in length, and packed with Celite CQ (100–120 mesh) coated with 3% methyl silicone gum (OV-1[2]). The column was conditioned for 12 hr under the following operating conditions: oven temperature, 150°; detector temperature, 150°; hydrogen pressure, 17 psi; air pressure, 12 psi; and nitrogen flow rate, 50 ml/min.

In the quantitative estimation of the alkaloid recovered from urine, the residue was dissolved in ether and diluted to 2 ml in a standard flask. A fixed volume, usually 5 μl, was injected into the column using a 10-μl syringe[3]. The amount of compound present was calculated from the area under the curve by comparison with the area of a standard amount of *d*-norpseudoephedrine subjected to a similar extraction procedure. Preliminary experiments were done to establish the percentage recovery of *d*-norpseudoephedrine from urine under specified conditions.

RESULTS

Only one compound was detected in urine from humans who had consumed *Catha* material or synthetic *d*-norpseudoephedrine, and this compound had the same R_f value (TLC) as reference *d*-norpseudoephedrine in all solvent systems used (Table I). Similarly, the retention time (GLC) for the basic compound isolated from urine was the same as that for *d*-norpseudoephedrine (Fig. 1). This basic compound was first detected in urine 30–50 min after ingestion of *d*-norpseudoephedrine, and trace amounts could still be detected 24 hr later when the experiment was discontinued.

The percentage recovery of *d*-norpseudoephedrine from control urine was 84.6 (6.2 *SD*). Since the amount of drug excreted in the urine within any specified interval was very small, it was necessary to combine two or three residue fractions for the purpose of quantitative estimation of the drug in urine with GLC. The peaks obtained when *d*-norpseudoephedrine was injected into the column were symmetrical and reproducible to within 10% for each urine sample. Approximately 40% of *d*-norpseudoephedrine was recovered in urine within the first 6 hr. The percentage of *d*-norpseudoephedrine excreted in urine varied considerably between the four individuals and even in the same individual at different times, but the values were always within the range of 30–40%.

Hydrolysis of urine (acidified with 2 *N* hydrochloric acid) by heating under reflux in a boiling water bath for 1 hr before extraction did not alter the results, an indication that conjugation is not an important metabolic pathway for *d*-norpseudoephedrine.

DISCUSSION

Since the basic residue recovered from urine of humans who consumed *Catha* material had the same R_f value as *d*-norpseudoephedrine in four different solvent systems and had the same retention time (GLC), it is logical to assume that the two compounds are identical.

d-Norpseudoephedrine is a polar basic compound; consequently, a large percentage of the free base would not be reabsorbed from the glomerular filtrate. This expectation is consistent with results obtained in the present work in which the drug was detected in urine 30–50 min after oral administration. Elimination of many drugs from the body follows a first-order kinetic rate; therefore, even though the drug might appear in urine soon after administration, the elimination process often continues for a long period. Research work has shown that amines of the ephedrine series are excreted unchanged (7). Excretion of drugs in urine after oral administration depends on the time course of absorption and distribution. In addition, urinary pH and output might influence the quantitative excretion of the drug. Since both urinary pH and output fluctuate considerably throughout the day, the amount of drug excreted also can be expected to fluctuate, even in the same individual.

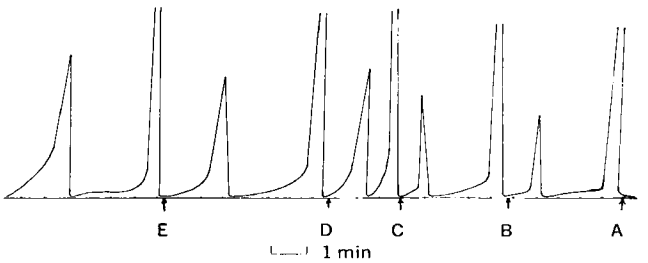

Figure 1—*Retention time for: A, basic residue from urine (4.1); B,* d-*norpseudoephedrine (4.1); C, amphetamine (1.6); D,* l-*ephedrine (4.7); and E,* d-*pseudoephedrine (4.7).*

Central stimulation by amphetamine-like substances is invariably accompanied by a compensatory phase of depression, but the exact mechanism by which central stimulation gives way to depression is not understood (8). Information received from people who chew *Catha* material indicates that the onset of mental fatigue sometimes occurs very suddenly. Continuous ingestion of the drug should forestall the onset of central depression, and this is apparently what happens in people who masticate *C. edulis* continuously, sometimes for more than 12 hr.

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[1] Pye Unicam gas chromatograph P.S. 104.
[2] Supplied by Pye Unicam Ltd., Cambridge CBI 2PX, England.
[3] Hamilton.

New Compounds: Fatty Acid and Long Chain Hydrocarbon Derivatives Containing a Strong Chelating Agent

WILLIAM C. ECKELMAN *‡x, STEPHEN M. KARESH *, and RICHARD C. REBA *‡

Abstract □ Fatty acid and long chain hydrocarbon analogs that contain a strong chelating group were synthesized as part of an effort to determine if the biological function of the fatty acid can be used to transport metallic isotopes to the myocardium.

Keyphrases □ Fatty acid analogs containing chelating groups—synthesis, potential radiopharmaceutical imaging agents □ Chelating agents—synthesis of fatty acid and long chain hydrocarbon analogs

Radiolabeled fatty acid analogs have been suggested as myocardial imaging agents for the detection of myocardial infarcts. Preliminary results with radioiodinated fatty acids indicate that infarcts can be detected by external counting of the emitted γ-rays (1–3). However, a combination of poor physical properties and expense has prevented the widespread use of any of the various isotopes of iodine in the compounds.

A technetium compound (^{99m}Tc, $t_{1/2}$ 6 hr, 140 kev gamma) would overcome these problems, because technetium possesses ideal physical properties and ready availability (4). However, the direct labeling of molecules with technetium usually does not create strong chemical bonds (5, 6). (Carboxymethyl)iminobis(ethylenenitrilo)tetraacetic acid (I) forms the most stable ^{99m}Tc chelate[1] known at this time (7), and even this chelate appears to dissociate to a small extent *in vivo* (8). Therefore, any chelating group that is less stable than the I group will create a weak chelate which would dissociate and result in complicated *in vivo* distribution. However, if I could be attached to a biological substrate such as a fatty acid in such a way as not to diminish the biological activity, then this new biological compound would possess both a functional group to bind technetium and a functional group to bring about localization in the myocardium.

It is not the intent to produce a strict analog of a fatty acid, but rather a molecule that is taken up in the myocardium as a fatty acid but is not completely metabolized as such. This type of molecule apparently could produce even higher concentrations of the radioisotope in the myocardium than the concentration reached by the rapidly metabolized fatty acid (9, 10). This hypothesis appears to be true for radioiodinated cholesterol localization, which is used for external visualization of the adrenal glands (11).

Previous work has shown that alkylating agents can successfully be delivered to hormone responsive tissue by binding the alkylating agent to steroid hormones (12–14).

The group of compounds in this study contain either I or an (ethylenedinitrilo)tetraacetic acid[2] (II)-like structure linked to a fatty acid or long chain hydrocarbon moiety. Ester derivatives of II have been prepared by the reaction of excess alcohol with an acid catalyst (15) and by the reaction of the dianhydride of II and an alcohol (16). No similar derivatives of I have been prepared. The preparation of monoester derivatives of II and I has not been reported. These monoderivatives would possess the optimal chelating strength.

Substitution on the nitrogen atom of I-like structures can be obtained by reacting ethylenediamine or diethylenetriamine with the appropriate alkyl bromide before carboxymethylation. The synthesis of (*N*-*n*-octyl)iminobis(ethylenenitrilo)tetraacetic acid has been reported (17).

Four classes of compounds have been synthesized (V–VII; IX and X; XI and XIII; and XIV), and all have been shown[3] to chelate strongly carrier-free radioisotopes such as ^{57}Co, ^{99m}Tc, and ^{113m}In.

EXPERIMENTAL

The physical properties[4] of the compounds are given in Table I; elemental analyses[5] are given in Table II.

***N*-*n*-Hexadecyldiethylenetriamine Trihydrochloride (III)**—A solution of diethylenetriamine (50 ml, 0.5 mole) and *n*-bromohexadecane (30.6 g, 0.10 mole) in ethanol (50 ml) was heated to reflux. A solution of sodium hydroxide (4.5 g) in water (6 g) was added dropwise over 5 min, and then the mixture was refluxed for 3 hr. The product was extracted into the organic layer after addition of *n*-butanol (40 ml), benzene (40 ml), and water (200 ml). The organic layer was washed twice with water, dried, and distilled at 180° at 0.05 mm pressure. The product was dissolved in ether (200 ml) and converted to the trihydrochloride with dry hydrogen chloride gas. The precipitated product was washed with ether.

***N*-*n*-Octadecyldiethylenetriamine Trihydrochloride (IV)**—A solution of diethylenetriamine (50 ml, 0.50 mole) and *n*-bromooctadecane (33.3 g, 0.10 mole) in ethanol (50 ml) was reacted according to the procedure for III.

(*n*-Dodecyl)iminobis(ethylenenitrilo)tetraacetic Acid Monohydrate (V)—A solution of sodium chloroacetate (15.4 g, 0.132 mole) and 4-*n*-dodecyldiethylenetriamine (10 g, 0.037 mole) was heated at 50° for 6 hr at pH 11.0 ± 0.5, maintained by the addition of sodium hydroxide (18). A 1.0-ml aliquot of the reaction mixture was titrated at pH 12 with 0.100 *N* calcium chloride solution to the permanent turbidity end-point, using sodium oxalate as the indicator. The reaction mixture was then adjusted to the chelation concentration that titrates 80 mg of calcium carbonate/ml of solution. Sulfuric acid (1:4) was added to the solution at 80° to a final pH of 2.6, and the solution was seeded with a crystal of I and cooled overnight at 5°. The precipitate was washed with water.

(*n*-Hexadecyl)iminobis(ethylenenitrilo)tetraacetic Acid

[1] ^{99m}Tc-Diethylenetriaminepentaacetic acid (DTPA).

[2] Ethylenediaminetetraacetic acid (EDTA).

[3] Unpublished data.

[4] Determined with the following instruments: Thomas-Hoover capillary melting-point apparatus (melting points are uncorrected), Perkin-Elmer model 337 grating infrared spectrophotometer, and Varian model A-60A spectrometer. Potentiometric titrations were performed using a Corning glass electrode, a Corning model 8 pH meter, and a Heathkit chart recorder.

[5] Performed by F. Kasler, University of Maryland.

Table I—Physical Properties of New Compounds

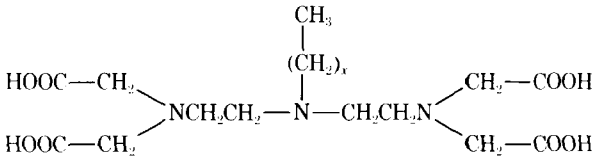
Compound	Yield, %	Recrystallization Solvent	Melting Point	IR Data, cm^{-1}
III	50	Water–isopropanol (4:1)	257–258°	1605, 2850, 2890
IV	37	Water–isopropanol (4:1)	251–254°	1605, 2850, 2890
V	55	Water–isopropanol (4:1)	159–160°	1640, 1730, 2850
VI	64	Water–isopropanol (4:1)	—	1650, 1730, 2848
VII	62	Water–isopropanol (4:1)	—	1650, 1730, 2848
VIII	95	Insoluble	193–195° dec.	1760, 1810, 1130
IX	49	Benzene	103–104°	1740, 1750, 2850
X	50	Benzene	105–106°	1740, 1750, 2850
XI	80	Methanol	149–151°	1600, 1730, 2850
XII	67	Ethyl acetate	94–95°	1685, 2850, 2910
XIII	72	Methanol	148–151°	1620, 1720, 2850
XIV	6	Water–isopropanol (4:1)	160–161°	1655, 1740, 2850
XV	84	Insoluble	182° dec.	1780, 1830, 2960

Table II—Elemental Analysis of New Compounds

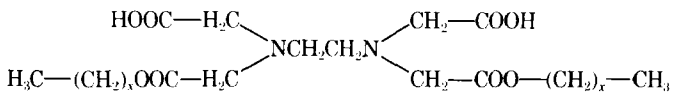
Compound	Molecular Formula	Calculated, %			Found, %		
		C	H	N	C	H	N
III	$C_{20}H_{48}Cl_3N_3$	54.97	11.07	9.61	55.25	11.22	9.30
IV	$C_{22}H_{52}Cl_3N_3$	56.82	11.27	9.04	56.97	11.24	8.78
V	$C_{24}H_{47}N_3O_9$	55.26	9.08	8.06	55.75	9.11	8.09
VI	$C_{28}H_{57}N_3O_{10}$	56.45	9.64	7.05	56.91	9.47	6.85
VII	$C_{30}H_{61}N_3O_{10}$	57.76	9.85	6.74	58.25	9.70	6.70
VIII	$C_{10}H_{12}N_2O_6$	46.87	4.73	10.93	46.68	4.88	10.82
IX	$C_{42}H_{80}N_2O_8$	67.38	10.74	3.93	67.41	11.02	3.80
X	$C_{46}H_{88}N_2O_8$	68.71	11.01	3.64	68.95	11.30	3.50
XI	$C_{40}H_{73}N_2O_{12.5}$	61.43	9.40	3.58	61.23	9.80	3.64
XII	$C_{16}H_{32}O_3$	70.52	11.84	—	70.66	12.05	—
XIII	$C_{42}H_{76}N_2O_{12}$	62.97	9.56	3.50	62.87	9.74	3.25
XIV	$C_{28}H_{51}N_3O_{11}$	55.52	8.49	6.94	55.47	8.58	7.18
XV	$C_{14}H_{19}N_3O_8$	47.06	5.36	11.74	46.25	5.37	11.46

Dihydrate (VI)—Compound III (23.1 g, 0.053 mole), sodium chloroacetate (37.5 g, 0.32 mole), and $Na_2CO_3 \cdot 10H_2O$ (30.5 g, 0.110 mole) were dissolved in water (100 ml) and heated at 95° for 24 hr (19). The product was isolated as for V.

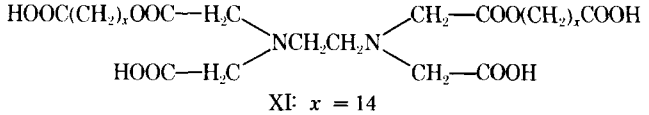
(*n*-Octadecyl)iminobis(ethylenenitrilo)tetraacetic Acid Dihydrate (VII)—A solution of IV (24.6 g, 0.053 mole), sodium chloroacetate (37.5 g, 0.320 mole), and $Na_2CO_3 \cdot 10H_2O$ (30.5 g, 0.110 mole) was dissolved in water (100 ml) and then heated at 95° for 24 hr. The product was isolated as for V.



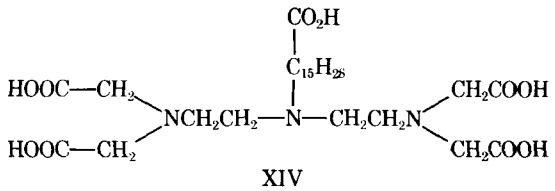
V: $x = 11$
VI: $x = 15$
VII: $x = 17$



IX: $x = 15$
X: $x = 17$



XI: $x = 14$
XIII: $x = 15$



XIV

Dianhydride of II (VIII)—Compound II (182 g, 0.71 mole) was suspended in pyridine (300 g), acetic anhydride (260 g, 2.54 moles) was added, and the mixture was stirred at 65° for 24 hr. The product was filtered, washed with acetic anhydride and ether, and dried.

***N,N'*-Dihexadecyl Ester of II (IX)**—A mixture of the dianhydride of II (32 g, 0.125 mole) and *n*-hexadecanol (60.5 g, 0.250 mole) in benzene (315 ml) was refluxed for 24 hr and then filtered hot. The collected precipitate was washed with boiling benzene, and the total filtrate was cooled to produce the crystalline product.

***N,N'*-Dioctadecyl Ester of II (X)**—A mixture of the dianhydride of II (32 g, 0.125 mole) and *n*-octadecanol (67.5 g, 0.250 mole) in benzene (315 ml) was refluxed for 24 hr, and the product was isolated according to the procedure for IX.

***N,N'*-Di(14-carboxytetradecyl) Ester of II (Hemihydrate) (XI)**—A mixture of 15-hydroxypentadecanoic acid (2.50 g, 0.010 mole) and the dianhydride of II (1.00 g, 0.004 mole) in benzene (15 ml) was refluxed for 18 hr. The precipitate was filtered, washed with boiling benzene, and dried.

16-Hydroxypalmitic Acid (XII)—A solution of juniperic acid lactone (9.1 g, 0.036 mole) in ethanol (10 g) was mixed with sodium hydroxide (5 *N*, 25 ml) and water (25 ml) and refluxed for 4 hr. The reaction mixture was washed with ethyl acetate and then acidified with 6 *N* HCl to pH 1.00. The product was then extracted into ethyl acetate.

***N,N'*-Di(15-carboxypentadecyl) Ester of II (XIII)**—A solution of XII (1.506 g, 0.006 mole) in benzene (15 ml) was mixed with the dianhydride of II (0.707 g, 0.003 mole) and refluxed for 16 hr. The resulting precipitate was filtered, washed with boiling benzene, and dried.

***N* - (15 - Carboxy - 9 - pentadecenyl)iminobis(ethylenenitrilo)-tetraacetic Acid Monohydrate (XIV)**—A solution of 16-bromo-9-hexadecenoic acid (10 g, 0.030 mole) and diethylenetriamine (16.5 ml, 0.150 mole) was mixed in ethanol (15 ml) and heated to reflux. A solution of sodium hydroxide (1.5 g) in water (1.8 g) was added dropwise over 5 min, and reflux was continued for 4 hr. The

product was isolated as the trihydrochloride according to the procedure for III. It was then carboxymethylated according to the procedure for V.

(Carboxymethyl)iminobis(ethylenenitrilo)tetraacetic Acid Dianhydride[6] (XV)—Compound I (39.3 g, 0.10 mole) was suspended in pyridine (50 g), and acetic anhydride (40.8 g, 0.40 mole) was added. The mixture was heated at 65° for 24 hr. The product was filtered, washed with acetic anhydride and ether, and dried.

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COMMUNICATIONS

Nonclassical Phase Transfer Behavior of Phenylbutazone

Keyphrases □ Phenylbutazone—nonclassical phase transfer behavior, pH and buffer effects, dissolution and ionization rates □ Dissolution—phenylbutazone, nonclassical phase transfer behavior □ Ionization—phenylbutazone, nonclassical phase transfer behavior

To the Editor:

Lovering and Black (1, 2) recently alluded to the nonclassical behavior of phenylbutazone in its transfer through a dimethylsiloxane membrane and through an everted rat intestine as a function of pH.

I measured the ionization rates of phenylbutazone in aqueous buffered solution (μ = 0.1) at 25 ± 0.2° using a stopped-flow spectrophotometer[1] and found the protonation of the phenylbutazone anion and the deprotonation of phenylbutazone to be *noninstantaneous*[2]. As expected, both protonation and deprotonation were highly dependent on the pH of the solution as well as buffer concentration.

As an example, the half-life for the deprotonation of phenylbutazone (taken to zero buffer concentration) at pH 5.5 was 55 msec and it was 67 msec at pH 7.0. The half-life for protonation at pH 3.5 was 10.3 msec, while at pH 4.0 it was 22.6 msec. Phenylbutazone is a carbon acid[3] of pKa 4.50–4.70 (3–5), and the ionization rates of carbon acids are slow relative to the approximately diffusion-controlled ionization rates of other acids (6–10).

Apart from the phase transfer anomalies noted here, the chemical properties of carbon acids often show anomalies when compared to other acids. For example, carbon acids show large negative deviations

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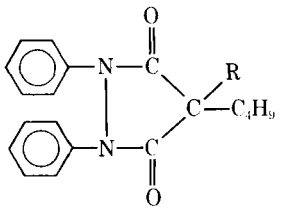

I: R = H
II: R = D

in Brønsted acid–base catalysis plots when compared to carboxylic acids, phenols, and other acids (13). These negative deviations also have been directly attributed to the fact that the protonation and deprotonation rates of carbon acids are slow relative to the approximately diffusion-controlled ionization of other acids (13). The reasons for these slow ionization rates are adequately discussed elsewhere (7).

Based on this realization of noninstantaneous ionization rates, I postulated that the carbon acid, phenylbutazone, may have a hindered dissolution due to simultaneous reversible noninstantaneous chemical reaction which must take place in the aqueous diffusion layer if the dissolution involves rate-determining diffusion through the aqueous diffusion layer. By hindered, I mean that the dissolution rate at pH's greater than the pKa of the compound could not be accounted for on the basis of the Noyes–Whitney equation when applying the appropriate correction for the ionization constant (11):

$$\left(\frac{dC}{dt}\right)_0 = K'C_0\left(1 + \frac{[H^+]}{K_a}\right) \qquad \text{(Eq. 1)}$$

where $(dC/dt)_0$ is the initial rate of dissolution; K' is a constant encompassing diffusivity, surface area, and diffusion layer thickness along with any corrections for the hydrodynamics of the system under study; C_0 is the saturation solubility of the free acid form of the solid; K_a is the dissociation constant of the acid; and $[H^+]$ is the hydrogen-ion concentration. The obvious limitations of this equation have been adequately discussed (11, 16). Ideally, the comparison here should be made to a model of an acidic drug undergoing dissolution with simultaneous instantaneous chemical reaction.

Other phase transfer phenomena that involve rate-determining diffusion through an aqueous diffusion layer and a simultaneous noninstantaneous reaction (reversible in the present case) are affected by the speed of such a reaction. In Lovering and Black's study (1, 2), the time-dependent interconversion of phenylbutazone to phenylbutazone anion and vice versa necessary for the phenylbutazone to pass into and out of the dimethylsiloxane membrane, as well as transfer through the aqueous diffusion layers, is a function of the pH and concentration of buffer components in the two respective compartments. At pH >4.5, in which Lovering and Black found the permeability coefficient to be pH dependent, the half-life for the approach to the ionization equilibrium was >30 msec; *i.e.*, the interconversions between the acid and base components took place in approximately the same time range as the residence time of the components in the diffusion layer (22). Another interesting fact was that boric acid, used as the sink buffer by Lovering and Black, also undergoes nonclassical noninstantaneous ionization behavior (12).

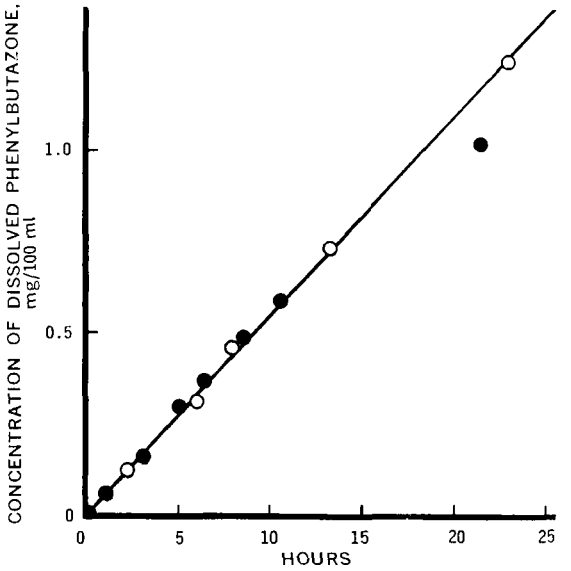

Figure 1—*Plot showing the dissolution of phenylbutazone (I, ○) and d_1-phenylbutazone (II, ●) into a 25% (v/v) ethanol–water solvent at 25°, ionic strength 0.1, pH 2.89 maintained by 0.05 M acetate buffer, from a constant-surface area pellet at a constant stirring rate of 50 rpm.*

The initial dissolution rate of phenylbutazone (I) and d_1-phenylbutazone[4] (II) into a 25% (v/v) ethanol–water solvent[5] at 25° and constant ionic strength (μ = 0.1 with sodium chloride) from a constant-surface area pellet was studied as a function of apparent pH, buffer concentration, and stirring rate. The objective of this work was to show the nonclassical behavior of phenylbutazone in the phase transport phenomenon of dissolution.

The dissolution of I and II into pH 2.89, 0.05 *M* acetate buffer and into pH 7.03, 0.05 *M* phosphate buffer is shown in Figs. 1 and 2. The identical and reproducible initial dissolution rates of I and II at pH 2.89, a pH below the pKa of phenylbutazone, represent a simple mass transfer phenomenon[6], showing that no quantitative differences in physical properties between I and II capable of affecting this simple mass transfer are present. The slower dissolution of II compared to I in the phosphate buffer (as well as the other buffers studied) can only be attributed to differences in the rate of deprotonation due to a pri-

[4] d_1-Phenylbutazone was formed by direct exchange in refluxing D_2O, mp 106°; deuteration, as determined by a Varian model T60 NMR spectrometer in $CDCl_3$, was >99% complete.

[5] Lovering and Black (1, 2) carried out all their work in purely aqueous solutions. In these ionization kinetic studies, all work again was carried out in purely aqueous media. The hydroalcoholic solvent used in the dissolution rate studies was necessary due to the poor aqueous solubility of phenylbutazone. It is recognized that a 25% (v/v) ethanol–water solvent may affect the apparent pKa of phenylbutazone (4.68 under the hydroalcoholic solutions used) and the ionization kinetics. However, it was not expected to have a major effect, especially since the dissolution rates of I and II were being compared as opposed to absolute differences. All pH values in the dissolution part of this work are apparent pH values.

[6] During this study the formation of phenylbutazone polymorphs was noted. The three polymorphs had melting points of 86, 96, and 106°, respectively. Polymorph III, mp 106°, the stable polymorph in aqueous solution, was the one studied.

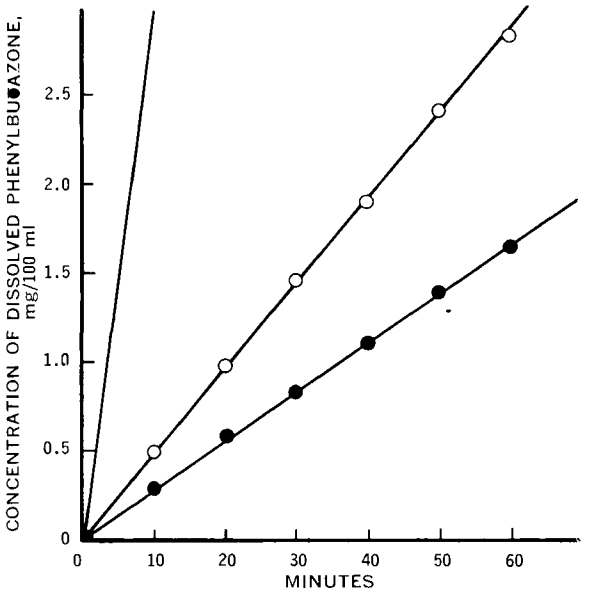

Figure 2—*Plot showing the dissolution of phenylbutazone (I, ○) and d_1-phenylbutazone (II, ●) into a 25% (v/v) ethanol–water solvent at 25°, ionic strength 0.1, pH 7.03 maintained by 0.05 M phosphate buffer, from a constant-surface area pellet at a constant stirring rate of 50 rpm. The solid line without experimental points is the line predicted by Eq. 1.*

mary isotope effect on the deprotonation rate between the two derivatives.

Also included in Fig. 2 is the theoretical line as defined by Eq. 1. It was felt that a demonstrated difference in dissolution rates between I and II at a pH greater than the pKa of phenylbutazone would be more meaningful than a comparison of the dissolution rate of I to a theoretical line, because the theoretical line itself is also model limited.

Various models have been proposed (14–18) for the dissolution of acids as a function of pH and basic components of a buffer. Similarly, theoretical models for the transport of neutral and acidic agents across theoretical membranes and the application of these models to the GI absorption of drug substances have been discussed (19–21). In all of these models, the assumption is made that all ionizations are *instantaneous, i.e.* the conversion of the acid to its basic component and vice versa take place much faster than residence times in the diffusion layer. The assumption is valid in most cases and may only be invalid in the examples noted here, namely carbon acids.

Higuchi *et al.* (22) observed the dissolution of 7-acetyltheophylline, which represents a case of dissolution with simultaneous irreversible noninstantaneous chemical reaction. They noted that increased dissolution of 7-acetyltheophylline occurred at pH's where the conversion of 7-acetyltheophylline to theophylline had a half-life of <3 sec. If the half-life was 3–30 msec or shorter, the dissolution became dependent on the diffusion of theophylline and theophyllinate from the solid–solvent interface.

The effect on dissolution behavior from solids of a reversible noninstantaneous reaction taking place within the diffusion layer film has not been formulated. Models for gas absorption accompanied by complex chemical reactions (23–28) are available, and I propose to modify these models to predict the effect of noninstantaneous reactions on the dissolution rates of solids, especially carbon acid pharmaceuticals such as oxyphenbutazone, phenindione, anisindione, diphenadione, and chlorindione.

I do not wish to imply that all anomalies in phenylbutazone behavior are directly attributed to its slow protonation and deprotonation rate. However, some of them, especially those involving phase transfer phenomena, may in part be attributed to its unusual ionization rate characteristics.

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Use of 24-hr Urinary Excretion Data to Assess Bioavailability of Griseofulvin in Humans

Keyphrases □ Griseofulvin—bioavailability, use of 24-hr urinary excretion data, humans ■ Bioavailability—assessment using 24-hr urinary excretion data, humans

To the Editor:

Clinical failure with griseofulvin therapy is most probably caused by the slow rate and low extent of absorption and by the appreciable intrasubject and intersubject variations in the bioavailability of this antifungal antibiotic from commercial products (1–3). Accordingly, in 1967, the Department of Health, Education, and Welfare's Task Force on Prescription Drugs recommended to the Food and Drug Administration that bioavailability studies be initiated on griseofulvin products (4). However, to date, bioavailability information only exists for drug products manufactured by the three major producers (5–10). To our knowledge, the literature is devoid of such information for any of the numerous generic products currently on the market. The available data indicate that *only* 50% of an oral dose of micronized griseofulvin is absorbed from the three innovators' products, irrespective of the type of dosage form administered, and that drug absorption is quite variable (5–7, 9).

After administration of a single therapeutic dose (500–1000 mg) of micronized griseofulvin to humans, plasma antibiotic levels are low (peak level of 1–3 μg/ml) and decline to undetectable levels in approximately 72 hr (5, 6, 10). In addition, drug absorption appears to occur over 30–40 hr (5–7, 9, 10). Consequently, one of the best methods for assessing single-dose bioavailability differences in humans among various commerical griseofulvin products is a urinary excretion method in which total 6-desmethylgriseofulvin (free and glucuronide conjugate), the major urinary metabolite, is determined (7–10). This method requires quantitative urine collections for 72–96 hr after drug administration. This relatively long collection period, which is based on an average biological half-life of 11–14 hr (5, 7, 9), increases the chances for a lack of compliance to the experimental protocol on the part of subjects participating in griseofulvin bioavailability studies. This communication describes the results of attempts to determine if the bioavailability of griseofulvin could be adequately assessed from 24-hr, rather than 72- or 96-hr, total 6-desmethylgriseofulvin urinary excretion data.

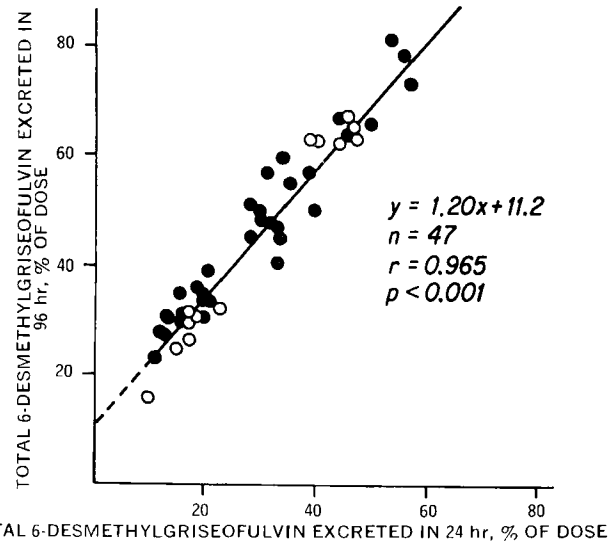

Figure 1—*Relationship between the 96- and 24-hr cumulative amounts of total 6-desmethylgriseofulvin excreted in the urine after administration of a 500-mg oral dose of griseofulvin in various dosage forms to humans. Key:* ●, *data from Refs. 9 and 11; and* ○, *data from Ref. 7.*

Figure 1 shows a plot of 96-hr *versus* 24-hr cumulative urinary major metabolite excretion data obtained after single-dose (500 mg) administration of various griseofulvin dosage forms to human volunteers. The 33 closed circles on this correlation plot represent the results obtained in our bioavailability studies after administration of micronized drug as an aqueous suspension, corn oil-in-water emulsion, and two different commercial tablets[1,2] to five fasting subjects (9) and as capsules of the anhydrous and chloroform solvate forms of the drug to four different subjects (11). The 14 data points denoted by open circles were obtained from a study conducted in two subjects (7) in which the absorption of griseofulvin from experimental tablet and capsule dosage forms of drug–polyethylene glycol 6000 coprecipitates was compared with that from a commercial microcrystalline drug tablet[1].

As indicated by the correlation coefficient and the level of significance (Fig. 1), excellent agreement was found to exist between the 24- and 96-hr cumulative urinary metabolite excretion data. Moreover, the overall variability in excretion was similar at the two collection intervals (*i.e.*, the pooled coefficients of variation were 27.9% at 24 hr and 20.4% at 96 hr). The excretion data obtained by Kabasakalian *et al.* (8) after single-dose administration to one subject of various griseofulvin doses and experimental tablet formulations under several study conditions (33 trials) were excluded from the correlation plot (Fig. 1) solely on the basis that only free 6-desmethylgriseofulvin was recovered from the urine whereas there is substantial evidence that the glucuronide conjugate of this metabolite is also excreted in human urine to an appreciable extent (7, 9–11). The fact that consideration of their findings together with those previously cited (Fig. 1) also resulted in a highly significant correlation[3] ($y = 1.39x + 3.31$, $n = 80$, $r = 0.970$, and $p < 0.001$) suggests that perhaps the composition of urine in the 24–96-hr period is constant. These correlations are encouraging since they provide the basis for possible utilization of 24-hr cumulative total 6-desmethylgriseofulvin excretion data as

[1] Grifulvin V, McNeil Lab.
[2] Fulvicin-U/F, Schering Corp.
[3] The cumulative excretion data of Kabasakalian *et al.* (8) tended to plateau in 72 hr. Estimates of 96-hr excretion values were only approximately 2–5% higher than the reported 72-hr values.

an index of griseofulvin bioavailability in humans. Recently, Greenblatt *et al.* (12) suggested that the single-dose bioavailability of digoxin preparations could be assessed in humans from 1-day, rather than 6-day, urinary excretion data.

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Epicillin and Ampicillin: Crystalline Modifications and Their Physicochemical Differences

Keyphrases □ Epicillin—crystalline modifications, physicochemical properties □ Ampicillin—crystalline modifications, physicochemical properties □ Antibiotics—epicillin and ampicillin, crystalline modifications and their physicochemical differences

To the Editor:

Epicillin (I), a new broad spectrum semisynthetic penicillin, is formed by the coupling of 6-aminopenicillanic acid with D-α-amino-1,4-cyclohexadiene-1-acetic acid (1). Its bacteriology (2, 3), pharmacology, and clinical applications have been reported (4–8). Epicillin is similar to ampicillin (II) in antimicrobial activity, although epicillin may exert a significantly greater bactericidal activity *in vitro* against certain Gram-negative bacteria than does ampicillin (2).

Although the molecular structure of epicillin differs only slightly from that of ampicillin, the difference being confined to the side chain, some physicochemical properties of epicillin differ significantly from those of ampicillin; these differences are reported herein.

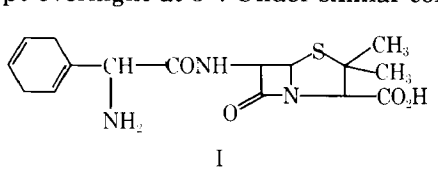
Table I—X-Ray Powder Diffraction Patterns of Epicillin Anhydrate and Epicillin Trihydrate

Epicillin Anhydrate		Epicillin Trihydrate	
d^a	$I/I_1{}^b$	d	I/I_1
11.3	0.17	11.8	0.21
10.7	0.49	8.0	0.11
10.2	0.09	7.8	0.14
6.55	0.06	7.2	0.88
6.15	0.25	5.82	1.00
5.92	0.16	5.44	0.21
5.62	0.22	5.13	0.25
5.48	0.80	5.07	0.21
5.10	0.28	4.90	0.89
4.57	0.04	4.56	0.56
4.41	0.24	4.47	0.28
4.32	0.11	4.40	0.24
4.22	0.20	4.10	0.36
4.11	0.45	4.01	0.41
3.95	0.62	3.95	0.21
3.85	1.00	3.82	0.50
3.75	0.12	3.76	0.59
3.51	0.14	3.60	0.17
3.45	0.12	3.48	0.32
3.40	0.24	3.45	0.52
3.35	0.12	3.32	0.61
3.13	0.01	3.21	0.19
3.07	0.38	3.17	0.15
2.96	0.12	3.08	0.62
2.90	0.12	3.03	0.29
2.85	0.09	2.97	0.24
2.81	0.12	2.91	0.14
2.76	0.11	2.80	0.34
2.69	0.11	2.74	0.18
2.62	0.21	2.69	0.18
2.58	0.12	2.66	0.16
2.48	0.11	2.60	0.21
2.41	0.05	2.58	0.25
2.30	0.11	2.51	0.20
2.25	0.19	2.26	0.23

[a] Interplanar spacing in angstrom unit. [b] Relative intensity visual estimation, radiation; Cu/Ni (Norelco, model IC 2000, Philips Electronic Instruments, Mount Vernon, N.Y.).

At room temperature (25°), epicillin trihydrate is about four times more soluble in water than is the anhydrate, 17.8 and 4.1 mg/ml, respectively. In contrast, ampicillin trihydrate is less soluble than is the anhydrate (9, 10), 7 and 10 mg/ml, respectively. In a study of ampicillin solubility at different temperatures, the solubility curves for the anhydrous and trihydrate forms intersected at about 50°, the transition temperature of these two crystalline forms (11); in a similar study, the transition temperature was 42° (10). No clearcut transition temperature is obtainable for the epicillin anhydrate–epicillin trihydrate system.

There is no evidence that epicillin anhydrate is converted to the trihydrate in an acidic slurry, even when kept overnight at 5°. Under similar conditions,



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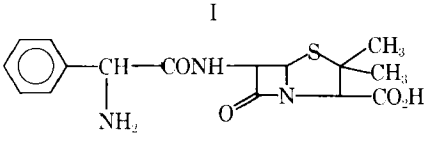

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3.40	0.24	3.45	0.52
3.35	0.12	3.32	0.61
3.13	0.01	3.21	0.19
3.07	0.38	3.17	0.15
2.96	0.12	3.08	0.62
2.90	0.12	3.03	0.29
2.85	0.09	2.97	0.24
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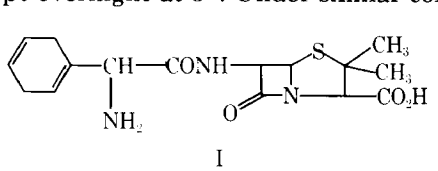

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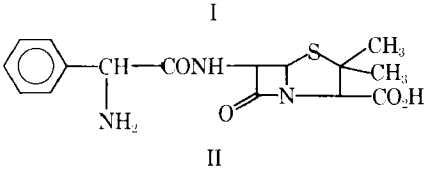

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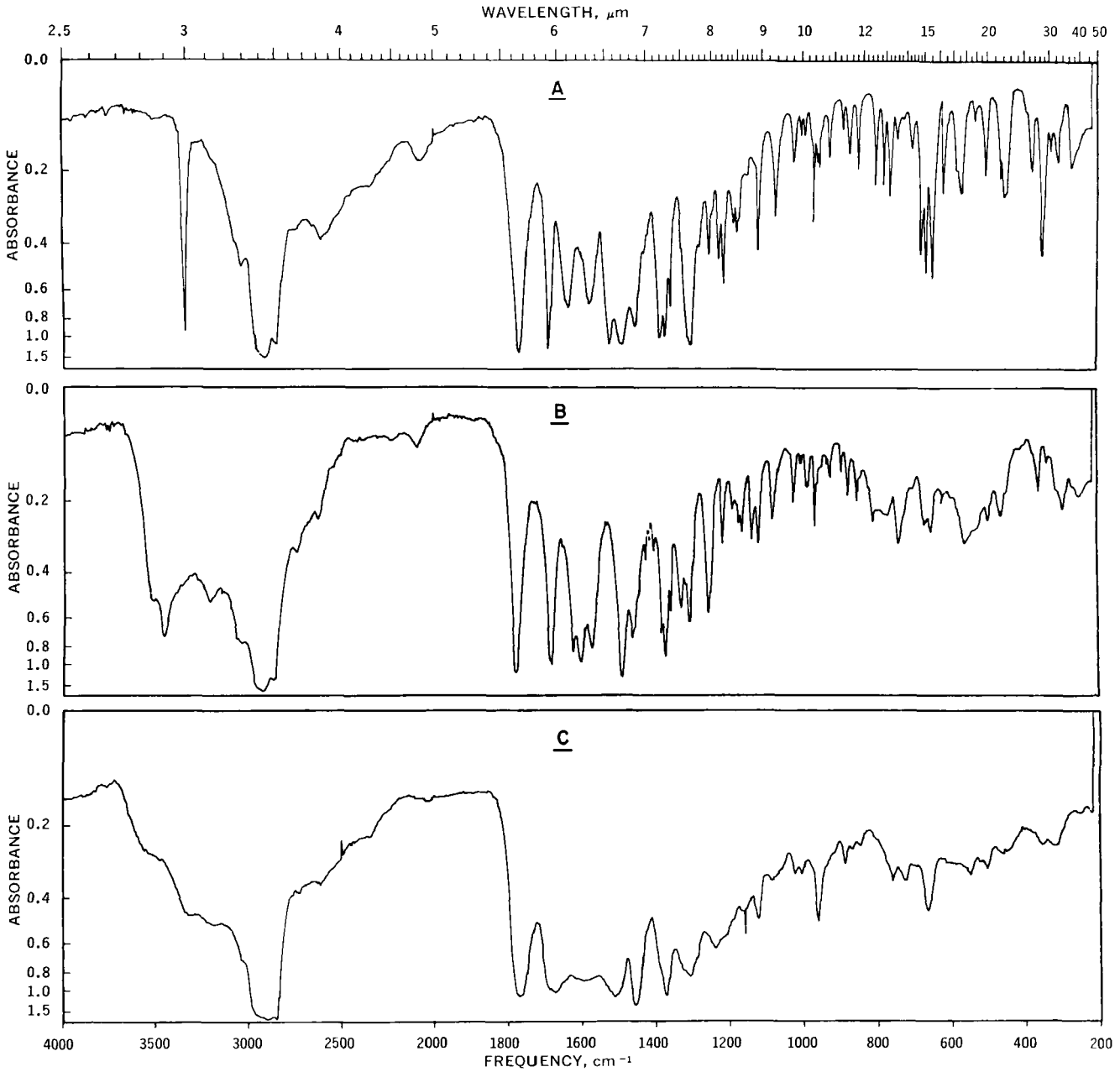

Figure 1—*IR spectra of polymorphic forms of epicillin: Key: A, anhydrate; B, trihydrate; and C, amorphous form.*

however, ampicillin anhydrate is converted to the crystalline needle-like trihydrate (12, 13) within 12 hr. Conversely, an aqueous slurry of epicillin trihydrate is converted to the anhydrate at ambient temperatures, and the conversion is accelerated if the slurry is heated to about 40°. In the case of ampicillin trihydrate, an aqueous slurry can be converted to the anhydrate only at 80–100° (11). Evidently, ampicillin trihydrate is more stable than the anhydrate, whereas the reverse is true for epicillin.

Epicillin is isolated more easily in the anhydrous form than as the trihydrate, although pure crystals of the latter can be obtained under controlled conditions, *i.e.*, acid–base recrystallization from an acidic aqueous solution when the pH is raised to between 3.5 and 4.5, preferably at 0–5°. If the crystals are dried without heating and under reduced pressure, their trihydrate form is maintained. The anhydrate is obtained when the temperature of an aqueous suspension of epicillin trihydrate is raised to 25–40° or higher or, alternatively, when the pH of an aqueous alkaline solution of epicillin is adjusted to about 5 by the addition of acid.

Although the existence of ampicillin monohydrate has been reported (14), crystalline epicillin monohydrate has not yet been isolated.

Prolonged drying of epicillin trihydrate at 50° under reduced pressure, in the presence of a dehydrating agent, results in formation of the amorphous form. This form of epicillin may be converted to either the anhydrous or trihydrate form by recrystallization.

Crystals of epicillin anhydrate are rectangular or plate-like, whereas those of the trihydrate are rod

shaped or needle-like. The X-ray powder diffraction patterns of the anhydrous and trihydrate forms of epicillin are distinguishable (Table I). The X-ray diffraction patterns of epicillin and ampicillin and of epicillin trihydrate and ampicillin trihydrate, respectively, are similar. The amorphous form of epicillin shows no X-ray diffraction pattern.

Epicillin trihydrate contains 13.3% of total volatiles or water, as determined by thermal gravimetric or Karl–Fischer analysis. Differential thermal analysis of the trihydrate shows characteristic endotherms at 92, 102, and 120° and an exotherm peak at about 213–218°; the anhydrate shows only an exotherm peak at about 215–220°. Differential thermal analysis of the amorphous form of epicillin shows only one inflection near 170°. Thermograms of ampicillin and ampicillin trihydrate resemble those of epicillin and epicillin trihydrate, respectively[1].

The solid polymorphic forms of epicillin may also be distinguished by their IR spectra (Fig. 1). Spectra of epicillin anhydrate (A) and epicillin trihydrate (B) differ in the NH and OH stretching regions. A very sharp isolated band at 2.99 μm is characteristic of the anhydrous form, whereas less sharp peaks at about 2.85 and 2.90 μm are found for the trihydrate. The anhydrate also shows distinct bands at 5.65, 5.92, 6.12, 6.32, 6.55, 6.71, 6.86, 7.20, 7.30, 7.37, 7.68, and 7.99 μm; the trihydrate shows corresponding bands at 5.64, 5.95, 6.15, 6.25, 6.36, 6.71, 6.85, 7.02, 7.08, 7.15, 7.25, 7.30, 7.52, 7.68, and 7.99 μm. There are, in addition, striking differences between the two crystalline forms of epicillin evident in the fingerprint region of the IR spectrum (beyond 8 μm). Spectrum C shows the typically poor resolution characteristic of the amorphous form of epicillin. The IR spectra of ampicillin and its hydrated forms were reported previously (11, 14).

Neither the trihydrate nor the anhydrate of epicillin is hygroscopic at 30° when exposed to an atmosphere of 85% relative humidity for 24 hr or to 100% humidity for 2 hr. The amorphous form of epicillin, however, increases in moisture content by 5–8% within 2 hr in air at ambient temperature.

When the epicillin trihydrate is heated *in vacuo* (dehydrated) at 50° for 4 days, the crystal lattice is ruptured and the compound is converted to the amorphous form; under these circumstances, loss of activity (11%) occurs. Similarly, ampicillin trihydrate and monohydrate showed losses in potency after rupture of their crystal lattices (9).

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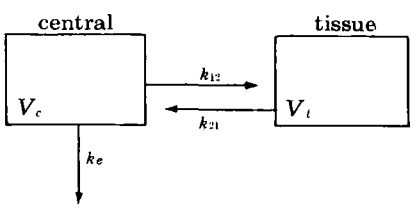
Changes in Pharmacokinetics of Cefazolin due to Stress

Keyphrases □ Cefazolin—pharmacokinetics, changes due to stress □ Pharmacokinetics—cefazolin, changes due to stress □ Stress—effects on pharmacokinetics of drugs, cefazolin

To the Editor:

Several investigators (1–4) have published cefazolin blood level data after intravenous infusion or bolus dose injection. In every case the pharmacokinetics of this drug have been described by a one-compartment model. Inspection of the data indicates that the decline in blood levels is a biexponential process and that the pharmacokinetics of this drug are more appropriately described by at least a two-compartment model, as was confirmed in our laboratories using normal human volunteers.

A 1-g dose of the drug was administered intravenously and serum samples were analyzed for cefazolin concentration as a function of time. The serum samples were assayed by absorbing 20 μl of suitably diluted serum on filter paper disks and placing the disks in bacto antibiotic medium No. 1 agar plates. Nine to 10 ml of agar, previously seeded with *Bacil-*



Scheme I

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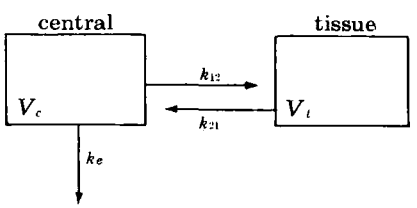

Scheme I

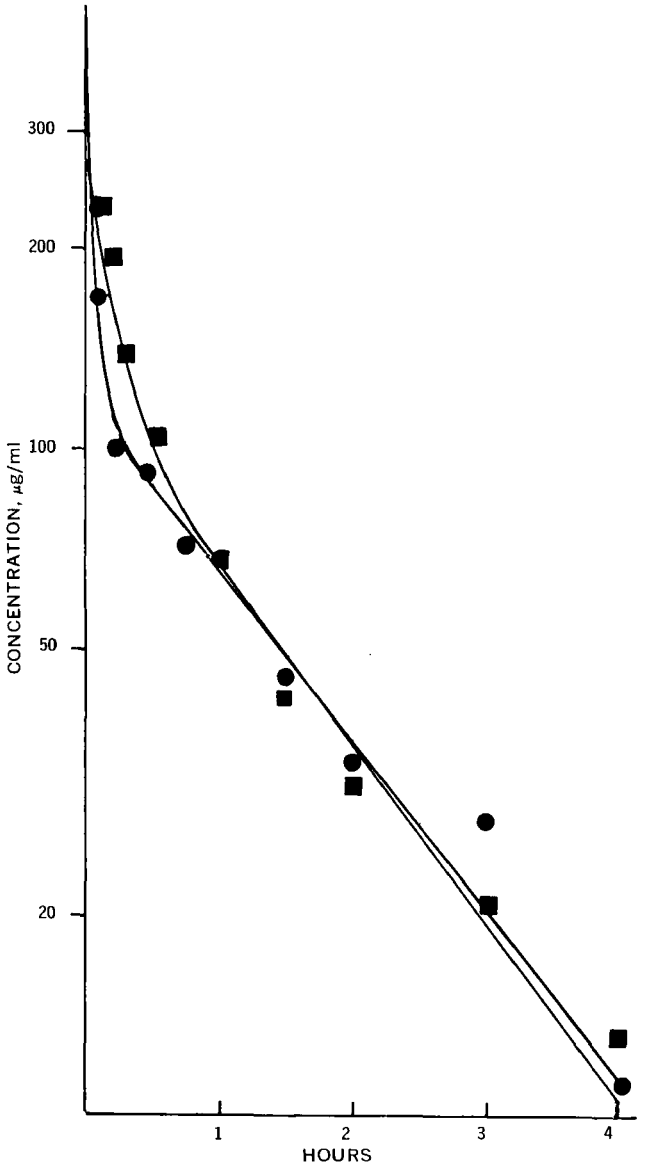

Figure 1—*Cefazolin serum concentrations. Key:* ■, *mean data, four subjects; and* ●, *Subject BEV.*

lus subtilis spores, was used in each 100-mm plate. The plates were incubated for 16–18 hr at 37°. Zones of inhibition were measured and compared to similarly assayed standards prepared in pooled human serum.

The data were fit to a two-compartment open model (Scheme I) with the aid of a nonlinear curve-fitting program (5). For such a model, the serum concentration at any time can be expressed by:

$$C_p = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

where:

$$C_p^0 = A + B \qquad \text{(Eq. 2)}$$

$$A = \frac{C_p^0(k_{21} - \alpha)}{\beta - \alpha} \qquad \text{(Eq. 3)}$$

$$B = C_p^0\frac{(k_{21} - \beta)}{\alpha - \beta} \qquad \text{(Eq. 4)}$$

and k_{12} and k_{21} are first-order distribution constants

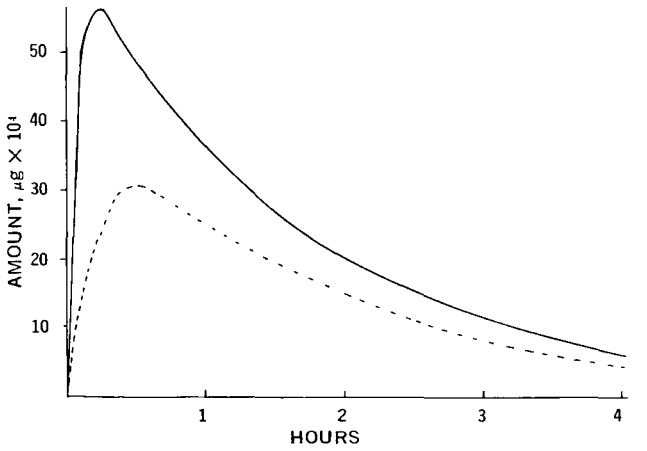

Figure 2—*Cefazolin tissue levels. Key: - - - -, mean data, four subjects; and ——, Subject BEV.*

and k_e is a first-order elimination rate constant [$(\alpha\beta)' = k_{21}k_e$, $\alpha + \beta = k_e + k_{12} + k_{21}$]. Then:

$$(V_d)_{ss} = V_c\left(\frac{k_{21} + k_{12}}{k_{21}}\right) = V_c + V_t \qquad \text{(Eq. 5)}$$

where V_t = volume of the tissue compartment, and V_c = volume of the central compartment = dose/(A + B). Then:

$$(V_d)_\beta = \frac{k_e V_c}{\beta} \qquad \text{(Eq. 6)}$$

$$V_{d(\text{extrap})} = \frac{\text{dose}}{B} \qquad \text{(Eq. 7)}$$

An excellent fit to the data was obtained as can be seen in Fig. 1. The correlation coefficient was never less than 0.996. Analysis of the available published data (1–4) gives pharmacokinetic parameters that are similar to the results of this study (Table I).

One patient (BEV), a white 27-year-old female weighing 56.3 kg, fainted upon drug administration and when blood samples were removed. It can be seen from Table I and Fig. 1 that this patient's data

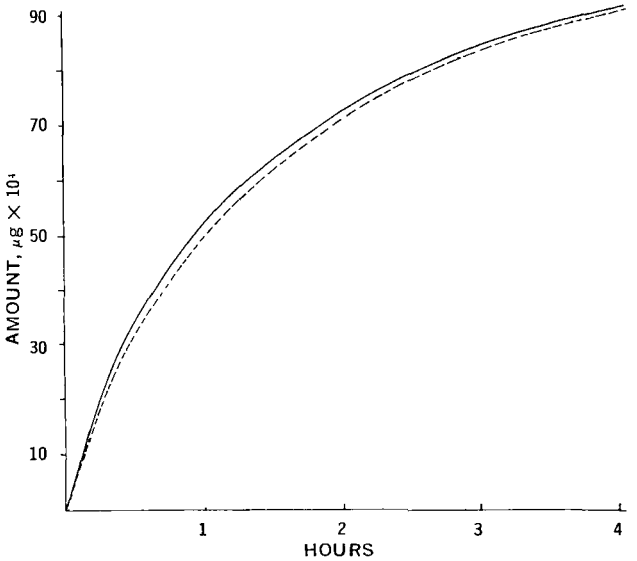

Figure 3—*Cefazolin excretion. Key: - - - -, mean data, four subjects; and ——, Subject BEV.*

Table I—Pharmacokinetic Parameters of Cefazolin[a]

Parameter	Present Study[b]	Literature Values[c]	Subject BEV
α, hr^{-1}	4.832 ± 1.784	3.451 ± 1.489	18.841
β, hr^{-1}	0.573 ± 0.116	0.434 ± 0.083	0.586
k_{12}, hr^{-1}	1.961 ± 0.943	1.291 ± 0.674	11.933
k_{21}, hr^{-1}	2.148 ± 0.914	1.683 ± 0.846	5.481
k_e, hr^{-1}	1.299 ± 0.226	0.907 ± 0.266	2.013
$(t_{1/2})_\alpha$, hr	0.167 ± 0.082	0.245 ± 0.142	0.037
$(t_{1/2})_\beta$, hr	1.248 ± 0.255	1.642 ± 0.322	1.184
V_c, ml	3,482 ± 357	4072 ± 1199	2260
$(V_d)_{ss}$, ml	6,791 ± 1873	6918 ± 1014	7181
$(V_d)_\beta$, ml	8,145 ± 2609	8082 ± 1157	7769
$(V_d)_{(extrap)}$, ml	10,113 ± 3755	9757 ± 670	8429
Clearance, ml/hr			
$(V_d)_\beta \times \beta$	4667	3508	4552
$V_c \times k_e$	4523	3693	4549

[a] Mean ± *SD*. [b] Mean of four subjects. [c] Data obtained from Refs. 1–4.

were significantly different from those of the other patients in this study. The value for α was nearly four times higher than normal, while the volume of the central compartment was reduced by approximately 50% to a value corresponding to the plasma volume for a female of BEV's weight and age (6), indicating that distribution to the tissue compartment was increased. This should be expected since fainting was probably due to decreased blood pressure or flow in the brain resulting from peripheral vasodilation.

If the muscle, a tissue associated with only moderate blood flow, is in the tissue compartment, peripheral vasodilation would result in a faster rate of drug distribution. One would not expect $(V_d)_{ss}$ to change under these circumstances, since this parameter represents the sum of the central and tissue compartment volumes ($V_c + V_t$). It can be seen from Table I that $(V_d)_{ss}$ for BEV was essentially identical to that of the other subjects in this study as well as to the mean values obtained from the literature. Similarly, peripheral vasodilation would not affect the overall elimination of cefazolin as is seen by the almost identical values for β. In addition, the total body clearance, which for this drug also represents renal clearance since the drug is virtually nonmetabolized, was not changed between BEV and the other patients in this study (Table I).

A theoretical plot of cefazolin tissue levels and the cumulative amount excreted into the urine, based on the data of Table I, is shown in Figs. 2 and 3. The tissue levels rose rapidly and peaked significantly sooner for BEV than for the other patients. Differences in urinary excretion, however, were not considered significant.

In terms of antibiotic therapy, the differences in pharmacokinetic parameters induced by this type of stress is probably not significant, because in actual therapy cefazolin must be administered in a constant infusion or multiple-dose regimen. This study illustrates that changes in drug distribution can occur in the clinical situation. These changes would be significant when therapeutic tissue levels must be obtained rapidly and the drug has a narrow therapeutic index, *i.e.*, in the treatment of cardiac arrhythmias. In these cases, the patient is under a stress situation and the clinician is advised to be aware of potential adverse effects due to drug "redistribution" as the patient's condition improves.

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REVIEWS

Strategy of Drug Design: A Molecular Guide to Biological Activity. By W. P. PURCELL, G. E. BASS, and J. M. CLAYTON. Wiley-Interscience, Wiley, New York, N.Y., 1973. 193 pp. 14.5 × 22.5 cm. Price $9.95.

Comprehensively yet concisely written in a step-by-step manner, this is a very useful book to introduce students, medicinal chemists, and drug designers to the structure–activity relationship study from a mathematical approach. The book consists of six chapters and four appendixes, with an author index and a subject index.

The introductory chapter presents a historical background of the development of various mathematical models. This is followed by three chapters on linear free energy related (extrathermodynamic) models, wherein the Hammett equation, Hansch calculations, Free–Wilson model, among others, are illustrated to correlate physicochemical properties (hydrophobic, steric, electronic, *etc.*) of a given molecule with its biological activity. Two following chapters on *de novo* model present a statistical approach for tabulating and ranking substituent group contributions to biological activity.

While correlations between calculated and observed biological activities in a closely related series of compounds are sometimes successful, the mathematical approach has presently found its usefulness mainly in correlating *in vitro* biological activities (enzyme inhibitory action, for example) and, in a limited extent, predicting semiquantitative and largely qualitative structure–activity relationships. The reason, as the authors wisely point out, is: "... that this approach does not represent a panacea to drug design and that biological systems are not ready to lie quietly while we dissect and probe with neat, mathematical models and laws of physics."

Although a molecule may exert its activity as such *in vitro*, it often undergoes various metabolic changes such as hydrolysis, hydroxylation, cyclization, esterification, reduction, oxidation, and/or ring cleavage, to name just a few. Furthermore, there are factors which can influence metabolism of a given compound or drug, *e.g.*, animal species, strain, age, sex, size of dose, route of administration, presence of other foreign compounds, temperature, time of day, and diet, which are rather difficult to consider and gather in a few equations. The authors very aptly review the efforts made to bring some order to a complex sequence of events by illustrating the nine hydrophobic, eight steric, 54 electronic, and eight miscellaneous parameters for the use in the linear free energy related model. However, for *in vivo* studies, these factors or parameters may not be additive.

Although the development of a few equations with some interesting correlations of experimental results is a far cry from "Drug Design," the mathematical approach is nevertheless a meaningful one which can be used and continuously developed for the study of structure–activity relationships. In this regard, the authors as well as other workers in this field are to be congratulated and encouraged for being involved in this undertaking.

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The Chemistry and Biochemistry of the Sulfhydryl Groups in Amino Acids, Peptides and Proteins. By MENDEL FRIEDMAN. Pergamon Press, Elmsford, NY 10523, 1973. vii + 485 pp. 18 × 26 cm. Price $22.50.

The main focus of the book is the sulfhydryl group (SH). The scientific literature is extensively reported and critical comment is offered. Division of the book is into 16 chapters, each devoted to a particular topic.

The first chapter deals with the pKa and K_a of the SH group in the aminothiol with greatest emphasis on methods used in their determination. A critical evaluation of the methods is offered.

Chapters 2–9 cover a wide variety of reactions of SH groups. Reactions of SH and other groups with transition metals are discussed in the second chapter. The affinity of SH for transition metals is related to the involvement of SH groups and metals in active sites of enzymes and in chelates.

In the next chapter the role of the SH group in protein structure is presented. Major emphasis is placed on the return of protein denatured by reduction of the disulfide bonds to the original structure by oxidation.

Reactions through which SH groups can be eliminated from protein and the products of the reactions are considered in Chapter 5. The importance of these reactions to food, wool, leather, and other technologies is illustrated.

Chapters 6–8 cover reactions through which SH groups may be modified. Chapter 6 deals with organic halides and epoxides. Chapter 7 emphasizes transfer of groups from S to N and S to S. Chapter 8 deals with the mechanisms through which simple thiols can be used to break disulfide bonds in protein. Practical application of the latter reactions are considered, for example, in relation to SH content of flour and bread quality.

Procedures for introducing thiol groups into proteins are discussed in Chapter 9. The primary areas of concern are synthesis of new cysteines, procedures used in thiolation, and methods used to protect SH groups during peptide synthesis.

The use of thiols as radioprotectives with various proposed mechanisms and conflicts is the focus of the next chapter. Consideration is given to factors such as the influence of pH and oxygen.

A number of methods for analysis for SH and S-S content of protein are included in Chapter 11.

Chapter 12 reports on the different reactivities of SH groups in proteins and the relation of enzyme activity and protein structure.

The involvement of SH groups in active sites of enzymes is considered next (Chapter 13). Major emphasis is on methods used to determine reaction mechanisms of SH enzymes.

Chapter 14 outlines several of the known biosynthesis routes for thiols. Involvement of SH in other aspects of metabolism is also discussed. Sulfhydryl groups are involved in certain cases in detoxification reactions. Mechanisms of reaction are considered.

The final chapter of the book is turned to the practical application of SH chemistry to disease and drug action.

The wide variety of topics covered in the book would make it of interest to any serious student of SH chemistry or biochemistry. Although discussion of the literature is often only fragmentary, entry into the literature is provided by extensive references. The critical evaluation of the literature provides insight into problems present in SH research and also points out conflicts which could lead to needed research.

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Frontiers in Catecholamine Research. Proceedings of the Third International Catecholamine Symposium Held at the University of Strasbourg, Strasbourg, France, May 20–25, 1973. Edited by E. USDIN and S. H. SNYDER, Pergamon Press, Elmsford, NY 10523, 1973. 1219 pp. 17.5 × 26 cm. Price $50.00.

This challenging title holds in 1219 pages eight broad topics with 210 papers which were presented at the Third International Catecholamine Symposium in May 1973. "The Impact of Monoamine Research on Drug Development" deals with mechanisms of drug action, *e.g.*, drugs affecting biosynthesis, the amine pump, intraneuronal storage, norepinephrine release, structure of the nerve terminal, receptors, and amine catabolism.

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Although a molecule may exert its activity as such *in vitro*, it often undergoes various metabolic changes such as hydrolysis, hydroxylation, cyclization, esterification, reduction, oxidation, and/or ring cleavage, to name just a few. Furthermore, there are factors which can influence metabolism of a given compound or drug, *e.g.*, animal species, strain, age, sex, size of dose, route of administration, presence of other foreign compounds, temperature, time of day, and diet, which are rather difficult to consider and gather in a few equations. The authors very aptly review the efforts made to bring some order to a complex sequence of events by illustrating the nine hydrophobic, eight steric, 54 electronic, and eight miscellaneous parameters for the use in the linear free energy related model. However, for *in vivo* studies, these factors or parameters may not be additive.

Although the development of a few equations with some interesting correlations of experimental results is a far cry from "Drug Design," the mathematical approach is nevertheless a meaningful one which can be used and continuously developed for the study of structure–activity relationships. In this regard, the authors as well as other workers in this field are to be congratulated and encouraged for being involved in this undertaking.

Reviewed by C. C. Cheng
Midwest Research Institute
Kansas City, MO 64110

The Chemistry and Biochemistry of the Sulfhydryl Groups in Amino Acids, Peptides and Proteins. By MENDEL FRIEDMAN. Pergamon Press, Elmsford, NY 10523, 1973. vii + 485 pp. 18 × 26 cm. Price $22.50.

The main focus of the book is the sulfhydryl group (SH). The scientific literature is extensively reported and critical comment is offered. Division of the book is into 16 chapters, each devoted to a particular topic.

The first chapter deals with the pKa and K_a of the SH group in the aminothiol with greatest emphasis on methods used in their determination. A critical evaluation of the methods is offered.

Chapters 2–9 cover a wide variety of reactions of SH groups. Reactions of SH and other groups with transition metals are discussed in the second chapter. The affinity of SH for transition metals is related to the involvement of SH groups and metals in active sites of enzymes and in chelates.

In the next chapter the role of the SH group in protein structure is presented. Major emphasis is placed on the return of protein denatured by reduction of the disulfide bonds to the original structure by oxidation.

Reactions through which SH groups can be eliminated from protein and the products of the reactions are considered in Chapter 5. The importance of these reactions to food, wool, leather, and other technologies is illustrated.

Chapters 6–8 cover reactions through which SH groups may be modified. Chapter 6 deals with organic halides and epoxides. Chapter 7 emphasizes transfer of groups from S to N and S to S. Chapter 8 deals with the mechanisms through which simple thiols can be used to break disulfide bonds in protein. Practical application of the latter reactions are considered, for example, in relation to SH content of flour and bread quality.

Procedures for introducing thiol groups into proteins are discussed in Chapter 9. The primary areas of concern are synthesis of new cysteines, procedures used in thiolation, and methods used to protect SH groups during peptide synthesis.

The use of thiols as radioprotectives with various proposed mechanisms and conflicts is the focus of the next chapter. Consideration is given to factors such as the influence of pH and oxygen.

A number of methods for analysis for SH and S-S content of protein are included in Chapter 11.

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The final chapter of the book is turned to the practical application of SH chemistry to disease and drug action.

The wide variety of topics covered in the book would make it of interest to any serious student of SH chemistry or biochemistry. Although discussion of the literature is often only fragmentary, entry into the literature is provided by extensive references. The critical evaluation of the literature provides insight into problems present in SH research and also points out conflicts which could lead to needed research.

Reviewed by William M. Hadley
College of Pharmacy
University of New Mexico
Albuquerque, NM 87131

Frontiers in Catecholamine Research. Proceedings of the Third International Catecholamine Symposium Held at the University of Strasbourg, Strasbourg, France, May 20–25, 1973. Edited by E. USDIN and S. H. SNYDER, Pergamon Press, Elmsford, NY 10523, 1973. 1219 pp. 17.5 × 26 cm. Price $50.00.

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"THE BUCK STOPS HERE!"

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Many feel that the pendulum has swung much too far, with the result that damages awarded are excessive, liability findings in some cases are unjustified, and many physicians have resorted to practicing "defensive medicine" in an effort to protect themselves. Be this as it may, the fact remains that medical malpractice lawsuits have become a major concern in the medical field.

Except perhaps for attorneys who specialize in food and drug law, relatively few people in the area of pharmaceutical production and distribution are even aware that a somewhat comparable situation potentially exists with respect to liability relative to the manufacture and distribution of pharmaceutical products.

To date, most action on the part of regulatory agencies is directed either at a specific product or a corporation. That is to say, a particular product is alleged to be adulterated or misbranded and is condemned or seized; or in other cases, a specific corporation is found to be in violation of good manufacturing practices and may be forced to close down.

For some time, however, questions have been asked as to whether this line of enforcement is either adequate or effective. Comments have been made to the effect that a certain major corporation when found guilty pays its $1000 fine and the next day resumes its multimillion dollar business with no real effort to correct the situation about which court action was initiated. In other instances, it has been felt that those responsible for supplying the public with pure food and with safe and effective drugs have not exercised sufficient concern, with the result that substantial injury—and at times, even death—have directly resulted. This experience has prompted thoughtful observers to suggest that enforcement-related action in more cases should be against *persons* within the corporation, and that such court action might even take the form of prosecution on the basis of criminal liability.

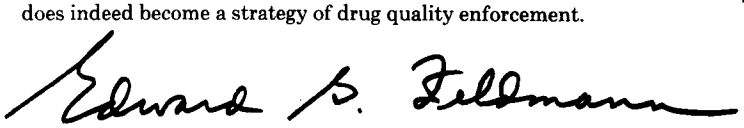
A case is now before the United States Supreme Court, *United States v. Park*, in which the president of a major food company has been held to be criminally responsible for not seeing that necessary steps were taken to prevent rodents from contaminating food stored in one of his corporation's warehouses. The outcome of this test case will undoubtedly have a significant impact on the degree of personal liability individuals may be held to have in the case of pharmaceutical manufacturing and distribution.

Indeed, it is reported that proposed revisions in good manufacturing practice (GMP) regulations for large-volume parenterals will place direct and specific responsibility on the designated quality assurance officer for certifying as to the qualifications of the manufacturing plant, all processes and procedures in the production of the article, and the suitability of the container and its enclosure before the product is released. Undoubtedly, many questions can be raised as to who *specifically* should bear the responsibility if criminal liability action is brought as the result of some defective product.

Although he did not originate it, Harry Truman made famous the comment "the buck stops here!" But again, where is "here"? Is it the person or persons who work out the formulation, or is it those who prepare the formulation, or is it those who are responsible for assuring the quality of the formulation, or is it those in some high strata of company management? These are questions which eventually the courts will decide.

In the meantime, it appears to us that the only prudent course of action for everyone involved in drug production is to perform in a manner as if such liability does rest directly on one's personal shoulders.

But, even putting the issue of personal liability aside, isn't such an attitude what we ought to expect from those who have assumed responsibility for producing and supplying our drug products? At times, it seems that a good deal of lip service is given to the term "a reputable manufacturer." Saying "we are sorry" after having a recall for a defective drug product really is not sufficient. A reputable drug manufacturer, in the true sense, is one which, to the extent possible, takes the necessary actions to anticipate and prevent the defect from occurring. It seems to us that companies as well as individual employees and corporation executives who perform with this philosophy will have little cause for concern in the event that criminal liability does indeed become a strategy of drug quality enforcement.



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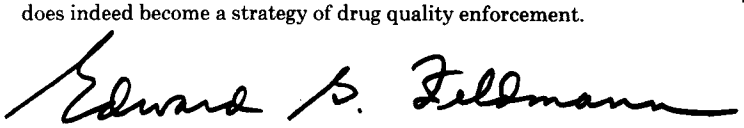

1975

May 19–21
National Research Council, Annual Scientific Meeting, National Academy of Sciences Auditorium, Washington, D.C.

May 22–24
International Congress on Patient Counseling, Amsterdam, The Netherlands (Contact K. Ellison Davis, Excerpta Medica Foundation, P.O. Box 1126, Jan van Gaelnstr., 335, Amsterdam, The Netherlands)

May 26–30
International Conference on Prostaglandins, Florence, Italy (Contact Dr. G. C. Folco, Scientific Secretary, Institute of Pharmacology and Pharmacognosy, University of Milan, via Andrea Del Sarto, 21-20129, Milan, Italy)

June 2–6
American Peptide Symposium, Delmonico Hotel, New York, N.Y. (Contact New York Academy of Sciences, 2 East 63rd St., New York, NY 10021)

June 12–13
USP Open Conference on Development of Compendial Standards, Statler-Hilton Hotel, Washington, D.C. (Contact Robert H. Henry, 301-881-0666)

June 15–18
Society for Economic Botany, Annual Meeting, University of Maryland, Baltimore, Md. (Contact Dr. Ralph N. Blomster, School of Pharmacy, University of Maryland, 636 W. Lombard St., Baltimore, MD 21201)

June 15–19
American Medical Association, Annual Meeting, Atlantic City, N.J. (Contact Dr. E. B. Howard, 535 N. Dearborn St., Chicago, IL 60610)

June 16–18
International Symposium on Mass Spectrometry in Biochemistry and Medicine, Alghero, Sardinia, Italy (Contact A. Frigerio, Istituto di Ricerche Farmacologiche, Mario Negri, via Evitrea 62, 20157, Milan, Italy)

June 16–20
International Symposium on Molecular Spectroscopy, Ohio State University, Columbus, Ohio (Contact K. M. Rao, Department of Physics, Ohio State University, Columbus, OH 43210)

June 18–20
Drug Information Association, Annual Meeting, Sheraton Boston Hotel, Boston, Mass.

June 22–26
ACS National Organic Chemistry Symposium, Division of Organic Chemistry, Colorado State University, Fort Collins, Colo. (Contact C. D. Gutsche, Department of Chemistry, Washington University, St. Louis, MO 63130)

June 27
Parenteral Drug Association, Bellevue Stratford Hotel, Philadelphia, Pa. (Contact Hubert Boyden, PDA, Broad & Chestnut Sts., Philadelphia, PA 19107)

July 6–11
IUPAC International Congress, Jerusalem, Israel (Contact Organizing Committee, P.O. Box 983, Jerusalem, Israel)

July 6–13
American Association of Colleges of Pharmacy, Annual Meeting, Concord Hotel, Kiamesha Lake, N.Y.

July 13–18
International Congress on Clinical Chemistry, Toronto, Ontario, Canada (Contact Dr. F. H. Sims, Women's College Hospital, Toronto, Ontario, Canada)

July 17–21
American Society for Pharmacology and Experimental Therapeutics, Annual Meeting, University of California, Davis, Calif.

July 22–August 2
American Society of Pharmacognosy, Joint Meeting with Die Gesellschaft für Arzneipflanzenforschung, University of Connecticut, Storrs, Conn. (Contact Ana Rother, School of Pharmacy, University of Connecticut, Storrs, Conn.)

August 11–15
Gordon Research Conference, Medicinal Chemistry, Colby Women's College, New London, N.H. (Contact Dr. Alexander M. Cruickshank, Gordon Research Conferences, University of Rhode Island, Kingston, RI 02881)

August 24–29
American Chemical Society, National Meeting, Chicago, Ill. (Contact A. T. Winstead, American Chemical Society, 1155 16th St., N.W., Washington, DC 20036)

September 1–5
International Congress of Pharmaceutical Sciences, Dublin, Ireland (Contact Congress Secretary, 35th International Congress of Pharmaceutical Sciences, 44 Northumberland Road, Ballsbridge, Dublin 4, Ireland)

September 7–10
AIChE National Meeting, Sheraton Hotel, Boston, Mass. (Contact Kenneth A. Smith, Department of Chemical Engineering, MIT, Cambridge, MA 02139)

September 7–12
British Pharmaceutical Conference, Norwich, England

September 8–13
International Conference of Women Engineers and Scientists, Cracow, Poland (Contact L. I. Pickup, 15284 Oak Dr., Renton, WA 98055)

September 22–24
Symposium on Assay of Drugs and Other Trace Substances in Biological Fluids, University of Surrey, Guildford, U.K. (Contact Dr. E. Reid, Wolfson Bioanalytical Centre, University of Surrey, Guildford GU2 5XH, U.K.)

October 1–5
American College of Apothecaries, Annual Meeting, Four Seasons Sheraton, Toronto, Ontario, Canada

October 6–8
ASTM Committee E-19 on Chromatography, Annual Meeting, Sheraton-Houston, Houston, Tex. (Contact Mr. Jack Stamm, Instrument Products Division, Photo Products Department, Du Pont Co., Wilmington, DE 19898)

October 13–16
Association of Official Analytical Chemists, Annual Meeting, Marriott Hotel, Twin Bridges, Washington, D.C. (Contact L. G. Ensminger, Association of Official Analytical Chemists, Box 540 Benjamin Franklin Station, Washington, DC 20044)

October 17
APhA Academy of Pharmaceutical Sciences, Annual Eastern Regional Meeting, Governor Morris Inn, Morristown, N.J. (Contact Dr. Daniel Mufson, USV Pharmaceutical Corp., 1 Scarsdale Road, Tuckahoe, NY 10707)

November 2–6
Society for Neuroscience, Annual Meeting, New York, N.Y. (Contact Neuroscience Meeting Headquarters, Room 200 A, Lee Building, 9650 Rockville Pike, Bethesda, MD 20014)

November 16–20
American Public Health Association, Annual Meeting, Chicago, Ill. (Contact William H. McBeath, APHA, 1015 18th St., N.W., Washington, DC 20036)

November 16–20
APhA Academy of Pharmaceutical Sciences, National Meeting, Marriott Motor Hotel, Atlanta, Ga.

November 21
Eastern Analytical Symposium, Statler-Hilton Hotel, New York, N.Y. (Contact Webster R. Hudgins, Allied Chemical, P.O. Box 1021R CRL, Morristown, NJ 07960)

November 29–December 4
American Medical Association, Clinical Meeting, Honolulu, Hawaii (Contact James H. Sammons, 535 N. Dearborn St., Chicago, IL 60610)

Items to be considered for inclusion or corrections should be addressed to:

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Journal of Pharmaceutical Sciences

MAY 1975
VOLUME 64 NUMBER 5

REVIEW ARTICLE

Potential Value of Plants as Sources of New Antifertility Agents II *

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Keyphrases □ Antifertility agents—review of plant sources, classified by anatomical mechanism and folkloric route of administration □ Medicinal plants—sources of antifertility agents, classified by anatomical mechanism and folkloric route of administration, review □ Plant extracts—potential sources of antifertility agents, review □ Contraceptives—plants with active constituents, review □ Abortifacients—plants with active constituents, review □ Oxytocics—plants with active constituents, review □ Estrogenic plants—review of active principles

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ESTROGENIC PLANTS AND THEIR ACTIVE PRINCIPLES

Historically, a number of plants have been used as sex hormones in native medicine. The pomegranate, *Punica granatum* (Punicaceae), was a symbol of immortality, fertility, and love in Oriental religions, and its legendary powers date back to Greek and Judeo-Christian mythology (921). In Egypt, the pollen grains of the date palm, *Phoenix dactylifera* var. *samani* (Palmae), were used to induce fertility in women; while moghat, *Clossostemon brugieri* (Sterculiaceae), is used as a postpartum hot beverage (922).

Phytochemical interest in plant estrogens, however, remained relatively dormant until the 1950's. The increased interest in phytoestrogens was, according to Biggers (923), due to at least four factors:

1. The recognition that infertility in animals and humans could follow excessive ingestion of plants rich in compounds possessing estrogenic activity.
2. The known existence of "spring flush" (increased yield of improved milk) in dairy cattle ingesting certain rapidly growing grasses that contained estrogenic substances.
3. The possibility that the demonstrated improvement in carcass quality, produced by the feeding of synthetic estrogens, might also be able to be produced by the feeding of plants rich in estrogens.
4. The possibility of obtaining estrogenic substances economically from plant sources.

The occurrence of substances in plants capable of inducing animal estrus was first demonstrated by Dohrn *et al.* (924) in 1926. The first isolation of an estrogen from plants was reported by Butenandt and Jacobi (925) in 1933, when they succeeded in isolating 18 mg of estrone from 50 kg of a botanically unspecified palm kernel press cake. In the same year, Skarzynski (926) reported on the isolation of 7.5 mg of estriol from 65 kg of female willow catkins. Thirty years elapsed before any concerted efforts were made in the phytochemical investigation of these compounds. Jacobsohn *et al.* (927), in 1965, raised the question as to the validity of claims for the presence of estrogens in plants when they failed to isolate estrone from any of four geographic varieties of the African oil palm, *Elaeis guineensis* (Palmae). *E. guineensis* may or may not have been the plant investigated by Butenandt and Jacobi (925), although by deductive reasoning Bradbury and White (928) thought that it certainly must have been.

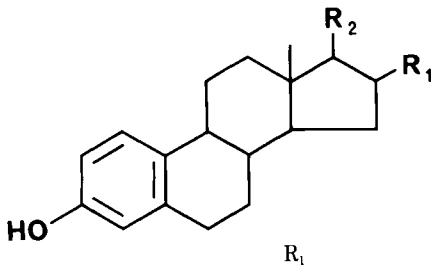
The question of the presence of estrogenic hormones in plants raised by Jacobsohn *et al.* (927) has been answered by work since 1965. The date palm, *P. dactylifera* var. *khadrawy* (Palmae), was

Editor's note: Part I of this article appeared in the April 1975 issue of the *Journal of Pharmaceutical Sciences*.

Table VI—Occurrence of Steroid Estrogens in Plants

Plant Name	Steroid Estrogen	Reference
Chenopodiaceae		
Beta vulgaris	β-Sitosterol[a] (CXLII)	1035–1037
Gramineae		
Avena sativa	Estrone (CLVIII)	1038
Oryza sativa	Estrone	1038
Triticum aestivum	Estrone	1038
Leguminosae		
Glycyrrhiza glabra	β-Sitosterol	1035–1037
	Estriol (CLVII)	1039
Phaseolus vulgaris	Estrone	1040
	Estriol	1040
	17α-Estradiol (CLV)	1040
Palmae		
Elaeis guineensis	Estrone	925
Hyphaene thebaica	Estrone	1041
Phoenix dactylifera	Estrone	929, 1042–1045
Phoenix dactylifera var. *samani*	Estrone	922
Punicaceae		
Punica granatum var. *nana*	Estrone	1046, 1047
Rosaceae		
Malus sylvestris	Estrone	1048
Salicaceae		
Salix caprea	Estrone	926
Sterculiaceae		
Clossostemon bruguieri	Estrone	922

[a] β-Sitosterol is a ubiquitous substance in plants, and no attempt has been made to document those plants in which it has been reported present. The plants indicated apparently contain a high enough percentage so that extracts exhibit estrogenic activity.



	R_1	R_2
CLV: 17α-estradiol	H	---OH
CLVI: 17β-estradiol	H	—OH
CLVII: estriol	---OH	—OH
CLVIII: estrone	H	=O

Structures of Estrogenic Sterols

demonstrated to contain estrone (CLVIII) by TLC; subsequently, the compound was isolated (929). A number of estrogenic sterols have since been reported as being derived from higher plants. A list of these compounds and their plant sources is presented in Table VI. A review of steroid estrogens in plants is available (921).

Biological and chemical interest in nonsteroidal estrogenic substances was spurred by the work of Bennetts and his coworkers (930, 931), who reported in 1946 that the serious decline in lambing rates in Australia was due to their grazing on the Dwalganup strain of subterranean clover *(Trifolium subterraneum)*, and the subsequent isolation of the isoflavone, genistein (CXCIII), from this plant (932). This compound was later demonstrated to be estrogenic (933). Since then, other isoflavones having estrogenic activity have been found in various forage plants and studies of environmental factors on the isoflavonoid content of these plants have been made (934–958). A large reduction in sperm numbers was observed on prolonged grazing of sheep on clover pasture (959); it is not known if this activity is also due to the estrogenicity of clover pastures. A list of estrogenic and potential estrogenic isoflavones and their sources is presented in Table VII.

In addition to the steroids and isoflavones, another group of chemical compounds found in forage plants that have estrogenic activity are the coumestans, having the skeletal structure of 6*H*-benzofuran(3,2-*c*)benzopyran-6-one. The best known compound in

Table VII—Plants Containing Isoflavonoids

Plant Name	Isoflavonoid	Reference
Amaranthaceae		
Iresine celosioides	Tlatlancuayin (CCXLVIII)	1049
Gramineae		
Bromus mollis	Genistein (CXCIII)	1050
Cynodon dactylon	Daidzein (CLIX)	1051
	Genistein	1051
Hyparrhena filipendula	Daidzein	1051
	Genistein	1051
Setaria ciliolata	Daidzein	1051
	Genistein	1051
Triticum aestivum	Genistein	1050
Iridaceae		
Belamcanda chinensis	Tectorigenin 7-*O*-glucoside (CCXXVI)	1052, 1053
Iris sp.	Irigenin 7-*O*-glucoside	1064
	Tectorigenin 7-*O*-glucoside	1064
Iris florentina	Irigenin (CCXXXIX)	1054
	Irigenin 7-*O*-glucoside (CCXL)	1055, 1056
	Irilone 4′-*O*-glucoside (CCXXVIII)	1057
	Irisflorentin (CCXLIX)	1054
	Irisolone (CCXXX)	1054
	Irisolone 4′-*O*-bioside (CCXXIX)	1057

Table VII—(*Continued*)

Plant Name	Isoflavonoid	Reference
	Iristectorigenin B (CCXXXVI)	1054
Iris germanica	Homotectoridin (CCXVII)	1058
	Irigenin 7-*O*-glucoside	1056
	Irilone (CCXXVII)	1059
	Tectorigenin 7-*O*-glucoside	1058
Iris nepalensis	Irisolidone (CCXXXV)	1060
	Irisolone	1061
Iris pallida	Irigenin 7-*O*-glucoside	1056
Iris tectorum	Irigenin 7-*O*-glucoside	1062
	Tectorigenin 7-*O*-glucoside	1062–1064
Leguminosae		
Adenocarpus complicatus	Genistein 7-*O*-glucoside (CXCV)	1065
Adenocarpus foliolosus	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein (CCII)	1066
Afrormosa elata	Afrormosin (CXC)	1067
Amphimas pterocarpioides	Afrormosin	1068
Andira parviflora	Biochanin A	1069
	5,7-Dihydroxy-4′-methoxyisoflavone (CCLXXXI)	1069
Astragalus austriacus	Biochanin A 7-*O*-glucoside (CCIV)	1070
Baphia nitida	Santal (CCXIII)	1071, 1072
Baptisia arachnifera	Afrormosin	1073
	Afrormosin 7-*O*-glucoside (CXCI)	1073
	Calycosin (CLXXVII)	1073
	Calycosin 7-*O*-glucoside (CLXXVIII)	1073
	Daidzein	1073
	Daidzein 7-*O*-glucoside (CLX)	1073
	Formononetin	1073
	Formononetin 7-*O*-glucoside (CLXIV)	1073
	Formononetin 7-*O*-rhamnoglucoside (CLXV)	1073
Baptisia australis	Afrormosin	1073–1075
	Afrormosin 7-*O*-glucoside	1073–1075
	Daidzein	1073, 1074
	Formononetin	1073–1075
	Formononetin 7-*O*-glucoside	1073–1075
	Genistein	1074
	Orobol (CCX)	1074
	Pseudobaptigenin (CLXXX)	1074
	Texasin (CLXXXVIII)	1073, 1076
	Texasin 7-*O*-glucoside (CLXXXIX)	1073, 1074
Baptisia bracteata	Afrormosin	1073
	Afrormosin 7-*O*-glucoside	1073
	Calycosin	1073
	Calycosin 7-*O*-glucoside	1073
	Daidzein	1073
	Daidzein 7-*O*-glucoside	1073
	Formononetin	1073
	Formononetin 7-*O*-glucoside	1073
Baptisia calycosa	Biochanin A	1073
	Biochanin A 7-*O*-rhamnoglucoside (CCV)	1073
	Calycosin	1073
	Daidzein	1073
	Formononetin	1073
	Formononetin 7-*O*-glucoside	1073
	Genistein	1073
	Genistein 7-*O*-rhamnoglucoside (CXCVIII)	1073
	Orobol	1073
	Orobol 7-*O*-rhamnoglucoside (CCXII)	1073
Baptisia cinerea	Afrormosin	1073
	Afrormosin 7-*O*-glucoside	1073
	Calycosin 7-*O*-glucoside	1073
	Daidzein	1073
	Daidzein 7-*O*-glucoside	1073
	Formononetin	1073
	Formononetin 7-*O*-glucoside	1073
	Genistein	1073
	Genistein 7-*O*-glucoside	1073
	Orobol	1073
	Orobol 7-*O*-glucoside (CCXI)	1073
Baptisia hirsuta	Biochanin A	1073
	Biochanin A 7-*O*-rhamnoglucoside	1073
	Genistein	1073
	Genistein 7-*O*-rhamnoglucoside	1073
	6-Hydroxygenistein (CCXXIII)	1073, 1077

(continued)

Table VII—(*Continued*)

Plant Name	Isoflavonoid	Reference
	6-Hydroxygenistein 7-*O*-rhamnoglucoside (CCXXIV)	1073, 1077
	Orobol	1073
	Orobol 7-*O*-rhamnoglucoside	1073
	Pseudobaptigenin	1073
	Pseudobaptigenin 7-*O*-rhamnoglucoside (CLXXXII)	1073
Baptisia lanceolata	Afrormosin	1073
	Afrormosin 7-*O*-glucoside	1073
	Calycosin	1073
	Calycosin 7-*O*-glucoside	1073
	Daidzein	1073
	Daidzein 7-*O*-glucoside	1073
	Genistein	1073
Baptisia lecontei	Afrormosin	1073
	Afrormosin 7-*O*-glucoside	1073
	Calycosin	1073, 1077
	Calycosin 7-*O*-glucoside	1073, 1077, 1078
	Calycosin 7-*O*-rhamnoglucoside (CLXXIX)	1073, 1077, 1078
	Daidzein	1073, 1078
	Daidzein 7-*O*-glucoside	1073, 1078
	Daidzein 7-*O*-rhamnoglucoside (CLXI)	1073, 1078
	Formononetin	1073, 1078
	Formononetin 7-*O*-glucoside	1073
	Genistein	1073, 1078
	Genistein 7-*O*-glucoside	1073
	Genistein 7-*O*-rhamnoglucoside	1073, 1078
	Genistein 7-*O*-rutinoside (CXCIX)	1078
	Orobol	1073, 1078
	Orobol 7-*O*-rhamnoglucoside	1073, 1078
	Pseudobaptigenin	1073, 1078
Baptisia leucantha	Orobol	1073
	Orobol 7-*O*-glucoside	1073
	Orobol 7-*O*-rhamnoglucoside	1073
Baptisia leucophaea	Afrormosin	1073
	Afrormosin 7-*O*-glucoside	1073
	Calycosin	1073
	Calycosin 7-*O*-glucoside	1073
	Daidzein	1073
	Daidzein 7-*O*-glucoside	1073
	Genistein	1073
	Genistein 7-*O*-glucoside	1073
	Orobol	1073
	Orobol 7-*O*-glucoside	1073
Baptisia megacarpa	Afrormosin	1073
	Afrormosin 7-*O*-glucoside	1073
	Afrormosin 7-*O*-rhamnoglucoside (CXCII)	1073
	Calycosin	1073
	Calycosin 7-*O*-glucoside	1073
	Calycosin 7-*O*-rhamnoglucoside	1073
	Daidzein	1073
	Daidzein 7-*O*-glucoside	1073
	Daidzein 7-*O*-rhamnoglucoside	1073
	Formononetin	1073
	Formononetin 7-*O*-glucoside	1073
	Formononetin 7-*O*-rhamnoglucoside	1073
	Genistein	1073
	Genistein 7-*O*-glucoside	1073
	Orobol	1073
Baptisia nuttalliana	Afrormosin	1073
	Afrormosin 7-*O*-glucoside	1073
	Calycosin	1073
	Daidzein	1073
	Formononetin	1073
	Formononetin 7-*O*-glucoside	1073
	Genistein	1073
	Genistein 7-*O*-glucoside	1073
	Orobol	1073
	Orobol 7-*O*-glucoside	1073
	Tectorigenin (CCXXV)	1073
	Tectorigenin 7-*O*-glucoside	1073
Baptisia perfoliata	Afrormosin	1073
	Calycosin	1073
	Daidzein	1073
	Daidzein 7-*O*-glucoside	1073
	Formononetin	1073
	Formononetin 7-*O*-glucoside	1073

Table VII—(*Continued*)

Plant Name	Isoflavonoid	Reference
	Orobol	1073
	Orobol 7-*O*-rhamnoglucoside	1073
Baptisia simplicifolia	Afrormosin	1073
	Afrormosin 7-*O*-glucoside	1073
	Afrormosin 7-*O*-rhamnoglucoside	1073
	Calycosin	1073
	Calycosin 7-*O*-glucoside	1073
	Calycosin 7-*O*-rhamnoglucoside	1073
	Daidzein	1073
	Daidzein 7-*O*-glucoside	1073
	Daidzein 7-*O*-rhamnoglucoside	1073
	Formononetin	1073
	Formononetin 7-*O*-glucoside	1073
	Formononetin 7-*O*-rhamnoglucoside	1073
Baptisia sphaerocarpa	Biochanin A	1073
	Biochanin A 7-*O*-rhamnoglucoside	1073
	Formononetin	1073
	Formononetin 7-*O*-glucoside	1073
	Genistein 7-*O*-rhamnoglucoside	1073
	Genistein 7-*O*-rutinoside	1073
	Orobol 7-*O*-rhamnoglucoside	1073
Baptisia tinctoria	Afrormosin	1073
	Afrormosin 7-*O*-glucoside	1073
	Biochanin A	1073
	Biochanin A 7-*O*-rhamnoglucoside	1073
	Genistein	1073
	Genistein 7-*O*-rhamnoglucoside	1073
	Orobol	1073
	Orobol 7-*O*-rhamnoglucoside	1073
	Pseudobaptigenin	1073
	Pseudobaptigenin 7-*O*-glucoside (CLXXXI)	1079–1081
	Pseudobaptigenin 7-*O*-rhamnoglucoside	1073
	Tectorigenin	1073
Calycotome spinosa	Genistein	1066
	5-*O*-Methylgenistein	1066
Calycotome villosa	Genistein	1066
	5-*O*-Methylgenistein	1066
Castanospermum australe	Afrormosin	1082
	Formononetin	1082
Chamaecytisus albus	Genistein	1066
Chamaecytisus smyrnaeus	Genistein	1066
	Daidzein	1066
Chamaespartium sagittale	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Chronanthus biflorus	Genistein	1066
	5-*O*-Methylgenistein	1066
Cicer arietinum	Biochanin A	1083–1094
	Biochanin A 7-*O*-glucoside	1095
	Daidzein	1091
	Formononetin	1083, 1085, 1088, 1092–1094, 1096–1099
	Homoferreirin (CCLXXXIV)	1099
	Pratensein (CCXIV)	1100
Cladrastis luteus	Afrormosin	1101
	Cladrastin (CCXVIII)	1101
	Cladrin (CLXXXIII)	1101
	Formononetin	1101
Cladrastis platycarpa	Fujikinetin (CCXX)	1102, 1103
	Fujikinin (CCXXI)	1103
Cordyla africana	6,7-Dimethoxy-3′,4′-methylenedioxyisoflavone (CCXXII)	1104
	6,7,2′,4′,5′-Pentamethoxyisoflavone (CCXLVII)	1104
	6,7,3′,4′-Tetramethoxyisoflavone (CCXIX)	1104
	6,7,3′-Trimethoxy-4′,5′-methylenedioxyisoflavone (CCXLVI)	1104
	Milldurone (CCXLIV)	1104
Cytisus battandieri	Daidzein	1066
	Genistein	1066
Cytisus commutatus	Daidzein	1066
	Genistein	1066
Cytisus eriocarpus	Genistein	1066
Cytisus laburnum	Genistein	1105
	5-*O*-Methylgenistein	1105, 1106
Cytisus proliferus	Daidzein	1066

(*continued*)

Table VII—(*Continued*)

Plant Name	Isoflavonoid	Reference
	Formononetin	1066
	Genistein	1066
Cytisus purgans	Daidzein	1066
	Genistein	1066
Cytisus scoparius	Genistein	1066
Cytisus welwitschii	Daidzein	1066
	Genistein	1066
Dalbergia barrentoana	Caviunin	1107
	Formononetin	1107
Dalbergia ecastophyllum	Daidzein	1108, 1109
	Formononetin	1108, 1110
Dalbergia lanceolaria	Biochanin A 7-*O*-apiosylglucoside (CCVII)	1111
	Pseudobaptigenin	1112
Dalbergia nigra	Caviunin (CCXXXVII)	1113
Dalbergia paniculata	Biochanin A	1114
	Caviunin	1115
	Dalpatein (CCXLII)	1115
	Dalpatin (CCXLIII)	1116
	Formononetin	1114
	Paniculatin (CCLXXIII)	1114
Dalbergia retusa	Retusine (CLXX)	1117
	8-*O*-Methylretusine (CLXXI)	1117
Dalbergia riparia	Afrormosin	1118
	Caviunin	1118
	Caviunin 7-*O*-glucoside (CCXXXVIII)	1118
	Fujikinetin	1118
	Tectorigenin	1118
	Tectorigenin 7-*O*-glucoside	1118
Dalbergia sisso	Biochanin A	1119
	Biochanin A 7-*O*-glucoside	1120
	7,4′-Dimethyltectorigenin (CCXXXIV)	1121
	7-*O*-Methyltectorigenin (CCXXXI)	1119
	7-*O*-Methyltectorigenin 4′-*O*-rhamnoglucoside (CCXXXII)	1122
	Tectorigenin	1119
Dalbergia villosa	Caviunin	1107
Dalbergia violacea	Di-*O*-methyldaidzein (CLXVIII)	1123
	Violanone (CCLXXXV)	1124
Derris malaccensis	Toxicarolisoflavone (CCLXV)	1125, 1126
Derris robusta	Derrubone (CCLII)	1127
	Derrustone (CCXVI)	1127
	Robustone (CCLVIII)	1127
	Robustone methyl ether (CCLIX)	1127
Derris scandens	Chandalone (CCLXXII)	1128
	Osajin (CCLXX)	1129
	5-*O*-Methylosajin (CCLXXI)	1129
	Scandenone (CCLX)	1128, 1129
	Scandinone (CCLXVIII)	1129
Erinacea anthyllis	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Ferreirea spectabilis	Biochanin A	1130
	Ferreirin (CCLXXXIII)	1130, 1131
	Homoferreirin	1130, 1131
Genista acanthoclada	Daidzein	1066
	Genistein	1066
Genista aetnensis	Daidzein	1066
	Formononetin	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista albida	Daidzein	1066
	Genistein	1066
Genista anglica	Daidzein	1066
	Genistein	1066
Genista arsitata	Daidzein	1066
Genista baetica	Daidzein	1066
Genista capitellata	Daidzein	1066
Genista carpetana	Daidzein	1066
Genista cinerea	Daidzein	1066
Genista corsica	Daidzein	1066
Genista cupanii	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista falcata	Daidzein	1066
Genista florida	Daidzein	1066
Genista hirsuta	Daidzein	1066
Genista hispanica	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066

Table VII—(*Continued*)

Plant Name	Isoflavonoid	Reference
Genista hystrix	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista germanica	Daidzein	1066
Genista lobelii	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista lydia	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista micrantha	5-*O*-Methylgenistein	1066
Genista morisii	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista nissana	Daidzein	1066
	Formononetin	1066
Genista obtusirama	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista pilosa	Daidzein	1066
Genista pumila	Daidzein	1066
Genista radiata	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista raetum	Genistein 7-*O*-glucoside	1132
Genista salzmanii	Daidzein	1066
	Formononetin	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista scorpius	Genistein	1066
Genista sericea	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista sessilifolia	Daidzein	1066
	Formononetin	1066
	Genistein	1066
Genista spartioides	Genistein	1066
Genista subcapitata	Daidzein	1066
	Formononetin	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista teritifolia	Daidzein	1066
	Formononetin	1066
Genista tinctoria	Daidzein	1066
	Genistein	1066
	Genistein 7-*O*-glucoside	1066, 1133, 1134
Genista triacanthos	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista tridens	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista ulicina	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Genista umbellata	Daidzein	1066
Genista valentina	Daidzein	1066
Genista villarsii	Daidzein	1066
	Formononetin	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Gleditschia triacanthos	Biochanin A	1135, 1136
Glycine max	Daidzein	995, 1137–1139
	Daidzein 7-*O*-glucoside	1099, 1139–1144
	Genistein	995, 1032, 1139, 1140, 1143, 1145, 1146
	Genistein 7-*O*-glucoside	995, 1032, 1145
	Glycitein (CLXXXVII)	1147
	6,7,4′-Trihydroxyisoflavone (CLXXXVI)	1148–1150
	Isoflavonoids	1151
Glycyrrhiza echinata	Formononetin	1152
Glycyrrhiza glabra	Formononetin	1153, 1154
Laburnum anagyroides	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Lathyrus montanus	Orobol	1156
Lupinus sp.	Genistein	1161
Lupinus luteus	Luteone (CCLI)	1157
Lupinus polyphyllus	Genistein	1158

(continued)

Table VII—(*Continued*)

Plant Name	Isoflavonoid	Reference
	Genistein 7-*O*-glucoside	1159
Lupinus termis	Biochanin A	1160
Lygos monosperma	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Lygos raetam	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Maackia amurensis	Pseudobaptigenin	1162
	Sophorol (CCLXXXVIII)	1163, 1164
Maackia amurensis var. *Buergeri*	7′,4-Dihydroxy-3′-methoxyisoflavone (CLXXVI)	1165
	Formononetin	1165
	Genistein	1165
Machaerium villosum	7′,4-Dihydroxy-3′-methoxyisoflavone	1166
	Formononetin	1166
	Isoformononetin (CLXII)	1166
	7,3′,4′,Trihydroxyisoflavone (CLXXV)	1166
Medicago sativa	Biochanin A	1167–1169
	Daidzein	1167, 1168, 1170–1172
	Formononetin	1167, 1168, 1170–1173
	Genistein	1167–1169
Milletia auriculata	Auriculatin (CCLXI)	1174
	Auriculin (CCLXII)	1175
	Isoauriculatin (CCLXIV)	1175
Milletia dura	Durlettone (CLXIX)	1176
	Durmillone (CCLXVI)	1176
	Milldurone	1176
Milletia ferruginea	Durmillone	1177
	Ferrugone (CCLXXV)	1177
Mundulea sericea	Mundulone (CCLXXVII)	1178
	Munetone (CCLXXXVI)	1179, 1180
Mundulea suberosa	Munetone	1181, 1182
Myrocarpus fastigiatus	Afrormosin	1183, 1184
	Cabreuvin (CLXXXIV)	1183
Myroxylon balsamum	Afrormosin	1184
	Cabreuvin	1183
Neorautanenia amboensis	Dehydroneotenone (CCLVI)	1185
	Neotenone (CCLV)	1185
	Pachyrrhizin (CCLVII)	1185, 1186
Neorautanenia edulis	Dehydroneotenone	1185
	Neotenone	1185
	Pachyrrhizin	1185, 1186
Pterocarpus dalbergioides	Calycosin	1214
Pterocarpus erinaceous	Pseudobaptigenin	1068
Pterocarpus indicus	Formononetin	1215
Pterocarpus osun	Prunetin	1216
Pterocarpus santalinus	Santal	1072, 1217, 1218
Pterocarpus vidalianus	Formononetin	1145
Pterodon pubescens	Milldurone	1206, 1219, 1220
	6,7,2′,3′,4′-Pentamethoxyisoflavone	1206, 1219, 1220
	6,7,3′,4′-Tetramethoxyisoflavone	1206, 1219, 1220
Pueraria mirifica	Miroestrol (CCLXXVIII)	1221, 1222
	Unknown isoflavone	1223
Pueraria pseudohirsuta	Puerarin (CLXXII)	1224
	Puerarin xyloside (CLXXIV)	1224
Pueraria thomsoni	Puerarin	1224
Pueraria thunbergiana	Daidzein	1224–1227
	Daidzein 7-*O*-glucoside	1227
	Genistein	1226
	Puerarin	1224, 1225, 1227
	Puerarin xyloside	1224, 1227
Pueraria tuberosa	Daidzein	1228
	Puerarin	1228
	Puerarin 4′,6″-diacetate (CLXXIII)	1228
Sarothamnus scoparius	Genistein 7-*O*-glucoside	1159
Sophora japonica	Genistein	1229–1232
	Genistein 4′-*O*-glucoside (CXCIV)	1064, 1230–1235
	Genistein 7-*O*-neohesperidoside (CXCVI)	1236
	Genistein 4′-*O*-rhamnoglucoside (CXCVII)	1233–1235
	Genistein 7-*O*-rhamnoglucoside	1230, 1237, 1238
Neorautanenia pseudopachyrrhiza	Dehydroneotenone	1186
	Neotenone	1186
	Nepseudin (CCLIV)	1186
	Pachyrrhizin	1185, 1186
Ononis spinosa	Formononetin 7-*O*-glucoside	1140, 1141, 1187–1191
Orobus tuberosus	Orobol 7-*O*-glucoside	1156, 1192
Ougeinia dalbergioides	Dalbergioidin (CCLXXXII)	1193, 1194

Table VII—(*Continued*)

Plant Name	Isoflavonoid	Reference
	Homoferreirin	1195
	Ougenin (CCLXXXVII)	1193–1195
Pachyrrhizus erosus	Dehydroneotenone	1186
	Neotenone	1186
	Pachyrrhizin	1196, 1197
Pericopsis sp.	Afrormosin	1198
	Formononetin	1198
Phaseolus aureus	Daidzein	1092, 1199
	7,2′,4′-Trihydroxyisoflavone (CCXLI)	1200
Piscidia erythrina	Ichthynone (CCLXVII)	1201
	Jamaicin (CCLXIII)	1202, 1203
	Piscerythrone (CCL)	1204
	Piscidone (CCLXXIV)	1204
Pisum sativum	Isoflavones	1205
Platymiscium praecox	Texasin	1206, 1207
Poecilanthe parviflora	5,7,4′-Trihydroxy-2′,3′-dimethoxy-isoflavanone (CCLXXXVI)	1208
	Mixture of isoflavones	1208
Psoralea esculenta	Isoflavones	1209
Psoralea rigida	Isoflavones	1209
Psoralea scaposa	Isoflavones	1209
Pterocarpus angolensis	7-*O*-Methyltectorigenin	1210
	Muningin (CCXXXIII)	1211, 1212
	Prunetin	1212, 1213
	Sophorol	1163
Sophora subprostrata	Daidzein	1239
	Genistein	1240
Stauracanthos genistoides	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Teline canariensis	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Teline congesta	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Teline linifolia	Genistein	1066
Teline monspessulana	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Teline rosmarinifolia	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Teline spachiana	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Teline stenopetala	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Tephrosia maxima	Maxima substance A (CCXV)	1241
	Maxima substance B (CLXXXV)	1242, 1243
	Maxima substance C (CCLIII)	1244
Tipuana tipu	Formononetin	1245
Trifolium alpestre	Biochanin A	1246
	Formononetin	1246, 1247
	Formononetin 7-*O*-glucoside	1246, 1247
	Genistein	1246
Trifolium anatolicum	Formononetin	1246
	Formononetin 7-*O*-glucoside	1246
	Genistein	1246
Trifolium baccarini	Biochanin A	1246
	Formononetin	1246
	Formononetin 7-*O*-glucoside	1246
	Genistein	1246
Trifolium batmanicum	Biochanin A	1248
	Formononetin	1248
	Genistein	1248
Trifolium campestre	Genistein	1249
Trifolium eriosphoerum	Biochanin A	1246, 1248
	Formononetin	1246, 1248
	Formononetin 7-*O*-glucoside	1246
	Genistein	1248
Trifolium globosum	Biochanin A	1246, 1248
	Formononetin	1246, 1248
	Formononetin 7-*O*-glucoside	1246
	Genistein	1248, 1249
Trifolium hybridum	Biochanin A 7-*O*-glucoside	1250
	Formononetin 7-*O*-glucoside	1250
	Genistein 7-*O*-glucoside	1250

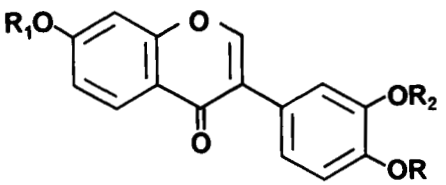
(*continued*)

Table VII—(*Continued*)

Plant Name	Isoflavonoid	Reference
Trifolium incarnatum	Biochanin A 7-*O*-glucoside	1250
	Formononetin 7-*O*-glucoside	1250, 1251
	Genistein 7-*O*-glucoside	1250
Trifolium isodon	Biochanin A	1246
	Formononetin	1246
	Formononetin 7-*O*-glucoside	1246
Trifolium israeliticum	Biochanin A	945, 1246, 1248
	Formononetin	945, 1246, 1248
	Formononetin 7-*O*-glucoside	1246
	Genistein	945, 1246, 1248
Trifolium lappaceum	Biochanin A	1246
	Formononetin	1246
	Formononetin 7-*O*-glucoside	1246
	Genistein	1246
Trifolium medium	Biochanin A	1252
	Daidzein	1252
	Formononetin	1252
	Formononetin 7-*O*-glucoside	1247
	Genistein	1247, 1252
Trifolium meduseum	Biochanin A	1248
	Formononetin	1248
	Genistein	1248
Trifolium ochroleucum	Biochanin A 7-*O*-glucoside	1250
Trifolium pannonicum	Biochanin A 7-*O*-glucoside	1250
Trifolium pauciflorum	Biochanin A	1246, 1248
	Formononetin	1246, 1248
	Formononetin 7-*O*-glucoside	1246
	Genistein	1246, 1248
Trifolium pilulare	Biochanin A	1246, 1248
	Formononetin	1246, 1248
	Formononetin 7-*O*-glucoside	1246
	Genistein	1246, 1248
Trifolium pratense	Biochanin A	940, 1167, 1246, 1253–1265
	Biochanin A 7-*O*-glucoside	1250, 1263, 1266–1268
	Biochanin A 7-*O*-glucoside 5-malonate (CCVI)	1263, 1267
	Daidzein	1167, 1168, 1258, 1263, 1264
	Daidzein 7-*O*-glucoside	1263
	Formononetin	940, 1013, 1167–1169, 1246, 1247, 1255, 1258–1260, 1262, 1263, 1265, 1269–1273
	Formononetin 7-*O*-glucoside	1246, 1247, 1250, 1263, 1266, 1268
	Formononetin 7-*O*-glucoside 6″-malonate (CLXVI)	1268
	Formononetin 7-*O*-glucoside 6″-methylmalonate (CLXVII)	1268
	Genistein	1050, 1167–1169, 1246, 1249, 1250, 1255, 1258, 1261, 1263, 1272
	Isoflavones	1274–1276
	Pratensein	1258, 1277–1279
Trifolium repens	Daidzein	1167, 1168
	Formononetin	1167, 1168, 1265, 1280
	Genistein	1050, 1167, 1168
	Isoflavones	1261
Trifolium sativum	Formononetin	1247
	Formononetin 7-*O*-glucoside	1247
Trifolium subterraneum	Biochanin A	934, 943, 945, 951, 1013, 1167, 1246, 1248, 1258, 1259, 1261, 1281–1288
	Biochanin A 7-*O*-glucoside	1250, 1268
	Biochanin A 7-*O*-glucoside 6″-malonate (CCVIII)	1268
	Biochanin A 7-*O*-glucoside 6″-methylmalonate (CCIX)	1268
	Daidzein	943, 1167, 1258, 1281–1283, 1285, 1286
	Formononetin	932, 934, 943, 945, 951, 1167, 1246, 1248, 1255, 1258, 1281–1287
	Formononetin 7-*O*-glucoside	1246, 1250, 1268
	Formononetin 7-*O*-glucoside 6″-malonate	1268
	Formononetin 7-*O*-glucoside 6″-methylmalonate	1268

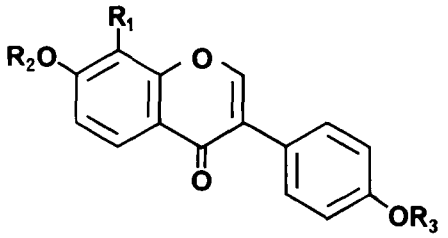
Table VII—*(Continued)*

Plant Name	Isoflavonoid	Reference
	Genistein	930, 932, 934, 943, 945, 951, 1013, 1167, 1246, 1248, 1249, 1255, 1258, 1259, 1261, 1281–1290
	Genistein 7-*O*-glucoside	1250
	Isoflavones	1274
Trifolium tembense	Biochanin A	1246
	Formononetin	1246
	Formononetin 7-*O*-glucoside	1246
Ulex bioivinii	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Ulex europaeus	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Ulex galli	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Ulex minor	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Ulex nanus	Genistein 7-*O*-glucoside	1065
Ulex parviflorus	Daidzein	1066
	Genistein	1066
	5-*O*-Methylgenistein	1066
Vigna sinensis	Isoflavones	1205
Wisteria brachybotrys	Afrormosin 7-*O*-glucoside	1291
Wisteria floribunda	Afrormosin 7-*O*-glucoside	1291
Wisteria sinensis	Isoflavones	1292
Moraceae		
Maclura pomifera	Osajin	1293, 1294
	Pomiferin (CCLXIX)	1294, 1295
Podocarpaceae		
Podocarpus spicatus	Genistein	1296
	Podospicatin (CCXLIV)	1296–1298
Rosaceae		
Prunus aequinoctalis	Genistein	1299
	Genistein 7-*O*-glucoside	1299
	Prunetin	1299
Prunus avium	Prunetin	1300
	Prunetin 4′-*O*-glucoside (CCI)	1064
Prunus emarginata	Prunetin	1300
	Prunetin 4′-*O*-glucoside	1064
Prunus mahaleb	Genistein	1301, 1302
	Prunetin	1301, 1302
Prunus maximowiczii	Genistein	1299
	Prunetin	1299
Prunus nipponica	Genistein	1299
	Genistein 7-*O*-glucoside	1299
	Prunetin	1299
Prunus puddum	Genistein	1303
	Padmakastein (CCLXXIX)	1304, 1305
	Padmakastin (CCLXXX)	1304
	Prunetin	1303, 1304
Prunus vericunda	Genistein	1308
	Prunetin	1308
Solanaceae		
Nicotiana tabacum	Isoflavonoids	1309



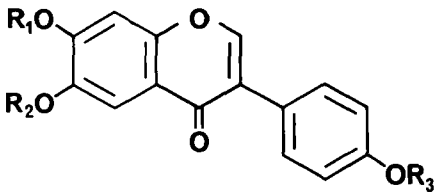
	R_1	R_2	R
CLXXV: 7,3′,4′-trihydroxyisoflavone	H	H	H
CLXXVI: 7,4′-dihydroxy-3′-methoxyisoflavone	H	CH_3	H
CLXXVII: calycosin	H	H	CH_3
CLXXVIII: calycosin 7-*O*-glucoside	Glu	H	CH_3
CLXXIX: calycosin 7-*O*-rhamnoglucoside	Rham·Glu	H	CH_3
CLXXX: pseudobaptigenin	H	—CH_2—	
CLXXXI: pseudobaptigenin 7-*O*-glucoside	Glu	—CH_2—	
CLXXXII: pseudobaptigenin 7-*O*-rhamnoglucoside	Rham·Glu	—CH_2—	
CLXXXIII: cladrin	H	CH_3	CH_3
CLXXXIV: cabreuvin	CH_3	CH_3	CH_3
CLXXXV: maxima substance B	—$CH_2CH{=}(CH_3)_2$	—CH_2—	

Structures of Isoflavonoids



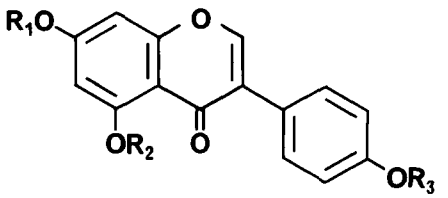
	R_1	R_2	R_3
CLIX: daidzein	H	H	H
CLX: daidzein 7-*O*-glucoside	H	Glu	H
CLXI: daidzein 7-*O*-rhamnoglucoside	H	Rham·Glu	H
CLXII: isoformononetin	H	CH_3	H
CLXIII: formononetin	H	H	CH_3
CLXIV: formononetin 7-*O*-glucoside	H	Glu	CH_3
CLXV: formononetin 7-*O*-rhamnoglucoside	H	Rham·Glu	CH_3
CLXVI: formononetin 7-*O*-glucoside-6″-malonate	H	Mal·Glu	CH_3
CLXVII: formononetin 7-*O*-glucoside-6″-methylmalonate	H	MeMal·Glu	CH_3
CLXVIII: di-*O*-methyldaidzein	H	CH_3	CH_3
CLXIX: durlettone	H	CH_3	$-CH_2CH=C(CH_3)_2$
CLXX: retusine	OH	H	CH_3
CLXXI: 8-*O*-methylretusine	OCH_3	H	CH_3
CLXXII: puerarin	Glu	H	H
CLXXIII: puerarin 4′,6″-diacetate	6-AcGlu	H	Ac
CLXXIV: puerarin xyloside	Glu	H or Xyl	Xyl or H

Structures of Isoflavonoids



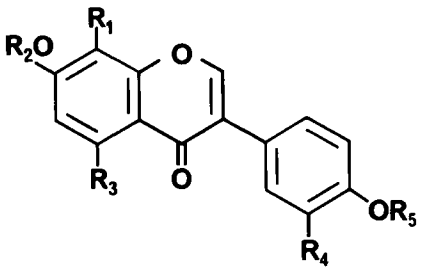
	R_1	R_2	R_3
CLXXXVI: 6,7,4′-trihydroxyisoflavone	H	H	H
CLXXXVII: glycitein	H	CH_3	H
CLXXXVIII: texasin	H	H	CH_3
CLXXXIX: texasin 7-*O*-glucoside	Glu	H	CH_3
CXC: afrormosin	H	CH_3	CH_3
CXCI: afrormosin 7-*O*-glucoside	Glu	CH_3	CH_3
CXCII: afrormosin 7-*O*-rhamnoglucoside	Rham·Glu	CH_3	CH_3

Structures of Isoflavonoids



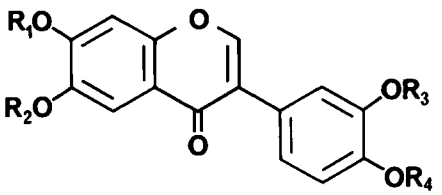
	R_1	R_2	R_3
CXCIII: genistein	H	H	H
CXCIV: genistein 4′-*O*-glucoside	H	H	Glu
CXCV: genistein 7-*O*-glucoside	Glu	H	H
CXCVI: genistein 7-*O*-neohesperiside	Neohesp	H	H
CXCVII: genistein 4′-*O*-rhamnoglucoside	H	H	Rham·Glu
CXCVIII: genistein 7-*O*-rhamnoglucoside	Rham·Glu	H	H
CXCIX: genistein 7-*O*-rutinoside	rutin	H	H
CC: prunetin	CH_3	H	H
CCI: prunetin 4′-*O*-glucoside	CH_3	H	Glu
CCII: 5-*O*-methylgenistein	H	CH_3	H
CCIII: biochanin A	H	H	CH_3
CCIV: biochanin A 7-*O*-glucoside	Glu	H	CH_3
CCV: biochanin A 7-*O*-rhamnoglucoside	Rham·Glu	H	CH_3
CCVI: biochanin A 7-*O*-glucoside 5-malonate	Glu	Mal	CH_3
CCVII: biochanin A 7-apiosylglucoside	Ap·Glu	H	CH_3
CCVIII: biochanin A 7-*O*-glucoside 6″-malonate	6-Mal·Glu	H	CH_3
CCIX: biochanin A 7-*O*-glucoside 6″-methylmalonate	6-MeMal·Glu	H	CH_3

Structures of Isoflavonoids



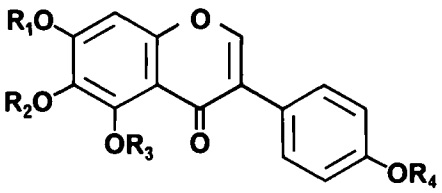
	R_1	R_2	R_3	R_4	R_5
CCX: orobol	H	H	OH	OH	H
CCXI: orobol 7-*O*-glucoside	H	Glu	OH	OH	H
CCXII: orobol 7-*O*-rhamnoglucoside	H	Rham·Glu	OH	OH	H
CCXIII: santal	H	CH_3	OH	OH	H
CCXIV: pratensein	H	H	OH	OH	CH_3
CCXV: maxima substance A	—OCH_2—		H	—CH_2O—	
CCXVI: derrustone	H	CH_3	OCH_3	—CH_2O—	
CCXVII: homotectoridin	OCH_3	Glu	OH	CH_3	H

Structures of Isoflavonoids



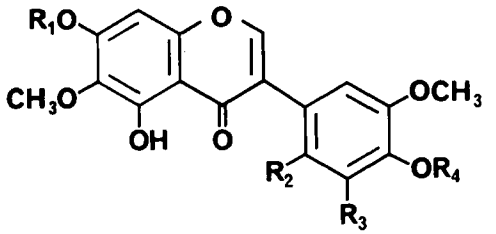
	R_1	R_2	R_3	R_4
CCXVIII: cladrastin	H	CH_3	CH_3	CH_3
CCXIX: 6,7,3′,4′-tetramethoxyisoflavone	CH_3	CH_3	CH_3	CH_3
CCXX: fujikinetin	H	CH_3	—CH_2—	
CCXXI: fujikinin	Glu	CH_3	—CH_2—	
CCXXII: 6,7-dimethoxy-3′,4′-methylenedioxyisoflavone	CH_3	CH_3	—CH_2—	

Structures of Isoflavonoids



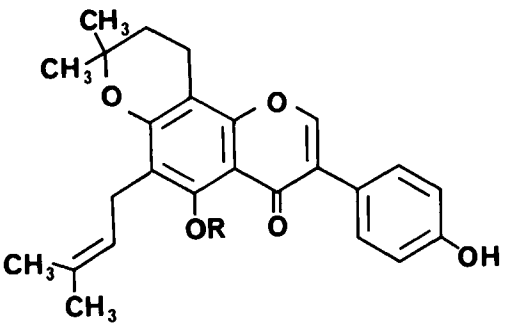
	R_1	R_2	R_3	R_4
CCXXIII: 6-hydroxygenistein	H	H	H	H
CCXXIV: 6-hydroxygenistein 7-*O*-rhamnoglucoside	Rham·Glu	H	H	H
CCXXV: tectorigenin	H	CH_3	H	H
CCXXVI: tectorigenin 7-*O*-glucoside	Glu	CH_3	H	H
CCXXVII: irilone	—CH_2—		H	H
CCXXVIII: irilone 4′-*O*-glucoside	—CH_2—		H	Glu
CCXXIX: irilone 4′-bioside	—CH_2—		H	Biose
CCXXX: irisolone	—CH_2—		CH_3	H
CCXXXI: 7-*O*-methyltectorigenin	CH_3	CH_3	H	H
CCXXXII: 7-*O*-methyltectorigenin 4′-*O*-rhamnoglucoside	CH_3	CH_3	H	Rham·Glu
CCXXXIII: muningin	CH_3	H	CH_3	H
CCXXXIV: 7,4′-dimethyltectorigenin	CH_3	CH_3	H	CH_3
CCXXXV: irisolidone	H	CH_3	H	CH_3

Structures of Isoflavonoids



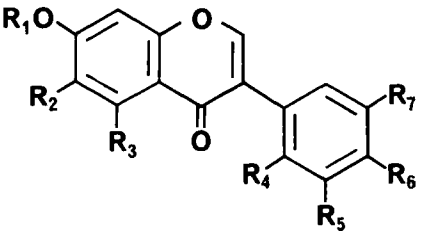
	R_1	R_2	R_3	R_4
CCXXXVI: iristectorigenin	H	H	H	H
CCXXXVII: caviunin	H	OCH_3	H	CH_3
CCXXXVIII: caviunin 7-*O*-glucoside	Glu	OCH_3	H	CH_3
CCXXXIX: irigenin	H	H	OH	CH_3
CCXL: irigenin 7-*O*-glucoside	Glu	H	OH	CH_3

Structures of Isoflavonoids



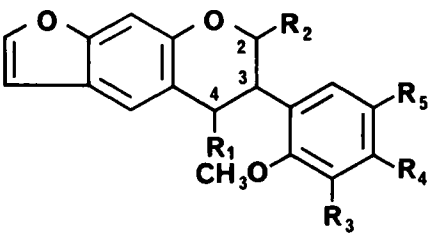
CCLXX: osajin, R = H
CCLXXI: 5-*O*-methylosajin, R = CH_3

Structures of Isoflavonoids



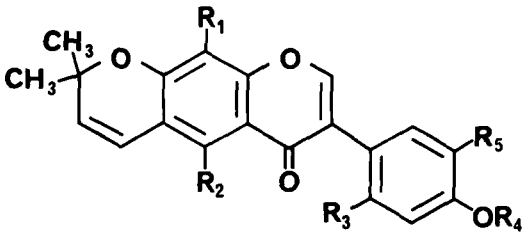
	R_1	R_2	R_3	R_4	R_5	R_6	R_7
CCXLI: 7,2′,4′-trihydroxyisoflavone	H	H	H	OH	H	H	H
CCXLII: dalpatein	H	OCH_3	H	OCH_3	H	—O—CH_2—O—	
CCXLIII: dalpatin	Glu	OCH_3	H	OCH_3	H	—O—CH_2—O—	
CCXLIV: milldurone	CH_3	OCH_3	H	OCH_3	H	—O—CH_2—O—	
CCXLV: podospicatin	H	OCH_3	OH	OH	H	H	OCH_3
CCXLVI: 6,7,3′-trimethoxy-4′5′-methylenedioxyisoflavone	CH_3	OCH_3	H	H	OCH_3	—O—CH_2—O—	
CCXLVII: 6,7,2′,4′,5′-pentamethoxyisoflavone	CH_3	OCH_3	H	OCH_3	OCH_3	OCH_3	H
CCXLVIII: tlatlancuayin	—CH_2—O—		OCH_3	OCH_3	H	H	H
CCXLIX: irisflorentin	—CH_2—O—		OCH_3	H	OCH_3	OCH_3	OCH_3
CCL: piscerythrone	H	H	OH	H	—CH_2CH=$C(CH_3)_2$	OH	OCH_3
CCLI: luteone	H	—CH_2CH=$C(CH_3)_2$	OH	OH	H	OH	H
CCLII: derrubone	H	—CH_2CH=$C(CH_3)_2$	OH	H	—O—CH_2—O—		H
CCLIII: maxima substance C	—CH_2CH=$C(CH_3)_2$	H	H	OCH_3	H	—O—CH_2—O—	

Structures of Isoflavonoids



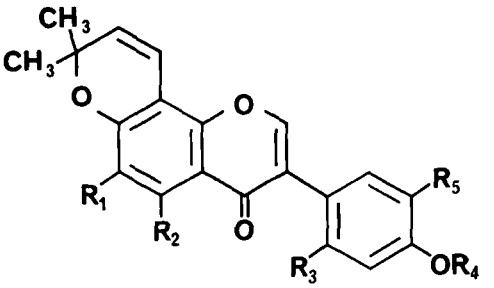
	R_1	R_2	R_3	R_4	R_5	other
CCLIV: nepseudin	=O	H_2	OCH_3	OCH_3	H	–
CCLV: neotenone	=O	H_2	H	—OCH_2O—		–
CCLVI: dehydroneotenone	=O	H	H	—OCH_2O—		$\Delta^{2,3}$
CCLVII: pachyrrizin	H	=O	H	—OCH_2O—		$\Delta^{3,4}$

Structures of Isoflavonoids



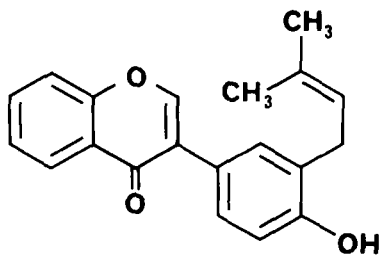
	R_1	R_2	R_3	R_4	R_5
CCLVIII: robustone	H	OH	H	—CH_2O—	
CCLIX: robustone methyl ether	H	OCH_3	H	—CH_2O—	
CCLX: scandenone	—CH_2CH=$C(CH_3)_2$	OH	H	H	H
CCLXI: auriculatin	—CH_2CH=$C(CH_3)_2$	OH	OH	H	H
CCLXII: auriculin	—CH_2CH=$C(CH_3)_2$	H	OH	CH_3	H

Structures of Isoflavonoids

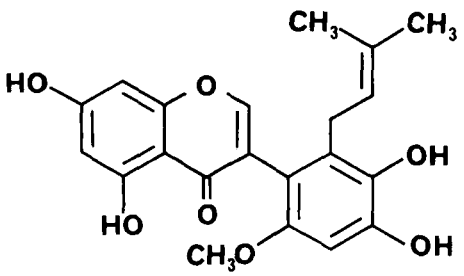


	R_1	R_2	R_3	R_4	R_5
CCLXIII: jamaicin	H	H	OCH_3	—CH_2O—	
CCLXIV: isoauriculatin	H	OH	OH	—CH_2CH=$C(CH_3)_2$	H
CCLXV: toxicarol isoflavone	H	OH	OCH_3	CH_3	CH_3
CCLXVI: durmillone	OCH_3	H	H	—CH_2O—	
CCLXVII: ichthynone	OCH_3	H	OCH_3	—CH_2O—	
CCLXVIII: scandinone	—CH_2CH=$C(CH_3)_2$	OCH_3	H	H	H
CCLXIX: pomiferin	—CH_2CH=$C(CH_3)_2$	OH	H	H	OH

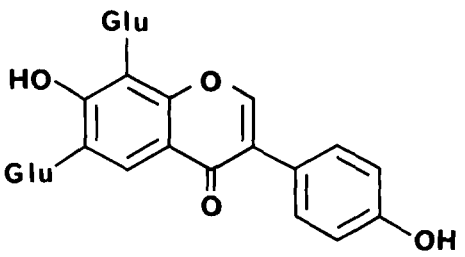
Structures of Isoflavonoids



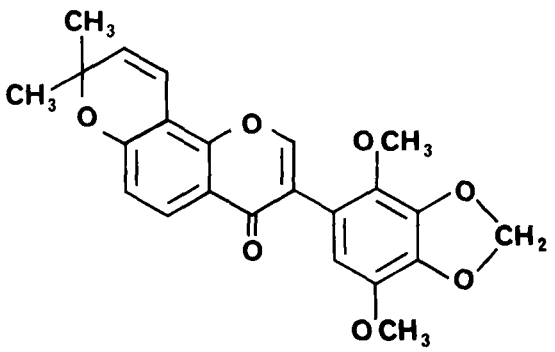
CCLXXII: chandalone



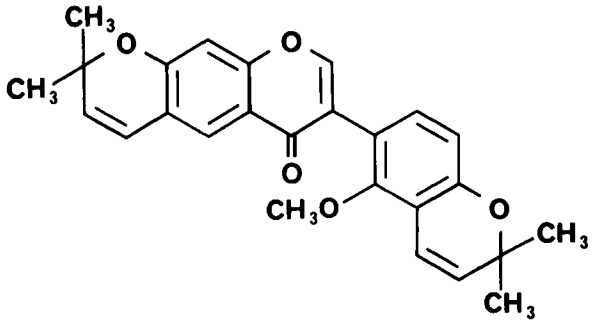
CCLXXIV: piscidone



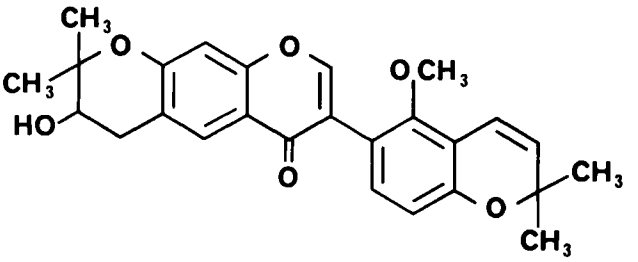
CCLXXIII: paniculatin



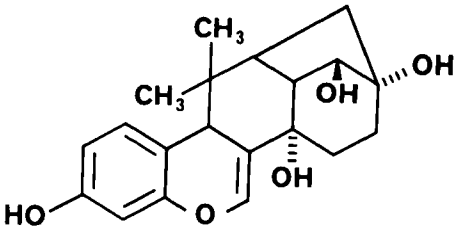
CCLXXV: ferrugone



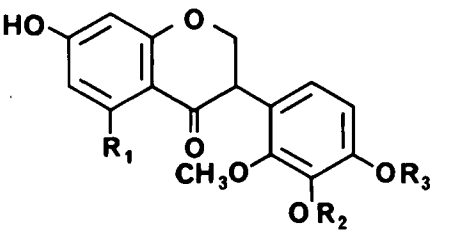
CCLXXVI: munetone



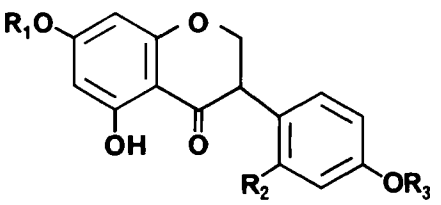
CCLXXVII: mundulone



CCLXXVIII: miroesterol

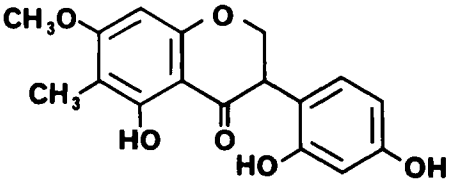


	R_1	R_2	R_3
CCLXXXV: violanone	H	H	CH_3
CCLXXXVI: 5,7,4′-trihydroxy-2′,3′-dimethoxyisoflavanone	OH	CH_3	H

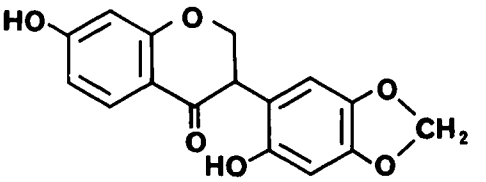


	R_1	R_2	R_3
CCLXXXIX: padmakistein	CH_3	H	H
CCLXXX: padmakistin	CH_3	H	Glu
CCLXXXI: 5,7-dihydroxy-4′-methoxyisoflavone	H	H	CH_3
CCLXXXII: dalbergioidin	H	OH	H
CCLXXXIII: ferreirin	H	OH	CH_3
CCLXXXIV: homoferreirin	H	OCH_3	CH_3

Structures of Isoflavonoids



CCLXXXVII: ougenin



CCLXXXVIII: sophorol

Structures of Isoflavonoids

Table VIII—Occurrence of Coumestans in Plants

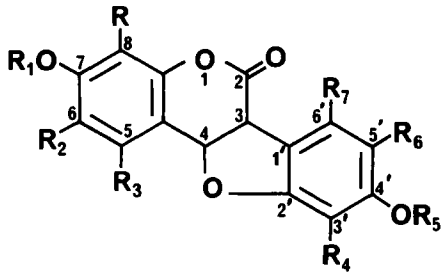
Plant Name	Coumestan	Reference
Compositae		
Eclipta alba	Wedelolactone (CCXCIX)	1310, 1311
	Demethylwedelolactone (CCXCVIII)	1311
Taraxacum officinale	Coumestrol	1312
Wedelia calendula	Wedelolactone	1313–1315
Gramineae		
Secale cereale	Coumestrol	1312
Leguminosae		
Cicer arietinum	Medicagol (CCC)	1316
	4′-*O*-Methylcoumestrol (CCXCI)	1316
Dalbergia decipularis	7,4′-Di-*O*-methylcoumestrol (CCXCIII)	1317
Glycine max	Coumestrol	1318, 1319
	Sojagol (CCCIII)	1318, 1319
Glycyrrhiza sp.	Isoglycyrol (CCCVII)	1320
	Glycyrol (CCCVI)	1320
	5-*O*-Methylglycyrol (CCCVIII)	1320
Maackia amurensis var. *buergeri*	Medicagol	1165
Medicago aculeata	Coumestrol	1321, 1322
Medicago arabica	Coumestrol	1321, 1322
Medicago arborea	Coumestrol	1321, 1322
Medicago blancheana	Coumestrol	1321, 1322

Table VIII—(*Continued*)

Plant Name	Coumestan	Reference
Medicago constricta	Coumestrol	1321
Medicago corulea	Coumestrol	1321, 1322
Medicago disciformis	Coumestrol	1321, 1322
Medicago dzhawakhetica	Coumestrol	1321, 1322
Medicago falcata	Coumestrol	1321, 1322
Medicago granadensis	Coumestrol	1321, 1322
Medicago hemicycla	Coumestrol	1321, 1322
Medicago hispida	Coumestrol	1323
Medicago intertexta	Coumestrol	1321, 1322
Medicago laciniata	Coumestrol	1321, 1322
Medicago littoralis	Coumestrol	1321, 1322, 1324, 1325
	4′-*O*-Methylcoumestrol	1325
	3′-Methoxycoumestrol (CCXC)	1325
Medicago minima	Coumestrol	1321, 1322
Medicago murex	Coumestrol	1321, 1322
Medicago orbicularis	Coumestrol	1321, 1322
Medicago polychroa	Coumestrol	1321, 1322
Medicago polymorpha	Coumestrol	1321, 1322
Medicago polymorpha var. *denticulata*	Coumestrol	1325
	4′-*O*-Methylcoumestrol	1325
	3′-Methoxycoumestrol	1325
Medicago praecox	Coumestrol	1321, 1322
Medicago rigidula	Coumestrol	1321, 1322
Medicago rotata	Coumestrol	1321, 1322
Medicago rugosa	Coumestrol	1321, 1322
Medicago sativa	Coumestrol	937, 942, 960, 1169, 1170, 1172, 1173, 1312, 1321–1323, 1326–1333
	5-Methoxy-4′-*O*-methylcoumestrol (CCXCII)	1170, 1334, 1335
	4′-*O*-Methylcoumestrol	1170, 1331, 1336
	Sativol (CCXCVI)	1170, 1331, 1337
	Medicagol	1170, 1331
	3′-Methoxycoumestrol	1331, 1338
	Trifoliol (CCXCVII)	1330, 1331
	Lucernol (CCXCIV)	1337
Medicago sauvagei	Coumestrol	1321, 1322
Medicago scutellata	Coumestrol	1321, 1322, 1325
	4′-*O*-Methylcoumestrol	1325
	3′-Methoxycoumestrol	1325
Medicago sogdiana	Coumestrol	1321, 1322
Medicago soleirolii	Coumestrol	1321, 1322
Medicago suffruticosa	Coumestrol	1321
Medicago tenoreana	Coumestrol	1321, 1322
Medicago tianshanica	Coumestrol	1321, 1322
Medicago tornata	Coumestrol	1321, 1322
Medicago truncatula	Coumestrol	1321, 1322, 1325
	4′-*O*-Methylcoumestrol	1325
	3′-Methoxycoumestrol	1325
Medicago turbinata	Coumestrol	1321, 1322
Medicago varia	Coumestrol	1321, 1322
Melilotus alba	Coumestrol	1322
Pachyrrhizus erosus	Erosnin (CCCIV)	1339, 1340
Phaseolus aureus	Coumestrol	1092, 1199, 1200, 1341
	Sojagol	1199
Psoralea corylifolia	Psoralidin (CCCV)	1342
Swartzia leiocalycina	6-Hydroxy-7-*O*-methylmedicagol (CCCI)	1343
	6-Hydroxy-5-methoxy-7-*O*-methylmedicagol (CCCII)	1343
Swartzia madagascariensis	7,4′-Di-*O*-methylcoumestrol	1344
Trifolium fragiferum	Coumestrol	960, 1323
Trifolium pratense	Coumestrol	1312, 1323, 1326, 1332
Trifolium repens	Coumestrol	960, 1280, 1312, 1323, 1326, 1345–1347
	4′-*O*-Methylcoumestrol	1346
	Trifoliol	1346, 1348
	Repensol (CCXCV)	1346
Trifolium subterraneum	Coumestrol	1281, 1282
Trifolium subterraneum var. *dwalganup*	Coumestrol	1323
Trigonella corniculata	Coumestrol	1322

this series is coumestrol (CCLXXXIX), and its isolation and estrogenic activity were first reported by Bickoff *et al.* (960). The coumestans are reported to have a higher order of estrogenic activity than the isoflavones (961). Table VIII gives a list of plants and their contained coumestans.

The synthetic stilbene derivative, diethylstilbestrol (CCCIX), is the prototype of the nonsteroidal estrogenic hormones. Of the natural stilbenes, rhaponticin (CCCX) is the only compound of this type reported to possess estrogenic activity. Knorr *et al.* (962) reported in 1956 that this compound could induce estrus in ovariectomized female rats. On the other hand, Klimek (963) reported in 1970 that rhaponticin produced only an insignificant increase in



		R	R_1	R_2	R_3	R_4	R_5	R_6	R_7
CCLXXXIX:	coumestrol	H	H	H	H	H	H	H	H
CCXC:	3′-methoxycoumestrol	H	H	H	H	OCH_3	H	H	H
CCXCI:	4′-*O*-methylcoumestrol	H	H	H	H	H	CH_3	H	H
CCXCII:	5-methoxy-4′-*O*-methylcoumestrol	H	H	H	H	H	CH_3	OCH_3	H
CCXCIII:	7,4′-di-*O*-methylcoumestrol	H	CH_3	H	H	H	CH_3	H	H
CCXCIV:	lucernol	H	H	OH	H	H	H	H	H
CCXCV:	repensol	H	H	H	H	H	H	H	OH
CCXCVI:	sativol	OH	CH_3	H	H	H	H	H	H
CCXCVII:	trifoliol	H	H	H	H	H	CH_3	H	OH
CCXCVIII:	demethylwedelolactone	H	H	H	OH	H	H	OH	H
CCXCIX:	wedelolactone	H	CH_3	H	OH	H	H	OH	H
CCC:	medicagol	H	H	H	H	H	$-CH_2-O-$		H
CCCI:	6-hydroxy-7-*O*-methylmedicagol	H	CH_3	OH	H	H	$-CH_2-O-$		H
CCCII:	6-hydroxy-5-methoxy-7-*O*-methylmedicagol	H	CH_3	OH	OCH_3	H	$-CH_2-O-$		H
CCCIII:	sojagol	H	H	H	H	$-CH_2CH_2-C(CH_3)_2-O-$		H	H
CCCIV:	erosnin	H	$-CH=CH-$		H	H	$-CH_2-O-$		H
CCCV:	psoralidin	H	H	$-CH_2-CH=C(CH_3)_2$	H	H	CH_3	H	H
CCCVI:	glycyrol	H	CH_3	$-CH_2-CH=C(CH_3)_2$	OH	H	H	H	H
CCCVII:	isoglycyrol	H	CH_3	$-CH_2CH_2-C(CH_3)_2-O-$	H		H	H	H
CCCVIII:	5-*O*-methylglycyrol	H	CH_3	$-CH_2-CH=C(CH_3)_2$	OCH_3	H	H	H	H

Structures of Coumestans

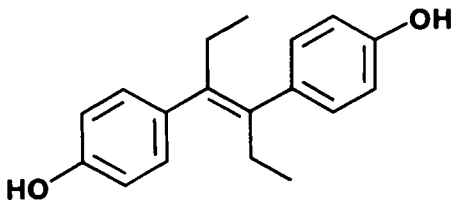
the uterine weight of immature mice. Therefore, the presence of estrogenic activity in this natural product remains to be demonstrated conclusively.

Another group of compounds has been reported to exhibit estrogenic activity. Wenzel and Rosenberg (964) reported that 6,4′-dihydroxyflavone (CCCXI) was estrogenic following subcutaneous injection, and Pretorius *et al.* (965) later found that quercetin (CXLVI) and robinin (CCCXII) showed slight estrogenic activity. Tricin (CCCXIII), from *Medicago sativa*, was also shown to have slight estrogenic activity (966). The estrogenic activity of quercetin has been questioned (967).

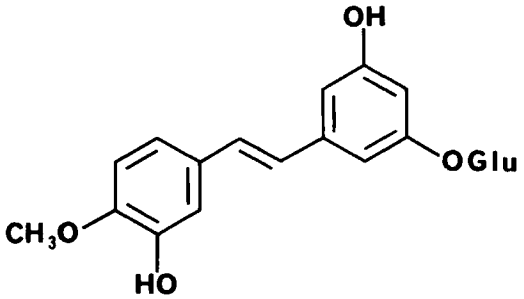
A number of reviews of plants containing estrogenic compounds have appeared (175, 928, 949, 968–1004).

A survey of 136 fodder plants for estrogenic activity appeared (1005) but was not available. Table IX presents a list of plants having estrogenic activity. A list of compounds obtained from plants that exhibit estrogenic activity is given in Table X.

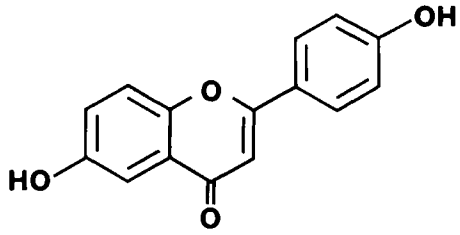
Humulus lupulus, *Avena sativa*, and *Oryza sativa* have been



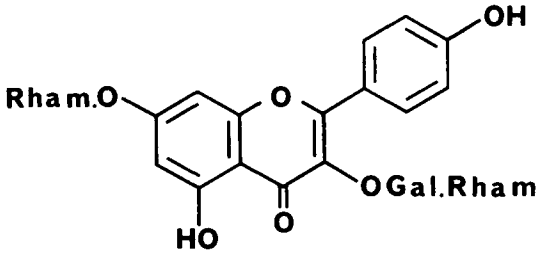
CCCIX: diethylstilbestrol



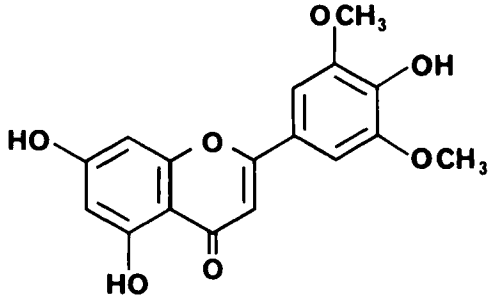
CCCX: rhaponticin



CCCXI: 6,4′-dihydroxyflavone



CCCXII: robinin



CCCXIII: tricin

shown to exhibit estrogenic properties (Table IX); these same plants have also been shown to induce ovulation (1006). Although there is no evidence of positive correlation between these two properties, it is of interest that the following plants also have been mentioned to induce ovulation: *Aralia cordata* (1006), *Citrus limon* (1006), *Lycium chinense* (1006, 1007), and *Raphanus sativus* (1006).

A number of plants have been reported from which products were obtained having properties similar to those of animal hormones. These products were not characterized, and no further details are available. Reviewers (928) have tended to assume that these products were estrogenic and have included them in tables of estrogenic plants. These claims may be unjustified and the plants are listed here rather than in Table IX, *i.e.*, *Convallaria majalis*,

Table IX—Plants Reported to Exhibit Estrogenic Activity

Plant Name		Reference
Agavaceae		
Agave americana		1349
Yucca aloifolia		1349
Anacardiaceae		
Mangifera indica		1350
Apocynaceae		
Funtumia sp.		1351
Holarrhena		1351
Araliaceae		
Panax ginseng		1352, 1353
Asclepiadaceae		
Asclepias tuberosa		263, 264, 1039
Balsaminaceae		
Impatiens balsamina		1354
Betulaceae		
Alnus glutinosa		968
Boraginaceae		
Lithospermum ruderale		177
Bromeliaceae		
Ananas comosus		194, 1355
Tillandsia aloifolia		1355
Tillandsia balbisiana		1355
Tillandsia circinata		1355
Tillandsia fasciculata		1355
Tillandsia juncea		1355
Tillandsia simulata		1355
Tillandsia tenuifolia		1355
Tillandsia usneoides		1356
Caprifoliaceae		
Sambucus niger		1357
Chenopodiaceae		
Beta vulgaris		924, 1358–1361
Compositae		
Calendula officinalis		1362
Carthamus tinctorius		1363
Chrysactinia mexicana		1364
Helianthus annuus		1365
Cruciferae		
Brassica napus		1365, 1366
Brassica pekinensis		1367
Cyperaceae		
Cyperus rotundus		1368
Euphorbiaceae		
Ricinus communis		1369
Gramineae		
Avena sativa		925, 968, 1038, 1370–1373
Agrostis tenuis		1374
Bromus mollis		1375–1378
Cynodon dactylon		1051
Dactylis glomerata	+	194, 1374, 1379–1384
	−	1382, 1383, 1385
Eragostris curvula		1386
Festuca pratensis		1371, 1374, 1387
Festuca rubra		1374, 1387
Festuca arundinacea		1383
Hordeum vulgare		1373, 1388
Hyparrhena filipendula		1051
Lolium perenne	+	194, 1374, 1379, 1380, 1383, 1389
	−	1383
Lolium rigidum	+	1390
	−	1391
Oryza sativa		1038, 1363, 1392
Phalaris arundinacea	+	1383
	−	1383
Phleum pratense	+	1374, 1381, 1384, 1393, 1394
	−	1374, 1379, 1395
Poa pratensis	+	1396
	−	1375, 1378, 1385, 1397
Poa trivialis		1374
Secale cereale	+	1385
	−	1385
Setaria ciliolata		1051
Triticum aestivum	+	1038, 1363, 1366, 1389, 1398–1400
	−	1375, 1378, 1385, 1397
Zea mays	+	1363, 1365, 1371, 1389, 1401

(continued)

Table IX—*(Continued)*

Plant Name		Reference
	−	1375, 1376, 1378
Labiatae		
Leonurus sibiricus		1402
Salvia officinalis		1403
Lauraceae		
Cinnamomum zeylanicum		1404
Leguminosae		
Arachis hypogaea		1363, 1365, 1405
Astragalus lentiginosus		1406
Astragalus miser		1407
Astragalus sinicus		1408
Butea superba		1223, 1409–1413
Butea sp.		1414–1417
Calopogonium muconoides		1405
Centrosema pubescens		1405
Ceratonia siliqua		1418
Cicer arietinum		1419
Crotalaria sp.		1405
Cytisus scoparius		1420
Glycine max	+	1365, 1375, 1389, 1405, 1408, 1421–1424
	−	1378
Glycyrrhiza glabra	+	1036, 1401, 1425–1433
	−	1434
Glycyrrhiza uralensis		1431
Glycyrrhiza sp.		1039
Leucaena glauca		1405
Lotus corniculatus	+	1383, 1389, 1396, 1435–1438
	−	1383
Lotus uliginosus		1439
Lupinus polyphyllus	+	1158
	−	1440
Lupinus termis		1441, 1442
Medicago falcata		1374
Medicago littoralis		1026, 1443
Medicago lupulina		1374, 1444
Medicago sativa		939, 1014, 1032, 1051, 1169, 1374–1377, 1381–1383, 1385, 1387, 1389, 1397, 1401, 1405, 1436, 1440, 1445–1478
Medicago tribuloides		1479
Medicago truncatula		1443
Melilotus officinalis		1473, 1475
Phaseolus aureus		1405
Phaseolus calcartus		1405
Phaseolus vulgaris		1040, 1480
Pisum sativum		1367, 1371, 1458, 1473, 1475
Pueraria javanica		1405
Trifolium alexandrium	+	1373, 1429, 1481, 1482
	−	1372
Trifolium fragiferum	+	1483
	−	1484
Trifolium hybridum		1374, 1383
Trifolium incarnatum		1374, 1444
Trifolium pratense	+	959, 975, 1000, 1011, 1013, 1032, 1034, 1169, 1258, 1260, 1367, 1371, 1374, 1379, 1381, 1383, 1384, 1387, 1389, 1393, 1394, 1397, 1401, 1436, 1437, 1439, 1444, 1445, 1450, 1467, 1471, 1473, 1475, 1476, 1485–1514
	−	1484
Trifolium repens	+	1290, 1345, 1374, 1376, 1383, 1389, 1395, 1396, 1411, 1412, 1435–1437, 1440, 1459, 1471, 1475, 1476, 1483, 1515–1521
	−	1383, 1397, 1473, 1483, 1484, 1510, 1511, 1522, 1523
Trifolium subterraneum		930, 931, 935, 936, 975, 1000, 1010, 1012, 1013, 1259, 1281, 1287, 1371, 1384, 1390, 1444, 1463, 1479, 1483, 1492, 1514, 1515, 1524–1549
Vicia americana		1407
Vicia angustifolia		1475
Vigna sesquipedalia		1405
Vigna sinensis		1405
Liliaceae		
Allium porrum		1349
Allium ursinum		1550
Chamaelirium luteum		1039
Phalangium liliago		1349
Tulipa gesneriana		1551, 1552
Veratrum californicum		153
Linaceae		
Linum usitatissimum		1363, 1365, 1389
Malvaceae		
Althaea rosea		1354
Meliaceae		
Melia azadirachta		1553
Moraceae		
Artocarpus integrifolia		1350
Ficus infectoria		1350
Ficus religiosa		1350
Humulus lupulus	+	962, 1353, 1367, 1554–1559
	−	1560
Morus sp.		1350
Moringaceae		
Moringa oleifera		1405
Myrtaceae		
Eucalyptus sp.		1561
Eugenia jambolana		1350
Nymphaeaceae		
Nuphar luteum		1354, 1562
Oleaceae		
Olea europaea		1363, 1365
Palmae		
Cocos nucifera		1363
Elaeis guineensis		925, 1365
Phoenix dactylifera		1043
Serenoa repens		1563
Pinaceae		
Pinus ponderosa	+	1564
	−	1565, 1566
Polygonaceae		
Rheum rhaponticum		968
Punicaceae		
Punica granatum		1567
Rosaceae		
Prunus cerasus		924
Prunus domestica		924
Rubiaceae		
Coffea arabica		1568
Ophiorrhiza mungos		1405
Salicaceae		
Salix babylonica		1349
Salix caprea		926, 968, 1354, 1366, 1388, 1562, 1569
Solanaceae		
Lycium chinense		1570
Solanum tuberosum		924, 1366
Sterculiaceae		
Theobroma cacao		1365
Tiliaceae		
Tilia cordata		1349
Tilia europaea		1357
Tilia platyflora		1349
Umbelliferae		
Daucus carota		1399, 1400, 1571
Foeniculum vulgare		1561
Heracleum sosnowskyi		1401
Levisticum officinale		1572
Petroselinum crispum		924
Pimpinella anisum		1428, 1429, 1561
Urticaceae		
Musanga cecropioides		1573
Urtica sp.		1357
Violaceae		
Viola odorata		1574
Zingiberaceae		
Costus speciosus		1575
Unidentified grasses		1371, 1447, 1547, 1576–1580

Table X—Compounds Reported to Have Estrogenic Activity

Compound Name		Reference
Anethole (CCCXIV)		1561
Anisole (CCCXV)		1561
Asiaticoside (CCCXVI)		1172
Biochanin A (CCIII)		999, 1011, 1031–1034, 1246, 1259, 1581
Cafesterol (CCCXVII)	+	1582, 1583
	−	1584–1587
Clupanodonic acid		1588
Colchicine (CXVIII)		1589
Coumestrol (CCLXXXIX)		994, 999, 1011, 1014, 1590–1598
Daidzein (CLIX)	+	1031–1034
	−	928
Deserpidine (CCCXVIII)		1599
Diosgenin (CCCXIX)		1600
17α-Estradiol (CLV)		1601
Estriol (CLVII)		1601
Estrone (CLVIII)		1601
Formononetin (CLXIII)	+	999, 1011, 1014, 1031–1034, 1259, 1273
	−	928, 972, 1034
Genistein (CXCIII)		930, 933, 999, 1011, 1014, 1031–1034, 1259, 1592, 1596, 1602–1604
Genistein 7-*O*-glucoside (CXCV)		1375, 1478, 1591
Genistein 4′-*O*-glucoside (CXCIV)		1605
Gibberellic acid (CCCXX)		1606, 1607
Miroestrol (CCLXXVIII)		994, 1608–1611
Nicotine (X)	+	1613–1615
	−	1616
Phloretin (CCCXXI)		1617
Pilocarpine (CXXVI)		1618
Podocarpic acid (CCCXXII)		1619
Prunetin (CC)		928
Reserpine (LXXXIX)	+	1620–1624
	−	1620–1629
Rhaponticin (CCCX)	+	962
	−	963
β-Sitosterol (CXLII)		1035, 1037
Glycyrrhiza glabra steroids		1427

Table XI—Relative Potency of Sterol Estrogens, Coumestrols, and Isoflavones

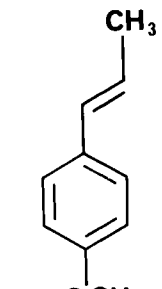
Compound	Quantity Required to Produce a 25-mg Uterus, μg	Relative Potency
Control	—	—
Diethylstilbestrol	0.083	100,000
Estrone	1.20	6,900
Coumestrol	240	35
Genistein	8,000	1.00
Daidzein	11,000	0.75
Biochanin A	18,000	0.46
Formononetin	32,000	0.26

Malus pumila, Petroselinum crispum, Prunus cerasus, Rheum rhaponticum, and *Solanum tuberosum* (1008).

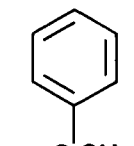
The metabolism of phytoestrogens in animals has been investigated in the sheep (1004–1014), rat (1015), cow (1016, 1017), fowl (1018–1021), other animals (1022), and liver enzyme systems (1023), and the effect of phytoestrogens on the blood in sheep has been studied (1024–1027).

If one inspects the structures of the estrogenic sterols (CXLII and CLV–CLVIII), isoflavones (CLIX–CCLXXXVIII), and coumestans (CCLXXXIX–CCCVIII), one can see a striking similarity of the skeletal structures of these compounds with the structure of the synthetic estrogen diethylstilbestrol (CCCIX).

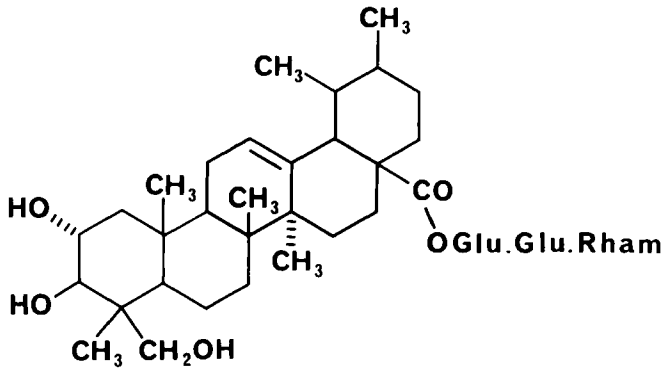
Recently, coumestrol (CCLXXXIX) and genistein (CXCIII) were shown to compete with 17β-estradiol (CLVI) for binding sites on a macromolecular component of the uterus from 6-day pregnant rabbits. The binding affinity of these compounds was related to their reported estrogenic potency; *i.e.*, one part by weight of 17β-estradiol, 70 of coumestrol, and 175 of genistein produced equivalent inhibition of the uptake *in vitro* of ^{3}H-17β-estradiol by the uterine receptor. Biochanin A (CCIII), formononetin (CLXIII), 4′-*O*-methylcoumestrol (CCXCI), sativol (CCXCVI), and medicagol (CCC) did not significantly inhibit 17β-estradiol binding, suggesting that free hydroxyl groups in positions 7 and 12



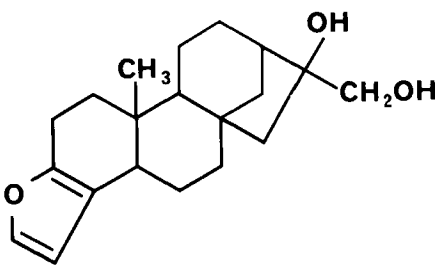
CCCXIV: anethole



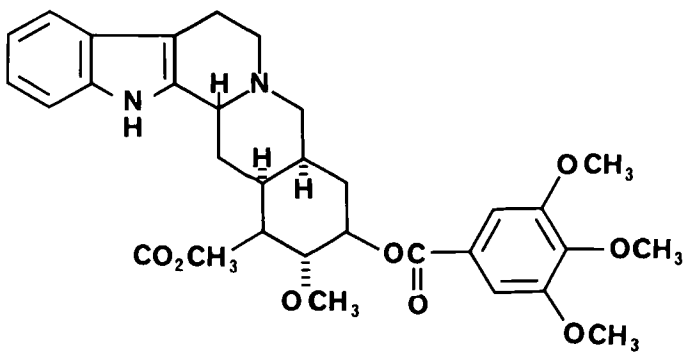
CCCXV: anisole



CCCXVI: asiaticoside

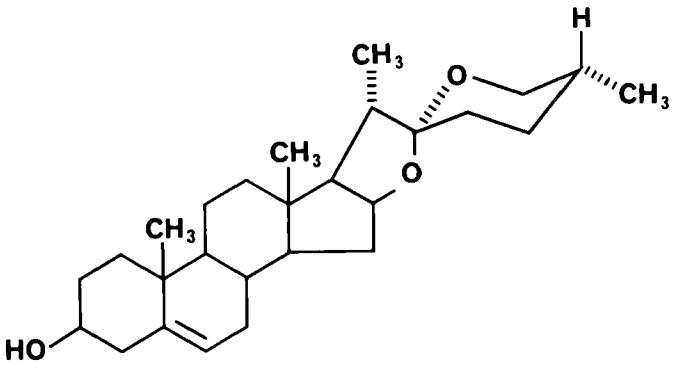


CCCXVII: cafesterol

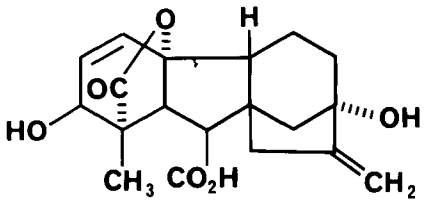


CCCXVIII: deserpidine

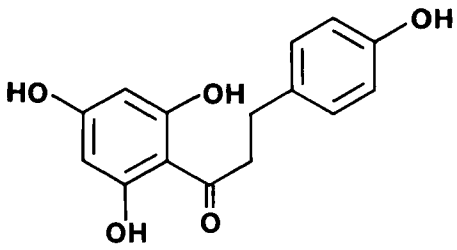
Structures of Additional Compounds Having Estrogenic Activity



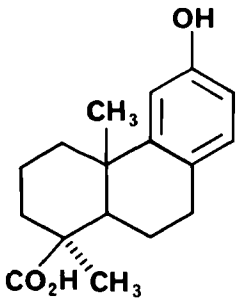
CCCXIX: diosgenin



CCCXX: gibberellic acid

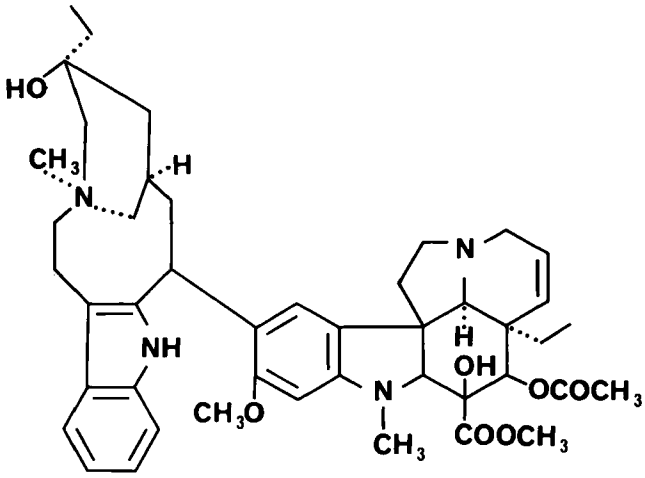


CCCXXI: phloretin

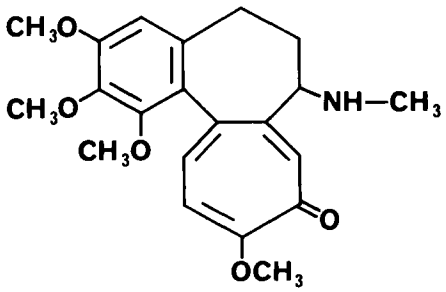


CCCXXII: podocarpic acid

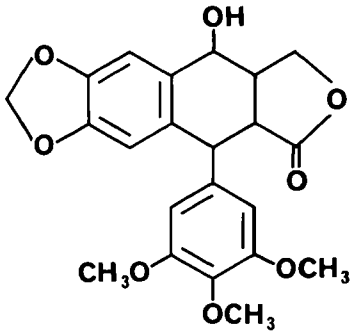
Structures of Additional Compounds Having Estrogenic Activity



CCCXXIII: vinblastine



CCCXXIV: demecolcine



CCCXXV: podophyllotoxin

of coumestans and isoflavones are essential for effective interaction with the estrogen receptor. The 7- and 4′-methoxycoumestans and isoflavones tested appeared to be proestrogens, able to bind to the uterine receptor only after *O*-demethylation *in vivo* (1029).

The order or degree of the biological activity of each of the three main groups of natural estrogens has been investigated (1030). The sterol estrogens were found to be of the highest order of activity, followed by the coumestrols and then the isoflavones. The relative potency of each substance, tested by the mouse-uterine weight bioassay method, with genistein arbitrarily chosen as the reference substance, is given in Table XI.

Other investigators studied the relative estrogenicity of the isoflavones and coumestan-type compounds. Comparison of the estrogenic activity of prunetin (CC), genistein (CXCIII), daidzein (CLIX), biochanin A (CCIII), and formononetin (CLXIII) led to the conclusion that the 5-hydroxyl group was essential for estrogenic activity (928). Genistein, biochanin A, and prunetin were estrogenic, while daidzein and formononetin were devoid of activity. Coumestrol (CCLXXXIX) was found to be many times more active than any of the isoflavonoid derivatives when administered intrarumenally to ovariectomized animals (1011). More recently, Leavitt and Meismer (999) reported that the relative estrogenic properties were in the order coumestrol > genistein > biochanin A > formononetin.

In addition to the work mentioned previously, some studies (1031–1034) have shown that daidzein was the most active isoflavonoid derivative in the mouse-uterine weight assay; biochanin A and genistein were of lesser but approximately equal activity, and

formononetin was essentially inactive. The other isoflavones listed in Table VII have not yet been examined for estrogenic activity, but they have been included because of potential estrogenic activity.

CYTOTOXIC AGENTS AS ANTIFERTILITY DRUGS

Certain naturally occurring cytotoxic agents have been shown to elicit an antifertility effect in laboratory animals, either by local or systemic routes of administration (70, 1630, 1631). For such a compound to be useful as an antifertility agent, it would have to be completely nontoxic, without teratogenic manifestation in marginal doses, and 100% effective (70). A number of plant products have been shown to elicit varying degrees of antifertility activity due to cytotoxicity. For example, vinblastine (CCCXXIII) from *Catharanthus roseus* (Apocynaceae), when administered to rabbits, reduced the percentage of normal fetuses at term to 0–33.3%; however, there were three fetal abnormalities produced in a group of 60 rabbits (70). An attempt was made to prevent this toxic effect by the concomitant administration of glutamic acid, which is known to prevent general toxic effects of vinblastine in mice. When administered prior to vinblastine, a 53.6% implantation rate was observed, with only 41.2% developing into normal fetuses (70).

Demecolcine (CCCXXIV), derived from several *Colchicum* species (Liliaceae), appears to act directly on the fetus and not on the placenta. This alkaloid, in doses of 2–8 mg/kg sc, destroyed the fetuses of rabbits 13–16 days pregnant. The drug was less effective when given orally (70). One or two doses of 2.5 mg/kg ip of demecolcine given to pregnant rats destroyed all litters when given between Days 11 and 19 of gestation. Fetuses showed marked edema, ascites, and hemorrhagic staining of internal organs and skin 6–12 hr after administration of the drug. No gross abnormalities were seen, but stunting was observed in a few surviving fetuses of rats treated with lower doses (70).

Podophyllotoxin (CCCXXV), a lignan derived from *Podophyllum* species (Berberidaceae), was administered to mice as a single subcutaneous dose of 0.25 mg, either immediately after detection of a vaginal plug or 3, 6, 12, or 14 days thereafter. Pregnancy did not continue in animals treated 3 days or more after copulation. Administered within 24 hr after copulation, podophyllotoxin was only occasionally effective (1631).

A number of plants listed in Tables II and III have a folkloric reputation as antifertility agents or have shown some degree of antifertility activity in laboratory animals. Table XII gives the names of those plants found in Tables II and III that are known to contain cytotoxic compounds, or crude extracts of which have shown cytotoxic or antitumor activity and thus could owe their antifertility activity to this mechanism. Known cytotoxic agents are also indicated for each plant in Table XII.

DISCUSSION

It can be seen in Table III that the effects, or lack thereof, of various plant extracts on the fertility of laboratory animals has been expressed in a number of ways by different workers. In the light of current knowledge concerning the reproductive cycles of the various laboratory species *(vide supra)*, it sometimes has not been clear from the procedures and data reported by some workers as to how they arrived at their conclusions that a particular mechanism was responsible for the antifertility effect produced by a given extract. Furthermore, lack of the use of control (vehicle-treated) animals in many studies leaves one to guess what would have been the expected rate of successful pregnancies in the strains and species used had no plant extract been administered. However, a clue was offered by Khanna *et al.* (103); in their estimation, an extract showing 100% "inhibition of implantation" had significant activity, one showing at least 50% inhibition had encouraging activity, and one showing less than 50% inhibition had no significant activity.

With such criteria in mind, it can be seen that some plants listed in Table III do not appear to have antifertility activity in spite of the fact that many of these same plants have a folkloric reputation for having contraceptive activity (Table II). If the fact that species variability *(vide supra)* could be at least partly responsible for the lack of correlation is ignored, it may be considered possible that such a lack could be due at least partly to the testing procedures used. For example, as indicated in the footnote to Table II, a number of plants (including *Calotropis gigantea, Carica papaya, Momordica charantia, Dendrocalamus strictus, Uraria lagopoides, Gloriosa superba, Piper longum,* and *Apium graveolens*) have been stated to have antifertility effects although it is possible that their activity might more narrowly fall into the abortifacient or emmenagogic categories. The plants just named, however, when tested in animals, were never administered past the 7th day of pregnancy; that is, they were administered until just barely beyond the time of implantation, probably not long enough to test for any abortifacient effect other than a very early acting one.

A few of the plants listed in Table II as having a folkloric reputation as antifertility agents are reported in Table III as having antiovulatory activity; these plants are *Apocynum androsaemifolium, Asclepias hallii,* and *Hibiscus rosa-sinensis. Semecarpus anacardium, Mallotus philippinensis, Ricinus communis, Abrus precatorius,* and *Plumbago zeylanica* also are listed in both Tables II and III. These plants, however, apparently have not been tested for antiovulatory activity. As indicated in Table III, extracts of these plants have been administered to animals following mating and have been shown to be ineffective in inhibiting pregnancy under these conditions, probably due to a lack of interceptive activity and/or anti-implantational activity *per se (vide supra)*. That these plants do not possess antifertility activity by means of an antiovulatory effect has not been shown.

Scheme I suggests a means by which one may test for the various possible mechanisms of antifertility activity in the female mammal. If one merely wishes to determine whether a given extract has contraceptive, interceptive, and/or abortifacient activity, the entire procedure can be carried straight through. If activity is demonstrated early in the scheme, obviously additional animals will be required to examine for later effects. As indicated earlier in this paper, a given compound may inhibit fertility by more than one mechanism and/or at more than one site. The offshoots at the right of the scheme indicate the means by which further details concerning mechanism(s) of action may be elucidated. The rat, which appears to be the most commonly used test species, is the species indicated in Scheme I; other species, of course, have been and can be used, but variations among their reproductive cycles (Table I and Ref. 4) should be considered.

It appears, then, that investigators interested in the further

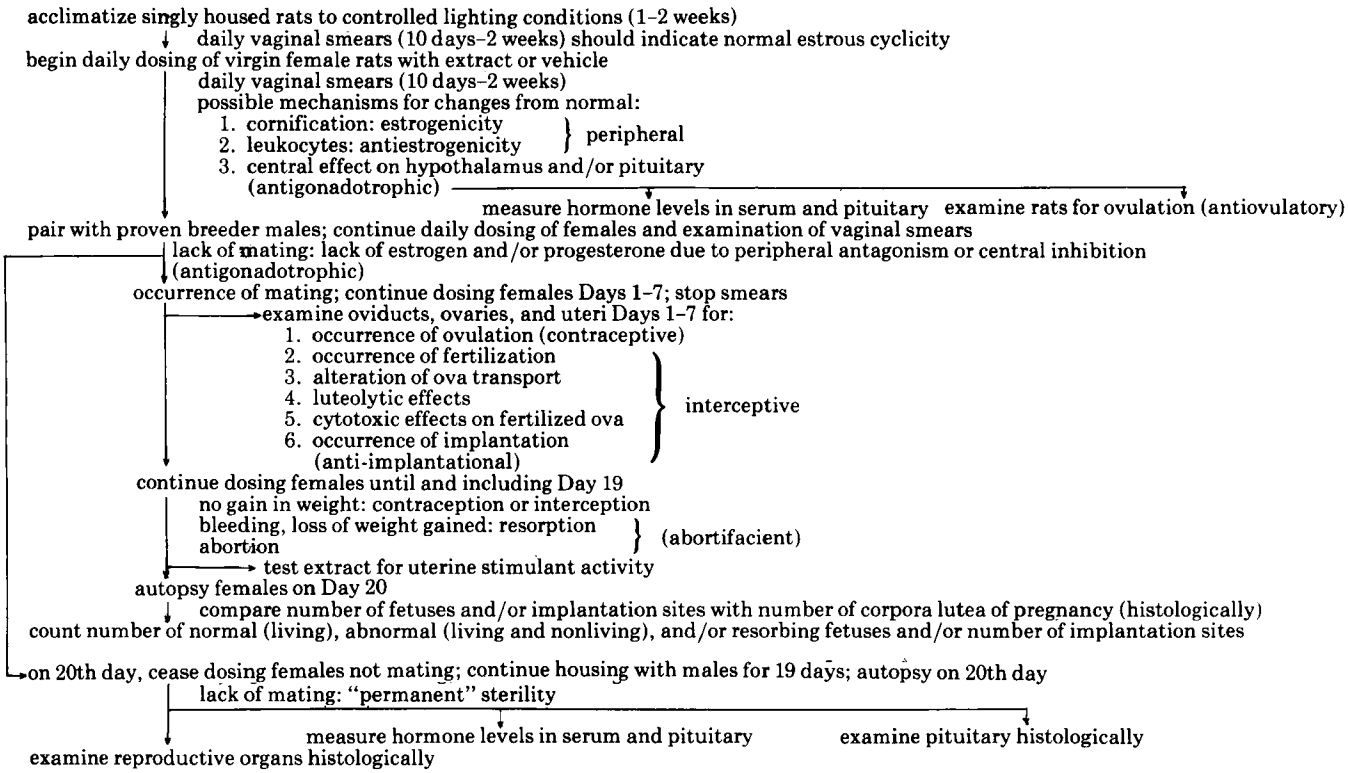
Table XII—Plants Whose Alleged or Demonstrated Antifertility Activity Could Be Explained on the Basis of Cytotoxicity

Plant Name	Cytotoxic Agent	Reference
Annonaceae		
Annona squamosa	Cytotoxicity	1640
Apocynaceae		
Apocynum androsaemifolium	Cardenolides	1632, 1633
Apocynum cannabinum	Apocannoside	1634, 1635
Catharanthus roseus	Vinblastine, vincristine, leurosine, leurosivine, rovidine, leurosidine	1636
Cerbera manghas	Cardenolides	1632
Nerium indicum	Cardenolides	1632
Rauvolfia serpentina	Reserpine	1671
Thevetia peruviana	Cardenolides	1632

(continued)

Table XII—(*Continued*)

Plant Name	Cytotoxic Agent	Reference
Aristolochiaceae		
Aristolochia clematitis	Aristolochic acid	1632, 1637
Aristolochia indica	Aristolochic acid	1632, 1637
Asarum canadense	Aristolochic acid	1632, 1635, 1637, 1638
Asclepiadaceae		
Asclepias hallii	Cardenolides	1632
Asclepias syriaca	Cardenolides	1632
Marsdenia cundurango	Cytotoxicity	1639
Berberidaceae		
Podophyllum peltatum	Podophyllotoxins	1632, 1641, 1642
Boraginaceae		
Lithospermum arvense	Cytotoxicity	1635
Symphytum officinale	Cytotoxicity	1643, 1644
Compositae		
Artemisia sp.	Sesquiterpene lactones	1632
Cruciferae		
Capsella bursa-pastoris	Cytotoxicity	1633
Raphanus sativus	Cytotoxicity	1645
Cucurbitaceae		
Citrullus colocynthis	Cucurbitacins	1632, 1646–1648
Cucumis sativus	Cytotoxicity	1649
Ecballium elaterium	Cucurbitacins	1632, 1646, 1647, 1650
Luffa cylindrica	Cucurbitacins	1632, 1646, 1648
Momordica charantia	Cucurbitacins	1639
Momordica tuberosa	Cucurbitacins	1632
Ericaceae		
Arctostaphylos uva-ursi	Cytotoxicity	1667
Euphorbiaceae		
Euphorbia resinifera	Cytotoxicity	1646
Excoecaria agallocha	Cytotoxicity	1651
Jatropha curcas	Jatrophone	1662
Mallotus philippinensis	Cytotoxicity	1652
Stillingia sylvatica	Cytotoxicity	1635
Leguminosae		
Abrus precatorius	Abrin	1653
Gleditsia horrida	Cytotoxicity	1654
Phaseolus aureus	Cytotoxicity	1640
Pisum sativum	Cytotoxicity	1655
Psoralea corylifolia	Cytotoxicity	1654
Liliaceae		
Asparagus officinalis	Cytotoxicity	1633
Colchicum autumnale	Colchicine, demecolcine	1656–1658
Loranthaceae		
Phoradendron flavescens	Cytotoxicity	1649, 1659, 1660
Lythraceae		
Lawsonia inermis	Lawsone	1651
Malvaceae		
Gossypium herbaceum	Gossypol	1659, 1661
Menispermaceae		
Cissampelos pareira	Cytotoxicity	1663
Stephania hernandifolia	Cytotoxicity	1656, 1664
Myristicaceae		
Myristica fragrans	Cytotoxicity	1654
Papaveraceae		
Chelidonium majus	Protopine	1665
Polygonaceae		
Polygonum multiflorum	Cytotoxicity	1654
Polypodiaceae		
Dryopteris filix-mas	Cytotoxicity	1652
Punicaceae		
Punica granatum	Cytotoxicity	1654
Ranunculaceae		
Aconitum napellus	Cytotoxicity	1639
Paeonia sp.	Cytotoxicity	1654, 1666, 1667
Rosaceae		
Sanguisorba officinalis	Cytotoxicity	1654
Rutaceae		
Citrus aurantium	Cytotoxicity	1654
Evodia rutaecarpa	Cytotoxicity	1660
Santalaceae		
Santalum album	Cytotoxicity	1654
Saxifragaceae		
Hydrangea arborescens	Cytotoxicity	1633
Scrophulariaceae		
Digitalis lanata	Cardenolides	1632
Rehmannia glutinosa	Cytotoxicity	1654
Simaroubaceae		
Simarouba amara	Cytotoxicity	1652
Solanaceae		
Solanum dulcamara	β-Solamarine	1669
Umbelliferae		
Siler divaricatum	Cytotoxicity	1654



Scheme I

study of plants as a source of new antifertility agents should note certain data that have been presented herein. First, a proper dosing schedule (Scheme I) should be followed in the testing that will allow the investigator to detect plants, the activity of which may be caused by constituents having a mechanism of interest. Second, it is apparent from published data that many studies have lacked adequate controls; vehicle-treated animals should always be studied concurrently so that the investigator can know the normal pregnancy rate for that particular strain and species of animal. Third, one should not rely on a single solvent extract for testing. For example, it has been shown in the case of *Medicago sativa* that the ether extract was estrogenic, that the chloroform extract was antiestrogenic, and that the acid–water extract interfered with seminal vesicle growth (141). In the case of *Ocimum sanctum*, the benzene extract showed 80% antifertility activity, the petroleum ether extract 60%, and the ethanol extract less than 50% (99).

To separate plant material for testing into fractions of differing polarity, it seems that each plant should be extracted first with petroleum ether. The marc should then be extracted with either ethanol or methanol, with this extract being partitioned between chloroform and water. The resulting three extracts, *i.e.*, petroleum ether, chloroform, and water, should then each be evaluated for antifertility effects; one should not rely on an evaluation of a single solvent extract alone.

Finally, the plant names presented in Tables II and III have been examined and compared with the data presented in Tables IV–XII. From this comparison, we have deleted all plant names in Tables II and III that have also been found in Tables IV–XII. This new list of suggested plants to investigate for antifertility activity is presented in Table XIII.

Table XIII—Plants Suggested to Be Reinvestigated for Antifertility Activity

Plant Name	Plant Part[a]	Reference
Aizoaceae		
Trianthema pentandra	—	192
Trianthema portulacastrum	—	192
Amaranthaceae		
Achyranthes aspera	—	192
Amaranthus retroflexus	PL	169
Amaranthus spinosus	—	171
Amaryllidaceae		
Stenomesson variegatum	—	92
Anacardiaceae		
Rhus trilobata	LF	92
Semecarpus anacardium	RT, SD	90, 103, 192
Semecarpus stellata	RT	92
Annonaceae		
Artabotrys odoratissimus	LF	104
Araceae		
Acorus calamus	RT	91
Anthurium tessmannii	IF	92, 93
Arisaema atrorubens	RZ	92
Ariasaema triphyllum	RT	90, 92
Philodendron dyscarpium	IF	92, 93

(continued)

Table XIII—(*Continued*)

Plant Name	Plant Part[a]	Reference
Urospatha antisylleptica	SP	92, 93
Araliaceae		
Fatsia horrida	PL	169
Hedera helix	FR, FL	90, 92
Asclepiadaceae		
Marsdenia cundurango	BK	171
Basellaceae		
Basella alba	RT	91
Berberidaceae		
Epimedium alpinum	LF, RT	90
Betulaceae		
Betula bhojpattra	SB	91
Bignoniaceae		
Dolichandrone falcata	—	192
Boraginaceae		
Anchusa officinalis	PL	170
Borago officinalis	PL	169
Cordia dichotoma	FR	91
Cordia quarensis	RT	90
Echium vulgare	PL	170
Lithospermum arvense	PX, PL	90, 169
Lithospermum croceum	PX, RT	169
Lithospermum distichum	PX, RT	169
Lithospermum latifolium	PX, RT	169
Lithospermum officinale	PX, RT, LF, PL	92, 114, 169, 188
Lithospermum ruderale	PX, RT	90, 92, 97, 111, 112, 169, 180, 188
Symphytum officinale	PL, RT	115
Bromeliaceae		
Tillandsia decomposita	FS	92
Buddlejaceae		
Buddleja asiatica	—	192
Capparidaceae		
Crataeva nurvala	SB	91, 92
Caprifoliaceae		
Lonicera ciliosa	LF	92, 117
Caryophyllaceae		
Dianthus superbus	PL	120, 121
Vaccaria pyramidata	PL	121
Celastraceae		
Celastrus paniculatus	—	192
Chenopodiaceae		
Chenopodium album	LF	127
	PL	90, 169
Combretaceae		
Terminalia catappa	—	171
Commelinaceae		
Aneilema scapiflorum	—	171
Compositae		
Achillea millefolium	PL	90
Ambrosia artemisifolia	PL	169
Artemisia maritima	—	171
Atractylis gummifera	RT	92
Chrysanthemum indicum	—	171, 192
Cnicus benedictus	PL	90, 169
Echinops echinatus	—	171
Eupatorium odoratum	RT	90
Franseria artemisioides	PL	92
Solidago odora	LF	171
Tanacetum umbelliferum	—	171, 192
Convolvulaceae		
Cuscuta reflexa	—	171, 192
Crassulaceae		
Crassula abyssinica	PL	98
Cruciferae		
Anastatica hierochuntica	—	171, 192
Brassica campestris	—	171
Brassica nigra	SD	91, 171
Isatis oblongata	PL	121
Lepidium sativum	—	171, 192
Raphanus sativus	PL	121
Dioscoreaceae		
Dioscorea sativa var. *rotunda*	TU	90
Ericaceae		
Arctostaphylos uva-ursi	PL	169
Rhododendron anthopogon	—	171
Euphorbiaceae		
Euphorbia neriifolia	RT	91
Euphorbia resinifera	—	192
Euphorbia tirucalli	—	171, 192

Table XIII—(*Continued*)

Plant Name	Plant Part[a]	Reference
Excoecaria agallocha	—	192
Stillingia sylvatica	RT	171
Gentianaceae		
Frasera speciosa	PL	90, 92
Gramineae		
Bambusa arundinacea	RT	91
Chusquea ramosissima	YS	92
Dendrocalamus strictus	LF	108, 192
Echinochloa frumentacea	—	171
Guttiferae		
Garcinia morella	—	192
Garcinia pedunculata	—	171
Labiatae		
Lycopus europaeus	LF	115
Lycopus lucidus	PL	121
Ocimum basilicum	LF	92
Ocimum sanctum	LF	99
Origanum majorana	LF	90
Rosmarinus officinalis	PL	90, 92, 115
Salvia plebeia	—	192
Lauraceae		
Cinnamomum cassia	—	192
Lecythidaceae		
Combretodendron africanum	SB	130
Leguminosae		
Astragalus glycyphyllus	PL	132
Cassia lanceolata	—	192
Clitoria ternatea	FR	121
Desmodium retroflexum	—	192
Entada scandens	SD	90, 92
Erythrina variegata var. *occidentalis*	—	171, 192
Gleditsia horrida	FR	137
Piliostigma thonningii	RT	92
Prosopis algarobilla	RT	92
Rhynchosia minima	—	192
Sesbania sesban	FL, LF	102, 171
Vigna phaseoloides	RT	92
Liliaceae		
Aloe barbadensis	LF, FP	102, 116, 192
Asagraea officinalis	SD	171
Asparagus acutifolia	FR	90, 92
Asparagus officinalis	FR	90, 92
Chamaelirium luteum	PL	169
Smilacina stellata	RT, LF	90, 92
Smilax bona-nox	RT	171
Loranthaceae		
Phoradendron flavescens	LF	90
Magnoliaceae		
Magnolia virginiana	BK	171
Malvaceae		
Hibiscus abelmoschus	FL	92
Hibiscus rosa-sinensis	PT	99, 171, 192
Hibiscus tiliaceus	FL	92
Sphaeralcea munroana	RT	92
Urena lobata	LF	90, 92, 192
Melastomataceae		
Memecylon amplexicaule	—	171
Menispermaceae		
Curarea tecunarum	ST	172
Stephania hernandifolia	RZ	102
Musaceae		
Ensete superbum	SD	155
Myristicaceae		
Myristica fragrans	—	192
Virola sp.	ST	101
Myrsinaceae		
Embelia ribes	RT, PL	91, 138
Myrtaceae		
Metrosideros collina	LF	105
Oleaceae		
Jasminum multiflorum	—	171
Palmae		
Cocos nucifera	SD, FJ	90, 92
Licuala sp.	RB	92
Pandanaceae		
Pandanus tectorius	—	171
Papaveraceae		
Argemone glauca	PL, IF	105, 121

(continued)

Table XIII—(*Continued*)

Plant Name	Plant Part[a]	Reference
Phytolaccaceae		
Phytolacca decandra	—	171
Piperaceae		
Piper latifolium	RT	171
Piper leptostachyum	—	171
Piper longum	LF, RT, FR	102, 138, 171, 192
Plantaginaceae		
Plantago lanceolata	PL	90
Polemoniaceae		
Phlox stansburyi	LF	92
Polygonaceae		
Eriogonum jamesii	RT	90, 92
Polygonum hydropiper	RT, PL	90, 157, 158
Polygonum multiflorum	PL	121
Polypodiaceae		
Asplenium adiantum-nigrum	—	90, 92, 171, 192
Dryopteris filix-mas	RT, SD	90, 92, 192
Ranunculaceae		
Aconitum heterophyllum	RT	91
Aconitum napellus	RT	171
Rosaceae		
Geum macrophyllum	LF	92
Hagenia abyssinica	LF, ST	119, 192
Prunus emarginata	WD	92, 117
Pyrus communis	SB	159
Quillaja saponaria	BK	171
Rubus idaeus	LF	169
Sanguisorba officinalis	PL	90, 121, 160
Rubiaceae		
Adinia cordifolia	LF	161
Anthocephalus cadamba	—	171
Anthocephalus indicus	LF, FL	91
Randia dumetorum	FR	103
Randia spinosa	FR	91
Rubia cordifolia	PL	121
Rutaceae		
Evodia rutaecarpa	PL	121
Citrus maxima	—	171
Citrus medica	FR	91
Salicaceae		
Populus alba	SB	90, 92
Santalaceae		
Santalum album	—	192
Sapindaceae		
Sapindus trifoliatus	—	192
Saxifragaceae		
Hydrangea arborescens	RT	171
Schizaeaceae		
Lygodium dichotomum	RT	90, 92
Scrophulariaceae		
Castilleja angustifolia	—	92
Rehmannia glutinosa	PL	121
Solanaceae		
Solanum nigrum	—	192
Tiliaceae		
Triumfetta bartramia	—	192
Umbelliferae		
Anethum sowa	FR	91
Carum carvi	SD, FR	91, 127
Carum roxburghianum	SD	91
Cicuta maculata	RT, RJ	90, 92
Ferula assa-foetida	R	138, 192
Leptotaenia reticulata	—	171
Siler divaricatum	PL	121
Trachyspermum roxburghianum	—	171
Verbenaceae		
Callicarpa macrophylla	—	171
Clerodendrum phlomidis	—	171
Clerodendrum serratum	RT	91
Gmelina asiatica	—	171
Stachytarpheta jamaicensis var. *indica*	—	171, 192
Verbena hastata	PL	171
Vitex lagundi	RT	92
Vitex negundo	SD, RB, RT	90, 91, 192
Zingiberaceae		
Globba marantia	—	92

[a] BK = bark, FJ = fruit juice, FL = flower, FP = fruit pulp, FR = fruit, FS = flower stems, IF = inflorescence, LF = leaf, PL = whole plant, PT = petals, PX = aerial parts, R = resin, RB = root bark, RJ = root juice, RT = root, RZ = rhizome, SB = stem bark, SD = seed, SP = spadix, ST = stem, TU = tuber, WD = wood, and YS = young stems.

ADDENDUM

Three recent papers have come to our attention since the compilation of data reported herein, which we believe should be included in this review.

Kapoor *et al.* (1673) tested extracts of *Areca catechu; Carica papaya, Daucus carota, Mentha arvensis,* and *Polygonum hydropiper,* which had previously been reported to possess antifertility activity, for anovulatory effects in female rabbits. The only plants of this group showing anovulatory activity were the ethanolic extract of *M. arvensis* leaves and the petroleum ether extract of *P. hydropiper* roots.

Similarly, various extracts and chromatographic fractions of the stems and leaves of *Argemone mexicana,* the seeds of *C. papaya,* the seeds of *Lawsonia inermis,* the leaves of *M. arvensis,* and the seeds of *Sapindus trifoliatus* were tested for antifertility activity in female albino rats (1674). The ethanolic extract of *M. arvensis* leaves, as well as certain chromatographic fractions from a methanolic fraction of *S. trifoliatus* seeds, showed high degrees of antifertility activity.

Petroleum ether and aqueous extracts of *Curcuma longa* (Zingiberaceae) rhizomes exerted a 100% antifertility activity in rats at a dose of 200 mg/kg; the effect was not due to anovulatory activity (1675).

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RESEARCH ARTICLES

Release of Corticoids from Oleaginous Ointment Bases Containing Drug in Suspension

Z. T. CHOWHAN [x] and R. PRITCHARD

Abstract □ Simplified methods for studying the release of drugs suspended in oleaginous ointment bases were developed. These procedures were used in studying the release rates of two corticoids, fluocinonide and flucloronide, from white petrolatum and petrolatum containing various adjuvants. A practical method for measuring drug solubilities was developed and used in determining solubilities of these corticoids in ointment bases. When using physical data obtained from model ointments, the release rates of drugs from modified ointment bases were predicted. Comparisons of the observed and predicted rates from ointments containing hydrophobic adjuvants indicated the usefulness of the physical model approach in predicting the release rates. For ointments containing emulsifying agents, the simple model used did not provide useful predictions.

Keyphrases □ Fluocinonide—suspension, release from oleaginous ointment bases containing various adjuvants, model predictions □ Flucloronide—suspension, release from oleaginous ointment bases containing various adjuvants, model predictions □ Ointment bases—release of corticoids in suspension □ Release rates—corticoid suspensions from oleaginous ointment bases containing various adjuvants

Percutaneous absorption involves two consecutive steps: the release of the drug from the vehicle and its subsequent penetration through the skin barrier. Generally, the latter step controls percutaneous absorption, because it is the slower of the two events. The release of the drug from the vehicle may play an important role in percutaneous absorption when the drug solubility and its diffusion constant in the vehicle are very small. When the skin barrier is in a damaged state due to disease or injury, drug release from the vehicle then controls percutaneous absorption.

Simplified equations describing the drug release from suspension- (1) and solution- (2) type vehicles have been in the literature for more than a decade. Numerous studies also have attempted to relate vehicle composition to observed changes in the *in vitro* release rate (3–9). Relatively little quantitative information appears in the literature correlating drug release data with variations in physical parameters produced by compositional changes in the formulations. In some cases, the drug release from the vehicles has been complicated by the use of a membrane barrier to separate the donor phase from the receptor phase. The use of dialysis membranes (3), filter membranes (4), membranes of animal origin (5, 6, 8), and dimethyl polysiloxane membranes (7) has been reported.

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Simplified equations describing the drug release from suspension- (1) and solution- (2) type vehicles have been in the literature for more than a decade. Numerous studies also have attempted to relate vehicle composition to observed changes in the *in vitro* release rate (3–9). Relatively little quantitative information appears in the literature correlating drug release data with variations in physical parameters produced by compositional changes in the formulations. In some cases, the drug release from the vehicles has been complicated by the use of a membrane barrier to separate the donor phase from the receptor phase. The use of dialysis membranes (3), filter membranes (4), membranes of animal origin (5, 6, 8), and dimethyl polysiloxane membranes (7) has been reported.

When a membrane is used in the *in vitro* release experiment, it may be difficult to explain the data using simple physical relationships. The membrane might alter the release rate profiles due to physical interactions with the drug molecule.

The purpose of this investigation was to develop relatively simple experimental procedures for studying the drug release from ointments. The solubility of the drugs in ointment bases was determined using data from the effective ointment–water partitioning experiments. The physical parameters determined for model ointments were used to predict the release rates of the modified ointments. Comparisons of the observed and predicted rates were expected to elucidate understanding the physical processes important in the release of drugs from ointments.

EXPERIMENTAL

Materials—Materials for the preparation of ointments were used as received from the manufacturers. The ointment base was white petrolatum[1], base number six (USP). The adjuvants were Amerchol CAB[2], glyceryl stearate[3], beeswax[4], cetyl alcohol[5], stearyl alcohol[5], coconut fatty acid[6], glycerol monolaurate[6], glycerol monostearate[7], hexadecyl alcohol[8] (cosmetic grade), hexadecyl stearate[9] (cosmetic grade), lecithin[10], polysorbate 60[11], sorbitan monostearate[12], soya fatty acid[6], stearic acid[6], and wool alcohol[13] (lanolin, anhydrous, USP). The solvents used were reagent grade chloroform[14] and methylene chloride[15] and spectrograde anhydrous methanol[14] and dioxane[16].

Procedures—*Preparation of Ointments*—Several methods for making ointments where the drug is uniformly suspended in the form of fine particles in the external phase were considered. The most commonly used procedure, in which the ointment ingredients were mixed together and the melt was cooled while mixing, produced a supersaturated solution of the drug in the ointment base. Approaching equilibrium solubilities from a supersaturated solution of corticoids in ointments was found to be a slow process. Besides supersaturation, heating produced some degradation of the corticoids unless the conditions were carefully controlled. Mechanical incorporation of the corticoid in the ointment base was also tried. The small quantities of the ointments needed for these experiments and the low drug concentration (50 μg/g) made it difficult mechanically to incorporate the drug uniformly.

For obtaining uniform suspensions and attaining equilibrium solubilities without degrading the drug, the following procedure was developed and used throughout this investigation. The radioactive and cold fluocinonide[17] and flucloronide[18] were coprecipitated from methylene chloride in a polypropylene beaker[4]. Contact of the ointments with glass surfaces was avoided, because these steroids adsorb onto glass surfaces. One gram of white petrolatum and a sufficient quantity of chloroform were added to the coprecipitated mixture to dissolve the drug and the white petrolatum completely. The solvent was then removed under a stream of nitrogen gas.

[1] Pennsylvania Refining Co., Butler, Pa.
[2] American Cholesterol Products, Edison, N.J.
[3] Atmul 84, ICI.
[4] Van Waters & Rogers, Brisbane, Calif.
[5] Ashland Chemical Co., Division of Ashland Oil, Columbus, Ohio.
[6] Emery Industries, Fatty Acid Division, Los Angeles, Calif.
[7] Armour Industrial Chemical Co., Chicago, Ill.
[8] Enjay Chemical Co., New York, N.Y.
[9] Wilson, Martin, Division of Wilson and Co., Inc., Philadelphia, Pa.
[10] Reheis Chemical Co., Chicago, Ill.
[11] Tween 60, Atlas Chemical Industries.
[12] Span 60, Atlas Chemical Industries.
[13] Malmstrom Chemical Corp., Linden, N.J.
[14] Mallinckrodt Chemical Works, St. Louis, Mo.
[15] J. T. Baker Chemical Co., Phillipsburg, N.J.
[16] Matheson, Coleman and Bell, Los Angeles, Calif.
[17] 6α,9α-Difluoro-16α-hydroxyprednisolone 16α,17α-acetonide 21-acetate, Syntex Research, Palo Alto, Calif.
[18] 6α-Fluoro-9α,11β-dichloro-16α,17α,21-trihydroxypregna-1,4-diene-3, 20-dione 16,17-acetonide, Syntex Research, Palo Alto, Calif.

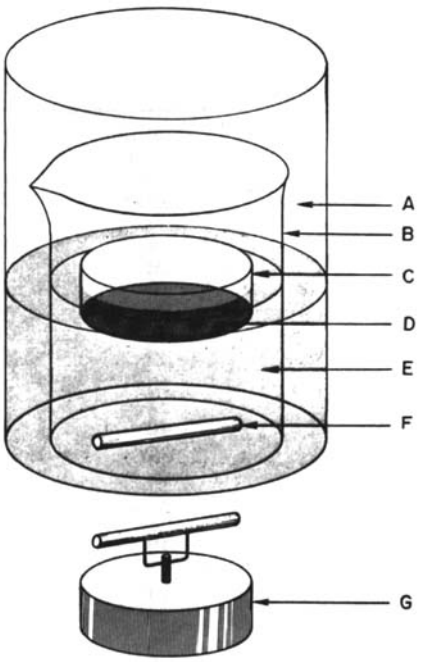

Figure 1—*Apparatus used in studying the release of drugs from an ointment layer containing suspended drug. Key: A, constant-temperature water bath; B, 250-ml beaker; C, Teflon dish; D, layer of ointment; E, sink; F, 5.0 × 0.9-cm (2 × 0.37-in.) magnetic stirring bar; and G, synchronous motor.*

All traces of solvent were removed by keeping the petrolatum stock suspension in vacuum for 5–7 days with occasional mixing by means of a spatula. The ointments were prepared from the petrolatum stock suspension and had the following composition: 0.05% corticoid, 5% adjuvant, and 95% white petrolatum. These ointments were well mixed with a spatula and allowed to equilibrate at room temperature before performing a release experiment.

Drug Release—The apparatus for studying drug release from ointments is shown in Fig. 1. It consisted of a Teflon dish [outer diameter 5.0 cm (2 in.)], which was allowed to float in 100 ml of distilled water in a 250-ml beaker. The water was stirred with a Teflon-coated magnetic stirring bar, which was driven by a synchronous motor at 60 rpm placed at the bottom of a water bath. The beaker was kept in a constant-temperature water bath maintained at 25°.

At the end of equilibration time, 300 mg of ointment was evenly spread in a thin layer at the bottom surface of a Teflon dish. Teflon served to hold the ointment layer because of similar surface properties. At zero time the dish was lowered onto the surface of water. This allowed direct contact between the donor ointment layer and the receptor phase. One-milliliter samples were withdrawn from the sink as a function of time and added to the liquid scintillation vials. To the latter, 15 ml of a liquid scintillation cocktail consisting of 50 mg of dimethyl 1,4-bis[2-(methyl-5-phenyloxazolyl)]benzene[19], 7 g of 2,5-diphenyloxazole[19], 50 g of naphthalene[15], 200 ml of methanol[14], and 800 ml of dioxane[16] was added. The radioactive counts were measured by a liquid scintillation counter[20]. The volume of the receptor phase was kept constant throughout the release run by replacing the removed sample with an equal volume of distilled water.

Partition Coefficient—For determining the ointment–water effective partition coefficient of the drug, the placebo ointments were prepared by melting the petrolatum and the adjuvant together and allowing them to cool to 25°. Exactly 2 g ointment was spread on the internal sides of a 50-ml polypropylene beaker. The radioactive drug was dissolved in distilled water, and 50 ml was added to the beaker. The aqueous solution was stirred with a Teflon-coated magnetic stirring bar, using a synchronous motor at 60 rpm placed under a water bath. The beaker was kept in a constant-

[19] Arapahoe Chemicals, Division of Syntex Corp., Boulder, Colo.
[20] Unilux II, Nuclear Chicago, Chicago, Ill.

Table I—Apparent Diffusion Constants of Fluocinonide and Flucloronide Calculated from Observed Release Rates and Apparent Solubilities after Equilibrating the Ointments for Different Times

Ointment Composition	Equilibrium Time, days	Apparent Diffusion Constant, $cm^2\ sec^{-1}$
Wool alcohol, 5%	9	2.2×10^{-11}
Petrolatum, 95%	23	2.3×10^{-11}
Fluocinonide, 0.05%	42	1.8×10^{-11}
Coconut fatty acid, 5%	9	6.86×10^{-11}
Petrolatum, 95%	23	5.75×10^{-11}
Fluocinonide, 0.05%	42	5.19×10^{-11}
Beeswax, 5%	9	0.77×10^{-11}
Petrolatum, 95%	23	0.88×10^{-11}
Fluocinonide, 0.05%	42	1.22×10^{-11}
Cetyl alcohol, 5%	9	1.1×10^{-11}
Petrolatum, 95%	23	1.4×10^{-11}
Fluocinonide, 0.05%	42	1.0×10^{-11}
Wool alcohol, 5%	5	0.15×10^{-9}
Petrolatum, 95%	19	0.18×10^{-9}
Flucloronide, 0.05%	33	0.15×10^{-9}
Coconut fatty acid, 5%	10	1.69×10^{-9}
Petrolatum, 95%	29	1.61×10^{-9}
Flucloronide, 0.05%	39	1.11×10^{-9}
Beeswax, 5%	5	0.68×10^{-9}
Petrolatum, 95%	19	0.72×10^{-9}
Flucloronide, 0.05%	33	0.74×10^{-9}
Cetyl alcohol, 5%	5	0.82×10^{-9}
Petrolatum, 95%	19	0.40×10^{-9}
Flucloronide, 0.05%	21	0.57×10^{-9}

temperature water bath at 25° until equilibrium was reached. One-milliliter samples were withdrawn from the aqueous phase and analyzed radiochemically.

THEORETICAL

An equation relating the amount of drug release from an ointment base containing drug in suspension to time and variables of the system was derived by Higuchi (1):

$$Q = \sqrt{2C_0DC_s^0t} \qquad \text{(Eq. 1)}$$

where Q equals the amount of drug released at time t per unit area of exposure, C_0 equals the concentration of drug expressed in units per cubic centimeter, C_s^0 equals the solubility of the drug as units per cubic centimeter in the external phase of the ointment, and D equals the diffusion constant of the drug molecule in the external phase.

In deriving the equation, it was assumed that: *(a)* the suspended drug is in a fine state such that the particles are much smaller in diameter than the thickness of the applied layer; *(b)* the amount of drug, C_0, present per unit volume is substantially greater than C_s, the solubility of the drug per unit volume of the vehicle; and *(c)* the surface area to which the drug ointment is applied is immiscible with respect to the ointment and constitutes a perfect sink for the released drug.

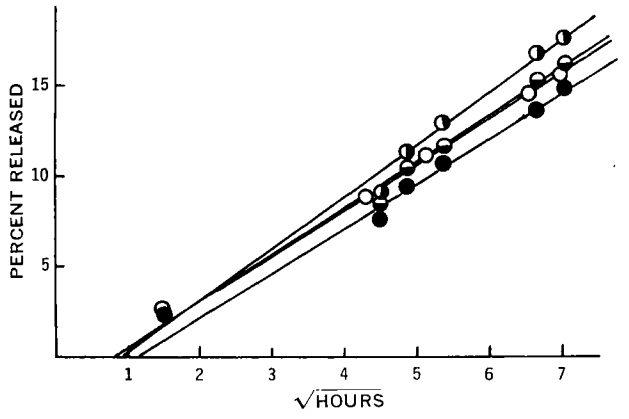

Figure 2—*Results of flucloronide release from oleaginous ointments prepared from white petrolatum. Key (in cm²/sec): ●, Batch I, run 1, D = 2.68 × 10⁻⁹; ◑, Batch I, run 2, D = 3.32 × 10⁻⁹; ◒, Batch I, run 3, D = 2.48 × 10⁻⁹; and ○, Batch II, run 1, D = 2.60 × 10⁻⁹.*

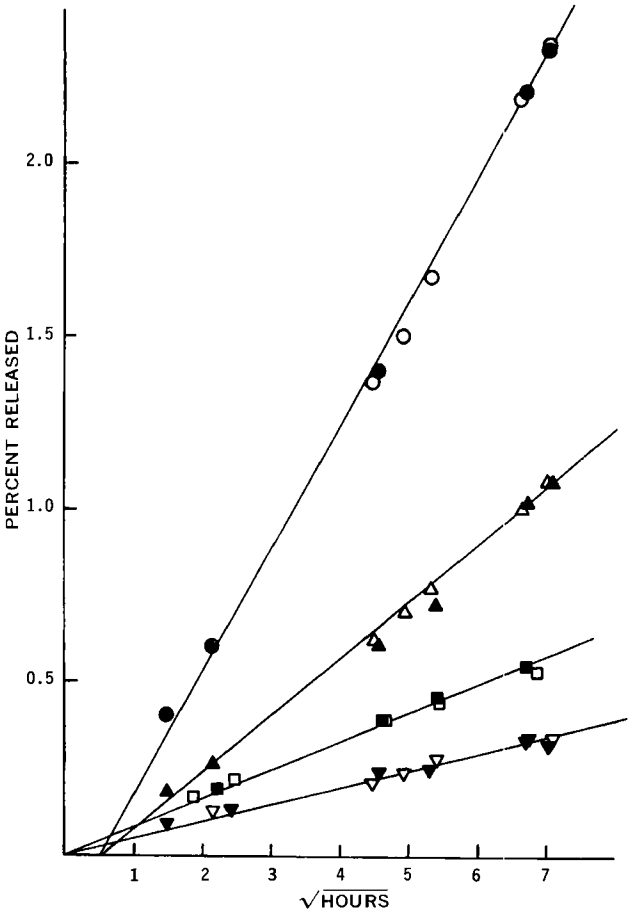

Figure 3—*Results of fluocinonide release from oleaginous ointments, showing the effect of the addition of various adjuvants to petrolatum. Empty and solid symbols represent data from two different runs. Key: ●, ○, 5% hexadecyl alcohol; ▲, △, 5% wool alcohol; ■, □, 5% Amerchol CAB; and ▼, ▽, 5% beeswax.*

Equation 1 may be rewritten to relate the percent drug release to time and variables of the system:

$$R = \sqrt{\frac{2 \times 10^4 DC_s^0t}{C_0h^2}} \qquad \text{(Eq. 2)}$$

where R is the percent drug release, and h is the thickness of the ointment layer.

The homogeneously dispersed drug particles should be in equilibrium with the drug in solution in the external phase of the ointment. Supersaturation or undersaturation of the drug in the external phase of the ointment might result, depending upon the manufacturing procedure. If the drug has not reached equilibrium within the ointment, different release profiles could result from the same ointment. When the initial concentration of the drug in the ointment is very low, changes in concentration due to nonhomogeneity or any other reason could also result in differences in release profiles. In light of these variables, release profiles showing the effect of adjuvants would have little meaning unless all of these factors have been accounted for.

Commonly used methods of determining the solubility of a drug in a liquid solvent cannot be used for solubility determinations in a semisolid ointment. For nonionic drugs that do not associate to form, for example, dimers in one of the two phases and for which the activity coefficients can be assumed to be unity in each phase,

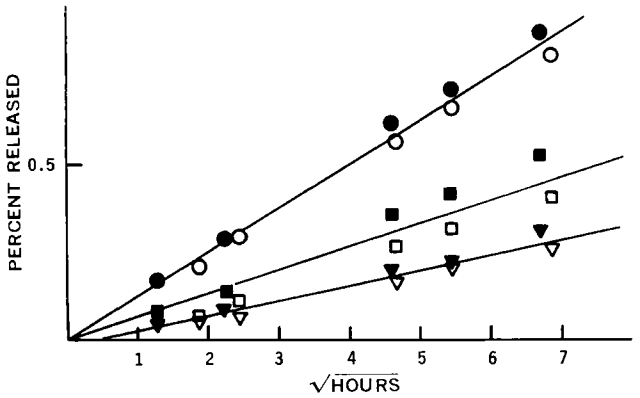

Figure 4—*Results of fluocinonide release from oleaginous ointments, showing the effect of the addition of various adjuvants to white petrolatum. Empty and solid symbols represent data from two different runs. Key: ●, ○, 5% coconut fatty acid; ■, □, 5% glyceryl stearate; and ▼, ▽, 5% glyceryl monostearate.*

the partitioning of the solute may be expressed in terms of the ratio of the concentrations in the two phases:

$$K = \frac{C_0}{C_a} \qquad \text{(Eq. 3)}$$

where K is the partition coefficient, and C_0 and C_a are the drug concentrations in the oil and aqueous phases, respectively.

When the amount of solute added is sufficiently small, the partition coefficient is relatively independent of the concentration. For solutes obeying Henry's law in each phase, changes in solute concentration would be expected to produce small changes in the partition coefficient. For these systems the effective partitioning of drugs near saturation between the two immiscible phases, such as ointment and water, can be expressed by:

$$K_e = \frac{A_0/W_0}{A_a/W_a} = \frac{(A_a^i - A_a)W_a}{A_a W_0} \qquad \text{(Eq. 4)}$$

where K_e is the effective partition coefficient; A_0 and A_a are the amounts of drug in the ointment and in the aqueous phase, respectively; A_a^i is the initial amount of drug in the water; and W_0 and W_a are the weights of the ointment and water, respectively.

Since the effective partition coefficient relates to the drug concentration in the two phases near saturation, the apparent solubility of the drug in an ointment can be determined from experimental drug solubility in water using:

$$C_s^0 = K_e C_s^w \qquad \text{(Eq. 5)}$$

where C_s^0 is the apparent solubility in the ointment, and C_s^w is the drug solubility in water.

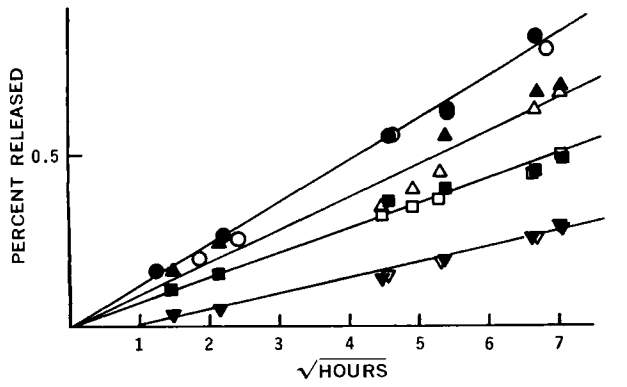

Figure 5—*Results of fluocinonide release from oleaginous ointments, showing the effect of the addition of various adjuvants to petrolatum. Empty and solid symbols represent data from two different runs. Key: ●, ○, 5% glyceryl monolaurate; ▲, △, 5% stearic acid; ■, □, 5% lecithin; and ▼, ▽, 5% cetyl alcohol.*

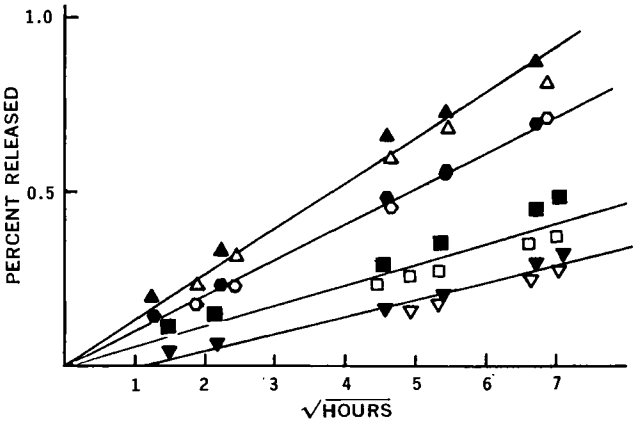

Figure 6—*Results of fluocinonide release from oleaginous ointments containing white petrolatum and adjuvants. Empty and solid symbols represent data from two different runs. Key: ▲, △, 5% Amerchol CAB + 2% sorbitan monostearate; ●, ○, 5% soya fatty acid; ■, □, petrolatum base six only; and ▼, ▽, 5% stearyl alcohol.*

RESULTS AND DISCUSSION

To determine the time required for reaching equilibrium solubilities of flucloronide and fluocinonide in different ointment bases, release runs were carried out after equilibrating the ointments for different time intervals. The apparent diffusion constants were calculated from the apparent drug solubilities in the ointments and the experimental release rates for each equilibrium time point. These data, given in Table I, indicate that the apparent solubility of the drugs had reached equilibrium as early as 5 days after preparation.

The results of flucloronide release from white petrolatum, showing the reproducibility of the experiment using the same ointment and using different ointments, are given in Fig. 2. These results indicate a lag time in all cases. Higuchi (1) pointed out that the initial lag time, L, corresponding to the time necessary for establish-

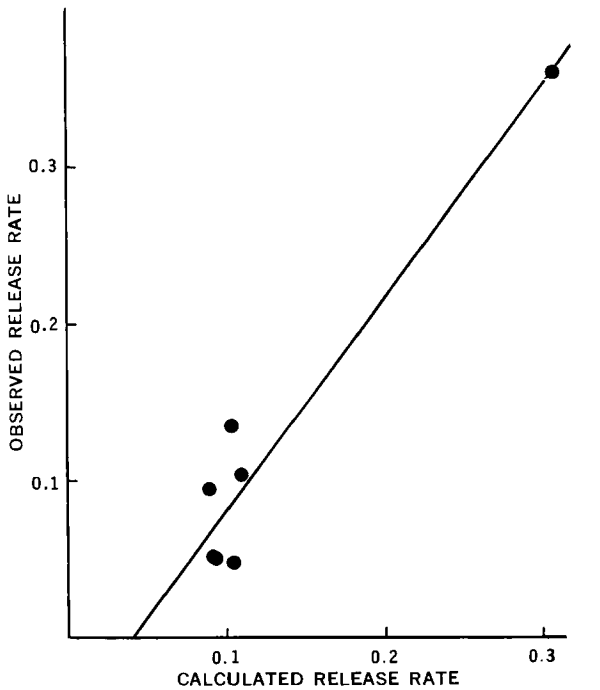

Figure 7—*Scatter diagram of the observed and calculated release rates of fluocinonide from Type I modified ointment bases. The line is the estimated regression line* $Y = 1.3461X - 0.0540$ *for solid circles.*

Table II—Parameter Values Determined from the Release and Partitioning Data of Fluocinonide for Ointments Prepared from Petrolatum and the Listed Adjuvants Using Eqs. 2 and 4

Adjuvant	K_e	C_s^0, μg/ml	Calculated Release Rate, %/$\sqrt{\text{hr}}$	Observed Release Rate, %/$\sqrt{\text{hr}}$
Type I				
Hexadecyl alcohol, 5%	10.61	4.46	0.308	0.360
Beeswax, 5%	1.01	0.42	0.094	0.049
Stearyl alcohol, 5%	0.96	0.40	0.092	0.050
Cetyl alcohol, 5%	1.25	0.52	0.105	0.047
Stearic acid, 5%	0.91	0.38	0.090	0.094
Hexadecyl stearate, 5%	1.26	0.53	0.106	—
Soya fatty acid, 5%	1.37	0.57	0.110	0.103
Coconut fatty acid, 5%	1.21	0.51	0.104	0.134
Type II				
Glyceryl monostearate, 5%	0.28	0.12	0.050	0.045
Glyceryl monolaurate, 5%	2.87	1.21	0.160	0.121
Wool alcohol, 5%	6.85	2.88	0.248	0.167
Amerchol CAB, 5%, + sorbitan monostearate, 2%	0.81	0.34	0.085	0.131
Amerchol CAB, 5%, + polysorbate 60, 2%	2.81	1.18	0.158	—
Lecithin, 5%	11.50	4.83	0.321	0.071
Glyceryl stearate, 5%	0.63	0.26	0.074	0.066
Amerchol CAB, 5%	0.86	0.36	0.087	0.083
None (petrolatum only)	0.39	0.16	—	0.058

ment of a quasistationary state would generally be less than:

$$L = \frac{(n\alpha)^2}{6D} \qquad \text{(Eq. 6)}$$

where α is the mean distance between the suspended particles, D is the diffusion constant of the drug in the external phase of the ointment, and n is the order of 2 or 3. The derivation of Eq. 1 assumed the particulate distance to be extremely small for the model system relative to the layer thickness, and thus the lag time was expected to be very short in comparison to the depletion period. The lag time seen in the present study may be explained on the basis of this assumption. It could be primarily due to the experimental difficulty in producing a suspension of fine particles containing 0.05% drug. Since the steady-state flux is reflected in the slope, any lag time seen in this study was neglected.

The effective partition coefficient data and the apparent solubilities of fluocinonide in ointments containing different adjuvants are given in Table II. The plots of percent fluocinonide release *versus* square root of time are given in Figs. 3–6. Table II also gives the observed and calculated release rates of fluocinonide in various ointment bases. The calculated release rates were obtained from Eq. 2 using the apparent solubilities of the modified ointment bases and the diffusion constant of the drug molecule in pure petrolatum.

A comparison of the observed and calculated release rates for ointments containing hydrophobic adjuvants (Type I) is given in Fig. 7. The correlation between the two rates was very good (r = 0.96). However, an examination of the observed and calculated rates (Table II) for ointments containing mainly emulsifying agents (Type II) indicated that knowledge of the calculated release rates offers little help in predicting the observed rates (r = 0.34).

Table III gives the effective partition coefficient data and apparent solubilities of flucloronide in ointments containing various adjuvants. The observed and calculated release rates of flucloronide

Table III—Parameter Values Determined from the Release and Partitioning Data of Flucloronide for Ointments Prepared from Petrolatum and the Listed Adjuvants Using Eqs. 2 and 4

Adjuvant	K_e	C_s^0, μg/ml	Calculated Release Rate, %/$\sqrt{\text{hr}}$	Observed Release Rate, %/$\sqrt{\text{hr}}$
Type I				
Hexadecyl alcohol, 5%	8.29	19.90	5.10	3.93
Beeswax, 5%	2.53	6.07	2.81	1.28
Stearyl alcohol, 5%	2.79	6.70	2.96	1.40
Cetyl alcohol, 5%	2.72	6.53	2.92	1.17
Stearic acid, 5%	3.76	9.02	3.43	3.13
Hexadecyl stearate, 5%	5.35	12.84	4.09	2.69
Soya fatty acids, 5%	6.23	14.95	4.42	3.02
Coconut fatty acid, 5%	5.17	12.41	4.02	3.10
Type II				
Glyceryl monostearate, 5%	2.86	6.86	2.99	—
Glyceryl monolaurate, 5%	6.30	15.12	4.44	3.56
Wool alcohol, 5%	8.11	19.46	5.04	1.24
Amerchol CAB, 5%	4.20	10.08	3.63	0.72
Amerchol CAB, 5%, + sorbitan monostearate, 2%	3.71	8.90	3.41	1.43
Amerchol CAB, 5%, + polysorbate 60, 2%	7.02	16.84	4.69	2.79
Lecithin, 5%	14.05	33.72	6.63	2.98
Glyceryl stearate, 5%	4.55	10.92	3.78	2.98
None (petrolatum only)	2.07	4.97	—	2.55

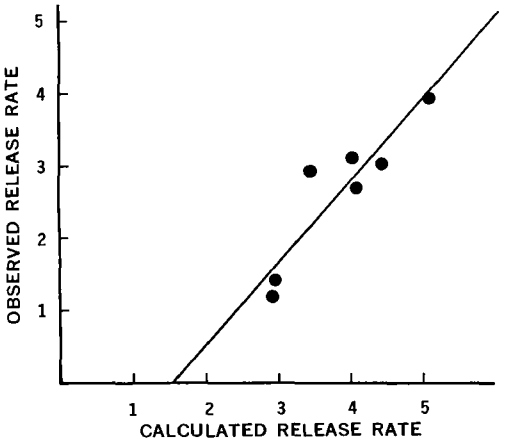

Figure 8—*Scatter diagram of the observed and calculated release rates of flucloronide from Type I modified ointment bases. The line is the estimated regression line* $Y = 1.1397X - 1.7732$ *for solid circles.*

are also given in Table III. For Type I adjuvants, these rates are plotted in Fig. 8. As in the case of fluocinonide, Fig. 8 indicates that the knowledge of calculated rates is helpful in predicting the release rates of flucloronide from ointments containing Type I adjuvants ($r = 0.90$). Type II adjuvants for flucloronide also gave a rather poor correlation between the observed and calculated rates ($r = 0.38$).

Negative intercepts were obtained in the observed *versus* calculated rate plots of Figs. 7 and 8. For the calculated rates, no association of the drug with the components of the ointment was assumed. If the drug has any tendency to bind with the ointment vehicle, the calculated rates would be higher than the observed rates. The negative Y intercept in Figs. 7 and 8 can thus be attributed to binding. In the calculations of release rates, it was also assumed that the diffusion constant remains constant when different adjuvants are added to petrolatum. Since the resistance to diffusion of drug molecules in the modified ointments is expected to vary depending on the adjuvant, some variations in the observed rates are expected due to changes in the diffusion constant of the modified vehicles.

Lack of correlation between the observed and calculated rates for Type II adjuvants indicates that the knowledge of calculated rates did not provide useful information in predicting the observed rates. Since Type II adjuvants are mainly emulsifying agents, during a release run the surfactant molecules are expected to orient at the ointment aqueous interface with their polar heads sticking in the aqueous phase and the hydrocarbon chain inside the ointment layer. Different surfactants are expected to dissolve and/or bind the drugs differently. Under these circumstances, the ointment–water interface and not the whole ointment layer may be the rate-determining step. The simple physical model used to calculate the release rates of drugs from ointment containing Type II adjuvants did not provide useful information in predicting the observed rates.

In summary, the results of this study show the usefulness of the physical model approach in helping to explain and predict the role of various factors in drug release from ointments containing suspended drug. In addition to drug solubility, initial drug concentration, and diffusion constant, drug release may be modified by unpredictable drug–excipient interactions. Surfactants are generally known to orient at the oil–water interface. The experimental observations of the ointment layer containing surface-active adjuvants before and after the release run indicated some changes in the ointment layer surface. Analysis of the data presented in this report has shown that the drug release from ointments containing surfactants as adjuvants cannot be explained solely by the simple equations used in this investigation.

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Comparison of Analgesic Effects of Isosteric Variations of Salicylic Acid and Aspirin (Acetylsalicylic Acid)

LEON THOMPKINS * and K. H. LEE ˣ

Abstract □ A reliable and sensitive method was used to compare the analgesic activities of salicylic acid and aspirin (acetylsalicylic acid) and several phenoxy substituted isosteric pairs. Those isosteric compounds studied did not show analgesic activity. The analgesic activity of aspirin was more than twofold greater than that of salicylic acid.

Keyphrases □ Aspirin—analgesic effects of isosteric variations, compared to salicylic acid isosteres □ Salicylic acid—analgesic effects of isosteric variations, compared to aspirin isosteres □ Analgesics—comparison of effects of isosteric variations of salicylic acid and aspirin □ Salicylates—comparison of analgesic effects of isosteric variations of salicylic acid and aspirin

Of the three major pharmacological actions of salicylic acid and aspirin (acetylsalicylic acid), their quantitative difference is most apparent in their analgesic effect. This difference in potency in experimental animals and in humans has been observed by several workers (1–5) and has been primarily responsible for the suggestion that aspirin's activity is not due to conversion to salicylic acid (6–8).

Although this suggestion was first made more than 50 years ago, there have been no attempts to resolve the question of whether aspirin is analgesic in its own right by using comparative analgesic studies of isosteric structural modifications. This seems especially significant in that isosteric substitution of $-CH_2-$ or —NH— for the phenoxy oxygen in aspirin would result in compounds stable or less susceptible to hydrolysis, which has been a major problem in assessing the relative effects of aspirin and salicylic acid on the biological system. Such studies might provide indications of the structural requirements for analgesic activity and information concerning the mechanism by which the salicylates exert their pharmacological effects.

Perhaps one reason that such studies were not carried out was the lack of reliable bioassay procedures for testing nonnarcotic analgesics. Aspirin and salicylic acid show activity as antinociceptive agents in many tests (9). However, most of them require high doses of salicylate to produce a minimal effect, are not clearly definitive, or are unreliable.

Guzman *et al.* (3) introduced a method that employs intraarterial injections of bradykinin in dogs, producing vocalization and other autonomic and behavioral reactions, to assess the activity of nonnarcotic and narcotic analgesics. In animals that respond, this method is superior to other analgesic tests if one considers overall reliability, sensitivity, and definitiveness. This method was later adapted to rats, and similar results were found (10). However, while Guzman *et al.* (3) used primarily the vocalization as the index to the pain response after the bradykinin injections in dogs, the latter workers described struggling, dextrorotation of the head, and occasional vocalization as indexes.

The effects of intraarterial injections of bradykinin into rats were used in this study to assess the analgesic activities of several phenoxy-substituted isosteric pairs of salicylic and acetylsalicylic acids. The analgesic effects of the following pairs were studied: acetylsalicylic acid (I) and salicylic acid (II), *N*-acetylanthranilic acid (III) and anthranilic acid (IV), acetylthiosalicylic acid (V) and thiosalicylic acid (VI), and 2-acetonylbenzoic acid (VII) and 2-methylbenzoic acid (VIII).

EXPERIMENTAL

Reagents—The following were used: thiosalicylic acid[1] (mp 163–165°), *N*-acetylanthranilic acid[1] (mp 184.5–187°), acetylsalicylic acid[2] (ACS reagent, spectrophotometrically pure), homophthalic acid anhydride[2] (mp 139–141°), salicylic acid[3], chloroform[4] (technical), anthranilic acid[5], pyridine[6] (reagent grade), 2-methylbenzoic (*o*-toluic) acid[7], and sterile sodium chloride injection[8] USP (0.0009 g/ml). All other chemicals were reagent grade.

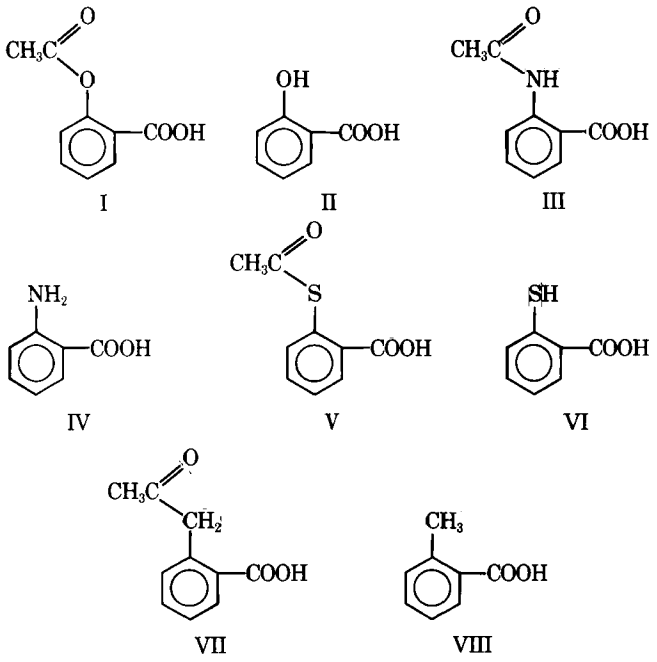
Purification Procedures—Thiosalicylic acid was recrystallized from ethanol and water as described in the literature (11). Pyridine was dried, distilled, and stored over sodium hydroxide pellets (12). Chloroform was purified, dried, and distilled (13). The distillate was used immediately.

Synthesis of Acetylthiosalicylic Acid—This compound was prepared using the method of Hinsberg (14). The crude product was dried over phosphorus pentoxide at room temperature under vacuum. The dried product was recrystallized several times from benzene until needles melting at 125° were obtained.

Synthesis of 2-Acetonylbenzoic Acid—This compound was prepared using the methods described by Schnekenburger (15, 16). These methods involve acetylation of homophthalic acid anhydride to obtain the 4-acetylhomophthalic acid anhydride, followed by alkaline hydrolysis with simultaneous decarboxylation of the product to 2-acetonylbenzoic acid (15). Colorless needles with the melting point of 2-acetonylbenzoic acid (122°), as described by Schnekenburger (16), were obtained.

Restraining Cage—For the measurement of analgesic activity, a restraining cage was designed in which the reactions of the animal to intraarterial injections of bradykinin could be observed and intraperitoneal injections of the test drug could be made (Fig. 1). The overall length of this cage was 20.3 cm (8 in.), and the distance between vertical supports was 7.6 cm (3 in.). The openings in the

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] City Chemical Corp., New York, N.Y.
[3] Baker analyzed reagent, J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] J. T. Baker Chemical Co., Phillipsburg, N.J.
[5] Baker grade, J. T. Baker Chemical Co., Phillipsburg, N.J.
[6] Eastman Organic Chemicals, Distillation Products Industries, Rochester, N.Y.
[7] Eastman grade, Eastman Organic Chemicals, Distillation Products Industries, Rochester, N.Y.
[8] Provided by the University of California Hospital Pharmacy, San Francisco, Calif.



front and rear supports were holes with a depth of 3.7 cm (1.47 in.) and a width of 4.4 cm (1.75 in.). The top was removable; once the animal was positioned, the top was secured with wingnuts to hold the rat firmly but comfortably (Fig. 2). This cage was found to be suitable for these experiments.

Measurement of Analgesic Activity—Male Sprague–Dawley or Wistar rats were used in all experiments. A modification of the procedure of Deffenu *et al.* (10) was used in the testing of the analgesic activity of the various isosteric pairs. The rats, 230–320 g, were anesthetized with ether in an open mask and the right common carotid artery was catheterized, using a catheter consisting of two fused lengths of heparinized polyethylene tubing with inside diameters of 0.58 (PE 50) and 0.28 (PE 10) mm.

The catheter was inserted centripetally, passing through the subcutaneous tissues so that the portion of the tubing with the smaller diameter was inserted into the artery while that with the larger diameter protruded from the top of the head. After the tubing was anchored and the incision was closed, the tubing was opened to check blood flow, filled with heparin (40 units/ml in sterile saline injection USP), shortened so that the rat could not reach it, and plugged with a metal plug. At least 24 hr was allowed for each rat to recover prior to testing.

Rats were placed in the cages, and polyethylene 50 tubing extensions were attached to the projecting catheter *via* a hollow metal tube prepared from a length of No. 23 hypodermic needle. The tubing was taped to the top of the cage so that the animal would be unaware of the experimenter's handling of the distant end. Then 0.2 ml of normal saline was injected very slowly from the carotid artery, and no reaction of the animal was observed. This was followed by an injection of 0.5 μg of bradykinin in 0.2 ml of normal saline to determine if the animal was responsive to bradykinin. In about 99% of the rats, vigorous struggling and dextrorotation of the head were observed. Rats that were unresponsive to this dose of bradykinin were not used further. The sodium salt of the drug to be tested was injected intraperitoneally in normal saline. The volume of the injection was 0.25 ml/100 g or the smallest volume that could completely dissolve the salt.

Ten minutes after the injection of the drug, the animals were challenged with another 0.5-μg dose of bradykinin, preceded by a normal saline injection, and the animals' responses were carefully watched. The same procedure was repeated at 10-min intervals until the bradykinin effect reappeared.

If no effect was seen with a dose level of 1000 mg/kg for a particular drug, intraarterial studies with a dose level of 100 mg/kg were made. The intraarterial injections of the drugs were made in normal saline (0.5 ml/rat) *via* the same catheter used for the bradykinin injection. Otherwise, the procedure was the same as for the intraperitoneal injections.

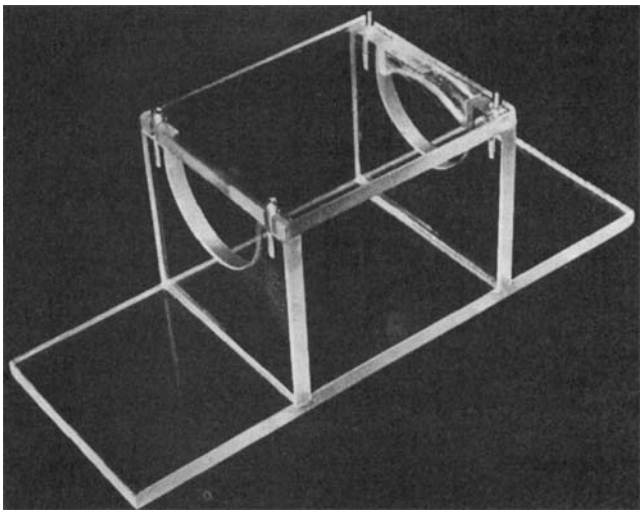


Figure 1—*Rat cage.*

The criterion chosen for evaluating protection was the disappearance of the bradykinin effect after two consecutive doses of bradykinin. The doses of drugs that showed no protection after five consecutive doses of bradykinin were assumed to be nonanalgesic in these studies. Each rat received one drug at one dose level, and five rats were used for each dose level. If a drug was found to be toxic, the LD_{50} was determined.

The ED_{50} and LD_{50} values were obtained using Thompson's method (17) of interpolation between moving averages.

RESULTS AND DISCUSSION

A summary of the data obtained in these experiments is contained in Table I, including the responses of the groups of rats to various doses of the drugs tested. Salicylic and acetylsalicylic acids showed clearcut analgesic activity *versus* bradykinin injections, intraperitoneally. Acetylsalicylic acid showed analgesic activity at a much lower dose level when intraarterial injections of the drug were made.

It is difficult to interpret the results obtained with *N*-acetylanthranilic and anthranilic acids. Both of the drugs seemed to afford protection *versus* bradykinin injections at lower dose levels intraperitoneally. However, at higher intraperitoneal dose levels and at a dose level of 100 mg/kg intraarterially, no analgesic activity was demonstrated. No other analgesic drugs demonstrating such anomalous behavior have been described. It is assumed that these two compounds have no analgesic activity as indicated in Table II, where the ED_{50} values for compounds showing analgesic activity and the LD_{50} values for compounds found to be toxic are reported.

Both Tables I and II show the toxicity of acetylthiosalicylic and thiosalicylic acids, the nonacetylated compound being more toxic. This toxicity was characterized by severe tremors and hyperventilation followed by a violent struggle just before death. All animals thus affected died within 50 min, and *rigor mortis* set in rapidly.

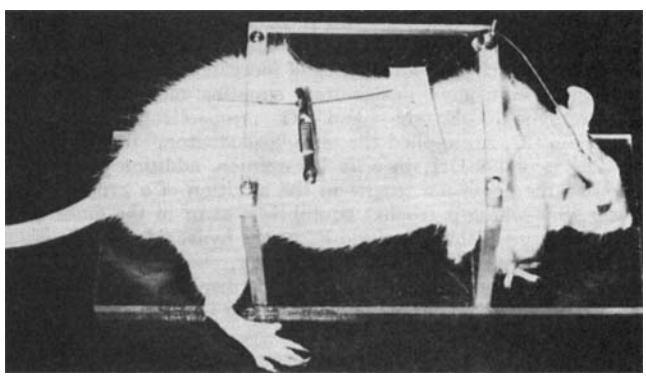


Figure 2—*Rat cage with rat.*

Table I—Effects of the Injection of Various Salicylate Isosteres *versus* Intraarterial Injections of Bradykinin in Rats

Drug	Number of Rats Tested	Dose of Drug, mg/kg	Route[a]	Number Protected *versus* Bradykinin	Number of Deaths
Acetylsalicylic acid	5	31.6	ip	1	0
	5	46.4	ip	1	0
	5	68.1	ip	2	0
	5	100.0	ip	5	0
	5	10.0	ia	4	0
Salicylic acid	5	68.1	ip	1	0
	5	100.0	ip	2	0
	5	147.0	ip	2	0
	5	215.0	ip	5	0
N-Acetylanthranilic acid[b]	5	100.0	ip	2	0
	5	215.0	ip	1	0
	5	464.0	ip	2	0
	5	1000.0	ip	0	0
	5	100.0	ia	0	0
Anthranilic acid[b]	5	100.0	ip	0	0
	5	215.0	ip	2	0
	5	464.0	ip	0	0
	5	1000.0	ip	0	0
	5	100.0	ia	0	0
Acetylthiosalicylic acid	5	147.0	ip	0	0
	5	215.0	ip	0	3
	5	316.0	ip	0	4
	5	464.0	ip	0	5
Thiosalicylic acid	5	100.0	ip	0	0
	5	147.0	ip	0	3
	5	215.0	ip	0	4
	5	316.0	ip	0	5
2-Acetonylbenzoic acid	5	100.0	ip	0	0
	5	464.0	ip	0	0
	5	1000.0	ip	0	0
	5	100.0	ia	0	0
2-Methylbenzoic	5	100.0	ip	0	0
	5	464.0	ip	0	0
	5	1000.0	ip	0	0
	5	100.0	ia	0	0

[a] ip = intraperitoneal, and ia = intraarterial. [b] See discussion.

In contrast to other methods, the bradykinin injection causes no injury and shows that the receptors for pain are chemosensitive (18). The sensitivity and reliability of this method are indicated by the fact that one could show the difference in potency of acetylsalicylic acid and salicylic acid (Table II). The procedure of injecting normal saline prior to injecting bradykinin allows one to compare the effects of nonstimulating and stimulating agents and, therefore, increases the sensitivity of the test.

The concept of isosterism has undergone several changes since its introduction in 1919. The term "isosteres" was first applied by Langmuir (19) to designate two molecules or ions having identical number and arrangement of electrons. Hinsberg (20) recognized even earlier that certain groups such as sulfur, viylene, and trivalent carbon and nitrogen could be interchanged in the aromatic ring without appreciably altering physical and chemical properties. Hinsberg's designation of such groups was "ring equivalents." Hückel (21) included other types of inorganic and organic groups under the equivalent designation, equating methyl, methylene, and methyne to —F, —N—, and —O—, respectively.

Grimm (22, 23) applied the term "pseudoatom" to groups such as $—NH_2$ and —OH since, in his opinion, addition of a proton (H^+) to the oxide ion results in the addition of a proton to the outer shell where it reaches equilibrium at or in the outer shell, and the result is thus a pseudo-F^- or the hydroxide ion. However, unlike the ring equivalents of Hinsberg, chemical properties of compounds obtained using the "hydride displacement law" might appear wholly different but may be similar in some physical properties.

Erlenmeyer (24) included both Grimm's concept of pseudoatoms and all elements of a particular group of the periodic table in his concept of isosteres, redefining the isosteres as "atoms, ions, or molecules in which the peripheral layers of electrons can be considered identical." Erlenmeyer was also one of the first to apply the isosteric principle to biological problems (25, 26).

Table II—LD_{50} and ED_{50} Values for Acetylsalicylic and Salicylic Acid Isosteres *versus* Intraarterial Injections of Bradykinin in Rats

Compound	Intraperitoneal LD_{50}, mg/kg	Intraperitoneal ED_{50}, mg/kg	Intraarterial, ED_{50}, mg/kg	95% Confidence Limits
Acetylsalicylic acid	—	64.9	—	47.9–88.00
Salicylic acid	—	138	—	84.1–226
N-Acetylanthranilic acid	—	1000	100	—
Anthranilic acid	—	1000	100	—
Acetylthiosalicylic acid	224	—	—	176–285
Thiosalicylic acid	153	—	—	120–194
2-Acetonylbenzoic acid	—	1000	100	—
2-Methylbenzoic acid	—	1000	100	—

Since the development of more sophisticated methods of treating atoms and molecules (*e.g.*, quantum mechanics), it is recognized that the concept of isosterism disregards some important factors such as size, shape, polarity, and hybridization, and the concept is of little theoretical importance to the pure chemist because of the unpredictability of its application. However, it has great value for the medicinal chemist, since biological properties of isosteres are more similar than their physical or chemical properties.

Since isosteres often have similar biological properties, Friedman (27) introduced the term bioisosterism to apply to compounds "which fit the broadest definition of isosteres and have the same type of biological activity." He included under this term isosteres with antagonistic activities, since these compounds may be acting by a similar mechanism. The broadest definition of the term isostere would, of course, include the ring equivalents of Hinsberg, the isosteres of Langmuir, the equivalents of Hückel, and the pseudoatoms of Grimm, all of which have been found to have utility in the search for more desirable, biologically active compounds. Thus, Erlenmeyer found that the isosteres *p*-aminodiphenylamine, *p*-aminodiphenylmethane, and *p*-aminodiphenyl ether all show similar antigen activity. Halogen isosteres of the antihistamine, tripelennamine, show greater antihistaminic activity when F and Cl are substituted for one aromatic hydrogen in the parent compound (28). Several other examples of the utility of the concept of isosterism in medicinal chemistry can be found in the literature (27, 29).

It is unusual that none of the isosteres was effective in producing analgesia, especially since most of the groups are closely related in size. Apparently, the receptors are very sensitive to the electronic and steric configurations of acetylsalicylic and salicylic acids. However, it has been demonstrated again that the analgesic activity of acetylsalicylic acid is more than twofold greater than that of salicylic acid.

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Abstract □ An intravenous dosage form of trichloromonofluoromethane, a common aerosol propellant, was formulated in polyethylene glycol 400 for single and multiple dosing to unanesthetized dogs. A three-compartment open model was proposed for disposition of this compound in dogs with average half-lives of 3.2, 16, and 93 min for three disposition phases. This finding is contrary to several reports where blood levels were monitored for shorter periods. A computer analysis of tissue compartment distribution following a single dose showed that about 2 hr was required to achieve pseudodistribution equilibration, following which more than 90% of the dose remaining in the body was retained in tissue compartments. Pulmonary clearance and volumes of distribution were calculated considering first-pass effect through the lung. The volume of distribution was approximately six times the body weight in terms of blood concentrations, and about 30% of the propellant was cleared from blood passing through the lung in each cycle. Disposition of propellant followed dose-independent kinetics after multiple dosing, and accumulation in tissues continued for a much longer period, resulting in high tissue compartment levels.

Keyphrases □ Trichloromonofluoromethane—pharmacokinetics following single and multiple dosing, dogs □ Fluorocarbon aerosol propellants—pharmacokinetics of trichloromonofluoromethane following single and multiple dosing, dogs □ Aerosols—pharmacokinetics of trichloromonofluoromethane following single and multiple dosing, dogs □ Propellants—pharmacokinetics of trichloromonofluoromethane following single and multiple dosing, dogs □ Pharmacokinetics—trichloromonofluoromethane, single and multiple doses, dogs

The volatile fluorocarbons have been widely used as aerosol propellants in commercial aerosol packages in this country. Although these compounds have been generally considered nontoxic and inert, several studies (1–3) have claimed that a variable degree of cardiac damage can be caused following their inhalation. An often used argument in favor of low toxicity of fluorocarbons is that these compounds are not absorbed to any significant extent when inhaled from a commercial aerosol product and that the small fraction absorbed is eliminated very fast from the body, decreasing the possibility of any toxic reaction (4–6). Unfortunately, the conclusions drawn from most previous studies monitoring blood levels of fluorocarbons following their inhalation from commercial aerosol products were not based on sound pharmacokinetic principles and do not reflect the true disposition pattern of these compounds as was recently discussed (7).

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The objective of the study reported here was to demonstrate that, contrary to the established belief, trichloromonofluoromethane, one of the most commonly used fluorocarbon propellants, has a longer biological half-life than previously thought and under-

goes extensive distribution in the body, an observation that might have an important bearing on the toxicity profile of this compound.

EXPERIMENTAL

Materials—Trichloromonofluoromethane[1] and polyethylene glycol 400[2] were obtained from commercial sources. A medical-grade silicone tubing[3] [0.3 cm (0.125 in.) i.d.] was used with 18–20-gauge cannulas[4] for venous cannulation. The infusion of solution was performed using a constant-rate infusion pump[5].

Formulation of Intravenous Dosage Form—Sterile solutions of fluorocarbons for intravenous infusion were prepared in polyethylene glycol 400, which was sterilized by heating at 100° for about 1 hr over a hot plate. The serum bottles (50-ml capacity) in which the solution was stored were also sterilized by heating at 110° for about 2 hr. After cooling, polyethylene glycol 400 was transferred to almost fill a preweighed, empty, sterile serum bottle, which was then quickly sealed with a lacquer-coated rubber stopper and an aluminum seal with the help of a manual crimper. The bottle was reweighed to determine the weight of polyethylene glycol transferred. The volume of polyethylene glycol was calculated through the density factor of 1.125 (8).

For the preparation of a solution of trichloromonofluoromethane, which exists in liquid form at refrigerator temperature (5°), 0.1–0.5 ml was transferred to the serum bottle using a precooled hypodermic syringe fitted with a 23-gauge needle. Caution was observed to avoid contact of the needle with the polyethylene glycol in the bottle. The bottle was then rotated gently for 15 min to dissolve the fluorocarbon and evenly disperse it. At the end of 15 min, a preweighed 25-gauge hypodermic needle was inserted just below the surface of the rubber stopper to release any excessive pressure and the bottle was reweighed before removing the needle. The weight of the fluorocarbon dissolved was calculated by subtracting the weight of the empty bottle, the polyethylene glycol 400, and the needle.

Analysis of Polyethylene Glycol Solutions—The analysis of the intravenous solutions was performed by transferring a small volume of solution (5–7 drops), using a hypodermic syringe fitted with an 18-gauge needle, to a preweighed and sealed serum bottle (15-ml capacity). The bottle was then reweighed to determine the exact weight of the solution transferred (normally 50–100 mg). The bottles were then rotated in all directions to spread the solution on the wall of the bottles. The bottles were then shaken in a vortex shaker for 10 min to allow complete equilibration between the liquid polyethylene glycol and the air phase.

Although the ratio of the air to liquid phase was very high, such as 300, for 0.05 ml of liquid in a 15-ml capacity bottle, the liquid phase may not be completely depleted of the fluorocarbon upon equilibration. For example, the partition coefficient between polyethylene glycol 400 and air was approximately 11 for trichloromonofluoromethane, which could result in about 3.6% underestimation of the content; proper correction was necessary. The concentration of fluorocarbon in the air phase was analyzed either by directly injecting a small sample of air from the bottle using a gastight syringe on a GC column or by first diluting 100 μl of the air phase in another 15-ml capacity bottle and then injecting a proper volume on the column. The concentration of trichloromonofluoromethane in the polyethylene glycol solution can easily be obtained using the following relationship:

$$C_{peg}\,(\text{mg/ml}) = \frac{C_a V_a D}{W_{peg}} C_a P \qquad \text{(Eq. 1)}$$

where C_a is the concentration of fluorocarbon in the air phase in milligrams per milliliter in the original bottle (if any dilutions are made), V_a is the volume of the air phase (capacity of the bottle, milliliters), D is the density of polyethylene glycol in grams per milliliter, W_{peg} is the weight of polyethylene glycol transferred to the bottle in grams, and P is the partition coefficient between

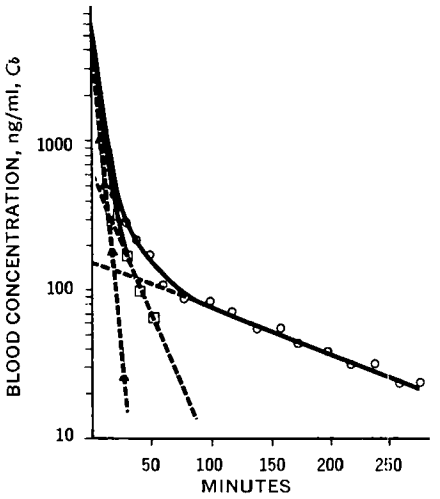


Figure 1—*Triexponential blood concentration decay following intravenous administration of 128.5 mg of trichloromonofluoromethane in 6.6 ml of polyethylene glycol 400 to a dog weighing 19.5 kg.* $C_b = 6000e^{-0.19800t} + 625e^{-0.04531t} + 152e^{-0.00693t}$.

polyethylene glycol 400 and air for trichloromonofluoromethane. Equation 1 is based on the assumption that the dilute solution of the propellant in polyethylene glycol 400 would not significantly affect the density of the polyethylene glycol solution.

The validity of this method of analysis was confirmed by almost perfect agreement between the values obtained gravimetrically (where the weight of fluorocarbon added was known) and those obtained by the described method.

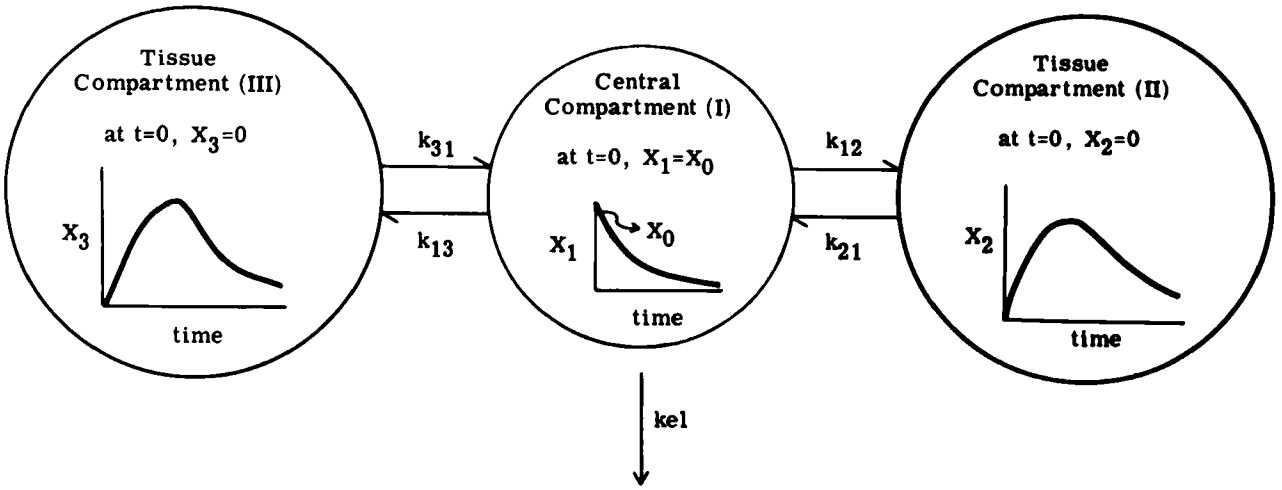
The concentration of trichloromonofluoromethane solutions prepared ranged from 7 to 36 mg/ml. A duplicate analysis in all instances showed a high degree of reproducibility. The analysis of these solutions was also performed just prior to their infusion to account for any loss during overnight storage. A freshly prepared solution (kept overnight) of fluorocarbon in polyethylene glycol was used for each study.

Infusion of Polyethylene Glycol Solutions—The solution stored in the 50-ml serum bottle was withdrawn in a 20- or 50-ml capacity glass syringe using a 16-gauge needle by applying a positive pressure inside the bottle by injecting 20–50 ml of air in the bottle and then slowly filling the syringe, avoiding the entry of air in the syringe. These precautions were necessary to minimize the loss of the fluorocarbon to the atmosphere during the transfer. The glass syringe was then connected to silicone tubing through a three-way stopcock and assembled in a constant-rate infusion pump.

Animal Procedures—Four male, mongrel, unanesthetized, conditioned dogs, 16.8–20 kg, were used. They were fasted for 16–18 hr prior to the experiments, and food and water were withheld during the experiments. The cephalic veins of the forelegs were cannulated using an 18-gauge cannula to which a three-way stopcock was attached. The syringe containing the fluorocarbon solution was connected to the cannula through silicone tubing. The solution was infused over 3 min at a constant rate of 2.2 ml/min for the single-dose study and for 2 min at a rate of 2.5 ml/min for the multiple-dose study. At the end of the infusion period, the cannula was flushed with 5 ml of heparinized sterile normal saline.

The blood samples (0.5–3.0 ml) during the single-dose study were collected from the other leg at 0, 1, 2, 3, 4, 6, 8, 10, 15, 20, 25, 30, 40, 50, 60, 70, 80, and 100 min and then up to 300 min at every 20 min. The zero time for sampling was assigned when the infusion was completed. A blank blood sample was obtained before the solution was infused to study the partition coefficients. The cannula through which the solution was infused was removed as soon as possible after the infusion was complete. In the case of multiple dosing, the cannula was left intact until the last dose was infused. The samples obtained during the multiple-dose study were less frequent except for the last dose, after which a pattern similar to that described for the single-dose study was followed. Every time a blood sample was withdrawn, the cannula was flushed with heparinized normal saline to avoid clotting.

[1] Supplied by E. I. duPont deNemours & Co., Wilmington, Del.
[2] Union Carbide Chemical Co., New York, N.Y.
[3] Dow Corning Corp., Midland, Mich.
[4] Medicut, Aloe Medical, St. Louis, Mo.
[5] Harvard Apparatus Co., Millis, Mass.



Scheme I—A three-compartment disposition model for trichloromonofluoromethane in dogs

During all studies, an adequate room ventilation was maintained and care was observed not to excite the animal since the cardiac output and breathing rate can affect the results significantly. No study was performed if the animal got highly excited during the process of cannulation. At least 1 week was allowed between studies in the same animal. A duplicate study was performed on Dog 2976.

Analysis of Trichloromonofluoromethane in Blood—Approximate volumes of blood samples, ranging from 0.5 to 3.0 ml, were quickly transferred using a 23-gauge needle to sealed preweighed and heparinized serum bottles of various capacities; a 27-gauge needle had been inserted to release the excessive pressure buildup due to the added volume of the blood. Both needles were removed quickly after the blood samples were transferred. The serum bottles were then reweighed to determine the weight of blood added to each bottle, which was then converted to volume using the experimentally determined density factor of 1.04. The blood samples were allowed to equilibrate in the sealed serum bottle with the air phase, which was then analyzed. Then the concentration of the blood samples was determined using the following relationship (9):

$$C_b = \frac{C_h(V_t - V_b)}{V_b} + C_hP \qquad \text{(Eq. 2)}$$

where C_b and C_h are the concentrations in the blood sample transferred to the serum bottle and the head space in the serum bottle with which it equilibrates, respectively; V_t and V_b are the volumes of the serum bottle and the blood sample transferred, respectively; and P is the partition coefficient between blood and air. Reference 9 should be consulted for a detailed description of the procedure.

The analyses were performed as soon as possible after withdrawing the blood samples to avoid any loss during storage. Duplicate analyses were performed on each sample and a high degree of reproducibility was always achieved.

RESULTS AND DISCUSSION

Dosage Form and Route of Administration—In most previous studies measuring blood concentrations of fluorocarbons following their inhalation, a large degree of variation was inevitably seen for several reasons. First, the uptake of gases largely depends on cardiac output and ventilation (10), both of which depend on such factors as levels of excitement, blood carbon dioxide tension, and pathological conditions. Second, the uptake of gases is further complicated by the rebreathing from the residual volumes in the lungs and from the atmosphere. For example, Shargel and Koss (11) found variations of more than 50-fold in blood concentrations of fluorocarbons following inhalation of equal doses in dogs. Such variations were also reported by Azar *et al.* (12) and were attributed to the reasons listed here.

One means of curtailing such large variations is to use a dosage form other than inhalations. For example, a solution of fluorocarbon in corn oil was used for oral administration to rats (13). How-

Table I—Pharmacokinetic Parameters of Trichloromonofluoromethane from Five Studies

Parameter[a]	Dog 3287	Dog 3288	Dog 3457	Dog 2976a	Dog 2976b
b_1	0.239	0.198	0.252	0.193	0.222
b_2	0.0504	0.0433	0.0590	0.047	0.0308
b_3	0.0084	0.00693	0.00825	0.00753	0.00660
C_1	0.714	0.885	0.81	0.573	0.897
C_2	0.220	0.0922	0.176	0.382	0.0849
C_3	0.0612	0.0224	0.00796	0.0446	0.0179
C_{12}	−0.330	−0.248	−0.306	−0.331	−0.348
C_{22}	0.333	0.231	0.301	0.309	0.319
C_{32}	0.049	0.0169	0.00555	0.0216	0.0287
C_{13}	−0.129	−0.183	−0.590	−0.0665	−0.125
C_{23}	−0.255	−0.107	−0.0620	−0.218	−0.115
C_{33}	0.384	0.290	0.121	0.284	0.239
k_{e1}	0.0679	0.102	0.139	0.0592	0.105
k_{21}	0.100	0.0589	0.0936	0.108	0.0487
k_{31}	0.0148	0.00993	0.00940	0.0108	0.00882
k_{13}	0.0404	0.0388	0.0175	0.021	0.0296
k_{12}	0.0742	0.0390	0.0594	0.0488	0.0673
D^0,[b] mg	209	128	241	116	49.2
AUC^∞, μg/ml, min	144	66.6	175	132	49.8

[a] The units for all b and k values are minutes^{-1}. [b] $X_0 = (1 - f)D^0$, where $(1 - f)$ is the fraction eliminated in the first pass through the lungs.

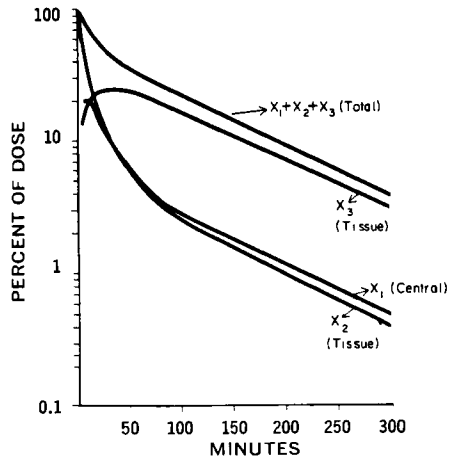


Figure 2—*Percent of absorbed trichloromonofluoromethane remaining in various body compartments based on a typical study (Dog 3287).*

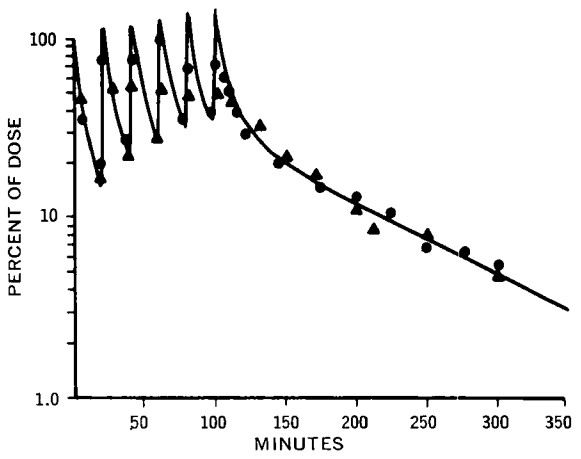


Figure 4—*Percent of absorbed dose remaining in the central compartment following six intravenous administrations of 43 mg (▲, Dog 3287) or 62 mg (●, Dog 3288) of trichloromonofluoromethane every 20 min. The solid curve represents the theoretical values based on a typical set of pharmacokinetic parameters obtained from a single-dose study (Dog 3287).*

ever, the biological availability problems in this case can complicate the situation. An intravenous dosage form offers the greatest advantages of control and ease of administration. Unfortunately, trichloromonofluoromethane presents the problem of extremely low solubility in water-miscible liquids that can be used as vehicles in intravenous dosage forms.

The choice of polyethylene glycol 400 was arrived at by the process of elimination of solvents that will have adequate solubility, show low toxicity when administered intravenously, and be stable to the sterilization procedure. This vehicle was previously used to formulate injections (14, 15). This vehicle, however, does not represent an ideal choice in view of a recent report showing that the administration of polyethylene glycol 400 increased the blood pressure response to 10 μg of epinephrine by an average of 22 mm Hg (27% increase over control). The polyethylene glycol 400 itself did not show any affect, but the response enhancement to epinephrine was dose dependent and lasted for about 1 hr following administration of a minimum dose producing an enhancement to epinephrine response. This observation, however, would not affect the utility of this dosage form in the present study where the main objective was to study the disposition of fluorocarbons rather than their toxic manifestations, which might possibly be complicated if polyethylene glycol 400 is used as a dosage vehicle. The amount of the fluorocarbon administered in this study was close to the total fluorocarbons that may be encountered clinically after inhalation.

Pharmacokinetic Model—The blood concentrations (venous) obtained at different time intervals following intravenous dosing of the fluorocarbon were plotted on semilogarithmic paper; the midpoint of infusion was assigned as zero time and a smooth curve was drawn through these points. A typical example is shown in Fig. 1. The feathering method (17) was then employed to isolate different exponents. In all instances, the blood concentration–time curves were characterized by a triexponential equation. A three-compartment open model for trichloromonofluoromethane is depicted in Scheme I. The two compartments besides the central compartment can be described as the group of tissues with distinct affinity and capacity (17) for trichloromonofluoromethane. The difference in affinity may be due to the solubility characteristics or to specific interactions such as protein binding or other complexations. A compartment can also be distinguished based on the blood supply to the tissues such as a vessel-rich group or a vessel-poor group (18).

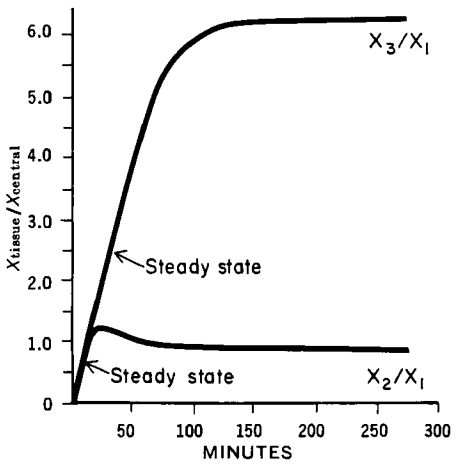


Figure 3—*Comparison of the relative amount of trichloromonofluoromethane remaining in tissue and central compartments based on a typical study (Dog 3287).*

For further analysis of this model, to calculate various rate constants as described in Scheme I, a computer program was generated based on the differential equation solutions described by Rescigno and Segre (17).

The fraction of dose remaining in various pharmacokinetic compartments can be described in terms of hybrid constants:

$$X_1/X_0 = C_1e^{-b_1t} + C_2e^{-b_2t} + C_3e^{-b_3t} \quad \text{(Eq. 3)}$$

$$X_2/X_0 = C_{12}e^{-b_1t} + C_{22}e^{-b_2t} + C_{32}e^{-b_3t} \quad \text{(Eq. 4)}$$

$$X_3/X_0 = C_{13}e^{-b_1t} + C_{23}e^{-b_2t} + C_{33}e^{-b_3t} \quad \text{(Eq. 5)}$$

where X_1, X_2, and X_3 are the amounts of fluorocarbon present in different compartments, and X_0 is the dose, which in the model proposed is the dose remaining in the body after the first pass through the lungs as described later.

The described pharmacokinetic parameters determined experimentally for trichloromonofluoromethane are reported in Table I.

Volume of Distribution and Pulmonary Clearance—The apparent volume of distribution, V_d, of a drug after reaching a pseudodistribution–equilibrium state following intravenous administration has been defined as (19):

$$(V_d)_{b_3} = \frac{D^0}{b_3 \times AUC} \quad \text{(Eq. 6)}$$

where AUC is the area under the blood concentration–time curve from zero to infinity, b_3 is the first-order disposition constant in the terminal phase, and D^0 is the dose administered.

Equation 6, however, cannot be used for the present situation where trichloromonofluoromethane was administered in the venous blood and where, therefore, a significant fraction may be eliminated from the lungs before the concentration in the venous blood is monitored, resulting in a serious overestimate of the volume of distribution if D^0 is assumed to be equivalent to the dose absorbed. Therefore, a correction factor is necessary to account for the fraction eliminated in the first pass through the lungs:

Table II—Volumes of Distribution and Clearance of Trichloromonofluoromethane

Dog	$(V_d)_{b_3}$, liters	V_c, liters	PC, liters/min	$(1 - f)$, %
3287	116.28	14.44	0.98	32.57
3288	169.33	11.51	1.17	39.11
3457	114.23	6.75	0.94	31.41
2976a	90.32	11.49	0.68	22.67
2976b	112.56	7.04	0.74	24.76
Mean ± *SEM*	120.54 ± 13.07	10.25 ± 1.47	0.90 ± 0.10	30.09 ± 2.95

$$(V_d)_{b_3} = \frac{f \times D^0}{b_3 \times AUC} \qquad \text{(Eq. 7)}$$

where f is the fraction of the administered dose retained in the body following the first pass through the lung. The fraction eliminated by the first pass can be calculated by the following equation in analogy to one proposed for the first-pass effect following oral dosing (20):

$$\text{fraction eliminated} = (1 - f)\frac{PC}{PFR} \qquad \text{(Eq. 8)}$$

where PFR is the pulmonary flow rate, which is almost equivalent to the cardiac output (an average value of 3 liters was used as reported in Ref. 21), and PC is the pulmonary clearance:

$$PC = (V_d)_{b_3} \times b_3 \qquad \text{(Eq. 9)}$$

Substitution of Eq. 9 into Eq. 8 and elimination of f from Eq. 7 lead to the following equation (22):

$$(V_d)_{b_3} = \frac{PFR \times D^0}{b_3(AUC \times PFR + D^0)} \qquad \text{(Eq. 10)}$$

This equation, however, assumes that the pulmonary clearance is the only route of elimination for the compound. The fluorocarbon studied in this investigation was shown to be primarily eliminated intact from the lung in rats (23). Therefore, Eq. 6 can be used as an approximation for the determination of the apparent volume of distribution and the pulmonary clearance. The volume of distribution for trichloromonofluoromethane (Table II) was calculated to be approximately six times the average body weight (16.8–20 kg), an indication that this compound is extensively distributed in the body tissues.

The pulmonary clearance values were calculated using Eq. 9 and are also reported in Table II. An average clearance of 900 ml/min corresponds to 30% clearance in each cycle through the lung, and an error of this magnitude would have been involved in the calculations of volumes of distribution if the correction due to the first pulmonary pass effect had not been made.

The volume of the central compartment, from where the actual elimination takes place, can be calculated easily since the clearance from the central compartment should equal the clearance from the body:

$$(V_d)_{b_3} \times b_3 = V_c \times k_{el} \qquad \text{(Eq. 11)}$$

where k_{el} is the elimination rate constant from the central compartment. Table II also shows the values for the volumes of the central compartment. Smaller volumes as compared to the total volumes of distribution indicate retention of a smaller fraction in the central compartment as compared to tissue compartments after the pseudodistribution phase.

Table III—Disposition Half-Lives of Trichloromonofluoromethane

Disposition Phase	Half-Life (Mean ± *SEM*), min (n = 5)
Initial	3.17 ± 0.16[a]
Intermediate	15.70 ± 1.83
Final	92.70 ± 4.39

[a] This value may be slightly different from the true value because the experimental value would be influenced by the duration of the intravenous infusion.

Disposition Half-Lives—Table III shows the half-lives for the three disposition phases of trichloromonofluoromethane. The terminal disposition half-lives reported here are much longer than previously reported (11, 12, 24–26). An obvious reason for this large discrepancy is that the blood concentrations were monitored previously for only short intervals. The longest period involved was about 60–70 min. However, as shown in Fig. 1, it took about 100 min to reach the terminal phase. Consequently, in all of the mentioned studies, the fluorocarbons had not yet equilibrated with the body tissues, resulting in much shorter apparent half-lives due to the distribution to the tissues.

An analysis of the data reported (11) for the disposition of fluorocarbons in dogs shows that during the 10–60-min time interval the half-life of elimination appeared to be about 20 min for trichloromonofluoromethane. The actual value will be lower if the terminal phase is feathered out. This value is in agreement with the value in this study for the intermediate disposition phase. An analogous scrutiny of other reported data (26) shows that the terminal half-life for trichloromonofluoromethane was about 90 min in a human subject, an observation that went unnoticed by these investigators.

The finding that trichloromonofluoromethane shows a multiexponential decay in the blood concentration can be utilized to study the true elimination rate from the body and to evaluate the degree of accumulation in the body following single or multiple exposures.

Retention following Single Intravenous Administration—Following administration of trichloromonofluoromethane through intravenous infusion, the concentration in the blood drops very sharply. Such an observation was also reported for fluorocarbons following their inhalation (25, 26). However, such sharp declines in blood concentrations do not necessarily follow a parallel loss of the drug from the body (27). The amount of the drug remaining in the body (addition of Eqs. 3–5) as a function of time can also be calculated using the following equation (27):

$$\text{fraction remaining in body} = \frac{(C_1/b_1)e^{-b_1 t} + (C_2/b_2)e^{-b_2 t} + (C_3/b_3)e^{-b_3 t}}{(C_1/b_1) + (C_2/b_2) + (C_3/b_3)} \qquad \text{(Eq. 12)}$$

Figure 2 shows a plot of the amount of trichloromonofluoromethane remaining in the body and in various tissue compartments as described earlier (Eqs. 3–5). For example, the amount of trichloromonofluoromethane in the central compartment dropped to about 15% of the absorbed dose (dose after the first pulmonary pass) in 25 min, but about 55% of the absorbed dose was still present in the body.

It is evident that a sharp buildup of the administered dose continues in the tissues until a steady state is reached when the rate of change of the amount present in the tissues equals zero; this steady state is then followed by a decline in the amounts present in the tissue compartments. The ratio of the fraction of the dose in the tissues over that in the central compartment, however, keeps increasing since the rate of loss from the central compartment is greater than from the tissue compartments until the pseudoequilibration stage is reached; then the ratio of the amount in the tissue compartments to the amount in the central compartment remains constant (18). To demonstrate this, a plot was made (Fig. 3) based on the data presented in Fig. 2. It is evident that although the

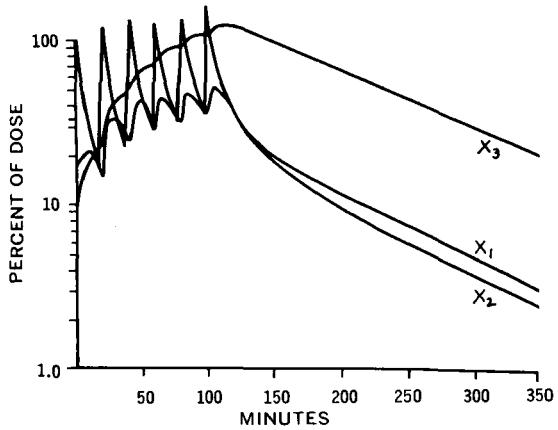


Figure 5—*Computer-generated curves for the distribution of trichloromonofluoromethane in various pharmacokinetic compartments following instantaneous absorption of six doses of 100 units each every 20 min. The pharmacokinetic parameters were obtained from a typical single-dose study (Dog 3287).*

steady state is reached rather quickly, as indicated by arrows in Fig. 3, the ratio of the amount in the tissue compartments over the central compartment keeps increasing until a pseudodistribution–equilibrium is reached. The retention of trichloromonofluoromethane, therefore, continues for a considerable period; for example, in the study reported in Scheme I, it took about 125 min to reach equilibration with the central compartment.

This finding is important because it shows for the first time that the claims made regarding fast elimination of fluorocarbons from the body (12, 25, 26) are probably erroneous and can be misleading in the evaluation of the toxicity of this compound.

In Compartment 2 (X_2), a peak was observed for the ratio of fluorocarbon in the tissue compartment over that in the central compartment. This kind of peak ratio was not observed for Compartment 3 (X_3). This difference can be explained on the basis of the capacity of the two compartments. Compartment 3 shows a much higher capacity for fluorocarbon retention compared to Compartment 2. In the initial phase of distribution, an "apparent supersaturation" of Compartment 2 may result due to the strong driving force from the central compartment because of the higher concentration. This apparent supersaturation might not exist in Compartment 3 because of its higher capacity.

The total amount of the dose retained following pseudodistribution–equilibrium is an important parameter, especially when extrapolating the single-dose study to multiple-dose studies. Table IV reports the retention of trichloromonofluoromethane in the tissue and central compartments after pseudodistribution–equilibrium has been reached. The fraction of the fluorocarbon retained in the tissue compartments compared to the central compartment shows that more than 90% of the total amount present in the body was retained in tissue compartments. This finding clearly shows that a sharp decline in the concentration in the central compartment does not necessarily parallel the decline in the amount in the body, an observation that was not well recognized in most studies reported earlier.

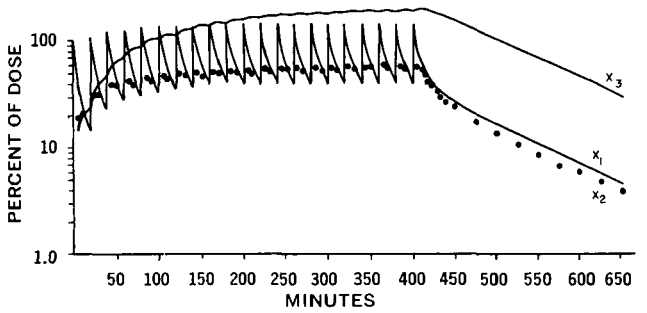


Figure 6—*Computer-generated curves for the distribution of trichloromonofluoromethane in various pharmacokinetic compartments following instantaneous absorption of 21 doses of 100 units each every 20 min. The pharmacokinetic parameters were obtained from a typical single-dose study (Dog 3287).*

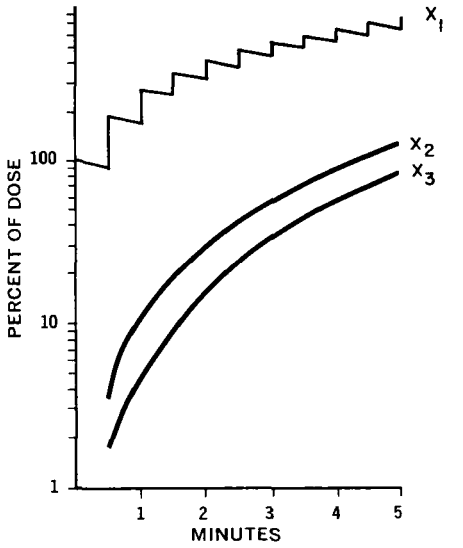


Figure 7—*Computer-generated curves for the distribution of trichloromonofluoromethane in various pharmacokinetic compartments following instantaneous absorption of 11 doses of 100 units each every 30 sec. The pharmacokinetic parameters were obtained from a typical single-dose study (Dog 3287).*

Multiple-Dose Kinetics—The pharmacokinetic parameters calculated following single-dose administration (Table I) can be used to simulate multiple-dose kinetics. A typical study, such as with Dog 3287, was used to generate multiple-dose disposition curves[6]. These curves were then superimposed by the experimental data obtained from two separate studies (Fig. 4). A good agreement between theoretical and experimental values provides the basis for the validity of the premise involved in the generation of multiple-dose curves that each dose decays independently of the amount present in the body or, in other words, that dose-independent kinetics are operative for the disposition of this compound in the dose range studied.

A significant aspect of this study is that it allows the assessment of the accumulation of trichloromonofluoromethane in the tissue compartments during multiple exposures in the frequent use of commercial products containing fluorocarbons as propellants. With fast intravenous infusion of a drug, it has often been shown (28) that a large fluctuation in the concentration in the central compartment is seen which is not necessarily paralleled in the tissue compartments because of the essential nature of the time-dependent process of distribution from the central compartment to the tissue compartments.

Figure 5 shows the calculated amount of trichloromonofluoromethane distributed to various tissue compartments following six multiple doses. The amount remaining in the tissue compartments shows much less fluctuation than is observed in the central compartment (the ordinate is in the logarithmic scale, which reduces the apparent variation). For example, Fig. 5 shows that the amount in Compartment 2 varied 1.5 times between the lowest level before administration and the peak level following the administration of last dose, whereas the amount in the central compartment varied almost four times. Compartment 3 showed the least fluctuation of about 1.13 times. Some correlation between the degree of fluctuation and the nature of the compartment can be drawn based on the data presented earlier regarding the capacity of various tissue compartments. The tissue compartment, which has a higher capacity to retain the fluorocarbon, would be expected to show less fluctuation in its highest and lowest levels during multiple-dose administration since a longer time may be required to equilibrate this compartment with the central compartment.

[6] IBM computer facility at the University of Illinois Medical Center.

Table IV—Average Retention ($\pm SEM$) of Trichloromonofluoromethane at 300 min following Pseudo-Distribution–Equilibrium in Various Compartments

Percent	$X_2 + X_3$ Compartments	X_1 Compartment
Percent of absorbed dose	3.05 ± 0.51	0.31 ± 0.23
Relative percent of total amount in the body	90.8 ± 15.2	9.2 ± 6.8

The magnitude of the problem of accumulation during multiple exposures to trichloromonofluoromethane is well emphasized in this study. For example, the total amount eventually accumulated in the tissues may well exceed the amount administered in one dose. This situation is shown in Fig. 5 where the total amount accumulated in two tissue compartments at the peak just after the last dose was about 1.65 times the dose that was administered every 20 min.

The computer-simulated curve for 21 consecutive doses at intervals of 20 min (Fig. 6) shows similar fluctuations of the amount in the central compartment as was observed for a six-dose study (Fig. 5). The fluctuation in the tissue compartments decreased gradually as the equilibrium was approached, and until then a buildup of trichloromonofluoromethane continued. The magnitude of the buildup would not be apparent if blood concentration alone were used to represent the body levels as was attempted by Dollery *et al.* (26). These investigators administered a mixture of fluorocarbons containing trichloromonofluoromethane by inhalation to humans every 10 min for 6 hr and failed to notice any significant rise in the blood concentration of trichloromonofluoromethane. They attributed the result to the possible lack of accumulation in the body.

The observations made in this study may have some bearing in hazard potential evaluation for professionals who come in frequent contact with aerosol packages containing fluorocarbon propellants.

The total accumulation in the body following multiple dosing depends on such factors as the dose administered and the frequency of administration. An interesting simulation of the accumulation pattern can be made to show the commonly observed excessive inhalation of bronchial aerosols by desperate asthmatic patients. A time interval of 0.5 min was chosen to administer 100 units of trichloromonofluoromethane through lungs in a typical study; the results in Fig. 7 show that in 5 min, during which 10 administrations were made, almost 96% of the trichloromonofluoromethane absorbed was retained in the body. Although a major fraction was present in the central compartment, the amount in the tissue compartments was more than twice the single dose administered. This massive buildup might also occur in youths who inhale aerosol packages to "turn on."

The toxicological aspects of the observations made here can be twofold. First, during excessive inhalation, it is possible that high tissue levels may be reached in spite of the high volatility of trichloromonofluoromethane. The sudden deaths reported following inhalation of aerosol packages can be attributed at least in part to threshold toxic limits, although no such direct and reliable data have been reported. Second, the accumulation following multiple exposures can result in much higher deep tissue compartment levels than are apparent from blood levels. Therefore, the possibility of chronic toxicity cannot be ruled out entirely.

The toxicological implications of the use of aerosol packages containing fluorocarbon propellants and the environmental pollution hazard from these compounds are matters of controversy, but the data presented here warrant against misleading toxicological interpretations that might result in the application of erroneous pharmacokinetic principles.

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[x] To whom inquiries should be directed.

Theoretical Considerations on Mononuclear Aggregations of Mixed Nucleosides in Dilute Aqueous Solutions Based upon Osmometric Measurements

HANNA N. BORAZAN

Abstract □ A theory is presented based on experimental work reporting shifts in the critical micelle-like concentrations of nucleosides and caffeine. An increase in these concentrations by multiple integers (called complexing capacity numbers) of the concentration of the vehicle compound, whose concentration was fixed, was observed for substances that tend to form complexes with each other and with some other compounds and to self-associate as well when their concentrations are varied. The physical model presented assumes the significance of the potential hydrogen bonding sites in determining the mode of aggregation as well as the magnitude of these complexing capacity numbers. This assumption was verified experimentally when reducing the hydrogen bonding by using 1,3-dimethyluracil. The mathematical model hypothesized has provided a means for calculating equilibrium constants for various consecutive processes as well as other quantities of theoretical interest.

Keyphrases □ Nucleosides, mixed—mononuclear aggregations, dilute aqueous solutions, osmometric measurements, theoretical considerations □ Aggregations, mononuclear—mixed nucleosides, dilute aqueous solutions, osmometric measurements, theoretical considerations □ Complexing capacity numbers—theoretical considerations, mononuclear aggregations of mixed nucleosides in dilute aqueous solutions based upon osmometric measurements

Goyan and Borazan (1) used an improved thermoelectric osmometer to study the colligative properties of dilute aqueous solutions containing caffeine and various substances of pharmaceutical interest. When a colligative property was plotted *versus* total molal concentration of caffeine (variable substance), pure or mixed with another substance kept at constant concentration (vehicle compound), two linear slopes could be used to represent all of the data. The points of break were referred to as the critical concentrations and were reported in each case.

The first slope of pure caffeine solution, expressed in terms of $\Delta R\ \Delta M^{-1}$, had a value similar to that determined for sucrose, indicating a monomeric behavior; a lower value was found for the second slope. Lower values were reported for both slopes of caffeine in the presence of a fixed concentration of a second substance which was used as a vehicle.

These critical concentrations were shifted to higher values on the molal scale of caffeine in the presence of a vehicle (1). When this increment in the critical concentration was divided by the vehicle concentration, a quotient approaching a simple integer was obtained. The investigators referred to these integers as the complexing capacity numbers; these numbers were assumed to be related to the stoichiometry of the complex. Later, the existence of the effect was confirmed with some biologically active nucleosides (2, 3), few of which were reported previously to form complexes and to self-associate (4, 5).

The purpose of this paper is to present theoretical, physical, and mathematical considerations that shed some light upon the origin of the shift in these critical micelle-like concentrations and the mechanism of the formation of these complexing capacity numbers.

EXPERIMENTAL

Instrumentation—The vapor phase thermoelectric osmometer used was described previously (1, 6–8). The measured colligative property is expressed in terms of ΔR, the difference between the resistances of the measuring thermistor when in contact with pure solvent and when in contact with the sample under study for a given molal concentration.

Solutions—Solutions were prepared with distilled water on a molal basis. 2′-Deoxyguanosine[1], 1,3-dimethyluracil[1], and 2′-deoxyadenosine[2] were obtained commercially. An assay for the percentage of water was made by drying over phosphorus pentoxide in a vacuum drying apparatus at the boiling point of absolute alcohol. This percentage was used to correct the weight of solid chemicals when preparing various concentrations.

Reagent grade sucrose crystals[3] were used to prepare the solutions used as the reference standard.

Standardization—The method was fully described previously (1–3). A value of 923.7 ohms/molal for the slope of the standard solution of sucrose is used for this work.

THEORY

To explain the origin of the shift in the critical micelle-like concentrations (1–3), the inhibition of self-association of the variable compound by a certain multiple integer of the vehicle concentration was investigated as a possible mechanism.

Pyrimidine Nucleosides—According to Table I, the self-association of pyrimidine nucleosides is modified by purine and pyrimidine nucleosides. When 2′-deoxyadenosine is used as a vehicle (3) (Table I), complexing capacity numbers of 6, 6, and 9 are obtained with thymidine, cytidine, and uridine, respectively, when they are used as variables. When 2′-deoxyguanosine is used as a vehicle, complexing capacity numbers of 7, 7, and 9 are obtained from these nucleosides, respectively, when they are used as variables.

A reasonable hypothesis is that these numbers represent the average number of molecules involved with one molecule of vehicle before the self-association takes place. The low bonding energy rules out firm complex formation and indicates that attachments must be regarded as arising from second-order effects. On the basis of this hypothesis, one can construct a model showing the possible orientation of these pyrimidine nucleosides relative to purine nucleosides. The model cannot represent a fixed structure but should show the most probable mode of aggregation having the lowest possible energy due to configuration. However, a comparison of the possible binding sites with the calculated complexing capacity numbers (Table I) provides clear evidence that there is some correlation between the possible potential hydrogen bonding sites and the arrangement of the molecules in solution.

Based on NMR data, Schweizer *et al.* (9) suggested the breakdown of purine and pyrimidine aggregates by pyrimidine nucleosides and/or their insertion between the purine stacks; a high concentration of purine was used. More recently, Raszka and Kaplan

[1] Nutritional Biochemicals.
[2] General Biochemicals.
[3] ACS Code 2376.

Table I—Comparison between the Number of Binding Sites on the Vehicle Molecule Relative to Variable Used and the Complexing Capacity Numbers with the Variable Molecule for Different Combinations of Nucleosides

Variable–Vehicle	Number of Binding Sites on Vehicle Molecule Relative to Variable Used	Complexing Capacity Numbers with Variables[a]
2′-Deoxyadenosine–thymidine (0.02 *M*)	4	1
2′-Deoxyadenosine–uridine (0.02 *M*)	4	1
2′-Deoxyadenosine–2′-deoxyguanosine (0.01 *M*)	7	4
2′-Deoxyguanosine–cytidine (0.003 *M*)	5	1
2′-Deoxyguanosine–2′-deoxyadenosine (0.003 *M*)	6	3
Uridine–2′-deoxyadenosine (0.015 *M*)	6	9
Uridine–2′-deoxyguanosine (0.012 *M*)	7	9
Cytidine–thymidine (0.03 *M*)	4	4
Cytidine–2′-deoxyadenosine (0.015 *M*)	6	6
Cytidine–2′-deoxyguanosine (0.012 *M*)	7	7
Thymidine–2′-deoxyguanosine (0.012 *M*)	7	7
Thymidine–2′-deoxyadenosine (0.015 *M*)	6	6
Thymidine–cytidine (0.02 *M*)	5	3
1,3-Dimethyluracil–2′-deoxyadenosine (0.015 *M*)	2	2
1,3-Dimethyluracil–2′-deoxyguanosine (0.012 *M*)	3	3

[a] Figures are approximate and were rounded to integers. They were calculated according to data taken from Ref. 3 and Table III.

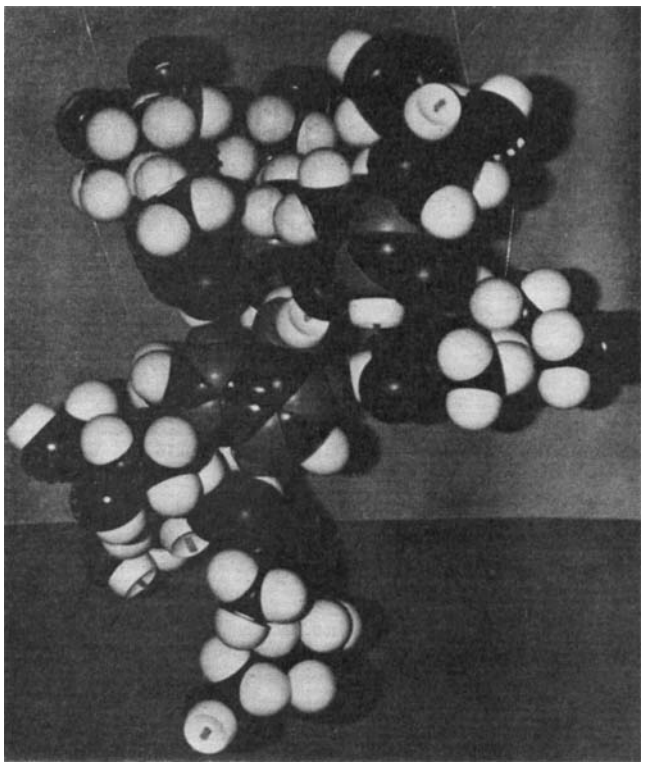

Figure 1—*Model representing hypothetical aggregation: a pictorial representation of a space-filling model showing the way the potential hydrogen bonding directs the mode of aggregation most effectively around the template (2′-deoxyadenosine). In reality, it is not a fixed rigid structure but a rather dynamic situation. The surrounding molecules (thymidine) crowd around the template molecule in such a dynamic way that an average partial parallel stacking is implied from this dynamic state. Approximate overall dimensions are 28 × 21 Å.*

(10) obtained evidence for association by hydrogen bonding of mononucleotides in aqueous solution based upon high-resolution PMR spectroscopy (220 MHz). The absence of self-association in water of some purine and pyrimidine base derivatives also was reported (11). On these bases, it is proposed that a spherical orientation might occur in which a purine nucleoside serves as a template surrounded by the smaller pyrimidine nucleosides so that the surrounding nucleosides orient themselves in the least hindered way. This orientation possibly can be by way of partial parallel stacking of their bases, with the ribose or deoxyribose directed away from the template molecule.

This picture is somewhat similar to that proposed for the mode of stacking of pyrimidine nucleosides based upon their osmometric measurements (9), and it is well illustrated in the model[4] shown in Fig. 1. The thymidine molecules are crowded around the template molecule and appear in a similar (but partial) way to the average parallel stacking of pyrimidine nucleosides (12). The potential hydrogen bonding sites also seem to play a role in directing the mode of aggregation of the nucleosides around the template at a distance of 3–4 Å (13) from each other.

Many factors seem to account for the smaller complexing capacity numbers associated with the inhibition of self-association of the pyrimidine nucleosides by other pyrimidines. These factors include the lack of aromaticity, the smaller size, the difference in steric effects, the smaller number of potential hydrogen bonding sites (Table I), and the differences in other physicochemical properties (14, 15).

Purine Nucleosides—The mechanism of inhibition of self-association and the mode of aggregation of the purine nucleosides induced by other purine or pyrimidine nucleosides seem very similar to those described for pyrimidine nucleosides. The differences observed in the complexing capacity numbers can be accounted for on the same basis (Table I). The orientation of purine nucleosides around another purine nucleoside, which serves as a template, seems to be similar to that described previously. The partial average parallel stacking (16) is also proposed. Several investigators (5, 17, 18) confirmed that the stacking is similar to the π-complex of benzene in chloroform (19, 20). However, the picture is not simple; the model presented represents the orientation in an infinitely small amount of time. Rapid exchange of bases and nucleosides has been reported (5), in connection with the break and reformation of the stacks, because the free energy of self-association and of complex formation is of the order of magnitude of the thermal kinetic energy (4, 5, 15, 21–24).

These results are in agreement with those presented by Jardetzky (12) for bases of purine and pyrimidine derivatives: "... that the rate of exchange of bases between the stacks and the free state is of an order of magnitude comparable to the rate of diffusion. Thus, the forces responsible for 'hydrophobic' bonding of nucleic acid bases appear to be sufficient to produce some preferred orientation of the bases in solution, but insufficient to maintain stable rigid arrays." This conclusion, from NMR studies, is in agreement with the dynamic picture hypothesized here of the absence of stable discrete complexes and the presence of aggregates, spherically oriented, in a dynamic state.

According to suggested models (5, 25, 26) and theoretical calculations (27), all possible orientations could exist. The lifetime is so short for each, about 10^{-9} sec (12), that what one deduces from NMR or other physical measurements is an average phenomenon of the entire dynamic picture. The contribution of one form might

[4] Overall dimensions were measured using CPK atomic models, Schwarz Bioresearch, Inc., Orangeburg, NY 10962

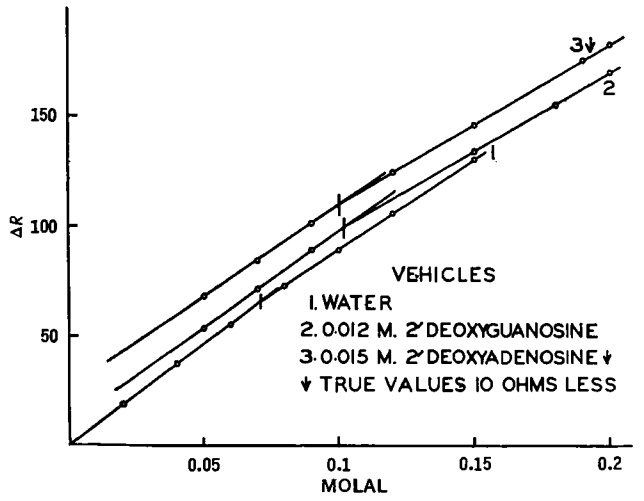

Figure 2—*Plot of* **ΔR** *in ohms* **versus** *molal concentration of 1,3-dimethyluracil. Arrow indicates displacement by 10 ohms to avoid crowding.*

be different from that of another because of various factors, including those that are inherent in the molecules themselves, such as size, aromaticity, polarizability, polarity, potential hydrogen bonding sites, and other physicochemical properties (14, 15).

The concentrations used for the study of self-association were all 0.1 molal and lower; the nucleoside mixtures were all 0.2 molal and lower (2, 3). Very dilute solutions were preferred over concentrated solutions to trace this effect and also to obtain a better picture of the behavior. Use of concentrated solutions could put constraints on the system and force one process over the other with the result that the actual picture could be deformed.

The present dynamic theory of these complexes is not in conflict with previous work on stacking but merely suggests that the potential hydrogen bonding, if present, might play a role in directing the mode of stacks of these aggregates, which are in a dynamic state. However, it is a reasonable hypothesis that the magnitude of the number of the potential hydrogen bonding sites can determine the total number of monomers in each aggregate (including both the bonded and nonbonded species), based upon the complexing capacity numbers each has with other similar base derivatives, *i.e.*, purine–purine and pyrimidine–pyrimidine. On these bases, the numbers in Table II are assigned for the nucleosides studied.

Based upon the physical interpretations of the described effect, the following mathematical model is proposed for calculating equilibrium constants for the multiple processes of mononuclear aggregation around a solute molecule:

$$\begin{aligned} A + B &= AB \\ AB + B &= AB_2 \\ &\vdots \\ AB_{j-1} + B &= AB_j \\ &\vdots \\ AB_{z-1} + B &= AB_z \end{aligned} \qquad \text{(Eq. 1)}$$

If the variable substance self-associates too, then:

$$\begin{aligned} B + B &= B_2 \\ B_2 + B &= B_3 \\ &\vdots \\ B_i + B &= B_{i+1} \\ &\vdots \\ B_\sigma + B &= B_{\sigma+1} \end{aligned} \qquad \text{(Eq. 2)}$$

In the presented models, it is assumed that no interactions among mixed and homogeneous associates, AB_j and B_i, respectively, take place; that the vehicle compound, A, does not self-associate; and that the binding sites on the nucleus molecule, A, for heterogeneous association processes are dependent and nonequivalent, *i.e.*, the binding of a free B to A can influence the binding of another B to the same nucleus.

Where z and σ are the maximum numbers of multiple equilibria, the corresponding equilibrium constants will be:

$$K_1 = (AB)/(A)(B) \qquad (AB) = K_1(A)(B) \qquad \text{(Eq. 3)}$$
$$K_2 = (AB_2)/(AB)(B) \qquad (AB_2) = K_2(AB)(B) \qquad \text{(Eq. 4)}$$
$$K_j = (AB_j)/(AB_{j-1})(B) \qquad (AB_j) = K_j(AB_{j-1})(B) \qquad \text{(Eq. 5)}$$
$$K_z = (AB_z)/(AB_{z-1})(B) \qquad (AB_z) = K_z(AB_{z-1})(B) \qquad \text{(Eq. 6)}$$
$$\beta_1 = (B_2)/(B)^2 \qquad (B_2) = \beta_1(B)^2 \qquad \text{(Eq. 7)}$$
$$\beta_2 = (B_3)/(B_2)(B) \qquad (B_3) = \beta_2(B_2)(B) \qquad \text{(Eq. 8)}$$
$$\beta_i = (B_{i+1})/(B_i)(B) \qquad (B_{i+1}) = \beta_i(B_i)(B) \qquad \text{(Eq. 9)}$$
$$\beta_\sigma = (B_{\sigma+1})/(B_\sigma)(B) \qquad (B_{\sigma+1}) = \beta_\sigma(B_\sigma)(B) \qquad \text{(Eq. 10)}$$

By substituting for (AB) of Eq. 3 into Eq. 4:

$$(AB_2) = K_1K_2(A)(B)^2 \qquad \text{(Eq. 11)}$$

If the same technique of substitution is carried out:

$$(AB_j) = K_1 \dots K_j(A)(B)^j \qquad \text{(Eq. 12)}$$
$$(B_{i+1}) = \beta_1 \dots \beta_i(B)^{i+1} \qquad \text{(Eq. 13)}$$

According to Eq. 1, the total concentration of the vehicle, (A_0), can be expressed:

$$(A_0) = (A) + (AB) + (AB_2) + (AB_3) + \dots + (AB_j) + \dots + (AB_z) \qquad \text{(Eq. 14a)}$$

$$(A_0) = (A) + \sum_{j=1}^{z}(AB_j) \qquad \text{(Eq. 14b)}$$

By combining Eqs. 12, 14*a*, and 14*b*:

$$(A_0) = (A) + \sum_{j=1}^{z}\left(\prod_{j=1}^{j}K_j\right)(A)(B)^j \qquad \text{(Eq. 15a)}$$

$$(A_0) = (A)\left[1 + \sum_{j=1}^{z}\left(\prod_{j=1}^{j}K_j\right)(B)^j\right] \qquad \text{(Eq. 15b)}$$

where:

$$\prod_{j=1}^{j}K_j = K_1K_2 \dots K_j \qquad \text{(Eq. 15c)}$$

The total concentration of the variable, (B_0), can be defined as follows:

$$(B_0) = (B) + (AB) + 2(AB_2) + \dots + j(AB_j) + \dots + z(AB_z) + 2(B_2) + 3(B_3) + \dots + (i+1)(B_{i+1}) + \dots + (\sigma+1)(B_{\sigma+1}) \qquad \text{(Eq. 16)}$$

$$(B_0) = (B) + \sum_{j=1}^{z}j(AB_j) + \sum_{i=1}^{\sigma}(i+1)(B_{i+1}) \qquad \text{(Eq. 17)}$$

Both (AB_j) and (B_{i+1}) can be substituted by their corresponding expressions (Eqs. 12 and 13, respectively) in Eq. 17:

$$(B_0) = (B) + \sum_{j=1}^{z}j\left(\prod_{j=1}^{j}K_j\right)(A)(B)^j + \sum_{i=1}^{\sigma}(i+1)\left(\prod_{i=1}^{i}\beta_i\right)(B)^{i+1} \qquad \text{(Eq. 18)}$$

Osmolal concentration, $(\bar{M})$, can be measured experimentally and defined according to the model presented:

$$(\bar{M}) = (A) + (B) + (AB) + (AB_2) + \dots + (AB_j) + \dots + (AB_z) + (B_2) + (B_3) + \dots + (B_{i+1}) + \dots + (B_{\sigma+1}) \qquad \text{(Eq. 19a)}$$

$$(\bar{M}) = (A) + (B) + \sum_{j=1}^{z}(AB_j) + \sum_{i=1}^{\sigma}(B_{i+1}) \qquad \text{(Eq. 19b)}$$

Table II—Possible Number of Molecules in Each Aggregate

Nucleoside	Monomers in Aggregate
2′-Deoxyadenosine	4
2′-Deoxyguanosine	5
Thymidine	5
Cytidine	4

The experimental slopes reported previously (1–3) and in this work (Table II and Fig. 2), which are expressed in terms of $\Delta R/\Delta M$, can be converted to osmolal slopes by dividing by a slope of an ideal substance expressed in the same units. The sucrose slope is used for this purpose. It can be calculated from measurements of a colligative property, ΔR, at concentrations lower than 0.2 molal (2, 3, 8).

By the application of simple analytical geometry, the first linear osmolal slopes of mixed nucleosides in water can be expressed:

$$S_1 = \frac{(\overline{M}) - (A_0)}{(B_0) - 0} = \frac{(\overline{M}) - (A_0)}{(B_0)} = \frac{\text{first experimental slope}}{\text{sucrose slope}} \qquad \text{(Eq. 20}a\text{)}$$

$$S_1 = \frac{\left[(B) + \sum_{i=1}^{\sigma}\left(\prod_{i=1}^{i}\beta_i\right)(B)^{i+1}\right]}{(B)\left[1 + \sum_{j=1}^{z} j\left(\prod_{j=1}^{j}K_j\right)(A)(B)^{j-1} + \sum_{i=1}^{\sigma}(i+1)\left(\prod_{i=1}^{i}\beta_i\right)(B)^{i}\right]} \qquad \text{(Eq. 20}b\text{)}$$

Then set:

$$\sum_{i=1}^{\sigma}\left(\prod_{i=1}^{i}\beta_i\right)(B)^{i+1} = \Phi(\beta_i, B) \qquad \text{(Eq. 21)}$$

$$\sum_{i=1}^{\sigma}(i+1)\left(\prod_{i=1}^{i}\beta_i\right)(B)^{i} = \frac{\partial\Phi}{\partial(B)} \qquad \text{(Eq. 22)}$$

$$1 + \sum_{j=1}^{z}\left(\prod_{j=1}^{j}K_j\right)(B)^{j} = \Psi(K_j, B) \qquad \text{(Eq. 23)}$$

and:

$$\frac{\partial\Psi}{\partial(B)} = \sum_{j=1}^{z} j\left(\prod_{j=1}^{j}K_j\right)(B)^{j-1} \qquad \text{(Eq. 24)}$$

Substituting for Φ of Eq. 21, $\partial\Psi/\partial(B)$ of Eq. 24, and $\partial\Phi/\partial(B)$ of Eq. 22 into Eq. 20:

$$S_1 = \frac{(B) + \Phi}{(B)\left[1 + (A)\frac{\partial\Psi}{\partial(B)} + \frac{\partial\Phi}{\partial(B)}\right]} \qquad \text{(Eq. 25)}$$

If one assumes that the self-association is inhibited at molal ratios of variable to vehicle of less than their corresponding complexing capacity numbers, according to the theory presented, then the function Φ and its derivative $\partial\Phi/\partial(B)$ should vanish; Eq. 25 then reduces to:

$$S_1 = (B)/(B_0) = \left[1 + (A)\frac{\partial\Psi}{\partial(B)}\right]^{-1} \qquad \text{(Eq. 26)}$$

By manipulating Eq. 26, the following can be written:

$$\frac{\partial\Psi}{\partial(B)} = \frac{1}{(A)}\left(\frac{1 - S_1}{S_1}\right) \qquad \text{(Eq. 27)}$$

Combining Eqs. 23, 15*a*, 15*b*, and 15*c*:

$$(A_0) = (A)\Psi \qquad \text{(Eq. 28)}$$

and substituting into Eq. 27:

$$\left(\frac{(A_0)S_1}{1 - S_1}\right)\frac{\partial\Psi}{\partial(B)} = \Psi \qquad \text{(Eq. 28}a\text{)}$$

Set:

$$\left(\frac{(A_0)S_1}{1 - S_1}\right) = \alpha_c = \text{constant} \qquad \text{(Eq. 29)}$$

Then:

$$\alpha_c\frac{\partial\Psi}{\partial(B)} = \Psi \qquad \text{(Eq. 30)}$$

This partial differential equation has the following solution:

$$\Psi = e^{(B)/\alpha_c}(\text{constant}) \qquad \text{(Eq. 31)}$$

While the constant has a value of 1, one may expand the function Ψ in the usual manner. By equating it with Eq. 23:

$$\Psi = 1 + K_1(B) + K_1K_2(B)^2 + \ldots + K_1 \ldots K_j(B)^j + \ldots + K_1 \ldots K_z(B)^z \qquad \text{(Eq. 32}a\text{)}$$

$$\Psi = 1 + \frac{(B)}{\alpha_c} + \frac{1}{2!}\left(\frac{(B)}{\alpha_c}\right)^2 + \ldots \frac{1}{j!}\left(\frac{(B)}{\alpha_c}\right)^j + \ldots \frac{1}{z!}\left(\frac{(B)}{\alpha_c}\right)^z + \ldots \frac{1}{\infty!}\left(\frac{(B)}{\alpha_c}\right)^{\infty} \qquad \text{(Eq. 32}b\text{)}$$

For large values of z and for low concentrations of B, the following can be set by excluding terms of higher order than z:

$$K_1 = 1/\alpha_c \qquad \text{(Eq. 33}a\text{)}$$
$$K_2 = 1/2\alpha_c \qquad \text{(Eq. 33}b\text{)}$$
$$K_j = 1/j\alpha_c \qquad \text{(Eq. 33}c\text{)}$$
$$K_z = 1/z\alpha_c \qquad \text{(Eq. 33}d\text{)}$$

Accordingly, the constant α_c will be referred to as the progression constant.

Calculations—The first slope, S_1, and the predetermined concentrations can be used to calculate the concentrations of various molecular species, osmotic coefficients, according to the following calculations.

Free Vehicle Concentration—Substituting for *(B)* of Eq. 26 into Eq. 31:

$$\Psi = e^{(1-S_1)(B_0)/(A_0)} \qquad \text{(Eq. 34)}$$

Then by setting:

$$(B_0)/(A_0) = \gamma \qquad \text{(Eq. 35)}$$

and combining Eqs. 34 and 28:

$$(A) = (A_0)e^{-\gamma(1-S_1)} \qquad \text{(Eq. 36)}$$

Concentration of AB_j—The concentration of AB_j is found by using Eqs. 26 and 35:

$$(B) = S_1\gamma(A_0) \qquad \text{(Eq. 37)}$$

Then, according to Eq. 33:

$$\prod_{j=1}^{j}K_j = \frac{1}{j!}\left(\frac{1}{\alpha_c}\right)^j = \frac{1}{j!}\left(\frac{1 - S_1}{(A_0)S_1}\right)^j \qquad \text{(Eq. 38)}$$

One may substitute for *(A)* of Eq. 36, *(B)* of Eq. 37, and the product of K's to calculate the concentration of AB_j according to Eq. 12:

$$(AB_j) = (A_0)\frac{\gamma^j}{j!}(1 - S_1)^j e^{-\gamma(1-S_1)} \qquad \text{(Eq. 39)}$$

Calculated Osmolal Concentration—In the absence of self-asso-

Table III—Data Falling on Two Linear Slopes[a]

Variable	Vehicle	Intercept[b], ohms	First Slope, $\Delta R/\Delta M$	Critical Concentration	Second Slope, $\Delta R/\Delta M$
1,3-Dimethyluracil	Water	0.03	921.8	0.068	830.0
1,3-Dimethyluracil	2′-Deoxyadenosine (0.015 *M*)	13.86	859.5	0.0987	736.1
1,3-Dimethyluracil	2′-Deoxyguanosine (0.012 *M*)	11.06	866.0	0.1015	723.9

[a] Measurements were at 25°. [b] The intercepts are not experimental points.

ciation, the calculated osmolal concentration can be obtained by substitution for *(A)* of Eq. 36, *(B)* of Eq. 26, and the concentration of AB_j of Eq. 39 into Eq. 19:

$$(\bar{M})_{calc} = S_1(B_0) + \sum_{j=0}^{z}(A_0)\frac{\gamma^j}{j!}(1 - S_1)^j e^{-\gamma(1-S_1)} \quad \text{(Eq. 40a)}$$

$$(\bar{M})_{calc} = S_1(B_0) + (A_0)e^{-\gamma(1-S_1)}\sum_{j=0}^{z}\frac{\gamma^j}{j!}(1 - S_1)^j \quad \text{(Eq. 40b)}$$

Osmotic Coefficient—The osmotic coefficient, ϕ, can be defined as the ratio of osmolal to the total molal concentrations. Thus:

$$\phi = (\bar{M})/[(A_0) + (B_0)] \quad \text{(Eq. 41)}$$

The following relationship can be arrived at by substituting for each term in Eq. 41:

$$\phi = [S_1\gamma + 1]/[\gamma + 1] \quad \text{(Eq. 42)}$$

RESULTS AND DISCUSSION

The experimental results obtained seem to fit the previous treatment for caffeine and nucleosides (1–3). Two linear slopes can also be used to express all of the data obtained, with ΔR as the ordinate and molal concentration as the abscissa.

Table III shows slopes, critical concentrations, and intercepts; the slopes pass through all points within the limits of experimental error and were calculated by the method of least squares. Table III also shows that these critical concentrations are shifted as a function of the nature and concentration of the vehicle used in much the same way as for caffeine and nucleosides (1–3).

Figure 2 shows clearly the shift along the variable molal scale. In each case, the amount of shift on the variable molal scale from that determined in pure water divided by the concentration of the vehicle used gave a certain number that also approaches an integer (Table I).

The importance of the potential hydrogen bonding sites in establishing the mode of aggregation is clearly indicated by the observation (Table I) that reducing the hydrogen bonding by using 1,3-dimethyluracil also reduces the complexing capacity numbers to 2 and 3 for 2′-deoxyadenosine and 2′-deoxyguanosine, respectively, when they are used as vehicles.

Borazan (2) and Borazan and Goyan (3) considered errors in standard and sample slopes which when combined introduced errors of about 1.0% in the osmotic coefficients. Accordingly, the progression constants calculated according to Eq. 29 are reported with percentage uncertainty in each case.

Table IV—Data Falling on a Linear Slope of Aqueous Solutions of Caffeine[a]

Variable	Vehicle	First Slope[b], $\Delta R/\Delta M$	Progression Constant[c], α_c (molal), %
Caffeine	*p*-Aminobenzoic acid (0.005 *M*)	743	0.0268 ± 9
Caffeine	*p*-Aminobenzoic acid (0.010 *M*)	659	0.0295 ± 6
Caffeine	*p*-Aminobenzoic acid (0.015 *M*)	605	0.0328 ± 4.5

[a] Measurements were at 25°. [b] Data taken from Ref. 1. [c] Progression constant = (first slope × vehicle concentration)/(sucrose slope − first slope), according to Eq. 29. Sucrose slope has a value of 882 in connection with this work.

Inspection of Table IV shows that by increasing the initial concentrations of the vehicle compound threefold, a slight increase in the values of the progression constant is produced. This increase is considered slightly above the values that can be accounted for on the basis of random error. A reasonable explanation for this finding is the various approximations made in the derivations such as the approximations made in deriving various equilibrium constants from Eq. 33. Also, empirical assumptions were made in the presented models, such as ignoring interactions among various associated forms. However, the results presented in Table IV are considered satisfactory and in agreement with the prediction of the equations.

The data of Borazan and Goyan (3) and of Table III of this work provided information used to calculate progression constants, α_c, for the equilibrium constants (Table V). Equilibrium constants can be calculated from osmolal slopes, determined from experimental data, using a simple mathematical model:

$$A + B = AB \quad \text{(Eq. 43)}$$

$$K = \frac{(AB)}{(A)(B)} \quad \text{(Eq. 44)}$$

where *(A)* and *(B)* are the concentrations of the free reactants, and *(AB)* represents the concentration of the complex. The osmolal, $(\bar{M})$, and the total molal, *(M)*, concentrations are then:

$$(\bar{M}) = (A) + (B) + (AB) \quad \text{(Eq. 45)}$$

$$(M) = (A) + (B) + 2(AB) \quad \text{(Eq. 46)}$$

By subtracting Eq. 45 from Eq. 46:

$$(AB) = (M) - (\bar{M}) \quad \text{(Eq. 47)}$$

$(\bar{M})$ can be determined from experimental slopes (1–3) (Tables III and IV) after dividing by the sucrose slope to get the first osmolal slopes, S_1, by the following relationship:

$$(\bar{M}) = S_1(B_0) + (A_0) \quad \text{(Eq. 48)}$$

while:

$$(M) = (A_0) + (B_0) \quad \text{(Eq. 49)}$$

By substituting for the osmolal and the total molal concentrations into Eq. 47:

$$(AB) = (B_0) - S_1(B_0) \quad \text{(Eq. 50)}$$

The free reactant species can be expressed as:

$$(A) = (A_0) - (AB) \quad \text{(Eq. 51)}$$

$$(B) = (B_0) - (AB) \quad \text{(Eq. 52)}$$

By substituting for *(AB)* of Eq. 50 into Eqs. 51 and 52:

$$(A) = (A_0) + S_1(B_0) - (B_0) \quad \text{(Eq. 53)}$$

$$(B) = S_1(B_0) \quad \text{(Eq. 54)}$$

Thus, Eq. 44 can be expressed in terms of measurable quantities as follows:

Table V—Equilibrium Constants Calculated According to Eq. 55, for Different $(B_0)/(A_0)$ Ratios and the First Equilibrium Constants (K_1) Calculated from the Progression Constant[a] (α_c) by Eq. 33, Together with the Standard Free Energies Based upon K_1 for Different Variable–Vehicle Nucleoside Combinations[b]

$(B_0)/(A_0)$	K, molal^{-1}
Thymidine–2′-Deoxyadenosine (0.015 *M*)	
0.1	12.25
0.5	13.07
1.0	14.23
1.33	15.10
2.0	17.40
3.0	22.40
4.0	42.40
5.0	51.75
6.0	146.30
$\alpha_c = 0.083 \pm 6.5\%$ molal; $K_1 = 12.04$ molal^{-1}; and $\Delta G^0 = -1490$ cal/mole	
2′-Deoxyadenosine–Thymidine (0.02 *M*)	
0.1	17.35
1.0	22.60
$\alpha_c = 0.0593 \pm 4\%$ molal; $K_1 = 16.84$ molal^{-1}; and $\Delta G^0 = -1690$ cal/mole	
2′-Deoxyadenosine–Uridine (0.02 *M*)	
0.1	10.4
1.0	12.3
$\alpha_c = 0.097 \pm 6\%$ molal; $K_1 = 10.30$ molal^{-1}; and $\Delta G^0 = -1400$ cal/mole	
2′-Deoxyadenosine–2′-Deoxyguanosine (0.01 *M*)	
0.1	38.6
4.0	82.6
$\alpha_c = 0.0267 \pm 3.5\%$ molal; $K_1 = 37.45$ molal^{-1}; and $\Delta G^0 = -2170$ cal/mole	
2′-Deoxyguanosine–Cytidine (0.003 *M*)	
0.1	10.30
1.0	10.60
$\alpha_c = 0.097 \pm 40\%$ molal; $K_1 = 10.30$ molal^{-1}; and $\Delta G^0 = -1400$ cal/mole	
2′-Deoxyguanosine–2′-Deoxyadenosine (0.003 *M*)	
0.1	38.0
1.0	40.2
3.0	51.3
$\alpha_c = 0.0276 \pm 10\%$ molal; $K_1 = 36.50$ molal^{-1}; and $\Delta G^0 = -2160$ cal/mole	
Uridine–2′-Deoxyadenosine (0.015 *M*)	
0.1	10.5
5.0	32.8
$\alpha_c = 0.0962 \pm 7.5\%$ molal; $K_1 = 10.40$ molal^{-1}; and $\Delta G^0 = -1405$ cal/mole	
Uridine–2′-Deoxyguanosine (0.012 *M*)	
0.1	7.80
5.0	13.43
$\alpha_c = 0.129 \pm 12.5\%$ molal; $K_1 = 7.75$ molal^{-1}; and $\Delta G^0 = -1227$ cal/mole	
Cytidine–Thymidine (0.02 *M*)	
0.1	4.68
4.0	7.05
$\alpha_c = 0.215 \pm 12\%$ molal; $K_1 = 4.65$ molal^{-1}; and $\Delta G^0 = -923$ cal/mole	
Cytidine–2′-Deoxyadenosine (0.015 *M*)	
0.1	10.10
5.0	28.46
$\alpha_c = 0.1005 \pm 8\%$ molal; $K_1 = 9.96$ molal^{-1}; and $\Delta G^0 = -1380$ cal/mole	
Cytidine–2′-Deoxyguanosine (0.012 *M*)	
0.1	9.02
5.0	17.40
$\alpha_c = 0.1105 \pm 10.5\%$ molal; $K_1 = 9.04$ molal^{-1}; and $\Delta G^0 = -1320$ cal/mole	
Thymidine–2′-Deoxyguanosine (0.012 *M*)	
0.1	11.86
5.0	30.40
$\alpha_c = 0.0855 \pm 8\%$ molal; $K_1 = 11.7$ molal^{-1}; and $\Delta G^0 = -1475$ cal/mole	
Thymidine–Cytidine (0.02 *M*)	
$\alpha_c = 0.2127 \pm 12\%$ molal; $K_1 = 4.7$ molal^{-1}; and $\Delta G^0 = -927$ cal/mole	
1,3-Dimethyluracil–2′-Deoxyadenosine (0.015 *M*)	
0.1	5.05
2.0	5.83
$\alpha_c = 0.201 \pm 14.5\%$ molal; $K_1 = 4.97$ molal^{-1}; and $\Delta G^0 = -960$ cal/mole	
1,3-Dimethyluracil–2′-Deoxyguanosine (0.012 *M*)	
0.1	5.60
3.0	7.03
$\alpha_c = 0.181 \pm 16\%$ molal; $K_1 = 5.52$ molal^{-1}; and $\Delta G^0 = -1027$ cal/mole	

[a] Calculated from data taken from Ref. 3 and Table III (for the combinations containing 1,3-dimethyluracil). [b] Measurements were at 25°.

$$K = \frac{(1 - S_1)}{[(A_0) + S_1(B_0) - (B_0)]S_1} \qquad \text{(Eq. 55)}$$

It is obvious from this relationship that, for slopes approaching unity and for low values of $(B_0)/(A_0)$, Eq. 55 reduces to:

$$K = \frac{(1 - S_1)}{(A_0)S_1} \qquad \text{(Eq. 56)}$$

This is essentially the same result obtained in calculating the first equilibrium constant from the model presented in Eq. 1. Therefore, one would not be surprised when getting different values of K from Eq. 55 along the molal scale of the variable compound (Table V). The magnitude of the K values increases with the increase in the total molal concentration of the variable compound, (B_0), while approaching K_1 values calculated from Eqs. 29 and 33 at a molal ratio of $(B_0)/(A_0)$ of less than 0.1.

These results indicate the significance of a simple equilibrium situation at low ratios of $(B_0)/(A_0)$. Thus, the linear experimental slopes indicate a rather complex equilibrium and a formation of complexes having stoichiometry different from 1:1.

One can also verify the validity of the derived equations and their consistency with experimental results by considering Eq. 40. For large values of z, the summation of terms in this equation approaches $e^{\gamma(1-S_1)}$; thus:

$$e^{-\gamma(1-S_1)} \sum_{j=0}^{z} \frac{\gamma^j}{j!}(1 - S_1)^j = 1 \qquad \text{(Eq. 57)}$$

and $(\bar{M})_{calc}$ equals osmolal concentration determined from the first osmolal slopes by the process of application of simple analytical geometry, *i.e.*, $S_1(B_0) + (A_0)$.

Another test for the validity of the derived relationships, α_c (Table V), is found to have approximately the same values in most cases studied, within the limits of accuracy, for the same combination when each is used as a variable and a vehicle.

According to the equilibrium constants calculated (Table V), the order of nucleoside heterogeneous association is as follows: purine–purine > purine–pyrimidine > pyrimidine–pyrimidine. This order is in agreement with previous findings (4, 13, 21).

Based upon configurational probabilities, Borazan (28) demonstrated that the self-association processes can induce identical, in-

dependent, and equivalent binding sites and that the overall equilibrium constants are all related to an intrinsic binding constant such that the following holds:

$$\beta_i = \left(\frac{i}{\sigma - i + 1}\right)k \qquad \text{(Eq. 58)}$$

where β_i is the overall equilibrium constant for the ith process, k is the intrinsic binding constant, and σ is the maximum number of association processes. Experimental curves were all generated according to the model.

The first equilibrium constants were smaller than the last in all cases studied and were the smallest constants for the heterogeneous association processes, based upon the assumption that the complexing capacity numbers determine the maximum number of multiple association processes. These results are consistent with the inhibition of the normal pattern of self-association hypothesized below a certain threshold concentration.

CONCLUSION

The theory presented in this paper gives physical and mathematical interpretations concerning the origin and the mechanism of the shift in the critical micelle-like concentrations of the nucleosides, whose concentrations are varied in the presence of a fixed concentration of another reacting nucleoside.

The theory was verified experimentally and found to be consistent with the inhibition of self-association of the compounds used in excess at variable–vehicle molal ratios equal to or less than their corresponding complexing capacity numbers.

A relationship was found between the number of the binding sites on the vehicle molecules and the magnitude of these numbers. Equilibrium constants for various mononuclear association processes are all related to one constant and their corresponding coordination number.

The present work has provided a tool for calculating concentrations of various molecular species which might otherwise be almost impossible to determine for such weak complexes. Other functions of theoretical interest can be determined from a measurable quantity, the slope of the curve when plotting osmolal *versus* molal concentrations, and predetermined concentrations.

Finally, it is hoped that the concept of the complexing capacity numbers and the determination of these numbers will add another thermodynamic parameter to the dimension of weak complexation problems.

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Ketonitrophenols from Mestranol and Related Compounds

JAMES W. CONINE

Abstract □ Mestranol (17α-ethynylestradiol 3-methyl ether), when placed on a carrier such as powdered silica gel and exposed to the atmosphere, is converted to a yellow product. The compound formed was shown to be 1α-ethynyltetrahydro-1β-hydroxy-4 - (2 - hydroxy - 5 - methoxy - 3 - nitrophenethyl) - 7a - methyl-5(4*H*)-indanone. The 3-methyl ethers of three other steroids having aromatic A rings yielded products of a similar type. Identical compounds were prepared from the respective steroids by treatment with nitrating agents in acetic acid. This reaction in acetic acid is light catalyzed. An independent synthesis of a model compound, 6-(2-hydroxy-5-methoxy-3-nitrophenyl)-3-hexanone, established the position of the constituents on the aromatic ring as well as the location of the carbonyl. The mechanism proposed for the formation of these products is an initial oxidation of the 1-substituted tetralin to form a hydroperoxide, which is ionically decomposed to form a ketophenol. The phenol is then nitrated in the *ortho*-position.

Keyphrases □ Mestranol—ketonitrophenols as decomposition products, mechanism □ Ketonitrophenols—isolated and prepared from mestranol on silica gel, mechanism □ Decomposition—preparation, isolation, and characterization of colored decomposition product from mestranol and similar products from related compounds

Tablets and dilute powder triturations of mestranol (I), when adequately protected from the atmosphere, show no loss in potency following long periods of storage[1]. However, when I was spotted on a TLC plate and exposed to the air in the laboratory for several days, it developed a cream color[2]. When the exposed TLC plate was then placed in an ammonia chamber or treated with aqueous alkali, the cream color changed to red-orange. The preparation, isolation, and characterization of this colored decomposition product from I and similar products from other related compounds are the subjects of this paper.

RESULTS AND DISCUSSION

Isolation and Characterization of Mestranol Decomposition Product—The decomposition product produced on the TLC plate was prepared on a larger scale by exposing a dried slurry of silica gel in a chloroform solution of mestranol to the air for several weeks. The organic compounds were washed off the silica gel with chloroform, and the phenolic products that had formed were extracted into aqueous alkali. The aqueous solution was acidified, the phenols were extracted back into chloroform, and the products were separated by preparative TLC.

The material that gave the yellow (407 nm) to red-orange (475 nm) color shift was extracted off the silica gel with chloroform. Evaporation of this solution yielded a bright-yellow oil, which crystallized upon scratching. The yellow crystals were subjected to high-resolution mass spectrometry[3]. The molecular weight of the compound was found to be 387.1656, corresponding within experimental error to the empirical formula $C_{21}H_{25}NO_6$.

The ions $C_8H_8NO_4$ and $C_9H_9NO_4$, two of the more abundant fragments in the spectrum, contain five and six elements of unsaturation, respectively, and probably arise from the chemically altered aromatic ring A of mestranol (I). The high degree of unsaturation as well as the three oxygen atoms and a nitrogen atom added to this portion of the molecule suggest that the ring contains a nitro group and a hydroxy. The concept that the added nitrogen atom has this character was substantiated by absorption bands in the IR spectrum at 1530 and 1315 cm^{-1}. A carbonyl absorption at 1700 cm^{-1} identified the remaining additional oxygen atom as a ketone (or aldehyde) functionality, located at some position other than ring A.

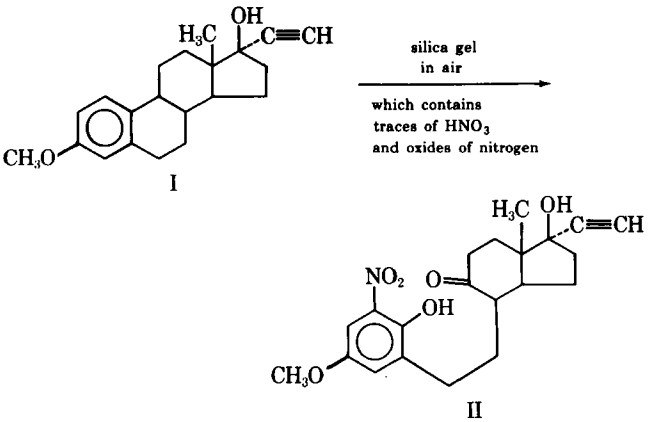
A moderately abundant fragment ion having the formula C_5H_5O is characteristic of ring D in ethynylestradiols and was taken to mean that there had been no alteration of that portion of the molecule.

The NMR spectrum indicated the presence of two aromatic protons instead of one, which would be the case if nitration and hydroxylation had occurred without any modification of the basic ring structure. Furthermore, the empirical formula found by mass spectroscopy contains 10 elements of unsaturation (rings and/or double bonds), but any derivative obtained by simply adding the list of functionalities to I would require 11, thus substantiating the NMR evidence that ring B has been opened.

The structural similarity of I to that of cumene (1) and tetralin (2) suggests that the yellow product resulted from oxidation followed by the nitration of the resulting phenol. This was borne out by the coupling constants of the two aromatic protons, which were seen to be *meta*, and the chemical shift of H-2, which indicated it to be adjacent to a nitro group rather than a hydroxy. Structure II [1α-ethynyltetrahydro-1β-hydroxy-4-(2-hydroxy-5-methoxy-3-nitrophenethyl)-7a-methyl-5(4*H*)-indanone] fits available data (Scheme I).

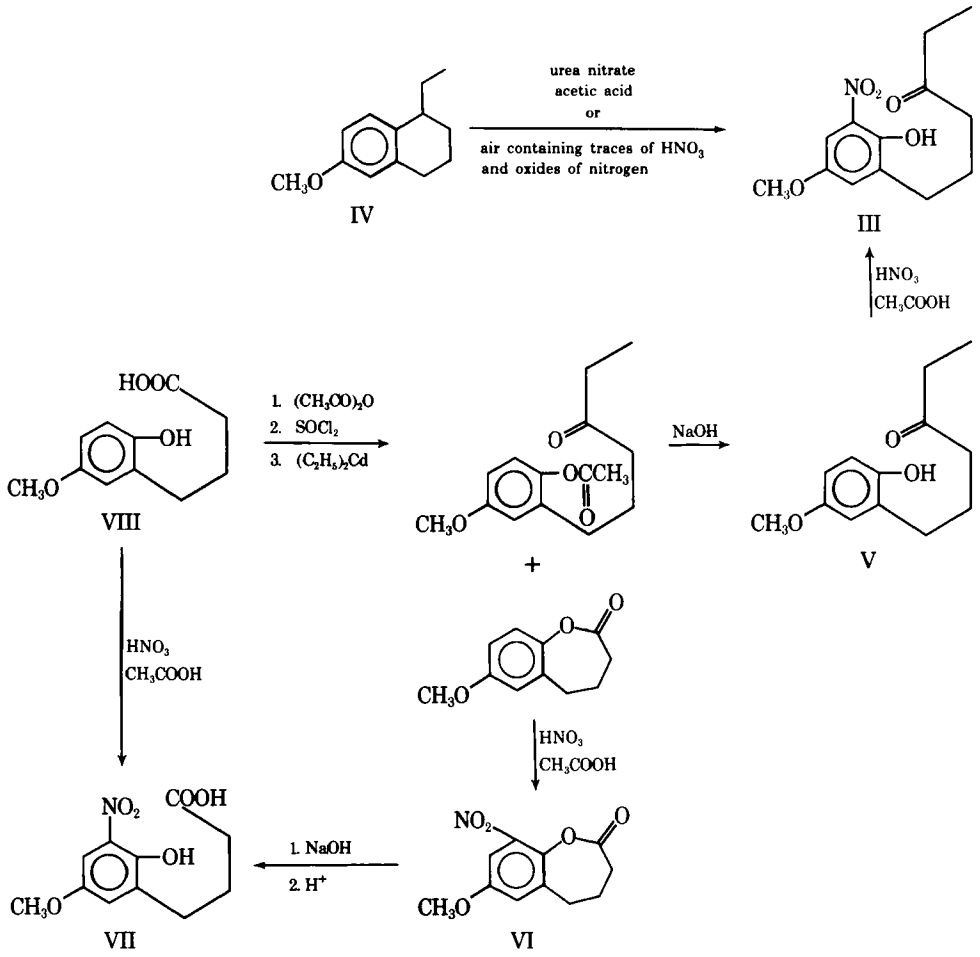
Preparation of Related Compounds—A further verification of the positions of the substituents on the aromatic ring, as well as confirmation of the location of the carbonyl, was made by the independent synthesis of 6-(2-hydroxy-5-methoxy-3-nitrophenyl)-3-hexanone (III), which was obtained from 1-ethyl-1,2,3,4-tetrahydro-6-methoxynaphthalene (IV) (Scheme II). Compound III was obtained by the exposure of IV to the air on silica and was identical to the product obtained by the nitration of the alkaline hydrolysis product (V) from the reaction of 4-(2-hydroxy-5-methoxyphenyl)butyryl chloride acetate (ester) and diethyl cadmium. In addition to III, a neutral compound, the lactone VI, was isolated following nitration of the unhydrolyzed fraction. Alkaline hydrolysis of VI yielded 4-(2-hydroxy-5-methoxy-3-nitrophenyl)butyric acid (VII), which was also obtained by direct nitration of VIII.

Several compounds closely related in structure to mestranol were also studied. Estrone 3-methyl ether, estradiol 3-methyl ether, and 3-methoxy-1,3,5-estratriene all yielded products similar



Scheme I

[1] Eli Lilly and Co., unpublished data.
[2] Paul E. Hartsaw, Eli Lilly and Co., personal communication.
[3] High-resolution mass spectrometer model 21-110, Consolidated Electrodynamics Corp.



Scheme II

to II. Estrone, which has a 3-hydroxy, was exposed to air on silica gel and yielded direct nitration products, 2-nitroestrone and 4-nitroestrone. These compounds were identified by comparison of IR spectra with those of authentic samples (3).

The nitration of estrone in acetic acid occurred instantaneously, resulting in products similar to those obtained on silica gel. These products had UV absorption peaks at 365 nm, which shifted to 400–410 nm in alkali.

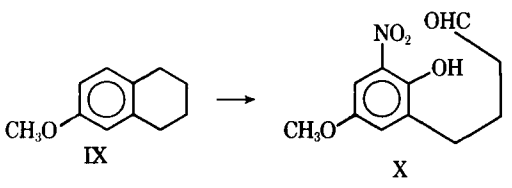
The minimum necessary structure for this reaction appears to be 1,2,3,4-tetrahydro-6-methoxynaphthalene (IX). Compound IX yielded two products in relatively small amounts, both of which had UV spectra similar to II. One of these compounds had a strong carbonyl absorption, while the other was much weaker. Complete separation of these two compounds was not accomplished. Mass spectra on a crude sample indicated a molecular weight of 239, which is in agreement with Structure X (Scheme III).

Mechanism of Reaction—The pathway to the formation of II and related compounds is most probably through a hydroperoxide intermediate. Extensive oxidation studies have been performed on cumene (4), tetralin (5–8), and closely related compounds (9). Both the conditions of the formation and the structures of the products obtained from mestranol and related compounds are similar to those produced from the simpler structures.

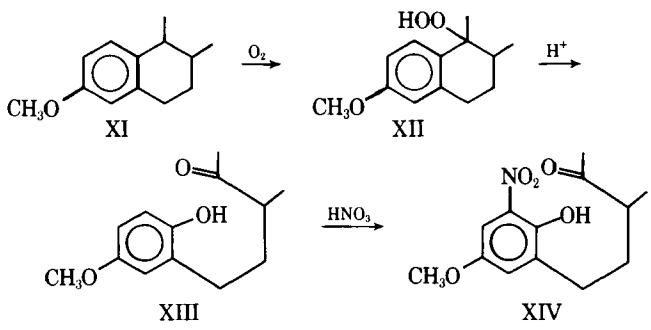
The 3-methoxyestratrienes can be considered to be 1,2-disubstituted 1,2,3,4-tetrahydro-6-methoxynaphthalenes (XI) (Scheme IV). Oxidation of XI in the air produced a hydroperoxide (XII). Ionic decomposition of XII either by acid or base yielded a (2-hydroxy-5-methoxyphenyl)alkanone (XIII), which was then readily nitrated to the *o*-nitrophenol (XIV). Nitration occurring on the silica gel carrier is facilitated because silica gel is an excellent scavenger for nitric acid and the oxides of nitrogen which are absorbed from the air (10).

The hindered position of the hydroxy group between the nitro and alkyl groups is responsible for the absorption in the visible spectrum at longer wavelengths than that for *o*-nitrophenol. The intense characteristic color of the 2-alkyl-4-methoxy-6-nitrophenols aids in their isolation. Although these compounds represent only a fraction of the starting compounds that have undergone a reaction on silica gel, they give strong evidence that the 3-methyl ethers of the estrogens are easily oxidized.

The addition of metal salts to the silica gel accelerated the formation of II. The rate of formation was about four times as fast in the presence of cupric ion as on the untreated carrier; it was also faster, but to a lesser extent, with ferric, manganic, nickelic, and co-



Scheme III



Scheme IV

Table I—Rate of Formation of II on Tricalcium Phosphate

Milligrams of I per Gram of Tricalcium Phosphate	Micrograms of II per Gram of Tricalcium Phosphate/100 hr	Micrograms of II per Milligram of I/100 hr
1.0	12.5[a]	12.5[a]
2.5	15.0[a]	6.0[a]
5.0	16.0	3.2
10.0	18.0	1.8
20.0	20.5	1.0

[a] Initial rate for first 50 hr.

Table II—Formation of II in Acetic Acid at 85° with Urea Nitrate

Reaction Time, min	Concentration of I, mg/ml	Concentration of II, μg/ml
0	9.5	0
15	9.8	70
30	8.75	115
60	—	350
90	8.12	500
180	6.5	310
240	5.9	65

baltic ions. The pH of the silica also had an effect on the formation rate and the type of product formed. Although the amount of II was initially the greatest at pH 9, the amount of II recovered from acidic silica gel was greater after several hundred hours of exposure. A large amount of unidentified dark material was found at the point of application of the material from alkaline silica gel, but only a small amount was found in this location with the acidic samples.

Other powders were tested as carriers. Next to silica gel, tricalcium phosphate was the most effective in promoting the formation of II. Due to its alkalinity, the tricalcium phosphate became pink instead of cream colored. The reaction occurred on or near the surface, so that an undisturbed sample became pink to a depth of 2–3 mm and remained colorless below that depth. In kinetic studies, the powder was mixed frequently and spread thinly to ensure that the reaction proceeded as rapidly as possible.

The effect of concentration of I on tricalcium phosphate on the rate of formation of II was studied (Table I). The reaction rate was not first order with respect to I, indicating that some other limiting factor such as surface phenomenon is involved. The rate of formation of II per milligram of I is the fastest at the lowest concentration of I.

Preparation of II in Solution—Compound II was also prepared by irradiating an acetic acid solution of I containing a small amount of nitric acid under fluorescent lamps at room temperature. Compound II formed rapidly and reached a peak concentration in less than 2 days. Exposure for a longer time brought about a rapid decrease in the concentration of II with a change in the solution color from bright yellow to brown. In the dark or in subdued light, the reaction proceeded at a much slower rate. A corresponding reaction mixture of the same composition, when heated on a steam bath for a few minutes, yielded no II even though the solution darkened.

When the oxides of nitrogen were destroyed by the addition of urea to the reaction mixture (11), the reaction proceeded more slowly both in the light and in the dark. After irradiation under a fluorescent lamp for 7 days, II was still present in appreciable amounts. By using urea nitrate as the nitrating agent, it was possible to prepare II by warming the reaction mixture in a water bath at 85°. The concentration of II reached a peak in about 90 min (Table II). The amount of II in the reaction was never large; at its maximum concentration, it was about 5% of the initial amount of I.

EXPERIMENTAL[4]

Compound II—*Preparation on Silica Gel*—One hundred grams of powdered silica gel[5] was added to a solution of 1.0 g of I in 110 ml of chloroform to make a slurry. The slurry was dried under a current of air, and the resulting powder was spread on a large filter paper. The powder was mixed and respread weekly to expose new surface to the atmosphere. After 2 months, the powder was mixed with 200 ml of chloroform and poured onto a buchner funnel. The silica gel was washed with chloroform until the washings were colorless.

The solution was then reduced in volume to 100–150 ml on a rotary evaporator. The chloroform solution was extracted with a 3% sodium hydroxide solution until the aqueous extracts were almost colorless. The red-brown aqueous layer was then washed once with a small amount of chloroform, acidified, and extracted with chloroform. The combined chloroform layers were washed once with water, dried over sodium sulfate, and evaporated to dryness on a rotary evaporator. The residue was dissolved in 2 ml of acetone and placed on a 1-mm silica gel G preparative TLC plate, which was developed with chloroform–ethyl acetate (9:1).

The orange-yellow layer, which moved about one-third of the way up the plate, was removed and extracted from the silica gel with chloroform, and the residue was rechromatographed again in the same system. The compound was identified by placing the TLC plate in an ammonia chamber and observing the shift in color from orange-yellow to deep red-orange. The product was eluted from the silica gel with chloroform. Evaporation of the solvent left a small amount of orange oil, which crystallized upon scratching. The yield was 44 mg of material, mp 130–133° with slight softening at 118°.

Light-Catalyzed Preparation in Solution—Mestranol (I) (0.4 g) was added to 50 ml of acetic acid containing 2 ml of concentrated nitric acid. The solution was placed in a 50-ml Pyrex flask and stored in a light cabinet 25.4 cm (10 in.) from two 20-w fluorescent lamps. A similar sample was stored in the dark. The sample in the light cabinet became yellow within 16 hr and changed to a brown in about 2 days. No trace of II was observed by TLC of the sample exposed for 2 days, although it was detected at 30 hr. It was still present in a sample after storage in the dark for 18 days.

The acetic acid solution was poured into five times its volume of water and extracted with chloroform until no color was apparent in the organic layer. The combined chloroform extracts were washed with water, and the isolation of the product was carried out in the same way as was described for the product obtained on silica gel. When the reaction mixture was diluted by increasing the amount of acetic acid to 200 ml, the product both formed and decomposed at a slower rate. When the amount of acetic acid was decreased to 12 ml, the sample became brown within 5 hr in the light and no II was detectable on TLC.

Preparation in Acetic Acid with Urea Nitrate—Mestranol (I) (1.27 g) was dissolved in 160 ml of acetic acid, and 3.2 g of urea nitrate was added. The solution was brought to 83–85° in a water bath and held there for 2 hr. The flask was removed from the bath and cooled. The acetic acid solution was worked up as previously described to yield 73.9 mg of II; 0.97 g of mestranol was recovered. No II was obtained when an identical reaction mixture was refluxed for 10 min, even though the solution developed an orange color.

Estrone 3-methyl ether (12), estradiol 3-methyl ether, and 1-ethyl-1,2,3,4-tetrahydro-6-methoxynaphthalene (13) were treated in the same manner as I and yielded products that were similar to II. 3-Methoxyestratriene prepared from 3-hydroxyestratriene (14) also yielded a similar product; but since the sodium and lithium salts of the ketonitrophenol were very soluble in chloroform, it could not be isolated by the method that was satisfactory for the other compounds. Removing the chloroform and replacing it with *n*-hexane[6] made possible the separation of the phenolic product from the starting material. The elemental analyses and melting points for these products are given in Table III.

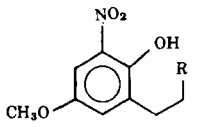
Compound VII—Two grams of 4-(2-hydroxy-5-methoxyphenyl)butyric acid (VIII) (15) was dissolved in 400 ml of acetic acid and 2 ml of concentrated nitric acid was added. After 1 min, an equal volume of water was added and the mixture was poured into

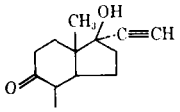
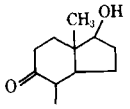
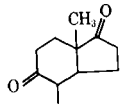
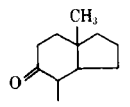
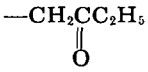
[4] Melting points were obtained on a Mel-Temp melting-point apparatus and are uncorrected.

[5] Both Syloid 63 (W. R. Grace and Co., Davison Chemical Division, Baltimore, Md.) and silica gel G (according to Stahl, E. Merck A.G., Darmstadt, Germany) were used with similar results.

[6] Skellysolve B.

Table III—Ketonitrophenols



Reactant	R	Empirical Formula	Molecular Weight	Melting Point	Analysis, % Calc.	Found
Mestranol		$C_{21}H_{25}NO_6$	387.42	130–133°	C 65.10 H 6.50 N 3.62	65.35 6.47 3.60
Estradiol 3-methyl ether		$C_{19}H_{25}NO_6$	363.40	99–101°	C 62.70 H 6.93 N 3.55	62.96 7.13 3.78
Estrone 3-methyl ether		$C_{19}H_{23}NO_6$	361.38	120–123°	C 63.14 H 6.42 N 3.88	63.33 6.67 4.09
3-Methoxy-1,3,5-estratriene		$C_{19}H_{25}NO_5$	347.40	107–110°	C 65.69 H 7.25 N 4.03	65.91 7.55 4.01
1-Ethyl-1,2,3,4-tetrahydro-6-methoxynaphthalene		$C_{13}H_{17}NO_5$	267.27	62–65°	C 58.42 H 6.41 N 5.24	58.13 6.66 5.24

1600 ml of water and extracted with chloroform. The chloroform solution was washed once with water and extracted with sodium bicarbonate solution. The alkaline solution was slowly acidified with dilute hydrochloric acid and then extracted with chloroform. The chloroform layer was washed with water and dried over sodium sulfate. The residue remaining after evaporation was recrystallized from acetone; 0.5 g, mp 126–129°, was obtained.

Anal.—Calc. for $C_{11}H_{13}NO_6$: C, 51.76; H, 5.13; N, 5.49. Found: C, 51.47; H, 5.03; N, 5.51.

Compound III—Two drops of concentrated sulfuric acid were added to a solution of 2 g of VIII in 10 ml of acetic anhydride. This solution was warmed to 40–45° and then was allowed to stand at room temperature for 30 min. The solution was poured into water and extracted with chloroform. After drying over sodium sulfate, the solvent and excess acetic anhydride were removed under reduced pressure. The residue was dissolved in thionyl chloride and warmed to start the reaction. When the reaction had subsided, the excess thionyl chloride was removed under reduced pressure. The residue was dissolved in ether and was added to a solution of diethyl cadmium (16). When the addition was completed, the mixture was refluxed for 1 hr. After cooling, cracked ice was added to the reaction mixture. Enough diluted sulfuric acid was then added to dissolve the white precipitate which had formed.

The ether layer was separated and extracted with sodium bicarbonate solution to remove any unreacted starting material. The ether solution was next extracted with sodium hydroxide solution. The sodium hydroxide solution was acidified and extracted with chloroform. The chloroform layer was washed with water, dried, and evaporated to dryness. The residue was dissolved in 10 ml of acetic acid, and the resulting solution was treated with a few drops of concentrated nitric acid. After 1 min, the solution was worked up as previously described. Five milligrams of a compound identified as III was obtained.

The ether layer was washed with water, dried over sodium sulfate, and evaporated to dryness. The oily residue was dissolved in 15 ml of methanol, and 5 ml of 3% sodium hydroxide in water was added. After 20 min, the solution was poured into water and extracted with chloroform. The chloroform solution was then extracted with sodium bicarbonate solution, washed with water, and dried. After removal of the solvent on a rotary evaporator, the residue was dissolved in acetic acid and treated with concentrated nitric acid. The reaction mixture yielded 27 mg of a material melting at 56–62°; the IR and NMR spectra of this material were identical to those of III prepared from IV.

When the neutral fraction was nitrated in acetic acid, one product was a material that moved at the solvent front on TLC. This product was isolated as an oil and was extracted from chloroform into sodium hydroxide solution. After acidification of the alkaline solution, the product was isolated as a yellow crystalline compound, mp 127–128°. The IR spectrum for this compound was identical with that of VII prepared by direct nitration of VIII.

Kinetic Studies—*Acetic Acid Solution*—Ten 5-ml glass-stoppered flasks containing 4 ml of acetic acid, 40 mg of I, and 200 mg of urea nitrate were placed in a water bath maintained at 83–85°. The flasks were removed at intervals between 15 min and 4 hr and immediately placed in an ice bath. The solutions were assayed for I using the colorimetric method described by Templeton *et al.* (17). Compound II was separated by TLC and eluted from the silica gel with chloroform. The concentration of II was determined by measuring the solution at 407 nm, using a solution of previously prepared II as a standard.

Dispersed on a Dry Powder Carrier—Mestranol was placed on tricalcium phosphate or silica gel at concentrations of 1–20 mg of I/g of carrier by making a chloroform slurry. The resulting dried powders were spread to a depth of about 0.5 cm in an open half of a petri dish and placed on a benchtop in the laboratory. The samples were mixed and respread frequently to ensure an even exposure to the atmosphere. From 0.5 to 2 g of the powder was removed periodically and placed in a tight screw-capped amber glass bottle. When all samples had been collected, II was isolated and the concentration was determined as previously described.

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Abstract □ Subcutaneous disappearance of lidocaine hydrochloride was followed as a function of time using a specially designed "closed" subcutaneous absorption cell affixed to anesthetized rats. Unbuffered, stirred lidocaine hydrochloride solutions in cells open to the atmosphere were previously shown to increase in pH with time because of carbon dioxide loss. The closed cell was designed to prevent this loss, but pH shifts still occurred, making the derivation of a simple pharmacokinetic absorption model impossible. Because the pH of the solution shifted to higher pH values, the data suggest that precipitation of lidocaine base may have occurred in some experiments.

Keyphrases □ Lidocaine hydrochloride—absorption from subcutaneous site, design of closed subcutaneous absorption cell □ Absorption—lidocaine hydrochloride from subcutaneous site, design of closed subcutaneous absorption cell, effect of pH changes □ Parenteral dosage forms—lidocaine hydrochloride absorption from subcutaneous site, design of closed subcutaneous absorption cell

Since the publication of Schou's (1) review of drug absorption from subcutaneous connective tissue, there has been an increasing interest in quantitatively measuring the absorption rates of drugs in aqueous solution from the subcutaneous site (2–8). Quantitative measurements of drug absorption rates should result in a better understanding of which of the many possible pharmacokinetic models are appropriate for describing subcutaneous drug absorption of various drugs and of what mechanisms are involved in subcutaneous drug absorption. To date, there has not been a study where different commercial products containing the same drug have been compared in their absorption behavior from the subcutaneous region under conditions where the drug was sampled periodically at the subcutaneous absorption site, where the solution was continuously stirred, and where the surface area for absorption was held constant.

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EXPERIMENTAL

Animals—Female Sprague–Dawley rats were used. The anesthesia and the method used for surgically exposing the subcutaneous tissue were described previously (3).

Reagents—Lidocaine hydrochloride was obtained from two manufacturers[1,2]. According to the label claims, each preparation contained (per milliliter) 10 mg of lidocaine hydrochloride, 7 mg of sodium chloride, 1 mg of methylparaben, and sodium hydroxide to adjust the pH. Both preparations conformed to the standards for lidocaine hydrochloride injection USP without epinephrine (9). Cyclizine hydrochloride[3] (10, 11) was used as an internal standard for the GC analysis at a concentration equivalent to 243 mg of cyclizine base/liter in 0.1 *N* HCl. The *n*-hexane used as the extraction solvent in the procedure was of spectrographic grade[4]. All water was double-distilled from dissolved potassium permanganate. All glassware except the microsyringe[5] was initially cleaned in concentrated nitric acid.

Subcutaneous Absorption Cell—The design of the absorption cell used in this study differed somewhat from that used in previous work (3). The glass cell was nearly hemispheric in shape, where the maximum distance from one inside point of the open end to another inside point was 19 mm and the distance between the top inside point and the plane across the open end of the cell was 10 mm. Two holes were made in the top region of the cell. One hole, used for the stirrer, was at the top center of the cell when the cell was placed flat on a horizontal surface. A glass tube was fused to the glass surrounding this hole, and the tube extended 3 mm up

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[2] Lot 2016923C, Invenex Pharmaceuticals, Grand Island, N.Y.
[3] Marezine, Burroughs Wellcome & Co., Research Triangle Park, N.C.
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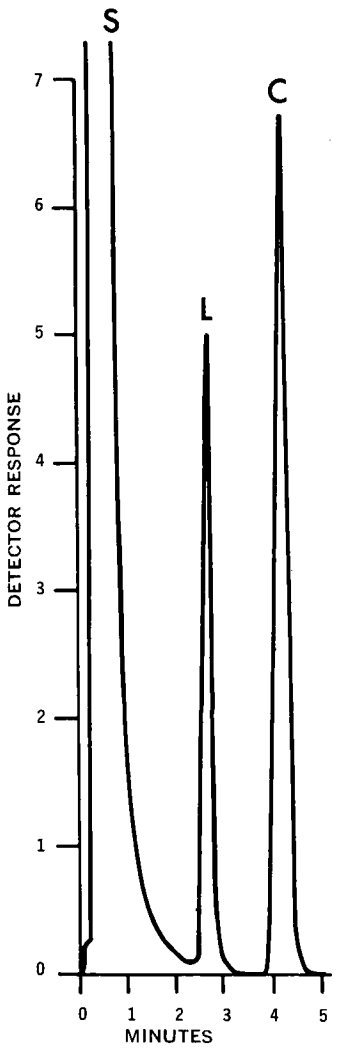

Figure 1—*Typical gas chromatogram of lidocaine (L) and cyclizine (C) internal standard in* n*-hexane solvent (S). The retention time of lidocaine is 2.7 min, and that for cyclizine is 4.2 min.*

from the top outside of the cell. The glass tube had an inside diameter large enough so that a polyethylene tube[6] could just pass through it snugly.

The second hole was used for sampling the cell's contents. The center of the second hole was about 7 mm from the center of the first hole and its inside diameter was 4 mm. It was ground flat so that it could be closed between sampling times by pressing a small piece of paraffin film[7] over it. The glass absorption cell was affixed to the moist subcutaneous tissue with a medical silicone adhesive, which was repackaged into an ophthalmic ointment tube as described previously (3).

The stirring of the cell's contents was accomplished by placing a 2-cm piece of polyethylene 100 tubing into the first hole so that one end was flush with the top inside region of the cell and the remaining portion passed through the glass tube and extended beyond it. The polyethylene 100 tube was affixed to the cell with a medical silicone adhesive in such a way that no hole existed between the glass and the polyethylene 100 tubing so as to prevent possible gas exchange between the inside and outside of the cell. A 30-cm piece of polyethylene 10 tubing was passed partially through the polyethylene 100 tubing. The end of the polyethylene 10 tubing nearest the inside of the absorption cell was softened by carefully heating it and was pressed flat with a pair of pliers to form a short paddle. The second end of the polyethylene 10 tubing was passed through 21 cm of glass tubing having an outside diameter of about 7 mm and then was placed into a chuck attached to a variable-speed motor[8].

After the absorption cell was attached to the subcutaneous tissue, the glass tubing was aligned and clamped in a straight line between the top of the absorption cell and the chuck. The glass tubing tended to prevent the polyethylene 10 tubing from kinking while it was being rotated. The amount of polyethylene 10 tubing remaining outside the cell was adjusted so that when the chuck attached to the variable-speed motor was rotated at about 14–16 rpm, the paddle inside the absorption cell would be free to rotate and not touch either the tissue or the cell. After the appropriate distances were established, a small amount of silicone stopcock grease[9] was placed at the region where the polyethylene 10 tubing was in contact with the external end of the polyethylene 100 tubing. In addition to its obvious lubrication function, the grease was used to maintain a nearly airtight seal between the two polyethylene tubes. Care was taken to keep the grease from entering the absorption cell.

GC—A gas chromatograph[10] equipped with dual flame-ionization detectors and a recorder[11] were employed. The chromatographic column consisted of glass tubing, 1.83 m long × 0.318 cm i.d., and a silicone stationary phase[12] on a 100–120-mesh solid support[13]. Conditioning was carried out by injecting a silylation reagent[14] and by maintaining the column at 250° for 24 hr with low nitrogen flow.

Operating temperatures for the lidocaine analysis were: column, 230°; injection port, 250°; and detector, 250°. The carrier gas was nitrogen at a flow rate of 30 ml/min. The hydrogen gas flow rate was 30 ml/min, and the air flow rate was 300 ml/min. The recorder speed was 50.8 cm/hr. An integrator[15] measured the area under the detector response *versus* time curve on recorder paper.

Assay—A 1.2-g sample of 1% lidocaine hydrochloride was transferred from the commercial 20-ml[1] or 30-ml[2] multiple-dose vial to the absorption cell through the second or side hole by means of a 2-ml syringe. The filled syringe was weighed on an analytical balance prior to adding the solution to the cell and then was reweighed just after the addition. Individual values for the weights added are listed in Table I. The mean weight added was 1.225 (±0.020) g, where the value in parentheses represents ±1 *SD* about the mean weight added to all cells.

Immediately after the 1.2-g sample of solution was added, a small piece of paraffin film was placed over the hole to prevent gas exchange. The 20-μl zero time sample was taken directly from the contents of the multiple-dose vial using a 25-μl syringe[5]. At other sampling times, 20-μl samples were withdrawn from the absorption cell. The accuracy of the sampling technique was checked. The syringe containing the aliquot was weighed on an analytical balance and reweighed after the sample was transferred to a 5-ml glass-stoppered tube[16]. In all cases the difference between the two weights corresponding to the volume of sample delivered (1 μl = 1 mg) agreed with that estimated from the syringe barrel markings within ±1%. One milliliter of the stock solution of cyclizine hydrochloride (internal standard) was added to rinse the drug sample down from the sides of the flask. The samples were then frozen and stored for future assay.

At the time of assay, the samples were allowed to reach ambient temperature and then 4 ml of *n*-hexane and 0.5 ml of 5 *N* NaOH were added to each. The solutions were shaken mechanically for 1 hr. About 2 ml of the *n*-hexane layer was decanted into another test tube. About 10 mg of anhydrous sodium sulfate was added to the *n*-hexane solution, which was allowed to stand for several hours to remove trace amounts of water from the organic solvent. A 1.2-μl sample was removed from each tube, using a 5-μl microsyringe[5], and injected into the gas chromatograph. Ten standard solutions of lidocaine hydrochloride were prepared, starting at 0.1% and increasing at 0.1% concentration increments up to 1.0%. Twenty-microliter samples were removed from the standard solutions and treated according to the procedures previously described.

[6] PE 100, Clay-Adams, Inc., New York, N.Y.
[7] Parafilm "M," American Can Co., Neenah, Wis.
[8] Electronic controller GT 21 laboratory mixer, Gerald K. Heller Co., Las Vegas, Nev.

[9] Stopcock grease, Dow Corning Corp., Midland, Mich.
[10] Varian Aerograph model 2100, Varian Instrument Division, Palo Alto, Calif.
[11] Varian model 20 recorder, Varian Instrument Division, Palo Alto, Calif.
[12] OV-17, Varian Instrument Division, Palo Alto, Calif.
[13] Gas Chrom Q, Applied Science Laboratories, State College, Pa.
[14] Silyl-8, Varian Instrument Division, Palo Alto, Calif.
[15] Disc Instruments, Santa Ana, Calif.
[16] Catalog Number 20908-040, Van Waters and Rogers, Brisbane, Calif.

Table I—Percent Unabsorbed Lidocaine Hydrochloride from Subcutaneous Absorption Cell as a Function of Time

Sampling Time, hr	Animal[a] (g)						Animal[b] (g)					
	A[c] (279)	B[c] (254)	C[c] (256)	D[c] (261)	E[c] (258)	F[c] (269)	G[c] (208)	H[c] (218)	I[c] (210)	J[c] (206)	K[c] (214)	L[c] (296)
0.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
0.5	59.0	98.1	87.9	80.5	92.0	74.0	63.4	77.0	68.2	91.6	95.4	95.6
1.0	56.4	87.1	75.0	90.0	77.4	63.3	68.4	72.0	54.1	77.6	70.0	58.0
1.5	38.0	97.1	70.5	77.1	54.6	50.7	50.7	53.0	45.7	61.0	52.4	55.7
2.0	23.8	66.7	57.8	37.8	47.4	37.2	37.6	31.2	41.6	46.0	38.0	29.4
2.5	15.6	63.0	44.8	24.2	36.0	33.1	36.6	23.1	37.8	27.7	24.8	24.3
3.0	11.8	44.5	39.8	14.2	24.7	22.1	34.5	19.2	34.5	15.4	15.2	11.4
3.5	4.7	35.2	24.4	9.3	13.3	14.5	23.4	11.0	26.1	2.9	13.6	5.8
4.0	3.7	31.8	23.2	1.1	15.9	12.4	13.6	7.4	17.7		10.5	2.5

[a] Lot 2016923C, Invenex. Initial cell pH for Animals A–F was 6.18. Final cell pH's for Animals A–F were 8.49, 7.89, 7.69, 7.99, 7.69, and 7.72, respectively. [b] Lot L 107770, Astra. Initial cell pH for Animals G–L was 6.15. Final cell pH's for Animals G–L were 7.40, 7.48, 7.48, 8.15, 7.92, and 7.84, respectively. [c] Solution weights in grams for Animals A–L were 1.218, 1.179, 1.222, 1.221, 1.227, 1.219, 1.249, 1.231, 1.221, 1.230, 1.220, and 1.261, respectively.

After GC analysis, the areas under the lidocaine curve, A_L, and under the cyclizine curve, A_C, were measured for each injected sample. A plot of the ratio, A_L/A_C, *versus* drug concentration was made. The standard curve was linear over the 0.1–1.0% lidocaine hydrochloride concentration range. Figure 1 shows a detector response *versus* time curve for the *n*-hexane extract of the sample taken from Animal E at the 0.5-hr time point. A pH meter[17] equipped with a miniature glass and reference electrode[18] system was used to determine the initial and final pH values of the solution in the absorption cell.

RESULTS AND DISCUSSION

Several points should be noted about the results shown in Table I. First, the pH of the solution measured before the drug solution was placed in the subcutaneous absorption cell was lower than the pH of the cell's contents measured after the experiment was concluded. Second, there was generally a decline in the percent of drug remaining in the subcutaneous cell as time increased; however, in several instances the percent of drug at a particular time point appeared to *increase* over that found at earlier time points. This apparent increase was then followed by another drop in the percent of drug remaining at later times. Third, if one plots the logarithm of the percent remaining in the subcutaneous absorption cell *versus* the time at which the samples were taken, there does not seem to be any convenient way to analyze the resulting curve in terms of either apparent mono- or biexponential loss of drug from the subcutaneous absorption cell.

It is not too surprising that the pH of the solution initially slightly greater than pH 6 should shift to higher pH values as the experiment progressed. According to the label statements, the commercial solutions have no added buffers. The pH of rat arterial blood (12) is 7.35 (range 7.26–7.44), and this system is known to be buffered. Thus, a small volume of a nonbuffered solution in the subcutaneous absorption cell adjacent to a larger volume of a buffered rat blood should eventually reach a pH value closely approximating that of the buffered system.

However, in a subcutaneous absorption cell open to the atmosphere, the pH often rose well above the expected maximum value of about 7.4. Levy and Rowland (8) noted that the pH of a nonbuffered isotonic saline solution in an open subcutaneous absorption cell rose from an initial value of 5.75 to about 8.2, lasting from 3 to 7 hr later. A major reason why the pH of the system open to the atmosphere rose substantially above pH 7.4 was that bicarbonate ion migrated into the subcutaneous absorption cell. Appropriate shifts in cations, such as K^+ and Na^+, and other anions, such as Cl^-, also occurred and electroneutrality in the subcutaneous cell was maintained. The loss of carbon dioxide from the bicarbonate-ion solution and the concomitant increase in the carbonate-ion concentration resulted in a cell pH greater than 7.4.

The nearly closed hemispherical subcutaneous absorption cell used in this work was designed to minimize gas exchange between the cell and the atmosphere. It can be seen from the data presented in Table I that there was considerable variation in the final pH of the solutions found in various subcutaneous cells. The exact cause for this pH variation is not known, but it is probably related to how freely gases could exchange between the cell and the atmosphere. With regard to the first point, therefore, it appears that considerably more work is needed to design an absorption cell that will allow the solution contained in it to be stirred, that will allow samples of solution to be removed periodically for analysis, and that will not allow any gases likely to influence the pH of the subcutaneous cell's contents away from the physiological range to be exchanged between the cell and the atmosphere.

The second point mentioned was that there was generally a decline in the percent lidocaine hydrochloride remaining in the subcutaneous absorption cell with respect to time (Table I). However, in some cases the apparent percent remaining at one time point *exceeded* that for a previous time point. For Animals B, D, and G, this phenomenon was observed somewhere between the 0.5- and 1.5-hr time points. The possibility that gross analytical or procedural errors had occurred was investigated, but no evidence of such errors could be found.

The most reasonable explanation for this phenomenon was that the shift in subcutaneous cell pH to increasing values caused some lidocaine base to precipitate. Since the contents of the cell were continuously stirred, fine crystals would have the tendency to stay suspended for some time. If a 20-μl sample removed from the subcutaneous cell contained crystals of lidocaine base, then that sample would give a higher reading for lidocaine content than a similar sample not containing suspended crystals. The pH at which the base just begins to precipitate, pH_p, is defined by Eq. 1, which is appropriate to physiological pH ranges (13, 14):

$$pH_p = pKa + \log_{10} \frac{[B]_{sol}}{[A^0] - [B]_{sol}} \qquad \text{(Eq. 1)}$$

where pKa is the negative logarithm of the ionization constant K_a for the protonated species of the base, $[B]_{sol}$ is the molar aqueous solubility of lidocaine base, and $[A^0]$ is the molar aqueous concentration of the protonated species in solution. At 24°, Levy (7) found that $[B]_{sol}$ was 9.184×10^{-4} *M* and that the pKa was 7.86. If these values are substituted into Eq. 1 and the values for $[A^0]$ are systematically varied, then the information contained in Table II can be obtained.

In Table II, one notes that, to avoid possible precipitation of lidocaine base, the pH of a 1% lidocaine hydrochloride solution must be pH 6.268 or less, which is approximately the pH of commercial 1% lidocaine hydrochloride products prior to their injection. If the pH of the system is increased to pH 7.4, for example, then the lidocaine hydrochloride concentration in the solution would have to be reduced according to Eq. 1 to about 0.097% to prevent the formation of a precipitate. Thus, if the pH in the subcutaneous absorption cell increased rapidly to about pH 7.4 during the experiment, then conditions were favorable for drug precipitation at the absorption site. Drug precipitation might not occur if supersaturation occurred or if drug interactions occurred with the proteins present in the biological fluids in the subcutaneous absorption cell (15), which might reduce the drug concentration below the point at which precipitation would occur.

The third point was that, by using the data shown in Table I,

[17] No. 7664, Leeds and Northrup, North Wales, Pa.
[18] No. 124138, Leeds and Northrup, North Wales, Pa.

Table II—Calculated pH values from Eq. 1 at which Precipitation of Lidocaine Base Will Just Begin to Occur from Various Concentrations of Lidocaine Hydrochloride at 24°

pH_p	Percent (w/v)	Molar
6.246	1.05	38.77×10^{-3}
6.268	1.00	36.93
6.367	0.80	29.54
6.497	0.60	22.16
6.683	0.40	14.77
7.013	0.20	7.39
7.399	0.097	3.58
7.515	0.08	2.95
7.711	0.06	2.22
8.077	0.04	1.48

plotting the logarithm of the percent of lidocaine present in the subcutaneous absorption cell *versus* sampling time resulted in plots that could not be resolved conveniently into a pharmacokinetic model that could be described by mono- or biexponential loss of drug from the cell. In contrast, Levy (7) found that the disappearance of lidocaine hydrochloride from an open subcutaneous cell *versus* time could be described by an apparent biexponential equation. The initial drug concentration used was 7×10^{-4} *M* and the aqueous isotonic system was buffered at pH 7.95, so that drug precipitation at the absorption site would not occur. The disadvantage of adding a buffer is that the commercial solution under study is not precisely the same as that formulated by the manufacturer. Also, the added buffer may cause initial precipitation of the free base.

When the subcutaneous absorption cell technique has been used (3, 7), absorption rate differences among animals have been attributed in part to surgical techniques resulting in differences in the thickness of the subcutaneous membrane through which the drug must pass and in part to microcirculation changes in the subcutaneous region as the experiment progresses, resulting from such factors as trauma, inflammation, anesthesia, and drug effects. These and other factors represent a limitation to the method when the results of this experimental design are related to actual subcutaneous drug administration, where most of these factors are absent or greatly reduced.

An "internal standard" method has been used (7); in a given experiment, it is the ratio of the half-life of the rate-limiting step for drug absorption divided by the half-life of the rate-limiting step for the internal standard. This ratio should be reasonably constant from experiment to experiment. A reason for not using an internal standard method in this study is that the solution under consideration would not be precisely the same as that formulated by the manufacturer. Also, it must be shown that neither the drug nor the internal standard will influence the absorption of the other.

In conclusion, the use of the subcutaneous absorption cell technique in evaluating drug absorption is most useful in studying salts of poorly soluble organic weak bases, such as lidocaine hydrochloride, when the drug base will not precipitate over the pH range studied and where some control of cell pH is maintained. The technique yields less satisfactory results even in a nearly closed cell when the solution administered has no added buffers and when the drug salt concentration is sufficiently high so that precipitation of uncharged drug is likely to occur if the pH shifts from that in the original solution to a more alkaline pH value.

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[19] *Note added in proof:* Additional information contained in Ref. 7 has appeared: R. H. Levy and M. Rowland, *J. Pharmacokin. Biopharm.*, **2**, 313(1974).

Fluorometric Assay of Bethanidine in Plasma

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Abstract □ A fluorometric method for determining bethanidine in blood plasma is described. The bethanidine is extracted into chloroform, a drug–dye complex with eosin Y is formed, and the fluorescence is measured (excitation, 535 nm; fluorescence, 560 nm). The assay detects 0.02 μM bethanidine (4 ng/ml) in plasma. The relative fluorescence of several body constituents and antihypertensive drugs is negligible. The plasma levels of bethanidine in four hypertensive patients receiving this drug were measured.

Keyphrases □ Bethanidine—fluorometric assay in plasma, complexed with eosin Y □ Fluorometry—analysis, bethanidine in plasma

Bethanidine is a new antihypertensive drug of the guanidine class (1). It is an adrenergic depleting agent similar to guanethidine, but the effect is of a shorter duration. Most potent antihypertensive compounds, including bethanidine, exhibit considerable variation in the dose necessary for acceptable control of blood pressure in different individuals. A simple fluorometric assay for bethanidine in plasma was developed in the present study. Bethanidine is extracted from plasma into chloroform, and the fluorescence then is determined using a drug–dye complex formed with eosin Y. This simple fluorometric assay will make it possible to study blood levels and pharmacokinetic parameters of bethanidine in hypertensive patients and to correlate blood levels with therapeutic efficacy.

EXPERIMENTAL

Analytical grade chloroform[1] stabilized with 0.75% ethanol was used without purification. The commercial source was important. Eosin Y[2] was stored in 0.1 M sodium phosphate buffer, pH 7.4, and was stable indefinitely. Bethanidine[3] was chromatographically and spectrophotometrically pure. Radioactive ^{14}C-bethanidine[4] (2.37 mCi/mmole) labeled at the benzyl carbon was purified by TLC on precoated silica gel plates[5]. In an ascending solvent system of butanol–acetic acid–water (3:1:1), the developing time was 6 hr at 25°. Unlabeled and ^{14}C-bethanidine had an R_f value of 0.53. There was a radioactive contaminant, R_f 0.57, in the ^{14}C-bethanidine. The 0.53 band was extracted into chloroform which then only contained ^{14}C-bethanidine. Fenfluramine[3], guanethidine[6], spironolactone[7] and its diethioacetylated-Δ^6 derivative[8], hydrochlorothiazide[9], and benzthiazide[3] were analytically pure. All other chemicals, reagents, and materials were of reagent grade[10].

Radioactivity was determined by liquid scintillation[11]. Plasma or urine (0.2–0.5 ml) was mixed with 1.5 ml of solubilizer. Then 0.5 ml of 30% H_2O_2 was introduced and allowed to incubate 30 min at 23°; finally, 15 ml of scintillation mix was added. Quenching was corrected by introducing ^{14}C-bethanidine as an internal standard after the initial counting.

Four hypertensive patients (age 45–52, weight 60–76 kg, three female and one male) were maintained on 50 mg of benzthiazide twice daily and 20 mEq of potassium chloride for 1 week before and during the bethanidine study. The patients gave informed written consent. At the first dose of bethanidine, they had a supine systolic blood pressure of 183 ± 16 mm Hg and a diastolic pressure of 119 ± 12 mm Hg. The patients are representative of a larger group under investigations for blood levels and pharmacokinetic parameters[12]. From a therapeutic standpoint, the bethanidine was given three times daily at varying doses and it was necessary to reduce the benzthiazide to 50 mg daily after 1 month.

Venous blood was collected in chilled 7-ml draw vacutainer tubes containing 10.5 mg ethylenediaminetetraacetic acid for plasma or into vacutainer clot tubes for serum and stored on ice. Preliminary studies utilized heparin tubes, which gave slightly higher blanks (2). Within 1 hr, the samples were centrifuged at 2000×g for 10 min and the supernates were transferred to specially cleaned test tubes and stored at −90°. The temperature and time were not critical for the bethanidine assay, but these conditions preserved the samples for analyses of other labile constituents.

Steps I–III of the assay were carried out in 1.6 × 14-cm, specially cleaned, glass-stoppered tubes protected from light with aluminum foil. The tubes and glassware were cleaned by boiling for 15 min in 0.1 N NaOH, rinsing, boiling for 15 min in 10% HCl, rinsing in water, boiling in distilled water for 15 min, rinsing three times in double-distilled water, and finally drying at room temperature. Furthermore, all steps (I–IV) were done in a cool (23–25°) dimly lighted room; the sample analyses were uniformly timed, within and between steps, to minimize adverse effects of chloroform degradation and of air and light exposure on net fluorescence and sensitivity. Reagents were prepared with double-distilled water.

Step I—The sample (2 ml) and 0.4 ml of 0.5 M sodium borate buffer, pH 10.5, are mixed and then 10 ml of chloroform is introduced. The tubes are tightly stoppered and wrapped in aluminum foil. Extraction is carried out for 10 min on a mechanical circular agitator. The stoppered samples are centrifuged at 500×g at 23° for 10 min; the upper aqueous portion contains an opalescent upper layer and a particulate layer at the chloroform interface.

Step II—The opalescent layer (1.5 ml) is transferred to a second tube containing 0.2 ml of 5 N NaOH, and then 7 ml of chloroform is added. The mixture is agitated and centrifuged as before.

Step III—The aqueous layer is aspirated and discarded, and 5 ml of the chloroform layer is transferred to a third tube containing 1 ml of 4×10^{-4} M eosin Y in 0.1 M sodium phosphate buffer, pH 7.4. The mixture is agitated and centrifuged as before.

Step IV—Fluorescence is measured[13] in 10 × 10 × 44-mm cells in a darkened room. Approximately 0.5 ml of the upper aqueous eosin layer is aspirated into a 5-ml pipet, and the tip is lowered to aspirate 3 ml of the chloroform layer containing the bethanidine–eosin complex. The contents of the pipet are transferred to the cell in such a way that the chloroform layer enters followed by the eosin layer; the chloroform layer must be completely covered by the eosin layer. The light path of the cell is visually inspected for small droplets of eosin, which can interfere with the recording. The fluorescence is determined at 560 nm with excitation at 535 nm. Readings must be made immediately after the sample is placed into the fluorometer due to rapid decay of fluorescence in the exci-

[1] Mallinckrodt Chemical Co., St. Louis, Mo.
[2] Aldrich Chemical Co., Milwaukee, Wis.
[3] A. H. Robins Co., Richmond, Va.
[4] New England Nuclear Corp., Boston, MA 02118
[5] E. Merck A. B., Darmstadt, Germany.
[6] Lot L-5746, [2-(hexahydro-1-(2H)-azocinyl)ethyl]guanidine sulfate, Ciba Pharmaceutical Co., Summit, NJ 07901
[7] Lot SC-9420, G. D. Searle & Co., Chicago, Ill.
[8] CPD No. SC-9376, Lot MA8-2604, G. D. Searle & Co., Chicago, Ill.
[9] Merck & Co., West Point, Pa.
[10] Fisher Scientific Co., Pittsburgh, Pa.
[11] Soluene-100 diluted 1:1 in isopropanol or Instagel in 0.5 N HCl 9:1 for scintillation, Packard Instruments Co., Downers Grove, IL 60515
[12] AHR-3050-15: Metabolic Disposition of Bethanidine, A. H. Robins Co., Richmond, Va.
[13] MKI spectrofluorometer–magnetic xenon arc stabilizer, Farrand Optical Co., Valhalla, NY 10595

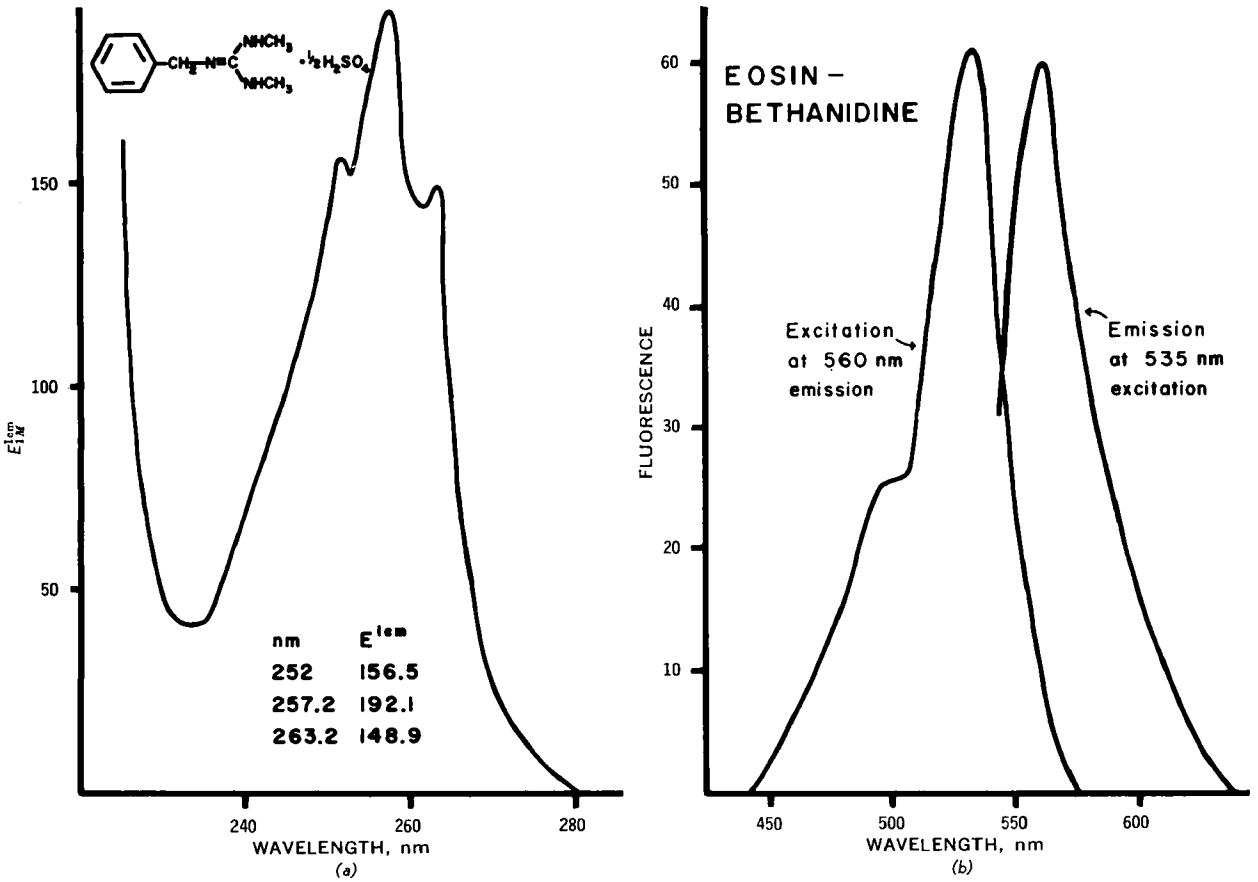

Figure 1—(a) *UV absorption spectrum of bethanidine sulfate in water at 23°.* (b) *Fluorescence excitation and emission spectrum of the bethanidine–eosin complex in chloroform. Bethanidine (0.7 m*M*) in 0.4 m*M *eosin, pH 7.4, was extracted into 5 volumes of chloroform (Steps III and IV).*

tation light path ($t_{1/2}$ = 2 min). The machine is standardized to a preset value before each individual reading with a sealed 1 μM aqueous eosin solution to eliminate small errors due to machine drift. The fluorescence of the eosin reference solution decays with a half-time of approximately 2 weeks.

Concentrations of bethanidine were calculated as follows. The water blank was determined daily (in triplicate) by adding 0.4 ml of borate buffer to 2 ml of water in Step I and assayed to give F_1. The fluorescence equivalent to 1.0 μM bethanidine in water was also determined in triplicate daily by using 0.4 ml of 5 μM bethanidine in borate buffer (diluted from 2.5 mM bethanidine in water stored at 4° for up to 9 months) to give F_2. The patient's prebethanidine plasma blank was determined from the fluorescence (F_3) of borate added to plasma. This plasma blank value was not repeated and was taken to represent the patient's blank throughout therapy (Eq. 1):

$$\text{micromolar in patient blank} = \frac{F_3 - F_1}{F_2 - F_1} \qquad \text{(Eq. 1)}$$

The apparent extraction efficiency (AEE) was determined in triplicate on each patient's prebethanidine plasma:

$$\text{percent } AEE = \left(\frac{F_4 - F_3}{F_2 - F_1}\right) \times 100 \qquad \text{(Eq. 2)}$$

Bethanidine in borate buffer was added to 2 ml of plasma (it is not necessary to preincubate) and assayed to give F_4. The apparent extraction efficiency was calculated and used to correct subsequent analyses on the patient's plasma, if necessary. The apparent extraction efficiency was 95 ± 2% for four patients; if ignored, it would not have much effect on the calculations. The bethanidine levels in the plasma of patients receiving bethanidine were determined in duplicate from the fluorescence (F_5) of plasma added in Step I and calculated from:

$$\text{micromolar} = \left[\left(\frac{F_5 - F_1}{F_2 - F_1}\right) - \text{micromolar in patient blank}\right]\left(\frac{100}{AEE}\right) \qquad \text{(Eq. 3)}$$

It should be kept in mind that due to variable factors that affect fluorescence, the F_1 and F_2 in Eqs. 1 and 2 will be different from the F_1 and F_2 of Eq. 3 unless F_3, F_4, and F_5 are all determined on the same run.

RESULTS

Radioactive ^{14}C-bethanidine (1 μM) in 0.1 N NaOH was extracted with chloroform by shaking the mixture in a stoppered test tube for 5 min. The chloroform layer was taken to dryness under nitrogen, and the radioactivity was determined. An aqueous chloroform ratio of from 1:4 to 1:8 gave a maximal extraction efficiency of 90%. Thus, in Steps I and II, a ratio of 1:5 was selected for the assay. In a second experiment, with this ratio of 1:5 less than 5% of the bethanidine was extracted into aqueous solutions with varying pH of 6–10. Above pH 10, the percentage of bethanidine extraction was maximal by pH 13.5. This finding is compatible with the high pKa of guanidine compounds (3).

In a third series of experiments, plasma containing no bethanidine and with varying pH of 6–13.5 was similarly extracted with chloroform. The chloroform extract was then subjected to the fluorescence development and measurement of Steps III and IV. The plasma blank was maximal below pH 8.0 and was unchanged at pH 9 and above. Thus pH 9.5–10.0 was chosen for Step I to extract maximally fluorescence material in the blank without losing bethanidine; pH 13.5 was chosen to extract maximally bethanidine in Step II.

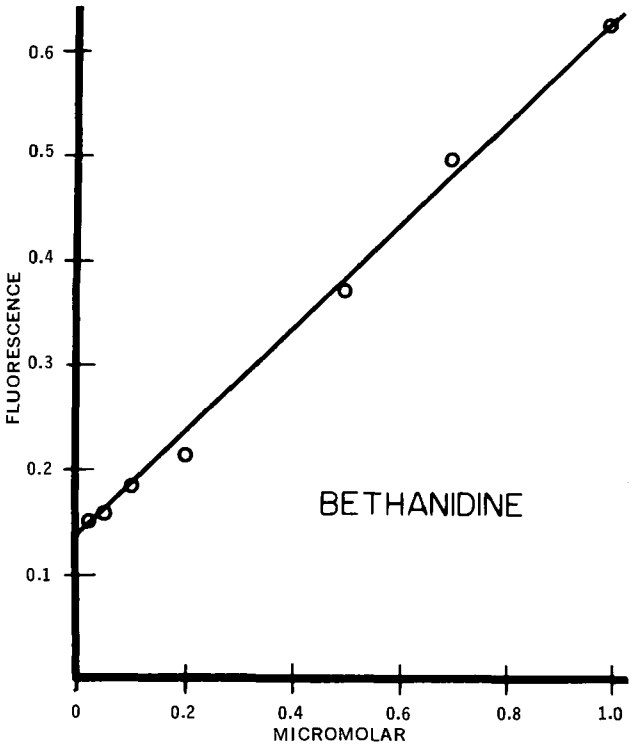

Figure 2—*Linearity of bethanidine–eosin fluorescence. Bethanidine sulfate in water was assayed by the standard procedure. A similar curve was obtained for bethanidine in plasma.*

The UV absorption spectrum exhibited by bethanidine sulfate in water is depicted in Fig. 1*a*. The molar extinction was too low for a sensitive assay utilizing absorption spectroscopy.

The fluorescence of the bethanidine–eosin complex (Fig. 1*b*) provided the necessary sensitivity for a chemically useful assay. Eosin has been complexed previously with alkaloids and quaternary ammonium compounds (4). The bethanidine–eosin complex formed at pH 7.4 in Step III is the most stable when measured fluorometrically in Step IV. The fluorescence is greater up to pH 8.0 and is less below 7.4. The concentration of eosin may be varied, but 4×10^{-4} *M* gives sufficient sensitivity with an acceptable blank.

In performing Steps I–IV, several precautions are necessary for reproducibility. Clean glassware is essential. Eosin may undergo quinoid–lactoid transformation (5). Chloroform is unstable in alkali (6). The fluorescence of the blank increases progressively as contact time with alkaline solutions is prolonged, and at the same time the net fluorescence exhibited by the bethanidine–eosin complex decreases. The mechanism may be due to the degradation

Table I—Relative Fluorescence[a]

Compound	Concentration	Relative Fluorescence
Bethanidine (n = 16)	1 μM	1.00 (0.02)
Guanethidine	1 μM	0.75
Hydrochlorothiazide	10 μM	0
Chlorothiazide	10 μM	0
Benzthiazide	10 μM	0
Urea	40 mM	0
Creatinine	1 mM	0
Uric acid	0.3 mM	0
Spironolactone	1 μM	0
Spironolactone metabolite (dethioacetylated Δ^6-derivative)	1 μM	0
Propranolol	10 μM	0
Aspirin	0.3 mM	0
Fenfluramine	10 μM	0

[a] The compounds were dissolved in water and carried through the standard assay. Relative fluorescence is expressed as the net fluorescence of the test substance in the assay compared to the net fluorescence of 1 μM bethanidine. The fluorescence in parenthesis is the standard deviation.

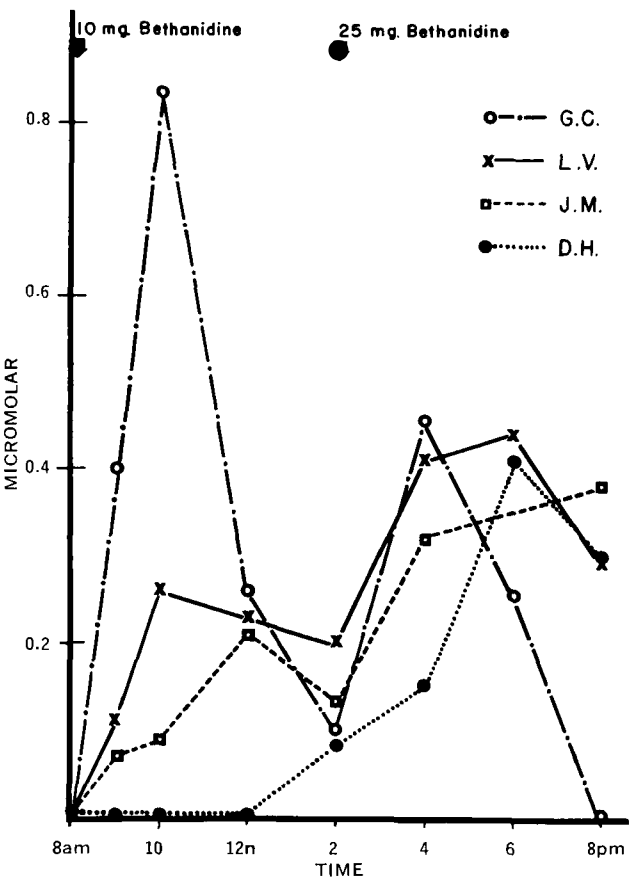

Figure 3—*Bethanidine in plasma. Bethanidine was administered orally to patients at the times indicated (see text).*

products of chloroform, but it has not been determined whether the fluorescence fails to develop or whether instead the developed fluorescence becomes extremely labile on exposure to light. Methylene chloride and ethylene dichloride were not acceptable substitutes for chloroform. The assay is more reproducible when conducted in a dimly lighted room and with aluminum foil-covered tubes.

The fluorescence in Step IV is stable in the dark for about 2 hr, but it is very labile in the excitation light path of 535 nm ($t_{1/2}$ = 2 min); it can be recovered with a $t_{1/2}$ of 20 min by allowing the bethanidine–eosin complex to stand in darkness. If the chloroform layer in Step IV is exposed to air, the fluorescence irreversibly decays ($t_{1/2}$ = 30 min). The 0.75% ethanol used as a stabilizer in the commercially available chloroform is optimal for the assay, and the commercial source is important. Several other sources of chloroform gave high blanks and low net fluorescence. The fluorescence is not affected between 10 and 30°, but at 4° the chloroform becomes supersaturated and aqueous droplets form. Other studies have also revealed a flat temperature coefficient for eosin in water (7). The entire assay should be uniformly timed within and between steps. Usually 16 samples can be analyzed in about 3 hr.

The fluorescence of 0–1 μM bethanidine in water is shown in Fig. 2. It is linear up to 10 μM bethanidine in water or plasma. The small daily variation in fluorescence of the complex is corrected by a standard of bethanidine in each run. The standard deviation of the net fluorescence of bethanidine in a single assay (n = 16) is 2%. The fluorescence of the reagent blank averaged 0.17 ± 0.02 μM, and the plasma blank in six patients was 0.14 ± 0.05 μM bethanidine. One can reproducibly detect about 0.02 μM (4 ng/ml). The assay readily detects guanethidine (Table I); however, the known metabolites of guanethidine have not been subjected to the assay. The fluorescence of some body constituents and drugs was also examined.

To compare the fluorometric assay with the radioactive assay, radioactive ^{14}C-bethanidine was given intravenously (1 μmole/kg) to an 11.2-kg male monkey (*Macaca mulatta*). The solution was standardized by radioactivity and fluorometry. The blood level of

bethanidine at 22 min was 1.01 μM as determined by both assays. Although the radioactive $t_{1/2}$ in plasma was 27 min with a prolonged phase of 4 hr, there was insufficient plasma for the fluorometric assay. Previous reports concerning cats and humans have not revealed any metabolites of bethanidine (8, 9). This matter has not been evaluated in chronic therapy, but the metabolites of bethanidine apparently do not interfere with the assay.

A study on hypertensive patients receiving bethanidine is underway in this laboratory. Bethanidine levels in plasma, correlation with efficacy during acute and chronic therapy, and pharmacokinetic parameters are being determined. Blood levels in four patients who received orally 10 mg followed by 25 mg were variable (Fig. 3). The values demonstrate that the fluorometric assay is readily applicable to analysis of blood levels under clinical conditions. After 3 weeks of therapy at an average dose of 20 mg given at 8 am, 2 pm, and 8 pm, the levels in the four patients were 0.39 ± 0.16 μM. Subsequent studies indicated that the therapeutic range on various dose levels is about 0.3–1.8 μM when determined 2 hr after the afternoon dose of drug[14].

DISCUSSION

This simple fluorometric assay of bethanidine is sufficiently sensitive for application in controlled clinical studies of bethanidine blood levels. Numerous drugs have been complexed previously by similar methods (4, 10). While several other dyes were examined in this study, only eosin Y was acceptable. This assay is not absolutely specific for bethanidine. However, under controlled conditions in which interfering substances (*i.e.*, guanethidine) are not administered and the differential extraction procedure is used, the assay is relatively specific.

Other assays for bethanidine have been described. It has been measured in urine with the alkaline hypobromite reaction (9). The reaction has a sensitivity of about 44 μM bethanidine, in contrast to 0.02 μM for the present fluorometric study. Another method for urine analyses utilized column chromatography followed by complexation of bethanidine with bromcresol green, and it had a limit of sensitivity of approximately 10 μM (11).

An elaborate method for urine and plasma involved a multistep extraction of the drug followed by GC (12). The final analysis was by flame ionization and multiple-ion detection. Bethanidine in plasma was not reported; however, guanethidine in plasma at 0.04 μM (20 ng/ml) gave a standard deviation of 9%. Assuming bethanidine acted similarly in that method, the sensitivity would be in a range approaching the fluorometric method.

Bethanidine is utilized in the therapy of severe hypertension and in acute exacerbations of blood pressure such as may occur in cerebrovascular accidents. The availability of the fluorometric assay described here should be valuable in pharmacokinetic and other clinical studies of this drug. In cases where antihypertensive effects are inadequate, it can be utilized for determining blood levels so that the extent of absorption of the drug or the problem of patient compliance can be examined. Six to eight samples can be analyzed in duplicate in about 3 hr by one person, and a minimum investment in equipment or technical assistance is required.

[14] Unpublished data.

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Two Antimicrobial Alkaloids from Heartwood of *Liriodendron tulipifera* L.

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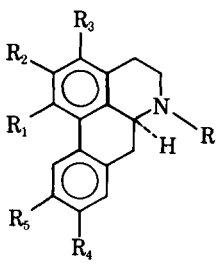
Abstract □ Alcoholic extracts of the heartwood of *Liriodendron tulipifera* have demonstrated antimicrobial activity against *Staphylococcus aureus, Mycobacterium smegmatis, Candida albicans,* and *Aspergillus niger.* The antimicrobial activity was associated only with the alkaloidal fraction. Separation of the active alkaloidal fraction by chromatography led to the isolation and identification of dehydroglaucine and liriodenine as the active components. Several other alkaloidal derivatives were prepared and tested. In addition to the active alkaloids, michelalbine was also identified in the tertiary nonphenolic base fraction along with the lignan, lirioresinol-B-dimethyl ether, and two *N*-acetylnoraporphine alkaloids from the nonbasic fraction.

Keyphrases □ *Liriodendron tulipifera* L.—isolation and identification of two antimicrobial alkaloids from heartwood □ Alkaloids, antimicrobial—isolation and identification of dehydroglaucine and liriodenine from heartwood of *Liriodendron tulipifera* □ Antimicrobial activity—dehydroglaucine and liriodenine, isolated and identified from *Liriodendron tulipifera* □ Liriodenine—isolated from *Liriodendron tulipifera,* screened for antimicrobial activity □ Dehydroglaucine—isolated from *Liriodendron tulipifera,* screened for antimicrobial activity

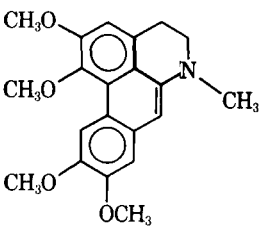
Liriodendron tulipifera (Magnoliaceae), the tulip tree or yellow-poplar tree, is one of two species of the genus *Liriodendron* (1). Chemical constituents isolated from this tree include a lignan diglucoside from the inner bark (2), alkaloids from the heartwood (3–5) and from the leaves (6), cytotoxic sesquiterpene lactones from the root bark (7) and leaves (8), and a cyclitol from the leaves (9).

As part of a program of random screening of higher plants for antimicrobial activity, it was observed that ethanolic extracts of the heartwood of *L. tulipifera* showed consistent and reproducible inhibitory activity against *Staphylococcus aureus, Mycobacterium smegmatis, Candida albicans,* and *Aspergillus niger.*

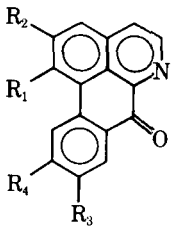
This paper reports the isolation and identification of the active antimicrobial alkaloids from the heartwood, the testing of several alkaloidal derivatives, and the isolation of two nonbasic aporphine alkaloids and a lignan from the nonbasic fraction of the heartwood.



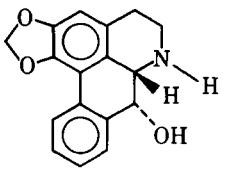
Ia: R = CH_3, $R_1 = R_2 = R_4 = R_5 = OCH_3$, $R_3 = H$
Ib: R = $COCH_3$, $R_1 = R_2 = R_3 = OCH_3$, $R_4 + R_5 = OCH_2O$
Ic: R = $COCH_3$, $R_1 = R_2 = OCH_3$, $R_4 + R_5 = OCH_2O$, $R_3 = H$



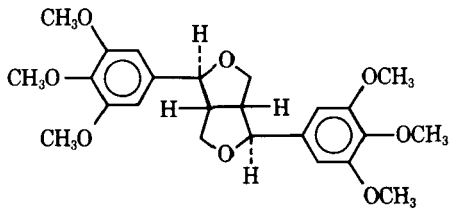
II



IIIa: $R_1 + R_2 = OCH_2O$, $R_3 = R_4 = H$
IIIb: $R_1 = R_2 = R_3 = R_4 = OCH_3$



IV



V

EXPERIMENTAL[1]

Plant Material—The yellow heartwood was separated from the sapwood of *L. tulipifera* and air dried before grinding[2]. Other plant parts (leaf, stem bark, root bark, root, fruit, and flower) were collected, but alcoholic extracts showed no antimicrobial activity.

Extraction and Separation of Alkaloids—The air-dried ground heartwood (2.2 kg) was extracted by percolation at room temperature with alcohol USP until a negative alkaloid test of the percolate was observed. Removal of the solvent at reduced pressure and at 40° left 71 g of residue that exhibited antimicrobial activity. A 35-g sample of the alcohol-soluble residue was partitioned between 125 ml each of ether and 2% citric acid. The ether layer was extracted twice more with 125 ml of 2% citric acid, filtered to remove some interfacial solids (5.8 g of alkaloid negative, no antimicrobial activity), dried (sodium sulfate), and evaporated to dryness, giving 8.6 g of ether solubles that had no antimicrobial activity.

[1] Melting points were determined on a Thomas-Hoover Uni-Melt melting-point apparatus and are uncorrected. Elemental analyses were performed by Midwest Microlabs, Ltd., Indianapolis, Ind. IR spectra were run in potassium bromide or chloroform, using a Perkin-Elmer 257 or Beckman IR-33 IR spectrometer. NMR spectra were recorded on a Jeolco C-60-HL spectrometer using deuterated chloroform as the solvent and tetramethylsilane as the internal standard; chemical shifts are reported in δ (parts per million) units. UV spectra were obtained in methanol on a Beckman Acta III spectrophotometer. Optical rotations were determined on a Perkin-Elmer 141 automatic polarimeter. Circular dichroism measurements were performed on JASCO J-40 recording spectrophotometer using methanol as the solvent. Mass spectral data were obtained on a DuPont-CEC 492 spectrometer.

[2] The tree was cut in the summer of 1973 in southern Lafayette County, Miss. The tree was identified by Dr. Maynard W. Quimby and a voucher specimen has been deposited in the Herbarium of the Department of Pharmacognosy, School of Pharmacy, University of Mississippi.

Table I—Chromatographic Separation of Tertiary Nonphenolic Base Fraction

Fraction Number	Eluent	Weight of Residue, mg	Remarks
1–11	$CHCl_3$	65	Nonalkaloidal, inactive[a]
12–19	$CHCl_3$	400	Crystalline residue, glaucine, dehydroglaucine, active
	1% CH_3OH in $CHCl_3$		
20–32	1% CH_3OH in $CHCl_3$	728	Amorphous residue, inactive[a]
33–40	1% CH_3OH in $CHCl_3$	118	Yellow solid, liriodenine, active
41–51	2% CH_3OH in $CHCl_3$	206	Crystalline residue, michelalbine, inactive[a]
52–65	2% CH_3OH in $CHCl_3$	84	Amorphous residue, inactive[a]
66–90	16% CH_3OH in $CHCl_3$	30	Amorphous residue, inactive[a]
Wash	50% CH_3OH in $CHCl_3$	300	Amorphous residue, inactive[a]

[a] No activity was observed against any of the test organisms.

The aqueous citric acid layers were combined, adjusted to pH 9–10 with ammonia, and extracted three times each with 1 liter of chloroform. The aqueous layer was neutralized, and a portion was evaporated to dryness and found to have no antimicrobial activity. The remaining aqueous layer was then acidified with acetic acid; a saturated aqueous solution of ammonium reineckate was added to a portion, but no precipitation occurred. No precipitate was noted with Valser's or Mayer's reagent.

The combined chloroform layers were dried (sodium sulfate) and evaporated to dryness to give 6.2 g of chloroform solubles that showed all of the antimicrobial activity.

The active chloroform-soluble residue (6.2 g) was separated into tertiary phenolic and nonphenolic fractions by dissolving the residue in 250 ml of chloroform and extracting three times each with 250 ml of 5% sodium hydroxide solution. After drying, the chloroform solution was evaporated to leave 4.7 g of tertiary nonphenolic alkaloids that possessed all of the antimicrobial activity.

The combined aqueous solution of the base layers was treated with an excess of ammonium chloride until a cloudy suspension was noted. This suspension was extracted three times with an equal volume of chloroform. The chloroform layer, after washing with water and drying (sodium sulfate), was evaporated to give 1.4 g of tertiary phenolic bases that had no antimicrobial activity.

Isolation of Antimicrobial Alkaloids from Tertiary Nonphenolic Base Fraction—A 2-g portion of the crude nonphenolic base fraction was dissolved in chloroform and chromatographed over 200 g of aluminum oxide (Woelm, neutral, grade III). The solvents used were 300 ml of chloroform, 500 ml of 1% methanol in chloroform, 300 ml of 2% methanol in chloroform, and 400 ml of 16% methanol in chloroform; finally the column was washed with 50% methanol in chloroform. The fractions (20 ml each) were evaporated in tared flasks, combined according to their weights and to their similarity on TLC[3], and then assayed (Table I).

(+)-Glaucine (Ia) and Dehydroglaucine (II) from Fractions 12–19—TLC analyses[3] of these fractions indicated the presence of two alkaloidal constituents. These were obtained pure by chromatography (2.4-g sample compiled from several columns as described previously) over 200 g of silica gel G[4] using ether as the eluent.

The first 125 ml of eluent contained no alkaloids, but the next 150 ml yielded a crude alkaloid (115 mg). This alkaloid was crystallized from alcohol to yield 59 mg of slightly colored plates, mp 113–115°. Subsequent recrystallizations raised the melting point to 121–122° (pale-yellow plates). The mass spectrum exhibited a parent ion at *m/e* 353. The UV spectrum showed maxima at 260 and 332 nm, while NMR indicated a 1H singlet at δ 9.60 (ArH) and a 3H singlet at δ 3.01 (NCH_3). These data are characteristic of dehydroaporphine alkaloids (10), and the physical data agree with those reported for dehydroglaucine (11). Direct comparison (melting point, TLC[3], UV, and IR) of a sample of dehydroglaucine prepared from glaucine by potassium permanganate oxidation (11) with that obtained from the separation confirmed the identity.

The next 1 liter of eluent yielded no alkaloids; but the following 1 liter yielded the second alkaloid (1.78 g), which was crystallized from alcohol–hexane to yield 0.855 g of needles, mp 119–120°. This was identified as (+)-glaucine by direct comparison (melting point, mixed melting point, TLC[3], IR, and circular dichroism) with an authentic reference sample of (+)-glaucine. (+)-Glaucine was reported previously to be the major alkaloidal constituent of the heartwood (3).

Glaucine methiodide was prepared by stirring a solution of glaucine in acetone with excess methyl iodide. After 30 min the crystalline methiodide was collected, mp 220–221° [lit. (12) mp 219°].

After the glaucine had been eluted, the column was washed with 50% methanol in ether. All fractions were assayed, and only the fraction containing dehydroglaucine was active. Dehydroglaucine was subsequently assayed and was shown to be the antimicrobial agent present in fractions 12–19 (Table II).

Liriodenine (IIIa) from Fractions 33–40—Crystallization of the residue of these fractions (118 mg) from chloroform yielded 85 mg of yellow needles, mp 280–281°. The melting point, IR, and UV data were consistent with that reported for the yellow alkaloid, liriodenine, previously reported from the heartwood (13). Direct comparison (melting point, mixed melting point, IR, and UV) with an authentic sample of liriodenine confirmed the identity. Antimicrobial assay showed liriodenine to be the active component present in these fractions (Table II).

Liriodenine oxime (13) and methiodide (14) were prepared as described in the references and were tested for antimicrobial activity (Table II).

Michelalbine (IV) from Fractions 41–51—Crystallization of the residue in these fractions from chloroform–hexane gave 92 mg of colored needles, mp 191–195°. Recrystallization yielded white needles, mp 199–200°. Chloroform solutions of this alkaloid were observed to undergo air oxidation very easily and become yellow. TLC analyses[3] indicated that liriodenine was formed.

By passing a stream of air through a solution of the alkaloid in *tert*-butanol (15), liriodenine was formed (TLC). Comparison of the physical data of the isolated compound with those of michelalbine (16, 17) indicated them to be the same. Direct comparison (melting point, mixed melting point, TLC[3], UV, IR, and circular dichroism) with an authentic sample of michelalbine confirmed the identity.

Dehydroglaucine (II) and 1,2,9,10-Tetramethoxyoxoaporphine (Oxoglaucine) (IIIb) from Oxidation of Glaucine (Ia)—To a stirred solution of 214 mg of glaucine in 15 ml of acetone was added a solution of 237 mg of potassium permanganate in 25 ml of acetone dropwise over 1 hr. After stirring at room temperature for an additional 4 hr, the suspension was filtered[5] and evaporated to dryness. TLC analysis (silica gel G, ether) showed the presence of mostly dehydroglaucine plus trace amounts of glaucine and oxoglaucine. The dehydroglaucine was obtained pure by chromatography over 10 g of silica gel G[4], using ether as the eluent. Crystallization from alcohol yielded 53 mg of dehydroglaucine, mp 122–123°; *m/e* 353.162 (calculated *m/e* for $C_{21}H_{23}NO_4$, 353.163). The physical data agree with that reported previously (11).

In another experiment, a crude fraction (2.4 g, one spot by TLC) of glaucine (in 75 ml of acetone) was oxidized by adding a solution of potassium permanganate in acetone (6 g/450 ml) dropwise over

[3] TLC analyses were carried out on aluminum oxide G-coated plates using 4% methanol in chloroform as the solvent, and the spots were visualized with Dragendorff's reagent.

[4] Silica gel G (for TLC according to Stahl) was slurried with water first, dried at 110° for 12 hr, and sieved through an 80-mesh sieve before use.

[5] Through Celite.

Table II—Antimicrobial Activity of Extracts, Fractions, and Compounds

Sample[a]	Zone Diameter, mm					
	Staph. aureus	*B. subtilis*	*M. smegmatis*	*C. albicans*	*S. cerevisiae*	*A. niger*
Alcohol extract	4	N.T.[b]	10	2	N.T.	5
Tertiary nonphenolic fraction	8	N.T.	12	5	N.T.	9
Fractions 12–19	3	N.T.	2	1	N.T.	—
Fractions 33–40	5	N.T.	10	3	N.T.	10
Liriodenine	5	8	11	3	5	11
Liriodenine methiodide	6	6	14	17	15	4
Dehydroglaucine	4	7	8	6	6	—
Oxoglaucine methiodide	9	9	10	10	11	—

[a] All samples were also tested against *E. coli* and *P. aeruginosa* and were found to be inactive. All other fractions also were assayed, but none showed any activity against any of the microorganisms listed in this table. Glaucine, glaucine methiodide, liriodenine oxime, michelalbine, and oxoglaucine were tested, but none showed any activity against any of the microorganisms. [b] N.T. = not tested.

2 hr, followed by stirring for 6 hr, and then adding another 6 g of potassium permanganate in 450 ml of acetone, followed by stirring for an additional 12 hr. The suspension was filtered[5] and the clear orange solution was evaporated to dryness. Crystallization from a small volume of chloroform yielded 0.578 g of orange needles of oxoglaucine, mp 224–225° dec. Recrystallization yielded orange needles, mp 229–230° dec., of oxoglaucine whose identity was confirmed by a direct comparison (melting point, mixed melting point, TLC[3], and IR) with an authentic sample[6].

Oxoglaucine methiodide was prepared by refluxing oxoglaucine in acetone with methyl iodide as described previously (18).

Chromatography of Nonbasic Fraction—When examined by TLC[7], the ether-soluble fraction indicated the presence of several alkaloidal spots that were different from any of the ones isolated from the basic fraction. The 8.69-g ether-soluble fraction was dissolved in chloroform and chromatographed over 200 g of silicic acid[8], using chloroform with increasing amounts of methanol as eluent. Elution with 2% methanol in chloroform yielded a 2.6-g fraction that was still a mixture. No other column fraction indicated the presence of alkaloids. A portion of this fraction (1.3 g) was further purified by chromatography over 65 g of silica gel G[4], using ether as the eluent. Fractions (8 ml) were collected, analyzed by TLC[7], and then combined.

Fractions 7 and 8 (382 mg) were combined, and crystallization from benzene–hexane gave nearly colorless crystals (200 mg), mp 112–114°. Recrystallization from alcohol two more times gave a pure sample, mp 118–120°. The mass spectrum showed a parent ion peak at *m/e* 446.194 (calculated *m/e* 446.190 for $C_{24}H_{30}O_8$). The formula was also supported by elemental analysis. The UV spectrum showed absorption maxima at λ_{max} 207 (log ϵ 4.79) and 268 (log ϵ 4.16) nm with a shoulder at 230 (log ϵ 4.16) nm. The NMR spectrum was extremely simple for a proton count of 30, suggesting a symmetrical molecule. The physical data were in agreement with the published data for lirioresinol-B-dimethyl ether (V) (2, 19–21), and a direct comparison (melting point, mixed melting point, TLC[7,9], and IR) with an authentic sample confirmed the identity.

Fractions 12–20 (130 mg) were treated with ether, and the crystalline material obtained was recrystallized from benzene–hexane and then alcohol to yield colorless needles (63 mg), mp 216–217°, for which Structure I*b* has been proposed (22).

Fractions 25–35 (217 mg) contained solid material which was crystallized from benzene–hexane and then alcohol to yield 90 mg of white needles, mp 283–284°. This alkaloid has been identified as (+)-*N*-acetylnornantenine (Ic) (22).

Antimicrobial Assay—Routine qualitative evaluation of antimicrobial activity of extracts, fractions, and pure compounds was accomplished using an agar well diffusion assay. All extracts and fractions were tested for activity against the following microorganisms: *Staphylococcus aureus* (ATCC 6538), *Escherichia coli* (ATCC 10536), *Mycobacterium smegmatis* (ATCC 607), *Pseudomonas aeruginosa* (ATCC 15442), *Candida albicans* (ATCC 10231), *Saccharomyces cerevisiae* (ATCC 9763), and *Aspergillus niger* (ATCC 16888). Purified compounds were also tested against *Bacillus subtilis* (ATCC 6633). Bacterial test organisms were cultured in eugonagar[10] and eugonbroth[10], and fungi and yeasts were cultured in mycophil[10] agar and broth.

Plates for assay were prepared by uniformly seeding sterile, partially cooled, molten agar with dilutions of test organisms grown in broth or suspensions of conidia produced on agar slants (*A. niger*). The seeded agar medium was dispensed into 100 × 15-mm sterile petri dishes (15 ml/dish). Cylindrical plugs were removed from the solidified agar plates, using a sterile cork borer, to produce wells having a diameter of approximately 11 mm. Then 100 μl of a solution or suspension of an extract, fraction, or pure compound was added to each well. The extracts and fractions were tested as solutions or suspensions in a concentration of 20 mg/ml; pure compounds were tested at 1 mg/ml. When solvents other than water were required to dissolve extracts or compounds, solvent blanks were run against each test organism.

Plates prepared as described were incubated as follows. Bacteria were grown at 37° for 24 hr before reading, except *M. smegmatis* which grows more slowly and requires incubation at 37° for 48 hr. Fungi or yeasts were incubated at 25° for 24 hr before growth was evident.

Antimicrobial activity was recorded as the width (in millimeters) of the clear zone of inhibition surrounding the agar well. Although no attempt was made to quantitate the diffusion assay rigidly, conditions were held sufficiently constant so that successful fractionation methods were evidenced by an increase in the size of the zones of inhibition as purification was achieved.

Quantitative assay of the pure compounds against selected test organisms involved utilization of a twofold serial dilution in eugonbroth or mycophil broth. The concentration of pure alkaloids in the initial dilution tube was 50 μg/ml. Streptomycin sulfate[11] (50 μg/ml in tube 1) and amphotericin B[12] (50 μg/ml in tube 1) were used as standard antibiotics for comparison with the activities of the alkaloids against bacterial and yeast species, respectively. Cultures used in the serial dilution assay included *Staph. aureus, B. subtilis, M. smegmatis, C. albicans,* and *S. cerevisiae.* The values reported in Table III represent readings taken after incubation times of 24 hr for all organisms except *M. smegmatis,* which was read at 72 hr. The concentration of the tube of highest dilution that was free from growth was recorded as the minimum inhibitory concentration (micrograms per milliliter).

RESULTS AND DISCUSSION

As part of a program of screening higher plants for antimicrobial activity, it was observed that an alcoholic extract of the heartwood of *L. tulipifera* showed inhibitory activity against several microorganisms (Table II). This activity has been traced to the alkaloids, dehydroglaucine (II) and liriodenine (III*a*), by systematic fractionation guided by antimicrobial assay.

Liriodenine previously was reported to be a constituent of the heartwood (12). Liriodenine was found to have a broad antimicro-

[6] Oxoglaucine was reported previously as a naturally occurring alkaloid of the heartwood (3, 13), but none was isolated from this collection. TLC examination showed only traces present.

[7] TLC analyses of this fraction were carried out on silica gel G-coated plates using ether or acetone–methanol–benzene (1:1:8) as the solvent.

[8] Mallinckrodt, 100 mesh.

[9] Lirioresinol-B-dimethyl ether gives a positive test with Dragendorff's reagent.

[10] BBL.

[11] Nutritional Biochemical.

[12] Calbiochem, "A" grade.

Table III—Minimum Inhibitory Concentration (Micrograms per Milliliter) of Active Compounds

Compound	*Staph. aureus*	*B. subtilis*	*M. smegmatis*	*C. albicans*	*S. cerevisiae*
Liriodenine	3.1	0.39	1.56	6.2	6.2
Liriodenine methiodide	6.2	3.1	3.1	0.78	3.1
Oxoglaucine methiodide	25	25	25	1.56	25
Dehydroglaucine	25	25	25	25	50
Streptomycin sulfate	3.1	1.56	0.78	—	—
Amphotericin B	—	—	—	0.78	0.78

bial spectrum, being active against Gram-positive bacteria, an acid-fast bacterium, and yeast-like and filamentous fungi (Table II). Liriodenine has activity comparable to streptomycin sulfate against *Staph. aureus, B. subtilis,* and *M. smegmatis* (Table III) under the same test conditions. Liriodenine methiodide was also prepared and tested. It has a similar spectrum of activity but is more active than liriodenine against *C. albicans* and *S. cerevisiae* and has activity comparable to that of amphotericin B. On the other hand, liriodenine oxime has no activity. It is apparent from the data in Table III that liriodenine and liriodenine methiodide show *in vitro* activity comparable to that of streptomycin sulfate and amphotericin B and thus warrant further investigation of their potential usefulness as antibiotics.

Dehydroglaucine, not previously reported from *L. tulipifera,* showed no activity against *A. niger.* Otherwise, it has a similar spectrum of activity to liriodenine and liriodenine methiodide but is not as potent.

1,2,9,10-Tetramethoxyoxoaporphine (oxoglaucine) (III*b*) has been reported as a constituent of the heartwood (3), but only traces were found[6]. However, oxoglaucine was prepared from glaucine; when tested, it showed no antimicrobial activity. On the other hand, oxoglaucine methiodide is active, particularly against *C. albicans.* Liriodenine methiodide was also more active than liriodenine against *C. albicans.*

Michelalbine (IV) was identified in the tertiary nonphenolic base fraction but showed no antimicrobial activity. Although it is a constituent of other plants of the family Magnoliaceae, this is the first report of it being a constituent of *L. tulipifera.*

The nonbasic fraction of the heartwood was also investigated and a lignan, lirioresinol-B-dimethyl ether (V), was identified along with two new naturally occurring, nonbasic aporphine alkaloids. The structure elucidation of these alkaloids was previously reported (22). One was identified as (+)-*N*-acetylnornantenine (I*b*), and Structure Ic has been proposed for the other.

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Bioavailability of Micronized Griseofulvin from Corn Oil-in-Water Emulsion, Aqueous Suspension, and Commercial Tablet Dosage Forms in Humans

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Abstract □ The purposes of this investigation were to determine and to compare the oral absorption characteristics of micronized griseofulvin (500 mg) after its administration to humans in the form of a corn oil-in-water emulsion containing dispersed drug, an aqueous suspension, and two different commercial tablets (A and B). The four dosage forms were administered in a random crossover fashion to five fasting subjects, and drug absorption was assessed from urinary excretion data for the major metabolite of the antibiotic (6-desmethylgriseofulvin). The drug was most rapidly, uniformly, and completely absorbed from the corn oil-in-water emulsion. As compared to either the aqueous suspension, Tablet A, or Tablet B, three- to fourfold increases in the maximum body levels and a twofold enhancement in the bioavailability of the antibiotic were observed after administration of the emulsion dosage form. A mechanism based on the ability of the linoleic and oleic acids liberated during the digestion of corn oil to inhibit GI motility and stimulate gallbladder evacuation may explain the marked enhancing effect of emulsified corn oil on griseofulvin absorption in humans. This new lipid-in-water emulsion dosage form of micronized griseofulvin appears to offer several clinical advantages in the treatment of fungal infections.

Keyphrases □ Griseofulvin, micronized—bioavailability from corn oil-in-water emulsion, aqueous suspension, and commercial tablet dosage forms, humans □ Emulsions, corn oil-in-water—bioavailability of micronized griseofulvin, humans □ Bioavailability—griseofulvin, emulsion, suspension, and tablet dosage forms, humans □ Dosage forms—micronized griseofulvin, bioavailability from corn oil-in-water emulsion, aqueous suspension, and commercial tablets, humans

Griseofulvin [7-chloro-2′,4,6-trimethoxy-6′β-methylspiro[benzofuran - 2(3*H*),1′ - [2] - cyclohexene]-3,4′-dione], a chemically neutral systemic antifungal antibiotic, is commonly used in the treatment of dermatophyte infections in humans (1, 2). As a result of its low aqueous solubility (~15 mg/liter at 37°), the drug is slowly, erratically, and incompletely absorbed from the GI tract of humans (3–6). Although oral administration of the micronized form of griseofulvin alone (7–9), with meals high in fat content (7, 9), or as a 1:9 (w/w) drug–polyethylene glycol 6000 solid dispersion (10) considerably enhances the bioavailability of the drug, its absorption from commercial tablet (11) and methylcellulose suspension (12) dosage forms is poor and quite variable.

An average of 52.7% of a 500-mg dose of micronized griseofulvin as a commercial tablet was slowly absorbed for as long as 30 hr after its oral administration to five fasting subjects (range of 38.5–52.3% of dose; coefficient of variation = 19.5%) (11). One to two months later, the absorption study was repeated in four of the five subjects and an average of 49.3% of the dose was absorbed (range of 27.0–72.5%; coefficient of variation = 40.6%). Similar results were also obtained after administration of a methylcellulose suspension of micronized griseofulvin to five fasting subjects (12). In this study, the average amount of drug absorbed was 50% of the dose (range of 31.0–63.0%; coefficient of variation = 23.2%).

As suggested by several investigators (5, 7, 8), the slow rate and low extent of absorption, as well as the appreciable intersubject differences in the amount of micronized griseofulvin absorbed from conventional dosage forms, are undoubtedly the most common reasons for subtherapeutic blood levels in some patients and, thus, for clinical failure with griseofulvin therapy. Therefore, a convenient, acceptable, and improved dosage form of micronized griseofulvin is needed from which the drug is uniformly, rapidly, and maximally absorbed in humans. Such a dosage form should assure that therapeutic blood levels of the antibiotic would be consistently attained in all patients.

Previous studies from these laboratories (13) indicated that one can markedly enhance the bioavailability and uniformity of absorption of micronized griseofulvin in rats by orally administering the drug dispersed in a corn oil-in-water emulsion vehicle as compared to administration in either an aqueous or corn oil vehicle. If similar results occur in humans, then an emulsion dosage form would offer obvious advantages in the treatment of fungal infections. The purposes of the present investigation were to determine and to compare the oral absorption characteristics of micronized griseofulvin after its administration to fasting subjects as a corn oil-in-water emulsion, as two different commercial tablets, and as a control aqueous suspension.

EXPERIMENTAL

Dosage Forms—Four dosage forms containing micronized griseofulvin (specific surface area of 1.32 m^2/g) were subjected to bioavailability testing in humans. The corn oil-in-water emulsion and control aqueous suspension dosage forms were prepared as described previously (13). Both preparations contained 300 mg of polysorbate 60[1] and 500 mg of suspended griseofulvin/30 g. The other two dosage forms were commercial tablets containing 500 mg of micronized griseofulvin (Tablets A[2] and B[3]). All dosage forms were assayed in triplicate for drug content by the spectrophotometric method described earlier (13).

Absorption Studies in Humans—Five healthy, nonobese, male subjects, 23–29 years old and weighing 61–82 kg (mean 69 kg), participated in this study. They were instructed not to consume any drug product or alcoholic beverage for 7 days prior to, and for 4 days following, griseofulvin administration. A single 500-mg oral

[1] Tween 60.
[2] Grifulvin V, Control No. SJ2901, McNeil Laboratories.
[3] Fulvicin-U/F, Control No. OAUG17P35531, Schering Corp.

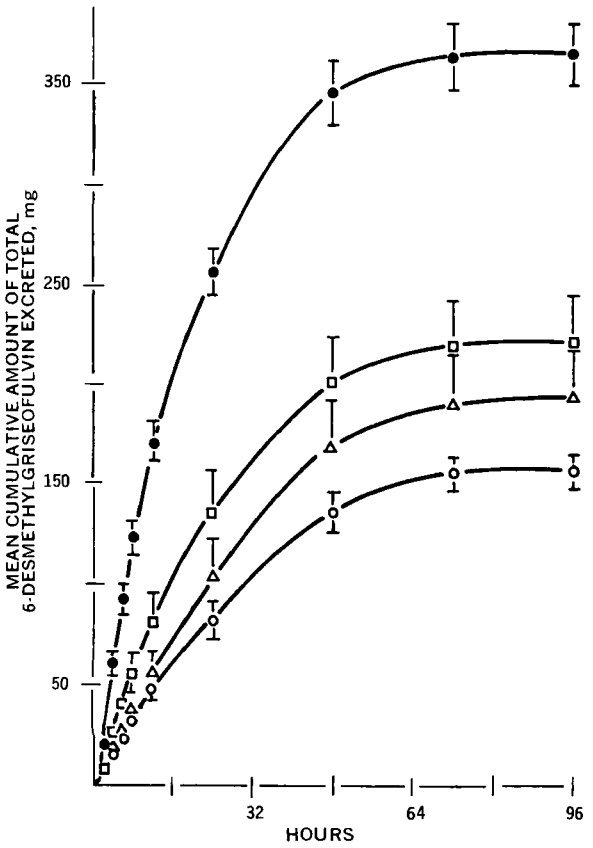

Figure 1—*Cumulative excretion of total 6-desmethylgriseofulvin as a function of time after a 500-mg oral dose of micronized griseofulvin as an aqueous suspension* (○), *as Tablet A* (△), *as Tablet B* (□), *and as a corn oil-in-water emulsion, 30 g* (●). *Each point represents the mean of five fasting subjects. Bars denote standard errors of the mean.*

dose of micronized griseofulvin in the form of the corn oil-in-water emulsion (30 g), aqueous suspension (30 g), and commercial Tablets A and B was administered at 8:00 am in a random crossover fashion to each subject after an overnight fast (at least 8 hr). At least 1 week was allowed between drug administrations.

A time zero urine specimen was collected immediately prior to drug administration. The dosage forms were ingested with 100 ml of water, and urine specimens were collected at 2, 4, 6, 8, 12, 24, 48, 72, and 96 hr after drug administration. The pH and volume of each specimen were recorded, and an aliquot was stored in the frozen state until assayed in duplicate for 6-desmethylgriseofulvin content. No food was permitted until 4 hr after drug administration.

By using the same protocol, the effects of the amount of emulsified corn oil administered and of the anticholinergic agent propantheline (a potent inhibitor of GI motility) on the absorption of micronized griseofulvin were assessed in Subject JAS. In the former preliminary studies, a 500-mg dose of the drug was administered in 7.5, 15, or 30 g of corn oil-in-water emulsion. These amounts correspond to 3, 6, and 12 g of emulsified corn oil, respectively. The volume of water ingested with the dose was appropriately adjusted so as to maintain the total fluid intake constant. In the latter studies, a single 30-mg oral dose of propantheline bromide[4] was administered 30 min prior to a 500-mg dose of the drug as the aqueous suspension dosage form. Drug absorption was also evaluated in the same subject after oral administration of a corn oil suspension dosage form (13). The dose of this suspension was such that the subject received 500 mg of micronized griseofulvin and 12 g of unemulsified lipid.

Free 6-desmethylgriseofulvin in the urine was determined by the double-extraction, differential spectrophotometric method of Rowland and Riegelman (14). Total 6-desmethylgriseofulvin (free and the glucuronide conjugate) was assayed by the same procedure after incubating an aliquot of the urine specimens with 500 units of β-glucuronidase[5] for 2 hr at pH 6.8 and 37°. Blank urine specimens collected over the previously mentioned time period after administration of the aqueous suspension and corn oil-in-water emulsion vehicles yielded zero absorbance readings when treated by this assay procedure. Only minor amounts of intact griseofulvin and its 4-desmethyl metabolite are excreted in human urine (12). However, they were found not to interfere with the determination of 6-desmethylgriseofulvin.

Differences among more than two mean values were statistically evaluated by an analysis of variance (15), and differences between any two means were compared using Tukey's multiple-range test (15).

RESULTS AND DISCUSSION

The urinary excretion rate of free and total 6-desmethylgriseofulvin in humans has been shown to be proportional to plasma griseofulvin levels (10, 12). A proportionality also exists between the amount of 6-desmethylgriseofulvin excreted and both the oral dose of griseofulvin (9) and the area under the griseofulvin plasma concentration–time curve (16). Therefore, the urinary excretion of 6-desmethylgriseofulvin may be used to assess the absorption characteristics of orally administered griseofulvin (9, 16). However, 6-desmethylgriseofulvin is a weakly acidic metabolite (pKa 4.27) and its elimination may be affected by changes in urinary pH and/

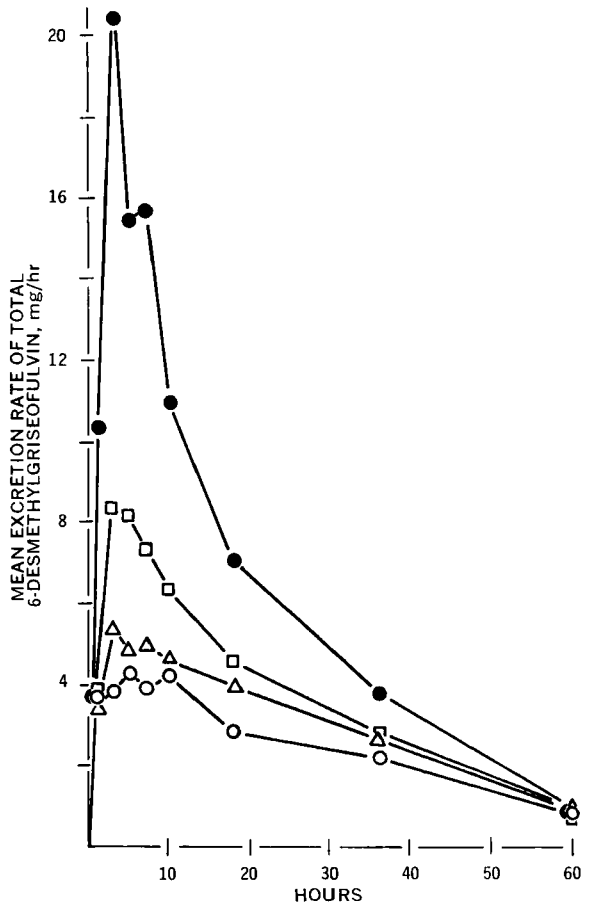

Figure 2—*Excretion rate of total 6-desmethylgriseofulvin as a function of time after a 500-mg oral dose of micronized griseofulvin as an aqueous suspension* (○), *as Tablet A* (△), *as Tablet B* (□), *and as a corn oil-in-water emulsion, 30 g* (●). *Each point represents the mean of five fasting subjects.*

[4] Two 15-mg Pro-Banthine tablets, Searle Co., Chicago, Ill.

[5] Bacterial-type II, Sigma Chemical Co., St. Louis, Mo.

Table I—Maximum Excretion Rate of Total 6-Desmethylgriseofulvin after Oral Administration of a 500-mg Dose of Micronized Griseofulvin in Four Dosage Forms

Subject	Maximum Excretion Rate of Total 6-Desmethylgriseofulvin, mg/hr				Ratio		
	Aqueous Suspension (AS)	Commercial Tablet A (CT-A)	Commercial Tablet B (CT-B)	Corn Oil-in-Water Emulsion (COE)	COE:AS	COE:CT-A	COE:CT-B
SIF	4.27	3.06	3.70	18.61	4.36	6.08	5.03
JPL	6.85	6.96	8.07	23.60	3.45	3.39	2.92
PJC	5.98	8.77	12.30	24.49	4.10	2.79	1.99
AVT	3.17	7.50	6.10	19.01	6.00	2.53	3.12
JAS	4.89	3.97	14.49	20.19	4.13	5.09	1.40
Mean	5.03	6.05	8.93	21.18	4.41	3.98	2.89
SE[a]	0.64	1.09	1.98	1.21	0.43	0.69	0.62
CV, %[b]	28.58	40.14	49.57	12.74	—	—	—

Statistical Comparisons

Differences among four dosage forms: $p < 0.001$ ($F^c = 31.8$; $df = 3,16$) (analysis of variance)
Difference between any two dosage forms [Tukey's allowable difference ($p = 0.05$; $df = 4,16$) = 5.35]:
COE − AS = 16.15; significant[d] AS − CT-A = 1.02; not significant
COE − CT-A = 15.13; significant[d] AS − CT-B = 3.90; not significant
COE − CT-B = 12.25; significant[d] CT-A − CT-B = 2.88; not significant

[a] Standard error of the mean. [b] Coefficient of variation. [c] Critical *F* value for 3 and 16 *df* at the 0.1% level is 9.00. [d] Also significant at 1% level.

or urine flow rate. Analysis of variance tests indicated that at the collection periods examined, neither urinary pH nor urine volume was significantly altered by the nature of the dosage form orally administered. Consequently, differences noted in the amounts of total 6-desmethylgriseofulvin excreted in the urine accurately reflect differences in the absorption profile of griseofulvin from the four dosage forms employed in this investigation.

The mean cumulative amount of total 6-desmethylgriseofulvin excreted in the urine as a function of time after oral administration of 30 g of the corn oil-in-water emulsion, 30 g of the aqueous suspension, and the two commercial tablets to the five fasting subjects is shown in Fig. 1. In all cases, a plateau value was reached in 72–96 hr, indicating that the experimental time period selected was adequate to assess completely the excretion and, thereby, the absorption characteristics of micronized griseofulvin.

The mean excretion rate profiles depicted in Fig. 2 indicate that micronized griseofulvin was absorbed at a considerably faster rate from the corn oil-in-water emulsion than from the aqueous suspension, Tablet A, or Tablet B. In addition, it is evident from an examination of the data presented in Table I that the maximum excretion rate of total 6-desmethylgriseofulvin, which is proportional to the maximum body level of griseofulvin, was substantially higher and less variable after administration of the corn oil-in-water emulsion (30 g) as compared to that observed after oral administration of any one of the other three dosage forms. The three- to fourfold increases experienced with the emulsion dosage form are highly significant, as determined by the method of Tukey (Table I). No significant differences in the mean maximum excretion rates of total 6-desmethylgriseofulvin exist between the control aqueous suspension and either tablet dosage form or between the two tablet formulations (Table I).

Analysis of the representative semilogarithmic excretion rate plots for total 6-desmethylgriseofulvin (Fig. 3) reveals that micronized griseofulvin was absorbed at a rapid rate during the first 3–7 hr after oral administration of the four dosage forms to Subject JPL and was then slowly absorbed over an additional period of approximately 29–33 hr. Subsequently, the drug was eliminated from the body in a monoexponential fashion. The terminal linear segments of the four plots are parallel, indicating that no significant absorption occurred after 40 hr irrespective of the dosage form orally administered to the subject. Similar prolonged absorption profiles were also observed in the other four subjects and are consistent with the results first reported by Rowland *et al.* (11) and later confirmed by Chiou and Riegelman (10) for micronized griseofulvin in humans.

The cumulative amounts of total 6-desmethylgriseofulvin ultimately excreted in the urine for each subject after each dosage form, expressed as percent of the oral dose of griseofulvin, are listed in Table II. The mean data indicate that the extent of absorption (bioavailability) of micronized griseofulvin from the corn oil-in-water emulsion (30 g) was, on the average, significantly and markedly enhanced by 134, 100, and 70% as compared to that observed with the control aqueous suspension, commercial Tablet A, and commercial Tablet B dosage forms, respectively. No statistical

Table II—Bioavailability of Micronized Griseofulvin after Oral Administration of a 500-mg Dose in Four Dosage Forms

Subject	Cumulative Amount of Total 6-Desmethylgriseofulvin Excreted in 96 hr, % of Dose				Ratio		
	Aqueous Suspension (AS)	Commercial Tablet A (CT-A)	Commercial Tablet B (CT-B)	Corn Oil-in-Water Emulsion (COE)	COE:AS	COE:CT-A	COE:CT-B
SIF	30.03	28.08	31.69	66.84	2.23	2.38	2.11
JPL	34.96	45.30	51.43	78.24	2.24	1.73	1.52
PJC	33.61	57.00	57.15	80.93	2.41	1.42	1.42
AVT	27.47	30.98	39.40	72.99	2.66	2.36	1.85
JAS	30.64	30.91	40.76	65.97	2.15	2.13	1.62
Mean	31.33	38.45	44.09	72.99	2.34	2.00	1.70
SE[a]	1.33	5.53	4.53	2.98	0.09	0.19	0.12
CV, %[b]	9.50	32.15	23.00	9.13	—	—	—

Statistical Comparisons

Differences among four dosage forms: $p < 0.001$ ($F^c = 21.6$; $df = 3,16$) (analysis of variance)
Differences between any two dosage forms [Tukey's allowable difference ($p = 0.05$; $df = 4,16$) = 15.92]:
COE − AS = 41.66; significant[d] AS − CT-A = 7.12; not significant
COE − CT-A = 34.54; significant[d] AS − CT-B = 12.76; not significant
COE − CT-B = 28.90; significant[d] CT-A − CT-B = 5.64; not significant

[a] Standard error of the mean. [b] Coefficient of variation. [c] Critical *F* value for 3 and 16 *df* at the 0.1% level is 9.00. [d] Also significant at 1% level.

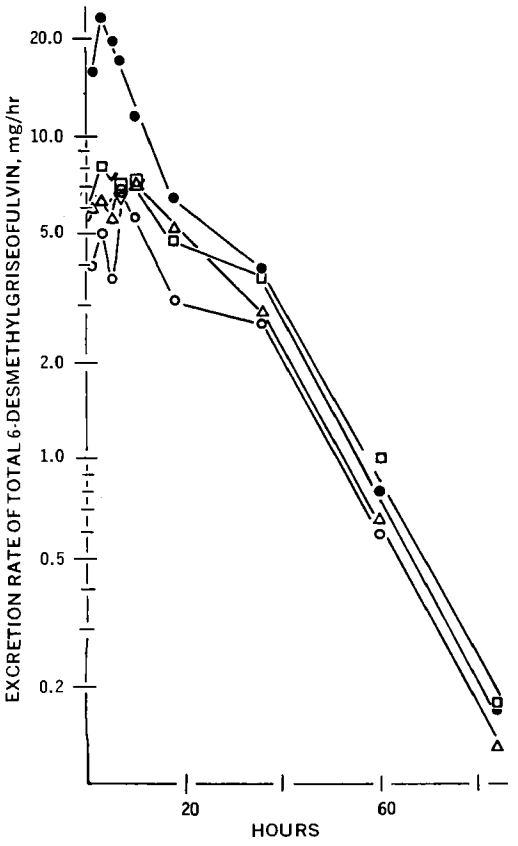

Figure 3—*Excretion rate of total 6-desmethylgriseofulvin as a function of time after administration of a 500-mg oral dose of micronized griseofulvin to Subject JPL as an aqueous suspension* (○), *as Tablet A* (△), *as Tablet B* (□), *and as a corn oil-in-water emulsion, 30 g* (●).

difference in bioavailability was found to exist between the aqueous suspension and either commercial tablet or between the two commercial tablets (Table II).

Chiou and Riegelman (10) reported that 65.3% of an intravenous dose of griseofulvin was ultimately excreted in the urine by one of their subjects as total 6-desmethylgriseofulvin, and Lin *et al.* (12) showed that total 6-desmethylgriseofulvin represented 84% (mean of five subjects) of the total radioactivity excreted in the urine after an oral dose of ^{14}C-griseofulvin. Based on these values and the mean 96-hr cumulative excretion data obtained in this investigation (Table II), it would appear that the oral dose of micronized griseofulvin was almost completely absorbed after administration as a corn oil-in-water emulsion.

Of considerable clinical importance is the fact that large intersubject variations were observed in the amount of micronized griseofulvin absorbed from the two commercial tablet dosage forms, as reflected by the high coefficients of variation listed in Table II. Similar variations in drug absorption from tablet formulations were reported by other investigators (11, 16). On the other hand, administration of the drug in the corn oil-in-water emulsion vehicle substantially reduced the degree of intersubject variability (Table II). In addition, the presence of emulsified corn oil in the body caused no appreciable change in the ratio of the amount of free to conjugated 6-desmethylgriseofulvin ultimately excreted in the urine (Table III). The mean ratios calculated from the corn oil-in-water emulsion and aqueous suspension data were quite similar (Table III) and were comparable to literature values of 1.24–1.86 (10, 12). The slightly, yet statistically significant, greater amount of 6-desmethylgriseofulvin glucuronide excreted by all subjects after administration of the corn oil-in-water emulsion is probably of no clinical significance but is worthy of additional study.

The most plausible major mechanism underlying the marked enhancement noted in the absorption of micronized griseofulvin following its oral administration to humans as a corn oil-in-water emulsion is similar to that established in these laboratories in the rat (13, 17). The inhibitory effect of lipid (emulsified corn oil) on the gastric emptying process (17–19) could have allowed only small amounts of undissolved drug to be emptied into the small intestine, as compared to the rapid emptying and, therefore, significantly larger amounts of drug emptied when administered in nonlipid-containing dosage forms (*i.e.*, the aqueous suspension and commercial tablets). The smaller amounts of drug present in the upper region of the small intestine at any particular time after oral administration of the corn oil emulsion, coupled with the inhibitory effect of lipid on proximal small intestinal motility (17, 18), allows the drug more time to dissolve in, and be absorbed from, the region of the intestinal tract where absorption is optimal (20).

It is also possible that the presence of emulsified corn oil in the intestinal tract stimulated bile secretion (21–24) which, because of the presence of increased micellar concentrations of physiological surfactants (bile salts and lysolecithin) in the intestinal fluids (22, 23, 25), increased the rate of dissolution (26, 27) and, thereby, the rate of absorption and bioavailability of this poorly soluble antibiotic. Additional support for this hypothesis was obtained from preliminary studies conducted in Subject JAS, which clearly demonstrate, apparently for the first time, the significant effect of GI motility on the absorption of micronized griseofulvin in humans. When a 30-mg oral dose of propantheline bromide, a potent inhibitor of GI motility, was administered 30 min prior to a 500-mg oral dose of the antibiotic in aqueous suspension, the time of occurrence and the magnitude of the maximum excretion rate of total 6-desmethylgriseofulvin were increased by 9 hr and by 165%, respectively (Table IV). The anticholinergic agent also caused a 1.53-fold increase in the amount of antibiotic absorbed from the aqueous suspension dosage form (Table IV). However, the absorption-enhancing effect of propantheline was less than that experienced with 30 g of the corn oil-in-water emulsion (Table IV).

In another series of experiments, the effect of varying the amount of emulsified corn oil administered to Subject JAS on the absorption of a 500-mg dose of micronized griseofulvin from the corn oil-in-water emulsion was examined (Table IV). As the amount of emulsified corn oil administered was increased from zero (aqueous suspension) to 12 g (equivalent to 30 g of corn oil-in-water emulsion), there was a progressive increase in both the maximum excretion rate and the cumulative 96-hr excretion of total 6-desmethylgriseofulvin, with both parameters reaching plateau values after 6 g of emulsified corn oil had been administered. Similar types of dose–response relationships also exist between the amount of fat or long chain fatty acid ingested and its effect on both GI motility (18, 28) and gallbladder evacuation (24) in humans. The doses of emulsified corn oil employed in this portion of the investigation are comparable to those necessary for initiation of the reflexes that result in decreases in GI motility and increases in gallbladder contraction.

In this connection, Card (18) reported that the minimum inhibitory effect of fat (arachis oil) on gastric motility can be rapidly (within 1–4 min) achieved in humans after administration of a 4-g

Table III—Ratio of the Amount of Free 6-Desmethylgriseofulvin to 6-Desmethylgriseofulvin Glucuronide Ultimately Excreted in the Urine after Oral Administration of a 500-mg Dose of Micronized Griseofulvin as an Aqueous Suspension and Corn Oil-in-Water Emulsion to Fasting Subjects

Subject	Aqueous Suspension	Corn Oil-in-Water Emulsion (30 g)
JPL	2.03	1.81
SIF	0.99	0.81
PJC	1.49	1.36
AVT	1.51	1.13
JAS	2.35	1.62
Mean	1.67	1.35
SE[a]	0.24	0.18
CV, %[b]	31.57	29.27
Statistical significance[c]	$p < 0.05$	

[a] Standard error of the mean. [b] Coefficient of variation. [c] Determined by paired *t* test.

Table IV—Factors Affecting Absorption of a 500-mg Oral Dose of Micronized Griseofulvin from Corn Oil-in-Water Emulsion and Aqueous Suspension Dosage Forms (Subject JAS, Fasting State)

Regimen	Maximum Excretion Rate of Total 6-Desmethylgriseofulvin, mg/hr	Cumulative Amount of Total 6-Desmethylgriseofulvin Excreted in 96 hr, % of Dose
Aqueous suspension	4.89 (1)[a]	30.64
Aqueous suspension, 30 min after a 30-mg oral dose of propantheline	13.01 (10)	46.96
Corn oil-in-water emulsion		
7.5 g (equivalent to 3 g of lipid)	15.39 (3)	50.17
15.0 g (equivalent to 6 g of lipid)	21.30 (3)	63.97
30.0 g (equivalent to 12 g of lipid)	20.19 (3)	65.97
Corn oil suspension		
13.1 g (equivalent to 12 g of lipid)	10.11 (3)	48.57

[a] Time of occurrence of maximum excretion rate (hours) in parentheses.

dose of unemulsified fat or after only a 2-g dose of fat emulsified with bile salts. Also, considerable emptying of the gallbladder of its bile content can occur in humans within 30–60 min after an oral dose of approximately 5 g of lipid (one egg yolk), with complete emptying occurring after a 25-g dose of lipid (five egg yolks) (24).

The total 6-desmethylgriseofulvin excretion patterns observed after oral administration of micronized griseofulvin (500 mg) to Subject JAS with 12 g of unemulsified corn oil (as a 13.1-g dose of corn oil suspension) and 12 g of emulsified corn oil (as a 30-g dose of corn oil-in-water emulsion) are compared with the excretion pattern for the control aqueous suspension in Table IV. An analysis of these data revealed that although the presence of 12 g of unemulsified lipid in the GI tract caused an appreciable increase in the rate and extent of absorption and maximum body levels of the antibiotic, an even greater enhancement in drug absorption was realized with the same amount of lipid in an emulsified form. In fact, the potentiating effect of 12 g of unemulsified corn oil on griseofulvin absorption is comparable to that observed with only 3 g of emulsified corn oil (Table IV).

These results, which are similar to those previously observed in the rat with the same dosage forms (13), are consistent with the fact that to stimulate the fat-sensitive receptors in the duodenum, which cause inhibition of gastric emptying in humans, the fat must be digested (29); the rapidity and efficiency of the digestion process are increased by increasing, through emulsification, the effective surface area of triglyceride exposed to the hydrolytic action of pancreatic lipase (30, 31). It has also been reported that some fat digestion must take place prior to the appearance of any significant effect on gallbladder evacuation and that the latter process is fat digestion rate limited (24).

The results obtained from this investigation suggest that to maximize the rate, extent, and, perhaps most importantly, uniformity of absorption of micronized griseofulvin, it should be administered in a lipid-in-water emulsion dosage form to patients with fungal infections for which the drug is indicated. The emulsion dosage form is also advantageous from the standpoint that only relatively small amounts (6–12 g) of emulsified, unsaturated lipid need be administered to the patient as compared to the considerably larger amounts (>60 g) needed when unemulsified lipid from dietary sources is used to enhance the absorption of micronized griseofulvin in humans (5, 9). Since the enhanced bioavailability of the antibiotic from the corn oil-in-water emulsion appears to be solely dependent on the effect of emulsified corn oil and/or its digestion products (oleic and linoleic acids) on the physiological processes of GI motility and gallbladder evacuation, it is not unreasonable to expect similar increases in drug absorption if a comparable amount of any vegetable oil containing long chain (C_{14}–C_{18}) saturated or unsaturated fatty acids is substituted for corn oil in the emulsion formulation. The applicability of the lipid-in-water emulsion as a dosage form vehicle for potentiating the bioavailability in humans of other poorly absorbed drugs is currently being ascertained in these laboratories.

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GLC Determination of Ibuprofen [*dl*-2-(*p*-Isobutylphenyl)propionic Acid] Enantiomers in Biological Specimens

GARRETT J. VANGIESSEN and DAVID G. KAISER ˣ

Abstract □ To study the behavior of the *d*- and *l*-isomers of ibuprofen in humans, a method for the determination of the individual enantiomers in plasma and urine was required. A specific procedure was developed based on *(a)* benzene extraction of the acidified specimens, *(b)* TLC of the benzene extract residue, *(c)* formation of the *l*-α-methylbenzylamides of the materials eluted from the chromatograms, and *(d)* quantification of the resulting diastereoisomeric amides by GLC in conjunction with flame-ionization detection. When using a 1-ml aliquot of the specimen, the method is sensitive to 1 μg of each enantiomer/ml of plasma or urine. As compared to simple aqueous solutions, the average recoveries of the enantiomers from plasma and urine ranged from 94 to 96%. Mass spectrometric analyses, in conjunction with GLC, confirmed the specificity of the method for the intact enantiomers. The procedure was applied successfully to drug absorption studies in humans. After oral administration of the racemic mixture, the predominant enantiomer in peripheral circulation and excreted in urine was of the *d*-configuration.

Keyphrases □ Ibuprofen—GLC determination of enantiomers in biological specimens □ GLC—determination, ibuprofen enantiomers in biological specimens

Ibuprofen[1] [*dl*-2-(*p*-isobutylphenyl)propionic acid] (I) is reported (1, 2) to be one of the most potent orally active anti-inflammatory, antipyretic, and analgesic agents of a large number of substituted 2-phenylalkanoic acids based on a variety of tests in animals. The extensive toxicological and biochemical studies (3–5) in animals and humans (6) have been comprehensively reviewed (7).

Paper chromatographic (8) and GLC (5, 9) methods have been used to study the absorption, metabolism, and excretion of I in animals and humans. None of these procedures separated the individual enantiomers of the intact drug from each other. After oral administration of I to humans, the major metabolites excreted in urine were found to be dextrorotatory (1, 5). To study the behavior of the *d*- and *l*-isomers of I in humans (10), a specific GLC method for determining the individual enantiomers of the intact drug in plasma and urine was developed.

EXPERIMENTAL

Reagents and Materials—The I used in this study was synthesized[2] and the *d*- and *l*-isomers were resolved. Tridecanoic acid[3], *l*-α-methylbenzylamine[4], and hydrocarbon-stabilized chloroform[5] were used as supplied. The stock solution of 1,1′-carbonyldiimidazole[4] (II) (65 mg/ml) in hydrocarbon-stabilized chloroform was prepared fresh daily. Stock solutions of I, the *d*-isomer of I (III), the *l*-isomer of I (IV), and *n*-tridecanoic acid (V) in ethanol (100 μg/ml) were stored in glass containers. All other solvents were analytical reagent grade. Phenyl methyl silicone fluid (OV-17) on 60–80-mesh Gas Chrom Q[3] and chromatography plates coated with a 250-μm layer of silica gel F-254[6] were used as supplied.

Instrumentation—A two-speed reciprocating shaker[7] was used for shaking the samples in a horizontal position. A mixer[8] was used to aid in preparing the α-methylbenzylamide derivatives. GLC measurements were made with a gas chromatograph[9], equipped with a hydrogen flame-ionization detector and a −0.2–1.0-mv recorder[10]. All cylinders of gases used for chromatography (*i.e.*, helium, hydrogen, and oxygen) were fitted with filters containing molecular sieve 4A.

TLC Conditions—All TLC was conducted on silica gel F-254; the plates were developed (ascending) in 10% (v/v) acetic acid in toluene. The separated materials were visualized by irradiation of the plates with a short wavelength (254 nm) UV lamp or by spraying with 0.05% (w/v) bromocresol green in isopropanol[6]. Under these conditions, I, III, IV, and V have similar R_f values, *i.e.*, 0.40–0.42.

GLC Conditions—GLC was conducted on a U-shaped glass column (1.5 m × 3 mm i.d.) of 3% (w/w) OV-17 on 60–80-mesh Gas Chrom Q. All newly prepared columns were preconditioned at 250° for 1 hr without carrier gas flow and for 16 hr with a carrier gas flow of 10 ml/min. During analysis, the column, injection port, and detector block were maintained isothermally at 220, 245, and 260°, respectively. Helium, hydrogen, and oxygen flow rates were 90, 70, and 350 ml/min, respectively. Under these conditions, the *l*-α-methylbenzylamide derivatives of III, IV, and V have retention times of 8.0, 9.0, and 12.4 min, respectively (Figs. 1 and 2).

Synthesis of Standard Materials—Place 200 mg of III in a 200-ml round-bottom flask containing 25 ml of benzene. Slowly add 25 ml of thionyl chloride and reflux for 1 hr. Remove the solvent and excess reagent under reduced pressure (15 mm) at 40°. Dissolve the residue in 25 ml of benzene and remove under reduced pressure (15 mm) at 40°. Repeat the addition and removal of benzene. Dissolve the residue in 25 ml of chloroform and add 10 ml of chloroform containing 1 ml of *l*-α-methylbenzylamine. Stir for 30 min at 24°. Transfer the solution to a separator and extract successively with 10 ml of 1 *N* aqueous hydrochloric acid and two 10-ml volumes of water. Transfer the chloroform layer to a fresh round-bottom flask and remove the solvent under reduced pressure. Recrystallize the residue from aqueous methanol.

Synthesize standard materials for identification of the *l*-α-methylbenzylamide derivatives of IV and V utilizing the same general reaction conditions as described for III. Recrystallize the residues from hexane.

Assay Procedure—*Preparation of Standards*—Pipet aliquots of the I ethanol stock solution equivalent to 10, 20, 35, 50, and 65 μg into glass-stoppered centrifuge tubes. Add an aliquot of the V stock solution equivalent to 50 μg and evaporate to dryness with a gentle stream of nitrogen gas. Add 1 ml of control plasma or urine to each centrifuge tube and mix well with a mixer. Prepare an appropriate blank. Extract all standards in the same manner as described later for the plasma or urine samples.

Preparation of Samples—Pipet 0.5 ml of the V stock solution (100 μg/ml) into a series of 15-ml glass-stoppered centrifuge tubes and evaporate to dryness with a gentle stream of nitrogen. Place 1 ml of plasma or urine in each tube. Add 0.25 ml of 1 *N* aqueous hy-

[1] Motrin, The Upjohn Co.; and Brufen, Boots Co., Ltd.
[2] Boots Drug Co., Nottingham, England.
[3] Applied Science Labs, State College, Pa.
[4] Aldrich Chemical Co., Milwaukee, Wis.
[5] Matheson, Coleman and Bell, Milwaukee, Wis.
[6] Brinkmann Instruments, Westbury, N.Y.
[7] Eberbach & Sons, Ann Arbor, Mich.
[8] Scientific Industries, Queen's Village, N.Y.
[9] F & M model 400, Hewlett-Packard Co., Avondale, Pa.
[10] Honeywell Electronik 15.

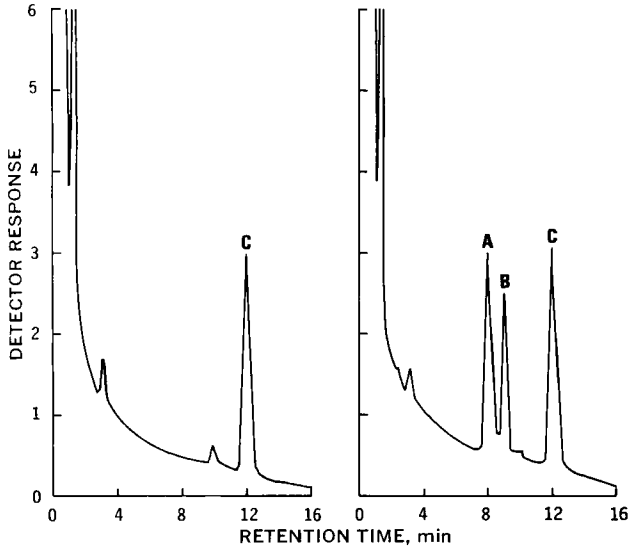


Figure 1—*Gas–liquid chromatograms of human plasma extracts. Left: normal plasma specimen. Right: plasma specimen from subject at 1 hr after single-dose oral administration of 800 mg of ibuprofen. Key: A,* d-*enantiomer; B,* l-*enantiomer; and C,* n-*tridecanoic acid internal standard.*

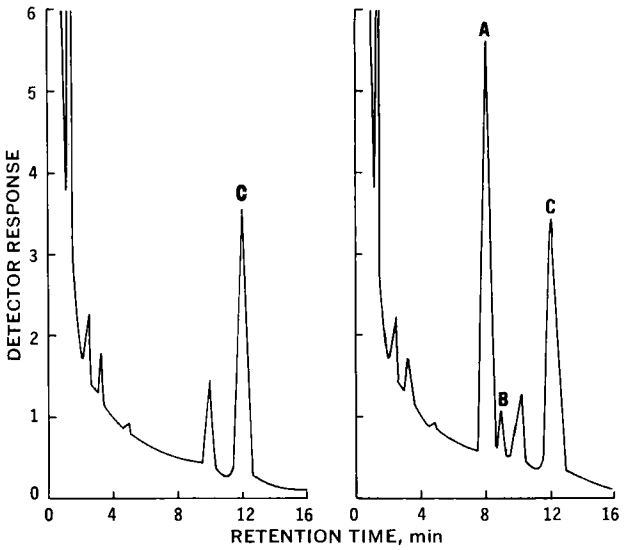


Figure 2—*Gas–liquid chromatograms of human urine extracts. Left: normal urine specimen. Right: urine specimen from subject during 0–4-hr time interval after single-dose oral administration of 800 mg of ibuprofen. Key: A,* d-*enantiomer; B,* l-*enantiomer; and C,* n-*tridecanoic acid internal standard.*

drochloric acid and 5 ml of benzene and shake in a horizontal position for 10 min. Centrifuge for 10 min at 2000 rpm. Transfer a 4-ml aliquot of the benzene layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with a gentle stream of nitrogen. Wash down the walls of the centrifuge tube with 0.5 ml of chloroform and evaporate to dryness with nitrogen.

Reconstitute the benzene extract residues from the standards and samples in 50 μl of chloroform and spot on the silica gel F-254 plates. Ascendingly develop each plate for a distance of 165 mm, air dry all chromatograms at room temperature (24°), and visualize the zones by irradiation with a 254-mm lamp. Scrape the zones corresponding to I and V into glass-stoppered centrifuge tubes. Add 1 ml of 1 *N* aqueous sodium hydroxide and mix thoroughly. Add 1 ml of 2 *N* aqueous hydrochloric acid and 5 ml of benzene and shake for 10 min in a horizontal position. Then centrifuge for 10 min at 2000 rpm. Transfer 3 ml of the benzene layer to a fresh glass-stoppered centrifuge tube and evaporate to dryness with nitrogen.

Add 0.1 ml of II reagent to each benzene extract residue. Rotate each tube to permit the reagent to contact the lower 2.5 cm of the centrifuge tube wall; allow the reagent to react for 5 min. Then add 10 μl of acetic acid and mix. Add 50 μl of *l*-α-methylbenzylamine, mix well, and allow the solution to react for 20 min. Inject a 1–5-μl aliquot for analysis into the chromatograph.

Calculations—The peak heights for the *l*-α-methylbenzylamide derivatives of III, IV, and V are measured. Peak height ratios are obtained by dividing the peak height of the III and IV amides by the peak height of the internal standard amide. Calibration curves from known concentrations of III and IV in plasma or urine are prepared by plotting peak height ratios *versus* free acid concentration, expressed as micrograms per milliliter of plasma or urine. Values for unknown concentrations of III and IV in plasma or urine specimens, obtained in the same manner, are then read directly from the graphs or calculated from the slopes of the standard curves.

Drug Administration to Humans—Informed written consent was obtained from each of three normal human male volunteers prior to participation in this study. All subjects were between 26 and 48 years; they ranged in body weight from 63.6 to 72.7 kg and in height from 1.62 to 1.81 m. All subjects were fasted for 16 hr prior to drug administration. Each then received an 800-mg dose of I as sugar-coated compressed tablets, and food was withheld for an additional 4 hr.

Blood specimens (10 ml) were withdrawn in heparinized syringes at 0, 1, 2, 4, and 8 hr after drug administration. The plasma was harvested and stored at −18°. Total urine specimens were collected for 12 hr prior to drug ingestion and at predetermined time intervals from 0 to 24 hr after drug administration. All specimens were stored at −18° until assay.

RESULTS AND DISCUSSION

Synthesis and Identification of *l*-α-Methylbenzylamide Derivatives of Ibuprofen Enantiomers and Internal Standard—Earlier investigations (9) showed that II was a useful reagent for the facile esterification of I in extracts of human plasma. The imidazolide intermediate of I formed very rapidly (≤1 min) and was highly reactive. A series of samples containing known amounts of III, IV, and V was prepared to determine the optimal reaction times for imidazolide formation and for amide formation from the imidazolides and *l*-α-methylbenzylamine. In studies with III and IV, known amounts of the *l*-α-methylbenzylamide derivative of V were added as the internal standard. Similarly, in studies with V, known amounts of the *l*-α-methylbenzylamide derivatives of III and IV were added as the internal standard.

The results indicated that imidazolide formation from III, IV, and V was complete within 1 min. Amide formation from the imidazolides of III and IV was complete within 20 min, while amide formation from the V intermediate was complete almost instanta-

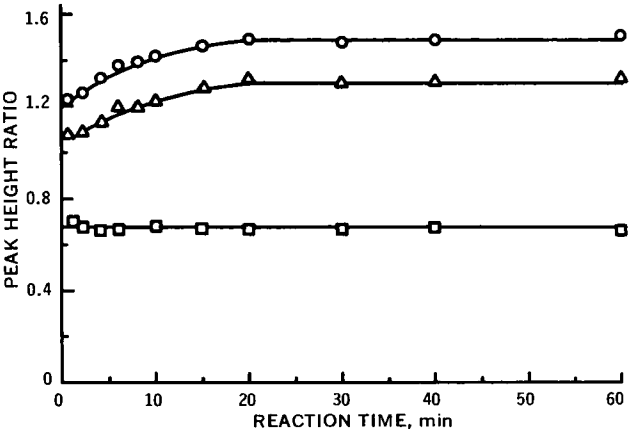
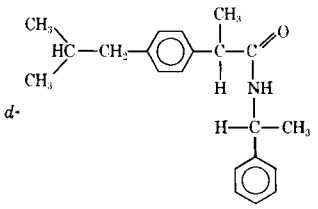
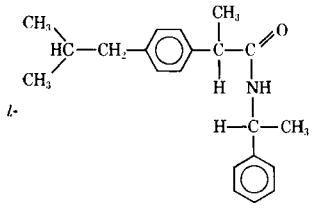
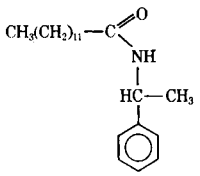


Figure 3—*Effect of reaction time* (l-α-*methylbenzylamine*) *on formation of amide derivatives of ibuprofen enantiomers and* n-*tridecanoic acid (reaction time of 1,1′-carbonyldiimidazole in chloroform = 5 min). Key:* ○, d-*enantiomer;* △, l-*enantiomer; and* □, n-*tridecanoic acid.*

Table I—Structural Formulas and Physical Properties of *l*-α-Methylbenzylamide Derivatives of Ibuprofen Enantiomers and *n*-Tridecanoic Acid

Structure[a]	Empirical Formula	Mol. Wt. Calc.	Mol. Wt. Found[b]	Analysis, % Calc.	Analysis, % Found	Melting Point	GLC Retention Time, min[c]
 *d*-	$C_{21}H_{27}NO$	309.43	309	C 81.51 H 8.79 N 4.52	81.45 8.70 4.39	109–110°	8.0
 *l*-	$C_{21}H_{27}NO$	309.43	309	C 81.51 H 8.79 N 4.52	81.48 9.03 4.46	96–97°	9.0
	$C_{21}H_{35}NO$	317.49	317	C 79.43 H 11.11 N 4.41	79.79 11.42 4.35	72–73°	12.4

[a] *d*- and *l*- refer to the optical rotation of the free acids used as starting materials. [b] Molecular weight of parent ion as determined by GLC in conjunction with mass spectrometry. [c] GLC conditions as described in *Experimental* section.

neously (Fig. 3). Reaction times of 5 min for imidazolide formation and of 20 min for amide formation were selected for convenience. Ancillary studies showed that at least 50 μl of *l*-α-methylbenzylamine was necessary to form the amides of III and IV quantitatively in the concentration range of 0–50 μg/ml of human plasma or urine (Fig. 4).

Synthesis of standard materials indicated that the *l*-α-methylbenzylamides of II, IV, and V were white crystals at room temperature (Table I). GLC, using a solid sample injector, indicated that the materials submitted for elemental analyses were greater than 99% pure. IR and mass spectrometric analyses, before and after GLC, supported the proposed structures and confirmed that the amides chromatographed as the intact molecules.

Identification of *N,N'*-Di-*l*-α-methylbenzylurea—During assay development, a major peak (R_T 10.1 min) unrelated to the individual enantiomers or internal standard was observed in the gas–liquid chromatograms from samples prepared with II and *l*-α-methylbenzylamine. Mass spectrometric analysis of the material(s) in the chromatographic peak suggested that the major component was the symmetrically disubstituted α-methylbenzyl derivative of urea. GLC and mass spectrometric analyses of *N,N'*-di-*l*-α-methylbenzylurea, synthesized directly from II and *l*-α-methylbenzylamine (11), showed that it was identical to the material found in the major peak. As described in the assay procedure, acetic acid (10 μl) was added to react with the excess II. Under these reaction conditions, little or no material responding in the assay as *N,N'*-di-*l*-α-methylbenzylurea was observed.

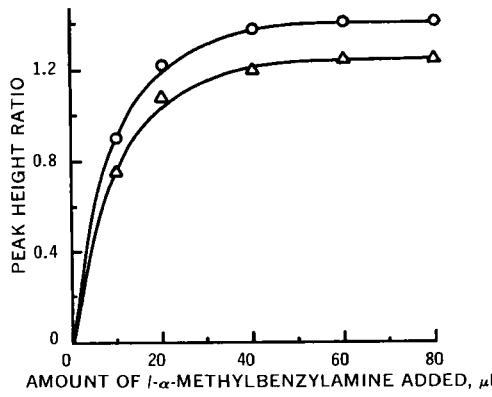


Figure 4—*Relationship between amount of* l-α-*methylbenzylamine added and formation of amide derivatives of ibuprofen enantiomers (reaction time of 1,1'-carbonyldiimidazole in chloroform = 5 min; reaction time of* l-α-*methylbenzylamine = 20 min). Key:* ○, d-*enantiomer; and* △, l-*enantiomer.*

Selection of Internal Standard—Pilot studies, using 2-(*p*-isobutylphenyl)acetic acid as an internal standard, showed that the *l*-α-methylbenzylamide derivative had a retention time of about 9.5 min as compared to 8.0 and 9.0 min for the *l*-α-methylbenzylamide of III and IV, respectively. As shown in Figs. 1 and 2, benzene extracts of plasma and urine specimens from normal human subjects contained interfering materials with approximately the same retention time (R_T 10.0 min) as the *l*-α-methylbenzylamide derivative of 2-(*p*-isobutylphenyl)acetic acid. Based upon *(a)* extraction efficiency into benzene, *(b)* mobility in the TLC solvent system, *(c)* formation of an *l*-α-methylbenzylamide derivative, and *(d)* GLC separation from III, IV, and endogenous materials present in extracts of plasma and urine, V was subsequently selected to replace the 2-(*p*-isobutylphenyl)acetic acid as the internal standard.

TLC of Plasma and Urine Extracts—Pilot studies were conducted with various TLC solvent systems to separate endogenous materials extracted from human plasma and urine that had relatively long GLC retention times (*i.e.*, 34 and 36 min, respectively). A solvent system composed of 10% (v/v) acetic acid in toluene separated the endogenous materials from I, III, IV, and V. The total time required for GLC of each plasma or urine sample was reduced from 40 to 16 min.

Assay Sensitivity and Specificity—At a sensitivity of 1.6×10^{-10} amp/mv, 0.87 μg of III and 0.95 μg of IV as the *l*-α-methylbenzylamides produced full-scale responses. However, under the assay conditions described, the lower limit of detection sensitivity for III and IV in extracts of human plasma or urine is 1.0 μg/ml of the original sample aliquot. This value is based on a sample signal equivalent to 2% of full-scale response. Under the assay conditions

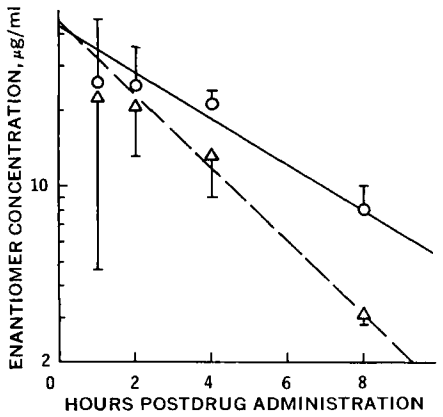


Figure 5—*Average* (±SD) *plasma concentrations of ibuprofen enantiomers* versus *time in humans* (n = *3*) *after single-dose oral administration of 800 mg of drug. Key:* ○, d-*enantiomer; and* △, l-*enantiomer. Bars indicate standard deviations.*

described, a linear relationship between detector response and concentration is obtained for III and IV over the range of 0–80 μg/ml. Quantification from a standard curve has been adequate. Analysis of plasma and urine specimens from drug-treated human subjects, using GLC in conjunction with mass spectrometry, has shown that the material responding to the assay is identical to known III- and IV-*l*-α-methylbenzylamide derivatives.

Recovery Experiments—Known amounts of III, IV, and V in ethanol were evaporated to dryness in centrifuge tubes; then water, plasma, or urine was added. The samples were thoroughly mixed and extracted with benzene. All extract residues were chromatographed on silica gel F-254, derivatized, and analyzed *via* GLC. The results indicated that, in the 5–35-μg/ml concentration range, recoveries of III and IV from plasma (94.7 ± 9.3 and 95.8 ± 11.1%, respectively) and urine (96.3 ± 2.7 and 93.8 ± 4.4%, respectively) were essentially quantitative as compared to simple aqueous samples.

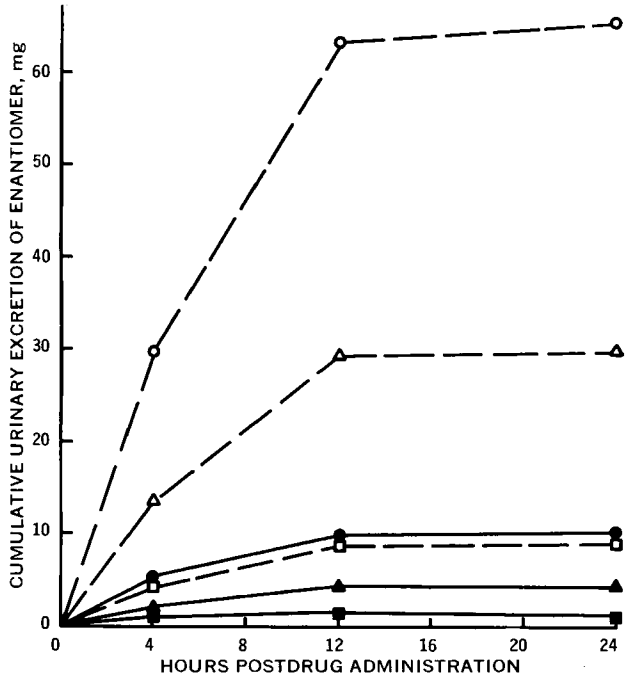


Figure 6—*Cumulative urinary excretion of ibuprofen enantiomers in humans after single-dose oral administration of 800 mg of drug (open symbols* = d-*enantiomer; closed symbols* = l-*enantiomer). Key:* ○,●, *Subject WW;* △,▲, *Subject PK; and* □,■, *Subject RT.*

Plasma and Urine Levels of Ibuprofen Enantiomers in Humans—Results from the measurement of plasma III and IV concentrations in three normal human subjects, after single-dose oral administration of 800 mg of I, demonstrated the utility of the analytical methodology (Fig. 5). Peak mean (±*SD*) levels of III (25.9 ± 19.6 μg/ml) and IV (22.6 ± 17.9 μg/ml) were observed at 1 hr after drug administration, indicating rapid drug absorption. As estimated graphically from the average plasma drug concentrations, the plasma drug disappearance half-lives for III and IV were 3.34 and 2.01 hr, respectively. The concentration ratio of III to IV increased progressively with time (from 1.17 at 1 hr to 2.65 at 8 hr), suggesting a stereospecific metabolism, excretion, or isomeric inversion.

Results from the measurement of III and IV in urine (Fig. 6) showed that III was the predominant isomer of the intact drug excreted. During the 0–24-hr time interval after drug administration, the concentration ratio of III to IV increased from 5.6 to greater than 9.0. The combined results from these investigations showed that the GLC method could be used for evaluating the pharmacokinetics and stereospecificity of metabolism, excretion, or isomeric inversion for the individual enantiomers of ibuprofen in humans.

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Viscosities of Acacia and Sodium Alginate after Sterilization by Cobalt-60

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Abstract □ Acacia and sodium alginate powders were sterilized by cobalt-60 irradiation to a total dose of 2.5 Mrad with no increase in residual radioactivity of the gums as a result of the treatment. Viscosity measurements showed an 11% reduction in the viscosity of acacia in water and a 16% decrease in the intrinsic viscosity in barium chloride solution due to the irradiation. Viscosities of sodium alginate solutions in 0.1 *N* sodium chloride showed a 70% decrease in intrinsic viscosity, indicating changes in the molecular structure corresponding to degradation to 30% of the original degree of polymerization. This dose of γ-radiation from cobalt-60 is not an appropriate method of sterilization for these gums.

Keyphrases □ Acacia—viscosity changes after sterilization by cobalt-60 □ Sodium alginate—viscosity changes after sterilization by cobalt-60 □ Irradiation, cobalt-60—effects on viscosity of acacia and sodium alginate □ Sterilization, cobalt-60—effects on viscosity of acacia and sodium alginate

Acacia, a dried exudate from species of *Acacia*, obtained by tapping the trees, is frequently sun bleached and sorted on the ground. Acacia consists primarily of a mixture of arabic acid salts which, on hydrolysis, yields L-arabinose, L-rhamnose, D-galactose, and glucuronic acid. Sodium alginate is extracted from brown seaweed by aqueous alkali and consists of a polymer of D-mannuronic and L-guluronic acids. Both of these vegetable gums, natural plant hydrocolloids, are subject to microbial attack and, because of their nature and method of production, contain microbes capable of causing spoilage of products incorporating them.

DISCUSSION

The primary use of these gums is to increase viscosity of aqueous systems. Solutions of gums, as with other high polymers, exhibit a great increase in viscosity over that of the solvent alone even when the concentration of the gum is small. It was suggested (1, 2) that viscosity could be quantitatively correlated to the molecular weight *(M)* of the polymer according to:

$$[\eta] = KM \qquad \text{(Eq. 1)}$$

where $[\eta]$ is the intrinsic viscosity, and K is a constant. This equation was later modified (3, 4) to the following expression:

$$[\eta] = K'M^a \qquad \text{(Eq. 2)}$$

where K' and a are also constants. Since these equations are based on empirical findings, this is not an absolute method for obtaining molecular weights. The method must be established for each particular polymer–solvent system at a definite temperature by reference to some other absolute method of molecular weight determination. Furthermore, the method is only applicable to linear polymers. Capillary viscometers are generally used, and the results are expressed as relative viscosity, η_r:

$$\eta_r = \eta/\eta_0 \qquad \text{(Eq. 3)}$$

where η is the viscosity of the polymer solution, and η_0 is the viscosity of the solvent. The older nomenclature is used here since the present work is based on the older literature. However, in determining the molecular weight, several other viscosities are used:

$$\text{specific viscosity} = \eta_{sp} = (\eta - \eta_0)/\eta_0 \qquad \text{(Eq. 4)}$$

$$\text{reduced viscosity} = \eta_{red} = \eta_{sp}/c \qquad \text{(Eq. 5)}$$

$$\text{intrinsic viscosity} = [\eta] = (\eta_{sp}/c)_{c=0} \qquad \text{(Eq. 6)}$$

and:

$$[\eta] = [(\ln \eta_r)/c]_{c=0} \qquad \text{(Eq. 7)}$$

where c is equal to grams of polymer/100 ml of solution. In effect, molecular weights are related to the reduced viscosity at infinite dilution. At finite concentrations of polymer, the reduced viscosity is increasingly dependent on concentration as the concentration increases. To obtain the intrinsic viscosity, one extrapolates to zero concentration both a plot of η_{sp}/c *versus* concentration and a plot of $(\ln \eta_r)/c$ *versus* concentration. The midpoint of the two intercepts is the intrinsic viscosity.

To obtain the constants in Eq. 2, the log of $[\eta]$ is plotted *versus* the log of the molecular weight of various fractions of the polymer (5). The slope of the linear relationship is equal to a and the intercept is K'. If the following expression is valid for a monodisperse polymer:

$$[\eta]_m = KM^a \qquad \text{(Eq. 8)}$$

where m indicates monodispersity, for a polydisperse polymer the relationship becomes:

$$[\eta]_p = K\left[\frac{\sum N_j M_j^{Ha}}{\sum N_j M_j}\right] = K\bar{M}_v^{\,a} \qquad \text{(Eq. 9)}$$

where p indicates polydispersity, and K and a have the same values as in Eq. 8 (6). The viscosity-average molecular weight is indicated by $\bar{M}_v$. For randomly coiled polymers, a varies from 0.5 in a θ solvent to a maximum of about 1, generally being between 0.6 and 0.8. Typical values of K' range from 0.5 to 5×10^{-4}. For many polymers, $\bar{M}_v$ is 10–20% lower than the weight-average molecular weight; but if $a = 1$, the viscosity-average molecular weight is the same as the weight-average molecular weight (7).

Polyelectrolyte polymers such as acacia and sodium alginate exhibit expanded conformation due to electrostatic repulsion of the charged sites as they lose mobile ions to the solution, and this expansion increases as the dilution increases. The expansion changes the frictional characteristics of the polymer; thus, while the reduced viscosity of a nonionic polymer gradually increases with increasing concentration, the reduced viscosity of a polyelectrolyte polymer markedly increases with decreasing concentration. The addition of simple salts to the polyelectrolyte solution creates a Donnan equilibrium, suppressing the loss of mobile ions from the polymer, preventing expansion, and enabling it to assume the random coiled configuration required for the use of Eq. 1.

In a previous study (8), it was shown that 2.5 Mrad of cobalt-60

Table I—Viscosity of Aqueous Acacia Solutions at 30° [a]

Concentration, g/100 ml	Nonirradiated, cps	Irradiated, cps	Change, %
2	1.707 ± 0.021	1.546 ± 0.015	−9.43
5	2.749 ± 0.033	2.448 ± 0.018	−10.95
10	4.955 ± 0.076	4.408 ± 0.033	−11.04

[a] Mean and relative standard derivation of five determinations.

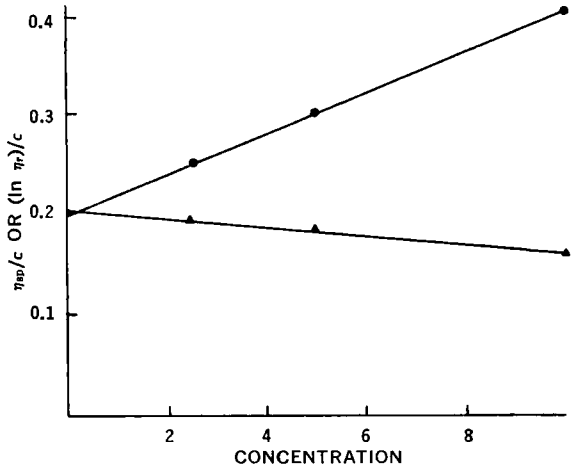

Figure 1—*Viscosity data for nonirradiated acacia. Key:* ●, η_{sp}/c; *and* ▲, $(\ln \eta_r)/c$.

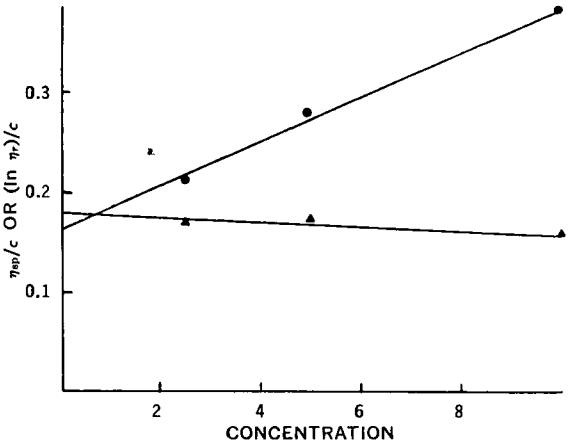

Figure 2—*Viscosity data for cobalt-60 irradiated acacia. Key:* ●, η_{sp}/c; *and* ▲, $(\ln \eta_r)/c$.

radiation was sufficient to sterilize selected colorants without causing a discernible change in their visible, UV, and IR absorption spectra. In the present study, acacia and sodium alginate powders were subjected to 2.5 Mrad of γ-irradiation from cobalt-60 and solutions of the treated gums were studied for effects on viscosity. A recently published report showed that sodium alginate powder subjected to 2.5 and 5 Mrad of cobalt-60 radiation gave solutions of lowered viscosity (9).

EXPERIMENTAL

The following materials were used: acacia USP, powder[1]; sodium alginate powder[1]; polysorbate 80 USP[2]; trypticase soy agar[3]; polyethylene vials, 4 dram with snap cap[4]; and ointment jars, 120 ml, opal glass with screw caps[5].

Irradiation—Gums were placed in polyethylene vials which had been ultrasonically cleaned. The vials were completely filled to eliminate excessive head-space and were irradiated[6] as previously described for approximately 10 hr to a total dose of 2.5 Mrad. Larger quantities were irradiated in completely filled jars. The cobalt-60 source consisted of 10 stainless steel clad cylinders of cobalt metal, each measuring 29.3 cm in length and 1.1 cm in diameter, arranged in a cylindrical array having a diameter of 14.0 cm.

The dose rate of the array was established by the National Bureau of Standards from a Fricke dosimeter placed in the center of the array, and current dose rates were calculated from this value using a half-life of 5.26 years for cobalt-60. The dose rate as of June 1973 was 2.53×10^5 rad/hr for samples, as in this study, placed in the same position as the dosimeter. Exposure time for a 2.5-Mrad dose in June 1973 was 9 hr and 53 min, and the length of irradiation for subsequent samples was adjusted according to the date of exposure.

Sterility Testing—A sample of gum was aseptically obtained and suspended in 10 ml of sterile saline solution (0.9% w/v) containing 1% (w/v) polysorbate 80. An aliquot of this suspension was transferred to a trypticase soy agar plate. Samples of nonirradiated sodium alginate were 0.2 g, and those of irradiated material were 0.3 g. Aliquots placed on the plates were 2.5 ml. The acacia samples were 1 g and the aliquots were 3 ml. Three irradiated and three nonirradiated samples of each gum were tested. Plates were incubated at 30° and checked daily for up to 14 days.

Induced Radiation—Radiation measurements[7] were made on 1-g samples of the gums, irradiated and nonirradiated. The counting period was 15 min, and each sample was counted three times.

Viscosity—Solutions of acacia, irradiated and nonirradiated, were prepared containing 2, 5, and 10 g/100 ml of solution in water and containing 2.5, 5, and 10 g/100 ml in 10 m*M* barium chloride solution. Solutions of irradiated and nonirradiated sodium alginate in 0.1 *N* sodium chloride solution were prepared containing 0.1, 0.3, and 0.5 g/100 ml of solution. All solutions were preserved by the addition of 0.1% methylparaben, and the solutions were allowed to solvate for 24 hr before viscosity measurements[8] were made, except for acacia in barium chloride solutions which were allowed to solvate for 2 weeks.

Viscosities of aqueous acacia solutions were determined at 30°, of acacia in barium chloride solutions at 42°, and of sodium alginate solutions at 25°; all temperatures were ±0.02°. Barium chloride and sodium chloride were used to suppress the extension of the polyelectrolytes. Donnan and Rose (10) found that the use of 0.1 *N* sodium chloride swamped the electroviscous effect of sodium alginate, while barium chloride was utilized in a viscosity study of acacia (11).

RESULTS

Sterility Tests—All plates containing the nonirradiated gums, 50 mg of sodium alginate or 300 mg of acacia, showed copious

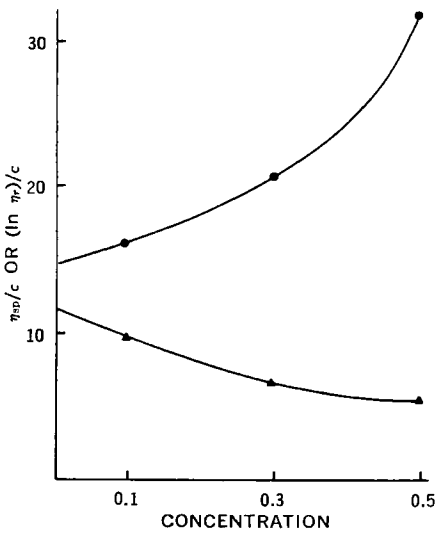

Figure 3—*Viscosity data for nonirradiated sodium alginate. Key:* ●, η_{sp}/c; *and* ▲, $(\ln \eta_r)/c$.

[1] Ruger Chemical Co., Irvington, N.J.
[2] Tween 80, I. C. I. America, Inc., Wilmington, Del.
[3] Baltimore Biological Laboratories, Baltimore, Md.
[4] Polyvials, Olympia Plastics, Los Angeles, Calif.
[5] Brockway Glass Co., Brockway, Pa.
[6] The cobalt-60 irradiation was performed at the Research Reactor Facility, Columbia, Mo.
[7] The measurements were made using a Nuclear Chicago model 1620 A scaler with an ultrathin window tube and a Nuclear Chicago model 1810 single-channel analyzer; Nuclear Chicago, Chicago, Ill.
[8] Obtained using Cannon-Fenske viscometers, Fisher Scientific Co., Pittsburgh, Pa.

Table II—Viscosities of Acacia in 10 m*M* Barium Chloride Solution at 42°[a]

Concentration, g/100 ml	η_r	η_{sp}	η_{sp}/c, g/100 ml	$(\ln \eta_r)/c$, g/100 ml
		Nonirradiated		
2.5	1.63 ± 0.01	0.627 ± 0.013	0.250 ± 0.005	0.195 ± 0.003
5	2.52 ± 0.02	1.52 ± 0.02	0.303 ± 0.005	0.185 ± 0.002
10	5.07 ± 0.08	4.07 ± 0.08	0.407 ± 0.008	0.162 ± 0.002
		Cobalt-60 Irradiated		
2.5	1.53 ± 0.01	0.528 ± 0.013	0.211 ± 0.005	0.169 ± 0.004
5	2.39 ± 0.02	1.39 ± 0.02	0.278 ± 0.004	0.174 ± 0.002
10	4.81 ± 0.03	3.81 ± 0.03	0.381 ± 0.003	0.157 ± 0.001

[a] Mean and relative standard deviation of five determinations.

growth within 72 hr. Plates containing the irradiated material, 75 mg of sodium alginate or 300 mg of acacia, showed no growth under the test conditions and were regarded as sterile.

Induced Radiation—No radioactivity of acacia and sodium alginate was induced by the cobalt-60 sterilization process.

Viscosity—Viscosities of acacia solutions in water (Table I) showed a decrease of about 10% as a result of irradiation, and the solutions of irradiated material exhibited a somewhat darker color. The viscosities of acacia in 10 m*M* barium chloride solution (Table II) showed a slight decrease for the irradiated material. The relationship of η_{sp}/c and $(\ln \eta_r)/c$ to concentration appears to be linear for both the nonirradiated (Fig. 1) and irradiated (Fig. 2) acacia in barium chloride solution. The plot of η_{sp}/c *versus* concentration has a correlation coefficient of 0.996, a slope of 0.0208, and an intercept of 0.1986 for the nonirradiated acacia. Similar values for the irradiated acacia are 0.996, 0.0224, and 0.1592. The plot of $(\ln \eta_r)/c$ against c gives values of −0.9869, −0.0043, and 0.2057 for nonirradiated acacia and −0.794, −0.0019, and 0.1779 for the irradiated gum.

Three experimental points on each line are not sufficient for establishing the intrinsic viscosity, $[\eta]$, but do provide enough information to estimate an apparent intrinsic viscosity, which being dependent on molecular structure is a good criterion for judging the suitability of this method of sterilization. In this respect, an apparent intrinsic viscosity would be a better criterion than a viscosity based on expanded conformation of the polyelectrolyte. If the further assumption is made that the relationship of Eq. 1 exists between the apparent intrinsic viscosity and the molecular weight (despite finding no such relationship in a perusal of the literature), the effects of the sterilization treatment on the acacia molecule may be compared to the effects on the sodium alginate structure. Thus, the apparent $[\eta]$ obtained is 0.202 for nonirradiated acacia and 0.169 for irradiated acacia, indicating that the irradiation sterilization with 2.5 Mrad of cobalt-60 results in the apparent depolymerization of acacia to the extent of about 16%.

Viscosities of the sodium alginate solutions in 0.1 *N* sodium chloride solution are shown in Table III. The decrease in values of the solutions of irradiated gum is quite apparent. The relationship of η_{sp}/c and $(\ln \eta_r)/c$ to concentration is not linear for nonirradiated alginate (Fig. 3), which is in agreement with the investigation of Donnan and Rose (10). These curves were extrapolated to the intercept, and the midpoint of the intercepts was taken as the best value for the apparent intrinsic viscosity. The linearity of these same plots for the irradiated alginate (Fig. 4) is indicative of apparent depolymerization as a result of the cobalt-60 treatment. By using the nine values, the plot of η_{sp}/c *versus* c was found to have a correlation coefficient of 0.992, a slope of 8.22, and an intercept of 3.75. The plot of $(\ln \eta_r)/c$ yielded values of −0.935, −1.35, and 3.89, respectively. The apparent $[\eta]$ obtained for the irradiated alginate was 3.82, while that of the nonirradiated gum was 12.8. Irradiated sodium alginate and its solutions also exhibit a greenish color.

The relationship of molecular weight and intrinsic viscosity of sodium alginate solution, using the data of Donnan and Rose (10), is shown in Fig. 5. This relationship is linear and therefore follows Eq. 1. When using $K = 7.97 \times 10^{-5}$ (12), calculations indicate the $\bar{M}_v$ of sodium alginate to be approximately 161,000 prior to the cobalt-60 irradiation and approximately 47,900 afterward. Donnan and Rose found the degree of polymerization (D.P.) to be related to the intrinsic viscosity by the following expression:

$$\frac{\text{D.P.}}{[\eta]} = 58 \qquad \text{(Eq. 10)}$$

By using Eq. 10 and an equivalent weight of 216, the D.P. and $\bar{M}_v$ of the nonirradiated alginate are found to be 742 and 160,000, respectively; the values for the irradiated material are calculated to be 222 and 47,900, which correlate well. The cobalt-60 sterilization

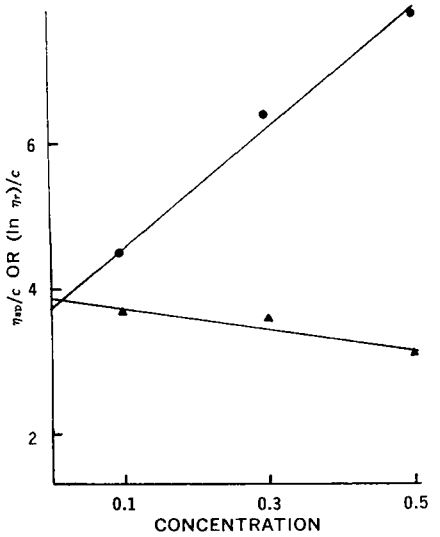

Figure 4—*Viscosity data for cobalt-60 irradiated sodium alginate. Key:* ●, η_{sp}/c; *and* ▲, $(\ln \eta_r)/c$.

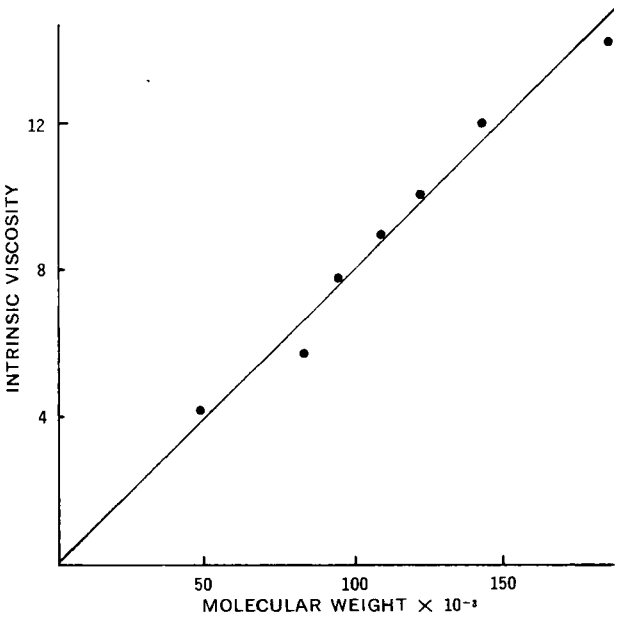

Figure 5—*Molecular weight and intrinsic viscosity relationship from the data of Donnan and Rose (10).*

Table III—Viscosities of Sodium Alginate in 0.1 *N* Sodium Chloride Solution at 25°[a]

Concentration, g/100 ml	η_r	η_{sp}	η_{sp}/c, g/100 ml	$(\ln \eta_r)/c$, g/100 ml
		Nonirradiated		
0.1	2.62 ± 0.01	1.62 ± 0.01	16.2 ± 0.11	9.62 ± 0.04
0.3	7.23 ± 0.08	6.23 ± 0.08	20.8 ± 0.27	6.59 ± 0.07
0.5	16.8 ± 0.11	15.8 ± 0.11	31.7 ± 0.21	5.65 ± 0.02
		Cobalt-60 Irradiated		
0.1	1.45 ± 0.02	0.449 ± 0.016	4.49 ± 0.16	3.71 ± 0.11
0.3	2.92 ± 0.05	1.92 ± 0.05	6.39 ± 0.16	3.57 ± 0.03
0.5	4.89 ± 0.07	3.89 ± 0.07	7.78 ± 0.15	3.17 ± 0.03

[a] Mean and relative standard deviation of three determinations.

treatment apparently has reduced the polymer to an average of 30% of its former degree of polymerization. The use of 216 for the equivalent weight takes into account the fact that sodium alginate exists in a hydrated form in which one molecule of water is associated with each mannuronide or guluronide unit.

The use of cobalt-60, 2.5 Mrad of γ-radiation applied over a 10-hr period, is not a suitable sterilization method for either of these vegetable gums. The apparent $[\eta]$ of acacia is reduced 16% and that of sodium alginate 70%. These viscosities represent a similar reduction in molecular weight; thus, the irradiated sodium alginate has the apparent $[\eta]$ of a fraction having a molecular weight 30% of the original molecule. Since the intent of this investigation was to determine the suitability of the use of cobalt-60 as a sterilization method, no study was made to ascertain what specific changes in the molecules actually occurred, such as depolymerization or changes in the carboxylic groups.

The reduction in apparent intrinsic viscosity for sodium alginate is over fourfold that occurring with acacia. Since each uronide unit in the alginate structure is accompanied by one molecule of water, it is possible that these water molecules actively participate in the greater depolymerization of sodium alginate. An alternative to depolymerization or extensive cleavage of the polymer is a change in the carboxylic groups present on the chain; these groups are more numerous in the alginate molecule, having an equivalent weight of 216, than in acacia, having an equivalent weight on the order of 1000–1200 (13). While the latter is not proposed as the mechanism for the greater reduction in viscosity of sodium alginate than of acacia as a result of the cobalt-60 sterilization process, it appears more plausible than chain cleavage by the water molecules.

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Development and Evaluation of Method for Simultaneous Determination of Phenobarbital and Diphenylhydantoin in Plasma by High-Pressure Liquid Chromatography

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Abstract □ A procedure for the simultaneous analysis of diphenylhydantoin and phenobarbital in plasma by high-pressure liquid chromatography was developed and evaluated. Separation is effected on a porous particle silicic acid column with chloroform–dioxane–isopropanol–acetic acid (310:9.7:1.0:0.1 by volume) and is monitored at 254 nm. Results of the procedure were compared with results of a GLC assay.

Keyphrases □ Phenobarbital–diphenylhydantoin—simultaneous high-pressure liquid chromatographic determination, plasma □ Diphenylhydantoin–phenobarbital—simultaneous high-pressure liquid chromatographic determination, plasma □ High-pressure liquid chromatography—simultaneous determination of phenobarbital and diphenylhydantoin in plasma, development and evaluation of method

The simultaneous determination of phenobarbital and diphenylhydantoin in plasma is of interest to pharmaceutical analysts because of the established efficacy of dosage manipulation based on their plasma concentration and the frequency with which they are concurrently administered as anticonvulsants.

Several techniques are available for this determination on a routine basis (1, 2), but the most widely accepted techniques involve GLC. The drugs may be subjected to direct GLC analysis, but several problems are often encountered. The extraction process may be involved (3), retention times of the drugs are usually long (4), and the drugs may reversibly or irreversibly adsorb to the solid support, causing peak tailing (5) or nonlinear calibration curves (6).

To overcome the disadvantages of direct GLC analysis, volatile derivatives of the drugs have been formed and determined. While initial procedures for the formation of anticonvulsant derivatives were complex (7), on-column methylation (8) overcame this objection. However, several uncertainties have arisen regarding the reproducibility of this technique. Column packing material is rapidly degraded by the methylating reagent (9). The recognized instability of phenobarbital in alkaline media results in degradation [and peaks called "early phenobarbital" (10)], the rate of which is erratic (11). Interpretation of a chromatogram of these degradation products is probably premature (11, 12). Pippenger (13), in reporting a retrospective study involving 5000 anticonvulsant samples, noted these and other uncertainties in anticonvulsant analysis by GLC.

High-pressure liquid chromatography (HPLC) has intrinsic advantages over GLC in this regard. The most obvious is that the volatility of the analyte is not a prerequisite to analysis, and problems related to heat instability or volatile derivative formation are thereby avoided. Also, the detection mechanism in HPLC affords possibilities for increased selectivity and thus a more direct extraction procedure. For example, an HPLC system may detect only molecules with appreciable absorption at 254 nm, whereas GLC with flame ionization forms an almost universal detection system (14).

Based on these considerations, a method for the simultaneous determination of phenobarbital and diphenylhydantoin in plasma by HPLC was developed and evaluated. The method was applied to the analysis of patient plasma, and results were compared with results of a GLC method. While these studies were in progress, a procedure for analysis of the two drugs by HPLC was reported (15). However, extraction and sample preparation were involved, no provision was made for use of an internal standard, and no data were presented relating results of phenobarbital analysis by HPLC to results obtained by other procedures.

EXPERIMENTAL

Apparatus—A liquid chromatograph[1] equipped with a 254-nm detector was used. The column was 30 cm × 4 mm (i.d.) stainless steel packed with 10-μm porous particle silicic acid[2]. The gas chromatograph[3] was equipped as previously noted (16).

Materials—Phenobarbital and diphenylhydantoin were obtained as USP reference standards. The remaining drugs, drug relatives, and metabolites were obtained from commercial suppliers and used without further treatment. TLC plates[4] were 5 × 20-cm glass, precoated with silica gel (0.25 mm) and fluorescent indicator.

Chromatographic Solvent Selection—*TLC*—TLC plates were spotted with 5 μl each of diphenylhydantoin and phenobarbital solutions (5 mg/ml) in methanol. Plates were developed in an ascending manner in a chamber previously equilibrated with the developing solvent. Developed plates were visualized with UV light (shortwave), and R_f values were determined.

HPLC—Solvent mixtures to be evaluated in the high-pressure system were pumped through the column for 6 hr (1.0 ml/min) for equilibration. Solutions of diphenylhydantoin and phenobarbital (10 μg/ml, 1.0 ml) in methanol were evaporated to dryness, reconstituted with the chromatographic solvent (50 μl), and injected into the chromatographic system. The flow rate was adjusted so that both peaks emerged in less than 10 min. Resolution was calculated by the formula $R = 2d/(w_1 + w_2)$, where d is the distance between peak centers, and w_1 and w_2 are the widths of each peak.

Chromatographic Conditions—The solvent system used for HPLC analysis was composed of chloroform–dioxane–isopropanol–acetic acid (310:9.7:1.0:0.1 by volume). The flow rate was 1.5 ml/min, and the pressure required to maintain this flow rate was approximately 70 kg/cm² (1000 psi). GLC operating conditions were as previously noted (16).

Preparation of Calibration Curves—Solutions (2.0 ml) of

[1] ALC 202, Waters Associates, Milford, Mass.
[2] μPorasil, Waters Associates, Milford, Mass.
[3] GC-4BP, American Instrument Co., Silver Spring, Md.
[4] Analabs, North Haven, Conn.

Table I—R_f Values of Phenobarbital and Diphenylhydantoin on Silicic Acid Gel TLC

Solvent Mixture	Solvent Composition (Parts by Volume)	R_f Values: Phenobarbital	R_f Values: Diphenylhydantoin	ΔR_f
1	Chloroform–ether (85:15)	0.391	0.318	0.073
2	Chloroform–ether (60:40)	0.688	0.594	0.094
3	Chloroform–isopropanol (90:10)	0.947	0.947	0.000
4	Chloroform–ethanol (90:10)	0.856	0.856	0.000
5	Chloroform–methanol–water (75:25:1)	0.675	0.688	0.013
6	Chloroform–ether–acetonitrile (48:9:3)	0.461	0.384	0.077
7	Chloroform–cyclohexane–acetonitrile (20:50:25:5)	0.102	0.082	0.020
8	Chloroform–cyclohexane–ether–acetonitrile (20:50:25:5)	0.382	0.274	0.108
9	Chloroform (100)	0.106	0.088	0.018
10	Dioxane (100)	0.822	0.801	0.021
11	Chloroform–dioxane (50:50)	0.676	0.640	0.036
12	Chloroform–dioxane (97:3)	0.466	0.300	0.166
13	Dioxane–methanol–ammonium hydroxide (84:10:5)	0.635	0.700	0.065
14	Dioxane–*n*-butanol–ammonium hydroxide (9:9:2)	0.462	0.613	0.151
15	*n*-Butanol–cyclohexane (10:200)	0.338	0.423	0.085
16	*n*-Butanol–ether–ammonium hydroxide (10:90:10)	0.100	0.262	0.162
17	Isopropanol–cyclohexane–ammonium hydroxide (65:25:10)	0.350	0.514	0.164

phenobarbital and diphenylhydantoin in chloroform containing 25.0, 37.5, 50.0, 62.5, 87.5, and 137.5 μg/ml were mixed with an aliquot (2.0 ml) of the internal standard solution [5-(4-methylphenyl)-5-phenylhydantoin, 50.0 μg/ml], and the solution was evaporated to dryness in a stream of dry nitrogen. At the time of analysis, the residue was reconstituted with chloroform (0.5 ml) and an aliquot (1–3 μl) was injected into the chromatograph. Ratios of the peak height of the drug to that of the internal standard were determined at each concentration.

Extraction and Evaluation of Extraction Efficiency—Drug-free plasma was spiked with sufficient quantities of the two drugs to give concentrations of 5.0, 7.5, 10.0, 12.5, 17.5, and 27.5 μg/ml. Centrifuge tubes containing the internal standard (5.0 μg) were prepared by evaporating an aliquot of a chloroform solution of the internal standard. A portion of spiked plasma (0.5 ml) was added to the tube with pH 6.8 phosphate buffer (0.5 ml), distilled water (0.5 ml), and methylene dichloride (5.0 ml). This mixture was placed on a vortex-type mixer for 1 min and centrifuged. The organic layer was separated, dried with sodium sulfate, and evaporated to dryness in a stream of dry nitrogen. The residue was reconstituted in chloroform (50 μl), and an aliquot (1–3 μl) then was injected into the HPLC system.

Extraction efficiency was evaluated by comparing peak height ratios obtained after extraction of spiked plasma to those obtained from corresponding concentrations in the preparation of calibration curves.

Analysis of Phenobarbital and Diphenylhydantoin in Patient Plasma—An aliquot (0.5 ml) of plasma was added to a centrifuge tube containing the internal standard (5.0 μg). The plasma sample was treated as described, and a portion of the reconstituted extract was injected into the HPLC system. An aliquot of a standard solution equivalent to 10 μg/ml of plasma for each of the drugs was injected immediately afterward. The ratio of the peak height of the drug to that of the internal standard was determined, and concentration of the drugs in the unknown sample was determined by the single-point standard method (17).

RESULTS AND DISCUSSION

Initially, R_f values for the two drugs were determined (Table I) on silicic acid plates in numerous solvent mixtures to select mixtures likely to effect a HPLC separation. On the basis of these data, Solvent Mixtures 12, 14, 16, and 17 appeared to merit investigation. When the latter three mixtures were applied to the HPLC system, however, the peak area of phenobarbital in each was approximately five times that of diphenylhydantoin when equimolar quantities were injected. Each of these three mixtures contains ammonium hydroxide, and the increased peak area is likely related to the stronger 254-nm absorption of phenobarbital in alkaline media (18). The therapeutic plasma level for these two drugs as anticonvulsants is still the subject of some controversy (19), but it is generally thought to be in the range of 10 μg/ml for phenobarbital (20) and 10–15 μg/ml for diphenylhydantoin (21). Because of these similarities, the large peak area difference in these three solvents was objectionable. Further HPLC investigations were based on Solvent Mixture 12, containing a preponderance of chloroform with dioxane.

The chloroform–dioxane mixture was modified in an attempt to optimize the system for separation of the two drugs. The ratio of chloroform to dioxane was increased until a decrease in the resolution was noted (35:1). A small amount of isopropanol served to increase the resolution, as did acetic acid. The relative amounts of these solvents in the solvent mixture were changed until maximum resolution was noted. Results of these alterations as indicated by relative resolution factors are presented in Table II. Thus, a solvent mixture of chloroform–dioxane–isopropanol–acetic acid (310:9.7:1.0:0.1 by volume) was selected for the remaining HPLC studies.

In the next phase of the study, retention volumes in the system were determined and resolution factors were calculated (Table III) for some structurally related drugs and drug relatives as well as for the two principal metabolites, 5-(4-hydroxyphenyl)-5-phenylhydantoin (22) and 5-ethyl-5-(4-hydroxyphenyl)barbituric acid (23).

Table II—Effect of Solvent System Changes on Resolution of Phenobarbital and Diphenylhydantoin by HPLC

Solvent Mixture, ml: Chloroform / Dixoane	Resolution	Solvent Mixture, ml: Chloroform / Dioxane	:	Isopropanol / (Chloroform + Dioxane)	:	Acetic Acid / (Chloroform + Dioxane)	Resolution
4.50	1.32	32.00	:	1.00	:	1.00	2.16
5.67	1.37	32.00	:	1.00	:	0.10	2.56
7.33	1.99	32.00	:	0.10	:	0.10	2.93
9.00	2.26	32.00	:	0.10	:	0.05	2.99
10.11	2.34	32.00	:	0.05	:	0.05	2.84
13.28	2.60	32.00	:	0.10	:	0.02	3.19
19.00	2.74	32.00	:	0.10	:	0.01	3.37
32.00	2.93						
35.00	2.74						

Table III—Retention Volumes and Resolution Factors for Selected Drugs and Metabolites

Compound	R_v[a]	Resolution[b]
Glutethimide	3.37	—[c]
Phensuximide	3.45	—
Trimethadione	3.49	—
Mephobarbital	3.90	—
Hexobarbital	4.24	—
Mesantoin	4.74	—
Butalbital	5.11	2.38
Secobarbital	5.50	2.33
Cyclobarbital	5.74	1.97
Ethotoin	5.86	1.87
5-Allyl-5-(2-cyclopenten-1-yl)barbituric acid	5.94	1.75
Amobarbital	6.12	1.56
Pentobarbital	6.24	1.51
Butethal	6.31	1.43
Allobarbital	6.37	1.26
Vinbarbital	6.42	1.23
Butabarbital	6.75	1.02
Bromisovalum	7.74	0.95
Primidone	8.50	1.40
5-(4-Methylphenyl)-5-phenylhydantoin	10.00	1.27
Phenacemide	13.00	1.94
Phenylethylmalonamide	24.60	—
5-(4-Hydroxyphenyl)-5-ethylbarbituric acid	26.00	—
5-(4-Hydroxyphenyl)-5-phenylhydantoin	39.00	—

[a] Flow rate was 1.5 ml/min. [b] Resolution *versus* phenobarbital (R_v = 8.02 ml). [c] Resolution factors not given were greater than 2.5. [d] Resolution *versus* diphenylhydantoin (R_v = 11.3 ml).

Since a resolution factor of 1.0 gives less than 3% error (24), only bromisovalum would interfere with the chromatographic interpretation. However, this finding does not preclude interference by metabolites of the products in Table III or by other structurally unrelated drugs. In addition to serving as an indication of potential interference, these data allowed a rational selection of the internal standard.

Standard curves for the two drugs were prepared seven times over 1 month. For diphenylhydantoin, the curve had a mean slope of 0.1255 ml/μg (2.14% *SD*), a mean correlation coefficient of 0.998, and a mean *Y*-intercept of −0.043. For phenobarbital, the mean slope was 0.1255 ml/μg (3.19% *SD*), the mean correlation coefficient was 0.999, and the mean *Y*-intercept was −0.070. These data indicate that the procedure is amenable to use of a single-point standard. There was no change in these parameters or in the number of theoretical plates during this study, indicating the stability of the column packing material.

Several investigators (6, 25) noted problems with standard solutions caused by the instability of these drugs in aqueous media and in organic solution. While degradation is slow (except at alkaline pH), standard solutions of the drugs and internal standard must usually be prepared fresh. To avoid this problem, the internal standard solution was prepared in chloroform, and aliquots were added to a number of extraction tubes and evaporated to dryness. In this state, no degradation was noted during the study. In addition, tubes containing aliquots of the standard solutions were prepared in the same manner, stored in the dry state, and reconstituted at the time of analysis. An aliquot of the reconstituted solution was injected, the solution was evaporated to dryness, and the tube was retained for later use. Since no more than 0.6% of the solution (3 μl of a 0.5-ml solution) was injected each time, this process could be repeated a number of times before new standards were required.

Table IV—Recovery of Phenobarbital and Diphenylhydantoin from Plasma as Determined by HPLC and GLC

Drug	Micrograms Added	Mean Recovery ± *SD*, % HPLC	GLC
Phenobarbital	5.0	101.60 ± 2.17	95.64 ± 6.42
Diphenylhydantoin	5.0	95.64 ± 2.44	97.23 ± 4.76
Phenobarbital	7.5	98.14 ± 1.50	92.16 ± 4.70
Diphenylhydantoin	7.5	101.5 ± 2.04	97.84 ± 5.30
Phenobarbital	10.0	96.74 ± 2.21	94.76 ± 3.76
Diphenylhydantoin	10.0	100.19 ± 1.13	102.41 ± 4.20
Phenobarbital	12.5	100.86 ± 2.84	100.34 ± 5.60
Diphenylhydantoin	12.5	99.55 ± 3.42	93.77 ± 4.91
Phenobarbital	17.5	96.74 ± 2.65	90.42 ± 7.74
Diphenylhydantoin	17.5	99.60 ± 3.27	94.93 ± 6.42
Phenobarbital	27.5	92.64 ± 4.97	87.26 ± 9.43
Diphenylhydantoin	27.5	97.45 ± 3.76	92.14 ± 6.07

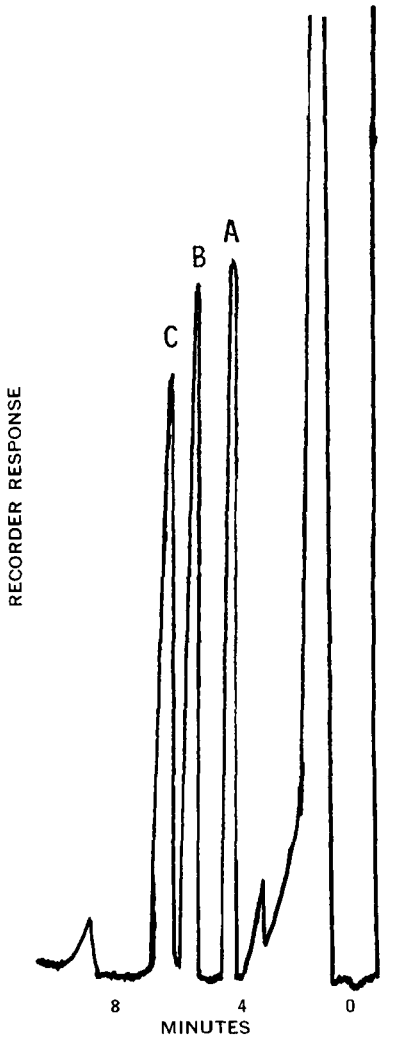

Figure 1—*Chromatographic trace of an extract of plasma found to contain 8.5 μg/ml of phenobarbital (A), 10.0 μg/ml of internal standard (B), and 9.2 μg/ml of diphenylhydantoin (C).*

Recovery of the two drugs from spiked plasma samples was determined (Table IV) at several concentrations by HPLC and by GLC. While mean results of the two procedures appear to be similar, the standard deviation of results obtained by GLC is larger. This increased spread of results may be attributed to the fact that the extraction process for GLC assay has several steps performed in an effort to clean up the chromatogram. However, determination is made in the HPLC assay by analysis of a concentrate of the initial chloroform extract since interpretation of the resulting chromatogram is sufficiently uncomplicated by extraneous peaks (Fig. 1). For both procedures, standard deviation increases at low and high concentrations of drug, reflecting the fact that precision is greatest in chromatographic methods when the drug and internal standard have the same peak area. In addition, precision is

Table V—Diphenylhydantoin and Phenobarbital Concentration in Plasma as Determined by HPLC and GLC

Sample	Phenobarbital, μg/ml		Diphenylhydantoin, μg/ml	
	HPLC	GLC	HPLC	GLC
1	21.4	22.5	20.3	20.1
2	14.9	15.5	11.0	11.2
3	24.0	23.4	5.0	5.3
4	11.8	12.3	17.9	17.8
5	24.5	26.0	6.2	6.0
6	7.9	7.8	2.3	2.4
7	9.3	9.7	4.8	4.8
8	15.2	14.8	8.9	9.3
9	27.3	27.7	7.9	8.1
10	7.0	7.2	23.6	24.0

greater for the analysis of diphenylhydantoin than for phenobarbital, since the internal standard is closely related to diphenylhydantoin and thus more effective in compensating for extraction and chromatographic irregularities.

Results of the analysis by GLC and HPLC of plasma of patients taking both drugs are presented in Table V. In light of the standard deviations of the results of the two procedures, the results are comparable. The HPLC procedure has since been used for determination of the two drugs in the plasma of more than 40 different patients, and no interferences have been apparent.

Several appealing features of this method may be noted. Sample preparation is rapid, no tedious pH adjustments are required, and extraction is quantitative. Internal standard and standard drug preparations are stable for long periods. The derived chromatogram is uncomplicated by extraneous peaks, and injections may be made every 7 min. Finally, the method requires only 0.5 ml of plasma, and analysis of smaller quantities of plasma should be possible by injecting larger volumes of the concentrated extract.

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Constituents of *Cannabis sativa* L. VIII: Possible Biological Application of a New Method to Separate Cannabidiol and Cannabichromene

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Abstract □ Synthetic and naturally occurring cannabidiol and cannabichromene were distinctly separated without derivation by GLC using a 6% OV-1 column; an artifact of cannabichromene, cannabicyclol, was separated from (−)-Δ^9-*trans*-tetrahydrocannabivarin. This procedure is versatile and applicable for the quantitation of *Cannabis* containing both cannabidiol and cannabichromene. Biological interaction among (−)-Δ^9-*trans*-tetrahydrocannabinol, cannabichromene, and other cannabinoids in natural *Cannabis* preparations can now be studied. In the phenyl methyl silicone polymer series, cannabidiol precedes cannabichromene on columns containing below a 50% phenyl-to-methyl ratio. Columns containing a 50:50 or greater ratio of phenyl to methyl reverse the separation order with cannabichromene preceding cannabidiol.

Keyphrases □ *Cannabis sativa* L.—constituents, method to separate cannabidiol and cannabichromene, GLC conditions discussed □ Cannabinoids—separation of cannabidiol and cannabichromene, GLC □ GLC—separation of cannabidiol and cannabichromene

Recently, numerous data supporting pharmacological interactions between cannabinoids have been published. Carlini *et al.* (1) suggested that cannabidiol (I) could possibly block some effects of (−)-Δ^9-*trans*-tetrahydrocannabinol (II). Karniol and Carlini (2) postulated that both I and cannabinol (III) may alter the action of II. More recently, Karniol and Carlini (3) reported that I blocked several effects of II in animal models and that I potentiated the analgesic effects of II. Borgen and Davis (4) found that I induced reduction of the hypothermic response to II in rats and rabbits and that I can attentuate other effects of II. Furthermore, Jones and Pertwee (5) reported that I pretreatment altered the metabolism of II. Therefore, quantitation of cannabinoids other than II must be accomplished to describe pharmacological parameters and to correlate these parameters with the cannabinoid(s) responsible for the activity.

This laboratory has recommended that each sample of *Cannabis sativa* L. be quantitated routinely for all possible cannabinoids so that pharmacologists may study the potentiation or antagonism of II activity by other cannabinoids (6, 7). To accomplish this task, much time and effort have been assigned to the development of techniques. Results have shown that when proper analytical techniques are used, quantitation of II in plant material is relatively simple (8, 9).

Cannabinol (III) can be quantitated, but care must be observed since a C_{29}-hydrocarbon has nearly the same relative retention time and can cause error in quantitating III (9). However, I is not easily quantitated in plant material.

Vree *et al.* (10), using GC–mass spectrometry, showed that a Brazilian sample of *Cannabis* contained no I, although a strong GC peak existed where I is normally found. The compound yielding the peak normally assigned to I was cannabichromene (IV), which supported de Faubert Maunder's (11) statement that I was definitely absent in some samples of *Cannabis*. This researcher used a TLC system. Moreover, Turner and Hadley (8) investigated a South African sample and found an absence of I but the presence of IV; this finding was confirmed by GC–mass spectrometry, TLC, and GC.

Although the OV-17 column[1] used in routine quantitation of cannabinoids has been reported as separating I and IV (12), this separation was based on a 5-sec time interval between peaks of pure I and IV and not a mixture of the two.

A clear and concise separation of synthetic I and IV, using a trimethylsilyl procedure, was reported (13); this procedure also afforded separation of I and IV in plant material (14). The silyl procedure in these laboratories provides excellent reproducibility in quantitating all cannabinoids when responsible analytical procedures are followed. However, when some silyl reaction mixtures are allowed to stand at ambient temperature for more than 1 hr, reproducibility

Table I—GC Columns and Conditions for the Separation of a Mixture of Cannabichromene and Cannabidiol[a]

Liquid Phase	Percent	Oven Temperature	Separation		
			No	Yes[b]	Yes[c]
OV-1	3	180°		x	
OV-1	3	200°	x		
OV-1	6	180°			x
OV-1	6	200°	x		
OV-1	8	180°			x
OV-1	8	190°		x	
OV-3	3	180°			x
OV-7	3	180°		x	
OV-7	3	200°	x		
OV-7	6	180°		x	
OV-11	3	200°	x		
OV-17	2	180°	x		
OV-17	2	210°	x		
OV-17	3	180°	x		
OV-17	3	210°	x		
OV-17	5	250°[d]	x		
OV-17	5	210°	x		
OV-25	2	210°	x		
OV-25	2	210°	x		
QF-1	3	210°	x		

[a] The mixture can be natural and/or synthetic. [b] Quantitation is possible using a GC–computer system. [c] Quantitative without computer. [d] Using 80–100 mesh and 4-mm i.d., 1.8-m (6-ft) column.

[1] High purity polar methyl silicone; approximately 30,000 mol. wt. used by National Institute on Drug Abuse for analyses of *Cannabis*.

Table II—Mass Spectra Data for Cannabichromene and Cannabidiol[a]

Compound	m/e														
I	314	299	286	271	—	260	259	258	—	246	—	232	231	230	229
IV	314	299	—	271	267	—	—	258	257	—	243	232	231	—	—

[a] Data obtained from combined GC–mass spectrometry as described under *Experimental*. Data were obtained at 70 ev.

diminishes. Also, silylation requires additional time and response factors. Therefore, the purpose of this present investigation was twofold: (*a*) to develop a method for the separation of I and IV without necessitating derivative formation, and (*b*) to eliminate the requirement for additional response factors.

EXPERIMENTAL

Analyses were performed using a gas chromatograph[2] equipped with hydrogen flame-ionization detectors and operated isothermally at 180°. The detector and inlet temperatures were 260 and 240°, respectively. Glass columns, 0.63 cm (0.25 in) o.d. and 2 mm i.d. × 2.43 m (8 ft), were packed with 6% OV-1 on 100–120-mesh Gas Chrom Q. Nitrogen was used as the carrier gas at a flow rate between 10 and 30 ml/min, depending upon instrument and column requirements.

The *Cannabis* samples used were from known seed stock[3] and prepared according to the modified Lerner extraction method (8). The synthetic standard solution was prepared by dissolving 5 mg of each standard [cannabichromene, cannabidiol, and androst-4-ene-3,17-dione (V)] in 1.5 ml of absolute ethanol. The resulting standard solution (1:1:1) was ultrasonically shaken for 30 sec, after which time 0.2–0.4 μl was injected. In these laboratories, quantitation reliability diminishes due to detector overload when any volume over 1 μl is injected. For additional analytical data on the analysis of *Cannabis*, see Refs. 6, 7, 9, and 14. Response factors were calculated using synthetic cannabinoids[4] and naturally occurring cannabinoids isolated in these laboratories.

RESULTS AND DISCUSSION

Gaoni and Mechoulam (12) reported pure I and IV to have retention times of 5 min 40 sec and 5 min 35 sec, respectively, using 2% OV-17 at 253°. This 5-sec time differentiation is insufficient for quantitating a mixture of I and IV. Additionally, de Zeeuw *et al.* (15) reported the separation of I and IV in a *Cannabis* sample using 5% OV-17, 5% OV-25, 3% QF-1, and 5% SE-30. The best separation was obtained on the 5% OV-17 column. Retention times reported, with the exception of SE-30, indicated that IV preceded I (15). However, these chromatograms from a 5-μl injection of a hex-

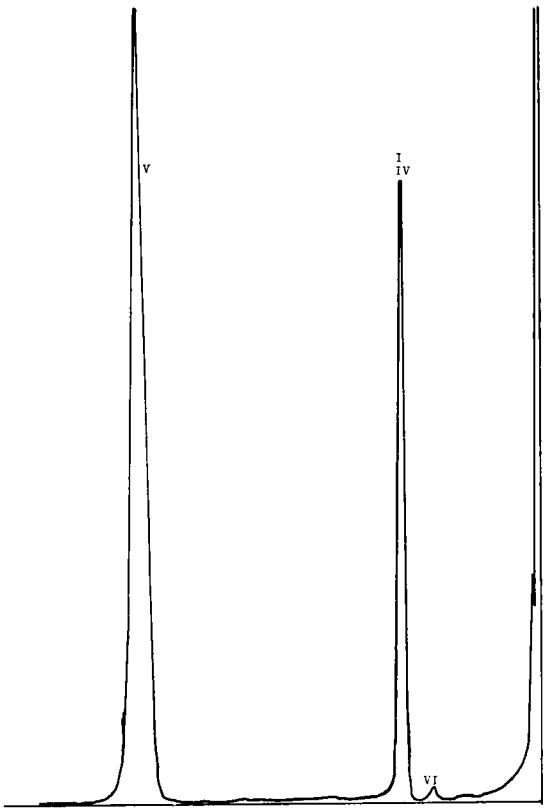

Figure 1—*Chromatogram showing the nonseparation of synthetic cannabidiol and cannabichromene on 2% OV-17. Key: VI, cannabicyclol; I, cannabidiol; IV, cannabichromene; and V, androst-4-ene-3,17-dione, the internal standard.*

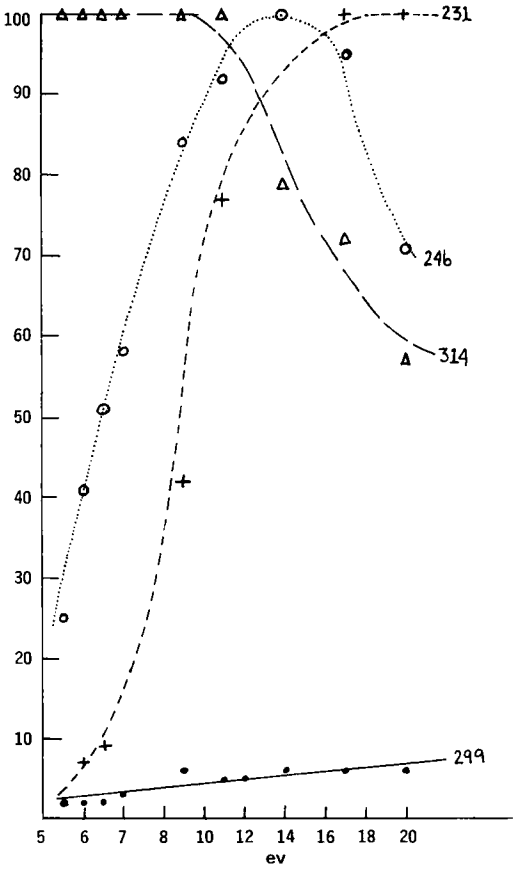

Figure 2—*Electron voltage–mass fragment intensity graph of cannabidiol. The point of intercept for mass fragments 231 and 314 is approximately 12 ev; in the system of Vree* et al. *(10), the intercept is approximately 13 ev. Although mass fragment intercepts are not identical, the patterns are identical.*

[2] Beckman GC-45, GC-65, interfaced to digital PDP-8 computer for data retrieval and determination of response factors.

[3] Grown at the University of Mississippi. *Cannabis* herbarium specimens are stored in the Herbarium, Department of Pharmacognosy, School of Pharmacy, University of Mississippi, University, MS 38677

[4] Obtained through the National Institute on Drug Abuse.

Table III—Relative Retention Times of Underivatized Cannabinoids and Other Components Found in *Cannabis*

Olivetol	0.04
Cannabidivarin (CBDV; VIII)	0.18
Tetrahydrocannabivarin (THC; VII)	0.26
Cannabicyclol (CBL; VI)	0.26
Cannabichromene (CBC; IV)	0.34
Cannabivarin (CBV; IX)	0.34
Cannabidiol (CBD; I)	0.34
Hexahydrocannabinol (HHC; X)	0.37
Cannabigerol monomethyl ether (CBGM; XI)	0.38
$\Delta^{9,11}$-Tetrahydrocannabinol (ETHC; XII)	0.41
Δ^{8}-Tetrahydrocannabinol (Δ^{8}-THC; XIII)	0.44
Cannabielsoin (CBE; XIV)	0.48
Δ^{9}-Tetrahydrocannabinol (Δ^{9}-THC; II)	0.49
Cannabigerol (CBG; XV)	0.57
Cannabinol (CBN; III)	0.63
C_{29}-Hydrocarbon (XVI)	0.67
Androst-4-ene-3,17-dione (Δ^{4}-dione; V)	1.00

ane extract of *Cannabis* contained only a trace amount of I and IV. Thus, it was difficult to ascertain if the separation reported allows for quantitation.

Duplication of the reported experimental conditions in these laboratories using synthetic cannabinoids did not separate a mixture of I and IV; however, cannabicyclol (VI), an artifact from IV, was separated and did procede the single peak observed for the mixture of IV and I. De Zeeuw *et al.* (15) did not mention the presence of VI, which is routinely observed when synthetic or naturally occurring IV is subjected to GC analyses (9). Therefore, the relative retention times reported by de Zeeuw *et al.* are indicative that VI is mislabeled and misidentified as IV.

Using another support phase, 3% OV-7 at 210°, Small and Beckstead (16) reported the separation of I and IV. Although no retention times were reported, a representative chromatogram showed

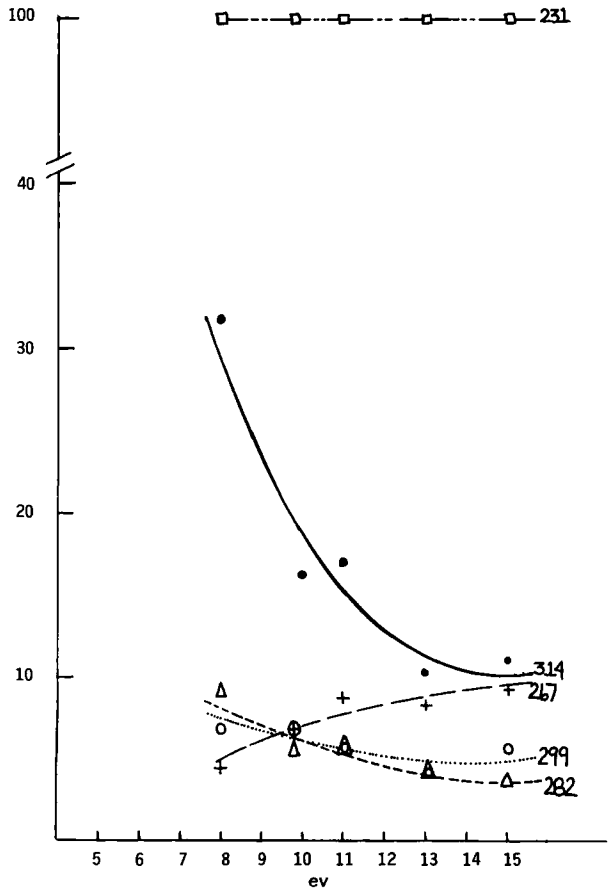

Figure 3—*Electron voltage–mass fragment intensity graph of cannabichromene.*

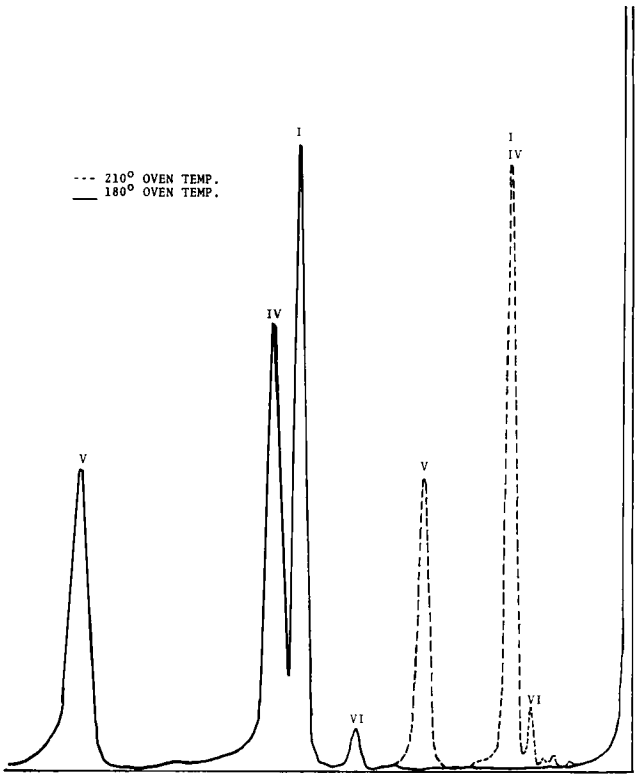

Figure 4—*Overlay of cannabidiol and cannabichromene on 6% OV-1 at oven temperatures of 210° (dotted line) and 180° (solid line). Key: VI, cannabicyclol; I, cannabidiol; IV, cannabichromene; and V, androst-4-ene-3,17-dione, the internal standard.*

IV preceding I. Investigation of the 3% OV-7 as a potential replacement for the 2% OV-17 column, which does not separate a mixture of IV and I, indicates that 3% OV-7 does not adequately separate I and IV for routine analyses.

When pure synthetic standards were used, IV did, indeed, precede I on OV-17 and OV-25 columns by seconds. However, these separations were not sufficient to provide separation of a synthetic mixture of I and IV (Fig. 1) in equal or unequal ratios. This finding was confirmed using GC–mass spectrometry[5] and, subsequently, "mass-fragmentograms" as described by Vree *et al.* (10) (Figs. 2 and 3). Additionally, no separation was obtained when chromatograms were produced at different temperatures (Table I).

Cannabidiol has been reported as preceding IV with a 5% SE-30 column (15), a methyl silicone as is OV-1. In the phenyl methyl silicone polymer series in these laboratories, I preceded IV when the percent phenyl-to-methyl ratio of the polymer was below 50% (*e.g.*, OV-1, 3, 7, and 11). However, VI precedes both I and IV (Fig. 4). This separation is based on pure synthetic samples. Mixtures were not separated if they are not reported in Table I. Thus, when the phenyl-to-methyl ratio was below 50%, the data from this study support de Zeeuw *et al.* (15) but are in direct conflict with data published by Small and Beckstead (16). When the phenyl-to-methyl ratio was 50:50 or greater, the separation of pure I and IV was reversed with IV preceding I. Cannabicyclol (VI) also precedes IV and I and is mislabeled IV in some publications (15–17)[6]. The OV-17 polymer is 50:50 phenyl to methyl, whereas OV-25 is composed of a higher percent phenyl-to-methyl ratio. As previously stated, this separation was based on pure synthetic samples, with no clear separation being obtained when a mixture of I and IV was analyzed (see Table III for relative retention times on OV-17).

Methyl silicone (6% OV-1) in these laboratories affords quanti-

[5] Varian Series 1400 interfaced to DuPont 21-492 high resolution interfaced with digital PDP-12.

[6] Since this manuscript was prepared, personal communications from Dr. Harry Beckstead revealed that Dr. Beckstead is aware of this problem. He agrees with our results: cannabicyclol is usually misidentified and labeled cannabichromene.

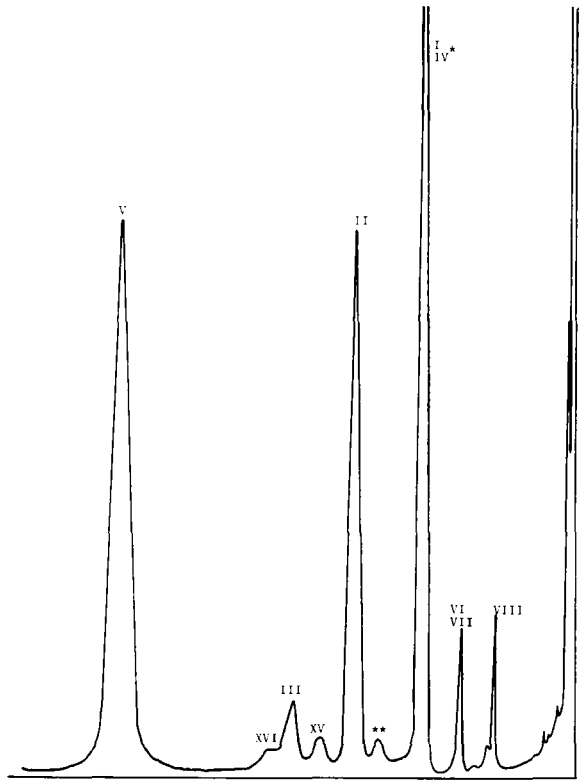

Figure 5—*Chromatogram of Indian* C. sativa *L., coded IN-B(2)/C-72, run on 2% OV-17. Key: VIII, cannabidivarin; VII,* (−)-Δ^9-trans-*tetrahydrocannabivarin; VI, cannabicyclol; I, cannabidiol; IV, cannabichromene; II,* (−)-Δ^9-trans-*tetrahydrocannabinol; XV, cannabigerol; III, cannabinol; XVI, C_{29}-hydrocarbon; and V, androst-4-ene-3,17-dione, the internal standard. *In certain samples, cannabivarin (IX) occurs under this peak. **This peak is normally labeled* (−)-Δ^8-trans-*tetrahydrocannabinol (XIII), but spectral data in these laboratories indicate that this particular peak is not XIII.*

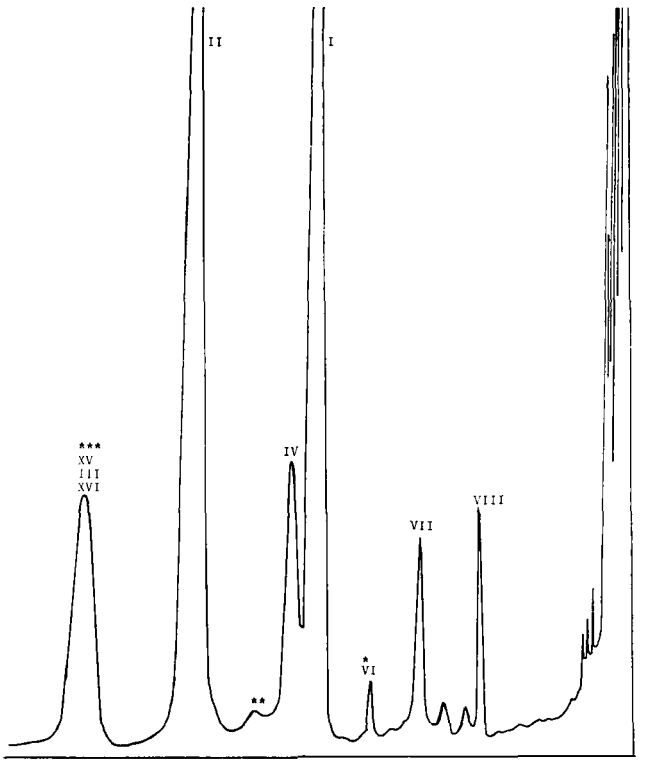

Figure 6—*Chromatogram of Indian* C. sativa *L., coded IN-B(2)/C-72, run on 6% OV-1. Key: VIII, cannabidivarin; VII,* (−)-Δ^9-trans-*tetrahydrocannabivarin; VI, cannabicyclol; I, cannabidiol; IV, cannabichromene; II,* (−)-Δ^9-trans-*tetrahydrocannabinol; XV, cannabigerol; III, cannabinol; and XVI, C_{29}-hydrocarbon. *When cannabivarin (IX) is present in the sample, it is under this peak. **Data indicate that this peak is probably the peak in question in Fig. 5 and is currently being investigated. ***Internal standard appears under this peak when present. This chromatogram is of a normalization analysis to determine the ratio of IV to I.*

tative separation of I and IV (Table II and Figs. 2 and 3). Figure 4 shows the separation of synthetic I and IV obtained on the 6% methyl silicone column at 180°. Preceding I is a small peak representing VI. Cannabicyclol (VI), an artifact, is routinely observed in chromatograms when IV is present (9).

Temperature was most critical in the separation of I and IV. When methyl silicone is used, 180° gives excellent separation; at 190°, the separation is less distinct; and above 200°, no separation occurs (Table I and Fig. 4). Synthetic I on methyl silicone has a retention time of 19 min 30 sec, and synthetic IV elutes at 21 min 15 sec. This separation is more than sufficient for quantitating I and IV. The separation and quantitation of (−)-Δ^9-*trans*-tetrahydrocannabivarin (VII) and VI are possible. When using a 2% OV-17 column, VI and VII occur under the same peak (Fig. 5 and Table III). Thus, for the separation and quantitation of I and IV and VI and VII without preparing derivatives (14), the methyl silicone is superior to those columns investigated in this experiment (Table I and Fig. 6). Eight percent methyl silicone and 3% OV-3 will separate I and IV, as does methyl silicone, but extended retention times diminish the separation advantages.

Fentiman[7], using 10% OV-101 at 190°, obtained a separation comparable to the separation on 6% OV-1, with cannabidiol and cannabichromene giving retention times of 1 hr 42 min and 1 hr 50 min, respectively.

Thus, in view of these findings, it appears that many reported procedures (12, 15–17) have not separated I and IV but have mistakenly identified VI and IV. Also, these findings and others (18) make the validity of a classification system for *Cannabis* using I, II, or I–III questionable. However, it is now possible to quantitate I and IV in any preparation of *Cannabis* without preparing derivatives. Additionally, in these laboratories, response factors from OV-1 and OV-17 columns are within experimental error.

It is the recommendation of this research group that routine *Cannabis* preparations analyzed on 2% OV-17 be supported by an analysis using methyl silicone, thereby enabling pharmacologists to extend and validate their findings on the potentiation and/or antagonism of II by other cannabinoids. Moreover, a recent recommendation by an United Nations working group on the chemistry of *Cannabis* and its components[8] that accurate analyses of I–III be included in all scholarly reports on *Cannabis* can become a reality. The biological significance and implications of these findings are far reaching but cannot be adequately documented at this time; however, preliminary data obtained from the methyl silicone column indicate that IV may be more abundant in nature than I. Since IV has been reported previously as I, alteration of the classical biological activity of II attributed to I may have been due to IV or to a combination of I and IV or of other cannabinoids in experiments where natural plant material was used.

SUMMARY

Cannabidiol and cannabichromene were separated and quantitated in *C. sativa* L. Most previous literature reporting the separation of cannabidiol and cannabichromene have mislabeled cannabicyclol, an artifact from cannabichromene, as cannabichromene. Cannabicyclol and (−)-Δ^9-*trans*-tetrahydrocannabivarin can be separated and quantitated on OV-1 but cannot be separated and quantitated on OV-17. Cannabichromene, often erroneously re-

[7] Dr. Al Fentiman, Battelle, Columbus, Ohio, confirmed results from these laboratories.

[8] United Nations Document MNAR/9/1974.

ported as cannabidiol, is more abundant in nature than previously thought; any previous classification of *C. sativa* L. based on cannabidiol, cannabinol, and (−)-Δ^9-*trans*-tetrahydrocannabinol must be questioned. Since it is known that synthetic cannabidiol antagonizes certain effects of (−)-Δ^9-*trans*-tetrahydrocannabinol, much work is now needed on variants of *Cannabis* containing various ratios of cannabidiol and cannabichromene in relation to (−)-Δ^9-*trans*-tetrahydrocannabinol.

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Electron-Capture GLC Determination of a New Antiarrhythmic Agent, α,α-Dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine, in Biological Fluids

ANTHONY G. ZACCHEI [x] and LINDA WEIDNER

Abstract □ A highly specific and sensitive GLC method was developed for the analysis of α,α-dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine, a new orally active antiarrhythmic drug, in biological fluids. The procedure involves the addition of an internal standard, 4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine, to the plasma or urine samples followed by extraction of the drugs into benzene at pH 8. The extracted amines are converted to the trifluoroacetyl derivatives (characterized by GLC–mass spectrometry), chromatographed, and detected with a ^{63}Ni electron-capture detector. The sensitivity of the method is such that 10 ng of α,α-dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine/ml of plasma can be analyzed. These levels are suitable for the analysis of samples obtained following a therapeutic dose.

Keyphrases □ α,α-Dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine—electron-capture GLC determination in biological fluids □ Antiarrhythmic agents—electron-capture GLC determination of α,α-dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine in biological fluids □ GLC, electron capture—analysis, α,α-dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine in biological fluids

α,α-Dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine (I) is a new orally effective agent for the treatment of ventricular arrhythmias resulting from myocardial infarction (1). The compound is one of the more potent agents in preventing or modifying the ventricular arrhythmia produced in anesthetized dogs by the intracoronary injection of a sclerosing agent (2, 3). The drug was designed as a prophylactic agent and has advantages over other drugs currently employed. Studies on the physiological disposition of the compound (4) have not included a specific method of assay.

Preliminary quantitative experiments in this laboratory using colorimetric, fluorometric, and GLC (flame-ionization detection) methods lacked the sensitivity required to detect clinical levels of the drug. The use of electron-capture techniques for the assay of low levels of drugs, has been reported (5–15). How-

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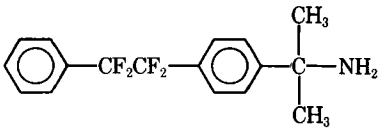
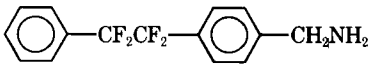


I



II

ever, many drugs, such as I, are not responsive to the electron-capture detector without prior derivatization to a compound that has electron-capturing properties. This report describes an extremely sensitive and specific GLC method for the determination of I using an internal standard (II), derivatization with trifluoroacetic anhydride, and an electron-capture detector.

EXPERIMENTAL

Reagents and Chemicals—The chemicals and reagents used were: α,α-dimethyl-4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine (I), the internal standard 4-($\alpha,\alpha,\beta,\beta$-tetrafluorophenethyl)benzylamine (II), pesticide grade benzene, trifluoroacetic anhydride[1], 5% ammonium hydroxide, and benzene containing 2.5% trifluoroacetic anhydride (freshly prepared).

Instrumentation—*GLC*—A gas chromatograph[2] equipped with a ^{63}Ni electron-capture detector and a 183 × 6-mm glass column containing 2% PPE-20 (polyphenyl ether) on 80–100-mesh Chromosorb W[3] (AW-DMCS) was employed for the analysis. The inlet and detector temperatures were 230° and the column temperature was 200°. Nitrogen was used as the carrier gas at a flow rate of 50 ml/min.

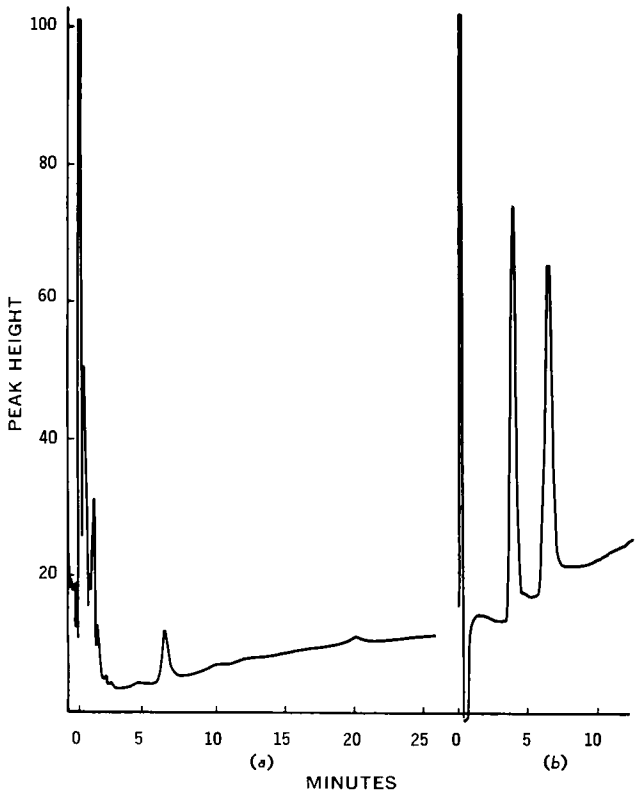


Figure 1—*Gas chromatograms of:* (a) *control dog plasma carried through extraction procedure, and* (b) *I and II as the trifluoroacetyl derivatives (500 pg of each compound). Five microliters was injected out of a total volume of 100 μl of benzene.*

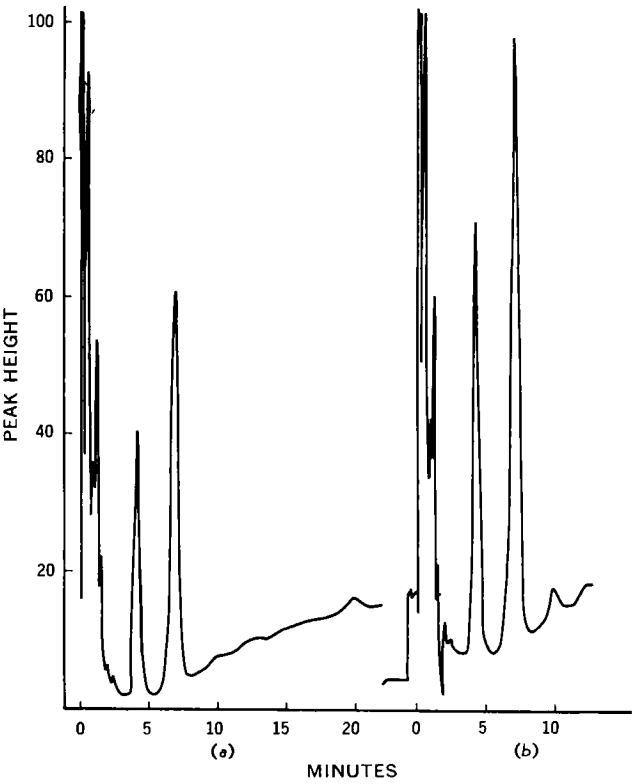


Figure 2—*Gas chromatograms of:* (a) *I and II added to control dog urine and carried through the extraction procedure, and* (b) *material obtained from a 0–24-hr dog urine sample following the administration of I. Compound II was added prior to extraction. Five microliters was injected in each case out of 100–500 μl of solvent.*

GLC–Mass Spectrometry—All mass spectra were obtained on a mass spectrometer[4] using the GLC inlet. A 183 × 5-mm glass column packed with 1% OV-17 on 80–100-mesh Supelcoport[3] and a 183 × 5-mm glass column packed with 2% PPE-20 were used. The gas chromatograph was operated isothermally at 190° with a helium flow rate of 30 ml/min. The mass spectrometer ionizing and accelerating potentials were 70 ev and 3.5 kv, respectively. The source, separator, and injection port temperatures were 270, 260, and 255°, respectively.

Measurement of I in Biological Samples—Compound I was determined in biological samples as follows. One milliliter of plasma or urine was added to a 50-ml glass-stoppered centrifuge tube containing 50 ng (calculated as the free base) of the internal standard as the hydrochloride in 1 ml of water, 1 ml of 0.2 *M* pH 8 phosphate buffer, and 25 ml of reagent grade benzene. The tube was shaken for 10 min and centrifuged, and at least 20 ml of the organic phase was transferred to a similar tube containing 5 ml of 0.1 *N* HCl. After shaking for 5 min, the tube was centrifuged and the organic phase was removed by aspiration. The pH of the aqueous phase was adjusted to greater than 8 by the addition of 0.5 ml of 1.25 *N* NaOH and 1 ml of 0.2 *M* pH 8 phosphate buffer.

The amines were extracted into 10 ml of pesticide-grade benzene. The benzene phase was transferred to a 25-ml glass-stoppered tube containing 1 ml of benzene (2.5% trifluoroacetic anhydride). The tube was then placed in a 65° warm water bath for 15 min. The tube was cooled and the contents were shaken with 1 ml of water (vortex, 1 min) and 1 ml of 5% NH_4OH. Following centrifugation, the benzene phase was transferred to a 13-ml tube for careful evaporation under nitrogen to about 100–500 μl (evaporation of the samples to dryness resulted in greater than 50% loss of detection response). Appropriate aliquots, usually 2–5 μl, were in-

[1] Aldrich Chemical Co.
[2] Packard model 7400.
[3] Supelco, Inc.
[4] LKB-9000S.

Table I—Recovery of I from Dog Plasma and Water

Amount Added, ng		Amount Recovered from Water[a]	Amount Recovered from Plasma[a]						Mean ± *SD* of Plasma Recovery
100	A	87.0	93.8	99.4	—[b]	105.6	99.4	109.2	101.5 ± 6.0
	B	87	94	99	—	106	99	109	
80	A	72.9	78.3	76.1	81.6	91.2	81.6	79.6	81.4 ± 5.2
	B	91	98	95	102	114	102	99	
60	A	60.1	59.4	66.5	70.6	77.5	68.6	—	68.5 ± 6.6
	B	100	99	111	118	129	114	—	
50	A	50.3	47.3	56.2	59.0	43.9	51.4	47.2	50.8 ± 5.8
	B	101	95	112	118	88	103	94	
30	A	30.6	32.9	35.7	35.0	37.7	35.0	—	35.3 ± 1.7
	B	102	110	119	117	125	117	—	
25	A	28.1	25	28.8	21.9	24.7	25.4	25.4	25.2 ± 0.9
	B	112	100	115	88	99	102	102	
15	A	16.6	16.6	13.7	18.5	15.1	15.8	11.9	15.3 ± 2.3
	B	111	111	91	123	101	105	79	
10	A	11.6	10.6	9.6	11.7	9.6	10.3	7.8	9.9 ± 1.3
	B	116	106	96	117	96	103	78	
5	A	10.1	3.7	3.4	5.5	5.5	4.8	3.5	4.4 ± 1.0
	B	202	74	69	110	110	96	70	

[a] Values in the A rows represent nanograms recovered; values in the B rows represent percent recovery. [b] No sample analyzed.

jected into the column. The retention times of I and II as the trifluoroacetyl derivatives were 4.0 and 6.6 min, respectively.

Standard water, urine, and plasma curves were constructed by plotting the peak height ratios (I/II) *versus* the weight ratios (I/II). The peak height ratio obtained from an unknown was then used to determine the amount of I present. The standard samples were run concurrently with the unknown samples as previously described.

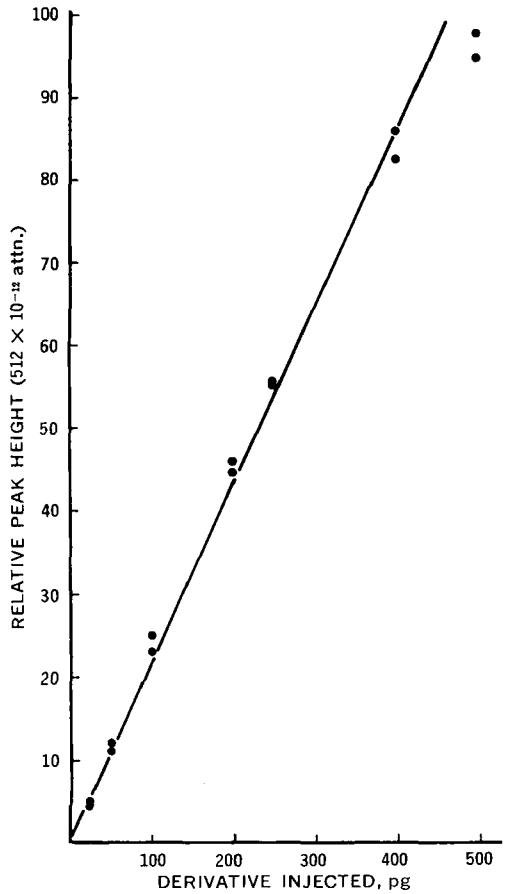


Figure 3—*Relationship between peak height and amount injected on a 2% PPE-20 column operated at 200°. Nitrogen flow rate was 50 ml/min. Five microliters was injected in duplicate in each instance.*

Absorption Studies—Four beagle dogs (7.0–10.3 kg) were administered a therapeutic dose of I (5 mg/kg) orally in a water solution. Blood samples were collected in heparinized tubes, the plasma was separated, and aliquots were removed for assay. Urine specimens were immediately frozen upon collection and remained frozen until assayed.

RESULTS AND DISCUSSION

A number of reagents (trifluoroacetic anhydride, heptafluorobutyric anhydride, *N*-trifluoroacetylimidazole, and pentafluoropropionic anhydride) were examined as potential derivatizing agents to act as electron-capturing groups to facilitate compound detection. Exploratory studies with I and three related analogs, 4-

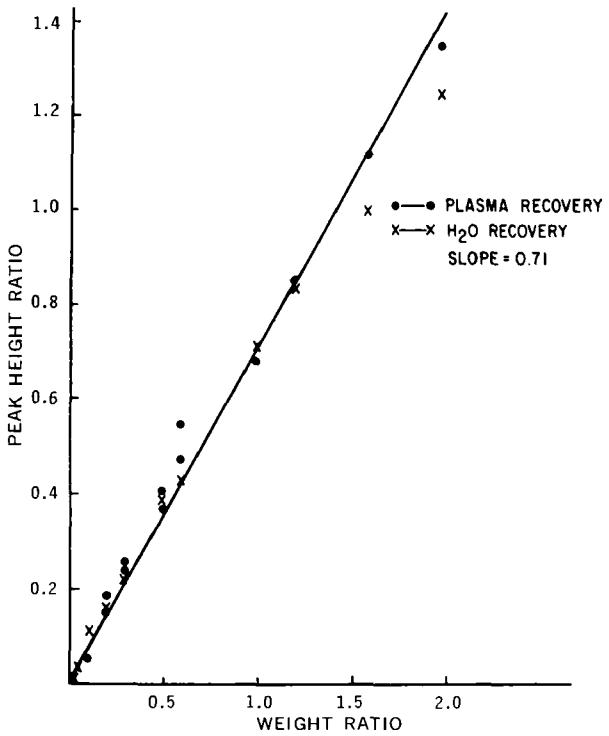


Figure 4—*Relationship between peak height ratio of the derivatives (I/II) and weight ratio (I/II). Five microliters was injected in each case out of 100–500 μl following appropriate recovery (water or plasma).*

Table II—Recovery of I from Human Urine

Amount Added, ng		Amount Recovered[a]							Mean ± *SD*
400	A	410	378	375	365	363	401	395	383 ± 18
	B	102	95	94	91	91	100	99	
200	A	212	228	208	202	201	—	196	207 ± 11
	B	106	114	104	101	101	—	98	
100	A	91	88	83	104	100	99	109	96 ± 9
	B	91	88	83	104	100	99	109	
50	A	43	47	54	57	54	39	60	50 ± 7
	B	86	94	108	114	108	78	120	
25	A	17	24	19	22	19	10	19	19 ± 4
	B	86	96	76	88	76	40	76	
12.5	A	10	13	9	10	11	14	11	11 ± 2
	B	80	104	72	80	88	112	88	
6.2	A	6	4	3	5	6	2	4	4 ± 1
	B	96	64	48	80	96	32	64	

[a] Values in the A rows represent nanograms recovered; values in the B rows represent percent recovery. Each value represents a separate recovery analysis.

(α,α,β,β-tetrafluorophenethyl)benzylamine, α,α-dimethyl-*N*-methyl-4-(α,α,β,β-tetrafluorophenethyl)benzylamine, and α-methyl-4-(α,α,β,β-tetrafluorophenethyl)benzylamine, led to the selection of trifluoroacetic anhydride and heptafluorobutyric anhydride as the best derivatizing reagents and 4-(α,α,β,β-tetrafluorophenethyl)benzylamine (II) as the best internal standard. Trial runs with each of the selected derivatives on a series of 183 × 6-mm columns packed with 1% SE-30, 1% OV-17, 1% QF-1, or 2% PPE-20 showed that the 2% PPE-20 column gave the highest resolution and separation of the I derivative from the internal standard (II) derivative and interfering peaks. The formation of each trifluoroacetyl derivative was confirmed by GLC–mass spectrometry.

Figure 1 presents gas chromatograms of the following samples: *(a)* control dog plasma carried through the procedure, and *(b)* I and II as the trifluoroacetyl derivatives (~500 pg of each compound injected directly into column). Figure 2 presents gas chromatograms of: *(a)* I and II added to control dog urine and carried through the extraction procedure, and *(b)* material obtained from a 0–24-hr dog urine sample. Compound II was added prior to extraction. As stated previously, the trifluoroacetyl derivatives of I and II had retention times of 4.0 and 6.6 min, respectively. As indicated in Fig. 1*a*, at a column temperature of 200°, the control plasma sample did give a very small peak which eluted (6.2 min) just prior to the internal standard peak (6.6 min). This peak did not present any problem in the analysis and could be separated from the peak of II by a slight decrease (4–5°) in column temperature. Several plasma and urine samples were adjusted to pH 6 prior to the initial extraction to remove or minimize endogenous peaks present. A linear relationship is observed when peak height is plotted *versus* amount injected (Fig. 3). Figure 4 presents a standard ratio curve obtained after various amounts of I (5–100 ng) and internal standard (50 ng) were carried through the entire procedure.

A summary of the recovery results of I obtained with dog plasma is presented in Table I. A similar set of recovery data from human urine is presented in Table II. Recoveries of I added to dog plasma

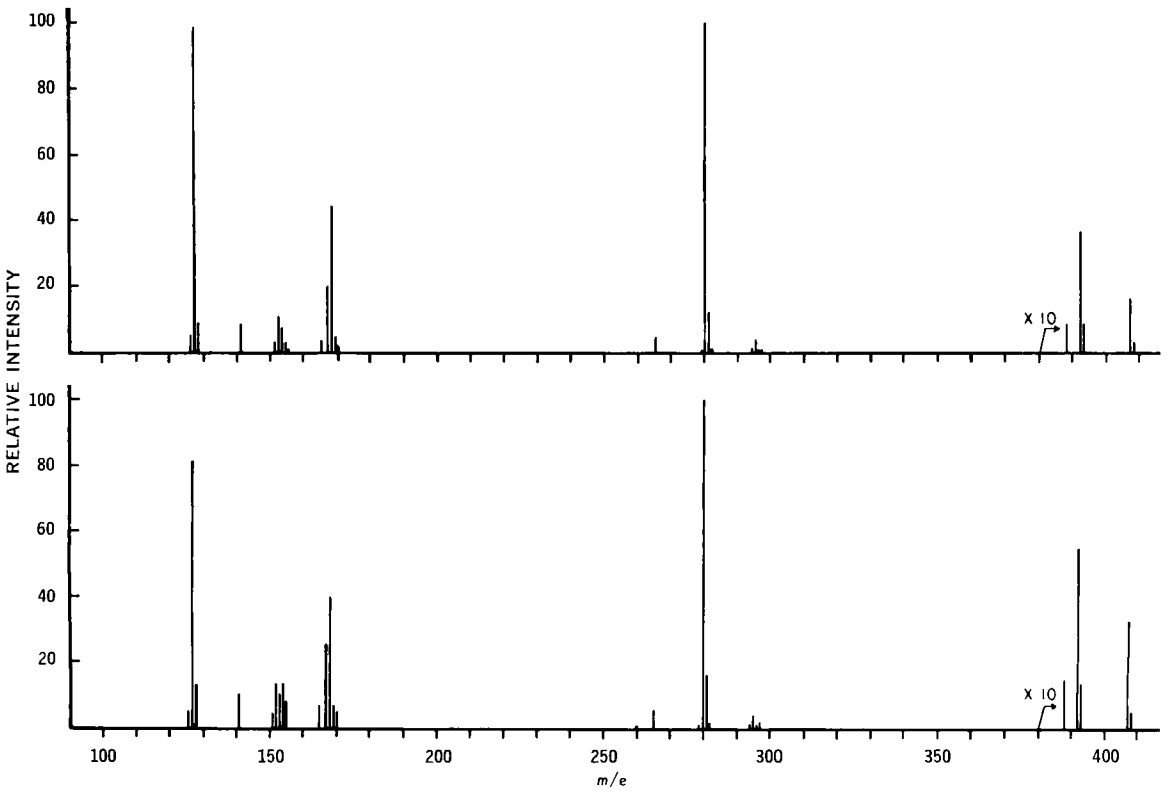


Figure 5—*Mass spectra of the trifluoroacetyl derivative of I (top) and the pooled material obtained from human plasma (bottom).*

Table III—Plasma Levels of I following Administration of I to Dogs at a Dose of 5 mg/kg

Hours	Nanograms of I per Milliliter of Plasma			
	Dog 1	Dog 2	Dog 3	Dog 4
0.25	40	10	26	23
0.5	81	21	61	37
0.75	103	30	87	65
1.0	108	33	88	93
1.5	99	38	96	85
2	98	58	95	86
3	81	60	96	78
4	78	58	82	66
6	59	55	83	57
24	28	18	23	23
30	12	12	15	17
48	—[a]	12	12	—[a]

[a] Less than 10 ng of I/ml.

in amounts of 5–100 ng ranged from 70 to 125% for analyses performed over several months. The mean recovery was 102.5 ± 13.5% over the entire concentration range. Recoveries of I added to human urine in amounts of 12.5–400 ng ranged from 72 to 114% for analyses performed over several months. The mean recovery was 93.7 ± 14.7% over the entire concentration range. Similar results were obtained when 100 ng of II was employed in the typical recovery studies. As evident, the GLC procedure for I in biological fluids is extremely sensitive and exhibits a high degree of accuracy and precision.

Typical I levels in dog plasma are seen in Table III. The plasma half-life of I in dogs has been shown to be 12–18 hr from these data and other data generated in these laboratories.

Confirmation of specificity of analysis was obtained when a pooled representative sample of several biological specimens was analyzed by combined GLC–mass spectrometry using the described technique. Figure 5 presents a comparison of the mass spectrum of the standard trifluoroacetyl derivative of I carried through the procedure with the material isolated from biological origin. The mass spectra were essentially identical in all respects for masses above 100. Below mass 100, small noncharacteristic peaks were seen at most masses in the spectrum of the isolated material.

A summary of the fragmentation pattern is presented in Table IV. The molecular ion (*m/e* 407) of the trifluoroacetyl derivative of I represents ~2% of the intensity of the base peak (*m/e* 280), which results from cleavage of the CF_2—CF_2 bond.

Studies have shown that caution must be exercised when the samples are evaporated. Samples should not be allowed to go to dryness since great losses (>50%) are encountered when this occurs. It is essential that an internal standard be carried through the entire procedure. There was no observable degradation of the trifluoroacetyl derivatives of either I or II when kept in solution (50–200 ng/ml) over a 2-week period.

CONCLUSION

A sensitive and accurate GLC method is presented for the detection of low levels of the new antiarrhythmic agent using derivatization and electron-capture techniques. The use of an internal standard eliminates any errors in quantitation as a result of sample manipulation (16). Although the electron-capture technique has proved to be extremely sensitive (can detect 10 pg of the trifluoroacetyl derivative of I when injected directly into the column), the amount of substance required for analysis is many times greater than the minimal detectable quantity since only a small portion of the total sample is usually injected into the chromatograph for analysis. The sensitivity of this method is such that 5–10 ng of material can be detected in 1 ml of plasma. GLC–mass spectrometric results from various pooled samples confirmed the specificity and identity of I.

Table IV—Summary of GLC–Mass Spectrometry Data on I and the Trifluoroacetyl Derivative

Fragmentation	Characteristic *m/e* Values	
	I	I-Derivative
M^+	—	407
$M^+ - 1$ $(-H)$	310	—
$M^+ - 15$ $(-CH_3)$	296[a]	392
$M^+ - 19$ $(-F)$	292	388
$M^+ - 34$ $(-CH_3; -F)$	277	—
$M^+ - 69$ $(-CF_3)$	—	338
$M^+ - 127$ $(-C_6H_5CF_2)$	184	280[a]
$M^+ - 127 - 15$ $(-C_6H_5CF_2; -CH_3)$	169	265
$M^+ - 184$ $[-CF_2C_6H_5C(CH_3)_2NH_2]$	127	—
$M^+ - 112$ $(-NHCOCF_3)$	—	295

[a] Base peak; retention time for I was 3.3 min and retention time for the derivative was 4.0 min under the GC–mass spectrometry operating conditions (refer to *Experimental* section).

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[x] To whom inquiries should be directed.

Effect of Aspirin on Fate of ^{14}C-Acetaminophen in Guinea Pigs

L. W. WHITEHOUSE [x], C. J. PAUL, and B. H. THOMAS

Abstract □ The interaction of ^{14}C-acetaminophen, 150 mg/kg (20 μCi/kg), and orally administered aspirin, 200 mg/kg, was studied in male guinea pigs. Aspirin-pretreated animals possessed higher ^{14}C blood levels than controls. Paper chromatography of 0–6-hr urines demonstrated that pretreated animals excreted significantly greater amounts of mercapturate than controls; however, it was only a minor metabolite, accounting for 1–3% of the counts in the urine. The major metabolite, the glucuronide, accounted for 90% of the counts, with free acetaminophen and its sulfate responsible for the remaining counts. Tissue distribution studies indicated that blood plasma and kidneys from aspirin-pretreated animals possessed statistically higher ^{14}C levels than did control tissues. Bile duct and ureter cannulation experiments indicated that aspirin inhibited the concentrating processes into the urine and bile.

Keyphrases □ Acetaminophen, radiolabeled—metabolism, effect of aspirin pretreatment, guinea pigs □ Aspirin—effect of pretreatment on ^{14}C-acetaminophen metabolism, guinea pigs

The restriction of phenacetin has caused acetaminophen, the principal metabolite of phenacetin, to come into widespread use. Since acetaminophen is now being used in combination with aspirin in some analgesic formulations, a study of the effect of aspirin upon acetaminophen was deemed desirable. A previous report (1) showed that, in rats, aspirin caused: *(a)* a reduction in the rate of acetaminophen absorption from the GI tract, *(b)* an enhanced blood level of radioactivity during the postabsorptive phase, *(c)* large changes in the proportions of acetaminophen and its metabolites excreted in the urine, *(d)* reduction in sulfate conjugation, and *(e)* an increase in glucuronide and mercapturate conjugation.

Amsel and Davison (2), studying the simultaneous metabolism of aspirin and acetaminophen in humans, found that aspirin had no effect on the formation of acetaminophen metabolites. This report confirmed earlier work in which a similar lack of effect was observed with lower doses (1.0 g) of sodium salicylate (3). In the present study the effect of aspirin upon the fate of acetaminophen in the guinea pig was investigated to determine if this species would serve as a better animal model than the rat for toxicological studies of this particular drug combination.

EXPERIMENTAL

Materials—^{14}C - Acetaminophen (*N* - acetyl - *p* - aminophenol-ring-UL-^{14}C)[1], with a specific activity of 44.64 μCi/mg, was custom-synthesized[2]. Aspirin[3], unlabeled acetaminophen[4], and gum tragacanth[5] were obtained commercially. Male Hartley strain guinea pigs, 250–350 g, were purchased locally[6].

[1] UL = uniformly labeled.
[2] Mallinckrodt, St. Louis, MO 63160
[3] B.D.H. Canada Ltd., Toronto, Canada.
[4] Matheson, Coleman and Bell, Norwood, Ohio.
[5] Fisher Scientific Co. Ltd., Toronto, Canada.
[6] High Oak Ranch, Goodwood, Ontario, Canada.

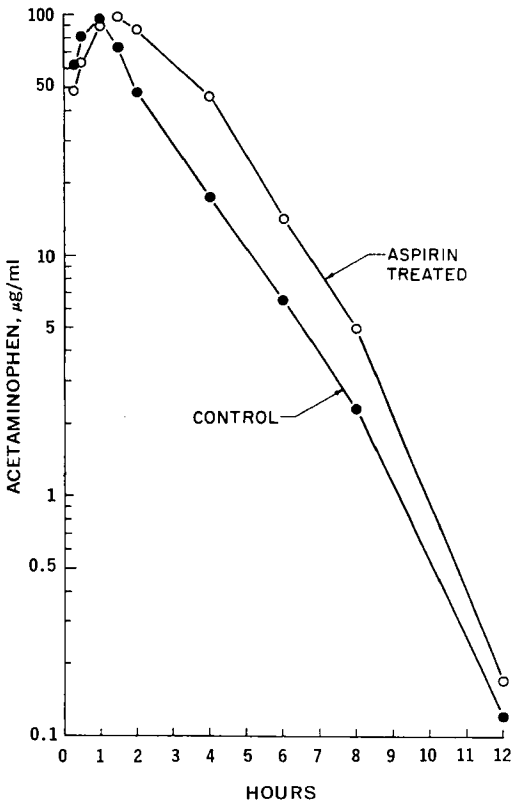

Figure 1—*Effect of aspirin (200 mg/kg) upon the blood profile of orally administered ^{14}C-acetaminophen (150 mg/kg). Blood concentrations of radioactivity are expressed as micrograms of acetaminophen per milliliter of blood. Values are means from five guinea pigs, with differences at 1.5, 2, and 4 hr being significant at* $p < 0.05$.

Methods—*Blood Profiles*—Guinea pigs were randomly divided into two groups of five animals each and deprived of food but not water for 16 hr. In the oral dosing study, all treatments were administered orally in volumes of 10 ml/kg as 0.25% gum tragacanth suspensions. One group of animals received aspirin (200 mg/kg) while the control group received 0.25% gum tragacanth. ^{14}C-Acetaminophen [150 mg/kg (20 μCi/kg)] was administered to both groups 30 min later.

Duplicate blood samples (10 μl) were collected from the toe at 0.25, 0.50, 1, 1.5, 2, 4, 6, 8, and 12 hr after dosing. The blood was digested and counted in a liquid scintillation counter as previously described (4). Urine was also collected at 6, 12, 24, and 48 hr after dosing and radioactivity was determined (4).

In the intraperitoneal dosing experiments, ^{14}C-acetaminophen [150 mg/kg (20 μCi/kg)] was administered in volumes of 7 ml/kg in a 10% ethanol solution, but the other routes of administration, dosing solutions, and procedures were otherwise identical to those used in the oral dosing study.

Urine Metabolites—Urines collected in the oral dosing experiments (0–6 hr) were chromatographed on medium flow rate chromatography paper[7]. Urines (10 μl) were applied as bands, and the chromatograms were developed using the alkaline paper chroma-

[7] Whatman No. 1.

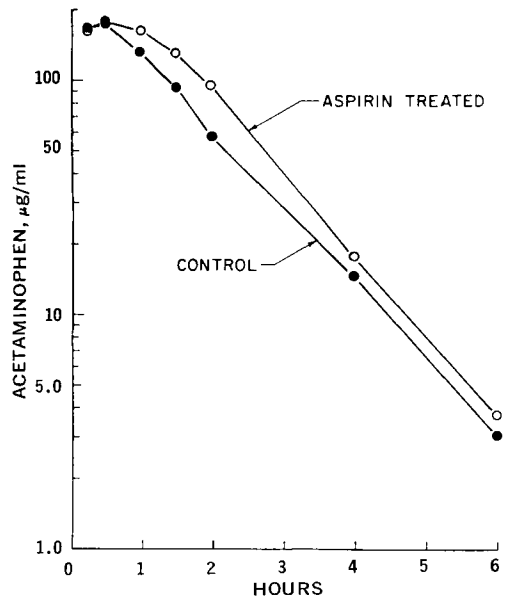

Figure 2—*Effect of aspirin (200 mg/kg) upon the blood profile of intraperitoneally administered ^{14}C-acetaminophen (150 mg/kg). Blood concentrations of radioactivity are expressed as micrograms of acetaminophen per milliliter of blood. Values are means from five guinea pigs, with differences at 1, 1.5, and 2 hr being significant at* $p < 0.05$.

tography system described by Shahidi (5, System C). The developed chromatograms were serially sectioned into 1-cm strips and radioactivity was determined by liquid scintillation counting.

Tissue Distribution—While employing the dosage regimen used in the intraperitoneal blood profile study, levels of radioactivity were estimated in duplicate blood, plasma, kidney, and liver samples collected 90 min following ^{14}C-acetaminophen administration. A previously described method for preparing samples for liquid scintillation counting was used (4). In the case of bile, 10-μl samples were treated similarly to blood for estimation of radioactivity.

Levels of ^{14}C in the intestinal contents were determined by making the intestinal contents and washings to a constant volume of 10

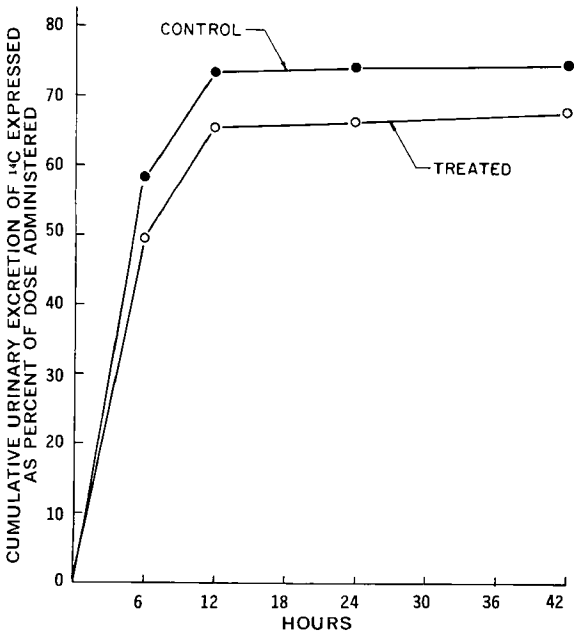

Figure 3—*Effect of aspirin (200 mg/kg) upon the cumulative urinary excretion of orally administered ^{14}C-acetaminophen (150 mg/kg). Values are means from five guinea pigs. A statistical difference of* $p < 0.05$ *was observed only at 12 hr.*

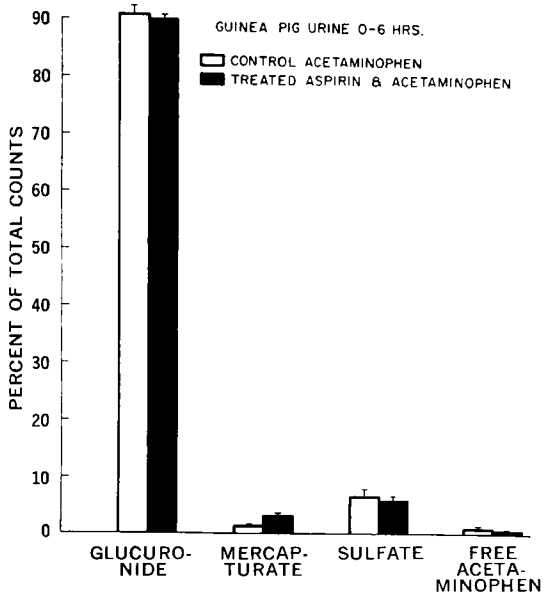

Figure 4—*Urinary excretion of acetaminophen and its metabolites in guinea pigs orally dosed with ^{14}C-acetaminophen (150 mg/kg). Values are means from five animals per group* ± SE.

ml with distilled water. Duplicate 0.5-ml aliquots were then dried in scintillation vials; the residue, digested with 1.0 ml of tissue digestant[8], was treated similarly to kidney and liver tissues.

Bile Duct and Ureter Cannulations—A median incision just below the xiphisternum was made in the abdomen of ether-anesthetized male guinea pigs. The common bile duct was exposed and surgical thread was positioned under the duct. A small incision was made in the upper part of the duct; polyethylene tubing[9] was inserted upward and tied into place. After stitching the abdominal incision, animals were placed in restraining cages and allowed to recover from the anesthesia.

Following recovery (at least 1 hr), animals were treated with acetaminophen and aspirin or gum tragacanth; the intraperitoneal dosage regimen used in the blood profile experiment was employed. Blood and bile were collected every 15 min for 180 min, and the concentration of radioactivity was determined by liquid scintillation counting using 10-μl samples.

In another group of animals, the left ureters were similarly exposed and cannulated while the right ureters were ligated close to the kidneys. Following recovery from anesthesia, animals were treated according to the intraperitoneal dosage regimen. Blood and urines were collected every 15 min, and the levels of ^{14}C in blood (10 μl) and urine (total) were determined as previously described (4).

RESULTS

Blood concentrations of radioactivity in treated and control animals dosed by the oral route are shown in Fig. 1. Animals pretreated with aspirin showed a 30-min delay in peaking of blood levels of radioactivity; only at 1.5, 2, and 4 hr did pretreated animals show significantly higher ^{14}C blood levels than did controls. A closer examination of the blood profiles indicated that aspirin did not change the half-life of ^{14}C-acetaminophen ($T_{1/2}$ = 1.40 hr, control; $T_{1/2}$ = 1.39 hr, aspirin pretreatment), but the total areas under the blood profile curves were significantly greater in pretreated animals (350.4 μg/ml × hr) than in controls (235.8 μg/ml × hr).

Initially, these results were attributed to inhibition of acetaminophen absorption from the GI tract by aspirin. An intraperitoneal study, in which acetaminophen was administered intraperitoneally while aspirin pretreatment was given orally, resulted in a blood profile (Fig. 2) similar to that obtained in the oral study (Fig. 1). This suggested that the delay in the blood profile was not caused

[8] Soluene, Packard Instrument Co., Downers Grove, Ill.
[9] PE-50, Clay-Adams, New York, N.Y.

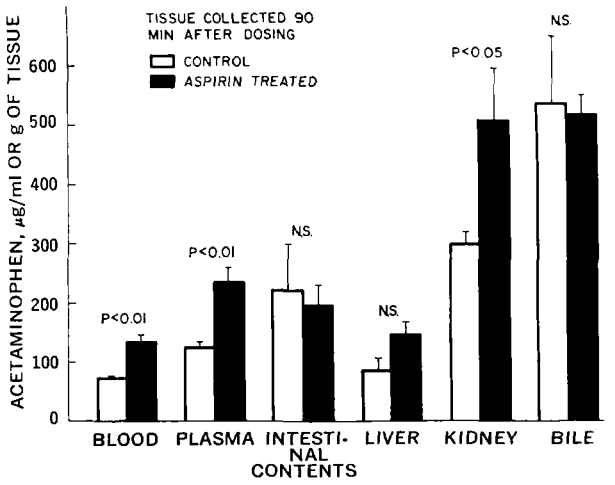

Figure 5—*Tissue distribution of radioactivity in control and aspirin-pretreated guinea pigs. Concentration is expressed as micrograms of acetaminophen per milliliter or gram of tissue. Values are means of five animals per group* ± SE.

by an inhibition of absorption due to a chemical interaction between the two chemicals in the GI tract.

The cumulative urinary excretion in orally dosed animals is presented in Fig. 3. Only at 12 hr did aspirin-pretreated animals excrete a significantly lower percentage of the administered dose than did controls. At the other time periods studied, the same trend was observed, although statistical significance at the 0.05 probability level could not be demonstrated.

Paper chromatography of the 0–6-hr urine samples collected from the oral dosing experiments separated the radioactivity into four distinct peaks, identified by R_f values from a previous study as the glucuronide, mercapturate, sulfate, and free acetaminophen (1). As shown in Fig. 4, the major metabolite in the urines of both treated and control guinea pigs was the glucuronide, which represented at least 90% of the total radioactivity excreted. Pretreatment with aspirin caused only a slight, but significant, increase in the percentage of mercapturate excreted in the urine.

A tissue distribution study of radioactivity (Fig. 5) showed that blood, plasma, and kidney levels of radioactivity were statistically higher in aspirin-pretreated animals than in controls. Livers showed the same trend, although the difference was not significant. The level of radioactivity in the intestinal contents and bile of aspirin-pretreated and control animals did not differ statistically, although the trend was for the control group to possess higher levels of ^{14}C than the pretreated group, suggesting that aspirin was inhibiting the biliary excretion of acetaminophen. A comparison of the cumulative biliary ^{14}C excreted in 180 min with the total area under the blood profile curve for the same period gave ratios for aspirin-pretreated animals that did not statistically differ from control ratios. However, a statistical difference was demonstrated in the bile to blood ratio of radioactivity at 60 min. Three control guinea pigs possessed a 7.07 ± 0.82- (mean ± *SE*) fold higher concentration of ^{14}C in the bile than in the blood, while three treated animals gave a ratio of 3.47 ± 0.40.

Because biliary excretion over the 3-hr period studied accounted for only 3–10% of the dose administered while urinary excretion over 6 hr accounted for as much as 50–60% (Fig. 3), the effect of aspirin on the urinary excretion of acetaminophen in ureter-cannulated animals was examined. Blood and urine were collected every 15 min for a total of 180 min. Cumulative radioactivity excreted in 180 min in the urine over the total area under the blood profile curve for the same period gave a mean ratio of 1.22 ± 0.08 for four control animals, which was significantly greater than 0.85 ± 0.07, the mean ratio obtained from six aspirin-pretreated animals.

DISCUSSION

The combination of orally administered aspirin and acetaminophen appeared to reduce the rate of acetaminophen absorption during the absorptive phase (Fig. 1). Despite the slower rate of absorption, peak blood levels of radioactivity were comparable in the control and aspirin-pretreated animals. Similarly, comparable peak blood levels of radioactivity were observed in the intraperitoneal study for the treated and control groups (Fig. 2).

Data from the urinary metabolite study (Fig. 4) indicated that aspirin had no effect upon the sulfation or glucuronidation of acetaminophen in guinea pigs, an observation previously reported for humans (2). A significant increase in the percentage of mercapturate excreted in the urines of aspirin-pretreated animals was observed. Since this metabolite only accounted for 1–3% of the ^{14}C in the urine samples, the difference in metabolism between treated and control animals was not considered a likely contributor to the observed effect of aspirin on the blood profile of radioactivity.

The most significant effect of aspirin was the short-term effect on biliary and urinary excretion of radioactivity. Bile to blood ratios were significantly lower in aspirin-pretreated animals, suggesting that plasma clearance of acetaminophen by the liver was being inhibited by the pretreatment. Since differences in the major metabolites in the urine could not be demonstrated (Fig. 4), reduction in clearance could not be attributed to the inhibition of hepatic metabolism as previously reported for the rat (1). The 180-min cumulative urinary excretion of ^{14}C, adjusted for differences in blood levels of radioactivity, indicated that excretion by the kidney was also being suppressed by aspirin pretreatment, an effect previously reported for the clearance of *p*-aminohippuric acid from human plasma (6).

Since aspirin, acetaminophen, and its major metabolites are acidic drugs excreted in part by the kidney tubules *via* an active excretory process (7, 8), the most likely explanation for the observed interaction is a competition of both drugs for a common anionic excretory mechanism. Because aspirin is a stronger acid, it is preferentially excreted, resulting in higher blood levels of acetaminophen.

In humans, aspirin does not appear to inhibit the glucuronide or sulfate conjugation of acetaminophen (2), but urinary excretion of acidic drugs is reduced (6). Since aspirin-pretreated guinea pigs appeared to respond similarly to humans and the proportions of glucuronide and sulfate resembled those in humans (9), this species probably would serve as a better animal model than the rat for further toxicological studies.

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Partition of an Organophosphorus Compound, Dichlorvos, between Liquid and Liquid Crystalline Phases

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Abstract □ The partition coefficients for an organophosphorus compound, dichlorvos (2,2-dichlorovinyl dimethyl phosphate), between pure liquids, micellar solutions, and liquid crystalline phases were determined by spectrometric analysis of the dichlorvos content after equilibration and separation of the phases. The results demonstrated the pronounced importance of associated colloid structures on the partition coefficient and showed that the partition coefficient did not display a simple concentration dependence.

Keyphrases □ *Dichlorvos—partition coefficients between pure liquids, micellar solutions, and liquid crystalline phases, effect of associated colloid structures* □ Partition coefficient of dichlorvos—determination in pure liquids, micellar solutions, and liquid crystalline phases, effect of associated colloid structures □ Organophosphorus compounds—partitioning of dichlorvos between liquid and liquid crystalline phases, effect of associated colloid structures

Many organophosphorus compounds are potent inhibitors of acetylcholinesterase and some are widely used as insecticides (1, 2). The most active members of the group are considered to be potential warfare agents (3). In addition to enzyme affinity, two other factors are important for the inhibitory efficiency of organophosphorus compounds: *(a)* the partition coefficients between different tissues with varying hydrophilic–lipophilic character (4), and *(b)* the metabolic degradation, mainly by hydrolysis, which causes detoxification (5).

Organophosphorus inhibitors have been found to show an unexpectedly high penetration into axons (6) in spite of having oil–water partition coefficients of only 0.1 (7). This fact has been considered a reason for the unexpected pharmacological response (8).

The purpose of this article is to draw attention to another factor of importance for the penetration of a drug in addition to its hydrophilic–lipophilic balance. Biological tissue is not, in general, a simple solution; the cellular membranes and the myelin part of the nerve substance are highly ordered systems with regular layer structures. Such structures (9) can be compared with the structure of a liquid crystalline phase (10). Liquid crystalline phases may actually assume many structures (11) (Fig. 1); the transition from a layer structure to a regular hexagonal packing of cylinders has been suggested as being responsible for the transport mechanism through membranes (12).

Since liquid crystalline structures appear to be of general importance for biological systems and since organophosphorus compounds have been found to penetrate axons in spite of low oil–water partition coefficients (7), an investigation of the possible effects on the partition of structuring elements such as

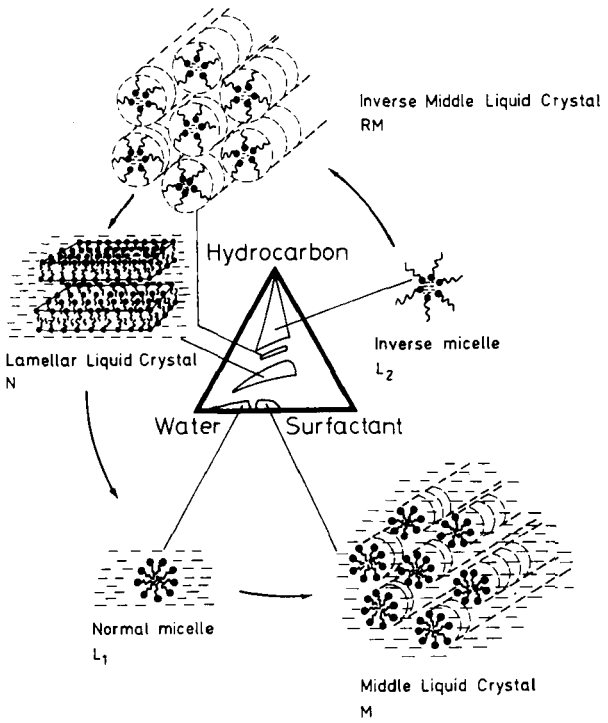

Figure 1—*When water, a hydrocarbon, and an amphiphilic compound are combined, micellar solutions with normal (L_1) and inversed (L_2) micelles and liquid crystalline phases [middle liquid crystal (M), lamellar liquid crystal (N), and inversed middle liquid crystal (RM)] are obtained.*

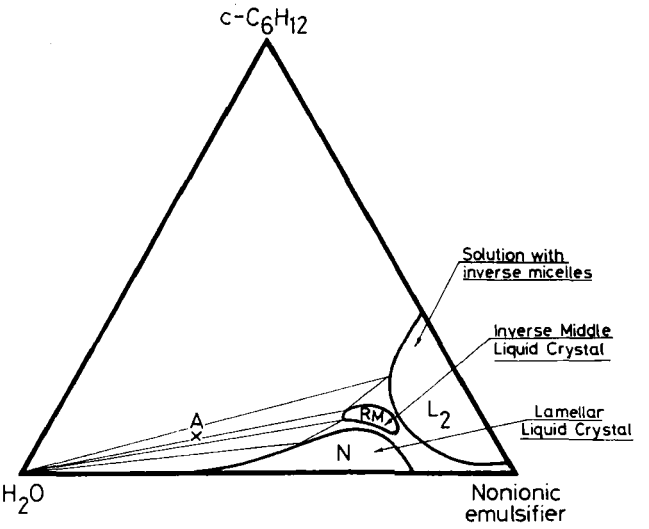

Figure 2—*Water, cyclohexane, and the nonionic emulsifier pentaoxyethylene dodecyl ether formed three liquid phases [pure water, pure cyclohexane, and a solution of cyclohexane in the emulsifier (L_2)] and two liquid crystalline phases [lamellar phase (N) and inversed middle phase (RM)].*

micelles in liquids and of the general longrange order characteristics of a liquid crystalline phase was considered to be of value. No earlier investigation of the distribution of organophosphorus compounds between phases containing associated structures was found in the literature. Dichlorvos (2,2-dichlorovinyl dimethyl phosphate) was selected for the investigation because its properties are well known (13) and its solubility in hydrocarbons and water is balanced.

Two different systems containing associated colloids were selected. In addition to water and cyclohexane, the first one contained two different liquid crystalline phases (RM and N) and one solution (L_2) with inverse micelles (Fig. 2). These three phases displayed comparatively small differences in their total composition and were judged well suited for the purpose of estimating the influence of different associated structures. The second system contained an alkylammonium carboxylate (Fig. 3) and was used to observe the influence on the partition of a gradual increase in the amount of associated structure units.

EXPERIMENTAL

Materials—The dichlorvos used was a commercial sample[1], which was purified by distillation with a spinning-band column (13 torr, 112°). Double-distilled water, cyclohexane, *p*-xylene, 1-aminooctane[2], 1-octanoic acid[3], and a commercial pentaoxyethylene dodecyl ether were used without further purification.

Separation of Phases—Between 0.5 and 1.5 wt. % of dichlorvos was dissolved in the mixture of water, hydrocarbon, and surfactant. The mixture was heated to 30° and allowed to cool slowly to 20°, at which temperature it was allowed to equilibrate for 24 hr. Centrifugation with an analytical centrifuge[4] (36,000 rpm for 16 hr) caused the phases to separate from each other.

Analysis—The system consisting of water, cyclohexane, and nonionic emulsifier did not show UV absorption at a level that would influence the determination of the dichlorvos at 205–207 nm. Separate calibration curves were determined for each phase involved in the equilibrium by addition of known amounts of dichlorvos to the separated phases.

For the system consisting of water, *p*-xylene, octanoic acid, and aminooctane, the determination of the amount of dichlorvos was done spectrophotometrically on the antimony phosphatomolybdate complex (14) after the phosphorus had been transformed to phosphate using sulfuric and nitric acids. Separate calibration curves also were determined for the different phases.

RESULTS

The regions for the different phases in the water–cyclohexane–surfactant system are given in Fig. 2. The nonionic surfactant used for the first system was soluble to less than 0.5% in water or in cyclohexane. In Fig. 2, the liquid phases of water and cyclohexane are only marked as points in the respective corners. Both water and cyclohexane were soluble in the emulsifier forming the L_2 area, which represented a solution with inverse micelles. This solution, the inverse middle phase (RM) (Fig. 2) containing a hexagonal array of cylinders (Fig. 1), and the lamellar neat phase (Fig. 1), denoted N in Fig. 2, showed small differences in total composition from each other and were judged suitable to demonstrate the influence of different associated structures.

The partition coefficient of dichlorvos between water and cyclohexane was determined separately using the pure compounds. This corresponds to a composition choice anywhere along the line H_2O–c-C_6H_{12} in Fig. 2. The results are given in Table I (Samples 4 and 5).

[1] Donated by Shell Co.
[2] Fluka.
[3] Eastman.
[4] Beckman.

Table I—Partition Coefficient of Dichlorvos between Different Phases and Water in the Water–Cyclohexane–Commercial Polyethylene Glycol Dodecyl Ether System

Sample	Cyclohexane	L_2	RM	N
1	—	4.7	5.9	—
2	—	4.6	5.7	—
3	—	—	5.8	5.5
4	2.2	—	—	—
5	2.2	—	—	—

The three phases H_2O, L_2, and RM could easily be separated from a total composition, denoted A in the diagram. The actual composition of the phases L_2 and RM in the three-phase region was not determined, but the fact that an increase of the cyclohexane content in the total composition A gave a sample containing only H_2O and L_2 was evidence of a very narrow three-phase region (approximately the one indicated in Fig. 2). The partition of dichlorvos between these three phases was determined in duplicate samples. The results are given in Table I (Samples 1 and 2) as partition coefficients for the two phases L_2 and RM against water.

The separation of the three phases H_2O, N, and RM was difficult, and it was considered sufficient to determine the partition between N and RM, which could easily be separated. The composition of the phase RM in equilibrium both with H_2O and N was necessarily close to its composition in equilibrium with H_2O and L_2. This is evident from an inspection of Fig. 2, in which the probable three-phase area containing H_2O, RM, and N is indicated. Regarding this similarity in composition of RM, it was judged reasonable to assume an RM–H_2O partition coefficient for the three phase H_2O, RM, and N very close to the corresponding coefficient for the three phases H_2O, RM, and L_2. The partition coefficients RM–H_2O and N–H_2O in Table I (Sample 3) were consequently calculated using an average value for the RM partition coefficient against water from Samples 1 and 2.

The partition coefficients are given in Table I. The results show that the dichlorvos actually showed a higher partition coefficient for the liquid crystalline and the L_2 phases than for the hydrocarbon phase. The difference between the two liquid crystalline phases was not significant.

The next system was constructed to answer the question of the importance of structure more generally; micelles were also taken into account. The system contained 1-octanoic acid and 1-aminooctane in a 1:1 molecular ratio. These two components form the salt octylammonium octanoate, which made the system contain a surfactant with a strong dipolar element similar to lecithin.

The water, *p*-xylene, and 1-octanoic acid–1-aminooctane (1:1) system (Fig. 3) consists of pure water, a *p*-xylene solution (L_2) containing the surfactant pair and solubilized water, and a lamellar liquid crystalline phase (N) (15). According to IR and NMR data, the *p*-xylene solution contains ion-pairs of the amphiphiles in a 1:1 ratio (16). When water is added, inverse micelles are formed simi-

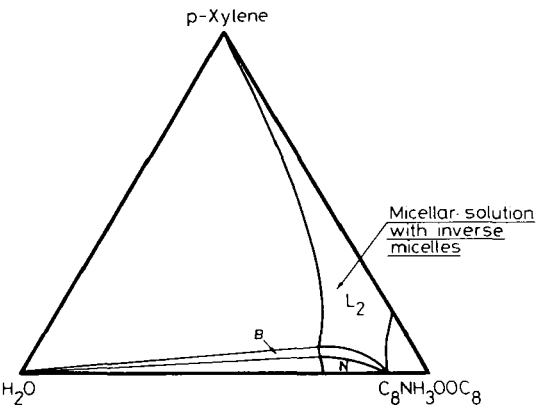

Figure 3—*Water, p-xylene, and the emulsifier octylammonium octanoate formed two liquid phases [pure water and a p-xylene solution (L_2) containing the emulsifier and solubilized water and liquid crystalline phase (N)].*

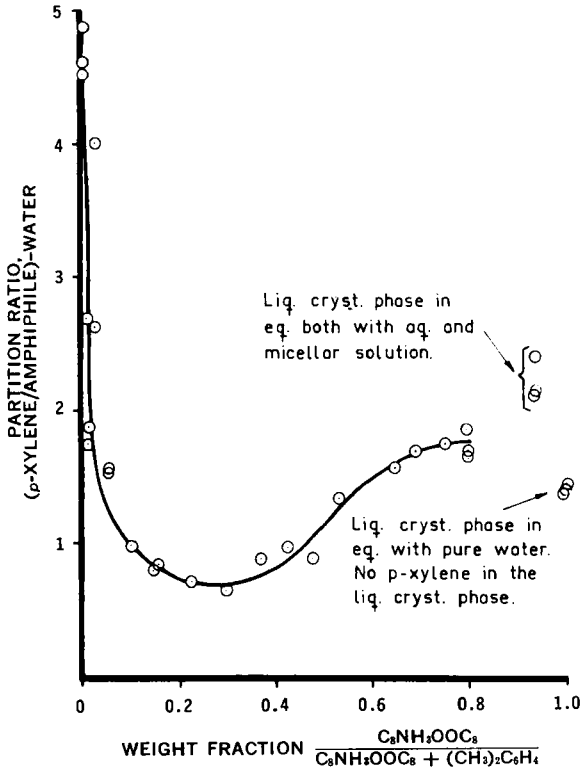

Figure 4—*Partition of dichlorvos between the phases in the water, p-xylene, and octylammonium octanoate system plotted against the weight fraction of the emulsifier.*

lar to those in the corresponding system in which the surfactant consisted of 1-aminooctane only (17).

The results (Fig. 4) show that the structure of the phases had a pronounced influence on the partition of dichlorvos between the phases. Small additions (~2%) of the two amphiphilic compounds reduced the partition coefficient to less than 50% of the initial value for *p*-xylene–water. When a weight basis fraction of added amphiphiles to *p*-xylene reached one-third, the partition coefficient reached a minimum, being 16% of the value for *p*-xylene–water. Further additions of the amphiphiles increased the coefficient. The three-phase region of water, isotropic liquid (L_2) phase with maximum amount of amphiphiles (B in Fig. 3), and liquid crystalline phase gave partition coefficients significantly higher for the liquid crystalline phase toward water (2.2) than the corresponding coefficient for the isotropic micellar solution and water (1.75).

Further reduction to zero of the *p*-xylene percentage within the liquid crystalline phase caused a reduction of the partition coefficient from 2.2 to 1.35.

DISCUSSION

The results obtained made evident that the associated colloid structures present in micellar solutions and in liquid crystalline phases had a pronounced influence on the partition of dichlorvos.

This influence was demonstrated by the drastic changes of the partition coefficient toward water when the initial addition of octylammonium octanoate to *p*-xylene was made. The initial steep reduction of the partition coefficient and its minimum at a weight fraction of about 0.3 are features that have to be explained from changes in the colloid and subcolloid associated structures. The exact determination of these structures was not within the realm of the present investigation, but a comparison with findings of earlier studies on similar systems may be of value, giving the general conditions that may be expected (17).

At low weight fractions of the salt, the salt is dissolved as separate ion-pairs with one or two water molecules attached to each pair. At higher concentrations, the addition of water gives rise to the formation of larger aggregates, the size of which determines if they are micelles. The onset of the formation of larger aggregates is seldom as sudden as in aqueous systems, where the critical micellization concentration is experienced as a narrow concentration range.

Considering these general statements, it appears probable that the minimum in the partition coefficient curve (Fig. 4) was related to the formation of larger aggregates. The increase of the partition coefficient may then be a rational consequence of the formation of colloid size volumes containing water and polar parts, which are then separated from the hydrocarbon parts of the solution (Fig. 1). The enhancement of the partition coefficient when the structure changed from the micellar solution to the liquid crystalline phase is in accordance with this opinion; the change implies an even stricter separation into polar and nonpolar colloid dimensions (Fig. 1).

The reduction of the partition coefficient when *p*-xylene was removed from the liquid crystalline phase was considerably higher than what could be expected with regard to the reduction of the nonpolar volume in the liquid crystalline phase. The reduction in nonpolar volume was less than 3%; the decrease of the partition coefficient was about 10 times that value. A continued investigation of possible changes in the localization of dichlorvos would be of interest with regard to this result.

For the system with a nonionic emulsifier, the partition coefficient between cyclohexane and water was lower than the coefficient between *p*-xylene and water. The liquid crystalline phases in the latter system, on the other hand, caused the value of the partition coefficient toward water to be higher than that between hydrocarbon and water; this finding was another example of the importance of structure conditions.

The importance of the results is not due only to the changes in partition coefficient. It is well known (18, 19) that variations in the polarity and hydrogen bonding capacity of the nonaqueous liquid generally give rise to a broad range of partition coefficient values. However, this fact is not easily applicable to ordered systems, since even the value of a liquid with the polarity and hydrogen bond capacity adjusted to average values for a liquid crystalline phase may be in error to a high degree. This is well displayed by the two systems used in the present investigation. Average properties calculated on the basis of the molecular composition are of no use for the determination of the partition coefficient when it passes through a maximum and a minimum; this was the case when the weight ratio of octylammonium octanoate to *p*-xylene was varied (Fig. 4). When these results are considered, the estimation of partition coefficients between nerve tissues and surrounding plasma from the coefficients of simple liquids appears questionable.

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Synthesis of Sparsomycin Analogs as Potential Antitumor Agents

R. J. DUBOIS ×, C.-C. L. LIN, and B. L. MICHEL

Abstract □ No information is available on the structural requirements for the antitumor activity of sparsomycin, an antibiotic obtained from the fermentation broth of *Streptomyces sparsogenes*. Its high *in vivo* and *in vitro* activity, novel structure, and uncommon mode of action have, therefore, suggested the synthesis of analogs. This report describes the preparation and screening of a series of *N*-substituted 3-aryl acrylamides which are closely related to sparsomycin. Three compounds exhibited some tumor inhibition but insufficient to warrant further testing.

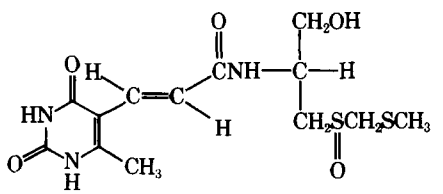
Keyphrases □ Antitumor agents, potential—synthesis and screening of sparsomycin analogs □ Cysteinol derivatives—synthesis and screening as possible anticancer agents □ 3-Aryl acrylamides, *N*-substituted—synthesis and pharmacological screening as possible anticancer agents □ Sparsomycin analogs—synthesis and screening as potential antitumor agents

The antibiotic sparsomycin (I) was first isolated in 1962 (1) from the fermentation broth of *Streptomyces sparsogenes*. Not until 1970, however, was the structure elucidation reported (2).

Following its isolation, sparsomycin was subjected to several preliminary biological tests where it displayed a broad spectrum of moderate *in vitro* activity against bacteria and moderate antifungal activity (3). Of greater interest was its very high activity against KB human epidermoid carcinoma cells (3). It also showed moderate to high inhibition in several *in vivo* tumor systems such as the Walker carcinosarcoma 256 and the sarcoma 180 solid tumor (3).

On the basis of this antitumor activity, sparsomycin was selected for Phase I clinical studies. It displayed severe eye toxicity, however, and the Phase I study was terminated (4).

Its biological activity appears to be primarily due to inhibition of protein synthesis, and this inhibition



I

Table I—NSC Numbers and Screening Data

Compound	NSC Number	Walker 256 Data, % T/C (Dose, mg/kg)
II	173109	49 (120)
III	177934	55 (40)
IV	181493	85 (50)
V	181494	64 (160)
VI	184712	96 (80)
VII	184714	107 (50)
VIII	159934	—
IX-HCl	166004	—
X	169798	—
XI	169799	—
XII	173110	—
XIII	174260	—
XIV	177936	—
XV	179898	—

has been substantiated (5, 6). Further work (7) indicated that its mechanism of action in the *Escherichia coli* system is on the 50S ribosome subunit, where it prevents peptide transfer by interfering with the function of the enzyme peptidyl transferase.

To date, no analogs of sparsomycin have been reported. Its high to moderate antitumor activity, novel structure, and somewhat uncommon mode of action have prompted the development of a structure–activity relationship by synthesis. This report describes some initial investigations.

RESULTS AND DISCUSSION

As an initial synthetic goal, the novel *N*-substituted 3-aryl acrylamides (II–VII) were chosen. These compounds represent analogs in which the uracil portion of sparsomycin has been replaced by other heterocyclic or aromatic moieties and the sulfoxide portion has been replaced by sulfur. It was believed that these compounds retained a sufficient portion of the sparsomycin structure to warrant their preparation and testing as potential antitumor agents.

Scheme I outlines the proposed synthetic approach. Condensation of the amino ester (IX) with the appropriate acrylic acid should yield esters (X–XV), which could then be selectively reduced to the desired compounds (II–VII). A synthetic pathway to the key intermediate (IX), involving the amino acid cystine, was developed (Scheme II). Since sparsomycin is optically active with a D-configuration at the asymmetric carbon atom, D-cystine would

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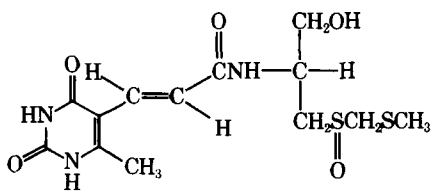

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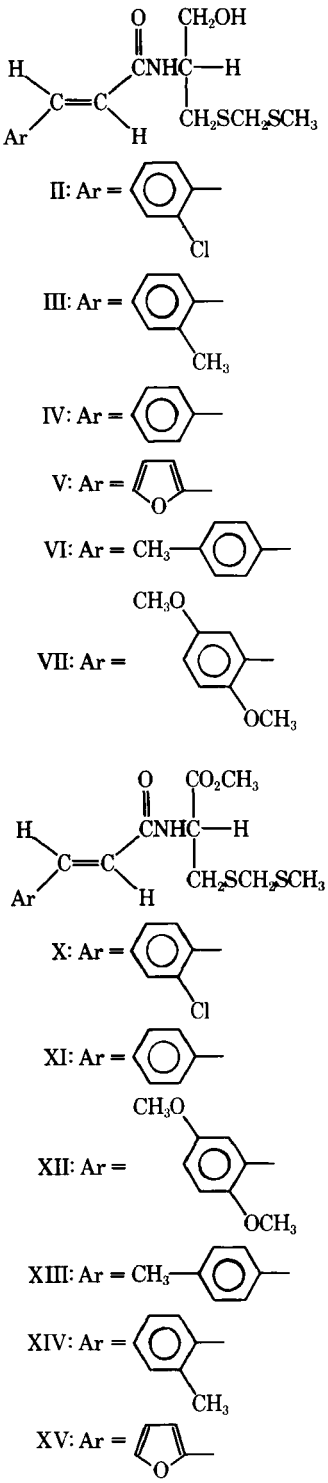
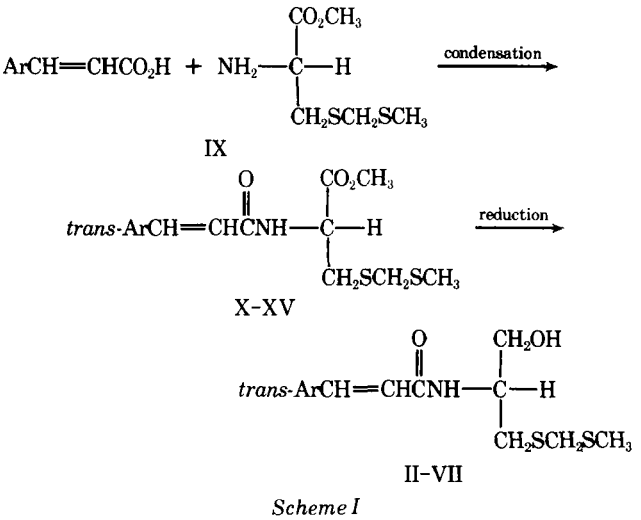


be the most desirable starting material. Its extremely high cost precludes its use, however, in the initial trial-and-error procedures necessary in development of a synthetic pathway. The DL-mixture and *meso*-form, on the other hand, would give no information on potential racemization during synthetic elaboration. Consequently, L-cystine was chosen to begin the synthesis. With the synthetic scheme established, it would be possible to substitute the D-enantiomer; this substitution would be made if subsequent evidence indicated that the antitumor activity of sparsomycin was dependent on absolute configuration.

As shown in Scheme II, reduction of L-cystine with sodium and liquid ammonia followed by addition of chloromethyl methyl sulfide yielded the amino acid (VIII). Compound VIII was then converted to the desired amino ester as the free base by treatment



Scheme I

with methanol saturated with hydrogen chloride[1], followed by base treatment with sodium bicarbonate.

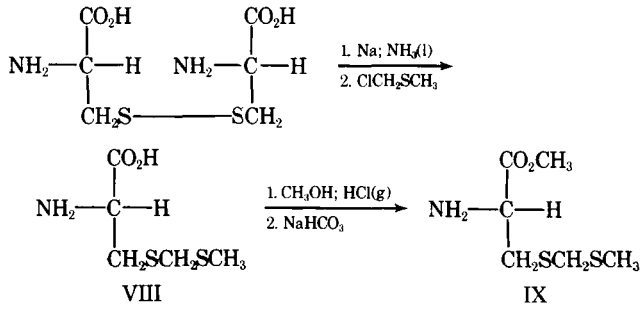
The desired intermediate was subsequently coupled with the appropriate acrylic acid (Scheme I) by means of the versatile reagent *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (8), allowing isolation of compounds (X–XV) in the *trans*-configuration. These compounds, in turn, were selectively reduced with lithium borohydride to the desired analogs (II–VII), also in the *trans*-configuration.

PHARMACOLOGY

The intermediates (VIII–XV) (see Table I for NSC numbers) were tested in BDF_1 mice in the L-1210 lymphoid leukemia system and P-388 lymphocytic leukemia system according to the standard protocol of the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health (9). These compounds were dissolved in saline or suspended in saline with polysorbate 80[2] and administered on Days 1, 5, and 9 or daily on Days 1–9. The compounds showed no significant activity as indicated by the ratio of the mean or median survival times of the test animals over those of the control animals expressed as a percentage (% T/C).

Since it had been shown (3) that the Walker carcinosarcoma 256 tumor system was sensitive to sparsomycin (I), Compounds II–VII (see Table I for NSC numbers) were evaluated in this system. The test was conducted according to the standard Walker 256 protocol (10), where an inoculum of tumor homogenate containing 10^6 viable cells was implanted intramuscularly in random-bred albino rats. The compounds were dissolved in saline or suspended in hydroxypropyl cellulose[3] and administered daily on Days 3–6. Mean tumor weight was the evaluation parameter expressed as % T/C wherein a value ≤42 was the criterion for activity.

Sparsomycin displayed antitumor activity in this protocol at a



Scheme II

[1] It was later found that thionyl chloride in methanol gave superior yields and purer products.
[2] Tween 80, Sigma Chemical Co.
[3] Klucel, Ben Venue Laboratories.

Table II—Experimental Data

Compound	Melting Point (Solvent)	Procedure	Yield, %	Optical Rotation $[\alpha]_D$ (Temperature, Concentration in g/100 ml, Solvent)	Formula	Analysis, % Calc.	Found
II	96.5–103.5° (Benzene)	B	44	−78.79° (28.0°, 0.84, ethanol)	$C_{14}H_{18}ClNO_2S_2$	C 50.67 H 5.46 N 4.22	51.02 5.14 4.15
III	73–77° (Benzene)	B	39	−95.54° (28.5°, 0.81, ethanol)	$C_{15}H_{21}NO_2S_2$	C 57.84 H 6.80 N 4.50	57.35 6.91 4.56
IV	65–70° (Benzene)	B	26	−111.40° (21.5°, 0.91, ethanol)	$C_{14}H_{19}NO_2S_2$	C 56.53 H 6.44 N 4.71	56.30 6.63 4.68
V	102–104.5° (Benzene)	B	42	−123.75° (20.8°, 0.75, ethanol)	$C_{12}H_{17}NO_3S_2$	C 50.15 H 5.96 N 4.88	50.21 6.06 4.77
VI	72–84° (Toluene)	B	49	−64.40° (21.0°, 0.69, ethanol)	$C_{15}H_{21}NO_2S_2$	C 57.84 H 6.80 N 4.50	57.76 6.72 4.47
VII	103–108°	B	30	−48.68° (22.3°, 0.66, dimethyl sulfoxide)	$C_{16}H_{23}NO_4S_2$	C 53.76 H 6.48 N 3.92	53.66 6.53 3.87
X	110–115° (Ethanol)	A	30	−73.79° (29.0°, 0.77, ethanol)	$C_{15}H_{18}ClNO_3S_2$	C 50.06 H 5.04 N 3.89	50.18 4.87 3.90
XI	75.5–78.5° (Isopropanol)	A	36	−95.17° (28.0°, 0.82, ethanol)	$C_{15}H_{19}NO_3S_2$	C 55.36 H 5.88 N 4.30	55.32 5.54 4.24
XII	105–108.5° (Ethyl acetate)	A	38	+1.45° (27.0°, 0.97, chloroform)	$C_{17}H_{23}NO_5S_2$	C 52.96 H 6.02 N 3.64	52.96 6.23 3.60
XIII	91–93° (*n*-Butanol)	A	40	−98.09° (22.5°, 0.68, ethanol)	$C_{16}H_{21}NO_3S_2$	C 56.60 H 6.23 N 4.12	56.44 6.13 4.10
XIV	101–105° (*n*-Butanol)	A	53	−82.54° (29.0°, 0.83, ethanol)	$C_{16}H_{21}NO_3S_2$	C 56.60 H 6.24 N 4.13	56.51 6.07 4.19
XV	80–82.5° (*n*-Butanol)	A	33	−125.61° (21.0°, 0.76, ethanol)	$C_{13}H_{17}NO_4S_2$	C 49.50 H 5.44 N 4.44	49.57 5.54 4.39

dose level of 0.8 mg/kg with % T/C = 19[4]. Therefore, the analogs (II–VII) were tested at dose levels ranging from 1.6 to 0.2 mg/kg, but no activity was displayed. At higher dose levels ranging from 160 to 40 mg/kg, only Compounds II, III, and V exhibited tumor inhibition (Table I), but it was not sufficient to meet the criterion for antitumor activity in this protocol.

EXPERIMENTAL[5]

Table II gives the experimental data for the individual compounds, and Tables III and IV give their NMR[6] and IR spectral characteristics. Chemical shifts are given in parts per million (δ) downfield from tetramethylsilane, the internal standard. IR spectra[7] were recorded as paraffin oil mulls. For dry column chromatography, 50 g of silica gel (activity III)[8] was used per 1 g of compound.

***S*-(Methylthiomethyl)cysteine (VIII)**—L-Cystine (25.0 g, 0.1 mole) in 500 ml of liquid ammonia was treated with small pieces of sodium metal until a blue color was retained for several minutes. Then 19.3 g (0.2 mole) of chloromethyl methyl sulfide was added dropwise. The solution was allowed to evaporate overnight, and the residue was dissolved in water and extracted with chloroform. The aqueous phase was then cooled to 5° and acidified to pH 5 with 6 *N* HCl. The solid was filtered and dried overnight at 36° under vacuum to give 23.9 g (66% yield) of VIII, mp 220° dec.; $[\alpha]_D^{28.5}$ −29.91° (c 1.06 g/100 ml in 6 *N* HCl); IR: 2900 (broad, NH_3), 2120 (NH_3), and 1600 (CO_2) cm^{-1}.

Anal.—Calc. for $C_5H_{11}NO_2S_2$: C, 33.13; H, 6.18; N, 7.73. Found: C, 33.00; H, 6.10; N, 7.92.

***S*-(Methylthiomethyl)cysteine Methyl Ester Hydrochloride (IX-HCl)**—To 17.5 g (0.094 mole) of the amino acid (VIII) slurried in 125 ml of methanol was added hydrogen chloride gas until saturated. The mixture was refluxed for 20 min and then stirred at ambient temperature for 2 hr. The solvent was removed under vacuum, and the residue was triturated with ether to give 14.0 g (64% yield) of solid. The crude product was recrystallized from isopropanol and dried under high vacuum at 46° for 1 hr to give an analytical sample of IX-HCl, mp 128–131° dec.; $[\alpha]_D^{28}$ −15.2° (c 1.14 g/100 ml in ethanol); NMR[9]: δ 2.14 (s, 3H, SCH_3), 3.20 (d, 2H, CH_2S), 3.78 (s, 3H, CO_2CH_3), 3.85 (s, 2H, SCH_2S), 4.28 (t, 1H, $HC—CH_2$), and 8.76 (broad, 3H, NH_3); IR: 2900 (broad, NH_3), 2000 (NH_3), and 1735 (C=O ester) cm^{-1}.

Anal.—Calc. for $C_6H_{14}ClNO_2S_2$: C, 31.10; H, 6.09; N, 6.04. Found: C, 31.32; H, 6.05; N, 6.18.

Alternative Procedure—To 15 ml of methanol at −25° was added dropwise 3.95 ml (0.055 mole) of thionyl chloride. Then 9.06 g (0.05 mole) of the amino acid (VIII) was added in small portions, while the temperature was maintained below −5°. The mixture was allowed to warm to room temperature, then heated at 45° for 4 hr, and finally allowed to remain at room temperature overnight. It was filtered and the filtrate evaporated under vacuum. The residue was then treated with ether to give 9.75 g (84% yield) of solid. Recrystallization from isopropanol gave a material identical to that obtained in the original procedure.

General Preparation of Compounds X–XV, Procedure A—

[4] Determined at these laboratories in a control experiment.
[5] Optical rotations were obtained with a Perkin-Elmer 141 polarimeter, and melting points (uncorrected) were obtained with a Thomas-Hoover capillary apparatus. Combustion analyses were performed by Galbraith Laboratories, Knoxville, Tenn.
[6] Varian A-60A spectrometer.
[7] Perkin-Elmer 521 spectrophotometer.
[8] Woelm, Waters Associates, Framingham, Mass.
[9] In dimethyl sulfoxide-d_6.

Table III—NMR Data (δ, Parts per Million) with Tetramethylsilane Reference and $CDCl_3$ as Solvent

Compound	SCH_3	CH_2S	OH[a]	SCH_2S	CH_2O	$HC-CH_2$	CH=CH	NH	CO_2CH_3	Aromatic	Other
II	2.16 (s)	2.95 (d)	3.50 (Broad)	3.72 (s)	3.88 (d)	4.24 (Broad)	6.48 (d) 8.01 (d) J = 16 Hz	6.72 (d)	—	7.32 (m)	—
III	2.17 (s)	2.95 (d)[b]	3.38 (Broad)	3.72 (s)	3.85 (d)	4.20 (Broad)	6.38 (d) 7.96 (d) J = 16 Hz	6.63 (d)	—	7.32 (m)	2.41 (s)
IV	2.13 (s)	2.94 (d)	4.0 (Broad)	3.70 (s)	3.83 (d)	4.0 (Broad)	6.50 (d) 7.62 (d) J = 16 Hz	6.98 (d)	—	7.30 (m)	—
V	2.18 (s)	2.95 (d)	3.75 (Broad)	3.71 (s)	3.84 (d)	4.25 (Broad)	6.40 (d) 7.42 (d) J = 16 Hz	6.81 (d)	—	6.50 (m)β 7.42 (s)α	—
VI	2.15 (s)	2.94 (d)	4.09 (Broad)	3.70 (s)	3.85 (d)	4.09 (Broad)	6.47 (d) 7.62 (d) J = 16 Hz	6.92 (d)	—	*AA'BB'* (q) 7.23 J = 8 Hz	2.33 (s)
VII[c]	2.15 (s)	2.89 (d of d)[b]	4.62 (t)	3.75 (s)	3.70 (m)	4.13 (m)	6.70 (d) 7.80 (d) J = 16 Hz	7.67 (d)	—	6.90 (m)	3.78 (s) 3.84 (s)
X	2.18 (s)	3.22 (d)[b]	—	3.72 (s)	—	5.08 (m)	6.67 (d) 8.09 (d) J = 16 Hz	7.42[d]	3.82 (s)	7.42 (m)	—
XI	2.18 (s)	3.22 (d)[b]	—	3.73 (s)	—	5.08 (m)	6.64 (d) 7.76 (d) J = 16 Hz	7.13 (d)	3.82 (s)	7.44 (m)	—
XII	2.14 (s)	3.16 (d)[b]	—	3.67 (s)	—	5.0 (m)	6.67 (d) 7.90 (d) J = 16 Hz	6.92[d]	3.78 (s)	6.92 (m)	3.78 (s)
XIII	2.13 (s)	3.19 (d)[b]	—	3.69 (s)	—	5.03 (m)	6.57 (d) 7.68 (d) J = 16 Hz	7.19[d]	3.77 (s)	*AA'BB'* (q) 7.26 J = 8 Hz	2.33 (s)
XIV	2.15 (s)	3.20 (d)	—	3.68 (s)	—	5.03 (m)	6.42 (d) 7.99 (d) J = 16 Hz	6.72 (d)	3.82 (s)	7.38 (m)	2.42 (s)
XV	2.14 (s)	3.18 (d)	—	3.67 (s)	—	5.0 (m)	6.48 (d) 7.47 (d) J = 16 Hz	6.97 (d)	3.78 (s)	7.43 (s)α 6.50 (m)β	—

[a] Exchangeable with D_2O. [b] Extra splitting due to the presence of an asymmetric center. [c] In $CDCl_3$–dimethyl sulfoxide-d_6 (5:1). [d] Resonates at the same frequency as the aromatic protons.

Compound IX-HCl was dissolved in water and treated with sodium bicarbonate until the foaming ceased and the pH was 8. It was then extracted with chloroform, dried over magnesium sulfate, and evaporated under vacuum to give IX as an oil.

To a solution of 0.034 mole of *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline in 230 ml of tetrahydrofuran was added 0.031 mole of the appropriate acrylic acid and 0.031 mole of IX. The mixture was then stirred at 35° for 4 days, filtered if necessary, and evaporated under vacuum. The residue was triturated with petroleum ether to a solid, which was then purified by recrystallization. In the case of XV, dry column chromatography over silica gel with carbon tetrachloride–benzene (1:3) preceded recrystallization.

General Preparation of Compounds II–VII, Procedure B—To 0.04 mole of lithium borohydride in 75 ml of tetrahydrofuran at room temperature was slowly added 0.02 mole of the appropriate ester (X–XV) in 75 ml of tetrahydrofuran. Cooling was used to maintain the temperature below 30°. After addition, the mixture was stirred at room temperature for 22 hr. The solution was then cooled in a water bath, and 23 ml of 6 *N* HCl was added dropwise while the temperature was maintained below 30°. After addition, the mixture was stirred at ambient temperature for 1 hr; then the tetrahydrofuran was removed under vacuum. The residue was diluted to 100 ml with water, extracted with chloroform, dried over potassium carbonate, and evaporated under vacuum to an oil. This oil was triturated with petroleum ether, and the residue was purified by recrystallization in the case of II. Dry column chromatography with silica gel followed by recrystallization provided Compounds III–VII.

Table IV—IR Data (Centimeters^{-1}) from Paraffin Oil Mulls

Compound	C=O Amide (Amide I Band)	Amide II Band	C=O (Ester)	NH	OH
II	1610	1520	—	3200–3500	3200–3500
III	1625	1545	—	3110–3510	3110–3510
IV	1620	1530	—	3100–3500	3100–3500
V	1615	1520	—	3100–3400	3100–3400
VI	1625	1535	—	3110–3510	3110–3510
VII	1630	1550	—	3110–3410	3110–3410
X	1625	1545	1740	3310	—
XI	1615	1525	1735	3330	—
XII	1620	1535	1735	3270	—
XIII	1615	1520	1750	3335	—
XIV	1620	1535	1740	3330	—
XV	1610	1520	1738	3330	—

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[x] To whom inquiries should be directed.

Structure Determination of the Anorexic Agent Mazindol

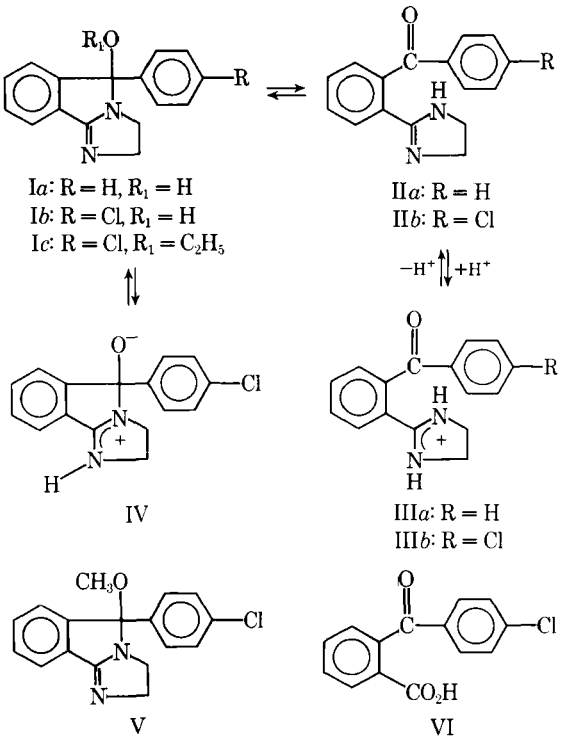
SANDOR BARCZA and WILLIAM J. HOULIHAN [x]

Abstract □ The anorexic agent mazindol was shown to exist as the carbinolamine tautomer 5-*p*-chlorophenyl-2,3-dihydro-5*H*-imidazo[2,1-*a*]isoindol-5-ol in solution and in the solid state. The latter was established by diffuse UV reflectance spectroscopy.

Keyphrases □ Mazindol—structure determination, diffuse UV reflectance spectroscopy □ Structure determination—mazindol in solution and solid state, diffuse UV reflectance spectroscopy □ UV spectroscopy, diffuse reflectance—structure determination, mazindol

It was reported (1) that condensation of 2-benzoylbenzaldehyde with ethylenediamine, followed by oxidation, gave a $C_{16}H_{14}N_2O$ product that can be formulated either as 5-phenyl-2,3-dihydro-5*H*-imidazo[2,1-*a*]isoindol-5-ol (I*a*) or the tautomeric form 2-(2-imidazolin-2-yl)benzophenone (II*a*). Near IR spectral measurement in chloroform gave OH and NH absorption in nearly equal amounts, indicating the presence of about a 1:1 mixture of I*a* and II*a* for the base form. UV study at varying pH in alcohol demonstrated that the protonated form exists as the benzophenone tautomer III. The X-ray single-crystal structure analysis (2) of the hydrobromide salt confirmed that III is also the preferred tautomeric form in the solid state (Scheme I).

In these laboratories the 4-chloro analog (mazindol) of III was prepared (3); and because of the commercial application[1] of the base form of this substance, it was of interest to determine the tautomeric form (I*b* or II*b*) in solution and the solid form.



Scheme I

DISCUSSION

As reference compounds, 5-chlorophenyl-5-methoxy-2,3-dihydro-5*H*-imidazo[2,1-*a*]isoindole[2] (V) was selected as a model for tautomeric form I*b* and 2-*p*-chlorobenzoylbenzoic acid (VI) was

[1] Mazindol is the active ingredient in the anorexic agent Sanorex.

[2] The structure of the deschloro analog of V, 5-methoxy-5-phenyl-2,3-dihydro-5*H*-imidazo[2,1-*a*]isoindole, was established by Metlesics *et al.* (1).

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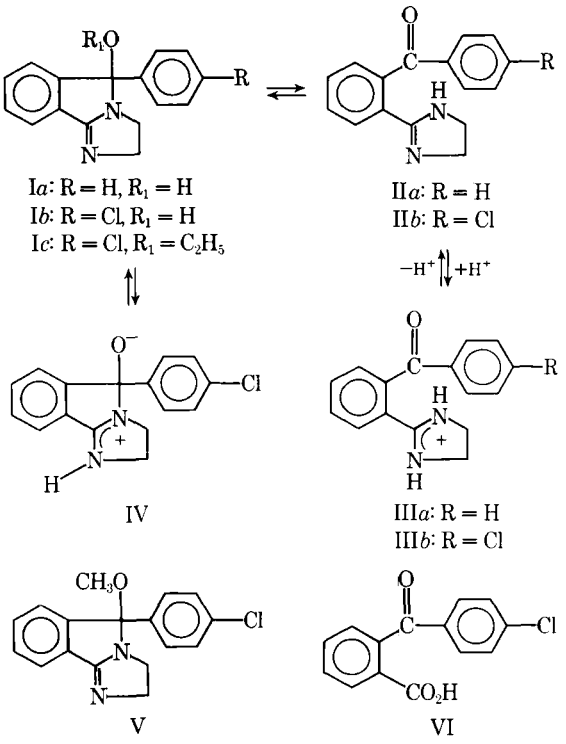

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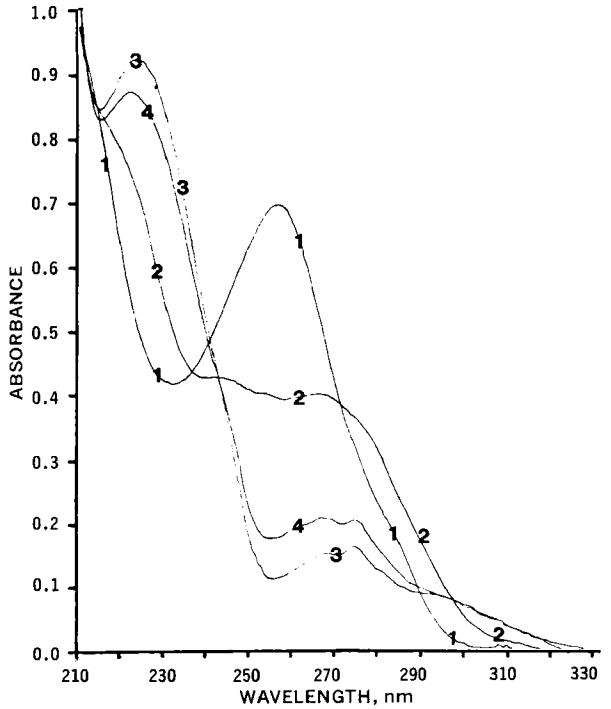

Figure 1—*UV spectra in 95% ethanol at 4.0 × 10^{-5} M of: 1, VI; 2, III*b; *3, V; and 4, I*b.

selected for tautomer II.

Figure 1 contains the UV spectra of mazindol (curve 4), its hydrochloride (curve 2), and the reference compounds V and VI at equimolar concentration in ethanol. Comparison of mazindol with V (curve 3) reveals that the spectra of both substances are nearly identical while the hydrochloride of mazindol exhibits a curve more similar to VI (curve 1). These data are in agreement with assignment of the base form of mazindol as the cyclic tautomer 5-*p*-chlorophenyl-2,3-dihydro-5*H*-imidazo-[2,1-*a*]isoindol-5-ol (I*b*).

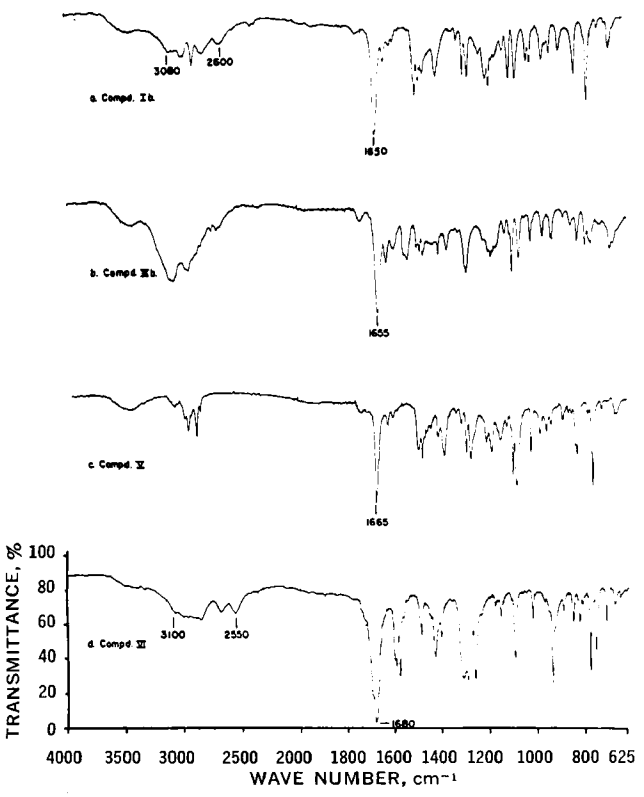

Figure 2—*IR spectra in potassium bromide.*

Table I—^{13}C-NMR Chemical Shifts

Carbon	Assignment[a], ppm[b]	
	I*b*[c]	V[d]
2	41.0*	42.2*
3	59.8*	61.0*
5	87.5	93.1
5a	154.0	150.5
6	121.9	123.2
7	131.2	131.8
8	128.6	129.8
9	123.6	124.4
9a	129.7	129.3
9b	166.3**	167.8
1′	140.0	138.2
2′,6′	127.8	128.2
3′,5′	128.1	128.9
4′	132.4	135.5**
OCH_3	—	50.6

[a] The assignments that are interchangeable are marked (*) while those that are uncertain are marked (**). All assignments are based on model compounds, ^{13}C-NMR shift theory, and intensity considerations. [b] Shifts are in parts per million downfield from tetramethylsilane and are accurate to ±0.1 relative and ±0.5 absolute. [c] Measured in dimethyl sulfoxide-d_6 at 17 mg/ml and 50%. The shifts are calculated from that of the central solvent peak, taken as 39.6 ppm. [d] Measured in $CDCl_3$ at 400 mg/ml and about 45°. The shifts are calculated from hexamethyldisiloxane internal standard, taken as 2.0 ppm.

The hydrochloride of mazindol appears to exist mainly as the benzophenone tautomer III*b*. The possible contribution by some protonated I*b* or the presence of some of the 5-ethoxy analog Ic cannot be excluded because of the relatively weak absorption in the "benzophenone" portion (about 260 nm) of the spectrum.

Additional evidence that the base form of mazindol exists in solution as the tautomer I*b* was obtained by comparing the ^{13}C-NMR spectra of mazindol with V. In $CDCl_3$ the C-5 signal of V was found at 93.1 ppm downfield from tetramethylsilane. Mazindol in dimethyl sulfoxide-d_6 gave a signal at 87.5 ppm that can be assigned to the C-5 atom of I*b*. No signals were found in the 160–200-ppm range of the spectrum, indicating the absence of the benzophenone[3,4] tautomer II*b* (Table I).

To obtain information on the tautomeric form in the solid state, the IR spectra of mazindol, III*b*, V, and VI in potassium bromide were obtained (Fig. 2). Inspection of the mazindol (I*b*) curve reveals an intense band at 1650 cm^{-1}, which could be due either to a C=O or a C=N stretching vibration (4). Unfortunately, no clear-cut distinction could be made between these possibilities since the hydrochloride II*b*[5], a substance containing a C=O group and a C=N group, absorbed at 1655 cm^{-1}, and V, a substance containing only the C=N group, absorbed at 1665 cm^{-1}.

The cluster of bands located at 3080–2600 cm^{-1} in the mazindol spectrum is similar in shape and position to the hydrogen-bonded OH bands at 3100–2550 cm^{-1} found in VI. These bands can be attributed to OH or NH hydrogen-bonded stretching vibrations (4) that could be present in either tautomer I*b* or II*b*, respectively. It is also possible that the zwitterionic form IV may be contributing to this cluster of bands since the overall shape is somewhat similar to the hydrochloride III*b*.

Since IR could not be used to determine the tautomeric form of mazindol in the solid form, diffuse UV reflectance was utilized[6]. The spectra of powdered mazindol, III*b*, and V from 400 to 270 nm are given in Fig. 3. For mazindol and V (curves 1 and 2), reflectance remained high from 400 to 355 nm where a smooth and steep drop occurred to about 0% reflectance at 325 nm. The hydrochloride (curve 3) gave a steep drop in reflectance at 380 nm with plateaus at 365, 355, and 335 nm and 0% reflectance at 310 nm. The

[3] The uncertainties of the values obtained by ^{13}C-NMR spectra are probably ±5%.

[4] The C=O atom of benzophenone is found at 194.8 ppm downfield from tetramethylsilane; G. C. Levy and G. L. Nelson, "Carbon-13 Nuclear Magnetic Resonance for Organic Chemists," Wiley–Interscience, New York, N.Y., 1972, p. 114.

[5] The free base I*a* and the hydrochloride of III*a* are reported to give an IR band in KBr at 1660 and 1665 cm^{-1}, respectively (1).

[6] Numerous unsuccessful attempts were made in this laboratory to obtain mazindol in a crystal form suitable for X-ray single-crystal analysis.

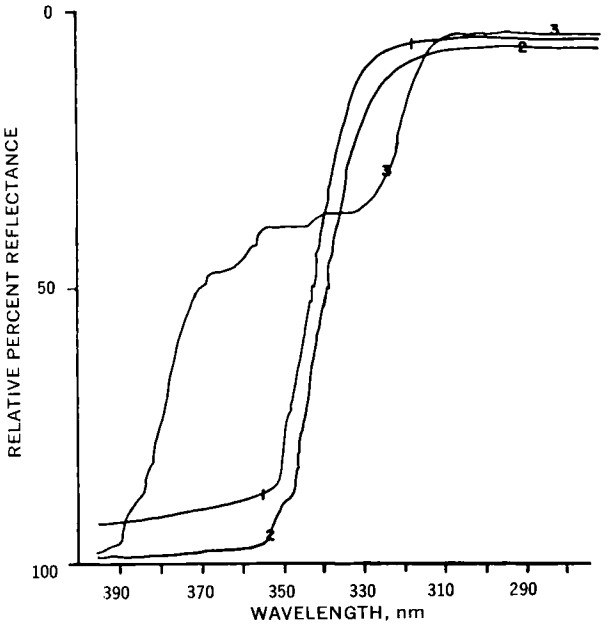

Figure 3—*Diffuse UV reflectance spectra of: 1, V; 2, Ib; and 3, IIIb.*

nearly superimposable spectrum of mazindol and V suggest that the former substance exists in the solid form solely as the imidazo[2,1-*a*]isoindole tautomer I*b*. The absence of any absorption in the 380-nm region rules out contribution by the benzophenone tautomer II*b*[7].

EXPERIMENTAL[8]

Apparatus—The diffuse reflectance spectra were measured on a recording spectrometer[9] equipped with a No. 1411 reflectance attachment that included an integrating sphere. The powdered samples were compressed at room temperature into the 2.54-cm (1-in.) diameter × 0.3-cm (0.125-in.) deep circular cavity of the aluminum reflectance attachment. Prior to running the samples, a new light source was installed and the baseline (100% reflectance) was established between 400 and 270 nm using magnesium oxide as a reference standard.

The solution UV spectra were determined with a recording spectrometer[10] between 210 and 400 nm at a scan speed of 5 Å/sec.

The ^{13}C-NMR spectrum of I*b* was taken on a spectrometer[11] equipped with a pulse-Fourier transform package[12]. The frequency was set at 25.2 MHz with broad band ^{1}H decoupling at δ 7 and a sweep width of 5000 Hz with 12-μsec pulse width. The internal D-lock on dimethyl sulfoxide-d_6 was at 15.4 MHz, and the spectrum was obtained from 32K scan over 10 hr.

The ^{13}C-NMR spectrum of V was taken on a spectrometer (Bruker) equipped with a pulse-Fourier transform accessory that included a signal averager and a computer providing 4K data points in the time domain. The frequency was set at 22.63 MHz with broad band ^{1}H decoupling at 90 MHz and a typical sweep width of 5000 Hz with 15-μsec pulse width. Hexafluorobenzene was added for locking, and hexamethyldisiloxane was used as the internal standard taken as 2 ppm downfield of tetramethylsilane.

5 - *p* - Chlorophenyl - 5 - methoxy - 2,3 - dihydro - 5*H* - imidazo[2,1-*a*]isoindole (V)—A stirred mixture of 56.0 g (0.20 mole) of I*b* and 1.0 liter of dry methanol was treated at room temperature with a stream of anhydrous hydrogen chloride gas until the solution was saturated. The solution was then refluxed for about 18 hr and concentrated *in vacuo* to about 500 ml. The concentrated solution was then slowly poured into 500 ml of a saturated sodium bicarbonate solution cooled in an ice bath. The mixture was extracted with four 200-ml portions of ether. The ether layer was dried with anhydrous potassium carbonate, filtered, and concentrated *in vacuo*. Crystallization from ether–pentane (1:1) gave 32.3 g (55%) of V, mp 104–105°; NMR[13] ($CDCl_3$): δ 3.15 (3H, s, OCH_3), 2.65–3.50 (2H, m, CH_2N), and 4.18–4.46 (2H, m, $=NCH_2$).

Anal.—Calc. for $C_{17}H_{15}ClN_2O$: C, 68.3; H, 5.1; Cl, 11.9; N, 9.1. Found: C, 68.5; H, 5.3; Cl, 12.0; N, 9.3.

4'-Chloro-2-(2-imidazolin-2-yl)benzophenone Hydrochloride (III*b*)—A solution of 5.0 g of I*b* in 100 ml of anhydrous methanol–tetrahydrofuran (1:1) was cooled in an ice bath and treated with a stream of anhydrous hydrogen chloride gas until it was saturated. The solution was then concentrated *in vacuo*, and the crystalline residue was filtered and washed with anhydrous ether. The solid was dried *in vacuo* at room temperature to give 5.2 g (92%) of III*b*, mp 175–177°.

Anal.—Calc. for $C_{16}H_{14}Cl_2N_2O$: C, 60.0; H, 4.4; Cl, 22.1; N, 8.8. Found: C, 60.1; H, 4.5; Cl, 22.2; N, 8.7.

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[7] A search of the chemical literature failed to uncover any examples where diffuse UV spectroscopy was used to distinguish tautomeric forms in organic solids. The relative ease of determining these spectra on solids, particularly those unsuitable for X-ray crystal analysis, warrants further application of this technique to tautomeric problems.

[8] Melting points were determined in a Thomas-Hoover capillary melting-point apparatus and are uncorrected.

[9] Cary model 14M.

[10] Cary model 15.

[11] Varian XL-100-15.

[12] Transform Technology Inc.

[13] The ^{1}H-NMR spectrum was obtained on a Varian Associates A-60 spectrometer.

Composite Membrane Estradiol Implant

RENÉ BLOCH ˣ, PERRY F. KRAICER*, HANA BINDER, and ESTHER LÖBEL

Abstract □ The diffusion and permeability of large organic molecules through polymeric films were investigated, specifically the passage of estrogen through films of polyethylene, silicone, or a composite of these two films. Such systems are of interest because of the possibility of constructing a biocompatible implant that releases a hormone at a controlled and desired rate. Diffusion and permeability coefficients through implants of a desirable size—successfully tested in rats—were measured by the "time lag" method of diffusion, and solubility coefficients were determined by the method of sorption kinetics. Independently derived values were in good agreement. An interesting observation was made with implications for the controlled release from membrane-based drug delivery systems. Permeability coefficients of estrogen are orders of magnitude higher when estrogen is present as a solid rather than in a dilute solution, while diffusion coefficients are equal. This finding is explained by the considerably higher partial vapor pressure of estrogen in the former case. In fact, control of the partial vapor pressure of the encapsulated drug is a powerful method for controlling its release rate.

Keyphrases □ Estradiol—polyethylene–silicone composite implant, diffusion, permeability, and solubility coefficients, release patterns □ Polymeric films—polyethylene–silicone composite, use as estradiol timed-release implant □ Estrogens—release patterns of estradiol from polyethylene–silicone composite implant □ Implants—estradiol polyethylene–silicone composite, use as a timed-release device

The development of an implant that would permit timed release of steroids has been the subject of numerous publications (1–7). In general, it has been found that the best encapsulating material for this application is a silicone elastomer. The physicochemical properties governing the rate of steroid release through a silicone membrane are most favorable (6–8). Other characteristics of the silicone that recommend its use for implants are its compatibility with tissues, mechanical strength, and flexibility.

For certain applications, however, the rate of release from a silicone elastomer implant is too great. For example, for estrogens it would be advantageous to restrict the rate of release to micrograms per day while retaining the outer coating of silicone elastomer with its excellent biocompatibility.

In this study, it was shown that a composite membrane of polyethylene coated with silicone elastomer is suitable for the fabrication of such implants. Estradiol (free alcohol) was chosen since it is the natural estrogen and therefore would be rapidly catabolized.

The diffusion behavior of estradiol in polyethylene and silicone membranes was evaluated by using both dry powdered estradiol and ethanolic solutions. On the basis of the diffusion behavior, implants suitable for rats were constructed and tested.

THEORETICAL

Transport across membranes as used in this study is governed by Fick's first law. From this law the release rate of a device containing a permeant at a defined concentration, C, can be computed by inserting the appropriate boundary conditions:

$$\frac{dM_t}{dt} = \frac{ADK\,\Delta C}{l} \qquad \text{(Eq. 1)}$$

where M_t is the mass of drug released, dM_t/dt is the steady-state release rate, A is the membrane surface, the product DK is the membrane permeability as D is the diffusion coefficient, and K is the partition coefficient.

The mathematical expression for the time lag, L (11), which can be derived from differentiation of the steady-state expression, is:

$$L = \frac{l^2}{6D} \qquad \text{(Eq. 2)}$$

By plotting M_t against time, the intercept L of the steady-state portion of the plot on the time axis gives the diffusion coefficient D. From the slope of the linear part dM_t/dt, the permeability coefficient DK can be computed.

Independently, the diffusion and solubility coefficients were determined by adsorption and desorption of steroid vapors into the membrane.

The following equation for sorption kinetics (11) limited to very short times was used:

$$\frac{M_t}{M_\infty} = \frac{4}{l}\frac{\sqrt{Dt}}{\sqrt{\pi}} \qquad \text{(Eq. 3)}$$

where M_t is the amount of drug adsorbed at time t, and M_∞ is the maximum amount of drug adsorbed into the membrane. From this equation, by plotting M_t/M_∞ *versus* t or $\sqrt{t}$, the diffusion coefficient can be computed from the initial slope.

The solubility, S, of the steroid in the membrane can be calculated from the maximum amount adsorbed, M_∞, per unit membrane (grams of steroid per gram of polymer).

In cases where a polyethylene membrane was coated with a silicone membrane, it was assumed that the diffusion through the silicone membrane could be neglected because the permeability through the polyethylene was a few orders of magnitude smaller than through the silicone and was, therefore, the rate-determining step.

EXPERIMENTAL

Diffusion and Sorption Experiments—Composite membranes of polyethylene[1] coated with a silicone rubber[2] were prepared by dissolving the silicone elastomer latex plus polymerization catalyst in chloroform at a concentration of 25% and by spreading a thin film on a polyethylene sheet with a membrane casting knife. A uniform layer was obtained with a dry thickness of 60–80 μm. Heat-sealed polyethylene bags were dipped in the same silicone coating solution and then floated overnight on a saturated aqueous sodium chloride solution, when polymerization of the silicone was complete.

The diffusion of estradiol[3] through silicone[4] rubber membranes, polyethylene films, and composite polyethylene membranes coated with silicone was measured by using a thermostated diffusion cell of Perspex in which the membrane separated two compartments. One compartment was filled with a dilute solution of estradiol in ethanol, and the other was filled with ethanol. Solutions were mag-

[1] Plastics U.S. Ltd., Holon, Israel.
[2] Silastic 382 medical grade elastomer, Dow Corning Corp., Midland, Mich.
[3] Ikapharm, Israel.
[4] Midland Silicones Ltd., Reading, U.K.

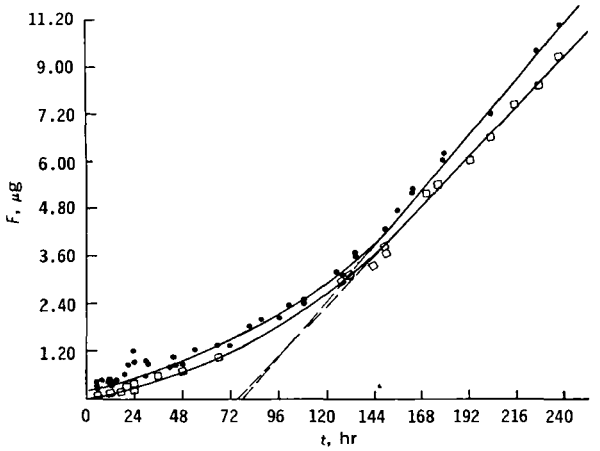

Figure 1—*Total amount, F, of estrogen diffusing through a polyethylene bag as a function of time. Key: ●,□, duplicate experiments with two polyethylene bags.*

netically stirred. The amount of estrogen appearing after diffusion into the second compartment as a function of time was analyzed. Diffusion experiments were made at 25°.

In a different type of experiment, 25 mg of estradiol was sealed by heat into a polyethylene bag with a surface area of 2 cm^2. The estrogen diffusing from the inside of the bag into a bath of alcohol as a function of time was analyzed. Some of these bags were coated with silicone.

Adsorption and desorption of estrogen on membranes were measured with an automatic recording vacuum ultramicrobalance[5] (with an accuracy of $\pm 1 \times 10^{-9}$ g) connected to a vacuum system containing solid estrogen. The membrane adsorbing or desorbing estrogen as a function of time was weighed. Estradiol was assayed by UV spectrophotometry[6] or by GC[7] of the diacetyl derivative (9, 10).

Animal Experiments—The ability of double-membrane encapsulated implants to provide physiological levels of estrogen was tested in ovariectomized adult albino females rats[8] (4–5 months of age). They were ovariectomized through a pair of lumbar incisions using ether anesthesia. Their vaginal smears showed persistence of leukocytes for 21 days.

A subcutaneous implant containing estradiol was made; after ether anesthesia, a small incision was made in the skin at the nape of the neck. The implant was placed under the scalp, between the ears.

RESULTS AND DISCUSSION

Diffusion and Sorption Kinetics—A typical time lag diffusion experiment showing the flux of estradiol through two polyethylene membranes as a function of time is illustrated in Fig. 1. The total

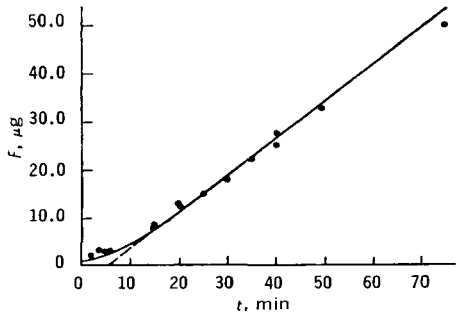

Figure 2—*Total amount, F, of estrogen diffusing through a silicone membrane as a function of time.*

[5] Cahn electrobalance.
[6] Beckman UV spectrophotometer.
[7] Packard model 803 gas chromatograph.
[8] University of Tel-Aviv breeding colony.

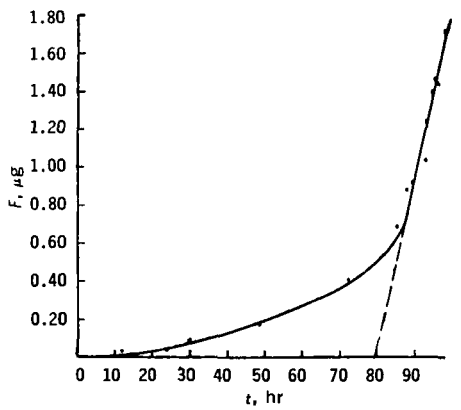

Figure 3—*Total amount, F, of estrogen diffusing through a polyethylene membrane coated with silicone as a function of time.*

areas of the polyethylene bags were 3.7 and 3.2 cm^2, respectively. The membrane thickness was 96 μm. From this plot, the diffusion coefficient, in addition to the solubility of estradiol in polyethylene (being equivalent to its partition coefficient from a saturated solution), was computed.

Figure 2 shows a similar experiment for a silicone membrane having an area of 19.6 cm^2 and a thickness of 70 μm. The same plot for a composite polyethylene–silicone membrane, the polyethylene being exposed to the estrogen solution, is shown in Fig. 3. A typical sorption kinetic experiment showing the fractional amount of estrogen adsorbed and desorbed in a silicone membrane as a function of time is illustrated in Fig. 4. Figure 5 shows the same data for a polyethylene film; the fractional release is plotted as a function of the square root of time and straight lines are observed. From these plots, diffusion coefficients and solubilities for estrogen in the various membranes used also were computed. In the case of sorption experiments, the estrogen was always in a solid form, but its relatively high vapor pressure (10^{-5} mm Hg) enabled it to be evaporated into the vacuum system connected to the microbalance, which was used to weigh the membranes during the experiment.

A summary of fluxes, diffusion coefficients, and solubilities com-

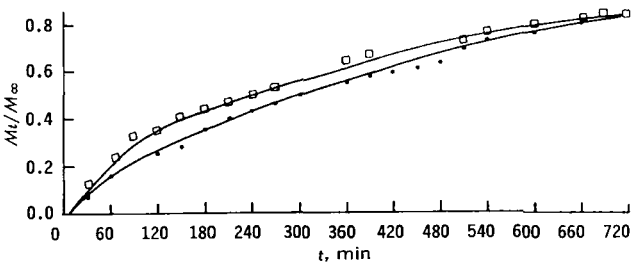

Figure 4—*Adsorption and desorption of estrogen on a silicone membrane as a function of time. Key: □, adsorption; and ●, desorption.*

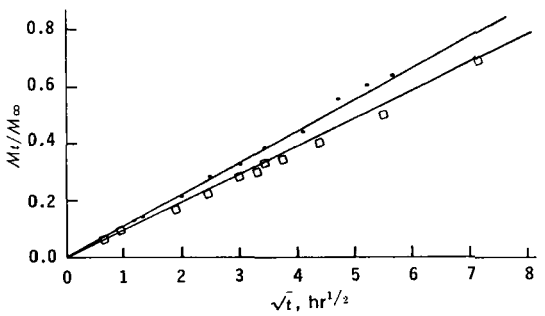

Figure 5—*Adsorption and desorption of estrogen on a polyethylene membrane as a function of time. Key: □, adsorption; and ●, desorption.*

Table I—Solubilities, Diffusion Coefficients, and Fluxes for Estrogen[a] through Silicone Membranes at Thickness l

Method[b]	$S \times 10^3$	$D \times 10^7$ cm²/min	$J \times 10^2$ μg/min cm²	C, %	l, μm	Temperature	System
s.k.	2.27	1.76			70	33°	Membrane–vapor
s.k.	2.55	1.45			70	30°	Membrane–vapor
s.k.	1.07	1.65			70	26°	Membrane–vapor
s.k.	0.99	1.05			70	25°	Membrane–vapor
t.l.		6.9	6	0.166	50	25°	Solution–membrane solvent
t.l.		4.7	5	0.128	70	25°	Solution–membrane solvent
t.l.		3.6	3.5	0.102	70	25°	Solution–membrane solvent

[a] The feed system is either an estrogen solution in alcohol or solid estrogen in powder form. [b] s.k. = sorption kinetics; t.l. = time lag method.

Table II—Solubilities, Diffusion Coefficients, and Fluxes for Estrogen[a] through Polyethylene Membranes of Thickness l

Method[a]	$S \times 10^4$	$D \times 10^9$ cm²/min	$J \times 10^5$ μg/min cm²	C, %	l, μm	Temperature	System
s.k.	0.94	1.28			96	25°	Membrane–vapor
s.k.	1.75	2.7			96	25°	Membrane–vapor
s.k.	1.34	3.7			96	25°	Membrane–vapor
s.k.	1.67	2.3			96	25°	Membrane–vapor
t.l.		3.4	0.77	0.102	96	25°	Solution–membrane solvent
t.l.		6.7	1.97	0.105	38	25°	Solution–membrane solvent
t.l.		4.4	1.4	0.105	53	25°	Solution–membrane solvent
t.l.		3.3	26		96	25°	Solid–membrane solvent
t.l.		3.4	28		96	25°	Solid–membrane solvent

[a] The feed system is either an estrogen solution in alcohol or solid estrogen in powder form. [b] s.k. = sorption kinetics; t.l. = time lag method.

puted from both time lag and sorption kinetic experiments is given in Tables I and II. It can be seen that the diffusion coefficients obtained from the time lag method and from sorption kinetics were different. From sorption kinetics, they were 2.5 times smaller than those found by the time lag method for silicone membranes and 1.3 times smaller for polyethylene membranes. The fact that different results for the diffusion coefficient have been obtained by different measurement methods is not extraordinary but in line with other findings (12, 13). A plausible explanation (12) is that nonstationary states of flow may not yield meaningful values of the interdiffusion coefficients for internally viscous polymeric media, since relaxation processes may take place in the polymer matrix which, in turn, influence the diffusion coefficient. In a steady-state measurement, all relaxation processes must have died away before a steady state can be established. The diffusion coefficient for estrogen through a silicone membrane depends on its concentration in solution.

Table III summarizes a comparison of fluxes of estrogen in a 0.1% alcohol solution through a polyethylene membrane and through a silicone-coated polyethylene membrane. It is apparent that coating with silicone does not affect the flux of the drug through polyethylene. Furthermore, solid estradiol yields a flux that is 35 times higher than that of estrogen in a solution of 0.1%.

Table III—Comparison of Fluxes of Estrogen through a Polyethylene Membrane and through a Polyethylene Membrane Coated with Silicone

J, g cm^{-2} min^{-1}	Diffusion System
7.7×10^{-6}	Solution–polyethylene–solvent
7.6×10^{-6}	Solution–polyethylene:silicone–solvent
2.6×10^{-4}	Solid–polyethylene–solvent
2.3×10^{-4}	Solid–polyethylene:silicone–solvent

Table IV—Diffusion Coefficients Obtained from Time Lag Experiments as a Function of Estrogen Concentration for Silicone Membranes with a Defined Thickness

l, μm	$D \times 10^7$, cm²/min	C, %
70	3.6	0.102
70	4.7	0.128
50	7	0.166

(A saturated solution of estradiol in alcohol is 0.23%.) This surprising result is explained by the higher vapor pressure of pure solid estradiol than of the 0.1% estrogen dissolved in alcohol and by the change of phase from a gas to a liquid system.

The fluxes of estradiol through the membrane as a function of its concentration in a solution and membrane thickness were also determined and found to be in agreement with Fick's law (Table IV). The distribution coefficient of estrogen between alcohol and silicone as computed from the time lag experiment was found to be 0.47; for polyethylene, it was 0.011.

Animal Experiments—In a preliminary study, 12 rats were used. Two days after insertion of silicone-coated polyethylene bags containing estradiol, their vaginal smears became estrous—cornified cells plus some nucleated epithelial cells. The smears remained persistently estrous for 6 months. At autopsy, the uteri were found to be large. The only sign of pathology was gross enlargement of the pituitary in eight of the 12 rats. The persistence of the estrous response indicated that the implants were capable of sustained release of effective quantities of estradiol.

In a second test, eight rats received estradiol implants; six others served as ovariectomized controls. From the 2nd day after implantation, all eight rats bearing implants had persistently cornified vaginal smears. The rats were killed 10 days after insertion of the implants. Weights of the uteri, pituitaries, and adrenals were recorded. The animals were also weighed on the day of ovariectomy, the day of implantation, and the day of autopsy.

The control animals weighed 178 ± 4.6 g on the day of ovariectomy. By the day of autopsy, they had gained 49.7 ± 2.3 g, an average of 1.4 g/day. Estrogen-treated rats weighed 187 ± 4.6 g initially; by 25 days after ovariectomy, they had gained 34.2 ± 2.7 g, an average of 1.4 g/day. During the 10 days following insertion of the implants, they lost 18.2 ± 2.7 g. This weight loss is believed to reflect the increased activity and decreased appetite associated with estrus. Ovariectomized rats are known to fatten due to relative inactivity.

Table V—Effect of an Estradiol Implant on Organ Weights (in mg/100 g Body Weight) of Ovariectomized Rats

Organ	With Implant	Control
Uterus (drained)	235 ± 15.4	47 ± 3.4
Pituitary	9.3 ± 0.36	6.1 ± 0.44
Adrenals	26 ± 4.4	21 ± 0.87

The estradiol released from the implant maintained the weights of the uteri, increased pituitary weights significantly, and had no effect on the adrenal weights (Table V). Five of the eight uteri from the estrogen-treated rats were distended with fluid at autopsy—further evidence of unopposed estrogen action. All of these findings confirm that the implants were able to maintain a physiologically significant level of estradiol in the treated rats.

No inflammations were observed after 8 months of subcutaneous residence.

CONCLUSIONS

The diffusion, flux, and solubility coefficients of estrogen are 100, 1000, and 10 times, respectively, higher through silicone than through polyethylene. The values of diffusion coefficients of estrogen through the membranes derived independently by the time lag method and by sorption kinetics were in good agreement. Coating of silicone on polyethylene had no influence whatever on the flux of estrogen through it.

An interesting phenomenon observed during this study was that permeability coefficients through the membranes were orders of magnitude higher when estrogen was present as a solid rather than in solution, while the diffusion coefficients found by the time lag method were equal in both cases. This finding can be explained by the fact that a larger concentration gradient within the membrane is established in the former case partly because the absolute concentration may be higher in the vapor phase above the solid and partly because of larger distribution coefficient in favor of the membrane. The existence of this phenomenon indicates that control of the partial vapor pressure of the encapsulated drug is a powerful method for controlling its release rate.

An implant of desirable size regulating the flux of estrogen through a polyethylene membrane, coated with silicone, was prepared based on these findings. This implant can be successfully used in rats.

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Hypothermic Response following Administration of 2-Amino-4-pentenoic Acid (Allylglycine)

R. L. LILIEDAHL, C. F. RYAN, and R. W. PIEPHO ˣ

Abstract □ Intraperitoneal administration of allylglycine resulted in a hypothermic response in rats. At a dosage of 90 mg/kg, a significant decrease in temperature was noted at 4 and 8 hr after administration, but no significant difference was noted at 12 hr. The same dose administered intraperitoneally to decapitated rats did not result in a hypothermic response, and intraventricular administration resulted in a rapid onset of hypothermia after 1 hr. These findings are indicative of a central site of action for the allylglycine-induced hypothermia. Quantitative assay of hypothalamic monoamines (norepinephrine, serotonin, and dopamine) did not show any significant changes at 4, 8, and 12 hr postadministration when compared to controls. A significant decrease in hypothalamic γ-aminobutyric acid was noted at each of these time points when compared to controls. These data suggest an important role for γ-aminobutyric acid in mammalian thermoregulatory control.

Keyphrases □ Allylglycine (2-amino-4-pentenoic acid)—effect of intraperitoneal and intraventricular administration on hypothermic response in rats □ Hypothermia—effect of intraperitoneal and intraventricular administration of allylglycine, rats □ Thermoregulation—effect of allylglycine intraperitoneal and intraventricular administration on hypothermic response, rats

Feldberg and Myers (1) first proposed that body temperature was regulated by a fine balance in the release of catecholamines and serotonin in the anterior hypothalamus. The hypothesis was based on the responses to intraventricular injection of these agents in the cat. Subsequent investigations showed that there is a distinct species variation in the temperature response, both in magnitude and direction of change, when these agents are administered by the intraventricular route. Feldberg and Lotti (2) showed that intraventricular administration of small doses of norepinephrine in the rat resulted in an increased body temperature, while larger doses had an opposite action. Intraventricular or intracisternal administra-

The estradiol released from the implant maintained the weights of the uteri, increased pituitary weights significantly, and had no effect on the adrenal weights (Table V). Five of the eight uteri from the estrogen-treated rats were distended with fluid at autopsy—further evidence of unopposed estrogen action. All of these findings confirm that the implants were able to maintain a physiologically significant level of estradiol in the treated rats.

No inflammations were observed after 8 months of subcutaneous residence.

CONCLUSIONS

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Hypothermic Response following Administration of 2-Amino-4-pentenoic Acid (Allylglycine)

R. L. LILIEDAHL, C. F. RYAN, and R. W. PIEPHO ˣ

Abstract □ Intraperitoneal administration of allylglycine resulted in a hypothermic response in rats. At a dosage of 90 mg/kg, a significant decrease in temperature was noted at 4 and 8 hr after administration, but no significant difference was noted at 12 hr. The same dose administered intraperitoneally to decapitated rats did not result in a hypothermic response, and intraventricular administration resulted in a rapid onset of hypothermia after 1 hr. These findings are indicative of a central site of action for the allylglycine-induced hypothermia. Quantitative assay of hypothalamic monoamines (norepinephrine, serotonin, and dopamine) did not show any significant changes at 4, 8, and 12 hr postadministration when compared to controls. A significant decrease in hypothalamic γ-aminobutyric acid was noted at each of these time points when compared to controls. These data suggest an important role for γ-aminobutyric acid in mammalian thermoregulatory control.

Keyphrases □ Allylglycine (2-amino-4-pentenoic acid)—effect of intraperitoneal and intraventricular administration on hypothermic response in rats □ Hypothermia—effect of intraperitoneal and intraventricular administration of allylglycine, rats □ Thermoregulation—effect of allylglycine intraperitoneal and intraventricular administration on hypothermic response, rats

Feldberg and Myers (1) first proposed that body temperature was regulated by a fine balance in the release of catecholamines and serotonin in the anterior hypothalamus. The hypothesis was based on the responses to intraventricular injection of these agents in the cat. Subsequent investigations showed that there is a distinct species variation in the temperature response, both in magnitude and direction of change, when these agents are administered by the intraventricular route. Feldberg and Lotti (2) showed that intraventricular administration of small doses of norepinephrine in the rat resulted in an increased body temperature, while larger doses had an opposite action. Intraventricular or intracisternal administra-

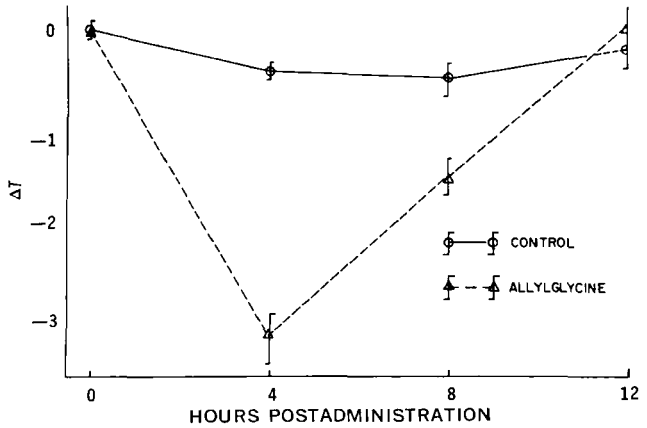

Figure 1—*Rectal temperature changes in unanesthetized rats for control and allylglycine-treated (intraperitoneally) groups. Brackets denote the standard error of the mean for each point* (n = 6).

tion of serotonin results in a decreased body temperature.

In 1971, Sgaragli and Pavan (3) reported that intracisternal administration of γ-aminobutyric acid could also affect thermoregulation by causing a decrease in body temperature. However, a previous study found that γ-aminobutyric acid injected into the hypothalamus in much smaller doses resulted in an increase in body temperature (4). Thus, γ-aminobutyric acid, which is believed to act as an inhibitory neurotransmitter at central synapses (5–7), also appears to play some part in mammalian thermoregulation.

Allylglycine[1] (2-amino-4-pentenoic acid) was shown (8) to possess convulsant activity when administered to rats. Dose–response relationships in mice (9) showed that a dose of 100 mg/kg was fatal to one out of 20 animals tested, while 17 of 20 died at 200 mg/kg. Subsequent studies (10) indicated that allylglycine, administered at a dose of 150 mg/kg to rats, caused an inhibition of glutamic acid decarboxylase and resulted in a 40% decrease in the concentration of γ-aminobutyric acid in the cerebral cortex.

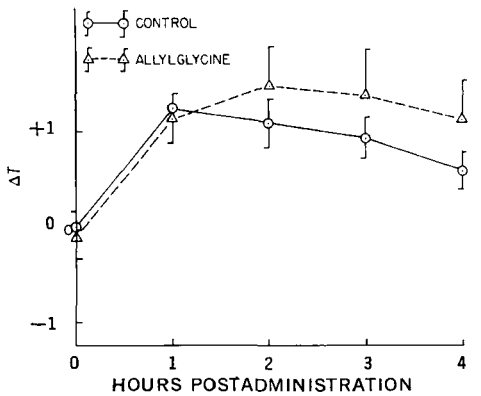

Figure 2—*Rectal temperature changes in decapitated rats for control and allylglycine-treated (intraperitoneally) groups. Brackets denote the standard error of the mean for each point* (n = 4).

[1] Sigma Chemical Co., St. Louis, Mo.

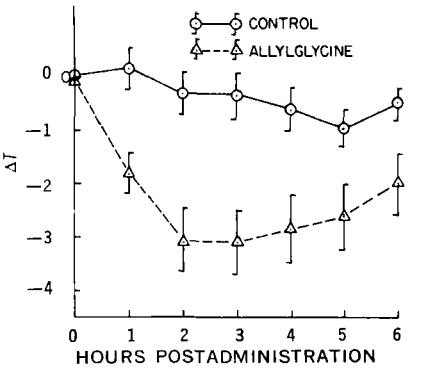

Figure 3—*Rectal temperature changes in unanesthetized rats for control and allylglycine-treated (23 μg intraventricularly) groups. Brackets denote the standard error of the mean for each point* (n = 6).

In light of these known effects of neurotransmitter alteration on thermoregulation, the purposes of this investigation were to: (*a*) administer allylglycine to the rat and study the effects of decreased γ-aminobutyric acid level on thermoregulation, and (*b*) correlate these effects with changes in the central levels of the neurotransmitters believed to be important in thermoregulation—*viz.*, norepinephrine, serotonin, and dopamine.

EXPERIMENTAL

Male albino rats[2], 200–250 g, were housed for 1 week and fasted overnight prior to experimentation. The experiments were conducted over a 12-hr period each day, from 7:30 am to 7:30 pm to avoid problems of circadian fluctuation; such fluctuations in hypothalamic levels of γ-aminobutyric acid are known to occur (11). Rats were housed individually on the day of the experiment to prevent interaction with other rats.

Ambient temperature (23.5 ± 0.5°) was maintained throughout the experiment. The colonic temperature of each rat was recorded every 4 hr over 12 hr *via* a rectal temperature probe[3] inserted 6 cm into the colon, allowing 2 min for adjustment to proper temperature while the animal was restrained in a small plastic cage. All drugs were administered in a volume of 0.1 ml/100 g body weight. Saline solution, 0.9%, served as a control.

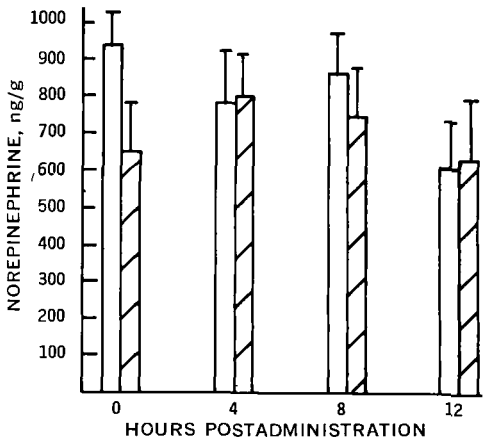

Figure 4—*Level of norepinephrine in the hypothalamus of control (□) and allylglycine-treated (intraperitoneally) (▨) groups. Brackets denote the standard error of the mean for each point* (n = 6).

[2] Sasco Co., Omaha, Neb.
[3] Yellow Springs Instrument Co.

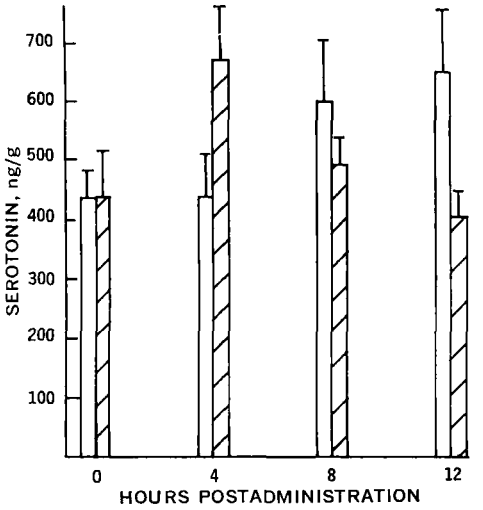

Figure 5—*Level of serotonin in the hypothalamus in control* (□) *and allylglycine-treated* (*intraperitoneally*) (▨) *groups. Brackets denote the standard error of the mean for each point* (n = 6).

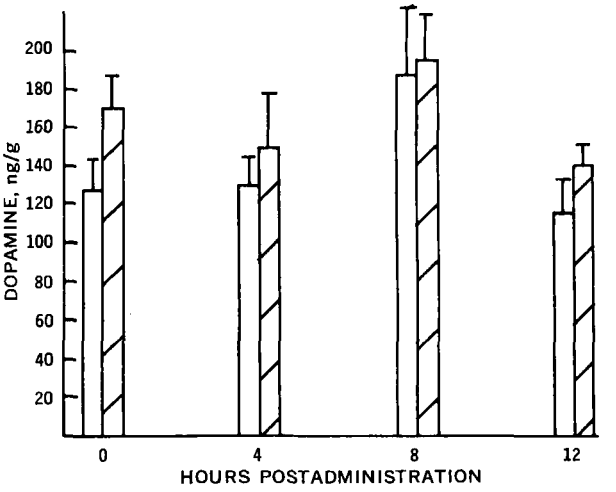

Figure 6—*Level of dopamine in the hypothalamus in control* (□) *and allylglycine-treated* (*intraperitoneally*) (▨) *groups. Brackets denote the standard error of the mean for each point* (n = 6).

To determine the central or peripheral sites affected by allylglycine in causing a temperature alteration, decapitated rats were used following the procedure of Weis (12). Rats were anesthetized with pentobarbital in a dose of 75 mg/kg ip. The trachea was cannulated and artificial respiration was initiated. The jugular veins and carotid arteries were ligated. The neck was clamped with a powerful hemostat to compress all ancillary vessels, and the head was cut off. The animals then were allowed to stabilize for 2 hr. Intraperitoneal injections of 0.9% saline or allylglycine were administered, and rectal temperature was monitored for 4 hr as previously described.

Cannulas were implanted into the lateral ventricles of anesthetized rats for the intraventricular administration of allylglycine. A 26-gauge needle, cut to proper length with a blunt end, was implanted stereotaxically into the ventricle at a point 2.5 mm lateral and 1 mm posterior to the bregma and secured to the skull with dental cement. A 30-gauge needle served as an injection cannula and occluding stylet. Then 1 week after the cannulas were implanted, 10 μl of either 0.9% saline or 0.2 *M* allylglycine was injected over 2 min and rectal temperature was monitored as previously described. Methylene blue was injected following the experiment for confirmation of the injection site.

Following the recording of rectal temperatures, 12 rats (six control and six allylglycine treated) were sacrificed by decapitation at 0, 4, 8, and 12 hr postadministration. The hypothalamus was removed following the procedure of Glowinski and Iversen (13), immersed in liquid nitrogen, and weighed. The tissue samples were wrapped in aluminum foil and stored at −20°. A method of solvent extraction coupled with alumina adsorption (14) was utilized for the assay of the monoamines. The fluorometric enzyme-coupled technique of Graham and Aprison (15) was used for γ-aminobutyric acid assay.

RESULTS

The intraperitoneal administration of allylglycine at 90 mg/kg caused a significant ($p < 0.05$) decrease in rectal temperature, $\Delta T = -3.1$ and $-1.1°$, when compared to the control group at 4 and 8 hr, respectively, postadministration (Fig. 1). A small, but insignificant, increase in rectal temperature was noted at 12 hr in the allylglycine-treated group when compared to the control.

At the dose of 90 mg/kg, compared to the 150-mg/kg dose used by Alberici *et al.* (10), no convulsions were seen and only three of 24 animals showed increased activity postadministration. All animals survived the 12-hr test period.

Intraperitoneal administration of allylglycine to the decapitated rat did not significantly decrease rectal temperature (Fig. 2). No significant change was noted at 1, 2, 3, and 4 hr after administration of either allylglycine or 0.9% saline.

Intraventricular administration of 10 μl of 0.2 *M* allylglycine resulted in a rapid decrease in temperature, with a significant ($p < 0.05$) decrease after 1 hr that lasted through 4 hr postadministration when compared to controls (Fig. 3). No significant temperature changes were noted in the control animals.

The data from the monoamine assay of the hypothalamic tissue show that allylglycine administration, 90 mg/kg ip, does not result in any significant changes in the levels of norepinephrine (Fig. 4), serotonin (Fig. 5), or dopamine (Fig. 6) at 4, 8, or 12 hr postadministration. However, a significant ($p < 0.05$) decrease in the hypothalamic γ-aminobutyric acid level does occur at 4, 8, and 12 hr postadministration (Fig. 7) when compared to control animals.

DISCUSSION

The absence of a significant decrease in rectal temperature in the decapitated animals treated with allylglycine suggests that this agent is causing its hypothermic action by acting at central thermoregulatory sites. With access to these sites eliminated by decapitation, any influence of the central thermoregulatory centers is removed, so the possibility that allylglycine causes hypothermia by affecting purely peripheral thermoregulatory mechanisms can be ruled out. This supposition of a purely central role in allylglycine

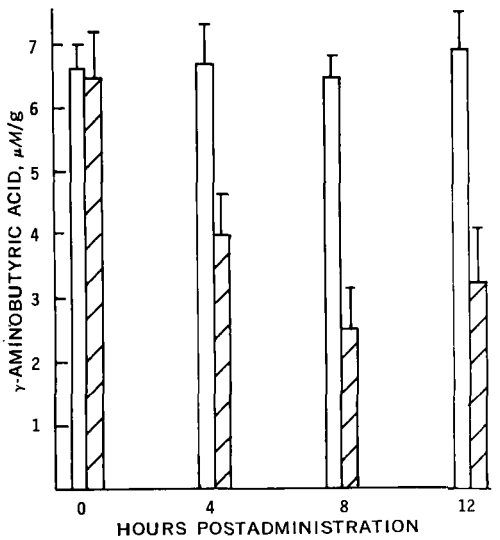

Figure 7—*Level of γ-aminobutyric acid in the hypothalamus in control* (□) *and allylglycine-treated* (*intraperitoneally*) (▨) *groups. Brackets denote the standard error of the mean for each point* (n = 6).

hypothermia is strengthened by the rapid onset of decreased temperature noted in intraventricularly injected rats (1 hr) as compared to intraperitoneally injected ones (4 hr).

The current theory of thermoregulatory control by the delicate balance of release of the two opposing monoamines, norepinephrine and serotonin, from the hypothalamus was mentioned previously. The results of this study indicate that no significant changes in the hypothalamic levels of norepinephrine or serotonin occurred at any time point after allylglycine and suggest that allylglycine affects monoamine turnover but causes no changes in content or that alteration of central γ-aminobutyric acid content produces this hypothermia. However, studies have shown that it is difficult to correlate a change in body temperature with a change in the hypothalamic levels of the putative neurotransmitters—*viz.*, norepinephrine and serotonin (16, 17). Here again, it may not be possible to propose a direct correlation between the temperature response seen and the hypothalamic tissue levels of the monoamines at the chosen time points of 4, 8, and 12 hr postadministration of allylglycine.

The studies of Alberici *et al.* (10) indicated that at 2–2.5 hr after administration of allylglycine, ultrastructural changes in the nerve endings of the cerebral cortex occurred, resulting in a reduction in the number of synaptic vesicles. Such alterations affected Fraction D of the glutamic acid decarboxylase-rich (nonaminergic) nerve endings obtained through subfractionation on a sucrose gradient (18). The fact that glutamic acid decarboxylase is inhibited *in vivo* and *in vitro* by allylglycine suggests that the decrease in γ-aminobutyric acid induced by this agent is directly related to the effect on the synthesizing enzyme, glutamic acid decarboxylase.

Allylglycine would also be expected to inhibit the high activity of this enzyme in the hypothalamus at the dose of 90 mg/kg as compared to the 150-mg/kg dose used by Alberici *et al.* (10), since significant ($p < 0.05$) decreases in the γ-aminobutyric acid content of the hypothalamic tissue were found in this study (Fig. 7). The greatest decrease in γ-aminobutyric acid, as compared to controls, was observed at 8 hr after allylglycine administration, followed by the 12- and 4-hr time points. Alberici *et al.* (10) completed their studies at 2–2.5 hr after allylglycine administration, since this was the time point noted for the greatest convulsive activity at the 150-mg/kg dosage. The time course of action of these effects on neuronal integrity of γ-aminobutyric acid neurons and glutamic acid decarboxylase is consistent with the time course of the allylglycine-induced hypothermia.

The greatest decrease in rectal temperature occurred 4 hr after allylglycine administration. The temperature of the animals then began to increase; at 12 hr, the allylglycine-treated group had an increased body temperature when compared to the control group. Thus, maximal allylglycine-induced hypothermia (at 4 hr) does not correlate with the greatest decrease in hypothalamic γ-aminobutyric acid levels (8 hr), but this maximum decrease at 4 hr may be caused by the ultrastructural changes in the nerve endings that could occur prior to the change noted at 4 hr. If the terminals are damaged and there is a reduction in the number of synaptic vesicles that store γ-aminobutyric acid, the initial release of γ-aminobutyric acid from the terminals may cause a postsynaptic stimulation of mechanisms that results in an inhibition of thermogenesis and/or an increased dissipation of body heat. The continued inhibition of glutamic acid decarboxylase by allylglycine can explain the further decrease in γ-aminobutyric acid levels at 8 and 12 hr. However, it would appear that after the 4-hr time point, a mechanism is brought into action to conserve the body heat and results in an increased body temperature, which results in the overcompensation noted at 12 hr after allylglycine administration.

In summary, the results indicate an involvement of γ-aminobutyric acid in mammalian thermoregulatory control. When synthesis of this putative neurotransmitter is impaired, a concomitant decrease in body temperature is noted. The mechanistic role of γ-aminobutyric acid in this system remains to be elucidated, although initial studies (19) point toward a modulatory role for this putative neurotransmitter in thermoregulation.

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Fluorescence Reactions of Fluorescamine with Levodopa and Its Derivatives: Fluorescence Assay of 3-Methoxy-4-hydroxyphenylalanine in Levodopa Dosage Forms

LAWRENCE K. CHENG, MICHAEL LEVITT *, and HO-LEUNG FUNG ˣ

Abstract □ A simple fluorometric method for the quantitation of 3-methoxy-4-hydroxyphenylalanine, either alone or in the presence of levodopa, is presented. Fluorescence is developed by reaction with fluorescamine. The interactions of this reagent with levodopa and a number of its derivatives under various experimental conditions were studied. Negligible fluorescence was obtained with levodopa, dopamine, levodopa benzyl ester, and 6-hydroxydopamine.

Keyphrases □ Levodopa and derivatives—reaction with fluorescamine, fluorometric analysis for 3-methoxy-4-hydroxyphenylalanine □ Fluorescamine—reactions with levodopa and derivatives, fluorometric analysis for 3-methoxy-4-hydroxyphenylalanine □ Fluorometry—reaction of fluorescamine with levodopa and derivatives, analysis for 3-methoxy-4-hydroxyphenylalanine

Fluorescamine[1], 4-phenylspiro[furan-2(3*H*),1-phthalan]-3,3-dione, is a new fluorogenic reagent recently introduced (1) for the assay of primary amines in the picomole range. This compound reacts rapidly, in milliseconds, with primary amino groups to produce an intense fluorophore while excess fluorescamine is hydrolyzed in seconds to form nonfluorescent products. At present, the fluorescence assays of catecholamines, including levodopa and dopamine, in biological fluids (2, 3) are rather tedious and are sensitive to changes in reaction conditions. It is, therefore, desirable to investigate whether simpler fluorescence procedures may be achieved by the reaction of fluorescamine with catecholamines.

The present report deals with studies on the fluorescence reactions of fluorescamine with levodopa and a number of its derivatives under various experimental conditions. It is shown that 3-methoxy-4-hydroxyphenylalanine, and possibly other *O*-methylated catecholamines, can be assayed with a very simple procedure using fluorescamine.

EXPERIMENTAL

Materials—Levodopa[2], 3-methoxy-4-hydroxyphenylalanine[3], dopamine hydrochloride[4], 6-hydroxydopamine[5], and glycine[6] were used without further purification. Levodopa benzyl ester was synthesized in this laboratory and confirmed by elemental analysis (C, H, N) and IR. All other reagents were analytical grade, except acetone, which was spectrophotometric grade.

Fluorescence Development—Stock solutions of test compounds were prepared in 0.1 *N* HCl in concentrations about 20-fold in excess of that in the final reaction mixture. All glassware used was cleaned by presoaking in 20% nitric acid for at least 24 hr. One hundred microliters of each test solution was added to 1.5 ml of buffer solution (0.05 *M* sodium borate except where indicated) in a test tube and mixed thoroughly. While the test tube was held on a mixer[7], 0.5 ml of fluorescamine in acetone (30 mg/100 ml) was added rapidly and the resultant solution was immediately mixed vigorously for at least 30 sec. Rapid addition of the reagent and thorough mixing thereafter are essential. The fluorescence of the solutions was read on a spectrophotofluorometer[8] at an excitation wavelength of 390 nm and an emission wavelength of 480 nm.

When commercial levodopa capsules were assayed, the contents of four capsules were emptied, mixed, and weighed. An equivalent amount of one capsule was weighed and dissolved in 250 ml of water. The solution was vigorously shaken for 10 min and filtered. Samples not subjected to alumina separation were directly assayed using the volumes described previously. Other samples, after a fivefold dilution, were treated with alumina to remove levodopa; the general procedure of Anton and Sayre (4) was used except that disodium (ethylenedinitrilo)tetraacetate was omitted. About 96% of levodopa was removed after this procedure. After alumina treatment, 0.5 ml of the resultant solution was used for fluorescence development.

RESULTS AND DISCUSSION

Table I shows the approximate net fluorescence readings obtained when the levodopa compounds[9] were separately mixed with fluorescamine at pH ~9.2. Both glycine and 3-methoxy-4-hydroxyphenylalanine reacted with the reagent to give significant fluorescence, whereas all other compounds gave readings only slightly higher than the blank (blank fluorescence = 0.3–0.4). Since it has been shown (1) that fluorescamine reacts indiscriminately with the primary amino group, whether it is present in a simple amine, an amino acid, a peptide, or a protein molecule, the inability of these compounds to form fluorophores with fluorescamine was thought to be due to a lack of reaction with the reagent rather than to the production of nonfluorescent reaction products.

At an alkaline pH of 9.2, the oxidation of unprotected catecholamine molecules to form indole-type compounds is extremely rapid. Therefore, it is possible then that the primary amino group in these compounds is effectively destroyed prior to reaction with fluorescamine. Careful deoxygenation of reagent solutions by flushing with nitrogen produced significant fluorescence from le-

[1] Fluram, Roche Diagnostics, Nutley, NJ 07110
[2] Supplied by INTERx Research Corp., Lawrence, KS 66044
[3] Supplied by Dr. A. Pletscher, Hoffmann–La Roche Co., Basel, Switzerland.
[4] Schwarz/Mann, Orangeburg, N.Y.
[5] Pfaltz and Bauer, Inc., Flushing, N.Y.
[6] J. T. Baker, through VWR Scientific, Buffalo, N.Y.
[7] Vari-Whirl mixer, Van-Lab, VWR Scientific, Buffalo, N.Y.
[8] Aminco-Bowman, Silver Spring, Md.
[9] The weak fluorescence (see Table I) developed with levodopa, dopamine, 6-hydroxydopamine, and levodopa benzyl ester might be partly attributed to impurities present in these samples. The readings, therefore, represent upper limits of fluorescence that could be developed with these compounds.

Table I—Approximate Fluorescence Readings of Dopa Compounds after Reaction with Fluorescamine

Compound[a]	Net Fluorescence
Levodopa	0.13
Dopamine	0.11
Levodopa benzyl ester	0.13
6-Hydroxydopamine	~0
3-Methoxy-4-hydroxyphenylalanine	2.90
Glycine	6.86
Catechol	~0

[a] All in a final concentration of about .1 μg/ml.

vodopa and dopamine solutions. However, these readings were highly sensitive to prevailing experimental surroundings and were not consistently reproducible. Unsuccessful attempts have been made to inhibit this possible oxidation by adding a metal chelating agent [disodium (ethylenedinitrilo)tetraacetate, up to 1% in the final reaction solution] or an antioxidant (sodium metabisulfite, up to 3% in the final reaction solution) or by substituting the borate buffer with a sodium bicarbonate buffer at the same pH. Helling and Bollag (5) reported that Pb^{+2} complexes with catechols to form insoluble salts. Addition of a 10-fold excess of lead acetate to a 1-μg/ml levodopa solution did not increase the fluorescence intensity. The fluorescence readings of reaction systems involving levodopa, dopamine, levodopa benzyl ester, and 3-methoxy-4-hydroxyphenylalanine were not significantly altered in the 7.8–9.2 pH range when a sodium borate–hydrochloric acid buffer was employed.

The intense fluorophore formed between fluorescamine and 3-methoxy-4-hydroxyphenylalanine under the present experimental conditions is in sharp contrast to the very weak ones obtained with levodopa and dopamine. The fluorescence spectra for the fluorophore are shown in Fig. 1. 3-Methoxy-4-hydroxyphenylalanine is a major metabolite of levodopa in animals and humans. In the rat, it demethylates to levodopa (6); because of its longer biological half-life compared to the parent compound in humans (7), there is intense interest in studying the feasibility of using 3-methoxy-4-hydroxyphenylalanine as a timed-release form of levodopa in the treatment of parkinsonism.

The present study provides a simple and rapid fluorometric method for the determination of the purity of 3-methoxy-4-hydroxyphenylalanine in the presence of impurities such as nonmethylated catecholamines and other nonamino catechol compounds. Figure 2 shows a fluorescence calibration plot for 3-methoxy-4-hydroxyphenylalanine. Good linearity was obtained up to a concentration of 1 μg/ml of drug in the final reaction mixture. This calibration plot holds even up to a concentration of 10 μg/ml without any significant self-quenching. A 0.1-μg/ml solution gives a fluorescence reading twice that of the blank. The present method compares favorably with the ferricyanide oxidation method (3) for the assay of 3-methoxy-4-hydroxyphenylalanine in the presence of

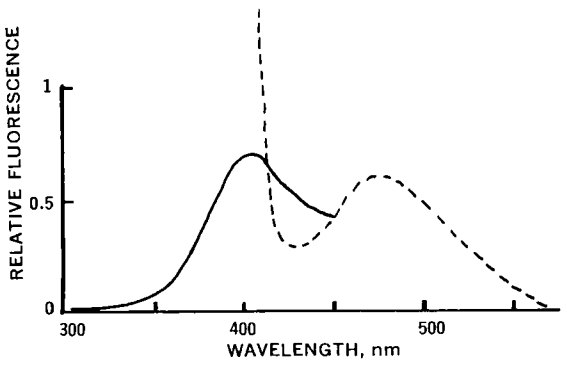

Figure 1—*Excitation (---) and emission (——) spectra (uncorrected) of fluorophore formed between 3-methoxy-4-hydroxyphenylalanine and fluorescamine. Emission wavelength = 480 nm, excitation wavelength = 390 nm, and 3-methoxy-4-hydroxyphenylalanine = 0.29 μg/ml of final reaction mixture.*

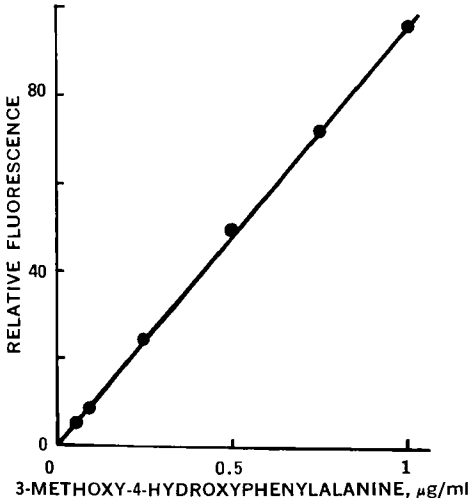

Figure 2—*Calibration plot for the reaction of 3-methoxy-4-hydroxyphenylalanine with fluorescamine.*

levodopa and dopamine in terms of simplicity, rapidity, and sensitivity to 3-methoxy-4-hydroxyphenylalamine. However, in the latter method, the interfering fluorescence due to the same catecholamines is less than that found in the present method.

The utility of the present technique can be illustrated in the assay of impurities in levodopa dosage forms. Watson (8), using a GLC assay, recently showed that 3-methoxy-4-hydroxyphenylalanine is the exclusive impurity in commercial levodopa dosage forms. Capsules of levodopa[10] were assayed for 3-methoxy-4-hydroxyphenylalanine using the present assay, both with and without a prior separation step involving alumina. The content of 3-methoxy-4-hydroxyphenylalanine in these capsules had been determined previously to be 0.35% by the GLC technique (8). Because of the relatively large errors involved in integrating the small peak generated by 3-methoxy-4-hydroxyphenylalanine in the proximity of the parent peak of levodopa, the standard deviation for this GLC-determined value was estimated to be at least 0.05%[11]. Using the present fluorometric method with prior alumina separation, the 3-methoxy-4-hydroxyphenylalanine impurity content was determined to be 0.43 ± 0.03% (mean ± standard deviation, four measurements). Without prior alumina separation, duplicate determinations gave values of 0.52 and 0.52% for 3-methoxy-4-hydroxyphenylalanine in the commercial levodopa capsules. Thus, it is evident that the present fluorometric method is accurate in determining the content of 3-methoxy-4-hydroxyphenylalanine in levodopa dosage form when an alumina separation step is employed before fluorescence development.

The present method possibly could be extended to assay for 3-methoxy-4-hydroxyphenylalanine in plasma and other biological fluids after it has been separated from catecholamines by alumina (4). Where the ratio of 3-methoxy-4-hydroxyphenylalanine to other catecholamines is high, alumina separation may not be required. For example, in the cerebrospinal fluids of parkinsonian patients receiving 3–6 g of levodopa daily, the average levodopa level was found to be 0.3–0.5 nmole/ml, whereas the level of 3-methoxy-4-hydroxyphenylalanine was 2–3 nmoles/ml (9). With this low concentration ratio of levodopa *versus* 3-methoxy-4-hydroxyphenylalanine, the present method could possibly be used directly to determine the concentration of the methylated metabolite in the presence of the parent drug without any significant interference.

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Systematic Identification of Drugs of Abuse I: Spot Tests

ASAAD N. MASOUD

Abstract □ More than 40 of the most commonly encountered street drugs were subjected to several spot tests. These tests were carried out in a special sequence leading to the construction of a flowsheet. Thus, with a limited number of simple tests, it is possible to identify tentatively or narrow down the drug. Since each drug investigated was subjected to all selected tests, whether such tests were developed for this type of compound or not, some unexpected and undocumented results were obtained.

Keyphrases □ Abuse drugs—systematic identification, spot tests, flow chart □ Drugs, abuse—systematic identification, spot tests, flow chart □ Spot tests—drugs of abuse, systematic identification

Spot tests still serve a valuable role in the identification of abused drugs today. Since many of the abused drugs are nonproprietary preparations and are prepared and marketed without any standards or quality control by any authority, they often contain substances other than what they are alleged to contain. Therefore, a fast simple battery of tests to provide preliminary information concerning the drug is needed.

In spite of the limitations of spot tests, such as the occurrence of false positives or false negatives, the lack of specificity, and the difficulty of interpreting some results, they still provide speedy answers to preliminary questions pertaining to these substances. The useful information provided by spot tests includes: *(a)* the definite absence of a compound or a group of compounds, and *(b)* the possible presence of a compound or compounds that belong to a certain group. The positive information provided by these tests will help in the selection of the specific confirmatory tests necessary.

The purpose of this work was to increase the usefulness of spot tests. More than 40 of the most common street drugs were subjected to nine different reagents. The results obtained provided a basis for the systematic classification of these drugs on an identification scheme. They also produced a number of unexpected and undocumented uses for conventional tests. Many false negatives and false positives were encountered and documented. These results increase the value of the tests once they are recognized and considered.

EXPERIMENTAL

Materials—The 43 drugs[1] investigated in this work are grouped in Table I according to their chemical nature and some of the results obtained in this work.

Preparation of Samples—The preparation of the standard references for the spot tests varied according to the test and the form of the drug provided. In general, the spot tests were performed directly on the powdered or liquid forms. For specific tests where no moisture is desired and where the drug has to be in a powdered form, solvents were removed from drugs available only in solution.

Generally, 1–2 mg was used for the tests; however, lysergide was detected in quantities as low as 5 μg. The tests were performed in 10-ml clear glass test tubes, and observation continued for approximately 30 min after the tests were completed.

Reagents and Procedures—The following reagents were prepared as indicated.

Mayer's Reagent (1)—This reagent consists of: mercuric chloride, 0.68 g; potassium iodide, 2.5 g; and distilled water to make 100 ml.

Dragendorff's Reagent (2)—Solution A consists of: bismuth subnitrate, 0.85 g; distilled water, 40 ml; and acetic acid, 10 ml. Solution B consists of: potassium iodide, 8.0 g; and distilled water, 20 ml. To prepare the concentrate, 5 volumes of A and 2 volumes of B are mixed. In this investigation, 20 ml of acetic acid was added to 10 ml of the concentrate, which was then diluted with 100 ml of distilled water (3).

Wagner's Reagent (4)—This reagent consists of: iodine, 1.27 g; potassium iodide, 2 g; and distilled water to make 100 ml. A small amount of the drug is dissolved in a few drops of 10% HCl. The reagent is then added dropwise to the acidic solution. A precipitate is

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Table I—Investigated Drugs

Alkaloids
Atropine
Caffeine
Cocaine hydrochloride
Codeine phosphate
Diacetylmorphine hydrochloride (heroin)
Ephedrine sulfate
Lysergide (lysergic acid diethylamide tartrate)
Mescaline hydrochloride
Morphine sulfate
Nicotine salicylate
Opium
Papaverine
Physostigmine salicylate
Psilocin
Psilocybin
Quinine sulfate
Scopolamine hydrobromide
Strychnine
Yohimbine hydrochloride
Compounds that Give Positive Alkaloidal Reaction
Lidocaine hydrochloride
Meperidine[a]
Methadone hydrochloride
Methaqualone
Methylphenidate hydrochloride
Pentazocine hydrochloride[b]
Phencyclidine hydrochloride
Procaine hydrochloride
Propoxyphene napsylate[c]
Methapyrilene hydrochloride[d]
Barbiturates
Amobarbital
Phenobarbital sodium
Secobarbital
Amphetamines
Amphetamine sulfate
Methamphetamine hydrochloride
Miscellaneous
Aspirin
Benzocaine
Cannabidiol
Cannabinol
Diphenylhydantoin sodium[e]
Glutethimide[f]
Meprobamate
Δ^9-Tetrahydrocannabinol (II)
Thiopental

[a] Demerol. [b] Talwin. [c] Darvon. [d] Dormin. [e] Dilantin. [f] Doriden.

formed with most alkaloids and some other organic nitrogenous compounds. This procedure is followed with the above three alkaloidal reagents.

Marquis Reagent (5)—This reagent consists of: concentrated sulfuric acid, 10 ml; and formaldehyde solution, 40%, 8–10 drops. It is freshly mixed prior to use. An intense purple color is considered a positive; this test is for opiates.

Cobalt Thiocyanate (5)—This reagent is an aqueous solution of 2% cobalt thiocyanate. A flaky blue precipitate is considered a positive; this test is for cocaine and related compounds.

Table II—Alkaloids that Give Negative Tests with One or More Reagents

Compound	Reagent		
	Mayer's	Dragendorff's	Wagner's
Caffeine	−	+	+
Heroin	+	+	−
Ephedrine	−	−	−
Lysergide	−	−	−
Mescaline	−	−	−
Morphine	+	+	−
Psilocin	−	−	+
Psilocybin	−	−	−

Table III—Nonalkaloids that Give Positive Alkaloidal Tests with One or More Reagents

Compound	Mayer's	Dragendorff's	Wagner's
Lidocaine hydrochloride	+	+	+
Meperidine	+	+	+
Methadone hydrochloride	+	+	+
Methaqualone	+	+	+
Methylphenidate hydrochloride	−	+	−
Pentazocine hydrochloride	+	+	+
Phencyclidine hydrochloride	+	+	+
Procaine hydrochloride	−	+	−
Propoxyphene napsylate	+	+	+
Methapyrilene hydrochloride	+	+	+

Zwikker's Test (6)—Solution A consists of 1% cobalt acetate in methanol, and Solution B consists of 5% isopropylamine in methanol. In the absence of water, the test is performed by adding to a small amount of the drug a few drops of A followed by a few drops of B. A blue-violet color is the positive test; this test is for barbiturates.

Mandelin's Test (7)—Ammonium vanadate, 1%, must be added gradually to concentrated sulfuric acid with continuous stirring. The reagent should acquire an orange color. In this investigation, only the formation of an instant conspicuous color is considered positive.

Ehrlich's Reagent (8)—A 0.2% solution of *p*-dimethylaminobenzaldehyde in 65% sulfuric acid is prepared, and 0.2 ml of 5% ferric chloride solution is added to each 100 ml. This reagent decomposes with light and should be prepared weekly. A purple to deep-blue color that develops slowly and becomes more intense with time is a positive; this test is for lysergide.

Duquenois Reagent (5)—Five drops of acetaldehyde and 0.4 g of vanillin are dissolved in 20 ml of alcohol USP. This reagent should be kept in a glass-stoppered bottle and protected from light; it should be discarded upon assuming a deep-yellow color.

To the drug or extract, a few drops of the reagent are added followed by a few drops of concentrated hydrochloric acid, and the mixture is shaken. Then a few drops of chloroform are added and the mixture is shaken again. The test is considered positive if a blue-violet color is developed upon the addition of the hydrochloric acid and the color migrates to the chloroform layer; this test is for marijuana and cannabinoids.

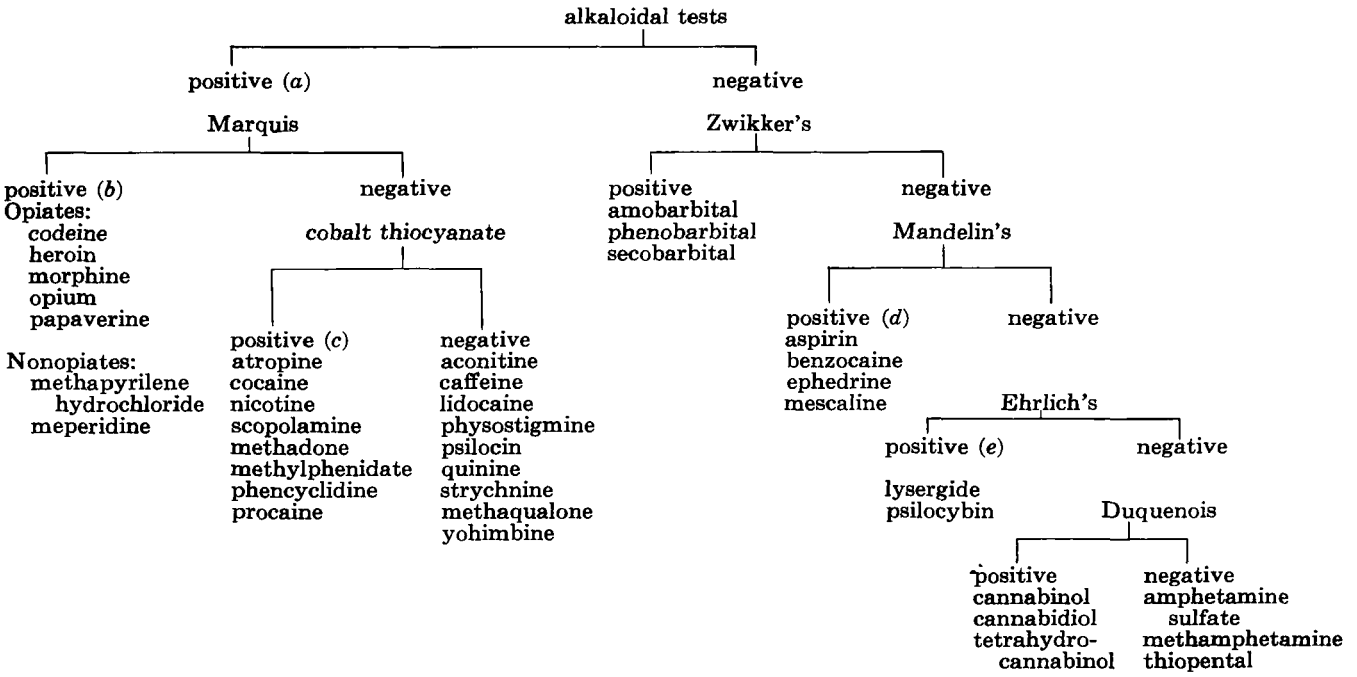
RESULTS AND DISCUSSION

Alkaloidal Reagents—All investigated drugs (Table I) were subjected to Mayer's, Wagner's, and Dragendorff's reagents, and a number of alkaloids gave negative reactions to one or more of them. These alkaloids are listed in Table II. All other alkaloids, namely atropine, cocaine, codeine, nicotine, opium powder, papaverine, physostigmine, quinine, scopolamine, strychnine, and yohimbine, gave a positive test to all three reagents.

A number of nonalkaloids gave positive reactions to one or more of the reagents used (Table III). These compounds have been designated as compounds that give a positive alkaloidal reaction. All

Table IV—Compounds that Give Positive Reaction with Cobalt Thiocyanate Reagent

Atropine	Meperidine
Cocaine hydrochloride	Methadone hydrochloride
Codeine phosphate	Methylphenidate hydrochloride
Heroin	Phencyclidine hydrochloride
Nicotine salicylate	Procaine hydrochloride
Opium	Methapyrilene hydrochloride
Scopolamine hydrobromide	Phenobarbital sodium



Scheme I—Systematic utilization of spot tests. Key: a, *compounds that gave a positive test with any of the three reagents used;* b, *other compounds given in text do not give positive alkaloidal tests;* c, *other compounds listed in Table IV do not give positive alkaloidal tests;* d, *see criteria for a positive Mandelin's reagent in the text; and* e, *psilocin also gives a positive for Ehrlich's reagent but it does give positive alkaloidal tests.*

other nonalkaloids tested gave negative results to these reagents.

Marquis Reagent—This reagent produces a characteristic intense purple color that becomes dark purple and then dark blue-violet with opiates (5–7). Such color was observed as expected with the five opiates tested, *i.e.*, codeine phosphate, heroin, morphine sulfate, papaverine, and opium powder. However, some nonopiates produced colors that are very similar to those produced by opiates. These false positives produced by ephedrine sulfate, methapyrilene hydrochloride, amphetamine sulfate, meperidine, and methamphetamine hydrochloride are not documented in other references known to the author and should be considered when interpreting the results of this test.

Cobalt Thiocyanate Test—This test is described (5) as an identification test for cocaine and procaine hydrochloride (5), but the characteristic blue flaky precipitate was also produced in this investigation with other drugs. Table IV lists all of the drugs that produced a positive test with this reagent. The lack of specificity of this test should be considered. However, these drugs are either alkaloids or compounds that give positive alkaloidal reaction with the exception of phenobarbital sodium.

Zwikker's Test—This test, also referred to as the alkaline cobalt test, was discussed in detail by Clarke (6). According to Clarke, a blue-violet color is produced by barbiturates as well as glutethimide. However, in this laboratory, utilizing the reagents described earlier, only the three barbiturates used (amobarbital, phenobarbital sodium, and secobarbital) produced such color. It was not possible to produce a positive test with glutethimide. The color produced by glutethimide reference standard was gray and was not considered a positive reaction. None of the other drugs investigated produced positive reactions with this test.

Table V—Easily Characterized Colors Produced by Mandelin's Reagent

Compound	Color Produced Instantly	Comments
Ephedrine sulfate	Brick red	Stable
Mescaline hydrochloride	Orange color	Changes to yellow, then green
Aspirin	Green with blue tint	Changes to red-violet
Benzocaine	Red-violet	Stable

Mandelin's Test—This test, also referred to as the ammonium vanadate test, has been described for the analysis of more than 150 different drugs (6, 7, 9–11). Most of these drugs show slowly developing colors that are yellow, orange, green, gray, or shades of each or that change from one to another. The interpretation of such results for the purpose of identification is very difficult and should not be relied upon. Of the drugs that gave negative alkaloidal tests in this investigation, only four compounds gave sharp conspicuous colors instantly (Table V).

Ehrlich's Reagent—This reagent is also referred to as Van Urk's reagent or simply given the name of its main ingredient, *p*-dimethylaminobenzaldehyde (6, 8). It produces a characteristic purple to deep-blue color with lysergide. However, in this study, two other indole alkaloids (psilocin and psilocybin) gave similar colors to the colors produced by lysergide. Moreover, other ergot alkaloids produced similar positive reactions.

Duquenois Test—Only the cannabinoids gave a positive test.

Systematic Utilization of These Tests—By using the results obtained from the individual tests performed, it was possible to construct a flowsheet (Scheme I) giving the specific order for performing these tests. By following this flowsheet, it is possible, in a short time and with a few simple tests, to identify tentatively or to narrow down the possibilities of the drug being investigated. Such results can be extremely informative in eliminating the possibility of a suspected drug or determining the confirmatory tests necessary for absolute identification.

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PHARMACEUTICAL TECHNOLOGY

Automated System for Analytical Microbiology V: Calibration Lines for Antibiotics

F. KAVANAGH

Abstract □ The accuracy of an automated system for the microbiological assay of antibiotics was increased by improvement attendant to connection to an on-line computer. The system was used to investigate the suitability of four forms of interpolation formulas by assaying for chlortetracycline and erythromycin. The calibration lines were prepared as point-to-point straight-line approximations and as cubic equations. Cubic equations through four calibration points were preferred. Since the automated system was a four-channel instrument, a separate response line was prepared for each channel. Combining the four response lines into one could substantially degrade the accuracy and precision of assays. A new general equation relating the response of the test organism to concentrations of active materials was used to account for factors in addition to the antibiotic upon the dose–response line. Some of these factors were: diluents, growth substances, relative proportions of mixed antibiotics, pH and buffer capacities of the sample solution and assay broth, salts, and organic compounds in samples and not in standard solutions. The equation was used to show under what conditions the dose–response lines of mixtures and single-component antibiotics could be the same. It could also account for the nonspecific nature of turbidimetric assays. The equation showed assay biases to be caused not by differences in composition of antibiotics in standards and samples but by differences in other substances affecting growth of the test organism. A new dose–response line applicable to assays using *Klebsiella pneumoniae* was described.

Keyphrases □ Microbiology, analytical—automated system, calibration lines for antibiotics □ Automated analysis—system for analytical microbiology, calibration lines for antibiotics □ Antibiotics—automated system for analytical microbiology, calibration lines □ Chlortetracycline—automated analysis, evaluation of interpolation formulas, calibration lines □ Erythromycin—automated analysis, evaluation of interpolation formulas, calibration lines

Automation of critical steps in the turbidimetric microbiological assay (1, 2) resulted in a significant increase in the accuracy and precision of assays. A further increase came when interpolation of sample potency was performed by a computer (3). A limitation of the latter was caused by the three-digit resolution of the digital voltmeter used to measure output of the spectrophotometer. Connection of the spectrophotometer to the computer dedicated to analytical services effected a further increase in accuracy and precision (4). The on-line computer had the further advantage of providing a typed report of assay results within 5 min after the last assay tube had been measured. Computerization of reading and recording of turbidity and calculations of potencies completed automation of the operational parts of the assay system.

Operational aspects of automated assays are now of such precision that the form of the calibration line used for interpolating potencies of samples can significantly affect accuracy and precision. Attention will be directed to consequences of using several forms of the lines. One with a theoretical basis and four empirical ones will be considered.

The philosophy guiding the design of the automated system (1, 2) was to minimize variances caused by operation of the electrical and mechanical parts. The same philosophy is applied in this report to treatment of the data to extract potencies with a minimum of computational errors.

EXPERIMENTAL

Preparation of Tests—The five dose–response lines were ap-

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F. KAVANAGH

Abstract □ The accuracy of an automated system for the microbiological assay of antibiotics was increased by improvement attendant to connection to an on-line computer. The system was used to investigate the suitability of four forms of interpolation formulas by assaying for chlortetracycline and erythromycin. The calibration lines were prepared as point-to-point straight-line approximations and as cubic equations. Cubic equations through four calibration points were preferred. Since the automated system was a four-channel instrument, a separate response line was prepared for each channel. Combining the four response lines into one could substantially degrade the accuracy and precision of assays. A new general equation relating the response of the test organism to concentrations of active materials was used to account for factors in addition to the antibiotic upon the dose–response line. Some of these factors were: diluents, growth substances, relative proportions of mixed antibiotics, pH and buffer capacities of the sample solution and assay broth, salts, and organic compounds in samples and not in standard solutions. The equation was used to show under what conditions the dose–response lines of mixtures and single-component antibiotics could be the same. It could also account for the nonspecific nature of turbidimetric assays. The equation showed assay biases to be caused not by differences in composition of antibiotics in standards and samples but by differences in other substances affecting growth of the test organism. A new dose–response line applicable to assays using *Klebsiella pneumoniae* was described.

Keyphrases □ Microbiology, analytical—automated system, calibration lines for antibiotics □ Automated analysis—system for analytical microbiology, calibration lines for antibiotics □ Antibiotics—automated system for analytical microbiology, calibration lines □ Chlortetracycline—automated analysis, evaluation of interpolation formulas, calibration lines □ Erythromycin—automated analysis, evaluation of interpolation formulas, calibration lines

Automation of critical steps in the turbidimetric microbiological assay (1, 2) resulted in a significant increase in the accuracy and precision of assays. A further increase came when interpolation of sample potency was performed by a computer (3). A limitation of the latter was caused by the three-digit resolution of the digital voltmeter used to measure output of the spectrophotometer. Connection of the spectrophotometer to the computer dedicated to analytical services effected a further increase in accuracy and precision (4). The on-line computer had the further advantage of providing a typed report of assay results within 5 min after the last assay tube had been measured. Computerization of reading and recording of turbidity and calculations of potencies completed automation of the operational parts of the assay system.

Operational aspects of automated assays are now of such precision that the form of the calibration line used for interpolating potencies of samples can significantly affect accuracy and precision. Attention will be directed to consequences of using several forms of the lines. One with a theoretical basis and four empirical ones will be considered.

The philosophy guiding the design of the automated system (1, 2) was to minimize variances caused by operation of the electrical and mechanical parts. The same philosophy is applied in this report to treatment of the data to extract potencies with a minimum of computational errors.

EXPERIMENTAL

Preparation of Tests—The five dose–response lines were ap-

plied to assays of erythromycin and chlortetracycline. *Staphylococcus aureus* (ATCC 9144) was the test organism for both tests. The automated turbidimetric system[1] was operated exactly as described previously (2). The turbidity of the suspension of bacteria in the assay tubes was measured photometrically by a spectrophotometer. The output of the spectrophotometer went directly to the computer for storage and subsequent processing. The samples were points of the standard curve and intermediate concentrations of standard for erythromycin assay or chlortetracycline extracted from animal feeds.

The erythromycin solutions were prepared in pH 7 buffer. Chlortetracycline was extracted by, and diluted to assay concentration with, methanol containing 1% concentrated hydrochloric acid solution. The automated diluter measured two 0.1-ml portions of sample into each of two contiguous assay tubes and diluted each to 10 ml with inoculated broth. The diluter also measured 0.15-ml portions of the sample into two assay tubes and diluted each to 10 ml with inoculated broth. The two sets of assay tubes, differing by 50% in quantity of sample and 0.5% in quantity of inoculated broth, constituted an assay of a sample.

Calibration Lines—The calibration line is the means by which responses to the samples are converted into equivalent concentrations of standard. The five lines discussed here are in daily use in pharmaceutical companies. The equations of the lines are given in generalized form. The first is the one derived from the dose–response line and is applicable to most antibiotic assays (5, 6):

$$\log A = E + BC \qquad \text{(Eq. 1)}$$

where A is the absorbance of the bacterial suspension, C is the concentration of the antibiotic in the sample tube, and B and E are constants.

The second is an empirical equation devised for manual interpolation (3) because of the ease by which it can be put into a nearly straight line on graph paper:

$$\log (D - T) = E + BC \qquad \text{(Eq. 2)}$$

where T is the transmittance of the bacterial suspension in the assay tube, and B, D, and E are constants.

The third equation is the dose–response line used for diffusion assays and, by some, for turbidimetric assays:

$$\log C = E + BT \qquad \text{(Eq. 3)}$$

In applying these expressions, four equations are used, one for each channel of the diluter. The average of the four potencies, one from each channel of the diluter, is taken to be the potency of the sample. Interpolation of sample potencies from the responses is done by the computer from the appropriate straight-line segment of the calibration lines drawn from point-to-point.

In a variant of Eq. 3, responses of the two 0.10-ml samples are averaged and the two responses from the 0.15-ml samples are averaged for each concentration, C, of standard. The averages are then used in constructing the standard curve. The concentrations of standard in the sample tubes are in the ratio of 1:2:4. To construct the standard curve, the 0.1-ml sample of one is assigned a value of 1, the 0.15-ml sample of one is assigned a value of 1.5, the 0.1 ml of two is assigned a value of 2, the 0.15 ml of two is assigned a value of 3, *etc.* Thus, one standard curve—not two—is made from the mean responses. Interpolation of sample potency is done from the individual responses. The two responses from the 0.15-ml amounts are divided by 1.5 before the mean of the four potencies is taken to represent the potency of the sample.

Cubic Equations—A graph of Eq. 3 usually has one inflection; therefore, it can be approximated by a cubic equation such as:

$$\log C = F + BT + DT^2 + ET^3 \qquad \text{(Eq. 4)}$$

A line used by the Food and Drug Administration (7):

$$A = K + L \log C \qquad \text{(Eq. 5)}$$

can be fitted by a cubic equation similar to Eq. 4.

[1] AUTOTURB System, Elanco Division of Eli Lilly and Co.

Table I—Responses to Standards and Samples of Erythromycin

Sample Number	Concentration, μg/ml	Responses, T, for Sample Volumes of 0.10 ml		0.15 ml	
1	0	54.65	54.70	54.86	54.86
2	1	60.40	60.49	64.66	64.86
3	2	67.49	67.44	72.86	73.11
4	3	72.56	72.83	77.96	78.01
5	4	76.04	75.97	81.08	81.03
6	5	78.83	78.94	83.46	83.49
7	3	72.67	72.80	78.01	78.09
8	2.5	70.50	70.70	75.79	75.82
9	3	72.60	72.62	77.72	77.82
10	3.5	74.45	74.62	79.82	79.79
11	4	75.96	75.93	81.07	80.95
12	2	67.41	67.65	72.92	72.91

In applying Eqs. 4 and 5, a separate equation was derived for each channel of the analyzer, as was done for point-to-point interpolations. These computations were done off-line, using data in Table I; therefore, unlike results for Eqs. 1 and 3, they would contain the rounding-off errors of data in the table. A cubic expression will pass through all four points of a four-point standard curve. When the number of standards is more than four, a line of best fit (least squares) is found. Such a line may not pass through all calibration points.

Two forms of a cubic equation were used to obtain potencies given in Table II. They are labeled 4(4) for Eq. 4 fitted to four calibration points and 4(5) for Eq. 4 fitted to all five points. A similar numbering system identified the four-point versions of Eqs. 1 and 5.

Computer Interpolation—The computer was programmed to compare sample responses with those of standards to decide what segment of the dose–response line to use for the interpolations. When sample responses were identical with those of a standard, the value of that standard was selected as the potency of the sample without further computation. When sample response was different from a standard, interpolation was from a line drawn between responses of the two standards bracketing the sample. The line was straight for the point-to-point lines and was a smooth curve for the cubic equations. Although the results in Table II are reported to four significant digits, the significance of the fourth is questionable. It was retained to avoid rounding-off errors.

Errors made in preparing the standards can cause a measurable error in response. The errors in concentrations of standards should

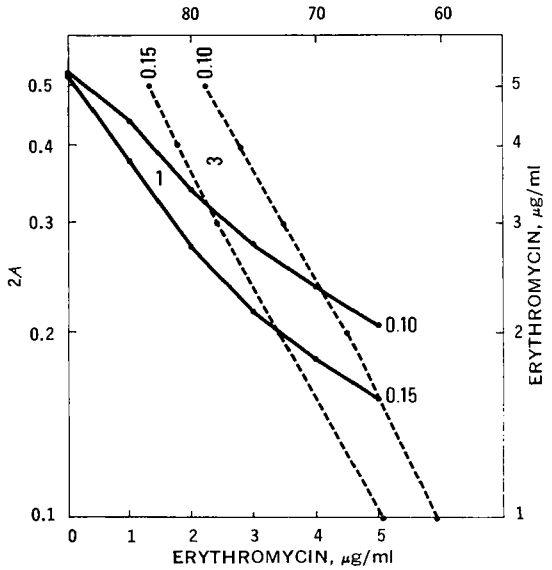

Figure 1—*Erythromycin dose–response lines for Eqs. 1 and 3. Only one of each pair of responses is shown. For convenience in plotting, 2A is used in place of A in Eq. 1.*

Table II—Assay of Erythromycin Standard Solutions (Potencies Obtained from the Indicated Interpolation Equations)

Sample	Equation[a]	Individual Potencies, μg/ml				Mean Potency, μg/ml	σ
		Sample Volume, ml					
		0.10		0.15			
8	1	2.578	2.588	2.553	2.532	2.563	0.025
	1(4)	2.546	2.536	2.506	2.483	2.518	0.029
	3	2.544	2.556	2.524	2.503	2.532	0.023
	3 (1 line)	2.500	2.537	2.613	2.620	2.567	0.059
	4(4)	2.549	2.546	2.510	2.487	2.525	0.032
	4(5)	2.559	2.586	2.524	2.504	2.543	0.036
	5(4)	2.532	2.477	2.486	2.455	2.487	0.032
9	1	3.011	2.958	2.949	2.958	2.969	0.028
	1(4)	3.008	2.950	2.936	2.948	2.960	0.032
	3	3.010	2.953	2.943	2.953	2.965	0.030
	3 (1 line)	2.916	2.921	3.060	3.085	2.996	0.090
	4(4)	3.009	2.950	2.939	2.950	2.962	0.032
	4(5)	2.883	3.004	2.959	2.974	2.955	0.052
	5(4)	3.008	2.944	2.933	2.944	2.957	0.034
10	1	3.529	3.558	3.580	3.574	3.560	0.025
	1(4)	3.501	3.514	3.542	3.541	3.525	0.020
	3	3.507	3.535	3.561	3.555	3.539	0.024
	3 (1 line)	3.449	3.506	3.623	3.614	3.548	0.095
	4(4)	3.496	3.503	3.541	3.536	3.519	0.023
1 standard	4(5)	1.000	1.001	1.000	1.000	1.000	0.000
2 standard	4(5)	1.997	1.986	1.995	1.995	1.993	0.005
3 standard	4(5)	3.014	3.053	3.020	3.024	3.028	0.017
4 standard	4(5)	3.980	3.921	3.969	3.964	3.959	0.026
5 standard	4(5)	5.036	5.066	4.982	5.005	5.022	0.037

[a] Results from four lines drawn point-to-point for both Eqs. 1 and 3. Calibration points for Eq. 3 (1 line) was obtained from responses to standards 1, 2, and 4 μg/ml. Results for Eqs. 4 and 5 were obtained from four cubic equations, one for each channel of the system. The equations were fitted exactly to four calibration points for 1(4), 4(4), and 5(4). A best cubic equation was fitted to five points for 4(5).

be less than 0.2% to cause an error in response not larger than the resolution (1 in 3400) of the analog-to-digital converter of the spectrophotometer. Class A volumetric pipets (≥2 ml) and volumetric flasks (≥100 ml) must be carefully used to avoid significant errors of concentrations.

RESULTS

Erythromycin—Concentrations of standards and samples and the corresponding responses are given in Table I for the erythromycin assay. The first six entries are standards and the last six are samples. The 2-, 3-, and 4-μg/ml samples are the same solutions as the corresponding standards. Standard curves are depicted in Fig. 1. Only one set of responses is given for each sample volume. The calibration lines are approximated by straight-line segments because the computer was so programmed. In this example, the log *C versus T* lines were less curved than the log *A versus C* lines. The latter could have been straightened in the region of significant curvature by modifying the expression to be log *(A − D) versus C*. When this straightening was done, the difference in answers was very small and the difference in precision was insignificant.

A comparison of the results of interpolating from four different dose–response lines is given in Table II. The differences between potencies interpolated from Eqs. 1 and 3 were small, and the standard deviations were the same. The single-line version of Eq. 3 was poorest, as indicated by the large standard deviations. Potencies computed from 1(4), 4(4), and 5(4), the four-point cubic equations, were nearly the same as those interpolated from straight-line versions of Eqs. 1 and 3. Equation 4(5) could give a computational bias of about 1%, as shown by results on the standard curve given in the lower part of Table II. These values were obtained by using responses of the standards to compute equivalent potencies. Similar computations using 1(4), 4(4), and 5(4) gave potencies of 3.000 for the 3-μg/ml standard whereas 3.028 was obtained from 4(5).

Chlortetracycline—The assay for chlortetracycline differs from most antibiotic assays in the large difference in turbidities between the assay tubes without antibiotic. Figure 2 shows the calibration lines for Eqs. 1 and 3 and for the single-curve version of Eq. 3. Both Eqs. 1 and 3 are more strongly curved than the lines for erythromycin. Therefore, the straight-line approximations are not quite as good as for erythromycin. The line for the single-curve version of Eq. 3 is rather unusual for a calibration line. The corresponding line for erythromycin and other antibiotics has the same general shape but is much less extreme.

Results for three samples are given in Table III. The single-curve version of Eq. 3 produced potencies lower than those obtained from Eqs. 1 and 3 and with significantly higher standard deviations. Computational biases are evident and are a principal cause of the large standard deviations. The errors are most pronounced at the lower concentrations, as the line in Fig. 2 indicates they should be. The last line in Table III shows the size of computational errors inherent in the procedure. These numbers were obtained by entering the responses for the 0.2-μg/ml standard in the section of the protocol devoted to samples and then calculating to obtain the indicated potencies.

The 0.120-μg/ml values were obtained from the segment of the calibration line lying between 0.1 and 0.15 μg/ml. The 0.1 point represented responses to 0.10 ml of the 0.1-μg/ml standard. The other end of the segment represented responses obtained from 0.15 ml of the 0.1-μg/ml standard. Thus, the values of 0.120 μg/ml were obtained by using responses to 0.10 ml of sample to interpolate from a calibration line quite different from that for 0.10-ml samples. In contrast to the 0.10-ml samples, the results for the 0.15-ml portions were interpolated from a region of the calibration line (very near a calibration point) where the slope had little influence. Results would be different away from one of the calibration points. Generally, the single-line version will bias the 0.10-ml samples low and the 0.15-ml samples high. The mean of such biased answers is not as near to the correct answer as the automated system is capable of producing.

DISCUSSION

Selection of Interpolation Formulas—Agreement between an answer and the expected result is not sufficient to permit selection of the best interpolation formula. Even procedures that minimize standard deviation may not be the best. Anyone who follows these guides may be trapped into selecting incorrect methods. The principle applied here is that "correct" answers are not obtained by procedures with inherent biases. In deciding which one to use, the assumption is made that responses of the standard curve are without error.

When sample responses are compared one-to-one with responses of the standard, the sample response indicates whether the sample

Table III—Assay for Chlortetracycline in Animal Feeds

Sample	Equation	Individual Potencies, μg/ml: Sample Volume, ml 0.10		0.15		Mean Potency, μg/ml	σ
2	1	0.467	0.471	0.469	0.464	0.468	0.003
	2	0.467	0.471	0.468	0.464	0.467	0.003
	3 (1 line)	0.452	0.459	0.467	0.470	0.462	0.008
9	1	0.356	0.346	0.335	0.325	0.341	0.013
	2	0.353	0.343	0.330	0.319	0.336	0.014
	3 (1 line)	0.284	0.279	0.350	0.346	0.315	0.038
11	1	0.186	0.159	0.150	0.150	0.163	0.016
	3 (1 line)	0.121	0.113	0.176	0.179	0.147	0.035
0.2 standard	3 (1 line)	0.125	0.124	0.201	0.200	0.162	0.043

measures more or less than the standard. Only those interpolation procedures are valid that always give potencies that deviate from the standard in the same direction as the responses indicate they should. This criterion, when applied to the four kinds of calibration lines, shows that all give acceptable results when a separate line is used for each channel of the diluter.

Cubic equations fitted to more than four points degrade accuracy and precision, as shown by lines 4(5) in Table II. The single-line version of Eq. 3 gives grossly erroneous results, as shown by those in Table III and confirmed by those in Table II. In Table II, Sample 9 is a standard solution assayed as a sample, the response of which can be compared with the responses of the 3-μg/ml standard. Potencies interpolated from Eq. 3 (one line) have only one result, 2.921, that deviates from Eq. 3 in the direction indicated by the responses; the three other results are biased. Inspection of the table reveals the large standard deviations of results obtained from the one-curve version of Eq. 3 to be caused by systematic errors.

Regardless of the form of the interpolation equation, the one that governs responses is Eq. A1 (see *Appendix*). Therefore, anything that affects coefficients of Eq. A1 also affects results interpolated from any other equation. Equations 1–3 and 5 represent convenient interpolation formulas—not dose–response lines.

A conclusion of interest is that in any assay corresponding to the assumptions (6) made in deriving Eq. A1, one antibiotic can be assayed in terms of another and the potency of the sample will be independent of dose. Lack of identity of standard and sample will not cause an obvious bias in the assay, even though potency of the sample may be grossly erroneous.

"Best" Calibration Lines—"Best" straight lines and curves of best fit are popular calibration lines without any evidence that the lines should be straight or that the best fit is such in other than a statistical sense. Use of an equation of best fit in place of one that goes through the actual calibration points or a point-to-point line implies that deviations of the calibration points from the best fit are caused by variances in responses.

Experience with the automated assay system indicates small variances when it is correctly operated. Each response may be assumed to be at or very near to the true response and to deviate, if at all, by a small unknown amount. Since deviation from true is unknown and unknowable for each point of a standard curve because each is determined by only one response, the simplest assumption is that of no error in the standard. This procedure attributes all error to the sample but does not, of course, necessarily increase the size of the error.

Equation 1, derived from the dose–response line (Eq. A1), should not be straight because of several approximations made in deriving it. To obtain Eq. 1, absorbance was substituted for N in Eq. A2 whereas, in reality, A and N were related by a quadratic expression (6). Light-scattering properties of the bacteria may not be independent of antibiotic concentration, thereby adding an additional nonlinearity to A. For these reasons, Eq. 1 is unlikely to be straight over an extended range of concentrations. Portions of the line may approximate a straight line as plotted, especially if specifications of accuracy are relaxed (Fig. 3).

Although Eq. 1 was derived for the simplest system, it was a satisfactory interpolation formula for more complex ones. Published data were used to show its applicability to antibiotics that bind to ingredients of the medium (8), to antibiotics enzymatically degraded in the biophase of the test organism (9), and to systems showing two phases in the drug-affected growth curve (10).

Equations 2, 3, and 5 exhibit degrees of curvature varying from assay to assay. Usually Eq. 2 can be put into a nearly straight line. There are two reasons not to use a single calibration line with the automated system: the two sample sizes always produce different dose–response lines because of the difference in initial concentrations of test organisms, and the menstrum effect (Eq. A1) may cause distortion (Fig. 2). Another defect in the single-line version of Eq. 3 is that the geometric spacing of concentrations of standards is too great. Concentrations of standards for turbidimetric assays should be uniformly spaced as in the erythromycin assay (Table I), regardless of the form of the calibration line used for obtaining potencies because the real dose–response line is Eq. A1 (or A2). The other forms (Eqs. 2–5) are simply convenient interpolation formulas with no theoretical foundations.

The best practice for more than four calibration points seems to be the point-to-point line whether the equation be 1, 2, 3, or 5. Whether 1, 3, or 5 is used for computer interpolations depends upon which gives lines with less curvature over the region of interest. When interpolation is done from a graph, the same criterion applies to Eqs. 1–3 and 5. Sometimes Eq. 5 is a little less curved than Eq. 3. If a cubic equation is used, it should be restricted to four points of the standard curve selected to cover the least curved portion of the line if such restriction meets the analytical needs. The cubic equation, when appropriate, probably gives the smallest error of interpolation.

Enough evidence has been given to establish the importance of considering consequences of Eq. A1 when selecting computational

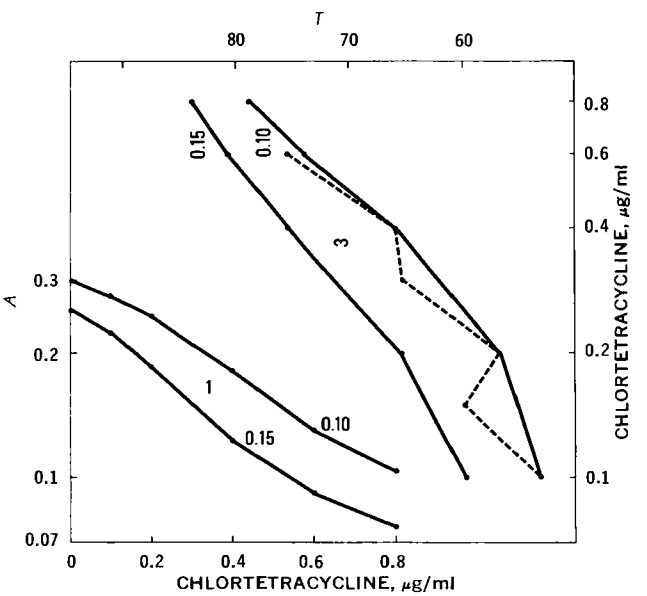

Figure 2—*Chlortetracycline dose–response lines for Eqs. 1 and 3. Only one of each pair of responses is shown. The dashed line is the single-line version of Eq. 3.*

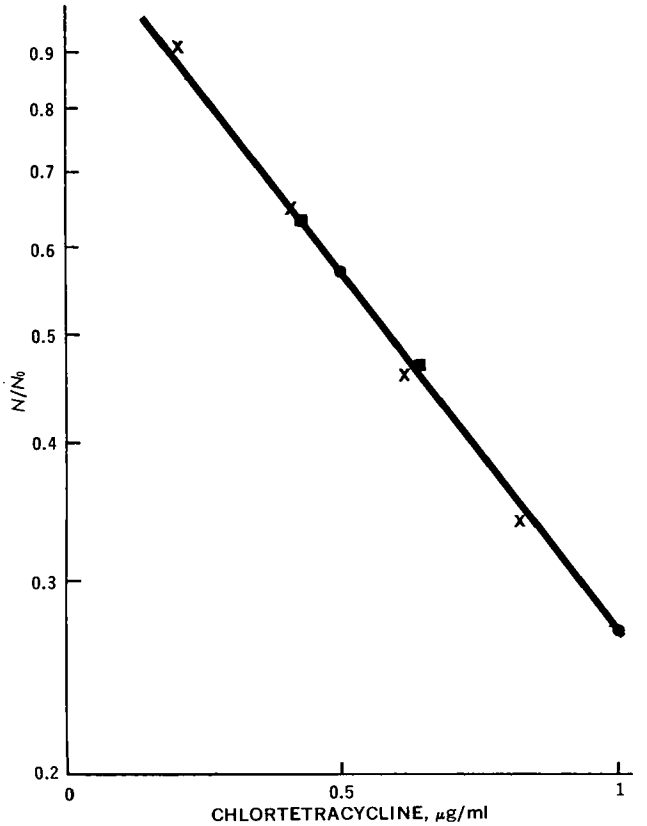

Figure 3—*Transformation of doses (Eq. A4) of erythromycin (■) and tylosin (×) to correspond to the chlortetracycline line. The erythromycin and tylosin lines were made to coincide with the chlortetracycline line at about 0.4 μg/ml. The multiplier of dose was 0.22 for erythromycin and 0.102 for tylosin.* N *was the concentration of bacteria in tubes with antibiotic, and* N_0 *was the concentration in tubes without antibiotic.*

procedures for the automated system. If only low accuracy and precision assays are wanted, the form of the dose–response line and computational procedures are not important. But the influence of $f(v)k_m$ and N_0 of Eq. A1 cannot be ignored if full accuracy and precision of the automated system are to be achieved. A separate interpolation equation for each channel of the diluter must be used to obtain potencies with minimum interpolation error. Thus, each assay requires construction of four interpolation lines or equations, an operation most conveniently done by a computer. If calculations must be done by hand, straight-line interpolation using Eq. 3 requires the fewest operations of a calculator[2].

In a turbidimetric assay, the test organism is exposed to all components of a mixture in the same proportion as they occur in the sample. This is in contrast to diffusion methods in which the several components may reach affected bacteria in ratios quite different from those in the sample. Such fundamental differences in behavior are part of the explanation for different potencies being obtained from the two methods applied to mixtures of antibiotics.

Influence of pH on Activity—The general equation for dose–response lines, Eq. A1 (see *Appendix*), may be used to study the effect of the pH of the assay broth upon activity. The equation is applicable to mixtures as well as to single-component antibiotics. Although the pH of the medium affects the concentration of the active form of the drug, attributing the changes in activity solely to changes in the size of the specific inhibitory coefficient (k_a) simplifies the discussion. The changes in k_a change the slope of the dose–response line, which shows as a change in activity. If the standard and sample contain the same single species of drug, the relative activity of the sample and standard is independent of pH.

Mixtures may give a different response. The k_a of one component of a mixture may change more with the pH of the assay broth than the k_a of another. The effect is to change the size of B, slope, in Eq. A4 more for certain samples than others, resulting in a change of relative potencies. A sample could be less active than the standard at one pH and more active at a different pH of broth. Consequently, potency of mixtures should be measured under carefully specified and attained conditions; otherwise, different assays may find significantly different answers.

Mixtures, unlike single-component antibiotics, do not have a potency directly translatable into weight except in the rare instances where the standard and sample have identical ratios of components. The potency of a mixture is equivalent to the activity in a particular assay of a certain concentration of standard and not to the concentration of standard. Samples of many acidic and basic antibiotics are mixtures not necessarily of the same relative composition as the standards. Assays of these antibiotics may show high interlaboratory variances in potency.

Indications of Invalidity—The second sample volume of 0.15 ml was introduced into the automated system solely to test the invalidity of the assay of a sample. It was not required for purely analytical purposes. If the response of the standard and sample falls on the same dose–response line, the potency of the sample is independent of the concentration at which it is assayed. In other words, if the standard and sample are indistinguishable, there is no evidence of invalidity. When the two sets of potencies (from 0.10 and 0.15 ml of sample) for a sample differ by more than experience shows is normal for the substance and type of sample assayed, potencies are of questionable validity.

In practice, especially with the assay of extracts of animal feeds, potencies obtained from the 0.1- and 0.15-ml volumes of samples may not be the same, thereby indicating bias. Such bias casts doubt upon the accuracy of the potency. The bias may be caused by inhibiting or stimulating substances in the extract, the $f(v)k_m$ effect (Eq. A1). Equation A1 indicates that antibiotics in the sample but not in the standard may not cause assay bias.

Once the nonantibiotic source of bias is recognized, steps can be taken to minimize it. For example, the inhibitory substance (acid methanol) in the chlortetracycline extract was not removed by evaporation because of the difficulty of being certain that standards and samples were treated in an identical manner. Instead, samples and standards were diluted in the same solution used to extract the antibiotic from the feeds. This procedure could be followed because the automated system measured acid methanolic solutions as accurately as aqueous ones. Acid methanol inhibited growth of *S. aureus*, as shown by the large difference in turbidity of the zero tubes in Fig. 2. Tubes of the lower line (Eq. 1) received 0.05 ml more acid methanol than those of the upper line.

Apparent bias can be caused by use of the wrong calibration line. For example, all results, except for Sample 2 in Table III, obtained from the one-curve version of Eq. 3 would be considered invalid or suspect. These invalidities were caused solely by erroneous computational procedures. Thus, biases caused by the single-line standard curve throw valid assays into doubt. Any single dose–response line constructed from responses obtained from more than one concentration of inoculum gives biased answers. Furthermore, such lines have no advantage when the data are processed either by computer or by hand.

Sources of Errors—Experience shows the absolute necessity of treating the sample and standard in an indistinguishable manner. The automated equipment will do its part to produce high accuracy assays by so processing standards and samples. The analyst must make certain that events external to the equipment do not degrade overall performance. The standard and sample must be in solutions of identical composition with respect to inorganic ions, organic solvents and other organic compounds, pH, and buffer capacity before high accuracy assays are possible. These conditions can be approximated for many samples but are not. Results of the theoretical treatment (Eq. A1) and extensive experience with diverse assay systems emphasize the importance of diluting standards and samples in *exactly* the same solvents. Not doing so has caused analysts to suffer much grief. Even using water for one and buffer for the other may affect assays not so accurate as those done with the automated equipment.

The mechanical operations are done so precisely by the automated system that external influences, not noticed in low accuracy assay, become apparent. Quite often, obvious deviations, first at-

[2] Hewlett-Packard model H-P 45 advanced scientific pocket calculator.

tributed to malfunction of equipment, are found to be caused by poorly controlled operations external to the equipment. The style of operations, considered adequate for manual assays, is soon found to be the source of error and variance. Some sources of error are: use of a water bath inadequate with respect to temperature uniformity, heating of the assay tubes in a steam bath instead of a water bath to stop growth at the end of the test, change in temperature of the inoculated broth during the diluting stage, dilution of standards and samples to assay concentrations by inaccurate means, too wide spacing of concentrations of standards, use of inappropriate calibration lines, measuring samples against a standard curve incubated in a different assay-tube carrier, use of different solvents for standards and samples, and use of degraded standards (high bias) or standards that increased in concentration (low bias of samples) because solvent evaporated during storage of the solution. These errors of operations affect results by influencing the size of one or more elements of Eq. A1.

APPENDIX

General Equation—The fallacy of a single calibration line when applied to the particular automated system or to any other in which different assay tubes receive different concentrations of inoculum is easily explained. Antibiotic assays of concern here are really growth rate assays. The simplified explanation is that the antibiotic reduces growth rate for a time of incubation which is the same for all tubes in an assay. The effect of lag period and other variants was discussed elsewhere (6) and will be ignored here. The assumptions stated there also apply to this treatment. Turbidity at the end of the incubation period is the integrated effect of growth rate over time.

The general equation can be written:

$$N_t = N_0 e^{(k_0 + f(v)k_m - k_a C)t} \qquad \text{(Eq. A1)}$$

where N_t is the bacterial concentration after incubation time, t, for an initial concentration of bacteria of N_0, at a concentration, C, of antibiotic which has an inhibitory coefficient, k_a, for the test organism; k_0 is the generation rate constant in the absence of antibiotic (11); k_m is the coefficient for menstruum effect; and $f(v)$ is a function of the volume of sample added to the assay tube.

The $f(v)k_m$ was introduced to recognize the influence of substances other than the antibiotic upon growth of the test organism. The term can account for the effect of such inhibitory agents as acid methanol in chlortetracycline assays as well as the effect of growth-promoting substances in extracts of animal feeds and of excipients in pharmaceutical dosage forms. Equation A1 reduces to the form of Eq. A2, as does the equation (5, 6) derived for the special case of $f(v)k_m = 0$.

Strictly, Eq. A1 holds only so long as growth is in the log phase, a good first approximation in practice. Since N_t is proportional to N_0, anything that affects N_0 changes N_t by the same proportion. As stated earlier, the diluter of the automated system dilutes each sample to 10 ml, causing one-half of the assay tubes to receive 9.9 ml of inoculated broth and the other half 9.85 ml of broth. Therefore, one-half of the assay tubes has a concentration of inoculum of N_0 and the other half has an inoculum concentration of 0.995 N_0. That difference of 0.5% in inoculum concentration is important when turbidity is as accurately measured as it is in the system. The difference in inoculum concentration is the principal cause of the differences of turbidities in the tubes without erythromycin in Table I. The extreme sensitivity to inoculum concentration explains failure of antibiotic assays inoculated, as in vitamin methods, by adding a drop of inoculum to each tube.

Other substances, in addition to antibiotic, can affect growth rate. If any inhibitory substance is added with the antibiotic, then one-half of the assay tubes in the automated system receives 50% more of it than the other half. The additional 0.05 ml of solution added by the 0.15-ml channels can have a profound influence upon growth rate. Examples are the acetone used as solvent in the monensin assay (12), the methanol–pH 8 buffer (40:60) in the tylosin assay (13), and the acid methanol used in the chlortetracycline assay (Fig. 2).

In Eq. A1, the algebraic sign of k_m is positive for growth-promoting substances such as amino acids, vitamins, purines, pyrimidines, other organic compounds, essential inorganic ions, and potentiating agents. The sign is negative when other inhibitory substances are added with the antibiotic. The k_m may be factored into at least three parts: one for growth, one for effect of diluent, and one for interaction between ions in the sample and the antibiotic. In a particular assay, from none to all three kinds of k_m may be involved. The numerical value of k_m (growth) is a function of nutritional requirements of the test organism, nutritional completeness of the assay medium, and composition of the sample solution.

For assays for penicillins, cephalexin, and erythromycin using *S. aureus* growing in antibiotic assay broth, k_m (growth) is zero or nearly zero for samples dissolved in pH 7 phosphate buffer. This is not true for the monensin assay, which is done in nutritionally limiting assay broth (12). The value of k_m (diluent) may be zero, as in the example above, a small number when acetone is the solvent, as in the monensin assay, or a much larger number, as in the chlortetracycline assay. If salts in the sample solution interfere with the activity of the antibiotic, then k_m (interaction) is not zero. Examples are reduction of activity of neomycin by aluminum, ferrous, and magnesium ions (14), potassium chloride, and potassium phosphate (15) and reduction of activity of novobiocin by magnesium ion (16). Thus, there are at least three k_m affecting an assay.

The $f(v)$ has at least three forms, all unknown: one form for growth-promoting substances, one for such inhibitory products as acetone and acid methanol, and one for substances that reduce activity of the antibiotic such as certain inorganic ions interacting with aminoglycoside antibiotics.

The apparent influence of the several $f(v)k_m$ depends upon the design of the assay. It is not apparent when one volume of standard solution (or sample) is added to a constant volume of inoculated assay broth. The effect of $f(v)k_m$ upon dose–response lines may be significant when more than one volume of sample is diluted to a constant volume with inoculated assay broth, as is done by the automated system used in this work. The lines for Eq. 1 in Fig. 2 illustrate this point (compare with equivalent lines in Fig. 1).

Although an exact Eq. A1 cannot be written, enough is known of the general form to use it to explain observations and to make predictions. Application of Eq. A1 will be made to tylosin and monensin assays. In the tylosin assay, k_m (growth) and k_m (diluent) may not be zero. The sign of k_m (growth) may be negative for some samples because of the presence of inhibiting concentrations of heavy metals. It was to remove such interferences that treatment with calcium phosphate was instituted (13). The methanol–pH 8 buffer (40:60) gave k_m (diluent) a negative sign. There was no evidence for k_m (interaction). The dose–response line for tylosin in feed extracts will always be affected by k_m (diluent) but usually not by k_m (growth).

The monensin assay (12) is more complex. Acetone in the extract reduced growth rate, fats in the extract increased growth rate, and potassium ion reduced activity of monensin. Activity of the fats in the feed extracts was compensated so that, in effect, k_m (growth) = 0. Problems with postassium ions were avoided by using acetone to extract monensin but not appreciable potassium ions. Thus, only k_m (diluent) remained to affect the assay.

Multiple-Factor Antibiotics—The logarithmic form of Eq. A1 may be written as:

$$\ln N_t = A - BC \qquad \text{(Eq. A2)}$$

where $A = k_0 t + f(v)k_m t + \ln N_0$ and $B = tk_a$; A is the logarithm of the concentration of test organism obtained in the absence of antibiotic. The value of A is affected by the initial concentration of bacteria in the assay tube, growth rate of the organism, incubation time, and the assorted influences represented by $f(v)k_m$. Temperature of incubation affects growth rate and, consequently, the value of A. In a test (one carrier of the automated system), t and k_0 are the same for all tubes and N_0 and $f(v)k_m$ should be the same for all tubes receiving the same volume of sample. The size of A is not independent of sample volume because N_0 is not and $f(v)k_m$ may not be.

Slope, B, of the line depends upon t and k_a. If the antibiotic has a single active component, k_a is the same in all tubes of an assay. If the antibiotic is a mixture, $k_a C$ has several components such as $k_{a1}C_1 + k_{a2}C_2 + k_{a3}C_3 \ldots$. Furthermore, the standard and sample may be different mixtures. Usually one factor, say 1, will be present in the greatest concentration and the concentrations of the other components can be expressed as a fraction of factor 1 such

as: $C_2 = bC_1$, $C_3 = cC_3$, ... In the same way, the several specific inhibitory coefficients can be related to that of component 1 by $k_{a2} = ek_{a1}$, $k_{a3} = gk_{a1}$, ... Therefore, the components of k_aC can be written as:

$$k_{a1}C_1 + ek_{a1}bC_1 + gk_{a1}cC_1 = (1 + eb + gc)k_{a1}C_1 = hk_{a1}C_1 \quad \text{(Eq. A3)}$$

Substitution in Eq. A2 leads to:

$$\ln N_t = A - hBC_1 \quad \text{(Eq. A4)}$$

Equation A4 differs from Eq. A2 written for a standard composed solely of factor 1 by the constant h. Therefore, Eq. A4 can be made identical with Eq. A2 by means of a change in the dose scale. This conclusion was tested by results from an assay containing responses of chlortetracycline (0–1 μg/ml), erythromycin (2–3 μg/ml), and tylosin (2–8 μg/ml). The agreement of the transformed erythromycin and tylosin doses with those of chlortetracycline (Fig. 3) giving the same responses are as good as can be expected considering the graphical conversions required to obtain values of N (6). Chlortetracycline was about five times as active as erythromycin and about 10 times as active as tylosin in the assay. The values of A in Eq. A2 were 2.08 for chlortetracycline, 2.05 for erythromycin, and 2.11 for tylosin. A consequence of the slopes of the standard and sample being related by a constant multiplier is that any mathematical transformation keeps the relationship unchanged. No form of interpolation line causes bias attributable to form. Of course, a line that does not fit the calibration points causes bias.

Differences in A (Eq. A2) between the standard and sample cause bias. If the differences are large enough, the validity of the assay is questioned. Differences in A are most likely to be caused by differences in $f(v)k_m$ because N_0, t, and k_0 should be the same for the standard and sample. If they and $f(v)k_m$ are not, the assay should be considered invalid. Within a test, if A is the same for the standard and sample, responses of the sample even at several doses cannot establish the identity of the sample with that of the standard.

Nothing written here should be considered a recommendation for assaying one antibiotic in terms of another. That always is a bad practice.

The assumption made in deriving Eq. A1, that inhibition of the growth rate of the test organism was proportional to the concentration of antibiotic, may not always be true. Certain combinations of test bacteria and antibiotics seem to show inhibitions proportional to a higher power of concentration of antibiotic such as the square. When this is true, Eq. A2 should be written:

$$\log N_t = M - PC^2 \quad \text{(Eq. A5)}$$

Assays following Eq. A5 are exemplified by those employing *Klebsiella pneumoniae* (ATCC 10031). The response with chlortetracycline, oxytetraycline, hygromycin A, dihydrostreptomycin, and streptomycin was studied. Examples of published data are those on the latter two antibiotics (17) and chlortetracycline alone and mixed with a constant proportion of neomycin (18). For these systems, graphs in the form of Eqs. 1–3 and 5 are highly curved. Interpolation from a point-to-point straight-line version of Eq. 1 causes a negative error in potency because of poor approximation to the highly curved calibration line. The size of the error depends upon the closeness of spacing of concentrations of antibiotic in the standard curve. The closer the spacing and the higher the concentration, the smaller is the error. Calculations from a model showed errors as large as 5% at the lower concentrations.

Although use of an incorrect calibration line (Eq. 1) can cause significant error, the relative size of the errors change so slowly with concentration that invalidity probably will not be indicated by the test applied to the automated system. Even though use of an inappropriate calibration line might not cause substantial error, such systems should be identified and a more nearly straight line (Eq. A5) should be used. When *K. pneumoniae* is the test organism in assays providing data required for calculating relative inhibitory coefficients (19) of families of antibiotics, calculations should be based upon Eq. A5—not Eq. A1.

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Soft Gelatin Capsules II: Oxygen Permeability Study of Capsule Shells

F. S. HOM ˣ, S. A. VERESH, and W. R. EBERT

Abstract □ A method is reported for studying the effects of several factors at room temperature on the oxygen permeability of soft gelatin capsule shell films. The method involves the use of a permeability cell assembly and the spectrophotometric determination of oxygen with an alkaline pyrogallic acid solution. Factors investigated were relative humidity, plasticizers, equilibrium water, an insoluble additive, and film thickness. Results from the effects of film thickness and oxygen partial pressure correlated well with the permeability equation. For greatest stability of encapsulated oxygen-labile materials, it is recommended that the soft gelatin capsule shells be prepared with a low plasticizer concentration and that the finished capsules be stored at low relative humidity.

Keyphrases □ Capsules, soft gelatin—oxygen permeability at room temperature, effects of relative humidity, plasticizers, equilibrium water, insoluble additives, and film thickness □ Soft gelatin capsules—oxygen permeability at room temperature, effects of relative humidity, plasticizers, equilibrium water, insoluble additives, and film thickness □ Oxygen permeability of soft gelatin capsules—effects of relative humidity, plasticizers, equilibrium water, insoluble additives, and film thickness

Information about capsule shell properties is of considerable importance to those concerned with the design and formulation of soft gelatin capsule dosage forms. In a previous paper (1), results and discussion of several factors affecting capsule shell dissolution rate were reported. Results from an investigation of the factors influencing the viscoelastic characteristics of soft gelatin films were presented by Castello and Goyan (2). The present study deals with oxygen permeability behavior of capsule shells. Oxygen uptake by certain drug preparations while in storage or on shelves can lead to undesirable changes.

An example is the USP vitamin A reference standard in soft gelatin capsules. Vitamin A is relatively unstable in air and light; however, the encapsulated contents show no significant loss of potency for 3 years or longer when stored and packaged under the prescribed conditions of temperature and humidity. Results are presented from investigations on factors that influence oxygen permeability of soft gelatin films that could be useful in the design and formulation of capsule shells for oxygen-labile drugs.

A survey of the literature revealed several methods used to measure gas permeability. The usual ASTM method (3) and most commercially available apparatus are not sufficiently sensitive. A commercially available one (4) and the method of Gilbert and Pegaz (5) lack the flexibility and simplicity demanded in the present study. The method developed by Williams *et al.* (6), which was used by Munden *et al.* (7) for their evaluation of polymeric materials, seemed applicable, but little experimental detail was given for the direct application of this procedure. The present study required a sensitive, simple, versatile, and leak-free technique. A method was developed with these criteria based on the method originated by Williams *et al.* (6).

THEORETICAL

The application of Fick's laws for diffusion and Henry's laws for partial pressure relations for gas solubility (8, 9) provides useful equations for gas flux across an intact polymeric film. When permeability occurs at a steady state in the absence of any interaction between gas and film toward the area of decreasing concentration, these equations are:

$$P = DS \qquad \text{(Eq. 1)}$$

$$P = qh/At(p_1 - p_2) \qquad \text{(Eq. 2)}$$

where P is a permeability coefficient, D is a diffusion coefficient, and S is a solubility coefficient or Henry's law constant. The quantity of gas (q) that passes through a given film at a given condition depends upon the area (A), thickness (h), partial pressure differential across the film $(p_1 - p_2)$, and time (t). Conventionally, the gas permeability coefficient has dimensions of ml × cm/(cm² × 24 hr × atm). It may be evaluated from the slope of the linear portion of the curve where permeability occurs at a steady state in a plot of milliliters of gas permeated *versus* time in hours. Extrapolation of the linear portion of the curve to the time axis yields the lag time (L). The lag time is related to the diffusion coefficient (10) as follows:

$$D = h^2/6L \qquad \text{(Eq. 3)}$$

Thus, all three terms in Eq. 1 may be evaluated.

EXPERIMENTAL

Materials—The following were used: gelatin[1] USP (Type B) with 195 Bloom gram gel strength (20–40 mesh size), glycerin USP, titanium dioxide USP, FD&C Yellow No. 5, Teflon film[2], polyethylene film[2], mercury, nitrogen, oxygen, plaster of Paris, silicone rubber septum (as used in GC), and reagent grade pyrogallic acid and sodium hydroxide.

Equipment—A permeability cell assembly[3], a gas microsyringe[4], a micrometer, a spectrophotometer[5], a vacuum pump, and vacuum desiccators were used.

Preparation of Film—The method used to prepare the soft gelatin films was reported earlier (1).

Relative Humidity Systems—The 31, 47, 58, 72, and 80% systems were saturated aqueous solutions of calcium chloride, potassium thiocyanate, sodium bromide, ammonium chloride–potassium nitrate, and ammonium chloride, respectively.

Preparation of Alkaline Pyrogallic Acid Solution—To prepare 2 liters of solution, introduce a magnetic stirrer, 8.0 g of pyrogallic acid, and 20.0 g of sodium hydroxide onto the bottom of the dry, 2-liter bottle without touching each other (Fig. 1). Pour 2 liters of freshly boiled distilled water into the separator on top of the bottle. Then flush the system with nitrogen until the boiled water is cooled to 30° without getting the reagents wet.

Close the system and stop the nitrogen gas. Evacuate the nitrogen gas trapped in the bottle by use of a vacuum pump. Close the

[1] Rousselot Corp., New York, N.Y.
[2] Cadillac Plastic and Chemical Co., Highland Park, Mich.
[3] Quality Glass Apparatus, Ann Arbor, Mich.; other parts were made by the Machine Shop, R. P. Scherer Corp.
[4] Hamilton Co., Whittier, Calif.
[5] Beckman model B.

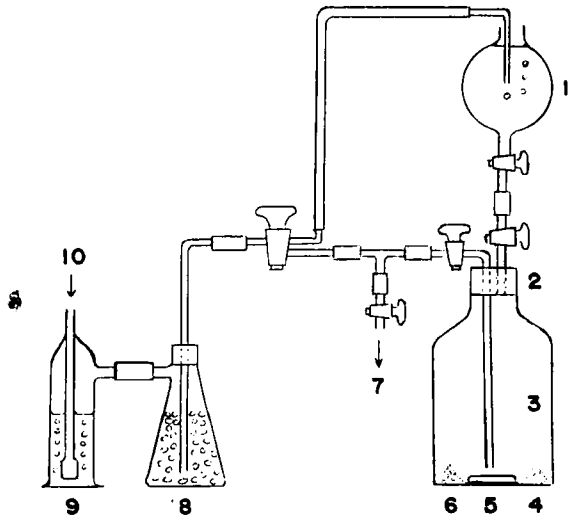

Figure 1—*Schematic diagram showing the preparation of alkaline pyrogallic acid solution. Key: 1, 2-liter funnel; 2, rubber stopper; 3, 2-liter thick wall Pyrex bottle; 4, pyrogallic acid; 5, magnetic stirrer, 5 cm; 6, sodium hydroxide; 7, vacuum; 8, moisture trap; 9, alkaline pyrogallic acid trap; and 10, nitrogen gas from tank. All connections are thick wall rubber hose.*

system and then allow water to enter the bottle slowly by controlling the stopcock. Mix the solution well by turning on the magnetic stirrer when all of the water has been added without allowing any air to enter the bottle. In this manner, a water clear, colorless solution will be obtained.

Assembling and Filling Permeability Cell—Assemble the

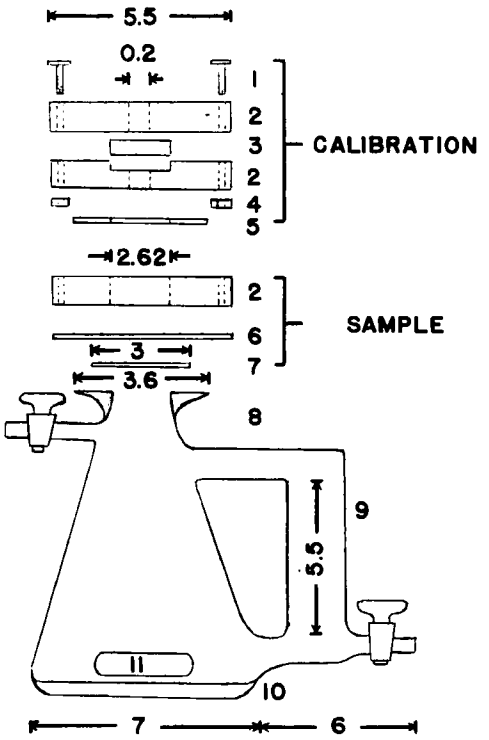

Figure 2—*Diagrams of calibration and permeability cell assembly in exploded side views with measurements in centimeters. Key: 1, bolt; 2, Plexiglas plate, 0.612 cm thick; 3, silicone rubber septum; 4, nut; 5, gelatin or lead gasket; 6, soft gelatin film; 7, Teflon film backing, 0.005 cm thick; 8, wide lip 125-ml filtering flask; 9, cell made from 1.6-cm i.d. test tube-type cell; 10, plaster of Paris for leveling and height adjusting; and 11, magnetic stirrer, 2.54 cm.*

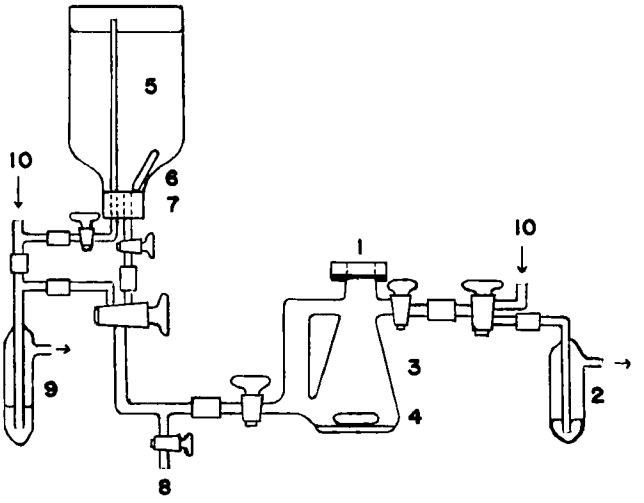

Figure 3—*Schematic diagram showing permeability cell filling operation. Key: 1, mercury in cavity; 2, mercury valve trap; 3, assembled permeation cell; 4, magnetic stirrer, 2.54 cm; 5, alkaline pyrogallic acid solution; 6, magnetic stirrer, 5 cm; 7, rubber stopper; 8, drain; 9, pressure relief valve; and 10, nitrogen gas from tank. All connections are thick wall rubber hose.*

cells as shown in Fig. 2, with gelatin film overlapping the outer edge of the Teflon film backing by at least 0.1 cm to serve as a gasket. The permeability area is equal to that of the 2.62-cm diameter machined hole. Fasten, with nylon strings, the assembled films and plate to the top of the flask containing a magnetic stirrer. Fill the cavity on top of the gelatin film with mercury and tape seal to keep the mercury from spilling. Weigh the assembled cell and record its tare weight. Then insert the cell into the system as shown in Fig. 3.

Flush out all traces of air with nitrogen gas by manipulating the several stopcocks. Fill the cell with alkaline pyrogallic acid solution to within 2 cm of the gelatin film. Weigh the filled cell to determine the weight of the solution and allow the filled cell to stand overnight at room temperature. Make an initial absorbance reading of the filled cell at 425 nm *versus* water after a thorough stirring with the magnetic stirrer. If the initial absorbance reading is less than 0.1, remove the mercury and then place the cell in a pure oxygen atmosphere until the cell solution reads between a 0.10 and 0.15 absorbance value. Replace the mercury on the filled cell and reestablish constancy of the absorbance reading. The filled cell is now ready for the leak test.

Leak Test—The absorbance of the solution in the filled cell was monitored at 425 nm for changes during a period of challenge by pure oxygen at 1 atm. The criterion for a leak-free system was no absorbance change after 2 days under such treatment.

Calibration and Correction Factor—Assemble the calibration cell with a silicone rubber septum between two Plexiglas plates. The hole in the middle of each plate is 0.2 cm in diameter. Fill the cavity on top of the septum with mercury and tape seal. Weigh the assembled cell and fill it with the solution as described in the filling procedure. Establish the weight and the initial absorbance of the solution and ensure that the system is leak free. By use of a gas microsyringe, slowly inject small increments of either pure oxygen or air samples through the mercury and septum into the nitrogen gas phase of the cell. Mix well with the magnetic stirrer until the color has reached equilibrium.

Place the cell in the predetermined position (previously adjusted with plaster of Paris) in the spectrophotometer and read the absorbance at 425 nm *versus* water. A typical standard curve is shown in Fig. 4, in which the change in absorbance at 425 nm is plotted against volume of oxygen added. However, the use of a standard calibration curve required a correction factor because the cells were not matched. A correction factor for each sample cell in relation to the standard cell was obtained from absorbance readings of a common, air-stable, FD&C Yellow No. 5 dye solution.

Permeability Study—After the weight, the absorbance of the solution at 425 nm, and the leak-free condition of the system have been established, remove the tape and mercury from the cell. Place the cell in a desiccator under controlled relative humidity condi-

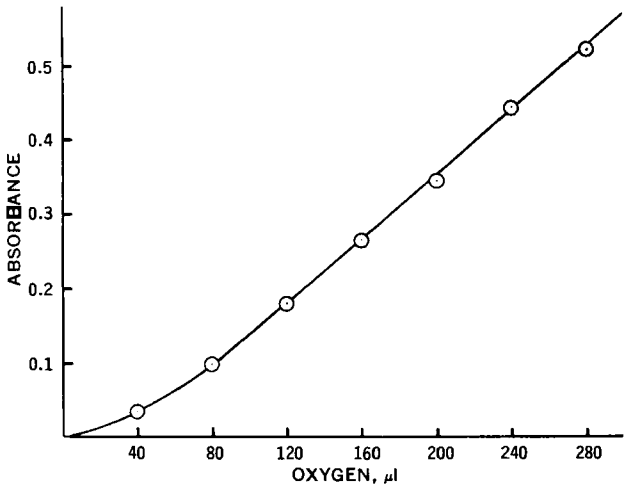

Figure 4—*Typical standard calibration curve in a plot of absorbance at 425 nm for 150 g of alkaline pyrogallic acid solution* versus *oxygen added in microliters.*

tions. Flush the trapped air out of the mercury valve with oxygen from a tank that has been passed through a salt solution system used for adjusting the desired humidity. At appropriate time intervals, remove the cell and make absorbance readings of the solution at 425 nm. At the end of the readings, place the cell in the desiccator and flush the air out with oxygen as before.

The volume of oxygen permeated across the intact film in t hours, V_t, may be calculated from the change in absorbance in t hours, A_t, the standard calibration curve, and the correction factor by use of Eq. 4:

$$V_t = A_t V_s W_u F / A_s W_s \qquad \text{(Eq. 4)}$$

where A_s is the change in absorbance of W_s grams of standard solution as elicited by V_s milliliters of pure oxygen at 1 atm, W_u is the weight of the sample solution in grams, and F is the ratio of absorbance readings of a common dye solution in the standard cell to that in the sample cell. Results are averages of duplicate runs.

RESULTS AND DISCUSSION

The permeabilities of dry gelatin or collagen films to dry oxygen are extremely low. Lieberman *et al.* (11) made experimental runs with oxygen that gave values consistently in the range of values associated with the permeability cell leakage rates. Hence, to obtain meaningful measurements of oxygen permeability through gelatin, the method used must be leak free, sensitive, and stable. The indigo carmine or FD&C Blue No. 2 method (12, 13) exhibited color instability over the period of an experiment. Preliminary work with the present method showed no indication of color instability but revealed a water vapor problem. Over time the water vapor from the alkaline pyrogallic acid solution rendered the study of the effect of relative humidity on permeation rate rather difficult. This problem was resolved by the use of a 0.005-cm thick Teflon film as a backing for the soft gelatin film. This complicated the determination of the gelatin film permeability coefficient somewhat, because it seemed that the observed value then had to be corrected for the Teflon contribution.

One may rationalize that if the Teflon film were to have an observable effect, it would manifest itself on the system with the highest permeation rate in a particular study. In line with this reasoning, permeation rates were determined in triplicate for a gelatin film made with 50% glycerin at 80% relative humidity with Teflon film thicknesses of 0.005 and 0.01 cm. There was no significant change in the observed oxygen permeation rates when the Teflon thickness was doubled. This observation is in agreement with results calculated by use of Eq. 5, given by Rogers *et al.* (14) for the Teflon film effect. Thus, oxygen permeation through the soft gelatin film is the rate-limiting factor. Hence, it is not necessary to correct for the Teflon film effect in the observed permeation rates as obtained under the conditions of the present study.

The present study demands that the permeation apparatus be leak free and requires such a test. The use of mercury as a seal adequately prevents oxygen from going through the permeation film. Therefore, there are three other possible sites for leakage: the gasket and the two stopcocks. Hand grinding of each individual stopcock and the use of a small amount of high vacuum stopcock grease help solve this problem. Teflon, polyethylene, and rubber gaskets were found to be inadequate in preventing oxygen leakage. Surprisingly, soft gelatin films make good gaskets and prevent oxygen leakage. Lead gaskets also may be used for this purpose.

Under the conditions of the experiments (film thickness used, *etc.*), the water permeability through the Teflon film is about 200 times slower than through the soft gelatin films. With the use of Teflon backing at the higher relative humidity conditions, the soft gelatin films swell to a measurable degree. For this reason, thickness measurements were made at the beginning and at the end of each run, but only the final thickness was used for permeability calculations.

When the alkaline pyrogallic acid solution is stored in a leak-free system, it is stable; the change in absorbance is reproducible as shown by standard calibrations done at the beginning and the end of an experiment that may last several weeks. For this reason, the cell systems were tested for leakage by mercury sealing the test film before each oxygen permeation study.

An elaboration of the chemistry of the reaction between oxygen and alkaline pyrogallic acid is beyond the scope of this paper. Apparently, there are at least two possible reaction pathways. The reaction is rapid, but the color produced is dependent on the oxygen flux. Rapid introduction of a larger bolus of air or oxygen into the system gave erratic results, probably from the formation of irreversible, nonstoichiometric dark-brown colors. In contrast, slow injections from a gas syringe of 40-μl increments of oxygen or an equivalent amount of air produced a reproducible yellow color; the results are typical of those shown in Fig. 4. Apparently, for a 150-g solution, about 80 μl of oxygen was expended before the color production was linear. Therefore, the absorbance of the filled cell was adjusted to between 0.10 and 0.15 before the cell was tested for leakage.

Oxygen permeability studies with Teflon and polyethylene films were designed to test the applicability of the equations, the correlation of experimental results with those available in the literature (15), and the effect of repetitive use of a Teflon film on its oxygen permeability. In preliminary runs with Teflon films, the use of pure oxygen was too rapid for accurate determination of the lag time and the permeability coefficient. Consequently, air was used. When humidity is not a factor, as in this case, air is convenient to use since an experiment can be started and stopped by manipulating the mercury seal on top of the film. However, the oxygen permeability coefficients were calculated for 1 atm of pure oxygen.

Figure 5 shows plots of milliliters of oxygen from air that has permeated through new and used Teflon films *versus* time in

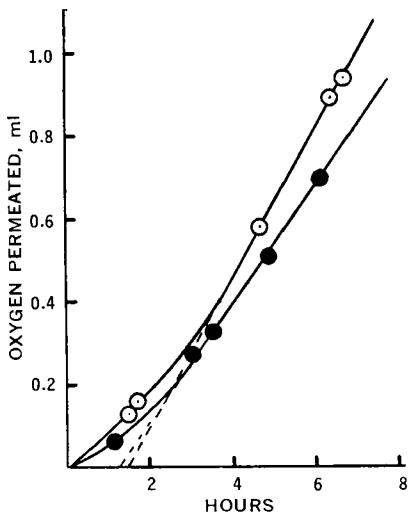

Figure 5—*Effects of new* (○) *and used* (●) *Teflon films on oxygen permeability rate at room temperature in a plot of milliliters of oxygen at 1 atm permeated across film* versus *time in hours*

Table I—Mean Oxygen Permeability Coefficients and Their Ranges from Duplicate Runs of Teflon and Polyethylene Films Along with Values Taken from Ref. 15

Film	Thickness × 10^{-3} cm	Permeability Coefficient, ml cm/(cm² 24 hr atm) × 10^{-3} Present Method	Literature (15) Value
New Teflon	5.334	4.18 ± 0.14	2.95
Used Teflon	5.334	3.15 ± 0.30	—
Polyethylene	7.620	1.80 ± 0.07	2.11

hours at room temperature. The extrapolated lag time in both cases is about 1.5 hr. The mean oxygen permeability coefficients with their ranges from duplicate runs of Teflon and polyethylene films are given in Table I along with those available from the literature (15). In consideration of the variability of the films and other parameters used, the results obtained with the present method agree well with the sparse data in the literature. However, the small difference between new and used Teflon film may be significant. It is reasonable to expect a slightly lower oxygen permeability coefficient value for a used film as compared to a new one.

The present study was limited to films made from one sample of gelatin, to a film thickness between 0.04 and 0.1 cm, to room temperature (21 ± 1°), to 32–63% initial glycerin concentrations, and to 31–80% relative humidity. Oxygen permeability measurements on soft gelatin films made with less than a 30% initial glycerin concentration and conditions of less than 30% relative humidity are extremely slow, time consuming, and inaccurate. Thus, the parameters chosen are such that an experiment usually can be completed in 2–3 weeks.

The mean results from duplicate runs are given in Table II. A typical plot is shown in Fig. 6 of milliliters of pure oxygen at 1 atm that had permeated across the test films, made with 32–63% initial glycerin, *versus* time in hours under a 72% relative humidity. Figures 5 and 6 resemble those pressure change *versus* time plots of Higuchi and Aguiar (10) from their study of permeability of fats, waxes, and other enteric coating materials to water vapor. They were able to obtain lag times precisely by extrapolation of the linear portion of the curve to the time axis. Presently, this is the case with Teflon films as shown in Fig. 5; however, an examination of Fig. 6 reveals that the lag times are erratic as compared to those obtained by using Teflon films alone. The reason is that at the beginning of each run the desired relative humidity conditions were

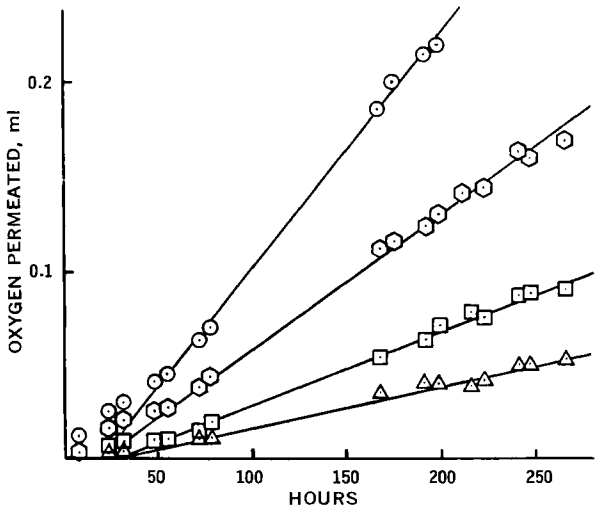

Figure 6—*Typical plot showing the rate at room temperature and 72% relative humidity of oxygen at 1 atm permeated across soft gelatin capsule shell films made with initial glycerin concentrations of 32* (△), *43* (□), *53* (○), *and 63* (⊙) % versus *time in hours.*

Table II—Mean Values from Duplicate Runs Showing Effects of Glycerin Concentration and Storage Relative Humidity on Oxygen Permeability Coefficients of Soft Gelatin Films at Room Temperature

Relative Humidity, %	Initial Glycerin, % 32	43	53	63
	Permeability Coefficient, ml cm/(cm² 24 hr atm) × 10^{-4}			
31	—	0.14[a]	—	1.04[a]
31	—	—	—	0.64
47	—	0.31	0.80	1.58
58	0.51	1.05	1.92	3.98
72	2.40	3.42	5.89	7.97
80	4.00	6.55	9.37	13.35

[a] Data obtained by Ox-Tran 100 method (4).

difficult to establish. In the leak test, the gelatin film was sealed with mercury for a number of days. During this time, the gelatin film was able to equilibrate with the water vapor from the solution that had permeated across the Teflon film. After mercury removal to commence the run, the gelatin film required several hours to reach equilibrium with the desired relative humidity conditions. The initial higher moisture gelatin film resulted in a shorter lag time, whereas the equilibrium process lengthened it. As a result, the lag time was neither reproducible nor accurate. However, this has no bearing on the permeability coefficient determination because it is calculated from the slope of the linear portion of the line when the system is at equilibrium and oxygen permeation has reached steady state.

Relative Humidity—Water is part of the composition of gelatin and also is contained in the soft gelatin film. It is reasonable to expect that relative humidity will have an effect on the physical and chemical properties of soft gelatin films. This part of the study was to determine the effect of relative humidity (percent) on the oxygen permeability of soft gelatin films. Figure 7 shows this effect in a plot of the logarithm of the oxygen permeability coefficient of soft gelatin films made with different initial glycerin concentrations *versus* relative humidity. The values obtained by two different methods correspond quite well. As can be seen, the logarithm of the oxygen permeability coefficient is a linear function of relative humidity. The higher the relative humidity, the exponentially higher is the oxygen permeability for the several initial glycerin concentrations. However, it is surprising that the straight lines, when extrapolated to 100% relative humidity, seem to converge into a single value.

In any event, soft gelatin capsules should be stored below 50% relative humidity in a cool place or at room temperature. Under such storage conditions, oxygen permeability across a soft gelatin capsule shell is limited to very low values under pure oxygen atmospheric conditions (Table II). When stored in air at atmospheric pressure and in a cool, dry place, the oxygen permeability coefficients are about one-fifth of these values or about 1×10^{-5} ml cm/cm² 24 hr atm.

Plasticizers—Glycerin is the most commonly used plasticizer in the manufacture of soft gelatin capsules. As part of the soft gela-

Table III—Mean Oxygen Permeability Coefficients from Duplicate Runs for Soft Gelatin Films Made with Several Different Plasticizers at an Initial 43% Concentration, 72% Relative Humidity, and at Room Temperature Along with Their Corresponding Equilibrium Water Concentration

Plasticizer	Permeability Coefficient, ml cm/(cm² 24 hr atm) × 10^{-4}	Water, % w/w
Glycerin	3.42 ± 0.00	23.6
Sorbitol–glycerin (1:1)	1.32 ± 0.04	22.3
Hexaglycerol	0.71 ± 0.13	20.0
Decaglycerol	0.67 ± 0.07	20.0

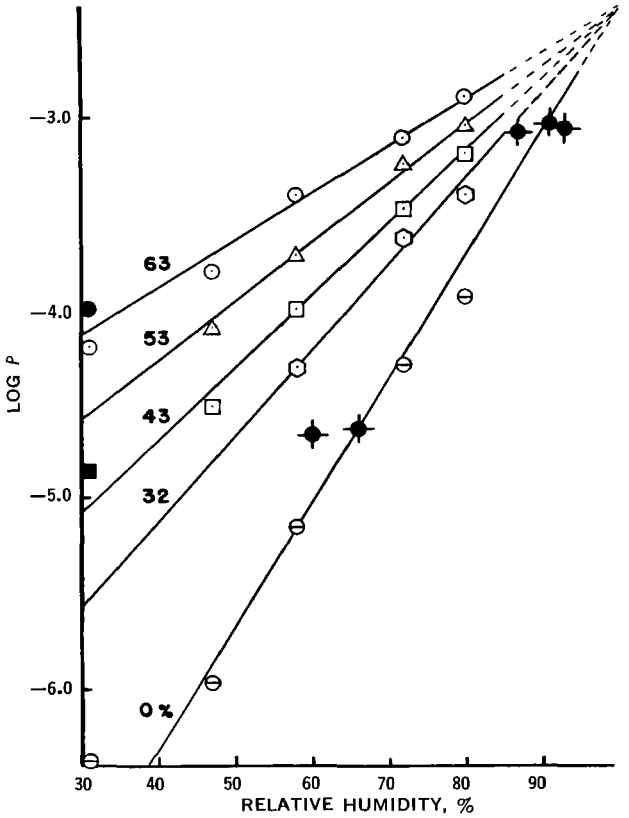

Figure 7—*Plot of the logarithm of the oxygen permeability coefficient,* P, versus *percent relative humidity at room temperature for soft gelatin capsule shell films made with several initial percent glycerin concentrations (given in figure). Solid circle and solid square data were obtained by a commercial method (4). Halved open circle data were obtained by extrapolating lines in Fig. 8 to 0% glycerin. Crossed solid circle data were taken from Ref. 11.*

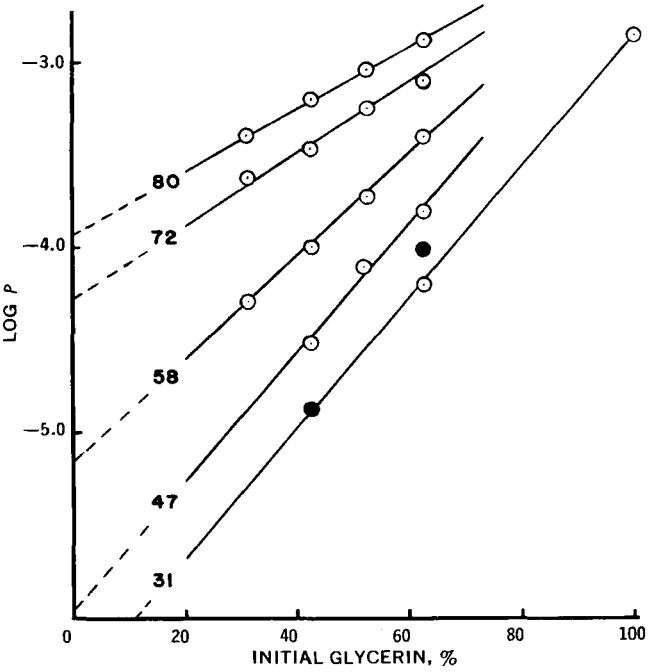

Figure 8—*Plot of the logarithm of the oxygen permeability coefficient,* P, versus *the initial percent glycerin concentration in soft gelatin capsule shell films at room temperature and relative humidity values from 31 to 80% (given in figure). Solid circle data were obtained by a commercial method (4).*

tin composition, it could be expected to affect significantly the oxygen permeability behavior of the capsule shells. Figure 8 is a plot of the logarithm of the oxygen permeability coefficients of soft gelatin films as a function of the initial glycerin concentration (percent) at the several relative humidity conditions. Again, the straight lines indicate that the logarithm of the oxygen permeability coefficients is a linear function of glycerin concentration in soft gelatin films. The higher the initial glycerin in the soft gelatin film, the exponentially higher their oxygen permeability coefficient values will be. However, the initial glycerin concentration of soft gelatin capsule shells is usually below 50%. If the soft gelatin capsules are stored under the recommended conditions (a cool, dry place in air), the oxygen permeability across the capsule shell will be limited to less than a value of 1.6×10^{-5} ml cm/cm^2 24 hr atm.

Table III lists the mean oxygen permeability coefficients and their ranges from duplicate runs of soft gelatin films made with glycerin, sorbitol–glycerin (1:1), hexaglycerol, and decaglycerol at an initial 43% plasticizer concentration, 72% relative humidity, and room temperature. If the permeability coefficient of the film made with glycerin is set at 100%, then the ones for sorbitol–glycerin (1:1), hexaglycerol, and decaglycerol will be 38.7, 20.8, and 19.5%, respectively. As can be seen, oxygen appears to be more permeable through gelatin films made with glycerin than with other plasticizers. Therefore, the use of plasticizers such as sorbitol and polyglycerols should be considered in the design and formulation of capsule shells for oxygen-labile drugs.

Equilibrium Water Concentration—Previous discussions on relative humidity and plasticizers could lead to speculation that equilibrium water concentration may be the common factor affecting oxygen permeability across soft gelatin films. In this study, pieces of soft gelatin films that were used in the permeation experiments were suspended with stainless steel wires in identical environmental conditions. The weight of each piece of film was monitored by periodic weighing. Constant weight was taken as the criterion for equilibrium. Equilibrium water concentration of each piece of soft gelatin film was calculated from knowledge of the final weight, final water content as determined by Karl-Fischer titration, and initial composition. These results are plotted in Fig. 9 for several initial glycerin concentrations and relative humidity conditions. The values for 0% glycerin films were taken from the paper by Bull (16).

For all relative humidity conditions, the equilibrium water concentration is relatively constant between 0 and 40% initial glycerin and then rises with increases in initial glycerin. There seems to be a minimum at about 35% initial glycerin for soft gelatin films stored below 50% relative humidity. This finding is interesting since most soft gelatin capsules in this area are manufactured with this amount of glycerin and stored at this relative humidity. The use of about 35% initial glycerin concentration was determined empirically many years ago. On a more theoretical basis, the glyc-

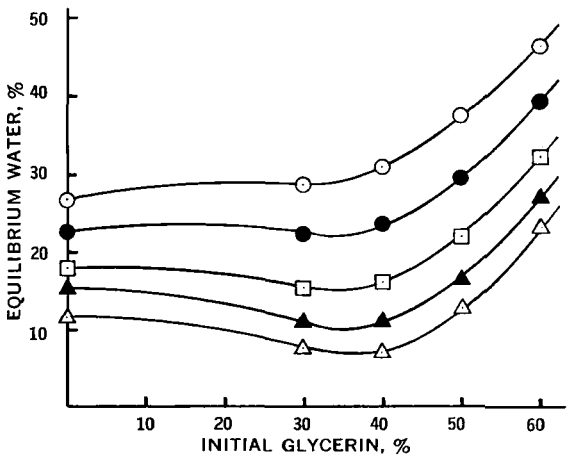

Figure 9—*Plot of percent equilibrium water* versus *percent initial glycerin in soft gelatin films at room temperature and relative humidity of 31* (△), *47* (▲), *58* (□), *72* (●), *and 80* (○) %.

Table IV—Mean Values and Ranges from Duplicate Runs Showing the Effects of a Solid Additive on Oxygen Permeability Coefficients of Soft Gelatin Films at 72% Relative Humidity

Initial Glycerin, %	Permeability Coefficient, ml cm/(cm² 24 hr atm) × 10^{-4}		Decrease, %
	0% TiO_2	5% TiO_2	
43	3.42 ± 0.00	2.61 ± 0.29	23.7
63	7.84 ± 0.51	6.00 ± 1.59	23.5

erin molecule solvates only one hydrophilic group of the gelatin molecule (17). By assuming that each amino acid subunit represents one hydrophilic group and knowing the gelatin composition, a near 1:1 molar ratio of glycerin to amino acid subunit can be calculated for the 35% initial glycerin soft gelatin film.

Without holding the relative humidity conditions constant, the effect of an equilibrium water concentration on oxygen permeability coefficients of soft gelatin films may be shown as in Fig. 10. Here the logarithm of the oxygen permeability coefficients are plotted as a function of the equilibrium mole percent water. The relationship is linear. The reason could be that as the amount of water in the films increases, the water becomes the dominant factor and the curve asymptotically approaches the oxygen permeability coefficient value of water. If the equilibrium water concentration (percent) in Table III were converted to mole percent and the results were plotted *versus* their respective log *P* values in Fig. 10, they would fall on top of the straight line. This indicates that the plasticizers control the equilibrium water concentration in a given soft gelatin film, but their respective equilibrium water concentration has the greatest effect on the oxygen permeability coefficient.

Insoluble Solid Additive—The addition of insoluble solid particles to soft gelatin capsule shells represents an increase in the tortuosity of the path that the permeating gas must flow. The end result is a decrease in the permeability coefficient value as compared to an identical film under the same conditions. The results from such a comparative study are given in Table IV for 0 and 5% titanium dioxide added to soft gelatin films made with 43 and 63% initial glycerin concentrations at 72% relative humidity. The decreases in the oxygen *P* values are 23.7 and 23.5% for the films made with 43 and 63% initial glycerin, respectively. There is little difference in the two results. However, the usual amount of titanium dioxide added to soft gelatin capsule shells is 1% or less. The use of such a small amount of an insoluble additive has a minimal effect on the oxygen permeability coefficient of a given soft gelatin capsule shell. Thus far, the main purpose for using insoluble solid additives in soft gelatin capsules is to obtain pharmaceutical elegance. This purpose may change in the future, especially when more suitable additives are found.

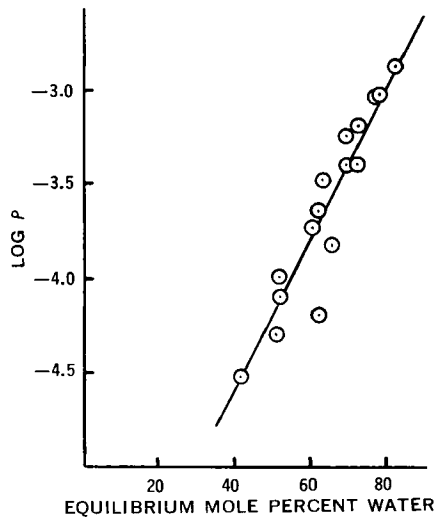

Figure 10—*Plot of the logarithm of the oxygen permeability coefficient,* P, versus *the equilibrium mole percent water in soft gelatin capsule shell films at room temperature.*

Table V—Nine Separate Runs Showing the Final Film Thickness Effect on Oxygen Permeability Coefficients of Soft Gelatin Films Made with 53% Initial Glycerin at 80% Relative Humidity

Final Film Thickness × 10^{-2} cm	Oxygen Permeated, ml/(cm² 24 hr atm) × 10^{-2}	Permeability Coefficient, ml cm/(cm² 24 hr atm) × 10^{-4}
4.01	2.26	9.06
4.27	2.17	9.27
5.46	1.72	9.39
5.79	1.59	9.21
6.05	1.58	9.56
7.11	1.24	8.82
7.62	1.33	10.13
9.88	1.06	10.47
9.93	0.85	8.44
	Mean ± *SD* =	9.37 ± 0.62

Film Thickness—A rearrangement of Eq. 2 shows that the volume of a gas permeated across a given film is directly proportional to the reciprocal of the film thickness. Table V presents the results from nine separate runs of oxygen volume permeated across soft gelatin films, at a thickness from 4.01 to 9.93 × 10^{-2} cm, made with a 53% initial glycerin concentration at 80% relative humidity. Figure 11 shows that the results obey the permeability equation in the form of a straight line going through the origin in a plot of the volume of oxygen permeated *versus* the reciprocal of the final film thickness. Table V also gives the average of the nine oxygen permeability coefficients and its standard deviation: 9.37 ± 0.62 × 10^{-4} ml cm/cm² 24 hr atm.

Oxygen Partial Pressure—With reference to Eq. 2, it can be shown that the volume of a gas permeated through a given film is directly proportional to the partial pressure difference (Δp) across the film. In the present method, Δp is equal to p_1 because p_2 is zero or approaches zero. For this reason, it is not difficult to demonstrate the oxygen partial pressure effect on permeability at room temperature. All that is necessary is to subject the same permeability cell system to air and then to pure oxygen. In this manner all variables are the same except the oxygen partial pressure.

Results from such an experiment are depicted in Fig. 12, where the amount of oxygen permeated in milliliters × 10^{-4} is plotted against the oxygen partial pressure in atmosphere for cm² × 24

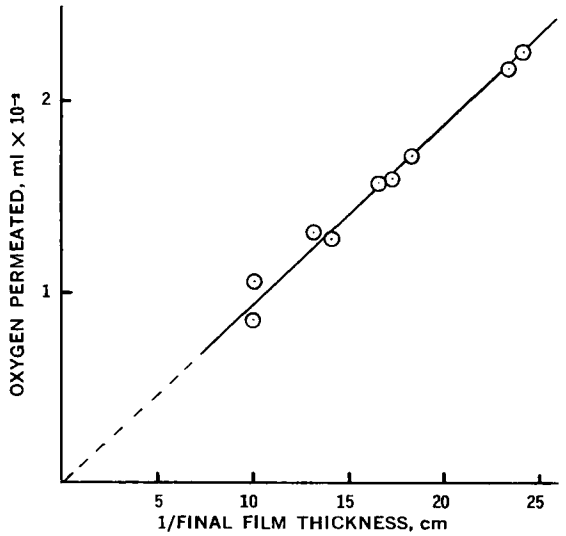

Figure 11—*Plot of volume of oxygen permeated* versus *the reciprocal of film thickness per 24 hr × cm² × atm for soft gelatin films made with 53% initial glycerin at 80% relative humidity.*

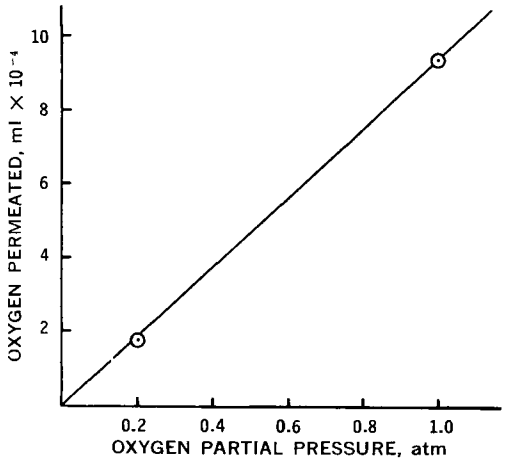

Figure 12—*Plot of the volume of oxygen permeated* versus *oxygen partial pressure per cm² × 24 hr/cm through soft gelatin films made with 53% initial glycerin at 80% relative humidity.*

hr/cm through soft gelatin films made with 53% initial glycerin at 80% relative humidity. In accordance with Eq. 2, the plot is a straight line going through the origin and the two points, 0.2 (air) and 1.0 (pure oxygen) atmosphere. Hence, by the use of this oxygen partial pressure effect, it is convenient, where desired, to speed up or to slow down a particular permeability study with the present method.

SUMMARY

A method is described for a study of the effects of various factors on oxygen permeability behavior of soft gelatin capsule shells. The method involves the use of a permeability cell assembly and the spectrophotometric determination of the amount of oxygen permeated into an alkaline pyrogallic acid solution. The validity of the method was confirmed by a correlation study with a commercially available method and with literature values.

The present method has enabled the investigation, at room temperature, of the influence of relative humidity (percent), types of plasticizers and their concentrations, equilibrium water concentrations, an insoluble solid additive (titanium dioxide), oxygen partial pressure, and film thickness on the oxygen permeability of soft gelatin films. The plasticizer and relative humidity control the equilibrium water concentration, with the latter having the greater effect on oxygen permeability. In a comparative study, the insoluble solid additive appeared to increase the tortuosity of the film and resulted in a decrease in the oxygen permeability coefficient value. The results from the study of the effects of film thickness of soft gelatin films and oxygen partial pressure correlated well with the permeability equation.

In conclusion, various factors have been demonstrated to influence oxygen permeability of soft gelatin capsule shell films. A knowledge of these relationships is helpful in the formation of soft gelatin capsule shells for the capsulation of oxygen-labile drugs and other materials. For the greatest stability against oxygen degradation of such encapsulated ingredients, it is recommended that the soft gelatin capsule shells be made with a lower concentration of plasticizer and that the finished capsules be stored in a dry, cool place.

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Synthesis and Antibacterial and Antifungal Activities of 5-Nitro-2-furfurylidene Polyhalophenoxyacethydrazides VIII

F. AFGHAHI ˣ, S. YAZDANY, and I. LALEZARI

Abstract □ Fifteen 5-nitro-2-furfurylidene phenoxyacethydrazides were synthesized, and the antibacterial and antifungal activities of the compounds prepared were determined against different microorganisms. The *o*-methoxy derivative was found to be the most active compound.

Keyphrases □ 5-Nitro-2-furfurylidene polyhalophenoxyacethydrazides—synthesis, antibacterial and antifungal activities □ Antibacterial and antifungal activities—5-nitro-2-furfurylidene polyhalophenoxyacethydrazides □ Antifungal and antibacterial activities—5-nitro-2-furfurylidene polyhalophenoxyacethydrazides

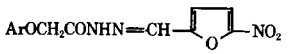
Recently, it was shown that 5-nitro-2-furfurylidene arylthioacethydrazides and 5-nitro-2-furfurylidene arylsulfonylacethydrazides (1) as well as 5-nitro-2-furfurylidene *p*-sulfonyl- and *p*-sulfamoylbenzhydrazides (2) displayed antibacterial activity. Several polyhalophenyl esters of substituted carbamic and carbanilic acids also were found to inhibit the growth of some fungi (3–6). On the basis of these observations, it was of interest to investigate the antibacterial and antifungal activities of compounds having both polyhalophenyl and 5-nitro-2-furfurylidene groups in their molecules.

A series of 5-nitro-2-furfurylidene polyhalophenoxyacethydrazides was prepared by interaction of 5-nitro-2-furfural and appropriate phenoxyacethydrazides. Several other 5-nitro-2-furfurylidene phenoxyacethydrazides were also synthesized for comparison of biological activity. *o*-Methoxy-5-nitro-2-furfurylidene phenoxyacethydrazide (Compound 5) was found to be the most active compound of this series. All compounds prepared are listed in Table I.

DISCUSSION

All compounds prepared were tested against *Bacillus subtilis* (NCTC 3610), *Klebsiella pneumoniae* (ATCC 10031), *Sarcina lutea* (ATCC 9341), *Staphylococcus aureus* (ATCC 6538), and *Bacillus cereus* (NCIB 9373). Nitrofurazone was used as a control. The compounds were dissolved in dimethylformamide at 0.5% concentration. Standard paper disks of 6-mm diameter were im-

Table I—5-Nitro-2-furfurylidene Polyhalophenoxyacethydrazide



Compound	Ar	Melting Point	Yield, %	Formula	Analysis, % Calc.	Found
1	C_6H_5	155°	83	$C_{13}H_{11}N_3O_5$	C 53.97	54.06
					H 3.80	3.70
2	*o*-$CH_3C_6H_4$	163–164°	72	$C_{14}H_{13}N_3O_5$	C 55.44	55.31
					H 4.29	4.33
3	*m*-$CH_3C_6H_4$	183–185°	78	$C_{14}H_{13}N_3O_5$	C 55.44	55.51
					H 4.29	4.28
4	*p*-$CH_3C_6H_4$	178°	80	$C_{14}H_{13}N_3O_5$	C 55.44	55.41
					H 4.29	4.22
5	*o*-$CH_3OC_6H_4$	176–179°	66	$C_{14}H_{13}N_3O_6$	C 52.66	52.69
					H 4.07	3.96
6	*m*-ClC_6H_4	198°	89	$C_{13}H_{10}ClN_3O_5$	C 48.22	48.20
					H 3.09	3.11
7	*p*-ClC_6H_4	195–198°	82	$C_{13}H_{10}ClN_3O_5$	C 48.22	48.20
					H 3.09	3.02
8	*p*-BrC_6H_4	190–191°	85	$C_{13}H_{10}BrN_3O_5$	C 42.39	42.42
					H 2.71	2.76
9	2,4-$Cl_2C_6H_3$	220–222°	91	$C_{13}H_9Cl_2N_3O_5$	C 43.57	43.50
					H 2.51	2.55
10	2,4,5-$Cl_3C_6H_2$	210°	71	$C_{13}H_8Cl_3N_3O_5$	C 39.74	39.69
					H 2.03	2.08
11	2,4,6-$Cl_3C_6H_2$	195°	80	$C_{13}H_8Cl_3N_3O_5$	C 39.74	39.70
					H 2.03	2.00
12	C_6Cl_5	255–260°	74	$C_{13}H_6Cl_5N_3O_5$	C 33.80	33.72
					H 1.32	1.39
13	*m*-$NO_2C_6H_4$	200°	69	$C_{13}H_{10}N_4O_7$	C 46.70	46.66
					H 2.99	3.05
14	*p*-$NO_2C_6H_4$	254°	71	$C_{13}H_{10}N_4O_7$	C 46.70	46.71
					H 2.99	2.90
15	Thymyl	160–162°	76	$C_{17}H_{19}N_3O_5$	C 59.13	59.19
					H 5.50	5.43

Table II—Antibacterial Activity

Compound	Zone of Inhibition, Average Size, mm				
	B. subtilis	*K. pneumoniae*	*S. lutea*	*Staph. aureus*	*B. cereus*
1	11	—	—	14[a]	15
2	11	—	—	15[a]	16
3	18	—	—	18[a]	21
4	14	15	—	19[a]	18
5	21	14	14	19	20
6	12	—	—	12	20
7	8	—	—	12[a]	20
8	13	13	—	15	20
9	—	—	—	15[a]	20
10	—	—	—	—	20
11	—	—	—	12[a]	20
12	13	8	15	15	15
13	12	12	8	15	18
14	—	—	—	—	—
15	12	12	16	12[a]	16
Nitrofurazone	23	20	17	20	21

[a] Inhibition zones were hazy.

mersed in solution and placed on an inoculated assay medium surface[1]. Disks containing pure dimethylformamide were used as controls and did not show antibacterial activity. There were four replicates for each compound. The antibacterial activity of the compounds prepared is reported in Table II.

All compounds prepared were also tested against *Candida albicans*[2] (1959-2), *Penicillium notatum*[2] (154-3), and *Aspergillus niger*[2] (A-23). Concentrations of 10, 25, and 50 μg/ml were used. The compounds were dissolved in dimethylformamide at concentrations of 5 mg/ml, and these solutions were diluted with hot sterile culture medium[3] to the desired concentrations. Griseofulvin was used as a control. A culture medium containing pure dimethylformamide was used and did not show antifungal activity. The antifungal activity of all tested compounds and griseofulvin was insignificant at concentrations of 10 and 25 μg/ml. The antifungal activity of the compounds at the 50-μg/ml concentration is reported in Table III.

EXPERIMENTAL[4]

Substituted Ethyl Phenoxyacetates—These compounds were prepared by interaction of the appropriate sodium phenolate and ethyl chloroacetate or ethyl bromoacetate in boiling ethanol as described by Haskelberg (7).

Pentachlorophenoxyacethydrazide—To a solution of 3 g (0.01 mole) of ethyl pentachlorophenoxyacetate (7) in 35 ml of ethanol, 2 ml (0.03 mole) of 80% hydrazine hydrate was added and the solution was allowed to stand for 8 hr at room temperature. After cooling, the crystalline mass was filtered and recrystallized from 95% ethanol to give 2.64 g (78%) of hydrazide as white crystals, mp 210–215°; molecular weight by mass spectrometry, *m/e* 337–339; IR: ν_{max} 3300, 1663, 1541, 1422, 1385, 1344, 1292, 808, and 707 cm^{-1}.

Anal.—Calc. for $C_7H_5Cl_5N_2O_2$: C, 28.36; H, 1.48. Found: C, 28.29; H, 1.53.

5-Nitro-2-furfurylidene Pentachlorophenoxyacethydrazide (Compound 12)—Pentachlorophenoxyacethydrazide (0.338 g, 1 mmole) was dissolved in 10 ml of alcohol and treated with a solution of 0.141 g (1 mmole) of 5-nitro-2-furaldehyde in 5 ml of alcohol. The reaction mixture was warmed gently on a water bath for 15 min. After cooling, the precipitate was filtered and recrystallized from dimethylformamide–water (7:3) to give 0.341 g (74%) of the desired compound, mp 255–260°; molecular weight by mass spectrometry *m/e* 461–462; IR: ν_{max} 3100, 1690, 1541, 1472, 1396, 1351, 1250, 1051, 955, 916, 783, and 710 cm^{-1}; UV_{max} 375 nm.

All other 5-nitro-2-furfurylidene derivatives were prepared similarly (Table I).

Table III—Antifungal Activity[a] of 5-Nitro-2-furfurylidene Polyhalophenoxyacethydrazides

Compound	*C. albicans*	*P. notatum*	*A. niger*
1	+	+	+
2	+	+	+
3	−	+	−
4	+	+	−
5	+	+	−
6	+	+	+
7	−	+	−
8	−	−	−
9	−	+	−
10	−	+	+
11	−	+	−
12	+	+	+
13	−	+	−
14	−	+	−
15	+	+	−
Griseofulvin	+	+	+

[a] + = complete inhibition, and − = no inhibition.

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[x] To whom inquiries should be directed.

[1] BBL antibiotic assay medium 1.

[2] Microorganisms were obtained from the Department of Parasitology, Public Health Institute, Tehran, Iran.

[3] Difco Sabouraud dextrose agar medium.

[4] Melting points were taken on a Kofler hot-stage microscope and are uncorrected. IR spectra were recorded on a Leitz model III spectrograph. UV spectra were obtained on a Varian Techtron 635 instrument. NMR spectra were taken on a Varian A60A instrument. Mass spectra were recorded on a Varian Mat 111 spectrograph. All compounds were subjected to IR, NMR, and mass spectrometry and the results were as expected.

Quantitative Analysis of Triclocarban in Blood

H. S. CHUN HONG ˣ, R. J. STELTENKAMP, and N. L. SMITH

Abstract □ A method is presented for quantitatively determining triclocarban in blood. Triclocarban is extracted from blood with ether, isolated by TLC, and measured through its UV absorption at 265 nm in methanol. This method is sensitive to 250 ng (50 ppb in 5 ml of blood) of free triclocarban with a relative standard deviation of 5.2%, correlated with a radiotracer analysis of ^{14}C-labeled triclocarban. It has been applied successfully to the analysis of triclocarban in human and rabbit blood.

Keyphrases □ Triclocarban—quantitative analysis in human and rabbit blood □ TLC—quantitative analysis, triclocarban in human and rabbit blood □ UV spectroscopy—quantitative analysis, triclocarban in human and rabbit blood

Recently, hexachlorophene was found to be absorbed into the blood after topical application (1–3). Other bacteriostatics used in skin products must also be examined for skin absorption. This requires detection methods sensitive to parts per billion (ppb) in blood. Triclocarban[1], a bacteriostatic currently used in personal care products, is effective against common Gram-positive bacteria (*e.g., Staphylococcus* type) and fungi (4, 5).

An attempt by the authors to measure triclocarban by GC[2] with electron capture was unsuccessful due to its low volatility. The authors were also unable to derivatize the possible enol (6) to increase volatility. Results are now presented concerning a method for quantitatively determining triclocarban in blood. This procedure employs the extraction of triclocarban from blood with ether, isolation by TLC, and measurement through its UV absorption in methanol.

EXPERIMENTAL

Reagents—The following were used: triclocarban[3] (99% pure as received), anhydrous ether (reagent grade), acetic acid (reagent grade), absolute methanol (reagent grade), and silica gel G-325[4] (325 mesh and above).

Procedure—A 5.0-ml blood sample (0.5% sodium citrate was added as an anticoagulant) was frozen in an ethylene glycol ethyl ether[5]–dry ice bath (−20°) for 10 min and then warmed to room temperature. The first extraction was made with 15 ml anhydrous ether on a mixer[6] for 5 min. Ten milliliters of the supernate was removed and 10 ml of fresh ether was added for the second extraction; this step was repeated for the third extraction. All ether extracts were collected, evaporated to 3.0 ml on a water bath[7] at 60°, mixed well with approximately 100 mg of silica gel G, and centrifuged. Then 2.5 ml of the supernate was transferred to a 2.5-ml centrifuge tube and evaporated to dryness.

The residue was taken up in 0.2 ml of methanol and spotted on a diethylaminoethylcellulose[8]-coated glass plate. On each plate, at least three standards of triclocarban were spotted along with unknown samples by adding known amounts of triclocarban stock solution (1 μg/μl of methanol) to each 5.0-ml blood sample and treating in the same way as other samples. The plate was developed with carbon tetrachloride to remove the interfering background from the original triclocarban spot. Finally, the plate was developed with 4.0% acetic acid in absolute methanol to approximately three-fourths of the length of the plate, and the triclocarban spot was located under shortwave UV light (254 nm). The spot was scraped off, collected in a 2.5-ml centrifuge tube, eluted with 0.3 ml of methanol for 10 min in a water bath at 60°, and centrifuged. The UV absorption spectrum of the eluate was recorded[9] using 50-μl capacity microcells[10]. Peak heights (absorbances) of the spectrum at 265 nm were used to determine the triclocarban.

Table I—Triclocarban Blood Levels by Different Analytical Methods (Rabbit Blood)

Standard Triclocarban, ppb	Radioisotope Technique: Counting Whole Blood, ppb	Radioisotope Technique: Counting Ether Extracts, ppb	TLC–UV Method, ppb
0	0	0	0
200	199	209	205
400	398	413	393
1000	969	1054	899
2000	2127	2100	1800
2400	2620	2382	2800
3000	3246	3225	2900
4000	4187	3881	4100
4000	4199	4278	4400
5000	5074	5336	5800

RESULTS AND DISCUSSION

The recovery of bacteriostatic from whole blood[11] is 89.8 ± 14.4% (average ± *SD*). This value was established by extracting blood samples with known amounts of triclocarban, from 100 to 50,000 ppb, and comparing with ether extracts to which the same amount of triclocarban was added after the extraction of blank whole blood. Although percent recovery was not compared below 100 ppb in this manner, it was possible to detect 50 ppb from blood samples inoculated with triclocarban, showing a good correlation with samples of higher concentrations. Recoveries were identical with both human and rabbit blood.

It was also established that the three repetitive extractions gave maximum recovery.

Recoveries decreased to 68% when samples were stored in a refrigerator for 6 weeks. However, samples stored for 1 month at 0° gave identical recoveries as fresh samples.

The linearity of the method was confirmed with blood samples through the standardization curves. Correlation coefficients (*r*) for linearity were calculated (7) for seven different sets of experiments, with three to five points for each set. They were found to be 0.999, 0.989, 0.995, 0.998, 1.000, 0.995, and 0.999, and all were very close to perfect linearity ($r = 1.000$).

The precision was determined by several replicate analyses of a

[1] 3,4,4′-Trichlorocarbanilide, TCC.

[2] Hewlett Packard model 7610 A, column OV-17, 2% on Chromosorb WHP with 5% methane in argon as flow and purge gas.

[3] 3,4,4′-Trichlorocarbanilide including radioactive compound (ring labeled as ^{14}C), Monsanto Co., St. Louis, Mo.

[4] Research Specialties Co., Richmond, Calif.

[5] Dowanol, Dow Chemical, Midland, Mich.

[6] Vortex Genie mixer, Scientific Industries, Mass.

[7] N-EVAP-A water bath with fitted test tube rack that has air flow device on the top, Organomation Associates Inc., Shrewsbury, MA 01545

[8] S&S material, 100–125 μm, 20 × 20 cm.

[9] Shimadzu MFP-50L, American Instrument Co., Silver Spring, Md., or Cary model 14, Cary Instrument Co., Division of Varian Co., Palo Alto, Calif.

[10] Beckman.

[11] Fresh human blood samples were donated by volunteers, and outdated human blood samples (3–6 weeks old) were obtained from Middlesex General Hospital, N.J. All samples were kept refrigerated until use.

human blood sample containing 500–1000 ppb of triclocarban. These blood samples were prepared by adding known amounts of triclocarban solution in absolute methanol (1.0 μg/μl). Four replicate samples for 500 ppb were spotted on one TLC plate and five replicate samples for 1000 ppb were spotted on another. The average relative standard deviation was 5.2%.

Samples for the radiotracer comparison were prepared by inoculating rabbit blood with a 0.1% methanol solution of ^{14}C-triclocarban. Ten samples ranged from 0 to 5000 ppb. Aliquots of each sample were analyzed for triclocarban using the present method (TLC–UV) and a radioisotope technique with both whole blood and their ether extracts. For isotope analysis, 100 μl of each blood sample was placed directly in 15 ml of 2,5-bis-2-(5,5-butylbenzoxazolyl)thiophene cocktail solution and counted in a liquid scintillation counter[12]. Ether extracts were evaporated to dryness, taken up in 1.0 ml of methanol, and counted in the same manner. The parts per billion values recovered for the unknown samples with three methods are compared in Table I. An analysis of variance (ANOVA test) showed no significant differences in recoveries among the three methods.

This method shows an average 90% recovery from blood with a good correlation with the amounts of triclocarban inoculated in blood. Results of *in vivo* studies with this method with animals following oral intubation and topical application of triclocarban will be published subsequently.

[12] Tricarb, Hewlett Packard.

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Clindamycin Phosphate: Neuromuscular and Blood Pressure Effects in Cats

R. J. SAMUELSON [*], A. H. GIESECKE, Jr., and F. T. KALLUS [x]

Abstract □ Three doses (10, 20, and 40 mg/kg) of clindamycin phosphate were tested in each of three feline neuromuscular preparations in a Latin-square sequence, and the muscular responses and blood pressures were compared. No statistically significant dose-related neuromuscular or blood pressure effects were observed.

Keyphrases □ Clindamycin phosphate—neuromuscular and blood pressure effects, cats □ Neuromuscular and blood pressure effects—clindamycin phosphate, cats □ Blood pressure and neuromuscular effects—clindamycin phosphate, cats

Clindamycin phosphate, a semisynthetic antibiotic produced by chlorination of lincomycin, is very similar in structure and antibacterial activity to its parent compound.

Neuromuscular depressive effects of lincomycin were demonstrated in experimental animals using doses comparable to those used clinically (1, 2). Lincomycin has been noted to interact with tubocurarine in a potentially clinically significant fashion to augment neuromuscular depression (3, 4).

Rapid administration of lincomycin has been associated with syncope, hypotension, and cardiac arrest (2, 5, 6).

Because of similarities between these drugs, this pilot experiment, employing the feline neuromuscular preparation, was undertaken to study the potential neuromuscular and blood pressure effects of clindamycin phosphate to determine if further studies were indicated.

Table I—Neuromuscular Effects

Dose, mg/kg	Depression of Time-Tension Integral Response, %
10	24 ± 15
20	15 ± 10
40	40 ± 30

EXPERIMENTAL

Three mongrel cats were anesthetized with halothane, nitrous oxide, and oxygen. A sciatic nerve–gastrocnemius muscle preparation was established as previously described (4, 7). Blood pressure was monitored *via* an arterial cannula and recorded electronically. Each animal received three doses (10, 20, and 40 mg/kg iv) of clindamycin phosphate according to a Latin-square design. Contractions were allowed to return to the control level prior to each administration. The data were examined by analysis of variance and the Student *t* test. The effect of calcium chloride (50 μg/kg) on recovery also was evaluated.

RESULTS

Neuromuscular depression in the cat was produced by all doses of clindamycin phosphate administered. The mean percent depression of the time–tension integral response with 1 *SD* for each dose is shown in Table I. Wide variation between the effects of the

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Table II—Blood Pressure Effects

Dose, mg/kg	Depression of Peak Systolic, %
10	15 ± 13
20	20 ± 31
40	29 ± 34

doses occurred; although a dose–response relationship is suggested, an analysis of variance revealed no significant differences in responses among the amount of drug given, the order of dose administration, and the responses of the individual animals to a given dose.

The suggestion of neuromuscular depressive activity prompts one to evaluate statistically muscle depression with control muscle contraction by comparison with the Student t test for significance of depression of all doses given ($0.2 < p < 0.3$). However, with the small number of animals used in this pilot study ($n = 3$) and the variability encountered, it is perhaps not surprising that statistical significance was not achieved. Attempts at reversal of these neuromuscular effects with calcium chloride administered after the nadir failed to alter the pattern of recovery (lincomycin block had been previously shown not to be reversed by calcium) (2).

Depression of peak systolic blood pressure was noted with all but one of the doses given. Mean responses with 1 *SD* (Table II) suggest that this blood pressure effect is dose related; however, an analysis of variance revealed the same lack of significant differences among responses to doses, dose order, and animal studied.

DISCUSSION

The manufacturer recommends that clindamycin phosphate be diluted (6 mg/ml) and administered slowly (not to exceed 1200 mg in any 1-hr period). The dose range is approximately 5–20 mg/kg (8). In this experiment, doses were in this range and also double the maximum recommended dose. Initial clinical studies with clindamycin used intravenously noted no acute neuromuscular depressive or cardiovascular effects (9–11). However, a summary of side effects in more than 1000 patients includes instances of hypertension, hypotension, and cardiac arrest, and the manufacturer warns of possible neuromuscular blocking properties (8). Nevertheless, even with the lack of dilution and the rapid use of up to twice the usual dose, this study revealed a lack of statistically significant dose-related neuromuscular depressive or blood pressure effects in the cat.

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Unexpected Sulfuration Reaction of 1-Substituted Azulenes

JOYE A. BILLOW * and TULLY J. SPEAKER ‡

Abstract □ Azulenes reacted unexpectedly and readily with thionyl chloride to give sulfonic acid chlorides and bisthioethers. The sulfonic acids but not the thioethers have antibacterial activity.

Keyphrases □ Azulenes, 1-substituted—reaction with thionyl chloride, antibacterial activity of resulting sulfonic acid derivatives and thioethers □ Antibacterial activity—sulfonic acid derivatives and thioether reaction products of 1-substituted azulenes with thionyl chloride □ Sulfuration reactions—1-substituted azulenes with thionyl chloride, antibacterial activity of resulting sulfonic acid derivatives and thioethers

While preparing some derivatives of azulene *via* Friedel–Crafts acylation (1, 2) using acid chlorides prepared *in situ* with thionyl chloride, the unexpectedly facile reaction of 1-carboxyethylazulene and thionyl chloride was noted. Under the reaction conditions, the primary product was apparently the 3-sulfinic acid chloride, which rapidly disproportionated giving rise to the corresponding bisthioether and sulfonic acid chloride.

The corresponding sulfonic acid is relatively unstable and could not be completely characterized, but its identity was inferred by formation of the more stable amide derivative upon treatment of the anhydrous system with gaseous ammonia. In seeking to confirm the course of the reaction utilizing the more reactive unsubstituted parent compound, azulene, bis(3-chloroazulyl)thioether was separated with difficulty from polymerization products.

The sulfonic acid and sulfonamide show selective

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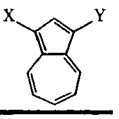
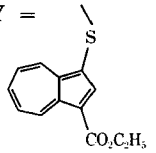
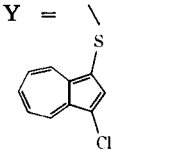
The sulfonic acid and sulfonamide show selective

Table I—Physical and Spectral Characteristics of New Azulene Derivatives



Substituents	Color and Form	Melting Point	Spectral Characteristics				Analysis[d], %	
			λ_{max}, nm[a]	log ε	IR, μ[b]	NMR, ppm[c]	Calc.	Found
X = $CO_2C_2H_5$ Y = SO_2NH_2	Red crystals	95–97°	Ethanol 229 251 (s) 284 (s) 289 301 468	 4.28 3.93 4.27 4.43 4.47 3.77	3.43 3.50 5.95 8.87 12.81 13.47	$CDCl_3$ 1.42 (t) 4.39 (q) 4.73 (s) 8.63 (s) 8.88 (d) 9.65 (d)	$C_{13}H_{13}NO_4S$: C 55.90 H 4.69 N 5.01 S 11.48	 54.07 4.91 4.88 11.02
X = $CO_2C_2H_5$ Y = SO_3H	Red crystals	70–72°	Ethanol 230 252 (s) 282 (s) 288 300 464	 3.72 3.35 3.69 3.81 3.90 3.19	3.02 3.43 5.96 8.27 9.60 12.82 13.43 15+	D_2O 1.40 (t) 4.30 (q) 8.43 (s) 8.49 (d)	Rapidly degrades at ambient temperature	
X = $CO_2C_2H_5$ Y = 	Green scales	127°	CCl 291[4] 302 (s) 318 (s) 373	 4.71 4.68 4.44 4.09	3.49 5.96 7.08 12.96 13.62	$CDCl_3$ 1.24 (t)[e] 4.23 (q) 8.08 (s) 8.68 (d)	$C_{26}H_{22}O_4S$: C 72.5 H 5.15 S 7.43	 72.67 4.83 7.43
X[f] = Cl Y = 	Green scales	57°	CCl_4 268 (s) 278 (s) 281 284 289 295 (s)	 4.33 4.45 4.47 4.48 4.49 4.48	3.48 12.72 13.63 14.51	9.36 (d)	$C_{20}H_{12}Cl_2S$: C 67.60 H 3.40 S 9.03	 67.64 3.21 8.91

[a] s = shoulder. [b] Major, correlated peaks only. [c] s = singlet, d = doublet, t = triplet, and q = quartet; complex patterns are not noted. [d] Elemental analyses were performed by Schwarzkopf Laboratories, New York, N.Y., and by Spang Microanalytical Laboratories, Ann Arbor, Mich. [e] A molecular ion of 430.12 amu was observed on the mass spectrum of this material; the spectrum was consistent with the identification of this compound as bis(3-carboxyethylazulyl)thioether. [f] Separated with difficulty from polymerization products.

antibacterial activity, but the highly insoluble thioethers do not.

EXPERIMENTAL

Materials—Azulene[1] was of 99+% purity; all other materials were of USP or reagent grade.

Physical Data—IR spectra[2] were obtained using potassium bromide disks. UV and visible spectra[3] were obtained using alcohol as solvent. NMR[4] spectra were obtained at ambient temperature using tetramethylsilane as the internal standard, and mass spectra[5] were obtained by direct probe methods. Melting points[6] are uncorrected.

General Chromatographic Procedure—Chromatographic separations were done on either alumina[7] or silica gel[8], utilizing the following procedure. Concentrated solutions of the reaction mixture, reaction mixture filtrates, or reaction mixture extracts were mixed with a small amount of adsorbent (5–15 g) and triturated until a dry, free-flowing powder was obtained. The adsorbate was packed above a 2 × 10-, 3 × 15-, or 4 × 40-cm column of the appropriate adsorbent and eluted with solvents of increasing polarity. The following solvents proved useful, either alone or in combination: petroleum ether, cyclohexane, carbon tetrachloride, toluene, chloroform, methylene chloride, *n*-butanol, 2-propanol, ethanol, and methanol.

[1] Henley and Co., New York, N.Y.
[2] Perkin-Elmer Infracord model 127 recording spectrophotometer.
[3] Cary model 15 recording spectrophotometer.
[4] Jeolco JNM-MH 6011 (60 MHz) recording spectrometer.
[5] Finnigan model 3000D mass spectrometer.
[6] Thomas–Hoover Unimelt, open capillary.
[7] Merck reagent aluminum oxide No. 71707.
[8] Mallinckrodt silicic acid powder No. 2847.

Chemistry—Physical and spectral data of these compounds are presented in Table I.

Thioethers—A solution of 5 mmoles of the appropriate azulene derivative and 3.57 g (30 mmoles) of thionyl chloride in 10 ml of benzene was refluxed for 1 hr and poured over crushed ice. The resultant biphasic system was made basic with ammonium hydroxide and stirred in an open vessel until the benzene evaporated spontaneously. Attempts to speed evaporation with heat drastically lowered yields. The solids were collected on a sintered-glass funnel and chromatographed on alumina using the general chromatographic method. Toluene eluted starting materials and several unidentified small bands; chloroform eluted the green thioethers. Typical yields averaged about 38% of theoretical.

3-Carboxyethylazulene Sulfonic Acid—A solution of 1.000 g (5 mmoles) of ethylazuloate and 3.57 g (30 mmoles) of thionyl chloride in 10 ml toluene was magnetically stirred for 24 hr in an open beaker contained in a sealed desiccator charged with solid sodium hydroxide. The reaction mixture was poured over crushed ice and made basic with ammonium hydroxide, and the phases were separated. The toluene phase was extracted once more with ammonium hydroxide.

The combined aqueous phases were made acidic with hydrochloric acid and extracted with *n*-butanol, and the *n*-butanol extract was subjected to chromatographic separation on silica gel. The material obtained from the major fraction eluted from the column by methanol was redissolved in water, made acidic with hydrochloric acid, and extracted into *n*-butanol. Evaporative concentration of the *n*-butanol yielded 3-carboxyethylazulene sulfonic acid as red crystals; yields averaged 12% of theoretical.

Treatment of the toluene phase as described under *Thioethers* affords small quantities of bis(3-carboxyethylazulyl)thioether and of 3-chloroethyl azuloate.

3-Carboxyethylazulene Sulfonamide—A solution of 1.000 g (5 mmoles) of ethyl azuloate and 3.57 g (30 mmoles) of thionyl chlo-

ride in 10 ml of toluene was magnetically stirred for 24 hr in an open beaker contained in a sealed desiccator charged with solid sodium hydroxide. The toluene solution was saturated with dry ammonia, allowed to stand in a closed vessel for an additional 72 hr, and poured over crushed ice. The resultant biphasic system was made alkaline with ammonium hydroxide and allowed to stand with stirring in an open vessel until the toluene had spontaneously evaporated. The aqueous suspension was then filtered through a sintered-glass funnel.

The filtrate was made acidic with hydrochloric acid and extracted with chloroform, and the chloroform extract was subjected to chromatographic separation on an alumina column. The material obtained from the major fraction eluted from the column with 2-propanol was redissolved in water, made acidic with hydrochloric acid, and extracted into chloroform. Evaporative concentration of the chloroform yielded 3-carboxyethylazulene sulfonamide as red crystals; yields averaged 16% of theoretical.

Chromatographic separation of the residue on the filter yielded small quantities of 3-chloroethyl azuloate and bis(3-carboxyethylazulyl)thioether.

Microbiological Testing—Preliminary screening of the compounds 3-carboxyethylazulene sulfonic acid, 3-carboxyethylazulene sulfonamide, bis(3-carboxyethylazulyl)thioether, and bis(3-chloroazulyl)thioether was accomplished by a simple bioautographic method (3). The preliminary screening was performed in duplicate using *Escherichia coli*[9] and *Staphylococcus aureus* (ATCC 6538) as test organisms. Erythromycin stearate[10] was employed as a positive control.

Because 3-carboxyethylazulene sulfonamide was more active, stable, and water soluble than the other test compounds, it was selected for additional screening. A sensitivity disk method (4) was used against eight microorganisms: *Corynebacterium xerosis* (ATCC 373), *Staph. aureus* (ATCC 6538), *E. coli, Pseudomonas aeruginosa* (ATCC 8308), *Proteus vulgaris, Streptococcus pyogenes* (ATCC 8668), *Candida albicans* (ATCC 1950), and *Mycobacterium fortuitum*[9]. All tests were performed in duplicate.

An aqueous solution of sodium bicarbonate was utilized as a negative control, and commercially available disks impregnated with sodium ampicillin[10] were utilized as a positive control. The equimolar stock solutions were sterilized prior to use by passage through sterile Swinney filters. The nutrient medium was brain–heart infusion agar[11]. Cultures were incubated for 24 and 48 hr, and the diameters of zones of inhibition were measured to the nearest millimeter.

RESULTS AND DISCUSSION

The facile route to the preparation of azulene sulfonic acids, the corresponding sulfonamides, and symmetrical azulylthioethers employing thionyl chloride is reported. Few methods for the sulfuration of the azulene nucleus have been reported (5), and all reported methods employ sulfur trioxide as the primary agent.

The proportions of thioether and presumed intermediate sulfonyl chloride formed in the reaction are sensitive to reaction conditions. The thioether predominates after reaction at 80° and above, while the sulfonyl chloride is the major product after reaction for an extended period at ambient temperature.

The physical and spectral properties and elemental analytical data are presented in Table I.

The free sulfonic acid and sulfonamide inhibited the growth of *Staph. aureus*, as evidenced by clear zones of inhibition in seeded culture medium overlaying the bands of these compounds on the thin-layer bioautographic plate. The thioethers, which are highly water insoluble, were inactive against this organism. No azulene compound showed activity against *E. coli*.

In the study employing the sensitivity disk method, 3-carboxyethylazulene sulfonamide produced zones of 11 and 12 mm on the *S. pyogenes* culture and zones of 13 and 14 mm on the *M. fortuitum* culture after 24 hr.

Further work is underway to obtain 50% inhibitory concentration values as well as kinetic growth curves for these and other members of several series of azulenoid derivatives possessing antibacterial activity.

Because these sulfonic acid derivatives do not possess amino functions, it appears that they exert their antibacterial properties independently of the folate synthetase system.

[9] The identity of cultures of *E. coli, P. vulgaris,* and *M. fortuitum* was verified by a combination of staining reactions, morphological features, and biochemical reactions.

[10] Abbott Laboratories, North Chicago, Ill.

[11] Difco Laboratories, Detroit, Mich.

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ˣ To whom inquiries should be directed.

Isolation and Partial Characterization of Toxins from the Dinoflagellate *Gymnodinium breve* Davis

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Abstract □ Three neurotoxins were isolated from unialgal cultures of the dinoflagellate *Gymnodinium breve* Davis. Of the three toxins, only one toxin (T_1) has hemolytic activity. The major toxin (T_2), in chromatographically pure form, appears to have a molecular weight of 725. The neurotoxin T_2 has no antiacetylcholinesterase activity.

Keyphrases □ *Gymnodinium breve* Davis—isolation and partial characterization of three neurotoxins □ Dinoflagellate (*Gymnodinium breve*)—isolation and partial characterization of three neurotoxins □ Toxins—isolation from *Gymnodinium breve*, partial characterization

The dinoflagellate *Gymnodinium breve* Davis has been reported to be responsible for the massive fish-kill associated with the so-called red-tide which occurs periodically along the west coast of Florida and the Gulf of Mexico. The dinoflagellate produces endotoxins which have been shown to be toxic to marine as well as to laboratory animals (1–4). A number of workers have reported the isolation and partial characterization of toxin(s) from *G. breve* (5–9). The physiological actions of the crude and partially purified toxin(s) have also been investigated (8, 10–12). Most reports about the purification indicate that the toxin(s) was contaminated with carotenoid(s). It is also significant that no two workers have reported the isolation of the same toxin from *G. breve* culture.

For the past few years this laboratory has been studying *G. breve* toxins and now wishes to report a modified method for the isolation of three toxins from the ether extract of *G. breve* culture. Some physical and physiological properties of the major toxin (T_2) are also reported.

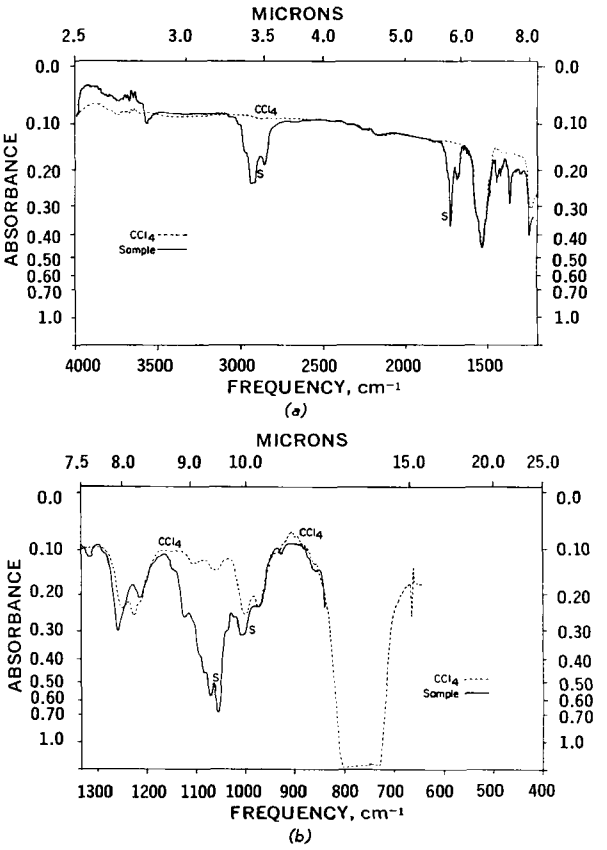

Figure 1—*IR spectrum of* G. breve *toxin T_2 in carbon tetrachloride. Concentration of T_2 is approximately 5 g %; 0.1-mm KBr cells were used with air as reference. The dotted line is the solvent; the solid line is the sample. Key:* (a), *frequency range of 4000–1200 cm^{-1}; and* (b), *frequency range of 1300–400 cm^{-1}.*

EXPERIMENTAL

G. breve[1] was cultured in modified NH-15 medium by the method of Gates and Wilson (13) and extracted by the method of Trieff *et al.* (7). The ether extract was evaporated to dryness *in vacuo*, and the residue was redissolved in a small volume of ether. Suspended particles were removed by centrifugation. The supernate was evaporated to dryness, and the residue was dissolved in 3 ml of benzene–ethyl acetate (2:1 v/v).

Column Chromatography—The *G. breve* extract in benzene–ethyl acetate (2:1 v/v) was placed on a column (7.5 × 7.5 cm), which was prepared by packing with a slurry of silicic acid[2] in benzene–ethyl acetate (2:1 v/v). The column was developed with benzene–ethyl acetate (2:1 v/v, 360 ml, fractions 1–9) followed by benzene–ethyl acetate (1:1 v/v, 360 ml, fractions 10–18) and finally with methanol (200 ml, fractions 19–23). In all, 23 fractions (40 ml each) were collected from column chromatography.

Each fraction was concentrated to 3–4 ml *in vacuo* on a rotating evaporator and spotted on a TLC plate to check its purity. On the basis of TLC in Solvent Systems I and II, fractions were combined to give a total of nine fractions. All column chromatography was carried out in the dark to avoid photochemical transformation.

TLC—Glass plates (20 × 20 cm) coated with silicic acid[3] (300 and 500 μm thickness) were used for qualitative and preparative work, respectively. Plates were activated for 0.5 hr at 110° just before use. TLC was carried out in the dark for the reason mentioned before. The solvent system used was benzene–ethyl acetate (2:1 v/v, Solvent System I; 1:1 v/v, Solvent System II). Location reagents used were 50% sulfuric acid, 1% aqueous potassium permanganate, and Dragendorff's reagent[4].

Combined fractions 7 and 8 were diluted with an equal volume of benzene and lyophilized and worked up to yield T_1 and T_2, a mixture of the two toxins being present in both combined fractions. The light-yellow residue in each case was dissolved in 2 ml of benzene and spotted on preparative TLC plates. The plates were developed in Solvent System II. After drying, the end portions of

[1] The unialgal starter culture was obtained from the culture collection of Dr. W. B. Wilson, Moody College of Marine Sciences and Maritime Resources, Texas A & M University, Galveston, TX 77550

[2] SilicAR 7GF for TLC, Mallinckrodt Chemical Works, St. Louis, Mo.

[3] Silica gel for TLC, D-5, Camag Inc., New Berlin, Wis.

[4] Dragendorff's reagent type QSR-D, Quantum Industries, Fairfield, N.J.

Table I—Weight, R_f Values, Toxicity, and Physiological Properties of the Toxins from *G. breve* Davis

	Fraction Number											
	Crude	1	2	3	4	5	6	7	8	9	T_1	T_2
Column chromatography eluting solvent		Benzene–ethyl acetate (2:1 v/v)					Benzene–ethyl acetate (1:1 v/v)			Methanol		
Weight of toxin(s), mg[a]	353.7	168.0	25.0	4.3	5.46	6.0	25.4	5.07	16.6	97.1	2.3	4.3
Phosphorus, % of weight	0.025	0.010	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.062	0.00	0.00
$R_f \times 100$ in Solvent System I	—	96, 90	90, 84	84, 71	71, 60	71, 60	43, 25	11, 08	08, 05	<05	08,	08,
	—	84, 71	71			43	12	05			05	05
$R_f \times 100$ in Solvent System II	—	—	—	—	—	92, 85	85, 74	60, 50	42, 35	26, 20	42	35
						74	70	42, 35	26, 08			
									04, 00			
Antiacetylcholinesterase activity	(−)[c]	(−)	(−)	(−)	(−)	(−)	(−)	—[d]	—[d]	(−)	(−)	(−)
Hemolytic activity[b], H_{50}, μg	100	(−)	(−)	(−)	(−)	(−)	(−)	—[d]	—[d]	(−)	25	(−)
Mouse toxicity, LD_{100}, mg/kg	5.9	>10	>10	>10	>10	>10	>10	0.30	0.49	0.20	0.30	0.25

[a] Average of 15 column chromatography fractionations. [b] Concentration of the toxin (in micrograms) that will produce 50% hemolysis of rabbit erythrocytes under the experimental conditions (16). [c] (−) stands for a negative value in either antiacetylcholinesterase or hemolytic activity. [d] Was not determined because fractions 7 and 8 had both toxins T_1 and T_2.

the plates were sprayed with aqueous potassium permanganate and with water. The regions reacting positive with potassium permanganate [yellow spot on a pink background, R_f 0.42 (T_1) and 0.35 (T_2)] and water [dense spot on a translucent background, R_f 0.42 (T_1) and 0.35 (T_2)] were scraped off and eluted with benzene–ethyl acetate (1:1 v/v). The eluate was concentrated *in vacuo*, diluted with benzene, and lyophilized. The procedure was repeated until the eluate gave a single compact spot on a TLC plate.

Physiological and Physical Testing[5]—Phosphorus was determined by the method of Mason *et al.* (14) and Bartlett (15). The effect of crude and purified toxins and fractions 1–6 and 9 on human blood serum cholinesterase activity was determined by a spectrophotometric method[6]. Hemolytic activities of the crude and purified toxins T_1 and T_2 and fractions 1–6 and 9 were determined by the method of Reich *et al.* (16). Toxicities to the mice of various fractions and crude and purified toxins T_1 and T_2 were determined by the method of Trieff *et al.* (7).

Acid hydrolysis of toxin T_2 (9.0 mg) was carried out by refluxing toxin T_2 in 50 ml of 2 *N* hydrochloric acid in methanol at 70° for 9 hr. After hydrolysis, the methanol was removed by distillation and the hydrolysate was partitioned between equal volumes of water and ether. The ether layer was separated and evaporated to dryness *in vacuo*. The residue was spotted on a TLC plate, which was then developed in Solvent System II.

RESULTS AND DISCUSSION

Table I shows the R_f values and weights of combined fractions 1–9. Only fractions 7, 8, and 9 were found toxic to mice[7]. Fractions 7 and 8, by repeated preparative TLC, gave toxins T_1 and T_2 with R_f values of 0.42 and 0.35, respectively. Toxin T_3, contained primarily in fraction 9, was purified by column chromatography on silicic acid[2] and was eluted with 5% methanol in chloroform. Toxin T_3 was contaminated with small amounts of pigment(s) and was not further characterized at this time.

All three toxins were toxic to mice and appear to be neurotoxic in nature. The major toxin (T_2), a white amorphous powder, showed a molecular ion at *m/e* 725 in its mass spectrum. The elemental analysis[5] of toxin T_2 showed the following: C, 68.2; H, 8.1; N, 0.72; and O, 22.6%. The IR spectrum of toxin T_2 (Fig. 1) had bands at 3000 (shoulder, CH unsaturated), 2895 (CH saturated), 1740 and 1710 (carbonyl), and a strong absorption band between 1120 and 1025 (C—O—C, tertiary amine) cm^{-1}. The absence of any band in the 3700–3200-cm^{-1} region indicates the absence of hydroxy and primary or secondary amino groups. The UV spectrum of the toxin T_2 had absorption maxima at 260 ($\epsilon M = 5.0 \times 10^3$), 267, and 270 ($\epsilon M = 3.8 \times 10^3$) nm.

The unsaturated nature of the toxin is evident from its reaction with aqueous potassium permanganate and from its IR spectrum. The ether-soluble hydrolytic product of the toxin T_2 gave a single spot (R_f 0.67) in Solvent System II. The IR spectrum of the hydrolytic product has a strong absorption at 3300 (O—H or N—H groups) and a weak absorption at 1750 (C=O group) cm^{-1}. On the basis of the IR spectrum of the ether-soluble hydrolytic product, it is evident that of the total oxygens in toxin T_2, at least two are present in a lactone ring or in an ester bond.

It may be noted from Table II that the present toxin T_2 is similar to the toxin T_2 of Spiegelstein *et al.* (9) and substance II of Martin and Chatterjee (6) in its hemolytic activity. The present toxin T_2 is different from the toxin T_2 of Spiegelstein *et al.* (9) in antiacetylcholinesterase activity but is similar to substance II of Martin and Chatterjee (6), who also reported the absence of antiacetylcholinesterase activity in their major toxin. However, the toxin T_2 now reported differs from the substance II of Martin and Chatterjee (6) in that it has no phosphorus. The physicochemical parameters of this toxin T_2 appear to be similar, although not identical, to those of ciguatoxin as described by Scheuer *et al.* (17), *i.e.*, IR bands at 3390 and 1742 cm^{-1}, UV absorption at 270 nm, no phosphorus, and Dragendorff positive. It is possible that certain species of marine life (grouper fish, moray eel, *etc.*) may take on toxins from a blue-green algae similar to *G. breve* toxins as has been previously postulated (18, 19) either directly through the gills or indirectly in the food chain. Toxins such as T_2 may thus be transformed by metabolic processes into ciguatoxin.

CONCLUSIONS

By a combined method of column chromatography and TLC,

Table II—Physiological Properties of *G. breve* Toxins

	Substance II of Ref. 6	T_2 of Ref. 9	Toxin T_2 of Present Study
Hemolytic activity	Not given	(−)	(−)
Antiacetylcholinesterase activity	(−)	(+)	(−)
Phosphorus	(+)	Not given	(−)
Nitrogen	(−)	Not given	(+)

[5] Visible and UV spectra were recorded in methanol solution (c 86.5 × 10^{-5} g %) on a Perkin-Elmer Coleman 124 spectrophotometer. The IR spectrum was recorded in carbon tetrachloride (c 0.05 g/ml) on a Perkin-Elmer 337 grating IR spectrophotometer. A mass spectrum was recorded on a CEC21-110B double-focusing instrument; the temperature was 380° and resolution was 8000 M/ΔM. Elemental analysis was performed by Huffman Laboratories, Inc., Wheat Ridge, Colo.

[6] See Sigma Technical Bulletin No. 420, Sigma Chemical Co., St. Louis, Mo.

[7] An LD_{100} of greater than 10 mg/kg was considered nontoxic.

three neurotoxins were isolated from unialgal cultures of the dinoflagellate *G. breve* Davis (the red-tide organism). Of the three toxins as assayed in mice, only one (T_1) has hemolytic activity. None of the toxins possesses antiacetylcholinesterase activity. The major toxin (T_2) appears to have a molecular weight of 725. This toxin is unsaturated and has no hydroxy groups, but it possesses a nitrogen (Dragendorff positive). The toxin has at least two oxygens in a lactone ring or in an ester bond. The physicochemical and toxicological properties of the toxin T_2 were contrasted with toxins isolated by other workers. Ciguatoxin may be a metabolic product of the major toxin T_2 or a closely related substance from blue-green algae.

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Determination of Sigma and Pi Constants of Quinolinium Acid Derivatives

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Abstract □ The apparent partition coefficients of 10 quinoline monocarboxylic acid derivatives in a chloroform–water system were determined. From these data, Hammett's σ and Hansch's π values were calculated.

Keyphrases □ Quinolinium acid derivatives—partition coefficients in chloroform–water system, sigma and pi constants □ Partition coefficients—10 quinoline monocarboxylic acid derivatives in chloroform–water system, sigma and pi constants □ Sigma constants—quinolinium acid derivatives □ Pi constants—quinolinium acid derivatives

By using Hammett's (1) equation (Eq. 1), considerable information concerning the effects of substituents on the reaction of given groups in the benzene series is available:

$$\log k - \log k_0 = \sigma\rho \qquad \text{(Eq. 1)}$$

where k is the rate or equilibrium constant for reactions of the substituents, k_0 is the corresponding rate or equilibrium constant of the unsubstituted compound, σ measures a change in electron density produced by a substituent, and ρ measures the susceptibility of the reaction in question to changes in electron density. The application of the Hammett equation has been relatively limited in heterocyclic compounds due to the presence of heteroatoms which themselves are capable of producing changes in electron density (2–8).

Furthermore, Hansch defined π as:

$$\pi = \log P_x - \log P_H \qquad \text{(Eq. 2)}$$

where P_H is the partition coefficient of a parent compound, and P_x is the partition coefficient of a derivative. It was shown by Hansch *et al.* (9–11) that a substituent constant, π, patterned after the Hammett σ constant, was useful in evaluating the lipohydrophilic character of a molecule upon which biological activity is dependent.

three neurotoxins were isolated from unialgal cultures of the dinoflagellate *G. breve* Davis (the red-tide organism). Of the three toxins as assayed in mice, only one (T_1) has hemolytic activity. None of the toxins possesses antiacetylcholinesterase activity. The major toxin (T_2) appears to have a molecular weight of 725. This toxin is unsaturated and has no hydroxy groups, but it possesses a nitrogen (Dragendorff positive). The toxin has at least two oxygens in a lactone ring or in an ester bond. The physicochemical and toxicological properties of the toxin T_2 were contrasted with toxins isolated by other workers. Ciguatoxin may be a metabolic product of the major toxin T_2 or a closely related substance from blue-green algae.

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Table I—pK_{app} Values of the Sodium Alkyl Sulfates of Some Esters of *N*-Methylquinolinium Acids

N-Methyliodides of	pK_{app} of Alkyl Sulfates				
	C_9	C_{10}	C_{11}	C_{12}	ΔC
Quinoline	2.79	3.24	3.78	4.20	3.5
2-Ethyl ester quinoline carboxylic acid	3.62	4.08	4.56	5.03	4.33
3-Ethyl ester quinoline carboxylic acid	3.41	3.89	4.90	4.76	4.10
4-Ethyl ester quinoline carboxylic acid	4.26	4.72	5.18	5.63	4.95
5-Ethyl ester quinoline carboxylic acid	3.87	4.39	4.97	5.57	4.70
6-Ethyl ester quinoline carboxylic acid	3.55	3.98	4.40	4.84	4.19
6-Methyl ester quinoline carboxylic acid	3.48	3.93	4.35	4.80	4.14
6-Propyl ester quinoline carboxylic acid	3.74	4.26	4.76	5.24	4.50
6-Butyl ester quinoline carboxylic acid	4.29	4.74	5.26	5.76	5.01
3-Propyl ester quinoline carboxylic acid	3.41	3.95	4.46	4.97	4.20
3-Acetamido ester quinoline carboxylic acid	3.95	4.38	4.83	5.30	4.61

The present investigation was confined to the determination of the apparent partition coefficients of the quinolinium acid derivatives and the calculation of the Hammett σ and Hansch π constants.

EXPERIMENTAL

Materials—The following were used: 2-ethyl-, 3-ethyl-, 4-ethyl-, 5-ethyl-, 6-ethyl-, 3-propyl-, 6-propyl-, 6-methyl-, and 6-butylquinolinic acids[1]; 3-acetamidoquinoline[1]; methyl iodide[1]; and chloroform[2].

Synthesis—The sodium alkyl sulfates were synthesized as previously described (12, 13). The syntheses of quinolinium acid derivatives are reported elsewhere (14).

Determination of Apparent Partition Coefficient (K_{app})—The K_{app}'s were determined by previously published methods (13, 15–17). That is, initial studies had indicated that the alkyl sulfates–*N*-methylquinolinium derivative salts exist as ion-ion-pair monomers in the organic phase. Therefore, a log–log plot of the concentration of ions in the chloroform phase would yield a slope of 2 with the *Y* intercept being equal to pK_{app} according to Eq. 3:

$$pK_{app} + 2 \log \text{concen}_{aq} = \log \text{concen}_{CHCl_3} \quad \text{(Eq. 3)}$$

By using Eq. 3, the pK_{app} values of the various alkyl sulfates of the quaternary derivatives of the quinolinic acids were calculated (Table I). The wavelength of the maximum absorbance and the molar absorptivity for these compounds are listed in Table II.

RESULTS AND DISCUSSION

The structures of the compounds listed in Table I were correlated by using the Hammett equation (Eq. 1).

The pK_{app} for the *N*-methylquinolinium alkyl sulfate was chosen to be k_0, and the pK_{app} for each *N*-methylquinolinium acid ester alkyl sulfate derivative was considered to be k. The ρ value of 2.113, as reported by Albert and Phillips (18), was used. By using the pK_{app} values reported in Table I, the Hammett function, σ, was calculated for each compound. Good agreement was achieved as illustrated in Fig. 1. Quinoline-5- and 6-carboxylic acids esters may be regarded as substituted benzoic acids in which the fused pyridine ring is considered as a substituent. In the quinoline-2-, 3-, and 4-carboxylic acid esters, the fused benzene ring may be considered as one substituent, and the electron-attracting ring nitrogen may be considered the second substituent.

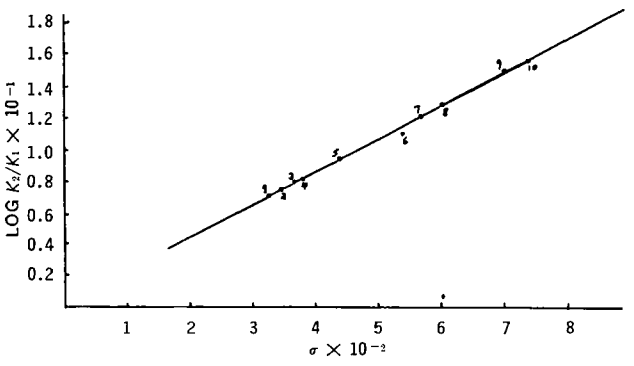

Figure 1—*Relationship between log* K_2/K_1 *and* σ *for various quinolinium acid derivatives. Key: 1, 3-*$COOC_2H_5$*; 2, 6-*$COOCH_3$*; 3, 6-*$COOC_2H_5$*; 4, 3-*$COOC_2H_5$*; 5, 2-*$COOC_2H_5$*; 6, 6-*$COOC_3H_7$*; 7, 3-*$CONH_2$*; 8, 5-*$COOC_2H_5$*; 9, 4-*$COOC_2H_5$*; and 10, 6-*$COOC_2H_5$.

Hammett (1) showed that a linear relationship exists between equilibrium and rate constants for practically all side-chain reactions of benzene derivatives containing *meta*- or *para*-substituents. Furthermore, a condition for such a linear relationship is that there be no significant change in entropy of activation. However, despite these assumptions, it is interesting that such a linear relationship exists (Fig. 1).

Hansch and Fujita (11) used partition coefficients in connection with the Hammett equation to rationalize the substituent effect on the growth-promoting activity of the phenoxyacetic acids and the bactericidal action of chloramphenicol derivatives on various bacteria. They showed that a substituent constant, π, patterned after

Table II—Wavelength of Maximum Absorbance and Molar Absorptivity of Some Esters of *N*-Methylquinolinium Acids

N-Methyliodides of	λ_{max}	Molar Absorptivity, $\times 10^3$
Quinoline	235	4.25
2-Ethyl ester quinoline carboxylic acid	248	2.60
3-Ethyl ester quinoline carboxylic acid	235	5.60
4-Ethyl ester quinoline carboxylic acid	237	2.70
5-Ethyl ester quinoline carboxylic acid	232	4.66
6-Ethyl ester quinoline carboxylic acid	245	3.70
6-Methyl ester quinoline carboxylic acid	242	5.75
6-Propyl ester quinoline carboxylic acid	246	3.50
6-Butyl ester quinoline carboxylic acid	246	3.10
3-Propyl ester quinoline carboxylic acid	246	3.08
3-Acetamido ester quinoline carboxylic acid	238	4.25

Table III—Hansch π Values of Some Esters of *N*-Methylquinolinium Acids

N-Methylquinolinium of	π Constant
2-Ethyl ester quinoline carboxylic acid	0.09
3-Ethyl ester qunoline carboxylic acid	0.07
4-Ethyl ester quinoline carboxylic acid	0.15
5-Ethyl ester quinoline carboxylic acid	0.13
6-Ethyl ester quinoline carboxylic acid	0.08
6-Methyl ester quinoline carboxylic acid	0.08
6-Propyl ester quinoline carboxylic acid	0.11
6-Butyl ester quinoline carboxylic acid	0.16
3-Propyl ester quinoline carboxylic acid	0.08
3-Acetamido ester quinoline carboxylic acid	0.12

[1] K & K Laboratories.
[2] Matheson, Coleman and Bell.

Table IV—pK_{app} Values per Carbon Atom in Alkyl Sulfates of Some Esters of *N*-Methylquinolinium Acids

N-Methyliodides of	pK_{app} per Carbon Atom in Alkyl Sulfates
2-Ethyl ester quinoline carboxylic acid	0.47
3-Ethyl ester quinoline carboxylic acid	0.45
4-Ethyl ester quinoline carboxylic acid	0.46
5-Ethyl ester quinoline carboxylic acid	0.57
6-Ethyl ester quinoline carboxylic acid	0.43
6-Methyl ester quinoline carboxylic acid	0.44
6-Propyl ester quinoline carboxylic acid	0.50
6-Butyl ester quinoline carboxylic acid	0.50
3-Propyl ester quinoline carboxylic acid	0.52
3-Acetamido ester quinoline carboxylic acid	0.45

the Hammett σ constant, was useful in evaluating the lipohydrophilic character of a molecule upon which biological activity is dependent (Eq. 2).

By using the quinolinium methyliodide as P_H and the different esters of quinolinium acids as P_x, values for π were calculated (Table III).

The change in pK_{app} per carbon atom in the alkyl sulfates of the esters of the quinolinium acid derivatives was found to have an average value of 0.48 (range 0.43–0.57) (Table IV), which is in reasonable agreement with the value of 0.44 for each CH_2 unit reported by Hansch *et al.* (11) and with the value of 0.46 reported by Plakogiannis (7).

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Oral Absorption Efficiency of Acid-Labile Antibiotics from Lipid–Drug Delivery Systems

SATISHCHANDRA P. PATEL ˣ and CHARLES I. JAROWSKI

Abstract □ The utility of cholesterol, cholesteryl acetate, and β-sitosterol in protecting and improving the oral absorption efficiency of acid-labile antibiotics is discussed. The potassium salts of penicillin G and penicillin V and erythromycin lactobionate were studied. The stability of the two penicillins in simulated gastric fluid was determined iodometrically. The rank order of acid protective activity was: cholesteryl acetate > β-sitosterol > cholesterol. Oral administration of erythromycin lactobionate coated with cholesteryl acetate produced a twofold increase in human urinary excretion of erythromycin when compared with the uncoated material. Potassium salts of penicillin G and penicillin V coated with cholesteryl acetate yielded 1.6- and 2-fold higher urine levels, respectively, as compared with the uncoated candidates.

Keyphrases □ Antibiotics, acid labile—oral absorption efficiency of penicillin G potassium, penicillin V potassium, and erythromycin lactobionate from cholesterol, cholesteryl acetate, and β-sitosterol protective carriers □ Penicillins G and V (potassium)—oral absorption efficiency from lipid–drug delivery systems □ Erythromycin lactobionate—oral absorption efficiency from lipid–drug delivery systems □ Lipid–drug delivery systems—oral absorption efficiency of penicillin G potassium, penicillin V potassium, and erythromycin lactobionate from cholesterol, cholesteryl acetate, and β-sitosterol protective carriers.

Solutions of penicillin G lose 50% of their potency in less than 1 min at pH 1 and in about 9 min at pH 2 (1). Solutions of penicillin V in 0.1 *N* hydrochloric acid at 37° were reported to have a half-life of 29 min (2). Chemical inactivation of penicillin G in the gastric fluid has been reported to be responsible for the

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Table I—Exposure of 100,000 Units of Plain and Lipid-Coated Penicillin G and Penicillin V to Simulated Gastric Fluid for 1 hr at 37°

Samples Exposed	Penicillin Activity Retained, units[a]		
	Units in Filtrate	Units in Residue	Percent[b] Retained
Potassium penicillin G			
Plain	18.8	—	0.47 ± 0.08
Plus cholesterol (1:1)	70	520.4	14.76 ± 2.01
Plus cholesterol (1:3)	52	657.8	17.74 ± 2.68
Plus cholesterol (1:6)	46	2259.6	57.67 ± 3.54
Plus cholesteryl acetate (1:1)	103	1507	40.27 ± 1.96
Plus cholesteryl acetate (1:3)	92	1798	47.26 ± 1.08
Plus cholesteryl acetate (1:6)	78	2716	69.85 ± 2.15
Plus β-sitosterol (1:1)	110	615.4	18.13 ± 1.56
Plus β-sitosterol (1:3)	86	847.7	23.31 ± 4.28
Plus β-sitosterol (1:6)	82	2328	60.25 ± 1.98
Potassium penicillin V			
Plain	532	—	13.30 ± 0.96
Plus cholesterol (1:1)	602	1121	43.07 ± 1.43
Plus cholesterol (1:3)	554	1709	56.15 ± 2.36
Plus cholesterol (1:6)	483	2320	70.10 ± 4.07
Plus cholesteryl acetate (1:1)	263	2241	67.60 ± 2.69
Plus cholesteryl acetate (1:3)	243	3010	81.33 ± 2.02
Plus cholesteryl acetate (1:6)	197	3135	83.30 ± 3.78
Plus β-sitosterol (1:1)	302	2058	58.99 ± 1.34
Plus β-sitosterol (1:3)	265	2755	75.50 ± 2.22
Plus β-sitosterol (1:6)	231	2867	77.45 ± 3.65

[a] Each value is an average of three determinations. [b] Mean value ±*SD*.

poor oral availability of this antibiotic. Attempts to minimize its destruction in the stomach by the use of enteric-coated preparations have failed since penicillin G is poorly absorbed beyond the duodenum (3). Juncher and Raaschou (4) conducted classic *in vitro* and *in vivo* studies with various salts of penicillin V and sodium penicillin G. The superior acid stability of the former was clearly indicated in dissolution studies at pH 2. The higher blood levels obtained after oral administration of potassium penicillin V could be explained on the basis of this greater acid stability.

Vicek and Vondracek (5) reported that various concentrations of stearic acid did not influence the oral absorption of penicillin V. However, these investigators reported that the addition of 2% stearic acid to tablets containing the potassium salt of penicillin G caused a fivefold increase in blood levels.

Solutions of erythromycin are highly unstable at gastric pH (6, 7). Degradation half-lives of less than 2 min have been reported for solutions of erythromycin and several of its ester derivatives (8). Laboratory and clinical investigations have shown that the biological availability of a series of erythromycin esters was inversely proportional to their dissolution rate in 0.1 *N* hydrochloric acid (9, 10). Approximately 2–5% of an orally administered dose was excreted in the urine whereas 12–15% was found in the urine after an intravenous dose (11).

The water-soluble lactobionate salt is suitable for intravenous administration. The stability of this salt has been studied (12).

The GI absorption of drugs administered in lipid-containing dosage forms has received little attention. Recently, Carrigan and Bates (13) reported that an oil-in-water emulsion of griseofulvin was absorbed 2.5-fold better than an aqueous suspension. Earlier studies in this laboratory (14) showed that the rate and extent of salicylate oral absorption were not impaired even when large excesses of cholesterol, cholesteryl acetate, or β-sitosterol were present. On the other hand, these lipid-coated salicylates exhibited slow rates of dissolution in simulated GI fluids at 37°. Such a marked difference between the *in vitro* dissolution and the *in vivo* rate of absorption was surprising. Cholesterol in cholesterol–hormone

Table II—Urine Recovery of Erythromycin in Two Human Adults after Oral Administration of Various Erythromycin Samples (500,000 μg of Activity)

Sample Administered	Subject	Erythromycin Activity Recovered in the Urine, μg			Percent Excreted
		0–12 hr	12–24 hr	Total 24 hr	
Erythromycin stearate film-coated tablet	C.I.J.	14,538	4,456	18,994	3.80
	S.P.P.	1,372	—[a]	1,372	0.27
Erythromycin lactobionate	C.I.J.	20,064	—[a]	20,064	4.01
	S.P.P.	—[a]	—[a]	—[a]	—[a]
Erythromycin lactobionate plus cholesteryl acetate (1:6)	C.I.J.	29,120	2,064	31,184	6.24
	S.P.P.	2,775	4,370	7,145	1.43

[a] Available data do not permit calculation since the erythromycin activity was less than the standard used.

mixtures enhanced the permeation of the latter through lipoidal membranes (15, 16).

These observations led to the present investigation of the utility of cholesterol, cholesteryl acetate, and β-sitosterol as protective carriers for acid-labile antibiotics. The antibiotics selected were the potassium salts of penicillin G and penicillin V and erythromycin lactobionate.

EXPERIMENTAL

Materials—The following were used: potassium benzylpenicillinate[1] (USP), potassium phenoxymethylpenicillinate[1] (USP), erythromycin lactobionate[2] USP, cholesterol[3] USP, cholesteryl acetate[3], β-sitosterol[3], erythromycin stearate film-coated tablets[2] USP, iodine solution[4] (0.1 *N*), soluble starch[4], sodium thiosulfate solution[4] (0.1 *N*), chloroform[5] NF, sodium hydroxide[5] (reagent grade), potassium phosphate[4], anhydrous monobasic (reagent grade), potassium phosphate[4] (anhydrous dibasic, reagent grade), and hydrochloric acid[6] (reagent grade).

Equipment—The following were used: a constant-temperature shaker bath[7], a pH meter[8], a microburet (50 divisions/ml), a gyrotory incubator shaker[9], brass sieves[10], an immersion filter[11] [1.7 × 5.1 cm (0.7 × 2 in.), medium porosity], a Swinny adaptor[12] (13 mm), and a filter disk[12] (13-mm diameter, 0.45 μm).

Preparation of Lipid-Coated Penicillins—Samples for the *in vitro* stability and *in vivo* bioavailability studies were prepared in drug–lipid ratios of 1:1, 1:3, and 1:6 by the following procedure. A weighed quantity of potassium penicillin G (252.36 mg = 400,000 units) or potassium penicillin V (266.66 mg = 400,000 units) was placed in a glass mortar. A weighed quantity of cholesterol, cholesteryl acetate, or β-sitosterol for an appropriate ratio was dissolved in a minimum quantity of chloroform to produce a saturated solution of lipid in chloroform. A 2-ml aliquot of this solution was poured onto the penicillin powder and gently triturated with a pestle.

During this procedure, a gentle flow of nitrogen gas was introduced to accelerate the evaporation of the chloroform. The sample was dried to a semisolid consistency. This procedure was repeated until the required quantity of lipid had been added. The final mixture of penicillin and lipid was doughy in consistency. The doughy mass was pressed through a 20-mesh sieve to form small granules. These granules were dried in a current of nitrogen and then dried[13] overnight at 37°. The dried granules were passed through a 20-mesh sieve and caught on a 40-mesh sieve. The 20–40-mesh fraction of penicillin–lipid granules was used for the *in vitro* stability and *in vivo* oral absorption studies.

Preparation of Lipid-Coated Erythromycin—Lipid-coated erythromycin lactobionate was prepared for an *in vivo* study as follows. A vial of erythromycin lactobionate powder for intravenous use containing 1 g of erythromycin activity and 180 mg of benzyl alcohol was quantitatively transferred to a glass mortar. To this powder mixture was added 6 g of cholesteryl acetate in chloroform in a similar manner to that already described. Dried, lipid-coated granules, 20–40 mesh, were obtained.

Procedure for *In Vitro* Stability Testing of Various Lipid-Coated Penicillins—An amount of granules corresponding to 100,000 units of penicillin activity was added to 25 ml of simulated gastric fluid (USP XVIII) without pepsin (pH 1.2) at 37° contained in 120-ml (4-fluid oz) amber-colored bottles. The bottles were tightly screw capped and kept in a 37° constant-temperature water bath with a reciprocating motion (stroke 5 cm, 100 times/min). After 1 hr, the undissolved penicillin was filtered off. The filtrate was passed through a 0.45-μm filter and assayed for penicillin by a modified iodometric method (17).

The residue remaining after filtration was dried in an oven at 37° overnight. The residue was dissolved in 10 ml of chloroform, and the chloroform solution was extracted with four 10-ml portions of pH 6 phosphate buffer. The four phosphate buffer extractions were combined and assayed iodometrically for penicillin (Table I).

Bioavailability of Erythromycin from Lipid-Coated Granules and Film-Coated Tablets—Two healthy adult male subjects were used for a bioavailability study of lipid-coated erythromycin lactobionate granules following oral administration after an overnight fast. Urine samples were pooled to represent collection intervals of 0–12 and 12–24 hr and were kept frozen in dry ice until they were analyzed for erythromycin microbiologically[14]. The following preparations were compared in a crossover study: erythromycin stearate film-coated tablets[2], 500 mg of activity; erythromycin lactobionate powder, 500 mg of activity; and erythromycin lactobionate granules containing cholesteryl acetate (1:6), 500 mg of activity (Table II).

Bioavailability of Penicillin G and Penicillin V from Lipid-Coated Granules—Initial screening for the bioavailability of the penicillins was conducted in two healthy human adults to ascertain the best lipid-coated candidates for the larger panel. The two subjects were dosed with 400,000 units of uncoated penicillin after an overnight fast and were instructed to fast an additional 3 hr after the dose was administered. Pooled urine samples represented 0–4- and 4–8-hr collections. Urinary recovery of penicillin from the test preparations was determined microbiologically using *Sarcina lutea* (ATCC-9341) as the test organism (17). Each of the three lipids, at an antibiotic–lipid ratio of 1:6, was then crossed over in the same two individuals (Table III).

In a similar fashion, a panel of six human adults was used to compare the bioavailability of the two penicillins coated with cholesteryl acetate (1:6) (Table IV).

Table III—Antibiotic Urine Recovery in Two Adults after Oral Administration of Plain and Lipid-Coated Potassium Salts of Penicillin G and Penicillin V (Single Dose of 400,000 Units)

	Urine Recovery, %			
	Subject C.I.J.		Subject S.P.P.	
Sample Administered	0–4 hr	4–8 hr	0–4 hr	4–8 hr
Potassium penicillin G				
Plain	19.95	3.99	18.20	1.89
Plus cholesterol (1:6)	25.11	7.01	22.84	4.10
Plus cholesteryl acetate (1:6)	30.11	8.70	25.03	8.91
Plus β-sitosterol (1:6)	29.94	3.00	24.90	2.96
Potassium penicillin V				
Plain	29.16	5.10	22.40	4.09
Plus cholesterol (1:6)	32.08	11.02	31.06	8.89
Plus cholesteryl acetate (1:6)	40.79	18.23	39.98	14.12
Plus β-sitosterol (1:6)	35.74	9.00	37.00	6.01

RESULTS

In Table I it can be seen that penicillin V is more stable than penicillin G in simulated gastric fluid. After 1 hr, 13.3 ± 0.96% of penicillin V was retained whereas only 0.47 ± 0.08% of penicillin G remained. The stabilizing effect of the lipid coatings is also apparent. As the lipid concentration is increased, the penicillin activity in the filtrate is diminished and the potency retained in the residue is increased. For example, in going from antibiotic–lipid ratios of 1:1, 1:3, and 1:6 in the case of the potassium salt of penicillin G–cholesteryl acetate, the filtrates contained 103, 92, and 78 units,

[1] Pfizer Inc., New York, N.Y.
[2] Abbott Laboratories, North Chicago, Ill.
[3] K and K Laboratories Inc., Plainview, N.Y.
[4] Fisher Scientific Co., Fair Lawn, N.J.
[5] Matheson, Coleman and Bell, Norwood, Ohio.
[6] J. T. Baker Chemical Co., Phillipsburg, N.J.
[7] Model W.B.R., New Brunswick Scientific Co., New Brunswick, N.J.
[8] Beckman Zeromatic, Beckman Instrument Co., Fullerton, Calif.
[9] Model G-27, New Brunswick Scientific Co., New Brunswick, N.J.
[10] U.S. Series designation [12.7 cm (5 in.) diameter]: No. 10, 2000 μm (9 mesh); No. 14, 1410 μm (12 mesh); No. 18, 1000 μm (16 mesh); No. 35, 500 μm (32 mesh); No. 60, 250 μm (60 mesh); and No. 140, 105 μm (150 mesh); Arthur H. Thomas Co., Chicago, Ill.
[11] Arthur H. Thomas Co., Chicago, Ill.
[12] Millipore Corp., Bedford, Mass.
[13] Drierite.
[14] The microbiological assays were conducted by Ruth Wallace, Pfizer Inc.

Table IV—Antibiotic Urine Recovery (Percent) in Six Adults after Oral Administration of Plain and Cholesteryl Acetate-Coated (1:6) Potassium Salts of Penicillin G and Penicillin V (Single Dose of 400,000 Units)[a]

	Potassium Penicillin G				Potassium Penicillin V			
	Plain		Lipid Coated		Plain		Lipid Coated	
Subject	0–4 hr	4–8 hr	0–4 hr	4–8 hr	0–4 hr	4–8 hr	0–4 hr	4–8 hr
S.P.P.	18.20	1.89	25.11	8.91	22.40	4.09	39.97	14.21
C.I.J.	21.78	3.09	30.11	8.70	29.16	5.10	40.79	18.24
F.S.	19.73	3.84	26.87	10.12	29.02	3.06	40.11	16.62
R.P.K.	20.82	3.74	28.82	10.57	31.82	3.97	42.29	19.09
M.S.	17.73	2.86	26.91	9.08	30.23	3.30	44.35	13.50
E.A.	16.14	2.98	21.97	7.83	26.15	4.09	37.27	16.92
Average	19.07	3.07	26.63	9.21	28.12	3.94	40.80	16.26
$\pm SD$	4.5	1.3	5.5	1.9	6.8	0.7	4.5	4.3

[a] Statistical evaluation of the data by the Student t test gave the following p values: potassium penicillin G, plain *versus* coated, 0–4 hr, $p < 0.05$, and 4–8 hr, $p < 0.001$; potassium penicillin V, plain *versus* lipid coated, 0–4 hr, $p < 0.01$, and 4–8 hr, $p < 0.001$.

respectively. The antibiotic potency retained in the residues, as anticipated, increased (1507, 1798, and 2716 units, respectively).

Cholesteryl acetate was the most protective of the three lipids studied. It was not surprising to find that the oral absorption efficiency for both the penicillins was best when cholesteryl acetate was employed as the lipid coating (Table III). The urine recovery data from a six-patient crossover study are shown in Table IV. The higher antibiotic potency recovered was statistically significant for either penicillin coated with cholesteryl acetate (1:6) when compared with the urine levels obtained with uncoated material.

In Table II it can be seen that the acid-labile erythromycin lactobionate yielded higher antibiotic urine levels when coated with cholesteryl acetate in a ratio of 1:6. In addition, for both subjects there was a greater urine recovery of erythromycin after oral administration of the lipid-coated lactobionate as compared with erythromycin stearate.

Thus, it has been shown that the acid-labile antibiotics, penicillin G, penicillin V, and erythromycin, can have their oral availability enhanced by the application of a protective lipid coating such as cholesteryl acetate. Furthermore, the significantly higher 4–8-hr urine recoveries of antibiotic from the lipid-coated penicillins are indicative of a more prolonged systemic action. It appears from the presented data that further exploration of the utility of lipids as drug delivery systems is warranted.

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Ultracentrifugal Study of Effect of Sodium Chloride on Micelle Size of Fusidate Sodium

ALFRED J. RICHARD

Abstract □ Apparent micellar molecular weights were determined with the antibiotic fusidate sodium by ultracentrifugation in varying counterion concentrations (Na^+). The effects of buffer salts, pH, sodium chloride concentration, and drug concentration were studied. The results strongly support the concept of the formation of primary micelles composed of five monomer units, followed by aggregation of the pentomers into larger micelles as salt concentration increases.

Keyphrases □ Fusidate sodium micelles—molecular weights determined by ultracentrifugation, effects of buffer salts, pH, and sodium chloride and drug concentrations □ Micelle size of fusidate sodium—determined by ultracentrifugation, effects of buffer salts, pH, and sodium chloride and drug concentrations □ Ultracentrifugation—determination of fusidate sodium micelle molecular weights in varying counterion concentrations □ Sodium chloride effect—micelle size of fusidate sodium

The counterion concentration has been shown to control the size of micelles in solutions of bile salts (1, 2) and also in solutions of the steroid-like antibiotic fusidate sodium[1] (I) (3–5). The critical micelle concentration (CMC) of I was found by surface tension measurements (3) to be 3–4 m*M* in solutions containing 0.01–0.6 *M* NaCl. Aggregation numbers of I micelles were not reported at counterion concentrations below 0.01 *M* nor above 0.6 *M* NaCl.

In view of the studies of Godtfredsen *et al.* (5) showing that I in humans is excreted through the bile and that rather large concentration changes occur during storage of the bile in the gallbladder, it was decided to study the apparent micelle molecular weights of I by ultracentrifugation through as wide a counterion concentration range as possible. In addition, the effect of I concentration and of rotor speed on micelle size was investigated. The results tend to confirm the postulate of Small (1) that primary micelles, perhaps pentomers, are formed through hydrophobic interactions of the steroid-like structure, followed at increasing ionic strength by the formation of secondary micelles through hydrogen bond-type interactions of the hydrophilic groups.

EXPERIMENTAL

No secondary spots were observed with I[2] by TLC (3, 4). All chemicals used in this work were analytical reagent grade. Molecular weights were determined with an analytical ultracentrifuge[3] equipped with schlieren optics and a temperature control. All determinations of molecular weight were made at 25°, with the camera lens focused at the two-thirds level of the cell, as is required (6) for aqueous solutions. Schlieren patterns were read on a two-dimensional microcomparator[4], followed by data analysis according to Method II of Van Holde and Baldwin (7).

The pH, when not controlled by buffers, varied between 8 and 9, depending on the I concentration. Compound I has a reported pKa of 5.35 (3). Tromethamine–hydrochloric acid buffer was made up at 0.1, 0.05, 0.025, and 0.0125 *M* tromethamine to pH 7.3. Sodium phosphate buffers also were made up at 0.1, 0.06, 0.03, and 0.015 *M* to pH 7.2. The partial specific volume of I was taken to be 0.774 ml/g, as reported previously (3).

RESULTS AND DISCUSSION

The effect of sodium chloride concentration on the apparent *z*-average molecular weight of I micelles is shown in Fig. 1, where $M_{z_{app}}$ is plotted against the molarity of sodium chloride for 18 m*M* I. Rotor speed effects were negligible in the 18 m*M* I solution; with the 0.6 *M* NaCl mixture, equilibrium was obtained at seven different speeds from 14,290 to 44,040 rpm with no resultant change in the calculated molecular weight. Overall, the data tend to fall on one curve with some scatter but extrapolate to the molecular weight of the I monomer of 538.

Also plotted in Fig. 1 are the results of Carey and Small (3) for 20 m*M* I at pH 10 in carbonate buffer, which extrapolate at zero added salt to about a molecular weight of 2500, *i.e.*, to the pentomer of I. Therefore, these investigators considered the pentomer as the size of primary micelles for I solutions.

Measurements of the molecular weight were also made on 18 m*M* I solutions in phosphate buffers and tromethamine buffers at pH 7.2 and 7.3, respectively. The plot shown in Fig. 1 for phosphate rises steeply and linearly from the monomer (538 daltons) at low phosphate molarity up to about 10,000 daltons when the total phosphate molarity reaches 0.1. The data end there, since increasing the phosphate content further resulted in slow gel formation up to 0.15 *M* phosphate and in rapid precipitation at any concentrations above 0.15 *M* phosphate. An attempt was made to study the effect on I micelles of sodium chloride added to 0.06 *M* phosphate buffer. However, independently of the order of mixing, I

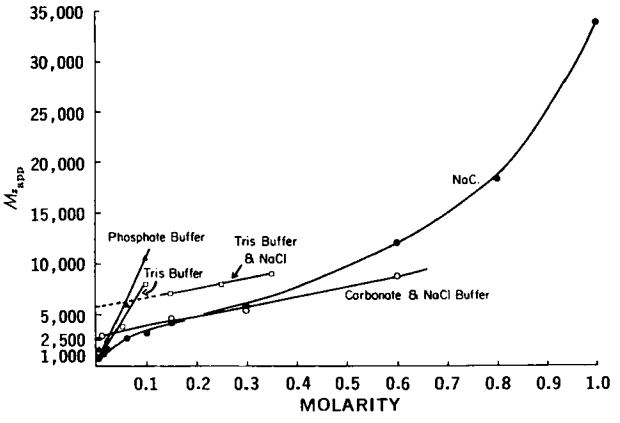

Figure 1—*Effect of various salt concentrations and pH on the apparent z-average micellar molecular weight of 18 m*M *I solutions. Key: ●, sodium chloride; ○, carbonate–bicarbonate buffer (0.01* M*) plus added sodium chloride, pH 10.0; ▲, phosphate buffer, pH 7.2; and □, tromethamine (tris) buffer alone and tromethamine (tris) buffer (0.05* M*) plus added sodium chloride, pH 7.3.*

[1] Sodium 3α,11α,16β-trihydroxy-29-nor-8α,9β,13α,14β-dammara-17(20),24-dien-21-oate 16-acetate.

[2] Lot 22151, supplied by Dr. W. O. Godtfredsen, Leo Pharmaceutical Products, Ballerup, Denmark.

[3] Model E, Beckman Instruments, Palo Alto, Calif.

[4] Nikon model 6, Nippon Kogaku, K. K. Japan.

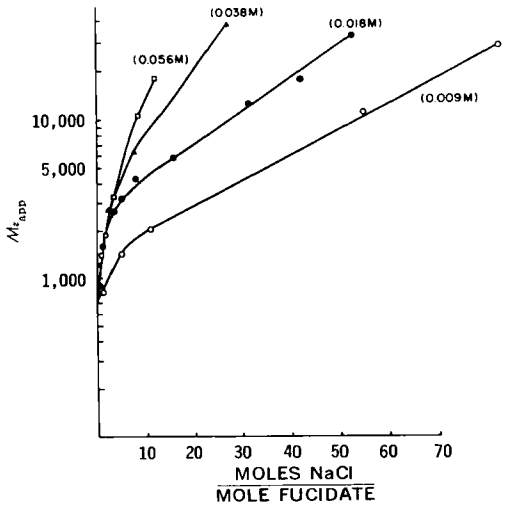

Figure 2—*Logarithm of the apparent z-average micellar molecular weight of I solutions as a function of the ratio of salt to drug for four different I concentrations.*

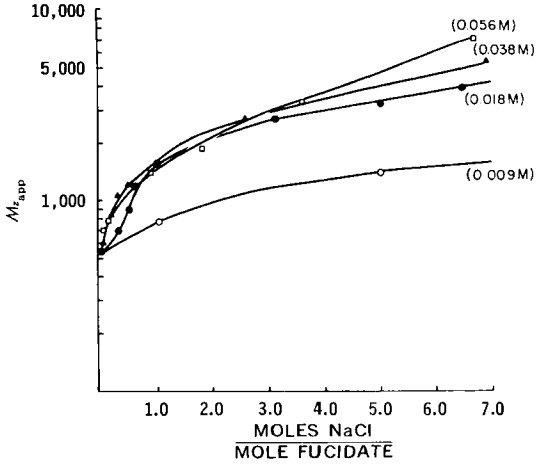

Figure 3—*Logarithm of the apparent z-average micellar molecular weight of I solutions as a function of the ratio of salt to drug in regions of low salt concentrations. Abscissa is expanded 10-fold over that of Fig. 2.*

would not dissolve in 0.06 *M* phosphate with added 0.1 *M* NaCl, and no data were obtained.

With increasing tromethamine buffer concentration at a constant pH of 7.3, a rapid linear increase in micelle size was seen, with slow gel formation again occurring at tromethamine concentrations above 0.1 *M*. However, addition of sodium chloride to the 0.05 *M* tromethamine buffer did not cause precipitation of I as it did with the phosphate system. A linear growth of micellar molecular weight was found that paralleled the data of Carey and Small (3) with carbonate–salt buffer at pH 10. Extrapolation to zero added salt, however, gave the decamer molecular weight rather than the pentomer obtained in carbonate buffer.

It has been stated (8, 9) that pH, counterion concentration, drug concentration, and temperature are important factors defining the size of micelles in solution. As shown by comparing the micelle molecular weight of I in phosphate and in tromethamine buffers at the same pH, however, care must be taken that interactions with the buffer salts themselves do not introduce additional micelle growth separate from any pH effect *per se*. Molecular models indicate that phosphate hydrogen bonds with the hydroxy groups of I. Thus, with phosphate bridging, I aggregates grow rapidly from the hydrophilic side (at low total buffer salt content) as well as from the hydrophobic side of the molecule. A similar argument, except for weaker hydrogen bond bridging than is present with phosphate, would explain the rapid growth of I micelles with the concentration of tromethamine buffer and also account for the continued solubility of I in the presence of added sodium chloride.

The sodium chloride curve in Fig. 1 suggested a logarithmic response of micellar weight to salt; therefore, various semilogarithmic graphs were attempted of the calculated molecular weights for four different I concentrations: *(a)* against molarity of sodium chloride, *(b)* against the square root of ionic strength, *(c)* against the mole fraction of sodium chloride, and *(d)* against the ratio of sodium chloride to I. Only the latter plots are shown in Fig. 2, because they contain linear portions that point out especially well the effect of sodium chloride concentration on micelle size. Excellent linearity was observed for all concentrations of I from 9 to 56 m*M* if the salt was present in 10-fold excess over the drug.

Unfortunately, the ultracentrifugal equipment available did not permit study at I concentrations in the vicinity of the CMC because of the limited instrument sensitivity with the standard 12-mm centrifuge cell depth.

To study the low salt region of Fig. 2 in more detail, the log $M_{z_{app}}$ was plotted against an abscissa expanded 10-fold (Fig. 3). It is clear from Fig. 3 that curves for 9, 18, and 38 m*M* I extrapolate to the monomer molecular weight at zero salt but that the curve for 56 m*M* I seems to extrapolate to a slightly higher value of M_z. Also, a rapid dissociation is indicated for the I micelles in 18 m*M* drug concentration as the salt to I ratio drops below unity. It is seen that an aggregation number of three (M_z = 1600) is not obtained for the 18, 38, and 56 m*M* I solutions until the salt to drug ratio reaches unity but that the curves seem to meet near that common point before diverging into higher molecular weight ranges.

To illustrate the effect of I concentrations on micellar size, the data from Figs. 2 and 3 were plotted in Fig. 4 as log $M_{z_{app}}$ against concentration of I at several different salt to drug ratios. Also shown is the micelle growth curve in the absence of a supporting electrolyte. Two effects are immediately obvious from Fig. 4. First, there exists a family of horizontal lines at salt to drug ratios from 0 to 2.5, where the apparent micellar weight is independent of I concentration after an initial rapid growth from the monomer to some constant molecular weight. The micellar weight does increase, however, with an increasing salt to drug ratio. It appears that no large micelles are formed until the salt to drug ratio is approximately three and until the extrapolated micelle molecular weight reaches 2600; that is, until pentomers are formed. Beyond that point, rapid logarithmic growth in micellar size occurs up to the occurrence of a phase change, often noted at molecular weights of 35,000 or greater as the separation of an oily layer from the solution.

Figure 4 strongly indicates that two mechanisms of aggregation are involved in the formation of micelles of I. First, with low salt to drug ratios, the salt reduces charge–charge repulsions of the car-

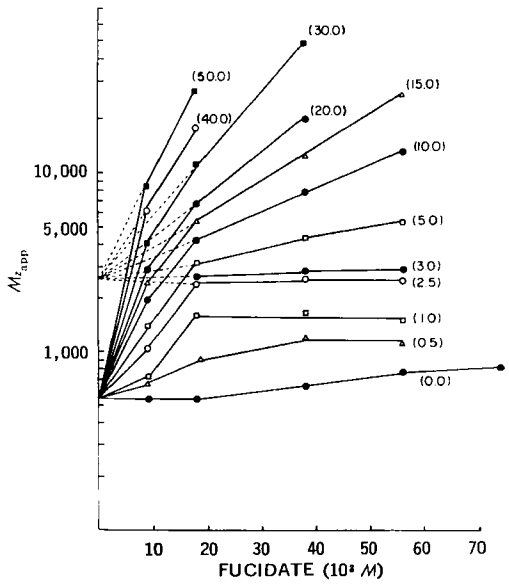

Figure 4—*Logarithm of apparent z-average micellar molecular weight of I solutions as a function of I molarity for several different sodium chloride to drug ratios. Notations on each curve indicate the salt to I molar ratio.*

boxyl group of I, thus making possible the approach and subsequent hydrophobic bonding of the steroid-like ring structure of neighboring molecules. This can continue until pentomers become the dominant species, after which increasing salt gives rise to secondary micelles. These are formed, as suggested by Small (1), by dehydration of the weaker nonionic groups and resultant hydrogen bond formation between primary micelles, analogous to a salting-out effect.

On a molar basis, the free energy of dimerization of I was calculated from the salt-free system. At the highest concentration studied, 74 m*M*, the molecular weight was 850. Thus, from both the expression for the *z*-average molecular weight (Eq. 1):

$$M_z = \frac{\sum_i m_i M_i^3}{\sum_i m_i M_i^2} = \frac{m_1 M_1^3 + m_2 M_2^3}{m_1 M_1^2 + m_2 M_2^2} \qquad \text{(Eq. 1)}$$

and the relationship between monomer and dimer concentrations (Eq. 2):

$$m_1 = m_{0_1} - 2m_2 \qquad \text{(Eq. 2)}$$

the concentration of monomer and dimer could be obtained, assuming ideal solution properties and the absence of larger aggregates. The equilibrium dimer concentration (m_2) was found to be 15 m*M*, and the monomer concentration (m_1) at equilibrium was 44 m*M*. The symbol used for initial monomer concentration was m_{0_1}. Then the equilibrium constant for the reaction:

$$2 \text{ fusidate} \rightleftharpoons \text{fusidate}_2$$

Scheme I

was given by:

$$K = \frac{[\text{fusidate}_2]}{[\text{fusidate}]^2} = 7.75 \qquad \text{(Eq. 3)}$$

and:

$$\Delta G^\circ_{298} = -RT \ln K = -1200 \text{ cal/mole} \qquad \text{(Eq. 4)}$$

On the other hand, with sodium chloride added to give a salt to I ratio of 0.5, dimerization was facilitated. A molecular weight of 900 was reached at a I concentration of only 18 m*M*, and repetition of the thermodynamic calculation for dimerization led to a value of 56 for *K* and of −2400 cal/mole for ΔG°_{298}. In this second computation, it was assumed, as shown by Carey and Small (3), that salt was not specifically or firmly bound to the dimer but acted only as a supporting electrolyte, that is, to mask charge at the anionic head of I. This value of ΔG°_{298} is in agreement with values calculated for dimerization of sodium lauryl sulfate (10) and in fair agreement with the −3400 cal/mole calculated for I from CMC data (3) at pH 10 in carbonate buffer, although in the latter work I dimers were not specified exclusively in the composition of the micelle. Work is continuing to interpret the multiple equilibria involved as the salt to I ratio leads to the formation of larger aggregates in solution.

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KISHOR B. PAREKH, WILLIAM H. SHELVER ˣ, AI-YU SHEN TSAI, and RICHARD REOPELLE

Abstract □ Eleven 2-substituted aminomethylnaphtho(2,3-*b*)-1,4-dioxans were synthesized. The nucleophilic displacement of 2-tosyloxymethylnaphtho(2,3-*b*)-1,4-dioxan by appropriate amines was carried out using dimethyl sulfoxide as the solvent. Preliminary pharmacological evaluation revealed a potentiation of norepinephrine at low doses and a noncompetitive antagonism at high doses in the rat vas deferens and a dose-related hypotensive action of short duration in the anesthetized rat.

Keyphrases □ Aminomethylnaphtho(2,3-*b*)-1,4-dioxans, 2-substituted—synthesized and screened as potential adrenergic blocking agents □ Dioxans—synthesis and screening of 2-substituted aminomethylnaphtho(2,3-*b*)-1,4-dioxans as potential adrenergic blocking agents □ Adrenergic blocking agents, potential—synthesis and screening of 2-substituted aminomethylnaphtho(2,3-*b*)-1,4-dioxans

Bovet and Simon (1) were the first investigators to demonstrate that 2-substituted aminomethyl-1,4-benzodioxans (I) possessed the ability to block certain actions of epinephrine. Piperoxan blocked norepinephrine by means of competitive antagonism in a study utilizing the rat vas deferens (2). The effects of

boxyl group of I, thus making possible the approach and subsequent hydrophobic bonding of the steroid-like ring structure of neighboring molecules. This can continue until pentomers become the dominant species, after which increasing salt gives rise to secondary micelles. These are formed, as suggested by Small (1), by dehydration of the weaker nonionic groups and resultant hydrogen bond formation between primary micelles, analogous to a salting-out effect.

On a molar basis, the free energy of dimerization of I was calculated from the salt-free system. At the highest concentration studied, 74 m*M*, the molecular weight was 850. Thus, from both the expression for the *z*-average molecular weight (Eq. 1):

$$M_z = \frac{\sum_i m_i M_i^3}{\sum_i m_i M_i^2} = \frac{m_1 M_1^3 + m_2 M_2^3}{m_1 M_1^2 + m_2 M_2^2} \qquad \text{(Eq. 1)}$$

and the relationship between monomer and dimer concentrations (Eq. 2):

$$m_1 = m_{0_1} - 2m_2 \qquad \text{(Eq. 2)}$$

the concentration of monomer and dimer could be obtained, assuming ideal solution properties and the absence of larger aggregates. The equilibrium dimer concentration (m_2) was found to be 15 m*M*, and the monomer concentration (m_1) at equilibrium was 44 m*M*. The symbol used for initial monomer concentration was m_{0_1}. Then the equilibrium constant for the reaction:

$$2 \text{ fusidate} \rightleftharpoons \text{fusidate}_2$$

Scheme I

was given by:

$$K = \frac{[\text{fusidate}_2]}{[\text{fusidate}]^2} = 7.75 \qquad \text{(Eq. 3)}$$

and:

$$\Delta G^\circ_{298} = -RT \ln K = -1200 \text{ cal/mole} \qquad \text{(Eq. 4)}$$

On the other hand, with sodium chloride added to give a salt to I ratio of 0.5, dimerization was facilitated. A molecular weight of 900 was reached at a I concentration of only 18 m*M*, and repetition of the thermodynamic calculation for dimerization led to a value of 56 for *K* and of −2400 cal/mole for ΔG°_{298}. In this second computation, it was assumed, as shown by Carey and Small (3), that salt was not specifically or firmly bound to the dimer but acted only as a supporting electrolyte, that is, to mask charge at the anionic head of I. This value of ΔG°_{298} is in agreement with values calculated for dimerization of sodium lauryl sulfate (10) and in fair agreement with the −3400 cal/mole calculated for I from CMC data (3) at pH 10 in carbonate buffer, although in the latter work I dimers were not specified exclusively in the composition of the micelle. Work is continuing to interpret the multiple equilibria involved as the salt to I ratio leads to the formation of larger aggregates in solution.

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Keyphrases □ Aminomethylnaphtho(2,3-*b*)-1,4-dioxans, 2-substituted—synthesized and screened as potential adrenergic blocking agents □ Dioxans—synthesis and screening of 2-substituted aminomethylnaphtho(2,3-*b*)-1,4-dioxans as potential adrenergic blocking agents □ Adrenergic blocking agents, potential—synthesis and screening of 2-substituted aminomethylnaphtho(2,3-*b*)-1,4-dioxans

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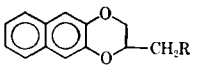
Table I—Physical Properties of the Hydrochloride Salts of 2-Substituted Aminomethylnaphtho(2,3-*b*)-1,4-dioxans



Compound	R	Melting Point	Yield, %	Analysis, % Calc.	Found
II*a*	$N(CH_3)_2$	225–227°	40	C 64.40 H 6.48 N 5.01	64.55 6.31 5.23
II*b*	$N(C_2H_5)_2$	226–227°	30	C 66.34 H 7.20 N 4.55	66.19 7.18 4.36
II*c*	$N(n\text{-}C_3H_7)_2$	198–200°	58	C 67.95 H 7.79 N 4.17	68.07 7.68 4.04
II*d*	$N(iso\text{-}C_3H_7)_2$	193–195°	22	C 67.95 H 7.79 N 4.17	67.84 7.76 4.04
II*e*	$N(n\text{-}C_4H_9)_2$	174–175°	70	C 69.31 H 8.30 N 3.85	69.48 8.42 3.68
II*f*		275–277°	60	C 67.60 H 6.93 N 4.38	67.63 7.07 4.20
II*g*		252–253°	79	C 66.77 H 6.59 N 4.58	66.96 6.71 4.45
II*h*		245–247°	70	C 63.45 H 6.26 N 4.35	63.58 6.40 4.15
II*i*		269–270°	82	C 58.32 H 6.51 N 7.54	58.34 6.60 7.41
II*j*	$HNCH_2CH(OH)C_6H_5$	244–245°	55	C 67.83 H 5.96 N 3.77	67.96 6.04 3.62
II*k*	$HNCH_2CH_2C_6H_5$	274–275°	52	C 70.88 H 6.22 N 3.94	71.00 6.34 3.77

structural variation in the dialkylaminoalkyl side chain, in the dioxan ring, and on the aromatic portion of the molecule have been reviewed (3). An extension of the aromatic area of the benzodioxan nucleus might provide an additional area for receptor binding by means of van der Waal's attraction. Additional binding would increase the potency of the compound.

Synthesis and biological evaluation of the 2-substituted aminomethylnaphtho(2,3-*b*)-1,4-dioxan (II) would indicate whether additional area on the receptor is available for this type of binding; therefore, 11 derivatives of 2-substituted aminomethylnaphtho(2,3-*b*)-1,4-dioxans were synthesized by means of the route shown in Scheme I. Previously, Augstein *et al.* (4) reported the synthesis of 2-guanidinomethylnaphtho(2,3-*b*)-1,4-dioxan.

EXPERIMENTAL[1]

2-Hydroxymethylnaphtho(2,3-*b*)-1,4-dioxan—To a stirring, refluxing mixture of 160 g (1.0 mole) of 2,3-dihydroxynaphthalene and 40 g (1.0 mole) of sodium hydroxide in 400 ml of 95% ethanol, 92 g (1.0 mole) of epichlorohydrin was added dropwise. The reaction mixture was refluxed for 4 hr and cooled. The precipitate was filtered, washed with ether and 95% ethanol, and recrystallized from benzene to yield 162 g (75%) of solid material, mp 156–157°. This compound was previously synthesized (4), but no physical or analytical data were reported.

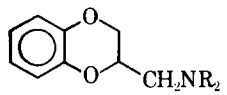
Anal.—Calc. for $C_{13}H_{12}O_3$: C, 72.21; H, 5.59. Found: C, 72.34; H, 5.66.

2-Tosyloxymethylnaphtho(2,3-*b*)-1,4-dioxan—One hundred and eight grams (0.5 mole) of the above alcohol was treated with 190 g (1.0 mole) of *p*-toluenesulfonyl chloride at 0° in anhydrous pyridine for 24 hr. The reaction mixture was poured onto ice, and the solid that separated was recrystallized from acetone–water to yield 157 g (85%) of a solid material, mp 127–128.5°.

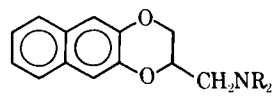
Anal.—Calc. for $C_{20}H_{18}O_5S$: C, 64.85; H, 4.89; S, 8.65. Found: C, 65.02; H, 5.01; S, 8.56.

2-Substituted Aminomethylnaphtho(2,3-*b*)-1,4-dioxans (Table I)—A solution of 10 g (0.027 mole) of 2-tosyloxymethylnaphtho(2,3-*b*)-1,4-dioxan in 100 ml of dimethyl sulfoxide was treated with an excess of the appropriate amine and heated to 80° for 36 hr. Then the reaction mixture was poured into ice, and the amine was isolated and converted to the hydrochloride salt.

Blood Pressure Experiments—Male Wistar rats (300 g) were anesthetized with 35 mg/kg of secobarbital, and the arterial blood pressure was monitored at the carotid artery by means of a linear core pressure transducer and recorded using a physiograph[2]. The drugs were injected into the femoral vein which had been previously cannulated. Normal blood pressure varied between 120 and 140



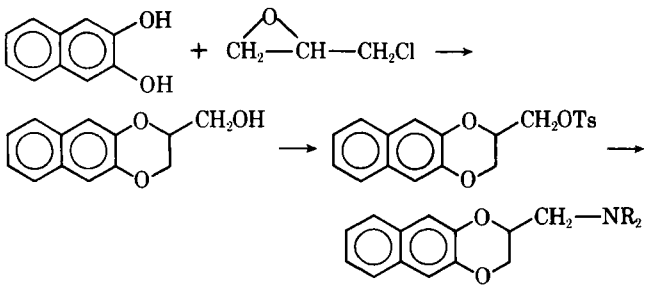
I



II

[1] The melting points of the compounds were determined on a Thomas-Hoover Uni-Melt apparatus and are uncorrected. IR spectra were obtained on a Perkin-Elmer model 337 IR spectrophotometer from potassium bromide pellets. NMR spectra were determined on a Varian model A-60A in $CDCl_3$ solution, using tetramethylsilane as a reference. Microanalyses were performed by Alfred Bernhardt mikroanalytische Laboratorium, West Germany.

[2] E&M electronic physiograph.



Scheme I

mm Hg. The data were plotted as the percent reduction of blood pressure *versus* dose of the drug (Fig. 1).

Vas Deferens Experiments—Male Wistar rats were killed by a blow on the head, and the vas deferens was dissected free. The vas deferens was placed in a tissue bath (23 ml) containing Tyrodes solution[3] and connected to an isotonic writing lever registering on a kymograph. The Tyrodes solution was maintained at 35° and aerated with 95% O_2–5% CO_2. The vas deferens was made to contract maximally with high doses of norepinephrine prior to beginning the cumulative response curves. Cumulative response curves were prepared by adding norepinephrine, allowing the muscle to equilibrate, adding twice the previous concentration of norepinephrine, and allowing the equilibration to occur until the muscle failed to contract further. The vas deferens was then washed with Tyrodes solution until the muscle relaxed to the original state.

Two cumulative curves of norepinephrine were obtained prior to beginning experiments on the naphthodioxans. The naphthodioxan was then added to the bath, and the response to norepinephrine was again determined. The minimal dose of naphthodioxan used was 4.4×10^{-6} mole/liter, and the maximum dose was 4.4×10^{-4} mole/liter. The percent response to the drugs was then compared to norepinephrine controls (Fig. 2).

PHARMACOLOGICAL EVALUATION

In a preliminary pharmacological study, the dimethylamino, diethylamino, dipropylamino, and the piperidino compounds were tested for their effects on blood pressure in rats. Each drug was tested on one rat in the preliminary screen. All of these compounds lowered the blood pressure of the rat, with the piperidino compound being the most active; however, the duration of the blood pressure response was extremely short.

Figure 1 shows the dose–response curve obtained with piperidinonaphthodioxan. When the piperidino compound was tested using the vas deferens preparation, the compound first potentiated

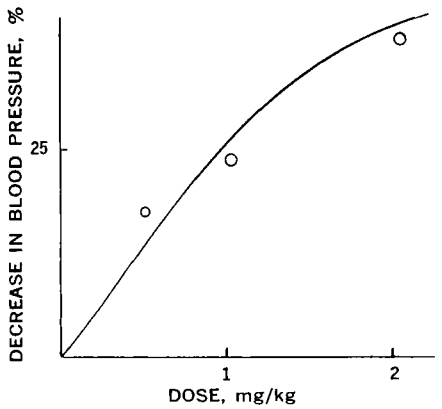


Figure 1—*Effects of 2-piperidinomethylnaphtho(2,3-b)-1,4-dioxan on the blood pressure of the rat. The points represent results on an individual rat, and the curve is arbitrarily drawn to go through the origin.*

[3] Containing (per liter) 8.0 g of NaCl, 0.1 g of KCl, 0.2 g of $CaCl_2$, 0.005 g of NaH_2PO_4, 1.0 g of $NaHCO_3$, and 1.0 g of glucose.

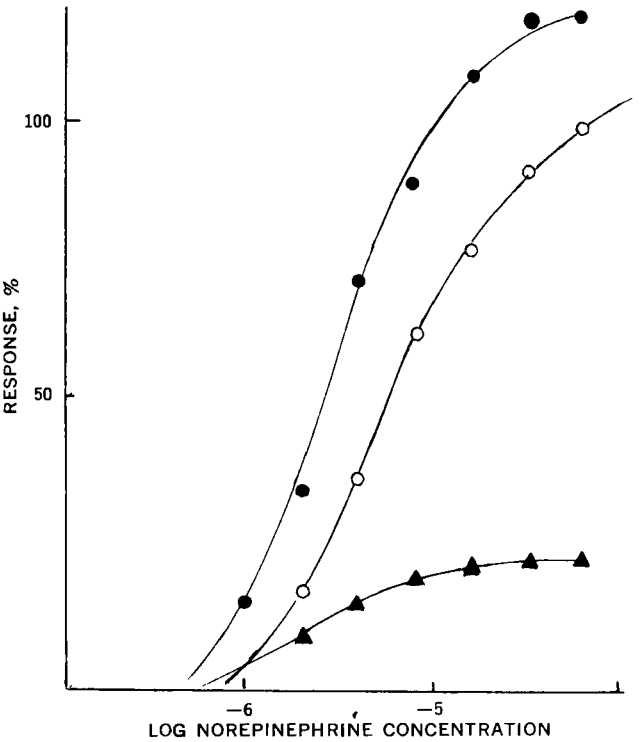


Figure 2—*Effects of 2-piperidinomethylnaphtho(2,3-b)-1,4-dioxan on the rat vas deferens. The points were obtained on an individual vas deferens. All concentrations are in moles per liter. Key:* ●, *4.4×10^{-6};* ○, *control; and* ▲, *4.4×10^{-4}.*

norepinephrine and then antagonized the effect of norepinephrine in a noncompetitive manner. The concentrations appropriate for the vas deferens experiment were found in a preliminary experiment, and a new vas deferens was utilized in the final test. Figure 2 shows the effect of piperidinonaphthodioxan relative to the response demonstrated by norepinephrine.

DISCUSSION

The synthesis of these compounds is straightforward and calls for little discussion except for the utilization of dimethyl sulfoxide as the solvent for the displacement of the tosylate with the amine. This solvent greatly improves yields and shortens reaction times in the synthesis of dialkylaminomethyl-1,4-benzodioxans.

The dose-related drop in blood pressure (Fig. 1) indicated that 2-piperidinomethylnaphtho(2,3-*b*)-1,4-dioxan was an extremely potent hypotensive agent, producing a noticeable drop of 28 mm Hg at 0.5 mg/kg. Unfortunately, the duration was extremely short, less than 30 sec, when the drug was given intravenously.

The experiments on the rat vas deferens indicate that these compounds act by a different mechanism than the model compounds, the benzodioxans (I). The benzodioxans act by competitive inhibition, shifting the norepinephrine dose–response curve to the right. The response curve of norepinephrine was shifted to the left and the maximum response was increased at low doses of piperidinomethylnaphtho(2,3-*b*)-1,4-dioxan whereas high doses depressed the maximum response. A similar type of response has been observed for protriptyline and desipramine (5). Avner and Triggle (6) observed an enhanced contraction to norepinephrine in the rat vas deferens exposed to 2-diethylamino-1,4-benzodioxans and attributed the enhancement to increased mobilization of calcium.

Two possible explanations exist for the naphthodioxans' ineffectiveness on the benzodioxan receptor. The transport of antagonists to the benzodioxan receptor may be partition coefficient dependent (Hansch-type relationship), or the naphthodioxans have a much greater lipid solubility than the corresponding benzodioxans and thus the naphthodioxans may not be transported to the receptor in an optimum fashion. An alternative and more attractive ex-

planation may be that the receptor for the benzodioxans will not accommodate the larger naphthodioxan.

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Influence of Particle Size on Rectal Absorption of Aspirin

EUGENE L. PARROTT

Abstract □ The rectal absorption of aspirin from theobroma oil suppositories was studied in seven human subjects using urinary excretion measurements. The effect of particle size on the excretion rate and cumulative amount of total salicylate excreted was demonstrated by the administration of a 600-mg dose as powdered aspirin and as aspirin disks having 0.023 as much surface as powdered aspirin. *In vitro* dissolution profiles of aspirin from the suppositories were studied. By the NF XIII Method II, the time required for 50% of the aspirin to dissolve from the suppository was 50 and 100 min for the powdered aspirin and the aspirin disks, respectively. In the bioavailability study, the diffusion equilibrium was attained at approximately 4–5 and 9–10 hr after the rectal administration of powdered aspirin and aspirin disks, respectively. No correlation was found between bioavailability and the dissolution profiles as determined by the USP XVIII dissolution method.

Keyphrases □ Aspirin—absorption from theobroma oil suppositories, effect of particle size □ Dissolution—aspirin from suppositories, effect of particle size □ Bioavailability—aspirin, effect of particle size, correlation with *in vitro* dissolution profiles □ Particle-size effect—aspirin absorption from suppositories □ Absorption, rectal—effect of particle size on aspirin absorption from suppositories

Since early studies (1, 2), numerous investigations have demonstrated the influence of particle size on bioavailability from oral dosage forms. For poorly soluble drugs, it is widely recognized that a smaller particle size (greater surface) brings about more rapid dissolution and more rapid GI absorption. If absorption is rate limited by slow dissolution so that the drug is not completely absorbed from a solid dosage form, the more rapid absorption obtained by increasing the surface may also cause an increase in the total amount of the drug absorbed from a given dose (3–6).

The purpose of this investigation was to demonstrate that the particle size of aspirin incorporated in a rectal suppository could influence absorption. As shown by plasma salicylate concentration (7) and urinary salicylate excretion (8), rectal administration of aspirin is as effective as oral administration. Wagner (9) cited certain general conclusions regarding absorption of drugs following rectal administration in humans. The emphasis in investigating suppositories has been on physical characteristics (10, 11), the influence of the base (12–14), and the *in vitro* release of the drug (15). By use of a simple aspirin and theobroma oil suppository, which melted at body temperature, the effect on absorption of approximately a 40-fold difference in surface area of the aspirin was studied.

EXPERIMENTAL

Protocol—Six healthy male and one healthy female subjects, 25–49 years of age and 60–82 kg, participated. Subjects were ambulatory and permitted to ingest food and fluids as desired. Urine samples were collected at hourly intervals after rectal insertion of the suppository. Volumes of the urine samples were measured, and aliquots were retained for analysis. Although the pH of each sample was measured, no attempt was made to control the pH. The formulations were coded, and a crossover technique was used with an interval of 1 week between bioavailability studies.

Preparation of Suppositories—When using mineral oil in the pycnometer, the density of powdered aspirin[1] was 1.40 g/cm^3. An 80–100-mesh fraction of the powdered aspirin was separated by a sieve shaker[2]. The average size of the fraction was assumed to be 163 μm. Aspirin powder of 163-μm size was quantitatively mixed with theobroma oil[3], placed in a suppository machine[4], and pressed into a suppository containing 600 mg of aspirin and 475 mg of theobroma oil.

Disks of aspirin were made by compressing the powdered aspirin with a 0.3175-cm (0.125-in.) flat-faced punch and die set. The length and diameter of each disk were measured using an optical micrometer[5]. Disks were selected so that 13 disks weighed 600 mg.

Suppositories containing the disks were prepared by the fusion method. The cavity of the mold was approximately one-third filled with melted theobroma oil, several disks were added, and the mass was allowed to congeal. This procedure was repeated until all disks had been added and the cavity was filled. The mold was immediately placed in a refrigerator so that the disks were not in contact

[1] USP, fine crystal, J. T. Baker Chemical Co.
[2] Allen-Bradley sonic sifter.
[3] Cocoa butter.
[4] Armstrong suppository machine No. 3.
[5] Gaertner Scientific Corp.

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EUGENE L. PARROTT

Abstract □ The rectal absorption of aspirin from theobroma oil suppositories was studied in seven human subjects using urinary excretion measurements. The effect of particle size on the excretion rate and cumulative amount of total salicylate excreted was demonstrated by the administration of a 600-mg dose as powdered aspirin and as aspirin disks having 0.023 as much surface as powdered aspirin. *In vitro* dissolution profiles of aspirin from the suppositories were studied. By the NF XIII Method II, the time required for 50% of the aspirin to dissolve from the suppository was 50 and 100 min for the powdered aspirin and the aspirin disks, respectively. In the bioavailability study, the diffusion equilibrium was attained at approximately 4–5 and 9–10 hr after the rectal administration of powdered aspirin and aspirin disks, respectively. No correlation was found between bioavailability and the dissolution profiles as determined by the USP XVIII dissolution method.

Keyphrases □ Aspirin—absorption from theobroma oil suppositories, effect of particle size □ Dissolution—aspirin from suppositories, effect of particle size □ Bioavailability—aspirin, effect of particle size, correlation with *in vitro* dissolution profiles □ Particle-size effect—aspirin absorption from suppositories □ Absorption, rectal—effect of particle size on aspirin absorption from suppositories

Since early studies (1, 2), numerous investigations have demonstrated the influence of particle size on bioavailability from oral dosage forms. For poorly soluble drugs, it is widely recognized that a smaller particle size (greater surface) brings about more rapid dissolution and more rapid GI absorption. If absorption is rate limited by slow dissolution so that the drug is not completely absorbed from a solid dosage form, the more rapid absorption obtained by increasing the surface may also cause an increase in the total amount of the drug absorbed from a given dose (3–6).

The purpose of this investigation was to demonstrate that the particle size of aspirin incorporated in a rectal suppository could influence absorption. As shown by plasma salicylate concentration (7) and urinary salicylate excretion (8), rectal administration of aspirin is as effective as oral administration. Wagner (9) cited certain general conclusions regarding absorption of drugs following rectal administration in humans. The emphasis in investigating suppositories has been on physical characteristics (10, 11), the influence of the base (12–14), and the *in vitro* release of the drug (15). By use of a simple aspirin and theobroma oil suppository, which melted at body temperature, the effect on absorption of approximately a 40-fold difference in surface area of the aspirin was studied.

EXPERIMENTAL

Protocol—Six healthy male and one healthy female subjects, 25–49 years of age and 60–82 kg, participated. Subjects were ambulatory and permitted to ingest food and fluids as desired. Urine samples were collected at hourly intervals after rectal insertion of the suppository. Volumes of the urine samples were measured, and aliquots were retained for analysis. Although the pH of each sample was measured, no attempt was made to control the pH. The formulations were coded, and a crossover technique was used with an interval of 1 week between bioavailability studies.

Preparation of Suppositories—When using mineral oil in the pycnometer, the density of powdered aspirin[1] was 1.40 g/cm^3. An 80–100-mesh fraction of the powdered aspirin was separated by a sieve shaker[2]. The average size of the fraction was assumed to be 163 μm. Aspirin powder of 163-μm size was quantitatively mixed with theobroma oil[3], placed in a suppository machine[4], and pressed into a suppository containing 600 mg of aspirin and 475 mg of theobroma oil.

Disks of aspirin were made by compressing the powdered aspirin with a 0.3175-cm (0.125-in.) flat-faced punch and die set. The length and diameter of each disk were measured using an optical micrometer[5]. Disks were selected so that 13 disks weighed 600 mg.

Suppositories containing the disks were prepared by the fusion method. The cavity of the mold was approximately one-third filled with melted theobroma oil, several disks were added, and the mass was allowed to congeal. This procedure was repeated until all disks had been added and the cavity was filled. The mold was immediately placed in a refrigerator so that the disks were not in contact

[1] USP, fine crystal, J. T. Baker Chemical Co.
[2] Allen-Bradley sonic sifter.
[3] Cocoa butter.
[4] Armstrong suppository machine No. 3.
[5] Gaertner Scientific Corp.

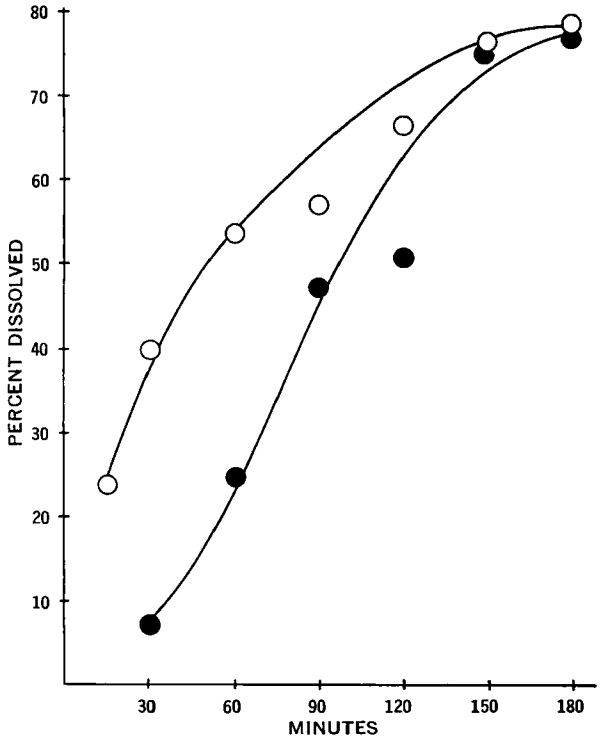

Figure 1—*Dissolution profile of aspirin from theobroma oil suppository using the NF Method II. Key:* ○, *powdered; and* ●, *disks.*

with the molten theobroma oil for more than 5 min. To determine if this brief exposure to the molten theobroma oil would alter the dimensions of the disk, disks were exposed to molten theobroma oil at 40° for 20 min and the dimensions were measured with an optical micrometer. No change in the diameter of the disk was detected. Each suppository contained 0.600 g of aspirin in the form of 13 disks and 1.50 g of theobroma oil.

All suppositories were stored in a refrigerator for 1 week before use.

Analytical Methods—The analysis of the urine samples for total salicylate was conducted by the method of Chiou and Onyemelukwe (16). For the measurement of dissolution from the suppository, an aliquot of the dissolution medium was subjected to alkaline hydrolysis. This solution was then acidified, appropriately diluted with 0.1 *N* hydrochloric acid, and assayed spectrophotometrically in terms of salicylic acid (17).

Dissolution—The percent of aspirin dissolved from the suppository was determined using 900 ml of distilled water at 37° in the NF Method II dissolution apparatus (18). Samples were removed by a pipet fitted with a filter, and the fluid was replenished with the same volume of distilled water. The samples were analyzed, and the percent dissolved at each interval of time was calculated (Fig. 1).

Similarly, the percent of aspirin dissolved from the suppository was determined using the USP dissolution apparatus (19) at 50, 100, and 200 rpm (Fig. 2).

RESULTS AND DISCUSSION

Bioavailability—The mean rates (±*SE*) of excretion of total salicylate for seven subjects are given in Table I. The initial excretion rates were greater for the aspirin powder with a surface of 320 cm^2 than for the aspirin disks with a surface of 7.5 cm^2, although both were incorporated in a theobroma oil base.

Even with the limited fluid available in the rectal canal, the suppository in which the aspirin had the greater surface dissolved at a faster rate and, consequently, this aspirin was absorbed and excreted more rapidly than the aspirin with approximately 0.023 as much surface.

Figure 3 shows the mean cumulative amount of total salicylate excreted as a function of time following rectal administration. In

Table I—Mean Rate of Total Salicylate Excretion (Milligrams per Hour ± *SE*) after the Administration of a Rectal Suppository Containing 600 mg of Aspirin in Seven Human Subjects

Collection Interval, hr	Powdered Aspirin, Surface = 320 cm^2	Disks of Aspirin, Surface = 7.5 cm^2
0–1	5.45 ± 1.87	1.67 ± 0.61
1–2	16.11 ± 4.22	4.36 ± 1.08
2–3	20.64 ± 5.83	5.79 ± 1.66
3–4	18.60 ± 5.51	7.55 ± 1.52
4–5	18.61 ± 4.54	10.35 ± 1.92
5–6	19.79 ± 5.15	11.38 ± 1.22
6–7	16.03 ± 3.75	13.09 ± 2.02
7–8	18.70 ± 5.18	14.91 ± 2.14
8–9	16.64 ± 3.86	15.28 ± 1.72
9–10	15.69 ± 3.68	16.16 ± 2.19
10–11	14.94 ± 4.30	14.62 ± 3.30
11–12	11.66 ± 3.06	15.28 ± 2.00

the 12-hr period, the total salicylate excreted after the administration of the suppository containing the powdered aspirin was 1.5 times that excreted after the administration of the suppository containing the aspirin disks. The data show that the particle size of aspirin incorporated in the theobroma oil influences the rectal absorption of aspirin.

In small doses of aspirin, the overall salicylate elimination proceeds by apparent first-order kinetics after the attainment of diffusion equilibrium. The rate of excretion *(dC/dt)* may be defined by (20):

$$\log (dC/dt) = -k_u t/2.3 + K \qquad \text{(Eq. 1)}$$

where t is time, and k_u is the specific velocity constant for elimina-

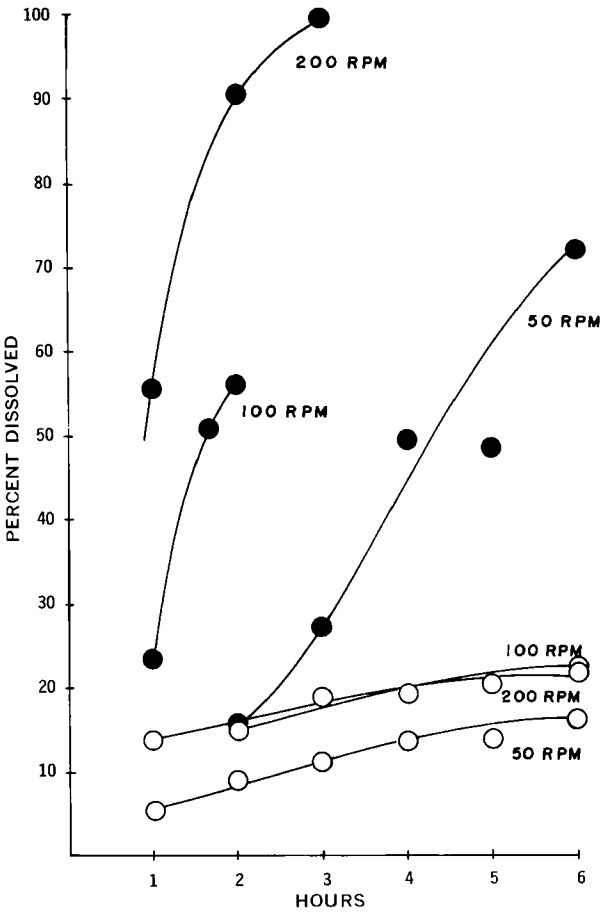

Figure 2—*Dissolution profile of aspirin from theobroma oil suppository using USP dissolution apparatus at 50, 100, and 200 rpm. Key:* ○, *powdered; and* ●, *disks.*

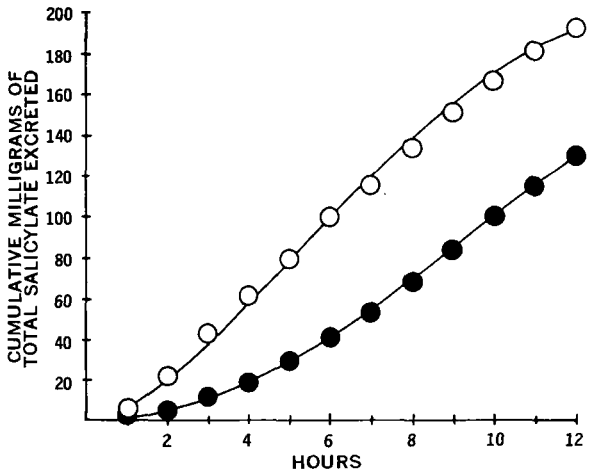

Figure 3—*Effect of particle size on the mean cumulative amounts of total salicylate excreted after the rectal administration of 600 mg of aspirin in seven human subjects. Key:* ○, *powdered; and* ●, *disks.*

tion of the drug based on urinary data. The biological half-life $(t_{1/2})_u$ based on urinary data is:

$$(t_{1/2})_u = 0.693/k_u \qquad \text{(Eq. 2)}$$

If the dose of aspirin is small, the half-life of salicylate elimination is from 4 to 6 hr (8, 21).

A semilogarithmic plot of the excretion rate against time according to Eq. 1 is shown in Fig. 4. The linear portion of the curve represents a $(t_{1/2})_u$ of 6 hr. With the powdered aspirin, the diffusion equilibrium is attained at approximately 4–5 hr; but with the aspirin disks having a limited surface, the diffusion equilibrium is not attained until approximately twice as much time has elapsed. Thus, the limited surface of the disks prolongs dissolution and aspirin is still being absorbed at 9–10 hr.

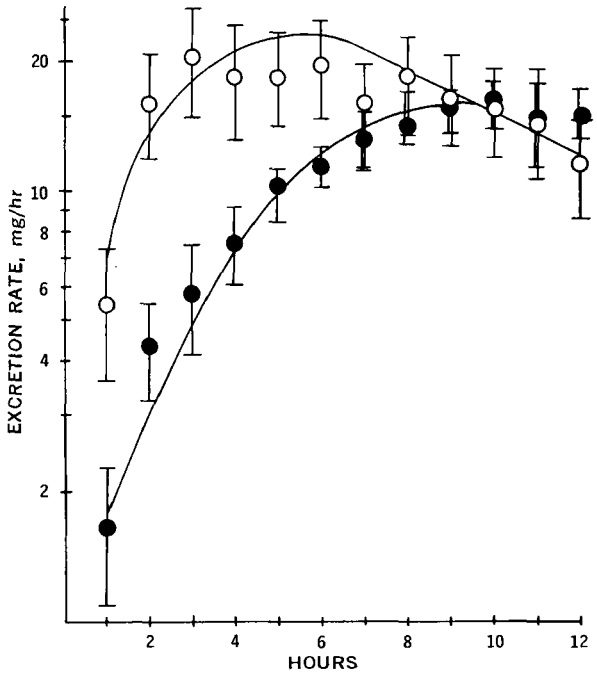

Figure 4—*Mean urinary excretion of total salicylates after rectal administration of 600 mg of aspirin in seven human subjects. Vertical bars represent standard error of the mean. Key:* ○, *powdered; and* ●, *disks.*

Dissolution—The usefulness of dissolution profiles for tablets has been demonstrated by the inclusion of dissolution specifications and methods in the USP and NF. It is interesting to apply these methods to other dosage forms such as the suppository. The dissolution profiles from suppositories containing powdered aspirin and aspirin disks are shown in Fig. 1 as determined by the NF XIII Method II. The time ($t_{50\%}$) required for 50% of the aspirin to dissolve from the suppository containing the powdered aspirin is one-half the $t_{50\%}$ for the suppository containing the aspirin disks. This *in vitro* comparison of the release from powdered aspirin and aspirin disks is similar to the ratio of *in vivo* attainment of the diffusion equilibrium from powdered aspirin and aspirin disks at 4–5 and 9–10 hr, respectively.

The USP dissolution apparatus was used similarly to show the effect of speed on the $t_{50\%}$. At 37°, the suppository melted within 5 min. The disks remained on the bottom of the basket and appeared to be free of theobroma oil. After the suppository containing the powdered aspirin melted, an oily mass of aspirin and theobroma oil remained on the bottom of the basket. If the apparatus was allowed to cool, the mass congealed to a brittle solid that was oily to the touch. Aspirin was very slowly released from the mass; at the speeds used, the $t_{50\%}$ was not reached after 7 hr in the apparatus. Obviously, the USP dissolution apparatus was not applicable to the evaluation of the suppository, while it appears that the NF Method II might be useful.

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Antiviral, Antibacterial, and Antifungal Activities of Isatin *N*-Mannich Bases

RAJENDRA S. VARMA * **and W. LEWIS NOBLES** ‡x

Abstract □ Preliminary antiviral, antibacterial, and antifungal screening results of a series of isatin *N*-Mannich bases are provided.

Keyphrases □ Isatin *N*-Mannich bases—synthesis, screened for antiviral, antibacterial, and antifungal activities □ Antiviral activity—synthesis and screening of isatin *N*-Mannich bases □ Antibacterial and antifungal activities—synthesis and screening of isatin *N*-Mannich bases

Isatin *N*-Mannich bases (1–3) were synthesized by the condensation of isatin(s), formaldehyde, and a suitable amine in equimolar proportions. Preliminary screening results for antiviral, antibacterial, and antifungal activities are described in this report. Antiviral activity in isatin *N*-Mannich bases was observed previously (2).

EXPERIMENTAL

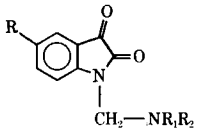
Antiviral screening was done using a modified[1] plaque suppression method (4, 5). Viruses used were poliomyelitis type II (RNA type), herpes simplex (DNA type), measles (RNA type), and parainfluenza-3 (RNA type). The viruses stock cultures were maintained in cultures of HeLa cells grown in Eagle's minimal essential medium. Confluent sheets of HeLa cells (a stable line of human adenocarcinoma cells) in petri dishes were heavily infected with the test virus and then overlaid with agar according to the Siminoff (5) procedure.

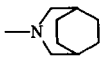
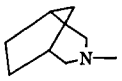
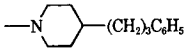
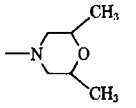
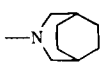
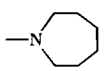
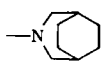
Filter paper disks (6.35 mm) saturated with 2 drops of a suspension of the test compound (20 mg/ml in ethanol) were then placed on the agar. After incubation for 2–4 days, the cells were stained with either neutral red or 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyltetrazolium chloride, which stained living cells only. Killing of the carcinoma cells was indicated by an unstained zone around the paper disk. Inhibition of the virus was indicated by a suppression of viral plaques in the vicinity of the chemical-saturated disk. Positive antiviral tests are shown in Table I. All other tests with these compounds showed no antiviral activity.

Antibacterial and antifungal screening was done with similar paper disks containing the test compounds. These disks were placed on plates of agar that had been freshly smeared with the test organism. Nutrient agar for bacteria and Sabouraud dextrose agar for yeasts and other fungi were used. Twelve[2] representative microbial types were chosen to observe possible drug activity against a wide spectrum of different organisms. The test organisms included Gram-positive human pathogens, Gram-negative human pathogens, acid-fast bacteria (representing the leprosy/tuberculosis group), common yeasts, and pathogenic fungi.

A clear zone around any test disk in a petri plate in which growth of the test microbe was confluent was interpreted to mean that the chemical on that disk had antimicrobial activity. The width of the zone of inhibition was not considered to be significant, since diffusion in agar varies with the solubility and other characteristics of the test compound. Results of the antibacterial and antifungal tests are given in Table II.

Table I—Antiviral Activity[a] of Isatin *N*-Mannich Bases



Compound	R	—NR_1R_2	Toxicity to Cancer Cells	Polio II
1	H		+	+
2	H		−	+
3	H		+	+
4	H		+	+
5	H		+	+
6	CH_3			−
7	CH_3		+	+
8	CH_3		+	−
9	Br			−
10	Br		+	+
11	Br		+	+
12	Br		+	+

[a] + = antiviral activity (or toxicity to cancer cells); − = no activity. Compound 1 was also active against parainfluenza-3, herpes simplex, and measles viruses. A blank indicates that this particular assay was not performed. All other antiviral tests gave negative results.

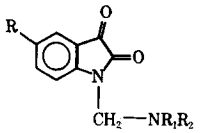
DISCUSSION

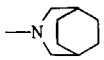
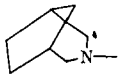
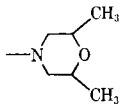
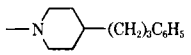
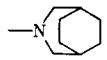
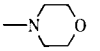
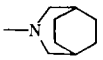
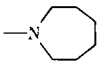
Twelve isatin *N*-Mannich bases incorporating dimethylamino, morpholino, piperidino, 3-azabicyclo[3.2.2]nonano, and 3-azabicyclo[3.2.1]octano moieties were subjected to antiviral screening procedures (Table I). Nine compounds showed activity against polio II virus. Compound 1 displayed activity against four different viruses; its effect was much more pronounced than that produced by

[1] Modifications suggested in a personal communication from Dr. Richard Stewart, Virology Laboratory, Smith Kline and French Co., Philadelphia, Pa.

[2] The microbial spectrum consisted of *Staphylococcus aureus* (K257)(+), *Escherichia coli* (ATCC 4157)(−), *Pseudomonas aeruginosa* (LBa 160)(−), *Klebsiella pneumoniae* (ATCC 8052)(−), *Proteus vulgaris* (LBa 155)(−), *Mycobacterium smegmatis* (Carolina Biological Supply Co.)(+), *Neisseria catarrhalis* (LB130)(−), *Saccharomyces* sp., *Candida albicans* (ATCC 10231), *Trichophyton mentagrophytes* (ATCC 9129), *Staphylococcus epidermidis*, and *Aspergillus niger* organisms. All cultures without identification numbers or source are from the University of Mississippi Department of Biology.

Table II—Antibacterial and Antifungal Activities[a] of Isatin *N*-Mannich Bases



Compound	R	—NR_1R_2	Acid-Fast Bacteria	Gram-Positive Bacteria	Gram-Negative Bacteria	Fungi and Yeasts
1	H		−	+	+	+
2	H		−	−	−	−
3	H		−	+	+	+
4	H		−	+	+	+
5	H		−	−	−	+
6	H		−	+	+	−
7	H		−	+	+	+
8	H		+	+	+	+
9	CH_3		−	+	+	+
10	CH_3		−	+	+	+
11	CH_3		−	+	+	−
12	Br		−	+	+	+
13	Br		+	+	+	+
14	Br	—$N(CH_3)_2$	−	+	+	+
15	Br		+	+	+	+
16	Br		−	+	+	+

[a] + = inhibition; − = no inhibition.

any other compound studied. Compounds showing toxicity to cancer cells may have anticancer significance.

Antibacterial and antifungal activities of 16 isatin *N*-Mannich bases are described in Table II. Three compounds (Compounds 8, 13, and 15) showed activity against all four types of organisms. Compound 2 did not show any activity.

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Synthesis and CMC Determination of a Series of Aliphatic Diamines

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Abstract □ Fifteen aliphatic diamines substituted with one long alkyl chain (C_{10}–C_{18}), a varying number of methylene groups separating the nitrogens (two to six), and various aliphatic substituents were synthesized and their CMC's were determined. While the length of the long alkyl chain plays a dominant role in influencing the CMC, the other *N*-alkyl substituents as well as the length of the methylene chain separating the two nitrogens of the diamine are significant factors.

Keyphrases □ Diamines, aliphatic—synthesis, determination of CMC values, effect of long alkyl and methylene chains on surface activity □ CMC—determined for 15 aliphatic diamines with one long alkyl chain and varying methylene chain

The bactericidal activity of aliphatic monoamines and diamines was shown to depend largely on their surface activity (1). This has been established in numerous model systems which have correlated antibacterial activity with surface tension lowering (2), critical micelle concentration (CMC) (3, 4), and penetration into monolayers (5). While the length of the aliphatic chain is the predominant factor in determining surface activity, the smaller but significant effects of changes at the protonated nitrogen have been noted (6).

To explore further the effect of structural variation on surface activity, a series of aliphatic diamines was synthesized and their CMC values were determined. This series allows variation of not only the length of the long alkyl chain (R_1) and the other nitrogen substituents (R_2, R_3, and R_4) but also the number of methylene groups *(n)* separating the two amino nitrogens.

EXPERIMENTAL[1]

***N,N*-Dimethyldiamine Dihydrochlorides (I–VIII and X–XIV)**—The appropriate acyl chloride was added to a stirred solution of the appropriate *N,N*-dimethyldiamine (1 equivalent) and triethylamine (1 equivalent) in benzene. The reaction mixture was refluxed for 2 hr, cooled, and filtered. The filtrate was washed with 5% $NaHCO_3$ and a saturated solution of sodium chloride and dried (sodium carbonate). The residue from the evaporation of the benzene was recrystallized from benzene–isopropanol to give the desired amide.

The amide was placed in an erlenmeyer flask connected to a three-necked round-bottom flask with a length of rubber tubing. From this container, the amide was added over 2 hr to a suspension of lithium aluminum hydride (twofold excess) in ether under dry nitrogen. After stirring for 12 hr at 25°, the reaction mixture was worked up in the usual method (7). The resulting filtrate was dried (sodium carbonate) and filtered. Then a saturated solution of dry hydrogen chloride in ether was added to the filtrate until no more salt precipitated. The precipitated salt was washed with ether and recrystallized from a mixture of ethyl acetate and methanol. IR spectra of the final compounds and intermediates were compatible with the structural assignments. The 2-hydroxyethyl derivatives, VII and XIV, were synthesized from V and XIII, respectively, using ethylene oxide in ether (8).

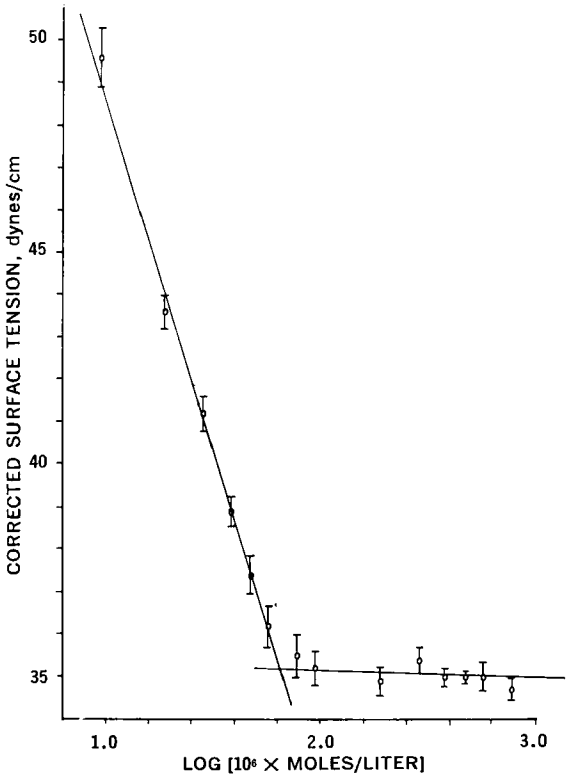


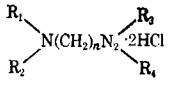
Figure 1—*CMC determination for* N′*-hexadecyl*-N,N-*dimethylbutanediamine dihydrochloride, XII. Vertical bars indicate the standard deviation* (n = 5).

***N*-Octadecyl-1,3-propanediamine Dihydrochloride (XV)**—3-Bromopropionyl chloride (18.6 mmoles, 3.18 g) was added dropwise to a cold (5–10°) solution of octadecylamine (18.6 mmoles, 5 g) and triethylamine (2.58 ml) in benzene (200 ml). The reaction mixture was allowed to warm to 25° and was stirred for 72 hr, during which time a product precipitated. The benzene was evaporated and the residue was washed well with water. The residue was recrystallized from methanol to give 5.1 g of the amide as white crystals, mp 90–91°. The IR spectrum was consistent with the structural assignment.

N-Octadecyl-3-bromopropionamide (12.4 mmoles, 4.0 g) and potassium phthalimide (13.6 mmoles, 2.29 g) were slurried in dimethylformamide (60 ml) and refluxed for 3 hr. The reaction mixture was cooled and poured into water, and the slurry was acidified with concentrated hydrochloric acid. The mixture was extracted with chloroform, the organic layer was dried (sodium sulfate), and the chloroform was removed. The white residue was washed with 2 *N* NaOH and recrystallized from methanol to give 2.5 g of the *N*-octadecyl-3-phthalimidylpropionamide as white crystals, mp 140–141°. The IR spectrum was consistent with the structural assignment.

The phthalimide (1.0 g) was dissolved in a refluxing solution of 95% ethanol (10 ml) and 85% hydrazine hydrate (0.5 ml). The reac-

[1] Melting points were determined on a Fisher-Johns apparatus and are uncorrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn. NMR spectra were determined in $CDCl_3$ (tetramethylsilane internal standard) solution on a Varian A-60A analytical spectrometer or a Hitachi-Perkin-Elmer 60-MHz spectrometer. IR spectra were determined on a Perkin-Elmer model 257 spectrophotometer.

Table I—Melting Points, CMC's, and Elemental Analyses for a Series of Alkyl Diamine Dihydrochlorides 

Compound	R_1	R_2	R_3, R_4	n	Melting Point	CMC (log concentration $\times 10^6$)	Analysis, % Calc.	Found
I	$C_{10}H_{21}$	H	CH_3	2	225–226°	—[a]	C 55.80	56.05
							H 11.37	11.43
							Cl 23.53	23.97
							N 9.30	9.45
II	$C_{12}H_{25}$	H	CH_3	2	216–219°	3.87	C 58.34	58.31
							H 11.63	11.54
							Cl 21.53	21.46
							N 8.50	8.49
III	$C_{14}H_{29}$	H	CH_3	2	212–215°	2.83	C 60.48	60.35
							H 11.84	11.68
							Cl 19.84	19.86
							N 7.84	7.90
IV	$C_{14}H_{29}$	H	CH_3	3	236–238°	2.94	C 61.43	60.99
							H 11.94	12.23
							Cl 19.09	18.98
							N 7.54	7.70
V	$C_{16}H_{33}$	H	CH_3	2	220–222°	1.65	C 62.31	62.12
							H 12.03	11.87
							Cl 18.39	18.48
							N 7.27	7.37
VI[b]	$C_{16}H_{31}O$	H	CH_3	2	69–70°	1.61	C 73.56	73.96
							H 12.96	13.27
							Cl —	—
							N 8.58	8.65
VII	$C_{16}H_{33}$	C_2H_4OH	CH_3	2	210–212°	1.49	C 61.51	61.93
							H 11.73	12.43
							Cl 16.51	16.21
							N 6.52	7.00
VIII	$C_{16}H_{33}$	H	CH_3	4	254° dec.	1.82	C 63.89	63.76
							H 12.18	12.56
							Cl 17.41	16.99
							N 6.77	6.62
IX	$C_{16}H_{33}$	H	CH_3	6	182–183°	2.10	C 65.27	65.25
							H 12.33	12.27
							Cl 16.06	16.12
							N 6.34	6.23
X	$C_{18}H_{37}$	H	CH_3	2	182–183°	1.72	C 63.89	63.79
							H 12.18	12.17
							Cl 17.14	17.18
							N 6.77	6.80
XI	$C_{18}H_{37}$	H	CH_3	3	190° dec.	1.98	C 64.61	64.53
							H 12.26	12.46
							Cl 16.58	16.38
							N 6.55	6.27
XII	$C_{18}H_{37}$	H	CH_3	4	210° dec.	1.80	C 65.27	65.49
							H 12.33	12.27
							Cl 16.06	16.12
							N 6.34	6.23
XIII	$C_{18}H_{37}$	H	C_2H_4OH	3	66–67°	1.36	C 61.58	61.73
							H 11.58	11.69
							Cl 14.54	14.44
							N 5.74	5.83
XIV	$C_{18}H_{37}$	C_2H_4OH	C_2H_4OH	3	88–90°	1.26	C 60.99	60.74
							H 11.37	11.82
							Cl 13.34	13.14
							N 5.27	5.50
XV	$C_{18}H_{37}$	H	H	3	212–214° dec.	0.96	C 63.13	63.25
							H 12.11	12.17
							Cl 17.74	17.58
							N 7.01	7.20

[a] No CMC was found below 10^{-2} *M*. [b] Palmitamide derivative, melting point and analysis are of the free base; hydrochloride, mp 120–121°.

tion mixture was refluxed for 12 hr and then cooled. The ethanol was evaporated and the residue was recrystallized from ethyl acetate to give 0.34 g of *N*-octadecyl-3-aminopropionamide, mp 108–110°. The IR spectrum was consistent with the spectral assignment.

The *N*-octadecyl-3-aminopropionamide (0.35 g) was added to a slurry of lithium aluminum hydride (0.5 g) in ether. The mixture was stirred overnight and worked up in the usual manner (7). The resulting ether solution was dried (sodium carbonate), and a saturated solution of anhydrous hydrogen chloride in ether was added. The precipitated dihydrochloride salt was recrystallized from a mixture of isopropanol–ethyl acetate to give 0.3 g of crystals, mp 214° dec. The IR spectrum was consistent with the structural assignment.

***N,N*-Dimethyl-*N'*-hexadecyldiaminohexane (IX)**—Caprolactone (30 ml, 0°) and cold (0°) dimethylamine (100 g) were added to a Parr bottle. The bottle was sealed and allowed to warm to 25° and then to stand for 1 week at 25°. The reaction vessel was cooled to 0° and the stopper was removed. Nitrogen was bubbled through the mixture to evaporate the excess dimethylamine. The

residue was distilled to give 30 g of oil (bp 188–190°/11 mm). IR and NMR indicated that it was pure *N,N*-dimethyl-6-hydroxyhexamide.

N,N-Dimethyl-6-hydroxyhexamide (5 g) and purified tosyl chloride (14 g) (9) were dissolved in dry pyridine (distilled from barium oxide) and stored for 3 days at 5°. The reaction mixture was poured into water (200 ml), and the resulting slurry was extracted with chloroform (2 × 150 ml). The combined chloroform solutions were washed with 1 *N* H_2SO_4 (3 × 100 ml), 5% $NaHCO_3$ (3 × 100 ml), and a saturated solution of sodium chloride. The chloroform solution was dried (magnesium sulfate), and the chloroform was evaporated to give an oil (7.7 g). NMR and IR indicated that it was >90% pure. This product was used without further purification.

N,N-Dimethyl-6-tosylhexamide (15.9 mmoles, 5 g) and potassium phthalimide (17.5 mmoles, 3.25 g) were slurried in dimethylformamide, and the mixture was refluxed for 3 hr. The reaction mixture was cooled and poured into water (150 ml), and the slurry was extracted with chloroform (3 × 100 ml). The chloroform was washed with 5% NaOH (2 × 100 ml), the organic layer was dried (magnesium sulfate), and the solvent was evaporated. The residual oil (2.5 g) was dissolved in 95% ethanol (25 ml), and 85% hydrazine hydrate (1 ml) was added. Then the mixture was refluxed for 12 hr. The ethanol was evaporated, and the residual oil was distilled through a short column to give 1 g of a clear oil. IR and NMR indicated that it was the desired *N,N*-dimethyl-6-aminohexamide.

A mixture of *N,N*-dimethyl-6-aminohexamide (4.74 mmoles, 750 mg), triethylamine (0.7 ml), and palmitoyl chloride (4.74 mmoles, 1.30 g) was refluxed for 2 hr in benzene and then allowed to stand for 12 hr at 25°. The reaction mixture was washed with water and a saturated solution of sodium chloride and dried (magnesium sulfate). Evaporation of the benzene gave a white residue (1.3 g). Then 1 g of this residue was reduced with lithium aluminum hydride and converted to the dihydrochloride salt in ether. The precipitate was washed with ether and recrystallized from ethyl acetate–methanol, mp 182–183° (yield 700 mg).

CMC Determinations—The CMC's were determined by the du Nouy ring method (10) in isotonic saline at 25° using a surface tensiometer[2]. The corrected surface tension, *S*, was calculated as $S = P \times (0.7250 + aP + b)$ dynes/cm, where *P* is the apparent surface tension (dial reading), $a = 4.0333 \times 10^{-4}$, and $b = 0.01378$ (11, 12). A minimum of 14 concentrations, five replicates each, were measured for each amine. The CMC was designated as the intercept of the linear segments from a plot of surface tension *versus* log (amine concentration). A typical plot is shown in Fig. 1.

RESULTS AND DISCUSSION

To determine the effect of changes in the length of the long alkyl chain (R_1) and the number of methylene groups separating the two nitrogens on the CMC, a series of *N,N*-dimethyl (R_3 and R_4) diamines was synthesized. These compounds were synthesized by converting the long chain carboxylic acid (commercially available in high purity) to the corresponding acyl chloride. The acyl chloride was then allowed to react with the appropriate *N,N*-dimethyldiamine. The resulting amide was reduced to the desired diamine by adding it, as a dry powder, to lithium aluminum hydride in ether. The addition of the amide as a dry powder was found to be more convenient than the soxhlet method recommended by Wilson and Stenberg (13) for amides of low solubility in ether.

It was necessary to synthesize the *N,N*-dimethylhexanediamine to obtain the hexamethylenediamine (IX). In contrast to the opening of γ-butyrolactone with amines, which gives a mixture of products (14), the opening of ε-caprolactone with dimethylamine gave a near quantitative yield of ω-hydroxy-*N,N*-dimethylhexamide. This compound was converted to the tosylate and then, by the Gabriel synthesis, to the corresponding 6-amino compound, which was acylated with palmitoyl chloride and reduced to the desired diamine with lithium aluminum hydride.

[2] Fisher.

The CMC decreased with increases in chain length from C_{10} to C_{16} as expected (Table I). The CMC's of C_{16} and C_{18} derivatives with the same substituents were similar (compare V and X and VIII and XII).

Increasing the N–N separation on the palmitoyl series (V, VIII, and IX) gave a consistent increase in CMC. This finding is in contrast to that with the stearoyl series (X, XI, and XII) which does not vary consistently. While this series does not contain a sufficient number of compounds to make definite conclusions on the effect of N–N separation, it appears that the CMC's vary in the order $n = 2 < n = 4 < n = 3 < n = 6$. This order perhaps reflects the ability of the compounds to form the hydrophilic shell of the micelle. In the case of the hexamethylene derivative, the *N'*-nitrogen must be in the center of the micelle or the hydrophobic hexamethylene chain must be on the surface of the micelle. Both of these possibilities appear to be unfavorable because they require a hydrophilic group in a lipoid region or a lipophilic group in an aqueous region.

Perhaps the most unexpected aspect of the data in Table I is the fact that the palmitamide derivative, VI, and the corresponding diamine, V, have essentially the same CMC. It is surprising that the second ionium ion in V does not have a more significant effect on the CMC. Substitution of the 2-hydroxyethyl substituent at R_2 resulted in a slight (20–25%) decrease in the CMC.

In comparing the CMC's of dimethyl-substituted, bis(2-hydroxyethyl)-substituted, and unsubstituted octadecylpropylenediamines (XI, XIII, and XV, respectively), the CMC increases as the *N*-substituent becomes more lipophilic. This order of increasing CMC is reasonable because increasing the polarity of the onium head and decreasing its steric bulk should make the formation of the hydrophilic shell of the micelle more favorable. In comparing the derivatives where R_2 is either H or C_2H_4OH (*i.e.*, V and VII and XIII and XIV), the more lipophilic hydroxyethyl substituent decreases the CMC. That is, increasing the lipophilicity of the internal amine makes the amine behave as if it has a longer alkyl chain.

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Nitrofurantoin Solubility in Aqueous Urea Solutions

Keyphrases □ Nitrofurantoin—solubility in aqueous urea solutions □ Urea—solubility of nitrofurantoin in aqueous solutions □ Solubility—nitrofurantoin in aqueous urea solutions

To the Editor:

Nitrofurantoin, 1-[(5-nitrofurfurylidene)amino]-hydantoin, is an antibacterial agent used to treat urinary tract infections. The drug is a weak acid (pKa 7.2) possessing relatively low aqueous solubility characteristics. Solubilities in water at 37° have been reported to be 190 mg/liter in pH 7 distilled water (1) and 125 mg/liter in pH 4.8 water (2). Bates and coworkers[1] (3) reported the solubilities of nitrofurantoin in pH 1.12 and 7.2 aqueous systems to be 154 and 379 mg/liter, respectively.

Much higher solubilities, however, have been reported for nitrofurantoin in urine. Nitrofurantoin concentrations of 250–500 mg/liter were found after administering 200 mg of drug every 6 hr to an 80-kg patient (4) and urine concentrations of 200–400 mg/liter were reported (5). Maximum urine nitrofurantoin concentrations from 158 to 372 mg/liter were reported in nine normal individuals, each of whom received a 100-mg tablet every 4 hr (6).

Although urine concentrations that represent saturation or supersaturation are routinely attained after normal usage, there are no reported incidents of crystalluria associated with nitrofurantoin therapy. These facts suggest that the normal urine contents might have some effect on the solubility of nitrofurantoin in urine. We wish to report our preliminary findings concerning the effect of urea on the aqueous solubility of nitrofurantoin.

An excess amount of nitrofurantoin (approximately 50 mg) was added to 40 ml of the appropriate test solution (0.0–5% urea in distilled water) in a screw-capped bottle of 45-ml capacity. The tightly closed container was placed in a constant-temperature water bath at 30 or 37 ± 0.1° and rotated[2] for at least 24 hr. Preliminary experiments indicated that equilibrium was established within 12–18 hr. After equilibrium, the test solutions were subject to filtration[3] (0.45-μm pore size), and the filtrate was diluted with deionized distilled water to make a solution of proper concentration (40–100 mg/liter) for spectrophotometric assay using the method of Conklin and Hollifield (7). At least five experimental runs were made for each individual test medium.

Figure 1 shows the effect of urea on the solubility of nitrofurantoin at two temperatures. The addition of urea to aqueous media had the paradoxical effect of first promoting increased nitrofurantoin solubility up to a maximum concentration level and then causing decreased solubility at higher urea concentrations. The change was abrupt, and nitrofurantoin solubility decreased to levels considerably lower than nitrofurantoin solubility in plain distilled water. The nitrofurantoin solubility was dependent on temperature, and the amount of urea needed to bring about maximum nitrofurantoin solubility was greater at 37° (2.25%) than at 30° (2.0%). The findings that urea first increased the aqueous solubility of nitrofurantoin might explain in part the elevated solubilities of this drug in urine (5, 6). This explanation is supported by the fact that concentrations of urea in human urine are normally in the neighborhood of 2% (8, 9).

The effect of urea on the aqueous solubility of organic compounds has been widely studied. Urea has been shown to increase the water solubility of chloramphenicol (10, 11) and acetaminophen (12) and to decrease the solubility of sulfathiazole (13). The solubility of methylene blue was reported to be 30 times higher in 10 *M* urea solution than in water (14). A recent investigation found that 1–5 *M* urea increased the water solubility of methyl *p*-hydroxybenzoate (15).

The effects of urea and thiourea on the solubility of benzoic and salicylic acids have been studied (16, 17). Altwein *et al.* (17) concluded that the interaction of urea with salicylic acid was relatively complex but that increases in the solubility of hydroxybenzoic acids were due to formation of soluble complexes. Feldman and Gibaldi (18), however, concluded that the enhanced solubility of benzoic and salicylic acids in urea and alkylurea solutions did not involve complexation. They proposed that urea and alkylureas solubilized benzoic and salicylic acids by "breaking up" water clusters surrounding the nonpolar molecule, increasing the entropy of the system, and producing a "driving" force for the solubilization.

In studying the effect of urea on hydrocarbon solubility, Wetlaufer *et al.* (19) suggested several mechanisms to explain the increase of hydrocarbon solubility in aqueous urea solutions: *(a)* the solute is solely dissolved by the urea, *(b)* urea alters the structure of water so as to enhance solvation of the solute by water molecules, and *(c)* solute molecules were solvated both by urea and water molecules. The proposed mechanisms have usually been associated with increased solubility of compounds in relatively high urea concentrations (1–5 *M*), and it appears that there is no conclusive evidence to support any one mechanism whereby urea changes solubilities.

In the present study, because of the paradoxical behavior of the system (first an increase followed by a considerable decrease in nitrofurantoin solubility), a single explanation of the phenomenon is certainly

[1] T. R. Bates, School of Pharmacy, State University of New York at Buffalo, personal communication.
[2] Menhold rotating apparatus, Lester, Pa.
[3] Millipore.

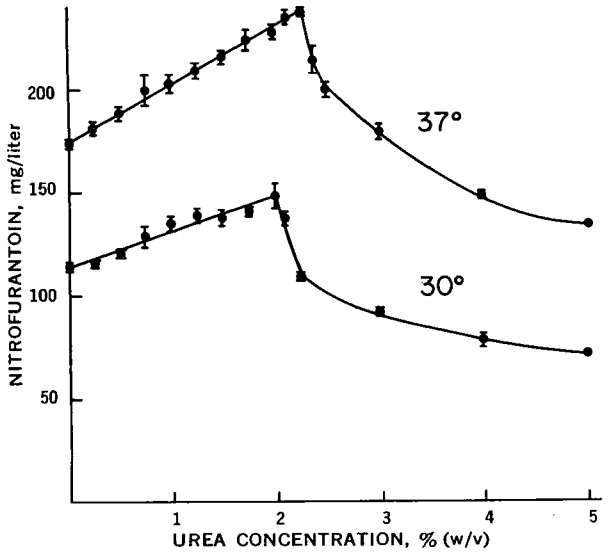

Figure 1—*Nitrofurantoin solubility in aqueous urea solutions at 30 and 37°. Bars mark off 1* SD *on either side of the average.*

not possible now. Preliminary spectral studies suggest some type of interaction between nitrofurantoin and urea molecules. This possible interaction may account for the increased solubility at low urea concentrations. The breakdown of water structure might account for the increase in solubility at lower urea concentrations; however, disruption of the water structure might not occur at the low urea concentrations used. The rapid decrease in nitrofurantoin solubility at higher urea concentrations may be due to a salting-out effect. However, the formation of an insoluble complex at higher urea concentrations might account for the abrupt decrease in solubility.

Further investigations are being conducted in these laboratories to determine the effect of urea and another urine component, creatinine, on nitrofurantoin solubility at various temperature and pH conditions and to elucidate the mechanism(s) of action of the solubilization phenomena.

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Ambrosin, Tumor Inhibitory Agent from *Hymenoclea salsola* (Asteraceae)

Keyphrases □ Ambrosin—tumor inhibitory agent from *Hymenoclea salsola*, isolation and identification □ *Hymenoclea salsola* (Asteraceae)—isolation and identification of ambrosin, a tumor inhibitory agent □ Antitumor agents—isolation and identification of ambrosin from *Hymenoclea salsola*

To the Editor:

As a result of the continuing search for plants having tumor inhibitory constituents, it was found that the chloroform extract of the leaves and stems of *Hymenoclea salsola* Torr. and Gray (Asteraceae)[1] showed inhibitory activity toward the P-388 lymphocytic leukemia test system (3PS)[2].

One of the two major constituents of the chloroform extract was shown to be ambrosin, previously isolated from many plants of the Asteraceae family including *H. salsola* (1). Isolation was effected by column chromatography, recrystallization, and preparative TLC[3]. Identification was achieved by IR, NMR, mass spectrometry, elemental analysis, and comparison with an authentic specimen[4].

Ambrosin demonstrated activities of 180, 158, 130, and 132% test/control (T/C) at 35, 22, 14, and 9.6 mg/kg, respectively, in the 3PS system. Activity in

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[3] In our plant extract, ambrosin crystallized as a 1:1 mixture with its 11,13-dihydro derivative, so preparative TLC was required to isolate pure ambrosin. Geissman and Toribio (1) detected none of the dihydro material in their plant extract.

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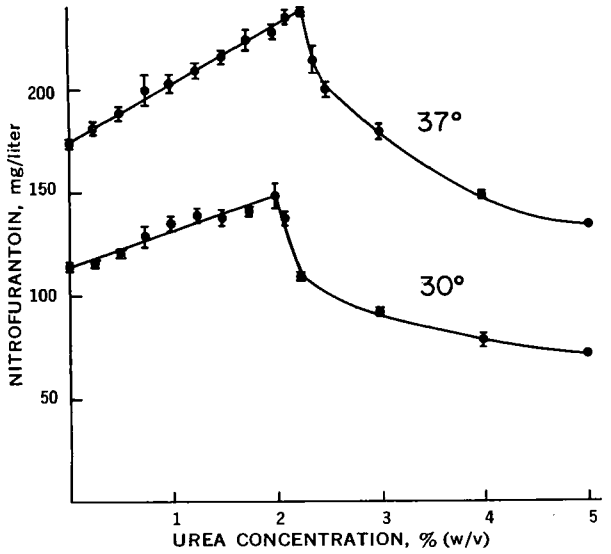

Figure 1—*Nitrofurantoin solubility in aqueous urea solutions at 30 and 37°. Bars mark off 1* SD *on either side of the average.*

not possible now. Preliminary spectral studies suggest some type of interaction between nitrofurantoin and urea molecules. This possible interaction may account for the increased solubility at low urea concentrations. The breakdown of water structure might account for the increase in solubility at lower urea concentrations; however, disruption of the water structure might not occur at the low urea concentrations used. The rapid decrease in nitrofurantoin solubility at higher urea concentrations may be due to a salting-out effect. However, the formation of an insoluble complex at higher urea concentrations might account for the abrupt decrease in solubility.

Further investigations are being conducted in these laboratories to determine the effect of urea and another urine component, creatinine, on nitrofurantoin solubility at various temperature and pH conditions and to elucidate the mechanism(s) of action of the solubilization phenomena.

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Chemical Constituents of Gentianaceae XVI: Antitubercular Activity of Xanthones of *Canscora decussata* Schult

Keyphrases □ *Canscora decussata* Schult—antitubercular activity of polyoxygenated xanthones □ Xanthones, polyoxygenated—activity against *Mycobacterium tuberculosis* □ Mangiferin—activity against *Mycobacterium tuberculosis* □ Medicinal plants—antitubercular activity of xanthones of *Canscora decussata* Schult

To the Editor:

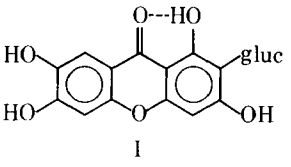
The extract of *Canscora decussata* Schult (Gentianaceae) is used in the treatment of certain mental disorders and of tuberculosis in the Indian system of medicine (1). Previously, the isolation, characterization, and pharmacological screening of about two dozen polyoxygenated xanthones of *C. decussata* were reported from this laboratory (2–5). The pharmacological profile of activities of the total xanthones (excluding mangiferin) and of the major glycoxanthone, mangiferin, was consistent with the reported uses of the plant extract in the treatment of affective disorders. We now wish to report the significant antitubercular activity of the free polyoxygenated xanthones of this medicinal plant.

After concentration, the ethanolic extract of the defatted plant material yielded mangiferin in high yield. The alcoholic mother liquor, after the separation of mangiferin, gave a syrupy mass with further concentration. The nitrogenous constituents were separated from it by aqueous acetic acid treatment in the usual way (2). The chloroform-soluble fraction of the acidic aqueous suspension gave a mixture of about a dozen polyoxygenated xanthones ("total xanthones"); their number and relative abundance were monitored by TLC and mass spectrometry of the mixture, its methyl ethers, and the acetates.

The major xanthonic constituent was mangiferin (I). The remaining total xanthones consisted of three 1,3,5-trioxygenated xanthones (II*a*–II*c*, relative proportion about 12%), four 1,3,5,6-tetraoxygenated xanthones (III*a*–III*d*, 32%), and four 1,3,5,6,7-pentaoxygenated xanthones (IV*a*–IV*d*, 56%), plus some unidentified minor xanthones. All of these compounds were screened for activity against *Mycobacterium tuberculosis* H37 RV using Youman's medium. The tube dilution method was used. The test compounds were added in different concentrations to Youman's medium tubes containing horse serum (10%). The tubes were then inoculated with the organism (about 10^6 million/ml). Thereafter, the tubes were incubated at 37° for 21 days.

After the incubation, the tubes were observed for growth of the microorganism. The minimum concentration of the compounds required for preventing the growth was recorded as the minimum inhibitory concentration (MIC). The total xanthones (II–IV) were found to be more active than mangiferin. The MIC (10 μg/ml) of the total xanthones was comparable to that of streptomycin. Mangiferin showed only weak inhibitory activity, its MIC being 200 μg/ml.

The activity of this class of compounds against *M. tuberculosis* was reported twice before in the literature. 1,3,8-Trihydroxyxanthone, a degradation product of sterigmatocystin (occurring in *Aspergillus versicolor*), was reported (6) to be active at a dilution of 1 in 80,000. Sterigmatocystin had virtually no tuberculostatic effect *per se*. Norswertianolin (3,5,8-trihydroxyxanthone-1-*O*-glucoside), occurring in a



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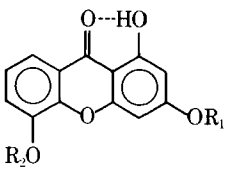

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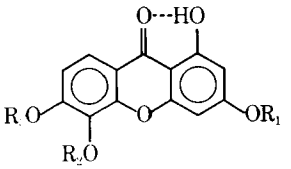

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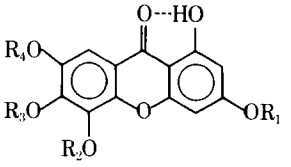

IV*a*: $R_1 = H$, $R_2 = H$, $R_3 = H$, $R_4 = H$
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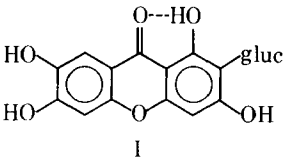

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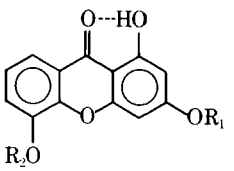

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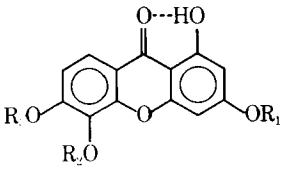

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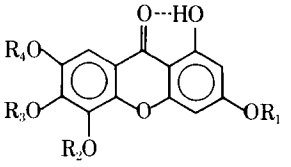

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Improved Method for Microencapsulation of Soluble Pharmaceuticals

Keyphrases □ Nylon (formalin treated) gelatin microcapsules—preparation, physical properties □ Sulfathiazole sodium—microencapsulation, dissolution □ Microencapsulation—soluble pharmaceuticals

To the Editor:

Interest in microencapsulation technology and its application to varied problems is increasing. In addition to many nonpharmaceutical applications (1), microencapsulation has been utilized pharmaceutically to increase product stability, modify drug release, overcome drug incompatibility in formulations, and improve certain physical characteristics of formulations such as compressibility and flow (2). The technology and applications of microencapsulation have been discussed in several monographs and reviews (1–6). However, as Luzzi stated (5), much of this information is found in the patent literature and the control and testing data essential for reproducibility are frequently lacking.

Table I—Percent Sulfathiazole Sodium Released from Microcapsules[a] Prepared by the Formalin-Treated Nylon Gelatin Technique (Formula I)[b] and with Nylon Alone (Formula II)[c] in 0.1 *N* HCl and 0.1 *M* Acetate Buffer (pH 5.6)

	Recoveries[d], %			
	0.1 *N* HCl		0.1 *M* Acetate Buffer (pH 5.6)	
Minutes	Formula I	Formula II	Formula I	Formula II
2	24.5	35.7	4.3	3.8
4	36.5	56.1	11.6	6.5
8	51.5	76.1	22.3	12.5
12	62.5	87.5	29.1	17.5
16	73.0	93.3	34.1	23.4
20	80.5	96.7	37.5	26.7
30	92.0	99.0	45.1	37.4
40	95.5	100.6	51.3	46.9
50	97.5	—	57.0	54.3
60	99.0	—	60.7	59.9
120	—	—	77.3	81.5

[a] One hundred percent of unencapsulated sulfathiazole sodium passed into solution in less than 2 min in 0.1 *N* HCl and 0.1 *M* acetate buffer (pH 5.6). [b] Drug content, 21% (w/w). [c] Drug content, 49% (w/w). [d] Average of duplicate assays upon the same batches.

Of interest is the work of Chang *et al.* (7), in which enzymes in semipermeable microcapsules of nylon, collodion, and heparin-complexed collodion were prepared for potential use in enzyme replacement therapy. More recently, Luzzi *et al.* (8) used a modification of their technique (7) to encapsulate a water-soluble barbiturate in nylon. To prevent loss of the barbiturate from the nylon capsules, the microcapsules were washed in chloroform prior to drying.

The present communication outlines an improved method for microencapsulating soluble drugs. Nylon was used to coat a gelatin matrix containing the drug, and the resulting microcapsules were then hardened with formalin. Sulfathiazole sodium was used as the encapsulated drug. The concentration of reactants required to form nylon and the volume ratios of aqueous and organic solvents were the same as those reported by Chang *et al.* (7). Gelatin USP (5 g) and sulfathiazole sodium (3 g) were dissolved in 60 ml of aqueous phase prior to the encapsulation step. After the nylon-coated capsules containing gelatin and sulfathiazole sodium were formed, 20 ml of formalin (formaldehyde solution USP, 37%) was added to the total reaction volume (approximately 700 ml) and this mixture was gently stirred for an additional 10 min.

The capsules were allowed to stand at 5° for 24 hr to ensure that the hardening of the gelatin within the capsules was complete (9). The microcapsules were separated from the organic phase by vacuum filtration and were then air dried at room temperature to remove formalin vapors, organic solvents, and water. Microcapsules passing through a 100-mesh screen were assayed for sulfathiazole sodium content (21% by weight) and used for dissolution studies. As controls, nylon microcapsules containing sulfathiazole sodium without gelatin were prepared using the same procedure (assay 49% by weight).

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Improved Method for Microencapsulation of Soluble Pharmaceuticals

Keyphrases □ Nylon (formalin treated) gelatin microcapsules—preparation, physical properties □ Sulfathiazole sodium—microencapsulation, dissolution □ Microencapsulation—soluble pharmaceuticals

To the Editor:

Interest in microencapsulation technology and its application to varied problems is increasing. In addition to many nonpharmaceutical applications (1), microencapsulation has been utilized pharmaceutically to increase product stability, modify drug release, overcome drug incompatibility in formulations, and improve certain physical characteristics of formulations such as compressibility and flow (2). The technology and applications of microencapsulation have been discussed in several monographs and reviews (1–6). However, as Luzzi stated (5), much of this information is found in the patent literature and the control and testing data essential for reproducibility are frequently lacking.

Table I—Percent Sulfathiazole Sodium Released from Microcapsules[a] Prepared by the Formalin-Treated Nylon Gelatin Technique (Formula I)[b] and with Nylon Alone (Formula II)[c] in 0.1 *N* HCl and 0.1 *M* Acetate Buffer (pH 5.6)

	Recoveries[d], %			
	0.1 *N* HCl		0.1 *M* Acetate Buffer (pH 5.6)	
Minutes	Formula I	Formula II	Formula I	Formula II
2	24.5	35.7	4.3	3.8
4	36.5	56.1	11.6	6.5
8	51.5	76.1	22.3	12.5
12	62.5	87.5	29.1	17.5
16	73.0	93.3	34.1	23.4
20	80.5	96.7	37.5	26.7
30	92.0	99.0	45.1	37.4
40	95.5	100.6	51.3	46.9
50	97.5	—	57.0	54.3
60	99.0	—	60.7	59.9
120	—	—	77.3	81.5

[a] One hundred percent of unencapsulated sulfathiazole sodium passed into solution in less than 2 min in 0.1 *N* HCl and 0.1 *M* acetate buffer (pH 5.6). [b] Drug content, 21% (w/w). [c] Drug content, 49% (w/w). [d] Average of duplicate assays upon the same batches.

Of interest is the work of Chang *et al.* (7), in which enzymes in semipermeable microcapsules of nylon, collodion, and heparin-complexed collodion were prepared for potential use in enzyme replacement therapy. More recently, Luzzi *et al.* (8) used a modification of their technique (7) to encapsulate a water-soluble barbiturate in nylon. To prevent loss of the barbiturate from the nylon capsules, the microcapsules were washed in chloroform prior to drying.

The present communication outlines an improved method for microencapsulating soluble drugs. Nylon was used to coat a gelatin matrix containing the drug, and the resulting microcapsules were then hardened with formalin. Sulfathiazole sodium was used as the encapsulated drug. The concentration of reactants required to form nylon and the volume ratios of aqueous and organic solvents were the same as those reported by Chang *et al.* (7). Gelatin USP (5 g) and sulfathiazole sodium (3 g) were dissolved in 60 ml of aqueous phase prior to the encapsulation step. After the nylon-coated capsules containing gelatin and sulfathiazole sodium were formed, 20 ml of formalin (formaldehyde solution USP, 37%) was added to the total reaction volume (approximately 700 ml) and this mixture was gently stirred for an additional 10 min.

The capsules were allowed to stand at 5° for 24 hr to ensure that the hardening of the gelatin within the capsules was complete (9). The microcapsules were separated from the organic phase by vacuum filtration and were then air dried at room temperature to remove formalin vapors, organic solvents, and water. Microcapsules passing through a 100-mesh screen were assayed for sulfathiazole sodium content (21% by weight) and used for dissolution studies. As controls, nylon microcapsules containing sulfathiazole sodium without gelatin were prepared using the same procedure (assay 49% by weight).

An approximation of the particle-size distributions for the two batches of microcapsules was determined using a light microscope and the calibrated counting field of a hemocytometer. The formalin-treated nylon gelatin microcapsules had an average diameter of 135 μm with a range of 70–197 μm. The particles encapsulated with nylon alone had an average diameter of 98 μm with a range of 40–170 μm.

Drug release characteristics of microcapsules containing 20 mg of drug were studied in 1500 ml of 0.1 *N* HCl and 0.1 *M* acetate buffer (pH 5.6). The dissolution apparatus consisted of a 2000-ml, three-necked, round-bottom flask maintained at 37°. A polyethylene stirring blade (7.6-cm diameter) was vertically centered and lowered to a depth of 2 cm above the bottom of the flask. The stirrer was attached to a synchronous motor and rotated at 100 rpm. The release of drug was followed spectrophotometrically at 280 nm for hydrochloric acid and at 283 nm for the acetate buffer. The reported data are the averages of duplicate runs on the same batch of material.

As can be seen from Table I, the release of sulfonamide in dilute acid from the nylon-coated and the formalin-treated nylon gelatin capsules was delayed only slightly. A greater retardant effect could be expected at the lower agitation rates used by Luzzi *et al.* (8), but it was felt that the stirring rate of 100 rpm provides results that are more realistic in terms of their release patterns. The release of drug from both nylon and formalin-treated nylon gelatin in acetate buffer at pH 5.6 is considerably slower than in dilute hydrochloric acid. A similar pattern of curves was also obtained in pH 7.6 phosphate buffer. As with dilute acid, the release rates into 0.1 *N* NaOH from both types of microcapsules were rapid and complete. Since unencapsulated sulfathiazole sodium readily passed into solution in all media tested, the reason for the slower release rate at pH 5.6 and 7.6 is unclear. Because several factors may be involved, further studies to determine the mechanisms are being conducted.

The microcapsules of formalin-treated nylon gelatin displayed ideal physical characteristics for formulation purposes. They were gritty and dense and, because of the nylon coating, they did not adhere together. The capsules had excellent flow properties and could be made of very small diameter by controlling the stirring speed during nylon formation.

Nylon microcapsules of sulfathiazole sodium containing unhardened gelatin, various cellulose gums, proteins, alginates, and other carrier materials were generally difficult to separate. In addition, they did not possess the superior physical characteristics of the formalin-treated nylon gelatin capsules.

Formalin-treated gelatin micropellets were prepared by Tanaka *et al.* (10). Such pellets have been reported to have timed-release properties in humans (11). Gelatin micropellets containing sulfathiazole sodium were prepared but showed poor flow properties even after several rinses in benzene. They tended to adhere together and were difficult to wet.

By combining the techniques of Tanaka *et al.* (10) and Chang *et al.* (7), we have successfully encapsulated a water-soluble drug in formalin-treated nylon gelatin microcapsules. Various drug–gelatin ratios are currently being studied to optimize drug release and, alternatively, to sustain the release of soluble drugs. The effects of different conditions using formalin are also being investigated.

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Relationship between pH of Saliva and pH of Urine

Keyphrases □ pH—saliva and urine, relationship □ Salivary pH—relationship to urinary pH □ Urinary pH—relationship to salivary pH

To the Editor:

A number of drugs appear in significant concentration in the saliva, and the ratio of their concentrations in saliva and plasma is relatively constant (1–7). It is feasible, therefore, to monitor the concentrations of these drugs in plasma indirectly by determining their concentrations in saliva (8). This noninvasive, convenient, painless, and safe method of indirect plasma concentration monitoring is particularly useful for children and for out-patients regardless of age.

Preliminary observations in this laboratory and by others[1] indicate that the saliva–plasma concentration ratio of certain weak acids and bases may be affected by the pH of the saliva, apparently because, among

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An approximation of the particle-size distributions for the two batches of microcapsules was determined using a light microscope and the calibrated counting field of a hemocytometer. The formalin-treated nylon gelatin microcapsules had an average diameter of 135 μm with a range of 70–197 μm. The particles encapsulated with nylon alone had an average diameter of 98 μm with a range of 40–170 μm.

Drug release characteristics of microcapsules containing 20 mg of drug were studied in 1500 ml of 0.1 *N* HCl and 0.1 *M* acetate buffer (pH 5.6). The dissolution apparatus consisted of a 2000-ml, three-necked, round-bottom flask maintained at 37°. A polyethylene stirring blade (7.6-cm diameter) was vertically centered and lowered to a depth of 2 cm above the bottom of the flask. The stirrer was attached to a synchronous motor and rotated at 100 rpm. The release of drug was followed spectrophotometrically at 280 nm for hydrochloric acid and at 283 nm for the acetate buffer. The reported data are the averages of duplicate runs on the same batch of material.

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To the Editor:

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Table I—Effect of Sodium Bicarbonate on pH of Saliva and Urine in Adults

Hours	Saliva pH[a]		Urine pH[a]	
	Control	Sodium Bicarbonate	Control	Sodium Bicarbonate
1	7.25 ± 0.19	7.26 ± 0.15		
2			6.44 ± 0.44	7.30 ± 0.23[b]
3	7.24 ± 0.25	7.41 ± 0.26[b]		
4			6.30 ± 0.67	7.25 ± 0.32[b]
5	7.26 ± 0.11	7.38 ± 0.09		
6			6.09 ± 0.62	6.84 ± 0.63[b]
7	7.19 ± 0.16	7.34 ± 0.16		
8			5.99 ± 0.70	6.89 ± 0.28[b]

[a] Mean of five subjects ± 1 *SD*. [b] Statistically significantly different from control value ($p < 0.05$ by paired t test).

other factors, this ratio is a function of the degree of ionization of such drugs in saliva and plasma (9). The pronounced effect of pH on the renal excretion of many weak acids and bases is well known. This study was carried out to determine the magnitude of pH variation in the mixed saliva of healthy adults, the relationship between the pH of saliva and urine, and the effect of a systemic antacid on saliva and urine pH.

Five healthy male volunteers, 22–39 years old, who had not taken any drugs for at least 1 week before the study, ate their usual meals and followed their usual activities. Each volunteer voided his bladder at 8 or 9 am and collected urine and saliva at 2-hr intervals for 8 hr. About 5 ml of saliva was collected each time by salivation into a small glass vial; saliva flow was stimulated by chewing on a piece of Parafilm. The pH of the saliva and urine samples was determined[2] immediately after collection.

Two of the subjects received 5 g of sodium bicarbonate in five gelatin capsules, size 00, with 100 ml water at the beginning of the experiment; the other three subjects received water only. The experiment was repeated at least 1 week later, with the subjects who had previously taken water only now receiving sodium bicarbonate and vice versa.

The results of the study are shown in Table I and Fig. 1. The pH values summarized in Table I were averaged as such, *i.e.*, without prior conversion to molar concentrations of hydrogen ion and subsequent reconversion. Sodium bicarbonate increased urine pH by an average of 0.75–0.95 unit; this increase was statistically significant at all sampling times. On the other hand, the antacid increased the average pH of saliva by only 0.01–0.17 unit, and this change was statistically significant at only one sampling time (Table I). In a total of 40 urine and saliva samples, the pH ranged from 5.10 to 7.66 in urine but only from 6.89 to 7.68 in saliva. There was no significant correlation ($r = 0.19$) between the pH of saliva and the pH of urine (Fig. 1).

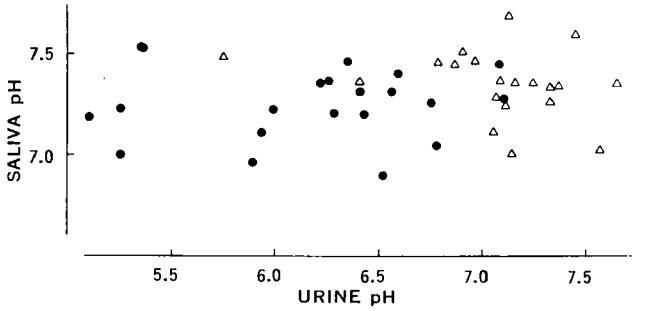

Figure 1—*Relationship between pH of saliva and pH of urine in five healthy adults. Each subject is represented by eight data points. Key: ●, control experiment; and △, after oral administration of 5 g of sodium bicarbonate.*

[2] Orion digital pH meter, model 601.

The results of this study show that:

1. Variations in the pH of saliva in normal subjects are much smaller than variations in the pH of urine.
2. It is apparently not possible to alter saliva pH significantly by acute administration of a systemic antacid.
3. There is no significant relationship between the pH of saliva and the pH of urine.

Since saliva pH varies less than urine pH, saliva concentrations may be more suitable than urinary excretion rates for an indirect estimation of the time course of plasma concentrations of certain weak acids and bases appearing in measurable concentrations in the saliva.

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Constituents of *Cannabis sativa* L. XI: Cannabidiol and Cannabichromene in Samples of Known Geographical Origin

Keyphrases □ *Cannabis sativa* L.—constituents, cannabidiol and cannabichromene in samples of known geographical origin □ Cannabidiol—analysis, samples of known geographical origin □ Cannabichromene—analysis, samples of known geographical origin □ Marijuana—constituents of *Cannabis sativa*, samples of known geographical origin

To the Editor:

Recent development of simplified methods for the separation of cannabidiol (I) and cannabichromene (II) as well as reports supporting the interactions between cannabinoids prompted this communication. We wish to report the presence of I and II in freshly grown *Cannabis* from known geographical origin.

The separation of pure I and II by conventional GLC techniques was reported (1), but this separation

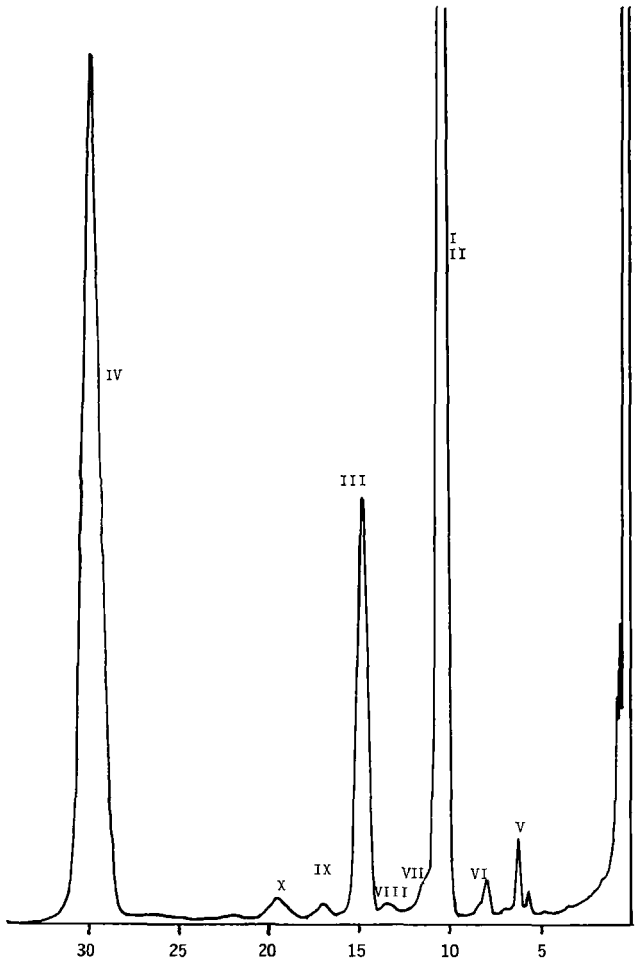

Figure 1—*Chromatogram of the Moroccan variant, MO-A male, showing: I, cannabidiol; II, cannabichromene; III,* (−)-Δ^9-trans-*tetrahydrocannabinol; X, cannabinol; and IV, androst-4-ene-3,17-dione, the internal standard. Also shown are: V, cannabidivarin; VI, combined cannabicyclol and* (−)-Δ^9-trans-*tetrahydrocannabivarin; VII, cannabigerol monomethyl ether; VIII,* (−)-Δ^8-trans-*tetrahydrocannabinol; and IX, cannabigerol.*

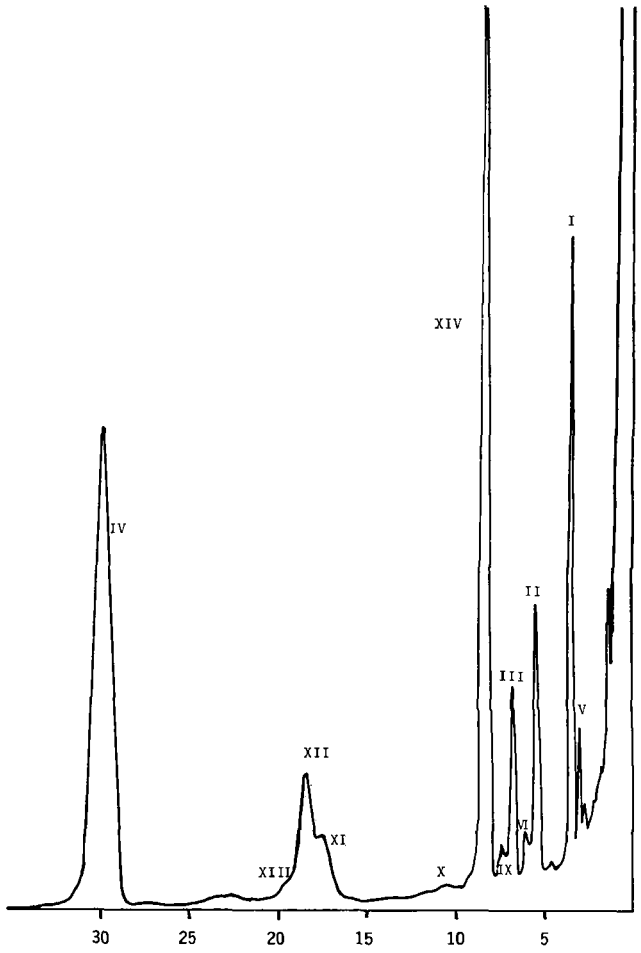

Figure 2—*Chromatogram of the silylated plant material MO-A, Moroccan male variant, showing the first method for the separation of cannabidiol (I) and cannabichromene (II). Also shown are: III,* (−)-Δ^9-trans-*tetrahydrocannabinol; IV, androst-4-ene-3,17-dione, the internal standard; V, cannabidivarin monosilylated; VI, cannabicyclol; IX, cannabigerol disilylated; XIV, cannabidiolic acid; X, cannabinol; XI, unknown; XII,* (−)-Δ^9-trans-*tetrahydrocannabinolic acid A; and XIII,* (−)-Δ^9-trans-*tetrahydrocannabinolic acid B.*

was insufficient for quantitation. Turner and Hadley (2) obtained a clear and discrete separation of I and II by the use of trimethylsilyl ether derivatives. Turner *et al.* (3) then described the use of silyl derivatives in routine analysis, which provided a method for quantitating I and II.

As early as 1970, Carlini *et al.* (4) observed that I could possibly block some effects of (−)-Δ^9-*trans*-tetrahydrocannabinol (III). More recently, I was reported to block several effects of III in animal models and to potentiate the analgesic effects of cannabinol (5). Cannabidiol induced reduction of the hypothermic response to III in rats and rabbits (6).

De Zeeuw *et al.* (7) reported that propyl cannabinoids seem to be more abundant in *Cannabis* grown in India, Nepal, and Pakistan. Turner and Hadley (8), using only samples of known geographical locations, confirmed and extended the previously thought abundance of propyl cannabinoids. Following this example, we investigated many variants of *Cannabis* from known geographical origins as to

Table I—Variants Grown in Mississippi

Geographical Origin	Seed Code	Sex[a]	I[b]	II	III
Afghanistan	AF-B	M	1.94	0.50	2.68
	AF-B	F	1.26	0.01	0.59
	AF-B(1)/C-71	F	0.19	0.83	2.11
	AF-B(1)/C-71	M	4.58	0.27	2.63
	AF-A(1)/C-71	M	0.11	0.77	4.41
	AF-C	Y	0.03	0.11	2.34
Brazil	BR-A	F	t	0.03	2.16
Canary Islands	CI-A	M	1.48	0.03	0.84
Czechoslovakia	CZ-A	F	1.22	0.04	1.11
	CZ-A	M	1.08	0.08	0.54
	CZ-B	F	0.29	0.02	1.03
	CZ-B	M	1.28	0.65	0.08
Ethiopia	ET-A	M	3.05	0.15	1.29
Fibriman	FI-A	F	0.94	0	0.05
	FI-A	M	1.12	0.18	0.06
France	FR-B	F	0.95	0.13	1.49
	FR-C	F	0.11	0.38	3.20
	FR-F	F	1.36	0	0.07
	FR-F	M	0.48	0.24	0.38
	FR-G	F	0.90	0	0.06
	FR-G	M	1.07	0	0.06
	FR-H	M	1.35	0.84	0.08
Ghana	GH-A	Y	0.01	0.08	1.68
	GH-B	Y	t	0.13	2.10
	GH-C	Y	0.02	0.09	2.60
India	IN-A	F	0.03	0.16	1.78
	IN-A	M	0.02	0.10	4.30
	IN-A	Y	0.01	0.15	0.72
	IN-A(1)/C-70	F	0.02	0.10	2.21
	IN-A(1)/C-70	M	0.3	0.20	1.70
	IN-A(2)/C-71	F	0.10	1.72	0.86
	IN-B(2)/C-71	F	0.02	0.26	2.72
	IN-B(2)/C-71	M	2.24	0	0.11
	IN-B(3)/C-72	F	t	0.64	1.95
	IN-B(3)/C-72	M	0	0.47	1.39
	IN-D	Y	0.01	0.40	1.68
	IN-E	F	0.02	0.52	3.31
	IN-F	M	0.03	1.36	0.72
	IN-F	Y	0.08	0.83	3.37
	IN-I	Y	3.40	0.26	0.99
Iowa	IO-A	F	1.70	0.02	0.10
	IO-A	M	1.66	0.04	0.09
	IO-A	Y	t	0.20	1.68
Iran	IR-A	Y	0.91	0	0.06
	IR-A(1)/C-71	F	0.73	0	0.72
	IR-A(1)/C-71	M	t	0.07	1.40
	IR-A(2)/C-72	F	0.07	0.01	0.33
	IR-A(2)/C-72	M	1.63	0.03	0.18
Jamaica	JA-A	Y	t	0.22	1.84
	JA-B	Y	0.01	0.41	2.20
	JA-C	Y	0.81	0.01	0.04
Kenya	KE-A	M	0.01	0.07	1.84
Korea	KO-A	Y	0.03	0.13	1.17
	KO-A(1)/C-70	Y	0.36	0.17	1.39
	KO-A(2)/C-71	M	0.02	0.91	3.83
	KO-A(2)/C-71	Y	t	0.09	0.34
	KO-B(1)/C-70	M	0.01	0.23	0.94
	KO-B(1)/C-70	Y	t	0.11	0.62
Lebanon	LE-A(1)/C-71	F	1.68	0.05	1.07
Manchuria	MN-A	F	1.93	0.23	1.99
	MN-A	M	0	0.56	1.48
Morocco	MO-A(1)/C-70	F	1.61	0	0.08
	MO-A(1)/C-70	M	0.95	0.38	0.39
	MO-B	F	1.84	0.02	0.54
	MO-C	F	0.70	0.11	0.71
	MO-C	M	0.37	0.08	0.16
Nepal	NE-C	Y	0.02	0.28	2.75
Nigeria	NI-D	Y	0.02	0.09	1.86
Pakistan	PK-A	F	t	0.05	1.32
	PK-A	M	0.40	0.19	1.51
	PK-A(1)/C-71	F	1.27	0.23	0.71
	PK-A(1)/C-71	M	1.20	0.04	1.37
Peru	PU-A	M	0.49	0.07	0.04
	PU-A	Y	t	0.08	2.06
Poland	PO-A	F	1.09	0.01	0.06
	PO-A	M	0.69	0.28	0.04
Russia	RU-A(1)/C-70	F	1.79	0.03	0.10
	RU-A(1)/C-70	M	0.04	0.21	1.05
Senegal	SE-A	Y	0.06	0.20	3.55
Sierra Leone	SL-A	F	0.02	0.03	1.23
South Africa	SA-A	X	0	0.11	1.60
	SA-A(1)/C-70	X	0	0.27	1.18
	SA-A(1)/C-71	F	0.03	0.61	3.02
	SA-A(2)/C-71	F	0.55	0.01	2.00
	SA-A(2)/C-71	M	0.05	0.68	2.89
	SA-A(2)/C-71	Y	0	0.06	0.84
	SA-D	F	t	0.15	1.84
	SA-D	Y	0.02	0.40	6.09
	SA-E	F	0.06	0.01	0.63
	SA-F	F	0.01	0.01	0.33
Sudan	SU-A	F	0.03	0.08	2.10
Thailand	TI-B(1)/C-Mon-70	F	0.02	0.42	2.91
	TI-B(1)/C-Mon-70	M	2.27	0.31	0.93
	TI-C(1)/C-Mon-70	F	2.40	0.06	1.36
	TI-C(1)/C-Mon-70	M	2.20	0.14	1.91
	TI-D(1)/C-Mon-70	F	1.25	0.11	1.56
	TI-D(1)/C-Mon-70	M	0.01	0.05	1.68
	TI-F	Y	0.87	t	0.33
	TI-G	Y	0.06	t	1.33
Turkey	TU-A	F	1.28	0.03	0.05
	TU-A	M	1.62	0.08	0.07
	TU-A(1)/C-68	F	1.91	0.04	2.79
	TU-A(2)/C-69	F	0.78	0.02	0.42
	TU-A(2)/C-69	M	2.79	0.16	1.59
	TU-A(2)/C-71	F	2.22	0.03	0.10
	TU-A(2)/C-71	M	1.87	0.24	0.84
	TU-A(3)/C-H-70	F	1.32	0.01	0.92
	TU-A(3)/C-H-70	M	2.02	0.14	0.09
	TU-C	Y	1.17	0.03	0.07
Viet Nam	VN-A(1)/C-71	M	0.54	0.23	3.23
	VN-A(1)/C-71	Y	t	0.04	0.99
	VN-B	Y	0.02	0.14	4.02

[a] F = female, M = male, Y = young plant, and X = mixture of male and female. [b] Data are reported as percent by dry weight in all tables. t = trace amounts (less than 0.01% by dry weight).

their abundance of I and II in freshly grown material.

Figure 1 shows the routine chromatogram used in determining the concentration of each of the cannabinoids. Here I and II are combined and are under one peak. The same Moroccan variant is shown in Fig. 2 after silylation (2), one method for the separation of I and II. However, with this method, four peaks must be taken into consideration: I monosilylated, I disilylated, cannabidiolic acid, and II. Silylated cannabichromenic acid has yet to be identified. Figure 3 again shows the Moroccan variant as produced by the 6% methyl silicone column described previously (3, 9) and shows excellent separation of I and II.

Data were obtained by a GLC computer[1] analysis based on relative retention times of routine *Cannabis* analyses and synthetic standards (Tables I–III). Table I consists of variants grown in Mississippi, and the variants included in Table II were grown in the listed countries. The data are presented as percentages by dry weight in all tables.

Analyses of Mexican material, coded ME-A[2], grown in Mississippi since 1968, are in Table III. The original ME-A seed was from Acapulco, Guerrero,

[1] Digital PDP-8 computer, interfaced with Beckman GC-45, 72-5, and 65.
[2] Used to supply the National Institute on Drug Abuse with research material.

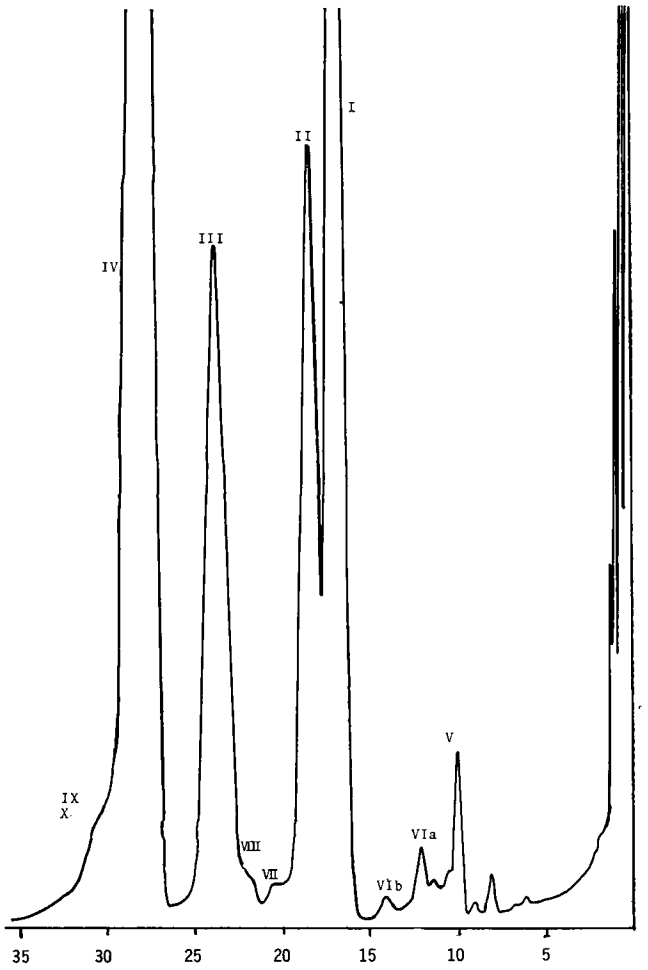

Figure 3—*The Moroccan variant, MO-A male, is shown on the 6% methyl silicone column. Cannabidiol (I) and cannabichromene (II) are adequately separated. (−)-Δ⁹-trans-Tetrahydrocannabinol (III), and the internal standard, androst-4-ene-3,17-dione (IV), are illustrated as well as: V, cannabidivarin; VIa, (−)-Δ⁹-trans-tetrahydrocannabivarin; VIb, cannabicyclol; VII, cannabigerol monomethyl ether; and VIII, (−)-Δ⁸-trans-tetrahydrocannabinol. Cannabigerol (IX) and cannabinol (X) are under the internal standard peak.*

Mexico, and analyses of the original plant material showed no I present. However, the fact that Mexican materials produced in our gardens do contain some I indicates that environmental factors affect the ratio of I to II or that some cross-pollination has occurred. The content of III has remained relatively constant when materials were harvested at the same age and time of day (10). A cyclic cannabinoid profile is observed in all variants with regard to age and hour collected; therefore, analyses reported are only for a particular sampling at a particular age and time of day (10).

As previously reported (8, 11), cannabinoid contents vary within each variant in regard to geographical origin and age. Therefore, the data in Tables I and II that show marked variation within a specific geographical location as well as geographical origin in general would be anticipated. Moreover, the presence of II and the absence of I in variants reputed to produce potent marijuana (Costa Rican and Mexican) seem to indicate that some interaction is occurring between II and III. If I antagonizes the action of III (4–6), it is highly possible that II may potentiate the action of HI. This area needs investigating using variants of varying amounts of I to III, II to III, and a mixture of I and II to III. However, it is now clear that II is more abundant in some variants than I and that II can no longer arbitrarily be considered a minor cannabinoid.

Table II—Variants Grown in Their Native Countries

Geographical Origin	Sample	I	II	III
Costa Rica	M-173	0	0.24	3.72
	M-174	0	0.09	1.60
	M-175	0	0.13	1.78
	M-176	0	0.16	1.87
	M-177	0	0.17	1.67
	M-236	0	0.20	1.19
	M-237	0	0.11	1.33
	M-238	0	0.14	2.04
	M-239	0.01	0.20	1.37
	M-240	0	0.17	1.01
Mexico	K24--1A	0	0.09	0.34
	K24--2B	0	0.10	0.14
	K24--3C	0.54	0.09	1.99
	K24--4D	0	0.07	0.59
	K24--5E	0	0.27	2.15
	K24--6F	0	0.15	2.65
	K24--7G	0	0.12	1.00
	K24--8H	0	0.13	1.30
	K24--9I	0	0.16	2.11
	K24--10J	0	0.16	2.97
	K24--11K	0	0.26	2.45
	K24--12L	0	0.24	1.15
	K24--13M	0	0.09	2.66

Table III—Mexican Variants Grown in Mississippi

Seed Code	Sex	I	II	III
ME-A	M	0.06	0.09	1.68
ME-A(1)/C-68	F	0.01	0.10	1.83
ME-A(2)/C-69	M	0.03	0.11	1.11
ME-A(2)/C-69	Y	0.01	0.10	1.35
ME-A(3)/C-71	Y	0.08	0.03	0.53
ME-A(3)/C-71	F	0.16	0.10	1.19
ME-A(2)/C-69	M	0.16	0.01	0.96
ME-A(2)/C-69	F	0.02	0.17	1.74

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Improved Method for Sampling Hepatic Venous Blood in the Rat

Keyphrases □ Blood sampling, hepatic venous—improved method, rats □ Hepatic blood samples, venous—improved method for sampling in the rat □ Elimination—method for sampling mixed hepatic venous blood proposed, rats

To the Editor:

In an attempt to study hepatic elimination of drugs *in vivo* in the rat, a method for sampling the mixed hepatic venous blood was devised. For estimation of hepatic extraction of drugs *in vivo*, simultaneous measurements of drug concentrations in hepatic arterial, portal venous, and hepatic venous blood are necessary. Extraction of propranolol by the dog liver was studied by direct measurement of hepatic venous concentrations of the drug *in vivo* (1). To obtain hepatic venous blood of the dog, a cannula was introduced into the right or left hepatic vein of the dog *via* a superficial jugular vein under fluoroscopic visualization. This cannulation applied in the dog is not practical for a hepatic vein of the rat, since hepatic veins of the rat are quite narrow and fragile and the cannulation is liable to cause trauma to the liver.

In this communication, we report a simple method for sampling the mixed blood draining from the hepatic veins of the rat in an *in vivo* study of hepatic drug binding and the subsequent drug metabolism. The method described here consists of introducing a cannula (Cannula A, Fig. 1) into the inferior vena cava *via* the external jugular vein so that the mixed blood from the hepatic veins may flow through an artificial channel in the inferior vena cava. Subsequent insertion of another cannula (Cannula B, Fig. 1) into the inferior vena cava makes it possible to sample the mixed hepatic venous blood.

A rat, 270–330 g, is placed in supine position and is anesthetized lightly with ether at suitable intervals. The abdomen is opened through a midline incision extending from the symphysis pubis to the xiphoid. The right lobe of the liver is reflected to the upper left, and the loops of the intestine are retracted downward to the abdominal cavity to expose the inferior vena cava between just above the right renal vein and the point where the vena cava becomes buried in the liver. The vena cava in this region is freed from its connective tissue, and a ligature is passed under the vena cava.

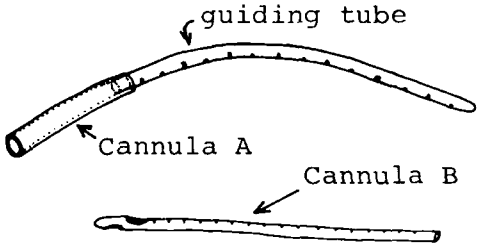

Figure 1—*Cannula A with a guiding tube and Cannula B. A guiding tube (polyethylene tubing, 0.2 cm o.d., 15 cm length) with its tip closed is inserted approximately 3 mm into Cannula A (polyethylene tubing, 0.2 cm i.d., 0.25 cm o.d., 3.2 cm length). The slight curve of the guiding tube is helpful in correcting false routes taken by the tip of Cannula A. Cannula A is filled with heparinized blood taken from another rat. Cannula B, with its tip closed, has two lateral holes near its tip. The guiding tube and Cannula B are put with centimeter markers throughout their length.*

The abdominal incision is then temporarily closed with two mosquito forceps. The right external jugular vein is exposed between the cephalic vein and the posterior external jugular vein and is ligated approximately 3 mm proximal to the junction of the posterior external jugular vein and the right external jugular vein. A small cut is made with scissors on the jugular vein, approximately 5 mm proximal to the ligature, while the jugular vein between the cut and the root of the cephalic vein is closed with a clamp to prevent bleeding.

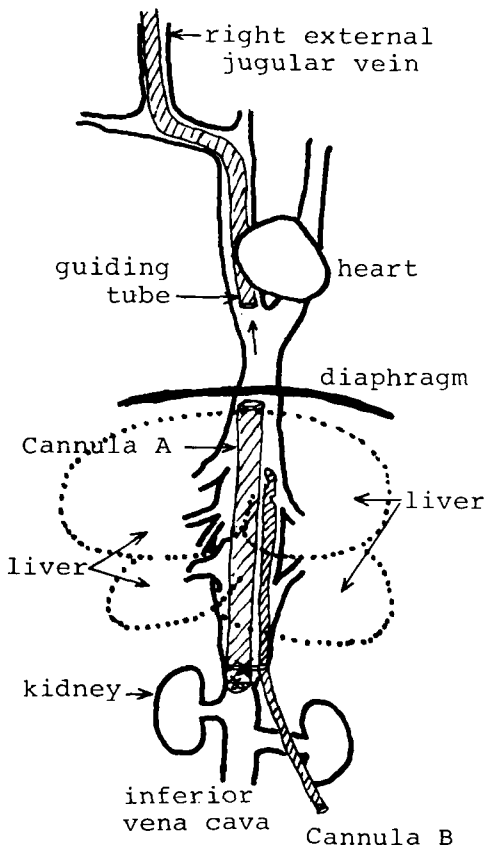

Figure 2—*Schematic representation of the inferior vena cava double cannulation for sampling mixed hepatic blood.*

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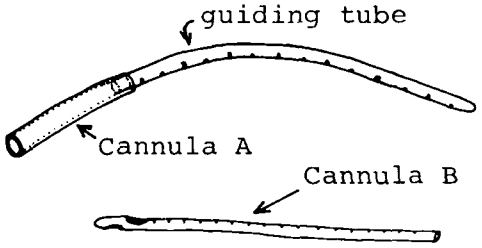

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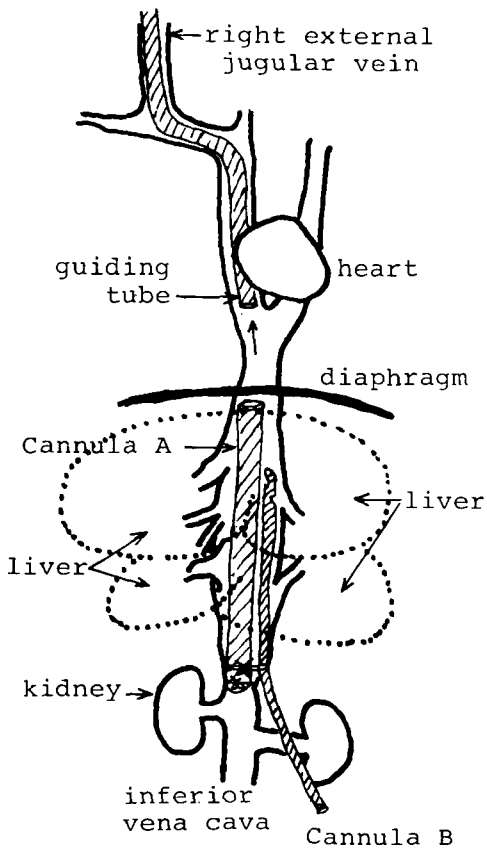

Figure 2—*Schematic representation of the inferior vena cava double cannulation for sampling mixed hepatic blood.*

Table I—Portal Venous (C_{pv}) and Hepatic Venous (C_{hv}) Concentrations[a] and Hepatic Extraction Ratios[b] (HER) following Intravenous Administration of Propranolol and Lithium Chloride

	Propranolol						Lithium Chloride		
	5 mg/kg			12.5 mg/kg			40 mg/kg		
Minutes	C_{pv}, ng/ml	C_{hv}, ng/ml	HER	C_{pv}, ng/ml	C_{hv}, ng/ml	HER	C_{pv}, mEq/liter	C_{hv}, mEq/liter	HER
5	1915 ± 600	7 ± 7	0.996	5224 ± 491	414 ± 162	0.921	1.58 ± 0.28	1.51 ± 0.27	0.04
30	1151 ± 476	1.7 ± 1.7	0.999	2061 ± 478	306 ± 93	0.852	0.96 ± 0.10	0.97 ± 0.08	0
60	639 ± 217	12 ± 12	0.981	1611 ± 404	104 ± 50	0.935	0.91 ± 0.07	0.91 ± 0.10	0
Mean			0.992			0.903			0.01

[a] Drug concentrations are expressed as mean ± *SE* for three rats. [b] Hepatic extraction ratios are expressed as the difference between the mean drug concentrations in the portal venous and hepatic venous blood divided by the concentration in the mean portal venous blood, since the drug concentrations in the portal venous blood and the hepatic arterial blood (estimated from the drug concentrations in the aortic blood) were not different significantly.

The operator starts to introduce Cannula A, provided with a guiding tube (Fig. 1), into the jugular vein from the cut toward the heart; at the same time, an assistant widens the cut carefully with dissection forceps and subsequently removes the clamp. The assistant ties softly a loose ligature, which is placed in advance around the jugular vein between the cut and the root of the cephalic vein to prevent loss of blood from the cut when the rear end of Cannula A passes the cut of the jugular vein. The cannula is pushed gently over a length of approximately 50 mm into the jugular vein until its tip is located near the diaphragm. Then the abdomen is again opened.

The assistant exposes the vena cava between the diaphragm and the upper margin of the liver so that one can observe the tip of Cannula A passing under the liver. The operator must guide Cannula A carefully near the right auricle, 30–35 mm from the cut, so as not to force it into the right auricle or the left superior vena cava. The operator must also be careful during the passage in the region of the junctions of the hepatic veins and the inferior vena cava. If Cannula A does not advance smoothly, one should not force it. This difficulty can usually be overcome by drawing Cannula A back slightly or rotating it slightly. The tip of Cannula A is then held approximately 5 mm proximal to the junction of the right renal vein and the inferior vena cava by tying the previously placed loose ligature (Fig. 2).

Immediately after ligation, the operator pulls the guiding tube out of Cannula A and extracts it from the vena cava and external jugular vein; the assistant holds Cannula A tightly over the vena cava with dissection forceps. It is important at this stage that the guiding tube be pulled out as quickly as possible after the ligation to relieve the obstruction of blood flow in the vena cava. Then the operator ligates the jugular vein just proximal to the cut to close the cut of the jugular vein.

The assistant reflects the right lobe of the liver to the left again and retracts the central and left lobes gently to expose the inferior vena cava between the lower margin of the liver and the right renal vein. Then the operator makes a venous puncture by a needle point, approximately 3 mm proximal to the ligature of the vena cava, and introduces Cannula B (Fig. 1) over a distance of approximately 15 mm toward the heart, picking up the vena cava near the cut by dissection forceps. Cannula B should be inserted up to 20–25 mm from the caudal tip of Cannula A to avoid reflux of the blood of the inferior vena cava into Cannula B. Usually no bleeding is encountered during this maneuver.

A syringe is attached to the other end of Cannula B, and it is then confirmed that the hepatic venous blood flows out in Cannula B on slight suction. Cannula B is secured by ligation, and the abdominal incision is closed. At the end of experiment the animals are sacrificed to make sure that the lateral holes of Cannula B are located in such a way as to draw the mixed hepatic venous blood. The duration of operation for this double-cannulation method is approximately 15 min.

The influence of this improved technique for sampling hepatic venous blood on hepatic blood flow was examined by the method usually employed in humans and dogs (2, 3). This method for calculation of hepatic blood flow is based on the Fick principle, using constant infusion of sulfobromophthalein, which is removed exclusively by the liver. The procedure used here was the same as that used by Chauvaud *et al.* (4) in the rat except for the sampling of hepatic venous blood. The result obtained with 30 rats after this operation showed a mean flow rate of 4.06 ± 0.22 *(SE)* ml/min/100 g of body weight. This value is comparable to reported values (5, 6). This finding indicates that the basal hepatic blood flow is not altered by the double cannulation into the inferior vena cava.

An extremely high extraction ratio during intravenous administration of propranolol was obtained in the dog by measurement of hepatic venous drug concentrations (1). Our previous result also suggests that the hepatic extraction of propranolol might be almost complete in the rat in a dose range of 2.5–12.5 mg/kg (7). The described double vena cava cannulation was used for estimation of the hepatic extraction ratio, using propranolol and lithium chloride as test compounds. Propranolol was administered to rats in doses of 5 and 12.5 mg/kg in 30 sec into the femoral

vein, and lithium chloride was administered in a dose of 40 mg/kg in the same manner. Hepatic extraction ratios of propranolol and lithium chloride were measured by obtaining blood samples simultaneously from the portal vein and the hepatic veins at various intervals after administration.

The portal venous blood of the rat can be taken through a cannula introduced *via* the pyloric vein into the hepatic portal vein (8, 9). Propranolol and lithium concentrations in whole blood were determined by the spectrophotofluorometric method of Shand *et al.* (10) and by the flame-photometric method of Amdisen (11), respectively. The hepatic extraction ratio was calculated from drug concentrations of the portal venous and hepatic venous blood samples taken simultaneously. The drug concentrations in the portal venous and hepatic venous blood and the mean hepatic extraction ratios are shown in Table I.

Propranolol concentrations in the mixed hepatic venous blood were always much lower than those in the portal venous blood, while lithium concentrations in the two venous blood samples were almost equal. Hepatic extraction of propranolol was essentially complete in the dose of 5 mg/kg, and it was more than 85% even in the dose of 12.5 mg/kg. On the other hand, lithium was essentially not extracted in the dose of 40 mg/kg.

A method for sampling hepatic venous blood may also be used for measurement of hepatic blood flow. In humans and in dogs, blood samples from a hepatic vein may be easily taken under direct fluoroscopic visualization by catheterization of a hepatic vein *via* the median basilic vein and the superficial jugular vein, respectively. However, in the rat, several technical problems arise on account of the small size of the vessels and liver. A major problem is to place a cannula in such a manner as to avoid trauma to the liver and to prevent contamination by the blood of the inferior vena cava.

For sampling hepatic venous blood, Dhumeaux and Berthelot (12) applied a catheter to one of the small hepatic veins through an incision made in the central lobe of the liver. The main disadvantage of the hepatic vein catheterization as well as this transhepatic vein catheterization is that one does not sample the mixed blood draining from all of the hepatic veins, since the hepatic veins empty themselves separately into the inferior vena cava. Drug concentrations in blood samples obtained by these methods are not always representative of the level in the entire hepatic venous outflow (13). Therefore, data obtained from such localized samplings may not be satisfactory for estimation of true hepatic extraction of a drug by the whole liver.

The technique of the double cannulation of the inferior vena cava presented here overcomes the disadvantage inherent in the previous methods, since the mixed hepatic venous blood, instead of blood from only one hepatic vein, can be obtained. This improved method for sampling the mixed hepatic venous blood is simple and practical. When applied in the rat, this method has proved useful for the determination of the drug hepatic extraction ratio in the study of *in vivo* drug metabolism as well as for measurement of hepatic blood flow in the rat. Details of these studies will be reported.

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Concentration Dependence of Ethanol Effect on Intestinal Absorption of Theophylline in Rats

Keyphrases □ Ethanol—effect on intestinal absorption of theophylline, concentration dependence, rats, □ Theophylline—intestinal absorption, concentration dependence of ethanol, rats □ Absorption, intestinal—theophylline, concentration dependence of ethanol, rats

To the Editor:

Koysooko and Levy (1) recently reported that the rate of absorption of theophylline from the perfused small intestine of anesthetized rats is increased significantly in the presence of a constant 2% concentration of ethanol and that there is a positive rank-order correlation between theophylline absorption from the ligated small intestine of rats and water net flux from

vein, and lithium chloride was administered in a dose of 40 mg/kg in the same manner. Hepatic extraction ratios of propranolol and lithium chloride were measured by obtaining blood samples simultaneously from the portal vein and the hepatic veins at various intervals after administration.

The portal venous blood of the rat can be taken through a cannula introduced *via* the pyloric vein into the hepatic portal vein (8, 9). Propranolol and lithium concentrations in whole blood were determined by the spectrophotofluorometric method of Shand *et al.* (10) and by the flame-photometric method of Amdisen (11), respectively. The hepatic extraction ratio was calculated from drug concentrations of the portal venous and hepatic venous blood samples taken simultaneously. The drug concentrations in the portal venous and hepatic venous blood and the mean hepatic extraction ratios are shown in Table I.

Propranolol concentrations in the mixed hepatic venous blood were always much lower than those in the portal venous blood, while lithium concentrations in the two venous blood samples were almost equal. Hepatic extraction of propranolol was essentially complete in the dose of 5 mg/kg, and it was more than 85% even in the dose of 12.5 mg/kg. On the other hand, lithium was essentially not extracted in the dose of 40 mg/kg.

A method for sampling hepatic venous blood may also be used for measurement of hepatic blood flow. In humans and in dogs, blood samples from a hepatic vein may be easily taken under direct fluoroscopic visualization by catheterization of a hepatic vein *via* the median basilic vein and the superficial jugular vein, respectively. However, in the rat, several technical problems arise on account of the small size of the vessels and liver. A major problem is to place a cannula in such a manner as to avoid trauma to the liver and to prevent contamination by the blood of the inferior vena cava.

For sampling hepatic venous blood, Dhumeaux and Berthelot (12) applied a catheter to one of the small hepatic veins through an incision made in the central lobe of the liver. The main disadvantage of the hepatic vein catheterization as well as this transhepatic vein catheterization is that one does not sample the mixed blood draining from all of the hepatic veins, since the hepatic veins empty themselves separately into the inferior vena cava. Drug concentrations in blood samples obtained by these methods are not always representative of the level in the entire hepatic venous outflow (13). Therefore, data obtained from such localized samplings may not be satisfactory for estimation of true hepatic extraction of a drug by the whole liver.

The technique of the double cannulation of the inferior vena cava presented here overcomes the disadvantage inherent in the previous methods, since the mixed hepatic venous blood, instead of blood from only one hepatic vein, can be obtained. This improved method for sampling the mixed hepatic venous blood is simple and practical. When applied in the rat, this method has proved useful for the determination of the drug hepatic extraction ratio in the study of *in vivo* drug metabolism as well as for measurement of hepatic blood flow in the rat. Details of these studies will be reported.

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Table I—Effect of a Constant Concentration of Ethanol in the Perfusion Solution on Theophylline Absorption from *In Situ* Perfused Rat Small Intestine

Ethanol Concentration, %	Water Net Flux[b], ml cm^{-1} hr^{-1}	Theophylline Intestinal Clearance, ml cm^{-1} min^{-1} × 10^3	Number of Rats	Body Weight, g
0	0.051 (0.024)[c]	5.25 (0.73)[c]	6	279 (50)[c]
0[a]	0.048 (0.017)	5.27 (0.62)	6	297 (41)
0.25 ± 0.03	0.070 (0.023)	6.66 (0.40)[d]	6	270 (56)
1.08 ± 0.27	0.133 (0.011)[d]	9.77 (0.70)[d]	4	252 (54)
1.88 ± 0.14	0.103 (0.014)[d]	9.02 (1.97)[d]	4	282 (43)
1.93 ± 0.35[a]	0.116 (0.013)[d]	8.33 (1.09)[d]	6	296 (29)

[a] Data from Ref. 1. [b] Slope of a plot of cumulative water net flux *versus* time during the first 60 min. [c] All results are expressed as means (standard deviation). [d] Statistically significant difference from control value ($p < 0.02$).

the intestine at initial ethanol concentrations of 0, 5, 10, and 20%. A subsequent study determined the concentration profile for the effect of ethanol on water flux and showed that ethanol concentrations as low as 0.05% can increase water net flux from the intestine (2). These findings suggested that even very low concentrations (<1%) of ethanol might increase theophylline absorption.

To investigate this possibility, we determined the effects of 0, 0.25, 1, and 2% ethanol on theophylline absorption from the *in situ* perfused small intestine of anesthetized rats, using the methodology described previously (1). The results of these investigations (Table I) show that ethanol in 1% and even in 0.25% concentrations significantly increases the absorption rate of theophylline from the rat small intestine. Results obtained previously with 0 and 2% ethanol (Table I) are in agreement with the results obtained in this study.

There is a perfect rank-order correlation between theophylline intestinal clearance and water net flux for the four sets of experiments in the present investigation. The results of studies concerning the effect of ethanol on the absorption of other drugs and of studies concerning the mechanism of the absorption-enhancing effect of ethanol and other alcohols will be described in subsequent reports.

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reader's labors and those legal requirements, and to call to the reader's attention the burgeoning scientific literature pertaining to drug control." In all three areas, a wealth of facts and information is presented from the viewpoint that can only be gained by many years of practical experience.

In the first section of the book, the legal origins and applications relevant to regulatory drug analyses are presented and the purposes, functions, and responsibilities of regulatory drug analyses delineated. In the second section, the relevant scientific documents and literature resources, including domestic and foreign official regulatory methods, nonofficial compilations, reference treatises and texts, and publications are presented with a description of each. In the third section, the constructional features of methods suitable for, and useful in, regulatory drug analysis are described in detail. Although methods of analyses are in a continual state of flux as new procedures are developed, instrumentation becomes more sophisticated, or specifications more stringent, the basic principles remain unchanged. These are discussed relative both to monographs in existing pharmacopeias and to personal experiences of the author and associates in laboratory problems.

The book should be particularly valuable for those chemists who are fairly new to the area of regulatory drug analyses, and at the same time be of interest to experienced personnel in emphasizing the wide scope of their laboratory activities.

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Amino-Acids, Peptides and Proteins. Volume 5. R. C. SHEPPARD, Senior Reporter. The Chemical Society, Burlington House, London, WIV OBN, England, 1974. 515 pp. 13 × 2.5 cm. Price £8.00.

It is indeed encouraging to see that the high standard which was established by Dr. Young has been faithfully maintained by Dr. Sheppard in presenting this fifth report in the series of the literature review in the field of amino acids, peptides, and proteins. The seven chapters of this volume provide a systematic and comprehensive review of papers appearing during 1972 and make an excellent addition to the earlier volumes.

The first four chapters are devoted to the detailed survey of the literature on amino acids, structural investigations of peptides and proteins, peptide synthesis, and peptides with structural features not typical of proteins. Of special interest is the subject matter of Chapter 5 concerned with chemical structure and biological activity as related to hormones and peptides, which have not been covered in this series since Volume 1. The detailed review of this area therefore includes references from 1971 and earlier literature, as does the subject of Chapter 6 (Metal Derivatives of Amino Acids, Peptides, and Proteins) which covers papers appearing in 1971 and 1972.

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The researchers in the area of amino acids, peptides, and proteins will greatly benefit from the indispensable service provided by this volume, however, it is sincerely hoped that such surveys of the literature in future volumes will be relatively more expedient.

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Staff Review

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The book should be particularly valuable for those chemists who are fairly new to the area of regulatory drug analyses, and at the same time be of interest to experienced personnel in emphasizing the wide scope of their laboratory activities.

Reviewed by W. N. French
Health Protection Branch
Department of National Health and Welfare
Ottawa, Ontario, Canada

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DRUG QUALITY: POTENTIAL PROBLEMS *VERSUS* ACTUAL EXPERIENCE

Scientists frequently are chagrined by the fact that the lay public will view technical subjects in a simplistic manner. For example, it is often said that the lay-person rarely understands or comprehends the fact that no drug is completely and absolutely "safe." In other words, the concept of benefit-to-risk, which ultimately applies in the case of every drug, appears to be a bit beyond general public comprehension.

However, those trained in the pharmaceutical and medical sciences—whether they be scientists or practitioners—should have sufficient knowledge and sophistication that they can be expected to recognize and understand such distinctions. Unfortunately, for some reason this is not always the case.

Specifically, over the years, APhA and Association officials have frequently pointed out that drug products must be formulated with care, that principles of good manufacturing practice must be followed, that specifications and standards must be adopted, and that drug marketing and distribution must be controlled and monitored. Moreover, APhA has taken the position that the absence of any of these vital components would give rise to serious questions about the suitability of the resultant drug products.

Subsequently, these APhA statements at times have been cited by various outside sources to support their claim or conclusion that the drug supply is of uncertain quality and that there is ample reason to be concerned about the safety and effectiveness of a very substantial portion of drugs on the market.

In turn, APhA has been surprised by such faulty reasoning and has been annoyed when efforts of this type are made to misconstrue the Association's position. Indeed, people who certainly should know better have alleged that, when APhA states that problems of therapeutic equivalence are of a very limited magnitude, the Association is "changing its position" or "reversing itself." These people appear to believe that the statements concerning *(a)* the potential for drug quality problems, the need for care in drug manufacturing, and the surveillance of products entering the market, and *(b)* the general low level of therapeutic inequivalence somehow are mutually contradictory positions. But such is not the case.

Simply stated, APhA has clearly recognized that drug products must be produced with appropriate care if they are to perform at the level of potency, safety, and uniformity expected of them; but at the same time, it is APhA's assessment that the comprehensive system of laws, regulations, programs, and other elements in the drug production, marketing, and surveillance network results in a very high level of drug quality experience. The net effect of this is that problems in general, and of therapeutic equivalency in particular, are rarely encountered.

But APhA also recognizes that our generally favorable experience to date is directly based upon the effective functioning of the overall drug regulatory system. And future success will be dependent upon continued functioning at the same level at least. For this reason, the Association maintains a close watch on the system itself, as well as on proposals to change or modify it. If it is felt that any such proposed change could have the effect of weakening this system, the Association has not hesitated to voice its concern.

On this basis—and again at some risk of being misunderstood or misinterpreted—APhA presented its concern most recently before the HEW Review Panel on New Drug Regulation this past May. In particular, the Association's comments were intended to ensure that no gap would be permitted to develop between the time that the abbreviated NDA system might be relaxed and the time that the so-called "old drug monograph" system is instituted and becomes effective. In short, APhA believes our present drug supply is of good quality, and we intend to do all that we can to see that it stays that way!

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Edward G. Feldmann

Transepidermal Water Loss

Ryan and Mezei[1] recently reported interesting and important data on the effect of polysorbate 85 on the transepidermal water loss from human skin. They found that the transepidermal water loss was increased by repeated treatments with a 10% blend of polysorbate 85 in petrolatum USP above that resulting from a control utilizing only petrolatum USP. The data failed to disclose whether or not the transepidermal water loss was reduced by repeated inunction with petrolatum USP, an effect that may obscure the significance of the data presented.

The significance of their findings is further complicated by the fact that the skin of the subjects was washed with "soap and water" prior to the measurement, because the degree of removal of petrolatum by soap and water may be influenced by the presence (or absence) of the nonionic surfactant. The fact that cosmetic *emulsions* have a tendency to increase the transepidermal water loss was reported as early as 1957 by Powers and Fox[2], and an explanation of this finding has been a problem for many years.

We recently attempted to explain[3] why a preparation that increases the transepidermal water loss *in vitro* relieves the drying–chapping syndrome during clinical testing. Until a valid explanation of these paradoxical findings is available, it appears premature to conclude that ". . . increased water loss through the skin . . ." may result in ". . . dry skin . . . ."

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[1] K. J. Ryan and M. Mezei, *J. Pharm. Sci.*, **64**, 671(1975).
[2] D. H. Powers and C. Fox, *Proc. Sci. Sect., TGA*, **28**, 21(1957).
[3] M. M. Rieger and D. E. Deem, *J. Soc. Cosmet. Chem.*, **25**, 253(1974).

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JUNE 1975

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REVIEW ARTICLE

Percutaneous Absorption

BERNARD IDSON

Keyphrases □ Percutaneous absorption—review of *in vitro* and *in vivo* penetration methods, skin transport, physicochemical factors, sorption promoters □ Absorption, percutaneous—review of *in vitro* and *in vivo* penetration methods, skin transport, physicochemical factors, sorption promoters □ Skin transport—review of percutaneous absorption

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The skin, the largest organ of the body, is under constant assault by numerous chemical and physical agents. New synthetic chemicals, toxic gases and liquids from chemical warfare research, environmental and industrial hazards, cosmetics, and topical medicaments penetrate the protective epidermis, producing either local or systemic toxicity.

Much work has been done to elucidate skin structure, physiology, barrier properties, and the mechanisms by which substances enter and cross the skin (1). Yet only in the last 2 or 3 decades has the scientific study of percutaneous absorption moved from emphasizing descriptive detail to correlating physicochemical factors in attempts to understand which molecules may be expected to breach the skin barrier. Studying the types of epidermis involved, the nature of the biochemical and physiological activities of the epidermis, and the epidermal barrier unit should indicate how to manipulate structure to cause selective and effective permeability.

In particular, valuable information on the influence of vehicles is just beginning to evolve. The potential exists for developing vehicles that will enable the drug to reach the site of action rapidly and maintain a sufficient concentration at the site for the required length of time. With increased understanding of percutaneous absorption, we can expect to see the advent of singularly effective new topical drugs—

drugs incorporated into precisely designed delivery systems which fully exploit the potentialities of the drug for treatment of specific disease states (2, 3).

A major problem in the study of percutaneous absorption is the interpretation of results of previous investigators, since the permeability of mammalian skin has interested scientists from differing disciplines (4). It is difficult and dangerous to draw valid conclusions and comparisons due to the use of various test systems, *in vitro* and *in vivo* models, various times and modes of application of material, and several species from mice to humans. The results have been expressed in many different ways; each worker has been anxious to express the data so as to elucidate his or her own problem without making assumptions about the mechanism.

This review attempts to draw together the more significant work. No attempt is made at cataloging the extensive literature. Reference 1 is the authoritative treatise up to 1954, and several fine reviews are available (2–18).

METHODS FOR STUDYING PERCUTANEOUS ABSORPTION

Percutaneous absorption has been studied in many ways, but the varied techniques do not give comparable quantitative data. Few of them determine the avenues of penetration, and rarely is the ultimate deposition of the penetrating substance accurately made clear. Progress in the area of transport through the skin has been slowed by the absence of good methodology. Since the amount of penetrating material is so small, good quantitative microtechniques must be available. Often a physiological yardstick, such as vasoconstriction or vasodilatation, has been used to quantitate penetration. Limited availability of tissue for *in vitro* experiments also has slowed progress. Yet the techniques are improving and have yielded valuable data on the routes of absorption, the barriers to absorption, the degree of systemic absorption after topical application, and the effects of various physical and chemical parameters. Numerous methods for studying percutaneous absorption have appeared (1, 2, 5, 7, 9, 14, 19–37).

The methods can be divided into *in vitro* and *in vivo* procedures involving two general types of test systems. The former involves excised skin in diffusion chambers; the latter employs the skins of the living animal or human subject *in situ*. Some investigators have access to surgical skin specimens or to freshly obtained skin of human cadavers and can obtain epidermis with its complete stratum corneum by separation from the dermis.

Those who lack these sources resort to the pressure-sensitive tape-stripping technique for removing sheets of stratum corneum, of about 15 μm thickness, for use in diffusion cells (38). It is rarely possible to remove the stratum corneum entirely in this way, but most of this layer can be obtained. The stripping technique enables one to work with human skin membranes whose resistance to penetration closely resembles that of the entire thickness of the epidermis. Cantharadin-blistered skin may be used if it contains no holes. Indeed, the intactness of any skin preparation should be confirmed by microscopic examination prior to and after conducting a diffusion study.

The disadvantage of the excised skin technique is that it does not reflect the role of the skin *in vivo;* but if the main barrier to percutaneous absorption is viewed as a semipermeable membrane, then the results obtained are acceptable. When comparable *in vivo* and *in vitro* experiments have been performed, almost comparable results have been obtained (39–41). Scheuplein (42) observed that when the extant data on skin permeability are considered in the light of both steady-state and transient diffusion, much apparently conflicting data can be resolved. *In vitro* measurements of skin permeability are invariably measurements of steady-state rates if for no other reason than it is usually impossible to detect concentrations within the first few minutes.

Thus, previous measurements (25, 35, 43) are all of steady-state fluxes and are in agreement about the paramount role of bulk diffusion through the unbroken stratum corneum. On the other hand, *in vivo* permeability measurements are rapid measurements and often depend on biological end-points sensitive to extremely small concentrations (44–46). The *in vivo* results show only that shunt diffusion is predominant in the early transient stage of diffusion. This area will be explored more fully in the *Skin Transport* section.

The advantage of *in vitro* procedures is the absolute control obtained of environment, allowing the demonstration of the importance of individual factors in determining percutaneous absorption of a particular substance. These *in vitro* results may point the way for further *in vivo* work. If their outcome supports animal and human experimental findings, they may have a useful place in the screening of new products without the difficulties usually involved with *in vivo* methods. Marzulli *et al.* (26) sought to develop data through excised human skin in diffusion cells first and then to study skin absorption in human subjects.

The main objection to the use of the living animal or human subjects is that this method measures skin penetration by indirect means. For example, the test substance is applied to the skin and penetration is estimated by what is recovered in the urine. In such a system, it is not possible to find out to what extent the penetrant agent is metabolized as it traverses the skin en route to the bloodstream. Furthermore, a correction must be made for the storage of the penetrant in body organs. This storage correction is often obtained by measuring urinary recovery of the penetrant after introducing a known amount of agent by a single intravenous injection. Thus, the organ storage value may have a sizable error in cases where significant percutaneous penetration takes place over several days. These limitations suggest that there is room for progress in methodology. Moreover, diverse but complementary data may be obtained from both *in vitro* and *in vivo* studies (26).

***In Vitro* Methods**—*Excised Skin*—Virtually all investigators use some type of diffusion cell, in which

animal or human skin is fastened to a holder and the passage of compounds from the epidermal surface to a fluid bath is measured (47). The simplicity of equipment has prompted widespread use of the techniques. For example, Cronin and Stoughton (48) stretched human skin over the mouth of a funnel, and Fritsch and Stoughton (49) developed a special glass chamber which allowed various refinements to be employed. With this technique, penetration rates can be quantitated. The area of spread of a radioactive agent on the surface is detected with autoradiographs of the surface, and the penetration of an agent can be expressed in terms of quantity per unit area per unit time.

Radioactive agents have not taken over completely. Many chemical agents penetrate in sufficient concentration to be determined by one of the many techniques of physical or chemical analysis. One objection to this technique is that it does not represent the *in vivo* problem of an efficient, if capricious, blood supply which would remove penetrating materials at various rates and thus change the concentration gradients across the membrane or, as in the case of steroids, induce vasoconstriction (which occurs *in vivo*) and thereby decrease the rate of removal of steroid in the corium. This process would increase the local concentration in the corium and decrease the penetration rate. Thus, both *in vivo* and *in vitro* measurements for a given agent are highly desirable (23).

Penetration into Models—This method involves penetration of some substance into a model designed to simulate skin. If a chemical in a given vehicle is applied to the surface of a model, the amount of that chemical transferred from the vehicle into the model may be measured with a certain degree of accuracy. By using various vehicles, the rates of transfer of a given chemical into the model can be determined. The most common model used to simulate skin was agar gel, but the data obtained were very crude and more suitable models were sought.

Recently, in attempts to develop practical vehicles, models have been devised for studying *in vitro* release from the base into immiscible solvents, acting as "sinks" or receptor skin phases (50–54). Results have established fair correlation with membrane testing. These models are discussed more fully in a later section.

***In Vivo* Methods**—The major *in vivo* techniques involve histological studies, use of tracers, elicitation of biological responses after topical application, and analysis of body fluids or tissues.

Histological Studies—Tissue changes in skin following application of various substances to the cutaneous surface can yield information about the specific tissues affected by any penetrant, so that not only absorption *per se* is revealed but also the route of penetration. The method is limited to dyes and to a few other substances that yield precipitable colored end-products in specific chemical reactions. An objection to the method is that evidence of routes of absorption using colored dyes cannot be accepted completely, because a dyed lipid applied to the skin may not penetrate intact and, indeed, the dye may combine with another lipid in the dermis or epidermis. Separation within the skin of the dyed substance and the dye is generally the rule.

Histochemical techniques have proved useful in determining routes of absorption but are limited by the numbers of drugs influencing enzyme systems. For instance, changes in epidermal sulfhydryl groups are easily detected, but the number of drugs producing these changes is limited (55–58).

Tracers—Following the movement of penetrants by means of dyes, fluorescence, or radioactive labeling represents the most frequently used method. This technique is always combined with other methods such as histological or chemical analysis of tissues or fluids.

The dye technique employs an oil-soluble dye to color the material being studied. Histological sections of the skin are then examined for the presence of the dye. This method presupposes that the tracer and the penetrant act similarly and remain together during penetration. Unfortunately, this may not always be the case.

The use of fluorescence techniques depends on the fact that substances that penetrate very readily show a more rapid diminution in the intensity of the fluorescence at the surface of the skin than do poor penetrators. The use of fluorescent compounds in penetration studies is open to the same criticism as when a dye is used as a tracer. One cannot be sure that the fluorescent material remains with the substance whose penetration is being studied. This objection is not valid if the penetrating substance itself is fluorescent. If used properly, the method is of considerable value, as when Baker and Kligman (59) used the fluorescent dye tetrachlorosalicylanilide to determine the permeability of the horny layer.

Radioactively labeled tracer compounds gave the greatest impetus to skin penetration studies. Radioisotopes have been used to study percutaneous absorption by measuring the urinary excretion of topically applied compounds (60–69), the decrease in the radioactivity of the skin surface (28, 70), or the accumulation of an isotope in the collecting fluid of a diffusion or continuous-flow cell in which a piece of excised skin was mounted (35, 43, 62, 71). The method of measuring the rate of disappearance from the skin requires much smaller amounts of radioactive material than does the determination by blood analysis of how much drug has passed into the circulation.

While the methods give information on the amount of the compound that moves across the skin, they yield little or no information on the route of penetration or on the localization of the penetrant within the structure of the skin. Actually, all work with radioisotopic compounds should be checked at some stage by chemical recognition of the compound concerned, because enzymatic splitting of the molecule may occur and the radioactivity counted may not represent the material under investigation.

Marzulli *et al.* (26), while studying the penetration of ^{14}C-testosterone, noted inconsistencies in permeability constants, which suggested that there was a tissue reaction. Such a reaction could affect the diffu-

sivity of the membrane or change the form in which testosterone penetrates the skin. The method also has the problem of a 5–15% error, which is almost impossible to avoid. Thus, it is difficult to measure penetration of a small percentage of applied material. ^{14}C-Agents pass into the horny layer where fewer emissions are recorded on the surface, giving a false impression of penetration.

If further information is desired about the routes of penetration, autoradiography is a useful procedure, but it is difficult to quantitate. The considerable scatter of the rays and showing are drawbacks (72). Some β-particle emitters darken areas up to 2–3 mm away from the localization of the molecule, giving one a mistaken impression of the location of the agent. Therefore, tritium-labeled agents are very useful because of their weak β emissions. Cutting, staining, and handling of tissue not only may displace agents but also may remove agents from the specimen, giving a false negative result.

Analysis of Body Fluids or Tissues—For body fluid or tissue analysis, urinary analysis is used most frequently and is probably the method most commonly used in studying percutaneous absorption. Small specimens of skin, obtained by biopsy either singly or in series, may be analyzed for the penetrant. In cases where drugs appear to have a greater affinity for a particular organ, the organ may be excised and analyzed for drug content. The chief analytical tool is radioactive labeling, followed by GC, fluorescence, and specific color reactions.

Feldmann and Maibach (63–65, 73–77) utilized urinary excretion for studying various compounds. The method is extremely valuable. However, although the chemical agent recovered in the urine will probably have penetrated the skin, the amount recovered in the urine will not necessarily be the original total amount to penetrate the skin. Some of the applied agent may go somewhere other than the urine, and some may be metabolized and, therefore, no longer be detectable. A steady state of balanced absorption and excretion needs to be reached before measurements can be accepted completely. This is time consuming and may need the resources of a metabolic ward. Measurement of the excretion rate of metabolites of labeled corticosteroids has been employed successfully (61, 70, 78). Another source of error in this method may be the ability of the living organism to alter the penetrant before it is measured quantitatively. When such alteration occurs, more of the chemical penetrates than can be retrieved by chemical analysis.

Under *in vivo* conditions, penetration often occurs from such a large amount of material, in comparison to the amount penetrating, that the concentration of the solute in the presenting material remains almost constant. Also, when the penetrating material reaches the bloodstream, it is probably removed so rapidly that the concentration in the bloodstream is always near zero. Hence, detection in blood is rarely attempted.

A useful modification involves "calibration" of the animal to be studied. Suppose that the blood is to be analyzed as a measure of penetration but that some material reaching the blood is known to be lost to other tissues. If the penetrant is administered slowly by the intravenous route and the level of this substance in the blood is determined at successive time intervals, a calibration curve can be constructed which relates the amount of penetrant introduced into the bloodstream to the amount of that substance actually present in the blood. If, subsequently, the penetrant is applied to a given area of the skin of this animal and the blood level of this substance is determined at successive intervals, the amount of penetrant reaching the blood *via* the percutaneous route can be determined from the calibration curve constructed from data obtained after intravenous administration. With a little experimentation, the rate of change in the blood level can be made quite similar for the two methods of administration. This method is probably one of the best available for quantitative measurement of percutaneous absorption in the living animal (21, 79).

Elicitation of Biological Responses—The use of agents that have biological activity when they reach the corium is a valuable method. With this technique, it is possible to demonstrate whether or not a substance capable of causing a physiological reaction can penetrate into the skin and, if it does penetrate, to determine the time required for the reaction to develop. The method is intriguing because of its simplicity and potential practical applications. In theory, such biological functions of skin as sebaceous gland growth and excretion, sweat gland secretion, pigment formation, vasoconstriction, vasodilation, vascular permeability, epidermal proliferation, and keratinization could be influenced by topical pharmacological agents.

Many responses (sweat secretion, vasoconstriction, vasodilation, pigmentation, and vascular permeability) can be reasonably accurately recorded by visual observation. Thus, when enough of a topically applied agent such as histamine reaches the vessels of the corium, there is a visible reaction. The amount needed to induce the reaction can be determined by intradermal injection of histamine in differing dilutions to find the minimum dilution necessary for a biological response (23). This same approach can be used for such drugs as norepinephrine, angiotensin, naphazoline, nicotinic acid (niacin), atropine, and glucocorticoids.

Quantitation of the actual amount of the agent penetrating is not as accurate as with *in vitro* methods, but comparative estimations of the penetration of a given agent in such varying environmental circumstances as humidity and temperature can be meaningful, and much information regarding comparative effectiveness of topically applied steroids has been derived from this approach (23). One sensitive test is based on the antigenic properties of the substance to be absorbed, because extremely minute traces penetrating to the site of tissue antibodies may elicit allergic reactions.

The elicitation of vasoconstriction or vasodilation by the topical application of various agents has been

successfully employed (44, 48, 49, 80). This technique led to the development of the vasoconstrictor assay for the efficacy of topical corticosteroids (81). The relative potency of various corticosteroids has been assayed with this *in vivo* technique. It seems to be one of the most valuable methods available for estimating clinical efficacy of various topical glucocorticoids. The most potent glucocorticoid will give blanching of the skin when applied topically in a concentration of less than 1 ppm. There is little doubt that a relationship exists between the ability of topical steroids to produce blanching and to alleviate inflammation in use (82).

A technique involving paired comparisons of vasoconstriction at adjacent sites was found to improve the assessment of relative vasoconstrictor activity (83). In addition to the vasoconstriction test, use is also made of the suppression of UV-induced inflammation, the suppression of chemical inflammation from croton oil, and the clinical suppression of psoriasis (84). The results obtained by the four test procedures not only correlate very well with each other but they are also in good accord with *in vitro* release data.

Vasoconstriction can be induced by topical application of other agents such as naphazoline, and this method has been used to study regional differences in penetration (85). Curtailment of sweat was blocked by topical applications of anticholinergic agents (86, 87). Vasodilation can be introduced by a number of drugs, including nicotinic acid and some of its derivatives. The amount per area necessary to give vasodilation with intradermal administration is compared to the amount per area to give vasodilation by topical application. A rough estimate can be made regarding the amount penetrating the skin (80). The elicitation of biological responses has permitted the construction of time–response curves, particularly those derived from the vasoconstrictor results in the evaluation of the absorption and activity of corticosteroids in an ethanol solution and in different bases (88).

Determination of the threshold concentrations of pharmacologically active substances, such as vasodilators and vasoconstrictors, that cause the skin to respond is possibly the easiest method, but it is indirect and not very precise. An important limitation is that the end-points express thresholds, not rates of penetration. Another drawback is that the method does not discriminate between the two major pathways a substance may take in entering the skin—*viz.*, *via* the hair follicles and sweat ducts (appendages), thus bypassing the horny layer, and *via* diffusion through the mass of the horny layer itself (59). The bioassay methods for measuring percutaneous absorption have been reviewed critically (89).

SKIN TRANSPORT

Skin is a structurally complex, thick membrane. Molecules moving from the environment across the intact skin of living humans must first penetrate the stratum corneum and any material of endogenous or exogenous origin on its surface. They must then penetrate the viable epidermis, the papillary dermis, and the capillary walls into the bloodstream or lymph channels, whereupon they are removed from the skin by the flow of blood or lymph. To move, molecules have to overcome a different resistance in each tissue. Transport across the skin membrane is obviously a complex phenomenon, challenging in analysis (10). To relate results on different penetrants, it is necessary to consider the transfer mechanism across skin. A recent symposium (90) was principally concerned with transport through the skin.

Epidermal Barrier—The currently most accepted theory of percutaneous absorption treats the skin as a uniform and invariant cellular matrix which produces a thin dead layer of impermeable membrane protecting the body (3). The surface lipid layer seems to offer relatively little resistance to the passage of water and other chemical compounds. The main barriers to absorption are the dead cells of the stratum corneum (horny layer), restricting the inward and outward movement of chemical substances and having a high electrical resistance (91, 92).

The stratum corneum is a heterogeneous tissue (93), composed of flattened keratinized cells. The outer layers of these cells are less densely packed than those adjacent to the underlying granular layer. Therefore, the epidermal barrier becomes more impermeable in the lower part, and this fact has led to the suggestion that a separate barrier exists at this level, the so-called stratum conjunctum (31, 94). No real evidence exists for the localization of the functional barrier. Analysis of penetration data, evidence from controlled stripping experiments, and the detailed picture of the stratum corneum, gained from electron microscopy, all support the idea that the barrier to penetration consists of the keratin–phospholipid complex in the dead and relatively dry cells of the entire stratum corneum (31, 35, 42, 93, 95–101).

Thus, as molecules move from the environment into the skin, the rate-limiting barrier, *i.e.*, the tissue that presents the greatest resistance to the movement of molecules, is the stratum corneum—the entire stratum corneum. One or two of the outermost cell layers may offer only limited resistance because the cells are desquamating and the layers are breaking up, but the rest of the stratum corneum may be considered relatively homogeneous as far as its resistance to the movement of molecules is concerned (102).

Kligman (93) showed that human stratum corneum can be removed intact in large sections in the form of a thin (10–15-μm) membrane and that this membrane is mechanically quite strong and resists chemical attack. The accessibility of stratum corneum and epidermal membranes makes possible the controlled physicochemical study of skin penetration *in vitro* (35).

Scheuplein (42) calculated the diffusion constants and geometrical data corresponding to appendages and to various regions near the surface of the skin. There are significant regional variations in thickness and number of cell layers as well as marked individu-

al variations within a region that are characteristic and specific for each individual. The number of cell layers appears to account for the variation in thickness (103).

Information is limited about the composition of the barrier. The main cellular components are proteins, lipids, and water, combined into an ordered structure (104). The cells are bound into a layered membrane, about 15 μm thick when dry but about 48 μm when fully hydrated (31). When hydrated, the stratum corneum contains approximately 75% water, 20% protein, and 5% lipid. While the surface lipids offer little resistance to the passage of compounds, studies of lipid removal from the cutaneous surface (105–110) indicate that lipids participate in epidermal water function.

Onken and Moyer (109) showed that the barrier function is restored when extracted lipids are returned to the skin. This finding suggests some possibility of marked variations in biological membrane permeability, dependent largely on the specific nature or distribution of the lipid contained in the cell membrane. Sweeney and Downing (110) recently applied a number of solvents to the epidermal side of hairless mice and human skin. All solvents increased the rate of diffusion of tritiated water, but there was no correlation between the type or amount of lipid extracted and the degree of alteration of the water barrier function.

The barrier to penetration by the horny layer holds for almost all compounds tested. For example, a 10% naphazoline concentration gives vasoconstriction when applied to normal skin, but a 0.001% concentration gives vasoconstriction when applied to skin where the horny layer has been removed by cellophane tape stripping (20). Skin with a disrupted epidermal barrier allows up to 80% of hydrocortisone to pass into the dermis. With a functionally intact epidermis, the absorption of steroid is about 1% (70).

The skin barrier is vulnerable to certain solvents and organic amines, as reflected in sharp increases in skin permeability (36). Solvents with such an effect are those with both polar and lipophilic properties. Apparently, they have the effect not only of removing skin lipids but also of breaking down the complex structure of the barrier, thus making it defective and possibly subject to entry by other chemical agents which do not penetrate intact skin. Substances such as dimethyl sulfoxide, dimethylformamide, and dimethylacetamide have the ability to produce a striking but reversible suppression of barrier resistance. It has been suggested that they alter the interrelationship of water, lipids, protein, and mucopolysaccharides regulating the epidermal water barrier function (112). The effects of these "accelerants" will be discussed more fully later.

Barrier Reservoir—The existence of a depot or "reservoir" within the stratum corneum was originally noted by Malkinson and Ferguson (60) and investigated in detail by Vickers (113). Vickers applied small quantities of fluorinated steroids to the surface of the skin and occluded the area with plastic film. As expected, vasoconstriction or blanching appeared when the film was removed after 16 hr (81). However, if the skin was then thoroughly washed and the subject was allowed to continue normal activity, the vasoconstriction faded in 10–16 hr. If areas were reoccluded at intervals as long as 12–14 days after the initial application of steroid, vasoconstriction reappeared, although no more steroid had been applied.

It was obvious, as a result of these observations, that a reservoir for steroids must exist somewhere in the skin. Clinical and radiobiological studies suggested strongly that the reservoir lay in the stratum corneum and that it was not just a surface film (114). It appears to lie in the deeper portions of the stratum corneum. The presence of an intact and normal stratum corneum is essential for the establishment of the reservoir (115). The amount of materials that can be retained within the reservoir is small, and their significance from a therapeutic point of view is open to question, although potent corticosteroids, such as fluocinolone, may be retained in sufficient quantity after a single application to influence epidermal cell division (116). Woodford and Barry (117) studied the bioavailability of betamethasone benzoate in a cream and gel base as well as other steroids. All formulations produced steroid retention in the stratum corneum for at least 8 days.

Garnier (88) discussed the parameters to be considered in devising the optimum base for topical preparations of corticosteroids. When the activity of the drug, as with steroids, depends on absorption into the vascular system in the dermis, the base may need to lower the interfacial barrier if the epidermis is intact or soften the superficial tissue if it has become excessively dry and thick.

Various factors that increase percutaneous absorption also appear to increase the quantity of material in the reservoir. Increasing temperature and humidity (49) result in increased penetration and in the establishment of a larger stratum corneum reservoir (113). Similarly, a solvent such as dimethyl sulfoxide enhances human skin penetration (118) and increases the reservoir (111). Dimethylacetamide and dimethylformamide are potent solvents which have been shown to increase the penetration of various steroids and griseofulvin and to establish, both *in vivo* and *in vitro*, a stratum corneum reservoir to a superior degree than such vehicles as ethanol or cream base (119).

Epidermal Diffusion—An understanding of the diffusion mechanism through skin may have even wider significance to the mechanism of membrane transport in general. The stratum corneum is an aggregate of tightly adherent cells, densely packed with lipid-enriched keratin fibrils, and presents a unique system for studying the chemical influence of naturally occurring protein–lipids on membrane transport. This is particularly significant when it is realized that many cell membranes are chemically quite similar to the stratum corneum and that transport through them is believed to be governed mainly by a lipid–penetrant interaction. In this restricted sense, the stratum corneum is a macroscopic analog of the unit-cellular membrane (35). Poulsen (120) reviewed

aspects of drug diffusion into skin.

Diffusion through the horny layer is a purely passive process, affected only by physical factors as determined by ambient conditions. Percutaneous absorption, on the other hand, is a more complicated process; epidermal diffusion is the first phase, and clearance from the dermis is the second. The latter depends on effective blood flow, interstitial fluid movement, lymphatics, and perhaps other factors such as combination with dermal constituents (35). A passive system has two main characteristics: *(a)* a delay period after the chemical is placed on the surface, during which the membrane itself becomes charged with the penetrant, and *(b)* a steady penetration rate after the delay period, which lasts as long as the chemical remains in adequate supply on the top surface and is removed from the lower surface. This steady rate is proportional to the concentration difference across the membrane; in the case of adequately perfused skin, the rate may be considered equal to the concentration applied (91). The ratio of the steady rate to the concentration applied should be, and is, a constant (termed the permeability constant); it is a measure of the permeability of the given skin to the chemical in the given solvent. If, and only if, results are expressed in terms of this parameter, they can be compared directly with each other (4). Physicochemical relationships involved in skin penetration have been derived (10, 21, 22, 31, 35, 43, 98, 102, 107, 108, 121–129).

There is little evidence to support specialized active transport systems for cells of the stratum corneum, except possibly for the work with water[1]. For water to move against a concentration gradient, an energy yielding "pump" is predicated in a viable epidermis. It is unlikely that such a reaction would exist in a nonviable tissue like the stratum corneum, and there is limited evidence for any transport across the stratum corneum against a concentration gradient. While the preponderence of evidence points to passive diffusion through a nonviable horny layer, Winkelman (90) was concerned that this assumption discounts the many rich variations of epithelial and mesenchymal structure which appear to have some role in the passage of material through the skin.

ROUTES OF SKIN PENETRATION

There are potentially three distinct routes of penetration through the stratum corneum: *(a)* the follicular regions, *(b)* the sweat ducts, and *(c)* the unbroken stratum corneum in between. But which is the principal route of penetration? There is little convincing evidence that eccrine sweat glands or ducts play any significant role in cutaneous permeability (5, 30). Material may enter the ducts and even the glands, but there appears to be no subsequent penetration to the dermis (130–132). Absorption of allergens and histamine through the palms and soles is very poor (44, 133). These are areas where, in view of the nature of the keratin, transepidermal absorption would be minimal; any absorption taking place must do so through the sweat glands because pilosebaceous glands are almost absent.

Hence, percutaneous absorption occurs through the cells of the stratum corneum, between the cells or *via* the pilosebaceous follicles. The particular route may be of major importance for one type of substance, but both routes probably play some part in the absorption of almost all compounds and the relative importance of one in contrast to the other depends almost entirely upon the physicochemical characteristics of the material being absorbed (2). In spite of specific literature statements, there are many difficulties in deciding the relative importance of each route in any one instance; material using the transfollicular route may diffuse in the dermis and appear in the subepidermal tissues, making it impossible to determine the route used. When considering skin penetration, it is helpful to keep in mind the following particularly relevant dimensions based upon reported values: thickness of stratum corneum, 14 μm; thickness of epidermis, 110 μm; and distance from surface to microcirculation, 150–200 μm (42).

For substances absorbed by the transepidermal route, penetration is fairly rapid, although slower than intestinal tract absorption, and is almost always accompanied by some degree of pilosebaceous penetration. For substances absorbed through both pathways, the transepidermal route is the principal portal of entry because of the total relatively small absorbing surface offered by the pilosebaceous units (5). The epidermis presents a surface area 100 or 1000 times greater than the other routes of absorption. The sweat glands and hair follicles are scattered throughout the skin in varying numbers but are comparatively sparse; their total cross-sectional area is probably between 0.1 and 1.0% of the area of the skin. The total volume available for transport, other than within the stratum corneum cells, is probably 0.01–0.1% of the volume of the stratum corneum. The evidence supports the belief that transient penetration occurs *via* the shunts and that steady-state transport occurs directly through the cells.

Drugs applied to the skin surface reach the orifices of the sweat glands and the hair follicles directly. Each hair follicle has one or more connecting sebaceous glands which empties its secretion into the follicular canal near the skin surface. These canals and duct systems are lined with stratified squamous epithelium, which is readily penetrated by medicaments. Therefore, the most likely pathway for drug penetration *via* the transfollicular route is through the microscopic spaces between the hair shaft and the follicular wall. These spaces permit the passage of substances into areas below the membrane barrier. Hence, the hair follicles were formerly thought to be the main route of percutaneous absorption. They may play a more conspicuous part in the penetration of particular substances, notably surfactants, which provide an increased wetting effect and allow close contact between incorporated substances and the follicular epithelium.

[1] See K. J. Buettner and F. F. Holmes, *J. Appl. Physiol.*, 14, 261, 276(1958).

Diffusion through the stratum corneum occurs with considerable difficulty even for small, soluble molecules (7). Steady-state penetration through the stratum corneum is not primarily intercellular or intraappendageal. The hydrated stratum corneum seems best described as a dense "semisolid" phase into which low molecular weight nonelectrolytes dissolve with strong chemical interaction and through which diffusion occurs very slowly. Before steady-state diffusion is established, small but possibly significant amounts of material may diffuse more rapidly through hair follicles and sweat ducts. The reasons for this seemingly anomalous behavior arise from the limited area of the skin surface occupied by these appendages, their relatively large diffusion constants, and the nonlinear character of diffusion prior to the steady state. The smaller the diffusion constant, the more important the role of the appendages or intercellular regions may become (124). Shunt diffusion may become dominant and control percutaneous absorption not only in the transient period but during steady state as well.

For molecules such as the steroids, which move very slowly through the stratum corneum and have long lag periods, the shunts may at all times be the major pathway into the skin (124, 134). Disease states, particularly those that affect the stratum corneum such as eczema and exfoliative dermatitis, undoubtedly result in many "artificial shunts" through the stratum corneum. Body fluids escape through such shunts, and topically applied medicaments can enter more easily through them than through normal skin (10).

In a study (43) of steady-state diffusion of alcohols from aqueous solutions, the high molecular weight, lipid-soluble alcohols had larger permeability constants than the low molecular weight, water-soluble alcohols. This finding indicates that the mechanism of steady-state diffusion of each of these alcohols cannot be only diffusion through relatively large, water-filled channels. If this were the mechanism, the permeability constant would change very little over the entire series; actually it would decrease slightly with increasing molecular weight. Low molecular weight molecules readily penetrate capillary walls and rapidly enter the systemic circulation. The rich vascularity of the superficial layers of the dermis limits the number of molecules free to diffuse deeper into the dermis, so lower lying layers of the dermis would not be expected to influence diffusion radically (42).

Polar and nonpolar substances diffuse through the stratum corneum by different molecular mechanisms (125). As the stratum corneum hydrates, water accumulates near the outer surface of the protein filaments. Polar molecules appear to pass through this immobilized water. In contrast, nonpolar molecules probably dissolve in, and diffuse through, the nonaqueous lipid matrix between the protein filaments. The activation energies for the diffusion of nonpolar molecules are somewhat lower than for the diffusion of polar molecules.

Scheuplein (42) maintained that it is probably wrong to assume that a principal route of penetration exists without specifying other conditions. Under the appropriate conditions, each contending route of permeability may be, in turn, overwhelmingly dominant. In particular, the transient diffusion occurring shortly after the application of a substance to the surface of the skin is potentially far greater through the appendages than through the matrix of the stratum corneum. After steady-state diffusion is established, the dominant diffusion mode is probably no longer intraappendageal but occurs through the matrix of the stratum corneum. Flux through shunts is difficult to measure experimentally, except possibly through hair. The recognition of transient diffusion occurring primarily *via* follicles and ducts and steady-state diffusion primarily through the intact stratum corneum results in a considerably more self-consistent and orderly treatment of the process of percutaneous absorption.

The role of intercellular spaces in cutaneous permeability has been pondered (43), but no one has seriously proposed these canals as an avenue for percolation of materials through the epidermis. The total volume of the intercellular space has been estimated to be 0.01–0.1% of the entire volume of the stratum corneum. Substances would have to move rapidly through the small intercellular space if this pathway were to contribute appreciably to the steady-state flux (53, 90). Few studies of the significance of this pathway have been attempted. It has been assumed that the principal route of entry is directly through cells rather than between them.

Once a substance passes through the superficial barrier, there is apparently no significant hindrance to penetration of the remaining epidermal layers and corium, following which there is ready entry into the circulation *via* the capillaries. However, for certain materials there may be a second barrier to absorption at, or near, the dermal–epidermal junction and possibly in relation to the basement membrane (38, 132, 135–137). A salient example of this barrier action, demonstrable with autoradiographs, is the almost complete arrest at the dermal–epidermal junction of certain electrolytes once the superficial barrier is passed (130–132). Thorium-X and ^{32}P-labeled sodium phosphate applied to the skin surface cannot be shown to be present in the dermis on autoradiographs, despite the fact that penetration into the epidermis and follicles occurs (132). There may be a double retention effect, one in the epidermis and a second in the dermis.

Menczel and Maibach (136, 138) recently noted considerable retention of testosterone in the dermis. While the role of dermal retention needs closer examination, the superficial barrier is the critical one for most substances; once it is breached, further passage into the epidermis, corium, and capillaries is virtually assured. The stratum corneum, the epidermis, and the uppermost part of the papillary layer of the dermis (*i.e.*, the portion subjacent to the basal layer of the epidermis and above the capillaries of the microcirculation) are together assumed to comprise the effective composite layer through which substances must diffuse to enter the bloodstream (42).

The factors that influence the penetration of the skin barrier are essentially the same as those that influence GI absorption. These factors can be divided into physiological and physicochemical variables (37, 139, 140). Additional variables are the condition of the skin, the skin age, the area of skin treated, the thickness of the skin barrier phase, the species variation, and the skin moisture content. The rate of diffusion of substances through the skin is dependent primarily on the physicochemical properties of the drug and only secondarily on the vehicle, pH, concentration, *etc.* The initial discussion will deal with physiological skin factors; physicochemical factor considerations will follow.

Skin Condition—"Intactness" of the skin is one of the most important factors preventing penetration. Injurious agents such as mustard gas, acids, and alkalies injure barrier cells and increase permeability. When the stratum corneum is damaged, diffusive water loss is increased. This has been amply demonstrated in experimental injury to the stratum corneum by cellophane tape stripping (97). Complete removal of the barrier by stripping enhances the absorption of almost any substance in contact with the skin surface. In human subjects, 78–90% of hydrocortisone-^{14}C penetrated stripped skin sites (70) while only 1–2% was absorbed from areas of normal skin (60, 141). Loeffler and Thomas (142) found that 50% of ^{89}Sr-labeled strontium chloride was absorbed through abraded rat skin in contrast to 10% absorption in intact skin sites. In diseases characterized by a defective horny layer, percutaneous absorption is also increased (5, 8, 43, 100, 143, 144).

Solvents other than water appear to cause marked alteration in the resistance of the skin barrier to penetration (108, 118, 121, 145, 146). The permeability of the skin to aqueous solutes, organic liquids, and solutes in organic liquids and solids has been reviewed (4). Pretreatment of the skin with organic solvents has variable effects on permeability. Treatment with ether did not alter the penetration rate of salicylates or surfactants (145), while the polar solvents acetone, alcohol, and hexane greatly increased the penetration of water into the skin (109). Excised stratum corneum is virtually "opened" by delipidization of the stratum corneum by holding it in a mixture of a polar and a nonpolar solvent such as chloroform–methanol. This procedure removes much of the lipid fraction of the stratum corneum and makes "holes" or artificial shunts in the membrane (10). Measurements of the activation energies show them to be comparable to diffusion through water-filled channels in delipidized membranes.

Allenby *et al.* (147), using electric impedance as their parameter, studied the permeability of excised human skin to various solvents. Some organic solvents including aliphatic acids, bases, and neutral compounds were shown to produce large changes in electrical impedance and, it is inferred, in water permeability. Dimethyl sulfoxide, dimethylformamide, and dimethylacetamide produced large and rapid changes. The changes in water permeability, which are inferred from the impedance changes and which are induced by the various organic solvents, are probably due to a combination of factors including solution of skin components, relaxation of the binding forces of the skin components, and swelling and hydration of the skin structure with the formation of additional channels for water permeation (147).

The role of sorption promoters such as dimethyl sulfoxide (77, 118, 148) and surfactants (41, 149–152) will be more fully discussed in a later section.

Skin Age—The relationship of age to skin permeability has been rarely investigated. Fetal and infant skin appears more permeable than adult skin (73). Percutaneous absorption of topical steroids occurs more readily in children than adults (153). The significant dermal atrophy and gross epidermal changes in the elderly denote absorption influence (2). The epidermis of the preterm infant has a higher permeability than that of older infants or has a less complete barrier function (154).

Increased Blood Flow—If blood flow through the dermal vessels increases, the rate of clearance of materials should also increase as the concentration decreases. This is particularly true of gas permeation (3). Whether the rate of passage through the barrier layer is altered is not quite so clear, although it seems possible that this may occur because the more rapid removal of material that has penetrated must alter the perfusion gradient across this area. Clinically, erythematous skin is usually diseased, and this condition may alter the rate of absorption for other reasons (2).

If one considers the opposite state of affairs, however, then the evidence is more clearcut. Vasoconstriction produced by the application of topical steroids, a process that it seems hard to believe would damage epidermal cells, undoubtedly slows penetration. The induction of vasoconstriction by the topical application of 6-methylprednisolone slowed the absorption of ^{14}C-testosterone applied after the vasoconstriction had appeared (70).

Regional Skin Sites—There are relatively few studies on the variations in absorption from one site to another and many are conflicting, with variable epidermal cell counts and histochemistry (29–36, 38–41, 131, 155, 156). In different normal individuals, there are wide variations in the absorption rate of a given substance through the same skin site, and penetration rates for the most permeable regions (posterior auricular skin) in some subjects are comparable to rates for the least permeable regions (plantar skin) in others (38). Anatomical differences in penetration rates may depend largely on differing thicknesses of the barrier layer, since equally thick layers of stratum corneum conjunctum from plantar skin and from the anterior forearm, for example, show equal resistance to penetration (38).

Variations in penetration rates have been demonstrated for full thickness cadaver skin, isolated from different sites, with rates increasing in the following anatomical order: plantar, anterior forearm, instep, scalp and ventral thigh, scrotum, and posterior auric-

ular (38). These permeation rates may be in direct proportion to the thickness of the area, because the penetration across skin, the flux, is inversely proportional to the thickness. The thickness of the stratum corneum from pieces of glabrous skin can be calculated from data obtained from desorption experiments (128).

The thickness of thick pieces of cornified epithelium (callus) can be measured directly, and the flux of water through these pieces can be determined (129). Fick's law, treated in a later section, governs passive diffusion and can be expressed as:

$$J_s = \frac{D_m}{\delta} \Delta C_s \qquad \text{(Eq. 1)}$$

where δ is the thickness of the stratum corneum, and D_m is the diffusion constant. The flux is proportional to the concentration gradient, ΔC_s, *i.e.*, the difference in concentration divided by the thickness.

Cronin and Stoughton (85), using the erythema reaction produced by vasodilators such as ethyl nicotinate and histamine, demonstrated that the forehead, presternal area, and back showed a greater response than the limbs and that the arm was more reactive than the leg. They concluded that the presence of more follicles in the forehead indicated increased penetration through sebaceous glands. In an earlier study with topical histamine, Shelley and Melton (44) did not observe any difference in urticaria among the arms, back, chest, thigh, and leg. Feldmann and Maibach (75), in studies of ^{14}C-hydrocortisone, noted that measurable absorption occurred through all regions of human skin except the heel. The absorption seemed greater in areas where follicles were large or more numerous, such as the forehead and scalp, and decreased where the stratum corneum was thicker, such as the foot.

Tregear (157), on the other hand, from studies of rapidly absorbed tributyl phosphate, concluded that hair follicles do not increase penetration. Laug *et al.* (158), working with rabbits, studied the penetration of mercury ointment through the skin and found no significant difference in penetration between the back and the belly. However, Smith *et al.* (155) observed a difference between the times taken for local anesthesia to develop on scrotal and abdominal skin after the topical application of lidocaine. With *in vitro* experiments, they also found other differences in the penetrability of skin between these two areas.

Species Variation—Humans and animals display wide differences in physical characteristics such as the number of appendageal openings per unit area and the thickness of the stratum corneum. These physical and structural differences obviously affect the penetration pathways and the penetration resistance of skin (26). The skin of rabbits, rats, and mice, which have been used frequently in work on percutaneous absorption, lacks sweat glands and abounds in hair and hair follicles in contrast to human skin. In addition, it is virtually impossible to apply small amounts of aqueous solution uniformly to hair-clipped animal skin. Moreover, when animals are used, some additional technical care must be taken to avoid unexpected disturbance of that part of the skin where a sample has been topically applied.

In spite of all these limitations, animals have been used in many studies on the percutaneous absorption of various substances, because biologically dangerous substances, including radioactive compounds, are under many restrictions as to the application to human subjects and the excision of normal human skin for the evaluation of any substance absorbed through the skin is hard to accomplish (44, 159–161). Yet, human skin, either living or excised, is easily obtained and usually more suitable than animal skin if the intent is to find out about the penetration capacity of a substance applied topically. Furthermore, biochemical differences between animal and human skin, even when subtle, may significantly alter skin reactions with penetrant chemicals (26).

Species variations of barrier permeability have been noted only in the broadest sense (3). The skin of common laboratory rodents is more permeable than human skin (4). The relationship between species is not consistent for different substances, but the average permeability order is rabbit > rat > guinea pig > humans. The largest deviation from the average is that human skin is very much less permeable to ions than is rabbit or guinea pig skin. While penetrability through rabbit skin is rapid, the structure of the epidermis or appendages does not appear to differ significantly from that of other animals more resistant to penetration (3, 162, 163). In certain studies, intact guinea pig skin has given results similar to human skin (164).

Notable studies include those of Stolar *et al.* (165), who used rabbits to study the absorption of sodium salicylate from hydrophilic ointment. Desgroseilliers *et al.* (69) used young domestic pigs for topical steroid absorption. Sweeney *et al.* (146) chose hairless mice for an investigation of the effects of dimethyl sulfoxide on water passage because of the absence of eccrine sweat glands and the presence of small-sized hair follicles. Penetration of fluorouracil through human and hairless mouse skin was measured *in vitro*, and the results were compared using radioactive-labeled drugs and a GC method specific for the free fluorouracil molecule (166). Significantly greater drug penetration was obtained in the mouse samples using both methods of detection.

Hydration—Hydration of the stratum corneum is among the most important factors in skin penetration, increasing the rate of passage of all substances that penetrate the skin. Hydration results from water diffusing from underlying epidermal layers or from perspiration accumulating after application of an occlusive vehicle or covering on the surface. Under occlusive conditions, the stratum corneum is changed from a tissue that normally contains very little water (5–15%) to one that may contain as much as 50% water. Permeability increases four- to fivefold.

Hydration apparently "opens" the compact substance of the stratum corneum, as can be demonstrated dramatically in rats by comparing the LD_{50} of percutaneously administered substances under oc-

cluded and unoccluded conditions. Brown (167) observed that the percutaneous LD_{50} of dinitrobutylphenol is 140 mg/kg when applied under occluded conditions and 1000 mg/kg under nonoccluded conditions. However, not all substances are influenced in the same way, and the same investigator found that the percutaneous LD_{50} of parathion was greater under occluded conditions than when the treated area was left uncovered.

Hydration of the epidermis has been shown to increase the percutaneous absorption of nicotinic acid (85) and salicylic acid (5). Wurster and Kramer (105) measured the rate of penetration of esters of salicylic acid through skin with dry and hydrated stratum corneum. When the tissue was hydrated, the rate of penetration of the most water-soluble ester increased more than that of the other esters studied. Working with aspirin in a temperature–humidity chamber, Fritsch and Stoughton (49, 118) showed the dual importance of these factors on the penetration of excised skin. Full hydration of the keratin, accomplished by layering water over ^{14}C-aspirin on the epidermal surface, dramatically increased the penetration when compared to conditions of 88% humidity at the same temperature. There is little doubt that heavy hydration of the horny layer drastically reduces the barrier efficacy.

Further evidence of the importance of hydration can be found in investigations employing occlusive plastic films in steroid therapy. Here the prevention of water loss from the stratum corneum and the subsequent increased water concentration in this skin layer apparently enhance the penetration of the steroid (81, 113, 168–170). McKenzie and Stoughton (81) showed that penetration of corticosteroids may be increased 100-fold by occluding the site of application and thus hydrating the stratum corneum. Vickers (113) demonstrated that occlusion not only enhances penetration of corticosteroids but also creates a depot effect in the stratum corneum.

The mechanisms of skin transport have already been discussed. The mechanism of transport of a drug through hydrated stratum corneum may be quite different from that through normal stratum corneum. Shelmire (171) suggested that the mechanism of hydration was to increase the size of the pores. There is not only a physical alteration of the tissue due to hydration, but at high water activities there are also changes in both the diffusion coefficient and activity coefficients of the penetrating agent (121). The important thing is the thermodynamic activity of water in the barrier phase, not just the amount there.

One would expect the rate of penetration of water-soluble drugs to be faster through hydrated than through normal stratum corneum (31). The low diffusion constant and high activation energy obtained for water and polar alcohols and the selective diffusion exhibited by molecules of varying polar character suggest that extensive hydration does not drastically affect the barrier function of the stratum corneum. The hydrated stratum corneum is one of the most water-impermeable biological membranes found in nature, although it is slightly more efficient before extensive hydration and presumably *in vivo* (35). A two-phase series model for the permeability behavior of the fully hydrated stratum corneum showed reasonable correlation between experimental permeability coefficients and partition coefficients (172).

Temperature—Relatively little attention has been paid to the effects of temperature on percutaneous absorption. Under normal *in vivo* conditions, substances penetrate the skin only within a very narrow temperature range. Clinical variations derive chiefly from occlusion. *In vitro* experiments may be conducted over a much wider range.

Blank and Scheuplein (31) observed little alteration of the permeability of the barrier from exposure for several hours to temperatures as high as 60°. However, Allenby *et al.* (147) showed that the stratum corneum undergoes irreversible structural changes when heated above 65° or when incubated in aqueous media at pH <3 or >9.

The dual effects of both temperature and humidity were shown clearly by Stoughton and Fritsch (118), who demonstrated considerable increases in the percutaneous absorption of aspirin and corticosteroids as the temperature rose and, especially, as humidity increased. *In vivo* work with occlusion (81, 113) and clinical studies (169) have corroborated the roles of temperature and humidity.

Blank and Scheuplein (31) studied the rates of penetration of ethanol and 1-pentanol within the 0–55° range. The flux, or the amount of alcohol penetrating per unit area in unit time, was an exponential function of the temperature. The energies of activation were determined by Arrhenius plots of the log of the permeability constant against the reciprocal of the temperature. The activation energies of the two alcohols differed measurably from one another and were higher than those obtained for diffusion of the same substances in solutions. The magnitude of the activation energies for the penetration of the low molecular weight alcohols through the skin indicated that penetration was a more complex process than diffusion through vehicle-filled channels.

Drug Concentration—The amount of drug percutaneously absorbed per unit surface area per time interval increases as the concentration of the drug in the vehicle is increased. Also, more drug is absorbed per time interval at a constant drug concentration if the drug is applied to a larger surface area. However, with a few compounds, increasing concentrations produce significant decreases in absorption rates. This finding has been recorded for substances that produce caustic effects on the skin in relatively high concentrations, thereby producing an artificial barrier—often with obvious crust formation—which impedes effective penetration. Such effects for substances that freely penetrate the barrier in low concentrations have been described for higher concentrations of phenol (173) and hydrogen sulfide gas (174).

The duration of contact with the skin as well as the concentration of the penetrant play important roles in skin diffusion. Tregear (4) showed that the perme-

ability constant of thioglycollic acid is dependent on concentration and probably on time of contact as well. The positive penetrative effects of increased concentrations of the steroids flucloronide (175), betamethasone (52), cortisone (76), hydrocortisone (76), testosterone (76), and androstenedione (76) have been demonstrated. In addition to the steroids, Maibach and Feldmann (76) showed penetration–concentration dependence for caffeine, salicylic acid, and benzoic acid.

Skog and Wahlberg (176) showed a definite increase in the absorption of various compounds with increasing concentration in guinea pigs. They noted increasing penetration up to a certain point, at which a plateau was reached. This finding may indicate that the barrier layer may not be influenced by diffusion gradients and may merely act as an absolute limiting step, limiting the total amount of any substance passing through in unit time. With true steady-state diffusion, permeability constants are independent of concentration. The neurological difficulties experienced in rats with topical administration of hexachlorophene prompted a series of investigations regarding its percutaneous absorption (177–181). Permeability constants, diffusion constants, and membrane–vehicle partition coefficients were determined (181).

One of the anomalies of the action of dimethyl sulfoxide on skin penetration is its unusual concentration dependence. Low concentrations are virtually without effect; as the concentration is increased, there is a rapid enhancement of percutaneous penetration (182). A direct relationship was obtained between the concentration of dimethyl sulfoxide and the rate of penetration of potassium methyl sulfate (183).

The effect of concentration as a factor controlling the rate of permeation of a drug through a membrane can be examined by reference to Fick's general law of diffusion which, in essence, states that the driving force causing the transfer of a substance from regions of high to regions of low concentration is proportional to the concentration gradient. Fick's law is commonly written as $J_s = K_p \Delta C_s$, where J_s is the flux across the membrane, K_p is the permeability constant, and ΔC_s is the difference in concentration on the two sides of the membrane.

By varying the concentration of the penetrant and observing the consistency of K_p, one can determine the extent to which Fick's law is applicable under the experimental conditions. When Fick's law is indeed applicable, the K_p value provides a satisfactory basis for comparing penetration rates obtained in different laboratories with different concentrations and techniques.

Over a wide concentration range, flux will be proportional to concentration only if the membrane remains unaffected by the concentrated solutions and if the partition coefficient remains constant over the entire concentration range (10, 91, 126). In a study of the penetration of low molecular weight alcohols (43), Fick's law held accurately only for relatively low concentrations. With high concentrations, the flux of some alcohols leveled off and then decreased.

Table I—Rating Chart for Human Skin Penetrants[a]

Penetrant Rating	Permeability Constant, μcm/min	Example
Very slow	<0.1	Aluminum salts
Slow	0.1–1.0	Tripropyl and tributyl phosphates, methyl-salicylate
Moderate	1.0–10.0	Trimethyl and triethyl phosphates, ethanol
Fast	10.0–100.0	*n*-Butanol, laurate
Very fast	>100.0	*n*-Pentanol

[a] From Ref. 26.

Marzulli *et al.* (26) proposed a scheme for rating substances as to their capacity for penetrating human skin according to their K_p value (Table I). Table I is based on permeability constants, most of which were compiled by Tregear (91) and which range from 0.02 to 200. Five categories are provided covering five orders of magnitude, with descriptive terms assigned to each category.

Solubility Characteristics of Penetrant—The solubility characteristics of a substance greatly influence its ability to penetrate biological membranes. The lipid–water solubility pattern of the applied material was recognized at the beginning of this century in the Meyer–Overton theory of absorption. This theory stated that, because the epidermal cell membrane consists of a mosaic pattern of lipid and protein molecules, substances soluble in lipids pass through the cell membrane owing to its lipid content while water-soluble substances pass after the hydration of the protein particles in the cell wall, which leaves the cell permeable to water-soluble substances. The validity of this theory and the importance of ether–water solubility coefficients have been shown on many occasions and in relation to many drug groups (2). In essence, the aqueous solubility of a drug determines the concentration presented to the absorption site, and the partition coefficient strongly influences the rate of transport across the absorption site (184).

Since the Meyer–Overton theory appeared, numerous papers have been published reporting the percutaneous absorption of various lipid-soluble substances. Since Minato *et al.* (160) comprehensively reviewed these studies, only recent notable studies will be included here. Tregear (4) reviewed the permeability of the skin to water, electrolytes, and organic solvents. Treherne (25) related the permeability constants of a series of compounds to their ether–water partition coefficients and suggested that a partition coefficient of unity might favor skin penetration.

A similar relationship between the vasodilator activity and lipid–water partition coefficient was demonstrated for a series of esters of nicotinic acid by Stoughton *et al.* (80). They also found a similar correlation between the benzene–water partition coefficients and the penetration of the epidermis by a series of closely related boronic acid derivatives. Cronin and Stoughton (48) estimated the penetration of nic-

otinic acid and one of its esters, ethyl nicotinate. They were able to show a very dramatic difference (37,000-fold) between the penetration rates of the two materials and postulated that the differing ether–water partition coefficients of the two compounds were at least in part responsible for the difference. They noted that ethyl nicotinate was some 10,000 times more volatile than nicotinic acid; since it is known that gases penetrate the epidermis very readily (185, 186), they postulated that the increased volatility was primarily responsible for the increased penetration.

The magnitude of the effect of moisture on the percutaneous diffusional rates of several salicylate esters has been shown to be proportional to the oil–water distribution coefficient and the water solubility of these closely related compounds (105). In the case of surfactants, the combination of hydrophobic and hydrophilic characteristics affect penetration (145, 183). Absorption profiles for a series of salicylates through intact rat skin showed decreases with increasing pH (187). Absorption apparently ceased if the degree of ionization was higher than 99.9% or if less than 0.1% of the drug was in the unionized form. For salicylic and *p*-aminosalicylic acids, absorption curves strictly followed the pH–partition hypothesis.

In the formulation of preparations for topical application, it is profitable to select or prepare compounds having the required solubility characteristics before attempting to promote their penetration by pharmaceutical manipulation. This is exemplified by the development of topical corticosteroid preparations. Triamcinolone possesses five times the systemic activity of hydrocortisone but only one-tenth of its topical activity. Conversion of triamcinolone to its acetonide enhances its topical activity 1000-fold (188). Similarly, betamethasone has 30 times the systemic activity of hydrocortisone but only 10 times its topical activity.

Of 23 esters of betamethasone examined (114), betamethasone 17-valerate possessed the highest topical activity and this coincided with the most balanced lipid–water solubility coefficient. The activity became less as the derivatives became more lipid and less water soluble. Compounds that were more soluble in water and less soluble in lipids were similarly less active on topical application. Katz and Shaikh (184), utilizing the vasoconstriction end-point for topical corticosteroids, secured results which seem to indicate that the efficiency of percutaneous absorption may be a function of the product of the partition coefficient and the square root of the aqueous solubility. The results are in agreement with developed theoretical considerations (121). This finding suggests that increases in topical corticosteroid anti-inflammatory activity, produced by molecular modifications, are in great measure proportional to changes in solubility and the partition coefficient. This effect of steroid solubility is a major factor in penetration from varied vehicles (51, 53, 54, 184, 189). Several cream and ointment vehicles were developed for flucloronide. Enhanced drug release and superior vasoconstrictor activity were demonstrated from vehicles where the drug was entirely solubilized (190).

Allenby *et al.* (147), in a study of the effect of various solvents on the electrical impedance of skin, found that dimethyl sulfoxide, dimethylformamide, and dimethylacetamide produced large and rapid changes. All of these compounds have aprotic molecules and form strong hydrogen bonds. They also have high lipid and water solubilities. Diethylformamide and tetramethylurea were without observable effect despite their strong solvent powers. Dimethyl sulfoxide–water mixtures containing less than 50% dimethyl sulfoxide were inactive, but more concentrated dimethyl sulfoxide mixtures produced impedance changes which increased with concentration. Accelerants will be treated further in the section on vehicles.

If a substance is much more soluble in the stratum corneum than in the vehicle in which it is dissolved, when presented to the stratum corneum the concentration in the first layers of the stratum corneum at equilibrium may be much higher than the concentration in the presenting solvent. The concentration in the lower layers of the stratum corneum remains near zero, since these layers are in contact with a fluid that is being continuously replaced or through which diffusion is relatively rapid. The flux, therefore, is more accurately related to the difference in concentration in the top and bottom layers of the stratum corneum than to the difference in concentration in the solutions on the two sides of the membrane (126). The concentration in the top layer of the stratum corneum is determined by the relative solubility of the penetrant in the stratum corneum and the vehicle, *i.e.*, the partition coefficient (K_m). Fick's law is now expanded to:

$$J_s = \frac{K_m D_m}{\delta} \Delta C_s \qquad \text{(Eq. 2)}$$

where δ is the thickness of the stratum corneum. (This term was treated previously.) It is now seen that:

$$k_p = \frac{K_m D_m}{\delta} \qquad \text{(Eq. 3)}$$

Thus, after k_p and K_m are determined experimentally and δ is known, D_m can be calculated. The nonhomogeneous nature of the stratum corneum and the complications and uncertainties attendant upon an accurate determination of K_m and δ would appear to pose serious limitations in developing a satisfactory diffusion constant (D) for human skin (126). Yet, the equations describe reasonably well the permeability of the skin to nonelectrolytes.

Blank and Scheuplein (31), in studies of homologous alcohols from methanol to octanol, noted that water solubility decreased and lipid solubility increased with increasing molecular weight and the rate of penetration increased as the molecular weight increased. The permeability constants were directly proportional to the partition coefficients. Thus, within a given molecular series [of constant or nearly constant diffusivity (D)], the observed steady-state flux

(J_s) and permeability constant (k_p) are directly proportional to the relative solubility of the substance in the skin, as expressed by the membrane–solvent partition coefficient (K_m) (124).

Solubility is certainly a very important factor in determining the rate at which a penetrant passes through a membrane. However, before solubility factors can be truly related to flux, the specific stratum corneum–vehicle partition coefficient rather than the ether–water partition coefficient must be known (31, 121). The positive correlations of the straight chain alcohols and of other compounds (25, 48, 80) with ether–water partition coefficients may be fortuitous. Lien and Tong (191), using computerized multiple-regression analysis, showed that, for many series of drugs, percutaneous absorption through intact skin is highly dependent upon the lipophilic character as measured by log partition coefficients. Electronic and steric terms appear to play minor roles.

The partition coefficient between the membrane itself and the solvent may be determined by allowing a weighed sample of stratum corneum to come to equilibrium with a small volume of a solution of a solute of known concentration. The loss in concentration is determined, and the concentration in the stratum corneum can be calculated from this loss (126).

Enough data are now available to indicate that not only the solubility of the penetrant in the stratum corneum but also the ease with which it diffuses through the stratum corneum influences the flux. As polar groups are added to the diffusing molecule, forces of attraction between these groups and polar sites within the stratum corneum increase and the diffusivity of the molecule therefore decreases (124, 126). The polarity is affected also by the pH. The best absorption takes place when the concentration of nonpolar molecules is greatest, thereby positively affecting the diffusion gradient of drug by mass action in a direct ratio.

When the penetrant passes through the stratum corneum, it encounters the much less compact tissues of the stratum germinativum and dermis. No satisfactory way has been found for isolating the stratum germinativum as an intact membrane for measurement of its permeability. The relatively few comparisons that have been made between the permeability of the stratum corneum and the entire epidermis indicate that the stratum germinativum offers very limited resistance to movement of molecules. The stratum corneum offered three orders of magnitude greater resistance to diffusion than did the dermis in the study of penetration of alcohols (10, 35).

Molecular Characteristics of Penetrant—While there is little doubt that factors such as hydration and solubility are uppermost, molecular features such as size and shape must play a part in penetration. Surprisingly little is known of the importance of the molecular characteristics of the applied agent. There are few published reports in which permeability coefficients are correlated with the size of penetrating molecules. Virtually nothing is known of the effect of molecular shape.

An inverse relationship appears to exist between absorption rate and molecular weight (4, 31, 62, 80, 91, 192). Small molecules penetrate more rapidly than large molecules, but within a narrow range of molecular size, there is little correlation between size and penetration rate. Diffusion constants through the hydrated stratum corneum for many low molecular weight compounds are approximately the same (124). The specific effect on the penetration rate of the size and shape of the penetrating molecules can be determined only if the effect of size and shape can be separated from the effect of solubility characteristics. The effect of polarity was discussed previously.

Molecular characteristics, yet unresolved, are involved in penetration. Feldmann and Maibach (73) determined the human urinary excretion of a series of topically applied molecules of relatively similar molecular weight (~200). There was a large difference in penetration of the compounds tested. The range for total absorption was greater than 250 times, while differences in the maximum absorption rate were greater than 1000-fold. Closely related compounds showed great differences in penetration. Benzoic acid was absorbed 200 times more than its glycine conjugate, hippuric acid. Nicotinic acid showed minimal penetration while 10% of its amide, nicotinamide, was absorbed. Compounds such as hippuric acid, nicotinic acid, and nitrobenzene support the generally held view of the effective barrier properties of the skin; others such as dinitrochlorobenzene, caffeine, and benzoic acid penetrate so extensively as to suggest that the human skin has little barrier properties to them.

Higher molecular weight materials also show variable penetration. Tregear (192) measured the rabbit and human skin penetration of large molecules such as serum albumin, polyvinylpyrrolidone, and two dextrans having molecular weights of 9400 and 153,000. The albumin penetrated the skin at a slower rate than it penetrated the excised skin. The labeled polyvinylpyrrolidone penetrated the slowest; the lower molecular weight dextran penetrated the fastest. Very large molecules such as proteins and polysaccharides went through very poorly, if at all. The use of dimethyl sulfoxide as a penetration carrier indicated that substances having molecular weights of 3000 or more cannot be transported into the skin (193).

Vehicles—The influence of formulation and selection of particular vehicles has been neglected from the point of view of their penetration of the skin. Few studies or techniques apparently can be used routinely to observe the role that a vehicle, or a particular component in a vehicle, will play on the overall percutaneous absorption of a drug. The lack of these studies can be partly related to the difficulties of routinely setting up percutaneous absorption studies and also to the emphasis given to other factors during the development of a topical vehicle or pharmaceutical product. The emphasis has generally been placed on the compatibility, stability, and appearance of the product rather than on the influence that the components in the vehicle may have on enhancing or hindering the movement of the drug through the skin

(123). It is only quite recently that experimental and clinical evidence has been produced to suggest that the vehicle can materially affect skin penetration (50–54, 84, 175, 184, 189, 194, 195). Particularly notable is the critical review of Poulsen (195) who analyzed the factors involved in optimizing drug availability from topical dosage forms.

The literature on the influence of vehicles on skin penetration is confusing and sometimes contradictory because: *(a)* a variety of experimental animals have been used, *(b)* many different methods of estimating skin penetration have been used, *(c)* there is a lack of awareness of possible drug–vehicle interactions and of the functions of different vehicles (10, 11, 196), and *(d)* there is a lack of consideration of the thermodynamics involved in the interpretation of results (10, 12, 121, 197).

Rothman (1) reviewed the literature on vehicles up to 1954, and Barr (7) reviewed this literature to 1962. More recent reviews have been prepared (2, 5, 8, 11, 50, 116, 196, 198, 199). The bulk of recent data on vehicular effects in percutaneous absorption has been derived from studies of corticosteroids (11, 17, 51, 63, 84, 116, 118, 119, 175, 194, 198, 200–205). Katz and Poulsen (17) discussed the interactions among the drug, the vehicle, and the skin. Their goal was the design of a dosage form that not only had good physical stability and patient acceptability but also that provided the optimum environment for the release of the corticoid from the vehicle and its penetration through the skin barrier.

The primary requirement for topical therapy is that a drug incorporated in a vehicle reach the skin surface at an adequate rate and in sufficient amounts. At one time, the primary factor influencing penetration through the skin was believed to be the vehicle itself. The bulk of evidence now indicates that unless an applied material is capable of passage through either the skin barrier or follicles, the vehicle is only of subsidiary importance. Hence, there are two general approaches to the problem of the development of vehicles that may increase penetration. One is to include agents in the vehicle that affect the barrier function of the epidermis so as to promote penetration of the therapeutic compound (111, 118, 119, 194). The other is to alter the physical characteristics of the vehicle and thus affect the diffusion of the drug from the vehicle into the skin (51, 54).

For the latter, it is advantageous to choose vehicles that do not bind the incorporated drug too strongly, because the drug has to separate from the base before it enters the cells. *In vivo* (52) and *in vitro* (31) studies have shown that the release of a substance will be favored by the selection of vehicles having a low affinity for the penetrant or in which the drug is least soluble. This finding is consistent with the view that the rate of release is governed by the vehicle to receptor phase (stratum corneum) partition coefficient.

For the first approach, penetration may be enhanced by a vehicle in which the drug is soluble if the solvent lowers the resistance of the skin to penetration either by producing changes in the transepidermal barrier or by modification of the follicular and sebaceous route, such as with dimethyl sulfoxide. Other drug–vehicle factors that are likely to be important are the particle size of poorly suspended drugs, the viscosity of the vehicle, and the drug concentration in the vehicle. A reduction in the particle size of fluocinolone acetonide has been shown to enhance its penetration (200, 201). Where a drug exists in more than one crystalline form, the one with the highest thermodynamic activity would be expected to penetrate most rapidly, provided it is stable (12, 121).

While vehicles appear to play a secondary role in transepidermal absorption, they are of considerable significance in enhancing absorption by the transappendageal route. Notable in this context is a vehicle consisting of water, propylene glycol, and wetting agents (44, 46). Increases in strictly perifollicular physiological responses to pilocarpine, histamine, and adrenalin were noted, with no significant changes in areas without follicles (44). While it is probable that wetting agents are the prime reason for the increase in transappendageal absorption, the physical nature of the base is also important.

The main factors in the physicochemical relationship of the penetrant to the vehicle appear to be the solubility of the penetrant in the vehicles or a constituent of the vehicle, the rate of diffusion of the penetrant within the vehicle, the rate of release of the penetrant from the vehicle, and the possible release of the penetrant in solubilized form together with a constituent of the vehicle (51, 195, 206). The one variable that can be altered is the thermodynamic activity of the drug in the vehicle. This thermodynamic activity is the product of the concentration of the drug and its activity coefficient in the vehicle. In a study of the effect of thermodynamic activity on the percutaneous absorption of methyl nicotinate from a water–glycerol mixture, the time of onset of erythema was the same for solutions of equal thermodynamic activity (207).

For most substances, the rate of penetration is limited by the impermeability of the skin. In such cases, the highest thermodynamic potential in the applied phase is necessary to obtain the maximum rate of penetration. Many investigators have indicated the importance of the drug concentration in a topical preparation, but few have indicated that the activity coefficient may be changed in many ways. Some investigators increased the concentration of the drug in the vehicle but actually lowered thermodynamic activity because they apparently were not aware of the effect of pH and other factors.

Higuchi (121) pointed out that the driving force behind the drug movement is the difference in the thermodynamic potential between the vehicle and the deeper tissues, and the direction of flow for systems is always from the higher thermodynamic potential to the lower thermodynamic potential. He derived the following equation describing the variables affecting the rate of release of solid drugs suspended in a vehicle:

$$\frac{dq}{dt} = \frac{1}{2}\sqrt{\frac{D(2A - C_s)C_s}{t}} \qquad \text{(Eq. 4)}$$

where:

dq/dt = rate of drug released per unit area of surface exposure
A = total concentration of the drug in the vehicle (suspended and dissolved drug)
C_s = solubility of the drug in the external phase of the vehicle
D = diffusion constant of the drug molecule in the external phase of the vehicle
t = time at which the amount of drug released is determined

When much more solid drug is present than is required to saturate the external phase of the vehicle ($A \gg C_s$), Eq. 4 can be simplified to:

$$\frac{dq}{dt} = \sqrt{\frac{ADC_s}{2t}} \qquad \text{(Eq. 5)}$$

Since the skin probably does not function as a perfect sink, absorption measurements would not be expected to agree initially with the rate of release and, consequently, the rate of absorption predicted by Eq. 5. However, once the rate of drug penetration across the major barrier reaches a steady state, absorption by the lower tissues should approximate the value predicted. Whether absorption by the epidermal and dermal phases would be proportional and whether absorption by either phase would agree with the absorption predicted by Eq. 5 would depend again on the relative affinity of the penetrating drug for each phase. There is evidence that absorption by the two phases of a drug suspended in systems such as described by Eq. 5 is not proportional (12, 121, 197, 208).

The permeability constant quantifies C_s and D as the product of the effective partition coefficient (PC) of the drug between the skin barrier and the vehicle and its diffusivity (D_b) in the skin or:

$$\text{permeability constant} = (PC)(D_b) \qquad \text{(Eq. 6)}$$

Using this equation, the rate of percutaneous absorption of drugs, applied as fine suspensions, can be described as a function of the product of the amount of drug released from the vehicle to the absorption site and its permeability constant for transport through the barrier phase. Combining the previous equations yields:

$$\frac{dQ}{dt} = \sqrt{\frac{ADC_s}{2t}}\,(PC)(D_b) \qquad \text{(Eq. 7)}$$

The release rate of a drug from the vehicle may be regulated by controlling A, D, and C_s. If an aqueous solution is the external phase of the vehicle, C_s can be varied by changing the effective pH of the vehicle for poorly soluble, weakly acidic and basic drugs. The activity coefficient of the molecular form of such drugs (the molecular or unionized form being assumed to be the species absorbed) is a rapidly changing function of pH for pH values greater than the pKa for acidic drugs and less than pKw − pKb for weakly basic drugs. The activity of the molecular species of a weakly acidic drug will be higher at low pH values and the activity of molecular species of a weakly basic drug will be higher at higher pH values (121). Thus, a weakly acidic compound, buffered to a weakly acid pH, will have a higher activity than if it were buffered at an alkaline pH and, consequently, its release will be dramatically improved. The converse is true of weakly basic compounds (11).

Table II—Permeability Constants (cm $hr^{-1} \times 10^3$) for Ethanol and Heptanol when Penetrating from Different Vehicles[a]

Alcohols	Vehicles		
	Water	Isopropyl Myristate	Mineral Oil
Ethanol	0.6	9.5	20.0
Heptanol	35.0	0.2	1.0

[a] From Ref. 35.

Equivalent relationships can be used to approximate the penetration of the barrier phase of the skin by a drug dissolved in a topical vehicle:

$$\frac{dQ}{dt} = (PC)C_v\frac{DA}{L} \qquad \text{(Eq. 8)}$$

$$\frac{dQ}{dt} = \frac{a_v DA}{\gamma_s L} \qquad \text{(Eq. 9)}$$

where:

dQ/dt = steady rate of penetration
(PC) = effective partition coefficient of the drug between the skin barrier and the vehicle
C_v = concentration of the drug in the vehicle
D = effective average diffusivity of the drug in the skin barrier
A = cross-sectional area of the application site
L = effective thickness of the skin barrier
a_v = thermodynamic activity of the drug in the vehicle
γs = effective activity coefficient of the drug in the skin barrier

If Eqs. 4 and 5 are considered with the objective of altering the penetration of a topically applied drug, it becomes apparent that only certain terms are readily susceptible to manipulation by the formulator. These terms are the partition coefficient (PC) and drug concentration (C_v) terms of Eq. 8 and, equivalently, the activity term (a_v) of Eq. 9 (51). Relationships between partition coefficients of various compounds and skin penetration have been reported (25, 80, 184, 209, 210). The partition coefficient can be defined as:

$$PC = \frac{\gamma_v}{\gamma_s} \qquad \text{(Eq. 10)}$$

where γ_v and γ_s are the activity coefficients of the drug in the vehicle and skin, respectively. While it may be necessary to regard the activity coefficient of the drug in the skin barrier phase as an unknown and unalterable value, γ_v can be modified simply by altering the composition of the vehicle. The mathematical analysis of permeability data has been reviewed (16, 206).

For a given concentration of drug in certain vehi-

cles, the activity coefficient of the drug and, consequently, the thermodynamic activity of the drug in the vehicle at that concentration may vary by a factor as much as a 1000-fold (121) from one vehicle to the next. Solutes held firmly by the vehicle, such as when the drug forms a soluble complex with the vehicle, exhibit low activity coefficients; hence, the release rate from such drug–vehicle combinations is slow. Solutes held "loosely" by the vehicle (less affinity of the vehicle for the drug or solute) exhibit high activity coefficients; therefore, the rate of release from such drug–vehicle combinations is faster (121).

Blank and Scheuplein (31) and Scheuplein (35) demonstrated the variability of permeability constants when alcohols, such as polar ethanol and nonpolar heptanol, are dissolved in solvents other than water (Table II). Ethanol penetrates better from the oils than from water, but the reverse is true for heptanol. Moreover, the penetration for both alcohols from the two oily vehicles varies. It is reasonable to assume for each of these alcohols that once the alcohol is transferred to the skin from any vehicle, the diffusivity through the stratum corneum (D_m) does not vary. Therefore, the change in flux for any alcohol from different vehicles must result from changes in the partition coefficient as the vehicle is varied. The polar alcohol tends to stay in the polar vehicle and not be transferred to the skin, but it is transferred from oily vehicles; the reverse is expected for the nonpolar alcohol.

Wagner (12) pointed out that thermodynamic considerations can help explain varied results with salicylic acid (165, 211–215) and sulfanilamide (211, 215) used as tracers in dermatological research. The activity coefficients of these tracers and their thermodynamic activities at a given concentration in the various vehicles would be expected to vary widely just on the basis of the nature of the vehicles and tracers employed in these studies. Polyethylene glycols complex salicylic acid; therefore, one would expect the thermodynamic activity of salicylic acid at a given concentration in polyethylene glycol ointment to be very low. Barrett *et al.* (196) noted that polyethylene glycol slowed down the penetration of methyl nicotinate and attributed this finding variously to an adverse diffusion coefficient in the vehicle, to an inability to hydrate the stratum corneum, or to an osmotic effect which would tend to dehydrate the horny layer. Commercially available formulations of corticoids were compared by the vasoconstriction test (216). Ointment vehicles were generally superior to creams or lotions.

The literature with regard to the advantage of one type of base over another is conflicting, yet the efficiency of various vehicles in aiding penetration can be reasonably predicted on the basis of their effect on *(a)* hydration of the stratum corneum or *(b)* the activity of water in the stratum corneum which influences the stratum corneum–vehicle partition coefficient.

Greases and oils are the most occlusive vehicles and induce the greatest hydration through sweat accumulation at the skin–vehicle interface (171). This process is accentuated by covering the surface with occlusive bandages or plastic. Emulsions of the water-in-oil type are somewhat less occlusive than greases. Substances in the vehicle that have a high affinity for water, such as humectants, tend to dehydrate the stratum corneum and decrease penetration. Similarly, powders increase surface area, increase the rate of evaporation of water, and decrease the extent of hydration (171).

The evidence of the role of a solvent, such as propylene glycol, in skin penetration is conflicting (46, 50, 51, 74, 175, 194, 201–205, 217). In combination with surfactants, propylene glycol was used to promote the penetration of water-soluble substances *via* the transfollicular route (46). It has been used as the preferred vehicle for the topical application of fluorouracil in the treatment of certain types of skin cancer. A concentration of 1% fluorouracil has been found to be as effective clinically as a minimum concentration of 5% in an ointment (217). Enhanced effects were noted in absorption of fluocinolone acetonide (201, 202, 205), betamethasone valerate (50), fluorometholone (204), flucloronide (175), and beclomethasone dipropionate (204). However, Feldmann and Maibach (74) failed to confirm these findings in a study of the percutaneous penetration of hydrocortisone.

Ostrenga *et al.* (206) showed that, at moderate concentrations, propylene glycol has no apparent effect on the permeability of the skin; but above 50%, the results suggest that the permeability of the barrier is decreased. The varying effect of propylene glycol on the penetration of different drugs is probably due to their different solubilities. Penetration is probably greatest when the drug is present at its maximal solubility concentration. Below this level, the affinity for the drug may be greater than that of the skin and penetration is prevented (199). Dimethyl sulfoxide and dimethylformamide increased the penetration of hydrocortisone three to four times, whereas dimethylacetamide and propylene glycol decreased it. These workers used undiluted solvents in their investigations, which may account for the apparent anomaly between their results and those of others (119, 194, 201, 202).

In a comparison of solvents, Brode (205) showed that, using ethanol as a standard, absorption of fluocinolone through rat skin was 9 for dimethyl sulfoxide, 5 for propylene glycol, and 2 for tetrahydrofurfuryl alcohol. Absorption from a polyethylene glycol base was less than 20% of that from ethanol solution; from both aqueous and oily creams, the absorption was 80%; and from white soft paraffin, it was 60%. These results are in agreement with those obtained in human subjects (194, 201).

Dempski *et al.* (189) noted that dexamethasone was released more rapidly from gelled isopropyl myristate than from petrolatum and two other nonaqueous vehicles. Their studies demonstrated that the *in vitro* release of medicinal agents from topical bases is a function of the solubility of that agent in both the base and its surrounding medium. The medicinal must be sufficiently soluble in a nonaqueous base to

allow for its release into an aqueous medium but not so soluble to remain in that base preferentially. If the drug is insoluble in its vehicle, only the drug particles available at the surface of the vehicle apparently dissolve into an aqueous medium. If the drug is partly soluble in the vehicle, it seems to dissolve and diffuse throughout the medium as it dissolves from the surface and then returns to the surface for release into the surrounding medium.

The vasoconstrictor activity of betamethasone 17-benzoate was studied in several ointment bases (218). Preparations containing the steroid in petrolatum–propylene glycol or in petrolatum–isopropyl myristate both gave rise to significantly higher total vasoconstriction than polyethylene glycol ointment BPC, the latter with varied amounts of propylene glycol, Plastibase, and a petrolatum–beeswax base (218).

The role of emulsions, particularly with regard to oil-in-water and water-in-oil-bases, is unresolved (see Refs. 1 and 7 for reviews through 1962). Salicylic acid has been studied frequently in various vehicles (165, 219–224), but the role of the vehicle is not yet understood. Barrett *et al.* (196) showed that methyl nicotinate penetrated the skin equally readily from an aqueous or oily cream. Using the steroid fluocinolone acetonide, Munro (116) demonstrated that a petrolatum ointment gave the best penetration, followed by a lotion and cream base and then by propylene glycol. Pure propylene glycol apparently inhibits penetration. This report confirmed the findings of Poulsen *et al.* (51) who indicated that maximum release of fluocinolone acetonide was obtained from vehicles containing the minimum amount of propylene glycol required to dissolve the steroid. Increasing the concentration of an effective solvent may, in fact, result in reduction of the amount released.

With betamethasone valerate, no marked difference in penetration was observed from an oil-in-water cream, water-in-oil cream, petrolatum ointment, or polyethylene glycol ointment (194). Glycols, such as propylene glycol and 1,3-butanediol, enhanced the action of ointments containing the same steroid (50). The potentiating action of glycols may be due to the droplets acting as an efficient reservoir of dissolved corticosteroid readily available to the oily phase. A mechanism whereby propylene glycol penetrates the skin and carries corticosteroid with it might also be involved. The results obtained with the steroids must be regarded with caution, since virtually all techniques measure the blanching of the skin produced by vasoconstriction. Assessment of the pallor can be influenced by the refraction and reflection of light by the vehicles.

The penetration of topical steroids through human abdominal skin was studied (225). Release, penetration, and *in vivo* data were compared as a function of vehicle composition. The similarity between the *in vivo* and *in vitro* composition profiles suggested that clinical efficacy can be predicted from *in vitro* data and from the physical properties of the steroids. The correlations indicated that the *in vivo* results were directly dependent upon penetrability.

The effect of vehicle composition on steroid release from ointments was studied (51, 88, 206, 226). Inferior release of fluocinonide was obtained in conventional ointments in which the drug was present as micronized particles. Greatly improved bioavailability was obtained from an ointment delivery system in which the steroid was completely dissolved. The ointment system consisted of a propylene glycol solution of the drug dispersed in petrolatum, which delivered the corticosteroid to the skin in a molecular form but retained the classical function of the ointment as an occlusive, protective covering. Similar improvements were noted with the use of an anhydrous fatty alcohol–propylene glycol cream base over a traditional two-phase emulsified cream (227). Time–response curves were constructed (88). These fatty alcohol–propylene glycol vehicles have the physical properties of gels but resemble creams in appearance and consistency.

All cited steroid studies dealt with the use of a skin membrane either *in vitro* or *in vivo*. Recently, solvents such as alcohol–water have been utilized as a "model" to simulate the skin phase (50–54, 207, 225). The vehicle has negligible solubility in the phase representing the skin, but the steroid is fairly soluble in it. A receptor phase or "sink" is used to receive the penetrant. Chloroform (50, 52) and isopropyl myristate (50, 51) have served as sinks. Since they are immiscible with the alcohol–water solvent, it is not necessary to introduce an artificial membrane to separate it from the vehicles.

Poulsen *et al.* (51) measured the release of fluocinolone acetonide and its acetate ester into an isopropyl myristate phase. The vehicles used were mixtures of propylene glycol and water gelled with carbomer[2] and diisopropanolamine. The studies showed that optimal release was obtained from vehicles containing the minimum concentration of propylene glycol required for complete solubilization of the corticosteroid. The poorest release rates were obtained with very high concentrations of propylene glycol. The results indicated that the important factors influencing the release of steroid into the receptor phase were the solubility in the vehicle and the partition coefficient of the steroid between the vehicle and the receptor phase. The findings appear to indicate that each compound requires individual formulation based on its solubility characteristics and that the formulation may also need modification for different concentrations of the agent to obtain maximal release rates (53, 206, 225). Similar studies using isopropyl myristate found striking correlations between *in vitro* release rates and *in vivo* results (206, 225).

Busse *et al.* (50) used both chloroform and isopropyl myristate as separate receptor phases in studies of betamethasone valerate in ointment bases. The rate of release of the steroid into the chloroform phase from a paraffin ointment was about 4.5 times that from a similar ointment containing 10% hydrogenated lanolin. Conversely, in the isopropyl myristate system, the reverse occurred. The *in vitro* results

[2] Carbopol 934.

suggest that the isopropyl myristate system more adequately represents the skin.

The data of the model systems can be considered in terms of the rate of solution of corticosteroid particles in the base and the rate of transfer of corticosteroid from the base (50). The latter rate is related to the partition coefficient between the base and the skin or phase representing the skin. When the partition coefficient is large due to the low solubility of the corticosteroid in the base, there is a greater tendency for transfer of corticosteroid to the skin. A second factor is related to the concentration of steroid dissolved in the base (50). Blank and Scheuplein (31) considered that differences in penetration from vehicles can be explained by differences in the stratum corneum–vehicle partition coefficient without assuming that one vehicle penetrates more readily than another. From work using receptor phases, it should be possible to predict *in vivo* effects with suitable models (50, 53, 206, 225).

Vehicles may also affect penetration by their ability to reduce water vapor loss on the skin surface. Experimentally, paraffin bases have been shown to suppress transepidermal water diffusion, whereas a number of other standard vehicles cause a lesser degree of transepidermal water loss suppression (228). Surprisingly, anhydrous wool fat failed to suppress water loss in this study. It was also postulated that the increased penetration of corticosteroids from vehicles containing solvents, such as tetrahydrofurfuryl alcohol and propylene glycol, was due to the influence of the latter on the barrier function of the horny layer. The evidence for this assumption is based on the increased water loss through the horny layer brought about by vehicles containing these solvents. Therefore, it appears that two contradictory mechanisms may be involved in vehicle effects. The occlusive nature of the vehicles is credited with retention of water and subsequent improved penetration, but an increase of water loss may be associated with reduced barrier function and, consequently, easier passage of the corticosteroid (198).

Lipophilic solvents have been reported to facilitate penetration. The absorption of water-soluble and lipid-soluble substances from terpenes and terpene derivatives was better from eucalyptol than from alcoholic solutions (229). A vehicle containing *p*-cymene was superior to others tested in promoting the penetration of hormones but had some irritating effects (230). Small differences were found in the penetration of physostigmine from alcoholic and ester solutions at a rate dependent on the aliphatic chain length (231).

SORPTION PROMOTERS

In the past 10 years, attention has focused on methods of increasing the rate of absorption of topically applied drugs. So far, the one method that has come into everyday use is the application of topical corticosteroids under thin plastic film (168, 169, 232, 233). This method now has widespread application in the treatment of recalcitrant psoriasis. Another method is to add materials that can combine with, or dissolve in, the structures of substances making up the barrier. These agents have come to be known as accelerants. To increase permeability, the accelerant causes the keratin to swell and leaches out essential structural material from the stratum corneum, thus reducing the diffusional resistance and increasing the permeability (36, 101, 182, 234, 235).

Varied agents have been reported as sorption promoters, such as the hydrophilic solvent propylene glycol or surfactants. However, the most effective accelerants are aprotic materials such as urea (236), dimethyl sulfoxide, dimethylformamide, and dimethylacetamide. Work with dimethyl sulfoxide and other accelerants is contributing to the understanding of the chemical nature of the skin barrier in relation to a specified penetrant and the transport mechanisms of various compounds across skin.

Dimethyl Sulfoxide—On application to the skin, the dipolar aprotic solvent dimethyl sulfoxide passes rapidly through the stratum corneum (20, 147, 237–239) and can aid in the penetration of numerous substances. This process does not change the structure of the skin. The role of dimethyl sulfoxide in enhancing percutaneous migration is well documented (6, 20, 77, 101, 118, 148, 240–242). A series of patents was issued for the topical use of dimethyl sulfoxide to aid tissue penetration (243).

In vivo, dimethyl sulfoxide has been found to accelerate the penetration through the skin of water (146, 241), dyestuffs (237), hexopyrronium bromide, naphazoline, fluocinolone acetonide (118), salicylic acid (244), tubocurarine hydrochloride, amphetamine sulfate, barbiturates (245), and other substances (93). *In vitro,* dimethyl sulfoxide accelerates the penetration of skin by hexopyrronium chloride and hydrocortisone (118), picric acid (182, 234, 235), fluocinolone acetonide, triamcinolone acetonide, hydrocortisone (20), griseofulvin and hydrocortisone (119), testosterone propionate (70, 77), hydrocortisone (77, 111, 113), triamcinolone acetonide (113), fluocinolone acetonide (111, 246), cortisone acetate and estradiol (247), fluorouracil (248), iodine (249), hexachlorophene (250), physostigmine and phenylbutazone (251), local anesthetics (252), antibiotics (244, 253), and quaternary ammonium compounds (254).

Substances having molecular weights of 3000 or more cannot be transported into the stratum corneum (255). The influence of pH on the percutaneous absorption of salicylic acid, with and without added dimethyl sulfoxide, from a hydrophilic ointment was studied in the rabbit (256). With those ointments containing dimethyl sulfoxide, salicylate levels were higher at each pH and time interval. Enhanced solubility of salicylic acid in the presence of dimethyl sulfoxide would appear to explain the results.

Dimethyl sulfoxide exerts variable influence even over structurally related compounds. The rate of migration of potassium methyl sulfate increases with an increasing concentration of dimethyl sulfoxide (183). Yet applications of the butyl and lauryl esters produces low penetration, independent of dimethyl sulfoxide concentration. In these latter cases, dimethyl

sulfoxide functions as a penetrant rather than as a vehicle (183, 257).

Dimethyl sulfoxide exhibits an unusual concentration dependence (146, 147, 182, 183, 257). The penetration rate of pure dimethyl sulfoxide depends on the concentration applied. At least 60% dimethyl sulfoxide is required for a measurable penetration rate. The rate rises to a maximum at about 3 hr and then declines; this is true for both the pure liquid and for dimethyl sulfoxide–water mixtures containing >70% dimethyl sulfoxide. The continued increase in the penetration rate of pure dimethyl sulfoxide with an increase in the applied quantity is probably a consequence of progressive barrier impairment. The decline in the peak rate after 3 hr is probably due to the back-diffusion of water with consequent dilution of the dimethyl sulfoxide (147).

Dimethyl sulfoxide can also establish a reservoir in the stratum corneum of compounds having a low water solubility and to which the epidermis is normally only slightly permeable (119). The related compounds dimethylacetamide and dimethylformamide also enhance cutaneous penetration (119) and retain agents in the stratum corneum reservoir (111, 258) but to a lesser degree than dimethyl sulfoxide (119).

Little information is available concerning the mechanism of action by which dimethyl sulfoxide, dimethylformamide, and dimethylacetamide enhance the rate of migration. They are all strongly hygroscopic, and it is likely that the presence of these substances in the stratum corneum greatly increases the hydration of the tissue and, therefore, its permeability. Two factors, the underlying dermal inflammation and the heat produced by the interaction of the solvent and water within the stratum corneum, would also lead to a small rise in the temperature and, therefore, the permeability of the horny layer (259). Neither of these factors could conceivably explain the magnitude of the increased diffusion. There is ample evidence of the ability of water (91, 146), and many other substances to move in an inward direction through the skin under the influence of dimethyl sulfoxide (51, 242). The accelerant effect observed *in vivo* is not due to increased skin circulation because this can be increased without increasing the penetration rate (91), because accelerants do not increase the skin clearance rate (101), and because the effect can be observed *in vitro* with isolated nonperfused skin preparations.

On a structural level, the possible reversible configurational changes in protein structure brought about by substitution of integral water molecules by dimethyl sulfoxide is worthy of special consideration (260). Also, dimethyl sulfoxide may function as a swelling agent (235). A combination of the latter two factors may explain in part the variable role of dimethyl sulfoxide since the swelling phenomenon may induce the formation of channels within the matrix of the stratum corneum, which favor the passage of various compounds (183), or lower the diffusional resistance of the stratum corneum. Dimethyl sulfoxide is able to extract soluble components from the stratum corneum, suggesting the possibility of ultrastructural modifications consistent with an increase in permeability (261). For a liquid to be a good accelerant, it must also release the penetrant readily to the aqueous milieu of the viable epidermis. This process could be hindered by an excessively unfavorable partition coefficient or by the extremely low water solubility of a penetrant (101). A possible explanation is that the flux of water into the dimethyl sulfoxide solution increases the thermodynamic activity of the drug and, hence, the penetration rate of drug into and through the skin (246).

Although the discovery of the rapid penetration through the skin of dimethyl sulfoxide suggested a major advance in therapeutics, the initial enthusiasm for this and similar solvents has not continued, partly because of their irritant action, odor, and toxicity.

Surfactants—Surfactants are often included in pharmaceutical formulations as emulsifying agents, but they probably have little effect on skin penetration. Yet, when the epidermis is treated with compounds having significant surface activity, its permeability to water alters (62, 262). One effect of surfactant action upon the skin may be to change the physical state of water in the skin in such a way as to permit greater freedom to the passage of charged, hydrophilic substances (62).

Several studies reported the effect of soap and surfactants on the loss of water (41, 149–152, 263–265). The role of surfactants in percutaneous absorption has been reviewed (7, 62, 71, 160, 240). Possible mechanisms whereby the skin is able to restrict the percutaneous migration of synthetic anionic surfactants have been well reviewed (145), and it was made clear that the factors involved may be of a complex nature. The irritant action of anionic materials, *e.g.*, "soap" or sodium lauryl sulfate, suggests that they must penetrate to susceptible tissue. It seems reasonable to assume that if a potential irritant is placed on the surface of the skin, penetration as far as the Malpighian layer of living cells is necessary for it to exert its irritant effect (149).

In the case of soap and other surfactants, the bulk of evidence indicates that the stratum corneum is an effective barrier (71, 102, 108, 127, 145, 183, 257, 266). While penetration is poor, there is evidence that anionic and cationic agents do penetrate the skin (71, 108, 127, 145, 150, 266), with anionics penetrating best followed by cationics and nonionics (134, 149).

Among anionic substances, the laurate ion is reported to have the greatest penetration and the greatest effect on the penetration of other solutes (150, 151). Soaps of different fatty acids have this property in varying degrees (149, 150). There is significant penetration by sodium salts of fatty acids with a chain length of 10 carbon atoms or less, and there is modest penetration by fatty acids with longer chains (62, 127, 145).

Previous investigations (127, 145) showed that none of a series of alkyl sulfates in the C_8–C_{18} range was able to penetrate whole skin samples until the samples had been pretreated with lipid solvents or the stratum corneum was mechanically removed.

This finding was corroborated for potassium lauryl sulfate (257). However, the lower molecular weight potassium methyl sulfate and potassium butyl sulfate are able to migrate across intact whole skin samples, with the methyl ester exhibiting the higher order of migration (183). Application of the lauryl ester and the butyl ester in dimethyl sulfoxide solutions did not alter the pattern of migration whereas the methyl ester showed a concentration-dependent accelerant relationship with increasing concentrations of dimethyl sulfoxide.

Sprott (62) found that alkyl sulfates did penetrate the skin of the rat, as did sulfonated fatty acids and carboxylic acid esters of isethionic acid. Measurements of the distribution of *n*-hexadecyl sulfate in the skin showed a very high concentration of this surfactant in the epidermis, suggesting that such anionic surfactants begin to penetrate into the dermis only after saturation of the epidermis. Hence, the inability of certain alkyl sulfates to migrate across whole skin samples is not a function of the whole class. The reduction of the alkyl chain length of these compounds leads to an increase in their rate of percutaneous migration. The sum effect of reduction in chain length is to increase the hydrophilic properties of the molecule and concomitantly reduce the lipophilic function. Anionic surfactants apparently bind strongly with skin protein and cause a reversible denaturation and uncoiling of the filaments, leading to a gross expansion of the tissue (267).

The penetration of fatty acid soaps has been shown to vary with pH (62, 127, 145). The penetration of fatty acid soaps varies inversely from pH 6 to 10.8. At higher pH, the action of the anionic surfactant upon the skin appears to be attenuated or overshadowed by the influence of the more alkaline pH itself. This result also suggests that any interpretation of surfactant action upon the skin must deal separately with these two phenomena, *i.e.*, pH and surfactant effect (62). However, Bettley (149), in a study of the penetration of varied surfactants, could find no correlation with pH.

Scala *et al.* (71) calculated permeability constants for a wide variety of materials including surfactants. The nonlinearity of diffusion curves (permeability constants *versus* time) for an anionic surfactant (sodium tetrapropylenebenzenesulfonate), a cationic surfactant (dodecyltrimethylammonium chloride), and soap probably means that the barrier to these surfactants is being altered by the surfactants themselves as they diffuse into and through the skin. A decrease in the barrier properties allows an increased diffusion, which results in a greater alteration of the barrier. Removal of the surfactant causes the process to stop at the stage of barrier alteration to which it has progressed.

Penetration of certain antimicrobial substances appears enhanced by addition of surfactants. Washing with sodium lauryl sulfate enhances the amount of hexachlorophene and tetrachlorosalicylanilide penetrating rat skin (62). The ability of anionic surfactants to promote absorption is confirmed by the observation that nickel salts compounded with anionic substances cause eczema, but nickel salts combined with nonionic or cationic substances do not. This difference was thought to be due possibly to an acanthotic effect or to a denaturing action on the epidermal protein by the anionic surfactants, thus resulting in the easier passage of the metal sensitizer through the skin (268).

The effects of the anionic surfactants on the penetration of water-soluble substances in the skin appear related to their ability to increase the permeability of the skin to water. Sprott (62) explained this effect in terms of a protein–surfactant interaction and its attendant effects. Thus, where surfactants attached to the skin proteins have brought about conformational changes upon the protein structure, the hydrophilic polar groups would have been forced into the interior of the protein helixes. Under those conditions, water and substances in aqueous solution, *e.g.*, iodide ions, would permeate the skin with enhanced facility since the individual water molecules in the vicinity of proteins so affected would be exposed to fewer electrovalently, *i.e.*, charged, polar groups.

Nonionic surfactants generally appear to have little effect in promoting skin penetration, although numerous reports on percutaneous absorption under the influence of nonionic substances have been published (212, 269–271). Penetration of hydrocortisone was enhanced by increasing concentrations of lauric diethanolamide, a nonionic surfactant, to a maximum at 7% and then was reduced, probably due to micellar trapping of the steroid (272).

The factor determining whether a nonionic surfactant will increase the absorption rate appears to be the configuration of the surfactant molecule rather than the hydrophilic–lipophilic balance or surface activity (273). Where the surfactant has several long hydrophilic chains (*i.e.*, more than five ethylene oxide units) rather than a single or several short ethylene oxide chains, drug absorption is not increased. This finding indicates that the effectiveness of the surfactant is due to the ease with which the surfactant molecules penetrate lipid membranes. Dugard and Scheuplein (274) also could not find a correlation between the hydrophilic–lipophilic balance and surfactant action, and they concluded that there was an action upon protein rather than lipid components of the stratum corneum.

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Polycyclic Aromatic Isothiocyanate Compounds as Fluorescent Labeling Reagents

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Abstract □ Polycyclic aromatic isothiocyanates were synthesized in an attempt to produce new fluorescent agents for protein labeling and for use in microanalytical techniques. The relative fluorescence of these reagents, the required intermediates, and the derivatives were established. Of the compounds evaluated, the 9-acridine derivatives were the most promising.

Keyphrases □ Fluorescent labeling agents—synthesis of polycyclic aromatic isothiocyanates, relative fluorescence □ Isothiocyanates, polycyclic aromatic—synthesized as fluorescent labeling agents, relative fluorescence

In 1950, Coon and Kaplan (1) introduced fluorescein isocyanate for the fluorescent labeling of proteins. The subsequent synthesis of fluorescein and rhodamine isothiocyanates (2, 3) greatly advanced this technique because of major improvements in reagent stability and purity. These latter compounds are widely used for protein labeling, especially in antibody–antigen studies (4).

There is continued interest in these laboratories concerning the synthesis of improved fluorescent agents of greater stability and of contrasting color as compared to fluorescein isothiocyanate. The synthesis and evaluation of stilbene isothiocyanates were described previously (5). The present study describes the synthesis and evaluation of isothiocyanate derivatives of polycyclic aromatic compounds for potential use as protein labeling reagents and in fluorescent microanalysis of amines (6).

EXPERIMENTAL[1]

2-Isothiocyanato-7-methoxyfluorene (I)—A mixture of 0.9 g (0.0043 mole) of 2-amino-7-methoxyfluorene, 0.22 g (0.0022 mole) of calcium carbonate, and 200 ml of benzene was refluxed, and 0.4 ml (0.005 mole) of thiophosgene in 50 ml of benzene was added slowly from a separator. The mixture was refluxed for 3 hr and filtered by suction in the hood. Air evaporation of the filtrate afforded 0.95 g (88%) of a pink-gray precipitate. The compound was recrystallized from acetone, mp 128–129°; IR (KBr): 2950 (CH_3), 2100 (broad, NCS), 1610 and 1470 (aromatic), and 1240 (aromatic ether) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 328 (log ϵ 4.41) and 210 (4.52) nm; NMR ($CDCl_3$): δ 3.66 (s, 2, CH_2), 3.82 (s, 3, OCH_3), and 6.82–7.64 (m, 6, aromatic H).

Anal.—Calc. for $C_{15}H_{11}NOS$: C, 71.11; H, 4.39; N, 5.53; S, 12.66. Found: C, 71.12; H, 4.37; N, 5.45; S, 12.65.

[1] Melting points were in open capillary tubes with a Mel-Temp electric block and are corrected. Spectra were recorded on Perkin-Elmer model 337 IR, Beckman DK-2A UV, Aminco-Bowman 4-8106 fluorescence, and Varian A-60-A NMR spectrometers. Tetramethylsilane was used as the internal reference in NMR measurements. Microanalyses were performed by Spang Microanalytical Laboratory, Ann Arbor, Mich.

1-Isothiocyanato-9-fluorenone (II)—A mixture of 1 g (0.005 mole) of 1-amino-9-fluorenone, 0.26 g (0.0026 mole) of calcium carbonate, 0.4 ml (0.005 mole) of thiophosgene, and 300 ml of benzene was treated in a similar manner as in the preparation of I. The solid was recrystallized from acetone to give 0.46 g (38%) of a yellow powder, mp 135–136.5°; IR (KBr): 2100 (broad, NCS), 1680 (C=O), and 1580 and 1560 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 340 (log ϵ 3.73), 284 (sh, 4.35), 268 (sh, 4.49), 252 (4.55), and 216 (4.64) nm; NMR ($CDCl_3$): δ 6.96–7.99 (m, aromatic H).

Anal.—Calc. for $C_{14}H_7NOS$: C, 70.86; H, 2.98; N, 5.90; S, 13.51. Found: C, 70.91; H, 3.06; N, 5.08; S, 13.61.

***N*-Benzyl-*N'*-(9-oxo-1-fluorenyl)thiourea (III)**—A mixture of 0.15 g (0.0006 mole) of II, 0.08 g (0.0007 mole) of benzylamine, and 30 ml of benzene was refluxed for 1.5 hr. Air evaporation of the solution yielded 0.1 g (45%) of a reddish-orange granular precipitate. It was recrystallized from ethanol–water, mp 184–185°; IR (KBr): 3270 and 3170 (secondary NH), 1650 (C=O), and 1570 and 1520 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 280 (sh, log ϵ 4.11), 260 (sh, 4.41), and 251 (4.46) nm; NMR [$(CD_3)_2SO$]: δ 4.78 (d, 2, J = 6 Hz, $CH_2C_6H_5$, 7.20–7.75 [m, 13 (12 aromatic H, 1 exchangeable NH)], and 9.55 (broad s, 1, NH).

Anal.—Calc. for $C_{21}H_{16}N_2OS$: C, 73.22; H, 4.69; N, 8.13. Found: C, 73.27; H, 4.79; N, 8.08.

***N*-Carboxymethyl-*N'*-(9-oxo-1-fluorenyl)thiourea (IV)**—A solution of 0.15 g (0.0006 mole) of II in 30 ml of acetone and 0.048 g (0.0006 mole) of glycine in 10 ml of 0.1 *M* $NaHCO_3$ was refluxed for 1.5 hr. The solution was cooled and a white precipitate was removed by filtration. The filtrate was adjusted to pH 4.0 with 10% HCl and was extracted with ether until the aqueous portion was almost colorless. The ether solution (dried over sodium sulfate) was evaporated, and the residue was recrystallized from acetone–water to give 0.12 g (60%) of an orange precipitate, mp 203.5–205° dec.; IR (KBr): 3300 (secondary NH), 1705 (COOH), 1645 (C=O), and 1580 and 1510 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 280 (sh, log ϵ 4.27) and 253 (4.63) nm; NMR [$(CD_3)_2SO$]: δ 4.28 (d, J = 5 Hz, 2, CH_2), 7.22–8.50 (m, 7, aromatic H), 9.24 (s, 1, NH), and 10.02 (s, 1, COOH).

Anal.—Calc. for $C_{16}H_{12}N_2O_3S$: C, 61.52; H, 3.88; N, 8.97. Found: C, 61.72; H, 3.92; N, 9.00.

4-Isothiocyanato-9-fluorenone (V)—Compound V was prepared from 4-amino-9-fluorenone in a similar manner as for II. A yield of 1.02 g (85%) was obtained from recrystallization from acetone–water, mp 140–141°; IR (KBr): 2100 (broad, NCS), 1690 (C=O), and 1570 and 1560 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 286 (sh, log ϵ 4.33), 271 (4.51), 248 (4.54), and 217 (4.87) nm; NMR ($CDCl_3$): δ 7.30–8.00 (m, aromatic H).

Anal.—Calc. for $C_{14}H_7NOS$: C, 70.86; H, 2.98; N, 5.90; S, 13.51. Found: C, 70.81; H, 2.97; N, 5.89; S, 13.36.

1-Isothiocyanato-4-methyl-9-fluorene (VI)—A mixture of 1 g (0.0048 mole) of 1-amino-4-methyl-9-fluorenone (7), 0.24 g (0.0024 mole) of calcium carbonate, 0.4 ml (0.005 mole) of thiophosgene, and 300 ml of toluene (dried over calcium hydride) was refluxed for 4 hr under the conditions used for preparing I. The filtrate was air evaporated in the hood, and the precipitate was recrystallized from acetone–water, affording 0.91 g (65%) of VI, mp 128–129°; IR (KBr): 2050 (broad, NCS), 1670 (C=O), and 1580, 1560, and 1540 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 284 (sh, log ϵ 4.06), 253 (4.39), and 217 (4.46) nm; NMR ($CDCl_3$): δ 2.49 (s, 3, CH_3) and 6.75–7.70 (m, 6, aromatic H).

Anal.—Calc. for $C_{15}H_9NOS$: C, 71.68; H, 3.62; N, 5.57; S, 12.76. Found: C, 71.58; H, 3.63; N, 5.48; S, 12.64.

***N*-Benzyl-*N'*-(4-methyl-9-oxo-1-fluorenyl)thiourea (VII)**—A mixture of 0.15 g (0.0006 mole) of VI, 0.08 g (0.0007 mole) of benzylamine, and 30 ml of benzene was refluxed for 1.5 hr. Air evaporation of the solution yielded 0.124 g (58%) of VII, which was recrystallized from acetone–water, mp 178–179°; IR (KBr): 3300 (secondary NH), 3020 (aliphatic CH), 1670 (C=O), and 1620 and 1550 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 282 (sh, log ϵ 3.92) and 254 (plateau, 4.40) nm; NMR [$(CD_3)_2SO$]: δ 4.80 (d, J = 6 Hz, 2, $CH_2C_6H_5$), 7.21–8.44 (m, 11, aromatic H), 9.47 (t, 1, NH), and 10.03 (broad s, 1, NH).

Anal.—Calc. for $C_{22}H_{18}N_2OS$: C, 73.71; H, 5.07; N, 7.82. Found: C, 73.66; H, 5.06; N, 7.82.

2-Isothiocyanato-9-acridone (VIII)—A mixture of 0.18 g (0.00086 mole) of 2-amino-9-acridone (8), 0.05 g (0.0005 mole) of calcium carbonate, 1 ml (0.013 mole) of thiophosgene, and 200 ml of toluene (dried over calcium hydride) was refluxed for 3 hr. The toluene was air evaporated in the hood. Then the residue, recrystallized from tetrahydrofuran, afforded 0.12 g (54%) of yellow-green plates, mp 338–340° dec.; IR (KBr): 3300 (NH), 2120 (broad, NCS), 1630 (C=O), and 1590, 1570, and 1520 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 327 (log ϵ 4.23), 314 (4.11), 293 (4.77), 247 (4.55), and 222 (4.35) nm.

Anal.—Calc. for $C_{14}H_8N_2OS$: C, 66.64; H, 3.20: N, 11.11. Found: C, 66.70; H, 3.45; N, 10.77.

***N*-(1-Butyl)-*N'*-(9-oxo-2-acridanyl)thiourea (IX)**—A mixture of 0.5 g (0.002 mole) of VIII and 1.5 g (0.02 mole) of *n*-butylamine in 300 ml of tetrahydrofuran was refluxed for 0.5 hr. The solvent was evaporated to dryness, and the residue was recrystallized from absolute alcohol to give 0.54 g (78%) of a yellow fluffy powder, mp 255° dec.; IR (mineral oil): 3050–3260 (broad, NH), 1635 (C=O), and 1600 and 1550 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 285 (log ϵ 4.61), 2.57 (4.68), and 213 (4.49) nm; NMR [$(CD_3)_2SO$]: δ 0.98 (t, J = 7 Hz, 3, CH_3), 1.25–2.02 (m, 4, CH_2), 3.33–3.85 (m, 2, CH_2), 7.17–8.38 [m, 8 (7 aromatic H and 1 exchangeable H), NH], 9.61 (broad s, 1, NH), and 12.12 (broad s, 1, NH).

Anal.—Calc. for $C_{18}H_{19}N_3OS$: C, 66.42; H, 5.90; N, 12.91. Found: C, 66.31; H, 5.96; N, 12.68.

***N*-Phenyl-*N'*-(9-acridinyl)thiourea (X)**—A solution of 0.5 g (0.0022 mole) of 9-isothiocyanatoacridine (9) and 0.25 g (0.0027 mole) of aniline in 100 ml of absolute alcohol was refluxed for 1 hr. The precipitate was filtered and washed with cold alcohol. Recrystallization from absolute alcohol afforded 0.63 g (90%) of product, mp 178–180°; IR (KBr): 3270 (secondary NH), 1630, 1560, and 1530 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 268 (log ϵ 4.61) and 241 (4.65) nm; NMR [$(CD_3)_2SO$]: δ 6.98–8.58 (m, aromatic H).

Anal.—Calc. for $C_{20}H_{15}N_3S$: C, 72.91; H, 4.60; N, 12.76. Found: C, 72.93; H, 4.68; N, 12.81.

***N*-Carboxymethyl-*N'*-(9-acridinyl)thiourea (XI)**—A modified literature procedure (10) was followed. A solution of 1.9 g (0.008 mole) of 9-isothiocyanatoacridine (9) in 100 ml of pyridine and 0.48 g (0.0064 mole) of glycine in basic water (20 ml of water adjusted to pH 9 with 1 *N* NaOH) was stirred and heated at 60° (water bath). The solution was maintained at pH 8–9 by adding 1 *N* NaOH dropwise until base consumption ceased (about 10 ml). Heating was continued for 0.5 hr longer. The excess isothiocyanatoacridine was extracted with benzene and the benzene extracts were discarded. The benzene content of the aqueous layer was reduced by air evaporation, and an equivalent of hydrochloric acid was added to precipitate crude XI. The precipitate was collected, dried, and recrystallized from methanol to give 1.3 g (55%) of product, mp 182–183°; IR (KBr): 3300 (secondary NH), 1720 (C=O), and 1630, 1590, and 1525 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 284 (sh, log ϵ 3.88), 253 (4.65), and 217 (4.30) nm; NMR [$(CD_3)_2SO$]: δ 4.28 (s, 2, CH_2) and 7.01–8.68 (m, 8, aromatic H).

Anal.—Calc. for $C_{16}H_{13}N_3O_2S$: C, 61.71; H, 4.22; N, 13.50. Found: C, 61.53; H, 4.24; N, 13.34.

***N,N*-Diethyl-*N'*-(9-acridinyl)thiourea (XII)**—A mixture of 0.14 g (0.0019 mole) of diethylamine and 0.5 g (0.0022 mole) of 9-isothiocyanatoacridine (9) in 100 ml of absolute alcohol was heated in a 60° water bath for 1 hr with occasional stirring. The solution was concentrated by air evaporation. Filtration and recrystallization of the precipitate from absolute alcohol afforded 0.51 g (76%) of a silky yellow precipitate, mp 184.5–186°; IR (KBr): 3080, 2980, and 2940 (aliphatic CH) and 1620, 1580, and 1520 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 285 (sh, log ϵ 4.24), 242 (4.67), and 220 (sh, 4.59) nm; NMR [$(CD_3)_2SO$]: δ 1.24 (m, 6, CH_3), 3.72 (q, 4, CH_2), 6.99–8.20 (m, 8, aromatic H), and 11.4 (s, 1, NH).

Anal.—Calc. for $C_{18}H_{19}N_3S$: C, 69.86; H, 6.20; N, 13.58. Found: C, 69.74; H, 6.18; N, 13.55.

***N*-(9-Acridinyl)thiourethane (XIII)**—A solution of 0.5 g (0.0022 mole) of 9-isothiocyanatoacridine (9) in 50 ml of absolute alcohol was treated with 10 ml of 10% HCl (aqueous). The solution was left at room temperature overnight, and a yellowish-orange precipitate was obtained. Recrystallization from acetone afforded 0.3 g (50%) of product as the hydrochloride salt, mp 138–140°; IR (KBr): 2800 (broad, HCl), 1590 and 1550 (aromatic), and 1220 (C=S) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 252 (log ϵ 5.17) nm; NMR [$(CD_3)_2SO$]: δ 1.40 (t, J = 7 Hz, 3, CH_3), 4.62 (q, J = 7 Hz, 2, CH_2), and 7.56–9.58 (m, 8, aromatic H).

Anal.—Calc. for $C_{16}H_{14}N_2OS \cdot HCl$: C, 60.27; H, 4.75; N, 8.79. Found: C, 60.54; H, 4.77; N, 8.70.

1-(9-Acridinyl)-4-benzyl-2-thiohydantoin (XIV)—The intermediate *N*-[α-benzyl(carboxymethyl)]-*N'*-(9-acridinyl)thiourea was prepared from 2 g (0.0085 mole) of 9-isothiocyanatoacridine (9), 0.7 g (0.0042 mole) of phenylalanine, and 10 ml of 1 *N* NaOH in a similar manner as for XI. A mixture of crude thiourea compound and 15 ml of acetic acid, saturated with hydrogen chloride gas, was stirred at room temperature for 6 hr in a stoppered erlenmeyer flask. The mixture was evaporated to dryness *in vacuo;* the residue, recrystallized from methanol, afforded 0.45 g (13%) of product, mp 176–178°; IR (KBr): 3320 (secondary NH), 1730 (C=O), and 1640, 1590, and 1530 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 253 (log ϵ 4.80) nm; NMR [$(CD_3)_2SO$]: δ 3.14 (d, J = 6 Hz, 2, CH_2), 5.20 (d, J = 6 Hz, 1, CH), 6.84–8.36 (m, 13, aromatic H), and 9.16 (s, 1, NH).

Anal.—Calc. for $C_{23}H_{17}N_3OS \cdot H_2O$: C, 68.80; H, 4.78; N, 10.47. Found: C, 68.55; H, 4.72; N, 10.44.

2-Thiono-3-(9-acridinyl) - 4-oxo-1,3-diazabicyclo[3.3.0]octane (XV)—A solution of 2.5 g (0.022 mole) of proline in 35 ml of Na_2HPO_4–H_3PO_4 buffer (pH 9.0) was treated with 1 g (0.0042 mole) of 9-isothiocyanatoacridine (9) in 35 ml of dioxane. The reaction mixture was stirred at room temperature for 10 hr and filtered. The filtrate was extracted with 10 100-ml portions of ethyl acetate (organic extracts were discarded), and the aqueous phase was made acidic with acetic acid until precipitation ceased (about 15 ml). Then the precipitate was filtered and dried in a vacuum desiccator to give the crude thiourea derivative. A mixture of the crude material and 10 ml of anhydrous acetic acid saturated with hydrogen chloride gas was stirred at room temperature for 10 hr in a stoppered erlenmeyer flask. The excess acid was evacuated *in vacuo,* and the remaining viscous material was recrystallized from methanol to give 0.21 g (15%) of product, mp 191–193°; IR (KBr): 2970 (aliphatic CH), 1720 (C=O), and 1630 and 1570 (aromatic) cm^{-1}; UV $\lambda_{max}^{(alcohol)}$: 256 (log ϵ 4.77) and 223 (sh, 4.48) nm; NMR [$(CD_3)_2SO$]: δ 1.40–2.12 (m, 6, CH_2), 3.75 (m, 1, CH), and 7.54–8.71 (m, 8, aromatic H).

Anal.—Calc. for $C_{19}H_{15}N_3OS \cdot 0.5H_2O$: C, 66.64; H, 4.72; N, 12.27. Found: C, 66.40; H, 4.97; N, 12.45.

4-Isothiocyanatobenzophenone (XVI)—A suspension of 10 g (0.042 mole) of 4-aminobenzophenone hydrochloride in 150 ml of chloroform was stirred with 50 ml of aqueous sodium hydroxide (6.12 g, 0.053 mole) solution until 4-aminobenzophenone dissolved in chloroform. The mixture was cooled to 0°, and 6.14 g (0.053 mole) of thiophosgene was added at once. Stirring was continued for 3 hr at 0° and then for 1.5 hr under reflux. The chloroform layer was removed, dried, and evaporated to dryness *in vacuo.* The crude product was kept in a vacuum desiccator over phosphorus pentoxide and potassium hydroxide and then was recrystallized from petroleum ether (bp 60–75°) (charcoal). The yield was 8.6 g (82.5%) of light-yellow crystals, mp 81–82°; IR (mineral oil): 2100 (NCS) and 1640 (C=O) cm^{-1}; NMR ($CDCl_3$): δ 7.22–7.89 (m, aromatic H).

Anal.—Calc. for $C_{14}H_9NOS$: C, 70.27; H, 3.79; N, 5.85. Found: C, 70.49; H, 3.93; N, 6.15.

***N*-Benzyl-*N'*-(4-benzophenone)thiourea (XVII)**—A solution of 2 g (0.0084 mole) of 4-isothiocyanatobenzophenone and 4.5 g (0.042 mole) of benzylamine in 10 ml of alcohol was refluxed for 2 hr. Excess ethanol was removed, and crystallization of the product was initiated by adding a few drops of water and cooling. The yield was 2.3 g (79%), mp 125–127°. Recrystallization from chloroform–

petroleum ether (bp 60–75°) gave 2.1 g, mp 127–128°; IR (mineral oil): 3400 and 3030–3200 (NH), 1640 (C=O), and 1190 (C=S) cm^{-1}; NMR ($CDCl_3$): δ 4.88 (d, J = 5 Hz, 2, CH_2), 6.87 (m, 1, NH), 7.32–7.83 (m, 14, aromatic H), and 8.76 (broad s, 1, NH).

Anal.—Calc. for $C_{21}H_{18}N_2OS$: C, 72.81; H, 5.23; N, 8.08. Found: C, 72.61; H, 5.21; N, 7.86.

2-Anilino-5-nitrobenzyl Chloride (XVIII)—A solution of 10 g (0.039 mole) of 2-anilino-5-nitrobenzoic acid (11) and 8 g (0.038 mole) of phosphorus pentachloride in 160 ml of benzene was refluxed on a water bath for 1 hr. The solution was treated with charcoal and filtered while still warm. Petroleum ether (bp 30–45°) was added to the filtrate. The product crystallized as yellow crystals, yielding 8.7 g (80.5%). Two recrystallizations from benzene–petroleum ether afforded an analytical sample, mp 157–158°; IR (mineral oil): 3300 (NH), 1710 (C=O), and 1620, 1600, and 1580 (aromatic) cm^{-1}; NMR ($CDCl_3$): δ 6.99–9.22 (m, 8, aromatic H) and 9.73 (broad s, 1, NH).

Anal.—Calc. for $C_{13}H_9ClN_2O_3$: C, 56.44; H, 3.28; Cl, 12.81; N, 10.12. Found: C, 56.56; H, 3.31: Cl, 12.74; N, 10.09.

2-Nitro-9-[2-(diethylamino)ethylamino]acridine Hydrochloride (XIX)—A literature procedure (12) was modified and applied. To a solution of 5 g (0.018 mole) of XVIII in 100 ml of warm benzene, 2.1 g (0.018 mole) of *N,N*-diethylethylenediamine was added and the solution was refluxed for 30 min. Then 6 ml (0.065 mole) of phosphorus oxychloride was introduced and refluxing was continued on an oil bath for 2 hr. Cooling caused the crude product (mp 220°) to separate from solution. It was collected on a filter, washed with benzene, and recrystallized from ethanol (400 ml) to give 6.2 g (92%) of yellow needles, mp 223–224°; IR (mineral oil): 3260 (NH), 1640 and 1580 (aromatic), and 1530 and 1330 (NO_2) cm^{-1}; NMR [$(CD_3)_2SO$]: δ 1.30 (t, J = 7 Hz, 6, CH_3), 3.05–3.80 (m, 8, CH_2), 7.13–8.72 (m, 7, aromatic H), 9.40 (broad s, 1, HCl), and 10.58 (broad s, 1, NH).

Anal.—Calc. for $C_{19}H_{22}N_4O_2 \cdot HCl$: C, 60.88; H, 6.18; Cl, 9.46; N, 14.94. Found: C, 60.94; H, 6.19; Cl, 9.47; N, 14.94.

2-Amino-9-[2-(diethylamino)ethylamino]acridine Monohydrate (XX)—A mixture of 4.1 g (0.011 mole) of XIX, 8.8 g (0.039 mole) of stannous chloride dihydrate, and 10 ml of concentrated hydrochloric acid was refluxed on an oil bath for 1 hr. The solution was made alkaline by the addition of 100 ml of 5 *M* sodium hydroxide and kept warm until the stannous hydroxide dissolved. A product separated as a yellow oil, which solidified after cooling. The solid was collected on a filter and washed with sodium hydroxide solution and then with water. The yield was 3.1 g (86.4%), mp 127°. Recrystallization from benzene (charcoal)–petroleum ether (bp 30–45°) gave 2.4 g (67%) of silvery-white crystals, mp 127–128°. A second recrystallization gave an analytical sample, mp 127–129°; IR (mineral oil): 3360, 3300, 3240, and 3180 (NH) and 1640, 1600, and 1580 (aromatic) cm^{-1}; NMR ($CDCl_3$): δ 0.98 (t, J = 7 Hz, 6, CH_3), 2.32–2.68 (m, 6, CH_2), 3.33 (t, J = 6 Hz, 2, CH_2), 3.53 (s, 2, NH_2), 6.55–7.34 [m, 9 (7 aromatic H and 2 exchangeable H), H_2O], and 8.00 (broad s, 1, NH).

Anal.—Calc. for $C_{19}H_{24}N_2 \cdot H_2O$: C, 69.91; H, 8.03; H_2O, 5.52; N, 17.16. Found: C, 69.98; H, 7.95; H_2O, 5.36; N, 17.23.

2-Isothiocyanato - 9-[2-(diethylamino)ethylamino]acridine Hydrochloride (XXI)—To a solution of 3.1 g (0.0095 mole) of XX in 50 ml of acetone, 1.15 g (0.01 mole) of thiophosgene was added and the solution was refluxed for 1.5 hr. The light-brown solution was filtered, and petroleum ether (bp 30–45°) was added to the filtrate to obtain 3.35 g (83%) of light-yellow product, mp 164–168° dec. Recrystallization from chloroform (charcoal)–ether gave 3 g (74.5%) of white product, mp 167–168°; IR (mineral oil): 3280 (NH), 2070 (NCS), and 1650 and 1580 (aromatic) cm^{-1}; NMR ($CDCl_3$): δ 1.45 (t, J = 7 Hz, 6, CH_3), 3.10–3.43 (m, 6, CH_2), 3.90 (m, 2, CH_2), 7.02–7.90 [m, 9 (7 aromatic H and 2 exchangeable H), H_2O], 8.99 (broad s, 1, HCl), and 9.76 (broad s, 1, NH).

Anal.—Calc. for $C_{20}H_{22}N_4S \cdot HCl \cdot 1.5H_2O$: C, 58.03; H, 6.33; Cl, 8.56; H_2O, 6.53; N, 13.53; S, 7.75. Found: C, 58.11; H, 6.32; Cl, 8.69; H_2O, 6.41; N, 13.56; S, 7.69.

***N* - Benzyl - *N'* - [9-[2-(diethylamino)ethylamino]-2-acridinyl]thiourea (XXII)**—To a solution of 0.42 g (0.001 mole) of XXI in 4 ml of ethanol, 0.21 g (0.002 mole) of benzylamine in 2 ml of alcohol was added and the mixture was refluxed in a water bath for about 10 min. The mixture was cooled and a few drops of ether were added. The yield was 0.38 g (82.5%) of white crystals, mp 172°. Recrystallization from alcohol gave a melting point of 172–173°; IR (mineral oil): 3290 and 3140 (NH), 1650, 1600, and 1580 (aromatic), and 1075 (C=S) cm^{-1}; NMR [$(CD_3)_2SO$]: δ 1.00 (t, J = 7 Hz, 6, CH_3), 2.39–2.72 (m, 6, CH_2), 3.24–3.47 (m, 2, CH_2), 4.88 (d, J = 6 Hz, 2, $CH_2C_6H_5$), 7.00–7.77 (m, 12, aromatic H), 8.33 (m, 3, NH and H_2O), 9.56 (s, 1, NH), and 9.60 (s, 1, NH).

Anal.—Calc. for $C_{27}H_{31}N_5S \cdot H_2O$: C, 68.18; H, 6.99; H_2O, 3.79; N, 14.72; S, 6.74. Found: C, 68.26; H, 6.93; H_2O, 3.75; N, 14.69; S, 6.78.

9-(4-Nitrobenzyl)acridine (XXIII)—This compound was prepared from 9-benzylacridine according to Hunters and Shaw (13), mp 217–218° [lit. (13) mp 195–198°]; IR (mineral oil): 1540 (NO_2) and 1600 (aromatic) cm^{-1}; NMR ($CDCl_3$): δ 5.07 (s, 2, CH_2) and 7.5–8.2 (m, 12, aromatic H).

Anal.—Calc. for $C_{20}H_{14}N_2O_2$: C, 76.42; H, 4.49; N, 8.91. Found: C, 76.22; H, 4.37; N, 8.96.

9-(4-Aminobenzyl)acridine (XXIV)—A solution of 1.7 g (0.0057 mole) of XXIII in 200 ml of 95% alcohol and 4.51 g (0.02 mole) of stannous chloride in 10 ml of concentrated hydrochloric acid was refluxed for 1 hr on a water bath. The solvent was removed *in vacuo*, and the residue was neutralized with 10% sodium hydroxide. The precipitate was collected on a filter, washed with water, and recrystallized from alcohol. The yield was 1.26 g (80%), mp 231–233°; IR (mineral oil): 3430 (NH) cm^{-1}; NMR ($CDCl_3$): δ 3.25 (s, 2, NH_2), 4.65 (s, 2, CH_2), and 6.2–8.1 (m, 12, aromatic H).

Anal.—Calc. for $C_{20}H_{16}N_2$: C, 84.48; H, 5.67; N, 9.85. Found: C, 84.31; H, 5.74; N, 9.90.

9-(4-Isothiocyanatobenzyl)acridine (XXV)—In 150 ml of acetone was dissolved 1 g (0.0034 mole) of XXIV, and 0.77 ml (0.01 mole) of thiophosgene was added at once. The solution was refluxed for 1 hr. The light-yellow solid was collected and washed with acetone, yielding 1 g (77%), mp 217–220° dec. One gram (0.0027 mole) of hydrochloride was dissolved in chloroform and converted to the free base by shaking with 10% ammonia. The chloroform was evaporated and the residue was recrystallized from cyclohexane to afford 0.7 g (79% yield) of product, mp 156–157°; IR (mineral oil): 3300 (NH) and 2120 (broad, NCS) cm^{-1}; NMR ($CDCl_3$): δ 4.81 (s, 2, CH_2) and 6.82–8.1 (m, 12, aromatic H).

Anal.—Calc. for $C_{21}H_{14}N_2S$: C, 77.28; H, 4.32; N, 8.58. Found: C, 77.23; H, 4.34; N, 8.53.

9-(4-Nitro-α-methylbenzyl)acridine (XXVI)—A solution of 2.5 g (0.0088 mole) of 9-(α-methylbenzyl)acridine (14) in 10 ml of 10 *N* nitric acid was heated to 60° for 10 min. The mixture was cooled and the solid was collected to yield 3 g of the nitrate of starting material, mp 165–168°. The product was dissolved in concentrated sulfuric acid (20 ml), which resulted in a rise in temperature to 50°. The solution was cooled, poured over ice, and made alkaline with concentrated ammonia. The resultant solid was collected, dissolved in alcohol (70 ml), decolorized with activated charcoal, and precipitated by addition of water (30 ml). The yield was 1.5 g (52%) of product, which was recrystallized from alcohol, mp 171–172°; IR (mineral oil): 1580 (NO_2) cm^{-1}; NMR [$(CD_3)_2SO$]: δ 2.02 (d, J = 7 Hz, CH_3), 6.33 (q, J = 7 Hz, 1, CH), and 7.41–8.33 (m, 12, aromatic H).

Anal.—Calc. for $C_{21}H_{16}N_2O_2$: C, 76.81; H, 4.91; N, 8.53. Found: C, 76.66; H, 4.97; N, 8.64.

9-(4-Amino-α-methylbenzyl)acridine (XXVII)—A solution of 1.69 g (0.005 mole) of XXVI in 250 ml of alcohol and 1.5 g (0.02 mole) of stannous chloride in 10 ml of concentrated hydrochloric acid was refluxed for 45 min. The solvent was removed *in vacuo* and the residue was neutralized with 10% sodium hydroxide. The precipitate was collected on a filter, washed with water, and recrystallized from 75% alcohol. The yield was 1.1 g (75%) of XXVII, mp 190–191°; IR (mineral oil): 3425, 3310, and 3180 (NH) and 1630, 1590, and 1520 (aromatic) cm^{-1}; NMR [$(CD_3)_2SO$]: δ 1.96 (d, J = 7 Hz, 3, CH_3), 3.49 (s, 2, NH_2), 5.67 (q, J = 7 Hz, 1, CH), and 6.55–8.31 (m, 12, aromatic H).

Anal.—Calc. for $C_{21}H_{18}N_2$: C, 84.53; H, 6.08; N, 9.39. Found: 84.37; H, 6.12; N, 9.42.

9-(4-Isothiocyanato-α-methylbenzyl)acridine (XXVIII)—To a solution of 0.75 g (0.0027 mole) of XXVII in 7 ml of acetone, 0.77 ml (0.01 mole) of thiophosgene was added at once. The solution was refluxed for 1 hr. The yellow solid was collected on a filter and washed with dry acetone to yield 0.9 g of solid. The solid was dissolved in chloroform and converted to the free base with 10% ammonia. The yield was 1 g (70%) of product, mp 110–114°; IR (mineral oil): 2120 (broad, NCS) cm^{-1}; NMR ($CDCl_3$): δ 2.02 (d, J

Table I—Fluorescence Properties

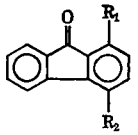
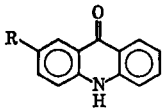
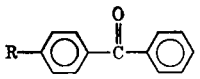
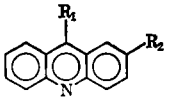
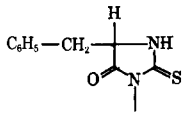
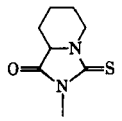
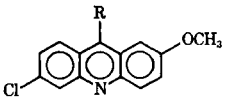
Compound	Compound Number or Source	pH[a]	Activation/ Fluorescence Wavelengths, nm	Relative Fluorescence[b]
Fluorenones				
R_1 / R_2				
NH_2 / H	—[c]	6.8	460/525	0.14
NCS / H	II	3.0	450/525	0.0044
$NHCSNHCH_2C_6H_5$ / H	III	11.0[d]	440/535	0.0096
$NHCSNHCH_2$-COOH / H	IV	10.0[d]	425/520	0.069
H / NCS	V	5.9	300/545	0.00026
NH_2 / CH_3	Ref. 7	5.9	470/535	0.23
NCS / CH_3	VI	5.9	360/535	0.0014
$NHCSNHCH_2C_6H_5$ / CH_3	VII	5.9	480/540	0.00029
Acridones				
R				
H	—[c]	9.0	400/425, 440	4.33
NH_2	Ref. 8	8.0	240/530	0.67
NCS	VIII	12.5	400/470, 480	0.49
$NHCSNHC_4H_9$	IX	9.0	400/470	0.039
Benzophenones				
R				
NH_2	—[c]	5.0	260/420	0.0021
NCS	XVI	7.0	257/420	0.0010
$NHCSNHCH_2C_6H_5$	XVII	2.0	302/405	0.0020
Acridines				
R_1 / R_2				
NH_2 / H	—[c]	5.9	270/440, 460	2.64
Cl / H	—[e]	3.0	270/440	0.088
NCS / H	Ref. 9	1.0	410/440, 460	0.67
$NHCSNHC_4H_9$ / H	Ref. 9	1.0	410/440, 460	0.013
$NHCSNHC_6H_5$ / H	X	1.0	400/440, 460	0.30
$NHCSNHCH_2$-COOH / H	XI	3.0	400/460	0.084
$NHCSN(C_2H_5)_2$ / H	XII	1.0	270/470	0.0069
$NHCSOC_2H_5$ / H	XIII	3.0	400/440	0.025
 / H	XIV	3.0	260/460	0.058
 / H	XV	12.5	400/490	0.046
$NHCH_2CH_2N(C_2H_5)_2$ / NCS	XXI	12.5	370/485	0.0033
$NHCH_2CH_2N(C_2H_5)_2$ / NHCSNH-$CHC_6H_5CH_2$	XXII	12.5	330/500	0.0016
$CH_2—C_6H_4—NH_2$ / H	XXIV	3.0	350/480	0.086
$CH_2—C_6H_4—NCS$ / H	XXV	7.0	360/430	0.20
$CH(CH_3)—C_6H_4—NH_2$ / H	XXVII	7.1	360/470	0.041
$CH(CH_3)—C_6H_4—NCS$ / H	XXVIII	8.0	365/430	0.17

Table I—(*Continued*)

Compound	Compound Number or Source	pH[a]	Activation/ Fluorescence Wavelengths, nm	Relative Fluorescence[b]
2-Methoxy-6-chloroacridines				
R				
$NHCH_2CH_2$—C_6H_4—NH_2	XXX	2.0	440/500	0.27
$NHCH_2CH_2$—C_6H_4—NCS	XXXI	11.0	270/490	0.03
$NHCH_2CH_2CH_2$—C_6H_4—NCS	XXXIV	11.0	430/500	0.40

[a] Compounds were measured in absolute alcohol–aqueous solutions (1:1) except where noted. Values given for pH are those of the aqueous solutions at maximal fluorescence. [b] Relative fluorescence is relative to a 1.60×10^{-6} *M* solution of fluorescein isothiocyanate at pH 10. [c] Aldrich Chemical Co. [d] Compounds measured in dioxane–aqueous solutions. [e] Eastman Organic Chemicals.

= 7 Hz, 3, CH_3), 5.71 (q, *J* = 7 Hz, 1, CH), and 7.18–8.38 (m, 12, aromatic H).

Anal.—Calc. for $C_{22}H_{16}N_2S$: C, 77.63; H, 4.74; N, 8.23. Found: C, 77.58; H, 4.84; N, 8.23.

2-Methoxy-6-chloro-9-[2-(4-nitrophenyl)ethylamino]acridine (XXIX)—One gram (0.0029 mole) of 2-methoxy-6-chloro-9-phenoxyacridine was dissolved in 4 g of phenol at 80°. Then 0.78 g (0.0042 mole) of 2-(4-nitrophenyl)ethylamine hydrochloride was added to the solution, which was heated at 140° for 7 hr. The mixture was cooled and poured into ether (10 ml), and the hydrochloride of XXIX was collected. The yield was 1.5 g, mp 255–258°. The base was liberated with cold 5% sodium hydroxide and recrystallized from methanol to give 1 g (82%) of yellow crystalline XXIX, mp 150–151°; IR[2]: 3300 (NH) and 1320 (NO_2) cm^{-1}; NMR [$(CD_3)_2SO$]: δ 3.18 (t, *J* = 7 Hz, 2, CH_2), 3.94 (s, 3, OCH_3), 4.10 (t, *J* = 7 Hz, 2, CH_2), and 7.21–8.38 [m, 11 (10 aromatic H and 1 exchangeable H), NH].

Anal.—Calc. for $C_{22}H_{18}ClN_3O_3$: C, 64.78; H, 4.45; N, 10.30. Found: C, 64.65; H, 4.51; N, 10.29.

2-Methoxy-6-chloro-9-[2-(4-aminophenyl)ethylamino]acridine (XXX)—A solution of 4.07 g (0.01 mole) of XXIX in 1 ml of methanol and 0.79 g (0.033 mole) of stannous chloride in 10 ml of hydrochloric acid was refluxed for 1.5 hr. The solvent was removed *in vacuo*, and the residue was neutralized with 5% sodium hydroxide to give 3 g (76%) of XXX. The product was recrystallized from 80% alcohol as yellow crystals, mp 130–131°; IR[2]: 3320 (NH_2) and 1600 (aromatic) cm^{-1}; NMR ($CDCl_3$): δ 2.70 (t, 2, CH_2), 3.58 (t, 2, CH_2), 3.63 (s, 3, OCH_3), 4.5 (broad s, 1, NH), and 6.16–7.62 (m, 10, aromatic H).

Anal.—Calc. for $C_{22}H_{20}ClN_3O$: C, 69.92; H, 5.33; N, 11.12. Found: C, 69.70; H, 5.43; N, 11.11.

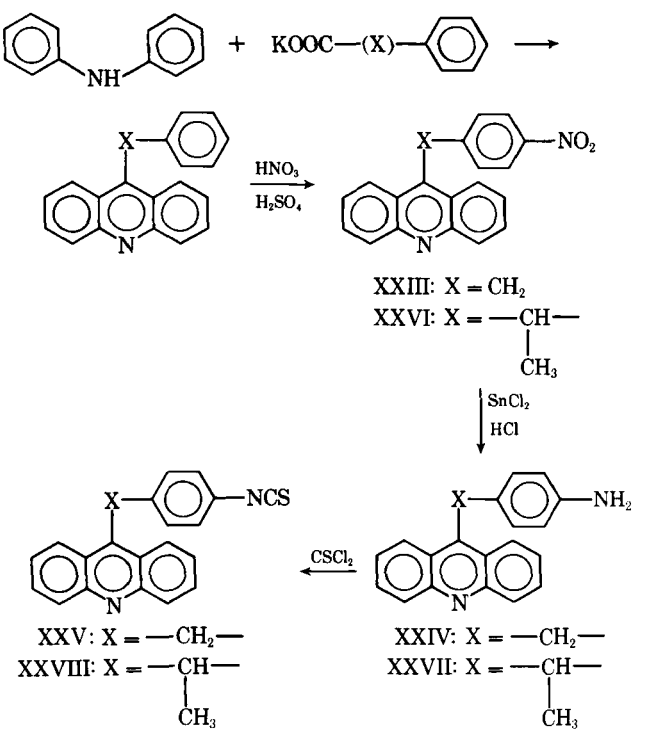
2 - Methoxy - 6 - chloro-9-[2-(4-isothiocyanatophenyl)ethylamino]acridine Hydrochloride (XXXI)—In 20 ml of acetone, 0.4 g (0.001 mole) of XXX was dissolved and 0.77 ml (0.01 mole) of thiophosgene was added at once. The solution was refluxed for 1.5 hr and the light-yellow precipitate was collected to yield 0.45 g (94%) of XXXI-HCl. It was recrystallized from methanol, mp 184–185°; IR[2]: 2120 (broad, NCS) cm^{-1}; NMR ($CDCl_3$): δ 3.15 (t, *J* = 7 Hz, 2, CH_2), 3.95 (s, 3, OCH_3), 4.2 (t, 2, CH_2), and 7.05–8.2 (m, 10, aromatic H).

Anal.—Calc. for $C_{23}H_{19}Cl_2N_3OS$: C, 60.54; H, 4.20; N, 9.21. Found: C, 60.31; H, 4.21; N, 9.20.

2 - Methoxy - 6 - chloro-9-[3-(4-nitrophenyl)propylamino]acridine (XXXII)—A quantity of 2.18 g (0.0065 mole) of 2-methoxy-6-chloro-9-phenoxyacridine (15) was dissolved in 9 g of phenol at 80°. Then 1.5 g (0.0065 mole) of 3-(4-nitrophenyl)propylamine hydrochloride (16) was added and heating was continued at 130° for 3 hr. The mixture was cooled and poured into ether (30 ml), and a solid was collected to give 3.2 g of XXXII-HCl, mp 229–231°. The free base was liberated with cold 10% sodium hydroxide. It was recrystallized from methanol to afford 2.5 g (91%) of product, mp 122–123°; IR[2]: 1330 (NO_2) cm^{-1}; NMR ($CDCl_3$): δ 2 (m, 2, CH_2), 2.7 (t, *J* = 7 Hz, 2, CH_2), 3.56 (t, *J* = 7 Hz, 2, CH_2), 3.78 (s, 3, OCH_3), and 6.90–8.0 (m, 10, aromatic H).

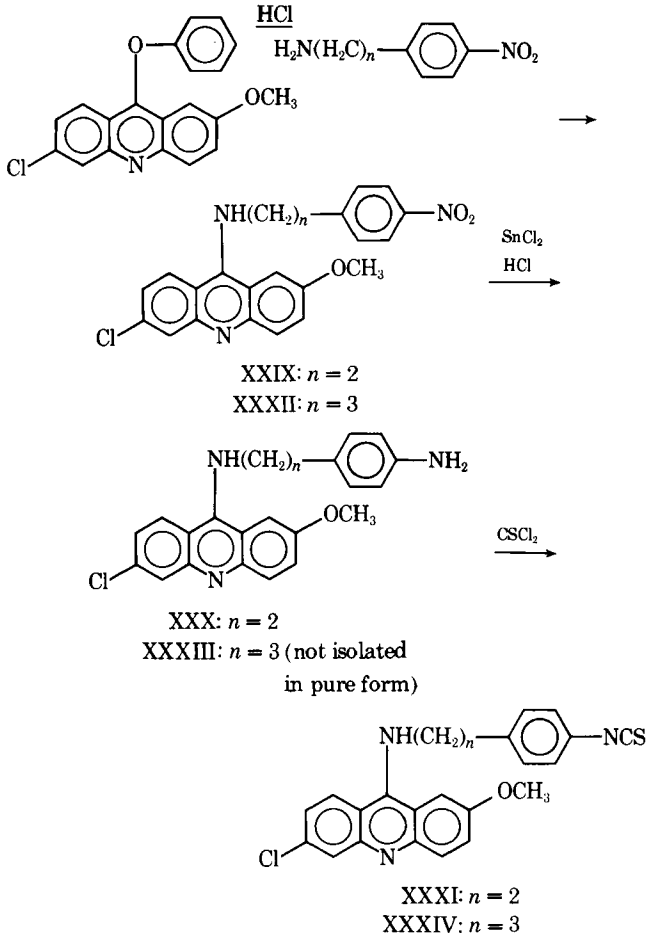
Anal.—Calc. for $C_{23}H_{20}ClN_3O_3$: C, 65.78; H, 4.78; N, 9.96. Found: C, 65.72; H, 4.79; N, 10.03.

2-Methoxy - 6-chloro-9-[3-(4-isothiocyanatophenyl)propylamino]acridine (XXXIV)—A solution of 4.2 g (0.010 mole) of nitro compound XXXII in 800 ml of methanol and 7.44 g (0.033 mole) of stannous chloride in 10 ml of concentrated hydrochloric acid was refluxed for 1.5 hr. It was refrigerated to yield 4 g of solid (87%), mp 275–278°. Conversion to the free base with 5% ammonia and recrystallization from 75% alcohol gave XXXIII, mp 90–92°. This solid was used without further purification as an acetone solution (1 g/50 ml), to which 1 ml of thiophosgene was added at once. The solution was refluxed for 1 hr, and the light-yellow solid was collected and washed with acetone to give 1 g of XXXIV-HCl, mp 230–235° dec. One gram of XXXIV-HCl was dispersed in chloroform and converted to the free base with aqueous 10% ammonia. The chloroform was evaporated and the residue was recrystallized from cyclohexane to afford 0.7 g (47%) of product as yellow needles, mp 132–133°; IR (mineral oil): 2120 (broad, NCS) cm^{-1};



Scheme I—Type A Compounds

[2] Fluorolube.



Scheme II—Type B Compounds

NMR ($CDCl_3$): δ 2.05 (m, 2, CH_2), 2.70 (t, J = 7 Hz, 2, CH_2), 3.65 (t, J = 7 Hz, 2, CH_2), and 7.0–8.05 (m, 10, aromatic H).

Anal.—Calc. for $C_{24}H_{20}ClN_3OS$: C, 66.42; H, 4.69; N, 9.68. Found: C, 66.23; H, 4.61; N, 9.71.

RESULTS AND DISCUSSION

The synthesis of polycyclic aromatic isothiocyanates appeared to be an attractive approach in the search for new microanalytical and protein labeling reagents. As rigid conjugated unsaturated structures, they presented the potential, after UV activation, for high fluorescence with minimal loss of energy by alternative pathways.

An initial survey of compounds was based upon the availability of suitable intermediates for the synthesis of isothiocyanate derivatives by reaction of the corresponding amines with thiophosgene, as previously described for the preparation of fluorescein isothiocyanate (17). Compounds I, II, V, and XVI were prepared in this manner from commercially available amines. Also, VI was prepared in the same manner from its corresponding amine synthesized in the laboratories for other purposes (7), and VIII was prepared from the amine described in the literature (8). In addition, 9-isothiocyanatoacridine was synthesized by reaction of the 9-chloro compound with silver thiocyanate as previously reported (9).

Previous studies with stilbene isothiocyanate (5) had indicated that the thiourea compounds formed by reaction of the isothiocyanates with amines, such as benzylamine, served as more suitable models for relative fluorescence studies than the reagents *per se*. In a similar manner, the *N*-benzyl and other thiourea derivatives were prepared from the aforementioned isothiocyanates (I, II, V, VI, VIII, and XVI) for comparison of fluorescence. The fluorescence characteristics of the compounds of interest are summarized in Table I. The fluorenes were not included because they lacked sufficient fluorescence for reliable measurement. Since 9-isocyanatoacridine (9) was among the more promising of the reagents synthesized, a further series of its derivatives (X–XV) was prepared and their relative fluorescence is also reported in Table I. It became evident that even with this more promising reagent, the derivatives of interest exhibited a considerable decrease in fluorescence.

Compound XXI was desired to examine the effect of isothiocyanato at the 2-position and to obtain increased water solubility as the amine hydrochloride salt. Compound XXI was prepared by the condensation of XVIII with *N,N*-diethylethylenediamine in the presence of phosphorus oxychloride to form XIX. Reduction with stannous chloride and hydrochloric acid yielded the 2-amino compound (XX), which was reacted with thiophosgene to give XXI as the hydrochloride salt. However, as indicated in Table I, this compound and its *N*-benzylthiourea (XXII) are not intensely fluorescent.

A study was initiated to remove the aromatic isothiocyanate moiety from conjugation with the acridine nucleus so as to retain, and not effect, the high fluorescence of the acridines. Examples of the following two types of isothiocyanates (A and B) were synthesized as indicated.

Type A compounds (XXIV, XV, XVII, and XVIII) (Scheme I) exhibited a moderate intensity of fluorescence (Table I). Type B compounds (XXX, XXXI, and XXXIV) (Scheme II) also showed moderate intensity. However, they were prone to cleave at the 9-position to yield 2-methoxy-6-chloro-9-acridone. This proved to be an important limitation in their synthesis and subsequent use.

Of the polycyclic compounds studied, the 9-acridine derivatives were the most promising reagents. Work has been most extensive with 9-isothiocyanatoacridine. However, its derivatives (X–XV) generally have a low intensity of fluorescence and use (6) depends upon a unique photochemical cyclization (9).

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Vehicle Effects on Ocular Drug Bioavailability I: Evaluation of Fluorometholone

JAMES W. SIEG and JOSEPH R. ROBINSON ˣ

Abstract □ The influences of drug concentration and vehicle composition on the corneal penetration of the steroid fluorometholone were studied in the albino rabbit. Aqueous dosing systems included a saturated solution and 0.1, 0.05, and 0.01% suspensions of micronized fluorometholone. Two different doses of a 0.1% oleaginous ointment were also studied. The results from the 0.1 and 0.05% suspensions show a peak aqueous humor steroid concentration at 30 min and a substantial sustaining effect with these two concentrations. The results also support the belief that moderate dilution of a suspension of a slowly soluble drug may not substantially lower the aqueous humor drug levels or, conversely, that use of a higher concentration suspension may not improve the aqueous humor drug concentration–time profile. The 0.01% suspension and the saturated solution did not produce a sustaining effect. The results demonstrate for the first time that the particles present in a dose of suspension are retained within the cul-de-sac of the eye and contribute significantly to the amount of steroid penetrating the cornea. This finding was confirmed by a study in which the eye was rinsed with saline solution 30 min after instillation of a dose of a 0.05% suspension. The rinsing procedure prematurely terminated the sustaining effect of the suspension. The results of the ointment studies show that partitioning of the lipophilic steroid from the oleaginous vehicle has a greater rate-limiting influence on corneal penetration than the dissolution rate parameter associated with the aqueous suspensions. Peak aqueous humor concentration was not achieved until 3 hr after dosing and was comparable to the 0.1 and 0.05% suspensions. Predosing of the eye with a saturated solution or the 0.1% suspension prior to dosing with ointment overcomes the inability of the ointment to provide adequate drug at short times following dosing. In this case, peak levels were achieved within 60 min and then maintained. The duration of aqueous humor levels and the amount penetrating from the ointment were greater than the suspensions, and these effects are discussed relative to the mechanism. Differences in aqueous humor levels produced by 25- and 50-mg doses of ointment were minimal. A discussion of the results from all studies is presented in the context of present theories regarding the role of the lipophilic epithelial layer of the cornea as a barrier to drug penetration.

Keyphrases □ Fluorometholone—effect of drug concentration and vehicle composition on corneal penetration, rabbits □ Ocular drug bioavailability, fluorometholone—effect of drug concentration and vehicle composition on corneal penetration, rabbits □ Bioavailability, ocular, fluorometholone—effect of drug concentration and vehicle composition on corneal penetration, rabbits

Steroids are widely used to treat various inflammatory disorders of the eye and to prevent progressive complications resulting from other ocular disease states. As a result, ophthalmic studies relating to the nature of topically administered steroids and to the extent to which they penetrate into ocular tissues have received increasing attention during the past 20 years. These investigations have provided information regarding the intraocular penetration of dexamethasone (1–6), prednisone (6–10), prednisolone (6–10), cortisone (11–13), hydrocortisone (11–13), and other related corticosteroids (12). Some studies have also included various derivatives of the parent compounds and have provided information pertaining to the influence of a drug's relative lipid and water solubilities on its ability to penetrate the epithelial layer of the cornea (5, 6, 9).

In addition, the dosing systems used in these studies have been formulated to include aqueous solutions (2–4, 10), suspensions (1, 7–9, 11–13), and various ointment vehicles (4, 5, 10). However, a mechanistic interpretation of the influence of these vehicles on the biological properties of the drug in ocular studies has not been explored in the literature. The present study examines the influence of vehicles on the intraocular penetration of the highly lipophilic steroid fluorometholone.

BACKGROUND

Whenever a true solution of a drug is administered to the eye, the ultimate aqueous humor drug concentration that may be attained is influenced by a number of controlling factors. These variables include the concentration of the drug in the vehicle (9, 10, 13), the volume of the instilled dose (14), the viscosity of the vehicle (15, 16), the influence of tear turnover and drainage on the instilled dose (14), and the absorption and elimination characteristics of the drug. For ophthalmic suspension systems, the additional parameters of intrinsic solubility and dissolution rate must be considered since the residence or contact time of the drug in the eye is usually brief.

The intrinsic solubility of the drug is important for suspension systems since it determines the amount of drug actually in solution and available for immediate absorption upon instillation of the dose. As the intrinsic solubility of the drug increases, the concentration of the drug in the saturated solution surrounding the suspended drug particles also increases. For this reason, any comparison of different drugs in suspension systems should include their relative intrinsic solubilities since the observed differences in their biological activities may be ascribed wholly or in part to their differences in this physical parameter.

The dissolution rate parameter must be considered for ophthalmic suspensions in light of the minimal residence time exhibited by most aqueous systems in the eye (14, 17). Upon instillation of the dose, drug from the saturated solution surrounding the suspended particles enters the lipophilic epithelium. It is then transported into the hydrophilic stromal layers of the cornea and ultimately penetrates the less lipophilic endothelium to the aqueous humor. As the initial saturated solution becomes depleted, the particles must dissolve to provide a further supply of drug. The requirement here is that the particles must undergo significant dissolution within the residence time of the dose in the eye if any benefit is to be gained from their presence in the dosing system.

For a drug whose dissolution rate is rapid, the dissolution requirement may present few problems; but for a slowly soluble substance, the dissolution rate becomes critical. If the dissolution rate is not sufficiently rapid to supply significant additional dissolved drug, there exists the possibility of a slowly soluble substance in suspension which provides no more drug to the aqueous humor than does a more dilute suspension or saturated solution of the substance in a similar vehicle (18, 19).

The particle size of the suspended drug affects the surface area available for dissolution. Apart from dissolution, particle size also plays an important role in the irritation potential of the dosing system. This consideration is important since irritation produces excessive tearing and rapid drainage of the instilled dose. It has been recommended that particles be less than 10 μm to minimize particle irritation to the eye.

Relative to an aqueous ophthalmic suspension or solution, the ultimate performance of an ointment system in the eye is controlled by a balance of positive and negative physicochemical considerations. On the positive side, there is the possibility of increased contact time for an ointment over that of an aqueous suspension or solution. In addition, for water-insoluble drugs, there exists a means of increasing the soluble drug concentration in the dosing system by choosing an oleaginous vehicle in which the drug is soluble. On the negative side, ophthalmic ointment systems present mixing problems between the ointment vehicle and the tears and also include an additional partitioning parameter for the drug between the ointment and the tear film.

The contact time of the ointment system with the precorneal tear film is largely determined by the viscosity of the vehicle and its other physical characteristics, in particular its miscibility with the ocular fluids. While the contact time can be extended by increasing the viscosity of the ointment vehicle or by decreasing its degree of miscibility with the tears, these same parameters also present mixing problems unfavorable for optimum partitioning and distribution of the drug from the ointment base to the tear film.

Mixing is an extremely important parameter since it determines the amount of surface area of instilled ointment that is available for partitioning within the time limitation imposed by drainage loss. The extent of this partitioning interface greatly influences the amount of drug released from the ointment and also affects the kinetics of drug distribution and the rate at which the drug partitions from the ointment to the precorneal tear film. At this point, it might seem reasonable to assume that an emulsion type of ointment base would be ideal in view of the large surface areas characteristic of this system. However, emulsion systems also have their own inherent problems involving stability, attainment of adequate viscosities, and the unpredictable effect of surfactants upon corneal drug transport and, most importantly, upon the integrity of the epithelial layer of the cornea (20, 21).

EXPERIMENTAL

Materials—Tritium-labeled fluorometholone preparations were formulated by the manufacturer[1] as a 0.1% aqueous micronized suspension and a 0.1% petrolatum-based ointment. The final products were of commercial quality, equivalent to other similar formulations currently available from the manufacturer. The specific activity of the two preparations was 40 μCi/mg of steroid.

Liquid scintillation counting vials and solutions were obtained from commercial sources[2,3]. All other chemicals used were either reagent or analytical grade, and water was double distilled from alkaline permanganate.

Male albino rabbits[4], 1.8–2.4 kg, were used. The rabbits were fed a regular diet with no restrictions on the amount of food or water consumed. Lighting and auditory stimulation (radio) were maintained in the caging facilities for 24 hr a day to provide a constant experimental environment.

Aqueous Humor Concentration *versus* Time Profile—*Fluorometholone Suspensions*—Male albino rabbits were placed into wooden restraining boxes to minimize movement. The normal upright posture of the animals was maintained at all times. Care was taken to permit normal eye movements, and head movement was only minimally restricted. The use of local and general anesthetics was avoided throughout the study. Recent work has demonstrated the effects of topical local anesthetics on tear dynamics and ocular drug bioavailability (22) and the influences of general anesthesia on lacrimal and instilled fluid dynamics (4) and on the corneal penetration of fluorometholone (19) in rabbits. Throughout each experimental run, the physical state of the test animals was as near normal as was experimentally feasible.

Fifty-microliter doses were administered, using a microliter syringe, by instillation directly onto the corneas of both eyes, collecting in the lower cul-de-sac. During instillation, the upper lid was slightly raised and the lower lid was pulled slightly away from the globe. The lids were immediately returned to their normal position after instillation was completed. Animals were then sacrificed at 20, 30, 60, 90, 120, 180, 240, and 300 min postinstillation by rapid injection of pentobarbital sodium into a marginal ear vein. The corneal surface was thoroughly washed with distilled water and dried with tissue. Aqueous humor samples were then removed from the anterior chamber using a 1-ml tuberculin syringe fitted with a 27-gauge needle.

One hundred-microliter samples of aqueous humor were transferred[5] to scintillation counting vials containing 5 ml of liquid scintillation solution. Vials were refrigerated at 0° for 24 hr prior to the addition of the samples and stored in the dark at room temperature for at least 72 hr before counting to minimize chemiluminescence. Ten-minute counts of each sample were made using a liquid scintillation spectrometer[6]. The final count rate obtained for each sample was then converted to a microgram of steroid per milliliter of aqueous humor basis by standardization techniques to facilitate the analysis and interpretation of results.

To investigate the effect of suspension concentration on the aqueous humor steroid levels, it was necessary to dilute the original 0.1% fluorometholone suspension to the desired concentrations. The potential influence of this procedure upon the final results will be discussed later. The 0.05 and 0.01% suspensions were prepared by diluting an aliquot of the original suspension with an appropriate volume of the suspension vehicle supplied by the manufacturer.

The diluted suspensions were then allowed to equilibrate on a shaker at room temperature for several days. Equilibration was verified by centrifuging samples taken from each suspension at various times and analyzing the supernatant liquids using liquid scintillation methods. Equilibration was assumed to be complete when the count rate for the supernatant samples reached a constant value.

Fluorometholone Saturated Solution—The saturated solution was prepared from the original 0.1% suspension of micronized fluorometholone. A laboratory clinical centrifuge[7] was used to separate the saturated solution and particulate components of the suspension. A tube containing 5 ml of the suspension was centrifuged at the highest speed setting until the supernate appeared clear by visual observation. The supernatant liquid was then removed and passed through a 0.22-μm biological filter[8]. The high initial specific activity of the original 0.1% fluorometholone suspension was required so that the saturated supernatant liquid obtained by centrifugation retained sufficient activity to give adequate counting rates in the aqueous humor studies.

Experimental runs using the saturated fluorometholone supernatant liquid were performed using the techniques previously described for the suspensions.

0.05% Fluorometholone Suspension Rinsed with Saline at 30 min Postinstillation of Dose—A modified suspension study was performed using a standard 50-μl dose of a 0.05% suspension. Thirty minutes after dosing, the eyes of the test animals were thoroughly flushed with isotonic saline injection containing no preservatives. A polyethylene wash bottle with a fine-drawn tip was used so that a stream of saline could be directed into all portions of the eye with sufficient force to remove any residual fluorometholone particles. The eyes were then left untouched and the animal was kept in a normal experimental posture for the completion of the run. No changes in the basic routine were made beyond the time of rinsing with saline.

0.1% Fluorometholone Ointment—Two different doses of fluorometholone ointment were used: 25 and 50 mg. The individual doses were weighed on an analytical balance prior to each experimental run. Dosing was carried out by means of a small laboratory microspatula. The weighed dose of ointment was carefully transferred to the spatula and then placed inside the center of the lower lid, with care being taken not to irritate the eye or touch the corneal epithelium with the instrument. The lower lid was gently moved upward to spread the dose uniformly over the corneal surface and then released. No further mechanical action was performed, and the remainder of the run was carried out using the same basic experimental techniques previously discussed.

[1] Allergan Pharmaceuticals, Irvine, Calif.
[2] Mini Vial, ICN Isotope and Nuclear Division, Cleveland, Ohio.
[3] Aquasol, New England Nuclear, Boston, Mass.
[4] Klubertanz, Edgerton, Wis.
[5] Biopette, Schwarz/Mann, Orangeburg, N.Y.
[6] Packard 2002, Packard Instrument Co., Downers Grove, Ill.
[7] Model CL, International Equipment Co., Needham Heights, Mass.
[8] Millipore, Millipore Corp., Bedford, Mass.

0.1% Fluorometholone Ointment (25 mg) Predosed with Fluorometholone Saturated Solution—This study involved a combination of the techniques described earlier for the fluorometholone saturated solution and the 0.1% fluorometholone ointment. Each run was initiated by dosing both eyes of the test animals with 50 μl of fluorometholone saturated solution. Ten minutes after instillation of the saturated solution, a 25-mg dose of 0.1% fluorometholone ointment was instilled and spread over the corneal surface as in the previous ointment study.

RESULTS

Aqueous Humor Steroid Levels following Dosing with Fluorometholone Saturated Solution—To determine the contribution of the micronized steroid particles to the aqueous humor drug levels, a study was conducted using only the saturated solution component of the suspension. These results would provide a baseline to evaluate the rate and extent to which the particles dissolve when instilled into the eye. Any observed differences in aqueous humor steroid levels between the saturated solution and the suspension could then be attributed to the particles, and the magnitude of this difference would provide some insight into their physical behavior in the eye.

The results of the study, using a 50-μl dose of saturated solution, are presented in Table I. The aqueous humor concentration *versus* time profile is depicted in Fig. 1. The results indicate that fluorometholone is a penetrating steroid which crosses the intact cornea to a significant extent within a short period following dosing, reaching a peak concentration in the aqueous humor at 30 min. The aqueous humor steroid levels then begin to decline rapidly. The apparent first-order rate constant for elimination was calculated to be 0.021 min^{-1}. A rigorous pharmacokinetic analysis of all data presented in this report will be presented in a subsequent publication.

Aqueous Humor Steroid Levels following Dosing with 0.1, 0.05, and 0.01% Fluorometholone Suspensions—Table I also presents the data from the studies using the fluorometholone suspensions. A comparison of the aqueous humor concentration *versus* time profiles obtained can be made by referring to Fig. 1. Error bars have been omitted for the sake of clarity. As in the case of the saturated solution, the peak time for each of the three suspension concentrations occurs at 30 min. However, in each case the peak aqueous humor steroid concentration was statistically higher than the baseline value obtained for the saturated solution. This finding may be attributed to the rapid dissolution and availability of small particles in the system or to protein binding of drug to the components of tears.

The 0.1% suspension maintains high aqueous humor levels for about 3 hr. Then the aqueous humor levels begin to decrease rapidly, indicating that the sustaining mechanism has lost its capacity to provide additional drug. A first-order kinetic analysis of the linear terminal portion of the curve results in an apparent elimination rate constant of 0.015 min^{-1}. This value is in good agreement with that obtained for the saturated solution.

The aqueous humor levels of fluorometholone presented in Table I for the 0.1% suspension are somewhat smaller than the values previously reported for a 0.1% suspension (19). The peak concentration at 30 min was approximately three times larger than the current value. These differences are due to the different methods of preparation of the two suspensions.

The previous work was performed using a suspension prepared in this laboratory, since a commercially prepared tritium-labeled suspension was not available. A 0.1% suspension was prepared by suspending 10 mg of micronized fluorometholone in 10 ml of the ophthalmic vehicle. An aliquot of tritium-labeled fluorometholone was then added, and the suspension was allowed to equilibrate on a shaker for several days. This method is similar to that used for the preparation of prednisolone suspensions (18). The suspension used in the current study was commercially prepared. The tritium label was first randomized by recrystallization. The steroid was then micronized to a particle size of not larger than 2.5 μm and reconstituted with ophthalmic vehicle according to the manufacturer's commercial specifications.

The results from the studies using the two suspensions demonstrate the importance of the physical properties of a suspension in regard to corneal drug penetration. While the qualitative aspects and the interpretation of the results of the previous study are not affected by these considerations, the values reported in the present study more accurately reflect the aqueous humor steroid levels achieved using a commercially available 0.1% fluorometholone suspension.

Table I—Concentration of Fluorometholone in Aqueous Humor following Topical Application of a Saturated Solution and 0.1, 0.05, and 0.01% Suspensions

t, min	Number of Eyes	Micrograms per Milliliter of Aqueous Humor
Fluorometholone Saturated Solution		
20	8	0.059 (0.003)[a]
30	6	0.077 (0.007)
60	8	0.043 (0.005)
90	9	0.017 (0.001)
120	6	0.014 (0.001)
0.1% Fluorometholone Suspension		
20	12	0.116 (0.010)
30	12	0.144 (0.016)
60	11	0.116 (0.016)
90	8	0.099 (0.010)
120	10	0.084 (0.012)
180	6	0.066 (0.010)
240	6	0.023 (0.005)
300	4	0.011 (0.001)
0.05% Fluorometholone Suspension		
20	10	0.097 (0.014)
30	8	0.120 (0.011)
60	6	0.103 (0.011)
90	8	0.085 (0.009)
120	8	0.073 (0.006)
0.01% Fluorometholone Suspension		
30	4	0.111 (0.007)
60	6	0.053 (0.006)
90	4	0.043 (0.007)
120	6	0.020 (0.003)

[a] Numbers in parentheses represent standard error of the mean.

The results of the study using the 0.05% fluorometholone suspension demonstrate that moderate dilution of the original suspension does not significantly lower the aqueous humor concentration of steroid. A statistical analysis of the data from the two studies failed to show a significant difference between the 0.1 and 0.05% suspensions. These results support the belief that higher concentrations of suspensions containing slowly soluble substances do not necessarily provide increased amounts of drug to the aqueous humor. Further work with more concentrated fluorometholone suspensions will be necessary to confirm this observed behavior over a wide range of concentrations.

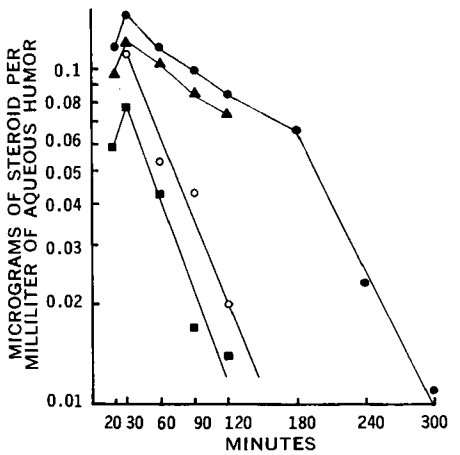

Figure 1—*Aqueous humor steroid concentrations following topical dosing with aqueous fluorometholone suspensions and saturated solution. Key:* ■, *0.05 ml of saturated solution;* ●, *0.05 ml of 0.1% suspension;* ▲, *0.05 ml of 0.05% suspension; and* ○, *0.05 ml of 0.01% suspension.*

Table II—Concentration of Fluorometholone in Aqueous Humor following Dosing with 0.05% Suspension and Rinsing with Saline at +30 min

t, min	Number of Eyes	Micrograms per Milliliter of Aqueous Humor
60	4	0.100 (0.017)[a]
90	8	0.065 (0.008)
120	6	0.043 (0.002)
180	7	0.014 (0.004)

[a] Numbers in parentheses represent standard error of the mean.

Although the experimental design for the 0.01% suspension was the same as that used for the 0.1 and 0.05% suspensions, the resulting aqueous humor concentration *versus* time profile is substantially different. The peak time and peak aqueous humor concentration are in good agreement with the two previous suspension concentrations, but Fig. 1 clearly indicates that the 0.01% suspension does not show the sustaining effect. Once the peak aqueous humor concentration is achieved, the 0.01% suspension undergoes a rapid decline in aqueous humor steroid levels. This decline is similar to that of the saturated solution and the linear terminal portion of the profile for the 0.1% suspension. First-order kinetic analysis of the curve for the 0.01% suspension gives an apparent elimination rate constant of 0.019 min^{-1}.

Aqueous Humor Steroid Levels following Dosing with 0.05% Fluorometholone Suspension and Rinsing with Saline—To confirm that the sustaining mechanism of the suspension systems is due to steroid particles retained within the cul-de-sac of the eye, a study was performed in which the eyes of the test animals were thoroughly rinsed with saline solution 30 min after instillation of 50 μl of the 0.05% fluorometholone suspension. The data obtained are presented in Table II.

The first sample point chosen for this study was 30 min after rinsing of the eyes with saline. This time corresponds to a sample time of 60 min postinstillation of the initial dose of suspension. It can be seen in Fig. 2 that the aqueous humor steroid concentration at this time point coincides with that obtained from the previous study using the 0.05% suspension. However, beyond this point the aqueous humor steroid levels begin a characteristic decline as observed in previous cases. The apparent first-order rate constant for elimination was calculated to be 0.016 min^{-1}, in good agreement with those values calculated for the 0.1 and 0.01% suspensions and the saturated solution.

Aqueous Humor Steroid Levels following Dosing with 0.1% Fluorometholone Ointment—Since studies with the aqueous fluorometholone suspensions demonstrated the importance of particle dissolution in achieving increased aqueous humor steroid levels over those produced by a simple saturated solution, it was considered worthwhile to investigate another physical parameter. Fluorometholone is a steroid with very low water solubility and strong lipophilic character. It was felt that incorporating this steroid in an oleaginous ointment vehicle would yield valuable information regarding its behavior in the eye. Partitioning of the drug from the oleaginous vehicle to the precorneal tear film would then be a potentially rate-limiting step in the corneal penetration process and would be reflected in the results of studies performed in the same manner as those using the aqueous suspensions.

Table III—Concentration of Fluorometholone in Aqueous Humor following Topical Application of 0.1% Ointment

t, min	Number of Eyes	Micrograms per Milliliter of Aqueous Humor
	50-mg Dose	
30	5	0.017 (0.007)[a]
60	4	0.065 (0.014)
120	4	0.105 (0.023)
180	4	0.136 (0.023)
240	4	0.114 (0.022)
300	4	0.106 (0.027)
360	4	0.062 (0.021)
420	4	0.026 (0.006)
480	4	0.009 (0.001)
	25-mg Dose	
120	4	0.102 (0.016)
180	4	0.146 (0.006)
240	4	0.102 (0.010)
300	4	0.053 (0.010)
360	4	0.029 (0.003)

[a] Numbers in parentheses represent standard error of the mean.

The results of the studies using two different doses of the 0.1% ointment are presented in Table III and illustrated by the curves in Fig. 3. Runs using the 50-mg dose gave a slow rise in aqueous humor steroid levels, with the peak concentration occurring at 180 min. The aqueous humor steroid concentration is then maintained at high levels for up to 360 min, giving the curve a broad bell shape in the interval from 120 to 360 min. The declining steroid levels beyond the 360-min time point were analyzed using first-order kinetics, and the linear terminal portion of the curve yielded an elimination rate constant of 0.018 min^{-1}. This value is again in accordance with the value obtained for the aqueous fluorometholone systems.

To assess the effect of smaller doses of ointment, a study was performed using a 25-mg dose of the same 0.1% fluorometholone ointment. A comparison of the aqueous humor concentration *versus* time profiles for the two doses can be made by referring to Fig. 3. The 25-mg dose produces statistically equivalent levels of aqueous steroid in the time interval from 120 to 240 min.

Aqueous Humor Steroid Levels of 0.1% Fluorometholone Ointment Predosed with Fluorometholone Saturated Solution—Since the results from the ointment studies indicated that partitioning of the lipophilic steroid from the oleaginous ointment base was limiting the rate at which peak aqueous humor steroid levels were being achieved, the effect of providing immediately available steroid to the precorneal tear film in conjunction with a dose of ointment was investigated. To effect this dosing requirement, a 50-μl dose of fluorometholone saturated solution was in-

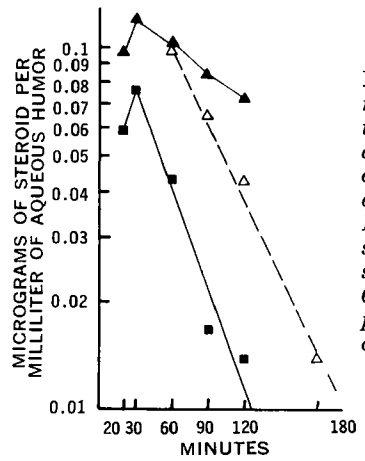

Figure 2—*Aqueous humor steroid concentrations following topical dosing with aqueous fluorometholone suspensions and saturated solution. Key: △, 0.05 ml of 0.05% suspension rinsed with saline at +30 min; ▲, 0.05 ml of 0.05% suspension; and ■, 0.05 ml of saturated solution.*

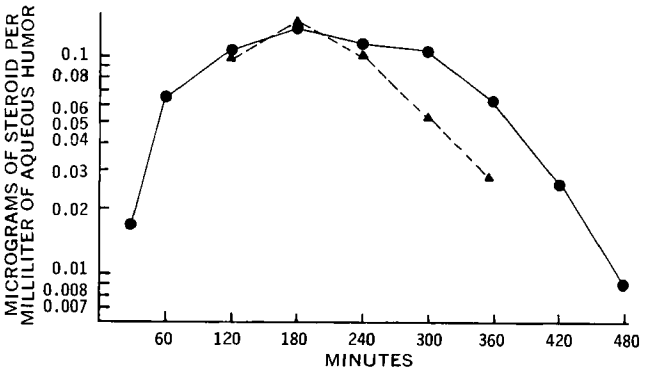

Figure 3—*Aqueous humor steroid concentrations following topical dosing with 0.1% fluorometholone ointment. Key: ●, 50-mg dose; and ▲, 25-mg dose.*

Table IV—Concentration of Fluorometholone in Aqueous Humor Using 0.1% Ointment Predosed with Fluorometholone Saturated Solution

t, min	Number of Eyes	Micrograms per Milliliter of Aqueous Humor
30	4	0.065 (0.013)[a]
60	4	0.130 (0.014)
120	4	0.100 (0.011)
180	4	0.098 (0.010)

[a] Numbers in parentheses represent the standard error of the mean.

stilled into the eyes of the test animals 10 min prior to the instillation of a 25-mg dose of the 0.1% ointment.

The results of this study are presented in Table IV. A comparison of the aqueous humor steroid concentration *versus* time profiles for this study and the two previous ointment studies can be made by referring to Fig. 4. It is clear that the immediate availability of the steroid from the saturated solution allows for a rapid rise in aqueous humor steroid levels relative to the other two ointment studies. The 30-min steroid level is significantly higher than that obtained for the nonpredosed ointments, and the peak aqueous humor steroid concentration is achieved 60 min after instillation of the dose—2 hr prior to the peak in the previous studies. The aqueous humor steroid concentration is then maintained at high levels as in the case of the nonpredosed ointment studies.

DISCUSSION

Contribution to Aqueous Humor Steroid Levels by Fluorometholone Particles in Aqueous Dosing Systems—The formulation of a drug as an aqueous suspension presumably has the advantage that the micronized particles persist within the cul-de-sac of the eye for prolonged periods. Particle dissolution then provides a sustaining source of drug to the precorneal tear film. Since fluorometholone possesses such a high degree of lipophilic character, one would not intuitively expect an aqueous dissolution rate rapid enough to provide significant amounts of additional steroid within the residence time of the particles in the eye. With this assumption, one would then expect that a suspension of this steroid would not provide significant increases in aqueous humor steroid levels over that of a simple saturated solution. In the absence of significant particle dissolution, the drug that penetrates the cornea would come only from the steroid actually in solution upon instillation of either dosing system. Since the solution surrounding any suspension of particles is in a state of saturation, the two dosing systems would be essentially equivalent in their ability to provide drug to the precorneal tear film.

The results of the suspension and saturated solution studies presented in this paper demonstrate that this is only partially correct. A comparison of the curves in Fig. 1 clearly shows that a significant amount of fluorometholone is provided by the particles in the suspensions. The aqueous humor steroid levels are statistically higher for the suspensions relative to the saturated solution. These results require an explanation as to the mechanism by which the particles are able to provide additional drug to the eye rapidly. The manner in which this dissolution process occurs can perhaps be explained on the basis of the extremely large surface areas associated with micronized suspensions. Considering the small volume of fluid actually in the eye (14) and the extensive surface area available for dissolution, it does not seem unreasonable that the suspension is able to provide additional steroid quickly.

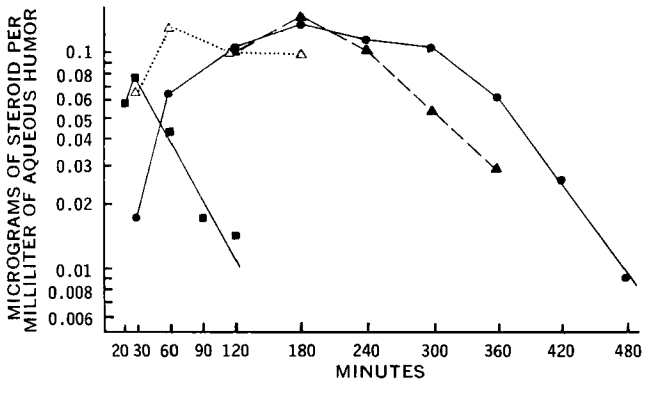

Figure 4—*Aqueous humor steroid concentrations of fluorometholone following topical dosing with 0.1% ointment and aqueous saturated solution. Key: △, 25 mg ointment predosed with 0.05 ml saturated solution; ●, 50 mg ointment; ▲, 25 mg ointment; and ■, 0.05 ml of saturated solution.*

The natural surface-active properties of the tears and the conjunctival mucin (23, 24) would also be expected to exert a favorable influence on the dissolution rate of the instilled particles. This additional steroid would then be available to enter the lipophilic epithelial layer of the cornea. Hence, one sees significant increases in aqueous humor steroid levels with the suspensions over those produced by the saturated solution, even at very short times following instillation of the doses. However, the interplay of contact time and dissolution rate is illustrated by the fact that the 0.1% suspension does not significantly improve the aqueous humor steroid levels over those produced by the 0.05% suspension.

The results obtained from the study using the saline-rinsed 0.05% suspension (Fig. 2) confirm for the first time that particles in an instilled dose of suspension are retained within the cul-de-sac of the eye. These particles are also responsible for the sustaining effect produced by the 0.1 and 0.05% suspensions. Evidence comes from the observation that the sustaining effect seen with the suspension is prematurely terminated by the rinsing process. The data for the rinsed suspension also show that steroid is provided to the aqueous humor for approximately 30 min beyond the point that the particles are removed from the cul-de-sac. This evidence strongly supports the existence of a reservoir somewhere in the cornea.

To explain the nonsustaining results from the 0.01% suspension study, it is worthwhile to note that this is an extremely dilute system and contains quite a small fraction of particles. Preliminary data from this laboratory indicate that this system contains only about 10 times the amount of steroid present in the saturated solution. A certain degree of particle contribution is evidenced by the fact that the 30-min point for the 0.01% suspension is statistically higher than that for the saturated solution. However, this contribution is not sufficient to sustain the aqueous humor levels. A reasonable explanation would be rapid removal of the small number of particles through the processes of drainage and tear turnover.

It is appropriate at this point to mention the possible influence on the results due to the method of preparation of the 0.05 and 0.01% suspensions. Since the 0.1% suspension was the only formulation available from the manufacturer at the time this study was performed, it was necessary to dilute the original suspension to produce the two lower concentrations. Although the vehicle itself was not altered in any way, subsequent equilibration after dilution was probably achieved at the expense of the smaller particles in the systems. Thus, the range of particle sizes was perhaps not consistent throughout the various suspension systems, with the lower concentrations possibly having a smaller percentage of the finer particles and a narrowed size distribution. This could have also influenced the behavior of the 0.01% suspension, since this system would have been affected the most by the dilution and equilibration process, especially if the sustaining mechanism is primarily maintained by the finer particles in the suspension.

If the method of preparation does have an effect on the results, the implication is that particle size has an influence on the aqueous humor drug levels produced by a suspension system. Current studies in this laboratory are being performed to investigate the influence of particle size on the corneal penetration of steroids and the results will be reported.

Effects of Vehicle on Aqueous Humor Levels of Fluorometholone—The epithelial layer of the cornea is highly lipophilic in character, whereas the stromal region tends to be more hydrophilic. It does not seem unreasonable to expect that a highly lipophilic steroid such as fluorometholone will tend to concentrate in the epithelial layer, perhaps even reaching a saturation concentration prior to passage through the hydrophilic layers of the cornea. Such a mechanism is not unlikely and was recently speculated upon for other steroidal compounds in a study using prednisolone acetate (9). In such a process, the rate and extent to which the epi-

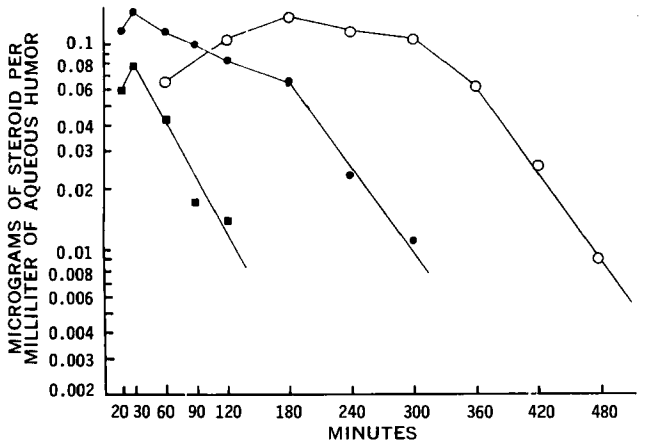

Figure 5—*Comparison of the aqueous humor steroid concentrations of fluorometholone following dosing with two aqueous dosing systems and 0.1% ointment. Key:* ■, *0.05 ml of saturated solution;* ●, *0.05 ml of 0.1% suspension; and* ○, *50-mg dose of ointment.*

thelial layer is saturated with steroid as well as the duration of this condition will influence the peak time, the peak concentration, and the maintenance of high steroid levels in the aqueous humor.

Figure 5 provides a perspective for a comparison of the results from the aqueous dosing systems and the oleaginous ointment. These results show several important aspects regarding the influence of the vehicles used in the present study:

1. The suspension does not obey strict sustained-release principles as evidenced by the absence of a true plateau in its aqueous humor concentration *versus* time profile.

2. The oleaginous vehicle causes an increase in the time required to reach peak steroid levels in the aqueous humor.

3. The area under the curve for the 0.1% ointment is considerably greater than that produced by a similar dose of the 0.1% suspension.

Upon instillation of a dose of the aqueous systems, the drug is immediately available from the steroid present in solution, as reflected by the relatively short peak time seen with these systems. For the suspensions, additional steroid becomes available through the process of particle dissolution. This process serves to maintain high levels in the aqueous humor but is not sufficient to provide a true sustaining mechanism. The ointment does sustain relatively constant levels of steroid but suffers from a delay in the time required to achieve these levels. Failure of the suspension to produce a true plateau indicates that a constant drug concentration in the tear film could not be maintained, due presumably to the slow dissolution rate of drug and/or drainage loss of drug.

The shift of the peak time seen with the ointment indicates a change in the rate-limiting mechanism. Unlike the aqueous systems, the ointment does not provide immediately available steroid. Partitioning of the steroid from the oleaginous vehicle decreases the availability of drug for a period of time following dosing. This partitioning process becomes the rate-controlling step in this case and causes a delay in the time to reach peak aqueous humor steroid levels. This delay can be easily overcome, as shown by the predosed ointment study. The dose of saturated solution compensates for the inability of the ointment to provide steroid immediately following dosing. This finding suggests that an oil-in-water or water-in-oil emulsion system might generate a better drug concentration–time profile. Furthermore, the delayed nature of the partitioning process for the ointment is reinforced by the fact that the 30-min steroid levels for the original saturated solution study and the predosed ointment study are essentially the same (Fig. 4).

Future studies will be performed in this laboratory using higher concentrations of fluorometholone ointment to investigate the influence of this parameter. Higher concentrations of steroid should provide greater amounts of steroid to the precorneal tear film (assuming it is less than saturated), and the early portion of the profile should show a steeper rise of higher levels of aqueous humor steroid at the earlier time points.

Although the peak time is extended in the case of the ointment, the duration of the aqueous humor levels is longer relative to the suspension. Increased contact time of the ointment is a possible explanation for this finding, but this cannot be stated with certainty. It is clear, however, that the area under the curve for the ointment is greater than that for the suspension. It must be concluded that the availability of fluorometholone from the ointment is greater. Therefore, it appears that for fluorometholone the greatest ocular bioavailability would be obtained by predosing the eye with a saturated solution or the 0.1% suspension followed by a dose of ointment.

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Radioimmunoassay of Chlordiazepoxide in Plasma

W. R. DIXON *x, J. EARLEY ‡, and E. POSTMA *

Abstract □ A simple, rapid, and sensitive radioimmunoassay was developed for the determination of chlordiazepoxide (7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide) in plasma. Antiserum capable of binding chlordiazepoxide-2-^{14}C was obtained from rabbits following immunization with an antigen prepared by coupling the reactive acyl azide of 7-chloro-5-(4-hydrazinocarbonylmethoxyphenyl) - 2 -methylamino-3*H*-1,4-benzodiazepine 4-oxide to bovine serum albumin. The radioimmunoassay allows for the specific determination of chlordiazepoxide directly in plasma without extraction and was compared with a differential spectrofluorometric assay for chlordiazepoxide. Both methods gave satisfactory agreement for the plasma levels of chlordiazepoxide in human subjects resulting from single and chronic oral doses of chlordiazepoxide hydrochloride.

Keyphrases □ Chlordiazepoxide—radioimmunoassay in plasma, compared to spectrophotofluorometric method □ Radioimmunoassay—chlordiazepoxide in plasma, compared to spectrophotofluorometric method

Chlordiazepoxide hydrochloride[1,2] (II), which was synthesized in the mid-1950's (1), was the first member of the 1,4-benzodiazepine class of compounds to be used clinically as an antianxiety agent in humans (2, 3) and is widely prescribed.

Three satisfactory methods have been developed for the determination of chlordiazepoxide in blood. In the most widely used technique, a spectrofluorometric assay (4) into which an earlier assay procedure (5) is incorporated, the level of chlordiazepoxide is obtained by subtraction of the fluorescence due to its metabolite, *N*-desmethylchlordiazepoxide, from the fluorescence due to both compounds. However, the determination of chlordiazepoxide is dependent on the accuracy of the assay of the metabolite. The method has a sensitivity limit of about 100 ng/ml using a 2-ml sample of plasma.

Recently, a polarographic method was developed which is specific for chlordiazepoxide (6). The technique involves plasma extraction and TLC isolation of chlordiazepoxide, which is then determined polarographically. The sensitivity limit is about 50 ng/ml using a 2-ml sample. An electron-capture GC technique has been reported (7) with a sensitivity limit of about 30 ng/ml using a 1-ml sample of plasma.

All of these methods require an extraction of chlordiazepoxide; in two instances, a chromatographic step is also employed. Such procedures limit the number of samples that may be analyzed in any one assay run because of the time required to carry out the assay.

The present report concerns the development of a radioimmunoassay for chlordiazepoxide directly in plasma without extraction. The method allows one person to analyze about 100 plasma samples over 2 days, using not more than a 0.1-ml sample of plasma and attaining a sensitivity limit of 20 ng/ml.

EXPERIMENTAL

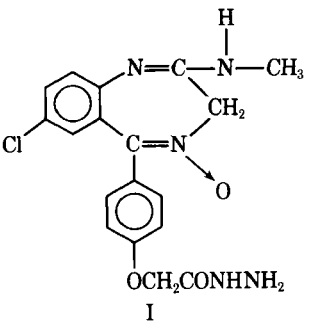
Preparation of Immunogen—7-Chloro-5-(4-hydrazinocarbonylmethoxyphenyl)-2-methylamino-3*H*-1,4-benzodiazepine 4-oxide (I) was converted to its reactive acyl azide and covalently coupled to bovine serum albumin[3] (8) in the following manner. Compound I (45 mg, 0.12 mmole), as a suspension in dimethylformamide (2 ml), was treated with 0.1 ml of 4.7 *N* hydrochloric acid in dioxane to give a pale-yellow solution. The mixture was cooled to −30°, and 0.16 ml of a 1:10 dilution of isoamyl nitrite (0.12 mmole) in dioxane was added. The solution was stirred at −30 to −40° for 30 min; then 0.1 ml of 0.5 *M* aqueous ammonium sulfamate was added and the stirring was continued for 10 min.

The cold azide solution was added slowly, dropwise with stirring, to a solution of bovine serum albumin (72 mg) in 4 ml of 0.16 *M* borate buffer (pH 8.5) at 0°. The pH was kept at 8.0–8.7 by the addition of 1 *N* NaOH. The resulting pale-yellow solution (pH 8.5) was kept at 4° for 36 hr and then dialyzed against 0.05 *M* tromethamine buffer (pH 8) for 3 days. After further dialysis for 2 days against distilled water, the immunogen was isolated by lyophilization.

Estimation of Number of Molecules of Hapten Coupled to Bovine Serum Albumin—A portion of the lyophilized hapten–protein conjugate was dissolved in buffer to give a solution of 1 mg/ml. A control solution of bovine serum albumin was similarly prepared. Each sample was then analyzed by differential pulse polarography (6) against a known amount of chlordiazepoxide. The control solution showed no peaks in its polarogram while the hapten–protein conjugate exhibited two peaks due to reduction of the *N*-oxide function and the azomethine bond in the $C_5{=}N_4$ position of the hapten molecule. On the basis of the peak heights relative to the standard of chlordiazepoxide, it was estimated that approximately eight molecules of the hapten had been covalently coupled to one molecule of the bovine serum albumin.

Immunization—The lyophilized immunogen was dissolved in 0.9% saline to give a solution of 2 mg/ml. After the addition to an equal volume of Freund's complete adjuvant[4], the mixture was emulsified to a thick paste using a blender[5] at 45,000 rpm.

Three New Zealand White female rabbits were injected intra-



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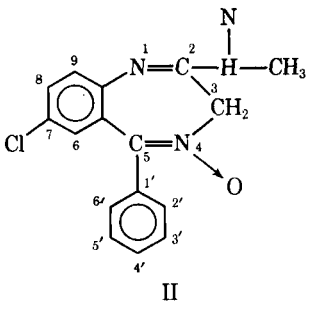

II

[1] The chemical names of the 1,4-benzodiazepines are: chlordiazepoxide, 7-chloro-2-methylamino-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide; *N*-desmethylchlordiazepoxide, 7-chloro-2-amino-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide; demoxepam, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide; diazepam, 7-chloro-1,3-dihydro-1-methyl-5-phenyl-2*H*-1,4-benzodiazepin-2-one; *N*-desmethyldiazepam, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one; and clonazepam, 5-(2-chlorophenyl)-1,3-dihydro-7-nitro-2*H*-1,4-benzodiazepin-2-one.

[2] Librium, containing chlordiazepoxide hydrochloride as its active ingredient, was administered throughout the study.

[3] Sigma Chemical Co., St. Louis, Mo.

[4] Grand Island Biological Co., Grand Island, N.Y.

[5] Virtis "S" 45, Virtis Co., Gardiner, N.Y.

Table I—Specificity of Antiserum

Compound	Cross-Reaction, %
Chlordiazepoxide	100
N-Desmethylchlordiazepoxide	5
Demoxepam	<1
N-Desmethyldiazepam	<1
Diazepam	<1
Clonazepam	<1

dermally on the flank with 0.1 ml of the emulsion at each of five separate sites; this process was repeated twice at 2-week intervals. Each animal then received 0.5 mg of the antigen subcutaneously as a solution in saline and bled from the ear artery 10 days later. Booster injections were given at monthly intervals.

Antiserums—The blood (20–30 ml) was allowed to clot at 37° for 2 hr and then kept at 4° overnight to allow for clot contraction. The serum was decanted, centrifuged at 3000 rpm, and divided into 1-ml aliquots. These aliquots were kept frozen at −20° until used.

Radioimmunoassay Method—The following stock reagents and materials were used: assay tubes[6] (12 × 75-mm disposable culture tubes); chlordiazepoxide-2-^{14}C (specific activity 200 μCi/mg)[7] in 0.001 *N* HCl to give 40,000 cpm/ml; antiserum diluted 1:50 with sodium phosphate buffer (0.01 *M*, pH 7.4) containing 0.1% sodium azide; and standard solutions (20, 50, 100, 200, 500, and 1000 ng/ml) of unlabeled chlordiazepoxide in 0.001 *N* HCl.

The assay procedure involved the addition of a constant volume of 20–100 μl of control human plasma to 0.1 ml of each standard of chlordiazepoxide to generate a calibration curve of 2–100 ng/tube. The same volume of the unknown plasma samples was added to tubes containing 0.1 ml of 0.001 *N* HCl. Two blanks were included by adding the control plasma to 0.1 ml of 0.001 *N* HCl. Then 0.1 ml (4000 cpm) of the chlordiazepoxide-2-^{14}C solution was added to each tube, followed by 0.1 ml of the antiserum solution to all tubes except one blank. The volume in each tube was brought to 1 ml with buffer. After mixing on a vortex, each tube was immersed in an ice water bath for 1 hr.

An equal volume (1 ml) of saturated ammonium sulfate was then added to precipitate globulin-bound chlordiazepoxide-2-^{14}C. After thorough mixing on a vortex and standing at 4° for 15 min, the tubes were centrifuged at 3000 rpm for 30 min at 4°. The supernate containing unbound chlordiazepoxide-2-^{14}C was decanted into a counting vial, and 10 ml of toluene scintillator[8] was added. The vial was then vortexed for 10 sec to extract the radioactive material into the organic phase and each sample was counted[9]. When many samples were to be analyzed, it was convenient to carry out the extraction on a reciprocating shaker for 10 min with the vials in a covered vial tray in the vertical position.

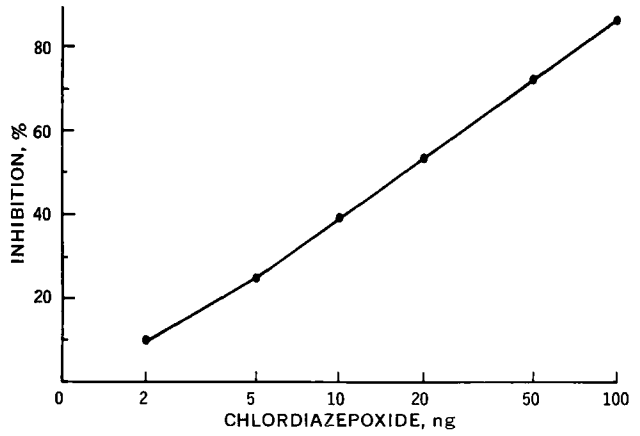

Figure 1—*Standard curve of chlordiazepoxide in 20 μl of human plasma.*

[6] diSPo, Scientific Products, McGaw Park, Ill.
[7] Supplied by Dr. A. Liebman, Hoffmann-La Roche Inc., Nutley, N.J.
[8] Omnifluor, New England Nuclear, Boston, MA 02118
[9] Packard Tri-Carb model 3380.

Table II—Chlordiazepoxide Plasma Levels in Different Subjects at Random Periods after Administration of Chlordiazepoxide Hydrochloride, Determined by the Radioimmunoassay and Spectrofluorometric Methods

Radioimmunoassay Method, μg/ml	Spectrofluorometric Method, μg/ml
Single Dose (30 mg)	
1.35	1.54
1.12	0.97
1.00	1.3
0.65	0.56
0.65	0.52
0.40	0.32
0.30	0.55
0.15	0.30
0.70	1.00
Chronic Dose (10 mg tid)	
1.85	1.50
1.35	1.23
0.95	1.11
0.23	n.m.[a]
0.80	0.90
0.95	0.85
1.25	1.47
0.78	1.0

[a] n.m. = not measurable.

All determinations, including the standards, were done in duplicate, and the average of each set of counts was used in the calculation.

RESULTS

Antibody Production—Each rabbit produced antiserum capable of binding chlordiazepoxide-2-^{14}C when compared to serum from nonimmunized rabbits. Almost identical and usable titers of antibody were obtained from each rabbit at the first bleeding. All data reported in this article were obtained with this first batch of antiserum, where 1 ml of a 1:500 dilution was capable of binding about 60% of the 4000 cpm of the tracer. After four monthly booster immunizations, it became possible to work with 1:1000 dilution of the antiserum.

Sensitivity of Radioimmunoassay—A typical standard curve obtained with chlordiazepoxide in 20 μl of normal human plasma is shown in Fig. 1. The percentage inhibition of binding was calculated by expressing the decrease in binding of the tracer due to unlabeled chlordiazepoxide as a percentage of the total amount bound in the absence of unlabeled chlordiazepoxide. The method appears readily applicable over a range of 2–100 ng/tube. Thus, the sensitivity limit is 20 ng/ml using a 0.1-ml sample of plasma.

Specificity—Several benzodiazepines were tested for their ability to compete with labeled chlordiazepoxide for the antibody. The competitors were tested at a concentration of 200 ng—10 times the concentration of chlordiazepoxide required to produce a 50% inhibition of binding (Fig. 1). The percentage cross-reaction was expressed as ng of chlordiazepoxide/200 ng × 100%, in which the amount of standard chlordiazepoxide and the 200 ng of competitor were read at the same percentage inhibition of binding. The results (Table I) indicate that the highest cross-reaction was 5% with *N*-desmethylchlordiazepoxide while demoxepam, *N*-desmethyldiazepam, diazepam, and clonazepam showed less than 1%.

Comparison of Radioimmunoassay with Spectrofluorometric Determination of Chlordiazepoxide in Plasma—Samples of plasma were obtained from subjects who had received both single and chronic oral doses of chlordiazepoxide hydrochloride. Duplicate 20-μl aliquots were analyzed for chlordiazepoxide by the radioimmunoassay procedure; single 1-ml aliquots were assayed by the spectrofluorometric method of Schwartz and Postma (4) (Table II). The joint determinations were subjected to a straight-line analysis. The fitted intercept and slope (−0.005 and 0.967, respectively) were not significantly different from 0 and 1 at the 0.05 level.

Precision—Since no extraction procedures were involved in the radioimmunoassay, thereby avoiding differences in extraction recoveries, the precision depended on the accuracy with which the

Table III—Reproducibility of Radioimmunoassay for Plasma Chlordiazepoxide

Sample[a]	Chlordiazepoxide, μg/ml[b]		
	Assay 1	Assay 2	Assay 3
1	0.98	1.1	1.0
2	0.62	0.64	0.65
3	0.44	0.40	0.38
4	1.30	1.35	1.25
5	1.2	0.93	0.95
6	0.27	0.23	0.22

[a] Samples obtained at random times from subjects receiving single and chronic doses of chlordiazepoxide hydrochloride. [b] Analyzed on three separate occasions several weeks apart.

operator pipetted the samples and reagents and decanted the "free" radioactivity into the counting vial. The mean coefficient of variation for six replicates at each point of the calibration curve was 4%. Six samples of plasma from subjects who had received chlordiazepoxide hydrochloride were analyzed for chlordiazepoxide on three separate occasions several weeks apart (Table III). The pooled standard deviation was 0.072 μg/ml (12 degrees of freedom) over a range of 0.22–1.35 μg/ml.

Chlordiazepoxide Plasma Levels in Humans—Table IV shows the plasma levels of chlordiazepoxide, as determined by radioimmunoassay, in a subject who received a single 30-mg oral dose of chlordiazepoxide hydrochloride.

DISCUSSION

A radioimmunoassay, developed for the determination of chlordiazepoxide directly in plasma, is simple, rapid, and easy to perform and it may be carried out using as little as 20 μl of plasma. The technique thus lends itself to the analysis of large numbers of samples. The radioimmunoassay might be employed in monitoring the blood levels of chlordiazepoxide in patients receiving the drug chronically and/or determining whether, in fact, the patient is taking the drug at all. Such monitoring of blood levels may help the practitioner in the optimization of drug therapy with chlordiazepoxide.

In designing the radioimmunoassay, several points were considered regarding the preparation of a suitable antigen. The ultimate goal of these investigations was to obtain antibodies of sufficient specificity to obviate the necessity of extracting chlordiazepoxide from plasma for its determination. Thus, the antibodies would have to be specific for chlordiazepoxide in the presence of all of its known plasma metabolites in humans, namely *N*-desmethylchlordiazepoxide (4), demoxepam (5), and *N*-desmethyldiazepam (9). Therefore, the antibodies would be required to distinguish structural changes occurring only in the 1,4-diazepine ring or "fingerprint" region. It is well recognized that antibody specificity for a particular fingerprint region of a hapten is enhanced by coupling the hapten to the protein at a position in the hapten well removed from the fingerprint region. For this reason, the 4′-position of the 5-phenyl group in chlordiazepoxide was chosen for linkage to the protein, thereby having the functional groups in the 1,4-diazepine ring relatively free to confer immunological specificity. This goal was achieved by immunization of rabbits with an immunogen prepared by covalently coupling an active acyl-azide at this 4′-position to the ε-amino groups of lysine on albumin (8). The antiserum obtained had a high degree of specificity for chlordiazepoxide. *N*-Desmethylchlordiazepoxide showed only about 5% cross-reactivity, while demoxepam and *N*-desmethyldiazepam showed less than 1%. This finding would suggest that the C-2 methylamino group was a critical antigenic determinant.

Since neither diazepam nor clonazepam cross-reacted, chlordiazepoxide apparently can be determined in the plasma of subjects receiving chlordiazepoxide in conjunction with diazepam and/or clonazepam.

The fact that the metabolite, *N*-desmethylchlordiazepoxide, cross-reacted with the antibody to about 5% did not significantly affect the accuracy of the assay for chlordiazepoxide. The plasma levels of *N*-desmethylchlordiazepoxide, as determined by the spectrofluorometric method (4), only sometimes exceeded those of chlordiazepoxide, by at the most a factor of two, at 36–48 hr after a single oral dose[10]. Thus, the maximum error in the determination of chlordiazepoxide due to *N*-desmethylchlordiazepoxide at these late periods of time cannot exceed a 10% overestimation.

Table IV—Plasma Levels of Chlordiazepoxide in Human Subject following a Single 30-mg Oral Dose of Chlordiazepoxide Hydrochloride

Hours	Chlordiazepoxide, μg/ml
0.5	0.35
1	2.30
2	1.80
3	1.65
4	1.55
6	1.30
8	1.22
12	0.84
24	0.48
30	0.37
48	0.19

In subjects receiving 10 mg tid or 30 mg/day of chlordiazepoxide hydrochloride, the average steady-state levels of *N*-desmethylchlordiazepoxide are about 50% of the chlordiazepoxide levels[10], thereby reducing the error in the overestimation of the steady-state levels of chlordiazepoxide by the radioimmunoassay to around 2.5%. The less than 1% cross-reactivity of demoxepam and *N*-desmethyldiazepam becomes insignificant since their levels have been found to be considerably less than those of chlordiazepoxide at all times following either single or chronic doses of chlordiazepoxide hydrochloride[10].

The specificity of the radioimmunoassay for chlordiazepoxide also was tested by comparison with an established spectrofluorometric method (4). Satisfactory agreement was obtained for the levels of chlordiazepoxide in subjects who had received both single and chronic doses of chlordiazepoxide hydrochloride. The greatest differences were noted with low levels of chlordiazepoxide. This finding is not too surprising when one considers that the sensitivity limit of the radioimmunoassay (100 ng/ml using a 20-μl sample of plasma) was twice that of the spectrofluorometric method (200 ng/ml using a 1-ml sample of plasma). Also, in the spectrofluorometric assay the level of chlordiazepoxide is dependent on the accuracy with which *N*-desmethylchlordiazepoxide is determined.

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Effect of Dilution on Reactivity and Structure of Aluminum Hydroxide Gel

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Abstract □ Aluminum hydroxide gel loses reactivity upon aging. However, a sharp decrease in reactivity occurs when the gel is diluted with double-distilled water. The loss of reactivity is directly related to the degree of dilution, but dilution with dioxane or the mother liquor has no effect on reactivity. It is hypothesized that dilution with water causes a change in the equilibrium between stabilizing ions incorporated in the gel structure and ions in solution. As the stabilizing ions leave the gel structure to reestablish the equilibrium, a loss of reactivity is observed until a new equilibrium is established.

Keyphrases □ Aluminum hydroxide gel—effect of dilution on reactivity and structure □ Gels—effect of dilution on reactivity and structure of aluminum hydroxide gel □ Acid reactivity—effects of dilution on aluminum hydroxide gel

Aluminum hydroxide gel is a complex material whose structure is not fully understood. Several factors involved in the manufacture of aluminum hydroxide gel have been found to affect acid reactivity, including precipitation temperature (1), pH (2), order of addition of reactants (1, 3), and washing (4). Changes in the acid reactivity of aluminum hydroxide gel were observed to occur following dilution of the gel. This report confirms this observation and proposes a mechanism by which the reactivity of aluminum hydroxide gel is affected by dilution.

EXPERIMENTAL

Materials—All chemicals used were either official or reagent grade. Double-distilled water was used.

Preparation of Aluminum Hydroxide Gel—Aluminum hydroxide gel was prepared by the reaction of aluminum chloride, sodium carbonate USP, and sodium bicarbonate USP at pH 6.5 (2). Aluminum hydroxide gel from two identical precipitations was used to study the effect of dilution. The final volume of each gel was adjusted to produce one gel containing 4.8% Al_2O_3 and a second gel containing 3.6% Al_2O_3.

A third gel was precipitated and, in place of the usual washing procedure, the mother liquor was immediately separated by vacuum filtration using a buchner funnel with No. 1 filter paper. The solids were maintained as a moist cake while the clear mother liquor was collected. A portion of the mother liquor was used to reconstitute the moist cake to prepare a gel containing 3.9% Al_2O_3.

Aluminum Hydroxide Gel Dilutions—The aluminum hydroxide gel containing 4.8% Al_2O_3 was used to study the effect of diluting the gel with double-distilled water. Three dilutions were prepared whereby 12.5, 25.0, and 50.0 g of gel were diluted to 100 ml with double-distilled water. Therefore, the samples contained 0.6, 1.2, and 2.4% Al_2O_3, respectively.

The gel containing 3.6% Al_2O_3 was used to prepare a series of gels to study the effect of dilution with water–dioxane mixtures. Fifty grams of gel was diluted to 100 ml with a water–dioxane mixture containing 0, 25, 50, 75, or 100% dioxane. All diluted gels contained 1.8% Al_2O_3.

The gel containing 3.9% Al_2O_3 was used to study the effect of dilution with the mother liquor. Fifty grams of the gel was diluted to 100 ml using the mother liquor, an equal mixture of mother liquor and double-distilled water, or double-distilled water. The samples contained 1.95% Al_2O_3.

Analytical Procedures—The aluminum oxide content was determined by ethylenediaminetetraacetic acid titration (5).

The acid reactivity was monitored by a pH-stat technique similar to the method described by Steinberg *et al.* (6). An automated technique utilizing a pH meter, a titrator, an autoburet, a titration assembly, and a recorder[1] was used.

An appropriate volume of water was added to the reaction vessel and brought to pH 3.0. A volume of sample containing 38 mg Al_2O_3 was then added. The volume of water was adjusted for each sample to produce a reaction volume of 22 ml. The instrument was set to maintain pH 3.0 by adding 1.0 *N* HCl from the autoburet. The instrument was activated simultaneously with the sample injection. The recorder plotted milliliters of 1.0 *N* HCl added *versus* time. The acid reactivity of each sample is expressed as T_{50}: the time required to add 50% of the total 1.0 *N* HCl needed to neutralize the aluminum hydroxide gel at 25°.

RESULTS AND DISCUSSION

Aluminum hydroxide gel becomes less reactive upon aging. However, as seen in Fig. 1, the increase in T_{50} was greatest during the first days after precipitation or dilution with double-distilled

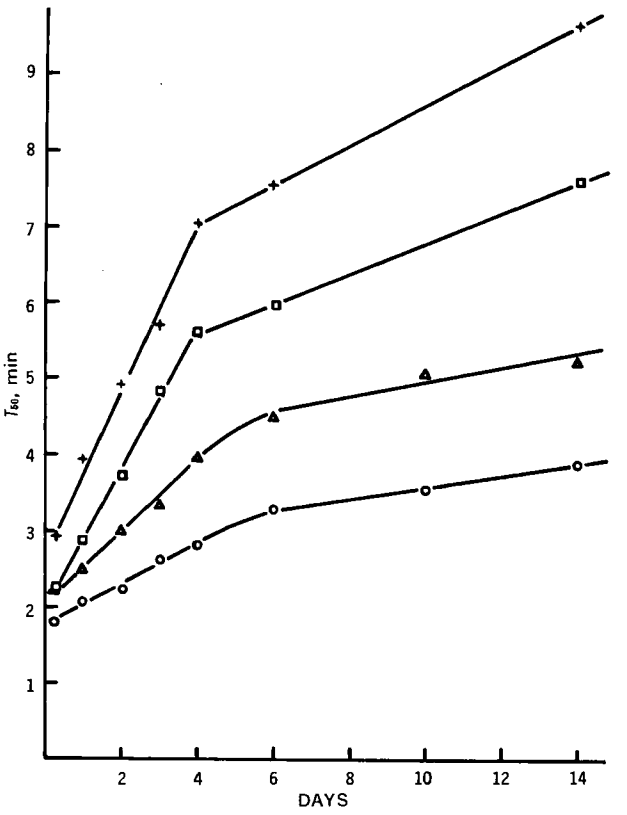

Figure 1—*Change in* T_{50} *during aging at 25°. Key:* ○, *undiluted gel containing 4.8%* Al_2O_3; *and gel diluted with double-distilled water to contain:* △, *2.4%* Al_2O_3; □, *1.2%* Al_2O_3; *and* +, *0.6%* Al_2O_3.

[1] pH meter PHM 26, titrator TTT 11, autoburet ABU 12 (2.5 ml), titration assembly TTA 3, and recorder SBR 2, all from Radiometer, Copenhagen, Denmark.

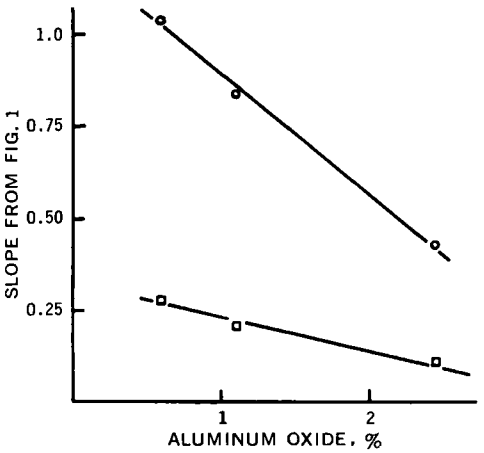

Figure 2—*Effect of dilution with double-distilled water on the rate of change of acid reactivity. Key: O, initial period; and □, long-term period.*

water. An initial sharp decrease in reactivity was observed during the first 4–6 days. The initial rate of loss of reactivity was directly related to the degree of dilution (Fig. 2). The gel diluted to 0.6% Al_2O_3 had the greatest increase in T_{50} during the first several days. The undiluted sample of the freshly precipitated gel also lost reactivity more sharply during the first several days following precipitation, but the increase in T_{50} during the first 4 days was from 1.8 to 2.8 min compared to an increase from 2.9 to 7.0 min for the most dilute sample.

A much smaller increase in T_{50} occurred for all dilutions as well as the undiluted gel after the initial period. The rate of loss of reactivity in this later period also was directly related to dilution (Fig. 2). The samples were monitored for 112 days, and the rate of loss of reactivity was constant for each gel after the initial period. The rate of loss of reactivity in the second period appears to represent the loss of reactivity normally observed during the aging of aluminum hydroxide gel.

The series of gels containing 1.8% Al_2O_3 but diluted with varying ratios of double-distilled water–dioxane produced the same pattern of increasing T_{50} (Fig. 3) as was observed when the gels were diluted with double-distilled water. It appears that dilution with dioxane has no effect on reactivity since the sample diluted with 100% dioxane had virtually the same reactivity profile as the freshly precipitated, undiluted gel (Table I). However, the concentration of water in the diluting solution was important. A greater initial increase in T_{50} was observed as the concentration of water in the diluting solution was increased.

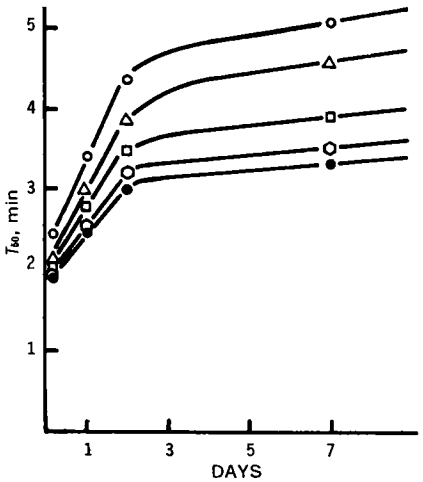

Figure 3—*Effect of dilution with varying double-distilled water–dioxane mixtures. Key: ●, 100% dioxane; ○, 25% water–75% dioxane; □, 50% water–50% dioxane; △, 75% water–25% dioxane; and O, 100% water.*

Table I—Acid Reactivity of Gel Diluted with Dioxane Compared to Undiluted Gel

Days	T_{50}, min: Undiluted Gel Containing 3.6% Al_2O_3	T_{50}, min: Gel Diluted with Dioxane to 1.8% Al_2O_3
0.25	2.0	2.0
1	2.5	2.5
2	3.3	3.0
7	3.4	3.3
14	4.5	3.9
28	4.7	4.6
56	5.2	5.4
112	8.1	8.9

The rate of change in T_{50} during the initial period was directly related to the concentration of water in the water–dioxane diluting mixtures (Fig. 4). A much smaller rate of change of acid reactivity occurred after the initial period. The later increase in T_{50} also was directly related to the concentration of water in the water–dioxane diluting mixtures (Fig. 5).

The pattern of a sharp decrease in reactivity followed by a slow rate of loss of reactivity following precipitation or dilution of aluminum hydroxide gel with water suggests the establishment of an equilibrium condition. Several reports (2, 7–9) indicated that ions present in the precipitation solution may be incorporated in the gel structure. These ions act to stabilize the aluminum hydroxide gel structure.

It was hypothesized that dilution with water alters the equilibrium between ions in the gel structure and in solution. When equilibrium is reestablished, the concentration of stabilizing ions in the gel structure is reduced. The reactivity of the gel reflects this change in concentration of stabilizing ions and a sharp decrease in reactivity occurs until a new structure is established. The new structure then changes at a rate related to the concentration of ions present in the gel structure and a constant, long-term loss of acid reactivity is observed.

A final series of dilutions was prepared to test this hypothesis. Fifty grams of the gel containing 3.9% Al_2O_3 was diluted to 100 ml with the mother liquor, an equal mixture of mother liquor and water, or double-distilled water. All sample dilutions contained 1.95% Al_2O_3. Figure 6 indicates that dilution with the mother liquor did not affect reactivity since the increase in T_{50} was virtually identical for the freshly prepared undiluted gel and for the sample diluted with mother liquor. The sample diluted with water had a sharp initial increase in T_{50}. The water–mother liquor dilution was intermediate in effect.

The unwashed gel was more reactive than either of the gels used for the water dilution or dioxane dilution experiments. The washing operation probably causes the loss of stabilizing ions from the

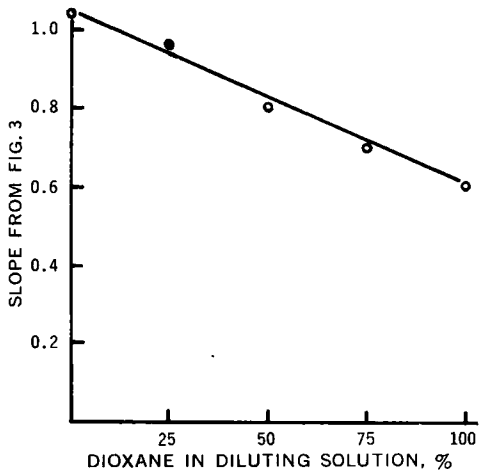

Figure 4—*Effect of dilution with varying double-distilled water–dioxane mixtures on the initial rate of loss of acid reactivity.*

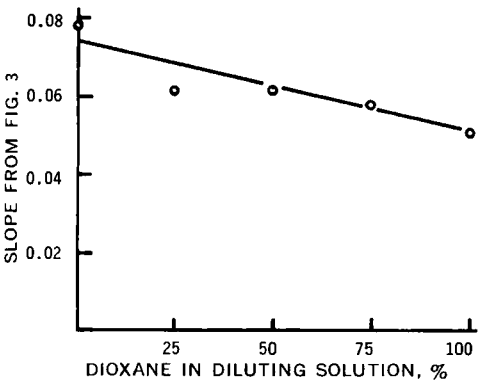

Figure 5—*Effect of dilution with varying double-distilled water–dioxane mixtures on the long-term rate of loss of acid reactivity.*

gel and results in a less acid reactive gel. Further experiments are in progress to determine the effect of washing on acid reactivity.

These results support the previous hypothesis, because the equilibrium between ions in the gel structure and ions in solution would not be affected by dilution with the mother liquor or dioxane. Dilution with water always caused a sharp loss of reactivity.

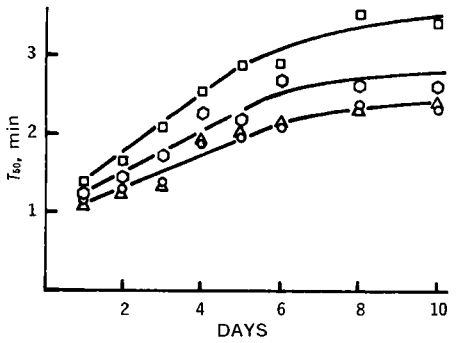

Figure 6—*Effect of dilution with mother liquor. Key:* ○, *undiluted gel containing 3.9% Al_2O_3; and gel diluted to 1.95% Al_2O_3 with:* △, *mother liquor;* ○, *50% mother liquor–50% double-distilled water; and* □, *double-distilled water.*

The change in reactivity was directly related to the amount of water added to the gel. The important stabilizing function of ions incorporated in the gel structure is illustrated by this study.

The composition of the solution used to dilute the gel has an important effect on the long-term acid reactivity of the aluminum hydroxide gel. A carefully designed diluting solution will minimize the loss of acid reactivity normally observed with aluminum hydroxide gels, resulting in increased efficacy and longer shelflife.

The composition of the solution and the technique used to wash the gel may likewise affect the acid reactivity of the gel.

This study emphasizes the complex structure of aluminum hydroxide gel. The preparation of aluminum hydroxide gel requires a carefully controlled manufacturing procedure to produce a highly reactive gel of uniform quality.

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Effect of Deoxyuridine Coadministration on Toxicity and Antitumor Activity of Fluorouracil and Floxuridine

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Abstract □ The addition of deoxyuridine (UDR) to fluorouracil (FU) or floxuridine (5-fluoro-2′-deoxyuridine) (FUDR) produced a substantial increase in their toxicity in BDF_1 mice. Antitumor assays using sarcoma 180 tumor-bearing mice showed a concomitant increase in tumor growth inhibition for the nucleoside–drug combination over identical doses of the single drug. However, no significant increase in antitumor activity with the combination treatment was demonstrated when equitoxic doses were given. Additional support for the therapeutic equality of the single and combination drug regimens was the similarity of the therapeutic indexes for each treatment regimen involving either fluorouracil or floxuridine. The results suggested that any therapeutic benefit achieved with the combination therapy could be duplicated with either fluorouracil or floxuridine at a higher dose.

Keyphrases □ Deoxyuridine—effect of coadministration on toxicity and antitumor activity of fluorouracil and floxuridine □ Fluorouracil—effect of deoxyuridine coadministration on toxicity and antitumor activity □ Floxuridine—effect of deoxyuridine coadministration on toxicity and antitumor activity □ Anticancer agents—effect of deoxyuridine coadministration on toxicity and antitumor activity of fluorouracil and floxuridine

Coadministration of deoxyuridine (UDR) with fluorouracil (FU) and floxuridine (5-fluoro-2′-deoxyuridine) (FUDR) was proposed by Windheuser and Jato (1) who observed a conversion of 5-fluorouracil to 5-fluoro-2′-deoxyuridine by human blood. Because this transformation reduces the steps needed to convert fluorouracil to the active form (2, 3), an enhancement of the antitumor activity of fluorouracil and floxuridine by deoxyribonucleosides was predicted. If this mechanism is operative in murine tumor therapy, the antitumor activity of fluorouracil or floxuridine should be increased in the presence of an excess of deoxyuridine. Preliminary experiments on transplanted mouse tumors, L-1210 leukemia and mammary adenocarcinoma 755, showed that deoxyuridine administered together with fluorouracil or floxuridine enhanced the antitumor activity of each drug over the same dose of each drug alone (4).

To understand the clinical significance of this enhancement, combinations of deoxyuridine with fluorouracil and floxuridine were examined in an animal tumor system (sarcoma 180) and compared to the single drugs using the parameters of antitumor activity and toxicity.

EXPERIMENTAL

Materials—*N*-Methylformamide[1], fluorouracil[2] (pH 9.0, solution), floxuridine[2] (powder), and deoxyuridine[3] (thymine and thymidine free) were used as received.

Animals—Female BDF_1 mice[4], 20–22 g, were maintained on a standard pellet diet and tap water *ad libitum*.

Toxicity Studies—Following a 7–10-day period of acclimatization before experimental use, the BDF_1 mice were randomized and divided into groups of 10, corresponding to the number of drug regimens to be tested plus a control group. Drugs were put into solution with sterile 0.9% NaCl 24 hr prior to the 1st day of injection. Each mouse received intraperitoneally 0.01 ml/g body weight of the drug regimen for 7 consecutive days; control animals received an equivalent volume of sterile 0.9% NaCl.

For the combination drug regimens, a constant amount of deoxyuridine (200 mg/kg/day) was coadministered with each dose of fluorouracil and floxuridine. This level of the nucleoside, an approximate fivefold excess of deoxyuridine over floxuridine, was used in previous *in vivo* studies (4). Average weights for each group were determined daily, and deaths were recorded throughout the 3-week observation period.

Implantation and Treatment of Sarcoma 180 Tumor—The sarcoma 180 tumor was maintained in the laboratory by subcutaneous transplant in female BDF_1 mice every 7 days. Aseptic conditions were maintained by using sterilized instruments and working under a hooded area. For tumor transplantation, the tumor was excised from the animal and placed in a petri dish containing cold saline. The tumor tissue was trimmed of necrotic and fibrous tissue and then minced with scissors into fragments; these fragments were implanted subcutaneously over the front shoulder using a 16-gauge trocar. Tumor fragments were tested for sterility by placing samples into two tubes containing thioglycollate broth[5] and checking for bacterial growth after 48 hr. If growth was found, the experiment was discarded.

The antitumor activities of fluorouracil, fluorouracil plus deoxyuridine, floxuridine, and floxuridine plus deoxyuridine were assayed in groups of 10 BDF_1 mice given implants on Day 0. Test mice were given daily intraperitoneal injections for 7 days with a volume of 0.01 ml/g body weight of the appropriate drug solution, starting 24 hr after implantation of the tumors, *i.e.*, Day 1. Control mice received 0.9% NaCl in an identical manner. *N*-Methylformamide (NSC-3051) was used as a positive control at a dose of 200 mg/kg/day for 7 days.

On Day 12 postimplantation, the mice were sacrificed and each tumor was excised and weighed. The mean tumor weight for each group was used to calculate the values for test/control (T/C). Percent inhibition of tumor growth was given by Eq. 1:

$$\text{percent inhibition} = 100(1 - T/C) \qquad \text{(Eq. 1)}$$

RESULTS AND DISCUSSION

Determination of LD_{10} and LD_{50}—In a previous study, Jato and Windheuser (4) suggested that the increased antitumor activity produced when deoxyuridine was combined with fluorouracil or floxuridine was associated with an increase in toxicity for the combination drug regimens. To examine the effects of the nucleoside on toxicity of the single drugs, a series of dosages of fluorouracil and floxuridine were coadministered with deoxyuridine in BDF_1 mice (10 mice/dose) and deaths were recorded over a 3-week period. A plot of log dose–percent survivors enabled the determination of LD_{10} and LD_{50} values for each combination regimen from their respective graphs (Fig. 1).

The LD_{10} values of 30 mg/kg/day for fluorouracil and 150 mg/

[1] Eastman Kodak Co., Rochester, N.Y.
[2] Hoffmann-La Roche, Nutley, N.J.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] A. R. Schmidt, Madison, Wis.
[5] Difco Laboratories, Detroit, Mich.

Table I—Lethal Doses for Fluorouracil plus Deoxyuridine and Floxuridine plus Deoxyuridine[a]

Drug	LD_{10}[b]		LD_{50}[c]	
	mg/kg/day	mmoles/kg/day	mg/kg/day	mmoles/kg/day
Fluorouracil plus deoxyuridine	12.0	0.092	16.0	0.123
Floxuridine plus deoxyuridine	58.0	0.236	67.0	0.272

[a] Drugs were given intraperitoneally for 7 consecutive days to groups of 10 BDF_1 mice/dose; the mice were observed for 2 weeks after the injection period; 200 mg/kg/day of deoxyuridine was coadministered with each drug. [b] Lethal dose for 10% of the mice. [c] Lethal dose for 50% of the mice.

kg/day for floxuridine have been reported (5). Because these values were obtained from a 5-day injection schedule, it was necessary to adjust the reported values to a 7-day injection period using the method described by Freireich *et al.* (6) before these values could be compared with the LD_{10} values obtained for the combination regimens in this study. The adjusted LD_{10} values were 21.4 mg/kg/day for fluorouracil and 107 mg/kg/day for floxuridine. A comparison with the experimentally determined values (Table I), 12 mg/kg/day for fluorouracil plus deoxyuridine and 58 mg/kg/day for floxuridine plus deoxyuridine, showed that the inclusion of deoxyuridine increased the toxicity of both fluorouracil and floxuridine approximately 100%.

In terms of relative toxicity, floxuridine plus deoxyuridine was less toxic than fluorouracil plus deoxyuridine on a milligrams per killogram per day and molar basis (Table I). This finding indicated that the addition of deoxyuridine, while enhancing the toxicity of the single drugs, did not alter the toxicity relationship; *i.e.*, floxuridine is less toxic than fluorouracil, as has been reported clinically (7) and demonstrated in mice (8).

Antitumor Activity—A preliminary study was performed in which a group of sarcoma 180-tumor bearing mice was injected for 7 consecutive days with varying doses of the nucleoside to determine if deoxyuridine exerted any antitumor activity. After 12 days postimplantation, the tumors were removed and weighed individually. The results of this study showed that deoxyuridine, even when administered at a dose 2.5 times greater than the level used in the combination treatment regimens, exhibited no antitumor activity.

The effect of deoxyuridine on the tumor growth-inhibitory activity of fluorouracil and floxuridine was then ascertained. When using the parameter of mean tumor weight on Day 12 postimplantation, the results in Table II show an increased antitumor activity for the combination drug regimens administered at dosages identical to those of the single drug regimens. These results, utilizing BDF_1 mice bearing sarcoma 180 tumors, corroborated the findings reported earlier in mice bearing L-1210 or adenocarcinoma 755 (4)—*viz.*, deoxyuridine increased the antitumor activity of fluorouracil and floxuridine.

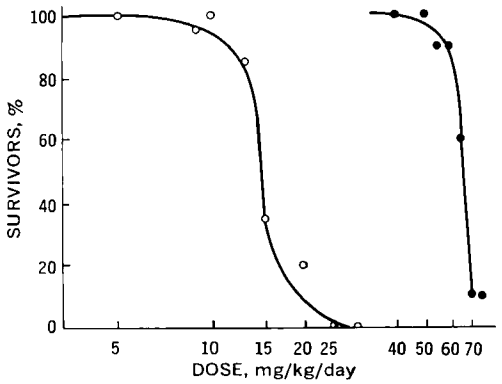

Figure 1—*Effect of deoxyuridine on lethality of fluorouracil* (○) *and floxuridine* (●). *Abscissa scale is logarithmic, but actual doses are indicated. Deoxyuridine was given at a dose of 200 mg/kg/day with each dose of the respective single drug.*

The loss in body weight that occurred with the higher dosages in the combination drug regimens suggested that the increased antitumor activity was achieved at the cost of a greater toxicity. The group that received 50 mg/kg/day of floxuridine had a net gain in weight of 1.2 g on Day 12 postimplantation, while the group that received 50 mg/kg/day of floxuridine in combination with 200 mg/kg/day of deoxyuridine experienced a net loss in weight of 2.0 g. A similar trend was produced by fluorouracil plus deoxyuridine at the highest dose (11 mg/kg/day).

Treatment of Sarcoma 180 with Equitoxic Levels—The preceding studies established that the addition of deoxyuridine to either fluorouracil or floxuridine resulted in an increase in the toxicity of the single drugs. Before a determination of increased antitumor efficacy could be ascribed to the combination drug regimens, it was necessary to compare the single drug regimen to the combination regimen at equitoxic levels rather than at identical dosages.

The results from such a study, using BDF_1 mice in which each drug regimen was given once daily for 7 consecutive days beginning 24 hr after implantation of sarcoma 180 tumor tissue subcutaneously, are shown in Table III. Each tumor was removed and weighed 12 days postimplantation; the mean tumor weight for each drug regimen was tabulated, and comparisons at equitoxic dosages were made between the single drug regimen and the corresponding combination drug regimen. The mean tumor weights of the mice that received deoxyuridine and fluorouracil were lower

Table II—Effect of Coadministered Deoxyuridine on Inhibition of Sarcoma 180 in BDF_1 Mice by Fluorouracil and Floxuridine[a]

Drug	Dose[b], mg/kg/day	Mean Body Weight Change[c], g	Tumors Evaluated	Mean Tumor Weight[d], g	T/C	Inhibition, %
Control (0.9% NaCl)	—	+0.8	41	6.16	—	—
Fluorouracil	5.5	+1.4	42	4.15	0.67	33
Fluorouracil plus deoxyuridine	5.5 / 200.0	+1.3	42	2.58	0.42	58
Fluorouracil	11.0	+1.4	42	4.27	0.69	31
Fluorouracil plus deoxyuridine	11.0 / 200.0	−0.1	41	0.73	0.12	88
Floxuridine	25.0	+1.2	42	2.85	0.46	54
Floxuridine plus deoxyuridine	25.0 / 200.0	+1.0	42	0.36	0.06	94
Floxuridine	50.0	+1.2	42	0.40	0.07	93
Floxuridine plus deoxyuridine	50.0 / 200.0	−2.0	40	0.03	0.01	99
N-Methylformamide	200.0	+0.8	42	1.13	0.18	82

[a] Tumor was implanted subcutaneously on Day 0. [b] Drugs were given intraperitoneally for 7 consecutive days starting on Day 1. [c] Between Days 1 and 12. [d] Tumors weighed on Day 12 postimplantation.

Table III—Effect of Equitoxic Levels of Single Drug and Combination Drug Regimens on Growth of Sarcoma 180 in BDF_1 Mice

Drug[a]	Dose Level[b]	Mean Tumor Weight[c], mg ± *SEM*		Mean Body Weight Change[d], g
Fluorouracil	3/4 LD_{10}	388 ± 74	NS[e]	+0.3
Fluorouracil plus deoxyuridine	3/4 LD_{10}	241 ± 25		−0.2
Fluorouracil	2/3 LD_{10}	467 ± 94	NS	+0.8
Fluorouracil plus deoxyuridine	2/3 LD_{10}	331 ± 71		+1.6
Fluorouracil	1/2 LD_{10}	582 ± 64	NS	+0.7
Fluorouracil plus deoxyuridine	1/2 LD_{10}	438 ± 66		+0.7
Floxuridine	2/3 LD_{10}	103 ± 17	NS	−0.6
Floxuridine plus deoxyuridine	2/3 LD_{10}	102 ± 18		−1.0
Floxuridine	1/2 LD_{10}	167 ± 41	NS	−0.1
Floxuridine plus deoxyuridine	1/2 LD_{10}	258 ± 38		+0.7
Floxuridine	1/4 LD_{10}	413 ± 76	NS	+0.9
Floxuridine plus deoxyuridine	1/4 LD_{10}	399 ± 84		0.0

[a] Drugs were given intraperitoneally for 7 consecutive days starting on Day 1. [b] Each group contained 15 BDF_1 mice; deoxyuridine was given at a dose of 200 mg/kg/day. [c] Tumors were weighed on Day 12 postimplantation. [d] Change in mouse body weight between Days 1 and 12. [e] NS = not significant ($p > 0.05$); significance value was determined by Student's *t* test.

than those that received only fluorouracil, but at no dosage level was the difference significant ($p > 0.05$). In the group that received floxuridine alone or in combination with deoxyuridine, there was much less of even an apparent difference in mean tumor weights between the two treatment regimens; again, no difference was significant.

No distinctive differences between the single drug and the combination drug regimens were evidenced in the net body weight changes over the 12-day experimental period. This finding suggested the equality of the regimens with regard to the toxicity produced at equal levels of antitumor activity.

The percent of tumor inhibition produced by each drug regimen was calculated from the mean tumor weight of each group and then plotted as a log dose–probit relationship (Figs. 2 and 3). By extrapolating from the appropriate figure, the effective dose that resulted in a 90% inhibition of tumor growth (ED_{90}) for each drug

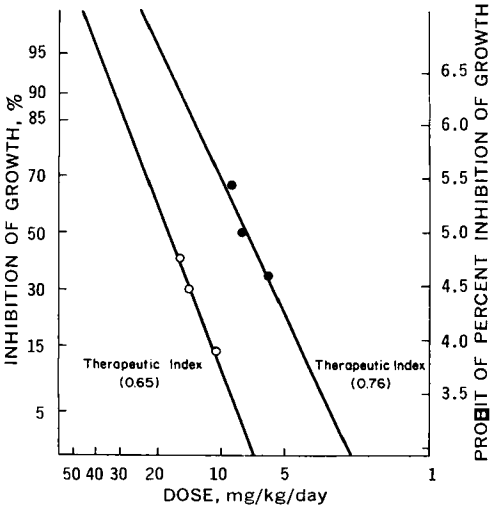

Figure 2—*Effect of fluorouracil and fluorouracil plus deoxyuridine on growth of sarcoma 180 in mice. Abscissa scale is logarithmic, but actual doses are indicated. Therapeutic index is represented by the ratio of LD_{10}/ED_{90}. Key: ○, fluorouracil; and ●, fluorouracil plus deoxyuridine (200 mg/kg/day).*

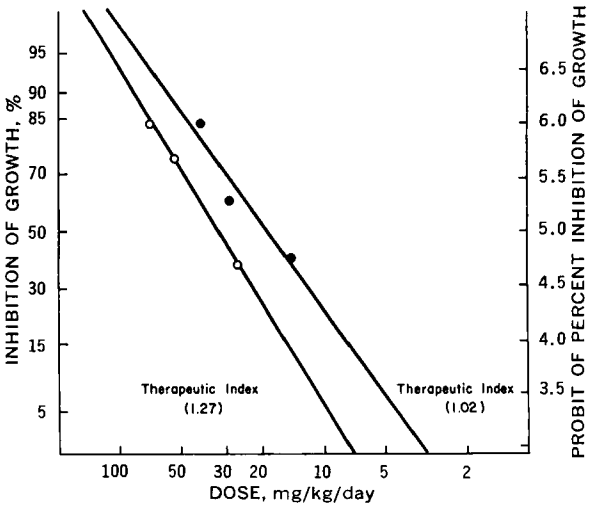

Figure 3—*Effect of floxuridine and floxuridine plus deoxyuridine on growth of sarcoma 180 in mice. Abscissa scale is logarithmic, but actual doses are indicated. Therapeutic index is represented by the ratio of LD_{10}/ED_{90}. Key: ○, floxuridine; and ●, floxuridine plus deoxyuridine (200 mg/kg/day).*

regimen was determined. By using these values, the therapeutic index, expressed by the ratio LD_{10}/ED_{90}, was calculated for each regimen.

The closeness of the calculated therapeutic indexes of fluorouracil and fluorouracil plus deoxyuridine, 0.65 and 0.76, respectively, indicated that the addition of deoxyuridine did not result in a therapeutic advantage for the combination drug regimen over the single drug regimen. This finding was reinforced by the therapeutic indexes of 1.27 for floxuridine and 1.02 for floxuridine plus deoxyuridine. Parenthetically, the superiority of both floxuridine and floxuridine plus deoxyuridine over the fluorouracil regimens was reflected by the larger therapeutic indexes, which denoted a greater margin of safety for the floxuridine regimens.

SUMMARY

When assayed against sarcoma 180, the combination of deoxyuridine (200 mg/kg/day) with fluorouracil or floxuridine produced an increase in antitumor activity over that produced by the identical dose of either single drug alone. Data from toxicity studies, however, revealed that the addition of deoxyuridine resulted in an increase in the toxicity of the combination regimens over the single drug regimens, as reflected primarily in mortality and secondarily in loss of body weight of BDF_1 mice.

When assayed at equitoxic dose levels, no significant increase ($p > 0.05$) in antitumor activity against sarcoma 180 was exhibited by either combination regimen over its respective single drug regimen. The addition of deoxyuridine to fluorouracil or floxuridine did not cause a notable change in either drug's therapeutic ratio (LD_{10}/ED_{90}). This finding suggested that any therapeutic benefit achieved with the combination drug regimen could be duplicated with either fluorouracil or floxuridine at a higher dose.

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Evaluation of a Dynamic Permeation Technique for Studying Drug–Macromolecule Interactions

F. BOTTARI, G. Di COLO, E. NANNIPIERI [x], M. F. SAETTONE, and M. F. SERAFINI

Abstract □ The applicability of a permeation rate technique to the determination of drug–macromolecule interactions was tested by measuring the extent of interaction of methylparaben with polyvinylpyrrolidone and polysorbate 80. Results were in agreement with literature data obtained by other techniques. The present method, although restricted to permeant molecules that diffuse readily through nonporous nylon membranes, is of potential value for investigations of drug binding by macromolecules not retained by porous dialysis membranes.

Keyphrases □ Permeation rate technique for determining drug–macromolecule interactions—tested using methylparaben with polyvinylpyrrolidone and polysorbate 80 □ Drug–macromolecule interactions—determination by permeation rate technique, compared to literature dialysis data

The phenomena of drug–macromolecule interactions have received wide attention in recent years in view of their relevance to drug absorption, transport, and overall availability. Dynamic dialysis[1] techniques have been successfully applied to the study of such interactions (1–3). These methods consist of the measurement of the rate of disappearance of a small molecule from a macromolecule-containing compartment under quasi-steady-state conditions. Their application purportedly offers some advantages (rapidity, economy, simplicity, *etc.*) over other time-honored techniques but is limited by the fact that porous dialysis membranes are pervious to many macromolecules of pharmaceutical interest.

Analogous techniques involving nonporous, lipid-like membranes have been proposed. Permeation rate methods (using nylon and dimethylpolysiloxane membranes) were applied to the study of complex formation between small molecules (4, 5). Nakano (6) investigated the interaction of chlorpromazine with several macromolecules, using a permeation rate method with dimethylpolysiloxane membranes, but his experimental system did not allow quantitative estimates of free and bound drug.

Nylon, whose permeability characteristics have been described (7), appeared to be an interesting membrane material for the study of drug–macromolecule interactions by a quasi-steady-state permeation rate technique. The purposes of this preliminary investigation were to evaluate the technique and to compare the results with literature data obtained by equilibrium dialysis.

EXPERIMENTAL

Materials—Methyl *p*-hydroxybenzoate[2] was recrystallized from methanol to a constant melting point of 127–128°. Polysorbate 80[3] and polyvinylpyrrolidone[4] were used as received. Nylon 6 (polycaprolactam) film from a single roll[5], in a labeled thickness of 0.5 mil (0.00127 cm), was used.

Apparatus—The permeation rate experiments were run with a specially designed cell (Fig. 1). The cell interior could be easily cleaned between experiments by removing the upper part (A) without disturbing the membrane. The nylon membrane was securely kept in place by a circular metal plate (C). Fluid tightness was ensured by two O-ring gaskets fitted in machined dies in the upper and lower part of the cell body (B). All parts in contact with the solution were either stainless steel or polytetrafluoroethylene[6] to avoid absorption of the diffusant by the cell material.

The approximate internal volume was 30 ml, and the diameter of the area available for diffusion was 6.0 cm. For use, the cell was placed in a jacketed beaker (internal height of 10.0 cm, internal diameter of 12.0 cm) connected to a thermostatted (30 ± 0.1°) water bath and circulator. Both the "internal" (cell) and "external" (beaker) solutions were stirred by synchronous motors[7]. One motor (60 rpm) was connected to the cell stirrer; the other (500 rpm) operated a magnetic stirrer.

To obtain reproducible results, newly cut membranes were soaked for at least 3 days in several changes of distilled water at 35°. Soaking at lower temperatures or for shorter times resulted in progressively decreasing permeation rates until an apparent stabilization occurred. This phenomenon resulted probably from insuf-

[1] The term dialysis is reserved here for a process involving diffusion through porous membranes, while permeation refers to diffusion through nonporous membranes.

[2] Carlo Erba, Milano, Italy.
[3] Tween 80, Atlas Europol SpA, Varese, Italy.
[4] Plasdone C, GAF Corp., New York, N.Y.
[5] Capran 77 C, Lot G/705283, Allied Chemical Corp., Morristown, N.J.
[6] Teflon, E.I. du Pont de Nemours & Co., Wilmington, Del.
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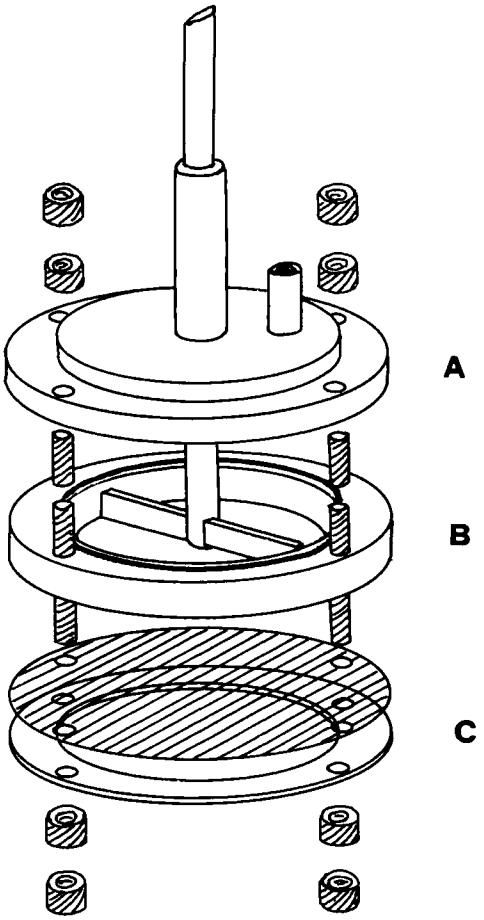

Figure 1—*Cell used for the permeation experiments; see text for details.*

ficient hydration and/or removal of leachable constituents from the membrane. An increase in the density of polycaprolactam fibers on washing, due to removal of low molecular weight components, was reported previously (8).

Permeation Rate Studies—The cell was positioned in the beaker containing 200 ml of distilled water. After equilibration, at t = 0, a 15-ml portion of prewarmed (30°) diffusing solution was introduced into the cell and stirring was initiated. A 100-ml portion of the external solution was periodically removed and immediately replaced with an equal amount of prewarmed water. To approximate sink conditions, care was taken that the external concentration never exceeded one-tenth of the concentration of the diffusant inside the cell.

The removed samples were analyzed spectrophotometrically[8] (255 nm) for methylparaben. The concentration of the diffusing solution (C_i) at different times was calculated by difference from a knowledge of the initial concentration (C_{i0}) and of the cumulative amount that had appeared in the receiving solution. In the present case, analysis of the diffusing solution at the end of several preliminary runs proved that absorption of the diffusant by the cell–membrane complex was minimal or at least within the range of experimental error. It was calculated, using data of Patel and Nagabushan (9) for the interaction of methylparaben with nylon film, that the amount of permeant sorbed by the membrane under the present experimental conditions should not exceed 2.6% of the total.

Data Treatment—Each experiment was repeated at least four times. In the case of linear plots, the data were fed to a desk electronic computer[9] fitted with a linear regression program for computation of regression parameters and statistics. All linear regressions were highly significant ($p < 0.005$), and deviations from linearity were not significant. The coefficient of variation for the slopes of replicate permeation experiments, carried out on consecutive days with the same membrane, was in all cases within the range of 4–11%, thus indicating a satisfactory reproducibility.

For nonlinear plots, the C_i *versus* time data were fitted to a four-parameter biexponential equation (*e.g.*, $C_i = ae^{-bt} + ce^{-dt}$) with the aid of a digital computer[10]. Differentiation of the equation allowed calculation of the concentration of unbound diffusant (C_f) at various values of C_i (*cf.*, Eq. 3). The procedure is essentially similar to that described by Meyer and Guttman (1).

RESULTS AND DISCUSSION

Figure 2 shows the results of permeation rate experiments in which methylparaben (initial concentration of 400 mg/liter, about 2.63×10^{-3} mole/liter) alone or in the presence of polyvinylpyrrolidone [concentration range of 1–4% (w/v)] was allowed to escape through nylon film. The plots are expressed as log C_i/C_{i0} *versus* time, C_i being the concentration of diffusant at time = t, and C_{i0} being the concentration at $t = 0$. A linear relationship between log C_i/C_{i0} and time was observed in all cases.

In the absence of the macromolecule, the following expression of Fick's law applied to the transfer of a solute through a nonporous membrane, under quasi-steady-state conditions, may be considered valid:

$$\ln (C_i/C_{i0}) = \frac{D'At}{V_iX} \qquad \text{(Eq. 1)}$$

where D', the permeability coefficient, is a function of the diffusion coefficient of the solute within the membrane and of the partition coefficient of the solute between the membrane and the solution; A and X are the area and thickness of membrane, respectively; and V_i is the volume of solution inside the cell.

The value of the permeability coefficient (D') of methylparaben through nylon film at 30°, calculated from the permeation data in the absence of a macromolecule using Eq. 1, is 37.4×10^{-9} cm²/sec, which is in good agreement with values reported by Kostenbauder *et al.* (7), $36.8–38.8 \times 10^{-9}$ cm²/sec.

As shown by the graphs in Fig. 2, in the presence of increasing concentrations of polyvinylpyrrolidone, a linear relationship between log (C_i/C_{i0}) and time was always observed; the lines showed progressively decreasing slopes with an increasing macromolecule concentration. Similar graphs, showing the effect of an increasing

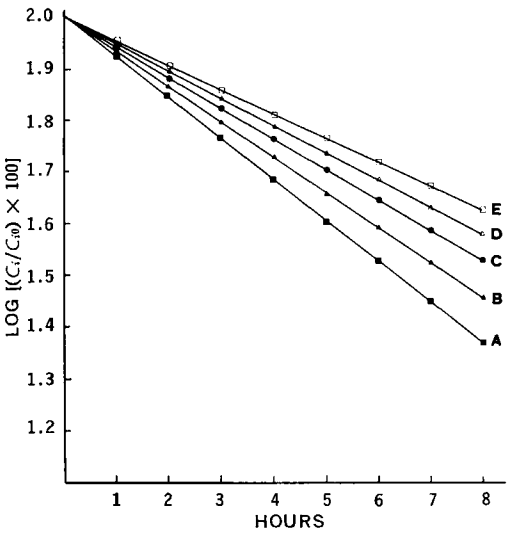

Figure 2—*Plots for the rate of permeation of methylparaben alone (A) and in the presence of 1.0% (w/v) (B), 2.0% (w/v) (C), 3.0% (w/v) (D), and 4.0% (w/v) (E) polyvinylpyrrolidone. The initial concentration of methylparaben was 2.63 × 10^{-3} mole/liter.*

[8] Beckman DU spectrophotometer.

[9] Olivetti Programma 101.

[10] IBM 370/158; nonlinear least-squares regression program BMDX85 (W. J. Dixon, "Biomedical Computer Program, X-Series Supplement," University of California Press, Berkeley, Calif., 1970).

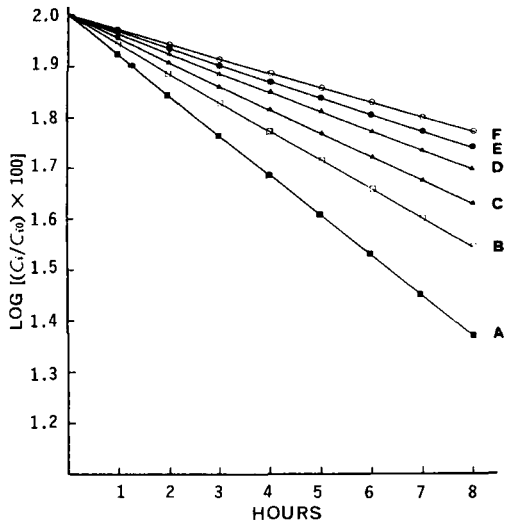

Figure 3—*Plots for the rate of permeation of methylparaben alone (A) and in the presence of 0.5% (w/v) (B), 1.0% (w/v) (C), 1.5% (w/v) (D), 2.0% (w/v) (E), and 2.5% (w/v) (F) polysorbate 80. The initial concentration of methylparaben was 2.63 × 10⁻³ mole/liter.*

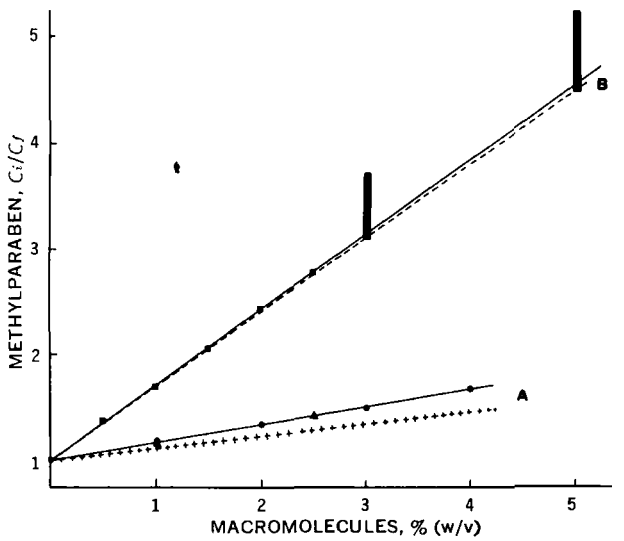

Figure 4—*Plots of the* C_i/C_f *ratio of methylparaben* versus *polyvinylpyrrolidone (A) and polysorbate 80 (B) concentrations. Key: +++, data of Jurgensen Eide and Speiser (11); ▲, data of Miyawaki* et al. *(10); ●, present experimental data; ----, data of Patel and Kostenbauder (12); and ■ and the vertical lines, present experimental data.*

polysorbate 80 concentration [0.5–2.5% (w/v)] on the permeation rate of methylparaben, at the same initial concentration (400 mg/liter), are presented in Fig. 3. The decreased slopes observed in the presence of macromolecules provide evidence of an interaction of the diffusant with the macromolecules. Indeed, the permeation rate in the absence of the macromolecule can be described by the following equation:

$$-dC_i/dt = K_eC_i \qquad \text{(Eq. 2)}$$

where $K_e = D'A/V_iX$ (Eq. 1), and C_i is the total concentration of methylparaben in the diffusing solution. In the presence of the macromolecule, Eq. 2 becomes:

$$-dC_i/dt = K_eC_f \qquad \text{(Eq. 3)}$$

where C_f is the concentration of unbound diffusant inside the cell. The experimental results in the presence of the macromolecule can be described by an equation of the type:

$$-dC_i/dt = K_e'C_i \qquad \text{(Eq. 4)}$$

where K_e' is the experimentally observed slope. Combination of Eqs. 3 and 4 gives the following:

$$C_f/C_i = K_e'/K_e \qquad \text{(Eq. 5)}$$

which shows the relationship existing between the relative amount of unbound diffusant (C_f/C_i) and the ratio (R) of the slope observed in the presence of the macromolecule to the slope observed in the absence of the macromolecule. The ratio of the concentrations of total to free *p*-hydroxybenzoate (C_i/C_f), calculated from the experimental data using Eq. 5, is plotted in Fig. 4 as a function of the concentration of polyvinylpyrrolidone and of polysorbate 80. Data obtained for polyvinylpyrrolidone (10, 11) and for polysorbate 80 (12) are also reported for comparison. The agreement between the present data and the previously reported data, obtained by equilibrium dialysis techniques, appears quite good. The somewhat lower binding values reported by Jurgensen Eide and Speiser (11) are probably due to the lower temperature (22°) of their experiments. The same investigators (11) observed an increasing binding tendency of propylparaben with polyvinylpyrrolidone with rising temperature.

The following expression, which describes the rate of disappearance of diffusant from the cell as a function of its total (C_i), bound (C_b), and free (C_f) concentrations can be obtained from Eq. 3, considering that $C_i = C_f + C_b$:

$$-dC_i/dt = \frac{K_eC_i}{1 + C_b/C_f} \qquad \text{(Eq. 6)}$$

As shown by Eq. 6, the permeation process in the presence of a binding macromolecule follows first-order kinetics only if C_b/C_f, the ratio of concentrations of bound to free permeant, remains constant during each experiment. The linearity of the graphs in Figs. 2 and 3 points, in fact, to a constant C_b/C_f ratio. In the case of the interaction of methylparaben with polyvinylpyrrolidone, this finding appears to agree with previous data (10, 11). Data for the interaction of methylparaben with polysorbate 80 (12), however, indicate a constant C_b/C_f ratio only at a low total permeant concentration (up to about 6.0×10^{-3} mole/liter). The observed constancy of this ratio in the present experiments might, therefore, depend on the low total concentration range of methylparaben explored in each permeation run.

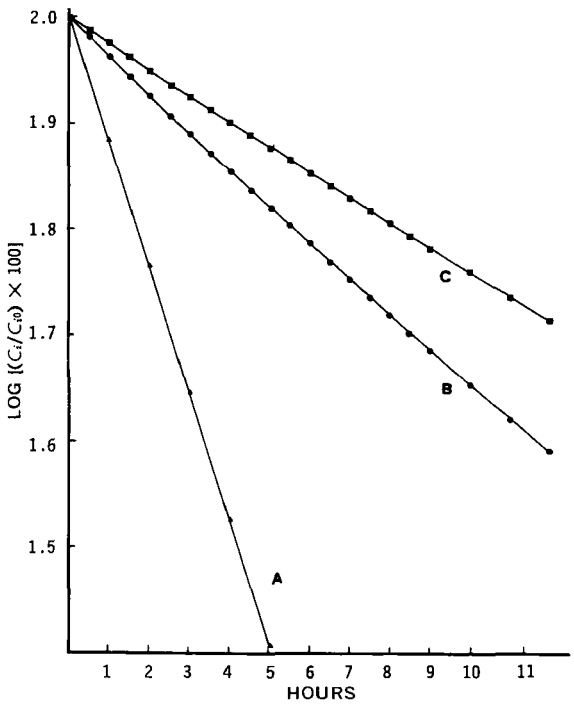

Figure 5—*Plots for the rate of permeation of methylparaben alone (A, initial concentration of 1.0 × 10⁻² mole/liter) and in the presence of 3.0% (w/v) (B) and 5.0% (w/v) (C) polysorbate 80. In the latter two cases, the initial concentration was 2.96 × 10⁻² mole/liter.*

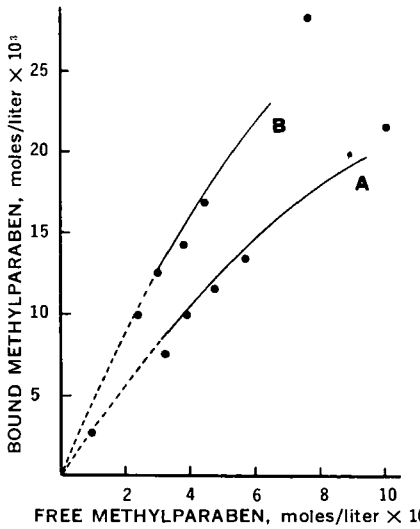

Figure 6—*A* C_b versus C_f *plot for methylparaben in the presence of 3.0% (w/v) (A) and 5.0% (w/v) (B) polysorbate 80. Key:* ●, *data of Patel and Kostenbauder (12); and* —, *present experimental data* (cf., *plots of Fig. 5*).

The results of permeation experiments, in which a higher initial concentration of methylparaben (1.0×10^{-2} mole/liter in the absence and 2.96×10^{-2} mole/liter in the presence of the macromolecule) and higher concentrations of polysorbate [3.0 and 5.0% (w/v)] were used, are illustrated in Fig. 5. As shown by the graphs, under these conditions a linear relationship between log (C_i/C_{i0}) and time was no longer observed. On the basis of Eq. 6, this finding indicates a variation of the C_b/C_f ratio in the course of the permeation experiments. The values of C_b and C_f corresponding to different values of C_i were calculated in this case by treating the data as indicated in the *Experimental* section. The values obtained are reported in Fig. 6 as a C_b *versus* C_f plot, together with the corresponding data from Patel and Kostenbauder (12). The agreement appears satisfactory.

The graphs in Fig. 6 show, for each permeation experiment in the presence of the macromolecule, a C_i/C_f range rather than the constant value observed in the experiments at lower permeant concentration. Such C_i/C_f ranges are indicated in Fig. 4 as vertical lines. It is quite possible that the C_i/C_f ratio might vary within a small range also at low methylparaben concentrations; the present experimental technique, however, could not detect such variation, as shown by the apparent linearity of the graphs in Figs. 2 and 3.

Graphs of the type illustrated in Fig. 4 have been proposed to facilitate the determination of the quantity of preservative to be added to systems containing known concentrations of surfactants. Appropriate use of such plots is made if the user recognizes that the C_i/C_f ratio is a linear function of the macromolecule concentration only at that fixed concentration of free preservative (C_f) that corresponds to the minimum effective concentration required for the desired biological activity. As shown, an apparent linear relationship between the C_i/C_f ratio and S, the surfactant concentration, may be observed only when the C_b/C_f ratio is practically constant for each given S value over a range of total preservative concentrations (C_i). This may occur either at low C_i values (as in the present case) or at constant C_f. Full discussions of the problems involved and the methods for expressing the preservative–surfactant interactions can be found in Refs. 13 and 14.

In conclusion, the present data point to the utility of quasi-steady-state permeation rate techniques for reasonably fast and accurate quantitative determinations of binding of drugs by macromolecules. These techniques, although restricted to permeants that diffuse readily through nonporous membranes, can be useful for studies of binding by macromolecules not retained by porous dialysis membranes. Further work, aimed at a fuller evaluation of the scope and limitations of this experimental approach, is now underway.

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Chlorpromazine Metabolism IV: Quaternization as a Key to Determination of Picomoles of Chlorpromazine and Other Tertiary Amine Drugs

ROLAND E. LEHR and PUSHKAR N. KAUL ˣ

Abstract □ Reaction of chlorpromazine with 9-bromomethylacridine under appropriate conditions yields a nonfluorescent quaternary ammonium derivative which, on subsequent photolysis, liberates fluorescence. The major component of this fluorescence is 9-methylacridine (86%), while two minor components are 9-acridinecarboxaldehyde (6%) and 9-acridinemethanol (8%). The mechanism of photolysis leading to formation of these products appears to involve homolytic as well as heterolytic cleavages of the quaternary salt. Both the quaternization and the photolysis are stoichiometric. Appropriate isolation of the fluorescence and its quantitative determination constitutes the basis of a new and highly sensitive assay applicable to chlorpromazine and other tertiary amine drugs.

Keyphrases □ Chlorpromazine—assay by quaternization and photolysis, picomole quantities □ Tertiary amine drugs—assay by quaternization and photolysis, picomole quantities □ Metabolism—chlorpromazine assayed by quaternization and photolysis, picomole quantities □ 9-Bromomethylacridine—quaternization reagent for picomole assay of chlorpromazine and tertiary amine drugs

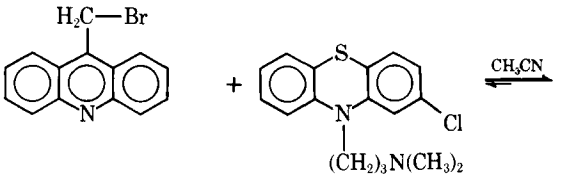
Currently increasing interest in generic bioequivalence of drugs and in drug dose–blood level–clinical response relationships of potent agents has created a general need for sensitive assay methods of high precision. Many tertiary amine drugs acting on the central nervous system exhibit a large volume of distribution and, therefore, yield low blood levels which are not amenable to available analytical methods. In some instances, GC or GLC alone or in conjunction with mass spectrometry has been successful in assaying levels less than 20–30 ng/ml. However, in the case of phenothiazine derivatives, one limitation of GC has been poor precision, and coupled GC–mass spectrometry is not easily available in most clinical laboratories. Therefore, there is a need for a sensitive assay applicable to potent tertiary amine drugs requiring routine monitoring in a clinical situation or warranting pharmacokinetic studies.

An approach of introducing a fluorescent tag into the tertiary amine molecule of chlorpromazine *via* a quaternization reaction was considered. On the bases of relatively high absorptivity and quantum yield of fluorescence of acridine, 9-bromomethylacridine was chosen for the purpose. However, the quaternary product obtained did not fluoresce as such, but on photolysis (Scheme I) it yielded fluorescence that could be determined fluorometrically. Preliminary attempts to apply this approach to assaying solutions and blood samples containing tertiary amine drugs were recently reported (1).

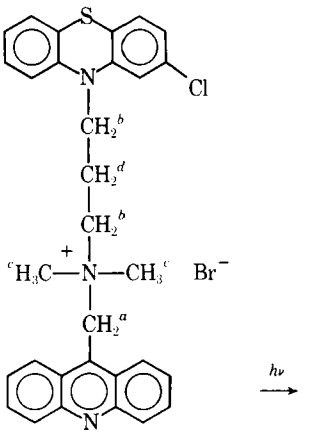
Other papers in this series have dealt with various analytical and pharmacological aspects of chlorpromazine metabolites (2–6). This paper describes the syntheses of various compounds involved in the quaternization of chlorpromazine and in confirming the nature of the fluorescent products resulting from the photolysis of the quaternary salt.

EXPERIMENTAL

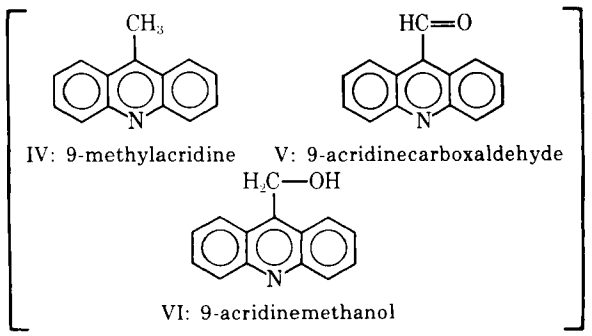
Synthesis of 9-Methylacridine—Two procedures, both based upon literature methods, were used. Method B has proven more successful than Method A for the preparation of large quantities of 9-methylacridine.



I: 9-bromomethylacridine II: chlorpromazine



III: quaternary salt of chlorpromazine



IV: 9-methylacridine V: 9-acridinecarboxaldehyde

VI: 9-acridinemethanol

Scheme I—Quaternization reaction of chlorpromazine and the fluorescent products resulting from the photolysis of the quaternary salt; a, b, c, *and* d *refer to the protons reflected in NMR spectrum*

Method A: From 9-Chloroacridine (7)—To 16.8 g (74.8 mmoles) of 9-chloroacridine in 48 ml of hot toluene was added ethyl sodiomalonate derived from the treatment of 18.7 g of diethyl malonate with 2.6 g of sodium in 48 ml of ethanol. The mixture was refluxed for 17.5 hr, and 7.0 g (48%) of 9-methylacridine was isolated according to the published procedure.

Method B: From Diphenylamine (8)—To 50 g (295 mmoles) of diphenylamine in a pressure bottle were added 30 ml of acetic acid and 85 g of anhydrous zinc chloride; the bottle then was heated at 220° for 14 hr. The crude product mixture was extracted with chloroform, and the chloroform layer was washed with water and evaporated under reduced pressure to yield the crude product. Although 9-methylacridine could be obtained by fractional crystallization from hexane, the remaining diphenylamine interfered with the crystallization. Thus, diphenylamine was separated from 9-methylacridine by column chromatography on silica gel[1], with toluene being used to elute the diphenylamine, which left 9-methylacridine on the column. Subsequent elution with acetone gave 17 g (30%) of 9-methylacridine, mp 113–115° [lit. (8) mp 115–116°].

Synthesis of 9-Bromomethylacridine—According to a modified literature procedure (7), 3.1 g (16.1 mmoles) of 9-methylacridine and 3.4 g (19.1 mmoles) of *N*-bromosuccinimide in carbon tetrachloride were mixed with a few milligrams of dibenzoyl peroxide. The suspension was refluxed to dissolve all components, and the appearance of 9-bromomethylacridine was monitored by TLC. Acetone–benzene (5:95) was used as the developing solvent in which 9-bromomethylacridine had a slightly higher R_f than that of 9-methylacridine. In some instances, refluxing did not initiate the reaction, but irradiation[2] of the mixture was successful. After 1 hr of reflux, the reaction mixture was filtered hot to remove succinimide. On cooling the filtrate, 9-bromomethylacridine crystallized and 3.4 g (12.5 mmoles, 78%) was collected by filtration. Recrystallization from carbon tetrachloride yielded 2.1 g (7.6 mmoles), mp 166–168° [lit. (9) mp 169–170° dec.].

Synthesis of 9-Acridinecarboxaldehyde—The selenium dioxide oxidation method (9) was followed, with 100 mg (0.51 mmole) of 9-methylacridine and 58 mg of selenium dioxide being added to 30 ml of xylene. After the mixture was refluxed for 1 hr, the organic phase was extracted repeatedly with 0.5 *N* HCl until the extracts appeared nearly colorless. The pooled aqueous phases were combined, made basic with sodium carbonate, and extracted with chloroform. The chloroform layer was dried over sodium sulfate. Filtration and removal of chloroform under reduced pressure afforded a crude product, from which 51 mg (48%) of 9-acridinecarboxaldehyde was obtained by preparative TLC[3], with acetone–benzene (5:95) being used as the developing solvent, mp 145–146° [lit. (10) mp 147°].

Synthesis of 9-Acridinemethanol—A new synthesis of the alcohol was devised. To 50 ml of methanol was added 455 mg (2.2 mmoles) of 9-acridinecarboxaldehyde. The solvent was heated to dissolve the aldehyde, and 100 mg of sodium borohydride was added. Some precipitate formed immediately. The reaction mixture was stirred for 30 min, treated with 10 ml of 2 *N* sodium hydroxide, and refluxed for 1 hr. The mixture, on cooling to room temperature, gave 280 mg (61%) of 9-acridinemethanol, mp 166–167.5° [lit. (7) 164–165°].

Quaternization Reaction—For quaternization on a preparative scale, 179 mg (0.56 mmole) of chlorpromazine in 5 ml of acetonitrile was added to a solution of 200 mg (0.73 mmole) of 9-bromomethylacridine in 45 ml of dry acetonitrile. The mixture was stirred at room temperature for 4 hr and concentrated under vacuum to 20 ml. On cooling in ice water, 198 mg (68%) of hygroscopic quaternary salt (III, Scheme I) was obtained, mp 178.5–179.5°; UV (ethanol): 254 (log ϵ 5.176) and 366 (3.987) nm; IR (KBr): major peaks at 1558, 1450, 1400, 1315, 1270, 1225, 1082, 1025, 920, 830, 801, 770, and 740 cm^{-1}. The NMR spectrum [$(CD_3)_2SO$] (Fig. 1) exhibited acridine aromatic protons at δ 8.52 (d, 2, J = 8 Hz), 7.98 (d, 2, J = 8 Hz), and 7.3–7.7 (m, 4); the chlorpromazine aromatic protons signaled at 6.6–7.1 (m, 7); other absorptions were at 5.57 (s, 2, H^a), 3.3–3.8 (m, 4, H^b), 2.8 (s, 6, H^c), and 1.9 (s, 2, H^d). The peak at δ 3.14 was found to be due to water, since it could be enhanced by addition of more water to the solution. The multiplet at δ 2.26 was inherent to dimethyl sulfoxide due to its incomplete deuteration.

Anal.—Calc. for $C_{31}H_{29}BrClN_3S\cdot0.5H_2O$: C, 62.05; H, 5.04; Br, 13.32; N, 7.00. Found: C, 61.83; H, 4.87; Br, 13.30; N, 6.95.

Photolysis of Quaternary Salt of Chlorpromazine—A solution of the quaternary salt (III) was spotted on a silica gel TLC plate, and the dried spot was exposed to UV light of 200–300-nm wavelength, with peak emission at 254 nm. The result of irradiation was the appearance of a fluorescent spot which, after elution with 0.01 *N* sulfuric acid containing 20% methanol, was subjected to spectrofluorometric determination.

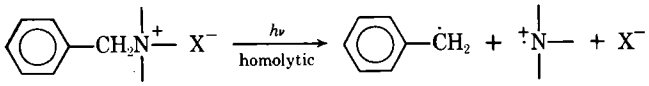
RESULTS AND DISCUSSION

The reaction of chlorpromazine with 9-bromomethylacridine under the conditions described to yield III appears to be stoichiometric. Assignment of the structure of the product as III is in accord with elemental analysis and NMR, UV, and IR spectra. Also consistent with the assignment of the product as a quaternary ammonium salt, as opposed to a sulfonium salt, is the reaction of promazine and chlorpromazine sulfoxide with 9-bromomethylacridine to form products with properties similar to those of III. Chlorpromazine *N*-oxide and chlorpromazine 5,*N*-dioxide do not react under the cited conditions to produce any quaternary products.

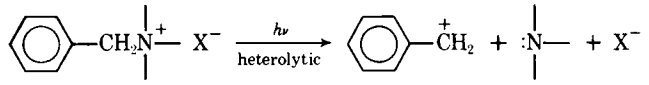
The quaternary product obtained is fairly stable as a solid or in organic solutions at temperatures up to 80°, but it is highly sensitive to light of a wide range of wavelengths. Effective photolysis occurs with a UV source emitting between 200 and 300 nm, with peak emission at 254 nm. With this source of light, the optimum photolysis of the quaternary salt occurred in 3 min, provided the distance between the source and the salt was maintained at 12 cm.

Photolysis of the quaternary salt III on a silica gel TLC plate produced a fluorescent spot exhibiting absorption and emission maxima at 350 and 474 nm, respectively. However, on development with methanol–benzene (1:9), this fluorescent spot separated into three fluorescent components. Of the total fluorescence, the major component contributed 86% while the minor spots were responsible for 6 and 8%, in order of their increasing R_f values, respectively. Structural assignments to these components were made by isolating sufficient quantities of the products following large-scale photolysis and comparing their physical, spectral, and TLC properties with those of the synthesized authentic samples. The major component was found to be 9-methylacridine, while the minor components were 9-acridinemethanol and 9-acridinecarboxaldehyde in order of their increasing R_f values. Based on calculations, the yields of the photolytic products obtained from the quaternary compound are as follows: 9-methylacridine, 26%; 9-acridinecarboxaldehyde, 1.0%; and 9-acridinemethanol, 0.6%.

Although a detailed mechanism of photolysis of III has not been studied, the products obtained are in line with the work of Ratcliff and Kochi (11), who presented evidence for competing solvolytic and radical processes in the photolysis of benzylammonium salts as shown in Schemes II and III.



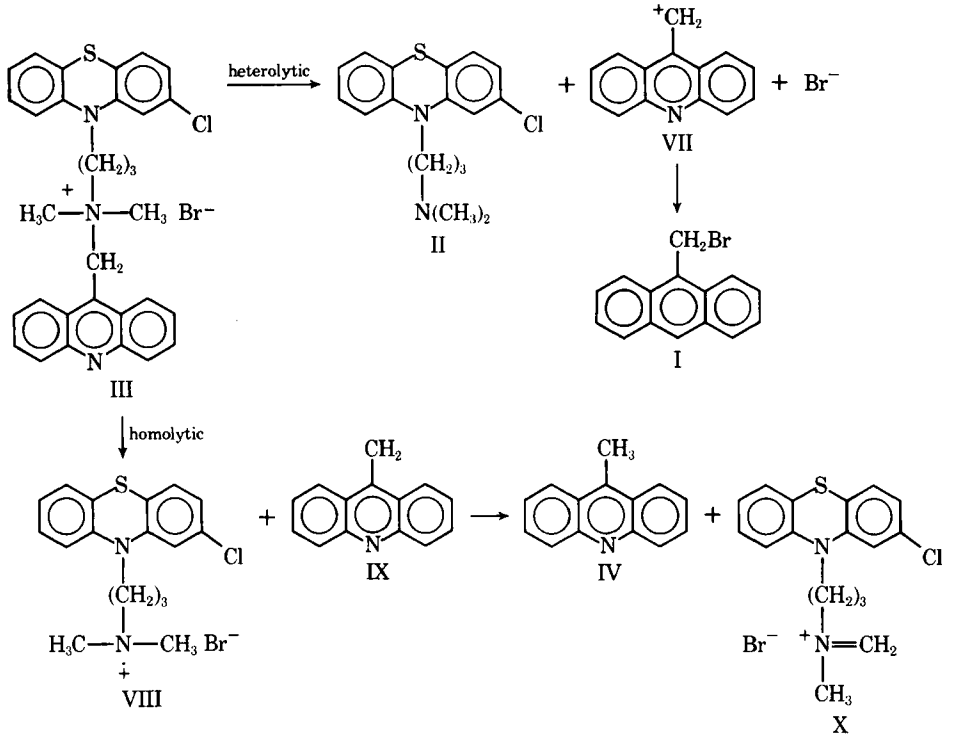
Scheme II



Scheme III

The homolytic pathway subsequently leads to formation of toluene by hydrogen abstraction from a N—CH_3 group by the benzyl radical. Scheme IV indicates an analogous manner in which the various products of photolysis of the chlorpromazine quaternary salt could arise. Thus, 9-methylacridine would reasonably arise by homolytic cleavage, followed by hydrogen abstraction by the 9-acridinyl radical (IX) from the chlorpromazine cation radical (VIII). One minor component, 9-acridinecarboxaldehyde, could result from a reaction of IX with oxygen, followed by decomposition. This latter pathway was not observed by Ratcliff and Kochi (11), but their experiments were carried out in solution whereas this photolysis was effected on silica gel. That 9-acridinecarboxal-

[1] Fisher Silicar CC-7.
[2] General Electric sunlamp.
[3] E. Merck silica gel $PF_{254+366}$.



Scheme IV—Postulated photolytic reaction mechanisms

dehyde is not a secondary photochemical product resulting from irradiation of 9-bromomethylacridine or 9-methylacridine was established by photolyzing pure samples of those two compounds adsorbed on silica gel. In neither instance was a detectable amount of 9-acridinecarboxaldehyde formed.

Heterolytic cleavage (Scheme II) of III should lead to formation of 9-bromomethylacridine (I), a product that is not observed under the reaction conditions. However, photolysis of I adsorbed on silica gel rapidly yields 9-acridinemethanol (VI), as shown by TLC comparison of the product with an authentic sample. This finding suggests that 9-bromomethylacridine is the precursor for 9-acridinemethanol observed in the photolysis of III and that both homolytic and heterolytic cleavages are occurring in the photolysis.

Although the structures of the phenothiazine nucleus-containing products have not been rigorously established, ferric chloride-active spots of R_f values [developing solvent of acetone–methanol–ethanolamine (100:30:1)] identical to those of chlorpromazine and

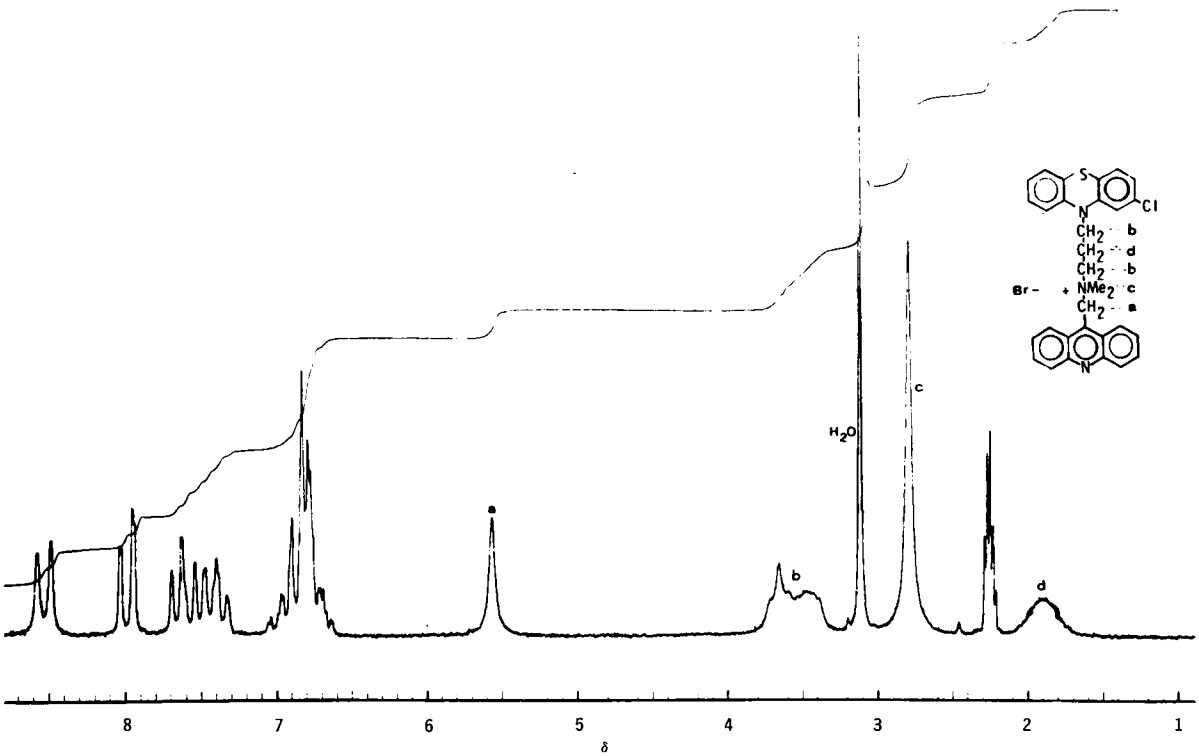
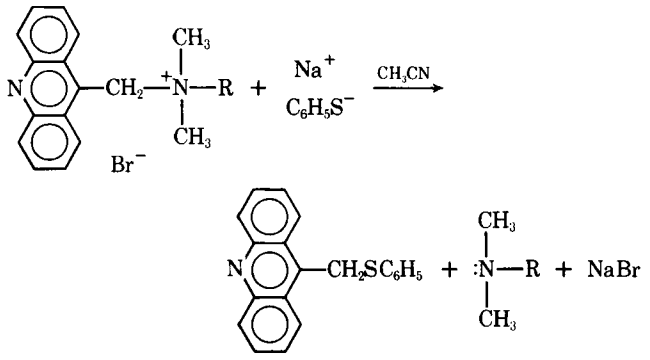


Figure 1—*NMR spectrum of 9-acridylmethyl quaternary salt of chlorpromazine in deuterated dimethyl sulfoxide;* a, b, c, *and* d *refer to the protons labeled in the structure of the quaternary salt shown.*



Scheme V

its monodemethylated derivative were observed. The latter should arise from hydrolysis of the chlorpromazine immonium salt (X) resulting from homolytic cleavage as shown in Scheme IV.

In applying the quaternization reaction to the assay of chlorpromazine (1), an aliquot of the reaction mixture after completion of the reaction is spotted on a silica gel TLC plate and developed first with methanol–methylal–benzene (2:2:1) and then with methanol–chloroform–benzene–ammonia (35:16:10:5). The first solvent system removes all nonquaternary products to the solvent front; the second system moves the quaternary product, which can now be photolyzed to yield determinable fluorescence. In the case of chlorpromazine, however, a minor secondary quaternary spot also arises with an R_f value slightly greater than that of the chlorpromazine quaternary. Although this minor product has not been rigorously identified, it is believed to be the quaternary salt resulting from the reaction of chlorpromazine sulfoxide with 9-bromomethylacridine on the basis of several pieces of indirect evidence. First, the spot also develops fluorescence upon photolysis. Second, the product has an R_f value identical to that of an independently prepared quaternary salt of chlorpromazine sulfoxide. Third, the quaternary salts of both chlorpromazine and its sulfoxide can be dequaternized with thiophenoxide according to Scheme V.

Pure, recrystallized III, upon dequaternization, yielded chlorpromazine as the only phenothiazine nucleus-containing product, as judged by TLC comparison with an authentic chlorpromazine sample. Similarly, upon dequaternization, the quaternary prepared from chlorpromazine sulfoxide yielded only chlorpromazine sulfoxide as the phenothiazine product. However, when chlorpromazine was reacted with 9-bromomethylacridine under the conditions of quaternization and an aliquot of the reaction mixture was treated with thiophenoxide for dequaternization, chlorpromazine as well as its sulfoxide was observed on TLC fractionation. Under identical conditions, but in the absence of 9-bromomethylacridine, chlorpromazine did not convert to any detectable amounts of its sulfoxide. This finding suggests that the chlorpromazine sulfoxide quaternary was produced under the reaction conditions of quaternization.

Furthermore, two-dimensional TLC has shown that the chlorpromazine quaternary can be converted to the sulfoxide product on the silica gel TLC plate, which is consistent with the reported (12) conversion of chlorpromazine to chlorpromazine sulfoxide on silica gel. On the whole, however, the generation of the sulfoxide product is generally limited to a total of <10%. In the actual assay, the calculations can be appropriately adjusted for this conversion.

Of significance are the facts that the quaternization reaction as well as the photolytically generated fluorescence is stoichiometric and that the fluorescing products can be successfully eluted from the TLC plate for fluorometric determination. This, in essence, constitutes the basis for the potential of the described quaternization reaction as a tool for a sensitive assay for chlorpromazine and other tertiary amine drugs. Several compounds other than chlorpromazine, including chlorpromazine sulfoxide, atropine, imipramine, chlorprothixene, codeine, morphine, and pilocarpine, were tested for reactivity with 9-bromomethylacridine; all yielded quaternary products and generated fluorescence on subsequent photolysis.

The fluorescence of the acridine moiety is totally quenched following introduction *via* quaternization into a tertiary amine molecule. Although no plausible explanation for this observation can be offered at present, a series of acridine derivatives is now being synthesized to determine whether or not the charge on the tertiary amine nitrogen is responsible for this total quenching of the acridine fluorescence or if there are other reasons for it.

The data presented and other preliminary experiments have confirmed that the overall approach of quaternization and photolysis is applicable to the determination of several tertiary amine drugs. In the case of chlorpromazine and its sulfoxide, for example, 50 ng and higher amounts have been quantitated (1). The amine concentration–fluorescence correlation is linear over a range of 0.05–1.00 μg. The detailed development and standardization of the specific assay for various tertiary amine drugs in aqueous solutions and human blood will be reported later.

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Synthesis of 5-(3,3-Disubstituted-1-triazenyl)imidazole-4-carbonitriles

Y. FULMER SHEALY [x] and C. ALLEN O'DELL

Abstract □ The 3,3-dimethyl-, 3-*n*-butyl-3-methyl-, 3-(2-hydroxyethyl)-3-methyl-, 3,3-bis(2-fluoroethyl)-, and 3,3-bis(2-chloroethyl)-1-triazenyl derivatives of imidazole-4-carbonitrile were prepared from 5-diazoimidazole-4-carbonitrile, a stable compound which produced a mass spectrum consistent with its structure. In contrast to the corresponding carboxamides, none of the triazenylimidazole-4-carbonitriles were active against lymphatic leukemia L-1210 in mice. Like the analogous carboxamide, the bis(2-chloroethyl)triazene readily cyclizes to the 1,2,3-triazolinium chloride.

Keyphrases □ Triazenylimidazoles—derivatives of imidazole-4-carbonitrile, synthesis □ Diazoimidazolecarbonitrile—synthesis and stability □ Triazenes—synthesis of 5-(3,3-disubstituted-1-triazenyl)imidazole-4-carbonitriles, screened against leukemia L-1210 □ Antileukemia activity—triazenylimidazolecarbonitriles screened

The effect of structural modifications on the antineoplastic activity of triazenes may be evaluated by varying the substituents on either the terminal nitrogen atom or the aryl (or heteroaryl) moiety. When the primary amide group of 5-(3,3-dimethyl-1-triazenyl)imidazole-4-carboxamide (I*a*) was replaced by ester groups (1–3), activity was retained. However, the esters, with the exception of the methyl ester (I*b*), were somewhat less effective against L-1210 lymphoid leukemia than was I*a*, and some of the ester analogs (I*c*) with terminal substituents identical with those of active congeners (I*d*) of I*a* did not show significant activity. Further alteration of the imidazole moiety of imidazole triazenes was effected by replacing the amide group of I*a* and a few of its active congeners (I*d*) with a nitrile group.

DISCUSSION

5-Aminoimidazole-4-carbonitrile (II) (4, 5) was prepared by dehydrating 5-aminoimidazole-4-carboxamide with phosphorus oxychloride (Scheme I) (6). The amino nitrile was diazotized in aqueous solution, and 5-diazoimidazole-4-carbonitrile (III) was isolated (80–90% yield) by extraction with ethyl acetate and was easily recrystallized. The IR spectrum included identifying bands at 3100 (imidazole CH), 2240 (CN), and 2185 (diazo group, strong) cm^{-1}. The mass spectrum of this diazoheterocycle was readily obtained and included prominent peaks arising from the molecular ion (*m/e* 119), loss of nitrogen (*m/e* 91), and loss of nitrogen and HCN (*m/e* 64), as well as metastable ions of *m/e* 69.6 (119 → 91) and 45.0 (91 → 64).

Coupling of III with the appropriate amine in ethyl acetate (or in ethyl acetate–ethanol) furnished the dimethyltriazenyl (IV*a*), *n*-butylmethyltriazenyl (IV*b*), and (2-hydroxyethyl)methyltriazenyl (IV*c*) derivatives. Reactions of III with bis(2-fluoroethyl)amine in aqueous solution or with bis(2-chloroethyl)amine in methylene chloride gave the bis(2-fluoroethyl)triazenyl (IV*d*) and bis(2-chloroethyl)triazenyl (IV*e*) derivatives, respectively. Triazenes IV*a*, IV*b*, and IV*d* were also obtained by diazotizing II and allowing the coupling reaction to proceed *in situ*.

Material isolated after reaction of III with bis(2-chloroethyl)amine contained 10–40% of the isomeric 1,2,3-triazolinium salt (V) as well as the triazene (IV*e*). Washing the isolated material with water reduced the amount of V to 5–10%. The triazolinium salt was isolated from the aqueous filtrate and was also prepared by stirring a mixture of IV*e* and aqueous methanol. The IR spectra of the triazenes (IV*d*–IV*e*) included a medium strong band at 1495–1500 cm^{-1}, whereas the triazolinium salt (V) produced a strong band at 1510 cm^{-1}. Although the analogous amide (NSC-82196) may be stored in the solid state at −15 to −20° for prolonged periods (7), triazene IV*e* slowly cyclizes to V under comparable conditions. The amount of V in a specimen of IV*e* stored at −15 to −20° had increased from about 10% to about 30% after 50 days. The hydrochloride of IV*e*, as well as the free base, cyclized to V rapidly in dimethyl sulfoxide solution.

The bis(2-fluoroethyl)triazene (IV*d*) was readily isolated and purified without the difficulties accompanying the obtainment of IV*e*. However, a UV absorption study showed that IV*d* changes rapidly at approximately pH 13 and indicated that the triazolinium fluoride analogous to V was formed. There was also evidence of a slower—presumably analogous—change in phosphate buffer.

All of the triazenes (IV*a*–IV*e*) and the diazoimidazole (III) were tested against lymphatic leukemia L-1210 in mice on the dosage schedules of Day 1, Days 1, 5, 9, and Days 1–9. Newly prepared specimens of IV*e* were used in these tests, and all compounds were injected within 5 min of the preparation of suspensions or solutions. No significant antileukemic activity was observed in any of these tests (Table I); in contrast, all of the corresponding imidazolecarboxamides (I*a* and I*d*) analogous to IV*a*–IV*e* are active against L-1210 leukemia (8–10). Except for the bis(2-fluoroethyl)triazene (IV*d*), triazenes IV*a*–IV*e* either were nontoxic (at doses up to 400 mg/kg) or were less toxic than the amide analogs. Although the failure of IV*e* to show activity may be due to its lability, the absence of observed activity by the remaining triazenes—and, perhaps, IV*e*—must be ascribed to replacement of the primary amide group by the nitrile group.

EXPERIMENTAL[1]

During all operations employed in the preparation, isolation, and purification of the triazenes (IV*a*–IV*e*) and the diazo derivative (III), the compounds were routinely protected from light. Specimens were routinely stored at 5° except for the bis(2-haloethyl)triazenyl derivatives, which were stored at −15 to −20°.

5-Aminoimidazole-4-carbonitrile (II) (4, 5)—This compound was prepared by the method of Suzuki *et al.* (6), except that the period of heating 5-aminoimidazole-4-carboxamide hydrochloride in phosphorus oxychloride was shortened to 2 hr to improve the yield (yields 40–50%); IR: 2220 and 2210 (CN) cm^{-1}; mass spectrum (direct-probe inlet temperature of 40°): *m/e* 108 (M), 81 (M − HCN), and 54 (81 − HCN).

5-Diazoimidazole-4-carbonitrile (III)—A solution of 5.20 g

[1] UV spectra were recorded with a Cary model 17 or model 14 spectrophotometer. UV maxima are in nanometers; sh = shoulder. Solutions for UV determinations were prepared by diluting a 5-ml aliquot of an ethanol solution (water for V) to 50 ml with 0.1 *N* HCl, phosphate buffer (pH 7), or 0.1 *N* NaOH; absorption maxima are reported as being at pH 1, 7, or 13, respectively. IR spectra were recorded with a Perkin-Elmer model 621 or model 521 spectrometer from samples in KBr disks. NMR spectra were determined with a Varian model A-60A spectrometer for observing proton resonance at 60 MHz. Chemical shift data (δ) are in parts per million downfield from tetramethylsilane, the internal reference. Mass spectral data were taken from low-resolution spectra determined with a Hitachi-Perkin-Elmer RMU-7 double-focusing instrument (70-ev electrons); M = molecular ion. Melting temperatures were determined in capillary tubes heated in a Mel-Temp apparatus and are not corrected. Unless otherwise indicated, TLC was performed on plates of silica gel H in chloroform–methanol (9:1) and spots were detected by: *(a)* UV light (254 and 365 nm) and *(b)* UV light (254 nm) after spraying the chromatogram with an optical whitening agent (Ultraphor WT, BASF Colors and Chemicals, Inc., Charlotte, N.C.). The quantity applied is shown parenthetically at appropriate places in the procedures.

Table I—Tests of 5-(3,3-Disubstituted-1-triazenyl)imidazole-4-carbonitriles against L-1210 Leukemia[a]

Compound	Dose, mg/kg[b] Day 1	Days 1, 5, 9	Days 1–9	Results[c]
IV*a*	400	400, 200, 100	200 (−2.8), 100	I, NT
			400 (6/6)	T
IV*b*	400 (6/6)			T
	300, 150, 75	300, 150, 75	300, 150, 75	I, NT
IV*c*	400, 200, 100	400, 200, 100	400[d], 200, 100	I, NT
IV*d*	150 (5/6)	75 (5/6)	37 (6/6)	T
	75 (−2.6)	37 (−2.7), 18	18 (−3.2), 9 (−1.9)	I, NT
IV*e*	400, 200, 100	400, 200, 100	400, 200, 100	I, NT
III	25 (4/6)			T
	12 (−2.0)	12 (−1.9)	12 (−3.5)	I, NT
		6 (−2.1), 3	6 (−1.9), 3	I, NT

[a] Tests were performed in accordance with protocols of the National Cancer Institute (11). These compounds were administered intraperitoneally in saline containing polysorbate 80 (Tween 80); the suspensions or solutions were prepared no more than 5 min prior to injection. [b] Numbers in parentheses give data either for deaths (treated/total) on or before Day 5 or for the difference (ΔW) in weight change (negative numbers, grams) on Day 5. Values for ΔW that were positive or were less than −1.8 g in magnitude are not shown. ΔW = average change in weight of treated mice minus average change in weight of control mice. [c] This summary of results applies to all of the doses listed on the same line in columns 2–4. I = inactive, T = toxic, and NT = nontoxic according to protocol criteria. [d] ΔW = −1.8 g.

(48 mmoles) of II in 50 ml of 2 *N* hydrochloric acid was added, dropwise, during 45 min to a stirred solution of 3.32 g (48 mmoles) of sodium nitrite in 50 ml of water at 0–5°. The red solution was stirred at 0–5° for 45 min and then extracted with ethyl acetate (5 × 100 ml). The organic solution was dried with magnesium sulfate, treated with activated charcoal, and concentrated *in vacuo* to a crystalline residue. The residue was triturated with cyclohexane, washed on a filter with cyclohexane, and dried *in vacuo* at 56° for 0.5 hr and at room temperature for 3 hr; the yield was 4.85 g (85%), mp 96–99°.

A specimen was recrystallized by treating a warm ethyl acetate solution of crude material with activated charcoal, diluting the hot filtrate with cyclohexane, and cooling the mixture, mp 97–100°; IR [medium and strong (s) bands in 3600–1000-cm^{-1} region]: 3100 (imidazole CH), 2240 (CN), 2185 s (diazo), 1465, 1425 s, 1320, 1285, 1260, 1225 s, 1150 s, and 1105 cm^{-1}; UV: λ_{max} 270 (sh) and 309 (ϵ 9000) nm in 0.1 *N* HCl; mass spectrum (direct-probe inlet temperature of 40°): *m/e* 119 (M), 91 (M − N_2), 69.6 (metastable, 119 → 91), 64 (91 − HCN), and 45.0 (metastable, 91 → 64).

Anal.—Calc. for C_4HN_5: C, 40.34; H, 0.85; N, 58.81. Found: C, 40.31; H, 1.17; N, 58.65.

5-(3,3-Dimethyl-1-triazenyl)imidazole-4-carbonitrile (IV*a*)—To a solution of 10 ml of anhydrous dimethylamine, 80 ml of ethyl acetate, and 10 ml of ethanol at 0° was added, portionwise and with stirring, 1.5 g of the diazoimidazole (III). Stirring at 0° was continued for 1 hr, and the solvents were evaporated *in vacuo*. The solid residue was triturated with ethyl acetate–cyclohexane (1:1), washed with ethyl acetate, and dried *in vacuo* at 56°; the yield was 1.98 g (87%), mp 186–189° dec.

A solution of the product in 100 ml of hot ethanol was treated with activated charcoal, filtered, diluted with 100 ml of cyclohexane, and stored at 5°; the yield of recrystallized IV*a* was 1.25 g (55%), mp 187–189° dec. (inserted at 100°, 3°/min); TLC: 1 spot (20 or 40 μg); IR: 2225 (CN) cm^{-1}; UV: λ_{max} 225 (ϵ 11,200) and 326 (ϵ 15,300) nm in phosphate buffer (pH 7) and 235 (ϵ 15,500) and 333 (ϵ 11,600) nm in 0.1 *N* NaOH; NMR (CF_3COOD): δ 3.48 (s, CH_3), 3.78 (s, CH_3), and 8.63 (s, imidazole CH).

Anal.—Calc. for $C_6H_8N_6$: C, 43.89; H, 4.91; N, 51.20. Found: C, 43.54; H, 4.82; N, 51.08.

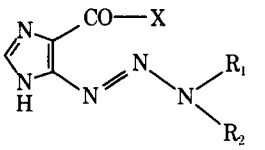
5-(3-Butyl-3-methyl-1-triazenyl)imidazole - 4-carbonitrile (IV*b*)—A solution of 173 mg of sodium nitrite in 4 ml of water was added slowly to a solution of 250 mg of the aminonitrile (II), 8 ml of water, and 2 ml of 2 *N* HCl at 0–5°. The mixture was stirred at 0–5° for 0.5 hr, filtered to remove a small amount of precipitate, and treated with sulfamic acid to decompose excess nitrite. Butylmethylamine (2 ml) was added to the cold solution, and the mixture was stirred for 1 hr at 0–5° and filtered to separate a precipitate (255 mg). Recrystallization of the crude product from a mixture of ethyl acetate (5 ml) and cyclohexane (12 ml) afforded white needles; the yield was 200 mg (42%), mp 103–105° dec. (inserted at 80°, 2°/min); TLC: 1 spot (40 or 80 μg); IR: 2225 (CN) cm^{-1}; UV: λ_{max} 226 (ϵ 11,600) and 328 (ϵ 15,600) nm in phosphate buffer (pH 7) and 236 (ϵ 16,600) and 334 (ϵ 11,500) nm in 0.1 *N* NaOH.

Anal.—Calc. for $C_9H_{14}N_6$: C, 52.41; H, 6.84; N, 40.75. Found: C, 52.71; H, 7.08; N, 40.62.

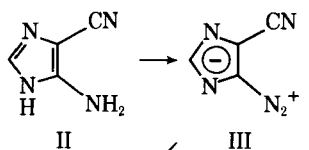
5-[3-(2-Hydroxyethyl)-3-methyl - 1-triazenyl]imidazole-4-carbonitrile (IV*c*)—This compound was prepared from III (476 mg) and *N*-methylethanolamine (1 ml) in ethyl acetate (20 ml) by the procedure used for IV*a* except that the reaction was carried out at room temperature. Sufficient methanol was added to dissolve a gummy material which precipitated in the reaction mixture, the solution was treated with activated charcoal, and the filtrate was concentrated *in vacuo* to a syrup. Stirring the residue with ethyl acetate (15 ml) containing methanol (1 ml) gave a crystalline solid (670 mg) which was recrystallized from ethyl acetate–cyclohexane (or, on a larger scale, from ethyl acetate–ethanol–cyclohexane); the yield was 570 mg (72%), mp 153–155° dec. (inserted at 60°, 3°/min); TLC: 1 spot (40 or 80 μg); IR: 2220 (CN) cm^{-1}; UV: λ_{max} 225 (ϵ 11,300) and 327 (ϵ 15,600) nm in phosphate buffer (pH 7) and 235 (ϵ 15,500) and 333 (ϵ 11,900) nm in 0.1 *N* NaOH.

Anal.—Calc. for $C_7H_{10}N_6O$: C, 43.28; H, 5.19; N, 43.28. Found: C, 43.24; H, 5.11; N, 43.19.

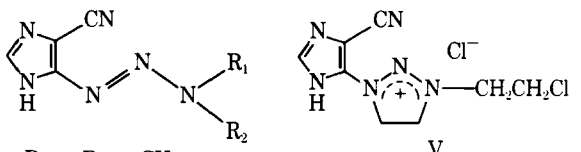
5-[3,3-Bis(2-fluoroethyl) - 1-triazenyl]imidazole-4-carboni-



Ia: X = NH_2, R_1 = R_2 = CH_3
Ib: X = OCH_3, R_1 = R_2 = CH_3
Ic: X = OR, R_1 = CH_3, R_2 = alkyl
Id: X = NH_2, R_1 = CH_3, R_2 = alkyl



II III



IVa: R_1 = R_2 = CH_3
IVb: R_1 = CH_3, R_2 = n-C_4H_9
IVc: R_1 = CH_3, R_2 = CH_2CH_2OH
IVd: R_1 = R_2 = CH_2CH_2F
IVe: R_1 = R_2 = CH_2CH_2Cl

V

Scheme I

trile (IV*d*)—A solution of 4.56 g of bis(2-fluoroethyl)amine hydrobromide (12), 20 ml of water, and 12 ml of 2 *N* aqueous sodium hydroxide was prepared at 0° and added in one portion to a solution of 1.0 g of the diazo derivative (III) in 30 ml of water at 0–5°. An orange precipitate formed immediately. The mixture was stirred at 0–5° for 15 min, and the precipitate was separated by filtration, washed thoroughly with water, and dried *in vacuo* at 55° for 45 min and at room temperature for 3 hr; the yield was 1.45 g, mp 125–128° dec.

A warm solution of the product in ethyl acetate (56 ml) was treated with activated charcoal, filtered, diluted with cyclohexane (20 ml), and chilled; the yield of recrystallized IV*d* was 1.2 g (62%), mp 129–131° dec. (inserted at 75°, 3°/min); TLC, 1 spot [40 μg, silica gel H, chloroform–methanol (95:5), detection by I_2, UV, and UV + whitening agent]; IR (KBr): 2220 (CN) cm^{-1}; UV: λ_{max} 226 (ε 9600) and 323 (ε 15,000) nm in 0.1 *N* HCl, 226 (ε 11,400) and 328 (ε 14,200) nm in phosphate buffer (pH 7), and unstable in 0.1 *N* NaOH; mass spectrum (direct-probe inlet temperature of 200°): *m/e* 228 (M), 120 [M − $N(CH_2CH_2F)_2$], 108 [$(FCH_2CH_2)_2N$+], and 92 (120 − N_2).

Anal.—Calc. for $C_8H_{10}F_2N_6$: C, 42.10; H, 4.42; N, 36.83. Found: C, 42.07; H, 4.43; N, 36.52.

A 10-ml aliquot of an ethanol solution of IV*d* was diluted to 200 ml with 0.1 *N* NaOH, and UV spectra of aliquots were determined at intervals up to 2.5 hr. The spectrum changed rapidly and within 20 min was essentially identical with that of V in 0.1 *N* NaOH: λ_{max} 234 (ε 10,500), 307 (ε 4200), and 412 (ε 13,300) nm.

5-[3,3-Bis(2-chloroethyl) - 1-triazenyl]imidazole-4-carbonitrile (IV*e*)—A solution of bis(2-chloroethyl)amine free base was prepared from 8.96 g of the hydrochloride and 150 ml (2 × 75 ml) of methylene chloride by the procedure described previously (7). To this solution at 15° was added 2.0 g of the diazoimidazole (III); the solution was stirred for 15 min, treated with activated charcoal, filtered, and diluted with 150 ml of cyclohexane. An oil precipitated and solidified when the mixture was chilled. The crystalline product was collected by filtration, washed with methylene chloride–cyclohexane (1:1), and dried *in vacuo* at room temperature; the yield was 3.97 g, mp 195–197° dec. (inserted at 70°, 3°/min).

The crude product was stirred in 50 ml of water in the dark for 5 min, separated by filtration, washed with water, and dried *in vacuo;* the yield was 3.76 g (86%); NMR (CF_3COOD): δ 4.17 (center of A_2B_2 multiplet, CH_2CH_2Cl) and 8.73 (s, imidazole CH). The NMR spectrum showed that this specimen was about 90% IV*e* and about 10% V (δ 5.1, 8.2). NMR analyses showed that the amount of V increased to about 15% after this specimen was kept for 20 days at −15 to −20° and that the amount of V in another specimen stored similarly had increased from about 10% to about 30% after 50 days. The melting temperature, 196–198° dec. (inserted at 70°, 3°/min), was the same as that of the triazolinium chloride (V). When a specimen was inserted at 130°, it melted immediately, resolidified, and remelted at 196–198° dec.

Anal.—Calc. for $C_8H_{10}Cl_2N_6$: C, 36.79; H, 3.86; N, 32.19. Found: C, 36.51; H, 3.65; N, 32.16.

The IR spectra of typical specimens of IV*e* containing 5–10% of V included the following bands in the 1600–1200-cm^{-1} region: 1570 m, 1510 sh, 1495 m, 1450 s, 1420 m, 1395 ms, 1375, 1355 ms, 1335 ms, 1285 ms, 1275, 1265 ms, 1220, and 1210 sh. A medium strong band at 1100 cm^{-1} and medium bands at 1160 and 1140 cm^{-1} were in a region where V showed only weak bands (at 1180, 1170, and 1135 cm^{-1}); other prominent bands in the spectra of IV*e* were at 2230 s (CN), 955, and 650 cm^{-1}. The shoulder at 1510 cm^{-1} in the spectra of specimens of IV*e* was probably due to the presence of the triazolinium salt (V). Attempts to correlate the results of NMR assays of IV*e* samples containing different amounts (5–40%) of V with IR spectra indicated that IV*e* was sometimes partly converted to V during the preparation of the potassium bromide disk.

A hydrochloride of IV*e* was prepared as follows. A mixture of 350 mg of IV*e*, 20 ml of ether, and 40 ml of methanol was filtered, the filtrate was treated with an excess of hydrogen chloride, the turbid mixture was filtered, and the filtrate was concentrated to a syrup *in vacuo*. Evaporation of several portions of ether from the residue converted it to a crystalline solid, which was washed with several portions of ether and dried *in vacuo;* the yield was 280 mg. The NMR spectra (CF_3COOD) of the hydrochloride and the free base were identical.

Anal.—Calc. for $C_8H_{10}Cl_2N_6 \cdot HCl$: C, 32.29; H, 3.73; N, 28.24. Found: C, 32.52; H, 3.48; N, 28.58.

1-(2-Chloroethyl)-3-(5-cyanoimidazol-4-yl)-Δ^2-1,2,3-triazolinium Chloride (V)—Another experiment performed by the procedure described for the reaction of III and bis(2-chloroethyl)amine afforded a crude product that contained (NMR analysis) about 40% of the 1,2,3-triazolinium salt (V). A suspension of this material (2.9 g) in 40 ml of water was stirred in the dark for 5 min. The insoluble fraction (1.88 g) was separated by filtration and was shown by NMR analysis to be the bis(2-chloroethyl)triazene (IV*e*), containing about 10% of V. The filtrate was treated with activated charcoal, filtered, and lyophilized.

The residual, yellow crystalline solid was triturated with ethyl acetate and dried *in vacuo* at 56°; the yield was 640 mg, mp 197–199° dec. (inserted at 70°, 3°/min); UV: λ_{max} 232 (ε 13,100), 252 (plateau), and 345 (ε 8800) nm in 0.1 *N* HCl; 233 (ε 11,300), 277 (ε 3750), and 375 (ε 8300) nm in phosphate buffer (pH 7); 233 (ε 10,500), 305 (ε 4200), and 410 (ε 13,600) nm in 0.1 *N* NaOH; IR (1600–1200-cm^{-1} region): 1575 ms, 1510 s, 1460 ms, 1440, 1420 s, 1365, 1350, 1325, 1300, 1280 s, 1270 s, and 1210 cm^{-1}; also medium strong IR bands at 3120, 2510 (broad), 2230 (CN), 930, and 865 cm^{-1}; NMR (CF_3COOD): δ 4.38 (center of A_2B_2 multiplet, CH_2CH_2Cl), 5.12 (multiplet approaching singlet, CH_2CH_2), and 8.25 (s, imidazole CH).

Anal.—Calc. for $C_8H_{10}Cl_2N_6$: C, 36.79; H, 3.86; Cl, 27.16; N, 32.19. Found: C, 36.71; H, 3.78; Cl, 27.18; N, 32.41.

Compound V was also prepared by stirring isolated IV*e* in 90% methanol; several days were required to complete the cyclization because of the sparing solubility of IV*e*.

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Interaction of Metronidazole with Metallic Ions of Biological Importance

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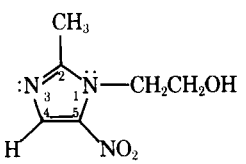
Abstract □ The possibility that metronidazole exerts several of its biological actions *via* interaction with important metal ions was investigated. NMR spectroscopy and polarography (ac) were used to test for any interaction, to locate the probable sites for complexation, and to determine the molecular stoichiometry of any complexes formed. Of the series of divalent metal ions tested, only cupric ion showed detectable interaction with metronidazole. The predominant site of interaction of cupric ion was the unsubstituted nitrogen atom (N-3) on the metronidazole molecule. The stoichiometry of the complex was $[Cu\text{-}(metronidazole)_4]^{+2}$. A likely structure for the complex is presented.

Keyphrases □ Metronidazole—interaction with divalent metal ions studied using NMR spectroscopy and ac polarography □ Metal ions, divalent—potential interaction with metronidazole studied using NMR spectroscopy and ac polarography □ Interactions, potential—metronidazole and divalent metal ions, studied using NMR spectroscopy and ac polarography

Metronidazole[1] (1-β-hydroxyethyl-2-methyl-5-nitroimidazole) (I) has been shown to be an effective agent against protozoal and anaerobic microbial infections (1–5). The antimicrobial activity of metronidazole against anaerobes is superior to that of its analogs as demonstrated by its minimum inhibitory concentration (MIC) (6, 7). On the basis of these results, it was proposed that metronidazole can act selectively as an electronic sink, inhibiting the metabolic activity of ferredoxin and hydrogenase systems involved in the phosphoroclastic reaction in the anaerobes. More recently, it was proposed that a favorable environment exists in anaerobic microorganisms which creates a concentration gradient for the entry of this drug into the microbial cell (8).

The purpose of this investigation was to test whether metronidazole, like other imidazole compounds, possesses an affinity for complexation with metallic ions that are known to be of biological significance. The biological importance of metalloproteins and metalloenzymes in living organisms is well documented (9). Since metronidazole might interact with such molecules, its activity in the presence of trace amounts of metal ions was examined in aqueous solutions. Studies were conducted with the aid of polarographic and NMR spectroscopic techniques to provide information on the type of complex as well as to detect the site of complexation on the imidazole nucleus.



I

[1] Flagyl, G. D. Searle & Co.

EXPERIMENTAL

Materials—All metallic salts used were analytical reagent grade. Metronidazole[2] was used as obtained.

NMR—Deuterium oxide[3], 99.7% pure, was used for the preparation of all solutions. Prior to the study, stock solutions of $CuCl_2$ (1.7×10^{-4} *M*), $SnCl_2$ (2.66×10^{-4} *M*), $MgCl_2$ (1×10^{-4} *M*), $FeCl_3$ (3.6×10^{-4} *M*), $FeCl_2$ (2.01×10^{-4} *M*), $CoCl_2$ (2.57×10^{-4} *M*), $NiCl_2$ (1.74×10^{-4} *M*), and $ZnCl_2$ (2.93×10^{-4} *M*) were prepared and stored in a refrigerator.

AC Polarography—Triple-distilled instrument mercury[4] was utilized in a dropping mercury electrode. For the preparation of all solutions, fresh deionized, triple-distilled water was used to prepare 0.2 *M* KCl solution. The KCl solution was utilized as a supporting electrolyte solution to prepare the solutions of $CuCl_2$ (8×10^{-4} *M*), metronidazole (8×10^{-4} *M*), and metronidazole–$CuCl_2$ combinations just prior to the study.

Procedure—*NMR*—A weighed amount (100 mg) of metronidazole was transferred to a 10-ml volumetric flask to which a known volume of a metallic salt stock solution was added. Then D_2O was added to make the volume 10 ml (metronidazole = 5.84×10^{-2} *M*). A portion of this solution was scanned on a NMR spectrometer[5] at 500-Hz sweep width. The operating temperature probe was held at a range of 37–40°, and 1% tetramethylsilane solution in $CDCl_3$ was used as an external standard. Each salt solution at a concentration of 10^{-4} *M* was run as a blank to ensure the optimal performance of the instrument.

AC Polarography—AC polarographic measurements were performed on a polarograph[6] equipped with mercury drop timer.

Six milliliters of supporting electrolyte solution or cupric-ion solutions with various metronidazole concentrations was deaerated with pure nitrogen gas for 5 min in a three-electrode cell prior to measurement. A nitrogen atmosphere was maintained above the solution during measurement to prevent oxygen contamination. The controls were preset as follows: modulation amplitude, 10 mv; ac frequency, 80 Hz; scan rate, 2 mv/sec; drop time, 0.5 sec; and sample duration, 10 msec.

RESULTS AND DISCUSSION

The effects of transition metal ions on the NMR spectra of the olefinic proton at C-4 and of the methyl proton at C-2 positions of metronidazole (I) are recorded in Table I. The effect of metallic ions on NMR peaks is represented by the broadening of $\Delta V_{1/2}$ (the linewidth at one-half the maximum peak height in cycles per second) and by the increase in relaxation rate ($1/T_2$) value. These two parameters are related to one another by the following relation (12):

$$\frac{1}{T_2} = \pi \Delta V_{1/2} \qquad \text{(Eq. 1)}$$

where $\pi = 3.1416$, and the $\Delta V_{1/2}$ value is estimated from the NMR spectrum.

None of the metallic ions studied, except cupric ion, showed any

[2] Lot 327, Searle Laboratories.
[3] Merck & Co., Rahway, N.J.
[4] Bethlehem Apparatus Co., Hellertown, Pa.
[5] Model A.60D, Varian Associates, Palo Alto, Calif.
[6] PAR electrochemistry system; model 170 equipped with model 172A mercury drop timer, Princeton Applied Research Corp., Princeton, N.J.

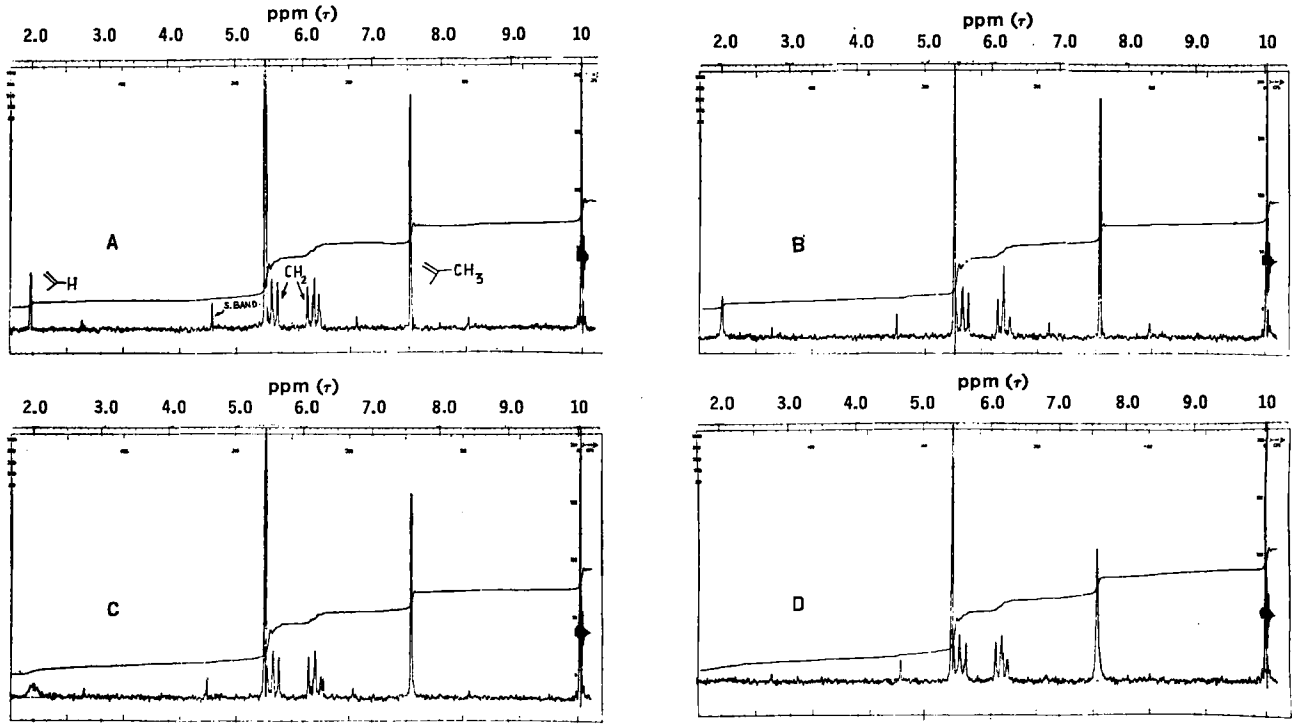


Figure 1—*Effect of cupric-ion concentration on the NMR spectrum of metronidazole. All of these spectra were run under standard operating conditions. Sweep time was set at 1000 sec. Key: A, metronidazole alone; B, metronidazole + 1.15 × 10⁻⁵ M $CuCl_2$; C, metronidazole + 1.15 × 10⁻⁴ M $CuCl_2$; and D, metronidazole + 2.30 × 10⁻⁴ M $CuCl_2$.*

substantial peak-broadening effect on the NMR spectra of metronidazole protons. The effect of cupric ion on both the olefinic proton peak and the methyl proton peak was negligible when the concentration of cupric ion was below 2.3×10^{-5} *M*. However, a significant effect was observed above this concentration. This finding may be explained by the fact that only cupric ion has an electrochemically favorable energy ground for electron acceptance from metronidazole.

Table II lists the redox processes and corresponding potentials for the metallic salts investigated. Cupric ion has a different redox potential profile than the other metallic ions and has a negative potential value which appears to be favorable for such an interaction. Also, cupric ion has a greater tendency to form stable complexes with electron-donating ligands as compared to the other metallic ions (9, 10).

Figure 1 shows that cupric ion caused a specific peak broadening of the imidazole ring proton (the olefinic proton at C-4) (for left-hand peak) and had a lesser effect on the peak for the methyl protons on C-2 position. Since there was no evidence of peak broadening for the protons on the hydroxyethyl group on the N-1 position, it may be concluded that this side chain was not directly involved in the complexation.

The proton relaxation rates ($1/T_2$) listed in Table I for both the olefinic proton and the methyl protons are plotted against the concentration profile of cupric ion. If the proton relaxation rates are dependent on cupric-ion concentration:

$$\frac{1}{T_2} \propto [\text{cupric ion}]^{\beta} \qquad \text{(Eq. 2)}$$

Then:

$$\log\left(\frac{1}{T_2}\right) = \text{constant} + \beta \log [\text{cupric ion}] \qquad \text{(Eq. 3)}$$

where β is the slope of the log ($1/T_2$) *versus* log [cupric ion] profile. Such a relationship is illustrated in Fig. 2 for the metronidazole–cupric-ion interaction. The effect of cupric ion on the olefinic proton peak is greater (β = 1.3) than that on the methyl protons (β = 0.26) when the cupric-ion concentration is higher than 2.3×10^{-5} *M*. At cupric-ion concentrations above 10^{-4} *M*, the NMR peak for the olefinic proton is eliminated. Cupric ion produced no observ-

Table I—Effect of Metallic Ions on the Peak Broadening ($V_{1/2}$) and Relaxation Rate ($1/T_2$) of Metronidazole[a] Protons

Metallic Ion	Metallic Ion Concentration, *M*	—H $\Delta V_{1/2}$, cps	—CH_3 $\Delta V_{1/2}$, cps	Relaxation Rate ($1/T_2$), cps H	Relaxation Rate ($1/T_2$), cps CH_3
None	—	1	1	3.142	3.142
Sn^{+2}	2.66×10^{-4}	1	1	3.142	3.142
Fe^{+2}	2.01×10^{-4}	1	1	3.142	3.142
Mg^{+2}	1.0×10^{-4}	1	1	3.142	3.142
Co^{+2}	2.57×10^{-4}	1.2	1	3.772	3.142
Ni^{+2}	1.74×10^{-4}	1	1	3.772	3.142
Zn^{+2}	2.93×10^{-4}	1	1	3.772	3.142
Cu^{+2}	1.76×10^{-6}	1.5	1	4.7	3.142
Cu^{+2}	2.3×10^{-6}	1.5	1	4.7	3.142
Cu^{+2}	4.25×10^{-6}	2.0	1.3	6.28	4.08
Cu^{+2}	7.0×10^{-6}	1.2	1	3.77	3.142
Cu^{+2}	8.5×10^{-6}	2.0	1.25	6.28	3.92
Cu^{+2}	1.15×10^{-5}	1.5	1.0	4.7	3.142
Cu^{+2}	1.76×10^{-5}	2.0	1.0	6.2	3.142
Cu^{+2}	2.3×10^{-5}	1.5	1.0	4.7	3.142
Cu^{+2}	8.5×10^{-5}	6.0	1.65	18.85	5.18
Cu^{+2}	1.15×10^{-4}	9.0	1.25	28.27	3.92
Cu^{+2}	1.76×10^{-4}	11.0	1.85	34.56	5.81

[a] 5.84×10^{-2} *M* metronidazole.

Table II—Oxidation–Reduction of Some Biologically Important Metallic Ions

Redox Process	Redox Potentials, v
$Cu^{+2} + 2e^- \rightleftharpoons Cu$	−0.337
$Sn^{+2} + 2e^- \rightleftharpoons Sn$	+0.140
$Ni^{+2} + 2e^- \rightleftharpoons Ni$	+0.230
$Co^{+2} + 2e^- \rightleftharpoons Co$	+0.280
$Fe^{+2} + 2e^- \rightleftharpoons Fe$	+0.440
$Zn^{+2} + 2e^- \rightleftharpoons Zn$	+0.763
$Mg^{+2} + 2e^- \rightleftharpoons Mg$	+2.370

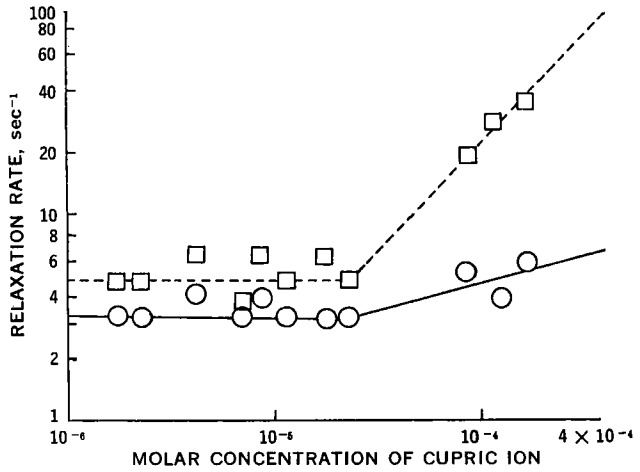


Figure 2—*Effect of cupric-ion concentration on the relaxation rate of olefinic proton (□) and methyl proton (○) peaks of the metronidazole molecule. Above a concentration of 2.3 × 10⁻⁵ M, cupric ion showed a broadening effect on both peaks. The effect on the olefinic proton (slope of 1.313) was fivefold greater than on the methyl proton (slope of 0.255).*

able effect on the proton relaxation rate of both peaks when its concentration was lower than 10^{-5} *M*. The instrument sensitivity (signal to noise ratio) may be inadequate to detect the occurrence of the cupric-ion–metronidazole molecule interaction at these concentrations.

To complex with cupric ion, the metronidazole molecule has to donate its electrons to occupy the outermost orbit of the cupric-ion hybrid. There is one pair of unshared electrons on each N-1 and N-3 atom. Therefore, it is reasonable that either one may be the potential site for metallic ion complexation. Since the electron-withdrawing nitro group at the C-5 position should outweigh the weak electron-donating property of the methyl group at the C-2 position, the availability of the lone pair electrons on the N-1 atom to cupric ion will be limited. In addition, the presence of the hydroxyethyl chain on the N-1 atom sterically hinders the approach of cupric ions.

The formal π-charge distribution around the metronidazole molecule was calculated by the Hückel method (Fig. 3). The positive charge of 0.0374 around the N-1 position, when compared to the negative charge (−1.0012) around the N-3 position, supports

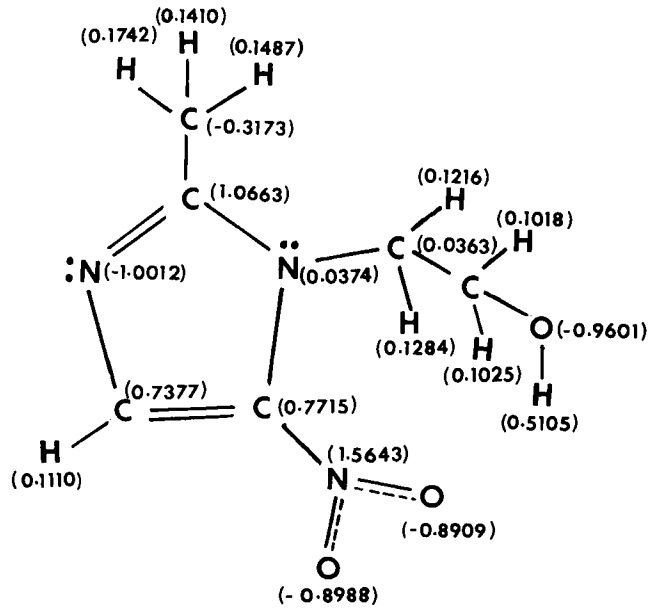


Figure 3—*Formal π-charge distribution (Hückel method) on the metronidazole molecule.*

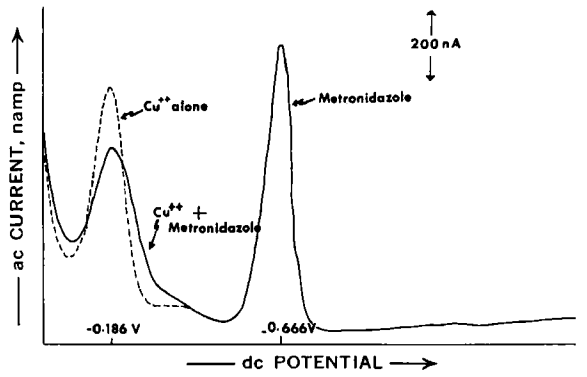


Figure 4—*An ac polarogram for cupric ion (8 × 10⁻⁵ M) alone (broken line) and with metronidazole (8 × 10⁻⁵ M, solid line). The peak at −0.186 v for cupric ion alone is decreased in the presence of metronidazole due to complexation. The peak at −0.666 v for metronidazole alone is not affected by the presence of cupric ion.*

the postulate that the latter is the site for cupric-ion complexation. This postulate is further supported by the NMR spectral data which show peak broadening for the olefinic proton on the carbon atom adjacent to the N-3 position.

Several investigators (12–16) demonstrated that NMR spectroscopy is useful for the detection of the complexation site between a ligand and a substrate. Li *et al.* (14) used this technique in identifying the site of cupric-ion complexation with amino acids and peptides. They showed that the selective broadening of a proton peak is due to a decrease in the relaxation time of the proton nuclei. Decreases of this type can be induced by the magnetic field of paramagnetic metallic ions undergoing a rapid exchange at a complexation site in the vicinity of that proton. The peak broadening of protons adjacent to the metallic ion binding site on polynucleotides was also reported (15, 16). The results presented here are in agreement with that observation.

To determine the stoichiometry of the complex, the cupric-ion–metronidazole interaction was investigated with ac polarography. The ac polarogram of cupric ion in the presence or absence of an equimolar concentration of metronidazole (8×10^{-5} *M*) is shown in Fig. 4 and demonstrates the applicability of ac polarographic techniques for the quantitative measurement of the cupric-ion–metronidazole complexation. Both metronidazole and cupric ion gave well-isolated ac peaks at −0.666 and −0.186 v, respectively. The magnitude of the ac peak for cupric ion was decreased considerably in the presence of an equimolar concentration of metronidazole, but the current height of the ac peak for metronidazole was constant and independent of the presence and concentration of cupric ion. This finding indicated that the nitro group on the metronidazole molecule did not participate in the complexation process since it is this group that is polarographically active (17, 18).

As demonstrated in Fig. 5, the free cupric-ion concentration decreased as the concentration of metronidazole increased. From this profile, it was calculated that 2.9×10^{-4} *M* of metronidazole was required to complex completely the 8×10^{-5} *M* of cupric ion. The

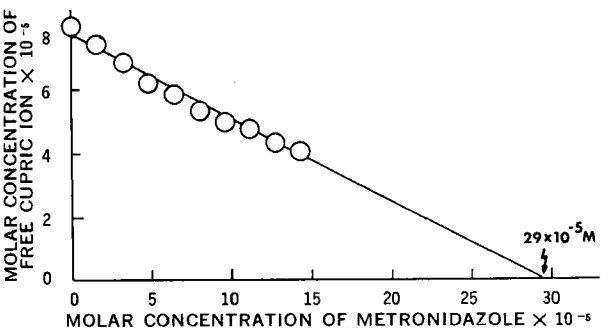
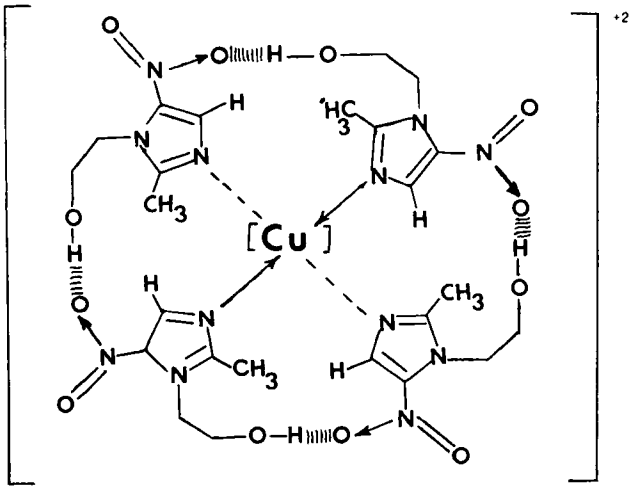


Figure 5—*Plot illustrating the linear decrease in polarographically assayable cupric-ion concentration as a function of increasing metronidazole concentration.*



II

coordination number for the complexation of metronidazole with cupric ion was calculated to be 3.63, which implies that four molecules of metronidazole are required for the complexation of one cupric ion. Cupric ion has an electronic configuration of dsp^2 and tends to form square planar complexes with electron-donating ligands, *e.g.*, $:NH_3$ (11). On the basis of the information available to date, a structure for a 4 to 1 square planar complex between metronidazole and cupric ion $[Cu\text{-(metronidazole)}_4]^{+2}$ is proposed (II). The intermolecular hydrogen bonding between the hydroxy proton on N-1 and the nitro group on the C-5 position was suggested by the formal π-charge distribution (Fig. 3) and would tend to strengthen the stability of a planar complex.

Fried (19) recently investigated the biochemical mode of metronidazole action against alcoholism. He found that the presence of excess zinc ions, which are essential at the active site of alcohol dehydrogenase, did not reverse the loss in enzymatic activity caused by metronidazole. This finding led to a conclusion that the inhibition of alcohol dehydrogenase activity by metronidazole is not *via* zinc–metronidazole complexation. This conclusion is consistent with the NMR data that metronidazole did not show any interaction with zinc ions. In testing the activity of metronidazole against uricase (contains cupric ion), it was found that the drug (at 10^{-3} *M*) completely inhibited enzyme activity (19). It is known that several metalloproteins that take part in cellular metabolic processes contain cupric ion at their active site (9). In view of the results on the selective complexation of cupric ion by metronidazole, it would be interesting to investigate the latter effect on these cupric ion-containing macromolecules.

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[x] To whom inquiries should be directed.

Linear Relationships between Plasma Binding and Lipophilicity of Disopyramide Derivatives

YIE W. CHIEN ˣ, HOWARD J. LAMBERT, and TENG K. LIN

Abstract □ The extent of plasma binding and the partition coefficient of disopyramide and 20 disopyramide derivatives were determined. Structural variations on the four functional groups around the tetrahedral carbon in the disopyramide molecule were found to influence both parameters to varying degrees. Three linear equations were developed to correlate the observed effects, depending on the type of chemical modification. The linear correlation between drug–plasma interaction and lipophilic character was analyzed theoretically. A simple model was derived to relate quantitatively the variation in the extent of plasma binding to the change in lipophilicity of disopyramide derivatives.

Keyphrases □ Disopyramide and 20 derivatives—linear relationship between plasma binding and lipophilicity determined □ Lipophilicity and structure of disopyramide and 20 derivatives—relationship to protein binding □ Plasma binding, disopyramide and 20 derivatives—determination, relationship to lipophilicity and structure □ Partition coefficients, disopyramide and 20 derivatives—determination

Much effort recently has been spent on separating the various physicochemical properties of drugs to correlate the partial contributions of hydrophobic, electronic, and steric parameters to their overall biological properties (1–3). Although the details of how drug action is related to physicochemical properties of drugs remain unclear, it is profitable to use the phenomenological approach to differentiate and classify such molecular properties (4).

It has long been recognized that most administered drugs interact to some degree with serum proteins (5). Recently, several successful attempts were made to correlate these protein binding data quantitatively with the hydrophobicity of drugs (6–10).

The reported antiarrhythmic activities of a series of disopyramide derivatives were investigated and appeared to be quantitatively dependent on the combined effect of the lipophilicity and carbonyl vibrational energy of the drug (3, 11). Disopyramide and its derivatives (Table I) contain an asymmetric carbon center with four varying substituent groups. The availability of this series of compounds permitted the study of which groups were the predominant sites for protein binding and how structural variations affected the extent and strength of interaction.

This paper reports on the correlation of lipophilicity and structure with the protein binding of disopyramide and 20 derivatives.

EXPERIMENTAL

Materials—Human plasma was collected by centrifuging whole blood at 2500 rpm and 4° for 20 min[1]. Approximately 50 ml of plasma was obtained from the centrifugation of 100 ml of whole blood.

The 20 derivatives of disopyramide[2] were synthesized[3] by previously described methods (12, 13).

Binding Studies—Drug solutions (16×10^{-5} *M*) were prepared in isotonic 0.1 *M* phosphate buffer (pH 7.4) immediately prior to binding measurements. From 2 to 5 ml of this solution was added to 5 ml of human plasma, and buffer was added to 10 ml. The resultant mixtures, after equilibrating at 37° for 1 hr, were poured into membrane ultrafilters[4] and centrifuged at 1250 rpm and 4° for 30 min. The filtrates were assayed spectrophotometrically from 240 to 360 nm[5]. The absorbance of the peaks in the range of 250–~280 nm was recorded for calculating the free drug concentration. Drug solution, in the absence of proteins, was also centrifuged at the same time to correct for drug loss by membrane absorption. The absorption sometimes accounted for up to 60% of drug, depending on the nature of drug species examined.

Plasma binding results are expressed as fraction of drug, β (%),

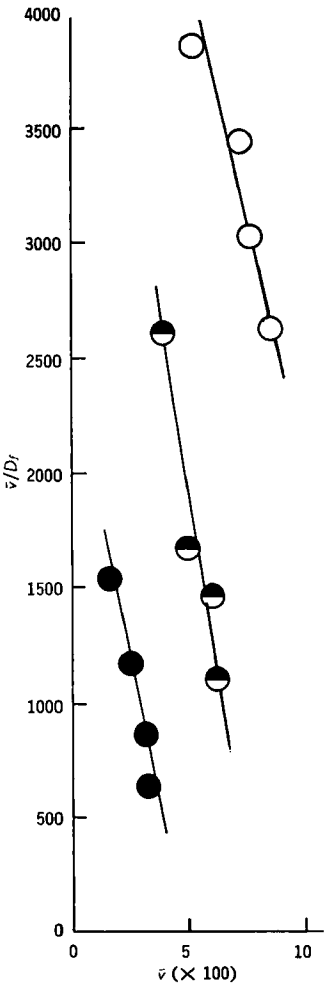


Figure 1—*Scatchard analysis for V* (○), *XVI* (◓), *and disopyramide* (●). *The linearity of the* $\bar{v}/D_f$ versus $\bar{v}$ *plots indicates that only one group of binding sites is involved in the interaction of disopyramide derivatives with plasma proteins in the concentration range* (*3.2–* ~ *8.0* × 10^{-5} M) *examined.*

[1] Sorvall RC-3 automatic refrigerated centrifuge.

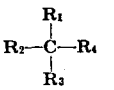
[2] SC-7031. The brand name for disopyramide phosphate (SC-13957) is Norpace (Searle Laboratories, Division of G. D. Searle & Co., Chicago, IL 60680).

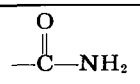
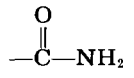
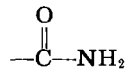
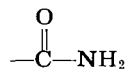
[3] Dr. J. W. Cusic, Dr. H. W. Sause, Mr. J. H. Yen, and Mr. P. K. Yonan, Chemical Research Department, Searle Laboratories.

[4] Centriflo, model CF 50A, Amicon Corp., Lexington, Mass.

[5] Coleman model 124 D spectrophotometer.

Table I—Relationship between Structural Variations around the Asymmetric Carbon Center of Disopyramide and the Extent of Drug–Plasma Interaction



Compound[a]	R_1	R_2	R_3	R_4	β, %	p.c.[b]
Disopyramide				$-CH_2CH_2N(iso\text{-}C_3H_7)_2$	27.3	0.66
I	H			$-CH_2CH_2N(iso\text{-}C_3H_7)_2$	58.5	11.97
II		H		$-CH_2CH_2N(iso\text{-}C_3H_7)_2$	4.6	0.0488
III			H	$-CH_2CH_2N(iso\text{-}C_3H_7)_2$	53.6	5.4
IV				H	27.3	6.87

[a] SC-7031, SC-26000, SC-21799, SC-5260, and SC-16571, respectively. [b] Partition coefficient.

bound to plasma:

$$\beta(\%) = \frac{(\text{drug concentration without plasma} - \text{drug concentration with plasma}) \times 100}{\text{drug concentration without plasma}} \quad \text{(Eq. 1)}$$

Partition Studies—Drug solutions, with a concentration of 8×10^{-5} *M*, were freshly prepared in 1-octanol-saturated phosphate buffer (0.1 *M*, pH 7.4, isotonic). Ten milliliters was agitated and equilibrated with 10 ml of phosphate buffer-saturated 1-octanol. The drug concentration in the phosphate buffer phase before and after partitioning was measured spectrophotometrically and utilized to calculate the magnitude of the partition coefficient.

RESULTS AND DISCUSSION

For plasma binding studies, four drug concentrations, ranging from 3.2×10^{-5} to 8.0×10^{-5} *M*, were examined. In this concentra-

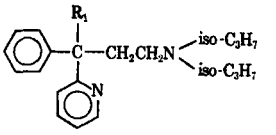
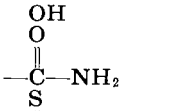
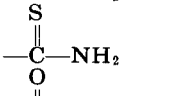
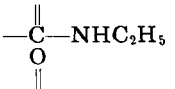
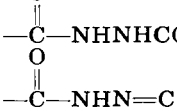
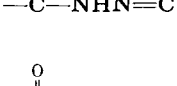
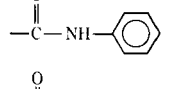
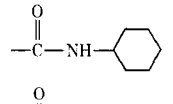
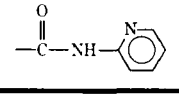


Table II—Structural Variation of the Amide Moiety of Disopyramide Derivatives

Compound[a]	R_1	log (p.c.)	log (D_b/D_f) Observed	log (D_b/D_f) Estimated Eq. 10	log (D_b/D_f) Estimated Eq. 13
I	H	1.078	0.149	0.206	0.234
V	OH	0.918	0.171	0.131	0.142
Disopyramide		−0.182	−0.425	−0.389	−0.493
VI		0.208	−0.091	−0.205	−0.268
VII		0.028	−0.299	−0.290	−0.372
VIII		−0.752	−0.656	−0.659	−0.822
IX		0.058	−0.321	−0.276	−0.355
X		1.468	0.316	0.391	0.459
XI		1.082	0.191	0.208	0.267
XII		1.568	0.520	0.438	0.517

[a] SC-26000, SC-26658, SC-7031, SC-25888, SC-26481, SC-26553, SC-26529, SC-27063, SC-29359, and SC-27071, respectively.

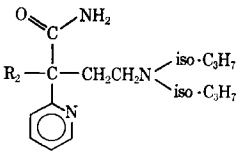
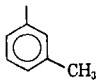
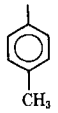
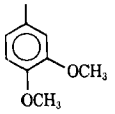


Table III—Structural Variation on the Phenyl Ring of Disopyramide Derivatives

Compound[a]	R_2	log (p.c.)	log (D_b/D_f) Observed	log (D_b/D_f) Estimated Eq. 11	log (D_b/D_f) Estimated Eq. 13
II	H	−1.310	−1.317	−1.209	−1.145
Disopyramide		−0.182	−0.425	−0.485	−0.493
XIII		0.174	−0.343	−0.257	−0.288
XIV		0.232	−0.203	−0.220	−0.254
XV		−0.788	−0.745	−0.874	−0.843
XVI		0.083	−0.294	−0.315	−0.340
XVII		0.603	−0.015	−0.018	−0.040

[a] SC-21799, SC-7031, SC-12875, SC-24874, SC-12857, SC-13234, and SC-13068, respectively.

tion region, the results of a Scatchard analysis (Fig. 1) demonstrated that only a primary binding site was involved in the interaction with disopyramide derivatives; a linear plot was observed. By following the Scatchard relationship (Eq. 2), the equilibrium binding constant, K_n, may be estimated from the slope of the linear $\bar{v}/D_f$ *versus* $\bar{v}$ plots:

$$\bar{v}/D_f = nK_n - \bar{v}K_n \qquad \text{(Eq. 2)}$$

Which of the four functional groups around the tetrahedral carbon center in this molecule was the predominant site for plasma binding? The results demonstrated that the omission of the amide or pyridyl groups (Table I) resulted in an approximately twofold increase in the extent of plasma binding (β values were increased from 27.3 to 58.5 or 53.6%, respectively). But the absence of the phenyl ring yielded a sixfold decrease in β (from 27.3 to 4.6%). The replacement of the diisopropylaminoethyl group produced neither gain nor loss in the extent of drug–plasma interaction. The results observed here indicate that the planar phenyl ring of the disopyramide intercalated into the plasma protein helix (14). An NMR study also provided evidence that the phenyl ring is the site binding to serum protein (15).

In addition, the observed partition coefficients demonstrated that the amide, pyridyl, and diisopropylaminoethyl groups increased the hydrophilicity of disopyramide derivatives. (Their absence resulted in increases in the partition coefficient from 0.66 to 11.97, 5.4, and 6.87, respectively.) On the other hand, the phenyl substitution added lipophilic character to the molecule. The replacement of this group substantially decreased the magnitude of the partition coefficient (from 0.66 to 0.049). The omission of the diisopropylaminoethyl group produced no change in the extent of plasma binding while the lipophilicity, as represented by the partition coefficient, was enhanced more than 10-fold (from 0.66 to 6.87). This observation may be explained by the fact that 90% of the diisopropylaminoethyl group (pKb = 8.36) is protonated at the physiological pH of 7.4, which may allow this group to project into the aqueous phase surrounding the protein molecule and not interact with the protein (8).

For a fuller understanding of the mechanisms by which disopyramide derivatives interact with plasma protein, the studies were expanded to additional derivatives in which the amide, phenyl, or pyridyl group was modified chemically while the other three functional groups were held constant. The results of these experiments are found in Tables II–IV along with estimated values for log (D_b/D_f) obtained with the following analysis.

At equilibrium (16), the partition coefficient (p.c.) for the partitioning of drug molecules from an aqueous phase to an organic phase may be related to the standard chemical potentials ($\mu_0°$ and $\mu_a°$) in organic and aqueous phases:

$$\mu_0° - \mu_a° = -2.303RT \log (\text{p.c.}) \qquad \text{(Eq. 3)}$$

The standard free energy change ($\Delta F°$) for the interaction of drug with plasma protein (16) may be related to the equilibrium binding constant, K_n, as follows:

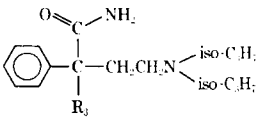
$$\Delta F° = \mu_p° - \mu_a° \qquad \text{(Eq. 4}a\text{)}$$

$$\Delta F° = -2.303RT \log K_n \qquad \text{(Eq. 4}b\text{)}$$

where $\mu_p°$, $\mu_a°$, and $\mu_0°$ are the standard chemical potentials for a drug species bound to a protein molecule and in the aqueous and organic phases, respectively. Substituting Eq. 3 for the $\mu_a°$ term in Eq. 4a and rearranging give Eq. 5:

$$\log K_n = \frac{\mu_0° - \mu_p°}{2.303RT} + \log (\text{p.c.}) \qquad \text{(Eq. 5)}$$

Table IV—Structural Variation on the Pyridyl Group of Disopyramide Derivatives



Compound[a]	R_3	log (p.c.)	log (D_b/D_f) Observed	log (D_b/D_f) Estimated Eq. 12	log (D_b/D_f) Estimated Eq. 13
III	H	0.732	0.063	0.061	−0.034
Disopyramide		−0.182	−0.425	−0.609	−0.493
XVIII		0.316	−0.141	−0.244	−0.206
XIX		0.055	−0.315	−0.436	−0.356
XX		1.393	0.482	0.546	0.416

[a] SC-5260, SC-7031, SC-12748, SC-12773, and SC-24115, respectively.

The Scatchard relationship (Eq. 2) may be expressed alternatively by Eq. 6

$$K_n = \frac{\bar{v}}{(\bar{n} - \bar{v})D_f} = \frac{D_b}{D_f} \times \frac{1}{(\bar{n} - \bar{v})P} \qquad \text{(Eq. 6)}$$

where $\bar{v}$ is the average number of drug molecules bound per molecule of plasma protein (D_b/P); $\bar{n}$ is the total number of binding sites in a protein molecule; D_b and D_f are the concentrations of drug bound to plasma protein and freely existing in the solution phase, respectively; and P is the overall concentration of plasma protein in the bloodstream. Equation 5 may be equivalently expressed as:

$$\log \frac{D_b}{D_f} = \frac{\mu_0^\circ - \mu_p^\circ}{2.303RT} + \log(\bar{n} - \bar{v})P + \log(\text{p.c.}) \qquad \text{(Eq. 7)}$$

Experimentally, it was observed that $\bar{n} = 3.51$ and $\bar{v} \leq 0.12$ for disopyramide in the drug concentration range investigated. So, Eq. 7 may be simplified to:

$$\log \frac{D_b}{D_f} = \text{intercept} + \log(\text{p.c.}) \qquad \text{(Eq. 8)}$$

where:

$$\text{intercept} = \frac{\mu_0^\circ - \mu_p^\circ}{2.303RT} + \log \bar{n}p \qquad \text{(Eq. 9}a\text{)}$$

$$\text{intercept} = \log(D_b/D_f)_0 = \text{constant} \qquad \text{(Eq. 9}b\text{)}$$

Equations 7 and 8 define a linear relationship between the degree of plasma binding (as represented by D_b/D_f) and the lipophilic character of the molecule (expressed as the partition coefficient). Under the condition of $\bar{n} \gg \bar{v}$, the magnitude of D_b/D_f is directly proportional to the equilibrium binding constant (K_n) with a proportionality of $(\bar{n} - \bar{v})P$ (Eq. 6). Therefore, the degree of plasma binding (D_b/D_f) may be directly estimated from a binding measurement without requiring a Scatchard analysis to calculate the magnitude of K_n.

A literature survey indicated that correlations between protein binding and the lipophilicity of drugs have usually employed a Hammett-type linear free energy relationship (6). In this treatment, log $(1/c)$ values are plotted against π, the lipophilicity of the drug. To do this, a series of drug–protein interaction measurements must be conducted at several drug concentrations to collect data points for estimating the molar concentration of drug necessary to produce a 1:1 drug–protein complex. Thus, a substantial workload is created when dealing with a large number of drugs. In such a situation, the development of a simple methodology with equivalent theoretical validity becomes necessary. In the present analysis, the protein binding (D_b/D_f) of all drugs investigated was done at a single, optimal, initial drug concentration (D_T). This practice allows biopharmaceutical screening of a large number of newly synthesized drugs.

The linear correlation of log (D_b/D_f) with π was successfully used in investigating (8) the binding of penicillins to serum protein. The linear log (D_b/D_f) *versus* π relationship was utilized empirically without the inclusion of a mathematical derivation from fundamental principles. The derivation of Eq. 8 from simple physicochemical laws (Eqs. 3 and 4) demonstrates how the degree of plasma binding [log (D_b/D_f)] is related theoretically to the lipophilicity (log p.c.) of the drugs examined.

On the basis of Eq. 8, the data on D_b/D_f (obtained from the β values) and the partition coefficients (Tables II–IV) were submitted to multiple-regression analysis. Three equations were obtained.

For the effect of structural variation of the amide moiety:

$$\log\left(\frac{D_b}{D_f}\right) = -0.303 + 0.473 \log(\text{p.c.}) \qquad \text{(Eq. 10)}$$

n	γ	s^2
10	0.986	0.0034

For the effect of structural variation of the phenyl ring:

$$\log\left(\frac{D_b}{D_f}\right) = -0.369 + 0.641 \log(\text{p.c.}) \qquad \text{(Eq. 11)}$$

n	γ	s^2
7	0.982	0.0059

For the effect of structural variation of the pyridyl group:

$$\log\left(\frac{D_b}{D_f}\right) = -0.476 + 0.734 \log(\text{p.c.}) \qquad \text{(Eq. 12)}$$

n	γ	s^2
6	0.923	0.0303

In Eqs. 10–12, n is the number of derivatives used in the analysis, γ is the correlation coefficient, and s^2 is the residual variance. Both the correlation coefficient (γ) and the residual variance (s^2) demonstrate that the degree of plasma binding of disopyramide derivatives is highly correlated to their lipophilicity. By using Eqs. 10–12, estimated D_b/D_f values for the disopyramide derivatives

Table V—Estimation of the Relative Lipophilicity (π) for Disopyramide Derivatives with Structural Variation on the Phenyl Ring

Compound	Estimated π Values[a]		
	Benzene System	Benzoic Acid System	Phenoxyacetic Acid System
II	0	0	0
Disopyramide	1.89	1.89	1.89
XIII	2.45	2.41	2.40
XIV	2.45	2.31	2.41
XV	1.85	2.11	1.97
XVI	2.03	2.08	2.04
XVII	2.60	2.76	2.69

[a] Calculated from Ref. 18.

were computed; they are shown in Tables II–IV together with the D_b/D_f values observed.

The slope of Eqs. 10–12 is a measure of the sensitivity of the protein binding system to the hydrophobicity of each series of disopyramide derivatives (4). The value of the intercept (Eqs. 10–12) will be a function of the sensitivity of the system and the intrinsic thermodynamic activity (Eqs. 9*a* and 9*b*) of a given series of drugs. It will also depend on the ratio of active sites to substrate molecules in position to react with these sites, $(\bar{n} - \bar{v})P$.

If the binding of disopyramide derivatives to plasma protein is nonspecific, as reported for most drugs (7), and the difference in plasma binding extent is only due to the change in their lipophilicity, then a good correlation should be found when disopyramide and all 20 derivatives (Tables I–IV) are submitted to the same multiple-regression analysis as in Eqs. 10–12, neglecting the differences in their sites of structural variation. The following correlation was obtained:

$$\log\left(\frac{D_b}{D_f}\right) = -0.388 + 0.577 \log(\text{p.c.}) \qquad \text{(Eq. 13)}$$

n	γ	s^2
21	0.955	0.018

Both the high correlation coefficient ($\gamma = 0.955$) and low residual variance ($s^2 = 0.018$) indicated that the extent of plasma binding of disopyramide and the 20 derivatives was highly correlated with their lipophilicity. The values of log (D_b/D_f) estimated from Eq. 13 were also determined; they are shown in Tables II–IV together with those values observed and computed from Eq. 10, 11, or 12. A comparison made between Eq. 13 and Eqs. 10–12 pointed out that the interaction of disopyramide derivatives with plasma protein is nonspecific in nature and is slightly dependent on the sites of structural variation (compare the data estimated to those observed in Tables II–IV). Overall, any modification in the molecular structure of disopyramide derivatives that leads to a higher lipophilicity will result in enhanced binding with plasma protein. In the practice of drug design, this fact should be considered when the pharmaceutical chemist attempts to improve the tissue permeability of a given drug by enhancing its lipophilic nature.

The values of log (p.c.) (Tables I–IV) for all of the disopyramide derivatives were obtained in an *n*-octanol–phosphate buffer (pH 7.4) system. The relative lipophilicity (π) of a given derivative (x) may be calculated as follows:

$$\pi(x) = \log(\text{p.c.})\, x - \log(\text{p.c.})_H \qquad \text{(Eq. 14)}$$

The magnitude of π values may also be estimated from information on the partition coefficient of the parent compound and the group contribution of the substituents added onto the derivative by using the rule of addition (8, 18). For example, Compound XIII (Table III) is the *m*-tolyl derivative of Compound II. Its relative lipophilicity may be estimated as follows:

$$\begin{aligned}\sum\pi(\text{XIII}) &= \pi(\text{disopyramide}) + \pi(m\text{-}CH_3) \\ &= \pi(\text{II}) + \pi(\text{phenyl}) + \pi(m\text{-}CH_3) \\ &= 0 + 1.89 + 0.56 \\ &= 2.45 \end{aligned} \qquad \text{(Eq. 15)}$$

By using this approach, the π values were calculated (Table V) for the other derivatives of II in reference to three systems, benzene, benzoic acid, and phenoxyacetic acid (18), and were quite close to each other.

The correlation of the extent of plasma binding of these disopyramide derivatives (Column 4 in Table III) with these three sets of estimated π values was also submitted to multiple-regression analysis. The results are represented by the following correlations:

With the benzene system as a reference source:

$$\log(D_b/D_f) = -1.352 + 0.462\,\pi \qquad \text{(Eq. 16)}$$

n	γ	s^2
7	0.948	0.016

With the benzoic acid system as a reference source:

$$\log(D_b/D_f) = -1.337 + 0.444\,\pi \qquad \text{(Eq. 17)}$$

n	γ	s^2
7	0.923	0.024

With the phenoxyacetic acid system as a reference source:

$$\log(D_b/D_f) = -1.350 + 0.456\,\pi \qquad \text{(Eq. 18)}$$

n	γ	s^2
7	0.941	0.018

A comparison made among Eqs. 11 and 16–18 indicates that the extent of plasma binding, log (D_b/D_f), of disopyramide derivatives is not only highly correlated to their lipophilicity, log (p.c.), measured experimentally but also to the magnitude of π estimated from literature data. The magnitude of the correlation coefficients (r) obtained only shows the trend on the change of log (D_b/D_f) as a function of the variation of the lipophilicity of a drug species. On the other hand, the residual variance (s^2) demonstrates the magnitude of difference between the observed log (D_b/D_f) value from that predicted.

The magnitude of the residual variances in Eqs. 16–18 is found to be three- to fourfold greater than that in Eq. 11. The results are illustrated and compared in Table VI. It was noted that Eqs. 16–18 failed to give good estimates of log (D_b/D_f) values when the phenyl ring contained methoxy, fluoro, and chloro groups, *i.e.*, electron-

Table VI—Comparison of the Observed and Calculated Extent of Plasma Binding of Disopyramide Derivatives

Compound	log (D_b/D_f)				
	Observed	Calculated			
		Eq. 11	Eq. 16	Eq. 17	Eq. 18
II	−1.317	−1.209	−1.352	−1.337	−1.350
Disopyramide	−0.425	−0.485	−0.480	−0.498	−0.489
XIII	−0.343	−0.257	−0.222	−0.268	−0.256
XIV	−0.203	−0.220	−0.222	−0.312	−0.251
XV	−0.745	−0.874	−0.499	−0.401	−0.452
XVI	−0.294	−0.315	−0.416	−0.414	−0.420
XVII	−0.015	−0.018	−0.152	−0.112	−0.124

withdrawing substituents. The measurement of lipophilicity, using experimental partition coefficient data, was more dependable than an estimation from literature data collected in a different system. The preference for actual measurement over the estimation of lipophilicity is even more obvious when one deals with drug molecules having a complicated molecular structure.

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Selecting Key Parameters in Pharmaceutical Formulations by Principal Component Analysis

N. R. BOHIDAR [x], F. A. RESTAINO, and J. B. SCHWARTZ

Abstract □ The role of principal component analysis in the selection of pharmaceutical formulations is presented. The objective and the procedure of the analysis are discussed in detail. The technique was successfully applied to a system consisting of 10 response variables (tablet properties). Analysis of the results showed that the first component (dissolution) and components one and two together (dissolution and disintegration) contributed 95.4 and 99.3%, respectively, to the overall information about the formulations and that eight of 10 response parameters contributed nothing further to the overall information. The results obtained from this method of analysis may be found useful for achieving economy in both cost and time of measuring responses. Principal component analysis also provides a basis for understanding the underlying mechanism of the system under consideration.

Keyphrases □ Pharmaceutical formulations—selecting key parameters by principal component analysis, examples □ Formulations, pharmaceutical—selecting key parameters by principal component analysis, examples □ Principal component analysis—selecting key parameters in pharmaceutical formulations

In the development of a drug delivery system, a research pharmacist usually measures several response parameters. For instance, 10 or more parameters were considered in the development of a pharmaceutical tablet formulation (1). Based on all of these parameters, one attempts to find those levels of the formulation factors (diluent ratio, compressional force, *etc.*) for which the system is considered optimum. Since a large number of interrelated response variables is generally involved, it is relevant to ask how the interrelation and covariation of these measurements might be represented and whether fewer measurements might not carry all the necessary information for accomplishing a specific objective.

When several formulations are available, the developmental pharmacist must determine how best to distinguish between them. When one is choosing between two or three, the trend may be obvious. For example, the formula changes made may cause no difference in tablet hardness but considerable difference in disintegration characteristics. But when a long list of formulations is available or, more precisely, when one has infinite possibilities (as in computer optimization) and is dealing with many parameters, the trend is less obvious.

One may have certain basic constraints, such as a minimum hardness value, but it is nevertheless important to know which property or properties can be used to distinguish between choices. Generally, an educated guess is made, based on experience with the system and with pharmaceutical systems in general.

But there is a mathematical method to select those variables that best distinguish between formulations and those variables that change most drastically from

withdrawing substituents. The measurement of lipophilicity, using experimental partition coefficient data, was more dependable than an estimation from literature data collected in a different system. The preference for actual measurement over the estimation of lipophilicity is even more obvious when one deals with drug molecules having a complicated molecular structure.

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When several formulations are available, the developmental pharmacist must determine how best to distinguish between them. When one is choosing between two or three, the trend may be obvious. For example, the formula changes made may cause no difference in tablet hardness but considerable difference in disintegration characteristics. But when a long list of formulations is available or, more precisely, when one has infinite possibilities (as in computer optimization) and is dealing with many parameters, the trend is less obvious.

One may have certain basic constraints, such as a minimum hardness value, but it is nevertheless important to know which property or properties can be used to distinguish between choices. Generally, an educated guess is made, based on experience with the system and with pharmaceutical systems in general.

But there is a mathematical method to select those variables that best distinguish between formulations and those variables that change most drastically from

one formulation to another and which should be the criteria upon which one selects a formulation.

A multivariate statistical procedure called "principal component analysis" can effectively be used to answer these questions. Basically, this method first finds the function of the observations that has the largest variance and then finds the function, independent of the first function, having the largest variance. It then finds the function, independent of the first two functions, with the largest variance and so on.

If p variates (responses) are observed, it is obvious that p of these could account for all of the variability in the original observations. The problem is whether fewer variates might be used. If so, some principal components might not contribute anything to the overall variability; thus, they would not help in distinguishing one formulation from another.

Large sets of data should not be subjected to a principal component analysis merely to obtain fewer variables to work with without regard to an overall objective. The objective should be established first and then the principal component model should be used only if it complements the objective. However, tremendous simplification of the data can often be effected for problems where principal component analysis is appropriate.

The primary purpose of this paper is to demonstrate the role of principal component analysis in pharmaceutical formulation development by applying the procedure to the data from an optimization experiment previously described (1). Ten response variables were measured on each of 27 formulations, and the variables substantially contributing to the overall information were identified.

The theoretical section is meant to serve as a guide for following the steps in the principal component analysis technique. However, computer programs can perform all of the indicated operations, and it is only necessary to feed in the raw data. A familiarity with the theory is useful for analysis of the results and for those who wish to modify available programs.

EXPERIMENTAL

The 10 parameters shown in Table I were measured on each of the 27 tablet formulations considered for the experiment[1] described in detail in Ref. 1. Thus, the data set to be subjected to principal component analysis contains 27 values for each response shown in Table I.

THEORY AND PROCEDURE

The structure of the data associated with the experiment is presented in the matrix form shown in Table II.

For the purpose of describing the procedure, let Y_{ik} ($i = 1, 2, \ldots, p$; $k = 1, 2, \ldots, N$) denote the numerical value associated with the kth experiment for the ith response variable, and let p and N represent the number of response variables and the number of experiments for each parameter considered, respectively. In this case, $p = 10$ and $N = 27$ in Table II. The value of Y_{ik} can be the mean of several measurements.

[1] The technique utilized here may be applied to any multivariate system and need not be limited to the statistically designed set of experiments considered here.

Table I—Response Variables

Symbolic Designation	Response Variable	Units
Y_1 (DT)	Disintegration time	Minutes
Y_2 (HD)	Tablet breaking strength	Kilograms
Y_3 (DR)	Dissolution	Percent released in 30 min
Y_4 (FR)	Friability	Percent weight loss
Y_5 (TH)	Thickness uniformity	*RSD*, %
Y_6 (PO)	Porosity	Milliliters per gram
Y_7 (MP)	Mean pore diameter	Micrometers
Y_8 (WT)	Weight uniformity	*RSD*, %
Y_9 (TB)	Tablet breakage	Number of chipped tablets
Y_{10} (GM)	Granulation mean diameter	Millimeters

The variance (S_{ii}) and the covariances (S_{ij}, $i \neq j$) associated with the p parameters are then calculated as follows:

$$\text{variance} = S_{ii} = \left[\sum_{k=1}^{N} Y_{ik}^2 - \left(\sum_{k=1}^{N} Y_{ik}\right)^2 N^{-1}\right](N-1)^{-1} \quad \text{(Eq. 1)}$$

where $i = 1, 2, \ldots, p$, and:

$$\text{covariance} = S_{ij}(i \neq j) = \left[\sum_{k=1}^{N} Y_{ik}Y_{jk} - \left(\sum_{k=1}^{N} Y_{ik}\sum_{k=1}^{N} Y_{jk}\right)N^{-1}\right](N-1)^{-1} \quad \text{(Eq. 2)}$$

$$= S_{ji}$$

where $i = 1, 2, \ldots, p$ and $j = 1, 2, \ldots, p$.

There would be a total of p variances and $\frac{1}{2}p(p-1)$ covariances associated with p parameters. There will be 10 variances and 45 covariances when 10 parameters are considered. The variance and covariance quantities are then arranged in a square matrix form as follows:

$$\Sigma = \begin{bmatrix} S_{11} & S_{12} & \ldots & S_{1p} \\ S_{21} & S_{22} & \ldots & S_{2p} \\ \cdot & & & \\ \cdot & & & \\ \cdot & & & \\ S_{p1} & S_{p2} & \ldots & S_{pp} \end{bmatrix} \quad \text{(Eq. 3)}$$

This matrix is called the variance–covariance matrix and is denoted by the greek letter Σ (sigma). The variances are arranged in the main diagonal of the matrix and the covariances are placed in their respective off-diagonal positions. Since $S_{ij} = S_{ji}$ ($i \neq j$), one has only $\frac{1}{2}p(p+1)$ distinct elements in the matrix. If there are p parameters, then the dimension of this matrix is $(p \times p)$ with p rows and p columns and there are p^2 elements in the matrix. When $p = 10$, the dimension of Σ is (10×10) with 100 elements in the matrix.

The determinant of the matrix reduces these p^2 (here 100) elements to a single number. This number represents the variance of the entire system, usually called the generalized variance. This statistic is helpful for comparing the variances of two different systems. The primary interest here, however, is in the magnitude of the variances of the individual components and their relative information within the system under consideration. So consider the determinant of the matrix $(\Sigma - \lambda I)$, where I is the identity matrix with ones in the main diagonal and zeros elsewhere and, expressed explicitly, gives:

$$|\Sigma - \lambda I| = \begin{vmatrix} S_{11} - \lambda_1 & S_{12} & \ldots & S_{1p} \\ S_{21} & S_{22} - \lambda_2 & \ldots & S_{2p} \\ \cdot & & & \\ \cdot & & & \\ \cdot & & & \\ S_{p1} & S_{p2} & \ldots & S_{pp} - \lambda_p \end{vmatrix} \quad \text{(Eq. 4)}$$

Table II—Data Matrix

Experiment Number (Formulation)	Response Variables									
	(DT)	(HD)	(DR)	(FR)	(TH)	(PO)	(MP)	(WT)	(TB)	(GM)
1	Y_{11}	Y_{21}	Y_{31}	Y_{41}	Y_{51}	Y_{61}	Y_{71}	Y_{81}	Y_{91}	$Y_{10,1}$
2	Y_{12}	Y_{22}	Y_{32}	Y_{42}	Y_{52}	Y_{62}	Y_{72}	Y_{82}	Y_{92}	$Y_{10,2}$
3	Y_{13}	Y_{23}	Y_{33}	Y_{43}	Y_{53}	Y_{63}	Y_{73}	Y_{83}	Y_{93}	$Y_{10,3}$
.	.	.	.	.	.	.	.	.	.	.
.	.	.	.	.	.	.	.	.	.	.
27	$Y_{1,27}$	$Y_{2,27}$	$Y_{3,27}$	$Y_{4,27}$	$Y_{5,27}$	$Y_{6,27}$	$Y_{7,27}$	$Y_{8,27}$	$Y_{9,27}$	$Y_{10,27}$

In this equation, the λ_i's ($i = 1, 2, \ldots, p$) are unknown. By setting:

$$|\Sigma - \lambda I| = 0 \qquad \text{(Eq. 5)}$$

and expanding the determinant on the left, one has a polynomial equation of pth order of the following form:

$$|\Sigma - \lambda I| = f(\lambda) = (-\lambda)^p + a_{p-1}(-\lambda)^{p-1} + \ldots + a_1(-\lambda) + a_0 = 0 \qquad \text{(Eq. 6)}$$

where the coefficients a_i's are, but for signs, the sum of all of the principal ith-order minors of the determinant of the variance–covariance matrix Σ. A pth-order polynomial would yield p roots (zeros of the polynomial). These roots (λ_i's) are known as characteristic roots, latent roots, or eigenvalues. The term "eigenvalue" will be used in the subsequent reference to the roots. These eigenvalues ($\lambda_1, \lambda_2, \ldots, \lambda_p$) represent the variances of each "orthogonal" component of the system. The variance–covariance matrix of the orthogonal system (Λ) has the following structure:

$$\Lambda = \begin{bmatrix} \lambda_1 & 0 & 0 & \ldots & 0 \\ 0 & \lambda_2 & 0 & \ldots & 0 \\ \cdot & & & & \\ \cdot & & & & \\ \cdot & & & & \\ 0 & 0 & 0 & \ldots & \lambda_p \end{bmatrix} \qquad \text{(Eq. 7)}$$

It is clearly seen in Eq. 7 that the original system has been transformed to an orthogonal system in which the covariances of the components have been reduced to zero (hence the term orthogonal system is used). The generalized variance of the original system is identically equal to the generalized variance of the orthogonal system, that is:

$$|\Sigma| = |\Lambda| \qquad \text{(Eq. 8)}$$

This clearly indicates the complete preservation of the total information of the original system in the transformed system. The orthogonal system provides an estimation of the relative contribution of each component to the overall information. Let $\theta = \lambda_1 + \lambda_2 + \ldots + \lambda_p$; then $100\lambda_1\theta^{-1}, 100\lambda_2\theta^{-1}, \ldots, 100\lambda_p\theta^{-1}$ are the respective relative contribution (in percent) of each of the p components of the system to the overall information.

Now consider the structure of each component. Associated with each eigenvalue λ_r ($r = 1, 2, \ldots, p$) is a set of p coefficients, $\nu_{r1}, \nu_{r2}, \ldots, \nu_{rp}$; $r = 1, 2, \ldots, p$. This set of coefficients, called the eigenvector of eigenvalue λ_r, is a solution of the equation:

$$(\Sigma - \lambda_r I)\nu = 0 \qquad r = 1, 2, \ldots, p \qquad \text{(Eq. 9)}$$

where I is the identity matrix with ones in the main diagonal and zeros elsewhere. This equation is expressed explicitly as follows:

$$\begin{bmatrix} S_{11} - \lambda_r & S_{12} & \ldots & S_{1p} \\ S_{21} & S_{22} - \lambda_r & \ldots & S_{2p} \\ . & & & \\ . & & & \\ . & & & \\ S_{p1} & S_{p2} & \ldots & S_{pp} - \lambda_r \end{bmatrix} \begin{bmatrix} \nu_{r1} \\ \nu_{r2} \\ . \\ . \\ . \\ \nu_{rp} \end{bmatrix} = \begin{bmatrix} 0 \\ 0 \\ . \\ . \\ . \\ 0 \end{bmatrix} \qquad \text{(Eq. 10)}$$

where $r = 1, 2, 3, \ldots, p$.

The magnitudes of the coefficients associated with those principal components that are substantially contributing to the overall information are of interest in the interpretation of the principal component analysis of the system under consideration.

The codes of a FORTRAN program for computing the eigenvalues and eigenvectors of a real symmetric matrix are available (2) and are suitable for adoption into any computer system without considerable effort.

The detailed mathematics of the procedure can be found in Ref. 3.

RESULTS AND DISCUSSION

The results of the principal component analysis associated with the system under consideration are presented in Tables III–V. The results presented in Tables III and IV pertain to the situation in which all 10 parameters were included in the analysis, as does Analysis I in Table V. The other results presented in Table V pertain to situations where two or more parameters were excluded from the system. This approach is not an essential feature of the principal component analysis. However, it is provided here to illustrate the results of the analysis in the presence or absence of cer-

Table III—Eigenvalues, Relative Information, and Cumulative Relative Information Associated with Each Component

Principal Component	Eigenvalues (λ_i)	Relative Information, % ($100\lambda_i\theta^{-1}$)	Cumulative Relative Information, %
I	578.4	95.4	95.4
II	23.9	3.9	99.3
III	2.2	0.4	99.7
IV	2.0	0.3	100.0
Total		100.0	

Table IV—Coefficients of Eigenvectors Associated with First Two Principal Components

Parameter	Principal Component I	Principal Component II
Dissolution (DR)	0.98	0.22
Porosity (PO)	0.00	0.00
Friability (FR)	0.00	0.00
Disintegration time (DT)	−0.22	0.97
Weight uniformity (WT)	−0.01	0.03
Thickness uniformity (TH)	0.00	0.00
Granular mean diameter (GM)	0.00	0.00
Tablet breakage (TB)	0.00	0.00
Mean pore diameter (MP)	0.00	0.00
Tablet breaking strength (HD)	0.01	0.12
Relative information, %	95.4	3.9

Table V—Relative Information (Percent) of Parameters for Five Principal Component Analyses

Serial Number	Analysis I		Analysis II		Analysis III		Analysis IV		Analysis V	
	Order of Parameters	Relative Information	Order of Parameters	Relative Information	Order of Parameters	Relative Information	Order of Parameters	Relative Information	Order of Parameters	Relative Information
1	(DR)	95.4	(DR)	95.4	—	—	—	—	—	—
2	(DT)	3.9	(DT)	3.9	—	—	—	—	—	—
3	(WT)	0.4	(WT)	0.4	(HD)	52.5	—	—	—	—
4	(HD)	0.3	(HD)	0.3	(WT)	44.9	—	—	—	—
5	(FR)	0.0[a]	(FR)	0.0	(FR)	2.3	(FR)	82.9	—	—
6	(MP)	0.0	—	—	(MP)	0.2	(MP)	10.0	(TB)	61.0
7	(TH)	0.0	—	—	(TH)	0.1	(TH)	4.5	(MP)	22.0
8	(TB)	0.0	—	—	(TB)	0.0	(TB)	1.4	(TH)	13.1
9	(GM)	0.0	—	—	(GM)	0.0	(GM)	1.1	(GM)	3.7
10	(PO)	0.0	—	—	(PO)	0.0	(PO)	0.1	(PO)	0.2
	Total	100.0		100.0		100.0		100.0		100.0

[a] Represents a rounded figure.

tain parameters. The information-analysis (variance-analysis) of the first four components is given in Table III. The structure-analysis of the first two components is presented in Table IV.

Table III lists the first four components, their respective eigenvalues, and the relative information calculated from them. An examination of these data shows that the total information contained in the system was contributed by these first four of the 10 principal components. In other words, the last six principal components did not contribute anything to the overall information. Furthermore, the first principal component contributed as much as 95.4% of the total information.

It is necessary now to conduct the structure-analysis of the components. The results presented in Table IV for principal component I reveal that "dissolution" was the predominant parameter of the component by sharing the largest value (0.98) among the coefficients associated with the component. Now, by relating the results of the variance-analysis and the structure-analysis, it may be inferred that dissolution accounted for most variabilities in the system. In other words, dissolution contained the most information concerning the power of distinguishing among the 27 formulations under consideration. If the objective is to achieve the optimum levels of the formulation factors (1), then constraining the dissolution parameter would lead to a faster selection of the optimum formulation than would constraining any other parameter considered.

Component II in Table III contributed only 3.9% of the total information. A structural analysis (Table IV) reveals that "disintegration time" was the predominant parameter associated with this principal component. The two parameters, dissolution and disintegration, yielded a cumulative relative information of 99.3%. The same procedure can be followed for each component.

In Table V, the parameters are presented in the order of the relative information contributed. The results of the principal component analysis involving all parameters in the system are shown in Analysis I. A discussion of these results was already presented. Analysis II shows the results of the principal component analysis involving only the first five parameters. The results of Analyses I and II are identical with respect to the order and magnitude of the relative importance of the first five parameters.

The results of the principal component analysis involving only the last eight parameters are presented in Analysis III; weight uniformity and tablet breaking strength become the predominant parameters in this particular system. The results of the principal component analysis involving only the last six parameters are presented in Analysis IV; friability, mean pore diameter, and tablet thickness become the predominant parameters in this system. The results of the principal component analysis involving only the last five parameters are presented in Analysis V; mean pore diameter, tablet breakage, and tablet thickness are the predominant parameters of this particular system. These types of analyses would provide a basis for a general understanding of the underlying mechanisms of the particular system considered.

A word of caution is in order. The results and the conclusions associated with a principal component analysis pertain only to the specific system to which the analysis was applied. Although the procedure may be applied to any multivariate system, any extrapolation of the results of one system to another is not appropriate. Each system must be analyzed separately and the results so obtained must be interpreted independently.

CONCLUSION

In this study it was clearly shown that principal component analysis can play an important role in pharmaceutical formulation by identifying parameters that are substantially contributing to the overall information associated with the system. It was observed that the first principal component contributed as much as 95.4% of the total information. The first two principal components together contributed as much as 99.3% of the overall information. Dissolution was identified as the predominant parameter of the system. Therefore, this parameter alone could effectively be used in the comparison of candidate formulations and in the constraining operation (1) for a faster selection of the optimum formulation.

Disintegration was identified as the next important parameter. All other parameters did not contribute substantially to the overall information. This information is vital in that one would be able to achieve economy in cost and time of measuring responses. Principal component analysis could also be used to provide a basis for understanding the underlying mechanism of the system.

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Aqueous Mixture of Pyrrolidone-5-hydroxamic Acid–Iron (III) Complexes: Isolation and Characterization of Tris(pyrrolidone-5-hydroxamato)iron (III)

M. PEĆAR ˣ, N. KUJUNDŽIĆ, B. MLINAREVIĆ, D. ČERINA, B. HORVAT, and M. VERIČ

Abstract □ The published procedure for the synthesis of pyrrolidone-5-hydroxamic acid was improved. The acidity constant of the pyrrolidone-5-hydroxamic acid was determined as pKa = 8.65. In an aqueous solution of iron (III) ions, pyrrolidone-5-hydroxamic acid binds ferric ion, forming a mixture of mono-, bis-, and tris(pyrrolidone-5-hydroxamato)iron (III) complexes. These complexes were studied by potentiometric and spectrophotometric methods. The tris compound was isolated as dark orange-red crystals and identified according to elemental analysis and IR spectral data as $C_{15}H_{21}FeN_6O_9 \cdot 6H_2O$, having the magnetic moment of 5.67 B.M.

Keyphrases □ Pyrrolidone-5-hydroxamic acid—synthesis, acidity, chelating properties □ (Pyrrolidone-5-hydroxamato)iron (III) complexes—synthesis, characterization, visible and IR spectral data, magnetic moment □ Complexes—iron (III) with pyrrolidone-5-hydroxamic acid, synthesis, characterization, visible and IR spectral data, magnetic moment

In spite of the great interest in iron (III)–hydroxamate complexes and their role in living systems (1–3), the data on heterocyclic aminohydroxamic acid–iron (III) complexes are rather scarce. Iron-containing hydroxamic acids play a functional role in the metabolism of microorganisms (4), and some of them have been patented as metallotherapeutics (5). Free hydroxamic acids are also of biological importance (1, 2, 6) and serve as antibiotics and bacteriostatics. Polyvinylpyrrolidone is used as a substitute for blood plasma (7). Since hydroxamic acids and their iron (III) chelates are of great interest for pharmaceutical sciences (6, 8–11), pyrrolidone-5-hydroxamic acid and its iron (III) complexes have been chosen from the nitrogen heteropentacyclic hydroxamic acids as a model system.

EXPERIMENTAL

Chemicals—Pyrrolidone-5-hydroxamic acid and iron (III) complexes were prepared as subsequently described. All other chemicals were of analytical reagent grade and were used without further purification. The water used in all experiments was distilled from aqueous acidified potassium permanganate solutions and stored in glass[1] containers. The methanol used contained about 0.04% water (determined by the Karl Fischer method).

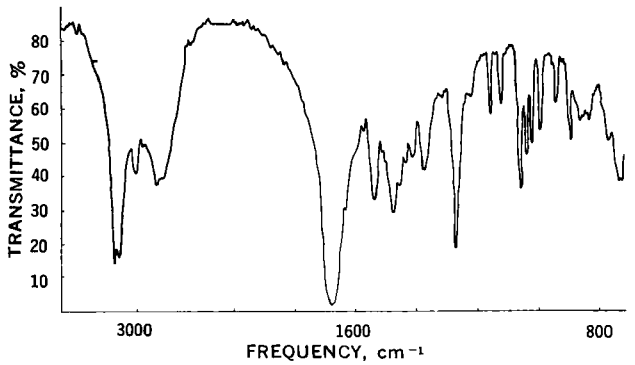
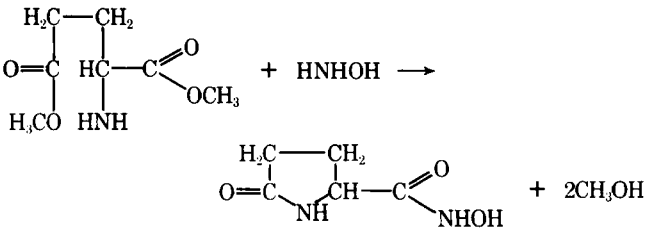


Figure 1—*IR spectrum of I.*



Scheme I

Preparation of Pyrrolidone-5-hydroxamic Acid (I)—The literature method (12) for the synthesis of I was modified as follows. Glutaminic acid, 100 g (0.68 mole), was suspended in 750 ml of anhydrous methanol; dry hydrogen chloride gas was introduced through a glass pipe[2], equipped with a sintered-glass filter, until white fumes appeared above the solution. Then 750 ml of methanol was added and refluxed for 3 hr along with bubbling of dry hydrogen chloride gas. Special care was taken to ensure nonaqueous conditions during the entire procedure since water interferes with the main reaction.

After removal of excess hydrochloric acid by evaporation under reduced pressure, the resulting oil-like liquid was diluted with 300 ml of methanol. The addition of the chloroform solution of ammonia liberated the dimethyl ester of glutaminic acid and precipitated ammonium chloride, which was filtered off. Methanol and chloroform were removed from the filtrate by distillation, and 95 g (1.36 mole) of hydroxylamine dissolved in methanol was added to the rest of the filtrate.

The mixture was allowed to stand in a refrigerator for 4 days. During that period, condensation as well as cyclization took place (Scheme I). The crystallized product was filtered, washed with cool methanol, and recrystallized from hot water; the total yield before recrystallization was 67 g (68%), mp 165.5–167.5°.

Anal.—Calc. for $C_5H_8N_2O_3$ (mol. wt. 144.14): C, 41.66; H, 5.59; N, 19.44. Found: C, 41.69; H, 5.35; N, 19.47.

Preparation of Tris(pyrrolidone-5-hydroxamato)iron (III) Complex—Ferric chloride hexahydrate, 1.35 g (0.005 mole), was

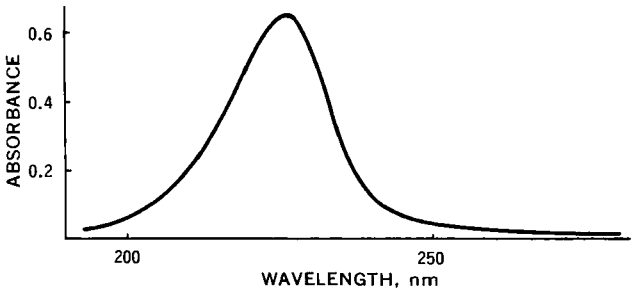


Figure 2—*UV spectrum of 0.003 M I at pH 6.7, using 1-cm quartz cells.*

[1] Pyrex.
[2] Jena.

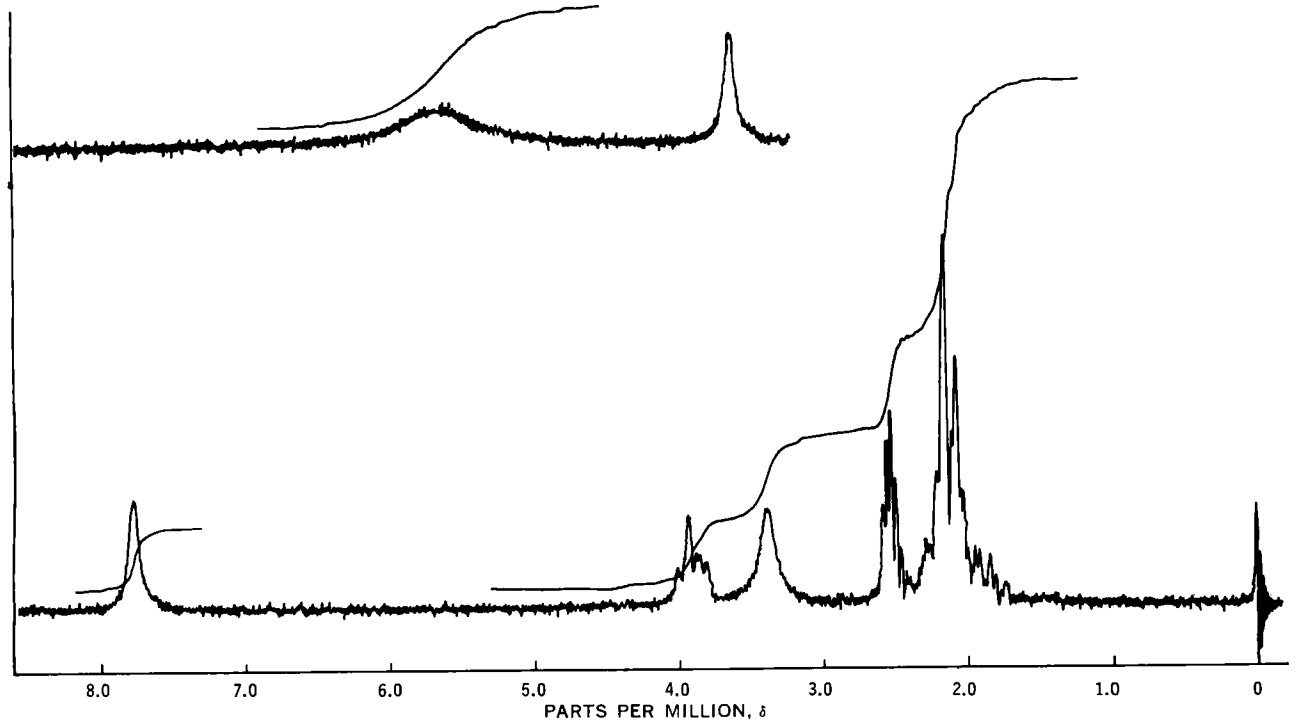


Figure 3—*NMR (60-MHz) spectrum of I in deuterated dimethyl sulfoxide.*

dissolved in 5 ml of water and mixed with 5 ml of solution containing 2.16 g (0.015 mole) of I and 0.60 g (0.015 mole) of sodium hydroxide. Acetone was then added slowly, with rigorous stirring, until the liquid over the resinous black mass turned colorless or pale yellow. The supernatant liquid was decanted from the solid, which was dried in air and finally vacuum desiccated at room temperature over phosphorus pentoxide, yielding 2.0 g (67%).

Anal.—Calc. for $C_{15}H_{21}FeN_6O_9 \cdot 6H_2O$ (mol. wt. 593.33): C, 30.35; H, 5.60; Fe, 9.42; N, 14.17. Found: C, 30.47; H, 4.37; Fe, 9.43; N, 14.08.

Physical Measurements[3]—To determine the acid strength of I, a potentiometric titration at 25° was performed and the pKa value of this acid was calculated from the plot of milliliters of base *versus* pH. By assuming the activity coefficient of unity for the neutral hydroxamic acid species and calculating the activity coefficient (f_{A^-}) for the pyrrolidone-5-hydroxamate anion from the Debye–Huckel equation, $f_{A^-} = -0.51\sqrt{\mu}$, where μ is ionic strength, the pKa value was found to be 8.65.

RESULTS AND DISCUSSION

Preparation and Characterization of I—The literature method (12) for the synthesis was modified since it started with a 10-fold increase in the amount of the reactants and the reaction mixture was allowed to stand much longer for cyclization and condensation; more importantly, anhydrous conditions were ensured all of the time. Finally, it may be noticed that dry hydrogen chloride gas was introduced through a sintered-glass filter, thus forming a large amount of bubbles. These modifications resulted in a higher yield (68 *versus* 52%) and a pure product.

The IR spectrum (Fig. 1) shows the characteristic functional groups in the I molecule. The UV spectrum of this acid (Fig. 2) shows an absorption peak at 225 nm, which can be assigned to the electronic transitions at the keto groups in the I molecule. This absorption peak is shifted to a longer wavelength in comparison with the analogous absorption maximum in the acetamide molecule at 208 nm (13), which suggests that the electron transfer in I takes place between closer π-energy levels than in the acetamide molecule.

The NMR spectrum of I (Fig. 3) shows two peaks of multiplet structure at 2.03 and 3.90 ppm, the first of which is exhibited by the four hydrogens of the two methylene groups and the second by a single proton attached to the ring carbon. The two other signals at 7.76 and 9.80 ppm are assigned to the single hydrogen attached to the ring nitrogen and to the two hydroxylamine protons, respectively. The NMR spectrum of I is in agreement with its proposed structure.

The acidity constant of I, pKa = 8.65, can be compared with the analogous values of benzhydroxamic acid, pKa = 8.79, and acethydroxamic acid, pKa = 9.37 (14). The increase of acidity in the sequence acethydroxamic acid < benzhydroxamic acid < I is in agreement with the fact that the pyrrolidone ring has a lower electron-donating ability than the phenyl and/or the methyl groups.

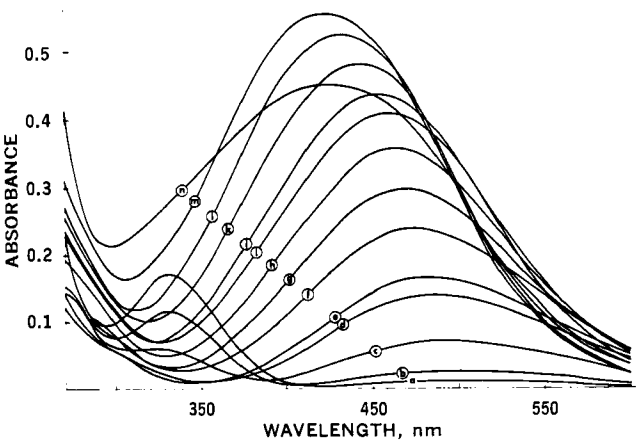


Figure 4—*Absorption spectra of 0.0002 M $FeCl_3$ and 0.002 M I using 1-cm quartz cells at the following pH values: a, 0.70; b, 1.25; c, 1.50; d, 1.72; e, 1.87; f, 2.20; g, 2.80; h, 3.13; i, 3.58; j, 3.96; k, 4.42; l, 5.00; m, 6.50; and n, 9.00.*

[3] The pH and potentiometric measurements were performed with a type MA 5701 ISKRA pH meter and a Radiometer type SRB2c/ABUlb/TTA3 titrigraph. IR spectra from 4000 to 400 cm^{-1} were obtained using a Perkin-Elmer model 457 grating spectrophotometer and KBr disks. The polystyrene spectrum was used for calibration. Proton magnetic resonance spectra were obtained with a Varian model T-60 NMR spectrometer. UV and visible spectra were obtained with a Opton PMQ II single-beam spectrophotometer and a Cary model 16K recording spectrophotometer equipped with quartz cells. Determinations of the magnetic susceptibility were made by means of the Gouy method. Elemental analyses were performed by the Research Institute of the Pharmaceutical and Chemical Works "Pliva," Zagreb.

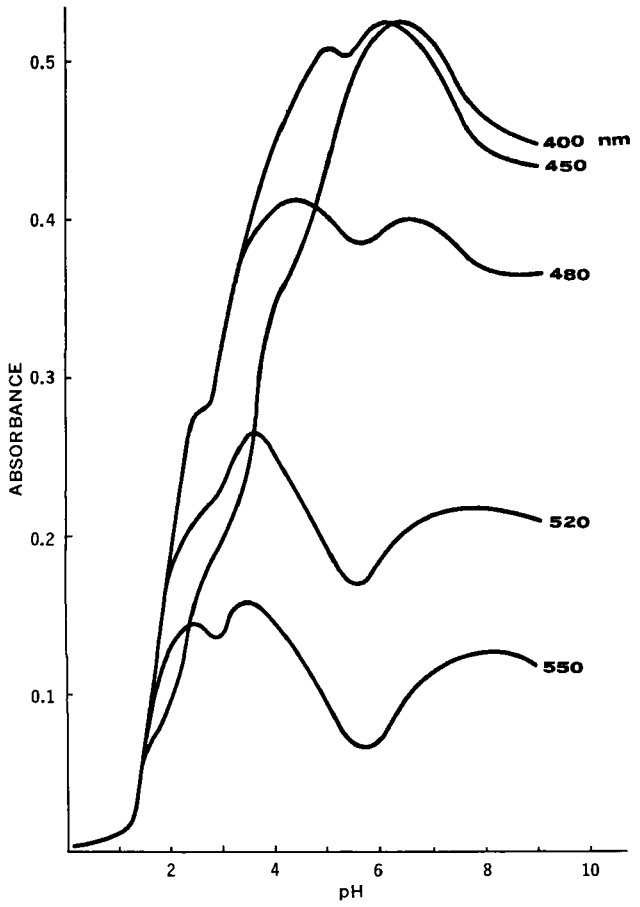


Figure 5—*Absorbance of 0.0002* M *$FeCl_3$ and 0.002* M *I* versus *pH at different wavelengths, using 1-cm quartz cells.*

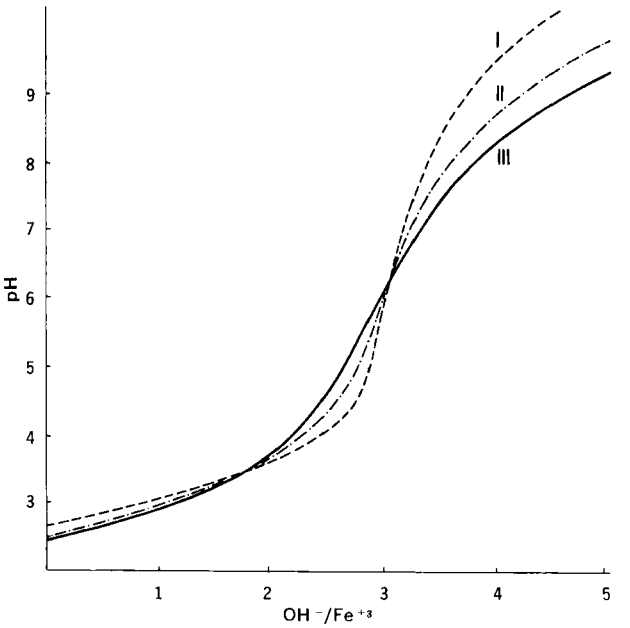


Figure 6—*Potentiometric titration of 0.002* M *$FeCl_3$ containing 0.002* M *I (curve I), 0.004* M *I (curve II), and 0.006* M *I (curve III) with 0.006* M *NaOH.*

Aqueous Mixture of Mono-, Bis-, and Tris(pyrrolidone-5-hydroxamato)iron (III) Complexes—The behavior of the solution containing 2×10^{-4} *M* ferric ions and 2×10^{-3} *M* I (mole ratio = 10) is represented in Fig. 4 over pH 0.70–9.00. Curve a, corresponding to pH 0.70, is similar to that of ferric ion with the absorption peak at 335 nm. However, a new peak at 500 nm appears, which probably indicates the formation of new absorbing species between the ferric ion and I. The increase in pH up to 6.50 results in the disappearance of the 335-nm peak and a shift of the absorption peak from 500 to 430 nm. This observation may suggest the formation of at least two absorbing species between the ferric ion and I. A further increase in pH causes a change of curve m (pH 6.50) to curve n (pH 9.00), which is accompanied by the precipitation of ferric hydroxide.

The pH dependence of the absorbance at several wavelengths of the solution with mole ratio = 10 is shown in Fig. 5. The appearance of the three peaks can be clearly recognized on each curve and may be tentatively assigned to the formation of the three different iron (III)–I species. The existence of these three different species is consistent with the visible color changes of the iron (III)–I solution; a pH decrease or ferric-ion concentration increase turns the solution violet, a pH increase to approximately 4 changes the violet color to red, and further pH increases to the neutral and slightly basic regions or an increase in the concentration of I turns the original red color to a golden yellow.

By following Job's (15) method of continuous variation, the plots of the absorbance *versus* iron fractions x, where x = [Fe]/[Fe] + [I], show the maxima at x = 0.5, 0.33, and 0.25. The assumption may be made that iron (III) forms complexes with I in the stoichiometric ratios of iron (III)–I of 1:1, 1:2, and 1:3. The acidic behavior of all three complexes is shown in Fig. 6, which shows potentiometric titration of the solution containing different I–iron (III) ratios of 1 (curve I), 2 (curve II), and 3 (curve III). All three potentiometric curves intersect at the joint inflection point, showing the existence of the three neutral species of the possible formulation $Fe(C_5H_7O_3N_2)(OH)_2$, $Fe(C_5H_7O_3N_2)_2(OH)$, and $Fe(C_5H_7O_3N_2)_3$.

On the basis of these observations, the behavior of the iron (III)–I solution may be tentatively represented by Scheme II.

$$Fe^{+3} + HC_5H_7O_3N_2 \rightleftharpoons Fe(C_5H_7O_3N_2)^{+2} + H^+$$

$$Fe(C_5H_7O_3N_2)^{+2} + HC_5H_7O_3N_2 \rightleftharpoons Fe(C_5H_7O_3N_2)_2^{+} + H^+$$

$$Fe(C_5H_7O_3N_2)_2^{+} + HC_5H_7O_3N_2 \rightleftharpoons Fe(C_5H_7O_3N_2)_3 + H^+$$

Scheme II

Tris(pyrrolidone-5-hydroxamato)iron (III)—The IR spectrum of the recrystallized product shows characteristic bands of I, including a rather broad band at 3000 cm^{-1} due to the crystal water.

The magnetic moment of tris(pyrrolidone-5-hydroxamato)iron (III) complex was 5.67 B.M.[4], which is very close to the full high-spin value of 5.92 B.M. for the five unpaired electrons per iron atom. The measured value of 5.67 B.M. is in agreement with the figures for the other high-spin iron (III)–hydroxamato complexes, such as ferrichrome (5.68 B.M.), ferrioxamine A (5.73 B.M.), and ferrioxamine B (5.86 B.M.)(2).

The spectral data and the elemental analyses given in the *Experimental* section confirm the formulation as $Fe(C_5H_7O_3N_2)_3 \cdot 6H_2O$.

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ker, New York, N.Y., 1969, p. 7.
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[x] To whom inquiries should be directed.

Specific TLC Tissue Residue Determination of Sulfadiazine following Fluorescamine Derivatization

CARL W. SIGEL [x], JOSEPH L. WOOLLEY, Jr., and CHARLES A. NICHOL

Abstract □ A spectrodensitometric method for the direct determination of sulfadiazine at the tissue residue level (0.1 ppm) is based upon the measurement of the fluorescence of a sulfadiazine–fluorescamine derivative formed directly on a TLC plate by dipping it into a fluorescamine solution. The linear dynamic range for the assay is about 150 from 200 to 0.2 ng, the lower limit of sensitivity. Recoveries from various spiked tissues including milk, eggs, liver, kidneys, muscle, skin, and fat varied with the tissue type but were reproducible. This assay technique has also been used for the assay of sulfamethoxazole and has been explored for use in specifically assaying sulfonamide mixtures.

Keyphrases □ Sulfadiazine—specific TLC tissue residue determination following fluorescamine derivatization □ Fluorescamine—used to prepare sulfadiazine derivative, specific TLC tissue residue determination of sulfadiazine □ TLC—tissue residue determination, sulfadiazine, fluorescamine derivatization

The antibacterial activity of trimethoprim is potentiated by administering it with a sulfonamide (1). Investigation of the use of the combination[1] of trimethoprim with sulfadiazine for treatment of infections in food-producing animals required the development of a method capable of determining sulfadiazine tissue concentrations down to 0.1 ppm. Previous work in this laboratory showed that quantitative TLC could be applied to the direct and specific determination of sulfamethoxazole extracted from plasma by measuring its absorbance on a silica gel plate (2). Although quite satisfactory for kinetic measurement of drug levels in body fluids, this procedure lacked the sensitivity needed for the determination of tissue residue levels of sulfadiazine. Therefore, derivatization on the TLC plate to form a fluorescing compound was considered as an alternative. Fluorescamine, which was first reported as a means of generating a fluorescing derivative of primary amino acids (3), was tested. The reagent was used for detecting amino acids after TLC development, but the fluorescing zones were not quantitated (4).

Sulfonamides and fluorescamine can form fluorescing derivatives directly on silica gel TLC plates, and the resulting zones can be quantitated using a scanning spectrodensitometer. These observations led to a sensitive and specific assay for sulfadiazine, which was applied to the determination of the drug at tissue residue levels. Determination of other sulfonamides, singly and in combination, was explored and will be discussed.

EXPERIMENTAL

Thin-Layer Plates—Silica gel 60 plates without fluorescent indicator (20 × 20 cm, 0.25 mm) were used[2].

Solvents and Solutions—All solvents were reagent grade[3] and all were distilled before use. The tissue homogenization buffer was 0.56 *M* sodium chloride, 0.01 *M* sodium phosphate dibasic, and 0.0013 *M* sodium phosphate monobasic (pH 7.4). Solutions of sulfadiazine (1.0 mg) in 100 ml of ethyl acetate and solutions of triethanolamine (1.25 ml) in 250 ml of chloroform were prepared each week. Fluorescamine[4] (25 mg) was dissolved in 250 ml of acetone. The solution was used for derivatization of not more than 15 plates and was not kept longer than 2 weeks.

Instrumentation—Plates were spotted with an automatic spotter[5] or by hand. Fluorescence was determined by scanning TLC plates with a spectrodensitometer[6], using the reflectance mode and only the sample beam with a secondary cutoff filter at 400 nm. Excitation was at 290 nm for the sulfadiazine–fluorescamine derivative, and total emission (above 400 nm) was determined using a density computer[7]. Peak areas were electronically integrated[8], and standard curves were calculated using the method of least squares.

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[3] Mallinckrodt.
[4] Fluram, Roche Diagnostics.
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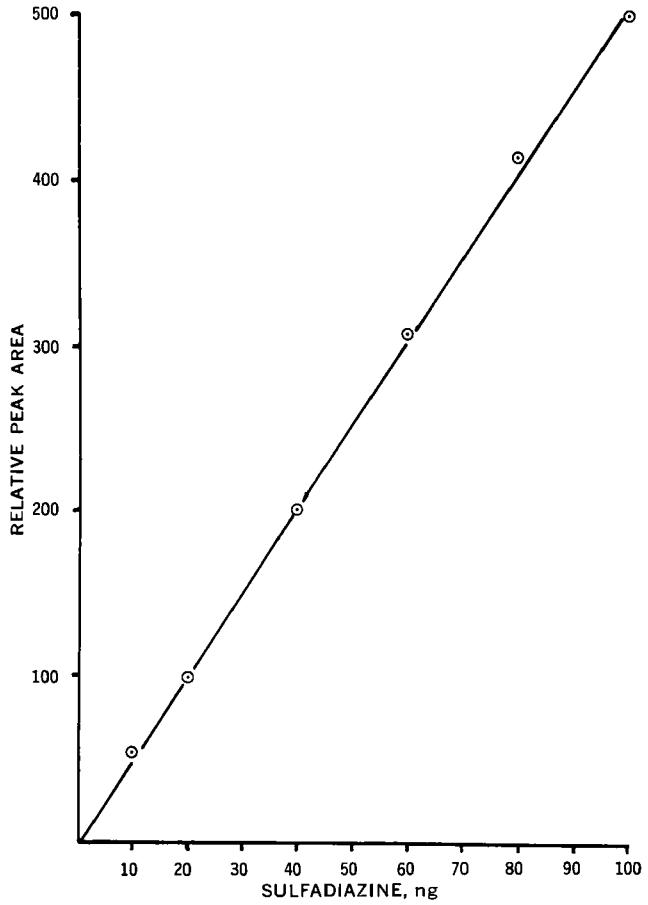


Figure 1—*Standard curve for sulfadiazine determination.*

extracts were dried by transferring them with a pipet into a 1 × 20-cm column containing anhydrous sodium sulfate (2 g).

Following elution, the column was rinsed with additional ethyl acetate (3 ml). Diethylaniline (5 μl) and 26.5% biphenyl in phenyl ether[10] solution (5 μl) were added to the combined eluates, and the sample was evaporated at 30° under a stream of dry nitrogen. The residue was redissolved in acetone (200 μl), and an aliquot (50 μl) was spotted on a scored silica gel TLC plate (20 channels/plate). Standards (0.01–0.1 μg) were spotted on the same plate. The plate was developed to 10–12 cm twice using diethyl ether in a tightly sealed solvent chamber.

The plate was air dried and dipped once quickly in a fluorescamine–acetone solution in a stainless steel tank (21.5 × 21.5 × 0.7 cm) to derivatize the sulfonamide. Then the plate was air dried for about 10 min and dipped once in a 0.5% triethanolamine–chloroform solution to stabilize the derivative (4). A second dipping into each of the solutions, respectively, did not cause either diffusion of the spots or diminution of the fluorescence. After air drying again, the plate was scanned with excitation wavelength at 290 nm and the total emission above 400 nm was measured. Sulfadiazine appeared at about R_f 0.40.

Table I—Recovery and Reproducibility of Sulfadiazine from Spiked Rat (R) and Chicken (C) Tissues and Cow's Milk

Tissue	Number of Samples	Amount Added, μg/g	Recovery, %	Deviation, %
Milk[a]	10	0.100	91.1	12.7
Egg (C)[a]	11	0.100	54.0	4.9
Muscle (R)	12	0.100	72.8	9.4
Liver (R)	10	0.100	76.3	15.0
Liver (C)[a]	6	0.100	71.7	10.4
Kidney (R)	11	0.100	59.8	10.3
Fat (C)[a]	10	0.100	59.8	7.7
Skin (C)[a]	5	0.100	81.6	4.2

[a] Obtained from commercial sources.

[10] Dowtherm A, added to prevent decomposition.

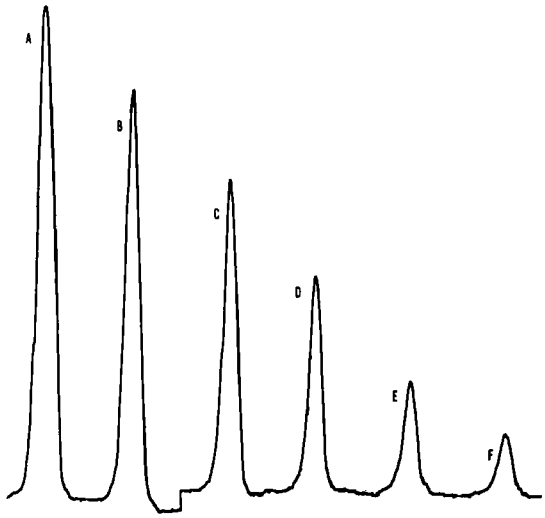


Figure 2—*Typical peaks observed when scanning the fluorescamine derivatives of sulfadiazine standards. Key: A, 0.1 μg; B, 0.08 μg; C, 0.06 μg; D, 0.04 μg; E, 0.02 μg; and F, 0.01 μg.*

Procedure for Eggs, Liver, Fat, Skin, and Kidneys—The tissue was homogenized and extracted as described for milk and muscle samples. Before drying with sodium sulfate, the combined ethyl acetate extracts were back-extracted with 2 *N* aqueous sodium hydroxide (1 ml). The pH of the aqueous solution was checked and, if less than 9, the organic extract was washed with additional base (1 ml). The combined alkaline washes were adjusted to pH 6.0 with 1 *M* H_3PO_4 and extracted with three 3-ml portions of ethyl acetate. The combined extracts were dried over anhydrous sodium sulfate and assayed as described for milk and muscle samples.

Procedure for Plasma—A modification of the procedure for sulfamethoxazole was used (2). An equal volume of buffer [0.5 *M* citric acid and 1 *M* sodium phosphate dibasic (pH 3.9)] was added to the plasma and extracted with two 3-ml volumes of ethyl acetate. Samples were assayed as described for milk and muscle samples.

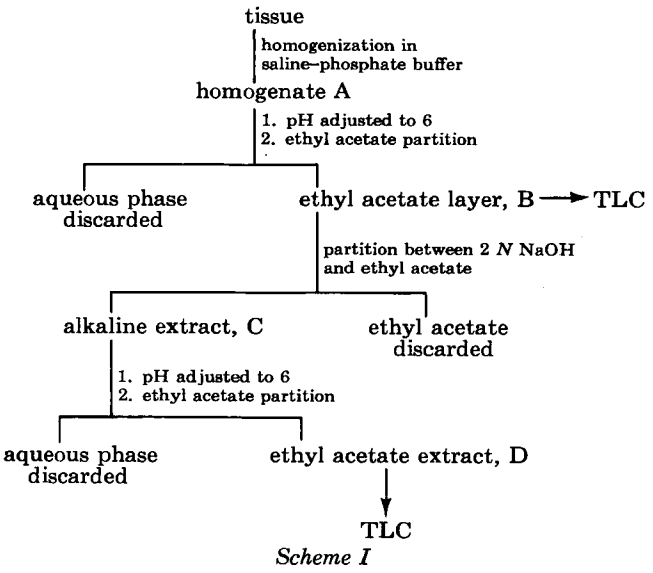
RESULTS

Sulfadiazine can be derivatized directly on a developed silica gel TLC plate with a fluorescamine solution by either spraying the plate or dipping it. The latter technique was more convenient and quicker, the sensitivity fourfold greater, the standard deviation lower, and the background more uniform. The derivative can be stabilized for about 5 hr by posttreating with triethanolamine (4). The standard deviation for the determination of nine samples of 0.05 μg of sulfadiazine was ±0.001 μg or 2%.

A calibration curve for the sulfadiazine assay is shown in Fig. 1. The linear dynamic range was found to be about 150 from 200 to 0.20 ng, the lower limit of sensitivity. Above 200 ng, linearity declined. Typical peaks observed on the recorder chart after scanning derivatized sulfadiazine standards in the 10–100-ng range are

Table II—Tissue Levels of Sulfadiazine in the Rat following a Single Oral Dose of Trimethoprim (4 mg/kg) and Sulfadiazine (20 mg/kg)

Rat	Hours	Plasma, μg/ml	Liver, μg/g	Muscle, μg/g
1	5	53.9	13.6	7.84
2	24	5.8	0.86	0.661
3	48	0.83	0.09	0.129
4	75	0.016	>0.006	>0.006



Scheme I

shown in Fig. 2. This range was used for assaying residue amounts for 1-g tissue samples.

Recovery data for extraction of sulfadiazine from spiked tissues are shown in Table I. Rat and chicken tissues were used for the recovery experiments. For milk and muscle, a simple "clean-up" procedure involving homogenization and a single extraction (Scheme I) could be used; however, for eggs, liver, kidneys, skin, and fat, a back-extraction into base was required to separate sulfadiazine from endogenous material partitioning into the ethyl acetate extract of the homogenate. The percent recovery of drug depended upon the tissue and whether a back-extraction was required but was considered reproducible for each tissue type.

These results were in the same range as reported by Crisp (5) who used a similar isolation scheme for a tissue residue assay based upon the GC determination of sulfaquinoxaline. In the current work, recoveries were improved by at least 30% by the addition of small aliquots of a solution of 26.5% biphenyl in phenyl ether and diethylaniline to the ethyl acetate extracts (B or D, Scheme I) prior to evaporation[11]. Addition of both components was necessary for recovery improvement. Treatment of the glassware with siliconizing fluid did not improve recoveries.

Careful adjustment of the pH of both the homogenate A and alkaline extract C was an important factor for reproducible recoveries. The relationship between partition coefficient and pH of the aqueous solution for sulfadiazine is shown in Fig. 3.

Four rats were dosed orally with the sulfadiazine–trimethoprim combination (20 and 4 mg/kg, respectively) and were sacrificed sequentially over 4 days. Plasma, muscle, and liver tissue levels of sulfadiazine were determined (Table II). Sulfadiazine appeared as a well-resolved symmetrical peak with no interference due to endogenous compounds, sulfadiazine metabolites, or trimethoprim or its metabolites.

A mixture of five sulfonamides also was chromatographed, derivatized with fluorescamine, and scanned (Fig. 4), thereby illustrating that the basic approach has potential for assaying sulfonamide mixtures.

DISCUSSION

The most widely used method for the determination of sulfonamides is a colorimetric procedure based upon the Bratton–Marshall reaction (6). For tissue concentrations, however, the method has limitations in terms of lack of specificity and background interference. With these considerations in mind, Crisp (5) developed a tissue residue procedure for sulfaquinoxaline based upon electron-capture GLC. The procedure was specific and extremely sensitive but time consuming. This TLC method for sulfadiazine,

[11] P. Flanagan, Wellcome Research Laboratories, Berkhamsted, England, personal communication, 1974.

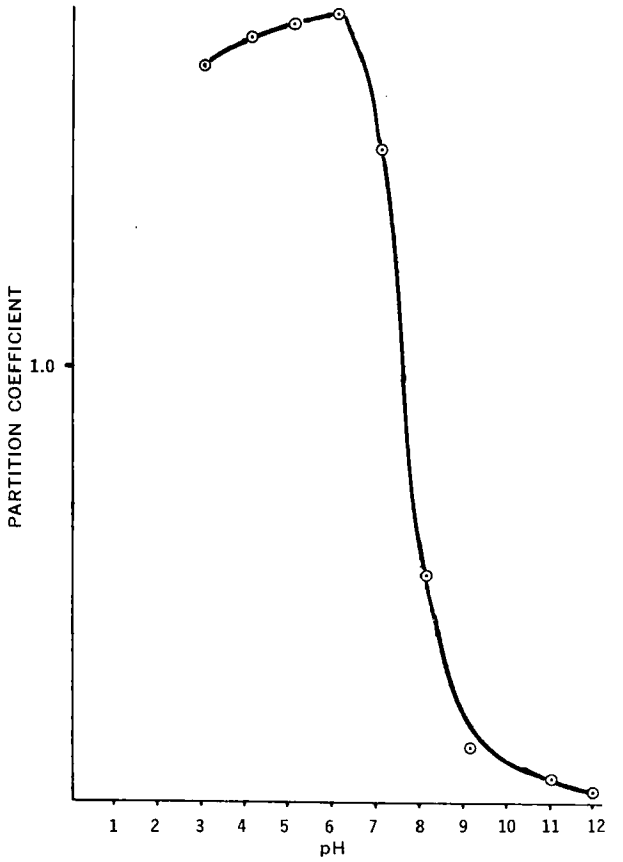


Figure 3—*Relationship between partition coefficient and pH for sulfadiazine.*

based upon fluorescence scanning, has the advantages that the sample preparation is simpler and the time required for analysis is shorter. The instrumentation is extremely reliable and easily operated. Very uniform TLC plates are commercially available. Even if there are plate or instrumental variations for different runs, these factors are eliminated by determining a series of standards on the same plate used for the unknown samples. This procedure still allows the analysis of at least 10 unknown samples on each plate. By using this procedure, about 8–10 tissue samples can be assayed by one person in 1 day.

The assay of sulfadiazine has been emphasized in this report. Sulfamethoxazole (following fluorescamine derivatization) has been assayed in vaginal washes[12] and other fluids and tissues down to 1 ng/ml by a modification of the basic TLC procedure previously reported from these laboratories (2). Presumably, the TLC method could be adapted for measurement of specific sulfonamide drug levels in cases where sulfonamide drug mixtures are used. TLC conditions for the separation of up to 15 different sulfonamides on one plate have been reported (7). The potential of this approach was demonstrated by derivatizing and scanning five sulfonamides following separation on a TLC plate. Of course, the method is specific for each of these compounds only insofar as there are no other molecules with primary amino moieties that comigrate with the drugs. Careful selection of the TLC solvent would obviate this problem.

CONCLUSIONS

Quantitative fluorescence scanning is a sensitive analytical technique which has the advantage of moving the drug away from interfering substances to an R_f on the chromatogram characteristic of the compound and the solvent system. Derivatization of sulfonamides with fluorescamine on the developed TLC plate amplifies the sensitivity beyond that attainable by absorbance scanning. A

[12] T. A. Stamey and M. Condy, to be published.

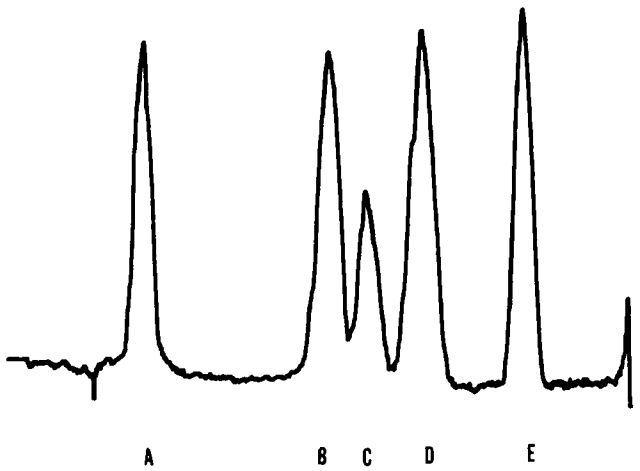


Figure 4—*Scan of derivatized sulfonamides separated on a silica gel TLC plate by development twice in diethyl ether (11 cm). Sulfonamides are: A, sulfathiazole; B, sulfadiazine; C, sulfamerazine; D, sulfadimidine; and E, sulfamethoxazole.*

sensitive and specific method utilizing these principles for the development of a tissue residue assay for sulfadiazine is described.

Fluorescamine was used previously as a means for detection of amino acids on TLC plates, but the quantitative aspects were not pursued (4). This report emphasizes the utility of this reagent as a useful analytical tool, especially for residue level determinations of sulfonamides. Undoubtedly, numerous extensions of this approach to include a broad range of drugs and pesticides can be made.

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Predicting Subjective Spreadability, Viscosity, and Stickiness

MARIA L. DeMARTINE and E. L. CUSSLER [x]

Abstract □ Subjective spreadability, viscosity, and stickiness perceived with the fingers were predicted from fluid mechanics. The correlation coefficients of these predictions were 0.95 for spreadability, 0.95 for viscosity, and 0.90 for stickiness. The two important assumptions in the predictions were that spreadability and viscosity were perceived as shear stress and that stickiness was perceived as time. When the finger geometry was approximated as two parallel plates, the predictions only required rheological data for the Newtonian and non-Newtonian liquids used. While these liquids covered a range of 10^6 in apparent viscosity, large variations in other fluid properties such as density and surface tension were not studied.

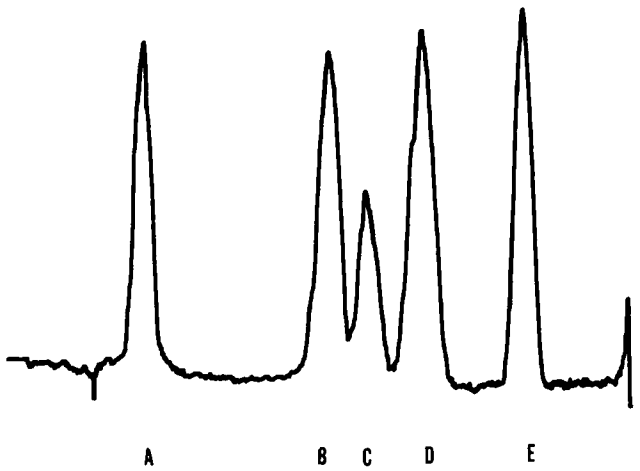
Keyphrases □ Spreadability, subjective—predicted from fluid mechanics, equations □ Viscosity, subjective—predicted from fluid mechanics, equations □ Stickiness, subjective—predicted from fluid mechanics, equations □ Liquid texture—predicting subjective spreadability, viscosity, and stickiness

Previous papers on liquid texture presented interesting empirical relationships between subjective attributes and rheological parameters (1–7). For example, Stevens and Guirao (2) found for Newtonian liquids that:

$$\begin{pmatrix}\text{subjective}\\ \text{viscosity}\end{pmatrix} \propto \begin{pmatrix}\text{objective}\\ \text{viscosity}\end{pmatrix}^{0.44} \qquad \text{(Eq. 1)}$$

where the exponent of 0.44 is found empirically. As a second example, empirical curves were found (4, 6, 7) giving the conditions under which fluids of different rheological properties have the same subjective texture. Why these curves have their peculiar shape remained uncertain.

This paper predicts some attributes of liquid texture without introducing empiricism. It depends on two key assumptions: *(a)* that subjective stickiness is perceived as the time during which the finger is pulled away from the sticky surface, and *(b)* that subjective spreadability and viscosity are perceived as the shear stress on the fingers. That these assumptions are sensible is easily tested. For example, when water and honey are rubbed between the fingers, the



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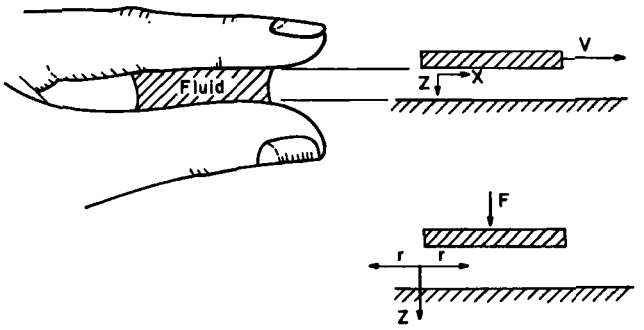

Figure 1—*Schematic representation of finger geometry as two parallel plates.*

fingers move in an oscillating motion with a constant period and amplitude; the finger velocity varies little with changes in objective liquid viscosity. Since the subjective viscosities of honey and water are very different, the subjective viscosity must be perceived not as velocity but as another quantity such as shear stress. This type of observation will be shown to lead to the successful prediction of liquid texture.

THEORETICAL

Finger Geometry and Fluid Rheology—There were three basic steps in developing predictions of spreadability, viscosity, and stickiness:

1. The finger geometry and fluid rheology were specified.
2. Equations for the thickness of the fluid film on the fingers as a function of time were developed.
3. How this fluid thickness leads to equations for predicting spreadability, viscosity, and stickiness was demonstrated.

In the first step, the actual geometry of the fingers was approximated by two parallel plates (Fig. 1). This approximation is reasonable only when the thickness of the sample is small relative to the radius of the finger. As suggested in Fig. 1, the shear force between the fingers is most easily found using Cartesian coordinates with the origin on one moving finger, but the thickness is most easily calculated in terms of cylindrical coordinates with the origin at the center of the film.

Just as parallel plates were used to approximate the finger geometry, the empirical power law model was assumed to approximate the fluid rheology (8):

$$\tau_{zx} = -m\left(\frac{\partial v_x}{\partial z}\right)^n \qquad \text{(Eq. 2)}$$

where τ_{zx} is the shear stress on a plane normal to the z direction and caused by motion in the x direction, v_x is the fluid velocity in the x direction, and m and n are rheological constants of this model. For a Newtonian fluid, n is unity and m is the viscosity; for the non-Newtonian fluids used in this work, n was always less than 1. The use of this equation required that the coordinate system be defined so that $(\partial v_x/\partial z)$ was always positive and τ_{zx} was not imaginary. Therefore, the coordinate system had to be redefined every time the velocity was reversed.

Thickness of Fluid Film—The second basic step in developing the desired predictions was to calculate fluid thickness for the approximate geometry and rheology. This problem was solved for Newtonian fluids by Stefan (9), yielding results frequently used in lubrication theory (10). For the power law fluids considered here, the equation of motion in cylindrical coordinates is:

$$\frac{\partial p}{\partial r} = -\frac{\partial \tau_{zr}}{\partial z} = m\frac{\partial}{\partial z}\left(\frac{\partial v_r}{\partial z}\right)^n \qquad \text{(Eq. 3)}$$

where p is the pressure and τ_{zr} is the stress on a plane normal to the z direction caused by motion in the r direction. The first boundary condition used with Eq. 3 was that the shear stress is zero in the exact center of the film; *i.e.*, that $\partial v_r/\partial z = 0$ when $z = 0$. The second boundary condition was that the velocity at the plates is zero; *i.e.*, that $v_r = 0$ when $z = \pm h/2$, where h is the film thickness between the fingers. Equation 3 was integrated using these conditions:

$$v_r = \left[\frac{1}{m}\frac{\partial p}{\partial r}\right]^{1/n}\frac{n}{n+1}\left[z^{(1/n)+1} - \left(\frac{h}{2}\right)^{(1/n)+1}\right] \qquad \text{(Eq. 4)}$$

The volumetric flow per unit arc, Q, was defined across a fluid element shaped like a piece of pie:

$$Q = \frac{2\int_0^{h/2}\int_0^{2\pi} v_r r\, d\theta\, dz}{\int_0^{2\pi} r\, d\theta} = 2\int_0^{h/2} v_r\, dz \qquad \text{(Eq. 5)}$$

Therefore, from Eqs. 4 and 5, Q was:

$$Q = 2\left[\frac{1}{m}\frac{\partial p}{\partial r}\right]^{1/n}\frac{n}{n+1}\int_0^{h/2}\left[z^{(1/n)+1} - \left(\frac{h}{2}\right)^{(1/n)+1}\right]dz \qquad \text{(Eq. 6)}$$

Then Q was related to the decrease of the fluid thickness by means of a mass balance on the same pie-shaped element already used:

$$-\frac{dh}{dt} = \frac{1}{r}\frac{\partial}{\partial r}(Qr) \qquad \text{(Eq. 7)}$$

Equations 6 and 7 were next combined and integrated using two more boundary conditions. The first of these was that the pressure is always finite or, in other words, that $p \neq \infty$ when $r = 0$. The second condition was that the pressure must be the atmospheric value p_0 at the edge of the fingers; *i.e.*, $p = p_0$ at $z = \pm h/2$ and $r = R$, where R is the effective radius of the fingers. The result of this integration was:

$$p = p_0 + \frac{m}{n+1}\left(\frac{2n+1}{4n}\right)^n\left(\frac{2}{h}\right)^{2n+1}\left(\frac{dh}{dt}\right)^n[R^{n+1} - r^{n+1}] \qquad \text{(Eq. 8)}$$

In this integration, dh/dt was assumed to be approximately constant. This pseudo-steady-state approximation took advantage of the fact that steady-state pressure gradients are approached rapidly.

Before Eq. 8 was finally integrated, it was rewritten in terms of the total normal force, F_0, acting on the plates:

$$F_0 + \pi R^2 p_0 = 2\pi\int_0^R pr\, dr \qquad \text{(Eq. 9)}$$

Combination of Eqs. 8 and 9 and integration over r yielded:

$$\frac{F_0}{R^{n+3}}\left(\frac{n+3}{2\pi m}\right)\left(\frac{n}{2n+1}\right)^n = \left(\frac{1}{h}\right)^{2n+1}\left(\frac{dh}{dt}\right)^n \qquad \text{(Eq. 10)}$$

a result similar to that of Parlato (11). Since the total normal force by the fingers was assumed a constant, the nth root of this equation could be integrated over time:

$$h = \frac{1}{\left[\frac{1}{h_0^{(n+1)/n}} + \left(\frac{F_0}{R^{n+3}}\frac{n+3}{2\pi m}\right)^{1/n}\frac{n+1}{2n+1}t\right]^{n/(n+1)}} \qquad \text{(Eq. 11)}$$

where h_0 is the initial film thickness at zero time. This equation shows how the thickness of the fluid film varies with time, finger geometry, and fluid rheology. It formed the basis for the predictions of subjective properties given in the next section.

Subjective Predictions—*Spreadability*—The third step in this development was the prediction of the subjective attributes. First, spreadability was assumed to be proportional to the reciprocal of the shear stress:

$$\begin{pmatrix}\text{subjective}\\ \text{spreadability}\end{pmatrix} \propto \frac{1}{\tau_{\text{spread}}} \qquad \text{(Eq. 12)}$$

For a thin film, combination of Eqs. 2 and 12 gave:

$$\begin{pmatrix}\text{subjective}\\ \text{spreadability}\end{pmatrix} \propto \frac{1}{m}\left(\frac{h}{V_{\text{spread}}}\right)^n \qquad \text{(Eq. 13)}$$

where V_{spread} is the steady velocity of the finger used in assessing

spreadability. This result was combined with Eq. 11 to give:

$$\begin{pmatrix}\text{subjective}\\ \text{spreadability}\end{pmatrix} \propto \frac{1}{mV_{\text{spread}}^{n}}\left[\frac{1}{h_0^{(n+1)/n}} + \left(\frac{F_0}{R^{(n+3)}}\frac{n+3}{2\pi m}\right)^{1/n} \times \frac{n+1}{2n+1}t_{\text{spread}}\right]^{-n^2/(n+1)} \quad \text{(Eq. 14)}$$

where t_{spread} is the average assessment time for spreading. Since all quantities on the right-hand side of this equation were measured by independent experiments, Eq. 14 provided a means of calculating spreadability. A plot of experimentally measured subjective spreadability *versus* the right-hand side of Eq. 14 should be linear.

Viscosity—Viscosity was assumed to be perceived as the shear stress, τ_{visco}, exerted on the finger:

$$\begin{pmatrix}\text{subjective}\\ \text{viscosity}\end{pmatrix} \propto \tau_{\text{visco}} = m\left(\frac{V_{\text{visco}}}{h}\right)^{n} \quad \text{(Eq. 15)}$$

where V_{visco} is the average finger velocity used on the thin fluid film. In fact, viscosity is assessed by rubbing the fingers with an oscillating motion, but the exact analysis of this motion is very elaborate (12). Combination of Eqs. 11 and 15 gave:

$$\begin{pmatrix}\text{subjective}\\ \text{viscosity}\end{pmatrix} \propto m(V_{\text{visco}})^{n}\left[\frac{1}{h_0^{(n+1)/n}} + \left(\frac{F_0}{R^{(n+3)}}\frac{n+3}{2\pi m}\right)^{1/n} \times \frac{n+1}{2n+1}t_{\text{visco}}\right]^{n^2/(n+1)} \quad \text{(Eq. 16)}$$

where t_{visco} is the assessment time for viscosity. All quantities on the right-hand side of Eq. 16 were determined experimentally. As a result, a plot of experimentally determined subjective viscosity *versus* the right-hand side of Eq. 16 should be linear.

For two special cases, the subjective viscosity could be predicted in simpler ways. First, for Newtonian fluids for which the initial thickness was large, $n = 1$, $m = \mu$, and $h \ll h_0$. Under these conditions, Eq. 16 is reduced to:

$$\begin{pmatrix}\text{subjective}\\ \text{viscosity}\end{pmatrix} \propto \mu^{1/2}\left[V_{\text{visco}}\left(\frac{4F_0 t_{\text{visco}}}{3\pi R^4}\right)^{1/2}\right] \quad \text{(Eq. 17)}$$

All quantities in brackets are characteristic of the assessment but experimentally independent of the fluid being judged. Thus, if subjective viscosity was, in fact, perceived as shear stress, a plot of the logarithm of subjective spreadability *versus* the logarithm of objective viscosity should have a slope of one-half.

The second special case for predicting subjective viscosity occurred when the film was mechanically prevented from thinning by means of a small sled holding the finger a constant distance from the surface (13). In this case, Eq. 15 assumed a very simple form for Newtonian fluids:

$$\begin{pmatrix}\text{subjective}\\ \text{viscosity}\end{pmatrix} \propto \tau_{\text{visco}} = \mu\left[\frac{V_{\text{visco}}}{h}\right] \quad \text{(Eq. 18)}$$

where h is now a constant of the small sled. The quantity in brackets is again characteristic of the assessment but independent of the fluid being judged. If subjective viscosity was truly perceived as shear stress, a plot of the logarithm of subjective viscosity assessed at constant film thickness *versus* the logarithm of objective viscosity should have a slope of unity.

Stickiness—Unlike spreadability and viscosity, which were assessed with a shearing motion, stickiness was assessed by a motion normal to the surface. First the finger was pressed against the sample being judged, and then the finger was pulled away. While the fluid mechanics in this situation were less well defined than for the previous two cases, the thickness of the film during this assessment can be approximately calculated from Eq. 11.

Stickiness was assumed to be perceived as the time during which the finger is pulled away from the sticky surface. This time varied inversely with the average velocity normal to the surface of the moving finger, dh/dt:

$$\begin{pmatrix}\text{predicted}\\ \text{stickiness}\end{pmatrix} \propto \frac{H}{dh/dt} \quad \text{(Eq. 19)}$$

where H is the distance at which the finger separates from the fluid. In general, H was a function of the fluid being tested. However, because the surface tension had approximately the same value for all of the fluids used, H was approximately a constant here.

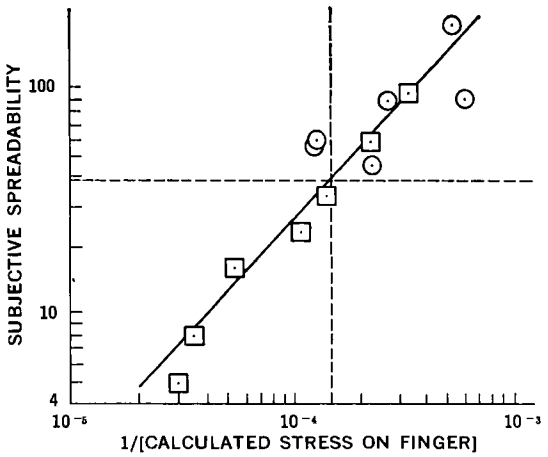

Figure 2—*Subjective spreadability* versus *[shear stress]*$^{-1}$. *The best line through the data has a slope of 1.05 ± 0.05, compared with a predicted value of 1.00. The circles and squares are Newtonian and non-Newtonian fluids, respectively.*

The velocity dh/dt can be calculated from Eq. 11 and combined with Eq. 19 to yield:

$$\begin{pmatrix}\text{subjective}\\ \text{stickiness}\end{pmatrix} \propto \frac{H(2n+1)}{n} \times \frac{\left[\frac{1}{h_1^{(n+1)/n}} - \frac{n+1}{2n+1}\left(\frac{F_1}{R^{n+3}}\frac{n+3}{2\pi m}\right)^{1/n} t_{\text{stick}}\right]^{(1+2n)/(1+n)}}{\left(\frac{F_3}{R^{n+3}}\frac{n+3}{2\pi m}\right)^{1/n}} \quad \text{(Eq. 20)}$$

where h_1 is the minimum film thickness after squeezing the film, F_1 is the normal force developed in pulling the finger upward, and t_{stick} is an average reference time. All quantities on the right-hand side of Eq. 20 were determined experimentally except for h_1, which was calculated from Eq. 11. Thus, a plot of experimentally measured subjective stickiness *versus* the right-hand side of Eq. 20 should be a straight line.

EXPERIMENTAL

Objective Measurements—The Newtonian fluids used in this work were silicone oils[1] ranging in viscosity from 2.6 to 85,000 cps. The non-Newtonian fluids included aqueous solutions of hydroxypropyl methylcellulose and aqueous solutions of polyacrylamide. Three grades of hydroxypropyl methylcellulose[2] were used as 2 wt. % solutions in water. The polyacrylamide solutions were prepared by polymerization of 2, 4, and 8% monomer in water. Polymerization was initiated with persulfate–bisulfite and continued at 60° for about 1 hr. Viscosities of the Newtonian fluids were measured with capillary viscometers[3], calibrated with Newtonian viscosity standards[4]. Rheological parameters of the non-Newtonian fluids were measured with a cone-and-plate viscometer[5].

In addition to these rheological properties, the predictions of spreadability, viscosity, and stickiness depended on various characteristics of the finger and its motion. Three of these characteristics were similar for all three subjective attributes: the average radius of the finger, R; the force used in thinning the film, F_0; and the initial film thickness, h_0. The average radius of the finger for 20 people was 0.75 cm, consistent with the measurements of Schaeffer and Chakravarti (14) who used a plate of 0.73 cm as a model of the second fingertip in the design of a commercial instru-

[1] Union Carbide.
[2] Methocel HG, labeled as 400, 8000, and 15,000 cps; Dow Chemical Co.
[3] Ubbelhode type, Fisher Scientific Co.
[4] Cannon Instrument Co., State College, Pa.
[5] Rheomat 15, Olkon Corp.

Table I—Spreadability for Eight Newtonian and Six Non-Newtonian Fluids[a]

Fluid	m	n	Measured h_0	Measured V_{spread}	Reciprocal Calculated Stress[b]	Subjective Spreadability
Water, 1 cps	0.01	1.0	0.013	7.4	12.3[c]	105.
Silicone oil, 20 cps	0.20	1.0	0.018	6.2	3.3	97.
Silicone oil, 70 cps	0.70	1.0	0.040	4.9	2.2	58.
Silicone oil, 936 cps	9.36	1.0	0.034	2.2	1.3_8	33.
Silicone oil, 1770 cps	17.70	1.0	0.046	2.0	1.0_7	23.
Silicone oil, 8450 cps	84.50	1.0	0.055	1.8_2	0.5_4	16.
Silicone oil, 23,300 cps	233.0	1.0	0.072	1.6_7	0.35	$7._9$
Silicone oil, 85,000 cps	850.0	1.0	0.068	0.97	0.30	$4._9$
Polyacrylamide, 2%	0.13	0.93	0.031	7.8	5.4	190.
Polyacrylamide, 5%	28.	0.52	0.056	4.8	6.0	90.
Polyacrylamide, 8%	390.	0.47	0.066	6.1	1.2_4	55.
Hydroxypropyl methylcellulose, 400 cps, 1.5%	3.4	0.79	0.057	7.3	2.7	88.
Hydroxypropyl methylcellulose, 8000 cps, 2%	93.	0.62	0.084	6.4	1.2_6	58.
Hydroxypropyl methylcellulose, 15,000 cps, 2%	630.	0.32	0.066	5.0	2.2	46.

[a] Units used here are as follows: m, dynes sec^n cm^{-2}; h_0, cm; V, cm sec^{-1}; and calculated spreadability, 10^{-4} cm^2 dyne^{-1}. Other quantities are dimensionless. [b] These calculations involve three additional experimentally measured quantities: the average normal force, F_0, of 89,000 dynes; the average finger radius, R, of 0.75 cm; and the average assessment time, t_{spread}, of 10 sec. [c] Not included in the regression analysis.

ment. The normal force for thinning the film was found by placing different samples on the pan of a magnetic balance and then by touching the fluids in an imitation of the subjective assessments. Each of 20 people used the same force for all fluids, independent of the fluid's viscosity. This force was about 89,000 ± 5000 dynes.

The initial thickness of the sample, h_0, was measured directly in the case of spreadability as the mass of fluid adhering to the finger divided by the finger area. These values did vary with viscosity and are reported in the *Results* section. The same values were also used in calculations of viscosity and stickiness but had little effect on the calculations. Indeed, assuming that h_0 always equaled 0.1 cm gave equivalent results in these two cases.

The basic equations for predicting spreadability, viscosity, and stickiness also contained characteristics of finger motion, which were specific for the attribute being studied. For spreadability, these characteristics were the velocity of the finger, V_{spread}, and the time of spreading, t_{spread}, both of which were measured with a stopwatch during the subjective experiments. The velocity varied with viscosity and is reported below, but the time of spreading was a constant for each subject and had an average value of 10 sec. For viscosity, the specific characteristics were the average velocity of finger motion, V_{visco}, and the time of assessment, t_{visco}. These quantities, also measured during the subjective experiments, were both constant, independent of viscosity. The velocity had a period of 1.0 ± 0.2 sec and an amplitude of 0.5 ± 0.1 cm. The time for the assessment of viscosity was found to be 5 ± 1 sec.

For stickiness, the specific characteristics necessary for the calculations were the force used in pulling up the finger, the time of squeezing the fluid, and the time of pulling the finger. The pulling force was measured with a balance (14). The time for squeezing, measured with a balance and a stopwatch during some of the subjective experiments, was a constant 3.0 ± 0.2 sec. The time of pulling was measured as 0.1 sec, but this measurement was approximate.

Subjective Measurements—For each textural characteristic, panels of between 15 and 24 members of both sexes, ranging in age from 20 to 50 years and not trained prior to the present work, were asked in written instructions (14) to assess the different fluid samples relative to the first sample. These samples were presented in random order on a plastic plate at room temperature. For spreadability, observers spread the samples along the plate with their index finger; for viscosity, observers often preferred to rub the samples between their fingers and were permitted to do so; for stickiness, observers touched the sample and then pulled their fingers away.

Whenever necessary, observers were allowed to go back to the standard for comparison. A judgment of zero was not accepted for any property; in that case, the concept of ratio scaling was clarified, and use of fractional numbers (1/10, 1/100, *etc.*) was encouraged. The subjective data were analyzed with linear regression analysis after a logarithmic transformation (15).

RESULTS

Spreadability—The agreement of the experimental and predicted values of the subjective assessments is clearest if the three attributes of spreadability, viscosity, and stickiness are discussed sequentially. The raw data for spreadability are shown in Table I. The first three columns give the fluid and its rheological parameters in terms of the power law model in Eq. 2. The next two columns give the initial thickness, h_0, and the spreading velocity, V_{spread}, both measured during the subjective assessments. The sixth column gives the reciprocal of the stress calculated from Eq. 14, using experimental quantities shown in the table. The final column gives the subjective spreadability measured by ratio scaling. The value for water deviates considerably from the trend shown by the other fluids; it probably lies outside the range of perception and was discarded when estimating the regression line.

The theory for spreadability predicts that a plot of the logarithm of subjective spreadability *versus* the logarithm of the right-hand side of Eq. 14 should be a straight line of slope unity. This prediction is in excellent agreement with the experimental slope of 1.05 ± 0.05 (Fig. 2). Phrased in other terms, the correlation coefficient between subjective spreadability and the reciprocal of the shear stress is 0.95. This correlation is impressive.

Equation 14 also predicts that two Newtonian fluids of different viscosities will never have the same subjective spreadability. In this case, n is always unity, so different values of $m = \mu$ imply dif-

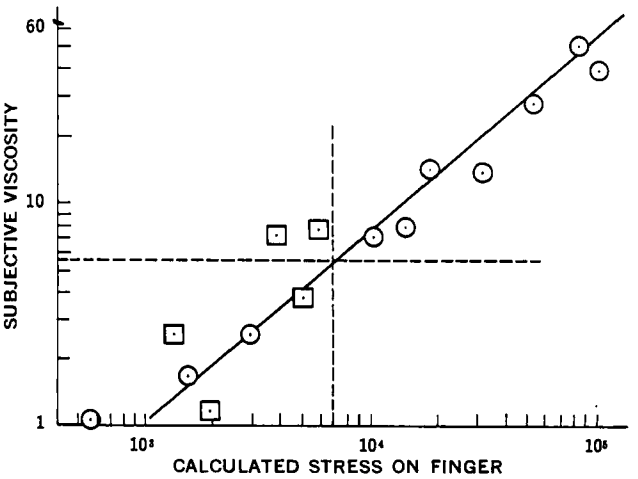

Figure 3—*Subjective viscosity* versus *shear stress. The best line through the data has a slope of 0.86 ● 0.02, compared with a predicted value of 1.00. The circles and squares are Newtonian and non-Newtonian fluids, respectively.*

Table II—Apparent Viscosity for 10 Newtonian and Six Non-Newtonian Fluids[a]

Fluid	m	n	h_0	Predicted Viscosity[b]	Subjective Viscosity
Silicone oil, 2.6 cps	0.026	1.0	0.015	0.56	1.0_6
Silicone oil, 20 cps	0.20	1.0	0.018	1.5	1.7_0
Silicone oil, 70 cps	0.70	1.0	0.040	2.9	2.6
Silicone oil, 936 cps	9.36	1.0	0.034	11.	7.2
Silicone oil, 1770 cps	17.7	1.0	0.046	15.	7.9
Silicone oil, 2805 cps	28.05	1.0	0.049	18.	14.2
Silicone oil, 8450 cps	84.50	1.0	0.055	32.	13.6
Silicone oil, 23,300 cps	233.0	1.0	0.072	54.	28.
Silicone oil, 51,500 cps	515.0	1.0	0.070	85.	51.
Silicone oil, 85,000 cps	850.0	1.0	0.068	110.	39.
Polyacrylamide, 2%	0.13	0.93	0.031	0.79	0.3_1
Polyacrylamide, 5%	28.	0.52	0.056	1.3	2.6
Polyacrylamide, 8%	390.	0.47	0.066	5.9	7.7
Hydroxypropyl methylcellulose, 400 cps, 1.5%	3.4	0.79	0.057	1.9	1.17
Hydroxypropyl methylcellulose, 8000 cps, 2%	93.	0.62	0.084	5.1	3.8
Hydroxypropyl methylcellulose, 15,000 cps, 2%	630.	0.32	.0.066	3.9	7.4

[a] Units used here are as follows: m, dynes sec^n cm^{-2}; predicted viscosities, 10^{-3} dynes cm^{-2}; and h_0, cm. Other quantities are dimensionless. [b] These predictions use four additional experimentally measured quantities: the average finger radius, R, of 0.75 cm; the average normal force, F_0, of 89,000 dynes; the average velocity, V_{visco}, of 3.1 cm sec^{-1}; and the average assessment time of 5 sec.

ferent spreadabilities. On the other hand, groups of non-Newtonian fluids can have the same spreadability when m and n differ. The only requirement is that the combination of m and n given by Eq. 14 results in the same predicted spreadability.

The results in Table I and Fig. 2 support this prediction. For example, silicone oil (936 cps) and silicone oil (1770 cps) are perceived subjectively as 33.0 and 23.0, respectively. However, the data confirm that a Newtonian fluid can show the same spreadability as a variety of non-Newtonian fluids with very different values of m and n. For example, silicone oil (70 cps), polyacrylamide (8%), and hydroxypropyl methylcellulose (8000 cps, 2%) all have about the same spreadability (58.0, 55.0, and 58.0, respectively), even though their rheological parameters are very different.

Viscosity—The data for subjective and predicted viscosity are shown in Table II. The first three columns give the rheological properties of the fluids in terms of Eq. 2. The initial film thickness, h_0, in the fourth column is not critical in the calculations; changes in these values by a factor of 2 have little effect on the results. The fifth column gives the predicted viscosity calculated from Eq. 16, using the values given in this table. The final column reports the subjective viscosity.

The theory predicts that a plot of the logarithm of subjective viscosity *versus* the logarithm of the right-hand side of Eq. 16 should be linear with a slope of 1. This predicted slope is in good agreement with the experimental value of 0.86 ± 0.02 (Fig. 3). The correlation coefficient between subjective viscosity and the calculated stress on the finger is 0.95. The agreement is not quite as close as that found for spreadability. However, in the derivation of the theory for viscosity, the major assumption was made that the periodic motion of the fingers can be approximated as a steady motion between parallel plates. In view of this approximation, the predictions of the theory are very good.

Other theoretical predictions for Newtonian fluids are tested in Fig. 4. The circles in this plot represent the logarithm of subjective viscosity plotted *versus* the logarithm of objective viscosity of the Newtonian fluid. The slope of the line drawn through these data is 0.40 ± 0.01, in good agreement both with other experimental studies of subjective viscosity (3) and with the value of 0.50 predicted by Eq. 17. Since the prediction requires only a measurement of Newtonian viscosity, additional properties like finger area and assessment time are constant and need not be measured experimentally.

These results and the others in this paper were unaffected by the temperature of the samples. For example, subjective Newtonian viscosity evaluated at skin temperature of 32° leads to an exponent of 0.41. This insensitivity is a consequence of ratio scaling. While viscosity changes significantly with temperature, the changes are similar percentages for all fluids used. Thus, the relative subjective viscosity measured by ratio scaling does not depend strongly on temperature.

The triangles in Fig. 4 represent the logarithm of subjective viscosity assessed at constant film thickness and plotted *versus* the

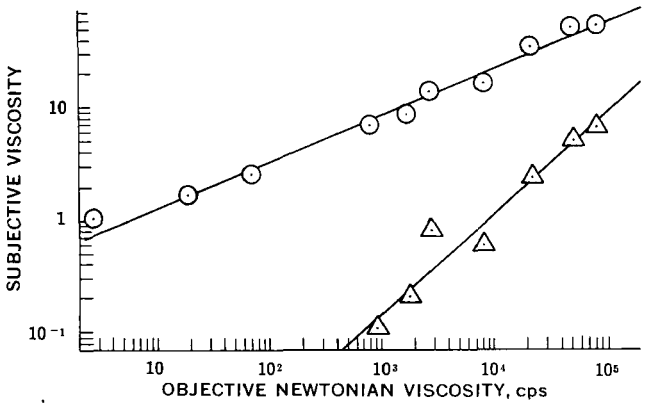

Figure 4—*Subjective viscosity* versus *objective Newtonian viscosity. The circles are values obtained when the thickness of fluid on the fingers varies during the assessments. The best line through these data has a slope of 0.40 ± 0.01, compared with 0.50 predicted by Eq. 17. The triangles represent assessments at constant fluid thickness; the best line through these values has a slope of 0.89 ± 0.06, compared with 1.00 predicted by Eq. 18.*

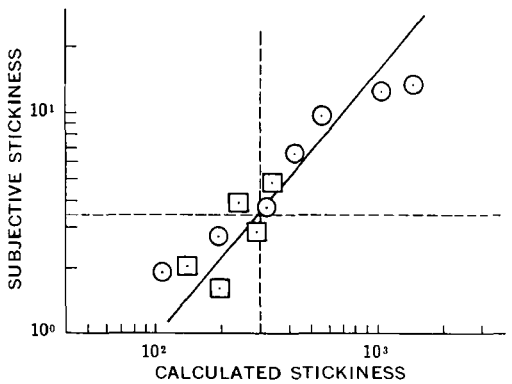

Figure 5—*Subjective stickiness* versus *stickiness calculated from Eq. 20. The best line through the data has a slope of 1.12 ± 0.08, compared with a predicted value of 1.00. The circles and squares are Newtonian and non-Newtonian fluids, respectively.*

Table III—Stickiness for Six Newtonian and Six Non-Newtonian Fluids[a]

Fluid	m	n	F_1	Calculated h_1[b]	Stickiness from Eq. 20[c]	Subjective Stickiness
Silicone oil, 936 cps	9.36	1.0	1.3	0.94	1.0_7	1.8_7
Silicone oil, 1770 cps	17.70	1.0	1.7	0.68	1.9_2	2.7
Silicone oil, 2805 cps	28.05	1.0	1.9	0.86	3.2	3.8
Silicone oil, 8450 cps	84.50	1.0	2.1	1.09	4.2	6.6
Silicone oil, 23,300 cps	233.0	1.0	2.7	1.29	5.7	9.8
Silicone oil, 51,500 cps	515.0	1.0	2.8	1.35	10.7	12.5
Silicone oil, 85,000 cps	850.0	1.0	3.4	1.35	15.0	13.2
Polyacrylamide, 2%	0.13	0.93	1.2	0.15	0.85	0.2_7
Polyacrylamide, 5%	28.	0.52	1.6	0.42	1.3_9	2.0
Polyacrylamide, 8%	390.	0.47	1.4	1.30	3.4	4.7
Hydroxypropyl methylcellulose, 400 cps, 1.5%	3.4	0.79	1.7	0.30	1.9_5	1.62
Hydroxypropyl methylcellulose, 8000 cps, 2%	93.	0.62	1.8	0.78	2.9	2.9
Hydroxypropyl methylcellulose, 15,000 cps, 2%	630.	0.32	2.1	1.30	2.4	3.9

[a] Units used here are as follows: m, dynes sec^n cm^{-2}; pulling force, F_2, 10^5 dynes; calculated h_1, 10^{-2} cm; and calculated stickiness, 10^2 sec cm^{-1}. Other quantities are dimensionless. [b] These predictions use four additional experimentally determined quantities: the average finger radius, R, of 0.75 cm; the average force thinning the film, F_0, of 89,000 dynes; the initial film thickness, h_0, given in Tables I and II; and the average time thinning the film of 3 sec. [c] These calculations use one additional experimentally estimated quantity: the average time for pulling up the finger, t_{stick}, of 0.1 sec.

logarithm of objective viscosity. These data were obtained by attaching to the finger a small sled with 0.06-cm runners, so that the finger touched the fluid but did not thin the fluid film. The best line through these data has a slope of 0.89 ± 0.06, in good agreement with the value of 1.00 predicted from Eq. 18. This slope is also consistent with earlier experiments showing that perceived force varies linearly with objective applied force (1). Both this result and the previous one illustrate how fluid mechanics, exemplified here by film thinning, influences the subjective perceptions.

Stickiness—The data for subjective and predicted stickiness are shown in Table III. The first three columns again give the fluid and its rheological properties. The final column gives the subjective stickiness as determined by ratio scaling.

The remaining data are connected with the calculations, which are more involved in this case than in spreadability and viscosity. The calculation has two main steps. First, one must find the film thickness, h_1, at which the film is thinnest. Because h_1 was not accurately measured experimentally, it must be calculated from Eq. 11. The key variable in this calculation is the time that the film is thinned by squeezing, which was found by experiment to be 3 sec. The second step is the calculation in Eq. 20, which depends on the minimum film thickness, h_1, calculated in the first step; the pulling force, F_1, measured experimentally; and the average time, t_{stick}, for pulling the finger upward. This time was estimated as 0.1 sec, but this estimation is not definitive.

The theory culminating in Eq. 20 predicts that a plot of the logarithm of subjective stickiness *versus* the logarithm of the right-hand side of Eq. 20 should have a slope of 1. This predicted slope is in good agreement with the experimentally determined slope of 1.12 ± 0.08 (Fig. 5). However, the agreement is not as good as that found in the two previous cases: the correlation coefficient between subjective and predicted stickiness is only 0.90. This poorer agreement is probably a consequence of the much more involved calculations.

Conclusions and Limitations—The picture emerging from this study has a beguiling symmetry. For attributes assessed by a shearing motion, such as spreadability and viscosity, the perception is of shear force with (approximately) constant velocity. For attributes assessed by a normal motion, like stickiness, the perception is proportional to time or velocity with (approximately) constant force. Calculations based on this picture give correlation coefficients of 0.90 or better between subjective and predicted assessments. These calculations are most definitive for Newtonian liquids, where no additional properties need be measured. They are also successful for spreadability, where the additional parameters involved are easily measured. The calculations are least successful for stickiness, where they are most complex.

However, the results given in this paper are limited by the fact that only the apparent viscosity of the fluids varies widely. This single variable is doubtless partially responsible for the well-defined conclusions that were reached. The effect of large changes in other physical properties may not be minor. For example, three physical properties that would alter the results are fluid elasticity, fluid finger wetting, and fluid thermal conductivity. The first of these properties, which requires a more complex rheological model than that in Eq. 3, is probably more important for subjective "slipperiness" than for the three attributes involved here (15). When studying elastic fluids, observers using ratio scaling frequently gave a value of zero to any nonelastic fluid, regardless of its viscosity.

Wetting between the fluid and the finger will strongly affect the total amount of fluid adhering to the finger and will change h_0 used in calculating both spreadability and viscosity. It also will affect H, the distance at which the finger separates from the fluid in the assessment of stickiness. Changes in fluid thermal conductivity are probably most important in the colloidal systems used in cosmetics, where the oil-in-water emulsion changes rapidly into a water-in-oil emulsion. This emulsion "break" causes a sudden 300% change in the heat lost by the finger. These effects should be much easier to predict than those of elasticity.

Because of these limitations, the results in this paper are a first step rather than an exhaustive picture. They allow the *a priori* prediction of some empirical exponents in Stevens' law. They give a firmer basis to the universal curves of equivalent texture (5–7). They suggest a rationale for ingredient substitutions and may aid in planning test panels, but they are certainly no substitute for these panels. Nevertheless, the results provide an intriguing indication that consumer reactions may be strongly influenced by physical restraints such as fluid mechanics.

NOMENCLATURE

F_0 = force used in thinning the film
F_1 = normal force developed in pulling the finger upward during assessment of stickness
h = thickness of the fluid sample at any time t
h_0 = initial thickness of the fluid sample
h_1 = minimum film thickness after squeezing during assessment of stickness
H = distance at which the finger separates from the fluid in assessment of stickiness
m,n = consistency and flow index in the power law model for non-Newtonian fluids
p = pressure
p_0 = atmospheric pressure
Q = volumetric flow per unit arc
R = radius of the finger
t = time
t_{spread}, t_{visco}, t_{stick} = times of assessments of spreadability, viscosity, and stickiness, respectively
v_x = velocity of flow in the x direction (Cartesian coordinates)

v_r = velocity of flow in the r direction (cylindrical coordinates)
V_{spread}, V_{visco} = average velocity with which the finger moves in assessment of spreadability and viscosity
μ = viscosity of Newtonian fluids
τ_{zx}, τ_{zr} = components of shear stress
τ_{spread}, τ_{visco} = stress exerted by the fluid on the finger during assessment of spreadability and apparent viscosity, respectively

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Electronic Spectra and Electronic Structures of Some Antimicrobials Derived from Proflavine

STEPHEN G. SCHULMAN [x], DATTA V. NAIK, ANTHONY C. CAPOMACCHIA, and TIMOTHY ROY

Abstract □ The shifts in the absorption and fluorescence spectra of 3-aminoacridine, proflavine, acridine orange, and acridine yellow were employed to show that the singly charged cations, the predominant species at biological pH, exist in the ground state in the amino form. In the lowest excited singlet state, however, the monocations of the diaminoacridines have the imino structure, a conclusion supported by the relative ground- and excited-state pKa values of the reactions of the monocation with H^+. The ground-state amino structure has its positive charge concentrated at the heterocyclic nitrogen atom, a fact that is of primary importance in determining the geometry of binding to DNA.

Keyphrases □ 3,6-Diaminoacridine derivatives—electronic spectra and electronic structures □ Proflavine derivatives—electronic spectra and electronic structures □ Antimicrobials—3,6-diaminoacridine derivatives, electronic spectra and electronic structures □ Electronic spectra and structure—diaminoacridine dyes, dependence upon solvent properties and state of protonation □ DNA—interaction with diaminoacridine dyes, electronic spectra and structure of dyes

Several dyes (acridine orange and acridine yellow) derived from proflavine (3,6-diaminoacridine) are employed as bacteriostatic agents, biological stains, and spectroscopic probes of the interactions of nucleic acids with small molecules (1). These substances are well known for their mutagenic effects arising from their interactions with nucleic acids (2). The singly charged cations, which are the prevalent prototropic species, at biological pH derived from proflavine (3,6-diaminoacridine), acridine orange [3,6-bis(dimethylamino)acridine], and acridine yellow (2,7-dimethyl-3,6-diaminoacridine) have been employed extensively in binding studies with nucleic acids (3). These dyes exhibit electronic absorption and fluorescence spectral changes upon binding to DNA.

The interactions of the diaminoacridines with DNA are generally believed to entail electrostatic association of the site of positive charge, in the singly protonated dye molecule, with a negatively charged phosphate ester linkage of the nucleic acid "backbone." In the case of proflavine, it has been shown that the remainder of the dye molecule is inserted between two base pairs of the double helix (or between individual bases in single-stranded nucleic acids), oriented toward the center of the helix and stabilized by hydrophobic interaction with the helix (2). There is, however, some disagreement as to whether the positive charge of the singly protonated diaminoacridine cations is predominately concentrated at the heterocyclic nitrogen atom of the acridine ring (the site of protonation) or at one of the amino nitrogen atoms. This argument arises from the possibility of writing the valence bond structures of the singly protonated cations of proflavine in either of two ways (I and II).

v_r = velocity of flow in the r direction (cylindrical coordinates)
V_{spread}, V_{visco} = average velocity with which the finger moves in assessment of spreadability and viscosity
μ = viscosity of Newtonian fluids
τ_{zx}, τ_{zr} = components of shear stress
τ_{spread}, τ_{visco} = stress exerted by the fluid on the finger during assessment of spreadability and apparent viscosity, respectively

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Electronic Spectra and Electronic Structures of Some Antimicrobials Derived from Proflavine

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Abstract □ The shifts in the absorption and fluorescence spectra of 3-aminoacridine, proflavine, acridine orange, and acridine yellow were employed to show that the singly charged cations, the predominant species at biological pH, exist in the ground state in the amino form. In the lowest excited singlet state, however, the monocations of the diaminoacridines have the imino structure, a conclusion supported by the relative ground- and excited-state pKa values of the reactions of the monocation with H^+. The ground-state amino structure has its positive charge concentrated at the heterocyclic nitrogen atom, a fact that is of primary importance in determining the geometry of binding to DNA.

Keyphrases □ 3,6-Diaminoacridine derivatives—electronic spectra and electronic structures □ Proflavine derivatives—electronic spectra and electronic structures □ Antimicrobials—3,6-diaminoacridine derivatives, electronic spectra and electronic structures □ Electronic spectra and structure—diaminoacridine dyes, dependence upon solvent properties and state of protonation □ DNA—interaction with diaminoacridine dyes, electronic spectra and structure of dyes

Several dyes (acridine orange and acridine yellow) derived from proflavine (3,6-diaminoacridine) are employed as bacteriostatic agents, biological stains, and spectroscopic probes of the interactions of nucleic acids with small molecules (1). These substances are well known for their mutagenic effects arising from their interactions with nucleic acids (2). The singly charged cations, which are the prevalent prototropic species, at biological pH derived from proflavine (3,6-diaminoacridine), acridine orange [3,6-bis(dimethylamino)acridine], and acridine yellow (2,7-dimethyl-3,6-diaminoacridine) have been employed extensively in binding studies with nucleic acids (3). These dyes exhibit electronic absorption and fluorescence spectral changes upon binding to DNA.

The interactions of the diaminoacridines with DNA are generally believed to entail electrostatic association of the site of positive charge, in the singly protonated dye molecule, with a negatively charged phosphate ester linkage of the nucleic acid "backbone." In the case of proflavine, it has been shown that the remainder of the dye molecule is inserted between two base pairs of the double helix (or between individual bases in single-stranded nucleic acids), oriented toward the center of the helix and stabilized by hydrophobic interaction with the helix (2). There is, however, some disagreement as to whether the positive charge of the singly protonated diaminoacridine cations is predominately concentrated at the heterocyclic nitrogen atom of the acridine ring (the site of protonation) or at one of the amino nitrogen atoms. This argument arises from the possibility of writing the valence bond structures of the singly protonated cations of proflavine in either of two ways (I and II).

Table I—Long Wavelength Absorption (λ_{1L_a} and λ_{1L_b}) and Fluorescence (λ_f) Maxima of 3-Aminoacridine and Several 3,6-Diaminoacridines in Various States of Protonation in Aqueous and Sulfuric Acid Media

Compound	Species[a]	λ_{1L_b}, nm	λ_{1L_a}, nm	λ_f, nm
3-Aminoacridine	D	353	402	480
	M	365	452	528
	N	353	408	522
3,6-Diaminoacridine (proflavine)	T	348	395	477
	D	361	458	535
	M	—	443	504
	N	—	393	514
3,6-Bis(dimethylamino)acridine (acridine orange)	T	351	395	480
	D	370	494	575
	M	—	490, 477	530
	N	—	435	535
2,7-Dimethyl-3,6-diaminoacridine (acridine yellow)	T	360	408	489
	D	372	460	532
	M	—	444	502
	N	—	394	498

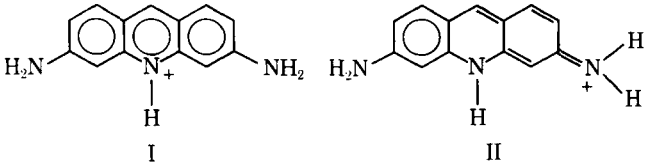
[a] T = triply charged cation, D = dication, M = monocation, and N = neutral species.

Consequently, two models for the geometry of the intercalative binding of proflavine-like dyes have been proposed: one with the heterocyclic nitrogen atom directed toward a phosphate ester group, leaving both exocyclic amino groups outside the helix, and one with an exocyclic nitrogen atom directed toward a phosphate ester group, having the other exocyclic group directed very nearly toward the center of the helix (3, 4). Löber (5) noted the similarity between the absorption and fluorescence spectra of singly protonated acridine orange and pyronine G, the xanthene analog of acridine orange, and suggested this similarity as evidence for the latter intercalation model. In pyronine G, the unprotonated molecule is singly charged and, because of the greater electronegativity of oxygen relative to nitrogen, Structure III is favored over Structure IV. Consequently, Structure V would, by spectral analogy, be favored over Structure VI for singly protonated acridine orange.

To elucidate the electronic structures of the diaminoacridine dyes and, ultimately, their interactions with DNA, the present study of the dependence of their electronic spectra upon solvent properties and state of protonation was undertaken.

EXPERIMENTAL

The pH measurements were made on a pH meter[1] with a combination silver–silver chloride glass electrode. Electronic absorption spectra were taken in 1-cm silica cells on a grating-type spectrophotometer[2]. Fluorescence measurements were taken on a fluorescence spectrophotometer[3] whose monochromators were calibrated against the line emission spectrum of xenon. Emission spectra were corrected for the wavelength response of the monochromators and phototube by means of a corrected spectra accessory employing a rhodamine-B quantum counter.



I

II

[1] Model 801, Orion Research, Inc., Cambridge, Mass.
[2] Model DB-GT, Beckman Instruments, Inc., Fullerton, Calif.
[3] Model MPF-2A, Perkin-Elmer Corp., Norwalk, Conn.

Table II—Long Wavelength Absorption (λ_{1L_a} and $\lambda^1_{L_b}$) and Fluorescence (λ_f) Maxima of 3-Aminoacridine and Several 3,6-Diaminoacridines in Various States of Protonation in Chloroform

Compound	Species[a]	λ_{1L_b}, nm	λ_{1L_a}, nm	λ_f, nm
3-Aminoacridine	M	368	462	530
	N	352	407	495
Proflavine	M	—	445	505
	N	—	397	497
Acridine orange	M	—	492	518
	N	—	432	498
Acridine yellow	M	—	446	503
	N	—	397	489

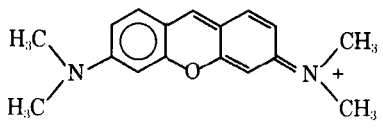
[a] M = monocation, and N = neutral species.

3-Aminoacridine was prepared by the deamination of proflavine according to the method of Martin and Tong (6). Proflavine[4], acridine orange[4], and acridine yellow[4] were purified by several recrystallizations of each from absolute ethanol.

Absorption and fluorescence spectra were taken on 1×10^{-5} *M* solutions of 3-aminoacridine, proflavine, and acridine yellow. However, because of the aggregation of acridine orange, which is appreciable at $\sim 10^{-5}$ *M*, the spectra of this compound were taken on 1×10^{-6} *M* solutions. The spectrophotometric titration procedures employed were described previously (7).

RESULTS

The electronic spectral maxima of 3-aminoacridine and of the diaminoacridines in various states of protonation, in aqueous and sulfuric acid media, are presented in Table I. The spectra of 3-aminoacridine are shown in Fig. 1. The spectral maxima of the neutral species and monocation derived from each compound, in chloroform, are presented in Table II. The pKa values (determined absorptiometrically) for the various prototropic reactions are listed in Table II. Also presented in Table II are the inflection points (pKa*) in the fluorometric titration curves corresponding to each measurable excited-state reaction.



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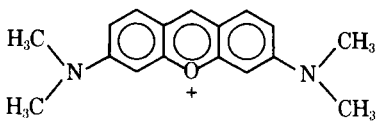

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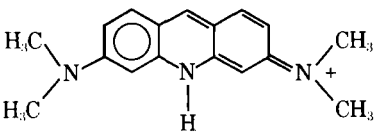

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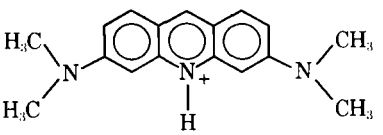


VI

[4] Matheson, Coleman and Bell, East Rutherford, N.J.

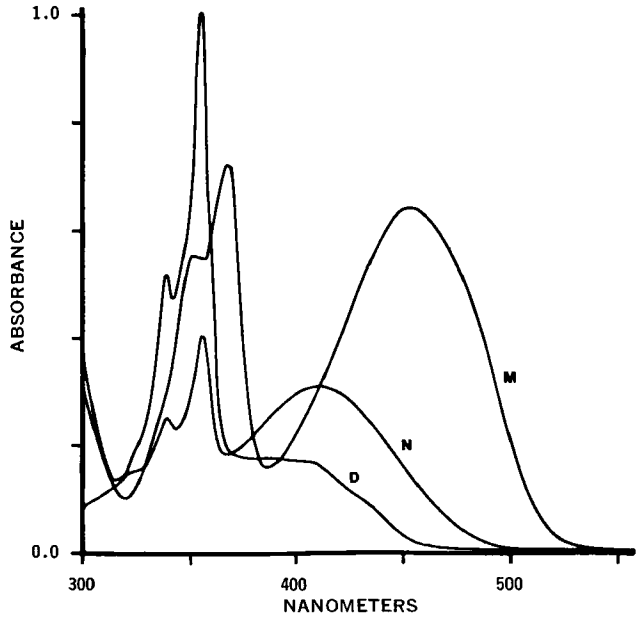


Figure 1—*Absorption spectra of 3-aminoacridine* ($\sim 5 \times 10^{-5}$ M). *Key: N, neutral; M, monocation; and D, dication.*

In each compound studied, only the neutral species ⇌ monocation interchange did not measurably occur during the lifetime of the excited state. The pKa* values for the trication ⇌ dication and dication ⇌ monocation excited-state reactions are believed to correspond very closely to the excited-state dissociation constants for these reactions.

DISCUSSION

The longest wavelength absorption bands of arylamines may be thought to originate from the transfer of electronic charge from the amino group to the vacant π^*-orbitals of the aromatic ring as a result of the interaction of the ground-state molecular electronic distribution with the electric vector of the exciting light (8). In the ground state, the aromatic ring is weakly conjugated with the amino group; but in the excited state the amino group is more extensively conjugated with the aromatic ring, resulting in stabilization of the excited state relative to the ground state. As a result, the longest wavelength absorption band of the aminoaromatic lies at a lower energy or longer wavelength than the longest wavelength absorption band of the parent hydrocarbon.

Protonation of the amino group removes the lone pair from conjugation with the aromatic ring in ground and excited states, resulting in an absorption spectrum very similar to that of the parent aromatic hydrocarbon but slightly displaced due to the polarizing influence of the protonated amino group. The second longest wavelength band of an arylamine originates from an essentially ring-localized transition and is relatively unaffected by protonation of the amino group (8). The fluorescence of an arylamine, which entails the spontaneous transfer of charge from the aromatic ring back to the amino group, lies at longer wavelengths than that of the parent hydrocarbon. However, the excited state from which fluorescence occurs and the ground state to which it occurs are somewhat different than the corresponding states involved in the longest wavelength absorption process.

The weakly conjugated amino group in the thermally equilibrated ground-state molecule is essentially tetrahedral in geometry. Because electronic transitions are somewhat faster than nuclear motions, the amino group in the electronically excited state, produced by absorption (the Franck–Condon excited state), is still predominately tetrahedral. However, subsequent to light absorption and prior to fluorescence, the amino group becomes planar (or nearly so) with respect to the aromatic ring to give the molecular configuration of lowest energy in accord with the highly conjugated excited amine. When fluorescence occurs, the light emission process is so rapid that the ground state in which the molecule initially arrives is thermally excited, retaining the planar amino group of the excited-state equilibrium geometry. Because the excited state involved in fluorescence is lower in energy than that involved in absorption and the ground state involved in fluorescence is higher than that involved in absorption, the fluorescence lies at longer wavelength than the longest wavelength absorption band. It can be concluded that, although both the absorption and fluorescence processes in question involve transition between the ground and first excited singlet states, the absorption process reflects the ground-state molecular structure while the fluorescence process reflects the excited-state atomic configuration.

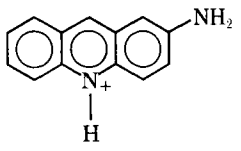
In nitrogen heterocycles such as acridine, the introduction of amino groups produces spectral effects similar to those just discussed but with some qualitative differences. The nitrogen atom is more electronegative than carbon and, therefore, exerts an attractive influence on the π-electron system of the aromatic ring as well as on the lone pair of an amino substituent (9). Because the excited states of aromatic rings and arylamines are generally more polar than their ground states, the attractive or stabilizing influence of the nitrogen atom is generally greater upon the excited state than upon the ground state. The result is that the longest wavelength absorption and fluorescence bands of *N*-heterocyclic amines lie at longer wavelengths than in the corresponding carbocyclic systems. This effect is much more pronounced in ring-protonated *N*-heterocyclic amines, in which the ring-protonated nitrogen is even more electronegative.

In aminoacridines, the longest wavelength absorption and fluorescence bands [the ^1L_a bands in the Klevens and Platt (10) nomenclature system] are extremely sensitive in position to the state of protonation of the heterocyclic nitrogen atom. The second absorption band (^1L_b) of an aminoacridine, however, is much less sensitive to protonation of the heterocyclic nitrogen atom and is indicative of the acridine ring system, lying at ~350–370 nm in monosubstituted acridines.

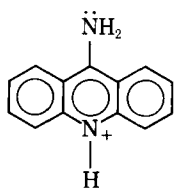
At this point, it is desirable to distinguish between two types of ring-protonated amino *N*-heterocycles: Type 1, those for which conjugation of the amino group with the aromatic ring cannot be represented by low energy canonical structures, *e.g.*, 2-aminoacridinium ion (VII); and Type 2, those for which conjugation of the amino group with the aromatic ring can be depicted by a formal low energy valence-bond representation, *e.g.*, 9-aminoacridinium ion (VIII and IX).

Protonated *N*-heterocyclic amines of the first type are weakly conjugated in the ground state because of orbital occupancy restrictions. However, they are substantially conjugated in the lowest excited state because the π^*-orbitals, which become occupied in the excited state, provide an electronic pathway between the amino group and the aromatic ring (7). Therefore, these compounds are characterized by spectroscopic behavior of the kind discussed up to this point. Moreover, the dissociation constants of these species are typical of the class, having pKa ~5–6 and pKa ~0–1 for dissociation from the ring nitrogen atom and for protonation of the amino group, respectively (7).

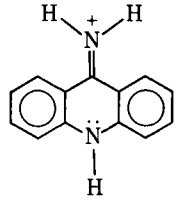
The second type of protonated *N*-heterocyclic amines may be extensively conjugated in the ground state because the canonical structure alternative to the protonated *N*-heterocyclic amine (the iminium ion) provides a low energy electronic distribution, which does not violate the restrictions imposed by the Pauli exclusion principle (7). The iminium structure may be regarded as the ex-



VII



VIII



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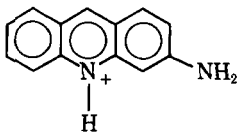
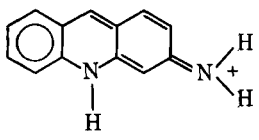


X



XI

treme limit of conjugation of the amino group with the protonated heterocyclic ring. However, in such a structure, it is clearly impossible for light absorption to transfer charge from the exocyclic group to the ring. Rather, because of the positive charge on the exocyclic group, the low energy π^*-orbital associated with this group (similar to a carbonyl group), and the lone pair localized on the heterocyclic nitrogen atom, absorption entails transfer of charge from the ring to the exocyclic group so that conjugation of the exocyclic group is actually less extensive in the lowest excited singlet state than in the ground state.

In this extreme situation, the ground state would be relatively more stabilized by the presence of the amino group and, as in the case of the 9-aminoacridinium ion, the ^1L_a absorption and fluorescence spectra would be expected to lie at shorter wavelengths than those of the acridinium cation (7). Moreover, the loss of aromaticity in the ground state should, as in the case of the 9-aminoacridinium monocation, shift the ^1L_b band (characteristic of aromatic ring size) to shorter wavelengths.

Dissociation or protonation of the monocation restores the aminoacridine or ammonium acridine structure, and either process thus shifts the spectra to longer wavelengths. The pKa values of these compounds also reflect the loss of positive charge at the ring nitrogen atom and the gain of positive charge at the exocyclic nitrogen atom in the ground state. For example, in 9-aminoacridinium monocation, pKa = 10.0 for the heterocyclic nitrogen atom and −8.5 (7) for the exocyclic nitrogen atom. Moreover, the dissociation constants of the excited species of the Type I ring-protonated heterocyclic amines are more basic at the ring nitrogen atom and less basic at the exocyclic nitrogen atom while those of the Type 2 compounds are in the opposite order, in accord with the opposite directions of flow of electronic charge in either type of ring-protonated heterocyclic amine (7).

As previously mentioned, the 9-aminoacridinium ion and the 2-aminoacridinium ion represent two extremes of conjugative behavior in ring-protonated *N*-heterocyclic arylamines. In the 9-aminoacridinium cation, the 9-iminoacridan cation form predominates because it is not only allowed electronically but also is electrostatically favored by the proximity between the heterocyclic and exocyclic nitrogen atoms as well as by the inherently low aromatic stability of the central ring of anthracene and acridine derivatives. However, in certain ring-protonated aminoacridines, such as 3-aminoacridine, the amino group is considerably more separated from the heterocyclic function so that the polarization of the amino lone pair of X to form XI is a weaker interaction than in the 9-aminoacridinium cation.

Moreover, the formation of XI from X entails the disruption of two aromatic rings, including one that is normally stabilized by an aromatic sextet, and is, therefore, less favorable electronically than the corresponding process in the 9-aminoacridinium cation. Under such circumstances, it is to be expected that the electronic distribution in the 3-aminoacridinium ion would be intermediate between Structures X and XI. In this case, the long wavelength electronic absorption band of the 3-aminoacridinium ion could entail transfer of electronic charge away from the amino group and toward the heterocyclic function if the actual structure is closer to the amino form (X), in the ground state, or transfer of electronic charge toward the imino group and away from the heterocyclic function if the structure is closer to that of the imino form (XI). If X more accurately represents the structure of the 3-aminoacridinium monocation, the long wavelength absorption band should shift to shorter wavelengths upon dissociation from the heterocyclic group or protonation of the amino group while the corresponding prototropic processes should result in absorption spectral shifts to longer wavelengths if XI is closest to the actual structure.

Similar arguments are applicable to the shifting of the fluorescence spectra of the 3-aminoacridinium monocation upon protonation or dissociation. However, because of the conformational relaxation that takes place in the excited state subsequent to absorption and prior to emission, it is possible that the electronic charge

Table III—Dissociation Constants for the Prototropic Equilibria in the Ground Electronic State (pKa) and in the Lowest Excited Singlet State (pKa*) (Where Measurable by Fluorometric pH Titrimetry) of 3-Aminoacridine and of the 3,6-Diaminoacridines[a]

Compound	$T \rightleftharpoons D + H^+$	$D \rightleftharpoons M + H^+$	$M \rightleftharpoons N + H^+$
3-Aminoacridine			
pKa	—	−1.4	8.0
pKa*	—	−5.6	
Proflavine			
pKa	−2.7	0.3	9.5
pKa*	−9.0	1.6	
Acridine orange			
pKa	−3.3	0.2	10.1
pKa*	−7.6	1.1	
Acridine yellow			
pKa	−3.0	0.5	8.9
pKa*	−7.3	1.5	

[a] T = triply charged cation, D = dication, M = monocation, and N = neutral species.

redistribution produced by going from the ground state to the lowest excited singlet state would convert the predominately amino-type ground-state monocation X into the predominately imino-type excited-state monocation (XI) or vice versa. The result of such an interaction would be that protonation and dissociation of the monocation would produce absorption and fluorescence shifts in opposite directions.

In 3-aminoacridine, the shifts of the ^1L_a absorption and fluorescence bands of the monocation upon dissociation to the neutral species or protonation to the dication, the presence of a well-defined ^1L_b band (between 350 and 370 nm) in all species, and the greater acidity of the dication in the lowest excited singlet state relative to the ground state all indicate that the monocation of 3-aminoacridine is predominately aminoacridine-like in ground and lowest excited singlet states (X).

In proflavine, the presence of the second amino group in the monocation results in a somewhat more complicated picture. The triply and doubly charged cations derived from proflavine are similar in electronic structure to the dication and monocation, respectively, derived from 3-aminoacridine. Because of the absence of lone pairs on both amino groups, the spectra of the trication of proflavine are similar to those of an unsubstituted acridinium ion (the iminium structure is impossible in the trication). Dissociation of one amino group to form the dication frees one lone pair for the interaction with the aromatic system. Because of the positive influence of the remaining protonated amino group, the charge-transfer interaction between the free amino group and the heterocyclic ring is enhanced, with the greatest stabilization effected on the excited state because of its great charge-transfer character. As a result, the ^1L_a and fluorescence bands of the dication lie at slightly longer wavelengths than those of the monocation of 3-aminoacridine (because of the lack of the $-NH_3^+$ group in the latter) as well as at much longer wavelengths than the corresponding spectra of the trication.

This behavior is anticipated for the spectrum of the amino form of the dication and is supported by the occurrence of the ^1L_b band typical of an acridine ring as well as by the fact that the free amino group is less basic in the first excited singlet state than in the ground state. If the imino form predominated, the loss of aromaticity in the acridine ring should have shifted the ^1L_b, ^1L_a, and fluorescence bands to shorter wavelengths while the imino structure of the exocyclic group should have resulted in enhanced basicity in the lowest excited singlet state.

In the monocation derived from proflavine, the ^1L_a absorption and fluorescence bands lie at wavelengths shorter than those of the corresponding spectral bands in both the monocation of 3-aminoacridine and the dication of proflavine. Moreover, the ^1L_b band in the proflavine monocation is not visible. The lack of appearance of the ^1L_b band is not in itself indicative, because this band could be extremely weak in intensity and buried under the tailing of the extremely intense ^1L_a band in the monocation of proflavine. However, the shifting of the ^1L_a absorption and fluorescence bands and the fact that pKa* > pKa for the dication–monocation intercon-

version suggest that at least the excited monocation is in the imino form (II).

Upon dissociation of the monocation of proflavine (from the heterocyclic nitrogen atom) to form the neutral species, the ^1L_a absorption band shifts to a shorter wavelength by an amount comparable to the corresponding shift in 3-aminoacridine. However, the fluorescence band of the monocation moves to a slightly longer wavelength upon dissociation from the heterocyclic nitrogen atom. These results indicate that, in the ground state, the monocation is predominately in the amino form (as 3-aminoacridine) and support the conclusion that in the lowest excited singlet state the monocation fluoresces as the imino valence tautomer. The neutral molecule is, of course, amine-like in ground and excited states, since an imino form in this species would be an inherently unstable zwitterion with positive and negative charges both borne by nitrogen atoms (*i.e.*, atoms of comparable electronegativity).

The small shift of the ^1L_a band of the dication to shorter wavelengths upon dissociation to the monocation can be rationalized in terms of the ground-state amino structures of both the dication and monocation as follows. Since the ^1L_a band arises from the promotion of an amino group lone pair electron to a π^*-orbital of the acridinium ring, the release of an additional lone pair of comparable energy, by dissociating the dication to the monocation, does not have much effect upon the orbital aspect of the transition. However, while the positive charge of the ammonium group of the dication enhances charge transfer in the excited state and thereby lowers the energy of the transition relative to that in the 3-aminoacridine monocation, the extra lone pair in the proflavine monocation causes greater electron repulsion in the charge-transfer excited state and thereby destabilizes the excited state with respect to the ground state. Hence, the ^1L_a band of the proflavine monocation lies at a shorter wavelength than that of the proflavine dication or the monocation of 3-aminoacridine, while the ^1L_a band of the neutral proflavine lies at a shorter wavelength than that of neutral 3-aminoacridine.

The spectroscopic and prototropic behavior of acridine orange is comparable to that of proflavine. However, the 2,7-dimethyl-substituted proflavine, acridine yellow, behaves differently in one way. The fluorescence of the neutral molecule is at slightly shorter wavelength than that of the monocation. However, the excited-state pKa* of the dication–monocation interconversion is more basic than the corresponding ground-state pKa, indicating that the imino structure of the monocation is in the excited state. The anomalous fluorescence shift observed upon going from the monocation to the neutral molecule in acridine yellow appears to be due to greater steric inhibition, by the methyl groups in the 2- and 7-positions, of conjugation in the excited state of the neutral species, resulting in an anomalously short fluorescence wavelength in this species. The apparently greater sensitivity to solvent polarity or hydrogen-bonding capacity of the fluorescences of the neutral proflavine and acridine orange molecules relative to that of the corresponding monocations also reverses the order of the fluorescence maxima of proflavine and acridine orange, although the amino and imino structures of the neutral molecules and monocations are, presumably, preserved in chloroform (Table II).

In light of the results of these experiments, it may be predicted that proflavine, acridine orange, and acridine yellow bind in their monocation forms to DNA in such a way that the positively charged heterocyclic nitrogen atom is oriented toward a phosphate ester group. However, upon excitation, the sites of predominant positive charge in the monocations shift to the exocyclic nitrogen atoms. Thus, the excited dye molecules bound to DNA will generally not be in the binding configuration of lowest electrostatic energy. They will tend to reorient themselves as best they can, before fluorescing, to achieve the lowest energy configuration. Although translational motions of the bound entities are severely restricted, the lifetime of a typical fluorescent excited state is more than adequate to allow for rotational and vibrational rearrangements. Consequently, electronic absorption spectroscopy appears to be a very powerful and readily available tool for the probing of drug–receptor interactions. However, much more work remains to be done before fluorescence spectroscopy can enjoy the same interpretive versatility.

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Effect of Surfactants on Absorption through Membranes III: Effects of Dioctyl Sodium Sulfosuccinate and Poloxalene on Absorption of a Poorly Absorbable Drug, Phenolsulfonphthalein, in Rats

SHAH N. MALIK, DONALD H. CANAHAM, and M. WAFIK GOUDA ˣ

Abstract □ The influence of dioctyl sodium sulfosuccinate and poloxalene on the GI absorption of phenolsulfonphthalein in the rat was studied. Urinary excretion data after oral administration of the drug to intact rats and loss of the drug from the whole small intestine as a loop were both utilized to assess the effect of the surfactants on absorption. Dioctyl sodium sulfosuccinate markedly increased the absorption of the drug, and the extent was dependent on the surfactant concentration. Maximum effect was observed at the reported ED_{50}, in rats, of the surfactant as a fecal softener. The mechanism responsible for absorption enhancement seems to be an alteration of the permeability of the intestinal membrane. Micellar complexation between the drug and the surfactant resulted in a lesser increase in absorption at the higher surfactant concentrations. Poloxalene did not increase drug absorption, but higher concentrations caused a decrease in absorption due to micellar entrapments of the drug molecules. The influence of dioctyl sodium sulfosuccinate on the peritoneal absorption of the drug was also investigated. Lower doses of the surfactant increased absorption of the drug by altering the membrane permeability. Higher doses decreased absorption due to unavailability of the drug molecules entrapped in the micelles.

Keyphrases □ Surfactants—effects of dioctyl sodium sulfosuccinate and poloxalene on absorption of phenolsulfonphthalein in rat □ Dioctyl sodium sulfosuccinate—effect on absorption of phenolsulfonphthalein in rat, urinary excretion and *in situ* intestinal loop data □ Poloxalene—effect on absorption of phenolsulfonphthalein in rat, urinary excretion and *in situ* intestinal loop data □ Absorption—effects of dioctyl sodium sulfosuccinate and poloxalene, rats

Dioctyl sodium sulfosuccinate USP (I) and poloxalene[1] are two surfactants used medicinally as fecal softeners. It was suspected that the presence of I in combination with oxyphenisatin acetate, a nonabsorbable peristaltic stimulant, resulted in promotion of intestinal absorption of oxyphenisatin and, therefore, caused jaundice in several patients (1, 2). A systematic study of the effect of these surfactants on the absorption of various drugs was undertaken. A previous report (3) showed that both surfactants enhanced the absorption of pentobarbital, a readily absorbable drug, through the goldfish membrane. Dioctyl sodium sulfosuccinate was also found to change the rate of absorption of pentobarbital from the rat intestine (4).

The purpose of the present investigation was to study the effect of both surfactants on the GI absorption of a poorly absorbable drug, phenolsulfonphthalein (phenol red), in the rat.

Although Lish and Weikel (5) reported that I enhances the absorption of phenolsulfonphthalein from the rat colon while poloxalene does not, these investigators used only one concentration of the surfactants. Since the action of surfactants on drug absorption is known to be concentration dependent, and since more recent reports (6) have shown that phenolsulfonphthalein, like most other drugs, is absorbed mainly from the upper GI tract, a more detailed study of the effect of various concentrations of the surfactants on the absorption of phenolsulfonphthalein from the intact rat and from *in situ* intestinal loops was deemed appropriate. The effect of I on the peritoneal absorption of phenolsulfonphthalein was also examined.

EXPERIMENTAL

Poloxalene, dioctyl sodium sulfosuccinate[2], and phenolsulfonphthalein[3] were all used as received. All other chemicals were either USP or reagent grade.

Absorption Studies in Intact Rats—Male Sprague–Dawley rats, 200–380 g, were starved for 18–20 hr with water allowed *ad libitum*. The experiments were performed in a crossover fashion with each rat serving as its own control. About half the number of animals received a control dose of 2 ml of phenolsulfonphthalein solution (0.75 mg/ml) in distilled water by gastric intubation. The other half received an equal volume of the same solution in distilled water containing one concentration of the surfactants studied. After a recovery period of 3 days, the procedure was reversed.

Urine was collected for 24 hr, and the amount of phenolsulfonphthalein excreted was determined spectrophotometrically at 560 nm following the method of Feldman and Gibaldi (7). The percent of the drug absorbed was calculated by means of a standard curve.

In a second set of experiments, the test animals were injected intraperitoneally in a crossover fashion with 0.5 ml of phenolsulfonphthalein solution (0.75 mg/ml) in distilled water or an equal volume of the same solution in distilled water containing one concentration of dioctyl sodium sulfosuccinate. The amount of the drug excreted in the urine was determined as already described, and the percent absorbed in the presence of various concentrations of the surfactant was calculated.

Absorption Studies from *In Situ* Intestinal Loops—Fasted Sprague–Dawley rats, 200–300 g, were used. The degree of intestinal absorption was measured using *in situ* single loop preparations (8, 9). The loop used was whole small intestine. The animals were anesthetized with ether, and a midline incision was made to expose the small intestine. Ligatures were placed about 1 cm distal to the pyloric junction and 1 cm proximal to the ileocecal junction. Care was taken not to occlude major blood vessels. Physiological saline was injected into the loop until the washings became clear.

The rats were kept under light anesthesia for 20–25 min, before closing the ileocecal end, to allow for absorption of any residual solution. Five milliliters of phenolsulfonphthalein solution (0.75 mg/ml) in distilled water or in various solutions of the surfactants was introduced into the intestines through the pyloric ligature by means of a syringe and a blunt needle. The midline incision was then closed and the animals were allowed to recover. The animals

[1] Ethylene oxide–propylene oxide polymer of average molecular weight 8350, obtained as Pluronic F-68, Wyandotte Chemicals Corp., Wyandotte, Mich.

[2] Aerosol O.T. 100%, Sargent-Welch Scientific Co.

[3] Analytical grade, J. T. Baker.

Table I—Total Urinary Excretion of Phenolsulfonphthalein after Oral Administration of 1.5-mg Dose with and without Dioctyl Sodium Sulfosuccinate[a]

Surfactant Dose, mg	Dose Absorbed, % ± *SD*
Control	4.8 ± 0.7
10	8.4 ± 0.9
Control	5.4 ± 1.0
20	11.1 ± 3.0
Control	4.5 ± 0.5
30	11.0 ± 1.7
Control	4.1 ± 0.7
40	8.2 ± 1.7

[a] Values represent mean of nine animals.

were sacrificed after 3 hr; the whole small intestine was removed, washed with saline to remove any blood adhering to its surface, homogenized, and assayed for drug content according to Lish and Weikel (5).

The percent of phenolsulfonphthalein absorbed was calculated by measuring the difference between the amount introduced into the loop and the amount recovered from the intestinal homogenate.

Solubility Studies—Equilibrium solubility was employed to determine the extent and the nature of possible interactions between the drug and the surfactants. Excess amounts of powdered phenolsulfonphthalein were placed in 25-ml ampuls with 10-ml solutions containing various surfactant concentrations. The ampuls were sealed and then rotated in a constant-temperature water bath at 37 ± 0.1°. After equilibrium was achieved, an aliquot was filtered through 0.45-μm filters[4]; the amount of the drug in solution (adjusted to pH 10 with sodium hydroxide) was determined spectrophotometrically at 560 nm.

Partition Studies—Solutions of phenolsulfonphthalein (0.05%) were prepared in a phosphate buffer (0.01 *M*, pH 6.0) or in the same buffer containing various concentrations of I. Ten milliliters of these solutions was added to an equal volume of *n*-octanol, previously saturated with the buffer solution, in a 50-ml erlenmeyer flask. The contents were equilibrated at 37 ± 0.1° for 2 days. After separation by centrifugation (at 37°), an aliquot of the aqueous phase was assayed spectrophotometrically for its drug content. The partition coefficient was calculated by difference. The organic phase, *n*-octanol, was chosen on the basis of its favorable nonpolar lipid character (10, 11). The pH of the solutions simulates the average pH of the small intestine.

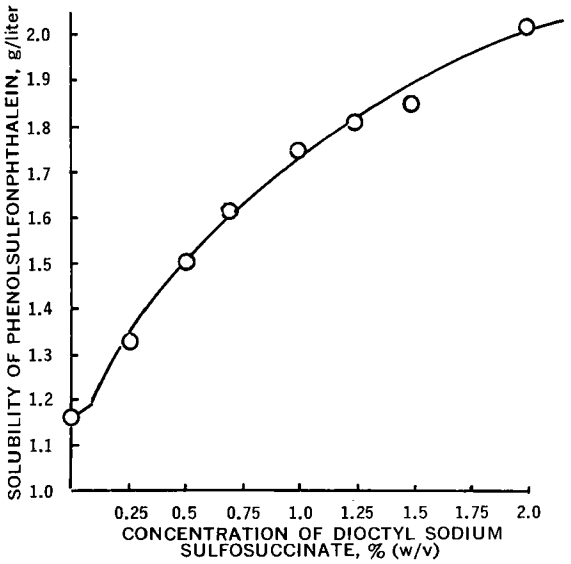

Figure 1—*Effect of dioctyl sodium sulfosuccinate on the equilibrium solubility of phenolsulfonphthalein at 37°.*

[4] Millipore.

Table II—Effect of Dioctyl Sodium Sulfosuccinate on the Absorption of Phenolsulfonphthalein from Rat Intestinal Loops[a]

Surfactant Concentration, % (w/v)	Dose Absorbed, % ± *SD*
Control	4.5 ± 0.4
0.25	17.5 ± 5.8
0.5	54.0 ± 20.0
1.0	73.6 ± 16.1
1.5	50.7 ± 11.4

[a] Values represent mean of at least six loops.

RESULTS AND DISCUSSION

Phenolsulfonphthalein is a poorly absorbable, weak acid (pKa 7.9). The poor absorption of the drug from the small intestine is attributed to the low lipid–water partition coefficient of the unionized form, while the poor absorption from the stomach can be attributed to the opening of the sulfolactone ring at low pH (≤2) and the generation of the benzenesulfonic acid group that becomes ionized at the stomach pH (11). Intestinal perfusion studies in rats have shown that phenolsulfonphthalein is equally absorbed in the proximal and distal regions of the small intestine (12). At the concentration employed in this study (0.75 mg/ml), phenolsulfonphthalein absorption from the rat intestinal tract occurs mainly by passive diffusion (13).

Effect of Dioctyl Sodium Sulfosuccinate on GI Absorption of Phenolsulfonphthalein—Table I shows the urinary recovery of phenolsulfonphthalein as the percent of dose after oral administration of 2 ml of a 0.75-mg/ml solution in the presence or absence of various concentrations of I. The percent of the drug absorbed in control animals was, on the average, about 5% of the dose administered. All doses of the surfactant significantly increased drug absorption. Differences between absorption from solutions containing I and control solutions are significant at $p < 0.001$ as determined by the Student *t* test for paired data.

The effect of I on the absorption of phenolsulfonphthalein from the intact rat was dependent on the amount of the surfactant administered. Maximum effect was observed at the dose level of 20–30 mg, corresponding to a 1–1.5% surfactant concentration in the administered solution. The dose that caused the maximum increase in drug absorption was approximately equal to 100 mg/kg, the ED_{50} reported for the fecal-hydrating effect of the surfactant (14).

The enhancement of phenolsulfonphthalein absorption in the intact rat due to coadministration of the surfactant could be the result of one or more mechanisms. For example, dioctyl sodium sulfosuccinate was reported to retard gastric emptying when administered intraduodenally (15). Such a delay in the GI transit rate could conceivably result in increased absorption of phenolsulfonphthalein when the two drugs are administered together. Other possible mechanisms include a possible interaction between the surfactant and the drug and/or a direct effect of the surfactant on the GI membrane with a resultant change in drug absorption.

Therefore, it was of interest to consider the absorption of the drug from a well-defined segment of the GI tract. The whole intestine as a loop is well suited for this purpose. This technique allows the study of the surfactant effect without the complicating problem of gastric emptying and permits a better control of the concentration of the surfactant at the absorption site. In addition, it was of interest to determine if *in situ* techniques correlate with absorption studies from intact animals.

The results obtained from intestinal loop experiments are presented in Table II. A significant increase in the absorption of phenolsulfonphthalein in the presence of the surfactant was observed. Differences between absorption from solutions containing I and control solutions were significant at $p < 0.001$ as determined by the Student *t* test for unpaired data. The intestinal loop experiments also indicated a dose-dependent effect of the surfactant on the intestinal absorption of phenolsulfonphthalein. Although there were quantitative differences in the extent of absorption between intestinal loops and intact rat experiments, the activity of the surfactant followed more or less the same pattern. These quantitative

Table III—Apparent Partition Coefficients for Phenolsulfonphthalein (0.05%) in the Presence of Various Concentrations of Dioctyl Sodium Sulfosuccinate

Surfactant Concentration, % (w/v)	Apparent Partition Coefficient (*n*-Octanol–Water)
Control	0.09
0.01	0.08
0.1	0.08
0.25	0.07
0.5	0.08
1.0	0.07
1.5	0.08

differences are to be expected in view of the differences in the effective concentration of both the drug and the surfactant at the absorption site and the differences in techniques. Nevertheless, the intestinal loop experiments proved to be in good qualitative correlation with intact animal absorption studies.

The results of the absorption studies from intestinal loops suggest that other factors besides delay in gastric emptying are mainly responsible for absorption enhancement. A significant change in the ability of the drug to permeate the GI membrane may result from an interaction between phenolsulfonphthalein and the surfactant to form a complex with a higher affinity for the membrane. The magnitude of interaction of the drug with the surfactant was determined by equilibrium solubility studies. Figure 1 shows that the solubility of the drug increases above the critical micelle concentration (CMC) of the surfactant, indicating micellar complexation.

The possibility that a complex of the drug and the surfactant has better affinity for lipoidal membranes was investigated by partition studies (Table III). Both premicellar and postmicellar concentrations of the surfactant did not increase the partitioning of phenolsulfonphthalein between *n*-octanol and water.

On the basis of the absorption, solubility, and partition studies, it is suggested that the enhancement of phenolsulfonphthalein absorption represents the sum of two main effects: modification of the permeability of the membrane and micellar complexation of the drug. The membrane effect of the surfactant is believed to increase absorption, while the micellar entrapment of the drug molecules usually decreases absorption. The results of the absorption studies indicate that the membrane effect is predominant. However, at higher concentrations of the surfactant (higher doses), the micellar entrapment results in a lesser increase in absorption. Several reports (4, 8, 16–20) attributed the absorption enhancement effect of various surfactants to a direct effect on the biological membrane.

The absorption studies in intact rats indicated that any effect of the surfactant on membrane permeability was reversible. Urinary excretion data obtained from control studies in rats, 3 days after each had received the surfactant, were essentially identical to those obtained from control studies in rats with no previous exposure to the surfactant.

Table IV—Total Urinary Excretion of Phenolsulfonphthalein after Intraperitoneal Administration of 0.375-mg Dose with and without Dioctyl Sodium Sulfosuccinate[a]

Surfactant Dose, mg	Dose Absorbed, % ± *SD*
Control	60.4 ± 6.8
0.05	68.8 ± 3.4
Control	51.8 ± 7.2
0.25	60.9 ± 4.6
Control	50.4 ± 4.0
0.5	55.6 ± 3.4
Control	49.0 ± 6.8
2.5	40.2 ± 8.6
Control	56.0 ± 4.5
5.0	35.4 ± 6.7
Control	57.0 ± 4.9
7.5	29.1 ± 6.1

[a] Values represent mean of five animals.

Table V—Total Urinary Excretion of Phenolsulfonphthalein after Oral Administration of 1.5-mg Dose with and without Poloxalene[a]

Surfactant Dose, mg	Dose Absorbed, % ± *SD*
Control	5.6 ± 1.6
10	5.6 ± 1.3
Control	4.5 ± 0.9
30	4.3 ± 0.8
Control	5.3 ± 1.2
50	5.3 ± 0.6
Control	5.0 ± 1.0
100	3.7 ± 1.0

[a] Values represent mean of at least five animals.

Effect of Dioctyl Sodium Sulfosuccinate on Peritoneal Absorption of Phenolsulfonphthalein—In view of the finding that the surfactant could change the permeability of the GI membrane, it was of interest to determine whether it has a similar effect on the peritoneal membrane. The results are shown in Table IV. The concomitant administration of the surfactant with the drug resulted in a small but significant ($p < 0.01$ as determined by the Student *t* test for paired data) increase in the drug absorption up to a 0.5-mg dose (corresponding to a 0.1% surfactant concentration). Above this dose, the drug absorption was significantly decreased. The effect of the surfactant on the peritoneal absorption of the drug was qualitatively similar to its effect on the GI absorption. The extent in increase in absorption, however, was less in the case of peritoneal absorption, and a decrease in absorption was observed at higher surfactant concentrations.

The mechanism by which the surfactant changes the peritoneal absorption of the drug seems to be basically similar to the one observed in the GI absorption studies. At lower surfactant concentration (lower dose), there was a potentiation of absorption through a direct effect on the membrane. At higher concentrations, the drug was entrapped in the micelles and was not readily available so a decrease in absorption was observed. The effect of dioctyl sodium sulfosuccinate on the peritoneal membrane and the resultant change in permeability reported in this study are in agreement with the findings of Mattocks and Penzotti (21). These investigators reported that the same surfactant accelerated peritoneal dialysis of both urea and sodium phosphate, and they attributed its effect to direct action on the structure of the mesentary membrane by removal of phospholipids.

Effect of Poloxalene on GI Absorption of Phenolsulfonphthalein—The total urinary excretion of the drug in the presence and absence of the surfactant is shown in Table V. No increase in absorption was noticed with the lower doses of the surfactant, while a significant decrease ($p < 0.05$) was observed at higher doses. The results of the intestinal loop experiments are presented in Table VI. Again, the same pattern of surfactant activity was observed. There was no significant change at lower concentrations, but a significant decrease in absorption ($p < 0.001$) was observed at higher concentrations.

The failure of poloxalene to increase drug absorption from the GI tract of the rat is in contrast to the absorption-enhancing effect of similar concentrations of the surfactant of pentobarbital in the goldfish (3). This nonionic surfactant apparently can change the

Table VI—Effect of Poloxalene on the Absorption of Phenolsulfonphthalein from Rat Intestinal Loops[a]

Surfactant Concentration, % (w/v)	Dose Absorbed, % ± *SD*
Control	4.5 ± 0.4
1.0	3.9 ± 0.7
2.0	4.0 ± 1.0
3.0	1.4 ± 1.1

[a] Values represent mean of six loops.

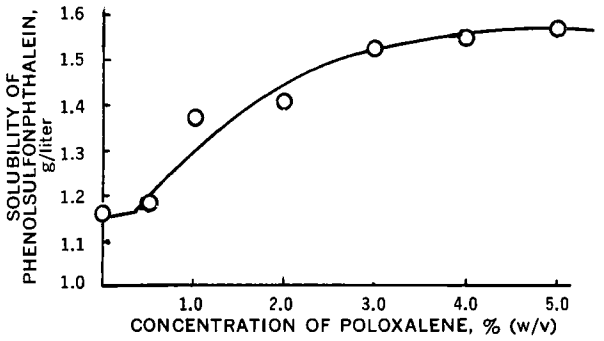

Figure 2—*Effect of poloxalene on the equilibrium solubility of phenolsulfonphthalein at 37°.*

permeability of the goldfish membrane but is unable to cause a significant change in the rat GI membrane. A similar nonionic surfactant, polysorbate 80, was also found to promote drug absorption in the goldfish but not in the rat (22). The lack of promotion of phenolsulfonphthalein absorption by poloxalene observed in this study is in agreement with the findings of Lish and Weikel (5) and the reported lower toxicity of nonionic surfactants toward biological membranes (23).

The decrease in absorption observed at higher concentrations of the surfactant is attributed to micellar entrapment of the drug molecules, as evidenced by the solubility curve shown in Fig. 2. It is suggested that the drug in the micellar phase is unavailable for absorption. Therefore, the effective concentration at the absorption site is less than the apparent concentration, resulting in a decrease in absorption. Higher concentrations of other nonionic surfactants were found by many investigators (24–27) to cause a similar decrease in drug absorption.

SUMMARY

Dioctyl sodium sulfosuccinate, an anionic medicinal surfactant, increased the absorption of phenolsulfonphthalein, a poorly absorbable drug, in the rat. Poloxalene, a nonionic medicinal surfactant, did not increase absorption of the drug; however, higher doses resulted in a decrease in absorption. The possibility that dioctyl sodium sulfosuccinate, a widely used fecal softener, causes a similar enhancement of drug absorption in humans is currently under investigation.

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Effect of Surfactants on Absorption through Membranes IV: Effects of Dioctyl Sodium Sulfosuccinate on Absorption of a Poorly Absorbable Drug, Phenolsulfonphthalein, in Humans

NAWAL KHALAFALLAH *, M. WAFIK GOUDA ‡x, and SAID A. KHALIL *

Abstract □ To explore the effect of dioctyl sodium sulfosuccinate on drug absorption in humans, the urinary excretion of a poorly absorbable drug, phenolsulfonphthalein, administered in solution with and without the surfactant was determined. Coadministration of a therapeutic dose of the surfactant with the drug solution resulted in a significant increase in the initial rate of absorption. A small increase in the extent of absorption was also observed. Pretreatment with the surfactant for 6 nights, followed by administration of the drug on the 7th day, did not significantly change the rate or extent of absorption. The surfactant is thought to have a direct effect on the GI membrane, resulting in a temporary change in its permeability. This effect appears to be reversible after a few hours.

Keyphrases □ Dioctyl sodium sulfosuccinate—effect on phenolsulfonphthalein absorption, humans □ Phenolsulfonphthalein absorption—dioctyl sodium sulfosuccinate effect, humans □ Surfactant effect—dioctyl sodium sulfosuccinate on phenolsulfonphthalein absorption, humans □ Absorption, drug—effect of surfactant (dioctyl sodium sulfosuccinate) on phenolsulfonphthalein, humans

Hepatic drug reactions are not uncommon. Various drugs were reported to cause such reactions, yet involvement of a laxative preparation in jaundice manifestations is a novelty (1). Recently, clinical observations describing hepatitis with jaundice manifestations in humans, during long-term administration of a widely used and generally assumed innocent laxative combination, were reported (1–3). This laxative product incorporates dioctyl sodium sulfosuccinate (I) as a wetting agent and a stool softener together with sodium carboxymethylcellulose and the cathartic oxyphenisatin acetate. A hypersensitivity to oxyphenisatin was suggested as the probable cause of hepatitis (3). These reports have drawn attention to the possibility of I being indirectly responsible for causing jaundice. The surfactant was suspected of producing a hyperabsorptive state by a direct action on the biological membrane, resulting in the absorption of toxic amounts of oxyphenisatin (4). Similarly, toxicity in rats (5) after oral administration of subtoxic doses of danthron (1,8-dihydroxyanthraquinone) with I was attributed to increased absorption of the former in the presence of the surfactant. Clinical data in humans (6) also support the observed toxicity in rats. Consequently, warning notes have appeared in the literature concerning the hazards of drug combinations containing I (4, 7).

A systematic study of the effect of I on drug absorption through biological membranes of varying complexities was undertaken. The purpose of such a study is to determine whether this seemingly inert surfactant (8) changes the absorption profile of representative drugs when both are given concomitantly. A previous report (9) showed that I enhances the absorption of pentobarbital, a readily absorbable drug, in the goldfish. Dioctyl sodium sulfosuccinate also enhanced the absorption of a poorly absorbable drug, phenolsulfonphthalein, in the rat (10). Since animal studies may only indicate similar effects in humans, a study of the effect of this surfactant on drug absorption in human volunteers was deemed appropriate. The effect of coadministration of the drug and the surfactant in solution on drug absorption under experimentally controlled conditions was examined. In addition, the effect of I on the absorption of the drug under clinically oriented conditions was determined.

EXPERIMENTAL

Twelve healthy male volunteers participated in the present study (Table I). The study was designed in a crossover fashion. The urinary excretion of orally administered phenolsulfonphthalein was determined subsequent to the administration of each of the following treatments:

A. 20 mg of phenolsulfonphthalein[1] (12 subjects).

B. 20 mg of phenolsulfonphthalein and 250 mg of dioctyl sodium sulfosuccinate[2] (11 subjects).

C. 20 mg of phenolsulfonphthalein and 500 mg of I (four subjects).

D. 20 mg of phenolsulfonphthalein alone on the morning of the 7th day following the daily administration of 200 mg of I, in the form of 50-mg capsules[3], taken after supper for 6 successive days (six subjects).

At least 1 week elapsed between two successive treatments in any one subject. Each dose was dissolved in 50 ml of water, and 20 mg of sodium bicarbonate was added to aid the solubility of phenolsulfonphthalein. Solutions containing the doses were administered to subjects in the morning on an empty stomach. No food was allowed for the next 4 hr. Fluid intake was regulated to permit hourly collection of urine, and no other drug was permitted throughout the experimental period. Doses containing the surfactant, when administered, produced temporary throat irritation, which explains the reduced number of volunteers in Treatment C.

Subsequent to dose administration, urine was collected hourly for 7–9 hr and then every 2–3 hr until the drug could no longer be detected in the urine. All urine samples were stored in a refrigerator and assayed within 24 hr according to McLeod *et al.* (11). The buffer solution, suggested by Ashley and Levy (12) to avoid underestimation of phenolsulfonphthalein particularly in acidic urine, was used to dilute the urine samples. Diluted urine was centrifuged, and the supernate absorbance was determined at 560 nm spectrophotometrically[4]. Necessary blanks were prepared using urine diluted with 0.1 *N* acetic acid.

[1] Phenolsulfonphthalein, Nutritional Biochemical Corp., Cleveland, Ohio.
[2] Aerosol O.T. 100%, Sargent-Welch Scientific Co.
[3] Colace capsules, supplied by Mead Johnson Laboratories, Evansville, IN 47721
[4] Unicam SP 500 spectrophotometer.

Table I—Effect of Dioctyl Sodium Sulfosuccinate on Phenolsulfonphthalein Absorption in Humans

Subject	Age, years	Weight, kg	1 hr Treatments A	B	C	D	24 hr Treatments A	B	C	D
			Amount of Phenolsulfonphthalein Excreted, mg							
GM	28	66	0.16	0.69	0.60	0.42	1.5	3.9	3.5	3.4
NK	31	55	0.22	0.69	0.47	0.48	1.8	2.7	3.2	1.9
SK	40	95	0.14	0.91	0.45	—[a]	4.1	4.2	3.3	—[a]
OA	26	90	0.30	—[a]	0.36	0.23	3.1	—[a]	2.3	1.8
SA	24	71	0.42	0.58	—[a]	0.53	2.3	2.1	—[a]	3.2
AE	24	67	0.20	0.50	—	0.19	1.8	2.0	—	2.2
AR	30	71	0.22	0.53	—	0.30	1.6	3.0	—	2.3
AN	23	85	0.46	0.32	—	—	2.0	1.6	—	—
AA	32	79	0.30	0.81	—	—	1.1	1.4	—	—
MM	27	86	0.40	0.48	—	—	3.0	3.5	—	—
WG	35	72	0.10	0.71	—	—	2.6	3.4	—	—
HE	31	80	0.45	0.45	—	—	2.1	2.0	—	—
Mean			0.28	0.61	0.47	0.36	2.3	2.7	3.1	2.5
RSD			45	29	21	23	37	35	17	27
Statistical significance[b]				$p < 0.005$	$p < 0.01$	N.S.		$p < 0.1$	N.S.	N.S.

[a] Subjects did not participate in this treatment. [b] Paired *t* test *versus* Treatment A (same subjects).

RESULTS AND DISCUSSION

The amounts of phenolsulfonphthalein excreted 1 and 24 hr following Treatments A–D are given in Table I. The two excretion parameters reported were chosen to reflect the rate and extent of phenolsulfonphthalein absorption. Plots of the time course of drug excretion following the treatments are shown in Fig. 1.

The average amounts of phenolsulfonphthalein excreted in 1 and 24 hr in the control treatment (Treatment A) were 1.4 and 11.3% of the dose, respectively. These values are in close agreement with those reported by other investigators (12). The urinary excretion pattern observed with the majority of subjects in the control study exhibited two excretion peaks at approximately 2 and 5 hr. Ashley and Levy (12) reported similar excretion patterns in humans following the same dose of phenolsulfonphthalein as a solution. The secondary excretion rate peak, after food ingestion, was attributed to transfer of residual drug from the stomach to the intestine or enhanced absorption due to discharge of bile into the intestinal lumen.

The urinary excretion data reported in Table I were statistically analyzed. Each set of data was compared with the control data from the same subjects using a paired *t* test (Table I). It can be observed from the 1-hr urinary excretion data that coadministration of 250 mg of the surfactant and the drug (Treatment B) resulted in a significant increase in the rate of phenolsulfonphthalein absorption. In the presence of the surfactant, 3% of the administered dose was excreted within 1 hr (Treatment B, average of 11 subjects) compared to a value of 1.4% of the dose in the absence of the surfactant (Treatment A, average of the same subjects). The dose of I administered in Treatment B, which resulted in the hyperabsorptive state, was within the usual prescribed oral dose [100–400 mg daily (13)]. Criticism has been raised against *in situ* data reporting surfactant effects on drug absorption. Such data are sometimes obtained under nonphysiological conditions, such as using high surfactant concentration (14), which prevents extrapolation of results to human therapy.

Coadministration of the drug and 500 mg of the surfactant resulted in a similar significant increase in the rate of absorption. Inspection of the 1-hr urinary excretion data following Treatment C suggests that doubling the dose of the surfactant did not increase the rate of drug absorption over what was observed following Treatment B. On the contrary, there seemed to be a lesser increase in the absorption rate at the higher surfactant dose. This concentration-dependent effect of the surfactant was also observed in similar studies in the rat (10). The 500-mg dose of the surfactant corresponds to an approximately 1% concentration in the administered solution. Previous studies (10) showed that such a concentration results in micellar entrapment of the drug molecules and, hence, the observed smaller increase in the absorption rate. The number of volunteers participating in both Treatments B and C was too small to permit statistical analysis of the difference between the two sets of data.

In Treatment D, where there was premedication with capsules containing 200 mg of the surfactant for 1 week, experimental conditions were selected to simulate a possible clinical situation where this stool softener surfactant is administered for some time and a poorly absorbable drug is administered during the same period. Such a clinical situation is very likely. Treatments B and C simulate another possible situation where patients may take the two drugs at the same time or where patients receive a combination dosage form containing the two drugs. It was a combination dosage form of I and the nonabsorbable oxyphenisatin acetate that resulted in hepatic toxicity (1–3).

The 1-hr urinary excretion data obtained following Treatment D indicates a small increase in the rate of absorption. The amount of phenolsulfonphthalein excreted increased from 1.3% (average of six subjects) to 1.8% of the dose (average of the same subjects). Statistical analysis of the data indicates that the difference between the two means is not significant. This result could be due to a decline in the surfactant effect during the time (overnight) that elapsed before drug administration. The effect of this surfactant (9, 10) and others (15, 16) on animal membranes was found to be reversible; membranes regained normal behavior after some time.

In the present study, the hyperabsorptive state described by the 1-hr urinary excretion data following Treatments B and C was most probably due to a direct action of the surfactant on the membrane. A similar mode of action was suggested in animal studies using phenolsulfonphthalein with I (10). The latter was also shown to accelerate peritoneal dialysis of urea and phosphates in rabbits (17). Various investigators attributed promotion of drug absorption in the presence of surfactants to a similar mechanism (15, 16, 18–20).

The exact mechanism of the surfactant-induced change in the membrane permeability is still unknown. The possibility of removal of phospholipids from the membrane by surfactants was suggested (17), as was alteration of the highly ordered barrier by such agents (21). Recently, Dujvone and Shoeman (22) reported that patients given therapeutic doses of dioctyl sodium sulfosuccinate excreted considerable amounts in the bile; therefore, the previous belief that this surfactant is not absorbed from the GI tract was invalidated.

It is likely that some of the surfactant molecules, in passing through the biomembrane, remain dissolved in the lipoidal cell wall. Plasticization results from the dissolution of low molecular weight compounds in high molecular weight materials. Physical properties, including permeability, undergo substantial changes. In the present study, dissolution of I in the lipoidal cell wall and a resultant permeability increase due to plasticization of the membrane was probably responsible for the observed increase in the rate of absorption of phenolsulfonphthalein. Lovering and Black (23) suggested a similar mechanism to explain the enhancement of permeation of phenylbutazone through everted rat intestines.

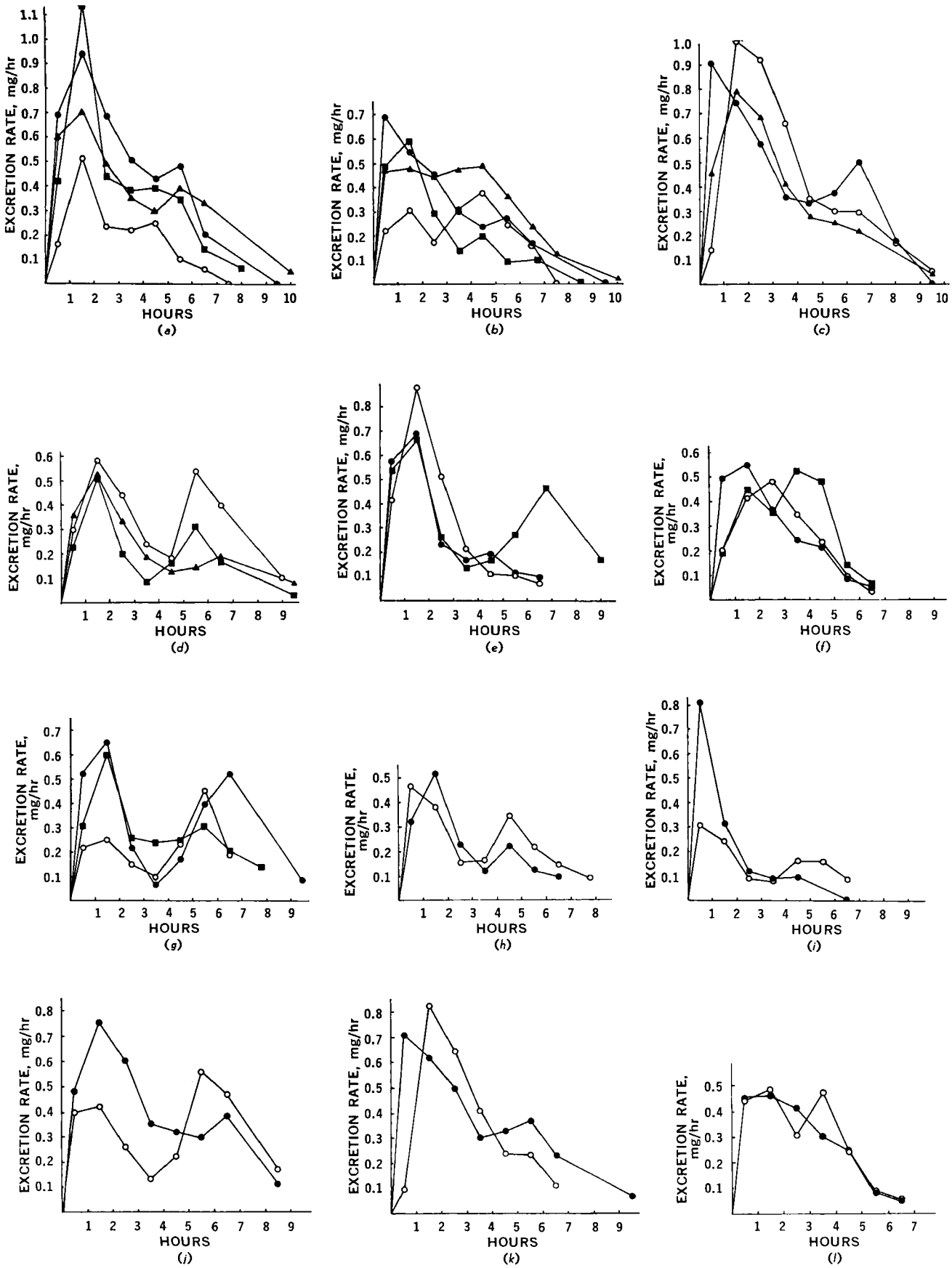

Figure 1—*Urinary excretion rates of orally administered phenolsulfonphthalein in human volunteers:* (a) *Subject GM,* (b) *Subject NK,* (c) *Subject SK,* (d) *Subject OA,* (e) *Subject SA,* (f) *Subject AE,* (g) *Subject AR,* (h) *Subject AN,* (i) *Subject AA,* (j) *Subject MM,* (k) *Subject WG, and* (l), *Subject HE. Key:* ○, *Treatment A;* ●, *Treatment B;* ▲, *Treatment C; and* ■, *Treatment D.*

Whatever the exact nature of this hyperabsorptiveness may be, it is generally agreed that it is reversible (9, 10, 15, 16). The membrane regains normal behavior toward drug permeation after some time, in contrast to the persistent hyperpermeability caused by fatty acids and aspirin (24).

Administration of 250 mg of the surfactant in phenolsulfonphthalein solution increased the total recovery of phenolsulfonphthalein from 10.9 to 13.5% (average of 11 subjects that participated in both Treatments A and B). This small increase was of borderline significance ($0.05 < p < 0.1$). Coadministration of 500 mg of the surfactant and premedication with capsules containing 200 mg of I did not significantly change the extent of absorption of phenolsulfonphthalein (Treatments C and D, Table I). Unavailability of the drug molecules for absorption due to micellar entrapment (10) could be the reason why a 500-mg dose of the surfactant did not increase the extent of absorption. The regaining of normal permeability characteristics by the membrane is probably responsible for the lack of increase in the extent of absorption following premedication with the surfactant capsules.

Two other factors should be considered for their possible implications on the results obtained in this study. The first factor is the mechanism of absorption of phenolsulfonphthalein. The drug is reported to be absorbed in animals and humans by both passive and specialized mechanisms (11, 25, 26). The latter has been described as a low capacity active transport process. Surfactants *have sometimes* been shown to retard the net absorption of drugs absorbed mainly by active mechanisms, in spite of the absence of drug micelle interactions (27–29). In the present investigation, although the coadministration of 250 mg of the surfactant and phenolsulfonphthalein resulted in an increase in net absorption, the increase was not as high as anticipated. Therefore, the mechanism of absorption of phenolsulfonphthalein could have exerted a damping effect on the increase in net absorption.

The second factor is that I has been reported to exert a pharmacological effect in animals that is not shared by other surfactants. It retards gastric emptying rate and inhibits gastric secretion in rats (30). These effects were only demonstrated at relatively high doses of the surfactant. Lish (30) reported retardation of gastric and intestinal motility using 100–1600 mg of I/kg in rats, while effects on gastric secretion were observed with 20–100 mg/kg in rats. In the present study, the hyperabsorptiveness was achieved with an average dose as low as 4 mg/kg in humans. Furthermore, the excretion pattern observed in Treatment B (Fig. 1) strongly suggests a direct action of I on the biomembrane and does not favor any of the previously discussed pharmacological effects of the surfactant. The sudden rise in urinary excretion rate of phenolsulfonphthalein during the 1st hr, with no apparent delay in peak excretion rate and no significant prolongation of absorption time, support the suggestion that the observed increase in absorption is due to a temporary change in membrane permeability.

In conclusion, the results of this study show that the initial rate of absorption of a poorly absorbable drug, phenolsulfonphthalein, from solution can be increased by the coadministration of a widely used medicinal surfactant, dioctyl sodium sulfosuccinate. The extent of absorption was only slightly increased. The results obtained also indicate that inclusion of this surfactant with the nonabsorbable laxative, oxyphenisatin acetate, could be one reason for the observed hepatic toxicity of the combination dosage form (4).

Therefore, it is imperative, as suggested by Tucker (31), that the possible effects of this and other surfactants on the absorption of each drug with which they are to be used be fully assessed. The drastic rise in excretion rate of phenolsulfonphthalein during the 1st hr (Subject WG, Treatment B, Table I) is strong proof of the possible hazards that may result from a sudden unintentional rise of the blood level of a potentially dangerous drug.

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Antimicrobial Evaluation of Diastereoisomeric 2-Dimethylaminomethyl-6-phenylcyclohexanols and Related Esters

J. R. DIMMOCK *x, W. A. TURNER *, and H. A. BAKER ‡

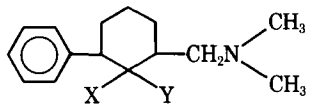
Abstract □ 2-Dimethylaminomethyl-6-phenylcyclohexanone was reduced to give the isomeric 2-dimethylaminomethyl-6-phenylcyclohexanols, which were then converted to a number of novel esters. These derivatives were screened against a wide range of microorganisms. The alcohol possessing an axial hydroxy group had high activity against *Candida albicans*, in contradistinction to the isomer having an equatorial hydroxy function, which was approximately 30 times less active. The esters from the isomeric alcohols demonstrated a low level of antimicrobial activity with the exception of the 10-undecenoyl ester of 2-dimethylaminomethyl-6-phenylcyclohexanol (possessing the equatorial hydroxy group), which showed significant activity against three pathogenic fungi.

Keyphrases □ 2-Dimethylaminomethyl-6-phenylcyclohexanone—conversion to isomeric alcohols and esters, screened for antimicrobial activity □ 2-Dimethylaminomethyl-6-phenylcyclohexanols and esters—screened for antimicrobial activity □ Antimicrobial activity—screening of diastereoisomeric 2-dimethylaminomethyl-6-phenylcyclohexanols and esters

It is well established that β-aminoketones (Mannich bases) have a wide range of chemotherapeutic properties (1–5) including antimicrobial activity (6–10). In addition, reduction of the keto group of Mannich bases has led to compounds with increased pharmacological activity (4). It was decided to prepare the Mannich base from 2-phenylcyclohexanone to compare the activity of this β-aminoketone (I) and related compounds with the corresponding acyclic derivative, 4-dimethylamino-1-phenyl-2-butanone, and its analogous derivatives.

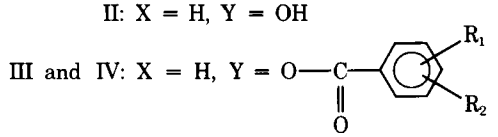
Reduction of I should lead to the formation of isomeric alcohols (II*a* and II*b*), the differences in stereochemistry of which may reflect different potencies against microorganisms. Furthermore, it was hoped to use the concept of latentiation (11) by preparing esters (III and IV) that would be predicted to undergo *in vivo* hydrolysis to the parent alcohols (II*a* and II*b*). The rate of hydrolysis in the series of benzoyl esters would be dependent on several factors, including the Hammett value of the nuclear substituents. In addition, steric impedance of the esters IV containing an axial ester group would be expected to be greater than in the series containing an equatorial ester configuration (III). The rate of hydrolysis of the esters, if carried out enzymatically, also may be dependent on the stereochemical requirements at a receptor site of the enzyme. Thus, a correlation between the rate of release of the alcohols and antimicrobial potency may emerge in this series of compounds.

Finally, it was proposed to synthesize the phenoxyacetyl and 10-undecenoyl esters of II*a* and II*b*, which may lead to the release of phenoxyacetic and 10-undecenoic acids *in vivo;* both of these compounds are known to possess antifungal activity.

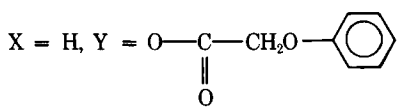


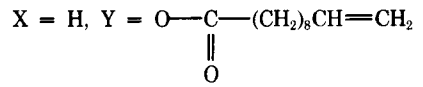
I: X, Y = O

II: X = H, Y = OH



$R_1 = R_2$ = H, Cl, NO_2, OCH_3





EXPERIMENTAL[1]

The preparation of the compounds described in this work was reported previously (12). The compounds in Table I were evaluated using an agar dilution method. In each case a stock solution of the compound was prepared by dissolving 20 mg of the sample in 1 ml of dimethyl sulfoxide, followed by the addition of 3 ml of water. Aliquots of the stock solution were pipetted into melted trypticase soy agar (BBL) to give final concentrations of the compound of 200, 50, 12.5, and 3.1 μg/ml. After hardening, the agar surface was inoculated with appropriately diluted suspensions of the test organisms using a multiple inocula replicating device[2]. The seeded plates were incubated (18 hr at 30° followed by 18 hr at 37°), and the plates were then observed for microbial growth.

The screening techniques for the compounds listed in Table II were as follows. In the antibacterial screen, 1 ml of stock solution (1000 μg/ml) was added to 9 ml of nutrient broth to give an initial concentration of 100 μg/ml and 1:1 dilutions were then added to nutrient broth. All tubes were inoculated with 0.1 ml of a 1:10 dilution of an 18-hr culture of the test organism. All tubes were incubated for 24 hr at 37° and then examined for growth.

The antifungal screen involved the addition of 1 ml of the stock solution (1000 μg/ml) to 9 ml of Sabouraud broth to give an initial concentration of 100 μg/ml and 1:1 dilutions were added to Sabouraud broth. A spore suspension (0.1 ml) of *Trichophyton granulosum* and *Microsporum gypseum* and 0.1 ml of a 1:100 dilution of a broth culture of *Candida albicans* were added to the test tubes containing the various concentrations of the test substances. Tubes containing *C. albicans* were incubated at 37° and tubes containing *M. gypseum* and *Tr. granulosum* were incubated at 28°. Tubes were examined for growth after 4 and 9 days of incubation.

In the antitrichomonal screen, 1 ml of the stock solution (1000 μg/ml) was added to 9 ml of Diamond medium to give an initial

[1] Antimicrobial evaluations were carried out by Dr. Joseph F. Pagano and staff at Smith Kline & French Laboratories, Philadelphia, Pa. (Table I) and by Ayerst Laboratories, Montreal, Quebec, Canada (Table II).

[2] Steers.

Table I—Screening of 2-Dimethylaminomethyl-6-phenylcyclohexanone and Related Alcohol and Esters against Various Microorganisms[a]

Microorganism[b]	I	IIa	IIIa	IIIb	IIIc	IIId	IIIe	IIIf	IIIg
Streptococcus faecalis	>200	>200	>200	200	>200	>200	>200	>200	>200
Staphylococcus aureus (R)[c]	>200	>200	>200	200	>200	>200	>200	>200	>200
Staphylococcus aureus (S)[d]	>200	>200	>200	200	>200	>200	>200	>200	>200
Klebsiella pneumoniae	>200	>200	>200	>200	>200	>200	>200	>200	>200
Pseudomonas aeruginosa	>200	>200	>200	>200	>200	>200	>200	>200	>200
Escherichia coli	>200	>200	>200	>200	>200	>200	>200	>200	>200
Salmonella typhimurium	>200	>200	>200	>200	>200	>200	>200	>200	>200
Trichophyton mentagrophytes	<12	>200	200	200	>200	>200	>200	>200	>200
Mycobacterium smegmatis	50	>200	>200	200	>200	>200	>200	>200	>200
Candida albicans	200	>200	>200	200	>200	>200	>200	>200	>200
Bacillus subtilis	200	>200	>200	50	>200	>200	>200	>200	>200
Fusarium oxysporum	50	>200	>200	200	>200	>200	>200	>200	>200
Penicillium citrinium	50	>200	>200	200	>200	>200	>200	>200	>200
Aspergillus niger	50	>200	>200	200	>200	>200	>200	>200	>200
Cryptococcus neoformans	50	>200	>200	200	>200	>200	>200	>200	>200
Blastomyces dermatididis	>200	>200	>200	200	>200	>200	>200	>200	>200
Xanthomonas vesicatoria	>200	>200	>200	200	>200	>200	>200	>200	>200
Streptococcus pyogenes	>200	>200	>200	50	>200	>200	>200	>200	>200
Sarcina lutea	>200	>200	>200	50	>200	>200	>200	>200	>200

[a] Figures in table are the minimum inhibitory concentrations of the compounds in micrograms per milliliter. [b] The strains of microorganisms in this table are identified by the following numbers: ATCC 9790, SK&F 24390, SK&F 23390, SK&F 4200, SK&F 11320, SK&F 12140, SK&F 11350, SK&F 17410, ATCC 101, SK&F 3470, ATCC 6633, ATCC 9848, ATCC 16040, SK&F 330, EK1, EK2, ATCC 11551, ATCC 8668, and ATCC 9341, respectively. [c] Strain resistant to penicillin G. [d] Strain sensitive to penicillin G.

concentration of 100 μg/ml and 1:1 dilutions were made. Tubes were inoculated with approximately 2.5×10^5 organisms/ml and examined microscopically for growth after 48 hr of incubation.

RESULTS AND DISCUSSION

The preparation and chemical properties of Compounds I–IV were described previously (12). Table I indicates that the β-aminoketone (I) had a good level of activity against the fungus *Trichophyton mentagrophytes* and low levels of activity against seven other microorganisms. Compound I was screened in the plaque inhibition test against rhinoviruses HGP, 28, 1059, and 33342 and was inactive at a concentration of 100 μg. In another screen, I was shown to reduce *Trichinella spiralis* parasites in mice by 24% at a dose of 50 mg/kg (25 mg/kg po and 25 mg/kg sc).

Reduction of I with lithium aluminum hydride gave a 3:1 mixture of isomeric alcohols (IIa and IIb), which was separated by column chromatography. Physicochemical determinations, principally PMR spectroscopy, provided evidence of the stereochemistry of IIa and IIb (Scheme I).

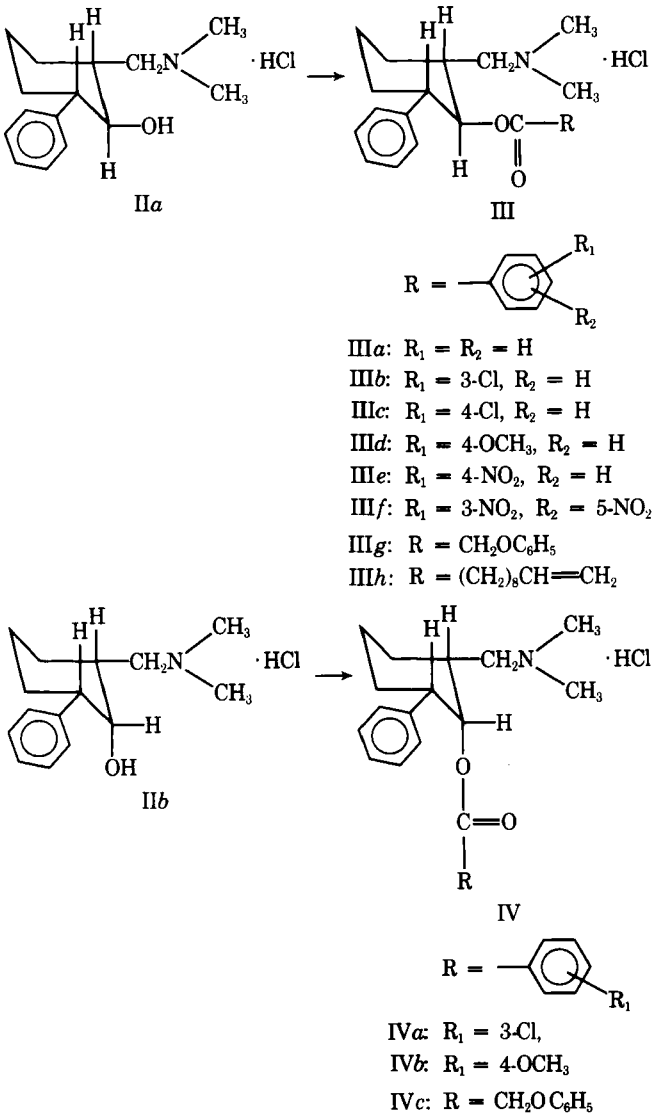
As can be seen from Tables I and II, IIa was virtually devoid of antimicrobial activity. However, alcohol IIb, while failing to show significant activity against most of the microorganisms listed in Table II, showed excellent activity against *C. albicans*. The large potency differences between IIa and IIb against this important pathogenic fungus are noteworthy, illustrating the potency variations between geometrical isomers (13). The lack of activity in IIa may possibly be due to its accessible equatorial hydroxy group, which may allow facile metabolism and detoxification to occur. In addition, the equatorial hydroxy group may form hydrogen bonds readily prior to reaching a site of action. In comparing the bioactivity of I, IIa, and IIb, the broader spectrum of activity of I is noteworthy. It is possible that I, being a Mannich base, can undergo deamination to the corresponding α,β-unsaturated ketone, which may be the active derivative (14). Neither IIa nor IIb would be expected to undergo such a biotransformation.

The masking of the polar hydroxy group by forming the benzoate esters of IIa was accomplished. However, the unsubstituted compound (IIIa) was virtually devoid of antimicrobial activity in the screens chosen. Substitution of the aromatic ester function by electron-withdrawing influences, to increase the predicted *in vivo* hydrolysis, yielded virtually inactive compounds (IIIb, IIIc, IIIe, and IIIf). The ester IIId, containing the electron-repelling methoxy group, would be expected to undergo slower hydrolysis than the other esters, but it failed to demonstrate a significant level of activity. The phenoxyacetyl ester (IIIg) showed virtually no activi-

Table II—Screening of the Isomeric 2-Dimethylamino-6-phenylcyclohexan-1-ols and Related Esters against Various Microorganisms[a]

Microorganism[b]	IIb	IVa	IVb	IVc	IIa	IIIb	IIId	IIIg	IIIh
Candida albicans	3.2	100	100	100	100	100	100	50	6.25[c]
Microsporum gypseum	100	50	25	50	50	100	100	100	3.2
Trichophyton granulosum	100	50	50	100	50	100	100	>100	6.25
Staphylococcus pyogenes (R)[d]	>100	—	—	—	—	>100	>100	>100	100
Staphylococcus pyogenes (S)[e]	>100	—	—	—	—	>100	>100	>100	100
Streptococcus faecalis	100	—	—	—	—	>100	>100	>100	100
Escherichia coli	100	—	—	—	—	>100	>100	>100	100
Aerobacter aerogenes	100	—	—	—	—	>100	>100	>100	100
Salmonella pullorum	100	—	—	—	—	>100	>100	>100	50
Pseudomonas aeruginosa	100	—	—	—	—	>100	>100	>100	100
Proteus mirabilis	100	—	—	—	—	>100	>100	>100	100
Proteus vulgaris	100	—	—	—	—	>100	>100	>100	100
Klebsiella pneumoniae	100	—	—	—	—	100	>100	>100	25
Serratia marcescens	100	—	—	—	—	100	>100	>100	50
Trichomonas vaginalis	100	—	—	—	—	25	100	100	50
Trichomonas foetus	—	50	>100	>100	100				

[a] Figures in table are the minimum inhibitory concentrations of the compounds in micrograms per milliliter. [b] The strains of microorganisms in this table are identified by the following numbers: AY-F-598, AY-F-605, AY-F-604, AY-B-353, AY-B-352, AY-B-355, ATCC 11229, AY-B-357, AY-B-358, AY-B-359, AY-B-360, AY-B-361, ATCC 10081, ATCC 9103, ATCC 30001, and ATCC 30003, respectively. [c] Activity values against resistant strains of *C. albicans* AY-F-610–619 were 20, 16, 32, 62.5, 32, 62.5, 62.5, 32, 32, and 32 μg/ml, respectively. [d] Strain resistant to penicillin G potassium. [e] Strain sensitive to penicillin G potassium.



Scheme I

ty at high doses, suggesting that the ester was not breaking down to yield the established antifungal agent, phenoxyacetic acid.

Attempts to prepare the analogous series of esters from II*b* were not completely successful. Only in the case of the phenoxyacetyl ester (IV*c*) was a crystalline ester obtained uncontaminated with unreacted alcohol. In other cases, a mixture of alcohol and ester was obtained; this mixture proved difficult to separate, although IV*a* and IV*b* were obtained in very low yields. By the time II*b* and its related esters were available for assessment as antimicrobial agents, the screen used to evaluate the previous compounds (Table I) became unavailable. However, the novel esters IV*a*, IV*b*, and IV*c* were evaluated elsewhere and compared to the analogous esters III*b*, III*d*, and III*g* along with the isomeric alcohols II*a* and II*b* (Table II). Esters IV*a*, IV*b*, and IV*c* did not show promising levels of activity, which may indicate failure for hydrolysis to occur under biological conditions.

Finally, attempts to prepare the undecenoyl esters of II*a* and II*b* were undertaken. While an attempt to form the ester from the sterically hindered alcohol II*b* was unsuccessful, II*a* reacted with undecenoyl chloride to give III*h* in good yield. Table II indicates that III*h* possesses high activity against three species of pathogenic fungi. Further studies of III*h* against 10 resistant strains of *C. albicans* showed activity between 16 and 62.5 μg/ml (Table II), considered to be too low a level to warrant further investigation. However, the fact that the undecenoyl ester (III*h*) showed far greater antifungal activity than the corresponding phenoxyacetyl derivative (III*g*) may indicate steric restraints of the aryloxyalkyl ester imposed by the bulky aromatic ring. The olefinic derivative (III*h*) may be able to undergo *in vivo* hydrolysis to undecylenic acid.

In summary, the work demonstrates that another Mannich base (I) shows antimicrobial potency as well as stereospecificity of the isomeric 2-dimethylaminomethyl-6-phenylcyclohexanols (II*a* and II*b*) toward *C. albicans*. Esters of II*a*, with one exception, prepared from II*a* failed to enhance the antimicrobial potency and all of the esters of IV failed to retain the activity against *C. albicans* shown by the precursor alcohol II*b*. The high activity of the undecylenic ester of II*a* may be due to its ability for facile hydrolysis to undecylenic acid, in which case the substituted cyclohexanol can be considered a novel carrier group for the active antifungal agent.

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Determination of (±)-1,2-Bis(3,5-dioxopiperazinyl)propane Plasma Levels in Rats, Rabbits, and Humans by GLC and Mass Fragmentography

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Abstract □ Specific assay procedures were developed to measure plasma concentrations of (±)-1,2-bis(3,5-dioxopiperazinyl)propane (I) by GLC using flame-ionization detection with a sensitivity limit of 5 μg/ml and by GLC–mass fragmentography with a sensitivity limit of 0.2 μg/ml. Applicability of the assay procedures was demonstrated in rats, rabbits, and humans. Plasma concentration–time curves of total ^{14}C activity and intact I were obtained in rats and rabbits following oral and intravenous administration of ^{14}C-I. Plasma elimination half-lives of I in the first 2 hr following intravenous doses in rats were 40 and 45 min in two rats. Plasma levels of I were measured over 6 hr after an intravenous dose in rabbits and followed a two-compartment open model with a terminal log-linear plasma half-life of 85 min. Significantly higher total ^{14}C levels compared to intact I plasma concentrations indicated rapid biotransformation in both rats and rabbits to unknown metabolites. The oral bioavailability appeared to be limited in both species relative to intravenous administration. Two patients receiving 3 g of I/m^2 in tablets orally showed plasma levels of I similar to those obtained after oral doses in rats and rabbits, with peak concentrations at 2 hr after the dose (3.8 μg/ml) and with still measurable levels 12 hr after the dose (0.4 μg/ml).

Keyphrases □ (±)-1,2-Bis(3,5-dioxopiperazinyl)propane—determination of plasma levels, rats, rabbits, and humans, GLC and mass fragmentography □ Dioxopiperazines—determination of (±)-1,2-bis(3,5-dioxopiperazinyl)propane plasma level, rats, rabbits, and humans, GLC and mass fragmentography □ GLC—determination, plasma levels of (±)-1,2-bis(3,5-dioxopiperazinyl)-propane, rats, rabbits, and humans □ Mass fragmentography—determination, plasma levels of (±)-1,2-bis(3,5-dioxopiperazinyl)-propane, rats, rabbits, and humans

The dioxopiperazine, (±)-1,2-bis(3,5-dioxopiperazinyl)propane[1] (I, Scheme I) (1), is under clinical investigation as a chemotherapeutic agent against various neoplasms (2, 3). Its interesting pharmacological properties include normalization of tumor vasculature and the prevention of metastases of Lewis lung tumor in mice (4–6). The plasma disappearance half-life of I, following intravenous injections of ^{14}C-I in rats, was found to be about 30 min when measured by a bioassay using cultured Chinese hamster cells; total ^{14}C activity declined less rapidly, indicative of the presence of metabolites (7). The bioavailability of oral ^{14}C-I doses in humans appeared to be inversely related to the amount administered as judged by ^{14}C urinary excretion (8).

This article reports a specific chemical assay using GLC with flame-ionization detection and, for low concentrations of I, GLC–mass fragmentography. These methods were previously applied in other pharmacokinetic studies, *e.g.*, plasma levels of fluorouracil[2].

Rats and rabbits were utilized as experimental animals to demonstrate the applicability of the assay procedures, since pharmacokinetic data using the bioassay were available in rats (7) and evaluation of the effect of combination therapy with adriamycin and I on adriamycin cardiac toxicity is being investigated in rabbits. No intravenous preparation of I is yet available for human use due to limited solubility of the racemic mixture. The isolated optical isomers are considerably more soluble (9) and may provide an intravenous preparation in the future.

[1] Designated ICRF-159 by the Chemotherapy Department of the Imperial Cancer Research Fund.

[2] C. Finn and W. Sadée, to be published.

EXPERIMENTAL

Instrumentation and Apparatus—The assay was carried out on a gas chromatograph[3] equipped with a flame-ionization detector and a 1.8-m (6-ft) × 2-mm (i.d.) glass column packed with 3% polymethylsiloxane[4] on 100–120-mesh silanized diatomaceous earth[5]. Flow rates were approximately 35 ml/min for nitrogen, 30 ml/min for hydrogen, and 300 ml/min for air. The injector, column, and detector were operated at 210, 210, and 250°, respectively.

The GLC–mass fragmentography assay was performed on a gas chromatograph[6] equipped with an identical glass column and interfaced with a mass spectrometer[7] by a separator[8]. Helium, at a flow rate of 20 ml/min, was used as the carrier gas. The ionizing voltage was 70 ev.

Reagents—Chemicals were of analytical grade with the exceptions noted[9].

An ethereal alcoholic solution of diazomethane was prepared by the reaction of potassium hydroxide with *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide[10] and stored at −10°. Because of its toxicity and explosiveness, all manipulations involving the generation or use of diazomethane were carried out in a well-ventilated hood behind a safety shield.

GLC—To 0.5 ml of plasma containing 5–50 μg of I were added 0.5 ml of saturated aqueous ammonium sulfate, 6.0 ml of ether–isopropyl alcohol (4:1 v/v), and 0.05 ml of a solution of 5 μg of methyl arachidate in methanol as an internal standard. Samples were mixed on a vortex-action high-speed mixer in stoppered test tubes for 4 min and centrifuged. The ethereal phase was pipetted into another test tube and evaporated to dryness with a stream of nitrogen at 40°. The residue was transferred to a 3.0-ml vial[11] with

[3] Varian Aerograph model 1400.

[4] OV-1.

[5] Gas Chrom Q.

[6] Varian Aerograph model 2700.

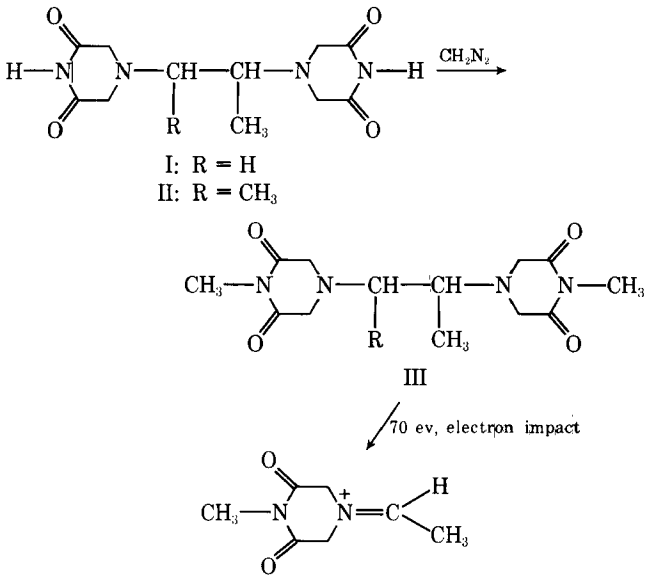
[7] Varian MAT CH-7.

[8] Biemann–Watson.

[9] Pure crystalline I (NSC-129943) was obtained from Dr. H. B. Wood, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute, Bethesda, Md. Compound II was supplied by Dr. K. Hellmann, Imperial Cancer Research Fund, London, England. ^{14}C-Ethylene-labeled I was provided by Dr. M. A. Leaffer, Isotope Synthesis Lab, Menlo Park, Calif. (Contract NIH-NIC 71-2161).

[10] Diazald, Aldrich Chemical Co., Milwaukee, Wis.

[11] Reactivial, Pierce Chemical Co., Rockford, Ill.



Scheme I

3 × 0.5 ml of methanol, and the solution was again evaporated with a stream of nitrogen at 65°.

The residue was dissolved in 0.2 ml of methanol, and about 1.5 ml of diazomethane solution was added. The sealed vial was kept at room temperature for 4 hr, and then excess diazomethane was evaporated by allowing the opened vial to stand in the hood for several minutes. The remaining solution was evaporated to dryness with a stream of nitrogen at 65°. Care should be taken to thoroughly dry the residue at this point. The residue was dissolved in 0.1 ml of methylene chloride, and a 2–3-μl aliquot of this solution was injected into the chromatograph.

At a column temperature of 210°, methyl arachidate and the dimethylated derivative of I had retention times of 6.5 and 8.0 min, respectively. Both chromatographic peaks were sufficiently separated from each other and from interfering peaks in the methylated plasma extract to allow the determination of their relative heights. A plot of peak height of I/peak height of methyl arachidate *versus* concentration of I was linear over the 5–50-μg/ml range of plasma. The column must be reconditioned at about 260° for 15 min after each injection to elute less volatile plasma components.

GLC–Mass Fragmentography Assay—Extraction and derivatizing procedures were identical to those in the GLC assay using flame ionization. In place of methyl arachidate, 0.1 ml of a solution containing 1 μg of II in methanol was added as the internal standard[12]. Aliquots of 2–3 μl were injected on the column. The fragment ion at *m/e* 155 was used to monitor both methylated I and II (Scheme I). At a column temperature of 230°, the dimethylated derivatives of I and II had retention times of 4.0 and 5.0 min, respectively. A standard curve was again constructed from peak height ratios by assaying plasma samples containing known amounts of I in the 0.2–2.0-μg/ml range. A plot of peak height of I/peak height of II *versus* concentration of I was linear in this range.

Radioactivity Analysis—Plasma samples (0.1 ml) were counted for ^{14}C activity in 10 ml of scintillation fluid[13] in polyethylene vials. The counter[14] had a ^{14}C efficiency of 75–85%, and counts were corrected by the channels ratio method. The ^{14}C activity was expressed as equivalents of ^{14}C-I.

Animal Procedures—Female Sprague–Dawley rats, 250–300 g, were used. Blood samples were collected from a carotid cannula, spun in a microcentrifuge[15], and frozen for subsequent analysis.

[12] Compound II could not be used as the internal standard for the flame-ionization GLC assay because it could not be adequately resolved from interfering plasma components.

[13] Aquasol, New England Nuclear.

[14] Unilux II, Nuclear Chicago.

[15] Beckman.

Table I—Mass Spectra of *N,N'*-Dimethylated I and II Obtained by GLC–Mass Fragmentography

N,N'-Dimethylated I		*N,N'*-Dimethylated II	
m/e	Relative Abundance, %	*m/e*	Relative Abundance, %
58	22	58	22
70	37	70	33
99	22	99	21
127	52	127	44
141	7.6	155	100
155	100	156	16
156	28	—	—
296 (M^+)	4.4	—	—

Compound I was stable in frozen plasma samples over several weeks. New Zealand white rabbits, ~2 kg, were also used and blood specimens were obtained from an ear artery.

Doses of I were prepared in water, and sufficient 1.0 *N* HCl was added to dissolve all crystals completely. ^{14}C-I was added to obtain a specific activity of 100 dpm/μg. All doses were administered orally by stomach intubation or intravenously by injection over 1 min at a level of 120 mg/kg in rats and 50 mg/kg in rabbits.

Clinical Protocols—Human subjects received oral doses of I (3 g/m^2) in tablets as part of their medical treatment in the Western Cancer Study Group. Blood samples were obtained by venipuncture into containers[16] containing ethylenediaminetetraacetate and centrifuged, and the plasma was frozen for subsequent analysis. The use of this container resulted in an interfering peak with the same retention time as I, invalidating the flame-ionization GLC assay data. Plasticizers and similar compounds may have been possible contaminants. No interference by this contaminant was noted when utilizing single ion detection at *m/e* 155 in the GLC–mass fragmentography assay.

RESULTS

Assay Procedures—Chemical analysis of I imposed several analytical problems due to its polarity, amphoteric character, and lack of UV absorbance. The aqueous lipid extraction procedure, using saturated ammonium sulfate, yielded about 70% recovery of the drug from plasma samples. Suitable GLC characteristics were obtained by derivatization with diazomethane to give the *N,N'*-dimethylated derivative (III, Scheme I). The sensitivity of the flame-ionization GLC procedure was limited to 5 μg of I/ml of plasma due to interfering endogenous substances. Thus, single ion detection by mass spectrometry was employed to increase both specificity and sensitivity. Due to its close chemical similarity, Compound II was selected as the internal standard to compensate for extraction losses and irreversible adsorption processes during analysis at low concentrations.

The dimethylated derivatives of I and II are separated by GLC; both give rise to one major fragment ion at *m/e* 155 (Table I), which can be used to quantitate I and II. It is difficult to prove specificity of the assay for I in the presence of its metabolites since their structures remain unknown. The following criteria for assay interference by metabolites have to be met: extractability into the organic phase, identical GLC retention time, and fragment ion at *m/e* 155 representing a major peak in the mass spectrum. These criteria do not apply to possible hydrolytic polar metabolites. Therefore, the described assay is specific for I commensurate with current standards of plasma drug analysis.

The detection limit of pure samples of I by monitoring the fragment ion at *m/e* 155 was in the low picogram range. The detection of I in plasma samples after extraction was limited to approximately 0.2 μg/ml due to endogenous compounds with a high interference probability at the relatively low mass setting of *m/e* 155. However, this sensitivity limit was sufficient to measure I in rat, rabbit, and human plasma following therapeutic doses.

Plasma Levels in Rats—The plasma concentration–time

[16] Vacutainers.

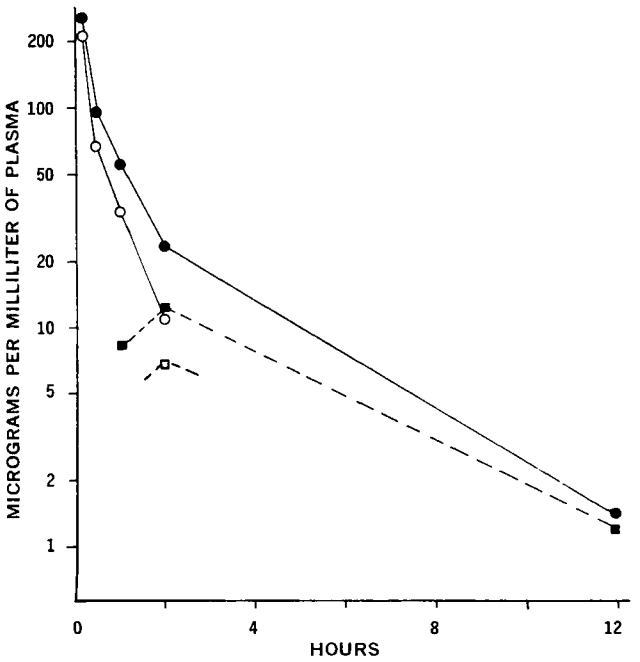


Figure 1—*^{14}C plasma activity and intact I plasma levels following a dose of 120 mg of I/kg in two rats. Key: ●, ^{14}C activity following intravenous dose; ■, ^{14}C activity following oral dose; ○, intact I levels following intravenous dose; and □, intact I levels following oral dose.*

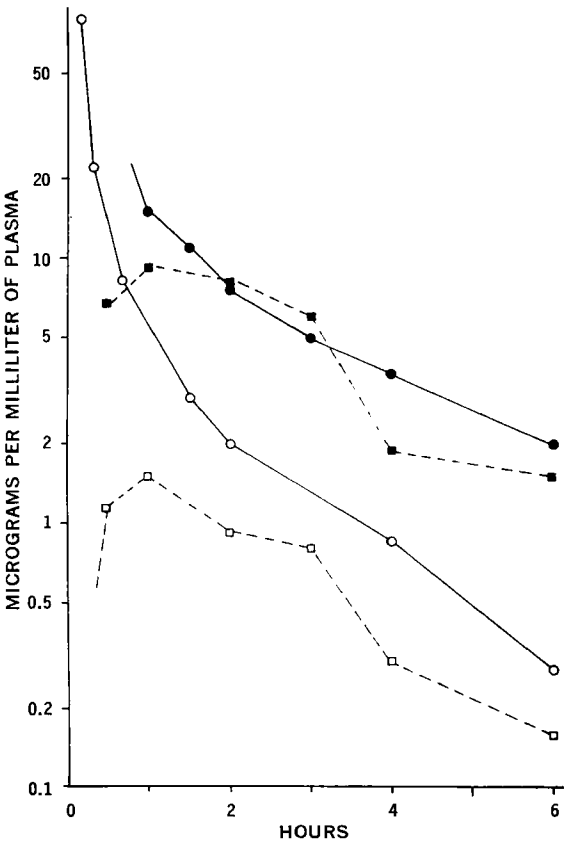


Figure 2—*^{14}C plasma activity and intact I plasma levels following a dose of 50 mg of I/kg in two rabbits. Key: ●, ^{14}C activity following intravenous dose; ■, ^{14}C activity following oral dose; ○, intact I levels following intravenous dose; and □, intact I levels following oral dose.*

curves measured by flame-ionization GLC and total ^{14}C activity resulting from an oral and intravenous dose of 120 mg of I/kg in two different rats are depicted in Fig. 1. Following intravenous administration, plasma levels of I rapidly disappeared during the initial distribution phase between 0 and 20 min and then declined with a half-life of about 40 min. A second rat showed a half-life of 45 min under identical conditions. The volume of distribution was obtained by extrapolating the apparent log-linear elimination phase to zero time and amounted to 120% of body weight in both rats. Total ^{14}C activity decreased less rapidly due to the formation of ^{14}C-labeled metabolites.

Following oral doses, peak plasma levels of 7 μg of I/ml were obtained after 2–4 hr, as measured by flame-ionization GLC (Fig. 1). Plasma levels of I were below the detection limit of this assay in all other samples.

Plasma Levels in Rabbits—^{14}C-I was orally and intravenously administered at a dosage of 50 mg/kg in two different rabbits (Fig. 2). Total ^{14}C plasma activity was again higher than the concentration of intact I, which was followed by GLC–mass fragmentation over 6 hr. The intravenous plasma concentration *versus* time curve of I could adequately be described by a two-compartment open model with a rapid α-phase ($\alpha = 4.15\ hr^{-1}$) and a slower β-phase ($\beta = 0.49\ hr^{-1}$, $T_{1/2} = 85$ min). The zero time intercepts were $A = 52$ μg/ml and $B = 5.5$ μg/ml.

Peak plasma concentrations of 1.5 μg/ml were obtained about 1 hr following oral administration and then declined parallel to the intravenous I concentration–time curve (Fig. 2), dropping below measurable levels 6 hr after administration. Total ^{14}C plasma activity remained as high as 0.6 and 0.5 μg/ml 24 hr after oral and intravenous administration, respectively, in these two rabbits.

Plasma Levels in Humans—The plasma concentration–time curve of I resulting from an oral dose of 3 g/m^2 (4.5 g) was measured by GLC–mass fragmentation (Fig. 3). Peak plasma levels of 3.8 μg of I/ml were obtained 2 hr following administration. Similar plasma concentrations were achieved in another patient under identical conditions. Twelve hours following administration of I, the plasma concentration in this patient had declined to 0.4 μg/ml and was below the detection limit (0.1 μg/ml) 24 hr after administration.

DISCUSSION

The specific assay for the determination of I in plasma samples was developed to evaluate the pharmacokinetics of this antineoplastic agent. The preliminary results are reported here.

Rat Studies—The plasma half-life of I measured by bioassay was about 30 min following an intravenous dose of 120 mg of I/kg (7). A plasma half-life of 40–45 min was found by the flame-ionization GLC assay under equivalent experimental conditions. Therefore, it is likely that no significant amounts of pharmacologically active metabolites were formed if one assumes that the bioassay is correlated with antineoplastic activity. Studies on the structure–activity relationships indeed demonstrate that pharmacological activity is very structure specific, and slight modifications of the chemical moiety of I usually abolish its antineoplastic activity (1, 10). However, it is difficult to assess pharmacological properties of I such as normalization of tumor vasculature, and formation of pharmacologically active metabolites cannot be ruled out.

The plasma half-life of 40–45 min for I may not represent the terminal log-linear elimination phase, since plasma levels were only followed over 2 hr by flame-ionization GLC.

Rabbit Studies—Plasma concentrations of I were measured by GLC–mass fragmentography over several half-lives. A log-linear β-phase for the elimination of I from plasma with a half-life of 85 min was reached 2 hr following intravenous administration. Initial plasma concentrations of I after intravenous injection were comparable to those obtained in rats when correcting for the lower dose in rabbits.

Plasma concentrations following oral doses were significantly lower than following intravenous doses in both species, indicating a limited oral bioavailability of I.

Human Studies—Only two patients were evaluated, each giving plasma concentration–time curves for I similar in order of magnitude to those obtained after oral doses in rats and rabbits. Therefore, it appears that both animal species may be useful as models for human pharmacological studies of I.

It cannot be determined at this point whether the limited bioavailability of I from oral preparations is due to low solubility or to

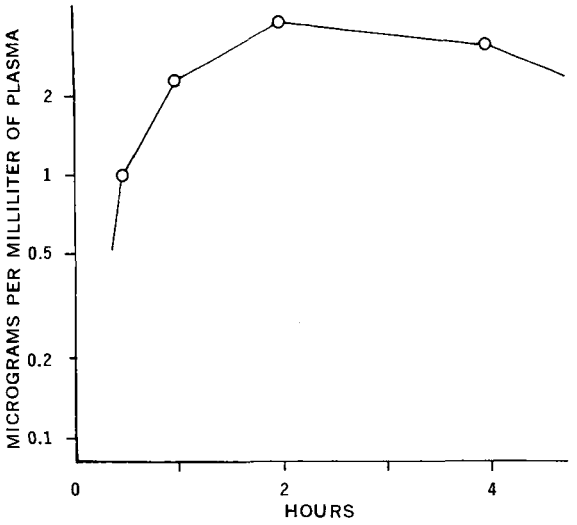


Figure 3—*Plasma concentrations of I in human subject following an oral dose of 3 g of I/m^2 in tablets.*

slow absorption through the GI mucosa. When given orally, I was slowly absorbed in all experiments, although an aqueous solution was used in rats and rabbits. The bioavailability of I is being studied.

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Synthesis of 2-(*N*-Arylcarboxamide)-3-substituted Ethoxyindoles and Their Monoamine Oxidase Inhibitory and Anticonvulsant Activities

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Abstract □ 2-(*N*-Arylcarboxamide)-3-substituted ethoxyindoles were synthesized by the reaction of 2-(*N*-arylcarboxamide)-3-hydroxyindoles, which were obtained by the cyclization of 2-carbomethoxyphenylglycine-substituted anilides. These 2-(*N*-arylcarboxamide)-3-substituted ethoxyindoles were evaluated for their *in vitro* monoamine oxidase inhibitory ability and *in vivo* monoamine oxidase inhibitory property as evidenced by reserpine reversal response. Their anticonvulsant activity also was determined against pentylenetetrazol-induced seizures. No definite correlation could be observed between chemical structure and biological activity.

Keyphrases □ 2-(*N*-Arylcarboxamide)-3-substituted ethoxyindoles—synthesis, monoamine oxidase inhibitory and anticonvulsant activities □ Monoamine oxidase inhibitory activity—synthesis and screening of 2-(*N*-arylcarboxamide)-3-substituted ethoxyindoles, rat brain homogenate □ Anticonvulsant activity—synthesis and screening of 2-(*N*-arylcarboxamide)-3-substituted ethoxyindoles, mice

Identification of serotonin in the brain as a possible central neurohumoral agent (1) and the widespread occurrence of the indole nucleus among both naturally occurring and synthetic psychoactive compounds (2) stimulated an investigation of indole analogs with potent central nervous system (CNS) activity. In addition, clinical efficacy of 3-(2-aminobutyl)indole for the treatment of some types of depression (3) and the ability of indole derivatives to inhibit rat brain and rat liver monoamine oxidase (4, 5) and to protect against pentylenetetrazol-induced seizures (6, 7) prompted the synthesis of some 2-(*N*-arylcarboxamide)-3-substituted ethoxyindoles and their evaluation for CNS effects.

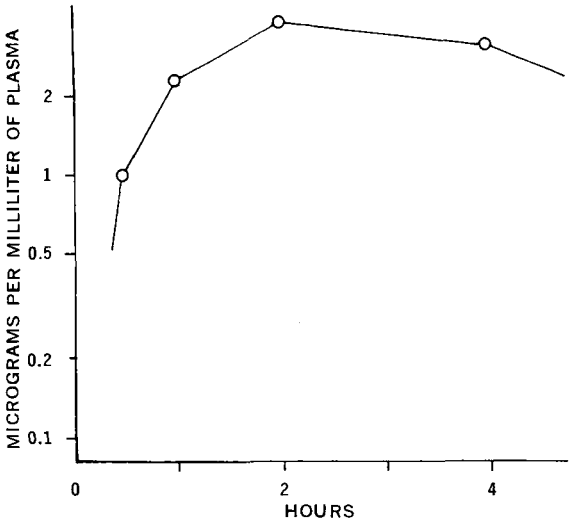

Figure 3—*Plasma concentrations of I in human subject following an oral dose of 3 g of I/m^2 in tablets.*

slow absorption through the GI mucosa. When given orally, I was slowly absorbed in all experiments, although an aqueous solution was used in rats and rabbits. The bioavailability of I is being studied.

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Synthesis of 2-(*N*-Arylcarboxamide)-3-substituted Ethoxyindoles and Their Monoamine Oxidase Inhibitory and Anticonvulsant Activities

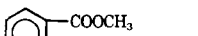
A. K. GUPTA [*], C. DWIVEDI [‡], T. K. GUPTA [*], SURENDRA S. PARMAR [*x], and RAYMOND D. HARBISON [‡]

Abstract □ 2-(*N*-Arylcarboxamide)-3-substituted ethoxyindoles were synthesized by the reaction of 2-(*N*-arylcarboxamide)-3-hydroxyindoles, which were obtained by the cyclization of 2-carbomethoxyphenylglycine-substituted anilides. These 2-(*N*-arylcarboxamide)-3-substituted ethoxyindoles were evaluated for their *in vitro* monoamine oxidase inhibitory ability and *in vivo* monoamine oxidase inhibitory property as evidenced by reserpine reversal response. Their anticonvulsant activity also was determined against pentylenetetrazol-induced seizures. No definite correlation could be observed between chemical structure and biological activity.

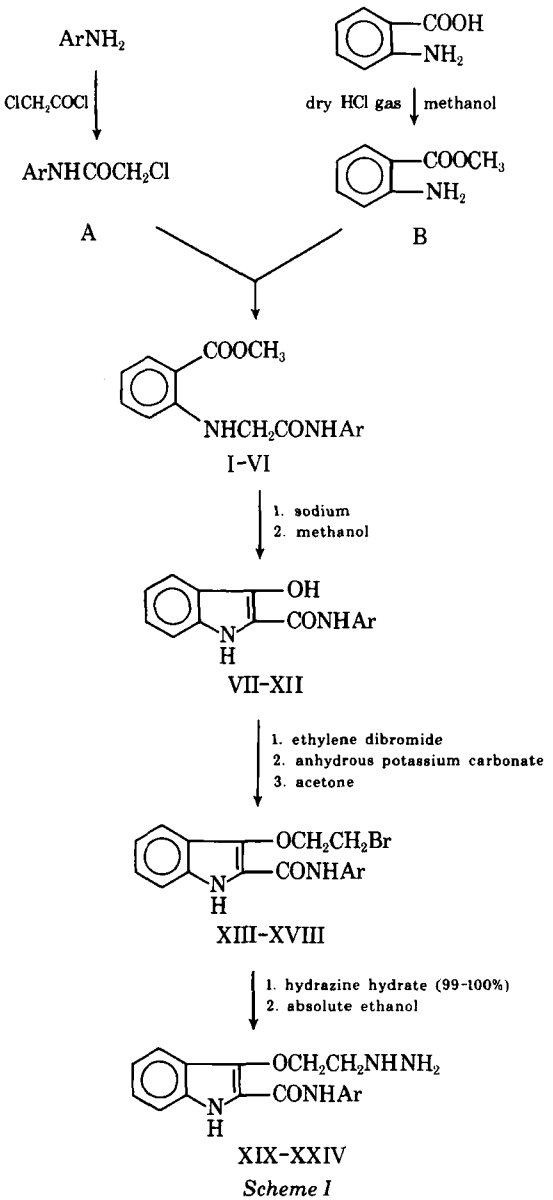
Keyphrases □ 2-(*N*-Arylcarboxamide)-3-substituted ethoxyindoles—synthesis, monoamine oxidase inhibitory and anticonvulsant activities □ Monoamine oxidase inhibitory activity—synthesis and screening of 2-(*N*-arylcarboxamide)-3-substituted ethoxyindoles, rat brain homogenate □ Anticonvulsant activity—synthesis and screening of 2-(*N*-arylcarboxamide)-3-substituted ethoxyindoles, mice

Identification of serotonin in the brain as a possible central neurohumoral agent (1) and the widespread occurrence of the indole nucleus among both naturally occurring and synthetic psychoactive compounds (2) stimulated an investigation of indole analogs with potent central nervous system (CNS) activity. In addition, clinical efficacy of 3-(2-aminobutyl)indole for the treatment of some types of depression (3) and the ability of indole derivatives to inhibit rat brain and rat liver monoamine oxidase (4, 5) and to protect against pentylenetetrazol-induced seizures (6, 7) prompted the synthesis of some 2-(*N*-arylcarboxamide)-3-substituted ethoxyindoles and their evaluation for CNS effects.

Table I—2-Carbomethoxyphenylglycine-substituted Anilides



Compound	Ar	Melting Point	Yield, %	Molecular Formula	Analysis, % Calc.	Found
I	α-$C_{10}H_7$	165°	62	$C_{20}H_{18}N_2O_2$	C 75.47	75.22
					H 5.66	5.51
					N 8.81	8.53
II	β-$C_{10}H_7$	171°	65	$C_{20}H_{18}N_2O_2$	C 75.47	75.32
					H 5.66	5.54
					N 8.81	9.17
III	2-$OC_2H_5C_6H_4$	128°	62	$C_{18}H_{20}N_2O_3$	C 69.23	69.01
					H 6.41	6.31
					N 8.97	9.22
IV	4-$OC_2H_5C_6H_4$	138°	70	$C_{18}H_{20}N_2O_3$	C 69.23	69.51
					H 6.41	6.23
					N 8.97	8.69
V	2,4-$(CH_3)_2C_6H_3$	155°	60	$C_{18}H_{20}N_2O_2$	C 72.97	74.13
					H 6.76	6.68
					N 9.46	9.17
VI	3,4-$(CH_3)_2C_6H_3$	140°	67	$C_{18}H_{20}N_2O_2$	C 72.97	72.69
					H 6.76	6.67
					N 9.46	9.73



Scheme I

In the present investigation, these indole derivatives were tested for their *in vitro* monoamine oxidase inhibitory property using kynuramine as a substrate and for their *in vivo* monoamine oxidase inhibitory property as evidenced by the reserpine reversal tests. Their anticonvulsant activity against pentylenetetrazol-induced seizures was also determined so that it could be correlated with their monoamine oxidase inhibitory property. These 2-(*N*-arylcarboxamide)-3-substituted ethoxyindoles were synthesized following the steps outlined in Scheme I.

EXPERIMENTAL

Chemistry—Arylamines, on treatment with chloroacetyl chloride, gave *N*-chloroacetyl derivatives (B) which, on reaction with methyl anthranilate (A), gave 2-carbomethoxyphenylglycine-substituted anilides (I–VI). These anilides were cyclized in the presence of sodium and methanol to the corresponding 2-(*N*-arylcarboxamide)-3-hydroxyindoles (VII–XII). These 3-hydroxyindoles were refluxed with ethylene dibromide in the presence of anhydrous potassium carbonate in acetone. This reaction gave 2-(*N*-arylcarboxamide)-3α-bromoethoxyindoles (XIII–XVIII) which, on treatment with hydrazine hydrate, yielded 2-(*N*-arylcarboxamide)- 3α-hydrazinoethoxyindoles (XIX–XXIV).

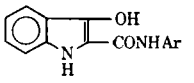
Analyses for carbon, hydrogen, and nitrogen were performed; melting points were taken in open capillary tubes.

***N*-Chloroacetyl Arylamines (A)**—A mixture of suitable arylamine (0.1 mole) and chloroacetyl chloride (0.11 mole) in dry benzene was refluxed on a steam bath for 4–5 hr. On cooling, the solid mass which separated was filtered, washed with water, dried, and recrystallized (8).

Methyl Anthranilate (B)—Anthranilic acid (0.20 mole) was introduced into absolute ethanol (100 ml) saturated with dry hydrogen chloride gas. The contents were refluxed for 2 hr, and the hot solution was poured into an excess of water. The pH of this solution was adjusted to 7.0 with sodium carbonate solution, and the solution was extracted with ether. The ether extract was dried, and removal of ether yielded methyl anthranilate, mp 23° (9).

2-Carbomethoxyphenylglycine-substituted Anilides (I–VI)—*N*-Chloroacetyl arylamine (0.03 mole) and methyl anthranilate (0.12 mole) were mixed and heated on a steam bath under anhydrous conditions. After 10 hr, 100 ml of dry benzene was added and the separated methyl anthranilate hydrochloride was removed by filtration. Benzene was removed by distillation from the filtrate. The residue was further heated for 8 hr, and the formed hydrochloride was again removed by filtration. The clear benzene solution was washed with 10% H_2SO_4, 10% Na_2CO_3, and water to re-

Table II—2-(*N*-Arylcarboxamide)-3-hydroxyindoles



Compound	Ar	Melting Point	Yield, %	Molecular Formula	Analysis, % Calc.	Found
VII	α-$C_{10}H_7$	235°	62	$C_{19}H_{14}N_2O_2$	C 75.50	75.73
					H 4.63	4.50
					N 9.27	9.03
VIII	β-$C_{10}H_7$	220°	65	$C_{19}H_{14}N_2O_2$	C 75.50	75.28
					H 4.63	4.70
					N 9.27	9.48
IX	2-$OC_2H_5C_6H_4$	153°	54	$C_{17}H_{16}N_2O_3$	C 68.92	68.68
					H 5.41	5.31
					N 9.46	9.65
X	4-$OC_2H_5C_6H_4$	210°	67	$C_{17}H_{16}N_2O_3$	C 68.92	68.73
					H 5.41	5.32
					N 9.46	9.73
XI	2,4-$(CH_3)_2C_6H_3$	204°	60	$C_{17}H_{16}N_2O_2$	C 72.86	72.62
					H 5.71	5.51
					N 10.00	10.29
XII	3,4-$(CH_3)_2C_6H_3$	225°	58	$C_{17}H_{16}N_2O_2$	C 72.86	73.13
					H 5.71	5.58
					N 10.00	9.72

move excess methyl anthranilate and dried. Excess benzene was removed by distillation; solid anilide, which separated on cooling, was collected by filtration, dried, and recrystallized. Crystals were characterized by their sharp melting points and elemental analyses (Table I).

2-(*N*-Arylcarboxamide)-3-hydroxyindoles (VII–XII)—To a solution of 2-carbomethoxyphenylglycine-substituted anilides (I–VI) (0.015 mole) in 15 ml of dry benzene with a few drops of methanol was added dry sodium (0.017 g-atom). This reaction mixture was refluxed on a steam bath for 2 hr under anhydrous conditions. The reaction mixture was allowed to cool, dry ether was added, and the residue was filtered and dissolved in cold water. The resulting solution was acidified with cold hydrochloric acid (50%), and the separated solid was filtered, dried, and recrystallized from suitable solvents. These crystals were characterized by their sharp melting points and elemental analyses (Table II).

2-(*N*-Arylcarboxamide)-3α-bromoethoxyindoles (XIII–XVIII)—To a solution of 0.011 mole of 2-(*N*-arylcarboxamide)-3-

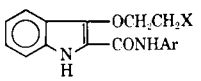


Table III—2-(*N*-Arylcarboxamide)-3-substituted Ethoxyindoles

Compound	Ar	X	Melting Point	Yield, %	Molecular Formula	Analysis, % Calc.	Found
XIII	α-$C_{10}H_7$	Br	100°	50	$C_{21}H_{17}BrN_2O_2$	C 61.61	61.42
						H 4.15	3.94
						N 6.85	7.11
XIV	β-$C_{10}H_7$	Br	113°	53	$C_{21}H_{17}BrN_2O_2$	C 61.61	61.87
						H 4.15	4.30
						N 6.85	6.58
XV	2-$C_2H_5C_6H_4$	Br	95°	48	$C_{19}H_{19}BrN_2O_3$	C 56.58	56.74
						H 4.71	4.61
						N 6.95	6.73
XVI	4-$C_2H_5C_6H_4$	Br	106°	55	$C_{19}H_{19}BrN_2O_3$	C 56.58	56.82
						H 4.71	4.64
						N 6.95	6.69
XVII	2,4-$(CH_3)_2C_6H_3$	Br	105°	50	$C_{19}H_{19}BrN_2O_2$	C 58.91	59.21
						H 4.91	4.79
						N 7.24	7.10
XVIII	3,4-$(CH_3)_2C_6H_3$	Br	138°	55	$C_{19}H_{19}BrN_2O_2$	C 58.91	58.63
						H 4.91	4.73
						N 7.24	7.51
XIX	α-$C_{10}H_7$	$NHNH_2$	125°	57	$C_{21}H_{20}N_4O_2$	C 70.00	70.28
						H 5.56	5.46
						N 15.56	15.31
XX	β-$C_{10}H_7$	$NHNH_2$	230°	69	$C_{21}H_{20}N_4O_2$	C 70.00	69.73
						H 5.56	5.66
						N 15.56	15.72
XXI	2-$C_2H_5C_6H_4$	$NHNH_2$	100°	45	$C_{19}H_{22}N_4O_3$	C 64.41	64.71
						H 6.22	6.13
						N 15.82	15.58
XXII	4-$C_2H_5C_6H_4$	$NHNH_2$	135°	58	$C_{19}H_{22}N_4O_3$	C 64.41	64.28
						H 6.22	6.08
						N 15.82	16.13
XXIII	2,4-$(CH_3)_2C_6H_3$	$NHNH_2$	145°	53	$C_{19}H_{22}N_4O_2$	C 67.45	67.73
						H 6.51	6.41
						N 16.57	16.33
XXIV	3,4-$(CH_3)_2C_6H_3$	$NHNH_2$	168°	55	$C_{19}H_{22}N_4O_2$	C 67.45	67.27
						H 6.51	6.38
						N 16.57	16.81

Table IV—Biological Activities of 2-(*N*-Arylcarboxamide)-3-substituted Ethoxyindoles[a]

Compound[b]	Approximate LD_{50}, mg/kg	Anticonvulsant Activity[c] Protection, %	Anticonvulsant Activity[c] 24-hr Mortality, %	Monoamine Oxidase Inhibition[d]
XIII	>1000	10	60	33.33 ± 1.04
XIV	>1000	0	80	45.24 ± 0.92
XV	>1000	20	80	23.81 ± 0.68
XVI	>1000	30	70	38.09 ± 0.62
XVII	>1000	20	80	28.57 ± 0.95
XVIII	>1000	20	80	33.34 ± 0.58
XIX	>1000	10	70	76.19 ± 1.21
XX	>1000	20	70	21.43 ± 1.10
XXI	1000	0	100	28.57 ± 0.82
XXII	1000	20	70	30.95 ± 0.77
XXIII	>1000	0	100	26.19 ± 0.58
XXIV	>1000	0	100	33.28 ± 0.49

[a] No reserpine reversal response was noted. [b] Compound numbers as given in Table III. [c] Compounds were administered at a dose of 100 mg/kg ip. [d] All values represent the mean with standard error calculated from three separate experiments done in duplicate. All compounds were dissolved in propylene glycol. An equivalent amount of propylene glycol was added to control tubes. Final concentrations of compounds and kynuramine were 2×10^{-4} and 1×10^{-4} *M*, respectively.

hydroxyindoles (VII–XII) in dry acetone was added 0.011 mole of ethylene dibromide and 0.011 mole of anhydrous potassium carbonate. This reaction mixture was refluxed on a steam bath for 15–20 hr. The reaction mixture was filtered, and excess acetone was removed by distillation. The crude product, which separated on cooling, was filtered, dried, and recrystallized from suitable solvents. These compounds were characterized by their sharp melting points and elemental analyses (Table III).

2-(*N*-Arylcarboxamide)-3α-hydrazinoethoxyindoles (XIX–XXIV)—A mixture of 0.004 mole of 2-(*N*-arylcarboxamide)-3α-bromoethoxyindole and 99–100% hydrazine hydrate (0.004 mole) in absolute ethanol was refluxed on a steam bath for 12–15 hr. Excess ethanol was removed by distillation. The crude product, which separated on cooling, was filtered, dried, and recrystallized from suitable solvents. These compounds were characterized by their sharp melting points and elemental analyses (Table III).

Determination of *In Vitro* Monoamine Oxidase Activity—Monoamine oxidase activity of rat brain homogenates, using kynuramine as the substrate, was determined by the spectrophotofluorometric method described by Dwivedi *et al.* (10). The 4-hydroxyquinoline formed during oxidative deamination of kynuramine was measured fluorometrically[1], using activating light of 315 nm and measuring fluorescence at the maximum of 380 nm. The percent inhibition was calculated from the decrease observed in absorbance.

Determination of *In Vivo* Monoamine Oxidase Inhibition by Reserpine Reversal Test—Male healthy mice, 20–25 g, were divided into groups of five and kept in separate cages. A group of five mice was used for each compound. The compounds were administered in a 5% suspension of gum acacia in a dosage of 200 mg/kg ip. Reserpine was administered (5 mg/kg ip) 4 hr after the administration of the compounds.

In the control group of mice, reserpine produced sedation, ptosis, and miosis; in animals pretreated with phenelzine and pargyline, known monoamine oxidase inhibitors, reserpine administration caused excitation, piloerection, mydriasis, and increased motor activity. Animals treated with test compounds were observed for these effects.

Determination of Anticonvulsant Activity—Groups of 10 male mice, 20–25 g, were used. Aqueous suspensions (5% gum acacia) of the test compounds were injected intraperitoneally at a dosage of 100 mg/kg. Pentylenetetrazol (80 mg/kg sc) was administered 4 hr after administration of the test compounds. Animals were observed 60 min for occurrence of seizures (10). The number of animals protected in each group was recorded and the percent protection was calculated. Mortality was recorded 24 hr after pentylenetetrazol treatment.

Determination of Approximate LD_{50}—The approximate LD_{50} was determined by the method of Verma *et al.* (11). All compounds were suspended in 5% aqueous gum acacia and administered intraperitoneally.

[1] Aminco Bowman spectrophotofluorometer.

RESULTS AND DISCUSSION

2-(*N*-Arylcarboxamide)-3-substituted ethoxyindoles inhibit brain monoamine oxidase activity (Table IV). All indole derivatives tested were found to inhibit rat brain monoamine oxidase at a concentration of 2×10^{-4} *M*. The various aryl substituents linked to a carboxamide moiety at position 2 of the indole nucleus have no significant effect on the degree of inhibition, except 2-(*N*-α-naphthylcarboxamide)-3α-hydrazinoethoxyindole (XIX) which produces a relatively higher degree of inhibition. The presence of α-bromoethoxy (XIII–XVIII) and α-hydrazinoethoxy (XIX–XXIV) groups at position 3 of the indole nucleus also had no significant effect on the degree of inhibition.

In vivo monoamine oxidase inhibitory activity of these compounds, as evidenced by the reserpine reversal test, is recorded in Table IV. These indole derivatives (XIII–XXIV) failed to produce a reserpine reversal response as observed with phenelzine and pargyline. These observations revealed that these compounds were inactive in producing *in vivo* monoamine oxidase inhibition.

The anticonvulsant activity of the indole derivatives ranged from 10 to 30% (Table IV). Compounds XIV, XXI, XXIII, and XXIV were completely devoid of anticonvulsant activity. These compounds also had lower protective ability against pentylenetetrazol-induced death. All indole derivatives had an approximate LD_{50} of either 1000 or >1000 mg/kg (Table IV). These data demonstrate the relatively low toxicity of these indole derivatives. No structure–activity relationships were noted.

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PHARMACEUTICAL ANALYSIS

GLC Trace Analysis of Dextromethorphan and Bromhexine Salts in Pharmaceutical Preparations

J. L. FABREGAS and A. MARGALET *

Abstract □ GLC proved to be particularly well suited for trace analysis of dextromethorphan and bromhexine salts in different pharmaceutical preparations, using diphenhydramine as the internal standard.

Keyphrases □ Dextromethorphan salts—GLC analysis in pharmaceutical preparations □ Bromhexine salts—GLC analysis in pharmaceutical preparations □ GLC—analysis, dextromethorphan and bromhexine salts in pharmaceutical preparations

Due to its bronchial mucolytic action, bromhexine has been incorporated in pharmaceutical preparations, especially since the preparation of *N*-(2-amino-3,5-dibromobenzyl)-*N*-cyclohexylmethylamine hydrochloride (1). Other studies reported its pharmacology (2), antitussive (3) and bronchosecretolytic activities (4), and metabolism (5, 6).

However, analytical procedures have been inadequate to determine bromhexine and dextromethorphan quantitatively in a pharmaceutical mixture, because of their similar solubilities, analogous UV absorptions (7, 8), and similar colorimetric reactions (9). Methods utilizing GLC analysis of dextromethorphan have been reported (10). None of these methods has been used for the quantitative determination of bromhexine. Under the conditions described here, GLC was particularly well suited for the analysis of dextromethorphan and bromhexine in pharmaceutical formulations containing antibiotics and essential oils.

Table I—Determination of Bromhexine and Dextromethorphan in Pharmaceutical Formulations

Formulation	Label Claim, mg	Range Found, mg	*SD*, mg	*CV*, %
Syrup				
Bromhexine	1.80	1.770–1.801	0.033	1.83
Dextromethorphan	2.90	2.904–2.940	0.037	1.28
Granulation				
Bromhexine	1.80	1.770–1.800	0.033	1.83
Dextromethorphan	2.90	2.860–2.901	0.042	1.45

EXPERIMENTAL

Chromatographic Conditions—A gas chromatograph[1] equipped with a −0.2–1.0-mv recorder[2] was used. A 1.5-m × 0.3-cm (5-ft × 0.125-in.) stainless steel column packed with 3% SE-30 (silicone rubber) on 100–120-mesh Varaport 30 was used. The column temperature was 180°, and the detector and injection port temperatures were 255 and 200°, respectively. The nitrogen carrier gas flow rate was 30 ml/min at 37 psi. A flame-ionization detector was used with a hydrogen flow rate of 30 ml/min and an air flow rate of 300 ml/min. The optimal working attenuation was 8×10^{-10} amp/mv.

Reagents—Carbon tetrachloride and chloroform[3], spectroscopic grade, were used.

Internal Standard—After a number of trials with other materials (codeine, ephedrine, chlorpromazine, and papaverine), diphenhydramine was chosen as the internal standard because of its commercial availability as a pure reagent[4] and its adequate separation from the other compounds, resulting in a symmetrical peak of low retention time (≃2.5 min).

For the internal standard solution, 30 mg of diphenhydramine hydrochloride was extracted with carbon tetrachloride, in alkaline media, following the procedure described for the sample preparation. The combined extracts were dried over anhydrous sodium sulfate and evaporated to dryness, and the residue was dissolved and diluted to 100 ml with carbon tetrachloride.

Preparation of Samples—An accurately weighed sample, containing dextromethorphan and bromhexine salts equivalent to

[1] Varian Aerograph 1740, Walnut Creek, Calif.
[2] Electronik 15, Honeywell, Inc., Philadelphia, Pa.
[3] Merck.
[4] Benadryl hydrochloride, Parke-Davis & Co., Detroit, Mich.

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Due to its bronchial mucolytic action, bromhexine has been incorporated in pharmaceutical preparations, especially since the preparation of *N*-(2-amino-3,5-dibromobenzyl)-*N*-cyclohexylmethylamine hydrochloride (1). Other studies reported its pharmacology (2), antitussive (3) and bronchosecretolytic activities (4), and metabolism (5, 6).

However, analytical procedures have been inadequate to determine bromhexine and dextromethorphan quantitatively in a pharmaceutical mixture, because of their similar solubilities, analogous UV absorptions (7, 8), and similar colorimetric reactions (9). Methods utilizing GLC analysis of dextromethorphan have been reported (10). None of these methods has been used for the quantitative determination of bromhexine. Under the conditions described here, GLC was particularly well suited for the analysis of dextromethorphan and bromhexine in pharmaceutical formulations containing antibiotics and essential oils.

Table I—Determination of Bromhexine and Dextromethorphan in Pharmaceutical Formulations

Formulation	Label Claim, mg	Range Found, mg	*SD*, mg	*CV*, %
Syrup				
Bromhexine	1.80	1.770–1.801	0.033	1.83
Dextromethorphan	2.90	2.904–2.940	0.037	1.28
Granulation				
Bromhexine	1.80	1.770–1.800	0.033	1.83
Dextromethorphan	2.90	2.860–2.901	0.042	1.45

EXPERIMENTAL

Chromatographic Conditions—A gas chromatograph[1] equipped with a −0.2–1.0-mv recorder[2] was used. A 1.5-m × 0.3-cm (5-ft × 0.125-in.) stainless steel column packed with 3% SE-30 (silicone rubber) on 100–120-mesh Varaport 30 was used. The column temperature was 180°, and the detector and injection port temperatures were 255 and 200°, respectively. The nitrogen carrier gas flow rate was 30 ml/min at 37 psi. A flame-ionization detector was used with a hydrogen flow rate of 30 ml/min and an air flow rate of 300 ml/min. The optimal working attenuation was 8×10^{-10} amp/mv.

Reagents—Carbon tetrachloride and chloroform[3], spectroscopic grade, were used.

Internal Standard—After a number of trials with other materials (codeine, ephedrine, chlorpromazine, and papaverine), diphenhydramine was chosen as the internal standard because of its commercial availability as a pure reagent[4] and its adequate separation from the other compounds, resulting in a symmetrical peak of low retention time (≃2.5 min).

For the internal standard solution, 30 mg of diphenhydramine hydrochloride was extracted with carbon tetrachloride, in alkaline media, following the procedure described for the sample preparation. The combined extracts were dried over anhydrous sodium sulfate and evaporated to dryness, and the residue was dissolved and diluted to 100 ml with carbon tetrachloride.

Preparation of Samples—An accurately weighed sample, containing dextromethorphan and bromhexine salts equivalent to

[1] Varian Aerograph 1740, Walnut Creek, Calif.
[2] Electronik 15, Honeywell, Inc., Philadelphia, Pa.
[3] Merck.
[4] Benadryl hydrochloride, Parke-Davis & Co., Detroit, Mich.

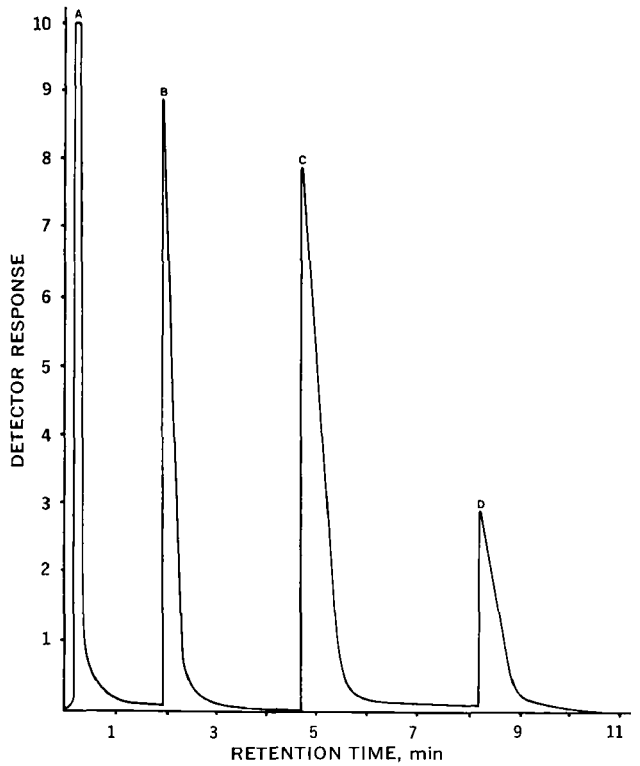

Figure 1—*Typical gas chromatogram. Key: A, chloroform; B, diphenhydramine; C, dextromethorphan; and D, bromhexine.*

2.3–3.2 and 1.4–2.5 mg of the respective bases, was extracted with five 35-ml portions of carbon tetrachloride in alkaline media. To the combined extracts, dried over anhydrous sodium sulfate, was added 5.0 ml of the internal standard solution. After evaporating to dryness, the residue was dissolved in 1.0 ml of chloroform (Solution A).

Preparation of Standards—For the working standard solution, a mixture was prepared of the same dextromethorphan and bromhexine salts[5] that were subjected to analysis and of known content as free bases. Four portions, equivalent to 110, 100, 90, and 80% of the formulation label claim, were accurately weighed and extracted following the procedure described for the sample preparation. To the extracts, dried over anhydrous sodium sulfate, 5.0 ml of the internal standard solution was also added. The resulting solutions were evaporated to dryness, and the four residues were dissolved in 1.0 ml of chloroform.

Assay Procedure—One microliter of the sample (Solution A) and standard solutions was injected into the gas chromatograph. The chromatograph was operated isothermally for the first 2 min, temperature-programmed at 2°/min, and switched to 12°/min just when the dextromethorphan peak reaches its maximum height. Under these conditions, the retention times relative to the internal standard were approximately 2.3 and 3.7 for dextromethorphan and bromhexine, respectively.

[5] The salts used in this study were prepared by the Research Division of Laboratorios Almirall, S.A., Barcelona, Spain.

Calculations—The peak areas were measured by multiplying their peak height times their width at half-height. Two standard curves were then established from the four standard tests by plotting the ratio of peak areas of dextromethorphan base to the internal standard against the weight of dextromethorphan base and the ratio of peak areas of bromhexine base to the internal standard against the weight of bromhexine base.

The concentrations of dextromethorphan and bromhexine bases in the sample were determined by computing the dextromethorphan–internal standard and bromhexine–internal standard peak area ratios, respectively, from the chromatogram and obtaining the corresponding weights from the standard curves. The values so obtained were then converted to percents of these two active ingredients in the original pharmaceutical formulation sample.

RESULTS AND DISCUSSION

To determine the recovery of bromhexine, samples were prepared containing eight different levels of known amounts of bromhexine base (1.55–2.55 mg); the precision of the chromatographic analysis was satisfactory. It was found that a linear relationship existed between the ratios of the observed peak areas of bromhexine base to the internal standard and the actual weight of bromhexine.

The results of this study led to the quantitative analysis of standard mixtures of bromhexine and dextromethorphan salts, with good agreement in the working ranges of 1.40–2.00 and 2.30–3.20 mg of the respective bases for 15 samples.

The results obtained under the experimental conditions and the good performance of the column during several months of continued use clearly indicate the column suitability for the proposed analytical system for conducting quality control assays of dextromethorphan and bromhexine in pharmaceutical preparations. The results are shown in Table I. A typical gas chromatogram is shown in Fig. 1.

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(8) *Ibid.*, p. 289.

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Quantitative GLC Determination of Conjugated Estrogens in Raw Materials and Finished Dosage Forms

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Abstract □ A GLC method is described for the quantitative analysis of conjugated estrogens. The procedure was used for the analysis of various estrogen mixtures obtained from formulations containing estrogen sodium sulfate salts. The method involves slurrying or dissolving a portion of the formulation in pH 5.2 buffer, hydrolysis with sulfatase enzyme, extraction with ethylene dichloride, and, finally, GLC separation and quantitation of the corresponding trimethylsilyl ethers on a 2.5% diethylene glycol succinate column.

Keyphrases □ Estrogen sodium sulfate salt formulations—enzymatic hydrolysis, GLC separation and analysis of conjugated estrogens □ Conjugated estrogen mixtures—enzymatic hydrolysis of estrogen sodium sulfate salt formulations, GLC separation and analysis □ GLC—separation, analysis, conjugated estrogens in estrogen sodium sulfate salt formulations

The analysis of conjugated estrogen mixtures has been the object of many studies. Various techniques such as TLC (1–3), liquid partition chromatography (4), colorimetric analysis (5), and GLC (6–9) have been used to separate and, in some cases, quantitate hydrolyzed estrogen mixtures of varying complexity. Gel permeation chromatography (10) also has been used in the partial resolution of some estrogen mixtures in conjugated form.

However, most reported methods are of limited use in completely separating and quantitating equine estrogen mixtures containing nine or more structurally similar estrogens. In many cases, these methods are not suitable for routine use because they may involve harsh hydrolysis techniques, lengthy extraction and purification steps, long elution times, and/or complex chemistry in the development of characteristic colors prior to quantitation.

These problems initiated the development of an assay method that would conveniently and completely hydrolyze the conjugated estrogens without creating artifacts and that would separate and quantitate the individual free estrogens. GLC appeared to offer the most promising method for separating and quantitating free estrogen mixtures. To accomplish this goal, it was necessary to develop a quantitative procedure for the complete conversion of the steroid conjugates to the free estrogens. After extensive investigation involving hot acid, solvolysis, and enzyme hydrolysis, the enzyme procedure was selected because it provided complete hydrolysis in the presence of tablet and other excipients without causing degradation.

Other criteria imposed and satisfied by this method are that it be relatively rapid, involve a limited number of steps, and offer a high degree of precision and accuracy. The method developed involves enzyme hydrolysis of the estrogen conjugates, a single extraction step, formation of the trimethylsilyl ether derivatives, and, finally, GLC separation on a very polar, selective column. The major estrogen peaks (up to 11) are then quantitated with disk or digital integration, using testosterone as the internal standard.

EXPERIMENTAL

Instrumentation—A research grade gas chromatograph[1] equipped with a U-shaped column and flame-ionization detector, together with a recorder[2] fitted with a disk integrator or equivalent, was used. A silanized (11) 1.8-m (6-ft) U-shaped glass column, 4 mm i.d., was packed with 2.5% diethylene glycol succinate[3] on 100–120 Gas Chrom Q[3]. This packing is prepared by using a 2.0–2.5% solution of the polyester in acetone and by making use of the absorptive properties of the solid support as described previously (12, 13).

Thorough degassing of the solid support under vacuum is also required to get a uniform liquid phase coating and to minimize tailing. The fluidized packing (14) is carefully packed into a column and conditioned with oxygen-free[4] nitrogen carrier gas at 200° overnight at 45 ml/min. Several injections of a silylating reagent[5] are made to achieve a stable baseline. After conditioning and when the column is not in use, the column oven temperature should be lowered to 175°.

Chromatographic Conditions—The following were utilized: column oven temperature, 195 ± 5°; detector temperature, 240°; injection port temperature, 225°; nitrogen flow rate, 45 ml/min; hydrogen flow rate, 40 ml/min; air flow rate, 1.8 S.C.F.H.; and range and attenuation, 100-10-1K (1×10^{-9} amp).

Reagents and Standards—The following required reagents and solvents were all of reagent grade quality or equivalent: 3A ethanol, ethylene dichloride, acetic acid, redistilled dry pyridine, anhydrous sodium sulfate, and anhydrous sodium acetate. The nine free estrogens used to determine chromatographic response factors relative to testosterone[6] were of reference standard quality[7].

Working Enzyme Solution—Sufficient enzyme concentrate[8] is taken and diluted to 50 ml with distilled water to give a working solution containing about 1000 ± 100 units of sulfatase enzyme/ml. All enzyme solutions should be stored at 4° until use.

Buffer Solution—The pH 5.2 buffer is prepared by combining 21.0 ml of 0.2 *M* acetic acid solution and 79.0 ml of 0.2 *M* sodium acetate solution. The solution should be checked on a pH meter and adjusted, if necessary, to pH 5.2 ± 0.1 unit.

Standard Steroid Solutions—Standard solutions of the steroids are prepared in 3A ethanol with the approximate desired concentrations (±10%) as follows: 17α-estradiol, 15 μg/ml; 17β-estradiol, 2 μg/ml; 17α-dihydroequilin, 45 μg/ml; 17β-dihydroequilin, 6 μg/ml; 17α-dihydroequilenin, 6 μg/ml; 17β-dihydroequilenin, 6 μg/ml; estrone, 180 μg/ml; equilin, 90 μg/ml; equilenin, 18 μg/ml; and testosterone, 100 μg/ml.

Standard Solution—One milliliter of each of the ten standards

[1] Bendix model 2500 and F&M model 400 were used in these studies.
[2] Honeywell 19 recorder.
[3] Hi-Eff 1B, Applied Science Laboratories, State College, Pa.
[4] Oxy-Sorb Trap, Regis Chemical Co., Morton Grove, Ill.
[5] Regisil [bis(trimethylsilyl)trifluoroacetamide plus 1% trimethylchlorosilane].
[6] Analytical grade, Nutritional Biochemicals Inc., Cleveland, Ohio.
[7] Ayerst Research Laboratories, Montreal, Quebec, Canada.
[8] Glusulase, Endo Laboratories, Garden City, N.Y.

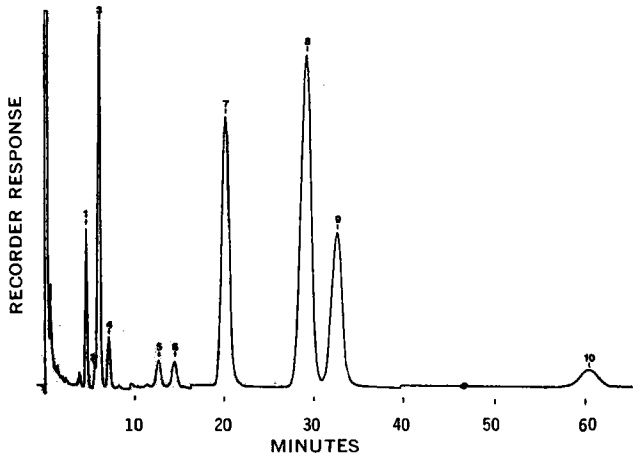


Figure 1—*Separation of the components of a nine-estrogen standard with testosterone as the trimethylsilyl ethers. The peaks with their relative retention times are: 1, 17α-estradiol (0.230); 2, 17β-estradiol (0.272); 3, 17α-dihydroequilin (0.299); 4, 17β-dihydroequilin (0.353); 5, 17α-dihydroequilenin (0.627); 6, 17β-dihydroequilenin (0.714); 7, testosterone (1.00); 8, estrone (1.452); 9, equilin (1.615); and 10, equilenin (2.988).*

is pipetted into a 12-ml centrifuge tube equipped with a Teflon-lined screw cap. Just prior to use, the solutions are evaporated to dryness at 40° in a water bath. The residue is dissolved in 15 μl of pyridine and 65 μl of silylating reagent[5], mixed well, and allowed to stand 15 min prior to injection. (The solution should be refrigerated when not in use and discarded after 24 hr.)

Preparation of Sample Extracts—*Tablets*—Randomly select 10 tablets from the sample and weigh to the nearest 0.1 mg. Place them in a mill[9] with one large and one small steel ball and mill them for 3 min. Collect the powder in an amber bottle and weigh a portion to the nearest 0.1 mg into a 60-ml (2-oz) extraction bottle fitted with a Teflon-lined screw cap. The sample weight should be approximately equivalent to one tablet so that from 0.3 to 2.5 mg of conjugated estrogens is taken for assay.

Add 15 ml of pH 5.2 buffer to the bottle and cap tightly. Shake mechanically for 0.5 hr, sonify for 30 sec in a sonic bath, and shake for an additional 0.5 hr. Add approximately 1000 units of sulfatase enzyme to the bottle and incubate for 20 min with shaking in a 50 ± 2° water bath. Immediately, without cooling the enzyme hydrolysate, pipet 15 ml of ethylene dichloride into the bottle, cap tightly, and shake mechanically for 15 min. Centrifuge the bottle at ~2000 rpm for 10 min and repeat if necessary to obtain a clear lower layer. Remove as much of this ethylene dichloride layer as possible and filter through a small amount of sodium sulfate (~5 g) in a 3.5-cm plastic funnel, containing a glass wool plug, into a 12-ml centrifuge tube.

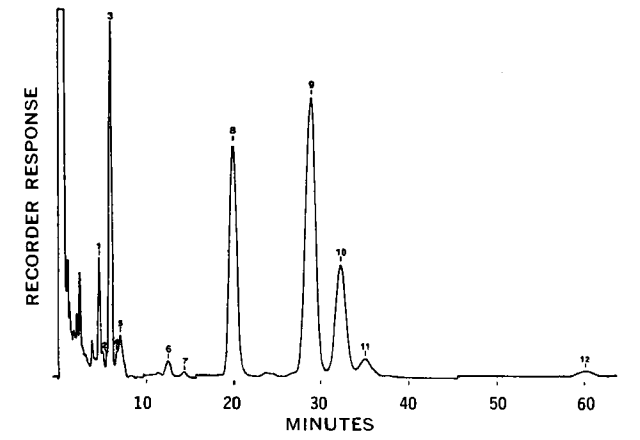


Figure 2—*Separation of a mixture of estrogen trimethylsilyl ethers derived from Sample A (see Table II). The peaks with their relative retention times are: 1, 17α-estradiol (0.235); 2, 17β-estradiol (0.280); 3, 17α-dihydroequilin (0.304); 4, iso-17α-dihydroequilin (0.337); 5, 17β-dihydroequilin (0.356); 6, 17α-dihydroequilenin (0.624); 7, 17β-dihydroequilenin (0.715); 8, testosterone (1.00); 9, estrone (1.443); 10, equilin (1.604); 11, Δ[8,9]-dehydroestrone (1.743); and 12, equilenin (2.980).*

[9] Spex.

Table I—Characteristics of Different Batches of Diethylene Glycol Succinate Liquid Phase

Measurement	Batch 1922	Batch 1989
IR, cm^{-1}	3530, 3460, 3260, 2960, 2880	3550, 3470, 2970, 2900
TGA	Two-step weight loss curve	Single-step weight loss curve
Viscosity[a], centistokes	0.646	0.739
Titration[b], mEq H^+/g	1.00	0.143
Water[c], %	1.03	0.86

[a] These are relative measurements obtained on a 5% solution of polymer in acetone at 25° using a Ubbelohde viscometer, model 1B-290. [b] The polymer was dissolved in dimethylformamide and titrated with tetrabutylammonium hydroxide in benzene–methanol (8:1). [c] The Karl Fischer method was used.

Injectable Vials—Carefully remove the caps from five lyophilized vials and add 5 ml of water to each vial. Recap, shake to dissolve the cake, pipet 2 ml from each vial into a 25-ml volumetric flask, and then dilute to volume. Pipet 1 ml into a 60-ml (2-oz) extraction bottle fitted with a Teflon-lined screw cap and add 15 ml of pH 5.2 buffer and 1000 units of sulfatase enzyme. Proceed with the hydrolysis and remaining steps described under *Tablets*.

Raw Materials—Given an approximate potency for the powdered sample, weigh one sample containing about 7.5 mg of conjugated estrogens into a 120-ml (4-oz) extraction bottle fitted with a Teflon-lined screw cap. Pipet 75 ml of pH 5.2 buffer into the bottle and shake mechanically for 10 min. Sonify the sample for 0.5 min in a sonic bath and then shake for an additional 5 min. Immediately pipet 15 ml of uniform suspension into a 60-ml (2-oz) extraction bottle. Add 1000 units of sulfatase enzyme and proceed with the hydrolysis and remaining steps under *Tablets*.

Instrument Standardization—Once the instrument has reached its operating temperature, the column should be conditioned with several injections of silylating reagent until a stable baseline is obtained. Inject about 2–4 μl of silylated standard and adjust the sample size and/or instrument attenuation so the major peaks (17α-dihydroequilin, testosterone, and estrone) are greater than 70% of full-scale deflection. To establish the identity of the standard estrogen peaks, compare their relative retention times (RRT) *versus* testosterone with those indicated in Fig. 1.

Measure accurately the areas of the estrogen and testosterone peaks using disk integration. Assume an exponential decay of the solvent peak and draw in this curve to meet the observed baseline points after 17α-estradiol and after 17β-dihydroequilin. Drop perpendiculars from each valley between the pertinent peaks to the projected baseline. This procedure will allow proper allocation of the peak areas to the estrogens involved.

Using the measured peak areas and known concentrations, calculate a response factor (RF) for each of the nine estrogens as follows:

$$RF = \left(\frac{A_t}{C_t}\right)\left(\frac{C_e}{A_e}\right) \qquad \text{(Eq. 1)}$$

where A_t = area of testosterone peak, C_t = micrograms of internal standard (testosterone) in standard, C_e = micrograms of estrogen in standard, and A_e = area of estrogen peak.

The standard is injected again following the last sample scan of

Table II—Compositions of Various Commercial Samples Containing Mixtures of Estrogen Sodium Sulfate Salts[a]

Component[b]	Sample											
	A[c]	B[c]	C[c]	D[c]	E[d]	F[d]	G[d]	H[e]	I[f]	J[g]	K[h]	L[h]
I	0.063	0.002	0.005	0.048	0.004	—	0.009	—	0.12	0.85	2.70	1.77
II	0.009	0.003	0.229	—	0.003	0.022	0.005	—	0.05	0.14	—	0.29
III	0.217	0.039	—	0.071	0.007	—	0.080	—	0.69	3.43	0.40	6.78
IV	0.028	0.005	—	—	—	—	0.011	—	0.11	0.37	—	0.94
V	0.019	0.013	—	0.036	0.006	—	0.006	—	—	0.49	2.60	0.58
VI	0.007	—	—	—	—	—	—	—	—	0.13	—	0.29
VII	0.747	0.786	0.806	0.653	0.488	0.792	0.612	0.788	13.21	13.74	271.0	22.82
VIII	0.395	0.335	0.329	0.330	0.100	0.040	0.028	0.116	6.75	7.17	27.7	12.11
IX	0.028	0.037	0.122	0.036	0.052	0.023	—	0.017	0.58	0.82	2.9	0.69
Total	1.513	1.240	1.491	1.174	0.660	0.877	0.751	0.921	21.51	27.14	307.3	46.27

[a] These numbers represent an average of two separate determinations. [b] All components are expressed as the estrogen sodium sulfate salts. [c] Values are reported as milligrams per tablet, and samples have a label claim of 1.25 mg of conjugated estrogens/tablet. [d] Samples have a label claim of 0.625 mg of conjugated estrogens/tablet, and all values are reported as milligrams per tablet. [e] Sample of international origin has a label claim of 1.00 mg of conjugated estrogens/tablet with values as milligrams per tablet. [f] An international sample with a label claim of 20 mg/vial with all values reported as milligrams per vial. [g] A sample of international origin with a label claim of 25 mg/vial with values reported as milligrams per vial. [h] Powdered raw material samples with values reported as milligrams per gram.

the day, and the RF's are calculated and averaged with the first run. The average RF's are then used in subsequent sample calculations.

Chromatographic Analysis—Inject a portion of silylated sample. The injection size and/or instrument attenuation should again be adjusted to keep the major peaks greater than 70% of full scale. The sample estrogen peaks can be identified by comparing their RRT's *versus* testosterone with those indicated on the sample GLC scans (Figs. 2–4). Accurately measure the areas of the estrogen and testosterone peaks using disk integration.

For the peak clusters that are not completely resolved, the areas are allocated by dropping a perpendicular from the minimum to a baseline projected across the base of the peak group. For the quantitation of two naturally occurring double bond isomers, iso-17α-dihydroequilin and $\Delta^{8,9}$-dehydroestrone, two approaches can be taken. They can be listed separately and quantitative values can be reported for each, or their areas can be added to the areas of 17α-dihydroequilin and equilin, respectively, since they are structurally closely related. For this study the second approach is used since it allows a more direct comparison of the GLC values obtained here with those values obtained using an official method (17). With this procedure, a single injection from each of four samples can be assayed in between two injections of the standard on a single column in 1 day.

The weight of the individual conjugated estrogens present in the samples is calculated from the response factors, the sample peak areas, and the constant; the weights of the individual species are then summed to get the total weight of conjugated estrogens present in the sample as follows:

$$\text{weight conjugated estrogens} = \text{constant } (RF_e)(A_e) = \frac{\text{mg conjugated estrogens}}{\text{unit dosage form (tablet, vial, grams)}} \quad \text{(Eq. 2)}$$

and:

$$\text{constant} = \frac{15 \text{ ml } (1.38)(10^{-3} \text{ mg}/\mu\text{g})(C_t)}{x \text{ ml } (y)(A_t)} \text{ (dilution factor)} \quad \text{(Eq. 3)}$$

where:

15 ml = extract volume of ethylene dichloride
1.38 = factor for converting "free" estrogens into their corresponding estrogen sodium sulfate salts
10^{-3} mg/μg = conversion factor for micrograms to milligrams
C_t = micrograms of testosterone in sample
dilution factor = inverse of any additional dilution of any sample
x ml = number of milliliters of ethylene dichloride extract taken for assay
y = equivalent number of dosage form units taken into the assay (tablets, vials, grams)
A_t = area of testosterone peak in sample

RESULTS AND DISCUSSION

Sample Preparation—During the early phases of this development work, attempts were made to extract the estrogen conjugates from tablet or formulation excipients. Initial results from recovery studies of estrone sodium sulfate from placebo tablets showed some small and unreproducible losses. Also, the multiple extractions required proved tedious and time consuming. Therefore, the technique of suspending a portion of the powdered formulation in buffer, in the presence of sulfatase enzyme, was tried. This straightforward simple procedure was found to give consistent, quantitative recoveries.

Various studies with mineral acid hydrolysis of estrogen conjugates (15, 16) have shown that side reactions and steroidal transformations frequently occur. Since interest was in measuring the original components of the mixture without creating artifacts during workup, acid hydrolysis was not used.

The level of enzyme necessary to achieve complete hydrolysis in the presence of excipients was also studied. Earlier work with very high levels of enzyme indicated that protein binding of the estrogens and thick emulsions would be problems. The lower levels of enzyme used in this method (~1000 units of arylsulfatase/0.3–2.5 mg of conjugated estrogens) give complete hydrolysis of the conjugated estrogens within a short time without causing sample preparation problems. A more detailed study of the enzyme procedure will appear elsewhere.

Approximately 45 tablet samples from almost as many different manufacturers were assayed with this method. For two samples, the enzyme levels specified here were not high enough to achieve

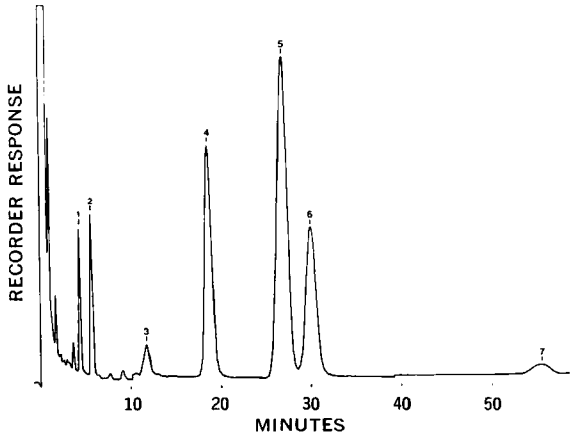
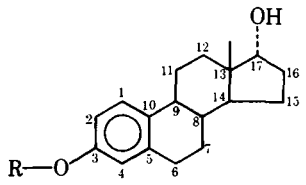
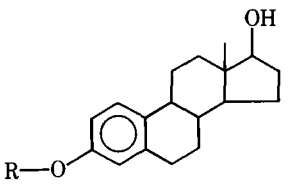


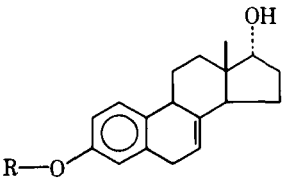
Figure 3—*Separation of a mixture of estrogen trimethylsilyl ethers derived from Sample D (see Table II). The peaks with their relative retention times are: 1, 17α-estradiol (0.233); 2, 17α-dihydroequilin (0.299); 3, 17α-dihydroequilenin (0.626); 4, testosterone (1.00); 5, estrone (1.441); 6, equilin (1.604); and 7, equilenin (2.957).*



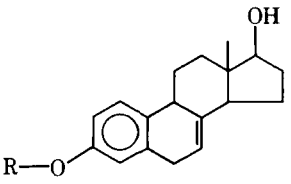
I: 17α-estradiol sodium sulfate



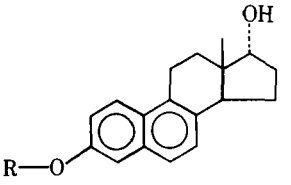
II: 17β-estradiol sodium sulfate



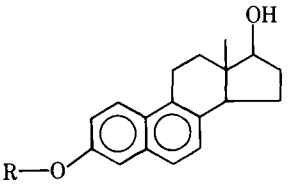
III: 17α-dihydroequilin sodium sulfate



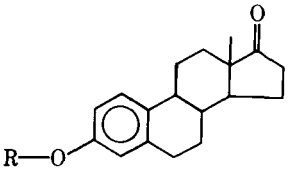
IV: 17β-dihydroequilin sodium sulfate



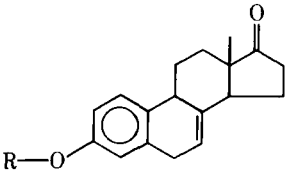
V: 17α-dihydroequilenin sodium sulfate



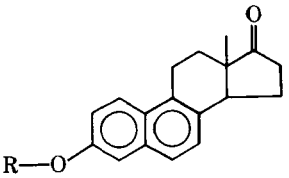
VI: 17β-dihydroequilenin sodium sulfate



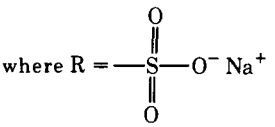
VII: estrone sodium sulfate



VIII: equilin sodium sulfate



IX: equilenin sodium sulfate

where R = 

complete hydrolysis, because of the presence of certain tablet excipients that inhibited enzyme activity. A 5–20-fold increase in enzyme activity was necessary to get complete hydrolysis and consistent recoveries in these two instances. Phosphate, a known inhibitor of sulfatase activity, was found in these two samples.

Chromatography—A systematic study of a large number of liquid phases was carried out in an attempt to resolve complex estrogen mixtures of the type obtained from pregnant mare's urine. These mixtures are known to contain estrone, equilin, equilenin, 17α- and 17β-estradiols, 17α- and 17β-dihydroequilins, and 17α- and 17β-dihydroequilenins (1); the structures of these steroids as the sodium sulfate salts are as shown (I–IX).

Only two commercial liquid phases were found, both of which were polyesters (ethylene glycol succinate and diethylene glycol succinate), that did separate the major components of the estrogen mixture. The diethylene glycol succinate, however, offered the most promise because of its slightly improved thermal stability and its ability to perform the separation of the estrogen trimethylsilyl ethers in a reasonable time. Two major estrogens, estrone and equilin, from urinary equine extracts are almost completely resolved on this column, and excellent quantitative results can be obtained. To the knowledge of the authors, a quantitative GLC method that allows the direct determination of estrone and equilin simultaneously has not been reported previously.

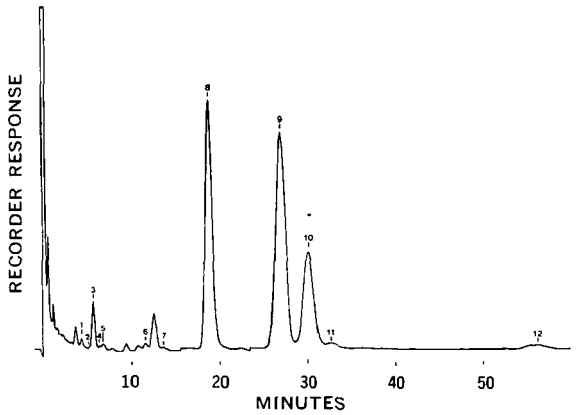


Figure 4—*Separation of a mixture of estrogen trimethylsilyl ethers derived from Sample I (see Table II). The peaks with their relative retention times are: 1, 17α-estradiol (0.232); 2, 17β-estradiol (0.281); 3, 17α-dihydroequilin (0.300); 4, iso-17α-dihydroequilin (0.333); 5, 17β-dihydroequilin (0.355); 6, 17α-dihydroequilenin (0.609); 7, 17β-dihydroequilenin (0.721); 8, testosterone (1.00); 9, estrone (1.427); 10, equilin (1.592); 11, $\Delta^{8,9}$-dehydroestrone (1.734); and 12, equilenin (2.969).*

The repeated preparation of diethylene glycol succinate columns showed that not all columns gave the same degree of peak resolution and peak symmetry. Some difference existed between the batches of liquid phase obtained from the same source. Therefore, IR, viscosity, thermogravimetric analysis (TGA), titration, and water measurements were made in an attempt to pinpoint the factors accounting for variations in the quality of the batches of polymer tested. The results are summarized in Table I for two batches of diethylene glycol succinate, one of which gave an excellent column packing (Batch 1922) and the other a poor packing (Batch 1989).

Interpretation of the data in Table I allows several conclusions to be made about the relative differences between the two batches of liquid phase tested. The batch of diethylene glycol succinate giving a high quality packing is more acidic (by both IR and titration) and has a lower viscosity; furthermore, the two-step TGA curve suggests that a wide range of molecular weight polymer units is present since a weight loss occurs at a temperature believed to be below the degradation temperature (~225°). These techniques and relative values are now being used on a routine basis to evaluate new batches of liquid phase prior to use.

In an attempt to control operator performance in reproducibly preparing the packings, TGA is used to monitor all new column packings. An acceptable weight range for the liquid phase loading varies from about 2.0 to 2.5%.

Quantitation—The quantitative composition of 12 different commercial samples containing conjugated estrogens is found in Table II. The table includes results for tablets of varying strengths together with raw material samples; the numbers represent an average of two individual determinations. The data clearly show the gravimetric quantities of all individual estrogenic components present in the samples for the first time. By summing these values, a total potency value is obtained which may differ significantly and is usually higher than the potency value obtained using an official method (17).

SUMMARY

A GLC method was developed for the quantitative analysis of complex mixtures containing conjugated estrogens. The method was used on both raw materials and finished dosage forms. It allows, for the first time, a complete delineation and quantitation of the conjugated estrogens present in extracts derived from preg-

nant mare's urine. Since the quality of the diethylene glycol succinate liquid phase and packed column is critical to the success of the method, details are provided relating to their use and conditioning.

Preliminary collaborative testing is underway to evaluate the use of this procedure as a routine control method. This method has also been used for the analysis of free estrogens, which might be present in formulations containing conjugated estrogens, by simply omitting the hydrolysis step and carrying out the balance of the procedure.

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Abstract □ A rapid and simple method for the assay of pholcodine in various syrup and linctus formulations, based on color reaction with *p*-dimethylaminobenzaldehyde, is suggested. At specified conditions, the results obtained show good reproducibility.

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Although pholcodine, like codeine, is a common component in syrups and linctus formulations, little has been written concerning the assay methods of pholcodine in pharmaceutical preparations. It is often the sole active component in a preparation[1] but may be combined with ephedrine hydrochloride, papaverine hydrochloride, promethazine, *etc.*

[1] Examples for such preparations are Copholco (Wade Co.), Falcodyl (Norton), Sancos (Sandoz), *etc.*

DISCUSSION

Pholcodine linctus formulations have appeared in the BPC (2). The official assay procedure involves extraction with chloroform from an alkaline solution followed by nonaqueous titration. The accuracy of the method depends on the purity of inactive components, such as sucrose, sorbitol, glycerol, citric acid, and chloroform spirit, used in the formulation. Since these components constitute the majority of the linctus weight, any impurity may introduce error. Theoretically, this difficulty would be overcome by running a blank linctus containing all components except pholcodine. However, since there are so many formulations and variations, running a blank cannot be considered a practical solution. This method also suffers from the use of a relatively dilute titrant (0.02 *N* $HClO_4$) which affects the sharpness of the end-point.

No method or modification has been proposed (2) for the assay of pholcodine combined with other drugs. The nonaqueous titration cannot be applied, since the free bases of most drugs commonly formulated with pholcodine are miscible in chloroform and would be titrated simultaneously with the pholcodine. This is the case with ephedrine, pseudoephedrine, oxomemazine, promethazine, codeine, promazine, papaverine, chlorpheniramine, trimeprazine, *etc.* Other methods of analysis of pholcodine in syrups are the reaction with hexamethylenetetramine–lactose reagent (3) (ephedrine interferes) and TLC (4, 5).

The method proposed in this paper utilizes the reaction of pholcodine with *p*-dimethylaminobenzaldehyde in strong acidic medium. Under the specified conditions, reproducible results are obtained in the assay of pholcodine when formulated alone or in combination with other drugs. The present method also has the

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The method proposed in this paper utilizes the reaction of pholcodine with *p*-dimethylaminobenzaldehyde in strong acidic medium. Under the specified conditions, reproducible results are obtained in the assay of pholcodine when formulated alone or in combination with other drugs. The present method also has the

Table I—Assay Results of Pholcodine in Commercial Pharmaceuticals

Preparation	Labeled Amount, mg/ml	Found, mg/ml	Mean	*SD*	Coefficient of Variation, %
1. Pholcodine	1.00	0.960, 0.980, 0.970	0.970	0.0100	1.031
Ephedrine hydrochloride	1.00				
Ammonium chloride	15.00				
2. Pholcodine	1.00	0.990, 0.970, 0.975	0.978	0.0104	1.063
Ephedrine hydrochloride	1.00				
Pyrilamine maleate	2.50				
Ammonium chloride	15.00				
3. Pholcodine	0.80	0.790, 0.795, 0.795	0.792	0.0032	0.400
Ephedrine hydrochloride	0.80				
4. Pholcodine	1.60	1.590, 1.600, 1.630	1.607	0.0208	1.294
Bromoform	0.03				
5. Pholcodine	1.00	0.965, 0.980, 0.995	0.980	0.0150	1.531
Ephedrine hydrochloride	1.60				
Chlorpheniramine maleate	0.40				
6. Pholcodine	1.00	0.960, 0.975, 1.010	0.982	0.0256	0.607
Papaverine hydrochloride	0.20				

advantage of determining pholcodine without preliminary separation from other combined drugs.

EXPERIMENTAL

Reagents and Chemicals—The following were used: dilute sulfuric acid, 10% (w/v); dilute ammonium hydroxide, 10% (w/v); chloroform (analytical reagent) and ether (analytical reagent); and pholcodine standard BP.

p-Dimethylaminobenzaldehyde reagent was prepared just prior to use by dissolving 1 g of *p*-dimethylaminobenzaldehyde (analytical reagent) in 100 ml of sulfuric acid, 70% (w/v).

Standard Solution—A solution of pholcodine standard, 0.4 mg/ml, was prepared by dissolving the solid in dilute sulfuric acid.

Sample Solution—An accurately weighed portion of syrup or linctus, equivalent to about 20 mg of pholcodine, was transferred into a 125-ml separator. The sample was made alkaline by addition of dilute ammonium hydroxide solution. The mixture was extracted successively with four 30-ml portions of chloroform. The chloroform extracts were combined, washed with 10 ml of distilled water, and evaporated to dryness. The residue obtained was dissolved in dilute sulfuric acid and diluted to a volume of 100 ml.

Procedure—Volumes of 0.5 ml of sample and standard solutions were separately transferred, in triplicate, into a series of test tubes. Dilute sulfuric acid (0.5 ml) was pipetted into another test tube to serve as a blank. *p*-Dimethylaminobenzaldehyde reagent (10 ml) was added; after vigorous shaking, the tubes were transferred into a water bath maintained at 60°. After 10 min, the tubes were removed and quickly cooled to room temperature. At 0.5 hr after removal from the bath and cooling to room temperature, the absorbance was read at a wavelength of 450 nm against blank reagents.

If the syrup contains orange extract, two preliminary extractions are necessary using a mixture of 15 ml of ether and 1 ml of dilute sulfuric acid. Then the procedure is followed beginning with "The sample was made alkaline . . ." under *Sample Solution*.

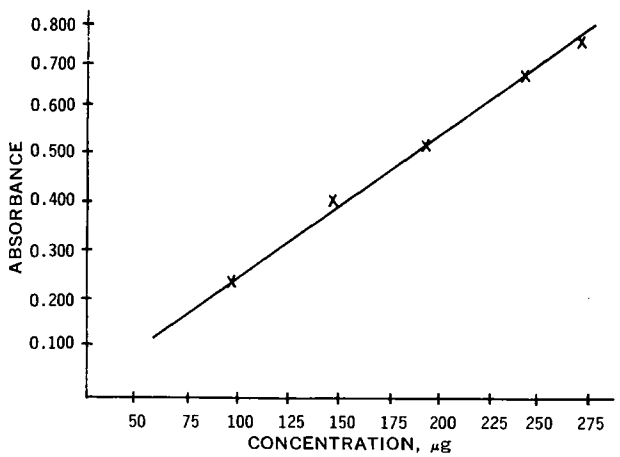

Figure 1—*Standard curve for pholcodine.*

RESULTS

When this method was tested on pholcodine samples containing various amounts of the drug, strict obedience to the Beer–Lambert law was achieved over the range of 50–250 μg of pholcodine (Fig. 1).

Both the accuracy and precision of the proposed method were checked on drug mixtures simulating existing commercial pharmaceuticals. Table I represents the composition of the pharmaceuticals, the labeled amounts of the active components, the amounts of pholcodine found by triplicate analysis, and the related interpretation of data. As shown, high accuracy and a small acceptable coefficient of variation were obtained. Table I also shows that the reaction of pholcodine with *p*-dimethylaminobenzaldehyde is specific as far as combination with common drugs is concerned.

The dependence of the absorbance on time, measured on samples just cooled to room temperature, is summarized in Table II. According to Table II, the color of the reaction product is sufficiently stable. The color reaction of pholcodine with *p*-dimethylaminobenzaldehyde is as sensitive as that of codeine with the same reagent (6, 7). The reaction mixture of codeine is not stable in light, but the reaction of pholcodine is not affected by such limitations.

Table II—Dependence of Absorbance on Time

Minutes	Absorbance	Change in Absorbance, %
0	0.485	—
5	0.485	—
10	0.488	0.62
20	0.492	1.44
30	0.496	2.27
60	0.505	4.13

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Determination of Iodochlorhydroxyquin and Corticosteroids in Pharmaceutical Formulations

ROBERT E. GRAHAM *x, CHARLES T. KENNER ‡, and E. R. BIEHL ‡

Abstract □ Iodochlorhydroxyquin was separated from various corticosteroids using an acetonitrile–diatomaceous earth column. Iodochlorhydroxyquin was eluted with cyclohexane, and the corticosteroid was eluted with chloroform. Iodochlorhydroxyquin was determined by both a UV absorbance method and a new complexiметric method using the nickel chelate of iodochlorhydroxyquin. The corticosteroid was determined by the blue tetrazolium and isoniazid procedures. The average percent recovery for these four methods was 100.8, 99.4, 100.7, and 99.9, respectively, for 10 known mixtures. The standard deviation for the absorbance for 10 determinations of the nickel complex was 0.002 absorbance unit (0.31%). Various characteristics of the nickel and other complexes were evaluated, including the sensitivity, solubility, and wavelength of maximum absorbance in 14 different solvents. The analyses of 23 typical products are reported, for which the standard deviation, expressed as a percentage of the amount declared, was 1.31% for the UV, 1.34% for the complexiметric, 1.49% for the blue tetrazolium, and 1.22% for the isoniazid procedures. Methods of determination in the presence of interferences are discussed.

Keyphrases □ Iodochlorhydroxyquin and corticosteroid formulations—partition chromatographic separation, complexiметric analysis of iodochlorhydroxyquin, blue tetrazolium analysis of corticosteroid □ Corticosteroid and iodochlorhydroxyquin formulations—partition chromatographic separation, complexiметric analysis of iodochlorhydroxyquin, blue tetrazolium analysis of corticosteroid □ Partition chromatography—separation, iodochlorhydroxyquin and corticosteroid formulations □ Nickel complex formation—analysis, iodochlorhydroxyquin after separation from iodochlorhydroxyquin and corticosteroid formulations

The "American Drug Index 1973" (1) lists 36 manufacturers of pharmaceutical formulations that contain iodochlorhydroxyquin (5-chloro-7-iodo-8-quinolinol) (I). Five of these manufacturers prepare products containing only I, while 33 manufacture products containing I plus a corticosteroid such as hydrocortisone, hydrocortisone acetate, or prednisolone.

USP XVIII (2) specifies an IR procedure that is specific for I in creams, ointments, and suppositories. NF XIII (3) requires the same IR technique to determine I in creams, lotions, and ointments containing I plus hydrocortisone and uses the blue tetrazolium method for the corticosteroid. IR methods specific for I in pharmaceutical products were described (4, 5), but these procedures are difficult to use due to the volatility and odor of the carbon disulfide and the small volumes required in the extraction step.

Other reported methods include UV absorption (3), gravimetry (3), combustion in an oxygen flask followed by titration (6), polarography (7), fluorescence (8), GLC (9), and spectrophotometry (10–12). Spectrophotometric methods depend upon the formation of a colored metallic complex with I. The iron (III) complex (10, 11) and the copper (II) complex (12) have been utilized for the determination of I in certain pharmaceutical products.

This paper reports a simple, rapid, quantitative method for the separation of I from corticosteroids by partition chromatography using the diatomaceous earth–acetonitrile column described previously (13, 14). After separation, I is determined by conversion to the nickel complex and measurement of the absorption at 406 nm while the corticosteroid is analyzed by the blue tetrazolium procedure of USP XVIII (15). Twenty-one different pharmaceutical formulations of creams, lotions, ointments, and suspensions were analyzed by the proposed method. When necessary, the method may be supplemented by the use of IR (3), GLC (9), or TLC (16) to detect the presence of impurities such as 8-hydroxyquinoline, 5-chloro-8-hydroxyquinoline, 5-iodo-8-hydroxyquinoline, 5,7-diiodo-8-hydroxyquinoline, and 5,7-dichloro-8-hydroxyquinoline.

EXPERIMENTAL

Equipment—The following were used: a UV–visible recording

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Determination of Iodochlorhydroxyquin and Corticosteroids in Pharmaceutical Formulations

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Abstract □ Iodochlorhydroxyquin was separated from various corticosteroids using an acetonitrile–diatomaceous earth column. Iodochlorhydroxyquin was eluted with cyclohexane, and the corticosteroid was eluted with chloroform. Iodochlorhydroxyquin was determined by both a UV absorbance method and a new complexiметric method using the nickel chelate of iodochlorhydroxyquin. The corticosteroid was determined by the blue tetrazolium and isoniazid procedures. The average percent recovery for these four methods was 100.8, 99.4, 100.7, and 99.9, respectively, for 10 known mixtures. The standard deviation for the absorbance for 10 determinations of the nickel complex was 0.002 absorbance unit (0.31%). Various characteristics of the nickel and other complexes were evaluated, including the sensitivity, solubility, and wavelength of maximum absorbance in 14 different solvents. The analyses of 23 typical products are reported, for which the standard deviation, expressed as a percentage of the amount declared, was 1.31% for the UV, 1.34% for the complexiметric, 1.49% for the blue tetrazolium, and 1.22% for the isoniazid procedures. Methods of determination in the presence of interferences are discussed.

Keyphrases □ Iodochlorhydroxyquin and corticosteroid formulations—partition chromatographic separation, complexiметric analysis of iodochlorhydroxyquin, blue tetrazolium analysis of corticosteroid □ Corticosteroid and iodochlorhydroxyquin formulations—partition chromatographic separation, complexiметric analysis of iodochlorhydroxyquin, blue tetrazolium analysis of corticosteroid □ Partition chromatography—separation, iodochlorhydroxyquin and corticosteroid formulations □ Nickel complex formation—analysis, iodochlorhydroxyquin after separation from iodochlorhydroxyquin and corticosteroid formulations

The "American Drug Index 1973" (1) lists 36 manufacturers of pharmaceutical formulations that contain iodochlorhydroxyquin (5-chloro-7-iodo-8-quinolinol) (I). Five of these manufacturers prepare products containing only I, while 33 manufacture products containing I plus a corticosteroid such as hydrocortisone, hydrocortisone acetate, or prednisolone.

USP XVIII (2) specifies an IR procedure that is specific for I in creams, ointments, and suppositories. NF XIII (3) requires the same IR technique to determine I in creams, lotions, and ointments containing I plus hydrocortisone and uses the blue tetrazolium method for the corticosteroid. IR methods specific for I in pharmaceutical products were described (4, 5), but these procedures are difficult to use due to the volatility and odor of the carbon disulfide and the small volumes required in the extraction step.

Other reported methods include UV absorption (3), gravimetry (3), combustion in an oxygen flask followed by titration (6), polarography (7), fluorescence (8), GLC (9), and spectrophotometry (10–12). Spectrophotometric methods depend upon the formation of a colored metallic complex with I. The iron (III) complex (10, 11) and the copper (II) complex (12) have been utilized for the determination of I in certain pharmaceutical products.

This paper reports a simple, rapid, quantitative method for the separation of I from corticosteroids by partition chromatography using the diatomaceous earth–acetonitrile column described previously (13, 14). After separation, I is determined by conversion to the nickel complex and measurement of the absorption at 406 nm while the corticosteroid is analyzed by the blue tetrazolium procedure of USP XVIII (15). Twenty-one different pharmaceutical formulations of creams, lotions, ointments, and suspensions were analyzed by the proposed method. When necessary, the method may be supplemented by the use of IR (3), GLC (9), or TLC (16) to detect the presence of impurities such as 8-hydroxyquinoline, 5-chloro-8-hydroxyquinoline, 5-iodo-8-hydroxyquinoline, 5,7-diiodo-8-hydroxyquinoline, and 5,7-dichloro-8-hydroxyquinoline.

EXPERIMENTAL

Equipment—The following were used: a UV–visible recording

Table I—Recovery (Percent) of Prepared Standards

Sample Number[a]	Iodochlorhydroxyquin		Hydrocortisone	
	Complexiometric Method	UV Method	Blue Tetrazolium Method	Isoniazid Method
1	99.8	101.1	100.7	100.6
2	99.0	100.6	100.8	99.2
3	99.2	100.8	100.8	100.4
4	99.8	101.3	100.3	100.4
5	99.5	100.5	100.6	99.7
6	99.2	100.8	100.2	99.6
7	99.4	100.5	100.3	100.0
8	99.5	101.1	100.4	99.7
9	99.6	100.6	101.6	100.8
10	99.4	101.1	99.3	98.6
Average	99.4	100.8	100.5	99.9
SD[b]	0.3	0.3	1.0	0.7

[a] Each standard mixture contained approximately 6 mg of iodochlorhydroxyquin and 1 mg of hydrocortisone. [b] Calculated from the range by the method of Dean and Dixon (18).

spectrophotometer[1] with 1-cm quartz cells, a glass chromatographic column (2.2 × 25 cm constricted at one end to 0.4 × 5 cm) plus aluminum tamping rod, and an electrobalance[2].

Reagents—*Solvents*—All solvents were spectrograde, analytical reagent grade, pesticide grade, USP, distilled-in-glass, or nanograde. Absolute ethanol, acetone, acetonitrile, alcohol USP, carbon tetrachloride, chloroform, cyclohexane, dimethyl sulfoxide, dioxane, acetic acid, *n*-heptane, *n*-hexane, methanol, methylene chloride, and 2,2,4-trimethylpentane were used.

Acetonitrile–Cyclohexane (Mutually Saturated)—Mix 20 ml of acetonitrile and 270 ml of cyclohexane (sufficient for two determinations plus a reagent blank) in a separator, agitate vigorously for 2 min, and allow to stand with occasional swirling until both layers are clear. These mutually saturated solutions must be used whenever acetonitrile or cyclohexane is called for in these directions.

Chloroform (Water Saturated)—Add 50 ml of water to 250 ml of chloroform in a separator, agitate for 2 min, and allow both layers to clarify. This water-saturated reagent is required when aqueous trap layers are inserted but should not be used otherwise.

Diatomaceous Earth[3]—Acid washed was used.

Nickel Reagent (0.010 M)—Dissolve 248.8 mg of nickel (II) acetate tetrahydrate in absolute ethanol with heating on a steam bath. Cool and dilute to 100.0 ml with absolute ethanol. This solution is stable for at least 6 months. Other metal-ion reagents (0.050 *M*) were prepared for method development studies using the acetates in ethanol or methanol.

Standards—All solid standards were USP or NF reference standards. Prepare corticosteroid standards (0.0100 mg/ml) in alcohol USP and I standards (0.0600 mg/ml) in cyclohexane. Individually weighed samples of the standards were used in some studies.

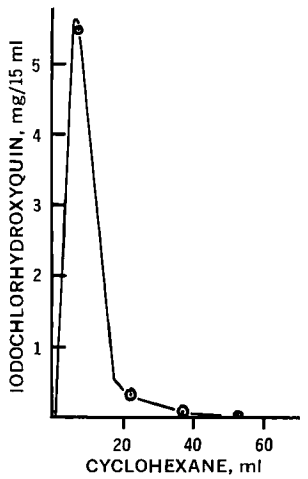

Figure 1—*Elution curve for iodochlorhydroxyquin.*

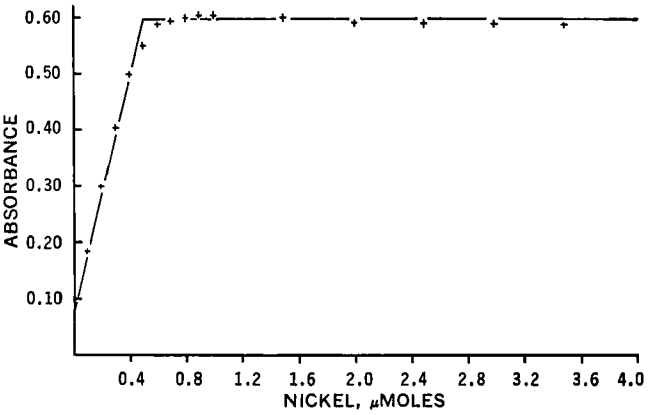

Figure 2—*Mole ratio study for nickel–iodochlorhydroxyquin complex.*

Sample Preparation—*Creams, Ointments, Lotions, Jellies, and Suspensions*—Prepare a composite sample by mixing thoroughly the contents of several containers and accurately weigh a sample equivalent to about 6 mg of I into a 100-ml beaker. Dissolve in 1.5 ml of acetonitrile plus 1.5 ml of cyclohexane with slight warming on a steam bath (1.5 ml of methanol plus 1.5 ml of water may be substituted for these solvents when necessary for complete solution of the formulation). Continue as directed under *Sample Layer*.

Standards—Accurately weigh (or pipet volumes that contain) about 1 mg of corticosteroid and 6 mg of I into a 100-ml beaker. Proceed as directed in *Creams, Ointments, Lotions, Jellies, and Suspensions*, beginning with "Dissolve in 1.5 ml of acetonitrile plus . . . ."

Column Preparation—*Acetonitrile Layer*—Insert a glass wool plug in the bottom of a chromatographic column. Thoroughly mix 4.0 g of diatomaceous earth with 4.0 ml of acetonitrile, transfer to the column, and pack firmly.

Trap Layer—Prepare and insert a neutral aqueous trap layer, as needed, according to Graham *et al.* (14).

Sample Layer—Thoroughly mix the dissolved sample or standard, prepared as directed under *Sample Preparation*, with 3.0 g of diatomaceous earth. Transfer to the column above the acetonitrile or trap layer and pack firmly. Dry wash the sample beaker, tamping rod, spatula, and funnel with 1 g of diatomaceous earth and transfer to the column. Dry wash the same equipment with

Table II—Characteristics of Complexes in Various Solvents

Solvent	Nickel Complex		Zinc Complex		Iron Complex	
	A	λ_{max}	*A*	λ_{max}	*A*	λ_{max}
Acetone	0.565	412	0.474	405	0.132	620
Acetic acid	0.574	378	—[a]	—[a]	0.133	630
Methylene chloride	0.574	401	0.509	395	0.229	620
Chloroform	0.592	402	0.468	398	0.239	620
Alcohol USP	0.592	400	0.507	400	0.301	645
Methanol	0.593	404	0.523	400	0.323	670
Dioxane	0.608	413	0.360	405	0.120	620
Ethanol (100%)	0.626	403	0.507	401	0.327	660
2,2,4-Trimethylpentane	0.636	405	0.463	405	0.146	605
Carbon tetrachloride	0.637	405	0.435	402	ND[b]	ND
Hexane	0.638	405	0.483	403	0.204	615
Acetonitrile	0.640	414	0.494	402	0.174	625
Cyclohexane	0.655	406	0.498	403	0.204	615
Dimethyl sulfoxide	0.666	418	0.531	407	0.065	645

[a] Complex does not form at this low pH. [b] ND = not determined.

[1] Cary model 15.
[2] Cahn model G2.
[3] Celite 545, Johns-Manville Product Corp., New York, NY 10016

Table III—Analysis of Typical Products

Sample Number[a]	Type of Product	Iodochlorhydroxyquin		Corticosteroid	
		UV Method	Complexiметric Method	Blue Tetrazolium Method	Isoniazid Method
		Average Amount Found, % of Declared[b]			
1	Suspension	104.2 (2)	102.2 (2)	116.3 (2)	113.0 (2)
2	Cream	104.1 (6)	102.5 (6)	107.1 (4)	105.8 (4)
3	Cream	107.4 (6)	106.0 (6)	98.7 (2)	103.6 (2)
4	Cream	121.9 (6)	119.4 (6)	114.1 (2)	110.9 (2)
5	Cream	107.2 (2)	106.8 (2)	99.6 (2)	103.6 (2)
6	Lotion	83.5 (6)	83.1 (5)	75.1 (6)	92.9 (6)
7	Jelly	101.7 (6)	101.0 (6)	101.2 (2)	101.3 (2)
8	Cream	83.3 (8)	86.3 (10)	107.5 (5)	116.6 (4)
9	Ointment	103.7 (4)	101.0 (4)	102.6 (4)	104.6 (4)
10	Cream	119.4 (2)	118.2 (2)	111.0 (2)	112.8 (2)
11	Cream	103.6 (2)	102.6 (2)	104.4 (2)	104.4 (2)
12	Ointment	101.6 (2)	98.2 (2)	102.5 (2)	99.8 (2)
13	Cream	119.7 (3)	119.0 (3)	111.5 (3)	113.3 (3)
14	Cream	106.8 (2)	104.2 (2)	102.5 (2)	101.1 (2)
15	Cream	105.1 (2)	102.6 (2)	102.1 (2)	104.8 (2)
16	Cream	107.9 (4)	106.4 (6)	102.8 (4)	103.7 (4)
17	Ointment	ND[c]	118.9 (4)	109.8 (4)	109.6 (2)
18	Cream	110.0 (4)	109.0 (4)	101.0 (4)	106.2 (2)
19	Cream				
	Tube 1	ND[c]	97.4 (4)	109.2 (4)	133.4 (2)
	Tube 2	ND[c]	153.0 (2)	129.9 (2)	163.2 (2)
20	Cream	105.7 (4)	105.9 (4)	108.9 (4)	110.4 (2)
21	Cream	99.2 (2)	99.2 (2)	94.6 (2)	98.6 (2)
22	Cream				
	Tube 1	104.0 (4)	103.5 (4)	52.0 (4)	53.6 (4)
	Tube 2	103.0 (2)	102.9 (2)	100.8 (2)	105.5 (2)
23	Cream				
	Tube 1	ND[c]	325.4 (4)	113.1 (4)	117.5 (4)
	Tube 2	ND[c]	324.9 (2)	116.0 (2)	118.9 (2)
SD[d]		1.31	1.34	1.49	1.22

[a] Components present in each sample are listed in Table IV. [b] Number in parentheses indicates number of determinations. [c] Not determined due to interference in UV spectra caused by a cyclohexane-soluble complex or an ingredient other than I. [d] Expressed as percent of amount declared calculated from the difference in duplicates (20).

glass wool, transfer to the column, and pack firmly. Retain the beaker for washing with cyclohexane and chloroform during the *Column Elution* step.

Column Elution—*Compound I*—Wash the beaker successively with six 20-ml portions of cyclohexane and pour into the column, maintaining the liquid head between 8 and 12 cm above the column bed and catching the effluent in a 100-ml volumetric flask since the column holds up approximately 23 ml. Allow the last wash to drain completely, wash the tip of the column with cyclohexane, make the effluent to volume, and retain for determination as described under *Determinative Procedures*.

Corticosteroids—Rinse the sample beaker with 125 ml of chloroform in several portions and pour each through the column, maintaining the liquid head as close to the top of the column as possible and catching the effluent in a 150-ml beaker. Allow the last portion to drain completely from the column and rinse the tip with alcohol USP. Carefully evaporate the effluent just to dryness on a steam bath in a hood with a current of air to ensure complete removal of the acetonitrile. Dissolve in alcohol USP and dilute accurately to a volume containing approximately 10 μg/ml of corticosteroid. Retain for determination as directed under *Determinative Procedures*.

Determinative Procedures for I—*Ultraviolet Method*—Mix the cyclohexane effluent thoroughly and scan from 500 to 280 nm *versus* cyclohexane as the reference. Measure net absorbance from the baseline to the absorbance maximum at 326 nm. Calculate by comparison with standards run concurrently.

Complexiметric Method—Pipet 5.00-ml aliquots of the cyclohexane effluents for each sample and standard into small glass-stoppered flasks. Prepare a reagent blank using 5.00 ml of cyclohexane. To each flask, add 0.1 ml of nickel reagent, swirl, let stand for at least 1 min, and then scan from 500 to 340 nm *versus* the reagent blank. Measure the net absorbance of the maximum at 406 nm and calculate by comparison with the standards run concurrently.

Determination Procedures for Corticosteroids—*Isoniazid Method*—The procedure of Umberger (17) was used, except that the concentration of hydrochloric acid was doubled to increase the sensitivity.

Blue Tetrazolium Method—The procedure given in USP XVIII (15) was followed without modification.

METHOD DEVELOPMENT

Column Characteristics—*Elution*—A column was prepared as directed under *Column Preparation*, using 6.050 mg of standard I and fitting the column with a separator to allow the maintenance of a 12-cm liquid head which ensured a constant flow rate of 7 ml/min. A total of 285 ml of cyclohexane was collected in 15-ml fractions. Each fraction was taken to dryness carefully under air on a steam bath, dissolved in and diluted to an appropriate volume with acetic acid, and scanned from 500 to 280 nm.

The elution curve shown in Fig. 1 indicates that all of I is completely eluted in the first 60 ml of cyclohexane. In the proposed procedure, the column holds up approximately 23 ml of the 120 ml of cyclohexane used so that the retention of 97 ml provides an adequate safety factor. In several cases, a second 100 ml of cyclohexane was poured through the column. No I was found in any of these effluents. Tests on several alcoholic extracts remaining from the corticosteroid determinations also were negative for I.

Separation and Recovery—Ten separate standard mixtures containing approximately 6 mg of I and 1 mg of hydrocortisone were analyzed by the proposed procedure using all four determinative methods (Table I). The overall average recovery was 100.2%, and the overall average standard deviation was less than 0.6%. Portions of each sample effluent were evaporated, dissolved in small volumes, spotted on TLC plates, and chromatographed. In each case, a single spot appeared, showing complete separation.

Characteristics of the Nickel–I Complex—*Beer's Law*—During the investigation, samples containing 0.030–0.83 mg of I/5.1 ml showed a linear relationship between absorbance and concentration.

Table IV—Sample Ingredients

Sample Number[a]	Ingredient Numbers[b]
1	(1), (7), (14), (15), (18), (19), (26), (36), (38), (39), (52)
2	(10), (17), (26), (28), (29), (31), (36), (37), (43), (52)
3	(5), (8), (14), (17), (24), (26), (41), (45), (48), (52), (53)
4	(15), (17), (26), (31), (32), (33), (37), (46), (47), (52)
5	(13), (17), (35), (49), (52)
6	(6), (10), (17), (23), (25), (26), (36), (52)
7	(9), (17)
8	(2), (3), (17), (22), (26), (31), (36), (37), (39), (42), (48), (52)
9	(8), (17), (37), (41), (52), (53)
10	(17), (26), (31), (36), (44), (48), (51), (52)
11	(4), (12), (17)
12	(4), (12), (17)
13	(17), (26), (31), (36), (44), (48), (51), (52)
14	(8), (16), (17), (26), (31), (36), (37), (39), (43), (47), (52)
15	(17), (26), (27), (36), (37), (39), (48), (52), (53)
16	(17), (26), (27), (36), (37), (39), (48), (52), (53)
17	(17), (27), (53)
18	(8), (15), (17), (20), (21), (26), (30), (36), (37), (45), (52)
19	(17), (26), (27), (36), (40), (52), (54)
20	(10), (17), (26), (28), (29), (31), (36), (37), (43), (52)
21	(4), (12), (17)
22	(13), (17), (35), (49), (52)
23	(10), (11), (14), (17), (26), (36), (41), (47), (48), (50), (52)

[a] Number listed in Table III. [b] Ingredient list: (1) acetic acid, (2) acetylated lanolin, (3) aluminum acetate, (4) hydrophilic ointment base [Aquaphor (Duke)], (5) beeswax, (6) carboxymethylcellulose, (7) carboxymethylene disodium ethylenediaminetetraacetic acid, (8) cetyl alcohol, (9) chlorobutanol, (10) citric acid, (11) coal tar solution, (12) cold cream, (13) free fatty alcohols, (14) glycerin, (15) glyceryl monostearate, (16) heather aroma, (17) hydrocortisone, (18) hydrocortisone acetate, (19) isopropyl myristate, (20) isopropyl palmitate, (21) lanolin, (22) lanolin alcohols extract, (23) lidocaine, (24) liquid petrolatum, (25) magnesium aluminum silicate, (26) methylparaben, (27) mineral oil, (28) mineral wax, (29) mono- and diglycerides of fat-forming fatty acids, (30) neomycin base, (31) petrolatum, (32) polyoxyethylene oxypropylene stearate, (33) polyoxyethylene stearate, (34) polyoxyl 40 stearate, (35) pramoxine hydrochloride, (36) propylparaben, (37) propylene glycol, (38) sodium acetate, (39) sodium bisulfite, (40) sodium borate, (41) sodium lauryl sulfate, (42) sorbitan sesquioleate, (43) sorbitol, (44) sorbitan fatty acid esters (Span 60), (45) spermaceti, (46) squalene, (47) stearic acid, (48) stearyl alcohol, (49) sulfonated and polyoxylated fatty alcohols, (50) triethanolamine, (51) hydrophilic ether esters (Tween 80), (52) water, (53) white petrolatum, and (54) white wax.

Mole Ratio—The mole ratio study (Fig. 2) showed that the complex contained 2 moles of I for each mole of nickel.

Absorbance Replication—Ten separate 5.00-ml aliquots of standard I in cyclohexane were pipetted into small glass-stoppered flasks, 0.1 ml of nickel reagent was added, and the resulting solutions were scanned from 500 to 340 nm. The average net maximum absorbance at 406 nm was 0.645 with a standard deviation calculated from the range (18) of 0.002.

Stability—The absorbance of a sample of I in cyclohexane was measured at 1-min intervals after the addition of the nickel reagent until the absorbance became constant and was then monitored periodically for 24 hr. The procedure was repeated several times and was also repeated using I in acetic acid. In all cases, the absorbance reached a maximum within 1 min and remained constant for 24 hr.

Volatility—Samples of the nickel complex with I were prepared in methanol and carefully evaporated under air on the steam bath just to dryness. Two of the dry samples were left on the bath for 40 min and two were left for 90 min, after which they were dissolved and the absorbance was measured. There was no loss due to volatility in either case.

Volatility of I—Twelve separate 5.00-ml aliquots of standard I were pipetted into small glass-stoppered flasks and placed under air on the steam bath. Eight aliquots were removed as soon as they reached dryness, two remained on the bath 30 min after dryness, and the last two remained on the bath 60 min after dryness. Each sample was dissolved in 5.00 ml of acetic acid, 0.10 ml of nickel reagent was added, and the solution was scanned from 500 to 280 nm *versus* a reagent bank. The recoveries for the first eight samples averaged 100.1% (range 98.6–101.2%) while the second two averaged 64% and the last two 49%. As a consequence, the cyclohexane effluent from the column should not be evaporated to dryness before the determination of I.

Metal Complexes with I (Solvent Effect)—Standard solutions of I were prepared in 14 different solvents. Separate 5.00-ml aliquots were treated with 0.1 ml of nickel reagent and scanned as in the proposed procedure against the reagent blank. The study was repeated with an iron (III) reagent (0.050 *M* in ethanol) and with a zinc (II) reagent (0.050 *M* in ethanol). The nickel and zinc solutions were scanned from 500 to 340 nm, and the iron solutions were scanned from 720 to 380 nm.

The net absorbance and the wavelength of maximum absorbance of each complex in each solvent are shown in Table II. The nickel complex with I was the most sensitive in all solvents, and the iron (III) complex was the least sensitive. The net absorbances for the nickel and the zinc complexes were at a maximum in dimethyl sulfoxide, but the iron complex showed minimum absorbance in this solvent and maximum absorbance in absolute ethanol. Cyclohexane appears to be a satisfactory solvent for all three complexes. The wavelength of maximum absorbance depended in part upon the solvent and varied from 378 to 418 nm for the nickel complex, from 395 to 407 nm for the zinc, and from 605 to 670 nm for the iron.

This experiment was repeated using only acetic acid as the solvent but using different metal reagents (0.050 *M* in ethanol or methanol). Of the metal ions tested, magnesium (II) and manganese (II) did not react, zinc (II) reacted partially, and copper (II), cobalt (II), and nickel (II) reacted completely. The reagent blanks were then scanned against acetic acid. There was a significant absorbance at the wavelength of maximum absorbance only in the case of the copper reagent blank.

RESULTS AND DISCUSSION

The analyses of 23 typical formulations containing both I and corticosteroids are shown in Table III. The standard deviation, expressed as the percentage of the amount declared, was 1.31% for the UV, 1.34% for the complexiметric, 1.49% for the blue tetrazolium, and 1.22% for the isoniazid procedures. Samples 6 and 22 were low in hydrocortisone while Samples 1, 19, and 23 were above the recommended limits set by USP. Samples 6 and 8 were low in I while Samples 4, 10, 13, 17, 19, and 23 were high. For Samples 3, 5, 6, 8, 18, 19, 21, and 22, comparison of the corticosteroid values by the blue tetrazolium and isoniazid methods indicates from 4 to 34% decomposition of the corticosteroid according to the test of Graham *et al.* (19). Application of the variation of absorbance with time test suggested in the same reference indicates interferences in the blue tetrazolium procedure with Samples 1, 4, and 5.

Samples 3, 4, 5, 7, 8, 10, 13, 14, 15, 17, 19, and 23 were varying shades of yellow, which was taken to indicate varying amounts of decomposition of I caused by the reaction with metal ions present in the ingredients or containers to form metal chelates. The presence of metal chelates of I in samples containing I often can be determined quantitatively by slight modifications of the proposed complexiметric procedure since all of the metal chelates studied are converted to the nickel complex upon addition of the nickel reagent. Two possibilities exist: *(a)* the metal chelate is not removed from the column with cyclohexane but is removed by the chloroform and *(b)* the metal chelate is removed from the column with the cyclohexane.

Sample 8, which contains aluminum acetate, is an example of the first type. When a sample from one tube was run by the proposed procedure, only 91.0% of the amount declared was found to be present. A portion of the chloroform eluate was concentrated by evaporation, the nickel reagent was added, and the solution was scanned against a blank of the nickel reagent in chloroform. Calculations showed that 16.0% of the amount of I declared was present in the chloroform eluate. A second sample from the same tube composite was dissolved directly in chloroform; total I was determined by adding the nickel reagent and was found to be 105.9%,

which agrees satisfactorily with the sum of 107.0% found separately. The presence of a metal–I complex of this type is indicated whenever the residue resulting from the evaporation of the chloroform fraction does not completely dissolve in the alcohol solvent used for the determination of the corticosteroid.

The presence of a cyclohexane-soluble metal complex of I is indicated when the amount of I cannot be determined by the UV procedure due to interference in the absorbance at 326 nm and when there is an absorbance peak close to 406 nm in the UV spectra. Sample 19 is an example of a formulation that contains both types of metal–I complexes. Analysis of a single tube composite by the proposed procedure indicated the presence of 88.1% by the UV method and 98.1% by the complexiметric method. The chloroform fraction was treated in the same manner as described for Sample 8 and was found to contain 46.1% of the amount of I declared. The total complexed and free I was determined by dissolving a second sample of the composite in chloroform, adding the nickel reagent, and scanning against a blank of the nickel reagent in chloroform. Total I was determined to be 149.8%, which compares satisfactorily with the 144.2% found by summing the values obtained separately.

Chloroform is not the solvent of choice for the determination of I by the complexiметric method since the nickel complex can exist in two different forms in chloroform; one absorbs at 402 nm and one absorbs at 470 nm. The equilibrium between these forms is affected greatly by the water concentration and causes the measurement at 402 nm to be lower than it should be whenever any water is present. The chloroform fraction is used to estimate the amount of the cyclohexane–insoluble metal–I complex since the solid metal–I complex remaining from the evaporation of the chloroform eluate was not sufficiently soluble in the 13 solvents listed in Table II. In the early phases of the investigation, the cyclohexane fraction was evaporated on a steam bath and the residue was dissolved in chloroform for the complexiметric determination. The trouble with the equilibrium between the two complex forms and the volatility of I led to the development of the direct measurement in the cyclohexane eluate.

The results for Samples 4, 7, 8, 19, 22, and 23 that were run on different days and/or from different containers did not agree satisfactorily, even though duplicates run at the same time were in close agreement for both I and the corticosteroid. This finding indicates nonuniformity in mixing during preparation and/or packaging.

Several products tested contained other pharmaceutically active ingredients such as lidocaine, neomycin sulfate, and pramoxine hydrochloride. No attempt was made to determine these components quantitatively.

Sample preparations and column elution for two samples require approximately 1.5 hr, and complete determination by all four determinative procedures can be completed in approximately 6 hr.

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Assay of Sulfacetamide Sodium Ophthalmic Solutions by High-Pressure Liquid Chromatography

MELVIN H. PENNER

Abstract □ A high-pressure liquid chromatographic method, using an adsorption column and sulfabenzamide as the internal standard, is proposed for the determination of sulfacetamide sodium and its principal hydrolysis product, sulfanilamide, in eye drops. It affords an average recovery of 100.9% of added sodium sulfacetamide with a relative standard deviation of 1.9%.

Keyphrases □ Sulfacetamide sodium—ophthalmic solutions, assay, high-pressure liquid chromatography □ Sulfanilamide—assay as hydrolysis product of sulfacetamide sodium in ophthalmic solutions, high-pressure liquid chromatography □ High-pressure liquid chromatography—assay of sulfacetamide ophthalmic solutions

Sulfacetamide sodium solutions have been shown to undergo hydrolysis to sulfanilamide and sodium acetate and oxidative discoloration (1–5). The nitrite titration method used for sulfacetamide sodium ophthalmic solution in USP XVIII (6) does not distinguish between sulfacetamide and sulfanilamide

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Sample preparations and column elution for two samples require approximately 1.5 hr, and complete determination by all four determinative procedures can be completed in approximately 6 hr.

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Assay of Sulfacetamide Sodium Ophthalmic Solutions by High-Pressure Liquid Chromatography

MELVIN H. PENNER

Abstract □ A high-pressure liquid chromatographic method, using an adsorption column and sulfabenzamide as the internal standard, is proposed for the determination of sulfacetamide sodium and its principal hydrolysis product, sulfanilamide, in eye drops. It affords an average recovery of 100.9% of added sodium sulfacetamide with a relative standard deviation of 1.9%.

Keyphrases □ Sulfacetamide sodium—ophthalmic solutions, assay, high-pressure liquid chromatography □ Sulfanilamide—assay as hydrolysis product of sulfacetamide sodium in ophthalmic solutions, high-pressure liquid chromatography □ High-pressure liquid chromatography—assay of sulfacetamide ophthalmic solutions

Sulfacetamide sodium solutions have been shown to undergo hydrolysis to sulfanilamide and sodium acetate and oxidative discoloration (1–5). The nitrite titration method used for sulfacetamide sodium ophthalmic solution in USP XVIII (6) does not distinguish between sulfacetamide and sulfanilamide

Table I—Response Factors for Standard Solutions[a]

Sulfonamide	Average Response Factor	*SD*	Variance	Coefficient of Variation, %
Sulfanilamide	6.4939	±0.2575	0.0663	±4.0
Sulfacetamide	0.8474	±0.0123	0.0001	±1.5

[a] Data calculated from three to five injections of each of four standard solutions.

Table II—Recovery of Sulfacetamide Sodium Added in Ophthalmic Preparations

Formulation	Recovery, %	Average Recovery, %	*RSD*, %
A	102.0, 100.3, 102.7, 102.3, 101.7, 100.0, 102.3, 102.0, 102.0, 102.0	101.7	±0.9
B	101.0, 101.3, 102.3, 104.3, 102.0, 102.7, 104.3, 100.0	102.2	±1.4
C	98.3, 96.7, 97.3, 99.3, 99.3, 99.0, 99.3, 100.3	98.7	±1.1

and is thus not stability indicating with respect to hydrolytic decomposition. A colorimetric assay was suggested based on the reaction of sulfacetamide sodium, an imide, with hydroxylamine and ferric chloride to form the ferric acethydroxamate complex (7). A quantitative TLC method was proposed in which sulfacetamide was separated from its degradation products with butanol–ammonia–water (9:1:8) followed by quantitation of the eluted zones *via* the Bratton–Marshall reaction (8). An alumina adsorption column and UV spectrophotometry were utilized for the separation and determination of sulfanilamide in ophthalmic solutions and tablets containing sulfacetamide (9); sulfacetamide is retained on the column.

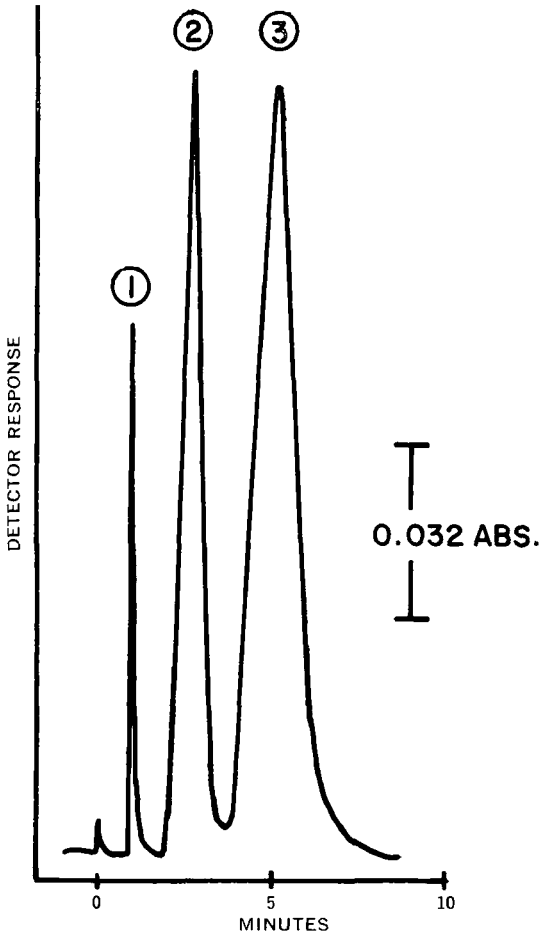

Figure 1—*Liquid chromatographic separation of the three sulfonamides. Key: 1, sulfanilamide; 2, sulfabenzamide (internal standard); and 3, sulfacetamide sodium.*

The recent availability of instrumentation for high-pressure liquid chromatography and reports (10, 11) on separations of sulfa drug mixtures using this technique with ion-exchange resins stimulated the work described here. A simple method was developed which requires only dilution of the sample to incorporate the internal standard and provides complete separation and quantitation of sulfacetamide and sulfanilamide within 10 min on an adsorption column. The method was used in a survey of the stability of several commercial sulfacetamide sodium formulations.

EXPERIMENTAL

Apparatus—A liquid chromatograph composed of a pump[1] (3000 psi maximum) with a pulse dampener and a UV monitor[2] (254 nm), a septumless injector[3], and a strip-chart recorder[4] outfitted with a potentiometric amplifier was used. A 70-cm × 2-mm i.d. stainless steel column packed with Corasil II, 37–50-μm particle size, was operated at 400 psi, with a 65% pump stroke resulting in a flow rate of about 2 ml/min. The mobile phase employed consisted of methylene chloride–isopropanol–concentrated ammonia (130:65:2.5), which was degassed by gentle heating prior to use. All injections were made with a 25-μl syringe[5].

Standard Preparation—Prepare individual stock solutions of sulfanilamide and sulfabenzamide internal standard at concentrations of 0.3 and 1.5 mg/ml of isopropanol, respectively. Accurately prepare a series of standard curve solutions to contain 0.9–1.65 mg of sulfacetamide sodium plus 0.15–1.5 mg of sulfanilamide/ml in 90% isopropanol. Each standard solution should contain 6.0 mg of internal standard/ml. The concentrations of sulfonamides represent a range of 60–110% sulfacetamide sodium and 1–10% sulfanilamide.

Sample Preparation—Dilute the sample solutions with isopropanol to obtain a concentration of 15 mg/ml. Pipet 1 ml of each sample into a 10-ml volumetric flask, add 4.0 ml of internal standard solution, and dilute to volume with isopropanol.

[1] Milton Roy.
[2] LDC.
[3] Varian.
[4] Heath.
[5] Precision Sampling Pressure-Lok liquid, Pierce Chemical Co.

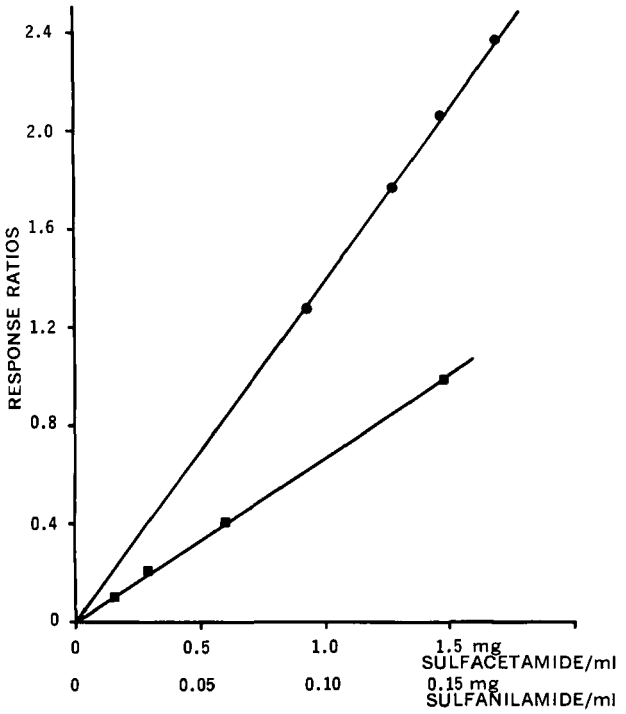

Figure 2—*Calibration curves indicating linearity of response of UV detector to sulfanilamide* (■) *and sulfacetamide sodium* (●).

Chromatographic Separation—With the UV monitor set at a suitable sensitivity range, inject 10 μl of each standard solution in duplicate. Sulfanilamide, sulfabenzamide, and sulfacetamide sodium appear as peaks after 1.0, 2.5, and 5.0 min, respectively. Compute the area of the sulfabenzamide and sulfacetamide sodium peaks arising from each standard injection, and determine the peak height of the sulfabenzamide and sulfanilamide peaks in each standard injection. Calculate the response factor, R, for sulfanilamide and sulfacetamide.

Inject each sample solution in duplicate, and calculate the concentrations of sulfacetamide sodium and sulfanilamide, if present, using the average response factors determined from the standards.

RESULTS AND DISCUSSION

All chromatographic runs were made employing a UV detector sensitivity of 0.32 absorbance unit full scale. A typical chromatogram is presented in Fig. 1. An internal standard was used to minimize the effects of small procedural variations. Sulfabenzamide was chosen as the internal standard since it eluted between the two sulfonamides of interest and, therefore, would not increase the analysis time.

The output of the UV detector was fed *via* an interface to a computer[6] programmed to determine peak heights and areas. Since sulfanilamide eluted as a rather narrow sharp peak, peak height rather than area was chosen as a more accurate measure of the concentration of this component. For the quantitation of intact sulfacetamide sodium, peak areas were employed. A linear response was obtained for the undegraded drug and its decomposition product over concentration ranges of 0–1.7 and 0–0.15 mg/ml, respectively (Fig. 2).

[6] IBM System 7.

Table III—Results of Replicate Assays of Sulfacetamide Sodium Ophthalmic Solutions

Solution	Sulfacetamide Sodium Found, %	Theory, %	Percent of Label Claim	Sulfanilamide, %
A	29.9	30.0	99.7	0.7
B	29.6	30.0	98.7	1.8
C	30.0	30.0	100.0	2.9
D	15.3	15.0	102.0	1.0
E	14.4	15.0	96.0	3.9
F	13.7	15.0	91.3	6.5
G	13.0	15.0	86.7	8.0
H	9.3	10.0	93.0	1.9
I	9.4	10.0	94.0	4.1
J	10.5	10.0	105.0	0.9

To compensate for day-to-day variations in any operating parameter, response factors for each sulfonamide were determined from a limited number of standard solutions each day the samples were analyzed. Since the response factors were linearly related to concentration, average response factors for sulfanilamide and sulfacetamide were calculated (Table I).

The results of adding known amounts of sulfacetamide sodium to each of three analytically prepared test formulations are shown in Table II. The data were obtained with replicate aliquots of each formulation injected in duplicate. Pooling the results of all 26 assays resulted in an average recovery of 100.9% with a relative standard deviation of 1.9%.

Sulfacetamide sodium ophthalmic preparations are usually available at solution concentrations of 30, 15, and 10%. Three different formulations[7] were assayed for each concentration, and two different lots of the same preparation were tested in one instance. The average results obtained for replicate aliquots of each formulation are presented in Table III. The only degradation product detected was sulfanilamide.

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[7] The formulations used were marketed preparations. None of the preparations with expiration dates was outdated.

GLC Analysis of Griseofulvin: A Collaborative Study

MICHEL MARGOSIS

Abstract □ A GLC method for the assay of griseofulvin in bulk and dosage forms was subjected to a wide and rigorous collaborative study. An overall recovery of 99.52 ± 2.33% for three samples from 19 participating laboratories was obtained. The success of this study is ascribed to the fact that strict performance requirements are specified for the operating system.

Keyphrases □ Griseofulvin—GLC analysis, collaborative study □ GLC—analysis, griseofulvin, collaborative study

The GLC method for the assay of griseofulvin in pharmaceutical preparations reported previously (1) was shown to be useful in characterizing the drug and has proved to be more specific, accurate, and reliable than the current official UV and microbial assay methods (2). As in the preceding study of chloramphenicol (3), it appeared advisable to subject this method to a wide collaborative study encompassing industry and academia, as well as national and supranational organizations, to demonstrate its merits and validate its practical applicability.

EXPERIMENTAL

Separate vials containing three "unknown" samples and one internal standard were sent to 24 laboratories[1]. The first unknown sample, Sample 1, was a commercial bulk, nominally pure; unknown Sample 2 was a commercial tablet; unknown Sample 3 was a tablet specifically prepared with known excipients for this study. The nominal values of the griseofulvin concentration in these samples were obtained from the manufacturers when applicable and were verified independently by the official UV procedure and this GLC procedure. These concentration values were then established at 100.00, 75.58, and 45.99% for Samples 1, 2, and 3, respectively.

Additional bulk materials were provided as working reference material and to allow the analyst to become familiar with the method and to optimize the instrumental conditions.

[1] The author gratefully acknowledges the gracious participation of the following collaborators: Siv Johansson and Bengt Ohrner, Apotekens Centrallaboratorium, Solna, Sweden; A. M. Monard and R. Bontemps, Association Pharmaceutique Belge, Service de Controle des Medicaments, Brussels, Belgium; David G. Prue and B. T. Kho, Ayerst Laboratories, Inc., Rouses Point, N.Y.; Sylvia Richens and C. A. Johnson, British Pharmacopoeia Commission, London, England; Jean-Pierre Guimbard and Rene C. Feibel, Ets Clin-Midy, Paris, France; Charlotte A. Yaciw, Food and Drug Administration, Office of Pharmaceutical Research and Testing, Division of Drug Chemistry, Washington, D.C.; Shirley S. Lyles, Food and Drug Administration, Office of Pharmaceutical Research and Testing, National Center for Antibiotic Analysis, Washington, D.C.; Ross D. Kirchhoefer, Food and Drug Administration, Office of Pharmaceutical Research and Testing, National Center for Drug Analysis, St. Louis, Mo.; A. A. R. Richardson (Ulverston Laboratories), D. S. Kaushik, and T. E. V. Horsley (Greenford Laboratories), Glaxo Laboratories Ltd., England; William L. Wilson, Health Protection Branch, Health and Welfare, Ottawa, Ontario, Canada; Gerard Janssen and Hubert Vanderhaeghe, Rega Institute, Katholieke Universiteit te Leuven, Belgium; Karin Worning and J. Roed Jensen, Leo Pharmaceutical Products Ltd, Ballerup, Denmark; Laurence Luberecki and Wm. R. Jones, McNeil Laboratories, Inc., Fort Washington, Pa.; D. H. Calam, National Institute for Medical Research, London, England; Richard Vivilecchia and Arthur F. Michaelis, Sandoz Pharmaceuticals, Hanover, N.J.; Bernard D. Rosen and Wilbur S. Felker, Schering Corp., Union, N.J.; Ida Mortensen and Jorgen Bang, Statens Serumsinstitut, Copenhagen, Denmark; Hadassah Nissenbaume and Jacob L. Zaidman, Tel-Aviv University Medical School, Beer Yaaqov, Israel; Ernest J. Kubiak and Marvin F. Grostic, The Upjohn Co., Kalamazoo, Mich.; Y. Nezaki, K. Ishimura, and K. Hotta, Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan.

Table I—Results for Griseofulvin Sample 1 (Bulk)

Laboratory	Number of Runs	Recovery Mean, %	Coefficient of Variation
1	8	100.28	3.32
2	4	100.23	0.21
3	12	97.91	2.45
4	4	101.05	0.98
5	4	96.52	2.26
6	2	98.21	0.04
7	8	100.34	0.72
8	4	97.98	0.75
9	4	95.65	1.05
10	4	100.09	0.40
11	2	98.79	1.45
12	4	100.97	0.81
13	10	99.74	0.65
14	4	99.49	1.14
15	4	98.93	1.33
16	6	99.23	2.29
17	4	96.96	1.42
18	12	99.33	2.29
19	4	99.41	1.22
Mean		99.01	1.50

The protocol of analysis was basically similar to that cited previously (1) and employed the identical curing and conditioning treatment recommended for practically all silicone columns. The calculations for potency (assay of griseofulvin), efficiency, resolution factor, and tailing factor were identical to those used in the collaborative study of chloramphenicol (3).

The analyst was requested to run duplicate injections of two separate weighings of each test sample and to compare the factor calculated from each to the average value obtained from the standard. When the coefficient of variation for five replicate injections of a standard solution exceeded a 2% limit, the entire system was to be tuned-up.

RESULTS[2]

Calculations and results from each collaborator were verified when sufficient data were available. These results include those submitted by the participants of this study and those calculated by the author from available chromatograms. Several sets of results were rejected outright. In two cases, precision values exceeding 5% in the replications of the standard were obtained. All results from another collaborator exceeded an acceptable sum rank limit by the Youden test (4), indicating a gross systematic error.

The final results were normalized to the nominal "labeled" concentration of the sample and are summarized in Tables I–IV for each sample and for the sum of the three samples. In Table V, "WD mean" is the result obtained through the weighted analysis of all observations from each collaborator, inclusive of "within sample" errors. "Mean" in Tables I–IV is the result obtained from the calculated mean from each collaborator; thus equal weight was attributed to each collaborator. The mean percent recoveries for Samples 1, 2, and 3 were 99.01 ± 1.50, 100.17 ± 2.05, and 99.37 ± 3.10, respectively, with an overall mean recovery of 99.52 ± 2.33% from 57 calculated means.

In Table V, results obtained from measurements by electronic integration are shown for comparison as well as to demonstrate the difference between the digital *versus* the combined digital and manual techniques. As expected, results derived from electronic

[2] All calculations, data processing, and statistical analyses were performed through the APL time-sharing system of an IBM 370/155 computer.

Table II—Results for Griseofulvin Sample 2 (Tablets)

Laboratory	Number of Runs	Recovery Mean, %	Coefficient of Variation
1	8	102.61	2.26
2	4	100.64	0.05
3	12	99.94	3.57
4	6	101.55	1.38
5	4	96.49	2.66
6	4	103.29	0.71
7	8	99.23	2.74
8	4	96.72	0.54
9	4	101.46	0.33
10	4	101.26	0.53
11	2	97.95	2.76
12	4	100.64	0.52
13	6	97.64	0.59
14	4	100.43	0.71
15	4	102.56	1.57
16	5	99.31	1.05
17	4	101.49	3.01
18	8	97.87	2.71
19	4	102.19	1.90
Mean		100.17	2.05

Table III—Results for Griseofulvin Sample 3 (Tablets)

Laboratory	Number of Runs	Recovery Mean, %	Coefficient of Variation
1	8	99.99	3.76
2	4	99.23	0.31
3	12	100.85	3.73
4	4	102.61	0.71
5	4	97.42	8.70
6	4	103.79	0.97
7	8	99.17	3.62
8	4	105.97	4.38
9	4	101.79	0.68
10	4	99.91	0.87
11	2	93.74	1.08
12	4	100.91	1.69
13	6	100.16	0.36
14	4	94.67	1.33
15	4	99.60	1.31
16	8	99.19	2.54
17	4	97.10	1.21
18	8	95.87	7.13
19	4	96.09	1.72
Mean		99.37	3.10

measurements were noticeably more precise, as indicated by the values of the range, variances, and standard deviations. All quantitative measurements were accomplished by one or more of six different procedures: electronic digital integration (11 sets), triangulation (six sets), peak height (four sets), disk integration (two sets), planimetry (one set), and cut and weigh (one set). These 25 sets of measurements were partitioned among the appropriate 19 participating laboratories as tabulated.

A computer plot of the results *versus* the frequency of results for each sample group numbering about 100, as well as for the combined groups of values totaling 303, yielded the typical S-shaped curve. A good fit to a normal distribution was indicated by the χ-square test for the results for each and for the combined samples. Because of its simplicity, a nonparametric sign test was performed; this indicated no detectable differences between the three samples at any confidence level.

The variances within each sample were found to be homogeneous by Bartlett's test, indicating similar reliability or precision from each collaborator at the 95% confidence level. Athough this test was not considered to be overly reliable within several sets of results because of the scarcity of observations, it may serve to attest that precision was more adversely affected by the manual techniques of peak measurements. A one-way analysis of variance was performed on all results calculated from each sample, and collaborator means were significantly different at $p < 0.05$ for all samples. However, when this analysis was performed on the means from each sample from each collaborator, no significant difference was noted between the laboratories ($F = 1.17$). This result was further verified in a two-way analysis of variance of the means, which indicated no significant differences between sample means ($F = 1.38$) and between laboratory means ($F = 1.26$).

A paired t-test was also employed to ascertain whether a statistically significant difference existed between the three samples. It was used to compare Samples 1 to 2, 2 to 3, and 1 to 3, but no such difference was detected at the 95% confidence level. A similar test comparing multiple groups of values (the Hotelling T square test) did indicate that the difference was not significant at the $p < 0.05$ level, but the calculation of this probability showed it to be near borderline (F of probability = 0.055, significant <0.05).

As mentioned before, Sample 3, made especially for this study, was formulated to contain 110 mg of griseofulvin in a 240-mg tablet containing lactose, starch, and magnesium stearate. The tablet weight varied somewhat (coefficient of variation = 1.25%), and the assay results on a per tablet basis gave a corresponding variation. To minimize this source of variation, the assay results in this study are reported on the basis of weight percent of granulation, which is indeed shown to be quite uniform.

Dechlorogriseofulvin was detected in the chromatograms by each collaborator but, because of its low concentration, it could be quantitated only by those 11 participants employing an electronic digital integrator. A 95% confidence limit of the mean for this component was calculated at 0.51–0.76% for the standard, 0.73–0.97% for Sample 1, 0.81–1.20% for Sample 2, 0.47–0.85% for Sample 3, or 0.70–0.87% for the combined total. The coefficient of variation for this measurement was estimated at about 30%, which may be acceptable for substances at this low concentration level and should also prove useful as an identity test for the product.

DISCUSSION

As in the earlier collaborative study, characteristics of column and operating conditions varied substantially from one laboratory to the next. Fifteen different models of GLC instruments from eight manufacturers were used; coiled or U-shaped columns ranged from 0.6 to 2 m in length and from 2 to 6 mm in diameter. Various supports were used with loading of 1–3% OV-17 in all but one case where 5% SE-30 was used. Except in one instance where argon was used, helium and nitrogen were used by about the same number of laboratories, with flow rates from 20 to 100 ml/min at column temperatures ranging from 245 to 280°.

Although wide flexibility was allowed in the selection of the analytical system, control of the method was attained by demanding that it meet certain performance requirements after subjecting the column to the proper temperature curing, sample conditioning, and suitability test as outlined in the protocol of analysis. These requirements included a measure of efficiency, resolution, a symmetry factor, and a maximum coefficient of variation of a number of replicate injections.

Table IV—Combined Results for Three Griseofulvin Samples

Laboratory	Number of Runs	Recovery Mean, %	Coefficient of Variation
1	24	100.96	3.25
2	12	100.03	0.65
3	36	99.57	3.45
4	14	101.71	1.21
5	12	96.81	4.94
6	10	102.48	2.32
7	24	99.58	2.59
8	12	100.22	4.93
9	12	99.64	3.03
10	12	100.42	0.84
11	6	96.82	2.91
12	12	100.84	1.03
13	22	99.28	1.18
14	12	98.20	2.86
15	12	100.36	2.08
16	19	99.24	2.05
17	12	98.51	2.92
18	28	97.93	4.35
19	12	99.23	3.02
Mean		99.52	2.33

Table V—Summary of Results[a]

Sample	A	B	C	D	AE	BE	CE	DE
Number of collaborators	19	19	19	19	11	11	11	11
Sample size	104	99	100	303	48	47	48	143
WD mean	99.11	100.14	99.49	99.57	99.50	99.74	99.96	99.73
Median	99.64	100.52	99.51	99.92	99.96	100.26	99.73	99.99
95% lower confidence limit	98.70	99.59	98.66	99.22	99.06	99.10	98.93	99.32
95% upper confidence limit	99.52	100.69	100.32	99.93	99.94	100.38	100.98	100.15
Maximum	104.03	106.91	111.48	111.48	103.22	104.31	111.48	111.48
Minimum	92.78	92.67	82.71	82.71	95.30	95.66	93.02	93.02
Range	11.25	14.24	28.77	28.77	7.92	8.65	18.46	18.46
Variance	4.47	7.51	17.45	9.86	2.29	4.75	12.43	6.45
SD	2.11	2.74	4.18	3.14	1.51	2.18	3.53	2.54

[a] Labels A, B, and C are Samples 1, 2, and 3, respectively, and D is the combined value of all three, calculated by all methods of integration; those labeled with E are the corresponding results derived from electronic integration.

The efficiencies of the columns used in this study ranged from a minimum of about 300 to a maximum of 2000 theoretical plates/m with a mean of 993 ± 37 plates/m. The symmetry factor on the OV-17 phase was calculated to be from 0.7 to 1.3 with most from 0.9 to 1.15; with the SE-30 phase, a factor of 1.6 was obtained. Although the retention volume is a more characteristic qualitative property of the solute, the operating conditions in the different systems used were too variable and almost impossible to replicate for accurate measurement. In all cases but one, the resolution factor exceeded a value of 4 with most factors lying between 6 and 8.

The internal standard may be used quite effectively in lieu of the retention volume for identification of the drug, particularly when compared to the behavior of an authentic sample. In this study, the retention time of griseofulvin relative to tetraphenylcyclopentadienone ranged from 1.87 to 2.12 with a mean of 2.01 ± 0.06 (2.9%) (95% confidence interval: 1.98–2.04) in 19 cases where OV-17 was used; with SE-30, the relative retention time was 2.95.

The statistical difference noted in the one-way analysis of variance of all observations was likely due to the overloading of the chromatographic column, which was noted by some analysts during the study. This overloading may be avoided by injecting about 1 μg of solute either by using a reduced volume or by diluting the final solution about 10-fold. This decrease in the amount of injected solute improves the overall performance characteristics of the column, as typically demonstrated by one collaborator who increased the efficiency of the column threefold and greatly improved the symmetry of the peaks.

One participant had great difficulty in the elution of the internal standard because of condensation in the exit line. However, another participant employing an identical chromatograph, which does not normally possess the capability of separate heating of the exit port, obtained adequate and reproducible data. It is presumed that this result was achieved through a combination of higher temperatures and higher carrier gas flow rates. This was the only major handicap reported.

CONCLUSION

The participants in this collaborative study proved this GLC method for griseofulvin to be reliable and efficient. The results reported, analyzed, and discussed amply validate the method. Therefore, because of its demonstrated superiority, this method has been proposed for inclusion in the "Code of Federal Regulations" and is recommended for primary compendial and regulatory usage.

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Drying Rates of Tablet Granulations II: Effect of Particle Size and Granular Densities

WILFRED O. OPANKUNLE *, BALDEV R. BHUTANI ×, and VISHNU N. BHATIA

Abstract □ The effects of the granule size and density on the drying rate kinetics of tablet granulations were studied using lactose and sulfathiazole granules prepared with acacia mucilage and povidone solution. The drying rate kinetics consisted of three distinct phases of drying when the drying rate was plotted against remaining moisture content: constant rate, first falling rate, and second falling rate periods. The effect of various statistical diameters and granular density on the drying rate during the first falling rate period was analyzed, and the mechanism of moisture migration during this phase of drying was identified.

Keyphrases □ Tablets—granulations, drying rates, effect of particle size and granular densities □ Granulations, tablet—drying rates, effect of particle size and granular densities □ Drying rates—tablet granulations, effect of particle size and granular densities, lactose and sulfathiazole granules prepared with acacia mucilage and povidone □ Particle size—effect on drying rates of tablet granulations □ Densities, granular—effect on drying rates of tablet granulations

In a preliminary investigation (1), a satisfactory technique was developed to study the kinetic rates of drying and to evaluate the effect of certain granulating adjuvants and fillers on the drying rates of tablet granulations.

It was observed that drying of granulation occurs through three distinct phases, as has been reported (2–6) for various materials in the chemical industry. The drying rate kinetic data vividly showed this drying as three straight lines when the drying rate (grams of moisture evaporated per hour per gram of dry solid) was plotted *versus* moisture content based upon dry solid. The first phase, generally termed the constant rate period, corresponded to the phase of drying during which the drying rate remained unchanged.

As long as the drying surface remained saturated with liquid moisture, the constant rate period existed. However, when the rate of drying exceeded the rate of moisture migration to the drying surface, the drying rate started falling, which initiated the first falling rate period. During this period, the drying rate depended more upon the physicochemical nature of the granulation and less on the external drying conditions such as temperature and air flow rate. During the second falling rate period, drying occurred by moisture evaporation within the granulation, followed by diffusion of water vapor from the interior of the granules to the drying air.

A major portion of the granulation drying takes place during the first falling rate period, so the drying rate during this phase has significant influence on the total drying time. Drying during the second falling rate period is usually extremely slow and utilizes proportionally larger amounts of energy. Thus drying during both falling rate periods can be significant in terms of the cost of the drying operation and the prolonged exposure of the active ingredients to heat.

This investigation is a detailed study of the effect of granule size on the drying rate. This parameter is related to other physical characteristics of granules such as density and porosity. An attempt was made to establish a relationship between the size and the density of granules and the drying rate during the first falling rate period. The first falling rate period was selected since the drying rate during this period is most affected by granulation properties and least by drying conditions. The effect of granules size and density has never been studied quantitatively for this phase of drying.

Lactose and sulfathiazole granules, prepared with acacia mucilage and povidone solutions as granulating agents, were selected as test substances.

EXPERIMENTAL

Equipment Design—The rates of drying of prepared granules were studied under standardized conditions. For each type of material, the temperature of inlet air, the velocity of air flow, and the method of granulation were kept constant. The apparatus developed by Bhutani and Bhatia (1) was used, but some modifications were made to enhance the reproducibility of the results obtained. The insulation of the heating chamber was improved by initially lining the outside of the chamber with glass wool followed by two layers of foam padding. This insulation kept the inlet air temperature more stable by eliminating heat loss. The second modification was related to the placement of the wet granulations in the drying tray. The bed thickness was reduced from 1.27 to 0.64 cm, and the granules were placed in the middle of the tray in the form of a square, 20 × 20 cm. This modification provided more direct and uniform contact between the granules and the drying air.

Granulating Process—On the basis of preliminary experiments, a standard granulating procedure was selected such that the variations in particle-size distribution due to variations in wet mixing time during granulation were completely avoided. A standard batch of granulation was prepared by granulating the test substance in a Glen mixer, using a definite quantity of concentrated solution of the binder and adding sufficient quantities of distilled water to obtain the wet mass. By keeping the amount of binder (dry weight) constant for a test substance, the particle-size distribution could be varied for a batch of granules by varying the amount of distilled water. The wet mass was then forced through a standard No. 8 mesh screen. Each granulation was prepared in quadruplicate, and the average results of the four batches were considered.

Lactose Granulation Studies—The drying chamber was preheated to about 42° with an inlet air temperature of 65° and the heating coil placed inside the dryer appropriately set to give the

Table I—Statistical Diameter[a], Granular Densities, Slopes for the First Falling Rate Period, and the Moisture Content of the Granulation at the First Critical Point for Batches of Test Granules

System	Batch	d_{geo}, μm	d_v, mm	d_s, mm	d_{vs}, mm	Density[b], g cm^{-1}	Slope[c]	σ_s[d]	Percent Moisture Content at First Critical Point
Lactose granulations with acacia mucilage	A	1600	0.778	0.612	1.25	1.37	2.95	0.26	7.8
	B	1050	0.482	0.371	0.82	1.42	3.99	0.25	7.9
	C	810	0.310	0.221	0.58	1.47	5.36	0.00	7.2
	D	640	0.239	0.170	0.46	1.51	6.02	0.41	4.0
	E	560	0.160	0.107	0.37	1.52	6.86	0.28	4.5
Lactose granulations with povidone aqueous solution	A	1580	0.588	0.436	1.13	1.36	2.43	0.06	11.1
	B	1050	0.490	0.380	0.82	1.43	3.48	0.03	10.1
	C	870	0.259	0.166	0.58	1.47	4.83	0.38	8.2
	D	680	0.197	0.130	0.45	1.51	6.54	0.00	5.1
	E	465	0.084	0.040	0.26	1.52	7.88	0.15	4.0
Sulfathiazole granulations with acacia mucilage	A	1180	0.436	0.313	0.86	1.43	2.17	0.12	22.0
	B	980	0.370	0.269	0.71	—	3.08	0.17	16.0
	C	720	0.260	0.190	0.52	1.52	3.80	0.16	14.0
	D	680	0.250	0.180	0.49	1.57	5.10	0.24	9.5
	E	610	0.230	0.160	0.44	1.59	6.30	0.50	7.0

[a] d_{geo} = geometrical mean diameter = 50% size from the logarithmic probability plot, d_v = mean volume diameter, d_s = mean surface diameter, and d_{vs} = mean volume surface diameter. [b] Densities represent average of three determinations. [c] Slopes of the first falling rate period calculated as g of moisture lost, g of dry solid × 100/hr, kg of dry solid, g of moisture still present. [d] σ_s = standard deviation for the slope of the first falling rate period obtained with 90% confidence limit.

desired temperature. The chamber was equilibrated at this temperature for 15 min prior to the introduction of granules. The initial moisture content of the granules was determined using a moisture balance[1] at 80° for 15 min. Sufficient sample of the wet granules was then spread uniformly to provide a 20 × 20-cm square bed of 0.64-cm thickness in a sample tray.

The tray was suspended inside the dryer from the weighing device and a thermocouple placed on the surface of the granules bed. The placement of the tray in the drying chamber requires the opening of the chamber door, which usually led to a slight drop in the drying chamber temperature. However, placement of the tray was done very quickly so that the drop in temperature was about 2° and the test granulations were immediately exposed to a temperature of 40 ± 1°.

The initial weight of the suspended system, *i.e.*, the weighing assembly and granules, was recorded along with the inlet air, granule surface, and outlet air temperatures every 30 min until the weight of the granulations became constant. At this point, the dryer was opened and the final moisture content of the granules was determined using the moisture balance.

Sulfathiazole Granulation Studies—The same procedure as described for lactose was used, except that the drying chamber was initially heated to about 53° with an inlet air temperature of 85°. The drying chamber was then equilibrated at 53° for 15 min. When placed in the dryer, the sulfathiazole granules were immediately exposed to a temperature of 50 ± 1°.

Determination of Mean Volume Diameter, d_v, Mean Surface Diameter, d_s, and Mean Volume-Surface Diameter, d_{vs}, of Granules—The statistical diameters were determined because of their physical significance and close relationship to the process of drying. Sieve analyses were performed on each of five batches of granules using standard U.S. sieve numbers 8, 10, 20, 40, and 60. Plots of the logarithm of sieve size against cumulative percent less than stated size (percent undersize) on a probability scale were obtained for each batch from sieve analysis data.

From log-probability plots, the geometric mean diameter, d_{geo}, and standard deviation, σ_{geo}, were calculated. Since a weight distribution was involved in this plot, the definitions of the statistical diameters in terms of weight distribution as derived by Hatch (7) were used for their determination:

$$\log d_v = \log d_{geo} - 3.4539 \log^2 \sigma_{geo} \quad \text{(Eq. 1)}$$

$$\log d_s = \log d_{geo} - 4.6052 \log^2 \sigma_{geo} \quad \text{(Eq. 2)}$$

$$\log d_{vs} = \log d_{geo} - 1.1513 \log^2 \sigma_{geo} \quad \text{(Eq. 3)}$$

[1] Model 6000, Ohaus Scale Corp., Union, N.J.

Determination of Granular Densities—Accurately weighed samples of dried granules (about 5 g) were placed in a known volume of xylene, and the volume of xylene displaced was carefully determined. The granular density was then calculated from the weight of the sample and the granular volume.

Determination of Slopes of First Falling Rate Period—The drying rate (grams hour^{-1} kilogram^{-1} of dry solid) was plotted against the percent moisture content of granules on a dry basis for each drying run. The drying rate curves followed three distinct linear drying phases, as previously reported for tablet granulation (1). For the present investigation, the slopes of the first falling rate period were of particular interest and were determined by linear regression (3). Four runs were made for each formulation, and four slopes were determined from them. The averages of the slopes were calculated and used in this study. The standard deviations for the slopes were determined with a 90% confidence limit for each batch of granulations.

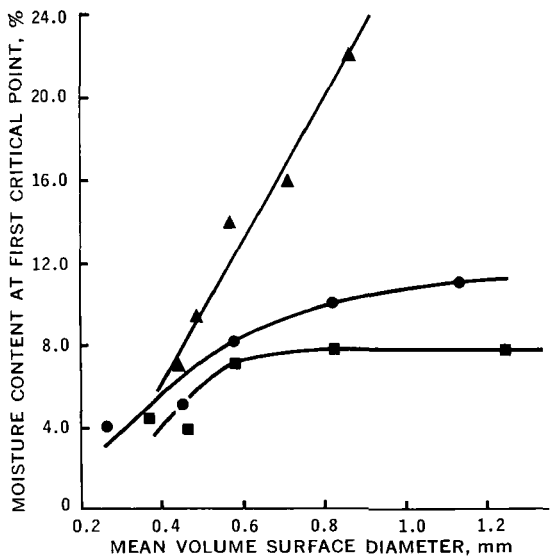

Figure 1—*Plot of the percent moisture content at first critical point* versus *mean volume-surface diameter of sulfathiazole and lactose granulations. Key:* ▲, *sulfathiazole granulations prepared using acacia mucilage as binder;* ●, *lactose granulations prepared using aqueous povidone solution as binder; and* ■, *lactose granulations prepared using acacia mucilage as binder.*

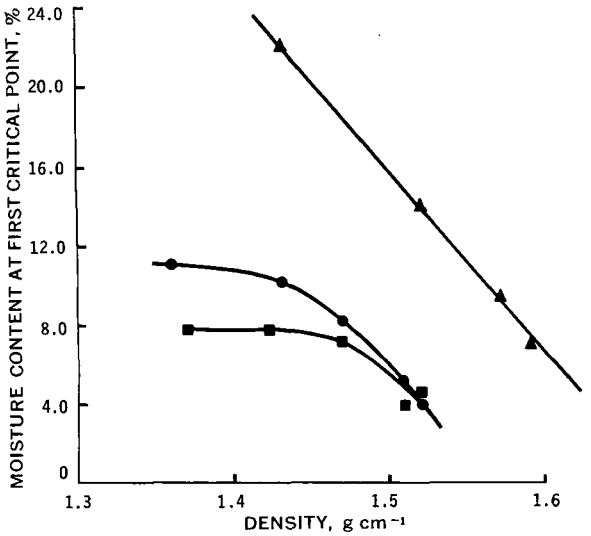

Figure 2—*Plot of the percent moisture content at first critical point* versus *density of sulfathiazole and lactose granulations. Key:* ▲, *sulfathiazole granulations prepared using acacia mucilage as binder;* ●, *lactose granulations prepared using aqueous povidone solution as binder; and* ■, *lactose granulations prepared using acacia mucilage as binder.*

RESULTS AND DISCUSSION

The various physical parameters for lactose and sulfathiazole granules, prepared using acacia mucilage and povidone solutions, are presented in Table I along with their corresponding values for the slopes of the first falling rate period and the moisture contents at which the first falling rate period started (first critical point). The statistical diameters of the granules affected the first critical points for these batches (Fig. 1). As the mean volume–surface diameters of the granules increased, the first critical point occurred at a higher moisture content. Since the moisture movement within a larger size granule would be more restricted, whether by diffusion or capillary flow, unsaturation of the granulation surface occurs at higher moisture content. Therefore, for larger granulations, more drying occurs during the falling rate periods and more time is needed to dry them to a given final moisture content.

There was a marked difference in the shape of the curves for lactose and sulfathiazole granules. For sulfathiazole granules, which are mostly porous and the moisture present is unbound since the material is water insoluble, the relationship was linear; for lactose granules, the curve indicated an apparent logarithmic relationship. This observation suggests that the moisture movement during this phase of the drying rate is directly proportional to the moisture content of the sulfathiazole granules and thus basically due to a

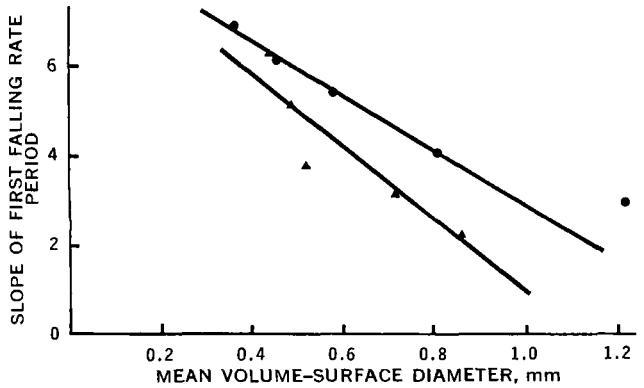

Figure 3—*Plot of the slope of first falling rate period* versus *mean volume–surface diameter of sulfathiazole and lactose granulations. Key:* ▲, *sulfathiazole granulations prepared using acacia mucilage as binder; and* ●, *lactose granulations prepared using acacia mucilage as binder.*

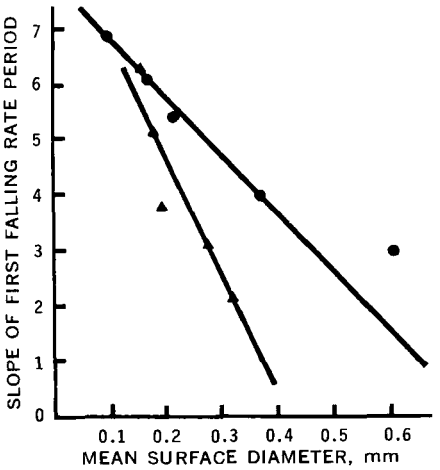

Figure 4—*Plot of the slope of first falling rate period* versus *mean surface diameter of sulfathiazole and lactose granulations. Key:* ▲, *sulfathiazole granulations prepared using acacia mucilage as binder; and* ●, *lactose granulations prepared using acacia mucilage as binder.*

capillary flow mechanism as reported for sand beds (6) and porous ceramic plates (5). For lactose granules, the moisture movement is due to a diffusion mechanism or to diffusion and a capillary flow mechanism, both occurring simultaneously as in the drying of clays and paper pulp (2).

Pitkin and Carstensen (9), while studying the final moisture content of sucrose granulations as a function of granule size, concluded that granules dry by a diffusional process. This conclusion is probably true for sucrose granules. However, in the present study, the mechanism of drying differed depending upon the nature of the ingredients present in the granulations.

The curves for the percent moisture content at the first critical point *versus* the mean volume diameter and the mean surface diameter were observed to be similar, and it was difficult to conclude if one statistical diameter gave a better correlation than the other two studied.

The density of the dried granules varied inversely with the granular size. Thus, opposite relationships were observed with densities of the granules compared with those observed with various statistical diameters. The curves for the percent moisture content at the first critical point *versus* densities of the granulations are shown in Fig. 2. As the granular density increased, the first critical point occurred at lower moisture contents. This relationship was linear for sulfathiazole granules; for lactose granules, an apparent logarith-

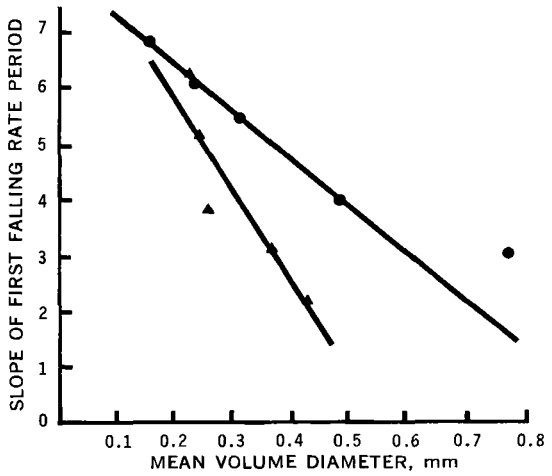

Figure 5—*Plot of the slope of first falling rate period* versus *mean volume diameter of sulfathiazole and lactose granulations. Key:* ▲, *sulfathiazole granulations prepared using acacia mucilage as binder; and* ●, *lactose granulations prepared using acacia mucilage as binder.*

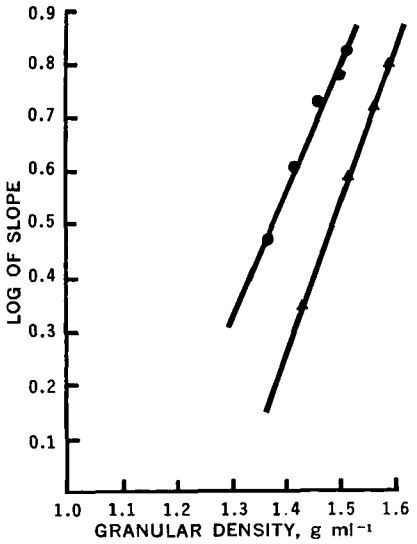

Figure 6—*Plot of logarithm of slope of first falling rate period* versus *granular density of sulfathiazole and lactose granulation. Key:* ▲, *sulfathiazole granulations prepared using acacia mucilage as binder; and* ●, *lactose granulations prepared using acacia mucilage as binder.*

mic-type relationship existed. This phenomenon could be easily explained based upon mechanism of moisture movement within the granules during this phase of drying.

Linear relationships were also observed between the slopes of the falling rate period and the statistical diameters of granulations prepared using the same binding agent (Figs. 3–5). A reduction in the size of the granules led to an increase in the slope because for smaller granules the first falling rate period starts at a lower moisture content so moisture is depleted at a faster rate and, furthermore, the moisture movement to the surface of the granules occurs faster.

A logarithmic linear relationship was observed between the slopes of the first falling rate period and the granular density for both lactose and sulfathiazole granules (Fig. 6). As the granular density decreased, the moisture movement within the granules became more restricted and the rate of moisture depletion from the surface increased. The granular density affects the first critical points as well as the slope of the first falling rate period and, therefore, the drying rate curve of the granulations. Having established the effect of physical parameters upon the drying rate during the constant rate period, falling rate periods, and critical points, one could draw a drying rate curve for a given formulation and determine the drying time from the initial to a desired final moisture content.

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Automated Method for Determining Calcium Disodium Edetate in Iodinated Contrast Media Parenterals

HOWARD W. ZIEGLER [x], WILMA L. ANDERSON, and JOHN T. SHADE

Abstract □ An automated method, based on the chelating reaction of calcium disodium edetate with zirconium and the subsequent determination of excess zirconium reacted with xylenol orange, was developed. The procedure is applicable to parenterals consisting of iodinated contrast media. Familiar modules of an automated analyzer were used, but the method can be performed manually if the sample load does not warrant automation. The pH should be controlled between 0.3 and 0.5. No interferences were encountered. Twenty samples per hour can be run on prepared sample solutions. The precision of a single determination, at the 95% confidence level, was ±0.008 mg/ml with a limit of detection near 0.40 mg/ml.

Keyphrases □ Calcium disodium edetate—automated analysis in iodinated contrast media parenterals □ Sequestering agents—automated analysis of calcium disodium edetate in iodinated contrast media parenterals □ Automated analysis—calcium disodium edetate in iodinated contrast media parenterals

Calcium disodium edetate USP (I) is commonly added to iodinated contrast media parenterals (*e.g.*, sodium and meglumine iothalamates USP) as a sequestering–stabilizing agent. It is also used as an antioxidant in some foods and beverages such as salad dressings, margarine, barbecue sauce, beer, and wine. As a sequestering agent, it is added to complex traces of metals, thus preventing oxidation (catalyzed by trace metals) or possible discoloration.

Several colorimetric methods have been reported

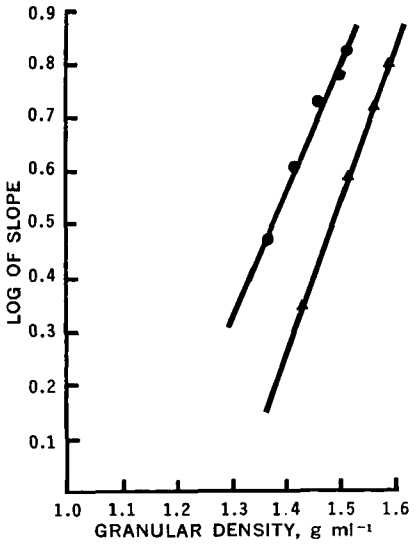

Figure 6—*Plot of logarithm of slope of first falling rate period* versus *granular density of sulfathiazole and lactose granulation. Key:* ▲, *sulfathiazole granulations prepared using acacia mucilage as binder; and* ●, *lactose granulations prepared using acacia mucilage as binder.*

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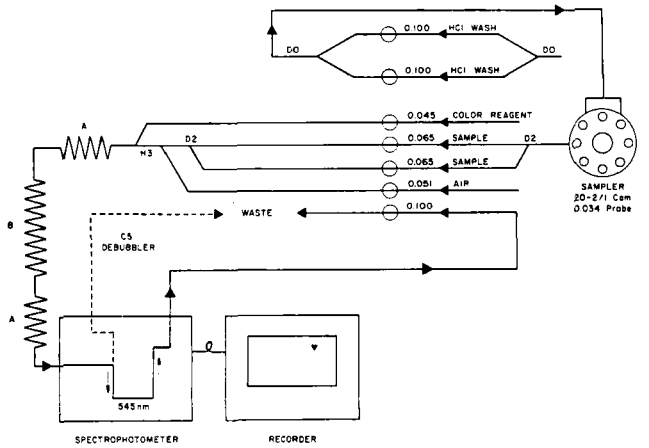

Figure 1—*Flow diagram of automated system for determining calcium disodium edetate. All tubing is standard. Key: A, 14-turn mixing coil, 2 mm i.d.; and B, 28-turn mixing coil, 2 mm i.d.*

for the determination of ethylenediaminetetraacetic acid (II). A method was reported that measures excess nickel with dimethylglyoxime after reaction with II (1). A procedure also was described using iron; the excess was reacted with thiocyanate (2). The use of a copper complex was reported (3), as was a procedure using chromium (4). The FDA "Food Additives Analytical Manual" (5) lists a method based on the chelating reaction of I on zirconium and subsequent determination of excess zirconium with xylenol orange. In the present study, this zirconium–xylenol orange (III) method, originally applied to the analysis of foods, was evaluated, adapted, and automated to analyze iodinated contrast media parenterals.

EXPERIMENTAL

Apparatus—A standard automated analyzer system[1] consisting of the following modules was used (Fig. 1): *(a)* a liquid sampler II[1] with 0.034 probe and 20-2/1 cam, *(b)* a proportioning pump II[1], *(c)* a spectrophotometer[2] modified with a 1-cm flowthrough cell, and *(d)* a strip-chart recorder[3].

Reagents and Solutions—*Zirconium Stock Solution*—Prepare by dissolving 1.77 g of zirconium oxychloride octahydrate in 100 ml of hydrochloric acid and dilute to 1000 ml with water in a volumetric flask.

Zirconium–Xylenol Orange (III) Color Reagent—Dissolve 50 mg of xylenol orange tetrasodium salt in 75 ml of water in a 500-ml volumetric flask. Add 5.0 ml of zirconium stock solution and 225 ml of 1 *N* HCl and dilute to volume with water. Age 24 hr and filter through a medium-porosity paper prior to use.

Hydrochloric Acid Wash Solution—Dilute 70 ml of concentrated hydrochloric acid to 2000 ml with water.

Calcium Disodium Edetate Stock Solution A—Prepare by dissolving 2.00 g of anhydrous (dried at 105° for 4 hr) calcium disodium edetate USP in water, adjust the pH to 7.0–7.5 with diluted sodium hydroxide or hydrochloric acid solution, and dilute to volume in a 100-ml volumetric flask with water.

Standard Solution B—Prepare by diluting 25.0 ml of Stock Solution A to 1000 ml with water

Diluted Standard Solution C (0.025 mg/ml of I)—Prepare by diluting 25.0 ml of Standard Solution B to 500 ml with water.

Transfer aliquots (usually five) of diluted Standard Solution C, ranging from 0.050 to 0.350 mg/100 ml, to 100-ml volumetric flasks; add 36 ml of 1 *N* HCl to each flask and dilute to 100 ml with water. Carry the standard solutions through the entire procedure with prepared sample solutions.

Procedure—Transfer a 2.0-ml aliquot of parenteral sample solution to a 100-ml volumetric flask. Add 20 ml of water and 40 ml of 1 *N* HCl, dilute to 100 ml with water, and mix. Let stand for at least 15 min with occasional swirling and allow the precipitate to settle. Filter a portion of the clear supernate through a dry, medium-porosity paper, discarding the first 10 ml. Fill an 8.5-ml turntable sample cup with filtrate and place in the sampler turntable.

Position the series of standard solutions in 8.5-ml sample cups ahead of the sample solutions and place one between every sixth sample. Insert the color reagent tube into the hydrochloric acid wash reservoir. Establish a baseline at zero absorbance by pumping hydrochloric acid wash through the system. Remove the color reagent tube and place it in the color reagent reservoir. Establish a steady "reagent blank" baseline, which will occur at about 0.3 absorbance unit.

Table I—Reproducibility of Automated Method for Calcium Disodium Edetate in Iodinated Contrast Media

Product	Day[a]	Calcium Disodium Edetate, mg/ml Lot[b] A	Lot B	Lot C	Lot D
C-Y	1	0.035	0.035	0.042	0.034
	2	0.045	0.045	0.045	0.043
	3	0.040	0.042	0.040	0.042
	4	0.038	0.038	0.043	0.036
	5	0.036	0.037	0.037	0.037
		Pooled *SD* of replications within lots = 0.00377			
A	1	0.098	0.095	0.095	0.107
	2	0.103	0.104	0.102	0.084
	3	0.106	0.099	0.101	0.082
	4	0.097	0.096	0.096	0.078
	5	0.099	0.097	0.099	0.080
		Pooled *SD* of replications within lots = 0.00348			
C	1	0.100	0.075	0.095	0.076
	2	0.110	0.086	0.109	0.087
	3	0.106	0.079	0.101	0.082
	4	0.102	0.076	0.097	0.078
	5	0.104	0.077	0.099	0.080
		Pooled *SD* of replications within lots = 0.00450			
C-4	1	0.115	0.085	0.082	0.093
	2	0.113	0.094	0.095	0.097
	3	0.111	0.090	0.090	0.092
	4	0.114	0.088	0.084	0.093
	5	0.107	0.088	0.087	0.090
		Pooled *SD* of replications within lots = 0.00366			

[a] A fresh sample was carried through the entire procedure each day. [b] Four different production lots of each product were arbitrarily lettered.

Table II—Recovery of Calcium Disodium Edetate from Iodinated Contrast Media[a]

Sample[b], ml	Detected, mg/ml	Added, mg/ml	Found, mg/ml	Recovery, %
1	0.102	0.050	0.152	100
0.5	0.060	0.050	0.105	88
1	0.111	0.050	0.156	90
1	0.108	0.050	0.158	100
0.5	0.038	0.050	0.089	102
1	0.066	0.050	0.114	96
			Average	96

[a] Determined by the manual procedure. [b] One-half- and 1-ml samples were taken to keep the total calcium disodium edetate content at approximately the level in the contrast media being analyzed.

[1] Technicon Autoanalyzer Model I, Technicon Corp., Ardsley, N.Y.
[2] Beckman model DB.
[3] Technicon Corp., Ardsley, N.Y.

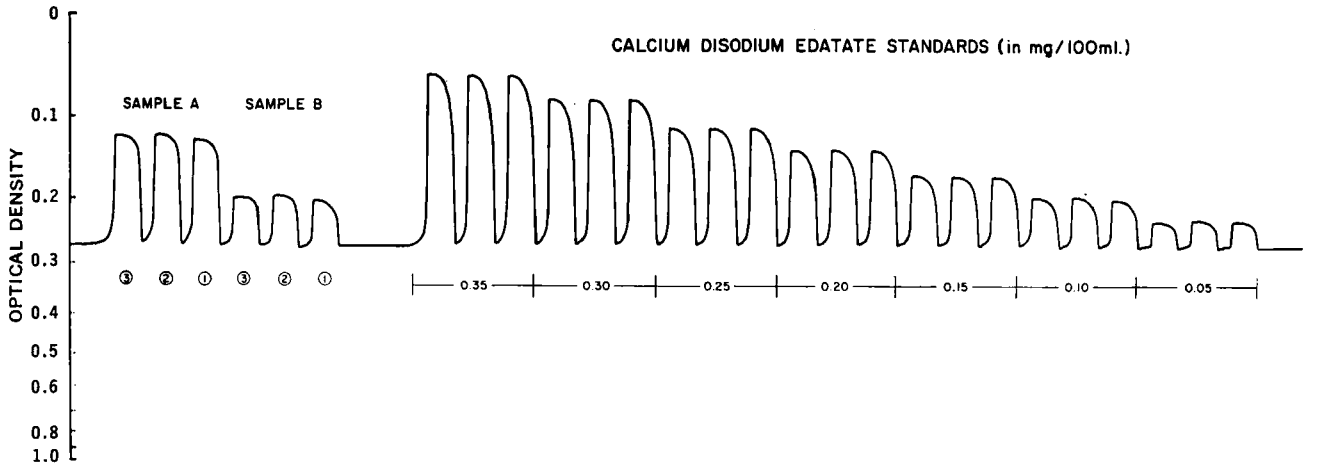

Figure 2—*Typical curves for the automated determination of calcium disodium edetate showing reproducibility of standards and actual readings of two different commercial parenterals on triplicate samples, individually prepared.*

Run the standards and sample solutions through the automated apparatus. Subtract the absorbances of the standards readings from the absorbance of the reagent blank baseline from the recorded peaks. Prepare a standard calibration curve by plotting the calculated absorbances *versus* the mg/100 ml of I taken for each standard. Calculate the milligrams per milliliter of I contained in a sample by dividing the milligrams read from a standard calibration curve by 2.

RESULTS AND DISCUSSION

Figure 2 shows a typical recording of a repeated series of standards. Typical curves for two commercial contrast media containing different levels of I are also shown in Fig. 2; triplicate samples, individually prepared, were used. Results of day-to-day reproducibility analyses are shown in Table I. A statistical evaluation of the results, from five replicates run on different days using four commercial products, indicates that the reproducibility at the 95% confidence level for a single determination is 0.008 mg/ml I (at the 0.1-mg/ml level).

The method is useful on formulations consisting of differing matrixes (*e.g.*, prepared with meglumine, sodium hydroxide, or saline). Several parameters were evaluated to ascertain if there were any interferences with the test. The experiments revealed the following:

1. The pH has an effect on optimum color development and should be controlled between 0.3 and 0.5. Alternatively, pH can be between 0.2 and 1.0 provided the pH of the sample and that of the standard solutions are identical. There is little or no color developed at pH <0.2. Investigations above pH 1.0 were not made.

2. The I level cannot be greater than 0.4 mg/100 ml, where full bleaching is achieved. This amount is equivalent to about 0.2 mg/ml in a typical iodinated contrast media parenteral solution or about double the level customarily added.

3. The wavelengths of the absorbance maximum shift slightly with concentration. The optimum wavelength was 545 nm, where a linear plot for all concentrations was obtained. The curve does not generally pass through the origin.

4. A phosphate salt is often added to parenterals as a buffer. No interference was found with as much as 0.2 mg/100 ml of sodium phosphate added to standard solutions.

5. Meglumine has no noticeable effect or interference on color development or other parameters of the test.

6. Fluoride ion interferes by bleaching and color complexation. If fluoride is suspected to be present, it can be masked by reaction with magnesium chloride (10 ml of a 25% solution) at pH 9 and then acidification with phosphoric acid (5 ml) (5) prior to color reaction.

7. Recovery studies using the manual procedure show an overall recovery of 96% (Table II).

The FDA procedure (5) calls for the preparation of separate xylenol orange and zirconium solutions to be used separately just prior to color measurement in standard and sample solutions. In this investigation, it was found that the reagents can be mixed and used as a single reagent. The mixed reagent should age about 24 hr and then be filtered. The solution is stable for several weeks. The mixed reagent is used essentially as a reagent blank to establish a working baseline in the automated procedure. This method is convenient since the reaction in the test is a bleaching of the highly colored solution. When using the technique, a positive slope can be obtained.

The method is fast, simple, and reproducible. Twenty prepared samples per hour can be run. The procedure can be performed manually as well. The method has been applied to other types of samples (*e.g.*, saline and plasma) with adaptations to remove or mask interferences. Meglumine iothalamate USP, sodium iothalamate USP, and combination formulas containing meglumine and sodium iothalamates and sodium acetrizoate have been run.

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Automated High-Pressure Liquid Chromatographic Analysis of Aspirin, Phenacetin, and Caffeine

PETER P. ASCIONE ˣ and GEORGE P. CHREKIAN

Abstract □ An automated high-pressure liquid chromatographic (HPLC) method for the separation and determination of aspirin, phenacetin, and caffeine in pharmaceutical dosage forms is described. Separation of these compounds for quantitation is achieved on a controlled pore glass support, utilizing a mixture of acetic acid and chloroform as the mobile phase. The method is specific, accurate, and simple and provides for the quantitation of each chromatogram in a continuous fashion every 7 min. HPLC separation of other analgesics was studied on a spherical siliceous support. The feasibility of determining free salicylic acid in analgesics also was established.

Keyphrases □ Aspirin, phenacetin, and caffeine—automated high-pressure liquid chromatographic analysis □ Phenacetin, aspirin, and caffeine—automated high-pressure liquid chromatographic analysis □ Caffeine, aspirin, and phenacetin—automated high-pressure liquid chromatographic analysis □ High-pressure liquid chromatography—automated analysis, aspirin, phenacetin, and caffeine

The separation and determination of analgesics in pharmaceutical dosage forms have been difficult and time consuming. Column chromatography has been used as an official method for determining aspirin, phenacetin, and caffeine (1, 2), and numerous other methods including solvent extraction techniques for separation of components were reported (3, 4). Other methods for determining the various analgesics involve UV spectrophotometry (5, 6), IR spectrophotometry (7, 8), titration (2), and NMR spectroscopy (9).

The utilization of high-pressure liquid chromatography (HPLC) in pharmaceutical analysis of analgesics was first reported by Henry and Schmidt (10). A recent paper (11) reported conditions for the routine separation of common components of analgesic tablets. The determination of analgesics by HPLC overcomes many shortcomings of the previously reported methods (1–9).

The present work describes the automation of HPLC as applied to the separation and determination of aspirin, phenacetin, and caffeine. It also includes the chromatography of other analgesics and/or combinations amenable to separation and analysis by automated HPLC. By modifying the chromatographic conditions, a rapid quantitative analysis of free salicylic acid can be made.

EXPERIMENTAL

Instrumentation and Reagents—*Apparatus*—The apparatus used in the automated HPLC was acquired from commercial sources and modified as required. The following were used: a solvent delivery pump[1]; a modified sample injection valve[2]; a UV detector equipped with optical path at 254 and 280 nm[3]; a modified liquid sampler II, proportioning pump, and assorted tubing and fittings[4]; a multiple-program timer to control the operation of both the sample injection valve and the liquid sampler[5]; pneumatic air valves, 90 psi capacity[6]; pneumatic piston[7]; a digital integrator[8]; and a recorder equipped with a multirange input and variable chart speed[9].

Columns—Columns are commercially available. They are 600 × 2.1-mm i.d. × 0.6-cm (0.25-in.) o.d. stainless steel tubing with end fitting containing a 2-μm filter bed support with the following packings: Column 1, controlled pore glass CPG-10-240 A, 200–400 mesh[10]; and Column 2, Corasil II[11], consisting of solid glass cores with a porous silica surface. Each column was dry packed with the support by feeding small increments slowly into the empty column while subjecting it to vibration and sharp vertical raps on a hard surface. The addition, vibration, and tapping were continued until the column was completely packed.

Solvents—Spectral grade and/or analytical reagent grade chloroform, methylene chloride, and acetic acid were used.

Mobile Phase—The mobile phase is acetic acid (up to about 10% v/v) in chloroform of such composition that the retention times, when the chromatograph is operated at a flow rate of 1.0 ml/min at ambient temperature, are: aspirin, 135–145 sec; phenacetin, 220–230 sec; and caffeine, 370–390 sec. The recommended mobile phase is 8% acetic acid in chloroform (v/v).

Aspirin, Phenacetin, and Caffeine Working Standard—Use reference standards of known purity. Weigh accurately about 58 mg of aspirin, 43 mg of phenacetin, and 8 mg of caffeine, and dissolve in 100 ml of mobile phase.

Preparation of Sample (Capsules)—Accurately weigh an amount of sample containing the equivalent of the working standard. Transfer to a 100-ml volumetric flask with the aid of 50 ml of mobile phase. Shake for 10 min and dilute to volume with mobile phase. Then filter a portion through fast flow paper.

Table I—Reproducibility of Peak Areas of Aspirin, Phenacetin, and Caffeine Using Automated HPLC

Sample	Peak Area, μv sec		
	Aspirin, × 10^4	Phenacetin, × 10^4	Caffeine, × 10^4
1	71.21	12.43	42.64
2	71.21	12.45	42.77
3	71.19	12.45	42.44
4	71.15	12.44	42.99
5	71.14	12.45	42.70
6	71.00	12.45	42.44
7	71.10	12.46	42.44
8	71.00	12.45	42.30
9	71.04	12.45	42.66
10	71.08	12.46	42.58
Mean	71.11	12.45	42.58
SD	±0.081	±0.0088	±0.163
RSD	0.11%	0.07%	0.38%

[1] Model 711, Laboratory Data Control, Riviera Beach, Fla.
[2] Model HPSV-20, Chromatronix, Berkeley, Calif.
[3] Model 220, Chromatronix, Berkeley, Calif., or Model 1285, Laboratory Data Control, Riviera Beach, Fla.
[4] Technicon Corp., Tarrytown, N.Y.
[5] Model 540, three poles, 2/3-rpm motor, Conrac Corp., Cramer Division, Old Saybrook, Conn.
[6] Schraeder Division of Scovill, Wake Island, N.C.
[7] Air-Mite Division, Chicago, Ill.
[8] Model 3370B, Hewlett-Packard, Avondale, Pa.
[9] Soltec Corp., North Hollywood, Calif.
[10] Electro-Nucleonics, Inc., Fairfield, N.J.
[11] Waters Associates, Framingham, Mass.

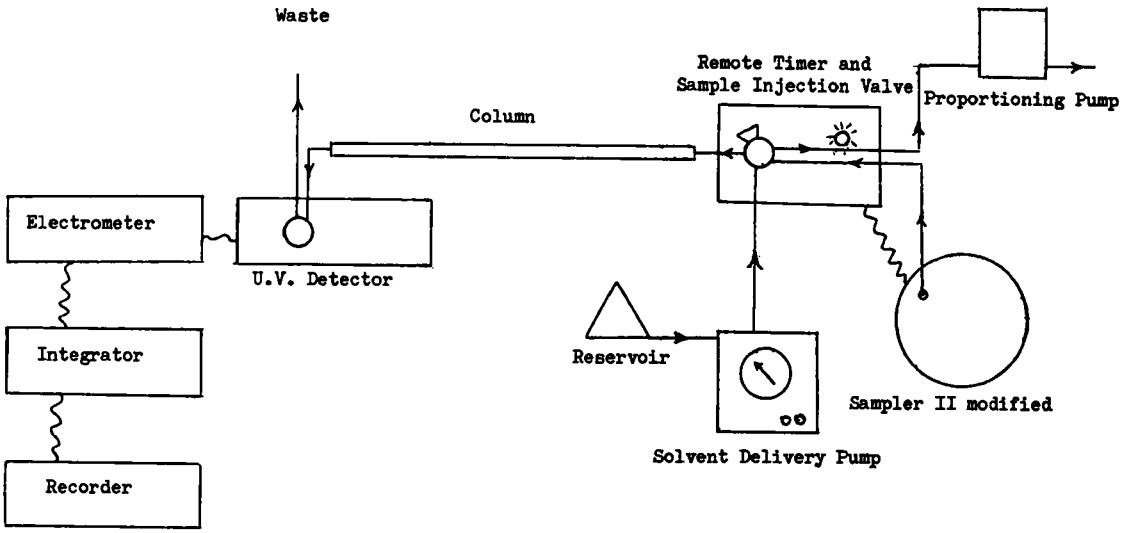


Figure 1—*Schematic diagram of the automated high-speed liquid chromatograph.*

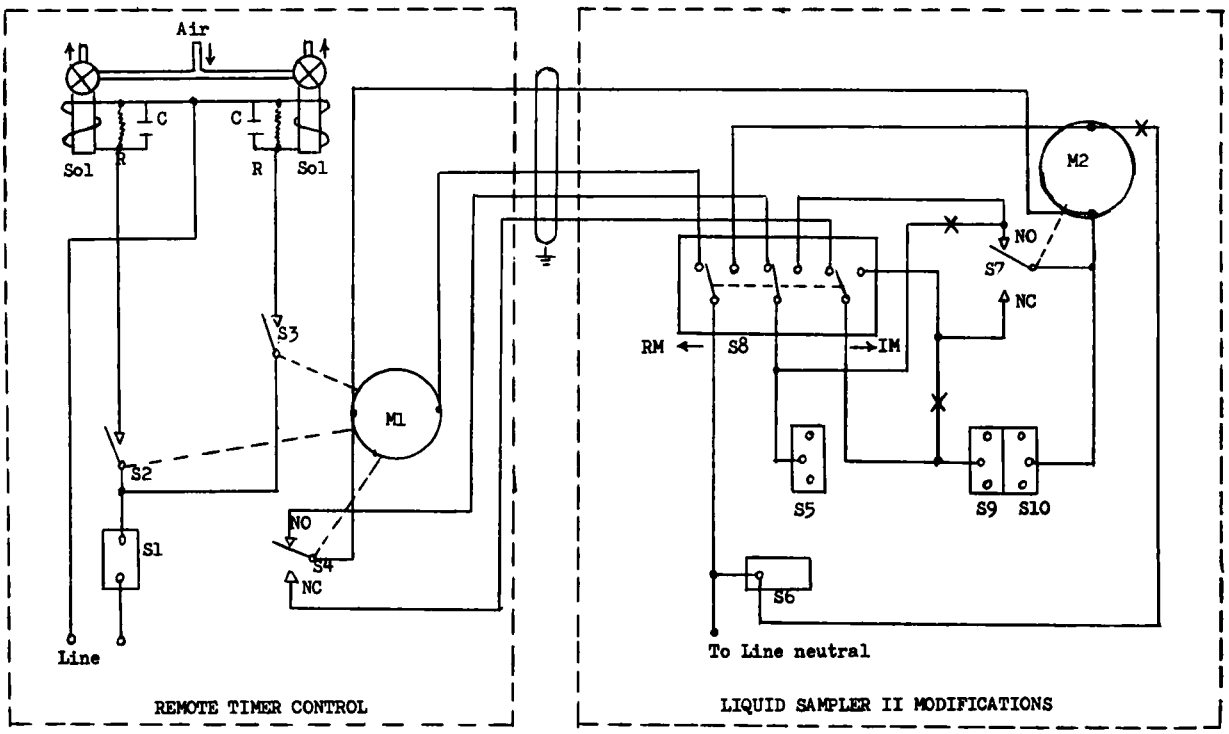
Automated Liquid Chromatographic Procedure—*Chromatographic Conditions*—A steady baseline is established with the following in operation: solvent delivery pump at a flow rate of 1 ml/min, Column 1, UV detector set at 280 nm and a range of 0.32 absorbance unit full scale, integrator settings, slope sensitivity up at 0.1 mv/min, slope down at 0.1 mv/min, peak level at 10 mv, shoulder control at front "on," shoulder at rear 1 mv, baseline reset delay at zero, and recorder with a chart speed of 5 mm/min.

Operating Conditions—Solutions of standards and samples are filled in glass vials, covered with aluminum foil, and placed in the sampler tray. At the same time the proportioning pump and air are turned on to transport the samples from the tray to the injection valve and to activate the pneumatic valves to inject the samples into the liquid chromatograph. Once the chromatographic and operating conditions are established, the control timer and integrator are activated to start the automated analysis. Integration of the chromatographic peak area of samples is compared to areas of standard peaks, and these ratios are used to calculate the analytical results.

RESULTS AND DISCUSSION

This paper describes the automation of HPLC and the chromatographic conditions that optimize the resolution of analgesics in a reasonable time. The automated HPLC system can be represented by the schematic diagram shown in Fig. 1.

The technique consists of a series of integral parts: HPLC sam-



S1-Line Switch
S2,S3-Timer switches SPST
S4-Timer switch SPDT
M1-Remote timer motor
R-22 ohms
C-0.5 microfarads, 600V
S5-Lower probe switch
S6-Alarm switch
S7-Cam switch
S8-Added 3 DT switch
S9-Upper probe switch (outer)
S10-Upper probe switch (inner)
M2-Sampler timer motor
X-Disconnect
RM-Remote Mode
IM-Internal Mode

Figure 2—*Electrical diagram of the remote timer control and liquid sampler II modifications.*

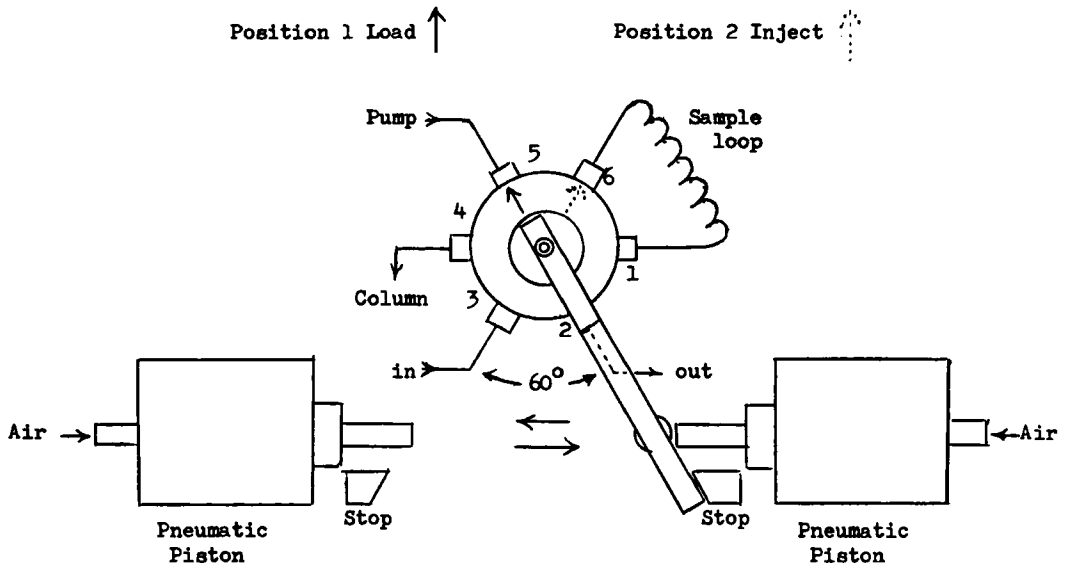


Figure 3—*Automated sample injection valve.*

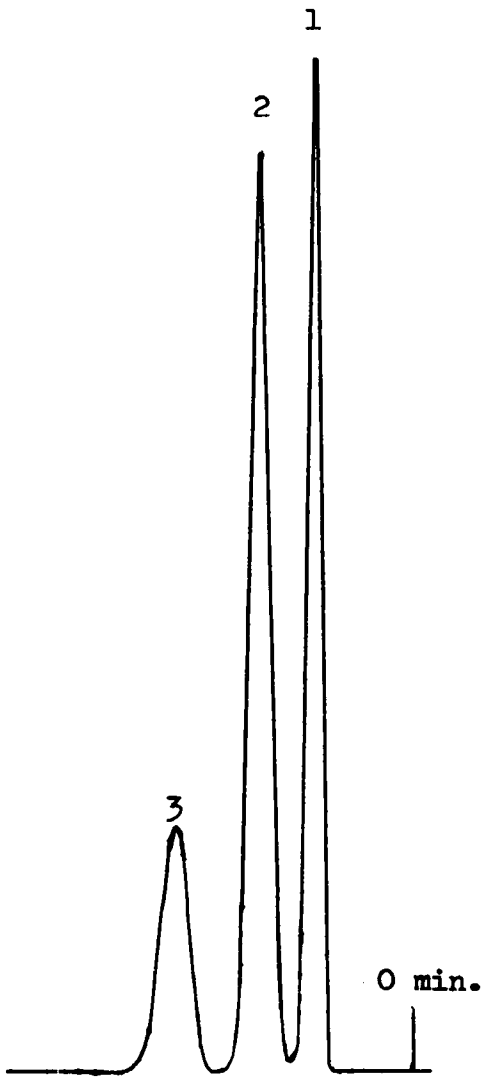


Figure 4—*Separation of aspirin (1), phenacetin (2), and caffeine (3). Chromatographic conditions were: Column 1; mobile phase, acetic acid–chloroform (8:92 v/v); flow rate, 1.0 ml/min; detection, 280 nm; and temperature, ambient.*

pler, sample transport system, automatic injection valve, remote timer control unit, and liquid chromatograph.

HPLC Sampler—A commercial sampler is modified to form a suitable sampler for the HPLC. A three-pole double-throw switch is installed in the sampler to permit a remote timer unit to control the operation of the sampler. This switch permits the use of the liquid sampler in a normal mode and/or by the remote timer control. These modifications are shown in Fig. 2.

Another modification consists of placing a thin sheet of metal on top of the turntable, which is held in place by locating pins. This modification allows glass vials[12] covered with aluminum foil, held in place by a suitable plastic ring, to be placed in the sample tray of the liquid sampler. At the start of the cycle, a sharp probe is lowered into the glass vial, piercing the aluminum foil. The sample is drawn from the vial to the sample injection valve. After injection of the sample, the probe is raised from the vial into a wash reservoir and the turntable is indexed to the next sample. The operation of the probe, turntable unit, and injection valve is controlled by a simple cam timer and microswitches.

Sample Transport System—Transporting samples from the glass vials in the sampler to the sample injection valve is accomplished with a channel peristaltic pump[13] with 0.040 red acidflex tubing. When the sample probe pierces the aluminum foil covering

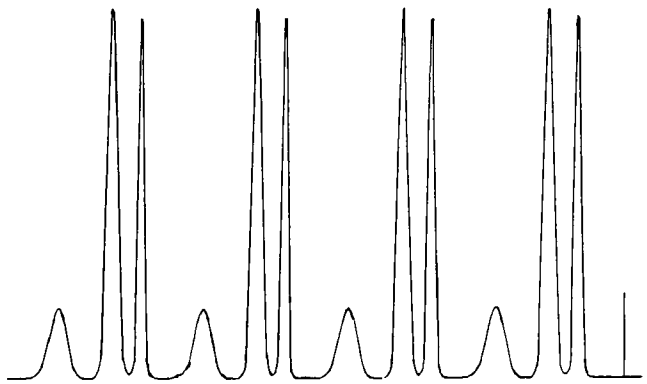


Figure 5—*Reproducibility of automated HPLC separation of aspirin, phenacetin, and caffeine using as a sample the working standard. Chromatographic conditions were: Column 1; mobile phase, acetic acid–chloroform (8:92 v/v); flow rate, 1.0 ml/min; detection, 280 nm; and temperature, ambient.*

[12] Article 60975 (14.5 × 45 mm, 1 dram), Kimble Products, Toledo, Ohio.
[13] Technicon 15.

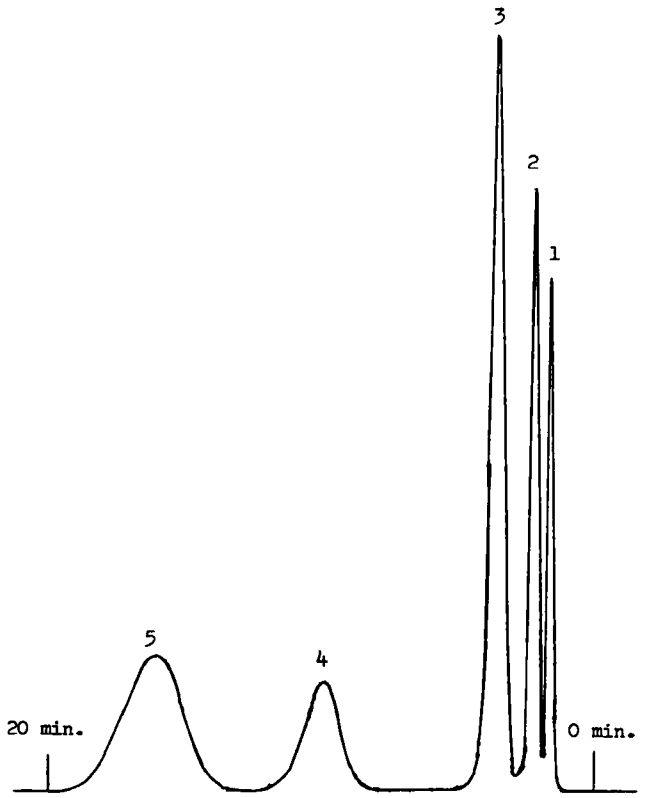


Figure 6—*Separation of five common analgesics. Key: 1, aspirin; 2, salicylamide; 3, phenacetin; 4, caffeine; and 5, acetaminophen. Chromatographic conditions were: Column 2; mobile phase, acetic acid–chloroform–methylene chloride (8:42:50 v/v/v); flow rate, 1.0 ml/min; detection, 254 nm; and temperature, ambient.*

the sample vial, the sample is drawn through the injection valve loop to waste by the peristaltic action of the proportioning pump.

Automated Sample Injection Valve (Fig. 3)—The sample application is accomplished by a rotary switching valve. Operation of the valve from one position to the other is accomplished by pneumatic pistons controlled by the cam timer and microswitch. The commercial sample injection valve is modified to form a suitable injection valve for liquid chromatography by replacing the ceramic rotor with a stainless steel rotor. This valve has an external sample loop, which determines the size of sample applied. Activation is accomplished by pneumatic piston-driven actuators, which position the rotor through the 60° movement. Stops are installed in front of the valve to ensure proper alignment of the flow when the automated valve is rotated fully against each stop (60° apart).

Remote Timer Control—The remote timer associated with the liquid sampler and automated injection valve is used to operate the function of the sampler and to initiate sample injection into the liquid chromatograph (Fig. 2). An electric timer[14] with cams and microswitches is used. Once the precise chromatographic conditions are established, operating procedures for sequential analysis employing the cycle timer are adjusted to control sampling and duration of chromatography.

Chromatography—HPLC is a fast, reliable method for the determination of aspirin, phenacetin, and caffeine. The advantages of this method include fast separation time, sensitive detection, minimum sample preparation time, and precision.

Adsorption, reverse phase, liquid–liquid, and ion-exchange chromatography were evaluated using various supports and mobile phases to obtain the desired separation. Several problems were encountered with these types of chromatography including column bleed, excessive tailing, interferences from sample matrix, and undesirable separation. Analgesics were best chromatographed by the adsorption technique where an acetic acid–chloroform solvent was used with a siliceous stationary phase.

[14] Conrac.

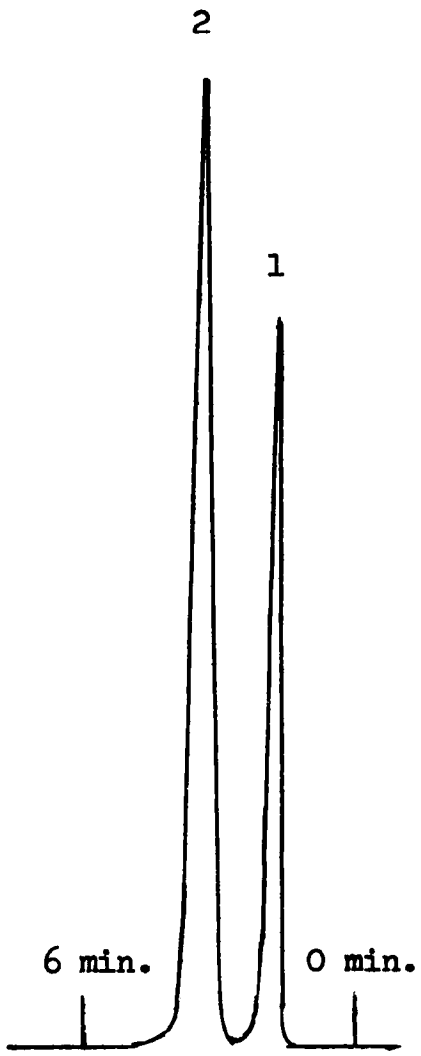


Figure 7—*Separation of salicylic acid (2) and aspirin (1). Chromatographic conditions were: Column 2; mobile phase, acetic acid–methylene chloride (1:99 v/v); flow rate, 1.0 ml/min; detection, 254 nm; and temperature, ambient.*

Figure 4 is a typical chromatogram depicting the separation of aspirin, phenacetin, and caffeine on Column 1. The chromatogram illustrates the range of structures that can be separated with symmetrical peaks using adsorption techniques. Separation is effected by varying the flow rate, changing the ratio of the mixture in the mobile phase, and using various controlled pore glass diameters. Hand-packed columns can be packed homogeneously with a reproducibility in performance of about ±10% within a given lot; however, there are variations in performance from lot to lot of a given packing material. Therefore, minor variations in chromatographic conditions may be necessary to reproduce the work described here.

To determine the precision of the automated procedure, the reproducibility of peak response was studied. A mixture of analgesics was prepared and repeatedly chromatographed. Figure 5 depicts 10 consecutive chromatograms, and a summary of results of integrator areas appears in Table I.

The detector sensitivity range for analgesics appeared to be adequate for most analyses. Linearity of the liquid chromatographic procedure was evaluated by running standard mixtures of various concentrations and plotting these concentrations against their corresponding peak areas. When measured peak areas were plotted, a linear relationship resulted between areas and concentration.

The separation and analysis of other analgesics in combination were applied to automated HPLC. Four common analgesics and caffeine were separated (Fig. 6) using the mobile phase of acetic

acid–chloroform–methylene chloride on Column 2. Aspirin eluted first, followed by salicylamide, phenacetin, caffeine, and acetaminophen. Since most analgesic combinations do not contain all five compounds, slight changes may be made in the mobile phase to reduce the elution time to less than 20 min.

The same chromatographic system can be used for rapid quantitative determination of free salicylic acid. Modifying the mobile phase accomplished the separation of free salicylic acid in analgesics containing aspirin (Fig. 7). This method of separation and detection, with minimum sample preparation, proved to be more rapid than literature methods.

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[x] To whom inquiries should be directed.

Timed-Release Tablets Containing Quinine Sulfate

NICOLAS H. CHOULIS [x] and HARRY PAPADOPOULOS [x]

Abstract □ The release rates of quinine sulfate from slowly eroding, timed-release tablets prepared with various amounts of a swellable gum, carbomer, and cellulose acetate hydrogen phthalate at different compaction pressures were attained. For the dissolution test of the prepared tablets, the method described in NF XIII was followed. The concentration of the released quinine sulfate was determined spectrophotometrically.

Keyphrases □ Quinine sulfate timed-release tablets—release rates, effect of carbomer and cellulose acetate hydrogen phthalate concentrations □ Carbomer—effect on timed release of quinine sulfate from slowly eroding tablets □ Timed-release quinine sulfate tablets—effect of carbomer concentration on release rate

It has been reported that continuously effective therapeutic drug levels can be achieved over a period of time by incorporating the medicament in a solid, edible, pharmaceutically and medically acceptable vehicle, consisting of a copolymer of glyoxal and partially degraded gelatin, and by compressing the resulting mixture into a tablet. The copolymer was claimed to swell and decompose slowly in the presence of aqueous solutions, liberating the active ingredient gradually (1). A process for the preparation of slowly eroding tablets, involving the compression of a medicinal agent and a hydrophilic swellable gum was described (2). This process was based on the discovery that a gelled zone is formed on the surface when such a tablet comes in contact with aqueous media, which delays entry of water into the interior of the tablet. The incorporation of water-insoluble medicament in a gel, formed from cellulosic gums in organic solvents, was used to produce stable tablet products with delayed release (3).

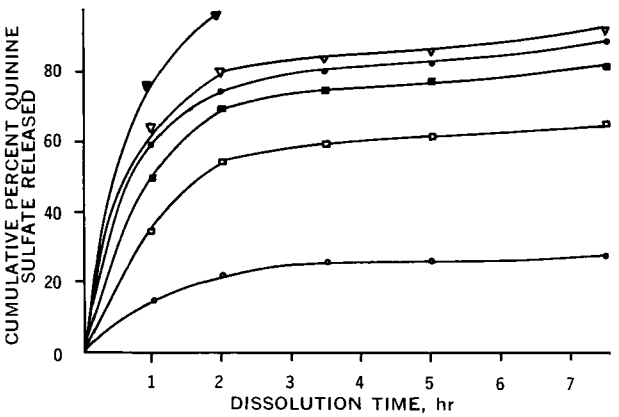

Figure 1—*Drug release rates from timed-release tablets containing decreasing amounts of carbomer. Key:* ○, *Formulation 1.1;* □, *Formulation 1.2;* ■, *Formulation 1.3;* ●, *Formulation 1.4;* ▽, *Formulation 1.5; and* ▼, *Formulation 1.6.*

Compressed tablets were prepared having sustained-release characteristics which contained, in addition to the medicinal agent, a water-insoluble, cross-linked polymer of acrylic acid and a particular, very slightly water-soluble, basic magnesium or calcium compound (4). The sustained mechanism for these polymer-containing tablets was thought to derive from the formation of a protective barrier of gelled material at the tablet surface due to the swelling of the polymer.

The complexity of factors influencing the release of medicament for this category of tablets did not allow the development of an expression to correlate the release rate with physicochemical parameters. It was

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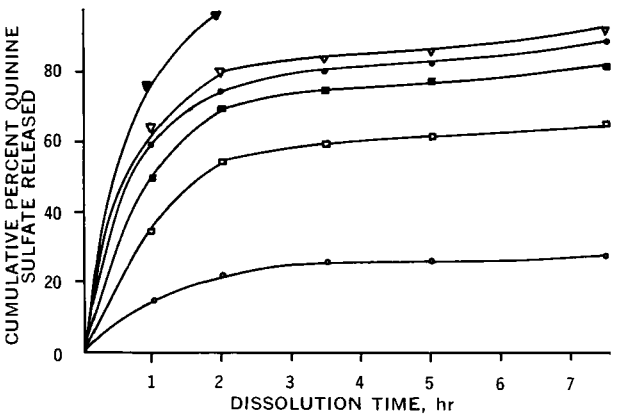

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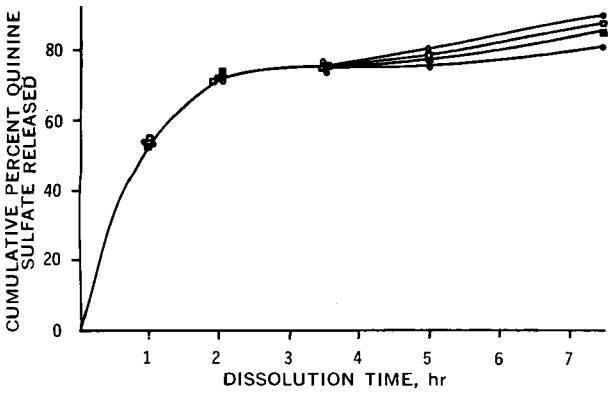

Figure 2—*Drug release rates from timed-release tablets containing increasing amounts of quinine sulfate and 40% carbomer. Key:* ○, *Formulation 2.1;* □, *Formulation 2.2;* ■, *Formulation 2.3; and* ●, *Formulation 2.4.*

reported that Higuchi's relationship adequately describes the dissolution process for tablets containing hydrophilic gums, although they cannot be considered as true matrix systems (5, 6).

In the present investigation the effect of the amount of a swellable gum, namely carbomer, on the release of quinine sulfate from slowly eroding, timed-release tablets is examined.

EXPERIMENTAL

Materials—Quinine sulfate[1], cellulose acetate hydrogen phthalate[2], and carbomer[3] were used. All other chemicals were analytical or USP grade, obtained from standard sources.

Procedures—Slowly eroding, timed-release tablets were formulated with a swellable gum, carbomer, to retard drug release. By adjusting the proportion of the gum, quantitative release of quinine sulfate was attained. Several formulations were prepared (Tables I–III).

The ingredients of each formulation, having the desired particle size less than 60 mesh, were mixed for 15–20 min with a twin-shell mixer[4]. Any aggregates formed during mixing were broken down with a spatula, thus producing additional shear mixing. Weighed amounts of the mixture of powders were compressed into tablets

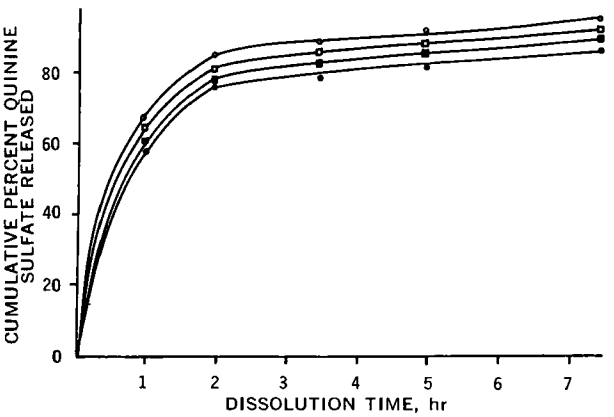

Figure 3—*Drug release rates from timed-release tablets containing increasing amounts of quinine sulfate and 25% carbomer. Key:* ○, *Formulation 3.1;* □, *Formulation 3.2;* ■, *Formulation 3.3; and* ●, *Formulation 3.4.*

[1] Fisher.
[2] Eastman.
[3] Commercially available as Carbopol 934, B. F. Goodrich.
[4] Patterson-Kelley Co.

Table I—Formulations of Timed-Release Tablets[a] Containing Various Proportions of Carbomer

Formulation Number	Ingredients	Amounts, parts %
1.1	Carbomer	75
	Quinine sulfate	25
1.2	Carbomer	50
	Cellulose acetate hydrogen phthalate	25
	Quinine sulfate	25
1.3	Carbomer	40
	Cellulose acetate hydrogen phthalate	35
	Quinine sulfate	25
1.4	Carbomer	30
	Cellulose acetate hydrogen phthalate	45
	Quinine sulfate	25
1.5	Carbomer	25
	Cellulose acetate hydrogen phthalate	50
	Quinine sulfate	25
1.6	Carbomer	20
	Cellulose acetate hydrogen phthalate	55
	Quinine sulfate	25

[a] Tablets were prepared by direct compression with a compaction force of 4000 lb.

by compression in a 13-mm diameter set of die and punches lubricated with magnesium stearate.

For the dissolution test of the prepared tablets, the method described in NF XIII (7) was modified as follows. One tablet was placed in each 90-ml screw-capped bottle containing 60 ml of pH 1.2 dissolution fluid, previously warmed to 37°. The bottles were capped tightly, placed in the clamps of the rotating-bottle apparatus, and rotated at 40 ± 2 rpm in a water bath maintained at 37 ± 0.5°.

At the end of 1 hr, the apparatus was stopped and the bottles were removed. The pH 1.2 dissolution fluid from each bottle was decanted through a 40-mesh metal screen; as much residue was retained in the bottle as possible. Any residue remaining on the screen was returned to the original bottle, and 60 ml of pH 2.5 dissolution fluid was added. The decanted pH 1.2 dissolution fluid was retained for assay.

The process was repeated at the end of 2, 5, and 7.5 hr using pH 4.5, 7.0, and 7.5 fluid, respectively.

For the chemical assay of quinine sulfate, aliquots of the samples were appropriately diluted and the pH's of the final solutions were adjusted to either 1.2 or 7.5. The concentration of quinine sulfate was then determined spectrophotometrically at 345 nm (for

Table II—Formulations of Timed-Release Tablets[a] Containing Various Proportions of Quinine Sulfate, Group I

Formulation Number	Ingredients	Amounts, parts %
2.1	Carbomer	40
	Cellulose acetate hydrogen phthalate	50
	Quinine sulfate	10
2.2	Carbomer	40
	Cellulose acetate hydrogen phthalate	40
	Quinine sulfate	20
2.3	Carbomer	40
	Cellulose acetate hydrogen phthalate	20
	Quinine sulfate	40
2.4	Carbomer	40
	Cellulose acetate hydrogen phthalate	10
	Quinine sulfate	50

[a] Tablets were prepared by direct compression with a compaction force of 4000 lb.

Table III—Formulations of Timed-Release Tablets[a] Containing Various Proportions of Quinine Sulfate, Group II

Formulation Number	Ingredients	Amounts, parts %
3.1	Carbomer	25
	Cellulose acetate hydrogen phthalate	65
	Quinine sulfate	10
3.2	Carbomer	25
	Cellulose acetate hydrogen phthalate	55
	Quinine sulfate	20
3.3	Carbomer	25
	Cellulose acetate hydrogen phthalate	35
	Quinine sulfate	40
3.4	Carbomer	25
	Cellulose acetate hydrogen phthalate	25
	Quinine sulfate	50

[a] Tablets were prepared by direct compression with a compaction force of 4000 lb.

solutions of pH 1.2) and 331 nm (for solutions of pH 7.5), using the appropriate medium as the blank.

RESULTS AND DISCUSSION

The relationship between the amount of carbomer included in the tablets and the release rates obtained with the dissolution test is shown in Fig. 1. The proportion of carbomer in the tablets ranged from 75% for Formulation 1.1 to 20% for Formulation 1.6. Release rates from tablets containing 10–50% quinine sulfate are shown in Figs. 2 and 3. The amount of carbomer included in Formulations 2.1–2.4 was 40%, while 25% was used for Formulations 3.1–3.4.

The release rates did not differ substantially for compaction pressures from 1000 to 5000 lb/1.3266 cm^2. However, higher pressures decreased considerably the release rates, probably due to entrapment of drug within the matrix. Pressure of 4000 lb/1.3266 cm^2 for matrixes containing carbomer produced tablets of sufficient hardness without hindering the release of drug. The lubricant magnesium stearate might retard tablet dissolution slightly; replacement with magnesium lauryl sulfate is possible, but its toxicity has not yet been established (8).

The mechanism of drug release of the eroding type could be explained by the fact that the formation of a hydrated and gelled zone on the surface acts as a barrier to the further penetration of the liquid into the tablet interior (9). As the gelled zone slowly dissolves in the medium, a fresh surface is exposed to the liquid, with subsequent formation of a new gelled zone. Drug is liberated from this zone through a combination of diffusion and attrition processes. Part of the drug is diffused through the swelled area while the remainder is liberated when the hydrated zone is dissolved.

In Fig. 1 the release of drug from timed-release tablets containing carbomer is shown. The amount of carbomer has a marked influence on the release of drug; as the proportion of carbomer decreases, the release rate increases. This effect would be expected since the swelled zone is more susceptible to attrition as the proportion of the gum decreases. Therefore, the proportion of carbomer can be adjusted so that all drug is released in 7.5 hr (Formulation 1.5). However, the release pattern is similar to that of matrix tablets; *i.e.*, most of the drug is liberated during the initial period of the dissolution process. It was also found that when the proportion of the gum was 20% or less (Formulation 1.6), rather rapid disintegration occurred within 2 hr (Fig. 1).

As a filler or diluent, cellulose acetate hydrogen phthalate was used to enhance the permeability of the hydrated area at higher pH values. This use was based on the property of cellulose acetate hydrogen phthalate to be insoluble in acidic environment but soluble in media of pH 5 or higher. It was postulated that this effect could counterbalance the increased viscosity of carbomer gel at higher pH values (10). The proportion of drug in the tablets containing 40% carbomer (Formulations 2.1–2.4) had a negligible effect on release rates expressed as percentages (Fig. 2). However, the absolute amounts of drug released will be proportional to the total drug content. Similar results were obtained when the proportion of carbomer was 25% (Formulations 3.1–3.4, Fig. 3). No significant differences in drug release were observed when the drug and carbomer contents were kept constant and the quantity of cellulose acetate hydrogen phthalate was varied at a given pH (pH 5 or higher).

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Comparison of Observed and Predicted Bioavailability of Nortriptyline in Humans following Oral Administration

MILO GIBALDI

Abstract □ The first-pass equation based on the dose, hepatic blood flow, and total area under the plasma level–time curve after oral administration was used retrospectively to predict the bioavailability of nortriptyline after oral administration of 1 mg/kg-single oral doses to monozygotic and dizygotic twin pairs. The predicted values of bioavailability ranged from 45 to 85%, consistent with experimentally derived estimates of nortriptyline availability.

Keyphrases □ Nortriptyline bioavailability in humans—predicted by first-pass equation, compared retrospectively to oral administration in twin pairs □ Hepatic metabolism, first-pass effect—comparison of observed and predicted nortriptyline bioavailability in humans

The availability of nortriptyline was assessed in three healthy subjects by comparing the areas under the plasma concentration *versus* time curves after oral and intramuscular administration of identical doses of the hydrochloride salt (1). The observed availability in these subjects ranged between 56 and 70% (mean of 64%). Complete GI absorption of nortriptyline is suggested by the fact that the recovery of the main urinary metabolite, 10-hydroxynortriptyline, was essentially the same following both routes of administration. The results suggest that, in humans, a significant fraction of the absorbed drug is lost to the systemic circulation during the first pass through the liver (1).

DISCUSSION

The following equation was developed to predict the degree to which a drug that is completely absorbed and eliminated exclusively by hepatoportal metabolism is subject to first-pass metabolism (2):

$$f = \frac{\text{flow rate}}{\text{flow rate} + \left(\text{dose} \Big/ \int_0^\infty C_0\, dt\right)} \qquad \text{(Eq. 1)}$$

where f is the fraction of the orally administered dose that actually reaches the systemic circulation (*i.e.*, the predicted availability), flow rate is the hepatic blood flow rate, and $\int_0^\infty C_0\, dt$ is the total area under the plasma concentration *versus* time curve after oral administration.

The value of Eq. 1 lies in the fact that it requires only concentration–time data after oral administration and a reasonable estimate of hepatic blood flow. This equation has been applied with reasonable success to studies in humans with propranolol (2) as well as with propoxyphene and alprenolol (3). Nortriptyline, like propranolol, propoxyphene, and alprenolol, is virtually completely metabolized in humans (4).

Extensive pharmacokinetic studies (5) in twin pairs receiving 1 mg/kg-single oral doses of nortriptyline hydrochloride afforded the opportunity to evaluate retrospectively the applicability of Eq. 1 as a predictor of nortriptyline availability in humans.

Table I—Apparent Clearance and Predicted Availability Values after Oral Administration of 1 mg/kg-Single Oral Doses of Nortriptyline Hydrochloride to Five Monozygotic Twin Pairs

Subject	Apparent Clearance[a], liters/min	Predicted Availability (f)
15	1.42	0.52
16	1.72	0.47
25	0.80	0.66
26	0.82	0.65
39	0.90	0.63
40	0.85	0.64
47	1.04	0.60
89	0.96	0.61
78	0.46	0.77
88	0.45	0.77
Mean ± 1 *SD*	0.94 ± 0.39	0.63 ± 0.09

[a] Calculated from Ref. 5.

EXPERIMENTAL

The term $(\text{dose}/\int_0^\infty C_0\, dt)$ in Eq. 1 is equivalent to the "apparent clearance" reported in Ref. 5 in terms of (liters per kilogram) (hour) and reexpressed in Tables I and II in terms of liters per minute. The predicted availability (f) of nortriptyline was calculated for each subject by substituting the individual apparent clearance and a mean blood flow rate of 1.53 liters/min (6) in Eq. 1. These values are also reported in Tables I and II.

RESULTS

As expected, the intrapair variation with respect to f averaged

Table II—Apparent Clearance and Predicted Availability Values after Oral Administration of 1 mg/kg-Single Oral Doses of Nortriptyline Hydrochloride to Six Dizygotic Twin Pairs

Subject	Apparent Clearance[a], liters/min	Predicted Availability (f)
9	1.09	0.58
10	0.94	0.62
27	0.66	0.70
28	0.39	0.80
31	1.90	0.45
32	1.22	0.56
68	0.63	0.71
81	0.78	0.66
74	0.64	0.71
80	0.27	0.85
92	0.88	0.63
94	0.48	0.76
Mean ± 1 *SD*	0.82 ± 0.44	0.67 ± 0.11

[a] Calculated from Ref. 5.

only 3% in the monozygotic twins; in fact, it was virtually negligible in four of five pairs. Intrapair variation with respect to predicted availability in the dizygotic twins averaged about 14%. The mean predicted availability was essentially identical in both groups. The predicted availabilities in all subjects ranged between 45 and 85%, which is consistent with the range of 56–70% observed in a study with three subjects (1). The mean predicted availability of 65% was in perfect agreement with the previously observed mean (1).

This retrospective study provides greater confidence in the reliability of Eq. 1 as a predictor of average bioavailability for drugs undergoing first-pass metabolism.

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Effects of Acids and Bases on Salicylic Acid–Cetrimide Systems

LUCY S. C. WAN

Abstract □ The effects of acids and bases on the viscosity of salicylic acid–cetrimide systems were investigated. A viscosity reduction was produced by the addition of acids and was independent of the degree of saturation of the cetrimide solution with salicylic acid. The incorporation of base followed by that of acid increased and decreased the viscosity, respectively. This viscosity behavior was demonstrated in undersaturated systems and was not governed by the relative amounts of base or acid used. In oversaturated systems, only a lowering of viscosity was observed.

Keyphrases □ Salicylic acid–cetrimide systems—effects of various acids and bases on viscosity □ Cetrimide–salicylic acid systems—effects of various acids and bases on viscosity □ Viscosity—salicylic acid–cetrimide systems, effects of various acids and bases

Since solutions of alkaline sodium salts have been shown to effect different changes in the viscosity of salicylic acid–cetrimide systems (1), a study of the reaction of the viscosity of the systems to acid additives was considered desirable. It was also the purpose of this investigation to determine whether the viscosity change arising from the addition of either acid or base is retained when these additives are added alternately to the same system. This approach is an attempt to test the sensitivity of the viscous property to these additives. A literature survey did not reveal information on this particular area of research.

EXPERIMENTAL

Recrystallized salicylic acid, mp 158–159°, and cetrimide[1] BP were the same as those described earlier (2). The acids were citric acid[2], tartaric acid[3], phosphoric acid[4], acetic acid[5], lactic acid[6], 1 *N* hydrochloric acid[2], and dilute hydrochloric acid BP. The alkalies were 1 *N* sodium hydroxide[3] and 1 *N* potassium hydroxide[3]. These additives were selected because they are representative of relatively weak acids, strong acids, and alkalies and are likely to be included in suspension formulation studies in which salicylic acid–cetrimide systems are involved.

The viscosity was measured at 25° as reported previously (2) and the pH[7] was determined.

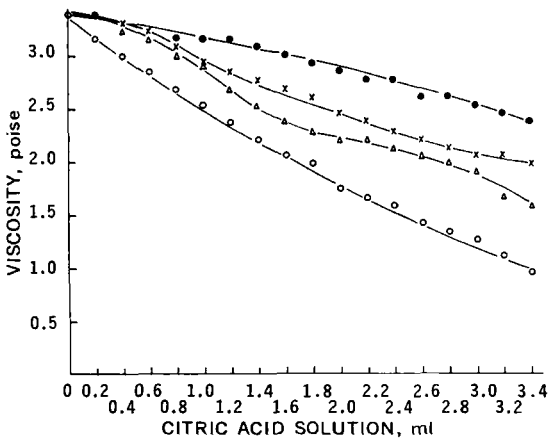

Figure 1—*Decrease in viscosity on addition of citric acid solutions of different concentrations to systems (50 g) containing 5% cetrimide and 1.4% salicylic acid at 25°. Key (citric acid, % w/v): ●, 1; ×, 4; △, 6; and ○, 10. Shear rate is 78.56 sec⁻¹.*

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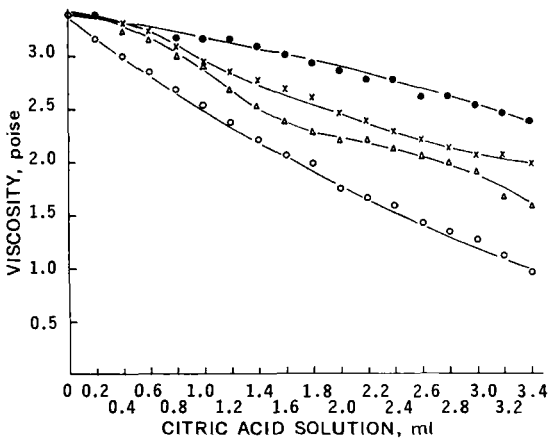

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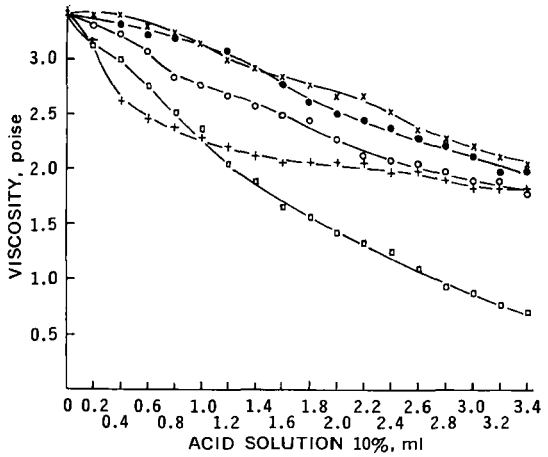

Figure 2—*Effect of 10% acid solutions on the viscosity of systems (50 g) containing 5% cetrimide and 1.4% salicylic acid at 25°. Key: ×, tartaric acid; ○, phosphoric acid; ●, acetic acid; +, dilute hydrochloric acid BP; and □, lactic acid. Shear rate is 78.56 sec^{-1}.*

RESULTS AND DISCUSSION

Figure 1 shows that the viscosity of salicylic acid–cetrimide systems was lowered in the presence of citric acid; a higher concentration of citric acid resulted in a greater lowering. Similarly, tartaric, acetic, phosphoric, lactic, and hydrochloric acids decreased viscosity (Fig. 2). The viscosity reduction was unrelated to the degree of saturation of the surfactant solution with salicylic acid, as can be seen in the action of an acetic acid solution on undersaturated and oversaturated systems (Fig. 3). This behavior also applied to the other acids employed. However, when the quantity of salicylic acid present in the system was low, no appreciable alteration in the viscosity was recorded (Fig. 3). Table I shows that the pH remained practically constant with increasing volumes of acid solution, with the exception of hydrochloric acid where a decrease in pH was observed. Therefore, it appears that the presence of most of these acids led to the formation of buffer systems.

Salicylic acid was found to interact with cetrimide to form a viscous product (3). It is believed that macromolecules resulting from this interaction are responsible for the high viscosity obtained. In the presence of other additives, the viscosity of salicylic acid–cetrimide systems was shown to vary according to the compounds added and the state of saturation of the system (2, 4, 5).

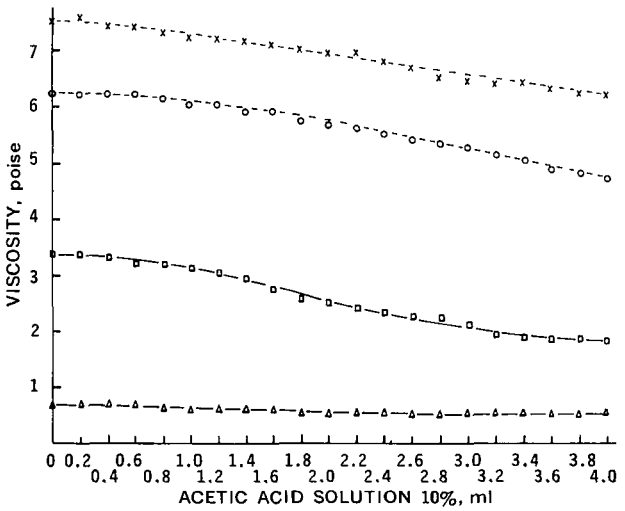

Figure 3—*Viscosity reduction by 10% acetic acid solution in dispersions (50 g) containing 5% cetrimide and such amounts of salicylic acid to form undersaturated and oversaturated systems at 25°. Key (salicylic acid, % w/v): ×, 1.8; ○, 1.6; □, 1.4; △, 1.2; —, undersaturated; and ----, oversaturated. Shear rate is 78.56 sec^{-1}.*

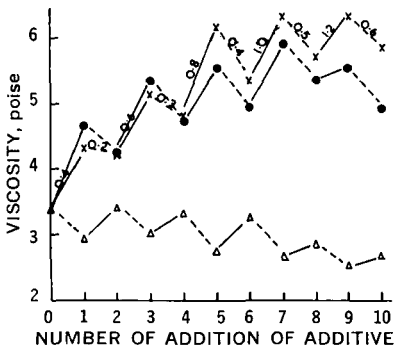

Figure 4—*Effect of various methods of adding base and acid to the viscosity of systems (50 g) containing 5% cetrimide and 1.4% salicylic acid at 25°. Key: ●, 0.6 ml of 1 N NaOH–0.3 ml of 1 N HCl; ×, 1 N NaOH–1 N HCl fixed at 2:1 but volume of additive varies as indicated in milliliters on graph; △, 0.6 ml of 1 N HCl–0.3 ml of 1 N NaOH; —, 1 N NaOH; and ---, 1 N HCl. Shear rate is 78.56 sec^{-1}.*

In preliminary experiments, it was observed that when the acid solutions were added to cetrimide solutions in the absence of salicylic acid, they had no effect on the viscosity. Hence, it was unlikely for this viscosity reduction to be brought about by a reaction between acid solutions and the surfactant. The viscosity decrease could not be attributed to a simple dilution effect, because the inclusion of water as an additive did not give a marked change in viscosity (4).

In the current investigation, the reduction in viscosity in both types of systems due to the added acids might arise from a breakup of the mesh-like structure of the system, the macromolecules becoming unstable in the presence of the acids. This condition indicates a high sensitivity of the macromolecules to these additives and the weakness of the links in the macromolecular network. The acid effect on viscosity is essentially the opposite to that of the sodium salts for undersaturated systems (1) but similar for oversaturated systems.

Instead of the usual method of adding the additive solution directly to the salicylic acid–cetrimide system, other procedures were adopted to ensure that the technique employed was not a contributing factor to the viscosity response obtained. First, the acid solution was added to the cetrimide solution and then the salicylic acid solution was added. Second, salicylic acid was added to the acid solution and this step was followed by the cetrimide solution addition. In both cases, a decrease in viscosity resulted. This finding suggests that the viscosity effect is not influenced by the manner of addition of the additive.

To examine further the mechanism of acid additive effect, a base was selected and, from preliminary studies, it was found that sodium hydroxide increased the viscosity of undersaturated systems but decreased that of oversaturated systems in the same way

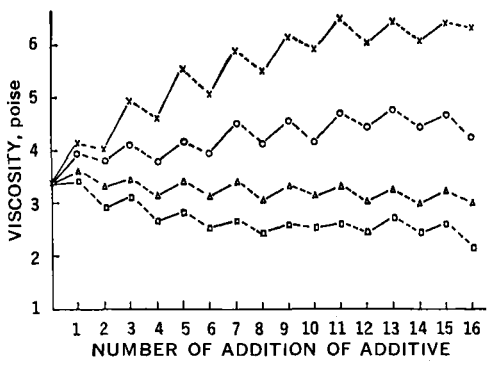

Figure 5—*Viscosity variation produced by different ratios of sodium hydroxide to citric acid in systems containing 5% cetrimide and 1.4% salicylic acid (50 g) at 25°. Key (1 N sodium hydroxide–7% citric acid): ×, 1:1; ○, 1:2; △, 1:4; □, 1:6; —, 1 N sodium hydroxide; and ---, 7% citric acid. Shear rate is 78.56 sec^{-1}.*

Table I—pH of Systems Containing 5% Cetrimide and 1.4% Salicylic Acid[a] in the Presence of Varying Volumes of 10% Acid Solutions

Acid Solution	Volume of Acid Solution, ml							
	0.2	0.4	0.8	1.2	1.6	2.2	2.8	3.4
Citric acid	2.45	2.45	2.45	2.45	2.45	2.40	2.40	2.35
Tartaric acid	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45
Acetic acid	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45
Lactic acid	2.35	2.35	2.35	2.35	2.35	2.35	2.35	2.35
Phosphoric acid	2.35	2.35	2.35	2.30	2.30	2.25	2.25	2.25
Dilute hydrochloric acid BP	2.35	2.25	2.15	2.10	2.05	1.95	1.90	1.85

[a] pH of system without additive is 2.45.

as the other sodium salts reported (1). Several approaches were made to determine whether the viscosity change brought about either by an acid or a base can be further influenced by a number of alternate additions of each additive. When the volumes of solutions of sodium hydroxide and of hydrochloric acid were kept constant so as to maintain a fixed ratio of base to acid, the viscosity increased and decreased with each addition of the basic and acidic solutions, respectively (Fig. 4). Keeping the same ratio but varying the additive volume did not alter the pattern of viscosity variation (Fig. 4). This behavior was not restricted either to a strong acid and a strong base or to one ratio.

By replacing hydrochloric acid with a weaker acid, citric acid (Fig. 5), each of the ratios used produced the typical viscosity reaction to sodium hydroxide and to citric acid. It was noted that the larger the ratio, the smaller the viscosity rise and the greater the viscosity fall. When the order of additive addition was reversed (Fig. 4), an increase and a decrease with respect to the additives were produced. From these results, it may be concluded that the changes in viscosity in these systems due to the added base and the acid can be predicted and that the viscosity effect exhibited by either additive can be reproduced in the presence of the other.

These procedures were applied to salicylic acid–cetrimide systems which were more than saturated with salicylic acid (Fig. 6).

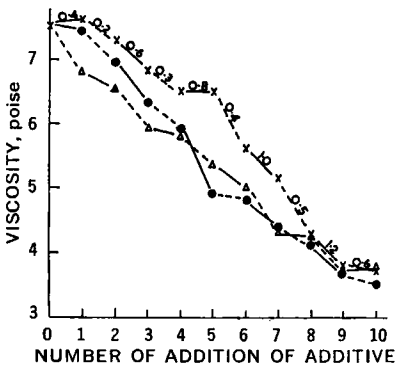

Figure 6—*Effect of different methods of adding base and acid on the viscosity of oversaturated systems (50 g) which contain 5% cetrimide and 1.8% salicylic acid at 25°. Key:* ●, *0.6 ml of 1 N NaOH–0.3 ml of 1 N HCl;* ×, *1 N NaOH–1 N HCl fixed at 2:1 but volume of additive is varied as indicated in milliliters on graph;* △, *0.6 ml of 1 N HCl–0.3 ml of 1 N NaOH;* —, *1 N NaOH; and* ---, *1 N HCl. Shear rate is 78.56 sec*$^{-1}$.

The normal rise and fall in viscosity (Figs. 4 and 5) were absent and only a lowering of viscosity occurred. Thus, two additives with contrasting properties have the same effect on these dispersions. Apparently, the viscosity behavior resulting from both types of additives was maintained. By varying the concentration of cetrimide but keeping the amount of salicylic acid constant and vice versa, systems undersaturated or oversaturated with salicylic acid were obtained. The effects of potassium hydroxide and hydrochloric acid solutions on such systems were also studied to ascertain that the observed viscosity changes were not limited to the sodium salt. The results were similar to those with sodium hydroxide and the same acid in corresponding systems.

The data presented show that the macromolecules in both types of salicylic acid–cetrimide systems are unstable to the additives. Recovery from a lower viscosity after the addition of acid additive to a greater value following the subsequent addition of alkali additive in an undersaturated system suggests that these systems are sensitive to these additives and that the viscosity change can be altered readily. This rapid recovery illustrates the degree of sensitivity.

This finding also supports the view that the bonding links involved in the formation of macromolecules are probably not strong and that there may exist a preferential reaction between sodium hydroxide and salicylic acid to produce sodium salicylate, which then interacts with the free cetrimide molecules, rather than between salicylic acid and cetrimide molecules, notwithstanding the previous presence of acid additive. Both reactions lead to an increase in viscosity. The insensitivity of the oversaturated systems may be due to the lack of free cetrimide molecules. Hence, the mechanism of macromolecule formation is not likely to involve complex interactions of such a nature as to give rise to a rigid structural system that is not easily affected by additives.

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(2) *Ibid.*, **60**, 939(1971).
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(5) *Ibid.*, **62**, 142(1973).

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Opium Alkaloids XV: Isolation of Stepholidine

E. BROCHMANN-HANSSEN [x] **and W. J. RICHTER** *

Abstract □ A protoberberine alkaloid was isolated from opium and characterized as (−)-2,10-dihydroxy-3,9-dimethoxytetrahydroprotoberberine by spectroscopic methods and by comparison with (−)-stepholidine.

Keyphrases □ Opium alkaloids—isolation and identification of (−)-stepholidine □ (−)-Stepholidine—isolation, identification as opium alkaloid □ Alkaloids, opium—isolation and identification of (−)-stepholidine □ Protoberberine alkaloids—isolation and identification of (−)-stepholidine from opium

In recent years, several new alkaloids have been isolated from opium and the opium poppy or detected by isotope dilution techniques based on established or probable biosynthetic pathways. The phenolic alkaloid fraction has been a particularly rich source of such alkaloids. The present article describes the isolation of a phenolic protoberberine from opium. Alkaloids of this group are found in several plant families. Scoulerine (I) (1) and isocorypalmine (II) (2, 3) were isolated from opium, while coreximine (VI) was detected by an isotope dilution method based on its biosynthesis from reticuline (4).

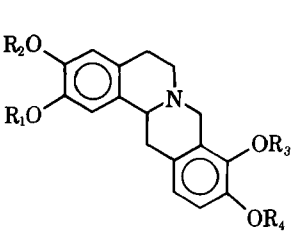
Preparative TLC of the opium fraction containing minor phenolic alkaloids revealed a new alkaloid, which was purified by column chromatography on neutral alumina and crystallized from methanol, mp 135–138°, $[\alpha]_D^{23}$ − 308° (ethanol).

DISCUSSION

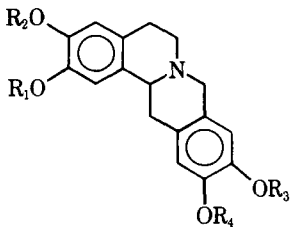
When subjected to GLC analysis[1], the new alkaloid produced a slightly tailing peak with a retention time of 23 min on a 3% OV-1 column at 230°. Silylation[2] gave a bis(trimethylsilyl) derivative (M = 471, determined by mass spectrometry[3]), which formed a sharp, symmetrical peak with increased retention time (31 min) under the same conditions, indicating that two active hydrogen atoms had been present. Correspondingly, the mass spectrum of the alkaloid displayed a molecular ion peak at *m/e* 327, for which high-resolution measurements[4] established a $C_{19}H_{21}NO_4$ composition.

The general fragmentation resembled the pattern characteristic of tetrahydroprotoberberine alkaloids (5–7), with the major fragments at *m/e* 178/177/176 and 150/149, suggesting a dihydroxydimethoxy compound having one hydroxy and one methoxy group in each of the rings A and D. In accordance with this finding, the NMR spectrum[5] in deuterochloroform with internal tetramethylsilane standard also revealed two methoxy singlets resonating at 3.80 and 3.86 ppm and four aromatic protons in the region of 6.5–6.85 ppm.

The optical rotatory dispersion spectrum[6] was very similar to spectra of (−)-scoulerine (I) (8), (−)-isocorypalmine (II)[7], and (−)-tetrahydropalmatine (III) (8). The small amplitude of the second Cotton effect (a = −144 in ethanol) was indicative of a



I: $R_1 = R_3 = H$, $R_2 = R_4 = CH_3$
II: $R_1 = H$, $R_2 = R_3 = R_4 = CH_3$
III: $R_1 = R_2 = R_3 = R_4 = CH_3$
IV: $R_1 = R_4 = H$, $R_2 = R_3 = CH_3$
V: $R_2 = R_4 = H$, $R_1 = R_3 = CH_3$



VI: $R_1 = R_4 = H$, $R_2 = R_3 = CH_3$

2,3,9,10-substitution pattern (8). This finding was confirmed by methylation of the alkaloid with diazomethane, which gave tetrahydropalmatine (III), identified by comparison with authentic material by TLC and GLC. In contrast to scoulerine, the new alkaloid gave no reaction with Gibb's reagent, suggesting that the phenolic functions had no unsubstituted *para*-position.

Mass spectrometric criteria recently developed for assignment of the substitution pattern of ring D of protoberberines, based upon the observation of $(M - OCH_3)^+$ fragments of due abundance[8], finally showed the methoxy group to be located at position 9. For this reason, the compound had to be either stepholidine (IV) (9) or its isomer with reverse substitution in ring A (V)[9]. Identity of the new opium alkaloid with stepholidine was borne out by comparison of the respective IR and mass spectra.

EXPERIMENTAL

Powdered opium of Indian origin [1.8 kg (4 lb)] was extracted, and a preliminary separation of alkaloid groups was carried out as described previously (10). The fraction containing the minor phenolic alkaloid was subjected to preparative TLC on silica gel with chloroform–methanol (9:1) and double development. A broad band, shown by analytical TLC to consist of several alkaloids, was collected, and the alkaloids were eluted from the silica gel with warm methanol. The solution was evaporated to dryness under reduced pressure, and the residue was chromatographed on a column of neutral alumina[10] with chloroform.

Twenty-milliliter fractions were collected with an automatic fraction collector. The first fraction contained porphyroxine, followed by scoulerine, *N*-methyl-14-*O*-desmethylepiporphyroxine, and an unknown alkaloid. The fractions containing this unknown alkaloid were combined and evaporated to dryness under reduced pressure, and the residue (25 mg) was crystallized from methanol, mp 135–138°[11] [lit. (9) 126–138° (*in vacuo*), 158–160° dec.], $[\alpha]_D^{23}$ − 308° (c = 0.192, ethanol) [lit. (9) $[\alpha]_D^{8}$ − 311° in ethanol]; λ_{max} (ethanol): 285 (log ϵ 3.77) and 226 (sh) (4.12) nm. Addition of alkali produced a bathochromic shift.

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(2) S. Pfeifer and J. Teige, *Pharmazie*, **17**, 692(1962).
(3) S. Pfeifer, *ibid.*, **21**, 492(1966).

[1] Varian Aerograph model 2100 gas chromatograph; glass column, 1.4 m (4.5 ft) × 2 mm i.d.
[2] Regisil-TMCS, Regis Chemical Co.
[3] CEC 21-110B mass spectrometer.
[4] The authors thank Dr. A. L. Burlingame and Mr. B. R. Simoneit of the Space Sciences Laboratory, University of California, Berkeley, Calif., for carrying out these measurements.
[5] Varian A-60A NMR spectrometer.
[6] JASCO model ORD/UV-5.
[7] E. Brochmann-Hanssen and K. Hirai, unpublished data.
[8] W. J. Richter and E. Brochmann-Hanssen, to be published.
[9] This isomer was recently shown to be identical with discretamine (W. J. Richter and E. Brochmann-Hanssen, to be published).
[10] Merck, activity 4.
[11] Micro-melting point, Kofler hot stage.

(4) E. Brochmann-Hanssen, C.-C. Fu, and G. Zanati, *J. Pharm. Sci.*, **60**, 873(1971).

(5) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Structural Elucidation of Natural Products by Mass Spectrometry," vol. I, Holden-Day, San Francisco, Calif., 1964, p. 181.

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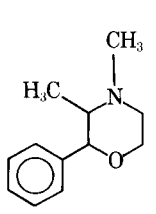
GLC Determination of Phendimetrazine in Serum

H. K. L. HUNDTˣ, E. C. CLARK, and F. O. MÜLLER

Abstract □ A sensitive, specific, and quantitative GLC method for the determination of phendimetrazine in serum is described. The procedure involves the addition of an internal standard to serum samples, followed by extraction at pH 13 into toluene. The extracted bases are back-extracted into 1 ml of 4 *M* hydrochloric acid and again into 100 μl of chloroform after making the 4 *M* hydrochloric acid extract basic with 1.5 ml of 4 *M* sodium hydroxide. The sensitivity of the method is such that 25 ng of material can be detected in 5 ml of serum

Keyphrases □ Phendimetrazine—GLC analysis in human serum □ GLC—analysis, phendimetrazine in human serum

Phendimetrazine (I) is an anorexigenic agent marketed in South Africa in the form of a standard formulation tablet. To satisfy the requirements of the local Drugs Control Council, the introduction of a timed-release preparation of I on the South African market necessitated the development of a sensitive quantitative method for the detection of I so that serum levels attained after administration of a single timed-release tablet (containing 105 mg of phendimetrazine bitartrate) could be compared with the levels attained after administration of a single tablet of the standard formulation (containing 35 mg of phendimetrazine bitartrate). Although various articles refer to the clinical and toxicological aspects (1–7) of I and its qualitative identification by TLC (8) and GC (9), only one reference dealing with the quantitative determination of phendimetrazine in plasma utilizing a ^{14}C-radioactive tracer technique (10) and one dealing with *in vitro* release studies of timed-release phendimetrazine (11) could be found.



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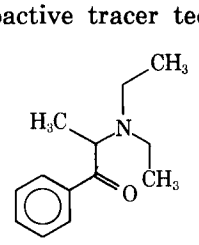

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This report describes a sensitive GLC method for I determinations, under conditions where only I was administered, using diethylpropion (II) as an internal standard.

Table I—Recovery of Phendimetrazine from Serum

Amount Added, ng/ml	Amount Recovered, ng/ml				Mean ± *SD*, ng/ml
20	20.2	20.7	20.9	19.4	20.30 ± 0.67
40	38.9	38.9	37.7	37.9	38.35 ± 0.64
60	59.0	61.2	61.8	62.3	61.08 ± 1.45
80	79.6	80.7	79.5	79.0	79.70 ± 0.71

EXPERIMENTAL

Reagents and Chemicals—The chemicals and reagents used were: 2 *M* sodium hydroxide[1], 4 *M* sodium hydroxide, 4 *M* hydrochloric acid[1], toluene[1], chloroform[1], phendimetrazine bitartrate[2], and diethylpropion hydrochloride[3].

Instrumentation—A gas chromatograph[4] equipped with a flame-ionization detector and a 183-cm × 6-mm glass column containing 3% SE-30 on Chromosorb W (AW HMDS), 80–100 mesh, was used. The injection port and detector were kept at 250°. The column temperature was kept at 140° for 8 min after injection and then programmed at 32°/min to 250°, where it was kept for 8 min. Nitrogen was used as the carrier gas at a flow rate of 80 ml/min. Quantitation was achieved by measuring peak heights. (Peak area calculation by a digital integrator was found to be less reliable.)

Measurement of I in Serum—To 5 ml of serum contained in a 20-ml glass-stoppered centrifuge tube were added 0.5 μg of internal standard (in 50 μl of water), 1 ml of 2 *M* sodium hydroxide, and 5 ml of toluene. The tube was shaken for 2 min (vortex) and centrifuged, and as much as possible of the organic phase was transferred to a 10-ml, glass-stoppered, tapered centrifuge tube contain-

[1] E. Merck, Darmstadt. Pro analysi.
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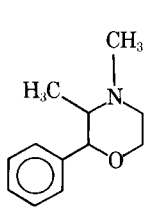

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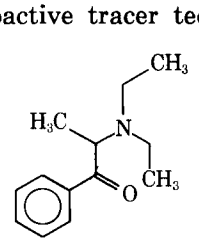

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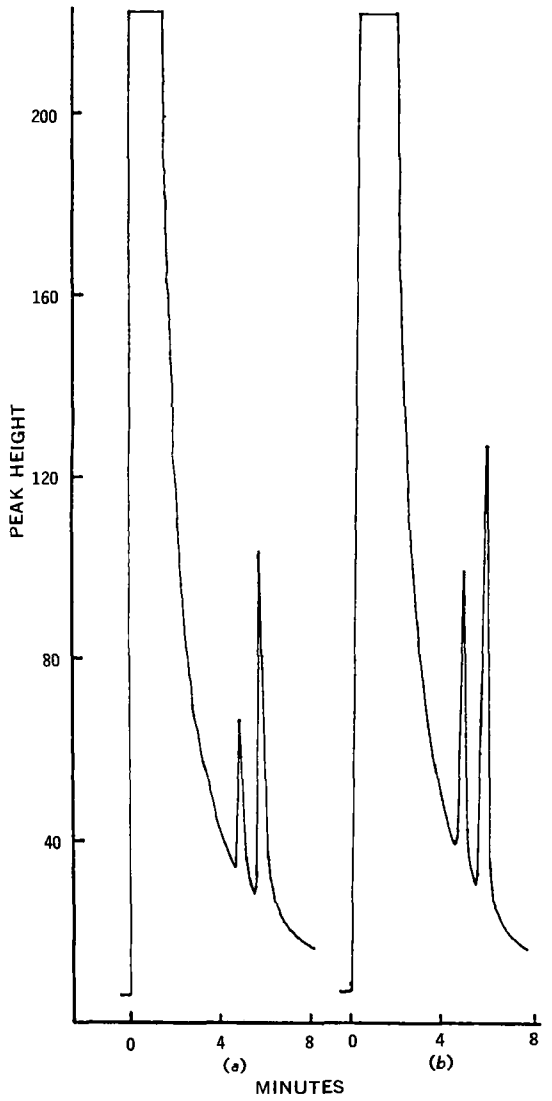

Figure 1—*Chromatograms of* (a) *I and II added to control serum and carried through extraction, and* (b) *material obtained from biological specimens after addition of II (internal standard).*

ing 1 ml of 4 *M* hydrochloric acid. After shaking for 2 min, the tube was centrifuged and the organic phase was removed by aspiration.

The aqueous phase was transferred to a special 5-ml glass-stoppered centrifuge tube equipped with a small nipple in which liquids denser than water can collect after centrifuging. The centrifuge tube was cooled for 2 min in an ice bath, the aqueous phase was made strongly alkaline by addition of 1.5 ml of 4 *M* sodium hydroxide, and the free bases were extracted into 0.1 ml of chloroform (vortex, 2 min). After brief centrifugation, 5 μl of the chloroform phase was injected into the gas chromatograph. The retention times of I and II were 292 and 345 sec, respectively.

Standard serum curves were constructed by plotting the peak height ratios (I/II) *versus* weight ratios (I/II) of standard samples which were run simultaneously with the unknown samples as previously described. The peak height ratio obtained from an unknown was then used to determine the amount of I present.

Absorption Studies—One tablet of the standard formulation was administered to each of five healthy human subjects who had fasted overnight. Blood was collected by venipuncture before administration of the drug and at intervals after administration. The serum was separated by centrifugation and frozen until assayed.

For assay, equal amounts of serum of each volunteer were pooled for each sample and the samples were analyzed in duplicate as previously described. After a washout period of 3 days, the same procedure was followed with the administration of one tablet of the timed-release preparation to the same volunteers.

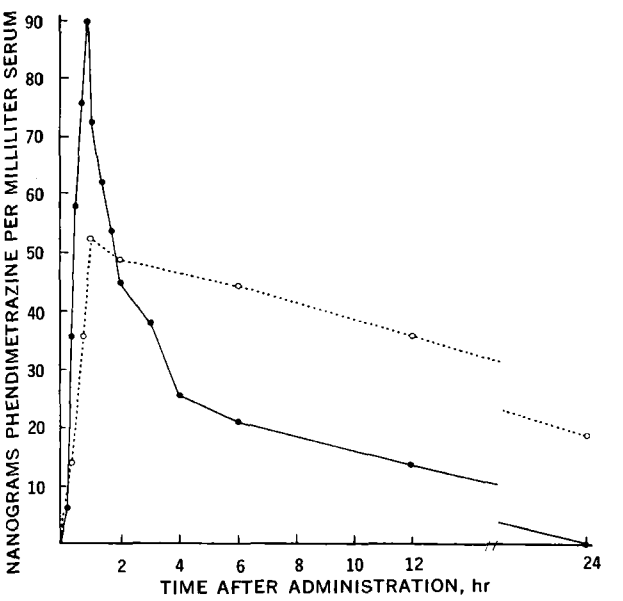

Figure 2—*Pooled serum levels of I after administration of standard formulation* (●) *and timed-release formulation* (○).

RESULTS AND DISCUSSION

Figure 1 represents the chromatogram of: *(a)* I and II added to control serum and carried through the extraction and GLC procedure, and *(b)* material obtained from biological specimens after the addition of II as the internal standard.

Figure 2 represents pooled serum levels of I after administration of the standard formulation and the timed-release formulation to the same five volunteers.

Gas chromatograms of control samples taken from the volunteers before any drug had been administered did not exhibit any peaks with retention similar to I or II. Possible interference by metabolites of I could not be checked because these metabolites were

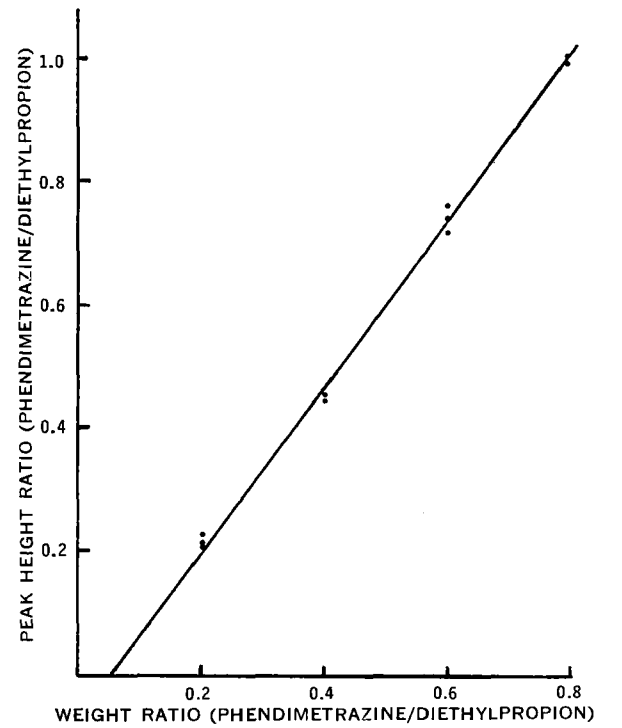

Figure 3—*Relationship between peak height ratio and weight ratio (phendimetrazine/diethylpropion). Five microliters was injected in each case out of 100 μl following extraction from serum.*

not available. However, a small peak with a retention time of 252 sec appeared occasionally in extracts of serum from volunteers but did not interfere with the assay; it was attributed to a metabolite.

A typical standard graph of peak height ratio (I/II) *versus* weight ratio (I/II) is presented in Fig. 3. A straight line with a slope varying between 1.31 and 1.35 and an intercept varying between 0.04 and 0.06 on the weight ratio axis was obtained by linear regression analysis over 1 month during which the analyses were performed. The intercept on the weight ratio axis corresponds to a loss of 0.8–1.2 ng of I on the GLC column during chromatography, thus imposing a lower limit of detection of I in serum by this method at about 5 ng/ml. A peak for I just begins to appear in the chromatogram at this concentration. A summary of the recovery results of I obtained with human serum when 20–80 ng/ml was added to the serum is presented in Table I.

This GLC method was applied to a large number of samples of human serum during a trial comparing serum levels attained after administration of a standard formulation with levels attained after administration of a timed-release formulation of I.

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Colorimetric Determination of Formaldehyde *via* Free Radical Formation

JAMES W. MUNSON [x] and THOMAS G. HODGKINS

Abstract □ Aliphatic aldehydes react with 2,3-dimethyl-2,3-bis(hydroxylamino)butane and sodium periodate to form colored free radicals. These radicals were stabilized with pyridine in aqueous solution. Low levels of formaldehyde in aqueous solutions were determined utilizing this reaction.

Keyphrases □ Formaldehyde—stabilized colorimetric analysis in aqueous solution *via* reaction with 2,3-dimethyl-2,3-bis(hydroxylamino)butane and sodium periodate □ 2,3-Dimethyl-2,3-bis(hydroxylamino)butane—colorimetric reagent for determination of formaldehyde in aqueous solution □ Colorimetry—analysis, formaldehyde in aqueous solution using 2,3-dimethyl-2,3-bis(hydroxylamino)butane

Aldehydes have been shown to form stable free radicals when reacted with 2,3-dimethyl-2,3-bis(hydroxylamino)butane (I) (1) (Scheme I). The anhydro product (II) can be isolated directly or may be converted to the free radical (III) by the action of lead dioxide or sodium periodate. The free radicals are red or blue in solution. The color of these free radicals suggests that this reaction may be suitable for colorimetric determination of aldehydes.

Preliminary data suggested that only aliphatic aldehydes and ketones react with I to form a colored product in aqueous solution. A spot test based on this reaction was developed that allows detection of as little as 0.2 μg of aliphatic aldehydes (2). Aliphatic ketones gave a much smaller response, with 100–200 μg being the minimum detectable quantity.

Initial attempts to utilize this reaction in a quantitative manner were plagued by the instability of the color of the free radical. The half-life for the disappearance of color was about 7 min at 25°. This paper reports the stabilization of the color and the adaptation of this reaction to the determination of formaldehyde at low concentrations in aqueous solution.

EXPERIMENTAL

Materials—Formaldehyde stock solutions were analyzed by peroxide oxidation (3). Acetaldehyde was redistilled and chilled. A portion of acetaldehyde was added to a tared flask, sealed, and reweighed. Water was then added to give a known concentration. All other aldehyde stock solutions were prepared by dissolving weighed amounts of the redistilled aldehyde in water. 2,3-Dimethyl-2,3-bis(hydroxylamino)butane sulfate[1] was recrystallized from a 2-propanol–water mixture, mp 185° dec.

Anal.—Calc. for $C_6H_{18}N_2O_6S$: C, 29.27; H, 7.32; N, 11.38. Found: C, 27.78; H, 7.54; N, 11.12.

Sodium periodate[2] and the organic solvents were reagent grade and were used directly.

Effect of Solvent on Stability of Color—An aqueous reaction mixture containing formaldehyde (1.34×10^{-3} *M*) and 2,3-dimethyl-2,3-bis(hydroxylamino)butane sulfate (Ia) (1.76×10^{-2} *M*) was prepared and allowed to react for 1 hr at 25°. One-milliliter aliquots of the reaction mixture were added to a tube containing 1 ml of the solvent being studied and 1 ml of sodium periodate (0.094 *M*).

[1] Eastman Organic Chemicals

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EXPERIMENTAL

Materials—Formaldehyde stock solutions were analyzed by peroxide oxidation (3). Acetaldehyde was redistilled and chilled. A portion of acetaldehyde was added to a tared flask, sealed, and reweighed. Water was then added to give a known concentration. All other aldehyde stock solutions were prepared by dissolving weighed amounts of the redistilled aldehyde in water. 2,3-Dimethyl-2,3-bis(hydroxylamino)butane sulfate[1] was recrystallized from a 2-propanol–water mixture, mp 185° dec.

Anal.—Calc. for $C_6H_{18}N_2O_6S$: C, 29.27; H, 7.32; N, 11.38. Found: C, 27.78; H, 7.54; N, 11.12.

Sodium periodate[2] and the organic solvents were reagent grade and were used directly.

Effect of Solvent on Stability of Color—An aqueous reaction mixture containing formaldehyde (1.34×10^{-3} *M*) and 2,3-dimethyl-2,3-bis(hydroxylamino)butane sulfate (Ia) (1.76×10^{-2} *M*) was prepared and allowed to react for 1 hr at 25°. One-milliliter aliquots of the reaction mixture were added to a tube containing 1 ml of the solvent being studied and 1 ml of sodium periodate (0.094 *M*).

[1] Eastman Organic Chemicals

[2] J. T. Baker Chemical Co.

Table I—Effect of Solvent on Stability of Color of Free Radical

Solvent	$10^3 k_{obs}$, sec^{-1}	Concentration of Added Solvent, M	k_{obs} (solvent)/k_{obs} (water)
Water	1.63	—	1.00
Methanol	1.70	8.2	1.04
2-Propanol	1.35	4.3	0.83
Hydrogen peroxide (30% w/v)	2.0	3.3	1.23
Dioxane	1.61	3.9	0.99
Tetrahydrofuran	0.60	4.1	0.37
Pyridine	0.028	8.2	0.017
Acetonitrile	0.85	6.3	0.52
Dimethyl sulfoxide	1.61	4.7	0.99
Dimethylformamide	1.36	4.3	0.83

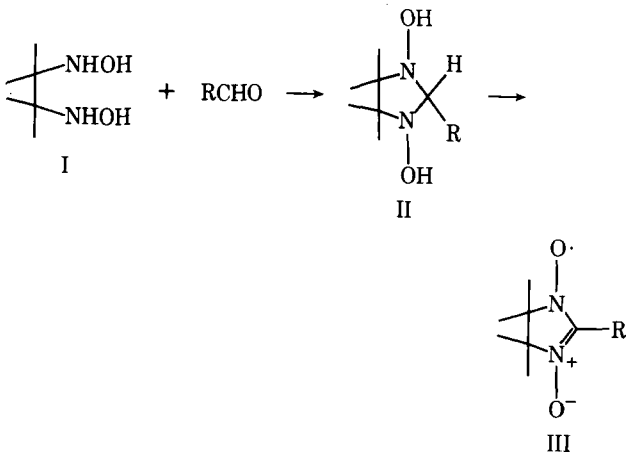
The absorbance[3] at 515 nm was measured as a function of time at 25°. Plots of $A_t - A_\infty$ *versus* time were linear for at least three half-lives of the color disappearance. Pseudo-first-order rate constants for this process were calculated from the slopes of these plots.

Effect of Pyridine Concentration on Stability—Reaction mixtures were prepared in the same manner as described in the previous section. The final solutions contained formaldehyde (4.47×10^{-4} *M*), Ia (8.22×10^{-3} *M*), and pyridine ranging from 0.41 to 8.24 *M*. Absorbance was measured as a function of time at 25°, and pseudo-first-order rate constants were calculated from plots of $A_t - A_\infty$ *versus* time.

Effect of Reaction Time on Extent of Color Formation—A reaction mixture containing Ia (1.32×10^{-2} *M*) and formaldehyde (2.76×10^{-3} *M*) was prepared and allowed to react at 25°. Aliquots (0.5 ml) were removed as a function of time and mixed with 0.5 ml of sodium periodate (3.7×10^{-2} *M*) and 2.0 ml of pyridine. The absorbance at 515 nm was measured at 1500 sec after the mixing of the final solution.

Effect of Ratio of Sodium Periodate to Formaldehyde on Extent of Color Formation—A reaction mixture was prepared containing Ia (1.32×10^{-2} *M*) and formaldehyde (2.68×10^{-3} *M*) and allowed to react for 5 min at 25°. Then 0.5 ml of the reaction mixture was added to 2.0 ml of pyridine and 0.5 ml of a sodium periodate solution. The absorbance was measured at 515 nm after 25 min.

Calibration Curve for Formaldehyde—Reaction mixtures were prepared containing Ia (1.32×10^{-2} *M*) and varying amounts of formaldehyde. The reaction mixture was allowed to stand at 25° for 5 min, and 0.5 ml was added to a cell containing 0.5 ml of sodium periodate (0.037 *M*) and 2.0 ml of pyridine. Absorbance at 515 nm was measured 25 min after the addition of the sodium periodate and pyridine.



I II III

Scheme I

[3] Absorbance measurements were made with a Cary 15 recording spectrophotometer equipped with a thermostatted cell compartment or a Gilford model 240 spectrophotometer.

Table II—Effect of Reaction Time on Extent of Color Formation[a]

Reaction Time, sec[b]	A_{515}[c]
10	0.525
20	0.540
80	0.561
120	0.590
300	0.594
600	0.588

[a] Formaldehyde substrate. [b] Time of reaction of Ia and formaldehyde before the addition of sodium periodate. [c] Absorbance measured 1500 sec after the addition of sodium periodate.

Reaction of Other Compounds—Reaction mixtures were prepared containing Ia (1.32×10^{-2} *M*) and some aldehydes and ketones. As a function of time, 0.5 ml of the reaction mixture was added to 0.5 ml of sodium periodate (0.037 *M*) and 2.0 ml of pyridine. Absorbance was measured at the appropriate wavelength after 25 min.

RESULTS AND DISCUSSION

Of the solvents tested (Table I), pyridine, acetonitrile, and tetrahydrofuran stabilized the color of the free radical. Only pyridine gave sufficient stability for analytical procedures. It was observed that the color increased immediately after the addition of the sodium periodate and then stabilized after 15 min. The color was quite stable after this time for at least 1 hr. During this span, the absorbance decreased at a rate of 0.8%/hr.

While these free radicals have been found to be stable in organic solvents, such as cyclohexane (1), their stability in primarily aqueous solution has not been reported. Although the solvents were not added in equimolar concentrations, an examination of the concentrations (Table I) does not suggest that any of the solvents approach the ability of pyridine to stabilize the color when the stability is compared on an equimolar basis.

Formaldehyde reacted very rapidly, the reaction being complete within 5 min at room temperature (Table II). Other aliphatic aldehydes reacted more slowly than formaldehyde under the same conditions (Table III). Acetone and benzaldehyde failed to react within 30 min under the same conditions and equivalent concentrations.

Increasing pyridine concentration in the final solution led to greater color stability (Table IV). A concentration of 8.2 *M* was chosen for convenience of sample handling. Furthermore, this concentration gave sufficient stability for the analytical procedure. The rate constants obtained for pyridine concentrations of 2.06 and 4.12 *M* were redetermined to check the apparent decrease in stability upon going from 2.06 to 4.12 *M* pyridine. These data are apparently real and at this time unexplained. All rate constants were determined at a single formaldehyde concentration.

The ratio of sodium periodate to formaldehyde (and by inference the anhydro product II) in the final solution was found to be

Table III—Rate and Extent of Reaction of Aldehydes and Ketones

Compound	Wavelength of Maximum Absorbance, nm	t_∞[a], sec	Log ϵ[b]
Formaldehyde	515	300	3.06
Acetaldehyde	538	600	3.28
Propionaldehyde	543	1000	3.20
Butyraldehyde	546	1000	3.16
Isobutyraldehyde	547	1400	3.13
Acetone		N.R.[c]	
Benzaldehyde		N.R.[c]	

[a] Time to reach maximum absorbance. [b] Molar absorptivity calculated on basis of formaldehyde concentration in sample (and by inference the concentration of free radical). [c] No reaction within 30 min.

Table IV—Effect of Pyridine Concentration on Color Stability[a]

Pyridine, M	$10^5 k_{obs}$, sec^{-1}
0.41	9.8
2.06	0.92
4.12	1.7
8.24	0.45

[a] Formaldehyde, 4.47×10^{-4} M; Ia, 8.22×10^{-4} M; sodium periodate, 3.12×10^{-2} M; 25°.

critical in that sodium periodate must be in at least a twofold excess for maximal color development (Table V). Therefore, a relatively high concentration of sodium periodate should be used when a wide concentration range of aldehyde is to be studied.

A calibration curve for the determination of formaldehyde gave excellent results. The slope of the line was 1156 liters $mole^{-1}$ cm^{-1} (1-cm cell used) with an intercept of 0.008 when calculated by linear regression analysis. The sample correlation coefficient was 0.999 with a standard error of the estimate of 0.009 ($S_{y \cdot x}$). Formaldehyde concentrations down to 4×10^{-5} M can be determined using a 1-cm cell and a spectrophotometer[4] (no scale expansion). It is quite conceivable that lower concentrations could be determined if enough sample were available to allow for the use of longer path length cells. Above concentrations of 10^{-3} M, deviation from Beer's law was observed.

Although other aliphatic aldehydes reacted more slowly than formaldehyde, each gave a similar color yield. It is not unexpected that acetone and benzaldehyde did not react since much higher concentrations of these compounds must be present to give a positive spot test using a variation of this reaction (2). Furthermore, on the basis of spot test data, no interference from a variety of carboxylic acid derivatives is expected (2).

[4] Cary 15.

Table V—Effect of Ratio of Sodium Periodate to Formaldehyde on Color Formation

Sodium Periodate: Formaldehyde[a]	A_{515}[b]
0.70	0
1.40	0.133
1.75	0.362
2.10	0.563
3.49	0.553
6.99	0.563
13.67	0.572

[a] Molar concentrations. [b] Absorbance measured 25 min after the addition of sodium periodate and pyridine.

This method represents a rapid and sensitive means for the determination of formaldehyde in aqueous solutions. Total analysis time for a single determination is 30 min, with as many as 30 samples being analyzed per hour.

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Nidation Inhibition by Simple Ergoline Derivatives

KAREL ŘEŽÁBEK *, JOHN M. CASSADY ‡, and HEINZ G. FLOSS ‡x

Abstract □ The ability of four ergoline-type compounds (elymoclavine, its *O*-benzoate and *O*-carbamate, and *N*-methyl-6,7-seco-elymoclavine) to inhibit nidation in rats was determined and found to parallel their prolactin-inhibiting activity.

Keyphrases □ Nidation—inhibition by four ergoline-type compounds, rats, relationship to prolactin-inhibiting ability □ Ergoline-related compounds—inhibition of nidation, relationship to prolactin-inhibiting ability □ Elymoclavine and related compounds—inhibition of nidation, rats

Research interest in the pharmacology of ergot alkaloids is currently focusing on two areas: their antihypertensive activity and their effect on hormone release from the anterior pituitary gland. The latter effect, primarily due to a direct inhibition of the synthesis and release of prolactin, manifests itself in the inhibition of some prolactin-dependent processes, *e.g.*, lactation in various animals and in humans, development of certain types of mammary tumors, and implantation of the fertilized egg (nidation) in rats and mice (1). While nidation inhibition in rats is generally thought to be predominantly or exclusively the result of prolactin inhibition, Flückiger and Wagner (2) obtained results that led them to conclude that 2-bromo-α-ergokryptine, a modified peptide ergot alkaloid which is undergoing clinical evaluation, has a specific nidation-inhibiting effect.

Since it has been shown (1, 3–5) that the peptide portion of these alkaloids is not required for activity, it was of interest to compare the ability of some simple ergolenes to inhibit prolactin release and nidation. For this comparison, four compounds were se-

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A calibration curve for the determination of formaldehyde gave excellent results. The slope of the line was 1156 liters $mole^{-1}$ cm^{-1} (1-cm cell used) with an intercept of 0.008 when calculated by linear regression analysis. The sample correlation coefficient was 0.999 with a standard error of the estimate of 0.009 ($S_{y \cdot x}$). Formaldehyde concentrations down to 4×10^{-5} M can be determined using a 1-cm cell and a spectrophotometer[4] (no scale expansion). It is quite conceivable that lower concentrations could be determined if enough sample were available to allow for the use of longer path length cells. Above concentrations of 10^{-3} M, deviation from Beer's law was observed.

Although other aliphatic aldehydes reacted more slowly than formaldehyde, each gave a similar color yield. It is not unexpected that acetone and benzaldehyde did not react since much higher concentrations of these compounds must be present to give a positive spot test using a variation of this reaction (2). Furthermore, on the basis of spot test data, no interference from a variety of carboxylic acid derivatives is expected (2).

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Keyphrases □ Nidation—inhibition by four ergoline-type compounds, rats, relationship to prolactin-inhibiting ability □ Ergoline-related compounds—inhibition of nidation, relationship to prolactin-inhibiting ability □ Elymoclavine and related compounds—inhibition of nidation, rats

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Since it has been shown (1, 3–5) that the peptide portion of these alkaloids is not required for activity, it was of interest to compare the ability of some simple ergolenes to inhibit prolactin release and nidation. For this comparison, four compounds were se-

Table I—Nidation Inhibition of Some Simple Ergolene Derivatives in Rats

	Series 1			Series 2				Series 3		
	Control	Elymoclavine Carbamate (1 mg)	*N*-Methylsecoelymoclavine (1 mg)	Control	D-6-Methyl-8-ergoline-1-yl-acetamide Tartrate (1 mg)	Elymoclavine Benzoate (1 mg)	Elymoclavine (1 mg)	Control	Elymoclavine Carbamate (0.1 mg)	Summarized Controls
Number of pregnant animals/total number of animals in group	6/8	0/7[a]	4/7[b]	7/7	0/6[a]	0/7[a]	0/7[a]	2/3	3/6[b]	15/18
Average number of fetuses per pregnant animal	9.7	—	10.5	9.2	—	—	—	8.5	8.7	9.3
Well-developed fetuses per pregnant animal	9.3	—	9.0	8.4	—	—	—	8.5	8.3	8.8
Resorbed fetuses per pregnant animal	0.3	—	1.5[b]	0.7	—	—	—	0.0	0.3	0.5
Percentage of resorbed fetuses/total number of fetuses	3.4/58	—	14.3/42[b]	7.8/64	—	—	—	0/17	3.8/26	5.0/139
Mean weight developed fetuses, g ± *SEM*	2.95 ± 0.05	—	3.55 ± 0.05[a]	—	—	—	—	—	—	—
Weight of pregnant animals at beginning of experiment, g ± *SEM*	179 ± 4	—	190 ± 9[b]	—	—	—	—	—	—	—

[a] Significantly different from the parallel and summarized controls ($p = 0.05$). [b] Difference from controls not statistically significant.

lected which had been assayed for prolactin inhibition in rats (5). Three of these compounds (elymoclavine, its benzoate, and its carbamate) were active (95, 66, and 66% of inhibition shown by ergocornine as reference); the fourth (*N*-methyl-6,7-secoelymoclavine), although structurally closely related, had only borderline activity (40% of reference).

EXPERIMENTAL

Nidation inhibition by these compounds was determined using virgin female rats of the Wistar strain weighing 170–230 g at the beginning of the experiment. They were caged with fertile males and their vaginal smears were examined each morning. The day of finding spermatozoa in the vagina was considered Day 1 of the experiment, and from this day on the animal was caged separately from males.

The compounds to be tested were dissolved in 0.2% aqueous tartaric acid solution at concentrations of 1 or 0.1 mg/ml (except in the case of elymoclavine benzoate which required addition of 10% ethanol for solution). They were administered, *via* stomach tube, to groups of six or seven animals once a day on Days 6 and 7. Each animal received 1 ml of solution/day, *i.e.*, 1 or 0.1 mg of drug, corresponding to approximately 5 or 0.5 mg/kg. The time of administration and the doses were chosen on the basis of previous experience (4). Control animals received 1 ml of 0.2% tartaric acid solution.

On Day 20, the animals were killed and the uterus was examined. The number of well-developed fetuses and of resorbed fetuses was determined, and the well-developed fetuses were weighed in some experiments (Table I).

RESULTS AND DISCUSSION

The experiments clearly show that elymoclavine, elymoclavine benzoate, and elymoclavine carbamate completely suppress the nidation of embryos and thus pregnancy in rats when given orally in doses of 5 mg/kg on the 5th and 6th days after copulation. When given at the lower dose of 0.5 mg/kg, elymoclavine carbamate did not significantly reduce the number of animals found pregnant. D-6-Methyl-8-ergoline-1-yl-acetamide tartrate, a known potent lactation and nidation inhibitor, was effective at both the higher (Table I) and the lower dose (6). *N*-Methylsecoelymoclavine, when given in the dose of 5 mg/kg in the same way as the other drugs, had no significant effect on nidation but caused an interesting, unexplained 20% increase in weight of the fetuses.

It follows from the results of these experiments that, in the simple ergolene series, the nidation-inhibitory activity parallels prolactin-inhibiting ability at least qualitatively.

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Positional Isomer of the Muscle Relaxant Dantrolene

THOMAS J. SCHWAN [x] **and K. O. ELLIS**

Abstract □ 3-[[5-(*p*-Nitrophenyl)furfurylidene]amino]hydantoin, a position isomer of dantrolene, was synthesized and evaluated for skeletal muscle relaxant activity.

Keyphrases □ Dantrolene positional isomer (3-[[5-(*p*-nitrophenyl)furfurylidene]amino]hydantoin)—screened for skeletal muscle relaxant activity □ 3-[[5-(*p*-Nitrophenyl)furfurylidene]amino]hydantoin (dantrolene positional isomer)—screened for skeletal muscle relaxant activity □ Skeletal muscle relaxant activity—3-[[5-(*p*-nitrophenyl)furfurylidene]amino]hydantoin (dantrolene positional isomer) screened

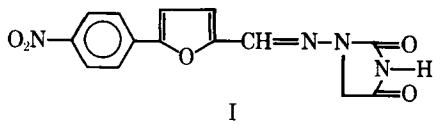
Dantrolene, 1-[[5-(*p*-nitrophenyl)furfurylidene]amino]hydantoin (I), was shown previously to demonstrate unique skeletal muscle relaxant activity (1, 2) by a direct action on skeletal muscle. It has been pharmacologically categorized as a skeletal muscle contraction antagonist (3). This report describes the synthesis of 3-[[5-(*p*-nitrophenyl)furfurylidene]amino]hydantoin (II), a positional isomer of I, and the evaluation of this compound for skeletal muscle relaxant activity.

DISCUSSION

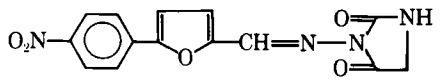
The reaction of 5-(*p*-nitrophenyl)-2-furaldehyde (III) with 3-aminohydantoin (IV) (4) by a previously described method (5) gave the target Compound II.

When using gross observational techniques similar to those described by Irwin (6), II did not produce any evidence of muscle relaxation or any other pharmacological activity at doses from 200 to 1600 mg/kg po. No toxicity was noted over the same dose range.

In the pithed rat gastrocnemius muscle preparation (7), a dose of 25 mg/kg iv administered in tetrahydrofurfuryl alcohol (0.75 ml/kg) caused a 25.3 ± 1.8% decrease in the twitch response. The solvent itself produced no significant effect on the twitch response, while dantrolene administered at the same dose caused a 70% decrease in the twitch response (7). This twitch inhibition indicates that II has slight contraction antagonist activity but that potency and effectiveness are less than that of dantrolene.



I



II

EXPERIMENTAL[1]

Compound II was prepared as follows. The reaction of III and IV by a previously described method (5) gave II, mp 233–234°. The analytical sample, mp 268–269°, was obtained by recrystallization from nitromethane; IR (μm): 5.61 and 5.80 (hydantoin C=O); NMR (dimethyl sulfoxide-d_6): δ 4.03 (s, 2, hydantoin CH_2), 7.28, 7.44 (doublets, J = 3.5 Hz, 2, furan C—H), 7.98, 8.28 (2 doublets, J = 9.0 Hz, 4, phenyl C—H), 8.28 (m, 1, N—H), and 9.23 (s, 1, =C—H).

Anal.—Calc. for $C_{14}H_{10}N_4O_5$: C, 53.50; H, 3.21; N, 17.83. Found: C, 53.51; H, 3.24; N, 18.03.

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[x] To whom inquiries should be directed.

[1] Melting points were determined on a Mel-Temp apparatus and are uncorrected. The IR spectrum was determined as a mineral oil mull using a Perkin-Elmer 137B spectrophotometer. The NMR spectrum was obtained on a Varian A-60A instrument and was compared with tetramethylsilane as an internal standard.

GLC Analysis of Metronidazole in Human Plasma

N. F. WOOD

Abstract □ A sensitive procedure was developed for metronidazole in human plasma. The metronidazole is extracted from the plasma with chloroform and determined as the trimethylsilyl derivative by GLC using a flame-ionization detector. Myristyl alcohol is used as the internal standard for quantitation by relative peak height.

Keyphrases □ Metronidazole—GLC analysis in human plasma □ GLC—analysis, metronidazole in human plasma

Metronidazole (2-methyl-5-nitroimidazole-1-ethanol) (I) is an antiprotozoal agent that has been widely used for many years in the oral treatment of trichomoniasis. The methods most frequently used for determining metronidazole in biological fluids have been polarography (1, 2) and colorimetry involving reduction of the nitro moiety (2, 3). A comparative study (2) found that these methods gave inconsistent results and that even a biological method was more reliable. Recently, more specific chromatographic methods were reported: a TLC method for metronidazole in serum (4) and a GLC method for metronidazole in plasma (5).

The GLC method described in the present work was developed before publication of the method of Midha *et al.* (5); although similar in approach, it has some advantages over the Midha *et al.* method.

EXPERIMENTAL

GLC—A gas chromatograph[1] equipped with a flame-ionization detector was used. A 183 × 0.04-cm column packed with OV-1 (3%) on Gas Chrom Q was operated at 160° with a carrier of nitrogen at 70 ml/min. Detector flow rates were: hydrogen, 45 ml/min, and air, 300 ml/min. Electrometer attenuations were 10 × 16 or 10 × 32. Injection size was 2 μl. Retention times of trimethylsilyl derivatives were as follows: metronidazole, 4.1 min; 2-methyl-5-nitroimidazole-1-ylacetic acid, 5.1 min; and myristyl alcohol, 8.1 min. After each plasma analysis, it was found preferable to raise the column temperature to 200° for about 10 min to eliminate the possibility of strongly retained substances interfering with subsequent analyses.

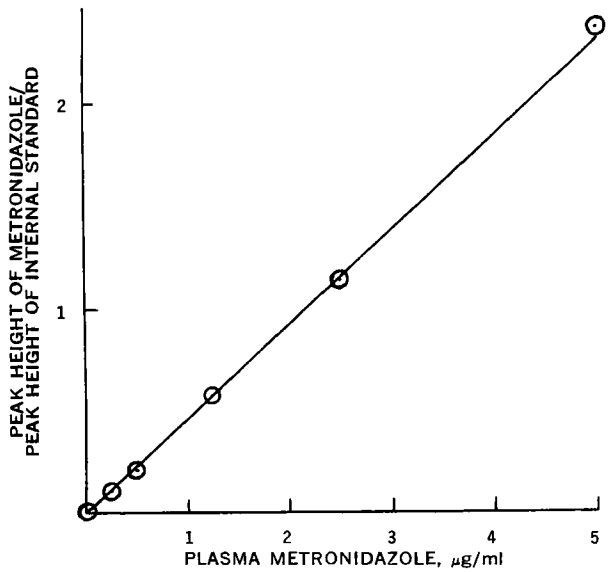


Figure 1—*Calibration chart for metronidazole.*

[1] Tracor MT-220.

Table I—Recovery of Metronidazole Added to Human Plasma

Metronidazole, μg/ml	Number of Experiments	Range of Results, μg/ml	Average Result, μg/ml	*RSD*, % ±	*RE*, %
0.5	2	0.48–0.60[a]	0.54	15.37	+8.00
1.5	3	1.51–1.65[a]	1.60	4.74	+6.67
2.5	4	2.37–2.53	2.49	3.09	−0.40
3.75	3	3.80–4.12	3.98	4.11	+6.13
5.0	2	4.62–4.80	4.71	2.70	−5.80
Average recovered		102.9%			

[a] Corrected for blank of 0.05 μg/ml.

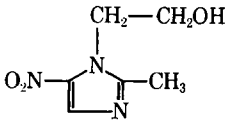
Method—Plasma (2 ml) adjusted to pH 8 was extracted with chloroform (1 × 20, 1 × 10, and 1 × 5 ml), and 100 μl of chloroform containing 5 mg/100 ml of myristyl alcohol was added to the extract. The extract was dried over anhydrous sodium sulfate for a few minutes and then decanted into an evaporation flask. After removal of chloroform using a rotary evaporator, the residue was transferred to a 1-ml vial with 0.5 ml of methanol. The methanol was removed in a stream of nitrogen, 50 μl of bis(trimethylsilyl)trifluoroacetamide was added, and the vial was immediately capped with a Teflon-coated rubber septum.

After standing overnight, the solution was examined by GLC and the ratio of the peak heights of the metronidazole and the myristyl alcohol derivatives was determined. Finally, the metronidazole was found by reference to a calibration chart relating the peak height ratio to plasma concentration. The calibration chart was prepared from standard mixtures of metronidazole with myristyl alcohol in bis(trimethylsilyl)trifluoroacetamide.

RESULTS AND DISCUSSION

The generally recommended dosage of metronidazole for women is one oral tablet of 250 mg three times daily. According to McFadzean (6), a single oral dose of 200 mg leads to a maximum serum level of 5 μg/ml after 1 hr; the serum level then decreases to 1 μg/ml after 24 hr. On this basis, the presently described method was developed so as to give good quantitation for metronidazole in plasma from 0.5 to 5 μg/ml.

GLC analysis of metronidazole as the trimethylsilyl derivative rather than as the parent compound itself was chosen because of superior peak shape and detector response. Moreover, it was anticipated that trimethylsilylation would improve the chances for detection of metronidazole metabolites, which would be expected to be more polar than metronidazole itself. Trimethylsilyl derivatives are usually sensitive to moisture and frequently unstable. Actually,



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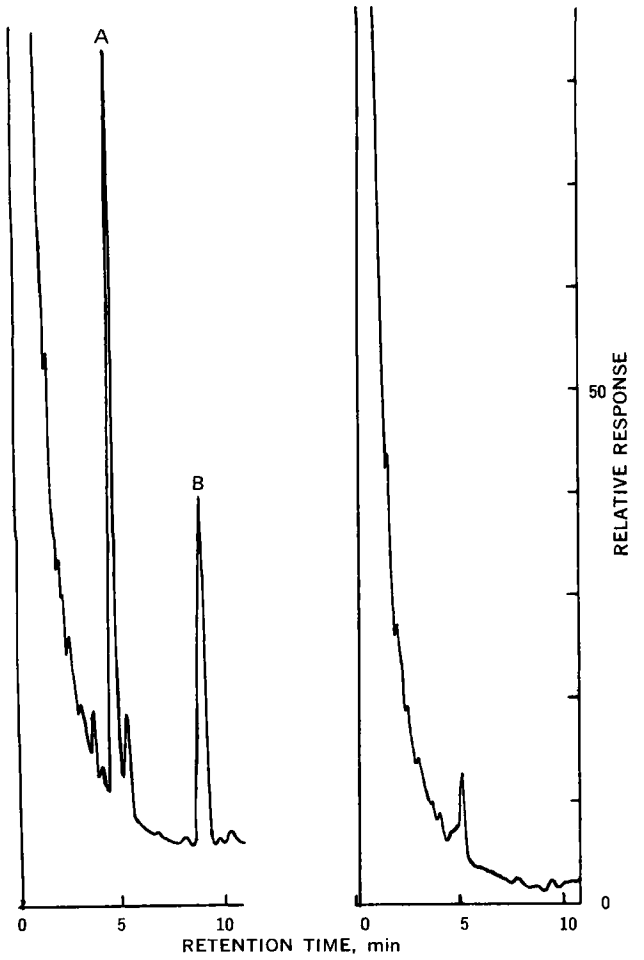
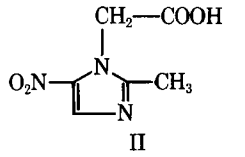


Figure 2—*Recovery of metronidazole from plasma. Chromatogram on left was obtained from a plasma sample with added metronidazole (5 μg/ml) and internal standard (5 μg/ml). Chromatogram on right was obtained from a control sample. Key: A, metronidazole; and B, internal standard.*

the derivatives of metronidazole and the chosen internal standard of myristyl alcohol are probably simple derivatives of primary alcohols (5) and, as such, do not suffer from these disadvantages. Indeed, the derivatives may be recovered from dimethylformamide solution by addition of water and extraction into hexane or chloroform. No peculiar behavior during GLC was noted.

A brief study was made of the conditions necessary for quantitative trimethylsilylation of a mixture of metronidazole and myristyl alcohol. In dimethylformamide–bis(trimethylsilyl)trifluoroacetamide (1:1), reaction was complete in less than 30 min; but it was obvious that at low levels of metronidazole, the tail of the dimethylformamide peak would interfere with quantitation. In bis(trimethylsilyl)trifluoroacetamide alone, reaction was complete in less than 1 hr at 50° and almost complete in 2 hr at room temperature. It was decided to trimethylsilylate the plasma extract by allowing it to stand in bis(trimethylsilyl)trifluoroacetamide at room temperature overnight.

Determinations of metronidazole were based on the ratio of the peak height of the metronidazole derivative to that of the internal



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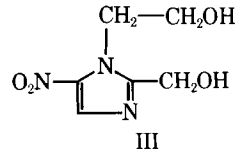

III

standard. Calibration charts relating this ratio to the concentration of metronidazole in plasma were prepared from standard mixtures of metronidazole with the internal standard in bis(trimethylsilyl)trifluoroacetamide. Such a chart is shown in Fig. 1, and it can be seen that a linear relationship exists in the 0–5-μg/ml range.

The results of some recovery experiments for standards of metronidazole added to human plasma are given in Table I. Typical chromatograms from a 5-μg/ml plasma sample and a control plasma sample are shown in Fig. 2.

Although 2-methyl-5-nitroimidazole-1-ylacetic acid (II) and 1-(2-hydroxyethyl)-2-hydroxymethyl-5-nitroimidazole (III) have been reported as metabolites of metronidazole in urine (7, 8), no clear evidence for metabolites of metronidazole in plasma was found in the present work during the analysis of several clinical samples. Compound III ought to lend itself to analysis since it is structurally related to metronidazole simply by having an additional primary alcohol moiety. Unfortunately, no sample of III was available. However, a submilligram sample of II was available, and some preliminary recovery experiments with plasma were performed. Results showed good recovery of II, giving a sharp chromatographic peak eluting immediately behind, and sharply resolved from, that of metronidazole.

The method described here is similar to that of Midha *et al.* (5). Midha *et al.* also carried out trimethylsilylation prior to analysis, but they used a reagent consisting of a mixture of hexamethyldisilazane and trimethylchlorosilane in acetonitrile rather than bis(trimethylsilyl)trifluoroacetamide. They were not able to analyze the resulting metronidazole derivative satisfactorily by GLC using a single liquid as the stationary phase as described here, but instead they had to resort to a mixture of three liquids. One practical difficulty in the application of the method of Midha *et al.* is that the internal standard, 1-(2-hydroxyethyl)-2-methoxy-5-nitroimidazole, may be difficult to obtain since it is quite a rare chemical and unavailable commercially.

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Determination of 17-Hydroxycorticosteroids in Human Urine

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Abstract □ A study was undertaken to develop a method that could be used as an indication of the absorption of steroids. It was demonstrated that 17-hydroxycorticosteroids in urine could be quantitatively determined *via* absorptivity values obtained through the use of standard hydrocortisone–alcohol solutions or standard solutions of hydrocortisone in urine utilizing the blue tetrazolium assay method. This method is dependent upon the hydrolysis of conjugated urinary steroids by beef liver glucuronidase. The resultant concentration of 17-hydroxycorticosteroids is determined colorimetrically using blue tetrazolium.

Keyphrases □ 17-Hydroxycorticosteroids—colorimetric analysis in human urine □ Steroids—colorimetric analysis of 17-hydroxycorticosteroids in human urine □ Colorimetry—analysis, 17-hydroxycorticosteroids in human urine

It has been shown by others that significant percutaneous absorption of steroids can take place from topical dosage forms. The percutaneous absorption that occurred when pharmaceuticals were applied over large body areas was investigated (1). The results from two patients treated with fluocinolone acetonide led to the conclusion that the depression of urinary steroid excretion was caused by suppression of endogenous adrenocorticosteroid production and that this suppression was a result of the absorption of the drug. One phenomenon associated with the administration of steroids is the decrease in concentration of 17-hydroxycorticosteroids in the urine.

Burch and Migeon (2) stated that this phenomenon presumably reflects a partial shutdown of adrenal gland activity because of the absorbed steroid. The extent of percutaneous absorption of this drug was noted to be dependent upon the lipid solubility of the drug. The biotransformation of the topically applied steroid to a compound of greater water solubility was studied by way of the urine concentration profiles of measured 17-hydroxycorticosteroids.

DISCUSSION

The 17-hydroxycorticosteroids that occur primarily in urine as conjugates of glucuronic acid can be hydrolyzed. Drekter *et al.* (3) developed a simplified method for the determination of urinary 17-ketosteroids by means of acid hydrolysis. They replaced the routine aqueous alkali wash with a solid alkali pellet wash to remove urine pigments, such as the acidic, phenolic, and pigmented constituents of hydrolyzed urine, which normally interfere with the results. Therefore, this technique reduces the blank readings significantly. In addition, they found that no interaction occurred between the solid sodium hydroxide pellets and the steroids present.

Several methods for hydrolyzing the steroid conjugates and extracting the free compounds were presented (4). It was noted that urine contains various reducing agents and the specificity of the assay method depends upon the efficiency of the extraction and the purification of the extract. The free 17-hydroxycorticosteroids resulting from the hydrolysis reaction can then be oxidized with sodium iodate or sodium bismuthate on the C_{20},C_{21}-dihydroxy position. The evolved formaldehyde then can be determined (5).

The oxidation reaction also produces 17-ketosteroids, which can be analyzed by the modified Zimmermann reaction (the ketosteroids are removed by borohydride reduction to the corresponding alcohols). The 17-ketogenic steroids are then oxidized by bismuthate and the resulting concentrations are determined either directly (6) or by the Pettenkoffer reaction (reaction between acetic acid solutions and the steroid when treated with furfural and sulfuric acid) (7). The normal range of 17-ketosteroids also gives this reaction.

The Kober reaction (involving acid hydrolysis, ether extraction, separation of ethers by chromatography, colorimetric measurement, and spectrophotometric correction for interfering chromogenic material) (8) is occasionally utilized for the analysis of free 17-hydroxycorticosteroids. However, all 17-hydroxyestrogens interfere with this reaction.

The only two specific reagents are those used in the Porter–Silber reaction (9), in which all 17,21-dihydroxy-20-one type steroids give a positive reaction, and the blue tetrazolium reagent (10), which is specific for primary ketol side chains. The Porter–Silber reaction requires 18 hr for color development and employs concentrated sulfuric acid to give increased values for the urine blank determination. The blue tetrazolium method employs no sulfuric acid and takes 90 min for color development (11).

The quantitative determination of 17-hydroxycorticosteroids in urine was studied using beef liver glucuronidase to hydrolyze conjugated urinary steroids and subsequently treating the sample with blue tetrazolium to measure the concentration of 17-hydroxycorticosteroids colorimetrically.

Table I—Absorbance as a Function of Hydrocortisone Concentration in Alcohol and Human Urine Using Blue Tetrazolium Assay

Alcohol		Human Urine	
Hydrocortisone, μg/ml	Net Absorbance (525 nm)	Hydrocortisone Added, μg/ml	Net Absorbance (525 nm)
1.667	0.210	0.62	0.075
3.333	0.408	1.24	0.150
5.000	0.620	1.86	0.250
8.333	1.020	3.10	0.350

EXPERIMENTAL

Test Solutions—For the blue tetrazolium solution, exactly 125 mg of blue tetrazolium was dissolved in 25 ml of absolute methanol. For the tetramethylammonium hydroxide test solution, 10 ml of a 10% tetramethylammonium hydroxide solution was diluted to 100 ml with absolute methanol.

Standard Preparation—Approximately 100 mg of hydrocortisone USP, accurately weighed, was dissolved in alcohol USP to make exactly 100 ml. Then 1 ml of the solution was diluted to exactly 100 ml with alcohol. This standard solution contained approximately 10 μg/ml.

Sample Preparation—Urine specimens were collected from normal, healthy adults, and the pH of the urine was immediately adjusted to 5.0 with acetic acid. The urine was divided into two portions; one portion was retained for use as the untreated specimen. The second portion was subdivided and to each aliquot were added varying amounts of hydrocortisone and urine to achieve final hydrocortisone concentrations of approximately 0.5, 1, 2, and 3 μg/ml.

Procedure—Aliquots of 2, 4, 6, and 10 ml of the standard solu-

tion were pipetted into separate 35-ml centrifuge tubes and the volume was brought to 10 ml with alcohol. To each standard solution and blank, 1 ml of blue tetrazolium solution was added. The tubes were mixed and exactly 1 ml of tetramethylammonium hydroxide test solution was added. The solution was mixed, allowed to stand for 90 min, and then placed into 1-cm silica cells. The absorbance of each standard solution was read against the blank in a suitable spectrophotometer at 525 nm (Table I).

Exactly 10 ml of the untreated urine from each specimen was pipetted into three 35-ml centrifuge tubes. A blank was prepared with 10 ml of purified water adjusted to pH 5.0 with acetic acid. Four milliliters of beef liver glucuronidase solution[1] was added to each sample and blank. These tubes were mixed, stoppered, and incubated overnight at 37°. After cooling to room temperature, 10 ml of carbon tetrachloride was added and the tubes were shaken for 5 min at very low agitation and centrifuged for 5 min. The aqueous phase was transferred to a second 35-ml centrifuge tube, and the extraction was repeated with 10 ml of carbon tetrachloride.

Twelve milliliters of the aqueous phase was transferred to a 125-ml separator, extracted with 50 ml of methylene chloride, and allowed to settle for 30 min. The organic phase was then transferred to a 125-ml flask. Thirty pellets of sodium hydroxide were added, the flask was shaken for 5 min, and the sample was then allowed to settle. The solution was filtered through filter paper[2] into a 50-ml centrifuge tube.

Twenty milliliters of the filtrate was pipetted into a 35-ml centrifuge tube and evaporated to dryness in a water bath at 30° with the aid of a gentle stream of air. The residue was dissolved in 10 ml of alcohol. The sample solutions and their blank were then treated with the blue tetrazolium solution and tetramethylammonium hydroxide solution, as indicated for the standard solution; the absorbance was read in a similar manner. The hydrocortisone-treated urine samples were assayed similarly, starting with the addition of the beef liver glucuronidase solution to 10-ml aliquots (Table I).

RESULTS

The analytical method (12) used for the determination of 17-hydroxycorticosteroids in urine was initially evaluated by adding known amounts of hydrocortisone to urine specimens. These evaluations indicated possible difficulties with the method. Therefore, this method was modified by omitting the sodium hydroxide backwash, substituting a solid sodium hydroxide wash, and utilizing the blue tetrazolium method in place of the Porter–Silber reaction. The results of the two standardizations are shown in Table I. These data demonstrate that there is no difference between the two standardization studies; that is, the absorptivity value for the hydrocortisone–blue tetrazolium reaction is essentially identical whether the solution is prepared in alcohol or urine.

Table II reports the results on 17-hydroxycorticosteroid recovery in untreated urine and is based on the absorptivity value calculated from the data presented in Table I. These studies demonstrate that 17-hydroxycorticosteroids in untreated human urine can be quantitatively determined *via* absorptivity values obtained through the use of standard hydrocortisone–alcohol solutions or standard solutions of hydrocortisone in urine.

[1] Ketodase solution (5000 Fishman units/ml), General Diagnostics, Division of Warner-Lambert Co., Morris Plains, N.J.

[2] Whatman No. 4.

Table II—17-Hydroxycorticosteroid Levels of Randomly Taken Urine Samples from Selected Subjects

Subject	Urine Voided, ml	Net Absorbance (525 nm)	17-Hydroxycorticosteroid	
			μg/ml	mg/500 ml
A (am)[a]	105	0.780	6.33	3.17
B (pm)	300	0.280	2.27	1.14
C (am)	270	0.570	4.62	2.31
D (pm)	250	0.192	1.56	0.78
E (am)	75	0.800	6.49	3.25
F (pm)	50	0.800	6.49	3.25

[a] Time of urine collection appears in parentheses.

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Fluorocarbon Aerosol Propellants V: Binding Interaction with Human Albumin

WIN L. CHIOU ˣ and JAUE-HUEY HSIAO

Abstract □ Binding of trichloromonofluoromethane, dichlorodifluoromethane, and dichlorotetrafluoroethane was studied in aqueous 5% human albumin solution, using the partition coefficient method in sealed serum bottles. The partition coefficient and the fraction of fluorocarbons bound were highly dependent on fluorocarbon concentrations. The average binding sites per molecule of albumin were 2.17, 0.30, and 0.42 and the binding association constants were 1.11×10^3, 1.73×10^3, and $5.06 \times 10^3\ M^{-1}$, respectively. At the lowest concentration studied, 62.3, 25.5, and 65.6% were found bound to albumin, respectively. This appears to represent the first extensive study on any gas–albumin interaction.

Keyphrases □ Fluorocarbon propellants—binding to human serum albumin determined using partition coefficient method in sealed serum bottles □ Trichloromonofluoromethane—binding to human serum albumin □ Dichlorodifluoromethane—binding to human serum albumin □ Dichlorotetrafluoroethane—binding to human serum albumin □ Binding of fluorocarbon propellants to human serum albumin—determined using partition coefficient method in sealed serum bottles □ Interactions, gas–albumin—binding of fluorocarbon propellants to human serum albumin

Fluorocarbons such as trichloromonofluoromethane (bp 23.7°), dichlorodifluoromethane (bp −29.8°), and dichlorotetrafluoroethane (bp 4.1°) have been widely used as aerosol propellants in various pressurized aerosol packages. In 1973, an estimated 3 billion units were manufactured in this country (1). The possible acute and chronic toxicities of these previously regarded "inert" propellants have been the subjects of intensive studies in recent years (1–6).

Based on the partition coefficient study, it was shown previously that these volatile or gaseous fluorocarbons were much more soluble in human plasma than in water or normal saline (7). It was postulated that such a solubility enhancement might be partly due to the binding of the fluorocarbons to plasma proteins. This postulation was subsequently confirmed by the fluorocarbon–human albumin binding study with one single concentration, using the partition coefficient method (8). In that study, a surprisingly high degree of binding of the three fluorocarbons to the purified human and bovine albumins was found. Since the possible effect of concentration variation on the extent of protein binding for numerous nonvolatile compounds has been well established, it was decided to extend the investigation by using a wide range of fluorocarbon concentrations. This approach also allows the number of the binding sites per molecule of protein and the protein-binding association constant to be calculated.

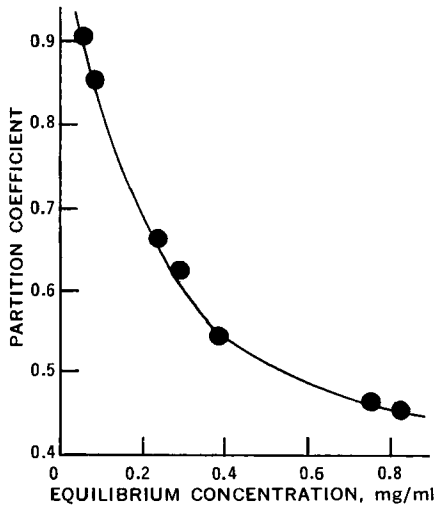

Figure 1—*Effect of concentration of trichloromonofluoromethane (both bound and unbound form) on its partition coefficient between 5% human albumin solution and air.*

EXPERIMENTAL

Materials—Only the three most widely used fluorocarbon propellants, trichloromonofluoromethane[1] (mol. wt. 137.4), dichlorodifluoromethane[1] (mol. wt. 120.9), and dichlorotetrafluoroethane[1] (mol. wt. 170.9), were studied. The crystallized and lyophilized human albumin (mol. wt. 69,000) was purchased commercially[2].

Preparation of Fluorocarbon–Albumin Solutions—The albumin solution (5% w/v) was freshly made by dissolving albumin into isotonic pH 7.4 phosphate buffer solution, prepared according to the method of Sorensen (9). One milliliter of this albumin solution was then pipetted into a 5-ml serum bottle[3], which was then sealed with a flange-type lacquer-coated stopper and aluminum cap (7). In the previous partition coefficient study (7, 8), the fluorocarbons were first dissolved in normal saline prior to introduction into plasma or protein samples. Due to their limited solubilities in normal saline or phosphate buffer and the high concentrations of the fluorocarbons used in the present study, their gaseous stock preparations were prepared by diluting the pure fluorocarbons from their liquid state (dichlorodifluoromethane and dichlorotetrafluoroethane were stored in pressurized steel cylinders at room temperature and trichloromonofluoromethane was stored in an aluminum container at 5°) into the sealed 50-ml empty serum bottles.

The albumin solutions, containing various amounts of the fluorocarbon, were prepared by transferring approximately 0.05–1 ml from the fluorocarbon stock preparations, using a gastight syringe[4], to the sealed 5-ml serum bottle containing 1 ml of the albumin solution. A 27-gauge disposable hypodermic needle was inserted briefly into the headspace of the serum bottle to reduce the pressure in the headspace to the atmospheric pressure. The three fluorocarbons were studied individually.

Determination of Partition Coefficients between Protein Solution and Air Phase—The fluorocarbon–albumin solution in the sealed serum bottle was shaken in a vortex mixer[5] for 30 min and allowed to stand at room temperature (25 ± 1°) for another 30 min. The preliminary study showed that the equilibration of the fluorocarbons between the protein solution and headspace was

[1] Supplied by E. I. du Pont de Nemours and Co., Wilmington, Del.
[2] Sigma Chemical Co., St. Louis, Mo.
[3] Wheaton Scientific, Millville, N.J.
[4] Hamilton.
[5] Vortex mixer, Fisher Scientific Co., Springfield, Mass.

reached under these conditions since the partition coefficient remained the same even after 1 hr of shaking. A much shorter period (less than 5 min) was required to obtain an equilibrium by the same method of shaking when the fluorocarbon dissolved in normal saline was first introduced into the protein solution as used previously (7, 8).

The concentration of the fluorocarbon in the headspace was analyzed directly by injecting an appropriate volume of the sample into the GC column using a 50- or 100-μl gastight syringe[4]. The GC operation conditions were identical to those reported previously (8, 10, 11). A proper dilution of the sample, using the sealed empty serum bottle, was made when the concentration of the fluorocarbon in the headspace was too high and the amount injected would have exceeded the linear response range of the electron-capture detector (10).

After determination of the fluorocarbon in the headspace, its concentration in the albumin solution was analyzed by transferring about 0.2 ml of the solution to a 3- or 5-ml vacuum tube[6] containing a known amount of cyclohexane. The tube was shaken in a shaker[7] for 5 min followed by centrifugation for 5 min in a clinical centrifuge[8]. The fluorocarbon concentration in the cyclohexane phase was determined by drawing 5–10 μl of the solution, using a 10-μl syringe[4], and injecting onto the GC column.

During the transferring of the fluorocarbon–albumin solution to the vacuum tube, the following special procedure was used to avoid loss of the fluorocarbon when the conventional pipet or syringe was employed. A 6.3-cm (2.5-in.) 22-gauge disposable hypodermic needle was cut to remove the hub and sharpened with a file. One end of the needle was inserted into the albumin solution of the inverted serum bottle, and the other end was then immediately inserted into the vacuum tube. When the liquid stopped flowing into the tube, another 23-gauge disposable needle was inserted into the headspace of the serum bottle to equilibrate the whole system to atmospheric pressure. The volume of the liquid transferred was measured by weighing after correction for its density. The exact volume (about 2.8 ml for a 3-ml tube and 4.8 ml for a 5-ml tube) of cyclohexane used in the extraction was also measured by weighing after correction for its density.

The extraction recovery of the fluorocarbons into the cyclohexane was essentially 100%. This recovery can be expected because of their high solubility in this solvent (7) and the relatively smaller volumes of the protein solution and headspace used. Throughout the present study, duplicate and excellent reproducible readings (less than 3% of variation) were obtained for each GC assay.

The partition coefficient, P_p, between the protein solution and the air phase was calculated according to the following equation:

$$P_p = \frac{C_p}{C_g} = \frac{C_c V_c}{C_g V_p} \qquad \text{(Eq. 1)}$$

where:

C_p = concentration of fluorocarbon in protein solution at the equilibrium state (weight per unit of volume)
C_g = concentration of fluorocarbon in air phase (*i.e.*, headspace) at the equilibrium state (weight per unit of volume)
C_c = concentration of fluorocarbon in cyclohexane extract
V_c = volume of cyclohexane used for extraction
V_p = volume of protein solution transferred for extraction

RESULTS AND DISCUSSION

Partition Coefficient Study—The partition coefficients of the three fluorocarbons between the 5% human albumin solution and the air phase were found to change with the total fluorocarbon concentration in the albumin solution at the equilibrium state. This finding is clearly demonstrated by a typical plot of the partition coefficient of trichloromonofluoromethane as a function of its total concentration in protein solution (Fig. 1). The partition coefficient decreased markedly and approached the value between the plain pH 7.4 phosphate buffer without protein and the air phase, P_f (8, 12). The P_f values for the three fluorocarbons (0.347 for trichloromonofluoromethane, 0.105 for dichlorodifluoromethane, and 0.032 for dichlorotetrafluoroethane) were independent of their total concentrations in the protein solution. These values are slightly higher than those previously reported for both water and normal saline systems (7). The discrepancies were caused by the depressing effect on the sensitivity of the electron-capture detector in the presence of water when the aqueous sample was injected directly onto the GC column (12) and by the inherent lack of a high degree of accuracy in the low partition media (such as partition coefficients less than 0.2) when the simple headspace method (7) was employed. This latter aspect will be dealt with in a future report.

Table I—Number of Fluorocarbon Binding Sites per Molecule of Human Albumin, n, and of the Binding Association Constant, K

Fluorocarbons	n	K, M^{-1}
Trichloromonofluoromethane	2.18	1.11×10^3
Dichlorodifluoromethane	0.30	1.73×10^3
Dichlorotetrafluoroethane	0.42	5.06×10^3

The concentration-dependent partition coefficient phenomenon could be rationalized by the classical drug–protein interaction. As the fluorocarbon concentrations increase, the protein binding sites become more saturated; therefore, the solubilities or partition coefficients tend to decrease. A detailed analysis of the binding properties will be discussed later. There appears to be some discrepancies between this study and the results published previously (7) that showed a concentration-independent partition coefficient phenomenon in the human blood and plasma systems. This previous finding could be attributed to the much lower concentrations of fluorocarbons used in that study. As will be shown later, the partition coefficients in the human albumin solution actually change only slightly in these lower concentration ranges.

Principles of Partition Coefficient Method to Study Protein Binding—Most reported protein binding studies were primarily limited to the nonvolatile compounds. The most common methods employed in the binding studies were equilibrium dialysis and ultrafiltration (13). Due to the existence of these fluorocarbons at the gaseous state at 25 or 37°, these conventional methods could not be easily adopted for their binding study. Since one can reasonably assume that only the free or unbound form is in equilibrium with the fluorocarbon in the headspace and that the partition coefficient is the same as that between the plain buffer solution without protein and the air phase, P_f, the data from the parti-

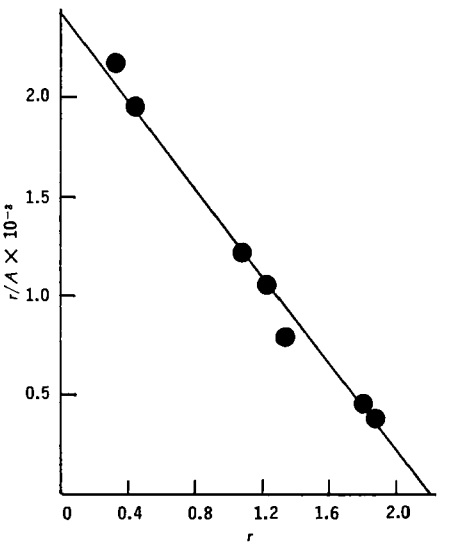

Figure 2—*Scatchard plot for the binding of trichloromonofluoromethane to human albumin.*

[6] Vacutainer, Becton-Dickinson Co., Rutherford, N.J.
[7] Catalog Number 65885, Precision Scientific Co., Chicago, Ill.
[8] Catalog Number 67477, Precision Scientific Co., Chicago, Ill.

tion coefficient study can be used to quantify the percentage of the fluorocarbon bound, the number of binding sites per molecule of protein, and the binding association constant. Mathematically, these values could be derived or determined as follows.

For the determination of the percent bound:

$$\% \text{ bound} = \frac{C_p - C_f}{C_p}(100) \qquad \text{(Eq. 2)}$$

where C_p and C_f are the total concentration (bound and unbound form) and the concentration of the unbound or free form in the protein solution, respectively.

Dividing Eq. 2 by C_g, the concentration in the headspace, one obtains (8):

$$\% \text{ bound} = \frac{C_p/C_g - C_f/C_g}{C_p/C_g}(100) \qquad \text{(Eq. 3)}$$

$$\% \text{ bound} = \frac{P_p - P_f}{P_p}(100) \qquad \text{(Eq. 4)}$$

To determine the number of fluorocarbon molecules bound per molecule of albumin *(r)*:

$$r = \frac{(C_p)(\text{fraction bound})(\text{mol. wt.}_{al})}{C_{al}(\text{mol. wt.}_f)} \qquad \text{(Eq. 5)}$$

where C_p and C_{al} are concentrations of the fluorocarbon and albumin expressed in the same unit, and mol. $wt._f$ and mol. $wt._{al}$ are their molecular weights, respectively.

The number of binding sites, n, and binding association constant, K, can be obtained from the Scatchard plot based on the following relationship (14):

$$\frac{r}{A} = nK - rK \qquad \text{(Eq. 6)}$$

where A is the molar concentration of the free form of the fluorocarbon. The plot of r/A *versus* r yields a straight line. The n value can be determined from the intercept on the abscissa and the nK value from the intercept on the ordinate.

Binding of Fluorocarbons with Human Albumin—Based on the partition coefficient data and Eq. 4, the percentages in the bound form for the three fluorocarbons at various concentrations can be calculated. The percentage bound decreased from 62 to 23, 23 to 8, and 60 to 24 as the total concentration increased from 0.06 to 0.83 mg/ml for trichloromonofluoromethane, from 0.03 to 0.27 mg/ml for dichlorodifluoromethane, and from 0.006 to 0.170 mg/ml for dichlorotetrafluoroethane. These concentration ranges are close to some reported fluorocarbon concentrations encountered in *in vitro* and *in vivo* toxicity studies (1, 15, 16).

A typical Scatchard plot for trichloromonofluoromethane is shown in Fig. 2. The two parameters, n and K, obtained from this and similar plots are summarized in Table I. All plots showed a straight line, which indicates that there are only primary binding sites for all three fluorocarbons. The highest binding constant found for dichlorotetrafluoroethane was not unexpected in view of its higher molecular weight and higher solubility in the lipoid medium (8), which may result in stronger van der Waals interaction and hydrophobic binding. Although the binding constant for dichlorodifluoromethane was slightly higher than for trichloromonofluoromethane, its binding capacity, n, was only about one-seventh of the latter. This finding explains why the percentages bound for dichlorodifluoromethane were much lower compared to trichloromonofluoromethane at similar concentrations.

When lower concentrations (about 130–8000 times lower) were used, the percentages bound were in good agreement with the predicted values, using the parameters listed in Table I. The binding data at these low concentrations are relevant because of the low blood levels that may be encountered clinically (17, 18) or in pharmacokinetic studies (10, 19, 20). The moderate to extensive protein binding found at these lower concentrations could explain in part the higher partition coefficients or solubilities of these fluorocarbons in blood and plasma of humans and other species (7, 20, 23). In light of these results, one can assume that these fluorocarbons may also be bound extensively to other macromolecules in various tissues of the body. This probably contributes partly to the high apparent volume of distribution of dichlorotetrafluoroethane in a dog estimated to be about eight times body weight based on the blood concentration (19). Similar findings for other fluorocarbons were also obtained with other dogs and will be reported.

Trichloromonofluoromethane has been shown *in vitro* to decrease significantly and irreversibly the invertase activity (21), and dichlorodifluoromethane has also been shown to inhibit markedly the *in vitro* o-diphenol oxidase activity (22). Hydrophobic binding and conformational modification of enzyme molecules have been proposed to explain the results, but no binding constants and binding sites were investigated in the two studies.

From a search of literature, it appears that no detailed binding studies on gas–protein interactions have been reported. The partition coefficient method proposed, using a sealed serum bottle and analyzing by the headspace and extraction method, appears to offer a simple and promising tool for studying the binding interactions between macromolecules and volatile or gaseous compounds.

There is some discrepancy in the percentage of dichlorodifluoromethane bound to the human albumin reported from this study and previous preliminary studies (8); the reason is unknown. Since the results from the previously reported concentration could not be reproduced and the present study employed a very wide range of concentrations, it is concluded that the value obtained from the present study is more reliable.

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Drug Release from a Lipophilic Ointment Base as Influenced by Chain Length of Added Surfactant

ANNE MÉRIAUX-BROCHU and J. PAIEMENT ˣ

Abstract □ The release of sulfathiazole and salicylic acid from white petrolatum containing ethoxylated surfactants of varying ethylene oxide chain length was evaluated by the dialysis method. The rate of release of the drugs increased with the ethylene oxide number of the added surfactant.

Keyphrases □ Ointment bases, lipophilic—effect of ethoxylated surfactant chain length on drug release □ Lipophilic ointment bases—effect of ethoxylated surfactant chain length on drug release □ Drug release from lipophilic ointment bases—chain length effect of added ethoxylated surfactant

The rate of release of drugs from ointment bases has received considerable attention during the last 10–15 years and interesting reviews are now available on the subject (1–13). Looking closely into the literature, however, one notes that if some aspects of this phenomenon have been extensively studied and are now well understood, others have been only partially explored. One such area is the effect of surfactants on lipophilic ointment bases.

This paper reports an attempt to verify how the variation of the length of the ethylene oxide chain of an added nonionic surfactant could influence the rate of release of water-soluble drugs from a typical lipophilic base such as white petrolatum. It was also thought that such simple systems would provide a good model to verify the possibility that a correlation exists between the rheological parameters of the ointments and the rate of liberation of drugs incorporated in them (14).

EXPERIMENTAL

The dialysis method (7) is a simple and convenient way to study the rate of diffusion of drugs in ointment bases. The significance of the information provided by that method has also been critically examined (15). In the present investigation, the setup suggested by Billups and Patel (9) was used with some modifications: *(a)* the receiving compartment of the dialysis cell was a double-walled beaker through which water could be circulated, permitting temperature maintenance of the aqueous solution (100 ml) of the receiving compartment at 25 ± 0.1°; *(b)* a small cavity was formed in the bottom of the beaker for a stirring bar with the beaker resting on a magnetic stirrer; and *(c)* the size of the jars was such that a relatively large area (~25 cm^2) of ointment (covered with cellulose paper) could be in contact with the aqueous phase. By proceeding in this way, the aliquots removed for analysis could be read directly on a spectrophotometer and then returned to the cell, thus eliminating the necessity of making volume corrections.

The absorbance of sulfathiazole was determined at 283 nm, and that of salicylic acid was determined at 302 nm. (In the acidic form, salicylic acid shows two peaks of absorbance at 237.5 and 302 nm; in both cases, Beer's law is obeyed.) As expected (16), spectrophotometric analysis failed to reveal any trace of surfactant which might have diffused along with the drugs.

The first drug, sulfathiazole[1] (mp 200–201°), was dialyzed into a phosphate buffer solution of pH 6.0 and ionic strength (μ) = 0.1. The second drug, salicylic acid[2], was dialyzed into a buffer solution of pH 1.85 (hydrochloric acid plus sodium chloride to μ = 0.1). In this second case, the conditions were, of course, far from being physiological but they permitted comparison of both drugs in the same conditions with respect to the proportion of nonionized drug in the aqueous solution (~93%). Since these drugs are weak acids, the buffer systems seemed mandatory so that the conditions in the receiving compartment would not change during an experiment. The aqueous solubilities of the nonionized portion of sulfathiazole and salicylic acid were evaluated at 25° (at pH's where less than 1% of the drugs was ionized).

The nonionic surfactants studied were some alkylaryl polyether alcohols[3]. They were completely dehydrated before incorporation into the melted bases[4] at a concentration of 1.592×10^{-3} mole/100 g of base (this value corresponds to 1 g/100 g in the case of the surfactant containing 9–10 ethylene oxide units[5]). The drugs were passed through a screen (BP 100) and were also dehydrated; this step was essential with sulfathiazole. Then they were dispersed into the bases, with a mortar and a pestle, at room temperature. Great care was exerted always to proceed in the same way (with respect to the agitation brought by the magnetic stirrer, the position of the inverted cup, *etc.*) so that the results could be compared.

For the rheological studies, a cone-plate rotational viscometer[6] was used. The experimental conditions adopted were similar to those used by Boylan (17) in his rheological study of white petrolatum.

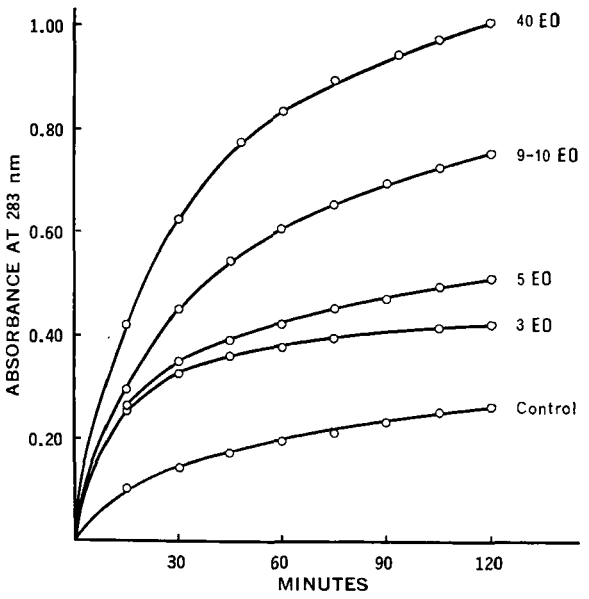

Figure 1—*Absorbance of sulfathiazole in the receiving compartment of the dialysis cell as a function of time (0.2 absorbance = 0.275 mg sulfathiazole/100 ml). Key: EO, ethylene oxide units of the added surfactant; and control, no surfactant added.*

[1] Matheson, Coleman & Bell.

[2] Analar, BDH Chemical Ltd.

[3] Tritons of the X-series, supplied by Rohm and Haas, West Hill, Ontario, Canada. The ethylene oxide units referred to in this investigation correspond to those given by the manufacturer.

[4] They were left overnight in an oven at 70° and then stored in a vacuum desiccator until their weight was found to be constant.

[5] Triton X-100.

[6] Ferranti-Shirley.

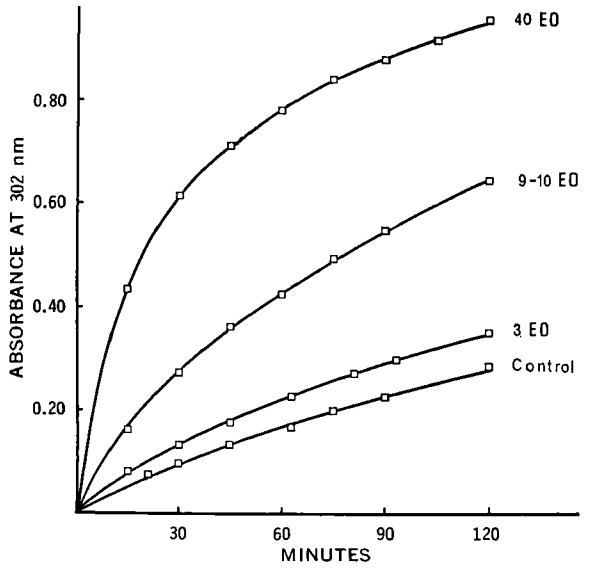

Figure 2—*Absorbance of salicylic acid in the receiving compartment of the dialysis cell as a function of time (0.2 absorbance = 0.80 mg salicylic acid/100 ml). Key: EO, ethylene oxide units of the added surfactant; and control, no surfactant added.*

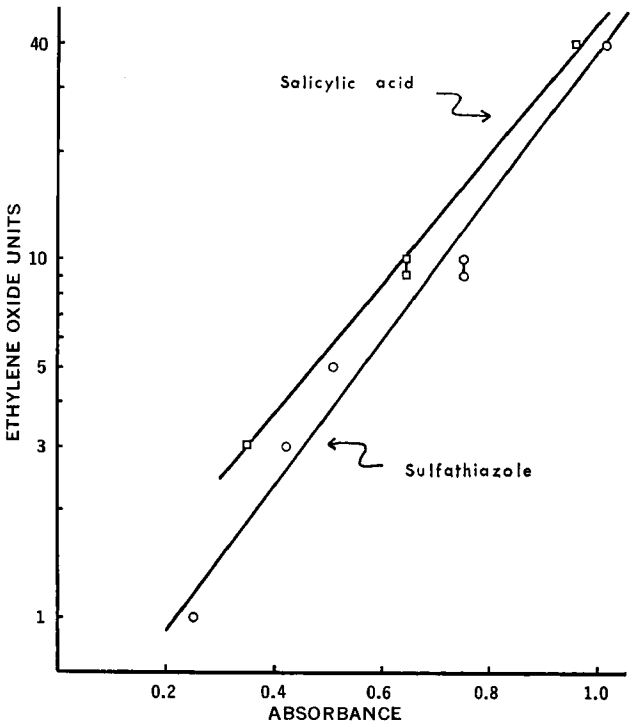

Figure 3—*Ethylene oxide number of the added surfactant (on a logarithmic scale)* versus *absorbance of the drug in the receiving compartment of the dialysis cell.*

RESULTS AND DISCUSSION

Figure 1 shows some representative curves for the release of sulfathiazole from white petrolatum containing surfactants of varying ethylene oxide chain lengths. As can be seen, the rate of release of the drug kept increasing up to the most hydrophilic surfactant studied (containing 40 ethylene oxide units). Since salicylic acid had been shown to diffuse best with mixtures of surfactants (sorbitan esters and polysorbates) having a low hydrophilic–lipophilic balance (HLB) value (12), it was thought that that drug would provide a good point of comparison for sulfathiazole. Figure 2 shows that the general pattern of diffusion obtained with salicylic acid was very close to the one obtained for sulfathiazole.

In Fig. 3, the amount of either sulfathiazole or salicylic acid dialyzed after 120 min was plotted as a function of the logarithm of the number of ethylene oxide units of the added surfactant. For sulfathiazole, it was verified that the relation was linear at any time in the dialysis runs so long as the curves had started to flatten. For salicylic acid at dialysis times less than 120 min, the relation was not linear; it can be observed (Fig. 2), however, that some diffusion curves had not then started to flatten.

The effect of surfactants on the release of drugs from hydrophilic ointment bases has been extensively studied (1, 2, 5, 6). Since those systems contained water (~30%), it is expected that oil-in-water or water-in-oil emulsions were formed, depending on the HLB of the mixture of surfactants studied, giving essentially different systems than those studied here. The diffusion behavior of drugs in such emulsified systems has been well explained (11).

In dealing with lipophilic bases modified by nonionic surfactants, it is believed that the comparisons should be made on a "pseudomolar" basis (*i.e.*, a given number of moles of surfactant added to a given weight of base or a given volume of preparation). The reason becomes apparent when one considers, for instance, the case of the following pair: sorbitan monooleate (mol. wt. ~453) and polysorbate 80 (mol. wt. ~1470). One notes then that, at any given percentage (identical for both surfactants), the concentration of the ethoxylated surfactant is, on a molar basis, about four times lower than that of the other. Since the concentration of the added surfactant is also important [the rate of diffusion increases with increasing concentration up to an optimum concentration and then decreases (11)], comparison of the effect of a large molar concentration of a surfactant having, for example, a middle HLB value to that of a low molar concentration of a surfactant having a high HLB value might obscure the real phenomenon.

It is also possible that nonionic surfactants of different classes (*i.e.*, sorbitan esters and ethoxylated surfactants) do not work in the same way (or to the same extent) when added to lipophilic bases. Hence, it has been observed that the addition of sorbitan monooleate to white petrolatum decreased the rate of diffusion of sulfathiazole while the addition of polysorbate 80 increased it (11). These explanations are given as suggestions to account for the fact that the diffusion of some drugs was shown previously to decrease above a certain HLB value of the added surfactants (3, 4, 12) while this phenomenon was not observed here.

In the three figures, the absorbance of the drugs instead of their concentration is presented to emphasize that the *pattern* of diffusion of both drugs is quite similar. However, the concentration of salicylic acid was evaluated using its peak of second maximum absorbance in the UV region (at 237.5 nm, the peak of maximum absorbance, volume corrections would have been necessary). Salicylic acid diffused to a greater extent than sulfathiazole (~3 times more on a molar basis) per unit of time. This observation may reflect that salicylic acid is about three times more soluble than sulfathiazole on a molar basis (87 mg *versus* 55 mg/100 ml with the unionized portions being considered). In conclusion, it is suggested that these solubility considerations and the relation shown in Fig. 3 (this relation will have, however, to be substantiated with other drugs and other surfactant series) might give a clue as to the mode of diffusion of water-soluble drugs in ointment bases containing ethoxylated nonionic surfactants.

In the rheological part of this study, only sulfathiazole preparations were considered. Rheograms were obtained with the modified bases only (without the drug) and with the complete preparations (with the drug). The study was also extended to white petrolatum containing varying concentrations of polysorbate 80 (11), with and without incorporated sulfathiazole. Various parameters [*e.g.*, thixotropy, yield value, and spur value (18)] were examined and compared to verify if a correlation could be found between the rate of release of the drug and any of the parameters. So far, no correlation has been found. It is possible that such correlations could be obtained if the bases investigated are gels made with water-soluble polymers. Further research in that direction is in progress.

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Antitubercular Activity of Substituted 5-Oxo-1-thiocarbamoyl-3-pyrazoline-4-alkanoic Acid Derivatives

ARTHUR A. SANTILLI [x], DONG H. KIM, and FRANCIS J. GREGORY

Abstract □ Several novel pyrazolin-5-ones prepared by the cyclization of variously substituted thiosemicarbazone derivatives of ethyl formylsuccinate, ethyl acetylsuccinate, and ethyl acetylglutarate were tested for antitubercular activity against *Mycobacterium tuberculosis*, human type, strain H37Rv, by a tube dilution technique. Minimum inhibitory concentrations (MIC) for these derivatives ranged from 0.05 to 100 μg/ml. The most active compound was ethyl 3-methyl-1-methylthiocarbamoyl-5-oxo-3-pyrazoline-4-acetate (MIC = 0.05–0.1 μg/ml).

Keyphrases □ 5-Oxo-1-thiocarbamoyl-3-pyrazoline-4-alkanoic acid derivatives—synthesis, antitubercular activity □ Pyrazolin-5-one derivatives—synthesis, antitubercular activity □ Antitubercular activity—synthesis and screening of substituted 5-oxo-1-thiocarbamoyl-3-pyrazoline-4-alkanoic acid derivatives

Various classes of thiosemicarbazones have been of interest to clinicians for their therapeutic value as antitubercular, antiviral, antileprotic, and antifungal agents (1–3). Recently, the synthesis of a group of variously substituted 5-oxo-1-thiocarbamoyl-3-pyrazoline-4-alkanoic acid derivatives (II*a*–II*l*), which were prepared by the cyclization of open-chain thiosemicarbazones (I) of diethyl formylsuccinate, diethyl acetylsuccinate, and diethyl acetylglutarate, was described (4). Spectral evidence indicates that these compounds also exist in their tautomeric 5-hydroxypyrazole form (II′*a*–II′*l*). Several of these derivatives were tested for their overall therapeutic value as anti-infective agents and were found to possess substantial antitubercular activity against *Mycobacterium tuberculosis*, human type. A summary of the biological test results in relation to chemical structure is reported here.

EXPERIMENTAL[1]

The open-chain thiosemicarbazones (I) were prepared by heating ethyl formylsuccinate, ethyl acetylsuccinate, or ethyl acetylglutarate with 1 equivalent of a suitably substituted thiosemicarbazide (Scheme I). Ring closures of these derivatives were carried out by warming the various thiosemicarbazones in ammonium hydroxide solution followed by acidification; the title compounds were obtained (Table I). The esters were readily converted to the corresponding acids by hydrolysis using sodium hydroxide solution.

Synthesis—The following example typifies the method used to prepare the title compounds. Further preparative details as well as the chemistry and spectral analyses for other compounds were previously published (4).

Diethyl Acetylsuccinate Thiosemicarbazone (I)—A mixture of 10.8 g of diethyl acetylsuccinate and 4.56 g of thiosemicarbazide in 250 ml of ethanol was heated under reflux for 17 hr. The ethanol solution was cooled in ice, and cyclohexane was added to precipitate the product. The product amounted to 13.1 g, mp 98–102°. The analytical sample (mp 98–100°) was obtained by recrystallization from ethanol–cyclohexane; IR (KBr): 2.90, 3.00, 3.12 (NH), 5.72, and 5.79 (ester C=O) μm.

Anal.—Calc. for $C_{11}H_{19}N_3O_4S$: C, 45.66; H, 6.62; N, 14.52; S, 11.08. Found: C, 45.64; H, 6.58; N, 14.65; S, 11.42.

Ethyl 3-Methyl-5-oxo-1-thiocarbamoyl-3-pyrazoline-4-acetate (IIa)—A mixture of 10 g of diethyl acetylsuccinate thiosemicarbazone and 250 ml of concentrated ammonium hydroxide solution was heated on a steam bath for approximately 30 min, at which

[1] Melting points were determined with a Thomas-Hoover capillary melting-point apparatus and are uncorrected. The IR spectra were determined in potassium bromide disks using a Perkin-Elmer model 21 spectrophotometer.

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Antitubercular Activity of Substituted 5-Oxo-1-thiocarbamoyl-3-pyrazoline-4-alkanoic Acid Derivatives

ARTHUR A. SANTILLI [x], DONG H. KIM, and FRANCIS J. GREGORY

Abstract □ Several novel pyrazolin-5-ones prepared by the cyclization of variously substituted thiosemicarbazone derivatives of ethyl formylsuccinate, ethyl acetylsuccinate, and ethyl acetylglutarate were tested for antitubercular activity against *Mycobacterium tuberculosis*, human type, strain H37Rv, by a tube dilution technique. Minimum inhibitory concentrations (MIC) for these derivatives ranged from 0.05 to 100 μg/ml. The most active compound was ethyl 3-methyl-1-methylthiocarbamoyl-5-oxo-3-pyrazoline-4-acetate (MIC = 0.05–0.1 μg/ml).

Keyphrases □ 5-Oxo-1-thiocarbamoyl-3-pyrazoline-4-alkanoic acid derivatives—synthesis, antitubercular activity □ Pyrazolin-5-one derivatives—synthesis, antitubercular activity □ Antitubercular activity—synthesis and screening of substituted 5-oxo-1-thiocarbamoyl-3-pyrazoline-4-alkanoic acid derivatives

Various classes of thiosemicarbazones have been of interest to clinicians for their therapeutic value as antitubercular, antiviral, antileprotic, and antifungal agents (1–3). Recently, the synthesis of a group of variously substituted 5-oxo-1-thiocarbamoyl-3-pyrazoline-4-alkanoic acid derivatives (II*a*–II*l*), which were prepared by the cyclization of open-chain thiosemicarbazones (I) of diethyl formylsuccinate, diethyl acetylsuccinate, and diethyl acetylglutarate, was described (4). Spectral evidence indicates that these compounds also exist in their tautomeric 5-hydroxypyrazole form (II′*a*–II′*l*). Several of these derivatives were tested for their overall therapeutic value as anti-infective agents and were found to possess substantial antitubercular activity against *Mycobacterium tuberculosis*, human type. A summary of the biological test results in relation to chemical structure is reported here.

EXPERIMENTAL[1]

The open-chain thiosemicarbazones (I) were prepared by heating ethyl formylsuccinate, ethyl acetylsuccinate, or ethyl acetylglutarate with 1 equivalent of a suitably substituted thiosemicarbazide (Scheme I). Ring closures of these derivatives were carried out by warming the various thiosemicarbazones in ammonium hydroxide solution followed by acidification; the title compounds were obtained (Table I). The esters were readily converted to the corresponding acids by hydrolysis using sodium hydroxide solution.

Synthesis—The following example typifies the method used to prepare the title compounds. Further preparative details as well as the chemistry and spectral analyses for other compounds were previously published (4).

Diethyl Acetylsuccinate Thiosemicarbazone (I)—A mixture of 10.8 g of diethyl acetylsuccinate and 4.56 g of thiosemicarbazide in 250 ml of ethanol was heated under reflux for 17 hr. The ethanol solution was cooled in ice, and cyclohexane was added to precipitate the product. The product amounted to 13.1 g, mp 98–102°. The analytical sample (mp 98–100°) was obtained by recrystallization from ethanol–cyclohexane; IR (KBr): 2.90, 3.00, 3.12 (NH), 5.72, and 5.79 (ester C=O) μm.

Anal.—Calc. for $C_{11}H_{19}N_3O_4S$: C, 45.66; H, 6.62; N, 14.52; S, 11.08. Found: C, 45.64; H, 6.58; N, 14.65; S, 11.42.

Ethyl 3-Methyl-5-oxo-1-thiocarbamoyl-3-pyrazoline-4-acetate (IIa)—A mixture of 10 g of diethyl acetylsuccinate thiosemicarbazone and 250 ml of concentrated ammonium hydroxide solution was heated on a steam bath for approximately 30 min, at which

[1] Melting points were determined with a Thomas-Hoover capillary melting-point apparatus and are uncorrected. The IR spectra were determined in potassium bromide disks using a Perkin-Elmer model 21 spectrophotometer.

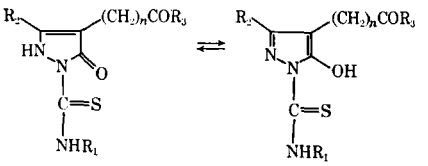
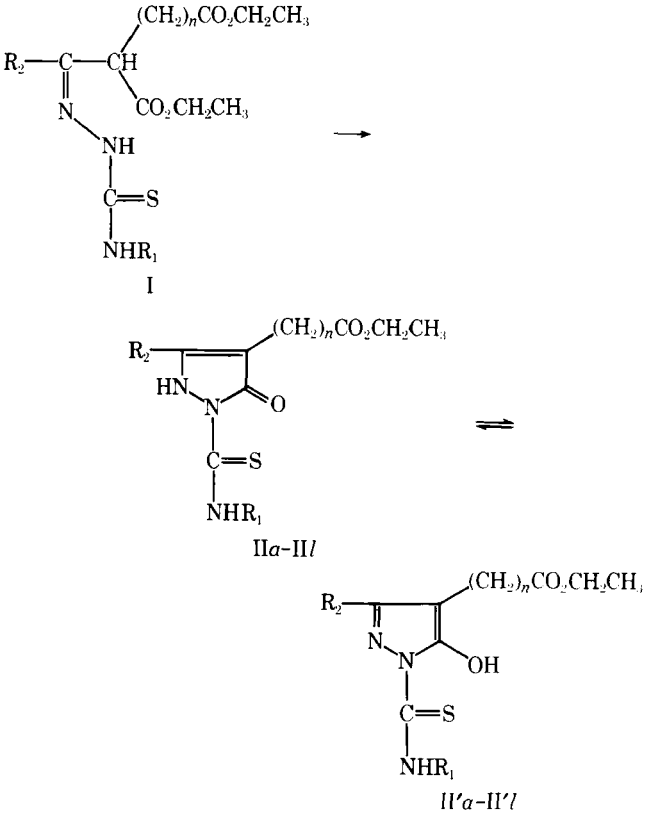


Table I—Substituted 5-Oxo-1-thiocarbamoyl-3-pyrazoline-4-alkanoic Acid Derivatives and Their *In Vitro* Activity *versus M. tuberculosis*, Human Type

Compound	n	R_1	R_2	R_3	Melting Point	Formula	Activity *versus* H37Rv, MIC, μg/ml
II*a*	1	H	CH_3	OCH_2CH_3	146–148°	$C_9H_{13}N_3O_3S$	0.5
II*b*	1	H	CH_3	OH	178–179°	$C_7H_9N_3O_3S$	50
II*c*	1	CH_3	CH_3	OCH_2CH_3	151–153°	$C_{10}H_{15}N_3O_3S$	0.05–0.1
II*d*	1	CH_3	CH_3	NH_2	181–183°	$C_8H_{12}N_4O_2S$	5.0
II*e*	1	CH_3	CH_3	OH	180–182°	$C_8H_{11}N_3O_3S$	50
II*f*	1	CH_3	H	OCH_2CH_3	149–151°	$C_9H_{13}N_3O_3S$	0.1
II*g*	1	CH_3	H	OH	192–194°	$C_7H_9N_3O_3S$	50
II*h*	1	H	H	OCH_2CH_3	141–142°	$C_8H_{11}N_3O_3S$	100
II*i*	2	H	CH_3	OCH_2CH_3	149–150°	$C_{10}H_{15}N_3O_3S$	0.5
II*j*	2	H	CH_3	OH	186–187°	$C_8H_{11}N_3O_3S$	50
II*k*	2	CH_2CH_3	CH_3	OCH_2CH_3	72–74°	$C_{12}H_{19}N_3O_3S$	—
II*l*	2	CH_2CH_3	CH_3	OH	152–153° dec.	$C_{10}H_{15}N_3O_3S$	10



Scheme I

time the reaction mixture was filtered free of some undissolved particles. The solution was cooled in ice and acidified with concentrated hydrochloric acid. The precipitate which formed amounted to 3 g. The analytical sample (mp 146–148°) was obtained by recrystallization from ethanol; IR (KBr): 3.08 (NH), 5.72 (ester C=O), and 6.02 (lactam C=O) μm.

Anal.—Calc. for $C_9H_{13}N_3O_3S$: C, 44.43; H, 5.38; N, 17.27; S, 13.18. Found: C, 44.44; H, 5.40; N, 17.64; S, 13.43.

Biological—The compounds described here were tested for their bacteriostatic activity against *M. tuberculosis*, human type, strain H37Rv, by a tube dilution technique. The medium employed was Dubos oleic acid liquid medium. Stock cultures were maintained on Dorset egg agar. Stock solutions of 1000 μg/ml of drug were prepared in distilled water or dimethylacetamide, and aqueous solutions were sterilized by passage through a Swinny filter. One-milliliter quantities of each dilution were incorporated into 9 ml of medium to give final concentration values of 0.01–100 μg/ml. Tubes were seeded with 0.1 ml of standardized bacterial suspension and incubated for 2 weeks at 37°. The minimum inhibitory concentration (MIC), expressed in micrograms per milliliter, is the least amount of material required for complete inhibition of growth of *M. tuberculosis*.

RESULTS AND DISCUSSION

Compound IIc (Table I) showed the highest order of activity of the compounds tested (MIC ≈ 0.05–0.1 μg/ml). Activity diminished considerably when this compound was tested as the free acid (II*e*) or as a carboxamide (II*d*). Replacement of the methylthiocarbamoyl group by a thiocarbamoyl group (II*a*) also resulted in diminished activity. Replacement of the 3-methyl group in IIc by a hydrogen afforded II*f*, which showed a high order of activity similar to that of IIc.

An activity comparison for II*a* and II*i* indicates that no gain in potency results when the alkanoic acid chain is extended by one methylene group. A comparison of IIc with 3-methyl-5-oxo-3-pyrazoline-4-acetate (4) (MIC = 0.5 μg/ml) indicates that the methylthiocarbamoyl group must contribute significantly toward activity in this series since potency diminished considerably in its absence. Similarly, removal of the alkanoic acid group appears to have a deleterious effect on activity. This supposition is based on the fact that a sharp decline in potency was observed when 3-methyl-5-oxo-1-methylthiocarbamoyl-3-pyrazoline (4) (MIC = 1 μg/ml) was compared with IIc. The clinical agent isoniazid was used as the standard for comparison. When tested in this assay, its MIC was 0.005–0.01 μg/ml.

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Preparation and Baeyer–Villiger Reaction of Certain 2-Carbalkoxycyclopropyl Methyl Ketones

JOSEPH G. CANNON ˣ and JOHN E. GARST

Abstract □ Treatment of mixed anhydrides derived from *cis*- and *trans*-2-carbobenzyloxycyclopropanecarboxylic acids and ethyl chloroformate with ethoxymagnesio di-*tert*-butyl malonate and subsequent treatment of the resulting adducts with *p*-toluenesulfonic acid afforded *cis*- and *trans*-benzyl 2-acetylcyclopropanecarboxylates in good to excellent yields, with retention of the original stereochemistry of the systems. These methyl ketones and an open chain congener, benzyl levulinate, were inert toward *m*-chloroperbenzoic acid. The *cis*-isomer and benzyl levulinate underwent normal Baeyer–Villiger reactions mediated by trifluoroperacetic acid, forming moderate yields of the acetate ester insertion products.

Keyphrases □ 2-Acetoxycyclopropanecarboxylic acid esters—synthesis from 2-carbalkoxycyclopropyl methyl ketones, Baeyer–Villiger reaction, retention of stereochemistry □ 2-Carbalkoxycyclopropyl methyl ketones—Baeyer–Villiger reaction, synthesis of stereospecific 2-acetoxycyclopropanecarboxylic acid esters

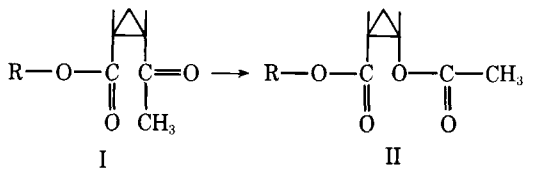
As a part of a synthetic program leading to partially rigid analogs of acetylcholine, a preparative route to *cis*-2-acetoxycyclopropanecarboxylic esters (II) was required. It was proposed that these systems should be attainable *via* Baeyer–Villiger oxidation of an appropriate methyl ketone (I) (Scheme I).

DISCUSSION

It was speculated that the malonic ester adduct (III) could be converted into I by decarboxylation of the malonic ester moiety and esterification of the ring carboxyl group. Treatment of *cis*-cyclopropane-1,2-dicarboxylic anhydride with sodio di-*tert*-butyl malonate gave a 3% yield of the expected product (III) (R = *tert*-butyl) and 33% of a neutral solid (IV), whose structure was assigned on the basis of spectral and analytical data and of literature precedent (1). Attempts to effect acid-catalyzed conversion of IV into a methyl ketone failed.

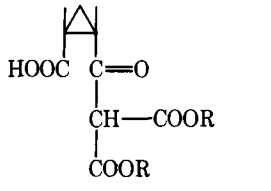
Treatment of *cis*-2-carbomethoxycyclopropanecarbonyl chloride with methoxymagnesio dimethyl malonate (V) gave a 24% yield of the predicted product (VI), whose NMR spectrum demonstrated broad resonance signals at δ1.10–2.80. These signals were typical of other *cis*-1,2-dicarbonyl cyclopropane systems encountered in the present work.

Repeated attempts to prepare *cis*-2-carbobenzyloxycyclopropanecarbonyl chloride led only to elimination of benzyl chloride and isolation of *cis*-cyclopropane-1,2-dicarboxylic anhydride. Reaction of the mixed anhydride (VII) with ethoxymagnesio di-*tert*-butyl malonate and treatment of the malonate adduct (VIII) with *p*-toluenesulfonic acid, according to a method of Fonken and Johnson (2), permitted good overall yields of the *cis*-methyl ketone (IX) (Scheme II). A similar reaction sequence on *trans*-2-carbobenzyloxycyclopropanecarbonyl chloride gave a 76% overall yield of the *trans*-isomer (X). Retention of the *cis*-geometry of IX was verified by comparison of its NMR spectrum with that of X. A reaction between the mixed anhydride (VII) and methylmagnesium iodide resulted in a mixture of the *cis*- and *trans*-isomers (IX and X).

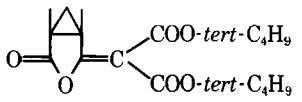


I II

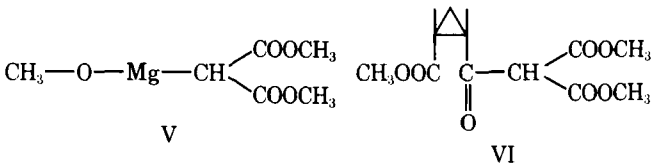
Scheme I



III



IV



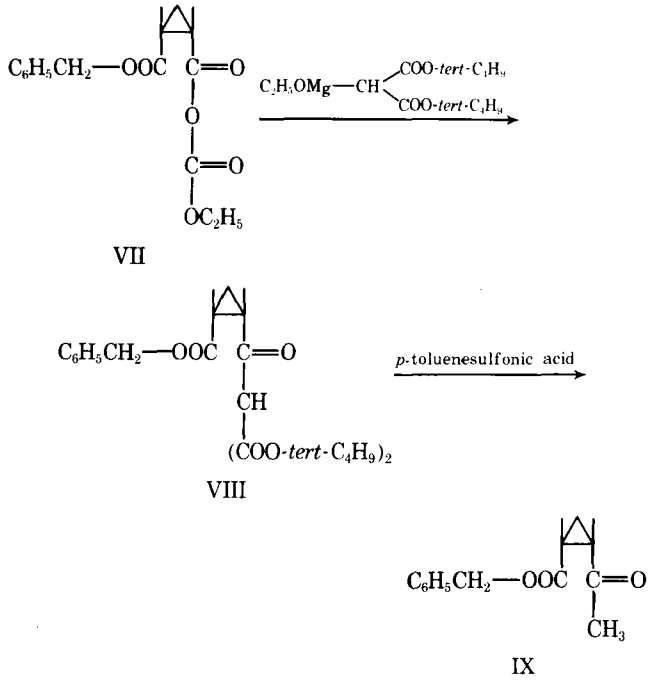
V VI

Sauers and Ubersax (3) reported a 20% yield of a mixture of Baeyer–Villiger products of methylcyclopropyl ketone, using *m*-chloroperbenzoic acid or trifluoroperacetic acid. The remaining 80% of the starting ketone was recovered unchanged. In the present work, *cis*-benzyl 2-acetylcyclopropanecarboxylate was unchanged (according to NMR analysis) after 10 days of stirring with *m*-chloroperbenzoic acid. Treatment of benzyl levulinate (XI), an open chain congener of the cyclopropane methyl ketone systems, with *m*-chloroperbenzoic acid failed to afford detectable reaction products after 10 days of stirring at room temperature. Baeyer–Villiger oxidation of benzyl levulinate into benzyl 3-acetoxypropionate (XIII) was achieved (in 65% yield) with trifluoroperacetic acid in an oxidant–ketone ratio of 4.4:1. This reagent, in a ratio of 8.8:1, converted *cis*-benzyl 2-acetylcyclopropanecarboxylate into the acetoxy insertion product (XII). When less oxidizing agent was employed, large amounts of unchanged starting ketone could be recovered.

EXPERIMENTAL[1]

Di-*tert*-butyl 2-Keto-3-oxa-[3.1.0]-bicyclohexyl-4-idene Malonate (IV)—Di-*tert*-butyl malonate (3) (65.1 g, 0.3 mole) in 200 ml of anhydrous benzene was added dropwise with stirring to 200 ml of anhydrous benzene containing 14.4 g (0.3 mole) of 50% sodium hydride in mineral oil, which had been washed with three 50-ml portions of hexane. The resulting mixture was refluxed for 2 hr, then 33.6 g (0.3 mole) of *cis*-cyclopropane-1,2-dicarboxylic anhydride (4) in 100 ml of anhydrous benzene was added dropwise, and this mixture was refluxed overnight. The solvent was removed under reduced pressure, and the solid residue was dissolved in 300 ml of water. This solution was extracted with ether and the extract was dried over magnesium sulfate. The water solution was reserved as Solution A and was utilized in the isolation of III. Evaporation of the ethereal solution gave a solid, which was recrystallized from hexane to yield 30.9 g (33%) of product, mp 117–119°; IR (potassium bromide): 3110 (cyclopropane CH), 2980 (*tert*-

[1] Boiling points are uncorrected. Melting points were determined in open glass capillaries, using a Thomas-Hoover Uni-Melt apparatus, and are corrected. Elemental analyses were performed by Galbraith Laboratories, Knoxville, Tenn., and by the Microanalytical Service, College of Pharmacy, University of Iowa. NMR spectra were recorded on a Varian Associates T-60 instrument relative to an internal standard of tetramethylsilane. IR spectra were obtained with a Beckman IR-10 instrument.



Scheme II

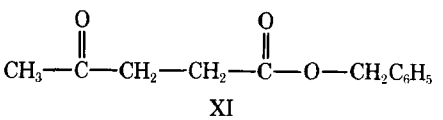
C_4H_9), 1814 (lactone C=O), 1720–1708 (ester C=O), and 1660 (C=C) cm^{-1}; NMR (deuterochloroform): δ 1.50 (s, 18H, *tert*-C_4H_9), 1.20–1.80 (m, 2H, ring CH_2), 2.10–2.60 (m, 1H, CHC=O), and 3.48–3.84 (m, 1H, CHC=C).

Anal.—Calc. for $C_{16}H_{22}O_6$: C, 61.92; H, 7.14. Found: C, 61.60; H, 7.14.

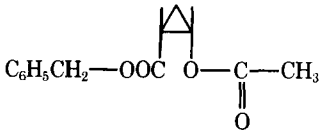
***cis* - 2 - [Di(carbo-*tert*-butoxy)acetyl]cyclopropanecarboxylic Acid (III)**—Acidification of Solution A from the preparation of IV with 6 *N* hydrochloric acid gave a solid, which was recrystallized from ether to afford 3 g (3%) of product, mp 129–130.5°; IR (mineral oil): 3350–2600 (acid OH), 1710 (acid C=O), 1734, and 1750 (ester C=O) cm^{-1}; NMR (deuterochloroform): δ 1.50 (s, 18 H, *tert*-C_4H_9), 1.15–1.60 (m, 2H, ring CH_2), 2.00–2.65 (m, 2H, ring CH), 4.39 [s, 1H, $CH(CO_2R)_2$], and 9.13 (broad s, 1H, CO_2H).

Anal.—Calc. for $C_{16}H_{24}O_7$: C, 58.55; H, 7.35. Found: C, 58.51; H, 7.44.

***cis*-Methyl 2-[Di(carbomethoxy)acetyl]cyclopropanecarboxylate (VI)**—Carbon tetrachloride (1 ml) was added to a mixture of 2.4 g (0.1 g-atom) of magnesium turnings, 42 ml of anhydrous methanol, and 13.2 g (0.103 mole) of dimethyl malonate. Within 3–5 min, the evolution of gas became vigorous and was moderated by external cooling. After the magnesium had dissolved, excess methanol was azeotroped with anhydrous benzene under reduced pressure. Fresh anhydrous benzene (100 ml) was added, and this solution was added dropwise with stirring to 15.9 g (0.098 mole) of freshly distilled *cis*-2-carbomethoxycyclopropanecarbonyl chloride (5); stirring was continued for 1 hr. After standing for 12 hr at room temperature, 200 ml of ether was added and the resulting solution was poured into 100 ml of 6 *N* hydrochloric acid. The organic layer was washed with water until the washings were neutral to litmus and was dried over magnesium sulfate. Evaporation of the volatiles left an oil, which was distilled at 120–124° (0.05 mm) to yield 6.0 g (24%) of product; IR (film): 1740



XI



XII

(ester C=O) and 1650 (C=C) cm^{-1}; NMR (deuterochloroform): δ 1.10–2.80 (m, 4H, ring H), 3.67 (s, 3H, CO_2CH_3), 3.80, 3.83 (2 s, 6H, malonate CH_3), 4.60 [s, 0.5H, $CH(CO_2R)_2$], and 13.71 (s, 0.5H, enol OH).

Anal.—Calc. for $C_{11}H_{14}O_5$: C, 51.16; H, 5.46. Found: C, 51.15; H, 5.16.

***cis*-Benzyl 2-Acetylcyclopropanecarboxylate (IX)**—To a stirred, cooled (0°) solution of 11 g (0.05 mole) of *cis*-2-carbobenzyloxycyclopropanecarboxylic acid (5) in 200 ml of anhydrous ether was added dropwise 5.1 g (0.05 mole) of triethylamine. After stirring for 10 min, 5.4 g (0.05 mole) of ethyl chloroformate was added dropwise over 5 min; stirring was continued at 0° for 0.5 hr. The reaction mixture was filtered and the filtrate was reserved as Solution A. Carbon tetrachloride (1 ml) was added to 13 g (0.06 mole) of di-*tert*-butyl malonate, 1.2 g (0.05 g-atom) of magnesium turnings, and 4 g (0.087 mole) of anhydrous ethanol. Evolution of gas began immediately, and 50 ml of anhydrous dimethoxyethane was added within 3–5 min. The temperature was maintained at 30–40°, and most of the magnesium had dissolved after 1 hr.

This reaction mixture was cooled to 0° and was added dropwise, with rapid stirring, to cooled (0°) Solution A. After stirring for 1 hr, the reaction mixture was allowed to come to room temperature overnight. It was then poured into 200 ml of cold (0°) 3 *N* hydrochloric acid, and this solution was extracted with three 100-ml portions of ether. The combined organic extracts were washed with water and extracted with 50 ml of 10% potassium carbonate solution and with three 100-ml portions of water. The organic phase was acidified by washing with 25 ml of 6 *N* hydrochloric acid and dried over magnesium sulfate, and the ether was removed under reduced pressure to afford 18.5 g of an oil. This oil was refluxed with 1.5 g of *p*-toluenesulfonic acid monohydrate in 100 ml of toluene for 5 hr; the solution was cooled and then extracted with 10% potassium carbonate solution until the washings were basic. The organic layer was washed with 20 ml of 6 *N* hydrochloric acid and then with several portions of water. After drying over magnesium sulfate, the volatiles were removed and the residue was distilled at 104–108° (0.05 mm) to afford 6.3 g (58%) of IX; IR (film): 1730 with a shoulder at 1700 (ester and ketone C=O) cm^{-1}; NMR (deuterochloroform): δ 0.92–1.40, 1.50–1.90, 2.00–2.35 (m, 4H, ring H), 2.20 (s, 3H, CH_3), 5.10 (s, 2H, CH_2Ar), and 7.34 (s, 5H, ArH).

Anal.—Calc. for $C_{13}H_{14}O_3$: C, 71.54; H, 6.46. Found: C, 71.72; H, 6.59.

***trans*-Benzyl 2-Acetylcyclopropanecarboxylate (X)**—Ethoxymagnesio di-*tert*-butyl malonate was prepared in the same manner as IX from 1 ml of carbon tetrachloride, 6.15 g (0.03 mole) of di-*tert*-butyl malonate, 0.7 g (0.029 g-atom) of magnesium turnings, 1.9 g (0.042 mole) of anhydrous ethanol, and 50 ml of anhydrous dimethoxyethane. The cooled (0°) solution was added dropwise over 10 min to 6.8 g (0.0285 mole) of *trans*-2-carbobenzyloxycyclopropanecarbonyl chloride (5) in 200 ml of anhydrous benzene. The reaction mixture was refluxed for 5 hr and was worked up as described for IX, yielding 4.7 g (76%), bp 99–102° (0.05 mm); IR (film): 1729 (ester C=O) and 1705 (ketone C=O) cm^{-1}; NMR (deuterochloroform): δ 1.25–1.55 (m, 2H, ring CH_2), 2.05–2.65 (m, 2H, 2CHCO), 2.27 (s, 3H, CH_3), 5.16 (s, 2H, $CH_2C_6H_5$), and 7.36 (s, 5H, ArH).

Anal.—Calc. for $C_{13}H_{14}O_3$: C, 71.54; H, 6.46. Found: C, 71.72; H, 6.63.

Benzyl 3-Acetoxypropionate (XIII)—Ninety percent hydrogen peroxide (11.4 g, 0.44 mole) was added dropwise over 0.5 hr to a stirred, cooled (0°) solution of 92 g (0.44 mole) of trifluoroacetic anhydride in 100 ml of methylene chloride. After cooling at 0° for 0.25 hr, the trifluoroperacetic acid solution was added dropwise to a rapidly stirred mixture of 120 g of anhydrous disodium phosphate, 200 ml of methylene chloride, and 20.6 g (0.1 mole) of benzyl levulinate (6). The resulting mixture refluxed within 0.5 hr and continued spontaneously for 2 hr. Refluxing was maintained for 4 hr total. The methylene chloride solution was filtered, and the solid on the filter was washed with two 300-ml portions of methylene chloride and then was refluxed for 10 min with an additional 300-ml portion of methylene chloride. The combined organic extracts were washed successively with 10% potassium carbonate solution, water, 6 *N* hydrochloric acid, and water and were dried over magnesium sulfate. The solvent was removed, and the residue was distilled through a 10-cm Vigreux column, bp 87–90° (0.04 mm). The yield was 10.2 g (63%); IR (film): 1741 (ester C=O) cm^{-1};

NMR (deuterochloroform): δ 2.00 (s, 3H, CH_3), 2.67 (t, 2H, CH_2CO_2R), 4.34 (t, 2H, CH_2OCOCH_3), 5.14 (s, 2H, $CH_2C_6H_5$), and 7.32 (s, 5H, ArH).

Anal.—Calc. for $C_{12}H_{14}O_4$: C, 64.85; H, 6.34. Found: C, 65.01; H, 6.27.

***cis*-Benzyl 2-Acetoxycyclopropanecarboxylate (XII)**—A procedure similar to that described for XIII was utilized with 10.9 g (0.05 mole) of IX and 57.2 g (0.44 mole) of trifluoroperacetic acid. The 3.3 g of crude oily product was treated with 4.8 g of Girard's reagent P[2] and 3 ml of acetic acid in 40 ml of refluxing methanol for 4 hr. Ice water (50 ml) was added to the hot solution, and it was brought to neutrality with solid potassium carbonate. This solution was extracted with methylene chloride, and the extract was washed with water and dried over magnesium sulfate. Removal of the solvent and distillation of the residual liquid at 113° (0.035 mm) gave 2.5 g (25%) of product; IR (film): 1730 (ester C=O) cm^{-1}; NMR (deuterochloroform): δ 1.08–2.23 (m, 3H, ring H), 1.93 (s, 3H, CH_3), 4.00–4.44 (m, 1H, $CHOCOCH_3$), 5.16 (s, 2H, $CH_2C_6H_5$), and 7.38 (s, 5H, ArH).

Anal.—Calc. for $C_{13}H_{14}O_4$: C, 66.66; H, 6.02. Found: C, 66.49; H, 6.18.

[2] Eastman.

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Abstract □ The syntheses of 2-(9-acridinyl)ethyl-*N*-substituted carbamates and their hydrochlorides and 10-*N*-oxides are reported along with biological results in the areas of antineoplastic, antimalarial, and CNS activity screening. The compounds showed negative biological activity in the areas tested.

Keyphrases □ 2-(9-Acridinyl)ethyl-*N*-substituted carbamates, hydrochlorides, and 10-*N*-oxides—synthesis, pharmacological activity □ Antineoplastic activity—synthesis and screening of 2-(9-acridinyl)ethyl-*N*-substituted carbamates, hydrochlorides, and 10-*N*-oxides

Acridine derivatives have been tested extensively for potential medicinal activity. Antibacterial (1, 2), antimalarial (3), anthelmintic (4), analeptic (5), and antineoplastic (6–10) activities have been reported for many substituted acridines. The synthesis of new acridine derivatives containing urea, thiourea, thiocarbamate, and carbamate groupings has been reported from this laboratory (11, 12). While possessing little or no anticancer activity, a general pharmacological screen revealed activity in antibacterial, metabolic, parasitologic, and GI screening procedures for some derivatives.

In a continuation of a study into the synthesis and potential biological activity of new acridine compounds, this paper reports the synthesis and antineoplastic screening data for 2-(9-acridinyl)ethyl-*N*-substituted carbamates and their hydrochlorides and 10-*N*-oxides. In addition, biological results for some compounds in antimalarial and central nervous system (CNS) activity screening procedures are reported.

DISCUSSION

The incorporation of the carbamate and acridine moieties into one structure is of special interest, since each structure is singly present in compounds that have shown various degrees of antineoplastic activity (6–10, 13–15). The interaction of acridines with nucleic acids and inhibition of nucleic acid synthesis is well documented (16, 17). Acridines tend to accumulate selectively in tumor tissue (18, 19) showing inhibition of tumor growth, reduction of rate of growth, and, in some cases, regression of tumor size. Nevertheless, no clinically useful agent has been found among the acridines tested, most of which have been substituted 9-aminoacridines. Structures containing carbamate esters also have been reported to have potent antitumor activity (13, 14). Ethyl carbamate itself has been used both for the production of tumors in experimental animals and for the treatment of chronic leukemia and multiple myeloma (15). Some evidence also indicates that carbamates are involved in alkylation of DNA (20).

The mechanism by which these new acridine carbamates were expected to exert their antineoplastic effect was by selective absorption in cancerous tissue followed by a two-pronged attack upon the nucleic acids of the cancer cells. The acridine ring would be expected to interact with the nucleic acid bases, thus holding the carbamate portion in close proximity to the bases to facilitate alkylation by the carbamate.

NMR (deuterochloroform): δ 2.00 (s, 3H, CH_3), 2.67 (t, 2H, CH_2CO_2R), 4.34 (t, 2H, CH_2OCOCH_3), 5.14 (s, 2H, $CH_2C_6H_5$), and 7.32 (s, 5H, ArH).

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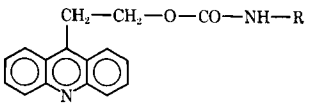
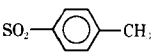
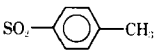
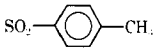
Table I—2-(9-Acridinyl)ethyl-*N*-substituted Carbamates, Hydrochlorides, and 10-*N*-Oxides



Compound	R	Melting Point	Method of Synthesis[a]	Yield, %	λ_{max} (ethanol), nm	Analysis, %	Calc.	Found
1	H	213–215°	A[b]	50	252 (2.0×10^5)	C	72.16	71.92
						H	5.30	5.51
						N	10.52	10.40
2	C_2H_5	125–126°	B	74	252 (1.2×10^5)	C	69.64	69.72
						H	5.84	5.88
						N	9.06	8.97
3	C_6H_5	183–184°	B	94	252 (8.1×10^4)	C	77.17	77.30
						H	5.30	5.39
						N	8.18	8.17
4		133–135°	B	92	250 (1.3×10^5)	C	63.29	63.07
						H	4.62	4.71
						N	6.42	6.28
	10-Hydrochlorides							
5	H	200° dec.	C	95	252 (1.7×10^5)	C	63.47	63.53
						H	4.99	5.04
						N	9.25	9.23
6	C_2H_5	126–130°	C	97	252 (8.2×10^4)	C	65.35	65.52
						H	5.79	5.86
						N	8.47	8.44
7	C_6H_5	159–160°	C	97	252 (1.4×10^5)	C	69.71	69.92
						H	5.05	5.15
						N	7.40	7.33
8		135° dec.	C	98	252 (1.4×10^5)	C	60.46	60.58
						H	4.63	4.77
						N	6.13	6.06
	10-*N*-Oxides							
9	H	169–171°	D	30	266 (8.0×10^4)	C	68.07	67.70
						H	4.99	4.94
						N	9.92	9.55
10	C_2H_5	142–145°	D	40	266 (4.9×10^4)	C	69.64	69.72
						H	5.84	5.88
						N	9.06	8.97
11	C_6H_5	218–220°	D	72	265 (9.0×10^4)	C	73.73	73.84
						H	5.06	5.19
						N	7.82	7.71
12		180–181°	D	10	266 (1.7×10^5)	C	63.29	63.07
						H	4.62	4.71
						N	6.42	6.28

[a] Letters refer to the experimental procedures. [b] Procedure modified from general method described in Ref. 17.

The hydrochloride salts of the various acridine carbamates were prepared to increase polarity of the molecules and, therefore, their water solubility. The 10-*N*-oxide derivatives were also synthesized, since it has been reported that oxidation of the acridine ring nitrogen reduces the toxicity of the agent by increasing renal clearance (21).

EXPERIMENTAL[1]

Chemistry—The carbamates were synthesized by reaction of 2-(9-acridinyl)ethyl alcohol with sodium cyanate, trifluoroacetic acid (22), and/or the respective isocyanate (23). The starting alcohol was obtained in a few synthetic steps from commercial materials (24, 25). The hydrochlorides were prepared from the respective carbamates with ethereal hydrogen chloride solution, and the 10-*N*-oxides were prepared using *m*-chloroperbenzoic acid. The synthesized compounds are shown in Table I, together with their melting points, UV maxima, and elemental analysis data.

2-(9-Acridinyl)ethyl Carbamate (Procedure A)—A solution of 4.5 g (0.02 mole) of 2-(9-acridinyl)ethyl alcohol in 25 ml of methylene chloride was treated with 2.6 g (0.04 mole) of sodium cyanate. The resulting suspension was slowly stirred while 3.2 ml (0.04 mole) of trifluoroacetic acid was added dropwise over 5 min. The reaction mixture was then stirred at room temperature for 2 hr. After washing with one 5-ml portion of water, the methylene chloride layer was separated and dried over anhydrous sodium sulfate. The filtered solution was then evaporated to dryness on a steam bath, followed by neutralization of the residue with a saturated solution of sodium bicarbonate. The resulting precipitate was recrystallized from aqueous ethanol to give the 2-(9-acridinyl)ethyl carbamate.

*2-(9-Acridinyl)ethyl-*N*-substituted Carbamates (Procedure B)*—The respective isocyanate (0.03 mole) was added to a solution of 4.5 g (0.02 mole) of 2-(9-acridinyl)ethyl alcohol in 100 ml of dry benzene. The solution was heated at reflux for 4 hr. The reaction mixture was evaporated to dryness, and the residue was recrystallized from aqueous ethanol (Compound 2), benzene–cyclohexane (Compound 3), or acetone–petroleum ether (Compound 4).

*2-(9-Acridinyl)ethyl-*N*-substituted Carbamate Hydrochlorides (Procedure C)*—A solution of 0.02 mole of the respective carbamate in 30 ml of ether was saturated with hydrogen chloride gas. A precipitate, which formed immediately, was collected by filtration and purified by trituration in acetone (Compounds 5–7) or ethanol (Compound 8) to yield the desired hydrochloride salt.

*2-(10-Oxido-9-acridinyl)ethyl-*N*-substituted Carbamates (Procedure D)*—A solution of 0.025 mole of *m*-chloroperbenzoic acid in 5–10 ml of chloroform was added to a solution of 0.02 mole of the respective carbamate dissolved in 25 ml of chloroform. The mixture was stirred for 3 hr at room temperature followed by evaporation to dryness. The resulting residue was washed with four 10-ml portions of ether followed by recrystallization of the residue with cyclohexane–chloroform.

[1] Melting points were taken in capillary tubes on a Thomas-Hoover apparatus and are uncorrected. Elemental analyses were obtained from Atlantic Microlabs, Atlanta, Ga. IR spectra were recorded on a Perkin-Elmer model 237 B spectrophotometer and were as expected. UV spectra were recorded on a Perkin-Elmer model 202 spectrophotometer.

Table II—Antineoplastic Screening Results

Compound	L-1210 Dose, mg/kg[a]	Survivors	Weight Difference	T/C, %
1[b]	400	3/3	−0.2	105
	200	3/3	+0.5	
	100	3/3	−0.5	105
2[c]	400	3/3	+0.4	94
	200	3/3	−0.3	97
	100	3/3	−0.6	105
3[c]	400	3/3	−1.0	90
	200	3/3	+0.6	94
	100	3/3	+0.5	94
4[d]	400	0/3	−1.2	
	200	3/3	−0.4	109
	100	3/3	−0.0	97
5[e]	400	3/3	−0.1	
	200	3/3	−1.2	103
	100	3/3	−0.1	93
	50	3/3	−0.3	93
	25	3/3	−0.5	96
6[f]	400	3/3	−1.1	97
	200	3/3	−0.1	90
	100	3/3	−0.3	102
7[g]	400	3/3	−1.0	97
	200	3/3	+0.3	94
	100	3/3	−0.4	
8[h]	400	0/3	−1.2	
	200	3/3	−2.0	97
	100	3/3	−0.2	97
9[c]	400	2/3	−0.7	95
	200	3/3	−0.8	98
	100	3/3	+0.2	95
	50	3/3	+0.5	98
10[c]	400[i]	5/6	+0.3	90
	200	6/6	−0.0	102
	100	6/6	+0.5	96
11[c]	400[i]	6/6	−1.0	102
	200	6/6	+1.4	99
	100	6/6	+0.2	91

[a] Doses were administered every 4th day for two doses unless otherwise noted. [b] Cell culture (KB): ED_{50} at 44 μg/ml. [c] Cell culture (KB): ED_{50} at 1.0×10^2 μg/ml. [d] Cell culture (KB): ED_{50} at 73 μg/ml. [e] Cell culture (KB): ED_{50} at 14 μg/ml. [f] Cell culture (KB): ED_{50} at 94 μg/ml. [g] Cell culture (KB): ED_{50} at 90 μg/ml. [h] Cell culture (KB): ED_{50} at 23 μg/ml. [i] Doses were administered every 4th day for three doses.

Biological[2]—Compounds 1–11 were tested against L-1210 leukemia according to the standard protocol[3] of the Division of Cancer Treatment, National Cancer Institute, National Institutes of Health. None of the compounds screened exhibited anticancer activity patterns considered adequate to justify expanded testing or further extension of the present group (Table II). Activity is defined as percent T/C (test/control) of 125 or greater. The term "survivors" in Table II is a measure of toxicity and was determined at Day 5 after the first injection.

Compounds 3 and 6 were tested for antimalarial activity. The activity was assessed against *Plasmodium berghei* in mice by the method of Osdene *et al.* (28). Both compounds were found to be inactive.

Compound 4 was tested for potential CNS activity by the method of Iturrian and Johnson (29). In the procedure, the carbamate was administered to audioconditioned mice at a level of 150 mg/kg. Data showed that the compound failed to block the running or tonic components of the audiogenic seizure.

Compound 12 was not submitted for biological testing due to insufficient quantities available from the synthetic program.

[2] The antineoplastic screening data were made available through courtesy of Drug Development Branch, Drug Research and Development, Division of Cancer Treatment, National Cancer Institute. The antimalarial test results were provided by the Walter Reed Army Institute of Research. The CNS activity data were provided by the Department of Pharmacology, School of Pharmacy, University of Georgia.

[3] For general screening procedure and data interpretation, see Refs. 26 and 27.

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Bioavailability of Aspirin from Commercial Suppositories

MILO GIBALDI ˣ and BARBARA GRUNDHOFER

Abstract □ A comparison of the bioavailability of salicylate from five brands of commercially available aspirin rectal suppositories in an adult panel is presented. All brands show slow absorption compared to oral administration of the drug in tablet form. At best, about 40% of the dose (on the average) was absorbed when retention time in the bowel was limited to 2 hr. However, four out of the five brands gave substantially lower absorption rates so that only about 20% of the aspirin is available.

Keyphrases □ Aspirin—bioavailability from commercial suppositories, humans □ Suppositories—bioavailability of aspirin, humans □ Bioavailability—aspirin from commercially available suppositories, humans

Salicylate absorption after rectal administration of an aspirin suppository[1] to eight children and four adults was recently studied (1). The absorption of the drug from the suppository was exceedingly slow, about an order of magnitude slower than that observed after oral administration of conventional tablets. Accordingly, the amount of drug absorbed was highly dependent on the time between insertion and the first defecation (retention time). Retention times as long as 4–5 hr resulted in absorption of only about 60% of the dose.

Several other brands of aspirin suppositories were available locally in community pharmacies and still another brand was used exclusively in a local hospital. Given the relatively poor bioavailability characteristics of suppository Product L, it was of interest to study salicylate absorption from other brands of aspirin suppositories. Accordingly, five products were compared in adult subjects. The bioavailability characteristics of the four other brands of aspirin suppository were substantially poorer than that observed with Product L.

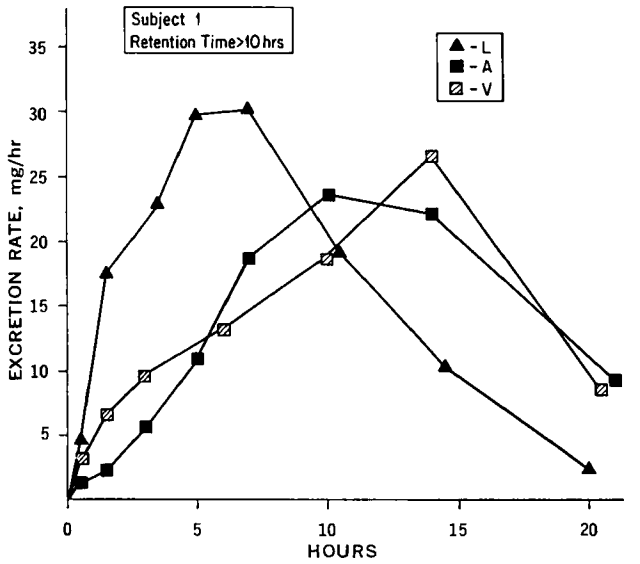

Figure 1—*Excretion rate of total salicylate* versus *time profiles after rectal administration and prolonged retention of various brands of 10-grain aspirin suppositories.*

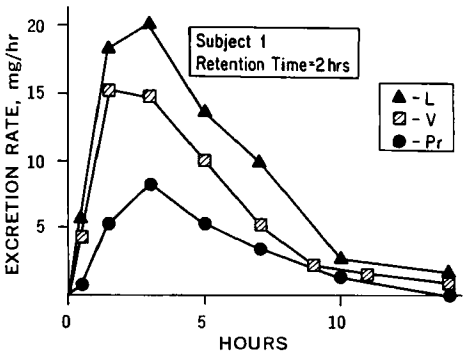

Figure 2—*Excretion rate of total salicylate* versus *time profiles after rectal administration of various brands of 10-grain aspirin suppositories.*

EXPERIMENTAL

The various aspirin suppositories were either purchased from local pharmacies or obtained from the pharmacy of a local hospital. The products were coded[2] A, L, Pp, Pr, and V. Each product claimed to contain 10 grains (600–650 mg) of aspirin. Two suppositories of each brand were assayed in duplicate for total salicylate. Expressed in terms of label claim, they were found to contain 103 and 105% (A), 108 and 105% (L), 106 and 86% (Pp), 117 and 115% (Pr), and 110 and 118% (V).

The adult panel consisted of four male volunteers, 24–35 years of age, three of whom received all five products. Each subject was instructed to insert a suppository shortly after arising in the morning and then either to evacuate his bowel as thoroughly as possible exactly 2 hr after insertion or to retain the suppository as long as possible. In the latter situation, retention times varied from 11 to >24 hr. In all cases, at least 1 week elapsed between studies.

Immediately prior to drug administration, the subjects voided their bladders and a specimen was obtained to serve as a blank. Urine was collected at appropriate intervals for 28–32 hr. The volume of each urine collection was determined and a sample was retained for drug analysis. Urine samples were hydrolyzed with an equal volume of concentrated hydrochloric acid in sealed ampuls overnight at 100°. The samples were then analyzed for total salicylate according to a modification of Brodie *et al.* (2) using carbon tetrachloride. Absorbance of the resulting ferric–salicylate complex was determined at 530 nm on a spectrophotometer[3] and corrected for blank values on an individual basis. Concentrations were determined from a previously constructed standard curve.

[1] A.S.A. suppositories, Lilly (USP) (coded L).

[2] A = APC suppositories (NDC-84-808-12, No. 808B), American Pharmaceutical Co., Bronx, N.Y.; L = Lilly A.S.A. suppositories (USP) (NDC-2-S16-16, Control 7HL14A), Lilly, Indianapolis, Ind.; Pp = Purepac aspirin suppositories (NDC-228-1028-12), Purepac Pharmaceutical Co., Elizabeth, N.J.; Pr = Premo aspirin suppositories (B11747), Premo Pharmaceutical Laboratories, Inc., South Hackensack, N.J.; V = Vitarine aspirin suppositories (127309), The Vitarine Co., Inc., New York, N.Y.

[3] Hitachi Perkin-Elmer model 139.

Table I—Initial Excretion Rates[a] (Milligrams per Hour) of Salicylate after Rectal Administration of 10-Grain Aspirin Suppositories to Adult Subjects

Hours	Product				
	L (4)[b]	A (3)	Pr (3)	Pp (3)	V (3)
0.5	6.4 ± 0.9	2.2 ± 0.4	1.4 ± 0.8	1.5 ± 1.3	3.2 ± 1.4
1.0	16.2 ± 3.7	5.6 ± 2.5	6.1 ± 2.8	5.6 ± 2.3	8.2 ± 2.9
1.5	25.6 ± 7.0	8.9 ± 4.9	10.9 ± 4.9	9.7 ± 3.9	13.2 ± 4.5

[a] Mean ± 1 *SD*. [b] Parenthetical values denote the number of subjects.

RESULTS AND DISCUSSION

Bioavailability is defined as the rate and extent of absorption of a drug from a dosage form. In the case of relatively low doses of aspirin (as used in the present study), it is well known that initial urinary excretion rates of total salicylate reflect the relative absorption rate of the drug and that the cumulative amount of total salicylate ultimately excreted in the urine serves as a quantitative index of the extent of absorption.

Table I shows the average initial excretion rates of total salicylate after rectal administration of the various products. The excretion rates at 0.5, 1.0, and 1.5 hr after insertion of Product L were at least two to three times greater than those observed with any other product. This difference is further illustrated in Fig. 1, where the urinary excretion rates of total salicylate are plotted as a function of time after administration of Products L, A, and V. The time of occurrence of maximum excretion rate was considerably less after Product L than that found after Products A and V. Moreover, the greatest peak excretion rate was observed after administration of L. Clearly, the absorption rate of aspirin must be considerably greater after rectal administration of Product L than after administration of the other suppositories studied. This finding is indeed remarkable when one considers how slowly aspirin is absorbed from Product L (1).

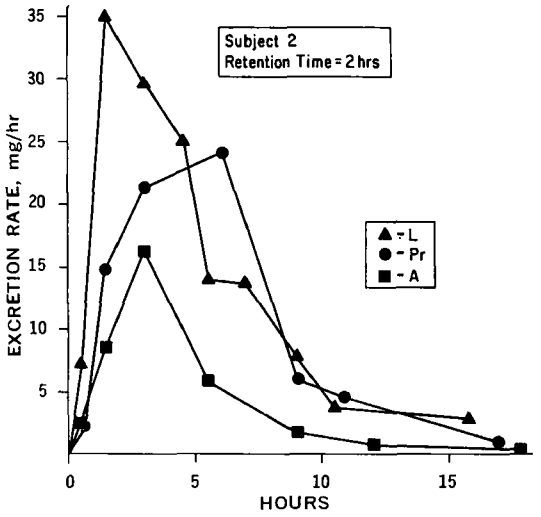

Figure 3—*Excretion rate of total salicylate* versus *time profiles after rectal administration of various brands of 10-grain aspirin suppositories.*

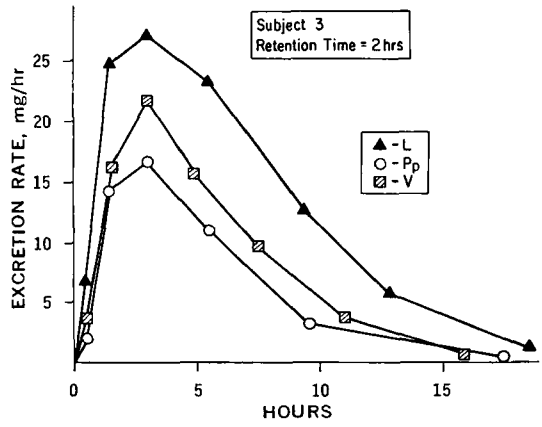

Figure 4—*Excretion rate of total salicylate* versus *time profiles after rectal administration of various brands of 10-grain aspirin suppositories.*

When drug products intended for rectal administration manifest such prolonged absorption patterns, the extent of absorption is obviously dependent on retention time. As shown in Table II, even after administration of the product from which aspirin is most rapidly absorbed (L), only about 40% of the dose was absorbed when the retention time was limited to 2 hr. During a similar 2-hr period, only about half that amount of aspirin was available, on the average, from Products A, Pp, Pr, and V, which released the drug still more slowly.

Differences between products with respect to the amount of aspirin absorbed in individual subjects are readily apparent in Figs. 2–4 when the total area under the excretion rate *versus* time plot is considered. In all three subjects, the cumulative amount of total salicylate ultimately excreted after a 2-hr retention of an aspirin suppository was consistently greater after administration of Product L. No consistent differences were obvious among the other four products.

The present results are readily extrapolated to permit one to conclude that the shorter the retention time the greater will be the difference between Product L and the other products tested with respect to the amount of drug absorbed, but in all cases the amount will be decreased. On the other hand, as shown previously (1) in studies with L, the greater the retention time the greater is the availability of aspirin. It follows that longer retention times tend to diminish differences among products with respect to the extent of drug absorption and that ultimately such differences disappear (Table II). When the aspirin suppository was retained for periods exceeding 10 hr, in most cases absorption of the drug was nearly complete and no differences among products were apparent.

Despite the substantial differences in bioavailability characteristics found to exist among various commercially available aspirin suppositories, the rate of absorption of aspirin from all products studied was sufficiently slow to raise considerable doubt as to whether efficacious body levels of aspirin or salicylate are attained after a single dose. The exceedingly slow absorption also suggests the possibility of a significantly reduced extent of absorption of the administered dose if the patient defecates even several hours after insertion of the suppository. It is anticipated that this prob-

Table II—Urinary Recovery of Salicylate after Rectal Administration of 10-Grain Aspirin Suppositories to Adult Subjects

Product	Retention Time	
	2 hr	>10 hr
L	38 ± 8[a] (4)[b]	81 ± 11 (4)
A	17 ± 6 (3)	74, 84
Pr	20 ± 10 (3)	93 ± 5 (3)
Pp	16 ± 4 (3)	88 ± 2 (3)
V	22 ± 6 (3)	80 ± 9 (3)

[a] Mean ± *SD*, expressed as percent of dose. [b] Parenthetical values denote the number of subjects.

lem would be most pronounced with Products A, Pp, Pr, and V. The slow absorption coupled with the inability to control the actual amount of drug available to a given patient raises serious questions as to the usefulness of the presently available aspirin suppository dosage forms for aspirin or salicylate therapy.

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Central *versus* Peripheral Anticholinergic Activity as Assessed by Two *In Vivo* Procedures in Mice

JEFFREY B. MALICK [x] and ALLEN BARNETT

Abstract □ The activity of tertiary and quaternary anticholinergic drugs was compared in two different test procedures designed to measure cholinolytic activity in mice. The four drugs utilized were atropine sulfate, atropine methylnitrate, scopolamine hydrobromide, and scopolamine methylnitrate. The results led to the conclusion that one of these test procedures, the induction of mydriasis (increase in pupil size), primarily measures peripheral anticholinergic activity whereas the other procedure, inhibition of physostigmine lethality, primarily measures anticholinergic activity in the CNS. These two test procedures can be utilized to characterize the nature of the cholinolytic properties of prospective therapeutic drug candidates.

Keyphrases □ Anticholinergic activity—determination of central *versus* peripheral cholinolytic activity, tertiary and quaternary derivatives of atropine and scopolamine, mice □ Mydriasis induction as measure of peripheral anticholinergic activity—tertiary and quaternary derivatives of atropine and scopolamine, compared to physostigmine-induced lethality inhibition (central activity), mice □ Physostigmine-induced lethality inhibition as measure of central anticholinergic activity—tertiary and quaternary derivatives of atropine and scopolamine, compared to mydriasis induction (peripheral activity), mice □ Atropine and scopolamine, tertiary and quaternary derivatives—central *versus* peripheral cholinolytic activity, mice □ Scopolamine and atropine, tertiary and quaternary derivatives—central *versus* peripheral cholinolytic activity, mice

The measurement of anticholinergic activity is important in the evaluation of drug candidates for humans. Peripheral anticholinergic liability is considered to be an undesirable side effect of many therapeutic agents, particularly with regard to antidepressants (1) and antihistamines (2). Central [*i.e.*, within the central nervous system (CNS)] anticholinergic activity may not be as troublesome (except at toxic doses) and, in fact, may even be desirable under certain circumstances. Central anticholinergic drugs (*e.g.*, scopolamine) produce sedation in humans, and amitriptyline, a potent central anticholinergic and sedative drug, may be the antidepressant of choice in cases of agitated depression, a condition in which sedative effects have utility (3).

In the present studies, two test procedures in mice were evaluated for their ability to detect central *versus* peripheral anticholinergic activity. To evaluate these procedures, tertiary and quaternary forms of atropine and scopolamine were utilized. The tertiary forms readily cross the blood–brain barrier whereas the quaternary forms are retarded by the blood–brain barrier and, consequently, are concentrated to a much lesser extent in the CNS than they are in the periphery following parenteral administration. Thus, by comparing the relative potency of these agents in the two procedures, the induction of mydriasis and the antagonism of physostigmine-induced lethality, the extent that each can predict central or peripheral anticholinergic activity was determined.

EXPERIMENTAL

Male CF No. 1-S mice, 18–22 g, were used. All drug doses were calculated in terms of milligrams per kilogram of free base, and all drugs were dissolved in distilled water. The volume of injection was 10 ml/kg for both the intraperitoneal and subcutaneous routes of administration. The drugs used were atropine sulfate, atropine methylnitrate, scopolamine hydrobromide, and scopolamine methylnitrate.

Induction of Mydriasis (Increased Pupil Size) in Mice—Mice were administered test drugs and were tested for mydriatic activity 30 min later. These studies were performed under double-blind conditions such that the investigator did not know what drugs or doses were being tested. Normal pupil size was scored as 0 and increases in pupil size were scored as 1, 2, and 3 (slight, moderate, and maximal increases in size, respectively). A score of 2 was approximately 50% of maximal pupil size, which was a modification of the scoring system of Janssen and Niemegeers (4).

Any mouse exhibiting a pupil size of 2 or greater was considered to be significantly affected. Mean pupil sizes for each group were also calculated. The dose intervals for each test drug were kept constant (0.5 $\log_{10}$ units). The MED_{50} (minimal effective dose) for producing a significant increase in mydriasis in 50% or more of the mice tested was determined for each test drug. The ED_{50}'s could not be calculated since there were very small intervals between doses at which no animals exhibited significant effects and doses producing effects in 100% of the mice.

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Inhibition of Physostigmine-Induced Lethality in Mice—

Table I—Induction of Mydriasis (Increase in Pupil Size) by Tertiary and Quaternary Anticholinergic Drugs in Mice

Treatment	Dose, mg/kg ip[a]	Mean Pupil Size[b]	Number of Mice Exhibiting Significant Mydriasis[c] / Number of Mice Tested	MED_{50}, mg/kg ip, for Inducing Mydriasis[d]
Saline control	1	0.0	0/30	—
Atropine sulfate	0.1	0.7	0/6	0.3
	0.3	2.3	5/6	
	1.0	2.8	6/6	
	3.0	3.0	6/6	
Atropine methylnitrate	0.01	0.0	0/6	0.1
	0.03	0.3	0/6	
	0.1	2.8	6/6	
	0.3	3.0	6/6	
Scopolamine hydrobromide	0.01	1.0	0/6	0.1
	0.03	1.3	2/6	
	0.1	1.7	5/6	
	0.3	2.7	6/6	
	1.0	2.5	6/6	
Scopolamine methylnitrate	0.003	0.2	0/6	0.03
	0.01	0.5	0/6	
	0.03	2.3	6/6	
	0.1	3.0	6/6	

[a] Drug administered 30 min prior to testing. [b] Pupil size scored as follows: 0 = normal size, 1 = slight increase, 2 = moderate increase, and 3 = marked increase. [c] Number of mice exhibiting a significant increase in pupil size (score of 2 or greater). [d] Minimal effective dose for producing significant mydriasis in 50% or more of the animals tested.

The method used was a modification of the technique reported by Collier *et al.* (5). Physostigmine salicylate (1.0 mg/kg sc) produced 100% lethality when administered to mice grouped 10 per cage [12.7 × 28 × 12.7 cm (5 × 11 × 5 in.)] 20 min after drug administration. Test agents were administered intraperitoneally 30 min prior to physostigmine. Inhibition of lethality was then calculated as percent of control. The ED_{50}, the dose that prevented lethality in 50% of the mice, was calculated for each drug by the Maximum Likelihood Probit Analysis Method (6).

RESULTS AND DISCUSSION

The induction of mydriasis by the four anticholinergic drugs is summarized in Table I. All four drugs produced dose-related increases in pupil size, as indicated by mean pupil size. The MED_{50}'s for induction of mydriasis by atropine sulfate, atropine methylnitrate, scopolamine hydrobromide, and scopolamine methylnitrate were 0.3, 0.1, 0.1, and 0.03 mg/kg ip, respectively. The quaternary forms of the anticholinergic drugs were more potent as mydriatics than their respective tertiary forms; both atropine methylnitrate and scopolamine methylnitrate were approximately three times as potent as their respective tertiary forms.

Table II summarizes the results of studies to determine the relative potency of the four anticholinergic drugs as inhibitors of physostigmine-induced lethality in mice. The ED_{50}'s for atropine sulfate, atropine methylnitrate, scopolamine hydrobromide, and scopolamine methylnitrate were 1.6, 12.8, 0.35, and 6.1 mg/kg ip, respectively. Although all four drugs inhibited the lethality produced by physostigmine, a cholinesterase inhibitor, the tertiary forms, in contrast to the results obtained in the mydriasis studies, were considerably more potent than their respective quaternary forms. Atropine sulfate was eight times more potent than atropine methylnitrate, and scopolamine hydrobromide was 18 times more potent than scopolamine methylnitrate in this procedure. This result was believed to be due to the blood–brain barrier impeding the passage of the quaternary derivatives into the CNS.

Table III summarizes the potency ratio comparisons of the various drugs in the two anticholinergic test procedures. As mentioned, there was a reversal of the potency relationships when appropriate tertiary and quaternary derivatives were compared such that the quaternary forms were more potent mydriatic agents whereas the tertiary forms were more potent inhibitors of physostigmine lethality. When scopolamine hydrobromide was compared to atropine sulfate, the former was approximately three to four times more potent than the latter in both procedures. The same relationship between the quaternary derivatives held true; *i.e.*, scopolamine methylnitrate was approximately two to three times more potent than atropine methylnitrate as an anticholinergic in both procedures (Table III).

From these results, it was concluded that the induction of my-

Table II—Antagonism of Physostigmine-Induced Lethality in Mice

Treatment[a]	Number of Mice Used	ED_{50}, mg/kg ip, for Antagonism of Physostigmine Lethality (95% Confidence Limits)[b]
Atropine sulfate	90	1.6 (1.2–2.3)
Atropine methylnitrate	150	12.8 (6.3–54.9)
Scopolamine hydrobromide	140	0.35 (0.19–0.40)
Scopolamine methylnitrate	100	6.1 (4.1–8.4)

[a] Drugs administered 30 min prior to physostigmine (1.0 mg/kg sc). [b] The ED_{50} and 95% confidence limits were calculated by Probit Maximum Likelihood Analysis (6).

Table III—Potency Ratio Comparisons of the Activities of Anticholinergic Drugs in Two Procedures in Mice

Comparison	Induction of Mydriasis Potency Ratio[a]	Antagonism of Physostigmine Lethality Potency Ratio (95% Confidence Limits)[b]
Atropine sulfate *versus* atropine methylnitrate	0.33	8.1 (5.0–14.7)
Scopolamine hydrobromide *versus* scopolamine methylnitrate	0.30	18.1 (12.3–27.7)
Scopolamine hydrobromide *versus* atropine sulfate	3.00	4.7 (3.2–7.2)
Scopolamine methylnitrate *versus* atropine methylnitrate	3.33	2.1 (1.3–3.5)

[a] Calculated from MED_{50}'s from Table I. [b] The potency ratio and 95% confidence limits were calculated by Maximum Likelihood Potency Probit Analysis (6) from data in Table II.

driasis was primarily a peripheral anticholinergic response since both the tertiary and quaternary derivatives of atropine and scopolamine were potent inducers of mydriasis. It also was concluded that inhibition of physostigmine-induced lethality was primarily a measure of central anticholinergic activity since the quaternary derivatives were much less potent inhibitors of physostigmine-induced lethality than their respective tertiary forms.

A careful evaluation of a drug's relative activity in these two procedures in the same species, the induction of mydriasis and the inhibition of physostigmine lethality, should predict its relative activity as a central and a peripheral anticholinergic in humans.

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Tissue Distribution of *N*-^{14}C-Azure C (Methylthionine) in the Rat

R. ENG, S. LINDBERG, K. WHITE, and L. A. SPITZNAGLE [x]

Abstract □ The distribution of radioactivity at 5-, 10-, and 15-min intervals following the intravenous administration of *N*-^{14}C-azure C was determined. The concentrations of radioactivity observed indicated that radioactive derivatives of azure C would not be useful pancreas or parathyroid scanning agents.

Keyphrases □ Azure C, radiolabeled—tissue distribution, considerations related to use as pancreas or parathyroid scanning agent □ Methylthionine, radiolabeled—tissue distribution, considerations related to use as pancreas or parathyroid scanning agent □ Scanning agents—tissue distribution of radiolabeled azure C

The report (1) that the intravenous administration of the phenothiazine dye tolonium chloride (toluidine blue O) caused a blue coloration of the pancreas and parathyroid glands but not of surrounding organs generated much interest and has been verified in experimental animals (2–4) and humans (5–7). Toluidine blue O has been used for the identification of the parathyroid gland at surgery (5–7) and as an aid in the diagnosis of small oral cancers (8), and it has been suggested as a potential parathyroid and pancreas scanning agent if labeled with a suitable radionuclide (2–4, 6, 7). Recent studies with an iodinated analog of toluidine blue O labeled with ^{131}I showed it to be useful for imaging parathyroid adenomas (9).

DISCUSSION

Kang and DiGiulio (3) found the highest concentrations of toluidine blue O in the parathyroids, heart, pancreas, kidneys, stomach, lungs, thyroid, muscles, liver, and blood, in that order. They extracted the excised tissues with ethanol and measured the dye concentrations colorimetrically. Mortenson and McRae (2) reported similar results using a similar assay. In both studies, rather low concentrations were observed in the organs surrounding the pancreas, suggesting the development of a pancreas scanning agent provided a suitable labeled derivative could be prepared.

Larose *et al.* (10) studied the distribution in rats of an iodinated derivative of toluidine blue O labeled with ^{125}I. Using an assay method that allowed the measurement of tissue radioactivity without extraction, they found the concentrations of radioactivity in the pancreas, liver, and kidneys to be similar for up to 60 min after intravenous administration. Likewise the parathyroid was observed to have concentrations of radioactivity similar to those found in the thyroid gland. They concluded that iodinated toluidine blue O was an unsatisfactory scanning agent for the pancreas and parathyroid glands.

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Preliminary work in this laboratory confirmed the results of Klopper and Moe (1); 30 min after the intravenous administration of toluidine blue O, extracts of the pancreas had 10 times the concentration of dye compared to extracts of the liver when measured colorimetrically (12). In a preliminary investigation of the distribution of ^{35}S-labeled toluidine blue O (12), concentrations of radioactivity in the pancreas, liver, and kidneys were found to be similar. These results seemed to confirm the conclusions of Larose *et al.* (10) and Archer *et al.* (11) that labeled derivatives of toluidine blue O were unsatisfactory pancreas scanning agents.

One possible explanation of the blue coloration of the pancreas consistent with these results is that the dye molecule may be

driasis was primarily a peripheral anticholinergic response since both the tertiary and quaternary derivatives of atropine and scopolamine were potent inducers of mydriasis. It also was concluded that inhibition of physostigmine-induced lethality was primarily a measure of central anticholinergic activity since the quaternary derivatives were much less potent inhibitors of physostigmine-induced lethality than their respective tertiary forms.

A careful evaluation of a drug's relative activity in these two procedures in the same species, the induction of mydriasis and the inhibition of physostigmine lethality, should predict its relative activity as a central and a peripheral anticholinergic in humans.

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Tissue Distribution of *N*-^{14}C-Azure C (Methylthionine) in the Rat

R. ENG, S. LINDBERG, K. WHITE, and L. A. SPITZNAGLE [x]

Abstract □ The distribution of radioactivity at 5-, 10-, and 15-min intervals following the intravenous administration of *N*-^{14}C-azure C was determined. The concentrations of radioactivity observed indicated that radioactive derivatives of azure C would not be useful pancreas or parathyroid scanning agents.

Keyphrases □ Azure C, radiolabeled—tissue distribution, considerations related to use as pancreas or parathyroid scanning agent □ Methylthionine, radiolabeled—tissue distribution, considerations related to use as pancreas or parathyroid scanning agent □ Scanning agents—tissue distribution of radiolabeled azure C

The report (1) that the intravenous administration of the phenothiazine dye tolonium chloride (toluidine blue O) caused a blue coloration of the pancreas and parathyroid glands but not of surrounding organs generated much interest and has been verified in experimental animals (2–4) and humans (5–7). Toluidine blue O has been used for the identification of the parathyroid gland at surgery (5–7) and as an aid in the diagnosis of small oral cancers (8), and it has been suggested as a potential parathyroid and pancreas scanning agent if labeled with a suitable radionuclide (2–4, 6, 7). Recent studies with an iodinated analog of toluidine blue O labeled with ^{131}I showed it to be useful for imaging parathyroid adenomas (9).

DISCUSSION

Kang and DiGiulio (3) found the highest concentrations of toluidine blue O in the parathyroids, heart, pancreas, kidneys, stomach, lungs, thyroid, muscles, liver, and blood, in that order. They extracted the excised tissues with ethanol and measured the dye concentrations colorimetrically. Mortenson and McRae (2) reported similar results using a similar assay. In both studies, rather low concentrations were observed in the organs surrounding the pancreas, suggesting the development of a pancreas scanning agent provided a suitable labeled derivative could be prepared.

Larose *et al.* (10) studied the distribution in rats of an iodinated derivative of toluidine blue O labeled with ^{125}I. Using an assay method that allowed the measurement of tissue radioactivity without extraction, they found the concentrations of radioactivity in the pancreas, liver, and kidneys to be similar for up to 60 min after intravenous administration. Likewise the parathyroid was observed to have concentrations of radioactivity similar to those found in the thyroid gland. They concluded that iodinated toluidine blue O was an unsatisfactory scanning agent for the pancreas and parathyroid glands.

Archer *et al.* (11) also studied the tissue distribution of a ^{125}I-labeled derivative of toluidine blue O. However, they extracted the tissues with ethanol and measured the radioactivity of the extracts. Their results differed from those of Larose *et al.* (10) in that the parathyroid extract contained approximately three times the concentration of extractable radioactivity as did the thyroid. The pancreas extract contained only a slightly higher concentration of radioactivity than the liver extract, indicating that iodinated toluidine blue O was an unsatisfactory scanning agent for the pancreas. However, these investigators observed that the percentage of the injected dose taken up by the pancreas was much greater when unlabeled toluidine blue O was given instead of iodinated toluidine blue O.

Preliminary work in this laboratory confirmed the results of Klopper and Moe (1); 30 min after the intravenous administration of toluidine blue O, extracts of the pancreas had 10 times the concentration of dye compared to extracts of the liver when measured colorimetrically (12). In a preliminary investigation of the distribution of ^{35}S-labeled toluidine blue O (12), concentrations of radioactivity in the pancreas, liver, and kidneys were found to be similar. These results seemed to confirm the conclusions of Larose *et al.* (10) and Archer *et al.* (11) that labeled derivatives of toluidine blue O were unsatisfactory pancreas scanning agents.

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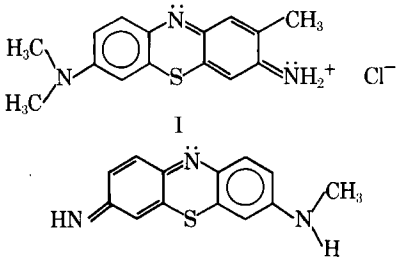

I

II

"decolorized" by the liver and kidneys but not by the pancreas.

An observation in this laboratory that toluidine blue O can easily be *N*-demethylated (13) suggested that the data might be explained by *N*-demethylation of toluidine blue O in the liver and kidneys but not in the pancreas.

If both *N*-methyl groups were removed from toluidine blue O (I) in the liver and the kidneys but not in the pancreas, then the resulting dye (2-methylthionine) would probably not be visible when the viscera was observed, nor could the concentration of the dye be measured accurately at 625 nm. Therefore, the distribution of a dye molecule labeled in the *N*-methyl group, rather than in the phenothiazine ring, was of interest. If such a dye were rapidly *N*-demethylated by the liver and kidneys but not the pancreas, it might be possible to produce a pancreas scanning agent by labeling such a dye with an ^{11}C-*N*-methyl group.

Since toluidine blue O contains two amino groups that are not symmetrical, it would be difficult to label the *N*-methyl groups. Therefore, the distribution of azure C (*N*-methylthionine, II), an aminophenothiazine dye with similar chemical properties to toluidine blue O which can easily be labeled with ^{14}C in the *N*-methyl group, was studied.

The purpose of the present study was to determine if the concentration of radioactivity in the pancreas and parathyroid glands following the administration of an aminophenothiazine dye labeled in the *N*-methyl group would be sufficient to warrant further efforts to develop pancreas and parathyroid scanning agents from such dyes.

EXPERIMENTAL

Chemicals—The dyes[1] were labeled 80–90% pure. All other chemicals were reagent grade and were used as received.

^{14}C-Azure C was prepared as previously reported (14), and TLC indicated the radiochemical purity to be greater than 89%. The dye was dissolved in water prior to injection.

Animals—The animals used were Sprague–Dawley rats, 200–250 g, and they were maintained on rat chow and water *ad libitum* prior to use.

Tissue Distribution—The animals were anesthetized with pentobarbital sodium, and 5 μCi of ^{14}C-azure C was injected *via* an exposed femoral vein. The animals were sacrificed after 5, 10, or 15 min. Thyroid, parathyroids, heart, pancreas, adrenals, liver, kidneys, spleen, stomach, and a sample of blood were removed as rapidly as possible. The organs were frozen immediately in dry ice to prevent dehydration. The tissue samples were weighed while still frozen.

Tissue samples too large to be dissolved directly were minced with a razor blade and then homogenized[2] in distilled water equal to the tissue weight. The tissues and aliquots of the tissue homogenates were dissolved by heating at 50–60° in liquid scintillation counting vials containing 1 ml of a solubilizer[3]. The dissolved samples were decolorized by slowly adding 30% H_2O_2. The dissolved and decolorized samples were then counted[4] at 5°. The sample counts were corrected for quenching by the external standard ratio method, which had been shown to give a linear relationship between external standard ratio and counting efficiency over the range of counting efficiencies obtained by this method of sample preparation.

[1] National Aniline and Eastman Kodak.

[2] Polytron homogenizer, Brinkmann Instruments, Westbury, N.Y.

[3] Protosol, Packard Instrumental Co., Des Plaines, Ill.

[4] In a Beckman LS-230 liquid scintillation counter, using Aquasol (New England Nuclear, Boston, MA 62118) as the counting solution.

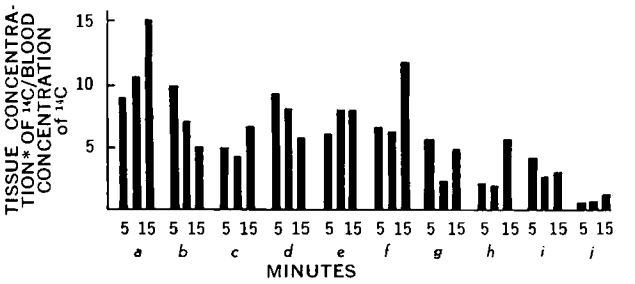

Figure 1—*Tissue distribution of ^{14}C activity at 5, 10, and 15 min after the intravenous administration of ^{14}C-azure C. (*Average from six animals at each time.)* Key: a, *adrenal;* b, *parathyroids;* c, *thyroid;* d, *lung;* e, *liver;* f, *kidney;* g, *heart;* h, *stomach;* i, *pancreas; and* j, *muscle.*

RESULTS

The ratios of tissue–blood radioactivity 5, 10, and 15 min after the intravenous administration of ^{14}C-azure C are shown in Fig. 1. The distribution of radioactivity is similar to that found after the administration of ^{35}S-toluidine blue O (12) or an ^{125}I-labeled analog of toluidine blue O (10). These results indicate that the blue coloration of the pancreas observed after administration of azure C is probably not due to rapid demethylation of the dye in the liver and kidneys. These results, when considered with the previous data obtained with toluidine blue (2, 3, 10–12), suggest that the pancreas turns blue after the dye administration because of its thickness and translucent appearance in comparison with surrounding organs. The results also indicate that ^{11}C-labeled azure C would not be a good parathyroid or pancreas scanning agent.

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Amino Acid Esters of Phenolic Drugs as Potentially Useful Prodrugs

Keyphrases □ Phenolic drugs—use of amino acid esters as prodrugs □ Amino acid esters of phenolic drugs—use as prodrugs □ Prodrugs—consideration of amino acid esters of phenolic drugs

To the Editor:

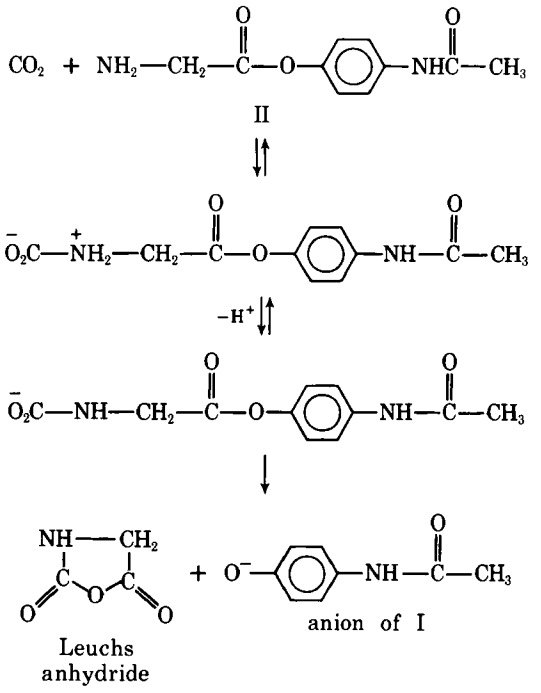
It is sometimes desirable to make temporary chemical modifications to drug molecules for the purpose of increasing their chemical stability, of changing their solubility or rate of dissolution, or of modifying their distribution within the body. If such chemically modified drugs are transformed to the parent drug before they elicit a pharmacological response, they are referred to as prodrugs (1).

It is frequently desirable to formulate phenolic drugs as prodrugs, because many of them have low water solubility as neutral molecules and they are all subject to oxidative degradations *in vitro* and conjugative metabolism *in vivo*. Amino acid esters of phenolic drugs appear to be attractive candidates for consideration as prodrugs for the following reasons:

1. Prodrugs with either higher or lower water solubility can be made following appropriate selection of the amino acid. For example, the aqueous solubilities at 25° of acetaminophen (*p*-acetamidophenol) (I), the hydrobromide salt of its glycine ester (II), and the hydrochloride salt of its β-aspartic acid ester (III) were calculated to be 0.10, 0.48, and 0.023 *M*, respectively.

2. Considerable differences in the stability against hydrolysis have been found for different amino acid esters of a particular phenol. Hence, the $t_{1/2}$ values (half-lives) for hydrolysis of II and III and for the α-aspartic acid ester of I (IV) in water at pH 7.0 and 25° were 49, 155, and 2.65 min, respectively. The $t_{1/2}$ values at pH 2.0 were 1850, 2850, and 76 min, respectively. These half-lives were observed when no general acids or bases or other catalytic species were present in the solution.

3. The hydrolyses of amino acid esters of phenols are likely to be catalyzed by general bases and nucleophilic species; even in the absence of any enzyme-facilitated hydrolysis, the hydrolyses are likely to be rapid within the body to yield the phenolic parent drug. The hydrolysis of the ester II was subject to general base and nucleophilic catalysis, and the catalytic rate constants (assuming that the rate-determining step of the reaction involves one molecule of the protonated form of the ester and one molecule of general base or nucleophilic species) were calculated to be $2.04 \times 10^{-3}\ M^{-1}\ min^{-1}$ for formate ion, $5.80 \times 10^{-3}\ M^{-1}\ min^{-1}$ for acetate ion, $120 \times 10^{-3}\ M^{-1}\ min^{-1}$ for dihydrogen phosphate ion, and $4120 \times 10^{-3}\ M^{-1}\ min^{-1}$ for tromethamine.



Scheme I

The rate laws for hydrolysis in glycine and tromethamine buffers also included a term that was second order in the amine. In addition, the rates of hydrolysis were dramatically catalyzed by dissolved carbon dioxide. Whereas the $t_{1/2}$ value for the hydrolysis of II in a $5 \times 10^{-2}\ M$ tromethamine buffer (pH 7.44) at 25° was 7.7 min, the $t_{1/2}$ value in a $2 \times 10^{-3}\ M$ solution of sodium bicarbonate in the same buffer (pH 7.44) was 4.9 min—the reaction rate constant had increased by more than 50%. Blood and other body fluids contain similar or larger concentrations of dissolved carbonic acid species (2). At pH 7.44, 6.75, and 6.00, the apparent catalytic rate constants for the total concentration of carbonic acid species (H_2CO_3, HCO_3^-, and CO_3^{-2}) were 23.07, 7.32, and 0.96 $M^{-1}\ min^{-1}$, respectively. These data can best be rationalized by postulating that carbon dioxide catalyzes the reactions *via* the sequence of reactions shown in Scheme I.

This reaction sequence is similar to one postulated by Hay and Main (3) to account for the effects of carbon dioxide on the hydrolysis of α-amino acid esters of *p*-nitrophenols. The only difference is that whereas Hay and Main concluded that the formation of the carbamate was the sole rate-determining step for hydrolysis of *p*-nitrophenol esters, the reactions of II can best be rationalized by concluding that both this step and the step that results in the formation of the Leuchs anhydride are rate determining.

The rate of hydrolysis of II was significantly increased over what was found in water at 25° when the solution temperature was increased to 37° and rabbit blood plasma was added to the solution. In a 3×10^{-2} *M* solution of tromethamine (pH 7.40) to which 1 and 3% blood plasma had been added, the $t_{1/2}$ values were

14 and 4.9 min, respectively, at 37°. Catalysis in these solutions is probably the result of a combination of general base, nucleophilic, carbon dioxide, and even some enzyme catalysis. Therefore, it is expected that many amino acid esters of phenolic drugs would be rapidly tranformed to the drug under *in vivo* conditions.

4. Many amino acids are normal dietary constituents or are substances with little toxicity. Thus, the compound formed along with the drug when the prodrug is hydrolyzed is unlikely to cause toxic reactions.

Detailed results of a kinetic study of the reactions of II, III, and IV in aqueous solutions together with some mechanistic considerations will be published subsequently.

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Phenol Formation from Alkylparabens by Bacteria

Keyphrases ◻ Phenol—formation from alkylparabens by bacteria ◻ Parabens, alkyl—role in formation of phenol by bacteria ◻ Preservatives—phenol formation from alkylparabens by bacteria

To the Editor:

Alkylparabens are widely used as preservatives for various pharmaceutical preparations such as injections, solutions, emulsions, and suspensions. They are also used in cosmetic preparations containing various fats and oils susceptible to microbial attack. Alkylparabens are also involved in gelatin preparation from animal sources to prevent proliferation of contaminated bacteria.

When a paste of gelatin containing alkylparabens was incubated at room temperature, we found that phenol formation was followed by the liquefaction of gelatin.

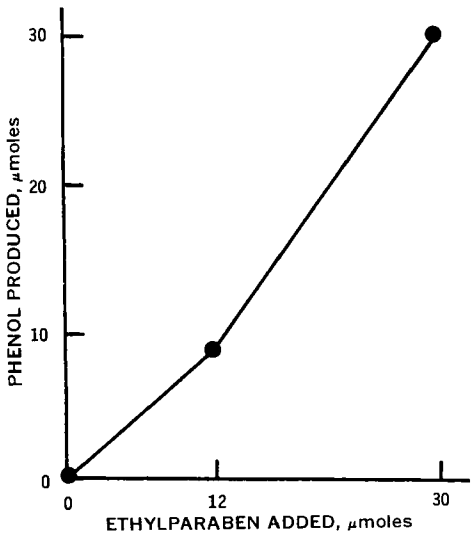

Figure 1—*Effects of various levels of ethylparaben on phenol formation, using resting cells as the enzyme source. Reaction mixtures contained ethylparaben and 7.6 × 10^{10} cells in a final volume of 12 ml. The mixed cell suspension containing strains 3, 4, and 5 was added. Reaction mixtures were incubated at 37° without shaking for about 110 hr. Viable cell counts in the reaction mixtures after incubation at the lowest and highest levels were estimated as 2.9 × 10^9 and 1.7 × 10^9, respectively. Each experiment was carried out with two flasks.*

With the advancement of the Good Manufacturing Practices, microbial contamination, even in nonsterile drugs in either final products or raw materials, has become of concern for the quality control of pharmaceutical manufacturing processes. This communication is concerned with the finding that alkylparabens are decomposed to phenol by the cooperative action of two different bacteria.

Three microorganisms were isolated from the pharmaceutical manufacturing processes using gelatin; they were identified as *Klebsiella aerogenes* (strain 3), *Pseudomonas aeruginosa* (strain 4), and *Pseudomonas aeruginosa* (strain 5). They are able to grow in a gelatin paste containing alkylparabens.

Each microorganism was grown on soybean casein digest agar slant medium (1) for 18 hr at 37° without shaking. The grown cells were harvested aseptically by centrifugation, washed once with sterile water, and suspended in sterile water. The cell suspensions thus prepared were kept in a refrigerator until use and were used for the following experiments.

Counting of viable cells was carried out by means of the plate method, using soybean casein digest agar as the test medium as described in USP XVIII (1). Various alkylparabens[1] and *p*-hydroxybenzoic acid[2] were obtained commercially. Other reagents used were of the best quality commercially available. TLC[3] was carried out according to the methods described by Sadamatsu *et al.* (2). Phenol was determined by literature methods (3, 4).

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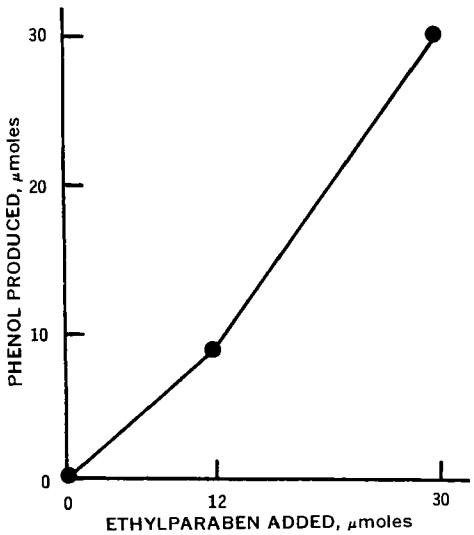

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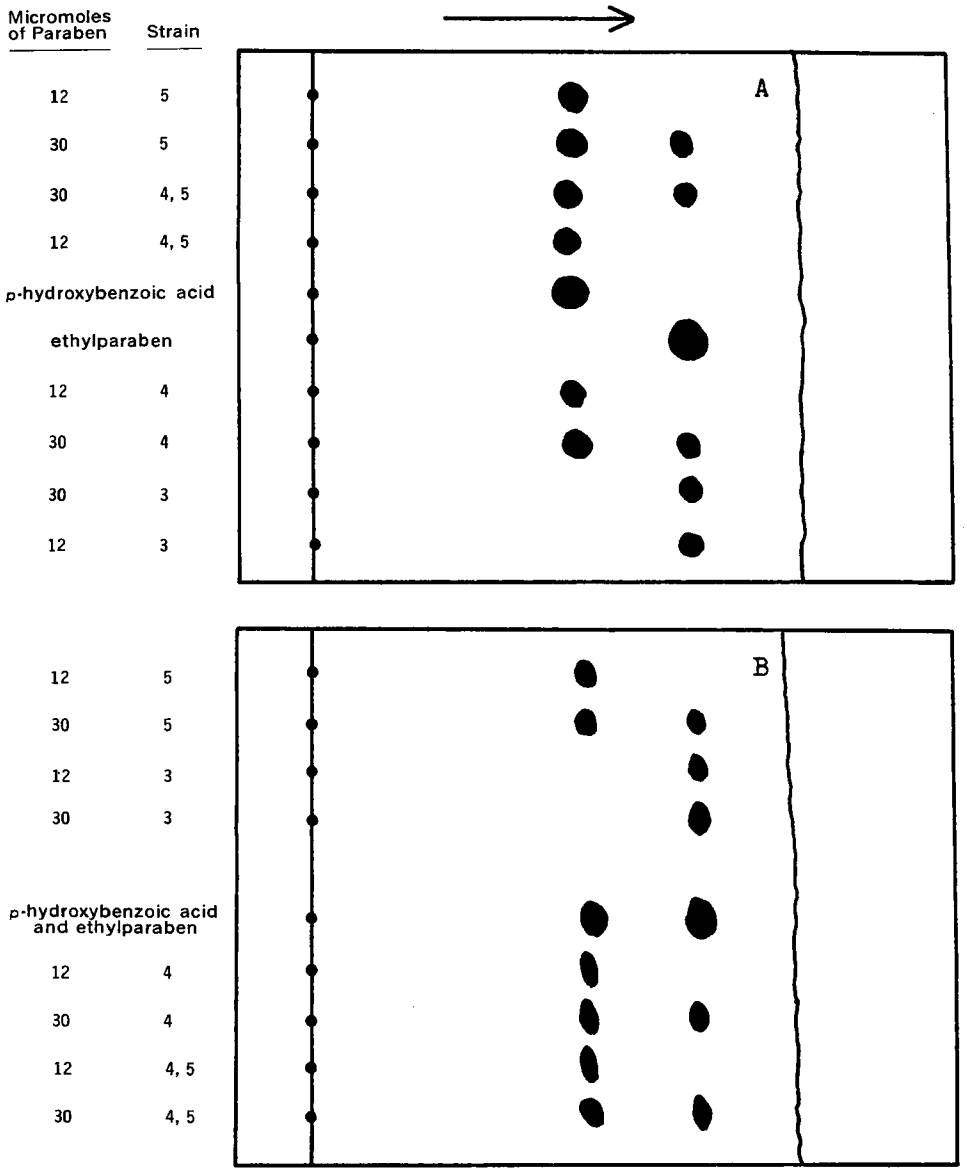


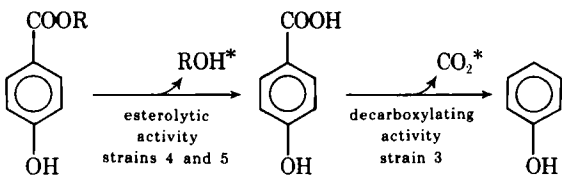
Figure 2—*TLC identification of esterolytic activity formed by strains 3 and 5. Reactions were carried out in the same manner as described in Table I. Developing solvents used were: A, chloroform–propionic acid (1:4); and B, benzene–dioxane–acetic acid (90:25:4). Spots were detected with a UV lamp.*

When the mixed cell suspensions of strains 3, 4, and 5 were added to the sterilized ethylparaben solution and incubated at 37° for about 110 hr, phenol was formed, depending on the amount of ethylparaben added (Fig. 1). Table I shows that neither strain 3, 4, nor 5 was capable of forming phenol from ethylparaben, but strain 3 was able to form phenol from *p*-hydroxybenzoic acid. It was also able to produce phenol from ethylparaben in combination with strains 4 and/or 5. Phenol was also formed from methyl-, propyl-, and butylparabens under the same conditions.

TLC patterns of the reaction mixture (Fig. 2) showed that *p*-hydroxybenzoic acid was formed from ethylparaben with strains 4 and/or 5, whereas ethylparaben remained unchanged with strain 3.

These findings can be explained if it is assumed (Scheme I) that both strains 4 and 5 have esterolytic activity and that strain 3 contains a decarboxylating system. It is also suggested that the rather rapid hydrolysis of alkylparabens by esterolytic activity of either strain 4 or 5 favors the survival of strain 3, which plays the leading role in the phenol formation from alkylparabens *via* *p*-hydroxybenzoic acid.

Previous studies (5) showed that *K. aerogenes* can form phenol from *p*-hydroxybenzoic acid, suggesting the presence of a decarboxylase system in the microorganisms. On the other hand, tyrosine phenol lyase



*Scheme I—Speculated mechanisms of phenol formation from alkylparabens (R = methyl, ethyl, propyl, and butyl; * = speculated)*

Table I—Effects of Various Parabens and *p*-Hydroxybenzoic Acid on Phenol Formation in the Presence of Single or Combined Cell Suspension as Enzyme Source[a]

Microorganisms Added (Strain)	Substrate, 12 μmoles (1.2 m*M*)					
	No Substrate	*p*-Hydroxybenzoic Acid	Methylparaben	Ethylparaben	Propylparaben	Butylparaben
3	0	11.4 12.2	—	0	—	—
4	0	0	—	0	—	—
5	0	0	—	0	—	—
3 and 4	0	7.4 10.0	—	9.4 9.0	—	—
3 and 5	0	9.9 9.7	—	9.4 9.3	—	—
4 and 5	0	0	—	0	—	—
3, 4, and 5	0	8.3 9.8	8.5 9.7	9.1 9.9	8.8 9.6	8.9 9.3

[a] Each figure in the table shows the amount of phenol formed (micromoles). Reaction conditions were those given in Fig. 1, except that each viable cell count of strains 3, 4, and 5 added was 5.9×10^{10}, 1.3×10^{10}, and 2.1×10^{10}, respectively, in a final volume of 10 ml.

has also been studied intensively by many investigators (6–10), who found that it catalyzes direct splitting of alanine from tyrosine to form phenol. In our studies with tyrosine as the substrate in place of alkylparabens, however, phenol was not produced.

Various alkylparabens are used or prescribed to prevent proliferation of contaminated microorganisms in pharmaceutical preparations. However, *P. aeruginosa* (NCTC 7244) was found to utilize alkylparabens as a carbon source and to grow well in an eye drop solution containing them (11). Furthermore, as described in the present communication, microorganisms such as strain 3 *(K. aerogenes)* metabolize *p*-hydroxybenzoic acid into phenol under restricted nutritional conditions.

Pseudomonas or *Klebsiella* species and other organisms have been isolated from various nonsterile pharmaceutical or cosmetic preparations and the production environment (12). It also has been shown that serious spoilage of drugs is brought about by these microorganisms. Although the mechanism of deterioration has not been elucidated from the standpoint of bacteriological metabolism, this communication presents a typical example concerning drug deterioration by microorganisms.

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Antiviral Activity of Gossypol and Apogossypol

Keyphrases □ Gossypol—screened for antiviral activity □ Apogossypol—screened for antiviral activity □ Antiviral activity—gossypol and apogossypol

To the Editor:

The pigment gland of the cotton seed contains an array of organic compounds including gossypol (I), which has been demonstrated to have antibacterial (1), antiviral (2–4), and antitumor (5) activities as well as mammalian toxicity (6, 7). Apogossypol (II), which has lower mammalian toxicity, is formed by deformylation of gossypol with base (8). The following report of preliminary findings establishes that apogossypol retains the potent antiviral activity of gossypol.

Gossypol can inactivate influenza virus infectivity *in vitro*, and influenza virus inactivated by treatment with gossypol was given to mice with a resultant 96–100% protection rate (2–4). Since little is known about either the mechanism of action or the activity spectrum of gossypol, an investigation of the *in vitro* inactivation of viruses by this compound and the less toxic apogossypol was initiated.

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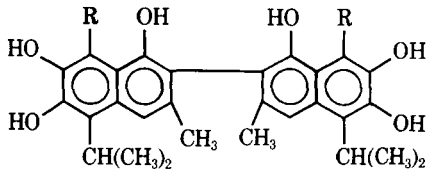

I: R = CHO
II: R = H

The toxicity of gossypol[1] and apogossypol[1] for HEp-2 cell cultures was determined to delineate the concentrations of gossypol that could be utilized in the antiviral assays. The HEp-2 cell cultures were incubated for 1 hr with varying concentrations (0.5–20 μg/ml) of gossypol in Hank's balanced salt solution; this solution then was removed and replaced with maintenance medium (Eagle's minimum essential medium containing 2% heat inactivated fetal calf serum). The cell cultures were observed for cytopathic effect and cell viability.

Gossypol concentrations of 5 μg/ml (9.6×10^{-6} *M*) and lower were not toxic for the HEp-2 cells, whereas cells incubated with gossypol at concentrations of 10 μg/ml and higher exhibited a rapid toxic effect accompanied by a decrease in cell viability. Under these conditions, apogossypol gave no indication of toxicity to HEp-2 cells at a concentration of 50 μg/ml (1.14×10^{-4} *M*).

The antiviral activity of gossypol was assayed against polio virus, parainfluenza-3 virus, and herpes simplex virus, and the antiviral activity of apogossypol was determined against poliovirus and herpes simplex. Virus suspensions in Hank's balanced salt solution were incubated for 30 min at 25° with gossypol or apogossypol; samples then were removed at intervals and assayed for infectivity by determining the number of plaque-forming units per milliliter (10). Parainfluenza-3 and herpes simplex were inactivated by incubation with apogossypol; however, there was no apparent activity exerted against poliovirus by either agent. As shown in Fig. 1, the potency of apogossypol against herpes simplex is somewhat less than that of gossypol.

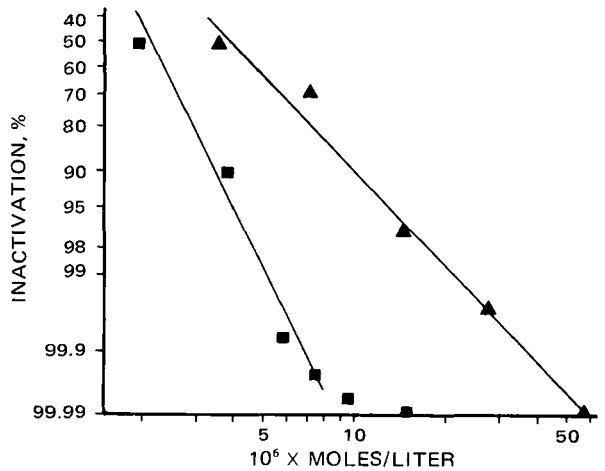

Figure 1—*Dose–response curves for gossypol (■) and apogossypol (▲). Data for gossypol are averages of two independent determinations (three replicates per determination). Data for apogossypol are averages of four independent determinations (three replicates per determination).*

Although the compounds have similar structures, the nature of the viral inactivation differs. This difference is reflected by the different slopes of the dose–response lines and the ability to inactivate the virus totally. Apogossypol can inactivate the virus completely (no plaque-forming units observed) at concentrations above 12.5 μg/ml (2.86×10^{-5} *M*) with no apparent toxicity to the HEp-2 cell cultures. Gossypol can decrease the number of plaque-forming units by a factor of 10^5, but the concentration required is also toxic to the HEp-2 cells. This toxicity probably accounts for the nonlinearity of the dose–response line at gossypol concentrations greater than 10^{-5} *M*.

Since the host cell would tolerate concentrations of gossypol and apogossypol that were antiviral *in vitro*, experiments were performed to determine whether treatment with gossypol could affect intracellular replication. Cell monolayers were infected with herpes simplex to produce approximately 100 plaques/plate. At the indicated intervals, 1 ml of Hank's balanced salt solution containing 5 μg of gossypol was added to each of three infected monolayers and incubated at 25° for 1 hr. Figure 2 shows that the antiviral effect of gossypol was apparent only during the initial stages of adsorption. Incubation with gossypol did not alter the result of infection after the virus entered the cell[2]. Similar results were observed using apogossypol in a concentration of 25 μg/ml.

These experiments demonstrate that:

1. Apogossypol is considerably (at least 10-fold) less toxic to HEp-2 cells in culture than is gossypol.
2. Apogossypol and gossypol both inactivate the enveloped virus parainfluenza-3 and herpes simplex *in vitro*.
3. The nonenveloped poliovirus is not affected by either agent.
4. Incubation of infected cells with gossypol or apogossypol does not alter subsequent plaque formation, indicating that the antiviral effect does not occur within the cell.

The molecular mechanism of viral inactivation by these compounds has not been determined. The presence of the reactive aldehyde substituents, which might react with free amino groups of proteins, has been postulated to be of importance in the biological activity of gossypol, including the mammalian toxicity (6). Likewise, previous investigators showed that gossypol inhibits the conversion of pepsinogen to pepsin by forming an imine with the ε-amino group of a lysine residue in pepsinogen (11–13). While a simi-

[1] Samples of gossypol (as the acetic acid complex) were provided by Dr. Walter Pons of the U.S. Department of Agriculture and by Dr. Harold Lewis of Cotton Inc. These samples were converted to free gossypol and recrystallized three times from chloroform, mp 199–200° [lit. (9) 200°]. Apogossypol was synthesized by the method of Clark (8) and stored in sealed (vacuum) tubes until assayed.

[2] The time required for the virus to enter the cell was established in a similar experiment using antiherpes serum rather than gossypol or apogossypol to inactivate the virus.

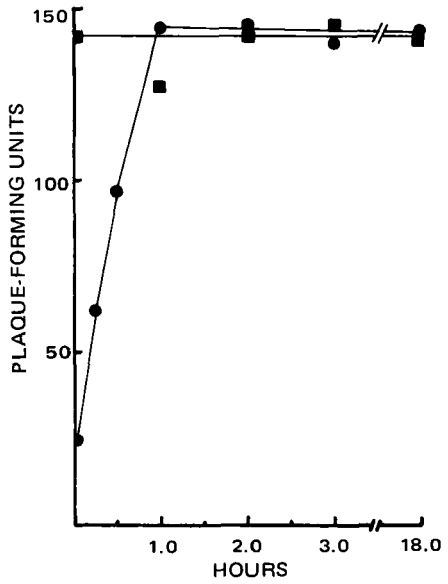

Figure 2—*Plot of time between inoculation with herpes simplex and treatment with gossypol (5 μg/ml)* versus *the plaque-forming units observed. Key:* ■, *control; and* ●, *gossypol treated.*

lar mechanism may be operative in viral inactivation, it does not appear likely due to the good activity of apogossypol.

An alternative mechanism of antiviral activity could be that these compounds bind to the virion envelope and cause the subsequent destruction of the integrity or loss of the envelope. If the membrane is the site of action, the basis for the selective toxicity to the viral particle might result from the structural differences between the envelope membrane of herpes and the outer membrane of the host cell (14). An alternative basis of the selective toxicity to the viral particle might be that the host cell has the capacity to repair membrane damage or inactivate gossypol and apogossypol. The viral particle, which is metabolically dormant, would not be expected to have either of these capabilities.

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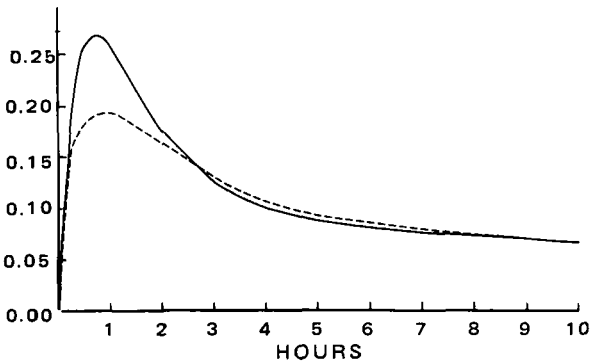
Demonstration of Claimed "Controlled-Release" Properties of a Drug Formulation

Keyphrases □ Controlled-release properties of drug formulations—claimed *versus* actual, blood level profiles compared to "plain-release" blood level profiles □ Timed-release formulations—claimed *versus* actual "controlled-release" properties, blood level profiles compared to "plain-release" blood level profiles

To the Editor:

The concept of a "controlled-release" formulation of a drug is, by now, a familiar one. The basic idea is that of delaying or prolonging the *in vivo* release of an orally administered drug. Controlled-release formulations are often termed "slow release," "sustained release," or "delayed release." The advantages of such formulations are obvious; a sustained-release formulation, for example, which prolongs blood levels over a greater period and eliminates high peaks of drug concentration, allows the possibilities of less frequent dosing and the elimination of side effects related to peaks in the blood level profile.

Two questions might reasonably be asked concerning a purportedly controlled-release formulation. First, is it efficacious in the dosage schedule recom-



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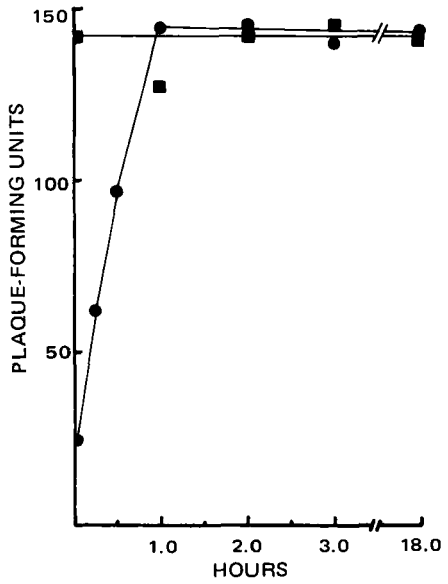

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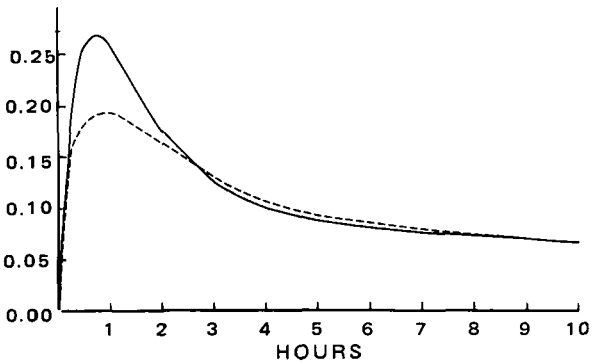

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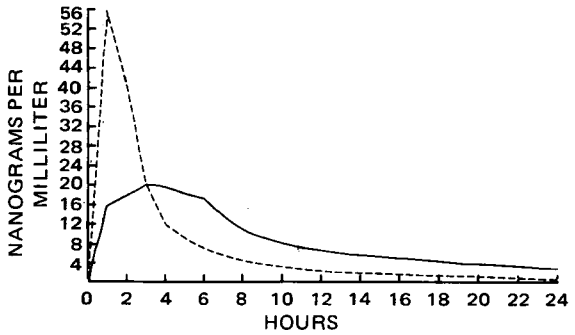

Figure 2—*Blood level curves from a 40-mg plain-release tablet of caramiphen (- - -) and from a 40-mg controlled-release formulation of caramiphen (—).*

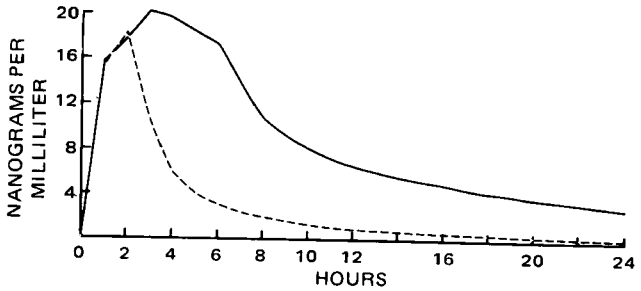

Figure 4—*Blood level curve from a 40-mg controlled-release formulation of caramiphen (—) and predicted blood level curve from a regimen of 11 mg of caramiphen at zero hr and 5 mg at 1 hr, both in plain-release tablets (- - -).*

mended? And second, does it indeed have the property of controlled release claimed for it? The fact that a once-a-day administration of a certain amount of drug in a controlled-release formulation proves to be as efficacious as a conventional regimen of the same amount of drug given in three divided doses taken over 24 hr is, in itself, no proof of controlled release. Figure 1 should make this clear. The single, plain dose might be highly effective therapeutically but never administered in this fashion because of, for example, the intolerable side effects associated with the high peak blood level. The purportedly controlled-release formulation might also be effective therapeutically in a once-a-day regimen without the accompaniment of side effects. However, one could hardly claim the controlled-release property for it on the basis of the blood level profile shown in Fig. 1. What appears to have happened is that the formulation simply makes the drug less available at the critical point where side effects might occur.

Apparently, the efficacy of a purportedly controlled-release formulation in the proposed regimen and its controlled-release character are independent questions requiring their own proof. Establishment of either one cannot be used to infer the presence of the other; the proof that a formulation possesses a controlled-release characteristic must be tackled directly. For example, if the drug in question generates a pharmacological response that can be measured with reasonable precision, a study measuring this response can be made the basis of a demonstration of the controlled-release property. In many cases, however, the most convenient approach will be through a pharmacokinetic study. A number of pharmacokinetic methods can be devised to demonstrate the controlled-release of a formulation; one that seems to represent the most generally satisfactory approach to the problem will be described here.

The method proposed is that of determining the regimen of divided doses to which the controlled-release formulation is equivalent in the sense that it generates an equivalent blood level profile over time. To illustrate the method, consider the blood level profiles (based on the mean from a number of subjects) obtained with two formulations of caramiphen: a 40-mg plain-release tablet and a 40-mg controlled-release formulation. Linear pharmacokinetics are assumed so that the predicted blood levels from any dose of caramiphen in the plain-release tablet can be obtained from the dotted line in Fig. 2 by scaling up or down by the appropriate factor.

One then determines what dose of plain-release drug given at $t = 0$ appears to match the beginning of the controlled-release blood level curve. Scaling the dotted line curve in Fig. 2, one finds that 11 mg given in a plain-release tablet appears to be a good match (Fig. 3). To match further the controlled-release blood level curve, one then finds that 5 mg in a plain-release form appears to be needed at 1 hr (Fig. 4). Further doses are added where required to match the controlled-release blood level curve until the allotted 40-mg dose is used up. The only principles involved are those of linear pharmacokinetics, involving scal-

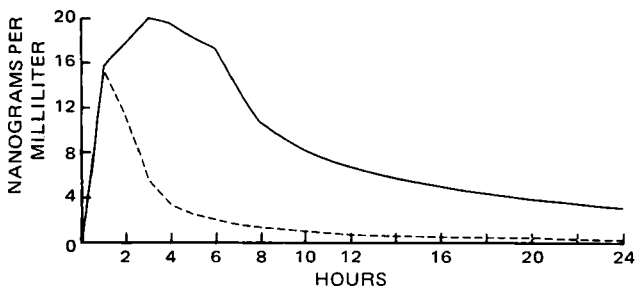

Figure 3—*Blood level curve from a 40-mg controlled-release formulation of caramiphen (—) and predicted blood level curve from 11 mg of caramiphen in a plain-release tablet administered at zero hr (- - -).*

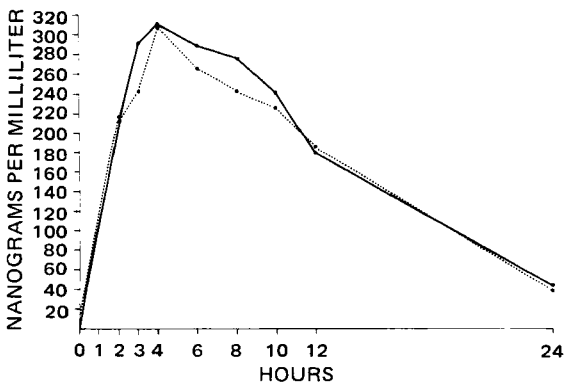

Figure 5—*Blood level curves from a 150-mg controlled-release formulation of phenylpropanolamine (—) and from a regimen totaling 150 mg of phenylpropanolamine given in various doses at 0, 1, 2, 3, 5, 6, and 7 hr (- - -).*

ing of curves, superpositioning, and adding of superposed curves.

Of course, one could fit a compartment model and proceed in a similar manner, but I have already expressed a preference for using simple empirical techniques (1). What is required to achieve the curve matching expeditiously is a small digital computer with an oscilloscope-type display[1]. One sets up the controlled-release blood level curve as background on the oscilloscope and then tries to match it by trial and error, which usually requires 5–10 min.

At this point, by *theoretical* pharmacokinetic computations, a regimen of plain-release doses has been derived which is predicted to match the controlled-release formulation with respect to its blood level curve. The next step is, of course, to verify the validity of these theoretical calculations with an actual comparative trial in human subjects. Figure 5 shows the actual mean blood level curves of phenylpropanolamine obtained in a crossover trial in 12 subjects. A controlled-release formulation of 150 mg was compared with the following regimen:

hours	dose, mg
0	52.4
1	21.4
2	23.8
3	23.8
5	9.6
6	9.6
7	9.4

This regimen was derived from pharmacokinetic computations of the type described. The blood level curves obtained in Fig. 5 appear to show a good match; apparently this regimen of divided doses does give rise to essentially the same blood level profile as the controlled-release formulation.

The question still exists as to whether the divided dose regimen is sufficiently dispersed over time to merit the title controlled release for the formulation that it appears to mimic. As an extreme example, the controlled-release description would probably not be merited for a formulation that appears to be equivalent to a divided dose regimen of 145 mg at zero hr and 5 mg at 1 hr. Obviously, some arbitrary rules are needed. Nevertheless, the proposed method offers a reasonable way of handling the problem of demonstrating controlled release.

So that no misunderstanding occurs, it should be noted that no claim is made that the divided dose regimen is a model of how the controlled-release formulation behaves *in vivo* but rather that it gives rise to blood levels equivalent to those generated by the controlled-release formulation.

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Antitumor Agents XIV: Molephantinin, a New Potent Antitumor Sesquiterpene Lactone from *Elephantopus mollis*

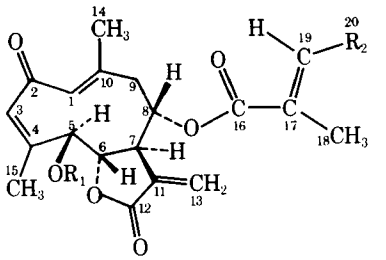
Keyphrases □ *Elephantopus mollis* H.S.K.—isolation and structure determination of molephantinin, antitumor activity □ Molephantinin—isolation, structure determination, and antitumor activity □ Antitumor agents, potential—molephantinin, constituent of *Elephantopus mollis* H.S.K. □ Structure-activity relationships—sesquiterpene lactones as antitumor agents

To the Editor:

We recently reported the isolation and structure determination of two novel cytotoxic germacranolides, molephantin (IV) (1) and phantomolin (2), from *Elephantopus mollis* H.S.K. This report[1] involves the isolation of an additional new sesquiterpene lactone, molephantinin (I), from the winter collection of this same plant. Molephantinin showed significant (T/C ≥ 125%) inhibitory activity against the Walker 256 carcinosarcoma in rats (T/C = 397%) at the 2.5-mg/kg level[2].

Molephantinin was isolated from the mother liquor after the removal of molephantin and phantomolin by silica gel column chromatography. Molephantinin, mp 223–225°, has the composition $C_{20}H_{24}O_6$ and shows an IR spectrum very similar to that of molephantin, indicating the presence of a hydroxy group (3420 cm^{-1}), a γ-lactone (1775 cm^{-1}), and the conjugated enone system (1713 and 1650 cm^{-1}). The NMR spectrum of molephantinin is superimposable with that of molephantin, except for the signal patterns in the acid portion of the ester. These differences were observed in molephantinin as the broad vinyl multiplets at δ 6.92 (1H, H-19) and the vinyl methyl multiplets at δ 1.83 (6H, H-18 and H-20), which are the typical signals for the tigloyl group (5, 6).

Added confirmation for these assignments was obtained by a comparison of the mass spectra of molephantinin and molephantin. The base peak in the mass spectrum of molephantinin is at *m/e* 83, due to



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[1] For Part XIII, see Ref. 3. For Part XII, see Ref. 2.
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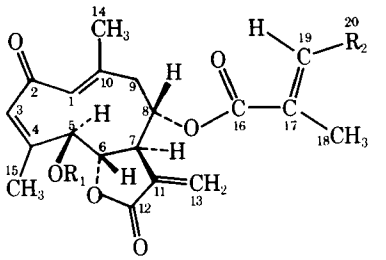

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Extensive NMR decoupling (100 MHz) led to the following assignment of protons, which was consistent with the structure of molephantinin acetate as depicted in II: δ 6.92 (1H, br m, H-19), 6.57 (1H, s, H-5), 6.36 (1H, s, H-1), 6.34 (1H, d, J = 2.5 Hz, H-13), 6.04 (1H, br s, H-3), 5.77 (1H, d, J = 2.0 Hz, H-13), 5.21 (1H, dt, J = 4.0, 10.5 Hz, H-8), 4.34 (1H, d, J = 3.5 Hz, H-6), 3.40 ([H, m, H-7), 2.62 (2H, m, H-9), 2.07 (3H, s, $OCOCH_3$), 1.98 (3H, d, J = 1.5 Hz, H-15), 1.83 (6H, m, H-18 and H-20), and 1.80 (3H, d, J < 1.0 Hz, H-14). For example, irradiation of the multiplet centered at δ 6.92 caused the six-proton multiplet at δ 1.83 to collapse to a singlet. Thus, the multiplet at δ 1.83 could be assigned to the methyl groups at C-18 and C-19.

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The foregoing evidence leads to the formulation of the complete structure of molephantinin as I. Studies on the structure–activity relationships among molephantinin-related sesquiterpene lactones are currently in progress.

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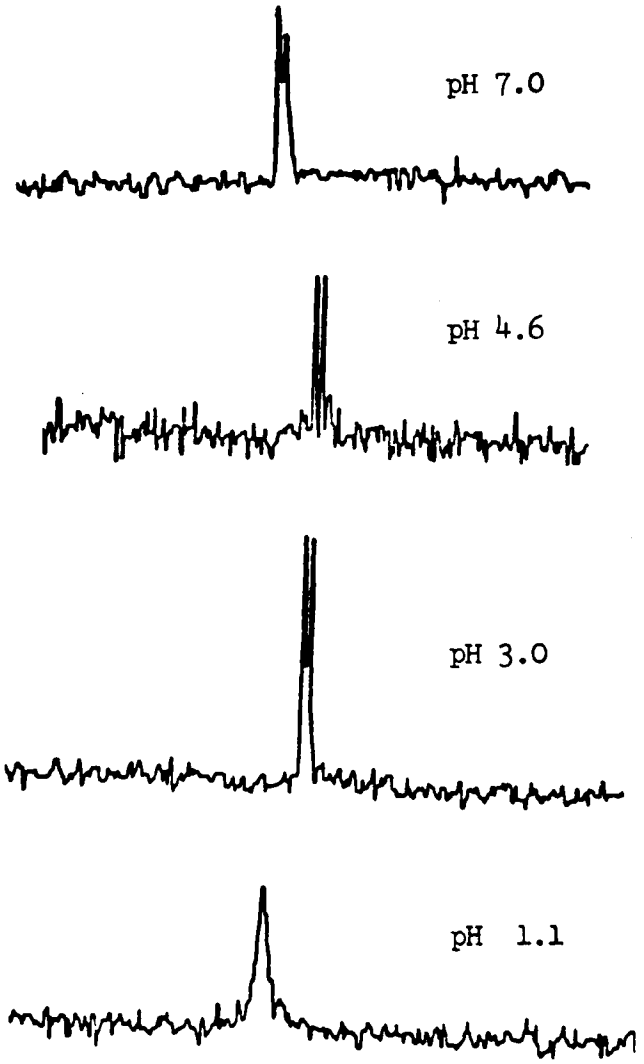

Figure 1—*^{13}C-NMR line shape dependence on pH of aqueous glutaraldehyde (carbonyl region).*

transient signal, such as sensitivity enhancement and digitization, can usually be avoided by peak area integration. However, equal numbers of carbon atoms in different sites can give dissimilar peak intensities due to a differing spin-lattice relaxation time, T_1, and nuclear overhauser enhancement due to proton decoupling (4). Whipple and Ruta (3) used a pulse delay and compared methylene carbon peaks to mitigate this problem. We used a smaller (2 μsec, 13°) pulse width with no delay and peak intensities of the carbons attached to oxygen at 209.2 and 208.4 (free aldehyde) and 91.0–95.2 (hydrated and cyclic forms) ppm.

Our results, at 38° in aqueous solution (with 10% D_2O for the heteronuclear lock[1]), are consistent with those of Whipple and Ruta when differences in temperature are taken into account. Since the relevant carbonyl peaks were of almost equal area, we found twice as much monohydrate as dialdehyde. The total free aldehyde content was equivalent to 28 free aldehyde groups/100 molecules of glutaraldehyde, compared to 24% found by Whipple and Ruta (3) at the lower temperature of 23°. This finding is in agreement with their predictions of the effects of temperature on the equilibria involved.

In the absence of the raw data of King *et al.* (1), we recalculated the proton NMR data of Hardy *et al.* (2), using our dialdehyde–monohydrate ratio of 1:2. We found that their intensity ratios can be adequately explained on this basis to give, on an average, 4% dialdehyde, 8% monohydrate, 54% dihydrate[2], and 34% hemiacetal. These results give about 16% free aldehyde group instead of the 43% calculated by Hardy *et al.* (2), which is in better agreement with the now available ^{13}C-NMR data. The 47% free aldehyde found by King *et al.* (1) would similarly decrease.

Finally, we wish to report preliminary evidence of pH-dependent linewidths in the ^{13}C-NMR spectrum of glutaraldehyde (Fig. 1). Sharp resonances are observed around pH 3–5, while line broadening is evident above and below this range. We attribute this line broadening to an increase in the rate of interconversion of one form into another. Previously, Wolman (5) suggested that the effectiveness of neutral buffered formalin in histological and histochemical work was due to the rapid conversion at neutral and alkaline pH of the polymerized form of HCHO to the monomer. Although this theory met with some skepticism (6), we feel that similar kinetic factors might best explain the pH-dependent activity of glutaraldehyde in the light of our ^{13}C-NMR findings.

Therefore, in view of the insignificance of pH and concentration dependence on the equilibria themselves, we suggest that the rates at which the equilibria are established (or maintained as the active components are depleted *in situ*) are important in considerations of antibacterial activity. This alternative interpretation of the pH dependence of antibacterial activity of commercial glutaraldehyde solutions is being investigated further.

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[1] Varian CFT 20 instrument.

[2] For comparison only with original data. Whipple and Ruta (3) indicated that peaks in this area have been misassigned to the dihydrate and should belong to *cis*- and *trans*-forms of the hemiacetal. This reassignment would not affect the analysis for free aldehyde.

Contact Angles on Human Skin

Keyphrases □ Contact angles—human skin, previously developed equations discussed □ Skin, human—determination of contact angles, equations

To the Editor:

Several reports of contact angle measurements of various liquids on human skin have appeared in recent years (1–4). Although these papers differed in the reported values of the contact angles, especially for water, there was general agreement that human skin behaves as a low energy solid. Rosenberg *et al.* (3) made their measurements on specially cleaned viable and excised skin and calculated the surface energy of skin due to dispersion forces (γ_S^D) using Eq. 1:

$$\cos\theta = \frac{-\gamma_L + 2\sqrt{\gamma_S^D\gamma_L^D} - \pi_S}{\gamma_L - \pi_L} \qquad \text{(Eq. 1)}$$

where θ is the contact angle, γ_L is the surface tension of the liquid, γ_L^D is the dispersion force component of the surface tension of the liquid, π_S is the reduction in surface energy of the solid due to adsorption of vapor from the liquid drop, and π_L is the reduction in surface tension of the liquid due to spreading of the solid. The investigators assumed π_S and π_L to be negligible, as is often done when dealing with low energy surfaces.

Similarly, El-Shimi and Goddard (4) neglected these spreading terms in their analysis of the dispersion force (γ_S^D) and polar (γ_S^P) contributions to the surface energy of skin, using Eq. 2:

$$\cos\theta = \frac{-\gamma_L + \dfrac{4\gamma_S^D\gamma_L^D}{\gamma_S^D + \gamma_L^D} + \dfrac{4\gamma_S^P\gamma_L^P}{\gamma_S^P + \gamma_L^P}}{\gamma_L} \qquad \text{(Eq. 2)}$$

In addition to the symbols previously defined, γ_L^P is the polar contribution to the surface tension of the liquid.

Skin presents a complex surface whose properties are influenced by a variety of endogenous, secreted, and excreted substances. Mono-, di-, and triglycerides and free fatty acids are among the lipids found most abundantly on the skin (5); these substances are known to spread on water, lowering its surface tension significantly (6). According to a recent report, sweat collected from thermally stimulated subjects had surface tension values lower than that of water by about 13–19 dynes/cm, depending on the skin region used (7).

There is, therefore, good reason to think that π_L may have a value other than zero in many cases. The value of π_L will be a function of the liquid, the nature of the skin area, the history (or pretreatment) of the skin sample, and the area of contact between the liquid drop and the skin. If the spreading process is time dependent, then the contact angle will decrease with time, an effect observed by one investigator (1). The large variation found in the contact angle of water on skin (1–4) is probably related to differences in lipid adsorption at both the water–air and skin–water interfaces.

Unfortunately, neither Eq. 1 nor 2 is adequate for quantitating the effect of skin surface lipids on the contact angle. Equation 2 does not contain π_L. Equation 1 does contain π_L, but no provision is made for changes in adsorption at the solid–liquid interface. For liquids whose contact angles are greater than 90°, Eq. 1 predicts that a rise in π_L will cause an increase in the value of the contact angle. Actually, the opposite takes place (8).

Thus, it appears that approaches that have been successful in characterizing other low energy surfaces are of only limited usefulness when applied to skin. More data on the adsorption of surface lipids would be helpful in understanding wetting and spreading on skin.

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(2) H. Schott, *J. Pharm. Sci.*, **60**, 1893(1971).

(3) A. Rosenberg, R. Williams, and G. Cohen, *ibid.*, **62**, 920(1973).

(4) A. El-Shimi and E. D. Goddard, *J. Colloid Interface Sci.*, **48**, 242(1974).

(5) N. Nicolaides, *Science*, **186**, 19(1974).

(6) G. L. Gaines, Jr., "Insoluble Monolayers at Liquid-Gas Interfaces," Interscience, New York, N.Y., 1966, pp. 208–254.

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Variability in System for Automated Determination of Dissolution Rate

Keyphrases □ Dissolution rates—automated determination, analysis of data, variability □ Automated analysis—dissolution rates, analysis of data, variability

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Recently, Johnson *et al.* (1) described a continuous-flow system for the automated determination of dissolution rates. The major obstacle encountered in any continuous system, *i.e.*, assuring the measurement of a clear solution free of extraneous particles which cause turbidity in the solution and cloud the surfaces of the flow cells, was overcome by the incorporation of filter units into the system, utilizing disposable Teflon or polyvinyl inserts.

The authors (1) reported excellent agreement between the dissolution rates obtained by an automated system and by manual measurements. According to these investigators, their instrumentation can

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The authors (1) reported excellent agreement between the dissolution rates obtained by an automated system and by manual measurements. According to these investigators, their instrumentation can

Table I—Range of Individual *In Vitro* Dissolution[a]

	USP—NF[b]				500-mg Levodopa Tablet[c]			
	500-mg Levodopa Tablet		2-mg Benzodiazepine Tablet		USP—NF		Levy	
Minutes	Average, %	Range, %	Average, %	Range, %	Average, %	Range, %	Average, %	Range, %
2.5	48	17	22	21	17	17	16	17
5	85	11	69	11	38	43	30	22
10	97	11	83	9	78	27	53	43
15	99	7	91	12	87	12	67	51
20	100	5	100	11	90	8	73	50
30	100	4	—	—	94	5	81	30

[a] Data from Ref. 1. [b] Table V of Ref. 1. Speed of agitation and number of determinations not specified. [c] Table VI of Ref. 1. Agitation speed of 60 rpm; six determinations.

Table II—Dissolution Rate of Salicylic Acid Tablets

	Percent Salicylic Acid Dissolved													
	Experiment												Average	
Minutes	1	2	3	4	5	6	7	8	9	10	11	12	%	Range, %
2.5	22	21	20	23	25	27	29	24	23	25	24	21	24	20—29 = 9
5	36	38	40	44	39	38	36	37	32	38	44	38	38	36—44 = 8
10	66	58	68	67	57	60	62	56	68	68	56	58	62	56—68 = 12
15	80	80	83	81	75	77	73	70	85	77	73	72	77	70—85 = 15
20	90	92	95	90	89	85	83	82	93	92	89	87	89	82—95 = 13
30	100	99	103	102	99	98	95	95	100	102	103	102	100	95—103 = 8

measure real differences in the dissolution rate of unit doses within a batch if these differences exceed ±2.8% of the average assay. However, analysis of their data (Table I) indicates that the intertablet variations gave rather large-range values. This study was undertaken to investigate the nature of the large-range values obtained with the automated apparatus.

Since the authors claimed the accuracy and reproducibility of the instrumentation by showing negligible variation caused by the electrical imbalance (<1%) and instrumentation, possible explanations for the large intertablet variation may be: (*a*) differences in the active ingredient content of the tablets, (*b*) differences in the disintegration properties of the tablets, and/or (*c*) uneven distribution of drug particle size among tablets.

An examination of Table I reveals that the range values were much higher in the period represented by 20–80% dissolution. This finding indicates that the disintegration properties, rather than content uniformity, of the tablets were largely responsible for the variations observed. For example, if some tablets in a given batch disintegrated much faster than the others, the faster disintegrating tablets would tend to yield faster dissolution rates as compared to the slower disintegrating tablets. Thus, the "average" dissolution rate of this batch of tablets would exhibit large intertablet variations.

To minimize differences due to content uniformity and manufacturing variables, a batch of 200-mg salicylic acid tablets was prepared by individually weighing 200 mg of salicylic acid for each tablet and compressing the tablets using a hydraulic press[1]. All tablets were prepared using identical compression force for the same time period, thus assuring that the tablets were uniform. Quick checks of the disintegration and hardness revealed that the tablets were identical in these respects.

Dissolution was followed in an automated apparatus[2] similar to the one described by Johnson *et al.* (1), using 900 ml of simulated gastric fluid (without enzymes), according to the USP–NF method (2, 3). A 1.9-ml/min flow rate[3] through a flow cell[4] and a recording spectrophotometer[5] were used. From the results (Table II), it is evident that the intertablet variations were somewhat greater than the accuracy and reproducibility claimed for the apparatus. Therefore, the large variations found by Johnson *et al.* could be due to differences in disintegration and other physical properties of the tablets as well as to the nonreproducibility of the apparatus. Further work is in progress to determine the factors responsible for these variations.

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[1] P-30 hydraulic press, Research and Industrial Co. Ltd., London, England.

[2] Model T1045-20x/53, Vanderkamp tablet dissolution tester, Van-Kel Industries, Chatham, N.J.

[3] Model 1201, Harvard Apparatus Co., Millis, Mass.

[4] Type 42 flow cell, Luminon, Inc., Irvington, N.J.

[5] Hitachi-Perkin-Elmer UV-VIS recording spectrophotometer model 139, Coleman Instruments Division, Maywood, Ill.

Synthesis and Neuromuscular Blocking Activity of a 3,3′-Bisbufotenidine Analog

Keyphrases □ 3,3′-Bisbufotenidine analog—synthesized and screened as possible muscle relaxant, neuromuscular blocking activity □ Bufotenidines—synthesis and screening of 3,3′-bisbufotenidine analog, neuromuscular blocking activity □ Neuromuscular blocking activity—3,3′-bisbufotenidine analog, effect of size □ Muscle relaxants, potential—synthesis and screening of 3,3′-bisbufotenidine analog, neuromuscular blocking activity

To the Editor:

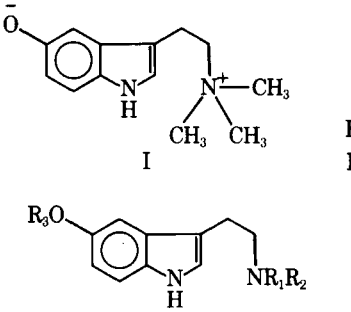
In correlating the neuromuscular blocking activity of some indole-3-alkylamines with their chemical structures, it was previously demonstrated (1–4) that the presence of two hetero atoms (O and N) in the molecule, intervened by six carbon atoms through the shortest route, is essential. It was also shown that the degree of activity, although not restricted to quaternary amines only, was a function of the pKa of the test compounds.

Accordingly, the neuromuscular blocking activity of bufotenidine (I) was found to be the most potent among the series of compounds (I–IV) tested. Bufotenidine itself, however, showed a significant difference in its activities *in vivo* and *in vitro*. Thus, the antiacetylcholine effect of I on the rectus abdominis muscle of the frog was almost equipotent to that of tubocurarine, while its effect on the reversal of neostigmine block on sciatic nerve–gastrocnemius muscle preparation of albino rats was five to six times less than the latter. The possibility that the size of the molecule may be responsible for this difference in activity was repeatedly considered theoretically (2–5) but not tested by observation. We have now obtained evidence to support this contention.

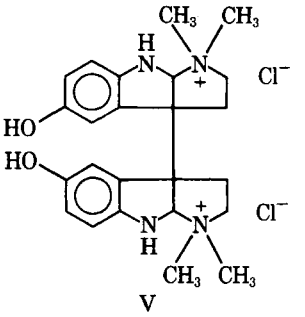
For these purposes, the bisbufotenidine analog (V) was synthesized from 5-*O*-benzylbufotenine (VI), and its neuromuscular blocking activity was tested both *in vitro* and *in vivo*. The synthesis was based on two previous reports: *(a)* that indolyl magnesium halides are ionic in tetrahydrofuran solution (6), and *(b)* that cyclization of an ethereal suspension of N_a-dipteryl magnesium iodide with anhydrous ferric chloride affords *rac*-chimonanthine (7).

Accordingly, 3-(2-*N*,*N*-dimethylaminoethyl)-5-benzyloxyindole (5-*O*-benzylbufotenine) (VI) was first subjected to ionization of N_a by formation of the Grignard reagent (VII), followed by oxidation with anhydrous ferric chloride to yield V as the major product. Methyl magnesium iodide was prepared from magnesium turnings (0.95 g) and methyl iodide (5.5 g) in anhydrous ether (300 ml) under a nitrogen cloud. A solution of 5-*O*-benzylbufotenine (11.5 g) in ether (500 ml) was added dropwise in 1 hr to the stirred solution of methyl magnesium iodide. The resulting suspension was stirred for a further 2-hr period, and then a solution of anhydrous ferric chloride (7 g) in ether (200 ml) was added dropwise.

The reaction was stirred for 12 hr and then treated with an aqueous solution of ammonium chloride to effect mild acidic hydrolysis of the product. The ethereal layer was separated, the aqueous solution was made basic (pH ~10), and the liberated bases were extracted with ethyl acetate. The gelatinous precipitate of ferrous hydroxide was adsorbed on powdered cellulose. The solids were removed by filtration, and the residue was again extracted with hot ethyl acetate. The total extractives (8.4 g), obtained from the ether and ethyl acetate extracts, revealed four major spots due to quaternary bases, in addition to the unreacted indolealkylamine (VI, 54%), on TLC analysis [silica gel G[1], benzene–ethyl acetate (4:1)].

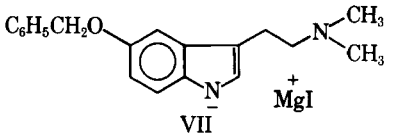


I



V

II: $R_1 = R_2 = CH_3$, $R_3 = H$
III: $R_1 = R_3 = H$, $R_2 = CH_3$
IV: $R_1 = R_2 = R_3 = H$
VI: $R_1 = R_2 = CH_3$, $R_3 = CH_2C_6H_5$



VII

The benzyl group was removed from the mixture of products by treatment with trifluoroacetic acid (20 ml) at room temperature for 18 hr (8). Column chromatography of the resulting mixture of phenolic bases over a magnesia silica gel[2] and a resin[3] gave the major product (1.72 g, 22%) as a microcrystalline solid. The compound, mp 210–214°, $C_{24}H_{32}N_4O_2Cl_2$ (elemental analyses and potentiometric titration), showed spectral properties, *e.g.*, UV: λ_{max} (ethanol) 238 (log ϵ 4.02) and 295 nm (log ϵ 3.61) [indicating the presence of a quaternary N_a—C—N_b^+ system since, on dequaternization with lithium aluminum hydride in tetrahydrofuran, the maxima were shifted to λ 248 and 307 nm (9)]; PMR (CD_3OD): δ 3.24–3.30 (12H, N^+—CH_3), 4.28–4.31 (2H, N—CH—N^+), and 6.8–7.4 (6H, Ar—H). The mass spectrum of the dequaternized product (phenolic tertiary base) showed, aside from the molecular ion peak at *m/e* 378 (14%), significant fragment ion peaks at *m/e* 188 (22), 187 (16), 146 (68), and 43 (base peak), characteristic of symmetrical scission of the dimeric indolic base. On the basis of these data, the major product was assigned Structure V.

The bisbufotenidine analog (V) exhibited the expected pronounced curariform activity on the rectus abdominis muscle of the frog, inhibiting acetylcholine-induced spasm without affecting the potassium

[1] E. Merck.
[2] Florisil.
[3] Amberlite CG-400 (Cl^-).

chloride-induced contractions. The inhibition was reversible and the spasmolytic ED_{50} was 0.82 μg/ml. Bufotenidine and tubocurarine, in parallel assays, showed spasmolytic ED_{50}'s of 1.45 and 1.38 μg/ml, respectively. Subsequently, the effect of the quaternary dimeric alkaloid (V) to hasten the reversal of tetanic response due to neostigmine (0.3 mg/kg iv) on sciatic nerve–gastrocnemius muscle preparation of albino rats (10) was studied. The response, in absence of the test compounds, started recovering in about 10–15 min and was almost completely recovered by 30–40 min. The reversal process was brought to completion by 10–15 min after the administration of bufotenidine (I, 0.2 mg/kg iv), bisbufotenidine analog (V, 0.01 mg/kg iv), or tubocurarine (0.015 mg/kg iv).

The curarimimetic activity of V was also tested in chicks according to the method described by Lewis (11). The test compounds were injected intravenously through the alar vein in groups of 10 chicks. All three compounds showed flaccid paralysis of the limbs with flexion of the head of chicks in doses of 50, 4, and 10 μg for I, V, and tubocurarine, respectively. From these results, it appears that the bisbufotenidine analog could be of potential therapeutic importance as a muscle relaxant. The nominal side effects encountered during its routine pharmacological screening may not pose any serious obstacle to its possible use as an adjuvant to anesthesia.

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BOOKS

REVIEWS

Pharmaceutical Calculations. By JOEL L. ZATZ. Wiley, 605 Third Ave., New York, NY 10016, 1973. 311 pp. 17 × 25.5 cm. Price $7.95.

Using the format of linear programming with occasional branched remedial sequences, this book adequately presents the most important aspects of pharmaceutical calculations. The book actively involves the student in the learning process by requiring frequent responses and solutions to short questions and problems. The material is presented in 11 chapters covering the interconversion of pharmaceutical systems of weights and measures, elementary prescription notation and drug dosing, formula adjustment, calculation of percentages and other expressions of concentration, stock solutions, dilutions and concentrations, and milliequivalents. Zatz is careful and thorough in his presentation of unit labels in the basic review of mathematical operations and in his treatment of scientific notation. Accuracy of measurement using pharmaceutical equipment is conveniently linked to a presentation of factors affecting percent error and ways of determining significant figures.

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chloride-induced contractions. The inhibition was reversible and the spasmolytic ED_{50} was 0.82 μg/ml. Bufotenidine and tubocurarine, in parallel assays, showed spasmolytic ED_{50}'s of 1.45 and 1.38 μg/ml, respectively. Subsequently, the effect of the quaternary dimeric alkaloid (V) to hasten the reversal of tetanic response due to neostigmine (0.3 mg/kg iv) on sciatic nerve–gastrocnemius muscle preparation of albino rats (10) was studied. The response, in absence of the test compounds, started recovering in about 10–15 min and was almost completely recovered by 30–40 min. The reversal process was brought to completion by 10–15 min after the administration of bufotenidine (I, 0.2 mg/kg iv), bisbufotenidine analog (V, 0.01 mg/kg iv), or tubocurarine (0.015 mg/kg iv).

The curarimimetic activity of V was also tested in chicks according to the method described by Lewis (11). The test compounds were injected intravenously through the alar vein in groups of 10 chicks. All three compounds showed flaccid paralysis of the limbs with flexion of the head of chicks in doses of 50, 4, and 10 μg for I, V, and tubocurarine, respectively. From these results, it appears that the bisbufotenidine analog could be of potential therapeutic importance as a muscle relaxant. The nominal side effects encountered during its routine pharmacological screening may not pose any serious obstacle to its possible use as an adjuvant to anesthesia.

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BOOKS

REVIEWS

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Reviewed by R. G. Mrtek
University of Illinois at the Medical Center
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The National Formulary, Fourteenth Edition. Prepared by the National Formulary Board with the approval of the Board of Trustees, by authority of the American Pharmaceutical Association. Published by the American Pharmaceutical Association, 1974. Distributed by Mack Publishing Co., 20th & Northampton Sts., Easton, PA 18042. lxxix + 1124 pp. 14.5 × 23.5 cm. Price $24.00 (domestic and foreign).

In view of the recent acquisition of the National Formulary by the United States Pharmacopeial Convention, Inc., this latest edition of the National Formulary (NF XIV) proves to be a fitting vehicle, even if not so originally designated, for the National Formulary Board of the American Pharmaceutical Association to conclude its traditional responsibilities in setting forth standards of identity, strength, quality, purity, packaging, storage, and labeling of drugs and related articles. Maintaining the policy established for the two previous editions, the recognition of drug substances provided for the admission of articles only on the basis of their therapeutic value.

Of the total of 1009 articles recognized as therapeutic agents or pharmaceutic aids in NF XIV, 318 are newly admitted. The articles newly admitted to NF XIV are broadly representative of classes of agents valuable in modern therapeutics including antibacterials (cephaloglycin), antihypertensives (pentolinium tartrate), antineoplastics (floxuridine), antipsychotics (thiothixene), diagnostic aids (macroaggregated iodinated I 131 serum albumin), and immunizing agents (inactivated mumps virus vaccine). In addition to the monographs, general test chapters, reagent specifications, and general information chapters comprise one-fifth of this edition.

As a result of the efforts of the USP–NF Joint Panel on Primary Requirements, the NF XIV and USP XIX General Notices and Requirements do not differ from each other in substance or intent with regard to the interpretation and application of the official standards, tests, assays, and other specifications—although major differences are noted in the section pertaining to the information included under *Categories and Doses* (*Categories, Doses, and Dispensing Information* in USP XIX). Substantial changes have been made, however, with respect to the General Notices of both previous compendia volumes.

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A new monograph format was adopted to provide a distinctive manner for setting aside the informational matter, such as category, dose information, sizes available, description, and solubility, that is of particular interest to pharmacists and other users of the compendium. The official definition follows this informational section; the monograph standards, test procedures, and specifications follow the official definition in the familiar two-column format which has been utilized in recent NF editions. However, those specifications and requirements of primary concern for pharmacy practitioners—those regarding packaging, storage, and labeling—have been removed from the end of the monograph (their placement in NF XIII) and prominently placed immediately below the official definition and preceding the chemical tests and assays.

Specific revisions in the compendial tests and assays include the extension of content uniformity requirements to sterile suspensions in addition to their application to tablets, capsules, and sterile solids; the adoption—with caution—of the concept of system suitability tests in procedures using complex instrumentation, as a means of establishing that the entire analytical system is functioning properly to successfully complete the specified test or assay; and the use of newer analytical techniques, such as nuclear magnetic resonance spectroscopy in the case of amyl nitrite, to ensure drug standardization. Where applicable, a new specification entitled *Reference standards* that describes the pertinent storage and drying conditions (unless otherwise indicated in the monograph) for the reference standard materials used in the monograph procedures has been added.

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Staff Review

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JULY 1975

VOLUME 64 NUMBER 7

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THE VALUE OF CONGRESSIONAL OVERSIGHT

Early June brought word that yet another U.S. Congressional subcommittee would soon begin investigating the Food and Drug Administration and its overall performance. With this latest entry, at least half-a-dozen Senate and House committees and subcommittees are now in some stage of investigation of the agency, its operation, and its decisions.

In our view such investigations are generally a good thing. They create a sense of accountability that is desirable in any system of government, and particularly in a government built on a series of checks and balances.

Indeed, there are many who believe that for too many years—during the 1950's and early 1960's especially—FDA escaped the eye of Congressional scrutiny. In the view of these critics, a loose and permissive climate developed which, in turn, resulted in the evolution of a comfortable bureaucracy staffed with officials engaged in activities which sometimes seemed suspicious or self-serving. This picture of incompetence or malfeasance led to allegations of various questionable practices, of sloppy methods of operation, of decisions not for the public benefit, and of serious conflicts of interest.

At that time, the agency was just a fraction of its current size. Hence, if the FDA had continued to operate freely as it had been doing, the nation might now be faced with a crisis of public confidence in the area of food and drug regulation—a crisis which could have dwarfed the recent scandals surrounding other governmental agencies.

But largely due to the spotlight of Congressional attention, the necessary housecleaning already had been attended to, and continued surveillance seems to have prevented any major reoccurrence of these problems.

Granted, then, that such review is a good thing, the next question is at what point does a "good thing" become too much. That is, as in any process, when a certain optimum limit is exceeded, we reach a point of diminishing returns.

In the case of Congressional committee investigations, witnesses must devote an enormous amount of time and effort to advance preparation for such testimony, as well as to the appearance itself and the subsequent follow-through. To do less is to run the real risk of appearing foolish at best and to court personal disaster at worst. Consequently, if this process of testifying is duplicated and even replicated due to multiple concurrent investigations, the energies of an organization or agency can be disproportionately spent—or even exhausted—in defending and justifying its past actions. When this occurs, the pendulum may swing too far, causing so much time and resources to be drained off that little in the way of new action can be undertaken.

We are concerned that FDA is in danger of reaching such a condition due to the recent proliferation of investigations of the agency by Congressional committees. If the Commissioner and his staff find themselves spending so much time answering to Congress that they are unable to undertake or initiate new programs, then the public will be poorly served. Indeed, if progress were to be halted in this manner, one might properly conclude that the public health would be as poorly served as agency critics claim when the agency was completely escaping Congressional oversight and enjoying a free-wheeling existence. In our view, this would represent a most unfortunate development.

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REVIEW ARTICLE

Pharmaceutical Sciences—1974: Literature Review of Pharmaceutics

JOHN K. HALEBLIAN *x and FRANK W. GOODHART ‡

Keyphrases □ Pharmaceutical sciences—1974 literature review of pharmaceutics □ Technology, pharmaceutical—sterility, dosage forms, cosmetics, packaging equipment □ Physical pharmacy—dissolution, solubility, permeability, complexation, surface phenomena □ Antibiotics—pharmaceutical aspects □ Radiopharmaceuticals—pharmaceutical aspects □ Biopharmaceutics—drug properties, formulation effects, absorption mechanisms and control □ Pharmacokinetics—literature review □ Literature review—pharmaceutical sciences 1974

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This review article is a continuation of the annual review of pharmaceutics begun in 1963. As in past reviews in this series (1–13), the scope has been limited to the overall area of pharmaceutics since other specific subjects in pharmaceutical sciences are pub-

lished elsewhere. The authors have used selected sections of *Chemical Abstracts* as well as 40 journals as the sources for this article.

It is hoped that this review will be of continued usefulness to the pharmaceutical scientist wherever pharmaceutical information is needed. The overall format remains essentially the same as that of the last several years; however, some changes have been made to reflect minor organizational improvements.

GENERAL PHARMACY

A four-part series (14–17) reported on a wide variety of pharmaceutical aspects considered during formulation. Topics discussed included dissolution and absorption of drugs, stability studies, physical pharmacy, microbiology, and tableting. A review article on the development of new drugs on a rational basis discussed the selection of factual leads, molecular modification, and perspectives on drug design (18). Two articles gave the current status of prostaglandins as potentially useful drugs (19, 20). Various properties of camptothecin were reviewed including synthesis, structure, and pharmacological activity (21). The pharmaceutical uses of polymers, both water soluble and insoluble, were discussed (22), and the characteristics and uses of gelatin in pharmacy and medicine were presented (23).

Preservatives—Good manufacturing practice guidelines were reviewed with respect to the prevention of bacterial and other contamination (24). The effectiveness of preservatives and their influences on different containers and on bacterial counts of 45 commonly used compounds in drug manufacturing were also discussed. A modified USP XVIII technique was used to test the efficacy of preservatives in emulsions, and the influence of pH on bacteriostatic activity was stressed (25). The choice of an appropriate preservative and the proper level for a cosmetic product in its final package were reviewed (26). The relationship between the antibacterial activity of glutaraldehyde and pH was studied (27). The antipseudomonal effect of polymyxin and phenylethanol was studied alone and in combination (28). The combination had an additive effect on sensitive *Pseudomonas aeruginosa,* while the behavior of resistant strains indicated that cross-resistance occurs between polymyxin and phenylethanol.

The binding of various preservatives, alone and in combination, to cetomacrogol was investigated (29). Data for the compounds alone indicated that all preservatives combined with two distinct classes of sites on the cetomacrogol micelle. A dynamic dialysis method for estimating the binding of preservatives to nonionic surfactants was studied (30), and Scatchard plots for the interaction of methylparaben and propylparaben with cetomacrogol at 25° indicated that there were two classes of binding sites. The minimum inhibitory concentrations (MIC) of five different mercurials against *Staphylococcus aureus* and *P. aeruginosa* were determined (31). Mercuric chloride was the most efficient bactericide, followed by the phenylmercuric salts; thimerosal was the least active.

Table I—Additional References on Preservatives

Reference	Topic
39	Methods for testing disinfectants and antiseptics
40	Preservation of shampoos and foam bathing agents
41	New preservatives for cosmetic and industrial use
42	Chemical and toxicological factors related to preservatives
43	Interaction of preservatives with surfactants and high molecular weight substances
44	Effects of formulation on preservative availability in fixed oil emulsified systems
45	Physicochemical and bactericidal properties of quaternary ammonium compounds in dispersed systems

A number of papers appeared on the preservation of creams and emulsions (32–37). The effect of ^{60}Co γ-irradiation on oil–water emulsion ointments and hydrogels, both contaminated with two test spores, was investigated (38).

Other papers on preservatives are listed in Table I.

Flavor, Aroma, and Color—Several compounds were classified as to odor type (46), but additional research into the field of olfaction and chemistry is needed to prove a definite relationship between structure and odor. The problems and methods involved in improving the taste and odor of drugs were reviewed (47). The behavior of perfumery ingredients in products was investigated by a new technique involving GLC of perfumery ingredients from product bases (48). The method was used to show how materials behave in soaps and laundry powders after various storage conditions. The formulation of floral perfume bases was given (49), and the perfumery of soap was discussed (50).

Model interaction energy calculations using the monopoles-bond polarizabilities method were used to explain the different sweetness levels in a series of 2-amino-4-nitrobenzenes (51). The taste-masking properties of sorbitol were compared to simple sugar syrup (52). The coverings of the bitter taste of quinine hydrochloride solutions, the salty taste of sodium chloride, and the astringent taste of alum by the two sweetening agents were compared. According to statistical evaluation, the taste-masking ability of sorbitol is equivalent to sugar syrup of the same concentration.

Coloring agents, including their legal requirements, for cosmetics were discussed (53). Another article reviewed the physical meaning of color, the chemical composition of dyes, and general and legal requirements of cosmetic dyes (54). Sixteen pharmaceutical coloring agents were studied with respect to solubility, light fastness, heat resistance, stability in relation to pH and oxidizing and reducing agents, and compatibility with drugs in common use (55).

The influence of the surfactants sodium lauryl sulfate, sodium salts of fatty acid methyltaurides, alkyltrimethylammonium chlorides, and betaines on the coloring of hair was investigated (56). The coacervation of alkyltrimethylammonium bromides by various colorants (tartrazine, amaranth, carmoisine, and

erythrosine), in concentrations of 0.15 and 15 mmoles/liter of the dyes and in the presence of certain salts, was studied (57). Both phase separation and microscopic techniques were used at 5–95°. Phase separation diagrams were prepared, the mechanism of interaction was discussed, and the limits of compatibility of dyes with surfactant were defined.

The influence of sunscreening agents on the color stability of tablets coated with FD&C Red No. 3 and FD&C Blue No. 1 was investigated (58, 59). The sunscreening agent giving the best protection against the fading of the red colorant was 2-ethoxyethyl *p*-methoxycinnamate, but none of the agents gave significant protective action for the blue dye. A general discussion of the stability of coloring agents in pharmaceuticals included the following topics: principal photochemical modifications of coloring agents, methods of measuring color changes, measurement of reflectance, and effects of additives for protection against UV and visible light (60).

Stability—Since fructose is sweeter than sugar (factor 173/100), syrups containing fructose were stability tested at various temperatures and found to be very stable at room temperature and also more resistant to microbial proliferation than normal syrups (61). The browning of dextrates in solid–solid mixtures containing dextroamphetamine sulfate was studied by using diffuse reflectance spectroscopy (62). The changes in the physical and chemical properties of aluminum hydroxide gel prepared by the modified Ivanov method were studied (63). The gel prepared by this method remained practically unchanged after 7 years of storage at 4–37°. The degradation of propantheline bromide in the presence of aluminum hydroxide was studied (64), and the decomposition was more rapid in the presence of aluminum hydroxide than in water. The effect of varying climatic conditions such as temperature, humidity, sunlight, and tropical areas on stability was discussed and examples were given (65).

Solid Dosage Forms—Aspirin tablet stability was studied in various plastic and glass containers, which are more commonly used overseas (66). The amount of salicylic acid in the tablets was determined after the containers were stored at 25° and humidities of 30, 60, and 90%. A series of three articles described various experimental attempts to stabilize aspirin including coacervation, recrystallization, and spraying (67–69). Poly(methyl methacrylate) and silicone oil improved the stability of aspirin in tablets, while the coacervation method using ethylcellulose was the most advantageous.

A study was reported concerning the effects of storage in typical British dispensing containers of the glyceryl trinitrate content in tablets of the British Pharmacopoeia (70). Gitoxin, digitoxin, and cordigit tablets were significantly less active after storage under UV light for 10 hr; however, abicine was not affected (71).

Solutions—Aspirin was found to be extraordinarily stable in silicone fluids (72). Its stability also was improved in a polyethylene glycol base by the use of citric and tartaric acids (73). The effect of six kinds of commercial polyphosphates in preventing the oxidation of ascorbic acid and sodium ascorbate solutions in the presence of Cu^{+2} was studied, and the polyphosphates were grouped in three classes according to their effectiveness (74). Nitroglycerin was stable only at a pH <5 unless alcohol was in high concentration (75).

The degradation of ergotamine tartrate in aqueous solutions was investigated at 100° in the 2.5–4.0 pH range (76). Based on this work, the pH of an injectable solution should be 3.6. The major decomposition products of phenylephrine hydrochloride were determined after storage over a wide pH range (77). Methyl and ethyl *p*-hydroxybenzoates were reported to be stable at pH 4 from 40 to 121°, but considerable hydrolysis occurred at pH 1 (78). The stability of prostaglandins E_1, E_2, and E_3 was studied in solvents useful for clinical trials as well as in solvents used for their isolation from biological systems (79). Tryptophan stability was studied in clear glass and amber vials at 5, 25, and 50°; this compound was found to be more stable in amber vials, decreasing only 4% in potency after 6 months at room temperature (80).

The effect of sodium hydrogen sulfite on the stability of epinephrine (adrenaline) solutions was studied (81). The hypothesis that orally administered lanatoside C was converted to digoxin in the gut partly by gastric acidity and partly by intestinal microflora was tested (82). Significant conversion of lanatoside C to digoxin took place at 37° *in vitro* below pH 3.

Ophthalmics—The kinetics of the hydroxide-ion-catalyzed hydrolysis and epimerization of pilocarpine in aqueous solution were investigated utilizing pH-stat titrimetry and NMR spectroscopy (83). Both hydrolysis and epimerization followed pseudo-first-order kinetics. The stability of *l*-epinephrine solution was strongly influenced by pH, with weakly acid solutions being stable for more than 1 year (84). Hydroxyquinoline sulfate, 0.01%, was found to be a satisfactory stabilizer. A mydriatic eye drop containing 0.5–1.0% cyclopentolate was stable for 1 year (85).

Injectable Products—Isoproterenol was inactivated in the presence of metallic ions and was very rapidly decomposed in Krebs solution, but the solution can be stabilized by the addition of edetic acid (86). A crystal growth study of chloramphenicol and prednisolone in injection suspensions led to the definition and the development of a reproducible method to control particle size and included number of particles to be counted, sample preparation, and method of dilution (87). The effects of pH, buffer components, magnesium and calcium salts, ethylenediaminetetraacetic acid, and a number of antioxidants on the stability of oxytetracycline solutions were studied (88). Formulas containing magnesium chloride, *p*-hydroxybenzoic acid, dimethylformamide, propylene glycol, and sodium sulfite at pH 7.5 were the most stable.

The stability of phenylbutazone sodium injectable solutions was improved in the presence of propylene glycol, sodium metabisulfite, and diethylenetriaminepentaacetic acid (89). The stability of urea solutions, which were heat sterilized to destroy pyrogenic

Table II—Additional References on Stability

Reference	Topic
106	Incompatibilities of intranasal drops and ointments
107	Comparative study of the cardiovascular effect of fresh and air-exposed dihydralazine sulfate–reserpine tablets (Adelphane, Ciba)
108	Effect of physical factors on drug stability
109	Stability of prostaglandins
110	Stability of heroin (diamorphine) hydrochloride and cocaine hydrochloride elixirs
111	Stability of belladonna alkaloid
112	Stability of sulfonamides
113	Method of stabilizing ^{125}I-insulin and ^{125}I-STH (growth hormone)
114	Stability and stabilization of phenothiazine derivatives
115	Solid-state thermal decomposition of *para*-substituted salicylic acids
116	Stability of dispensed medicines
117	Decomposition and stabilization of aminoglycol alkanesulfonates
118	Stability of thiabenzenes
119	Acidic hydrolysis of 2-acylamidobenzamide
120	Physicochemical properties, stabilities, and analysis of iodoxamic acid
121	Stability of rutin in artificial gastric juice
122	Stability of tetrahydrocannabinols
123	Stability of amino acids in a parenteral nutrition solution
124	Hydrolysis of meclofenoxate and its methiodide
125	Coloration and stabilization of aminopyrine preparations
126	Reaction of aminopyrine and iodide compounds
127	Review of stabilities of some common drugs
128	Stability of urokinase preparations
129	Stability of androstenedione 3-enol glucosiduronate in solution
130	Stability of monofluorophosphate and fluoride ions in a dentifrice containing calcium carbonate

impurities, was studied; it was found that urea was partially destroyed (90). One percent solutions of morphine hydrochloride and 2% omnopone in polyethylene injection vials were stabilized by use of 6% glycerin and 0.05% disodium edetate (91). The effect of ^{60}Co irradiation on strychnine nitrate and neostigmine (proserine) injection solutions was investigated; frozen solutions were very stable and the decomposition products were less toxic than the starting compounds (92).

The stability of sulfathiazole sodium and sulfamethazine (sulfadimidine) sodium solutions for injection was investigated (93). Thermal degradation rates of reserpine were obtained by measuring the remaining intact drug and found to follow first-order kinetics at various concentrations (94). Further work by the same investigators included the effects of ionic strength, dielectric constant, inert gas, and stabilizers on reserpine stability (95). A proposed formula contains propylene glycol, phosphoric acid, sodium dihydrogen phosphate dihydrate, and sodium sulfite.

Other stability studies on injectable products concerned Ringer–lactate concentrate in ampuls (96), stability of glaucine hydrochloride (97), and securinine nitrate (98).

Semisolids—The concentration of prednisolone in an ointment was decreased by the addition of phenol and diphenhydramine and, especially, by adsorption to zinc oxide or potato starch (99). The degradation of 1% hydrocortisone in polyethylene glycol ointment USP was investigated at several temperatures (100). Decomposition at the C-17 side chain as well as ring A was followed by two different analytical procedures, and the side chain decomposed at the faster rate. Anthralin was found to deteriorate in zinc oxide paste, but this was remedied by the addition of salicylic acid which reacts with the surface zinc oxide to form zinc salicylate (101).

Several studies appeared on the stability of drugs in suppositories. The stability of chloral hydrate in various bases, including polyethylene glycol and estarine D (102), was studied. The effect of various factors on the oxidation of promethazine suppositories was investigated (103). Stabilized vitamin A in suppositories with massa estarinum C showed a similar but higher stability than vitamin A palmitate (104). The decomposition of hexahydroadiphenine hydrochloride in suppositories prepared with either aged or normal cocoa butter and aged Witespol H_{15} converted the drug to its base and *N*-oxide (105). The mechanism of the decomposition, independent of the type of suppository base, was discussed in detail.

Additional references on stability are given in Table II.

Kinetics—The effect of pH (2.75–9.16) and temperature (40 and 50°) on the hydrolysis of methyl-, propyl-, and *n*-butylparabens was studied at 70° in 0.1 *M* phosphate buffer solution at an ionic strength of 0.3; kinetic constants were calculated (131). The influence of cetrimonium bromide on base-catalyzed hydrolysis of *para*-substituted ethyl benzoates was investigated and led to the hypothesis that the observed rate modifications were dependent not only on the *para*-substituent and the surface pH of the micelle but also on the dielectric constant at the surface of the micelle (132).

The kinetics of the alkaline hydrolysis of benzocaine and some homologs in the presence of a nonionic surfactant were studied (133). It was concluded that these compounds were probably associated with the polyoxyethylene portion of the micelle and that the observed change in the rate of hydrolysis was attributed to both a decrease in the rate of hydrolysis and an increase of solubility within the micellar phase. Ethinyl estradiol did not undergo thermal decomposition at 110° over 72 hr, and decomposition depended on excipients such as lactose, starch, polyvinylpyrrolidone, talc, alcohols, and parabens (134). Thermal decomposition under the conditions established produced estrone, estradiol, and doisynolic acid.

The *in vitro* hydrolysis of oxazepam succinate half-ester by a stereospecific soluble esterase from different animal species was reported (135). The kinetics of hydrolysis of salicylamide were investigated under varying conditions of temperature, pH, and ionic strength (136). The hydrolytic degradation of the drug was found to be first order throughout the entire pH range. The conversion of a steroid oxime, 17α-acetoxy-6α-methyl-4-pregnen-3,20-dione 3-oxime, to the corresponding diketone in acidic media

Table III—Additional References on Stability Kinetics

Reference	Topic
139	Stabilization of aspirin in solid drug forms
140	Degradation of aspirin in methoxypolyethylene glycol 350, 550, and 5000 and unsubstituted polyethylene glycol 400
141	Topochemical decomposition patterns of aspirin
142	Kinetics of hydrolysis of aspirin binary systems of water and organic solvents
143	Kinetics of hydrolysis of (1'-ethoxy)ethyl 2-acetoxybenzoate
144	Stabilities of two aspirin preparations containing aspirin and quinine
145	Hydrolytic decomposition of hexobarbital in frozen systems
146	Oxidative destruction of α-tocopherol and ascorbic acid in methyl linoleate
147	Clinical significance of pilocarpine hydrolysis
148	Kinetics of β-lactamase inactivation of penicillins, and effect of competitive inhibitors
149	Solid-state stability of digoxin as a function of temperature and humidity
150	Decomposition of carbazochrome sodium sulfonate in acid and alkaline media
151	Acid–base catalysis of carbazochrome sodium sulfonate decomposition
152	Kinetics of metronidazole hydrolysis
153	Kinetics and mechanisms of solvolysis of 5-iodocytosine
154	Kinetics and reaction mechanism of phenoxybenzamine decomposition in aqueous and aqueous ethanolic solutions

was found to be first order at 37° (137). The effects of incorporating ester groupings at the oxime function or at C-17 position and modifications in the B ring were also investigated; only the length of the ester chain had a profound effect on the rate constant.

A simple experimental procedure was described to obtain estimates of the activation energy and preexponential factor for drug decomposition from a single experiment (138). A computer program was written involving numerical integration, and the method was illustrated using the decomposition of riboflavin in alkaline solution.

Other general kinetic studies are listed in Table III.

Antibiotics—The stability of procaine and potassium salts of penicillin G in oleaginous systems containing colloidal silica was studied (155). Results indicated that instability of penicillin G in these bases was due to the surface acidity of the silica in conjunction with the ester constituents of the oils. The formation of hydrolysis products of Bulgarian penicillin G was dependent on temperature, pH, and storage time; the development of the degradation products, penicillenic and penicilloic acids, was the cause of allergic reactions (156). The kinetic degradation of methicillin, penicillin V (phenoxymethyl penicillin), and their penicillenic acids was studied in an effort to explain their role in penicillin hypersensitivity phenomena (157).

Another paper also described the kinetics of penicillin stability because of the proposed role of the degradation products in causing allergic response (158). Papers also appeared describing the stability of semisynthetic penicillins and 6-aminopenicillanic acid in aqueous solution (159), as well as carbenicillin degradation in aqueous solutions (160). Three ampicillin studies were concerned with the effect of pH on the stability of ampicillin sodium solutions (161), the stability of ampicillin in aqueous suspension (162), and the stability of betacillin in aqueous solution (163).

Various factors affecting the stability of tetracycline hydrochloride in ointments were studied; included were the effects of preservatives, aseptic processing, light, temperature, and the type of base (hydrophilic or lipophilic) (164). A number of tetracyclines was studied by differential thermal analysis and differential gravimetric analysis (165). A review article on the chemical stability of tetracyclines in aqueous solution listed references on the formation of epimers, anhydro compounds, isomers, and various oxidation products (166). The addition of solubilizers, urea, thiourea, polysorbate 20, and polyethylene glycol 6000 to tetracycline solutions significantly reduced epimerization of the drug (167); it was suggested that occasional kidney damage may be caused by the epimerization.

Stability kinetic studies of oxytetracycline hydrochloride in solutions were performed (168). The effect of different pH's and buffers on the solubility and stability was determined. Also the effect of magnesium, calcium, edetate (EDTA), pyridine derivatives, aromatic oxyacids, water-soluble antioxidants, and inert gases on the stability of the drug in solution was studied. Two stable injection formulations were presented. Another study found that the addition of glycerin, propylene glycol, polyethylene glycol 400, dimethyl sulfoxide, and water was detrimental to oxytetracycline in ointments (169). Rolitetracycline was found to hydrolyze rapidly in dilute aqueous solution (170), and the base was less stable than the nitrate salt. Other workers reported that rolitetracycline was hydrolyzed to tetracycline and 4-epitetracycline in aqueous solutions at 25° (171); both the base and salts were >50% hydrolyzed in 3 hr and the degradation was probably first order. The dehydration kinetics of epitetracycline in solution to form epianhydrotetracycline were investigated (172), and the reaction was found to be first order with respect to epitetracycline and hydronium-ion concentrations.

Table IV—Additional References on Antibiotic Stability

Reference	Topic
175	Decomposition of chloramphenicol stored in frequently opened vessels
176	Effects of aging and relative humidity on drug release of chloramphenicol capsules
177	Physical compatibility and chemical stability of cephapirin sodium in combination with antibiotics and large volume parenteral solutions
178	Stability of frozen solutions of cephapirin sodium
179	Compatibility of antibiotics with gels of semisynthetic and synthetic products
180	Transformation of the factor M of virginiamycin in acid medium
181	Stability of auricular stamycin solutions
182	Stability of nisin preparations
183	Stability of parenteral solutions of cefazolin sodium

Two papers appeared on the intravenous solution stability of amphotericin B stored in 5% dextrose–water solution (173, 174). No appreciable loss in activity was found with the antibiotic alone or in the presence of hydrocortisone and heparin, even when exposed to light; however, sodium and chloride ions were very deleterious to stability of amphotericin, reducing the potency 25% in 4 hr.

Other references relating to antibiotic stability are listed in Table IV.

Vitamins—A number of factors improved the stability of multivitamin oral liquid preparations (184). These factors included the use of glass-distilled water, 0.01% disodium edetate, 0.05% sodium glutamate, and 0.01% cysteine hydrochloride; purification of sucrose with activated carbon; and protection from light and oxygen. Two other articles discussed general vitamin stability in pharmaceutical preparations (185, 186).

The effect of two nonionic surfactants on the aerobic oxidation of ascorbic acid and thermodynamic properties was obtained for vitamin C solutions containing 1% surfactant (187). On storage at 37° and 100% relative humidity, the stability of ascorbate salts was Ca > Mg > Na > K, although they were much less stable than the free acid and the stability was even lower in talc and silica formulations (188). The effect of pH on the rate of coloration of 1% ascorbic acid in 60% sorbitol solution at 37 and 47° was studied, and kinetic constants were calculated (189).

The compatibility of vitamin B_1 preparations in combination with other drugs was studied. Vitamin C did not affect various B_1 derivatives, but those containing B_{12} were decomposed (190). Tablet stability of vitamin U was determined (191, 192), and the effect of fillers on the stability was reported (193). Excipients such as mannitol, lactose, and dicalcium phosphate imparted high stability to vitamin U, but aluminum hydroxide and magnesium hydroxycarbonate had adverse effects on the stability of tablets in storage.

Additional references on vitamin stability are listed in Table V.

Table V—Additional References on Vitamin Stability

Reference	Topic
194	Transformation of vitamin A aldehyde in hydroalcoholic and other aqueous cosolvent solutions
195	Stability prediction of thiamine and L-ascorbic acid in multivitamin tablets and capsules
196	Stability of thiamine, pyridoxine, and hydroxocobalamin hydrochloride in parenteral form
197	Effects of hydrotropic salts on stability of menadione
198	Determination and stability of pantothenates in drugs
199	Compatibility of vitamin B_{12} preparations in combination with other drugs
200	Stability of water-soluble vitamin P
201	Decomposition of L-ascorbic acid in the presence of D-araboascorbic acid
202	Hydrolysis of benfotiamine in aqueous solutions
203	Effect of low storage temperatures on stability of medicinal cod liver oil
204	Stability of parenteral solutions containing amino acids, vitamin B, sugar alcohol, and commercial mineral mixtures

PHARMACEUTICAL TECHNOLOGY

New drug forms resulting from present technological developments, with emphasis on microencapsulation, were reviewed (205). The clinical and design aspects of medicines for oral administration were discussed (206–208). Preparations of gastric antacids were checked for onset and duration of buffer effect against artificial gastric fluid by an *in vitro* test method (209).

Sterile Products—A new sterile products laboratory incorporating the latest developments in the field was described (210), and the anticipated revisions in current good manufacturing practice regulations were reviewed (211).

Several papers concerning particulate contaminant problems were published. An assay procedure utilizing the scanning electron microscope for counting asbestos and other inorganic fibers in fluids was described, along with a method for testing the membrane filter retention of asbestos fibers (212). Examination of 22 sterile solid products for particulate matter using the standard membrane filtration technique indicated that this class of products contained an appreciable number of particles, of which more than 95% are 5–50 μm in size; small volume injections in a similar manner contained approximately half the total number of particles on a per vial basis (213).

Polyvinyl chloride and glass large volume parenteral containers were examined to determine the effects of agitation, storage, and additive handling on the generation of subvisual particulate matter, and it was found that shaking and temperature generated small particles in the polyvinyl chloride containers (214). Particle contaminants from saline and dextrose intravenous solutions were sized and counted using the Prototron instrument (215). Various particle contaminants were identified and these included salt crystals, starch grains, glass fibers, mica flakes, and metal shavings. The general problem of asbestos and other fibers in parenteral solutions was reviewed (216–218), and the particulate contamination of dextran for intravenous use was studied (219).

The incidence and potential for microbial contamination of glass bottle and plastic bag systems used for intravenous fluid administration were estimated from results obtained in a study simulating both hospital and centralized admixture program use (220). Two other articles discussed the microbial contamination of packaged fluids after sterilization (221, 222).

The methods of particle counting in infusion fluids were discussed (223). Another article compared the Coulter counter, the HIAC automatic counter, and a microscopic membrane method in their ability to measure different types and sizes of contamination (224). The use of the Silting index was suggested as an alternative to particle counting (225); this method

Table VI—Additional References on Sterile Products

Reference	Topic
233	Method for aseptic filling of unit dose syringes
234	Preparation of glycerol solutions for intravenous administration
235	Aqueous injection solutions of vitamin A
236	Formulation and stabilization of phenylbutazone sodium injectable solution
237	Limulus test for *in vitro* pyrogen detection
238	Preparation and stability of menadione injection
239	Production of sterile sodium alginate solution
240	Parenteral administration of ethoxypolysiloxane oil
241	Preparation of injectable solution of nivalidine methiodide
242	Solubilization, emulsification, and dispersion with surfactants in parenteral preparations
243	Incompatibility and other problems involved in adding drugs to intravenous fluids
244	Drug additive compatibility
245	Side effects from glucose injections traceable to nitrogen content
246	Behavior of erythrocytes in water–monohydric alcohol solutions
247	Quality control in manufacture of parenteral solutions
248	Control of infusion sets according to requirements in the Nordic Pharmacopoeia

is based upon the tendency of particles to clog a filter, and the flow rate decrease is proportional to the particulate contamination in the test fluid.

Preparative methods for purifying water were described including vapor pressure distillation (226), reverse osmosis (227), ozonation (228), and filtration (229). Removal of pyrogen in physiological saline solution was studied by filtration through Chamberland 25C, Seitz Sheet EKS-I, and activated-carbon Chamberland (230).

A report pointed out the possibility of the precipitation of poorly water-soluble drugs contained in a nonaqueous vehicle when the solution was mixed with human plasma (231). The compatibility of certain vitamins combined with other parenteral solutions was studied (232).

Other papers concerning sterile products are listed in Table VI.

Ophthalmics—The action and uses of a number of topical ophthalmic drugs were discussed (249). The development of radiosterilized tetracycline ointment was reported, and the cost savings feature of cobalt 60 γ-ray sterilization in the final package made it an attractive alternative to conventional aseptic manufacturing and packaging (250). It was found that the amount and rate of drug loss through drainage for a single drop of topically applied ophthalmic solution increased with increasing volume of instilled solution (251). However, the concentration of drug in the precorneal tear film immediately after installation of drug was higher with larger instilled volumes. Ocular absorption of drugs using various vehicle modifications was reported (252, 253). The formulation of a semisolid oleaginous ointment base employing a polyethylene[1], fused micronized silica, and sodium stearate was studied (254, 255).

[1] Epolene C-10, Eastman-Kodak.

Table VII—Additional References on Ophthalmics

Reference	Topic
256	Stability of palmatine eye solutions preserved by a quaternary amine hydrochloride
257	Effect of polyvinylpyrrolidinone on stability of sodium sulsaphiazole eye drops
258	Formulation of a stable pilocarpine hydrochloride solution
259	Determination of steroid level in aqueous humor after application using hydrophilic contact lenses
260	Formulation of ophthalmic suspensions containing steroids

Other references on ophthalmics are listed in Table VII.

Sterility and Sterilization—An approach to the establishment of parenteral solution sterilization cycles was offered (261). A comparative study of seven media and two systems for sterility testing was presented (262). The use of ethylene oxide as a sterilant was the topic of many reports. Articles sterilized with ethylene oxide released the gas at rates depending on materials and methods of manufacture (263). A mixture of ethylene oxide and acetic acid (1:1) was as effective as a mixture of ethylene oxide and carbon dioxide but was more rapidly desorbed. Extracts of polypropylene syringes that had been sterilized with ethylene oxide did not inhibit growth of *Staph. aureus*, were not toxic to mice after intravenous injection, and did not cause hemolysis upon addition to erythrocyte suspensions (264).

Naturally contaminated samples of sodium alginate treated with ethylene oxide showed a significant drop in viable microbe content without affecting viscosity (265). Ethylene oxide sterilization procedures were discussed as potential generators of chemical toxicities by way of an alkylating agent reaction mechanism (266). Detection procedures for ethylene oxide, ethylene glycol, and ethylene chlorohydrin residues in sterilized materials were reviewed, and 65 references were given. The role of packaging on ethylene oxide permeability was reported. Both 100% ethylene oxide and a dichlorodifluoromethane–ethylene oxide mixture were studied in conjunction with the following materials: 4-mil polyethylene, polyethylene/tyvek, paper, and paper/polyethylene terephthalate (Mylar) (267). Both the packaging and the sterilization cycle played a role in effective sterilization using ethylene oxide. It was found that hard and soft polyvinyl chloride as well as cellophane were sometimes more gas permeable for the mixture of ethylene oxide and methyl formate than pure ethylene oxide (268).

A discussion on the principal factors affecting sterilization, *i.e.*, gas concentration, temperature, humidity, and time, was presented and a detailed sterilization cycle was given (269). Various polyvinyl chloride and rubber materials were sterilized with ethylene oxide, and the residual amount of gas was determined as well as the biological compatibility (270). It was found that the desorption rate was markedly faster at 50° than at ambient temperature. The treatment of naturally contaminated materials was inves-

Table VIII—Additional References on Sterility and Sterilization

Reference	Topic
289	Sterilization of polymers for medical devices
290	Dosimetric substantiation of sterilization of radioactive pharmaceutical preparations
291	Review of sterility testing methods
292	Short-time sterilization of glass materials under ultraclean conditions
293	Microbiological sterilizing characteristics of 1,5-pentanediol aqueous solutions
294	Influence of thermal sterilization and storage on quality of nonstabilized and stabilized fixed oils
295	Optimal conditions for sterilization of buffered glucose solutions
296	Continuous automatic sterilization of parenteral solutions
297	Detection of fungi and yeasts in presence of preservatives
298	Sterilization of particulated atmosphere
299	Control of sterile medical equipment
300	Leakage of spray cooling water into topical water bottles
301	Bactericidal capacity of sodium dichloroisocyanurate formulations used for sterilization of infant feeding bottles and teats
302	Aeration of medical plastics
303	Sterility of a sterile powder-filling line

tigated (271), and the inactivation kinetics of ethylene oxide were discussed with respect to concentration, temperature, relative humidity, and other effects (272).

A number of papers discussed radiation sterilization of various plastics. Samples of plasticized polyvinyl chloride were irradiated with 2.5-Mrad doses of γ-radiation from ^{60}Co and 2.8–3.4-Mrad doses of β-radiation from a linear electron accelerator (273). Electron beam sterilization could be used for this plastic if it was not to be in direct contact with blood. Another paper pointed out that γ-irradiation prior to sterilization with ethylene oxide enhanced 2-chloroethanol formation in a surgical, hospital grade, polyvinyl chloride tubing (274). Another study indicated that polymethacrylate, cellulose derivatives, and polytetrafluoroethylene products were unsuitable for radiation sterilization but that polyvinyl chloride, polyethylene, polypropylene, polyethylene terephthalate, polyamides, polystyrene, silicone rubber, and rubber materials were sterilized satisfactorily (275).

Six kinds of polystyrene were irradiated with 4–5 Mrad of γ-radiation from ^{60}Co, and a study of their chemical and physical–chemical properties showed no harmful effect from this treatment (276). The practical application of radiosterilization of an ophthalmic and nasal ointment of neostigmine bromide, 1%, and dexpanthenol, 5%, with ^{60}Co as the radiation source was studied (277). Two levels, 2.5 and 4.5 Mrad, were used; sterility was achieved with the higher dose. The chemical stability of these drugs was satisfactory, but the tubes exhibited gas formation and an increase in volume and leakage. Two papers reported the sterilization of dry ampuls containing chlordiazepoxide hydrochloride (278, 279).

A series of selected colorants composed of eight FD&C dyes, seven lakes, and four D&C dyes was subjected to 2.5 Mrad of γ-irradiation from a cobalt 60 source over approximately 10 hr (280); no changes were found in these materials and they were all shown to be sterile. Another study reported that colorants were not affected by thermal or radiation sterilization, but decomposition occurred when they were tested in an experimental tablet mass (281). Other papers reported on the use of high frequency sterilization (282) and formaldehyde gas as a sterilant (283). Some drugs were decomposed by the high frequency method.

Three papers discussed experiences using biological indicators (284–286). The use of isopropyl myristate as a solvent in the sterility testing of oleaginous-based ointments was investigated (287, 288).

Other references pertaining to sterility and sterilization are listed in Table VIII.

Tablets and Capsules—Content uniformity criteria, including tests currently in the USP and NF, were studied using computer simulation (304). The results were compared with selected tests by variables for reliability, flexibility, and simplicity. Another paper reviewed the quality control aspects of compressed tablets (305). The formulation of effervescent aspirin tablets was investigated (306). Various methods, *i.e.*, sugar coating and press coating, were studied along with other factors to prepare a tablet containing pepsin and pancreatic enzymes (307).

Tablets containing pancreatin and barium sulfate were subjected to the USP XVIII disintegration test with and without plastic disks (308). The same tablets were given to two groups of people, one with normal stomachs and the other with reduced stomachs; the fate of the tablets in the digestive tract was radiographically documented. General background information and some advantages of formulation into soft gelatin capsules were published (309). A review on formulation of products in hard gelatin capsules discussed the rate of dissolution, release of the active ingredient as a function of particle size, porosity of the active ingredient, and addition of surface-active agents, diluents, and lubricants (310).

Additional general references on tablets are: *in vitro–in vivo* correlation and aging studies of commercial hydrochlorothiazide tablets (311), design of tests for content uniformity (312), degree of disintegration and solubility of tablets as characteristics of their high quality (313), and review of apparatus and methods for improving tablet formulations (314).

Comminution, Mixing, Granulating, and Drying—The milling of pharmaceutical solids was reviewed (315). Another report discussed the problem of obtaining fine particle sizes and the measurement of tensile strength, hardness, and breaking strength of particles, as well as the types of mills used (316). Various physical factors affecting blending of solids were presented together with methods of measuring mixedness (317). The mixedness of 10% ^{113m}In–phenobarbital triturations using three different fillers was evaluated (318). A mixture of fine and crystalline lactose was better than crystalline lactose alone or starch (corn) mixed with fine lactose.

The homogeneity of multicomponent powder mixtures was studied, and a numerical value for mix-

Table IX—Additional References on Comminution, Mixing, Granulating, and Drying

Reference	Topic
331	Homogeneity of pharmaceutical dispersed systems
332	Measurement of mixing of pharmaceutical powders using indium-113m
333	Review of various aspects of granulation
334	Effects of milling on granulation particle-size distribution
335	Manufacture and stability of tablet containing L-ascorbic acid and ferrous nicotinate
336	Influence of different factors on *in vitro* dissolution rate of tolbutamide from tablets

edness was mathematically derived (319). A twin-shell blender was used to investigate the blending procedures used in mixing aspirin–lactose (1:99) blends (320). Powders having good flow were less apt to show unmixing tendencies, and better blends were obtained when the amount of solvent was limited since this prevented excessive drug migration. A vertical cone mixer was used to mix a single active component, fenfluramine hydrochloride, with a number of diluents used for production-scale tablet preparations (321). The mixing operation was followed by the analysis of the drug in a number of samples equivalent to the final tablet size.

Reserpine was spray coated onto calcium sulfate granules of different porosities, and the relation between the macroporous nature of the granules and their reserpine content was investigated (322). The effect of fines on the physical properties of a starch–lactose granulation and the corresponding tablets was reported (323). An apparatus was made to measure granulating pressure so that it might be related to the amount of binder used as well as to its surface tension (324). Mathematical calculations were made relating power consumption during granulation of viscoplastic materials in a blade-type mixer (325). Mathematical formulations were also derived for calculating the dynamic coefficient of friction for materials used in the wet granulation process in a blade-type granulator, and the accuracy was checked by experimental runs with various drugs (326).

It was reported that compression of a barbital sodium granulate was greatly affected by moisture content (327). Various solvents were evaluated for use in granulating fusidate sodium (328). The relationship between moisture content and the quality of finished tablets of various drugs was investigated (329), and the optimum conditions of drying urodan granules in a fluid bed dryer were studied (330).

Other references on comminution, mixing, granulating, and drying are listed in Table IX.

Powder Characteristics—The flow of powders and granular solids in an excentric tablet press with the punches removed was measured (337), and the results varied widely compared with those obtained with a flowmeter. Six kinds of granules from four powdered materials, lactose, calcium carbonate, barium sulfate, and zinc oxide, were prepared by extrusion and crushing procedures (338). Physical properties were measured and related to flow rate. The influence of particle shape, hygroscopicity, electrostatic charge, and particle size on powder flow was reviewed (339). A statistical study of tablet uniformity in which the drug medazepam was mixed with three fillers at two particle sizes and two dosage levels was reported (340).

The cohesive force effect of magnesium stearate addition (0.2–1.0%) to salicylic acid was investigated (341). An equation was developed to estimate accurately the flow rates of powders from circular orifices in which flow is dependent on orifice opening, specific gravity of the powder, and a fluidity index obtained by the variable rotating cylinder method (342). Magnesium carbonate, encapsulated with various polymers, showed variations in resultant physical properties such as angle of repose and flow and could be tableted (343). The effect of the choice of excipient on the mixture quality of the unmilled drug, cyclopenthiazide, was investigated, and segregation of the drug mixture could be avoided by using wheat or maize starch in place of lactose (344). The effects of certain process variables were determined for the spheronization process (345).

Various methods of measuring the tensile strength of compacted powders were described including transverse compression using squares and disks as well as the direct tensile stressing of lightly pressed powder (346). The failure properties of lactose, calcium carbonate, and ultramarine were determined, and it was found that narrow size fractions behaved as simple powders (347). The packing properties of moist bulk solids were studied using glass powder, potassium chloride, and calcium phosphate (348). Powder bed densities were found to be dependent on the concentration of calcium stearate and the cumulative number of revolutions in a V-type blender (349).

Compression—The compressibility of sulfanilamide granules and starch was studied by means of a rotary tablet machine fitted with strain gauges (350). The effect of various factors including the method of granulation, residual moisture content, and binder concentration was reported. A follow-up study determined the effect of nonionic surfactants on the compressibility of sulfanilamide granules (351). The strength of compressed tablets was markedly improved in the presence of 1% silica (Aerosil), whereas certain cellulosic materials improved disintegration times (352). Photomicrographs of potential tablet disintegrants in polarized light before and after water treatment revealed the relationship between swelling factors and tablet disintegration. A variety of dextrose (Celutab) was found to be suitable for the preparation of tablets containing aspirin, phenacetin, and caffeine citrate by direct compression (353). Physical changes due to high frequency and tray drying of tablet cores containing a strongly moisture binding antibiotic, which had different coatings in selected solvents, were studied (354). Drying was shown to result in improved stability of aspirin-containing tablets.

No constant relationship was found between tablet compressive force and the tablets *in vivo* and *in vitro* properties (355). A timed-release salicylamide tablet

Table X—Additional References on Compression

Reference	Topic
364	Regulation of structural–mechanical properties of tableted substances
365	Factors influencing physical properties of tablets
366	Influence of water content of pharmaceutical powders on compacting phenomenon
367	Compression under constant upper punch pressure, and behavior of particles filling in voids
368	Influence of particle shape on cohesion of particulate solids
369	Influence of tablet weight on compaction pressure–tablet density relationships
370	Effect of rate on pressure of pressing tablets
371	Compaction of chemical–pharmaceutical powders
372	Solid-state distribution analysis of sulfisoxazole tablets
373	Physics of solid state as related to dosage form design
374	Pressure transmission to lower punch during compression of potassium chloride
375	Log-normal distribution of tablet pore diameters

was made using two types of sodium carboxymethylcellulose; compressive force affected release only slightly with one type of the cellulose polymer but was slowed with the other type (356). The dissolution rate and surface area of phenacetin tablets made with wheat starch and talc were investigated (357).

The relationship between compression stress and displacement of the upper punch was studied to clarify the viscous behavior of powder when a compressed sample was recompressed (358). There appeared to be a hysteresis loop in the stress–displacement diagram; from the loop area, loss energy, depending on the viscosity of the sample, was calculated. The relationships among axial pressure, radial die wall pressure, and density changes in beds of powder undergoing compression were studied using a single-punch instrumented tablet machine (359); data were developed for aspirin, sodium chloride, acetaminophen (paracetamol), and sucrose. Tablets formed from dendritic sodium chloride crystals, which had a larger shape index than cubic crystals of the same size fraction, were stronger than tablets formed from cubic crystals (360).

A new method for determining the tensile strength of tablets was proposed utilizing two fulcrums and a knife edge (361). The results agreed well with tensile strength values obtained from diametral compression. A report on the use of an industrial scale compactor compared the results of various granulation procedures using aspirin, potassium chloride, and a sulfonamide (362). The criteria for using different materials for the dies of rotary tablet machines were discussed, and recommendations for the types of steels for press-molding machines were given (363).

Other references on compression are listed in Table X.

Effects of Excipients—A compressible starch (STA-Rx 1500) was compared as a compression vehicle with potato, maize, and rice starches, Dry Flo starch, cellulose (Solka-Floc), microcrystalline cellulose (Avicel pH 101), and anhydrous lactose USP in aspirin tablets (376). The resulting tablet properties were measured; the compressible starch (STA-Rx 1500) was found to be a good vehicle, but magnesium stearate adversely affected tablet properties. The use of potato starch as a disintegrant was reviewed, and water contents were given after drying and after storage at various humidities (377). High concentrations of starch were used in hard gelatin capsules to improve the release of phenobarbital (phenobarbitone) (378).

Two special directly compressible blends of microcrystalline cellulose with starch and calcium sulfate were investigated (379). It was shown that hydroxypropyl cellulose could be a useful binder in direct compression tablet formulations when properly combined with potato starch and lactose (380). It was demonstrated that tensile strength of lactose tablets decreased as the fatty acid composition of tablets increased (381). The presence of glucose in industrial lactose at various stages of purification and the effect of glucose on the quality of lactose for pharmaceutical use were determined (382). The pore structure of tablets made from wet massed granules and from crystalline granules was compared (383). Tablets made from crystalline granules showed a monosized pore structure regardless of granule size or compression pressure. Direct compression formulations containing microcrystalline lactose, dicalcium phosphate, microcrystalline cellulose, and starch derivatives were characterized using an instrumented rotary tablet machine (384).

L-Leucine and L-isoleucine were investigated as tablet lubricants and compared to other widely available lubricants (385). The addition of 5% L-leucine and 1–5% L-isoleucine gave almost the same effect as 1% magnesium stearate. The use of magnesium lauryl sulfate in tableting was evaluated using ejection force and compressibility measurements (386). A calcium carbonate–starch paste granulation was suggested as a diluent for tablets (387).

The effect of the binders, cellulose acetate phthalate, ethylcellulose, and shellac on the release of thioridazine from tablets was investigated (388). The effect of disintegrants and lubricants on the antimicrobial activity of tetracycline hydrochloride and chloramphenicol after tableting was determined (389, 390). These components caused activity losses of these antibiotics to varying degrees.

A method for the rapid evaluation of the effect of excipients on color fading was suggested; it involved the use of a fadeometer to accelerate light-induced fading followed by spectrophotometric evaluation (391). A photographic technique was developed to measure the degree of mottling of calcium phosphate tablets colored with FD&C Blue No. 1 (392). Optimization of the photographic process was described, and mottling values calculated by this method correlated well with the visual appearance of the tablets. Inclusion of wheat and potato starch (20%) in granule formulations reduced mottling and increased elasticity, suggesting that the elasticity of the granules enables them to retain their original shape after deformation under pressure (393).

Table XI—Additional References on Effects of Excipients

Reference	Topic
394	Effect of disintegrating agents on texture of tablets
395	Effects of formulation factors on dissolution rate of sulfamethazine (sulfadimidine) tablets
396	Relationships between amounts of binders and maximum granulating pressure
397, 398	Dissolution and disintegration of experimental phenindione tablets
399	Effects of disintegrant concentration on disintegration and compression characteristics of two insoluble, direct compression systems
400	Evaluation of two types of cellulose for direct compression

Other references on the effects of excipients are listed in Table XI.

Tablet Coating—Acrylate-based products were suitable for the manufacture of film tablets and other coated dosage forms (401). The use of polyvinyl alcohol in preparing tablet coatings with and without sugar was described (402). Drug coating materials soluble in the small intestine were prepared from succinate monoesters or succinate maleate monoesters of cellulose ethers (403). Various quantities of cellulose acetate phthalate were applied to barium sulfate tablets, and the homogeneity of the films was assessed by a statistical study of thickness (404). The results showed a linear relation at constant pH between the resistance time and the quantity of the film applied per tablet.

Cellulose acetate phthalate was used to coat soft gelatin capsules repeatedly by dipping, and the resultant product was resistant to gastric fluid for 2 hr and to intestinal fluid for 20 min (405). A dipping experiment was also successful for enteric coating tablets (406). A fluidized-bed apparatus was used to apply various aqueous coatings such as cellulose acetate in ammonia solution, methylcellulose, and hydroxypropyl methylcellulose (407). The effects of solids loading on moisture permeability of methylcellulose–ethylcellulose films with two mineral talcs and titanium oxide were investigated (408). Water vapor transmission reduction was directly proportional to the amount of either talc added but reached a minimum at an intermediate solids loading value for titanium dioxide.

Table XII—Additional References on Tablet Coating

Reference	Topic
409	Gas permeability through poly(methyl methacrylate) films containing special fillers
410	Technological control of tablet coating
411	Polymer coating of tablets including acrylic tablet coatings
412	Formulation and process considerations of film coating
413	Review of manufacturing control of dragees and coated tablets
414	Review of various coating techniques including compression, film, and sugar coatings
415	Coating with acrylic resins
416	Coating with aqueous dispersions of acrylic resins

Additional references on tablet coating are listed in Table XII.

Suspensions—The techniques of suspension formulation and stabilization were reviewed; the general topics discussed were particle size and shape, surface-active agents, deflocculating agents, and the principles of rheology (417). The flocculation, dispersion, and caking in sulfonamide suspensions by a cationic agent were studied (418). A monolayer adsorption was reported for sulfisomidine, whereas multilayer adsorption was found for sulfadiazine, sulfaphenazole, and sulfameter (sulfamonomethoxine). The effects of two electrolytes, aluminum chloride and monobasic potassium phosphate, as well as two cationic surfactants, on the stability of a monodisperse system of calcium phosphate were investigated (419). The proper flocculating agent and concentration needed were established.

The flocculating properties of xanthan gum on suspensions of zinc oxide, sulfaguanidine, bismuth subcarbonate, and bismuth subnitrate were investigated under various conditions and in the presence of 1% potassium chloride, 1% calcium chloride, 25% ethanol, and 25% ethanol–1% potassium chloride (420). The studies indicated that xanthan gum acts *via* polymer bridging as an effective flocculating agent for some suspensions and that flocculation occurs at polymer concentrations well below those needed to produce a viscous suspending medium.

Additional articles on suspensions are listed in Table XIII.

Table XIII—Additional References on Suspensions

Reference	Topic
421	Effect of proportion of active ingredient added to a pomade suspension on extensibility
422	Effect of excipient on extensibility of pomade suspensions
423	Effect of powder level on extensibility of pomade suspensions
424	Preliminary study of use of glucomannan as a suspending agent
425	Calculation of kinetics of suspension coagulation
426	Homogeneity of pharmaceutical dispersed systems
427	Electrical properties of particle surfaces in pharmaceutical suspensions
428	Coarse dispersions, suspensions, emulsions, and lotions
429	Chloramphenicol (Levomycetin) suspension formulation for treatment of chronic dysentery

Emulsions—A review and discussion with 13 references on the experimental determination of the hydrophilic–lipophilic balance (HLB) value for a system and the selection of an emulsifier were presented (430). Another review with 40 references was given on the HLB, the phase inversion temperature, and the cohesive energy ratio systems for emulsifier selection (431). The problems posed by emulsion manufacture, especially the technical variables involved, were reviewed (432). A technique was described in which the addition of a thickener to an emulsion at the critical HLB allowed the preparation of stable emulsions

Table XIV—Additional References on Emulsions

Reference	Topic
448	Techniques of emulsification and equipment
449	Physicochemical discussion of emulsion instability
450	Structure of emulsions induced by nonionic emulsifiers
451	Review of emulsions in pharmacy including biopharmaceutical and cutaneous absorption aspects
452	Review of medicinal emulsions (146 references)
453	Effectiveness of emulsified sunscreen agents
454	Problems posed by emulsion technology
455	Survey of theory and practice of emulsions
456	Review of emulsions (72 references)
457	Transparent solutions of oils, perfumes, and aromas
458	Emulsion technology
459	Review of emulsions
460	Emulsion theory
461	Microemulsions
462	Microemulsion formation

(433). Linear HLB was defined, and its use in the preparation of emulsions and their evaluation were explained (434).

HLB values were determined by the Huebner method for 24 surface-active agents involving the following groups of compounds: oxyethylenated wool wax alcohols, fractions of liquid lanolin, oxyethylenated castor oil, oxyethylenated C_{10-18} aliphatic alcohols, and oxyethylenated alcohols of sperm whale oil (435). Phase inversion temperatures were determined for mixtures of several systems including cyclohexane–sorbitol–mono- and diglycerides of fatty acids (Arlacel 186) (436). Various methods used to determine the type and stability of emulsions were reviewed including phase coloring, the drop method, the dilution method, the conductivity method, and microscopic examination (437).

The influence of different thickeners on the characteristics of petrolatum emulsions at a critical HLB value was investigated; stable creams and ointments were prepared with stearyl alcohol or with carbomer 961 as adjuvants (438). The properties of emulsions containing liquid paraffin, glycerol, water, and blends of polysorbate 60 and sorbitan monooleate were studied over a temperature range of 25–80° (439). The formation of clear solutions at 70° depended on the polysorbate–sorbitan ratio and oil content, and the micelle diameter (13–45 nm) increased with increasing oil content and decreasing surfactant–glycerol ratio. A similar study was carried out on microemulsions containing sodium oleate, amyl alcohol, water, and liquid paraffin (440). The relative viscosity relationships for some of the microemulsions (440) were determined (441).

Polysorbate 20 and polysorbate 81 were compared as emulsifiers for carbon tetrachloride and C_5, C_6, C_7, C_{10}, and C_{16} saturated alkyl hydrocarbons (442). Microscopic examination and particle-size measurements were made, and polysorbate 20 was found to be a better emulsifier than polysorbate 81 on oil-in-water emulsions as the carbon number of the hydrocarbons in the dispersed phase became smaller. Nonaqueous emulsions of glycerin and mineral oil were formed using anionic, cationic, and nonionic surfactants (443). Although stable emulsions were prepared by employing surfactants from all three groups, more stable emulsions were obtained when ionic surfactants were used.

A method of measurement of the spontaneity of self-emulsifiable oils was developed (444). The system involved light scattering by dispersed oil droplets and collection of the scattered light by fiber optics onto an array of silicon photodiodes. A very stable emulsion of benzyl benzoate was prepared using triethanolamine oleic acid (445). The physical properties and the capacity of gas transport of fluorocarbon emulsions were reported (446). The manufacture and application of essential oil emulsions in microbiology were discussed (447). Thyme and verbena essential oils were used for the study.

Additional references on emulsions appear in Table XIV.

Semisolids—The quality and stability of mass-produced ointments were assessed (463), and the quality of rectal ointment bases was evaluated (464). Paraffin oil gels made with 5% polyethylene as a gel-forming agent were studied (465). The gels have low flow points and a low degree of thixotrophy, and their viscosities are only slightly influenced by temperature. The effect of adding up to 20% of six different sorbitan emulsifiers on the rheological properties of a microcrystalline paraffin and a white wax–cetyl stearyl alcohol mixture was determined (466). There were small differences in the rates of flow, deformations under load, and restoring tendency between the two sample ointment bases and between materials based on solid *versus* liquid emulsifiers.

Approximate solubilities in common ointment bases were given for salicylic acid, chloramphenicol, prednisolone and its hydrate, thiosinamine, and neomycin sulfate (467). Solubilities in components such as yellow petrolatum, emulsifying alcohols, paraffin waxes, and polyethylene glycols 600 and 1500 were also determined. Bubbles formed in ointments during manufacturing and their enlargement during storage were investigated (468). The influence of the concentration of polyethylene oxide of different molecular weights on the rheological index (degree of penetration) was studied (469). The use of propylene glycol in oral preparations, as a dermatological vehicle, and as a parenteral vehicle was reviewed (470). Its physical and chemical properties were given and its toxicology was discussed.

Ointments containing drugs in suspension were studied for dispersity by a photometric method and a microscopic method (471). The former procedure permitted determination of changes in dispersion caused by comminution, generally gave better precision, was simple and rapid, and required less time and effort. Separation of fluid from ointments was investigated by means of a test used for lubricating greases involving cone mesh filtration (472). The experimental data followed a mathematical relationship and correlated well with bleeding that occurred in the container during room temperature storage. The bleeding of hydrocarbon ointment bases was af-

fected by both the dispersing methods and the concentration of polyethylene (473).

The melting characteristics of ointments and creams were investigated by differential thermal analysis, and an interpretation of their structure was made (474). Another paper using similar methodology gave a further explanation of the melting behavior of semisolids (475). Two papers were concerned with the absorption of materials by gel bases. The consistency of petrolatum and polyethylene glycol gels was studied after addition of peanut oil and camphor (476). The amount of water uptake in mineral oil gels after addition of varying amounts of nonionic emulsifier was determined (477). Radiation had a relatively weak effect on yellow petrolatum, with viscosity being significantly reduced and penetrability and exudation being increased (478). With synthetic petrolatum, penetrability decreased and viscosity increased; polyethylene gels were more strongly affected than the petrolatums. The crystallinity of the solid phases in artificial petrolatum containing either paraffin or polyethylene was changed by extraordinary cooling rates (479). In every case where slow cooling was used, the gels showed microcrystalline structure of the solid phase and good ointment properties; these data were obtained by using a polarization microscope.

A general method using the solubility parameters of polymers was helpful for predicting the relative *in vitro* diffusion rate of an agent dissolved in a carbomer-based gel (480). A review on the selection of semisolid formulations, especially of corticosteroid compounds, discussed how to achieve maximum therapeutic effectiveness while maintaining an elegant and stable product (481). Models used in the description of diffusion processes were applied to drug release from semisolids (482). Two models, an exponential model for finite systems and a nonexponential model for semifinite and infinite systems, were evaluated for their suitability in describing literature data for the release of substances from semisolids.

The dynamics of drug release from ointment bases using the system of 2% salicylic acid, petrolatum, sorbitan monooleate, and 10% dimethyl sulfoxide were reported (483). The releases of a wide variety of common antibiotics from 26 ointment bases were studied and bases were recommended for each product (484). The release of benzocaine from oleaginous, absorption, emulsion, and water-soluble ointment bases *via* dialysis through a cellulose membrane to an aqueous sink was studied at 37.5° (485). The release of benzocaine from commercial and experimental preparations was compared. The diffusion of fluorescein from ointment bases containing methylcellulose was reported (486). Topical availability of hydrocortisone from different ointment bases was determined by the stripping method and reduction of erythema; brand products as well as experimental formulations were evaluated (487). In general, polyethylene bases, hydrogels, and petrolatum bases are biopharmaceutically poor vehicles compared to either oil-in-water or water-in-oil emulsions.

The effects of rheological properties of an ointment base and size of salicylic acid particles on its release from the ointment and absorption by skin were determined (488). Both *in vitro* and *in vivo* tests showed that the release rate and absorption of salicylic acid were mostly affected by the particle size and to a lesser extent by rheological properties. The release of papain from various ointments was increased by the addition of 5% sodium lauryl sulfate (489). The addition of 0.1% chlorocresol and 0.1% sodium metabisulfite suppressed oxidation.

Other references on semisolids are listed in Table XV.

Table XV—Additional References on Semisolids

Reference	Topic
490	Comparative vasoconstrictive activity of topical corticosteroid preparations
491	Sensory firmness and viscousness of cream base substances
492	Ointment bases containing methylcellulose solution
493	Alternative base for calamine cream
494	Cosmetic transparent gels
495, 496	Gel structure of ointments
497	Effect of additives on gelatin gel strength
498	Formulation and clinical evaluation of protective epidermal barriers
499	Ointment base containing polyethylene
500	Pastes, liniments, plasters, and similar drug mixtures known by names of their originators

Suppositories—Factors that affect absorption of rectally administered drugs and the considerations to be taken in suppository formulation were reviewed (501). Formulation and pharmacokinetic studies of suppositories, with particular emphasis on factors influencing drug absorption through the anorectal tract, physical–chemical properties, use of surfactants, and active ingredient concentration, were reviewed (502). Cumidin red was used to test the release rate of suppositories made with polyethylene glycols with stearin base and emulsifiers (503). The consistency of lipophilic suppository bases and drug-containing suppositories was evaluated by determination of the cone flow point, the breaking strength, and the disintegration time (504). Zinc oxide labeled with ^{113m}In was used as a tracer to determine the degree of mixing with cocoa butter (505). The degree of drug release from 10 suppository bases was determined using an apparatus consisting of a semipermeable membrane and peristaltic pump to provide circulation of dialyzing fluid (506).

The aminophylline content of commercial and of laboratory prepared suppositories, all kept at 18–27°, was analyzed periodically; all commercial products were chemically and physically unstable (507). The best results were observed in a laboratory composition containing hydroxylamine hydrochloride as a stabilizer in anhydrous suppositories. The preparation of teturam suppositories with fatty bases was reported (508). The oral absorption and rectal absorption of cystamine hydrochloride were compared, and the rate of drug release from various suppository bases was also determined (509, 510). Work on *in vitro* and *in vivo* drug availability from digitalis sup-

Table XVI—Additional References on Aerosols

Reference	Topic
525	Partition coefficients of fluorocarbon propellants in water, normal saline, cyclohexane, chloroform, human plasma, and human blood
526	Suitability of some plastics for preparing parts of aerosol containers
527	Cosmetic aerosol development: semisolid type of pressurized systems
528	Classification of pharmaceutical aerosols
529	Review of gas dispersions (aerosols and sprays)
530	Medicated dry foam for local therapy in infected burns

positories was reported (511, 512). The consistency of suppository bases containing polysorbate 20 and polysorbate 61 was evaluated (513).

Aerosols—A wide ranging review of pharmaceutical and cosmetic aerosols was published (514). An evaluation of inhalation aerosols using a simulated lung apparatus was reported, and the compatibility of ^{14}C-labeled phenylephrine hydrochloride in polyethylene nasal spray containers was presented (515). Pharmaceutical quality control of aerosol foams was discussed (516, 517). A review of aerosol medicinal foams with respect to structure, properties, and classification was reported (518); quick-breaking aerosol foams and their relation to cosmetics also were reviewed (519). Major factors affecting the time for a foam to break were: water–alcohol ratio, total alcohol content, type and amount of surfactant, and type of propellant. The development of pharmaceutical and cosmetic aerosol emulsions was explained, and the type of material obtained, foam or droplets, was dependent on whether the propellant was in the internal or external phase of the emulsion (520). Various factors, such as powder concentration, surface activity, propellant concentration, and orifice diameter, influencing the diameter of solid particles of an aerosol were investigated and a mathematical equation was developed (521).

The development and evaluation of an inhalation aerosol of nitroglycerin were reported (522). An *in vivo* method for determining effectiveness of spray-on bandages containing anti-infectives was developed (523). Standardized, contaminated wounds in guinea pigs served as the testing system, and the inflammatory response of the wounds was evaluated using several indexes including skin temperature, degree of induration, and presence of pus. A multistage liquid impinger was used to determine the particle-size distribution of aerosols generated by six pressurized inhalers containing isoproterenol (isoprenaline) (524). Inhalers of the suspension type had a higher percentage of size ranges likely to penetrate to the lower airways than the solution type.

Additional references on aerosols are listed in Table XVI.

Timed-Release Preparations—Various aspects of pharmaceutical preparations designed to give a prolonged effect were reviewed (531). The report included a discussion of depot preparations, methods of drug molding, biopharmaceutical and pharmacokinetic aspects of sustained release, and release by zero- and first-order kinetics from insoluble matrix compounds. Formulation of oral, long-acting, solid dosage forms was the topic of another article (532).

A number of papers discussed various mechanisms used to obtain controlled-drug release. Further work was reported on preparations obtained by flocculating an acrylic latex with a solution of an amine drug (533). The interaction between methapyrilene and latexes was studied, and it was found that a compressed mixture of the drug and polymer provided sustained release in GI fluids. The influence of shape factors on the kinetics of drug release from matrix tablets was reported in two papers (534, 535). The rate of drug release varied with tablet shape, but rate constants that were independent of shape were included in the expression. Therefore, it was possible to predict drug release from tablets having identical compositions but varying widely in shape and size. The release of salicylic acid and ephedrine was measured *in vitro* after compounding into tablets containing stearyl alcohol, and it was found that release profiles could be described by a nonlinear expression for both cylindrical and biconvex tablets.

Tablets containing quinidine compressed in a polyvinyl chloride matrix were exposed to γ-radiation, and the drug release was then determined (536). The rate of drug release was proportional to the radiation dose and was correlated to the degree of cross-linking of the matrix. Timed release of acetaminophen was observed from spherical polystyrene beads (537). Factors influencing the rate of release of ethynodiol diacetate, a synthetic progestin, from solid silicone polymer vaginal devices were evaluated, and the cumulative amount of drug released was proportional to the square root of time (538). The release of methapyrilene, pentobarbital, and salicylic acid dispersed in films composed of different ratios of hydroxypropyl cellulose and polyvinyl acetate was investigated (539). The results indicated that drug release followed a diffusion-controlled matrix model, where the quantity released per unit area was proportional to the square root of time. Release rates were also proportional to drug concentration but independent of film thickness.

Two articles reported on the use of microencapsulation for sustained or controlled release of a drug (540, 541). The method whereby an injectable product could be prolonged was reviewed (542). Two depot-type vitamin B_{12} preparations were studied *in vivo* over 2 years (543). One preparation gave satisfactory levels when injected once every 8 weeks, but the other preparation required more frequent dosing.

Dissolution studies were made on seven samples of propantheline tablets from six manufacturers (544). Considerable variation occurred between individual tablets of two samples, and one sample exhibited great delay (over 2 hr) in disintegration as compared to 4–34 min for the other samples. The effect of three factors—surfactant type, surfactant concentration, and time—on the release of sulfosalicylic acid from polyvinyl chloride matrixes was studied (545). Drug

Table XVII—Additional References on Timed-Release Preparations

Reference	Topic
550	Availability of phenformin in a sustained-release oral pharmaceutical form
551	Release of salicylic acid and sodium salicylate from tablets
552	Tetracycline sustained-release tablets
553	Nitrofurantoin release from polyvinyl acetate–crotonic acid copolymers
554	Prolonged-action sulfonamide preparations
555	Preparation of sustained-release ferruginous hematinics
556	Sustained-release tablet containing atropine sulfate, scopolamine (hyoscine) hydrobromide, and phenobarbital (phenobarbitone)
557	Delayed release of niacin (nicotinic acid) in compressed tablets
558	Sustained release for fertility control

release was proportional to the square root of time and could be controlled by the use of surfactants. Sustained-release pellets and capsules prepared with polyglycerol esters indicated that these compounds had significant dissolution-retarding properties (546).

A nonirritating coated tablet of potassium chloride containing a dissolution-retarding additive from aqueous solution was developed (547). Retardant materials evaluated were carnauba wax, agar, alginic acid, stearic acid, cellulose acetate phthalate, hydroxypropyl methylcellulose, polyethylene glycol 6000, and cetostearyl alcohol. The sintering of matrix tablets prepared from a vinyl acetate–vinyl chloride copolymer increased the tensile strength of the tablets but also increased the rate of release of potassium chloride from the matrix (548). Electron-scanning photomicrographs showed that sintering at 100° for 1 hr caused the polymer particles, which had been deformed during compression, to regain their shape. The strength of plastic matrix tablets (Duretter) was correlated to their likelihood of being recovered in the feces after administration to human subjects (549).

Other references on timed-release preparations, particularly on specific drugs, are listed in Table XVII.

Cosmetics—*Microbiological Contamination*—The uses of X-irradiation and chemical compounds for the preservation of cosmetic preparations were discussed (559). Chemical preservatives were examined in detail, and a table of dissociation constants of acidic preservatives was given. Various *Staphylococcus* strains were isolated from cosmetic creams and lotions, and the pathogenicity was determined by culturing in various media (560). Larger amounts of *Staphylococcus* with higher pathogenicity were isolated from less expensive products. Factors that affect microbial growth in various cosmetic and toilet preparations were discussed; these included temperature, pH, oxygen, surface tension, and water quality (561). A study concerned with the resistance of bacteria compared the relative merits of "in use" and laboratory methods for the evaluation of antimicrobial products (562).

Formulation and Technology—The uses, properties, and formulations of dermatological skin lotion vehicles were reviewed (563). Cosmetic dermatitis factors were discussed along with case histories of typical cosmetic products causing irritation and allergenic reaction (564). The pharmaceutical aspects of skin therapy including the management of skin disorders, dermatological vehicles, skin penetration, and percutaneous absorption were reviewed (565). Various problems of black skin were considered, and recommendations for lotion formulations were made (566). The relationships of estrogens and androgens to sebaceous gland function and various kinds of hair growth were explained (567). A review article on the substantivity of cosmetic ingredients to skin, hair, and teeth discussed the advantages and disadvantages of substantive effects, various ways of achieving them, and methods for detection and assessment (568). The necessary properties for the obtainment of a protective epidermal skin barrier were given (569). Three classes of formulations were evaluated: a gelled oil, a water-in-oil emulsion, and an oil-in-water emulsion.

By using basic diffusion theory, mathematical models were obtained that correlate moisture loss to the thickness of topical substances during actual field usage (570). Under these conditions, there was a thickness below which the product did not have a perceptible effect on transepidermal moisture loss rate. Two factors, elastic modulus and stress relaxation, were used to characterize the mechanical properties of the stratum corneum (571). Both properties depended on the moisture content of the stratum corneum or the ambient relative humidity. Water vapor absorption and water vapor transmission were also dependent on relative humidity. In another report by the same investigators, it was shown that cosmetic humectants increase transepidermal water loss *in vitro*, and an attempt was made to explain this phenomenon (572). A depilometer was designed to simulate practical use conditions as closely as possible (573). Thus, important formulation variables could be studied quickly; in-use tests gave good correlation with the depilometer findings.

The cuticle of human hair was isolated in bulk by a new method involving vigorous agitation of fibers in water (574). The cuticle fractions were shown to be of high morphological purity using various techniques of electron microscopy, and the significance of amino acid analyses was discussed. Papain and dithiothreitol were used to separate the A-layer and cell membrane complex from human hair cuticle (575). The digestion was followed gravimetrically, and an electron microscope examination of the digested hair sections demonstrated that the components were clearly separated. The gross and fine structures of hair were discussed, and excellent scanning electron microscope microphotographs were included with the article (576). Scanning electron microscopy was used to evaluate hair care products while the hair was still attached to the head (577).

The effect of critical micelle concentration (CMC) on the detergent action of shampoos at equivalent micellization indexes was studied (578). The amounts

Table XVIII—Additional References on Formulation and Technology of Cosmetics

Reference	Topic
588	Technical aspects in preparation of toothpaste
589	Harmful metals in a cosmetic product containing inorganic pigments
590	Efficiency of chlorhexidine in mouthwashes
591	Formulation and properties of lipsticks
592	Shampoo preparation made from diethanolamine salt of synthetic fatty acids
593	Significance of vitamins for healthy and diseased skin
594	Composition of some natural waxes
595	Crystallization of cetyl alcohol from cosmetics
596	Alkaline and acid hand cleaners
597	Skin lotions in aerosol forms
598	Reconstituted triglycerides in cosmetic lotions
599	Poloxamers (Pluronic polyols) in skin lotions
600	Dermatological skin lotion vehicles
601	Progress in perfumery materials
602	Beeswax–borax reaction in cold creams
603	Brominated salicylanilides and their structure–activity relationships
604	Moisturization of stratum corneum
605	Cationic lotion formulation
606	Sunscreen lotions containing various natural oils
607	Skin lotions for black skin
608	Protein derivatives in cosmetics
609	Pearl pigments in skin lotions
610	Lanolin derivatives in skin lotions
611	Fatty acid amido alkyldimethylamines as cationic emulsifiers in skin formulations
612	Use of propoxylates in skin lotions
613	Use of reconstituted triglycerides in cosmetic lotions
614	Skin lotions in aerosol form
615	Dermatological skin lotion vehicles and formulations
616	Cosmetic gel systems from licorice root
617	Vegetable oils in lotions
618	Sucrose esters in skin lotions
619	Nonionic surfactants in skin lotions
620	Formulation of pigmented cosmetic lotions
621	Use of glucam methyl glucoside in skin lotions
622	Carbomer resins in moisturizing lotions
623	Use of fatty alcohols in skin lotion formulation
624	Properties of mineral oils and petrolatums used in skin lotions
625	Alkanolamine use in skin lotions
626	Natural protein from oats in cosmetic formulations
627	Review of solvents used in cosmetics
628	Emulsion formation with mixed nonionic surfactants
629	Gum usage in drug, cosmetic, and food industry
630	Use of natural products in cosmetic and toiletry preparations
631	Panthenol in cosmetics
632	Lecithin composition, properties, and use in cosmetic formulations
633	Isostearics in cosmetic formulation
634	Effect of simple aluminum compounds on mammalian epidermis
635	Technological properties and quality assessment of petrolatums
636	Surfactants and emulsifiers used in dermatology and cosmetics

of surfactants required to clean greasy hair at physiological standards were calculated (579). Two modes of analysis were utilized to evaluate antidandruff shampoos on groups as small as 10 persons (580). One method was subjective, and the amount of scaling was scored on a 0–10 scale; the second method involved the counting of the number of horny cells produced using a hemocytometer.

Two techniques were used to demonstrate the penetration of proteins into hair strands (581). One method involved chemical analyses of hair scrapings, and the other method involved staining cross sections of hair with ninhydrin. A second paper on factors controlling the action of hair spray investigated the adhesion of hair spray resins to hair fibers (582). The properties of the resin and solvent system were important for the ability of the hair to withstand impact and bending.

A standardized swabbing technique was developed to evaluate changes of axillary microflora, and seasonal differences were found in levels of some bacteria (583). A new method of deodorization was suggested; it involved clathrate formation using zinc ricinoleate and 0.1–2% synergistic additives such as polyhydroxylated fatty acids or resin acids (584, 585).

The history of sunscreen preparations was given, and several natural and synthetic compounds were evaluated and compared (586). A new sunscreen containing 4-(3′,4′-methylenedioxobenzylidene)-1-benzyl-2,3-dioxopyrrolidine in combination with mexenone (Uvistat 2211), titanium dioxide, and burnt sugar solution was suitable for patients who display abnormal sensitivity to UV irradiation (587).

Additional references on formulation and technology of cosmetics are listed in Table XVIII.

Packaging—The requirements for quality, packaging, and transportation of active materials and adjuvants used in the pharmaceutical industry were presented (637). Another review discussed the types of materials that should be considered for pourable liquids, sterile products, and semisolid and solid dosage forms in bulk and unit dose containers (638). Use of glass, metal, and plastics was explored; each group was described with reference to their advantages and disadvantages. The progress in the development of child-resistant packaging systems was explored (639). Three papers discussed various aspects of the use of polyvinyl chloride and plastics as packaging materials. Physicochemical control of plastic materials used in the packaging of unit dose drugs was reviewed (640). The toxic effect of various additives used in the manufacture of polyvinyl chloride was discussed (641), and the importance of physical and chemical tests was stressed (642). A paper from the Netherlands reviewed pharmacopeial requirements, including chemical and toxicological testing, of plastic materials used as containers for intravenous injections (643).

The economics of blister and skin packaging were reported (644), and the technology of shrink wrapping, particularly for toiletries, was discussed (645). A comparative test for the evaluation of the tightness of various prescription containers was developed (646). The test consisted of placing a weighed amount of calcium chloride in a container, closing it, and following the weight gain after storage in an 86% relative humidity chamber. Glass vials with lined phenolic screw-capped closures provided the best protection, while polystyrene vials with a child-resistant closure were the least effective. The unit packaging of solid dosage forms, powders, and granules in blisters or flexible pouches was discussed (647). A

new method of hermetically packaging solid drugs was tested and found to be satisfactory (648). The method involved "cold welding" of aluminum foil under pressure. The packaging of suppositories using various plastic materials was reported (649). A survey of cartoning equipment and a buyer's guide to equipment now on the market were presented (650).

Plastic packaging of infusion and injection solutions was discussed, with particular reference to polyethylene, polypropylene, and polyvinyl chloride (651). The major factors involved in the design and manufacture of packaging to preserve sterility of the contents were enumerated; these included the suitability of the packaging material for sterilization, the resistance of the material to bacteria, the type and strength of the package, and the type of package opening (652). Several approaches to meeting the needs of users of small volume, prefilled, syringe products were presented (653). Development aspects unique to small volume, prefilled, disposable syringes were enumerated. Two-compartment injection systems were described together with their inherent advantages for powder or lyophilized formulations (654).

Instruments and Equipment—A continuing series of articles concerned with pharmaceutical engineering presented the fundamentals of laminar flow (655) and the principles of fluidized beds (656). The ideal physical plant conditions of temperature and humidity in a number of specific pharmaceutical operations were presented (657). The quantity and quality of the air in the plant were discussed with respect to particle content and the means of controlling it.

Various papers were concerned with particular pharmaceutical processes such as drying, coating, and metal particle detection. An accurate automatic control for dryers was devised (658). Equipment available for the polymer film coating of tablets by coating pan, fluidized-bed, spraying, and dipping techniques was examined and compared (659). Tablets and granules of any shape were coated using a new machine equipped with two brush rollers (660). Coating material was fed to the brushes by a roller which was in contact with a reservoir; the equipment was capable of coating tablets with as little as 1 mg of coating material/tablet.

A survey of pumping technology was presented, and some recent developments were of particular importance to the pharmaceutical industry (661). An instrument was developed that automatically detected small metallic particles possessing ferro-, para-, or diamagnetic properties (662). A review of the practical applications of spray drying was presented with a detailed description of the spraying agents used (663). A report appeared on the spray drying of aspirin (664). Scanning electron microscope and electron diffraction studies of spray-dried aspirin with methylcellulose revealed that a noncrystalline amorphous sample of the drug was formed at a 70% polymer concentration. Increased amounts of aspirin in the product led to the formation of crystalline products.

The developments in mixing and grinding technology were examined, and machine and design changes were emphasized (665). A Nauta mixer was evaluated by mixing a tablet preblend containing 10% phenobarbital (phenobarbitone), 1% secobarbital (quinalbarbitone), and 1% butethal (butobarbitone) with 88% lactose (666). Each component behaved in a unique manner during mixing, with adequate mixing occurring at different times.

An instrument was described that will produce a standardized skin abrasion test for skin irritants (667). The same degree of abrasion can be reproduced without bleeding. Further work on the evaluation of vaginal odors by means of GLC was reported (668). Refinements in methodology allowed the technique to be sufficiently sensitive to indicate significant ($p < 0.05$) changes in 10 subjects.

Additional references on instruments and equipment are listed in Table XIX.

Table XIX—Additional References on Instruments and Equipment

Reference	Topic
669	Industrial monitoring of particle size of antibiotic powders
670	Comparison of final filtration devices
671	Heat production by ultrasonic equipment
672	Validation and bioengineering aspects of implantable glucose sensor
673	Continuous intragastral pH measurement after administration of antacid drugs

PHYSICAL PHARMACY

Several review articles were devoted to polymorphism where various polymorphs were characterized by X-ray diffraction patterns, IR spectrophotometry, and dissolution rates. Dissolution rate is the most important characteristic in pharmaceutical formulations because it affects the bioavailability of various formulations (674, 675). The thermal analysis of pharmaceuticals including solvation, polymorphism, impurities, interactions, stability, and compatibility was also discussed (676). The relationships of various polymorphic forms of pharmaceuticals to their biological activity were reviewed (677). Phase solubility in combination with IR spectrophotometry was used for studying the polymorphism of progesterone, cortisone acetate, and prednisolone (678).

Upon crystallization from various solvents, barbital, amobarbital, butethal (butobarbital), hexobarbital, and phenobarbital were found to be polymorphic by thermomicroscopy, microsublimation, and X-ray diffraction (679). The effect of polymorphism on the dissolution behavior and GI absorption of chlortetracycline hydrochloride was studied (680). After oral administration to humans, the cumulative amounts of chlortetracycline hydrochloride excreted by two polymorphs were compared. The results suggest that the more soluble β-form was absorbed much faster than the α-form and was more bioavailable than the α-form. Depending on the method of preparation, hydroflumethiazide crystallized as an anhydrous form, a desolvated crystal species, and an ethanolic solvate, which, under normal atmospheric conditions,

converted to the desolvated crystal, losing all except a small fraction of the solvent from the crystal lattice (681).

Two new polymorphic modifications and a solvent-containing form of indomethacin were described and characterized by their thermal data, IR spectra, and solubilities (682). Solid-state studies were done on the polymorphs of methylprednisolone (683). The metastable crystalline form underwent a transformation to a form other than the stable Form I upon exposure to high humidities, moderately high temperatures, or UV radiation.

Cameroni and coworkers studied the polymorphism of progesterone. In their first paper, they reported on the preparation and characterization of the various polymorphs (684). They studied the use of differential calorimetry with various progesterone crystalline forms (685), and the solubility and thermodynamic data of two crystalline forms were also developed (686). The densities, refractive indexes, and X-ray diffraction measurements of four polymorphic forms of sulfanilamide were studied (687). The data suggested that the forms have very similar structures.

Effects of some process conditions on the particle size and polymorphic form of phenobarbital (phenobarbitone) prepared by acid–base precipitation were studied (688). The presence of water profoundly influenced the behavior of the solid product, and the significance of this phenomenon was discussed. More than 20 progesterone steroids and 30 testosterone-type compounds were tested for hydrate formation to evaluate their stability in solutions and in pharmaceutical preparations (689). Hydrates were observed with 21-acetoxypregnenolone, fluorogestone acetate, 4-chlorotestosterone, testosterone, and stanozolol.

A review of mass transport phenomena and models covered passive diffusion, diffusion in an isotropic medium, diffusion through laminated structures, the influence of some specific permeant and barrier properties on mass transport including diffusant solubility as a flux-limiting factor, and factors affecting diffusivity (690). The effect of sodium carboxymethylcellulose on sodium salicylate transport was studied (691). The drug release rate out of the polyelectrolyte solution was found to be faster than that without carboxymethylcellulose. This transport-enhancing action of sodium carboxymethylcellulose was probably due to the electrical charge between the coion and the polyelectrolyte.

By means of a diffusion cell consisting of two compartments partitioned with a cellophane membrane, the influence of surfactants, thickening agents, and pH on the rate of drug transport of three flavonoid compounds was studied (692). Association and complexing of oxytetracycline in its solutions at pH 1.65 were studied by diffusion transport through inert membranes (693). Association occurred in solutions $>5 \times 10^{-3}$ *M*. The diffusion of aerosols was tested at low temperatures where classical Brownian movement theory is not applicable (694). At room temperature, the diffusion coefficient calculated from experimental penetrations agreed with the theoretical coefficients. However, at −16 and −72°, the experimental coefficients were 150–500% greater than the theoretical values.

Table XX—Additional References on Physical Pharmacy

Reference	Topic
697	Physicochemical properties of amitriptyline hydrochloride
698	Physicochemical properties of digitoxin
699	Physicochemical properties of diphenhydramine hydrochloride
700	Physicochemical properties of echothiophate iodide
701	Physicochemical properties of nifurpipone
702	Physicochemical properties of oxazepam
703.	Physicochemical properties of phenylephrine hydrochloride
704	Physicochemical properties of trimethaphan camsylate
705	Physicochemical properties of tolbutamide
706	Physicochemical properties of tropicamide
707	Review of particulate dispersions and colloids
708	Review of nonaqueous systems
709	Dissociation constants of oxazolidines of ephedrine and pseudoephedrine
710	Influence of anions on physical properties of butyrophenone-type molecules
711	Characterization of phenobarbital (phenobarbitone) samples
712	*In vitro* investigations of antacids
713	Potentiometric study of hydrogels of carbomer 940
714	Factors affecting pharmacodynamic activity of drugs in ointments
715	Physicochemical study of phenobarbital–urea system
716	Effect of washing on physicochemical properties of aluminum hydroxide gel
717	Comparison between theoretical and experimental electric dipole moments of selected *N,N*-dimethylaniline derivatives
718	Measurement of drug displacement by continuous ultrafiltration
719	Interconversion of ampicillin and hetacillin *in vitro*
720	Role of physical properties in formulation and manufacture of drugs

Microencapsulation of solid stearyl alcohol particles by complex coacervation was studied (695). Wall thickness decreased with increasing ratios of solid particles and increased as the particle diameters increased. Microencapsulated aspirin with ethylcellulose was examined as to its dissolution rate in pH 1.1–8.3 media (696). The rate was slower in acidic pH. Release of aspirin from the microcapsule was also examined microscopically. Aspirin leached out of the capsule, leaving the capsule membrane almost intact.

Additional references relating to physical pharmacy are listed in Table XX.

Dissolution—Phenylpropanolamine hydrochloride was found to be released from a typical wax matrix by a diffusion mechanism (721). After an initial rapid release of drug from the tablet, the amount dissolved was proportional to the square root of time. Dissolution of human gallstones was evaluated in a water solution containing a mixture of limonene with polyoxyethylene hydrogenated castor oil and ethanol; the solution was able to dissolve completely 1.4–3.2 g of cholesterol-containing human gallstones in 2 hr *in vitro* (722). Bile salt solutions slowly dissolved cholesterol gallstones *in vitro*, chenodeoxycholate and

deoxycholate being about fourfold more potent than cholate (723).

Experimental results suggested that various lots of drug formulations that meet compendial dissolution requirements may contain fractions of poorly soluble tablets (724). If the assumption is made that dissolution behavior and drug absorption *in vivo* are related for certain drugs, such formulations give rise to suboptimal therapy. A modification of the compendial test was presented with the objective of increasing the probability of identifying lots containing a fraction of poorly soluble tablets. The dissolution rate of nitrofurantoin from commercial suspensions and tablets containing microcrystalline drug particles and from capsules containing macrocrystalline drug particles was determined at 37° in simulated gastric and intestinal fluids using the stirrer-flask method (725). The dissolution rate of nitrofurantoin from the suspension and capsule dosage forms at pH 7.2 was significantly faster than at pH 1.12. In contrast, the drug from the tablet dosage dissolved in the pH 1.12 dissolution medium at a rate twice that observed at pH 7.2.

A nonlinear expression describing the dissolution of drug particles under sink conditions and low agitation intensity was presented (726). By using the beaker method, the dissolution of salicylic acid particles having a relatively narrow size distribution was followed to nearly complete solution. It was found that the process could be described by the nonlinear expression and that a dissolution rate constant having the dimension of reciprocal time could be obtained. Dissolution rates of salicylic acid in micellar solutions of polysorbate 20 were determined at pH 1–4 (727). At any one pH, as the concentration of the polysorbate 20 was increased up to 12% (w/v), the dissolution rate increased; but the further increase up to 20% (w/v) polysorbate 20 only slightly increased the dissolution rate. At any one concentration of polysorbate 20, the dissolution rate increased linearly as the pH was increased.

The effects of particle size on dissolution and GI absorption rates of drugs were reviewed (728). When the influence of particle size on dissolution behavior of sulfonamides was evaluated, it was found that at a particle size below 300 μm, the lines obtained by plotting dissolution rate *versus* time had positive intercepts; at above 300 μm, the lines passed closely through the origin (729). The findings indicated that the critical particle size is at about 300 μm in the initial dissolution process.

It was shown that dissolution rates and equilibrium solubilities of digoxin samples before and after dry grinding in a mortar mill may influence dissolution behavior (730). The need for a specification for digoxin that not only controls surface area but also exercises some control of the crystal properties was suggested. The influence of the tablet manufacturing method and drug particle size on the dissolution rate was studied (731). The rate of dissolution of the active ingredient was faster with a particle size of 50 μm than for formulations made with particle sizes of 180–200 μm.

Dissolution characteristics of three crystal forms of aspirin were evaluated (732). The dissolution process of the highest melting form could be represented by the general Noyes–Whitney equation, which assumes that diffusion is the rate-controlling process; the dissolution of the lower melting forms did not obey this model so it was necessary to postulate that the interfacial reaction was not instantaneous. Bundgaard (733) claimed that the differences in the rates of dissolution of aspirin were due to differences in the content of the acetylsalicylic anhydride impurity. When using the rotating-disk method, it was shown that the dissolution rate of aspirin was independent of crystal growth, salicylic acid content, habit, and particle size (734). The dissolution of the eutectic mixtures of aspirin and urea, when evaluated in 0.1 *N* hydrochloric acid, was about the same as pure aspirin; in the 35% solid solution, the dissolution was appreciably enhanced (735).

Shah and Ochs (736) designed and evaluated the rotating-filter–stationary basket *in vitro* dissolution test apparatus. An automated procedure for the determination of *in vitro* dissolution rates from two-component (outer layer plus sustained-release core) sulfonamide tablets was described (737). The dissolution rates *in vitro* for film-coated tablets were dependent on the solubility of the film. An automated dissolution rate apparatus, meeting requirements of the USP–NF dissolution test and applicable to various other agitation systems in common usage, was described (738). The equipment allows simultaneous determination of the dissolution *rate of six* unit doses, enabling the evaluation of a statistically significant number of samples.

Dissolution rates of eight products containing levodopa in three strengths, made by two different manufacturers, were determined using a continuous-flow dissolution apparatus (739). Significant difference was observed in the 250-mg capsules made by different manufacturers. A significant difference also was observed in a 250-mg tablet and a 250-mg capsule of the same manufacturer. Comparisons were made between different methods for the determination of dissolution rates of drugs from tablets using the USP XVIII method and the beaker method (740). The reproducibility of the USP method was better than or equal to the reproducibility of the beaker method. Comparisons of dissolution profiles of tablets and capsules were made using the USP, Levy, and three different size magnetic basket methods (741). The five different dissolution methods produced significantly different dissolution profiles at selected times for both the tablets and capsules studied.

By using the USP method and the shaking beaker method, the dissolution rate of methandrostenolone and phenobarbital tablets was studied (742). The USP method gave a greater dissolution rate. A theoretical model of dissolution was developed in terms of the diffusion layer theory (743). This model was programmed on an analog computer, and theoretical dissolution curves were obtained. Experimental dissolution curves were compared with simulated curves, and the fit of the data allowed the estimation of the

Table XXI—Additional References on Dissolution

Reference	Topic
744	Dissolution kinetics of sulfathiazole Form I
745	Method of testing applicability of diffusion layer dissolution model
746	Influence of dissolution rate of lithium tablets on side effects
747	Influence of drug concentration on aggregation and *in vitro* dissolution rate of tablets
748	Use of porous matrix substances to increase dissolution rate
749	Increasing the dissolution rates of some corticosteroids utilizing glass dispersions and partial solid solutions
750	*In vivo* significance of dissolution test procedures for evaluation of drug products
751	Dissolution profile of log-normal powders, and dissolution before critical time
752	Dissolution of lithium and magnesium from lithium carbonate capsules containing magnesium stearate
753	Influence of dispersion method on dissolution rate of digoxin–lactose and hydrocortisone–lactose triturations
754	Thermodynamics of dissolution of salicylamide by a nonionic surfactant
755	Rate studies on dissolution and enzymatic hydrolysis of chloramphenicol palmitate
756	Methods for determination and criteria for expression of dissolution rate
757	Solvent effects on comparative dissolution of pharmaceutical solvates
758	Digoxin tablet dissolution
759	Dissolution rate of aminopyrine–barbital molecular compounds
760	Dissolution–dialysis and absorption aspects of some pharmaceutical systems
761	Dissolution and dialysis aspects of molecular complexes
762	Liquid–liquid mass transfer studies, and their relationship to solid dissolution

drug concentration in the diffusion layer.

Table XXI lists additional references on dissolution.

Solubility–Solubilization Phenomena—Cholesterol solubility in model bile systems was evaluated (763). It was found that there were significant behavioral differences in the rate of solution with the various crystalline forms. The solubility of the equilibrium species, hydrated cholesterol, and of fresh human gallstones was found to be approximately 5 mole %. The surface areas for 73 aliphatic hydrocarbons and alcohols, for which solubility and boiling point data are available, were calculated (764). The solubility of the alcohols and hydrocarbons could be quantitatively related to a combination of the hydrocarbon and hydroxy surface areas of the molecules.

The solubility of antipyrine derivatives was determined in water and several organic solvents (765). Aminopyrine and isopropylantipyrine exhibited solubility minimums near 25 and 15°, respectively, corresponding to a zero heat of solution. The rates of solution in butanol of compressed plates of modifications I–III of sulfanilamide were determined along with the solubilities at various temperatures (766). A method of calculating heats of transition from the rates of solution was also described. Differential scanning calorimetry was used to determine the solubility of the metastable form of testosterone (767).

Selected surfactants, above and below the CMC, were evaluated for their solubilizing effects on suspensions of aspirin crystals (768). Ranked in order of decreasing solubilizing effectiveness were: cetylpyridinium chloride > polysorbate 20 > benzalkonium chloride > polysorbate 80 > dioctyl sodium sulfosuccinate. The effects of sodium chloride and urea, separately and together, were evaluated on the solubility of methyl *p*-hydroxybenzoate in water (769). Sodium chloride decreased and urea increased the solubility. Solubilizers formed soluble and/or insoluble complexes with tetracycline and its derivatives (770). In all cases, oxytetracycline interacted less than tetracycline. The solubilization of the surfactants was assumed to be due to normal micellar solubilization rather than complexation.

The cosolvent capacity of 1,2-propylene glycol, 1,3-butanediol, and 2,3-butanediol in the solubilization of phenylethylbarbituric acid was studied (771). The solubilizing effect of the aqueous dispersions obtained with these polyols was mainly due to the modification of the dielectric constant of the water, with existence of a complex mechanism, rather than to the direct action of the polyol itself. Solubilities of certain barbiturates in poly(oxyethylene) monoalkyl ethers of varying hydrophobic chain length were studied (772). Solubilization increased with the increasing hydrophobic chain length of the solubilizer due to formation of larger micelles. The effect of monosaccharides on the solubility of barbituric acid and its derivatives was studied (773). The solubility of barbituric acid increased with the increasing monosaccharide concentration, the increase being more pronounced in solutions containing galactose. Calcium *p*-aminosalicylate formed an isonicotinic acid hydrazide–calcium chelate in aqueous solution with isonicotinic acid hydrazide (isoniazid) and resulted in monocalcium *p*-aminosalicylate with increased solubility (774).

Other papers concerning solubility–solubilization phenomena are listed in Table XXII.

Membrane Permeation and Release—The per-

Table XXII—Additional References on Solubility–Solubilization Phenomena

Reference	Topic
775	Solubility of amorphous phase of caffeine, theobromine, theophylline, morphine, and heroin (diacetylmorphine) in water
776	Solubility of sulfanilamide in water
777	Solubilization of liquid paraffin by mixed nonionic surfactant system
778	Solubilization of cresol, chloroxylenol, oils, and vitamin preparations
779	Solubilization, emulsification, and dispersion with surfactants in parenteral preparations
780	CMC and solubilizing capacity of some surfactants
781	Solubilization of cholesterol and cholesterol esters by synthetic surfactants
782	Solubilization of perfume oil with HLB principle
783	Effects of polyvinylpyrrolidone on solubility and dissolution rate of allopurinol
784	Thermodynamic parameters for solubilization of some steroids by nonionic surfactants
785	Thermodynamic stability of solubilized solutions
786	Relationship of composition of nonaqueous binary solvent systems and dielectric constant

meability of films of ethylcellulose and polyethylene glycol to caffeine was studied (787). The rate of caffeine transfer across the films increased linearly with the reciprocal of thickness and caffeine concentration. The permeability of plastics, in particular, in relation to drug products with regard to physical factors and to intrinsic and total permeability was evaluated (788). Gravimetric, manometric, and hygrometric methods were used for the permeability evaluation. Comparisons of dimethylpolysiloxane and trifluoropropylmethylpolysiloxane systems were made, using nicotine base as the permeating species (789). The drug permeability through dimethylpolysiloxane was >20 times more rapid than through the trifluoropropylmethylpolysiloxane system.

The permeation of phenylbutazone through polydimethylsiloxane was studied under conditions of varying membrane thickness, stirrer speed, and pH (790). The results were described in terms of the two-phase model of general transport theory and provided experimental evidence of its usefulness. The influence of surfactants on the transfer of salicylate and salicylamide was studied in everted rat intestine (791). Sodium tauroglycocholate, sodium oleate, sodium lauryl sulfate, polysorbate 20, and sorbitan monolaurate increased the transfer rate of both sodium salicylate and salicylamide across the everted rat intestine.

The effects of pH and various substances on the permeation of phenylbutazone through the everted rat intestine and polydimethylsiloxane were studied (792). The classical pH–partition hypothesis was not obeyed. Phenylbutazone permeation rates in one or both membranes decreased in the presence of gelatin, methylcellulose, polyvinylpyrrolidone, skim milk, albumin, sodium lauryl sulfate, polysorbate 80, and cetrimonium bromide. The effects of excipients and various additives on the permeation of chlordiazepoxide through polydimethylsiloxane membranes also were studied (793). No effect on the permeability coefficient was observed in the presence of starch, calcium hydrogen phosphate, gelatin, lactose, silica, sucrose, cholesterol, or porcine mucin. The coefficient increased in the presence of magnesium stearate and decreased with talc, sodium saccharin, skim milk, egg lecithin, beef albumin, egg albumin, polysorbate 80, cetrimonium bromide, and sodium lauryl sulfate, indicating an interaction of these substances with the drug.

The effect of hyaluronidase on the activity of two ointments containing antibiotics was studied by the evaluation of the active transport of sodium through the isolated skin of frog and by the measurement of the current necessary to cancel the electrical potentials of the membrane covered with ointments (794). The antibiotics evaluated were tetracycline, chloramphenicol, neomycin, erythromycin, nystatin, streptomycin, kanamycin, griseofulvin, and penicillin. Interrelationships between skin, vehicle, and drug during penetration into skin were reviewed (795).

The permeation of water vapor through cellulose acetate phthalate polymer films applied in various ways to hygroscopic solid surfaces was determined at several different temperatures (796). The data indicated that the activation energy of the absorption processes was determined by the film, while the method of film application affected the actual rates of permeation at a given temperature.

Models used in the description of diffusion processes were applied to the drug release from semisolids (797). Two models, an exponential model for finite systems and a nonexponential model for semi-infinite and infinite systems, were evaluated for their suitability in describing literature data for release of substances from semisolids. The exponential model derived for finite systems was found applicable in all of the literature cases, while the nonexponential model had restricted applicability. The effects of systematic alteration of vehicle composition on the release rate of drug from their vehicle were studied (798). Equations were developed that quantitatively predicted the rate of transport of a drug across a membrane separating two identical binary aqueous solvents. Separate equations were derived for solutions and for suspensions; they accounted for both the resistance of the membrane and the resistance of the solvent to drug diffusion.

The release of sodium lauryl sulfate and cetrimide from ointment bases containing complex emulsifiers was studied (799). Cetostearyl alcohol blocked the release of sodium lauryl sulfate into agar, but doubling the sodium lauryl sulfate concentration enhanced its release. A stabilized shea butter in the form of an ointment base was developed and found to release medicaments at a faster rate than the British Pharmacopoeia ointment bases. The assessment of release of medicaments by microbiological method and through a dialysis membrane gave promising results (800). The rate of drug release from a gel medium was determined by placing a salicylic acid-containing gel in a 1-cm diameter hole in a ferric chloride-containing agar plate and measuring the colored ring formed. Two gels were used, one based on liquid paraffin and the other on castor oil. The gel based on castor oil was more viscous but released salicylic acid faster.

The effects of various sorbitan surfactants on drug release were determined (801). The drug release increased with increased HLB value and decreased with increased chain length. Effects of polysorbate 80, sorbitan monostearate, water, and physicochemical structure of the ointment on potassium iodide release from lard and white petrolatum were studied (802). Addition of 3% polysorbate 80 retarded potassium iodide release from lard but accelerated the release from white petrolatum. A reverse effect was observed with a 3% addition of sorbitan monostearate. Release of iodine in a potassium iodide solution was studied from ointment bases, comprised of petroleum or a melt of petroleum with sunflower oil and various oxyethylenated aliphatic alcohols as emulsifiers, into an agar gel (803). The bases were water-in-oil emulsions. Release of iodine increased with the increasing water content of the base and decreased with the increasing number of carbon atoms in the hydrophobic portion of the molecule of an emulsifier.

The releasing ability of four different ointment bases containing salicylic acid was evaluated by an *in vivo* test on rats and by four different *in vitro* tests (804). The salicylic acid releases from the various bases in the *in vivo* test decreased in the following order: Unguentum leniens, Unguentum emulsificans hydrosum, Unguentum alcoholum lanae hydrosum, and Unguentum macrogoli.

The effects of small-scale preparation techniques on the diffusion of salicylic acid from various ointment bases were studied (805). The method of mechanical incorporation of the drug in cold petrolatum base, using a spatula, appeared to result in higher rates of salicylic acid diffusion than those encountered with ointments prepared by fusion, regardless of drug concentration and the presence or absence of a surfactant. The drug release pattern of micronized ethynodiol diacetate from silicone devices was investigated in polyethylene glycol containing elution media with a wide range of solubility and partition properties (806). When high drug solubility was maintained, the drug release pattern was matrix controlled. As the drug solubility in the elution medium was decreased, the drug release process shifted from matrix controlled to partition controlled.

The *in vitro* release pattern of nitrofurantoin embedded in polyvinyl acetate–crotonic acid copolymers was studied in artificial intestinal juice (807). *In vivo* studies were also done on conventional and slow-release nitrofurantoin tablets. Both *in vivo* and *in vitro* results indicated that the copolymer with 8% crotonic acid was effective for prolonging the release and excretion of nitrofurantoin. The water solubility and chemical reactivity of hydrocortisone and hydrochlorothiazide were studied, and the type of matrix useful for their preparation as a sustained-release dosage form was determined (808). Acetohexamide incorporated into silicone rubber disks was released into water in microgram quantities for over 6 months (809). Based on these data, these disks were recommended as sustained-release polymeric capsules for acetohexamide.

The effect of some variables involved in the manufacture of nylon microcapsules *via* an interfacial polymerization on the release rate of core material from the microcapsules was evaluated (810). The presence of emulsifiers in the external phase had no significant effect on the release of core material; sodium hydroxide, as an acid acceptor, did not enhance the retentive properties of the microcapsules; and the choice of organic solvent not only affects the release of the core material but may govern the collection method.

Additional references on membrane permeation and release are listed in Table XXIII.

Table XXIII—Additional References on Membrane Permeation and Release

Reference	Topic
811	Properties and drug permeabilities of poly(vinyl alcohol)–oxystarch membranes
812	Drug permeation through artificial membranes
813	Problems on ocular penetration of antibiotics
814	Correlation between *in vitro* and *in vivo* availability of salicylic acid for membrane transfer from polysorbate 20 solutions
815	Reverse osmosis separation of solutes from aqueous solution
816	Model transport studies utilizing lecithin spherules
817	Rate of dialysis of chemicals through semipermeable membrane with varying concentrations of polyvinylpyrrolidone
818	Influence of surfactants on dialysis of drugs through artificial membranes
819	Release of boric acid from a base with silicone, paraffin, and olive oil
820	Release of salicylic acid, chloramphenicol, and prednisolone from ointment bases
821	Influence of packing on the release of salicylic acid from dimethyl sulfoxide-containing ointment bases
822	Effects of various factors on velocity of drug release from isoniazid repository tablets
823	Drug liberation of meprobamate–dihydroergocristine from retard tablets
824	Influence of solute properties on release of *p*-aminobenzoic acid esters from silicone rubber

Complexation—Studies related to complexation phenomena are categorized into: *(a)* interactions of drugs with biological substances, and *(b)* interactions of drugs with nonbiological substances.

Interactions of Drugs with Biological Substances—A quantitative comparison of various methods of fundamental binding parameter estimation was presented (825). For optimal reliability, the choice of a particular approach should be made on a critical assessment of the experimental procedure and various calculated statistical criteria rather than on the ease of data treatment. By using nonlinear regression, an improved parameter estimate in drug protein binding studies was devised. In addition, a kinetic approach to drug–protein binding was developed (826). Computer analysis of drug–protein binding data was evaluated (827). A stepwise equilibrium model was recommended as the standard procedure for the investigation of drug–protein interactions.

The binding constants and the number of binding sites for the binding of 11 sulfonamide and seven penicillin derivatives to bovine serum albumin were determined using a fluorescence probe method (828). The results demonstrated that these drugs bind to hydrophobic sites of the serum albumin. New fluorescence probes for drug–protein binding studies were developed (829). The binding of various ingredients was studied, and the results showed that the fluorescence of these acridine derivatives was essentially a combination of fluorescence of acridine and the substitution effects. A dynamic dialysis technique, utilizing a constant flow of buffer solution, yielded the same drug–protein binding information as did equilibrium dialysis (830).

The application of membrane ultrafiltration to the determination of protein binding of drugs was studied (831). Reproducibility between the experiments was good, as was agreement between the binding data obtained by this method and those obtained by equilibrium dialysis. The characterization of drug–protein interactions by classical methods was reviewed (832). Methods discussed were dialysis, ultrafiltration, and gel filtration. The mechanisms of drug–protein interactions also were reviewed (833). The ef-

fects of binding on drug elimination and drug displacement were discussed.

The binding of ascorbic acid and fatty acid ascorbyl esters to bovine serum albumin was studied (834). The analysis of the binding data showed that the albumin possessed a single binding site for these chemicals and that there were marked differences in their binding strength, which increased in the order of ascorbic acid, benzoate, octanoate, and decanoate. The binding of atropine to albumins or γ-globulins from human blood serum was studied (835). The binding significantly increased with an increase in pH above 6.0 or 7.1, respectively. The *in vitro* binding of phenytoin (diphenylhydantoin) to the protein in plasma from 97 volunteers was studied using ultrafiltration (836). The protein binding of phenytoin was significantly decreased in patients with renal disease, hepatic disease, or hepatorenal disease.

Comparative binding of disopyramide phosphate and quinidine sulfate to human plasma proteins was studied (837); the two drugs did not compete for the same binding sites on protein molecules. Glisoxepide, a new antidiabetic drug, was 93% bound by human serum proteins, independent of its concentration (838). When evaluating the competitive binding capacity with phenprocoumon, it was found that glisoxepide did not displace it. Protein binding of indomethacin was studied (839). Binding of indomethacin to human plasma albumin was increased by the presence of phenylbutazone and decreased by the presence of ibuprofen and salicylate. Binding coefficients of various antibiotic affinities to blood protein in a kinetic–biological model suggested that low protein binding was associated with good antibiotic activity (840).

In balanced dialysis experiments, scopolamine hydrobromide interacted with blood serum albumin and γ-globulin (841). An increase in the pH of the solutions from 6.9 to 7.3–7.8 was accompanied by a significant increase in the alkaloid binding with the proteins. The interaction of tolbutamide, glyburide (glibenclamide), chlorpropamide, and tolazamide with serum albumin was studied (842). Glyburide, the most strongly bound of the four compounds, was bound to only one class of sites. The other three compounds were bound to at least two sites. When the interactions of sulfonylureas with plasma proteins were studied, glipizide and glyburide bound to different but closely located sites on the protein with greater affinities (843).

The binding of tolbutamide and glyburide to human serum albumin was examined in varying concentrations of phosphate and tromethamine buffer and in phosphate containing sodium chloride (844). Binding of tolbutamide was affected little by changes in phosphate concentration but was particularly sensitive to the presence of tromethamine and chloride ions. The interaction of tetracycline with human serum lipoproteins and albumin was studied (845). Albumin possessed two binding sites for tetracycline, one a high affinity, low capacity site and the other a low affinity, high capacity site. Tetracycline appeared to dissolve in the lipophilic portion of the lipoprotein molecule rather than to be associated with specific binding sites.

Table XXIV—Additional References on Interactions of Drugs with Biological Substances

Reference	Topic
852	Binding of two β-adrenergic receptor antagonists, alprenolol and metoprolol, to human serum proteins
853	Binding of β-adrenergic receptor antagonists to human serum albumin
854	Binding of *p*-aminohippuric acid, hydrochlorothiazide, and cyclopenthiazide to serum proteins of rats of different ages
855	Spectrofluorometric study of association of ampicillin–lysozyme with diverse medical preparations
856	Binding of antibiotics to a soluble protein from rat liver
857	Influence of binding on pharmacological activity of antibiotics
858	Determination, physicochemical bases, and pharmacokinetic relevance of binding of drugs to plasma proteins
859	Binding of certain antibiotics by blood serum protein
860	Interaction of pharmaceuticals with β-cyclodextrin
861	Plasma protein binding of carbamazepine
862	Binding of chlorpromazine to human serum albumin
863	Binding of diazoxide and benzothiadiazines to human albumin
864	Plasma protein binding of digitoxin and digoxin
865	Comparative study of plasma protein binding of phenytoin (diphenylhydantoin)
866	New approach to determination of protein-bound bilirubin displacement and its applications
867	Doxycycline and albumin-binding capacity of serum from neonates
868	Complex formation between macromolecules and drugs
869	NMR studies of binding of drugs to macromolecules and cellular structures
870	Protein binding of β-methyldigoxin
871	Salicylate interaction with penicillin and secobarbital binding sites on human serum albumin
872	Binding of pyrazolone and pyrazolidine derivatives to bovine serum albumin
873	Binding of (+)- and (−)-Δ^1-tetrahydrocannabinols and (−)-7-hydroxy-Δ^1-tetrahydrocannabinol to blood cells and plasma proteins in humans
874	Effects of drugs on thyroxine-binding capacity of blood proteins
875	Distribution pattern of a series of tricyclic and bicyclic thymoleptics compared with their lipophilic properties and binding to plasma proteins
876	Binding of warfarin sodium to plasma albumin, and its displacement by phenylbutazone
877	Binding of zinc to human serum proteins
878	Circular dichroic investigations into binding of some drugs to human serum albumin
879	Review of plasma protein binding
880	Conformational changes of human serum albumin by binding of small molecules
881	Physicochemical aspects of drug–protein interactions

Nazareth *et al.* (846, 847) studied the binding of L-thyroxine to serum albumins. They studied the use of 2-(4′-hydroxybenzeneazo)benzoic acid to mirror L-thyroxine binding to, and displacement from, serum albumin. Free fatty acid concentrations within the physiological range were found to affect drug

binding to plasma albumin in two ways: at low molar ratios of free fatty acid to albumin, an allosteric effect predominated; at higher molar ratios, there were both allosteric and competitive binding effects (848).

The binding of calcium and magnesium ions to human erythrocyte membranes was investigated (849). At least three different kinds of sites were concerned in the binding of calcium or magnesium to the membrane. Binding of pentazocine to human whole blood, plasma, and blood cells was investigated in 20 normal subjects and 22 patients by means of equilibrium dialysis (850). Plasma protein binding was 56–66% in the control subjects and 48–75% in the patients. In control subjects, 48% of the total amount of pentazocine in whole blood was present in blood cells and 33% was bound to plasma proteins. The remaining 19% was in the plasma water. A constant degree of binding of quinidine to a red blood cell hemolysate preparation was found for a clinically significant range of concentrations at 37° using the method of equilibrium dialysis and ultracentrifugation (851). Quinidine appeared to be bound to a single binding site.

Additional references on interactions of drugs with biological substances are given in Table XXIV.

Interactions of Drugs with Nonbiological Substances—Moorhatch and Chiou (882) evaluated the interactions between drugs and plastic intravenous fluid bags. They studied the adsorption characteristics of 17 drugs, of which three (vitamin A acetate, warfarin sodium, and methohexital sodium) were found to be significantly adsorbed. They also studied the leaching of chemicals from commercial polyvinyl chloride intravenous fluid bags. Absorbances of media such as distilled water, normal saline, 5% dextrose solution, 5% alcohol solution, and aqueous buffer solution with pH values ranging from 3.5 to 9.5 were all quite low within 48 hr of study, indicating insignificant leaching of UV-absorbing material. Absorbances of 0.01–0.04% of polysorbate 20 and 80 solutions were quite high, indicating leaching of a significant amount of chemicals from the bags. The preliminary study indicated that the plasticizer, bis(2-ethylhexyl) phthalate, or other type of phthalate plasticizer may be a major leached chemical (883).

Chloramphenicol seemed to be solubilized by complexation with polysorbate 80 throughout the neutralization range of the interaction (884). Maximum complexation took place at around pH 3.0, the disappearance of the complex began beyond pH 3.4, and interaction ceased at pH 10. The interaction of theophylline with sodium benzoate was examined by NMR (885). Chemical shifts of theophylline protons were determined as a function of sodium benzoate concentration in deuterium oxide at 30°. Signals of both methyl groups underwent significant upfield shifts, indicating vertical or plane-to-plane stacking. The decomposition of aqueous solutions of common antimicrobial agents, sodium metabisulfite, and vitamins was accelerated by storage in polyethylene containers, indicating interactions between the container and its contents (886).

The *in vitro* binding of drugs by colestipol hydrochloride depended not only upon ionic strength, pH, and type of competing ion but also upon whether association could occur with other molecules (887). Where feasible, the initial input ratio of drug to binding agent was equivalent to the ratio if both were administered orally and in therapeutically effective amounts. The water-soluble drugs ascorbic acid, aspirin, salicylic acid, phenobarbital-^{14}C, sulfadiazine, penicillin G, and lincomycin hydrochloride were less than 30% bound to colestipol hydrochloride, while warfarin and tetracycline hydrochloride were bound 59 and 30%, respectively. The interaction of phenobarbital and ethanol given intraperitoneally to mice was studied (888). Dose–response relationships were established for incidence of death, sleeping time, and time of onset of sleep. When the two drugs were administered together, sleep occurred more rapidly than was calculated as likely on the basis of the effects of the drugs given individually. On the other hand, sleep was of shorter duration and the incidence of death was less than anticipated.

The interaction between the preservative 8-hydroxyquinoline sulfate and macromolecules present in tuberculin purified protein derivative solutions

Table XXV—Additional References on Interactions of Drugs with Nonbiological Substances

Reference	Topic
892	Interaction between preservatives, plastics, and rubbers
893	Interaction between water-soluble phosphated nonylphenol ethoxylate and oil-soluble phosphated fatty alcohol ethoxylate
894	Physicochemical study of khellin–urea binary systems
895	Interaction between chlorpromazine and polysorbate 80
896	Complex formation of nicotinamide with copper (II) in solution
897	Interactions between gelatin–surfactant–water systems
898	Interaction of theophylline and phenobarbital in solution
899	Interaction of insulin with medicinal polymers
900	Interaction of sulfonamides with β-cyclodextrin
901	Complexes of disubstituted sulfonylureas with aliphatic and araliphatic amides
902	Interaction of chloroquine and carrageenan
903	Interaction of cetyltrimethylammonium bromide and sodium dodecylbenzenesulfonate with polyvinyl alcohol
904	Interactions between 5-*n*-butyl-1-cyclohexyl-2,4,6-trioxoperhydropyrimidine and sulfonamides in dogs
905	Interactions between sulfinpyrazone and sulfonamides and between oxyphenbutazone and sulfonamides in dogs
906	Interactions of chlorpheniramine–ethanol combinations
907	Relationship of *in vitro* binding of digoxin to its intestinal absorption in rats
908	Adsorption of chlorpheniramine maleate from aqueous solution by activated charcoal
909	Drugs known to react with coumarin-type anticoagulants
910	Solid dispersions for formulation of pharmaceutical compositions containing slightly soluble drugs
911	Complex formation of caffeine by sodium benzoate and sodium salicylate
912	Interaction of atropine sulfate with sodium lauryl sulfate

such as tuberculoprotein, nucleic acid, and polysaccharide were studied (889). The degree of binding was a function of the concentration of these macromolecules. Interactions of iodine with ethylene glycol, diethylene glycol, triethylene glycol, and polyethylene glycols of molecular weight between 200 and 11,000 in ethanol and in carbon tetrachloride were investigated (890). The absorbance of an ethanol solution of iodine at 360 nm increased by the addition of polyethylene glycols. A new peak was observed at 380 nm by the addition of polyethylene glycols of a molecular weight above 300 to the carbon tetrachloride solution of iodine. The complexes of polyethylene oxide with guanidine hydrochloride and with phenobarbital were subjected to differential scanning calorimetry, polarized IR spectroscopy, and X-ray fiber photography, confirming the formations of the complexes; the structures of polyethylene oxide molecules in the complexes were discussed in comparison with a polyethylene oxide–urea complex or with original samples of polyethylene oxide (891).

Additional references on interactions of drugs with nonbiological substances are listed in Table XXV.

Surface Phenomena—The publications dealing with surface phenomena are divided into four major categories: *(a)* interface studies, *(b)* adsorption studies, *(c)* general properties of surfactants, and *(d)* micelle studies. However, because of the obvious overlap among these categories, the reader with special interest in this field should consider the entire section.

Interface Studies—Studies were done on surface films of sorbitan esters at the air–water interface (913). The effects of continuous and discontinuous compression methods on the surface pressure–area isotherms were studied. The slower, continuous compression method gave surface pressure–area isotherms that tended toward a smaller area than the faster, discontinuous method of compression. Cumulative results derived from the effects of compression–decompression studies on hysteresis areas, surface pressure relaxation with time from preselected pressures, and recycling experiments were used to explain molecular behavior at the air–water interface (914). A physical model was developed that considered bulk diffusion, interfacial resistance, interfacial area, and oil–water partition coefficient. The model was used to analyze the data obtained from the rate experiments and to determine the interfacial permeability coefficients. Results of the experiments on the transport of sterols from aqueous sodium taurocholate–lecithin and aqueous cholate–lecithin systems into hexadecane strongly suggested that the kinetics of transport of cholesterol, desmosterol, and hydroxycholesterol were interfacially controlled and involved a two-step process. First there was a collision of the solute–micelle complex with the oil–water interface, and this step was followed by the release of the solute from the micelle in a largely polar environment at the interface (915).

Aggregation and interfacial properties of association colloids of benzalkonium chloride and macromolecular colloids of sodium carboxymethylcellulose and guar gum were studied (916). From the data, it seems that a different drag-reducing mechanism existed for sodium carboxymethylcellulose than for guar gum. Sodium chloride significantly reduced the drag-reducing property of sodium carboxymethylcellulose but did not seem to affect drag retention by guar gum. The effects of variations in temperature on drag reduction were different with sodium carboxymethylcellulose than with guar gum.

Table XXVI—Additional References on Interface Studies

Reference	Topic
921	Enhancing effect of calcium ions on transport of cholesterol from aqueous sodium taurocholate–lecithin micellar phase to oil phase
922	Influence of surfactants on structure of solid–liquid interface
923	Wettability of modified silica surfaces
924	Studies on titanium dioxide solutions in presence of surfactants
925	ζ-Potential of magnesium carbonates in inorganic electrolytes
926	Entropy of transfer of molecular benzoic acid from pure liquid to aqueous solution
927	Interfacial phenomena of pharmaceutical systems
928	New apparatus for measurements of dynamic interfacial properties

The interfacial tension of 10 polar liquids against hexane and tetradecane and the contact angle against paraffin, polyethylene, and polytetrachloroethylene were measured (917). Work-of-adhesion values against any one nonpolar phase, calculated from these data, were independent of the polar liquid used. The kinetic and thermodynamic aspects of *in vitro* interphase transfer of sulfonamides were studied (918, 919). Based on the kinetic and thermodynamic parameters, possible mechanisms for interphase transfer of unionized sulfonamides were proposed. The influence of phospholipids on the transport of antibacterial agents across nonaqueous barriers was studied (920). The results indicate that binding of penicillin G and ampicillin with phospholipids occurred at the oil–water interface, which influenced the further transport of the antibiotics through the nonaqueous phase.

Additional references on interface studies are listed in Table XXVI.

Adsorption Studies—The uptake of digoxin and digitoxin by some antacids was studied (929). Magnesium trisilicate showed the highest adsorptive effect, the extent of adsorption being up to 99% for the two glycosides. Antacid preparations containing magnesium trisilicate adsorbed digoxin from a pediatric elixir to the extent of about 95%. The effect of antacids, when concurrently administered with oral digoxin, on the bioavailability of the drug was discussed. Activated charcoal was capable of adsorbing organic vapors commonly used in anesthesia (930). The stability of activated charcoal after the adsorption of halothane was determined despite the passage of high and low halothane free gas flows through the canister.

Physicochemical studies were done on the adsorption of polysiloxane polymers onto hydroxyapatite

Table XXVII—Additional References on Adsorption Studies

Reference	Topic
939	Kinetics of adsorption of radioactive isotopes from ionic solutions
940	Adsorption of hydrocortisone from aqueous solutions using swelling ion-exchange resins
941	Theory of stabilization of spherical colloidal particles by nonionic polymers
942	Investigations into mechanism of adsorption of cationic surfactants
943	Effect of surface reactions of titanium dioxide on usefulness of drug preparations
944	Adsorption of polymers on clays
945	Properties of finely dispersed silicas with specific adsorptivity with respect to given substances
946	NMR studies of water adsorbed on silica surfaces
947	Adsorption of narrow molecular weight fractions of poly(vinyl alcohol) at the polystyrene–water interface
948	Water vapor adsorption by cellulose acetate
949	Adsorption properties of colloidal silicic acids for homologous polyethylene glycols and polyethylene glycol derivatives
950	Effects of manufacturing methods on surface properties of magnesium silicates
951	Determination of specific surface areas of silicates dispersed in dimethyl sulfoxide by negative adsorption
952	Particle size and adsorption properties of aluminum hydroxide used as depositing substance
953	Role of bridging in colloid flocculation
954	GC study of adsorption and intermolecular interactions
955	Use of GC to determine adsorption isotherms
956	Surface reactivity of microcrystalline carbons
957	Adsorption of vapors and gases at high pressures
958	Effect of temperature on adsorption of binary liquid mixtures on silica gels
959	Relationship among adsorbability on carbon black from aqueous solution, protein binding, and pharmacological activities of barbituric acid derivatives and sulfonamides
960	Review of physical adsorption
961	Review of theory of chemisorption
962	Adsorption of vitamin A palmitate into polyethylene from solubilized solution
963	Determination of specific surface by adsorption from solution
964	Determination of specific surface by the Brunauer, Emmett, and Teller method
965	Physicochemical behavior of poly(methacrylic acid) derivatives
966	Desorption of epinephrine (adrenaline) with carboxyl-containing fibers

(931). The molecular configuration of the polymer adsorbed onto a solid surface was essentially a function of: *(a)* the strength of the polymer adsorption onto the solid surface, *(b)* the degree of polymer solvation by the solvent, and *(c)* the extent to which the solvent could compete with the polar anchoring segments of polymer molecules for the surface sites. The adsorption of benzoic acid on sulfamethazine particles was found to be pH dependent (932). At pH values above 4.2, a gradual suppression of the adsorption occurred; at pH 4.9 and above, no adsorption was noted. The effect of sodium citrate on adsorption was attributed to pH and ionic strength effects.

Adsorption of phenothiazines from aqueous solution by silica (Aerosil) was studied (933). The results obtained were compared with the adsorption on carbon black. The adsorption of benzocaine by nylon 6 powder from aqueous solution was examined under varying environmental conditions (934). The extent of adsorption increased with an increase in electrolyte concentration and with pH up to about 4. Increasing temperature reduced the amount of drug adsorbed. In all cases, the adsorption isotherms were linear over the concentration ranges studied. The adsorption of atropine and scopolamine by magnesium trisilicate was studied (935). At relatively low initial concentrations, the adsorption data were shown to fit a Langmuir plot; values for monolayer adsorption were 14.9 and 3.8 mg/g for atropine and scopolamine, respectively.

A method was developed for determining the adsorption rate of water-soluble dyes by soft gelatin capsules (936). This method was used for determining the diffusivities and activation energies of five FD&C water-soluble dyes. The activation energies of these dyes were in either of two groups, which had ranges of 25.1–25.7 and 34.8–36.0 kcal/mole. The adsorption behavior of steroids on silicic acid surfaces was studied (937). Hydrocortisone acetate was bound to the extent of 0.16 mg to 1 g of silica (Aerosil 200) from a saturated solution. The corresponding adsorption value of ethinyl estradiol was 0.26 mg/g of silica (Aerosil 200).

The adsorption isotherms of water vapors on sodium chloride crystals were plotted (938). True adsorption occurred only when the vapor pressure was less than the pressure over saturated sodium chloride solutions at the corresponding temperatures. With great water vapor pressures, a saturated solution film formed on the sodium chloride crystal surface, and the apparent water adsorption was due to isothermal condensation. The true adsorption of water vapor on sodium chloride crystals was caused by physical forces.

Additional references on adsorption studies are listed in Table XXVII.

General Properties of Surfactants—A study of variations of the surface tension coefficient of chlorpromazine aqueous solutions in relation to concentration and UV irradiation showed that the isotherm had an exponential curve characteristic of negative surfactant substances (967). The wetting behavior of aqueous sodium lauryl sulfate solutions on polyethylene terephthalate, nylon 11, graphite, and aluminum powders was studied (968). By using the Washburn equation for the flow of liquid through a capillary, it was possible to calculate, from the rate of penetration, the magnitude of the advancing contact angle, and the work of adhesion between the liquid and the powder. A linear dependence of HLB values of ethoxylated aliphatic nonionic surfactants on molecular weights was found on the basis of two simplifying assumptions. Surfactants of different polyoxyethylene chain lengths attached to the same hydrocarbon chain, and surfactants of different hydrocarbon chain lengths attached to the same polyoxyethylene chain (969).

The chromatography and spectroscopic analytical methods useful for the separation, identification, and determination of anionic and nonionic surfactants in

Table XXVIII—Additional References on General Properties of Surfactants

Reference	Topic
976, 977	Wettability of some low energy surfaces such as air–liquid–solid interface and oils on solids submerged in water
978	Spreading of solid glycerides and phospholipids at air–water interface
979	Calculation of rate of coagulation of hydrophobic colloids in nonsteady state
980	Basis of theoretical correction factors for use with drop weight method of surface tension measurement
981	Colloidal and surface chemical aspects of dosage forms
982	Cloud point determination of nonionic surfactants
983	Properties of aqueous solution of surfactants under high pressure
984	Effect of shape of micelles and temperature on hydration of nonionic surfactants
985	Velocity gradient dependence of flow birefringence in highly concentrated aqueous solutions of anionic surfactants
986	Interrelation of surface and bulk properties of surfactant solutions
987	Alkyl phosphate surfactants
988	Foam concentration of synthetic surfactants
989	Effect of surface phenomena in pharmaceutical systems
990	Temperature variation of surface tension of water–sodium chloride system
991	Temperature variation of surface tension of water
992	Statistical theory of electrical double layer
993	Surfactant and rheological properties of sodium monoalkyl maleate in aqueous solutions
994	Calculation of capillary constant taking into account the contact angle of wetting in a capillary method for determining surface tension
995	Contact angles and their temperature dependence
996	Surface tension and electric potential of aqueous solutions of some sodium sulfonates
997	Wettability of powders

mixtures were reviewed (970). An optical method for the determination of surface tension was described; the method was based on the determination of the slope of the tangent lines to menisci on the plane vertical walls immersed in the liquid (971). The method was independent of wetting phenomena and gave results in agreement with literature data. The surface tension and wetting capacity of biodegradable preparations such as Azolate A_2 and alkyl sulfate or their compounds with metasilicate and sodium tripolyphosphate were evaluated at 480° and a pressure of 20 atmospheres (972). The surfactants had adequate surface tension and wetting capacity at high temperatures and pressures and were thus recommended for use in crude oil extraction from deep-seated strata.

The manufacture of carbohydrate-based surfactants and their use in food, cosmetics, and pharmaceuticals were reviewed (973), as were the softening, washing, emulsifying, and foam-stabilizing properties of surfactants (974). Also reviewed were the various methods used in determining and calculating the HLB of surfactants and oils (975).

Additional studies on general properties of surfactants are listed in Table XXVIII.

Micelle Studies—Experiments were done to obtain new information on the solubility behavior of nonionic surfactants in nonpolar media, glycerol, and sorbitan monofatty acid esters (998). The results obtained were discussed on the basis of the thermodynamic functions calculated from the temperature dependence on the critical aggregating or micellar concentration and from the phase diagrams constructed with both the solubility and the aggregating or micellar concentration curves. The effect of the HLB on micelle formation for a homologous series of nonionic surfactants was studied (999). The HLB value increased as the aggregation number decreased, indicating a reduced tendency for micelle formation.

The CMC's of a series of polysorbates were determined using a surface tension method (1000). The CMC values obtained did not vary to any appreciable extent from those determined by other techniques, indicating that the procedure adopted was reliable, simple, and fast. Phase studies and particle-size analysis of solutions formed with nonionic surfactants were evaluated (1001). The micellar size was increased by increasing the oil content and by decreasing the surfactant–glycerol ratio. Also studied were the phase diagram and particle-size analysis of solutions formed with an ionic surfactant. Phase diagram studies were carried out on oil-in-water solubilized micellar solutions containing sodium oleate, amyl alcohol, water, and varying amounts of liquid paraffin (1002). Light-scattering measurements indicated an increase in micellar diameter following an increase in the concentration of liquid paraffin and a decrease in the surfactant–alcohol ratio.

Viscosity studies were carried out on a series of oil-in-water solubilized micellar solutions of liquid paraffin, glycerol, and water and blends of sorbitan monooleate and polysorbate 60 (1003). The effect of the surfactant concentration on the viscosity was discussed in terms of interaction between the polyoxyethylene chains at the surface of the micelles. The micellar properties of the antihistamine drugs diphenhydramine hydrochloride, bromodiphenhydramine hydrochloride, chlorcyclizine hydrochloride, and diphenylpyraline hydrochloride were studied in aqueous solutions (1004). The ζ-potentials of the micelles were calculated from their electrophoretic mobilities, as determined by a dye-tracer technique.

The solution properties of a series of phenothiazine hydrochlorides were studied using light-scattering, viscosity, conductivity, dialysis, and NMR techniques (1005). The aggregates formed were considered to be true micelles, which in water are probably compounds of about 10 monomers vertically stacked with the alkyl side chain and nitrogen group alternating to form an approximately spherical unit in solution. The usefulness of an analytical centrifuge in investigating micellar properties of drugs was evaluated (1006). Various examples of micelle forms of drugs were discussed, as was the working hypothesis that the same binding forces that hold together the aggregate found at high concentrations may be responsible for the binding of monomer to biological structures. The addition of ethanol, propanol, butanol, dioxane, or urea to aqueous solutions of hexaethylene glycol monoundecyl ether, heptaethylene glycol

Table XXIX—Additional References on Micelle Studies

Reference	Topic
1010	Effect of structural modifications on CMC and HLB of various surfactants
1011	Stability of micelles in apolar media
1012	Effect of polyoxypropylene chain length on CMC of propylene oxide–ethylene oxide block copolymers
1013	Study of solvent properties near surface of a surfactant micelle by merocyanine dye
1014	CMC of sodium lauryl sulfate–bivalent metal lauryl sulfate mixtures in aqueous solutions
1015	Influence of micelle formation on internal chemical shift of aromatic protons of solutions of a cationic surfactant
1016	Effect of aqueous alcoholic solvents on counterion binding to cetrimonium bromide micelles
1017	Distribution equilibria in micellar solutions
1018	Dielectric relaxation of micelle-forming anionic paraffin chain salt, sodium lauryl sulfate
1019	Effect of poly(ethylene oxides) on structure formation in aqueous montmorillonite and palygorskite dispersions
1020	Micelle formation by ionic surfactants
1021	Mechanism of micelle formation
1022	Micelle formation of ester-containing surfactants
1023	Effect of counterion on CMC of surfactants in nonaqueous media
1024	Review of CMC of high polymer surfactants in nonaqueous solutions
1025	Nature of species giving spectral changes in an azo dye on interaction with cationic surfactants below CMC
1026	Effect of anions on dissolution and association of nonionic surfactants
1027	Role of entropy and enthalpy changes in micelle formation and solubilization in water–surfactant systems
1028	Review of micelle formation mechanism, cloud point, and phase inversion temperature of various emulsifiers
1029	Review of micelle formation mechanism, and the determination of the HLB of various surfactants
1030	Use of comparative calculation methods during study of aqueous solutions of surfactants, and critical concentrations of micelle formation
1031	Effect of electrolytes on critical concentration of micelle formation and detergent action of sodium lauryl sulfate and sodium laurate
1032	Micellar properties of drugs
1033	Charge transfer phenomena and CMC's of surfactants
1034	Thermodynamics of micelle formation, and prediction of micelle size and size distribution
1035	Effects of electrolytes on micelle formation in solutions of synthetic detergents
1036	Effect of temperature on critical concentration of micelle formation of sodium *n*-alkane sulfonates
1037	Micellar systems in aqueous solutions
1038	Micelle formation of surfactants in mixed solvents
1039	Study of water structure in micellar solutions and mesomorphic gels of potassium laurate–water system by near-IR spectrophotometry
1040	Potentiometric determination of CMC and activity coefficient of cationic surfactants
1041	Determination of critical concentration of micelle formation of surfactants

monodecyl ether, or OP-10 increased the critical concentrations of micelle formation of these surfactants (1007). The alcohols, which stabilized the aggregates of water molecules, decreased the mobility of the surfactant molecules and diminished the number of collisions in unit time.

The kinetics of the formation of sodium lauryl sulfate micelles between 7.5 and 20° were studied by a pressure jump apparatus (1008). The theoretically predicted $1/\tau$ linear increase with increasing sodium lauryl sulfate concentration could not be confirmed, but the relaxation process became detectable at concentrations well below the CMC. Ultrasonic relaxation data were used to determine the kinetics of micellization of ionic surfactants sodium pentyl sulfate, sodium nonane sulfonate, and octylammonium chloride in aqueous solution, and a two-state kinetic model for micellization was used to describe the interchange of monolayers with the micelles by an adsorption mechanism governed by the Langmuir adsorption theory (1009).

Additional references on micelle studies are given in Table XXIX.

Dispersion Stabilization—Mixtures of phosphated nonylphenol ethoxylate, phosphated fatty alcohol ethoxylate, and *n*-hexane formed "oils," which may spontaneously emulsify when added to water (1042). However, the degree of spontaneity and the stability of the resulting dispersions varied according to the constitution of the system. Studies of the equilibrium phase diagrams suggested that the anomalies may be attributed to the presence of liquid crystalline phases of different types formed in the presence of water. The stabilization of emulsions by solid emulsifiers was evaluated (1043). The effects of surface-active compounds, which are often found in hydrocarbon oils, also were studied (1044). The surface-active compounds interacted with the solid emulsifiers and were related to the stabilization of the emulsion by coagulation structure formation. To determine the optimal HLB of oils, oil-in-water emulsions of castor and paraffin oils were prepared with complex emulsifiers, such as polysorbates and sorbitan esters, in appropriate ratios according to the Griffin formula. The optimum HLB depended upon the concentration of the oil phase (20 or 40%) and was not specific for a certain oil.

The stabilization of pharmaceutical suspensions was evaluated by checking the ζ-potential of suspensions of erythromycin propionate and troleandomycin (1045). Rising concentrations of the dispersed phase led to a reduction of the value of the ζ-potential. Suspensions prepared with surfactant solutions with high HLB values characteristically showed high ζ-potential values. Calcium caproate was studied as an emulsifier in the stabilization of xylene in water emulsions (1046). The effect of inorganic electrolytes on the emulsion flocculation was studied by observing ζ-potentials. The potential barriers and stability of the emulsions were high.

Stabilization of disperse systems by surfactants was studied in relation to the formation of structure and electrostatic repulsion (1047). The oil phase was *o*-xylene. The monomers were styrene and vinyl acetate. The emulsifiers used were the ionized sodium salt of butylnaphthalenesulfonic acid and alkyl benzenesulfonic acid and nonionized polyoxyethylated alkyl phenols and polypropylene oxide. Studies of the emulsions showed that the stabilization was due to

Table XXX—Additional References on Dispersion Stabilization

Reference	Topic
1049	Mechanism of formation of so-called microemulsions studied in connection with phase diagram
1050	Antifoam activity of dimethylpolysiloxanes
1051	Mechanism of stabilization of emulsions
1052	Stability of colloidal dispersions, and theory for interaction between particles dispersed in a regular mixture
1053	Effect of electrolytes and surfactants on emulsion separation of colloids collected by synthetic surfactants
1054	Use of hydrocolloids for stabilization of emulsion systems
1055	Phase inversion temperature and cohesive energy ratio systems as modern methods of selecting emulsifiers
1056	Phase inversion temperature of emulsions stabilized with active nonionic surfactants
1057	Dispersive and cohesive actions of surfactants
1058	Review of classification of mixtures and stabilization of disperse systems
1059	Electrokinetic potential and stability of emulsions
1060	Effect of impeller diameter on liquid–liquid dispersion
1061	Stabilization of dispersed systems by nonionic surfactants
1062	Structure of emulsions stabilized by nonionic emulsifiers

the formation of structure at interfaces. By using phase diagrams, the effects of the polarity of oils and the structure of emulsifier on emulsions stability were studied in oil-in-water formulations (1048). The liquid crystal phases, in particular the lamellar phase, formed from the three components of the emulsion appeared to have definite influence on the stability of emulsions.

Additional references on dispersion stabilization are given in Table XXX.

Rheology—The physicochemical principles involved in rheological measurements were reviewed (1063). The use of instrumentation including the Brookfield viscometer, the Brookfield viscometer with Helipath, the Brookfield plate and cone viscometer, and the Rotovisco viscometer were evaluated. Comparative data were presented for typical lotion, cream, and suspension products as measured with each of these instruments. The practical application of such data to formulations, stability, and processing studies was illustrated.

Sulfonamide drug suspensions were aged for 1 year at room temperature, and their rheological behavior was found to be similar to that of freshly prepared suspensions (1064). Drug content and particle-size distributions of aged and fresh preparations were similar. The data obtained from stability controls were in agreement with predictions based on rheological data. When measurements were made with and without the Helipath stand, the flow curves of bentonite gel, determined at 21°, showed that the area and shape of the hysteresis loop were different, emphasizing the importance of considering the basis and exact way by which rheological values were obtained (1065).

The effects of lemon and rose perfume oils, sodium chloride, and various ethoxylated oleyl and lanolin esters on the viscosity of a standard foam bath were studied (1066). Addition of 4% lemon and rose perfume plus an emulsifier sharply decreased the viscosity, while the rose perfume alone caused inhomogeneity. Sodium chloride increased the viscosity, with a maximum viscosity at ~2% sodium chloride. The suitability of the Hoeppler consistometer for testing ointment consistency was evaluated (1067). The Hoeppler consistometer determination of dynamic viscosity gave absolute values (in centipoises). Davis (1068) reviewed the Weissenberg rheogoniometer and digital transfer function analyzer and found them to be the equipment of choice for studies of mucoid fluids and mucolytic activities. Saliva and gastric mucin were useful in *in vitro* systems for screening mucolytic agents. Bottari (1069) reviewed Newtonian, plastic, pseudoplastic, dilatant, and thixotropic flow instrumental methods for the determination of rheological properties and their applications to cosmetics.

Table XXXI—Additional References on Rheology

Reference	Topic
1070	Determination of viscosities
1071	Relationships between kinematic viscosity and concentrations in aqueous solutions of disodium dodecyl phenoxybenzenedisulfonate
1072	Properties and evaluation of oleogels
1073	Rheological properties of feed slurries containing sodium salicylate and salicylic acid particles
1074	Microrheology of disperse systems

Additional references on rheology are provided in Table XXXI.

PHARMACEUTICAL ASPECTS

Antibiotics—The solubilities of the sodium and potassium salts of benzylpenicillin were determined in butanol and in butanol containing 0.5–3% water (1075). Also determined was the hygroscopicity of salts. Results of storage at 82.5 and 95.5% relative humidity showed the degradation products formed faster with the sodium salt. Oxytetracycline preparations in propylene glycol were incubated at 50, 60, and 70°, and changes in color and biological activity were measured at certain time intervals (1076). These data were used to calculate a low limit of antibacterial activity after 3.4–3.6 years of 30%. Appearance of an undesirable intense color was predicted after 4.73 years for the propylene glycol solution.

Polysorbate 80 decreased the biological activity of levorine but not that of amphotericin B, when added at 1.6 mg/mg of antibiotic before a 100-hr storage period at 40° (1077). In experiments with *Torula utilis* cultures, the rate of amphotericin B diffusion from ointments containing polysorbate 80 was greater than that from ointments containing lanolin of wool and wax alcohols. Polysorbate 80 also increased the resorption of the antibiotic by the skin of mice. The use of several flocculants and wetting and suspending agents was studied in the development of an oral troleandomycin suspension (1078). The influence of water, dimethyl sulfoxide, propylene glycol, polyeth-

ylene glycol 400, glycerol, and ethanol on the release of tetracycline hydrochloride from white petrolatum was determined and compared with a tetracycline–urea complex (1079). Water was most effective with tetracycline hydrochloride and dimethyl sulfoxide with tetracycline–urea complex.

Bauer (1080) reviewed the formulation of antibiotics including appropriate dosage form, route of administration, and physiological and morphological conditions at the site of resorption. Also important was the nature of the active ingredient such as its chemical, physical, pharmacokinetic, and toxicological properties. The effects of particle size and shape on the flow and failure properties of procaine penicillin powders were studied (1081). The effects of moisture content and gelatin binding agent on the mechanical and failure properties of an oxytetracycline formulation were also evaluated (1082).

Radiopharmaceuticals—The rapid incorporation of short-lived cyclotron-produced radionuclides into radiopharmaceuticals was reported (1083). Production methods were described for two sterile, pyrogen-free preparations of 10-min ^{13}N and were used clinically. It was found that cobalt 60 was a constant contamination of pharmaceutically prepared iron 59. For research studies, where the iron 59 preparation has decayed several half-lives, establishment of the level of cobalt 60 contamination prior to use seems desirable (1084). The chemistry of ^{99m}Tc, used as a tracer in medicine, was discussed; this information might help achieve a better understanding of the use of radiopharmaceuticals containing ^{99m}Tc and a more rapid development of additional useful formulations (1085). The mechanism of labeling of human serum albumin with ^{99m}Tc was also discussed. Sterile ^{43}KCl was prepared from $^{43}CaCl_2$ by neutron bombardment (1086). The chlorides were taken up in tetralithium edetate (Li_4EDTA), lithium hydroxide was added to pH 10, and the salts were separated on an ion-exchange resin (Dowex 50x12Li^+).

Aulagner *et al.* (1087) reviewed the phenomenon of radioactivity, the detection of and protection against ionizing radiation, the application of radiopharmaceuticals to diagnosis and therapy, and the use of preparations obtained from isotopic generators for organ scanning. The method of preparation of albumin labeled with ^{99m}Tc and its application to other radiopharmaceuticals were discussed (1088). Stability studies were made on 4-iodoantipyrine labeled with iodine 131 when mixed with sodium nitrate (1089). The quantity of sodium nitrate required for stabilization depended on the specific activity of the preparation.

The preparation of ^{113}In-SMAA was described (1090). This radiopharmaceutical was free from side effects and was the most appropriate indium compound for lung scintigraphy. A simple laboratory method of preparation of ^{99m}Tc-labeled iron hydroxide aggregates was described (1091). The formulation was used for lung scanning. The development of radiopharmaceuticals for application in the evaluation of diseases of the kidney and urinary tract was reviewed (1092). A portable, external, two-channel radiotelemetrical GM-detector unit, for measurements of radionuclide tracers *in vivo*, was developed (1093). The equipment was suggested for local tracer studies when continuous long-term measurements are needed.

Table XXXII—Additional References on Radiopharmaceuticals

Reference	Topic
1094	Effect of ionizing radiation on pharmaceuticals
1095	Effect of cobalt 60 γ-radiations on biological activity and some physical and chemical properties of oxytetracycline
1096	Freeze-dried kits for preparing radiopharmaceuticals from technetium-99m
1097	Dosimetrical importance of radiochemical purity of radioactive diagnostic preparations
1098	Radioactive dosage forms
1099	Continuous monitoring of ^{14}C-activity in blood
1100	Survey of radiopharmaceuticals and their current status
1101	Effect of ionizing irradiation on changes in gelatin of pharmaceutical quality
1102	Studies on quality and test method of radioactive iodine-labeled insulin, IgE, and poly(vinylpyrrolidone)
1103	New rapid synthesis of ^{11}C-labeled norepinephrine hydrochloride
1104	Review of radioactive isotopes in pharmaceuticals
1105	Review of radioactive pharmaceuticals
1106	Stability of a stabilizer-free technetium sulfur colloid preparation

Additional references on radiopharmaceuticals are found in Table XXXII.

BIOPHARMACEUTICS

The various publications dealing with biopharmaceutics were subdivided according to the area of special interest. However, because of the obvious overlap in subject matter, the reader seeking a thorough review should consider the entire section.

As in previous years, the bioavailability of digoxin attracted the most attention in the field of biopharmaceutics. In a series of five papers, Greenblatt and coworkers evaluated the various aspects of digoxin bioavailability. They recommended intravenous digoxin as a bioavailability standard and found that slow infusion was preferable over rapid injection as an intravenous standard in bioavailability testing (1107). They also evaluated the bioequivalence of digoxin as an elixir and a rapid dissolution tablet (1108). A comparison of 1- and 6-day urinary digoxin excretion in single-dose bioavailability studies was made; 24 hr of urine collection was sufficient for single-dose studies of digoxin bioavailability (1109). The same authors also evaluated the digoxin bioavailability in single-dose studies. They compared the intravenous infusion, intramuscular injection, oral elixir, and oral tablet. The area under the 8-hr serum concentration curve correlated with cumulative urinary excretion but showed greater between subject variation. The intramuscular digoxin and digoxin elixir were significantly more bioavailable than the tablet but not sufficiently absorbed to serve as bioavailability standards (1110).

The bioavailability of digoxin tablets and elixir in the fasting and postprandial states was evaluated. In

the same individual, variations in bioavailability between fasting and postprandial states were similar in magnitude to those after repeated fasting administration. Postprandial administration of digoxin did not significantly alter completeness of absorption but altered the rate (1111). In correlating the plasma levels of digoxin in cardiac patients with dose and measures of renal function, it was found that the readily obtainable parameters did not allow accurate prediction of plasma concentration of digoxin in the individual patient, based on the data collected in a panel of cardiac patients. Therefore, serial measurements of plasma concentration of digoxin were necessary in individual patients taking digoxin (1112). The effect of route of administration on the uptake and elimination of digoxin and the relationship of serum digoxin levels to cardiac arrhythmias were investigated in 15 children with congenital cardiac defects who were receiving maintenance doses of digoxin (1113). The highest serum digoxin concentrations were noted 1 hr after oral and parenteral administration. In the limited number of patients studied, there was no apparent relationship between the serum digoxin level and the onset or persistence of cardiac arrhythmias.

Shaw (1114) evaluated the various digoxin tablets marketed in the United Kingdom. He found that they differed markedly in the fraction of the dose adsorbed. Hazards to the patient were not entirely avoided by brand name prescribing, and it was suggested that new pharmacopeial standards were needed for uniform potency. The bioavailability characteristics of a rapidly dissolving digoxin–hydroquinone complex were evaluated, and the complex was found to be faster dissolving than digoxin alone (1115). Four commercially available digoxin tablet brands were characterized by determination of dose uniformity, dissolution rate of digoxin, and tablet disintegration time (1116). Large differences in the dissolution rate of digoxin were observed. Two batches of one brand were found to have a low dose uniformity, whereas the others were well within the pharmacopeial limits.

The comparative bioavailability of two oral preparations of digoxin in healthy volunteers was investigated (1117). They showed no differences with respect to disintegration time, content uniformity, peak serum levels, and cumulative areas under the curve for 8 hr after oral administration of 0.25-mg doses to healthy subjects. Evaluation of pharmacological response intensities showed evidence for variable digoxin absorption (1118). Ball milling or Muller milling digoxin with a 20-fold excess of lactose, sucrose, dibasic calcium phosphate, or microcrystalline cellulose significantly enhanced its dissolution rate (1119). Tablets prepared with such triturations also exhibited superior rates of dissolution. Those prepared with the digoxin–lactose trituration gave plasma levels in rats that were 89.6% of the values from an oral solution of digoxin.

A method was described for the reduction of digitalis glycoside intoxication by rational dosing procedures (1120). Variations in digoxin bioavailability also were evaluated by mean serum digoxin levels obtained 0–6 hr after oral administration of 0.5-mg digoxin tablets (1121). There was a twofold difference in the mean serum peak concentration and the urinary excretion of the two brands studied. Low serum digoxin levels were found in eight institutionalized patients with congestive heart failure despite daily administration, in standard dosage, of a digoxin preparation for 4 weeks (1122). All eight patients developed serum levels within the accepted therapeutic range after receiving another digoxin preparation for 1 week. Analysis revealed that the first preparation did not meet USP requirements of content uniformity. *In vitro* testing demonstrated marked differences in dissolution characteristics between preparations.

Relative bioavailabilities of commercial digoxin tablets were investigated, using a digoxin elixir (Lanoxin) as the most available oral dosage form (1123). Bioavailability of digoxin tablets was calculated from the 0–12- and 0–24-hr areas under the serum level–time curve and from 48-hr cumulative urinary excretion data. Results obtained by the three different methods of calculation were in agreement with each other. Digoxin was reproducibly absorbed from the elixir, as shown by relatively small intrasubject and intersubject variations.

Bioavailability of 14 nitrofurantoin products was evaluated *in vitro* and *in vivo* (1124). All products tested met USP XVIII specifications for drug content, disintegration time, and dissolution rate. Statistically significant differences were observed in bioavailability, as determined from crossover urinary excretion studies in 14 human volunteers. Bioequivalency of generic and brand name chlorpromazine was evaluated (1125). Because of consistent similarities between generic and brand name chlorpromazine for the wide ranges of dimensions evaluated, it was concluded that the two compounds were bioequivalent.

A four-way crossover study was performed to establish the bioavailability of phenytoin (diphenylhydantoin) (1126). Single 100-mg doses of phenytoin sodium were administered as an aqueous solution and compared with a new phenytoin 100-mg capsule and a commercially available 100-mg capsule. Phenytoin capsules from both manufacturers gave plasma levels that were not significantly different at any sampling time. Although phenytoin in solution form was absorbed more rapidly, higher initial plasma levels were produced with the solution form. Plasma level curves of oral doses of naproxen ranging from 125 to 900 mg were studied in normal subjects (1127). Areas under plasma concentration–time curves increased linearly with dose increments up to 500 mg twice a day, but larger doses resulted in a plateau effect. Experiments with tritium-labeled naproxen showed that there was no difference in the fraction of drug excreted in the stools whether the dose was 250 or 900 mg, eliminating incomplete absorption as a factor. Accelerated renal clearance at high doses because of disproportionate increases in the amount of unbound drug appeared to be the most likely explanation for the plateau effect.

The disposition of propoxyphene and its major

Table XXXIII—Additional References on Biopharmaceutics

Reference	Topic
1141	Review of biopharmaceutics
1142	Review on biopharmaceutical criteria for nonoral administration
1143	Review of bioavailability
1144	Bioavailability testing and clinical significance
1145	Biopharmacy and drug effectiveness
1146	*In vitro* evaluation of bioavailability of oral solid formulations
1147	Biopharmaceutics in preformulation stages of drug development
1148	Review of bioavailability
1149	Bioavailability of oral solid dosage forms, and clinical response to drug therapy
1150	Bioavailability and dose–response correlation
1151	Physiological availability of medicinal agents in tablets, and an appraisal of their quality tests
1152	Equipment for studying *in vitro* absorption of orally administered drugs
1153	Bioavailability and pharmacokinetic requirements for registration of new drug products
1154	Corneal penetration of aceclidine into rabbit eyes using perfusion technique
1155	Rate of dissolution and biological availability of coated aspirin
1156	Urinary excretion of amitriptyline *N*-oxide in humans
1157	Comparison of plasma levels of antipyrine, tolbutamide, and warfarin after oral and intravenous administration
1158	Gold kinetics following aurothiomalate therapy
1159	Bioavailability of chloramphenicol in minipigs
1160	Chlorphenesin carbamate serum levels during subchronic administration to normal human volunteers
1161	Bioavailability of clofibrate
1162	Bioavailability of volatile or gaseous compounds after peripheral intravenous dosing
1163	Factors affecting excretion of ^{14}C-dicumarol in rat bile
1164	Di(2-ethylhexyl) phthalate (DEHP) metabolism in animals and posttransfusion tissue levels in humans
1165	Serum levels of gentamicin and tobramycin after slow intravenous bolus injection
1166	Fate of intravenous ^{3}H-glycopyrrolate in humans
1167	Role of sweat in accumulation of orally administered griseofulvin in skin
1168	Correlation of plasma ibuprofen levels with biological activity
1169	Serum levels, excretion, and biotransformation of ketoprofen in animals and humans
1170	Disposition and metabolism of 4-methyl-2-(4-phenylbenzyl)-2-oxazoline-4-methanol in rats and dogs
1171	Bioavailability of methaqualone
1172	Fate of ^{14}C-naproxen
1173	Plasma half-life of niacin (nicotinic acid) in humans
1174	Ocular penetration of ^{14}C-oxyphenbutazone
1175	Equivalence of oxytetracycline tablets BP
1176	Biological availability of oxytetracycline hydrochloride capsules
1177	Use of animal model to predict bioavailability of penicillins
1178	Penicillin blood levels after intramuscular administration of Ampiclox
1179	Biliary excretion kinetics of phenolphthalein glucuronide after intravenous and retrograde biliary administration
1180	Absorption of penicillin V potassium (potassium *S*-phenoxymethyl penicillin)
1181	Biopharmaceutical study of colloidal association of scopolamine *N*-butylbromide and sodium lauryl sulfate
1182	Effect of mucin on bioavailability of tetracycline from GI tract, and *in vivo–in vitro* correlations
1183	Comparison of tetracycline preparations for oral use
1184	Bioavailability of thiazinamium methylsulfate after oral and intramuscular administration
1185	Influence of polymers and pH on transcorneal biophasic availability and mydriatic response of tropicamide
1186	Biopharmaceutical study of vitamin U
1187	*In vitro* testing of drug availability with Stricker apparatus
1188	Penetration of fluorouracil in excised skin
1189	Evaluation of activity of dermopharmaceutical preparations with corticosteroid base
1190	Percutaneous penetration of methotrexate

biotransformation product norpropoxyphene was studied in normal subjects following a single 130-mg oral dose (1128). The peak plasma level of propoxyphene occurred at 2 hr, followed by a rapid elimination with an average apparent half-life of 3 hr. The plasma level of norpropoxyphene reached a peak at 4 hr and slowly decayed with an apparent half-life of 16.8 hr. The data indicated that individual differences in absorption and biotransformation may have been responsible for the substantial variation of the plasma propoxyphene levels seen in subjects receiving the same dose.

A single-dose, crossover, comparative study of 25- and 100-mg spironolactone tablets was undertaken in 39 healthy males (1129). Following a total dose of 200 mg of each dosage form, the primary discriminators for comparing the two dosage forms were found to be the plasma metabolite levels at 3 and 4 hr, peak plasma metabolite level, and total 24-hr area under the plasma level curve. Total serum iodine levels were measured for 24 hr in human subjects following a single, oral, therapeutic dose of organically bound iodine administered as iodinated glycerol (1130). Peak levels were obtained 1–2 hr after administration of a single dose, with a gradual fall to almost basal levels at 24 hr. The experiment with continued dosing for 6 days indicated that once peak blood levels were reached, they continued at this concentration until the drug was discontinued.

The concentration of theophylline in the plasma and saliva of seven normal adults receiving single oral doses of 200-mg theophylline/m^2 of body surface area was determined spectrophotometrically over 8 hr (1131). There was an excellent linear relationship between theophylline concentrations in plasma and saliva over a plasma concentration range of 4–14 μg/ml. The results suggested that theophylline determination in saliva may be a convenient, painless, and noninvasive method for routine monitoring of theophylline levels. To determine bioavailability of theophylline in a new sustained-release form, plasma drug concentration–time curves were determined for five normal male subjects after an intravenous dose of 4 mg/kg and after an oral dose of 700 mg as two oral tablets (1132). The absolute bioavailability of the oral dosage was 77.1 ± 5.4%.

Chrai (1133) studied the influence of precorneal factors on the bioavailability of topically applied drugs. Urinary excretion data were used to determine the bioavailability of crystal forms II and III of sulfameter in humans (1134). Agreement was observed between the ratio of absorption parameters of the two forms determined in the present study and those previously obtained from blood level data. Although the urinary data revealed a significant difference in the rate of absorption of the two forms, no significant difference was observed in the extent of absorption of both forms as indicated by the 72-hr urinary excretion data.

The *in vivo* dissolution rates of prednisolone from five commercially available 5-mg prednisolone tablets were determined in distilled water (1135). A two-way crossover bioavailability study was also preformed in 12 human male adult volunteers, comparing the fastest and the slowest dissolving brands. Statistical analysis of the data for the two brands showed no significant differences between average plasma levels of prednisolone at any sampling time. The results suggested that on the average the *in vivo* rates of dissolution of the two brands were essentially the same. Wagner and Johnson (1136) reviewed the relationship between the rate of drug dissolution *in vitro* and biological availability and suggested that the dissolution rate may be the rate-limiting step in drug absorption, hence limiting its bioavailability from solid dosage forms. Correlation of *in vitro* dissolution measurements with *in vivo* parameters makes it easier to control the factor of bioavailability.

A modified *in vitro* permeability technique permitted the hydration of the stratum corneum to be maintained very close to its natural state (1137). Penetrant molecules were applied by adding a few microliters of acetone solution to the stratum corneum surface. The acetone quickly evaporated, leaving a residue of solid penetrant. The time course of penetration of the solid material through the epidermis was observed as a function of applied dose, mild hydration, and solvent contact time. Remarkedly steady and uniform penetration persisting over several weeks was observed. Diffusion through the stratum corneum rather than sorption of the solid at the surface appeared to be rate limiting.

The development and evaluation of ointment and cream vehicles for a new topical steroid, flucloronide (fluclorolone acetonide), were described (1138). Enhanced drug release and superior vasoconstrictor activity were demonstrated by flucloronide when prepared in vehicles in which it was entirely solubilized. A gel or cream base containing 0.025% betamethasone benzoate was a suitable formulation for topical treatment of steroid responsive dermatoses in comparison with other steroids (1139). The cream was significantly less efficacious than the gel. Percutaneous absorption of escin-^{3}H in mice and rats was studied (1140). A horizontal diffusion was observed in the skin of mice. Only small amounts of escin were found in inner organs, bile, and urine. Higher escin concentrations were detected only in the part of the muscular system beneath the site of application.

Additional references on biopharmaceutics are listed in Table XXXIII.

Effects of Physicochemical Properties—Microcrystals of aspirin of 100 × 25 × 2-μm size, given orally to volunteers, were excreted in urine more rapidly than aspirin crystals of 1000 × 300 × 250-μm size (1191). Apparently, smaller crystals were more readily absorbed by the digestive tract than were larger ones. The influence of three granulometric fractions of aspirin on rectal absorption in rabbits was examined (1192). Particle size of the drug influenced the absorption, as had been established for GI absorption of drugs. A correlation between dissolution rate and release of the drug from suppositories was established. The particle-size effect of sulfathiazole on availability after subcutaneous administration was evaluated (1193). The particle size influenced the absorption and dissolution rates when sulfathiazole was administered as aqueous suspensions subcutaneously.

Structure–activity relationships of some organic hydroxy acids with reference to their choleretic activity were studied (1194). Of the nine hydroxy acids tested, 3,4-dimethoxycinnamic acid was the most potent choleretic. Acids that possessed an unsaturated carbon–carbon bond and a methoxy group possessed a stronger action, and acids that contained a free hydroxy group possessed a weak action. A study was made to determine the relationship between the structure of phenylalkylamines and their psychotomimetic activity (1195). Optimum activity was associated with: *(a)* an isopropylamine side chain, with a *R*-(−) configuration at the carbon atom alpha to the amino group; and *(b)* 2,5-dimethoxy substitution, together with an alkyl or halo group at position 4, which is probably limited in bulk to *n*-propyl or bromo.

The anticancer activities of some dihydrofolate reductase-inhibiting 2,4-diaminopyrimidine derivatives, solapalmitine-related *N*-acylamines, bis(1-aziridinyl) phosphinyl carbamate derivatives, and aromatic nitrogen mustards were correlated with substituent constants (1196). For the 2,4-diaminopyrimidines, activity correlated with Taft's steric constants for substituents in the 5-position, demonstrating that anticancer activity in these compounds was related to the steric bulk of the 5-substituent. The carbonic anhydrase inhibitory activities of 17 sulfonamides were compared with their structures, based on quantum chemical calculations performed within the framework of the extended Hückel theory (1197). Correlations were found between the activity of molecules and the reduced Mulliken overlap populations relative to the SO_2 group. Screening of 300 chalcone, flavanone, flavone, flavonol, and acetophenone derivatives showed that 44 compounds had hypotensive, analgesic, anti-inflammatory, antithrombic, sedative, or antifungal activity (1198). Halosubstituted flavones were more active than corresponding flavanones, but all flavanone derivatives were inactive, suggesting that pharmacological activity was dependent on the presence or absence of a double bond in the 2–3 position.

Table XXXIV—Additional References on Effects of Physicochemical Properties

Reference	Topic
1205	Structure–activity correlations of actinomycins
1206	Structure–activity correlations of β-adrenergic receptor antagonists
1207	Structure–activity correlations of apomorphine analogs
1208	Structure–activity correlations of anti-inflammatory 2-arylbenzoxazoles
1209	Structure–activity correlations of aureolic acid group of antibiotics
1210	Structure–activity correlations of cardioactive steroids
1211	Structure–activity correlations of chlorohydrins as orally active male antifertility agents
1212	Structure–activity correlation in series of compounds related to diphenhydramine
1213	Structure–activity correlations of isoarecaidine derivatives
1214	Structure–activity correlations of mitomycins
1215	Structure–activity correlations of *N*-substituted narcotic antagonists
1216	Structure–activity correlations of pyridine derivatives as myorelaxants
1217	Structure–activity correlations of tricyclic antidepressants
1218	Structure–activity correlations of *dl*-α-tocopherol having different isoprene chains
1219	Structure–activity correlations of zygosporin derivatives
1220	Relation between chemical structure and pharmacodynamic activity
1221	Absorption of iron from ferrous salts
1222, 1223	Review of effect of physicochemical properties on bioavailability
1224	Effect of physicochemical and pharmacokinetic properties of barbiturates on induction of drug metabolism
1225	Comparative study of absorption and distribution of quinidine salts
1226	Effect of physicochemical properties on intramuscular absorption of drugs from oily solutions in rats
1227	Influence of molecular structure on intestinal absorption of fat-soluble vitamins
1228	Relation of drug effectiveness to physicochemical properties
1229	General influence of physicochemical properties on drug–receptor combination
1230	Comparative clinical pharmacology of three ampicillins and amoxicillin administered orally
1231	Physicochemical factors influencing drug absorption from intramuscular injection site
1232	Effects of physicochemical properties on biological availability of drugs
1233	Comparison of availability of ions from sodium salicylate and salicylic acid tablets
1234	Absorption and *in vitro* dissolution of phenytoin and its sodium salt
1235	Comparative serum levels and urinary recovery of cefazolin, cephaloridine, and cephalothin in humans

Based on the theory of quantum statistics, a method was developed for the correlation of drug action and chemical structure (1199). It was applied to the interaction of distinguishable biological receptors and in distinguishable drug molecules. Calculations for several local anesthetic drugs illustrated the usefulness of the method. A Hansch analysis of the anabolic activities in the rat of butyrate to undecanoate normal fatty acid ester of nandrolone was made (1200). Two peaks of activity were observed in plots of organ weight against time for each ester and were assigned to release into the blood from the site injection and from body fat, respectively. Anabolic activities were measured as an increase in weight of levator ani over control weight, multiplied by duration of response. A binomial relationship was obtained between log anabolic activity and the log ethyl oleate–water distribution coefficient. Kasuya (1201) studied the structure–activity relationships of androgens and evaluated the metabolism and target organ uptake of the various compounds.

An aqueous suspension of ampicillin, when compared to ampicillin sodium, yielded a higher and longer lasting blood level and was eliminated less in urine (1202). The bile concentration was two to three times higher than the blood level in rabbits injected with ampicillin intramuscularly. The bioavailability of aminosalicylic acid and its sodium, potassium, and calcium salts was evaluated in humans (1203, 1204). Absorption of aminosalicylic acid and its three salts was essentially complete. Dissolution of aminosalicylic acid appeared to be the rate-limiting factor in its absorption, and the type of salt administered affected the rate of absorption. Although absorption of all four compounds was complete, the areas under the plasma concentration–time curve, or bioavailable drug, were dependent on the rate of absorption. The data presented showed evidence of rate-limited metabolism, especially in the first pass.

Additional studies on the effects of physicochemical properties are listed in Table XXXIV.

Effects of Formulation—The effects of formulations in various digoxin preparations were evaluated. Binnion (1236) studied tablets from four different manufacturers and found that the disintegration times for these tablets were different and did not correlate with the plasma levels in subjects who took them. The tablet with the slowest disintegration rate produced satisfactory plasma digoxin concentrations. Another tablet, with slow dissolution rate, failed consistently to produce a therapeutic plasma level. The author recommended that all packages containing digoxin tablets should be labeled with data on their dissolution characteristics but that none should be sold unless these data are consistent with biological availability. Also the *in vitro* dissolution was correlated with bioavailability of various oral digoxin preparations (1237). Plasma concentrations were measured by radioimmunoassay. For rapidly releasing preparations, the dissolution rate did not control absorption. Absorption from slowly releasing tablets was reduced but only partially controlled by dissolution rate.

By using various methods of estimating digoxin absorption, the absolute bioavailability of 0.25-mg digoxin tablets was found to be 62%, and an equivalent dose of digoxin solution was determined to be 77% (1238). Of the various methods studied, the urinary excretion of digoxin was the most valid measure of bioavailability of oral digoxin preparations. The bioavailability and dissolution properties of two commercial digoxin tablets and an elixir were evaluated (1239). The elixir form gave much higher blood levels than either tablet during the first two sampling

times, but the two tablets showed nearly identical blood levels and relative bioavailabilities. A previously reported dissolution rate test method showed wide differences between the two tablets and thus failed to correlate with the similar bioavailabilities observed *in vivo*.

The *Federal Register*, for the first time, reported some guidelines for marketing oral digoxin tablets (1240). It was proposed that digoxin tablets be withheld from distribution until released by the Food and Drug Administration after testing to meet USP XVIII requirements and dissolution tests and that the quantity of digoxin dissolved in 1 hr should not be more than 95% of the assayed amount or that the quantity dissolved at 15 min should not be more than 90% of that assayed amount.

The effect of (−)-Δ^9-*trans*-tetrahydrocannabinol was studied in mice following four routes of administration: oral, subcutaneous, intraperitoneal, and intravenous (1241). For each route, four different vehicles were used: bovine serum albumin, polysorbate 80, polyvinylpyrrolidone, and propylene glycol. The anticonvulsant activity of the parent drug was strongest in the propylene glycol vehicle with subcutaneous and intraperitoneal routes and equal in the propylene glycol and polyvinylpyrrolidone vehicles with the oral route. With these three routes, bovine serum albumin and saline appeared to be inadequate vehicles for studying the anticonvulsant activity of (−)-Δ^9-*trans*-tetrahydrocannabinol.

A correlation between the ear temperature-lowering effect and blood level was investigated in rabbits and dogs after oral administration of two different dosage forms of ethylphenylephrine: an uncoated tablet, which showed a rapid dissolution rate *in vitro*, and a sustained-release capsule, which showed a gradual dissolution rate (1242). Absorption of the drug from the uncoated tablet was more rapid than from the sustained-release capsule. Plasma concentration and urinary excretion of proxyphylline were studied in healthy volunteers after the administration of the drug intravenously and orally as ordinary tablets and slow release tablets of the insoluble matrix type (1243). The rate of absorption from the slow release tablets was limited by the dissolution from the tablets, and the *in vitro* release agreed closely with the calculated absorption. Administration of slow release tablets twice a day for 5 days gave about the same plasma levels as ordinary tablets given three times a day, and the bioavailability was the same from both preparations.

A competitive protein-binding method was used to evaluate two prednisolone preparations: a conventional form and an enteric-coated formulation (1244). Except for the delay due to the coating, they were completely comparable, since maximum levels reached, areas under plasma concentration–time curves, and apparent disappearance half-lives were almost identical. Diethylpropion 25-mg capsules and 75-mg sustained-release tablets were tested in humans under acidic conditions, and the results compared with those obtained previously after administration of the drug in aqueous solution (1245). The bioavailabilities were comparable in each case, and the metabolic fate of the drug was not modified by these pharmaceutical formulations. Under conditions of uncontrolled urinary pH, the recoveries of diethylpropion and all basic metabolites except norepinephrine were greatly reduced.

Table XXXV—Additional References on Effects of Formulation

Reference	Topic
1251	Relative bioavailability of commercial ampicillin formulations in humans
1252	Effects of formulation on aspirin bioavailability
1253	Relative bioavailability of chloral hydrate after rectal administration of different dosage forms
1254	Effects of formulation on clomethiazole bioavailability
1255	Effects of formulation on availability of cyclazocine
1256	Effects of formulation on griseofulvin and riboflavin bioavailability
1257	Effects of formulation on topical bioavailability of griseofulvin
1258	Effects of formulation on isoniazid bioavailability
1259	Effects of formulation on nitrofurantoin bioavailability
1260	Effects of formulation on phenobarbital bioavailability
1261	Effects of formulation on potassium chloride bioavailability
1262	Effects of formulation on procainamide bioavailability
1263	Effects of formulation on sulfadiazine availability
1264	Effects of formulation on theophylline bioavailability
1265	Effects of drug–adjuvant interactions on drug bioavailability
1266	Drug adsorption properties of different activated charcoal dosage forms *in vitro* and in humans
1267–1269	Review of effects of formulation on drug bioavailability
1270	Effects of formulation on bioavailability of injectables

The dissolution rates of oxyphenbutazone in two commercial samples of sugar-coated tablets and one soft gelatin capsule were determined (1246). Clinical trials to compare tolerance indicated that the soft gelatin capsules led to less complaints of gastric irritation. The bioavailability of two experimental ampicillin capsule formulations was greater than 85% of the standard product, indicating that the processes involved in the manufacture of these formulations (dry granulation *versus* direct encapsulation) had little influence on ampicillin systemic availability (1247). In two test panels of 10 subjects each, plasma levels and urinary excretion of two drugs, acetaminophen and nitrofurantoin, were measured to compare bioavailability when the drugs were formulated as commercial tablets and as specially formulated gelatin capsules (1248). No significant differences in the relative efficiency of absorption were obtained between tablets and capsules with either drug.

Pagay and coworkers (1249, 1250) studied the rectal absorption of acetaminophen from an aqueous solution and from several polyethylene glycol base dosage forms. A relationship was observed between acetaminophen bioavailability and the dielectric properties of the vehicles utilized. The *in vitro* dissolution

rates of acetaminophen in these dosage forms were also studied and were somewhat related to the time of occurrence of the maximum urinary excretion rate.

Additional references on effects of formulation are found in Table XXXV.

Absorption Control and Alteration—Segre *et al.* (1271) studied the pharmacokinetic interactions between aspirin and other nonsteroidal anti-inflammatory drugs including naproxen. The results showed that simultaneous oral administration of aspirin and naproxen in humans resulted in a small, but statistically significant, lowering of plasma levels of naproxen. This effect was not mediated by competition for drug absorption but was associated with enhanced renal clearance of naproxen or its metabolites. The bioavailability of digoxin was evaluated in the presence of antacids containing magnesium trisilicate (1272). The results indicated that antacids may impair the bioavailability of the drug.

The effects of 1,25-dihydroxycholecalciferol on calcium absorption, muscle weakness, and bone disease in chronic renal failure was investigated (1273). 1,25-Dihydroxycholecalciferol given for 4–8 days increased intestinal calcium absorption without causing large changes in plasma calcium or phosphate. Effects of caffeine on the absorption and analgesic efficacy of acetaminophen (paracetamol) were studied in rats (1274). There was a dose-dependent decrease in the GI absorption of acetaminophen. The administration of aluminum hydroxide gel delayed and depressed the GI absorption of isoniazid in rats and patients with active tuberculosis, whereas magaldrate had less pronounced and more variable effects (1275). The depressed absorption of isoniazid was probably related to delayed gastric emptying as a result of the presence of Al^{+3} in the stomach.

Tetracycline in aqueous solution containing 50 mg of iron caused an ~80 and ~65% inhibition of iron absorption in subjects with normal iron stores or depleted iron stores, respectively, by complex formation (1276). This effect was decreased when tetracycline was given before and iron was given after the meals. The effects of potassium ion on the intramuscular absorption of some anionic drugs were examined using the rat thigh muscle clearance method (1277). In the presence of potassium ion, a specific and reversible inhibition was noted in the absorption of anionic drugs such as isonicotinic acid, aminobenzoic acid, and sulfa drugs. The effect of ethanol on intestinal absorption of theophylline was evaluated (1278). Theophylline absorption was significantly increased by 5% ethanol and was decreased by 20% ethanol.

The effect of salicylic acid on antibacterial activity and distribution of sulfadimethoxine was studied in rabbits (1279). The antibacterial activity of sulfadimethoxine following intravenous administration of sulfadimethoxine was significantly increased by the concurrent administration of salicylic acid. The effects of the polysorbates on the intramuscular absorption of the water-soluble, micelle-free drugs were investigated in the rat (1280). The presence of a low concentration of the polysorbates caused a pronounced decrease in the absorption rates of drugs, and the reduction was reflected in their plasma levels. Absorption rates of the drug in the muscle were dependent on the concentration rather than the absolute amount of the surfactant. The GI absorption of 1% phenobarbital or phenobarbital sodium, administered orally to rats, was enhanced by polysor-

Table XXXVI—Additional References on Absorption Control and Alteration

Reference	Topic
1290	Absorption of aspirin and acetaminophen (paracetamol) in patients with achlorhydria
1291	Effect of concurrent administration of aspirin and indomethacin on serum concentrations
1292	Effect of acidic drugs on absorption of aminopyrine from rat small intestine
1293	Effect of gastric emptying on absorption of aminopyrine in rats
1294	Effects of drug pretreatment on antipyrine levels in blood and tissue
1295	Effect of nonionic surfactants on absorption of aprobarbital sodium from suppositories in rabbits
1296	Inhibitory effect of high dietary zinc on copper absorption in rats
1297	Effect of salicylate ion on absorption of ^{3}H-dextromethorphan hydrobromide in rats
1298	Digoxin malabsorption due to altered renal function
1299	Absorption of phenytoin (diphenylhydantoin) in humans and its inhibition by phenylbutazone
1300	Effect of phenobarbital on bioavailability of erythromycin
1301	Effect of food on *in vivo* release of propranolol from a polyvinyl chloride matrix tablet in dogs
1302	Effect of sodium cholate on intestinal absorption of sulfa drugs
1303	Absorption studies of sulfamethoxazole in presence of trimethoprim in young adults
1304	Absorption studies of sulfamethoxazole in presence of trimethoprim in humans
1305	Interactive effect of (+)-fenfluramine and dextroamphetamine on feeding in rats
1306	Effect of potassium ion on *in situ* rat intestinal absorption of several drugs
1307	Effect of short-chain fatty acids on intestinal absorption of drugs in rats
1308	Effect of phenylpyrazolone derivatives on gastric emptying rate and drug absorption in rats
1309	Effect of γ-radiation on intestinal absorption of sulfanilamide
1310	Effects of complex formation on drug absorption
1311	Enhancement of drug absorption after administration by an automatic injector
1312	Influence of tablet hardness and food intake on absorption of drugs
1313	Effect of HLB values of surfactants on ephedrine absorption and release from emulsified systems after oral administration to dogs
1314	Effect of dialkylpropionamide on *in vitro* and *in situ* absorption of prednisolone
1315	Effect of nonionic surfactants on absorption of drugs
1316	Effect of drug interactions on absorption
1317	Effect of acid production by stomach on absorption of levodopa
1318	Relation between viscosity of digestive fluid and disintegration time of tablets
1319	Effect of retention time on rectal absorption of aspirin suppositories in children and adults
1320	Availability of three antibiotics after intramuscular injection into thigh and buttock
1321	Effects of dimethyl sulfoxide and trimethylphosphine oxide on percutaneous absorption of corticosteroids in rats
1322	Effect of vehicles on reduction of brain norepinephrine by α-methyltyrosine

bate 80 and sodium lauryl sulfonate, especially at doses lower than the CMC, and was depressed by polyvinylpyrrolidone and methylcellulose (1281).

The effect of polysorbate 80 on the intestinal absorption of nine micelle-free drugs was examined in the rat (1282). Regardless of the ionic nature of the compounds, two levels of the effect were observed, namely an absorption-enhancing effect at a relatively low concentration range and a small inhibitory effect at high concentrations of the surfactant. The effects of surfactants on absorption through membranes were studied (1283). Pentobarbital absorption was enhanced in the goldfish by dioctyl sodium sulfosuccinate and poloxalene.

A prodrug approach to the enhancement of the rate of dissolution of allopurinol was studied (1284). These prodrugs had lower melting points, higher solubilities, and, consequently, greater rates of dissolution than did allopurinol. The prodrug concept was reviewed as a method of overcoming pharmaceutical problems (1285). The effect of food intake and sleep on the absorption of acetaminophen was studied (1286). The rate of absorption of acetaminophen was found to be more rapid in human subjects under fasting conditions than when it was given immediately following food. Differences in absorption rate were confirmed by the excretion rate, but the overall elimination of drug was not affected by urine flow nor was it altered during the sleep period.

The effect of food on the absorption characteristics of nitrofurantoin from a commercial capsule dosage form containing macrocrystalline drug and a commercial tablet dosage form containing microcrystalline drug was assessed in human subjects by a urinary excretion method (1287). In fasting subjects, less nitrofurantoin was absorbed and at a slower rate; lower body levels were obtained when the capsule rather than the tablet dosage form was orally administered. When each was administered with food, the absorption of nitrofurantoin was appreciably delayed. Food in the GI tract produced an increase in the peak body levels of nitrofurantoin from the macrocrystalline form.

The effect of fasting on the oral absorption and excretion of sodium salicylate and aspirin was studied in rabbits (1288). The attainment of the maximum blood levels of these drugs in the fasted rabbits was somewhat slower than in the nonfasted rabbits. However, the extents of absorption were the same in the fasted rabbits as in the nonfasted rabbits. The peak serum levels of amoxicillin and ampicillin were detected 2 hr after oral administration of 500 mg of either drug to a nonfasting subject, the peak values being 5.9 and 2.1 μg/ml, respectively (1289). In contrast, the peak serum level of amoxicillin was found at 3 hr after an oral dose of amoxicillin plus probenecid.

Additional references on absorption control and alteration are listed in Table XXXVI.

Absorption Mechanisms—The effects of surfactants, such as sodium glycocholate, on the absorption and metabolism of the model compounds *O*-benzoylthiamine disulfide and thiamine disulfide were evaluated by both *in situ* and *in vivo* experiments using rats (1323). The *in situ* results indicated that, in the presence of a biosurfactant, the increasing or decreasing effect of a synthetic surfactant on drug absorption and metabolism could be canceled by the possible formation of new mixed micelles consisting of the drug and both surfactants. Results were confirmed by oral experiments using the drug–micellar solution in rats. Based on *in vitro* and *in vivo* studies in rats, it was established that α-tocopherol deficiency affected the passive absorption of barbital, phenol red, and salicylate, probably by altering the GI membrane (1324).

Bromphenol blue and *p*-acetylaminohippuric acid were studied with rats to understand the basic principles of the hepatobiliary transport process (1325). The results suggested that these organic anions were transported from blood into bile by at least two processes (hepatic uptake and biliary excretion) and that the hepatic uptake of these compounds was mainly due to the binding to substances in the liver cells. Atropine sulfate, when recirculated through the rat intestine *in vivo*, was absorbed as a function of pH but not concentration (1326). Similar results were observed when atropine sulfate was instilled in rat intestinal loops for 30–120 min. Thus, atropine did not inhibit its own absorption in the intestine, where the blood vessels are not supplied with cholinergic vasodilators. The difference in absorption at different pH's might indicate that the diffusion of the drug was dependent on the degree of its ionization.

The effects of altered urinary pH on the renal excretion of doxycycline were studied in humans (1327). Alkalinization of the urine resulted in higher cumulative amounts of doxycycline excretion during both single- and multiple-dose regimens. The increased excretion was reflected in larger renal clearances and shorter half-lives for both regimens when the alkaline condition was compared to the control condition. A model was developed to study the GI absorption of drugs and dosage forms in the unanesthetized rhesus monkey (1328). The absorption of an ionizable acidic drug, an ionizable basic drug, and a nonionized drug from the stomach and the intestines was compared. The nonionized form of the drug was

Table XXXVII—Additional References on Absorption Mechanisms

Reference	Topic
1330	Contribution of solvent drag to intestinal absorption of acidic drugs, benzoic acid and salicylic acid, from rat jejunum
1331	Contribution of solvent drag to intestinal absorption of basic drugs, aminopyrine (amidopyrine) and antipyrine, from rat jejunum
1332	Proposed mechanism for gentamicin transport
1333	Sublingual resorption of trapidil in humans
1334	Effect of physiological factors on bioavailability of oral dosage forms
1335	Kinetics of sulfathiazole (norsulfazole) absorption in isolated intestine of rats
1336	*In vitro* hydrolysis and absorption in animals and humans of a new synthetic ester, thiamphenicol palmitate
1337	Review of absorption mechanisms of drugs
1338	Models for studying drug absorption *in vitro*

absorbed faster than the ionized form at any particular site in the GI tract.

A general physical model was described for the simultaneous active and passive transport of conjugated and unconjugated bile acids from nonmicellar solutions in the ileum (1329). It reduced to the passive absorption mechanism in the duodenum and the jejunum. Theoretical computations indicated that the stagnant water layer may be the rate-determining barrier.

Additional references on absorption mechanisms are listed in Table XXXVII.

PHARMACOKINETICS

A modern view of pharmacokinetics was presented, suggesting that both linear and nonlinear systems must be included (1339). Special attention was given to the equations used to describe nonlinear kinetics, the recognition of nonlinearities, nonlinear models, and the fitting of data. Seven guidelines were presented for use in possible future pharmacokinetic studies involving drug kinetics. After reviewing the present status of pharmacokinetics, the methods for pharmacokinetic analysis were outlined (1340). Special emphasis was given to the determination of drug bioavailability from various dosage forms. Use of pharmacokinetics in determining sites of drug action and in determining a clinical metabolic profile was suggested.

The effect of the route of administration on drug disposition was reviewed (1341). The authors suggested that while the first-pass effect is a fairly generally understood phenomenon in the gross sense, it is not commonly appreciated that the rate at which the drug is delivered to the liver can influence the fraction of the administered dose that reaches the fluids of distribution. The drug molecules being absorbed *via* the hepatic portal vein intermix with previously absorbed drug recycling in the hepatic artery. The concentration of the drug returning *via* the hepatic artery is affected by the volume of distribution of the drug into body tissues.

The importance of tissue distribution in pharmacokinetics was discussed (1342). Mechanisms of drug distribution were summarized, and the differences between intracellular and extracellular pH, active transport system for drugs, distribution of drugs between fat and water in adipose tissue, reversible binding of drugs to phospholipids and to various macromolecules including proteins, nucleic acid, and melanin were presented. Examples were presented of pharmacokinetic analysis which influence the practice or principles of practical therapeutics. The action of the organism on the drug was summarized in terms of pharmacokinetic parameters: absorption, distribution, metabolism, and excretion. These parameters were examined with respect to drug accumulation, relation of pharmacokinetic data to therapeutic effects, saturation phenomena, effect of kidney disease, variation of pharmacokinetic parameters in individual patients due to age, pH of body fluids, and states of wakefulness (1343).

A general consideration of cancer chemotherapy was presented, consisting of a brief description of cell kinetics and some biochemical events (1344). Models of local tissue and cell uptake of drugs were presented, including various diffusion and mass transport steps. The complex interactions of saturable transport, cell binding, and other pharmacokinetic parameters on the concentration levels of methotrexate in rat bone marrow and other tissues were studied.

A simple equation was presented by which the first-order rate constant for drug absorption into the central compartment of any linear mammillary pharmacokinetic model can be calculated (1345). No specific knowledge of the distribution or elimination characteristics of the drug was required. The meaning of clearance for drugs that are exclusively eliminated from a peripheral compartment of a multicompartment mammillary system was examined (1346). The utility of the various pharmacokinetic parameters employed to interpret drug elimination was discussed. Practical pharmacokinetic techniques for drug consultation and evaluation were described (1347). Psychotherapeutic drugs were used as prototypes for illustrating some considerations in pharmacist-generated dosage regimens.

Pooling of Michaelis–Menten equations for models having parallel paths for formation of two or more metabolites was discussed (1348). A theory that explains phenomena exhibited by pooled nonlinear pharmacokinetic systems and equations relating pooled Michaelis–Menten constants (V_p, K_p) to microscopic constants (V_i, K_i) were presented. The suitability of this type of pooling for use in pharmacokinetic modeling was discussed, as was the use of pooling concepts in the design of clinical studies. Ballard (1349) reviewed the effect of temperature on pharmacokinetics. He discussed the effects of body and environmental temperature on human physiological changes and physicochemical properties, absorption, distribution, and metabolism of drugs.

Pharmacokinetic parameters of amikacin and kanamycin were compared in a crossover study using healthy adult male volunteers (1350). Apparent volumes of distribution, serum half-lives, and renal clearance were compared and were found to be virtually identical. The pharmacokinetics of intravenously administered amobarbital and its major metabolite hydroxyamobarbital were studied (1351). The metabolism of amobarbital was a saturable process and largely zero order over the entire dose range studied.

Pharmacokinetic studies in amphetamine-dependent subjects, using large intravenous doses, were undertaken to relate measurable pharmacokinetic parameters to the clinical manifestations of amphetamine abuse (1352). Subjects having an acidic urine exhibited a relatively mild psychosis, while the psychotic symptoms were aggravated in patients with an alkaline urine. Chlorphenesin carbamate serum levels during subchronic administration to normal human volunteers were studied (1353). A one-compartment open model appeared to have predicted adequately the equilibrium serum concentrations of chlorphenesin carbamate.

Analog and digital computing techniques were

used to elucidate the pharmacokinetic parameters involved in the metabolism and excretion of diethylpropion and its metabolites in humans (1354). Rate constants for the various processes were evaluated for the complex reaction scheme. The values of the rate constants were used as the basis for discussion of the relative importance of some metabolic routes.

Several papers were presented on the pharmacokinetics of digoxin. During chronic therapy with 0.25 mg of digoxin, mean plasma digoxin levels measured by radioimmunoassay were 0.7 ng/ml and the daily urine excretion of digoxin was about 44% of the daily given dose (1355). When the levels of orally given 0.25-mg digoxin tablets were measured by peak serum digoxin concentration as well as by area under serum digoxin concentration–time curve, the bioavailability of digoxin appeared to be higher in the fasted subjects than in fed ones (1356). After intravenous administration, the pharmacokinetics of digoxin were described by a two-compartment open model. Digoxin was strongly distributed to tissues, as reflected by a mean ratio of k_{el}/B = 9.24 and an average volume of distribution at steady state of 5.95 liters/kg (1357).

Pharmacokinetic design of digoxin dosage regimens in relation to renal function was studied (1358). Calculation of a dosage schedule to produce predetermined steady-state serum concentrations of digoxin was dependent on four major variables: bioavailability, biotransformation rate constant, renal clearance, and apparent volume of distribution. Loading and maintenance doses of digoxin in patients of normal renal function and those with severely impaired renal function were studied (1359). It was found that loading doses for patients with severely impaired renal function should be half those for patients with normal renal function.

A dose–effect curve constructed from ventricular rate slowing and oral maintenance doses for digoxin provided evidence for assuming that occupation theory correctly described drug–receptor site interaction (1360). The influence of allopurinol on drug metabolism was studied in humans. Its administration in conjunction with the anticoagulant warfarin did not alter their dose requirements (1361).

Furosemide, as tablets or aqueous solution, was taken orally by normal subjects during a fast and postprandially (1362). Comparative studies were conducted using intravenous furosemide. No significant differences were found between the tablets and the aqueous solution. During fasting, detectable drug levels appeared in the serum within 10 min. The postprandial studies displayed delayed appearance and low peak serum concentration. Salivary and urinary excretion of lithium was studied in three healthy male subjects after oral administration of two or three different doses (1363). In all individuals, the concentration of lithium in the salivary fluid was 2.2–3.3 times as high as the concentration in plasma. Thus, once the saliva–plasma concentration ratio is established, the measurement of saliva concentrations can provide all of the pharmacokinetic information necessary for rational dosage regimens.

Serum levels of methaqualone were determined in eight unfasted subjects following single- and multiple-dose administration (1364). The data were analyzed by a two-compartment open model. The significance of pharmacokinetic parameters in relation to bioavailability and the biological disposition of single- and multiple-dose methaqualone administration was discussed. Acetaminophen (paracetamol) was administered intraperitoneally or intravenously to male rats (1365). Its availability in the systemic circulation after intraperitoneal administration was 34% of that after intravenous administration. This finding suggested a high first-pass metabolism of intraperitoneally administered drug, which was confirmed using the rat isolated perfused liver.

The absorption of orally administered pivampicillin, an ester that hydrolyzes to ampicillin *in vivo*, was studied in healthy males and compared to ampicillin administered intravenously, intramuscularly, and orally (1366). Renal clearances and serum half-lives for pivampicillin were the same as those for ampicillin. The clinical efficacy, tolerance, and pharmacokinetics of a new aminoglycoside antibiotic, sisomicin, were studied in elderly male patients suffering from complicated urinary tract infections (1367). After intramuscular injection, serum levels of the antibiotic declined, with a half-life of about 3 hr after the initial dose. The pharmacokinetics of thiothixene was studied in psychotic patients on long-term therapy (1368). Drug plasma concentrations reached an early peak following an oral dose and declined slowly thereafter. Despite considerable differences in doses, peak plasma concentrations fell within a relatively narrow range, indicating that, during earlier adjustments of dose to achieve therapeutic control, optimum plasma concentrations were also being achieved.

Matin *et al.* (1369) studied the pharmacokinetics of tolbutamide and found a good linear relationship between tolbutamide concentration in saliva and plasma; salivary levels were 1.2% of plasma levels. The pharmacokinetics and pharmacodynamics of enantiomers of warfarin were studied in humans (1370). In nine subjects, the plasma half-life of *R*-warfarin after a single oral dose was significantly longer than that of *S*-warfarin. Similar pharmacokinetic studies on the warfarin enantiomers were conducted with rats (1371).

There has been a new stress on determining the various pharmacokinetic parameters of different drugs used during the diseased state. Jackson and McLeod (1372, 1373) discussed the dosing concentration of antimicrobial agents when used in patients with renal impairment. They discussed the pharmacokinetics of the following drugs: bacitracin, chloramphenicol, chloroquine, clindamycin, dapsone, erythromycin, lincomycin, neomycin, vancomycin, the urinary tract anti-infectives, sulfonamides, and trimethoprim.

The pharmacokinetics of cefazolin were studied in normal and uremic patients after single and multiple intramuscular doses (1374). In normal subjects, cefazolin gave maximum serum concentrations of 30–50

μg/ml 1 hr after a single 500-mg injection, with a serum half-life of about 2 hr. Higher serum levels were obtained after identical doses to uremic subjects, and serum half-lives increased with decreasing renal function. Also studied were the pharmacokinetics of cefazolin in the presence of normal and impaired renal functions (1375). The mean serum half-life of cefazolin sodium was 1.6 hr in normal patients and about 42 hr in patients with impaired renal function.

The pharmacokinetics of furosemide in normal subjects and functionally anephric patients were studied (1376). The plasma half-life was increased from 29.5 min in normal patients to 80.7 min in anephric patients. The pharmacokinetics of furosemide in advanced renal failure, where the renal furosemide clearance was reduced (1377), were also discussed. The anticoagulant half-life of heparin in human subjects with normal and impaired renal functions was studied (1378, 1379). The anticoagulant half-life of heparin was found to be dose related. The normal renal function group gave a mean anticoagulant half-life of 36.8 min for the 0.6-unit/ml dose and 22.7 min for the 0.3-unit/ml dose. The chronic renal failure population had a mean half-life of 47.5 min for the 0.6-unit/ml dose and a mean half-life of 32.6 min for the 0.3-unit/ml dose.

The pharmacokinetics of metolazone were studied in normal adult volunteers after oral or intravenous administration and after oral administration in patients requiring diuretic therapy for severe renal failure, for moderate renal failure, and for heart disease (1380). Clearance of metolazone from the central compartment was approximately equal to creatinine clearance and ranged from 110 ml/min in normal subjects to 20 ml/min in severe renal failure. The absorption was 64% in normal controls, but as little as 40% of the oral dose was absorbed in some patients. The plasma half-life of phenylbutazone in patients with impaired liver function was studied (1381). Preliminary evidence was presented for a significant correlation between the apparent elimination rate constant of phenylbutazone and the galactose elimination capacity in cirrhotic patients.

Eight volunteers with normal renal function and 11 patients with chronic renal insufficiency were each given propranolol while in the fasting state (1382). Absorption was rapid in both groups. In the patients, propranolol appeared in the serum within 0.5 hr of oral administration and maximum concentrations, with a mean of 154.6 ng/ml, appeared in 1.5 hr; in the control group, maximal concentrations, with a mean of 51.1 ng/ml, developed in 2 hr. The pharmacokinetics of the antibacterial combination sulfamethoxazole and trimethoprim in patients with normal or impaired kidney function were studied (1383). Following oral administration, the elimination rate of sulfamethoxazole and, to a lesser extent, trimethoprim was decreased by renal impairment. Also studied were the pharmacokinetics of 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile in normal and polyarthritic rats (1384).

Additional references on pharmacokinetics are listed in Table XXXVIII.

Table XXXVIII—Additional References on Pharmacokinetics

Reference	Topic	Reference	Topic
1385	Pharmacokinetics of acetylkidamycin	1401	Pharmacokinetics of 1β-D-arabinofuranosylcytosine in humans
1386	Pharmacokinetic properties of β-adrenergic receptor blocking drugs	1402	Pharmacokinetics of 1β-D-arabinofuranosylcytosine deamination in several species
1387	Influence of pharmacokinetic variations on the pharmacological properties of adriamycin	1403	Pharmacokinetic aspects of elimination from plasma and distribution to brain and liver of barbiturates in rat
1388	Blood levels, tissue distribution, and clinical effects of adriamycin	1404	Absorption and metabolism of orally administered beclomethasone dipropionate
1389	Kinetics and bioavailability of two formulations of amiloride in humans	1405	Pharmacokinetics of 1-[4,6-bis(allylamino)-*s*-triazin-2-yl]-4-(di-*p*-fluorobenzhydryl)piperazine bis(methanesulfonate)
1390	Urine data analysis for pharmacokinetics of aminopyrine	1406	Pharmacokinetics, drug availability, and clinical pharmacology of bromhexine
1391	Antibacterial activity, absorption, excretion, and clinical applications of amoxicillin	1407	Pharmacokinetics of the diuretic bumetanide
1392	Pharmacokinetics of amoxicillin	1408	Pharmacokinetic study of local anesthetics, bupivacaine and etidocaine, in humans
1393	Distribution of parenteral ampicillin and cephalosporins in late pregnancy	1409	Pharmacokinetics of burimamide and metiamide
1394	Pharmacokinetics of ampicillin potassium in healthy subjects and patients with glomerular renal insufficiency	1410	Pharmacokinetics of butaperazine
1395	Comparative studies on absorption of ampicillin trihydrate and ampicillin potassium	1411	Kinetics of human urinary excretion results for butethal (butobarbitone) and its metabolites
1396	Comparative study of serum concentrations and urinary excretion of ampicillin, pivampicillin, and amoxicillin after oral application to fasted subjects	1412	Butethal (butobarbitone) metabolism in humans: identification of 3-ketobutobarbitone
1397	Absorption, distribution, and excretion of anisodamine	1413	Pharmacokinetics of calcium dobesilate in humans
1398	Relevant pharmacokinetics of antimicrobial drugs	1414	Pharmacokinetics of microdispersed calcium oleate
1399	Pharmacokinetics of antineoplastic preparations	1415	Distribution, metabolism, and excretion of caprylohydroxamic and nicotinohydroxamic acids
1400	Pharmacokinetics and biodegradation of aprindine in humans	1416	Pharmacokinetics of carbenicillin in high dosage

Table XXXVIII—*Continued*

Reference	Topic
1417	Pharmacokinetics of cefazolin compared with four other cephalosporins
1418	Cefazolin plasma concentrations and urinary excretion in patients with renal impairment
1419	Pharmacokinetics and therapeutic uses of cephaloridine
1420	Determination of half-life, clearance, and dialyzability of sodium salt of cephalosporanic acid in humans
1421	Dose equation for continuous intravenous infusion based on pharmacokinetics and its clinical applications on cephalothin
1422	Pharmacokinetics of cephanone in healthy adult volunteers
1423	Pharmacokinetics of chlorpromazine metabolites
1424	Absorption, distribution, and excretion of 6-chloro-5-cyclohexylindane-1-carboxylic acid in rats
1425	Absorption, distribution, and excretion of 8-chloro-6-phenyl-4*H*-*s*-triazolo(4,3-*a*)(1,4)-benzodiazepine in rats
1426	Species differences in metabolism of 8-chloro-6-phenyl-4*H*-*s*-triazolo(4,3-*a*)(1,4)benzodiazepine
1427	Multicompartmental analyses of bi- and triexponential plasma disappearance curves after intravenous injection of ^{14}C-cholic acid, sulfobromophthalein, and ^{14}C-bilirubin, and their significance for quantitative studies of the hepatic excretory function
1428	Distribution and excretion of iodochlorhydroxyquin (clioquinol) in animals
1429	Pharmacokinetics of clonazepam in humans
1430	Pharmacokinetic profiles of clonazepam in dogs and humans and of flunitrazepam in dogs
1431	Comparative pharmacokinetics of coumarin anticoagulants; relationship between distribution, elimination, and anticoagulant action of warfarin
1432	Comparative pharmacokinetics of anticoagulant effect of coumarin drugs in humans and rats
1433	Comparative pharmacokinetics of coumarin anticoagulants; relationship between distribution, elimination, and anticoagulant action of dicumarol
1434	Pharmacokinetics of cyclohexylamine in humans
1435	Distribution of daunomycin and adriamycin in mice
1436	Pharmacokinetics of diatrizoate sodium and iodipamide sodium under hemodialysis
1437	Absorption, excretion, and distribution of 3′,4′-dideoxykanamycin B in rats
1438	Absorption, excretion, and distribution of 3′,4′-dideoxykanamycin B in rabbits and dogs
1439	Pharmacokinetic analysis of 3′,4′-dideoxykanamycin B in pediatric field
1440	Pharmacokinetics of ^{14}C-dimethothiazine
1441	Pharmacokinetics of 4-[3-(dimethylamino)-propylidene]-4,9-dihydrobenzo[*e*]thieno[2,3-*b*]thiepine-10-^{35}S (dithiadene-10-^{35}S)
1442	Metabolic fate of *N*,*N*-dimethyl-*N*′-(*p*-phenoxyphenyl)sulfamide in rats, dogs, and humans
1443	Fate and metabolism of dimethyl sulfoxide
1444	Pharmacokinetic studies of phenytoin (diphenylhydantoin)
1445	Half-life of diphenylpyraline in humans
1446	Diazepam metabolism in normal humans; serum concentrations and clinical effects after intravenous, intramuscular, and oral administration
1447	Diazepam metabolism in normal humans; serum concentration and clinical effect after oral administration and accumulation of diazepam
1448	Pharmacokinetic analysis of 3′,4′-dideoxykanamycin B in humans
1449	Review on pharmacokinetics and bioavailability of digitalis
1450	Absorption, metabolism, and excretion of cromoglycate disodium in nine animal species
1451	Effect of phenobarbital on pharmacokinetics of erythromycin
1452	Pharmacokinetics of fluorocarbon aerosol propellants
1453	Pharmacokinetic studies on flupentixol and flupentixol decanoate in animals and humans
1454	Pharmacokinetics and metabolism of fominoben in humans
1455	Pharmacokinetics of gentamicin in cerebrospinal fluid and eye compartments
1456	Pharmacokinetics of glibornuride
1457	Pharmacokinetics and pharmacodynamics, as well as metabolism, following orally and intravenously administered ^{14}C-glipizide
1458	Pharmacokinetics of glipizide in humans
1459	Pharmacokinetics of radioactively labeled glisoxepide in animals
1460	Pharmacokinetics and metabolic spectrum of glisoxepide in humans
1461	Absorption, distribution, metabolism, and excretion of glycopyrrolate in mice
1462	Pharmacokinetics of glymidine and tolbutamide in acute and chronic liver diseases
1463	Absorption, metabolism, and excretion of ^{14}C-griseofulvin in humans
1464	Absorption and elimination of ^{35}S-gutimine following intraperitoneal administration
1465	Metabolism of heptabarbital (heptabarbitone)
1466	Disposition of hydralazine in humans, and a specific method for its determination in biological fluids
1467	Comparative studies on distribution, excretion, and metabolism of hydroxyzine-^{3}H and its methiodide-^{14}C in rats
1468	Imipramine protein binding and pharmacokinetics in children
1469	Pharmacokinetics of isoniazid in Caucasian race
1470	Pharmacokinetics of isoniazid in humans
1471	Pharmacokinetic and metabolic studies with lanatoside C, α- and β-acetyldigoxin, and digoxin in humans
1472	Pharmacokinetics of levodopa and its metabolites
1473	Hypothesis for rapid attainment and maintenance of lidocaine plasma levels
1474	Pharmacokinetic studies with maprotiline
1475	Pharmacokinetics of menadione and menadione sodium bisulfite in animals
1476	Pharmacokinetic properties of meprobamate in dogs; an example of dose dependency in elimination kinetics
1477	Pharmacokinetic studies after administration of ^{14}C-dipyrone (^{14}C-metamizole) to rats, dogs, and humans
1478	Pharmacokinetics and availability of methanesulfonic acid derivative of dapsone (*p*,*p*′-diaminodiphenylsulfone) in rabbits
1479	Review of pharmacokinetics of methotrexate
1480	Absorption, metabolism, and distribution of ^{14}C-*O*-methyldopa and ^{14}C-levodopa after oral administration to rats
1481	Pharmacokinetics of antimicrobial agent mydecamycin
1482	Pharmacokinetic studies on mydecamycin
1483	Pharmacokinetics of ^{3}H-naftazone in humans
1484	Pharmacokinetics of nalidixic acid: serum and urinary levels with normal renal function
1485	Pharmacokinetics of naloxone
1486	Metabolism of nealbarbital (nealbarbitone)
1487	Absorption of neomycin from alimentary tract of a pig

(continued)

Table XXXVIII—*Continued*

Reference	Topic
1488	Pharmacokinetic and genetic studies of nortriptyline and desipramine in human subjects
1489	Aspects of pharmacokinetics of opiates
1490	Compartmental study on pharmacokinetics of D-penicillamine
1491	Metabolism of D-penicillamine
1492	Pharmacokinetics of dihalo-substituted isoxazolyl penicillins
1493	Pharmacokinetic evaluation of penicillin and cephalosporin derivatives in serum and milk of lactating cows and ewes
1494	Pharmacodynamic and pharmacokinetic study of modern semisynthetic penicillins
1495	Pharmacokinetics of pentazocine and its optical isomers
1496	Analysis of intersubject variation in pentazocine metabolism
1497	Dose and dosage form-dependent pharmacokinetics of phenacetin and its metabolite in humans
1498	Pharmacokinetic studies on 2-phenyl-4-(*p*-chlorophenyl)thiazol-5-ylacetic acid
1499	Urinary metabolites of phylloquinone and α-tocopherol
1500	Metabolism and kinetics of piribedil in humans and rats
1501	Absorption and distribution of 4-prenyl-1,2-diphenyl-3,5-pyrazolidinedione in rats
1502	Excretion of probenecid and its metabolites in bile and urine of rats
1503	Pharmacokinetics of procainamide
1504	Pharmacokinetics as a guideline for procainamide and theophylline dosing regimens
1505	Computerized dosage regimens for procainamide
1506	Pharmacokinetic studies on propanidid
1507	Clinical investigations on pharmacokinetics of propiram-^{14}C fumarate
1508	Chemical study on pharmacokinetics of propiram fumarate
1509	Influence of dose and infusion rate on propranolol bioavailability
1510	Pharmacokinetics of distribution and elimination of sodium di-*n*-propylacetate in mice and dogs
1511	Pharmacokinetic studies on proxyphylline administered intravenously and orally to humans
1512	Pharmacokinetics and tolerance of quinidine sulfate and dihydroquinidine gluconate in horses and dogs
1513	Pharmacokinetic disposition of quinidine in rhesus monkeys
1514	Absorption and clearance of secobarbital, heptabarbital, methaqualone, and ethinamate
1515	Use of a mathematical model of streptomycin pharmacokinetics for providing necessary blood levels under conditions of intravenous infusion
1516	Plasma levels and urinary excretion of K-strophanthoside-^{3}H administered rectally to humans
1517	Pharmacokinetics of sulfadiazine–nitrofurantoin during normal kidney function
1518	Dosage regimens of sulfonamides based on pharmacokinetic parameters
1519	Pharmacokinetic study on metabolism and excretion of sulpyrine in rats
1520	Distribution, excretion, and metabolism of N^1-(2-tetrahydrofuryl)-5-fluorouracil
1521	Pharmacokinetics and metabolism of ^{3}H-thebaine
1522	Pharmacokinetics of thiamphenicol: behavior in acute and chronic hepatic insufficiency
1523	Pharmacokinetics of thiamphenicol: behavior in renal insufficiency under dialysis conditions
1524	Absorption, distribution, excretion, and metabolism of tinidazole
1525	Pharmacokinetic profile of trimethoprim–sulfamethoxazole in humans
1526	Pharmacokinetic studies with trimethoprim and different doses of sulfadiazine in healthy subjects
1527	Absorption and urinary excretion of trimethoprim, sulfamethoxazole, and trimethoprim–sulfamethoxazole
1528	Pharmacokinetics of trimethoprim and sulfamethoxazole in normal subjects and in patients with renal failure
1529	Metabolism of trimethoprim in humans, and measurement of a new metabolite
1530	Excretion of trimethoprim and sulfamethoxazole in patients with renal failure
1531	Similarity of warfarin half-lives in human subjects
1532	Review of pharmacokinetic factors affecting epidermal penetration and percutaneous absorption
1533	Review of pharmacokinetics and dosage regimens
1534	Review of one-compartment open model pharmacokinetics comparing excretion following intravenous administration with and without metabolism
1535	Review of pharmacokinetics of one-compartment open model at constant intravenous infusion and after a single dose
1536	Review of pharmacokinetic and biopharmaceutical fundamentals
1537	Contribution of pharmacokinetics to drug design
1538	Significance of pharmacokinetics in drug therapy
1539	Mathematical aspects of pharmacokinetics and toxicokinetics
1540	Pharmacokinetics in relation to age
1541	Apparent biological half-life values determined by drug administration by methods other than rapid intravenous injection
1542	Mathematical procedures for evaluating pharmacokinetic studies
1543	Dose-dependent drug metabolism during absorptive phase
1544	Pharmacokinetic considerations in clinical drug trials
1545	Pharmacokinetics as foundation for drug therapy
1546	Application of pharmacokinetic principles to elucidation of polygenically controlled differences in drug response
1547	Kinetics of drug–drug interactions
1548	Structural effects in drug distribution using whole animal pharmacokinetics
1549	Statistical estimations in pharmacokinetics
1550	Importance of use of appropriate pharmacokinetic model to analyze *in vivo* enzyme constants
1551	Critical evaluation of use of effective protein fractions in developing pharmacokinetic models for drug distribution
1552	Safe method for rapidly achieving plasma concentration plateaus
1553	Linearity and superposition in pharmacokinetics
1554	Importance of type of dosage form and saturable acetylation in determining bioactivity of aminosalicylic acid
1555	Simulation of blood level of drugs by digital computer
1556	Significance of blood levels of drugs
1557	Design and analysis of comparative blood level trials
1558	Pharmacokinetic consequences of plasma protein binding of drugs
1559	Age-related differences of pharmacokinetic behavior of short-lasting sulfonamides in

Table XXXVIII—*Continued*

Reference	Topic	Reference	Topic
	rats, with special reference to protein binding rate		drug concentrations in blood
1560	Calculation of plasma level *versus* time profiles for variable dosing regimens	1562	Effect of antacids on pH of urine and its effect on kinetics of elimination of certain acidic and basic drugs
1561	Factors causing interindividual variations of		

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RESEARCH ARTICLES

Kinetics and Mechanisms of Degradation of the Antileukemic Agent 5-Azacytidine in Aqueous Solutions

ROBERT E. NOTARI ˣ and JOYCE L. DeYOUNG *

Abstract □ The hydrolytic degradation of 5-azacytidine was studied spectrophotometrically as a function of pH, temperature, and buffer concentration. Loss of drug followed apparent first-order kinetics in the pH region below 3. At pH <1, 5-azacytosine and 5-azauracil were detected; at higher pH values, drug was lost to products which were essentially nonchromophoric if examined in acidic solutions. The apparent first-order rate constants associated with formation of 5-azacytosine and 5-azauracil from 5-azacytidine are reported. Above pH 2.6, first-order plots for drug degradation are biphasic. Apparent first-order rate constants and coefficients for the biexponential equation are given as a function of pH and buffer concentration. A reaction mechanism consistent with the data is discussed together with problems associated with defining the stability of the drug in aqueous solutions. At 50°, the drug exhibited maximum stability at pH 6.5 in dilute phosphate buffer. Similar solutions were stored at 30° to estimate their useful shelflife. Within 80 min, 6×10^{-4} *M* solutions of 5-azacytidine decreased to 90% of original potency based on assumptions related to the proposed mechanisms.

Keyphrases □ 5-Azacytidine—kinetics and mechanisms of degradation in aqueous solutions, effect of pH, temperature, and buffer concentration □ Hydrolysis, 5-azacytidine in aqueous solutions—kinetics and mechanisms, effect of pH, temperature, and buffer concentration □ Antileukemic agents—5-azacytidine, kinetics and mechanisms of degradation in aqueous solutions

The synthesis of 5-azacytidine (I) was reported in 1964 (1). The compound has antimicrobial and antitumor activities (2–4) and is currently being evaluated clinically in the treatment of human leukemias.

The clinical use of this drug makes it desirable to determine kinetic data on its reactivity in aqueous solutions. Such information is especially important in consideration of its relative instability when compared to cytidine itself. Especially notable are the ease with which the triazine ring hydrolyzes and the lability of the sugar–triazine bond (5). Although most hydrolysis products of I have been identified (5), lit-

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RESEARCH ARTICLES

Kinetics and Mechanisms of Degradation of the Antileukemic Agent 5-Azacytidine in Aqueous Solutions

ROBERT E. NOTARI ˣ and JOYCE L. DeYOUNG *

Abstract □ The hydrolytic degradation of 5-azacytidine was studied spectrophotometrically as a function of pH, temperature, and buffer concentration. Loss of drug followed apparent first-order kinetics in the pH region below 3. At pH <1, 5-azacytosine and 5-azauracil were detected; at higher pH values, drug was lost to products which were essentially nonchromophoric if examined in acidic solutions. The apparent first-order rate constants associated with formation of 5-azacytosine and 5-azauracil from 5-azacytidine are reported. Above pH 2.6, first-order plots for drug degradation are biphasic. Apparent first-order rate constants and coefficients for the biexponential equation are given as a function of pH and buffer concentration. A reaction mechanism consistent with the data is discussed together with problems associated with defining the stability of the drug in aqueous solutions. At 50°, the drug exhibited maximum stability at pH 6.5 in dilute phosphate buffer. Similar solutions were stored at 30° to estimate their useful shelflife. Within 80 min, 6×10^{-4} *M* solutions of 5-azacytidine decreased to 90% of original potency based on assumptions related to the proposed mechanisms.

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The synthesis of 5-azacytidine (I) was reported in 1964 (1). The compound has antimicrobial and antitumor activities (2–4) and is currently being evaluated clinically in the treatment of human leukemias.

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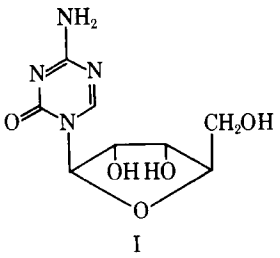


I

tle has been done concerning the kinetics and mechanism of its degradation.

The purpose of this report is to describe the results of studies on the hydrolysis of I and the related compounds 5-azacytosine (II) and 5-azauracil (III).

EXPERIMENTAL

Analytical Methods—Beer's law plots were constructed for 5-azacytidine, 5-azacytosine, and 5-azauracil in 0.1 *N* HCl using a recording spectrophotometer[1]. Molar absorptivities were 2.36×10^3 for 5-azacytidine at 255 nm; 3.35×10^3, 4.33×10^3, and 5.98×10^3 for 5-azacytosine at 230, 235, and 250 nm, respectively; and 3.67×10^3, 3.80×10^3, and 1.50×10^3 for 5-azauracil at 230, 235, and 250 nm, respectively.

Concentrations of components in mixtures of 5-azacytosine and 5-azauracil were calculated using:

$$10^3[\text{II}] = 0.217A_{250} - 0.088A_{230} \quad \text{(Eq. 1)}$$

$$10^3[\text{III}] = 0.353A_{230} - 0.198A_{250} \quad \text{(Eq. 2)}$$

which were derived from simultaneous equations for total absorbance, *A*, at 230 and 250 nm for mixtures of 5-azacytosine and 5-azauracil in 0.1 *N* HCl. Good agreement was obtained between the results of the assay and the known concentrations in synthetic mixtures.

Mixtures of 5-azacytidine and the two bases were assayed utilizing the differential stability of the components. The absorbance at 255 nm of solutions containing all three components in 0.1 *N* HCl was determined. These solutions were then made 0.05 *N* in sodium hydroxide and allowed to stand at room temperature for 40–50 min. This period was sufficient to degrade 5-azacytidine completely to nonchromophoric products without loss of 5-azacytosine or 5-azauracil. Samples were reacidified with 0.1 *N* HCl, and the absorbances at 235, 250, and 255 nm were determined. The 235- and 250-nm values were used to calculate the concentrations of 5-azacytosine and 5-azauracil:

$$10^3[\text{II}] = 0.234A_{250} - 0.092A_{235} \quad \text{(Eq. 3)}$$

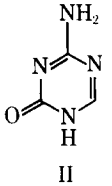
$$10^3[\text{III}] = 0.368A_{235} - 0.267A_{250} \quad \text{(Eq. 4)}$$

After correcting for dilution, the absorbances of the sample before and after treatment with alkali were used to calculate the 5-azacytidine concentration:

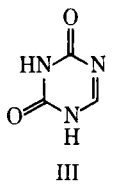
$$\text{I} = \Delta A_{255} / \epsilon_{255} \quad \text{(Eq. 5)}$$

This assay method was shown to be satisfactory using synthetic mixtures.

Kinetics of 5-Azacytosine and 5-Azauracil Hydrolysis in Hydrochloric Acid Solutions—Reaction solutions containing 2×10^{-4} *M* 5-azacytosine or 3×10^{-4} *M* 5-azauracil in 0.1 and 0.25 *N* HCl were placed in constant-temperature baths. Samples of the 5-azacytosine reactions were withdrawn as a function of time and assayed for substrate and 5-azauracil as described previously. The spectra of the 5-azauracil reactions decreased with time, with no evidence of formation of a new chromophore. Rate constants for its loss were calculated directly from absorbance data.



II



III

[1] Cary 15.

Table I—Apparent First-Order Rate Constants (hour^{-1}) for Hydrolysis in Hydrochloric Acid at 70°

Substrate	[HCl], *N*	k_{obs}	k_{23}	k_{25}	k_{36}
5-Azacytosine[a]	0.10	0.376	0.121	0.255	0.0495[b]
	0.25	0.238	0.119	0.119	0.0636[b]
	[HCl], *N*	k_{obs}	k_{12}	k_{13}	k_{14}
5-Azacytidine[c]	0.10	1.98	0.111	0.110	1.77
	0.25	1.05	0.136	0.101	0.810

[a] See Scheme I. Concentration is 2×10^{-4} *M*. [b] Determined from hydrolysis of 5-azauracil (3×10^{-4} *M*). [c] See Scheme II. Concentration is 2×10^{-4} *M*.

TLC of Reaction Solutions of 5-Azacytosine and 5-Azauracil—Samples of the reactions of approximately 0.01 *M* 5-azacytosine and 0.01 *M* 5-azauracil in 0.10 *N* HCl at 70° were taken after about 50 and 100% of the substrate had reacted. These samples were neutralized and spotted along with authentic samples of 5-azacytosine and 5-azauracil on TLC plates coated with 0.25 mm silica gel GF_{254}. The plates were developed in 1-butanol–acetic acid–water (100:10:30) for a distance of about 10 cm, air dried at room temperature, and examined with a shortwave UV lamp.

Kinetics of 5-Azacytidine Hydrolysis in Hydrochloric Acid Solutions—Reaction solutions containing 5-azacytidine in excess hydrochloric acid were prepared as shown in Tables I and II. The absorbance of reactions in solutions less concentrated than 0.1 *N* HCl was determined by diluting aliquots of the reaction with hydrochloric acid to give a final concentration of 0.25 *N* HCl. In these solutions, the UV absorption spectrum of 5-azacytidine decreased with time without the appearance of any new absorbances. Absorbance data were used directly to calculate the apparent first-order rate constants.

In solutions containing 0.1 *N* or stronger hydrochloric acid, there was evidence that a fraction of the 5-azacytidine decomposed to form 5-azacytosine and 5-azauracil. In these cases, the reactions were assayed using the described differential stability method. At 70°, Eqs. 3, 4, and 5 were used to assay for 5-azacytidine, 5-azacytosine, and 5-azauracil, respectively. In the 50° reactions, the differential absorbance due to 5-azacytidine (Eq. 5) was used to calculate the apparent first-order rate constants for loss of substrate.

Kinetics of 5-Azacytidine Hydrolysis in Buffers—Reaction solutions containing 5-azacytidine in excess buffer were prepared as shown in Table III. Solutions were placed in constant-temperature baths; aliquots were removed at appropriate times, chilled, and diluted with an equal volume of hydrochloric acid to give a final concentration of 0.25 *N* HCl. The absorbance of these solutions was measured at 255, 260, and 265 nm. Since absorbance values decreased with time to become zero when the reaction was complete, absorbance data were used directly in the calculation of

Table II—Apparent First-Order Rate Constants for Hydrolysis of 5-Azacytidine[a] at 50° in Hydrochloric Acid

[HCl], *N*	pH[b]	k_{obs}, hr^{-1}
0.80	0.22	0.0595
0.50	0.44	0.0764
0.25	0.73	0.118
0.097	1.12	0.239
0.0097	2.06	1.12
0.005	2.39	1.39

[a] Reactions in 0.80, 0.50, 0.25, and 0.005 *N* HCl contain 4×10^{-4} *M* substrate. Substrate concentration in 0.097 and 0.0097 *N* HCl reactions is 6×10^{-4} *M*. [b] The pH of hydrochloric acid solutions was calculated using activity coefficient values from the literature (6).

Table III—Experimental Conditions and Apparent First-Order Rate Constants for Loss of 6×10^{-4} *M* 5-Azacytidine in the Presence of Buffers in the 7.6–2.6 pH Range at 50°; $\mu = 0.5$

pH	Buffer			k_a[a], hr^{-1}	k_b[a], hr^{-1}	A[a]	B[a]
	$[NaH_2PO_4]$	$[Na_2HPO_4]$	[NaCl]				
7.59 ± 0.08	0.015	0.15	0.035	15.0	0.74	0.17	0.83
	0.010	0.10	0.19	13.2	0.62	0.17	0.83
	0.005	0.05	0.345	11.2	0.50	0.20	0.80
	0.003	0.03	0.407	10.6	0.44	0.18	0.82
		Unbuffered		9.42[b]	0.37[b]	0.18[c]	0.82[c]
6.52 ± 0.04	0.10	0.10	0.10	5.49	0.29	0.19	0.81
	0.08	0.08	0.18	5.05	0.24	0.21	0.79
	0.05	0.05	0.30	4.38	0.18	0.20	0.79
	0.02	0.02	0.42	3.34	0.12	0.18	0.82
		Unbuffered		2.91[b]	0.076[b]	0.20[c]	0.80[c]
5.42 ± 0.01	0.30	0.03	0.11	5.51	0.41	0.28	0.72
	0.20	0.02	0.24	4.87	0.29	0.25	0.75
	0.10	0.01	0.37	5.19	0.18	0.21	0.79
	0.06	0.006	0.42	5.03	0.13	0.22	0.78
		Unbuffered		5.15[c]	0.065[b]	0.24[c]	0.76[c]
	$[CH_3COOH]$	$[CH_3COONa]$	[NaCl]				
5.60 ± 0.04	0.040	0.40	0.10	4.39	0.15	0.26	0.74
	0.024	0.24	0.26	4.83	0.12	0.23	0.77
	0.016	0.16	0.34	4.72	0.099	0.24	0.76
	0.008	0.08	0.42	4.38	0.080	0.25	0.75
		Unbuffered		4.58[c]	0.064[b]	0.24[c]	0.76[c]
4.62 ± 0.04	0.400	0.400	0.100	2.15	0.48	0.60	0.40
	0.240	0.240	0.260	3.04	0.41	0.47	0.53
	0.144	0.144	0.356	3.70	0.37	0.32	0.68
	0.080	0.080	0.420	4.11	0.27	0.32	0.68
		Unbuffered		4.57[b]	0.25[b]	0.23[b]	0.77[b]
				k_{obs}			
3.58 ± 0.02	0.40	0.040	0.460	1.82[d]			
	0.24	0.024	0.476	1.79[d]			
	0.16	0.016	0.484	1.80[d]			
	0.08	0.008	0.492	1.80[d]			
		Unbuffered		1.80[c]			
	[HCOOH]	[HCOONa]	[NaCl]				
2.58 ± 0.06	0.40	0.040	0.460	1.57[e]			
	0.24	0.024	0.476	1.58[e]			
	0.16	0.016	0.484	1.60[e]			
	0.08	0.008	0.492	1.69[e]			
		Unbuffered		1.61[c]			

[a] See text for explanation of these constants and the method used to calculate them. Average of results for two or three wavelengths. [b] Obtained from intercept of plot of *k versus* buffer concentration. [c] Average of values in presence of buffer. [d] It was not feasible to separate the two phases for analysis. The rate constants given were obtained from the first 17% of the reaction. See text for further details. [e] Reaction appears first order over at least two half-lives.

rate constants. In phosphate buffers of pH 7.6 and 6.5, spectra of nonacidified samples were also obtained. At wavelengths greater than 240 nm, there was eventual loss of the UV absorption spectrum. Absorbance data in this region were used directly for the calculation of rate constants.

In two additional experiments, samples of 5-azacytidine (10^{-2} *M*) were reacted in buffer for a sufficient time to ensure that the initial rapid phase of the reaction was completed. Both reactions were conducted at 70°, one in 0.33 *M* phosphate buffer at pH 7.59 for 10 min and the other in 0.04 *M* phosphate buffer at pH 6.52 for 1 hr. Aliquots from each reaction were added to 10 times their volume of hydrochloric acid at 70° to yield a final reaction condition of 0.1 or 0.25 *N* HCl (Table IV). These reactions were then followed at 70° using the differential assay for 5-azacytidine described earlier (Eq. 5).

Evidence for 5,6-Addition to 5-Azacytidine—The UV spectra of 5-azacytidine and 5-azacytosine were recorded at each of three conditions: 0.1 *N* HCl, 0.001 *N* HCl, and pH 6.5 phosphate buffer. Each solution was then altered to match one of the others so that the sequence represents all possible combinations of the three conditions. Precautions were taken to eliminate any changes in absorbance due to passage of time or dilution of sample. In this way, UV spectra for solutions of authentic samples were compared to those that had been previously prepared and recorded at one or more of the other conditions as described under *Results*.

Solutions of 5-azacytidine (6×10^{-5} *M*) and 5-azacytosine (1.5×10^{-5} *M*) were prepared in 0.025, 0.005, and 0.001 *M* aqueous bisulfite previously adjusted to pH 3.67 ± 0.06. A fourth solution of 5-azacytidine in 0.001 *M* bisulfite containing 0.024 *M* NaCl was also prepared. Immediately after dissolution of the substrate, UV spectra were recorded as a function of time.

Spectrophotometric Determination of pKa Values for 5-Azacytosine—The pH of a solution containing 10^{-4} *M* 5-azacytosine in buffer (0.08 *M* acetic acid–0.008 *M* sodium acetate–0.492 *M* sodium chloride) was measured, and the UV spectrum of the solution was obtained. The pH was then adjusted using hydrochloric acid or sodium hydroxide to obtain a series of spectra ranging from the cationic form of 5-azacytosine to the anionic form. To check for degradation, a sample at high pH was adjusted to a previously obtained lower pH value and the two spectra were compared to show that no irreversible changes had occurred.

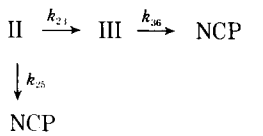
Table IV—Apparent First-Order Rate Constants[a] ($hour^{-1}$) in Hydrochloric Acid at 70° for 5-Azacytidine Compared to Those Obtained when 5-Azacytidine was Previously Reacted in Phosphate Buffer[b]

[HCl], *N*	Unreacted Sample	Phosphate Buffer[b]	
		pH 6.5	pH 7.6
0.10	1.98	1.86	1.95
0.25	1.05	1.07	—

[a] Average of values obtained at two or three wavelengths. [b] See *Experimental* for reaction conditions.

RESULTS

Apparent First-Order Rate Constants in Hydrochloric Acid Solutions—Good first-order plots were obtained when ex-



Scheme I

perimental data were graphed according to:

$$\ln X = \ln X_0 - k_{obs}t \quad \text{(Eq. 6)}$$

where t is time, X is the concentration of unreacted 5-azacytidine or 5-azacytosine, and X_0 is the initial concentration. When appropriate, data were plotted according to:

$$\ln A = \ln A_0 - k_{obs}t \quad \text{(Eq. 7)}$$

where A is the absorbance due to unreacted substrate at 255, 260, or 265 nm for 5-azacytidine or at 230 or 250 nm for 5-azauracil. The apparent first-order rate constants for loss of 5-azacytidine and 5-azacytosine (k_{obs}) and 5-azauracil (k_{36}) are listed in Table I.

TLC of Reaction Solutions of 5-Azacytosine and 5-Azauracil in 0.10 *N* Hydrochloric Acid—The 50 and 100%-reacted samples of 5-azacytosine showed faint spots at the same R_f value as an authentic sample of 5-azauracil. The 100%-reacted sample showed no trace of starting material. The 5-azauracil reaction showed a disappearance of the spot due to the uracil without development of any other compounds absorbing UV light. The R_f values obtained were 0.38 for 5-azacytosine and 0.58 for 5-azauracil.

Kinetics of 5-Azacytosine and 5-Azauracil Hydrolysis in Hydrochloric Acid Solutions—The observed spectral changes during the reactions and the results of the TLC experiments pointed to the applicability of Scheme I to 5-azacytosine hydrolysis. Nonchromophoric products are designated as NCP. A value for k_{36} may be obtained directly from 5-azauracil hydrolysis, while k_{23} and k_{25} were obtained from k_{obs} by:

$$k_{obs} = k_{23} + k_{25} \quad \text{(Eq. 8)}$$

and:

$$k_{23} = f k_{obs} \quad \text{(Eq. 9)}$$

where f is the fraction of 5-azacytosine that deaminates to 5-azauracil. The method used to calculate f is described below. In the case of a sequential reaction with parallel first-order loss, as in Scheme I, the concentration of III as a function of time is given by:

$$[III] = \{k_{23}[II]_0/(k_{36} - k_{23} - k_{25})\}\{e^{-(k_{23}+k_{25})t} - e^{-k_{36}t}\} \quad \text{(Eq. 10)}$$

where $[II]_0$ is the initial concentration of 5-azacytosine. The area under a plot of [III] *versus* time is found by integrating Eq. 10 from zero to infinity:

$$(\text{area III})_1 = f[II]_0/k_{36} \quad \text{(Eq. 11)}$$

where f is the same as in Eq. 9. Similarly, for the simple first-order loss:

$$III \xrightarrow{k_{36}} NCP \quad \text{(Eq. 12)}$$

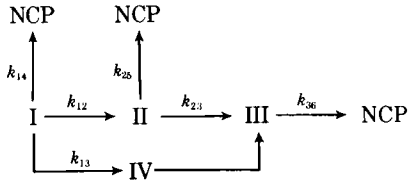
Integrating the equation for III yields:

$$(\text{area III})_2 = [III]_0/k_{36} \quad \text{(Eq. 13)}$$

Application of Eqs. 11 and 13 to experimental data allows the calculation of f. The concentration of 5-azauracil produced from 5-azacytosine was plotted as a function of time. During formation from 5-azacytosine, the area under this curve was estimated by the method of trapezoids. After the 5-azacytosine was exhausted, the remaining area was calculated from:

$$(\text{area})_{t\to\infty} = [III]_t/k_{36} \quad \text{(Eq. 14)}$$

where $[III]_t$ is the concentration of 5-azauracil at time t, and k_{36} is the apparent first-order rate constant for 5-azauracil loss under



Scheme II

identical conditions. The sum of the two areas provides the total area, $(\text{area III})_1$. The area under the concentration *versus* time curve for hydrolysis of an equimolar solution of 5-azauracil, $(\text{area III})_2$, was also calculated from Eq. 14. Since $[II]_0 = [III]_0$, Eqs. 11 and 13 may be combined to yield:

$$(\text{area III})_1/(\text{area III})_2 = f \quad \text{(Eq. 15)}$$

The rate constants obtained are listed in Table I. These were used in an analog computer[2] program to simulate the time course of the various reaction components. A typical plot is shown in Fig. 1, which shows good agreement between observed and computer-generated values.

Kinetics of 5-Azacytidine Hydrolysis in Hydrochloric Acid Solutions—The proposed path for 5-azacytidine hydrolysis in 0.1–0.8 *N* HCl is shown in Scheme II. The apparent first-order rate constants k_{12}, k_{14}, and k_{13} were determined in a manner similar to that used for k_{23} and k_{25}. For example:

$$k_{12} = f(k_{obs}) \quad \text{(Eq. 16)}$$

where f is the fraction of 5-azacytidine that hydrolyzes to 5-azacytosine. This fraction is obtained using Eq. 15, replacing $(\text{area III})_1$ with the area under the concentration curve for 5-azacytosine produced from 5-azacytidine and replacing $(\text{area III})_2$ with the area starting with 5-azacytosine itself.

Since the values for k_{25}, k_{23}, k_{36}, and the time course for II are all known (Scheme II), they can be employed to define the time course for $[III]_{II}$ (the 5-azauracil arising from 5-azacytosine). The difference between the observed values for III as a function of time, [III], and the expected values, $[III]_{II}$, defines the time course for III arising from IV *via* k_{13}:

$$[III]_{IV} = [III] - [III]_{II} \quad \text{(Eq. 17)}$$

The fraction of I converted to III by this route was used to calculate the value for k_{13}; this value, in turn, was employed to calculate k_{14} from:

$$k_{14} = k_{obs} - k_{12} - k_{13} \quad \text{(Eq. 18)}$$

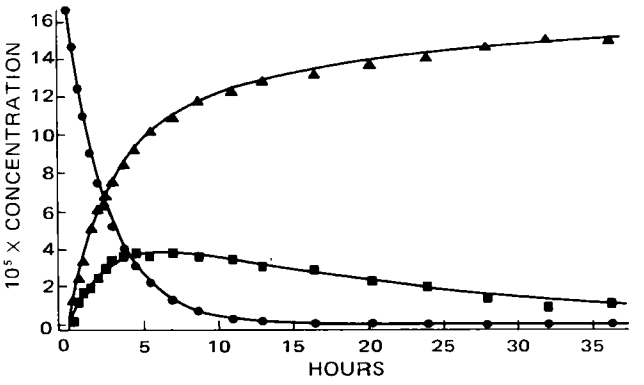


Figure 1—*Time course for 5-azacytosine (●), 5-azauracil (■), and nonchromophoric products (▲) during the hydrolysis of 5-azacytosine in 0.1* N *HCl at 70°. The lines were generated using an analog computer programmed with rate constants listed in Table I.*

[2] EAI, model TR 20.

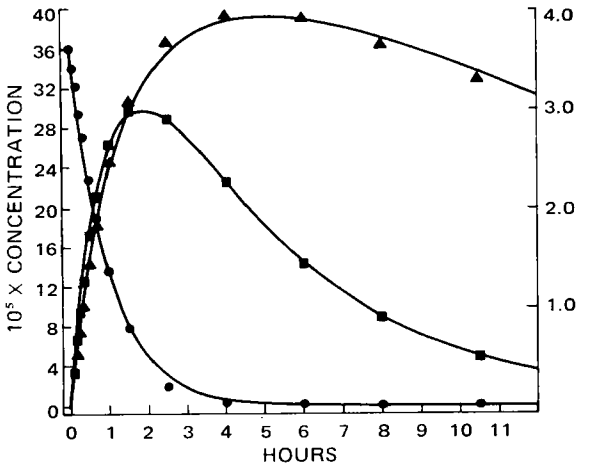


Figure 2—*Time course for 5-azacytidine (●), 5-azacytosine (■), and 5-azauracil (▲) during the hydrolysis of 5-azacytidine in 0.25 N HCl at 70°, showing the agreement between the experimental points and the computer-generated lines. The scale for 5-azacytosine and 5-azauracil is given on the right ordinate.*

These analyses were carried out for the reaction conditions listed in Table I. Typical plots are shown in Fig. 2. The k_{obs} values obtained at 50° are listed in Table II.

Kinetics of 5-Azacytidine Hydrolysis in Buffer Solutions—In buffers at pH 4.6 or higher, the plots of absorbance *versus* time were distinctly biphasic. Acidified samples gave plots similar to Fig. 3, showing a rapid initial decrease in absorbance, followed by a much slower second phase. When the spectra of buffered samples were determined, there was a rapid initial increase in absorbance followed by a slower decrease (Fig. 4).

The biphasic curves were analyzed by feathering the data using commonly employed methods[3]. Treated in this way, each reaction produced two linear first-order plots (Figs. 3 and 4). If the data are normalized relative to the initial absorbance values, a single equation, wherein the algebraic sign for the coefficient A is negative in buffer and positive in acid, may be used to describe the biphasic

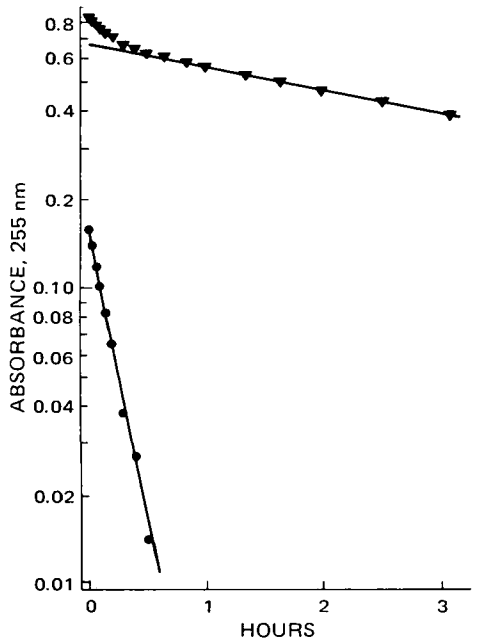


Figure 3—*Absorbance of acidified samples at 255 nm as a function of time (▼) for 5-azacytidine hydrolysis at 50°, pH 6.52, showing lines of slope* k_a *(●) and* k_b *(▼) obtained by feathering.*

[3] For a typical example of this method of data analysis, see Ref. 7.

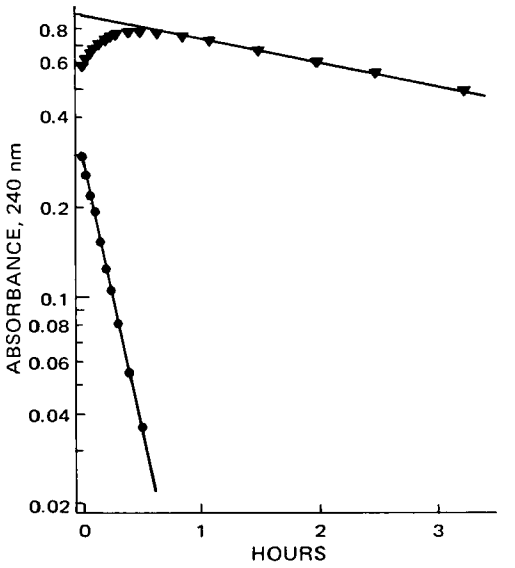


Figure 4—*Absorbance at 240 nm as a function of time (▼) for nonacidified samples of 5-azacytidine at 50°, pH 6.52, showing lines of slope* k_a *(●) and* k_b *(▼) obtained by feathering.*

curves:

$$F(A) = \pm Ae^{-k_a t} + Be^{-k_b t} \qquad \text{(Eq. 19)}$$

where $F(A)$ is the fraction of absorbance remaining; A and $-k_a$ are the intercept and slope of the rapid phase, respectively; and B and $-k_b$ are the intercept and slope of the slow phase, respectively. Rate constants are independent of the pH of analysis. Their values, together with the coefficients in acid, are given in Table III.

Table IV compares first-order rate constants for hydrolysis of 5-azacytidine in hydrochloric acid at 70° to those obtained for 5-azacytidine previously reacted in phosphate buffer. Although the buffer reactions had proceeded into the second phase, the observed rate constants after transfer to hydrochloric acid were the same as those for authentic samples.

At pH 3.58, the plots of ln (A) *versus* time showed definite curvature but were not distinctly biphasic. In fact, the plots were adequately described by a first-order equation over approximately the first half-life. Rate constants, estimated using data representing the first 17% of the reaction, are reported in Table III.

Hydrolysis was apparent first order at pH 2.58, and the rate constants were obtained using Eq. 7 (Table III).

Effect of Buffer on Hydrolysis Rate—Plots of k_a, k_b, or k_{obs}

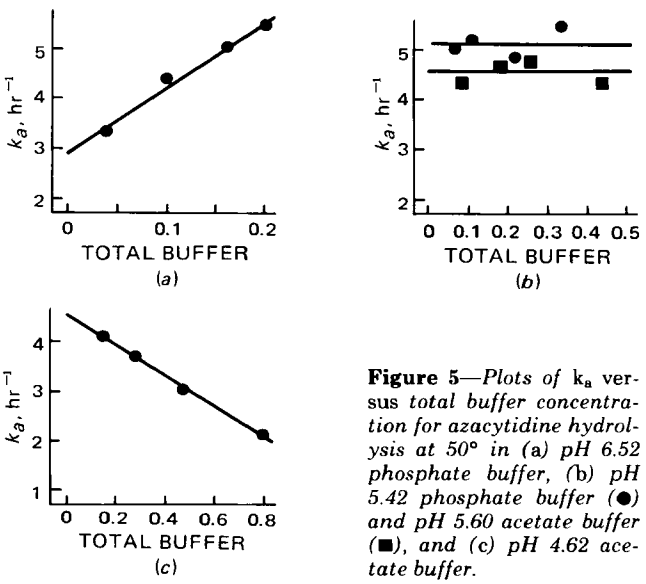


Figure 5—*Plots of* k_a *versus total buffer concentration for azacytidine hydrolysis at 50° in (a) pH 6.52 phosphate buffer, (b) pH 5.42 phosphate buffer (●) and pH 5.60 acetate buffer (■), and (c) pH 4.62 acetate buffer.*

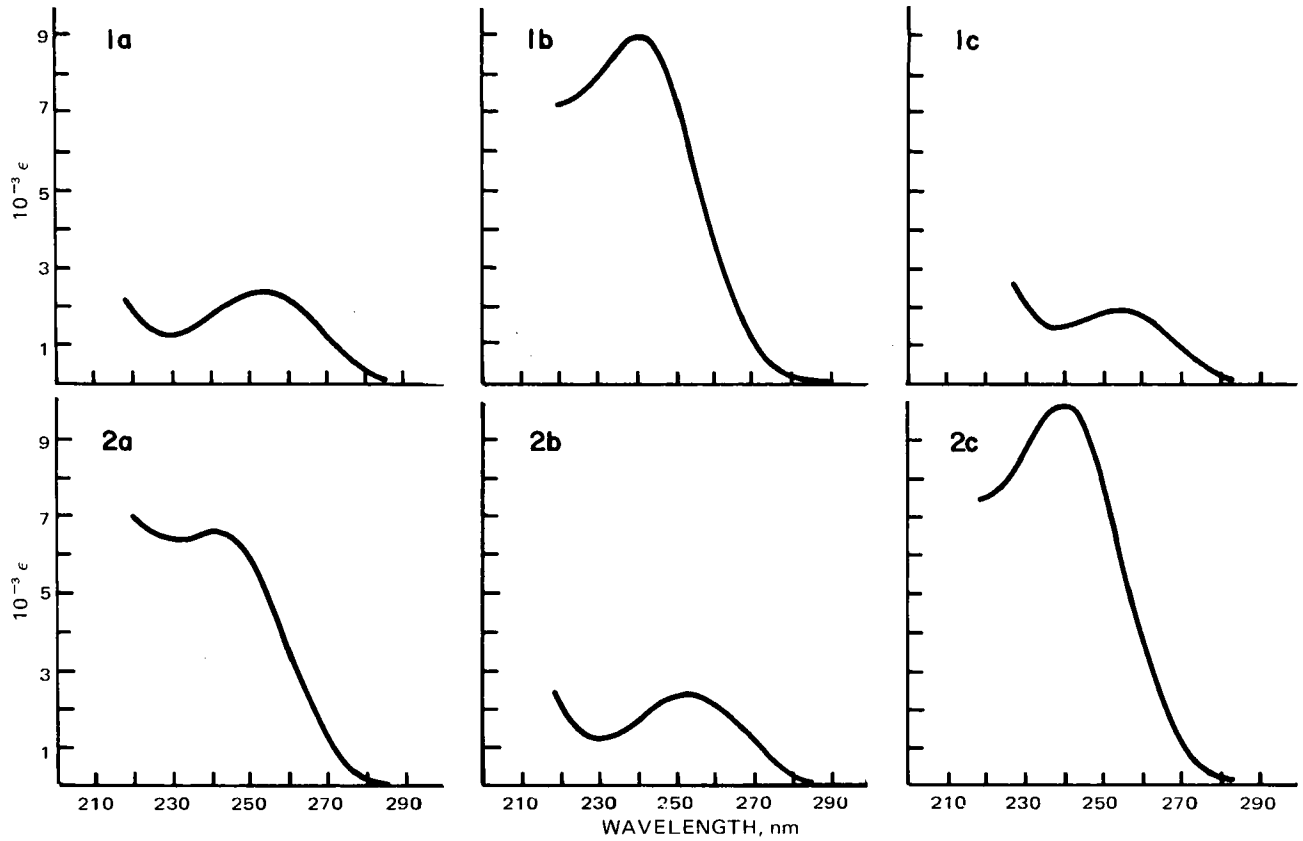


Figure 6—*Plots of apparent molar absorptivity* versus *wavelength for 5-azacytidine in solutions that were originally acid (1a), then adjusted to neutrality (1b), and reacidified (1c), or that were prepared at neutral pH (2a), were acidified (2b), and were reneutralized (2c).*

versus buffer concentration were extrapolated to zero buffer using least-squares linear regression analysis to obtain the values for these constants in the absence of buffer (Table III). In all cases, buffer plots of k_b were linear and showed a positive slope. But plots for k_a had positive slopes at pH 6.52 and 7.59, showed no buffer catalysis at pH 5.60 and 5.42, and indicated inhibition by the buffer at pH 4.62 (Fig. 5). No buffer sensitivity was seen for k_{obs} at pH 3.58 or 2.58.

Evidence for Addition across 5,6-Double Bond of 5-Azacytidine—If the UV spectrum of a 5-azacytidine solution is recorded as a function of pH, that spectrum is dependent on the order of the pH adjustment as well as on the pH itself (Fig. 6). For example, a neutral solution (2*a* in Fig. 6) has a spectrum that is less intense than that of a solution of equal concentration and pH that has been prepared from a previously acidic solution (1*b* in Fig. 6).

Clearly, acid at least partially converts the 5-azacytidine to a form that has a greater absorptivity at neutral pH than the 5-azacytidine itself. However, this rapid conversion does not occur at neutral pH since acidification of an originally neutral sample results in a solution whose spectrum is identical to that of an authentic sample in acid. Furthermore, if the neutral solution that was made acidic is readjusted to neutrality, the spectrum resembles one from an authentic sample in acid adjusted to neutrality (1*b* and 2*c* in Fig. 6).

The degree of difference between a neutralized solution (such as 1*b* in Fig. 6) and a neutral solution (2*a* in Fig. 6) depends upon the acidity of the initial solution. In Fig. 6 the initial acid concentration was 0.1 *N* HCl, and the apparent absorptivity at the absorption maximum for the solution adjusted to pH 6.5 was 9.03×10^3. When the initial acidity was 0.001 *N* HCl, the maximum absorptivity at pH 6.5 was 7.37×10^3. It was consistently observed that solutions made neutral and then reacidified showed decreased maximum absorptivities compared to the original acidic spectra as illustrated for the example in Fig. 6. These changes cannot be attributed to time or dilution factors. This decrease was not observed when solutions were adjusted from 0.1 to 0.001 *N* HCl and back to 0.1 *N*.

When experiments identical to those illustrated in Fig. 6 were carried out using 5-azacytosine, all spectra in 0.1 *N* HCl were identical, as were all spectra at pH 6.5.

Increasing concentrations of bisulfite brought about a progressive loss in intensity of spectra of both 5-azacytidine and 5-azacytosine. When spectra changed with time, initial absorbances were estimated by extrapolating plots of absorbance *versus* time to zero time. When these estimates were used, 5-azacytidine showed apparent molar absorptivities at 240 nm of 5.78×10^3, 3.60×10^3, and 1.38×10^3 in 0.001, 0.005, and 0.025 *M* bisulfite, respectively. Increasing the ionic strength by addition of sodium chloride did not alter the spectrum of the 0.001 *M* solution. In 0.001, 0.005, and 0.025 *M* bisulfite, 5-azacytosine had apparent molar absorptivities of 3.65×10^3, 1.55×10^3, and 0.37×10^3, respectively.

Spectrophotometric Determination of pKa Values for 5-Azacytosine—Absorbances at λ_{max} for the anionic form were plotted *versus* pH. Absorbances were corrected for dilution caused by the pH adjustment using:

$$A_{corr} = A_{exp}(V/V_0) \qquad \text{(Eq. 20)}$$

where V is the volume after addition of acid or base, and V_0 is the original volume. Figure 7 shows the plot obtained. The two pKa values were estimated from the midpoints of the curves and by use of Eqs. 21 and 22 for the pKa's of the conjugate acid and neutral molecule, respectively (8):

$$\text{pKa} = \text{pH} + \log[(A_I - A)/(A - A_N)] \qquad \text{(Eq. 21)}$$

$$\text{pKa} = \text{pH} + \log[(A - A_N)/(A_I - A)] \qquad \text{(Eq. 22)}$$

The absorbance of the solution when all the substrate is ionized is represented by A_I; A_N represents a solution of the neutral molecule; and A is the observed absorbance at the given pH. Two or three pH values within 0.5 pH unit of the pKa's were used. The average pKa values were 2.64 for the protonated molecule and 8.10 for the neutral species.

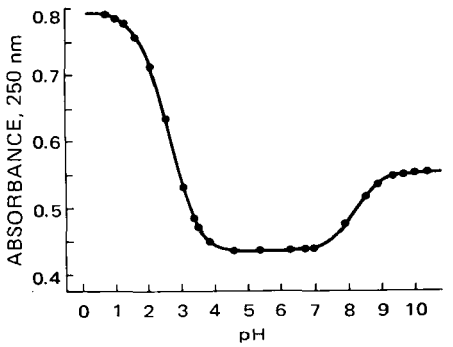


Figure 7—*Absorbance at 250 nm* versus *pH for 1 × 10⁻⁴ M 5-azacytosine.*

DISCUSSION

Kinetics of 5-Azacytidine Hydrolysis in Hydrochloric Acid Solutions—Scheme II outlines the proposed path for 5-azacytidine loss in acid. The formation of 5-azacytosine and 5-azauracil was expected from previously reported results (5) and was confirmed by UV assay. TLC and spectral changes demonstrated that 5-azauracil was produced from the hydrolysis of 5-azacytosine. This was the expected source for the 5-azauracil in the nucleoside hydrolysis.

However, as may be seen in Fig. 2, the data indicate the immediate and rapid production of 5-azauracil. If it were produced solely from 5-azacytosine, a lag phase would be evident. Therefore, it was necessary to postulate the production of 5-azauracil by another, more rapid route. The most logical path involves formation of 5-azauridine as a reactive intermediate. It is proposed to have only a transitory existence and a low concentration and may be said to be in the steady state. This is supported by the absence of a lag time for the formation of 5-azauracil from 5-azacytidine. A lag phase would be expected if 5-azauridine had an appreciable lifetime. Moreover, there was no spectral evidence for the existence of 5-azauridine in the reaction samples.

The steady-state assumption requires that 5-azauridine be very unstable in the reaction system. This assumption is reasonable in view of what is known about the reactivity of the molecule. It is reported to cleave easily in hydrochloric acid at room temperature to form 5-azauracil and ribose. In dilute aqueous ammonia, 1β-D-ribofuranosylbiuret is formed. At pH 5 at room temperature, the UV spectrum of a solution disappeared in less than 1 hr (1).

The absence of 5-azauridine UV absorption was substantiated in the following manner. In neutral solution and as a solid, 5-azauridine actually exists in a form that exhibits no absorbance in the near UV (1). In ~0.1 *N* HCl, solutions have an apparent absorptivity of ~1000 at the λ_{max} of 241 nm (1, 9). Therefore, significant amounts in the reaction would be detected in the UV spectra of the reaction samples. This was tested by using the concentrations calculated for 5-azacytidine, 5-azacytosine, and 5-azauracil at various times during the reaction to generate spectra for the reaction mixtures. These calculated spectra were identical to the actual spectra in the region examined, 235–270 nm. This could not occur in the presence of an appreciable concentration of 5-azauridine unless its spectrum exactly matched that of one of the other components or their absorptivities differed by a constant factor at all wavelengths. Since 5-azacytosine, 5-azauracil, and 5-azacytidine have λ_{max} values of 247, 233, and 255 nm, while 5-azauridine is reported to have its λ_{max} at 241 nm, neither of these conditions can be met.

Since all of the 5-azacytidine loss could not be accounted for by the production of 5-azacytosine and 5-azauracil, it was necessary to include a third path for loss of the substrate to produce nonchromophoric products, probably resulting from fission of the triazine ring (5) (represented by k_{14} in Scheme II). While it is possible that 5-azauridine also contributes to the formation of nonchromophoric products, this pathway would be kinetically equivalent since 5-azauridine is in the steady state.

Data for 5-azacytosine hydrolysis were used to complete Scheme II. Rate constants shown in Scheme II were calculated at 70° (Table I). The observed first-order rate constant for loss of 5-azacytidine decreased at lower pH. This trend was seen also at 50°, as shown in Table II. As the acid concentration was increased, however, a larger fraction of 5-azacytidine formed 5-azacytosine and 5-azauracil (24% in 0.25 *N* HCl as opposed to 11% in 0.1 *N* HCl). 5-Azacytosine showed a similar pH dependency. Only 32% of the 5-azacytosine formed 5-azauracil in 0.1 *N* HCl, and 50% followed this route in 0.25 *N;* the overall rate constant for loss decreased in the more concentrated acid.

Therefore, it appears that increased acid concentration decreases ring opening reactions that produce nonchromophoric products, thereby forcing more substrate to undergo deamination and, in the case of 5-azacytidine, sugar loss. Since these latter two reactions are relatively insensitive to pH changes over the region examined, the overall rate of substrate loss decreased.

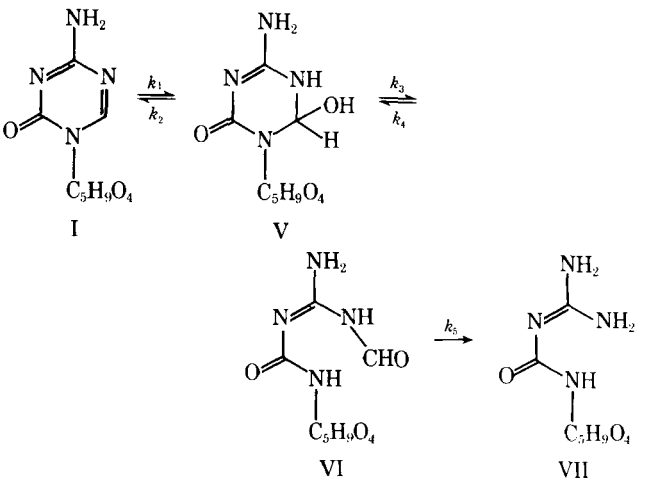
5-Azacytidine Hydrolysis in Presence of Buffers—In formic acid buffer, pH 2.6 and 50°, the loss of absorbance of a 5-azacytidine solution follows first-order kinetics, with the final absorbance being essentially zero. This same behavior is seen in hydrochloric acid solutions more dilute than 0.1 *N*. However, at pH 3.6 the absorbance plots of acidified samples are no longer linear. From pH 4.6 to 7.6, the absorbance plots are biphasic (showing a rapid initial drop in absorbance) and can be separated into two phases by the method of feathering (7) (Fig. 3). Reactions at pH 7.6 and 6.5, followed using nonacidified samples, were also biphasic. In this case the initial change was a rapid increase in absorbance, which was then followed by a slower decrease. An example is shown in Fig. 4. The apparent first-order rate constants obtained by feathering were the same whether acidified or nonacidified samples were used.

Several possible causes of this biphasic behavior were considered: (*a*) the presence of an impurity; (*b*) an A → B → C reaction where the initial phase represents the loss of A to B and the second phase represents the loss of B to C; and (*c*) a reaction with an initial reversible step (or steps) such as A ⇄ B → C (or A ⇄ X ⇄ B → C). The first possibility was eliminated by running a pair of reactions using 5-azacytidine purchased from two suppliers[4]. The absorbance *versus* time plots were identical, making an impurity an unlikely cause for the nonlinear behavior.

Samples taken during the second phase of the reactions, diluted in acid as shown in Table IV, and allowed to react at 70° showed rate constants that agreed with those found for 5-azacytidine. In addition, samples taken throughout the reaction and acidified had spectra that were essentially identical with that of 5-azacytidine at wavelengths longer than 240 nm. Therefore, it appears that 5-azacytidine is present throughout the reaction. This indicates that the formation of B must be reversible, thus eliminating the second reaction scheme.

A pathway with a reversible step is supported by the work of other investigators who reported the reversible formation of the *N*-formyl derivative of ribosylguanylurea (VI in Scheme III) in the decomposition of 5-azacytidine in buffer solutions (5).

By using results of previous work with 5-azacytidine and what is



Scheme III

[4] Schwarz/Mann and Aldrich.

known about the hydrolysis of imines, together with the experimental observations reported here, it is possible to develop a reaction scheme for 5-azacytidine in the presence of buffers as depicted in Scheme III.

Studies of imine hydrolysis (10) indicate that water attack to form a carbinolamine intermediate (V) is fast at low pH since attack proceeds by way of the protonated imine. Loss of free amine (V → VI) becomes the rapid step at higher pH values. This is consistent with the results of pH change experiments, which indicate that formation of an intermediate is rapid in acid but slow at neutral pH, while the reaction of this intermediate to form a third compound is rapid in buffer. This interpretation is also strengthened by the fact that more intermediate appeared to form at pH ~1 (0.1 *N* HCl) than at pH ~3 (0.001 *N* HCl). Based upon what is known about cytosine and its nucleosides (11), the pKa of 5-azacytidine should be little different from the 2.59 found for 5-azacytosine. Therefore, at pH 1, the sample, being nearly all in the protonated form, should contain more hydrate than the pH 3 sample, where more than half of the compound is not protonated.

If water adds across the 5,6-double bond, it seems reasonable that a better nucleophile, such as bisulfite, that is known to attack double bonds of heteroaromatic molecules (12, 13) would also add. Indeed, it was found that with increasing concentrations of bisulfite, the absorbance of 5-azacytidine solutions became progressively less intense, indicating an essentially nonchromophoric product as might be expected from an addition that brings about a loss of conjugation (14).

Although bisulfite adds to 5-azacytosine under the same conditions used for addition to 5-azacytidine, no evidence of hydrate formation was seen when 5-azacytosine was carried through the same pH adjustment experiments as the nucleoside. This finding suggests that the sugar group may be important in the hydration reaction. The 5′-hydroxy could act as a general base, making water a better nucleophile. It is especially well suited to act through a "concerted" mechanism, such as that proposed (15) for the addition of alcohols to phthalimidium cations.

The lack of significant hydrate formation with 5-azacytosine is also demonstrated by the spectral curve for pKa determination (Fig. 7). The protonated form of the molecule has a higher absorptivity than the neutral form. Conversely, 5-azacytidine absorbs less in acid than at neutral pH, presumably due to formation of the nonabsorbing intermediate. This loss of absorbance can be used to estimate the fraction of 5-azacytidine present in the hydrated form in acidic solution. Cytosine nucleosides and bases typically absorb more in the protonated than in the neutral form.

For example, the maximum absorbance of protonated forms of 6-methylcytidine and 6-methylarabinosylcytosine is about 1.7 and 1.6 times greater than that of the neutral molecules at the same wavelength. The protonated form of the base 6-methylcystosine absorbs 1.8 times as strongly as the uncharged form (11). At the wavelength of maximum absorption, protonated 5-azacytosine absorbs 1.6 times more than its neutral form. Without hydration, 5-azacytidine would be expected to exhibit the same pattern. Comparison of the observed absorptivity of 5-azacytidine in acid to that projected from the absorptivity of the unprotonated form (using the typical ratios just described) implies that roughly 70% of the 5-azacytidine exists as the hydrate in 0.1 *N* HCl.

Although the structure for Compound VI in Scheme III was proposed previously, it was neither successfully isolated from the reactions nor synthesized (5). Attempts at synthesizing compounds of this type by formylation of guanylurea led to recovery of either starting material or 5-azacytosine. Formation of 5-azacytosine in this way confirms the reversibility of the ring-opening step. The strong absorbance of analogous compounds such as *N*-formyl-*N*′-cyanoguanide was cited as evidence for formation of a compound like VI, since it could thereby account for the initial increase in absorbance observed in buffered samples (5). The fact that this increase occurs with no apparent lag phase emphasizes that loss of V to VI is rapid compared to formation of V from I.

Pithova *et al.* (5) identified ribosylguanylurea (VII) as a product of the hydrolysis of 5-azacytidine and showed that VII further decomposes to yield guanidine. In the present experiments, a λ_{max} of 221 nm at pH 7.6, observed in spectra for reaction mixtures, was attributed to the formation of VII. First-order plots using absorbance indicated that VII did not show appreciable absorbance at wavelengths longer than 240 nm in acid or buffer.

Table V—Results of Analog Computer Simulation of Scheme III

k_1	k_2	k_3	k_4	k_5	k_a[a]	k_b[a]	k_{obs}[a]
0.9	0.05	5.0	5.0	0.3	0.95	0.14	
					0.83[c]	0.14[c]	
0.7	0.1	7.0	7.0	0.1	0.70	0.044	
					0.72[c]	0.044[c]	
0.7	0.4	7.0	7.0	0.1	0.83	0.037	
					0.84[c]	0.036[c]	
					0.87[b]	0.038[b]	
0.7	0.7	7.0	7.0	0.1	1.01	0.031	
					0.93[c]	0.031[c]	
					1.03[b]	0.031[b]	
7.0	7.0	0.7	0.7	0.1	1.07	0.031	
1.0	7.0	0.7	0.7	0.1	0.86	0.010	
7.0	1.0	0.7	0.7	0.1	1.34	0.044	
7.0	7.0	0.5	0.5	0.5	1.10	0.105	
7.0	7.0	0.2	0.2	0.1	0.42	0.029	
7.0	7.0	0.2	0.2	0.5	0.72	0.066	
7.0	7.0	0.2	0.2	1.0			0.079
7.0	7.0	0.2	0.2	5.0			0.093
7.0	7.0	0.2	0.2	9.0			0.095

[a] All rate constants were obtained from semilog plots of I + V *versus* time unless otherwise noted. Apparent first-order rate constants are expressed as time^{-1}. [b] Obtained from data for I only. [c] Obtained from plots of 0.3 (I + V) + VI.

As stated under *Results*, the values of k_a and k_b obtained experimentally were the same using UV absorption data for the buffered reactions with or without acidification. It was of interest to determine whether Scheme III could provide data that would behave in this manner. Therefore, Scheme III was programmed using an analog computer. The values of the microconstants used are listed in Table V. Various components or combinations of components were studied as a function of time to simulate the spectral changes seen during the reactions.

According to Scheme III, I, V, and VI are present throughout the reaction. Using acidified samples, VI does not absorb at the wavelengths used for kinetic studies. When samples are acidified, I and V equilibrate so that the absorbance obtained actually reflects the sum of their concentrations in the reaction, although V has very little, if any, absorbance. In buffered samples, I and VI are responsible for the largest part of the absorbance, with VI absorbing more strongly than I.

With these considerations in mind, the analog program was adjusted to provide various kinds of data. The time course of I + V *versus* time was used to represent acidified samples. To simulate spectral changes seen with buffered samples, 0.3 (I + V) + VI was generated. The factor 0.3 was arbitrarily chosen to simulate the effect of differences in absorptivity. The time course of I alone was

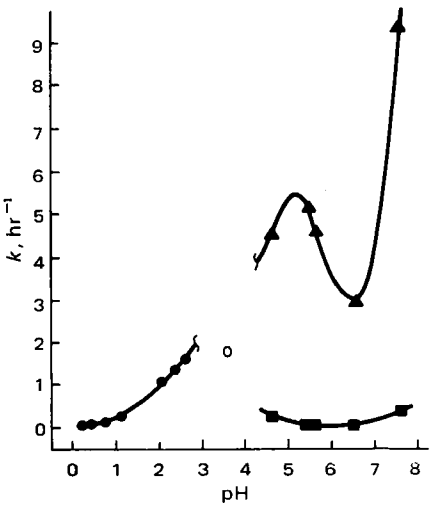
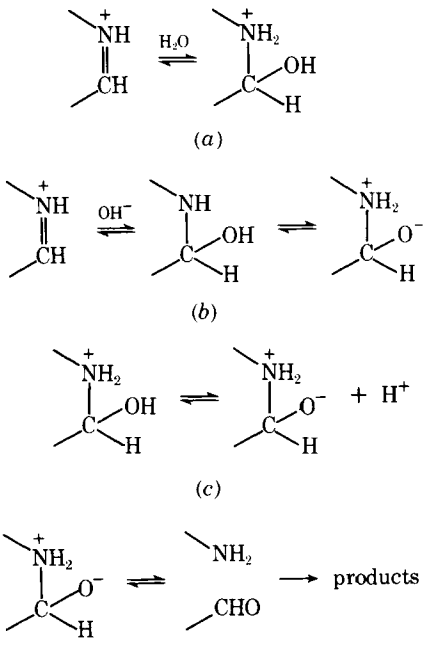


Figure 8—*Apparent first-order rate constants* k_a (▲), k_b (■), *and* k_{obs} (●) *for hydrolysis of 5-azacytidine at 50° as a function of pH. The point at pH 3.58 (○) was obtained as described in the text.*



Scheme IV

also defined. Plotting and feathering the data as described earlier gave estimates for k_a and k_b. These values were the same regardless of which simulation was used (Table V).

Hydrolysis of 5-Azacytidine as a Function of pH—Figure 8 shows a plot of observed first-order rate constants for 5-azacytidine hydrolysis as a function of pH. It is obvious from Scheme III that these are complex kinetic constants composed of several microconstants. This situation precludes an unequivocal interpretation of the pH–rate profile. However, Scheme III can be used in conjunction with present data to suggest mechanisms consistent with observed pH effects.

Both rate constants k_a and k_b are shown for the biphasic region (pH >4) in Fig. 8. Based on Scheme III, k_b may be defined as:

$$k_b = fk_5 \qquad \text{(Eq. 23)}$$

where f is the fraction of (I + V + VI) present as VI. The values for k_5 cannot be calculated from the observed constant k_b, since the value for f is not known. However, it is apparent from Fig. 8 that the product fk_5 is relatively independent of pH. Conversely, k_a is pH dependent. Although it is recognized that k_5 can affect the value of k_a, the observed pH dependency is most likely due to changes in the relative rates of the reversible steps for hydrolysis of I to VI. In the 4.5–6.5 pH region, hydrolysis of the triazine ring may be visualized as a series of steps analogous to those postulated for imine hydrolysis (Scheme IV).

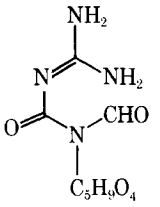
As in imine hydrolysis (10, 16), formation of protonated carbinolamine or neutral carbinolamine (Scheme IV, *a* and *b*) *via* the reactive cationic substrate (IH^+) would be the slowest reversible step at pH 6.5. As the pH decreases, the concentration of IH^+ increases; therefore, the apparent first-order rate constant increases. This increase would be expected to continue until protonation of I was complete, but increasing acidity inhibits formation of the zwitterion (Scheme IV, *c*) so less of it is available to react. Thus, near pH 5 the observed rate constant begins to decrease.

As the pH decreases further, first-order plots become linear instead of biphasic. Although the rate of formation of protonated V (VH^+) from IH^+ becomes faster, the fraction in the reactive form (zwitterion) decreases. The apparent first-order rate constant for loss of IH^+ is now attributed to cleavage of the carbinolamine (Scheme IV, *d*) as described by Scheme V.

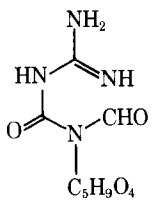
$$IH^+ \underset{\text{fast}}{\rightleftharpoons} VH^+ \underset{\text{slow}}{\rightleftharpoons} VI \underset{\text{fast}}{\longrightarrow} VII$$

Scheme V

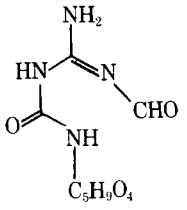
Since the hydration of substrate to form VH^+ is fast, the overall loss appears to be simple first order. The observed constant continues to decrease with increasing acidity due to the decreasing fraction of reactive species in the form of zwitterion. Eventually, loss of IH^+ by this path becomes sufficiently slow that formation of 5-azacytosine and 5-azauracil becomes important at a pH of about 1, but k_{obs} continues to decrease in this region, presumably *via* the reduction of zwitterion.



VIII



IX



X

The observed increase in k_a at pH 7.6 may result from a pathway involving base-catalyzed water attack on the neutral molecule as observed for certain imines (10).

Scheme IV shows the result of 5,6-addition of water. Cleavage of the ring would then result in Compound VIII. Addition across the 1,6-bond would be necessary to obtain Structure VI, which was suggested previously (5). Such addition appears possible starting from the resonance form of IH^+, which has the positive charge at C-6. Addition of water could then occur across the 5,6- or the 1,6-bond. Since a positive identification of the compound was not made (5) and both VI and VIII yield the same product upon hydrolysis of the amide, both routes are possible. Initial protonation could also occur at the 3-nitrogen, leading to Compounds IX and X, which differ from VI and VIII only in the position of a proton.

Implications for Stability Prediction—This report is the first one to suggest the formation of the proposed 5,6-hydrate of 5-azacytidine. While the hydrate was not isolated, considerable indirect evidence has been presented in support of its existence. Regardless of the exact structure for the proposed intermediate, its facile formation in acidic solutions of 5-azacytidine is readily apparent. The undetermined biological significance of this previously unreported intermediate, along with the complexity of the observed first-order rate constants (as illustrated by the analog simulations), indicates the difficulty in predicting stability of 5-azacytidine potency in solutions.

For example, although the apparent rate constants for loss in acidic solutions are less than the k_a values obtained in buffers, a large fraction (about 0.7) of the drug forms the proposed hydrate immediately upon dissolution in acid. The rate constant observed in acid applies only to the loss of drug after the initial equilibration between drug and hydrate has taken place. Therefore, if the hydrate is not itself biologically active or is not bioreversible to drug, most potency is lost long before predictions based on the observed rate constant would have indicated significant hydrolysis.

Equation 19 describes the absorbance changes for 5-azacytidine in buffer solutions. The rate constant values are the same with or without acidification, but the values of the coefficients A and B differ. Provided that Scheme III applies, it is assumed that neither VI nor VII absorbs in acid at the wavelengths used in this study. It is also assumed that the proposed hydrate (V) does not accumulate to a significant extent at neutral pH. Although these assumptions were not proven, they were justified in previous discussion. By using these assumptions, the absorbance of acidified samples of reactions run in the buffer region may be attributed to 5-azacytidine and Eq. 19 may be written:

$$\text{percent remaining} = 100\,(Ae^{-k_a t} + Be^{-k_b t}) \qquad \text{(Eq. 24)}$$

Table VI—Times to Reach 90, 80, and 70% of Original Concentration of 5-Azacytidine in Buffer Solutions at 50° (Predicted using Eq. 24)

pH	Total Buffer[a]	Minutes		
		90%	80%	70%
	Phosphate			
7.59	0.615	2.5	7	14
	0.033	3.5	10	22
6.52	0.20	6	15	32
	0.04	11	32	81
	(0.04	80	225)[b]	
5.42	0.33	3.5	9	17
	0.066	6	18	52
	Acetate			
5.60	0.44	6	15	34
	0.088	6.5	18	56
4.62	0.80	4	10	16
	0.16	5	11	20

[a] Adjusted to μ = 0.5 with sodium chloride (Table III). [b] At 30°.

This equation may be used to calculate the percent drug remaining at any time by employing data for acidified samples, provided the above conditions are met. Failure of the first condition would lead to inapplicability of the biexponential equation. If the second assumption does not hold, the actual percent remaining will be less than that calculated from Eq. 24.

The percent drug remaining can be calculated using the appropriate constants in Eq. 24, keeping in mind the assumptions involved. For example, the values for A, k_a, B, and k_b listed in Table III were used to estimate the time required to lose 10, 20, and 30% in a variety of buffers at 50°. Results are shown in Table VI, where it can be observed that each succeeding 10% loss requires a longer time. This result is due to the transition from the rapid initial phase to the slower terminal phase. It is apparent that 5-azacytidine is decidedly unstable under these conditions. The maximum time required to lose 10% of the drug is 11 min, and 20% is lost in 32 min under these conditions.

To estimate the loss of potency under potential clinical conditions, a reaction was studied at 30° at the pH of maximum stability observed at 50° (0.02 M Na_2HPO_4–0.02 M NaH_2PO_4–0.42 M NaCl, pH 6.5). Within 30 min, 5% of the drug was lost; after 80 min, 10% was lost; and 20% was gone in 225 min. This apparent instability of 5-azacytidine suggests that it would be prudent to prepare solutions shortly before use to avoid significant degradation. However, it requires more than 2 hr for potency to decrease from 90 to 80% and the decrease from 80 to 70% would take even longer. It is likely that solutions used within 6 hr contain at least 70% of the original drug.

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Determination of Dicumarol Metabolites in Bile of Rats

WALTER D. CONWAY ˣ, FRANCIS H. LEE, and LAWRENCE NEUFELD

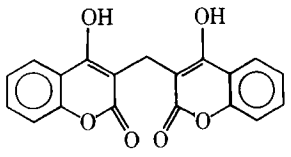
Abstract □ After intravenous administration of dicumarol-^{14}C to rats, the bile excreted over the next 24 hr contained from 32 to 46% of the administered radioactivity. At least three primary metabolites and a small amount of unchanged dicumarol were present in the bile. Over 91% of the primary metabolites was converted to dicumarol and 7-hydroxydicumarol by hydrolysis with β-glucuronidase. Some primary metabolites were hydrolyzed simply by acidification to pH 3 or by treatment under the acidic conditions utilized in the enzymatic hydrolysis. The three primary metabolites contain carboxylic acid groups, as indicated by their electrophoretic mobility–pH profiles, and some are simple glucuronides of dicumarol and 7-hydroxydicumarol. The possibility that others are derivatives of these compounds in which a coumarin lactone ring is opened cannot be ruled out. When the metabolites released by either acidification or enzymatic hydrolysis were chromatographed in *n*-butanol–3 *M* ammonia, artifacts were produced, presumably as a result of decomposition of 7-hydroxydicumarol. The question is raised whether a previously reported metabolite (B055) is an artifact.

Keyphrases □ Dicumarol and metabolites—biliary excretion after intravenous administration of dicumarol-^{14}C, rats □ Biliary excretion—dicumarol and metabolites after intravenous administration of dicumarol-^{14}C, rats

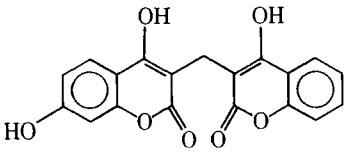
The anticoagulant drug dicumarol (I) was prepared in the early 1940's and is still utilized in therapy (1, 2). It is known to be almost totally metabolized in the body, and several investigators reported separation of from four to seven metabolites in the feces, urine, or bile of rats by paper chromatography (3–9). None of these metabolites has been identified, and it is not known whether any possesses physiological activity (10). While biliary excretion has been recognized as a major pathway for elimination of dicumarol, few studies have been made on bile (8, 9, 11, 12), and in only two of these (8, 9) were the metabolites separated.

The most extensive study (7) of the structure of a metabolite concerned a metabolite isolated from the feces of rats and designated as B055 to signify its mobility of 0.55 in the paper chromatographic system of *n*-butanol–3 *M* ammonia (1:1). This metabolite contained a hydroxy group in position 7 of one coumarin ring; it was neither a glucuronide nor identical with 7-hydroxydicumarol (II).

In the present study, the excretion of radioactivity in the bile, urine, and feces of rats was measured after intravenous administration of dicumarol. Metabolites were separated by high-voltage paper electrophoresis and by paper chromatography. The amounts of I and II were measured by the reverse isotope dilution technique in aliquots of 0–24-hr bile treated alternatively as follows: (*a*) with β-glucuronidase, (*b*) by incubation with buffer in the absence of enzyme, and (*c*) by chloroform extraction of unhydrolyzed bile.



I



II

EXPERIMENTAL

Dicumarol-4,4′-^{14}C (specific activity 0.74 μCi/mg) was synthesized by a modification of the method of Underwood (13) and was administered to one rat. Dicumarol-methylene-^{14}C (specific activity 11.8 μCi/mg) was prepared by the method of Lee *et al.* (14, 15) and administered to two rats. Only one radioactive spot was observed when electrophoresis and paper chromatography were carried out in the systems to be described. 7-Hydroxydicumarol was prepared by the method of Abramovitch and Gear (16) and was identical with an authentic sample[1].

Electrophoresis—Separations by high-voltage paper electrophoresis were run on Whatman 3 MM paper for 30 min at 5000 v at 20° using an enclosed apparatus with two cooling platens[2]. The electrophoregrams were dried and the radioactive zones were detected by scanning in a gas flow counter using methodology previously described (17). Scanner settings were 50 cm/hr, time constant 10 sec, and 5 × 50-mm open window.

Paper Chromatography—Bile samples were examined by applying aliquots of bile as a streak across the entire width of 4 × 40-cm strips of Whatman No. 1 (10-μl aliquot) and No. 3 MM (15-μl aliquot) papers, followed by descending development using *n*-butanol–3 *M* ammonia (1:1, v/v) (3–9). Aliquots (10 μl) of 0.2% solutions of dicumarol and 7-hydroxydicumarol in 0.1 *N* NaOH were applied to the same paper strips as reference compounds. The reference compounds were located by their fluorescence under short wavelength (254 nm) UV light. Radioactive zones were located by scanning in the same manner as for electrophoregrams.

Animal Treatment—Polyethylene 10 cannulas were secured with ligatures in the proximal and distal portions of the bile ducts of male Sprague–Dawley rats (~200 g) under ether anesthesia. The bile duct was then ligated in two places between the cannulas and severed between the ligatures. The cannulas were led through puncture wounds in the backs of the animals, and the exteriorized ends were connected with a 1.5-cm length of 27-gauge needle tubing, thereby permitting normal circulation of bile. The procedure is similar to that recently described by Rietbrock and Abshagen (18). Animals were housed in a restraining cage and given water and food *ad libitum*.

Twenty-four hours after surgery, the cannula was opened to permit collection of bile. Approximately 1 ml of a solution of dicumarol in 0.1 *N* NaOH was administered *via* the femoral vein. Samples of urine and bile were collected at 24-hr intervals. The bile collection vessel was immersed in ice throughout the collection period, and samples were counted and analyzed as soon as possible after collection. In one rat the 0–48-hr feces were collected on a screen.

Enzymatic Hydrolysis—Samples of bile were hydrolyzed with β-glucuronidase using the following microprocedure. A 100-μl aliquot of bile was diluted with water to 1 ml, and 100 mg of monobasic potassium phosphate was added followed by approximately 1 mg of the appropriate nonradioactive carrier (either dicumarol or 7-hydroxydicumarol) in approximately 75 μl of 0.2 *N* NaOH. The

[1] The gift of 7-hydroxydicumarol from Dr. R. A. Abramovitch is gratefully acknowledged.

[2] Shandon model 2550.

Table I—Elimination of Radioactivity in Bile, Urine, and Feces after Intravenous Administration of Dicumarol-^{14}C to Rats

Rat Number	Dose, mg/kg	Hours Postmedication	Percent of Administered ^{14}C Eliminated in			
			Bile	Urine	Feces	Total
7	24[a]	0–24	45.8	21.8	Not measured	67.6
		24–48	6.5	3.1	—	9.6
		0–48	52.3	24.9	—	77.2
9	24[b]	0–24	42.6	20.6	—	63.2
		24–48	7.8	2.7	—	10.5
		0–48	50.4	23.3	3.2	76.9
10	16[b]	0–24	31.8	18.2	Not measured	50.0
		24–48	5.5	8.4	—	13.9
		0–48	37.3	26.6	—	63.9

[a] Specific activity 0.74 μCi/mg. [b] Specific activity 11.8 μCi/mg.

sodium hydroxide was added in 5-μl increments under the control of a microglass electrode to a final pH of 5.4. Then 5 μl of 4 *M* acetate buffer (prepared to yield a solution of pH 5.4 when diluted 25-fold) containing 6.3 mg/ml of bovine serum albumin and 100 μl of a β-glucuronidase preparation[3] were added. The sulfatase contained in the β-glucuronidase preparation is inhibited by the phosphate incorporated into the mixture (19). The sample was incubated at 37° in a water bath for 24 hr. When larger quantities of bile were hydrolyzed, the volumes of reagents were increased proportionately.

Reverse Isotope Dilution—The sample of hydrolyzed bile was extracted with two 20-ml portions of chloroform, and a 100-μl aliquot of each extract was counted to determine the recovery of radioactivity. One hundred milligrams of the appropriate carrier (either dicumarol or 7-hydroxydicumarol) was added to the combined chloroform extracts, and the solution was evaporated to dryness. The residue was recrystallized four to six times from 20 ml of acetic acid. Samples of solid were removed after each recrystallization, dried, and dissolved in 1–2 ml of boiling acetic acid, which, in turn, was dissolved in a dioxane-based scintillation fluid for assay of radioactivity.

Total radioactivity associated with the added carrier was calculated from the mean count rate of samples from the last two recrystallizations after constant specific activity had been reached. Samples of untreated bile or bile incubated without enzyme were similarly treated. In some cases, aliquots of bile or hydrolyzed bile were simply evaporated *in vacuo*, carrier was added, and the residue was recrystallized from acetic acid.

Liquid Scintillation Counting—Total radioactivity in urine, bile, and other solutions was assayed by dissolution of aliquots in a scintillation fluid consisting of dioxane (946 ml), water (50 ml), 2,5-diphenyloxazole (9.5 g), 1,4-bis[2-(5-phenyloxazolyl)]benzene (0.3 g), and naphthalene (142 g) and counting at ambient temperature using a liquid scintillation spectrometer[4].

Feces were homogenized with an approximately equal weight of 2% polysorbate 80[5]; a weighed aliquot was dried and prepared for scintillation counting by a modification of the plastic bag combustion technique (20). Samples of urines and combusted feces were counted for 10 min, providing over 10,000 accumulated counts, and were corrected for efficiency by the internal standard technique using a 109,000-dpm aliquot of toluene-^{14}C counted for 1 min.

RESULTS AND DISCUSSION

Excretion of Radioactivity—In early studies in this laboratory using anesthetized rats, the excretion of radioactivity in bile appeared to be somewhat less than would be expected from the reported excretion in feces of normal, conscious rats (6). Therefore, in subsequent experiments, the drug was administered to conscious animals after recuperation from surgery.

To allow animals sufficient time to recover from surgery and at the same time prevent the depletion of bile salts, which would occur with a simple exteriorized cannula, two cannulas were installed, as described in the *Experimental* section. This system permitted normal circulation of bile during the 24-hr recuperation. The drug was then administered intravenously and bile, urine, and feces were collected from the conscious animals.

Substantial amounts of radioactivity were obtained in the 0–24- and 24–48-hr bile (Table I), although these quantities are still somewhat less than the mean excretion of 57.2 and 10.6% in the 0–24- and 24–48-hr feces, respectively, of three normal rats dosed intravenously by Christensen (6). The excretion observed in urine was quite comparable to his values of 17.7 and 3.0% for 0–24- and 24–48-hr periods, respectively. This finding suggests that biliary excretion slows down as bile constituents are depleted over the long collection period.

Analysis by Electrophoresis—Analysis of 0–24-hr bile samples by direct application to paper, without extraction, and electrophoresis at pH 6.25 (Fig. 1) demonstrates the presence of at least three major components along with minor amounts of 7-hydroxydicumarol and dicumarol. Similar results were obtained by electrophoresis at pH 3.70 and 8.10.

Portions of the pH–mobility profiles of these metabolites in the bile of Rat 9 are presented in Fig. 2. Similar results were obtained for Rats 7 and 10. Lack of mobility below pH 2.7 rules out the possibility of a sulfuric acid ester metabolite (17), which would be ionized at low pH and would migrate substantially. The pKa in the region of 3.2, indicated by the pH–mobility profiles of three metabolites, is characteristic of carboxylic acids. The profile of the slowest moving component, accounting for about 9% of the radioactivity, is consistent with the profile observed for dicumarol.

After hydrolysis of bile (Rat 10) with β-glucuronidase, direct electrophoretic analysis of the hydrolysate (Fig. 3) demonstrates the absence of the metabolites originally present and the appearance of incompletely resolved zones in the vicinity of reference spots of authentic dicumarol and 7-hydroxydicumarol. Lack of exact correspondence of the zones observed by scanning with the positions of the authentic reference compounds should not be regarded as evidence of nonidentity. Under the conditions used for the electrophoresis, in which the authentic reference compounds

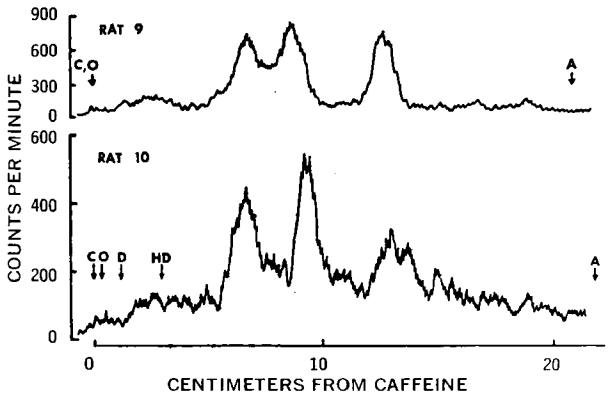


Figure 1—*Electrophoretic separation of metabolites in 10 μl of 0–24-hr biles of Rats 9 and 10, using phosphate buffer, pH 6.25, ionic strength 0.05. Positions of origin (O), caffeine (C), p-nitrobenzenesulfonic acid (A), dicumarol (D), and 7-hydroxydicumarol (HD) reference spots are indicated.*

[3] Glusulase, Endo Products, Inc., Richmond Hill, N.Y.
[4] Packard Tri-Carb model 2202.
[5] Tween 80.

Table II—Composition of 0–24-hr Bile as Measured by Reverse Isotope Dilution Analysis prior to and following Hydrolysis with β-Glucuronidase[a]

Rat Number	Percent of Radioactivity in Bile Present as: Dicumarol	7-Hydroxydicumarol	Total
After Treatment with β-Glucuronidase, 24 hr, 37°, pH 5.4			
7	50.8	40.7	91.5[b]
9	24.8, 23.8	69.7	94.5
10	39.9	55.2	95.1
After Incubation with Buffer Only, 24 hr, 37°, pH 5.4			
9	24.9	23.5	48.4
10	7.7	11.1	18.8
No Incubation with Buffer			
9	1.5	2.3	3.8[c]
After Evaporation of Bile and Recrystallization of Residue and Carrier from Acetic Acid			
7	11.6	21.4	33.0[b]

[a] One hundred microliters of bile was incubated with buffer or buffer plus β-glucuronidase and extracted with two 20-ml portions of chloroform; 100 mg of carrier was added to the chloroform, recovered, and recrystallized from acetic acid. [b] Four milliliters of bile was evaporated *in vacuo*, and 100 mg of carrier was added and recrystallized from acetic acid. [c] Not incubated with buffer but extracted and treated as in Footnote *a*.

were run in a channel adjacent to the bile on the electrophoregram, a discrepancy in relative mobility of the magnitude observed in Fig. 3 is commonly observed. The reference compound typically runs slightly faster than the corresponding compound in the biological fluid.

Analysis by Reverse Isotope Dilution—The composition of 0–24-hr bile samples, as determined by reverse isotope dilution analysis, is summarized in Table II. After hydrolysis with β-glucuronidase, 91.5–95.1% of the radioactivity in the 0–24-hr bile was accounted for as dicumarol and its 7-hydroxy derivative. Very little dicumarol or its 7-hydroxy derivative was present in the bile prior to subjecting it to the conditions for enzymatic hydrolysis. This finding is particularly evident from the results on the bile from Rat 9 where the bile was simply extracted with chloroform; 20% of the radioactivity in the bile was extractable into chloroform, but only a small portion of this amount, corresponding to 1.5 and 2.3% of the bile radioactivity, was characterized as dicumarol and 7-hydroxydicumarol, respectively, by reverse isotope dilution.

Either incubation with buffer alone or direct recrystallization of bile and carrier from acetic acid converts a significant portion of the nonhydroxylated metabolites to dicumarol, apparently all of them in the case of Rat 9. From 20 to 52% of the 7-hydroxylated metabolites are converted to 7-hydroxydicumarol by similar treatment.

If it is assumed that some conjugates are labile under acidic conditions, the data are consistent with the conclusion that the metabolites excreted in bile are predominantly simple glucuronides of

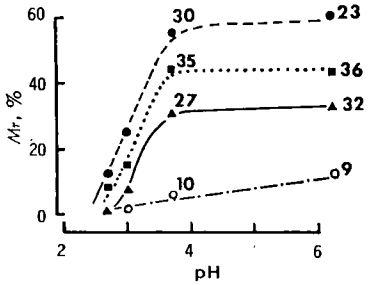


Figure 2—*Mobility* (M_r) *of metabolites in 0–24-hr bile of Rat 9 in the pH 2–6 region expressed as percent of mobility of* p-*nitrobenzenesulfonic acid. Numerals indicate percentage of bile radioactivity associated with the zone. Symbols* (○, ▲, ■, *and* ●) *represent the four zones observed.*

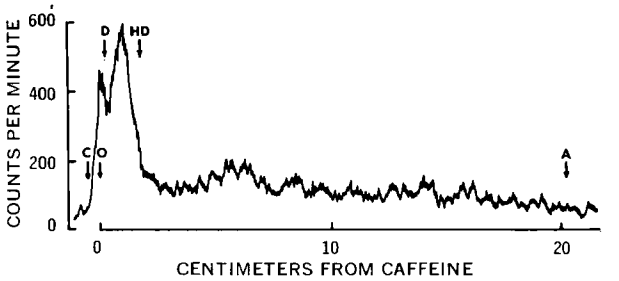


Figure 3—*Electrophoretic separation of components in the 0–24-hr bile of Rat 10 after hydrolysis with β-glucuronidase, using phosphate buffer, pH 6.25, ionic strength 0.05. Positions of origin (O), caffeine (C),* p-*nitrobenzenesulfonic acid (A), dicumarol (D), and 7-hydroxydicumarol (HD) reference spots are indicated.*

dicumarol and 7-hydroxydicumarol. At least 50% of the 7-hydroxylated metabolites, equivalent to at least 20% of the radioactivity in bile, appear to be simple glucuronides of 7-hydroxydicumarol. The nonhydroxylated metabolites may include a fairly labile glucuronide as well as other substances that can be converted to dicumarol under acidic conditions. Compounds similar to III, a metabolite of ethyl biscoumacetate, reported by Pulver and Kaulla (21) would behave in this manner.

Analysis by Paper Chromatography—Paper chromatography in *n*-butanol–3 *M* ammonia (1:1) has been utilized extensively in studies of the metabolic fate of dicumarol (3–9). The evidence presented here indicates that several artifacts are generated by chromatography of dicumarol metabolites in this system. The resulting confusion is no doubt responsible for the protracted period over which the disposition of dicumarol has been studied.

When bile is applied directly to paper and chromatographed in *n*-butanol–3 *M* ammonia (1:1), substantially all radioactivity remains near the origin (upper curve, Fig. 4). Little or no radioactivity is seen in the regions of control spots of dicumarol or 7-hydroxydicumarol or in the region expected for the reported metabolite B055. Acidification to pH 3 and extraction with ethyl acetate, as described by Buttar *et al.* (9), removed more than 95% of the radioactivity from bile. Chromatography of the ethyl acetate extract in the butanol–ammonia system revealed at least five components (lower curve, Fig. 4).

Similar chromatograms were obtained from the β-glucuronidase hydrolysate of 0–24-hr bile from Rat 10 by direct application of the hydrolysate to the paper (upper curve, Fig. 5) and from an extract (lower curve, Fig. 5) prepared by adjustment of the hydro-

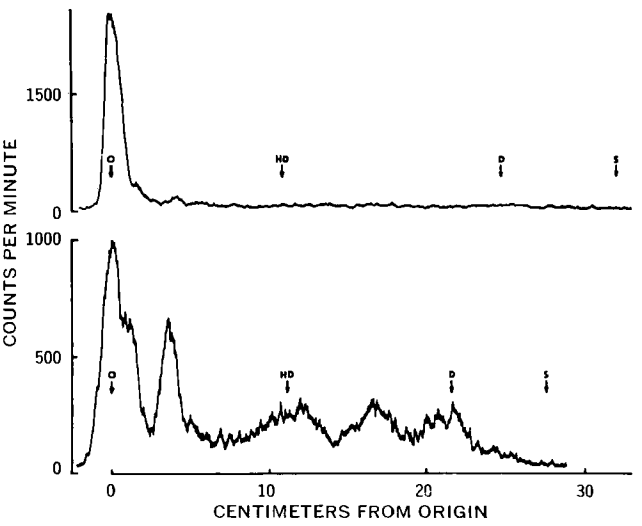
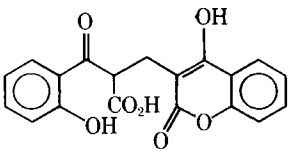


Figure 4—*Paper chromatography of 0–24-hr untreated bile from Rat 10 (upper curve) and ethyl acetate extract (lower curve) obtained from Rat 10 bile adjusted to pH 3 as described by Buttar* et al. *(9). Positions of origin (O), solvent front (S), and reference spots of dicumarol (D) and 7-hydroxydicumarol (HD) are indicated. The system is* n-*butanol–3* M *ammonia (1:1).*



III

lyzed bile to pH 3 and extraction with ethyl acetate (9).

The R_f values for the peaks and the percentages of bile radioactivity represented by the areas designated a, b, c, d, and e in the chromatograms shown in Fig. 5 are compared in Table III with the corresponding values obtained by Buttar *et al.* (9) by chromatography of an ethyl acetate extract of β-glucuronidase-hydrolyzed bile. The present results are in good agreement with those of Buttar *et al.* and confirm their conclusion that component e cochromatographs with dicumarol. The data presented here indicate that component c cochromatographs with 7-hydroxydicumarol.

The suggestion of the presence of at least five metabolites by the chromatographic data is at variance with the conclusion clearly indicated by reverse isotope dilution that virtually all radioactivity in bile is accounted for by dicumarol and its 7-hydroxylated derivative. This inconsistency arises only when the bile is hydrolyzed with enzyme (Fig. 4) or is simply acidified prior to extraction and chromatography (lower curve, Fig. 4) in the alkaline system. The additional components are not generated by chromatography of bile, which is neither acidified nor extracted (upper curve, Fig. 4). The reverse isotope dilution data (Table II) do suggest that some conjugates in bile are labile under acidic conditions.

Chromatography of dicumarol-^{14}C added to bile collected from an unmedicated rat chromatographed as a single zone, which corresponded to a reference spot of dicumarol in the *n*-butanol–3 *M* ammonia system. This finding eliminated the possibility of dicumarol decomposition or binding to biliary components to yield multiple spots.

Chromatography of nonradioactive 7-hydroxydicumarol produced two spots at R_f 0.38 and 0.65. The lower R_f spot was judged to be the unchanged 7-hydroxy compound by the similarity of the color produced on spraying with diazotized *p*-nitroaniline to that of the unchromatographed compound and also by the similarity of the R_f value to the value of 0.33 reported for it in this system by Christensen (7). After 3 days of standing in 3 *M* ammonia prior to chromatography, the spot at R_f 0.65 disappeared and at least five additional spots formed, ranging in R_f from 0.1 to 0.78. In contrast, nonradioactive dicumarol appeared to be stable after 3 days of standing in 3 *M* ammonia. Chromatography in the *n*-butanol–3 *M* ammonia system required at least 6 hours, which provided ample time for hydrolytic, oxidative, and possibly photochemical decomposition to occur.

Therefore, several metabolites observed by previous investigators apparently are artifacts resulting from the decomposition of 7-hydroxydicumarol by the widely used *n*-butanol–3 *M* ammonia solvent system. The 7-hydroxydicumarol can be released from some of its conjugates simply by acidification. The major artifact (Figs. 4 and 5) exhibits an R_f value in the range of the B055 metabolite (7). This raises the question of whether B055 is actually an artifact derived by decomposition of 7-hydroxydicumarol in the *n*-butanol–3 *M* ammonia solvent system in which the biological material was repeatedly chromatographed during the isolation procedure (7).

Table III—R_f Values and Relative Amounts of Radioactivity in Components Separated by Paper Chromatography in *n*-Butanol–3 *M* Ammonia (1:1) from the Enzyme-Hydrolyzed Bile of Rats Given Dicumarol-^{14}C Intravenously

	Upper Curve, Fig. 5[a]		Lower Curve, Fig. 5[b]		Buttar *et al.* (9)[c]	
Component	R_f	%	R_f	%	R_f	%
a	0.03	15.1	0.04	11.3	0.09	11.6
b	0.13	15.0	0.14	10.0	0.22	9.3
c	0.47	26.4	0.46	29.5	0.50	19.3
d	0.66	32.3	0.66	38.1	0.66	36.0
e	0.76	11.5	0.81	11.1	0.80	23.9
7-Hydroxydicumarol[d]	0.47	—	0.44	—	—	—
Dicumarol[d]	0.76	—	0.77	—	0.80	—

[a] β-Glucuronidase-treated 0–24-hr bile applied directly to chromatogram. [b] Ethyl acetate extract of acidified, β-glucuronidase-hydrolyzed 0–24-hr bile. [c] Ethyl acetate extract of acidified, β-glucuronidase-hydrolyzed 0–6-hr bile; mean values from Table 3 of Ref. 9. [d] Reference compounds run in adjacent channels on electrophoregram.

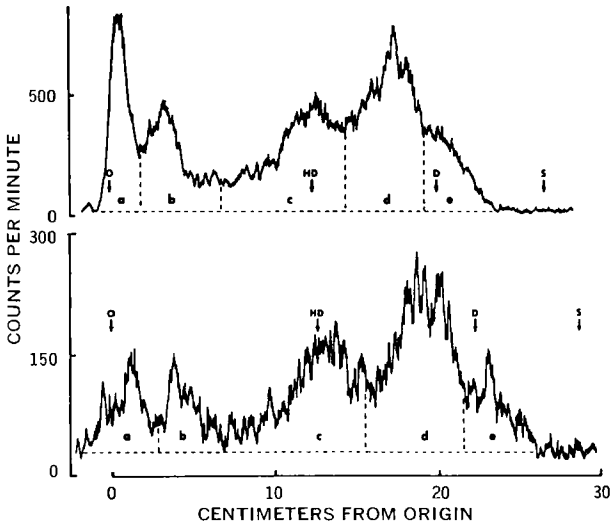


Figure 5—*Paper chromatography of β-glucuronidase hydrolysate (upper curve) of 0–24-hr bile from Rat 10 and paper chromatography of an ethyl acetate extract (lower curve) of the hydrolysate adjusted to pH 3 as described by Buttar* et al. *(9). Positions of origin (O), solvent front (S), and reference spots of dicumarol (D) and 7-hydroxydicumarol (HD) are indicated. The system is n-butanol–3* M *ammonia (1:1).*

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Correlations between Physical and Drug Release Characteristics of Polyethylene Glycol Suppositories

IAN W. KELLAWAY and CHRISTOPHER MARRIOTT [x]

Abstract □ The mechanical strength and elastic moduli of blocks of polyethylene glycol with a range of molecular weights were determined. A rotating-basket dissolution test was used to measure the release characteristics of prednisolone from similar blocks. The effects of blending bases of different molecular weight and of the addition of water also were determined. Linear relationships were found for the mechanical strength, molecular weight, and release rate, but no simple relationship could be observed for the elastic moduli.

Keyphrases □ Polyethylene glycol suppositories—relationships between physical characteristics (mechanical strength, molecular weight, and elastic moduli) and drug (prednisolone) release rates □ Suppositories, polyethylene glycol—relationships between physical characteristics and drug release rates □ Drug release characteristics of polyethylene glycols—relationship to polymer molecular weight, mechanical strength, and elastic moduli

Polyethylene glycols are among the most widely used of the hydrophilic polymer suppository bases. Drug liberation occurs as a result of base dissolution into the aqueous environment of the rectum, differing radically from the classical lipophilic bases which melt at body temperature and act as a lipid reservoir from which drug molecules partition prior to absorption.

Salicylate release from polyethylene glycol bases has been examined by several workers (1–5). High plasma levels of salicylic acid and sodium salicylate have been achieved (1) and the *in vitro* release of two different salicylates did not differ significantly (2). To achieve rapid drug release, polyethylene glycols have been recommended for aspirin formulations whereas cocoa butter (theobroma oil) is the base of choice for both sodium salicylate (4) and choline salicylate (3). The use of polyethylene glycols in some instances has produced similar plasma levels to an equivalent oral dose (4, 5). It has been suggested that salicylate liberation and absorption from polyethylene glycol suppositories may be influenced by salicylate–polyethylene glycol complexation (6–8).

Studies on drugs incorporated into a polyethylene glycol matrix include those on acetaminophen (9), iodoform and 2,4,6-triiodophenol (10), thiazinamium and indomethacin (11), chloramphenicol (12, 13), sulfonamides (14), antipyrine (phenazone) and sodium barbital (15), diphenhydramine and its hydrochloride salt (16), oxytetracycline (17), and other antibiotics (18). When selecting a suppository base, it is generally true that lipophilic drugs are best formulated in hydrophilic bases (19) and water-soluble compounds are best formulated in lipophilic bases (20) for rapid and complete release.

Rheological investigations of suppository bases have been confined to the molten or semisolid state (21–25) and determinations of "hardness" of the solid bases (4, 26–29). Baichwal and Lohit (25) ob-

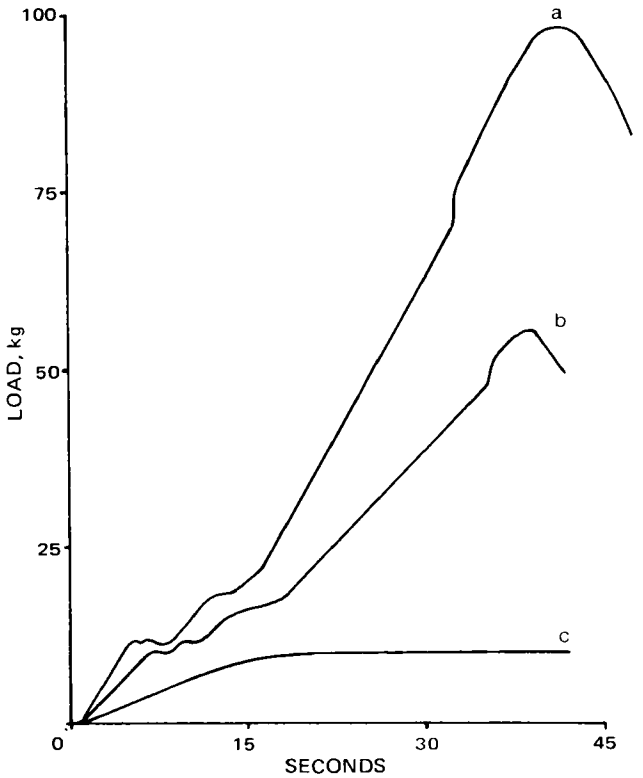

Figure 1—*Transmitted load-time curves for: (a) polyethylene glycol 6000, (b) 60% polyethylene glycol 6000 and 40% polyethylene glycol 1000, and (c) polyethylene glycol 1000. The rate of strain was 60 mm hr*$^{-1}$.

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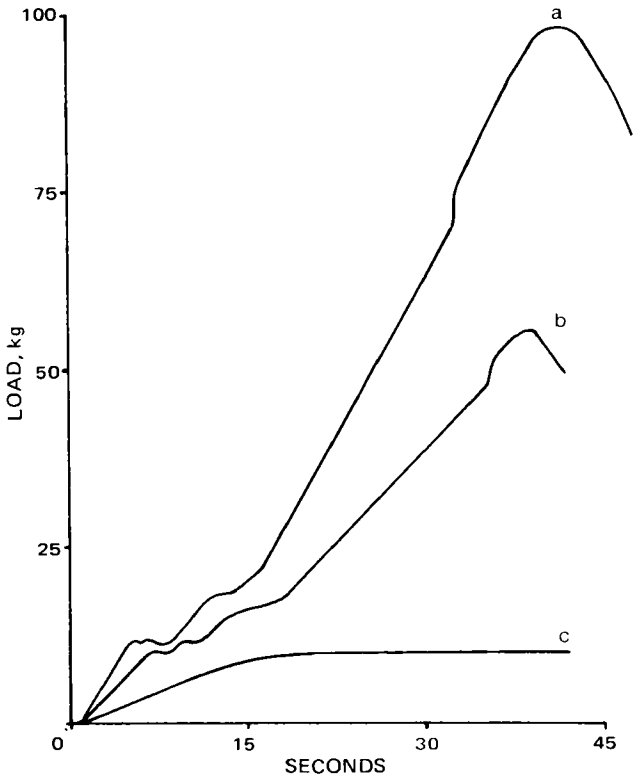

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Table I—Characterization of Polyethylene Glycols $CH_2(OH)(CH_2OCH_2)_mCH_2OH$

Polyethylene Glycol	m	Molecular Weight Range	Mean Molecular Weight	Melting Point
400	8—10	380—420	400	<10°
1000	20—23	950—1050	1000	33.3—33.4°
1540	28—36	1300—1600	1450	43.1—43.3°
4000	69—84	3100—3700	3400	57.4—57.6°
6000	115—160	6000—7500	6750	60.7—61.0°

served that drug release from cocoa butter (theobroma) bases was inversely related to the consistency, expressed as a viscosity index. Sharova *et al.* (29) found that breaking strength increased with molecular weight for polymers of polyethylene oxide.

The purpose of this study was to examine drug release characteristics from polyethylene glycols in relation to polymer molecular weight and as a function of breaking load and elasticity of the bases.

EXPERIMENTAL

Preparation of Suppositories—Polyethylene glycols 400, 1000, 1540, 4000, and 6000 were used[1]. Cylindrical "suppositories," 15 mm in diameter and length and approximately 3 g in weight, were prepared by pouring the molten base into a brass mold mounted on a glass plate. Prednisolone BP, micronized[2] (0.625%, w/w), was dissolved in the molten base to produce blocks for drug release testing; water, if required, was added immediately prior to pouring. All blocks were removed from the molds 1 hr after pouring and stored in well-closed containers at 5°. The blocks were allowed to attain room temperature prior to testing.

Prednisolone Release Determinations—A rotating-basket dissolution apparatus was used and enabled determinations at 37 ± 0.2° to be made simultaneously on four independent blocks. Each block was placed vertically in a basket rotating at 125 rpm. Two liters of distilled water was used as the dissolution medium for each block, and 5-ml samples were withdrawn every 120 sec from a fixed point in the medium. Each sample was analyzed for prednisolone content at 247 nm in a UV spectrophotometer[3].

Breaking Load and Elasticity Determinations—The blocks were placed face on in a compression unit against a 100-kg load cell[4] at 22 ± 0.5°. Compression was carried out at a constant strain rate of 60 mm hr^{-1}. The breaking load was measured as the transmitted force in kilograms at failure, and the elastic modulus was determined from the initial slope of the stress–strain curve (Fig. 1). Division of the load by the area of the block face produces the stress, and the corresponding strain can be derived by dividing time by the recorder speed. The elastic modulus can then be calculated from the ratio of stress to strain:

$$\text{elastic modulus } (Nm^{-2}) = \frac{\text{load (kg)}}{\text{area of block } (m^2)} \times \frac{\text{recorder speed } (m\,sec^{-1})}{\text{seconds}} \quad \text{(Eq. 1)}$$

A recorder[5] speed of 200 mm min^{-1} was used for all compression tests. A minimum of three tests was performed on each formulation.

Table II—Effect of Pouring Temperature on the Breaking Load of Polyethylene Glycol 6000

Pouring Temperature	Breaking Load, kg
60°	93
65°	96
70°	96
75°	96
80°	96
85°	96

RESULTS AND DISCUSSION

The release of prednisolone from polyethylene glycols 1000, 1540, 4000, and 6000 is shown in Fig. 2. Unlike the curves for salicylate release (4), no linear region was observed. Since semilogarithmic plots were also nonlinear, the release of prednisolone from

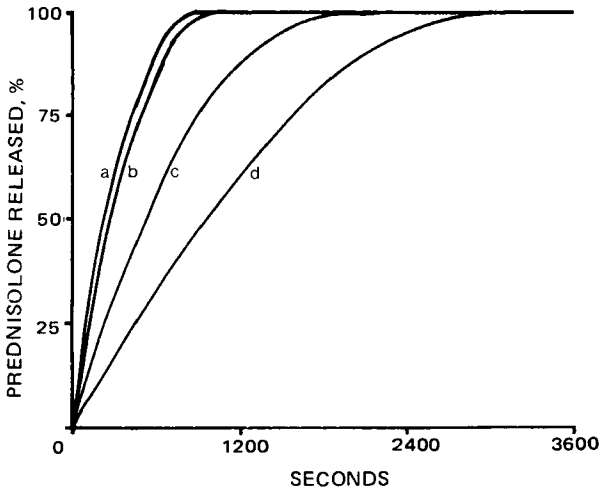

Figure 2—*Release curves of prednisolone from: (a) polyethylene glycol 1000, (b) polyethylene glycol 1540, (c) polyethylene glycol 4000, and (d) polyethylene glycol 6000 at 37°.*

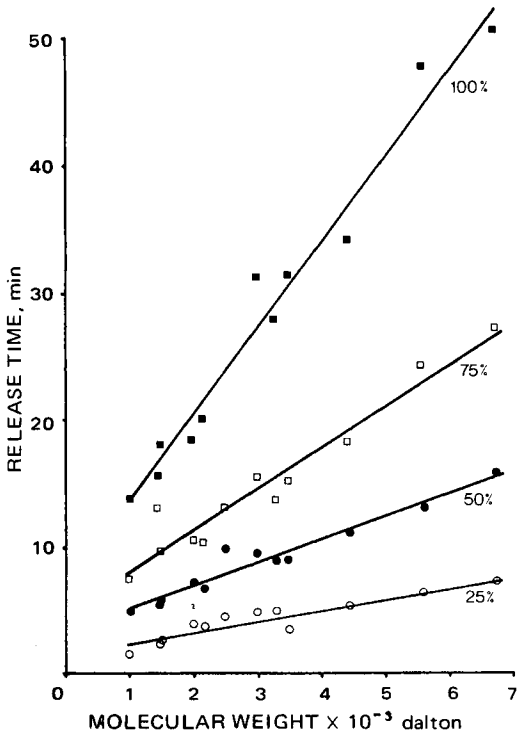

Figure 3—*Release times (t_{25}, t_{50}, t_{75}, and t_{100}) as functions of base molecular weight.*

[1] Koch-Light Laboratories Ltd., Colnbrook, Bucks, United Kingdom.
[2] The Boots Co. Ltd., Nottingham, United Kingdom.
[3] Cecil model CE272, Cecil Instruments Ltd., Cambridge, United Kingdom.
[4] DC 12/C, Transducers (C.E.L.) Ltd., Reading, United Kingdom.
[5] Metrohm Labograph E478, Metrohm AG, Ch-9100 Herisau, Switzerland.

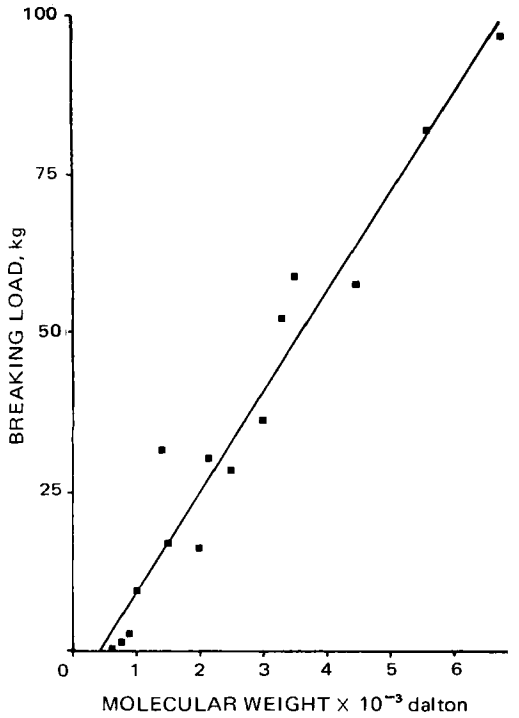

Figure 4—*Breaking load as a function of base molecular weight.*

polyethylene glycol suppositories follows neither zero-order nor first-order kinetics.

The times for release of 25, 50, 75, and 100% (t_{25}, t_{50}, t_{75}, and t_{100}, respectively) of the prednisolone were determined graphically from the dissolution–time plots. When these times were plotted against the "average" molecular weights (Table I), significant linear relationships ($p > 0.999$) were obtained (Fig. 3). Extrapolation to the ordinate was not undertaken, since release times corresponding to liquid polyethylene glycols would have dubious practical significance. Figure 3 includes data for the "pure" compounds and bases formulated by blending polyethylene glycol 1000 with both 4000 and 6000.

The molecular weights of the blends were calculated as weight averages according to:

$$\bar{M}_w(\text{blend}) = \frac{N_1M_1^2 + N_2M_2^2}{N_1M_1 + N_2M_2} \qquad \text{(Eq. 2)}$$

where M_1 and M_2 are the "average" molecular weights of the two polyethylene glycols, and N_1 and N_2 are the numbers of moles of each in the blend. Therefore, it appears feasible that any desired release rate of prednisolone from polyethylene glycol suppositories can be achieved by the selection of a suitable base or blend of bases to produce the appropriate mean molecular weight.

Table III—Effect of Storage Time on the Breaking Load of Polyethylene Glycol 1540

Storage Time, hr	Breaking Load, kg
2	33
5	31
21	32
43.5	32
72	32
120	32

Collins *et al.* (30) suggested that the appearance and mechanical properties of polyethylene glycol suppositories are dependent upon the temperature of pouring and recommended the use of a temperature just above the congealing point to prevent glazing of the surface. Krowczynski (31), however, using 24 different bases, concluded that the preparative technique had no effect on the drug release characteristics. To examine the effect of the preparation procedure on breaking strength, polyethylene glycol 6000 blocks were prepared by pouring the base at different temperatures above the congealing point. The results (Table II) indicate that the breaking strength is independent of pouring temperature, except when this temperature is close to the congealing point. It is possible that crystallization may be occurring prior to pouring, giving rise to a nonhomogeneous polymer matrix within the block interior.

Aging effects were monitored in blocks of polyethylene glycol 1540 stored at 5° in well-closed containers for between 2 and 120 hr. No significant differences could be observed in the breaking loads over this aging period (Table III). All other tests, however, were carried out on blocks that had been aged for between 4 and 24 hr.

Figure 4 illustrates the linear relationship that existed between the breaking load and mean molecular weight of the base. Bases with molecular weights less than 1000 were produced by blending polyethylene glycol 1000 with polyethylene glycol 400. The computed line intersects the abscissa at a molecular weight corresponding closely to the transition between solid and liquid polyethylene glycols at 22°. Therefore, knowledge of the approximate molecular weight serves as a useful guide to base hardness.

When the elastic moduli of the bases were calculated from the compression tests, no simple linear correlation with molecular weight was evident. This finding may be due to the relatively in-

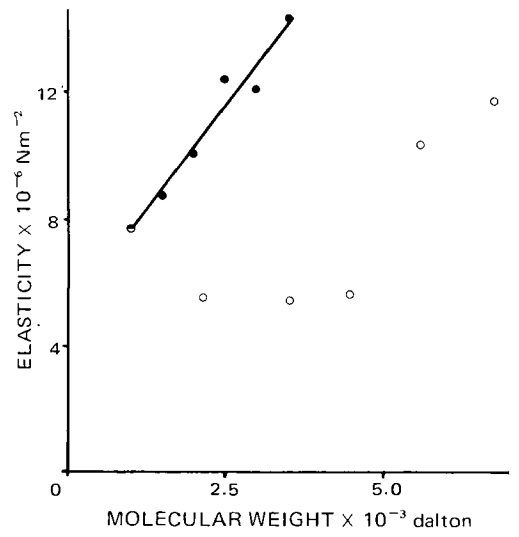

Figure 5—*Elasticity-molecular weight relationships for 4000–1000 blends (●) and 6000–1000 blends (○).*

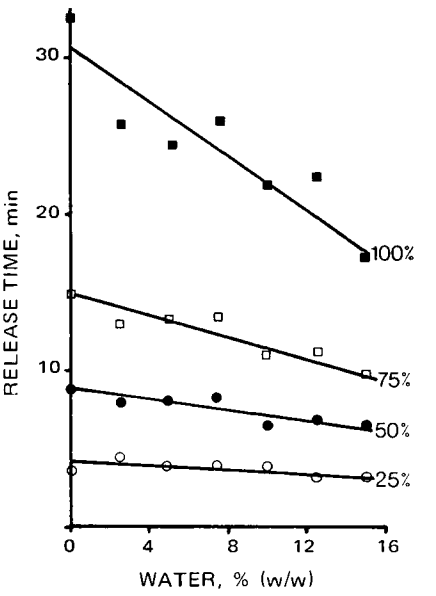

Figure 6—*Effect of water addition to polyethylene glycol 4000 on* t_{25}, t_{50}, t_{75}, *and* t_{100}.

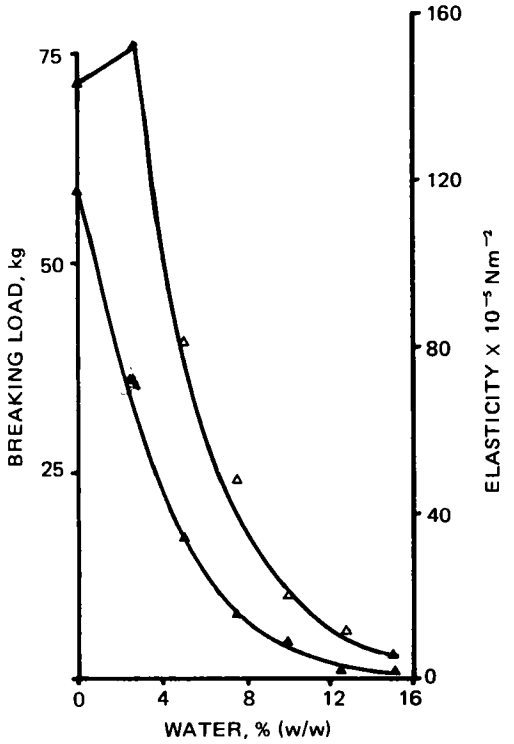

Figure 7—*Effect of water addition to polyethylene glycol 4000 on breaking load (▲) and elasticity (△).*

sensitive technique used. Figure 5 illustrates the effect of blending polyethylene glycol 1000 with polyethylene glycols 4000 and 6000; the former resulted in a linear plot, the latter in a curvilinear one. An explanation of the different behavior exhibited by 6000–1000 blends could involve differences in the matrix structure of polyethylene glycol 6000 when compared with those of the 4000 and 1000 bases. The observation that, with the nonblended bases, polyethylene glycol 6000 was the only compound to produce a clean fracture when compressed to failure may support this suggestion.

It is common pharmaceutical practice to add water to polyethylene glycols when they are used as suppository bases in an attempt to counteract the irritant action on the rectal mucosa occasionally encountered due to water removal by osmosis. The effect of the addition of water to polyethylene glycol 4000 on prednisolone release (Fig. 6), breaking load, and elastic modulus (Fig. 7) were examined. A linear decrease in release times with increasing water content was observed. Breaking load and elastic modulus were also found to decrease but in a nonlinear manner to the maximum water content of 15% (w/w). Concentrations exceeding this figure produced blocks that could not be ejected whole from the molds.

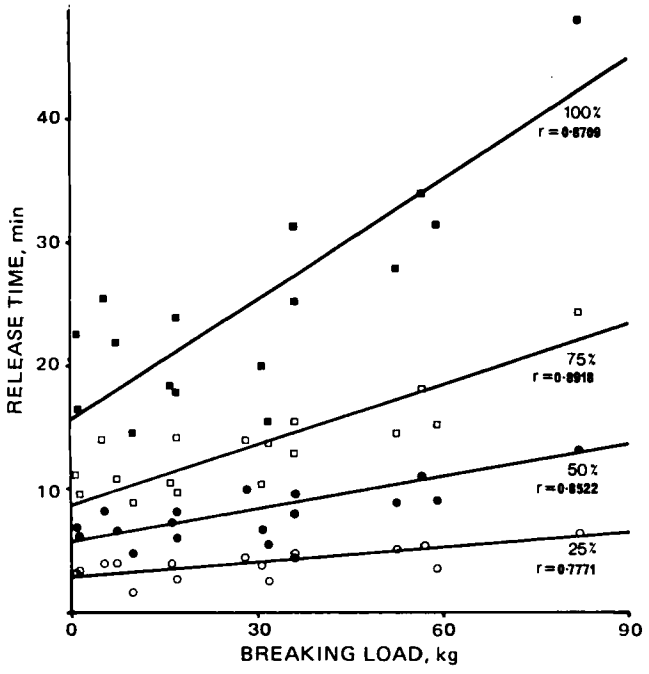

Figure 8—*Significant (>0.999) linear relationships existing for* t_{25}, t_{50}, t_{75}, *and* t_{100} *as a function of breaking load.*

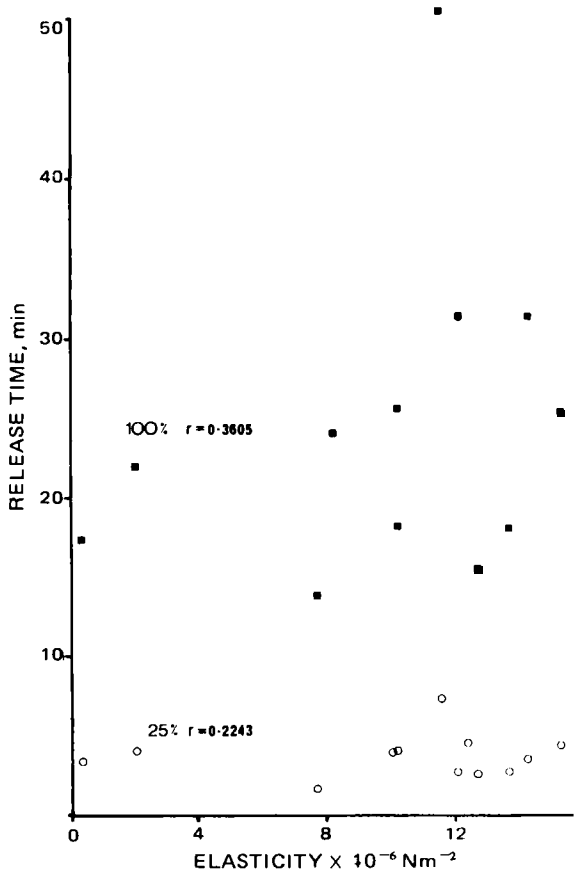

Figure 9—*Nonsignificant (<0.90) correlation between* t_{100} *and* t_{25} *and elasticity.*

It was also possible to relate prednisolone release times to the breaking load of the base when linear relationships were observed (Fig. 8). The significance of the correlation coefficient was >0.999 but was reduced to <0.90 when the elastic modulus was substituted for the breaking load (Fig. 9). Only 25 and 100% release data are included in this latter figure for the sake of clarity. It is considered that the relationship demonstrated between breaking load and release times may prove useful as a formulation guide for lipophilic drugs in polyethylene glycol suppository bases.

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STEVEN L. NAIL [*], JOE L. WHITE [‡], and STANLEY L. HEM [*x]

Abstract □ Data are presented which demonstrate that IR spectrophotometry is more sensitive than X-ray diffraction to structural changes occurring in aluminum hydroxide gel during aging. By examining changes in peak shape and position in the IR spectrum, evidence is seen for an increasing degree of order as the gel ages. The increased order may be responsible for the loss of acid reactivity observed during aging. IR analysis is also recommended for routine monitoring of aluminum hydroxide gels.

Keyphrases □ Aluminum hydroxide gel—monitoring of structural changes during aging, IR and X-ray diffraction methods compared, relationship to decreased acid reactivity □ IR spectrophotometry—monitoring, structural changes in aluminum hydroxide gel during aging, compared to X-ray diffraction method □ X-ray diffraction—monitoring, structural changes in aluminum hydroxide gel during aging, compared to IR spectrophotometric method

Aluminum hydroxide gel is an effective antacid, although many aspects of its structure and acid reactivity are not fully understood. The initial precipitate resulting from the reaction of a soluble aluminum salt with a base is probably a highly random structure. With aging, structural rearrangement occurs to form a more thermodynamically stable system. For example, a polymerization-like process may produce a highly ordered system resistant to attack by acid (1–4). The rate of this process has been related to many factors including pH of precipitation (5), type and concentration of ions present (6–8), temperature (9), and order of addition of the reactants (10). The end-product of the aging process is usually a crystalline form of aluminum hydroxide (1–4, 6–8, 11, 12). These crystalline structures are resistant to attack even by concentrated acid.

It has been demonstrated that aluminum hydroxide gels, which are essentially nonreactive as measured by the USP acid-consuming capacity test, retain an amorphous X-ray diffraction pattern (5). Since X-ray diffraction appears to be relatively insensitive to the structural changes occurring during aging that result in a diminished acid reactivity, a more sensitive method is needed to study these structural changes. The purposes of this report are to demonstrate the usefulness of IR spectrophotometry

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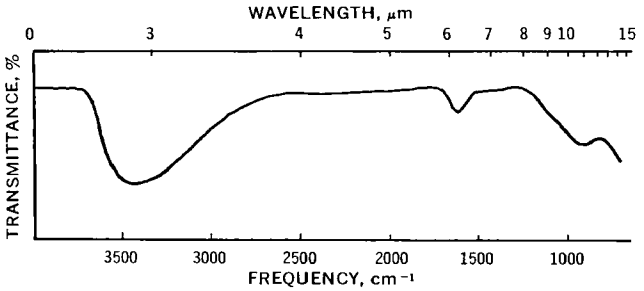

Figure 1—*IR spectrum of freshly prepared aluminum hydroxide gel.*

in studying the structural characteristics of aluminum hydroxide gel and to point out its application in relating structural changes to decreased acid reactivity.

EXPERIMENTAL

Materials—All chemicals used were either official or reagent grade.

Preparation of Aluminum Hydroxide Gel—Aluminum hydroxide gel was prepared by the reaction of aluminum chloride and strong ammonia solution at pH 7.0 (5).

Analytical Procedures—The acid-consuming capacity test (11) was modified by titrating to pH 3.5 rather than using bromophenol blue TS, as given in the USP. Duplicate determinations were made for each sample. The antacid property of the aluminum hydroxide gel is given as a percentage of the theoretical acid-consuming capacity.

For X-ray analysis, a sample of gel was air dried at room temperature and ground to a fine powder with an agate mortar and pestle. Powder mounts were prepared using aluminum McCreery mounts. The diffraction pattern was recorded from 6 to 30° 2θ[1] under the following conditions: CuK_α radiation, 40 kv, 20 mamp, 500 cps full scale, and 2°/min scan speed.

IR spectra[2] of gel samples were obtained by air drying a film of the gel on a zinc sulfide[3] window. Spectra of gibbsite[4], bayerite[4], and boehmite[4] standards were obtained by pressing a disk containing 1 mg of the standard material with 300 mg of potassium bromide.

RESULTS

The IR spectrum shown in Fig. 1 is typical of a freshly prepared aluminum hydroxide gel. The broad diffuse peak centered at about 3400 cm^{-1} corresponds to the O—H stretching frequency of structural hydroxy and adsorbed water. The band at 1640 cm^{-1} is the H—O—H bending vibration of adsorbed water. The broad peak at about 900 cm^{-1} represents an O—H deformation vibration. A peak at 1410 cm^{-1} is sometimes seen and is due to ammonium ion that was not totally removed by washing. Gels containing carbonate have a peak in the 1400-cm^{-1} region as well as a peak at 1550 cm^{-1}.

The O—H stretching and O—H bending frequencies are markedly affected by their chemical environment. The broad absorption band in the 2900–3700-cm^{-1} region of the IR spectrum of the fresh gel (Fig. 1) indicates O—H groups in many environments, thus indicating the highly disordered nature of the fresh gel (13).

The presence of adsorbed water causes some obliteration of structural detail in the O—H stretching region of the spectrum. For this reason, it is best to prepare the sample as an air-dried film on zinc sulfide or silver chloride windows. The use of a potassium bromide disk is not advisable due to the hygroscopic nature of potassium bromide. For the highly crystalline standard materials, the use of the potassium bromide pressed disk technique is satisfactory because the absorption bands are sufficiently well defined that small amounts of adsorbed water do not affect the spectrum. Air-dried films are also suitable for a vacuum heating treatment or deuteration to remove water.

[1] Norelco, Phillips Electronic Instruments, Mount Vernon, N.Y.
[2] Model 421, Perkin-Elmer Corp., Norwalk, Conn.
[3] Irtran 2, Eastman Kodak Co., Rochester, N.Y.
[4] Reynolds Metal Co., Richmond, Va.

Table I—O—H Vibrations in Stretching and Bending Regions for Crystalline Aluminum Hydroxides

Phase	Frequency, cm^{-1} O—H Stretching Region	Frequency, cm^{-1} O—H Bending Region	
Gibbsite	3612 3520 3440 3395	1030 970	
Bayerite	3650 3540 3460	1010 970	doublet
Boehmite	3300 3095	1150 1070	

The hydroxy groups in the aluminum trihydroxides, gibbsite and bayerite, are part of a highly ordered structure and have characteristic absorption bands in both the O—H stretching and O—H deformation regions. These crystalline aluminum hydroxides are easily identified by their IR spectra (Table I). Gibbsite has well-defined bands at 3612, 3520, 3440, and 3395 cm^{-1} in the O—H stretching region. An intense peak occurs at 1030 cm^{-1} with a smaller peak at 970 cm^{-1}, both of which are O—H deformations.

The general features of the bayerite spectrum are similar to gibbsite with peak positions shifted to 3650, 3540, and 3460 cm^{-1}. A doublet at 1010 and 970 cm^{-1} occurs in the O—H deformation region.

Band positions for boehmite, an aluminum oxide hydroxide, are also shown in Table I. The O—H stretching frequency region is characterized by two broad peaks at 3300 and 3095 cm^{-1}. The O—H deformation frequencies are at 1150 and 1070 cm^{-1}.

Figure 2 shows X-ray diffraction patterns for the gel at various

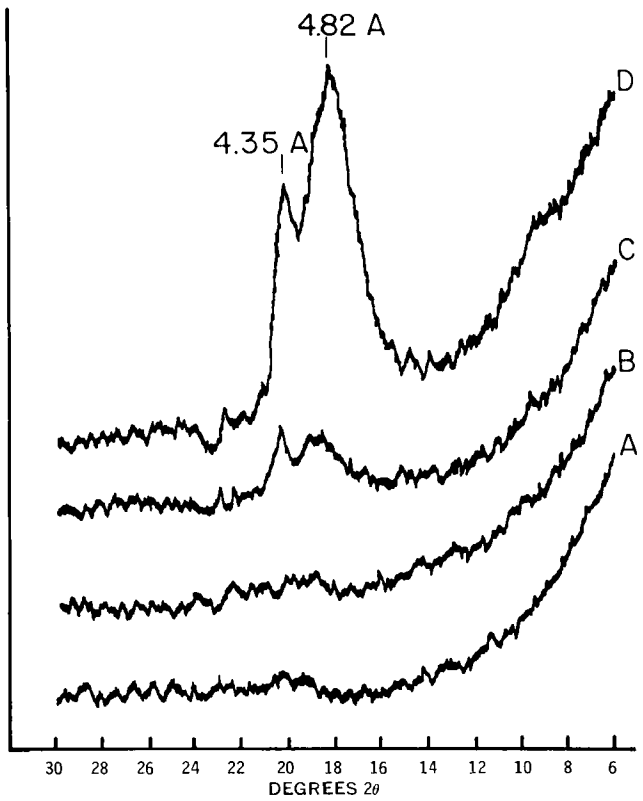

Figure 2—*Change in X-ray diffraction pattern of aluminum hydroxide gel during aging at 25°. Key: A, 57 days; B, 65 days; C, 71 days; and D, 124 days.*

Table II—X-ray Powder Diffraction Patterns of the Five Most Intense Peaks for Crystalline Aluminum Hydroxides[a]

Gibbsite		Bayerite		Boehmite	
Interplanar Spacing, Å	Relative Intensity, %	Interplanar Spacing, Å	Relative Intensity, %	Interplanar Spacing, Å	Relative Intensity, %
4.84	100	4.72	100	6.11	100
4.34	40	4.36	70	3.16	65
4.30	20	3.19	25	2.34	53
2.37	20	2.21	67	1.86	32
2.03	12	1.71	26	1.85	27

[a] Taken from K. Wefers and G. M. Bell, Alcoa Research Laboratories Technical Paper No. 19, 1972, p. 38.

aging times. The gel retains an amorphous pattern until about 70 days after preparation, at which time a rather broad peak is seen from 18.5 to 19.2° 2θ (4.77–4.62 Å) with a sharper peak at 20.4° 2θ (4.35 Å). After 124 days of aging, well-defined peaks are seen at 18.3° 2θ (4.82 Å) and at 20.4° 2θ (4.35 Å).

Table II lists the predominant X-ray diffraction peaks for gibbsite, bayerite, and boehmite. A comparison of the X-ray diffraction pattern of the 70-day sample with the spacings in Table II clearly indicates that a crystalline aluminum hydroxide phase has formed, although the X-ray diffraction pattern is too diffuse to identify the crystalline phase clearly. With further aging, the crystalline phase is clearly identified as gibbsite.

The O—H stretching frequency region of the IR spectrum is shown for various aging times in Fig. 3. The gel aged 5 days has an IR absorbance pattern characteristic of a highly disordered structure. As the gel ages, an inflection point occurs at 3520 cm^{-1}. This shoulder develops into a well-resolved peak. A shoulder is seen developing at 3612 cm^{-1} along with a peak at 3470 cm^{-1}.

The spectral changes in the O—H deformation region are shown in Fig. 4. The fresh gel shows a broad peak centered at 900 cm^{-1}. As the gel ages, a shoulder develops at 1020 cm^{-1} and eventually becomes a well-resolved peak.

A plot of the log of the acid-consuming capacity of the gel *versus* time at room temperature is shown in Fig. 5. The loss in acid reactivity follows apparent first-order kinetics. The slope and y-intercept are -5.93×10^{-3} day^{-1} and 93.5%, respectively, by linear regression analysis. The linear correlation coefficient is −0.99.

DISCUSSION

Based on X-ray diffraction, the first evidence of crystallinity is seen after about 70 days of aging. It is not possible at that time to identify the polymorph present; however, the characteristic X-ray diffraction pattern of gibbsite developed in later samples.

By examining the same gel by IR spectrophotometry, a slight inflection is seen at 3520 cm^{-1} after 42 days and it clearly develops into a gibbsitic peak after less than 70 days.

The O—H deformation region of the spectrum is also sensitive to the degree of order of the gel. The deformation peak undergoes a marked shift to higher frequency, and a sharpening of the band occurs as the gel ages. An absorption pattern develops characteristic of gibbsite.

The IR spectrum indicates that gibbsite or a precursor of gibbsite is the major crystalline phase that is forming as the gel ages. The sharp peak at 3650 cm^{-1} characteristic of bayerite and the doublet at 970 and 1010 cm^{-1} are not seen in the IR spectrum, indicating that the developing order is leading to gibbsitic material.

The value of IR analysis of aluminum hydroxide gel is also seen

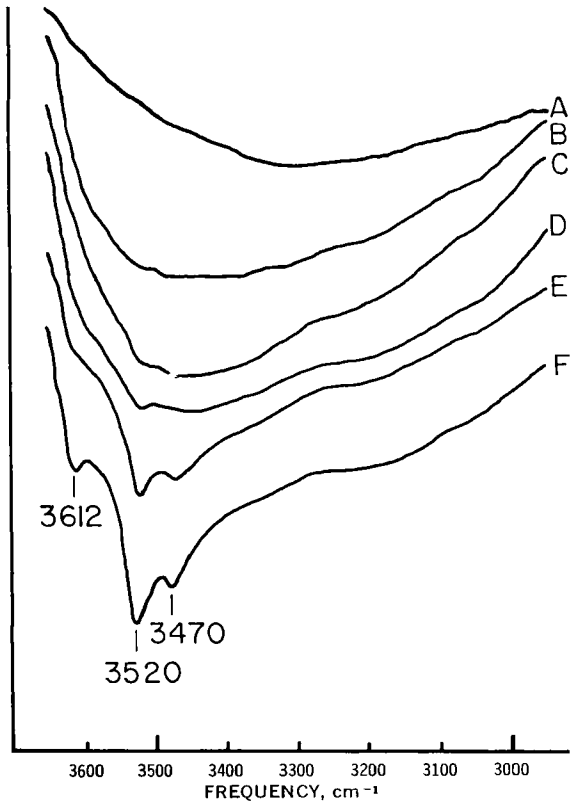

Figure 3—*Change in O—H stretching frequency region of IR spectrum of aluminum hydroxide gel during aging at 25°. Key: A, 5 days; B, 42 days; C, 54 days; D, 61 days; E, 69 days; and F, 124 days.*

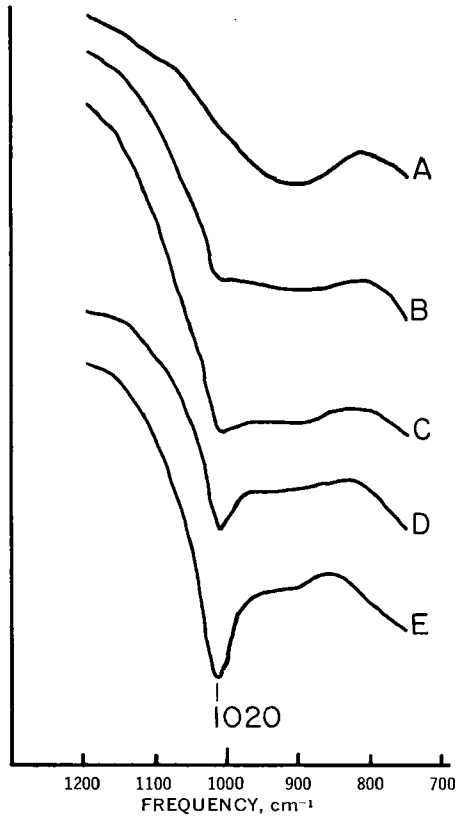

Figure 4—*Change in O—H bending frequency region of IR spectrum of aluminum hydroxide gel during aging at 25°. Key: A, 5 days; B, 54 days; C, 61 days; D, 70 days; and E, 124 days.*

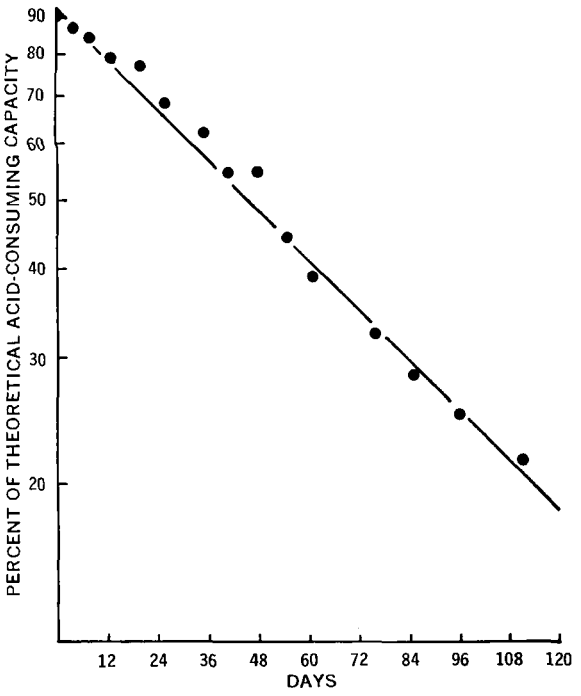

Figure 5—*Change in acid-consuming capacity of aluminum hydroxide gel during aging at 25°.*

when the degree of acid reactivity is compared to IR and X-ray spectral changes. When using IR spectrophotometry, evidence of crystallinity is first seen when the gel is about 50% reactive. X-ray diffraction does not indicate any structural change until the gel is less than 35% reactive. The X-ray diffraction pattern is too diffuse at this point to permit identification of the crystalline phase.

The IR spectra also yield information concerning structural changes occurring during aging. A freshly prepared gel has a diffuse band in both the O—H stretching and deformation regions, indicating a highly unordered structure with many types of environments associated with O—H. As the gel ages, definite absorption bands develop as the hydroxys become part of an ordered structure. Increasing degrees of ordering are apparent from the shape and position of the bands in comparison to the reference IR spectra. This type of analysis yields insight into the mechanism responsible for reactivity losses that occur upon aging.

IR analysis offers additional advantages over X-ray diffraction in terms of lower cost of instrumentation and more universal access to IR spectrophotometers. It is suitable for routine monitoring of aluminum hydroxide gels.

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Potential Fibrinolytic Agents: Versatile Synthetic Route to Novel 4-(Branched alkyl)benzoic Acids *via* β-Monosubstituted or β,β-Disubstituted Styrene-4-carboxylic Acids

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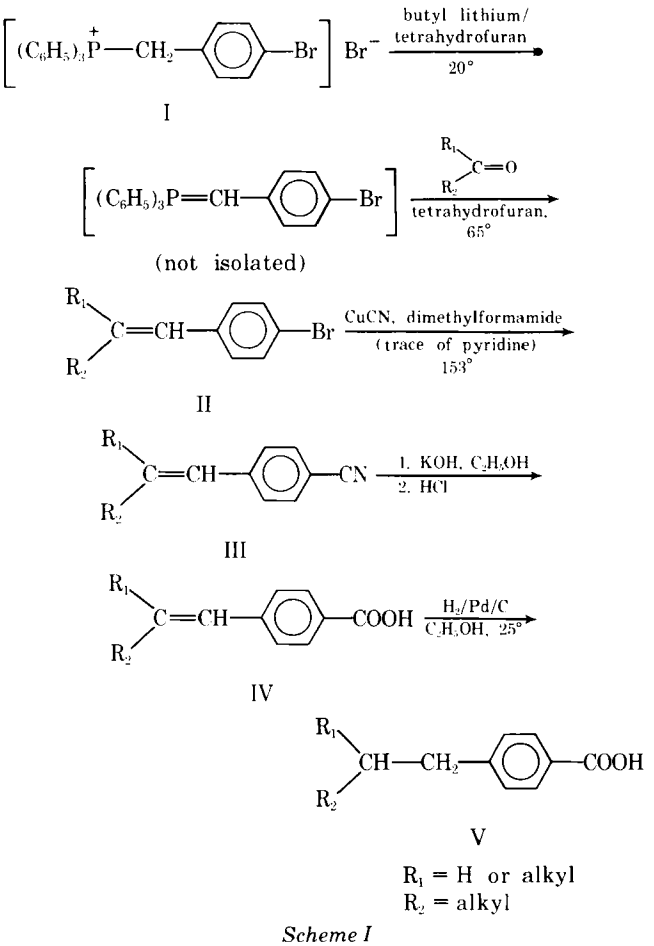
Abstract □ A versatile and unambiguous synthetic route to some novel 4-(branched alkyl)benzoic acids *via* their β-monosubstituted or β,β-disubstituted styrene-4-carboxylic acid precursors is described. The procedure starts with readily available aldehydes or ketones and the common intermediate *p*-bromobenzyltriphenylphosphonium bromide in a Wittig reaction and proceeds in good yield through *p*-bromostyrene and *p*-cyanostyrene intermediates. Several benzoic acid derivatives were of potential biological interest as fibrinolytic agents.

Keyphrases □ 4-Alkylbenzoic acids, branched—synthesis *via* β-mono- or β,β-disubstituted styrene-4-carboxylic acids, potential fibrinolytic agents □ Styrene-4-carboxylic acids, β-mono- and β,β-disubstituted—precursors in synthesis of 4-(branched alkyl)benzoic acids, potential fibrinolytic agents □ Fibrinolytic agents, potential—synthesis of 4-(branched alkyl)benzoic acids *via* β-mono- or β,β-disubstituted styrene-4-carboxylic acids

This report describes an unambiguous synthetic route to some novel 4-(branched alkyl)benzoic acids and their β-monosubstituted or β,β-disubstituted styrene-4-carboxylic acid precursors, starting from readily available symmetrical or unsymmetrical dialkyl ketones or carboxaldehydes and *p*-bromobenzyltriphenylphosphonium bromide in a Wittig reaction (Scheme I). Such compounds were of interest because of anticipated activity in the activation of the fibrinolytic system for the dissolution of fibrin blood clots. This interest was generated by observations that many compounds with similar features (*i.e.*, aromatic acids with bulky substituent groups such as *o*-thymotic acid) exhibited fibrinolytic activity in an *in vitro* test system involving plasminogen activation (1). Test results, however, subsequently indicated activity for derivatives IV and V only at levels judged too high for potential therapeutic utility (see *Experimental*).



Scheme I

DISCUSSION

An interest in benzoic acids substituted in the *para*-position with a branched hydrocarbon moiety quickly led to the finding that very few such compounds had been reported. In fact, nearly all *p*-alkylbenzoic acids previously reported are *n*-alkyl compounds (2–5), simple three- to five-carbon branched alkyl derivatives (3, 6), or, in a few cases, highly symmetrical larger branched chain compounds (such as that with a 2,2,4,4-tetramethylpentyl substituent) (3, 6, 7).

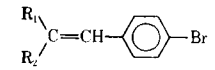
The only generally applicable synthetic procedures for preparing saturated compounds appear to be those involving Friedel–Crafts *para*-acetylation of alkylbenzenes, followed by oxidative degradation of the acetyl group to a carboxyl group or *para*-acylation (or alkylation) of toluene, followed by oxidation of the methyl group to a carboxyl group. As expected, these procedures may be complicated in the Friedel–Crafts step by benzene ring position isomerization, dialkylation or dealkylation, or isomerization in the side chain (8–11). Also less than desirable are low yield oxidation procedures and often high temperature catalytic oxidations (3, 4, 6, 7).

The synthesis of unsaturated styrene carboxylic acids usually involves dehydration of an alcohol, which, with β-substituted compounds, might proceed in more than one manner to give contaminating double bond isomers.

The synthetic route (Scheme I) starts with the phosphonium salt *p*-bromobenzyltriphenylphosphonium bromide (I). Treatment of I in tetrahydrofuran with butyl lithium at 25° affords the orange phosphorane solution to which either a straight chain or branched aldehyde or unhindered straight chain or branched ketone is added, giving the *p*-bromostyrene (II), normally after refluxing several hours in tetrahydrofuran. Compounds and yields prepared by this method are shown in Table I.

Conversion of the bromo compounds (II) to the corresponding nitriles (III) with cuprous cyanide in refluxing dimethylformamide (trace of pyridine) proceeds smoothly, affording products and yields listed in Table II. Hydrolysis of the nitriles (III) to the unsaturated acids (IV) is readily accomplished with potassium hydroxide in refluxing ethanol (Table III). Where the unsaturated acids (IV) are *cis–trans*-isomer mixtures (*i.e.*, $R_1 = R_2$) (not shown

Table I—Properties of β-Substituted 4-Bromostyrenes



Compound	R_1	R_2	Empirical Formula	Boiling Point (mm)	Yield, % from I	Purity by Vapor Phase Chromatography
II*a*	Ethyl	Ethyl	$C_{12}H_{15}Br$	88.5–89° (0.7)	65.3	98
II*b*	*n*-Propyl	*n*-Propyl	$C_{14}H_{19}Br$	101° (0.6)	52.0	97
II*c*	*n*-Butyl	*n*-Butyl	$C_{16}H_{23}Br$	136–139° (1.2)	42.0	99
II*d*	Pentamethylene		$C_{13}H_{15}Br$	103–104° (0.4)	61.5	96
II*e*	*n*-Propyl	*n*-Pentyl	$C_{16}H_{23}Br$	135–136° (1.0)	39.7	99[a]
II*f*	Hydrogen	3-(*n*-Heptyl)	$C_{15}H_{21}Br$	125–128° (1.3)	75	87, 99[a]
II*g*	Hydrogen	3-(*n*-Pentyl)	$C_{13}H_{17}Br$	114–116° (2.5)	60 (estimated accidental loss)	99+[a]
II*h*	Hydrogen	Cyclohexyl	$C_{14}H_{17}Br$	120–145° (1.0)	61.3	92, 100[a]
II*i*	Hydrogen	α-Naphthyl	$C_{18}H_{13}Br$	mp 103–107° ethanol	~20	Homogeneous[a] TLC
II*j*	Hydrogen	β-Naphthyl	$C_{18}H_{13}Br$	mp 70–74° ethanol	~32	Homogeneous[a] TLC

[a] *cis–trans*-Isomer mixtures.

in Table III), these materials are hydrogenated directly to the saturated acids (V) (Table IV), as are the purified unsaturated acids (IV). Nearly quantitative yields are observed in the hydrogenation step.

Several points concerning this synthetic scheme and the compounds prepared thereby deserve special mention. Of primary significance is the fact that the route outlined is simple and completely unambiguous with regard to the position of the double bond in the unsaturated acids produced; moreover, it allows for no isomerization of alkyl groupings. Therefore, it appears to have distinct advantages over routes utilizing Friedel–Crafts reactions or possible ones involving alcohol formation through a Grignard-type reaction, followed by formation of a double bond through elimination of water or a halogen acid. Success of the latter approach seems unlikely. Great difficulty was experienced in preparing Grignard reagents from secondary alkyl halides (particularly with large alkyl groups) to add to *p*-bromobenzaldehyde. Likewise, the successful use of alkyl ketones or aldehydes to add to a 4-substituted benzyl magnesium halide appears doubtful because of a complicating *para*-substituent (*i.e.*, halogen, cyano, or ester).

With bromo intermediates (II), nitriles (III), and unsaturated acids (IV), where $R_1 = R_2$, varying mixtures of *cis–trans*-isomers were observed, and relative proportions could be quantitatively estimated by vapor phase chromatography in the cases of II and III. No attempt was made to separate isomers on a preparative scale; however, monitoring of distillation fractions indicated that this would probably be feasible in most cases.

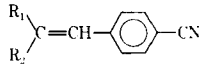
Attempted use of the phosphonium salt 4-cyanobenzyltriphenylphosphonium bromide as the Wittig reactant in place of the 4-bromo compound failed completely, presumably because of the deleterious effect of the electron-withdrawing cyano group on the nucleophilic reactivity of the ylid carbon atom. The use of ketones branched on the α-carbon atoms (*i.e.*, 3,5-dimethyl-4-heptanone or phenyl *n*-heptyl ketone) retarded the reaction under conditions employed (refluxing tetrahydrofuran) to such an extent that synthetic utility was eliminated.

Preparation of the unsaturated acid (IV*a*) by reaction of *p*-bromostyrene (II*a*) with magnesium and subsequent carbonation afforded small amounts of product; however, dissolution of magnesium was very sluggish and this route was abandoned.

Attempted hydrolysis of *p*-cyanostyrenes (III) with hydrochloric acid rather than base led rapidly to extensive decomposition of the starting material and none of the desired products.

It appears from the examples discussed that this synthetic route

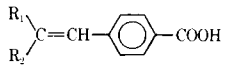
Table II—Properties of β-Substituted *p*-Cyanostyrenes



Compound	R_1	R_2	Boiling Point (mm)	Yield, % from II	Purity by Vapor Phase Chromatography	Empirical Formula	Analysis, %[a] Calc.	Found
III*a*	Ethyl	Ethyl	85–86° (0.1)	84.1	99+	$C_{13}H_{15}N$	C 84.28 H 8.16 N 7.56	84.07 7.93 7.70
III*b*	*n*-Propyl	*n*-Propyl	115–117° (0.3)	70.5 72	100	$C_{15}H_{19}N$	—	—
III*c*	*n*-Butyl	*n*-Butyl	135–136° (0.4)	58.5	96–98	$C_{17}H_{23}N$	—	—
III*d*	Pentamethylene		128–129° (0.8) mp 37–38°	55.9	99+	$C_{14}H_{15}N$	C 85.23 H 7.66 N 7.10	84.97 7.47 7.21
III*e*	*n*-Propyl	*n*-Pentyl	128–130° (0.1)	84.2	—[b]	$C_{17}H_{23}N$	—	—
III*f*	Hydrogen	3-(*n*-Heptyl)	105–112° (0.2)	79	87[b]	$C_{16}H_{21}N$	—	—
III*g*	Hydrogen	3-(*n*-Pentyl)	108–114° (0.7)	70.3	100[b]	$C_{14}H_{17}N$	C 84.37 H 8.60 N 7.03	84.46 8.25 7.18
III*h*	Hydrogen	Cyclohexyl	110–115° (0.1)	51	100[b]	$C_{15}H_{17}N$	C 85.26 H 8.11 N 6.63	85.19 7.92 6.78
III*i*	Hydrogen	α-Naphthyl	Crude solid not purified	80	—[b]	$C_{19}H_{13}N$	—	—
III*j*	Hydrogen	β-Naphthyl	Crude solid not purified	79	—[b]	$C_{19}H_{13}N$	—	—

[a] Satisfactory analytical data (±0.4% for C, H, and N) were reported for III*a*, III*d*, III*g*, and III*h*. Other compounds were converted to the acids (IV). [b] *cis–trans*-Isomer mixtures.

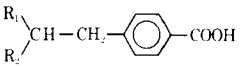
Table III—Styrene-4-carboxylic Acids (IV)[a]



Compound	R_1	R_2	Melting Point	Yield, % from III	Empirical Formula	Analysis, %[b] Calc.	Found
IV*a*	Ethyl	Ethyl	129–133°	73.5	$C_{13}H_{16}O_2$	C 76.44 H 7.90	76.42 7.72
IV*b*	*n*-Propyl	*n*-Propyl	107.5–109°	73.7	$C_{15}H_{20}O_2$	C 77.55 H 8.68	77.47 8.32
IV*c*	*n*-Butyl	*n*-Butyl	97.5–99°	85.7	$C_{17}H_{24}O$	C 78.42 H 9.29	78.16 8.95
IV*d*	Pentamethylene		180–182°	76.5	$C_{14}H_{16}O_2$	C 77.75 H 7.46	77.99 7.40

[a] Contains only purified "symmetrical" acids (*i.e.*, $R_1 = R_2$). [b] Satisfactory analytical data (±0.4% for C and H) were reported for all new compounds in the table.

Table IV—Saturated 4-Alkylbenzoic Acids (V)



Compound	R_1	R_2	Melting Point	Yield, % from III or IV	Empirical Formula	Analysis, %[a] Calc.	Found
V*a*	Ethyl	Ethyl	134.5–136°	91.5[b]	$C_{13}H_{18}O_2$	C 75.69 H 8.79	76.00 8.69
V*b*	*n*-Propyl	*n*-Propyl	142.5–144°	83.5[b]	$C_{15}H_{22}O_2$	C 76.88 H 9.46	77.27 9.09
V*c*	*n*-Butyl	*n*-Butyl	121–122°	88.8[b]	$C_{17}H_{26}O_2$	C 77.81 H 9.99	77.42 9.71
V*d*	Pentamethylene		180.5–182°	90+[b]	$C_{14}H_{18}O_2$	C 77.03 H 8.31	77.54 8.06
V*e*	*n*-Propyl	*n*-Pentyl	106.5–107.5°	75.1[c]	$C_{17}H_{26}O_2$	C 77.81 H 9.99	78.17 9.46 78.25 9.99
V*f*	Hydrogen	3-(*n*-Heptyl)	81–82°	57.7[c]	$C_{16}H_{24}O_2$	C 77.37 H 9.74	77.51 9.48
V*g*	Hydrogen	3-(*n*-Pentyl)	125.5–126.5°	76.3[c]	$C_{14}H_{20}O_2$	C 76.32 H 9.15	76.45 8.93
V*h*	Hydrogen	Cyclohexyl	178.5–180°	71.0[c]	$C_{15}H_{20}O_2$	C 77.55 H 8.68	77.83 8.57
V*i*	Hydrogen	α-Naphthyl	175–176°	50.5[c]	$C_{19}H_{16}O_2$	C 82.58 H 5.84	82.44 5.83
V*j*	Hydrogen	β-Naphthyl	206.5–208°	66.4[c]	$C_{19}H_{16}O_2$	C 82.58 H 5.84	82.51 5.93

[a] See Footnote *b*, Table III. [b] From IV. [c] From III.

will be general for aldehydes, including those branched in the α-position, and for ketones, except those with α-branching, at least under the reaction conditions employed in these experiments. It seems reasonable that *meta*-substituted acids also could be readily produced by this method, starting with *m*-bromotoluene.

It may be possible to employ this method for the synthesis of 4-alkylbenzoic acids or styrene carboxylic acids with branching on the α-position of the *para*-substituent, starting with an α-branched *p*-bromobenzyl bromide. However, α-substituents, particularly if large, may hinder successful completion of the Wittig reaction.

EXPERIMENTAL[1]

Chemical Synthesis—*p*-Bromobenzyl bromide, mp 60–61.5°, was prepared from *p*-bromotoluene[2] according to the method of Weizmann and Patai (12). The reaction of this material with triphenylphosphine[2] in toluene, according to the procedure of Hoffmann (13), gave the phosphonium salt (I), mp 278°, which was dried *in vacuo* and stored over phosphorus pentoxide. All ketones and aldehydes were freshly distilled, their purity was determined by vapor phase chromatography, and they were stored under nitrogen over 4A molecular sieves. The solvents used (tetrahydrofuran and dimethylformamide) were distilled and stored over 4A molecular sieves.

Wittig Reaction: 4-(2-Ethyl-1-buten-1-yl)bromobenzene (IIa)—To a stirred suspension of 32.8 g (64 mmoles) of *p*-bromobenzyltriphenylphosphonium bromide (I) in 80 ml of dry distilled tetrahydrofuran (under nitrogen) was added, over 30 min, 40 ml of 1.60 *N* *n*-butyl lithium in hexane (64 mmoles); the temperature was kept between 20 and 25°. The highly colored (orange-yellow) suspension was stirred for an additional 1 hr, 100 mg of benzoic acid was added, and 6.55 g (76 mmoles, 20% excess) of freshly distilled diethyl ketone was added dropwise over 30 min. Little if any temperature increase was observed.

The mixture was then heated to reflux for 24 hr under nitrogen, during which time the color gradually faded. The mixture was neutralized with a small amount of dilute hydrochloric acid, the solvent was removed *in vacuo*, and the residue was steam distilled until the distillate was clear. The combined distillates were extracted with ether; the extracts were dried over magnesium sulfate, filtered, and evaporated to a yellow oil. Vacuum distillation afforded a small amount of starting diethyl ketone in early fractions and then 10.0 g (65.3%) of a colorless oil, bp 88.5–89° (0.7 mm). The material was estimated to have a purity of 98% by vapor phase chromatography; IR (near): 1630 (weak, double bond) cm^{-1} and no evidence of carbonyl absorption.

[1] All melting points were determined using a Thomas-Hoover Uni-Melt capillary melting-point apparatus and are uncorrected. NMR spectra were obtained using a Varian A60-A NMR spectrometer. Chemical shifts (δ) are reported in parts per million (ppm) downfield from an internal tetramethylsilane standard. Vapor phase chromatography was done on an F&M model 810 instrument, employing a 0.6-cm × 1.8-m (0.25-in. × 6-ft) glass column of 5% QF-1 on acid-washed dimethylchlorosilane on Chromosorb BG (100–120 mesh).

[2] Aldrich Chemical Co.

Although all Wittig reaction mixtures were refluxed for about 24 hr, reactions with aldehydes appeared to proceed quite rapidly, even at room temperature, as judged by the fading of the phosphorane color. With ketones, the addition of a trace of benzoic acid appeared to catalyze the reaction significantly. All *p*-bromostyrenes were separated from the by-product triphenylphosphine oxide by steam distillation except II*i* and II*j*, which were not volatile. In these cases, materials were purified by fractional crystallization from ethanol or chloroform–hexane mixtures. The purity of the *p*-bromostyrenes (II*a*–II*h*) was estimated by vapor phase chromatography. Good resolution of *cis*–*trans*-isomers was achieved with II*e*–II*h*, and relative quantities of each in various distillation fractions could be estimated readily from peak areas.

*Nitrile Preparation: 4-(2-Ethyl-1-buten-1-yl)benzonitrile (III*a*)*—A mixture of 4.78 g (20 mmoles) of II*a* (98% pure by vapor phase chromatography), 2.0 g (22 mmoles) of cupric cyanide, 20 ml of dry dimethylformamide, and 12 drops of pyridine was heated to reflux in an oil bath (180°) for 20 hr under nitrogen. The dark mixture (suspension) was cooled and poured into a mixture of 40 ml of concentrated ammonium hydroxide and 40 g of ice. The mixture was extracted thoroughly with ether, and more cold aqueous ammonium hydroxide was added until the solids were completely dissolved. The combined ether extracts were washed thoroughly with dilute ammonium hydroxide, water, 3 *N* HCl, saturated sodium bicarbonate, and water and then were finally dried over magnesium sulfate.

Evaporation of the filtered ether extracts left a brown liquid, which was distilled *in vacuo*, giving a main fraction of 3.12 g (84.1%) of nearly colorless liquid, bp 102–103° (0.6 mm). Vapor phase chromatography indicated greater than 99% purity. The material was redistilled for analysis, bp 85–86° (0.1 mm); IR (KBr): 1635 (C=C, s) and 2220 (C≡N, vs) cm^{-1}.

Anal.—Calc. for $C_{13}H_{15}N$: C, 84.28; H, 8.16; N, 7.56. Found: C, 84.07; H, 7.93; N, 7.70.

The nitriles prepared were examined for homogeneity by vapor phase chromatography (Table II). In III*f*–III*h*, it was readily possible also to resolve *cis*- and *trans*-isomers and to estimate relative percentages of each in crude materials and various distillation fractions. NMR spectra of nitriles supported fully the assigned structures.

*Nitrile Hydrolysis to Unsaturated Acids: 4-(2-Ethyl-1-buten-1-yl)benzoic Acid (IV*a*)*—To a solution of 3.0 g of potassium hydroxide in 15 ml of ethanol were added 1.50 g (8.1 mmoles) of III*a* and 10 mg of hydroquinone, and the mixture was heated to reflux in a stainless steel flask overnight. After the addition of 20 ml of water, ethanol was removed *in vacuo* and the aqueous solution was chilled and acidified with 6 ml of concentrated hydrochloric acid. The copious white precipitate was extracted into several portions of ether, and the combined extracts were dried over magnesium sulfate.

Filtration and evaporation of the filtrates left 1.57 g of white solid, mp 121–128°. Recrystallization from acetonitrile and finally sublimation at 90–100° (0.1 mm) gave the analytically pure material, mp 129–133° (1.21 g, 73.5%); TLC [fluorescent silica, benzene–dioxane–acetic acid (90:25:4)]: R_f 0.52; NMR (deuterochloroform): δ 0.93 (t, 3H, J = 7 Hz, CH_3), 0.97 (t, 3H, J = 7 Hz, CH_3), 2.09 (q, 2H, J = 7 Hz, CH_2), 2.15 (q, 2H, J = 7 Hz, CH_2)[3], 6.15 (s, 1H, vinyl proton), 7.18 (d, 2H, J = 8 Hz, protons *ortho* to alkyl group), 7.97 (d, 2H, J = 8 Hz, protons *ortho* to carboxyl group), and 12.43 (s, 1H, carboxyl) ppm; IR (KBr): 1670 (COOH) and 1645 (sh) (double bond) cm^{-1}.

Anal.—Calc. for $C_{13}H_{16}O_2$: C, 76.44; H, 7.90. Found: C, 76.42; H, 7.72.

All "symmetrical" unsaturated acids (IV*a*–IV*d*) were highly crystalline and were purified easily by sublimation or recrystallization from acetonitrile or ethanol. Nonsymmetrical unsaturated acids all appeared to be mixtures of *cis*–*trans*-isomers and were noncrystalline or broadly melting solids. Their purity, however, was judged as good by TLC and all were hydrogenated readily to crystalline saturated acids (V*e*–V*j*). Attempted hydrolysis of unsaturated nitriles with strong mineral acid rather than base led to extensive decomposition and was completely unsuccessful.

[3] These two pairs of triplets and quartets overlap each other, being displaced from each other by only about 4–5 Hz. This presumably arises because one ethyl group is *trans* to the phenyl ring while the other is *cis* to it.

*Hydrogenation of Unsaturated to Saturated Acids: 4-(2-Ethyl-1-butyl)benzoic Acid (V*a*)*—To a solution of 760 mg (3.7 mmoles) of IV*a* in 50 ml of 95% ethanol was added 100 mg of 5% Pd/C[4]. The mixture was shaken at room temperature on a Paar apparatus under an atmosphere of hydrogen at about 2.1 kg/cm^2 (30 $lb/in.^2$). After 2 hr, the catalyst was removed by filtration through sintered glass, and the filtrate was evaporated *in vacuo*, leaving a white solid, mp 132.5–135°. Sublimation at 120° (0.5 mm) gave 700 mg (91.5%) of material, which was further purified by recrystallization from acetonitrile and sublimation, mp 134.5–135°; TLC [fluorescent silica, benzene–dioxane–acetic acid (90:25:4)]: R_f 0.62; NMR (deuterochloroform): δ 0.7–1.8 (m, complex, 11H, $CH_3CH_2CHCH_2CH_3$), 2.62 (d, 2H, J = 6 Hz, CH_2), 7.23 (d, 2H, J = 8 Hz, aromatic protons *ortho* to alkyl substituent), 8.03 (d, 2H, J = 8 Hz, aromatic protons *ortho* to carboxyl group), and 12.28 (s, 1H, COOH) ppm; IR (KBr): 1670 (COOH) cm^{-1}.

Anal.—Calc. for $C_{13}H_{18}O_2$: C, 76.69; H, 8.79. Found: C, 76.00, H, 8.69.

All saturated acids reported in Table IV were readily purified by sublimation and/or recrystallization from acetonitrile or ethanol. NMR spectra of all saturated acids supported completely the assigned structures.

Fibrinolytic Testing—Saturated and unsaturated compounds (IV and V) were tested utilizing the hanging clot method described by Baumgarten *et al.* (14). Measurable lytic activity was observed at levels of 1 mg/ml but not at lower levels normally required for activity in *in vivo* systems and for clinical utility (1).

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Technique for Assessment of Local Effects of Substances Found in Bile upon Opening Pressure of Choledochoduodenal Junction

MARTIN F. TANSY ˣ, DAVID L. INNES, JOHN S. MARTIN, and FRANK M. KENDALL

Abstract □ An assessment of the localized influences of various chemicals found in bile was made by applying them to the interior of the canine choledochoduodenal junction. Test agents were isolated by air bubbles and introduced into the terminal lumen of the intramural portion of the common bile duct *via* the pressure measurement catheter; they remained in the duct for approximately 1.5 min. The system was flushed and opening pressures were then measured. Responses were measured in terms of alterations in ductal opening pressures generated by a linear pressure ramp. Histamine, serotonin, and bethanechol markedly increased ductal opening pressures, whereas epinephrine and norepinephrine decreased opening pressures. None of the agents affected either cardiovascular or small intestinal motor activity in the vicinity of the sphincter when administered in this manner. The results suggest that the presence in bile of certain neurohumoral transmitters and neurohumoral-like agents may directly affect the canine choledochoduodenal sphincter function.

Keyphrases □ Choledochoduodenal junction, canine—effect of neurohumoral transmitters and neurohumoral-like agents present in bile on opening pressure of choledochoduodenal sphincter □ Bile neurohumoral transmitters and neurohumoral-like agents—technique for determining effect on opening pressure of choledochoduodenal junction, dogs

Experimentally, it has been difficult to evaluate discrete drug-induced effects on the sphincter of Oddi *in situ* without encountering the disturbing influence of the surrounding smooth musculature of the duodenum. For anatomical reasons (1), this difficulty has been found to be particularly true in the dog.

The effects of intravenous and intraarterial injections of drugs on the canine sphincter of Oddi have received much attention, but repeated results have sometimes been inconsistent. Presumably, the reason for injecting drugs into the arterial supply to the terminal bile duct is to affect the sphincter of Oddi selectively. However, because of the close anatomical relation between the terminal bile duct and the duodenum, it is probable that an injected drug would reach the surrounding duodenum even though it reached the terminal bile duct first. Of course, drugs injected intraarterially also may exert their effects on the sphincter of Oddi indirectly by affecting the vasculature.

An alternative way of selectively affecting the sphincter of Oddi would be to introduce drugs into the lumen of the terminal bile duct. This proposal is in conformity with an interesting series of experiments on the action of some antibiotics on the extrahepatic biliary tract (2–5). In one experiment (3), intraductal erythromycin increased the motility and tone of the terminal bile duct in the guinea pig, cat, dog, and monkey. In subsequent experiments (6), intraductal aminosiderin decreased the motility and tone of the normal and hypertonized choledochus in the same species of animals. The consensus was that antibiotics eliminated in the active form in the bile can interfere with both the tone and motility of the extrahepatic biliary tract.

Published work so far has dealt primarily with intraductal antibiotics. However, biliary excretion into the lumen of the intestine is a major pathway of elimination of a whole host of drugs and drug metabolites. An excellent summary of the literature data relating to biliary excretion of drugs in animals and humans is available (7).

It was considered worthwhile to determine whether various normal constituents of bile, as well as pharmacological agents known to be excreted in the bile, can produce a discrete, localized effect upon the choledochoduodenal junction, which may or may not be similar to their action when administered intragastrically or systemically.

EXPERIMENTAL

Animals and General Procedure—Thirty-four adult mongrel dogs were used. All experiments were undertaken after the animals had been fasted overnight with water *ad libitum*. The animals were anesthetized at first with intravenous injections of thiopental sodium[1] (25 mg/kg), followed by maintenance with a mixture of α-chloralose (5%, dissolved in polyethylene glycol 200) and urethan (50% in 0.9% saline solution). Further injections of the chloralose–urethan mixture were given when necessary.

Surgical and Recording Procedure—Along with a tracheotomy, a thoracotomy and laparotomy were performed in each dog through a long midline incision. A tracheal cannula was routinely inserted. Systemic pressure was measured from a cannula inserted into a femoral artery. Through an ileostomy, an open-tip catheter with a balloon attachment was introduced into the upper jejunum. For recording biliary flow, another catheter was inserted into one hepatic duct toward the liver and the other end was connected to a photoelectric cell drop counter.

Measurements of sphincter opening pressures were conducted as follows. A polyethylene catheter was inserted into the common bile duct *via* the fundus of the gallbladder. The end of the catheter was advanced through the duct toward the duodenum until palpation indicated that it was *juxta* the sphincter of Oddi. The catheter was secured by tying it at its insertion into the fundus of the gallbladder. A longitudinal duodenotomy was performed at a point opposite the opening of the choledochoduodenal junction to permit visualization of the sphincter.

Duodenal stabilization was effected in such a manner that the sphincter opening could be completely immersed in a pool of clear saline. The perfusion system was filled with warmed normal saline containing a blue food dye. It was thus possible to confirm an opening of the sphincter by direct visualization of a thin stream of blue fluid in the clear pool of saline covering the aperture. The

[1] Pentothal.

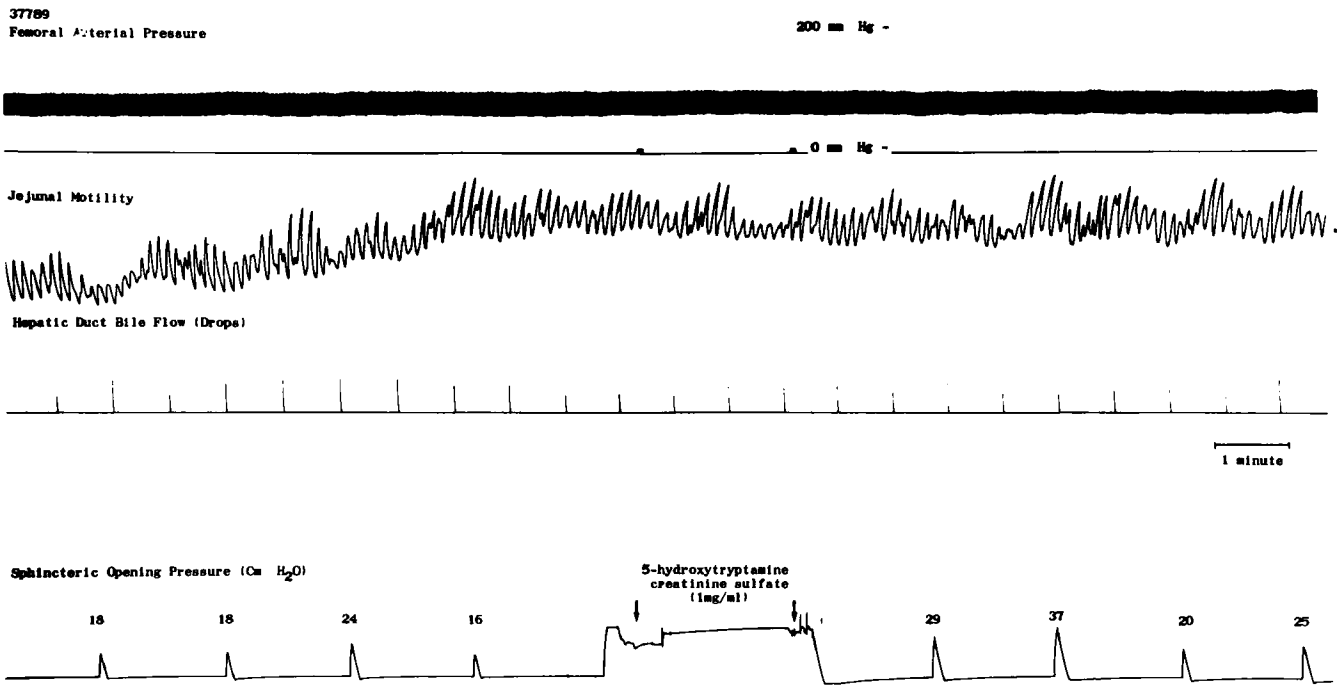

Figure 1—*Polygram depicting the time course of femoral arterial blood pressure, intrajejunal pressure activity, bile flow rate as measured with a drop recorder, and ductal opening pressure in a bilaterally vagotomized dog. Serotonin was introduced into the terminal bile duct during the period indicated by arrows. The extraductal events were not altered during or following drug administration. The pressure limb rise and plateau configuration seen in this figure represent the measurement system pressure during the introduction and subsequent stasis of a test agent. The drop in pressure limb represents the rapid washout of the small fluid volume (0.2 ml) and the reversal of the infusion pump.*

dyed saline flowing through the choledochus was drained outside the duodenum by a large outflow tube inserted into the duodenal lumen.

The perfusing catheter was connected to a constant-flow infusion pump through a "T" tube. The side arm was connected by valves to a fluid reservoir, pressure transducer, and tall manometer tube. The pressure transducer was simply calibrated in terms of the height of the column of saline in the manometer tube. In essence, the compliance represented by the fluid column is so large with respect to the lumped compliances of both the terminal common duct and the polyethylene catheter that the entire system compliance is substantially that of the manometer. Thus, infusion at a constant flow results in a linear rate of pressure rise in the duct.

Initial experiments (8) had shown that a pressure ramp value of 7 cm/sec was satisfactory. This value is in substantial agreement with that utilized previously (9, 10). Femoral blood pressure, upper jejunal pressure, hepatic duct flow, and sphincter opening pressures were recorded with an ink-writing polygraph.

Chemicals—The following were used: histamine dihydrochloride[2], epinephrine hydrochloride[2], norepinephrine hydrochloride[2], 5-hydroxytryptamine creatinine sulfate[2], magnesium sulfate[2], papaverine[2], secretin[2], bethanechol chloride[3], morphine sulfate[3], lidocaine hydrochloride[4], penicillin G potassium[5], cephaloridine[5], chloramphenicol succinate[6], rifampin[7], and cholecystokinin[8].

All drugs were administered intraductally. Drugs were introduced into the region of the sphincter by administration through the pressure measurement catheter and allowed to remain for a definite period. Both ends of the solution column were marked by air bubbles. It was thus possible to determine when the drug arrived at its intended site of action, because the blue marker dye abruptly disappeared. Similarly, removal of the test agent was also clearly marked by reappearance of the dye marker. Drug sequences were randomized among the various experiments. Following intraductal stasis, each drug was flushed from the ductal lumen with copious amounts of saline to ensure that chemical or functional interactions would be minimized.

Each series of experimental opening pressure determinations following drug administration was immediately preceded by a control series of determinations. Mean opening pressures for each posttreatment series were statistically compared to the mean values of the preceding pretreatment values under the null hypothesis that mean posttreatment opening pressures were the same as pretreatment values. The alternative hypothesis stated that posttreatment mean values were different, with rejection of the null hypothesis being made at the 5% level of confidence for a two-tailed test.

RESULTS AND DISCUSSION

Figure 1 is a complete polygram depicting arterial blood pressure, intrajejunal pressure activity, and biliary flow preceding, during, and following the intraductal administration of serotonin. It can be readily seen that these parameters were unaffected. Sphincter opening pressures measured after washout of the drug were increased.

Table I summarizes the mean opening pressures observed prior to and subsequent to the intraductal administration of histamine, norepinephrine, epinephrine, serotonin, and bethanechol. Histamine, serotonin, and bethanechol administrations were subsequently associated with elevated mean opening pressures, while norepinephrine and epinephrine were associated with subsequently lowered mean opening pressures. In all cases, the differences in mean values were significant.

The data shown in Table I were obtained from polygrams that also recorded vascular activities and jejunal pressure activity as depicted in Fig. 1. Although not shown, these polygrams revealed that in no case was the intraductal administration of any of these agents effective in altering either ongoing cardiovascular activity or intestinal motor function in the near vicinity of the choledochoduodenal junction.

Preliminary experiments with other agents revealed that rifampin (12 trials in two dogs) was also associated with apparent in-

[2] Sigma.
[3] Merck.
[4] Astra.
[5] Lilly.
[6] Parke-Davis.
[7] Ciba.
[8] Karolinska Institute.

Table I—Comparison of Mean Forward Ductal Opening Pressures after Intraductal Drug Administration

Dog Number[a]	Pretreatment Opening Pressures[b]	Posttreatment Opening Pressures
Histamine Dihydrochloride (0.65 mg/ml)		
37600	16.8 ± 2.99 (6)	26.6 ± 2.96 (7)
36557	14.9 ± 2.73 (7)	30.0 ± 4.36 (6)
38230	18.8 ± 3.60 (6)	29.2 ± 2.06 (6)
38236	14.9 ± 3.18 (7)	25.0 ± 2.25 (9)
36672	15.7 ± 1.50 (12)	29.3 ± 3.37 (6)
36489	12.2 ± 2.36 (10)	28.4 ± 2.85 (10)
11880	16.1 ± 4.84 (14)	38.2 ± 3.91 (6)
11949	15.3 ± 2.83 (6)	27.5 ± 3.02 (6)
Norepinephrine Hydrochloride (0.45 mg/ml)		
37600	20.3 ± 2.87 (7)	10.3 ± 2.97 (6)
37604	20.3 ± 2.89 (8)	13.4 ± 2.64 (8)
67491	18.4 ± 3.78 (6)	11.8 ± 1.71 (6)
36557	31.7 ± 7.66 (6)	14.7 ± 2.81 (6)
11880	20.6 ± 3.01 (6)	13.8 ± 2.38 (8)
Epinephrine Hydrochloride (0.45 mg/ml)		
39593	19.0 ± 1.26 (6)	11.7 ± 1.51 (6)
37789	21.4 ± 4.90 (8)	13.8 ± 2.40 (6)
37600	17.2 ± 2.56 (6)	10.6 ± 1.26 (8)
11949	19.5 ± 3.39 (6)	12.2 ± 2.36 (8)
11411	24.5 ± 3.99 (6)	12.8 ± 2.10 (6)
5-Hydroxytryptamine Sulfate (1 mg/ml)		
37789	18.2 ± 2.82 (12)	27.7 ± 1.74 (8)
11880	19.0 ± 2.36 (8)	28.3 ± 2.16 (6)
67491	16.3 ± 3.92 (8)	26.5 ± 4.97 (6)
36218	16.0 ± 0.63 (10)	25.5 ± 1.52 (8)
Bethanechol Chloride (5 mg/ml)		
11411	23.8 ± 2.35 (10)	86.8 ± 14.40 (8)
14788	17.8 ± 3.43 (8)	80.7 ± 8.94 (6)
44599	18.6 ± 4.71 (6)	90.8 ± 11.35 (8)
37600	26.3 ± 3.25 (8)	79.1 ± 6.47 (8)

[a] Difference in mean opening pressures are significant in each experiment. [b] Opening pressure measurements are in centimeters of saline; ± indicates 1 *SD*; parentheses contain number of observations.

creases in opening pressures. The following agents did not appear to produce any qualitative changes in sphincter opening pressures when administered intraductally: lidocaine (18 trials in two dogs), magnesium sulfate (18 trials in two dogs), papaverine (18 trials in two dogs), morphine sulfate (22 trials in five dogs), cephaloridine (12 trials in two dogs), penicillin G potassium (12 trials in two dogs), chloramphenicol (12 trials in two dogs), secretin (18 trials in four dogs), and cholecystokinin (15 trials in two dogs).

A histological description of the terminal ductal mucosa probably illuminates the observations made with respect to the marked elevation of mean opening pressures produced by the intraductal administration of histamine and bethanechol. According to Bhatnagar *et al.* (11), the mucosal coat of the interior of the canine common bile duct is continuous with the lining membrane of the hepatic ducts and gallbladder and also with that of the duodenum. The epithelium is of columnar variety.

This description is characteristic of highly vascularized tissue, and the effect noted in the present study was probably due to a parasympathomimetic edematous effect which is similar to the increase in upper airway resistance common in such reactions as allergic rhinitis. The inhibitory effect of intraductal norepinephrine, and to a lesser extent epinephrine, was probably produced by local vasoconstriction, a phenomenon that is analogous to shrinking the edematous nasal membrane with this type of agent.

The intraductal administration of serotonin also significantly elevated mean opening pressures. Although the functional significance of this observation is not at all clear, the observation itself again points directly to the as yet baffling question of just what is the functional significance of the serotonin content of the large enterochromaffin cell population of the intramural portion of the common duct or, for that matter, of the pancreatic duct (12).

A few of the remaining results were generally supportive of data collected previously by other investigators, and only a few points require discussion. The observation that the intraductal administration of lidocaine was ineffective in altering mean opening pressures supports the observations of McCarthy and Picazo (13) in humans. The observation that rifampin tended to increase ductal opening pressures is in agreement with the results of Benzi *et al.* (3), who noted that intraductal administration of rifampin (rifamycin SV) induced a hypertonus of the canine choledochus.

In light of these findings, it is believed that intraductal administration of various test agents may have revealed an interesting facet to the evaluation of various experimental observations relative to ductal control mechanisms. Thus, the observation that intraductal administration of cholecystokinin has no effect upon mean ductal opening pressures is in opposition to the experimental findings of others that intravenous administration of the same substance lowers mean opening pressures. However, intravenous administration of cholecystokinin also causes relaxation of the duodenal musculature in the region of the ampulla (14). Undoubtedly, the relaxing action of cholecystokinin on the duodenum would increase flow through the terminal duct in this species. The difference in effects accompanying the administration of an agent by different routes indicates that a procedure that introduces an agent *via* a route permitting widespread systemic action is dangerous if the purpose of the study is to determine whether a highly discrete and localized effect does occur *in situ*.

The same argument may be advanced with respect to the systemic administration of other agents such as morphine or epinephrine, especially when the *in vivo* and *in vitro* sphincter effects are already known to be different. For example, according to Persson (15), *in vivo* morphine is active locally on the cat choledochoduodenal junction but inactive when added to the bath fluid of the isolated feline sphincter of Oddi. On the other hand, isolated spiral strips cut from the terminal bile duct respond to epinephrine by contracting (16). However, when the terminal bile duct remains surrounded by the wall of the duodenum into which it is inserted, the action of intravenous epinephrine seems to depend on the tone and motility of the duodenum (17).

CONCLUSIONS

It appears that the intraductal technique of administering drugs permits a very localized and quantitative estimation of the effects of various test agents on the tone and motility of the choledochoduodenal junction. This technique may eventually prove of value in the evaluation of the effects of therapeutic agents and/or their metabolites that are secreted in the bile if it is applied systematically, as part of well-defined protocols, so that adequate experience can be obtained to permit a judgment of its merit.

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Binding Study of Sulfonylureas and Phenothiazines to Bovine Serum Albumin Using Difference Spectrophotometry

H. ZIA[*§] and JAMES C. PRICE [‡x]

Abstract □ 2-(4′-Hydroxybenzeneazo)benzoic acid is a spectrophotometric probe which shows absorption spectrum changes upon binding to protein. Difference absorption spectra of this probe were used as an indirect measurement of the binding of selected sulfonylurea and phenothiazine drugs to bovine serum albumin. The results obtained using the spectrophotometric probe were similar to data obtained from other methods, especially fluorescent methods. Of the four sulfonylureas studied, tolbutamide showed the highest binding affinity, followed by glyburide, glipizide, and acetohexamide, in that order. The data collected for phenothiazine drugs indicated that chlorpromazine has the highest affinity, followed in order by trifluoperazine, perphenazine, fluphenazine, and promazine. Correlation of these results with chemical composition indicated that the interaction of phenothiazine drugs with bovine serum albumin was of a hydrophobic nature.

Keyphrases □ Sulfonylureas and phenothiazines—binding to bovine serum albumin, difference spectrophotometry using 2-(4′-hydroxybenzeneazo)benzoic acid □ Phenothiazines and sulfonylureas—binding to bovine serum albumin, difference spectrophotometry using 2-(4′-hydroxybenzeneazo)benzoic acid □ Spectrophotometry, difference—determination, binding of sulfonylureas and phenothiazines to bovine serum albumin using 2-(4′-hydroxybenzeneazo)benzoic acid □ 2-(4′-Hydroxybenzeneazo)benzoic acid—used to determine binding of sulfonylureas and phenothiazines to bovine serum albumin, difference spectrophotometry

The phenomena of drug–protein binding and of competitive binding of drugs for available protein sites have been the subjects of many investigations and have been reviewed (1). Various experimental procedures and analysis methods have been used to study drug–protein interactions. These include equilibrium dialysis (2), ultrafiltration (3), gel filtration (4), NMR rate measurements (5), and fluorescence techniques (6).

Compared to dialysis or ultrafiltration techniques, spectrophotometric and/or fluorescence probe techniques are capable of providing data similar to what could be obtained through dialysis or ultrafiltration studies but are less time consuming, simpler, and more reproducible. It also may be possible to estimate the nature of binding and binding sites from the chemical structure and spectral properties of the probes used (7).

In spite of their usefulness in protein binding studies, fluorescence probes cannot be used successfully in certain instances such as the lack of a fluorescence change upon binding and/or a change of fluorescence due to a mechanism other than binding (*e.g.*, photoxidation).

Although fluorescence probes have been explored in recent years, little information has been generated concerning spectrophotometric probes. In 1968, Moriguchi and coworkers (8–11) studied the binding of the 2-(4′-hydroxybenzeneazo)benzoic acid probe to bovine serum albumin. Recently, Nazareth *et al.* (12) used this probe as an agent that reflects the binding of L-thyroxine to serum albumin. However, in this study they employed ultrafiltration to separate the free probe from serum albumin to calculate the ratio of bound drug to protein.

The usefulness of spectrophotometric probes in the study of the binding of drugs to protein has not been adequately explored. In addition, certain procedures and methods of data treatment reported for fluorescence probes (13, 14) appear to be useful for the spectrophotometric probe technique. This study was made to explore the usefulness of 2-(4′-hydroxybenzeneazo)benzoic acid as a spectrophotometric probe in studying the interaction of sulfonylurea and phenothiazine drugs with bovine serum albumin and to define, if possible, the binding parameters of some of these drugs.

EXPERIMENTAL

Materials—Bovine serum albumin[1] (crystalline), 2-(4′-hydroxybenzeneazo)benzoic acid[2], tolbutamide[3], acetohexamide[4], glipizide[5], glyburide (glibenclamide)[3], chlorpromazine hydrochloride[6], trifluoperazine hydrochloride[6], promazine hydrochloride[7], perphenazine[8], and fluphenazine hydrochloride[8] were used as obtained without further purification. The solvents used were spec-

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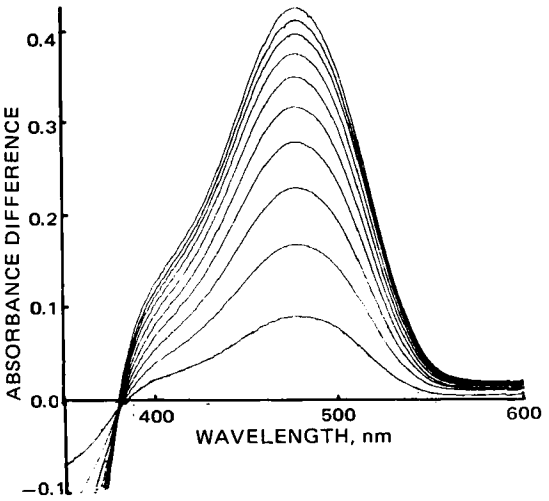


Figure 1—*Typical absorption difference spectra of bovine serum albumin solution (6.9 × 10^{-5} M) with successive increments of 2-(4′-hydroxybenzeneazo)benzoic acid.*

tral grade, and all other chemicals were reagent grade.

Apparatus—Absorption spectra and difference absorbance measurements were made with a double-beam spectrophotometer[9] equipped with tandem cell holders.

Absorbance Difference Titrations—The absorbance difference titrations in the absence and presence of drugs were carried out manually with microsyringes[10]. The probe was dissolved in methanol at a concentration of 1 × 10^{-2} *M*. The bovine serum albumin solutions were buffered at pH 7.4 with 0.05 *M* phosphate for sulfonylureas and 0.05 *M* tromethamine for phenothiazines.

Two 3.00-ml quantities of bovine serum albumin solution were pipetted into two cells and two 3.00-ml quantities of buffer solution were pipetted into two other cells; these cells were placed in the reference and sample beams in such a manner that a buffer cell and a protein solution cell were in tandem in each beam. After running a baseline, the contents of the buffer solution cell in the reference beam and the protein solution cell in the sample beam were titrated with successive additions of 3 μl of probe solution and absorbance differences were measured.

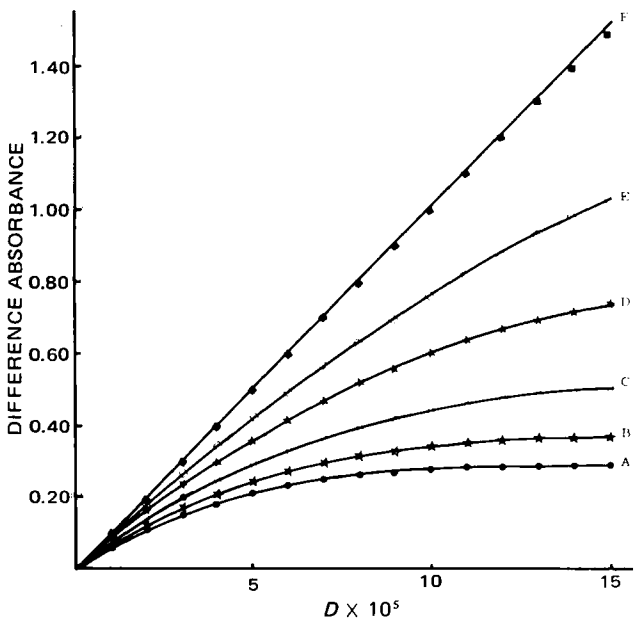


Figure 2—*Absorbance differences as a function of the 2-(4′-hydroxybenzeneazo)benzoic acid concentration, D, at various bovine serum albumin concentrations: A, 3.45 × 10^{-5} M; B, 4.6 × 10^{-5} M; C, 6.9 × 10^{-5} M; D, 13.8 × 10^{-5} M; and E, 55.2 × 10^{-5} M. The theoretical line (F) is based on calculated values of absorbance difference for high concentrations of bovine serum albumin.*

[9] Cary 118C, Varian Associates, Palo Alto, Calif.
[10] Hamilton Co., Reno, Nev.

Because of low solubility in water, all sulfonylureas were dissolved in 0.1 *N* NaOH solution with the exception of glyburide, which was dissolved in methanol. The addition of the microliter quantities of the sodium hydroxide solutions of the drugs did not change the final pH of the cell solutions more than 0.05 pH unit. The phenothiazine drugs were dissolved in tromethamine buffer with the exception of perphenazine, which was dissolved in methanol. All absorbance difference measurements were made at ambient temperature.

Data Treatment—The absorbance difference observed upon addition of the probe to bovine serum albumin was measured for each successive addition in the absence and presence of the competitive drugs at 482 nm. These data were used to calculate the fraction of bound probe, *X*, using the following equation:

$$X = \frac{\Delta A_l}{\Delta A_h} \qquad \text{(Eq. 1)}$$

where ΔA_l and ΔA_h refer to the absorbance difference of a given concentration of probe in solutions of low and high protein concentration, or the absorbance difference in the absence and presence of excess sites, respectively. To determine the value of ΔA_h for a given concentration of probe, absorbance difference titrations were carried out at several different protein concentrations and the values of ΔA_h were taken to be values extrapolated to the intercepts of plots of $1/\Delta A$ *versus* $1/[P]$, where $[P]$ represents the total concentration of protein.

After values for the fraction of bound probe were found for all points along the titration curve, the data were treated according to the Scatchard (15) equation:

$$\bar{V}/[D] = nK_a - \bar{V}K_a \qquad \text{(Eq. 2)}$$

where $\bar{V}$ is the number of moles of bound probe per mole of protein, $[D]$ is the concentration of free probe, n is the number of binding sites on the protein, and K_a is the intrinsic association constant of the complex. In addition, plots of $\log \bar{V}/(n - \bar{V})[D]$ *versus* $\bar{V}$ were drawn (13).

The competitive binding of two different groups of drugs, sulfonylureas and phenothiazines, were studied using 2-(4′-hydroxybenzeneazo)benzoic acid as the indicating probe. The binding of the probe to bovine serum albumin was determined in the presence and absence of competitive drugs by varying the concentration of the probe at constant albumin and drug concentrations. The binding constants of competitive drugs were calculated by using the equation of Klotz *et al.* (16):

$$K_b = \frac{n[P_t]K_a[D] - K_a[D][PD] - [PD]}{[B_t]K_a[D] - n[P_t]K_a[D] + K_a[D][PD] + [PD]} \times \frac{K_a[D]}{[PD]} \qquad \text{(Eq. 3)}$$

where:

K_b = association constant for competitor
K_a = association constant for probe
$[D]$ = concentration of free probe
$[PD]$ = concentration of bound probe
n = number of binding sites
$[P_t]$ = total concentration of protein
$[B_t]$ = total concentration of competitor

RESULTS AND DISCUSSION

The usefulness of a dye probe for the study of drug–protein binding comes from the fact that the absorption spectrum of the dye changes as a result of binding to the albumin. Figure 1 shows typical absorption difference spectra at constant serum albumin concentration with several concentrations of 2-(4′-hydroxybenzeneazo)benzoic acid. These spectra have a maximum at 482 nm,

Table I—Association Constants of Sulfonylureas to Serum Albumins

Compound	Blood Fraction	Method	Association Constants, M^{-1}	Reference
Acetohexamide	HSA[a]	Dialysis	$K^b = 3.39 \times 10^4$	19
	BSA[c]	Fluorescence	$K^d = 3.76 \times 10^4$	20
	BSA	Spectrophotometry	$K = 1.60 \times 10^4$	This study
Glipizide	BSA	Fluorescence	$K^d = 6.88 \times 10^4$	20
	BSA	Fluorescence	$K^d = 2.45 \times 10^4$	20
	BSA	Spectrophotometry	$K = 4.90 \times 10^4$	This study
Glyburide	BSA	Fluorescence	$K^d = 3.26 \times 10^5$	20
	BSA	Spectrophotometry	$K = 6.13 \times 10^4$	This study
Tolbutamide	HSA	Dialysis	$K^b = 4.05 \times 10^4$	19
	HSA	Dynamic	$K_1^e = 2.16 \times 10^5$	21
		Dialysis	$K_2^d = 1.71 \times 10^2$	
	BSA	Fluorescence	$K^d = 9.04 \times 10^4$	20
	BSA	Spectrophotometry	$K = 1.65 \times 10^5$	This study

[a] HSA = human serum albumin. [b] The pH and temperature of the solution were 7.4 and 40°. [c] BSA = bovine serum albumin. [d] The pH and temperature of the solution were 7.4 and 25°. [e] The pH and temperature of the solution were 7.4 and 37°.

and the absorbance difference increases as the concentration of the probe increases.

The absorbance difference data measured at 482 nm for probe–bovine serum albumin are plotted as a function of probe concentration in Fig. 2. The values of the points on the theoretical line are taken from the extrapolated values of the plots of $1/\Delta A$ *versus* $1/[P]$ (see *Data Treatment*).

Scatchard plots for the binding of probe to bovine serum albumin are shown in Fig. 3. At various concentrations of protein, the Scatchard plots gave lines that, like fluorescence results, are not truly linear in all ranges of concentration (13). The curvatures of Scatchard plots are generally considered to indicate that probe molecules bind to more than one class of sites on the protein. Recently, based on fluorescence results, it was suggested that the intrinsic association constants may not be equal even in the same class of binding sites (13).

Although these considerations are likely in some instances, another possibility is suggested by the spectrophotometric data collected here. It can be postulated that, in the range of concentration studied, the spectral changes are due to the formation of a charge transfer complex of only one probe molecule with one serum albumin molecule. This possibility is indicated by the fact that the value of $\bar{V}$ in the range of concentration studied never exceeded 1 and, as seen in Fig. 3, that line *C* with the lowest concentration of albumin starts to fall off as $\bar{V}$ exceeds the value of 0.85.

It has been reported from equilibrium dialysis studies that dyestuffs may bind to as many as 11 and/or 22 sites on serum albumin (17, 18). Although physical interpretation of these sites is questionable, it is possible that there are additional binding sites for probe molecules that do not cause spectral changes and that cause negative deviations from linearity of Scatchard-type data plots.

Figure 4 shows the results as plotted according to a modified Scatchard equation (13). In this figure, the values of log $\bar{V}/(n - \bar{V})[D]$, where n is assumed to be 1, are plotted against $\bar{V}$. The lines are linear except at the extremes of concentration.

For the purpose of competitive binding, the fact that the curves of Fig. 3 are not completely linear in all ranges of concentration need cause little concern, because the probe molecules are only used as a relative measure of the binding of drugs and the albumin may not be molecularly homogeneous with respect to its binding properties. It may only be necessary either to select a range of concentration within which the binding data can be mathematically treated or to substitute corresponding values of K for each point along the Scatchard plot into Eq. 3 to calculate the binding constants of the competitor drugs. In fact, for fluorescence data where probe molecules are said to bind to one class of sites, the low values of $\bar{V}$ have been disregarded to obtain a straight line with a high significance factor (13, 16). It is evident that the same treatment can be applied to the data in Fig. 3 to find the values of K and n for the range of concentration within the linear portion of the plot.

Moriguchi *et al.* (8) studied the binding of 2-(4′-hydroxybenzeneazo)benzoic acid to bovine serum albumin Fraction V and expressed their results by a modified Langmuir-type equation:

$$\log [\bar{V}(n - \bar{V})] = m \log [D] + m \log K \quad \text{(Eq. 4)}$$

where m is an empirical parameter and varies with factors such as protein concentration, buffering agent, and temperature.

By this method and by using an estimated value of molar absorptivity that is known to be dependent on factors such as solvent and temperature, the value of n was determined by trial and error so that the plot of log $[\bar{V}(n - \bar{V})]$ *versus* log $[D]$ was linear. In this way, the authors found the values of n and log K to be equal to 2 and 4.2 ± 0.1, respectively, while the value of m varied from 0.96 to 0.86 for albumin concentrations of 0.25×10^{-4}–1×10^{-4} *M*.

Nazareth *et al.* (12) used the same probe to reflect the binding

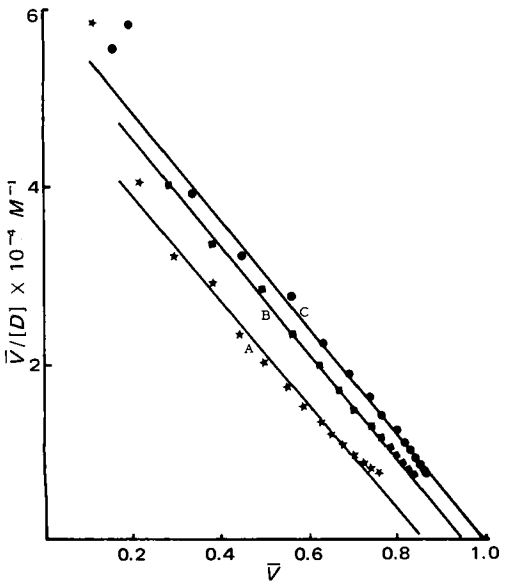


Figure 3—*Scatchard plots of 2-(4′-hydroxybenzeneazo)benzoic acid binding to various bovine serum albumin concentrations in 0.05* M *phosphate buffer, pH 7.4. Key:* ●, 3.45×10^{-5} M; ■, 4.6×10^{-5} M; *and* ★, 6.9×10^{-5} M.

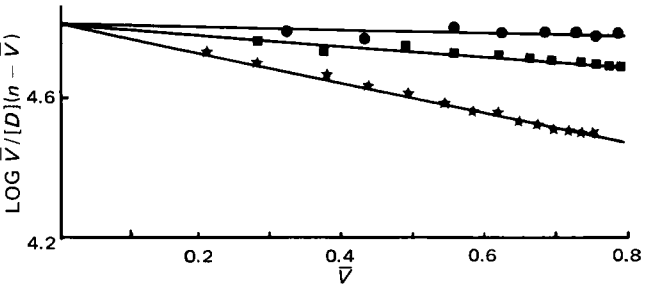
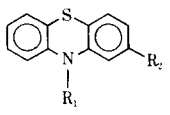


Figure 4—*Plots of* $\bar{V}/[D](n - \bar{V})$ versus $\bar{V}$ *for the binding of 2-(4′-hydroxybenzeneazo)benzoic acid to various bovine serum albumin concentrations in 0.05* M *phosphate buffer, pH 7.4. Key:* ●, 3.45×10^{-5} M; ■, 4.6×10^{-5} M; *and* ★, 6.9×10^{-5} M.

Table II—Association Constants of Selected Phenothiazines to Bovine Serum Albumin



Generic Name	R_1	R_2	Association Constant $\times 10^{-4}\ M^{-1}$
Chlorpromazine hydrochloride	Cl	$-CH_2CH_2CH_2N(CH_3)_2$	2.57
Trifluoperazine hydrochloride	CF_3	$-CH_2CH_2CH_2N\langle\rangle NCH_3$	2.32
Perphenazine	Cl	$-CH_2CH_2CH_2N\langle\rangle NCH_2CH_2OH$	1.96
Fluphenazine hydrochloride	CF_3	$-CH_2CH_2CH_2N\langle\rangle NCH_2CH_2OH$	1.82
Promazine hydrochloride	H	$-CH_2CH_2CH_2N(CH_3)_2$	1.04

of L-thyroxine to serum albumin. Ultrafiltration was used to determine the ratio of bound drug to protein, and low values of $\bar{V}$ were utilized to draw reciprocal plots to show the binding and displacement of probe to and from primary sites of albumin.

A comparison of the spectrophotometric method reported here with the methods of Moriguchi *et al.* (8) and/or Nazareth *et al.* (12) together with the results obtained from each method illustrates the usefulness and simplicity of the spectrophotometric method. It is recognized that the determination of binding parameters may depend on the experimental methods used. For some classes of components, the results obtained from spectrophotometric and fluorescence techniques are different from those of other methods, probably because only the primary (strong) binding sites causing spectral changes are detected, whereas the results obtained from other methods such as dialysis often show larger numbers of binding sites.

Competitive Binding between 2-(4′-Hydroxybenzeneazo)benzoic Acid and Sulfonylureas—A typical difference absorption spectrum in the presence and absence of a drug (glyburide) is shown in Fig. 5; the addition of competitive drug decreased the absorbance differences, presumably because the bound probe was displaced from the protein. The results of difference absorbance measurement at 482 nm in the presence and absence of fixed amounts of each sulfonylurea are shown as Scatchard plots in Fig. 6. The bovine serum albumin and sulfonylurea concentrations were held constant at 6.9×10^{-5} and 5×10^{-5} *M*, respectively, while the concentration of probe was varied from 1×10^{-5} to 11×10^{-5} *M*.

The binding constants calculated using the equation of Klotz *et al.* (16) are shown along with literature data in Table I. It can be seen from Table I and Fig. 6 that, as the association constants of the competitor sulfonylureas increase, the slopes of Scatchard plots decline. These findings, together with the fact that binding of probe decreases when it is in competition with sulfonylureas, appear to indicate that these drugs bind to the bovine serum albumin *via* the same site or closely located sites on the protein.

The conclusion to be drawn from Table I is that the binding constants found by the spectrophotometric probe technique are comparable to fluorescence data but are different from dynamic dialysis where more than one class of sites is detected. Among the four sulfonylureas studied, tolbutamide had the highest binding affinity followed in order by glyburide, glipizide, and acetohexamide.

Displacement of 2-(4′-Hydroxybenzeneazo)benzoic Acid by Phenothiazines—The determination of binding constants of phenothiazine drugs to serum albumin by the usual fluorescence probe technique is somewhat difficult because of rapid photoxidation and the consequent rapid change in fluorescence. Equilibrium dialysis is also ruled out because of the length of time required during which drugs are oxidized. Since the spectrophotometric probe technique utilizes very low light levels (compared to the fluorescence technique) and the analysis time is short, it has strong advantages over the other methods for this class of drugs.

In this study, the bovine serum albumin and phenothiazine concentrations were held constant at 4.6×10^{-5} and 1.5×10^{-4} *M*, respectively, while the concentration of the probe was varied from 1×10^{-5} to 15×10^{-5} *M*. Figure 7 shows Scatchard plots of the ef-

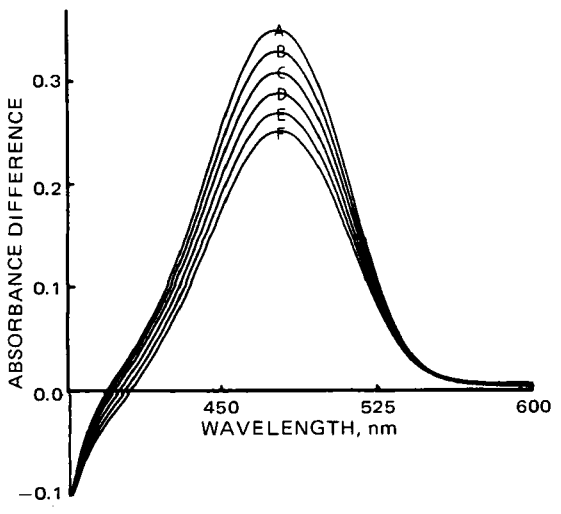


Figure 5—*Absorbance difference spectra for solutions containing bovine serum albumin (6.9×10^{-5} M) and 2-(4′-hydroxybenzeneazo)benzoic acid (6.5×10^{-5} M) in absence (curve A) and presence (curves B, C, D, E, and F) of 1.25×10^{-5} M increments of glyburide.*

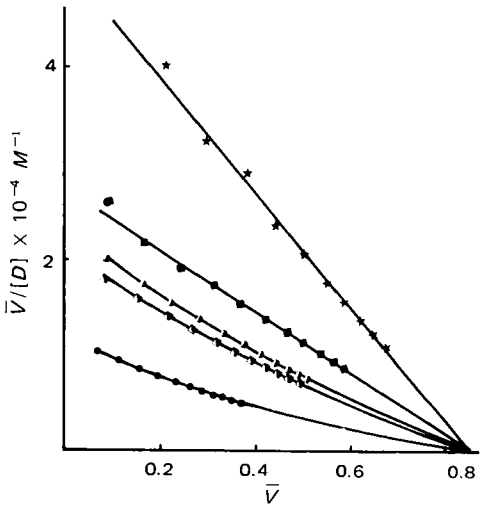


Figure 6—*Scatchard plots of 2-(4′-hydroxybenzeneazo)benzoic acid binding to bovine serum albumin in 0.05 M phosphate buffer, pH 7.4. Key: ★, in absence of drug; ■, in presence of 5×10^{-5} M acetohexamide; ◑, in presence of 5×10^{-5} M glipizide; ◪, in presence of 5×10^{-5} M glyburide; and ●, in presence of 5×10^{-5} M tolbutamide.*

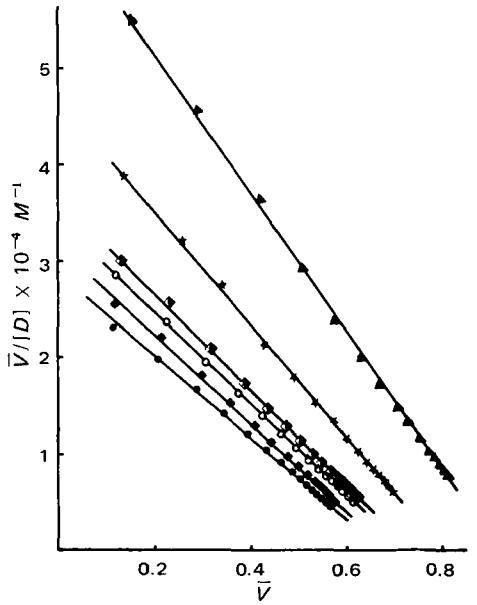


Figure 7—*Scatchard plots of 2-(4'-hydroxybenzeneazo)benzoic acid binding to bovine serum albumin in 0.05 M tromethamine buffer, pH 7.4. Key: ▲, in absence of drug; ★, in presence of 1.5 × 10^{-4} M promazine; ◪, in presence of 1.5 × 10^{-4} M fluphenazine; ○, in presence of 1.5 × 10^{-4} M perphenazine; ■, in presence of 1.5 × 10^{-4} M trifluoperazine; and ●, in presence of 1.5 × 10^{-4} M chlorpromazine.*

fect of adding different phenothiazine drugs to probe–bovine serum albumin solutions, and Table II shows calculated association constants. Chlorpromazine showed the greatest displacement of probe, followed by trifluoperazine, perphenazine, fluphenazine, and promazine. Furthermore, the binding was enhanced by direct or indirect halogen substitution on the phenothiazine moiety. Promazine, with no substitution at the 2-position, had the lowest value of K; chlorpromazine, having one chloride in the 2-position, had the highest value of K.

In addition, the side chain substitution at the 10-position also has considerable effect on the binding of these drugs. This is shown by trifluoperazine and fluphenazine, which have the same substitution at the 2-position but differ by their side chain at the 10-position. The hydroxy group on the fluphenazine appears to reduce the binding capacities. These results seem to indicate that interaction of phenothiazine drugs with bovine serum albumin is hydrophobic in nature. This conclusion is supported by the fact that the greater the hydrophobicity of the drug molecule, the greater is the value of the association constant.

The fact that Scatchard plots in the presence of phenothiazine drugs do not converge at the same intercept probably indicates that the site of binding for these drugs is not identical to that of the probe. It is likely that the binding of these drugs alters the conformation of the protein, thereby causing displacement of the probe. It is hoped that further work with these drugs will help explain the significance of these results.

SUMMARY

Difference spectrophotometry was used to show the displacement of a spectrophotometric probe [2-(4'-hydroxybenzeneazo)-benzoic acid] from bovine serum albumin by two classes of drugs, sulfonylureas and phenothiazines. It was shown that the difference absorbance data obtained could be treated in a manner similar to those obtained from fluorescence probe measurements.

Among the four sulfonylureas studied, tolbutamide showed the largest displacement of probe and the highest calculated binding affinity followed in order by glyburide, glipizide, and acetohexamide. These results closely paralleled reported results obtained by other techniques.

Among the phenothiazines, chlorpromazine showed the highest probe displacement and calculated binding affinity followed in order by trifluoperazine, perphenazine, fluphenazine, and promazine. These results indicated that the order of binding affinity was based on the hydrophobicity of the phenothiazine, with the more hydrophobic molecules being more strongly bound.

It is concluded that the use of difference spectroscopy to study indirectly the binding of drugs by measuring the displacement of a spectrophotometric probe offers several advantages over other methods. These advantages include convenience and fewer problems of oxidation and decomposition because of the short analysis time required and the low light intensities used. Interference from the fluorescence of drug molecules is also less of a problem than when fluorescent probes are used.

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Effect of Uridine Diphospho-*N*-acetylglucosamine and Sodium Salicylate on L-Glutamine–D-Fructose-6-phosphate Aminotransferase Activity from Rat Gastric Mucosa

JAMES CHAN and K. H. LEE ˣ

Abstract □ Mechanisms of inhibitory action of sodium salicylate on L-glutamine–D-fructose-6-phosphate aminotransferase, prepared from rat gastric mucosa, were studied. Sodium salicylate at lower concentrations (10–20 m*M*) inhibited reversibly aminotransferase activity by competing with fructose-6-phosphate. At higher concentrations, sodium salicylate inactivated the enzyme irreversibly, with an inactivation rate following first-order kinetics with respect to the enzyme concentration. Uridine-5′-diphospho-*N*-acetylglucosamine is an endogenous feedback inhibitor. It inhibited the aminotransferase-catalyzed reaction also by competing with fructose-6-phosphate but with an inhibiting activity 1000 times that of sodium salicylate. Uridine-5′-diphospho-*N*-acetylglucosamine reduced the salicylate inhibition of the enzymic reaction and protected the enzyme from salicylate-induced irreversible inactivation. At a fixed concentration of uridine-5′-diphospho-*N*-acetylglucosamine (7×10^{-6} *M*), an increase of salicylate concentration produced an increase in enzyme activity as compared with the control.

Keyphrases □ L-Glutamine–D-fructose-6-phosphate aminotransferase activity—inhibition by sodium salicylate and uridine-5′-diphospho-*N*-acetylglucosamine, rat gastric mucosa □ Sodium salicylate—inhibition of L-glutamine–D-fructose-6-phosphate aminotransferase activity from rat gastric mucosa □ Uridine-5′-diphospho-*N*-acetylglucosamine—inhibition of L-glutamine–D-fructose-6-phosphate activity from rat gastric mucosa □ Gastric mucosa—effect of uridine diphospho-*N*-acetylglucosamine and sodium salicylate on L-glutamine–D-fructose-6-phosphate aminotransferase activity

Sodium salicylate has been reported to inhibit the activities of many enzymes involved in mucopolysaccharide synthesis including L-glutamine–D-fructose-6-phosphate aminotransferase (EC 2.6.1.16) (1–4), acetylcoenzyme A synthetase (EC 6.2.1.1) (5, 6), uridine-5′-diphospho-*N*-acetylglucosamine-uridine-5′-diphospho-*N*-acetylgalactosamine epimerase (EC 5.1.3.1) (7), uridine-5′-diphosphoglucose dehydrogenase (EC 1.1.1.22) (8), and uridine-5′-diphospho glucuronyltransferase (EC 2.4.1.17) (9).

L-Glutamine–D-fructose-6-phosphate aminotransferase is located at a branch point in carbohydrate metabolism and competes for fructose-6-phosphate with other pathways utilizing hexose monophosphates such as glycolysis, glycogenesis, and the hexose monophosphate shunt. Depending on the tissue, 1.5–20% of the fructose-6-phosphate is converted to hexosamine (10, 11). The aminotransferase enzyme has the lowest extractable activity of the enzymes in the hexosamine pathway (12) and has been reported to be the rate-limiting enzyme in hexosamine biosynthesis (10).

In addition, several reports indicated that hexosamine biosynthesis was subjected to feedback regulation by uridine-5′-diphospho-*N*-acetylglucosamine (13–16). This feedback inhibition is on the initial enzyme of the pathway, catalyzing the apparent irreversible formation of glucosamine. Perrey (2) suggested that there may be a possible correlation between the inhibition of the aminotransferase enzyme in the rat gastric mucosa by sodium salicylate and gastric lesions.

In view of the potentially important role of this enzyme and implications resulting from its inhibition, this study presents the mechanisms of action of sodium salicylate and uridine-5′-diphospho-*N*-acetylglucosamine on L-glutamine–D-fructose-6-phosphate aminotransferase prepared from the rat gastric mucosa and the interaction between the two inhibitors.

EXPERIMENTAL

Materials and Chemicals—The following were used: D-glucose-6-phosphate sodium salt, D-fructose-6-phosphate sodium salt, L-glutamine hydrochloride, uridine-5′-diphospho-*N*-acetylglucosamine[1], sodium salicylate[2], and cross-linked dextran gel[3].

Preparation of L-Glutamine–D-Fructose-6-phosphate Aminotransferase—The enzyme was prepared by the method described by Bates and Handschumaker (16) with some modifications. Sprague–Dawley male rats[4] (120–150 g) were decapitated, and the stomachs were removed and placed into an ice-cold extraction medium containing 10 m*M* glucose-6-phosphate, 20 m*M* glu-

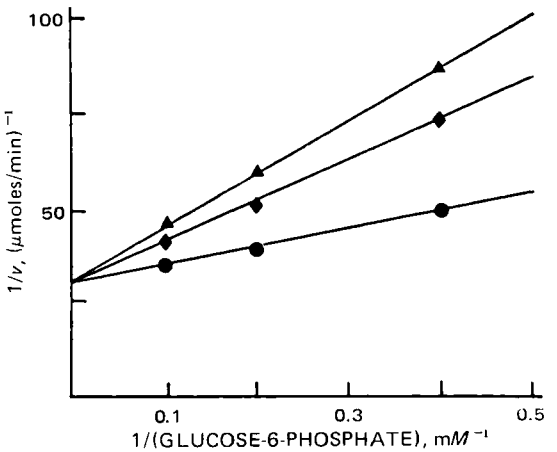

Figure 1—*Effect of salicylate on* L-*glutamine–*D-*fructose-6-phosphate aminotransferase activity as a function of the concentration of glucose-6-phosphate;* v *is expressed as micromoles of glucosamine formed per minute. Key:* ●,- *control;* ♦, 1×10^{-2} M *sodium salicylate; and* ▲, 1.5×10^{-2} M *sodium salicylate.*

[1] Sigma Chemical Co., St. Louis, Mo.
[2] J. T. Baker Chemical Co., Phillipsburg, N.J.
[3] Sephadex G-25, Pharmacia Co., Uppsala, Sweden.
[4] Simenson Laboratories, Gilroy, Calif.

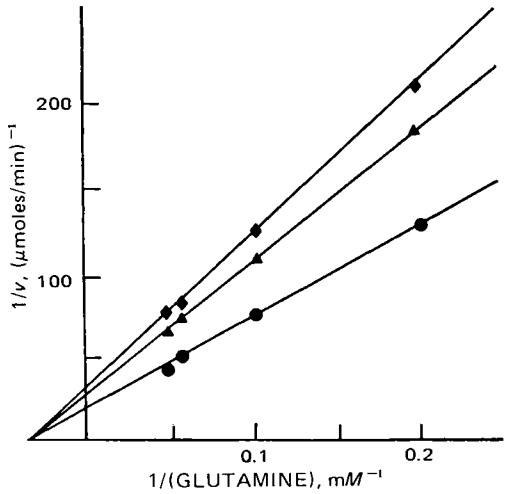

Figure 2—*Effect of salicylate on aminotransferase activity as a function of the concentration of glutamine. Key:* ●, *control;* ▲, *1* × 10^{-2} M *salicylate; and* ♦, *1.5* × 10^{-2} M *salicylate.*

tamine, 85 m*M* potassium phosphate buffer (pH 7.4), 0.1% mercaptoethanol, and 1 m*M* ethylenedinitrilotetraacetic acid disodium salt.

Each stomach was cut along its greater curvature and spread on a watchglass placed over ice, and the mucosal layer was scraped with a small spatula. The mucosal scrapings were homogenized in a hand homogenizer[5] with the volume to weight of the extraction medium. The homogenate was centrifuged at 35,000×*g* for 60 min in a refrigerated ultracentrifuge[6]. The clear supernatant fraction was passed through columns (20 × 1.5 cm) of cross-linked dextran gel[3] previously equilibrated with the extraction medium and maintained between 0 and 4°. The protein-containing fraction containing the enzyme was eluted with the extraction medium.

L-Glutamine–D-Fructose-6-phosphate Aminotransferase Assay—The activity of the enzyme was determined by the rate of formation of glucosamine. The reaction mixture contained, in 4 ml, 85 m*M* potassium phosphate buffer (pH 7.4), 1 m*M* ethylenedinitrilotetraacetic acid disodium salt, 0.1% mercaptoethanol, 1 ml of the eluted mucosal supernatant protein fraction (0.4–0.5 mg of protein/ml), and graded concentrations of glucose-6-phosphate, L-glutamine, sodium salicylate, and uridine-5′-diphospho-*N*-acetylglucosamine as indicated. The incubation was carried out in a 25-ml flask on a Dubnoff shaker at 37° for 45 min.

Enzyme activity was terminated by heating the samples in boiling water for 2 min. After cooling to room temperature, the mixtures were centrifuged at 35,000×*g* for 15 min, and the supernatant fraction was removed for glucosamine determination using the method described by Benson and Friedman (17). Protein concentrations were determined by the biuret method (18).

Inactivation Studies—The enzyme was preincubated with sodium salicylate in the absence of glucose-6-phosphate. The preincubation mixture contained, in 3 ml, 85 m*M* potassium phosphate buffer (pH 7.4), 1 m*M* ethylenedinitrilotetraacetic acid disodium salt, 0.1% mercaptoethanol, 20 m*M* glutamine, and 1 ml of the mucosal supernatant protein fraction. After the preincubation period, the samples were passed through columns (20 × 1.25 cm) of the dextran gel; the columns had been equilibrated with a solution containing 85 m*M* phosphate buffer (pH 7.4), 20 m*M* glutamine, 1 m*M* ethylenedinitrilotetraacetic acid disodium salt, and 0.1% mercaptoethanol. The procedure was carried out between 0 and 4°.

The protein fraction, characterized by a pink band, was collected and 1 ml was added to 2 ml of the incubation medium containing 85 m*M* phosphate buffer (pH 7.4), 20 m*M* glutamine, 1 m*M* ethylenedinitrilotetraacetic acid disodium salt, 0.1% mercaptoethanol, and fructose-6-phosphate to give a final concentration of 10 m*M*. This mixture was incubated for 30 min, and enzyme activity was determined by glucosamine assay.

Protection Studies—Fructose-6-phosphate, glutamine, and uridine-5′-diphospho-*N*-acetylglucosamine were studied as compounds that could afford some protective effect on salicylate-induced inactivation of the enzyme. The enzyme was preincubated with salicylate and one of these compounds. The preincubation mixture contained, in 3 ml, 85 m*M* phosphate buffer (pH 7.4), 1 m*M* ethylenedinitrilotetraacetic acid disodium salt, 0.1% mercaptoethanol, 1 ml of the mucosal supernatant protein fraction, and one of the following: 20 m*M* glutamine, 10 m*M* fructose-6-phosphate, or 3.5 × 10^{-6} *M* uridine-5′-diphospho-*N*-acetylglucosamine.

After preincubation, the samples were treated as described under *Inactivation Studies*. In experiments where preincubation in the absence of glutamine and/or fructose-6-phosphate was indicated, they were not present in the homogenizing medium and were added only to the final incubation mixture for glucosamine synthesis.

Interaction Studies—In studies involving the interaction of sodium salicylate and uridine-5′-diphospho-*N*-acetylglucosamine, the reaction mixture contained, in 4 ml, 10 m*M* glucose-6-phosphate, 20 m*M* glutamine, 85 m*M* potassium phosphate buffer (pH 7.4), 1 m*M* ethylenedinitrilotetraacetic acid disodium salt, 0.1% mercaptoethanol, 1 ml of the mucosal supernatant protein fraction, and varying concentrations of salicylate and uridine-5′-diphospho-*N*-acetylglucosamine. The incubation was carried on for 30 min, and the enzyme activity was determined by glucosamine assay.

No correction was applied for the fact that glucosamine-6-phosphate, on a molar basis, gave 85% of the color produced by glucosamine (19). Throughout this work, it was assumed that the isomerization of glucose-6-phosphate to fructose-6-phosphate was not rate limiting due to the presence of hexose isomerase in the enzyme extracts (16). In fact, Pogell and Gryder (20, 21) found that glucose-6-phosphate was a better substrate for the crude enzyme. Experiments using glucose-6-phosphate and fructose-6-phosphate gave essentially identical results. The production of glucosamine-6-phosphate was proportional to time for 60 min under experimental conditions, and the presence of inhibitors did not alter this linearity of product formation.

RESULTS

Figure 1 shows the effect of salicylate on L-glutamine–D-fruc-

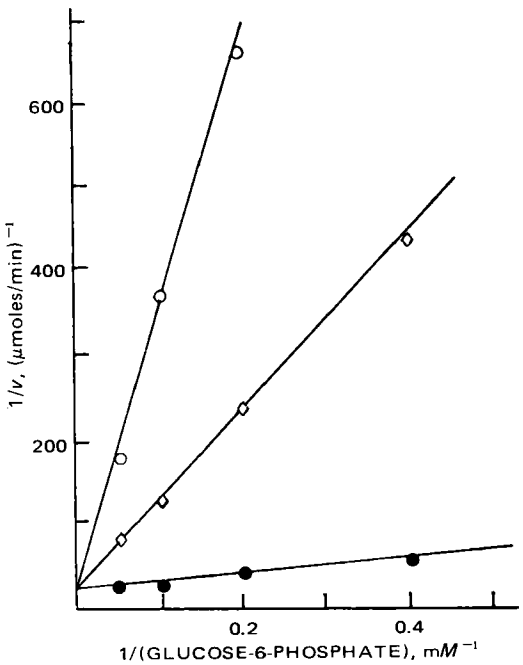

Figure 3—*Effect of uridine-5′-diphospho-*N*-acetylglucosamine on aminotransferase activity as a function of the concentration of glucose-6-phosphate. Key:* ●, *control;* ◊, *1* × 10^{-5} M *uridine-5′-diphospho-*N*-acetylglucosamine; and* ○, *5* × 10^{-5} M *uridine-5′-diphospho-*N*-acetylglucosamine.*

[5] Corning Glass Works, Corning, NY 14830
[6] Spinco, Class A, Beckman Instruments, Palo Alto, CA 94304

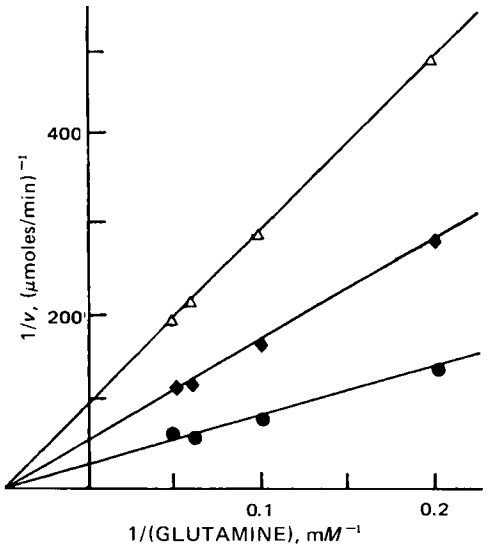

Figure 4—*Effect of uridine-5′-diphospho-*N*-acetylglucosamine on aminotransferase activity as a function of the concentration of glutamine. Key:* ●, *control;* ♦, *4* × 10^{-6} M *uridine-5′-diphospho-*N*-acetylglucosamine; and* △, *1* × 10^{-5} M *uridine-5′-diphospho-*N*-acetylglucosamine.*

tose-6-phosphate aminotransferase activity as a function of the glucose-6-phosphate concentration. The resultant double reciprocal plot indicated that salicylate acted as a competitive inhibitor with respect to glucose-6-phosphate. A double reciprocal plot of the aminotransferase activity as a function of the glutamine concentration is shown in Fig. 2. The results indicated that salicylate

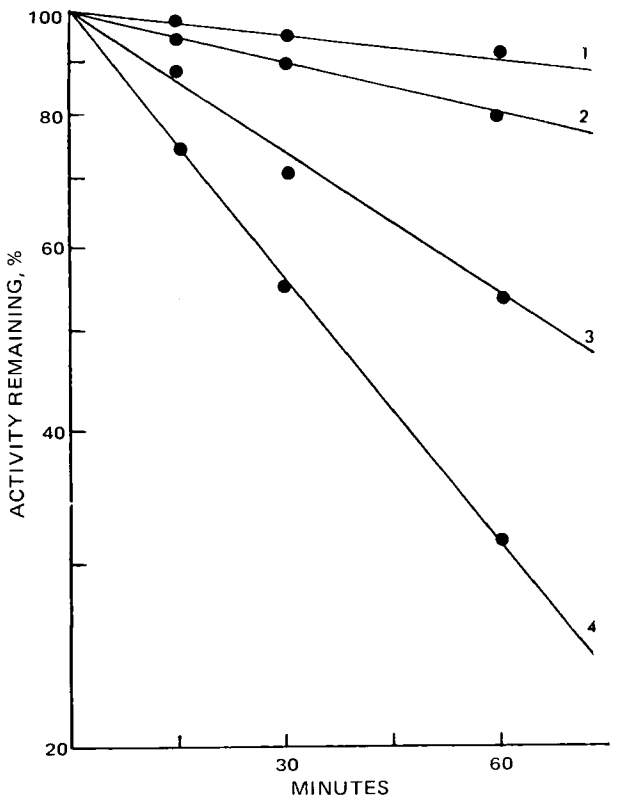

Figure 5—*Effect of varying concentrations of salicylate on the aminotransferase activity as a function of the preincubation time. Concentrations of salicylate are as follows: curve 1, 15 m*M; *curve 2, 25 m*M; *curve 3, 30 m*M; *and curve 4, 35 m*M. *Enzyme activity is expressed as percent of the control.*

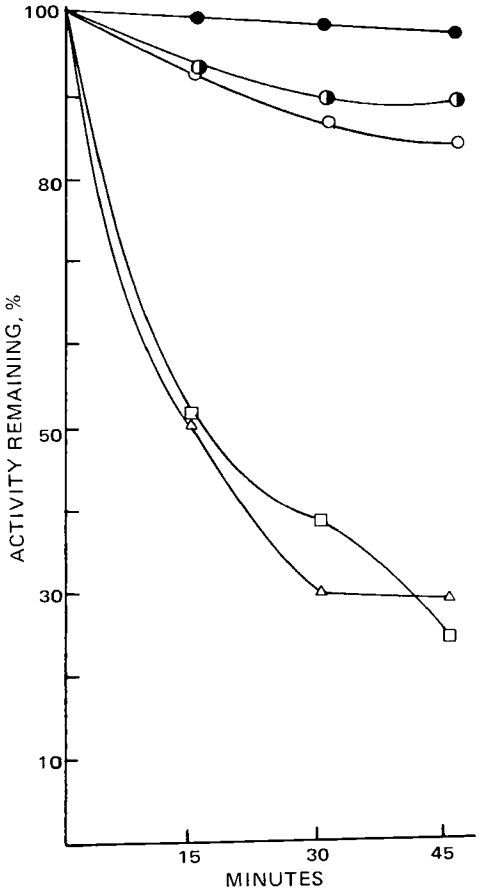

Figure 6—*Protective effect of* D-*fructose-6-phosphate,* L-*glutamine, and uridine-5′-diphospho-*N*-acetylglucosamine on salicylate-induced enzyme inactivation. Enzyme activity is expressed as percent of the control. Key:* ●, *control;* ◑, *40 m*M *salicylate and 3.5* × 10^{-6} M *uridine-5′-diphospho-*N*-acetylglucosamine;* ○, *40 m*M *salicylate and 10 m*M *fructose-6-phosphate;* □, *40 m*M *salicylate and 20 m*M *glutamine; and* △, *40 m*M *salicylate.*

acted as a noncompetitive inhibitor with respect to glutamine. Using uridine-5′-diphospho-*N*-acetylglucosamine as the inhibitor, similar double reciprocal plots depict this compound to be a competitive inhibitor with respect to glucose-6-phosphate (Fig. 3) and a noncompetitive inhibitor with respect to glutamine (Fig. 4).

It appears that both sodium salicylate and uridine-5′-diphospho-*N*-acetylglucosamine acted as competitive inhibitors of the enzyme with respect to glucose-6-phosphate and as noncompetitive inhibitors with respect to glutamine. The inhibitor dissociation constants (K_i) for competitive inhibition for salicylate and uridine-5′-diphospho-*N*-acetylglucosamine were 7.5 × 10^{-3} *M* and 1 × 10^{-6} *M*, respectively, indicating about a 700,000-fold difference in activity.

Salicylate was found to be capable of inactivating L-glutamine-D-fructose-6-phosphate aminotransferase at higher concentrations and longer exposure times. This inactivation was apparently irreversible since passage through columns of the dextran gel did not restore original enzyme activity. The results indicated that the rate of inactivation (Fig. 5) appears to follow first-order kinetics with respect to the enzyme and a much higher order with respect to the salicylate concentration. The results further indicate that under the conditions where reversible inhibition was studied (salicylate concentrations of 10–15 m*M* and 45-min exposure time), no significant inactivation of the enzyme occurred. Therefore, in Figs. 1 and 2 the observed inhibition resulted only from a reversible inhibition of the enzymatic reaction.

The two substrates, fructose-6-phosphate and glutamine, as well as the endogenous feedback inhibitor, uridine-5′-diphospho-*N*-acetylglucosamine, were studied as compounds that may afford protection for the enzyme from inactivation by sodium salicylate.

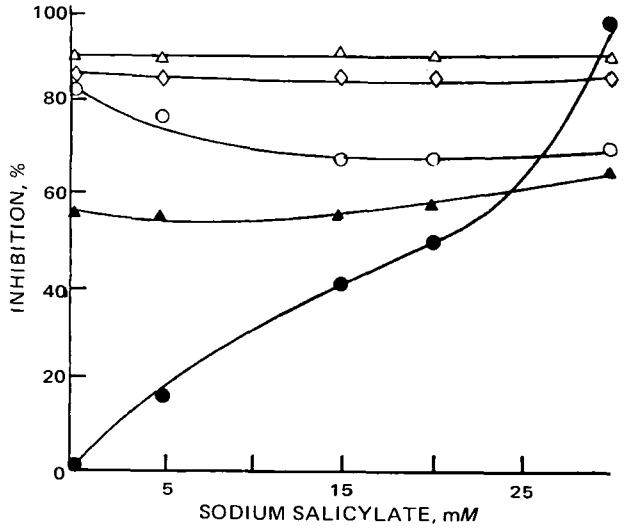

Figure 7—*Effect of sodium salicylate on the activity of* L-*glutamine*–D-*fructose-6-phosphate aminotransferase activity in the absence and presence of uridine-5′-diphospho-*N*-acetylglucosamine. Concentrations of uridine-5′-diphospho-*N*-acetylglucosamine are as follows:* ●, *none;* ▲, *3.5* × 10^{-6} M; ○, *7* × 10^{-6} M; ◇, *1.4* × 10^{-5} M; *and* △, *2.8* × 10^{-5} M.

The results (Fig. 6) indicated that fructose-6-phosphate at 1 × 10^{-2} *M* and uridine-5′-diphospho-*N*-acetylglucosamine at 3.5 × 10^{-6} *M* can protect the enzyme against inactivation by 40 m*M* of salicylate. Glutamine up to 20 m*M* showed no ability to protect the enzyme.

The addition of sodium salicylate to a system inhibited by uridine-5′-diphospho-*N*-acetylglucosamine modified the inhibition (Fig. 7). At a fixed concentration of uridine-5′-diphospho-*N*-acetylglucosamine (7 × 10^{-6} *M*), an increase in salicylate concentration gave rise to activation. Since both salicylate and uridine-5′-diphospho-*N*-acetylglucosamine inhibited the enzyme in a similar manner, such an activation effect caused by two inhibitors acting in concert may be interpreted as a competition for a common site.

DISCUSSION

The results indicated that sodium salicylate can inhibit L-glutamine–D-fructose-6-phosphate aminotransferase either by inhibiting the enzyme-catalyzed reaction by a reversible competitive inhibition with respect to fructose-6-phosphate or by an irreversible inactivation of the enzyme *via* a yet undefined mechanism. In the range of 10–15 m*M*, the inhibition observed was reversible; at concentrations of 30 m*M* or greater, irreversible inactivation was observed also.

Fructose-6-phosphate and uridine-5′-diphospho-*N*-acetylglucosamine were capable of protecting the enzyme against salicylate-induced inactivation as well as of modifying the reversible inhibition of the enzyme. The results further indicated that uridine-5′-diphospho-*N*-acetylglucosamine and sodium salicylate apparently bind to the same site on the enzyme. Based on the consideration of the chemical structures of the two compounds, one can conceive of salicylate binding to the same site occupied by the pyrimidine ring of uridine. Other uridine diphospho sugars such as uridine diphospho galactose and glucose have been reported to inhibit the enzyme at concentrations $\frac{1}{100}$ that of uridine-5′-diphospho-*N*-acetylglucosamine (13).

Kornfeld (13) was able partially to desensitize the enzyme to inhibition by uridine-5′-diphospho-*N*-acetylglucosamine, suggesting that the inhibitor site was distinct from the active site. This finding further suggests that the binding of salicylate and uridine-5′-diphospho-*N*-acetylglucosamine to the "inhibitor" site resulted in the increase in K_m of fructose-6-phosphate, which is characteristic of competitive inhibition (22). The enzyme has a reported molecular weight of 360,000–400,000 but does not exhibit the sigmoid dependence of velocity and modifier concentration, a property in common with the classical allosteric enzymes (23).

This study indicates that for salicylate to inhibit L-glutamine–D-fructose-6-phosphate aminotransferase *in vivo*, the *in vivo* concentration of the endogenous feedback inhibitor, uridine-5′-diphospho-*N*-acetylglucosamine, as well as of fructose-6-phosphate and salicylate would be important. Work by Hardingham and Phelps (10) and Winterburn and Phelps (14) indicated that the enzyme was inhibited in excess of 90% by the feedback inhibitor *in vivo* in the rat skin and rat liver. If this type of inhibition occurred in the general gastric mucosa, it would mean that the binding site for salicylate would be occupied by the feedback inhibitor *in vivo*. In addition, the presence of a feedback loop tends to correct for any deviation from the normal metabolic flux of uridine-5′-diphospho-*N*-acetylglucosamine. If salicylate should inhibit the formation of glucosamine-6-phosphate, it would reduce the level of uridine-5′-diphospho-*N*-acetylglucosamine which, in turn, would release more enzyme from inhibition, restoring the original metabolic flux of glucosamine-6-phosphate. These factors seem to suggest that L-glutamine–D-fructose-6-phosphate aminotransferase would not be a sensitive site for salicylate inhibition *in vivo*.

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Formulation of *De Novo* Substituent Constants in Correlation Analysis: Inhibition of Dihydrofolate Reductase by 2,4-Diamino-5-(3,4-dichlorophenyl)-6-substituted Pyrimidines

CORWIN HANSCH *, CARLO SILIPO *, and EDWARD E. STELLER

Abstract □ A correlation equation based solely on *de novo* constants was formulated for 105 2,4-diamino-5-(3,4-dichlorophenyl)-6-substituted pyrimidines acting as inhibitors of dihydrofolate reductase. An equation with seven indicator variables gives a correlation with a correlation coefficient of 0.903 and a standard deviation of 0.229. The technique used is a modification of the Free–Wilson approach. The results indicate that correlation equations with fewer parameters than the theoretical required to account for all molecular changes may often be encountered. It is also shown that cross-product terms can be used to establish the significance of cooperative substituent effects.

Keyphrases □ Correlation analysis—formulation of *de novo* substituent constants, inhibition of dihydrofolate reductase by 2,4-diamino-5-(3,4-dichlorophenyl)-6-substituted pyrimidines, equation with seven indicator variables □ Dihydrofolate reductase—inhibition by 2,4-diamino-5-(3,4-dichlorophenyl)-6-substituted pyrimidines, correlation analysis, formulation of *de novo* substituent constants, cross-product terms □ Pyrimidines, 2,4-diamino-5-(3,4-dichlorophenyl)-6-substituted—inhibitors of dihydrofolate reductase, correlation analysis, formulation of *de novo* substituent constants, cross-product terms

During the past decade, the use of computers and statistical techniques in the correlation of chemical structure with biological activity has been developing at a continually increasing rate. It is difficult now even for specialists in structure–activity work to keep up with new techniques. Medicinal chemists are beginning to work with tools developed in the fields of psychology and economics—areas long plagued by multivariable problems. Discriminate analysis (1), pattern recognition (2), factor analysis (3), cluster analysis (4), multidimensional scaling (5), and regression analysis (6, 7) are being applied to various structure–activity problems. These techniques will be coupled with the computerized manipulation of organic structural data to develop increasingly more powerful analytical tools (8) for drug design.

BACKGROUND

Regression analysis has been extensively employed in two ways. The most experience has been obtained in relating biological activity of sets of congeners to physicochemical properties using constants from model systems (6, 7). An alternative method, first used by Bruice *et al.* (9) and then more fully developed by Free and Wilson (10), derives *de novo* substituent constants directly from the biological activities of a set of congeners. The Free–Wilson approach was succinctly stated as (11):

$$A_n = \sum_p \sum_s a_{n,ps} + \mu \qquad \text{(Eq. 1)}$$

where A is a biological response, μ is the activity contributed by the constant portion (parent structure) of the congeneric set, and a is the contribution of activity by each substituent s located on position p of the parent structure. Equation 1 can be used to account for any structural feature in a set of congeners contributing to the total activity of each congener in a strictly additive fashion. The use of Free–Wilson-type constants, which can be regarded in a general sense as discrete variables [indicator variables (12)], can be combined with continuous variables such as π or σ to extend greatly one's ability to formulate correlation equations (13–17)[1].

Following the Free–Wilson approach strictly, one would need a discrete variable for every specific structural change present in a set of congeners. This requirement places one in a dilemma; one must either make a rather large number of derivatives to be certain of formulating meaningful constants or use relatively few derivatives to obtain rather unreliable constants. The authors have found that, in some cases, changes that might seem rather different from a chemist's point of view can be covered by a single indicator variable (14)[1]; this opens up new possibilities in the development of correlation equations.

It was found in the present study of the congeners listed in Table I that the continuous variables π, MR (molar refractivity), and σ were of no value in the formulation of the structure–activity relationship. However, a quite good correlation equation for 105 of the congeners of Table I could be formulated (Eq. 7)[2] using only seven indicator variables. The distinct structural features of the compounds of Table I are summarized in Table II.

By taking certain liberties with Eq. 1, matters can be simplified greatly by assuming that a given substituent Z or SO_2F always makes the same contribution to A, regardless of the length and geometry of the two bridges connecting the two phenyl rings to the parent structures. Even with this simplification, to follow Eq. 1 strictly, one would need to formulate 36 *de novo* constants, with many based on one or two data points. Previous experience has shown that enzyme inhibitors do not always show such high specificity or, to put it another way, test systems are not always sensitive enough to detect small biological responses.

After first discovering that the functions π and MR were of no value in correlating the data of Table I, the indicator variables of Table III were studied. The features of Table III were parameterized by assigning a value of 1 for their presence or of 0 for their absence. Some attempts were made to combine certain variables; I-18 was used to account for I-12 + I-13, I-21 for I-8 + I-9, and I-22 for I-10 + I-11. These combinations proved to be less significant than the single variables.

Three variables (I-19, I-20, and I-27) were studied to test the possibility that enzymes from different sources showed different activity. Only I-20 was necessary in the final analysis; that is, for practical purposes, enzyme from liver, L-1210/FR8, or L-1210/DF8 behaved in the same way.

The irreversible activating effect of the various inhibitors is also listed in Table I. Log $1/C$ values are for reversible inhibition. No obvious relationship is seen between the two types of inhibition.

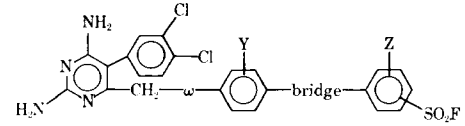
The degree of independence of the important variables is given in Table IV; collinearity is not a problem. The biological data are from Refs. 18–26.

RESULTS

A reasonable alternative in starting an analysis with such a large number of variables, where it is impossible to derive equations with all possible linear combinations of variables, is to first formu-

[1] C. Silipo and C. Hansch, to be published.
[2] Equations 2–12 are shown in Table V.

Table I—Inhibition Constants and Indicator Variables for the Inhibition of Dihydrofolate Reductase by 2,4-Diamino-5-(3,4-dichlorophenyl)-6-substituted Pyrimidines



Number	Substituents[a] Y	Substituents[a] —bridge— Z, SO2F	Log 1/C Obs.[b]	Log 1/C Calc.[c]	|Δ Log 1/C|	I-1	I-2	I-4	I-7	I-8	I-9	I-10	I-13	I-15	I-17	I-20	Inactivation[d], %	Enzyme Source[e]	Reference for Inhibition Data
1	2-Cl	4-NHCONHC6H4—4′-SO2F	5.48	5.941	0.46	0	1	0	1	1	0	0	0	1	0	0	58	2	20
2[f]	2,6-(CH3)2	4-NHCONHC6H4—3′-SO2F	5.48	6.161	0.68	0	0	0	1	1	0	0	0	0	0	0	17	2	19
3		4-NHCONHC6H4—4′-SO2F	5.68	5.941	0.26	0	0	0	0	1	0	0	0	1	0	0	82	4	20
4		4-NHCONHC6H3—3′-CH3,4′-SO2F	5.70	5.941	0.24	0	0	0	0	1	0	0	0	1	1	0	45	4	20
5	3-Cl	4-NHCONHC6H3—3′-Cl,4′-SO2F	5.74	5.941	0.20	0	0	0	1	1	0	0	0	1	1	0	82	2	20
6	2-Cl	4-NHCONHC6H4—3′-SO2F	5.85	6.161	0.31	0	1	0	1	1	0	0	0	0	0	0	98	2	19
7		4-NHCONHC6H4—3′-SO2F	6.00	6.161	0.16	0	0	0	0	1	0	0	0	0	0	0	50	4	19
8	3-Cl	4-NHCONHC6H4—4′-SO2F	6.01	5.941	0.07	0	0	0	1	1	0	0	0	1	0	0	90	2	20
9	3-Cl	4-NHCONHC6H3—3′CH3,4′-SO2F	6.01	5.941	0.07	0	0	0	1	1	0	0	0	1	1	0	87	2	20
10		4-NHCONHC6H3—3′-Cl,4′-SO2F	6.04	6.306	0.27	1	0	0	0	1	0	0	0	1	1	0	76	2	21
11		4-NHCONHC6H4—4′-SO2F	6.05	6.306	0.26	1	0	0	0	1	0	0	0	1	0	0	76	2	21
12		4-NHCOC6H4—3′-SO2F	6.09	6.390	0.30	0	0	0	0	0	1	0	0	0	0	0	94	2	18
13	3-Cl	4-NHCONHC6H3—2′-Cl,5′-SO2F	6.17	6.161	0.01	0	0	0	1	1	0	0	0	0	1	0	100	2	19
14		4-NHCONHC6H3—2′-Cl,5′-SO2F	6.25	6.161	0.09	0	0	0	0	1	0	0	0	0	1	0	50	4	19
15	2-Cl	4-NHCOC6H4—3′-SO2F	6.28	6.390	0.11	0	1	0	1	0	1	0	0	0	0	0	82	2	21
16	3-Cl	4-NHCONHC6H3—3′-OCH3,4′-SO2F	6.28	5.941	0.34	0	0	0	1	1	0	0	0	1	1	0	63	2	20
17	2-Cl	4-NHCOC6H3—4′-CH3,3′-SO2F	6.30	6.390	0.09	0	1	0	1	0	1	0	0	0	1	0	51	2	21
18		4-NHCONHC6H3—2′-OCH3,5′-SO2F	6.32	6.161	0.16	0	0	0	0	1	0	0	0	0	1	0	50	4	19
19		4-NHCONHC6H3—4′-OC2H5,3′-SO2F	6.35	6.161	0.19	0	0	0	0	1	0	0	0	0	1	0	55	4	19
20[f]		4-CH2NHCONHC6H3—2′-Cl,5′-SO2F	6.37	7.174	0.80	0	0	0	0	0	0	1	0	0	1	0	100	2	22
21		4-CH2NHCONHC6H3—3′-CH3,4′-SO2F	6.37	6.954	0.58	0	0	0	0	0	0	1	0	1	1	0	71	2	22
22[f]		4-CH2NHCOC6H4—3′-SO2F	6.43	7.174	0.74	0	0	0	0	0	0	0	0	0	0	0	100	2	22
23[f]		4-CH2NHCOC6H3—4′-CH3,3′-SO2F	6.46	7.174	0.71	0	0	0	0	0	0	0	0	0	1	0	70	4	22
24	3-Cl	4-NHCONHC6H4—3′-SO2F	6.47	6.161	0.31	0	0	0	1	1	0	0	0	0	0	0	100	2	19
25[f]		3-CH2NHCONHC6H3—2′-Cl,5′-SO2F	6.48	7.174	0.69	0	0	0	0	0	0	1	0	0	1	0	93	1	25
26		4-CH2NHCOC6H3—2′-CH3,5′-SO2F	6.49	7.174	0.68	0	0	0	0	0	0	0	0	0	1	0	84	2	22
27	3-CH3	4-NHCONHC6H4—4′-SO2F	6.51	6.615	0.11	0	0	1	1	1	0	0	0	1	0	0	50	4	20
28	2-Cl	4-NHCOC6H4—4′-SO2F	6.51	6.684	0.17	0	1	0	1	0	1	0	0	1	0	1	39	3	21
29	3-CH3	4-NHCOC6H4—3′-SO2F	6.55	6.390	0.16	0	0	1	1	0	1	0	0	0	0	0	94	2	21
30	3-Cl	4-NHCONHC6H3—2′-OCH3,5′-SO2F	6.55	6.161	0.39	0	0	0	1	1	0	0	0	0	1	0	55	2	19
31	3-Cl	4-NHCONHC6H3—4′-OCH3,3′-SO2F	6.68	6.161	0.52	0	0	0	1	1	0	0	0	0	1	0	95	2	19
32		4-NHCOC6H3—4′-SO2F	6.74	6.684	0.06	0	0	0	0	0	1	0	0	1	0	1	33	3	21
33		4-NHCONHC6H4—4′-OCH3,3′-SO2F	6.77	6.674	0.10	0	0	0	0	1	0	0	0	0	1	1	80	3	19
34		4-CH2NHCONHC6H4—4′-SO2F	6.77	6.954	0.18	0	0	0	0	0	0	1	0	1	0	0	50	4	22
35		4-(CH2)2NHCOC6H4—4′-SO2F	6.80	6.954	0.15	0	0	0	0	0	0	0	0	1	0	0	76	1	25
36	3-CH3	4-CH2NHCOC6H4—4′-SO2F	6.80	6.954	0.15	0	0	1	1	0	0	0	0	1	0	0	98	1	24
37	2-Cl	4-NHCOC6H3—2′-Cl,5′-SO2F	6.80	6.904	0.10	0	1	0	1	0	1	0	0	0	1	1	53	3	21
38	3-CH3	4-NHCONHC6H3—3′-CH3,4′-SO2F	6.80	6.615	0.19	0	0	1	1	1	0	0	0	1	1	0	84	4	20
39		4-(CH2)2NHCONHC6H4—4′-SO2F	6.82	6.954	0.13	0	0	0	0	0	0	1	0	1	0	0	73	1	25
40	2-Cl	4-CH2NHCONHC6H3—4′-CH3,3′-SO2F	6.82	7.174	0.35	0	1	0	1	0	0	1	0	0	1	0	62	1	24
41		4-CH2NHSO2C6H4—4′-SO2F	6.82	6.954	0.13	0	0	0	0	0	0	0	0	1	0	0	85	2	22

(continued)

Table I—*Continued*

Number	Substituents[a] Y	Substituents[a] —bridge— (Z, SO_2F)	Log $\frac{1}{C}$ Obs.[b]	Log $\frac{1}{C}$ Calc.[c]	$\lvert\Delta \text{ Log } \frac{1}{C}\rvert$	*I*-1	*I*-2	*I*-4	*I*-7	*I*-8	*I*-9	*I*-10	*I*-13	*I*-15	*I*-17	*I*-20	Inactivation[d], %	Enzyme Source[e]	Reference for Inhibition Data
42		4-$CH_2NHCONHC_6H_4$—3′-SO_2F	6.82	7.174	0.35	0	0	0	0	0	0	1	0	0	0	0	100	2	22
43		4-$NHCOC_6H_3$—4′-CH_3,3′-SO_2F	6.82	6.904	0.08	0	0	0	0	0	1	0	0	0	1	1	35	3	21
44		4-$CH_2NHCOC_6H_3$—4′-$CH(CH_3)_2$,3′-SO_2F	6.85	7.174	0.32	0	0	0	0	0	0	0	0	0	1	0	85	2	22
45	3-CH_3	4-$NHCONHC_6H_3$—2′-OCH_3,5′-SO_2F	6.85	6.835	0.02	0	0	1	1	1	0	0	0	0	1	0	100	4	19
46	2-Cl	4-$CH_2NHSO_2C_6H_4$—4′-SO_2F	6.89	6.954	0.06	0	1	0	1	0	0	0	0	1	0	0	12	1	24
47	3-CH_3	4-$NHCONHC_6H_3$—2′-Cl,5′-SO_2F	6.89	6.835	0.06	0	0	1	1	1	0	0	0	0	1	0	100	4	19
48	2-Cl	4-$CH_2NHCONHC_6H_3$—3′-CH_3,4′-SO_2F	6.92	6.954	0.03	0	1	0	1	0	0	1	0	1	1	0	78	1	24
49	2-Cl	4-$CH_2NHCOC_6H_3$—4′-CH_3,3′-SO_2F	6.92	7.174	0.25	0	1	0	1	0	0	0	0	0	1	0	77	1	24
50	3-CH_3	4-$NHCOC_6H_4$—4′-SO_2F	6.92	6.684	0.24	0	0	1	1	0	1	0	0	1	0	1	45	3	21
51		3-$CH_2NHSO_2C_6H_4$—3′-SO_2F	6.96	7.174	0.21	0	0	0	0	0	0	0	0	0	0	0	25	1	25
52		4-$(CH_2)_2NHCONHC_6H_4$—3′-SO_2F	6.96	7.174	0.21	0	0	0	0	0	0	1	0	0	0	0	48	1	25
53		4-$(CH_2)_2NHSO_2C_6H_4$—4′-SO_2F	6.96	6.954	0.01	0	0	0	0	0	0	0	0	1	0	0	60	1	25
54	3-CH_3	4-$CH_2NHSO_2C_6H_4$—4′-SO_2F	6.96	6.954	0.01	0	0	1	1	0	0	0	0	1	0	0	94	1	24
55		4-$NHCONHC_6H_3$—2′-Cl,5′-SO_2F	6.96	7.039	0.08	1	0	0	0	1	0	0	0	0	1	1	82	3	21
56		4-$CH_2NHCONHC_6H_3$—4′-CH_3,3′-SO_2F	7.00	7.174	0.17	0	0	0	0	0	0	1	0	0	1	0	81	2	22
57	3-CH_3	4-$NHCOC_6H_3$—2′-Cl,5′-SO_2F	7.00	6.904	0.10	0	0	1	1	0	1	0	0	0	1	1	91	3	21
58	2-OCH_3	4-$CH_2NHCONHC_6H_4$—4′-SO_2F	7.00	6.954	0.05	0	0	0	1	0	0	1	0	1	0	0	77	1	24
59		4-$CH_2NHCOC_6H_3$—2′-Cl,5′-SO_2F	7.02	7.174	0.15	0	0	0	0	0	0	0	0	0	1	0	90	2	22
60	3-CH_3	4-$CH_2NHCOC_6H_4$—3′-SO_2F	7.04	7.174	0.13	0	0	1	1	0	0	0	0	0	0	0	100	1	25
61	2-Cl	4-$CH_2NHCOC_6H_3$—2′-Cl,5′-SO_2F	7.05	7.174	0.12	0	1	0	1	0	0	0	0	0	1	0	65	1	24
62	2-OCH_3	4-$CH_2NHSO_2C_6H_4$—4′-SO_2F	7.07	6.954	0.12	0	0	0	1	0	0	0	0	1	0	0	37	1	24
63	3-CH_3	4-$CH_2NHCONHC_6H_4$—4′-SO_2F	7.07	6.954	0.12	0	0	1	1	0	0	1	0	1	0	0	68	1	24
64		3-$CH_2NHCONHC_6H_4$—4′-SO_2F	7.07	6.954	0.12	0	0	0	0	0	0	1	0	1	0	0	95	2	25
65[f]		4-$NHCOC_6H_3$—2′-Cl,5′-SO_2F	7.07	6.390	0.68	0	0	0	0	0	1	0	0	0	1	0	58	2	21
66		3-$CH_2NHCONHC_6H_4$—4′-SO_2F	7.08	7.319	0.24	1	0	0	0	0	0	1	0	1	0	0	93	1	25
67	2-OCH_3	4-$CH_2NHCONHC_6H_4$—3′-SO_2F	7.12	7.174	0.05	0	0	0	1	0	0	1	0	0	0	0	83	1	24
68		3-$CH_2NHCONHC_6H_4$—3′-SO_2F	7.17	7.174	0.00	0	0	0	0	0	0	1	0	0	0	0	93	2	25
69		3-$CH_2NHCOC_6H_4$—4′-SO_2F	7.18	6.954	0.23	0	0	0	0	0	0	0	0	1	0	0	85	2	25
70	3-CH_3	4-$NHCONHC_6H_4$—3′-SO_2F	7.20	7.349	0.15	0	0	1	1	1	0	0	0	0	0	1	100	3	19
71	2-Cl	4-$CH_2NHCONHC_6H_4$—3′-SO_2F	7.20	7.174	0.03	0	1	0	1	0	0	1	0	0	0	0	88	1	24
72	2-Cl	4-$CH_2NHCONHC_6H_4$—4′-SO_2F	7.21	6.954	0.26	0	1	0	1	0	0	1	0	1	0	0	46	1	24
73	3-CH_3	4-$CH_2NHSO_2C_6H_4$—3′-SO_2F	7.22	7.174	0.05	0	0	1	1	0	0	0	0	0	0	0	78	1	24
74		3-$CH_2NHSO_2C_6H_4$—4′-SO_2F	7.22	7.319	0.10	1	0	0	0	0	0	0	0	1	0	0	69	1	25
75		4-$CH_2NHSO_2C_6H_4$—3′-SO_2F	7.22	7.174	0.05	0	0	0	0	0	0	0	0	0	0	0	51	2	22
76		3-$CH_2NHCOC_6H_4$—3′-SO_2F	7.24	7.174	0.07	0	0	0	0	0	0	0	0	0	0	0	46	1	25
77		4-$NHCOC_6H_4$—3′-SO_2F	7.24	7.269	0.03	1	0	0	0	0	1	0	0	0	0	1	56	3	21
78		4-$(CH_2)_6NHCOC_6H_4$—3′-SO_2F	7.25	7.174	0.08	0	0	0	0	0	0	0	0	0	0	0	55	1	25
79		3-$CH_2NHSO_2C_6H_4$—4′-SO_2F	7.25	6.954	0.30	0	0	0	0	0	0	0	0	1	0	0	17	1	25
80		3-$CH_2NHSO_2C_6H_4$—3′-SO_2F	7.25	7.539	0.29	1	0	0	0	0	0	0	0	0	0	0	73	1	25
81		4-$CH_2NHCOC_6H_4$—4′-SO_2F	7.26	7.468	0.21	0	0	0	0	0	0	0	0	1	0	1	82	3	22
82		3-$CH_2NHCONHC_6H_3$—4′-CH_3,3′-SO_2F	7.27	7.174	0.10	0	0	0	0	0	0	1	0	0	1	0	93	2	25
83	3-CH_3	4-$NHSO_2C_6H_4$—4′-SO_2F	7.28	7.373	0.09	0	0	1	1	0	0	0	1	1	0	0	46	1	25
84	2-OCH_3	4-$CH_2NHCOC_6H_4$—3′-SO_2F	7.28	7.174	0.11	0	0	0	1	0	0	0	0	0	0	0	64	1	24
85		4-$(CH_2)_2NHSO_2C_6H_4$—3′-SO_2F	7.31	7.174	0.14	0	0	0	0	0	0	0	0	0	0	0	23	1	25
86		4-$NHSO_2C_6H_4$—4′-SO_2F	7.31	7.373	0.06	0	0	0	0	0	0	0	1	1	0	0	93	2	21
87	2-Cl	4-$CH_2NHCOC_6H_4$—3′-SO_2F	7.32	7.174	0.15	0	1	0	1	0	0	0	0	0	0	0	33	1	24
88	2-Cl	4-$NHSO_2C_6H_4$—4′-SO_2F	7.33	7.373	0.04	0	1	0	1	0	0	0	1	1	0	0	57	1	25

89	2-OCH_3	4-$CH_2NHCOC_6H_4$—4'-SO_2F	7.33	6.954	0.38	0	0	0	1	0	0	0	1	0	0	68	1	24
90		4-$CH_2NHCOC_6H_3$—4'-Cl,3'-SO_2F	7.33	7.174	0.16	0	0	0	0	0	0	0	0	1	0	100	2	22
91	2-Cl	4-$CH_2NHCOC_6H_4$—4'-SO_2F	7.34	6.954	0.39	0	1	0	0	0	0	0	1	0	0	56	1	24
92		4-$(CH_2)_2C_6H_4$—4'-SO_2F	7.34	7.319	0.02	1	0	0	0	0	0	0	1	0	0	97	2	26
93		3-$CH_2NHCOC_6H_4$—4'-SO_2F	7.38	7.319	0.06	1	0	0	0	0	0	0	1	0	0	82	1	25
94	2-Cl	4-$CH_2NHSO_2C_6H_4$—3'-SO_2F	7.39	7.174	0.22	0	1	0	0	0	0	0	1	0	0	20	1	24
95		4-$NHCOC_6H_4$—4'-SO_2F	7.39	7.049	0.34	1	0	0	1	0	0	0	1	0	1	49	3	21
96		4-SO_2F	7.40	7.539	0.14	1	0	0	0	0	0	0	0	0	0	0	1	26
97	2-OCH_3	4-$CH_2NHSO_2C_6H_4$—3'-SO_2F	7.41	7.174	0.24	0	0	0	1	0	0	0	0	0	0	37	1	24
98	2-Cl	4-$CH_2NHCONHC_6H_3$—2'-Cl,5'-SO_2F	7.44	7.174	0.27	0	1	0	0	1	0	0	0	1	0	60	1	24
99		4-$(CH_2)_2C_6H_4$—3'-SO_2F	7.44	7.539	0.10	1	0	0	0	0	0	0	0	0	0	92	2	26
100	3-CH_3	4-$CH_2NHCONHC_6H_4$—3'-SO_2F	7.52	7.174	0.35	0	0	1	0	0	0	0	0	0	0	100	2	24
101		4-$CH_2NHCONHC_6H_4$—4'-SO_2F	7.52	7.319	0.20	1	0	0	0	1	0	0	1	0	0	80	2	23
102		4-$CH_2NHCONHC_6H_4$—3'-SO_2F	7.52	7.539	0.02	1	0	0	0	1	0	0	0	0	0	75	2	23
103	2-Cl	4-$NHSO_2C_6H_4$—3'-SO_2F	7.54	7.593	0.05	0	1	0	0	0	1	0	0	0	0	23	1	25
104		4-$CH_2NHCOC_6H_3$—4'-CH_3,3'-SO_2F	7.57	7.539	0.03	1	0	0	0	0	0	0	0	1	0	94	2	23
105		4-$CH_2NHCOC_6H_4$—3'-SO_2F	7.60	7.539	0.06	1	0	0	0	0	0	0	0	0	0	100	2	23
106		3-$(CH_2)_2C_6H_4$—4'-SO_2F	7.62	7.319	0.30	1	0	0	0	0	0	0	1	0	0	82	2	26
107		4-$CH_2NHCOC_6H_4$—4'-SO_2F	7.66	7.319	0.34	1	0	0	0	0	0	0	1	0	0	68	2	23
108		4-SO_2F	7.68	7.174	0.51	0	0	0	0	0	0	0	0	0	0	43	2	26
109	3-CH_3	4-$NHSO_2C_6H_4$—3'-SO_2F	7.70	7.593	0.11	0	0	1	1	0	0	0	1	0	0	97	1	25
110		4-$CH_2NHCOC_6H_3$—2'-Cl,5'-SO_2F	7.70	7.539	0.16	1	0	0	0	0	0	0	0	1	0	80	2	23
111		4-$NHSO_2C_6H_4$—3'-SO_2F	7.74	7.593	0.15	0	0	0	0	0	0	1	0	0	0	42	3	21

[a] The function w is either CH_2 or O; this is parameterized by $w - 1 = 1$ for $w = CH_2$. [b] From Refs. 18–26. [c] Calculated from Eq. 8. [d] Percent irreversible inactivation from Refs. 18–26. [e] 1 = enzyme from liver, 2 = enzyme L-1210/DF8 leukemia, 3 = L-1210/0 leukemia, and 4 = L-1210/FR8 leukemia. [f] These points not used in deriving Eqs. 2–12.

late an equation with the largest number of terms possible. Often all variables cannot be considered because high collinearity of certain vectors yields singular matrixes which cannot be solved for the regression coefficients. In the present instance, 22 variables of Table III (*I*-3, *I*-6, *I*-16, *I*-18, *I*-21, *I*-22, and cross-product variables not included) gave a correlation equation with $s = 0.240$ and $r = 0.910$. Adding four cross-product terms (*I*-4·*I*-8, *I*-4·*I*-17, *I*-8·*I*-17, and *I*-4·*I*-8·*I*-17) gave a 26-variable equation with $s = 0.212$ and $r = 0.934$. This equation constitutes a kind of upper limit, which one would hope to approach with fewer terms. Equations 8 and 11 come close to this type of approach.

Such brute force methods cannot be used with cross-product terms. The enormous number of such terms is given by the expression $2^n - (n + 1)$, where n is the number of primary terms. Hence, for only 10 variables, there would be 1013 possible cross-product terms! The possibilities are truly enormous for interactions of a complex inhibitor with a macromolecule.

To search for patterns of cooperative activity among the different structural units, the authors explored the 15 cross-product terms of Table III. These possibilities were selected simply by study of the residuals of the best equations. It seems unlikely that a mechanical machine approach can be developed to uncover the significant interactions among such a large number of variables. The human mind is the most efficient pattern recognizer at this level of complexity.

After considering thousands of regression equations, the following variables were found to be the most significant: *I*-1, *I*-2, *I*-7, *I*-8, *I*-9, *I*-10, *I*-13, *I*-15, *I*-20, *I*-4·*I*-8, and *I*-8·*I*-17. Equations with all possible linear combinations of these variables were derived (2047). The "best" equation (that with the lowest standard deviation) in each class is listed in Table V. The terms appear quite independent and fall into place in regular order. All of the terms in the equations of Table V are significant as judged by the *F* test, except Eq. 11 ($F_{1,60\ \alpha 0.001} = 12.0$; $F_{1,60\ \alpha .05} = 4.1$; $F_{1,60\ \alpha .1} = 2.8$). There is little reduction in the variance in the equations above Eq. 8:

$$\log 1/C = 0.365(\pm 0.12)I\text{-}1 - 1.013(\pm 0.12)I\text{-}8 - 0.784(\pm 0.19)I\text{-}9 + 0.419(\pm 0.20)I\text{-}13 - 0.220(\pm 0.09)I\text{-}15 + 0.513(\pm 0.18)I\text{-}20 + 0.674(\pm 0.23)I\text{-}4\cdot I\text{-}8 + 7.174(\pm 0.07)$$

n	r	s	
105	0.903	0.229	(Eq. 8)

The *F* statistic also drops sharply at this point. Equation 8 would seem to be the best point from which to make projections.

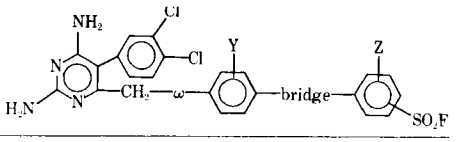
It is interesting that the cross-product term *I*-4·*I*-8 plays a significant role in Eq. 8. The positive coefficient with this term means that the cooperative activity of a 3-CH_3 and a 4-NHCONH— bridge adds significantly to the activity. This is not a "pure" cross-product term, since *I*-4 does not occur in Eq. 8 except in the product form. The 3-CH_3 appears to make a significant contribution to activity only when the 4-NHCONH— bridge is present. When the 3-CH_3 is present with other bridges, the data points are well fit (except Compound 100), although there are no variables in Eq. 8 to account for this function. If *I*-4 is used alone, the correlation is poorer than when *I*-4·*I*-8 is used; use of this cross-product with *I*-4 gives an equation with a low coefficient with *I*-4. The 3-Cl substituent does not behave in this fashion.

In Table V, it is seen that the two most significant variables reflect structural changes that produce lower activity (*I*-8 and *I*-9). A most interesting point is Compound 96 (an alternative parent compound), which is the only congener showing only reversible activity; this highlights the unusual specificity of the SO_2F function.

Six points in Table I were not used in the regression analysis. Four of these (Compounds 20, 22, 23, and 25) contain the bridge —CH_2NHCO— or —$CH_2NHCONH$—; however, the use of *I*-10, *I*-11, and *I*-22 and the study of many cross-products failed to improve the correlation of these compounds since other congeners containing these functions are well fit. It seems strange that these four are so poorly fit and one wonders if it could be due to experimental error. Data point 2, which is also poorly fit, is unique in that it contains di-*ortho*-substitution.

If Compound 108 is taken as the parent structure, it can be seen from Table I that only three congeners with slightly greater activi-

Table II—Structural Features of Congeners of Table I



w	Number of Occurrences	Y	Number of Occurrences	Bridge	Number of Occurrences	Z	Number of Occurrences	SO_2F	Number of Occurrences
O	92	2-Cl	20	4-NHCONH—	27	2′-Cl	13	5′-	17
CH_2	19	3-Cl	8	4-NHCO—	13	3′-Cl	2	4′-	45
		2-CH_3	1	4-CH_2NHCONH—	15	2′-CH_3	1	3′-	47
		3-CH_3	16	4-CH_2NHCO—	20	3′-CH_3	5		
		6-CH_3	1	3-CH_2NHCONH—	5	4′-CH_3	7		
		2-OCH_3	6	3-CH_2NHCO—	3	2′-OCH_3	3		
		4-SO_2F	2	4-CH_2CH_2NHCO—	2	3′-OCH_3	1		
				4-CH_2CH_2NHCONH—	2	4′-OCH_3	2		
				4-$NHSO_2$—	6	4′-OCH_2CH_3	1		
				4-CH_2NHSO_2—	7	4′-$CH(CH_3)_2$	1		
				4-$CH_2CH_2NHSO_2$—	2				
				3-CH_2NHSO_2—	4				
				3-CH_2CH_2—	1				
				4-CH_2CH_2—	2				

ty were discovered and these differences are probably not significant. The conclusion from the analysis is that a significantly more potent inhibitor is not likely to be found by working with the set of structural features of Table II. New departures are needed for further advances with this type of inhibitor.

Table III—Indicator Variables (=1) Studied in the Formulation of Eqs. 2–12

Indicator Variable	Substituent Studied
I-1	$w = CH_2$
I-2	Y = 2-Cl
I-3	Y = 3-Cl
I-4	Y = 3-CH_3
I-5	Only substituent 3-position
I-6	Only substituent 4-position
I-7	Substituent in 4 plus other substituent in 2, 3, or 6
I-8	Bridge 4-NHCONH—
I-9	Bridge 4-NHCO—
I-10	Bridge 4-CH_2NHCONH—, 4-CH_2CH_2NHCONH—, or 3-CH_2NHCONH—
I-11	Bridge 4-CH_2NHCO—, 4-CH_2CH_2NHCO—, or 3-CH_2NHCO—
I-12	Bridge 4-CH_2NHSO_2—, 4-$CH_2CH_2NHSO_2$—, or 3-CH_2NHSO_2—
I-13	Bridge 4-$NHSO_2$—
I-14	Bridge 4-CH_2CH_2— or 3-CH_2CH_2—
I-15	4′-SO_2F
I-16	3′-SO_2F
I-17	SO_2F plus other substituent on the last ring
I-18	*I*-12 + *I*-13
I-19	L-1210/FR8 enzyme
I-20	L-1210/0 enzyme
I-21	*I*-8 + *I*-9
I-22	*I*-10 + *I*-11
I-23	Z = 2′-Cl
I-24	Z = 3′-CH_3
I-25	Z = 4′-CH_3
I-26	3′- or 5′-SO_2F
I-27	Liver enzyme
I-28	Z = 2′-OCH_3

Cross-Product Terms Studied

I-4·*I*-21	*I*-15·*I*-22
I-4·*I*-8	*I*-16·*I*-10
I-8·*I*-17	*I*-16·*I*-11
I-15·*I*-5	*I*-16·*I*-22
I-15·*I*-6	*I*-6·*I*-22·*I*-16
I-15·*I*-10	*I*-6·*I*-22·*I*-17
I-15·*I*-11	*I*-4·*I*-8·*I*-17
I-15·*I*-21	

DISCUSSION

In designing the inhibitors considered in this study, Baker and coworkers (18–26) drew on their experience gained from the study of a large number of heterocyclic inhibitors of dihydrofolate reductase. The 3,4-dichlorophenyl moiety in the 5-position probably provides close to optimal lipophilic interaction with the lipophilic pocket in this area of the enzymes (13)[1]; however, this point is worthy of more study. Substituents in the 6-position of the pyrimidine ring do not appear to interact with lipophilic space (no correlation with π), nor does MR account for substituent effects in this part of enzymic space. Substituents Y and Z have little or no effect on activity. It seems that most of the substituents of the 6-position must project into a very loosely structured part of the enzyme or into the surrounding aqueous phase.

The more flexible bridges, those containing a CH_2 unit in addition to an amide moiety, have little or no effect on activity either way. These more flexible bridges allow maximum freedom (from negative steric effects) to the second phenyl ring and its substituents. Two of the rigid bridges, —NHCONH— and —NHCO—, have very deleterious effects on activity; this result seems to be connected to the positioning of the second phenyl ring. Strangely, the 4-$NHSO_2$— bridge (*I*-13) does make a small contribution to activity. Congeners with this function are, on the average, 10 times as active as those having the 4-NHCO— bridge. What property of $NHSO_2$ is responsible for this activity is not obvious. Similar hydrophobicity is shown by $\pi_{NHSO_2C_6H_5} = 0.45$ and $\pi_{NHCOC_6H_5} = 0.49$. The electronic effect of SO_2— on the phenyl ring can be ruled out since CH_2NHSO_2 does not show special activity. This leaves one with the conclusion that the geometry of the two groups may be the critical factor.

One must bear in mind that structural features not parameterized by specific variables do not make significant contributions to the activity μ (Eq. 1) of the parent structure. One value of the present analysis is that it clearly indicates, in an objective way, structural features that are not worth further study. While these features are often carried in the mind of the active investigator in a qualitative sense, they are quite difficult for a person new to the work to discover. Correlation equations quickly bring the salient features of a very complex set of data into focus.

The value of the present analysis does not reside in its ability to suggest new congeners for synthesis; rather, it demonstrates that a highly complex set of data, whose structural properties produce a set of discontinuous perturbations in a macromolecule, can be brought into coherent order with a relatively small number of variables. This constitutes a valuable modification of the Free–Wilson approach.

Moreover, the results of this analysis suggest that the Free–Wilson approach can be further extended by the use of cross-product terms. This use of cross-product terms may prove to be of considerable value in establishing the significance of special patterns or

Table IV—Squared Correlation Matrix for Indicator Parameters Used in the Correlation Study[a]

	I-1	*I*-2	*I*-4	*I*-7	*I*-8	*I*-9	*I*-10	*I*-13	*I*-15	*I*-17	*I*-20
I-1	1.00	0.05	0.04	0.19	0.01	0.00	0.00	0.01	0.01	0.01	0.00
I-2		1.00	0.04	0.25	0.02	0.02	0.01	0.01	0.00	0.00	0.00
I-4			1.00	0.21	0.01	0.01	0.01	0.02	0.00	0.00	0.01
I-7				1.00	0.02	0.01	0.00	0.01	0.00	0.00	0.00
I-8					1.00	0.04	0.08	0.02	0.00	0.14	0.00
I-9						1.00	0.03	0.01	0.00	0.00	0.39
I-10							1.00	0.02	0.00	0.07	0.00
I-13								1.00	0.00	0.03	0.01
I-15									1.00	0.07	0.00
I-17										1.00	0.01
I-20											1.00

[a] Numbers show the percent correlation (r^2) between each variable.

Table V—Equations Correlating Log 1/*C* with 11 Most Significant Variables[a]

Equation Number	Intercept	*I*-8	*I*-9	*I*-20	*I*-4·*I*-8	*I*-1	*I*-15	*I*-13	*I*-8·*I*-17	*I*-7	*I*-2	*I*-10	s^b	r^c	$F_{1,x}{}^d$
2	7.13	−0.82											0.377	0.683	90
3	7.20	−0.89	−0.48										0.348	0.742	19
4	7.19	−0.96	−0.86	0.59									0.317	0.795	22
5	7.19	−1.08	−0.84	0.55	0.63								0.293	0.830	18
6	7.13	−1.05	−0.79	0.50	0.68	0.31							0.269	0.860	20
7	7.21	−1.05	−0.82	0.51	0.67	0.33	−0.21						0.248	0.884	18
8	7.17	−1.01	−0.78	0.51	0.67	0.37	−0.22	0.42					0.229	0.903	18
9	7.17	−1.16	−0.78	0.51	0.69	0.36	−0.21	0.42	0.23				0.223	0.909	6.2
10	7.11	−1.18	−0.82	0.54	0.63	0.42	−0.22	0.40	0.24	0.18			0.218	0.915	5.7
11	7.12	−1.18	−0.81	0.53	0.58	0.42	−0.22	0.40	0.21	0.18	−0.15		0.213	0.919	5.2
12	7.14	−1.20	−0.83	0.53	0.58	0.42	−0.22	0.38	0.21	0.18	−0.15	−0.06	0.213	0.920	1.0

[a] Figures in each column are the regression coefficients for the indicated variable. [b] Standard deviation. [c] Correlation coefficient. [d] *F* statistic for addition of each successive term.

constellations of atoms not normally parameterized in structure–activity relationships.

The importance of correlation equations can only be fully appreciated when X-ray crystallographic studies of inhibitor binding with the enzyme are undertaken. The terms of the correlation equations should be of great help in interpreting the interactions of micro- and macromolecules.

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Dissolution Profile in Relation to Initial Particle Distribution

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Abstract □ A general equation was derived describing the complete and exact dissolution profile of powders under sink conditions. It is applicable to powders having any initial particle-size distribution, with particles dissolving according to any explicit equation. It was applied to develop an equation for the dissolution of log-normal powders that is more generally applicable than previous approaches. The effect of change in initial particle-size distribution parameters on the dissolution profile is illustrated.

Keyphrases □ Dissolution profile—powders under sink conditions, general equation, relationship of initial particle-size distribution, applicability to dissolution of log-normal powders □ Particle-size distribution—effect on dissolution profile, general equation, applicability to dissolution of log-normal powders □ Powders—dissolution profile under sink conditions, general equation, relationship of initial particle-size distribution, applicability to dissolution of log-normal powders

Several investigators (1–4) considered the problem of exactly describing the dissolution profile of powders in relation to their particle-size distribution. These authors have been concerned with powders initially consisting of particles with log-normal size distribution. Earlier attempts made use of approximations (1) or computer simulations (2). More recently, Brooke (3) developed an equation that permits calculation of the dissolution profile of such powders without the aid of a computer. This equation was later presented in a form to account for truncated log-normal distributions (4), but in this paper it is shown not to be generally applicable for all timelengths.

A general equation that exactly describes the entire dissolution profile of powders under sink conditions would be of considerable value. Such an equation should be valid for particles having any initial size distribution and dissolving according to any explicit equation. In particular, it should be applicable to the dissolution of log-normal powders, which are frequently encountered.

THEORY

Consider a powder consisting of particles which initially ($t = 0$) has a weight density (probability) distribution $f_0(w_0)$. Let the particles dissolve independently of each other according to:

$$w = g(w_0,t,A) \qquad \text{(Eq. 1)}$$

where w and w_0 are the particle weights at time t and $t = 0$, respectively; and A represents collectively the dissolution parameters such as solubility, particle density, and the particle shape factor. The inverse dissolution function is defined as:

$$w_0 = g^{-1}(w,t,A) \qquad \text{(Eq. 2)}$$

Using the rules of transformation of independent variables (5), the particle weight density function at time t becomes:

$$h(w) = f_0[g^{-1}(w,t,A)]\frac{d}{dw}g^{-1}(w,t,A) \qquad \text{(Eq. 3)}$$

For Eq. 3 to hold, the following conditions for g must be satisfied: *(a)* g^{-1} must be a strictly increasing function of w for all t values, *(b)* g must decrease strictly with time until equal to zero, and *(c)* g must remain equal to zero beyond that time. The latter two conditions ensure that the dissolution function reflects the actual physical conditions of the dissolution process. The first condition will rarely be violated because, in application, g is nearly always a strictly increasing function of w_0 for all t values. It is obvious that the second condition must be met by any dissolution equation.

The third condition is not satisfied for most equations in the literature (1, 6, 7). To overcome this problem, it is necessary to redefine the particle weight density function such that it is generally applicable:

$$f(w) = \frac{h(w)}{\int_0^\infty h(w)dw} \qquad \text{for } w > 0 \qquad \text{(Eq. 4a)}$$

$$f(w) = 0 \qquad \text{for } w \leq 0 \qquad \text{(Eq. 4b)}$$

where division by the integral is necessary to satisfy the condition that the total integral (from $-\infty$ to $+\infty$) of $f(w)$ must be equal to 1.

The weight of undissolved powder, W, at any time, t, is equal to the product of the number of particles remaining, N_t, and the mean particle weight, which for a large number of particles is the same as the expected value of w, $E_t(w)$. Therefore, the following general equation can be written:

$$W = N_t E_t(w) \qquad \text{(Eq. 5)}$$

The number of particles remaining at time t is:

$$N_t = N_0 \int_0^\infty h(w)dw \qquad \text{(Eq. 6)}$$

where the initial number of particles, N_0, is equal to the initial powder weight, W_0, divided by the initial mean particle weight:

$$N_0 = \frac{W_0}{\int_0^\infty w_0 f_0(w_0)dw_0} \qquad \text{(Eq. 7)}$$

The mean particle weight at time t is given by:

$$E_t(w) = \int_{-\infty}^{\infty} w f(w)dw \qquad \text{(Eq. 8)}$$

which, according to Eq. 4*a*, can be written:

$$E_t(w) = \frac{\int_0^\infty w h(w)dw}{\int_0^\infty h(w)dw} \qquad \text{(Eq. 9)}$$

Substituting Eqs. 6, 7, and 9 into Eq. 5 yields:

$$\frac{W}{W_0} = \frac{\int_0^\infty w h(w)dw}{\int_0^\infty w_0 f_0(w_0)dw_0} \qquad \text{(Eq. 10)}$$

This equation relates to unbounded particle weight distributions. In practice, the distribution is always bound, so the limits of integration must be changed accordingly.

Let m_0 and M_0 denote the initial weights of the smallest and largest particle, respectively[1]. These values then represent the lower and upper boundaries, respectively, of $f_0(w_0)$; $f(w)$ will be correspondingly bounded by $Pg(m_0,t,A)$ and $Pg(M_0,t,A)$. The operator P is introduced to ensure that the limits are never negative. Therefore, by definition, P is equal to 1 in the time period before the operand becomes zero and is equal to zero beyond that time. When these limits are introduced into Eq. 10 and $h(w)$ is written according to Eq. 3, the following expression is obtained:

$$\frac{W}{W_0} = \frac{\int_{u=Pg(m_0,t,A)}^{u=Pg(M_0,t,A)} w f_0[g^{-1}(w,t,A)] \frac{d}{dw} g^{-1}(w,t,A) dw}{\int_{m_0}^{M_0} w_0 f_0(w_0) dw_0} \qquad \text{(Eq. 11)}$$

If $g^{-1}(w,t,A)$ and w_0 in the integrals are considered as dummy variables and called x, Eq. 11 simplifies to:

$$\frac{W}{W_0} = \frac{\int_{L_1}^{L_2} g(x,t,A) f_0(x) dx}{\int_{m_0}^{M_0} x f_0(x) dx} \qquad \text{(Eq. 12}a\text{)}$$

Because of this transformation and the properties of the operator P, the limits of integration, L_1 and L_2, have the following values:

$L_1 = m_0$ for t such that $Pg(m_0,t,A) > 0$ (Eq. 12b)

$L_1 = g^{-1}(0,t,A)$ for t such that $Pg(m_0,t,A) = 0$ (Eq. 12c)

$L_2 = M_0$ for t such that $Pg(M_0,t,A) > 0$ (Eq. 12d)

$L_2 = g^{-1}(0,t,A)$ for t such that $Pg(M_0,t,A) = 0$ (Eq. 12e)

The time at which $g(m_0,t,A) = 0$ is the critical time, that is, the time when the dissolving particles begin to disappear. When $g(M_0,t,A) = 0$, all particles are dissolved. Beyond that time, $L_1 = L_2$, thus making W/W_0 equal to zero.

Equation 12a requires knowledge of the initial weight distribution $f_0(w_0)$, but more often the initial size distribution is of greatest interest. Consider particles that are spherical and remain so during the dissolution; in this case, $w = \rho\pi a^3/6$, where ρ and a are the particle density and diameter, respectively[2]. Transformation in Eq. 11 from the initial weight distribution to the initial size distribution is then easily achieved; and by a similar procedure to that used to obtain Eq. 12a from Eq. 11, the following equation is derived:

$$\frac{W}{W_0} = \frac{\int_{R_1}^{R_2} g\left(\frac{\rho\pi}{6} x^3,t,A\right) l_0(x) dx}{\int_{d_0}^{D_0} \frac{\pi\rho}{6} x^3 l_0(x) dx} \qquad \text{(Eq. 13}a\text{)}$$

where:

$R_1 = d_0$ for t such that $Pg\left(\frac{\rho\pi}{6} d_0^3,t,A\right) > 0$ (Eq. 13b)

$R_1 = \left[\frac{6}{\rho\pi} g^{-1}(0,t,A)\right]^{1/3}$ for t such that $Pg\left(\frac{\rho\pi}{6} d_0^3,t,A\right) = 0$ (Eq. 13c)

$R_2 = D_0$ for t such that $Pg\left(\frac{\rho\pi}{6} D_0^3,t,A\right) > 0$ (Eq. 13d)

$R_2 = \left[\frac{6}{\rho\pi} g^{-1}(0,t,A)\right]^{1/3}$ for t such that $Pg\left(\frac{\rho\pi}{6} D_0^3,t,A\right) = 0$ (Eq. 13e)

[1] These values are not intended to be absolute but rather represent limits giving the best fit when the actual particle distribution is approximated by any particular function. Therefore, they also represent truncation limits of the function.

[2] The derivation is valid for particles of other shapes as long as they remain unchanged during dissolution and an appropriate shape factor is used in place of $\pi/6$.

In Eq. 13a, d_0 and D_0 are initial diameters of the smallest and largest particles, respectively[1]; l_0 is the initial particle-size density function.

Equations 12a and 13a rigorously describe the entire dissolution profile of any powder if its initial particle weight density function, f_0, or initial particle-size density function, l_0, is known, together with the particle dissolution function, which can be any explicit expression. With appropriate choice of limits of integration, the equations are applicable to truncated as well as "ideal" distributions. The time-dependent integral in the numerator of Eqs. 12a and 13a reduces to the constant integral in the denominator at zero time, thus making the ratio W/W_0 equal to 1 as expected.

RESULTS AND DISCUSSION

The general mathematical models expressed by Eq. 12a or 13a require the use of a computer for numerical evaluation because they are in integral form. However, if the initial particle weight or particle-size distribution can be approximated by some simple function, then the model can often be solved in terms of an expression suitable for evaluation without the use of a computer.

Consider a powder consisting of spherical particles of initial diameters a_0, distributed such that $\ln a_0$ approximates a normal distribution with mean μ and standard deviation σ, truncated at $\ln d_0 = \mu - i\sigma$ and $\ln D_0 = \mu + j\sigma$, where i and j are truncation parameters. The density function of $\ln a_0$ is then given by:

$$u(\ln a_0) = \frac{N(\ln a_0,\mu,\sigma)}{\int_{\ln a_0=\mu-i\sigma}^{\ln a_0=\mu+j\sigma} N(\ln a_0,\mu,\sigma) d \ln a_0}$$

$$\mu - i\sigma \leqslant \ln a_0 \leqslant \mu + j\sigma \qquad \text{(Eq. 14)}$$

where the normal density function N is defined as $N(x,\mu,\sigma) = [1/(\sigma\sqrt{2\pi})]\exp[-(x-\mu)^2/2\sigma^2]$. Because the distribution is truncated, the integral in Eq. 14 is introduced to satisfy the requirement that the total integral of $u(\ln a_0)$ must be equal to 1. By using standard transformation techniques (5), the initial particle-size density function, l_0, then becomes:

$$l_0(a_0) = \frac{a_0^{-1} N(\ln a_0,\mu,\sigma)}{\int_{d_0}^{D_0} a_0^{-1} N(\ln a_0,\mu,\sigma) da_0} \qquad d_0 \leqslant a_0 \leqslant D_0 \qquad \text{(Eq. 15)}$$

Let the particles dissolve according to the Hixson–Crowell (6) cube root law:

$$w = (w_0^{1/3} - kt)^3 \qquad \text{(Eq. 16)}$$

where k is a positive constant. The inverse dissolution function is then:

$$w_0 = (w^{1/3} + kt)^3 \qquad \text{(Eq. 17)}$$

After the initial particle-size distribution (Eq. 15) and the particle dissolution equation (Eq. 16 and its inverse Eq. 17) are defined, the relationship giving the dissolution profile can then be derived by means of Eq. 13a (for derivation, see *Appendix*):

$$W/W_0 = \left[\frac{F\left(\frac{T_2-\mu}{\sigma} - 3\sigma\right) - F\left(\frac{T_1-\mu}{\sigma} - 3\sigma\right)}{F(j-3\sigma) - F(-i-3\sigma)}\right]$$

$$- 3(Kt)\left[\frac{F\left(\frac{T_2-\mu}{\sigma} - 2\sigma\right) - F\left(\frac{T_1-\mu}{\sigma} - 2\sigma\right)}{F(j-3\sigma) - F(-i-3\sigma)}\right] e^{-\mu+5\sigma^2/2}$$

$$+ 3(Kt)^2\left[\frac{F\left(\frac{T_2-\mu}{\sigma} - \sigma\right) - F\left(\frac{T_1-\mu}{\sigma} - \sigma\right)}{F(j-3\sigma) - F(-i-3\sigma)}\right] e^{-2\mu+1\sigma^2}$$

$$- (Kt)^3\left[\frac{F\left(\frac{T_2-\mu}{\sigma}\right) - F\left(\frac{T_1-\mu}{\sigma}\right)}{F(j-3\sigma) - F(-i-3\sigma)}\right] e^{-3\mu+9\sigma^2/2} \qquad \text{(Eq. 18}a\text{)}$$

where:

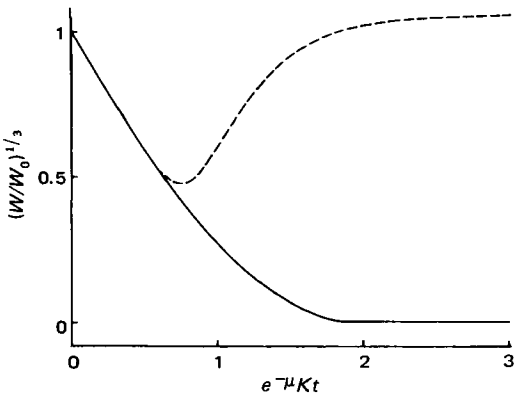

Figure 1—*Plot of the cube root of fraction undissolved* versus $e^{-\mu}Kt$ *for hypothetical log-normal powder, with* $\sigma = 0.3$ *and truncated at* $\mu \pm 2\sigma$ *(*i = j = 2*). The unbroken curve is calculated according to Eq. 18a, and the stippled curve is calculated according to an equation given by Brooke (4).*

$$T_1 = \mu - i\sigma \quad \text{for } \ln Kt \leqslant \mu - i\sigma \quad \text{(Eq. 18b)}$$

$$T_1 = \ln Kt \quad \text{for } \ln Kt > \mu - i\sigma \quad \text{(Eq. 18c)}$$

$$T_2 = \mu + j\sigma \quad \text{for } \ln Kt \leqslant \mu + j\sigma \quad \text{(Eq. 18d)}$$

$$T_2 = \ln Kt \quad \text{for } \ln Kt > \mu + j\sigma \quad \text{(Eq. 18e)}$$

The function, F, is the commonly tabulated area under the standard normal curve function, defined by:

$$F(x) = \int_{-\infty}^{x} \frac{1}{\sqrt{2\pi}} e^{-x^2/2}\, dx \qquad \text{(Eq. 19)}$$

The positive constant, K, is used for simplification in place of the expression $(6/\rho\pi)^{1/3}k$. The change in T_1 at timelength $Kt = e^{\mu - i\sigma}$ (critical time) corresponds to the time when the smallest particles, initially having a diameter $d_0 = e^{\mu - i\sigma}$, begin to disappear. The change at $Kt = e^{\mu + j\sigma}$ $(= D_0)$ signifies the end of the dissolution process, so W/W_0 becomes zero after that timelength.

Equation 18*a* describes the complete dissolution profile of log-normal powders and any sieve fraction of such powders. It assumes that the particles dissolve according to the cube root law (Eq. 16), which also can be written in the form $a = a_0 - (6/\rho\pi)^{1/3}kt$ or $a = a_0 - Kt$ for spherical particles, where a_0 and a are the particle diameters at times zero and t, respectively. This relationship is similar to the equation $a = a_0 - \tau$ used previously (2–4) in which τ denotes the timelength.

Brooke (4) derived an equation similar to Eq. 18*a* for log-normal powders. Directions were given for the changes required in the equation at the critical time. However, in his equation the first term is constant, equal to 1. Therefore, his equation is incorrect if applied to dissolution *after* the critical time. The error so introduced becomes quite substantial for large timelengths and large values of σ.

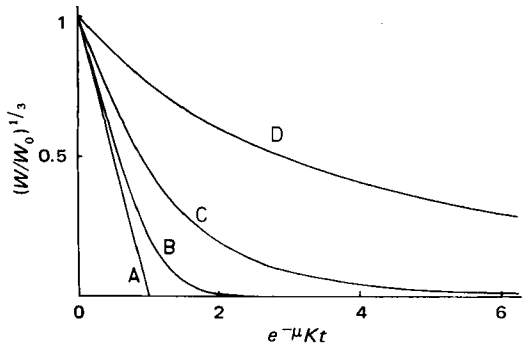

Figure 2—*Effect of the particle-size distribution parameter,* σ*, on plots of the cube root of fraction undissolved* versus $e^{-\mu}Kt$*, calculated according to Eq. 18a for hypothetical ideal* (i = j = *10) log-normal powders. Key: A,* $\sigma = 0$*; B,* $\sigma = 0.2$*; C,* $\sigma = 0.4$*; and D,* $\sigma = 0.7$

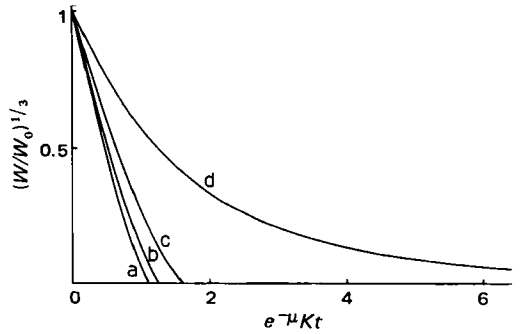

Figure 3—*Effect of upper end truncation on the dissolution profile of powders having a log-particle diameter distribution that is normal, with* $\sigma = 0.5$*, and truncated at* $\mu - 10\sigma$ *and* $\mu + j\sigma$*, where* j = *0.25 (a), 0.5 (b), 1 (c), and 10 (d).*

Brooke calculated values of W/W_0 for various values of τ/e^{μ} (the latter terminology corresponds to $e^{-\mu}Kt$ used here). This procedure represents an ingenious method of "scaling time" (by the factor $e^{-\mu}K$) so the dissolution profile becomes independent of the parameters μ and k (Eq. 16) and only depends on σ, enabling the effect of σ alone to be assessed. In this work, however, cube root-type plots of $(W/W_0)^{1/3}$ *versus* $e^{-\mu}Kt$ are used for better comparison with the fundamental particle dissolution equation (Eq. 16), which obeys the cube root law.

Figure 1 shows such a plot for powders truncated at $\mu \pm 2\sigma$ $(i = j = 2)$ and having $\sigma = 0.3$. The curvature of the unbroken line, calculated according to Eq. 18*a*, is logically expected. The stippled line represents the dissolution profile calculated according to Brooke's equation (Eq. 4 of Ref. 4)[3]. The two profiles are, as expected, identical until critical time ($e^{-\mu}Kt = 0.5488$), but the later part clearly demonstrates the limitation of his equation.

Figure 2 demonstrates the effect of σ on the dissolution profile for a powder initially having an ideal distribution $(i = j = 10)$.

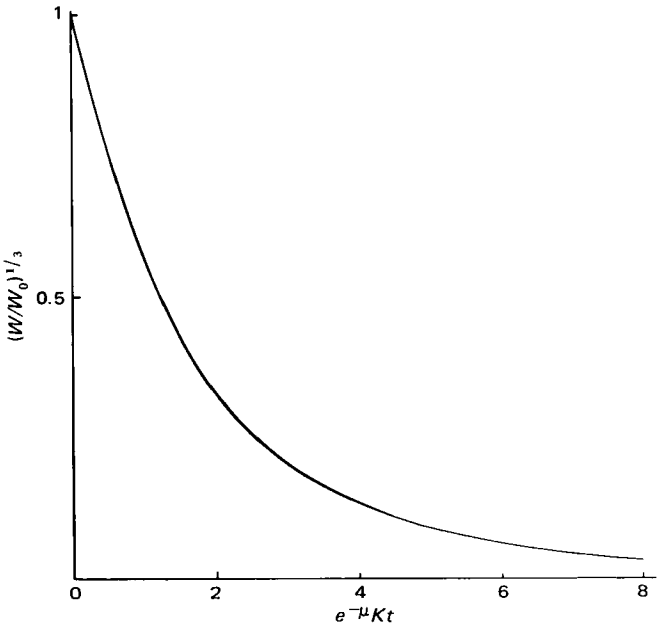

Figure 4—*Effect of lower end truncation on the dissolution profile of powders having a log-particle diameter distribution that is normal, with* $\sigma = 0.5$*, and truncated at* $\mu - i\sigma$ *and* $\mu + 10\sigma$*, where* i = *0.25, 0.5, 1, and 10. The four curves are essentially coincidental.*

[3] A Cyber 76 digital computer equipped with Calcomp plotter was used for calculations and plots. Numerical evaluations were tested to six digits.

Powders of uniform particle size, *i.e.*, $\sigma = 0$, give linear cube root plots as expected, while the deviation from linearity is significant for larger σ values. An increase in σ results in a decrease in the initial slopes of the curves, which is consistent with calculations made by Brooke (4). Among powders having the same logarithmic mean diameter, μ, those with broadest distribution will have the slowest initial release rate.

Figures 3 and 4 show the effect of truncation on the dissolution profile when the initial log (particle diameter) – distribution approximates a normal distribution ($\sigma = 0.5$) with various degrees of upper (Fig. 3) and lower (Fig. 4) end truncation. Comparison of the two figures indicates that the effect of truncation at the low end is very small and considerably less than that of truncation at the upper end. The curves for $i = 10$, 1, 0.5, and 0.25 are so close that they are coincidental in the plot (Fig. 4), while they are clearly separate for $j = 10$, 1, 0.5, and 0.25 in Fig. 3. Figure 3 also shows that the magnitude of the slopes of the curves increases with increasing truncation. The most marked effect of truncation at the upper end is that the time for complete dissolution is drastically reduced.

APPENDIX

After inserting Eqs. 15, 16, and 17 into Eq. 13*a*, the integral in the numerator, I_n, of Eq. 13*a* becomes:

$$I_n = \frac{\int_{R_1}^{R_2}\left[\left(\frac{\rho\pi}{6}\right)^{1/3} w - kt\right]^3 w^{-1}N(\ln w,\mu,\sigma)dw}{\int_{d_0}^{D_0} w^{-1}N(\ln w,\mu,\sigma)dw} \quad \text{(Eq. A1)}$$

It was indicated previously that $W/W_0 = I_n/(I_n)_{t=0}$. By using this fact, W/W_0 can be written:

$$\frac{W}{W_0} = \frac{\int_{R_1}^{R_2}\left[w - \left(\frac{6}{\rho\pi}\right)^{1/3} kt\right]^3 w^{-1}N(\ln w,\mu,\sigma)dw}{\int_{d_0}^{D_0} w^{2}N(\ln w,\mu,\sigma)dw} \quad \text{(Eq. A2)}$$

In Eq. A2, the time-independent integral in the denominator is equal to the time-dependent integral in the numerator evaluated at zero time. To evaluate the numerator of Eq. A2, employing a technique similar to that used to derive the moment-generating function for a normal distribution (5), the following useful equation can be obtained, which is similar to Eq. 12 given by Brooke (3):

$$\int_q^r x^s N(\ln x,\mu,\sigma)dx = \left[F\left(\frac{\ln r - \mu}{\sigma} - (s+1)\sigma\right) - F\left(\frac{\ln q - \mu}{\sigma} - (s+1)\sigma\right)\right] e^{[s+1)[\mu+(s+1)\sigma^2/2]} \quad \text{(Eq. A3)}$$

where the function, F, is the area under the standard normal curve function given by Eq. 19. Thus, to evaluate the numerator, I, in Eq. A2, $(w - Kt)^3 w^{-1}$ is expanded as $w^2 - 3(Kt)w + 3(Kt)^2 - (Kt)^3 w^{-1}$ [letting $K = (6/\rho\pi)^{1/3}k$ for simplicity]. The formula is then applied term by term by putting $s = 2$, 1, 0, and -1; $r = R_2$; and $q = R_1$ and by treating t as a constant for purposes of integration. Equation 18*a* is then derived using $W/W_0 = I/(I)_{t=0}$ and noting that $\ln d_0 = \mu - i\sigma$ and $\ln D_0 = \mu + j\sigma$.

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Microdetermination of Ergocalciferol (Vitamin D_2) in Pharmaceutical Preparations by Differential Spectroscopy Using Trifluoroacetic Acid in the Absence of Vitamin A

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Abstract □ A differential spectroscopy method for the determination of microgram quantities of ergocalciferol, using trifluoroacetic acid and hydrogen peroxide, was applied to commercially available pharmaceutical preparations. Tablets were powdered and extracted with methanol after being made basic with diethylamine. The methanol was concentrated, chloroform was added, and the mixture was chromatographed on neutral alumina using chloroform as the eluent. Ergocalciferol was determined in an aliquot of the eluate by differential spectroscopy and was compared with a reference ergocalciferol standard treated similarly. The method applied to low potency formulations (2.5–6 μg/tablet) is simple and quantitative, needs less than 5 hr for completion, and has a precision of <±2%. A simple and accurate chromatographic method for the determination of ergocalciferol in stabilized powders and high potency tablets, which precludes the necessity of solvent–solvent extraction, was also developed. The results are comparable with those obtained by the rat bioassay and the USP XVIII chemical assay.

Keyphrases □ Ergocalciferol in pharmaceutical preparations—differential spectroscopic analysis using trifluoroacetic acid, absence of vitamin A □ Vitamin D_2 in pharmaceutical preparations—differential spectroscopic analysis using trifluoroacetic acid, absence of vitamin A □ Differential spectroscopy—analysis, ergocalciferol in pharmaceutical preparations not containing vitamin A

Ergocalciferol (vitamin D_2), one of the fat-soluble vitamins (1), is essential for the prevention of rickets. The determination of ergocalciferol in nutritional and pharmaceutical products and biological materials has been the subject of extensive research work. Studies on the metabolism of ergocalciferol, its toxicity when taken in excess (2), and its consideration as a drug if assimilated in quantities greater than 400 IU/day (40 IU = 1 μg) (3) have made its evaluation even more necessary.

The rat bioassay for ergocalciferol (4) is time consuming, expensive, and imprecise. Several chemical and physicochemical methods of determination are applicable to pure ergocalciferol (5), but a tedious cleanup is usually necessary before quantitation. Saponification in an alcoholic medium followed by partition with organic solvent (6) and column chromatography (7) is the usual route taken for the extraction and purification of ergocalciferol from the sample. The main difficulty in obtaining quantitative recoveries of the vitamin is its potential instability due to the effects of heat, oxygen, and water (8).

Kobayashi and Adachi (9) determined ergocalciferol and its isomers in mixtures by GLC after separation by TLC. Mulder *et al.* (10) evaluated the chemical analysis of ergocalciferol in concentrates (≥25,000 IU/g) by the most widely used antimony trichloride colorimetric method. Kneczke and Andersson (11) modified the USP XVIII chemical method, using hydroquinone as the antioxidant, and compared the results with and without saponification of different preparations containing ergocalciferol with a bioassay method.

"Reversed-phase partition" high-pressure liquid chromatography was used for the separation and quantitation of the fat-soluble vitamins and the D vitamins (12). The technique was applied to multivitamin capsules and tablets, but the study was for chromatographic reproducibility and not for the reproducibility of the extraction methods. Fat-soluble vitamins were separated by gel filtration, and vitamin D was determined in the presence of vitamin A and α-tocopherol (13).

Recently, a stable and sensitive differential spectroscopy method for the determination of ergocalciferol, using trifluoroacetic acid and hydrogen peroxide, was developed (14). This paper deals with the application of this procedure to the determination of ergocalciferol when present in microgram quantities in commercially available pharmaceutical preparations containing no vitamin A. A simple chromatographic procedure also was developed for preparations containing milligram quantities using the trifluoroacetic acid colorimetric method recommended by Clements *et al.* (15).

EXPERIMENTAL

Instrumentation and Apparatus—A spectrophotometer[1], a rotary evaporator, a stir-hot plate, an electric micromill, and a chromatographic glass column (250 × 25 mm i.d. with a 250-ml capacity reservoir) were used.

Reagents and Chemicals—The following were used: ethanol[2] (anhydrous), methanol[3] (anhydrous), chloroform[4], diethylamine[5], trifluoroacetic acid[6] (extremely corrosive acid), hydrogen peroxide (30%), aluminum oxide (neutral)[7], silicic acid[8], and ergocalciferol (USP reference standard). Hydroquinone[6] solution (0.1%) was prepared by dissolving hydroquinone in a minimal amount of ether and diluting to volume with chloroform.

Procedure A: For Low Potency Preparations—Carry out all operations in subdued artificial light.

Preparation of Alumina Column—Pour about 20 ml of methanol into the chromatographic column and lightly tamp a pledget of

[1] Coleman 124, Perkin-Elmer.
[2] U.S. Industrial Chemicals Co.
[3] Mallinckrodt, O.R.
[4] Mallinckrodt, A.R.
[5] Matheson Coleman & Bell, ACS.
[6] Matheson Coleman & Bell.
[7] Woelm.
[8] SilicAR CC7, Mallinckrodt.

cotton into the bottom, ridding the cotton of air bubbles. Weigh 15 g of neutral aluminum oxide and transfer to a small flask. Add about 20 ml of methanol, swirl to eliminate air bubbles, and transfer completely to the prepared column using 20–30 ml of methanol. Drain the methanol to about 0.5 cm above the alumina. Add 50 ml of chloroform, level the surface of the alumina by tapping the sides of the column, and drain the solvent to about 1 cm above the alumina. Wrap the stem of the column with aluminum foil and set aside until needed.

Preparation of Standard Solution—Accurately weigh about 20 mg of reference standard ergocalciferol from a freshly opened vial into a 100-ml volumetric flask. Dissolve in and dilute to volume with chloroform (Standard Solution A-I). Dilute 5.0 ml of this solution to 100 ml with chloroform (Standard Solution A-II). Prepare a third solution by diluting 5.0 ml of Standard Solution A-II to 100 ml with chloroform (Standard Solution A-III).

Determination of Ergocalciferol in Standard Solution—Accurately pipet 10.0 ml of reference standard ergocalciferol solution in chloroform (A-III, 0.5–0.7 μg/ml) in a small flask and add 1 drop of diethylamine. Evaporate the mixture completely on a rotary evaporator at 30–35°, add 5 ml of anhydrous ethanol, and evaporate again completely. Then add 5 ml of chloroform and evaporate completely.

Flush the flask for a few seconds with a stream of nitrogen to eliminate the last traces of chloroform. Pipet 1.0 ml of 0.1% hydroquinone solution to dissolve the residue. Using a fast delivery pipet, add exactly 1.0 ml of trifluoroacetic acid, swirl quickly two or three times, and transfer to a 1-cm cell within 30 sec (cover the cell).

Record the absorbance at 490 nm as a function of time, using a mixture of chloroform and trifluoroacetic acid (1:1) as the reference solution. Determine the highest absorbance value obtained within the first 3 min. Record the spectrum between 520 and 480 nm to ensure that the peak is at 490 ± 2 nm. Add 2 drops of hydrogen peroxide from a disposable pipet to the sample solution in the cell and shake once to mix. Record the absorbance value at 490 nm obtained 2 min ± 10 sec after the addition of the hydrogen peroxide. The difference between the two absorbance values (A_{std}) is proportional to the amount of ergocalciferol in the standard solution.

Preparation of Sample Solution and Determination of Ergocalciferol—Accurately weigh a number of tablets and determine the average tablet weight. Grind the tablets to a powder, using the electric micromill for 40–50 sec or a mortar and pestle. Accurately weigh sufficient powdered tablets to contain a total of about 25 μg of ergocalciferol and transfer to a 125–250-ml amber-colored erlenmeyer or extraction flask. Add 25 ml of methanol and 0.1 ml of diethylamine. Reflux the mixture in a water bath at 65–70° for 2 hr, using a magnetic stirrer for additional agitation. Remove the magnet and rinse it with chloroform by using a disposable pipet; add the rinse to the flask. Add 20 ml of chloroform and concentrate the mixture to about 10 ml with a rotary evaporator at 30–35°. Cool under the tap and add 30 ml of chloroform.

Transfer the mixture quantitatively through a wide mouth funnel to the prepared alumina column, rinsing the flask and funnel with about 10 ml of chloroform. Wash the sides of the column with about 5 ml of chloroform and then allow the mixture to stand for about 5 min to permit the powder to settle in the column. Allow the solvent to flow slowly (80–120 drops/min) into an amber-colored, 100-ml volumetric flask. Drain the column to the level of the powder and then carefully add 10-ml portions of chloroform, draining the column each time (use at least 30 ml of additional chloroform for elution). Collect exactly 100 ml of eluate. The colored zone, due to dyes in the tablets, if present should not have progressed more than halfway through the alumina.

Pipet 20.0 ml of the eluate into a 50-ml, amber-colored erlenmeyer flask and proceed as directed for Procedure A under *Determination of Ergocalciferol in Standard Solution*, starting with "Evaporate the mixture completely on a rotary evaporator at 30–35°, . . ." The difference between the two absorbance values (A_{spl}) is proportional to the amount of ergocalciferol present.

Calculations—The amount of ergocalciferol is calculated using:

$$\mu g \text{ ergocalciferol / tablet} = \frac{A_{spl}}{A_{std}} \times \text{weight of standard (mg)} \times \frac{\text{average tablet weight (g)}}{\text{sample weight (g)}} \times 1.25 \qquad \text{(Eq. 1)}$$

Procedure B: For High Potency Preparations—Carry out all operations in subdued artificial light.

Preparation of Silica Column—Pour about 40 ml of chloroform into the column and lightly tamp a small pledget of cotton into the bottom. Weigh 5 g of silicic acid[8] in a 50-ml erlenmeyer flask, add 15 ml of chloroform, mix well, and quickly pour through a funnel into the column. Wash the flask and the sides of the column with 2 × 10 ml of chloroform. Withdraw 40–45 ml of solvent, wrap the column with aluminum foil, and set aside until needed.

Preparation of Standard Solution—Accurately weigh about 35–40 mg of reference standard ergocalciferol from a freshly opened vial into a 100-ml volumetric flask. Dissolve in and dilute to volume with chloroform (Standard Solution B-I). Dilute 10 ml of Standard Solution B-I to exactly 100 ml with chloroform (Standard Solution B-II).

Determination of Ergocalciferol in Standard Solution—Accurately pipet 8.0 ml of reference standard ergocalciferol Solution B-II in a small flask and add 2.0 ml of trifluoroacetic acid. Swirl the mixture three or four times and transfer to a 1-cm cell. Record the absorbance at 403 nm as a function of time, using a mixture of chloroform and trifluoroacetic acid (4:1) as the reference solution. Determine the highest absorbance value (A_{std}) obtained within 10 min; the absorbance value will depend on the quality of the trifluoroacetic acid and may vary from day to day, which necessitates running a standard.

Determination of Ergocalciferol in Sample Solution—Weigh accurately an amount of sample (powder or powdered tablets) to contain 3.5–4.0 mg of ergocalciferol and transfer to a small flask. Add water (2.5 times the amount of sample) and mix well for 3–4 min to get a homogeneous slurry containing no lumps. Wash the sides of the flask with ethanol equal in volume to one-third the amount of water and mix gently for 2–3 min. Swirl the mixture, add silicic acid[8] in an amount equal to four times the volume of water, and mix quickly with a spatula to get a homogeneous mobile powder within 60 sec. Immediately add chloroform in an amount equal to four times the amount of the silica and mix well.

Transfer the slurry to the previously prepared silica column through a wide mouth funnel and wash the flask, funnel, and sides of the column with 2 × 10 ml of chloroform. Allow the solvent to drip fast into an amber-colored, 100-ml volumetric flask until it reaches the level of the adsorbent. Wash the flask and sides of the column with 10-ml portions of chloroform, draining each time. Collect exactly 100 ml of eluate. Pipet 8.0 ml of eluate in a small flask and continue as directed for Procedure B under the *Determination of Ergocalciferol in Standard Solution*, starting with "add 2.0 ml of trifluoroacetic acid."

Calculations—The amount of ergocalciferol is calculated as follows:

$$\text{mg ergocalciferol/g} = \frac{A_{spl}}{A_{std}} \times \frac{\text{standard weight (mg)}}{\text{sample weight (g)}} \times \frac{1}{10} \qquad \text{(Eq. 2)}$$

RESULTS AND DISCUSSION

Due to the difference in the procedure for analysis, pharmaceutical preparations containing ergocalciferol can be divided into two categories: those containing vitamin A, which are numerous, and those devoid of it, which are few. The latter can be considered as high potency or low potency preparations according to the content of ergocalciferol and its administration per day relative to the daily requirement.

The problems of extraction of ergocalciferol in microgram quantities with minimal loss, especially from sugar-coated tablets (16), with subsequent purification and choice of a sensitive method of determination usually afford a challenge to the pharmaceutical analyst. In this laboratory the need arose for a simple quality control and stability assay for the determination of ergocalciferol in a vitamin–mineral supplement containing only 3.33–6 μg/sugar-coated tablet weighing 1.3–1.4 g (2.4–4.4 ppm).

Saponification with alcoholic potassium hydroxide or aqueous-alcoholic potassium hydroxide and extraction with ether or petro-

Table I—Ergocalciferol Content of Low Potency Preparations: Comparison of Results by Procedure A and the Biological Rat Assay

Product	Procedure A			Biological Assay[a]
	Number of Assays	Ergocalciferol, μg/Tablet	Average Deviation, %	Ergocalciferol, μg/Tablet
A[b]: Lot 1	4	5.32	0.45	5.63
2	4	4.28	1.29	5.06
3	2	4.46	0.00	4.26
4	2	4.31	0.39	5.06
5	2	4.53	1.47	6.09
6	2	4.68	1.07	—
B[c]	2	3.88	0.51	3.65
C[d]	3	3.58	1.92	3.15

[a] Assays carried out by private testing laboratories. [b] Theoretical amount 5 μg/tablet. [c] Label claim 3.12 μg/tablet. [d] Label claim 2.5 μg/tablet.

Table II—Ergocalciferol Content of High Potency Preparations: Comparison of Results by Procedure B, the Biological Rat Assay, and the USP Chemical Method

Product	Procedure B			USP Method[a]	
				Ergocalciferol, mg/g	
	Number of Assays	Ergocalciferol, mg/g	Average Deviation, %	Biological	Chemical
A[b]: Lot 1	6	2.98	1.23	3.22	2.75
2	2	2.91	0.86	3.05	2.92
3	2	3.17	1.89	2.68	3.02
B[c]	8	5.90	2.60	6.45	6.00

[a] Assays carried out by private testing laboratories. [b] Label claim >2.5 mg/g. [c] Label claim 6.25 mg/g.

leum ether, or a combination of both followed by subsequent purification by alumina and/or silica chromatography, proved to be tedious and gave erratic and nonquantitative results. The presence of water and the dye in the sugar coating of the tablets were the main sources of difficulties in obtaining an extract pure enough for spectral analysis. The application of Procedure B and many modifications of it proved unsuccessful for the determination, because substances in the eluate interfered by giving an absorbance near 403 nm. Moreover, Procedure B was inadequate for the analysis due to the small amount of the vitamin in comparison to the bulk of the material and thus the need for relatively large quantities of water and adsorbent. It was the development of the stable and sensitive differential spectroscopy method at 490 nm that paved the way for an adequate analysis.

Procedure A proved to be simple, quantitative, and adequate for quality control purposes, with a precision of <±2%. The application of the method for stability studies is currently under investigation. The small amount of diethylamine is added before extraction to ensure that the methanolic milieu is on the slightly basic side to avoid destruction of the vitamin. Concentration of the methanol before chromatography is recommended, because higher percentages of methanol in the solvent contributed to the increase of interfering substances in the eluate. Interferences were detected by the increase in the value of absorbance at 490 nm after the addition of hydrogen peroxide.

The preliminary cleaning of the alumina with methanol and then chloroform is necessary to remove substances that affect the colorimetry. The slow flow of the solvent through the alumina is essential in adsorbing the dyes used in the sugar coating of the tablets; these dyes appear as a colored band in the upper part of the column. Anhydrous ethanol is added to the residue of the aliquot from the eluate and evaporated to remove mainly all traces of diethylamine. The low absorbance obtained at 490 nm after the addition of hydrogen peroxide (<0.08 as compared to 0.02–0.04 for reference standard ergocalciferol) shows that minimal interferences are eluted and are accounted for by the use of differential spectroscopy.

Procedure A, carried out on placebo tablets, verified that no significant interference was encountered. Quantitative recoveries within experimental error (±2%) of reference standard ergocalciferol added to placebo tablets showed that there was no loss through the assay procedure. The addition of sodium ascorbate or hydroquinone as an antioxidant before extraction did not improve the yield and sometimes interfered with the colorimetry.

Table I shows the results obtained with Procedure A for different samples compared with results obtained by the biological rat assay (17). The proposed method was also applied to commercially available tablets containing low potency amounts of ergocalciferol.

Variation in the results for the different lots of Product A (86–106% of the theoretical amounts) was expected due to the differences in the amounts of ergocalciferol in the lots of stabilized powder used in the tablets. In addition, low assay values could be attributed to the complex effect of heat, pressure, and long exposure to air and light during the manufacturing procedure of granulation, drying, compression, subbing, and sugar coating. Product C contained a comparatively large amount of salicylamide per tablet, which affected the accuracy of the differential spectroscopy analysis. The main bulk of the salicylamide was eliminated by concentration of an aliquot from the eluate, precipitation by addition of hexane, and filtration through cotton.

Although Procedure A takes 4–5 hr for completion, four assays can be carried out within 8 hr. The main advantages of the method are that the inconvenience of liquid–liquid partition extraction is avoided and the effect of spectral interferences can be accounted for. The differential spectroscopy method is currently under investigation for use in preparations containing vitamin A.

Ergocalciferol could be obtained commercially in the dry state, suitable for the preparation of granulations, as a stabilized form containing >2.5 mg ergocalciferol/g of powder. This stabilized powder contains mainly dicalcium phosphate, acacia, coconut oil, and preservatives as excipients. Procedure B was developed for quality control analysis of the powder as a raw material. The powder is mixed with water, which is necessary to dissolve the acacia and release the vitamin. A suitable amount of ethanol is added to dissolve the vitamin without precipitating the acacia, which would occlude some of the vitamin and thus cause a loss. Addition of the silicic acid is used to adsorb the water and also to distribute the vitamin on a large surface area and thus facilitate its extraction.

Chloroform quantitatively elutes ergocalciferol from the column. The trifluoroacetic acid colorimetric method recommended by Clements *et al.* (15) was chosen due to its simplicity and obvious advantages over the antimony trichloride method.

Procedure B was also applied to commercially available tablets containing milligram quantities of ergocalciferol. The results obtained (Table II) were compared with those obtained for samples from the rat bioassay (17) and the USP XVIII chemical method (7). Recoveries of reference standard ergocalciferol added to the stabilized powder in quantities of 0.3 and 3.0 mg for each gram of powder were 99 and 97%, respectively, which are within experimental error of the assay.

The results of Procedure B (Table II) are mostly slightly higher than those of the USP chemical method. A loss through the long procedure of saponification, solvent–solvent extraction, and column chromatography in the latter method is a possibility. The biological assay gave higher results than Procedure B for most samples. Procedure B (average deviation ≤2.6%) takes only 1.5 hr for completion; for quality control purposes, it is a suitable assay for high potency ergocalciferol preparations.

SUMMARY

Ergocalciferol could be assayed when present in microgram quantities in tablets by refluxing followed by column chromatography and differential spectroscopy using trifluoroacetic acid and hydrogen peroxide. The simplicity and accuracy of Procedure A make it adequate for quality control analysis. Preparations containing milligram quantities of ergocalciferol could be assayed for their ergocalciferol content (Procedure B) by partition/extraction chromatography followed by spectrophotometry using trifluoroacetic acid.

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Quantitative Determination of Phenol in Phenolated Calamine Lotion USP

V. DAS GUPTA [x] and KATHRYN A. BOMER

Abstract □ A method for the quantitative analysis of phenol in phenolated calamine lotion USP is described. The method is based on spectrophotometrically measuring the color produced by reacting phenol with either ferric chloride or ferric nitrate. Beer's law is followed. The effect of ferric-ion concentration on the sensitivity of the assay method is reported.

Keyphrases □ Calamine lotion, phenolated—colorimetric analysis of phenol □ Phenolated calamine lotion—colorimetric analysis of phenol

Phenolated calamine lotion is an official USP (1) product. Although phenol is a potent therapeutic agent, the USP does not require its quantitative analysis in phenolated calamine lotion.

Kiposki and Allen (2) reported a bromometric method for the analysis of phenol. Stanko and DeKay (3) recommended a complicated colorimetric method for the quantitative analysis of phenol in phenolated calamine lotion based on reaction with copper sulfate. This report describes a simple colorimetric assay method for the analysis of phenol in phenolated calamine lotion USP. The method is based on an identification test with ferric chloride as described for phenol USP (4).

EXPERIMENTAL

Reagents and Chemicals—All chemicals and reagents used were USP, NF, or ACS grade. $FeCl_3 \cdot 6H_2O$[1], $Fe(NO_3)_3 \cdot 9H_2O$[2], and phenol loose crystals[3] were purchased and used without further purification.

[1] Mallinckrodt Chemical Works.
[2] J. T. Baker Chemical Co.
[3] Matheson, Coleman and Bell.

Chloroform quantitatively elutes ergocalciferol from the column. The trifluoroacetic acid colorimetric method recommended by Clements *et al.* (15) was chosen due to its simplicity and obvious advantages over the antimony trichloride method.

Procedure B was also applied to commercially available tablets containing milligram quantities of ergocalciferol. The results obtained (Table II) were compared with those obtained for samples from the rat bioassay (17) and the USP XVIII chemical method (7). Recoveries of reference standard ergocalciferol added to the stabilized powder in quantities of 0.3 and 3.0 mg for each gram of powder were 99 and 97%, respectively, which are within experimental error of the assay.

The results of Procedure B (Table II) are mostly slightly higher than those of the USP chemical method. A loss through the long procedure of saponification, solvent–solvent extraction, and column chromatography in the latter method is a possibility. The biological assay gave higher results than Procedure B for most samples. Procedure B (average deviation ≤2.6%) takes only 1.5 hr for completion; for quality control purposes, it is a suitable assay for high potency ergocalciferol preparations.

SUMMARY

Ergocalciferol could be assayed when present in microgram quantities in tablets by refluxing followed by column chromatography and differential spectroscopy using trifluoroacetic acid and hydrogen peroxide. The simplicity and accuracy of Procedure A make it adequate for quality control analysis. Preparations containing milligram quantities of ergocalciferol could be assayed for their ergocalciferol content (Procedure B) by partition/extraction chromatography followed by spectrophotometry using trifluoroacetic acid.

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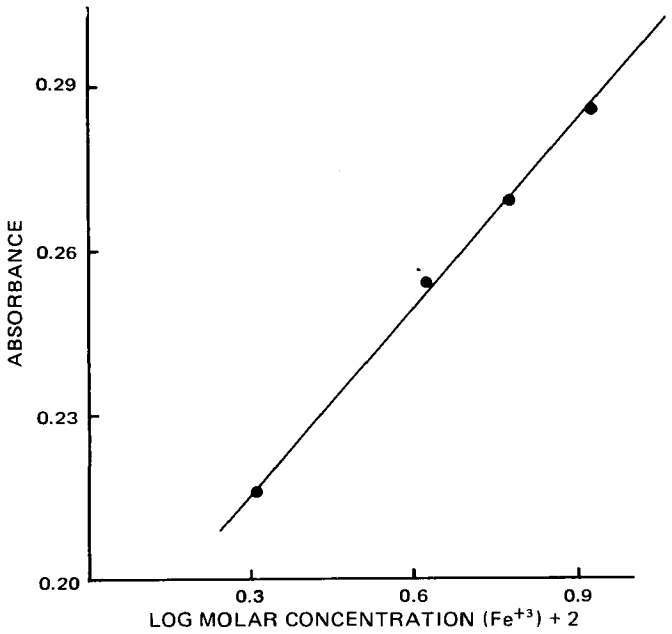

Figure 1—*Plot of log ferric-ion concentration* versus *absorbance.*

Table I—Assay Results on Phenolated Calamine Lotion and Calamine Lotion USP

Assay Number	Results, %	
	Phenolated Calamine Lotion	Calamine Lotion
1	100.2	0.0
2	100.5	0.0
3	100.2	0.0
Average deviation ± 0.13%		

Preparation of Solutions—All solutions of phenol, ferric chloride, and ferric nitrate were prepared (w/v) in distilled water using a simple solution method.

Preparation of Phenolated Calamine Lotion USP—A phenolated calamine lotion was prepared according to the USP (1).

Selection of Wavelength of Maximum Absorption—A 2.0-ml quantity of phenol solution (0.5%) was mixed with 2.0 ml of ferric chloride solution (4%), and the mixture was brought to volume (10.0 ml) with distilled water. The solution was scanned in the visible range using a spectrophotometer[4], and a broad peak was recorded between 528 and 556 nm. For further investigations, a wavelength of 550 nm was selected.

Effect of Ferric-Ion Concentration on Sensitivity of Assay Method—A 2.0-ml quantity of phenol solution (0.05 *M*) was mixed with 2.0, 4.0, 6.0, or 8.0 ml of ferric chloride solution (0.1 *M*), and the mixture was brought to volume (10.0 ml) with distilled water. The absorbance of the solution was measured at 550 nm against a reagent blank, prepared by substituting distilled water for phenol solution (Fig. 1).

Preparation of Calibration Curves—A 2.0-ml quantity of ferric chloride[5] solution (either 2 or 4%) was mixed with 2.0, 2.5, 3.0, 3.5, or 4.0 ml of phenol solution (0.5%), and the mixture was brought to volume (10.0 ml) with distilled water. The absorbance of each solution was measured as already described (Fig. 2).

Assay Procedure for Phenolated Calamine Lotion USP—A 10.0-ml quantity of phenolated calamine lotion was transferred on a filter paper. The filtrate was collected in a suitable container, and the precipitate on the filter paper was washed with 3 × 10-ml portions of distilled water. The precipitate was then rinsed with small portions of distilled water to bring the filtrate to volume (50.0 ml). It was mixed and refiltered when necessary. Then 8.0 ml of the clear filtrate was mixed with 2.0 ml of ferric chloride solution (4%), and the absorbance was measured at 550 nm as already described. The results, calculated by using the standard curve, are presented in Table I.

[4] Beckman model DB.

[5] Ferric nitrate may be substituted for ferric chloride on a mole per mole basis.

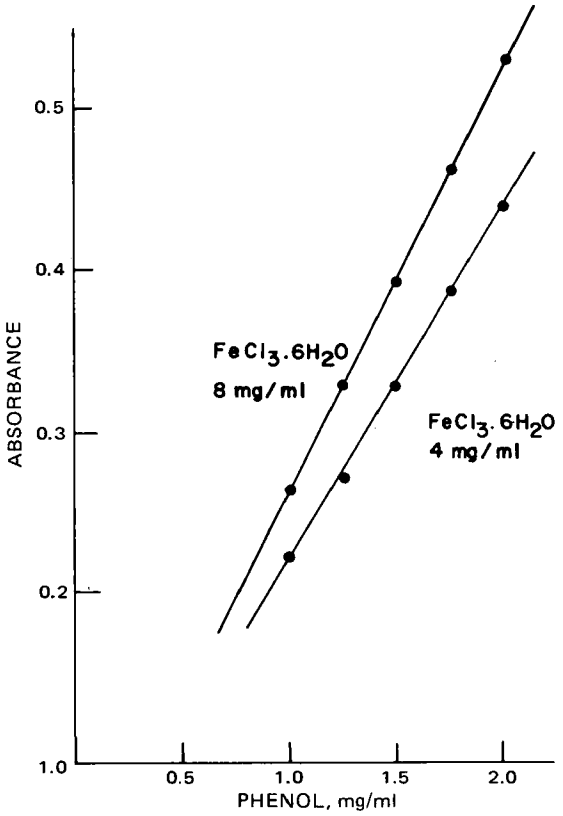

Figure 2—*Standard curves for phenol.*

Interference from Other Ingredients—The described procedure was repeated using calamine lotion USP. The results are presented in Table I.

DISCUSSION AND CONCLUSIONS

The results (Table I) show that phenol can be assayed in phenolated calamine lotion USP using a simple method (reaction with ferric chloride or ferric nitrate). Beer's law is followed (Fig. 2) at different concentrations of ferric ion. The concentration of ferric ion may be increased for more sensitivity in the assay technique, and the blank value is increased also. The log of the ferric-ion concentration is linearly related to absorbance (Fig. 1).

The reaction between phenol and ferric ion may be an oxidation–reduction or the formation of a complex. There is no interference from the other ingredients (Table I). It is important to keep the ferric-ion concentration constant since the sensitivity is dependent on this factor (Fig. 1). A 4.00% solution of ferric chloride is recommended for routine use for the quantitative analysis of phenol in phenolated calamine lotion USP.

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Liquid Chromatography in Pharmaceutical Analysis III: Separation of Diuretic–Antihypertensive Mixtures

I. L. HONIGBERG ˣ, J. T. STEWART, A. P. SMITH, and D. W. HESTER

Abstract □ High-pressure liquid chromatography was used to optimize the resolution of therapeutic agents commonly found in antihypertensive preparations. Seven widely prescribed drugs were investigated. The compounds were chromatographed on reversed-phase octadecyltrichlorosilane (C_{18}) or diphenyldichlorosilane (phenyl) columns, using mixtures of acetonitrile or absolute methanol and distilled water buffered with ammonium acetate, ammonium chloride, or ammonium carbonate. By calculating approximate resolution values, the separation of selected drug mixtures can be predicted.

Keyphrases □ Diuretic–antihypertensive mixtures—high-pressure liquid chromatographic separation, effect of solvent composition, pH, and stationary phase polarity □ Antihypertensive–diuretic mixtures—high-pressure liquid chromatographic separation, effect of solvent composition, pH, and stationary phase polarity □ Multicomponent dosage forms—high-pressure liquid chromatographic separation of diuretic–antihypertensive mixtures □ High-pressure liquid chromatography—separation, diuretic–antihypertensive mixtures

High-pressure liquid chromatography (HPLC) has been successfully applied to the analysis of multicomponent pharmaceutical dosage forms in this laboratory (1, 2). In a continuation of the study of multicomponent dosage forms, this paper reports an investigation of the parameters associated with the separation and detection of diuretic–antihypertensive agents.

The compounds studied are included in listings of most prescribed drugs used singly or in combination for the treatment of hypertension. The drugs are chlorothiazide, hydrochlorothiazide, triamterene, hydralazine hydrochloride, guanethidine sulfate, methyldopa, and reserpine. A preliminary report on the sequential quantitative analysis of a chlorothiazide–reserpine mixture was published (2).

Chlorothiazide and hydrochlorothiazide in single entity and combination dosage forms have been analyzed by many methods including absorption spectroscopy (3–5), colorimetry (6), amperometry (7), bromatometric and bromo–chlorometric titrimetry (8), complexometric titrimetry (9), and nonaqueous titrimetry (10, 11). Triamterene can be measured by absorption spectroscopy, fluorescence, and TLC fluorometry (12–14), while hydralazine and guanethidine have been assayed by various titrimetric methods (15, 16). Fluorescence methods have been utilized for the determination of methyldopa (17) and reserpine (3, 18). Other procedures reported for these latter drugs include polarography (19) and colorimetry (20, 21).

The HPLC operating parameters investigated were limited to solvent composition, pH, and stationary phase polarity. The use of these data can be readily applied to the separation of selected multidrug dosage forms. This use has been exemplified by the separation and quantification of chlorothiazide–reserpine at unit-dosage levels (2).

EXPERIMENTAL[1]

Reagents and Chemicals—Powdered samples of chlorothiazide[2], hydrochlorothiazide[2], triamterene[3], hydralazine hydrochloride[4], guanethidine sulfate[4], methyldopa[2], and reserpine[4] were used in the analytical procedure. All other chemicals and solvents were the highest grade commercially available.

Mobile Phases—The mobile phases consisted of various concentrations of acetonitrile or absolute methanol mixed with aqueous solutions of 0.1 or 1% ammonium acetate, 0.5 or 1% ammonium chloride, and 0.2 or 1% ammonium carbonate. The solutions were prepared fresh daily.

Drug Solutions—Each drug was prepared as a 0.5% solution using the appropriate mobile phase.

Chromatographic Separation—The degassed mobile phase was pumped through columns containing either monomolecular layers of octadecyltrichlorosilane (C_{18}) or diphenyldichlorosilane (phenyl) chemically bonded to a high-efficiency pellicular packing, consisting of solid glass cores with a porous silica surface[5]. A flow rate of 1.4 ml/min (1000–2000 psig) was used until a stable baseline was obtained. The column and solvents were kept at ambient temperature. Replicate 20-μl injections of the sample solutions were made using a 25-μl syringe[6]. The chart recorder provided a record of the elution of the drugs from the column as peaks on a chromatogram.

RESULTS AND DISCUSSION

The purpose of this study was to determine the operating conditions for HPLC that would optimize the resolution of chemically unrelated therapeutic agents most commonly found in antihypertensive preparations. Three operating parameters were studied: *(a)* variations in solvent composition containing acetonitrile or absolute methanol with changing concentrations of distilled water; *(b)* variations in pH within the 5.5–9.0 range using ammonium acetate, ammonium chloride, or ammonium carbonate as inorganic buffers; and *(c)* variations in the nonpolar (reversed phase) stationary phase in which the pellicular packing differed in polarity.

The effects of these parameters on retention times and peak widths of the drugs are shown in Table I. In addition, multiple entries in Table I indicate those solvent systems (Table II) that generate multiplets for a component. These multiplets may arise from decomposition of the drug and/or the varied species of the drug possible at the mobile phase pH used. Significant tailing of single-drug entities is also noted.

The following generalizations can be inferred from the data in Table I. Retention time for many of the drugs increases as the polarity of the mobile phase increases. This generalization can be obscured when the solute travels as a multiple species such as methyldopa. It is also difficult to observe this effect for drugs that travel close to the void volume of the column and for which it is difficult to measure small differences in retention time. This situation is exemplified by chlorothiazide and hydrochlorothiazide. When

[1] A Waters Associates liquid chromotograph (model ALC 202), equipped with a M-6000 pump, UV (254 nm) and differential refractive index monitors, and Waters packed columns, 1.22 m long × 2.3 mm i.d., was used.

[2] Merck Sharp and Dohme, Rahway, N.J.

[3] Smith Kline and French Labs., Philadelphia, Pa.

[4] Ciba, Summit, N.J.

[5] Corasil/C_{18} and Corasil/phenyl, 37–50 μm, Waters Associates, Milford, Mass.

[6] Model B-110, Precision Sampling Corp., Baton Rouge, La.

Table I—Effect of Mobile Phase Composition on Retention Times[a]

Mobile Phase Composition[b]	Chlorothiazide		Hydrochlorothiazide		Triamterene		Hydralazine		Guanethidine[c]		Methyldopa		Reserpine	
	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl
A		94[d] (26)[f]		95 (33)		107 (28)		131 (64)		372[e] (137)		102 (37) 136 (56)		99 (25)
B		101 (24)		115 (32)		105 (30)		159[e] (222)		449[e] (204)		116[e] (93)		130 (42)
C	90 (20)		95 (24)		108 (20)		102 (52) 141 (44) 333[e] (167)		—[g]		94 (30)		192 (49)	
D		95 (22)		100 (32)		123 (27)		109 (44) 132 (50) 168 (90)		425[e] (123)		105 (63)		219 (66)
E		99 (19)		108 (26)		154 (38)		220 (79)		587[e] (231)		125[e] (105)		—[g]
F	96 (20)	102 (23)	96 (24)	102 (25)	145 (44)	129 (36)	224 (155)	108 (20) 153 (128) 261[e] (715)	185 (67)	103 (39) 483[e] (233)	109 (37) 140 (63)	115 (33) 139 (30) 169 (240)	1950 (800)	1440[e] (1000)
G	92 (22)	92 (18)	92 (27)	92 (22)	100 (24)	101 (39)	123 (92)	98 (23) 112 (43) 135 (46)	125 (37) 153 (56)	150 (35) 243 (57)	98 (82)	95 (24)	98 (23)	99 (20)
H	95 (20)	92 (21)	95 (25)	95 (25)	100 (24)	101 (27)	137[e] (240)	142 (115) 172 (130)	94 (18) 122 (38)	95 (24) 119 (25)	93 (20)	117[e] (138)	138 (35)	120 (30)
I		98 (24)		100 (24)		145 (70)		128 (80) 508[e] (250)		90 (27) 141 (57)		139[e] (140)		—[g]
J	99 (20)	102 (25)	99 (25)	101 (27)	126 (45)	124 (40)	140 (80)	188[e] (100)	99 (27) 180 (96)	102 (30) 299[e] (160)	102 (28)	109 (27)	374[e] (168)	224 (138)

K	113 (25)	108 (37)	271[e] (152)	306[e] (133)	108[e] (30) 435[e] (172)	118 (40)	—[g]					
L	82 (20)	90 (27)	98 (21)	106 (31) 192 (53)	—[g]	91 (22) 115[e] (92)	148 (37) 214 (62)					
M	98 (20)	97 (26)	101 (24)	105 (25)	137 (43)	121 (30)	106 (26) 126[e] (81)	135 (60) 169 (73)	—[g]	—[g]	101 (30) 143[e] (73)	99 (22) 125 (37) 235[e] (298)	104 (75) —[g]	132 (44) 1047 (920)
N	98 (20)	105 (24)	119 (27)	113 (36) 367[e] (443)	—[g]	104 (45) 783[e] (960)	107 (40) 621 (580)					

[a] Time elapsed between injection and attainment of the chromatographic peak maximum. The eluted peaks were monitored using UV detection unless otherwise noted. [b] Solvent compositions and pH are given in Table II. [c] The eluted peaks were monitored using a differential refractive index detector. [d] Retention time expressed as seconds. Variability is ±12 sec. [e] Significant tailing was observed. [f] Peak width expressed as seconds. [g] Retention time greater than 2400 sec.

Table II—Solvent Compositions and pH

Mobile Phase	Components	Solvent Ratio	pH
A	Acetonitrile	80	7.35
	0.1% Ammonium acetate	20	
B	Acetonitrile	60	7.35
	0.1% Ammonium acetate	40	
C	Acetonitrile	50	7.35
	0.1% Ammonium acetate	50	
D	Acetonitrile	40	7.35
	0.1% Ammonium acetate	60	
E	Acetonitrile	20	7.35
	0.1% Ammonium acetate	80	
F	Absolute methanol	50	7.10
	1% Ammonium acetate	50	
G	Acetonitrile	80	6.10
	1% Ammonium chloride	20	
H	Acetonitrile	50	5.57
	1% Ammonium chloride	50	
I	Acetonitrile	20	5.56
	1% Ammonium chloride	80	
J	Absolute methanol	50	5.60
	0.5% Ammonium chloride	50	
K	Absolute methanol	20	5.60
	0.5% Ammonium chloride	80	
L	Acetonitrile	50	8.80
	0.2% Ammonium carbonate	50	
M	Absolute methanol	50	8.45
	0.2% Ammonium carbonate	50	
N	Absolute methanol	50	8.70
	1% Ammonium carbonate	50	

the pH of the mobile phase is adjusted so that the unionized species of the drug predominates, as calculated from its pKa value (15, 22, 23)[7], an increased retention time is noted for many of the drugs. Guanethidine, methyldopa, and triamterene are exceptions to this effect. A comparison of solvents containing equivalent amounts of acetonitrile–water and methanol–water showed that retention times are greater in the methanol-containing solvents. This finding is consistent with the observation of the effect of mobile phase polarity on retention time. The interplay of mobile phase pH and polarity and their effects on either the ionizability of the traveling solute or the ability to solvate the solute may evert the outlined generalizations.

Modifications in the polarity of stationary and mobile phases provided the following observations for the drugs examined. Chlorothiazide and hydrochlorothiazide traveled close to the solvent front in all systems. Triamterene could be separated from the thiazide diuretics in Solvent Systems E and K (Table III)[8]. The remaining solvent systems, such as exemplified by mobile phases I and N, did not adequately resolve these compounds. The drug was resolved best using the more polar mobile phases.

Hydralazine gave multiplets in most solvents. The exceptions were Solvents A, B, E, J, and K. Solvents A and E gave symmetrical peaks while B, J, and K showed tailing.

The aliphatic character of guanethidine precluded its detectability by UV absorption. Thus, differential refractometry was used to monitor the column effluent. Sensitivity was in the range of 50–100 μg for guanethidine. Acetonitrile–water mixtures were most effective in producing single symmetrical peaks for guanethidine at pH 7.35. However, as the pH of the mobile phase was made acidic, a doublet was observed.

Methyldopa was the most difficult to resolve from the other drugs in this study. In systems where the retention time of methyldopa was increased by the addition of water, excessive tailing was obtained. In mobile phases with pH >8, multiple peaks were obtained indicative of the several species of methyldopa possible in basic media. At pH <7, no multiplets were observed. In solvent systems where methyldopa eluted as a single peak, it could not be

[7] Guanethidine pKa value supplied by Dr. J. Mollica, Ciba-Geigy, Suffern, N.Y., personal communication, 1974.

[8] It is possible to calculate the approximate resolution *(Rs)* of two components by the equation: $Rs = 2(t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 are retention times, and w_1 and w_2 are base peak widths of Compounds 1 and 2, respectively. Experience indicates that two components with an *Rs* value >1.5 show satisfactory resolution by this analytical technique.

Table III—Calculation of Approximate Resolution (*Rs*)[a] Values for Triamterene—Thiazide Mixtures

Drug Mixture	Mobile Phase[b] E	K	I	N
Triamterene	154[c]	271	145	119
	(38)[d]	(152)	(70)	(27)
Chlorothiazide	99	113	98	98
	(19)	(25)	(24)	(20)
Rs =	1.9	1.8	1.0	0.89
Triamterene	154	271	145	119
	(38)	(152)	(70)	(27)
Hydrochlorothiazide	108	108	100	105
	(26)	(37)	(24)	(24)
Rs =	1.4	1.7	0.96	0.55

[a] See footnote 8. [b] Letters refer to solvent compositions in Table II. [c] Retention time on phenyl column from Table I. [d] Peak width on phenyl column from Table I.

Table IV—Calculation of Approximate Resolution (*Rs*)[a] Values for Hydrochlorothiazide—Hydralazine—Reserpine Mixture

Drug Mixture	Mobile Phase[b] E	F	K
Hydrochlorothiazide	108[c]	102	108
	(26)[d]	(25)	(37)
Hydralazine	220	224	306
	(79)	(155)	(133)
Rs =	1.95	1.36	2.3
Hydralazine	220	224	306
	(79)	(155)	(133)
Reserpine	>2400	1950	>2400
		(800)	
Rs =	>6.0[e]	3.6	>5.7[e]

[a] See footnote 8. [b] Letters refer to solvent compositions in Table II. [c] Retention time data from Table I. [d] Peak width data from Table I. [e] Calculated value based on retention time of 2400 sec and peak width of 800 sec for reserpine.

resolved from the thiazides. However, solvents systems such as D, J, and K could resolve methyldopa from reserpine; D and K could resolve methyldopa from both guanethidine and reserpine.

At high concentrations of acetonitrile, reserpine traveled with the solvent front. Its retention time was increased as the polarity of the solvent increased. In many instances, the retention time of reserpine was much greater than that of the remaining drugs tested and resulted in considerable band spreading. However, the reserpine peaks were symmetrical and would be suitable for quantification. In solvent systems with pH >8, several additional peaks were detected in addition to the reserpine band. This finding may be due to rapid decomposition of reserpine in alkaline media (24). Table IV illustrates resolution values for the three-component mixture of hydrochlorothiazide, hydralazine, and reserpine in some mobile phases. Solvent Systems E and K seem most suitable for separating the mixture, but E is better since hydralazine exhibits considerable tailing in mobile phase K. A flow programming procedure (changing flow rate with time) would be necessary with Solvent System E to decrease the retention time for reserpine.

Information contained in Table I presents operating parameters for HPLC that can be used to optimize the resolution of the multifarious drugs found in antihypertensive dosage forms. With the calculation of approximate *Rs* values as described previously, the separation of selected drug mixtures can be predicted.

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Identification and Quantitation of Impurities in Dapsone Preparations

G. R. GORDON ˣ, D. C. GHOUL, and J. H. PETERS

Abstract □ Chromatographic and fluorometric procedures were developed to isolate and quantitate small amounts of 2,4′-diaminodiphenyl sulfone and 4-aminodiphenyl sulfone in pharmaceutical preparations of the antileprosy drug 4,4′-diaminodiphenyl sulfone (dapsone). Identification was accomplished by comparison with authentic compounds employing UV absorption, fluorometry, and mass spectrometry in addition to TLC and high-pressure liquid chromatography.

Keyphrases □ Dapsone—isolation, identification, and quantitation, pharmaceutical preparations □ TLC—determination, dapsone impurities in pharmaceutical preparations □ High-pressure liquid chromatography—determination, dapsone impurities in pharmaceutical preparations

Dapsone (4,4′-diaminodiphenyl sulfone, I) has emerged as the most efficacious drug in the therapy of leprosy (1). It is also of value in the suppression of chloroquine-resistant malaria (2, 3).

During studies on the purity of I, some samples were found to be contaminated[1] with 2,4′-diaminodiphenyl sulfone (II), 4-aminodiphenyl sulfone (III), and 4-amino-4′-chlorodiphenyl sulfone (IV). The purpose of this study was to isolate, identify, and quantitate any such impurities in several pharmaceutical preparations of I. These preparations were being used at that time by various clinical collaborators for the treatment of leprosy patients.

EXPERIMENTAL

Reagents and Standards—All reagents were of analytical grade except ethylene dichloride, which was purchased as a high purity solvent[2]. Compound I was technical grade[3] purified by crystallization from 95% ethanol. Compounds II–IV were used as received[1].

TLC and Microbore Column Chromatography—One dapsone tablet of the various pharmaceutical preparations (Table I) was triturated in 100 ml of 70% ethanol, and the insoluble excipients were removed by centrifugation at 2600×*g*. An aliquot of the clarified solution (containing approximately 250 μg of I) was streaked on a 250-μm thick silica gel plate[4]. The chromatogram was developed with chloroform. After air drying, the plate was viewed with a shortwave (254 nm) UV lamp[5]. The plate was then sprayed with a solution of 1 g of *p*-dimethylaminobenzaldehyde in 100 ml of ethanol containing 1.0 ml of concentrated hydrochloric acid.

Microbore column chromatography was performed using a glass column, 300 × 2.75 mm i.d.[6]. The column was slurry packed in small increments with silica gel[7] in ethyl acetate. A mixture of chloroform–carbon tetrachloride (70:30 v/v) was then pumped[8] through at a rate of 0.65 ml/min and 35.15 kg/cm² (500 psi). The absorbance of the column effluent liquid was measured at 280 nm using a UV monitor[9].

For chromatography, aliquots of each tablet solution (equivalent to 300 μg of I) were evaporated to dryness at 50° under a stream of nitrogen[10]. The residue was dissolved in 100 μl of chloroform, and 50 μl was injected into the solvent stream of the microbore column using a pneumatically actuated valve[11]. Individual fractions (≃0.3 ml) containing II and III were collected manually every 30 sec. After 42 fractions were collected, an additional 30-ml aliquot of the effluent liquid containing I was collected in a 50-ml volumetric flask. The latter collection was subsequently adjusted to 50 ml with additional solvent.

The first 42 fractions and 50 μl of the last collection were evaporated to dryness using nitrogen as previously described. To each residue was added 3 ml of ethylene dichloride, and the relative fluorescence was determined[12]. Fractions 1–24, containing III, were measured at 295/405 nm (activation/emission wavelengths); fractions 25–42, containing II, were measured at 295/375 nm; and the aliquot containing I was measured at 285/350 nm. The quantity of each compound was calculated by comparison to reference standards prepared for each chromatographic separation. Recoveries of the reference compounds from the chromatographic column were quantitative.

Spectrometry—UV spectra[13] of II and III isolated from dapsone preparations were obtained by pooling the respective fractions from several separations, evaporating the samples to dryness, and dissolving the residue in 95% ethanol. Reference spectra of authentic II and III were obtained in the same solvent. The fluorescent activation and emission spectra of II and III isolated by column chromatography were obtained from small aliquots of the ethanolic solutions used for UV spectrometry. After evaporating the ethanol, the residue was dissolved in ethylene dichloride. Similar spectra were obtained for authentic II and III. The mass spectra of II and III (authentic samples and isolated compounds) were obtained by subjecting samples to GC and then introducing the pure sulfone into the mass spectrometer[14] as it emerged from the gas chromatograph.

RESULTS

After TLC, Compounds I, II, III, and IV exhibited R_f values of 0.00, 0.11, 0.17, and 0.20, respectively. Compounds I and II exhibited a blue fluorescence, and Compounds III and IV yielded a yellowish-blue fluorescence under UV light. All yielded a yellow-to-orange color with the spray reagent. When aliquots of the various preparations of I were chromatographed, only bands corresponding to I, II, and III were detected. The retention times of I, II, and III after column chromatography were 44, 14, and 8 min, respectively. Each compound exhibited a linear relationship between peak height and quantity injected over the range of 0.5–2.0 μg. Only three peaks were found in the pharmaceutical preparations tested; they exhibited retention times identical to those of I, II, and III.

Because the fluorescence of all sulfones was considerably greater than their UV absorption per unit weight, all quantitative estimations were based upon fluorescent measurements. Figure 1 shows a

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[7] Silica gel 7, J. T. Baker Co., Phillipsburg, N.J.
[8] Milton-Roy minipump, Milton Roy Co., St. Petersburg, Fla.
[9] Model UA-2, Instrumentation Specialties Co., Lincoln, Nebr.
[10] N-Evap model 105, Organomation Associates, Shrewsbury, Mass.
[11] Model SVA-8031, Chromatronix, Inc., Berkeley, Calif.
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[13] Model 220 spectrophotometer, Gilford Instrument Laboratories, Oberlin, Ohio.
[14] Model 9000 gas chromatograph–mass spectrometer, LKB Instruments, Rockville, Md.

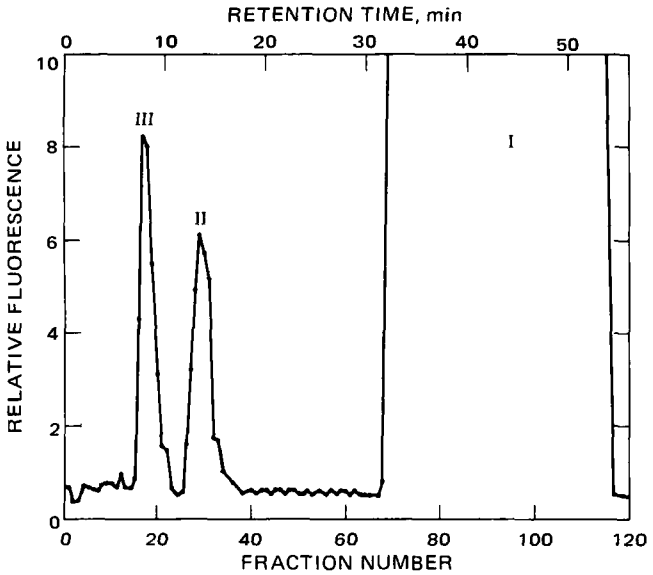

Figure 1—*Typical chromatographic separation and fluorometric quantitation of I, II, and III found in a pharmaceutical preparation of dapsone. The fluorescence of fractions 14–24 was determined at 295/405 nm (activation/emission wavelengths), of fractions 25–42 at 295/375 nm, and of fractions 67–116 at 285/350 nm.*

typical elution pattern of a dapsone preparation when the fluorescence of each fraction was measured. During each separation, I was always present in large amounts (150 μg) so the elution volume containing I (fractions 65–120) was routinely collected as a single sample beginning after fraction 42.

Figure 2 shows calibration curves obtained for I, II, and III in ethylene dichloride *via* fluorometry. The sensitivities (fluorescent intensity units per nanogram per milliliter) were 0.372, 0.236, and 0.173, respectively. Coefficients of variation for these sensitivity factors were 4.6, 3.9, and 6.3%, respectively, for 1 week of daily determinations. The lower limit of detection of II and III in 150 μg of I was about 0.05%.

Compound I exhibited fluorescence maxima of 285/350 nm (activation/emission) in ethylene dichloride (4). The recovery of I from the six pharmaceutical preparations averaged 96.9% (range 91.8–99.2%).

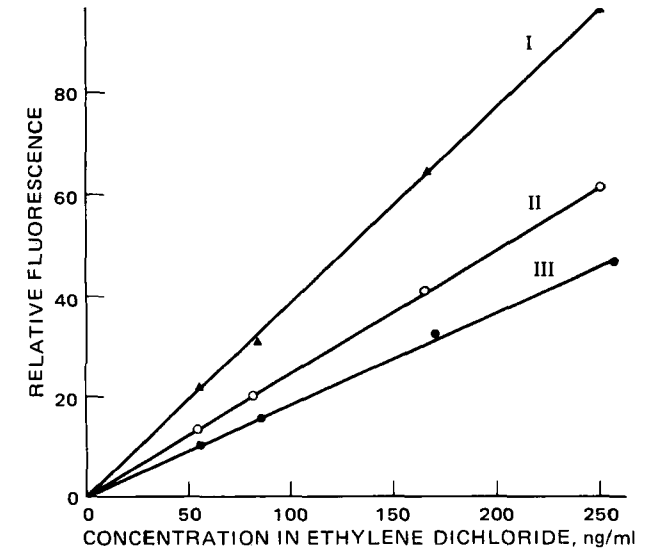

Figure 2—*Fluorescence calibration curves for I, II, and III. The activation/emission wavelengths were 285/350, 295/375, and 295/405 nm, respectively.*

Table I—Quantities of Impurities in Dapsone Preparations

Manufacturer[a]	Formulation	Percent of Impurity[b] II	III
1	25-mg tablet	0.16	0.64
1	50-mg tablet	0.31	0.76
2	25-mg tablet	0.49	0.65
2	50-mg tablet	0.80	0.99
3	100-mg tablet	0.35	0.51
4	100-mg tablet	0.20	0.27
5	Crystalline	3.3	2.7
6	Crystalline, technical grade	<0.05	0.70
6	Crystalline, technical grade, recrystallized	<0.05	0.18

[a] 1, P.C.B., Brussels, Belgium; 2, Laboratoire Lafran, Paris, France; 3, Ayerst Laboratories, New York, N.Y.; 4, Imperial Chemical Industries, Cheshire, England; 5, K&K Laboratories, Hollywood, Calif.; and 6, Merck & Co., Rahway, N.J. [b] Relative to the quantity of dapsone.

Compound II exhibited a UV_{max} at 280 nm, and the uncorrected maximum fluorescent wavelengths were 295/375 nm. The characteristics of the peak eluting at 14 min were identical to II, and its mass fragmentation spectrum was also identical to II. Since two other isomers of I, 3,4′- and 3,3′-diaminodiphenyl sulfone, yield mass spectral fragmentation patterns significantly different from I and II, possible confusion among these isomers and I and II is eliminated.

Compound III exhibited a UV_{max} at 290 nm, and its fluorescent characteristics were 295/405 nm. The peak that eluted at 8 min yielded these same characteristics in addition to exhibiting the same mass fragmentation pattern as authentic III.

The percentages of II and III found in several preparations of I are shown in Table I.

DISCUSSION

In the six tablet preparations of I, the quantity of II averaged 0.38% while the level of III averaged 0.64%. In one crystalline preparation (not pharmaceutical grade), the amounts of II and III were found to be considerably greater. The uncrystallized technical grade preparation contained 0.70% III, and the level of II was below the sensitivity of the procedure. On recrystallization, the level of III in this preparation was reduced to 0.18%.

The antimicrobial activities of II and III were compared with I. Using an *in vitro* assay method employing *Mycobacterium* sp. 607, Colwell *et al.* (5) showed that neither compound was as effective in preventing growth at concentrations at which I was very active. Likewise, the *in vivo* mouse foot pad test system showed that II and III were inactive in suppressing the multiplication of *M. leprae*[15] wherein I is very active (6).

The therapeutic regimen for the treatment of leprosy is generally 50 or 100 mg of I/day. Thus, preparations of I containing the levels of II and III found in these studies would result in a daily ingestion of less than 1.0 mg of either II or III. Although these quantities appear to be small compared with the daily dose of I, the long-term effects may be important because therapy using I in leprosy patients usually extends for many years and, in some cases, for the lifetime of the patients.

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Rapid Fluorometric Determination of Procainamide Hydrochloride Dosage Forms

HENRY S. I. TAN [x] and CHERYL BEISER

Abstract □ A fluorometric procedure for procainamide hydrochloride was developed, and it offers improvements in ease, speed, and sensitivity over the official method. The new procedure is based on the reaction with fluorescamine in aqueous medium at pH 7.5 to form a fluorophore, with activation and emission wavelengths of 400 and 485 nm, respectively. The fluorescence is linear (r = 0.999) over the 0.04–1-μg/ml concentration range and is stable for at least 2 hr. Recovery data appeared to be accurate, quantitative, and reproducible. The overall recovery was 99.8% with a standard deviation of ±1.14 (n = 5). The method was successfully applied to commercially available dosage forms.

Keyphrases □ Procainamide hydrochloride—rapid fluorometric determination, bulk and dosage forms, fluorescamine reagent □ Fluorometry—analysis, procainamide hydrochloride dosage forms □ Fluorescamine—reagent in fluorometric analysis of procainamide hydrochloride dosage forms

The USP XVIII (1) assay procedure for procainamide hydrochloride capsules is time consuming, since it is based on a prior separation of the drug from the dosage form followed by a diazotization titration of the compound with standard sodium nitrite at low temperatures and starch iodide paper as the external indicator. This method is subject to variations between individuals in determination of the end-point. The assay method (2) for procainamide hydrochloride injections is also lengthy, since the sample must be evaporated and dried before the drug can be analyzed by nonaqueous titrimetry with standard perchloric acid.

Recently, it was reported (3–5)[1] that 4-phenylspiro[furan-2(3*H*),1′-phthalan]-3,3′-dione (fluorescamine) is a reagent for the detection of primary amines in the picomole range. Its reactions with amines is almost instantaneous at room temperature in aqueous solutions. The resulting fluorophores are fluorescent, whereas the reagent and its degradation products are nonfluorescent. This report presents a sensitive, simple, and accurate method for the assay of procainamide hydrochloride and its dosage forms with fluorescamine in an aqueous medium.

EXPERIMENTAL

Instruments—The following were used: a fluorescence spectrophotometer[2] with 1-cm cells, a mixer[3], and an analytical balance[4]. The response of the fluorescence spectrophotometer was calibrated daily, using a 0.5-μg/ml quinine sulfate solution in 0.1 *N* sulfuric acid, at activation and emission wavelengths of 350 and 445 nm, respectively.

Materials and Reagents—The following were used: procainamide hydrochloride[5], acetone[6], a 0.15% (w/v) solution of fluorescamine[7] in acetone, and pH 7.5 phosphate buffer. This buffer solution was prepared by adding 40.8 ml of 0.2 *M* sodium hydroxide to 50.0 ml of 0.2 *M* monobasic potassium phosphate in a 200-ml volumetric flask and diluting to volume with water. The pH of the resulting buffer solution was checked with a pH meter. All chemicals were analytical grade.

Preparation of Standard Curve—A stock solution of procainamide hydrochloride was prepared by dissolving 50.0 mg of the compound in 1000 ml of water. Further dilutions were made to obtain procainamide hydrochloride standard solutions containing 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 μg/ml. One milliliter of each solution was utilized for fluorescence development as described under *Assay Procedure for Procainamide Hydrochloride*. The stock and standard solutions were stable for at least 3 weeks.

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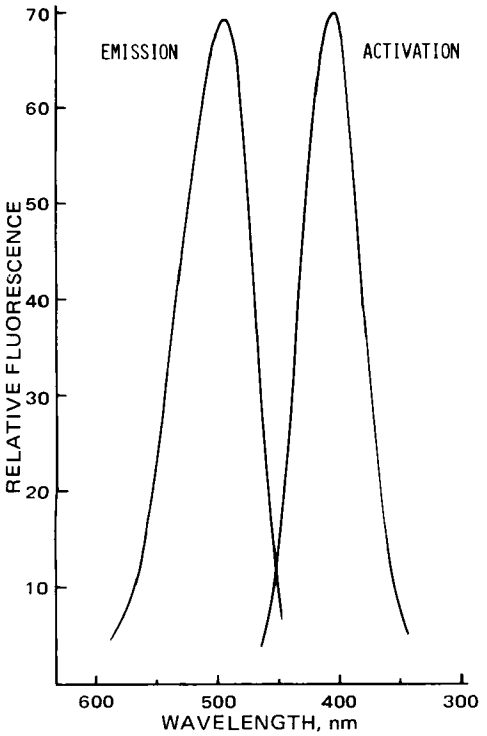
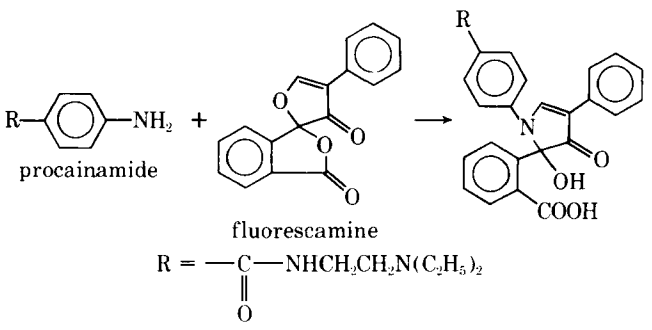


Figure 1—*Activation and emission spectra of procainamide hydrochloride–fluorescamine fluorophore.*

rescamine solution *directly into the whirling solution.* After 10 sec, measure the fluorescent intensity at activation and fluorescence wavelengths of 400 and 485 nm, respectively, against a blank prepared similarly but without the procainamide hydrochloride. Read the concentration of procainamide from a calibration curve.

Assay Procedure for Procainamide Hydrochloride Capsules—Weigh, as completely as possible, the contents of 20 capsules. Weigh accurately a portion of the powder, equivalent to about 100 mg of procainamide hydrochloride, and transfer to a 1000-ml volumetric flask[8]. Add water to the mark and shake well for a few minutes. Filter the mixture through fluted filter paper, rejecting the first 10 ml of filtrate. Pipet 1.0 ml of the filtrate into a 100-ml volumetric flask and dilute to volume with water. Pipet 1.0 ml of this solution into a 25 × 200-mm test tube and proceed as described under *Assay Procedure for Procainamide Hydrochloride*, beginning with "To this solution, add . . . ."

Assay Procedure for Procainamide Hydrochloride Injections—Pipet 1.0 ml of a 10% procainamide hydrochloride injection into a 1000-ml volumetric flask and dilute to volume with water. Transfer 1.0 ml of this solution into a 100-ml volumetric flask and dilute to volume with water. Pipet 1.0 ml of the resulting solution into a 25 × 200-mm test tube and proceed as described under *Assay Procedure for Procainamide Hydrochloride*, beginning with "To this solution, add . . . ."



Scheme I

[8] The portion of the powder must come from a representative distribution of the contents of the 20 capsules.

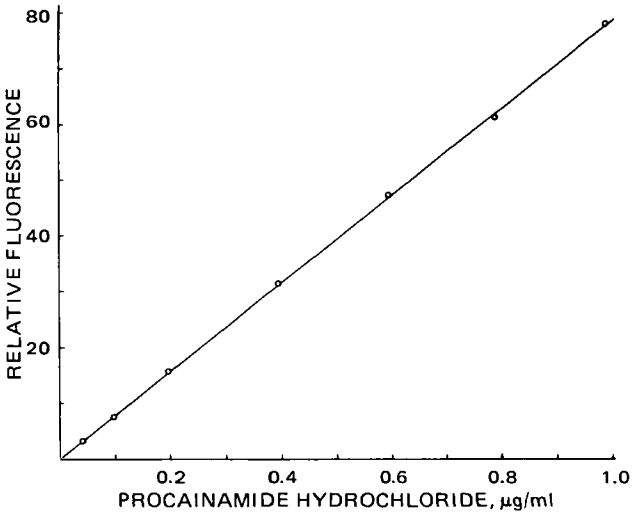


Figure 2—*Relationship between fluorescence and concentration of procainamide hydrochloride.*

Table I—pH Dependency of Fluorophore Using 0.3 μg/ml Procainamide Hydrochloride

pH	Relative Fluorescence Intensities
Borate buffer	
10.0	5.5
9.5	8.0
9.1	13.0
8.6	15.5
8.1	23.5
Phosphate buffer	
8.0	29.7
7.8	28.7
7.5	28.7
7.0	28.7

Table II—Effect of Time on Fluorescence Intensity of Fluorophore[a]

Minutes	Relative Intensity[b]
3	32.5
15	33.0
30	32.8
45	32.0
60	31.8
75	31.8
90	31.5
105	31.5
120	31.2

[a] Procainamide hydrochloride concentration about 0.1 μg/ml. [b] Instrument settings: $\lambda_{activation}$, 400 nm; $\lambda_{emission}$, 485 nm; selector, X1; and sensitivity, 4.

Table III—Effect of Fluorescamine Reagent Solution on Fluorescence Intensity of Fluorophore[a]

15% (w/v) Fluorescamine in Acetone, ml	Relative Intensity[b]
0.17	3.2
0.25	24.0
0.50	28.2
1.00	12.5

[a] Procainamide hydrochloride concentration about 0.08 μg/ml. [b] Instrument settings: $\lambda_{activation}$, 400 nm; $\lambda_{emission}$, 485 nm; selector, X1; and sensitivity, 4.

Table IV—Reproducibility of Fluorophore Development of Replicate Procainamide Hydrochloride Samples Containing 0.4 μg/ml

Solution	Relative Intensity[a]
1	54.2
2	56.5
3	54.4
4	54.8
5	55.1
6	55.9
7	56.0
8	54.6
9	54.5
Average	55.1
Standard deviation	0.822
Coefficient of variation	1.49%

[a] Instrument settings: $\lambda_{activation}$, 400 nm; $\lambda_{emission}$, 485 nm; selector, X1; and sensitivity, 4.

was $y = 0.161x - 0.643$ with a standard error of the estimate of y on x of 1.36 and standard errors of the estimate of the intercept and slope of 1.06 and 0.0018, respectively. The fluorophore produced appeared to be stable for at least 120 min (Table II).

The concentration optimum of the fluorescamine reagent was determined by adding varying volumes of 0.15% fluorescamine in acetone to a series of 25 × 200-mm test tubes, each containing 1.0 ml of 0.5 μg/ml procainamide hydrochloride, and measuring the fluorescence against a blank[10] after diluting each solution to a total volume of 2.5 ml with pH 7.5 phosphate buffer. The results (Table III) indicated that 0.5 ml of the fluorescamine reagent gave the highest response. It was also established that, at constant volume, within certain limits the amount of pH 7.5 phosphate buffer did not affect the fluorescence.

The precision of the assay procedure was determined by running replicate studies on nine 1.00-ml aliquots of a standard procainamide hydrochloride solution containing 1 μg/ml. Each solution was assayed by the proposed procedure. The coefficient of variation for the nine replicate samples was 1.49% (Table IV).

Table V—Results of Analysis of Known Concentrations of Procainamide Hydrochloride

Amount Weighed, mg	Analyzed at Concentration Level, ng/ml	Amount Found[a], mg		Recovery, %	
		Proposed Method	USP XVIII Method	Proposed Method	USP XVIII Method
40.6	162	40.0		98.5	
52.4	210	51.8		98.9	
123.8	495	123.2		99.5	
189.5	758	191.3		100.9	
221.8	887	224.0		101.0	
205.9			200.4		97.3
203.1			198.6		97.8
Overall recovery, %				99.8	97.6
Standard deviation				1.14	

[a] Average of duplicate assays.

Table VI—Results of Analysis of Commercially Available Procainamide Hydrochloride Dosage Forms

Dosage Form	Amount of Procainamide Hydrochloride Claimed, mg/Capsule or mg/ml	Amount Found[a], mg		Percent of Claim	
		Proposed Method	USP XVIII Method	Proposed Method	USP XVIII Method
Capsule	250	241.2	237.9	96.5	95.2
Capsule	500	494.6	492.8	98.9	98.6
Injection	100	99.4	97.8	99.4	97.8

[a] Average of duplicate assays.

RESULTS AND DISCUSSION

The reaction between procainamide and fluorescamine (Scheme I) presumably proceeds in several steps. There is a Michael addition of the amine to the acrylic function of fluorescamine, followed by ring closure and opening of the lactone ring.

The fluorophore obtained from the interaction between procainamide hydrochloride and fluorescamine shows activation and fluorescence wavelengths of 400 and 485 nm, respectively (Fig. 1), between pH 7.0 and 10.0. However, the fluorescence intensity is a function of the pH when alkaline borate buffer (pH 8.0–10.0) is used. In this case, the intensities decrease with increasing pH (Table I). When the phosphate buffer (pH 7.0–8.0) is used, the fluorescent intensities appear to be constant and are of greater magnitude than those in borate buffer (Table I). Based on these findings, the reaction was carried out in pH 7.5 phosphate buffer.

Under the experimental conditions, a linear relationship existed between fluorescent intensity and concentration over the 0.04–1-μg/ml concentration range (Fig. 2), with a correlation coefficient of 0.999. Regression analysis[9] showed that the regression equation

As little as 0.043 μg of procainamide hydrochloride can be assayed by the proposed method. The method, however, is reliable only for concentrations above 0.05 μg. Data presented in Table V indicate the accuracy of the procedure. The overall recovery for the six samples was 99.8% with a standard deviation of ±1.14. In comparison, the average recovery for two samples analyzed by the USP XVIII method was 97.6%.

Results obtained by applying the proposed fluorescence procedure to commercially available procainamide hydrochloride dosage forms are presented in Table VI. Comparison of the experimental data with those obtained by the compendial method shows a good correlation. Particularly in the analysis of the parenteral solution, the proposed procedure offers considerable improvement in speed over the USP XVIII method. Evaporation of the sample, which requires at least 60–75 min, and drying of the residue as directed by USP XVIII are completely eliminated.

It was also established that in the assay of the capsules the emptied capsules need not be soaked and agitated for 15 min as directed by the USP procedure. As long as care was taken to empty the

[9] Programa 101 Minicomputer, Olivetti Underwood Corp.

[10] Each blank contained the same amount of fluorescamine as in the solution studied but contained no procainamide hydrochloride.

250- or 500-mg capsules as completely as possible, the recovery appeared to be satisfactory (Table VI). Another advantage of the present method is the ease of performing the assay. The fluorescamine reagent is stable for several weeks. In contrast, the USP XVIII method for the capsules requires freshly prepared sodium nitrite solutions.

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GLC Determination of 17α-Ethynylestriol 3-Cyclopentyl Ether

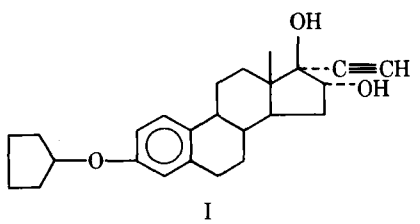
RAFIK H. BISHARA [x], BONNIE S. RUTHERFORD, and ALAN DINNER

Abstract □ A rapid, sensitive, and accurate GLC method of analysis of a new estrogenic drug, 17α-ethynylestriol 3-cyclopentyl ether, was developed. The drug and the internal standard, tetratriacontane, are dissolved in chloroform, and an aliquot is heated with *N*-trimethylsilylimidazole at 80° for 30 min. The silylated sample is chromatographed using a column packed with 1% methyl vinyl silicone gum on Gas Chrom Q. Quantitation is achieved by computer calculation of the peak area ratios. The observed peak is the 16α,17β-bistrimethylsilyl derivative of the new drug substance. The GLC method was applied to the quantitative determination of the estrogenic compound in a tablet formulation containing 25 μg/tablet.

Keyphrases □ 17α-Ethynylestriol 3-cyclopentyl ether—GLC analysis as trimethylsilyl derivative □ Estrogens—GLC analysis of 17α-ethynylestriol 3-cyclopentyl ether □ GLC—analysis, 17α-ethynylestriol 3-cyclopentyl ether

A new potent estrogenic hormone, 17α-ethynylestriol 3-cyclopentyl ether (I), useful in the treatment of spontaneous or induced menopausal syndrome, estrogen deficiency, or conditions when estrogen may be used therapeutically, has been synthesized (1, 2).

In the process of developing a GLC method for analyzing I, the literature concerning the GLC determination of estrogens was screened. Numerous publications deal with the GLC determination of estriol, both as the pure compound (3–6) and in biological materials (7–29). Although there are several reports of the GLC of free estriol (4, 5, 15, 17, 27), the use of estriol derivatives is preferred to prevent irreversible adsorption on the column and/or thermal decomposition of the underivatized hormone (30, 31). Higher volatility of the steroid derivative results in a shorter retention time, sharper peak, and improved sensitivity (31, 32).



I

The trimethylsilyl (21, 22, 25, 28, 33), acetate (19, 23, 24, 29, 33), heptafluorobutyrate (14, 34), and trifluoroacetate (6, 35, 36) derivatives are the most frequently used. The advantages of the trimethylsilyl derivatives relative to the acetate derivatives were discussed (37, 38). Other derivatives that were also reported for the GLC determination of estriol include the 3-methyl ether (39, 41), monochloroacetate (42), chloromethyldimethyl ether (43), chlorodifluoroacetate (43), 3-methyl-16,17-diacetate (44–46), 3-methyl-16,17-diheptafluorobutyrate (7), and 3-methyl-16,17-ditrimethylsilyl (13, 20, 39, 44) and iodomethyldimethylsilyl ethers (47). Several reported procedures were tried with no success for the analysis of I in this study. The purpose of this paper is to report the trimethylsilyl derivatization and GLC conditions for the quantitation of I.

EXPERIMENTAL

Equipment—A gas chromatograph[1] equipped with a flame-ionization detector was used. The detected signals were fed to a computer[2] for peak area integration and to a potentiometric recorder[3] with a chart speed of 38.1 cm (15 in.)/hr and a 1-sec full-scale response. Helium, with a flow rate of 60 ml/min, was the carrier gas. The flow rates of hydrogen and oxygen were adjusted to optimum sensitivity and low noise background in the electronic integrator and were found to be 40 and 300 ml/min, respectively.

[1] F&M model 402, Hewlett-Packard, Avondale, Pa.
[2] Hewlett-Packard 2100.
[3] Honeywell Electronic 16, Honeywell, Philadelphia, Pa.

250- or 500-mg capsules as completely as possible, the recovery appeared to be satisfactory (Table VI). Another advantage of the present method is the ease of performing the assay. The fluorescamine reagent is stable for several weeks. In contrast, the USP XVIII method for the capsules requires freshly prepared sodium nitrite solutions.

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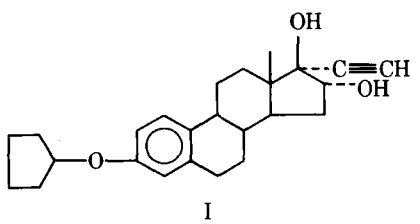

I

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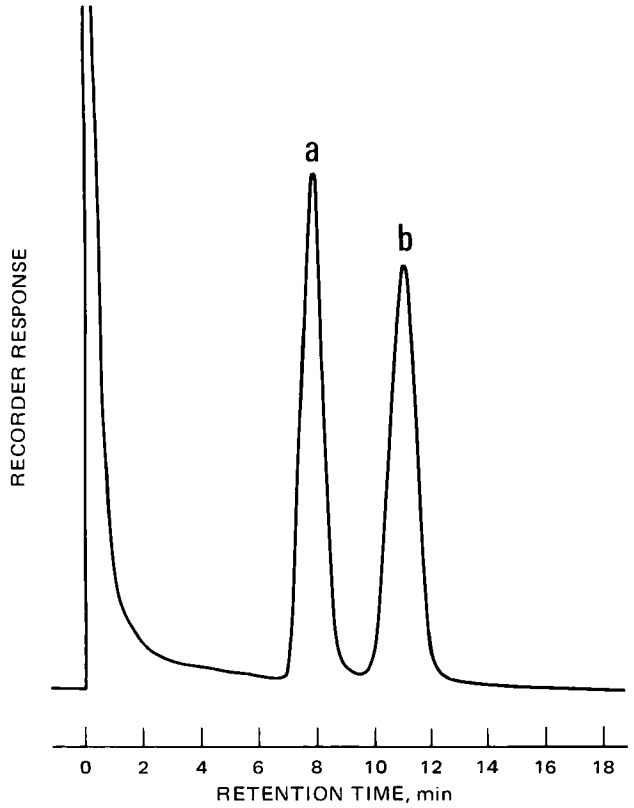

Figure 1—*Typical gas chromatogram of trimethylsilyl-derivatized Compound I. Key: a, 3-(cyclopentyloxy)-16α,17β-bis[(trimethylsilyl)oxy]-19-nor-17α-pregna-1,3,5(10)-trien-20-yne; and b, tetratriacontane.*

The U-shaped glass column (61 cm × 3 mm i.d.) was packed with 1% methyl vinyl silicone gum (UC-W98) on 80–100-mesh Gas Chrom Q[4]. The operating temperatures were: injection port, 260°; column, 225° (isothermal); and detector, 280°. All injections were made using a 10-μl syringe[5].

Reagents—The silylating reagent was *N*-trimethylsilylimidazole[6]. For the tablet assay, the reagent was diluted 1:5 with methylene chloride[7] previously dried over anhydrous sodium sulfate. The reference standard solution was prepared in chloroform[8]. Spectroquality carbon tetrachloride[9] was used for extracting the tablets.

Internal Standard Solution—*n*-Tetratriacontane[10], 2.4 mg/ml in chloroform, was used.

Reference Standard Solution—A I solution containing 2.0 mg/ml of chloroform was prepared. One-milliliter aliquots of the reference standard and internal standard solutions were pipetted and mixed together in a small screw-capped vial sealed with a Teflon septum. One milliliter of the silylating reagent was added by a tuberculin syringe[11], and the vial was heated for 30 min at 80° in a constant-temperature heating block[12]. The vials were then removed from the heating block, cooled to room temperature, and submitted for GLC.

Raw Material Solution—The raw material sample was prepared exactly as the reference standard solution.

Tablet Solution—The average tablet weight of 20 tablets was determined. A portion of the powdered tablet material, equivalent to 25 μg of I, was suspended in 10 ml of 0.05 *N* hydrochloric acid and extracted with three 30-ml portions of carbon tetrachloride.

[4] Custom-made nontested packing, Applied Science Inc., State College, Pa.
[5] Hamilton No. 701, Reno, Nev.
[6] Pierce Chemical Co., Rockford, Ill.
[7] ACS reagent, Matheson, Coleman and Bell, Norwood, Ohio.
[8] Analytical reagent, Mallinckrodt, St. Louis, Mo.
[9] Matheson, Coleman and Bell, Rockford, Ill.
[10] Analabs, North Haven, Conn.
[11] B-D Yale tuberculin, Becton, Dickson and Co., Rutherford, N.J.
[12] Temp-Block module heater, holes with 1.5-cm diameter, Lab-Line Instruments, Melrose Park, Ill.

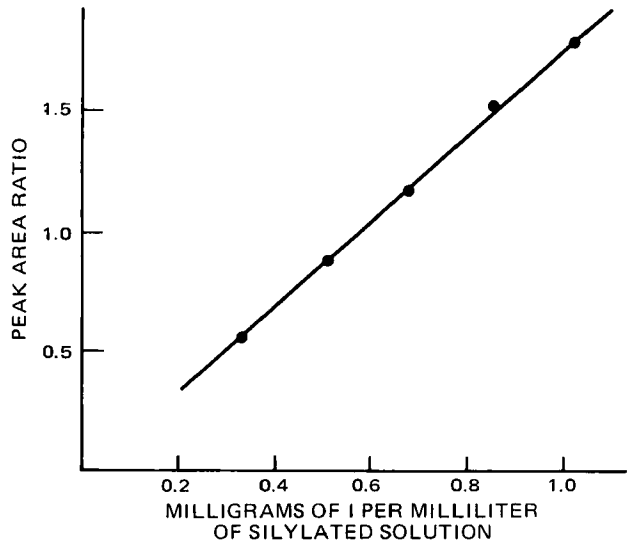

Figure 2—*Calibration curve demonstrating linear response of different amounts of trimethylsilyl Compound I to a constant concentration of tetratriacontane.*

One milliliter of internal standard solution (25 μg/ml) was added, and the solution was evaporated to near dryness. Then the remaining solution was transferred quantitatively to a small glass vial[6], 3.5-ml capacity, and evaporated.

A 0.25-ml aliquot of the silylating mixture was added to the residue, and the vial was sealed and heated as described for the reference standard. The reference standard solution used for the tablet assay was 25 μg/ml. One milliliter of this solution was mixed with 1 ml of the internal standard solution (25 μg/ml) in a small glass vial. After evaporation, 0.25 ml of the silylating mixture was added; then the vial was sealed and heated.

GLC—Four 3-μl portions of the standard solution were injected into the chromatograph to prime the column, and the instrument parameters were adjusted to obtain optimum response of the two peaks. The retention time was approximately 8.0 min for silylated I and 11.0 min for the internal standard, tetratriacontane.

When optimum conditions were obtained, duplicate injections were made of the standard solution, the sample solution, and the standard solution again. The peak areas obtained from the electronic integrator were used for the calculation of the percent purity of the raw material sample as shown in Eq. 1. Tablet potency was calculated as in Eq. 2.

GLC–Mass Spectrometry—A 70-ev electron-impact mass spectrum of the observed GLC peak was obtained[13].

Calculation—The ratio of the sample peak to the internal standard peak is calculated for each injection, and the duplicate ratios are averaged:

$$\%\ \text{purity} = \frac{R_{sa}}{R_{std}} \times \frac{\text{standard weight (mg)}}{\text{sample weight (mg)}} \times \text{standard purity} \times 100 \quad \text{(Eq. 1)}$$

where R_{sa} is the ratio of the silylated I peak area to tetratriacontane peak area in the sample solution, and R_{std} is the average ratio of the silylated I peak area to the tetratriacontane peak area of the standard solution before and after sample injections.

The potency of I in micrograms per tablet is calculated as follows:

$$\text{micrograms per tablet} = \frac{R_{sa}}{R_{std}} \times \frac{\text{standard weight } (\mu g)}{\text{sample weight (mg)}} \times \text{standard purity} \times \text{average tablet weight (mg)} \quad \text{(Eq. 2)}$$

where R_{sa} and R_{std} are defined as in Eq. 1.

[13] LKB-9000S gas chromatograph–mass spectrometer, LKB-Produkter AB, Bromma, Sweden.

Table I—Identity and Chemical Names of Some Closely Related Steroids to Compound I

Compound Identity[a]	Chemical Name
P	3-Hydroxyestra-1,3,5(10)-trien-17-one (estrone)
P	Estra-1,3,5(10),16-tetraene-3,17-diol diacetate
P	16α,17α-Epoxyestra-1,3,5(10)-triene-3,17β-diol diacetate
P	3,16α-Dihydroxyestra-1,3,5(10)-trien-17-one diacetate
P	19-Nor-17α-pregna-1,3,5(10)-trien-20-yne-3,16α,17-triol
P	19-Norpregna-1,3,5(10)-trien-20-yne-3,16α,17α-triol
P	16α,17α-(Isopropylidenedioxy)-19-norpregna-1,3,5(10)-trien-20-yn-3-ol
PDP	3-(Cyclopentyloxy)-17β-hydroxyestra-1,3,5(10)-trien-16-one
PDP	3-(Cyclopentyloxy)-2',3'-dihydroxyestra-1,3,5(10)-trieno-(16α,17α-*b*)-furan-17β-ol
RFS	3-Methoxy-19-nor-17α-pregna-1,3,5(10)-trien-20-yn-17-ol
RFS	19-Nor-17α-pregna-1,3,5(10)-triene-3,16α,17-triol
RFS	3-(Cyclopentyloxy)-16α,17α-(isopropylidenedioxy)-19-norpregna-1,3,5(10)-trien-20-yne

[a] P = synthetic precursor, PDP = probable degradation product, and RFS = related foreign steroids.

RESULTS AND DISCUSSION

A typical gas chromatogram is shown in Fig. 1. The first peak is the trimethylsilyl derivative of I, which is identified as 3-(cyclopentyloxy)-16α,17β-bis[(trimethylsilyl)oxy]-19-nor-17α-pregna-1,3,5(10)-trien-20-yne (II) by GLC–mass spectrometry. The mass spectrum of II shows a molecular ion at *m/e* 524, indicating silylation of the two hydroxy groups on ring D at the 16- and 17-positions. The observed fragmentation pattern is analogous to that described for the trimethylsilyl ether derivatives of estriol and other related steroids (48, 49). The second peak in the chromatogram is tetratriacontane, the internal standard. Relative to the internal standard peak, the retention time of II is 0.73.

Figure 2 shows the linear relationship of various amounts of I (0.34–1.02 mg/ml) to a constant amount of tetratriacontane (0.8 mg/ml).

Twelve related steroids were chromatographed using this method. Table I shows the chemical names of these related compounds and identifies them as precursors, possible degradation products, or related foreign steroids. All 12 tested compounds showed shorter retention times than that of II. Thus, the synthetic precursors and the known degradation products of I do not interfere with its GLC analysis.

Duplicate injections of five different raw material samples by a single analyst give a standard deviation of 0.004 and a relative standard deviation (*SD*/mean × 100) of 0.322%. The accuracy and precision of the tablet assay were determined by analyses of five replicate samples. A relative standard deviation of 0.80% and a relative error of −0.20% were obtained.

Aged and degraded samples of tablets containing I were evaluated by this method. The assay values show a decrease on aging for 1 year at high temperature (50°). No interferences or extraneous peaks were observed on the chromatograms.

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PHARMACEUTICAL TECHNOLOGY

Effect of Physical Properties on Compression Characteristics

E. SHOTTON [x] and B. A. OBIORAH [*]

Abstract □ The transmission of force to the die wall was measured by a piezoelectric sensor, and the compression cycles of lactose granules of different shapes were compared. In addition, a nearly spherical fraction of spray-dried lactose was similarly compared with a crystalline sample. Better tablets were formed when the conversion of axial to radial pressure was high and the residual pressure on the die wall remained after removal of the top punch. With acetaminophen and phenacetin, the pressure on the die wall was low, as was the residual pressure, and capping occurred in both cases. With direct compression acetaminophen, higher die wall pressure was produced and capping did not occur. It is considered that these results can be explained by the ease with which the more nearly isodiametric particle can rearrange under pressure and by the elastic properties of the solid.

Keyphrases □ Lactose granules—effect of physical properties on compression characteristics □ Compression of lactose granules—effect of physical properties compared □ Die wall pressures—compared for various lactose granules, effect on tablet compressibility

The effect of crystal habit and particle shape upon the properties of cubic and dendritic crystals of sodium chloride and the tablets produced from these crystals was previously examined (1). For any particular material, the difference between applied and lower punch pressure depends on the coefficient of friction at the die wall and on the radial pressure.

Working with sodium chloride, aspirin, and hexamine (methenamine), Shotton and Ganderton (2) postulated that capping of hexamine was due to the failure of the material induced by axial elastic recovery after compression force was removed, and this capping occurred when the interparticulate bonds were sufficiently strong to resist separation of the crystals.

EXPERIMENTAL

The following materials were used: phenacetin[1], acetaminophen[2], direct compression acetaminophen[3], spray-dried lactose[4], and crystalline lactose[5]. Lactose granules[6] with the following composition by weight were also used: lactose, 50%; sucrose, 33%; maize starch, 16%; and magnesium stearate, 1%.

A vibratory sieving machine was used to obtain 30–40-mesh lactose granules, acetaminophen crystals, and phenacetin crystals, and an air jet sieve was used to obtain a 75-μm fraction of direct compression acetaminophen. The 40–45-μm fraction of the spray-dried and crystalline lactose powders was separated using a zig-zag classifier[7]. All materials were dried at 60° for 4 hr in a hot air oven and stored in wax-sealed screw-capped jars.

Different shape fractions of the lactose granules were obtained from the shape-sorting table described by Ridgway and Rupp (3). In Table I, 1, 4, 8, and 12 refer to the fractions obtained at these stations of this machine. The smaller the number of the station, the more spherical was the shape of the particles; the larger the number of the station, the more angular were the particles. The shape coefficient for the shape fractions of lactose granules was calculated from the average values of the particle volume, surface area, and projected diameter using the method of Heywood (4) as modified by Ridgway and Rupp (3).

Shape factor determinations were not carried out for the lactose powders because it was not possible to count accurately such fine particles. Microscopic examinations, however, showed that the spray-dried lactose was composed of spheroidal particles and that the crystalline lactose was highly angular. The shape-sorting table was unsuitable for the needle-shaped crystals of acetaminophen and phenacetin because these were broken down by vibration. The values of shape coefficient for lactose granules are presented in Table II.

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[2] Paracetamol crystals BP, Graesser Salicylates Ltd.
[3] Graesser Salicylates Ltd.
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Table I—Details of Tableting Parameters[a] at Highest Machine Setting

Material	Sieve or Particle Size	*Fa*	*Fb*	*Fd*	*Fe*	*Fc*	Porosity	*R*
Spray-dried lactose	40—45 μm	19,044	18,400	644	878	82	15.8	0.9662
Crystalline lactose	40—45 μm	17,638	16,844	794	822	78	16.1	0.9550
One lactose granules	−30—+40	21,120	20,795	325	685	72	13.8	0.9894
Four lactose granules	−30—+40	21,096	20,756	340	703	65	13.9	0.9559
Eight lactose granules	−30—+40	20,584	20,184	400	722	63	13.9	0.9479
Twelve lactose granules	−30—+40	20,298	19,858	440	745	61	13.8	0.9422
Acetaminophen crystals	−30—+40	17,246	14,527	2719	567	—	—	0.8423
Phenacetin crystals	−30—+40	17,950	14,826	3124	573	—	—	0.8260
Direct compression acetaminophen	+200 mesh	19,713	18,005	1708	721	19	15.5	0.9134

[a] *Fa, Fb, Fd, Fe,* and *Fc* are applied force, transmitted force, force lost to the die wall, ejection force, and crushing force, respectively, expressed in newtons (N). Porosity values are expressed in percentage. Punch diameter is 12 mm. *R* is the ratio of *Fb/Fa.*

Table II—Values of Shape Coefficient for Lactose Granules (30—40 mesh)

Station[a]	Shape Coefficient
1	14.5
4	15
8	17
12	21.5

[a] Refers to the stations from the shape-sorting table; a sphere would have a shape coefficient of 6.

The powders were compressed in an instrumented single-punch machine. The top and bottom punches were instrumented as described by Shotton and Ganderton (5). The radial pressure was measured by inserting a piezoelectric transducer[8] through the die wall, and forces were recorded as previously described (1). Five tablets were prepared from each powder sample at approximately 45, 90, 135, and 180 MN m^{-2} compression pressures. Once the tableting pressure had been chosen for a particular material, the same machine setting was used for the compression of different shape fractions or forms where applicable.

To condition the die, the first two tablets of any compression exercise were rejected. The weight of material in each case, calculated from its true density, was sufficient to give a tablet of 4-mm length at zero porosity using a 12-mm diameter punch and die set. The material was introduced by hand into a die previously lubricated by applying, with a small camel-hair brush, a 2% solution of stearic acid in a mixture of equal parts of acetone and carbon tetrachloride and the solvents were allowed to evaporate.

The porosity of the tablet was obtained from the dimensions of the ejected tablet. In all cases, the crushing strength of the tablet was determined after 24 hr using the diametral crushing strength test described by Shotton and Ganderton (6).

RESULTS

Compacts that showed signs of capping or that were too fragile to be measured were regarded as unsatisfactory. Both the crystalline acetaminophen and phenacetin yielded unsatisfactory tablets, so it was not possible to determine the values of crushing strength and porosity. Tablets produced from direct compression acetaminophen, lactose powders, and lactose granules were satisfactory.

The results for the different tableting parameters at the highest compression pressure used are presented in Table I. With lactose, at the highest machine setting, greater values of applied force *(Fa)* were obtained for the spheroidal spray-dried powder than with the crystalline form. Similarly, the lactose granules gave greater values of applied force for the more nearly spherical particles than for the more angular ones. Also, the more spherical-shaped lactose granules and the spheroidal spray-dried lactose gave stronger tablets on compression than the more irregular granules and the crystalline powder.

[8] Kistler, 601H.

The force lost to the die wall *(Fd)* increased with increasing departure from the spherical, *i.e.*, increasing shape coefficient; thus, lower values of *R*, the ratio of transmitted and applied force, *Fb/Fa*, were obtained for the less spherical particles (Table I). The values obtained at four axial pressures are shown in Fig. 1 for the four shape fractions of lactose granules. These findings show that the force lost to the die wall $[Fd = (Fa - Fb)]$ increases as the particles depart increasingly from the spherical.

However, for the lactose powders, the crystalline form, which was highly angular, gave a smaller value for the ejection force *(Fe)*. The converse was true for the lactose granules.

The compression cycle plots for the various materials are illustrated in Figs. 2–4.

Recordings for pressure applied to the top punch and the corresponding pressures exerted on the die wall were made continuously during the down and up strokes of the top punch by a UV spot galvanometer on photographic paper. The die wall pressure was then plotted against the corresponding axial pressures to obtain the pressure cycle. The compression cycles for the lactose powders and granules as well as for direct compression acetaminophen had the same general form and seem to indicate that the crystals behave similarly to a Mohr body as described by Long (7).

The shape of the cycles obtained for acetaminophen and phenacetin crystals differed in some respects. The residual pressure on the die wall after the axial pressure returned to zero was lower than with lactose granules and powder and with direct compression acetaminophen. None of the materials gave a pressure cycle similar to that of a body with constant yield stress in shear since the value of the second upward slope of the line would be constant and equal to 1 for such a material.

DISCUSSION

In cases where a greater axial force, *Fa*, is recorded, the particles are probably more difficult to deform. Data obtained by Higuchi *et al.* (8) show that materials associated with high residual die wall pressure give correspondingly high ejection force. They concluded that residual elastic compressional energy remaining after partial recovery in the die appears to determine the amount of work required to remove the finished tablet. This conclusion is in agreement with friction theory.

Train and Hersey (9) postulated that the value of the force lost to the die wall *(Fd)* is the product of the shear strength of the compressed material and the true area of contact between compact and the die wall. Carrington (10) showed that a maximum value for *Fd* can only be obtained when the whole compact is moved relative to the die at the point of maximum compression.

In every case, a satisfactory tablet resulted when there was a good conversion of the applied pressure to the die wall. In such cases the residual die wall pressure, after the compression pressure returned to zero, was greater than that for the materials yielding unsatisfactory tablets. Thus, lactose powders and granules and direct compression acetaminophen gave satisfactory tablets. Versano and Lachman (11) found a direct proportionality between binding and the contact area of the solid surfaces on which these forces play a role. Materials such as lactose, which showed plastic flow from the pressure cycles, gave correspondingly good tablets due to a greater contact area being formed whereas acetaminophen and

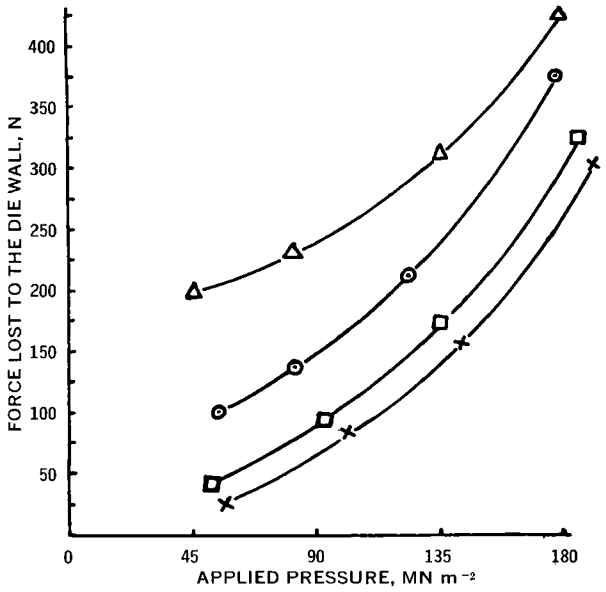

Figure 1—*Force lost to die wall for lactose granules at four machine settings. Key: ×, No. 1 shape fraction; □, No. 4 shape fraction; ○, No. 8 shape fraction; and △, No. 12 shape fraction.*

phenacetin crystals, in which recovery was mainly elastic, produced weak tablets. Also, the acetaminophen and phenacetin crystals were essentially needle-like and could be readily fractured.

None of the materials used gave pressure cycles identical with the theoretical pressure cycles described by Long (7), since these pressure cycles were for models of solid, isotropic bodies under uniaxial compression, assuming that die wall friction was absent. Leigh *et al.* (12) reported that materials giving the Mohr-type body of pressure cycle are prone to capping and lamination. In this study, there was no tendency to cap or laminate at the pressure used for lactose and direct compression acetaminophen, although these behaved like a Mohr body.

With the crystalline acetaminophen and phenacetin, the pressure cycles were more akin to those of an elastic body. With these latter materials, the initial transmission of pressure to the die wall was very small, probably due to difficulties encountered by the particles to rearrange. The values for the initial slopes were broadly in agreement with the Poisson ratio of the materials, and this ratio determines the degree of lateral expansion a body undergoes when subjected to a normal axial force. The values for the Poisson ratio obtained for acetaminophen and phenacetin from the slope were low, and the residual pressure on the die wall was also low after the axial pressure was removed. Thus, the compact had recovered axially and contracted radially, inducing considerable strain within the compact, because during the recovery process the

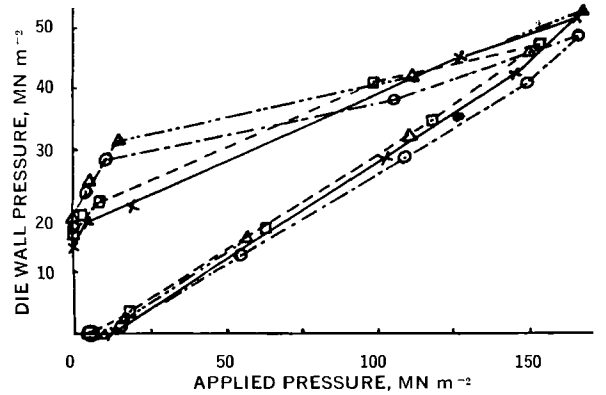

Figure 2—*Pressure cycles for four shape fractions of lactose granules (30–40 mesh) at the highest machine setting. Key: ×, No. 1 shape fraction; □, No. 4 shape fraction; ○, No. 8 shape fraction; and △, No. 12 shape fraction.*

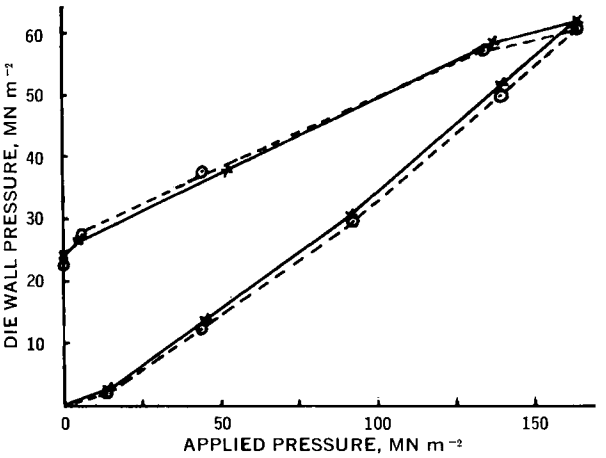

Figure 3—*Pressure cycles for spray-dried and crystalline lactose, 40–45 μm, at the highest machine setting. Key: ×, spray-dried lactose; and ○, crystalline lactose.*

tablet was subjected to a residual pressure acting from the die wall and friction restricted peripheral movement. Under these conditions, separation or capping can occur along the stress loci.

Mohr's theory of failure can be used to explain this process. An isotropic solid material fails during compression by slip along a certain surface when shear stress acting along the surface reaches a limiting value, which depends on the major principal stress. The radial pressure is the major stress when the axial pressure returns to zero. This assumes that failure occurs initially while the compact is still in the die.

The dissipation of radial pressure for acetaminophen and phenacetin was greater than for the direct compression acetaminophen and was probably due to a greater axial recovery. This finding also indicates that the direct compression acetaminophen was subjected to more permanent deformation than the crystals; with the direct compression material, it is possible that the gelatin films may interfere with the bonding between the solid particles and that the gelatin films may yield under elastic recovery to give relaxation at the bond.

CONCLUSIONS

Certain predictions can be made by evaluating the die wall pressures. The indications are that materials that permit good conversion of applied pressure to the die wall yield satisfactory tablets. This finding may prove to be a useful tool in the search for materials that can be directly compressed to yield tablets. Furthermore, materials giving a low residual die wall pressure yield tablets that are too weak or that cap on ejection.

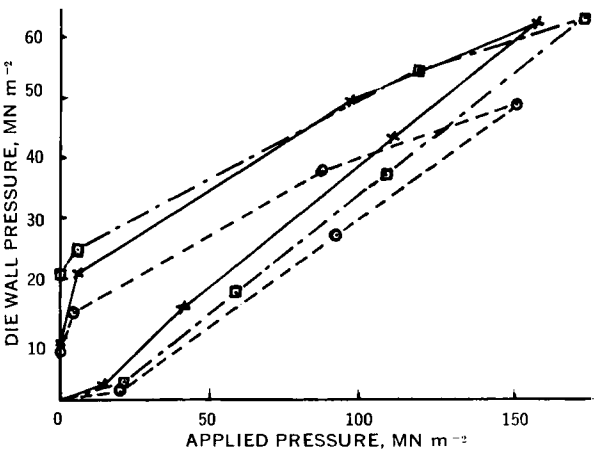

Figure 4—*Pressure cycles for direct compression acetaminophen, acetaminophen crystals, and phenacetin crystals. Key: ○, phenacetin (30–40 mesh); ×, acetaminophen (30–40 mesh); and □, direct compression acetaminophen.*

The acetaminophen and phenacetin crystals, which are essentially needle shaped, have a tendency to fracture easily and yield weak tablets. Investigations must be conducted with a much wider range of materials.

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Application of Powder Failure Testing Equipment in Assessing Effect of Glidants on Flowability of Cohesive Pharmaceutical Powders

PETER YORK

Abstract □ Powder failure testing equipment was used successfully to study the effect of glidants on the flowability of two cohesive pharmaceutical powders, lactose and calcium hydrogen phosphate, using the flow factor as the flowability parameter. Fine silica, magnesium stearate, and purified talc were investigated as glidants; for each host powder–glidant mixture, an optimum concentration of glidant was observed beyond which no further increase in flowability occurred. The order of efficiency of glidants for both host powders was fine silica > magnesium stearate > purified talc. The mode of action of the three glidants is discussed.

Keyphrases □ Flow properties of cohesive powders—application of powder failure testing equipment (shear cell and tensile tester) □ Glidant—effect on flow factor for powder mixtures, optimum concentration, mode of action □ Powders—effect of glidants on flowability determined using powder failure testing equipment

Over recent years the increasing use of fine powders has emphasized the need for more information regarding their handling and mechanical properties. Fine powders, in general, exhibit nonfree-flowing (*i.e.*, cohesive) properties, which can cause serious problems such as caking of powders in storage and bridging in hoppers. Such problems can lead to the uneven flow of powders and to difficulties in filling operations in various pharmaceutical processes.

To improve the flowability of powders and granulations, a small amount of a second agent, or glidant (1, 2), is often added, usually in powder form. Examples of glidants commonly used in the pharmaceutical industry are talc, magnesium stearate, starch, and fine silica (3). Several postulates have been proposed to explain the mechanism of action of glidants:

1. Removal of electrostatic charges on the surface of the host powder (3, 4).

2. Distribution of glidant through the host powder, and the collection of very fine cohesive host particles onto glidant particle surfaces (5).

3. Selective adsorption of gases and vapors otherwise adsorbed onto the host powders (5).

4. Reduction of van der Waals forces by separation of host powder particles (3).

5. Reduction of interparticular friction and surface rugosity by glidant particles adhering to the surface of the host powder (3, 4).

Several techniques have been employed to study the effects of the addition of glidant to host powders and granulations. Several investigators measured the rate of flow of powder particles from hoppers through circular orifices and measured the flow rate under dynamic conditions (6–8). Other methods have included tablet weight variation (9), vibrating funnel (10), and angle of repose (6, 11, 12). In the latter test, the maximum angle between the surface of a heap of powder and the horizontal plane is measured and values reflect the magnitude of the static coefficient of friction between particles.

All of these tests are most successfully employed for assessing the flowability of relatively coarse powders and granules in the 100–400-μm range (13), and optimum glidant concentrations were derived for particular systems (3, 14). However, the results obtained are significantly affected by the conditions of the test. Angular tests, such as the angle of repose, only provide a qualitative basis for assessing the flow of powders (14). In addition, these techniques do not lend themselves to the study of the flowability of fine cohesive powders, because the measurements become inaccurate (5).

The mechanical properties of cohesive powders can

The acetaminophen and phenacetin crystals, which are essentially needle shaped, have a tendency to fracture easily and yield weak tablets. Investigations must be conducted with a much wider range of materials.

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Application of Powder Failure Testing Equipment in Assessing Effect of Glidants on Flowability of Cohesive Pharmaceutical Powders

PETER YORK

Abstract □ Powder failure testing equipment was used successfully to study the effect of glidants on the flowability of two cohesive pharmaceutical powders, lactose and calcium hydrogen phosphate, using the flow factor as the flowability parameter. Fine silica, magnesium stearate, and purified talc were investigated as glidants; for each host powder–glidant mixture, an optimum concentration of glidant was observed beyond which no further increase in flowability occurred. The order of efficiency of glidants for both host powders was fine silica > magnesium stearate > purified talc. The mode of action of the three glidants is discussed.

Keyphrases □ Flow properties of cohesive powders—application of powder failure testing equipment (shear cell and tensile tester) □ Glidant—effect on flow factor for powder mixtures, optimum concentration, mode of action □ Powders—effect of glidants on flowability determined using powder failure testing equipment

Over recent years the increasing use of fine powders has emphasized the need for more information regarding their handling and mechanical properties. Fine powders, in general, exhibit nonfree-flowing (*i.e.*, cohesive) properties, which can cause serious problems such as caking of powders in storage and bridging in hoppers. Such problems can lead to the uneven flow of powders and to difficulties in filling operations in various pharmaceutical processes.

To improve the flowability of powders and granulations, a small amount of a second agent, or glidant (1, 2), is often added, usually in powder form. Examples of glidants commonly used in the pharmaceutical industry are talc, magnesium stearate, starch, and fine silica (3). Several postulates have been proposed to explain the mechanism of action of glidants:

1. Removal of electrostatic charges on the surface of the host powder (3, 4).

2. Distribution of glidant through the host powder, and the collection of very fine cohesive host particles onto glidant particle surfaces (5).

3. Selective adsorption of gases and vapors otherwise adsorbed onto the host powders (5).

4. Reduction of van der Waals forces by separation of host powder particles (3).

5. Reduction of interparticular friction and surface rugosity by glidant particles adhering to the surface of the host powder (3, 4).

Several techniques have been employed to study the effects of the addition of glidant to host powders and granulations. Several investigators measured the rate of flow of powder particles from hoppers through circular orifices and measured the flow rate under dynamic conditions (6–8). Other methods have included tablet weight variation (9), vibrating funnel (10), and angle of repose (6, 11, 12). In the latter test, the maximum angle between the surface of a heap of powder and the horizontal plane is measured and values reflect the magnitude of the static coefficient of friction between particles.

All of these tests are most successfully employed for assessing the flowability of relatively coarse powders and granules in the 100–400-μm range (13), and optimum glidant concentrations were derived for particular systems (3, 14). However, the results obtained are significantly affected by the conditions of the test. Angular tests, such as the angle of repose, only provide a qualitative basis for assessing the flow of powders (14). In addition, these techniques do not lend themselves to the study of the flowability of fine cohesive powders, because the measurements become inaccurate (5).

The mechanical properties of cohesive powders can

Table I—Relevant Physicochemical Properties of the Host Powders and Glidants

Property	α-Lactose	Calcium Hydrogen Phosphate	Fine Silica	Magnesium Stearate	Purified Talc
Average particle size, μm	10.10	5.69	0.012[a]	3.61	8.64
Particle density, g/cm³	1.51	2.30	2.20	1.03	2.75
Surface area, m²/g	0.48	0.69	200[a] ± 25	3.65	0.75
Moisture content, % (w/w)	0.04	0.14	0.02	0.08	0.02

[a] Manufacturer's values.

be investigated using powder failure testing equipment (13), *i.e.*, a shear cell and tensile tester, and their flow characteristics can thus be studied in a more scientific and quantitative manner. This investigation was conducted to determine whether this type of equipment can also be employed to study the effect of glidants on the flowability of cohesive pharmaceutical powders and to establish whether optimum glidant concentrations exist for such systems. Clearly, it would be beneficial to be able to select glidants on a scientific basis when they are required to improve the flow properties of cohesive powder formulations.

For comparison, the angle of repose test was also performed using the same powder–glidant mixtures.

EXPERIMENTAL

Materials—The host powders used, α-lactose monohydrate[1] and calcium hydrogen phosphate[2], were reagent grade and were selected as representative cohesive organic and inorganic pharmaceutical powders. The three glidants used were fine silica[3], magnesium stearate[4] BP, and purified talc[5] BP. The relevant physicochemical properties of the powders are listed in Table I.

Average particle sizes were determined using optical microscopy and surface area measurements by an air permeability technique[6], except for fine silica where the particle-size and surface area values in Table I are those quoted by the manufacturer. Particle densities were determined using the specific gravity bottle technique with appropriate immersion fluids.

All powders were dried in thin layers in a hot air oven at appropriate temperatures and were then stored over calcium chloride granules in desiccators.

Preparation of Powder Mixtures—Binary mixtures of lactose containing 0.25, 0.50, 1.0, 2.0, and 4.0% (w/w) of each of the three glidants were prepared by placing the weighed amounts of the powders together in a cylindrical jar and rotating the jar for 20 min to ensure adequate mixing. Similar binary mixtures were prepared to contain calcium hydrogen phosphate and glidant. After mixing, the powder systems were stored over calcium chloride granules in desiccators.

Angle of Repose Measurements—The angle of repose was determined using the funnel method (5). A funnel was positioned centrally over a shallow circular dish of known radius, r, and the powder system was poured evenly through the funnel until the circumference of the dish was touched by the heap of powder. The length of the side of the heap was measured using calipers, h, and the angle of repose, θ, was calculated from the expression $\cos \theta = h/r$. At least five measurements were recorded for the powders and powder mixtures.

Tensile Strength Measurements—The tensile tester was similar to that described by Ashton *et al.* (15); the cell was 9.5 cm (i.d.) and 1.0 cm deep. The mode of operation of the cell was described elsewhere (16), and tensile measurements of the two host powders and the powder–glidant mixtures were made at room temperature. The results for each system were subjected to least-squares regression analysis to enable the tensile strength of any system at a given bulk density to be predicted.

Annular Shear Cell Measurements—An annular-type shear cell was composed of a base, with an annular trough into which the powder was placed, and a close fitting lid containing 18 flights at 20° pitch, the depth of which determined the shear plane level in the powder bed. The inner and outer radii of the annular trough measured 5.6 and 7.6 cm, respectively.

Figure 1 shows a diagram of the cell with a powder sample ready for testing. The base, mounted on a turntable, was rotated at 1 revolution/hr, and the strain-gauge bar was restrained by pins, thus creating a torque in the bar. The developed torque was measured

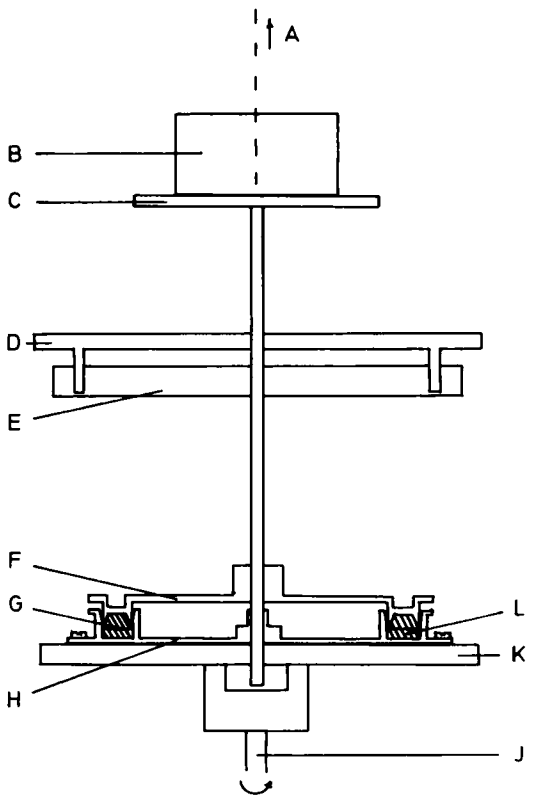


Figure 1—*Diagrammatic representation of annular shear cell with powder bed prepared for testing. Key: A, attachment for counterpoise weight; B, normal load; C, weight platform; D, restraining bar with restraining pins; E, strain-gauge bar; F, shear cell lid; G, flight; H, shear cell base; J, drive shaft (1 revolution/hr); K, platform; and L, powder bed.*

[1] Whey Products Ltd., Crewe, England.
[2] Hopkins and Williams Ltd., Chadwell Heath, Essex, England.
[3] Aerosil 200, De Gussa, Frankfurt, West Germany.
[4] Wilfred Smith Ltd., Edgware, London, England.
[5] Evans Medical Ltd., Speke, Liverpool, England.
[6] Rigden apparatus, Griffin and George Ltd., Wembley, London, England.

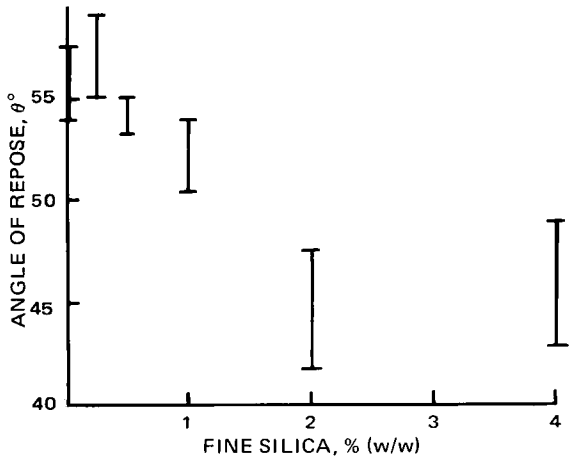


Figure 2—*Representative graph of the angle of repose* versus *the percentage fine silica in lactose–fine silica mixtures. The bar lines represent ±1* SD *from the mean value for each set of data.*

from the output of four silicon semiconductor strain gauges[7] mounted on the bar to form a Wheatstone bridge circuit. Values of shear stress were estimated from the torque developed (17), since the strain gauges had been previously calibrated with known torques using a pulley system.

The bed was prepared by introducing powder in thin even layers into the annular trough in the base and then pressing lightly with an annular disk to ensure a level bed. The lid was then lowered onto the prepared bed, and a normal load was applied using the weight platform. The cell was then rotated with the strain bar restrained. Consolidation of the sample was achieved causing the sample to fail under a normal consolidating load until a steady state, as measured from the output of the strain gauges on a chart recorder, was observed. The lid was then returned manually to its original position, and the sample was sheared under a reduced normal load.

This procedure was repeated after consolidation using different normal loads to obtain a powder yield locus, *i.e.*, a graph of shear stress at failure *versus* normal stress for a given density condition. The failure stress under steady-state conditions for the consolidating normal load was taken to represent the end-point of the locus. Results were only used if the height of the bed did not differ by more than ±2% from the original value, and the bulk density of the sample was calculated from the weight and volume of the sample. Four yield loci were obtained for each powder system by using four different consolidating loads.

The annular-type shear cell has certain advantages over the cylindrical type (18) in that smaller quantities of powder are required (50–80 g) and measurements can be made in a much shorter time. However, the annular cell suffers from the fact that at any particular angular deflection, powder at the outer wall of the cell's annulus is strained more than that at the inner wall so there is a small variation in shear stress across the width of the sample. For this investigation, this effect was minimized by making the annular width small and by employing a low rate of shear (1 revolution/hr).

RESULTS AND DISCUSSION

The angle of repose is not a fundamental property of a powder, since the value obtained partly depends upon the technique of measurement (13). For a static heap of powder, the angle of repose has a limiting value. Particles in the surface layer of the heap exist in an equilibrium situation, with the gravitational force balanced by interparticular attractive and frictional forces. Any particle that lies above this layer will slide down the heap since the gravitational force will predominate (5).

[7] Strain-gauge types 2A-1A-120P and 2A-1A-120N, Ether Ltd., Stevenage, England.

Table II—Powder Flow Classification by Flow Factor (18)

Flow Factor	Powder Flow Classification
>10	Free flowing
4—10	Easy flowing
1.6—4	Cohesive
<1.6	Very cohesive

Representative results of angle of repose measurements are illustrated in Fig. 2 for the lactose–fine silica mixtures, and the graph shows a general decrease in the angle of repose with increasing glidant concentration. Significant scatter of results can be observed due to such factors as the nonuniformity of heap and the formation of a steep-sided peak at the end of the test resulting in a concave surface. Similar scatter of results was observed for the other host powder–glidant mixtures in the study. This type of angular test thus appears to be generally unsatisfactory for any accurate assessment of the flowability of cohesive powder–glidant mixtures.

Powder failure tests, however, provide an alternative approach. Of the several flowability parameters that can be derived from the results of failure tests, *e.g.*, cohesion (4), angle of internal friction (19), and index of flow (19, 20), it is essential to select the most relevant factor. Since the problems related to the gravity flow of cohesive powders, *e.g.*, flow from hoppers and flow into dies, reduce the efficiency of many pharmaceutical processes, for the present investigation the flow factor (F.F.) (18) was used to assess flowability. Increasing values of the flow factor indicate that a powder or powder mixture is becoming more free flowing under the effect of gravity (18) (Table II).

To obtain the flow factor from a powder yield locus, the tensile strength value was calculated at the bulk density of the locus (Fig. 3) and then data from each locus were analyzed by regression analysis, using the logarithmic form of the Warren Spring Laboratory yield locus equation (20):

$$\log \tau = K + \frac{1}{n} \log(\sigma + T) \qquad \text{(Eq. 1)}$$

$$K = \log \frac{C}{T^{1/n}} \qquad \text{(Eq. 2)}$$

where:

σ = normal stress (grams per square centimeter)
τ = shear stress at failure (grams per square centimeter)
C = cohesion (grams per square centimeter)
T = tensile strength (grams per square centimeter)
n = index of flow

Flow factors were then obtained using the locus equations by estimating f_c (unconfined yield stress) and σ_{max} (maximum normal stress) for each locus (18) (Fig. 3). These stresses are derived by drawing two Mohr semicircles tangential to the yield locus, one passing through the origin and intersecting the abscissa at f_c and the second passing through the end-point of the locus and giving σ_{max}. A computer program was written to carry out this analysis. The flow factor is then defined as the reciprocal of the slope of graphs of f_c *versus* σ_{max} for a number of loci obtained for an individual powder or mixture. In this present investigation, these graphs have a curvature, as illustrated in Fig. 4, and the flow factor

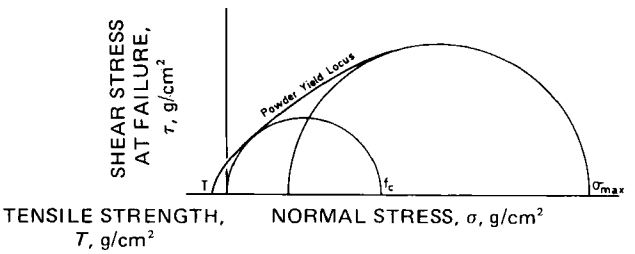


Figure 3—*Diagram illustrating the derivation of the unconfined yield stress,* f_c, *and maximum normal stress,* σ_{max}, *from a powder yield locus.*

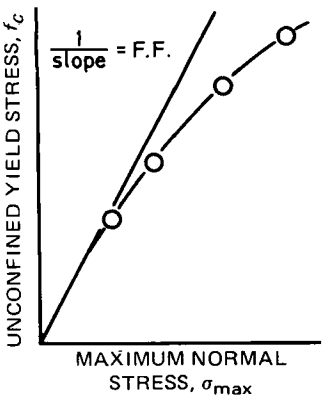


Figure 4—*Diagram illustrating the derivation of the flow factor (F.F.) from a graph of the unconfined yield stress,* f_c, versus *the maximum normal stress,* σ_{max}.

is determined as the reciprocal of the slope of the tangent to the graph at the origin (18).

Graphs of the flow factor *versus* the percentage (w/w) glidant in the mixtures with both lactose and calcium hydrogen phosphate are shown in Figs. 5, 6, and 7 for fine silica, magnesium stearate, and purified talc, respectively. The largest changes in the flow factor are produced by the addition of fine silica, with increases from 1.9 to 10.4 for lactose and from 3.9 to 10.5 for calcium hydrogen phosphate. Both increases represent a change in classification from cohesive to free flowing according to Table I. The optimum concentrations of fine silica, estimated from Fig. 5, were 0.85% (w/w) for lactose and 1.45% (w/w) for calcium hydrogen phosphate. Addition of further glidant to the host powder beyond these concentrations produced no additional increase in flow factor.

It has been proposed that the mechanism whereby fine silica operates to improve flowability is adherence to the surface of host powder particles (14). Approximate calculations of the concentration of glidant required to form an adsorbed layer one particle thick can be made, using the surface area measurement of the host powder and the average particle size of the glidant, assuming that the glidant particles are spherical and uniformly closely packed over the surface of the host powder. Such estimates are in close agreement with the optimum percentage concentration of fine silica as found by experiment (Table III), and this finding seems to support the proposed mechanism of action. If the fine silica particles were adsorbed onto host powder particles, a smoothing out of the host particle surfaces would take place, thus decreasing both friction and mechanical interlocking of particles during flow. In addition, the host–host interactions at particle contact points would be replaced by weaker glidant–glidant forces.

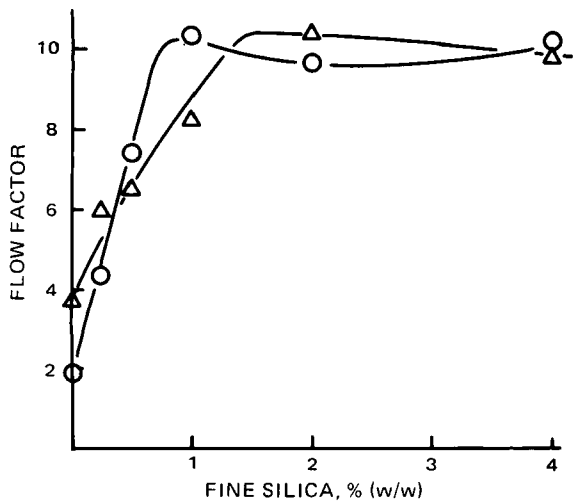


Figure 5—*Graphs of changes in the flow factor of host powder–fine silica mixtures at different concentrations of fine silica. Key:* ○, *lactose–fine silica mixtures; and* △, *calcium hydrogen phosphate–fine silica mixtures.*

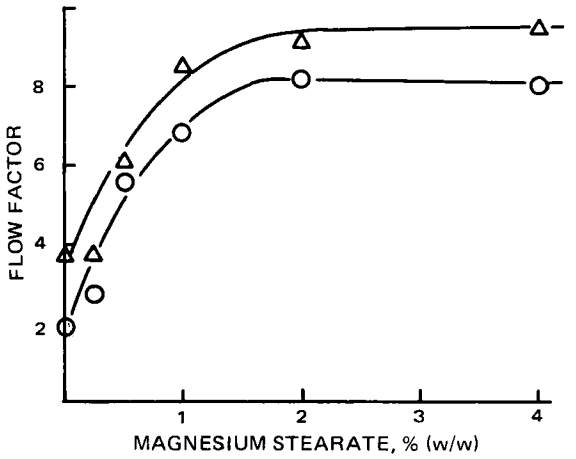


Figure 6—*Graphs of changes in the flow factor of host powder–magnesium stearate mixtures at different concentrations of magnesium stearate. Key:* ○, *lactose–magnesium stearate mixtures; and* △, *calcium hydrogen phosphate–magnesium stearate mixtures.*

Table III—Optimum Glidant Concentrations

	Optimum Glidant Concentration, % (w/w)			
	Fine Silica		Magnesium Stearate,	Purified
Host Powder	Experimental	Theoretical	Experimental	Talc, Experimental
α-Lactose	0.85	0.96	1.75	2.25
Calcium hydrogen phosphate	1.45	1.38	2.20	2.45

Magnesium stearate and purified talc do not produce such large increases in the flow factor as does the fine silica (Figs. 6 and 7), although the former glidant does improve the flowability of both lactose and calcium hydrogen phosphate from cohesive to easy flowing (Table II). In all cases, an optimum percentage glidant concentration can be estimated (Table III). Magnesium stearate, unlike fine silica, is hydrophobic and appears to function as a glidant by accumulating in voids between hydrophilic host particles (14), thereby reducing interparticular friction and attractive forces between host particles. To achieve a significant improvement in the flowability of cohesive powders, a larger percentage of magnesium stearate is required compared with fine silica, since the optimum concentrations of magnesium stearate are 1.75% (w/w) for lactose and 2.20% (w/w) for calcium hydrogen phosphate.

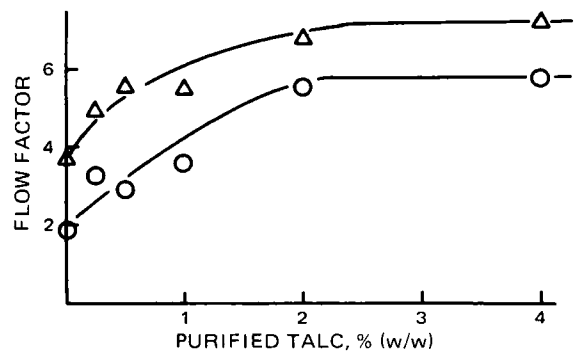
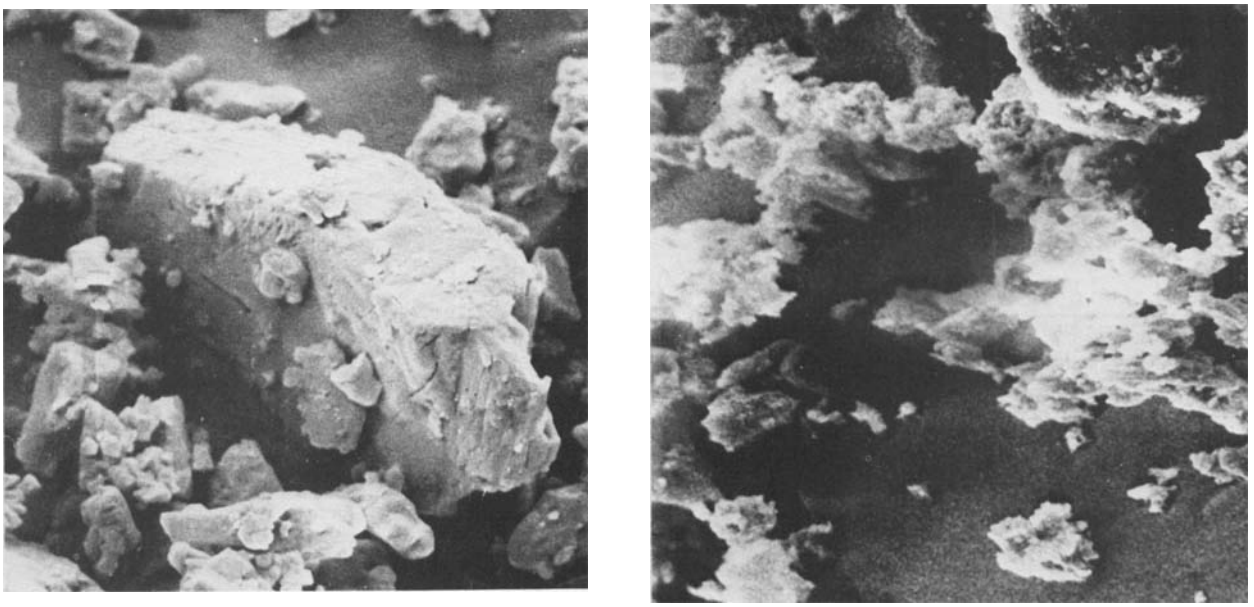


Figure 7—*Graphs of changes in the flow factor of host powder–purified talc mixtures at different concentrations of purified talc. Key:* ○, *lactose–purified talc mixtures; and* △, *calcium hydrogen phosphate–purified talc mixtures.*



A B

Figure 8—*Electron scanning microscope photographs of host powder particles. Key: A, α-lactose (about 6500×); and B, calcium hydrogen phosphate (about 3250×).*

Figure 7 indicates that purified talc is the least effective of the three glidants studied, both in terms of the increase in the flow factor and in requiring the largest concentrations to achieve maximum improvement in flowability. Indeed, the increases in the flow factor do not appear to be sufficient to impart free-flowing properties to either of the host powders.

A possible mode of action for purified talc has been proposed in terms of its laminar crystalline structure which, when subjected to the low shearing forces created when powders flow, rolls up into a spherical structure (21). The presence of such spheres would then improve the flowability of the host powder.

The differences in optimum percentage concentration obtained for the three glidants with both host powders can be attributed to differences in the mechanism of action of the glidants, the physical and chemical nature of the materials, and the relative particle sizes of the host and glidant particles. Indeed, it is conceivable that if purified talc had an average particle size similar to that of the fine silica, it might also function by adsorbing to form a monoparticular layer over the host powder, thereby improving its efficiency as a glidant.

Higher values of optimum glidant concentration were derived for calcium hydrogen phosphate than for lactose with all three glidants studied. This finding can be explained not only by the larger surface area per gram of the calcium hydrogen phosphate but also by the different surface structure of the two powders (Fig. 8).

Electron scanning microscope photographs reveal that the lactose particles have a relatively smooth and angular structure, while calcium hydrogen phosphate particles possess a more convoluted and rougher surface. Thus, calcium hydrogen phosphate would be expected to require the presence of relatively more glidant particles to improve its flowability.

From this discussion, it is evident that powder failure testing equipment can be successfully used to assess the efficiency of glidants in improving the flowability of cohesive powders and also to select optimum glidant concentrations. This kind of equipment could be usefully employed in pharmaceutical formulation in cases where cohesive powders and mixtures cause powder flow problems.

SUMMARY

1. The angle of repose test was unsuitable for assessing the flowability of cohesive lactose and calcium hydrogen phosphate powders and their mixtures with glidants.

2. Powder failure testing equipment, *i.e.*, annular shear cell and tensile tester, were successfully employed to study the effects of three glidants (fine silica, magnesium stearate, and purified talc) on the flowability of cohesive lactose and calcium hydrogen phosphate powders. Flowability was assessed using the flow factor parameter.

3. For both host powders, the order of efficiency in increasing the flow factor was in the order fine silica > magnesium stearate > purified talc.

4. In all systems, optimum glidant concentrations were observed. For the same glidant, these values were higher for calcium hydrogen phosphate than for lactose; this finding is attributed to differences in the surface area and structure of the two powders.

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Rapid, Sensitive Colorimetric Method for Determination of Ethinyl Estradiol

MOHAMED A. ELDAWY ˣ, A. S. TAWFIK*, and S. R. ELSHABOURI

Abstract □ A colorimetric procedure, based on the formation of an azo dye by condensation of diazotized 5-chloro-2,4-dinitroaniline with ethinyl estradiol, was developed. An alkaline solution of ethinyl estradiol is reacted with the reagent, and the resulting color is measured at 450 nm. Absorbance *versus* concentration is linear up to 10 μg/ml; the lower limit of detection is 1 μg/ml under the conditions studied. Replicate analysis showed good agreement, and an average recovery of 99.6 ± 0.3% was obtained for analyses of synthetic mixtures. Vitamins and minerals likely to be present along with ethinyl estradiol in certain geriatric formulations, as well as ordinary tablet excipients and coating materials, do not interfere with the precision of the method or development of the color. The method is applicable to progestin–estrogen preparations. Assay results on various single-component as well as contraceptive commercial samples are reported.

Keyphrases □ Ethinyl estradiol in progestin–estrogen and vitamin/mineral geriatric preparations—colorimetric analysis □ Progestin–estrogen contraceptive tablets—colorimetric analysis of ethinyl estradiol □ Geriatric formulations containing vitamins, minerals, and estrogens—colorimetric analysis of ethinyl estradiol □ Colorimetry—analysis, ethinyl estradiol in progestin–estrogen and vitamin/mineral geriatric preparations

Several methods for the analysis of ethinyl estradiol are available in the literature, including UV (1), GLC after suitable derivatization (2, 3), and colorimetric methods (4–8). The USP XVIII colorimetric procedure (9), which is a modification of the Kober reaction (4), suffers from several disadvantages exhibited by the rather limited solubility of ethinyl estradiol in isooctane. These disadvantages might be circumvented by using an isooctane–chloroform mixture instead as modified in the first supplement to USP XVIII. The Kober reaction, in spite of many modifications (10–12), is time consuming, and the color development is critically dependent upon reagent composition, reaction time, and temperature. Interference by other nonphenolic steroids also can occur (13).

The suitability of diazotized 4-amino-6-chloro-*m*-benzenedisulfonamide for the determination of a number of estrogens has been investigated (14). The method, although suitable for routine analysis, has rather low sensitivity. The lower limit of detection is 0.05 mg of estrogen/ml of sample solution. Commercially available dosage forms, especially geriatric formulations, are usually of very low dosage (0.008–0.05 mg/tablet or capsule). Consequently, the sensitivity of this procedure is such that single-tablet analysis of most commercial dosage forms is not feasible.

In spite of its high sensitivity, the Liebermann–Burchard reaction-based fluorometric method of James (15), with 0.5 μg/ml as a lower limit for detection, is not applicable to progestin–estrogen preparations. Progestational steroids produce a color in the Liebermann–Burchard reaction that quenches the fluorescence of ethinyl estradiol.

It has been observed in these laboratories that diazotized 5-chloro-2,4-dinitroaniline couples with phenolic compounds to form products with extremely stable colors; this finding offers the basis for a rapid and sensitive method for the determination of pharmaceuticals containing a phenolic hydroxy group (16). The suitability of this reagent for the determination of ethinyl estradiol was investigated, and a rapid and sensitive colorimetric method for the determination of ethinyl estradiol was developed. This method eliminates the disadvantages of the Urbanyi and James methods, as well as those of the Kober reaction.

EXPERIMENTAL[1]

5-Chloro-2,4-dinitroaniline—This compound was prepared by a reported procedure (17). Several crystallizations from ethanol yielded an analytical sample, mp 174°.

Reagents and Solutions—The following were used: 5-chloro-2,4-dinitroaniline (0.2% ethanolic solution), sodium nitrite (3% aqueous solution), 1 *N* hydrochloric acid, 2 *N* sodium acetate, 0.1 and 1 *N* sodium hydroxide, and absolute ethanol.

[1] A Hungarian-made Spektromom 203 spectrophotometer was used.

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Rapid, Sensitive Colorimetric Method for Determination of Ethinyl Estradiol

MOHAMED A. ELDAWY ˣ, A. S. TAWFIK*, and S. R. ELSHABOURI

Abstract □ A colorimetric procedure, based on the formation of an azo dye by condensation of diazotized 5-chloro-2,4-dinitroaniline with ethinyl estradiol, was developed. An alkaline solution of ethinyl estradiol is reacted with the reagent, and the resulting color is measured at 450 nm. Absorbance *versus* concentration is linear up to 10 μg/ml; the lower limit of detection is 1 μg/ml under the conditions studied. Replicate analysis showed good agreement, and an average recovery of 99.6 ± 0.3% was obtained for analyses of synthetic mixtures. Vitamins and minerals likely to be present along with ethinyl estradiol in certain geriatric formulations, as well as ordinary tablet excipients and coating materials, do not interfere with the precision of the method or development of the color. The method is applicable to progestin–estrogen preparations. Assay results on various single-component as well as contraceptive commercial samples are reported.

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It has been observed in these laboratories that diazotized 5-chloro-2,4-dinitroaniline couples with phenolic compounds to form products with extremely stable colors; this finding offers the basis for a rapid and sensitive method for the determination of pharmaceuticals containing a phenolic hydroxy group (16). The suitability of this reagent for the determination of ethinyl estradiol was investigated, and a rapid and sensitive colorimetric method for the determination of ethinyl estradiol was developed. This method eliminates the disadvantages of the Urbanyi and James methods, as well as those of the Kober reaction.

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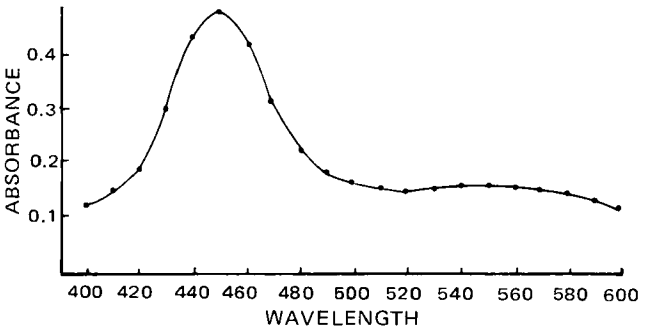

Figure 1—*Absorption spectrum of ethinyl estradiol with diazotized 5-chloro-2,4-dinitroaniline.*

Standard Ethinyl Estradiol Solution—Weigh accurately about 20 mg of ethinyl estradiol USP into a 10-ml volumetric flask and dissolve in ethanol. Dilute to volume with ethanol and then dilute 1 ml of this solution to 10 ml with 0.1 *N* sodium hydroxide. Pipet appropriate volumes so as to give solutions containing 1–10 μg/ml after color development.

Preparation of Assay Solutions—Extract an accurately weighed sample of the powdered synthetic mixture or tablets equivalent to about 0.5 mg of ethinyl estradiol with three 5-ml portions of ethanol. Centrifuge each portion, filter the ethanol extracts into a small beaker, and evaporate the ethanol to dryness. Transfer the contents of the beaker quantitatively with 0.1 *N* sodium hydroxide to a 10-ml volumetric flask and dilute to volume with additional 0.1 *N* sodium hydroxide. This solution is the assay solution.

For Contraceptive Tablets—Prepare as directed for the assay solutions. Transfer the contents of the beaker quantitatively with 0.1 *N* sodium hydroxide to a 10-ml volumetric flask, shake vigorously, transfer to a centrifuge tube, and centrifuge for 10 min. The clear solution is the assay solution for contraceptive tablets.

For Geriatric Formulations Containing Ethinyl Estradiol—Extract an accurately weighed sample of the powdered synthetic mixture equivalent to about 0.5 mg of ethinyl estradiol three times each with 5 ml of alcohol-free chloroform (18), centrifuge for 10 min, collect the extracts in a small beaker, and evaporate to dryness. Transfer the contents of the beaker quantitatively with 0.1 *N* sodium hydroxide to a 10-ml volumetric flask, and dilute to volume with additional 0.1 *N* sodium hydroxide. This solution is the assay solution.

Color Development—Into separate 10-ml volumetric flasks, pipet successively 1.0 ml each of 5-chloro-2,4-dinitroaniline, sodium nitrite, and hydrochloric acid solutions and mix well. Allow to stand for 2 min and add 1 ml of ethanol and 1 ml of standard solution, 1 ml of the assay solution, or 1 ml of 0.1 *N* sodium hydroxide solution to separate flasks. Add 2.0 ml of sodium acetate solution to each flask, and allow to stand for 20 min. Dilute each flask to volume with 1 *N* sodium hydroxide. Allow to stand for 30 min and determine the absorbance of the standard and sample solution at 450 nm in 1-cm cells against the blank using a suitable spectrophotometer.

RESULTS AND DISCUSSION

Initial investigations using lower concentrations of sodium nitrite solution resulted in inconsistent and nonreproducible spectral intensities for standard solutions. Accordingly, a study of the effect of sodium nitrite concentration on diazotization of 5-chloro-2,4-dinitroaniline and subsequent coupling with ethinyl estradiol was carried out using 1, 2, 3, 4, and 5% sodium nitrite solution. Use of the 3% sodium nitrite solution gave the highest color intensity. Destruction of the excess nitrous acid was not necessary since no interference in the subsequent color development and measurement was observed.

The normality of the sodium hydroxide used affected both the development of the color and the reproducibility of the method. Higher concentration of alkali resulted in lower absorption intensities. The most intense and reproducible absorption was obtained when ethinyl estradiol was dissolved in 0.1 *N* sodium hydroxide, coupled, and diluted to volume with 1 *N* sodium hydroxide.

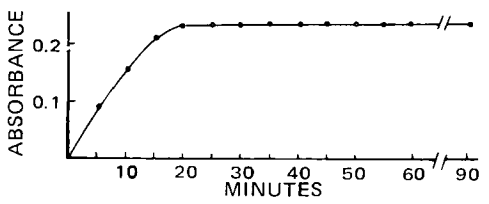

Figure 2—*Effect of coupling time on the absorbance of the final solution.*

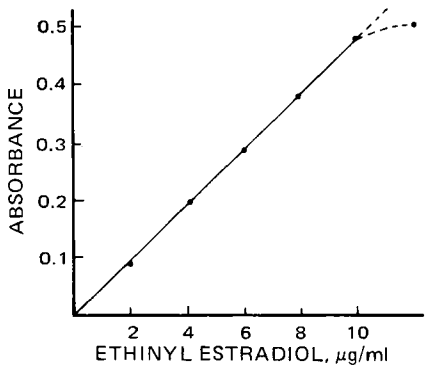

Figure 3—*Standard calibration curve for ethinyl estradiol with diazotized 5-chloro-2,4-dinitroaniline.*

The addition of 1 ml of ethanol to the diazotized solution of 5-chloro-2,4-dinitroaniline was necessary to avoid the turbidity observed in the absence of ethanol. The alcohol probably helps keep the coupling product in solution.

The absorption spectrum of the alkaline solution of the coupled product, resulting from the reaction of ethinyl estradiol with diazotized 5-chloro-2,4-dinitroaniline, is shown in Fig. 1 (absorption maximum at 450 nm). The molar absorptivity as calculated from the curve is 11.284×10^3.

The effect of coupling time under these conditions, after the addition of the alkali, on the absorbance of the final solution at 450 nm is shown in Fig. 2. Color intensity increased during the first 20 min and remained constant for at least 90 min.

The relationship between absorbance at 450 nm and concentration was linear up to 10 μg of ethinyl estradiol/ml of sample solution (Fig. 3).

The specificity of the method for ethinyl estradiol in the presence of several frequently encountered excipients is shown in Table I. A placebo was prepared containing some common tablet excipients (*e.g.*, lactose and starch); several different weights of the excipients, each approximating the sample weight of commercial tablets, were spiked with a known amount of ethinyl estradiol and the assay was performed. The average recovery was 100 ± 0.20%.

Multiple analyses were performed on different commercial samples of varying potencies. Agreement between replicate analyses was good, and all samples assayed 99.6% or better (Table II).

The method presented here is applicable to progestin–estrogen preparations. Synthetic mixtures, in which the ratio of the progestin norethindrone acetate to ethinyl estradiol was that of the average found in commercial formulations, as well as commercial coated contraceptive tablets containing this progestin along with ethinyl estradiol were satisfactorily analyzed by this method. Percentage recoveries were 99.86 ± 0.06%.

Other phenols will, of course, interfere in the determination of ethinyl estradiol by this method, as they would in the Kober reaction and the Urbanyi method. Although such interferences would not normally be expected, interference due to pyridoxine hydrochloride, which possesses a similar phenolic hydroxy group, was encountered. This problem was circumvented by extraction of ethinyl estradiol with alcohol-free chloroform in which pyridoxine hydrochloride is almost insoluble (19). Ascorbic acid does interfere with the development of the color and this interference is removed by the same method. Accordingly synthetic mixtures containing the minerals and vitamins shown in Table I, which are likely to be

Table I—Determination of Ethinyl Estradiol in the Presence of Common Excipients, Minerals, Vitamins, and Norethindrone Acetate[a]

	Ethinyl Estradiol		Ethinyl Estradiol
Lactose		Thiamine hydrochloride	
Added, mg/g	2.00	Added, μg/5 mg	10.0
Found, mg/g	2.00	Found, μg/5 mg	10.0
Recovery, %	100 ± 0.1	Recovery, %	100 ± 0.2
Starch		Niacinamide	
Added, mg/g	2.00	Added, μg/5 mg	10.0
Found, mg/g	2.00	Found, μg/5 mg	9.98
Recovery, %	100 ± 0.2	Recovery, %	99.8 ± 0.7
Norethindrone acetate		Pyridoxine hydrochloride	
Added, mg/50 mg	2.00	Added, μg/1.0 mg	10.0
Found, mg/50 mg	1.99	Found, μg/1.0 mg	9.98
Recovery, %	99.5 ± 0.3	Recovery, %	99.8 ± 0.4
Ascorbic acid		Cobalt sulfate	
Added, μg/50 mg	10.0	Added, μg/120 μg	10.0
Found, μg/50 mg	9.98	Found, μg/120 μg	10.05
Recovery, %	99.8 ± 0.4	Recovery, %	100.5 ± 0.2
Copper sulfate		Manganese sulfate	
Added, μg/1.26 mg	10.0	Added, μg/1.55 mg	10.0
Found, μg/1.26 mg	10.1	Found, μg/1.55 mg	9.98
Recovery, %	101 ± 0.2	Recovery, %	99.8 ± 0.6
Ferrous sulfate		Magnesium sulfate	
Added, μg/20 mg	10.0	Added, μg/2 mg	10.0
Found, μg/20 mg	9.98	Found, μg/2 mg	10.05
Recovery, %	99.8 ± 0.3	Recovery, %	100.5 ± 0.2
Zinc sulfate			
Added, μg/1 mg	10.0		
Found, μg/1 mg	10.0		
Recovery, %	100 ± 0.6		

[a] Values are averages of three determinations.

Table II—Determination of Ethinyl Estradiol in Single-Component and Contraceptive Tablets

Sample Type	Ethinyl Estradiol Declared, mg	Amount Found, mg/Tablet[a]	Recovery, %
Single component	0.050	0.0496	99.6 ± 0.4
Contraceptive	0.050	0.0499	99.96 ± 0.06

[a] Average of four determinations.

present along with ethinyl estradiol in certain geriatric formulations (20), were satisfactorily analyzed by this method. The amounts chosen were the averages found in commercial preparations (20).

The sensitivity of the method is such that single-tablet analysis, even at the lowest dosage, is feasible; this sensitivity is an improvement over the diazotized 4-amino-6-chloro-*m*-benzenedisulfonamide method (14). Another advantage is the applicability of the method to progestin–estrogen combinations, which is an improvement over the fluorometric method (15).

These improvements make this method more suitable for the determination of ethinyl estradiol and related estrogens in biological fluids, as well as for the determination of other compounds of pharmaceutical interest containing a hydroxy group (to be discussed in a future report).

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Microbiological Diffusion Assay II: Design and Applications

F. KAVANAGH

Abstract □ Application of new equipment and new techniques was made to antibiotic diffusion assays. Accumulation of data and computation of potencies were made by an on-line computer. The system was tested by assaying cephalexin with the aid of *Bacillus subtilis* in an FDA single-dose design modified by reducing the number of standards to two. The influence of the thickness of the base layer and the form of the dose–response line were tested. Zone diameter was measured with a resolution of 0.01 mm. The potency of samples was measured with an error usually less than 3%. An error of 0.1 mm in measuring zone size would cause an error of 3% of potency. The usual calibration line was inadequate for extrapolation beyond a twofold range. A dose–response line derived from the Cooper equation was better for standard curves spanning more than a twofold range of concentrations. Precision was better on the plates with the 20-ml base layer. The two-dose method of assaying gave larger errors than the single-dose method. Large variances in measuring zone diameters are a reason for the low precision of diffusion assays and set an inherent limit on precision.

Keyphrases □ Antibiotics—microbiological diffusion assay, design and application □ Microbiology—diffusion assay of antibiotics, design and application □ Diffusion assay—microbiological assay of antibiotics, design and application

Most petri dish diffusion assays done in the United States for quantitation of antibiotics are variants of two Food and Drug Administration (FDA) designs. One is the Knudsen and Randall (1) two-dose assay, and the other is the alternate or single-dose method. The latter is usually thought of as the FDA method (2).

In the single-dose design, the calibration line is assumed to be straight. Sample responses are assumed to fall on a line parallel to the standard curve. Responses of samples and references are assumed to be influenced equally by factors, known and unknown, that affect zone size. If these assumptions are true, then sample responses can be corrected for the several factors, other than concentration of drug, causing plate-to-plate variations in zone size. As shown previously (3), the FDA correction is a good first approximation to complete correction for such variations.

During the last 25 years, the single-dose method has undergone evolution with respect to the spacing of concentrations of the standards but other details have not changed. For example, the 1949 (4) method for penicillin and streptomycin had 10 standards closely spaced on a linear scale covering a 2.5-fold range. The same concentrations were given by Grove and Randall (5). The number of concentrations was later reduced to five, with the middle one used as reference and spaced on a logarithmic scale to facilitate computation of the "best" straight line (6, 7).

Thus the method had assumed its present form by 1957. The number of concentrations of standards and their spacing were selected more for mathematical convenience than for analytical necessity. The full range of the tests was either 2.44- or fourfold. The best straight line was computed through the points by calculating the low point and the high point of the line by means of an unweighted least-squares procedure.

The practice of using many closely spaced points to determine a straight line originated when location of a point on the standard curve was uncertain and shape of the dose–response line had no theoretical basis. A line determined by a number of points was considered more precisely located than one established by only the end-points. Now that the theory of the method has been established and the precision of assays can be much higher than it once was, the intermediate points are redundant. Since the calibration line is straight by definition, any point on it (*e.g.*, either end concentration) could be used as the reference.

Three plates, each holding three zones of each of two concentrations of standards, are sufficient to establish the standard curve, thus reducing the method to the minimum. Such an assay was designed and applied to samples of known concentration. Limits to the precision of the assay were ascertained during the work. To do so required the determination of the precision of measuring zone size and the influence of the form of the dose–response line upon accuracy and precision. Concepts (3) described earlier were applied to these assays to minimize influence of operations upon accuracy and precision. Some data were used to investigate the two-dose method (1).

EXPERIMENTAL

Equipment and Operations—As shown previously (3), variations in agar thickness had only a second-order influence upon zone size attributable to thickness but a first-order influence caused by effective change in the concentration of bacteria of two layer plates. Variations in agar thickness were reduced by employing automatic pipetting equipment and placing the petri dishes containing molten agar on a level surface to cool. A flat slate slab installed on a bench top and carefully leveled with the aid of a machinist's level provided the level surface.

Plastic dishes were placed on the slab, and molten agar was added from an automatic pipetting machine at the rate of 60 or more/min. Both double- and single-layer plates were prepared; when a second layer was added, it was put on the first layer without moving the plates from the slab. Single-layer plates were preferred, but the type of plates that gave the more sharply defined zone edge was used.

The poured plates were not refrigerated because using plates of different temperatures can affect zone size (8) and increase variances.

Most cylinders used in the United States are made of stainless steel. The ends of the cylinders may be flat, finished with a radius, or have an inside bevel. Cylinders of the same type of end were used because the effective inside diameters are not the same for flat, rounded, or beveled ends. Cylinders must not leak due to either rough ends or rough handling of plates.

Table I—Duplicate Measurements of Zone Sizes at Four Cephalexin Concentrations

Base Layer	Plate Number	Cephalexin 4 μg/ml Measurement 1	4 μg/ml Measurement 2	8 μg/ml Measurement 1	8 μg/ml Measurement 2	16 μg/ml Measurement 1	16 μg/ml Measurement 2	32 μg/ml Measurement 1	32 μg/ml Measurement 2
		Diameters, mm							
10 ml	1	15.38	15.43	18.24	18.20	20.52	20.43	22.56	22.48
	2	15.28	15.27	18.51	18.45	20.44	20.35	22.47	22.37
	3	15.35	15.27	17.94	17.92	20.35	20.30	22.63	22.62
	Mean	15.34	15.32	18.23	18.19	20.44	20.36	22.55	22.49
	σ	0.051	0.092	0.285	0.265	0.085	0.0656	0.082	0.125
20 ml	1	13.15	13.08	15.84	15.88	18.32	18.42	20.59	20.66
	2	13.32	13.32	16.07	16.11	18.71	18.79	20.68	20.69
	3	13.00	13.01	15.84	16.03	18.54	18.65	20.78	20.86
	Mean	13.16	13.14	15.92	16.01	18.52	18.62	20.68	20.74
	σ	0.16	0.16	0.133	0.12	0.195	0.19	0.095	0.11

The cylinders were added by means of a semiautomatic dropper to a group of plates. Filling was by means of an automatic pipet delivering 0.2 ml of sample. The disposable tips of the pipets were changed between samples. One advantage of the automatic pipet was the impossibility of filling the cylinders to overflowing.

The effect of differences in heating rates upon assays was considered earlier (3). As Cooper and Linton (9) showed, the plates in the center of a stack of five heated much slower than those at the ends. A special incubator was designed to receive plates in a single layer and to incubate them at uniform temperature, thus avoiding the problems caused by incubating plates in a stack. The prototype incubator was constructed from chill-plates through which water circulated from a constant-temperature bath at 20 liters/min through the three shelves connected in series. The walls of the incubator were constructed from 5.1-cm (2-in.) thick, rigid, polystyrene foam insulation. Later, larger electrically heated versions were made and had six shelves measuring 41 cm (16 in.) deep × 86.3 cm (34 in.) long spaced 5.1 cm (2 in.) apart. Each shelf had its own thermoregulator and door and was a closed chamber isolated from all others.

The incubator shelves were so close together that the petri dishes could not be put on them individually. A holder designed for a different system (10) proved ideal. Each holder held three dishes. When the holder was placed on the shelf, each dish had the same air space between its bottom and the shelf as all others. Heat transfer, the same for all, was limited by transfer through the air film.

Zone diameters were measured with a modified antibiotic zone reader (11). Resolution of the measuring system was about 0.01 mm when recorded by computer. Four calibration lines were used. Equation 1 was used as a short-range interpolation formula following FDA practice, as a point-to-point line through an eightfold range of concentrations of standard, and as a best straight line through the same four points:

$$\log C = A + Bd \qquad \text{(Eq. 1)}$$

The fourth line was derived from the Cooper equation (12) as:

$$\log C = E + FX^2 \qquad \text{(Eq. 2)}$$

where X, the distance diffused, was computed from $X = \frac{1}{2}(d - 5.8)$. In this work, d was the zone diameter in millimeters, and 5.8 mm was the inside diameter of the cylinders.

The operational elements of the assay—plate pouring, cylinder dropping, cylinder filling, and incubation—were integrated into one system by using the concept of a work station. The operator sits at the work station, drops the cylinders, fills the cups of three plates in a carrier, covers the plates, inserts the carrier into the incubator, and proceeds to the next carrier. The 18 cylinders of a carrier can be filled in 1 min, a time too short to have much effect by reason of the different prediffusion times. Each shelf has space for seven carriers or a total of 21 plates.

If the modified FDA single-dose design is used, one carrier suffices for standards and the remaining six carriers on a shelf are for six samples.

Design of Assays—As already described, the assay is a slight modification of the single-dose method of FDA (7). One carrier of three plates holds the standards, which have the same number of responses per concentration as in the FDA design. The mean of the high standard and the mean of the low standard determine the calibration line. The higher concentration is the reference standard. There are three plates (one carrier) for each sample. One-half of the zones are reference and one-half are sample. The sample is diluted to fall between the two standards.

The reference concentration in these procedures as in that of FDA is one of the standards. This need not be so. The only restriction placed upon the reference is that it be the same on all plates. The reference concentration could be unknown. It is made one of the standards solely in the interest of efficiency. The reference is used to correct for certain variations in operations; it cannot correct for blunders, bad diluting, or misreading of zone sizes.

On the sample plates, the mean of the nine sample zones is corrected for deviation of the mean of the nine reference zones on the sample plates from the mean reference (high standard) on the standard plates. The corrected sample zone is used to interpolate its potency from the standard curve. Interpolation may be done

Table II—Replicate Measurements of Zone Diameters of Standard Curves

Base Layer	Plate Number	Cephalexin 4 μg/ml Measurement 1	4 μg/ml Measurement 2	8 μg/ml Measurement 1	8 μg/ml Measurement 2
		Diameters, mm			
10 ml	1	15.84	15.93	18.32	18.27
		15.93	16.00	18.57	18.47
		15.85	15.87	18.22	18.15
	2	15.68	15.70	18.43	18.47
		15.93	15.87	18.09	17.98
		15.63	15.67	18.25	18.23
	3	15.55	15.55	18.33	18.41
		15.96	15.97	18.42	18.37
		15.44	15.54	17.67	17.66
	Mean	15.757	15.789	18.255	18.223
	σ	0.188	0.177	0.259	0.265
20 ml	1	13.63	13.57	16.19	16.20
		13.53	13.57	16.18	16.09
		13.42	13.39	16.19	16.22
	2	13.37	13.41	16.20	16.21
		13.21	13.22	16.08	16.02
		13.48	13.43	16.04	16.17
	3	13.45	13.38	16.15	16.16
		13.47	13.54	16.07	16.08
		13.36	13.35	15.97	15.91
	Mean	13.436	13.429	16.119	16.118
	σ	0.118	0.115	0.082	0.103

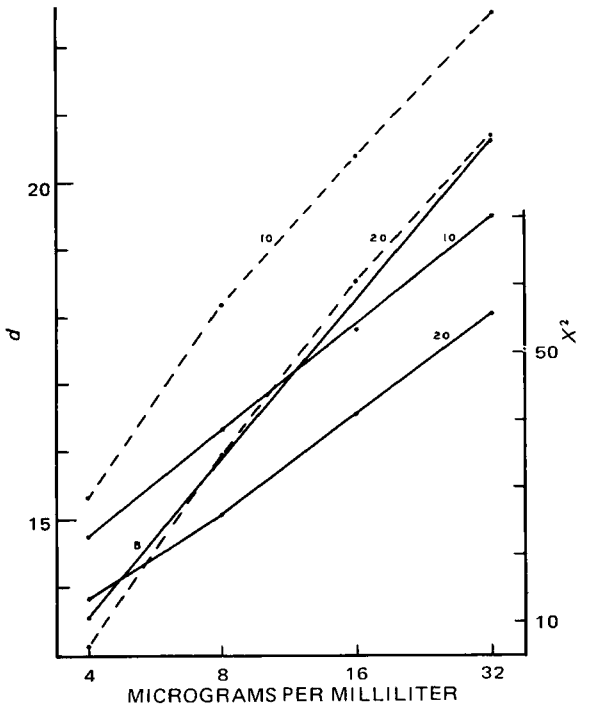

Figure 1—*Two forms of dose–response lines of a cephalexin*–B. subtilis *assay. The dashed lines are for* d versus *log* C, *and the continuous lines are for* X^2 versus *log* C, *where* d *is the zone diameter in millimeters and* X^2 *is the square of the distance diffused computed from* $X^2 = \frac{1}{4}(d - 5.8)^2$. *The numbers on the lines refer to volumes of the base layers. The straight line labeled B is the best straight line through the points for the 20-ml base layer as in the FDA method.*

manually or by computer. Only mean values are used in the calculations, and only one answer is obtained from the nine responses of a sample.

The zone sizes of the standards are not subjected to an FDA correction because none is required. The low concentration of the standard could be corrected for the plate difference between the plate high standard and the mean reference (high standard). The result would be the same as the mean of the nine responses of the low standard.

Application of the concepts and designs discussed in this and the previous paper (3) was made to an assay for cephalexin by a cylinder-plate method, employing *Bacillus subtilis* (ATCC 6633) as the test organism. This assay was selected because the antibiotic was stable and the zone edges were sharp. Zone sizes were accumulated and processed by an on-line computer system. Diameters are reported to 0.01 mm in the tables.

Two base layers (10 and 20 ml) were used with 5 ml of top layer. The plates were poured from the same bottle of molten agar, had the top layers poured from the same bottle of inoculated agar, and received the same solutions. All work was done by one person. Two extended standard curves were prepared at the same time from the same bottles of agar.

The precision of the measurement of zone sizes was obtained by having the same operator measure the same set of plates a second time. One person produced the data in Table I, and another produced the data in Table II.

RESULTS AND DISCUSSION

Single-Dose Assays—Results of the measurement of zone diameters are given in Tables I and II. Table I contains data for an eightfold standard line obtained on plates with either a 10- or 20-ml base layer. Data for the replicate measurements of the standard curve for the single-dose method of assay are given in Table II for the two base layers.

Several conclusions can be drawn from the data. Means of the two sets of measurements of zone diameters differed by 0.1 mm or less; one set of mean diameters is as good as the other. One-half of the comparisons differed by 0.06 mm or less. Only three differences, or one-twentieth of the total, were as great as 0.1 mm, which is the resolution of the manual zone reader when it is fitted with a millimeter scale. These results, as well as many others, indicate that measuring zone diameters with a resolution of 0.01 mm can give useful information. Precision of measurement was somewhat greater for zones formed on a 20-ml base layer.

The zone edge of *B. subtilis* inhibited by cephalexin was sharp enough for most measurements of diameter to be replicated within 0.08 mm or within 2% of potency. Most assay systems are less precise.

The standard deviations in Tables I and II include variances in actual zone diameters as well as measuring errors. One zone of 8-μg/ml concentration on Plate 3 of the 10-ml base layer was responsible for the rather large standard deviation. Zone diameters were more variable on plates with the 20-ml base layers.

Standard lines representing the data in Table I are plotted in Fig. 1 in three ways:

1. As zone diameter (*d*) *versus* logarithm of concentration (usual way for FDA type of assays).
2. As $\frac{1}{4}(d - 5.8)^2$ *versus* logarithm of concentration.
3. As the best straight line through the points obtained on the plates with the 20-ml base layers.

The second relationship was derived from the Cooper equation (12), assuming the inside diameter of the cup (5.8 mm) to be equivalent to a hole of that size. If the Cooper equation is a valid representation of the facts, the first set of lines should curve toward the concentration axis and the second set should be straight. Figure 1 shows that the Cooper expression is followed by the cephalexin–*B. subtilis* assay from 4 to 32 μg/ml. A low concentration of 6–7 μg/ml would give better zone edges than 4 μg/ml gave on the plates with 20 ml of base layer and would straighten the line.

The lines in Fig. 1 show quite clearly that for high accuracy assays, the usual dose–response relationship (Eq. 1) would add a small error for a narrow (twofold) range assay and a larger error to a wider range assay because the line is curved and not straight as assumed.

An assay for potency of three samples is given in Table III. Standards are at the top of the table. The six zones for one plate are on one line. The relative standard deviation (*RSD*) of zone sizes is reported as percentage. The values of *RSD* are not used in any computation and are given solely as a measure of quality of the work. A large *RSD* should alert the analyst to a problem. The computer program interpolated potencies of the unknowns from a line of the form *d versus* log *C*. The samples were the 8-μg/ml standard and two concentrations approximately 10% above and below it. These latter samples bracket the concentrations of most samples analyzed by a quality control laboratory.

A notable feature of the assays reported in Table III is the plate-to-plate uniformity of adjusted mean responses. The worst was the second plate of Sample 5. Its adjusted mean was 18.11 mm, which indicated a potency of 7.640 instead of the expected 8.00. Thus, the worst plate gave an error of −4.5%. Most mean plate deviations from the true values were less than 2% of potency.

Results of the assays are given in Table IV. The maximum error was +3%. Such an error would be caused by a 0.1-mm error in mean zone size. The difference between the 8-μg/ml standard used as samples and the values found could be caused by an error of 0.025 mm in the mean of the zone diameters. Better agreement is not to be expected because 0.02–0.03 mm is about the size of the difference in means between two sets of measurements of the same set of zones.

The resolution of the zone measurements was 0.01 mm, which was equivalent to 0.022 μg/ml at 8 μg/ml, or 1 part in 400 of concentration, or 1 part in 1800 of diameter, or 1 part in 600 of distance diffused. Therefore, rounding the potencies to three digits (Table IV) introduced little error even in the high accuracy assays reported here. Such rounding should be done in assays to avoid the appearance of unreal and unrealizable accuracy.

The results in Table IV indicate accuracy to be substantially independent of thickness of the base layer in the range from 10 to 20 ml. Extrapolation by less than 10% did not degrade accuracy greatly. However, the lines in Fig. 1 show that the usual extrapolation (Eq. 1) beyond the 8-μg/ml point with the slope determined by the 4–8-μg/ml region will cause error in potency. These errors come

Table III—Assay for Cephalexin Standards in Micrograms per Milliliter, 10-ml Base Layers

	Standards					
	4 μg/ml			8 μg/ml		
	15.84	15.93	15.85	18.32	18.57	18.22
	15.68	15.93	15.63	18.43	18.09	18.25
	15.55	15.96	15.44	18.33	18.42	17.67
Mean		15.76			18.26	
RSD, %		1.19			1.42	

Samples

Number	Sample Zones, mm			Reference Zones, mm			Adjusted Mean	Result
1	18.18	18.39	18.65	18.23	18.71	18.38	18.23	7.944
	18.01	18.34	18.10	18.33	18.61	17.81		
	18.18	18.37	18.45	18.11	18.46	18.26		
Mean		18.30			18.32			
RSD, %		1.08			1.46			
2	18.09	18.50	18.26	18.40	18.23	18.37	18.16	7.788
	17.89	18.27	18.00	18.29	18.42	18.06		
	18.02	18.48	18.31	18.27	18.57	18.08		
Mean		18.20			18.30			
RSD, %		1.18			0.89			
3	18.52	18.72	18.12	18.33	18.50	17.82	18.51	8.590
	18.25	18.75	18.27	18.45	18.15	18.02		
	18.46	19.03	18.09	18.62	18.13	17.88		
Mean		18.47			18.21			
RSD, %		1.73			1.54			
4	17.81	18.32	18.20	18.12	18.46	18.09	18.07	7.603
	18.08	18.28	18.23	18.33	18.39	18.46		
	17.72	18.46	17.96	18.21	18.59	18.06		
Mean		18.12			18.30			
RSD, %		1.36			1.03			
5	18.36	18.17	18.36	18.09	18.11	18.47	18.23	7.946
	17.82	18.02	18.30	18.03	18.45	18.12		
	18.02	18.39	18.33	18.04	18.44	18.24		
Mean		18.20			18.22			
RSD, %		1.11			1.01			
6	18.97	18.86	18.87	18.75	18.52	18.46	18.53	8.641
	18.47	18.46	19.04	18.02	18.56	18.39		
	18.14	18.69	18.26	18.03	18.66	17.87		
Mean		18.64			18.36			
RSD, %		1.73			1.70			

from use of inappropriate standard lines and are not inherent in the assay.

Potencies of the 8.70-μg/ml samples were also obtained from lines drawn point to point to include the 4–16-μg/ml region of the standard curve.

The rationale for correcting the FDA and Cooper types of lines for plate-to-plate variations is fundamentally different. The FDA correction assumes slope of lines of standards and sample to be identical (intercept changes). This is the same as saying that the value of A in Eq. 1 may not be the same for all plates whereas B is the same.

According to the Cooper equation, factors (not concentration of antibiotic) causing first-order changes in zone size do so by affecting the slope of the line (intercept constant). Thus, in Eq. 2, F is affected by operational and environmental variations. In reality, B of Eq. 1 is also affected by the same factors. When using Eq. 2 to correct for operational variances (Table IV), the value of E is computed from the responses of the standard curve. The value of F is computed from the mean of the responses of the reference on the set of three plates in question and then the equation is used to interpolate values of sample concentrations. A different value of F is computed for each set of three plates. Results of these interpolations are given in Table IV for the 8.7-μg/ml sample.

If the extrapolated portion of the calibration line is to be used without degrading accuracy, the slopes of the lines above or below the adjacent standard must be the same. This restriction, when ap-

Table IV—Assay of Samples of Known Concentrations (Data from Table III)

Base Layer	Concentration of Samples, μg/ml	Form of Equations			
		d versus log *C*		$1/4(d - 5.8)^2$ *versus* log *C*	
		Potencies, μg/ml	Error, %	Potencies, μg/ml	Error, %
10 ml	7.55	7.79, 7.60	3.2, 0.66	7.76, 7.55	2.78, 0.00
	8.00	7.94, 7.95	−0.75, −0.63	7.93, 7.93	−0.88, −0.88
	8.70	8.59, 8.64	−1.26, −0.69	8.68[b], 8.73[b]	−0.21, 0.34
	8.70	8.65[a], 8.71[a]	−0.57, 0.11	8.64[c], 8.68[c]	−0.69, −0.23
20 ml	7.55	7.62, 7.58	0.93, 0.40	7.57, 7.52	0.26, −0.40
	8.00	8.08, 7.94	1.00, −0.75	8.10, 7.93	1.25, −0.88
	8.70	8.74, 8.74	0.46, 0.46	8.86[b], 8.88[b]	1.88, 2.09
	8.70	8.77[a], 8.77[a]	0.80, 0.80	8.68[c], 8.70[c]	−0.23, 0.00

[a] Interpolated from a three-point calibration line drawn point to point; same data as on line above. [b] Obtained from extrapolated standard curve for the 4–8-μg/ml region. [c] Line computed for the 8–16-μg/ml region; same data as for line above.

Table V—Two-Dose Assays of 8-μg/ml Standard

Agar Layer	Sample Number	Potency, μg/ml	*SD*	Error	Error, %
10 ml	1	8.274	0.34	+0.274	+3.43
	2	7.615	0.37	−0.385	−4.81
20 ml	1	7.851	0.11	−0.149	−1.86
	2	7.819	0.12	−0.181	−2.26

plied to the examples, requires that the lower standard be 4 μg/ml for plates with 10 ml of base layer and 8 μg/ml for 20-ml base layer plates and that Eq. 2 be used. If Eq. 1 is the interpolation formula, then the standard curve should be drawn point to point. The worst possible line is the best straight line through four or five widely spaced points (B in Fig. 1).

In diffusion assaying where at least four types of calibration lines are used, compensation of errors may occur through the use of an incorrect line. Therefore, agreement with an expected answer is not proof of correctness. The answer must also be obtained by procedures free from obvious technical errors.

The results in Table IV show that extrapolation can lead to significant errors because of changes in slope of the calibration line. Equation 2 probably is better than Eq. 1 as an interpolation formula and requires no more work when a computer is available. For assays done without special precautions and a computer, the FDA procedure is adequate. The data also show that potency of the sample should fall between the ends of the standard line and that the span of the line should, as a general rule, be restricted to twofold when the FDA method is used.

Previous theoretical treatment (3) showed that the unique feature of the FDA single-dose design, the method for correcting for plate-to-plate variations, gives a good first approximation to reality and is essential to high accuracy assays (Table IV).

Although errors of several percentages caused by manual interpolation of potencies from graphed calibration lines were avoided by having the computer make the interpolations mathematically, a further advantage was the practical one of removing restrictions upon the form of the calibration line. Although interpolation from the *d versus* log *C* plot on semilogarithmic paper is more conveniently done manually than from a plot of X^2 *versus* log *C*, this is not true for the computer.

The data in Tables I and II were subjected to an analysis of variance. Sources of variation were considered to be dose, plate, thickness of base layer, and measurement of zone size. The first three sources were significant ($p < 0.0005$); no interaction terms were significant. The first must be significant in order to have a test. A significant plate-to-plate variation indicates the necessity for correcting the variation in high accuracy assays. Significant variation caused by different thicknesses of agar layer was expected. The standard deviation of measurement was 0.05 mm. There was no difference in operators. Although the plate-to-plate differences in zone sizes were small, the analysis of variance found them to be significant because of the high resolution and consistency of the measurements.

The accuracy of diffusion methods is severely limited by several factors about which little, if anything, can be done. Among these is the inability of the operator to determine reliably the edge of the zone to better than 0.02 mm, even with a very favorable system. If the uncertainty in measuring zone size is independent of zone size, the percentage of uncertainty in the potency estimate is independent of potency and decreases as the slope of the calibration line increases.

Equation 2, not Eq. 1, is the dose–response line. Equation 1 is only a convenient empirical calibration line for short-range assays. The low precision of measuring distance diffused limits the accuracy of diffusion assays. Zone edges of certain essential assay systems never are sharp. Add to these the generally poor control over operations and accuracy becomes considerably poorer than that obtained here.

Diffusion assays are particularly susceptible to technique. Careless, indifferent, or improperly trained technicians produce assays of low accuracy and precision. Contributing to the degradation of technique is the belief that diffusion assays are biological and, as such, are inherently variable. Therefore, care is not needed because of the large "biological variation." The evidence to date supports the earlier (13) statement that the causes of "biological variation" in microbiological assaying have primarily a macro- and not a microbiological source.

Two-Dose Assays—The first official FDA microbiological assay for penicillin was the two-dose cylinder-plate method (1, 2). Both standard and sample are present on each plate at two concentrations. The ratio of concentrations is the same for standard and sample and can be either 1:2 or 1:4. Each plate has four zones, and four plates constitute the assay of a sample. Each plate contains a complete calibration line and, thus, becomes a complete assay of the sample.

This assay is statistically symmetrical; standard and sample are treated alike. The procedure for calculating the potency of the sample is that for a parallel line assay in which standard and sample lines have identical slopes. The identity of the slope is assured by using the responses from the standard and sample to compute a mean slope. The potency of the sample is the mean of the four potencies obtained from the four plates.

The data on standard curves given in Table II can be considered to be a two-dose assay in which a standard and two samples are on each plate. Three plates, not four, constitute assay of the samples in this example. For purposes of computation, the zone diameters on the first line under each plate number was assigned to the standards, the next row to Sample 1, and the third row to Sample 2. Thus, three potencies for each sample were obtained, one from each plate. The mean of the three values was reported as the potency of the sample in Table V.

All potencies differed from the true value of 8 by more than 1.5%. The standard deviations of the assays performed on plates with a 20-ml base layer were substantially less than those of plates with 10 ml of base layer. The assays on plates with a 20-ml base layer had the smaller errors. Comparison with the assays of the 8-μg/ml samples given in Table IV shows the latter to have the smallest errors.

One difference between the two types of assays is the amount of replication. Nine responses (one concentration) of sample were used in the single-dose method whereas only six (three at two concentrations) were used in the two-dose assay. The number of responses of standard was also smaller in the two-dose method. An assumption underlaying the two-dose method was the identity of slopes of the two dose–response lines. Departure from ideality was assumed to be caused by variances and not biases. Only two of the 12 pairs of slopes were identical. The ratio of the slope of the standard line to the sample line ranged from 0.94 to 1.3 for the plates with 10 ml of agar base layer and from 0.97 to 1.1 for the 20-ml plates.

The two-dose method has the theoretical advantage that each petri dish is a complete test; thus, the influence of plate-to-plate variations in zone size upon precision and accuracy is removed. Plate-to-plate variations in zone diameter could be large at the time (before 1945) when the method was developed. The method was not widely used and was soon abandoned in favor of the single-dose assay. One reason for abandoning it was the lack of identity of slopes of standards and samples, as illustrated by the results of this investigation. Were the differences in slopes real was the unanswerable question. Results of this investigation, which was done under much more favorable conditions than existed in 1945, indicate that the method was inferior to the single-dose method.

SUMMARY

Facilities and equipment used in this work were designed for plate assaying and were superior to most. Zone diameters were measured with a resolution of 0.01 mm. Control of other operations was such that the corrected mean zone diameter for a sample on one plate was indistinguishable from the corrected mean sample zone diameters on the other two of the set of three plates.

Acquisition and processing of the data by a computer not only reduced drudgery and error associated with manual operations but also removed practical restrictions upon form of the standard line. A calibration line of the form X^2 *versus* log *C* was better than the usual *d versus* log *C*. The principal assay used was the single-dose design of FDA modified to employ only two standards. The test assay, cephalexin–*B. subtilis*, had such sharp zone edges that most

measurements of diameter were replicated within 0.08 mm or within 2% of potency.

Using the equipment, procedures, and designs described here, proper computational procedures, and a very favorable assay system, a single determination (three plates) may be expected to be within 2% of the true value. Furthermore, no increase in effort or expense is required. As shown by the consideration of the influence of error of zone measurement upon error in sample potency, the tests cannot be greatly improved. The modified method requires fewer plates and less operator time than the standard FDA method. The accuracy of this plate method should be compared with the five- to 10-fold greater accuracy obtainable from an automated method for turbidimetric assays (14). Such differences in inherent accuracy is one reason to prefer turbidimetric methods when they are applicable.

The two-dose design of FDA was a less satisfactory method than the single-dose assay because it seemed to be excessively sensitive to the slope of the calibration line.

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Automated Constant-Current Coulometric Assay System for Ascorbic Acid and Sodium Ascorbate

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Abstract □ The performance of an automated constant-current coulometric system for the assay of ascorbic acid and sodium ascorbate is described. After loading, it is capable of analyzing 25 samples and printing out the titer values with no operator attention for 2.5 hr. Under optimum conditions, ascertained by evaluating various electrochemical parameters, the accuracy and precision (95% ts) were found to be ±0.3%.

Keyphrases □ Ascorbic acid and sodium ascorbate—automated constant-current coulometric assay system □ Coulometry, constant current, automated—analysis, ascorbic acid and sodium ascorbate

Coulometric analysis of ascorbic acid, using both controlled-potential and constant-current methods, was reported previously. The constant-current method with iodine generated at the anode was used for the determination of small quantities (about 0.5 mg) of ascorbic acid with biamperometric ("dead-stop") end-point detection (1). The reproducibility was found to be ±0.1% *SD*. A platinum anode controlled at +1.1 v *versus* the saturated calomel reference electrode was used to oxidize 15–100 mg of ascorbic acid within 0.5–1 hr, with an average accuracy of ±0.7 mg (2).

The constant-current method employing electrogenerated iodine affords the electrochemical equivalent to the USP assay and offers a time advantage over the potentiostatic technique. Therefore, it was chosen for automation. A modular system (Fig. 1) consisting of an electrode station, electrolyte dispenser, current source, sample changer, control module, and

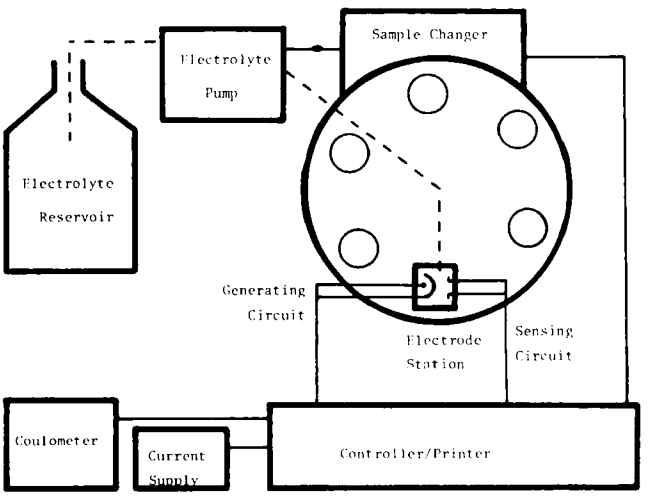

Figure 1—*System block diagram and interrelation of components.*

measurements of diameter were replicated within 0.08 mm or within 2% of potency.

Using the equipment, procedures, and designs described here, proper computational procedures, and a very favorable assay system, a single determination (three plates) may be expected to be within 2% of the true value. Furthermore, no increase in effort or expense is required. As shown by the consideration of the influence of error of zone measurement upon error in sample potency, the tests cannot be greatly improved. The modified method requires fewer plates and less operator time than the standard FDA method. The accuracy of this plate method should be compared with the five- to 10-fold greater accuracy obtainable from an automated method for turbidimetric assays (14). Such differences in inherent accuracy is one reason to prefer turbidimetric methods when they are applicable.

The two-dose design of FDA was a less satisfactory method than the single-dose assay because it seemed to be excessively sensitive to the slope of the calibration line.

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Automated Constant-Current Coulometric Assay System for Ascorbic Acid and Sodium Ascorbate

S. A. MOROS ˣ, C. M. HAMILTON, J. E. HEVERAN, J. J. DONAHUE, and S. OLIVERI-VIGH

Abstract □ The performance of an automated constant-current coulometric system for the assay of ascorbic acid and sodium ascorbate is described. After loading, it is capable of analyzing 25 samples and printing out the titer values with no operator attention for 2.5 hr. Under optimum conditions, ascertained by evaluating various electrochemical parameters, the accuracy and precision (95% ts) were found to be ±0.3%.

Keyphrases □ Ascorbic acid and sodium ascorbate—automated constant-current coulometric assay system □ Coulometry, constant current, automated—analysis, ascorbic acid and sodium ascorbate

Coulometric analysis of ascorbic acid, using both controlled-potential and constant-current methods, was reported previously. The constant-current method with iodine generated at the anode was used for the determination of small quantities (about 0.5 mg) of ascorbic acid with biamperometric ("dead-stop") end-point detection (1). The reproducibility was found to be ±0.1% *SD*. A platinum anode controlled at +1.1 v *versus* the saturated calomel reference electrode was used to oxidize 15–100 mg of ascorbic acid within 0.5–1 hr, with an average accuracy of ±0.7 mg (2).

The constant-current method employing electrogenerated iodine affords the electrochemical equivalent to the USP assay and offers a time advantage over the potentiostatic technique. Therefore, it was chosen for automation. A modular system (Fig. 1) consisting of an electrode station, electrolyte dispenser, current source, sample changer, control module, and

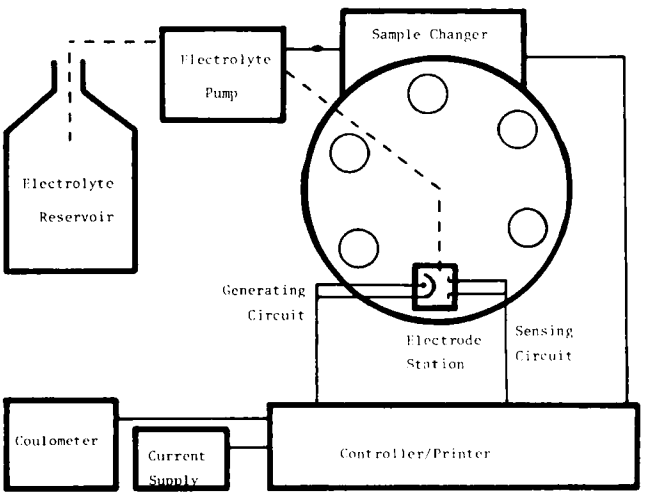

Figure 1—*System block diagram and interrelation of components.*

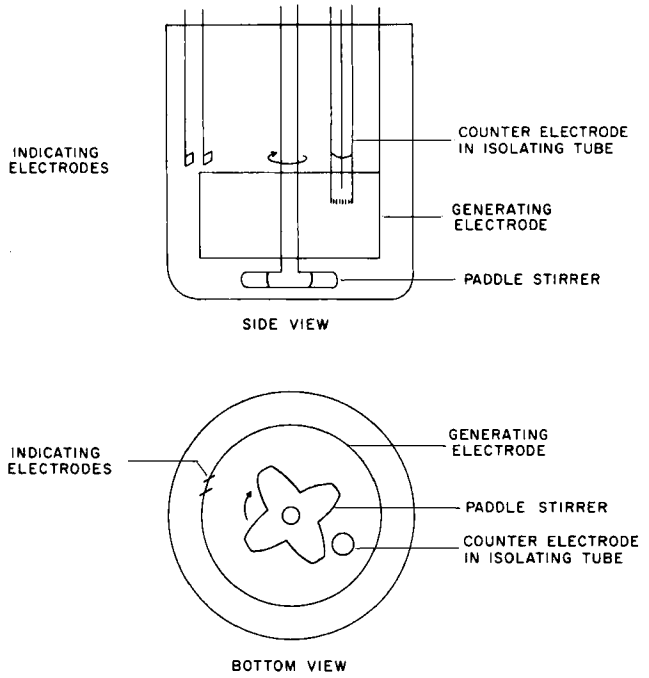

Figure 2—*Cell geometry.*

printer was assembled, and a study of various electrochemical parameters was undertaken to define the optimum conditions for the assay.

EXPERIMENTAL

Electrode Station—The electrode station was a cluster of components including the electrolyte delivery tip, the stirrer, and the generating and sensing electrodes, all mounted on a stationary arm bearing the stirring motor and appropriate electrical wiring. The glass stirrer consisted of four 2 mm-thick blades or paddles (each ~10-mm diameter) fused to the end of a 5-mm diameter glass rod to form a flat cloverleaf. At 560 rpm, efficient stirring was obtained without a vortex being formed or air entrained as observed with propeller-shaped stirrers.

The relative positions of the stirrer and electrodes, *i.e.*, cell geometry, are indicated in Fig. 2. The generating electrode (anode) was a cylinder of platinum screen (40-mm diameter × 25-mm height) coaxial with the shaft of the stirrer whose blades were positioned just below this electrode. The counter electrode (cathode) was a 5-mm length of platinum wire immersed in a few milliliters of electrolyte contained in a sintered-glass isolation tube located within the anode cylinder. The indicating electrode pair (5-mm platinum foil squares with the faces set parallel and 2 mm apart) was positioned outside the field between the anode and cathode.

Electrolyte Dispenser—A metering pump[1] delivered the requisite volume (80 ml) of electrolyte from a reservoir prior to each titration. The electrolyte, 0.5 *M* KI in 0.4 m*M* H_2SO_4, was treated with a steady stream of nitrogen passing through a submerged sintered-glass gas-dispersion tube to prevent air oxidation.

Current Source—A constant-current source, based on conventional operational amplifier circuitry, provided 25–200 mamp with an accuracy of ±0.2% at a voltage compliance of 18 v. The 10-μamp polarizing current for the indicating electrodes was similarly obtained from conventional transistor circuitry.

Sample Changer—By means of an elevator mechanism, a turntable[2] with 50 positions for 100-ml beakers presented 25 preweighed samples and rinse water alternately to a fixed electrode station where the electrolysis was carried out in the sequence given under *System Operation.* As each revolution was completed, the entire turntable was automatically displaced relative to the electrode station to present beakers in the next circle. After the last circle of samples was presented, the turntable returned to the starting position and the "end of cycle" signal was displayed.

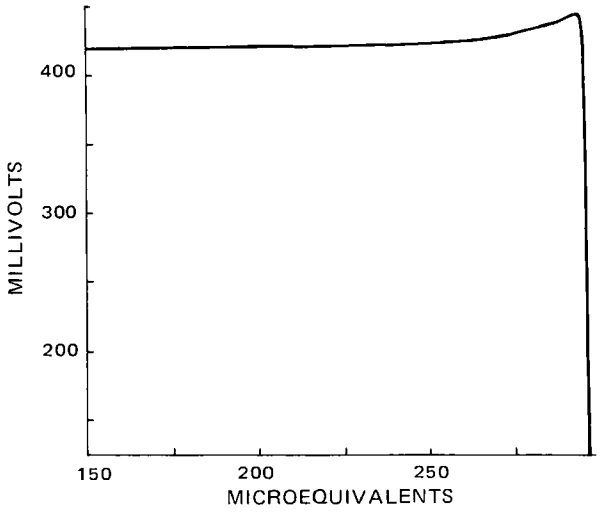

Figure 3—*End-point region of titration curve.*

Control Module and Printer—The operation of the entire system was controlled by a printing coulometer[3] incorporating the aforementioned current sources, a sensing and control circuit, a coulometer, and a printer. The sensing circuit displayed the potential across the indicating electrodes and compared it with a preset end-point potential, ending the titration when the potentials became equal. An adjustable time delay of up to 180 sec in the sensing circuit precluded false end-point signals, which could result from switching transients occurring at the start of the titration. An electronic integrator, accurate to 0.1%, provided a continuous digital readout in microequivalents and drove a printer for the final readout.

System Operation—The listed components formed a constant-current coulometric system operating in the following sequence. As each sample was presented to the electrode station, the electrolyte dispenser delivered electrolyte and the stirrer was activated. After 30 sec of stirring to dissolve the sample, the generating current was started to begin the titration. At the end-point, the control module stopped the generating current, printed both the sample number and titer in microequivalents, reset the integrator and printer, and sent a signal to the sample changer. This procedure resulted in the titrated sample being replaced by a beaker of rinse water for washing the electrodes and the next preweighed sample being presented to the electrode station.

Electrochemical Parameters—The generating current was selected to provide the highest titration rate possible consistent with 100% current efficiency for iodine generation. This current corresponded to a current density of 5 mamp/cm^2 of anode area in the 0.5 *M* KI electrolyte employed and did not increase significantly at higher concentrations. Above this current, the precision of the titration decreased and gas formation became evident at the anode.

The remaining electrochemical parameters were dictated by the titration curve; the end-point region is depicted in Fig. 3. The potential between the indicating electrodes rises gradually (20–30 mv) during the titration and more sharply just before excess iodine appears, whereupon the potential falls rapidly almost to zero. For the end-point, the comparator voltage was set 40 mv below the peak potential after several trials. This choice allowed for some variation in electrode response during a run but minimized the value and variability of the blank correction required for a larger difference.

The response of the indicating electrodes became less stable and the potential difference between them decreased during a series of titrations of ascorbic acid unless the electrolyte was acidified. The response was degraded during titration of sodium ascorbate to the

[1] Vision Laboratories Beaker Butler.
[2] Vision Laboratories Li'l Squirt.
[3] Ryaby Associates.

Table I—Precision Studies

Sample	Weight, mg	Current, mamp	Electrolyte Sulfuric Acid Concentration, m*M*	Degrees of Freedom	Precision, % (95% Confidence Level)
Ascorbic acid					
A	30	200	0	46	0.4
B	30	200	0	11	0.4
A	30	100	0	17	0.3
B	30	100	0	67	0.3
C	30	120	0	30	0.3
B	100	120	0	8	0.1_5
D	30	125	0.4	22	0.2
D	400	(USP manual titration)		14	0.6
Sodium ascorbate					
E	30	120	0.1	26	0.5
F	30	120	0.1	17	0.9
E	30	120	0.4	50	0.3

extent that only a few samples could be titrated. In both cases, the formation of a coating on the indicating electrodes appeared to be involved; rinsing with a few drops of concentrated sulfuric acid restored the original electrode condition. Incorporating a trace of acid in the electrolyte minimized electrode contamination, so a full load of 25 samples could be titrated with the same end-point setting.

Other Analytical Parameters—The blank could not be directly determined because the potential obtained in acidified potassium iodide electrolyte was substantially lower than that obtained in the presence of sample. Instead, the blank was determined by extrapolating to zero sample weight, having titrated various weights of sample. Typical blank values were equivalent to 0.5% assay, but variations in these values were obtained with lots and suppliers of potassium iodide. These values were confirmed by manual titration with 0.1 *N* I_2 solution; the values ranged from 2 to 6 microequivalents of iodine/80 ml of electrolyte, values that would, if neglected, adversely affect the assay values.

Stability studies of acidified 0.5 *M* KI sparged with nitrogen showed no significant deterioration for up to 3 weeks.

A 30-mg sample size represents a compromise among such factors as titration time, weighing error, blank variability, and sample homogeneity. When using the maximum current for the electrochemical conditions (electrode area, stirring, temperature, *etc.*) employed, 5 min was required for titrating samples of the size recommended.

RESULTS AND DISCUSSION

Numerous determinations of the precision of the method were made on replicate samples of various lots of ascorbic acid or sodium ascorbate (Table I). Generating current levels of 200 mamp afford somewhat poorer precision than 120-mamp levels in the titration of ascorbic acid. Increasing the sample size to 100 mg (from the recommended 30 mg) improves the precision at the cost of prolonged titration time. The addition of acid permits the titration to be carried out in a reasonable time with excellent precision, ±0.2% (95% probability). Similarly, sodium ascorbate titrations are best carried out at the 0.4 m*M* acid concentration recommended. In this case, the use of a slightly lower acid concentration significantly reduces the precision of the measurement. In summary, the precision of the recommended method exceeds that of the USP XVIII (3) manual titration (different operators weighed and titrated the samples to avoid bias).

The automated system, standardized electronically, was compared to chemical standardization by ascorbic acid and arsenic trioxide to determine the accuracy of the system. Ascorbic acid, assayed as 100.0% by manual iodometric titrations, gave a coulometric value of 99.7%. Arsenic trioxide (National Bureau of Standards), when dissolved in base, acidified, and titrated, gave an average of 100.2% for 12 determinations.

The automated system described is capable of analyzing 25 samples in less than 4 hr, apportioned as follows: electrolyte and rinse preparations, 30 min; sample weighing, 35 min; titrator operation (100 mamp), 2.5 hr; cleanup, 15 min; and calculations, 10 min. The total time can be reduced by approximately 30 min if the remaining samples are weighed as the first few are being titrated. In any event, the operator's attention is required for only 90 min to carry out the auxiliary functions.

This automated system is similar in principle to the USP method and: *(a)* is equivalent to, or exceeds, the manual method in precision and accuracy; *(b)* is significantly less time consuming than the manual method; and *(c)* eliminates the need for preparation, standardization, and storage of the titrant.

While the prototype system is being evaluated for other applications, an equivalent system is in daily operation for monitoring the quality control of ascorbic acid production. Incorporating automatic weighing equipment and digital output capability would permit calculations to be performed by a computer, virtually eliminating all sources of human error.

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Automated Spectrophotometric Assay of Cefazolin

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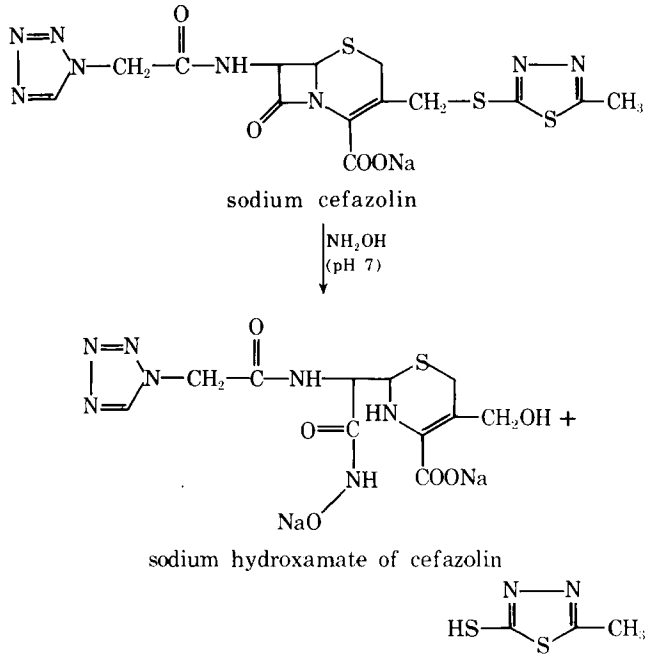
Abstract □ An automated, stability-indicating, UV spectrophotometric assay for cefazolin is presented. The method employs a reaction with hydroxylamine and derives its stability-indicating power through comparison of reacted and unreacted aliquots of the sample. A double-probe sampling procedure is used. Good agreement with microbiological assays is obtained, and the coefficient of variation is about 1%.

Keyphrases □ Cefazolin—automated spectrophotometric assay, stability indicating, double-probe sampling procedure □ Automated methods—spectrophotometric assay of cefazolin, stability indicating, double-probe sampling procedure □ Spectrophotometry, automated—assay of cefazolin, stability indicating, double-probe sampling procedure

Cefazolin sodium, a potent new cephalosporin, is used parenterally and exhibits a broad spectrum of antibiotic activity (1–3). It is a certifiable antibiotic, and two methods of analysis have been approved by the Food and Drug Administration for certification. One method is an agar diffusion microbiological procedure (4), and the other is a manual colorimetric hydroxylamine procedure (4).

While such methods are adequate for certification, a more rapid method is desirable for production and stability studies where the number of samples becomes large. For this reason, an automated assay procedure that is both rapid and stability indicating was developed. The method employs the reaction between cefazolin and hydroxylamine to derive its stability-indicating characteristics. Hydroxylamine reacts with the β-lactam function of all cephalosporins and penicillins in an alkaline medium to produce a corresponding hydroxamic acid salt (5) (Scheme I).



Scheme I

In the case of cefazolin, the UV absorption spectra of the intact cephalosporin and its corresponding hydroxamic acid are different (Fig. 1). This difference makes it possible to assay for intact cefazolin in the presence of breakdown products or other UV-absorbing background materials which do not react with hydroxylamine. The procedure is to make a stock solution of the sample to be tested and then remove two identical aliquots. One aliquot is diluted to a suitable concentration for spectrophotometry, while the other is first reacted with hydroxylamine and then diluted.

The absorbance of both solutions is measured at 271 nm, and the difference is calculated. It is this absorbance difference that is proportional to the con-

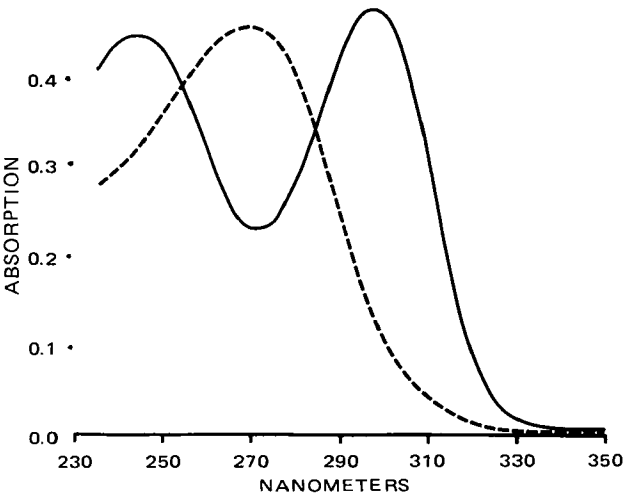


Figure 1—*Absorption spectra of cefazolin (- - -) and its hydroxamic acid salt (—).*

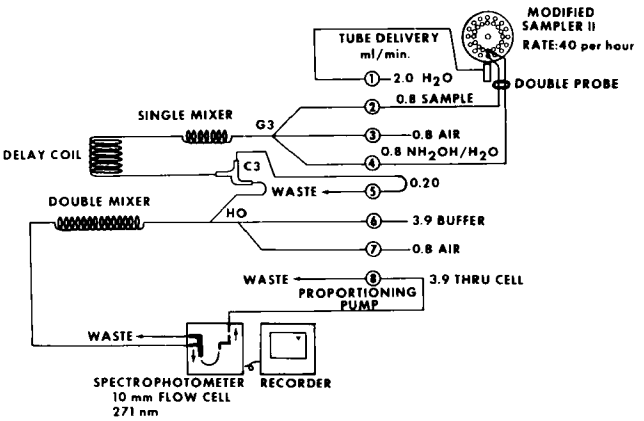


Figure 2—*Flow diagram of automated spectrophotometric assay for cefazolin.*

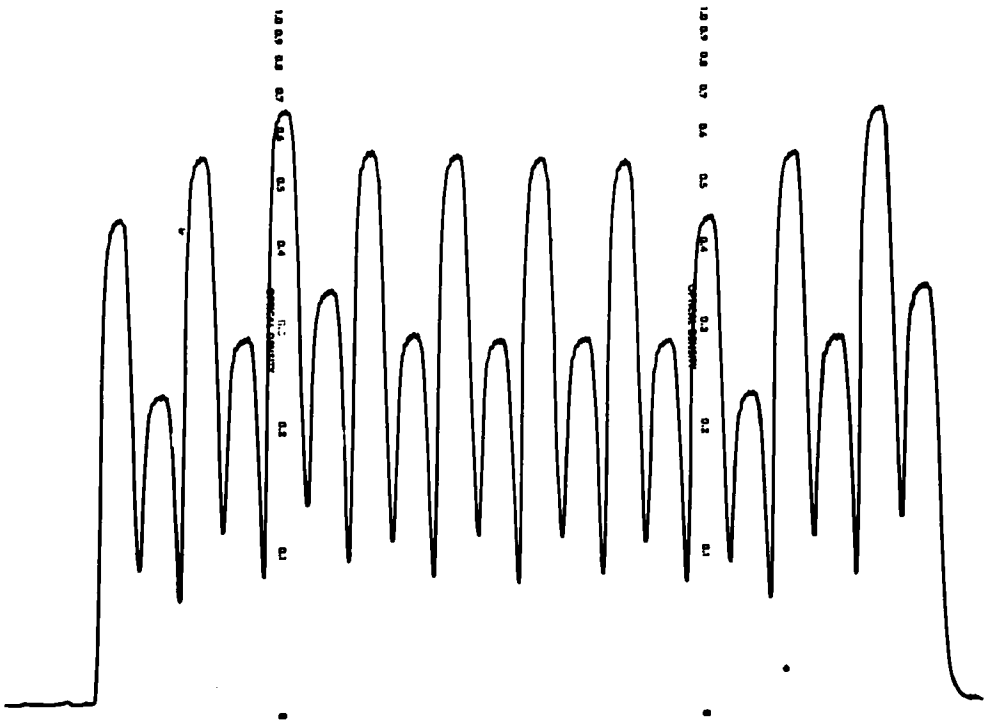


Figure 3—*Typical recording showing four samples preceded and followed by three standards.*

centration of intact β-lactam, cefazolin. The actual concentration is calculated by comparison with a reference standard run in the same manner.

EXPERIMENTAL

Reagents—The following were used:

1. A 0.5 *M* sodium bicarbonate aqueous solution.
2. Buffer, pH 4—Mix equal volumes of aqueous 0.1 *M* sodium acetate and 0.1 *M* acetic acid and adjust to pH 4.0.
3. Alkaline sodium acetate solution—Dissolve 34.6 g of sodium hydroxide and 4.1 g of sodium acetate in water and dilute to 1000 ml.
4. Hydroxylamine reagent—Mix 100 ml of aqueous 5 *M* hydroxylamine hydrochloride and 500 ml of alkaline sodium acetate solutions.

Standard Solutions—*Stock Standard Solution*—Weigh accurately about 100 mg of cefazolin reference standard and transfer to a 100-ml volumetric flask. Add 10 ml of 0.5 *M* sodium bicarbonate solution and swirl to dissolve the cefazolin. Dilute to volume with water and mix well.

Working Standard Solutions—Transfer 20, 25, and 30 ml of stock standard solution to separate 50-ml volumetric flasks. Dilute to volume with water and mix well.

Sample Preparation—Weigh accurately a portion of sample expected to contain about 100 mg of cefazolin and transfer to a 200-ml volumetric flask. Add 10 ml of 0.5 *M* sodium bicarbonate solution and swirl to dissolve the cefazolin. Dilute to volume with water and mix well.

Instrumentation—The following were used: a standard, continuous-flow, analyzer proportioning pump[1] with a manifold platter conforming to the flow diagram shown in Fig. 2 (6); a UV spectrophotometer[2] equipped with a low-volume flow cell[3]; strip-chart recorder suitable for recording the output of the spectrophotometer as a function of time; and a sampler modified to allow the installation and use of two solution pick-up probes, with its turntable drilled to accommodate two standard 2-ml sample cups side by side at each position.

The modification of the sampler allows the solution to be aspirated from two side-by-side cups simultaneously. Thus, two identical aliquots of sample can be aspirated into the analytical system, with one being reacted with the simultaneously aspirated hydroxylamine and the other simply being mixed with a volume of water equivalent to that of the hydroxylamine. This principle has utility in other situations where an unreacted sample blank is required. It has been applied in this laboratory to the iodometric procedure for cephalosporins as well.

This approach, first used by Strandjord and Clayson (7) for enzyme assays, is superior to the commonly used blank procedures which either make a separate run without one reagent for the blank or set up a parallel duplicate system devoid of one reagent. In the first method, instrumental drift or changes in pumping characteristics of the manifold tubes may introduce errors; the second method requires perfect matching of two systems, which is quite difficult to achieve. The double-probe technique overcomes both these problems, since sample and blank run through the exact same system side by side, virtually eliminating any effect of drift.

Procedure—A reagent baseline is established with the reagents flowing according to the flow diagram of Fig. 2. The sample cups are arranged in two concentric circles: an outer circle and an inner circle. Two cups of each cefazolin-containing solution, either standard or sample, are placed in neighboring positions in the outer circle of cups. A cup containing hydroxylamine reagent is placed in the position in the inner circle alongside one of the pair of cefazolin-containing cups; alongside the other is placed a cup of water. Thus, at one sampler position, sample solution plus hydroxylamine reagent is aspirated into the analytical system while another aliquot of the sample solution plus water is aspirated at the next position.

This procedure produces two peaks for each sample (or standard); one has been reacted with hydroxylamine and one has not. The difference between absorbances represented by these two peaks is proportional to the concentration of intact cefazolin in the solution. A typical recording is shown in Fig. 3. The concentration of the sample solution may be determined by plotting the absorbance difference *versus* concentration of the standards and reading the sample concentration from this graph.

[1] Technicon AutoAnalyzer, Technicon Instruments, Tarrytown, N.Y.
[2] Hitachi-Perkin-Elmer-Coleman 111, Arthur H. Thomas, Philadelphia, Pa.
[3] Arthur H. Thomas microflow cell, Arthur H. Thomas, Philadelphia, Pa.

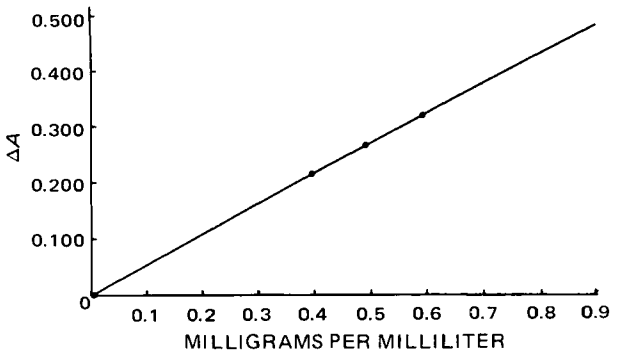


Figure 4—*Relationship between absorbance difference and cefazolin concentration.*

Table I—Replicate Determinations on a Single Sample

Milligram of Cefazolin per Milliliter	Milligram of Cefazolin per Milliliter
0.493	0.503
0.505	0.501
0.500	0.502
0.503	0.501
0.505	0.491

Mean = 0.500
SD = 0.0047
CV = 0.94%

Table II—Day-to-Day Variation

Sample	Percent Purity		
	Day 1	Day 2	Difference
1	78.5	80.0	1.5
2	90.3	90.4	0.1
3	66.4	66.5	0.1
4	67.7	68.7	1.0
5	93.5	94.0	0.5

Mean difference = 0.64
SD = 0.59
CV = 0.74%

Table III—Comparison between Automated Chemical and Microbiological Procedures

Lot	Percent Purity		
	Chemical	Microbiological	Difference
A	94.3	92.7	1.6
B	99.5	99.4	0.1
C	93.5	93.3	0.2
D	94.6	94.7	0.1
E	94.4	95.4	1.0
F	94.0	95.2	1.2
Mean	95.1	95.1	0.7

Alternatively, since such a graph is linear (Fig. 4), an average response factor may be calculated from the standards and used to calculate sample concentration. If the automated system is equipped with an automatic peak detection device, the calculations can be done directly by computer. This mode of operation has been used successfully in this laboratory.

RESULTS AND DISCUSSION

As can be seen from Fig. 2, the method is extremely simple and requires only two reagents: the alkaline hydroxylamine reagent and the acidic buffer used to quench the reaction. The only critical part of the assay is the time of reaction between the cefazolin and the alkaline hydroxylamine. An experiment was carried out to determine the optimum reaction time, and the result was a reaction time of 6 min (Fig. 5). This time is achieved by a delay coil consisting of 10.5 turns of 2-mm i.d. glass tubing wound with a 14-cm diameter.

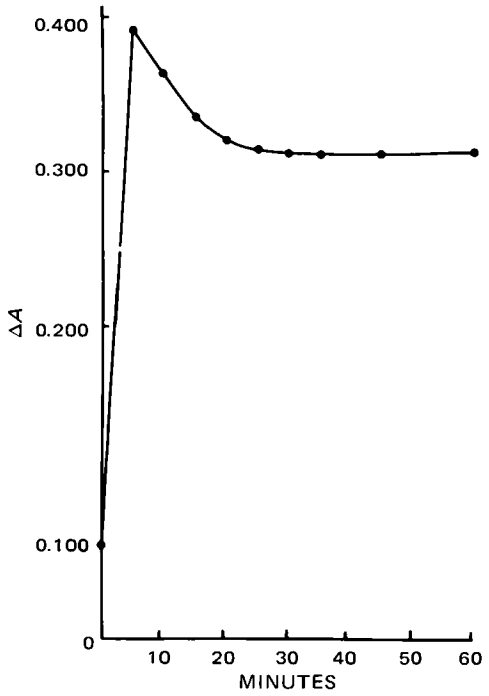


Figure 5—*Absorbance difference* versus *time of reaction between cefazolin and hydroxylamine.*

Table I shows the results of 10 successive determinations of the same sample solution run at one time. The standard deviation is 0.0047 mg/ml or 0.94%. Table II shows the results obtained by repeating five samples on two different days; the coefficient of variation of 0.74% is the same order of magnitude as that obtained on a single day. Table III shows a comparison of results obtained by this procedure with those of a microbiological procedure (8). These results show excellent agreement, as indicated by the mean difference of 0.7%.

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Genetic Effects of Povidone-Iodine

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Abstract □ Povidone-iodine is capable of specifically altering the DNA of living cells. This alteration may result in the induction of mutations of the base-substitution type. Because of the known relationship between mutagenic potential and the ability to induce cancer in animals, the present findings raise serious questions concerning the safety of this topical disinfectant.

Keyphrases □ Povidone-iodine—genetic effects □ Mutagenicity—povidone-iodine □ Genetic effects—povidone-iodine

Povidone-iodine[1], an organic iodine complex reported to contain 1% free iodine (1), is widely used as a topical disinfectant. Because halogens, including iodine, are capable of reacting with nucleic acids and their constituents (2–5) and in view of the known relationship between the ability to react with DNA and mutagenesis and/or carcinogenesis (6–8), the potential genetic effects of povidone-iodine were investigated. The ability of povidone-iodine to modify intracellular DNA and to induce mutations in *Salmonella typhimurium* is now reported.

EXPERIMENTAL

Mutagenicity Testing—A series of histidine-requiring mutants of *Salmonella typhimurium* was constructed, which can be used to detect and classify mutagens. Two of these strains, TA1530 and TA1538, were used in this study; they can be induced to revert to histidine independence by base substitution and frameshift mutagens, respectively (9–11). To determine the mutagenicity of povidone-iodine, two procedures were used.

In the standard mutagenicity assay (9), bacteria are incorporated into an agar overlay. When the agar surface has solidified, a paper disk impregnated with the test agent is deposited on it. The plates are incubated for 54 hr at 37°, and the number of mutants (revertants to histidine independence) is determined. The diameters of the zones of growth inhibition are also measured.

A second, more quantitative, procedure was also used. Bacteria in liquid medium (Columbia base-broth-BBL) were chilled to 4°, and povidone-iodine was added. At intervals, portions of the cells were removed, the povidone-iodine was decomposed by addition of ascorbic acid (final concentration 0.1 *M*), and 10-fold serial dilutions of the treated bacteria were spread onto the surface of agar plates for the determination of the number of revertants to histidine independence (mutants) as well as of the number of viable cells. The medium for scoring mutants was a minimal medium supplemented with biotin (9) and containing a trace of histidine sufficient to support approximately two or three divisions (9). For the determination of the total number of viable cells, a nutrient broth base medium was used. Plates were incubated in the dark for 54 hr, and the number of colonies was counted. The first dilution tube used to make 10-fold serial dilutions for the determination of the number of viable cells also contained ascorbic acid (0.1 *M*); ascorbic acid is devoid of mutagenic activity and has no effect on the viability of the bacteria (12).

[1] Betadine.

Table I—Preferential Inhibition of DNA Polymerase-Deficient *E. coli*

Agent	Amount, μl	Diameter of Zone of Inhibition, mm: pol A^+	pol A_1^-
Povidone-iodine solution	20	73.0	>86
	10	50.6	81.4
	5	26.5	31.0
	3.3	19.2	25.6
	2	16.6	18.9
	1	14.2	15.7
Methyl methanesulfonate	10	37.4	55.1
β-Propiolactone	10	36.5	58.7
Propyleneimine	10	11.5	21.5
Chloramphenicol	30 μg	31.8	31.9

DNA Modifying Activity—Following exposure to agents capable of altering DNA, cells attempt to overcome this damage to their genetic material by a variety of DNA repair systems. One reaction involves the excision of the modified DNA segment, followed by "patching" of the removed section. The enzyme DNA polymerase I is active in several of these DNA repair processes (13). Therefore, mutant cells deficient in this enzyme (pol A_1^- bacteria) are more sensitive than the pol A^+ parents from which they were derived to the lethal action of agents capable of modifying the cellular DNA (13). Indeed, this prediction was fulfilled when DNA polymerase-deficient *Escherichia coli* (pol A_1^-) became available (14, 15).

Accordingly, a simple bioassay system was developed for detecting agents with the potential for altering cellular DNA. Numerous known mutagens and carcinogens gave positive results in this system; *i.e.*, they preferentially inhibited the growth of the pol A_1^- bacteria (15 and unpublished results). The technical aspects of the procedure were described in detail elsewhere (15) and basically involve the spreading of *E. coli* pol A^+ and *E. coli* pol A_1^- onto the surface of agar plates. When the surface of the agar has dried, paper disks impregnated with the test agent are deposited on top of the bacterial layer. The cultures are incubated at 37° for 12 hr, and the diameters of the zones of inhibition are measured. Agents known to modify cellular DNA produce a larger zone of growth inhibition on the pol A_1^--containing plate than on the corresponding pol A^+ one. Substances known to interfere with structures other than the DNA inhibit the two strains to the same extent (15).

RESULTS AND DISCUSSION

Povidone-iodine preferentially blocked the growth of the DNA polymerase-deficient *E. coli* strain (Table I), a property it shared with known mutagens and carcinogens (methyl methanesulfonate, β-propiolactone, and propyleneimine), which are known to derive activity from their ability to react with the cellular DNA. Chloramphenicol, which affects protein synthesis, inhibited the two strains to the same extent (Table I).

The mutagenicity of povidone-iodine for *S. typhimurium* could not be demonstrated in the standard mutagenicity assay (Table II), even though there was a zone of growth inhibition around the disks impregnated with this disinfectant. A number of known mutagens capable of inducing base substitution (methyl methanesulfonate, β-propiolactone, and ethyl methanesulfonate) and frame-

Table II—Mutagenicity Testing Using a Standard *S. typhimurium* Assay System[a]

Agent	Amount, μl	TA1530 Zone of Inhibition, mm	TA1530 Mutants per Plate	TA1538 Zone of Inhibition, mm	TA1538 Mutants per Plate
Povidone-iodine solution	0.2	0	17	0	13
	2	11.3	16	11.5	14
	20	31.0	22	32.3	16
Ethyl methanesulfonate	10	0	8000	0	7
Methyl methanesulfonate	10	22.2	530	24.2	14
β-Propiolactone	10	31.0	2652	25.9	13
2-Nitrofluorene	250 μg	0	13	0	87
Water (control)	10	0	17	0	12

[a]The "standard" procedure developed by Ames (9) was followed. Bacteria were incorporated into the agar overlay while the test substance was present in a paper disk which was placed on the surface of the inoculated plates. The plates were incubated in the dark at 37° for 54 hr whereupon the diameters of the zones of inhibition were measured and the number of mutants (revertants to histidine independence) was determined.

shift (2-nitrofluorene) mutations were active in this standard assay (Table II).

Recent studies have detected a number of agents that act in the same manner as povidone-iodine; *e.g.*, they are positive in assays designed to detect *in vivo* modifications of the DNA but they do not respond in the standard *Salmonella* mutagenesis assay. Substances behaving in this manner include nitrofurans (16 and unpublished results), sodium hypochlorite (12, 17), and some haloalkanes (18). It was demonstrated that this behavior is due to the fact that agents in this group are strongly bactericidal [as evidenced by large zones of growth inhibition (Table II)], and the frequency of mutations is not detectable due to devitalization of the cells (12, 18).

In this connection, it must be stated that results in the standard mutagenicity assay are expressed as mutants per number of bacteria added to the plate and not as mutants per surviving cell. This difficulty was overcome readily if the cells were pulsed for a short duration with the potential mutagen, the latter was then removed, and the cells were processed for the enumeration of mutants and survivors by plating on selected media. Moreover, cellular survival could be increased by carrying out the procedure in an ice bath (4°). The mutagenicity of nitrofurans (unpublished results), haloalkanes (18), and hypochlorite (12) was demonstrated by this procedure.

When using the modified assay, the mutagenicity of povidone-iodine for *S. typhimurium* TA1530 was easily demonstrated. This effect was a function of both the povidone-iodine concentration and the duration of exposure (Fig. 1, curves A and B). Under experimental conditions similar to those described in Fig. 1A, povidone-iodine had no mutagenic effect on strain TA1538. At the dilutions plated, no mutations were detected at all (*i.e.*, even less than in untreated control). This finding presumably reflected the devitalizing effect of povidone-iodine. When the concentration of the latter was diminished (*i.e.*, situation similar to Fig. 1B) so as to increase the number of survivors, histidine-independent mutants could be scored. Their frequency, however, was not significantly different from that of the untreated control (Fig. 1C). These findings indicate that in *S. typhimurium*, povidone-iodine induces mutations of the base substitution but not of the frameshift type.

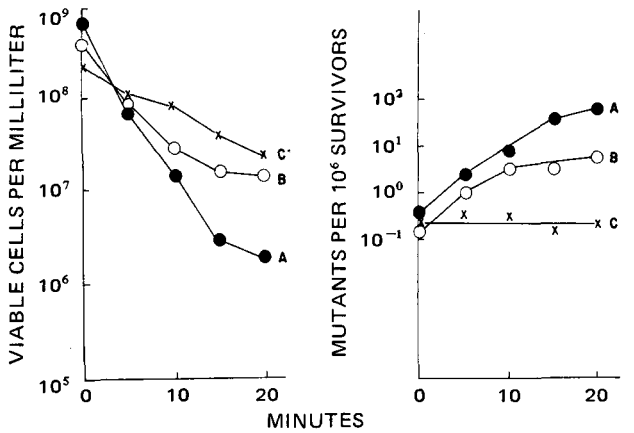

Figure 1—*Mutagenicity of povidone-iodine for* S. typhimurium. *The effects of povidone-iodine on the viability of* Salmonella *are given in the left panel, while the numbers of mutants induced per 10^6 survivors are given in the panel on the right. Key: A, effect of a 1/250 dilution of povidone-iodine solution on strain TA1530; and B and C, effects of a 1/1000 dilution of povidone-iodine solution on strains TA1530 and TA1538, respectively.*

CONCLUSIONS

The present results indicate that povidone-iodine, a widely used disinfectant, is capable of modifying the DNA of living cells and this modification, in turn, may result in mutagenic events. In view of the known relationship between mutagenic potential and the ability to induce cancer in mammals, the present findings raise some serious questions about the safety of povidone-iodine, especially in view of the residual iodine deposited at the site of application which may generate active iodine for prolonged periods. An evaluation of the potential genetic and carcinogenic hazards posed by the widespread use of povidone-iodine appears to be in order. These hazards will have to be balanced against the availability of a disinfectant of equal effectiveness and less potential toxicity.

The present findings reemphasize the importance of including tests that detect DNA modifications as well as mutations in the battery of standard microbial assays used to detect potential environmental mutagens and carcinogens.

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GLC Determination of Heroin and Its Metabolites in Human Urine

S. Y. YEH ˣ and R. L. McQUINN

Abstract □ Heroin and its metabolites, 6-monoacetylmorphine, morphine, and normorphine, were determined in human urine with a GLC procedure. Heroin was extracted with chloroform at pH 4.5 and chromatographed at a temperature programmed from 200–250° by 8°/min. 6-Monoacetylmorphine and morphine were extracted with ethylene dichloride containing 30% isopropanol at pH 8.5, and normorphine was extracted at pH 10.4 with the same solvent. The extract was derivatized with trimethylsilylimidazole and chromatographed at 230° for the determination of 6-monoacetylmorphine and morphine and at 220° for normorphine and morphine.

Keyphrases □ Heroin and metabolites—GLC analysis in human urine □ Morphine, normorphine, and 6-monoacetylmorphine (heroin metabolites)—GLC analysis in human urine □ GLC—analysis, heroin and metabolites in human urine

Previous studies on laboratory animals *in vitro* and *in vivo* have indicated that heroin (3,6-diacetylmorphine) is rapidly deacetylated to 6-monoacetylmorphine and then to morphine (1–7). The major portion of a given dose of heroin can be accounted for in the urine as free morphine and morphine conjugate (8–13). Heroin, 6-monoacetylmorphine, and morphine have previously been estimated in biological materials with a methyl orange dye procedure, a Folin–Ciocalteu phenolic reagent (3), radioactive tracers (5), paper chromatography (7, 14), and GLC (12). This article presents an improved GLC procedure for the estimation of heroin and its metabolites, 6-monoacetylmorphine, morphine, and normorphine.

EXPERIMENTAL

Materials—Heroin hydrochloride[1] (containing about 5% 6-monoacetylmorphine as an impurity, as determined with the GLC procedure described here), normorphine hydrochloride[1], and morphine sulfate[2] USP were obtained.

Gas Chromatograph—A gas chromatograph[3] equipped with dual flame-ionization detectors and a dual pen recorder[4] was used. A 0.9-m (3-ft) × 2-mm glass column (Column 1) was packed with 3% OV-17 coated on Gas Chrom Q (60–80 mesh) and conditioned at 270° by passing nitrogen (30 ml/min) for 1 hr, then at 340° without nitrogen for 4 hr, and finally at 290° with nitrogen (16 ml/min) for 72 hr. A 1.5-m (5-ft) × 2-mm stainless steel column (Column 2) was packed with 3% SE-30 coated on Varaport (100–120 mesh). The temperatures of the injector and detector were set at 255 and 295°, respectively.

Determination of Heroin—A 5-ml aliquot of urine was placed in a 40-ml centrifuge tube, adjusted to about pH 4.5 with acetic acid, buffered with 1 ml of 1 *M* sodium acetate buffer at pH 4.5, salted with 1.6 g sodium chloride, shaken with 15 ml of chloroform in a shaker[5] at 280–300 oscillations/min for 10 min, and centrifuged at 2000 rpm for 10 min. After removal of the aqueous phase by aspiration, a 13-ml aliquot of the organic phase was transferred to a 15-ml conical centrifuge tube and evaporated to dryness under a stream of nitrogen in a water bath at 60–70°.

The tube was rinsed with 1 ml of chloroform by mixing on a mixer[6] for about 10 sec, and the chloroform was evaporated to dryness. To the residue was added 50 μl of cholestane solution (0.05 mg/ml in ethyl acetate) as an internal standard, and 1 μl of the solution was injected onto Column 2. The temperature of the column was programmed from 200 to 250° at 8°/min. A standard curve was prepared by adding authentic heroin hydrochloride (0.25–10 μg) to 5 ml of drug-free cigarette smoker's urine and analyzing by the described procedure.

Determination of 6-Monoacetylmorphine and Morphine—A 5-ml aliquot of urine was placed in a 40-ml centrifuge tube, adjusted to pH 8.0–8.5 with sodium hydroxide solution, buffered with 1 ml of 1 *M* phosphate buffer at pH 8.5, salted with 1.6 g of sodium chloride, shaken with 15 ml of ethylene dichloride containing 30% isopropanol in a shaker[5] at 280–300 oscillations/min for 10 min, and centrifuged at 2000 rpm for 10 min. After removal of the aqueous phase by aspiration, a 13-ml aliquot of the organic phase was transferred to a 40-ml centrifuge tube.

The alkaloids in the organic phase were back-extracted into the aqueous phase with 5 ml of 0.1 *N* HCl by shaking in the shaker for 5 min and centrifuging for 5 min. The organic phase was removed by aspiration without removing any of the acid phase (leaving a small drop of the organic phase in the bottom of the tube). The acidic phase was adjusted to pH 8.0–8.5, buffered, salted with 1.0 g of sodium chloride, and extracted with the organic solvent as already described. After removal of the aqueous phase by aspiration, a 13-ml aliquot of the organic phase was transferred carefully (so as not to include any aqueous phase) to a 15-ml conical centrifuge tube and evaporated to dryness under a stream of nitrogen in a water bath at 60–70°.

[1] Through the courtesy of Dr. Everette May, National Institutes of Health.

[2] Commercial product.

[3] Varian Aerograph, Series 2700.

[4] Model A-25, Varian.

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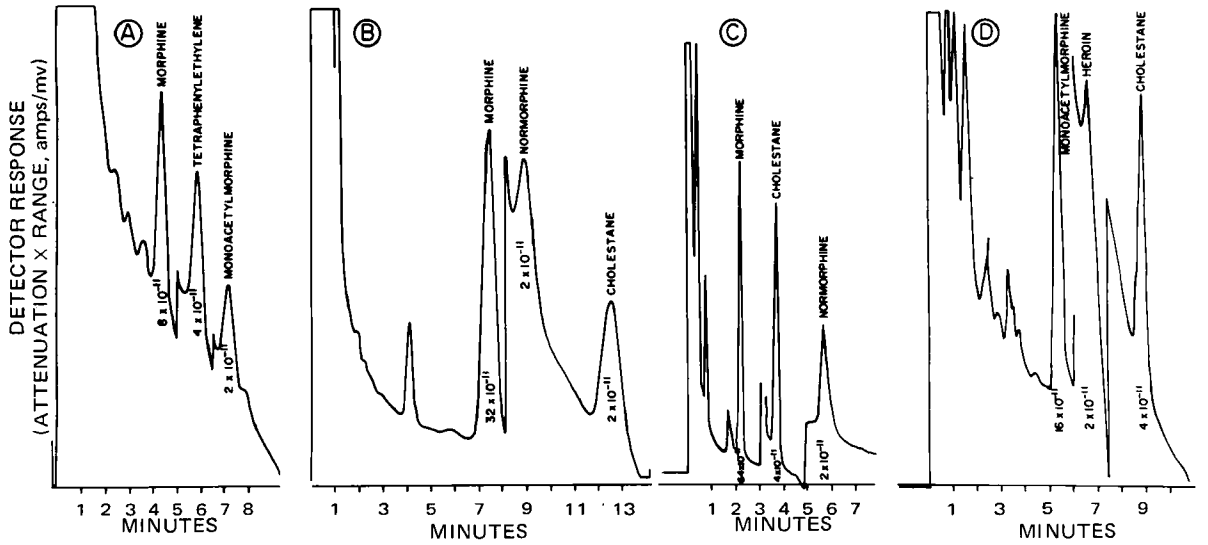

Figure 1—*Chromatograms of heroin, 6-monoacetylmorphine, morphine, and normorphine obtained from the extract of human urine after intravenous administration of 10 mg/70 kg of heroin hydrochloride.*

The tube was rinsed with 1 ml of methanol by mixing on a mixer[6] for about 10 sec and was then evaporated to dryness. The residue was used for GLC determination of 6-monoacetylmorphine and morphine after silanization with 25% trimethylsilylimidazole in pyridine[7]. Standard curves were prepared with five concentrations of 6-monoacetylmorphine and morphine added to 5 ml of drug-free cigarette smoker's urine and analyzed as already described.

Determination of Morphine and Normorphine—The procedure for extraction of normorphine and morphine was described previously (15). It was essentially the same as the procedure for extraction of 6-monoacetylmorphine and morphine, except that the urine samples were adjusted to about pH 10, buffered at pH 10.4 with 1 ml of 40% phosphate buffer (37% K_2HPO_4 and 3% K_3PO_4), and extracted with ethylene dichloride (glass distilled) containing 30% isopropanol by shaking for 30 min each time.

The residue of the urine extract was dissolved in 0.3 ml of methanol, 50 μl of internal standard solution was added, and the tube was mixed[6] for about 10 sec. Tetraphenylethylene (50 μg/ml in ethyl acetate) was used for the determination of 6-monoacetylmorphine and morphine, and cholestane solution (50 μg/ml in ethyl acetate) was used for the determination of normorphine. The solution was transferred to an acylation tube[8], and the centrifuge tube was rinsed with 0.1 ml of methanol and transferred to the acylation tube. The mixture was evaporated to dryness, and the residue was either silanized or acetylated.

Acetylation was accomplished by heating the residue with 0.2 ml of acetic anhydride and 0.1 ml of pyridine in a sealed acylation tube at 60–70° for 0.5 hr. The excess acetic anhydride was removed by evaporation and the residue was dissolved in 50 μl of ethyl acetate; 1 μl of the solution was injected onto Column 2. Silanized derivatives were prepared by mixing the contents of the tube with 50 μl of 25% trimethylsilylimidazole in pyridine and heating the mixture in an oil bath at 90–95° for 1 hr. One microliter of the solution was injected onto Column 1.

The ratios of the peak heights of heroin, 6-monoacetylmorphine, morphine, and normorphine to the peak height of internal standard were calculated. A linear relationship was found by plotting the peak height ratios *versus* concentrations. Heroin, 6-monoacetylmorphine, morphine, and normorphine concentrations in the urine sample were determined from the standard curves. The amount of injected heroin was converted to equivalent amounts of 6-monoacetylmorphine and morphine for calculation of the percentage of excretion.

Estimation of Free and Conjugated Morphine and Normorphine—Total morphine and normorphine in the urine were estimated, according to the precedure of Yeh and Woods (16), by autoclaving the specimen in a final concentration of 2.2 *N* HCl (20% by volume of concentrated hydrochloric acid) in a steam-jacketed autoclave at 15 lb of pressure and 115° for 30 min. After cooling, the sample was adjusted to about pH 10 and then extracted for GLC by the described procedure. Free morphine and normorphine were estimated from the unhydrolyzed specimens. Conjugated morphine was calculated as the difference between the total morphine and the free drug of morphine and 6-monoacetylmorphine.

RESULTS AND DISCUSSION

The chromatograms of heroin and its metabolites (6-monoacetylmorphine, morphine, and normorphine) extracted from the first urine samples of subjects after intravenous administration of 10 mg of heroin hydrochloride/70 kg are shown in Fig. 1. Normorphine, either in free or conjugated form, would be expected to be a metabolite of heroin since free and conjugated normorphine have been found in the urine of morphine-dependent subjects (15). The chromatograms of free 6-monoacetylmorphine and free morphine Fig. 1(A) were obtained from a silanized extract and chromatographed on Column 1 at a temperature of 230°. The retention times of silylmorphine, tetraphenylethylene, and silyl-6-monoacetylmorphine were 4.3, 5.8, and 7.1, respectively.

Chromatograms B and C show total morphine and total normorphine extracted from acid-hydrolyzed urine at pH 10.4. Chromatogram B was obtained from the extract that was silanized and chromatographed on Column 1 at 220°. The retention times of silylmorphine, silylnormorphine, and cholestane under these conditions were 7.4, 8.8, and 12.5 min, respectively. Chromatogram C was obtained from the extract that was acetylated and chromatographed on Column 2 at 250°. The retention times of diacetylmorphine, cholestane, and triacetylnormorphine were 2.3, 3.7, and 5.7 min, respectively.

Chromatogram D shows 6-monoacetylmorphine and heroin obtained from the extract chromatographed on Column 2, temperature programmed from 200 to 250° at 8°/min. The retention time of heroin and its metabolites changed slightly from column to column but the basic pattern remained the same. Free normorphine concentration was too low to be detectable in a 5-ml urine aliquot but was detected from larger samples extracted with XAD-2 resin[9].

The sensitivity of the procedure for determination of heroin was

[7] Tri-Sil-Z, Pierce Chemical Co., Rockford, Ill.
[8] Regis Chemical Co., Chicago, Ill.
[9] Amberlite XAD-2 resin, Rohm & Haas Chemical Co., Philadelphia, Pa.

0.2 μg/5 mg of urine. The extraction efficiency of the procedure could not be established, because the volatility of heroin hydrochloride appears higher than that of heroin (presumably heroin acetate) extracted with the solvent. The peak height of heroin hydrochloride (2–10 μg) was less than that of the same amount of heroin after extraction when chromatographed on Column 1. Column 2 seemed more sensitive than Column 1 when extracted heroin was chromatographed, although heroin hydrochloride did not emerge from Column 2.

The extract obtained from a single extraction contained many impurities, but these did not interfere with the detection or measurement of the heroin peak. Attempts to clean the extract by extraction of heroin into the aqueous phase with 0.1 *N* HCl and then back into the organic phase with chloroform at pH 4.5 were unsuccessful. 6-Monoacetylmorphine was also extracted with chloroform from the urine sample buffered at pH 4.5.

6-Monoacetylmorphine is quite stable under the conditions for extraction and silanization, as evidenced by the fact that a morphine peak was not observed in the chromatogram when authentic 6-monoacetylmorphine alone was added to the urine. Attention must be paid to the adjustment of the pH of the sample. Oberst (10) reported that 88% of heroin was hydrolyzed at 26° in 0.5 *M* sodium carbonate solution in 10 min. The biological sample obtained after administration of heroin should be frozen and analyzed as soon as possible. 6-Monoacetylmorphine was detected in 10 out of 10 frozen human urine samples and only in three out of 10 refrigerated urine samples after heroin administration.

The overall recoveries for 6-monoacetylmorphine and morphine in concentrations of 2–15 μg/5 ml of urine were 68–70 and 60–62%, respectively. The sensitivity of this procedure for the determination of 6-monoacetylmorphine hydrochloride and morphine sulfate was 0.25 μg/5 ml of urine. Morphine can be extracted from the sample buffered to pH 8.5 or 10.4 and determined simultaneously with either 6-monoacetylmorphine or normorphine.

The optimal conditions of reaction time and temperatures for derivatization of morphine and normorphine were studied with two silanizing reagents, bis(trimethylsilyl)trifluoroacetamide plus 10% trimethylchlorosilane and 25% trimethylsilylimidazole in pyridine. A clean chromatogram was obtained from the extract of a drug-free urine sample when trimethylsilylimidazole was used. The results from silanization of 100 μg of morphine sulfate and normorphine hydrochloride with 50 μl of 25% trimethylsilylimidazole in pyridine heated at 90–95° for 15, 30, and 60 min (six samples for each time period) were not significantly different. However, the chromatogram of drug-free urine extracts heated for 15 and 30 min with the silanizing reagent showed a small peak interfering with morphine (0.2 min after morphine peak). The peak height ratios for morphine were not significantly different when the extract was silanized at 60–70 or 90–95°, but the peak height ratios for normorphine were smaller at the lower temperature. Therefore, reaction at 90–95° for 60 min was used in the final procedure.

The sensitivity of silanized and acetylated morphine is comparable but the sensitivity for determination of silanized normorphine is somewhat greater than that for acetylated normorphine.

Cholestane interferes with the retention time of 6-monoacetylmorphine, so it is not suitable for use as an internal standard for 6-monoacetylmorphine determination. Tetraphenylethylene was used as an internal standard for the determination of both 6-monoacetylmorphine and morphine.

This procedure is currently being used in this laboratory for studying the urinary excretion of heroin and its metabolites following intravenous administration of heroin in humans.

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Synthesis, Spectral Properties, and Antibacterial Activity of Synthetic Precursors of Macrocyclic Oxa- and Thia-Substituted Benzolactones and Benzoketones

PAUL R. JONES ˣ, CECIL E. MALMBERG, and CAROL McGRATTAN

Abstract □ Terminally difunctional compounds were synthesized by alkylation of salicylic acid, thiosalicylic acid (*o*-mercaptobenzoic acid), or their derivatives. Whereas methyl salicylate and thiosalicylic acid were smoothly etherified, salicylic acid was alkylated at the carboxyl. Characteristic IR and NMR spectral patterns in the synthesized compounds can be attributed to intramolecular hydrogen bonding or substituent effects and are consistent with observations already reported for similar compounds. Three synthesized compounds exhibited low but reproducible inhibitory effects on *Bacillus subtilis* growth.

Keyphrases □ Salicylic and thiosalicylic acid derivatives—synthesis, spectra, and antibacterial activity as precursors of macrocyclic oxa- and thia-substituted benzolactones and benzoketones □ Antibacterial activity—synthesis and screening of salicylic and thiosalicylic acid derivatives as precursors of macrocyclic oxa- and thia-substituted benzolactones and benzoketones

Metabolites possessing a macrocyclic ring *ortho*-fused to an aromatic nucleus are few in number (1). The four known *ortho*-fused macrocyclic lactone metabolites are curvularin, monorden (radicicol), zearalenone, and lasiodiplodin. The first three, containing a keto group as well, fit the general structure of the macrolides (2); lasiodiplodin is devoid of such an additional function.

Although curvularin shows no antibacterial or antifungal activity (3), *S*-zearalenone (4) and the saturated *S*-zearalanone (5) exhibit uterotrophic activity; monorden displays strong antifungal properties with low toxicity (6). Biological testing of lasiodiplodin has apparently not yet been reported.

Previous synthetic approaches to curvularin, zearalenone, and their close structural relatives have involved closing of the aliphatic macrocyclic ring by way of an aromatic, difunctional precursor. In the case of curvularin, this procedure required a troublesome Friedel–Crafts acylation to form the aryl–carbon bond (7). The approach to preparing analogs of the naturally occurring, *ortho*-fused metabolites was to choose an aromatic starting material, with appropriate *o*-substituents already in place, and, by means of efficient standard synthetic steps, to attach one or two functionally substituted long chains at these *o*-positions.

The choice of ethers or thioethers as one of the ring-forming functional groups seemed particularly attractive. They could be derived from readily available phenols or thiophenols by the Williamson synthesis and, once formed, would be inert to a wide variety of reaction conditions. Thus, by this approach, I and II with an array of substituents X and Y would be synthetically achievable. They, in turn, would be precursors to heterocyclic analogs (IV) of the *ortho*-fused metabolites.

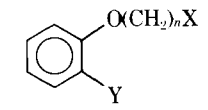
EXPERIMENTAL[1]

The following are representative preparations of compounds of types I, II, and III.

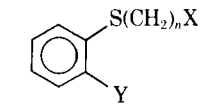
11-(2-Carboxyphenoxy)hendecan-1-ol (I: *n* = 11, X = OH, Y = COOH)—To a magnetically stirred mixture of 3.04 g (0.02 mole) of methyl salicylate, 6.0 g of anhydrous potassium carbonate, and 250 ml of dried, distilled dimethylformamide was added a solution of 5.0 g (0.02 mole) of 11-bromohendecan-1-ol (8) dissolved in 70 ml of dimethylformamide. During the addition, which required 25 min, the temperature from external heating was allowed to rise from 33 to 60°. After the mixture had been stirred and heated at 60–70° for 2.5 hr, it was filtered and the filtrate was allowed to evaporate at room temperature for 2 days and was then partitioned between 100 ml of water and 2 × 60 ml of chloroform.

The combined organic phase was washed with 2 × 50 ml of 5% NaOH, 2 × 100 ml of water, 100 ml of dilute hydrochloric acid, and 2 × 100 ml of water, dried over magnesium sulfate, and concentrated to 5.2 g of oil; this oil was distilled (112°, 0.05 torr) to remove the starting bromo alcohol. The 3.6 g of residual oil was purified by column chromatography (silica gel, chloroform eluent) to afford 3.3 g (51%) of the hydroxy ester as a light-tan oil; IR (film): 3650–3200 (OH) and 1730 ($COOCH_3$) cm^{-1}; NMR (CCl_4): δ 7.75 (m, 1, ArH), 7.18 (broad m, 3, ArH), 4.0 (t, J = 7 Hz, 1.8, $ArOCH_2$), 3.85 (s, 2.7, $COOCH_3$), 3.54 (t, J = 7 Hz, 1.8, CH_2OH), 3.0 (s, 1.0, CH_2OH), and 1.9–1.2 (m, 18.2, $—(CH_2)_9—$) ppm.

The hydroxy ester (1.5 g) was saponified with potassium hydroxide–ethanol to give 1.1 g (78%) of 11-(2-carboxyphenoxy)hendecan-1-ol, mp 81.0–81.5°; IR ($CHCl_3$): 3615 (free OH), 3530–3200 (bonded OH), and 1735 (COOH) cm^{-1}; NMR ($CDCl_3$): δ 8.32 (m, 1, ArH), 7.70 (m, 1, ArH), 7.25 (m, 2, ArH), 6.0–5.5 (broad m, 1.8, COOH and CH_2OH), 4.3 (t, J = 7 Hz, 1.9, $ArOCH_2$), 3.7 (t, J = 7 Hz, 1.9, CH_2OH), and 2.0–1.2 (m, 18.3, $—(CH_2)_9—$) ppm. The



I



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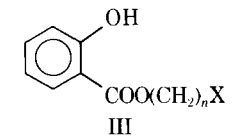
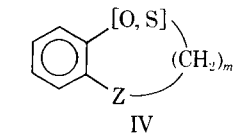


III



IV

X = OH, COOH, COOR
Y = COOH, COOR
Z = COO, CO, CH(OH)CO

[1] IR spectra were determined as films or solutions with a Perkin-Elmer 337 grating spectrophotometer. NMR spectra were measured in solution with a Varian A-60 or a Jeol JNM-MH-100 spectrometer. All spectra are on file in the Department of Chemistry, University of New Hampshire. Microanalyses were obtained at the University of New Hampshire with an F&M model 185 analyzer or by Galbraith Laboratories, Knoxville, Tenn. Melting points, determined with a Thomas-Hoover model 6406-H apparatus, are corrected.

Table I—Summary of Synthetic Compounds and Antibacterial Assay

Compound	Melting Point	Molecular Formula	Analysis, % Calc.	Analysis, % Found	Concentration, mg/ml Ethanol 1	Concentration, mg/ml Ethanol 0.1
					Net Zone of Inhibition, mm	
I: n = 3, X = OH, Y = COOH	107.5—109.0°	$C_{10}H_{12}O_4$	C 61.21 H 6.17	61.30 5.97	0[a]	—
I: n = 11, X = OH, Y = COOH	81.0—81.5°	$C_{18}H_{28}O_4$	C 70.10 H 9.15	70.26 9.14	0[a]	—
I: n = 10, X = $COOC_2H_5$, Y = $COOCH_3$	28.0—28.5°	$C_{21}H_{32}O_5$	C 69.20 H 8.85	69.32 8.95	0[a]	—
II: n = 11, X = OH, Y = COOH	108.5—109.0°	$C_{18}H_{28}O_3S$	C 66.63 H 8.69	66.63 8.69	9[b]	0[a]
II: n = 10, X = $COOC_2H_5$, Y = $COOC_2H_5$	29.0—29.5°	$C_{22}H_{34}O_4S$	C 66.98 H 8.67	67.14 8.72	0[a]	—
II: n = 10, X = $COOC_2H_5$, Y = COOH	72—73°	$C_{20}H_{30}O_4S$	C 65.54 H 8.25	65.65 8.36	11[b]	3[a]
III: n = 11, X = OH	41—42°	$C_{18}H_{28}O_4$	C 70.10 H 9.15	70.15 9.33	5[b]	0[a]
Salicylic acid	—	—	—	—	0[a]	—
Thiosalicylic acid	—	—	—	—	0[a]	—

[a] Average of three determinations. [b] Average of nine determinations.

broad multiplet at 6.0–5.5 ppm disappears with the addition of D_2O. The acid proton was assigned on the basis of integration. Similar behavior occurs with 11-(2-carboxythiophenoxy)hendecan-1-ol.

Ethyl 11-(2-Carboxythiophenoxy)hendecanoate (II: n = 11, X = $COOCH_2CH_3$, Y = COOH)—To a stirred mixture of 4.35 g (0.028 mole) of thiosalicylic acid and 10 g of anhydrous potassium carbonate in 300 ml of dimethylformamide was added a solution of 7.50 g (0.026 mole) of ethyl 11-bromohendecanoate (9, 10) in 100 ml of dimethylformamide over 2.8 hr; the temperature ranged from 40 to 55°. Stirring at 60° was continued for an additional 4 hr. The mixture was filtered, and the filtrate was allowed to evaporate at room temperature until a yellow solid remained. This solid was partitioned between 100 ml each of chloroform and hydrochloric acid.

The aqueous layer and residual solid material were extracted twice with 75 ml of ether. The chloroform and ether extracts were combined and concentrated. The residual solid was washed with benzene and then recrystallized from hexane to afford 3.25 g (34%), mp 72–73°; IR (CCl_4): 3300–2500 (COOH), 1734 (ester), and 1689 (acid) cm^{-1}; NMR (CCl_4): δ 12.19 (s, 1.04, COOH), 8.18 (m, 1, ArH), 7.34 (broad m, 3, ArH), 4.08 (q, 1.82, J = 6 Hz, $COOCH_2$), 2.88 (t, 1.82, J = 6 Hz, CH_2S), 2.21 (t, 1.82, J = 6 Hz, CH_2COO), and 1.90–1.00 (m, 19.6, $-(CH_2)_8-$ and CH_2CH_3) ppm.

11-Hydroxyhendecyl Salicylate (III: n = 11, X = OH)—Addition of 7.0 g (0.027 mole) of 11-bromohendecan-1-ol in 100 ml of dimethylformamide to a stirred mixture of 3.84 g (0.0278 mole) of salicylic acid, 9 g of anhydrous potassium carbonate, and 260 ml of dimethylformamide was carried out as described for II. After being stirred and heated for 7 hr, the mixture was filtered and the filtrate was evaporated to a tan solid. After being washed with water, the solid was taken up in chloroform, which was washed sequentially with 2 × 20 ml of 5% $NaHCO_3$, 2 × 20 ml of 5% NaOH, 3 × 20 ml of water, dried (magnesium sulfate), and concentrated to 3.8 g (45%) of crude oil product.

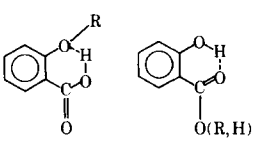
Column chromatography (silica gel, chloroform eluent) afforded 1.5 g of oil which gave, after solidification in an ice bath and three recrystallizations from hexane, 1.1 g (13%) of white crystalline solid, mp 41–42°; IR ($CHCl_3$): 3630 (free OH), 3500–3150 (bonded OH), and 1675 (ester) cm^{-1}; NMR (CCl_4): δ 10.65 (s, 0.77, ArOH), 7.80 (m, 1, ArH), 7.40 (m, 1, ArH), 6.85 (m, 2, ArH), 4.34 (t, J = 6 Hz, 1.8, $ArCOOCH_2$), 3.56 (t, J = 6 Hz, 1.8, CH_2OH), and 1.90–1.10 (m, 19.5, $-(CH_2)_9-$ and CH_2OH) ppm. Both the phenolic proton signal at 10.65 ppm and the alcohol proton signal, appearing as a singlet at 1.45 ppm, disappear with the addition of D_2O.

Antibacterial Assay—Inhibition of *Bacillus subtilis*[2] growth was measured by placing a paper disk containing 20 μl of solution on an agar surface seeded with the organism and noting zones of growth inhibition after 20 hr of incubation (11). The "net zone of inhibition" (Table I) is the total diameter of the inhibition zone (millimeters) minus the diameter of the paper disk. All compounds were also tested against the green alga *Chlorella pyrenoidosa*[3] (No. 395) (12) and found to be inactive.

RESULTS

Synthesis—Whereas the synthesis of II by alkylation of thiosalicylic acid at the sulfur proceeded smoothly, attempts at alkylating salicylic acid led, unexpectedly, to III rather than to I. In fact, this tendency of salicylic acid to undergo alkylation at the carboxyl rather than the hydroxyl was noted by Herzig in 1894 (13). Later, it was reported (14) that the benzyl ether of salicylic acid could be obtained in a Williamson synthesis, provided a large excess of alkali was employed. Apparently the dianion of salicylic acid, which would lead to alkylation at the phenolic site, is formed only with difficulty. Nozu *et al.* (15) showed that salicylic acid behaves as a typical monobasic acid on titration. Compounds of type I were obtained by alkylating salicylate esters, a reaction that proceeded as expected. Saponification of the ethereal esters led to I.

Spectral Properties—Unusual IR spectral characteristics were encountered in some of the synthetic compounds. For example, in



I (Y = COOH) III

Table II—IR Stretching Frequencies of Salicylic Acid Derivatives

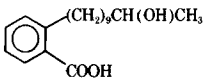
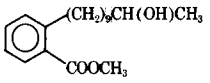
Compound	OH, cm^{-1}	C=O, cm^{-1}
o-Ethoxybenzoic acid[a]	3290	1740
11-(2-Carboxyphenoxy)-hendecan-1-ol (I: n = 11, X = OH, Y = COOH)	3615, 3530, 3200	1735
Salicylic acid, hexyl ester[b]	3175	1670
11-Hydroxyhendecyl salicylate (III: n = 11, X = OH)	3630, 3500–3150	1675

[a] "Sadtler Standard Spectra, Prism," Sadtler Research Labs., Inc. Philadelphia, Pa., 1965, No. 36382. [b] *Ibid.*, No. 3871.

[2] Difco Laboratories.

[3] Obtained from Indiana Algal Culture Collection, R. C. Starr, Bloomington, Ind.

Table III—Characteristic NMR Chemical Shifts in the Aromatic Region of *o*-Substituted Benzoic Acids and Esters

Compound	Solvent	NMR Shift δ_1[a]	NMR Shift $\delta_{2,3}$[a]	$\Delta\delta[\delta_1-(\delta_{2,3})]$
o-Methoxybenzoic acid	$CDCl_3$[b]	8.17	7.37	0.80
Methyl salicylate	CCl_4[c]	7.70	7.03	0.67
I: n = 11, X = OH, Y = COOH	$CDCl_3$	8.32	7.47	0.85
I: n = 11, X = OH, Y = $COOCH_3$	CCl_4	7.75	7.15	0.60
I: n = 10, X = $COOC_2H_5$, Y = $COOCH_3$	$CDCl_3$	8.12	7.48	0.64
III: n = 11, X = OH	CCl_4	7.80	7.12	0.68
	$CDCl_3$[d]	8.10	7.35	0.75
	$CDCl_3$[d]	7.80	7.30	0.50
I: n = 3, X = OH, Y = COOH	$(CH_3)_2SO$-d_6	7.67	7.20	0.47
I: n = 3, X = OH, Y = $COOCH_3$	$(CH_3)_2SO$-d_6	7.62	7.12	0.50
II: n = 10, X = $COOC_2H_5$, Y = COOH	CCl_4	8.18	7.34	0.84
II: n = 10, X = $COOC_2H_5$, Y = $COOC_2H_5$	CCl_4	7.95	7.25	0.70
II: n = 11, X = OH, Y = COOH	$(CH_3)_2SO$-d_6	7.98	7.42	0.56

[a] δ_1 = midpoint of low-field multiplet proton (1H), and $\delta_{2,3}$ = midpoint of up-field multiplet (3H). [b] "High Resolution NMR Spectra Catalog," vol. 1, Varian Associates, Palo Alto, Calif., 1962, No. 195. [c] "Sadtler Nuclear Magnetic Resonance Spectra," Sadtler Research Labs., Inc., Philadelphia, Pa., 1965, No. 7214M. [d] Reference 17.

I, where Y=COOH, both the OH and C=O stretching vibrations were unusually high in the regions expected for a monomeric carboxylic acid (16). This finding can be attributed to extensive intramolecular hydrogen bonding of carboxyl with *ortho*-phenolic ether oxygen. As expected, the IR spectra of the corresponding thio compounds (II) were normal, with the OH appearing as a broad band at 3300–2500 cm^{-1} and the C=O appearing at 1690 cm^{-1}. The ester carbonyl stretching band in III was, on the other hand, at an unusually low frequency, probably because of another mode of intramolecular hydrogen bonding. Precedents for these spectral features are to be found in related compounds (Table II).

The proton NMR spectra displayed distinctive features in the aromatic region. In essence, the aromatic (Ar—H) proton *ortho* to a carboxyl or ester function was shifted downfield relative to the three remaining aromatic protons. This behavior, already noted in dideoxyzearalane and two related compounds (17), seems to be generally characteristic. The extent of shift, expressed as $\Delta\delta$ (*i.e.*, separation between the lowest field Ar—H proton and the remaining three aromatic protons), is dependent on the nature of the carbonyl function, being greater for carboxylic acid than for carboxylic ester. Although the shifts are undoubtedly dependent on other factors such as solvent and additional substituents, which have not yet been clarified, the trend can be clearly seen from the examples assembled in Table III, taken from this work and published literature. This NMR pattern has been valuable in corroborating the identity of isomeric Structures I and III.

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Binding of Cupric Ions to Bovine Serum Albumin

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Abstract □ The binding of cupric ions to bovine serum albumin was investigated by using a cupric-ion-specific electrode. When using a modified form of the Scatchard equation, it was determined that there are at least two classes of binding sites on bovine serum albumin for cupric ions. One class has three binding sites of relatively strong affinity, with an average binding constant of 3.0×10^6. The other class has about 16 binding sites of relatively weak affinity, with an average binding constant of 2.0×10^4.

Keyphrases □ Cupric ions—binding to bovine serum albumin, determination of number and classes of binding sites, specific ion electrode □ Copper–protein binding interactions—studied using specific ion electrode □ Serum protein binding—binding of cupric ions to bovine serum albumin, studied using specific ion electrode

In the normal human body, the blood serum contains about 114 μg of copper/100 ml of serum. About 94% of this copper is tightly bound to the serum copper–protein ceruloplasmin, an α-globulin with a molecular weight of approximately 151,000. The function of ceruloplasmin is unknown, but it does possess polyphenol oxidase activity (1, 2); it may function in the oxidation of serotonin (3) and epinephrine (4). The remaining 6% of serum copper is loosely bound to serum albumin. The red blood cells also contain about 115 μg/100 ml of packed red blood cells. It has been suggested that there are at least two different fractions of copper in erythrocytes (5), one of which is labile and can be exchanged easily between plasma and red blood cells. The other fraction is present as a copper–protein called erythrocuprein, which contains about 0.34% copper, by weight, tightly bound to the protein.

A hereditary deficiency or absence of ceruloplasmin (6–8) often results in Wilson's disease (hepatolenticular degeneration). This disease results from inordinately high concentrations of copper in many organs and tissues due to a lack of control in absorption of dietary copper or excretion of copper that already has been absorbed or both.

Even though the amount of copper present in the different parts of the blood is known, the individual binding of copper to each component is not well understood. Klotz and Curme (9) studied the thermodynamics of the copper–bovine serum albumin interaction at pH 4.83 and reported that there are 16 binding sites. They had also determined $\Delta F°$ values for the binding of individual Cu^{+2} ions to the protein. However, the analytical method they used to calculate the Cu^{+2} ions in the copper–protein system was sensitive only to about 1×10^{-4} *M* of Cu^{+2} ions. Therefore, it was desirable to reinvestigate the binding of Cu^{+2} to bovine serum albumin with a more sensitive analytical method for the determination of free Cu^{+2} ions. In this regard, the copper-ion-specific electrode seemed very attractive because of its ease of operation. This report presents the use of the specific ion electrode to study the copper–protein binding interaction.

EXPERIMENTAL

Chemicals—The protein solution was prepared by dissolving crystalline bovine serum albumin[1] in distilled deionized water. A 0.100 *M* copper nitrate standard solution[2] was used to prepare the cupric-ion stock solution.

Apparatus—A cupric-ion-specific electrode[3], a single-junction reference electrode[4], and a digital pH/mv meter[5] were used for measurement of the electric potential from which the cupric-ion concentrations were calculated.

Procedure—Fifty milliliters of 5.0×10^{-6} *M* bovine serum albumin (molecular weight assumed to be 69,000), prepared in deionized distilled water, with ϵ at 280 nm equal to 4.60×10^4 liters $mole^{-1}$ cm^{-1} (10), was placed in a 100-ml beaker. The reference electrode, the cupric-ion electrode, and a thermometer were dipped into this solution, which was stirred magnetically. The temperature was maintained at 25.0° using a constant-temperature bath.

Copper nitrate solution, 1.0×10^{-2} *M*, was added to the bovine serum albumin in 10-μl increments with a micropipet[6]. Both the bovine serum albumin and the copper nitrate solutions were adjusted to pH 5.2 with trace amounts of sodium hydroxide or sulfuric acid, as appropriate. For samples below 10^{-3} *M* in total ionic strength, the difference in total ionic strength between samples and standards may be as large as a factor of 5 without resulting in serious errors (11). The 5.0×10^{-6} *M* protein and 1.0×10^{-2} *M*

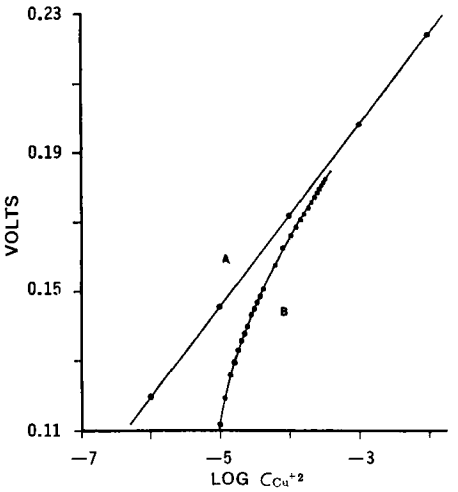

Figure 1—*Specific ion electrode response, in volts, as a function of formal cupric-ion concentration in the absence of bovine serum albumin (A) and in the presence of 5.0 × 10⁻⁶ M bovine serum albumin (B).*

[1] Calbiochem, San Diego, Calif.
[2] Orion Research Inc., Cambridge, Mass.
[3] Model 94-29, Orion Research Inc., Cambridge, Mass.
[4] Model 90-01, Orion Research Inc., Cambridge, Mass.
[5] Model 801, Orion Research Inc., Cambridge, Mass.
[6] Centaur Chemical Co., Stamford, Conn.

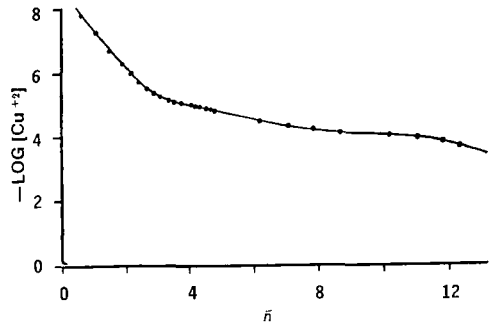

Figure 2—*Plot of* $-log\ [Cu^{+2}]$ versus $\bar{n}$ *for the bovine serum albumin–*Cu^{+2} *system at pH 5.2.*

copper nitrate solutions in distilled deionized water had a pH around 5, so only trace amounts of sulfuric acid or sodium hydroxide were needed to adjust the pH to 5.2. Such small quantities of acid or base are not expected to change the ionic strength enough to affect the measurement of the cupric-ion concentration. Ideally, the binding study should be carried out at the biological pH, which is 7.4; but at a pH of 6 or higher, some Cu^{+2} is hydrolyzed to hydroxo complexes, which may or may not precipitate. Hence, pH 5.2 was chosen for the present study.

The instrument was standardized before and after each titration, using various exact concentrations of pure copper nitrate solutions. By knowing the total amount of cupric ion added and the concentration of free cupric ions, the amount of copper bound was calculated.

RESULTS AND DISCUSSION

The potentiometric data for the titration of bovine serum albumin with copper nitrate are presented graphically in Fig. 1. Curve A is the standard curve for the known concentrations of cupric ions in the absence of the protein. Curve B represents the electromotive force in volts during the bovine serum albumin–copper nitrate titration as a function of total Cu^{+2} added.

The binding of cupric ions to bovine serum albumin, *P*, can be represented by a series of equilibria (Scheme I):

$$P + Cu^{+2} \overset{k_1}{\rightleftharpoons} P(Cu^{+2})$$
$$P(Cu^{+2}) + Cu^{+2} \overset{k_2}{\rightleftharpoons} P(Cu^{+2})_2$$
$$\vdots \qquad\qquad \vdots \qquad\qquad \vdots$$
$$P(Cu^{+2})_{i-1} + Cu^{+2} \overset{k_i}{\rightleftharpoons} P(Cu^{+2})_i$$

Scheme I

where $P(Cu^{+2})_{i-1}$ and $P(Cu^{+2})_i$ represent protein–copper complexes with $i-1$ and i cupric ions, respectively. The number i may vary from 1 to N, the maximum number of cupric ions that can bind to the protein. The k's are the respective equilibrium constants corresponding to each equilibrium.

The number of cupric ions bound per mole of bovine serum albumin, $\bar{n}$, at any point in the titration is related to the N equilibrium constants by the expression (12):

$$\bar{n} = \frac{\sum_1^N i\left(\prod_1^i k_i\right)[Cu^{+2}]^i}{1 + \sum_1^N \left(\prod_1^i k_i\right)[Cu^{+2}]^i} \qquad \text{(Eq. 1)}$$

where $[Cu^{+2}]$ indicates the equilibrium concentration of free cupric ions, and k_i is the equilibrium constant for the binding of ith cupric ion. If the individual equilibrium constants differ widely from each other (by at least two orders of magnitude) such that only one equilibrium is experimentally detectable at any point in the titration, Eq. 1 predicts that a plot of $-\log [Cu^{+2}]$ *versus* $\bar{n}$ should show N distinct inflection regions. The midpoint of these inflections should correspond to the respective equilibrium constants. Moreover, it also predicts that as $\bar{n}$ approaches N, $-\log [Cu^{+2}]$ should decrease sharply.

Figure 2 shows a plot of $-\log [Cu^{+2}]$ *versus* $\bar{n}$ for the bovine serum albumin–Cu^{+2} system. There are no clear inflection regions, a result that indicates strongly overlapping successive equilibria. The fact that a definitive saturation plateau has not been reached even at $\bar{n} = 12$ indicates that there are more than 12 binding sites. Therefore, the binding data should be analytically fitted to Eq. 1 (which will have $N + 1$ unknowns) to obtain precise binding parameters. However, if the N binding sites for cupric ions on bovine serum albumin are electrostatically noninteracting, and if the protein has identical affinity for each of the N cupric ions (*i.e.*, $k_1 = k_2 = k_3 \ldots = k_N$), then the relationship of $\bar{n}$ to the overall binding constant, K, becomes simpler and can be expressed by the Scatchard equation (13):

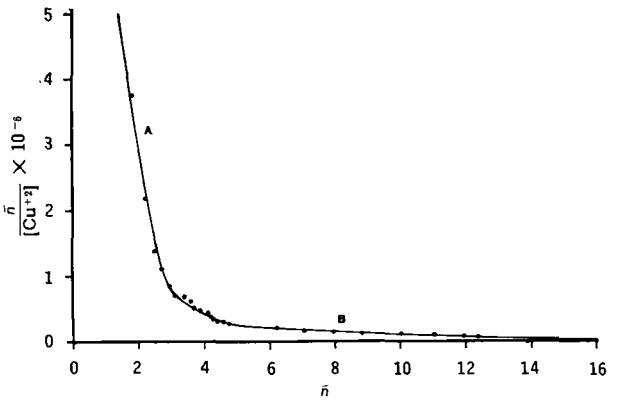

Figure 3—*Scatchard plot for the binding of* Cu^{+2} *ions to bovine serum albumin.*

$$\bar{n} = Nk[Cu^{+2}]/(1 + k[Cu^{+2}]) \qquad \text{(Eq. 2)}$$

which, on rearrangement, gives:

$$\bar{n}/[Cu^{+2}] = Nk - \bar{n}k \qquad \text{(Eq. 3)}$$

According to Eq. 3, a plot of $\bar{n}/[Cu^{+2}]$ *versus* $\bar{n}$ (known as a Scatchard plot) should be a straight line with the slope $= -k$ and the X intercept $= N$. Figure 3 shows the Scatchard plot for the bovine serum albumin–Cu^{+2} system. Clearly, these data do not fit Eq. 3. Klotz and Curme (9) also found that their data for the bovine serum albumin–Cu^{+2} system did not fit this linear expression. However, if it is assumed that there are two classes of binding sites that are widely different from each other in their binding affinities, the Scatchard Eq. 2 can be modified to:

$$\bar{n} = \{N_1k_1[Cu^{+2}]/(1 + k_1[Cu^{+2}])\} + \{N_2k_2[Cu^{+2}]/(1 + k_2[Cu^{+2}])\} \qquad \text{(Eq. 4)}$$

where N_1 is the number of binding sites of the first kind with a binding constant of k_1, and N_2 is the number of binding sites of the second kind with a binding constant of k_2. In this case, the ideal Scatchard plot, instead of being a straight line, is a hyperbola composed of two straight-line portions.

The binding data of the bovine serum albumin–Cu^{+2} system do yield a hyperbola made up of two straight-line portions (Fig. 3), indicating that there are at least two classes of binding sites on bovine serum albumin for cupric ions. The straight-line segment, A, of Fig. 3, gives an X intercept of about 3, indicating one class of binding has three sites. The slope of this line, which is equal to the average binding constant, is 3×10^6. The second straight-line segment, B, gives an X intercept of about 16, revealing that the second class of binding has about 16 sites. From the slope of segment B, the average binding constant for the latter sites is 2×10^4.

The number of binding sites for the second class of binding is the same as that reported by Klotz and Curme (9). However, they calculated the change in free energy of the individual 16 equilibria, the first of which had a k value of 1.8×10^4 ($\Delta F^\circ = -5908$ cal/mole at 25°). This value agrees with the value obtained from the Scatchard plot.

Thus, it appears that cupric ions bind to bovine serum albumin through at least two different classes of binding sites; one accom-

modates only about three cupric ions, which are relatively tightly bound, while the other accommodates about 16 cupric ions, which are rather weakly bound. It is possible that the first three cupric ions bind intimately by penetrating the protein while the rest of the cupric ions bind weakly to the surface of the protein.

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Keyphrases □ 2-(4-Arylthiosemicarbazidocarbonylthio)benzthiazoles—synthesis, characterization, monoamine oxidase inhibitory and anticonvulsant activities □ Monoamine oxidase inhibitory activity—substituted benzthiazoles, relationship between enzyme inhibitory and anticonvulsant activities, rat brain homogenate □ Anticonvulsant activity—substituted benzthiazoles, relationship to monoamine oxidase inhibitory activity, mice

Benzthiazoles have been reported to possess diverse pharmacological properties (1, 2). Recent studies indicated anticonvulsant and monoamine oxidase [EC 1.4.3.4 monoamine: O_2 oxidoreductase (deaminating)] inhibitory properties of substituted benzthiazoles (3). In addition, the ability of hydrazines (4), semicarbazides (5, 6), and thiosemicarbazides (7) to inhibit monoamine oxidase and the anticonvulsant activities possessed by the inhibitors of monoamine oxidase (8) led to the synthesis of 2-(4-arylthiosemicarbazidocarbonylthio)benzthiazoles. In the present study, all substituted benzthiazoles possessed anticonvulsant activity and inhibited monoamine oxidase activity of rat brain homogenate.

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2-(Ethoxycarbonylthio)benzthiazole—A mixture of 2-mercaptobenzthiazole (0.01 mole) in dry acetone, containing anhydrous potassium carbonate (0.011 mole) and ethyl chloroformate (0.01 mole), was refluxed on a steam bath for 16–18 hr. The mixture was filtered and the excess acetone from the filtrate was removed by distillation. The solid mass, which separated on cooling, was collected by filtration, washed with water, and recrystallized from ethanol, mp 63° [lit. (9) mp 64°].

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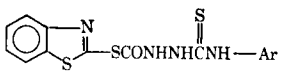
[1] All compounds were analyzed for their carbon, hydrogen, and nitrogen content. Melting points were taken in open capillary tubes with partial immersion thermometer and are corrected.

Table I—Physical Constants of 2-(4-Arylthiosemicarbazidocarbonylthio)benzthiazoles



Compound	Ar	Melting Point[a]	Yield, %	Formula	Analysis, % Calc.	Found
I	C_6H_5	241°	68	$C_{15}H_{12}N_4OS_3$	C 50.00	50.24
					H 3.33	3.28
					N 15.55	15.59
II	o-$CH_3C_6H_4$	250°	55	$C_{16}H_{14}N_4OS_3$	C 51.33	51.25
					H 3.74	3.68
					N 14.97	14.91
III	m-$CH_3C_6H_4$	258°	58	$C_{16}H_{14}N_4OS_3$	C 51.33	51.42
					H 3.74	3.82
					N 14.97	14.98
IV	p-$CH_3C_6H_4$	244°	85	$C_{16}H_{14}N_4OS_3$	C 51.33	51.28
					H 3.74	3.75
					N 14.97	14.85
V	o-$OCH_3C_6H_4$	242°	52	$C_{16}H_{14}N_4O_2S_3$	C 49.23	48.28
					H 3.59	3.56
					N 14.36	14.28
VI	p-$OCH_3C_6H_4$	230°	86	$C_{16}H_{14}N_4O_2S_3$	C 49.23	49.32
					H 3.59	3.62
					N 14.36	14.31
VII	p-ClC_6H_4	255°	65	$C_{15}H_{11}ClN_4OS_3$	C 45.63	45.48
					H 2.79	2.76
					N 14.19	14.15
VIII	p-BrC_6H_4	240°	90	$C_{15}H_{11}BrN_4OS_3$	C 41.00	41.42
					H 2.56	2.51
					N 12.76	12.66
IX	p-IC_6H_4	256°	75	$C_{15}H_{11}IN_4OS_3$	C 37.04	37.34
					H 2.26	2.23
					N 11.52	11.47
X	α-$C_{10}H_7$	260°	70	$C_{19}H_{14}N_4OS_3$	C 55.61	55.58
					H 3.41	3.37
					N 13.66	13.55

[a] Melting points were taken in open capillary tubes with a partial immersion thermometer and are corrected.

2-(Hydrazinocarbonylthio)benzthiazole—To a solution of 2-(ethoxycarbonylthio)benzthiazole (0.01 mole) in absolute ethanol was added 99–100% hydrazine hydrate (0.02 mole), and the mixture was refluxed on a steam bath for 10–12 hr. Excess ethanol was distilled and the precipitated crude product was collected, dried, and recrystallized from ethanol, mp 199–200° [lit. (9) mp 201°].

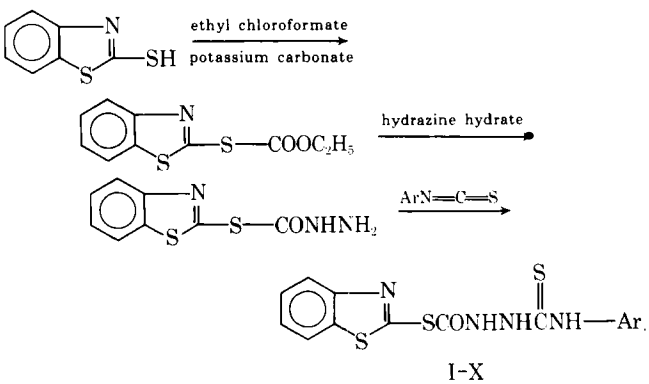
2-(4-Arylthiosemicarbazidocarbonylthio)benzthiazoles (I–X)—Equimolar portions of 2-(hydrazinocarbonylthio)benzthiazole and the suitable arylisothiocyanate were mixed in dry benzene, and the mixture was refluxed on a steam bath for 6 hr. The mixture was concentrated by removing benzene by distillation. The crude product, which separated on cooling, was collected by filtration; it was washed first with ether, then with dilute hydrochloric acid, and finally with water and was recrystallized from dimethylformamide (Table I).

Determination of Monoamine Oxidase Activity—*Spectrophotofluorometric Method*—Male albino rats, 100–150 g, were allowed food and water *ad libitum* until they were sacrificed by decapitation. The brains were removed immediately and homogenized[2] in the ratio of 1:9 (w/v) in ice-cold 0.25 *M* sucrose. The monoamine oxidase activity of rat brain homogenate was determined by incubation at 37° in air for 30 min, using kynuramine as the substrate (10). The reaction mixture in a total volume of 3 ml consisted of 0.5 ml of phosphate buffer (0.2 *M*, pH 7.5), 0.1 m*M* kynuramine, and 0.5 ml of brain homogenate (equivalent to 10 mg wet weight of the tissue).

The various 2-(4-arylthiosemicarbazidocarbonylthio)benzthiazoles were dissolved in propylene glycol (100%) and added to the brain homogenate to produce a final concentration of 0.5 m*M*. An equivalent amount of propylene glycol was added to the control tubes, and the mixture was incubated for 10 min before the addition of kynuramine. After the addition of kynuramine, the mixture was further incubated for 30 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid (w/v), and the precipitated proteins were removed by centrifugation. Suitable 1-ml aliquots of the supernatant solution were taken in 2 ml of 1 *N* NaOH solution and were assayed for 4-hydroxyquinoline, formed during oxidative deamination of kynuramine by rat brain homogenate.

The 4-hydroxyquinoline formed was measured fluorometrically[3], using activating light of 315 nm and measuring fluorescence at the maximum of 380 nm. An increase in absorbance provided a direct measurement of 4-hydroxyquinoline formation, which was taken as an index of the monoamine oxidase activity. The percentage inhibition was calculated from the decrease observed in absorbance, and this value provided an index of the inhibitory property of these substituted benzthiazoles.

Warburg Manometric Method—The monoamine oxidase activity of rat brain homogenate was determined by the conventional Warburg manometric technique, using tyramine and 5-hydroxytryptamine as the substrates (10). The decrease in oxygen uptake for 1 hr during oxidative deamination of tyramine or 5-hydroxytryptamine in the presence of the substituted benzthiazoles was used as an index of enzyme inhibition. The reaction mixture in a final concentration consisted of 50 m*M* phosphate buffer (pH 7.5),



Scheme I

[2] Potter–Elvehjem homogenizer.

[3] Aminco Bowman spectrophotofluorometer.

Table II—Monoamine Oxidase Inhibitory and Anticonvulsant Activity of 2-(4-Arylthiosemicarbazidocarbonylthio)benzthiazoles

Compound	Monoamine Oxidase Inhibition[a], %			Anticonvulsant Activity[b], % Protection	Pentylenetetrazol Mortality[c], % after 24 hr
	Kynuramine	Tyramine	5-Hydroxytryptamine		
I	30.0 ± 1.0	12.1 ± 0.7	14.8 ± 0.9	20	40
II	71.4 ± 0.8	29.2 ± 1.0	18.8 ± 0.9	20	60
III	61.4 ± 0.8	39.0 ± 0.9	28.3 ± 0.7	30	50
IV	74.3 ± 0.7	28.2 ± 0.9	19.2 ± 0.8	30	50
V	87.1 ± 0.6	24.6 ± 0.6	29.3 ± 1.0	50	50
VI	70.0 ± 1.0	29.6 ± 0.7	49.9 ± 0.8	10	90
VII	68.6 ± 1.0	23.8 ± 0.7	30.1 ± 0.7	50	70
VIII	65.0 ± 0.8	17.8 ± 0.9	26.0 ± 0.5	50	40
IX	77.9 ± 0.5	36.5 ± 0.6	46.9 ± 0.9	30	50
X	34.3 ± 0.8	7.9 ± 0.8	8.7 ± 1.1	60	40

[a] The reaction mixture and assay procedures are described in the text. Each experiment was done in triplicate, and the values are the means of three separate experiments ± *SE*. [b] Screening procedures for the determination of anticonvulsant activity are described in the text. [c] Mortality in pentylenetetrazol-treated mice was observed during 24 hr.

10 m*M* tyramine or 5-hydroxytryptamine, and rat brain homogenate (equivalent to 250 mg of fresh tissue weight) in a total volume of 3 ml.

The various substituted benzthiazoles were dissolved in propylene glycol (100%), used at a final concentration of 1 m*M*, and were incubated with the enzyme preparation for 20 min before tyramine or 5-hydroxytryptamine was added. The enzyme system was then incubated for an additional hour at 37°, using oxygen as the gaseous phase. Readings of oxygen uptake were made every 10 min.

Determination of Anticonvulsant Activity—Anticonvulsant activity of substituted benzthiazoles was determined (11) in mice, 25–30 g, of either sex. The mice were divided in groups of 10, keeping the group weights as near the same as possible. Each test compound was suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v) and was injected in a group of 10 mice at a dose of 100 mg/kg ip. Four hours after the administration of the substituted benzthiazoles, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose of pentylenetetrazol not only produced convulsions in almost all untreated mice but also exhibited 100% mortality during 24 hr. No mortality was observed during 24 hr in animals treated with 100 mg/kg alone of the test compounds.

The mice were observed 60 min for seizures. An episode of clonic spasm that persisted for a minimum of 5 sec was considered a threshold convulsion. Transient intermittent jerks and tremulousness were not counted. Animals devoid of threshold convulsions during 60 min were considered protected. The number of mice protected in each group was recorded, and the anticonvulsant activity of 2-(4-arylthiosemicarbazidocarbonylthio)benzthiazoles was represented as percent protection. The mice were then observed for 24 hr, and their mortality was recorded.

RESULTS AND DISCUSSION

The inhibitory effects of 2-(4-arylthiosemicarbazidocarbonylthio)benzthiazoles on the monoamine oxidase activity of rat brain homogenate during oxidative deamination of different substrates and their ability to afford protection against pentylenetetrazol-induced convulsions are recorded in Table II. The results indicated greater sensitivity of these substituted benzthiazoles toward monoamine oxidase during oxidative deamination of kynuramine, as reflected by higher inhibition of monoamine oxidase compared to inhibition observed when tyramine or 5-hydroxytryptamine was the substrate. Similar changes in the degree of monoamine oxidase inhibition also have been observed with other inhibitors during oxidative deamination of different substrates (7, 12).

The presence of substituents on the phenyl group of the thiosemicarbazide moiety influenced their ability to inhibit monoamine oxidase. Introduction of a substituent in the phenyl ring of these substituted benzthiazoles increased the degree of monoamine oxidase inhibition, with the exception of α-naphthyl substitution (X), which caused lowering of inhibition when tyramine or 5-hydroxytryptamine was the substrate. Among halogen-substituted benzthiazoles (VII–IX), the iodo-substituted benzthiazole (IX) possessed maximum monoamine oxidase inhibitory activity.

All 2-(4-arylthiosemicarbazidocarbonylthio)benzthiazoles possessed measurable anticonvulsant activity and exhibited some protection against pentylenetetrazol-induced 24 hr mortality in experimental mice (Table II).

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Cardiotonic Steroids II: 3-Deoxycardenolides and 3-Deoxycardanolides

THOMAS R. WITTY *, WILLIAM A. REMERS *x, and H. R. BESCH, Jr. ‡

Abstract □ A series of compounds related to 3-deoxydigitoxigenin was prepared and assayed for inhibition of myocardial Na^+,K^+-adenosine triphosphatase. Although the relatively high activity of 3-deoxydigitoxin was confirmed, the corresponding 3β,4β-epoxide and a mixture of 2,3-olefins and 3,4-olefins were less active. 3-Deoxy compounds with variations at the 14-position and the butenolide ring were much less active than the corresponding 3β-hydroxy analogs. Thus the activity of 3-deoxydigitoxigenin appears to be particularly susceptible to structural changes elsewhere in the molecule.

Keyphrases □ 3-Deoxydigitoxigenin analogs—synthesis and inhibition of Na^+,K^+-adenosine triphosphatase □ Cardenolide and cardanolide derivatives—synthesis of 3-deoxydigitoxigenin analogs, inhibition of Na^+,K^+-adenosine triphosphatase □ Cardiotonic steroids—synthesis of 3-deoxydigitoxigenin analogs, inhibition of Na^+,K^+-adenosine triphosphatase □ Structure–activity relationships—3-deoxydigitoxigenin analog inhibition of Na^+,K^+-adenosine triphosphatase

For many years it was accepted that a 3β-oxygen function was indispensable to the activity of cardiotonic steroids (1). Useful oxygen functions were exemplified by glycosides, ketals, esters, and the free hydroxy group. However, recent reports that 3-deoxydigitoxigenin (Ia) shows cardiotonic activity comparable to that of digitoxigenin (IIa) in the isolated frog heart (2) and in a Na^+,K^+-adenosine triphosphatase inhibition assay (3) appeared to refute this concept. These reports prompted a more extensive investigation on 3-deoxydigitoxigenin analogs, including compounds where the 14-substituent and the butenolide ring were varied (4).

One purpose of this investigation was to determine if the 3-deoxydigitoxigenin structure was more sensitive in biological activity to changes elsewhere in the molecule than was digitoxigenin. Another purpose was to generate some parent cardenolide and cardanolide structures (*e.g.*, IVa and IVb) since these compounds, although unlikely to have potent cardiotonic activity, might serve as starting points for structure–activity relationships.

RESULTS AND DISCUSSION

Synthesis—3-Deoxydigitoxigenin (Ia) was prepared from a mixture of 2,3- and 3,4-dehydrodigitoxigen isomers as described previously (2). This olefin mixture also was converted by *m*-chloroperbenzoic acid into two compounds, but only one could be purified. It is considered to be the 3β,4β-epoxide (V) for the following reasons. The NMR spectrum showed a broad, unsymmetrical pattern of peaks for the protons attached to the epoxide ring. This pattern is consistent with a 3,4-epoxide but not a 2,3-epoxide. Since the α-side of the 3,4-olefin from which V is derived has significant steric crowding (as determined from Dreiding models), *m*-chloroperbenzoic acid should form preferentially the β-epoxide. Literature precedent also exists for the selective formation of β-epoxides from ring A olefins in 5β-steroids (5).

Treatment of Ia with thionyl chloride gave the 14-olefin (IIIa), which was converted into 14β,15β-epoxide (VI) by *N*-bromoacetamide (Scheme I). Catalytic hydrogenation of IIIa gave 5β,14α-cardenolide (IVa) when palladium-on-barium sulfate was the catalyst, but the corresponding cardanolide [IVb, mixture of 20(*R*)- and 20(*S*)-epimers] was obtained when platinum was the catalyst. The pure 20(*R*)-epimer was reported previously (6). Hydrogenation of Ia in the presence of platinum gave 14β-hydroxycardanolide [Ib, mixture of 20(*R*)- and 20(*S*)-epimers], which afforded the 14-olefin (IIIb) upon treatment with thionyl chloride.

Table I—K_I Values for the Inhibition of Na^+,K^+-Adenosine Triphosphatase

Compound	K_I, μM
IIa (digitoxigenin)	0.043
Mixture of 2- and 3-dehydro-3-deoxydigitoxigenin	0.16
Ia (3-deoxydigitoxigenin)	0.076
V	0.65
IIIa	17.0
VI	100.0
IVb	>100.0
Ib	25.0
IIb (20,22-dihydrodigitoxigenin)	2.3

Biological Testing—Certain cardenolides and cardanolides were tested for their ability to inhibit a Na^+,K^+-stimulated, Mg^{+2}-dependent adenosine triphosphatase isolated from calf or canine myocardium (7). The ability of a large number of cardenolides to inhibit specifically this adenosine triphosphatase has been shown to correlate well with their positive inotropic activity (8, 9).

As recorded in Table I, the relatively high potency of Ia was confirmed. In this assay, it had 0.57 times the potency of digitoxigenin. No other compound had comparable activity, although the olefin mixture (2) was 0.22 times as potent as digitoxigenin. The 3β,4β-epoxide (V) had 0.066 times the activity of digitoxigenin, whereas the 14β,15β-epoxide (VI) had very poor activity (0.0004 times digitoxigenin). The 14-olefin (IIIa) was somewhat more active than VI, in contrast to the relative order of activity of 14-ene and 14β,15β-epoxide in the digitoxigenin-3-acetate series (4).

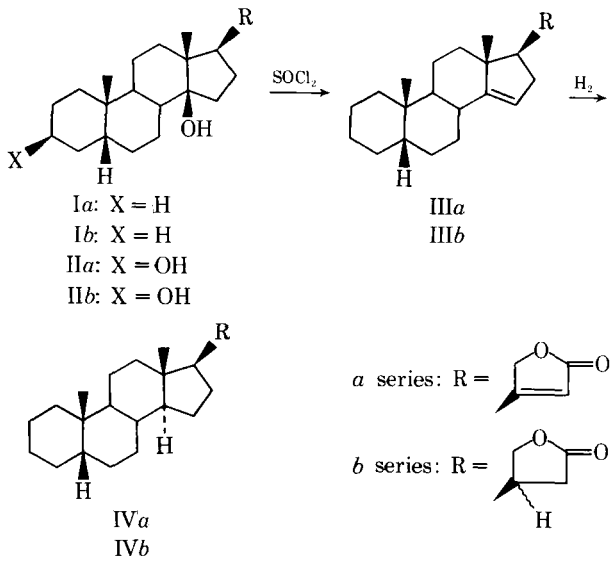
Of the saturated lactone compounds, 20,22-dihydrodigitoxigenin (IIb) (10) had 0.019 times the activity of digitoxigenin, but the 3-deoxy analog (Ib) had only 0.0017 times its activity. Thus, it appears that changes in the structure of 3-deoxydigitoxigenin result in much greater loss of activity than those occurring when corresponding changes are made in the digitoxigenin structure.

The parent 5β,14α-cardanolide (IVb) was too inactive to give a 50% inhibition value in the assay. Compounds IVa and IIIb lacked sufficient solubility for evaluation of their activities.

EXPERIMENTAL[1]

14-Hydroxy-3β,4β-oxido-5β,14β-card-20(22)-enolide (V)—A solution of 356 mg of the mixture of isomers, 14-hydroxy-5β,14β-carda-2,20(22)-dienolide and 14-hydroxy-5β,14β-carda-3,20(22)-dienolide (2), in 2.5 ml of methylene chloride was treated with 173 mg of *m*-chloroperbenzoic acid. After 2 hr, a small amount of sodi-

[1] Melting points were determined on a Mel-Temp apparatus and are uncorrected. IR absorption spectra were recorded on a Perkin-Elmer 237B spectrophotometer using $CHCl_3$ solutions or KBr disks. NMR spectra were determined in $CDCl_3$ using tetramethylsilane as the reference on a Varian XL-100 spectrometer. Mass spectra were determined on a Consolidated Electronics Corp. 21-110B mass spectrometer. Microanalyses were carried out by the Microanalytical Laboratory, Department of Chemistry, Purdue University. A Guilford recording spectrophotometer with a Hoake constant-temperature bath was used for the biological assay.



Scheme I

um bisulfite was added followed by 5% sodium bicarbonate solution. The organic layer was washed with water, dried (magnesium sulfate), and concentrated.

Chromatography of the crude product on silica gel, with chloroform–acetone (6:1) as the solvent, gave starting material followed by two new components. The first of these compounds, after two recrystallizations from acetone–petroleum ether, gave 70 mg of needles, mp 206–209°; IR (KBr): 5.75 (butenolide) μm; NMR: δ 5.84 (m, 1, vinyl of butenolide), 5.02 and 4.76 (each m, 1 each, methylene of butenolide), complicated asymmetrical multiplet with main peaks at 3.22, 3.19, 3.12, 3.07, 3.00, and 2.96 (2, protons on epoxide ring), 2.78 (m, 1, allylic 17-proton), and 0.89 (6, angular methyls) ppm.

Anal.—Calc. for $C_{23}H_{32}O_4$: C, 74.16; H, 8.66. Found: C, 74.22; H, 8.73.

The second component gave, upon concentration, a small amount of glassy solid, mp 75–87°, after recrystallization from acetone–hexane. It was not investigated further.

5β-Carda-14,20(22)-dienolide (IIIa)—A solution of 360 mg of 3-deoxydigitoxigenin (Ia) (2) in 24 ml of pyridine was cooled to −20° and treated dropwise with 1.2 ml of thionyl chloride in 12 ml of pyridine. After the mixture was stirred for 1 hr at 0°, it was worked up in the usual way (4) to give 270 mg (79%) of white crystals, mp 164–164.5°, after recrystallization from ethyl acetate–methanol.

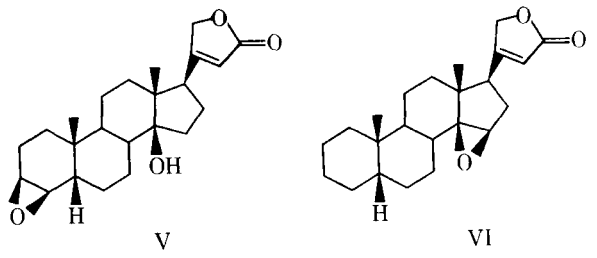
Anal.—Calc. for $C_{23}H_{32}O_2$: C, 81.13; H, 9.47. Found: C, 81.06; H, 9.52.

14β,15β-Oxido-5β,14β-card-20(22)-enolide (VI)—A solution of 200 mg of IIIa in 8 ml of acetone containing 0.8 ml of water was treated with 200 mg of *N*-bromoacetamide. The mixture was kept in the dark, and 60-mg portions of *N*-bromoacetamide were added at 24 and 48 hr. After 84 hr, the mixture was worked up in the usual way (4) and the product was purified by chromatography on silica gel with chloroform–acetone (6:1) as the solvent. Recrystallization of the product from ether gave colorless needles, mp 220–222°; the mass spectrum showed a molecular ion at M^+ 356.

Anal.—Calc. for $C_{23}H_{32}O_3$: C, 77.49; H, 9.05. Found: C, 77.24; H, 8.82.

5β,14α-Card-20(22)-enolide (IVa)—A mixture of 100 mg of 5β-carda-14,20(22)-dienolide (IIa), 20 ml of acetic acid, and 100 mg of 5% palladium-on-barium sulfate was shaken with hydrogen at an initial pressure of 1.1 kg/cm² (15 psi) for 5 hr. It then was filtered and concentrated to a solid, which was recrystallized from methylene chloride–methanol and then from methanol to give 33 mg of colorless needles, mp 154–155°; IR (KBr): 5.75 (butenolide) μm; NMR: δ 5.85 (m, 1, butenolide vinyl proton) ppm. The analytical sample was recrystallized once more from methanol, mp 166–166.5°.

Anal.—Calc. for $C_{23}H_{34}O_2$: C, 80.65; H, 10.01. Found: C, 80.40; H, 9.72.



5β,14α-Cardanolide [20(*R,S*)] (IVb)—A mixture of 150 mg of IVa, 35 ml of ethanol, and 100 mg of platinum oxide was shaken with hydrogen at an initial pressure of 0.7 kg/cm² (10 psi) for 1 hr. It then was filtered and concentrated to give 91 mg of a solid, mp 174–175°, after two recrystallizations from ethyl acetate–methanol; IR (KBr): 5.62 (saturated γ-lactone) μm. The NMR spectrum showed an angular methyl group at δ 0.66 ppm, which confirms the saturated lactone (11).

Anal.—Calc. for $C_{23}H_{36}O_2$: C, 80.18; H, 10.53. Found: C, 80.34; H, 10.70.

14-Hydroxy-5β,14β-cardanolide [20(*R,S*)] (Ib)—A mixture of 100 mg of Ia, 25 ml of ethanol, and 100 mg of platinum oxide was shaken with hydrogen at an initial pressure of 0.7 kg/cm² (10 psi) for 1 hr. It then was filtered and concentrated to a solid (101 mg), mp 170–172°, after recrystallization from ethyl acetate–methanol. Repeated recrystallizations from methanol failed to separate the mixture of 20(*R,S*)-epimers. The analytical sample had a melting point of 185–190° with softening at 175°; IR (KBr): 5.62 (saturated γ-lactone) μm. The NMR spectrum showed approximately equal peaks at δ 0.94 and 0.92 ppm, the 18-methyl groups for the epimers.

Anal.—Calc. for $C_{23}H_{36}O_3$: C, 76.62; H, 10.06. Found: C, 76.52; H, 9.98.

5β-Card-14-enolide [20(*R,S*)] (IIIb)—A solution of 90 mg of Ib in 3 ml of pyridine was treated at −20° with 0.3 ml of thionyl chloride. The solution was stirred for 1 hr at 0° and then worked up in the usual way (4). The product (26 mg) had a melting point of 166–167° after two recrystallizations from methylene chloride–methanol; IR (KBr): 5.62 (saturated γ-lactone) μm; NMR: δ 5.15 (m, 1, 15-vinyl proton) and 0.89 (s, 6, 18-methyl and 19-methyl) ppm.

Anal.—Calc. for $C_{23}H_{34}O_2$: C, 80.65; H, 10.01. Found: C, 80.63; H, 9.88.

Biological Assay—The Na^+,K^+-adenosine triphosphatase activity was determined by continuously monitoring at 340 nm the oxidation of NADH linked *via* a series of enzymatic reactions to the hydrolysis of ATP (12). This assay was described in detail previously (4).

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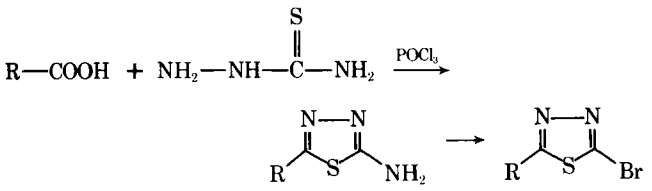
Synthesis and Pharmacological Activity of 5-Substituted 2-(*N,N*-Dialkylaminoethyl)amino- and 2-*N*-Methylpiperazinyl-1,3,4-thiadiazoles

I. LALEZARI *ˣ, A. SHAFIEE *, A. BADALY *, M. M. SALIMI ‡, M. A. KHOYI ‡, F. ABTAHI ‡, and M. R. ZARRINDAST ‡

Abstract □ 5-Substituted 2-amino-1,3,4-thiadiazoles were transformed to their corresponding 2-bromo derivatives. The reaction of the 5-substituted 2-bromo-1,3,4-thiadiazoles with *N,N*-dialkylaminoethylamines or *N*-methylpiperazine afforded the corresponding amino-1,3,4-thiadiazole derivatives. All prepared compounds displayed antihistaminic, anticholinergic, and norepinephrine-potentiating activities.

Keyphrases □ 1,3,4-Thiadiazoles, 5-substituted 2-(*N,N*-dialkylaminoethyl)amino and 2-*N*-methylpiperazinyl—synthesis, antihistaminic, anticholinergic, and norepinephrine-potentiating activities □ 2-(*N,N*-Dialkylaminoethyl)amino-1,3,4-thiadiazoles, 5-substituted—synthesis and pharmacological activity □ 2-*N*-Methylpiperazinyl-1,3,4-thiadiazoles, 5-substituted—synthesis and pharmacological activity □ Antihistaminic, anticholinergic, and norepinephrine-potentiating activities—synthesis and screening of 5-substituted 2-(*N,N*-dialkylaminoethyl)amino- and 2-*N*-methylpiperazinyl-1,3,4-thiadiazoles

In continuing studies on the chemistry and pharmacological activity of 1,3,4-thiadiazoles and their derivatives, it was of interest to introduce the =N—CH_2CH_2—N= moiety, the important group in known compounds with antihistaminic activity, to the 1,3,4-thiadiazole ring system. A previously reported method was used for the preparation of 2-amino-5-substituted-1,3,4-thiadiazoles (1, 2); the 2-amino compounds were then transformed to their corresponding 2-bromo compounds by a modified Sandmeyer reaction (3) (Scheme I).

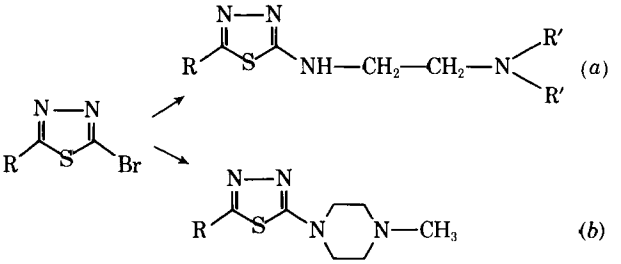


Scheme I

The 2-bromo-5-substituted-1,3,4-thiadiazoles thus obtained were subjected to a nucleophilic substitution reaction with *N*-methylpiperazine or asymmetrical dialkylaminoethylamine (Scheme II).

EXPERIMENTAL

Synthesis of 5-Substituted 2-*N*-Methylpiperazinyl- and 2-*N,N*-Dialkylaminoethylamino-1,3,4-thiadiazole Hydrochlorides—5-Substituted 2-bromo-1,3,4-thiadiazole (0.01 mole) and 2 *M* portions of *N*-methylpiperazine or asymmetric dialkylethylenediamine were refluxed for 8 hr in 40 ml of dry benzene. After cooling, the solid hydrobromide of the starting amine was separated by



Scheme II

1*a*: R = *o*-$CH_3C_6H_4$, R′ = CH_3
2*a*: R = *m*-$CH_3C_6H_4$, R′ = CH_3
3*a*: R = *p*-$CH_3C_6H_4$, R′ = CH_3
4*a*: R = *m*-$CH_3C_6H_4$, R′ = C_2H_5
5*a*: R = *p*-$CH_3OC_6H_4$, R′ = CH_3
6*a*: R = *m*-$NO_2C_6H_4$, R′ = CH_3
7*a*: R = *m*-$NO_2C_6H_4$, R′ = C_2H_5
8*a*: R = *p*-$NO_2C_6H_4$, R′ = CH_3
9*a*: R = *p*-$NO_2C_6H_4$, R′ = C_2H_5

1*b*: R = CH_3
2*b*: R = C_2H_5
3*b*: R = CF_3
4*b*: R = C_6H_5
5*b*: R = *o*-$CH_3C_6H_4$
6*b*: R = *m*-$CH_3C_6H_4$
7*b*: R = *p*-$CH_3C_6H_4$
8*b*: R = *m*-$CH_3OC_6H_4$
9*b*: R = *p*-$CH_3OC_6H_4$
10*b*: R = *p*-FC_6H_4
11*b*: R = *o*-ClC_6H_4
12*b*: R = *m*-ClC_6H_4
13*b*: R = *p*-ClC_6H_4
14*b*: R = *m*-BrC_6H_4
15*b*: R = *p*-BrC_6H_4
16*b*: R = *m*-$NO_2C_6H_4$
17*b*: R = *p*-$NO_2C_6H_4$

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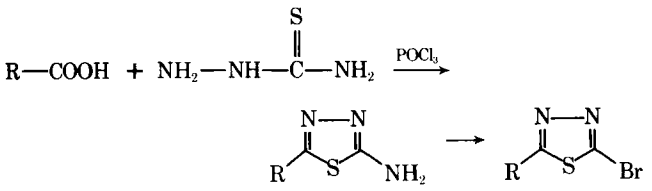
Synthesis and Pharmacological Activity of 5-Substituted 2-(*N,N*-Dialkylaminoethyl)amino- and 2-*N*-Methylpiperazinyl-1,3,4-thiadiazoles

I. LALEZARI *ˣ, A. SHAFIEE *, A. BADALY *, M. M. SALIMI ‡, M. A. KHOYI ‡, F. ABTAHI ‡, and M. R. ZARRINDAST ‡

Abstract □ 5-Substituted 2-amino-1,3,4-thiadiazoles were transformed to their corresponding 2-bromo derivatives. The reaction of the 5-substituted 2-bromo-1,3,4-thiadiazoles with *N,N*-dialkylaminoethylamines or *N*-methylpiperazine afforded the corresponding amino-1,3,4-thiadiazole derivatives. All prepared compounds displayed antihistaminic, anticholinergic, and norepinephrine-potentiating activities.

Keyphrases □ 1,3,4-Thiadiazoles, 5-substituted 2-(*N,N*-dialkylaminoethyl)amino and 2-*N*-methylpiperazinyl—synthesis, antihistaminic, anticholinergic, and norepinephrine-potentiating activities □ 2-(*N,N*-Dialkylaminoethyl)amino-1,3,4-thiadiazoles, 5-substituted—synthesis and pharmacological activity □ 2-*N*-Methylpiperazinyl-1,3,4-thiadiazoles, 5-substituted—synthesis and pharmacological activity □ Antihistaminic, anticholinergic, and norepinephrine-potentiating activities—synthesis and screening of 5-substituted 2-(*N,N*-dialkylaminoethyl)amino- and 2-*N*-methylpiperazinyl-1,3,4-thiadiazoles

In continuing studies on the chemistry and pharmacological activity of 1,3,4-thiadiazoles and their derivatives, it was of interest to introduce the =N—CH_2CH_2—N= moiety, the important group in known compounds with antihistaminic activity, to the 1,3,4-thiadiazole ring system. A previously reported method was used for the preparation of 2-amino-5-substituted-1,3,4-thiadiazoles (1, 2); the 2-amino compounds were then transformed to their corresponding 2-bromo compounds by a modified Sandmeyer reaction (3) (Scheme I).

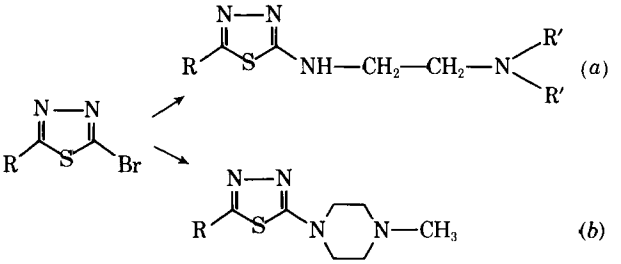


Scheme I

The 2-bromo-5-substituted-1,3,4-thiadiazoles thus obtained were subjected to a nucleophilic substitution reaction with *N*-methylpiperazine or asymmetrical dialkylaminoethylamine (Scheme II).

EXPERIMENTAL

Synthesis of 5-Substituted 2-*N*-Methylpiperazinyl- and 2-*N,N*-Dialkylaminoethylamino-1,3,4-thiadiazole Hydrochlorides—5-Substituted 2-bromo-1,3,4-thiadiazole (0.01 mole) and 2 *M* portions of *N*-methylpiperazine or asymmetric dialkylethylenediamine were refluxed for 8 hr in 40 ml of dry benzene. After cooling, the solid hydrobromide of the starting amine was separated by



Scheme II

1*a*: R = *o*-$CH_3C_6H_4$, R′ = CH_3
2*a*: R = *m*-$CH_3C_6H_4$, R′ = CH_3
3*a*: R = *p*-$CH_3C_6H_4$, R′ = CH_3
4*a*: R = *m*-$CH_3C_6H_4$, R′ = C_2H_5
5*a*: R = *p*-$CH_3OC_6H_4$, R′ = CH_3
6*a*: R = *m*-$NO_2C_6H_4$, R′ = CH_3
7*a*: R = *m*-$NO_2C_6H_4$, R′ = C_2H_5
8*a*: R = *p*-$NO_2C_6H_4$, R′ = CH_3
9*a*: R = *p*-$NO_2C_6H_4$, R′ = C_2H_5

1*b*: R = CH_3
2*b*: R = C_2H_5
3*b*: R = CF_3
4*b*: R = C_6H_5
5*b*: R = *o*-$CH_3C_6H_4$
6*b*: R = *m*-$CH_3C_6H_4$
7*b*: R = *p*-$CH_3C_6H_4$
8*b*: R = *m*-$CH_3OC_6H_4$
9*b*: R = *p*-$CH_3OC_6H_4$
10*b*: R = *p*-FC_6H_4
11*b*: R = *o*-ClC_6H_4
12*b*: R = *m*-ClC_6H_4
13*b*: R = *p*-ClC_6H_4
14*b*: R = *m*-BrC_6H_4
15*b*: R = *p*-BrC_6H_4
16*b*: R = *m*-$NO_2C_6H_4$
17*b*: R = *p*-$NO_2C_6H_4$

Table I—5-Substituted 2-(*N*,*N*-Dialkylaminoethyl)amino-1,3,4-thiadiazoles

Compound	Molecular Formula	Molecular Weight	Melting Point	Yield, %	Analysis, % Calc.	Found
1*a*	$C_{13}H_{18}N_4S$	262	173°	22	C 59.54 H 6.87	59.62 6.80
2*a*	$C_{13}H_{18}N_4S$	262	198°	50	C 59.54 H 6.87	59.48 6.89
3*a*	$C_{13}H_{18}N_4S$	262	218°	47	C 59.54 H 6.87	59.46 6.86
4*a*	$C_{15}H_{22}N_4S$	290	171°	6	C 62.06 H 7.58	61.93 7.52
5*a*	$C_{13}H_{18}N_4OS$	278	152°	10	C 56.11 H 6.47	56.06 6.53
6*a*	$C_{12}H_{15}N_5O_2S$	293	234°	9	C 49.14 H 5.11	49.22 5.05
7*a*	$C_{14}H_{19}N_5O_2S$	321	201°	53	C 52.33 H 5.91	52.38 5.94
8*a*	$C_{12}H_{15}N_5O_2S$	293	257°	26	C 49.14 H 5.11	49.10 5.07
9*a*	$C_{14}H_{19}N_5O_2S$	321	273°	49	C 52.33 H 5.91	52.30 6.08

Table II—5-Substituted 2-*N*-Methylpiperazinyl-1,3,4-thiadiazoles

Compound	Molecular Formula	Molecular Weight	Melting Point	Yield, %	Analysis, % Calc.	Found
1*b*	$C_8H_{14}N_4S$	198	250°	56	C 48.48 H 7.07	48.59 7.02
2*b*	$C_9H_{16}N_4S$	212	149°	39	C 50.92 H 7.55	50.87 7.50
3*b*	$C_8H_{11}F_3N_4S$	252	234°	57	C 38.10 H 4.37	38.11 4.43
4*b*	$C_{13}H_{16}N_4S$	260	276°	80	C 60.00 H 6.15	60.12 6.21
5*b*	$C_{14}H_{18}N_4S$	274	220°	87	C 61.31 H 6.57	61.28 6.50
6*b*	$C_{14}H_{18}N_4S$	274	283°	71	C 61.31 H 6.57	61.30 6.64
7*b*	$C_{14}H_{18}N_4S$	274	292°	69	C 61.31 H 6.57	61.39 6.63
8*b*	$C_{14}H_{18}N_4OS$	290	217°	58	C 57.93 H 6.21	57.86 6.29
9*b*	$C_{14}H_{18}N_4OS$	290	233°	68	C 57.93 H 6.21	57.92 6.12
10*b*	$C_{13}H_{15}FN_4S$	278	283°	38	C 56.12 H 5.40	56.20 5.44
11*b*	$C_{13}H_{15}ClN_4S$	294.5	231°	94	C 52.97 H 5.09	52.90 5.01
12*b*	$C_{13}H_{15}ClN_4S$	294.5	296°	82	C 52.97 H 5.09	52.98 5.16
13*b*	$C_{13}H_{15}ClN_4S$	294.5	291°	64	C 52.97 H 5.09	52.89 5.08
14*b*	$C_{13}H_{15}BrN_4S$	339	304°	56	C 46.03 H 4.42	46.09 4.36
15*b*	$C_{13}H_{15}BrN_4S$	339	277°	49	C 46.03 H 4.42	46.13 4.50
16*b*	$C_{13}H_{15}N_5O_2S$	305	265°	60	C 51.15 H 4.92	51.18 4.83
17*b*	$C_{13}H_{15}N_5O_2S$	305	246°	20	C 51.15 H 4.92	51.04 4.98

filtration and the benzene solution was extracted with 5% hydrochloric acid. The aqueous layer was evaporated under reduced pressure, and the solid residue was recrystallized from absolute ethanol.

The free base of 5-phenyl-2-*N*-methylpiperazinyl-1,3,4-thiadiazole was prepared as follows. The hydrochloride was dissolved in water and made basic with concentrated sodium hydroxide, and the solid was separated and recrystallized from ethyl acetate, mp 112°.

Physical properties of the synthesized compounds are found in Tables I and II.

Antihistaminic and Anticholinergic Activities—Isolated guinea pig ileum was prepared according to the method of Magnus (4) and suspended in a 10-ml bath filled with Tyrode solution, which was aerated at 30°. The isotonic contractions were magnified sixfold and recorded with a frontal writing lever on smoked drum. The pA_2 values were determined by the method of Schild (5).

Table III—Antihistaminic, Anticholinergic, and Norepinephrine-Potentiating Effects of 5-Substituted 2-(*N,N*-Dialkylaminoethyl)amino- and 2-*N*-Methylpiperazinyl-1,3,4-thiadiazoles[a]

Compound	pA_2 Values: Antihistaminic	pA_2 Values: Anticholinergic	Increase of Maximum Contraction, %
1*a*	4.55 ± 0.26 (4)	4.17 ± 0.19 (4)	—
2*a*	4.81 ± 0.5 (4)	4.72 ± 0.06 (4)	—
3*a*	4.80 ± 0.08 (4)	4.61 ± 0.11 (4)	—
4*a*	4.96 ± 0.04 (4)	5.01 ± 0.07 (4)	100 ± 19.14
5*a*	4.89 ± 0.09 (5)	4.84 ± 0.02 (4)	—
6*a*	4.75 ± 0.11 (4)	4.80 ± 0.32 (4)	—
7*a*	5.37 ± 0.05 (4)	5.06 ± 0.06 (4)	33.87 ± 2.78
8*a*	5.09 ± 0.07 (4)	4.89 ± 0.11 (4)	22.05 ± 1.76
9*a*	5.41 ± 0.12 (4)	5.34 ± 0.08 (4)	120.25 ± 13.44
1*b*	3.75 ± 0.10 (4)	3.38 ± 0.07 (4)	—
2*b*	3.91 ± 0.09 (4)	3.96 ± 0.16 (4)	—
3*b*	4.31 ± 0.05 (3)	4.43 ± 0.22 (4)	17.95 ± 21.93
4*b*	3.87 ± 0.12 (3)	3.68 ± 0.07 (4)	—
5*b*	4.01 ± 0.13 (4)	4.02 ± 0.05 (4)	—
6*b*	3.96 ± 0.10 (4)	4.04 ± 0.06 (4)	—
7*b*	4.22 ± 0.13 (4)	4.26 ± 0.14 (4)	—
8*b*	4.34 ± 0.02 (4)	4.37 ± 0.03 (4)	—
9*b*	3.72 ± 0.11 (4)	3.95 ± 0.11 (4)	—
10*b*	4.35 ± 0.12 (4)	4.21 ± 0.01 (4)	—
11*b*	4.03 ± 0.08 (4)	4.12 ± 0.12 (4)	—
12*b*	4.34 ± 0.11 (4)	4.33 ± 0.13 (4)	—
13*b*	4.50 ± 0.05 (4)	4.32 ± 0.07 (4)	34.92 ± 8.49
14*b*	4.64 ± 0.09 (4)	4.67 ± 0.09 (4)	39.97 ± 5.25
15*b*	4.43 ± 0.07 (4)	4.37 ± 0.06 (4)	—
16*b*	4.32 ± 0.10 (5)	4.34 ± 0.08 (4)	—
17*b*	4.94 ± 0.26 (4)	4.60 ± 0.11 (4)	24.07 ± 5.94
Atropine	—	9.84 ± 0.17 (4)	—
Diphenhydramine	9.74 ± 0.42 (4)	—	—
Cocaine	—	—	28.95 ± 10.83

[a] Values are expressed as mean ±*SE*. Numbers in parentheses indicate the number of experiments. When studying the norepinephrine-potentiating effect, Compounds 7*a*, 8*a*, and 9*a* were used in a concentration of 1×10^{-6} *M* and the other compounds were used in a concentration of 1×10^{-5} *M*.

Histamine and acetylcholine were used as agonists, and diphenhydramine and atropine were tested for comparison.

Norepinephrine-Potentiating Effect—Preliminary experiments revealed that some of these compounds potentiated the effect of norepinephrine on vas deferens; therefore, Compounds 4*a*, 7*a*, 8*a*, 9*a*, 3*b*, 13*b*, 14*b*, and 17*b* were tested on isolated rat vas deferens. The tissue was mounted in the bath under 1 g of tension. Isometric contractions were amplified and recorded[1] through a photosensitive myograph transducer. The test compounds were added 3 min before norepinephrine administration, and results were compared with contraction induced by norepinephrine in the absence of the compounds.

LD_{50} Determination—The toxicity of the sufficiently available compounds was determined in ordinary mice by the moving average method (6). The compounds were injected intraperitoneally; six animals were used for each dose.

RESULTS AND DISCUSSION

The potency of antihistaminic and anticholinergic activities is given in Table III and compared with diphenhydramine and atropine. The pA_2 values show that dialkylaminoethylamine derivatives possessed approximately three to four times more antihistaminic and anticholinergic potency than *N*-methylpiperazine derivatives.

Nitrophenyl, bromophenyl, and methoxyphenyl as well as trifluoromethyl compounds were found to be more active. Compounds substituted in the *para*-position were more active than the corresponding *ortho*- and *meta*-substituted derivatives. 5-Trifluoromethyl-2-*N*-methylpiperazinyl-1,3,4-thiadiazole was much more active than the corresponding 5-methyl, 5-ethyl, and 5-phenyl derivatives.

In the ethylenediamine series, compounds having a diethyl group were two to three times more active as compared with dimethyl derivatives, probably due to an increase in hydrophobicity.

It is also suggested that the lower activity in the piperazine series is due to the large size of the piperazine moiety of the molecule.

The potentiation of the effect of norepinephrine on α-receptors of vas deferens by these compounds, particularly 9*a*, is remarkable as compared with that of cocaine. This effect was significantly greater with the ethylenediamine series as compared with the piperazine series (Table III).

The LD_{50}'s of Compounds 2*b*, 3*b*, 4*b*, and 7*a* were 124.4 (107.4–144), 51.7 (44.2–60.5), 261.7 (222.7–307.5), and 148.2 (120.5–182.4) mg/kg, respectively. The trifluoromethyl group increased the toxicity of the compounds.

The animals showed hyperexcitability and tonic convulsions before death.

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[1] E. & M. physiograph.

Interaction of Substituted Benzoic Acids with Polysorbate 20 Micelles

J. H. COLLETT [x] **and L. KOO**

Abstract □ Equilibrium solubilities of a series of substituted benzoic acids in different concentrations of polysorbate 20 at controlled pH were measured. The maintenance of pH was achieved using a pH-stat assembly. A linear relationship was found between the amount of benzoic acid solubilized and surfactant concentration. As solubilizate polarity increased, the amount solubilized also increased. Solubility data were analyzed, and the interaction between solubilizate molecules and micelles was calculated in terms of partition coefficients of ionized and unionized molecules between aqueous and micellar phases. A linear relationship between π values (log partition coefficients) of functional groups and aqueous–micellar partition coefficient was found.

Keyphrases □ Benzoic acid and derivatives—solubilization in polysorbate 20 at controlled pH, partition coefficients between aqueous and micellar phases □ Polysorbate 20—effect on solubilization of benzoic acid and derivatives at controlled pH, partition coefficients between aqueous and micellar phases □ Micellar–aqueous partition coefficients—benzoic acid and derivatives in polysorbate 20 □ Solubilization, benzoic acid and derivatives—effect of various polysorbate 20 concentrations at controlled pH

There are various methods of studying solubilization including equilibrium dialysis, molecular sieving, potentiometry, and solubility measurement. Although they all have limitations (1), they have been used to provide useful data of micellar solubilization of benzoic acid derivatives (2–5) and barbiturates (6) by micelles of nonionic surfactants. None of the reports has given information of the interactions of the ionized and unionized solubilizate moieties with the surfactant. Such information can be obtained for weakly ionizing compounds from analysis of equilibrium solubility data in surfactant solutions at different pH values (7).

This report describes the solubilization of a series of substituted benzoic acids in polysorbate 20. Partition ratios of ionized and unionized molecules between aqueous and micellar phases were calculated, and correlations between these values and substituent parameters of the solubilizate were examined.

EXPERIMENTAL

Materials—Polysorbate 20 (polyoxyethylene 20 sorbitan monolaurate) was used as received. It was characterized by its mass spectrum and proton magnetic resonance spectrum (8) and found to contain 21 oxyethylene units/molecule.

Benzoic acid and its 2-hydroxy, 3-hydroxy, 4-hydroxy, 4-chloro, 4-bromo, 4-iodo, 4-nitro, and 4-methoxy derivatives[1] were used as solubilizates. They were chosen because they represent typical weak acids with low aqueous solubility and are capable of being absorbed from the stomach in an unionized form.

Assay—Benzoic acids were assayed by UV absorption spectroscopy. The presence of polysorbate 20 at the concentration in the diluted solutions did not interfere with the assay either by absorption at the wavelengths used or by shifting the absorption peak.

Solubility—An excess of benzoic acid or its derivatives was added to 30-ml volumes of aqueous solutions containing different concentrations of polysorbate 20. The pH of the solution was adjusted by the addition of sufficient hydrochloric acid or sodium hydroxide. The volume of adjusting solution added was known, thereby permitting correction of polysorbate concentrations due to dilution. The pH was maintained by the addition of acid or alkali by the automatic control of a pH-stat assembly[2]. Solutions were stirred in a water-jacketed vessel at 25° during the initial pH adjustment.

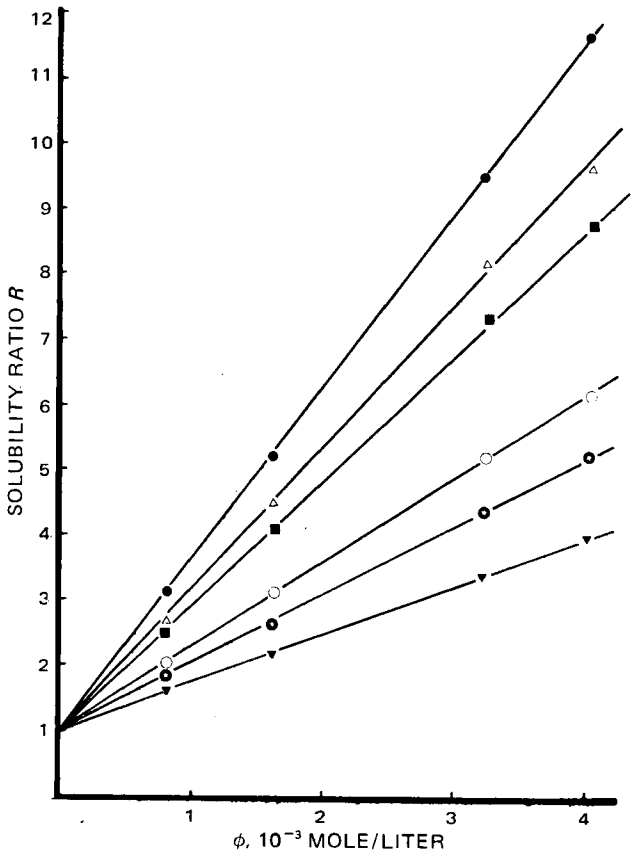

Figure 1—*Solubility of 4-chlorobenzoic acid in different concentrations of polysorbate 20 at different pH's. Key:* ●, *3.00;* △, *3.40;* ■, *3.62;* ○, *4.00;* ❂, *4.15; and* ▼, *4.40.*

After pH adjustment, the solutions were placed in 50-ml erlenmeyer flasks[3] and then shaken for 48 hr in a water bath at 25°; the pH was checked periodically. In the presence of surfactant, the addition of electrolytes not exceeding an ionic strength of 0.2 mole/liter had no effect on the solubility of the benzoic acids. This ionic strength is greater than that produced by the addition of hydrochloric acid or sodium hydroxide to control pH. After equilibration, aliquot samples were filtered through 0.45-μm filters[4] and assayed for benzoic acid content.

Density—Apparent densities of polysorbate 20 in 1, 2, 4, and 5% (w/v) aqueous solutions were determined at 25° using 50-ml density bottles.

[1] B.D.H., Ltd.

[2] Radiometer, Radiometer Ltd., Copenhagen, Denmark.
[3] Quickfit.
[4] Millipore.

Table I—Molar Ratios and Solubility Ratios of Benzoic Acid Derivatives to Polysorbate 20 Concentration at Various pH Values

Compound	(a)[a]	(b)[b]	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)	(a)	(b)
Benzoic acid	pH 3.20		pH 3.60		pH 3.85		pH 4.20		pH 4.35		pH 4.55	
	0.120	0.397	0.121	0.342	0.124	0.282	0.126	0.205	0.124	0.160	0.131	0.118
2-Hydroxy-	pH 2.30		2.80		3.00		3.15		3.40		3.65	
benzoic acid	0.110	0.728	0.112	0.470	0.115	0.378	0.120	0.298	0.105	0.177	0.134	0.144
3-Hydroxy-	pH 3.10		3.45		3.70		4.00		4.25		4.45	
benzoic acid	0.164	0.163	0.162	0.221	0.175	0.208	0.172	0.150	0.209	0.130	0.190	0.084
4-Hydroxy-	pH 3.50		3.85		4.10		4.40		4.65			
benzoic acid	0.141	0.286	0.137	0.247	0.135	0.211	0.151	0.179	0.165	0.128		
4-Chloro-	pH 3.00		3.40		3.62		4.00		4.15		4.40	
benzoic acid	0.00918	2.568	0.00914	2.095	0.00945	1.880	0.00962	1.243	0.00996	1.029	0.01024	0.722
4-Bromo-	pH 3.00		3.40		3.60		3.90		4.20		4.40	
benzoic acid	0.00655	4.047	0.00647	3.480	0.00673	3.130	0.00672	2.327	0.00701	1.601	0.00711	1.101
4-Iodo-	pH 3.00		3.30		3.60		3.90		4.20		4.40	
benzoic acid	0.00568	6.291	0.00534	5.074	0.00650	4.656	0.00602	3.316	0.00583	2.444	0.00500	1.418
4-Nitro-	pH 2.45		2.80		3.05		3.40		3.60		3.80	
benzoic acid	0.00904	0.819	0.00943	0.749	0.00967	0.648	0.01038	0.531	0.00994	0.353	0.01062	0.257
4-Methoxy-	pH 3.50		3.80		4.10		4.40		4.70		5.00	
benzoic acid	0.113	0.601	0.111	0.542	0.115	0.474	0.123	0.366	0.122	0.225	0.118	0.160

[a] Column (a) represents the slope of solubility curves expressed in terms of molar ratios of solubilizate to polysorbate 20 concentration. [b] Column (b) represents the slope of solubility ratios obtained from plots of R against ϕ.

RESULTS AND DISCUSSION

Solubilities of the substituted benzoic acids in solutions containing graded concentrations of polysorbate 20 were measured at different pH values between 0.5 pH unit above and 1.0 pH unit below the pKa of the substituted benzoic acids. Typical solubility curves of 4-chlorobenzoic acid in solutions of different concentrations of polysorbate 20 at different pH values are shown in Fig. 1. At any one pH, solubility increased linearly with surfactant concentration. This observation is in agreement with other reports (1–4) and is attributed to solubilization of the acid by micelles of the surfactants.

The slopes of solubility curves for substituted benzoic acids at different pH values in graded concentrations of surfactant are shown in Table I. In each case, a linear relationship was observed, as would be expected for the solubilization of polar solubilizates. For each compound, the slope of molar ratio of solubilizate to polysorbate 20 concentration was similar at all pH values. The slope of the curves for each compound increased with decreasing polarity. A similar observation, that the amount of substituted benzoic acid solubilized by a surfactant increased as the polarity of the drug increased, has been reported (6).

Increased solubilization may have resulted from the smaller size of the benzoic acids. Although the polarity of solubilizate molecules is usually the most important factor determining their degree of solubilization, the molar volume of solubilizate has also been considered to be a significant factor. The volume of various hydrocarbons solubilized by potassium laurate was found to be inversely related to the molar volume of hydrocarbons (9). Alternatively, there may be some solubilization within the polyoxyethylene chains, and increased solubilizate polarity will result in an increased amount of solubilization at this site.

A treatment of solubility data (7, 10) based on the pseudophase model of surfactant solutions permits the individual quantification of the interactions of both ionized and unionized solubilizate molecules with surfactant. The concentration of solubilizate in the micellar phase is related to its concentration in the aqueous phase by:

$$P = \frac{(C_m)}{(C_a)} \qquad \text{(Eq. 1)}$$

where C denotes concentration; subscripts a and m refer to aqueous and micellar phases, respectively; and P is the micellar–aqueous partition coefficient. If concentrations are expressed in terms of the total volume of the system rather than the volumes of individual phases, then:

$$P = \frac{(C_m)}{(C_a)}\left(\frac{1}{\phi}\right) \qquad \text{(Eq. 2)}$$

where ϕ, the volume fraction of surfactant, is small (ϕ is the volume of the surfactant as a fraction of the total volume of the solution containing the surfactant).

The ratio, R, of solubility in surfactant to solubility in water is given by:

$$R = \frac{(C_m^-) + (C_m^0)}{(C_a^-) + (C_a^0)} + 1 \qquad \text{(Eq. 3)}$$

where superscripts 0 and − denote unionized and ionized solubilizate, respectively. A plot of R against volume fraction, ϕ, of surfactant has an intercept on the R axis of unity. The slope, S, of the plot is:

$$S = \frac{(C_m^-) + (C_m^0)}{(C_a^-) + (C_a^0)}\left(\frac{1}{\phi}\right) \qquad \text{(Eq. 4)}$$

Table I gives values of S obtained from plots of R against ϕ at several pH values. The slope, S, for any one compound decreases with an increase in pH. The higher solubilizing capacity of polysorbate 20 at pH values where the solute is predominantly unionized indicates that this form of the acid is solubilized to a greater extent than the ionized form.

Values of S at different pH's can be plotted against the fraction, fi, of ionized solubilizate present. At $fi = 1.0$, Eq. 4 becomes:

$$S = \frac{(C_m^-)}{(C_a^-)}\left(\frac{1}{\phi}\right) \qquad \text{(Eq. 5)}$$

and the value of S at $fi = 1.0$ is the partition coefficient of ionized molecules between surfactant micelles and water.

The micellar–aqueous partition coefficients of unionized benzoic acid derivatives and their respective lipophilicity expressed in terms of the hydrophobic–lipophilic constant, π (13), of the functional groups are presented in Table II (π is defined as $\pi = \log P_X - \log P_H$, where P_X is the partition coefficient of a derivative and P_H is the partition coefficient of the parent compound).

Table II—Partition Coefficient of Unionized Species and π (13) Values of the Respective Functional Group of Benzoic Acid Derivatives

Compound	Partition Coefficient of Unionized Moiety	π
Benzoic acid	43.5	0
2-Hydroxybenzoic acid	83.0	—
3-Hydroxybenzoic acid	28.5	−0.38
4-Hydroxybenzoic acid	30.8	−0.30
4-Chlorobenzoic acid	277.0	0.87
4-Bromobenzoic acid	445.0	0.98
4-Iodobenzoic acid	678.0	1.14
4-Nitrobenzoic acid	92.7	0.02
4-Methoxybenzoic acid	66.5	0.08

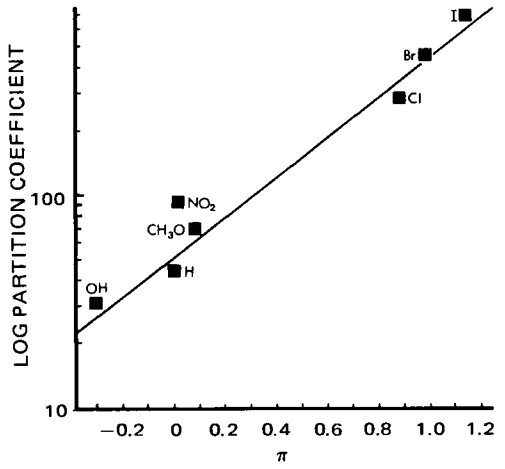

Figure 2—*Micellar–aqueous partition coefficients of substituted benzoic acids and π values of the functional groups.*

It can be seen in Fig. 2 that as the lipophilicity of the benzoic acid derivatives increases, the partition coefficient of the corresponding unionized molecules increases. 2-Hydroxybenzoic acid and 3-hydroxybenzoic acid are excluded from the plot because the hydroxy group is at positions other than the *para*-position; factors such as steric and electronic effects may contribute to the variations in their partition coefficient. The relationship between log partition coefficient and π is linear, with a slope of 0.885 and a correlation of 0.981.

The partition coefficient of 4-nitrobenzoic acid indicates a higher degree of solubilization than expected due to the negative inductive effect of the functional group, nitro, as compared with the positive effect of other groups (11), resulting in a greater interaction between the solubilizer and the solubilizate. When 4-nitrobenzoic acid is excluded, a better relationship can be obtained, with a slope value of 0.929 and a correlation of 0.994, indicative of a direct relationship between the micellar–aqueous partition coefficient of the unionized species and the π value of the substituent group. This relationship suggests that the unionized species is solubilized in a lipophilic region of the micelles.

Ismail *et al.* (6) reported an increase in the amount of substituted barbituric acids solubilized by polyoxyethylene stearates as solubilizate polarity decreased. The amount of each barbituric acid solubilized showed a rank-order correlation with their partition coefficients between 1-octanol and water. However, their work and that of Kakemi *et al.* (12) were limited to systems at pH 1.0.

The present data are particularly useful since, with a knowledge of micellar–aqueous partition coefficients of unionized parent compounds and of substituent π values, it is possible to calculate the amount of free drug present in micellar solution for other substituted derivatives. These calculations may find application in the interpretation of results from drug stability studies with micellar solutions and in drug absorption studies with micellar solutions.

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Confirmation of Iminoacridan Structure of Singly Protonated 9-Aminoacridine

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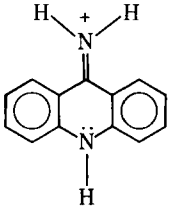
Abstract □ The occurrence of the second absorption band of singly protonated 9-aminoacridine, which arises from an acridinium ring-localized transition, at anomalously short wavelength indicates the disruption of the aromaticity of the central ring of the singly charged cation. The shifting of the two longest wavelength absorption bands and the fluorescence band of 10-methyl-9-aminoacridine monocation, a model of singly protonated 9-aminoacridine which is constrained to dissociate from the exocyclic nitrogen atom, to shorter wavelengths, upon dissociation, indicates the imine-like nature of the 10-methyl derivative. These observations support the conclusion that the predominant site of positive charge in singly protonated 9-aminoacridine is the exocyclic nitrogen atom, even though the heterocyclic nitrogen atom is the site of protonation of the neutral molecule.

Keyphrases □ 9-Aminoacridine—singly protonated, confirmation of iminoacridan structure □ Iminoacridan—confirmed as structure of singly protonated 9-aminoacridine □ Acridine derivatives—confirmation of iminoacridan structure of singly protonated 9-aminoacridine

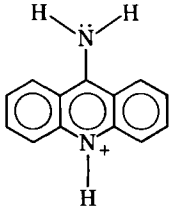
In a recent paper (1), it was shown that singly protonated 9-aminoacridine, the predominant prototropic species derived from 9-aminoacridine at physiological pH, existed in aqueous solution as the protonated iminoacridan (I) form rather than as the protonated aminoacridine (II). This finding is of pharmacological significance because of the ionic interaction of 9-aminoacridine and presumably other antimicrobials derived from 9-aminoacridine with DNA, an interaction whose geometrical characteristics are determined by the site of positive charge in the acridine derivative.

Previously (1), the anomalous shifting of the long wavelength absorption and fluorescence bands of singly protonated 9-aminoacridine with protonation, dissociation, and solvent polarity relative to the corresponding shifts of the singly protonated cation of 2-aminoacridine, a "typical" *N*-heterocyclic amine, was suggested to indicate that I was closest to the actual electronic structure of the 9-aminoacridine monocation.

Recently, in this laboratory, the *N*-methyl monocation of 9-aminoacridine (10-methyl-9-aminoacridinium iodide) was prepared and the pH dependences of its absorption and fluorescence spectra were studied. Moreover, the second absorption band of 9-aminoacridine, whose position in the electromagnetic spectrum is indicative of the extension of aromaticity in the aromatic ring (acridine), was examined as a function of pH. The results of these experiments confirm the assignment of Structure I as most representative of the electronic structure of singly protonated 9-aminoacridine.



I



II

EXPERIMENTAL

The pH measurements were made on a pH meter[1] with a combination silver–silver chloride glass electrode. Electronic absorption spectra were taken in 1-cm silica cells on a grating-type spectrophotometer[2]. Fluorescence measurements were taken on a fluorescence spectrophotometer[3] whose monochromators were calibrated against the line emission spectrum of xenon. Emission spectra were corrected for the wavelength response of the monochromator and phototube by means of a corrected spectra accessory employing a rhodamine-B quantum counter.

10-Methyl-9-aminoacridinium iodide was prepared by methylating 9-aminoacridine[4] according to the literature method (2). Spectra were taken of solutions that were 1×10^{-5} *M* in 9-aminoacridine or 10-methyl-9-aminoacridine. The spectrophotometric titration procedure employed was described previously (1).

RESULTS AND DISCUSSION

The longest wavelength absorption bands of acridines may be considered to be derived from the short axis polarized transition [$^1L_a \leftarrow {}^1A$ in Platts' nomenclature system (3)] of the parent hydrocarbon, anthracene. This band generally is most sensitive to substituents in what corresponds to α-positions of anthracene (4) (positions 1, 4, 5, 8, 9, and 10 in acridine) and, therefore, to protonation of the heterocyclic nitrogen atom of acridine. The second absorption bands of acridines are derived from the long axis polarized transition of anthracene ($^1L_b \leftarrow {}^1A$), are generally more structured and narrower than the ^1L_a bands, are sensitive in position to β-type substituents (4) (positions 2, 3, 6, and 7 in acridine), and are relatively insensitive to α-substituents (*e.g.*, a 9-amino group or protonation of the heterocyclic nitrogen atom) in acridine.

Thus, while the ^1L_a bands of α-substituted acridines appear in the absorption spectrum anywhere from 380 to >500 nm, the ^1L_b bands occur from 350 to 370 nm (5). Even in 2-aminoacridine, which has a β-substituent and no α-functional group other than the heterocyclic nitrogen atom, the O–O feature of the ^1L_b band always occurs within the latter interval, at 354, 370, and 356 nm in the dication, monocation, and neutral molecule, respectively. Hence, while the ^1L_a band in acridines is an "intramolecular charge-transfer band" whose position is determined by the interactions of the functional groups with the aromatic ring as well as by the size of the aromatic ring itself, the ^1L_b band in acridines is essentially a "ring-localized $\pi \rightarrow \pi^*$ transition" whose position is largely "fixed" by the size of the aromatic ring system. In anthracenes and the corresponding heterocyclic molecules, the ^1L_b band occurs between 350 and 370 nm; in naphthalenes, it occurs between 315 and 340 nm; and in benzenes, it occurs between 255 and 300 nm.

The ^1L_b bands of the various prototropic species derived from 9-aminoacridine indicate that the dication and anion are "well-behaved" acridine derivatives. The neutral molecule also is on the

[1] Model 801, Orion Research, Cambridge, Mass.
[2] Model DB-GT, Beckman Instruments, Fullerton, Calif.
[3] Model MPF-2A, Perkin-Elmer Corp., Norwalk, Conn.
[4] Pfaltz and Bauer, Flushing, N.Y.

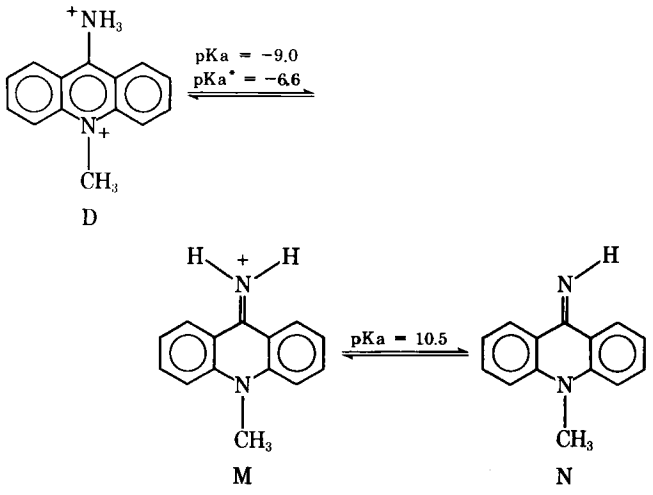
Table I—The O–O Vibronic Maxima of the Two Longest Wavelength Absorption Bands (λ^1L_a and λ^1L_b) and the Fluorescence Bands (λ) of the Various Prototropic Species Derived from 9-Aminoacridine and 10-Methyl-9-aminoacridinium Iodide

	λ^1L_b, nm	λ^1L_a, nm	λf, nm
9-Aminoacridine			
Dication ($H_0 - 10.0$)	366	440	475
Monocation (pH 1.0)	324	423	430
Neutral molecule (pH 12.0)	348	426	445
Anion (H_17.5)	353	443	470
10-Methyl-9-amino-acridinium iodide			
Dication ($H_0 - 10.0$)	368	445	480
Monocation (pH 1.0)	327	431	433
Neutral molecule (pH 12.5)	312	408	431

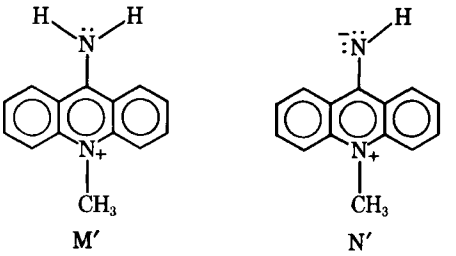
borderline of being a "typical" acridine derivative. However, the ^1L_b band of the monocation is at a much shorter wavelength than that indicative of an acridine ring system and suggests disruption of the aromaticity of the tricyclic system. Actually, the ^1L_b maximum at 324 nm is reasonable for an extended benzenoid system as represented by Structure I.

The 10-methyl-9-aminoacridinium ion has an electronic structure very much like that of the 9-aminoacridinium cation (Table I). The methylated cation can be protonated or dissociated according to Scheme I. An excited-state equilibrium occurs for the dication (D)–monocation (M) pair but not for the monocation–neutral (N) pair. The pKa* and pKa, as well as the spectral shifts occurring upon going from the dication to the monocation of the methylated derivative, are similar to the corresponding quantities measured for the dication–monocation interconversion of 9-aminoacridine (1) and therefore support the validity of using the *N*-methyl derivative as a model of the 9-aminoacridinium ion; which cannot dissociate from the heterocyclic function.

The dissociation of the monocation to form the neutral *N*-methyl derivative results in shifts of the long wavelength absorption (^1L_a) and fluorescence bands to shorter wavelengths. This result is expected for dissociation from a structure such as M to form one such as N because it indicates the electron-withdrawing nature of the exocyclic imino group (similar to but weaker than that of a carbonyl group).



Scheme I



If the alternative amino structures of M′ and N′ are taken to represent M and N, a shift of the ^1L_a and fluorescence bands to longer wavelengths would be expected upon dissociation, because of the increased lone-pair repulsion on the optical electron (stabilization of the charge-transfer excited state of N′ relative to that of M′) produced by dissociation of the electron donor amino group. These conclusions are supported by the positions of the ^1L_b bands of the 10-methyl monocation and neutral species at 327 and 312 nm, respectively. Both are at anomalously short wavelengths for acridinium ions (*i.e.*, M′ and N′) and also rule out the possibilities that the 10-methyl monocation is in the form M′ and that the neutral species is in the form N, a circumstance that might also be expected to yield a shift of the ^1L_a and fluorescence bands to shorter wavelengths upon dissociation.

The loss of acridine-like aromaticity in the monocation derived from 9-aminoacridine, as reflected in the short wavelength of the ^1L_b band and the imine-like dissociation and spectral behavior of the *N*-methylated monocation, supports the assignment of Structure I as that of singly protonated 9-aminoacridine. This conclusion is also supported by a recent X-ray diffraction study of 9-aminoacridine hydrochloride monohydrate (6), in which it was shown that the length of the bond joining the amino group to the aromatic ring is indicative of a double bond as represented in Structure I.

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Anti-Inflammation Mechanism of Extract from *Eisenia bicyclis* (Kjellman) Setchell

DAVID M. WHITAKER and GARY P. CARLSON ˣ

Abstract □ An extract from *Eisenia bicyclis*, previously shown to possess anti-inflammatory activity, was found to stabilize lysosomal membranes *in vitro* as determined by measurement of inhibition of the marker enzyme β-glucuronidase. Some anti-inflammatory activity was also due to counterirritancy.

Keyphrases □ *Eisenia bicyclis* extract—mechanism of anti-inflammatory activity, lysosomal stabilization □ Anti-inflammatory activity—*Eisenia bicyclis* extract, lysosomal stabilization

Although a number of important steroidal and nonsteroidal agents are presently in use for the treatment of inflammation, the search continues for new anti-inflammatory drugs. Previous studies from this laboratory (1) indicated that an extract from the alga *Eisenia bicyclis* (Kjellman) Setchell, collected from the shores of Korea, contains a compound possessing anti-inflammatory activity. This material has been reported (2) to be a complex polymer which, on chemical degradation, yields glucose, phloroglucinol, and trimethylamine.

Several methods were used in the original studies (1) to demonstrate anti-inflammatory activity, including the inhibition of the denaturation of bovine serum albumin and the prevention of carrageenan-induced edema in the rat paw. However, the material was ineffective in suppressing rat paw edema due to the administration of complete Freund's adjuvant. Little could be determined from these initial studies regarding the mechanism of the anti-inflammatory action other than that it was not due to a thermogenic effect and that it did not involve the pituitary–adrenal axis.

It was of interest, therefore, to determine if the compound might be active by virtue of its ability to stabilize lysosomal membranes and thereby inhibit the release of lysosomal enzymes from the organelle. The release of these enzymes and subsequent tissue damage have been implicated in several types of inflammation such as chemical burns (3) and arthritis (4). That stabilization of the membrane could be achieved by anti-inflammatory drugs was demonstrated utilizing the release of the lysosomal marker enzymes β-glucuronidase and acid phosphatase (5). Comparison of the ability to stabilize the membranes was made with phenylbutazone[1], a well-known anti-inflammatory agent. In addition, it was important to determine if all or some of the anti-inflammatory effect might be due to counterirritancy.

Table I—Effect of Drugs on Lysosomal Membrane Stability *In Vitro*

Drug	Enzyme Activity[a] ± *SE* (4)[b]	
	Acid Phosphatase	β-Glucuronidase
None	4.46 ± 0.16[c]	0.66 ± 0.02[c]
Phenylbutazone (0.309 mg/ml)	4.13 ± 0.08[c,d]	0.20 ± 0.01[d]
Eisenia extract (3.087 mg/ml)	3.66 ± 0.22[d]	0.24 ± 0.03[d]
Eisenia extract (0.309 mg/ml)	3.64 ± 0.16[d]	0.24 ± 0.01[d]
Eisenia extract (0.031 mg/ml)	3.83 ± 0.22[d]	0.64 ± 0.02[c]

[a] Expressed as μmoles of product/ml of lysosomal fraction/20 min. [b] Number of animals. [c,d] Groups not having same superscript are significantly different, $p < 0.05$.

EXPERIMENTAL

Materials and Methods—Adult male albino rats[2] were housed in air-conditioned rooms and allowed food and water *ad libitum*. The procedures for the preparation of hepatic lysosomal fractions and the determination of the activities of lysosomal β-glucuronidase and acid phosphatase were basically those of Ignarro (5). A 10% liver homogenate was prepared in 0.25 *M* sucrose–0.02 *M* tromethamine acetate buffer, pH 7.4, and centrifuged at 600×*g* for 5 min at 4° in a refrigerated centrifuge[3]. The supernate was decanted, mixed with an equal volume of the buffer, and recentrifuged at 3500×*g* for 15 min.

The resulting pellet was rinsed gently twice with 0.45 *M* sucrose–0.02 *M* tromethamine acetate buffer, pH 7.4, and resuspended in 4.0 ml of the buffer. The suspension was warmed to 25°, and 0.20-ml aliquots were added to 20-ml beakers containing 2.0 ml of 0.18 *M* sucrose–0.04 *M* tromethamine acetate buffer, pH 7.4, with either 0.1 ml of drug solution or water. Incubation was carried out at 37° for 0 or 15 min in a metabolic shaker[4] and terminated by centrifugation at 27,000×*g* for 15 min at 4° in polyethylene tubes.

To determine if the drug had any direct effect on the marker enzymes, the drug was added after the incubation period. To determine the total enzyme activity, 2.0 ml of 0.2% octoxynol[5] in 0.04 tromethamine acetate buffer, pH 7.4, replaced the buffer.

Acid phosphatase was determined by measuring the formation of *p*-nitrophenol from *p*-nitrophenyl phosphate, and β-glucuronidase activity was determined by measuring the formation of phenolphthalein from phenolphthalein glucuronide (5). One milliliter of the high-speed supernate and 1 ml of water were added to 1 ml of 0.3 *M* citrate buffer, pH 4.8. Incubation was initiated by the addition of 0.04 ml of substrate [*p*-nitrophenyl phosphate (79 mg/ml) or phenolphthalein glucuronide (63 mg/ml)]. Acid phosphatase incubations were terminated with 0.2 ml of 4 *N* NaOH, and absorbances were read at 405 nm. β-Glucuronidase assays were stopped with 0.4 ml of 2.2 *M* glycine–sodium hydroxide buffer, pH 12, and read at 540 nm.

Measurement of anti-inflammatory activity was made using a carrageenan-induced rat paw edema test system. The rat hindpaw was injected in the subplantar region with 0.1 ml of 1.0% carrageenan in saline, and the paw volume was determined by a plethysmographic method described by Van Arman *et al.* (6). The volume was measured 3 hr later, and edema formation was calculated as the difference.

Statistical analysis of the data from the lysosomal stabilization data comparing various doses was carried out using Duncan's new

[1] Butazolidin.
[2] Charles River Breeding Laboratories.
[3] Sorvall RC2-B.
[4] Dubnoff.
[5] Triton X-100.

Table II—Effect of Buffering *Eisenia* Extract on Carrageenan-Induced Rat Paw Edema

Treatment[a]	Mean Edema, $mm^3 \pm SE$ (6)[b]
Saline	1174 ± 99
Buffered[c] *Eisenia* extract	678 ± 176[d]

[a] Administered intraperitoneally 1 hr before carrageenan. [b] Number of animals. [c] Buffered at pH 7.4 with 0.2 *M* KH_2PO_4–Na_2PO_4. [d] $p < 0.05$.

multiple-range finding test. Comparison of the anti-inflammatory activity data utilized the Student two-tailed *t* test (7).

RESULTS AND DISCUSSION

The results of testing the activity of the *Eisenia* extract for ability to stabilize lysosomal membranes are presented in Table I. They have been corrected for any direct inhibition of the enzyme. Acid phosphatase was a poor indicator of lysosomal membrane condition, since the powerful stabilizing agent phenylbutazone, which was used as a positive control, inhibited enzyme release by only 7%. However, phenylbutazone (0.309 mg/ml) and the extract at a concentration of either 3.087 or 0.309 mg/ml offered significant inhibition of release of β-glucuronidase. At the lower extract concentration of 0.031 mg/ml, there was no protection.

Since some compounds have been shown to be anti-inflammatory because of their irritant properties (8), it was important to determine if the compound might be acting as an anti-inflammatory agent by virtue of its acidity; it normally was injected at pH 4.5. When the material was buffered at pH 7.4 and anti-inflammatory activity was assessed (Table II) using the carrageenan-induced rat paw edema model, there was only 42% inhibition of inflammation. This value is somewhat lower than the expected 88% (1), indicating that the protection afforded by the *Eisenia* extract is due in part to a nonspecific counterirritancy.

It appears that there are two mechanisms for the anti-inflammatory activity of the complex polymer isolated from *E. bicyclis* (Kjellman) Setchell. One is its ability to stabilize the membranes of lysosomes and inhibit the release of the destructive lysosomal enzymes, and the other is a counterirritancy effect.

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Synthesis and Antimicrobial Activity of *N*-Substituted *N'*-Cyano-*S*-(triorganostannyl)isothioureas

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Abstract □ Six *N*-substituted *N'*-cyano-*S*-(trimethylstannyl)isothioureas were synthesized by the reaction of (trimethylstannyl)cyanamide with various organic isothiocyanates. The IR spectrum of each compound was obtained over the 4000–30-cm^{-1} range, and some bands were assigned. The six new compounds and five previously synthesized *N*-substituted *N'*-cyano-*S*-(triphenylstannyl)isothioureas were tested for and were found to exhibit antifungal activity. *N*-Phenyl-*N'*-cyano-*S*-(triphenylstannyl)isothiourea was also investigated for antibacterial activity and was observed to be especially inhibitory toward Gram-positive species. The antimicrobial activity of two compounds was compared to that of the oxygen analogs of these compounds.

Keyphrases □ *N'*-Cyano-*S*-(triorganostannyl)isothioureas, *N*-substituted—synthesis, IR spectra, antimicrobial activity □ Antifungal activity—synthesis of six and evaluation of 11 *N*-substituted *N'*-cyano-*S*-(triorganostannyl)isothioureas □ Antibacterial activity—evaluation of *N*-phenyl-*N'*-cyano-*S*-(triphenylstannyl)isothiourea

Recently, the synthesis of 13 *N*-substituted *N'*-cyano-*O*-(trimethylstannyl)isoureas (I*a*) and 12 *N*-substituted *N'*-cyano-*O*-(triphenylstannyl)isoureas (I*b*) was described (1). Six of these compounds were tested for and were found to exhibit antifungal activity; one (I*b*, R' = C_6H_5) was also investigated for an-

Table II—Effect of Buffering *Eisenia* Extract on Carrageenan-Induced Rat Paw Edema

Treatment[a]	Mean Edema, $mm^3 \pm SE$ (6)[b]
Saline	1174 ± 99
Buffered[c] *Eisenia* extract	678 ± 176[d]

[a] Administered intraperitoneally 1 hr before carrageenan. [b] Number of animals. [c] Buffered at pH 7.4 with 0.2 *M* KH_2PO_4–Na_2PO_4. [d] $p < 0.05$.

multiple-range finding test. Comparison of the anti-inflammatory activity data utilized the Student two-tailed *t* test (7).

RESULTS AND DISCUSSION

The results of testing the activity of the *Eisenia* extract for ability to stabilize lysosomal membranes are presented in Table I. They have been corrected for any direct inhibition of the enzyme. Acid phosphatase was a poor indicator of lysosomal membrane condition, since the powerful stabilizing agent phenylbutazone, which was used as a positive control, inhibited enzyme release by only 7%. However, phenylbutazone (0.309 mg/ml) and the extract at a concentration of either 3.087 or 0.309 mg/ml offered significant inhibition of release of β-glucuronidase. At the lower extract concentration of 0.031 mg/ml, there was no protection.

Since some compounds have been shown to be anti-inflammatory because of their irritant properties (8), it was important to determine if the compound might be acting as an anti-inflammatory agent by virtue of its acidity; it normally was injected at pH 4.5. When the material was buffered at pH 7.4 and anti-inflammatory activity was assessed (Table II) using the carrageenan-induced rat paw edema model, there was only 42% inhibition of inflammation. This value is somewhat lower than the expected 88% (1), indicating that the protection afforded by the *Eisenia* extract is due in part to a nonspecific counterirritancy.

It appears that there are two mechanisms for the anti-inflammatory activity of the complex polymer isolated from *E. bicyclis* (Kjellman) Setchell. One is its ability to stabilize the membranes of lysosomes and inhibit the release of the destructive lysosomal enzymes, and the other is a counterirritancy effect.

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Synthesis and Antimicrobial Activity of *N*-Substituted *N'*-Cyano-*S*-(triorganostannyl)isothioureas

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Abstract □ Six *N*-substituted *N'*-cyano-*S*-(trimethylstannyl)isothioureas were synthesized by the reaction of (trimethylstannyl)cyanamide with various organic isothiocyanates. The IR spectrum of each compound was obtained over the 4000–30-cm^{-1} range, and some bands were assigned. The six new compounds and five previously synthesized *N*-substituted *N'*-cyano-*S*-(triphenylstannyl)isothioureas were tested for and were found to exhibit antifungal activity. *N*-Phenyl-*N'*-cyano-*S*-(triphenylstannyl)isothiourea was also investigated for antibacterial activity and was observed to be especially inhibitory toward Gram-positive species. The antimicrobial activity of two compounds was compared to that of the oxygen analogs of these compounds.

Keyphrases □ *N'*-Cyano-*S*-(triorganostannyl)isothioureas, *N*-substituted—synthesis, IR spectra, antimicrobial activity □ Antifungal activity—synthesis of six and evaluation of 11 *N*-substituted *N'*-cyano-*S*-(triorganostannyl)isothioureas □ Antibacterial activity—evaluation of *N*-phenyl-*N'*-cyano-*S*-(triphenylstannyl)isothiourea

Recently, the synthesis of 13 *N*-substituted *N'*-cyano-*O*-(trimethylstannyl)isoureas (I*a*) and 12 *N*-substituted *N'*-cyano-*O*-(triphenylstannyl)isoureas (I*b*) was described (1). Six of these compounds were tested for and were found to exhibit antifungal activity; one (I*b*, R' = C_6H_5) was also investigated for an-

Table I—*N*-Substituted *N'*-Cyano-*S*-(trimethylstannyl)isothioureas

$RN{=}CSSn(CH_3)_3$
|
NHCN

Compound	R	Yield, %[a]	Melting Point[b]	Formula	Analysis, % Calc.	Found
1	CH_3	76	127–128°[c]	$C_6H_{13}N_3SSn$	C 25.93	26.01
					H 4.71	4.64
					N 15.12	15.18
					S 11.54	11.27
					Sn 42.70	42.83
2	cyclo-C_6H_{11}	66	149–150°[d]	$C_{11}H_{14}N_3SSn$	C 38.18	38.46
					H 6.12	6.09
					N 12.14	12.30
					S 9.27	9.52
					Sn 34.30	34.69
3	$C_6H_5CH_2$	76	106–107°[c]	$C_{12}H_{17}N_3SSn$	C 40.71	40.99
					H 4.84	4.84
					N 11.86	12.06
					S 9.05	9.01
					Sn 33.52	33.45
4	C_6H_5	61	125–126°[d]	$C_{11}H_{15}N_3SSn$	C 38.85	38.89
					H 4.45	4.21
					N 12.36	12.46
					S 9.43	9.38
					Sn 34.90	34.62
5	*p*-FC_6H_4	65	129–130°[d]	$C_{11}H_{14}FN_3SSn$	C 36.91	37.13
					H 3.94	4.08
					N 11.74	11.92
					S 8.96	8.78
					Sn 33.15	32.89
6	*p*-$O_2NC_6H_4$	68	140–141°[c]	$C_{11}H_{14}N_4O_2SSn$	C 34.31	34.20
					H 3.67	3.75
					N 14.55	14.41
					S 8.33	8.12
					Sn 30.82	30.68

[a] Based on material that melts within 5° of the analytical sample. [b] Analytical sample. [c] Recrystallized from ether. [d] Recrystallized from chloroform–*n*-pentane.

Table II—IR Spectra of *N*-Substituted *N'*-Cyano-*S*-(trimethylstannyl)isothioureas[a]

Compound	NH	C≡N	C=N	SnS	$Sn(CH_3)_3$ ν_{as}	ν_s
1	3322 (m)	2198 (s)	1515 (s)	390 (s)	541 (s)	472 (m)
2	3300 (m)	2179 (s)	1515 (s)	360 (m)[b]	541 (s)	481 (w)
3[c]	3289 (m)	2183 (s)	1536 (s)[d]	—[e]	541 (s)	513 (s)
4[f]	3236 (m)	2174 (s)	1520 (s)[d]	370 (m)[g]	541 (s)	488 (m)
5[h]	3236 (m)	2183 (s)	1517 (s)[d]	328 (s)[i]	535 (s)	500 (s)
6[j]	3390 (m)	2193 (s)	1538 (s)[d]	335 (m)[k]	541 (s)	490 (m)

[a] The SnS values were obtained using CsI pellets; the other values were obtained using KBr pellets. Values are expressed in centimeters^{-1}; s = strong, m = medium, and w = weak. [b] A band was present at 337 (s); in mineral oil, bands were present at 360 (w) and 335 (s). [c] A band was present at 556 (w). [d] This assignment is uncertain due to the presence of aromatic C=C bands in this region. [e] The only band observed in the 400–300-cm^{-1} region was at 306 (m) (303 m in mineral oil). [f] A band was present at 565 (w). [g] A band was present at 330 (w). [h] A band was present at 562 (w). [i] At 330 (s) in mineral oil. [j] A band was present at 553 (m), [k] A band was present at 315 (m). The same bands were present in mineral oil.

tibacterial activity and was found to be inhibitory toward Gram-positive species.

The purpose of the present study was to evaluate the antimicrobial activity of some *N*-substituted *N'*-cyano-*S*-(triorganostannyl)isothioureas (II*a* and II*b*), which are the sulfur analogs of I*a* and I*b*, respectively.

$R'N{=}COSnR_3$
|
NHCN

I*a*: R = CH_3
I*b*: R = C_6H_5

$R'N{=}CSSnR_3$
|
NHCN

II*a*: R = CH_3
II*b*: R = C_6H_5

EXPERIMENTAL[1]

***N*-Benzyl-*N'*-cyano-*S*-(trimethylstannyl)isothiourea (Compound 3)**—A mixture of (trimethylstannyl)cyanamide (2) (2.048 g, 0.01 mole), benzyl isothiocyanate (1.492 g, 0.01 mole), and ether (25 ml) was refluxed for 72 hr and filtered to give 2.7 g (76%) of Compound 3, mp 104–105°. Two recrystallizations from ether gave the analytical sample, mp 106–107°.

The other compounds in Table I were prepared in a similar manner.

Biological Methods—The organotin compounds were individually dissolved in tetrahydrofuran except for Compounds 7–10, which were solubilized in chloroform. The preparation of sterile

[1] Melting points were determined with a Mel-Temp capillary melting-point apparatus and are uncorrected. IR data were obtained using KBr pellets with a Beckman IR 8 spectrophotometer. The far IR data were obtained with a Perkin-Elmer model FIS-3 IR spectrophotometer (CsI pellets or mineral oil) and with a Perkin-Elmer model 21 double-beam IR spectrophotometer fitted with a cesium bromide prism and purged with nitrogen (KBr pellets). Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y.

Table III—Antifungal Activity of *N*-Substituted *N'*-Cyano-*S*-(triorganostannyl)isothiourea Compounds

RN=C(SSnR'$_3$)–NHCN

Compound	R	R'	*Aspergillus niger* (ATCC 12845)			*Chaetomium globosum* (ATCC 6205)			*Cladosporium carpophilum* (ATCC 12117)			*Fusarium moniliforme* (ATCC 10052)			*Myrothecium verrucaria* (ATCC 9095)			*Penicillium notatum* (ATCC 9179)			*Rhizopus stolonifer* (ATCC 10404)			*Saccharomyces cerevisiae* (ATCC 9896)			*Trichoderma viride* (ATCC 8678)			*Trichophyton mentagrophytes* (ATCC 9129)		
			1[a]	10	100	1	10	100	1	10	100	1	10	100	1	10	100	1	10	100	1	10	100	1	10	100	1	10	100	1	10	100
1	CH_3	CH_3	–	–	2+	–	+	2+	–	2+	2+	–	+	+	–	+	2+	–	+	2+	–	–	–	–	+	+	–	–	–	2+	+	+	2+
2	cyclo-C_6H_{11}	CH_3	–	–	+	–	+	+	–	–	2+	–	+	+	–	+	2+	–	+	2+	–	–	–	–	+	+	–	–	–	2+	+	+	2+
3	$C_6H_5CH_2$	CH_3	–	+	2+	–	–	+	–	2+	2+	–	+	+	–	+	2+	–	+	2+	–	–	–	–	+	+	–	–	–	+	+	+	+
4	C_6H_5	CH_3	–	+	+	–	+	+	–	2+	2+	–	+	+	–	+	2+	–	+	+	–	–	–	–	+	+	–	+	+	+	+	+	2+
5	p-FC_6H_4	CH_3	–	–	+	–	–	+	–	2+	2+	–	+	+	–	+	+	–	+	+	–	–	–	–	+	+	–	–	–	+	+	+	2+
6	p-$O_2NC_6H_4$	CH_3	–	+	+	–	–	2+	–	+	2+	–	–	+	–	+	2+	–	+	+	–	–	–	–	+	+	–	–	–	+	+	+	2+
7	C_2H_5	C_6H_5	–	+	+	–	+	+	–	+	+	–	–	+	–	+	+	–	+	+	–	+	+	–	+	+	–	–	–	+	+	+	+
8	$C_2H_5CH_2$	C_6H_5	–	–	+	–	–	+	–	–	+	–	–	+	–	–	+	–	+	+	–	+	+	–	+	+	–	–	–	+	+	+	+
9	C_6H_5	C_6H_5	–	+	2+	–	+	+	–	–	–	–	+	+	–	+	+	–	+	+	2+	+	+	–	+	+	–	–	–	+	+	+	+
10	p-$C_2H_5OC_6H_4$	C_6H_5	–	+	+	–	–	+	–	–	–	–	–	+	–	+	+	–	+	+	+	–	+	–	+	+	–	–	–	+	+	+	+
11	p-$O_2NC_6H_4$	C_6H_5	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+	–	+	2+	+	+	+	–	+	+	–	+	+	+	+	+	2+

[a] Indicates concentration of compounds employed in micrograms per milliliter; – indicates no inhibition of growth, + indicates partial inhibition of growth, and 2+ indicates complete inhibition of growth.

Table IV—Antibacterial Activity of *N*-Phenyl-*N'*-cyano-*S*-(triphenylstannyl)isothiourea (Compound 9)

Organism[a]	Concentration, μg/ml		
	1	10	100
Bacillus subtilis	2+[b]	2+	2+
Escherichia coli	–	–	+
Micrococcus agilis	2+	2+	2+
Staphylococcus aureus	2+	2+	2+

[a] Obtained from the culture collection of the Department of Biology, St. John's University. [b] – indicates no inhibition of growth, + indicates partial inhibition of growth, and 2+ indicates complete inhibition of growth.

solutions of the organotin compounds, the fungi employed, the antimicrobial testing procedures, and the determination of growth inhibition were reported previously (1).

One organotin compound, *N*-phenyl-*N'*-cyano-*S*-(triphenylstannyl)isothiourea (Compound 9), was also investigated for antibacterial activity according to the procedure reported earlier (1).

RESULTS AND DISCUSSION

Synthesis—The five *N*-substituted *N'*-cyano-*S*-(triphenylstannyl)isothioureas (II*b*) used in this study were described previously (3). The six *N*-substituted *N'*-cyano-*S*-(trimethylstannyl)-isothioureas (I*b*) are new compounds and were prepared by allowing (trimethylstannyl)cyanamide to react separately with six different organic isothiocyanates (1:1 mole ratio) in refluxing ether (Table I). The IR spectra of these compounds are summarized in Table II. The IR spectra contained both the ν_{as}(SnC) band and the ν_S(SnC) band, indicating that the trimethyltin group may be nonplanar in these compounds (4).

Biological Results—The 11 *N*-substituted *N'*-cyano-*S*-(triorganostannyl)isothiourea compounds were investigated for inhibition of growth of 10 fungal species. One compound was also investigated for antibacterial activity. Table III shows the antifungal activity of the compounds tested. The data presented indicate that the trimethyltin compounds (Compounds 1–6) were significantly more active than the triphenyltin compounds (Compounds 7–11). The incidence of total inhibition of fungal growth, for example, was six times greater with the trimethyltin compounds as compared to the triphenyltin compounds. In addition, the latter compounds did not exhibit total inhibition at a level of 10 μg/ml, whereas the trimethyltin compounds did in four instances.

Of the 11 compounds tested, the most effective antifungal activity, as indicated by total inhibition of growth, was exhibited by *N*-methyl-*N'*-cyano-*S*-(trimethylstannyl)isothiourea (Compound 1). From the data compiled, it was possible to compare the growth-inhibiting properties of specific trimethyltin compounds with their triphenyltin analogs. Thus, for example, *N*-benzyl-*N'*-cyano-*S*-(trimethylstannyl)isothiourea (Compound 3) completely inhibited four of 10 fungi, whereas the triphenyltin analog (Compound 8) displayed only partial inhibition of growth. The latter compound, however, did possess activity against *Rhizopus stolonifer*, which was lacking in the trimethyltin derivative. Similarly, *N*-phenyl-*N'*-cyano-*S*-(trimethylstannyl)isothiourea (Compound 4) displayed greater overall antifungal activity than its triphenyltin counterpart (Compound 9).

The superior antifungal activity of the trimethyltin compounds was even more evident when *N*-*p*-nitrophenyl-*N'*-cyano-*S*-(trimethylstannyl)isothiourea (Compound 6) was compared with its triphenyltin analog (Compound 11). The former compound completely inhibited five of the 10 fungi whereas the latter compound displayed total inhibition against only two of the fungi. Of interest was the fact that the trimethyltin compound partially inhibited *Saccharomyces cerevisiae*, an activity shared by only one other compound, Compound 1.

Further comparisons were possible between two of the organotin compounds reported in the present investigation and their oxygen analogs, the antimicrobial activities of which were described previously (1). For example, Compound 4 completely inhibited the growth of three of the 10 fungi, whereas *N*-phenyl-*N'*-cyano-*O*-(trimethylstannyl)isourea (1) totally inhibited only one fungus.

Secondly, *N*-phenyl-*N'*-cyano-*S*-(triphenylstannyl)isothiourea (Compound 9) and its oxygen analog (1) both exhibited total inhibition of two of the 10 fungi tested. The former compound completely inhibited *Aspergillus niger* and *R. stolonifer* at a concentration of 100 μg/ml. The oxygen analog, on the other hand, was most active against *Myrothecium verrucaria* and *S. cerevisiae* at a similar concentration. The latter activity is noteworthy in that none of the 11 compounds reported in the present investigation completely inhibited *S. cerevisiae*.

The antibacterial activity of Compound 9 is shown in Table IV. This compound was remarkably active against the three Gram-positive species, displaying complete inhibition at a concentration of 1 μg/ml. In addition, partial inhibition of the Gram-negative organism, *Escherichia coli*, was evident. This latter activity contrasts with that of the oxygen analog, *N*-phenyl-*N'*-cyano-*O*-(triphenylstannyl)isourea, which was previously reported to be ineffective against *E. coli* (1). In addition, among the four Gram-positive species tested, total inhibition by the oxygen analog was accomplished only against *Micrococcus agilis* (1).

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New Synthesis of *(RS)*-Carnitine Chloride

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Abstract □ A four-step synthesis of *(RS)*-carnitine chloride was developed using extremely mild reaction conditions and versatile intermediates. Crotyl chloride was converted to *tert*-butyl 3-butenoate using *tert*-butyl alcohol and triethylamine in ether. Treatment of *tert*-butyl 3-butenoate with *m*-chloroperbenzoic acid in chloroform afforded *tert*-butyl 3,4-epoxybutyrate. Reaction of this compound with trimethylamine hydrochloride in methanol, followed by mild acid hydrolysis of the *tert*-butyl ester, afforded *(RS)*-carnitine chloride.

Keyphrases □ *(RS)*-Carnitine chloride—four-step synthesis under mild reaction conditions from crotyl chloride □ Crotyl chloride—starting material in four-step synthesis of *(RS)*-carnitine chloride

The importance of *(R)*-(−)-carnitine chloride in the transport of fatty acids across membranes was demonstrated previously (1). Specifically, carnitine chloride catalyzes the oxidation of long chain fatty acids by participating in the enzyme-mediated transport of activated fatty acids from the cytoplasm to the β-oxidation sites within the mitochondria (2, 3). The objectives of this investigation were to develop a convenient and versatile synthetic route that could be readily adapted to the synthesis of not only carnitine chloride itself but also to carnitine analogs. The availability of carnitine analogs could provide valuable biochemical tools for investigating the structural requirements of carnitine chloride as a catalyst of fatty acid oxidation (4).

DISCUSSION

In 1966, Ozeki and Kusaka (5) described the synthesis of numerous 3-butenoate esters from crotyl chloride (I) and the appropriate alcohol in the presence of a variety of tertiary amines. It appeared that proper functionalization of the olefinic bond in one of the 3-butenoate esters would provide a convenient and relatively straightforward synthesis of *(RS)*-carnitine chloride (VI) and, thus, carnitine analogs. The *tert*-butyl ester was selected because of the extremely mild reaction conditions required for conversion to the free carboxylic acid (dilute hydrochloric acid at room temperature). Triethylamine was selected as the base because it provided the highest yield of the desired 3-butenoate ester, with only small amounts (3.1%) of the unwanted *cis*- and *trans*-2-butenoate esters (determined by GC).

Since the previous report (5) provided few experimental details and the reaction was not discussed from the standpoint of providing an extremely versatile synthetic intermediate, it was necessary to develop the appropriate reactions conditions to afford the desired *tert*-butyl 3-butenoate (II) in a yield that would be useful for completing the synthesis of VI. The small amount of the *cis*- and *trans*-2-butenoate esters produced during the preparation of II were of little concern because the following step in the synthetic sequence involved epoxidation of the olefinic bond. The mildest conditions possible were used that would eliminate the possibility of isomerization of the 3-butenoate ester to a mixture of the *cis*- and *trans*-2-butenoate esters during exposure to the *m*-chlorobenzoic acid.

It is also well known that α,β-unsaturated carbonyl compounds do not normally undergo epoxidation when subjected to the same reaction conditions as isolated olefinic bonds. The desired epoxy ester (III) was initially converted to the iodohydrin (IV) using the method of Cornforth (6). Compound IV was subsequently treated with trimethylamine in methanol to afford *tert*-butyl 4-dimethylamino-3-hydroxybutyrate methiodide (V*a*) which, when subjected to ion-exchange chromatography, afforded V*b*. The *tert*-butyl protecting group was then removed by the mild reaction conditions described to yield VI.

It was later found, after much experimental effort, that III could be directly converted into the ester V*b*, using trimethylamine hydrochloride in methanol, in a yield high enough to make the reaction synthetically feasible, thus eliminating one step in the synthetic sequence as well as the ion-exchange chromatography.

Secondly, *N*-phenyl-*N'*-cyano-*S*-(triphenylstannyl)isothiourea (Compound 9) and its oxygen analog (1) both exhibited total inhibition of two of the 10 fungi tested. The former compound completely inhibited *Aspergillus niger* and *R. stolonifer* at a concentration of 100 μg/ml. The oxygen analog, on the other hand, was most active against *Myrothecium verrucaria* and *S. cerevisiae* at a similar concentration. The latter activity is noteworthy in that none of the 11 compounds reported in the present investigation completely inhibited *S. cerevisiae*.

The antibacterial activity of Compound 9 is shown in Table IV. This compound was remarkably active against the three Gram-positive species, displaying complete inhibition at a concentration of 1 μg/ml. In addition, partial inhibition of the Gram-negative organism, *Escherichia coli*, was evident. This latter activity contrasts with that of the oxygen analog, *N*-phenyl-*N'*-cyano-*O*-(triphenylstannyl)isourea, which was previously reported to be ineffective against *E. coli* (1). In addition, among the four Gram-positive species tested, total inhibition by the oxygen analog was accomplished only against *Micrococcus agilis* (1).

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New Synthesis of *(RS)*-Carnitine Chloride

SHARON G. BOOTS and MARVIN R. BOOTS ˣ

Abstract □ A four-step synthesis of *(RS)*-carnitine chloride was developed using extremely mild reaction conditions and versatile intermediates. Crotyl chloride was converted to *tert*-butyl 3-butenoate using *tert*-butyl alcohol and triethylamine in ether. Treatment of *tert*-butyl 3-butenoate with *m*-chloroperbenzoic acid in chloroform afforded *tert*-butyl 3,4-epoxybutyrate. Reaction of this compound with trimethylamine hydrochloride in methanol, followed by mild acid hydrolysis of the *tert*-butyl ester, afforded *(RS)*-carnitine chloride.

Keyphrases □ *(RS)*-Carnitine chloride—four-step synthesis under mild reaction conditions from crotyl chloride □ Crotyl chloride—starting material in four-step synthesis of *(RS)*-carnitine chloride

The importance of *(R)*-(−)-carnitine chloride in the transport of fatty acids across membranes was demonstrated previously (1). Specifically, carnitine chloride catalyzes the oxidation of long chain fatty acids by participating in the enzyme-mediated transport of activated fatty acids from the cytoplasm to the β-oxidation sites within the mitochondria (2, 3). The objectives of this investigation were to develop a convenient and versatile synthetic route that could be readily adapted to the synthesis of not only carnitine chloride itself but also to carnitine analogs. The availability of carnitine analogs could provide valuable biochemical tools for investigating the structural requirements of carnitine chloride as a catalyst of fatty acid oxidation (4).

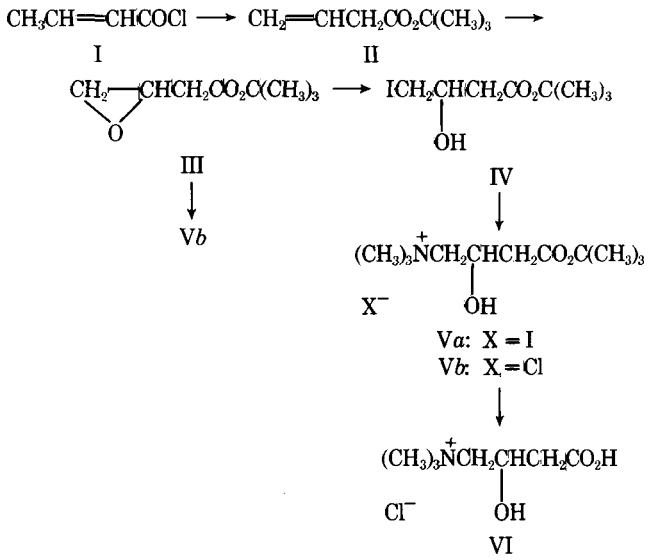
DISCUSSION

In 1966, Ozeki and Kusaka (5) described the synthesis of numerous 3-butenoate esters from crotyl chloride (I) and the appropriate alcohol in the presence of a variety of tertiary amines. It appeared that proper functionalization of the olefinic bond in one of the 3-butenoate esters would provide a convenient and relatively straightforward synthesis of *(RS)*-carnitine chloride (VI) and, thus, carnitine analogs. The *tert*-butyl ester was selected because of the extremely mild reaction conditions required for conversion to the free carboxylic acid (dilute hydrochloric acid at room temperature). Triethylamine was selected as the base because it provided the highest yield of the desired 3-butenoate ester, with only small amounts (3.1%) of the unwanted *cis*- and *trans*-2-butenoate esters (determined by GC).

Since the previous report (5) provided few experimental details and the reaction was not discussed from the standpoint of providing an extremely versatile synthetic intermediate, it was necessary to develop the appropriate reactions conditions to afford the desired *tert*-butyl 3-butenoate (II) in a yield that would be useful for completing the synthesis of VI. The small amount of the *cis*- and *trans*-2-butenoate esters produced during the preparation of II were of little concern because the following step in the synthetic sequence involved epoxidation of the olefinic bond. The mildest conditions possible were used that would eliminate the possibility of isomerization of the 3-butenoate ester to a mixture of the *cis*- and *trans*-2-butenoate esters during exposure to the *m*-chlorobenzoic acid.

It is also well known that α,β-unsaturated carbonyl compounds do not normally undergo epoxidation when subjected to the same reaction conditions as isolated olefinic bonds. The desired epoxy ester (III) was initially converted to the iodohydrin (IV) using the method of Cornforth (6). Compound IV was subsequently treated with trimethylamine in methanol to afford *tert*-butyl 4-dimethylamino-3-hydroxybutyrate methiodide (V*a*) which, when subjected to ion-exchange chromatography, afforded V*b*. The *tert*-butyl protecting group was then removed by the mild reaction conditions described to yield VI.

It was later found, after much experimental effort, that III could be directly converted into the ester V*b*, using trimethylamine hydrochloride in methanol, in a yield high enough to make the reaction synthetically feasible, thus eliminating one step in the synthetic sequence as well as the ion-exchange chromatography.



Scheme I

A four-step synthesis of VI is described (Scheme I). It uses extremely mild reaction conditions and provides versatile intermediates, which can readily be used for the preparation of a variety of carnitine analogs. No other published synthesis of VI (7) that the authors are aware of utilizes such mild reaction conditions and such versatile intermediates[1].

EXPERIMENTAL[2]

***tert*-Butyl 3-Butenoate (II)**—The procedure of Ozeki and Kusaka (5) was modified. To a solution of 28 ml (0.3 mole) of *tert*-butyl alcohol and 28 ml (0.2 mole) of triethylamine in 75 ml of ether was added dropwise with stirring, while maintaining the temperature at 25–30°, a solution of 18.8 ml (0.2 mole) of freshly distilled crotyl chloride in 10 ml of ether. After the addition was completed, the mixture was allowed to stir at 25° for 1 hr.

The mixture was then filtered, and the solid was washed thoroughly with ether. The filtrate was washed with a cold 5% sodium bicarbonate solution, water, a cold 5% hydrochloric acid solution, water, a cold 5% sodium bicarbonate solution, water, and then a saturated sodium chloride solution. The organic phase was dried over anhydrous sodium sulfate, and the solvent was removed at atmospheric pressure with a minimal amount of heating.

The residue was distilled to give 16.9 g (60%) of a colorless liquid, bp 52–54° (20–24 mm); IR (liquid film): 1730 (ester C=O) and 1642 (C=C) cm^{-1}; NMR ($CDCl_3$): δ 4.9–6.2 (m, 3H, CH_2=CH—), 2.9–3.0 (m, 2H, —CH_2—), and 1.45 [s, 9H, —$C(CH_3)_3$] ppm.

Anal.—Calc. for $C_8H_{14}O_2$: C, 67.6; H, 9.9. Found: C, 67.8; H, 9.9.

***tert*-Butyl 3,4-Epoxybutyrate (III)**—To an ice-cold solution of 8.4 g (0.06 mole) of II in 40 ml of chloroform was added dropwise, with cooling and stirring, a solution of 18.2 g (0.09 mole) of 85% *m*-chloroperbenzoic acid in 250 ml of chloroform. The solution was allowed to stand at 4° for 4 days. The resulting precipitate was removed by filtration, and the filtrate was stirred with 300 ml of a freshly prepared aqueous 10% sodium sulfite solution for 30 min (negative starch-iodide test). The organic phase was washed with a 5% sodium bicarbonate solution, water, and a saturated sodium chloride solution and then dried over anhydrous sodium sulfate.

The solvent was removed under reduced pressure to afford a yellow liquid, which was distilled to give 6.3 g (66%) of III as a colorless liquid, bp 40–42° (0.3 mm); IR (liquid film): 1728 (ester C=O) cm^{-1}; NMR ($CDCl_3$): δ 2.38–3.38 (m, 5H, CH_2—$CHCH_2$—) and 1.47 [s, 9H, —$C(CH_3)_3$] ppm.

Anal.—Calc. for $C_8H_{14}O_3$: C, 60.8; H, 8.9. Found: C, 60.5; H, 8.8.

***tert*-Butyl 4-Iodo-3-hydroxybutyrate (IV)**—The procedure of Cornforth *et al.* (6) was used. A mixture of 0.53 g (3.5 mmoles) of sodium iodide, 0.24 g (2.5 mmoles) of sodium acetate, 0.45 ml of acetic acid, and 2.5 ml of propionic acid was cooled to −30°. A 0.375-g (2.5-mmole) sample of III was added to the cooled solution. The mixture was stirred at from −30 to −20° for 30 min and then at 0° for 30 min. The mixture was poured into ether and an ice-cold 5% sodium bicarbonate solution. The organic phase was washed with water, a 10% sodium thiosulfate solution, water, and a saturated sodium chloride solution and then dried over anhydrous sodium sulfate.

The solvent was removed under reduced pressure to afford 0.616 g (88%) of a pale-yellow liquid, which was used immediately without further purification due to its apparent instability; IR (liquid film): 3420 (OH) and 1725 (ester C=O) cm^{-1}; NMR ($CDCl_3$): δ 2.5–4.2 [m, 6H, —$CH_2CH(OH)CH_2$—] and 1.46 [s, 9H, —$C(CH_3)_3$] ppm.

***tert*-Butyl 4-Dimethylamino-3-hydroxybutyrate Methiodide (V*a*)**—To a solution of 0.616 g (2.2 mmoles) of IV in 10 ml of methanol was added 5 ml (16 mmoles) of a 25% solution of trimethylamine in methanol. The mixture was allowed to stand at 25° for 19 hr. The solvent was then removed under reduced pressure to afford a white solid. The solid was slurried with anhydrous ether, filtered, and then dried under high vacuum at 25° to yield 0.711 g (95%) of V*a*, mp 138–139°.

Recrystallization from acetone–ether afforded an analytical specimen as white prisms, mp 146–148°; IR (mineral oil): 1715 (ester C=O) cm^{-1}; NMR (D_2O): δ 4.30–4.80 (m, 1H, —CH—), 3.50 [d, 2H, $(CH_3)_3$—$^+$NCH_2—], 3.26 [s, 9H, $(CH_3)_3{}^+$N—], 2.60 [d, 2H, —$CH_2CO_2C(CH_3)_3$], and 1.47 [s, 9H, —$C(CH_3)_3$] ppm.

Anal.—Calc. for $C_{11}H_{24}INO_3$: C, 38.3; H, 7.0; N, 4.1. Found: C, 38.0; H, 6.8; N, 3.9.

***tert*-Butyl 4-Dimethylamino-3-hydroxybutyrate Methochloride (V*b*)**—A 0.500-g (1.45-mmole) sample of V*a*, dissolved in 5 ml of methanol, was passed through a column (2-cm diameter) containing 8.1 g (4.3 mEq/g, dry basis) of resin[3] (chloride form, 50–100 mesh), which had previously been washed with 100 ml of water and then with 100 ml of methanol. Elution with 50 ml of methanol afforded 0.350 g (95%) of V*b* as a white solid. All attempts to purify this material by recrystallization failed because the isolated crystalline form proved to be extremely hygroscopic. (See later experimental details for analytical and spectral data for this compound.)

Preparation of V*b* Directly from III—A mixture of 0.656 g (4.2 mmoles) of III, 0.320 g (3.30 mmoles) of trimethylamine hydrochloride, and 2.0 ml of anhydrous methanol was allowed to stand at 25° for 5 days in a glass-stoppered flask. The methanol was removed under reduced pressure, and the residue was added to ether and water. The organic phase was washed an additional time with water, and the aqueous extracts were combined. The water was removed under high vacuum at approximately 30–40°, and then the traces of water remaining were removed by azeotropic distillation with four 10-ml portions of *tert*-butyl alcohol under reduced pressure. The resulting white solid was dried for 19 hr at 25° under high vacuum; the yield was 0.724 g (73%), mp 203–204°.

Recrystallization from methanol–acetone afforded an analytical specimen of V*b* as white prisms, mp 210–211°; IR (mineral oil): 1715 (ester C=O) cm^{-1}; NMR (D_2O): δ 4.30–4.60 (m, 1H, —CH—), 3.50 [d, 2H, $(CH_3)_3{}^+$NCH_2—], 3.26 [s, 9H, $(CH_3)_3{}^+$N—], 2.60 [d, 2H, —$CH_2CO_2C(CH_3)_3$], and 1.47 [s, 9H, —$C(CH_3)_3$] ppm.

Anal.—Calc. for $C_{11}H_{24}ClNO_3$: C, 52.1; H, 9.5; N, 5.5. Found: C, 52.1; H, 9.5; N, 5.5.

***(RS)*-Carnitine Chloride (VI)**—A solution of 0.114 g (0.45 mmole) of V*b*, 8 ml of water, and 0.4 ml of concentrated hydrochloric acid was stirred at 25° for 17 hr. The water was removed at 30–40° under high vacuum; then four 10-ml portions of *tert*-butyl alcohol were added, as already described, to remove the remaining water azeotropically. The resulting white solid was dried under high vacuum at 25° for 2 days; the yield was 0.0874 g (98%) of white prisms, mp 195–197° [lit. (8) mp 196°]. The IR (mineral oil)

[1] For a discussion of previous methods used for the synthesis of *(RS)*-carnitine chloride, see Ref. 7.

[2] Melting points were determined with a Thomas-Hoover capillary melting-point apparatus and are uncorrected. Elemental analyses were obtained from Galbraith Laboratories, Knoxville, Tenn. IR spectra were determined using a Perkin-Elmer model 237 spectrophotometer. NMR spectra were determined on a Perkin-Elmer model R-24 spectrometer either in deuterochloroform, using tetramethylsilane as the internal reference, or in deuterium oxide, using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the internal reference.

[3] Dowex 1-X2.

and NMR (D_2O) spectra were superimposable with an authentic specimen of *(RS)*-carnitine chloride[4].

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[x] To whom inquiries should be directed.

COMMUNICATIONS

Bioavailability of Digoxin Tablets in Relation to Their Dissolution *In Vitro*

Keyphrases □ Digoxin tablets—dissolution data compared to bioavailability □ Bioavailability of digoxin tablets—relationship to dissolution

To the Editor:

The bioavailability of digoxin tablets has been claimed to be related to their dissolution *in vitro*. Many investigators have correlated the dissolution rate of digoxin tablets to concentration of digoxin in serum or plasma and to values for areas under the serum level–time curves of tablets (1–6). They have concluded that the dissolution rate *in vitro* is adequate for predicting the bioavailability of commercial digoxin tablets. It has been reported recently, however, that despite the different dissolution rates of two different brands of digoxin tablets, the total amount of digoxin absorbed from these tablets was quite similar to that from digoxin elixir USP (7). The present paper reports that the dissolution test according to the USP (8) is not always effective for evaluating the bioavailability of commercial digoxin tablets.

The experiment was carried out with eight healthy volunteer female subjects, ranging in age from 23 to 50 years and in weight from 57 to 68 kg. The subjects had no history of GI, liver, or kidney disease, and none admitted to taking any medication regularly. One digoxin tablet[1] (0.25 mg) or 10 ml of digoxin standard solution[2] (0.025 mg/ml) was given with 100 ml of water to each subject at 9 am for 10 days under laboratory supervision in a crossover manner. Tablets were determined to meet USP specifications as to purity, disintegration time, and content uniformity.

After taking the drug, the subjects were not allowed to eat for 2 hr and were asked to abstain from alcoholic beverages during the experiment. For measuring serum digoxin levels, venous blood samples were taken on the 8th, 9th, and 10th mornings just previous to the next dose of the drug. After the last dose, samples were drawn at intervals of 0.5, 1, 2, 4, 8, and 24 hr.

[1] Digoxin Novum, Star Ltd., Pinnink. 53, SF-33100 Tampere 10, Finland.

[2] Digoxin, 0.00025 g (International Chemical Reference Substance Digoxin, WHO Centre for Chemical Reference Substances, Apotekens Centrallaboratorium, Solna 3, Sweden); propylene glycol, 0.4 g; alcohol, 0.1 g; and sterile water, a sufficient quantity to make 10 ml.

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The experiment was carried out with eight healthy volunteer female subjects, ranging in age from 23 to 50 years and in weight from 57 to 68 kg. The subjects had no history of GI, liver, or kidney disease, and none admitted to taking any medication regularly. One digoxin tablet[1] (0.25 mg) or 10 ml of digoxin standard solution[2] (0.025 mg/ml) was given with 100 ml of water to each subject at 9 am for 10 days under laboratory supervision in a crossover manner. Tablets were determined to meet USP specifications as to purity, disintegration time, and content uniformity.

After taking the drug, the subjects were not allowed to eat for 2 hr and were asked to abstain from alcoholic beverages during the experiment. For measuring serum digoxin levels, venous blood samples were taken on the 8th, 9th, and 10th mornings just previous to the next dose of the drug. After the last dose, samples were drawn at intervals of 0.5, 1, 2, 4, 8, and 24 hr.

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Table I—Percentage of Labeled Digoxin Found in Solution after 60 min, Basal Concentration of Serum Digoxin (Mean of the Last 4 Experimental Days), and Areas under the Serum Level–Time Curves (for 24 hr) on the 10th Experimental Day (Values are Means ± *SE*)

	Percent of Labeled Amount in Solution (n = 12)	Basal Concentration of Serum Digoxin (n = 8)		Areas under the Serum Level–Time Curves (n = 8)	
		ng/ml	Compared with Standard, %	ng/ml × hr	Compared with Standard, %
Digoxin tablets	57.8 ± 1.6	0.59 ± 0.05	95.2 ± 10.6	4.43 ± 0.69	95.0 ± 8.9
Digoxin standard solution	98.4 ± 0.7	0.62 ± 0.04		4.60 ± 0.46	
Probability level	$p < 0.0005$	$p > 0.3$[a]		$p > 0.6$[a]	

[a] Not significant.

The serum samples were analyzed for digoxin radioimmunologically utilizing specific antiserum[3], ^{3}H-digoxin[4], and a liquid scintillation spectrometer[5]. The bound radioactivity separated from free ^{3}H-digoxin during the assay procedure was counted for 10 min/sample. The dissolution of digoxin tablets was performed according to the USP method (8). Twelve tablets were dissolved individually in a cylindrical stainless-steel basket[6], which was dipped into 500 ml of diluted hydrochloric acid (3 in 500) and rotated for 60 min at 120 rpm. After dissolution medium samples were filtered with a filter of 0.45-μm porosity[7], digoxin concentration was measured fluorometrically[8].

The percentage of digoxin dissolved after 60 min from the tablets was compared with the percentage of drug recovered from the dissolution medium containing dissolved digoxin (10 ml of standard solution) equivalent to the labeled amount in the tablets. For this comparison, the Student t test was used. Data from *in vivo* experiments were tested by a t test for paired observations. Areas under the serum level–time curves were determined by a planimetrical technique.

The amount of digoxin dissolved after 60 min from the tablets corresponded to 57.8 ± 1.6% (mean ± *SE*) of the declared amount and varied from 49.0 to 69.5% (Table I). The respective percentage of drug recovered from the dissolution medium containing standard solution (10 ml) was 98.4 ± 0.7% (from 94.0 to 101.5%). Despite the rapid disintegration of each tablet in this medium (1–2 min), four tablets dissolved less than the required 55% of the labeled amount of drug.

The basal digoxin concentration in serum and areas under the serum level–time curves during the last experimental day are shown in Fig. 1 and Table I. During the treatment with tablets, the basal digoxin level was 95.2 ± 10.6% of that caused by the standard solution (mean of the last 4 experimental days), and the area under the serum level–time curve describing digoxin absorption from tablets in the alimentary tract was 95.0 ± 8.9% of that of the standard solution. There was no statistically significant difference between tablets and standard solutions in these parameters.

Previously, Klink *et al.* (7) demonstrated that the bioavailability of digoxin tablets may be excellent despite the poor dissolution *in vitro*. They did not, however, report whether their tablets disintegrated rapidly during the dissolution test or whether digoxin was released slowly from the surface. In the present study, the poor dissolution percentage may be partly explained by the fact that during the test an essential part of the grains of the disintegrated tablets fell through the wall of the rotating basket into a nonagitated part of the apparatus where the release of digoxin from the grains probably was negligible or too slow.

Although the tablets tested did not meet USP specifications for dissolution (8), the basal digoxin concentration in serum and the area describing digoxin absorption during the treatment with tablets did not differ significantly from those caused by the standard solution. These results agree with the concept of Klink *et al.* (7) that a single dissolution test may not show a good correlation between the GI ab-

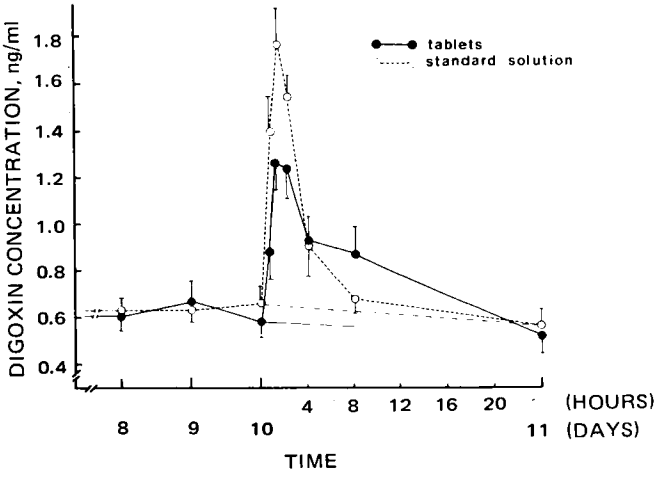

Figure 1—*Basal concentration of serum digoxin during the last 4 experimental days and the serum digoxin levels at each time of collection on the 10th experimental day following 10 consecutive daily administrations of 0.25 mg of digoxin (n = 8; values are means ± SE).*

[3] Laboratory Kvantti, Hallitusk. 28-30, SF-90100 Oulu 10, Finland.
[4] Digoxin 12-^{3}H, New England Nuclear, Boston, MA 02118
[5] Packard Tri-Carb scintillation spectrometer, Packard Instrument Co., Downers Grove, IL 60515
[6] A 40-mesh woven wire cloth formed into a cylinder 3.66 cm high and 2.5 cm in diameter; Hanson Research Corp., Northridge, CA 91324
[7] Sartorius-Membranfilter Ltd., Weender Landstrasse 94-102, 34 Göttingen, Germany.
[8] Aminco-Bowman spectrophotofluorometer, American Instrument Co., Inc., Silver Spring, MD 20910

sorption of digoxin from all commercially available compressed tablets and their dissolution *in vitro*. The results also indicate the poor applicability of the USP (8) dissolution test in predicting the bioavailability of this particular brand of commercial digoxin tablets.

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BOOKS

REVIEWS

Proceedings of the USP Conference on Radiation Sterilization, The United States Pharmacopeia, Inc., Rockville, MD 20852, 224 pp. 16 × 22 cm. Price $10.00.

This volume consists of 15 papers devoted to an area of pharmaceutical and biomedical technology in which the United States has lagged behind several European nations, largely because of strict controls on testing of safety and efficacy imposed by federal agencies. Among the subjects considered in detail are instrumentation and experimental conditions employed in radiation sterilization, chemical dosimetry, the effects of ionizing radiations on microorganisms, and the evaluation of microbiological control systems for the determination of the efficacy of radiation sterilization.

Ionizing radiation offers an advantage over heat in the sterilization of thermally degradable materials. However, the high doses and dose rates required to effect sterilization, especially against radio-resistant bacteria, represent a problem because of the susceptibility of many materials to chemical and structural alterations as a result of their interactions with ionizing radiations. The treatment of bacteria-containing samples with UV light prior to high-energy irradiation provides a solution to this problem, at least in some cases, as bacteria preirradiated with UV light appear to require smaller doses of ionizing radiations to produce the same degree of sterilization observed in untreated bacterial samples. The cobalt-60 γ-ray source which, historically, was the first sterilization source employed on a wide scale is still preferred over other isotopic sources because of its high radiation flux, long-term stability, and relatively low cost and is favored over electronuclear sources such as the linear accelerator because of the high cost of the latter. It is suggested by several contributors to the volume that the cobalt-60 source is expected to maintain its preeminence for the forseeable future. It is also suggested that the search for and evaluation of better dosimeters and microbiological standards will be a major area of endeavor for some time to come. The need for improved microbiological controls is particularly acute because much of the testing of the efficacy of radiation sterilization has been carried out with bacteria which are unrepresentatively sensitive to radiation damage.

"Proceedings of the USP Conference on Radiation Sterilization" is a comprehensive, authoritative, and very readable account of a subject which is probably the major industrial application of ionizing radiations. Although it deals primarily with applications to biological test systems and surgical products, it is worthwhile reading for all pharmaceutical and biological scientists.

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The United States Pharmacopeia, Nineteenth Revision. Prepared and Published by The United States Pharmacopeial Convention, Inc., Distributed by Mack Publishing Co., 20th & Northampton Sts., Easton, PA 18042, 1974. l + 824 pp. 21.5 × 28 cm. Price $25.00.

This latest revision of the *United States Pharmacopeia* (USP XIX) continues its respected tradition by providing legally recognized standards for identity, strength, quality, and purity for nearly 1300 important and well-established drugs and their dosage forms in use in the United States. Additionally, about one-fourth of this volume is devoted to general chapters, reagent specifications, and tables.

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Received January 24, 1975.
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BOOKS

REVIEWS

Proceedings of the USP Conference on Radiation Sterilization, The United States Pharmacopeia, Inc., Rockville, MD 20852, 224 pp. 16 × 22 cm. Price $10.00.

This volume consists of 15 papers devoted to an area of pharmaceutical and biomedical technology in which the United States has lagged behind several European nations, largely because of strict controls on testing of safety and efficacy imposed by federal agencies. Among the subjects considered in detail are instrumentation and experimental conditions employed in radiation sterilization, chemical dosimetry, the effects of ionizing radiations on microorganisms, and the evaluation of microbiological control systems for the determination of the efficacy of radiation sterilization.

Ionizing radiation offers an advantage over heat in the sterilization of thermally degradable materials. However, the high doses and dose rates required to effect sterilization, especially against radio-resistant bacteria, represent a problem because of the susceptibility of many materials to chemical and structural alterations as a result of their interactions with ionizing radiations. The treatment of bacteria-containing samples with UV light prior to high-energy irradiation provides a solution to this problem, at least in some cases, as bacteria preirradiated with UV light appear to require smaller doses of ionizing radiations to produce the same degree of sterilization observed in untreated bacterial samples. The cobalt-60 γ-ray source which, historically, was the first sterilization source employed on a wide scale is still preferred over other isotopic sources because of its high radiation flux, long-term stability, and relatively low cost and is favored over electronuclear sources such as the linear accelerator because of the high cost of the latter. It is suggested by several contributors to the volume that the cobalt-60 source is expected to maintain its preeminence for the forseeable future. It is also suggested that the search for and evaluation of better dosimeters and microbiological standards will be a major area of endeavor for some time to come. The need for improved microbiological controls is particularly acute because much of the testing of the efficacy of radiation sterilization has been carried out with bacteria which are unrepresentatively sensitive to radiation damage.

"Proceedings of the USP Conference on Radiation Sterilization" is a comprehensive, authoritative, and very readable account of a subject which is probably the major industrial application of ionizing radiations. Although it deals primarily with applications to biological test systems and surgical products, it is worthwhile reading for all pharmaceutical and biological scientists.

Reviewed by Stephen G. Schulman
College of Pharmacy
University of Florida
Gainesville, FL 32610

The United States Pharmacopeia, Nineteenth Revision. Prepared and Published by The United States Pharmacopeial Convention, Inc., Distributed by Mack Publishing Co., 20th & Northampton Sts., Easton, PA 18042, 1974. l + 824 pp. 21.5 × 28 cm. Price $25.00.

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Staff Review

Sprowls' American Pharmacy, 7th Edition. Edited by LEWIS W. DITTERT. Lippincott, Philadelphia, PA 19105, 1974. 511 pp. 18 × 26 cm. Price $19.50.

For all those educators familiar with the series of editions of the "American Pharmacy" texts little can be said of the latest high quality revision in the 7th Edition. Although we now see a new Editor-in-Chief in Professor Lewis W. Dittert and a change in names of some of the chapter contributors from the previous 6th Edition, upon complete review, we see a finished product in keeping with the tradition of excellence brought to us by the previous contributors and the late Editors-in-Chief Rufus A. Lyman and Joseph B. Sprowls.

In the first five editions of this series, the attention was focused on compounding of medicinals primarily discussing specifics of the then official preparations. With the 6th Edition the attention was turned to the manufacturing of dosage forms at the industrial level. This appears to be the goal of the present 7th Edition as well. To this end, the goal is reached in a better than adequate format since the theory and practice given in this text are most appropriate to the industrial formulator. In no way should this detract from its use in preparing a dispensing pharmacist for a future role since some exposition is given for compounding on the extemporaneous level as well. Similarly, pharmaceutical heritage of the various dosage forms is given either as an historical overview in the first chapter or as an introduction to the chapters dealing with the particular groups of dosage forms.

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sive tables of currently official solutions classed on method of preparation. A table of important formerly official solutions is also included. Chapter 4 covers waters, syrups, and juices whereas Chapter 5 then expounds on spirits, elixirs, and extracted products.

Chapter 6 illustrates the use of some timely physicochemical elements into the text which is employed in subsequent chapters as it discusses the colloidal state in theory and practice (including rheology) along with elements of surface chemistry. This is expanded upon in Chapter 7 as it focuses on suspensions, emulsions, and lotions. It is in this chapter that micromeritics is inserted rather than in a later chapter covering solids.

The next two chapters discuss semisolids. Chapter 8, for example, includes coverage of ointments, creams, and pastes with an introduction to percutaneous absorption, whereas Chapter 9 deals solely with suppositories, rectal anatomy, and rectal absorption.

Chapter 10 introduces the student to powdered dosage forms. Here large and small scale handling of powders is treated along with the preparation of folded powders and capsules. An extensive chapter on tablets naturally follows in Chapter 11. It is here that greatest emphasis is placed on the industrial procedures and equipment.

The format continues in its progressive orientation to industrial pharmacy by concluding with three highly specialized chapters. That is, Chapter 12 covers gas dispersions (aerosols and sprays) while Chapter 13 deals with radiopharmaceuticals and finally Chapter 14 concludes the text by treating sterile products.

All in all, it is a well-organized and easy to read text in pharmaceutics and although its progression of treatment may not fit an individual syllabus in pharmaceutics, all pertinent points are covered. In particular, it is gratifying to see the extensive use of tables of official products being preceded by general principles. The main drawback is that it has been published four years after the publication of USP and NF.

Reviewed by Bartley J. Sciarrone
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Rutgers—The State University
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1975

July 17–21
American Society for Pharmacology and Experimental Therapeutics, Annual Meeting, University of California, Davis, Calif.

July 22–August 2
American Society of Pharmacognosy, Joint Meeting with Die Gesellschaft für Arzneipflanzenforschung, University of Connecticut, Storrs, Conn. (Contact Ana Rother, School of Pharmacy, University of Connecticut, Storrs, Conn.)

August 11–15
Gordon Research Conference, Medicinal Chemistry, Colby Women's College, New London, N.H. (Contact Dr. Alexander M. Cruickshank, Gordon Research Conferences, University of Rhode Island, Kingston, RI 02881)

August 24–29
American Chemical Society, National Meeting, Chicago, Ill. (Contact A. T. Winstead, American Chemical Society, 1155 16th St., N.W., Washington, DC 20036)

September 1–5
International Congress of Pharmaceutical Sciences, Dublin, Ireland (Contact Congress Secretary, 35th International Congress of Pharmaceutical Sciences, 44 Northumberland Road, Ballsbridge, Dublin 4, Ireland)

September 7–10
AIChE National Meeting, Sheraton Hotel, Boston, Mass. (Contact Kenneth A. Smith, Department of Chemical Engineering, MIT, Cambridge, MA 02139)

September 7–12
British Pharmaceutical Conference, Norwich, England

September 8–13
International Conference of Women Engineers and Scientists, Cracow, Poland (Contact L. I. Pickup, 15284 Oak Dr., Renton, WA 98055)

September 9
Pharmaceutical Processing Symposium, American Institute of Chemical Engineers, Sheraton-Boston Hotel, Boston, Mass. (Contact Al Heiser, Abbott Laboratories, North Chicago, IL 60064)

September 22–24
Symposium on Assay of Drugs and Other Trace Substances in Biological Fluids, University of Surrey, Guildford, U.K. (Contact Dr. E. Reid, Wolfson Bioanalytical Centre, University of Surrey, Guildford GU2 5XH, U.K.)

October 1–5
American College of Apothecaries, Annual Meeting, Four Seasons Sheraton, Toronto, Ontario, Canada

October 6–8
ASTM Committee E-19 on Chromatography, Annual Meeting, Sheraton-Houston, Houston, Tex. (Contact Mr. Jack Stamm, Instrument Products Division, Photo Products Department, Du Pont Co., Wilmington, DE 19898)

October 13–16
Association of Official Analytical Chemists, Annual Meeting, Marriott Hotel, Twin Bridges, Washington, D.C. (Contact L. G. Ensminger, Association of Official Analytical Chemists, Box 540 Benjamin Franklin Station, Washington, DC 20044)

October 17
APhA Academy of Pharmaceutical Sciences, Annual Eastern Regional Meeting, Governor Morris Inn, Morristown, N.J. (Contact Dr. Daniel Mufson, USV Pharmaceutical Corp., 1 Scarsdale Road, Tuckahoe, NY 10707)

October 19–21
Pharmaceutical and Analytical Technology 1975 as Related to Current Official Regulations, Society of Pharmacists in Industry Seminar, St. Louis Sheraton Westport Inn, St. Louis, Mo. (Contact Mr. Chris Christensen, Rexall Drug Company, 3901 N. Kingshighway, St. Louis, MO 63115)

October 20–22
Canadian Conference on Medical Devices in Health Protection, Government Conference Centre, Rideau Street, Ottawa, Ontario, Canada (Contact Dr. A. K. DasGupta, Health Protection Branch, Tunney's Pasture, Ottawa, Ontario K1A OL2, Canada)

November 2–6
Society for Neuroscience, Annual Meeting, New York, N.Y. (Contact Neuroscience Meeting Headquarters, Room 200 A, Lee Building, 9650 Rockville Pike, Bethesda, MD 20014)

November 16–20
American Public Health Association, Annual Meeting, Chicago, Ill. (Contact William H. McBeath, APHA, 1015 18th St., N.W., Washington, DC 20036)

November 16–20
APhA Academy of Pharmaceutical Sciences, National Meeting, Marriott Motor Hotel, Atlanta, Ga.

November 21
Eastern Analytical Symposium, Statler-Hilton Hotel, New York, N.Y. (Contact Webster R. Hudgins, Allied Chemical, P.O. Box 1021R CRL, Morristown, NJ 07960)

November 29–December 4
American Medical Association, Clinical Meeting, Honolulu, Hawaii (Contact James H. Sammons, 535 N. Dearborn St., Chicago, IL 60610)

November 30–December 5
American Chemical Society, National Meeting, Mexico City, Mexico (Contact Meetings Department, 1155 16th St., N.W., Washington, DC 20036)

December 7–11
American Society of Hospital Pharmacists, Midyear Meeting, Washington, D.C.

1976

February 18–24
American Association for the Advancement of Science, Annual Meeting, Boston, Mass. (Contact Ms. Elizabeth Zeutschel, AAAS, 1776 Massachusetts Ave., Washington, DC 20036)

April 3–8
APhA Annual Meeting, New Orleans, La.

April 4–7
Proprietary Association, Annual Meeting, The Breakers, Palm Beach, Fla.

April 26–May 1
Third International Symposium on Detection and Prevention of Cancer, Americana Hotel, New York, N.Y. (Contact Dr. Herbert E. Nieburgs, Mount Sinai School of Medicine, City University of New York, Fifth Ave. and 100th St., New York, NY 10029)

May 19–21
Belgian Chemical Society, International Meeting on Drug Receptor and Drug Enzyme Interactions, Namur, Belgium (Contact F. Durant, Department of Chemistry, Facultes Universitaires de Namur, 61, Rue de Bruxelles, B-5000 Namur, Belgium)

Items to be considered for inclusion or corrections should be addressed to:

J. Pharm. Sci. Calendar
2215 Constitution Avenue, N.W.
Washington, DC 20037

Journal of Pharmaceutical Sciences

AUGUST 1975

VOLUME 64 NUMBER 8

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THE FIFTH FREEDOM: SCIENTIFIC COMMUNICATION

The United States is about to celebrate its 200th birthday. In case this fact had somehow escaped anyone, the month of July was filled with a host of commemorative activities which served to launch a year-long period of such features intended to culminate on July 4, 1976.

This editorial will serve as our way of contributing to the bicentennial effort, and adding our voice in reaffirmation of the ideals which prompted the American colonists to establish "a new nation, under God, with liberty and justice for all."

One of the basic freedoms or rights which these founding fathers established as a fundamental feature of the new nation was freedom of the press. This concept was novel in its time and even today has not gained acceptance through much of the world. We are proud to be able to say that provision for such expression is embodied in this Journal, not only through our editorials but more broadly through the Open Forum page. This Journal feature constitutes a vehicle for any reader to "sound off" and offer comments and opinions—which, within the most reasonable restrictions, are assured publication without being subject either to editing by the staff or to review by referees.

Mention of the press and the Revolutionary War period also brings to mind the major role played by the public press during colonial times in stimulating thinking, nurturing new concepts, and spreading ideas. Indeed, one can justifiably speculate that in the absence of the public press, the American Revolution might never have come about. Even the most casual or brief reading of history of that period dramatically reveals the tremendous impact of contemporary publications and the importance attached to them by the colonists.

Many other examples could be cited as to the power of the written word in stimulating thought processes and in communicating information whereby progress can be achieved. Not the least of these, by far, is the system which has been employed for the reporting of and dissemination of results of research in the basic and applied sciences. The most prominent element in this system has been the technical journal, of which this Journal is a typical representative.

But in recent years the scientific press generally has fallen upon hard times. Constricting subscription budgets, broad availability of photocopy machines, dropping circulation figures, soaring paper and printing costs, and rising postage rates are just some of the factors which have combined to put a financial squeeze on most technical journals and their publishers.

It is our guess that we shall see at least two effects of this squeeze within the coming decade. First, there will be a major "shake-out" in which most of the weaker journals will fall by the wayside and cease to exist. Second, among surviving journals there will be a substantial change in the mechanical way that they package and transmit the information intended for delivery to the reader; for example, journals may publish only abstracts of papers with the full text on deposit at some central source, or, instead of being printed on paper, journals may be issued entirely on microfiche.

Whatever the shape of things to come, it appears certain that significant changes will occur. In considering these new approaches, in adapting to them, and in accepting them, it is important to recognize that it is only through this means that viable communication can be maintained within the scientific community. To cling to our present system indefinitely will spell doom for the communication of research results generally.

If the scientific press strangles economically, it would be just as disastrous for the future of research as a political edict shutting down the public press would be in abruptly halting the advance of civilization. Hence, it is important for us to recognize that our basic goal is the survival of a system of scientific communication, whatever its eventual format may happen to be. —EGF

Journal of Pharmaceutical Sciences

AUGUST 1975

VOLUME 64 NUMBER 8

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Osmolarity Values

Interest in the quality of drug products has continued to increase during the 5-year preparation of USP XIX. Several new discerning techniques of determining the identity, quality, purity, and strength have been included[1]. High-pressure liquid chromatographic methods, X-ray diffraction spectrometric methods, and atomic absorption spectrometric methods have been included as new pharmacopeial tests. Other strides in the new compendial requirements include particulate matter standards on large volume parenterals, pharmaceutical container standards, and expiration dates on drug products.

During the past revision period, the USP advisory panel on electrolytes and parenteral therapy unanimously recommended that the osmolarity value be included in the labeling for parenteral electrolytes and large volume parenterals which are commonly given intravenously. The objective of this recommendation was to alert physicians to the fact that they may be injecting solutions that are markedly hypotonic or hypertonic[2].

A brief survey of USP XIX indicates that for several preparations the osmolarity value must be stated on the label in milliosmoles per liter. Furthermore, different sizes and concentrations of these preparations are commercially available.

An array of concentration ranges of different parenteral preparations are official in USP XIX—*viz.*, calcium chloride (2–10%), calcium gluconate (1%), calcium levulinate (10%), dextrose (2.5–50%), dextrose and sodium chloride (2.5–25% and 0.11–0.9%, respectively), Ringers injection (0.85% sodium chloride, 0.3% potassium chloride, and 0.33% calcium chloride), magnesium sulfate (10–50%), mannitol (5–25%), mannitol and sodium chloride (5–15% and 0.45%, respectively), potassium chloride (8–24%), sodium bicarbonate (1.4–8.4%), sodium chloride (0.9%), and sodium lactate (0.19–5.6%). In view of the multitude of commercially available preparations, ranging from very dilute to saturated and/or supersaturated concentrations, the label requirement of the osmolarity value will become somewhat confused. Not only are these relative values of different preparations impractical to measure in concentrated solutions using a conventional osmometer, but they also markedly deviate from the expected values. In principle, the osmotic relationships can be considered in relation to other colligative properties and are indeed valid only for dilute solutions.

Additionally, our experience has demonstrated that some concentrated solutions are out of the measurable limits, some do not freeze, and some show deviations from calculated values when subjected to the normal operating conditions of the osmometer.

In view of the apparent controversy and confusion, additional guidelines are warranted if the manufacturer is to serve the community in a meaningful manner and the desired objectives are to be achieved. Unfortunately, the USP did not indicate any statement(s) in regard to this requirement. It is hoped that more uniform labeling can be developed for transmitting important information to various health-care professionals. Finally, it is surprising that this problem failed to attract our attention in the comment-proof stage of USP XIX.

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Grand Island, NY 14072

Received June 2, 1975.

[1] "The United States Pharmacopeia," 19th rev., Mack Publishing Co., Easton, Pa., 1975 and its 1st supplement.
[2] Dr. Daniel Banes, United States Pharmacopeia, Rockville, Md., personal communication.

Propylene Glycol USP—Harmful or Fatal If Swallowed!

A major supplier[1] of propylene glycol clearly indicates on the container label that the product is of USP quality. On the same label in red letters, half the size of the name of the product, is the word "CAUTION!" followed by the statements "Harmful or fatal if swallowed. Do not take internally." I am aware that propylene glycol is used as a solvent for vitamins taken orally as well as for other products administered by the oral route. A paper by Zaroslinski *et al.*[2] refers to propylene glycol having a relatively low order of toxicity and possessing pharmacological inertness. I know of at least one major hospital pharmacy that uses propylene glycol as a solvent for certain oral medications.

I do not understand how a well-known purveyor of a product which purports to meet the specifications of the USP can be allowed to include false, misleading, and patently incorrect information on a container label.

Martin I. Blake
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Received June 19, 1975.

[1] Sargent-Welch Scientific Co., Skokie, IL 60076
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AUGUST 1975

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REVIEW ARTICLE

Characterization of Habits and Crystalline Modification of Solids and Their Pharmaceutical Applications

JOHN K. HALEBLIAN

Keyphrases □ Solids and their pharmaceutical applications—review of crystal habit, amorphous solids, polymorphs, solvates, and clathrates □ Crystalline aspects of pharmaceutical solids—review of crystal habit, amorphous solids, polymorphs, solvates, and clathrates □ Pharmaceutical applications of solids—review of crystal habit, amorphous solids, polymorphs, solvates, and clathrates □ Polymorphs—occurrence, dissolution kinetics, and pharmaceutical application, review □ Solvates—preparation, characterization, properties, and pharmaceutical applications, review □ Clathrates—crystal structure, preparation, characterization, properties, and pharmaceutical application, review □ Amorphous solids—preparation, characterization, and pharmaceutical application, review

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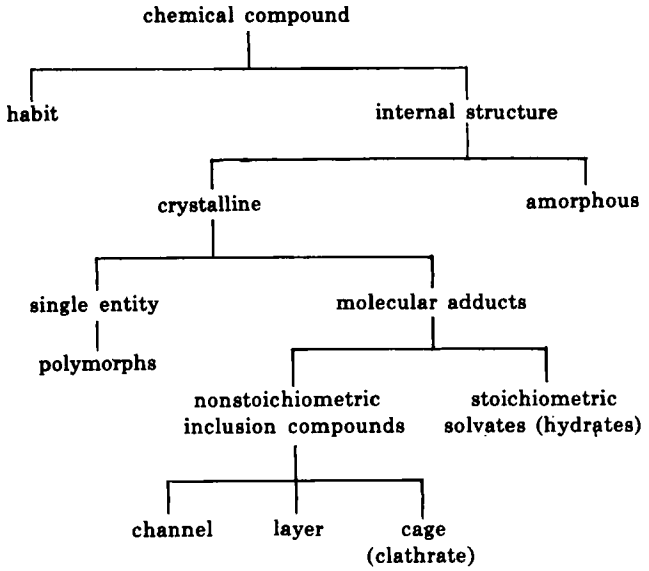
Once the molecular chemistry of a potential drug is established and understood, most chemists feel that the compound is ready for formulation and clinical testing. Yet, every year, numerous papers appear showing that the performance of different drugs depends on, among other parameters, the habit and crystalline modifications of the active drugs. One crystal habit of a drug may tablet well while another may cause trouble, but both have the same melting point and apparently the same X-ray pattern. One crystalline modification may show five to 10 times the absolute solubility and bioavailability of another polymorph of the same drug. A drug marketed by Company A gives a stable suspension; the same com-



Scheme I—Outline Differentiating Habit and Crystal Chemistry of a Chemical Compound

pound, marketed by Company B, is stable only for a short time before the suspension is caked. These cases are not unusual. The majority of drugs marketed in various dosage forms probably can exist in different habits and crystalline modifications.

Since 1969, a series of review articles has dealt with polymorphism and its pharmaceutical application (1–5), but there is still confusion in the terminology used to identify different crystalline modifications of solids deviating from the basic definition of polymorphs (6–8). The intent of this work is not to review again the field of polymorphism but rather to review, in a broader sense, the different crystalline modifications of solids of pharmaceutical importance and their pharmaceutical application. Scheme I represents the outline to be followed in this review to differentiate habits and crystalline modifications of solids, *e.g.*, noncrystalline amorphous solids, polymorphs, solvates, and clathrates.

PHYSICAL APPEARANCE OF SOLIDS-HABITS

Definition—Habit is the description of the outer appearance of a crystal. If the environment of a growing crystal affects its external shape without changing its internal structure (which affects the polymorphism of a crystal), a different habit results. These

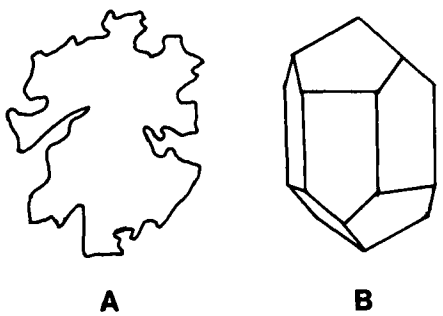


Figure 1—*Anhedral (A) and euhedral (B) quartz crystals. (Reproduced, with permission, from Ref. 9.)*

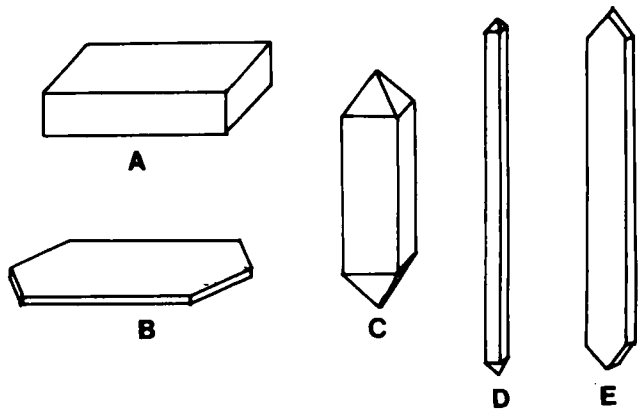


Figure 2—*Different habits of crystals. Key: A, tabular; B, platy; C, prismatic; D, acicular; and E, bladed. (Reproduced, with permission, from Ref. 9.)*

alterations are caused by the interference with the uniform approach of crystallizing molecules to the different faces of the crystal.

Crystal growth may be impeded by adjacent crystals growing simultaneously or contacting container walls. As a result, the development of plane faces may be inhibited or, in the case of late crystallizing crystals, an irregularly shaped crystal may occur since it is constrained to occupy only the spaces left between substances already crystallized. Such irregularly shaped crystals are described as anhedral or allotriomorphic (A, Fig. 1); those bound by plane faces are termed euhedral or idiomorphic (B, Fig. 1) (9). Anhedral crystals, although irregularly shaped, have a regular arrangement of building units which may be proved by X-ray diffraction.

Euhedral crystals are shown in Fig. 2:

A. Tabular crystal—moderate development of a pair of parallel faces, at the expense of the others, produces a tabular crystal.

B. Platy crystal—excessive development of the parallel faces as described in the tabular habit produces a platy crystal.

C. Prismatic crystal—crystal has a columnar form.

D. Acicular crystal—prism is elongated so much as to be needle like.

E. Bladed crystal—acicular crystal is flattened.

Factors that May Affect Crystal Habits—*Supersaturation*—The degree of supersaturation of the mother liquor or a supersaturation difference on opposite sides of the growing crystal may affect crystal habits. As supersaturation is increased, the crystal form tends to change from granular to needle like. A thin needle or dendrite loses less heat by conduction than a thicker crystal, so it grows faster. The effect of supersaturation on the change in the external form is given by a curve of high order which is characteristic of the substance:

$$y/x = k\Delta G^n \qquad \text{(Eq. 1)}$$

where y/x is the ratio of crystal length to breadth; k is a coefficient of proportionality which depends on the conditions of crystallization, especially on diffusion; G is the degree of supersaturation, in moles/

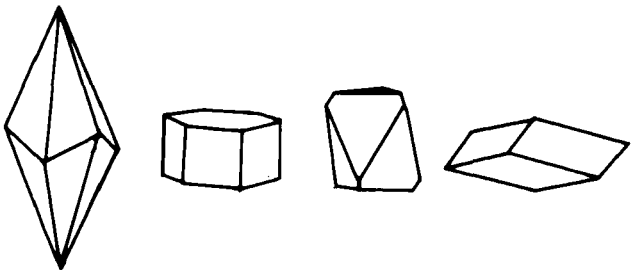


Figure 3—*Crystal habits of calcite. (Reproduced, with permission, from Ref. 9.)*

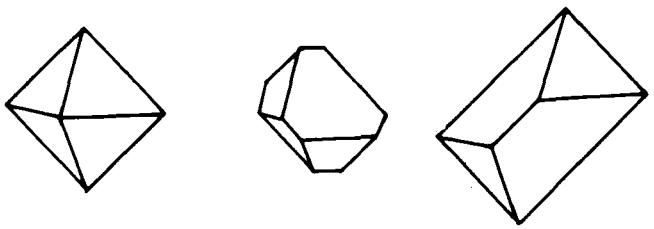


Figure 4—*Unevenly developed octahedra of magnetite. (Reproduced, with permission, from Ref. 9.)*

1000 moles of solvent, at the moment of formation of the nuclei of crystallization; and n is a number, above unity, dependent on the crystallographic classification and chemical composition of the substance (10).

Rate of Cooling and Degree of Solution Agitation—When naphthalene is rapidly crystallized by rapid cooling from ethanol or methanol, it gives thin plates; when it is slowly crystallized by evaporation, it yields compact crystals. The rate of cooling is effective in altering crystal habits because of its influence on the degree of supersaturation (11).

Nature of Crystallizing Solvent—The interaction between the solute and the solvent is important in controlling the crystal habit. For example, resorcinol crystallizes from benzene into fine needles, but it crystallizes from butyl acetate into squat prisms. Similarly, iodoform crystallizes as hexagonal bipyramids from aniline and as prisms from cyclohexane. This difference is due to the affinity of a solvated solvent to be adsorbed on certain crystal faces and thus to inhibit the growth of those particular crystalline faces (12).

Presence of Cosolutes, Cosolvents, and Adsorbable Foreign Ions—The addition of any new substance in a crystallizing medium may affect the habit of the crystal formed. Sodium chloride, which normally develops only cubic {100} faces, develops octahedral {111} faces when grown in the presence of urea (13, 14). Whetstone (15–18) studied crystal habit modifications due to the addition of impurities as crystal poisons. He found that the crystal habit change by dyes depended on anionic and cationic substituent groups and the nature of substitution. For further examples, the reader is referred to the review works of Tipson (11) and Biles (19).

Constancy of Conditions—Because any small change in these variables may affect the habit of the growing crystal, duplication of the habit of any crystal requires crystallization under identical conditions.

An excellent example of different habits of the same compound can be seen in calcite, $CaCO_3$ (20), where crystals can occur in acute or obtuse rhombs, hexagonal prisms, and many types of scalenohedra (Fig. 3). All of these geometric forms are built from the same unit rhombohedron of calcite, which can be shown readily by their optical properties and X-ray diffraction patterns.

Although habit is the description of the outer appearance of a crystal, crystals showing the same habit may appear different superficially. A good example is the three geometric forms of magnetite, Fe_3O_4, represented in Fig. 4, where all are octahedra of the cubic system but with uneven developments. Measurement of the interfacial angles would reveal true symmetry. Such measurements are the only morphological bases for crystal classification and identification of habits.

Characterization of Habits—The angle between two crystal faces can be described in two ways: (*a*) included or edge angle between two faces, and (*b*) interfacial or polar angle, the angle between the normals to the faces of the crystal (Fig. 5). The interfacial angle is commonly used in optical crystallography.

The interfacial angles of crystals are measured by instruments known as goniometers (9, 21). The simplest, the contact goniometer, is a semicircular protractor with a movable arm pivoted at the circle center. When the two faces enclosing the angle to be measured are fitted closely to the base and the movable arm, the value of the angle may be read off directly from the graduated half circle. Contact goniometers are used only on large crystals with dull faces for which the reflecting goniometer cannot be used.

In the reflecting goniometer (Fig. 6), the crystal to be measured is mounted on a stout needle. The needle is attached to the spindle of a graduated rotatable

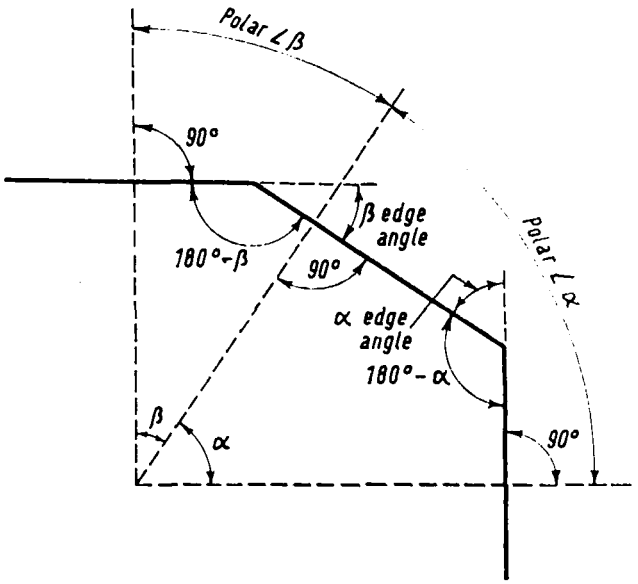


Figure 5—*Horizontal section across three vertical faces to show the relationship between included (edge) and polar angles. (Reproduced, with permission, from Ref. 9.)*

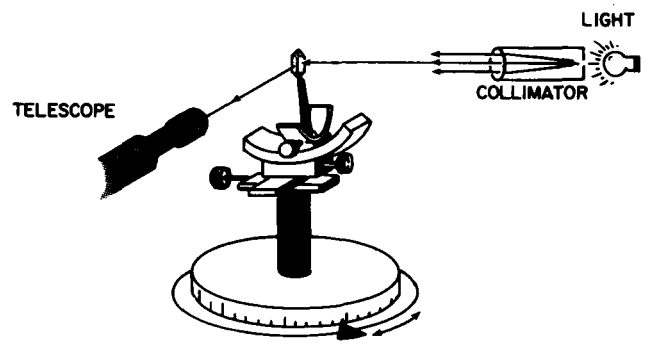


Figure 6—*Schematic of the reflecting goniometer.*

drum, which can be read to 1 min of arc by means of a vernier. The needle is provided with lateral and tilting adjustments, so that any zone of the crystal may be brought parallel to the axis of rotation of the drum. After this is done for one zone, parallel light from a collimator, fitted with a slit, is directed at the crystal and, by turning the drum, is reflected from each face of the zone in turn into a telescope provided with crosshairs. The reading is taken whenever the image of the slit coincides with the intersection of the crosshairs. The differences between these readings supply the angles between the normals to the faces (9).

Pharmaceutical Application of Habits—Crystal habits may influence several pharmaceutical characteristics.

Suspension Syringeability—The influence of suspension syringeability is mostly mechanical. For example, a suspension of plate-shaped crystals may be injected through a small needle with greater ease than one with needle-shaped crystals of the same overall dimensions (22).

Tableting Behavior—Shell (22), in his work on X-ray and crystallographic applications in pharmaceutical research, showed the effect of crystal habit on tablet properties. To evaluate the tableting behavior as influenced by crystal habit, he quantitatively described crystal habits by measurement of preferred orientation and related this parameter to compression characteristics of the powder. He took the ratio of the relative peak intensities of critical lines in the X-ray diffraction pattern and used them as average habits of crystals. In the compound studied, he found that the higher the 001 orientation ratio to the 010 orientation, the better was the tableting behavior.

Dissolution of Crystalline Material—If a crystal changes its habit due to crystal poisoning by a dye, then, in an indirect way, as reported by Piccolo and Tawashi (23), the adsorbed dye may inhibit the dissolution of drug crystals, which, in turn, may affect the bioavailability of the material.

Although the concept of habits of solids has been recognized since ancient times, the internal structure of solids, *i.e.*, the arrangements of atoms, was not understood until the introduction of X-ray crystallography by William and Lawrence Bragg. Solids in general are divided into two categories: (*a*) amorphous, where there is no regularity in the structure, and (*b*) crystalline, where the atoms are arranged in regular arrays. The crystalline form is the most frequent of the two and includes the metals and most minerals.

NONCRYSTALLINE SOLIDS-AMORPHOUS FORM

Definition—The most common amorphous solid is glass. Its atoms are put together in a nonuniform array compared to its crystalline form. Figure 7 represents the two forms of silica, which exists either as a crystal or as a glass. Cristobalite is the high-temperature form of quartz (SiO_2), while the glass structure looks like a distorted structure of cristobalite.

Randall and coworkers (24–28) reported that when a substance is capable of existing in both amorphous and crystalline forms, then the X-ray pattern given by the amorphous form may be regarded as a very diffuse version of the crystal pattern. There is, in fact, no sharp distinction between crystalline and amorphous states. If, starting with a coarsely crystalline solid, the size of the crystals could be reduced by stages and an X-ray diffraction photograph could be taken at each stage, the photographs would become diffuse when the crystal size fell below about 10^{-5} cm. With reduction of crystal size, the reflections become increasingly diffuse until the limit is reached at 10^{-7}–10^{-8} cm, the region of atomic dimensions, where the word crystal, with its implication of precise pattern repetition, ceases to be appropriate.

One cannot speak of a crystal only one unit cell in diameter, because the term unit cell implies repetition; this is the justification for the use of the term amorphous in describing glass-like substances, although the word noncrystalline might be preferable.

Preparation of Amorphous Solids—Preparation of amorphous solids, if feasible, does not follow a routine procedure. Methods mentioned by previous workers include lyophilization of fluprednisolone in *tert*-butanol (29), rapid quenching of chloramphenicol palmitate solution in hydrophilic solvents (30), and rapid quenching of melted chloramphenicol palmitate in the refrigerator to −10° (31). Precipitation is also used to prepare the amorphous prompt insulin zinc suspension of USP XVIII (32). It is prepared by precipitation of insulin and zinc chloride at a specified pH.

Characterization of Amorphous Solids—The only positive way to differentiate amorphous from crystalline solids is by means of X-ray powder diffraction. The X-ray powder diffraction of amorphous

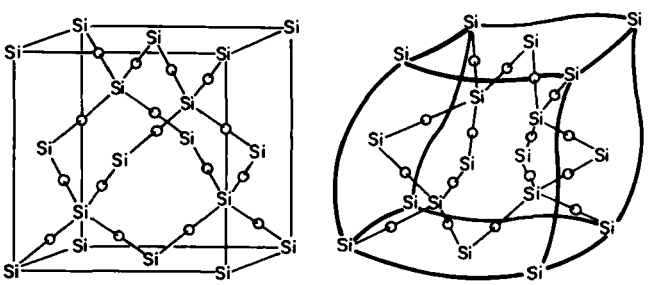

Si
Si
Si

Table I—Novobiocin Plasma Levels in Dogs following Oral Administration of Different Solid Forms[a]

Hours after Dose	Novobiocin Sodium, μg/ml Plasma	Amorphous Novobiocin Acid, μg/ml Plasma	Crystalline Novobiocin Acid
0.5	0.5	5.0	N.D.[b]
1	0.5	40.6	N.D.[b]
2	14.6	29.3	N.D.[b]
3	22.2	22.3	N.D.[b]
4	16.9	23.7	N.D.[b]
5	10.4	20.2	N.D.[b]
6	6.4	17.5	N.D.[b]

[a] Dose = 12.5 mg/kg. [b] Not detectable.

solids gives very diffuse reflections where the *d* distances, the distance between parallel planes in which the atoms of the crystal lie, cannot be determined as is done with crystalline solids.

Pharmaceutical Application of Amorphous Solids—Although it is feasible to prepare amorphous solids, there are few instances where advantage has been taken of this class of compounds. One main reason is their thermodynamic instability, since they are the most energetic form and tend to revert to a more stable form. This is particularly true when the formulation is in aqueous suspension.

Mullins and Macek (33), working on pharmaceutical properties of novobiocin, identified two forms of novobiocin: crystalline and amorphous. In tablet and capsule formulations, novobiocin is used as the sodium salt, which is active orally but is unstable chemically in a solution. The insoluble forms of novobiocin acid are more stable chemically. But the crystalline novobiocin acid is poorly absorbed and does not provide therapeutically adequate systemic levels following oral administration. The amorphous acid is readily absorbed and is therapeutically active. This difference in availability is due to differences in solubility in aqueous systems.

When an excess of crystalline or amorphous novobiocin acid in less than 10-μm size was shaken in 0.1 *N* hydrochloric acid at 25°, the amorphous solids were at least 10 times more soluble than the crystalline acid. This difference in solubility might be expected to favor the absorption of the amorphous solid from the GI tract. Data showing differences in novobiocin plasma levels following oral administration of 12.5 mg/kg each of amorphous novobiocin acid, crystalline novobiocin acid, and the sodium salt are shown in Table I.

Unless special precautions are taken to maintain the solid in suspension in the amorphous state by the addition of materials to suppress crystallization, amorphous novobiocin converts slowly to a crystalline form. The formulation becomes less and less absorbable and finally loses therapeutic effect entirely. A search was made for additives that would significantly retard or even prevent crystallization of aqueous suspensions of amorphous novobiocin, and some agents provided adequate protection for significant periods. The best agents found were methylcellulose, polyvinylpyrrolidone, and several alginic acid derivatives such as sodium alginate and propylene glycol algin.

In formulating injectable insulin, the duration of the action is controlled by its crystallinity. Insulin precipitates as an insoluble complex when reacted with zinc chloride and, depending on the pH, it may precipitate either as an amorphous or crystalline phase. Prompt insulin zinc suspension USP consists of amorphous insulin zinc complex. It is readily absorbed when injected and has a relatively short duration of action.

Extended insulin zinc suspension USP is made up of crystalline zinc complex. It is very slowly absorbed and has a longer duration of action. Insulin zinc suspension USP is made up of a mixture containing seven parts of crystalline and three parts of amorphous insulin zinc complex, and it is intermediate in duration of action. Another difference in the formulations is their particle sizes; prompt insulin is made up of small particles, and extended insulin is made up of large particles. This is another example where the rate of absorption and duration of action are determined both by particle size and degree of crystallinity (32).

Crystalline solids produced by the different methods of crystallization may crystallize out as single entities or as molecular adducts, where the adduct is one of the solvents of crystallization. The solid crystalline phase of a single entity is said to be polymorphic when it is crystallized at least in two different arrangements of the molecules.

SINGLE-ENTITY CRYSTALLINE SOLIDS-POLYMORPHS

Haleblian and McCrone (1) reviewed the pharmaceutical applications of polymorphism. This section presents some papers dealing with the pharmaceutical application of polymorphism appearing since 1969.

One welcome addition to the field of polymorphism is Kuhnert-Brandstätter's book on "Thermomicroscopy in the Analysis of Pharmaceuticals" (34). To show how frequently polymorphism occurs in pharmaceuticals and how numerous the modifications of a substance may be, the author described three representative groups in detail. Of the barbiturates used medicinally, she found that about 70% were polymorphic. A fairly large number of these barbiturates possessed four or five crystalline modifications.

The other groups described were the steroid hormones and sulfonamides; she found 42 polymorphic steroids in the former group and 30 polymorphic sulfonamides and related compounds in the latter group. She also compiled the microscopic behavior of hundreds of pharmaceuticals when subjected to heat and identified the presence of any existing polymorphs.

Another important paper is Buerger's review (35) of the basics of transformation theory and the forms involved in the transformation, namely the polymorphs. He discussed the reasons why some poly-

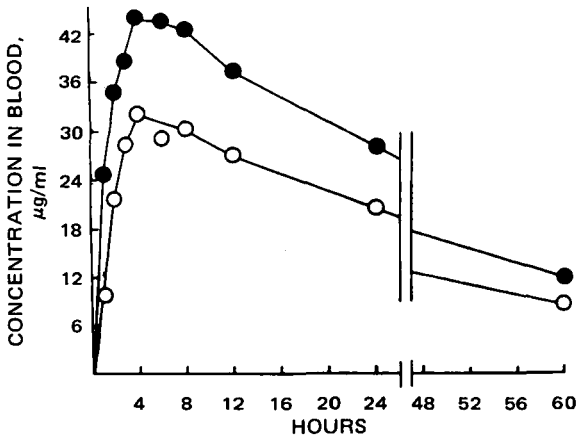


Figure 8—*Mean blood concentration curves of sulfameter crystal Forms II and III. Key: ●, Form II; and ○, Form III. (Reproduced, with permission, from Ref. 49.)*

morphic changes happen faster than others. Kinetic theory predicts that, if the transformation from one polymorphic structure to another is opposed by a potential barrier of magnitude B, the speed of transformation is proportional to $1/e^{B/kT}$.

For some structural changes, when the temperature reaches the transformation temperature, many or all of the atoms can immediately surmount the barrier. Therefore, the transformation is rapid. In other structural changes, few atoms have the thermal energy to surmount the barrier. Appreciable time then is needed for one structure to change to the other through temperature variation; the transformation is relatively sluggish (35).

Examples of Polymorphism—A recent major advance in the field of polymorphism has been the X-ray crystallographer's interest in compounds exhibiting polymorphism. Investigations using single crystals have, for the first time, given the absolute configuration of different compounds exhibiting polymorphism. Perrin and Michel (36, 37) solved the structure of p-chlorophenol where the α-form, the stable modification, is a monoclinic crystal while the β-form is a metastable crystal stable at $-10°$ but also belongs to the monoclinic system. Kahn *et al.* (38), working with cyclohexane, solved the structures of both Phases I and II, the stable phases, but could not solve the structure of Phase III. Phase III is a metastable form, and single crystals are not obtainable.

Of special pharmaceutical importance is the work of Busetta *et al.* on estrone (39). They found that Phases I and II crystallize as orthorhombic crystals while Phase III crystallizes as a monoclinic crystal. This reviewer believes that the method developed by Busetta *et al.*, although tedious, is the ultimate method for establishing whether a polymorph exists and should be used whenever the polymorphism of a compound is in question.

The search for different polymorphs of pharmaceuticals has now spread all over the world, with sulfonamides being extensively studied (40–43).

The p-amino group, the acidic N^1-hydrogen atom, and the oxygens of the sulfonamide group were implicated in the various hydrogen bonding arrangements that distinguish one polymorphic form from another (43). It was postulated that electron-withdrawing and electron-donating groups at the N^1-position influence the strength of the hydrogen bonds formed, hence the tendency of compounds to exist in more than one crystalline form.

The thermal behavior of four crystalline phases of sulfanilamide and of sulfanilamide-d_4 were compared; the deuterated modifications exhibited smaller heats of transition and heats of fusion than the corresponding undeuterated forms (44). Further work was done on the different crystal forms of sulfathiazole (45–47).

The crystal forms of sulfameter were studied and identified as three polymorphs, two solvates, and an amorphous phase (48, 49). The GI absorption of Forms II and III was studied in humans. One gram of either crystal phase was suspended in a mixture of 25 ml each of 20% mucilage of acacia and simple syrup. The mean blood concentration curves are shown in Fig. 8. The energetic crystal Form II had an absorption rate about 1.4 times as great as that of the water-stable Form III. Marketed pharmaceutical preparations of sulfameter were also studied and contained mainly Form III. As a result of the absorption study, the use of Form II, the more biologically available crystal form, was recommended, provided adequate measures are taken to prevent its transformation.

Borka (50, 51), using both differential thermal analysis and IR spectrophotometry, identified the different polymorphs of chloramphenicol palmitate and studied their solid and solution phase transformations. He found that Form B, although metastable, has unusual stability, which, combined with its low solubility in water, assures very reliable drug preparations not only in the dry form as capsules but also when suspended in water.

Aguiar (52) continued his work on the different polymorphs of chloramphenicol palmitate and determined their dilatometric behavior. Aguiar and Zelmer (53) also studied the relative dissolution and solubility of the three polymorphs of chloramphenicol palmitate. From a comparison of the *in vivo* absorption data in humans and thermodynamic parameters, they postulated that when the free energy differences between the polymorphs are high, they might affect the absorption profiles. However, if the free energy differences are not high, as in mefenamic acid, there are no significant differences in their absorptions as measured by blood levels.

Banerjee *et al.* (54) followed the absorption of amorphous and polymorph A chloramphenicol palmitate suspensions and found that the absorption of the amorphous form was "definitely superior" in children. Miyamoto *et al.* (55) also studied the differential scanning calorimetry of chloramphenicol palmitate and its phase transitional behavior during the preparation of its oral suspension.

Finally, after billions of aspirin tablets have been produced and consumed, Tawashi (56) reported that aspirin is dimorphic. He also studied the GI absorption of these two forms in humans; 600 mg of each phase dispersed in water was ingested by each volun-

teer and the total serum salicylates were measured. The salicylate concentration–time curve is given in Fig. 9 (57). The disclosure by Tawashi generated one of the most concentrated efforts in the field of polymorphism, and the outcome is yet far from the fate of Derjaguin's "polywater."

Summers *et al.* (58) agreed that aspirin is polymorphic and proposed six different forms; Borka (59), using data obtained from solution phase transformations, agreed that aspirin is at least dimorphic. The major opponents to the polymorphism of aspirin have been Mitchell and coworkers (60, 61), who suggested that differences seen in different aspirins crystallized from different solvents are due to crystal defects (60), aging and ripening of the crystals, or poisoning by impurities other than salicylic acid (61).

Questions (62–64) raised on crystal habits, crystal size, or salicylic acid impurities, coupled with the mentioned controversies, will surely generate more work on the crystal chemistry of aspirin until a final agreement is reached. This author believes that the best way to solve the questions will be by doing a total structure determination using single-crystal X-ray crystallography, which has already been successfully used with other polymorphic substances.

Other compounds reported to exhibit polymorphism are water with nine polymorphs (65), sulfur with 12 crystalline phases (66), chlordiazepoxide hydrochloride (6), methylchloromethane (67), cholesteryl palmitate (68), adiphenine hydrochloride (69); benzocaine picrate (70), phenobarbital (71), meprobamate (72), and erythromycin free base (73). The polymorphism of 3-(3-hydroxy-3-methylbutylamino)-5-methyl-*as*-triazino[5,6-*b*]indole (74) and 1-(2,3-dihydro-5-methoxybenzo[*b*]furan-2-ylmethyl)-4-(*o*-methoxyphenyl)piperazine hydrochloride (75) also was reported.

Dissolution Kinetics of Polymorphs—Kuhnert-Brandstätter and Burger (76–78) developed mathematical derivations on dissolution kinetics of both crystalline and amorphous phases of drugs and applied them to crystalline sulfathiourea and pyrithyldione and to crystalline and amorphous phases of calcium D-pantothenate. Dissolution studies were also carried on with different polymorphs of phenobarbital (79). Phase transitions between different crystalline forms were followed for methylprednisolone in the solid form (80), barbital (81), and sulfameter (sulfamethoxydiazine) (82) suspended in water.

Dissolution studies were taken one step further with the development of a mathematical derivation to determine the transition temperature of sulfathiazole polymorphs and the transition temperature between hydrate and anhydrous forms of phenobarbital by measuring their initial dissolution rates (83, 84). This method was in good agreement with values obtained by the conventional solubility equilibrium method.

Pharmaceutical Application of Polymorphism—*Polymorphism and Tableting Behavior of Powders*—Simmons *et al.* (85) reported that tolbutamide exists as Forms A and B, and they evaluated their respective tableting behavior. A preliminary evaluation on a rotary press revealed that Form B was responsible for both powder bridging in the hopper and extensive capping problems during tableting. This behavior was due to the platy habit of Form B and could be corrected by using nonplaty Form A raw material.

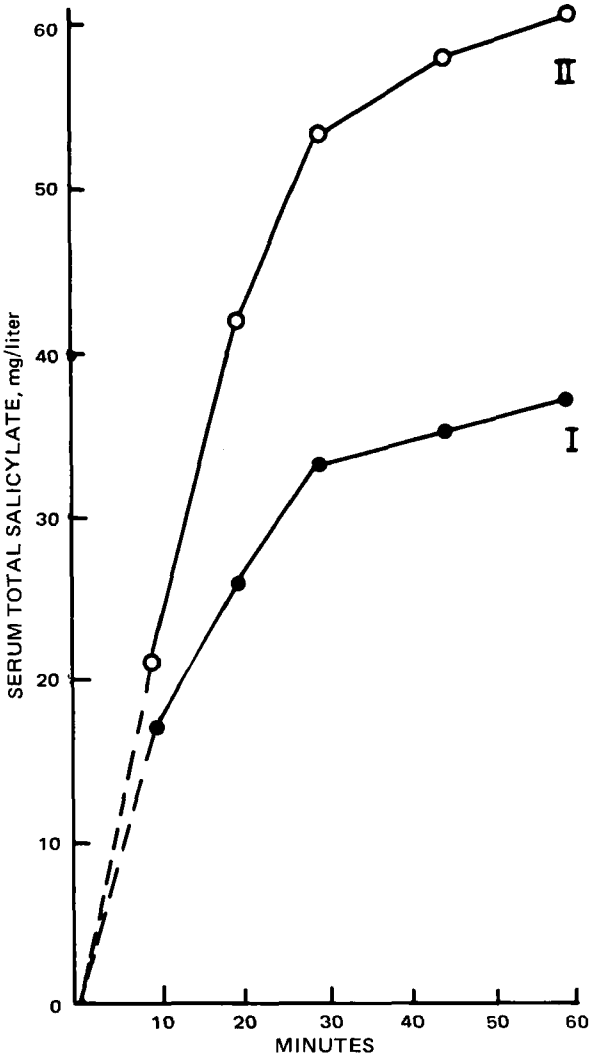


Figure 9—*Serum total salicylate concentration after oral ingestion of aspirin Forms I and II. (Reproduced, with permission, from Ref. 57.)*

Polymorphism and Physically Stable Dosage Forms—Pearson and Varney (86) followed the growth of oxyclozanide crystals in quiescent suspensions with the use of a particle counter[1]. They found that there was an increase in the particle size as a result of an isothermal, solvent-mediated phase transition between two polymorphs. The rearrangement of molecules in the crystal occurs through selective dissolution and redeposition between crystals of different chemical potential. By this mechanism, the less soluble phase grows at the expense of the more soluble phase. To form a stable suspension, the initial use of a less soluble polymorph is mandatory. However, as Ebian *et al.* (87) reported in their work with sulfameter (sulfamethoxydiazine), the addition of transformation-retardation agents, *e.g.*, polyvinylpyrrolidone, might be useful.

[1] Coulter.

Polymorphism and Chemical Stability—Munshi and Simonelli (88), working with two polymorphs of methylprednisolone, found that Phase II degraded when exposed to various temperatures and relative humidity while Phase I was stable. They also found that this surface reaction was catalyzed by heat, UV light, and humidity.

As a result of the scientific evaluation of different polymorphs of drugs, there is a totally new awareness in this field. This awareness was demonstrated with the introduction of a new section on X-ray diffraction in NF XIII (89) and special X-ray diffraction patterns for different polymorphs of some drugs being included in the compendia. It is encouraging that this awareness of different polymorphs of different chemicals is now reflected in drug catalogs (90). This author's hope is that this kind of information will be available for any marketed chemical.

Molecular adducts of chemicals and the solvent of crystallization can be in either a stoichiometric proportion, as in the case of solvates, or a nonstoichiometric proportion, as in the case of inclusion compounds.

STOICHIOMETRIC ADDUCTS-SOLVATES

Definition—During crystallization from a solution, crystals separating may consist of a pure component or be a molecular compound. Molecular compounds may contain two or more constituents that have completely satisfied classical "valence forces" and are crystallized together as a new single crystalline entity. Solvates are molecular complexes that have incorporated the crystallizing solvent molecule in their lattice. When the solvent incorporated in the solvate is water, it is called a hydrate.

To distinguish solvates from polymorphs, which are not molecular compounds, the term pseudopolymorph is used (91). All solvates are formed with stoichiometric proportions between the compound and solvent of crystallization (92). Just as different chemical compounds can have different polymorphs, solvates of different compounds can exhibit polymorphism too. Kuhnert-Brandstätter and Gasser (91), in their work with fluocortolone, found dimorphism of the ethanolic solvates. Similarly, polymorphism was also reported for hydrates of fluprednisolone (29) and succinylsulfathiazole (93).

Examples of Solvates—The pharmaceutical literature on examples of solvates is relatively new, but an extensive amount has been published in the last 20 years. Kuhnert-Brandstätter and coworkers (91, 92, 94–96) worked extensively on solvates of steroids and found that solvate formation is independent of polymorphism. Of all the steroids examined, they found that estradiol formed solvates with all 30 solvents tested, the highest number of solvates so far recorded (92). Other steroids that have been observed to form solvates include hydrocortisone acetate (97), cortisone acetate (98), fluprednisolone (29), the *tert*-butylacetates of prednisolone and hydrocortisone (99), and fludrocortisone (fluorohydrocortisone) acetate and cholesterol (93).

Many antibiotic–antibacterials can also form solvates, *e.g.*, erythromycin (100), gramicidin (101), furaltadone (nitrofurmethone) (102), ampicillin (103, 104), cephaloridine (105), chloramphenicol (106), griseofulvin (107), sulfanilamide (108), sulfabenzamide (43), sulfaguanidine (43), succinylsulfathiazole (93), sulfameter (sulfamethoxydiazine and sulfamonomethoxine) (48, 109). Other miscellaneous compounds also forming solvates include caffeine, theophylline, glutethimide (93), ouabain (110), phenobarbital (111), triazinoindoles (74, 112), and at least 90 other hydrates included in USP XVIII (32) and NF XIII (89).

Preparation of Solvates—Most solvates are prepared by crystallization of the solvated compound from the respective solvent of solvation. In some cases where mixed solvents are used, solvates might be formed from either or both solvents. Holden and Singer (113), in their book on crystal growing, discussed salt hydrate formation where a salt is added to water which weakens the electric forces between the ions and also attracts the ions away from the solid. Once ions are free, they gather water molecules around them. Positive ions gather water molecules to them more readily than negative ions. When these hydrated ions arrive at the surface of a growing crystal, they crystallize into a salt hydrate, carrying some or all of their attached water molecules into the solid composition (Fig. 10).

Even when the number of water molecules is definite and the hydrated ions are combined into a crystalline solid (an orderly arrangement of ions and water molecules), they may, nonetheless, be able to combine in several ways to form different solids. For each hydrate, the number of water molecules per ion

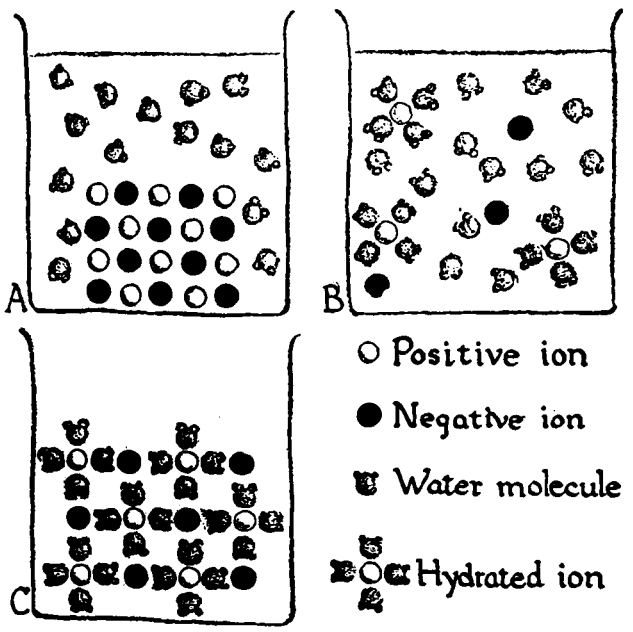


Figure 10—*Formation of a salt hydrate. When the anhydrous salt comes in the presence of water (A), it dissolves and the positive ions become hydrated (B). When crystallizing from solution again, the ions may remain hydrated and then the water molecules play an orderly role in the crystalline salt hydrate (C). (Reproduced, with permission, from Ref. 113.)*

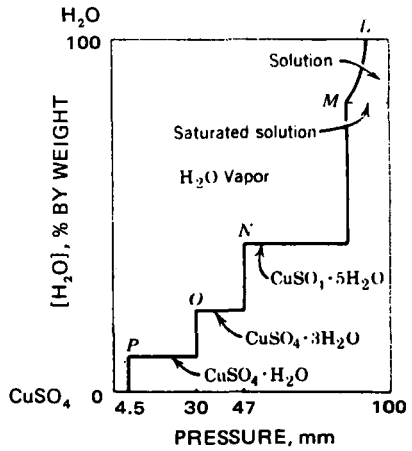


Figure 11—*Pressure–concentration curve for the cupric sulfate–water system at 50°. (Reproduced, with permission, from Ref. 114.)*

has a definite value. Solvates of a compound with different moles of solvent can be prepared in different ways, either by holding the temperature constant or by maintaining the pressure constant in the concentration–temperature–pressure systems.

Figure 11 represents the concentration–pressure curve at 50° for the cupric sulfate–water system (114). If a dilute solution of cupric sulfate is placed in an evacuated desiccator at 50° and water vapor is removed so slowly that an equilibrium is maintained, the water vapor pressure changes along the *LMNOP* line. As water is removed, the cupric sulfate concentration increases; the vapor pressure of the solution then decreases along line *LM*. When the solution becomes saturated, the pressure remains constant (vertical line at *M*), while the water is removed.

The gross composition of the system changes as the water is removed, and the relative amounts of saturated solution and $CuSO_4 \cdot 5H_2O$ change. However, since the compositions of the two phases do not change, the vapor pressure remains constant. When all of the solution has disappeared, the pressure drops abruptly to 47 mm at *N*. The pressure over a $CuSO_4 \cdot 5H_2O$ and $CuSO_4 \cdot 3H_2O$ mixture remains constant at 47 mm. When all the $CuSO_4 \cdot 5H_2O$ has been dehydrated to $CuSO_4 \cdot 3H_2O$, the partial pressure of water drops abruptly to 30 mm at *O*. It remains constant until 2 more moles of water/mole of cupric sulfate is lost at *P*. Then the water vapor pressure drops to 4.5 mm and remains constant until dehydration is complete (114).

Table II—Water Content and Temperature of Isolation of Ouabain Hydrates

Hydrate	Temperature of Isolation
Ouabain·$9H_2O$	0–15°
Ouabain·$8H_2O$	15–28°
Ouabain·$4\frac{1}{2}H_2O$	20–30°[a]
Ouabain·$4H_2O$	20–30°[b]
Ouabain·$3H_2O$	20–30°[c]
Ouabain·$2H_2O$	28–90°
Ouabain	150°[d]

[a] From 95% ethanol. [b] From 97% methanol. [c] From dioxane containing 6% water. [d] Obtained by heating ouabain recrystallized from ethanol in abderhalden apparatus at 150°.

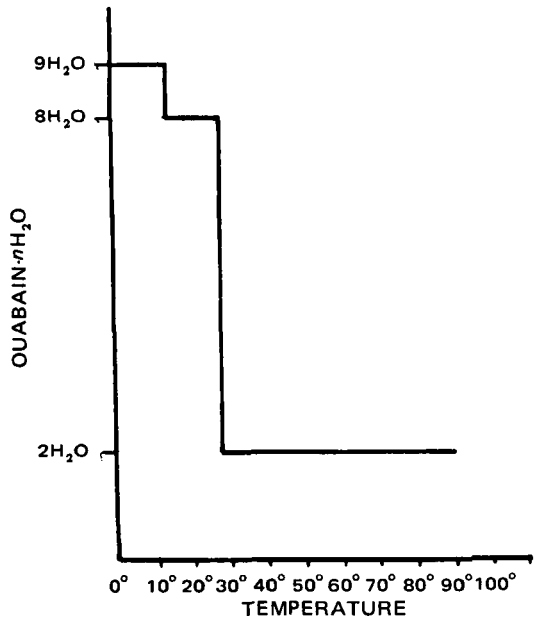


Figure 12—*Temperature–water composition phase diagram for ouabain hydrates. (Reproduced, with permission, from Ref. 110.)*

Trivedi *et al.* (110), using the alternative method of holding pressure constant, obtained hydrates of ouabain with different molecules of water by adding excess amounts of ouabain to the solvent for recrystallization and heating it above the crystallization temperature. The solutions were then allowed to cool to the temperature desired for crystallization. After a sufficient amount of ouabain had crystallized, the containers were closed to help equilibrate the system without evaporation. In this manner, ouabain was crystallized at 0° and at intervals of 10° from 20 through 90°. Six different ouabain hydrates were obtained, depending on the temperature of crystallization. Table II shows the water content and tempera-

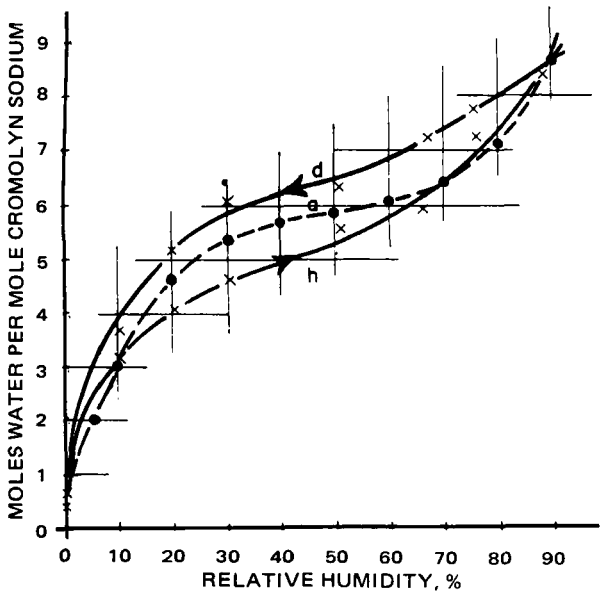


Figure 13—*Water content as a function of relative humidity during hydration (h) and dehydration (d), and an average curve for all data determined (a). (Reproduced, with permission, from Ref. 115.)*

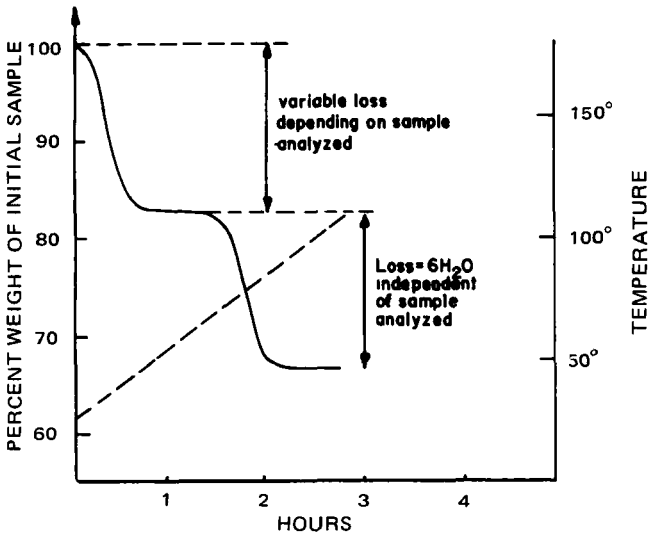


Figure 14—*Thermogravimetric analysis thermogram of tetracycline base with a linear increase in temperature. (Reproduced, with permission, from Ref. 116.)*

ture of isolation. The temperature–water composition phase diagram for the hydrates isolated from water is illustrated in Fig. 12.

Cox *et al.* (115), in their work on solid-state chemistry of cromolyn sodium, obtained a continuous series of interstitial solid solutions or lyotropic mesophases with water by changing the relative humidity. Figure 13 represents the change of water content as a function of relative humidity. The hydration curve shows that, although there is a region of slow increase of moisture content with increasing humidity, there are no sharp plateaus corresponding to fixed hydrates. This absorbed water, amounting to as much as 24 wt. % at about 90% relative humidity, resulted in reversible expansion of the lattice, especially along

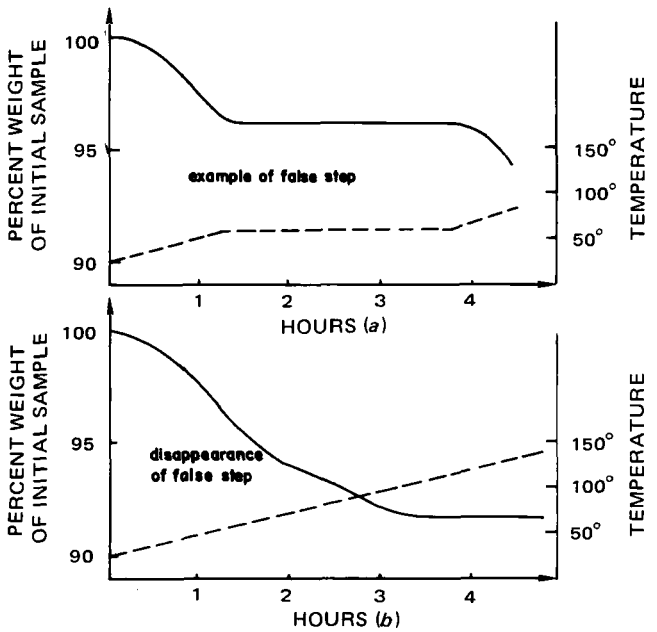


Figure 15—*Thermogravimetric analysis thermogram of SD-1223-01. Key:* a, *using a constant temperature of 60°; and* b, *with a linear increase in temperature. (Reproduced, with permission, from Ref. 116.)*

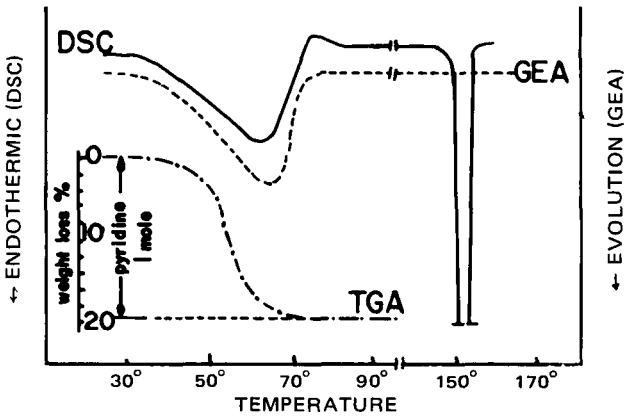


Figure 16—*Differential scanning calorimetry–gas evolution analysis and thermogravimetric analysis thermogram of chloramphenicol pyridinate. (Reproduced, with permission, from Ref. 106.)*

the *b* crystallographic direction. This, in turn, caused changes in X-ray diffraction patterns, density, and other physical properties.

Characterization of Solvates—Different analytical methods have been used for identification of either the solvates or the solvent of the molecular complex.

Methods Used to Identify Solvates—Hirtz *et al.* (116), using thermogravimetric analysis, studied the solvation of organic compounds with pharmaceutical interest. Thermograms of different solvates show different steps of desolvation, which correspond, at a certain interval of temperature and pressure, to the stable solvate. The distinction of the steps on the thermogram could be due to real solvation or to pseudosolvation, which is due to the special setup of the experiment.

Real solvation for tetracycline base is illustrated in Fig. 14. A linear increase of temperature produces a loss of initial weight, which varies from sample to sample and is due to volatilization of "free water." This loss is followed with a second loss of weight, in this case equivalent to 6 moles of water and independent of sample analyzed, indicating that the original sample was a hexahydrate. In the case of thermograms that are the results of special sets of experimental conditions, one can see, as in Fig. 15, that when the temperature increase is not linear, a false step develops on the thermogram which disappears during a linear temperature increase.

Using differential thermal analysis, Brancone and Ferrari (109) were able to demonstrate that sulfameter is a solvate. Similarly, Sekiguchi *et al.* (107) used differential thermal analysis to identify different hydrates of phenobarbital, theophylline, and other organic pharmaceuticals.

Differential scanning calorimetry, a method similar to differential thermal analysis, was also used by Sekiguchi *et al.* (107) to identify the chloroform solvate of griseofulvin.

Gas evolution analysis can be done simultaneously with differential scanning calorimetry. Himuro *et al.* (106), in their work on pyridine solvate of chloramphenicol, plotted differential scanning calorimetry–

gas evolution analysis and thermogravimetric analysis thermograms (Fig. 16). There is a very close correlation of the three different methods used in identifying the solvate.

Trivedi *et al.* (110), using the petrographic microscope, determined optical crystallographic data for ouabain hydrate. These data included the crystal system, optic orientation, optic sign, axial angle, dispersion, common orientation, and refractive indexes. Kuhnert-Brandstätter and Grimm (95) used thermomicroscopy to identify different solvates and polymorphic modifications of different steroids.

Most X-ray diffraction work reported employed powder diffraction. This method was used to differentiate fluprednisolone polymorphs from fluprednisolone solvates (29), to study pseudopolymorphism of furaltadone (102), and to differentiate chloramphenicol from its pyridinate solvate (106). Moustafa *et al.* (48) used powder diffraction to differentiate polymorphs and solvates of sulfameter. If the purity of a sample is established, powder X-ray diffraction is one of the best methods available to differentiate any crystalline modification, be it polymorphs, solvates, or clathrates. Busetta *et al.* (117), continuing their work on single-crystal X-ray crystallography of pharmaceuticals, determined the crystal structure of methanol plus water, of ethanol, and of dimethyl sulfoxide solvates of diethylstilbestrol. They found that all three phases are triclinic crystals. Single-crystal X-ray diffraction also was used to determine the structure of different pharmaceutical hydrates (118, 119).

IR spectrophotometry is much more useful for distinguishing between solvates and anhydrous forms than for identifying polymorphs because of the addition of new stretching frequencies resulting from solvation. Biles (99) used IR spectrophotometry to differentiate crystalline modifications of the *tert*-butylacetates of prednisolone and hydrocortisone. Haleblian *et al.* (29) used IR spectrophotometry in their work on characterization of different solid phases of fluprednisolone. Kuhnert-Brandstätter and Grimm (94, 95) also used this method for identification of solvates of some of the different steroids studied, although they felt that it was difficult and often impossible to distinguish crystal forms containing solvent from polymorphic modifications (94).

Methods Used to Identify Solvent of Solvation—Most solvents of solvation are determined by using some method that produces the release of the solvent, which, in turn, is trapped and identified. If the solvent of solvation is water, *e.g.*, hydrates, some of the USP XVIII (120) methods for water determination can be used. Of these, the most important is the Karl Fischer titrimetric method. This method was used to identify the hydrates of fluprednisolone (29) and ouabain (110).

IR spectrophotometry was used to identify the solvents of solvation that are readily vaporized and give satisfactory spectra at low concentrations in conventional gas cells (121). The sample was heated in polyethylene glycol 300, a liquid of high boiling point, which dissolves the sample, and the vapor was examined in an evacuated gas cell for the presence of absorption spectra characteristic of a particular solvent.

The GC method described in the British Pharmacopoeia 1968 was also used to identify solvents (121).

Investigation of hydrated crystals using proton magnetic resonance was pioneered by Pake (122) and advanced by McGrath and Silvidi (123) and Pedersen (124). Chapman *et al.* (105), in their work on cephaloridine, obtained quantitative information on the different solvated solvents by using proton magnetic resonance.

Differential scanning calorimetry has also been used. Different amounts of water in cortisone acetate hydrates were measured by calibrating the effluent analyzer, using $CuSO_4 \cdot 5H_2O$ as the standard (98).

Properties of Solvates—*Dissolution Rate of Solvates*—Shefter and Higuchi (93), in their work on the dissolution behavior of crystalline solvated and nonsolvated forms of some pharmaceuticals, were the first to treat mathematically the dissolution rate of solvates. The dissolution of a solvate can be expressed in water as in Scheme II:

$$A{:}\,nB_{\text{solid}} \rightleftharpoons A_{\text{aqueous}} + nB_{\text{aqueous}}$$

Scheme II

where A is the drug component and B is the solvent that is solvated. The rate of dissolution can be given by:

$$G = \frac{dC_A^*}{dt} = kD_A(C_A - C_A^*) = \frac{1}{n}\frac{dC_B^*}{dt} = \frac{kD_B}{n}(C_B - C_B^*) \quad \text{(Eq. 2)}$$

where C_A^* and C_B^* are the concentrations of the two species in the bulk of the solution, C_A and C_B are their respective concentrations at the crystal surface, D_A and D_B are the respective diffusivities, and k is a combined geometric and agitation factor.

For the case where $n = 1$ and $D_A = D_B$, Eq. 2 can be simplied to:

$$G = \frac{dC_A^*}{dt} = kD_A(\sqrt{Ksp} - C_A^*) \quad \text{(Eq. 3)}$$

where $[C_A][C_B]^n = Ksp$.

From Eq. 3, it can be seen that C_A^* may be able to build above the solubility of A itself in water. If the concentration of C_B^* is increased much more than C_A^* by the addition of the solvating solvent in the dissolution medium, then:

$$G = \frac{KD_A Ksp - kD_A C_A^*}{C_B^*} \quad \text{(Eq. 4)}$$

which indicates that the dissolution rate for the solvate will decrease with an increase of B in solution.

Nogami *et al.* (111), in their work on the dissolution of organic medicinals involving simultaneous phase changes, defined dissolution rate as:

$$\frac{dC}{dt} = k_t\{C_{SA}e^{-k_t t} + C_{SH}[1 - e^{-k_t t}] - C\} \quad \text{(Eq. 5)}$$

where C is the concentration in the bulk liquid, C_{SA}

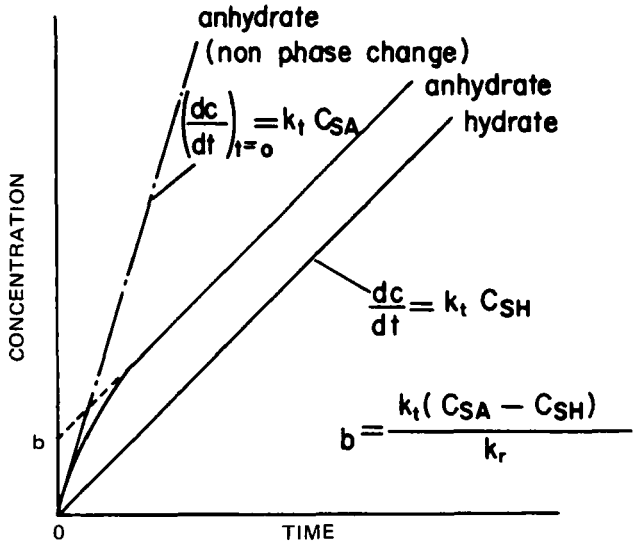


Figure 17—*Schematic illustration for relationship between concentration and time in initial time of dissolution according to Eqs. 8 and 10. (Reproduced, with permission, from Ref. 111.)*

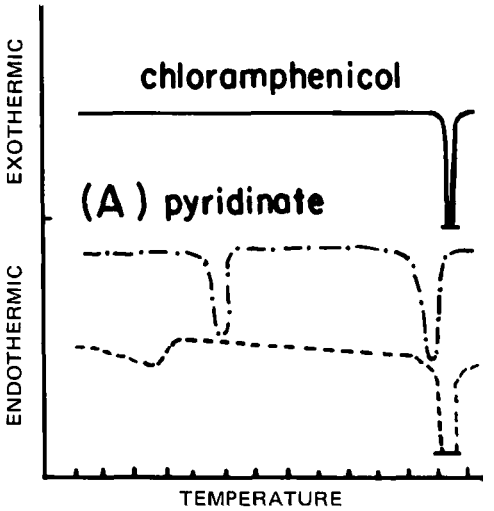


Figure 18—*Differential scanning calorimetry thermogram of chloramphenicol pyridinate solvate. (Reproduced, with permission, from Ref. 106.)*

is the saturated concentration of the anhydrous phase, C_{SH} is the saturated concentration of the hydrate, and k_r and k_t are the rate constants of crystallization and transport processes, respectively. Since $C = 0$ and $t = 0$, Eq. 5 is integrated to:

$$C = \frac{k_t(C_{SA} - C_{SH})}{k_t - k_r}(e^{-k_r t} - e^{-k_t t}) + C_{SH}(1 - e^{-k_t t}) \quad \text{(Eq. 6)}$$

This equation expresses the general dissolution curve involving the phase change caused by hydration. Since C_i, the effective concentration on the solid surface, is initially greater than C, Eq. 5 is modified to:

$$\frac{dC}{dt} = k_t(C_{SA}e^{-k_r t} + C_{SH}[1 - e^{-k_r t}]) \quad \text{(Eq. 7)}$$

Under the initial condition $C = 0$ at $t = 0$, Eq. 7 is integrated to yield:

$$C = \frac{k_t(C_{SA} - C_{SH})}{k_r}[1 - e^{-k_r t}] + k_t C_{SH} t \quad \text{(Eq. 8)}$$

Since t may be sufficiently long, even in the initial stage, Eq. 8 gives a linear relationship with the gradient of $k_t C_{SA}$. Then, when the linear portion of Eq. 8 is extrapolated, the intercept, b, is given by:

$$b = \frac{k_t(C_{SA} - C_{SH})}{k_r} \quad \text{(Eq. 9)}$$

Equation 9 predicts how much the dissolution data of the anhydrous phase undergoing phase transition deviate from the dissolution curve expected from the hydrate alone.

The initial dissolution rate, $(dC/dt)_t = 0$, is derived from Eq. 7 with:

$$\left(\frac{dC}{dt}\right)_{t=0} = k_t C_{SA} \quad \text{(Eq. 10)}$$

which corresponds to the dissolution rate of the anhydrous phase undergoing no phase transition. The general patterns of dissolution behavior according to Eqs. 8 and 10 are illustrated in Fig. 17 (111).

The rate of dissolution of fluprednisolone above a certain temperature may be faster than the rate of transition (anhydrous → hydrous) and not noticeable in the concentration *versus* time plots (125). Ways of calculating different thermodynamic parameters were derived for the anhydrous–hydrated systems, *e.g.*, enthalpy of hydration, free energy of hydration, entropy change, and transition temperatures (93).

Melting Behavior of Solvates—Clear solvate crystals, when observed by transmitted light under a hot stage microscope, lose their solvent of crystallization and become either cloudy or opaque crystals, a process known as pseudomorphosis. Sometimes a solvate first melts and then resolidifies as the temperature rises to give the solvate-free form, which finally again melts. This phenomenon is illustrated by the differential scanning calorimetry curves of chloramphenicol pyridinate (Fig. 18) (106). The differential scanning calorimetry curve consists of three peaks (Fig. 18) (106):

1. The first endothermic peak is due to the desolvation of pyridine, because the shape and the temperature of peak decline are strongly influenced by experimental conditions.
2. The small exothermic rise is due to crystallization of amorphous solid or a metastable melt.
3. The second sharp endothermic peak represents melting of desolvated chloramphenicol.

Brancone and Ferrari (109) found, using differential thermal analysis, that the shallow endotherm of sulfameter at 120° is due to a solvent. They confirmed this observation by running another thermogram under reduced pressure where the endotherm, due to the solvent, had shifted to 66°.

A classical method for distinguishing solvates from polymorphs involves observation of the melting behavior of crystals embedded in silicone oil, where, upon heating, bubbles of solvent are generated by solvates. In the case of polymorphs, no such generation occurs.

This reviewer's opinion is that during heating of crystals embedded in silicone oil, the temperature at

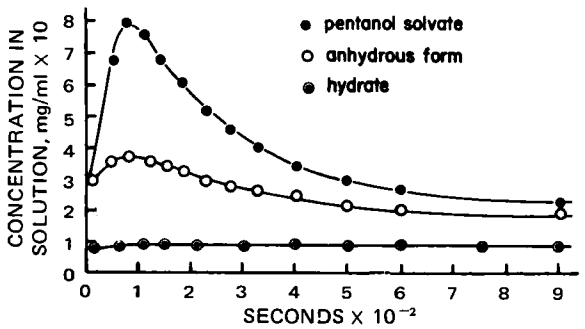


Figure 19—*Dissolution behavior of different forms of succinylsulfathiazole in ~0.001 N sulfuric acid solution at 20°. (Reproduced, with permission, from Ref. 93.)*

which gas bubbles generate is in the vicinity of the boiling point of the solvating solvent. In the case of clathrates (discussed later), this temperature is much higher than the boiling point of the clathrated solvent and very near the melting point of the clathrate.

Pharmaceutical Application of Solvates—*Dissolution*—As anticipated from the theoretical properties of solvates, the dissolution behavior of crystalline solvated and nonsolvated forms of some pharmaceuticals agreed with the theory (93). Figure 19 shows the dissolution behavior of anhydrous, hydrated, and pentanol-solvated forms of succinylsulfathiazole. These curves confirm the theoretical prediction that the dissolution rate of a solvate may be many times greater than the anhydrous form. On the other hand, the dissolution rate of the hydrate is less than the anhydrous form.

Another interesting confirmation of theory (93) is the effect of the concentration of the solvating solvent in the dissolution medium. When measuring the dissolution of the pentanol solvate of fludrocortisone acetate, the dissolution rate was retarded by the addition of pentanol in the dissolution medium. According to this study, it is conceivable that solvates will find broad use in dosage form development "due to their higher temporary solution concentrations and rates of solution which can be obtained by their use instead of the anhydrous form. This is because the system utilizes the free energy of dilution of the complexing agent to raise the solubility of the drug." (93).

Bioavailability—The physicochemical factors influencing the absorption of the anhydrous and trihydrate forms of ampicillin were studied (103). The aqueous solubility of the anhydrous form was 20% greater than the trihydrate at 37° (10 and 8 mg/ml, respectively). The effect of the observed solubility differences on the *in vitro* availability of the drug was also studied. The T_{50} (time required for 50% of the labeled amount to appear in solution) was 7.5 and 45 min for the anhydrous and trihydrate forms, respectively.

In vivo experiments were also done with dogs and humans, where the anhydrous and the trihydrate forms of the drug were given as oral suspensions or capsules. The anhydrous form produced higher and earlier peaks in the blood serum than the trihydrate form. This result was more pronounced in the suspension formulations. Figure 20 shows the mean blood serum concentration in human subjects after oral administration of a 250-mg dose of the suspension. With both formulations, the area under the blood serum level *versus* time curve was greater with the anhydrous form, indicating that the anhydrous form is more efficiently absorbed.

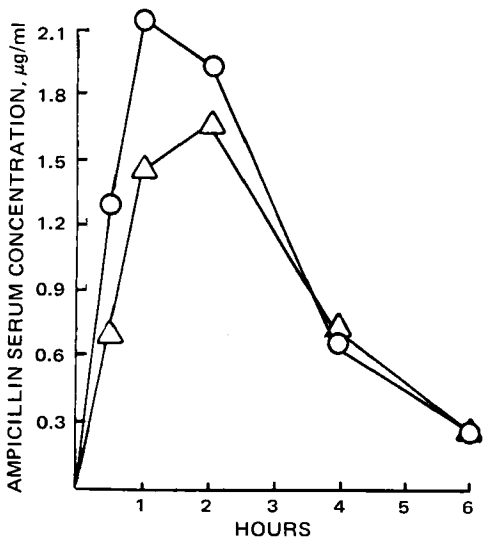


Figure 20—*Mean blood serum concentrations of ampicillin in human subjects after oral administration of 250-mg doses of oral suspension. Key: ○, anhydrous; and △, trihydrate. (Reproduced, with permission, from Ref. 103.)*

Hill *et al.* (126) questioned the conclusion (103) that the differences seen in ampicillin bioavailability were due to the hydration of ampicillin, since they found that the solubilities of the two phases in dilute hydrochloric acid at 37° were very similar. As a result of their data, they suggested that differences in the bioavailability are related to formulation factors rather than the hydration state of the raw material.

Ballard and Biles (127) studied the *in vitro* absorption rates of the *tert*-butylacetates of hydrocortisone and prednisolone and their solvates by the pellet implantation technique. They found that solvates affect the absorption rate from solid drug implants (Table III). With prednisolone tebutate pellets, for example, the monoethanol solvate (Phase II) had an absorption rate 4.7 times greater than that of the anhydrous phase (Phase I). The hemiacetone solvate (Phase IV) had practically the same mean absorption rate as the

Table III—Absorption Rate of the *tert*-Butylacetates of Hydrocortisone and Prednisolone

Crystal Modification		Absorption Rate per Unit Area, mg/hr/cm²
	Prednisolone *tert*-Butylacetates	
Phase I	Anhydrous phase	1.84×10^{-3}
Phase II	Monoethanol solvate	8.70×10^{-3}
Phase III	Hemiacetone solvate	2.20×10^{-3}
	Hydrocortisone *tert*-Butylacetates	
Phase I	Monoethanol solvate	1.83×10^{-3}
Phase II	Monoethanol solvate	7.32×10^{-3}
Phase III	Hemichloroform solvate	7.40×10^{-3}
Phase IV	Anhydrous	4.74×10^{-3}

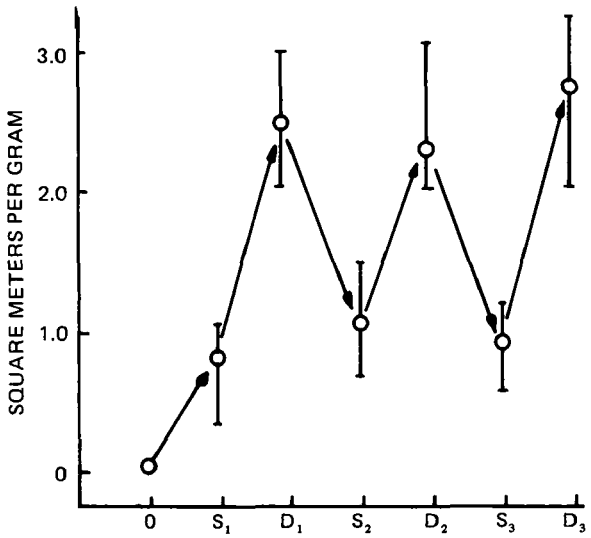


Figure 21—*Effect of repeated solvation and desolvation upon specific surface area of griseofulvin and its chloroformate. Key: O, ordinary crystal; S_1, solvated crystal; and D_1, desolvated crystal. (Reproduced, with permission, from Ref. 107.)*

anhydrous phase. With hydrocortisone *tert*-butylacetate pellets, the mean absorption rate for the solvates were all significantly different from the mean rate of the anhydrous phase (Phase IV). Also one monoethanol solvate (Phase II) had 4.0 times the mean absorption rate of the other monoethanol solvates (Phase I). These results indicate that drug solvates may exhibit dimorphism and each form may exhibit different *in vitro* absorption rates.

In a comparison of the physical properties and biological activities of some crystalline phases of fluprednisolone, Haleblian *et al.* (125) isolated one amorphous and six crystalline phases. Two phases were dimorphic solvates containing 1 mole of water. One existed as the *tert*-butylamine disolvate and three were anhydrous trimorphs. Using the Wruble apparatus, they determined the *in vitro* aqueous dissolution rates (6 rpm and 23°) of the anhydrous and hydrated forms. The dissolution rate ratio for the highest and lowest energetic phases was 2.24, where Form I was the most energetic phase and the α-monohydrate was the least energetic phase.

Similar results were obtained by comparing the *in vivo* dissolution rates of pellets of each crystalline phase, implanted subcutaneously by the method of Ballard and Nelson (128) into males rats. The *in vivo* dissolution rate ratio between the highest and lowest energetic phases was 1.61. Thus, the biological uptake of Form I of fluprednisolone in pellet form was 1.61 times that of the α-monohydrate phase. This value illustrates the effect that crystalline phases may have on biological activity. The *in vivo* and *in vitro* dissolution rates were also correlated with a pharmacological response–adrenal cortex atrophy of the rats. The adrenal cortex atrophy resulting from the uptake of Form I from pellet implants was 1.46 times that of the α-monohydrate.

Physically Stable Suspensions—When aqueous topical suspensions of two anhydrous forms of two different triazinoindoles were formulated, both produced large crystals upon aging. When these crystals were isolated, both were found to be monohydrates of the respective compounds. When the air-milled monohydrate of triazinoindole I, which is labile but does not lose water during milling, was used, suspensions resulted which were physically stable for 2 years.

Table IV—Effect of 3 hr of Exposure to Direct Sunlight or 1 hr of Exposure to 85° of Different Crystals of Coenzyme Form Vitamin B_{12} (Average of Two Runs)

	Percent Remaining	
	After 3 hr of Exposure to Direct Sunlight	After 1 hr of Exposure to 85°
Conventional crystals	74.85	23.70
Hydrated Form A	98.75	72.70
Hydrated Form B	73.75	37.25

Particle-Size Reduction of Medicinal Compounds—Sekiguchi and coworkers (106, 107, 129, 130), working with griseofulvin and chloramphenicol, developed a method of crystal-size reduction involving phase conversion. The technique required use of a proper solvent which forms a solvate with a medicinal compound and which also can be easily desolvated. The effects of repeated solvation and desolvation on the specific surface area of griseofulvin and its chloroformate particles were examined using the Brunauer, Emmett, and Teller (131) method with nitrogen as the adsorbing gas. When ordinary crystals were sorbed with chloroform vapor under vacuum, the apparent volume of the crystal mass was increased by about 50%. But continued desorption and sorption did not change the volume.

A zigzag curve was obtained, and the size reduction was practically finished by one cycle of solvation and desolvation (Fig. 21) (107).

The specific surface area after each desolvation exceeded 2 m^2/g; that of the solvated crystals was about 1 m^2/g after the second solvation. Although no liquid was observed visually at the end of sorption of vaporous chloroform, a minute amount of liquid probably was formed between particles by such a capillary condensation. Therefore, the primary particles are bridged, so surface area is decreased considerably. It was concluded that repeated applications of the method influence the specific surface area only slightly. However, when a solid substance forms a molecular compound with a gas, its surface area increases continuously by repetition of sorption and desorption of the gas (107).

Solvates and Chemical Stability—A patent was granted (132) for light- and heat-stable hydrate crystals of the coenzyme form of vitamin B_{12}. The coenzyme form of vitamin B_{12} can exist as conventional crystals (not specified if anhydrous or not) and as two hydrates, A and B, containing 15% weight of water. Table IV shows the effect of 3 hr of exposure to direct sunlight and also of 1 hr of exposure to 85°. From these data, it can be seen that the hydrate form A is chemically more stable than the other two forms.

Hydrocortisone 21-*tert*-butylacetate can spontaneously oxidize in air and change from 11β-ols to 11-

ones (133). This reaction requires molecular oxygen and produces water. The reaction is accelerated by heat and greatly accelerated by free-radical initiators or UV light. Three crystalline phases of the compound were studied. Phase A is a form in which the crystallizing solvent is held within the crystal in nonstoichiometric proportion; it can be completely desolvated in a vacuum without changes in the X-ray diffraction pattern. Phase B is a stoichiometric solvate. Desolvation is accomplished only at elevated temperature and is accompanied by changes in the X-ray diffraction pattern. Phase C is a nonsolvated form. Esters subject to air oxidation are Phase A, while Phases B and C are resistant to oxidation. Prednisolone tebutate behaved similarly to a lesser degree.

Inclusion compounds are a specialized form of molecular compounds; they do not exhibit stoichiometry. The title "inclusion compounds" for this class of complexes was first used by Schlenk (134, 135). Inclusion compounds vary from the other molecular compounds in that hydrogen bonding plays a minor role or is even nonexistent (136). In all three general types of inclusion compounds, a guest molecule is physically entrapped in a host molecule. The constituents are usually present in constant, but not necessarily stoichiometric, proportions.

The formation of these inclusion compounds[2] or adducts are dependent on the respective molecular size and shape of the constituents. The hollow space in which the guest molecule is enclosed may be a channel, a layer, or a cage.

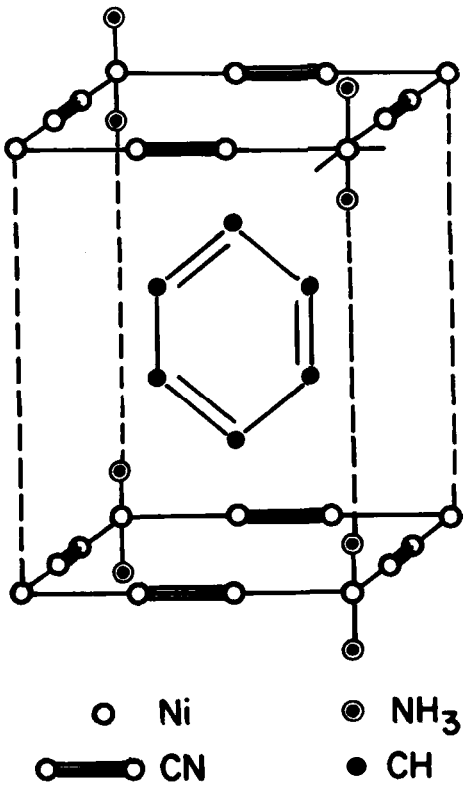


Figure 22—*Schematic representation of the clathrate of benzene with an ammonia–nickel cyanide complex* $[Ni(CN)_2NH_3—C_6H_6]$. *[Reproduced, with permission, from* Nature, ***163,*** *566(1949).]*

NONSTOICHIOMETRIC ADDUCTS-CLATHRATES

Definition—Clathrates are inclusion compounds. They were given this name by Powell (138) since the guest is enclosed or protected by crossbars of a grating. According to one source (139), a clathrate is a single-phased solid with two distinct components: the host and the guest. The guest is retained in closed cavities provided by the crystalline structure of the host. Generally, a cage and its enclosed molecule(s) are taken as a unit cell.

Figure 22 illustrates a typical clathrate where the guest, benzene, is clathrated in the host, ammonia–nickel cyanide. Since a crystalline structure cannot be easily deformed, a particular cage retains only molecules or atoms within a definite size and shape range. The lower limit of size is determined by the openings in the cage walls through which the guest components could escape. Two series of clathrates may have a common host component, but different cage structures, and contain physically different guest components. Moreover, the unit cells of some clathrates contain cages of two different sizes. A single clathrate of this type may have two different guest components.

The two components of a clathrate do not react chemically with each other, and a chemical equation implying bonds and chemical stoichiometry is inapplicable. Instead, a simple clathrate may be designated by a maximum-composition formula of $(nC)(mM)$, where C and M are the host and guest components, respectively; n is the number of C molecules per unit cage cell; and m is the maximum number of M molecules that can be accommodated in a single cage. The formula $(n/m)(CM)$ may also be used (139).

A special case of clathrate is a self-clathrate, built by incorporation of two identical but independent frameworks of the same compound. The polymorph VI of ice is built this way, where two independent tetrahedral frameworks interpenetrate but are not interconnected by hydrogen bonds. Each framework fills the void space in the other and, in reality, forms a self-clathrate (65).

Crystal Structures and Examples of Clathrates—Crystal structures of different clathrates have one thing in common; that is, they have enclosed spaces, which may be empty, partially occupied, or totally occupied with one or more kinds of guest molecules. On the other hand, there is no common structural characteristic of the enclosing part. The guest molecules, which have nonbonded distances with the host, have a nonstoichiometric relationship with each other. These guest molecules also have some interaction with their cage but cannot be firmly trapped in a cage that is not itself firmly constructed. This implies that the component parts of a host cage should be strongly bound together. The binding between the host atoms may be classified as covalent bonds, ionic bonds, metallic bonds, hydrogen bonds, or van der

[2] The general subject of inclusion compounds will be reviewed later (137).

Waals bonds. In any cage structure, more than one of these types may be involved.

The major classes of clathrates are hydroquinone clathrates, water clathrates[3], phenol clathrates, and clathrates of Dianin's compound. The only official monograph of a pharmaceutical clathrate is that of sodium warfarin clathrate in USP XVIII (140). This clathrate consists principally of sodium warfarin, isopropyl alcohol, and water, where the molecular proportions vary between 8:4:0 and 8:2:2. The expenditure of more time and effort will surely result in the discovery of more pharmaceutical clathrates as has happened with pharmaceutical polymorphs and solvates during the last 15 years.

Preparation of Clathrates—Preparation of most clathrates can be done easily under ordinary laboratory conditions. Most clathrates are generally prepared by recrystallization from solution, where the composition of the clathrate depends on the availability of the guest component at the site of crystal growth. Whenever a host is soluble in the guest, the preparation of the clathrate proceeds without difficulty, and a maximum clathrate composition is realized. Some problems may arise where a common solvent for both host and guest must be used. For example, when the guest concentration is low, slow crystallization with stirring is advised to ensure adequate concentration of the guest at the site of crystallization. Another problem is the choice of the common solvent, which should be inert as far as clathrate formation with the host.

In the case of clathrates formed with gases, high pressure is used to increase the concentration of the second component; at ordinary pressures, the solubility of the gas in the crystallization medium is not sufficient. This method was used to prepare hydroquinone clathrates of the inert gases argon (141), krypton (142), and xenon (143). A most unusual way of obtaining clathrates of substances that might not enclathrate has been suggested (138). This method involves the enclathration of molecules which will decompose within the host cage. Two factors must be satisfied: (*a*) the enclathrated compound must be capable of decomposition by the action of heat, UV light, or X-rays; and (*b*) the cage component must be stable under the decomposition conditions. For more details of the formation of clathrates, the reader is directed to the excellent review by Hagan (144).

Characterization of Clathrates—*Methods Used to Identify Clathrates*—X-Ray diffraction studies on a single crystal is the only sure way to construct the structure of clathrates totally. This is true for any crystalline modification of pharmaceuticals, be it polymorphs, solvates, or clathrates. Rayner and Powell (145, 146), using single crystals, found the crystal structure of the clathrate of benzene with an ammonia–nickel cyanide complex (Fig. 22) and also the crystal structure of hydrated nickel cyanide ammoniate.

X-Ray powder diffraction, although not as powerful a tool as the single-crystal X-ray studies, is a very potent tool for identification purposes. McAdie (147), in his work on urea–*n*-paraffin inclusion compounds, used this technique to differentiate urea from the urea–*n*-paraffin inclusion compound. This technique was not sensitive enough to differentiate the different chain-length paraffins. This finding might mean that once the host cage of an inclusion compound is formed, be it channel, cage, or layer type, X-ray powder diffraction does not vary with the change of the guest molecule. Another interesting observation was made by Mesley (148), who found that the X-ray powder diffraction of clathrates formed by chloroform with hydrocortisone, dexamethasone acetate, and prednisone did not change with various chloroform contents and only reverted to the pure steroid diffraction pattern when all chloroform was removed by heating.

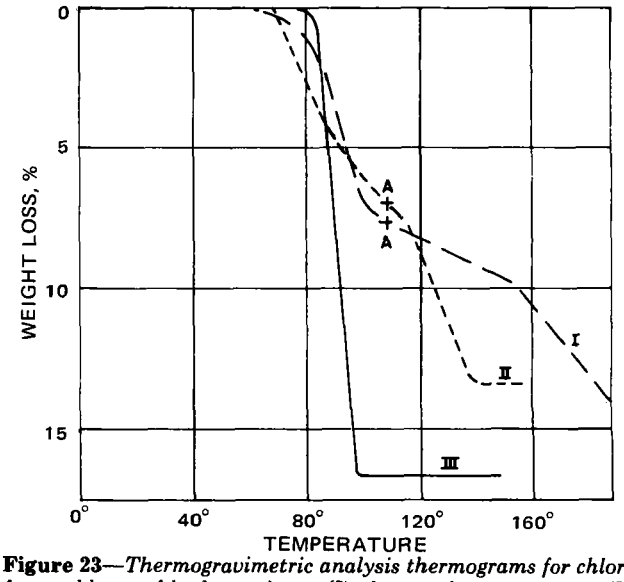


Figure 23—*Thermogravimetric analysis thermograms for chloroform adducts of hydrocortisone (I), dexamethasone acetate (II), and prednisolone (III). (Reproduced, with permission, from Ref. 148.)*

Due to the change of habits of different clathrates, microscopy, both plain and polarized, can be used to identify clathrates. Powell (141), in his work with clathrates of hydroxyquinone with argon, differentiated the large rhombohedra crystals of the clathrate from needle-shaped crystals of α-hydroxyquinone.

Using thermogravimetric analysis, Mesley (148), showed the thermogravimetric curves of hydrocortisone, dexamethasone acetate, and prednisone clathrates with chloroform (Fig. 23). According to this investigator, the chloroform adducts of hydrocortisone and dexamethasone acetate differed from that of prednisone in two respects. The thermogravimetric curves showed two distinct stages of weight loss compared with the rapid and complete decomposition of the prednisone compound. If the thermal decomposition of hydrocortisone and dexamethasone acetate is interrupted at points A in Fig. 23, the materials are still in the crystal form of the clathrate but contain relatively little chloroform. Thus, the initial weight loss is due to removal of chloroform from the lattice, while the second stage corresponds to the

[3] This group should not be confused with crystalline hydrates, as discussed in the solvate section. Here the water is the host of a clathrate host–guest system; in hydrates, water is the solvent of solvation.

breaking up of the lattice. In the case of hydrocortisone, this is followed immediately by steroid decomposition (148).

McAdie (147), working with urea–*n*-paraffin inclusion compounds, used differential thermal analysis. Although these were not clathrates as such, he differentiated the different chain lengths of the *n*-paraffins in the inclusion compounds. This result was just the opposite of that from X-ray powder diffraction, where he was unable to differentiate the inclusion compounds.

One common method used to identify clathrates has been IR spectrophotometry. Using IR, Mesley (148) was able to distinguish the chloroform both in the lattice and on the surface of hydrocortisone and dexamethasone acetate clathrates. Staveley (149) reviewed all of the different spectroscopic studies used in identifying clathrates including IR spectra, nuclear quadrupole resonance spectra, paramagnetic resonance studies, and NMR studies.

Methods Used to Identify the Guest Molecule—Most methods involve breaking the cage structure of the host to liberate the guest molecule for identification purposes.

Lowther and Williams (121) used a grating IR spectrophotometer with a conventional gas cell for identification of the inclusion compound in several clathrated pharmaceuticals found in the British Pharmacopoeia of 1968. This was done by liberating the guest by heat, trapping in a gas cell, and recording the absorption spectrum of the vapor. They were also able to calculate the percentage of solvent found in the clathrate.

Lowther and Williams (121) also used the GC method described in the British Pharmacopoeia of 1968 to identify the guest solvent in several pharmaceuticals.

Using mass spectrometry, Mandelcorn *et al.* (150), while working with the sulfur hexafluoride clathrate of Dianin's compound, calculated the amount of sulfur hexafluoride by melting the clathrate and determining the amount of gas released with a mass spectrometer.

Baker *et al.* (151) were able to use elemental analysis of the adduct when it contained a high proportion of nitrogen, sulfur, and halogen.

Titration can also be used to identify the guest molecule. If the adduct is an acid, the clathrate is dissolved in a suitable solvent and titrated with a suitable alkali (151).

Methods Used to Analyze Host–Guest Physical–Chemical Relationships—There is no doubt that a total X-ray diffraction analysis using single crystals is the only way to identify the host–guest physical–chemical relationships. This is illustrated by work on the crystal structure of the clathrate of benzene with an ammonia–nickel cyanide complex (145) and by work on the chlorine clathrate of water (152).

Other than X-ray diffraction, IR spectrophotometry is also used to check the host–guest physical–chemical relationships. Mandelcorn *et al.* (150), using this technique, were able to find that the interaction between sulfur hexafluoride and its enclosing Dianin's compound cage is very weak.

Properties of Clathrates—Clathrate compounds exhibit the general behavior of molecular compounds. They might have some properties that are those of the host, while others might indicate the presence of the guest. Also, some properties may be seen that are variations resulting from the mutual effect of the host and guest in close proximity.

Molecular Properties—According to Mandelcorn, the host of a clathrate is characterized by one or more permanent dipoles and/or an awkward molecular shape. Molecules not possessing such characteristics are usually arranged, in the crystalline state, as closely packed aggregates; the intermolecular distances are so small that the empty crystalline spaces cannot accommodate molecules larger than those of hydrogen. These molecular properties of the host cause intermolecular interactions and orientations in the crystalline state that lead to the open molecular arrangements essential for a clathrate structure (139). The clathrate structure of Dianin's compound is an example of the effects of both a permanent dipole and an awkward molecular shape (153).

Some weak interactions between a guest molecule and its surrounding cage are similar to those found in physical adsorption. These interactions range from very weak van der Waals intermolecular attractions to highly oriented dipole contacts. The resultant forces contribute to the stability of a clathrate. However, molecules whose interactions with the host are great enough to create new molecular species cannot form clathrates. Hydrogen sulfide interacts very weakly with water; it therefore forms a clathrate with water because of its appropriate molecular size. However, hydrogen chloride reacts chemically with water to form hydronium and chloride ions rather than remain undissociated in a water clathrate structure which accommodates molecules of that size (139).

Thermodynamics—Van der Waals (154) gave a statistical mechanical description of clathrate compounds of nonpolar gases. He first derived the general form of the partition function for a clathrate crystal, while leaving the potential field within a cavity unspecified, and then discussed the thermodynamic consequences of the general formulation. He calculated the potential field within the cavities using the Lennard-Jones and Devonshire method. This method permits the expression of thermodynamic properties of clathrates in terms of a few simple parameters.

Thermal Decomposition—According to Mandelcorn (139), the thermal decomposition of a clathrate into its host and guest at temperatures below its melting point may involve one or more of the following:

1. Sublimation of the host.
2. Change of the clathrate structure to the nonclathrate form of the host.
3. Diffusion of guest molecules through the lattice structure of the clathrate.
4. Desorption of the guest from the surface of the solid clathrate.

All of these processes, except Process 2, are characterized by kinetic expressions that include the geom-

etry of the decomposing clathrate particles. The activation energies of Processes 1 and 2 are determined by the crystalline lattice energies of the clathrate. The activation energy of Process 3 is determined by the lattice energy and guest molecule–cage interaction energy, and the activation energy of Process 4 is determined by the forces of attraction of the guest molecule to the surface of the solid clathrate. A kinetic interpretation of decomposition data can be extremely difficult when more than one of these processes is occurring (139).

Pharmaceutical Application of Clathrates—Although most applications of clathrates are not useful pharmaceutically, a brief review will be given of uses with potential application.

Purification—Evans *et al.* (155) purified benzene of one of its usual contaminants (thiophene) by clathrate formation. Although both thiophene and benzene form clathrates with monoamminenickel cyanide, benzene is more firmly held in the cage structure, so it is preferentially clathrated. The solid clathrate formed between benzene and monoamminenickel cyanide is separated from the solution by filtration. On dry distillation of the clathrate, the thiophene-free benzene is readily recovered.

Separation of Rare Gases—Rare gases were separated by selective clathration (156). Argon is separated from neon by adjusting the pressure conditions under which the hydroquinone–argon clathrate is formed; under the same pressure condition, neon does not form a clathrate with hydroquinone.

Separation of Optical Isomers—Another inclusion complexing substance that will separate optical isomers is tri-*o*-thymotide. This molecule is nonplanar and lacks both a mirror plane and a center of symmetry. It forms two stereochemical configurations, which resemble a three-bladed propeller and are optically active and interconvertible by heating. Slow crystallization provides the chance formation of either the *l*- or *d*-form. These forms have inclusion cavities that are mirror images. Thus, crystallization of trithymotide from a reactive solvent that is also a racemic mixture results in the preferential inclusion of either the *l*- or *d*-form of the solvent. This agent has been used to achieve the optical resolution of *sec*-butyl bromide (157).

Storage of Inert Gases—Hydroquinone–inert gas clathrates are granular materials whose gaseous constituents may be about 10% of the total weight. When the solid volume is taken into account, this gas concentration would exert a pressure of 90 atm in a similar free volume space. The gas can be released by heating or dissolving the clathrate. Therefore, these clathrates may be used for convenient storage of inert gases or to introduce such gases into fairly inaccessible locations. A predetermined amount of clathrate may be sealed into a container. Then, when heat is applied, the container is filled with a desired amount of gas (139, 141). This concept of storage of gases on a commercial basis was patented by Mandelcorn *et al.* (158).

Handling of Dangerous Materials—Cross *et al.* (159) found that dimethylmercury, which is a volatile and extremely toxic compound, can form a stable clathrate with 4-*p*-hydroxyphenyl-2,2,4-trimethylthiochroman as a host. This clathrate is very convenient for preparing solutions of known concentration of dimethylmercury; the preweighed quantity of the adduct is just dissolved in the appropriate solvent. Controlled release of guests that are difficult or hazardous to handle in the free state can be of considerable value in, for example, organic synthesis.

Mode of Action of Anesthetics—Pauling (160, 161), in his hydrate–microcrystal theory of anesthesia, proposed that nonhydrogen-bonding anesthetics work primarily due to the clathrate formation of the molecules of the anesthetic agent with the water contained in the neurons and around the neural network, both in the cells of the neurons and the synaptic regions between the neurons. These water molecules may trap some electrically charged side chains of neuronal proteins and interfere with the movement of ions in the synapses and neurons in such a way as to increase the impedance to the reverberating electric oscillations, thus reducing electrical activity to the point at which consciousness is lost (162).

CONCLUSIONS

Different techniques of identifying habits and characterization of amorphous or crystalline solids, along with their pharmaceutical application, have been discussed. Special emphasis has been given to differentiate polymorphs from molecular adducts, which can occur both stoichiometrically as solvates or nonstoichiometrically as clathrates. The rewards to formulators who characterize the habits and crystalline modification of active drugs and study their cause–effect relationships as part of preformulation studies include: *(a)* fewer processing and shelflife problems, along with rapid solution to problems that do arise; *(b)* the best bioavailable formulations; and *(c)* controlled production of the most efficacious dosage forms.

With all of these examples of the effects of habits, polymorphs, solvates, and clathrates on optimizing pharmaceutical formulations, this reviewer's hope is that crystal chemistry will become a routine part of every pharmaceutical company's preformulation program.

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Linear Drug Release from Laminated Hydroxypropyl Cellulose–Polyvinyl Acetate Films

SAUL BORODKIN ˣ and FLORENCE E. TUCKER

Abstract □ Release of drug from a single-layer film containing dispersed drug follows a diffusion-controlled matrix model, where the quantity released per unit area is proportional to the square root of time. The kinetics may be made linear with time (zero order) by laminating a second film without drug to the releasing side of the film with dispersed drug. In this manner, the drug layer serves as a reservoir and controls the duration of drug release, while the nondrug layer functions as a rate-controlling membrane. Zero-order drug release was demonstrated in such laminated films using 18–45% pentobarbital, methapyrilene, or salicylic acid contained in hydroxypropyl cellulose as the reservoir layer and mixtures of hydroxypropyl cellulose and polyvinyl acetate as the membrane layer. Inverse relationships between the release rate and membrane thickness and between the logarithm of the rate and the percentage of polyvinyl acetate in the membrane layer were observed. Of the three drugs tested, salicylic acid gave the fastest release rates while pentobarbital gave the slowest.

Keyphrases □ Drug release (methapyrilene, pentobarbital, salicylic acid) from laminated hydroxypropyl cellulose–polyvinyl acetate films—linear drug release, effects of membrane thickness and polyvinyl acetate content □ Polymer films, laminated hydroxypropyl cellulose–polyvinyl acetate—linear drug release (methapyrilene, pentobarbital, salicylic acid) □ Laminated hydroxypropyl cellulose–polyvinyl acetate films—linear drug release □ Hydroxypropyl cellulose–polyvinyl acetate films, laminated—linear drug release

The release kinetics of drugs dispersed in films composed of different ratios of hydroxypropyl cellulose and polyvinyl acetate were recently described (1). Drug release followed a diffusion-controlled matrix model, originally suggested by Higuchi (2, 3), where the quantity released per unit exposed area, Q, is proportional to the square root of time, t:

$$Q = \left[\frac{D\epsilon}{\tau}(2A - \epsilon C_s)C_s\right]^{1/2} t^{1/2} \qquad \text{(Eq. 1)}$$

where D and C_s refer to the diffusivity and solubility of the drug in the permeating fluid, respectively; A is the initial drug concentration; and τ and ϵ describe the tortuosity and porosity of the matrix, respectively. Although the release rates could be drastically altered by changing the polymer ratio and drug concentration, the Q *versus* $t^{1/2}$ linear relationship was inviolate.

For many dosage applications, linearity with time (zero-order release) would be desirable. Capsules (4), bandages (5), and tapes (6) that provide long-term uniform availability of drugs and pesticides have been described. Zero-order release from these devices appears to be obtained by maintenance of a constant concentration gradient through a membrane from a controlled concentration at the inner surface to zero concentration at the outer surface. This mechanism was recently reviewed (7), and it may be described by the following equation after an initial lag time:

$$\frac{dQ}{dt} = \frac{DC_0}{h} \qquad \text{(Eq. 2)}$$

where D is the diffusion constant through the membrane, C_0 is the concentration at the internal membrane surface, usually at saturation, and h is the membrane thickness. Analogously, zero-order release from a film should be attainable by laminating a polymer layer without drug to the releasing face of a film containing dispersed drug. In this way, the nondrug layer can serve as the rate-controlling membrane, while the drug-containing layer serves as a reservoir to maintain C_0 and the constant gradient.

This paper describes studies of drug release from such laminated films. Pentobarbital, methapyrilene, and salicylic acid contained in hydroxypropyl cellulose were used as the reservoir layers; the membrane layers were composed of different ratios of hydroxypropyl cellulose and polyvinyl acetate.

EXPERIMENTAL

Chemicals—Hydroxypropyl cellulose[1] was a food or pharmaceutical grade with an average molecular weight of 100,000, which gives a viscosity of 75–150 cps as a 5% solution in water. Polyvinyl acetate[2] had an average molecular weight of 500,000 and a viscosity of 90–110 cps as an 8.6% solution in benzene. Pentobarbital sodium and salicylic acid were USP grade, while methapyrilene hydrochloride was NF grade.

The pH 7.0 buffer used in all release rate studies was prepared by dissolving 28.9 g of $Na_2HPO_4 \cdot 7H_2O$ and 8.05 g of $NaH_2PO_4 \cdot H_2O$ in enough water to give 1 liter of solution.

Film Preparation—The reservoir layer films were cast from solutions containing 10% solute (drug plus hydroxypropyl cellulose), with a methylene chloride–methanol mixture (9:1) as the solvent. Hydroxypropyl cellulose was added as a dry powder by slow addition to the vigorously stirring solution. Salicylic acid was added as the dry powder, while pentobarbital and methapyrilene were incorporated from stock methylene chloride solutions prepared from the drug salts.

The pentobarbital stock solution, containing 60.8 mg/ml, was prepared by dissolving 12.0 g of pentobarbital sodium in 50 ml of water, adding 5 ml of concentrated hydrochloric acid, extracting three times with 50-ml portions of methylene chloride, and adjusting the volume of the combined extract to 180 ml. The methapyrilene base solution, containing 58.5 mg/ml, was prepared by dissolving 12.0 g of methapyrilene hydrochloride in 60 ml of water, adding 10 ml of 5 N NaOH, extracting three times with methylene chloride, and adjusting the volume of the combined extract to 180 ml. Films were cast from the solutions at various wet thicknesses

[1] Klucel LF, Hercules, Inc., Wilmington, DE 19899
[2] Gelva V-100, Monsanto Co., St. Louis, MO 63166

(0.64–2.54 mm) by spreading with a knife[3] on Teflon-coated plate glass and allowing the films to air dry at least 24 hr before combining with the membrane layer.

The membrane layers were prepared separately by slowly adding hydroxypropyl cellulose and polyvinyl acetate to a methylene chloride–methanol mixture (9:1) with vigorous stirring to give a solution containing 10% solutes. The solutions were spread on Teflon-coated plate glass with a knife[3], and the films were allowed to air dry at least 24 hr before lamination to the reservoir layer.

The laminated films were prepared by cutting a section (approximately 5 × 5 cm) of the drug reservoir film, measuring its thickness in five places with a micrometer[4], placing it on Teflon-coated plate glass, and spraying one side with methylene chloride. A slightly larger section of the membrane layer, of which the thickness had been previously measured in five places with a micrometer, was immediately pressed on the tacky side of the drug film. The laminated film was allowed to air dry for 24 hr and inspected to assure adhesion between the two layers before use in release rate testing.

Release Rate Determinations—Rectangular films, 2.2 × 4.0 cm (8.8 cm^2), were obtained by cutting a section of the laminated film with a razor blade; a microscope cover glass was used as a template. A thin coating of high vacuum silicone lubricant[5] was applied to a 2.54 × 7.62-cm microscope slide and the exposed face of the drug reservoir layer. The film was then carefully pressed onto the slide with the membrane layer exposed, making sure that all edges adhered and were below the silicone lubricant level but that no lubricant touched the exposed surface. The water repellency of the silicone lubricant covering the film edges prevented any drug release from the edges of the reservoir layer. Studies in which the reservoir layer surface was coated with silicone lubricant and exposed to release conditions showed only insignificant drug dissolution after 7 hr.

The slide was placed film side up at about a 45° angle into a 250-ml beaker in a 37° water bath containing 200 ml of pH 7.0 buffer preheated to 37°. A nonagitated system was selected to eliminate any effect of turbulence on the release rate as well as to prevent any disruption of the film. Periodic assay samples (generally hourly for 8 hr) were obtained by removing the slide, stirring the solution, and pipetting a 5-ml sample. The slide was quickly reinserted, making sure that the film remained completely immersed throughout the release study. The beaker was kept covered[6] except during sampling to prevent evaporation. After 24 hr, the run was terminated and the solution was assayed.

All samples were assayed by UV spectrophotometry[7] after the required dilution in the specific solvent used for each drug. Pentobarbital was assayed at 240 nm in 0.1 *N* NH_4OH, methapyrilene was assayed at 312 nm in 0.1 *N* HCl, and salicylic acid was assayed at 297 nm in 0.1 *N* NaOH.

RESULTS AND DISCUSSION

Drug Release Kinetics—Drug release kinetics from films containing dispersed drug could be changed from linearity with the square root of time to linearity with time (zero order) by laminating a membrane layer to the releasing surface. This effect was demonstrated in many such laminated films using a hydroxypropyl cellulose matrix in the reservoir layer and hydroxypropyl cellulose–polyvinyl acetate composite mixtures in the membrane layer. Figures 1–3 compare drug release from films containing dispersed drug with and without membrane layers. The results of all release rate studies from the laminated films are summarized in Tables I–III. The high correlation coefficients obtained from linear regression analysis (9) of the *Q versus t* treatment attest to the zero-order release kinetics.

The slopes obtained by plotting the amount released per area *versus* time are designated in the tables by an apparent zero-order rate constant, k_0, with the units of milligrams centimeters^{-2} hours^{-1}. Integration of Eq. 2 and evaluation of the integration constant in terms of the lag time (intercept on the time axis obtained when steady-state rate is extrapolated), t_{lag}, yield:

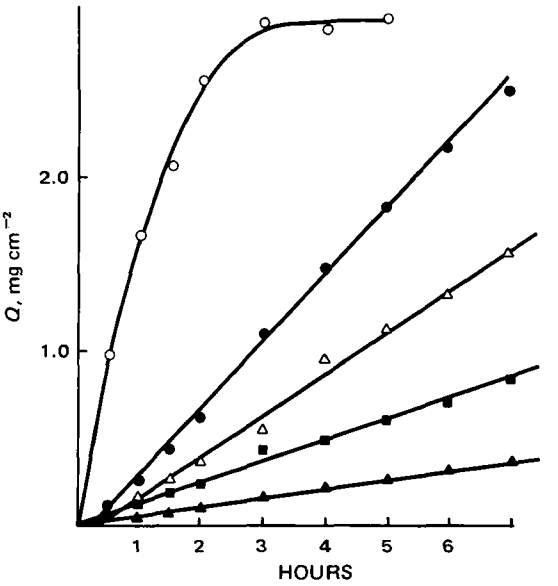

Figure 1—*Drug release from films containing 18.2% pentobarbital in hydroxypropyl cellulose as the reservoir layer. Key:* ○, *no membrane layer;* ●, *0.091-mm hydroxypropyl cellulose–polyvinyl acetate (9:1) membrane;* △, *0.137-mm hydroxypropyl cellulose–polyvinyl acetate (8:2) membrane;* ■, *0.128-mm hydroxypropyl cellulose–polyvinyl acetate (6:4) membrane; and* ▲, *0.093-mm hydroxypropyl cellulose–polyvinyl acetate (4:6) membrane.*

$$Q = \frac{DC_0}{h}t - \frac{DC_0}{h}t_{lag} \qquad \text{(Eq. 3)}$$

For a given film in which the membrane remains intact, D, C_0, and h are constant through most of the release, and the apparent zero-order rate constant may be defined as:

$$k_0 = \frac{DC_0}{h} \qquad \text{(Eq. 4)}$$

and:

$$Q = k_0 t - k_0 t_{lag} \qquad \text{(Eq. 5)}$$

Generally, the lag times were less than 15 min. However, in some cases, especially with methapyrilene as the drug, lag times of greater than 30 min were observed before attainment of steady-state release.

No correlation could be seen between the membrane thickness and lag time, as might be expected from the theoretically developed equation (7):

$$t_{lag} = \frac{h^2}{6D} \qquad \text{(Eq. 6)}$$

It appears likely that other factors, such as hydration of the film or attainment of saturation conditions within the reservoir layer, were more dominant in establishing steady-state conditions and the lag time.

The zero-order relationship would be expected to break down before total drug release when the reservoir layer is no longer able to sustain C_0 at the interface of the two layers. As reflected by the half-lives shown in Tables I–III, none of the laminated films gave complete release in the 7–8-hr release times generally studied. However, in a number of films, the release went well beyond the half-life with no observed deviation from zero-order linearity. In all cases where release was calculated to be incomplete at 24 hr ($t_{1/2} > 12$ hr), the 24-hr assays were in line with those projected by k_0.

Lamination of a membrane layer to a film containing dispersed drug results in slower drug release, as well as a change in kinetics, due to the barrier nature of the membrane layer. This does not mean that all laminated films must release drug at slower rates than nonlaminated films and single- and double-layer films with comparable rates, but different release patterns can be fabricated. Figure 4 compares the release obtained from two salicylic acid films: one a single-layer film following the Q *versus* $t^{1/2}$ linear rela-

[3] Gardner Laboratories, Bethesda, MD 20014
[4] L. S. Starrett Co., Athol, Mass.
[5] Dow Corning Corp., Midland, MI 48640
[6] Parafilm M, American Can Co., Neenak, Wis.
[7] Coleman model 124, Perkin-Elmer Co., Norwalk, Conn.

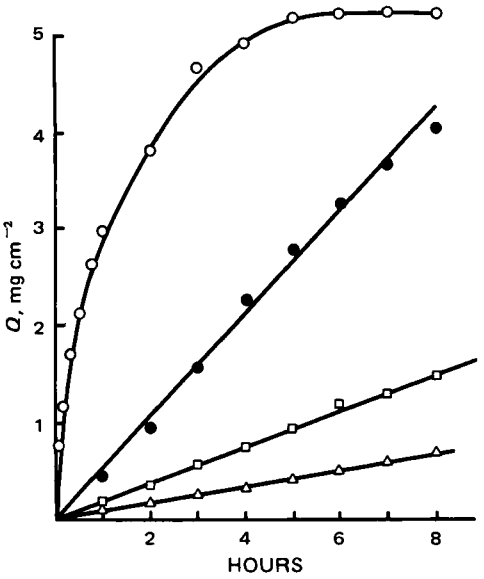

Figure 2—*Drug release from films containing 20% salicylic acid in hydroxypropyl cellulose as the reservoir layer. Key: ○, no membrane layer; ●, 0.164-mm hydroxypropyl cellulose–polyvinyl acetate (8:2) membrane; □, 0.204-mm hydroxypropyl cellulose–polyvinyl acetate (6:4) membrane; and △, 0.164-mm hydroxypropyl cellulose–polyvinyl acetate (4:6) membrane.*

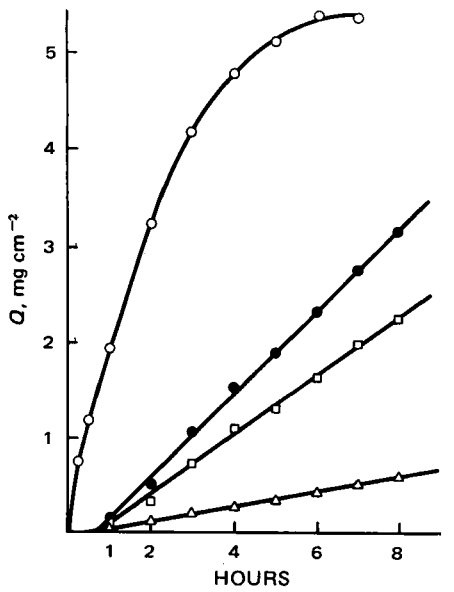

Figure 3—*Drug release from films containing 26.3% methapyrilene in hydroxypropyl cellulose as the reservoir layer. Key: ○, no membrane layer; ●, 0.171-mm hydroxypropyl cellulose–polyvinyl acetate (9:1) membrane; □, 0.160-mm hydroxypropyl cellulose–polyvinyl acetate (7:3) membrane; and △, 0.210-mm hydroxypropyl cellulose–polyvinyl acetate (5:5) membrane.*

tionship and the other a laminated type following zero-order kinetics. Although both gave approximately 60% release in 6 hr, the release kinetics were quite different.

Effect of Membrane Thickness—Equation 4 indicates that the apparent zero-order rate constant, k_0, should be inversely proportional to the membrane layer thickness. Figure 5 shows this relationship using three different thicknesses of pure hydroxypropyl cellulose as the membrane layers; Fig. 6 shows similar results with the much slower releasing hydroxypropyl cellulose–polyvinyl acetate (5:5) membranes.

The linearity of the observed rate constant with the reciprocal of the measured membrane thickness allows calculation of a thickness-independent zero-order constant, k_0', with the dimensions milligrams centimeters^{-1} hours^{-1}, where:

$$k_0' = k_0 h \qquad \text{(Eq. 7)}$$

These values, which should approximate DC_0, are also included in Tables I–III. Their independence of membrane thickness makes them more useful in comparing films of different composition.

The hydrophilic nature of the membrane layer would indicate that the fully hydrated swelled membrane would be the true barrier layer. This would still be consistent with the zero-order release model, although the rate of attaining full hydration might affect the lag time.

During dosage development, it may be beneficial or desirable to increase the initial release of drug. This approach may be necessary to overcome a lag time or to establish a useful therapeutic level which might be followed by a maintenance rate. This can con-

Table I—Zero-Order Release Rates Obtained from Laminated Pentobarbital Films

Membrane: Hydroxypropyl Cellulose–Polyvinyl Acetate Ratio	Membrane: h, mm	Reservoir[a]: Drug, %	Reservoir[a]: h, mm	k_0[b], mg cm^{-2} hr^{-1}	k_0', mg cm^{-1} hr^{-1} × 10^3	$t_{1/2}$, hr	Correlation Coefficient
10:0	0.088	45.5	0.147	0.496 ± 0.028	4.36	8.1	0.998
10:0	0.090	45.5	0.192	0.503 ± 0.030	4.52	10.4	0.998
10:0	0.111	45.5	0.137	0.408 ± 0.040	4.52	8.2	0.994
10:0	0.143	18.2	0.172	0.304 ± 0.013	4.36	6.1	0.999
9:1	0.075	36.4	0.138	0.428 ± 0.043	3.21	6.8	0.994
9:1	0.091	18.2	0.187	0.385 ± 0.021	3.50	5.3	0.998
8:2	0.063	36.4	0.141	0.431 ± 0.036	2.71	6.3	0.993
8.2	0.074	18.2	0.167	0.418 ± 0.031	3.10	4.2	0.997
8:2	0.114	18.2	0.161	0.304 ± 0.030	3.47	5.7	0.995
8:2	0.137	18.2	0.164	0.245 ± 0.020	3.35	7.3	0.996
6:4	0.049	18.2	0.218	0.296 ± 0.010	1.45	8.7	0.999
6:4	0.128	18.2	0.204	0.121 ± 0.008	1.54	14.7	0.997
6:4	0.136	27.3	0.149	0.094 ± 0.013	1.28	23.3	0.990
6:4	0.142	36.4	0.118	0.102 ± 0.008	1.44	22.6	0.995
6:4	0.137	45.5	0.173	0.077 ± 0.002	1.06	52.8	0.999
5:5	0.136	18.2	0.183	0.037 ± 0.002	0.51	51.2	0.999
5:5	0.092	27.3	0.142	0.063 ± 0.002	0.57	33.3	1.000
5:5	0.128	27.3	0.134	0.037 ± 0.001	0.47	51.0	0.999
5:5	0.197	27.3	0.150	0.028 ± 0.002	0.56	76.8	0.998
5:5	0.230	27.3	0.141	0.021 ± 0.001	0.48	96.6	0.999
4:6	0.051	18.2	0.181	0.074 ± 0.003	0.37	24.1	0.999
4:6	0.093	18.2	0.245	0.054 ± 0.003	0.50	40.3	0.998

[a] Reservoir layer composed of pentobarbital in hydroxypropyl cellulose. [b] Slope of Q *versus* t plot ± 95% confidence limits (9).

Table II—Zero-Order Release Rates Obtained from Laminated Salicylic Acid Films[a]

Membrane Layer: Hydroxypropyl Cellulose–Polyvinyl Acetate Ratio	h, mm	k_0[b], mg cm^{-2} hr^{-1}	k_0', mg cm^{-1} hr^{-1} × 10^3	$t_{1/2}$, hr	Correlation Coefficient
10:0	0.176	0.543 ± 0.054	9.56	5.4	0.995
9:1	0.171	0.540 ± 0.058	9.25	5.4	0.994
8:2	0.164	0.530 ± 0.048	8.69	5.5	0.996
7:3	0.169	0.327 ± 0.017	5.53	8.9	0.999
6:4	0.204	0.190 ± 0.012	3.88	15.3	0.998
5:5	0.187	0.156 ± 0.010	2.92	18.7	0.998
4:6	0.164	0.083 ± 0.005	1.36	35.2	0.998
3:7	0.177	0.059 ± 0.001	1.04	49.5	1.000

[a] Reservoir layer composed of 20.0% salicylic acid in hydroxypropyl cellulose with a thickness of 0.256 ± 0.012 mm. [b] Slope of Q *versus* t plot ± 95% confidence limits (9).

Table III—Zero-Order Release Rates Obtained from Laminated Methapyrilene Films[a]

Membrane Layer: Hydroxypropyl Cellulose–Polyvinyl Acetate Ratio	h, mm	k_0[b], mg cm^{-2} hr^{-1}	k_0', mg cm^{-1} hr^{-1} × 10^3	$t_{1/2}$, hr	Correlation Coefficient
10:0	0.173	0.441 ± 0.018	7.63	8.8	0.999
9:1	0.171	0.432 ± 0.019	7.38	9.0	0.999
8:2	0.151	0.495 ± 0.035	7.48	7.8	0.998
7:3	0.160	0.307 ± 0.019	4.91	12.6	0.998
6:4	0.222	0.097 ± 0.011	2.14	40.1	0.996
5:5	0.210	0.071 ± 0.003	1.49	54.5	0.999
4:6	0.179	0.041 ± 0.004	0.74	93.9	0.996
3:7	0.193	0.022 ± 0.005	0.42	176.6	0.987

[a] Reservoir layer composed of 26.3% methapyrilene in hydroxypropyl cellulose with a thickness of 0.259 ± 0.006 mm. [b] Slope of Q *versus* t plot ± 95% confidence limits (9).

ceivably be accomplished by including a low level of drug in the membrane layer.

Reservoir Layer—To achieve the constant concentration gradient in the membrane layer required for zero-order release, the drug layer must continually provide sufficient drug to maintain a constant concentration, C_0, at the inner membrane surface. This condition may be achieved if C_0 is equivalent to saturation and the transfer rate from the reservoir is rapid enough to maintain saturation. Alternatively, C_0 may remain constant through partitioning from a constantly maintained saturated solution phase in the reservoir layer. Such a condition can exist if the reservoir layer contains a high dispersed drug concentration relative to the solution phase level ($A \gg \epsilon C_s$ in terms of Eq. 1) and transfer of drug through the reservoir matrix is faster than loss through the membrane.

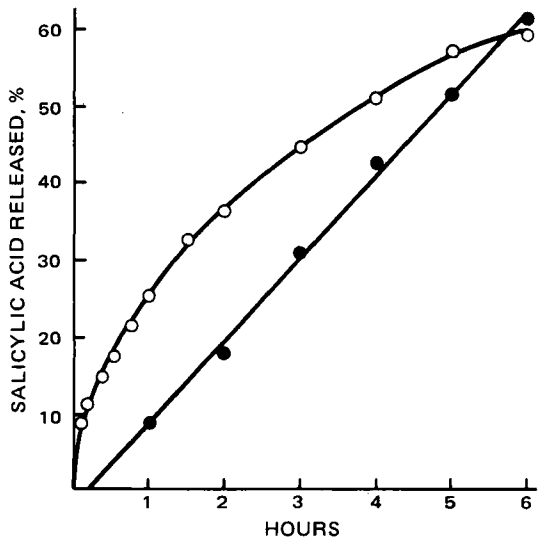

Figure 4—*Comparison of salicylic acid release from single-layer and laminated films. Key: ○, single-layer film composed of 20% drug in hydroxypropyl cellulose–polyvinyl acetate (3:7) (0.156 mm); and ●, laminated film, reservoir is 20% drug in hydroxypropyl cellulose (0.256 mm), and membrane is hydroxypropyl cellulose–polyvinyl acetate (9:1) (0.171 mm).*

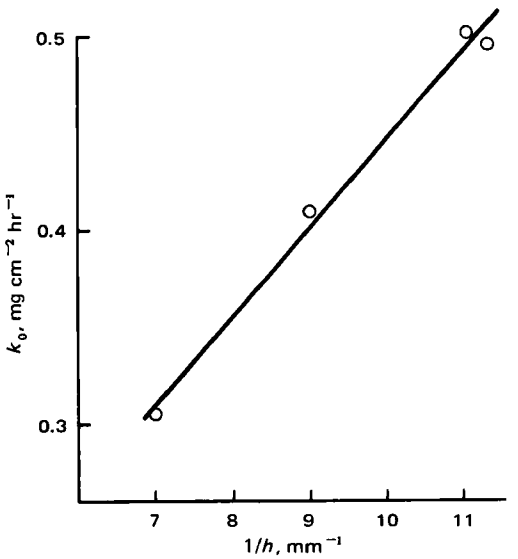

Figure 5—*Relationship of pentobarbital release rate to the reciprocal of membrane layer thickness from laminated films containing pure hydroxypropyl cellulose as the membrane layer.*

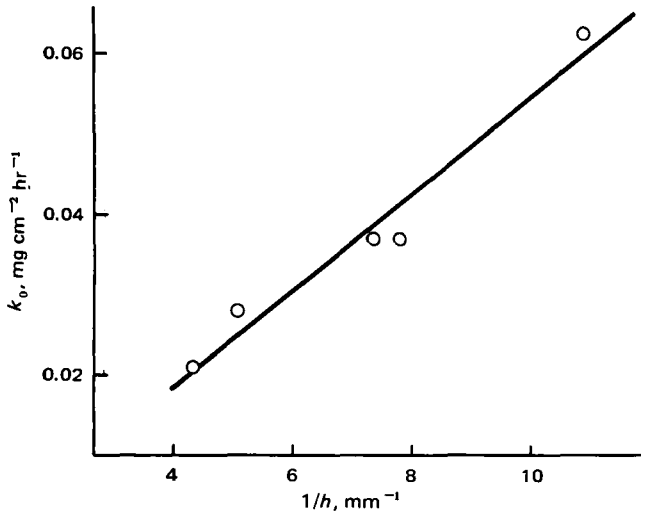

Figure 6—*Relationship of pentobarbital release rate to the reciprocal of membrane layer thickness from laminated films containing hydroxypropyl cellulose–polyvinyl acetate (5:5) as the membrane layer.*

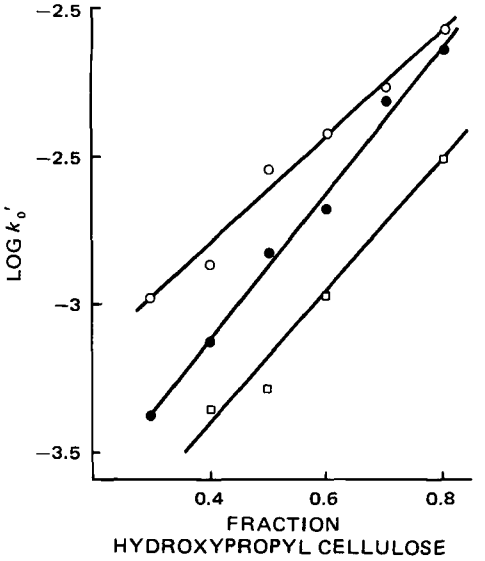

Figure 7—*Relationship of log k_0' to the fraction of hydroxypropyl cellulose in the membrane layer. Key: ○, salicylic acid; ●, methapyrilene; and □, pentobarbital.*

The conditions conducive to maintaining C_0 and zero-order release were achieved by using drug concentrations of greater than 18% and pure hydroxypropyl cellulose as the matrix. This matrix had been shown previously to give the fastest diffusion rates (1). Matrixes with slower diffusion rates were not tested.

The drug concentration in the reservoir layer should not affect the release rate as long as it exceeds the level necessary for maintaining saturation. The data in Table I show that similar k_0' values were obtained for laminated films with similar membrane matrixes but different pentobarbital reservoir concentrations. In studies using less than 10% drug concentration in the reservoir layer and fast releasing membrane layers, zero-order release was not obtained.

Although the drug concentration above the critical level should not affect the release rate, it controls the duration of drug release. The more concentrated the reservoir, the longer it will be able to supply drug to maintain C_0. Similarly, the duration of release, as reflected by the half-life, may be controlled by the thickness of the reservoir layer.

Polymer Ratio in Membrane—As shown in Tables I–III, all three drugs behaved similarly with regard to the hydroxypropyl cellulose–polyvinyl acetate ratio in the membrane layer. Increasing the polyvinyl acetate concentration from 0 to 20% affected the release rate only slightly. Beyond 20%, there was a marked decrease in release rate with further increases in the polyvinyl acetate content, with each 10% reducing the rate by approximately one-third to one-half.

Attempts to relate mathematically the release rate to the hydroxypropyl cellulose–polyvinyl acetate ratio yielded the best results when the logarithm of the thickness-independent rate contant, k_0', was plotted against the fraction of hydroxypropyl cellulose in the membrane (Fig. 7). The relationship may be depicted by the following empirical equation:

$$\log k_0' = k_R F_H + \log (k_0')_p \qquad \text{(Eq. 8)}$$

where F_H is the fraction of hydroxypropyl cellulose in the membrane layer, k_R is the slope specific for a given drug and reservoir, and $(k_0')_p$ is the extrapolated rate constant for a pure polyvinyl acetate membrane ($F_H = 0$). In practice, pure polyvinyl acetate membranes that could be incorporated into laminated films could not be fabricated without plasticizers. Table IV lists the calculated values obtained using Eq. 8. This logarithmic relationship between the rate constant and fraction of hydroxypropyl cellulose in the membrane is quite similar to that found earlier with homogeneous films containing dispersed drug (1).

The greater barrier characteristics obtained with increased polyvinyl acetate percentages in the membrane layer may be attributed to its lower hydrophilicity relative to hydroxypropyl cellulose. Weight gain studies with films suspended in a 100% relative humidity atmosphere at 37° showed very rapid equilibration, with the percentage moisture absorbed increasing with hydroxypropyl cellulose content from 4% with pure polyvinyl acetate to 32% with pure hydroxypropyl cellulose. The lower hydration could cause reduced drug release rates by lower drug solubility, less porosity, or increased tortuosity.

Table IV—Linear Relationship of Log k_0' to Fraction of Hydroxypropyl Cellulose in Membrane Layer

Drug	Concentration in Reservoir Layer, %	Slope (k_R)	$(k_0')_p$, mg cm^{-2} hr^{-1} × 10^3	Correlation Coefficient
Salicylic acid	20.0	1.88	0.282	0.991
Methapyrilene	26.3	2.54	0.074	0.997
Pentobarbital	18.2–45.5	2.29	0.048	0.979

With pure hydroxypropyl cellulose as the membrane layer, it might appear that the membrane would simply be a depleted matrix from a single-phase film and the zero-order release model would not be applicable. However, all results showed zero-order release well beyond the half-life. Apparently, the presence or absence of initial drug imparts sufficiently different characteristics so that the two layers retain individual behavior, with drug saturation in the reservoir layer predominating longer than drug release through the membrane layer.

Drug Effect—With similar polymer compositions, the relative release rates for different drugs in laminated films would be governed primarily by the drug solubility and diffusion coefficient in the membrane layer. The drug solubility in the reservoir layer might also affect C_0 and the release rate through partitioning.

Of the three drugs tested, salicylic acid gave the fastest release rates while pentobarbital gave the slowest. The relatively slower release rates with pentobarbital can be most easily attributed to its much lower solubility in pH 7.0 buffer at 37° (3 mg/ml *versus* greater than 600 mg/ml for methapyrilene and salicylic acid, which are primarily in the ionized form). Although the drug solubility in the external solution should not directly affect the release rate under sink conditions, permeation of buffer into the hydrated membrane layer could give some buffering effect within the membrane.

The faster release rates obtained with salicylic acid relative to methapyrilene may be due primarily to its lower molecular weight (138.1 *versus* 261.4). Since the diffusion coefficient is inversely proportional to the cube root of the molecular weight (10), salicylic acid would give a diffusion coefficient approximately 1.24 times greater than methapyrilene with all other variables equal.

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Pharmacokinetics of Polychlorinated Biphenyl Components in Swine and Sheep after a Single Oral Dose

R. E. BORCHARD [*], M. E. WELBORN, W. B. WIEKHORST, D. W. WILSON, and L. G. HANSEN [x]

Abstract □ Single-dose oral administration of a commercial polychlorinated biphenyl product containing 54% chlorine provided data with which to plot the time course of total polychlorinated biphenyl and individual components in the blood of swine and sheep. Pharmacokinetic parameters describing absorption from the gut and elimination from a two-compartment body system were determined for the components in swine and sheep. The absorption half-time for total polychlorinated biphenyl in swine was 1.13 hr while that for sheep was 3.83 hr. The half-time for disposition of total polychlorinated biphenyl from the central compartment was 4.4 hr in swine and 7.7 hr in sheep; the apparent biological half-life was 62.4 hr in swine and 78.8 hr in sheep. Individual components varied significantly from each other and from total polychlorinated biphenyl in all parameters.

Keyphrases □ Polychlorinated biphenyl components—pharmacokinetics in swine and sheep after single oral dose □ Pharmacokinetics—polychlorinated biphenyls in swine and sheep after single oral dose

The ecological persistence of polychlorinated biphenyls and their continual release into the environment in recent years have created a situation in which contamination of animal feed as well as human food is a constant threat. Since commercial preparations are mixtures of some 200 possible isomers and any given residue probably represents more than a single polychlorinated biphenyl mixture, it becomes important to evaluate the individual components as well as total polychlorinated biphenyl. Furthermore, such a mixture permits the simultaneous comparison of several different compounds in the same animal, and studies using single purified components can be used to assess the extent of interactions within the mixture.

One means of assessing exposure to polychlorinated biphenyls and persistence of the chemicals in the body is by way of blood sampling. Previous studies characterized the polychlorinated biphenyl mixture used (1), developed and refined methods of extraction and analysis (2), and determined pharmacokinetic parameters of individual components in swine and sheep after intravenous administration (3). Reports of investigations of pharmacokinetic parameters after feeding contaminated feed are in preparation.

This paper deals with the time course of polychlorinated biphenyls in the animal body as determined by blood sampling after a single oral dose of a commercial polychlorinated biphenyl containing 54% chlorine[1] to swine and sheep.

EXPERIMENTAL

Three sheep and three swine were given a calculated oral dose of a polychlorinated biphenyl containing 54% chlorine by weight[1] in a gelatin capsule. The dosage was 30 mg/kg for the sheep and 15 mg/kg for the swine; pilot studies indicated that these levels were necessary to provide adequate blood concentrations for analysis.

The sheep were mature ewes of mixed mutton-type breeding and weighed 53.5, 57.0, and 66.5 kg; the swine were of Hampshire-Yorkshire breeding and weighed 48.0, 59.5, and 57.2 kg. Both sheep and swine were maintained in individual steel cages with slot floors. Blood samples were obtained from the sheep by jugular puncture for 7 days and from the swine by femoral venous cannulas, fitted 24 hr prior to dosing, for 2 days.

The levels in swine blood collected after 48 hr were not adequate to permit accurate quantitation, and most of the β values were estimated from only one or two points along with the computer-generated tangent; therefore, the parameters for elimination from the tissue compartment in swine should be viewed with reservation. Further investigations will be conducted to determine these values more accurately.

[1] Aroclor 1254, Monsanto electrical grade No. KB-05-612.

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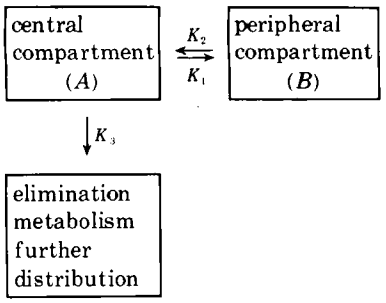


Scheme I

Blood samples were extracted with hexane-saturated acetonitrile and analyzed for polychlorinated biphenyls by electron-capture GLC as previously described (2).

Quantitation of components as well as total polychlorinated biphenyl for each blood sample was accomplished by the methods previously utilized (1–3).

The blood concentrations of each component and total polychlorinated biphenyl were plotted and fit to a two-compartment model utilizing a computer program (4) for the elimination phase and a graphical method for the absorption phase. By using estimates obtained from data plots, the computer program generated four parameters (A, α, B, and β) which describe elimination for a two-compartment open model (Scheme I), where K_1 and K_2 are first-order distribution rate constants between the central compartment *(A)* and the peripheral compartment *(B)*; K_3 is the overall elimination rate constant.

The integrated mathematical equation for Scheme I is:

$$C_t = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

where:

C_t = blood concentration at time t
A = blood zero time concentration intercept for the rapid disposition curve
B = blood zero time concentration intercept for the slow disposition curve
α = first-order rate constant for disposition from the central compartment to the peripheral compartment
β = apparent elimination rate constant

It was established previously that the peripheral compartment consists, at least in part, of fatty tissues in sheep and swine[2].

With oral administration, one can modify the model as shown in Scheme II, where G is the enteric reservoir (gut) from where absorption occurs, and K_a is the rate constant of absorption from the gut.

The integrated mathematical model approximates:

$$C_t = Ae^{-\alpha t} + Be^{-\beta t} - C_0e^{-K_a t} \qquad \text{(Eq. 2)}$$

where $C_0 = A + B$ (generally determined from intravenous elimination data).

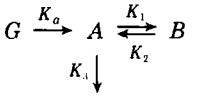
In this paper, the absorption rate constants, K_a, of the components and total polychlorinated biphenyl were determined by a method similar to the conventional one for one-compartment models. The value of K_a was determined from the slope[3] of the line when $(\bar{C}_t - C_t)$ is plotted semilogarithmically against time, where $(\bar{C}_t - C_t)$ = the difference between the predicted elimination line, extrapolated back to zero time, and the actual absorption data points.

The approximate integration of the mathematical model, as used here, then becomes:

$$C_t = Ae^{-\alpha t} + Be^{-\beta t} - Xe^{-K_a t} \qquad \text{(Eq. 3)}$$

where X = the zero time intercept of line $(\bar{C}_t - C_t)$ determined from oral elimination data.

[2] Unpublished data from this laboratory.
[3] Determined with a Wang 500 calculator exponential regression program.



Scheme II

Other parameters were calculated as follows:

$$t\tfrac{1}{2}\alpha = \frac{0.693}{\alpha} \qquad \text{(Eq. 4)}$$

$$t\tfrac{1}{2}\beta = \frac{0.693.}{\beta} \text{ (biological half-life)} \qquad \text{(Eq. 5)}$$

$$t\tfrac{1}{2} \text{ absorption} = 0.693/K_a \qquad \text{(Eq. 6)}$$

$$K_1 = \alpha + \beta - K_2 - K_3 \qquad \text{(Eq. 7)}$$

$$K_2 = \frac{A\beta + B\alpha}{A + B} \qquad \text{(Eq. 8)}$$

$$K_3 = \frac{\alpha\beta}{K_2} \qquad \text{(Eq. 9)}$$

Where applicable, component results were compared statistically by multiple-range and paired t tests (5).

RESULTS

The absolute blood concentrations for components and total polychlorinated biphenyl after a single oral dose are given in Table I for swine and in Table II for sheep. Absolute component concentrations were determined by utilizing the component percentage of the commercial mixture and concentrations relative to the standard mixture as previously discussed (3).

The components were first detected in swine blood at 0.5–1.0 hr after administration, and maximum blood concentrations were seen in 4–8 hr. As seen in Table III, components of a longer relative retention time and a larger percentage of chlorination (1) took longer to attain maximum concentrations. Component 286 attained a significantly greater ($p < 0.05$) relative blood concentration (and blood–dose ratio) than total polychlorinated biphenyl and all other components except Component 332.

The components were first detected in sheep blood at 2–4 hr after administration, and maximum blood concentrations occurred at 8–24 hr. As seen in Table IV, components of a longer retention time and a larger percentage of chlorination attained maximum concentrations later, just as in the swine. Components 286 and 332 attained significantly greater ($p < 0.05$) relative blood concentrations (and blood–dose ratios) than Components 70, 99, and 105 and total polychlorinated biphenyl. Component 286 was also greater than 84.

By comparing blood–dose ratios of swine to those of sheep (Table IV), one sees a significantly greater ratio for all components and total polychlorinated biphenyl in swine. Thus, for a given dose, one would expect to see higher blood concentrations in swine, which is consistent with results obtained after feeding a ration containing the same commercial mixture[2].

The blood concentrations of each commercial mixture component and total polychlorinated biphenyl were plotted as the mean concentration for swine and sheep at each sampling time. The data representing the elimination phase were fit to the two-compartment model for elimination, and the data representing the absorption phase were utilized for calculation of absorption rates, based on the extrapolated elimination curve. Examples of the data plots are given for both swine and sheep in Figs. 1–4 for Component 127. Figures 1 and 3 show the absorption phase of Component 127, and Figs. 2 and 4 show the complete elimination phase.

Fitted elimination curves for all components and total polychlorinated biphenyl are shown for swine in Fig. 5 and for sheep in Fig. 6. The slopes for each component in the figure for swine or sheep are the same as those resulting from plotting the data in Tables I and II; their positions on the graph vary depending on whether the values relative to the commercial mixture (Figs. 5 and 6) or the absolute values (Tables I and II) were used. For example, the relative concentration of Component 286 (Fig. 5) was greater than all others, but the absolute concentration (Table I) was only greater than Component 332.

Table I—Mean Absolute Blood Concentrations (Parts per Billion) of Polychlorinated Biphenyl Components after a Single Oral Dose in Swine

RRT[a]	Hours											
	1	2	3	4	5	6	8	12	18	24	30	48
70	8	59	158	202	185	120	79	45	13	10	6	4
84	15	58	169	241	261	217	176	111	49	39	27	22
99	6	32	90	120	144	116	92	63	30	22	15	12
105	5	37	101	121	149	118	101	54	18	12	9	6
127	8	61	184	255	297	253	213	133	55	31	20	16
149	7	47	134	195	219	196	186	120	63	41	23	14
176	5	22	71	114	122	130	145	93	46	30	24	16
208	1	5	15	25	34	32	36	23	12	7	5	4
253	1	4	16	23	31	32	37	24	12	8	6	5
286	3	3	8	8	11	13	19	15	10	7	5	3
332	1	3	5	6	10	12	14	10	6	4	3	2
Total polychlorinated biphenyl	60	331	951	1310	1463	1239	1098	691	314	211	143	104

[a] Aroclor 1254 component relative retention time; 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) = 100.

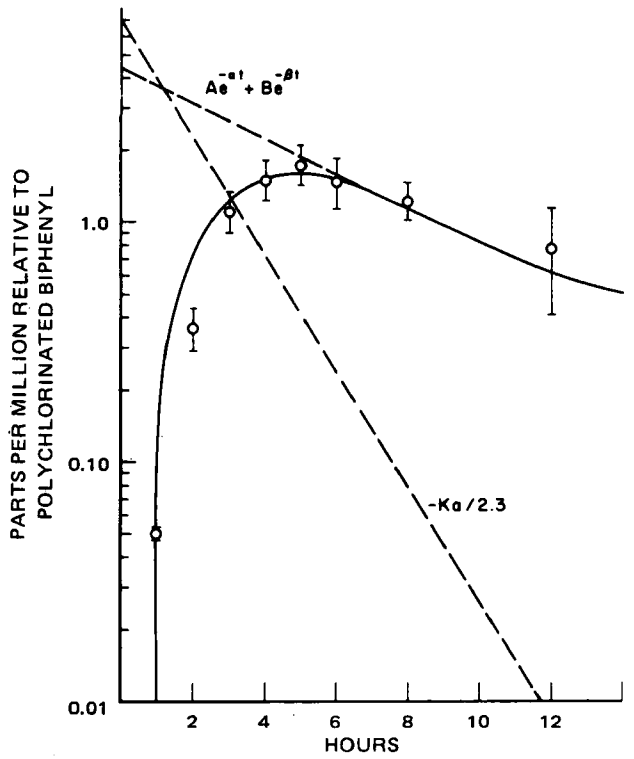


Figure 1—*Absorption phase of Component 127 in swine. The circles represent the mean blood concentrations relative to the standard mixture (concentration of standard necessary to cause the same detector response), the brackets around the circles represent the standard deviations of the means, the broken line designated by* $Ae^{-\alpha t} + Be^{-\beta t}$ *is the extrapolation of the fitted elimination curves, the broken line designated by* $-K_a/2.3$ *is the line describing absorption, and the solid line is the fitted curve for absorption and elimination.*

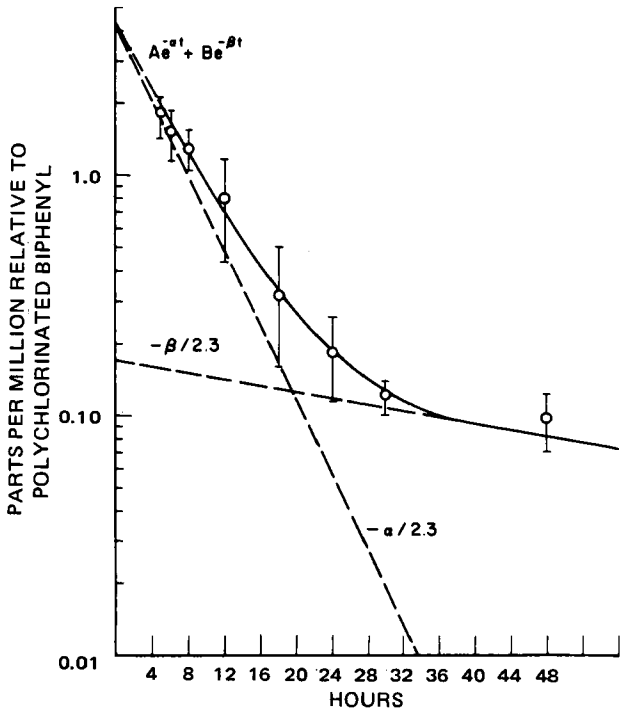


Figure 2—*Elimination phase of Component 127 in swine. The circles and brackets have the same designation as in Fig. 1, the broken line designated* $-\alpha/2.3$ *is the line describing distribution from the central compartment, and the broken line designated* $-\beta/2.3$ *is the extrapolated line describing the slower elimination phase.*

Pharmacokinetic parameters determined for components and total polychlorinated biphenyl after a single oral dose to swine and sheep are listed in Tables V and VI. Parameters A, α, B, and β were generated by the computer program from the elimination phase of the data plots. The parameters were determined as described under *Experimental.*

The pharmacokinetic parameters K_a, α, β, and K_3 were compared statistically. In swine, Component 70 was absorbed and eliminated at a faster rate ($p < 0.05$) than were most other components. In sheep, absorption of Components 70 and 105 was the most rapid, while the elimination of Components 99 and 105 was the most rapid; several components of longer retention time, as well as Component 84, were eliminated at a slow rate relative to the total polychlorinated biphenyl content.

The rate constants K_a, α, β, and K_3, in general, were greater for swine than for sheep. This comparison is consistent with results obtained from studies in which swine and sheep were fed a ration containing the commercial polychlorinated biphenyl.

DISCUSSION

This paper has attempted to provide some basic information on the time course of a polychlorinated biphenyl mixture when administered as a single oral dose to swine and sheep. Previously discussed methods (1–3) were utilized in sample and data analysis. A more precise method for the determination of absorption rate constants may be more desirable and may be adaptable in future studies when more basic information on the distribution of commercial polychlorinated biphenyl mixtures from the gut of swine and sheep is known.

Table II—Mean Absolute Blood Concentrations (Parts per Billion) of Polychlorinated Biphenyl Components after a Single Oral Dose in Sheep

	Hours													
RRT[a]	4	6	8	12	24	36	48	60	72	84	96	120	144	168
70	37	63	79	66	35	21	14	12	12	9	9	—	—	—
84	44	80	111	110	81	48	29	24	24	19	21	18	17	—
99	22	37	49	43	28	20	11	11	10	—	—	—	—	—
105	14	22	28	21	11	8	5	5	4	4	—	—	—	—
127	55	105	147	147	122	67	48	42	39	31	28	26	22	21
149	25	77	99	133	114	72	50	47	42	37	32	29	27	26
176	26	50	78	84	66	41	27	25	24	18	18	17	17	15
208	5	11	19	19	13	7	5	4	4	3	3	3	2	—
253	6	12	21	24	23	16	12	12	10	8	8	7	7	6
286	3	5	8	11	12	9	6	6	5	4	3	3	3	—
332	2	4	7	11	11	8	6	6	5	4	4	3	3	3
Total polychlorinated biphenyl	239	466	646	669	516	317	213	194	179	137	126	106	98	71

[a] Aroclor 1254 component relative retention time; 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) = 100.

Table III—Concentration of Polychlorinated Biphenyl Components in the Blood of Swine after a Single Oral Dose

		Maximum Concentration in Blood			
RRT[a]	Absolute Dose, mg/kg	Relative, ppm	Absolute, ppm	Hours	Blood[b] / Dose
70	2.41	1.260	0.202	4	0.084
84	2.46	1.587	0.261	5	0.106
99	1.51	1.423	0.144	5	0.095
105	1.51	1.477	0.149	5	0.099
127	2.50	1.780	0.297	5	0.119
149	2.05	1.600	0.219	5	0.107
176	1.24	1.760	0.145	8	0.117
208	0.31	1.707	0.036	8	0.116
253	0.29	1.900	0.037	8	0.128
286	0.10	2.960	0.019	8	0.190
332	0.10	2.207	0.014	8	0.140
Total polychlorinated biphenyl	15.00	1.463	1.463	5	0.098

[a] Aroclor 1254 component relative retention time; 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) = 100. [b] Absolute maximum concentration per dose.

Published methods for the determination of absorption rate constants exhibiting two-compartment open-model characteristics utilize at least two parameters generated from intravenous data

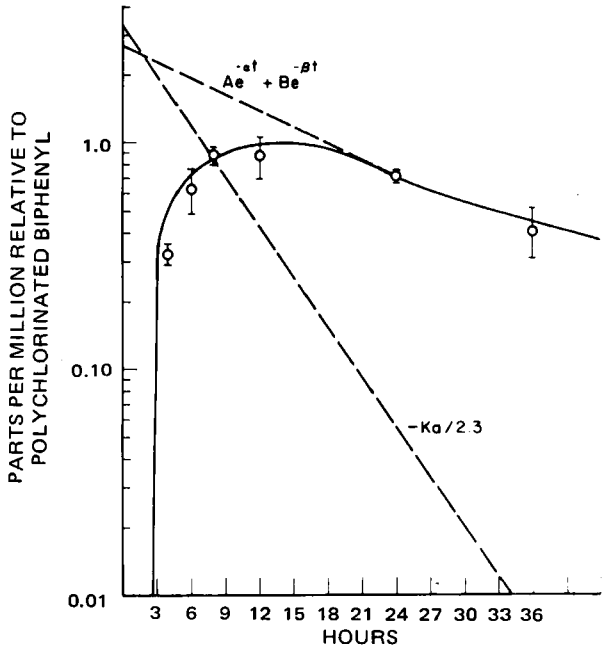


Figure 3—*Absorption phase of Component 127 in sheep. The circles, brackets, and lines have the same designations as in Fig. 1.*

Table IV—Concentration of Polychlorinated Biphenyl Components in the Blood of Sheep after a Single Oral Dose

		Maximum Concentration in Blood				
RRT[a]	Absolute Dose, mg/kg	Relative, ppm	Absolute, ppm	Hours	Blood[b] / Dose	Swine[c] / Sheep
70	4.82	0.493	0.079	8	0.016	5.25
84	4.92	0.673	0.111	8	0.023	4.61
99	3.02	0.487	0.049	8	0.016	5.94
105	3.02	0.273	0.028	8	0.009	11.00
127	5.00	0.880	0.147	8	0.029	4.10
149	4.10	0.973	0.133	12	0.032	3.34
176	2.48	1.020	0.084	12	0.034	3.44
208	0.62	0.893	0.019	12	0.031	3.74
253	0.58	1.233	0.024	12	0.041	3.12
286	0.20	1.833	0.012	24	0.060	3.17
332	0.20	1.720	0.011	24	0.055	2.55
Total polychlorinated biphenyl	30.00	0.667	0.667	12	0.022	4.45

[a] Aroclor 1254 component relative retention time; 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) = 100. [b] Absolute maximum blood concentration per dose. [c] Accumulation (*i.e.*, blood/dose) comparison.

Table V—Comparison of Pharmacokinetic Parameters for Polychlorinated Biphenyl Components in Swine

RRT[a]	A, ppm	α, hr^{-1}	B, ppm	β, hr^{-1}	$t_{1/2}\ \alpha$, hr	$t_{1/2}\ \beta$, hr	$t_{1/2}$ Absorption, hr	K_a, hr^{-1}	K_1, hr^{-1}	K_2, hr^{-1}	K_3, hr^{-1}
70	3.858	0.283	0.135	0.0405	2.45	17.11	0.62	1.112	0.040	0.049	0.234
84	3.054	0.151	0.138	0.0005	4.59	1386.00	1.01	0.685	0.134	0.007	0.011
99	2.949	0.180	0.220	0.0014	3.85	495.00	1.19	0.585	0.149	0.014	0.018
105	3.546	0.184	0.092	0.0080	3.77	86.63	1.26	0.552	0.062	0.012	0.118
127	4.124	0.175	0.170	0.0150	3.96	46.20	1.24	0.560	0.046	0.021	0.123
149	2.796	0.140	0.340	0.0260	4.95	26.45	1.20	0.580	0.032	0.039	0.094
176	5.390	0.161	0.333	0.0108	4.30	64.17	1.87	0.371	0.063	0.020	0.087
208	4.778	0.148	0.281	0.0110	4.68	63.00	1.80	0.386	0.053	0.019	0.086
253	5.422	0.151	0.310	0.0059	4.59	117.46	1.90	0.364	0.040	0.008	0.109
286	5.052	0.103	0.900	0.0145	6.73	47.79	2.96	0.234	0.036	0.028	0.053
332	4.808	0.126	0.540	0.0100	5.50	69.30	2.19	0.317	0.056	0.022	0.057
Total polychlorinated biphenyl	2.929	0.156	0.170	0.0110	4.44	62.43	1.13	0.611	0.057	0.019	0.090

[a] Aroclor 1254 component relative retention time; 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) = 100.

Table VI—Comparison of Pharmacokinetic Parameters for Polychlorinated Biphenyl Components in Sheep

RRT[a]	A, ppm	α, hr^{-1}	B, ppm	β, hr^{-1}	$t_{1/2}\ \alpha$, hr	$t_{1/2}\ \beta$, hr	$t_{1/2}$ Absorption, hr	K_a, hr^{-1}	K_1, hr^{-1}	K_2, hr^{-1}	K_3, hr^{-1}
70	0.723	0.068	0.090	0.0057	10.19	121.58	0.78	0.883	0.031	0.013	0.030
84	2.664	0.084	0.152	0.0026	8.25	266.54	3.98	0.174	0.049	0.007	0.031
99	0.606	0.061	0.150	0.0082	11.36	84.51	1.02	0.681	0.024	0.019	0.026
105	0.379	0.079	0.069	0.0077	8.77	90.00	0.82	0.841	0.035	0.018	0.034
127	2.422	0.065	0.253	0.0043	10.66	161.16	4.10	0.169	0.031	0.010	0.028
149	2.141	0.057	0.309	0.0030	12.16	231.00	3.71	0.187	0.033	0.010	0.017
176	2.554	0.066	0.286	0.0025	10.50	277.20	3.57	0.194	0.042	0.009	0.018
208	1.643	0.051	0.133	0.0015	13.59	462.00	2.37	0.292	0.033	0.005	0.015
253	2.217	0.046	0.498	0.0028	15.07	247.50	4.03	0.172	0.026	0.011	0.012
286	2.772	0.036	0.730	0.0043	19.25	161.16	4.41	0.157	0.015	0.011	0.014
332	2.782	0.037	0.600	0.0027	18.73	256.67	4.15	0.167	0.020	0.009	0.011
Total polychlorinated biphenyl	2.347	0.090	0.302	0.0088	7.70	78.75	3.83	0.181	0.037	0.018	0.044

[a] Aroclor 1254 component relative retention time; 1,1-dichloro-2,2-bis(*p*-chlorophenyl)ethylene (DDE) = 100.

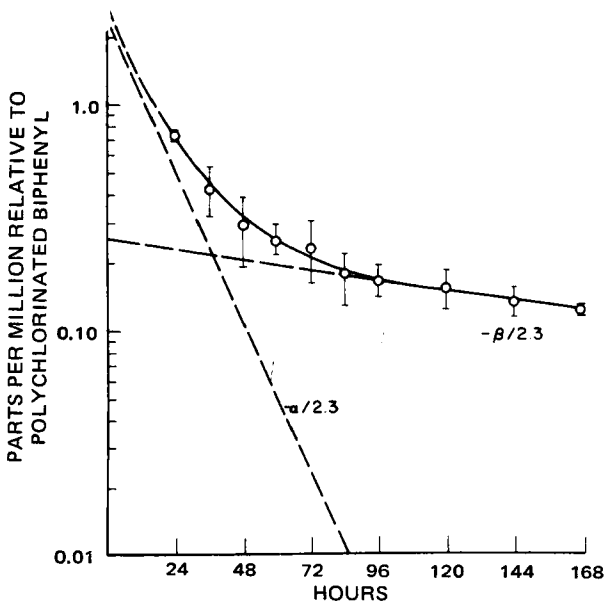


Figure 4—*Elimination phase of Component 127 in sheep. The circles and brackets have the same designations as in Fig. 1. The lines have the same designations as in Fig. 2.*

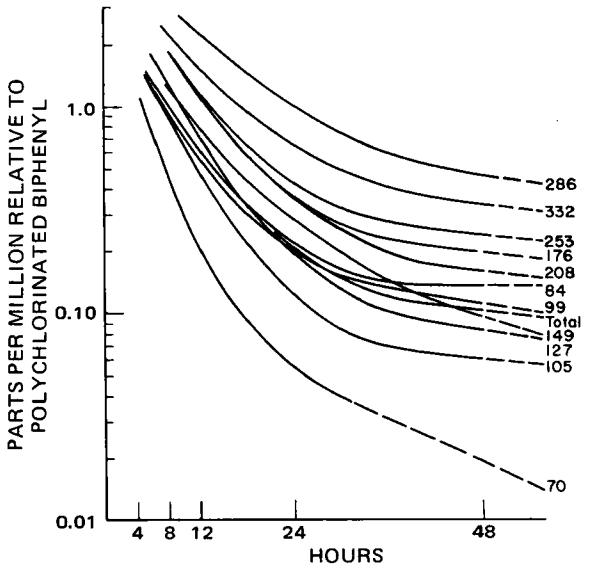


Figure 5—*Elimination of polychlorinated biphenyl components in swine (relative values).*

from the same animals (6, 7). Repeated experiments with a polychlorinated biphenyl mixture in the same animal are impractical because of slow excretion, tissue residues, and low metabolic conversion. Similar groups of animals can be utilized for the extrapolation of intravenous data to oral data if the intravenous and oral kinetics are well correlated in terms of dosage, compartmentalization, and extent of absorption. Since the correlation between intravenous and oral kinetics has not been well established for polychlorinated biphenyl mixtures, a method for the determination of absorption rate constants based only on oral dosage data seems to

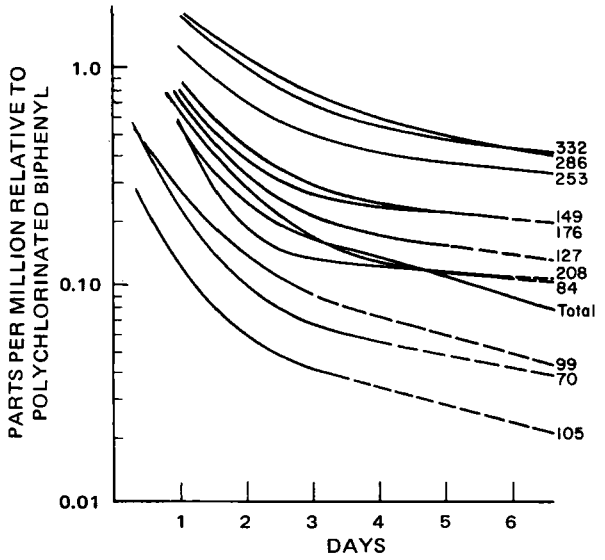


Figure 6—*Elimination of polychlorinated biphenyl components in sheep (relative values).*

be most appropriate, at least for comparison of the components within the commercial mixtures.

The comparisons made between swine and sheep are somewhat predictable, because of obvious species differences in GI physiology, and generally consistent with results obtained from other studies being prepared for publication. The components were absorbed faster, reached higher blood concentrations, and were eliminated faster in swine than sheep. There is a tendency for higher chlorinated components to be absorbed slower but to reach higher maximum concentrations relative to amounts present in the dose.

It is difficult at this time to attempt correlation of kinetic data within each species for each type of administration of the commercial mixture. Variations between intravenous parameters (3) and the oral parameters determined in this study pose questions concerning distribution and dose dependence. Although polychlorinated biphenyls are left intact after treatment with strong acids, their extractability from biological materials such as egg yolk is dependent on pH. Polychlorinated biphenyls, especially the higher chlorinated species, are not readily extractable from aqueous solutions at acid pH; neutralization of the acid can increase recovery up to 10-fold[2]. If this dependence applies to the gastric portions of the central compartment, the redistribution of polychlorinated biphenyl to the blood would reflect a lower K_3 and $t_{1/2}\,\beta$. Although a longer period of sampling after intravenous dosing might reveal closer half-lives, both areas will have to be investigated to resolve this discrepancy.

For total polychlorinated biphenyl, K_3 and $t_{1/2}$ *(B)* were 0.189 hr^{-1} and 15.1 hr, respectively, for swine administered 3 mg/kg iv; the same parameters for sheep were 0.194 hr^{-1} and 14.7 hr, respectively (3). In this study, the values were 0.091 hr^{-1} and 62.4 hr for swine receiving 15 mg/kg and 0.044 hr^{-1} and 78.8 hr for sheep receiving 30 mg/kg. In intravenous studies, however, the blood samples were collected for only 24 hr and the oral dose level in swine should have been followed for a longer duration in the present study. It is obvious that oral and intravenous investigations approximating the same C_0 are required and that the duration of the study needs to be expanded.

A previous report indicated similar poor absorption of the same mixture from the gut of growing pigs, but dilution rather than elimination was responsible for reducing tissue concentrations (8). It is difficult to compare these studies, however, since tissue concentrations were not determined at peak levels in the previous study and analytical methods limited results to an estimation of total polychlorinated biphenyls so that individual components were not assayed.

Accumulation of data from future studies along with analyses of data from studies already undertaken will undoubtedly help to assemble the total picture of polychlorinated biphenyl mixture distribution in the animal body.

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Acylation of Ascorbic Acid in Water

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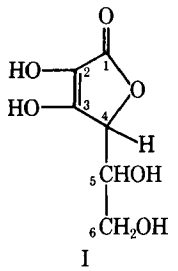
Abstract □ The acylation of ascorbic acid by acetic anhydride was studied in water at 25°. The results indicate that the initial products of reactions between these two compounds were acetic acid and 3-*O*-acetylascorbic acid. The latter product then underwent two parallel reactions: hydrolysis to ascorbic and acetic acids and an intramolecular O → O acyl migration to yield 2-*O*-acetylascorbic acid. The former reaction was predominant at pH values below 4, whereas the latter reaction predominated at pH values between 4 and 7. These results are used as a basis for questioning the structural assignments previously made to some ascorbic acid esters.

Keyphrases □ Ascorbic acid—acylation by acetic anhydride in water, kinetics and products *via* hydrolysis and rearrangement □ Acylation of ascorbic acid in water using acetic anhydride—kinetics and products *via* hydrolysis and rearrangement

A kinetic and product analysis is described for reactions that occur in aqueous solutions initially containing ascorbic acid (I) and acetic anhydride. The purposes of this study were to determine which hydroxy groups of I were acetylated and in what proportion and then to investigate the stability of the formed esters with respect to intramolecular O → O acyl transfers and hydrolysis. Intramolecular O → O acyl transfers were previously reported to occur for ascorbic acid esters (1), and they are well-known reactions of glycerol esters (2) and hydroxyacids (3, 4). The results of these studies will prove useful in the characterization of the structures of mono-, di-, and triacyl derivatives of ascorbic acid existing in water and in understanding the reactivity of these derivatives with respect to hydrolysis and rearrangement.

Considerable interest exists in the synthesis and properties of acyl derivatives of I. The major reasons for this interest are: *(a)* the resistance of ascorbic acid to autoxidation can be increased by esterifying one or both of the hydroxy groups in the enediol system [—(HO)C=C(OH)—] (1), and *(b)* esters can be conferred with almost any desired amount of lipophilic character by appropriate selection of acyl moieties.

Two advantages that esters of ascorbic acid of I might possess are that lipophilic derivatives can be included in nonaqueous preparations [*e.g.*, the use of 6-*O*-palmitoylascorbic acid (6) or 6-*O*-stearylascorbic acid (7) as antioxidants in nonaqueous formulations] and that they may be absorbed percutaneously more efficiently (8)[1]. Administration of ascorbic acid through the skin has been suggested (9) as a means of facilitating wound healing. However, it is believed (10) that both the antioxidant properties and antiscorbutic activity of I depend on the presence within the molecule of the enediol system. Consequently, it is important to know whether a particular ester contains an intact enediol system or whether the intact enediol system will be regenerated in the biological environment.



I

EXPERIMENTAL

Equipment—Spectrophotometric[2] and spectropolarimetric[3] studies were performed using instruments with thermostated cell compartments. All thermostated baths were regulated within ±0.1°.

Reagents—All reagents used were of analytical grade, unless otherwise stated. Acetic anhydride was distilled over magnesium turnings, and the fraction boiling within 137.8–138.2° was collected. Ascorbic acid was recrystallized from its concentrated solution in hot methanol by adding sufficient petroleum ether to make the solution turbid and keeping the resulting mixture overnight at room temperature.

The water used was redistilled from acid permanganate using an all-glass still. 5,6-Isopropolidine-2,3-di-*O*-acetylascorbic acid was synthesized following the Vestling and Rebstock (11) procedure. Unless otherwise mentioned, all experiments were carried out in aqueous solutions.

Measurement of Acetylation—Three milliliters of a solution of I (0.05 *M*) was combined with 2.0 ml of water and titrated with a standard solution of sodium hydroxide to the phenolphthalein end-point. The volume of the required titrant, *a* ml, was noted. Three milliliters of a solution of I (0.05 *M*) was reacted with 0.3 ml of 0.6 *M* solution of acetic anhydride (in dioxane) in a closed vial for 1 hr at 25°. The reaction mixture was then diluted with 2.0 ml of water and titrated to the same end-point. The volume of the titrant, *b* ml, was likewise noted. An equivalent amount of acetic anhydride was titrated after hydrolysis in 5.0 ml of water to the same end-point. The volume of the consumed titrant, *c* ml, was also recorded.

The ratio of ester to acetic anhydride and the percent acetylation were calculated from *a*, *b*, and *c* according to:

$$\frac{\text{ester}}{\text{acetic anhydride}} = \frac{(a + c) - b}{c} \times 2 \qquad \text{(Eq. 1)}$$

$$\%\ \text{acetylation} = \frac{(a + c) - b}{c} \times 100 \qquad \text{(Eq. 2)}$$

Determination of k_1^{obs}—The second-order rate constant (k_1^{obs}) for the initial attack of anhydride on ascorbic acid (Scheme I) was determined by following the loss of UV absorption at 275 nm. The theory is described under *Results and Discussion*. Acetate buffers (2×10^{-1} *M*) containing 1×10^{-4} *M* edetate (to minimize any ca-

[1] This reference describes an absorption study done with ascorbic acid 3-phosphate.

[2] Using Cary 15 and Cary 16 spectrophotometers, Varian Instruments, Inc.

[3] Using a Cary 60 spectropolarimeter, Varian Instruments, Inc.

talysis of the oxidation of ascorbic acid by trace elements) were used in these runs.

The solutions were adjusted to an ionic strength of 1.0 M with potassium chloride, and temperature was maintained at 25.0 ± 0.1°. The amount of ascorbic acid used was such as to give an initial absorption of about 0.9 unit (5×10^{-5} M at pH > 4.5, $1–2 \times 10^{-4}$ M at pH < 4.5). The amount of acetic anhydride added (as a solution in dioxane) was sufficient to produce a 100–400-fold excess of acetic anhydride concentration over that of ascorbic acid.

The loss of absorbance at 275 nm was recorded for about 1 min. About 20 sec (accurately measured) elapsed between mixing the reagents and taking the first reading in the spectrophotometer. The smoothness of the absorbance curve after about 15 sec indicated that the 20 sec allowed for the first point was sufficient for an accurate measurement of absorbance.

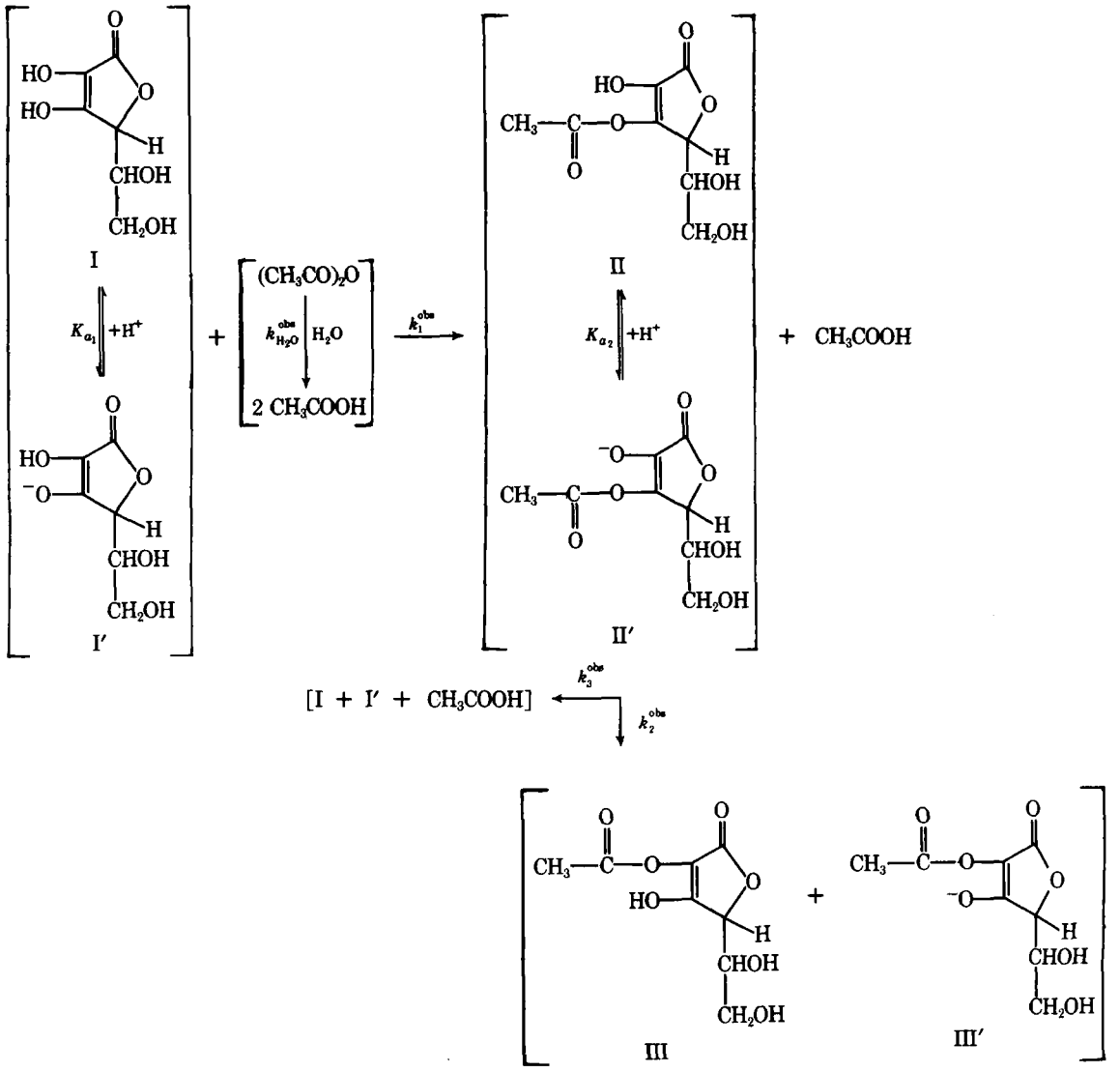
Determination of k_2^{obs}—The first-order rate constants for the loss of 3-*O*-acetylascorbic acid were determined by following the increase of UV absorption at the λ_{max} of 2-*O*-acetylascorbic acid. Citrate buffers (0.01 M) were used to minimize any catalysis of oxidation of ascorbic acid by trace elements (citrate complexes with the metal ions) and also to utilize the broad range of the buffering potential of the citric acid system. The solutions were adjusted to an ionic strength of 0.1 M with potassium chloride, and temperature was maintained at 25.0 ± 0.1°.

Concentrated solutions of 3-*O*-acetylascorbic acid formed *in situ* (see *Results and Discussion*) were diluted in the specified buffers (final concentration of ascorbic acid and its derivatives about 5×10^{-5} M–1×10^{-4} M), and the increase in UV absorbance was recorded until a plateau value was reached. Similarly, hydrolysis rate constants of 5,6-isopropolidine-2,3-di-*O*-acetylascorbic acid were determined by diluting a concentrated methanolic solution in the specified buffers and following the increase in UV absorbance at the λ_{max} of the 3-*O*-acetyl species.

RESULTS AND DISCUSSION

Several pieces of evidence suggest that the major series of reactions that occur when acetic anhydride (initial concentration $1–5 \times 10^{-2}$ M) is added to aqueous solutions of I (initial concentration $1–50 \times 10^{-2}$ M) at 25° are those shown in Scheme I. A qualitative picture of the events following the addition of acetic anhydride to solutions of I comes from the changes of optical rotation (at 350 nm) of the reaction solution as a function of time (Fig. 1). At pH values between 2 and 7, the changes in optical rotation were characterized by an initial rapid decrease followed by a slower increase. At pH values above 4.0, the final optical rotation was greater than the initial value. At lower pH values, the initial and final optical rotation values were similar.

It is believed that the initial decrease in optical rotation is caused by the conversion of I to 3-*O*-acetylascorbic acid (II) and



Scheme I

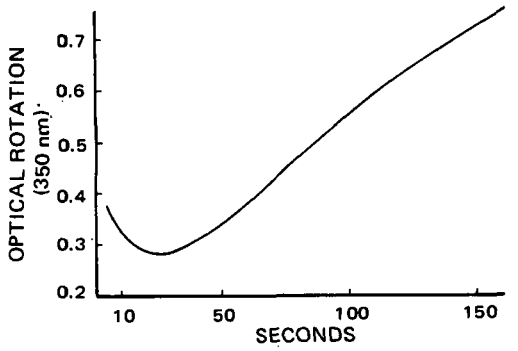


Figure 1—*Change in optical rotation at 350 nm as a function of time for the reactions of acetic anhydride (initial concentration 0.02 M) in a solution of ascorbic acid (initial concentration 0.25 M) (0.2 M acetate buffer, pH 5.25, I = 0.5 M, T = 27°).*

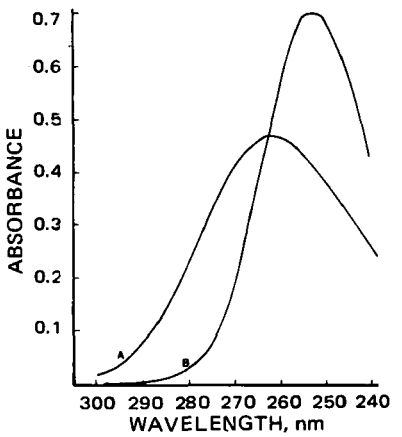


Figure 2—*UV spectra of: A, 5 × 10⁻⁵ M solution of ascorbic acid; and B, reaction mixture containing 5 × 10⁻⁵ M ascorbic acid and 1 × 10⁻² M acetic anhydride after 40 min. The solvent for both solutions was a 1 × 10⁻¹ M acetate buffer at pH 4.65.*

that the subsequent increase in optical rotation results from both the rearrangement of II to 2-*O*-acetylascorbic acid (III) *via* an O → O acyl migration and hydrolysis of II to I and acetic acid. These conclusions concerning the reaction mechanism are in part based on the assumption that the rate-determining step in the acylation reaction in water involves a reaction between an anionic form of I and acetic anhydride. It was previously found (4) that neutral molecules of alcohols, phenols, and hydroxycarboxylic acids are not significantly acylated by acetic anhydride in water whereas their anions, at least in the case of hydroxycarboxylic acids, are rapidly acetylated.

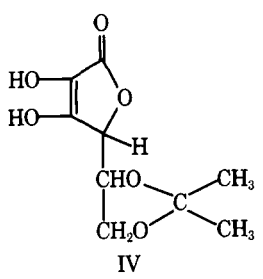
Hence, because the 3-hydroxy group is the most acidic in I (12), it is expected that it would be the first to be acetylated between pH 2 and 7. The large change in optical rotation that accompanies the initial reaction is consistent with the proposed reaction sequence because the 3-hydroxy group is adjacent to a rigid asymmetric carbon atom (C-4) and its derivatization would be expected to result in a relatively large change in optical activity. Additional evidence for this first reaction was obtained from a kinetic study and will be discussed later.

If III were simply hydrolyzed in a subsequent reaction to yield I and acetic acid, the initial and final optical activities of the reaction solutions would be the same. However, apart from the fact that the initial and final optical activities did differ by a small but significant amount, several other pieces of evidence indicate that the final reaction mixture contained at least one compound in addition to I (and I') and acetic acid. Evidence for this conclusion is:

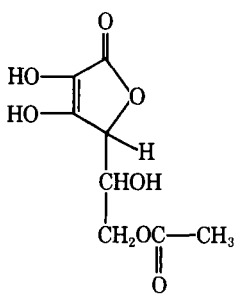
1. The UV spectrum of the initial and final reaction solutions differed significantly (Fig. 2).

2. Titration of the final reaction mixture with sodium hydroxide to the phenolphthalein end-point yielded a smaller titer than that obtained for a mixture containing ascorbic acid and fully hydrolyzed acetic anhydride. For example, 4.8 ml of 0.1 *N* NaOH was required to titrate 3.3 ml of a reaction mixture that originally contained 0.15 m*M* I and 0.18 m*M* acetic anhydride and had stood at 25° for 1 hr. If each mole of acetic anhydride had reacted to yield 2 moles of acetic acid, the titer would have been 5.1 ml.

The major product following these reactions is believed to be III rather than the 5-*O*-acetyl or 6-*O*-acetyl derivatives of ascorbic acid for several reasons. First, 5,6-isopropolidineascorbic acid (IV) and 6-*O*-acetylascorbic acid (V) reacted in a similar manner (both qualitatively and quantitatively) to I in aqueous solutions of acetic



IV



V

anhydride (the 6-hydroxy group cannot be acetylated in either compound and both the 5-hydroxy and 6-hydroxy groups are masked in IV). Second, potentiometric titration of the final reaction mixture of I and acetic anhydride with hydrochloric acid revealed that it contained a compound with a pKa of approximately 2 [it seems unlikely that 5-*O*-acetyl- or 6-*O*-acetylascorbic acid would have significantly different pKa values from that of I (pKa = 3.90 at I = 0.10 *M*)]. And third, titration of a 1-hr-old reaction mixture, made by adding 0.1 ml of a 0.6 *M* solution of acetic anhydride in dioxane to 3.0 ml of a 0.1 *M* solution of sodium ascorbate (at pH 6.0) with 2,6-dichloroindophenol or iodine, gave titers accounting for only between 82 and 84% of the original amount of ascorbic acid. Since both of these reactants detect molecules that contain an intact enediol system (13), the low titers indicate that at least some of the originally added ascorbic acid was derivatized at the 2-hydroxy or 3-hydroxy groups.

The yield of III in the final reaction mixtures could also be calculated from the titers of sodium hydroxide that were required to reach the phenolphthalein end-point. Details of this method were given previously (4). It depends on the fact that esterification of 1 mole of I by acetic anhydride produces 1 mole of acetic acid and 1 mole of ester, whereas hydrolysis of 1 mole of acetic anhydride yields 2 moles of acetic acid.

The experimental details for calculating yields of ester are given under *Experimental*, and a plot of yield of ester $[III]_e$ relative to the initial acetic anhydride $[Ac_2O]_0$ concentration against initial ascorbic acid concentration (at pH 6.0) is shown in Fig. 3. The plot in Fig. 3 reveals that the yield of ester relative to initial anhydride concentrations increases with increasing concentrations of I when these are low but becomes independent of the concentrations of I when these were above 1 × 10⁻¹ *M*. Furthermore, the maximum yield of ester never exceeded 87%. A possible explanation would be that at high concentrations of ascorbic acid relative to initial acetic anhydride concentrations, virtually all of the acetic anhydride reacted with I to yield II. It would then be necessary to conclude that II participated in two parallel reactions, rearrangement to III and hydrolysis, and that the ratio of the rate constants for these

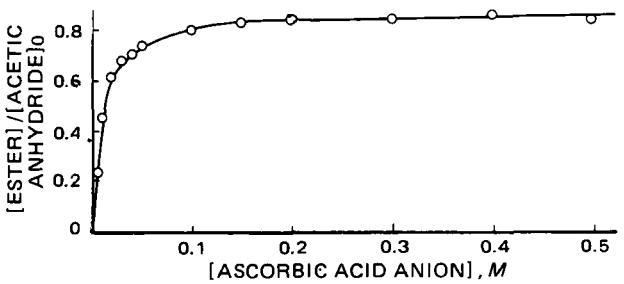


Figure 3—*Plot of yield of mono-O-acetyl ascorbate against initial concentration of sodium ascorbate ($[Ac_2O]_0$ = 0.02 M, pH 8.5, I = 1.5 M, T = 25°).*

reactions was insensitive to concentrations of I. This is considered to be the most likely explanation.

An alternative explanation for the results in Fig. 3 would be that ascorbate ion is catalyzing the hydrolysis of acetic anhydride by acting as a general base as well as by reacting to yield the ester, II. Although this possibility cannot be ruled out completely, evidence is presented subsequently to establish that hydrolysis and O → O acetyl transfer reactions both occur in II. Hence, any general base catalysis by ascorbate or hydrolysis of acetic anhydride will change the reaction sequence only slightly, and the paramount reactions appear to be those described. A strong suggestion that II would be likely to hydrolyze quite rapidly came from the observation that the closely related 5,6-isopropolidine-2,3-di-*O*-acetylascorbic acid hydrolyzed to the corresponding 2-*O*-acetyl derivative, with half-lives ($t_{1/2}$ values) of the order of 10 min in the 3.5–6.5 pH range.

The calculation of rate constants for the reactions in Scheme I is complicated by the fact that hydrolysis of acetic anhydride to acetic acid competes with the formation of I. Estimates of the rate constants for the various reactions were made from the results of experiments designed on the basis of the following considerations:

1. The rate law for the conversion of I and I′ (I + I′ = I_T) to II and II′ would be:

$$-\text{rate}_{[I]_T} = k_1^{obs}[Ac_2O][I]_T \qquad \text{(Eq. 3)}$$

2. Most of the acetic anhydride would be consumed by hydrolysis if the initial acetic anhydride concentration $[Ac_2O]_0$ was greatly in excess of the initial concentration of $[I]_T$. Hence, because the hydrolysis of acetic anhydride in aqueous buffers is a pseudo-first-order reaction:

$$[Ac_2O] = [Ac_2O]_0 e^{-k_{H_2O}^{obs}t} \qquad \text{(Eq. 4)}$$

The $k_{H_2O}^{obs}$ value in Eq. 4 is the pseudo-first-order rate constant for hydrolysis of acetic anhydride in a particular buffer solution at a particular pH value. If I and I′ do not significantly affect the rate of hydrolysis (*e.g.*, by acting as general acids or general bases), then the $k_{H_2O}^{obs}$ value in a reaction solution containing $[I]_T$ would be identical to that in the same solution without $[I]_T$. In experiments designed to study the rate of formation of II, the concentration of $[I]_T$ was approximately 5×10^{-5} *M*. It was assumed that these low concentrations would not significantly affect the value of $k_{H_2O}^{obs}$ and that the value of this latter parameter could be determined from experiments in which no ascorbic acid was included in the reaction solutions. Values of $k_{H_2O}^{obs}$ at several pH values are listed in Table I.

3. Substitution of Eq. 4 in Eq. 3 yields:

$$-\text{rate}_{[I]_T} = k_1^{obs}[Ac_2O]_0[I]_T e^{-k_{H_2O}^{obs}t} \qquad \text{(Eq. 5)}$$

4. If the conversion of I to II and the hydrolysis of acetic anhydride were the only reactions occurring, and if II and acetic anhydride did not absorb UV energy appreciably at a wavelength where I absorbed strongly, then the absorbance at that wavelength would vary with time in accordance with Eq. 6:

$$\log A = \log A_0 + \frac{k_1^{obs}[Ac_2O]_0}{2.303k_{H_2O}^{obs}}(1 - e^{-k_{H_2O}^{obs}t}) \qquad \text{(Eq. 6)}$$

Hence, plots of log *A* against $e^{-k_{H_2O}^{obs}t}$ should be linear with slopes equal to $(k_1^{obs}[Ac_2O]_0/2.303k_{H_2O}^{obs})$, and the slopes of these lines should be linearly related to $[Ac_2O]_0$ and should equal zero when $[Ac_2O]_0$ equals zero. This type of dependence of log *A* on the value of $e^{-k_{H_2O}^{obs}t}$ was observed during the first 1.0 min following the addition of acetic anhydride (initial concentration 0.5–2 × 10^{-2} *M*) to solutions of ascorbic acid (initial concentration ~5 × 10^{-5} *M*).

Plots of log *A* against $e^{-k_{H_2O}^{obs}t}$ yielded straight lines, and Fig. 4 shows a typical plot of the slopes of these lines against $[Ac_2O]_0$. Although acetic anhydride did absorb slightly at 275 nm, it was included in equal concentrations in both sample and reference cells. Hence, the changes in absorbance caused by the hydrolysis of acetic anhydride were minimized. The assumption that II did not absorb UV energy appreciably at 275 nm was justified on the basis that the absorptivity of 5,6-isopropolidine-2,3-di-*O*-acetylascorbic acid was very low at this wavelength (ϵ = <50). Both this latter compound and II do not have a free 3-hydroxy group. It is believed that the conjugated pathway between this group and the C-1 carbonyl group is the major chromophore in I and its derivatives.

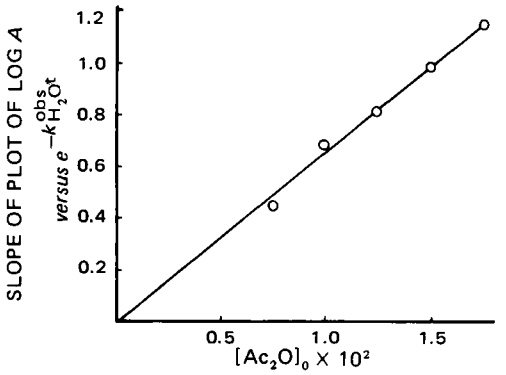


Figure 4—*Plot of slopes of plots of log* A versus $e^{-k_{H_2O}^{obs}t}$ *against initial acetic anhydride concentration (0.2* M *acetate buffer, pH 5.5, I = 1.0* M, T = 25°).

Kinetic evidence will be presented shortly to establish that very little conversion of II to III or back to I occurs during 1 min. Hence, it is believed that these experiments can be analyzed to obtain values of k_1^{obs}, as was done in the present study; values of k_1^{obs} are shown plotted against pH in Fig. 5. The solid line in this figure was calculated on the basis that the major reaction leading to formation of II involves acetic anhydride and the monoanion of I (*i.e.*, I^-). This finding is consistent with the postulate that the 3-hydroxy group of I is the first to be acylated in water at pH values between 2 and 6.

5. Because the reaction leading to the formation of II is a second-order reaction, its rate is very concentration dependent. On the other hand, the rearrangement and hydrolysis of II are postu-

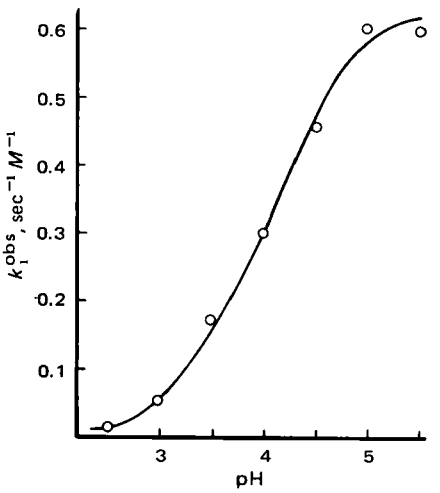


Figure 5—*The pH–rate profile of the second-order rate constants* (k_1^{obs}) *for the initial attack of acetic anhydride on ascorbic acid in a 0.2* M *acetate buffer (I = 1.0* M, T = 25°).

Table I—Rate Constants for Hydrolysis of Acetic Anhydride in 0.2 *M* Acetate Buffers at 25° (*I* = 1.0 *M*)

pH	$t_{1/2}$, min	$10^3 k_{H_2O}^{obs}$, sec^{-1}
2.5	4.55	2.49
3.5	5.12	2.24
4.0	4.80	2.33
4.5	4.73	2.44
5.0	4.26	2.71
5.5	3.72	3.04

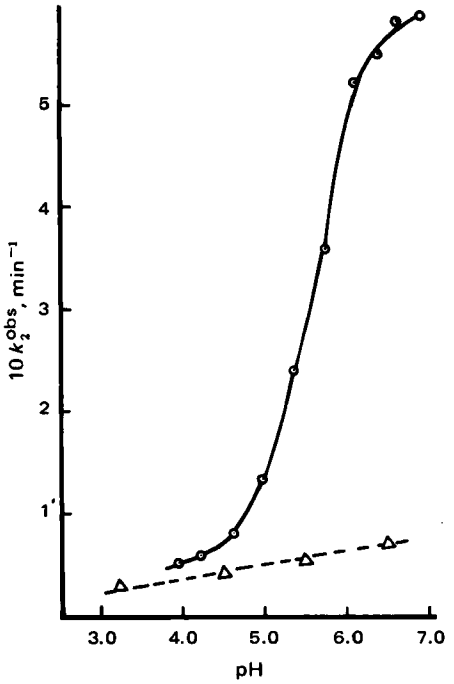


Figure 6—*The pH–rate profile of the overall rate of loss of 3-O-acetylascorbic acid (⊙) and rate of hydrolysis of 5,6-isopropolidine-2,3-di-O-acetylascorbic acid (△) (0.01 M citrate, I = 0.1 M, T = 25°).*

lated to be first-order (or pseudo-first-order) reactions in aqueous buffers, and their rates should be independent of the concentration of II. These postulates are the bases for the experiments employed to estimate the rate of conversion of II to III and I. A solution that was initially 10^{-2} *M* in both I and acetic anhydride and was buffered at pH 4.0 was prepared and kept at 25° for 4 min. The major reactions occurring under these conditions were the hydrolysis of acetic anhydride and the formation of II.

After 2 min, an aliquot of the solution was diluted ~200-fold into a buffer, and changes in UV absorbance at λ_{max} were recorded. Plots of log $(A - A_\infty)$ against time were linear for at least four half-lives, and these plots were used to calculate first-order rate constants, k^{obs} values, for the reactions that were occurring. A plot of k^{obs} values against the pH values of the buffers in which the reactions took place are displayed in Fig. 6.

Upon dilution of the original reaction mixture, any second-order reactions between acetic anhydride and $[I]_T$ (or possibly II or III) would be greatly slowed down. Hence, it is believed that the absorbance changes were caused by both the rearrangement of II to III (with a first-order rate constant k_2^{obs}) and hydrolysis of II (with a pseudo-first-order rate constant k_3^{obs}). On this basis, k^{obs} values would be related to the rate constants for the various reactions by:

$$k^{obs} = k_2^{obs} + k_3^{obs} \qquad \text{(Eq. 7)}$$

The fact that the pH profile in Fig. 6 has an inflection point at pH 5.5 suggests that the substrate has a pKa value of 5.5. This value is believed to be the pKa value of II. Although the second pKa value of I (*i.e.*, ionization of the 2-hydroxy group) is 11.5, the pKa value of II is expected to be much lower because it has an electron-withdrawing *O*-acetyl group adjacent to the ionizing group rather than a negatively charged oxygen atom.

The magnitudes of the rate constants k_2^{obs} and k_3^{obs} could not be calculated from values of k^{obs} alone. However, the fact that the yield of III is 87% at pH 6.0 suggests that the ratio k_2^{obs}/k_3^{obs} has a value of approximately 7 at this pH. Also, the observation that the UV spectra of final reaction mixtures were much closer to those of the initial solutions at pH <4 than at pH 4–6 suggests that the value of the ratio k_2^{obs}/k_3^{obs} is lower at pH 4 than it is at pH 6. Hence, it appears likely that the rearrangement reaction occurs most favorably in the ionized form of II (II′).

Although the ester III was much more stable than II, it slowly degraded in aqueous buffers. After standing at 25° for several days, solutions that originally contained III had identical UV spectra to solutions that contained equivalent amounts of I. Hence, it appears that III is slowly hydrolyzed ($t_{1/2} \simeq 24$ hr at pH 4.5) to ascorbic and acetic acids. Rate constants for the various reactions that were calculated from changes in optical rotation or by using a pH-stat technique were very close to those calculated from measurements of changes in UV absorbance.

No evidence was found for any migration of acyl groups from the 2-hydroxy or 3-hydroxy groups to the 5-hydroxy or 6-hydroxy groups as previously reported (1). These migrations would only be likely at pH values where the 5-hydroxy and 6-hydroxy groups are appreciably ionized. Because these hydroxy groups are so weakly acidic, much higher pH values than 7.0 would be required.

During this study, some questions arose about structural assignments that had been previously made to some ascorbic acid esters. Nomura and Sugimoto (1) synthesized a di-*O*-benzoylascorbic acid, which had a melting point within the 149–151° range and a pKa in methanol of 4.00. It was suggested that this ester was 3,6-di-*O*-benzoylascorbic acid. However, based on the finding in the present study that II has a pKa in water (which probably would be lower than that in methanol) of about 5.5, whereas III has a pKa in water of about 2, it seems likely that Nomura and Sugimoto's compound was the 2,6-di-*O*-benzoyl derivative.

Furthermore, it was reported by Nomura and Sugimoto (1) that 2,6-di-*O*-benzoylascorbic acid rearranged to the 3,6-isomer in base. Again, the present study leads to the conclusion that it was the 3,6-isomer that isomerized to yield the 2,6-isomer. Other workers (13) synthesized a di-*O*-benzoyl ester of ascorbic acid, which had a melting point of 151–152°, and assigned it the structure of 2,6-di-*O*-benzoylascorbic acid. This compound is probably the same one that Nomura and Sugimoto (1) synthesized.

Vestling and Rebstock (11) carried out an acetylation of 5,6-isopropolidineascorbic acid in acetone with ketene. These authors isolated a compound which melted at 113–115° and had the elemental analysis of: C, 51.44%; H, 5.55%. It was suggested that this compound was the 3-*O*-acetate of 5,6-isopropolidineascorbic acid. The theoretical values for the elemental analysis of this latter compound are: C, 51.2%; H, 5.42%. This synthesis was repeated in the present study and a compound was obtained with a melting point of 113–115° and a similar elemental analysis to that found by Vestling and Rebstock (11). However, this product is believed to be 5,6-isopropolidine-2,3-di-*O*-acetylascorbic acid. The reasons for reaching this conclusion are:

1. The theoretical elemental analysis figures for the diacetate are: C, 52.0%; H, 5.33%.
2. The mass spectrum of the compound had a peak at *m/e* 300, which is the molecular weight of the diacetate.
3. The NMR spectrum of the compound in $CDCl_3$ did not contain singlet proton peaks at or near δ 10.8 or 8.0 (which is where the 2-hydroxy and 3-hydroxy protons of I and 5,6-isopropolidineascorbic acid resonate), but it contained two peaks at δ 2.35, which integrated for six protons. These peaks would be caused by the six protons on the two acetyl peaks of 5,6-isopropolidine-2,3-di-*O*-acetylascorbic acid.

When this compound was dissolved in aqueous buffers with pH values between 2 and 7, the solution developed a UV spectrum very similar to that of III. Hence, it is believed that the diacetate hydrolyzes to 2-*O*-acetylisopropolidineascorbic acid, in agreement with the assignment of Sumiki *et al.* (5).

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Keyphrases □ *Eleutherine bulbosa* (Iridaceae)—extraction of eleutherin and eleutherol, pharmacological screening □ Eleutherol and eleutherin—extracted from *Eleutherine bulbosa*, pharmacological screening □ Medicinal plants—extraction of eleutherin and eleutherol from *Eleutherine bulbosa*, pharmacological screening

In 1959, unknown bulbs, collected in the Peruvian part of the Amazonian jungle along the Ucayali River near Pucallpa[1], were sent to Europe[2]. Some bulbs, which arrived presumably a few months after collection, were planted in northern Italy near Milan at about 220 m above sea level, while others were subjected to biological screening.

Following the procedure described in this paper, a crystalline yellow substance was extracted from the dry bulbs. The substance proved to be a mixture of eleutherol and eleutherin[3]. The two compounds were separated, crystallized, and biologically assayed. Through specimens of bulbs and flowers obtained from the plant grown in Italy, the plant was identified[4] as *Eleutherine bulbosa* (Miller) Urb. [*E. plicata* (Ser.) Herb.].

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EXPERIMENTAL[5]

Bulbs grown in Amazonia were used in the early phases of the research, while bulbs grown in Italy were used subsequently. The only apparent difference between them was that the yellow crystalline material on the outer scales of dry bulbs was more abundant on those of Amazonian origin than on those of Italian origin. The bulbs planted in Italy were allowed to grow for about 6–7 months; they then were harvested, separated from the aboveground parts, and air dried for about 3 months at room temperature.

Dry bulbs (1 kg) were sliced and left to macerate with 1 liter of 95% ethanol for 2 days; the procedure was repeated once. The filtered alcoholic extracts were combined and brought to dryness at 50° under reduced pressure. The residue was dissolved in warm acetone, and one volume of ether and six volumes of water were added to it. The aqueous phase was separated and extracted four times with a total amount of ether corresponding to 1.5–2 times the volume of the water. All ether extracts were combined, dried over anhydrous sodium sulfate, filtered, and brought to dryness under reduced pressure.

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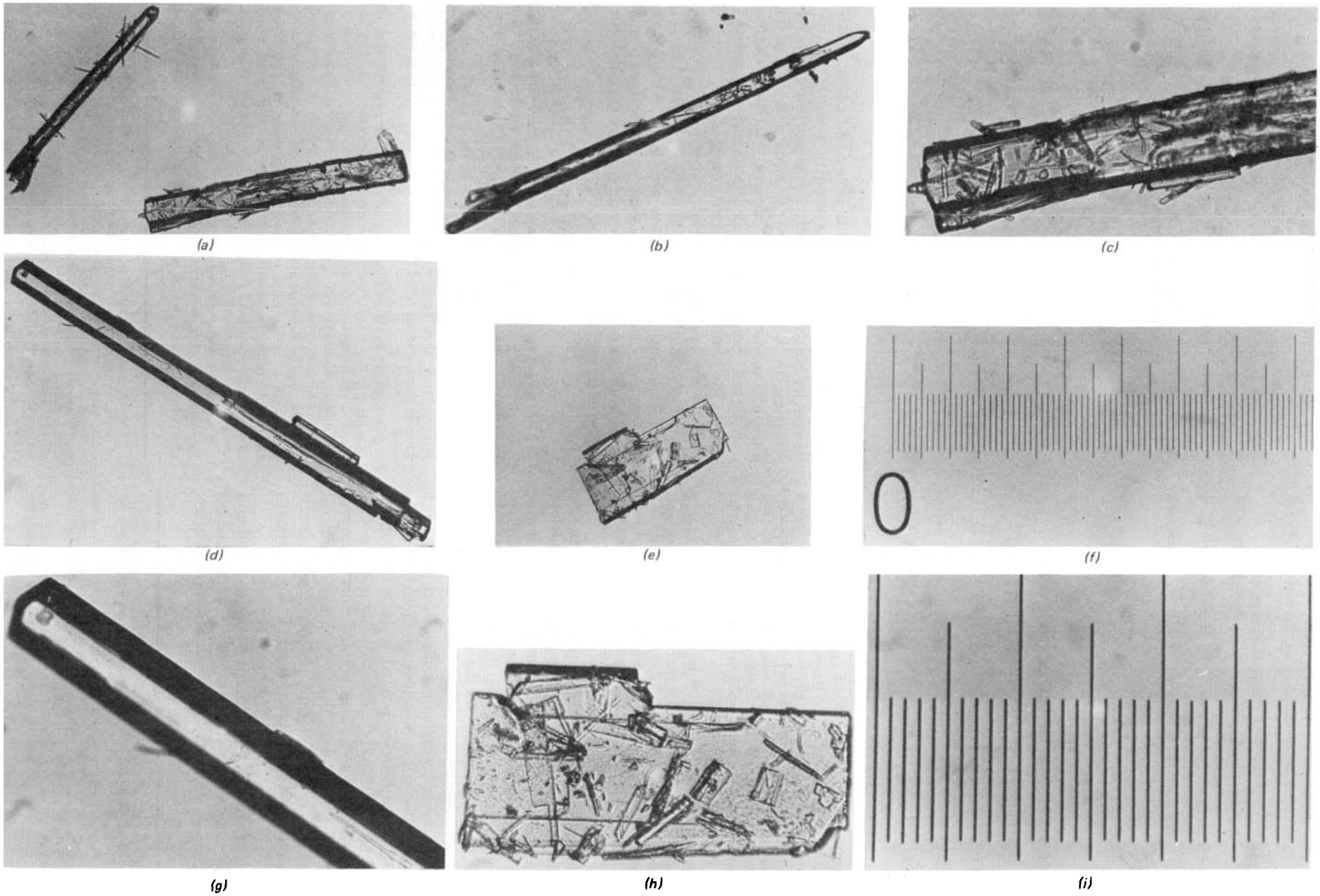
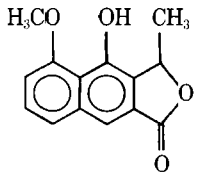


Figure 1—*Photomicrographs of crystals of eleutherol and eleutherin. Key: (a) crystals of eleutherol and eleutherin before fractionation, 40×; (b) crystal of eleutherol before fractionation, 100×; (c) crystal of eleutherin before fractionation, 100×; (d) crystal of eleutherol after fractionation, 40×; (e) crystal of eleutherin after fractionation, 40×; (f) scale 40× (1/10 of a millimeter is subdivided in 10 parts); (g) crystal of eleutherol after fractionation, 100×; (h) crystal of eleutherin after fractionation, 100×; and (i) scale 100× (1/10 of a millimeter is subdivided in 10 parts).*



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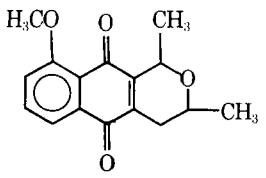

II

alcohol, once from acetone, and twice from *n*-butanol, yielding 500 mg, mp 145–147°; $[\alpha]_D^{20}$ + 221° (c. 0.1, chloroform). The material turned red when treated with concentrated sulfuric acid.

TLC showed the presence of two major blue spots, R_f 0.73 and 0.57 with Solvent A and R_f 0.51 and 0.40 with Solvent B. In addition, six minor spots and a tail were seen when Solvents A and B, respectively, were used.

Isolation of Eleutherol—The crystalline material was dissolved in warm benzene (500 mg in 30 ml at 50°). An equal amount of petroleum ether was added to the solution after the undissolved resins were separated by centrifugation. On refrigeration, white crystals precipitated from the solution. They were washed twice with benzene–petroleum ether (1:1) and crystallized twice from methanol, yielding white crystals (120 mg), mp 202–203°; $[\alpha]_D^{20}$ + 91.4° (c 0.1, chloroform). The material turned yellow when treated with concentrated sulfuric acid (1).

TLC showed the presence of a major spot at R_f 0.73 with Solvent A and at R_f 0.51 with Solvent B and only trace amounts of one undetermined substance. The UV spectrum and the analytical data corresponded to those published by Schmid *et al.* (1) for eleutherol (I), mp 202–203°; $[\alpha]_D^{20}$ + 90° in chloroform.

Isolation of Eleutherin—The benzene–petroleum mother liquors remaining from the initial crystallization of eleutherol were taken to dryness under reduced pressure. The residue was crystallized twice from *n*-butanol to give yellow crystals (280 mg), mp 165–170°; $[\alpha]_D^{20}$ + 280° (c 0.1, chloroform). These crystals turned red when treated with concentrated sulfuric acid (3). TLC showed the presence of one major spot at R_f 0.57 with Solvent A and at 0.40 with Solvent B, as well as trace amounts of I and three undetermined substances. The UV spectrum corresponded to that published by Schmid *et al.* (3) for eleutherin (II), except for the presence of a maximum at 313 nm, which indicates the presence of I.

The analytical data are compatible with those for II published by Schmid *et al.* (3), mp 175°; $[\alpha]_D^{15}$ + 346° in chloroform. This sample of eleutherin differed from that described previously (3) in that it had a lower melting point and optical rotation. Such differences may be related to the presence of traces of eleutherol and other unidentified products in the eleutherin sample.

Photomicrographs of the isolated materials are shown in Fig. 1.

Due to the preliminary scope of the biological investigations, the samples of I and II were screened without further purification.

Antibacterial and Cytotoxicity Testing—For testing antibacterial and cytotoxic properties *in vitro*, stock solutions of II were made up in methanol (10 mg in 1 ml of warm methanol diluted to 40 ml with distilled water). The antibiotic activity was tested on *Bacillus subtilis* (ATCC 9466) with the agar plate diffusion method, using medium 1 (15). The antidehydrogenase activity was tested according to Miyamura (16) on sarcoma 180 cells surviving in agar.

General Pharmacological Testing—For testing general pharmacological properties in mice and rats *in vivo*, II was finely ground, sieved through a 200-mesh stainless steel screen, suspended in an aqueous suspending medium [0.1% carboxymethylcellulose (w/v) and 1% polysorbate (v/v)] and given intraperitoneally in the constant volume of 0.2 ml/10 g (mice) or 0.5 ml/100 g (rats).

Mice—Experiments were performed on groups of albino male mice of 20–22 g (five to 10 animals/group).

The general activity was tested according to Irwin (17) on animals observed for 3 hr after treatment. Rectal temperature was measured with an electrothermometer[6] and a rectal probe (RM 6) for instant measuring (18). Motor coordination was controlled for 5 min on a rough-surfaced rod (22 mm diameter) rotating at 7.5 rpm (19, 20).

The response to a noxious stimulus was controlled with the tail pinch method of Bianchi and Franceschini (21) and the hot-plate method (22). The mydriatic effect was tested with the Pulewska method (23, 24), using a binocular microscope and controlled illumination. The diameter of the pupil was expressed in arbitrary units (20 units = 1 mm; basal value of 40 control mice = 11.2 units). The experiments were carried out 1 hr after dosing the animals.

Anticonvulsant properties were tested in animals convulsing after pentylenetetrazol (125 mg/kg sc), strychnine (3 mg/kg sc), or electric shock (15 mamp for 0.2 sec) (25, 26). Antitremor properties were tested in animals showing sustained tremors after oxotremorine (2.5 mg/kg sc) (24), antireserpine properties were tested in animals showing ptosis and hypothermia after reserpine (2 mg/kg sc) (26), and depressant properties were tested in aggregated animals showing an increased sensitivity toward the toxic (26) and motor stimulant (27) effects of amphetamine (25 mg/kg sc using 10 mice/group and 1 mg/kg ip using five mice/group, respectively) and in animals showing loss of the righting reflex following pentobarbital administration (40 mg/kg ip) (28).

The effects on the autonomic nervous system were tested in animals treated with lethal doses of epinephrine (2.5 mg/kg iv) (26) and with doses of oxotremorine (2.5 mg/kg sc) (24) and carbacholine (100 μg/kg sc) (24) provoking parasympathetic overstimulation (salivation, diarrhea, diuresis, and lacrimation). The effects on the intestinal motility were tested with the charcoal bolus method (29). An interval of 60 min was allowed between the administration of II and the tests.

An additional test for analgesia was carried out on animals showing abdominal writhings following intraperitoneal administration of phenylquinone [0.1 ml/10 g of a 0.02% (w/v) solution in 6% (v/v) ethanol] (30). In this test, II was given subcutaneously 1 hr before phenylquinone.

Rats—The anti-inflammatory and analgesic properties were tested in groups of albino male animals of 100–110 g (10 animals/group) showing edema and hyperalgesia in an hindfoot after a subplantar injection of carrageenan (0.1 ml/rat of a 2% solution) (31). The determinations were carried out before and at hourly intervals for 5 hr after the administration of carrageenan. Compound II was given orally 30 min before carrageenan.

The effect on blood pressure was determined in six unanesthetized male animals of 300–400 g. Before and at intervals after II was given intraperitoneally (15, 30, and 60 min), blood pressure was measured by means of a pneumatic cuff and a piezocrystal pulse pick-up applied to the root of the tail. An automatic blood pressure recorder[7] (32) was used.

The Quick prothrombin time (33) was determined on the plasma of male animals of 180–200 g (three or four animals/group) at intervals after II was given intraperitoneally and orally.

Rabbits—The effects on blood pressure and respiration were determined in three male rabbits of about 3 kg, anesthetized with urethan (1 g/kg iv) and with the vagal nerves severed. Blood pressure was recorded by means of a pressure transducer connected to a carotid artery; respiration was measured by means of an impedance pneumograph connected to needle electrodes placed on the chest. A physiograph[8] was used to record the variations in blood pressure and respiration. Epinephrine (3 μg/kg), acetylcholine (3 μg/kg), and carotid occlusion were the methods used to assess the reactivity of the blood pressure-regulating mechanisms. Compound II dissolved in ethanol and normal saline (10 mg in 3 ml of 95° ethanol diluted with saline to 10 ml) was given slowly (0.3–0.6 ml/kg iv).

Isolated Heart—Amplitude, rate of contractions, and coronary flow in Langendorff rabbit isolated heart were recorded in three preparations perfused with II. Isoproterenol (0.04 μg) was used to increase the rate and force of contractions of the heart (34).

Due to a shortage of material, I was tested only for antibiotic and cytotoxic properties *in vitro* and for the general activity in mice *in vivo*. The methods used for the preparation of the necessary solutions or suspensions and for the execution of the assays were as already described.

The solutions or suspensions of I and II were used as soon as they were prepared.

RESULTS

Antibacterial and Cytotoxicity Testing—Compounds I and II were assayed at concentrations ranging from 7.17 to 125 μg/ml.

[6] Ellab, Electrolaboratoriet, Copenhagen, Denmark.

[7] W & W Electronic, Basel, Switzerland.

[8] EMI.

Compound I failed to inhibit the development of *B. subtilis* (ATCC 9466) up to 125 μg/ml. Compound II was found to inhibit the development at various concentrations down to 62.5 μg/ml. Tetracycline hydrochloride was active at doses ranging from 0.3 to 0.5 μg/ml. Compounds I and II, up to 125 μg/ml, did not show any antidehydrogenase activity on sarcoma 180 cells.

General Pharmacological Testing—*Mice*—Compound I was given intraperitoneally to six groups of mice (five animals/group) at various doses (800, 400, 200, 100, 50, and 25 mg/kg) without provoking overt toxic effects within 3 hr of observation. It only provoked abdominal writhing at all doses and hypothermia (mean fall in rectal temperature about 2°) at 800 and 400 mg/kg. The central nervous system, the autonomic nervous system, and the muscular system were not affected by the compound as judged by a direct observation of the treated mice. As indicated under *Experimental*, the compound was not tested further due to the shortage of material.

Compound II was given intraperitoneally at various doses (400, 200, 100, 50, and 25 mg/kg) to groups of mice (five animals/group); the animals were observed for 3 hr after treatment. At 400 mg/kg, II provoked the death of one animal; reduced spontaneous motility and curiosity; did not provoke Straub tail, mydriasis, salivation, lacrimation, diuresis, diarrhea, and piloerection; and did provoke abdominal writhings, twitches, and convulsions. At the same dose, it provoked hypothermia (about 2°), motor incoordination, and analgesia (tail pinch method and hot-plate method). At 200 mg/kg and at lower doses down to 25 mg/kg, it produced abdominal writhings in all mice, with the effect lasting about 30 min.

Compound II, given intraperitoneally at 200 mg/kg to groups of mice (five to 10 animals/group), did not show anticonvulsant properties (pentylenetetrazol, strychnine, and electric seizures), anti-chemotremor properties (oxotremorine tremors), and antireserpine properties (reserpine ptosis and hypothermia). It did not modify the hypnotic effects of pentobarbital, the stimulant properties of amphetamine in aggregated mice (toxicity and hypermotility), the parasympathetic stimulant properties of oxotremorine and carbacholine, the lethal effects of epinephrine, and the intestinal motility. Compound II, 200 mg/kg sc, did not modify the writhing syndrome brought about by an intraperitoneal injection of phenylquinone.

Rats—Compound II, 100 mg/kg po given to groups of rats (10 animals/group), did not show anti-inflammatory (carrageenan foot edema) or analgesic effects in edematous hyperalgesia (Randall–Selitto method using carrageenan as hyperalgic compound). The anti-inflammatory and analgesic tests were performed following oral administration of II, because irritant drugs may simulate anti-inflammatory effects when administered parenterally (35). Compound II has irritant properties, as shown by the writhing syndrome brought about by the compound when administered intraperitoneally in mice.

Compound II, 200 mg/kg po and ip, shortened the Quick prothrombin time from the control value of 13.2 to 8.3 sec (po) and to 8.1 sec (ip). The effect was manifest 15 min after treatment but not 30 min and 1, 4, and 24 hr after treatment. Menadione sodium bisulfite, 200 mg/kg po and ip, was still effective 24 hr after treatment (prothrombin time 10.7 sec po and 10.5 sec ip).

Compound II, 100 mg/kg ip, increased blood pressure 15 and 30 min after dosing (initial blood pressure of 175 mm Hg; increase of 15–20 mm Hg); blood pressure returned to normal within 60 min. Abdominal writhings were present for about 20 min after dosing. Reserpine, 1 mg/kg ip, provoked a long-lasting fall in blood pressure (initial blood pressure of 165 mm Hg; fall of about 30 mm Hg).

Rabbits—Compound II, 300–600 μg/kg, provoked an occasional and modest fall in blood pressure which lasted a few seconds only, but it failed to modify blood pressure considerably and for long periods. It did not alter the responses of the animals to epinephrine, acetylcholine, and carotid occlusion. At higher doses (2 mg/kg), II provoked the arrest of respiration and death.

Isolated Heart—Compound II, perfused at the concentration of 1 μg/ml, did not alter the rate and amplitude of contractions and coronary flow of the Langerdorff rabbit isolated heart and did not modify the positive inotropic and chronotropic effects of isoproterenol.

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GLC Analysis of Hydrochlorothiazide in Blood and Plasma

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Abstract □ GLC with electron-capture detection was successfully applied to the analysis of hydrochlorothiazide in human blood and plasma with a sensitivity (0.05 μg/ml) suitable for use with persons on therapeutic dosage levels. On-column methylation with trimethylanilinium hydroxide in methanol was employed to convert hydrochlorothiazide and its bromo analog, which served as the internal standard, to their tetramethyl derivatives.

Keyphrases □ Hydrochlorothiazide—GLC analysis in human blood and plasma □ GLC, electron-capture detection—analysis, hydrochlorothiazide in human blood and plasma

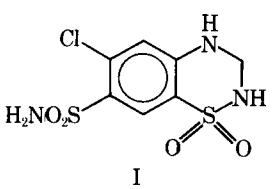
Hydrochlorothiazide[1] (I), 6-chloro-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide, is a potent saluretic agent widely used in the treatment of hypertension, congestive heart failure, and other edematous conditions. Although a colorimetric method permits the estimation of urinary levels of this drug (1), it is not sufficiently sensitive for analysis of blood and plasma samples from patients receiving therapeutic doses.

GLC, especially with electron-capture detection, has proven to be a useful method for the determination of submicrogram quantities of biologically active compounds in small volumes of body fluids. Direct GLC analysis of polar compounds at such levels is usually not possible, and derivatization techniques are normally employed to convert these compounds to less polar derivatives with improved chromatographic properties. On-column methylation with trimethylanilinium hydroxide in methanol has been found to effect this conversion by transforming hydrochlorothiazide to its tetramethyl derivative.

This paper describes the successful application of GLC combined with electron-capture detection to the analysis of hydrochlorothiazide in human blood and plasma with a sensitivity (~0.05 μg/ml) suitable for use with persons on medication at normal dosage levels.

EXPERIMENTAL

GLC was carried out with an instrument[2] equipped with an automatic sampler[3] (fitted with a 10-μl syringe[4]) and an electron-capture detector (8 mCi ^{63}Ni, 50-μsec pulse width, 310°). Commercially available, acid-washed support[5] was sieved (80–100 mesh) and then acid washed and silanized (2). This material was coated, using the filtration coating technique (2), with a 1% solution [toluene–chloroform (1:1)] of a mixture of OV-1 and polycyclohexanedimethanol adipate (6:1).



I

The two-component stationary phase approach (3) was employed because the inclusion of a small proportion of polyester resulted in improved chromatographic behavior[6]. Because of the difference in bleed rates between the polyester and the less volatile OV-1 siloxane polymer, the column temperature was reduced to <190° when not in use, resulting in a useful column lifetime of approximately 150 hr. Column conditions were: 1.2-m (4-ft) × 2.5-mm i.d. glass U-tube; 235°; argon–methane (95:5) carrier gas, 60 ml/min; and injector temperature, 280°. The retention time for tetramethyl-I was ~15 min.

On-column conversion of I and the internal standard, 6-bromo-3,4-dihydro-2*H*-1,2,4-benzothiadiazine-7-sulfonamide 1,1-dioxide (II), to the tetramethyl derivatives at the nanogram level was effected by dissolution and injection in a solution of trimethylanilinium hydroxide[7] in methanol (0.005 *M*). Preparative scale methylation of these two benzothiadiazines was carried out independently by dissolving 1 mg of the compound in 0.2 ml of 0.5 *M* trimethylanilinium hydroxide, removing the methanol *in vacuo*, and heating the residue in a sealed tube under nitrogen at 250° for 30 min.

The total reaction mixture was partitioned between ethyl acetate and water, and the crude reaction product was obtained by evaporation of the ethyl acetate solution. Purification was achieved by preparative scale TLC (silica gel G) with benzene–ethyl acetate (90:10) (tetramethyl-I, R_f 0.50) or benzene–ethyl acetate (70:30) (tetramethyl-II, R_f 0.46) and elution of the appropriate zone with ethyl acetate. Purity and identity were established by GLC–mass spectrometry.

The detailed procedure devised for isolation of the drug (and internal standard) in a form suitable for GLC analysis is presented here. The main steps are: (*a*) addition of the internal standard, (*b*) removal of extraneous substances with benzene extraction, (*c*) extraction with ethyl acetate, (*d*) back-extraction into ammonium hydroxide, (*e*) adjustment to pH 3.7 and extraction with ethyl acetate, (*f*) evaporation of the extract to dryness, (*g*) dissolution of the residue in trimethylanilinium hydroxide in methanol, and (*h*) GLC.

One-half milliliter of blood (heparinized) or plasma is placed in a 1.5-ml plastic centrifuge tube[8] to which previously has been added 0.15, 0.20, or 0.30 μg of the internal standard (II) in methanol (taken to dryness with nitrogen). The sample is thoroughly agitated to ensure dissolution of the internal standard. The blood or plasma is extracted with 0.5 ml of reagent grade benzene (a process blank should be run to ensure that none of the solvents or reagents cause interferences at the retention times of the drug and internal standard), the mixture is centrifuged[9], and the benzene layer is removed (disposable pipet) and discarded; this step is repeated. Then the sample is extracted with 0.5 ml of reagent grade ethyl acetate, the mixture is centrifuged, and the ethyl acetate layer is isolated (disposable pipet); this step is repeated.

The ethyl acetate extracts are combined in a second 1.5-ml plas-

[1] HydroDIURIL, Merck & Co.
[2] Hewlett-Packard model 7610A.
[3] Hewlett-Packard model 7671A.
[4] Hamilton 701N.
[5] Gas Chrom P, Applied Science Laboratories.
[6] Following submission of this manuscript, it was found that 3% OV-61 packing (Gas Chrom Q, 100–120 mesh, Applied Science Laboratories) also serves as a useful stationary phase; Dr. George O. Breault, personal communication.
[7] Fluka.
[8] Eppendorf Reaktionsgefässe microtube 3810.
[9] Brinkmann Eppendorf centrifuge 3200.

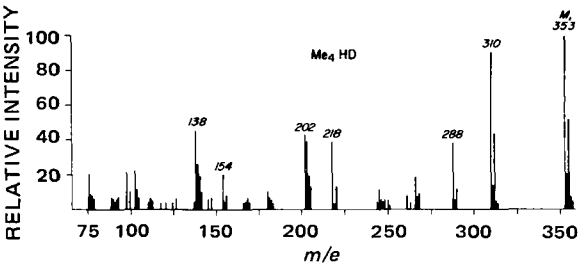


Figure 1—*Mass spectrum of tetramethyl-I as determined by combined GLC–mass spectrometry. Spectrometer conditions were: LKB model 9000; electron energy, 70 ev; source temperature, 270°; accelerating potential, 3.5 kv; and trap current, 60 μamp.*

tic centrifuge tube, and 0.5 ml of 0.1 *N* ammonium hydroxide is added. After thorough and immediate mixing and centrifugation, the ethyl acetate layer is discarded. The aqueous phase is taken to pH ~3.7 with 0.05 ml of acetic acid and extracted with 0.5 ml of ethyl acetate. Following centrifugation, the ethyl acetate layer is isolated; ethyl acetate extraction of the aqueous phase and isolation of the organic phase are repeated.

The ethyl acetate extracts are combined in a 5-ml glass centrifuge tube and taken to dryness with nitrogen (water bath, 45°). The residue is dissolved in 0.05 ml of 0.005 *M* trimethylanilinium hydroxide in methanol and transferred, using a disposable pipet, to a tapered 0.2-ml sampling vessel[10] for automated injection, GLC analysis (~1.8 μl of the final solution is injected).

A working curve for quantitation is obtained as follows. One-half-milliliter portions of control blood or plasma (preferably from the patient prior to medication) are added to 1.5-ml plastic centrifuge tubes containing 0.15, 0.20, or 0.30 μg of internal standard (added as methanol solution and taken to dryness) and sufficient I to result in levels of 0, 0.05, 0.20, 0.40, and 0.60 μg of drug/ml of blood or plasma. These samples are then carried through the isolation procedure and analysis, and the resulting peak height ratios (drug–internal standard) are plotted *versus* micrograms per milliliter of added I. The peak height ratio resulting from analysis of blood or plasma from a patient is then compared to a working curve (obtained as described) to give the concentration of drug in micrograms per milliliter.

RESULTS AND DISCUSSION

Preliminary experiments indicated, not unexpectedly, that I itself is not detected by GLC using column conditions suitable for compounds of similar molecular weight but possessing less polarity. Since derivatization techniques are often employed to allow or improve GLC of polar compounds of moderate molecular weight, this approach was investigated. Trimethylsilylation was found not to be fruitful, nor was methylation with diazomethane.

MacGee (4) reported that a number of polar drugs can be successfully analyzed by GLC by employing an on-column methylation, using a solution of tetramethylammonium hydroxide in methanol as both solvent and reagent. Similarly, Brochmann-Hanssen and Oke (5) described an on-column methylation method for the GLC of polar compounds, using trimethylanilinium hydroxide in methanol. VandenHeuvel *et al.* (6) reported the use of this reagent in a heavy isotope tracer study involving GLC–mass spectrometry. An on-column methylation approach for the determination of the drug bumetanide in urine also was reported (7).

Initial experiments at the microgram level using this reagent with flame-ionization detection were highly encouraging. Compound I was converted to a single product with satisfactory chromatographic properties; this product was shown to be the tetramethyl derivative (mol. wt. 353) by combined GLC–mass spectrometry (Fig. 1). The requirements of an assay for I in human blood and plasma are such that a sensitivity of at least 0.1 μg/ml is needed. Successful derivatization, chromatography, and detection of submicrogram quantities of drug are needed in a reproducible procedure.

The presence of a chlorine atom in I would be expected to lead to moderately good electron-capture sensitivity for the tetramethyl derivative of this drug, and a value of 1.75×10^3 coulombs/mole (8) was found under the described conditions. This sensitivity is sufficient to allow detection and quantification at the nanogram (injected) level; linearity of detector response is observed up to 12 ng with the instrument conditions employed. On-column conversion of I to its tetramethyl derivative is quantitative, since injection of 4 ng of drug in trimethylanilinium hydroxide in methanol results in the same electron-capture detector response as injection of an equivalent amount of authentic tetramethyl-I dissolved in methanol.

Analogous experiments with ^{14}C-labeled I and authentic tetramethyl-I-^{14}C, involving collection and counting of the carbon dioxide resulting from combustion of the appropriate GLC component, using the method of Trenner *et al.* (9), demonstrated that equivalent amounts of radioactivity were eluted from the column in both cases. Only 75% of the theoretically available radioactivity was collected from the tetramethyl-I peaks, however, indicating some loss on the column during chromatography. Experiments with mass standards and I-^{14}C indicated that the yield of drug carried through the entire isolation procedure is ~50%.

The molarity of the methanolic trimethylanilinium hydroxide solution used for on-column methylation is important (10). At reagent concentration of <0.0005 *M* and I concentrations of 1–5 ng/μl, methylation drops off sharply and no peak is observed for tetramethyl-I (or even trimethylhydrochlorothiazide). Maximal tetramethyl-I formation is observed at reagent molarities of ~0.005–0.01 *M*; at higher molarity, the size of the tetramethyl-I peak diminishes until essentially no derivative peak is observed at 0.5 *M*.

At the optimal reagent molarity, the flash heater temperature of the instrument used in this study can be varied from 240 to 320°

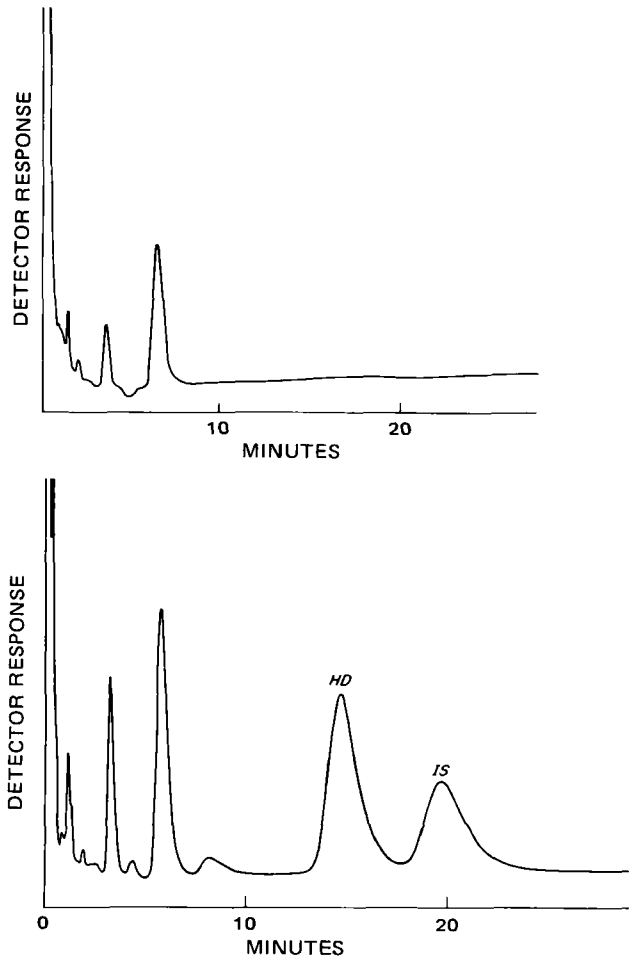


Figure 2—*Gas chromatograms resulting from analysis of control blood (top) and of control blood spiked with I and the internal standard (0.40 μg/1.0 ml each) (bottom). Isolation and chromatographic conditions are as given in the* Experimental *section.*

[10] Hewlett-Packard glass vials, 4330-0540.

Table I—Hydrochlorothiazide Levels in Blood and Plasma

Subject	Dose, mg	Hours Postdose	Blood Level, μg/ml	Plasma Level, μg/ml
W	50	3	0.62	—
		24	0.11	—
W	25/50	2	0.22/0.51	0.10/0.31
W[a]	50	1.5	0.36	—
		4	0.64	—
		6	0.44	—
		24	0.09	—
K	50/100	1.5	—	0.23/0.23
		2	—	0.35/0.36
		3	—	0.28/0.43
		4	—	0.24/0.29
L	50/100	1.5	—	0.05/0.15
		2	—	0.11/0.34
		3	—	0.29/0.33
		4	—	0.18/0.22
G	100	1.5	—	0.71
		2	—	0.51
		3	—	0.35
		4	—	0.35

[a] These values were obtained without use of the internal standard.

with little effect upon the size of the tetramethyl-I peak. The temperature normally employed, 280°, is similar to that (270–275°) reported for on-column methylation of uric acid with trimethylanilinium hydroxide in methanol (11).

Automated injection is of obvious value in GLC assays. The samples are usually sealed in sampling vials, but occasionally a vial may not be sealed tightly enough to prevent the evaporation of a relatively volatile solvent such as methanol at the temperature (40–45°) of the automatic injector and an increase in sample concentration results. This should not be a problem when an internal standard is employed for the assay; but if the concentration of reagent increases sufficiently, this effect of molarity may be encountered. It is desirable to keep the length of time I is exposed to the basic reagent to a minimum.

The bromo analog of I, II, was chosen as an internal standard. The bromo compound is converted upon injection in methanolic trimethylanilinium hydroxide to its tetramethyl derivative (mol. wt. 397). The retention time of this compound is sufficiently greater than that of the tetramethylated drug to allow its use as an internal standard, and no interfering peaks (for drug or internal standard) are observed when control blood or plasma is carried through the isolation procedure (Fig. 2, top). The chromatogram resulting from analysis of control blood spiked with I and II is illustrated in Fig. 2 (bottom).

Linear relationships are observed when mixtures of these two compounds, containing constant amounts of the internal standard and varying amounts of I, are spiked into control blood or plasma and carried through the assay and the resulting peak height ratios are plotted against micrograms of spiked I per milliliter blood or plasma. Such plots are established each day and are used as working curves in the quantification of blood and plasma levels (see *Experimental*).

A sensitivity limit of ~0.05 μg/ml of blood and plasma is found using the isolation procedure and instrument conditions described in the *Experimental* section. Satisfactory replica of the entire assay results and precision of the GLC analysis were obtained. For example, quadruplicate assay of a spiked plasma sample gave a value of 0.66 ± 0.02, and quintuplicate GLC analysis of one of these isolates gave a value of 0.67 ± 0.01 μg/ml.

The chromatograms resulting from the assay of blood samples from a patient (W) receiving hydrochlorothiazide therapy, but who had ceased medication 4 days prior to resumption, are presented in Fig. 3. Blood drawn immediately after resumption of medication (single dose, 50 mg) gave an insignificant peak at the retention time of tetramethyl-I (Fig. 3, top; referred to as 0 hr but actually 96 hr postdose). The tetramethyl-I peak in the 3-hr postdose chromatogram is greatly enhanced (Fig. 3, middle), whereas this peak is markedly reduced in intensity with the 24-hr sample (Fig. 3, bottom). The concentrations calculated for these two samples are 0.62 and 0.11 μg/ml, respectively. The corresponding 3- and 24-hr urinary concentrations of I, as determined with the GLC method

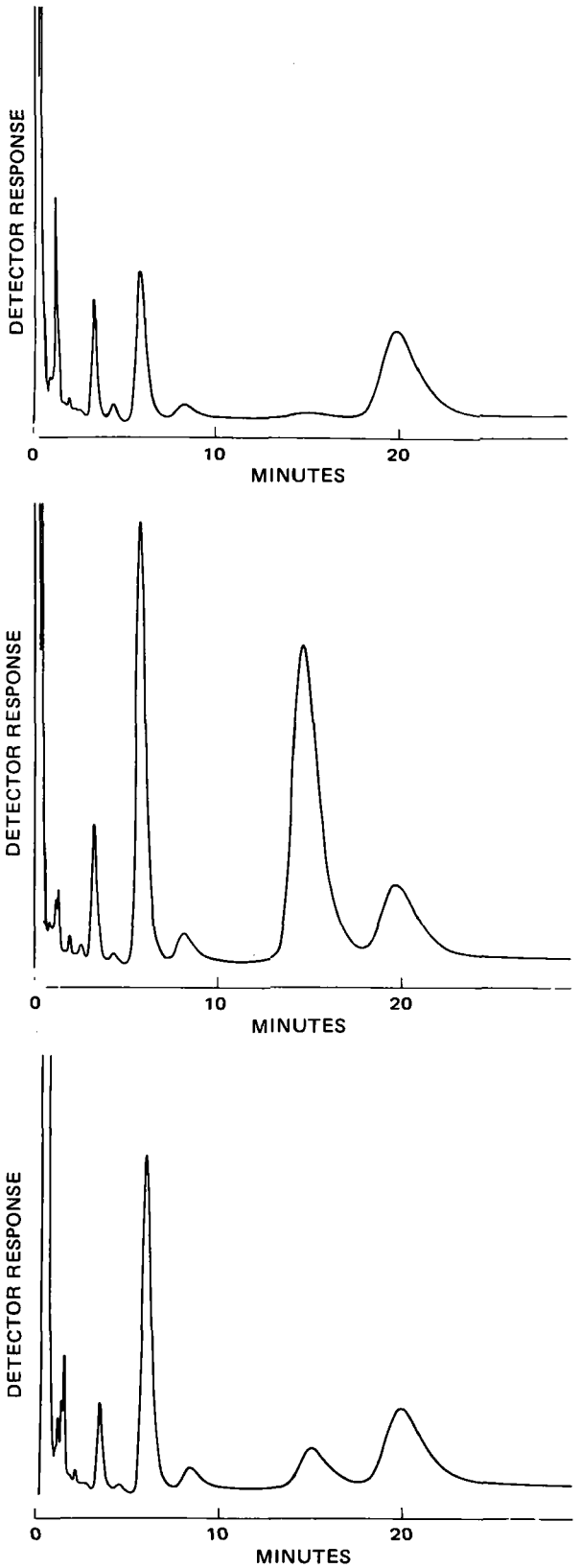


Figure 3—*Gas chromatograms resulting from analysis of blood from Subject W (0 hr but actually 96 hr postdose) (top), blood from Subject W 3 hr postdose (50 mg I) (middle), and blood from Subject W 24 hr postdose (bottom).*

(simply substituting urine for blood), were 19.8 and 6.4 μg/ml, respectively.

The level of drug in the blood of Subject W 2 hr postdose (50

mg) on a single day during an extended medication period was 0.51 μg/ml, in line with earlier findings. The corresponding value during a period in which the dosage was only 25 mg/day was 0.22 μg/ml. Plasma levels corresponding to these two values were 0.31 and 0.10 μg/ml, respectively. These values are presented in Table I, along with plasma levels for three individuals (Subjects G, K, and L) at dosage levels of 50 and 100 mg. Blood levels for Subject W (50 mg) at 1.5, 4, 6, and 24 hr, determined prior to introduction of the internal standard approach, are also presented in the table.

This paper presents a method for the determination of hydrochlorothiazide in small volumes of blood and plasma from persons receiving therapeutic levels of this drug. Drug levels are reported for a limited number of patients, demonstrating the adequacy of the method for measuring the drug in blood and plasma at clinical doses. The method described in this paper should provide the means for carrying out pharmacokinetic and bioavailability studies.

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Abstract □ The flow properties and viscosity of the vehicle into which drugs are incorporated can be determining factors in the bioavailability of topically applied ophthalmic drugs. It is shown, in rabbits, that when polyvinyl alcohol and methylcellulose are compared on a viscosity basis, there is essentially no difference in the two vehicles with regard to their influence on ocular drug bioavailability. Moreover, the rate of drainage loss for polyvinyl alcohol solutions, as determined by the radioactive technetium technique, compares favorably to methylcellulose solutions of similar viscosity. The relationship between viscosity and contact time or drainage loss of a drug is not a direct one, but an optimum viscosity range exists for polyvinyl alcohol solutions. This optimum range of 12–15 cps in rabbits is identical to that found for methylcellulose and differs considerably from the commonly employed viscosity in commercial preparations. Based on the methylcellulose–polyvinyl alcohol comparison, it appears that vehicles exhibiting or approximating Newtonian flow properties show comparable effects as ophthalmic vehicles. Finally, a discussion of non-Newtonian vehicles and their expected behavior in the eye is presented.

Keyphrases □ Polyvinyl alcohol—effect of vehicle viscosity on ocular drug bioavailability in rabbits, compared to methylcellulose □ Ocular bioavailability—effect of vehicle viscosity, polyvinyl alcohol and methylcellulose solutions, rabbits □ Ophthalmic vehicles—evaluation of polyvinyl alcohol, compared to methylcellulose, optimum viscosity range, rabbits

Great time and effort have been expended on determining the optimum liquid vehicle in which to incorporate drugs for instillation into the eye. Most efforts have been based on the premise that a highly viscous solution would prolong contact time of the drug with eye tissues, thus enabling a greater amount of drug to be absorbed into the desired area.

Recently, Adler *et al.* (1) made the important observation that high viscosity solutions do not greatly increase corneal contact time in humans and have only a small effect on bioavailability. An extensive study on methylcellulose solutions (2), however, has shown that there is considerable prolongation of contact time in rabbits and a two- to threefold improvement in bioavailability. This inconsistency apparently is due to differences between humans and rabbits with respect to the effect of medium viscosity solutions in the eye.

The present study explores the influence on ocular drug bioavailability of various polyvinyl alcohol solutions. Methylcellulose and polyvinyl alcohol are both used extensively as vehicles for ophthalmic drugs and the literature is confusing as to which polymer is best for ocular use. The aim of this report is to resolve that confusion as well as to provide a rational basis for predicting ophthalmic vehicle viscosity effects in general.

EXPERIMENTAL

Materials—Water was double distilled from alkaline permanganate in an all-glass distillation apparatus. Technetium solutions

mg) on a single day during an extended medication period was 0.51 μg/ml, in line with earlier findings. The corresponding value during a period in which the dosage was only 25 mg/day was 0.22 μg/ml. Plasma levels corresponding to these two values were 0.31 and 0.10 μg/ml, respectively. These values are presented in Table I, along with plasma levels for three individuals (Subjects G, K, and L) at dosage levels of 50 and 100 mg. Blood levels for Subject W (50 mg) at 1.5, 4, 6, and 24 hr, determined prior to introduction of the internal standard approach, are also presented in the table.

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Materials—Water was double distilled from alkaline permanganate in an all-glass distillation apparatus. Technetium solutions

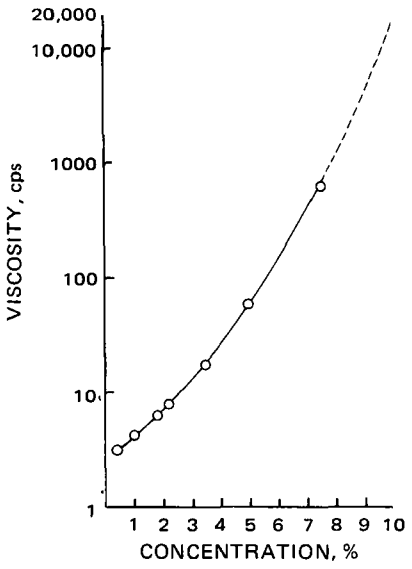

Figure 1—*Viscosity of polyvinyl alcohol solutions as a function of concentration at 30°. The dotted line represents extrapolated values for concentrations greater than 7.5%.*

were prepared using a package of solutions[1] and equipment used to prepare technetium, ^{99m}Tc, colloidal suspensions. The preparative procedure was described previously (3).

Polyvinyl alcohol[2], 20–90 grade, was used. Tritiated pilocarpine was obtained commercially[3] and was purified immediately before use. The reasons for the purification and the methods used were reported earlier (4). The specific activity of pilocarpine was 6.95 Ci/mmole. All other chemicals were either reagent or analytical grade.

Male, albino rabbits[4], 1.8–2.4 kg, were fed a regular diet with no restrictions on food or water intake.

Methods—*Determination of Instilled Solution Drainage Rate*—The general procedure for the determination of drainage rate by the nonsampling method was reported previously (3). In the present studies, twice the desired concentration of polyvinyl alcohol was diluted with an equal volume of technetium suspension to obtain the final concentration. The resulting solution was equilibrated in a water bath at 30° prior to instillation. Then 25 μl of polyvinyl alcohol–technetium solution was instilled with a microliter syringe[5], the probe was positioned, and the experiments were carried out as described previously (3).

Viscosity Studies—Viscosity profiles of the various polyvinyl alcohol solutions were obtained using a viscometer[6] and a No. 1 spindle. Solutions were maintained at 30 ± 0.1° throughout the viscosity measurements.

Aqueous Humor Drug Studies—The experimental details for aqueous humor drug concentration studies were reported previously (5). Unanesthetized rabbits were used in all experiments.

RESULTS

Viscosity Studies—Dilute polymer solutions generally exhibit or approximate Newtonian flow properties (6), and this was the case in the methylcellulose studies mentioned previously (2). It is well known that aqueous solutions of polyvinyl alcohol exhibit non-Newtonian viscosity (7). However, over the concentration range employed, the results approximated Newtonian behavior.

The relationship between viscosity and concentration of polyvinyl alcohol solutions at 30° is illustrated in Fig. 1. It is clear that dilute solutions of polyvinyl alcohol (<3%) show relatively small increases in viscosity with concentration. As the concentration of polyvinyl alcohol is further increased, however, viscosity changes may cover several orders of magnitude. It is known (8) that aqueous solutions of polyvinyl alcohol greater than approximately 1% contain entangled aggregates of hydrogen-bonded molecules. Such solutions may vary in their viscosities with age and with applied shear due to gelling.

[1] Collokit, Abbott Radio-Pharmaceuticals, North Chicago, Ill.
[2] Supplied by Allergan Pharmaceuticals, Irvine, Calif.
[3] New England Nuclear, Boston, Mass.
[4] Klubertanz, Edgerton, Wis.
[5] Hamilton Co., Reno, Nev.
[6] Brookfield model LVT.

Table I—Drainage Rate Constant of Polyvinyl Alcohol Solutions as a Function of Concentration and Viscosity

Concentration, %	Viscosity, cps	k, min^{-1}	Number of Determinations
0.0[a]	1.0	0.54	3
0.5	3.1	0.32 (0.05)[b]	5
1.1	4.2	0.25 (0.03)	5
1.9	6.1	0.25 (0.03)	10
2.25	7.7	0.31 (0.04)	5
3.5	17.5	0.08 (0.02)	7
5.0[c]	57.8	0.14 (0.02)	9

[a] Refers to data obtained using pure technetium suspension (2). [b] Values in parentheses refer to standard error of the mean. [c] Considerable difficulty was encountered in instilling this solution as well as in mixing it with the technetium suspension. These factors may limit the reliability of the drainage rate data.

Drainage Rate of Polyvinyl Alcohol Solutions and Its Relationship to Viscosity and Concentration—Loss of an instilled drug solution through drainage can account for a tremendous decrease in the amount of drug available for absorption (3). Thus, a small drainage rate would favor increased drug absorption. The drainage rates of various concentrations of polyvinyl alcohol were determined as described under *Experimental*. Table I lists these data as well as the viscosities of the various polyvinyl alcohol solutions.

A very dilute (0.5%) solution of polyvinyl alcohol causes a significant decrease in the drainage rate constant over that of an aqueous solution. This decrease occurs over a threefold increase in solution viscosity. From 0.5 to 2.25% polyvinyl alcohol, another two- to threefold increase in viscosity occurs and the drainage rate constant remains essentially unchanged. Somewhere beyond 3% polyvinyl alcohol, the drainage rate undergoes another sharp decrease and then remains fairly constant.

Figure 2 illustrates the drainage rate *versus* viscosity data. Drawing a smooth curve through the points results in a major change in the slope of the line at about 12–15 cps, which is shown as the hatched area in Fig. 2. This area corresponds approximately to a 3% solution (see Fig. 1). The conclusion then is that approximately a 3% polyvinyl alcohol solution is the optimum because large increases in viscosity result in only minor decreases in drainage rate beyond this concentration. The concentration of polyvinyl alcohol commonly used commercially is 1.4%; presumably other factors, such as comfort, account for the difference.

Aqueous Humor Levels of Philcarpine Nitrate in Polyvinyl Alcohol Vehicle—It seemed reasonable to assume that a viscous

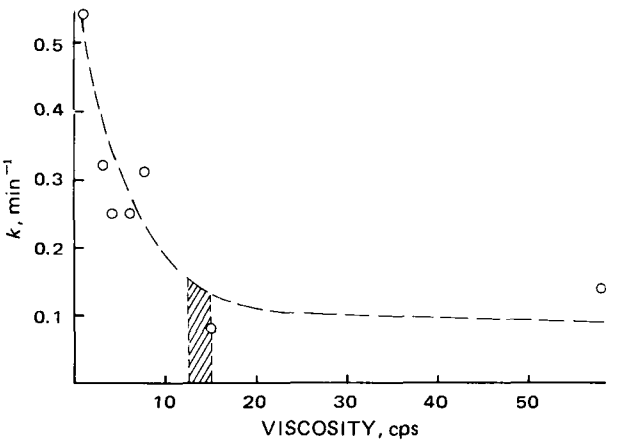

Figure 2—*Drainage rate of various polyvinyl alcohol solutions as a function of solution viscosity.*

Table II—Aqueous Humor Levels of Pilocarpine Nitrate from Various Viscosity Polyvinyl Alcohol Solutions

Polyvinyl Alcohol, %	Aqueous Humor[a], μg/ml		
	20 min	30 min	60 min
0	1.20 (0.10)[b]	0.66 (0.06)	0.67 (0.05)
0.5	1.16 (0.14)	1.38 (0.08)	
1.1	1.26 (0.20)	0.94 (0.06)	0.34 (0.03)
1.9	1.14 (0.09)	2.03 (0.31)	
2.25	1.11 (0.07)	1.28 (0.11)	0.27 (0.04)
3.5	1.65 (0.22)	1.48 (0.20)	
5.0	2.22 (0.20)	2.01 (0.28)	
10.0	5.41 (0.38)	3.18 (0.64)	2.05 (0.19)

[a] At least six determinations at each point. [b] Numbers in parentheses refer to standard error of the mean.

solution would prolong contact time of drug with the eye through decreased drainage loss and would provide the potential for increased drug absorption into and through the cornea. Since the decrease in drainage rate had already been shown for various polyvinyl alcohol solutions, the effect on aqueous humor drug concentration remained to be demonstrated.

Table II shows the results of various polyvinyl alcohol concentrations on aqueous humor drug levels at selected time points. There appears to be little if any improvement in aqueous humor levels until something beyond 2.25% polyvinyl alcohol is used. A rather dramatic increase is noted with 10% polyvinyl alcohol, but the solution is so viscous at this concentration that it probably blocks the drainage duct and would also cause marked blurring of vision. These drawbacks probably would make it impractical for commerical use.

There is greater scatter in some of the data than was experienced in the past; based on experimental technique, an explanation for the deviation in the data cannot be offered. Nevertheless, trends can be discussed. For example, the 3.5, 5.0, and 10.0% polyvinyl alcohol solutions are all consistently higher than the lower concentrations.

DISCUSSION

To discuss viscosity effects, one must first have some understanding of the types of flow exhibited by various systems as well as an appreciation as to how these different systems behave in animal or human studies. For example, rabbits blink considerably less frequently than humans and therefore exert less stress on a polymer system.

The flow properties of any given system can have, in theory, a significant effect on the behavior of that system when instilled into the eye. By knowing the flow properties of a system, it should be possible to make some statements regarding its expected activity in the eye, as well as to account for possible differences in humans and animals. This information is summarized in Table III.

As mentioned, numerous studies have been conducted on methylcellulose and polyvinyl alcohol, both in rabbits and in humans, in an effort to determine the ideal ophthalmic vehicle. Almost all of these studies considered only vehicle contact time or bioavailability and ignored the very important aspects of patient acceptability and desirable properties for pharmaceutical handling, *e.g.*, sterile filtration. Many of these studies were confusing, contradictory, and, at best, incomplete. If, however, these studies are discussed in terms of the information in Table III and the findings reported here, the picture begins to become somewhat clearer.

In one early study, homatropine hydrobromide in 1% methylcellulose was administered to human subjects; this combination induced greater cycloplegia and mydriasis than a simple aqueous solution (9). The increased effectiveness was attributed to an increased contact time of drug with the eye. It was concluded that about double the quantity of drug could be expected to penetrate by increasing the viscosity of the instilled solution.

This effect was substantiated in a study in rabbits where a solution of 0.5% methylcellulose (4000 cps) increased contact time by 334% over an aqueous solution (10). Haas and Merrill (11), using human subjects, determined that a solution of pilocarpine in 0.5% methylcellulose produced greater miosis than a nonmethylcellulose solution of equal drug concentration.

Krishna and Brow (12), comparing vehicles, found that 1.4% polyvinyl alcohol in isotonic saline afforded greater surface contact time than 0.5% methylcellulose in isotonic saline alone. The results of this study, performed in rabbits, seem rather strange since 0.5% methylcellulose has a much higher viscosity than a 1.4% polyvinyl alcohol solution, assuming the standard grades of each were used.

It was found that in rabbits the increase in contact time of methylcellulose ophthalmic vehicles was about proportional to the

Table III—Various Viscosity Types and Their Expected Behavior in the Eye

Flow Type	Characteristics	Example	Potential Ocular Effects
Newtonian	Constant viscosity at constant temperature and pressure; viscosity independent of rate of shear	Simple liquids such as water and glycerin	Drainage loss of drug solution should be inversely proportional to viscosity; due to shear independence, blinking should have no effect on viscosity and such systems should behave the same in rabbits and humans
Non-Newtonian Pseudoplastic	Stress increases more rapidly at low rates of shear than at high rates; the apparent viscosity decreases as shear stress (shear thinning) increases	Concentrated dispersions of small particles and solution of long chain molecules	If the system undergoes shear in the eye, this system would be poor; in humans, if blinking causes shear, the system will thin and drain from the eye; in rabbits, such a system will probably resemble a Newtonian system
Plastic	Resembles pseudoplastic system, but the rate of shear does not acquire a finite value until the stress exceeds a certain yield value	Modeling clay	This system would be good as long as the yield value is not exceeded; in rabbits, this may be true, but in humans the system thins as with pseudoplastic once the yield value is exceeded
Dilatant	The opposite of pseudoplastic; the force increases faster than the rate of shear	Wet sea sand	If blinking in humans produces shear, the system will thicken; this system has good potential in humans, although the advantages of such a system would probably not be observed in rabbits
Thixotropic	A reversible and noninstantaneous decrease in apparent viscosity upon shear; the effect increases with the rate of shear; time-dependent shear thinning	Bentonite magma	Probably not a desirable situation in humans or in rabbits since the system will thin with time

Table IV—Comparison of Methylcellulose and Polyvinyl Alcohol Vehicles in Rabbit Eyes

Methylcellulose (1)		Polyvinyl Alcohol	
From 1 to 15 cps, drainage rate constant decreases by a factor of 3; from 15 to 100 cps, drainage rate constant further decreases by a factor of 3		From 1 to 7.7 cps, drainage rate constant decreases by a factor of 2; another two- to threefold decrease in k from 7.7 to 57.8 cps	
A rapid decrease in drainage rate with a small increase in viscosity, followed by a smaller decrease in drainage with a large increase in viscosity		True with polyvinyl alcohol also	
cps	k, min^{-1}	cps	k, min^{-1}
4.2	0.36	4.2	0.25
12.5	0.19	17.5	0.08
40.0	0.14	57.8	0.14
Approximately a 1.6-fold increase in aqueous humor concentration at 20 min from 1 to 12.5 cps, followed by a 1.2-fold increase in concentration from 12.5 to 100 cps		Approximately a 1.4-fold increase in aqueous concentration at 20 min from 1 to 17.5 cps, followed by a 1.4-fold increase from 17.5 to 57.8 cps	

viscosity of the solution in the lower viscosity region (5–25 cps) and leveled off at 55 cps or above (13). Even though methylcellulose is strictly a pseudoplastic system, Table III predicts Newtonian behavior in rabbits, making the 5–25-cps findings consistent. When a methylcellulose solution at 55 cps was used, mydriasis from a 0.02% solution of scopolamine hydrobromide was doubled.

Linn and Jones (14) contradicted the results of Krishna and Brow (12) by finding that the length of time needed for 0.5% hydroxypropyl methylcellulose to pass through the drainage apparatus was about twice that of 1.4% polyvinyl alcohol. This work, however, was carried out in humans whereas the earlier work was carried out in rabbits.

Bach *et al.* (15), using rabbits, and Waltman and Patrowicz (16), using human subjects, confirmed the superiority of 0.5% methylcellulose over 1.4% polyvinyl alcohol, the former in preventing ocular infections with neomycin sulfate and the latter in producing corneal and aqueous humor fluorescein concentrations. Again, these findings appear reasonable due to the higher viscosity imparted by a 0.5% methylcellulose solution over a 1.4% polyvinyl alcohol solution. Other studies (17, 18) also confirmed this finding.

A comprehensive evaluation of methylcellulose solutions in improving ocular drug bioavailability was conducted (2). Methylcellulose delayed solution drainage from the eye, and the major change occurred in the lower viscosity range. Moreover, it was found that pilocarpine aqueous humor levels could be approximately doubled through the use of methylcellulose solutions.

Despite some of the seeming contradictions in these studies, it does seem reasonable to assume that a viscous solution would prolong the contact time of drug with the eye and provide the potential for greater drug absorption into the desired area. Recently, however, this belief was challenged by Adler *et al.* (1) who concluded that increasing vehicle viscosity does not prolong ocular contact time nor markedly improve ocular drug bioavailability in humans. If the vehicles under consideration are behaving as Newtonian systems, these findings appear intuitively incorrect (Table III); however, if the vehicles are, in fact, pseudoplastic, the shear created by blinking would cause them to thin so that a large increase in instilled solution viscosity might provide only slight increases in contact time and aqueous humor levels. This is exactly what was found.

In almost all studies comparing polyvinyl alcohol and methylcellulose, the conclusion is reached that one vehicle is superior to the other based on contact time or bioavailability. But in many cases, the authors are basing their conclusions on polymer concentrations rather than viscosity. It is intuitively clear that as long as two vehicles exhibit the same flow properties, solutions with the same viscosity should exhibit the same drainage behavior in the eye. The important factors to consider then are the flow properties of the vehicle in question and its viscosity—*not* the concentration. There is no reason to suspect that methylcellulose should behave any differently from polyvinyl alcohol in the eye when a comparison is made on an equal viscosity basis.

A comparison between data obtained in this laboratory for methylcellulose (2) and the present report on polyvinyl alcohol seems to bear out this conclusion. Table IV summarizes the more important points of the polyvinyl alcohol–methylcellulose comparison. Of course, the basis for distinguishing the superiority of one vehicle over another must include factors other than drainage and bioavailability such as patient acceptability, influence on wound healing, and pharmaceutical handling.

It is clear from most of the work that has been conducted in humans (9, 18) or animals (13, 15), as well as from the methylcellulose and polyvinyl alcohol studies in this laboratory, that contact time is *not* proportional to drug bioavailability. Even with as much as a 100-fold increase in viscosity and a 10-fold decrease in drainage rate, the maximum improvement in drug activity, be it miosis, inhibition of infection, or aqueous humor levels, is about twice that of an aqueous solution.

The explanation for this is not clear. However, it is possible that a shear is created in the eye, even in rabbits, which causes these systems to thin. Thus, the responses obtained are not proportional to what would be expected from a consideration of the viscosity of the instilled solutions. Of course, more than a twofold increase in aqueous humor levels was obtained with pilocarpine in 10% polyvinyl alcohol. However, as pointed out earlier, the 10% polyvinyl alcohol is probably not practical because of the blurring of vision which would accompany its use. The relatively large increase in drug levels is probably due to a blockage of the drainage duct.

Large decreases in the drainage rate of instilled solutions can be obtained with relatively minor alterations in solution viscosity, and these large decreases do not lead to significant improvement in aqueous humor levels. With both methylcellulose and polyvinyl alcohol, it appears that the optimum viscosity solution to use would be in the range of 12–15 cps. Further increases in viscosity above this level do not appear to give proportional increases in drug levels. In addition, viscosities greater than 15 cps may present problems with respect to accuracy of instillation and blurring of vision. For these reasons, using polyvinyl alcohol, methylcellulose, or any system which approximates Newtonian behavior, a viscosity of 12–15 cps seems to be the optimum value in rabbits. Further work needs to be done to clarify why the substantial lowering of drainage rate and the corresponding increase in contact time with low to medium viscosity solutions is not reflected in a proportional increase in drug bioavailability.

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EXPERIMENTAL

Materials—I-9-^{14}C and I-3-^{14}C were synthesized using a recently patented procedure (2). Since I has asymmetric carbon atoms at positions 2 and 3a and is obtained as a mixture of diastereoisomers, the chromatographic systems employed (Table I) were chosen carefully in order not to resolve the diastereoisomeric pairs.

Absorption in Rats—The small intestine of male Sprague–Dawley rats, 230–250 g, was rapidly filled with a solution of 90 μg/ml of I-9-^{14}C (2.13 μCi/ml), using a previously described technique (3). Constant blood volume was maintained by transfusion with blood from a donor animal. At appropriate intervals, samples were taken of hepatic portal and systemic blood, and portions of the contents of the intestinal lumen were removed. These materials were assayed for radioactivity and subjected to TLC. The developed plates were scanned for radioactivity, and the area under the trace was determined by integration to measure the ^{14}C in each zone.

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Concentration in Circulating Blood—A solution containing 5 mg of I-9-^{14}C (129 μCi) dissolved in 0.5 ml of polyethylene glycol 400 was administered by stomach tube to each of six male Sprague–Dawley rats, weighing an average of 293 g. Six other rats received 0.5 mg of I-3-^{14}C (7.43 μCi) dissolved in 0.5 ml of the same vehicle. Blood samples were taken from the tail vein at appropriate intervals, and radioactivity was determined by liquid scintillation counting (4).

Beagle hounds, 7.6–10.2 kg, received single oral doses of I-9-^{14}C of 30.8 mg/kg (three animals, 42.9 μCi each), 66 mg/kg (one animal, 495 μCi), and 490 mg/kg (one animal, 1.98 μCi). At suitable intervals, blood was taken from the jugular vein and the plasma radioactivity of each sample was determined.

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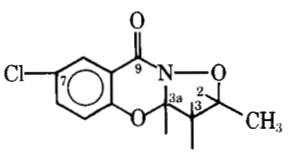

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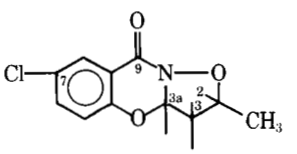


I

Table I—TLC of Urinary End-Products of I

Compound	R_f[a] Solvent System A[b]	Solvent System B[c]
I	0.58	0.63
5-Chlorosalicylic acid	0.47	0.47
5-Chlorosalicyluric acid	0.35	0.16
5-Chlorosalicylic acid glucuronide	—	0.06

[a] Silica gel (Merck). [b] Benzene–methanol–acetic acid (180:32:16). [c] *n*-Butanol saturated with 2 *N* ammonia.

Urine was collected from each subject for 48 hr. A urine aliquot was adjusted to pH 6.4 with hydrochloric acid; then four volumes of 0.07 *M* pH 6.4 phosphate buffer, containing 100 units of β-glucuronidase[1], was added. The mixture was incubated for 18 hr at 37° and then assayed for 5-chlorosalicylic acid.

GLC Assays—The amount of I present in plasma was measured in concentrations of 1–10 μg/ml. Chloroform, 0.2 ml, containing 1.2 μg of dibutyl phthalate as the internal standard, was added to a 1-ml aliquot of plasma. The mixture was agitated on a vortex mixer for 30 sec and centrifuged for 10 min. The aqueous phase was discarded, and the remaining emulsion was disrupted by freezing in dry ice–acetone. The liquid phases were separated by centrifugation, and 1.4 μl of the chloroform extract was injected into a gas chromatograph[2] equipped with a flame-ionization detector.

The GLC conditions were: column, 1.2-m (4-ft) glass, 3-mm i.d. packed with 3.8% W-98 on Diatoport S, 80–100 mesh; column temperature, 198°; flash heater temperature, 250°; detector temperature, 252°; helium flow rate, 86 ml/min; hydrogen flow rate, 34 ml/min; oxygen flow rate, 600 ml/min; and range, 10 with an attenuation of 16×.

Freshly prepared samples of plasma containing known amounts of I were used as standards. The 5-chlorosalicylic acid content of plasma and urine samples was determined by GLC. The conditions for the extraction and assay were reported separately (5).

Stability in Blood and Plasma—Heparinized rat blood was obtained by cardiac puncture. A solution of 60 μg of I-9-^{14}C (136 μCi), dissolved in 1.0 ml of 0.07 *M* pH 7.4 phosphate buffer, was added to 4.0 ml of whole rat blood; the resulting mixture was incubated at 37° with stirring. Aliquots, 1.0 ml, were taken at the start and after 4 hr. The cells were removed by centrifugation, washed, and assayed for radioactivity. An equal volume of acetone was added to the plasma, and the mixture was again centrifuged. The clear supernatant fluid, containing essentially all radioactivity, was examined by TLC. Only one radioactive zone, corresponding to I, was detected.

One-milliliter aliquots of a solution of I in normal dog plasma, 10 μg/ml, were placed in stoppered tubes and stored at 4° or frozen and stored at −18°. At intervals, samples were removed and assayed for I by the described GLC procedure.

Tissue Distribution in Rats—A male Sprague–Dawley rat, 215 g, received 4.0 mg of I-9-^{14}C (95.8 μCi), dissolved in 1.0 ml of polyethylene glycol 400, by stomach tube. A second rat, 198 g, received a solution containing 2.9 mg of I-3-^{14}C (39.8 μCi) orally. Each animal was placed in a metabolism chamber for 24 hr and then sacrificed. The excreta, soft tissue organs, and remainder of the carcass were processed and analyzed for radioactivity as previously reported (4). Respiratory carbon dioxide was collected in 2.5 *N* sodium hydroxide, and the ^{14}C content of an aliquot of this solution was determined.

Isolation of 5-Chlorosalicylic Acid—Nonradioactive I was administered, 50 mg/kg ip, to two male beagle hounds. Pooled 24-hr urine was continuously extracted with carbon tetrachloride for 18 hr. The organic phase was separated, washed with water, and concentrated, and the residue was examined for unchanged I by TLC; no free drug was detected.

The urine remaining from the initial extraction was acidified with concentrated hydrochloric acid to pH 1 and continuously extracted for an additional 24 hr with carbon tetrachloride. The organic phase was separated, washed with acidified water, and evaporated to dryness under vacuum. The residue, which was recrystallized from methanol–water and then from water, was identified as 5-chlorosalicylic acid by melting point (171–172°), TLC characteristics, and IR spectrum, which were identical to those of authentic 5-chlorosalicylic acid.

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Hewlett-Packard model 402.

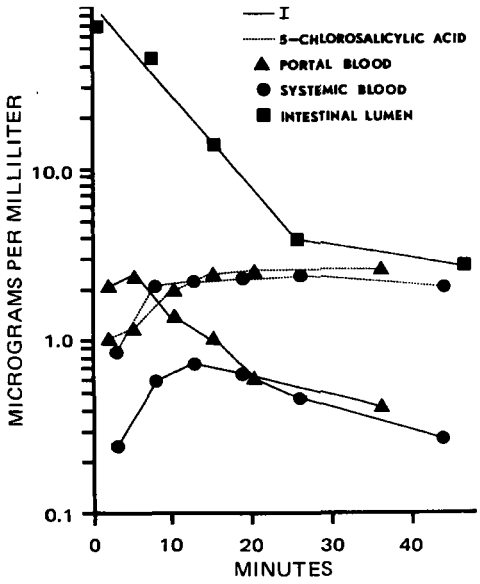


Figure 1—*Disappearance of I-9-^{14}C from rat intestine and the concentration of I and 5-chlorosalicylic acid in systemic and hepatic portal plasma, as determined by the area under a radiochromatogram trace.*

Inverse Isotope Dilutions—Inverse isotope dilution studies were performed using the urine of animals that had received labeled I. Typical procedures are described here.

I-9-^{14}C—A male Sprague–Dawley rat, about 250 g, received 2.5 mg of I-9-^{14}C (63.1 μCi), dissolved in 0.5 ml of polyethylene glycol 400, by stomach tube. Urine was collected for 24 hr and its radioactivity was measured. Inverse isotope dilutions were carried out on separate aliquots of urine.

1. Nonradioactive I, 1000 mg, was added to a 1.0-ml aliquot. Sodium carbonate solution, 5%, was added to adjust the pH to 9–10 and to prevent the extraction of 5-chlorosalicylic acid; the mixture was extracted three times with six volumes of chloroform. The chloroform phases were combined and evaporated to dryness. The

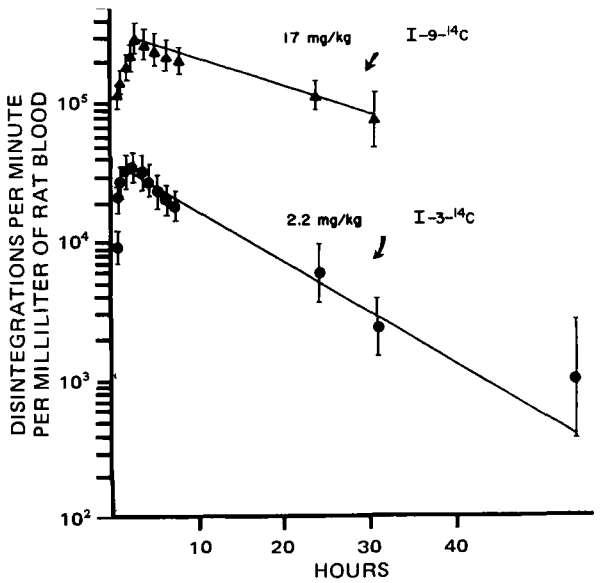


Figure 2—*Blood radioactivity after oral administration of I-^{14}C to rats.*

Table II—Comparison of Theoretical and Actual Concentrations of I in Dog Plasma

Hours	I, μg/ml[a]	
	Observed[b]	Calculated[c]
0	0	0
0.5	0.6	216
1	2.2	761
2	2.6	886
4	4.8	1034
7	8.0	830
24	0.6	341
48	0	88
72	0	3

[a] Dog received a capsule containing I-9-^{14}C, 490 mg/kg, 1.98 μCi. [b] By a GLC procedure. [c] From the amount of radioactivity in the plasma.

residue, consisting of crude I, was repeatedly crystallized from ethyl acetate to constant specific activity, mp 150–151°.

2. Details of the procedures for quantitating 5-chlorosalicylic acid and 5-chlorosalicyluric acid by the isotope dilution technique were reported previously (6).

3. The metabolites in the urine present in a bound form were converted to the free form by hydrolysis. An aliquot was treated with an equal volume of concentrated hydrochloric acid, and the mixture was heated on a steam bath for 10 min. Nonradioactive carrier was then added, and the inverse isotope dilutions were carried out as with the free metabolites. Conjugated urinary end-products were calculated by difference.

4. Similar studies were performed on the urine of a dog that had received 502.2 mg of I-9-^{14}C orally.

I-3-^{14}C—A male Sprague–Dawley rat, 251 g, received 4.6 mg of I-3-^{14}C (62.6 μCi), dissolved in 1.0 ml of polyethylene glycol 400, by stomach tube. Urine was collected for 24 hr and assayed for radioactivity.

1. Nonradioactive sodium β-hydroxybutyrate, 1000 mg, was added to a 0.5-ml aliquot. A homogeneous solution resulted from the addition of 5 ml of water, 4 drops of 1 *N* hydrochloric acid, and 10 ml of ethanol. 2,4′-Dibromoacetophenone, 1.0 g, was added, and the mixture was heated at reflux for 2 hr. The resulting *p*-bromophenacyl ester was precipitated by the addition of water, washed with water, dried, and recrystallized from ethyl acetate–heptane to constant specific activity, mp 82–83°. The specific activity was corrected for the difference in the formula weight between the crystalline derivative and the sodium β-hydroxybutyrate added. This ester, *p*-bromophenacyl β-hydroxybutyrate, has not previously been reported.

Anal.—Calc. for $C_{12}H_{13}BrO_4$: C, 47.86; H, 4.35. Found: C, 48.11; H, 4.29.

2. Nonradioactive succinic acid, 1000 mg/kg, was added to another 0.5-ml aliquot of urine, and 10 ml of ethanol was added to give a homogeneous solution. This solution was evaporated to dryness under vacuum, the residue was triturated with hot ethyl acetate, and the insoluble material was discarded. The addition of heptane to the ethyl acetate solution separated crystals of succinic acid. These crystals were removed by filtration and recrystallized from ethyl acetate–heptane to constant specific activity, mp 185–186°.

3. The techniques for the isolation of α-ketoglutaric, fumaric, malic, and citric acids were similar to those for succinic acid.

Plasma Binding—The extent of binding was determined using a previously described procedure (3), except that the dialysis bags were placed in a solution of either 0.4 mg of I-9-^{14}C or 0.5 mg of 5-chlorosalicylic-7-^{14}C acid dissolved in 5 ml of buffer. The flasks were shaken at 20 ± 2° for 18 hr to ensure equilibration. TLC confirmed the stability of both I and 5-chlorosalicylic acid under the experimental conditions.

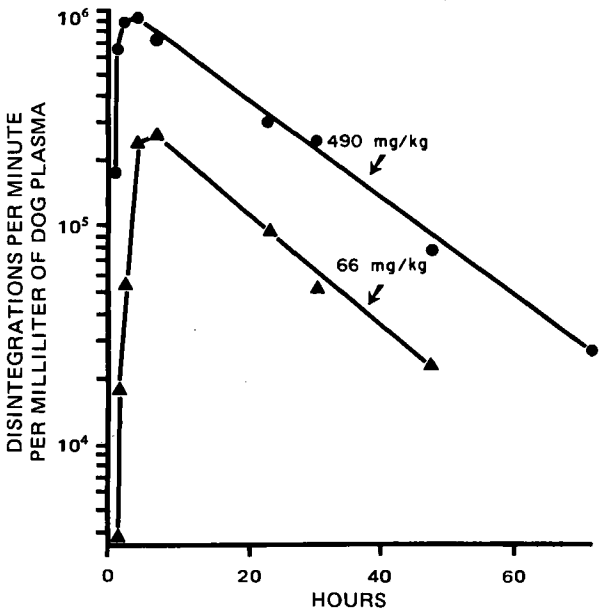


Figure 3—*Blood radioactivity after oral administration of I-9-^{14}C to dogs.*

Table III—Comparison of Theoretical and Actual Concentrations of 5-Chlorosalicylic Acid in Dog Plasma

Hours	5-Chlorosalicylic Acid, μg/ml					
	Dog 695[a]		Dog 795[a]		Dog 727[a]	
	Observed[b]	Calculated[c]	Observed[b]	Calculated[c]	Observed[b]	Calculated[c]
0	0	0	0	0	0	0
0.25	0	0	0	0	2	3
0.5	0	0	8	9	9	10
1	7	8	19	19	16	16
2	14	16	28	31	14	16
4	18	18	35	36	14	17
6	14	16	31	34	15	17
24	10	13	7	9	10	13

[a] Dogs received capsules containing I-9-^{14}C, 30.8 mg/kg, 42.9 μCi each. [b] By a GLC procedure. [c] From the amount of radioactivity in the plasma.

RESULTS

Absorption in Rats—The disappearance of radioactivity from the lumen of the intestine of an anesthetized rat, which had been rapidly infused with a solution of I-9-^{14}C, followed apparent first-order kinetics with a half-life of about 6 min. Results with a typical rat are shown in Fig. 1. After 25 min, more than 95% of the radioactivity of the administered dose had been absorbed and the clearance of ^{14}C from the gut lumen declined thereafter. The intestinal lumen contained intact drug for at least 20 min. After 46 min, in a single rat, less than 13% of the I remaining in the intestine had been hydrolyzed.

When the contents of the hepatic portal vein were investigated,

Table IV—Effect of β-Glucuronidase Treatment on Human Urine

Subject	Urine Sample Time Period, hr	5-Chlorosalicylic Acid, mg		
		Initial	After Treatment	Difference
1	0—24	38.3	57.5	19.2
	24—48	21.5	33.3	11.8
2	0—24	48.0	67.2	19.2
	24—48	38.8	46.7	7.9
3	0—24	15.5	62.1	46.6
	24—48	10.7	26.7	16.0
4	0—24	45.9	72.1	26.2
	24—48	9.6	10.6	1.0
Average	0—24	36.9	64.7	27.8
	24—48	20.2	29.4	9.2

Table V—Tissue Distribution of Radioactivity in Rat after Oral Administration of I-^{14}C[a]

Specimen	Radioactivity Recovered			
	I-9-^{14}C		I-3-^{14}C	
	dpm/g	% of Dose[b]	dpm/g	% of Dose[c]
Urine	13,470,000[d]	61.1	3,190,000[d]	62.0
Carcass	324,000	26.8	76,000	13.5
GI tract (including contents)	610,000	5.55	308,000	6.21
Liver	828,000	4.28	321,000	2.51
Kidneys	870,000	1.03	302,000	0.61
Lungs	793,000	0.56	143,000	0.18
Heart	500,000	0.21	131,000	0.13
Spleen	276,000	0.13	146,000	0.13
Respired Carbon Dioxide	—	0.08	—	16.1
Feces	189,000	0.003	377,000	0.29
Recovery, %	99.7		101.6	

[a]Animals sacrificed 24 hr after drug administration. [b]Animal received 4.0 mg of I-9-^{14}C, 95.8 μCi. [c]Animal received 2.9 mg of I-3-^{14}C, 39.8 μCi. [d]Disintegrations per minute per milliliter.

however, substantial quantities of I had been converted into 5-chlorosalicylic acid. About 8 min after the drug solution filled the intestine, the portal plasma contained equal concentrations of the metabolite and I. Thereafter, more 5-chlorosalicylic acid than intact I was present. Examination of the blood in the systemic circulation showed that the concentration of intact I was less in systemic blood than in portal blood during the initial 20 min. However, after absorption from the intestine had declined, the concentrations of I in the systemic circulation and in the hepatic portal vein were the same (Fig. 1). These results suggest that the I was metabolized during its passage through the intestinal wall. The ability of a preparation of minced intestine to convert 41.5% of the I present into 5-chlorosalicylic acid during a 90-min incubation period confirmed the metabolic activity of intestinal tissue.

Concentration in Circulation—Peak levels of blood radioactivity were found about 2 hr after the oral administration of I-9-^{14}C to the rat. The clearance of ^{14}C from the bloodstream followed apparent first-order kinetics, with a half-life of about 18 hr (Fig. 2). Maximal blood radioactivity values were attained within 90 min of the oral administration of I-3-^{14}C to rats, and the first-order half-life was about 8 hr (Fig. 2).

The absorption and elimination of the radioactivity of 9-labeled drug in two beagle hounds are shown in Fig. 3. Apparent first-order kinetics were followed for the decline in blood radioactivity. After a 0.5-g dose, 66 mg/kg, the half-life was about 11.5 hr; the feces contained only about 5% of the ^{14}C of the dose after 48 hr. After a 5.0-g dose, 490 mg/kg, the half-life was about 13.2 hr; only 27% of the radioactivity of the dose was found in the feces after 48 hr.

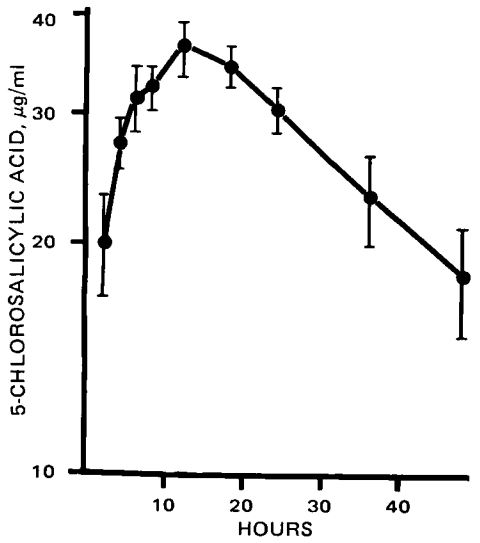


Figure 4—*Concentration of 5-chlorosalicylic acid in human plasma after oral administration of I (average of six subjects).*

Plasma samples from the dog that had received the larger dose were found by the GLC procedure to contain small amounts of I (Table II). Three other beagle hounds received 30.8 mg/kg of I-9-^{14}C; five of the samples, chosen at random, contained less than 1 μg/ml of intact I, as determined by GLC. The radioactivity of the samples was used to calculate the amount of 5-chlorosalicylic acid that would be present theoretically if all of the radioactivity were in that chemical form. The actual concentration of 5-chlorosalicylic acid was determined by GLC; the agreement between the theoretical and actual values is shown in Table III, which vividly demonstrates the extent of degradation of I to 5-chlorosalicylic acid.

Human Studies—Nonradioactive I was readily absorbed after oral administration to humans. No intact I could be detected in the plasma from any of the six subjects at either 2 or 4 hr after drug administration. However, the clearance of 5-chlorosalicylic acid, the metabolite of I, followed apparent first-order kinetics, with an estimated half-life of 33 hr (Fig. 4).

Incubation of the urine from four men who had ingested I with β-glucuronidase resulted in substantially higher levels of 5-chlorosalicylic acid, as determined by GLC (Table IV). Incubation of urine with buffer, in the absence of enzyme, had no effect. The nature of the bond between glucuronic acid and 5-chlorosalicylic acid was not determined.

GLC Assays—The relationship between peak height and the concentration of I was linear in the 1–10-μg/ml range. The repro-

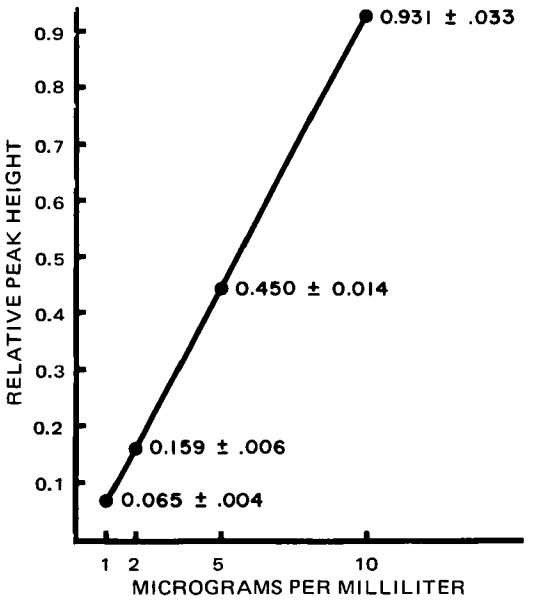
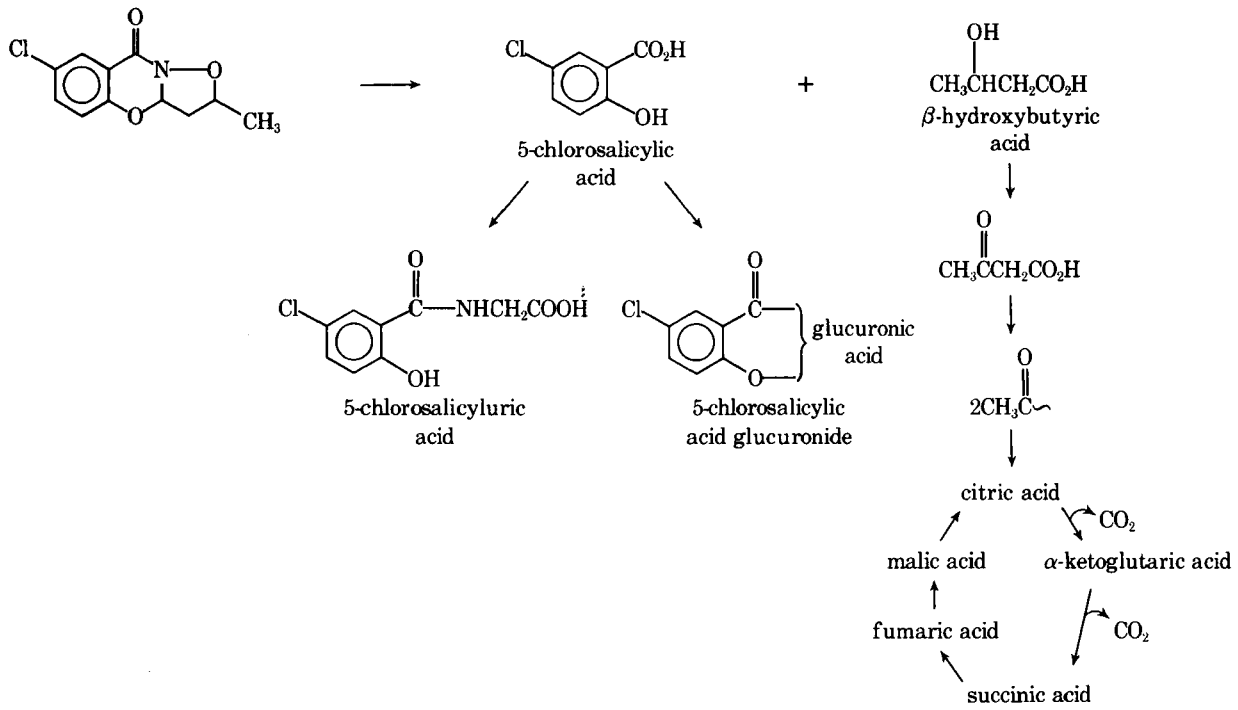


Figure 5—*Relationship between relative peak height and plasma concentration of I. Results are the means of quadruplicate determinations and are shown with their standard errors.*



Scheme I

ducibility of the technique was shown by the standard errors of quadruplicate analyses (Fig. 5).

Stability in Blood and Plasma—Compound I was not degraded by incubation with whole rat blood for 4 hr. Furthermore, the compound was stable in dog plasma for at least 51 days when stored in the freezer and for at least a week when refrigerated.

Tissue Distribution in Rats—A study of the distribution of radioactivity after the oral administration of I-9-^{14}C showed that the kidneys were the major route of elimination of ^{14}C within the 24-hr experimental period. Significant amounts of radioactivity were also found in the carcass, GI tract, and liver at that time (Table V).

A similar study with the 3-labeled drug showed that the radioactivity of the isoxazole moiety is also eliminated in rat urine. About one-sixth of the ^{14}C was exhaled as respired carbon dioxide. The location of the remainder of radioactivity is shown in Table V.

Identification and Quantitation of Urinary End-Products—Four radioactive substances were detected by TLC in the urine of dogs that had received I-9-^{14}C (Table I). Assignment of the glucuronide structure to the 5-chlorosalicylic acid conjugate was based upon the liberation of additional compound upon treatment of the urine with β-glucuronidase.

The identities and amounts of the urinary metabolites of I-9-^{14}C in the rat and dog are shown in Table VI. The major end-product was 5-chlorosalicylic acid. In the dog, this metabolite occurred in an unbound form and accounted for three-quarters of the urinary radioactivity. In the rat, there were about equal amounts of free 5-chlorosalicylic acid and its glucuronic acid conjugate. In both species, lesser amounts of the glycine derivative of 5-chlorosalicylic acid, 5-chlorosalicyluric acid, were found. Small quantities of unchanged drug were excreted in the urine of both species.

Isotope dilution experiments on the urine of a rat that had received I-3-^{14}C showed the presence of β-hydroxybutyric acid and other acids of the tricarboxylic acid cycle (Table VII). The presence of these compounds suggests that the labeled carbon dioxide found in respired air (Table V) came from the decarboxylation of the oxidation products of β-hydroxybutyric acid by Krebs' cycle enzymes.

Plasma Binding—Compound I was bound appreciably to the plasma proteins of all species studied (Table VIII), but it was bound less extensively than 5-chlorosalicylic acid.

DISCUSSION

Part of orally administered I is hydrolyzed upon passage through the intestinal wall during the absorption of the drug. The ability of the gut to hydrolyze I was verified by *in vitro* studies. No intact drug could be detected in the systemic circulation, unless the administered dose of I was very large; at a dose of 490 mg/kg, the maximum plasma concentration attained was only 8 μg/ml.

Since I was stable in blood and plasma, the unaltered drug that was absorbed into the circulation and subsequently converted into 5-chlorosalicylic acid was not hydrolyzed by plasma enzymes but was probably degraded by liver enzymes. In any event, labeled 5-chlorosalicylic acid was the metabolite of I-9-^{14}C that was present in the bloodstream, and this metabolite accounted for virtually all of the radioactivity of the administered drug.

Table VI—Quantitation of I-9-^{14}C Urinary End-Products in Rat and Dog

Urinary End-Product	Urinary Radioactivity, % Rat[a]	Dog[b]
5-Chlorosalicylic acid		
Free	46.1	73.6
Conjugated	47.7	7.3
5-Chlorosalicyluric acid		
Free	1.3	4.2
Conjugated	n.d.[c]	5.4
I	0.3	6.2
Total radioactivity recovered	95.4	96.7

[a] This rat received 2.5 mg of drug orally. [b] The dog received 502.2 mg of I-9-^{14}C orally. [c] None detected.

Table VII—Quantitation of I-3-^{14}C Urinary End-Products in Rat

Urinary Metabolite	Urinary Radioactivity[a], %
β-Hydroxybutyric acid	19.1
Citric acid	10.9
α-Ketoglutaric acid	24.9
Succinic acid	15.8
Fumaric acid	18.4
Malic acid	12.9
Total radioactivity recovered	102.0

[a] This rat received 4.6 mg of I-3-^{14}C orally.

Table VIII—Binding of I and 5-Chlorosalicylic Acid to Plasma

Species	Percent Bound[a]	
	I[b]	5-Chlorosalicylic Acid[c]
Human	80.5	99.4
Sprague—Dawley rat	54.6	71.1
Rhesus monkey	83.2	97.2
Beagle dog	78.9	97.5
Control, buffer only	−1.7	0.3

[a] Average of duplicates. [b] Initially there was 0.4 mg of I to be partitioned between 5 ml of plasma and 5 ml of buffer. [c] Initially there was 0.5 mg of 5-chlorosalicylic acid to be distributed between 5 ml of plasma and 5 ml of buffer.

In all three species, rat, dog, and human, cleavage of the oxazine ring of I yielded 5-chlorosalicylic acid, which was excreted into the urine in the free form and as conjugates with glucuronic acid and glycine.

The radioactivity from 3-labeled I appeared as respiratory carbon dioxide in exhaled breath and as β-hydroxybutyric acid and its metabolites fumaric, citric, α-ketoglutaric, succinic, and malic acids in the urine.

The metabolic fate of I is summarized in Scheme I.

CONCLUSIONS

The present study showed that I was converted to 5-chlorosalicylic acid by rats, beagle hounds, and humans and that the chemical entity present in the systemic circulation after the oral administration of I was not the intact drug but was the metabolite, 5-chlorosalicylic acid. The latter compound was excreted through the kidneys either free or conjugated with glucuronic acid and glycine. Elements of the isoxazole ring were converted to β-hydroxybutyric acid, which was metabolized by enzymes of the citric acid pathway.

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Keyphrases □ 7-Chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-*b*)(1,3)-benzoxazin-9-one—anti-inflammatory, analgesic, and antipyretic activities compared to 5-chlorosalicylic acid, rats □ 5-Chlorosalicylic acid—anti-inflammatory, analgesic, and antipyretic activities compared to 7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-*b*)(1,3)-benzoxazin-9-one, rats □ Anti-inflammatory activity—7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-*b*)-(1,3)-benzoxazin-9-one and 5-chlorosalicylic acid compared in rats □ Analgesic activity—7-chloro-3,3a-dihydro-2-methyl-2*H*,-9*H*-isoxazolo-(3,2-*b*)(1,3)-benzoxazin-9-one and 5-chlorosalicylic acid compared in rats □ Antipyretic activity—7-chloro-3,3a-dihydro-2-methyl-2*H*,9*H*-isoxazolo-(3,2-*b*)(1,3) - benzoxazin-9-one and 5-chlorosalicylic acid compared in rats

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Table I—Oral Activity and Toxicity of Compound I and 5-Chlorosalicylic Acid in the Carrageenan-Induced Edema Test in Rats

Drug	Carrageenan		Acute Toxicity[a]		Therapeutic Index, LD_{50}/ED_{50}
	ED_{50} (95% C.L.), mg/kg	mM[b]	LD_{50} (95% C.L.), mg/kg	mM[a]	
I	34.5 (23.3–51.1)	0.144	1710.0 (1335.0–2190.0)	7.16	49.6
5-Chlorosalicylic acid	25.8 (18.8–38.3)	0.150	261.0 (193.0–352.0)	1.52	10.0

[a] LD_{50} values for both the 24-hr and 7-day observation periods following single-dose administration were identical. [b] ED_{50} or LD_{50} values are expressed as millimolar.

Table II—Prophylactic Effect of Compound I and 5-Chlorosalicylic Acid on Adjuvant-Induced Polyarthritis in Rats

Treatment Group[a]	[Drug] in Diet, %	Actual Mean Dosage Consumed, mg/kg/day (mM)	Day 21 Measurements, Mean ± SE				
			Foot Volume, ml		Body Weight Net Gain, g, Mean ± SE	Erythrocyte Sedimentation Rate, mm/hr	Serum Albumin/ Globulin
			Injected	Uninjected			
Nonarthritic controls	—	— —	2.50 ± 0.08[b]	2.49 ± 0.08[b]	139.0 ± 3.1	2.8 ± 0.7	1.40 ± 0.09[b]
Arthritic controls	—	— —	4.79 ± 0.38	3.68 ± 0.25	61.0 ± 2.7[c]	6.0 ± 0.9	0.37 ± 0.05
I	0.1	76.5 (0.32)	3.66 ± 0.19[b]	2.70 ± 0.19[b]	91.5 ± 12.1[c]	4.0 ± 0.3	0.65 ± 0.10[b]
	0.2	150.4 (0.63)	2.93 ± 0.18[b]	2.40 ± 0.17[b]	73.5 ± 8.4[c]	2.6 ± 0.4[b]	0.86 ± 0.16[b]
5-Chlorosalicylic acid	0.1	74.3 (0.43)	3.03 ± 0.10[b]	2.44 ± 0.12[b]	82.5 ± 9.0[c]	2.6 ± 0.8[b]	1.26 ± 0.20[b]
	0.2	150.0 (0.87)	2.53 ± 0.07[b]	1.96 ± 0.06[b]	39.6 ± 6.1[c]	2.2 ± 0.8[b]	1.97 ± 0.19[b]

[a] n = eight rats per treatment group. [b] $p \leq 0.05$ when compared with arthritic control group. [c] $p \leq 0.05$ when compared with nonarthritic control group.

rat and dog, orally administered I was converted to its metabolite 5-chlorosalicylic acid by the intestinal wall during absorption. In addition, Edelson *et al.* (2) revealed that very little intact I could be detected in the systemic circulation. Moreover, these investigations (2) showed that absorption of I from the GI tract is not rate limiting. Since 5-chlorosalicylic acid appears to be the major metabolite of I, it was of interest to determine to what extent it might be contributing to the pharmacological properties of I. This report describes the results of a direct comparative study of I and 5-chlorosalicylic acid in various test systems.

EXPERIMENTAL

Animals—Male rats of the Sprague–Dawley strain[2] were used. They were allowed to acclimate to the laboratory environment for at least 5 days prior to use, with food and water allowed *ad libitum*.

Carrageenan-Induced Edema—A modification of the method of Winter *et al.* (3) was used. At least six rats, 100–120 g, were used at each dose level tested. All doses of I and 5-chlorosalicylic acid were prepared as suspensions in a 1% acacia vehicle for this and subsequent experiments unless otherwise stated.

Drugs were administered orally by gavage in a volume of 0.5 ml/100 g of body weight. One hour after dosing, 0.05 ml of a 1% solution of calcium carrageenan was injected subcutaneously into the plantar surface of the right hindpaw of each rat. The volume of the injected foot was measured by water displacement to the lateral malleolus immediately before drug administration and again 3 hr after carrageenan injection. The difference between the two was called the edema volume.

The mean edema volume was determined for each experimental group. An ED_{50} value, defined as that dose at which the edema volume was decreased by 25% or greater in 50% of the rats studied, was determined by the method of Litchfield and Wilcoxon (4).

Adjuvant-Induced Polyarthritis—Polyarthritis was induced in rats, 150–170 g at the start of the experiment, by injection of heat-killed *Mycobacterium tuberculosis* organisms (5). Each animal received 0.1 ml of adjuvant suspension (0.5% in heavy mineral oil) injected subcutaneously into the plantar surface of the right hindfoot. At least eight individually caged rats were used in each test group.

Appropriate concentrations of I and 5-chlorosalicylic acid were blended into a commercial diet, and the resulting mixtures were fed continuously to the test animals beginning immediately after injection of *M. tuberculosis*. The animals were maintained on this drug–diet admixture for the duration of the experiment, *i.e.*, 21 days. Nonarthritic (normal) and arthritic control animals received only the commercial diet. Beginning on Day 0 and subsequently on Days 7, 14, and 21 of the experiment, the following parameters were measured: drug–diet consumption, injected and uninjected hindpaw volumes, and body weight.

Group mean drug dosage was calculated from food consumption and body weight data. Following all measurements on Day 21, each rat was sacrificed by decapitation and a 2- or 3-ml blood sample was collected in heparinized vials. Erythrocyte sedimentation rate (6) and serum albumin/globulin ratios (7) were determined.

Analgesic Activity—The paw pressure method described by Randall and Selitto (8) was used to measure the analgesic effectiveness of I and 5-chlorosalicylic acid in rats (100–120 g). Inflammation was produced in the right hindpaw of each rat by injection of 0.1 ml of a 20% suspension of brewer's yeast in saline. One hour later, all rats (eight per group) received the test drug or vehicle orally (0.5 ml/100 g of body weight).

Pain threshold was measured[3] 1 hr following drug administration by applying pressure to the inflamed paw at a steadily increasing rate of 14 g/sec. The end-point, or pain threshold, was defined as that pressure necessary to cause the animals to struggle and/or vocalize. An analgesic effect was denoted if the individual reaction thresholds were 2 SD or greater than the mean threshold for the vehicle-treated control group (9). An ED_{50} value was determined (4) based on this all-or-none response.

Antipyretic Activity—Eighteen hours before oral drug administration, rats (~200 g) were rendered pyretic by subcutaneously injecting a 15% suspension of brewer's yeast (1.0 ml/100 g of body weight) according to the method of Smith and Hambourger (10). At least eight rats were used for each dose of drug and for fevered and nonfevered control groups.

Rectal temperatures were determined before the yeast injection and immediately before (designated the 0-hr reading) and at 2 and

[2] Charles River, Inc.

[3] Analgesy-Meter, Ugo Basile, Milan, Italy.

Table III—Oral Analgesic Effectiveness of Compound I and 5-Chlorosalicylic Acid in Rats as Measured by the Randall–Selitto Paw Pressure Test

Drug	Analgesia		Acute Toxicity		Therapeutic Index, LD_{50}/ED_{50}
	ED_{50} (95% C.L.), mg/kg	m*M*[a]	LD_{50} (95% C.L.), mg/kg	m*M*[a]	
I	84.0 (46.4—152.0)	0.35	1710.0 (1335.0—2190.0)	7.16	20.4
5-Chlorosalicylic acid	182.0 (92.0—360.0)	1.06	261.0 (193.0—352.0)	1.52	1.4

[a] ED_{50} or LD_{50} values are expressed as millimolar.

4 hr after oral drug administration. These measurements were recorded on a telethermometer[4] from a thermistor probe inserted a constant depth of 5.0 cm.

Drug-Induced Gastric Lesions—Ulcerogenic activity was studied by a method similar to that described by Domenjoz (11). Rats (~200 g), in groups of at least six, received the test drug or vehicle orally at 9 am and 4 pm on the test day. These animals were fasted for 18 hr prior to dosing, but they had access to water throughout the experiment beginning 0.5 hr prior to drug administration.

Each rat was sacrificed by chloroform inhalation 24 hr after the first dosing; its stomach was removed, opened along the greater curvature, and examined with a dissecting microscope for the presence of gastric ulcers. Animals with stomachs exhibiting at least one ulcer were considered positive responders. Statistical comparison among groups was carried out using the χ-square test with the Yates correction.

Toxicity—Single-dose oral LD_{50} values for I and 5-chlorosalicylic acid were determined in nonfasted rats (150–170 g) 24 hr and 7 days following drug administration. Ten rats per treatment group and at least three doses were used in this study. The number of animals dead in each group at each time interval was tallied and LD_{50} values were calculated (4).

RESULTS

Carrageenan-Induced Edema—Compound I and 5-chlorosalicylic acid were orally effective inhibitors of carrageenan-induced hindpaw edema in rats (Table I). When a comparison of their ED_{50} values was made, both compounds were equally potent anti-inflammatory drugs. The acute oral LD_{50} values for these compounds and their corresponding therapeutic index (LD_{50}/ED_{50}) were also calculated. Lethalities following a single oral administration of various doses of I or 5-chlorosalicylic acid occurred within the first 24 hr; hence, LD_{50} values for this period and for the 7-day observation period were identical. Although not depicted graphically, this comparison is valid since the slopes of the dose–response curves to determine the ED_{50} and LD_{50} of each drug are parallel. Table I reveals that although both compounds did not differ in their anti-inflammatory potency, I possessed a therapeutic index nearly five times greater than that observed for the metabolite.

Assuming that I (mol. wt. 239) was completely biotransformed to 5-chlorosalicylic acid (mol. wt. 172), justification for transformation of ED_{50} and LD_{50} values to millimolar units was warranted. Although the ED_{50} for I was somewhat, but not statistically significantly, greater than 5-chlorosalicylic acid, its corresponding millimolar value (0.144) was identical to that of its major metabolite (0.150).

Adjuvant-Induced Polyarthritis—Results of a comparative study of the prophylactic effect of I and 5-chlorosalicylic acid on adjuvant-induced polyarthritis in rats are shown in Table II. Dietary concentrations of 0.1 and 0.2% (approximately 75.0 and 150.0 mg/kg/day) of each drug significantly inhibited the development of adjuvant-induced polyarthritis. Enlargement of the injected foot (primary lesion) was markedly inhibited by each drug in a dose-related manner. However, the metabolite was significantly more effective than identical doses of I, with the highest concentration (0.2% or 150.0 mg/kg/day) of the former completely suppressing edema formation.

Similarly, a dose-dependent reduction of swelling in the contralateral, uninjected hindpaw (secondary lesion) was observed for each drug. The high concentration of I (0.2% or 150.4 mg/kg/day), as well as the low concentration of 5-chlorosalicylic acid (0.1% or 74.3 mg/kg/day), completely abolished this secondary involvement associated with adjuvant disease. On the other hand, the volume of the uninjected hindpaw of rats receiving the 0.2% drug–diet admixture of 5-chlorosalicylic acid was markedly less than the nonarthritic control group.

When the milligram per kilogram doses of I and 5-chlorosalicylic acid are converted to millimolar values (Table II), it is apparent that the millimolar doses of the latter compound are approximately 35% greater. This fact most likely accounts for the slightly greater activity of 5-chlorosalicylic acid against adjuvant-induced polyarthritis.

Another prominent manifestation of adjuvant-induced polyarthritis was a significant attenuation of body weight gain over the 21-day period, *e.g.*, 61.0 g for the arthritic controls compared to 139.0 g for the nonarthritic controls or approximately a 56.2% reduction (Table II). Suppression of body weight gain in arthritic animals treated with I or 5-chlorosalicylic acid was not restored to the rate of gain displayed by the nonarthritic control group. However, a trend toward normal body weight gain was noticed in the 0.1% I-treated (91.5 g) and 5-chlorosalicylic acid-treated (82.5 g) groups, since they gained significantly more weight than the arthritic control rats. But 0.2% of 5-chlorosalicylic acid further retarded body weight gain.

Table II also reveals that the erythrocyte sedimentation rate was markedly increased while the serum albumin/globulin ratio was severely lowered in adjuvant-induced polyarthritis. Compound I, as a 0.2% drug–diet admixture, significantly reversed the elevated erythrocyte sedimentation rate to the level of the nonarthritic control group. This effect was dose related, since the lower concentration (0.1%) of I did not significantly affect this measurement, although a trend was apparent.

Both concentrations of 5-chlorosalicylic acid were equally effective in completely reversing this elevated erythrocyte sedimentation rate. Likewise, the altered serum albumin/globulin ratio was partially restored by each concentration of I, whereas the 0.1% drug–diet admixture of the metabolite completely protected rats from this phenomenon. When the concentration of 5-chlorosalicylic acid in the diet was raised to 0.2%, serum albumin/globulin ratios were significantly elevated beyond the value for nonarthritic control rats.

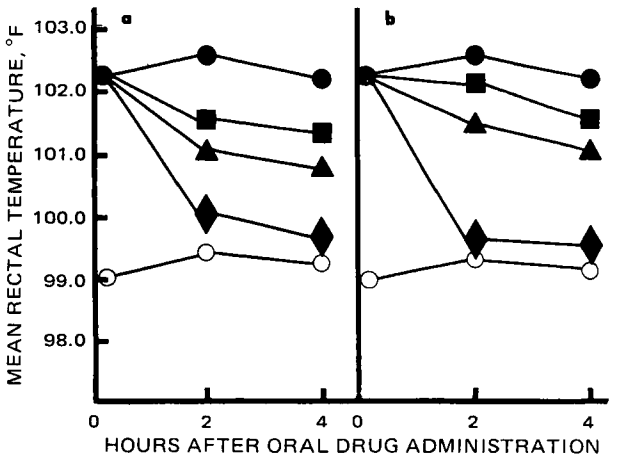

Figure 1—*Antipyretic activity of Compound I (a) and 5-chlorosalicylic acid (b) in rats with yeast-induced fever. Key: ●, fevered controls; ○, nonfevered controls; ■, 25.0 mg/kg; ▲, 50.0 mg/kg; and ♦, 100.0 mg/kg.*

[4] Yellow Springs Instrument Co., Yellow Springs, Ohio.

Table IV—Comparative Ulcerogenic Activity of Compound I, 5-Chlorosalicylic Acid, and Phenylbutazone in Rats[a]

Drug	Oral Dose, mg/kg (m*M*) bid	Number of Rats with Stomach Ulcers: Number of Animals Studied	Mortality
1% Acacia	—	0/44	0/44
I	139.0 (0.58)	0/15	0.15
	208.5 (0.87)	0/15	0/15
5-Chloro-	100.0 (0.58)	1/38	0/38
salicylic acid	150.0 (0.87)	11/28**	10/28*
Phenylbutazone	150.0 —	16/18***,†	0/18‡

[a] *$p < 0.05$, **$p < 0.02$, ***$p < 0.001$ when compared to the group receiving the high dose of I (208.5 mg/kg bid). †$p < 0.01$, ‡$p < 0.02$ when compared to the group receiving the high dose of 5-chlorosalicylic acid (150.0 mg/kg bid).

Analgesic Activity—Compound I and 5-chlorosalicylic acid were both orally effective analgesic agents when evaluated in rats using the Randall–Selitto paw pressure test (Table III). However, I was approximately 2.2 and 3.9 times more potent than its metabolite on a milligram per kilogram and millimolar basis, respectively. When the acute lethality of these compounds is considered, I has a substantially higher therapeutic index (20.4) than 5-chlorosalicylic acid (1.4).

Antipyretic Activity—When administered at oral doses (25.0, 50.0, and 100.0 mg/kg) which did not affect rectal temperature in nonpyretic rats, I and 5-chlorosalicylic acid significantly reduced fever 2 and 4 hr after dosing (Fig. 1). This activity was dose related for each compound, with the 100.0-mg/kg dose returning rectal temperature back to normal (nonfevered control). Moreover, the overall antipyretic effectiveness of I and 5-chlorosalicylic acid was nearly identical.

Drug-Induced Gastric Lesions—Following oral administration of markedly effective anti-inflammatory doses (total doses of 278.0 and 417.0), I did not produce gastric ulcers nor was it fatal to rats (Table IV). 5-Chlorosalicylic acid produced ulcers in 39% and killed 36% of the rats receiving a total dose of 300.0 mg/kg, which was equimolar to the high dose of I. Likewise, a 300.0-mg/kg total dose of phenylbutazone, a nonsteroidal anti-inflammatory agent with a propensity for ulcerogenesis, produced ulcers in 89% of the rats dosed but was not lethal. These data indicate that phenylbutazone is significantly more ulcerogenic than 5-chlorosalicylic acid and that each of these compounds is excessively ulcerogenic compared to I.

DISCUSSION

The results of the present investigation clearly demonstrate that both I and 5-chlorosalicylic acid, its major biotransformation product, were effective anti-inflammatory, analgesic, and antipyretic agents in the rat. On a milligram per kilogram basis, I and its metabolite were equally effective inhibitors of carrageenan-induced edema and brewer's yeast-induced pyresis. However, I was slightly, but significantly, less effective than 5-chlorosalicylic acid in the adjuvant-induced polyarthritis test when both were studied as 0.1 and 0.2% drug–diet admixtures. On the other hand, I was approximately 2.2 times as potent as 5-chlorosalicylic acid in its analgesic effectiveness.

A toxic manifestation shared by most clinically useful nonsteroidal antiarthritic compounds, including the salicylates, administered acutely or for long periods to laboratory animals (12) and humans (13) is a propensity to induce GI disturbances such as ulceration. Results obtained with I (Table IV) reveal that total doses as high as 417.0 (or 208.5 mg/kg bid) given orally did not induce ulcer formation in fasted rats. However, an equimolar dose of 5-chlorosalicylic acid (total dose of 300.0 or 150.0 mg/kg bid) not only caused ulcers to occur but proved fatal to a substantial number of the rats dosed. Likewise, phenylbutazone was markedly ulcerogenic but not lethal at 300.0 mg/kg. In a previous study (1), ulcer formation as well as lethality did occur following oral administration of a higher total daily dose of 600.0 mg/kg (or 300.0 mg/kg bid) of I.

Similarly, inspection of the LD_{50} values obtained following a single oral dose of I or its metabolite (Tables I and III) to nonfasted rats points out another striking difference in the toxicity of these two compounds. 5-Chlorosalicylic acid (261.0 mg/kg) was approximately 6.5 times as toxic as I (1710.0 mg/kg). The calculated LD_{50} for I in the present investigation closely correlates with the value of 1660.0 mg/kg for the nonfasted rat obtained in earlier experiments (1). Moreover, all deaths following oral administration of these compounds occurred within the first 24 hr.

The LD_{50} data reported for the nonfasted rat in this study, along with lethality data obtained previously (1) for nonfasted and fasted rats, clearly suggest that I is more toxic to fasted rats. This finding is not unreasonable since Zbinden (14) pointed out that many compounds are more toxic in fasted as compared to nonfasted rats. Therefore, this point may explain the discrepancy observed between the doses that were lethal and induced ulcer formation in the previous study (1) and the findings obtained in this study.

Based on the results of this investigation, it appears reasonable to speculate that 5-chlorosalicylic acid, the major metabolic product of I (2), may be largely responsible for the anti-inflammatory and antipyretic properties of this compound and, to a lesser degree, for its analgesic effectiveness. However, the profound ulcerogenic and lethal activity of 5-chlorosalicylic acid is avoided when this compound is metabolically generated following administration of I. As pointed out previously (2), orally administered I was converted to 5-chlorosalicylic acid as it passed through the intestinal wall. Therefore, I most likely functions as a carrier or unique delivery system for 5-chlorosalicylic acid *in vivo*, permitting manifestation of its beneficial pharmacological properties with complete avoidance of its deleterious GI effects.

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Pharmacokinetic Model for a Primary Antibody Response

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Abstract □ Antigen distribution and clearance studied *in vivo* may be viewed as a pharmacokinetic problem complicated by the intervention of the immune response. A model characterizing the clearance of the simple antigen ϕX174 from the bloodstream of various experimental animals, as well as the subsequent serum antibody response, was developed. The present model is the simplest of the possible models and no doubt will have to be modified when considering more complex antigens, additional distribution modes, and differences in antibody reactivity. For the primary (IgM) immune response, however, early events in the immunological response can be adequately accounted for in a consistent and quantitative manner.

Keyphrases □ Antibody response, primary—pharmacokinetic model developed □ Antigens—distribution and clearance, pharmacokinetic model described for primary antibody response □ Pharmacokinetic models—primary antibody response

The time course during which drugs are absorbed, distributed, metabolized, and excreted is commonly studied by using pharmacokinetic models (1, 2). A pharmacokinetic model takes into account the various ways by which a drug may be distributed between different body compartments and attempts to account quantitatively for the disposition of drug in any one compartment at any time. Particular compartments, such as blood or urine, may be explicitly identified because there are the biophases assayed for drug content.

However, other compartments, such as adipose tissue, have their presence implied from the nature of the drug concentration–time profile. A model is constructed depicting the transfer of drug from one compartment to another, the transfer being characterized by one or more rate constants, and subsequently a mathematical expression is derived to replicate the observed drug concentration–time profile. As a number of models may serve to fit the same data, it is usual to prefer the scheme that involves the fewest number of possible compartments and is compatible with experimental results.

In certain immunological studies, it is the practice to inject an antigen into a test animal and to assay blood samples, withdrawn at timed intervals, for remaining antigen and for the presence of antibody. Antigen distribution and clearance, when studied in this manner, may be viewed as a pharmacokinetic problem complicated by the intervention of the immune response. With this perspective, a pharmacokinetic model was developed which accounts for the major events taking place in the initiation of the primary (IgM) immune response. This model deals only with early events in the primary antibody response; that is, IgM is considered to be the sole antibody involved in the removal of antigen from the circulation.

In constructing the model, use is made of generally accepted concepts regarding the nature of the primary antibody response. It is shown that this model characterizes the primary immune response toward the bacteriophage ϕX174 in different mammalian species. With antigens more complex than ϕX174, a more involved pharmacokinetic model may be necessary.

MODEL

Construction—Injection of small amounts of an antigen, such as ϕX174, into the circulatory system of guinea pigs (3, 4), mice (5, 6), or fetal lambs (7) provides a means of following the primary antibody response in a quantitative manner. The elimination of antigen from the circulation occurs in two phases (Fig. 1). The first and slower elimination is due to nonimmune catabolism, while the second and rapid elimination is interpreted as being caused by the production and release into the circulation of specific antibody (3, 4). Designating A as the amount of antigen and B as the amount of antibody present at a particular time t, antigen elimination may be represented by either of three generalized competing pathways:

$$A \xrightarrow{k_p} \text{nonimmune elimination}$$

$$A + B \xrightarrow{k_c} \text{immune elimination}$$

Scheme I

$$A \xrightarrow{k_p} \text{nonimmune elimination}$$

$$A + B \xrightarrow{k_c} AB \xrightarrow{k_a} \text{immune elimination}$$

Scheme II

$$A \xrightarrow{k_p} \text{nonimmune elimination}$$

$$A + B \underset{k_r}{\overset{k_f}{\rightleftharpoons}} AB \xrightarrow{k_a} \text{immune elimination}$$

Scheme III

where k_p, k_c, k_a, k_f, and k_r are rate constants and AB represents the amount of antigen–antibody complex. Both the antigen and the antigen–antibody complex are thought to be removed through the reticuloendothelial system (8), which suggests that the pathways (Schemes I–III) should have a common elimination compartment. However, since blood levels of antigen are monitored and not contents of the elimination compartment, it is permissible to view the nonimmune and immune clearances as separate competing elimination modes.

Scheme I shows the more general clearance model; it simply designates that antigen may be removed from the circulation either by nonimmune catabolism or by reaction with antibody. The rate law appropriate to elimination of antigen by this pathway is given by:

$$-\frac{dA}{dt} = k_pA + k_cA(B) \qquad \text{(Eq. 1)}$$

Scheme II designates that, in the immune elimination, the antigen–antibody complex is formed in an irreversible manner and that the complex is eliminated. So long as only antigen blood levels are monitored, the rate law for this pathway is indistinguishable from Eq. 1. Scheme III is similar to Scheme II insofar as the antigen–antibody complex is the species eliminated due to the immune response. However, it differs from Scheme II by considering the formation of the antigen–antibody complex to be reversible. In this instance, the rate law appropriate to antigen elimination, under steady-state conditions, is given by:

$$-\frac{dA}{dt} = k_pA + \left(\frac{k_fk_a}{k_r + k_a}\right)A(B) \qquad \text{(Eq. 2)}$$

Equation 2 differs from Eq. 1 by having a more complex form to the second-order rate constant, but Eqs. 1 and 2 are kinetically indistinguishable in practice. For practical purposes, then, antigen elimination may be represented by the rate law:

$$-\frac{dA}{dt} = k_p A + k_o A(B) \qquad \text{(Eq. 3)}$$

where k_o is the experimental second-order rate constant.

Equation 3 can serve as a basis for interpreting ϕX174 clearance curves if an expression for B can be arrived at that allows integration. This problem may be approached by noting that antigen stimulates lymphocytes toward replication and differentiation into mature plasma cells. Mature plasma cells are nonreplicating, and the number of these cells ultimately produced seems to depend on the amount of initially administered antigen (3, 4, 9). The population P of these nonproliferating antibody-producing cells at any time t may then be denoted by the expression:

$$P = P_A(1 - e^{-k_d t}) \qquad \text{(Eq. 4)}$$

in which P_A is the optimum population of these cells achieved for a particular initial dose of antigen, and k_d is a parameter characterizing the rate of differentiation.

For extended periods, much longer than the time period of present concern, the tendency for mature plasma cells to "die off" after having attained their optimum number must be considered. This can be done by representing the population of antibody-producing cells P as subject to the consecutive process:

$$U \xrightarrow{k_d} P \xrightarrow{k_i} \text{nonantibody producing cells}$$

Scheme IV

where U is the population of replicating cells from which differentiation is occurring, and k_i is a constant characterizing the inactivation of mature plasma cells. By presuming that both differentiation and inactivation are apparent first-order processes, the population of antibody-producing cells is represented by:

$$P = \frac{k_d U_A}{k_i - k_d}(e^{-k_d t} - e^{-k_i t}) \qquad \text{(Eq. 5)}$$

where, by this representation, U_A is viewed as the total number of lymphocytes stimulated to divide by a given dose of antigen. Earlier models for Scheme IV (10, 11) were not as explicit as Eq. 5.

Within the timespan during which Eq. 4 is useful in characterizing the population of antibody-producing cells, it may be presumed that the amount of antibody liberated from the antibody-producing cells is reflective of their population (11). In the simplest instance, the amount of antibody produced is directly proportional to the number of antibody-producing cells:

$$B = k_b P \qquad \text{(Eq. 6)}$$

where k_b is a constant characterizing the average amount of antibody produced per cell. This relationship seems reasonable, since it has been demonstrated that the amount of antibody present has no effect on the production of antibody or on the proliferation of antibody-producing cells (12).

Substitution of Eq. 4 into Eq. 6 provides an expression for the average amount of antibody present at any time:

$$B = B_A(1 - e^{-k_d t}) \qquad \text{(Eq. 7)}$$

where $B_A = k_b P_A$. Upon introducing Eq. 7 into Eq. 3 and solving, the resulting expression is:

$$\ln\left(\frac{A}{A_0}\right) = -(k_p + k_A)t + \frac{k_A}{k_d}(1 - e^{-k_d t}) \qquad \text{(Eq. 8)}$$

Here $k_A = k_o B_A$, and A_0 is the initial amount of antigen in the blood. Equation 8 thus represents the amount of antigen in the circulation at any time.

Application—A typical clearance curve for the elimination of the bacteriophage ϕX174 from the blood of a mouse (Swiss male) is shown in Fig. 1. Equation 8 allows this curve to be readily interpreted. There is minimal production of antibody, implying $k_A = 0$,

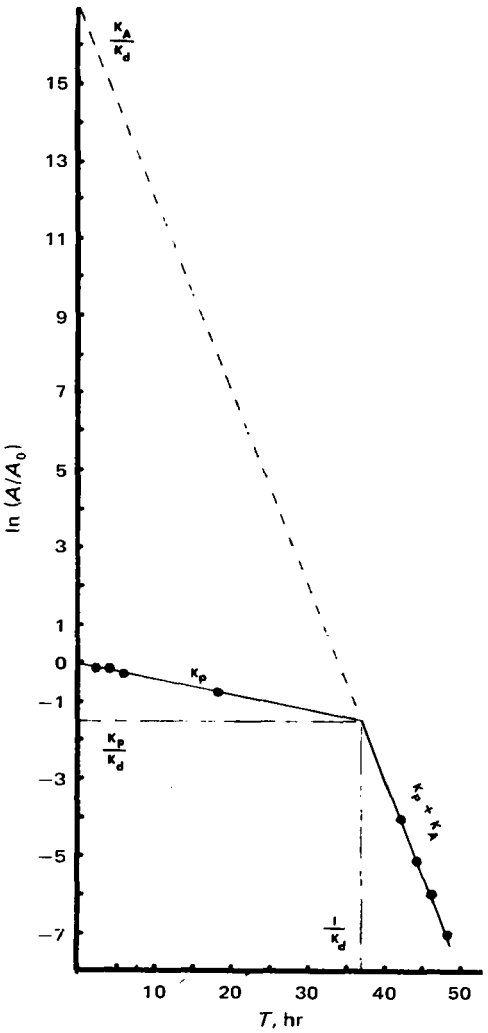

Figure 1—*Clearance curve for the elimination of the bacteriophage ϕX174 from the blood of a mouse (Swiss male).*

in the time period immediately following injection of antigen. During this stage, the elimination of antigen can be characterized by:

$$\ln\left(\frac{A}{A_0}\right) = -k_p t \qquad \text{(Eq. 9)}$$

As antibody production increases, the competition between nonimmune and immune antigen elimination modes becomes evident by a curvature in the clearance curve (not shown in Fig. 1). This competitive elimination is characterized by Eq. 8. When antigen-stimulated antibody production is at its optimum, the exponential term in Eq. 8 becomes negligible and Eq. 8 reduces to:

$$\ln\left(\frac{A}{A_0}\right) = -(k_p + k_A)t + \frac{k_A}{k_d} \qquad \text{(Eq. 10)}$$

Computer simulations showed that, with k_d in the 0.02–0.40 range, Eq. 8 reduces to Eq. 10 well within the time of a clearance experiment.

The linear equations (Eqs. 9 and 10) provide a basis for obtaining the individual rate constants from the clearance curves. A least-squares fit of the experimental points for the nonimmune and immune clearances of antigen is made to gain estimates for the slopes and intercepts represented in the respective relations. Nonimmune clearance provides the slope k_p, while immune clearance gives a slope of $k_p + k_A$. The difference between the two slopes provides an estimate for k_A. The reciprocal of the intercept for the immune clearance least-squares equation is identified as k_d/k_A. Hence, multiplication by k_A provides an estimate of k_d.

An alternative, and more precise, means of gaining an estimate

for k_d involves equating the two least-squares equations for nonimmune and immune clearances to determine the point of intercept. Equating the doses leads to an estimate of the inflection time:

$$t_i = \frac{1}{k_d} \qquad \text{(Eq. 11)}$$

while equating the times leads to an estimate for the inflection dose:

$$\ln\left(\frac{A_i}{A_0}\right) = -\frac{k_p}{k_d} \qquad \text{(Eq. 12)}$$

These estimates are shown schematically in Fig. 1. The reciprocal of the inflection time provides k_d.

RESULTS AND DISCUSSION

Table I summarizes values for the rate constants k_p, k_A, and k_d, which have been estimated from Fig. 1 and from similar studies (3–7). When literature values were used for the estimates, only those clearance curves having at least three experimental values describing nonimmune and immune clearances were analyzed by least squares. A comparison of the values suggests the following:

1. The rate of nonimmune clearance, as measured by k_p, is essentially independent of the administered dose of antigen and seems to differ from one mammalian species to another. This is consistent with the well-known species variation in the phagocytic index (13). The agreement between the values of k_p for the mouse should be noted, because the clearance curves were obtained in three independent laboratories. Comparison between the results gained in separate laboratories is possible since the magnitude of experimental slopes, and not points, is compared. Therefore, while the level of response of an animal toward clearing antigen may vary, the time course of clearance as measured by k_p should be essentially the same, animal to animal, if a similar clearance mechanism operates in each instance.

2. The rate of differentiation to antibody-producing cells, as reflected by k_d, is essentially the same for all mammalian species considered. The average value is 0.027 hr^{-1}. There seems to be a limited trend between the value of k_d and the dose of antigen administered, which can be represented by least-squares equations:

$$k_d(\text{mouse}) = -8.12\,(\pm 0.22) \times 10^{-3} \ln A + 6.80\,(\pm 0.11) \times 10^{-2} \qquad \text{(Eq. 13)}$$

n	r	s
3	0.999	0.00025

$$k_d(\text{guinea pig}) = -5.64\,(\pm 0.92) \times 10^{-3} \ln A + 6.74\,(\pm 0.92) \times 10^{-2} \qquad \text{(Eq. 14)}$$

n	r	s
3	0.986	0.00267

where n is the number of data points, r is the correlation coefficient, s is the standard error of the estimate, and the values in parentheses following each coefficient are the standard errors of the estimate of the slope and intercept, respectively. However, in view of the low values for the slopes of these relationships, one may, to a good approximation, consider k_d to be essentially independent of the dose of antigen administered.

3. The rate of immune clearance, as measured by k_A, seems to differ from one mammalian species to another. This finding is not unexpected, considering that the reticuloendothelial system is involved in both immune and nonimmune clearances (8) and that rates of nonimmune clearance differ among species. No apparent relationship between k_A and the initial dose of antigen is readily discerned. More precise data, involving a larger number of data points for the immune clearance, may make discernible a relationship between k_A and the initial dose of antigen. With the data at hand, however, one may presume that k_A is essentially constant for each species. Under this presumption, the ratio k_A/k_p provides a measure of the magnitude of the immune response for each species. This ratio is about 10.7, 5.9, and 1.8 for the mouse, fetal lamb, and guinea pig, respectively. Of the species considered, the mouse is the most and the guinea pig is the least immunologically sensitive animal toward ϕX174.

Table I—Pharmacokinetic Parameters from ϕX174 Clearance Curves

Species	Antigen Dose, PFU/ml[a]	k_p, hr^{-1}	k_A, hr^{-1}	k_d[b], hr^{-1}	Reference
Mouse	1.5×10^4	0.037	0.371	0.034	5
Mouse	9×10^4	0.033	0.329	0.028	6
Mouse	6×10^5	0.039	0.460	0.021	—
Guinea pig	5×10^4	0.115	0.200	0.042	3
Guinea pig	6×10^6	0.104	0.251	0.027	3
Guinea pig	6×10^8	0.163	0.251	0.019	3
Fetal lamb (116 days)	5×10^6	0.060	0.368	0.023	7
Fetal lamb (103 days)	1×10^{10}	0.064	0.368	0.021	7

[a] PFU = number of plaque-forming units. [b] Determined using point of intercept method.

As is well known (3, 4), there is no detectable specific antibody to ϕX174 (IgM) in the blood during the immune clearance of the antigen; but shortly after antigen clearance, an exponential build-up of antibody is observed and it reaches an optimum value approximately 96 hr after immunization. This build-up of antibody can be expressed by a rearranged form of Eq. 7:

$$\ln\left(\frac{B_A}{B_A - B}\right) = k_d' t \qquad \text{(Eq. 15)}$$

Early (72–96 hr after injection) serum antibody responses toward ϕX174, interpolated from published (3, 5, 7) figures, are essentially linear when plotted in accord with Eq. 15 (Fig. 2). This linear representation is limited in the respect that, when serum antibody levels B are considerably less than the optimum B_A, information content is lost since the ratio $B_A/(B_A - B)$ is near 1 in these instances.

An ambiguity is associated with the determination of k_d' values from serum antibody responses as opposed to k_d values from antigen clearance curves, and it seems related to the immunological sensitivity of the test animal. If the model applies to both antigen clearance and serum antibody response, one expects the k_d values determined from each experiment to agree within the limits of ex-

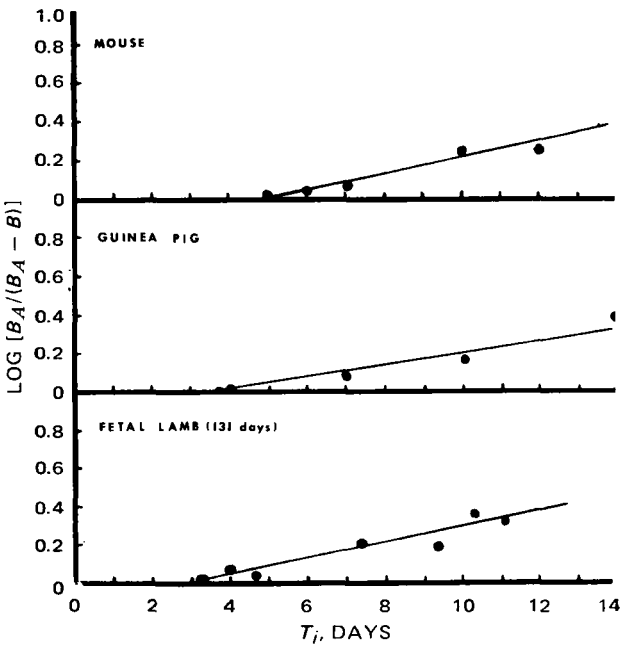

Figure 2—*Linear representation of serum antibody response.*

Table II—Comparison of k_d Values

Species	Antigen Dose, PFU/ml	Optimum Antibody Produced (Estimated)	k_d, hr^{-1} Antigen Clearance	k_d, hr^{-1} Serum Antibody Response	Reference
Guinea pig	2×10^9	50[a]	0.023	0.019	3
Fetal lamb (131 days)	6×10^8	10[a]	0.022	0.0038	7
Mouse	3×10^9	80×10^2[b]	0.021[c]	0.0056	6

[a] Assay in terms of K values. [b] Assay in terms of SD_{50} values. [c] Initial dose of antigen, 6×10^5 PFU/ml.

perimental uncertainty. This appears to be so with the guinea pig as the test animal (Table II). However, for the two other species, mouse and fetal lamb (131 days), the k_d' value determined from the serum antibody response is about 20 times less than the k_d value determined from antigen clearance curves.

Evidently, immunological sensitivity implies that antigen stimulates not only the production of antibody-producing cells but also the production of antibody from these cells. On this basis, animals such as the guinea pig, which are relatively insensitive immunologically toward ϕX174, respond to antigen primarily through the production of antibody-producing cells. Values of k_d determined from clearance curves and from serum antibody responses are then expected to be in agreement. However, animals that are immunologically sensitive toward ϕX174 have their antibody-producing cells stimulated by antigen so as to produce more antibody. Serum antibody responses thus lead to k_d' values characterizing the rate of production of antibody in the absence of antigen, while antigen clearance curves lead to estimates of k_d characterizing the rate of production of antibody in the presence of antigen.

CONCLUSIONS

The developed pharmacokinetic model enables an interpretation of cellular events from *in vivo* experiments on the time course of the primary antibody response toward ϕX174. A modified model may be necessary for more complex antigens, especially in relation to their distribution between blood and body tissues. However, the present model generally describes the major events occurring in the initiation of the primary antibody response. It does so in a quantitative manner, without straining or forcing an interpretation. It provides a basis for comparing results from differing sources, a framework into which kinetic information from the study of isolated events in the immunological initiation process may be fitted, and offers a means of gaining a preliminary mechanistic interpretation.

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Antihemolytic and Anticonvulsant Activities of 1-(2,4-Dichloro/2,4,5-Trichlorophenoxyacetyl)-4-alkyl/arylthiosemicarbazides and Their Inhibition of NAD-Dependent Oxidations and Monoamine Oxidase

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Abstract □ Several 1-(2,4-dichloro and 2,4,5-trichlorophenoxyacetyl)-4-alkyl/arylthiosemicarbazides were synthesized and characterized by their sharp melting points and elemental analyses. All substituted thiosemicarbazides protected *in vitro* hypoosmotic hemolysis of dog red blood cells. These thiosemicarbazides selectively inhibited nicotinamide adenine dinucleotide (NAD)-dependent oxidation of pyruvate and α-ketoglutarate, while NAD-independent oxidation of succinate was not affected. These compounds inhibited the activity of monamine oxidase in rat brain homogenate, and the degree of inhibition ranged from 26.5 to 89.2% at a final concentration of 0.03 m*M*, with kynuramine as the substrate. Almost all thiosemicarbazides possessed anticonvulsant activity; protection against pentylenetetrazol-induced convulsions in mice ranged from 10 to 70% at a dose of 100 mg/kg ip. These results provided evidence of some similarity between the membrane-stabilizing property of these substituted thiosemicarbazides with their ability to exhibit selective inhibition of NAD-dependent oxidations and inhibition of monoamine oxidase. On the other hand, the anticonvulsant activity possessed by these substituted thiosemicarbazides was unrelated to their *in vitro* antihemolytic and enzyme inhibitory properties.

Keyphrases □ Thiosemicarbazides, 1-(2,4-dichloro and 2,4,5-trichlorophenoxyacetyl)-4-alkyl/aryl—synthesis, antihemolytic and anticonvulsant activities, and relationship to inhibition of cellular respiratory activity and inhibition of monoamine oxidase □ Structure–activity relationships—thiosemicarbazides, antihemolytic activity, anticonvulsant activity, enzyme inhibitory properties □ Antihemolytic activity—thiosemicarbazides synthesized and tested □ Cellular respiratory activity—thiosemicarbazides, selective inhibition of NAD-dependent oxidation of pyruvate and α-ketoglutarate, monoamine oxidase, rat brain homogenate □ Monoamine oxidase inhibition—thiosemicarbazides, relationship to antihemolytic (membrane stabilizing) and anticonvulsant properties, inhibition of cellular respiratory activity, selective inhibition of NAD-dependent oxidation

Various hydrazines (1) and semicarbazides (2, 3) have been shown to inhibit monoamine oxidase [EC 1.4.3.4 monoamine O_2 oxido-reductase (deaminating)]. Monoamine oxidase inhibitors also have been shown to possess anticonvulsant properties (4). Furthermore, studies have indicated a relationship between the *in vitro* monoamine oxidase inhibitory property of α-benzoylamino-*N*-[(4-aryl semicarbazide/thiosemicarbazide)benzoyl]-4-substituted cinnamides and their ability to afford protection against pentylenetetrazol-induced convulsions (5). Numerous psychotropic agents have been proposed to affect the physicochemical properties of cell membranes which account for the basis of their mechanism of action (6), and various drug responses have been indicated as the probable result of drug–receptor interactions (7).

Stabilization of the red cell membranes has also been observed in various central nervous system depressants and local anesthetics (8, 9), tricyclic tranquilizers (9, 10), nonphenothiazine tranquilizers (8), and biogenic amines (11). Recently, the synthesis of substituted anilino-[3-methoxy-4-(4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidines was reported, and attempts were made to correlate their monoamine oxidase inhibitory effectiveness and their ability to afford protection against hypoosmotic hemolysis in dog erythrocytes with their anticonvulsant property (12). A decrease in the cellular respiratory activity of brain tissue by the inhibition of the mitochondrial membrane-bound oxidase systems was observed with hypnotics (13–15) and anticonvulsants (16, 17), where these changes are presumably responsible for reduced energy production, leading ultimately to depressed neuronal activity.

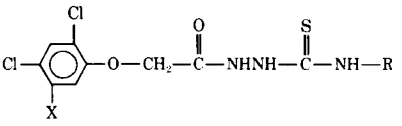
In the present study, various 1-(2,4-dichlorophenoxyacetyl)-4-substituted thiosemicarbazides and 1-(2,4,5-trichlorophenoxyacetyl)-4-substituted thiosemicarbazides were synthesized and evaluated for: (*a*) their *in vitro* ability to stabilize the red cell membrane; (*b*) their effects on the cellular respiratory activity during oxidation of pyruvate, α-ketoglutarate, NADH, and succinate by rat brain homogenates, and (*c*) their effects on the activity of rat brain monoamine oxidase. The ability of these substituted thiosemicarbazides to provide protection against pentylenetetrazol-induced seizures was also investigated in an attempt to correlate the anticonvulsant activity exhibited by these compounds with their ability to inhibit various enzyme systems as a basis of their biochemical mechanism of action.

EXPERIMENTAL[1]

Preparation of 1-(2,4-Dichlorophenoxyacetyl- or 2,4,5-Trichlorophenoxyacetyl)-4-substituted Thiosemicarbazides—The various 1-(2,4-dichlorophenoxyacetyl)-4-alkyl/arylthiosemicarbazides and 1-(2,4,5-trichlorophenoxyacetyl)-4-alkyl/arylthiosemicarbazides were synthesized by refluxing a mixture of an appropriate substituted chlorophenoxyacethydrazide (0.01 mole) and alkyl/aryl isothiocyanate (0.01 mole) in 25 ml of absolute ethanol for 2 hr. The solid mass, which separated on cooling, was collected by filtration, washed with petroleum ether (bp 40–60°), and recrystallized from ethanol (Table I).

[1] All compounds were analyzed for their carbon, hydrogen, and nitrogen content. Melting points were taken in open capillary tubes with a partial immersion thermometer and are corrected.

Table I—Physical Constants of 1-(2,4-Dichlorophenoxyacetyl)-4-alkyl/arylthiosemicarbazides and 1-(2,4,5-Trichlorophenoxyacetyl)-4-alkyl/arylthiosemicarbazides



Compound	X	R	Melting Point[a]	Yield, %	Formula	Analysis, % Calc.	Found
I	H	C_2H_5	148°	50	$C_{11}H_{13}Cl_2N_3O_2S$	C 41.01 H 4.07 N 13.05	40.98 4.00 12.83
II	H	n-C_4H_9	126°	65	$C_{13}H_{17}Cl_2N_3O_2S$	C 44.58 H 4.89 N 12.00	44.44 4.78 11.94
III	H	n-C_7H_{15}	152°	55	$C_{16}H_{23}Cl_2N_3O_2S$	C 48.98 H 5.86 N 10.71	48.87 5.87 10.48
IV	H	o-$CH_3C_6H_4$	210°	45	$C_{16}H_{15}Cl_2N_3O_2S$	C 50.01 H 3.94 N 10.94	49.90 3.89 10.80
V	H	m-$CH_3C_6H_4$	162°	45	$C_{16}H_{15}Cl_2N_3O_2S$	C 50.01 H 3.94 N 10.94	50.00 3.87 10.80
VI	H	p-BrC_6H_4	183–185°	55	$C_{15}H_{12}BrCl_2N_3O_2S$	C 40.11 H 2.69 N 9.36	40.07 2.65 9.29
VII	H	p-FC_6H_4	162°	50	$C_{15}H_{12}Cl_2FN_3O_2S$	C 46.41 H 3.12 N 10.83	46.32 3.00 10.54
VIII	H	CH_2—CH=CH_2	158°	40	$C_{12}H_{13}Cl_2N_3O_2S$	C 43.13 H 3.92 N 12.58	43.00 3.91 12.20
IX	H	C_6H_{11}	198°	60	$C_{15}H_{19}Cl_2N_3O_2S$	C 47.88 H 5.09 N 11.17	47.85 5.07 11.20
X	Cl	C_2H_5	206°	65	$C_{11}H_{12}Cl_3N_3O_2S$	C 37.05 H 3.39 N 11.79	36.96 3.37 11.40
XI	Cl	n-C_4H_9	168°	55	$C_{13}H_{16}Cl_3N_3O_2S$	C 40.59 H 4.19 N 10.93	40.54 4.00 10.58
XII	Cl	n-C_7H_{15}	205–207°	40	$C_{16}H_{22}Cl_3N_3O_2S$	C 45.03 H 5.20 N 9.85	45.00 5.09 9.63
XIII	Cl	o-$CH_3C_6H_4$	165°	50	$C_{16}H_{14}Cl_3N_3O_2S$	C 45.90 H 3.37 N 10.04	45.85 3.29 9.90
XIV	Cl	m-$CH_3C_6H_4$	185°	60	$C_{16}H_{14}Cl_3N_3O_2S$	C 45.90 H 3.37 N 10.04	45.87 3.36 9.98
XV	Cl	p-BrC_6H_4	195°	45	$C_{15}H_{11}BrCl_3N_3O_2S$	C 37.25 H 2.29 N 8.69	37.20 2.19 8.53
XVI	Cl	p-FC_6H_4	160°	55	$C_{15}H_{11}Cl_2FN_3O_2S$	C 42.62 H 2.62 N 9.94	42.59 2.60 10.00
XVII	Cl	CH_2—CH=CH_2	166°	50	$C_{12}H_{12}Cl_3N_3O_2S$	C 39.10 H 3.28 N 11.40	39.02 3.19 11.28
XVIII	Cl	C_6H_{11}	175°	55	$C_{15}H_{18}Cl_3N_3O_2S$	C 43.86 H 4.42 N 10.23	43.56 4.41 10.04

[a] Melting points were taken in open capillary tubes and are corrected.

Determination of Hypoosmotic Hemolysis—Assay of hypoosmotic hemolysis was carried out by following a slightly modified published procedure (10) in which blood was used without oxygenation. Fresh heparinized blood of healthy mongrel dogs was used. To a 0.1-ml aliquot of dog blood was added 3 ml of a buffer solution (0.425% Na_2HPO_4–NaH_2PO_4 buffer, 5 m*M*, pH 7.4; total osmolality 135 mosmoles/liter) containing the test compounds. The tubes were shaken gently two or three times and were allowed to stand at room temperature (26–28°) for exactly 10 min. These tubes were then centrifuged for 5 min at 1000×*g* to separate the cells.

Under these conditions, hemolysis of the blood cells occurred but in no case exceeded 50%. The absorbance of the supernate was read at 540 nm in a colorimeter. Percent protection was calculated by comparing the absorbance values observed in the presence of the test compounds with those observed in the control tubes without the test compounds. The control value was represented as 100% hypoosmotic hemolysis. Absorbance values for the blank containing 3 ml of 0.85% NaCl (normal saline) were subtracted from the values for both the control and experimental tubes.

All test compounds were dissolved in the buffered saline solution of pH 7.4 (osmolality 135 mosmoles/liter) and were used at a final concentration of 0.05 or 0.1 m*M*. The effective dose$_{50}$ (ED_{50}), the concentration of the the test compound exhibiting 50% antihemolytic activity, was evaluated graphically using different concentrations of these substituted thiosemicarbazides, and this procedure provided comparative effectiveness of the test compounds. From the relationship between the concentration of the test compound and its relative antihemolytic activity, it was possible to determine, either by extrapolation or interpolation, the ED_{50} values

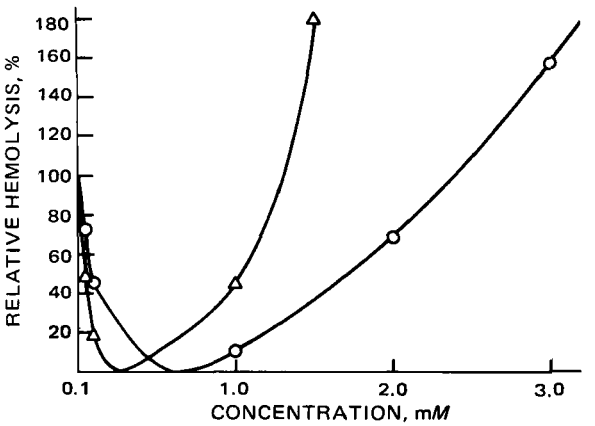


Figure 1—*Biphasic response of varying concentrations of 1-(2,4-dichlorophenoxyacetyl)-4-*m*-tolylthiosemicarbazide (V, △) and 1-(2,4,5-trichlorophenoxyacetyl)-4-ethylthiosemicarbazide (X, ○) on relative hypoosmotic hemolysis of red blood cells of dog. Assay procedures and contents of the reaction mixture are as indicated in the text.*

of these substituted thiosemicarbazides.

Assay of Respiratory Activity of Rat Brain Homogenate[2]—Male albino rats[3], kept on *ad libitum* diet, were used. Rats weighing 150–200 g were sacrificed by decapitation. The brains were taken out immediately and homogenized[4] in the ratio of 1:9 (w/v) in 0.25 *M* cold sucrose. Respiratory activity was determined by measuring the oxygen uptake by the conventional Warburg manometric method at 37° with air as the gas phase (16). Fresh brain homogenate of healthy albino rats, equivalent to 125 mg of wet tissue weight, was used in each flask.

The reaction mixture, in a final volume of 3 ml, consisted of 20 m*M* Na_2HPO_4 buffer (pH 7.4), 6.7 m*M* $MgSO_4$, 1 m*M* AMP (sodium salt), 33 m*M* KCl, and 500 μg of cytochrome c. The central well contained 0.2 ml of 20% KOH solution. Pyruvate, α-ketoglutarate, and succinate were used at a final concentration of 10 m*M*, and the final concentration of NADH was 0.5 m*M*. All substituted thiosemicarbazides were dissolved in propylene glycol (100%) and were used at a final concentration of 1 m*M*. An equivalent amount of propylene glycol was added to the control vessels.

Determination of Monoamine Oxidase Activity—A spectrophotofluorometric method was used for the determination of monoamine oxidase activity of rat brain homogenate, using kynuramine as the substrate (18). The 4-hydroxyquinoline formed during oxidative deamination of kynuramine was measured fluorometrically in a spectrophotofluorometer[5], using activating light of 315 nm and measuring fluorescence at the maximum of 380 nm.

Male albino rats[3], 150–200 g, were allowed food and water *ad libitum* and were sacrificed by decapitation. The brains were removed immediately and homogenized in the ratio of 1:9 (w/v) in ice-cold 0.25 *M* sucrose. The reaction mixture, in a total volume of 3 ml, consisted of phosphate buffer (0.5 ml, pH 7.5; 0.2 *M*), 0.1 m*M* kynuramine, and 0.5 ml of brain homogenate (equivalent to 10 mg of wet brain weight). The monoamine oxidase activity of rat brain homogenate was determined by incubation for 30 min at 37° in air.

The various substituted thiosemicarbazides were dissolved in propylene glycol (100%), added to the brain homogenate at a final concentration of 0.33 m*M*, and then incubated for 10 min before the addition of kynuramine. After the addition of kynuramine, the mixture was incubated for an additional 30 min. The reaction was stopped by the addition of 1 ml of 10% trichloroacetic acid solution (w/v), and the precipitated proteins were removed by centrifugation.

Suitable 1-ml aliquots of the supernate were taken in 2 ml of 1 *N* NaOH solution and were assayed for 4-hydroxyquinoline. An increase in absorbance provided a direct measurement of 4-hydroxyquinoline formation, which was taken as an index of the monoamine oxidase activity. The percentage inhibition was calculated from the decrease observed in absorbance, and this value provided an index of the inhibitory property of these substituted thiosemicarbazides.

Table II—Protection of Hypoosmotic Hemolysis of Dog Erythrocytes by 1-(2,4-Dichlorophenoxyacetyl)-4-alkyl/arylthiosemicarbazides and 1-(2,4,5-Trichlorophenoxyacetyl)-4-alkyl/arylthiosemicarbazides

Compound	Protection[a], % 0.05 m*M*	0.1 m*M*	ED_{50}[b], m*M*
I	22.6 ± 0.38	48.1 ± 1.11	0.103
II	17.4 ± 0.58	39.5 ± 0.95	0.125
III	13.9 ± 0.77	28.3 ± 0.48	0.180
IV	22.3 ± 0.48	43.5 ± 0.73	0.118
V	51.5 ± 0.21	82.3 ± 1.25	0.048
VI	31.5 ± 0.35	44.4 ± 1.40	0.130
VII	35.6 ± 0.76	58.6 ± 0.88	0.078
VIII	40.0 ± 0.66	71.5 ± 0.64	0.064
IX	52.1 ± 0.99	82.5 ± 0.22	0.047
X	26.8 ± 0.41	53.4 ± 1.41	0.094
XI	41.3 ± 0.62	69.0 ± 0.85	0.063
XII	24.3 ± 0.59	44.2 ± 0.63	0.117
XIII	59.6 ± 0.30	85.3 ± 0.58	0.040
XIV	29.4 ± 0.99	63.4 ± 0.66	0.080
XV	41.3 ± 1.21	66.3 ± 0.73	0.066
XVI	48.9 ± 0.87	80.3 ± 0.11	0.052
XVII	42.5 ± 0.78	74.6 ± 1.7	0.061
XVIII	46.5 ± 0.55	80.1 ± 1.33	0.054

[a] Each experiment was done in duplicate. Assay conditions and the contents of the reaction mixture were as reported in the text. All values represent mean values of percent protection with ±*SE* calculated from three separate experiments. [b] Represents the concentration calculated graphically that would reduce the relative hypoosmotic hemolysis to one-half of the hemolysis of dog erythrocytes.

Determination of Anticonvulsant Activity—Anticonvulsant activity was determined against pentylenetetrazol-induced seizures in albino mice[3] of either sex weighing 25–30 g. The mice were divided into groups of 10, and the group weights were kept as near the same as possible. All substituted thiosemicarbazides were suspended in 5% aqueous gum acacia to give a concentration of 0.25% (w/v). The test compounds were injected intraperitoneally in a dose of 100 mg/kg to one group of 10 mice. Four hours after the administration of these compounds, the mice were injected with pentylenetetrazol (90 mg/kg sc). This dose of pentylenetetrazol has been shown to produce convulsions in almost all untreated mice and to exhibit 100% mortality in these animals during 24 hr. No mortality was observed during 24 hr in mice treated with 100 mg/kg of the test compounds alone.

The mice were observed for 60 min for the occurrence of convulsions. An episode of clonic convulsion persisting for a minimum of 5 sec was considered a threshold convulsion. Transient intermittent jerks and tremulousness were not counted. Animals devoid of threshold convulsions during 60 min were considered protected. The number of mice protected in each group was recorded, and the anticonvulsant activity of these substituted thiosemicarbazides was represented as the percentage protection. The animals were then observed for 24 hr and the mortality was recorded.

RESULTS

The membrane-stabilizing property of various 1-(2,4-dichlorophenoxyacetyl)-4-substituted thiosemicarbazides and 1-(2,4,5-trichlorophenoxyacetyl)-4-substituted thiosemicarbazides (Table I) was evaluated by determining the decrease in the degree of hypoosmotic hemolysis of dog red blood cells. All substituted thiosemicarbazides protected hypoosmotic hemolysis when used at the final concentrations of 0.05 and 0.1 m*M*.

As is evident from Table II, an increase in the ability of 2,4-dichloro-substituted thiosemicarbazides to protect against hypoosmotic hemolysis was mostly observed by the introduction of an ad-

[2] Commercial chemicals were used. Sodium pyruvate, sodium α-ketoglutarate, sodium succinate, NADH, adenosine monophosphate (AMP), cytochrome c, and kynuramine were obtained from Sigma Chemical Co., St. Louis, Mo.

[3] Animal Supply House, Lucknow, India.

[4] Potter-Elvehjem homogenizer.

[5] Aminco-Bowman.

Table III—Effect of 1-(2,4-Dichlorophenoxyacetyl)-4-alkyl/arylthiosemicarbazides and 1-(2,4,5-Trichlorophenoxyacetyl)-4-alkyl/arylthiosemicarbazides on the Cellular Respiratory Activity of Rat Brain Homogenate

Compound	Inhibition[a], %			
	Pyruvate	α-Ketoglutarate	NADH	Succinate
I	53.6 ± 1.2	58.5 ± 1.2	50.7 ± 0.7	Nil
II	81.5 ± 0.3	80.0 ± 0.9	60.3 ± 0.8	Nil
III	47.8 ± 1.3	52.3 ± 1.5	41.2 ± 0.6	Nil
IV	20.9 ± 1.3	31.3 ± 0.9	19.4 ± 0.2	Nil
V	29.1 ± 0.5	35.8 ± 0.7	25.3 ± 0.4	Nil
VI	37.2 ± 0.6	45.1 ± 0.4	29.2 ± 0.7	Nil
VII	49.9 ± 1.9	55.5 ± 1.1	32.8 ± 0.7	Nil
VIII	79.4 ± 0.9	84.7 ± 0.8	64.8 ± 1.0	Nil
IX	89.3 ± 1.5	87.8 ± 1.6	69.2 ± 1.5	Nil
X	51.8 ± 0.2	55.7 ± 0.5	45.8 ± 0.8	Nil
XI	79.9 ± 1.1	82.4 ± 1.8	69.3 ± 0.8	Nil
XII	56.1 ± 1.6	60.3 ± 1.0	55.8 ± 0.8	Nil
XIII	91.0 ± 1.2	94.3 ± 0.9	78.4 ± 0.4	Nil
XIV	35.0 ± 1.3	42.1 ± 1.2	32.0 ± 1.1	Nil
XV	79.7 ± 1.6	85.1 ± 1.1	66.4 ± 1.7	Nil
XVI	81.4 ± 0.3	86.2 ± 1.0	71.8 ± 1.5	Nil
XVII	76.0 ± 1.2	78.1 ± 1.5	65.4 ± 1.3	Nil
XVIII	84.2 ± 1.5	90.2 ± 1.3	72.1 ± 1.7	Nil

[a] Each experiment was done in triplicate. Assay conditions and vessel contents were as reported in the text. All values represent mean values of percent inhibition with ±*SE* of the mean calculated from three separate experiments. Inhibition was determined by the decrease in the oxygen uptake with 125 mg wet weight of brain/hr. Pyruvate, α-ketoglutarate, and succinate were used at a final concentration of 10 m*M*, while the concentration of NADH was 0.5 m*M*. All substituted thiosemicarbazides were used at a final concentration of 1 m*M*.

ditional chloro substituent (2,4,5-trichloro-substituted thiosemicarbazides) at position 5 of the phenyl nucleus. In the present study, greatest antihemolytic activity was observed with Compounds V, IX, and XIII while 1-(2,4-dichlorophenoxyacetyl)-4-*n*-heptylthiosemicarbazide (III) was the least effective compound. With the use of higher concentrations of two of these thiosemicarbazides (V and X), an increase in hypoosmotic hemolysis, rather than in protection of hemolysis, of the red blood cells was observed (Fig. 1). In the present study, both antihemolytic (Table II) and hemolytic (Fig. 1) properties of these substituted thiosemicarbazides were concentration dependent.

Effects of these substituted thiosemicarbazides on the cellular respiratory activity of the rat brain homogenate are shown in Table III. All thiosemicarbazides at a final concentration of 1 m*M* elicited various degrees of inhibition during oxidation of pyruvate, α-ketoglutarate, and NADH whereas NAD-independent oxidation of succinate remained unaffected. Introduction of a chloro substituent at position 5 of the phenyl nucleus of the 2,4-dichloro-substituted thiosemicarbazides (IV–VII) resulted in an increased inhibitory effectiveness of the corresponding 2,4,5-trichloro-substituted thiosemicarbazides (XIII–XVI). No change in the inhibitory effectiveness was observed by the introduction of the chloro substituent on 4-alkyl- and cyclohexyl-substituted thiosemicarbazides (I–III, VIII–XII, XVII, and XVIII).

The monoamine oxidase inhibitory activity of these substituted thiosemicarbazides and the anticonvulsant activity possessed by these compounds are recorded in Table IV. All thiosemicarbazides inhibited *in vitro* monoamine oxidase activity of rat brain homogenate during oxidative deamination of kynuramine. In the present study, the 2,4,5-trichlorophenoxy-substituted thiosemicarbazides (X, XI, and XIII–XVII) produced greater inhibition of monoamine oxidase than their corresponding 2,4-dichlorophenoxy-substituted derivatives (I, II, and IV–VIII) when used at a final concentration of 0.33 m*M*, with the exception of heptyl- (III and XII) and cyclohexyl- (IX AND XVIII) substituted thiosemicarbazides where the 2,4-dichlorophenoxy-substituted thiosemicarbazides (III and IX) produced higher degree of monoamine oxidase inhibition.

The anticonvulsant activity possessed by these substituted thiosemicarbazides, as evidenced by the protection afforded by these compounds against pentylenetetrazol-induced seizures at a dose of 100 mg/kg, ranged from 10 to 70% (Table IV). Maximum anticonvulsant activity was observed with 1-(2,4-dichlorophenoxyacetyl)-4-*p*-bromophenylthiosemicarbazide (VI) while 1-(2,4-dichlorophenoxyacetyl)-4-*o*-tolylthiosemicarbazide (IV) showed no protection against pentylenetetrazol-induced convulsions. In general, 2,4,5-trichlorophenoxy-substituted thiosemicarbazides afforded greater protection than their corresponding 2,4-dichlorophenoxy-substituted thiosemicarbazides (Table IV). The data on anticonvulsant activity of these compounds and their 24-hr pentylenetetrazol-induced mortality did not indicate clearly an association between increased protection from convulsions and decreased mortality in experimental animals (Table IV).

DISCUSSION

The antihemolytic property of phenothiazines (9), reserpine (9), haloperidol (9), imipramine (10), amitriptyline (10), barbiturates (11), and amphetamine (11) has been proposed to reflect their effects on cell membrane functions. Thus, these thiosemicarbazides presumably prevent or diminish various processes in the cell membranes during protection of hypoosmotic hemolysis. As is evident from Fig. 1, these compounds exhibit a biphasic effect on erythrocyte membrane stabilization and lysis, as has been observed with psychotropic agents (19–21). Such biphasic changes are not abrupt in nature, since membrane stabilization and lysis were found to be concentration dependent and lysis was observed only after a peak of 100% stabilization.

Other studies have indicated that the cells with higher area to volume ratios are associated with a lower osmotic fragility and, consequently, less hypoosmotic hemolysis (22). Thus, the rigidity of the cell membranes may presumably account for the biphasic action of thiosemicarbazides; during the protection of hypoosmotic hemolysis, the surface area to volume ratio is not high enough to warrant the dispersing of the membrane molecules to cause lysis. An increase in the concentration of thiosemicarbazides possibly dominates the dispersing effect which leads to the lysis of the red blood cells.

These thiosemicarbazides seem to possess all necessary structural requirements of other membrane-stabilizing compounds for an

Table IV—Monoamine Oxidase Inhibitory and Anticonvulsant Properties of 1-(2,4-Dichlorophenoxyacetyl)-4-alkyl/arylthiosemicarbazides and 1-(2,4,5-Trichlorophenoxyacetyl)-4-alkyl/arylthiosemicarbazides

Compound	Monoamine Oxidase Inhibition[a], %	Anticonvulsant Activity[b], % Protection	Pentylenetetrazol Mortality[b], %
I	44.1 ± 0.78	10	70
II	51.6 ± 0.33	20	80
III	71.2 ± 0.25	10	50
IV	26.5 ± 0.88	0	60
V	55.4 ± 1.10	20	50
VI	43.3 ± 1.00	70	10
VII	44.1 ± 0.98	10	70
VIII	46.5 ± 0.35	10	80
IX	61.7 ± 0.25	30	60
X	55.4 ± 0.66	30	80
XI	64.9 ± 0.78	40	50
XII	47.3 ± 0.22	10	90
XIII	89.2 ± 1.22	30	50
XIV	70.2 ± 0.77	20	60
XV	82.1 ± 0.81	30	90
XVI	80.6 ± 0.72	40	50
XVII	67.6 ± 0.45	30	70
XVIII	35.2 ± 0.35	40	60

[a] Assay procedure and the contents of the reaction mixture were as indicated in the text. All substituted thiosemicarbazides were used at a final concentration of 0.33 m*M*. Each experiment was done in duplicate, and figures indicate mean values of three separate experiments with ±*SE* of the mean. [b] Screening procedure was as indicated in the text. Each thiosemicarbazide was used at the dose of 100 mg/kg ip, and pentylenetetrazol-induced mortality was observed during 24 hr. In the present study, administration of an equivalent amount of 5% gum acacia solution was found to possess no anticonvulsant activity.

effective antihemolytic agent. These include a basic hydrazino group at one end, a chain of carbon atoms, and a phenyl ring structure at the opposite end. Introduction of an electronegative chloro substituent at position 5 of the phenyl nucleus of 2,4-dichlorophenoxy-substituted thiosemicarbazides in general resulted in a greater antihemolytic property, as reflected by their lower ED_{50} values (Table II). An increase in the number of carbon atoms in the alkyl chain of 2,4-dichlorophenoxy-substituted thiosemicarbazides caused a simultaneous decrease in their antihemolytic effectiveness (I–III); in 2,4,5-trichlorophenoxy-substituted thiosemicarbazides, an increase in activity was observed with *n*-butyl substitution (XI) and further extension of the alkyl chain decreased their antihemolytic activity (XII). Halogen substitution was shown to play some role in enhancing the activity, while thiosemicarbazides possessing a hydrophobic cyclohexyl group (IX and XVIII) possessed significant antihemolytic activity.

The ability of thiosemicarbazides to cause selective inhibition of NAD-dependent oxidations has indicated possible inactivation of the electron-transport chain at the site of the transfer of the electrons from NADH to FAD (17, 23). Introduction of a chloro substituent at position 5 of the 2,4-dichlorophenyl nucleus caused greater inhibition of the cellular respiratory activity by these 2,4,5-trichlorophenoxy-substituted thiosemicarbazides. This increased inhibition of the oxidation of pyruvate, α-ketoglutarate, and NADH was only observed with aryl-substituted compounds since no significant change was observed with alkyl-, allyl-, and cyclohexyl-substituted thiosemicarbazides.

The monoamine oxidase inhibitory effectiveness of 2,4-dichlorophenoxy-substituted thiosemicarbazides was increased by the introduction of a chloro substituent at position 5 of their phenyl nucleus to form 2,4,5-trichlorophenoxy-substituted thiosemicarbazides, with the exception of XII and XVIII where a decrease in enzyme inhibition was observed (Table IV). An increase in the length of the alkyl chain in 2,4-dichlorophenoxy-substituted thiosemicarbazides alone resulted in a gradual increase in their enzyme inhibitory activity. On the other hand, elongation of the alkyl chain from *n*-butyl to *n*-heptyl showed lowering of monoamine oxidase inhibition in 2,4,5-trichlorophenoxy-substituted thiosemicarbazides. The low anticonvulsant activity of these thiosemicarbazides, with the exception of VI, failed to reflect the structure–activity relationship of these compounds.

These results have provided indications of some similarity between the membrane-stabilizing property of the thiosemicarbazides and their ability to inhibit cellular respiratory activity selectively and monoamine oxidase. The membrane-stabilizing property may be unspecific, and some functional groups in thiosemicarbazide molecules could presumably share common properties for cell membrane attachment to exhibit enzyme inhibitory and antihemolytic properties.

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Assay of Flucytosine and Furosemide by High-Pressure Liquid Chromatography

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Abstract □ A high-pressure liquid chromatographic method for the analysis of flucytosine and furosemide concentrations in biological fluid is described. The separations were carried out on a pellicular cation-exchange resin eluted with an ammonium phosphate buffer. Detection of elution peaks was by UV absorption at 280 nm and fluorescence monitoring. Advantages of the method are specificity, minimal preanalysis sample workup, and small sample size.

Keyphrases □ Flucytosine and furosemide—high-pressure liquid chromatographic analysis in plasma and urine □ Furosemide and flucytosine—high-pressure liquid chromatographic analysis in plasma and urine □ High-pressure liquid chromatography—analysis, flucytosine and furosemide in plasma and urine

In recent years, high-pressure liquid chromatography (HPLC) has gained increasing use in the analysis for drugs in biological fluids, *e.g.*, barbiturates (1), phenytoin (diphenylhydantoin) (1), and phenacetin (2). The fact that many drugs are highly soluble in water or polar organic solvents but are nonvolatile (troublesome problems for GC analysis and many extraction procedures) is advantageous in HPLC, especially when using aqueous elution from ion-exchange resins. HPLC also has the advantages of specificity, small sample size, and minimal preanalysis sample workup.

These advantages led to the development of an HPLC analysis system for two drugs: flucytosine and furosemide. Flucytosine was recently recognized as a useful antifungal agent in the treatment of systemic *Candida albicans, Cryptococcus neoformans* (3–6), and *Cladosporium trichoides* (7). While studying the pharmacokinetics of both flucytosine and furosemide in normal and uremic volunteers, a rapid and sensitive assay procedure applicable to serum and urine samples was needed to enable the administration of a low drug dosage as an extra margin of safety to the patients. Since 80–95% of flucytosine is excreted unchanged in the urine (8) and dose-related hepatotoxicity has been reported (3), a rapid analysis for plasma drug concentration in clinical samples, particularly in patients with renal insufficiency, was desirable. An HPLC method for the determination of flucytosine concentrations in both serum and urine is reported. With minor modifications of the conditions, an analysis for furosemide for use in pharmacokinetic studies can also be conducted. This method replaces the fluorometric technique reported earlier (9). Although not generally useful for furosemide for clinical purposes, the HPLC method for the pharmacokinetic study of both drugs has shown decided advantages over existing methods (9–15) for the determination of furosemide and flucytosine concentrations in both serum and urine.

EXPERIMENTAL

Flucytosine[1] (5-fluorocytosine) (for assay development), furosemide[2] (4-chloro-*N*-furfuryl-5-sulfamoylanthranilic acid), 4-chloro-5-sulfamoylanthranilic acid[2] (I), ^{35}S-furosemide[2] (for standard and clinical studies), and probenecid sodium[3] were used. All other chemicals were analytical reagent grade.

The high-pressure liquid chromatograph[4] was equipped with an absorbance monitor[5]. The absorbance monitor was equipped with an optical unit[6], a 280–310-nm filter, and high-pressure microflow cells[7], and the output was recorded[8]. For the analysis of furosemide, the eluant from the absorbance monitor was passed through a fluoromonitor[9] (excitation at 320–400 nm; emission at 400–700 nm) and the signal output was recorded on a dual-pen recorder[10]. A pellicular cation-exchange resin, prepared in 304.8 × 0.1-cm i.d. stainless steel tubing[11] with a precolumn[12] containing the same packing material, was used for both serum and urine.

Five-microliter aliquots of urine or serum were injected directly onto the column resin bed (stop-flow technique) with a 10-μl syringe[13] equipped with a 7-cm needle.

For the analysis of flucytosine, the column was eluted with a monobasic ammonium phosphate buffer solution (16 m*M*) with the pH adjusted to 2.00 with phosphoric acid. The column pressure was 2000 psi, the eluent flow rate was 0.24 ml/min, and the column temperature was 78°. Recordings were made at a range of 0.01 absorbance unit and a recorder span of 1 v full scale.

Standard flucytosine samples were prepared in concentrations ranging from 0 to 10 mg/liter in water, serum, and urine. Aqueous samples were stable for at least 1 month at ambient temperature.

Furosemide working standards were prepared fresh each day from injectable furosemide (10 mg/ml) from the same lot number used in each study and was, in turn, standardized against a solution of the pure compound. Compound I standards were prepared from chemically pure compound dissolved in water. All standards, urines, and serums containing furosemide were protected from light.

The column was eluted with the monobasic ammonium phosphate buffer solution (50 m*M*) with the pH adjusted to 2.50 with phosphoric acid. The column conditions were: pressure, 1300 psi; eluent flow rate, 0.33 ml/min; and temperature, 74°. Recordings were made at a range of 0.02 absorbance unit with a recorder span of 10 mv.

Drug concentrations were determined by measuring the net peak height above the baseline. Baselines were determined by a trough-to-trough tangent method. Drug concentrations were calculated by a computer-assisted, unweighted, least-squares fit of the standard concentrations and peak heights to a straight line. When serum samples were analyzed, a preadministration sample was also analyzed to determine if extraneous compounds, which might interfere, were already present in the serum. A preadministration urine sample, suitably diluted in proportion to the increased urine

[1] Hoffmann-La Roche, Inc., Nutley, NJ 07110
[2] Hoescht Pharmaceutical Co., Cincinnati, OH 45229
[3] Merck Sharp and Dohme Research Laboratories, West Point, Pa.
[4] Varian model LCS-1000, Varian Aerograph, Walnut Creek, CA 94598
[5] ISCO model UA-5, Instrument Specialties Co., Lincoln, NE 68505
[6] Type 6.
[7] Series 0080-014.
[8] Houston Omniscribe recorder model 51112, Houston Instrument, Bellaire, TX 77401
[9] LDC model 1209, Laboratory Data Control, Inc., Riviera, FL 33404
[10] Varian model A-25, Varian Aerograph, Walnut Creek, CA 94598
[11] Varian No. 07-000036-00, Varian Aerograph, Walnut Creek, CA 94598
[12] Varian No. 07-000038-00, Varian Aerograph, Walnut Creek, CA 94598
[13] No. 801 SN, Hamilton Co., Whittier, CA 90608

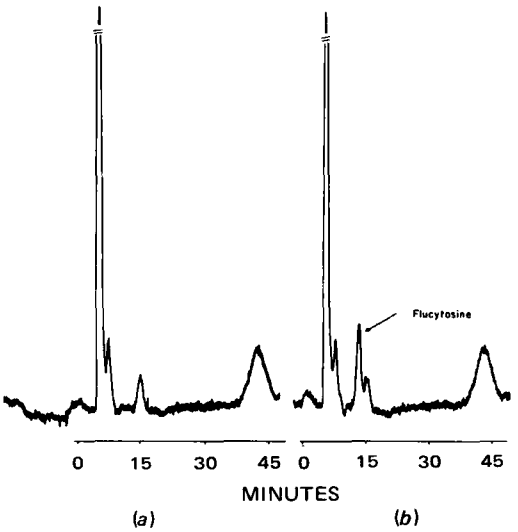

Figure 1—*HPLC elution pattern (280 nm) for serum without (a) and with (b) flucytosine (2.1 mg/liter), depicting the separation of flucytosine from other materials in serum which absorb at 280 nm.*

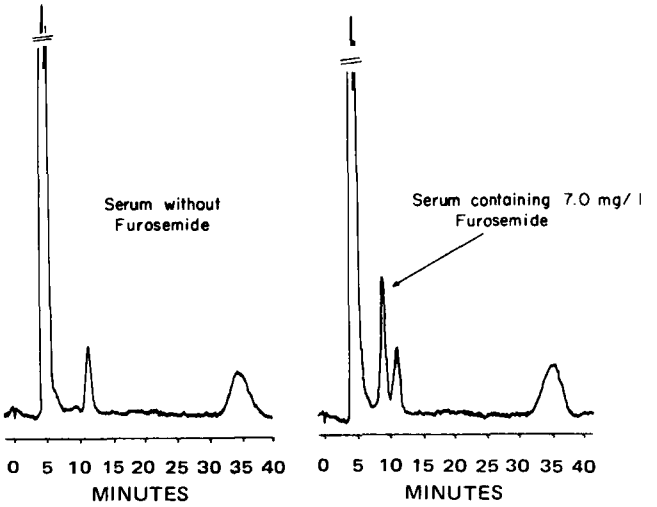

Figure 3—*HPLC elution patterns (280 nm) for serum without and with furosemide, depicting the separation of furosemide (elution time of 9 min) from other serum constituents that absorb at 280 nm.*

flow rate in the case of furosemide (9), was analyzed as a check for interfering substances.

Liquid scintillation counting and quench correction of samples containing ^{35}S-furosemide were carried out as reported earlier (9). Drug concentrations were calculated from the disintegrations per minute resulting from a dilution of the prepared injection solution of known specific activity, which was counted simultaneously.

Due to the high concentration of flucytosine in the urine samples following administration of the drug, both pre- and postadministration urines were diluted by a factor of either 20 or 100, depending on the actual flucytosine concentration, to obtain concentrations within the range of the standard curve.

RESULTS AND DISCUSSION

Following the described chromatographic methods, pure flucytosine or pure furosemide, when added to samples of serum or urine, was particularly well resolved from the other constituents in serum and urine (Figs. 1–4).

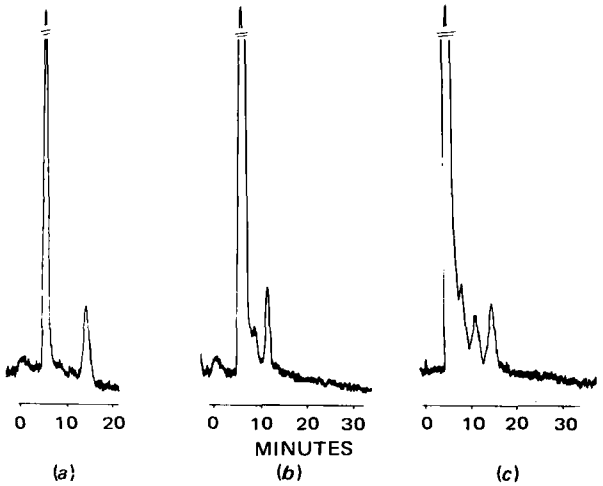

Figure 2—*HPLC elution pattern (280 nm) for patient urine with and without flucytosine. Different dilutions show effect on chromatogram peak height. Peak at 11 min in 1:20 dilution is a urine constituent that is almost diluted out in the 1:100 dilution. Flucytosine concentrations are those calculated after dilution. Key: (a), urine diluted 1:100 containing flucytosine (2 mg/liter); (b), urine diluted 1:20 without flucytosine; and (c), urine diluted 1:20 containing flucytosine (1.5 mg/liter).*

Flucytosine—With the reported conditions, flucytosine elutes from the column 13.5 min after sample injection (Fig. 1). Altering the buffer molarity to a higher concentration reduces the elution time but also tends to cause shifts of elution time, resulting in some overlap of the flucytosine peak with the small serum constituent peak, which normally elutes at approximately 15 min. Increasing the pH to 2.80 has little effect on the elution of flucytosine; however, with pH's higher than 2.00, resolution of flucytosine and the 15-min serum peak disappears.

The time involved to analyze a single serum sample (*i.e.*, for all peaks to elute) is 55–60 min; however, the flucytosine concentration may be calculated within 15 min after injection, making this technique relatively rapid for monitoring patient therapy. Since a large flat area in the elution pattern exists between the 15- and the

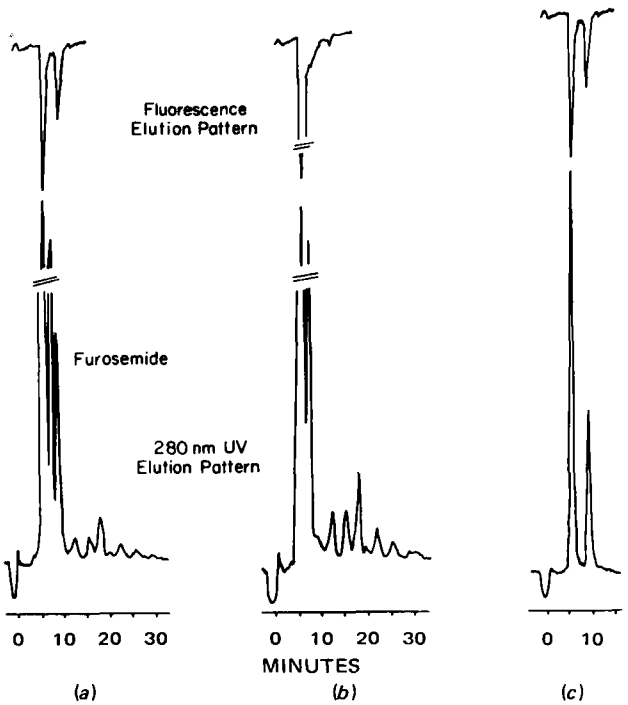

Figure 4—*HPLC elution patterns (280 nm) for urine with and without furosemide. Fluorescence pattern (top) shows clear separation from other fluorescent material in urine. Key: (a), urine diluted 1:20 containing furosemide (15 mg/liter); (b), urine diluted 1:10; and (c), urine from normal volunteer receiving furosemide.*

Table I—Comparison of HPLC Method with a Microbiological Method for Flucytosine in Serum (B) and Urine (U)

Sample	Flucytosine Concentration, mg/liter	
	HPLC Method	Microbiological Method
B_0	0	0
B_4[a]	0.8	0
B_5	6.7	6.8
B_6[a]	3.2	3.2
B_7	6.48	4.5
B_9	5.5	6.1, 5.6[b]
B_{11}	5.8	9.5
B_{12}	5.3	5.4
B_{15}	2.3	3.2
B_{17}[a]	1.5	1.8
B_{19}[a]	0.6	0
U_1	659	651
U_1[a]	108	164
U_2[a]	392	651
U_3	126	41

[a] Samples from a second study. [b] Duplicate sample to check reproducibility of the microbiological method.

55-min serum peaks, a second sample can be injected 25–30 min after the first injection, thereby allowing the analysis of at least two samples/hr. The urine samples from normal volunteers treated with flucytosine and diluted 1:20 showed no significant peaks (Fig. 2) due to urine constituents following the elution of flucytosine, thereby allowing a second injection 15 min after the first. Thus, four urine samples may be analyzed per hour.

The differences between peak heights for each set of standards prepared from pure flucytosine added to water, serum, and urine were not outside the relative standard deviation for repetitive injections of the same standard aqueous solution. Therefore, aqueous standards were used for calculation of serum and urine flucytosine concentrations in the clinical studies. The HPLC method was utilized for 3 months for the analysis of flucytosine in serum and urine samples. A standard curve for aqueous standards is shown in Fig. 5. The day-to-day relative standard deviation in the slope of the standard curve was 8%, and daily analysis of the same aqueous standard showed a relative standard deviation of 5.5% at 10 mg/liter. The within-day relative standard deviation for repetitive injections of the same aqueous standard solution was 2% at a concentration of 10 mg/liter.

The results from the HPLC method were also checked against a microbiological method performed by an independent laboratory[14] (Table I). The serum samples correlated reasonably well with a few exceptions, whereas the correlation between methods for urine samples was only fair at best.

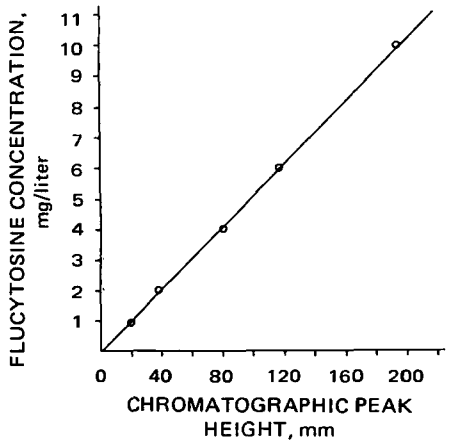

Figure 5—*Standard curve for aqueous flucytosine standards ranging from 1 to 10 mg/liter (slope = 0.052, SE = 0.061, and Y intercept = 0.042).*

[14] Bio-Science Laboratories, Van Nuys, CA 91405

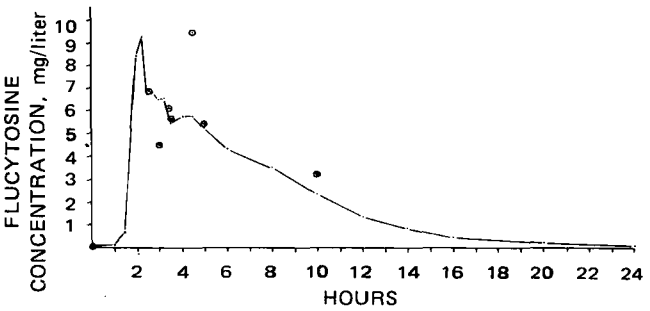

Figure 6—*Pharmacokinetic curve for a normal volunteer receiving 500 mg of flucytosine orally after a meal, comparing duplicate samples assayed by the HPLC method (•—•) and by a microbiological method (⊙).*

When the microbiological assay values are plotted on the same graph as those by HPLC from a complete patient study (Fig. 6), the plot depicts the greater precision of the HPLC method; this increased precision leads to more accurate values of the fitted parameters for the pharmacokinetic curve. When measured by the microbiological method, the quantity of recovered flucytosine for Sample U_2 (Table I) alone was 114% of the ingested dose compared to a total of 135% recovered overall; this example depicts the magnitude of the gross error in the microbiological method. In contrast, 80.5% of the ingested dose was recovered overall when the

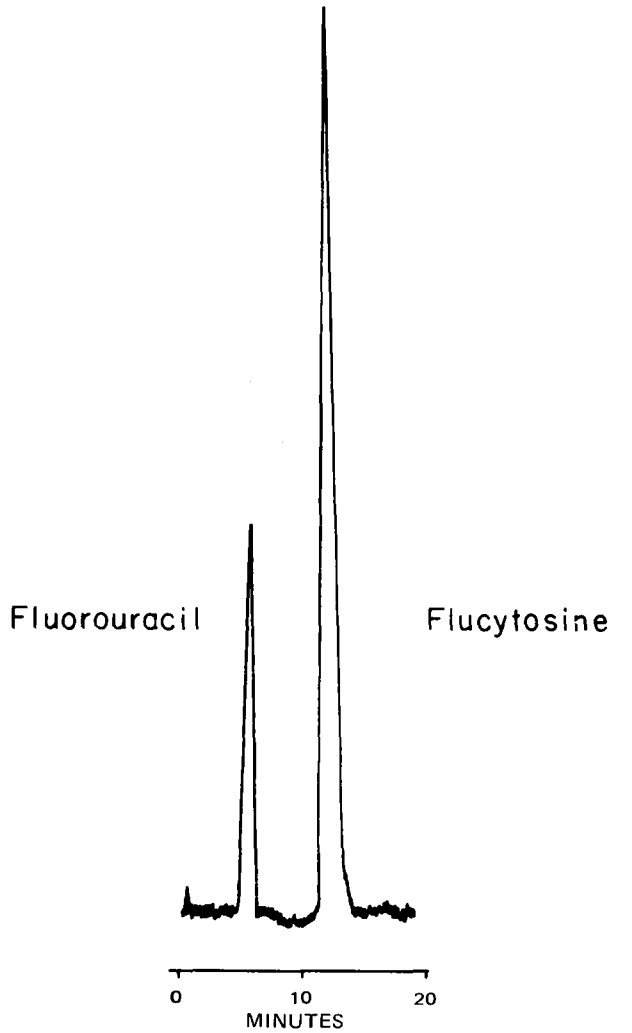

Figure 7—*HPLC elution pattern for an aqueous solution of flucytosine (10 mg/liter) and fluorouracil (10 mg/liter), depicting the clear separation of these two compounds.*

Table II—Comparison of Furosemide Concentrations (Milligrams per Liter) in Urine (U) and Plasma (P) as Determined by the HPLC and Radiochemical Techniques after an Oral Dose of 242 mg of Furosemide (Specific Activity 13.186 μCi/μmole)

Sample	Minutes	HPLC	Radiochemical
U_0	0	0	0
U_1	35	2.3	1.5
U_2	52	5.9	5.4
U_3	64	7.3	7.4
U_4	75	7.5	7.3
U_5	89	8.6	8.5
U_6	97	9.3	8.3
U_7	105	9.5	10.5
U_8	124	10.4	10.9
U_9	137	11.6	11.9
U_{10}	152	10.9	10.0
U_{11}	166	10.1	9.3
U_{12}	176	7.5	7.3
U_{13}	188	5.8	6.0
U_{14}	207	4.9	5.0
U_{15}	219	5.3	5.8
U_{16}	233	3.6	4.1
U_{17}	250	3.0	3.0
U_{18}	260	2.6	2.6
U_{19}	273	2.2	2.3
U_{20}	282	2.1	2.3
U_{21}	294	1.6	1.9
U_{22}	308	1.4	1.7
U_{23}	323	1.3	1.7
U_{24}	333	1.3	1.5
U_{25}	348	1.3	1.5
P_0	0	0	0
P_1	11	0	0
P_2	23	0	0
P_3	33	0	0.6
P_4	42	1.6	1.2
P_5	50	2.0	1.4
P_6	60	2.0	1.4
P_7	70	2.4	2.0
P_8	80	2.7	2.6
P_9	100	3.2	3.4
P_{10}	120	3.4	3.8
P_{11}	150	3.9	3.2
P_{12}	180	2.4	2.0
P_{13}	210	1.7	1.2
P_{14}	240	0.5	0.6

same urine samples were assayed by the HPLC method.

The HPLC method for the analysis of flucytosine has certain advantages over existing reported methods (13–15). This method requires no lengthy workup of serum or urine samples. Serum is applied directly to the column, and urine is simply diluted before application to the column. The sample size is small (5 μl), which facilitates analysis of samples derived from uremic patients, who normally have low hematocrits, and from infants. Pharmacokinetic and toxicity studies in small animals would also be facilitated by this method. The method is simple and fast.

Although flucytosine was reported not to be metabolized to fluorouracil in mammalian tissue (4, 16), it was of interest to show that fluorouracil does not interfere with the analysis of flucytosine, for instance, in concomitant therapy with these two drugs. In an aqueous solution under the reported conditions, fluorouracil elutes from the column 6 min postinjection, well resolved from the flucytosine peak (Fig. 7).

Furosemide—Using the chromatographic conditions described for furosemide, this drug was well resolved from other serum and urine constituents (Figs. 3 and 4). Furosemide was also completely resolved from its major breakdown product (I) (Fig. 8), both in biological samples and in aqueous solution. A separation of the two compounds in aqueous solution was shown in Fig. 8. Injectable furosemide is normally alkaline (pH 10.00) to prevent acid-catalyzed degradation to I. Urine, however, may be acidic, and furosemide will be quickly degraded unless alkalinized. Since the reaction is also light catalyzed, urine samples are also protected from light. As a further precaution, the samples are usually analyzed immediately. At the pH of analysis (pH 2.50), furosemide is highly fluorescent, whereas I is only weakly so (9, 10). This attribute allows

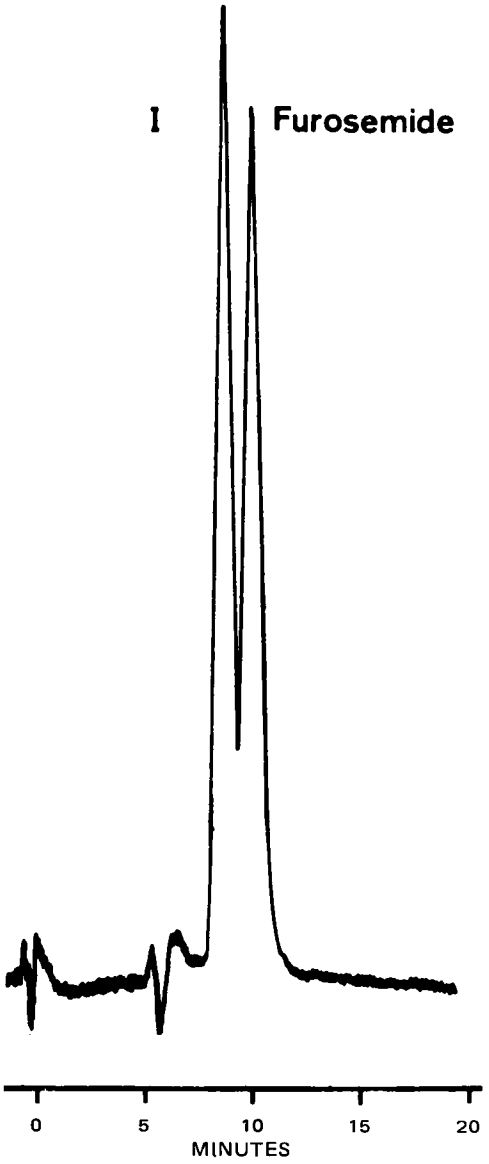

Figure 8—*HPLC elution pattern for an aqueous solution of furosemide and its major breakdown product, 4-chloro-5-sulfamoylanthranilic acid (I), showing that I does not interfere with the determination of the furosemide peak height.*

furosemide to be distinguished from other constituents in urine and plasma. Normally, only two fluorescent peaks are seen in elution patterns, one due to the breakthrough peak and the other due to furosemide. There is no peak in nonfurosemide-containing serum or urine, which exhibits both absorbance and fluorescence, and also elutes at the position corresponding to furosemide shown in Fig. 4.

The urine of patients who respond normally to furosemide shows only one urine peak at the solvent front due to the diluting effect of the drug. In the more concentrated urines from patients who do not respond to furosemide with a diuresis or from the normal concentrated urine from an untreated individual, there are a number of small unidentified peaks (Fig. 4). This figure shows, however, that the furosemide peak is, in all cases, uniquely identified and well resolved from any other fluorescent or absorbing material in the urine.

In these cases, the pH of the monobasic ammonium phosphate buffer is very critical. Changes of the monobasic ammonium phosphate buffer pH affect the retention time of furosemide only minimally over several pH units. However, changes on the order of 0.05 pH unit drastically alter the retention time of the other urine constituents. As a result, analyses during pharmacokinetic studies of

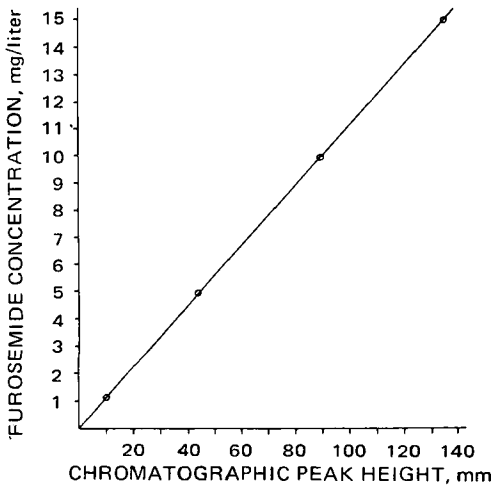

Figure 9—*Standard curve for aqueous furosemide standards ranging from 1 to 15 mg/liter (slope = 0.112, SE = 0.043, and Y intercept = 0.038).*

patients resistant to the diuretic action of furosemide require more careful monitoring of the buffer pH to maintain the separation of furosemide from other urine peaks. This phenomenon is also important in the analysis of urine samples in species such as mice, which do not exhibit a diuretic response to furosemide, even when extremely high doses of up to 400 mg/kg body weight are used (17).

Increasing the buffer molar concentration causes a shortening of the retention time of furosemide. The retention times of many compounds contained in the urine are also greatly affected by changes in buffer molarity, since some compounds undergo ion-exchange processes while others are being ion excluded. These other urine components have not been identified. Thus, while the effects on the elution profile of varying the buffer molarity are more subtle, they turn out to be less predictable than the effects of changing only the eluent pH.

The HPLC method was used for more than 9 months to measure furosemide levels in clinical studies. A standard curve for aqueous standards is shown in Fig. 9. The day-to-day relative standard deviation in the slope of the standard curve was 2.1%, and the day-to-day variation in the analysis of the same aqueous standard showed a relative standard deviation of 1.5%. The relative standard deviation of the concentration of repetitive injections of the same aqueous standard solution (within day variation) was 1.2% at a concentration of 15 mg/liter. Results from repetitive analysis of serum and urine samples containing 10 mg/liter of furosemide on the same day were 3 and 2%, respectively.

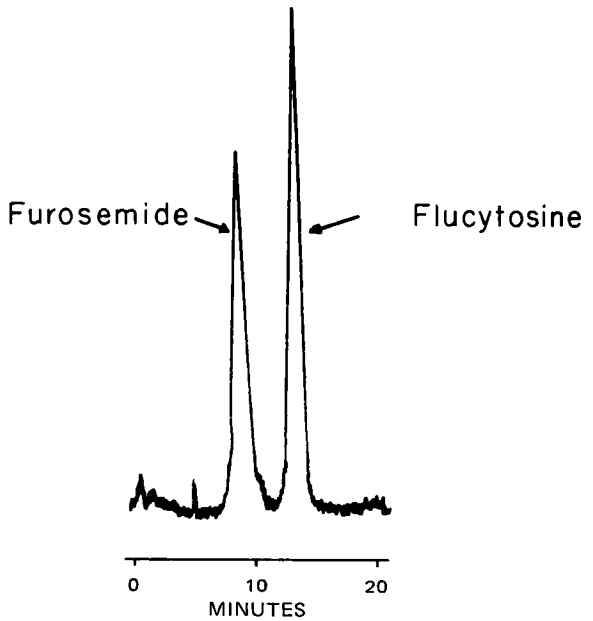

Figure 10—*HPLC elution pattern for an aqueous solution of flucytosine (5 mg/liter) and furosemide (5 mg/liter), using the conditions for separation of flucytosine from serum and urine.*

Table III—Comparison of Furosemide Concentrations (Milligrams per Liter) in Urine (U) and Plasma (P) as Determined by the HPLC and Radiochemical Techniques after an Intravenous Injection of 232 mg of Furosemide (Specific Activity 11.588 μCi/μmole)

Sample	Minutes	HPLC	Radiochemical
U_0	0	0	0
U_1	14.5	93.8	92.2
U_2	25.2	70.0	64.8
U_3	37.5	41.5	44.0
U_4	47.4	28.0	30.6
U_5	57.5	22.0	24.3
U_6	68.0	17.3	18.9
U_7	82.8	15.3	16.5
U_8	103.0	12.0	12.4
U_9	116.6	10.0	10.8
U_{10}	135.0	8.5	9.1
U_{11}	152.0	7.2	8.0
U_{12}	168.3	6.7	7.3
U_{13}	201.0	7.0	7.6
U_{14}	245.1	8.5	9.7
P_0	0	0	0
P_1	11.9	29.5	32.3
P_2	20.2	20.4	23.6
P_3	30.5	13.6	18.4
P_4	40.0	10.4	13.3
P_5	50.0	9.8	18.4
P_6	60.0	7.3	9.1
P_7	70.0	5.2	7.1
P_8	80.5	4.9	6.6
P_9	100.6	3.5	4.7
P_{10}	121.0	4.2	3.8
P_{11}	150.0	1.1	3.0
P_{12}	181.8	1.1	2.1

To estimate the accuracy of the HPLC method, it was compared to the measurement of ^{35}S-furosemide concentration determined radiochemically by means of the ^{35}S-label after administration to a normal subject by both oral and intravenous routes. In a normal subject, the drug virtually disappeared from the serum by 4 hr. The results (Tables II and III) demonstrate good agreement between the HPLC measurement and that made radiochemically, indicating that little metabolism of furosemide took place.

As with any chromatographic assay method, care must be taken to ensure that other drugs do not interfere with the determination of the drug to be analyzed. In initial studies using a combination of probenecid and furosemide, these two drugs were found to have the same elution time under the conditions described. However, by lowering the column temperature to 60°, furosemide was resolved from the probenecid peak. This lowering of the temperature results in a longer elution time and a 20% reduction in the furosemide peak height, which makes the assay somewhat less sensitive at low drug concentrations.

The size of the sample required for analysis (5 μl) makes this assay particularly attractive in clinical research studies for measuring serum concentrations of furosemide in infants and anemic patients and for pharmacokinetic and metabolism studies in small animals. In the analysis of rat serum and urine, no interferences during measurement of furosemide levels were found. By using the conditions for the separation of flucytosine from serum or urine, furosemide, when it is present, is well resolved from the flucytosine peak. Furosemide elutes at 9 min and flucytosine elutes at 13.5 min (Fig. 10). Thus, the analysis of flucytosine would not be interfered with by concomitant furosemide therapy.

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The authors thank Ms. B. Maxwell and Ms. M. O'Neill for technical assistance.

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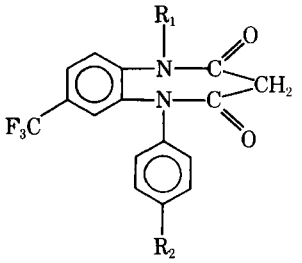
High-Pressure Liquid Chromatographic Analysis of Triflubazam and Its Metabolites in Human and Animal Blood and Urine

RICHARD E. HUETTEMANN [x] and ARVIN P. SHROFF

Abstract □ A high-pressure liquid chromatographic method is described for analyzing triflubazam [1-methyl-5-phenyl-7-trifluoromethyl-1*H*-1,5-benzodiazepine-2,4(3*H*,5*H*)-dione] and its primary metabolites in blood and urine. Adsorption chromatography, using pellicular silica gel as the stationary phase and dioxane–isooctane as the mobile phase, permitted rapid sample analysis. After extraction of blood and urine samples with toluene, quantitation is achieved using liquid chromatography with an internal standard. The method is sensitive above 50 ng/ml of triflubazam and its known metabolites. Recoveries for all compounds from blood or urine averaged above 95%. The specificity of the method was established by collecting samples separated by liquid chromatography and characterizing them by mass spectrometry. Human and animal data are presented to illustrate the utility of the method.

Keyphrases □ Triflubazam (a 1,5-benzodiazepine) and its metabolites—high-pressure liquid chromatographic analysis in human and animal blood and urine □ 1,5-Benzodiazepines—high-pressure liquid chromatographic analysis of triflubazam and its metabolites in human and animal blood and urine □ High-pressure liquid chromatography—analysis, triflubazam (a 1,5-benzodiazepine) and its metabolites in human and animal blood and urine

The quantitative analysis of drugs and their metabolites in physiological fluids is important for correlating pharmacological activity. Various methods requiring preliminary purification and/or lengthy analysis time to obtain the sensitivity and specificity needed to generate an accurate profile of the drug's metabolism have been described. Polarography (1), colorimetry (2), UV spectrophotometry (3), GLC (4), TLC (5), and liquid chromatography (6) have been employed to analyze the benzodiazepine class of compounds in blood and urine. A comprehensive review (7) of all methods for the analysis of benzodiazepines was published recently.



	R_1	R_2
I:	CH_3	H
II:	H	H
III:	CH_3	OH
IV:	H	OH

The methodology presented in this paper for the analysis of triflubazam [1-methyl-5-phenyl-7-trifluoromethyl-1*H*-1,5-benzodiazepine-2,4(3*H*,5*H*)-dione], Compound I[1], and its metabolites (II–IV) in blood and urine by high-pressure liquid chromatography (HPLC) exemplifies the advantages of this technique to this area of research.

EXPERIMENTAL

Reagents—All solvents were analytical reagent quality and were used without further purification.

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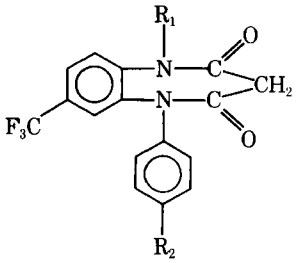

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EXPERIMENTAL

Reagents—All solvents were analytical reagent quality and were used without further purification.

[1] Synthesized by Boehringer-Ingelheim G.m.b.H., Ingelheim, Germany; referred to as ORF-8063.

Table I—Recoveries from Serum (Human)

Sample	I Added, μg	I Found, μg	Recovery, %	II Added, μg	II Found, μg	Recovery, %
1	1.00	0.96	96.0	1.00	1.03	103.0
2	1.00	0.98	98.0	1.00	1.01	101.0
3	1.00	0.99	99.0	1.00	0.99	99.0
4	1.00	1.01	101.0	1.00	0.99	99.0
5	1.00	0.98	98.0	1.00	1.04	104.0
		$\bar{X}$ = 98.0			$\bar{X}$ = 101.2	
1	0.10	0.096	96.0	0.10	0.103	103.0
2	0.10	0.101	101.0	0.10	0.095	95.0
3	0.10	0.101	101.0	0.10	0.094	94.0
4	0.10	0.100	100.0	0.10	0.102	102.0
5	0.10	0.095	95.0	0.10	0.102	102.0
		$\bar{X}$ = 99.0			$\bar{X}$ = 99.2	

Sample	III Added, μg	III Found, μg	Recovery, %
1	1.00	1.01	101.0
2	1.00	0.95	95.0
		$\bar{X}$ = 98.0	

Sample	IV Added, μg	IV Found, μg	Recovery, %
1	1.00	1.06	106.0
2	1.00	1.05	105.0
		$\bar{X}$ = 105.5	

Apparatus—A liquid chromatograph[2] fitted with a standard 254-nm detector[3] was used. The column was a tube (1.0 m × 2.1 mm i.d.) with a stationary phase of pellicular silica gel[4]. The column was operated at 35 kg/cm^2 (500 psi) at 30°, with a mobile phase of 15% dioxane in isooctane at a flow rate of 3 ml/min.

Procedure—Stock solutions (10 μg/ml) of I–IV and 11α-hydroxy-17α-methyltestosterone were prepared in chloroform. A stock standard solution (A) was prepared to contain 1 μg of each of I–IV and 10 μg of internal standard/5 μl of chloroform. Exactly 1.0 ml of the internal standard solution was pipetted into a 10-ml glass-stoppered extraction tube and evaporated to dryness in a 65° water bath under a stream of nitrogen. Exactly 1.0 ml of blood or 5.0 ml of urine was added to the tube. Five milliliters of toluene was added, and the tube was shaken for 5 min.

The tubes were then centrifuged for 2 min and cooled in a dry ice–acetone bath for 20 sec, and the toluene was decanted into a conical centrifuge tube. One milliliter of toluene was added to the extraction tube; the contents were mixed for 30 sec, centrifuged, and cooled in the dry ice–acetone bath; and the toluene was decanted into the respective conical tube. The toluene was evaporated to dryness in a 65° water bath under a stream of nitrogen.

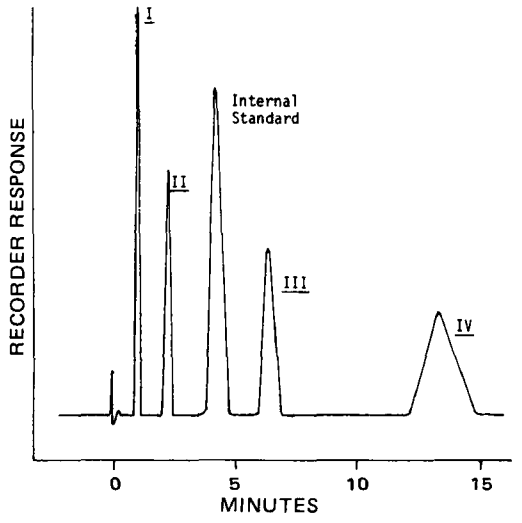

Figure 1—*Chromatogram depicting retention time of internal standard and I–IV.*

The residue was treated with acetone (200-, 50-, and 10-μl portions) and quantitatively transferred to a microflex tube[5]. Five microliters of chloroform was added to the evaporated residue in the microflex tube, mixed[6] for 15 sec, and saved.

Five microliters of Standard Solution A was injected into the liquid chromatograph, and the compounds eluted as shown in Fig. 1. The sample microflex tube contents were injected next. Quantitation was achieved by dividing the ratio of any compound detected in the sample by the ratio of the same compound in the stan-

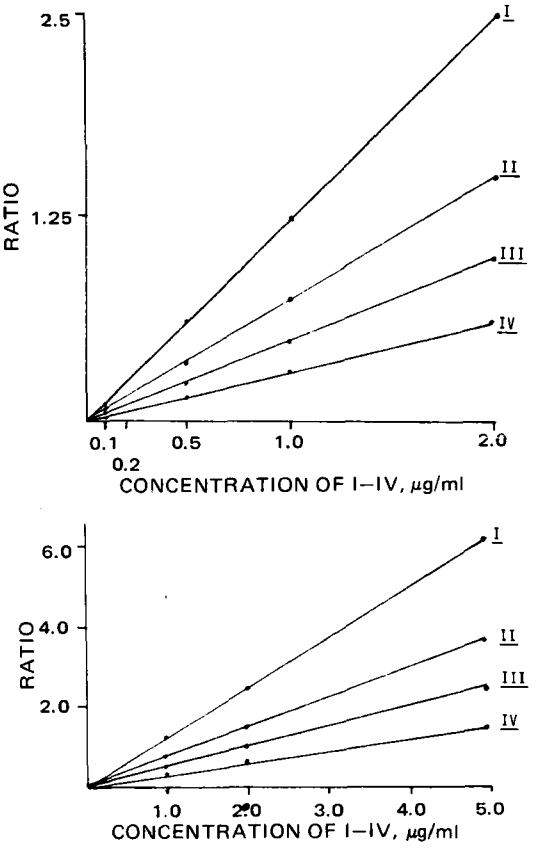

Figure 2—*Plot of concentration* versus *peak height ratio.*

[2] Dupont model 830.
[3] Dupont.
[4] Pelosil HC, Reeve Angel.
[5] Kontes Glass Co.
[6] Vortex Genie.

Table II—Recoveries from Urine (Human)

Sample	I Added, μg	I Found, μg	Recovery, %
1	1.00	1.03	103.0
2	1.00	1.06	106.0
3	1.00	1.05	105.0
4	1.00	1.01	101.0
5	1.00	0.99	99.0
		$\overline{X}$ = 102.8	
Sample	II Added, μg	II Found, μg	Recovery, %
1	1.00	1.09	109.0
2	1.00	0.95	95.0
3	1.00	0.99	99.0
4	1.00	0.99	99.0
5	1.00	1.02	102.0
		$\overline{X}$ = 100.8	
Sample	III Added, μg	III Found, μg	Recovery, %
1	1.00	1.02	102.0
2	1.00	1.05	105.0
		$\overline{X}$ = 103.5	

dard (the ratio is the height of the compound peak divided by the height of the internal standard peak) to give micrograms per milliliter of any compound in blood or micrograms per 5 ml in urine. Typical standard curves demonstrating the detector's response to I–IV over the concentration ranges of 0.1–2.0 and 1.0–5.0 μg/ml are shown in Fig. 2.

Liquid Chromatography–Mass Spectrometry—Compound identification was achieved by evaporating the appropriate aliquot from liquid chromatography and obtaining the mass spectrum[7] of the residue at 70 ev.

RESULTS AND DISCUSSION

The described method using HPLC allows for simultaneous cleanup and analysis and, if necessary, for more rapid identification of sample peaks than is obtained with other methods.

The method used to analyze for I and its metabolites, II–IV, was developed after several stationary phases and solvent systems were investigated to give an ideal separation in a short period. Adsorption chromatography employing a stationary phase[4] and a mobile phase of dioxane–isooctane allowed samples to be analyzed within 15 min. Quantitation was accomplished using an internal standard technique to minimize errors caused by the loss of sample during extraction and transfer steps.

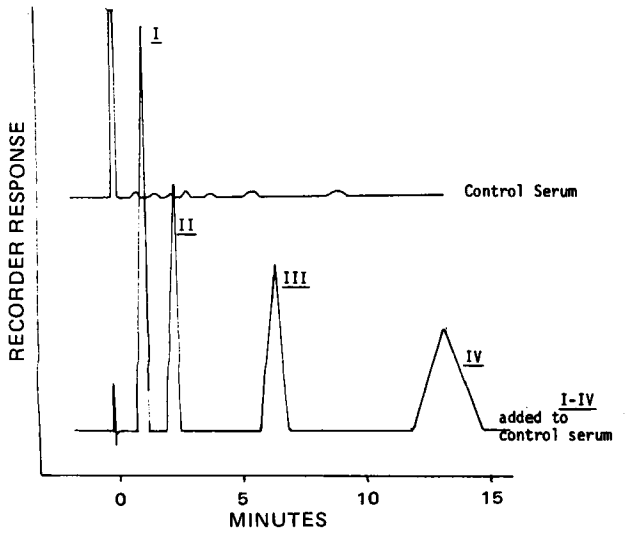

Figure 3—*Liquid chromatogram of I–IV at 1 μg (8×) and a human control serum sample (2×).*

[7] Finnigan model 1015 GC-MS.

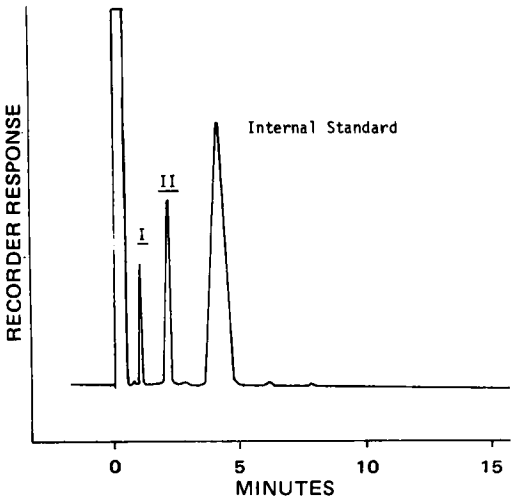

Figure 4—*Chromatogram of a typical human serum sample.*

11α-Hydroxy-17α-methyltestosterone was an ideal internal standard, as demonstrated in Fig. 1. The response of known standard concentrations of I–IV was found to obey Beer's law (Fig. 2). Since studies showed that these slopes varied from day to day due to the quality of the solvent and/or temperature, the daily use of a standard solution is recommended.

The accuracy and precision of the method were determined by the addition of known quantities of I–IV to control human serum and urine samples as well as to rat, rabbit, and dog control serum samples. The samples were then analyzed according to the outlined procedure. Table I summarizes the recoveries from human serum at various levels, Table II summarizes the human urine studies, and Table III summarizes the animal serum studies. All recovery studies were well within acceptable levels. A typical liquid chromatogram obtained by analyzing both spiked and controlled human serums is shown in Fig. 3. No interferences were noted from control serum or urine samples.

To demonstrate the suitability of this methodology for biological materials, blood and urine samples were obtained from patients participating in a clinical trial of I. Table IV depicts the results obtained by both HPLC and TLC densitometry (5) methods for blood samples, while Table V shows the results with urine samples. Both blood and urine samples were obtained 24 hr after dosing. Figure 4 illustrates the chromatogram obtained from typical human serum in the same study.

To obtain a better understanding of the absorption characteristics of this drug, a monkey study was initiated. Blood samples were withdrawn over a 12-hr period, and urine samples were collected

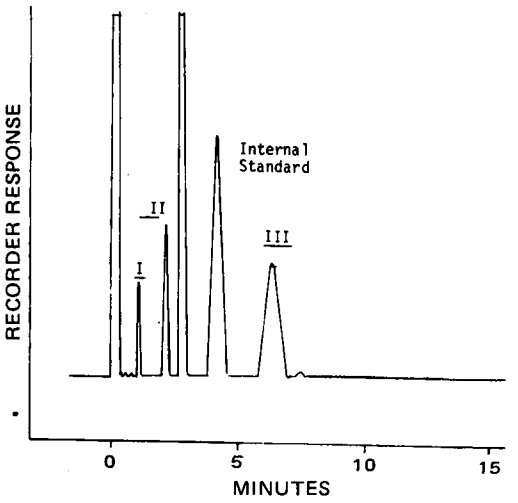

Figure 5—*Chromatogram of a typical monkey urine sample.*

Table III—Recoveries from Serum of Different Species

Species	I			II		
	Added, μg	Found, μg	Recovery, %	Added, μg	Found, μg	Recovery, %
Rat						
1	1.00	1.00	100.0	1.00	0.92	92.0
2	1.00	1.01	101.0	1.00	0.95	95.0
		$\bar{X}$ = 100.5			$\bar{X}$ = 93.5	
Rabbit						
1	1.00	1.05	105.0	1.00	0.98	98.0
2	1.00	1.00	100.0	1.00	1.01	101.0
		$\bar{X}$ = 102.5			$\bar{X}$ = 99.5	
Dog						
1	1.00	1.01	101.0	1.00	1.00	100.0
2	1.00	1.06	106.0	1.00	1.08	108.0
		$\bar{X}$ = 103.5			$\bar{X}$ = 104.0	

Table IV—Serum Levels of I and II in Six Patients by TLC and HPLC

Patient	I, μg/ml		II, μg/ml	
	TLC	HPLC	TLC	HPLC
1	0.94	0.92	0.94	1.00
2	0.84	0.85	0.89	0.90
3	0.57	0.59	1.02	1.05
4	N.D.[a]	N.D.	N.D.	N.D.
5	1.40	1.51	0.86	0.80
6	1.79	1.80	1.73	1.81

[a] N.D. = not detected.

Table V—Urine Levels of I and II in Three Patients by TLC and HPLC

Patient	I, μg/ml		II, μg/ml	
	TLC	HPLC	TLC	HPLC
1	0.15	0.12	0.19	0.19
2	0.20	0.22	0.30	0.35
3	0.68	0.60	1.51	1.63

every 24 hr. Table VI lists the serum results, while Table VII lists the urine results. Figure 5 illustrates the chromatogram of a typical monkey urine extract showing peaks corresponding to I–III. From this study, it appears that I is slowly absorbed and rapidly converted to II in the blood, while urine data suggest slow elimination from the body.

Fractions from the HPLC corresponding to I–IV and any other unknown peaks from any study can be quickly collected, evaporated, and submitted for mass spectrometry to ensure specificity and allow for rapid elucidation of unknown peaks if they appear. The actual cleanup occurs while quantitative analyses are being performed. The HPLC column exhibited good column life even after several weeks of continuous operation.

Table VI—Serum Results of the Monkey Study

Hours	Monkey I		Monkey II	
	I, μg/ml	II, μg/ml	I, μg/ml	II, μg/ml
Predose	N.D.[a]	N.D.	N.D.	N.D.
0.15	N.D.	N.D.	N.D.	N.D.
0.30	N.D.	N.D.	N.D.	N.D.
0.45	N.D.	N.D.	N.D.	N.D.
1	N.D.	N.D.	N.D.	N.D.
2	N.D.	N.D.	N.D.	N.D.
3	N.D.	N.D.	N.D.	N.D.
4	0.29	0.16	0.60	0.55
5	0.24	0.16	0.56	0.59
6	0.23	0.20	0.57	0.62
12	0.91	2.11	0.35	0.49

[a] N.D. = not detected.

Table VII—Urine Results[a] of the Monkey Study

Monkey	Day	I, μg/2 ml Urine	II, μg/2 ml Urine	III, μg/2 ml Urine
1	1	5.84	19.56	N.D.[b]
1	3	3.88	6.22	1.17
1	4	N.D.	7.83	N.D.
1	5	N.D.	0.72	N.D.
2	1	1.90	5.85	5.17
2	2	0.70	2.83	4.07
2	3	2.00	1.63	—
2	4	2.30	1.06	1.10
2	5	1.61	1.28	1.03

[a] Urine was not hydrolyzed prior to extraction. [b] N.D. = not detected.

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Synthesis and Cytotoxicity of 6-Selenopurine Arabinoside and Related Compounds

SHIH-HSI CHU ˣ, CHYNG-YANN SHIUE, and MING-YU CHU

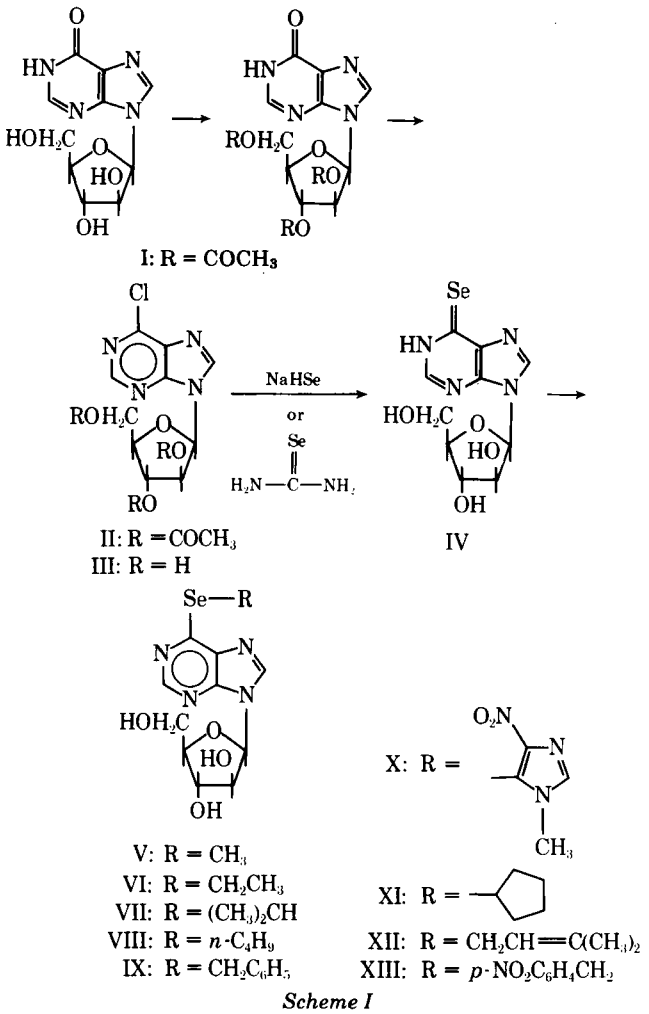
Abstract □ 6-Chloro-9-(β-D-arabinofuranosyl)purine served as an intermediate for the chemical synthesis of a series of 6-substituted selenopurine arabinosides. In an *in vitro* test using murine leukemic cells (L-5178Y) these 6-substituted selenopurine arabinosides showed some cytotoxicity. Lengthening the side chain had no effect on their cytotoxicity. Selenourea was a useful reagent for synthesizing selenopurines, selenopurine nucleosides, and selenopurine arabinoside under mild conditions.

Keyphrases □ 6-Selenopurine arabinoside and related compounds—synthesis and cytotoxicity □ Selenium analogs—synthesis and cytotoxicity of 6-selenopurine arabinoside and related compounds □ Cytotoxicity—6-selenopurine arabinoside and related compounds

Mercaptopurine and thioguanine have been employed widely in the clinical treatment of various types of leukemia (1–5). The selenium isostere, 6-selenopurine (6), was found (7) to inhibit mouse leukemia L-1210 as effectively as mercaptopurine. Unfortunately, it is not a useful compound due to its instability *in vivo*. Subsequently, selenoguanine, an analog of thioguanine, was synthesized (8, 9). It is as effective an inhibitor as thioguanine on the growth of several experimental tumors, is less toxic to the host, and has a superior therapeutic index (9).

Numerous selenium analogs were synthesized to reexplore their biochemical behavior including 6-selenopurine riboside (10), 6-seleno-*N*-alkyl purines (11), ethyl 6-selenoguanine-9-carboxylate (12), 6-selenoguanosine (13, 14), (α,β)-2′-deoxy-6-selenoguanosine (15–17), alkyl-selenopurine ribosides (14, 18, 19), 6-selenoguanosine 5′-monophosphate (20), and 8-substituted selenoadenosines and their cyclic nucleotides (21). 6-Selenoguanosine and β-2′-deoxy-6-selenoguanosine were active inhibitors of several experimental tumors *in vitro* (14, 15) and *in vivo* (17, 20).

Cytarabine (1β-D-arabinofuranosylcytosine) is a potent inhibitor of the growth of tumor cells in culture and also causes striking regression of tumors in mice (22). 6-Mercapto-9-(β-D-arabinofuranosyl)purine was found to have both antitumor (23) and immunosuppressive (24) properties. 6-Mercapto-9-(β-D-arabinofuranosyl)purine also was a competitive inhibitor of L-1210 adenosine deaminase when 9-β-D-arabinofuranosyladenine was used as the substrate. The phosphorylation of adenosine, 9-β-D-arabinofuranosyladenine, and 6-methylthio-9-(β-D-ribofuranosyl)purine did not appear to be affected by 6-mercapto-9-(β-D-arabinofuranosyl)purine (25). These results prompted the synthesis of 6-selenopurine arabinoside and certain related compounds and the evaluation of their cytotoxicity.



Scheme I

RESULTS AND DISCUSSION

Chemical—Acetylation of 9-(β-D-arabinofuranosyl)hypoxanthine with acetic anhydride in pyridine gave 9-(2′,3′,5′-tri-*O*-acetyl-β-D-arabinofuranosyl)hypoxanthine (I) (26). Chlorination of I with phosphorus oxychloride–*N*,*N*-diethylaniline at refluxing temperature gave 6-chloro-9-(2′,3′,5′-tri-*O*-acetyl-β-D-arabinofuranosyl)purine (II) (27). Deacetylation of II with methanolic ammonia at room temperature gave 6-chloro-9-(β-D-arabinofuranosyl)purine (III). Compound III was used for further transformation without purification. Treatment of III with sodium hydrogen selenide in refluxing methanol gave 6-selenoxo-9-(β-D-arabinofuranosyl)purine (IV) in 52% yield.

Compound IV can be synthesized alternatively by the reaction of III with selenourea. This method was found to be a convenient way to synthesize various seleno compounds. Compound IV was relatively unstable; its half-life was 3 days in water at room temperature.

Kikugawa *et al.* (28) reported that lengthening the side chain of 2-thioadenosine increases its activity as an inhibitor of platelet aggregation; therefore, several derivatives of IV were synthesized. Treatment of IV with methyl iodide, ethyl bromide, isopropyl bromide, *n*-butyl bromide, benzyl bromide, 5-chloro-1-methyl-4-nitroimidazole, cyclopentyl bromide, 3,3′-dimethylallyl bromide, and

Table I—Physical Properties of 6-Selenopurine Arabinoside and Related Compounds

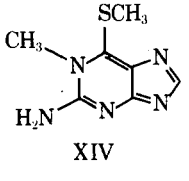
Compound	λ_{max}, nm ($\epsilon \times 10^{-3}$) pH 1	pH 11	H_2O	R_f[a]
IV	231 (8.9)	240 (11.2)	234 (10.1)	0.52
	350 (15.7)	328 (13.7)	347 (15.6)	
V	231 (12.0)	230 (10.5)	228 (8.8)	0.71
	315 (14.9)	302 (13.8)	302 (13.1)	
VI	312 (11.5)	229 (8.1)	229 (8.2)	0.71
		303 (11.4)	304 (11.7)	
VII	310 (10.8)	229 (10.6)	228 (10.5)	0.62
		304 (13.7)	304 (15.1)	
VIII	313 (12.6)	229 (10.9)	229 (8.7)	0.45
		305 (15.1)	305 (15.0)	
IX	307[b]	233 (16.2)	—	0.21
		305 (14.4)	305 (14.3)	
X	289 (17.2)	288 (15.4)	288 (15.3)	0.73
XI	237[b]	231 (8.9)	231 (8.1)	0.42
	320	306 (11.7)	306 (11.4)	
XII	314 (12.6)	221 (14.7)	306 (11.7)	0.32
		306 (13.4)		
XIII	299 (22.8)	301 (21.0)	300 (21.4)	0.11
XIV	228 (9.5)	233 (16.1)[c]	230 (17.6)	0.3
	253 (5.2)	284 (3.9)	283 (3.8)	
	336 (8.1)	340 (6.0)	340 (8.0)	
XX	266 (7.6)	238 (17.0)	228 (14.6)	0.32
	370 (17.8)	354 (17.2)	360 (17.6)	
XXI	273 (3.1)	230 (19.3)	—	0.84
	379 (15.6)	306 (11.4)	349 (13.7)	

[a] TLC was run on a polygram CEL 300 PEI and developed with water. [b] The solubilities of these compounds are low. [c] The half-life from the height of the 340-nm peak in pH 11 at room temperature was about 1 hr.

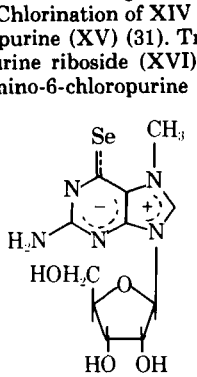
p-nitrobenzyl bromide gave the corresponding purine arabinoside derivatives of 6-(methylseleno) (V), 6-(ethylseleno) (VI), 6-(isopropylseleno) (VII), 6-(*n*-butylseleno) (VIII), 6-(benzylseleno) (IX), 6-[(1-methyl-4-nitro-1*H*-imidazol-5-yl)seleno] (X), 6-(cyclopentylseleno) (XI), 6-[(3-methyl-2-butenyl)seleno] (XII), and 6-(*p*-nitrobenzylseleno) (XIII), respectively (Scheme I).

Alkylations with primary halides were performed in aqueous basic solution, but alkylations with secondary halides or relatively active halides were carried out in anhydrous media. Alkylation of IV with secondary halides in aqueous basic solution gave low yields of products. The low yields may arise from the hydrolysis of these secondary halides into the corresponding alcohols. The 6-selenopurine arabinosides were purified by continuous extraction with ether or by recrystallization from water. The physical properties of the 6-selenopurine arabinosides are shown in Table I. The products were identified by elemental analysis and by a comparison of their UV spectra with known thio analogs (26).

Selenourea has been reported to react with 6-chloropurine, ethyl 2-amino-6-chloropurine-9-carboxylate, and 7-chloro-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine to give 6-selenopurine, ethyl 2-amino-6-selenopurine-9-carboxylate, and 7-selenoxo-3-(β-D-ribofuranosyl)pyrazolo[4,3-*d*]pyrimidine (6, 12, 29), respectively. To test the limitation of this procedure, several substituted purines were synthesized. Methylation of 1-methyl-6-thioguanine (30) with methyl iodide in sodium hydroxide solution gave 2-amino-1-methyl-6-(methylthio)purine (XIV). Chlorination of XIV in methanol gave 2-amino-6-chloro-1-methylpurine (XV) (31). Treatment of XV, 2-amino-6-chloro-7-methylpurine riboside (XVI) (32), 6-chloropurine riboside (XVII), 2-amino-6-chloropurine riboside (XVIII), and 2-amino-6-chloropurine (XIX) with selenourea in refluxing methanol or at room temperature gave the corresponding seleno compounds (XX–XXIV) in good yield. The advantages of this procedure are that handling of hydrogen selenide gas is avoided and reaction conditions are milder than in the conventional sodium hydrogen selenide method.



XIV



XXI

Biological Effects—The analogs were evaluated in two studies for their cytotoxicity, using L-5178Y murine leukemic cells grown in culture.

Long-Term Studies—These studies were conducted for 72 hr over a wide range of concentration (10^{-4}–10^{-7} *M*). As shown in Table II, 6-thioguanosine, IV, and X exhibit 50% inhibition at concentrations of 5.6×10^{-6}, 6.6×10^{-5}, and 1.9×10^{-5} *M*, respectively. Compounds V–IX, XI, and XII caused less than 50% inhibition at the highest concentration (10^{-4} *M*) studied, so percent inhibition at 10^{-5} or 10^{-4} *M* was determined as shown in columns 3 and 4 of Table II.

Short-Term Studies—As shown in Table III, L-5178Y cells in the exponential phase of growth were treated with the inhibitory compounds (10^{-4}–10^{-7} *M*) for 2 hr and cell viability was determined by colony formation in dilute agar (33). The cloning efficiency of the untreated cells was approximately 75%. All values were normalized to 100% acute cell kill after 2 hr of incubation.

Compound X, which was shown to be active by long-term and short-term studies, was also evaluated for its ability to cause acute cell death by exposure of cells for 1–5 hr at two concentrations (10^{-4} and 10^{-5} *M*); cell viability was determined by colony formation in dilute agar (33). The results (Table IV) demonstrated that the logarithmic cell kill by X was 29%/hr at 1×10^{-4} *M* and 15%/hr at 1×10^{-5} *M*.

EXPERIMENTAL[1]

9 - (2′,3′,5′ - Tri-*O*- acetyl-β-D- arabinofuranosyl)hypoxanthine (I)—This compound was prepared by the method of Reist *et al.* (26) with 9-(β-D-arabinofuranosyl)hypoxanthine (1.5 g, 0.57 mmole) and acetic anhydride (2.1 ml, 2.25 mmoles) in 90 ml of pyridine in 60% yield.

6-Chloro-9-(2′,3′,5′-tri-*O*-acetyl-β-D-arabinofuranosyl)purine (II)—Into a mixture of 35 ml of phosphorus oxychloride and 2 ml of *N,N*-diethylaniline, 6.0 g (15.8 mmoles) of I was added. The suspension was refluxed rapidly for 3 min. The resulting clear brown solution was evaporated *in vacuo*, and the residue was added to chloroform and ice water. The solution was extracted with chloroform (2 × 50 ml). The organic layer was extracted with 1 *N* HCl (2 × 60 ml) and then water until the aqueous layer was neutral to pH paper. The organic layer was dried and evaporated to dryness to give 3.8 g of crude II.

6-Chloro-9-(β-D-arabinofuranosyl)purine (III)—Into 70 ml of absolute methanol saturated with ammonia, 3.8 g (9.6 mmoles) of II was added. The solution was stirred at room temperature for 6 hr and then evaporated to dryness. The residue was dried *in vacuo* at 65° for 1 day to give 2.4 g (92.7%) of III.

6-Selenoxo-9-(β-D-arabinofuranosyl)purine (IV)—*Method I*—Condensed hydrogen selenide[2] (1.0 ml, 24 mmoles) was bubbled through a solution of 0.2 g (8.7 mmoles) of sodium in 50 ml of absolute methanol. Compound III (2.2 g, 7.7 mmoles) in 10 ml of absolute methanol was added, and the suspension was refluxed for 40 min. The resulting yellow precipitate was filtered by suction, dissolved in 20 ml of 3% Na_2CO_3, and filtered. The filtrate was acidified with acetic acid to pH 4 and cooled. The yellow solid was collected, washed with a small amount of cold water, and dried *in vacuo* to give 1.4 g (52%) of IV, mp 155–157° dec.

Anal.—Calc. for $C_{10}H_{12}N_4O_4Se \cdot H_2O$: C, 34.64; H, 4.04; N, 16.04. Found: C, 34.80; H, 4.07; N, 16.01.

Method II—The suspension of 100 mg (0.36 mmole) of III and 45 mg (0.36 mmole) of selenourea (34) in 5 ml of methanol was refluxed for 20 min. The yellow solution was filtered, cooled in an ice

[1] Melting points were determined on a Gallenkamp melting-point apparatus and are uncorrected. UV spectra were determined on a Perkin-Elmer model 402 spectrophotometer. Elemental analyses were performed either by Midwest Microlab, Indianapolis, Ind., or Robertson Lab, Florham Park, N.J. TLC was run on a polygram CEL 300 PEI and developed with water. Evaporations were accomplished using a Buchler flash evaporator under reduced pressure with a bath temperature of 40°.

[2] Hydrogen selenide, 98.0% minimum purity, from Matheson Co., East Rutherford, NJ 07073

bath, and then poured into 50 ml of ether. The precipitate was held in the refrigerator overnight, filtered, and then dried *in vacuo* to give 45 mg (38%) of yellow solid. The nucleoside isolated was established by UV and TLC to be identical with IV prepared by Method I.

6-(Methylseleno)-9-(β-D-arabinofuranosyl)purine (V)—To a solution of 288 mg (0.82 mmole) of IV in 2.2 ml (0.87 mmole) of 0.4 *N* NaOH, 0.11 ml of methyl iodide was added. The suspension was stirred at room temperature for 2 hr. The solid was filtered by suction, recrystallized from water, and dried *in vacuo* to give 200 mg (84%) of V, mp 171–173°.

Anal.—Calc. for $C_{11}H_{14}N_4O_4Se \cdot H_2O$: C, 36.39; H, 4.45; N, 15.42. Found: C, 36.60; H, 4.66; N, 15.45.

6-(Ethylseleno)-9-(β-D-arabinofuranosyl)purine (VI)—By following the procedure described for V, VI was synthesized from IV and ethyl bromide in 36% yield, mp 160–162°.

Anal.—Calc. for $C_{12}H_{16}N_4O_4Se$: C, 40.12; H, 4.49; N, 15.59. Found: C, 40.56; H, 4.66; N, 15.83.

6-(Isopropylseleno)-9-(β-D-arabinofuranosyl)purine (VII)—To a solution of 145 mg (0.41 mmole) of IV in 5 ml of dry dimethylformamide, 150 mg (1.42 mmoles) of anhydrous sodium carbonate and 65 mg (0.5 mmole) of isopropyl bromide were added. The suspension was stirred at room temperature for 4 hr and partitioned with ethyl acetate–water. The organic layer was dried over anhydrous magnesium sulfate, condensed to a small volume, and partitioned with chloroform–water. The organic layer was dried and evaporated to a small volume. This solution was added dropwise to 200 ml of vigorously stirred hexane. The resulting precipitate was filtered and dried to give 20 mg of VII. The aqueous layer was held at room temperature overnight, and the crystals were collected and dried *in vacuo* to give 40 mg of VII, mp 160–161.5°. The total yield of VII was 60 mg (35%).

Anal.—Calc. for $C_{13}H_{18}N_4O_4Se \cdot H_2O$: C, 39.91; H, 5.15; N, 14.31. Found: C, 39.60; H, 4.88; N, 13.99.

If the reaction was run in aqueous solution at room temperature overnight, the compound isolated was mostly starting material as indicated by UV and TLC.

6-(*n*-Butylseleno)-9-(β-D-arabinofuranosyl)purine (VIII)—To a solution of 288 mg (0.82 mmole) of IV in 2.2 ml of 0.4 *N* NaOH, 120 mg (0.85 mmole) of *n*-butyl bromide was added. The solution was stirred at room temperature overnight and then continuously extracted with ether to give 110 mg (34%) of VIII. The analytical sample was recrystallized from water, mp 142–144°.

Anal.—Calc. for $C_{14}H_{20}N_4O_4Se$: C, 43.42; H, 5.31; N, 14.46. Found: C, 43.02; H, 5.24; N, 14.37.

6-(Benzylseleno)-9-(β-D-arabinofuranosyl)purine (IX)—Compound IX was synthesized in 70% yield from IV and benzyl bromide by the method described for V. The analytical sample was recrystallized from methanol–water, mp 182–184°.

Anal.—Calc. for $C_{17}H_{17}N_4O_4Se \cdot H_2O$: C, 46.48; H, 4.59; N, 12.75. Found: C, 46.48; H, 4.49; N, 12.75.

6-[(1-Methyl-4-nitro-1*H*-imidazol-5-yl)seleno]-9-(β-D-arabinofuranosyl)purine (X)—To a solution of 290 mg (0.82 mmole) of IV in 2.2 ml of 0.4 *N* NaOH, 130 mg (0.81 mmole) of 5-chloro-1-methyl-4-nitroimidazole in 10 ml of methanol was added. The solution was stirred at room temperature for 7 hr and evaporated to dryness, and then 3 ml of water was added. The solid was collected, washed with water, and dried to give 250 mg (64%) of X. The analytical sample was recrystallized twice from water, mp 179–180.5°.

Anal.—Calc. for $C_{14}H_{15}N_7O_6Se \cdot H_2O$: C, 35.46; H, 3.61; N, 20.67. Found: C, 35.89; H, 3.32; N, 20.73.

6-(Cyclopentylseleno)-9-(β-D-arabinofuranosyl)purine (XI)—Compound XI was synthesized in 20% yield from IV and cyclopentyl bromide by the method described for V except that the reaction mixture was stirred at room temperature for 6 days. The analytical sample was recrystallized from methanol–water, mp 96–99°.

Anal.—Calc. for $C_{15}H_{20}N_4O_4Se \cdot H_2O$: C, 43.17; H, 5.31; N, 13.42. Found: C, 43.31; H, 5.27; N, 13.47.

6-[(3-Methyl-2-butenyl)seleno]-9-(β-D-arabinofuranosyl)-purine (XII)—Compound XII was synthesized in 25% yield from IV and 3,3-dimethylallyl bromide by the method described for VII. The analytical sample was recrystallized from methanol–water, mp 113–116° dec.

Anal.—Calc. for $C_{15}H_{20}N_4O_4Se$: C, 45.12; H, 5.05; N, 14.02. Found: C, 44.82; H, 5.20; N, 13.79.

Table II—Inhibition of Reproduction of L-5178Y by 6-Selenopurine Arabinoside and Related Compounds[a]

Compound	Concentration Causing 50% Inhibition	Concentration Causing <50% Inhibition: Concentration	Concentration Causing <50% Inhibition: % Inhibition
6-Thioguanosine	5.6×10^{-6} *M*		
IV	6.6×10^{-5} *M*		
X	1.9×10^{-5} *M*		
V	—[b]	10^{-5} *M*	17
VI	—[b]	10^{-4} *M*	10
VII	—[b]	10^{-5} *M*	10
VIII	—[b]	10^{-4} *M*	24
IX	—[b]	10^{-5} *M*	12
XI	—[b]	10^{-4} *M*	31
XII	—[b]	10^{-4} *M*	45

[a] The leukemic cells L-5178Y were grown from an inoculum of 4×10^3 cells/ml for 72 hr in the presence of different levels (10^{-4}–10^{-7} *M*) of 6-selenopurine arabinoside and its derivatives. The final number of cells were determined in a Coulter particle counter, model B, and the 50% inhibition of each drug was determined as the amount of drug required to cause a 50% decrease in the number of doublings undergone by the cell population when compared with the untreated controls. [b] Compounds V–IX, XI, and XII caused less than 50% inhibition at the highest concentration (10^{-4} *M*) studied.

6-*p*-Nitrobenzylseleno-9-(β-D-arabinofuranosyl)purine (XIII)—To a solution of 144 mg (0.41 mmole) of IV in 1.1 ml of 0.4 *N* NaOH, 94 mg (0.44 mmole) of *p*-nitrobenzyl bromide in 5 ml of methanol was added. The solution was stirred at room temperature for 4 hr. The mixture was evaporated to dryness, and the residue was washed with 50 ml of ether and then with 7 ml of water. The solid was recrystallized from methanol–water and dried *in vacuo* to give 115 mg (60%) of XIII, mp 118–120°.

Anal.—Calc. for $C_{17}H_{17}N_5O_6Se$: C, 43.79; H, 3.68; N, 15.01. Found: C, 43.42; H, 3.72; N, 15.26.

2-Amino-1-methyl-6-(methylthio)purine (XIV)—To a solution of 365 mg (2 mmoles) of 1-methyl-6-thioguanine in 5 ml of 0.4 *N* NaOH, 1.4 ml of methyl iodide was added. The mixture was stirred at room temperature for 30 min. The white precipitate was filtered by suction, washed with water, and dried *in vacuo* to give 290 mg (66%) of XIV, mp 242–245°.

Anal.—Calc. for $C_7H_9N_5S \cdot 1.4H_2O$: C, 38.14; H, 5.40; N, 31.77. Found: C, 37.79; H, 5.24; N, 32.19.

When XIV was heated at 140° *in vacuo*, it dehydrated to give $C_7H_9N_5S$.

Anal.—Calc. for $C_7H_9N_5S$: C, 43.07; H, 4.65; N, 35.86. Found: C, 43.34; H, 4.65; N, 35.73.

1-Methyl-6-selenoguanine (XX)—*Method I*—A mixture of 60 mg (0.27 mmole) of XIV, 300 mg (3.0 mmoles) of potassium bicarbonate, and 0.1 ml (2.6 mmoles) of hydrogen selenide in 3 ml of water was kept at 105° overnight. The solution was cooled to room temperature. The solid was filtered, washed with water, dissolved

Table III—Inhibition of L-5178Y Cells by 6-Selenopurine Arabinoside and Related Compounds[a]

Compound	Control	Survival, % at 1×10^{-7} *M*	at 1×10^{-6} *M*	at 1×10^{-5} *M*	at 1×10^{-4} *M*
IV	100	87 ± 4.08	85 ± 9.98	69 ± 2.53	68 ± 2.08
V	100	87 ± 3.74	83 ± 1.89	79 ± 5.72	73 ± 5.74
VI	100	89 ± 3.56	82 ± 1.89	75 ± 2.06	70 ± 2.63
VII	100	92 ± 7.81	82 ± 6.35	78 ± 4.04	70 ± 2.0
IX	100	92 ± 1.26	82 ± 6.85	75 ± 0.96	63 ± 1.41
X	100	87 ± 8.89	86 ± 3.61	78 ± 3.79	67 ± 1.15

[a] The L-5178Y cells (2×10^5/ml) in the exponential phase of growth were incubated with 6-selenopurine arabinoside and related compounds singly (10^{-4}–10^{-7} *M*) for 2 hr. Cell viability was determined by the dilute agar colony method. Each observation represents the mean value of three experiments with four replicates per experiment. A minimum of 200 colonies was counted for each group. The cloning efficiency of the untreated cells was approximately 75%. All values were normalized to 100%.

Table IV—Inhibition of L-5178Y Cells by X at Different Time Intervals[a]

Concentration, *M*	Survival, %				
	1 hr	2 hr	3 hr	4 hr	5 hr
0	100	100	100	100	100
1×10^{-5}	98 ± 3.9	80.2 ± 2.39	71 ± 6.69	61 ± 3.67	49 ± 1
1×10^{-4}	81 ± 5.36	60.4 ± 2.7	45 ± 5.24	31.2 ± 5.22	18.5 ± 0.71

[a]The L-5178Y cells (2×10^5/ml) in the exponential phase of growth were incubated with X at different time intervals. Cell viability was determined by the dilute agar colony method. Each observation represents the mean value of three experiments with four replicates per experiment. A minimum of 200 colonies was counted for each group. The cloning efficiency of the untreated cells was approximately 75%. All values were normalized to 100%.

in 5 ml of 3% Na_2CO_3 solution, and then acidified with acetic acid to pH 4. The precipitate was collected, washed with cold water, and dried *in vacuo* to give 42 mg (66%) of XX, mp 275–278° dec.

Anal.—Calc. for $C_6H_7N_5Se \cdot 1.1H_2O$: C, 29.07; H, 3.74; N, 28.24. Found: C, 28.81; H, 3.85; N, 28.44.

Method II—A suspension of 93 mg (0.46 mmole) of XV (31) and 61 mg (0.5 mmole) of selenourea in 5 ml of methanol was stirred at room temperature for 1 hr. The yellow precipitate was filtered by suction, washed with 5 ml of methanol, and dried *in vacuo* to give 101 mg (89%) of yellow solid, which was identified as XX by TLC and UV.

2-Amino-7-methyl-6-selenoxo-9-(β-D-ribofuranosyl)purine (7-Methyl-6-selenoguanosine) (XXI)—*Method I*—A solution of 1 g (3.02 mmoles) of 2-amino-6-chloropurine riboside and 1 g (70.4 mmoles) of methyl iodide in 6 ml of dimethylformamide was stirred at room temperature for 40 hr. Then 0.8 g (6.5 mmoles) of selenourea was added, and the solution was stirred at room temperature for an additional 2 hr. At the end of the reaction, the solution was neutralized with methanolic ammonia and diluted with 120 ml of acetone. The precipitate was collected, recrystallized from methanol, and dried *in vacuo* to give 50 mg (6%) of XXI, mp 175° dec.

Anal.—Calc. for $C_{11}H_{15}N_5O_4Se$: C, 36.68; H, 4.20; N, 19.43. Found: C, 36.39; H, 4.14; N, 19.40.

Method II—A solution of 500 mg of XVI in 2.5 ml of dimethylformamide was added to a solution of 50 mg of sodium and 0.5 ml of hydrogen selenide in 5 ml of methanol. The solution was stirred at room temperature for 1 hr and then neutralized with methanolic ammonia. The yellow precipitate was collected and recrystallized from methanol to give 50 mg (10%) of yellow solid, which was verified by UV and TLC to be identical with XXI prepared by Method I.

6-Selenoxo-9-(β-D-ribofuranosyl)purine (XXII)—A suspension of 100 mg (0.36 mmole) of XVII and 45 mg (0.36 mmole) of selenourea in 5 ml of methanol was refluxed for 20 min. The yellow solution was filtered, cooled in an ice bath, and then poured into 50 ml of ether. The precipitate was held in a refrigerator overnight, filtered, and dried *in vacuo* to give 60 mg (50%) of yellow solid, which was identified as XXII (10) by UV and TLC.

2-Amino-6-selenoxo-9-(β-D-ribofuranosyl)purine (6-Selenoguanosine) (XXIII)—A suspension of 200 mg (0.61 mmole) of XVIII and 90 mg (0.72 mmole) of selenourea in 10 ml of methanol was refluxed for 20 min. The solution was filtered, cooled in an ice bath, and poured into 100 ml of ether. The precipitate was filtered and dried *in vacuo* to give 160 mg (76%) of yellow compound, which was identified as XXIII (13, 14) by UV and TLC.

2-Amino-6-selenopurine (6-Selenoguanine) (XXIV)—A suspension of 170 mg (1 mmole) of XIX and 123 mg (1 mmole) of selenourea in 5 ml of methanol was refluxed for 30 min. The yellow precipitate was cooled, filtered, and dried *in vacuo* to give 210 mg (98%) of a yellow solid, which was identified as XXIV (9) by UV and TLC.

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Maternal–Fetal Transfer of ^{14}C-Di-2-ethylhexyl Phthalate and ^{14}C-Diethyl Phthalate in Rats

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Abstract □ ^{14}C-Di-2-ethylhexyl and ^{14}C-diethyl phthalates were administered intraperitoneally to pregnant rats on either Day 5 or 10 of gestation. Rats were sacrificed at 24-hr intervals starting on Days 8 and 11, respectively; maternal blood, fetal tissue, amniotic fluid, and placentas (whenever possible) were obtained. The ^{14}C-activity of each sample was determined by scintillation counting. It was found that both diesters and/or their metabolic products were present in each of these compartments throughout the gestation period, thus suggesting that the embryo–fetal toxicity and teratogenesis reported previously could be the results of a direct effect of the compound (or its metabolites) upon developing embryonic tissue. Additionally, the reduction in concentration of ^{14}C from these tissues as a function of time was found to fit a first-order excretion curve. From this model curve, the half-life for both compounds was calculated; the average was about 2.33 days for di-2-ethylhexyl phthalate and 2.22 days for diethyl phthalate.

Keyphrases □ Phthalate diesters, radiolabeled—distribution and persistence in maternal blood, fetal tissue, amniotic fluid, and placenta, intraperitoneal administration, rats □ Maternal–fetal transfer—phthalate diesters (radiolabeled), distribution and persistence in maternal blood, fetal tissue, amniotic fluid, and placenta, intraperitoneal administration, rats □ Toxicity—phthalate diesters (radiolabeled), maternal–fetal transfer, rats

Phthalic acid esters are the major plasticizers used by the plastics industry, and di-2-ethylhexyl phthalate (I) is the most widely used plasticizer. Compound I is contained in many plastic products (primarily polyvinyl chloride) used in medical, dental, and paramedical applications as well as in household and vehicular furnishings. Some of these plasticizers also are used as solvents in commercial formulations such as perfumes and insect repellents.

The acute and chronic toxicities of these esters have been found to be rather low by conventional routes of administration (oral, intraperitoneal, intravenous, and inhalation) (1–6); their industrial uses have presented few confirmed toxicological problems, although some adverse effects have been suggested (7–9). Jaeger and Rubin (10) reported finding I in the tissues of two patients who had received transfusions of blood stored in plastic bags. *In vitro* experiments, which they conducted using the perfused rat liver, indicated that this organ would accumulate I but would not hydrolyze it. A related plasticizer, butyl glycolyl-butyl phthalate, was hydrolyzed by this system.

Teratogenic effects of the phthalates were reported in chick embryos when the phthalates were injected into the yolk sac of the developing embryo (11–13). Recently, demonstration of mammalian teratogenicity was reported from intraperitoneal injections of phthalates into pregnant rats (14).

When considering potential teratogenic effects, physicochemical properties of the compound often play a highly significant role. It is generally believed that a compound with a molecular weight of less than 1000 (unless highly bound to plasma proteins or other large molecules) can pass through the placental barrier to reach the developing fetus, where it may exert a direct effect (15). Passage through the placenta, however, is not the sole criterion for teratogenicity, since many drugs are known to reach the fetus without having any adverse effect upon its development. Moreover, a compound that does not pass this barrier may cause metabolic disturbances in the mother which secondarily interfere with development of the fetus (15).

The purposes of this study were to investigate the maternal–fetal transfer in rats of two phthalic acid esters and to determine whether they and/or their metabolites pass through the placental barrier to the developing fetus where they may elicit potential direct embryo–fetal toxicity and/or teratogenic effects.

EXPERIMENTAL

Materials—Carboxy-labeled ^{14}C-di-2-ethylhexyl phthalate (^{14}C-I) and ^{14}C-diethyl phthalate (^{14}C-II)[1] were mixed with unlabeled I and II[2], respectively, to provide the desired level of radioactivity. Test animals were adult virgin female rats of the Sprague–Dawley strain[3], weighing 175–225 g. Male rats of the same strain and age were used as the "stud pool."

Methods—Female rats were selected for experimentation only after observation of at least two complete 4- or 5-day estrus cycles. Females were bred and vaginal smears were obtained each morning. The date of appearance of sperm in the vaginal smear was recorded as Day 0 of pregnancy, as previously described (14).

A group of 13 pregnant rats was injected with 5 ml/kg ip of ^{14}C-I (4.0123×10^7 dpm/ml or 7.16×10^{-3} mCi/mmole) on Day 5 of gestation. A second group of 10 pregnant rats was injected intraperitoneally with the same dosage of ^{14}C-I on Day 10 of gestation. A similar group of pregnant rats was injected with 1.0116 ml/kg ip of ^{14}C-II (1.121×10^8 dpm/ml or 1.00×10^{-2} mCi/mmole) on Day 5 of gestation, and another group was injected on Day 10.

From groups injected on Day 5 of gestation, one rat was sacrificed with an overdose of ether by inhalation 72 hr after the ^{14}C-I or ^{14}C-II injection and then every 24 hr through Day 20 of gestation to obtain specimens for analysis. From the groups injected on Day 10, one rat was sacrificed every 24 hr through Day 20 of gestation.

About 4–5 ml of maternal blood was collected by cardiac puncture using a heparinized syringe, and amniotic fluid was aspirated from the gravid uterus. The whole fetuses and, whenever possible, the placentas were removed by uterine incision and isolated from the maternal tissue to avoid cross-contamination during subsequent dissection. Whole fetuses (and placentas when collected) were blotted gently with a surgical sponge and transferred to chilled, tared bottles for weighing. The tissues (whole fetuses and placentas) were minced with scissors and diluted three- to fivefold with distilled water. The diluted tissues were then homogenized[4]

[1] Mallinckrodt Nuclear.
[2] Matheson, Coleman and Bell, Norwood, Ohio.
[3] Sprague-Dawley, Inc., Madison, Wis.
[4] Virtis model 23 homogenizer.

Table I—Distribution of Radioactivity in Rats from ^{14}C-Di-2-ethylhexyl Phthalate Injected on Day 5 of Gestation[a]

Gestation Day	Maternal Blood			Placenta			Amniotic Fluid			Fetal Tissue		
	Counts, dpm/g	Total Counts in Tissue[b]	Percent of Injected Dose[c]	Counts, dpm/g	Total Counts in Tissue	Percent of Injected Dose[c]	Counts, dpm/g	Total Counts in Tissue	Percent of Injected Dose[c]	Counts, dpm/g	Total Counts in Tissue	Percent of Injected Dose[c]
8	6654	95,098	0.240	—	—	—	2577	2577	0.007	2003	470	0.001
9	4338	70,285	0.178	—	—	—	2046	1177	0.003	1392	556	0.001
10	2870	46,297	0.118	—	—	—	1047	200	—[d]	1226	641	0.002
11	1367	22,439	0.057	—	—	—	1321	539	0.001	1058	890	0.002
12	1260	20,326	0.052	1140	1180	0.004	510	375	—[d]	736	176	—[d]
13	1403	27,993	0.072	786	1386	0.004	410	683	0.002	485	579	0.001
14	1593	26,937	0.068	937	1672	0.004	155	225	—[d]	284	437	0.001
15	659	11,656	0.030	484	1215	0.003	167	254	—[d]	149	453	0.001
16	610	10,401	0.026	590	2732	0.007	125	346	—[d]	213	1453	0.003
17	505	8,003	0.021	314	1723	0.004	199	352	—[d]	187	2223	0.005
18	349	6,000	0.015	329	2075	0.005	73	239	—[d]	136	1802	0.004
19	377	6,402	0.017	207	1327	0.003	87	182	—[d]	158	1938	0.005
20	237	4,192	0.011	165	1146	0.003	73	232	—[d]	99	1641	0.003

[a] Mean counts of one to five samples for each tissue. [b] Total blood in the rat was assumed to be 7% of its body weight. [c] Percent of injected dose was based upon quantity of radioactivity administered and total radioactivity estimated for each organ. [d] Less than 0.001% of injected dose.

Table II—Distribution of Radioactivity in Rats from ^{14}C-Di-2-ethylhexyl Phthalate Injected on Day 10 of Gestation[a]

Gestation Day	Maternal Blood			Placenta			Amniotic Fluid			Fetal Tissue		
	Counts, dpm/g	Total Counts in Tissue[b]	Percent of Injected Dose[c]	Counts, dpm/g	Total Counts in Tissue	Percent of Injected Dose[c]	Counts, dpm/g	Total Counts in Tissue	Percent of Injected Dose[c]	Counts, dpm/g	Total Counts in Tissue	Percent of Injected Dose[c]
11	6002	114,656	0.290	—	—	—	6186	6186	0.016	9416	13,136	0.033
12	6433	118,338	0.300	—	—	—	1601	983	0.002	3301	5,461	0.013
13	3960	67,243	0.170	2679	4447	0.011	507	616	0.002	1454	1,263	0.003
14	1585	30,278	0.076	1259	3023	0.008	378	660	0.002	703	1,224	0.003
15	1858	31,813	0.080	1449	5224	0.013	472	1022	0.003	683	2,550	0.006
16	1481	26,510	0.066	1980	7551	0.019	396	965	0.002	581	2,971	0.008
17	1326	19,139	0.049	851	3940	0.010	231	562	0.001	280	2,519	0.006
18	1221	24,793	0.063	471	3821	0.010	209	733	0.002	299	4,705	0.013
19	755	15,491	0.039	387	2914	0.008	241	458	0.001	132	1,526	0.003
20	304	6,022	0.016	483	3085	0.008	241	275	—[d]	66	726	0.002

[a] Mean counts of one to five samples for each tissue. [b] Total blood in the rat was assumed to be 7% of its body weight. [c] Percent of injected dose was based upon quantity of radioactivity administered and total radioactivity estimated for each organ. [d] Less than 0.001% of injected dose.

at medium speed while the flask was immersed in dry ice.

Aliquots of blood, amniotic fluid, placentas, or fetal tissues (usually 100–150 mg) were solubilized[5]. The tissues (only) were warmed for about 2 hr at 50° in a water bath to minimize chemiluminescence. The samples were bleached when necessary with benzoyl peroxide[6] (up to 3 ml) and adjusted to pH 6.4 with acetic acid (67%). The samples treated with benzoyl peroxide exhibited significant chemiluminescence, which was minimized by storing the prepared samples in the dark for 24 hr. Ten milliliters of preblended liquid scintillation cocktail[7] was added to the samples of blood, amniotic fluid, or homogenized placentas or fetal tissues. Finally, these prepared samples were counted in a liquid scintillation spectrometer[8].

After the samples were counted, a standard aliquot of ^{14}C-toluene[9] (5000 dpm) was added to each, and these samples were recounted to obtain an estimate of the counting efficiency. The absolute radioactivity of the samples was calculated based upon the specific activity of the internal standard, ^{14}C-toluene.

Counts, counting efficiency, and original weight of sample were used to calculate the radioactivity of each specimen in terms of disintegrations per minute per gram or disintegrations per minute per organ. These values were used in conjunction with the specific activity of the injected ^{14}C-I and ^{14}C-II to estimate their molar concentrations (assuming all radioactivity was still present as the diesters) in the maternal blood, embryonic fluid, placentas, and whole fetuses. For this purpose, the total blood in the rat was assumed to be 7% of its body weight.

[5] With 2 ml of TS-1 solubilizer (0.6 *N* solution in toluene), Research Products International Corp., Elk Grove Village, Ill.
[6] Research Products International Corp., Elk Grove Village, Ill.
[7] 3a40, Research Products International Corp., Elk Grove Village, Ill.
[8] Model LS-100C, Beckman Instruments, Fullerton, Calif.
[9] Beckman Instruments, Fullerton, Calif.

RESULTS

A total of 46 pregnant rats and their fetuses were studied following ^{14}C-I and ^{14}C-II administration on Days 5 or 10 of gestation. Radioactivity was detected in maternal blood, placentas, amniotic fluid, and developing fetuses at all gestational stages investigated. No grossly obvious toxic effects were seen in the pregnant females during the gestation period after injection of either radioactive phthalate. Tables I and II present a summary of the mean counts per gram of tissue, estimated counts per organ, and percent of injected dose taken up by various tissues after ^{14}C-I was injected on Day 5 or 10 of gestation. Similar data following ^{14}C-II injection are shown in Tables III and IV.

Both radioactive phthalates and/or their metabolites were widely distributed and were found in maternal blood, placentas, amniotic fluid, and fetal tissues. Less than 1% of the injected dose was present in these tissues at any of the measured times. Generally, the level of radioactivity in the placenta, amniotic fluid, and whole fetus seemed to be related to the concentration in the maternal blood, but none of these tissues exhibited a consistently higher level than that found in the maternal blood for either compound.

Radioactivity in the maternal blood increased, reaching a peak during the first 48 hr following injection of ^{14}C-I and during the first 24 hr after radioactive II injection. The concentration of radioactivity then diminished quickly for both compounds. A similar pattern was observed in the amniotic fluid and fetal tissues. Some radioactivity was detected in all tissues examined throughout the experimental period (10–15 days postinjection).

Table III—Distribution of Radioactivity in Rats from ^{14}C-Diethyl Phthalate Injected on Day 5 of Gestation[a]

Gestation Day	Maternal Blood			Placenta			Amniotic Fluid			Fetal Tissue		
	Counts, dpm/g	Total Counts in Tissue[b]	Percent of Injected Dose[c]	Counts, dpm/g	Total Counts in Tissue	Percent of Injected Dose[c]	Counts, dpm/g	Total Counts in Tissue	Percent of Injected Dose[c]	Counts, dpm/g	Total Counts in Tissue	Percent of Injected Dose[c]
8	1584	29,811	0.100	—	—	—	421	11	—[d]	1403	333	0.001
9	1372	27,180	0.085	—	—	—	416	21	—[d]	1108	562	0.002
10	1064	19,422	0.066	—	—	—	621	61	—[d]	724	372	0.001
11	644	9,204	0.040	668	447	0.002	285	131	—[d]	1375	145	—[d]
12	747	14,376	0.046	465	390	0.001	263	198	—[d]	694	124	—[d]
13	361	6,258	0.028	301	412	0.002	63	128	—[d]	170	107	—[d]
14	385	7,491	0.023	274	572	0.002	77	159	—[d]	121	210	—[d]
15	275	4,806	0.017	157	613	0.002	82	231	—[d]	70	228	0.001
16	224	3,804	0.014	184	1245	0.005	46	167	—[d]	68	507	0.002
17	234	3,444	0.015	164	1313	0.006	74	176	—[d]	68	672	0.003
18	207	3,661	0.012	146	1347	0.005	52	166	—[d]	65	947	0.003
19	146	2,479	0.008	108	831	0.003	36	122	—[d]	56	916	0.003
20	185	3,848	0.012	87	965	0.003	47	116	—[d]	69	1185	0.003

[a] Mean counts of one to five samples for each tissue. [b] Total blood in the rat was assumed to be 7% of its body weight. [c] Percent of injected dose was based upon quantity of radioactivity administered and total radioactivity estimated for each organ. [d] Less than 0.001% of injected dose.

Table IV—Distribution of Radioactivity in Rats from ^{14}C-Diethyl Phthalate Injected on Day 10 of Gestation[a]

Gestation Day	Maternal Blood			Placenta			Amniotic Fluid			Fetal Tissue		
	Counts, dpm/g	Total Counts in Tissue[b]	Percent of Injected Dose[c]	Counts, dpm/g	Total Counts in Tissue	Percent of Injected Dose[c]	Counts, dpm/g	Total Counts in Tissue	Percent of Injected Dose[c]	Counts, dpm/g	Total Counts in Tissue	Percent of Injected Dose[c]
11	2625	50,145	0.164	1575	871	0.003	1077	471	0.002	3685	317	0.001
12	1892	41,497	0.118	1516	1434	0.004	904	824	0.002	905	216	—[d]
13	1815	33,645	0.113	1033	1549	0.005	448	428	0.001	509	332	0.001
14	1532	27,423	0.095	682	1424	0.005	225	390	0.001	249	333	0.001
15	1343	27,081	0.085	234	824	0.002	283	898	0.003	202	887	0.003
16	1032	18,546	0.065	216	799	0.003	253	526	0.002	150	664	0.002
17	543	11,257	0.033	151	800	0.003	169	583	0.002	162	1808	0.006
18	302	6,068	0.018	112	1775	0.005	98	364	0.001	112	1820	0.005
19	193	3,892	0.012	200	1473	0.004	84	257	—[d]	117	1695	0.006
20	134	2,674	0.008	125	856	0.003	51	101	—[d]	70	1176	0.003

[a] Mean counts of one to five samples for each tissue. [b] Total blood in the rat was assumed to be 7% of its body weight. [c] Percent of injected dose was based upon quantity of radioactivity administered and total radioactivity estimated for each organ. [d] Less than 0.001% of injected dose.

A first-order rate equation was fitted to that portion of the experimental data where radioactive counts were declining with respect to time. Least-square slopes were obtained from the logarithm of radioactivity *versus* time in days for maternal blood, amniotic fluid, and fetal tissue compartments. For three of the four cases (administration of ^{14}C-I at Days 5 and 10 and of ^{14}C-II at Day 10), the 95% confidence intervals of the least-square slopes were overlapping for all of these compartments. In the other case (^{14}C-II administration on Day 5), the confidence intervals of the least-square slopes failed to overlap, but the gap was present only in the third decimal place. From this treatment of the experimental data, it is not possible to determine that the slopes (or rate constants) are significantly different from one another.

DISCUSSION

Previous studies (14) revealed embryo–fetal toxicity and teratogenicity in rats from intraperitoneal injections of these phthalate esters. This study clearly demonstrated the presence of phthalates and/or their metabolites in the developing fetus from early embryogenesis to birth, including the critical period of teratogenic sensitivity. The embryo–fetal toxicity and fetal abnormalities produced by these phthalates, together with the demonstration of their presence in the early embryo, suggest that phthalate esters and/or their metabolites may act directly on embryonic tissues in the induction of teratogenicity.

Using ^{14}C-radioactivity data, a half-life was calculated for each compound. The half-life of II was 2.22 days; for I, it was 2.33 days. These values are in reasonably good agreement with those reported by Daniel and Bratt (18), who gave a half-life of 1–2 days for I in rat liver and of 3–5 days for fat.

These studies showed that radioactivity from ^{14}C-II and ^{14}C-I is transmitted across the placenta from mother to fetus in pregnant rats and that the radioactivity is detectable for at least 15 days postinjection. Although the exact chemical nature of the radioactive compound(s) transmitted to the fetus was not determined, some of them probably were mixtures of parent compounds, monoesters, and phthalic acid.

Studies on absorption, distribution, metabolism, and excretion of carboxy-labeled ^{14}C-I in rats were reported previously (16–18). The three studies found that increased water-soluble products were formed and excreted primarily in the urine and feces. Albro *et al.* (17) suggested there is probably an initial hydrolysis of the compound to the monoester, mono-2-ethylhexyl phthalate (III), with subsequent side-chain oxidation to the alcohol, ketone, and acids or hydrolysis to phthalic acid. Daniel and Bratt (18) reported finding only the diester in the liver of treated animals, while Schultz and Rubin (16) found both water-soluble and organic-extractable fractions in the liver at 1 and 24 hr after intravenous administration of ^{14}C-I.

Studies[10] with ^{14}C-I in mice revealed the presence of ^{14}C-III and other metabolites in tissues of the treated animals as well as in urine and feces. Dillingham and Autian (19) reported that approximately 83% of an intraperitoneal dose of ^{14}C-I was excreted in the urine of mice in 14 days and that no significant radioactivity was found in the mice after 35 days. In another study[10], both mice and rabbits metabolized and excreted ^{14}C-II in a fashion somewhat similar to that for I.

Autian (20) reported inhibition of mammalian cell growth

[10] Unpublished data, Materials Science Toxicology Laboratories.

(mouse fibroblasts or L-cells) in culture from both I and II. Using a protein assay system for quantitation of cell growth, 3×10^{-3} mole/liter of II reduced growth by 50%; a similar effect was observed for I at a concentration of 5×10^{-5} mole/liter. These original data also revealed that approximately 10% growth inhibition was produced by II at a level of 5×10^{-4} mole/liter and that a similar effect was elicited by 4.7×10^{-5} mole/liter of I. In the present study, fetal concentrations of II (based upon ^{14}C counts) ranged from 1.5×10^{-4} to 2.8×10^{-6} mole/kg; similar ranges of I were from 5.9×10^{-4} to 4×10^{-6} mole/kg. Thus, these data suggest the phthalates may be reaching developing fetal tissues in concentrations comparable to those that have been demonstrated to affect growth of mammalian cells in culture.

It was found that maternal blood levels reached a peak radioactive concentration in 24 hr or less after intraperitoneal administration of ^{14}C-II and within 48 hr after ^{14}C-I. Counts (disintegrations per minute per gram) obtained 48 hr or more after ^{14}C-I injection were invariably higher in maternal blood than in fetal tissues; concentrations in maternal blood also appeared to influence the concentrations in the amniotic fluid and placenta.

Radioactivity following a single injection of the ^{14}C-phthalate was observed in maternal blood and fetal tissues throughout the observation period (10–15 days). The relatively long *in vivo* half-life (about 2.2–2.3 days) for these compounds suggests that repetitive maternal exposures during pregnancy might lead to accumulation of toxicologically significant quantities of such compounds within the major organs of the fetus. To explore this potential problem further, a comparative study should be conducted to quantitate the phthalate and/or its metabolites in various major maternal and fetal organs. Concurrently, investigations should be conducted to determine the effects of varying levels of the phthalate esters (and/or metabolites) upon developmental structures and functions of the various organs.

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Metabolism of Triamcinolone Acetonide-21-phosphate in Dogs, Monkeys, and Rats

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Abstract □ The absorption, distribution, and metabolic fate of triamcinolone acetonide-^{14}C-21-phosphate were studied in the dog, monkey, and rat. A comparison of levels of radioactivity in blood or plasma, reached after intramuscular or intravenous administration, indicated that the drug was completely absorbed from the site of intramuscular injection within 10–15 min in all three species. Within 1–5 min after intramuscular or intravenous administration, the 21-phosphate ester was completely hydrolyzed to triamcinolone acetonide, which was present in the blood. The radioactivity was eliminated rapidly ($t_{1/2}$ = 1–2 hr) from plasma (dogs, monkeys, and rats) and tissues (rats) after intramuscular or intravenous administration. In the three species, the major route of excretion was *via* the bile; however, the ratio of biliary to urinary excretion among the species varied considerably (from 1.5 to 15). In rats, excretion of radioactivity as expired carbon dioxide accounted for only 2–3% of the dose. 6β-Hydroxytriamcinolone acetonide was the major metabolite in urine of the three species. Hydrolytic cleavage of the acetonide group did not appear to be significant.

Keyphrases □ Triamcinolone acetonide-21-phosphate, ^{14}C-labeled—absorption, distribution, and elimination after intravenous and intramuscular administration, dog, monkey, rat □ Absorption—triamcinolone acetonide-^{14}C-21-phosphate after intravenous and intramuscular administration, dog, monkey, rat □ Distribution—triamcinolone acetonide-^{14}C-21-phosphate after intravenous and intramuscular administration, dog, monkey, rat □ Elimination—triamcinolone acetonide-^{14}C-21-phosphate after intravenous and intramuscular administration, dog, monkey, rat □ Metabolism—triamcinolone acetonide-^{14}C-21-phosphate after intravenous and intramuscular administration, dog, monkey, rat

Esterification of steroid alcohols has a marked influence on their absorption and excretion. After intramuscular administration, water-soluble esters (*e.g.*, phosphates, succinates, and glycinates) are absorbed and excreted rapidly (1–4), whereas water-insoluble esters (*e.g.*, acetates) are absorbed and excreted more slowly (1, 5). After administration, the steroid esters seem to be readily hydrolyzed to the corresponding steroid alcohols.

Hydrolysis of the phosphate esters of hydrocortisone, prednisolone, and dexamethasone appears to be rapid and complete, possibly as a result of the activity of phosphomonoesterases in plasma (2). The steroid alcohol resulting from such hydrolysis may then be metabolized as if it had been administered directly. In dogs, 6-methylprednisolone-^{3}H-21-acetate was metabolized in a manner similar to that of prednisolone (5).

No data are available for the relative blood levels obtained after intravenous and intramuscular administration of water-soluble esters of synthetic anti-inflammatory steroids, nor has the distribution of such drugs in tissues been reported. It is important to determine the distribution in blood and other tissues at various times after administration, because the increased water solubility of a steroid ester may alter its distribution in the body and, hence, influence its concentration at the target site.

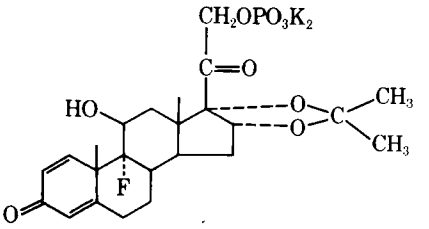
Triamcinolone acetonide-21-dipotassium phosphate (I) is a water-soluble 21-phosphate ester of triamcinolone acetonide (9-fluoro-11β,16α,17,21-tetrahydroxypregna-1,4-diene-3,20-dione cyclic 16,17-acetal with acetone, 21-phosphate, dipotassium salt). It is about 80 times more active than cortisone acetate in the liver glycogen deposition assay[1], and 10–50 times more active than hydrocortisone acetate in the antigranuloma assay in the rat[2].

Metabolic transformations of triamcinolone acetonide-21-phosphate may be similar to those of triamcinolone acetonide. Triamcinolone was metabolized in dogs and humans to the 6β-hydroxy compound (6), and triamcinolone acetonide was converted in rat liver *in vitro* to 6β-hydroxytriamcinolone acetonide (7). However, biotransformation *in vivo* of triamcinolone acetonide or of its phosphate ester has not been reported.

The 17-hydroxy group, considered necessary for the glucocorticoid activity of hydrocortisone, is blocked by the presence of the 16,17-acetonide group in the triamcinolone acetonide molecule. Inasmuch as triamcinolone acetonide has greater glucocorticoid activity than does hydrocortisone, it is of interest to find out whether the 16,17-acetonide group is hydrolyzed *in vivo* to give rise to a 17-hydroxy group. The present studies were carried out to determine this hydrolysis as well as the absorption, excretion, tissue distribution, and biotransformation of triamcinolone acetonide 21-phosphate after intramuscular or intravenous administration to dogs, monkeys, and rats.

EXPERIMENTAL

Measurement of Radioactivity—All samples were counted in a liquid scintillation spectrometer[3], using the method of automatic external standardization. Feces were homogenized in a volume of



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[3] Packard Tri-Carb model 3375 or 3380.

methanol equivalent to half the weight of the sample. After portions of fecal homogenate, blood, plasma, and tissues were combusted in an oxygen atmosphere and the resulting carbon dioxide was trapped in a solution containing phosphor, the samples were counted.

Portions of bile and urine were counted in Bray's solution (8). Nonaqueous samples were counted in a solution containing 5 g of 2,5-diphenyloxazole and 0.3 g of 1,4-bis(4-methyl-5-phenyloxazolyl)benzene in 1 liter of spectral grade toluene. Portions (0.5 ml) of solutions containing trapped, expired $^{14}CO_2$ were counted after the addition of 2 ml of a solubilizer[4] and 15 ml of toluene phosphor solution.

Purity and Specific Activity of Triamcinolone Acetonide-^{14}C-21-phosphate—Triamcinolone acetonide-21-phosphate (dipotassium salt), labeled with ^{14}C in the 2-position of the acetonide group, was synthesized[5] and had a specific activity of 5.8 μCi/mg and a radiochemical purity of 96%, as determined by paper chromatography[6] in *n*-butanol–ethanol–2 *N* ammonium hydroxide (10:2:4).

Extraction of "Unconjugated" Metabolites—Plasma samples were extracted once with 10 volumes of methylene chloride. Urine and bile samples were extracted four times with an equal volume of ethyl acetate; the pooled extracts were washed with 0.1 volume of water.

Extraction of "Conjugated" Metabolites—Conjugated metabolites were separated from the extracted urine or bile by use of a nonion-exchange resin[7], according to the procedure described by Bradlow (9). Hydrolysis of the conjugated steroid fraction was carried out by incubation at 37° for 48 hr in 0.1 *M* acetate buffer, pH 4.6, containing 10% (v/v) of a solution containing β-glucuronidase[8].

After incubation, samples were extracted with ethyl acetate by the procedure described for urine and bile. The ethyl acetate extracts represented hydrolyzed metabolites originally present as glucuronides. The resulting aqueous fraction was subjected to solvolysis (10) to cleave and isolate the steroids originally present as sulfate conjugates.

Chromatographic Methods—TLC was carried out on precoated 0.25- and 2.0-mm silica gel H and silica gel F-254 plates[9]. Prior to use for preparative purposes, the plates were predeveloped, first in benzene and then in methanol. Three solvent systems were used for developing the thin-layer chromatograms: A, methanol–chloroform (8:92); B, methanol–ethyl acetate (2:98); and C, acetone–benzene (35:65). The radioactive zones on TLC plates were located by use of a scanner[10].

Paper chromatography[6] was carried out with 50% aqueous methanol–benzene (1:1) as the developing solvent. Radioactive zones were located by uniform sectioning of a lengthwise portion of the paper chromatogram, followed by liquid scintillation counting of each section in Bray's solution.

Silica gel[11] (100–200 mesh) was used for column chromatography. The eluting solvents were 10-ml portions of mixtures that contained increasing amounts of ethyl acetate in benzene (5% increase per successive 10-ml portion up to 100%). The elution was then continued with similar mixtures containing increasing amounts of methanol in ethyl acetate. Portions of the fractions collected were counted to locate the labeled compounds.

Partition chromatography[12] was carried out in a 1.5 × 60-cm column, with chloroform–toluene–ethylene glycol (1:6:7) as the solvent system. Fractions (10 ml) were collected at a rate of three per hour. Portions of the fractions collected were counted to locate the labeled compounds.

Drug Administration—The ^{14}C-labeled drug was dissolved in a vehicle formulated for the injectable product. The vehicle consisted of saccharin sodium, 40 mg; sodium citrate, 10 mg; sodium bisulfite, 3 mg; polyethylene glycol 300, 250 mg; benzyl alcohol, 15 mg; and water for injection to make 1.0 ml. Triamcinolone acetonide-21-phosphate was stable in this vehicle.

Purebred beagles (9.2–9.6 kg), female rhesus monkeys (*Macacca mulatta*, 3.6–5.6 kg), and Sprague–Dawley rats[13] (0.2 kg) were used. Dogs and rats were placed in individual metabolism cages, and monkeys were seated in restraining chairs[14]. Dogs and monkeys were fitted with urinary catheters. All animals were deprived of food, but not of water, for 24 hr before dosing.

At 60 and 30 min prior to drug administration, the dogs and monkeys were hydrated by oral administration of 20 ml of water/kg. A bile-cannulated dog was maintained under gaseous anesthesia (methoxyflurane) and infused with isotonic saline at a rate of 3 ml/min; thiamylal sodium[15], 0.4 ml of 4% solution/kg iv, was used as the preanesthetic. Intramuscular doses were injected into the gastrocnemius muscle of dogs, monkeys, and rats. The saphenous vein of dogs and monkeys and the tail vein of rats were used as the sites of intravenous injections.

Sampling Procedure—Heparinized syringes were used to withdraw blood (5–10 ml) from the jugular vein of dogs or the saphenous vein of monkeys. A portion of the blood sample was saved for radioassay, and the remainder was centrifuged under refrigeration at 2000 rpm for 10 min to separate the plasma.

During the first 8 hr after administration to dogs, urine was collected through catheters; thereafter, urine was collected in metabolism cages. After ligation of the gallbladder of the dog, bile was collected through a catheter inserted into the common bile duct. With monkeys, urine was collected through catheters throughout the experiment. Feces were collected every 24 hr from dogs and monkeys.

Urine and feces from rats were collected from the metabolism cages. To measure the $^{14}CO_2$ evolved, each rat was placed in a plastic metabolism cage enclosed in a plastic bag. Air was drawn from the cages through two carbon dioxide-absorbing traps, connected in series with a vacuum line and containing a solution of monoethanolamine and 2-methoxyethanol (1:3). Tissues were excised from rats after decapitation.

Incubation with Dog Blood—Triamcinolone acetonide-^{14}C-21-phosphate (0.2 mg) was incubated with 50 ml of freshly drawn heparinized blood at 37° with gentle shaking. Blood samples (5 ml) were removed at various times and centrifuged to separate plasma. Portions of plasma (1 ml) were extracted with 5 ml of methylene chloride. Portions of blood, plasma, extracted plasma, and methylene chloride extracts were assayed for radioactivity. Triamcinolone acetonide-^{14}C-21-phosphate was incubated with phosphate buffer (0.15 *M*, pH 7.4) as a control.

Incubation with Rat Muscle—Femoral muscles from four female rats were minced in a meat grinder, and a 10% suspension of this minced muscle in 0.25 *M* sucrose was prepared. Muscle homogenate was prepared from a portion of the minced muscle suspension (11). Minced muscle suspension or muscle homogenate, each containing the equivalent of 1.2 g of minced muscle and 0.2 μmole of glucose, was incubated with 40 μg of triamcinolone acetonide-^{14}C-21-phosphate at 37° for 24 hr. An equivalent amount of muscle homogenate, immersed for 30 min in boiling water, was used as a control.

Portions of each mixture (1 ml) were removed at various times during the incubation period. Each portion was extracted with 5 ml of methylene chloride, and 0.5 ml of each methylene chloride extract was assayed for radioactivity.

Isolation of Triamcinolone Acetonide (Metabolite 1) and 6β-Hydroxytriamcinolone Acetonide (Metabolite 2) from Urine—*Dog*—An ethyl acetate extract of the first 8-hr urine sample from Dog Q9-191 was subjected to preparative TLC in Solvent System B. Metabolite 1 was eluted from silica gel in an area corresponding in R_f value to that of authentic triamcinolone acetonide and was purified by paper chromatography.

Metabolite 2 was isolated by preparative TLC (Solvent System A) of an ethyl acetate extract of an 8-hr urine sample from a bile-cannulated dog (Q9-156). The radioactive material, isolated from an area corresponding to R_f 0.3 (triamcinolone acetonide, R_f 0.45), was purified by TLC in Solvent System B.

Monkey—Silica gel column chromatography of the combined ethyl acetate extracts of the 24-hr urine samples (from the four ex-

[4] NCS, Amersham-Searle.
[5] By Dr. L. High, Squibb Institute for Medical Research.
[6] Whatman No. 1.
[7] Amberlite XAD-2.
[8] Ketodase, Warner-Chilcott.
[9] Brinkmann Instruments.
[10] Actigraph III, Nuclear Chicago.
[11] Matheson Coleman & Bell.
[12] Celite Filter-Aid, Johns-Manville.
[13] Charles River.
[14] PCP-1208 primate chair, BRS Foringer.
[15] Surital, Parke-Davis.

Table I—Plasma Levels (Micrograms per Milliliter) of Radioactivity[a] in Dogs and Monkeys after Intravenous or Intramuscular Administration of Triamcinolone Acetonide-^{14}C-21-phosphate[b]

	Dogs		Monkeys			
Minutes	Q9-191, Intramuscular	Q9-156, Intravenous	MF-65, Intramuscular	MF-66, Intramuscular	MF-65, Intravenous	MF-66, Intravenous
5	—[c]	—	—	0.82	3.48	—
10	0.61	—	0.48	1.47	0.96	1.13
15	—	—	—	1.63	0.55	—
20	—	0.68	—	—	—	—
30	0.60	—	—	—	—	—
40	—	—	—	0.52	—	—
50	0.42	0.31	—	—	—	—
60	—	—	0.36	0.43	0.39	0.28
70	0.29	0.23	—	—	—	—
90	0.22	0.19	—	—	—	—
120	0.15	0.14	—	0.28	0.29	—
180	—	—	0.16	—	—	0.13
240	0.044	0.04	—	0.12	0.18	—
360	0.023	0.021	0.063	0.07	0.09	0.05
480	0.017	0.013	0.044	0.06	0.07	0.04

[a] Expressed as microgram equivalents of triamcinolone acetonide. [b] Dose: 0.9 mg/kg for dogs and 1.3 mg/kg for monkeys. [c] Indicates no sample taken.

periments with Monkeys 65 and 66) yielded two peaks of radioactivity. Metabolites 1 and 2 were isolated by TLC in Solvent System A from the fractions corresponding to the first and second peaks off the column, respectively. Metabolite 2 was further purified by TLC in Solvent System B.

Rat—For isolation of metabolites from urine, 10 mg of triamcinolone acetonide-^{14}C-21-phosphate was administered intramuscularly daily to each of four female rats (0.25–0.30 kg) for 4 days. Urine collected for 4 days from the four rats was pooled. TLC (Solvent System A) indicated that radioactive metabolites in this pooled urine were the same as those in the urine collected after administration of a single dose (1.5 mg/kg).

The pooled urine was extracted with ethyl acetate. After evaporation of the solvent from the ethyl acetate extract, the residue was partitioned between benzene and water. The aqueous fraction was then extracted with ethyl acetate (ethyl acetate fraction). Metabolite 1 and a portion (20%) of Metabolite 2 were isolated from the benzene extract by preparative TLC in Solvent System A. Metabolite 1 was crystallized from methanol. Metabolite 2, isolated from the benzene fraction, was purified by silica gel column chromatography and crystallized from ethyl acetate. The major portion of Metabolite 2 (80% of the total) was isolated from the ethyl acetate fraction by silica gel column chromatography and then crystallized from ethyl acetate.

NMR and Mass Spectrometry—The 100-MHz NMR spectra were obtained on an NMR spectrometer[16] homolocked to the protons in the tetramethylsilane reference. All chemical shifts are given in tau (τ) values. The low- and high-resolution mass spectra were obtained on a mass spectrometer[17], using the direct insertion probe. The source temperature was maintained at 180° above ambient; data were collected on a frequency-modulated analog tape recorder and were subsequently processed on a computer using Squibb programs (12).

RESULTS

The dose of triamcinolone acetonide-21-phosphate and the levels of radioactivity are expressed as triamcinolone acetonide equivalents.

Hydrolysis of Triamcinolone Acetonide Phosphate to Triamcinolone Acetonide by Incubation with Dog Blood—Based on an hematocrit value of 0.44 and relative concentrations in blood and plasma, it was established that at least 98% of the radioactivity in blood was present in the plasma. One minute after incubation of triamcinolone acetonide-^{14}C-21-phosphate with blood (4 μg/ml), 46% of plasma radioactivity was extractable into methylene chloride. No radioactivity was extracted into methylene chloride from the phosphate buffer control incubation. During the 2-hr incubation, methylene chloride extracts of plasma samples, obtained at nine different times, contained 44–62% of the radioactivity in plasma, whereas extracts of control incubation contained no more than 3% of the total radioactivity.

TLC (Solvent System A) of the methylene chloride extracts of plasma samples showed a single peak of radioactivity, which had the same R_f value (0.45) as did triamcinolone acetonide.

A portion (8000 dpm) of the combined methylene chloride extracts of plasma samples was mixed with unlabeled triamcinolone acetonide (8.0 mg), and the mixture was crystallized from ethyl acetate three successive times. The specific activity of each successive batch of crystals was 930, 1005, and 962 dpm/mg.

These results demonstrate that triamcinolone acetonide-21-phosphate is hydrolyzed to triamcinolone acetonide by plasma esterases.

Hydrolysis of Triamcinolone Acetonide-21-phosphate to Triamcinolone Acetonide by Incubation with Rat Muscle—One minute after incubation of triamcinolone acetonide-^{14}C-21-phosphate with rat minced muscle or rat muscle homogenate (33 μg/g of muscle), 3% of the radioactivity in the incubation mixture could be extracted with methylene chloride. Successively greater quantities were extractable: 28% in 2 hr and 42% after 4 hr. The percentage of radioactivity extracted from a control incubation with a boiled muscle homogenate was 0.6–1.1% during the first 4 hr of incubation.

Methylene chloride extracts of the incubation mixtures contained only one radioactive compound, which had the same R_f as did triamcinolone acetonide (Solvent System A).

These results show that hydrolysis of the 21-phosphate ester of triamcinolone acetonide could occur in the rat muscle. The rate of hydrolysis in the rat muscle incubations appeared to be much slower ($t_{1/2}$ = 4.5 hr) than that in plasma, in which case 46% was hydrolyzed after 1 min of incubation.

Plasma Levels—Levels of radioactivity in the plasma of two dogs and four monkeys are shown in Table I. In addition, plasma levels in a bile-cannulated dog (Q9-156)[18] are shown in Fig. 1. Plasma samples collected during the first 10 or 15 min after intravenous administration to Dog Q9-156 (bile cannulated) and Monkey 65 showed a rapid and precipitous drop in the level of radioactivity during this interval, indicating rapid distribution of the drug into the extravascular compartments. The plasma half-life of radioactivity during this distribution phase was calculated[19] to be 1 min for the dog and 1.5 min for the monkey.

After the first 10 or 15 min, elimination of radioactivity from plasma of the three dogs and the four monkeys followed a biphasic pattern. The half-life values, calculated by the method of residuals, are shown in Table II. The plasma half-life during the first phase (10–240 min) was 35 min in each of the three dogs and 65–85

[16] Varian Associates XL-100.
[17] Model MS-902, Associated Electrical Industries.
[18] This dog had been used as an intact dog 7 weeks earlier.
[19] Calculated by methods of residuals as shown in Fig. 1 for Dog Q9-156.

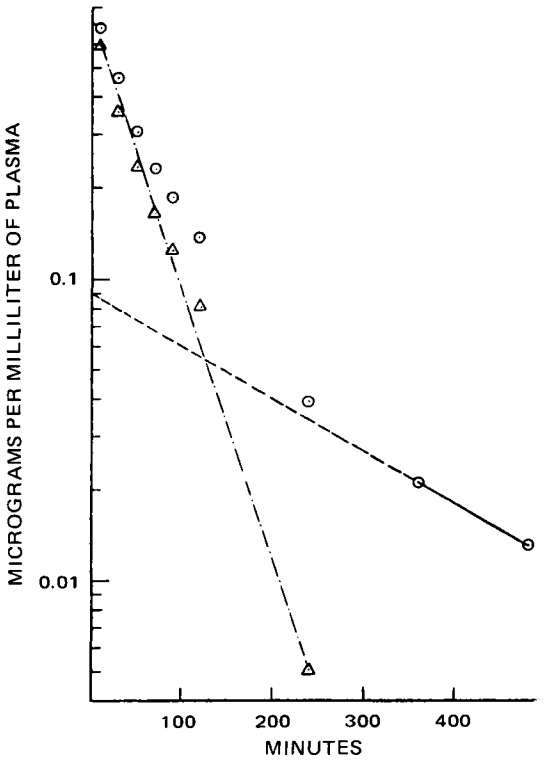


Figure 1—*Levels of radioactivity in plasma of Dog Q9-156 after intravenous administration (0.9 mg/kg) of triamcinolone acetonide-^{14}C-21-phosphate. Key: O, experimental values; and Δ, residual values, calculated by subtracting from the experimental value the value obtained at the corresponding time on the extrapolated part of the curve. The ordinate values are expressed as microgram equivalent of triamcinolone acetonide based on radioassay.*

min in the four monkeys. In the same animals, the rate of disappearance during the second phase (240–480 min) was slower and more variable than that during the first phase. The half-life values during the second phase were 170–270 min in the dogs and 250–720 min in the monkeys; however, the levels of radioactivity during this phase were less than 5% of the initial radioactivity in plasma observed after intravenous administration to dogs or monkeys.

Levels of radioactivity in rats after intramuscular or intravenous administration are shown in Tables III and IV (with tissue distribution data). The elimination of radioactivity from plasma followed first-order kinetics during the interval of 30–360 min after dosing. The half-life for disappearance of radioactivity during this phase was 95 or 110 min after intramuscular or intravenous administration, respectively[20]. After this initial phase, the plasma levels declined at a slower rate ($t_{1/2}$ = 420 min) between 360 and 1440 min. Plasma levels during the latter phase were less than 3% of the initial levels seen after intravenous administration.

In all three species, peak levels of radioactivity in plasma were observed 10–15 min after intramuscular administration, and the subsequent plasma levels were substantially the same as those observed after intravenous administration. These results indicate a rapid and complete absorption of the drug from the site of intramuscular injection.

Distribution in Rat Tissues—Concentrations of the labeled drug in different tissues at various times between 1 and 1440 min after intravenous or intramuscular administration are shown in Tables III and IV. After intramuscular administration, peak levels of radioactivity were measured at 15 min in the plasma, liver, adrenals, ovary, and stomach and at 30 min in other tissues (except the intestine plus contents). The levels of radioactivity between 30 and 1440 min after intramuscular or intravenous administration were approximately equal in all tissues, indicating rapid and complete distribution of the drug when given by the intramuscular route.

[20] Due to insufficient data points on the plasma level curves, the half-life values were calculated from the slope of the semilogarithmic plots and not by the method of residuals.

Table II—Half-Life Values for Disappearance of Radioactivity from Plasma of Dogs and Monkeys after Intramuscular or Intravenous Administration of Triamcinolone Acetonide-^{14}C-21-phosphate

Animal	Route of Administration	Dose, mg/kg	Half-Life, min: Between 10 and 240 min	Half-Life, min: Between 240 and 480 min
Dog Q9-191	Intramuscular	0.9	35	270
Dog Q9-156	Intravenous	0.9	35	250
Dog Q9-156[a]	Intravenous	1.2	35	170
Monkey 65	Intramuscular	1.3	65	250
Monkey 66	Intramuscular	1.3	65	720
Monkey 65	Intravenous	1.3	85	380
Monkey 66	Intravenous	1.3	70	360

[a] Anesthetized bile-cannulated dog; 7 weeks after previous dosing.

At 1 min after drug administration (intravenous or intramuscular), as one might expect, plasma had a higher concentration of radioactivity than did any of the tissues examined. At subsequent times, the concentrations in liver were 3–10 times greater than those in plasma; those in adrenals were 1.3 to 8 times greater. Concentrations in the kidneys increased from 0.7 to 2 times those in plasma. The concentration in the brain was the lowest (less than one-tenth the concentration in plasma) of all tissues. The amount of radioactivity in the intestines and their contents increased continuously throughout the 1440-min period, suggesting biliary excretion of the drug; this suggestion was confirmed by excretion studies.

The half-life values for disappearance of labeled drug from tissues were calculated. Although the concentrations in various tissues varied widely, the half-life values for disappearance of labeled drug from all tissues (except the intestine plus contents) were in good agreement with the half-life in plasma. Between 120 and 360 min, the half-life values after intravenous or intramuscular administration ranged from 96 to 120 min in plasma and from 84 to 180 min in the tissues other than intestine plus contents.

The half-life for transfer of radioactivity from the site of injection after intramuscular administration was 12 min during the first 30 min; this half-life represents the rate of drug transfer. After 120 min, when most of the injected drug would be expected to have left the injection site, the half-life observed, *i.e.*, 102 min, was in good agreement with the measurements of half-lives in the other tissues.

Characterization of Radioactive Material in Plasma—The percentage of radioactivity extracted into methylene chloride from plasma samples obtained at various times from the three species is shown in Table V. When triamcinolone acetonide-^{14}C-21-phosphate was added to plasma (4 μg/ml) *in vitro*, no radioactivity was extracted into methylene chloride; however, under the same conditions, triamcinolone acetonide-^{14}C was completely extractable. For the plasma samples obtained within the first 10 min after administration of the labeled drug, 91–98% of the radioactivity in plasma was extracted into methylene chloride; with subsequent samples, the extractable radioactivity generally decreased with time.

Radioscans after TLC of methylene chloride extracts of plasma samples, obtained during the first 60 min after dosing of dogs, monkeys, and rats, showed one major peak (90–95% of the radioactivity in the extracts), corresponding in R_f values to triamcinolone acetonide in Solvent Systems A (R_f 0.45) and B (R_f 0.40). At later times (up to 24 hr), smaller peaks with R_f values lower than that of triamcinolone acetonide were also seen; these peaks accounted for 15–25% of the radioactivity in the plasma extracts.

The radioactivity present in the first 60-min plasma samples of each species was eluted with ethyl acetate from the silica gel plates. The isolated material for each species (approximately 4000–8500 dpm) was mixed with an authentic sample of unlabeled triamcinolone acetonide (6–7 mg) and crystallized from ethyl acetate to constant specific activity. For labeled material isolated from dog plasma, the specific activity of each successive batch of

Table III—Levels of Radioactivity[a] Found in Tissues of Female Rats after Intravenous Administration of Triamcinolone Acetonide-^{14}C-21-phosphate[b]

Tissue	Micrograms per Gram[c]				
	1 min	30 min	120 min	360 min	1440 min
Adipose	0.57	0.93 ± 0.09	1.84 ± 0.08	0.14 ± 0.02	0.04 ± 0.01
Adrenals	4.65	2.52 ± 0.70	2.41 ± 1.22	0.91 ± 0.28	0.12 ± 0.10
Bone	1.18	0.43 ± 0.01	0.34 ± 0.0	0.10 ± 0	0.01 ± 0
Brain	0.48	0.12 ± 0.01	0.08 ± 0.02	0.02 ± 0	0
Eye	1.04	0.34 ± 0.10	0.18 ± 0.01	0.05 ± 0	0
Heart	2.99	1.18 ± 0.04	0.71 ± 0.21	0.19 ± 0.01	0.02 ± 0
Intestine plus contents	0.74	3.20 ± 0	9.40 ± 6.70	8.75 ± 1.60	13.7 ± 8.00
Kidney	4.41	1.97 ± 0.05	1.10 ± 0.03	0.39 ± 0.03	0.06 ± 0.01
Liver	3.56	5.93 ± 0.20	5.32 ± 1.62	2.13 ± 0.37	0.42 ± 0.04
Lung	3.38	1.19 ± 0.01	0.81 ± 0.26	0.28 ± 0.02	0.04 ± 0.01
Skeletal muscle	0.61	0.64 ± 0.03	0.44 ± 0.12	0.13 ± 0.01	0.02 ± 0
Skin	0.44	0.66 ± 0.06	0.42 ± 0.34	0.16 ± 0.03	0.03 ± 0.01
Spleen	0.53	0.73 ± 0.03	0.49 ± 0.16	0.18 ± 0.01	0.04 ± 0.01
Stomach	0.39	0.69 ± 0.50	0.44 ± 0.02	0.21 ± 0.08	0.08 ± 0.03
Ovary	2.21	1.11 ± 0.05	1.20 ± 0.02	0.23 ± 0.03	0.04 ± 0.03
Thymus	0.85	0.69 ± 0.01	0.50 ± 0.13	0.18 ± 0.02	0.03 ± 0.01
Uterus	0.90	1.15 ± 0.11	0.65 ± 0.13	0.22 ± 0.01	0.03 ± 0.01
Plasma[d]	18.63	1.96 ± 0.01	0.97 ± 0.15	0.26 ± 0.04	0.04 ± 0
Blood[d]	11.08	1.26 ± 0	0.75 ± 0.11	0.18 ± 0.03	0.04 ± 0

[a] Expressed as micrograms of triamcinolone acetonide per gram of tissue. [b] 1.5-mg equivalents of triamcinolone acetonide/kg. [c] Each value shown is the average and range of the mean for two determinations, each in a separate rat, with the exception of the 1-min values. [d] Micrograms per milliliter.

crystals was 729, 736, 709, and 710 dpm/mg; calculated specific activity was 731 dpm/mg. Similar values obtained in plasma samples from the monkey were 1290, 1245, 1179, and 1220 dpm/mg; those for the rat were 954, 942, and 941 dpm/mg. Calculated values for specific activity were 1214 and 1095 dpm/mg for the monkey and rat, respectively.

Based on chromatography in the two solvent systems and on isotope dilution, the major radioactive metabolite found in the plasma of dog, monkey, and rat was identified as triamcinolone acetonide.

These results show that as early as 1–10 min after its administration, triamcinolone acetonide-21-phosphate is almost completely hydrolyzed to triamcinolone acetonide, probably by plasma esterases, and is then further metabolized. Other water-soluble esters of corticosteroids have also been shown to undergo hydrolysis to corresponding steroid alcohols after being administered to humans (1–4).

Excretion in Urine and Feces of Dogs, Monkeys, and Rats—Table VI shows the data for excretion in urine and feces. Elimination of radioactivity from the body took place rapidly. In all but one animal (Monkey 65 after intravenous or intramuscular administration), two-thirds of the total excretion in urine occurred during the first 6–8 hr after administration. For Monkey 65, 24 hr was required for elimination of two-thirds of the total excretion in urine.

In all species, excretion in urine was essentially complete after 48 hr and excretion in feces was essentially complete between 48 and 96 hr. Total excretion in urine and feces accounted for 95, 85, and 87% of the intravenously or intramuscularly administered dose in dogs, monkeys, and rats, respectively. Excretion of radioactivity

Table IV—Concentration of Radioactivity[a] Found in Tissues of Female Rats after Intramuscular Administration of Triamcinolone Acetonide-^{14}C-21-phosphate[b]

Tissue	Micrograms per Gram[c]					
	1 min	15 min	30 min	120 min	360 min	1440 min
Adipose	0.18 ± 0.06	0.77 ± 0.06	0.81 ± 0.05	0.32 ± 0.15	0.10 ± 0.01	0.07 ± 0.04
Adrenals	0.31 ± 0.10	3.10 ± 1.20	2.64 ± 0.18	1.30 ± 0	0.39 ± 0.08	0.23 ± 0
Bone	0.11 ± 0.10	0.46 ± 0.01	0.53 ± 0.08	0.30 ± 0.02	0.07 ± 0.01	0.01 ± 0
Brain	0.04 ± 0.02	0.07 ± 0.01	0.10 ± 0.01	0.07 ± 0.01	0.01 ± 0	0
Eye	0.22 ± 0.06	0.25 ± 0.03	0.27 ± 0.04	0.14 ± 0.01	0.05 ± 0	0
Heart	0.24 ± 0.07	0.97 ± 0.05	1.15 ± 0.10	0.71 ± 0.07	0.13 ± 0.02	0.02 ± 0
Intestine plus contents	0.04 ± 0.03	0.92 ± 0	1.69 ± 0.36	5.60 ± 0.50	5.20 ± 1.40	23.0 ± 7.5
Kidney	0.39 ± 0.01	1.40 ± 0.02	1.80 ± 0.08	1.28 ± 0.12	0.28 ± 0.03	0.26 ± 0.01
Liver	0.40 ± 0.31	8.60 ± 0.90	6.73 ± 0.68	4.39 ± 0.07	1.24 ± 0.12	0.26 ± 0.01
Lung	0.21 ± 0	0.97 ± 0.08	1.15 ± 0.11	0.77 ± 0.06	0.17 ± 0.02	0.06 ± 0.01
Muscle at site of injection	40.4 ± 1.70	18.4 ± 2.60	7.35 ± 0.67	0.68 ± 0.08	0.14 ± 0.03	0.02 ± 0
Skeletal muscle	0.05 ± 0.03	0.45 ± 0.02	0.63 ± 0.07	0.45 ± 0.05	0.08 ± 0.02	0.03 ± 0.01
Skin	0.03 ± 0.02	0.47 ± 0	0.69 ± 0.07	0.49 ± 0.03	0.10 ± 0.02	0.03 ± 0
Spleen	0.03 ± 0.03	0.57 ± 0.01	0.73 ± 0.09	0.51 ± 0.06	0.14 ± 0	0.05 ± 0
Stomach	0.03 ± 0.03	1.30 ± 0.60	0.42 ± 0.15	0.68 ± 0.02	0.10 ± 0.02	0.05 ± 0
Ovary	0.12 ± 0.06	1.14 ± 0.54	1.03 ± 0.04	0.67 ± 0.02	0.18 ± 0	0.03 ± 0.01
Thymus	0.05 ± 0.01	0.42 ± 0.02	0.65 ± 0.03	0.51 ± 0	0.11 ± 0.01	0.04 ± 0.02
Uterus	0.11 ± 0.04	0.72 ± 0.07	1.03 ± 0.01	0.66 ± 0.09	0.23 ± 0.06	0.08 ± 0.03
Plasma[d]	1.60 ± 0.20	1.90 ± 0.15	1.88 ± 0.18	1.04 ± 0.08	0.17 ± 0.03	0.03 ± 0
Blood[d]	0.81 ± 0.13	1.13 ± 0.03	1.28 ± 0.17	0.71 ± 0	0.12 ± 0.02	0.04 ± 0.01

[a] Expressed as micrograms of triamcinolone acetonide per gram of tissue. [b] 1.5-mg equivalents of triamcinolone acetonide/kg. [c] Each value shown is the average and range of the mean for two determinations, each in a separate rat. [d] Micrograms per milliliter.

Table V—Percentage of Radioactivity Extracted into Methylene Chloride after Intravenous or Intramuscular Administration of Triamcinolone Acetonide-^{14}C-21-phosphate to Dogs, Monkeys, and Rats[a]

Time after Administration, min	Percent Extracted					
	Dogs		Monkeys		Rats	
	Intravenous	Intramuscular	Intravenous	Intramuscular	Intravenous	Intramuscular
1	—	—	—	—	98	95
5	—	—	—	98	—	—
10	95	91	98	—	—	—
15	—	—	93	89	90	—
30	79	87	—	—	92	92
40	—	—	—	81	—	—
50	78	75	—	—	—	—
70	69	64	—	—	—	—
90	63	89	—	—	—	—
120	49	90	81	74	94	92
240	53	72	72	55	—	—
360	—	77	58	35	92	92
480	—	86	48	37	—	—
1440	—	—	52	41	76	51

[a] Dose: 0.9 mg/kg for dogs (male), 1.2 mg/kg for monkeys (female), and 1.5 mg/kg for rats (female).

in feces was always greater than that in urine. The ratio of fecal to urinary excretion varied among the species, however; it was 15:1 for dogs, 4.7:1 for monkeys, and 1.4:1 for rats. The results indicate that excretion *via* bile is the major route of elimination in all three species.

Excretion in Bile and Urine of a Dog—Excretion of radioactivity in bile and urine after intravenous administration (1.2 mg/kg) of triamcinolone acetonide-^{14}C-21-phosphate is shown in Table VII. A total of 81% of the dose was accounted for during the 8-hr period, 10% in urine and 71% in bile. The apparent half-life for excretion of radioactivity in bile and urine was 2 hr during the first 3 hr and 5 hr between 3 and 8 hr after dosing. The results show that excretion *via* bile is a major route of elimination of this drug in the dog.

Based on the findings that *(a)* the plasma $t_{1/2}$ of radioactivity in the intact dogs and the bile-cannulated dog was the same (Table II) and *(b)* the ratio of excretion of radioactivity in urine to that in feces was no higher for intact dogs than for the bile-cannulated animal, it appears that the drug is not reabsorbed after excretion *via* bile in the canine species.

Excretion of Radioactivity as Respiratory $^{14}CO_2$ in Rats—During 48 hr after intravenous and intramuscular administration (1.5 mg/kg) of triamcinolone acetonide-^{14}C-21-phosphate to rats, the administered radioactivity found in the expired air was 2.6 and 2.4%, respectively. Of this total radioactivity expired as $^{14}CO_2$, 90% was eliminated during the first 8 hr.

Table VII—Excretion of Radioactivity in Urine and Bile of Dog Q9-156[a] after Intravenous Administration of Triamcinolone Acetonide-^{14}C-21-phosphate (1.2 mg/kg)

Collection Period, min	Cumulative Percent of Dose	
	Urine	Bile
0–6	0	0
6–15	0	1.7
15–30	1.1	7.2
30–45	—	19.0
45–60	2.0[b]	27.0
60–90	3.8	39.0
90–180	6.2	56.0
180–300	8.0	65.0
300–420	9.3	69.0
420–480	9.8	71.0

[a] Seven weeks after the previous dose. [b] A 30–60-min collection.

The triamcinolone acetonide-21-phosphate used in these studies was labeled with ^{14}C in the acetonide group (2-position). The appearance of only 2 or 3% of the administered radioactivity as $^{14}CO_2$ indicates that hydrolysis and subsequent oxidation of the acetonide group are not significant metabolic reactions in the rat. In addition, because the total recovery in urine and feces (in 4 or 7 days) accounted for 95 and 85% of the dose in dogs and monkeys, respectively, elimination of radioactivity as $^{14}CO_2$ and, hence, degradation of the acetone-derived portion also may not be significant in these species.

Isolation and Identification of Metabolites in Urine—TLC (Solvent System A) of urine or ethyl acetate extract of urine from the dog, monkey, and rat showed three peaks of radioactivity, A, B, and C. Peak A had the same R_f value (0.45) as that of triamcinolone acetonide, peak B had an R_f value of 0.30, and peak C remained at the origin. TLC of ethyl acetate extracts of urine, collected sequentially from dogs and monkeys, revealed a pattern of change in the levels of each peak with time. The data in Table VIII show the percent of urinary radioactivity associated with peaks A and B. In addition, Table VIII shows the percent of radioactivity as X, which represents the radioactivity in peak C plus that not extracted with ethyl acetate.

In the rat, TLC was carried out on urine samples prior to any extraction. The mean values (intramuscular or intravenous administration) for percent of urinary radioactivity associated with peaks A, B, and C were 17 ± 1, 73 ± 1, and 10 ± 1%, respectively, in the 0–6-hr urine, and 10 ± 2, 76 ± 4, and 15 ± 1% in the 6–24-hr urine, respectively. The pattern of urinary metabolites in each species, as seen in these chromatograms, was the same after intravenous or intramuscular administration.

In characterization studies of metabolites in the test species, pooled urine was collected during the first 8 hr from Dog Q9-156 (bile cannulated) and during the first 24 hr from each monkey (65 and 66, four experiments) and each of four rats; these samples

Table VI—Excretion of Radioactivity by Dogs, Monkeys, and Rats after Administration of Triamcinolone Acetonide-^{14}C-21-phosphate[a]

Animal	Route of Administration	Cumulative Percent of Dose					
		Urine			Feces		Total
		0–8 hr	0–24 hr	0–48 hr	0–24 hr	0–48 hr	0–96 or 0–144 hr[b]
Dog Q9-156	Intravenous	4	5	6	82	88	96
Dog Q9-191	Intramuscular	3.6	6	6	83	86	94
Monkey 65[c]	Intravenous	6.6	14	19	2	19	102
Monkey 66[c]	Intramuscular	7.1	9	11	0.1	56[d]	75
Monkey 65	Intramuscular	4.8	8	12	1	56	78
Monkey 66	Intravenous	7.8	9	10	11	63	76[e]
Rat 1	Intravenous	24	38	38	43	47	85
Rat 2	Intramuscular	26	29	29	49	52	81
Rat 3	Intramuscular	28	35	36	47	49	74

[a] Dose: 0.9 mg/kg for dogs (male), 1.2 mg/kg for monkeys (female), and 1.5 mg/kg for rats (female). [b] 0–96 hr for dogs and rats, and 0–144 hr for monkeys. [c] Received second dose 7 weeks later. [d] 0–72 hr. [e] Feces sample between 48 and 72 hr was lost.

Table VIII—Percent Distribution of Radioactivity[a] in Urine Collected at Various Times after Administration of Triamcinolone Acetonide-^{14}C-21-phosphate to Dogs, Monkeys, and Rats

Time of Urine Collection	Intravenous Administration			Time of Urine Collection	Intramuscular Administration		
	A	B	X		A	B	X
Minutes		Dog Q9-156		Minutes		Dog Q9-191	
0–1	96.5	0	3.5	0–20	25.3	13.7	61.0
1–3	38.5	0	61.5	20–40	25.7	19.7	60.1
3–7	26.6	5.4	68.0	40–60	13.0	22.5	64.5
7–20	13.6	25.4	61.0	60–80	11.9	26.0	62.6
20–30	9.8	33.4	56.8	80–105	14.5	19.9	65.6
30–50	6.8	29.5	63.8	105–180	10.1	21.3	68.6
50–90	0	30.3	69.7	180–300	10.8	22.1	67.1
90–180	11.1	21.8	67.1	300–420	4.6	29.2	66.3
180–300	0	38.8	61.2	420–480	1.6	10.6	87.8
300–420	0	24.9	75.1				
420–480	0	35.5	64.5				
Hours		Monkey 66		Hours		Monkey 65	
0–4	5.6	27.8	66.8	0–4	5.4	30.7	63.9
4–8	5.0	42.0	53.0	4–8	1.2	41.7	57.1
8–24	3.9	41.6	54.5	8–24	1.8	46.6	51.6
24–48	1.8	26.2	72.0	24–48	2.0	15.4	82.6
48–72	0.5	10.6	88.9	48–72	1.4	7.0	91.6

[a] A and B represent percent of urinary radioactivity associated with peaks A and B (described in text) calculated by measuring areas under the peaks seen in radioscans after TLC (Solvent System A) of ethyl acetate extracts of urine; X represents percent radioactivity associated with peak C in the same radioscans plus radioactivity in urine not extracted in ethyl acetate.

were extracted with ethyl acetate. The ethyl acetate extract accounted for 53% of the radioactivity in urine of the dog. In the monkeys and rats, the ethyl acetate urinary extracts accounted for 41 ± 7 and 90 ± 3% of the radioactivity, respectively.

Radioscans after TLC (Solvent System A) of these ethyl acetate extracts showed the three peaks of radioactivity noted previously, *i.e.*, A, B, and C. Peak B was the major peak in all three species. Based on the area under the peak, peak B accounted for 30, 29, and 72% of the radioactivity in the urine of the dog, monkey, and rat, respectively. Similarly, peak A accounted for 8, 2, and 17% in the dog, monkey, and rat, respectively.

Metabolites 1 and 2, associated with peaks A and B, respectively, were isolated from the urine of the three species, as described under *Experimental*.

Chromatography of a portion of Metabolite 1, isolated from urine samples of each species, showed a single peak of radioactivity, with R_f values of 0.45 and 0.40 in Solvent Systems A and B, respectively. Authentic triamcinolone acetonide had R_f values of 0.45 and 0.40 in Solvent Systems A and B, respectively.

Samples of Metabolite 1, isolated from urine of each species, were mixed with an authentic sample of triamcinolone acetonide and crystallized from ethyl acetate to constant specific activity (Table IX).

A low-resolution mass spectrum of Metabolite 1, isolated from the urine of rats, exhibited a fragmentation pattern similar to that of authentic triamcinolone acetonide. A molecular ion peak at *m/e* 434 and a base peak at *m/e* 375, resulting from the loss of $C_2H_3O_2$ (side chain) through cleavage between carbon atoms 17 and 20, were seen in the mass spectra of both triamcinolone acetonide and Metabolite 1. The mass spectrum of triamcinolone acetonide was discussed by Florey (13).

On the basis of data from chromatography, isotope dilution, and mass spectrometry, Metabolite 1 was identified as triamcinolone acetonide.

TLC of Metabolite 2 from the urine of rats showed a single radioactive peak with R_f values of 0.30 and 0.28 in Solvent Systems A and B, respectively. A triamcinolone acetonide standard had R_f values of 0.45 and 0.40 in these systems, respectively.

A mass spectrum of Metabolite 2 from the urine of rats showed a molecular ion peak at *m/e* 450 and an intense peak at *m/e* 391. If the peaks resulting from low molecular weight impurities (*m/e* 41–44) are excluded, the peak at *m/e* 391 was the most intense in the spectrum. The relative intensity of the molecular ion peak at *m/e* 450 was 6% that of the peak at *m/e* 391. Compared with the mass spectrum of triamcinolone acetonide, the spectrum of Metabolite 2 showed an increase of 16 amu which suggested addition of one oxygen atom. The mass spectrum also demonstrated an M−18 ion at *m/e* 432, corresponding to the loss of a hydroxy group through dehydration on electron impact. The mass spectrum of triamcinolone acetonide did not exhibit a significant M−18 ion, even though there are two hydroxy groups in the compound (13).

The 100-MHz NMR spectrum of Metabolite 2 was obtained in deuteromethanol containing tetramethylsilane as internal reference. Based on this analysis, the following proton assignments were made: C-1H, 2.63 τ (doublet, 10 Hz); C-2H, 3.72 τ (multiplet, 10 Hz); C-4H, 3.80 τ (multiplet); C-18H, 9.07 τ; C-19H, 8.25 τ; α-acetonide methyl, 8.59 τ; and β-acetonide methyl, 8.85 τ. The NMR spectrum of triamcinolone acetonide was discussed by Florey (13). Comparison of the two spectra indicated that the C-19 proton resonance had been shifted downfield by 0.21 ppm. The introduction of a hydroxy group in the *cis* 1,3-axial position to the axial methyl group will cause a downfield shift of this magnitude (14).

In a C-19 steroid, four positions can have a 1,3-diaxial relationship to the C-19 methyl group: C-2, C-6, C-8, and C-11. In triamcinolone acetonide, C-2 is not available and there already is a C-11 hydroxy in a 1,3-diaxial relationship to the C-19 methyl group. C-8 is excluded, because the C-8 β-hydroxy group causes a downfield

Table IX—Specific Activity after Isotopic Dilution of Metabolite 1 Isolated from Urine after Administration of Triamcinolone Acetonide-^{14}C-21-phosphate to Dogs, Monkeys, and Rats

Source of Metabolite	Metabolite 1, dpm	Unlabeled Triamcinolone Acetonide, mg	Calculated Specific Activity	Specific Activity after Crystallization, dpm/mg			
				1	2	3	4
Dog	4,000	7.3	545	624	469	456	470
Monkey	10,050	9.9	1015	1025	1010	1015	—
Rat	10,470	9.0	1115	1140	1125	1135	—

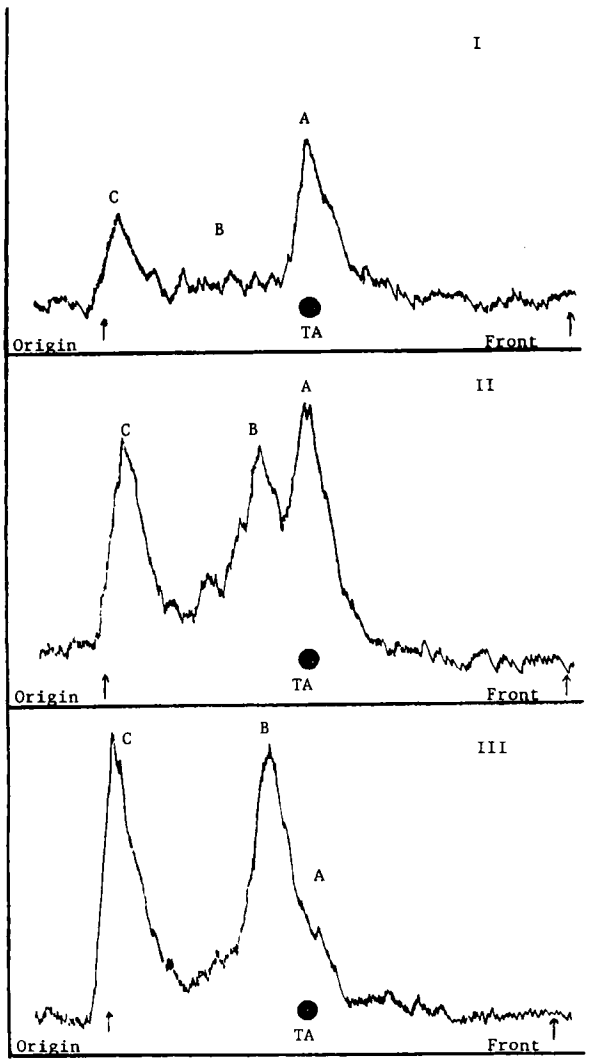
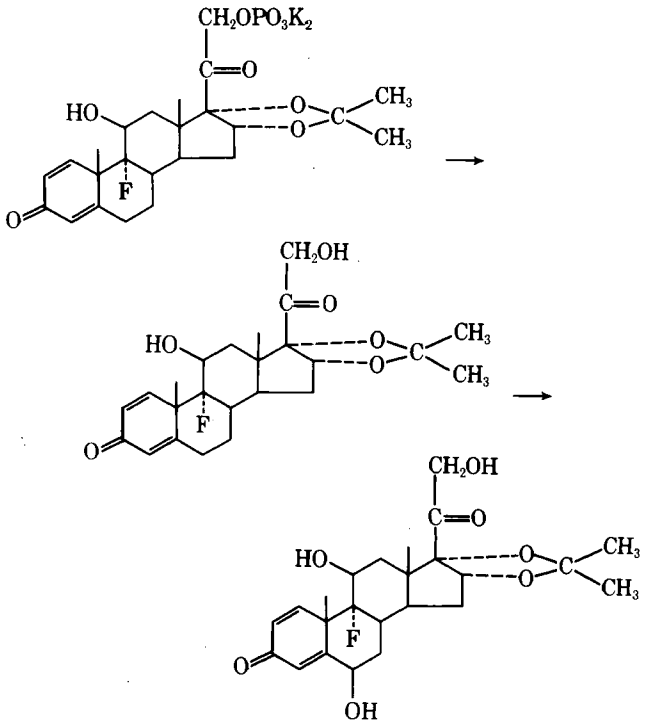


Figure 2—*Radioscans after TLC (Solvent System A) of ethyl acetate extracts of bile collected at various times after intravenous administration of triamcinolone acetonide-^{14}C-21-phosphate (1.2 mg/kg) to Dog Q9-156. Time of bile collection after dosing was: I, 6–15 min; II, 30–45 min; and III, 60–90 min. TA represents authentic triamcinolone acetonide chromatographed in the same solvent system.*



Scheme I—Biotransformations of triamcinolone acetonide-21-dipotassium phosphate

shift of both the C-18 and C-19 protons (15). Therefore, the downfield shift (0.21 ppm) of the C-19 protons must be due to a hydroxy group in the 6β-position.

Based on evidence from mass and NMR spectra, Metabolite 2, isolated from the urine of rats, was identified as 6β-hydroxytriamcinolone acetonide. Fifty-nine percent of the total urinary radioactivity was associated with the isolated compound, but this value is a minimum based only on the material recovered.

TLC of Metabolite 2 from the urine of the monkey showed a single radioactive peak in Solvent Systems A (R_f 0.30) and B (R_f 0.28). These R_f values were similar to those of 6β-hydroxytriamcinolone acetonide isolated from the rat urine. A portion of Metabolite 2 (6000 dpm) isolated from the monkey urine was mixed with an equal amount (6000 dpm) of 6β-hydroxytriamcinolone acetonide isolated from the rat urine. TLC of the mixture in Solvent Systems A, B, and C showed only a single radioactive peak in each system.

The mass spectrum of Metabolite 2 from the monkey urine was similar to that of 6β-hydroxytriamcinolone acetonide from the rat urine; both spectra showed a molecular ion peak at *m/e* 450 and a base peak at *m/e* 391.

On the basis of chromatographic characteristics and mass spectral analysis, Metabolite 2 from the monkey urine was identified as 6β-hydroxytriamcinolone acetonide. A total of 12% of the radioactivity in urine was recovered in the isolated 6β-hydroxytriamcinolone acetonide.

Metabolite 2 isolated from the urine of the dog had the same R_f values as did 6β-hydroxytriamcinolone acetonide in Solvent Systems A, B, and C. This metabolite was not obtained in a sufficiently pure form to allow analysis by mass spectrometry.

6β-Hydroxylation was reported (6) to occur in dogs and humans after dosing with triamcinolone and in studies *in vitro* (rat liver) with triamcinolone acetonide as the substrate (7). The results of the present study show that triamcinolone acetonide undergoes this biotransformation (6β-hydroxylation) in the rat and monkey and, apparently, in the dog as well.

Metabolites present in the urine as glucuronide conjugates accounted for 21, 6, and 4% of the radioactivity in urine of dogs, monkeys, and rats, respectively. In each species, incubation of the glucuronide fraction with β-glucuronidase yielded equal amounts of radioactive metabolites corresponding to peaks B and C (Solvent System A). Sulfate conjugates, which accounted for 11% of the radioactivity in the dog urine, remained at the origin when chromatographed in Solvent System A.

Characterization of Metabolites in Dog Bile—TLC (Solvent System A) of ethyl acetate extracts of bile samples that had been collected sequentially (Dog Q9-156) showed a pattern of radioactive peaks that was similar to that of ethyl acetate extracts of urine samples from the same dog; *i.e.*, with time, peak A decreased, whereas peaks B and C increased. This finding is illustrated by radioscans shown in Fig. 2 for bile samples collected at 15, 45, and 90 min after dosing.

Metabolites extracted into ethyl acetate (unconjugated fraction) accounted for 23% of the radioactivity in total bile collected during the first 8 hr after dosing. Another 23% of the remaining radioactivity was extracted into ethyl acetate after incubation with β-glucuronidase (glucuronide fraction). Radioscans after TLC (Solvent System A) showed peaks A, B, and C in the ratio of 1:3:2 for the unconjugated fraction and peaks B and C (4:1) for the glucuronide fraction. The TLC (Solvent System A) of radioactive material not extracted by ethyl acetate showed only peak C.

From the unconjugated fraction, Metabolite 1, which corresponded to peak A, was isolated by column chromatography, followed by preparative TLC (Solvent System A) of the radioactive material eluted in fractions 32–62 from the column. Metabolite 1 was identified as triamcinolone acetonide, based on evidence from TLC (Solvent Systems A and B), mass spectrometry, and isotope

dilution. Three percent of the total radioactivity in bile was associated with the isolated triamcinolone acetonide.

A metabolite corresponding to peak B in the unconjugated fraction was eluted from the silica gel plate; it had the same R_f value as that of 6β-hydroxytriamcinolone acetonide in Solvent Systems A and B.

The sequence of biotransformations is shown in Scheme I.

CONCLUSIONS

Within 10–15 min after intramuscular administration of triamcinolone acetonide-^{14}C-21-phosphate to dogs, monkeys, and rats, levels of radioactivity in blood and other tissues were approximately equal to those obtained after intravenous administration. These results indicate that triamcinolone acetonide-21-phosphate is absorbed rapidly from the site of intramuscular injection.

Plasma half-life values of 0.5–2 hr in dogs, monkeys, and rats and the rate of excretion in a bile-cannulated dog ($t_{1/2}$ = 2 hr) suggest a short biological half-life for triamcinolone acetonide-21-phosphate after intramuscular or intravenous administration. Excretion *via* the bile seems to be the major route of drug elimination; in all species studied, excretion in the feces after intramuscular or intravenous administration was higher than that in the urine, although the ratio of fecal to urinary excretion varied among the species. Reabsorption of the drug after its excretion in the bile did not appear to take place in the dog.

In the rat, only 2–3% of the ^{14}C label in the acetonide portion of the molecule appeared as $^{14}CO_2$. Most of the remaining radioactivity (81–94%) was recovered in the urine and feces. These results indicate that hydrolysis of triamcinolone acetonide to give acetone-^{14}C and, therefore, a subsequent yield of $^{14}CO_2$ is not a significant metabolic reaction in the rat. Although $^{14}CO_2$ was not measured in dogs or monkeys, 95 and 85%, respectively, of the labeled dose was recovered in urine and feces, suggesting the existence of a metabolic pathway similar to that in the rat.

Triamcinolone acetonide-21-phosphate was rapidly and extensively hydrolyzed to triamcinolone acetonide after intramuscular or intravenous administration. This hydrolysis apparently occurred prior to any other metabolic transformation. Unchanged triamcinolone acetonide-21-phosphate was not found in plasma, urine, or bile samples collected as early as 1–5 min after its administration to dogs, monkeys, and rats.

Hydrolysis of triamcinolone acetonide phosphate also could be determined *in vitro* in dog plasma and rat muscle. Triamcinolone acetonide was identified in the plasma and urine of all species studied and in the bile of the dog. 6β-Hydroxytriamcinolone acetonide was a major metabolite in the urine of rats and monkeys and apparently in the urine of dogs as well.

The presence of 6β-hydroxytriamcinolone acetonide as a major metabolite in the urine of rats and monkeys and the absence of any metabolites resulting from the reduction of Δ^4-3-keto or C-20-keto groups in any of the species studied indicate that the Δ^1, 9α-fluoro, and 16,17-acetonide substituents may block the normal reduction reactions undergone by hydrocortisone. Similar results were reported by other investigators for different corticosteroids possessing Δ^1 or 9α-fluoro substituents (16, 17).

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Metabolism of Minoxidil, a New Hypotensive Agent I: Absorption, Distribution, and Excretion following Administration to Rats, Dogs, and Monkeys

RICHARD C. THOMAS ˣ, RICHARD S. P. HSI, HARRY HARPOOTLIAN, and RAY W. JUDY

Abstract □ Minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide), a potent new hypotensive agent, was ^{14}C-labeled in the 2-position of the pyrimidine ring in 17% yield from $Ba^{14}CO_3$. This material was used to study the absorption, distribution, and excretion of the drug in rats, dogs, and monkeys. Following oral administration of a single dose, the drug was rapidly and well absorbed and rapidly eliminated by each species as judged by plasma levels and urinary excretion of unchanged drug and total drug-related materials. Chronic oral administration of the drug at a high level (10 mg/kg) for 30 days slightly increased the rate of clearance of minoxidil and minoxidil-related material from circulation. Water diuresis, resulting from water loading of dogs, caused an even greater increase in the rates of disappearance of the drug and drug-related material. Whole-body autoradiography studies in rats showed that minoxidil was rapidly distributed following its oral and intravenous administration. It was subsequently concentrated, primarily in the excretory system. Minoxidil-related material was detected in aorta walls, but not in the CNS, following both routes of drug administration.

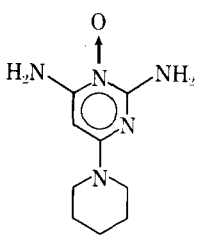
Keyphrases □ Minoxidil, ^{14}C-labeled—absorption, distribution, and excretion after oral and intravenous administration, rats, dogs, and monkeys □ Absorption—^{14}C-labeled minoxidil after oral and intravenous administration, rats, dogs, and monkeys □ Distribution—^{14}C-labeled minoxidil after oral and intravenous administration, rats, dogs, and monkeys □ Excretion—^{14}C-labeled minoxidil after oral and intravenous administration, rats, dogs, and monkeys

Minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide, I) is a potent, orally active hypotensive agent. Studies in laboratory animals indicate that its activity is due primarily to a direct relaxant effect on peripheral vascular smooth muscle (1). It has been shown to be orally active in humans (2, 3).

As part of the development of this drug, studies were undertaken in several animal species to determine which most resembles the human in its metabolism of minoxidil. This report is concerned with the preparation of ^{14}C-labeled minoxidil and a study of its disposition, as shown by measurements of unchanged drug and total radioactivity, in three animal species: the rat, dog, and monkey.

EXPERIMENTAL

Preparation of ^{14}C-Labeled Drug—Labeled minoxidil was prepared by a six-step synthetic sequence from barium carbonate-^{14}C in 17% overall yield. Barium carbonate-^{14}C was converted to guanidine-^{14}C hydrochloride *via* barium cyanamide-^{14}C in 84% yield by the method of Bennett (4). Guanidine-^{14}C hydrochloride was then converted to 2,4-diamino-6-hydroxypyrimidine-2-^{14}C monohydrate in 77% yield by the method of VanAllan (5).



I

The ^{14}C-labeled hydroxypyrimidine was heated with phosphorus oxychloride, essentially as described by Israel *et al.* (6) to prepare 2,4-diamino-6-chloropyrimidine-2-^{14}C. By carefully heating the reaction mixture in an oil bath at 95–100° rather than under reflux, a reproducible yield of 69% was obtained in this step. The ^{14}C-labeled chloropyrimidine was oxidized with an equimolar quantity of *m*-chloroperbenzoic acid in ethanol at 3° for 3 hr.

The resulting precipitate of 2,4-diamino-6-chloropyrimidine-2-^{14}C 3-oxide, as the *m*-chlorobenzoic acid salt, was filtered, washed successively with cold ethanol and ether, and air dried. This material, having a specific activity of 1.71 mCi/mmole, was obtained in 68% yield. Its melting point was 199–200° dec. and its IR, UV, and NMR spectra were consistent with the proposed structure.

Anal.—Calc. for $C_{11}H_{10}Cl_2N_4O_3$: C, 41.66; H, 3.18; Cl, 22.36; N, 17.67. Found: C, 41.42; H, 3.41; Cl, 22.25; N, 17.71.

The *m*-chlorobenzoic acid salt of ^{14}C-labeled 2,4-diamino-6-chloropyrimidine 3-oxide (2.72 g) was mixed with 25 ml of piperidine, and the mixture was heated with stirring under nitrogen in an oil bath at 130° to give a clear solution. The solution was refluxed for 1.5 hr, during which time a precipitate formed. After standing overnight at room temperature, the precipitate was filtered. The crude product was washed three times in the filter funnel with 1.5 *N* sodium hydroxide, followed by repeated washing with water. After air drying, 1.10 g (62%) of ^{14}C-minoxidil, 2,4-diamino-6-piperidinopyrimidine-2-^{14}C 3-oxide, having a specific activity of 1.72 mCi/mmole, was obtained. Its melting point was 262–262.5° dec. and its IR, UV, and NMR spectra corresponded to those of authentic minoxidil.

Anal.—Calc. for $C_9H_{15}N_5O$: C, 51.66; H, 7.23; N, 33.46. Found: C, 51.42; H, 7.36; N, 33.42.

When chromatographed in paper and thin-layer systems, the product and its associated radioactivity had mobilities identical to that of authentic minoxidil.

Dosage of Animals and Collection of Samples—Each of three female rats[1], weighing approximately 200 g, was given 1.01 mg (8.2 μCi) of ^{14}C-minoxidil in 1 ml of water by oral intubation. One hour prior to and immediately following administration of the drug, each rat was given 10 ml of deionized water by oral intubation. The

Table I—Urinary Excretion of Minoxidil and Total Radioactivity following Oral Administration of ^{14}C-Minoxidil to the Rat[a]

Hours	Average Percent of Dose Excreted in Urine ± *SD*	
	Total Radioactivity	Minoxidil
0–1	27.8 ± 1.1	15.1 ± 2.4
1–5	38.4 ± 9.9	12.2 ± 7.5
5–11	17.4 ± 11.8	3.0 ± 3.3
11–24	3.49 ± 1.3	0.6 ± 0.6
24–48	0.79 ± 0.74	—
48–72	0.23 ± 0.09	—
Total	88.1 ± 2.6	—

[a] Three animals.

[1] Sprague–Dawley (Upjohn strain).

rats were housed in metabolism cages, designed for the separation and collection of urine and feces, and samples were collected at predetermined intervals for 72 hr. Water and food[2] were permitted *ad libitum*.

In a separate study, each of two 120-g male rats[1], which had been fitted, under ether anesthesia, with bile-duct cannulas [polyethylene (PE-10) tubing], was given 2.55 mg (20.8 μCi) of ^{14}C-minoxidil in 2 ml of water by oral intubation. The rats were placed in restraining cages, and bile was collected continuously for 40 hr in 4-hr fractions. Water was permitted *ad libitum*.

Each of three female dogs[3], weighing approximately 9 kg, was given 5.0 mg (41.0 μCi) of ^{14}C-minoxidil in 10 ml of water by oral intubation. One-half hour before, immediately following, and 1 and 2 hr following administration of the drug, each dog was given 100 ml of deionized water by oral intubation. The dogs were housed in metabolism cages, designed for the separation and collection of urine and feces, and samples were collected at predetermined intervals for 168 hr.

Urine was obtained by catheter up to and including the 16-hr sample and combined with the appropriate voided sample. Blood samples (7 ml) were collected by jugular venipuncture in tubes containing potassium oxalate as an anticoagulant at predetermined times for 168 hr. Except for a 4-hr period following drug administration, water was permitted *ad libitum*. Food[4] was given 12 hr following drug administration and once a day thereafter.

In a separate study, each of four dogs[3], weighing approximately 11 kg, was given a 1.00-mg/kg dose of ^{14}C-minoxidil (8.2 μCi/mg) in 10 ml of water by oral intubation. On each of the next 30 days, each dog was given a 10-mg/kg oral dose of nonradioactive minoxidil in a hard gelatin capsule. This dose was followed on the 31st day with another 1.00-mg/kg dose of ^{14}C-minoxidil. The dogs were kept in metabolism cages during the entire period.

Separate urine collections 0–12 and 12–24 hr following administrations of the labeled drug were made for each dog. Blood samples were collected at predetermined times for 12 hr following labeled drug administrations. Fecal collections were not made.

Each of three male rhesus monkeys, weighing approximately 4 kg, was given a 1.00-mg/kg dose of ^{14}C-minoxidil (8.2 μCi/mg) in 10 ml of water by intubation through a nose tube. The monkeys were placed in restraining chairs, designed for the separate collection of urine and feces, and samples were collected at predetermined intervals for 72 hr.

Blood samples (5 ml) were collected by venipuncture of the femoral vein in the inner midthigh region at predetermined times for 48 hr. The blood was transferred from the syringe to a tube containing potassium oxalate immediately after collection. Water was permitted *ad libitum*, and food[5] was given 8 hr after drug administration and once a day thereafter.

Measurement of Radioactivity in Biological Samples—Blood samples were centrifuged at 1100×*g* for 30 min at 5° to obtain plasma. Feces samples were homogenized with water to a suitable consistency using a blender[6] or homogenizer[7]. Plasma, urine, feces, and bile samples were stored in the frozen state prior to analysis. All samples were counted in liquid scintillation spectrometers[8] interfaced to keypunches[9]. Computations were performed with a computer[9].

Duplicate 0.1- or 0.5-ml aliquots of urine and bile were counted in 15 ml of counting solvent [toluene–dioxane–methanol (350:350:210 v/v) containing 73 g of naphthalene, 4.6 g of 2,5-diphenyloxazole, and 0.080 g of 1,4-bis-2-(5-phenyloxazolyl)benzene/liter]. Counting efficiencies were determined by the internal standard technique using toluene-^{14}C. Duplicate 0.2- or 0.5-ml blood plasma samples and duplicate aliquots, approximately 0.5 g each, of feces homogenates were dried in cellophane sacs at room temperature, combusted[10] (8), and counted. Counting efficiencies were determined by the external standard technique.

[2] Purina rat chow.
[3] Beagle (Upjohn strain).
[4] Purina dog chow.
[5] Purina monkey chow.
[6] Waring Blendor, Waring Products Division, Dynamics Corporation of America, New Hartford, Conn.
[7] Virtis "45," The Virtis Co., Gardiner, N.Y.
[8] Model 3375, Packard Instrument Co., Downers Grove, Ill.
[9] Models 026 and 029 keypunches and model 360/50 computer, International Business Machines, White Plains, N.Y.
[10] With a Peterson-NIH oxidizer.

Table II—Fecal Excretion of Radioactivity following Oral Administration of ^{14}C-Minoxidil to the Rat[a]

Hours	Average Percent of Dose Excreted in Feces ± *SD*
0–24	5.24 ± 1.03
24–48	0.37 ± 0.07
48–72	0.21 ± 0.16
Total	5.8 ± 1.2

[a] Three animals.

Table III—Biliary Secretion of Minoxidil and Total Radioactivity following Oral Administration of ^{14}C-Minoxidil to Bile Duct-Cannulated Rats[a]

	Average Percent of Dose Secreted in Bile	
Hours	Total Radioactivity	Minoxidil
0–4	8.8	0.34
4–8	2.25	0.057
8–12	1.03	—
12–16	0.383	—
16–20	0.182	—
20–24	0.0478	—
24–28	0.0215	—
28–32	0.0167	—
32–36	0.0135	—
36–40	0.0125	—
Total	12.8	—

[a] Two animals.

Preparation of Samples for Chromatography—Samples of urine, blood plasma, and bile containing sufficient radioactivity were applied directly to the paper for chromatography, usually as 2.5-cm streaks. Samples containing lower levels of radioactivity were lyophilized, and the residues were reconstituted at a greater concentration with water or were leached three times with methanol.

The methanol was removed with a nitrogen stream, and the residue was dissolved in a small volume of 1-butanol–methanol (1:2 v/v) for application to paper in a manner similar to that used for whole urine and blood plasma. The leaching procedure resulted in chromatographic metabolite patterns quantitatively identical to those obtained by direct chromatography of whole urine, blood plasma, and bile.

Paper Chromatography and TLC—Paper chromatography was carried out on 86 × 15-cm sheets[11] by a descending technique in the 1-butanol–piperidine–water (82:2:16 v/v) system (System I). Occasionally, the 1-butanol–acetic acid–water (4:1:1 v/v) system (System II) was used. TLC was carried out on 20 × 5-cm films of silica gel, 0.25 mm thick, in the benzene–methanol–ammonium hydroxide (1:1:0.01 v/v) system (System III).

Standard minoxidil was run on each chromatogram. In certain cases, minoxidil was mixed with samples prior to chromatography to act as an internal standard. Following solvent development, the chromatograms were viewed under UV light to locate the standard and, when possible, drug-related materials by their UV absorption.

Quantification of Minoxidil by Radiochromatogram Scanning—Paper and thin-layer chromatograms, when they contained sufficient radioactivity, were scanned for radioactivity using chromatogram scanners[12] having Geiger detectors. The resulting chart-strip tracings were integrated with a mechanical planimeter[13] or with an electronic digitizer system[14] to determine quantitatively the relative distribution of radioactivity along the length of the chromatogram.

When a chromatogram did not contain sufficient radioactivity for quantification by Geiger detection, it was cut into sequential

[11] Whatman No. 2 paper, W. and R. Balston, Ltd., London, England.
[12] Model 880 paper and model 885 thin-layer scanners, Vanguard Instrument Co., North Haven, Conn.
[13] Model 700AR Lasico, Los Angeles Scientific Instruments Co., Los Angeles, Calif.
[14] Model 9864 digitizer and model 9810 calculator, Hewlett-Packard Corp., Loveland, Colo.

Table IV—Plasma Levels and Areas under Plasma Level Curves for Minoxidil and Total Radioactivity following Oral Administration of ^{14}C-Minoxidil to the Monkey at a Level of 1 mg/kg[a]

Hours	Average Plasma Concentration ± *SD*, μg/ml		Cumulative Average Area under Plasma Level Curve ± *SD*, hr × μg/ml	
	Total Radioactivity	Minoxidil	Total Radioactivity	Minoxidil
0.25	0.343 ± 0.144	0.037 ± 0.021	0.0429 ± 0.0181	0.005 ± 0.003
0.5	0.490 ± 0.221	0.037 ± 0.014	0.147 ± 0.062	0.014 ± 0.007
1	0.371 ± 0.100	0.028 ± 0.002	0.340 ± 0.180	0.026 ± 0.011
4	0.123 ± 0.046	0.013 ± 0.005	1.17 ± 0.33	0.102 ± 0.033
8	0.0324 ± 0.0160	0.0028 ± 0.0025	1.48 ± 0.43	0.133 ± 0.048
14	0.0058 ± 0.0029	—	1.58 ± 0.69	—
24	0.0014 ± 0.0010	—	1.62 ± 0.70	—
48	0.0006 ± 0.0002	—	1.64 ± 0.72	—

[a] Three animals.

segments (1 cm in the case of paper chromatograms and 0.5 cm in the case of thin-layer chromatograms). Then the segments, each in a vial with 10 ml of counting solvent containing 3% water, were counted by liquid scintillation spectrometry. The resulting raw data were processed by the computer system to give a plot of the distribution of radioactivity along the length of the chromatogram, in a format similar to that of a chart-strip tracing, together with a tabular printout of derived data. Based on the plot, the tabular data were used to quantify the relative distribution of radioactivity along the length of the chromatogram.

Whole-Body Autoradiography—Each of 10 male rats[1], weighing approximately 110 g, was given 2.25 mg (18.2 μCi) of ^{14}C-minoxidil, five by oral intubation and five by intravenous administration in the tail vein. At intervals of 0.5, 2, 4, 8, and 24 hr following oral administration and 1 and 10 min and 0.5, 2, and 4 hr following intravenous administration, the rats were quickly frozen by immersion in dry ice-cooled acetone while under ether anesthesia. The rats were then embedded by freezing in blocks of 4% aqueous carboxymethylcellulose sodium and sectioned for whole-body autoradiography essentially as described by Ullberg (7).

Sections 30 μm thick were collected on cellophane tape[15] as they were being cut with a microtome[16] equipped with a 55° tungsten carbide-tipped knife[16] at −20° in a cryostat[17]. The sections were allowed to dehydrate in the cryostat and then were placed in contact with X-ray film[18] for 1–6 weeks at −15°. The film was developed by standard methods.

RESULTS

As shown in Tables I and II, the rat eliminated minoxidil rapidly, primarily *via* urine. Following a 5-mg/kg oral dose of ^{14}C-minoxidil, 88% of the radioactivity was excreted in urine and 5.8% was excreted in the feces. More than 80% of the dose was accounted for in urine during the 11 hr immediately following drug administration. Approximately 35% of the drug-related material excreted in urine was minoxidil. Bile duct-cannulated rats (Table III) secreted an average of 12.8% of a 21-mg/kg oral dose of ^{14}C-minoxidil in bile over 40 hr. Most of this amount (11% of the dose) was secreted during the 8 hr following drug administration. Less than 4% of the drug-related material secreted in bile during this 8-hr period was minoxidil.

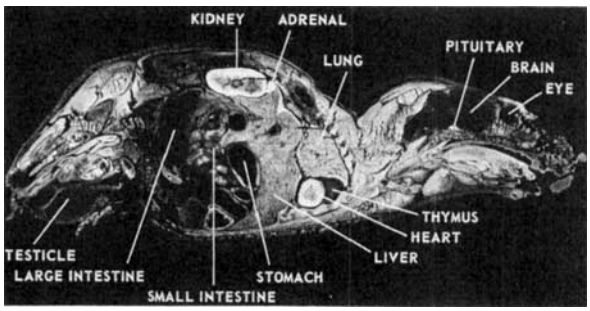


Figure 1—*Distribution of radioactivity (light areas) in a rat 1 min following intravenous administration of ^{14}C-minoxidil. Film was exposed to the tissue slice for 1 week.*

Plasma levels of minoxidil, total drug-related material, and areas under plasma level curves (expressed as microgram-equivalents of drug) following oral administration of 1 mg of minoxidil/kg to monkeys are presented in Table IV. An average peak plasma level of 0.037 μg of minoxidil/ml was attained 0.25–0.5 hr after drug administration. Minoxidil and total radioactivity levels in plasma decreased rapidly thereafter. Unchanged drug accounted for only about 10% of the total drug-related material in circulation.

Minoxidil disappeared from plasma with a 1.9-hr average half-life during the 8-hr interval following attainment of the peak plasma level. Excretion of minoxidil by the monkey also was rapid and primarily *via* urine (Tables V and VI). Approximately 94% of the dose was excreted in urine and 2.68% was excreted in feces. More than 90% of the dose was excreted in urine during the 14 hr immediately following drug administration. Approximately 10% of the drug-related material excreted in urine was minoxidil.

Plasma levels of minoxidil, total drug-related material, and areas under plasma level curves (expressed as microgram-equivalents of drug) following oral administration of 0.55 mg ^{14}C-minoxidil/kg to water-loaded dogs are presented in Table VII. An average peak plasma level of 0.37 μg of minoxidil/ml was observed at the first sampling time, 15 min after drug administration.

Minoxidil disappeared from plasma with a 0.8-hr average half-life during the 5-hr period following the peak plasma level. Excretion of radioactivity by the dog was rapid and primarily *via* urine (Tables VIII and IX). Nearly 91% of the dose was excreted in urine

Table V—Urinary Excretion of Minoxidil and Total Radioactivity following Oral Administration of ^{14}C-Minoxidil to the Monkey[a]

Hours	Average Percent of Dose Excreted in Urine	
	Total Radioactivity	Minoxidil
0–4	66.9 ± 6.8	5.4 ± 1.6
4–8	18.5 ± 6.3	3.6 ± 2.4
8–14	7.1 ± 1.4	0.66 ± 0.47
14–24	1.13 ± 0.23	—
24–48	0.47 ± 0.04	—
48–72	0.055 ± 0.018	—
Total	94.2 ± 0.3	—

[a] Three animals.

Table VI—Fecal Excretion of Radioactivity following Oral Administration of ^{14}C-Minoxidil to the Monkey[a]

Hours	Average Percent of Dose Excreted in Feces ± *SD*
0–24	0.006 ± 0.008
24–72	1.99 ± 0.35
72–96	0.69 ± 0.18
Total	2.68 ± 0.16

[a] Three animals.

[15] Scotch Magic mending tape, No. 810, 3M Co., St. Paul, Minn.
[16] Jung, Model K, William J. Hacker and Co., West Caldwell, N.J.
[17] Harris Manufacturing Co., Cambridge, Mass.
[18] Kodak No-Screen X-ray film, Eastman Kodak Co., Rochester, N.Y.

Table VII—Plasma Levels and Areas under Plasma Level Curves for Minoxidil and Total Radioactivity following Oral Administration of ^{14}C-Minoxidil to the Dog at a Level of 0.55 mg/kg[a]

Hours	Average Plasma Concentration ± *SD*, μg/ml		Cumulative Average Area under Plasma Level Curve ± *SD*, hr × μg/ml	
	Total Radioactivity	Minoxidil	Total Radioactivity	Minoxidil
0.25	0.675 ± 0.093	0.37 ± 0.07	0.084 ± 0.012	0.047 ± 0.008
0.75	0.584 ± 0.053	0.19 ± 0.01	0.399 ± 0.048	0.187 ± 0.024
1.25	0.494 ± 0.036	0.11 ± 0.02	0.67 ± 0.06	0.261 ± 0.017
1.75	0.398 ± 0.038	0.083 ± 0.024	0.89 ± 0.07	0.310 ± 0.011
2.25	0.322 ± 0.038	0.048 ± 0.020	1.07 ± 0.09	0.343 ± 0.018
2.75	0.251 ± 0.035	0.031 ± 0.014	1.21 ± 0.11	0.363 ± 0.026
3.5	0.175 ± 0.025	0.013 ± 0.005	1.37 ± 0.12	0.379 ± 0.032
5	0.098 ± 0.020	0.0053 ± 0.0041	1.58 ± 0.15	0.303 ± 0.039
8	0.0385 ± 0.0048	0.0016 ± 0.0011	1.78 ± 0.18	0.403 ± 0.046
12	0.0174 ± 0.0017	—	1.89 ± 0.19	—
16	0.0104 ± 0.0017	—	1.95 ± 0.19	—
24	0.0050 ± 0.0003	—	2.01 ± 0.19	—
36	0.0021 ± 0.0006	—	2.06 ± 0.19	—
48	0.0012 ± 0.0002	—	2.08 ± 0.19	—
72	0.0006 ± 0.0001	—	2.10 ± 0.19	—
96	0.0004 ± 0.0002	—	2.11 ± 0.19	—
120	0.0006 ± 0.0002	—	2.13 ± 0.19	—
144	0.0005 ± 0.0001	—	2.14 ± 0.19	—
168	0.0004 ± 0.0001	—	2.15 ± 0.19	—

[a] Three animals.

and 7.1% was excreted in feces. More than 80% of the dose was excreted in urine during the 12 hr immediately following drug administration. Approximately 13% of the drug-related material excreted in urine was minoxidil.

Plasma levels of minoxidil, total drug-related material, and areas under plasma level curves (expressed as microgram-equivalents of minoxidil) following oral administration of 1 mg of ^{14}C-minoxidil/kg to dogs before and after 30 successive 10-mg/kg daily oral doses of minoxidil are presented in Table X. Before chronic drug treatment, an average peak plasma level of 0.84 μg of minoxidil/ml was observed at the first sampling time, 15 min after drug administration; the corresponding value after chronic drug treatment was 0.535 μg/ml and occurred about 45 min following administration of the labeled drug.

For approximately 5 hr following attainment of the peak plasma level, minoxidil disappeared with an average half-life of 1.2 hr prior to, and 1.0 hr following, chronic drug treatment. The corresponding areas under the plasma level curves are presented in Table XI. The cumulative areas under the minoxidil-level curves during the 12 hr following drug administration were 1.27 hr × μg/ml prior to, and 1.08 hr × μg/ml following, chronic drug treatment.

Urinary excretion of radioactivity during this chronic study is presented in Table XII. Both before and after chronic drug treatment, approximately 89% of the radioactive dose was excreted in urine during the 24 hr following administration of the labeled drug. In each case, more than 80% of the dose was excreted during the first 12 hr. Approximately 18% of the drug-related material excreted in urine was minoxidil in both cases.

Within 1 min after injecting the rat intravenously with ^{14}C-minoxidil, radioactivity had penetrated into most tissues (Fig. 1). The distribution was relatively uniform except for a notable lack of radioactivity in the brain, testicles, stomach, large intestine, spleen, and bone. The level of radioactivity was low in the thymus. Ten minutes after intravenous injection (Fig. 2), levels of radioactivity were highest in the kidneys, liver, bladder, and aorta walls, although most other tissues, including the testicles and stomach, contained moderate concentrations of radioactivity. Little, if any, radioactivity was present in the brain and large intestine.

One-half hour following intravenous injection (Fig. 3), radioactivity levels were relatively high in the liver, kidneys, small intestine, bladder, and aorta walls. Little radioactivity remained in other tissues, including blood, and none was apparent in the brain. Four hours following intravenous administration, levels of radioactivity were relatively high in the large intestine and bladder, moderate in the liver, low in the kidneys, and not detectable in most other tissues, including the brain and blood.

One-half hour after oral administration of ^{14}C-minoxidil to the rat, the radioactivity (Fig. 4) was most concentrated in the kidneys, stomach, bladder, and small intestine. The liver and aorta walls had nearly as high a concentration. Other tissues had a rather uniform concentration but to a lesser degree. The brain was de-

Table VIII—Urinary Excretion of Minoxidil and Total Radioactivity following Oral Administration of ^{14}C-Minoxidil to the Dog[a]

Hours	Average Percent of Dose Excreted in Urine ± *SD*	
	Total Radioactivity	Minoxidil
0–0.5	11.3 ± 2.7	4.0 ± 0.9
0.5–1	12.6 ± 3.0	3.0 ± 0.8
1–1.5	13.0 ± 1.1	1.9 ± 0.5
1.5–2	8.9 ± 0.9	1.0 ± 0.3
2–2.5	6.7 ± 0.4	0.5 ± 0.2
2.5–3	5.9 ± 0.6	0.4 ± 0.3
3–4	7.4 ± 1.6	0.4 ± 0.3
4–6	8.4 ± 1.3	0.2 ± 0.2
6–8	4.20 ± 0.79	0.03 ± 0.1
8–12	4.50 ± 0.54	0.05 ± 0.1
12–16	2.85 ± 0.95	0.05 ± 0.1
16–24	2.29 ± 0.36	—
24–36	1.10 ± 0.98	—
36–48	0.90 ± 0.73	—
48–72	0.41 ± 0.21	—
72–96	0.103 ± 0.024	—
96–120	0.065 ± 0.026	—
120–144	0.038 ± 0.010	—
144–168	0.042 ± 0.032	—
Total	90.7 ± 2.3	—

[a] Three animals.

Table IX—Fecal Excretion of Radioactivity following Oral Administration of ^{14}C-Minoxidil to the Dog[a]

Hours	Average Percent of Dose Excreted in Feces ± *SD*
0–24	0.016 ± 0.014
24–48	5.92 ± 2.34
48–72	0.89 ± 0.82
72–96	0.127 ± 0.099
96–120	0.068 ± 0.077
120–144	0.044 ± 0.046
144–168	0.019 ± 0.020
Total	7.1 ± 3.1

[a] Three animals.

Table X—Plasma Levels of Minoxidil and Total Radioactivity following Oral Administration of ^{14}C-Minoxidil at a Level of 1 mg/kg to the Dog before and after Chronic Administration of Minoxidil[a]

	Average Plasma Concentration ± *SD*, μg/ml			
	Before		After	
Hours	Total Radioactivity	Minoxidil	Total Radioactivity	Minoxidil
0.25	1.29 ± 0.14	0.84 ± 0.27	0.466 ± 0.227	0.333 ± 0.159
0.75	1.20 ± 0.14	0.51 ± 0.05	1.07 ± 0.21	0.535 ± 0.081
1.25	1.05 ± 0.10	0.35 ± 0.04	1.07 ± 0.13	0.390 ± 0.038
1.75	0.93 ± 0.12	0.23 ± 0.07	0.96 ± 0.10	0.260 ± 0.032
2.25	0.80 ± 0.16	0.18 ± 0.03	0.82 ± 0.07	0.190 ± 0.037
2.75	0.71 ± 0.18	0.14 ± 0.03	0.68 ± 0.05	0.135 ± 0.041
3.5	0.63 ± 0.21	0.10 ± 0.01	0.51 ± 0.057	0.080 ± 0.030
5.0	0.417 ± 0.168	0.039 ± 0.005	0.293 ± 0.052	0.030 ± 0.015
8.0	0.172 ± 0.077	0.007 ± 0.002	0.118 ± 0.029	0.007 ± 0.004
12.0	0.067 ± 0.028	0.001 ± 0.001	0.050 ± 0.009	0.001 ± 0.002

[a]Four animals.

Table XI—Areas under Minoxidil and Total Radioactivity Plasma Level Curves following Oral Administration of ^{14}C-Minoxidil to the Dog before and after Chronic Administration of Minoxidil[a]

	Average Cumulative Area under Plasma Level Curve ± *SD*, hr × μg/ml			
	Before		After	
Hours	Total Radioactivity	Minoxidil	Total Radioactivity	Minoxidil
0.25	0.161 ± 0.055	0.105 ± 0.034	0.058 ± 0.028	0.042 ± 0.020
0.75	0.784 ± 0.195	0.444 ± 0.113	0.442 ± 0.137	0.258 ± 0.079
1.25	1.35 ± 0.25	0.662 ± 0.122	0.98 ± 0.22	0.489 ± 0.102
1.75	1.84 ± 0.30	0.809 ± 0.133	1.48 ± 0.27	0.652 ± 0.106
2.25	2.27 ± 0.35	0.911 ± 0.148	1.93 ± 0.30	0.764 ± 0.103
2.75	2.65 ± 0.43	0.092 ± 0.160	2.30 ± 0.30	0.846 ± 0.098
3.5	3.15 ± 0.57	1.08 ± 0.163	2.75 ± 0.31	0.926 ± 0.089
5.0	3.94 ± 0.84	1.19 ± 0.165	3.36 ± 0.30	1.01 ± 0.088
8.0	4.82 ± 1.20	1.26 ± 0.166	3.97 ± 0.30	1.06 ± 0.097
12.0	5.30 ± 1.41	1.27 ± 0.161	4.31 ± 0.33	1.08 ± 0.104

[a]Four animals.

void of radioactivity. The relative distribution of radioactivity 2 hr following oral drug administration (Fig. 5) was similar to that at 30 min. However, the levels of radioactivity in the stomach and kidneys had decreased somewhat.

Four hours following oral drug administration (Fig. 6), radioactivity levels were relatively high in the bladder, the large intestine, and some sections of the small intestine; moderate in the stomach, liver, and kidneys; very low in most other tissues; and lacking in the brain. Eight hours following oral administration, the large intestine had the highest levels of radioactivity relative to other tissues. The liver and kidneys, as well as the compact portion of bone, had somewhat lower levels. Most other tissues contained very little radioactivity; none was detectable in the brain.

Twenty-four hours following oral drug administration (Fig. 7), the large intestine contained the highest level of radioactivity. Somewhat lower levels were observed in the stomach and in the compact portion of bone. The liver and kidneys had very low levels; other tissues, including the brain, appeared to be devoid of radioactivity.

DISCUSSION

As judged by plasma levels and excretion of minoxidil and by total radioactivity, labeled minoxidil, administered as a single oral dose, was well absorbed and rapidly cleared from the circulation and the body by the rat, dog, and monkey. Each species absorbed nearly 90% or more of the drug based on the amount of radioactivity excreted in urine. More than 80% of the administered drug was cleared from the body of each species within 12 hr. With the monkey and dog, peak plasma levels of minoxidil were attained 15–45 min following drug administration. The disappearance of minoxidil from plasma was rapid, corresponding to average half-lives of 1.9 and 1.2 hr for the monkey and dog, respectively. This difference in half-lives is significant ($p < 0.10$ by t test).

Minoxidil was at least as well absorbed by the human (9), with 97% of the radioactivity associated with an orally administered dose of labeled drug appearing in urine. It was cleared somewhat more slowly from circulation by the human, with a plasma half-life

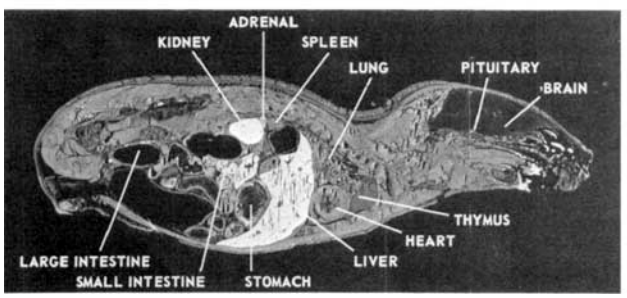


Figure 2—*Distribution of radioactivity (light areas) in a rat 10 min following intravenous administration of ^{14}C-minoxidil. Film was exposed to the tissue slice for 1 week.*

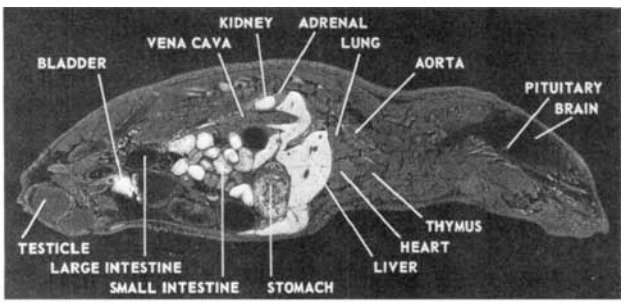


Figure 3—*Distribution of radioactivity (light areas) in a rat 30 min following intravenous administration of ^{14}C-minoxidil. Film was exposed to the tissue slice for 1 week.*

Table XII—Urinary Excretion of Minoxidil and Total Radioactivity following Oral Administration of ^{14}C-Minoxidil to the Dog before and after Chronic Administration of Minoxidil[a]

	Average Percent of Dose Excreted in Urine ± *SD*			
	Before		After	
Hours	Total Radioactivity	Minoxidil	Total Radioactivity	Minoxidil
0–12	81.0 ± 2.9	17.1 ± 5.1	81.3 ± 4.5	18.5 ± 2.2
12–24	8.17 ± 1.90	0.2 ± 0.2	7.34 ± 2.39	0.3 ± 0.1
Total	89.2 ± 3.0	17.3 ± 5.3	88.7 ± 3.2	18.8 ± 2.2

[a] Four animals.

of 4.2 hr, and more slowly from the body, with 70% of the dose appearing in urine within 12 hr after drug administration.

Oral administration of 30 successive daily doses of minoxidil, at a level of 10 mg/kg, to dogs resulted in an apparent small increase in the rate of removal of minoxidil from circulation. When 1.00-mg/kg doses of labeled minoxidil were administered, the average half-life for removal of minoxidil from plasma and the area under the plasma minoxidil level *versus* time curve both were 20% lower following, as compared to immediately preceding, chronic drug treatment. The average half-life changed from 1.2 to 1.0 hr, and the area changed from 1.27 to 1.08 hr × μg/ml. These small changes ($p < 0.05$ for half-life and $p < 0.20$ for area differences by paired t tests) could have resulted from an increase in the rate of drug metabolism, caused by stimulation of an enzyme system metabolizing minoxidil, or from an increase in urinary excretion rates for the drug and its metabolites.

The dogs used in the single-dose kinetic study, in which a 0.55-mg/kg dose of labeled minoxidil was used, were water loaded to obtain frequent urine samples. The dogs used in the chronic study, however, were not water loaded since frequent urine sampling was not planned. The water diuresis resulting from water loading appears to have increased the rate of removal of minoxidil-related material from circulation. The half-life for removal of minoxidil from plasma and the area under the plasma minoxidil level curve (0–8 hr) for the water-loaded dogs both were approximately 35% lower than the corresponding values obtained following the first dose of radioactive drug in the chronic study ($p < 0.10$ for half-life and area differences by paired t tests). This effect is even greater than that due to chronic drug administration. This effect could be due to an increase in urinary excretion rates for the drug and its metabolites caused by their decreased renal reabsorption under conditions of water diuresis.

Biliary secretion of minoxidil-related materials following oral administration of the drug to the rat was significant, accounting for 13% of the dose. Since only 6% of an oral dose (in a separate study) was accounted for in feces of normal rats, there is some indication of enterohepatic recirculation of the drug and its metabolites by the rat.

As determined by autoradiography, radioactivity associated with labeled minoxidil was very rapidly and widely distributed in the body of the rat following intravenous and oral drug administration. Shortly thereafter, radioactivity began concentrating, primarily in the digestive and excretory system. These systems, particularly the liver, kidneys, bladder, stomach, and intestines, generally contained the highest relative concentrations of radioactivity at increasing times following drug administration. These concentrations were relative to other tissues of the body, since previous excretion studies showed that at least 38, 41, 57, 76, and 92% of the administered radioactivity had been eliminated by the rat 1, 2, 4, 8, and 24 hr, respectively, following oral administration of the labeled drug.

Although accumulation of a drug (and, particularly, only the radioactivity initially associated with the drug) in a certain tissue does not necessarily indicate the site of pharmacological action, certain observations are informative. Little, if any, minoxidil-related material was detected in the central nervous system relative to other tissues at any time following drug administration. Radioactivity concentrated in aorta walls, but not in nonsmooth vascular

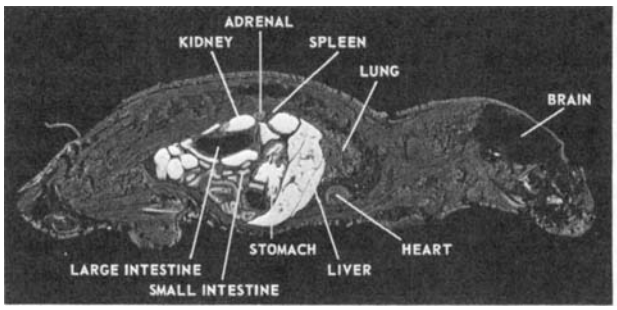


Figure 4—*Distribution of radioactivity (light areas) in a rat 30 min following oral administration of ^{14}C-minoxidil. Film was exposed to the tissue slice for 1 week.*

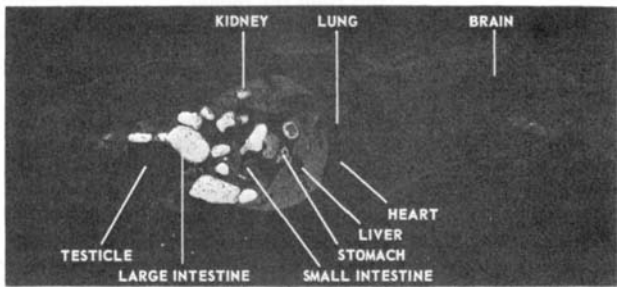


Figure 6—*Distribution of radioactivity (light areas) in a rat 4 hr following oral administration of ^{14}C-minoxidil. Film was exposed to the tissue slice for 2 weeks.*

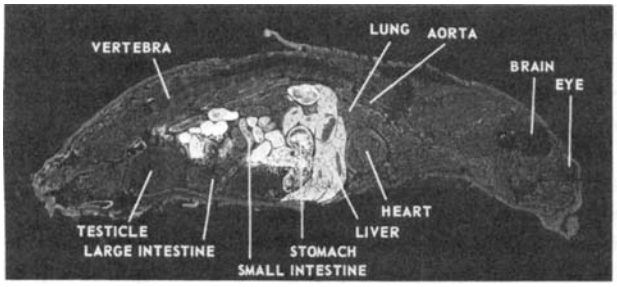


Figure 5—*Distribution of radioactivity (light areas) in a rat 2 hr following oral administration of ^{14}C-minoxidil. Film was exposed to the tissue slice for 2 weeks.*

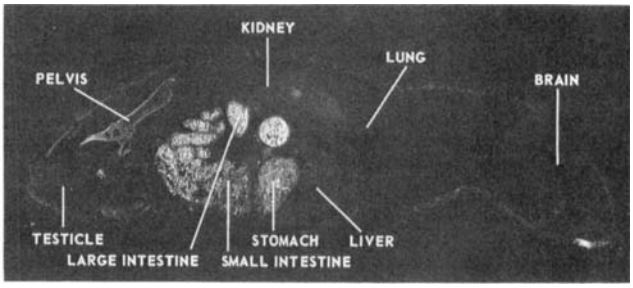


Figure 7—*Distribution of radioactivity (light areas) in a rat 24 hr following oral administration of ^{14}C-minoxidil. Film was exposed to the tissue slice for 6 weeks.*

muscle such as the myocardium, for at least 2 hr following drug administration. This concentration may well have continued beyond 2 hr, as reported by Pluss *et al.* (10), without being detected because of the relatively low sensitivity of whole-body section autoradiography. In any event, these observations are consistent with minoxidil having a direct relaxant effect on peripheral vascular smooth muscle.

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Metabolism of Minoxidil, a New Hypotensive Agent II: Biotransformation following Oral Administration to Rats, Dogs, and Monkeys

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Keyphrases □ Minoxidil—biotransformation in rat, dog, and monkey after oral administration, compared to reported human metabolism □ Metabolism, minoxidil—biotransformation in rat, dog, and monkey after oral administration, compared to reported human metabolism

As part of a program to develop minoxidil (2,4-diamino-6-piperidinopyrimidine 3-oxide, I) as an orally active hypotensive agent, metabolism studies with the drug were undertaken in three animal species: the rat, dog, and monkey. Previous reports described studies that showed that minoxidil, following its oral administration, was rapidly and well absorbed, rapidly and widely distributed, and rapidly eliminated, primarily *via* urine, by the rat, dog, and monkey (1) and by the human (2). Tissue distribution studies in the rat using ^{14}C-minoxidil were reported previously (1, 3).

These results, based on measurements of unchanged drug and total drug-related material, were not sufficient for selecting one species as being most like the human in its disposition of minoxidil. Such a selection was of particular importance for this drug because it causes an apparently species-specific, right-atrial lesion in the dog (4). Therefore, studies were undertaken to compare the biotransformation of minoxidil in the three animal species and to see if at least one of them was similar to the human in this aspect of the drug's disposition. These studies were extended to identify the metabolites of minoxidil produced by each animal species and to determine the kinetics of absorption, distribution, and excretion of the unchanged drug and its metabolites by each species.

This report is concerned with comparing the biotransformation of minoxidil in the three animal species and humans, including characterization, isolation, identification, and quantification of metabolites in urine and blood plasma. A subsequent report will deal with the kinetics of absorption, distribution, and excretion of minoxidil and its metabolites by each animal species.

muscle such as the myocardium, for at least 2 hr following drug administration. This concentration may well have continued beyond 2 hr, as reported by Pluss *et al.* (10), without being detected because of the relatively low sensitivity of whole-body section autoradiography. In any event, these observations are consistent with minoxidil having a direct relaxant effect on peripheral vascular smooth muscle.

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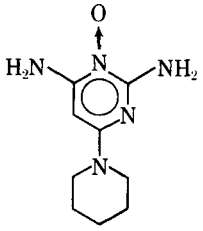


I

EXPERIMENTAL

Radioactivity Measurements—Samples were counted with liquid scintillation spectrometers[1] interfaced to keypunches[2]. Routine calculations were made with a computer[3]. Duplicate (usually 0.1- or 0.5-ml) aliquots of solutions were counted in 15 ml of counting solvent [toluene–dioxane–methanol (350:350:210 v/v) containing 73 g of naphthalene, 4.6 g of 2,5-diphenyloxazole, and 0.080 g of 1,4-bis-2-(5-phenyloxazolyl)benzene/liter]. Counting efficiencies were determined by the internal standard technique using toluene-^{14}C.

Radiochromatogram Scanning—Paper and thin-layer chromatograms, when they contained sufficient radioactivity, were scanned for radioactivity using chromatogram scanners[4] having Geiger detectors. The resulting chart-strip tracings were integrated with a mechanical planimeter[5] or with an electronic digitizer system[6] to determine quantitatively the relative distribution of radioactivity along the length of the chromatogram.

When a chromatogram did not contain sufficient radioactivity for quantification by Geiger detection, it was cut into sequential segments (1 cm in the case of paper chromatograms and 0.5 cm in the case of thin-layer chromatograms). Then the segments, each in a vial with 10 ml of counting solvent containing 3% water, were counted by liquid scintillation spectrometry. The resulting raw data were processed by the computer system to give a plot of the distribution of radioactivity along the length of the chromatogram, in a format similar to that of a chart-strip tracing, together with a tabular printout of derived data. Based on the plot, the tabular data were used to quantify the relative distribution of radioactivity along the length of the chromatogram.

Preparation of Samples for Chromatography—Samples of urine and blood plasma containing sufficient radioactivity were applied directly to the paper or thin-layer medium for chromatography, usually as 2.5-cm streaks. Samples containing lower levels of radioactivity, as well as all enzyme incubation samples, were lyophilized and the residues were reconstituted at a greater concentration with water or were leached three times with methanol. The methanol was removed with a nitrogen stream, and the residue was dissolved in a small volume of 1-butanol–methanol (1:2 v/v) for application to the chromatography medium in a manner similar to that for whole urine and blood plasma. The leaching procedure resulted in chromatographic metabolite patterns quantitatively identical to those obtained by direct chromatography of whole urine and blood plasma.

Enzyme Hydrolysis—Samples of whole urine and extracts of urine and blood plasma were incubated with β-glucuronidase[7] at a concentration of approximately 1000 Fishman units/ml in 0.05 *M*, pH 5.5 sodium acetate buffer for 24 hr at 37°. Occasionally, samples were hydrolyzed under similar conditions with a mixture of β-glucuronidase and sulfatase[8].

Paper Chromatography and TLC—Paper chromatography was carried out on 86 × 15-cm sheets[9] by a descending technique in the 1-butanol–piperidine–water (82:2:16 v/v) system (System I). Occasionally, the 1-butanol–acetic acid–water (4:1:1 v/v) system (System II) was used. TLC was carried out on 20 × 5-cm films of silica gel, 0.25 mm thick, in the benzene–methanol–ammonium hydroxide (100:100:1 v/v) system (System III). Occasionally, solvent systems containing 2% piperidine in water and 10% methanol in chloroform (Systems IV and V, respectively) were used.

Appropriate standard compounds were run on each chromatogram. In certain cases, standard compounds were mixed with samples prior to chromatography to act as internal standards. Following solvent development, the chromatograms were viewed under UV light to locate standards and, when possible, drug-related materials by the UV absorption. In addition, certain chromatograms were sprayed with a 10% solution of ferric chloride in water. Minoxidil and its metabolites containing the *N*-oxide group produced a blue color (against a yellow background), whereas minoxidil metabolites not containing an *N*-oxide group produced no color.

Preparation of 2,4-Diamino-6-(4′-hydroxypiperidino)pyrimidine 3-Oxide (III)—A mixture of 0.160 g (1 mmole) of 2,4-diamino-6-chloropyrimidine 3-oxide and 0.505 g of 4-hydroxypiperidine was heated with stirring under nitrogen in an oil bath at 130° for 2.5 hr. After standing at room temperature overnight, the viscous oil was leached with acetone. The acetone leaching was evaporated *in vacuo* and the residue was taken up in methanol and applied to a 4 × 15-cm silica gel bar[10]. The silica gel bar was developed by the ascending technique in a mixture of acetone–methanol (70:30 v/v).

The UV light-absorbing zone near the origin of the bar was removed and eluted with methanol, the methanol was evaporated *in vacuo*, and the residue was recrystallized from acetone–methanol to yield 0.050 g of a crystalline solid. Its IR, UV, and NMR spectra were reasonable for the subject compound. Mass spectrometry revealed a molecular ion of 225 and a fragmentation pattern consistent with that expected for the subject compound.

Anal.—Calc. for $C_9H_{15}N_5O_2$: C, 47.98; H, 6.71; N, 31.09. Found: C, 47.67; H, 6.55; N, 30.65.

Chromatography in Systems I and III revealed a single UV-absorbing zone in each case.

Preparation of 2,4-Diamino-6-(3′-hydroxypiperidino)pyrimidine 3-Oxide (IV)—Compound IV was prepared from 3-hydroxypiperidine in a manner identical to that described for the 4′-hydroxypiperidino compound; 0.030 g of product was obtained. Its IR, UV, and NMR spectra were reasonable for the subject compound. Mass spectrometry revealed a molecular ion of 225 and a fragmentation pattern consistent with that expected for the subject compound.

Anal.—Calc. for $C_9H_{15}N_5O_2$: C, 47.98; H, 6.71; N, 31.09. Found: C, 47.07; H, 6.53; N, 30.14.

Chromatography in Systems I and III revealed a single UV-absorbing zone in each case.

Isolation of Dog Metabolites—The 0–2-hr urine of a dog which had received a tracer dose of ^{14}C-minoxidil was mixed with the urine of four dogs which had received two successive 10-mg/kg oral doses of nonradioactive minoxidil (1). The urine mixture was adjusted to pH 10 with ammonium hydroxide and passed through a 4.8 × 68-cm column of polystyrene polymer resin[11]. The column then was rinsed with three column volumes of water and eluted with three column volumes of acetone.

The acetone eluate was evaporated to dryness *in vacuo*, and the residue was taken up in methanol and applied to a 8 × 90-cm column of silica gel[12] packed with a mixture of benzene–methanol–ammonium hydroxide (75:25:1 v/v). The column was eluted with 3 mixtures of benzene–methanol–ammonium hydroxide while collecting 50-ml fractions as follows: 10 liters of a 75:25:1 (v/v) mixture, 4 liters of a 70:30:1 (v/v) mixture, and 8 liters of a 60:40:1 (v/v) mixture.

Two major zones of radioactivity emerged. Chromatographically, the first zone was a mixture of minoxidil and a more polar material and the second zone was essentially the more polar material alone. The fractions comprising each zone were combined and

[1] Model 3375, Packard Instrument Co., Downers Grove, Ill.
[2] Models 026 and 029, International Business Machines, White Plains, N.Y.
[3] Model 370/145, International Business Machines, White Plains, N.Y.
[4] Model 880 paper and model 885 thin-layer scanners, Vanguard Instrument Co., North Haven, Conn.
[5] Model 700AR Lasico, Los Angeles Scientific Instruments Co., Los Angeles, Calif.
[6] Model 9864 digitizer and model 9810 calculator, Hewlett-Packard Corp., Loveland, Colo.
[7] Ketodase, Warner-Chilcott.
[8] Glusulase, Endo Laboratories.
[9] Whatman No. 2 paper, W. and R. Balston, Ltd., London, England.
[10] Unibar, Analtech Inc., Wilmington, Del.
[11] XAD-2, Rohm and Haas Co., Philadelphia, Pa.
[12] Fifty to 200-μm particles. Available from Brinkmann Instruments, Westbury, N.Y.

Table I—Urinary Excretion of Minoxidil and Metabolites for the 0–24-hr Period following Oral Administration of ^{14}C-Minoxidil to the Rat, Dog, and Monkey

Species	Percent of Total Drug-Related Material in Urine						
	Total Polar	Carboxy Metabolites (VI/VII)	Minoxidil Glucuronide (V)	4′-Hydroxy-minoxidil (III)	3′-Hydroxy-minoxidil (IV)	Minoxidil (I)	Reduced Minoxidil (II)
Rat[a]	38	—[f]	6	12	1	43	<1
Rat (chronic)[b]		—[f]					
Before	43	(34)[g]	4	14	1	29	4
After	38	(28)[g]	2	9	1	35	10
Dog[c]	42	—[f]	5	29	4	13	<1
Dog (chronic)[d]							
Before	27	—[f]	2	47	4	19	<1
After	33	—[f]	4	35	4	18	3
Monkey[e]	21	—[f]	51	8	0	10	<1

[a] A single 5-mg/kg radioactive dose to each of three rats. [b] A 15-mg/kg radioactive dose to each of six rats before and after nine successive, daily, 150-mg/kg nonradioactive doses. [c] A single 0.55-mg/kg radioactive dose to each of three dogs. [d] A 1-mg/kg radioactive dose to each of four dogs before and after 30 successive, daily, 10-mg/kg nonradioactive doses. [e] A single 1-mg/kg radioactive dose to each of three monkeys. [f] Included in Total Polar but not differentiated. [g] Included in Total Polar.

evaporated to dryness *in vacuo*, and the residues were further purified by chromatography on 4 × 12-cm silica gel bars[10] in a benzene–methanol (70:30 v/v) mixture. The material corresponding to the first zone of the silica gel column was recrystallized from ethanol–water, water, and methanol–water (with the aid of charcoal[13]) to yield 0.015 g of white crystals. This material corresponded to minoxidil according to UV, IR, and mass spectrometry.

Anal.—Calc. for $C_9H_{15}N_5O$: C, 51.66; H, 7.23; N, 33.47. Found: C, 51.55; H, 7.19; N, 34.03.

Chromatography in Systems I and III–V revealed a single UV-absorbing zone corresponding to minoxidil. The material corresponding to the second zone on the silica gel column was recrystallized three times from methanol–acetone (with the aid of charcoal[13]) to yield 0.030 g of white crystals. This material corresponded to 2,4-diamino-6-(4′-hydroxypiperidino)pyrimidine 3-oxide according to UV, IR, and mass spectrometry.

Anal.—Calc. for $C_9H_{15}N_5O_2$: C, 47.98; H, 6.71; N, 31.09. Found: C, 48.02; H, 6.76; N, 30.72.

Chromatography in Systems I and III revealed a single UV-absorbing zone corresponding to the 4′-hydroxypiperidino compound. NMR spectrometry showed that the carbinol hydrogen was not at the C-2′, C-3′, C-5′, or C-6′ position of the piperidino ring, so it must have been at the C-4′ position. The coupling to the C-3′ and C-5′ methylene hydrogens of the piperidino ring indicated that the hydroxy group was equatorial.

Isolation of Rat Metabolites—Each of six female rats[14], weighing approximately 200 g, was given a 3-mg (24.4 μCi) dose of ^{14}C-minoxidil, nine successive 30-mg daily doses of nonradioactive minoxidil, and a final 3-mg (24.4 μCi) dose of ^{14}C-minoxidil by oral intubation of a solution or suspension in water. The rats were placed in individual metabolism cages, and urine was collected at 24-hr intervals and frozen for the duration of the study. Small samples of each urine collection for the 0–24-hr period following administration of the radioactive doses were set aside for analysis. The bulk of the radioactive urine collections was combined with the nonradioactive collections for metabolite isolation.

The composite urine was adjusted to pH 9 with ammonium hydroxide and passed through a 2.8 × 77-cm column of polystyrene resin[11]. The column was rinsed with three column volumes of water, which had been adjusted to pH 9, and then eluted with three column volumes of 50% methanol in water. The methanol eluate was evaporated to dryness *in vacuo* and set aside. The aqueous wash was adjusted to pH 4 with acetic acid and passed through a 2.8 × 77-cm column of polystyrene resin[11]. The column was rinsed with three column volumes of water, which had been adjusted to pH 4, and then eluted with three column volumes of 50% methanol in water. The rinse water was discarded and the methanol eluate was evaporated to dryness *in vacuo*.

A 10% aliquot of the pH 9 polystyrene resin[11] column eluate in 5 ml of benzene–methanol–ammonium hydroxide (50:50:1 v/v) was applied to a 2.5 × 51-cm column of silica gel[15]. The column was eluted with the same solvent mixture while collecting 10-ml fractions. Two major zones of radioactivity emerged. Chromatographically (Systems I–V), the first zone corresponded to 2,4-diamino-6-piperidinopyrimidine and the second zone corresponded to minoxidil. The contents of the tubes corresponding to the second zone were combined and evaporated to dryness, and the residue was recrystallized three times from ethanol–water (with the aid of charcoal[13]) to yield 0.010 g of crystals. This material corresponded to minoxidil according to UV spectrometry and chromatography in Systems I and III–V.

Anal.—Calc. for $C_9H_{15}N_5O$: C, 51.66; H, 7.23; N, 33.47. Found: C, 51.67; H, 7.48; N, 32.94.

This material gave a positive *N*-oxide test with ferric chloride solution, whereas the material that corresponded chromatographically to 2,4-diamino-6-piperidinopyrimidine did not.

The pH 4 polystyrene resin[11] column eluate was subjected to countercurrent distribution in the 1-butanol–acetic acid–water (9:1:10 v/v) system; 100 ml of each phase was used per tube for 20 transfers of the lower phase. The contents of tubes 11–16, which contained the major radioactive zone, were combined and evaporated to dryness in a nitrogen stream. The residue was recrystallized twice from acetone–water (with the aid of charcoal[16]) to obtain 0.026 g of crystals. Mass spectrometry, using an electron-impact technique, revealed an apparent molecular ion of 225 mass units and a fragmentation pattern consistent with 2,4-diamino-6-(4′-carboxy-*n*-butylamino)pyrimidine. UV spectrometry supported this structure.

NMR spectrometry suggested that the material was actually a mixture of the proposed compound and its 3-oxide (in a ratio of approximately 2 to 1) on the basis of comparisons with the spectra of 2,4-diamino-6-piperidinopyrimidine and its 3-oxide (minoxidil). This finding was supported by resolution of the material into two radioactive and UV-absorbing zones, only one of which gave a positive *N*-oxide test with ferric chloride solution, when subjected to chromatography in Systems I (for 6 days) and IV. In this case, the ratio of *N*-oxide to the reduced form was found to be nearly 1:1 based on radioactivity.

Mass spectrometry with a different instrument, using a field-desorption technique[17], revealed two molecular ions, one at 225 mass units, attributable to the initially proposed compound, and the other at 241 mass units, attributable to its 3-oxide. Use of an electron-impact technique and careful regulation of the probe temperature with this same instrument[17] revealed an apparent molecular ion at 241 mass units and a fragmentation pattern consistent with a mixture of the two materials.

Anal.—Calc. for a 1:1 mixture of $C_9H_{15}N_5O_2$ and $C_9H_{15}N_5O_3$: C, 46.40; H, 6.49; N, 30.03. Found: C, 46.08; H, 6.52; N, 30.41.

[13] Darco G60, Atlas Powder Co., Wilmington, Del.
[14] Sprague–Dawley (Upjohn strain).
[15] Ten to 40-μm particles containing 13% $CaSO_4$. Available from Brinkmann Instruments, Westbury, N.Y.
[16] Nuchar C-190, West Virginia Pulp and Paper Co., New York, N.Y.
[17] Model CH-5, Varian MAT/USA, Springfield, N.J.

Table II—Areas under Minoxidil and Metabolite Plasma Level Curves for the 0–8-hr Period following Oral Administration of ^{14}C-Minoxidil to the Dog and Monkey

Species[a]	Percent of Total Drug-Related Area				
	Total Polar	Minoxidil Glucuronide (V)	4′-Hydroxy-minoxidil (III)	3′-Hydroxy-minoxidil (IV)	Minoxidil[b] (I)
Dog	14	<1	46	3	23
Dog (chronic)					
Before	12	<1	49	<1	26
After	17	<1	46	<1	27
Monkey	17	64	7	<1	9

[a]See Table I for drug dosages and numbers of animals. [b]Includes possible reduced minoxidil (II).

RESULTS AND DISCUSSION

Initial studies involved comparative paper and thin-layer radiochromatography of urine from rats, dogs, and monkeys which had received ^{14}C-minoxidil orally. In each case, radioactivity zones were detected, indicating extensive metabolism of minoxidil. One of these zones for each species had the same chromatographic mobility as minoxidil. The other zones were attributed to then unidentified metabolites of minoxidil. Judging from the relative amount of radioactivity in each zone, the major metabolite was different for each species. However, each species produced certain amounts of the major metabolites of the others. The urinary metabolite patterns for the three animal species are illustrated in Fig. 1; the metabolite pattern for the human will be discussed later. The peak designated I/II had the same mobility in System I as standard minoxidil.

Attention was next turned to identifying the urinary excretion products of minoxidil in the three animal species. In the case of the dog, the peak designated I/II in Fig. 1 was tentatively identified as unchanged minoxidil on the basis of its paper and thin-layer chromatographic mobilities. Subsequently, mass spectrometry of this material, isolated by preparative paper chromatography and TLC, gave spectra that were identical to that of minoxidil.

Finally, this material was isolated in crystalline form from dog urine and identified as minoxidil by elemental and spectral analyses. Material of the mobility designated I/II in Fig. 1 also was isolated in crystalline form from rat urine and identified as minoxidil. In the case of the monkey, the characterization of peak I/II in Fig. 1 was based entirely on paper chromatography and TLC.

During isolation of the rat urinary minoxidil, a radioactive material was detected with the same mobility as minoxidil in System I but with a greater relative mobility in Systems III–V. In contrast to minoxidil, it did not produce a blue color when treated with ferric chloride solution and, therefore, was considered to lack an *N*-oxide function. Since it had the same mobility in Systems I and III–V as 2,4-diamino-6-piperidinopyrimidine (II), it was characterized as this reduced form (II) of minoxidil. That particular study involved chronic administration of minoxidil to rats [labeled Rat (chronic) in Table I].

In another rat study, involving single 50-mg/kg doses of ^{14}C-minoxidil, II also was found in urine. However, in a third study, using single 5-mg/kg doses of ^{14}C-minoxidil in rats, II was not detected in urine. Thus, at least when large or chronic doses of minoxidil were administered to rats, II was excreted in urine. This also was the case with the dog. This metabolite was not detected in monkey urine.

Mass spectrometry of the material designated III in Fig. 1, isolated by preparative paper chromatography and TLC of urine from dogs that had received ^{14}C-minoxidil, indicated that this material was a hydroxypiperidino form of minoxidil. Therefore, both 2,4-diamino-6-(4′-hydroxypiperidino)pyrimidine 3-oxide (III) and 2,4-diamino-6-(3′-hydroxypiperidino)pyrimidine 3-oxide (IV) were prepared and characterized as standards. Paper chromatography and TLC showed that the 4′-hydroxy standard had the same mobility as the major peak designated III and the 3′-hydroxy standard had the same mobility as the minor peak designated IV.

Subsequently, III was isolated from dog urine in crystalline form and identified as such by elemental and spectral analyses. NMR was particularly valuable in confirming the 4′-position for the hydroxy group and showing that it was equatorial. With the rat and monkey, characterization of peak III in Fig. 1 was based entirely on paper chromatography and TLC. The characterization of IV in Fig. 1 for the dog and rat was based solely on this small peak having the same mobility as standard IV in System I. This metabolite was not detected in monkey urine.

Characterization of the peak designated V in Fig. 1 was based principally on results obtained by incubating urine containing this radioactive metabolite with β-glucuronidase. For each animal species, such incubation resulted in loss of peak V and a concomitant and equivalent increase in peak I/II. Mass spectrometry of eluates of the zone corresponding to I/II from preparative thin-layer chromatograms, following enzyme incubation of monkey urine, confirmed that minoxidil was being released. Metabolite V was thereby characterized as a glucuronic acid conjugate of minoxidil. It is likely that conjugation was with the *N*-hydroxy tautomeric form of minoxidil (Scheme I), giving an *O*-glucuronide[18]. Never-

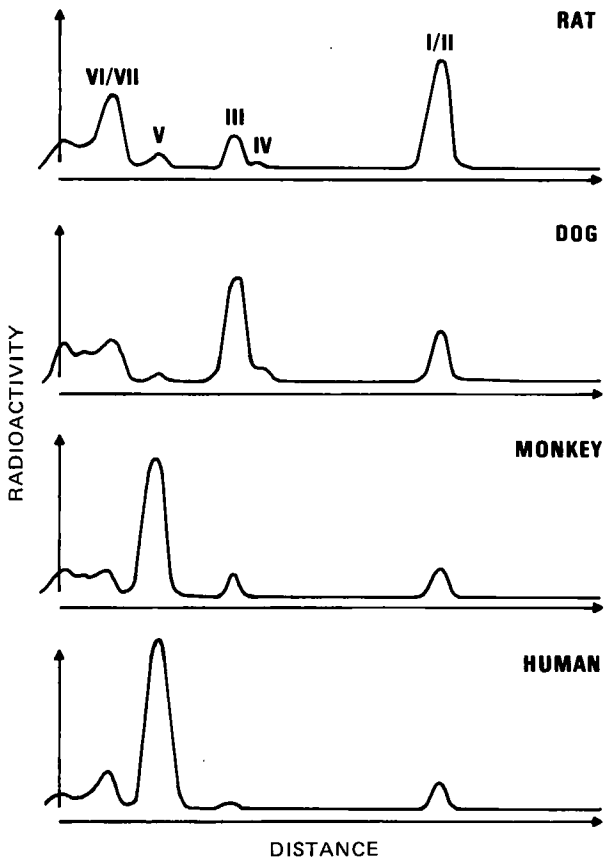
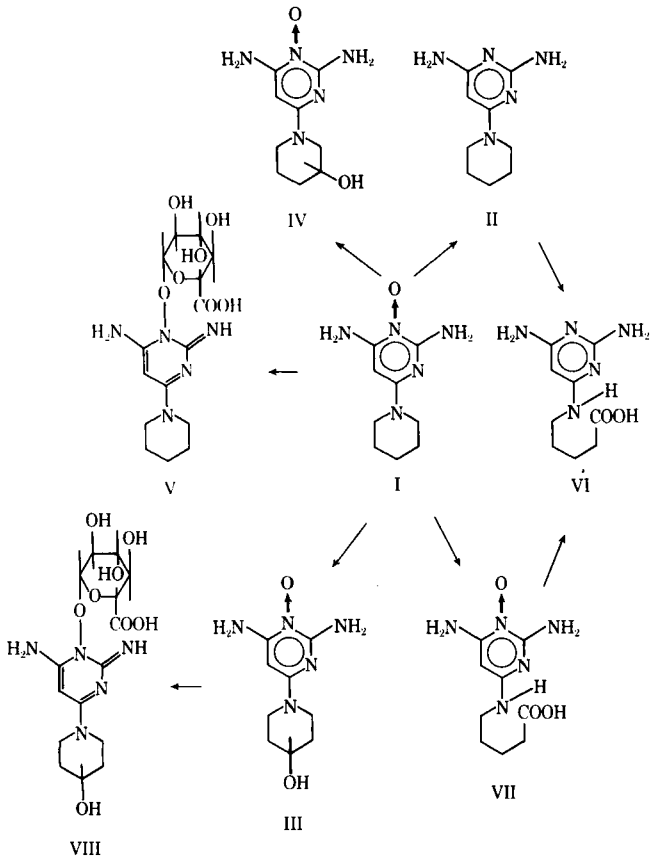


Figure 1—*Composites of radiochromatograms (System I) representing 0–24-hr urine collections for the rat, dog, monkey, and human.*

[18] The glucuronide conjugate is stable to hydrolysis in 0.1 *N* HCl at 35°; *N*-glucuronides generally are hydrolyzed by such treatment.



Scheme I—Biotransformation of minoxidil (the points of attachment of the glucuronic acid moiety to V and VIII have not been definitely established)

theless, conjugation through one amine nitrogen to give an *N*-glucuronide has not been completely ruled out.

Preliminary characterization of the metabolite peak designated VI/VII in Fig. 1 was accomplished by mass spectrometry of appropriate eluates of preparative paper and thin-layer chromatograms of urine from rats that had received ^{14}C-minoxidil. These results indicated that this metabolite was 2,4-diamino-6-(4′-carboxy-*n*-butylamino)pyrimidine (VI), derived from minoxidil *via* oxidative cleavage of the piperidine ring at the C—N bond. Subsequently, the metabolite(s) was isolated in crystalline form from rat urine and shown by elemental analysis and spectrometry to be a mixture of VI and 2,4-diamino-6-(4′-carboxy-*n*-butylamino)pyrimidine 3-oxide (VII) in a ratio of 1:1 or 2:1. The only evidence that these materials were metabolites of minoxidil in dog and monkey urine was that each species produced a urinary metabolite of minoxidil having a mobility corresponding to the carboxy metabolites in System I. Studies are in progress to determine the importance of these metabolites in the dog and monkey.

Evidence for urinary excretion of a glucuronide conjugate (VIII) of the 4′-hydroxy metabolite by the dog was obtained. Incubation with β-glucuronidase of urine from dogs that had received ^{14}C-minoxidil resulted in loss of radioactivity from the origin and a concomitant and equivalent appearance of radioactivity in peak III when chromatographed in System I. This could be an *O*-glucuronide of the *N*-hydroxy group or the C-hydroxy group or perhaps an *N*-glucuronide of one amino group of the 4′-hydroxy metabolite. This material was not detected as a metabolite of minoxidil in rat and monkey urine.

The results described thus far are summarized in Fig. 1 and Scheme I. In Fig. 1, an attempt was made to present true, quantitative relationships among the drug and its metabolites for the various species. Scheme I shows the structures of the various metabolites produced by the three animal species as well as their possible relationships to one another. The points of attachment of the glucuronic acid moiety to minoxidil and one of the metabolites are somewhat speculative, but there is substantial evidence in each case for the structure of the aglycone and for its conjugation with glucuronic acid.

Once chromatographic profiles of urinary minoxidil metabolites were obtained and most metabolites were identified, it was possible to quantify the biotransformation of minoxidil for each animal species. Minoxidil and its metabolites in urine were quantified by determining the amount of radioactivity associated with the drug or a particular metabolite following separation by paper chromatography and TLC. The results presented in Table I and Fig. 1 clearly show that the principal drug-related material excreted by the rat was unchanged minoxidil, closely followed by the carboxy metabolites. With the dog, the 4′-hydroxy metabolite was the principal excretion product. The monkey excreted predominantly the glucuronide conjugate of minoxidil.

The various drug-related materials in the circulation of the dog and monkey were characterized and quantified by radiochromatography of blood plasma samples taken sequentially following oral administration of ^{14}C-minoxidil. The results (Table II) are presented as the percent contribution each drug-related material makes to the total area under the plasma radioactivity level *versus* time curve during the 8-hr period following drug administration. This method should take into account the persistence, as well as the concentration, of each drug-related material in plasma.

As can be seen from Table II, the principal circulatory material in the dog was the 4′-hydroxy metabolite; in the monkey, it was the glucuronide of minoxidil. Substantial amounts of minoxidil and the polar group of metabolites were circulated by both species. The major drug-related material in circulation for the human was not identified by Gottlieb *et al.* (2), but it was not minoxidil. Since the major urinary excretion product in the human and the monkey was the glucuronide of minoxidil, it seems reasonable that this was also the major circulatory material in the human.

Gottlieb *et al.* (2) reported that the human excretes four minoxidil-related products in urine following oral administration of ^{14}C-labeled drug. They were characterized as minoxidil, a glucuronide of minoxidil, and two unidentified metabolites, designated M-II and M-I. Their relative amounts in 0–24-hr urine collections were 12, 48, 18, and 21%, respectively, as determined by radiochromatography in System I.

Experience with paper chromatography of monkey urine and urine extracts containing the minoxidil glucuronide has shown this glucuronide to have two apparent migration rates in System I. Although the glucuronide usually migrated to one or the other of two similar R_f values (0.2 and 0.3), at times it would appear as a doublet occupying both locations. Certain samples, particularly those with relatively great solids content, were more susceptible than others to this variation in migration, including doublet formation. Replicate paper chromatograms of such a sample over time could result in the glucuronide displaying either, or both, R_f value unpredictably.

Regardless of the particular chromatographic behavior, however, hydrolysis of each sample resulted in complete loss of the glucuronide and a concomitant and equivalent appearance of minoxidil. The existence of two glucuronides of minoxidil (such as an *O*-glucuronide and an *N*-glucuronide) would not explain these observations. At the same time, these results do not preclude the presence of two such materials. These results can be explained best by a single glucuronide which, depending on its environment, either can be partially modified by application to the paper or can exist in several ionic forms having different migration rates in System I.

Chromatography in System I of human urine samples, collected by Gottlieb *et al.* (2), resulted in separation of three major zones of radioactivity rather than four. These zones corresponded, in order of increasing polarity, to minoxidil, the glucuronide of minoxidil, and a very polar zone, sometimes resolvable into two components. Integration of these chromatograms showed that our glucuronide accounted for the sum of the glucuronide and Metabolite M-II or M-I described by Gottlieb *et al.* (2). Hydrolysis of these urine samples with β-glucuronidase resulted in the complete loss of the material herein designated as the glucuronide and a concomitant and equivalent appearance of minoxidil, as judged by chromatography in System I. Similar hydrolysis studies using System III showed

that minoxidil, not 2,4-diamino-6-piperidinopyrimidine, was released from the glucuronide.

It appears that, as with monkey urine, a single glucuronide best explains the results with human urine. In addition to minoxidil, the glucuronide of minoxidil, and the very polar minoxidil-related material in human urine, small (less than 3%) amounts of 2,4-diamino-6-(4′-hydroxypiperidino)pyrimidine 3-oxide and, in a few samples, traces of 2,4-diamino-6-piperidinopyrimidine were found. The more mobile component of the very polar zone of radioactivity corresponded to the migration of the carboxy metabolites of minoxidil. The urinary metabolite profile for the human is based on the results reported by Gottlieb *et al.* (2) modified by our chromatographic results with human and monkey urine containing the glucuronide of minoxidil as discussed.

The urinary metabolite profile for minoxidil in the human is compared to those in the rat, dog, and monkey in Fig. 1. This comparison shows that each species, including the human, excreted substantially the same metabolites but in quite different relative amounts. The monkey and human exhibited similar metabolite profiles, whereas the dog and rat were quantitatively different from each other and from the monkey and human. Thus, of the three animal species studied, the monkey is most like the human in its biotransformation and disposition (1) of minoxidil.

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Antiradiation Compounds XV: Condensations of Carbon Disulfide with Amino, Chloro, Cyanomethyl, and Sulfonamido Heterocycles

WILLIAM O. FOYE [x], JOEL M. KAUFFMAN, JOSEPH J. LANZILLO, and EDWARD F. LaSALA

Abstract □ Condensations of carbon disulfide were carried out with amino, chloro, and diamino heterocycles to give condensed ring thiazoline-2-thiones and imidazoline-2-thiones, with cyanomethyl heterocycles to give dithio acid derivatives, and with heterocyclic sulfonamides to give sulfonyldithiocarbamates. Of several examples tested, pyrido[3,2-*d*]thiazoline-2-thione, disodium 2-(5-chloro-2-thienyl)-3,3-dimercaptoacrylonitrile, triethylammonium 4-sulfamoylphenyldithiocarbamate, ammonium β-phenethyldithiocarbamate, and methyl *N*-(thiophene-2-sulfonyl)dithiocarbamate, only the last-named compound showed any radiation protection for mice. Several compounds gave negative tests for antimalarial activity.

Keyphrases □ Antiradiation compounds—condensations of carbon disulfide with amino, chloro, cyanomethyl, and sulfonamido heterocycles □ Carbon disulfide—condensation products with amino, chloro, cyanomethyl, and sulfonamido heterocycles, potential antiradiation agents □ Heterocycles (amino, chloro, cyanomethyl, and sulfonamido)—condensation products with carbon disulfide, antiradiation activity

Condensation products of carbon disulfide with amines (1, 2) and mercaptans (3), as well as with active methylenes adjacent to cyano groups (4), have all shown some degree of protection for animals against ionizing radiation. With the dithiocarbamates, the presence of heterocyclic rings, particularly when strongly basic (5), provided greater protective properties. In the case of the dimercaptoacrylonitriles derived from active methylene compounds, the presence of heterocyclic rings on the carbon alpha to the dimercapto acid function generally resulted in hygroscopic products and sometimes compounds of low stability.

The attempt accordingly has been made to obtain condensation products of carbon disulfide with heterocyclic rings that have greater stability and also a mercapto group two or three atoms distant from a basic nitrogen or heteroatom, to provide an analogy with the more protective aliphatic mercapto amines.

DISCUSSION

Condensations with Amino and Chloro Heterocycles—The finding (6) that 4-chloro-5,6-diaminopyrimidine gave a thiazoline-2-thione (I) with replacement of amino and chloro groups and not the imidazoline-2-thione prompted a trial of this reaction on several suitably substituted heterocycles (Scheme I).

The pyridine analog (II) was prepared previously by Yamamoto and Takahashi (7), who reported a decomposition point of 294° and stated no percentage yield. Their product was obtained from 2-chloro-3-nitropyridine, sodium sulfide, hydrogen sulfide, and carbon disulfide; sulfide reduction of the nitro group was involved. In the present research, II was obtained in 73% yield, mp 310–314° dec., from 3-amino-2-chloropyridine and carbon disulfide, with dimethylformamide as the solvent.

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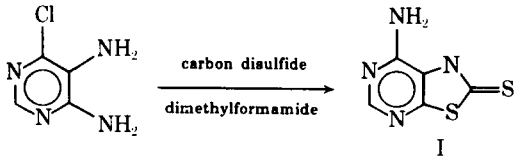
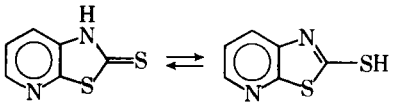


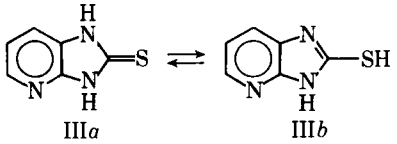
I

Scheme I



IIa IIb

Scheme II



IIIa IIIb

Scheme III

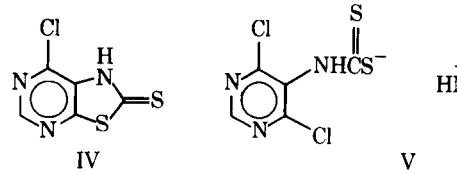
Most evidence indicates that 2-mercaptothiazole (8) and 2-mercaptobenzothiazole (9) are present in neutral media mainly as the thiones (8). The pyridine derivative has a melting point 150° higher than the benzo analog, dissolves instantly in cold, aqueous alkali, and is insoluble in cold, dilute, mineral acid. This behavior can be explained by the existence in neutral media of the thione form as a hydrogen-bonded dimer, in equilibrium with a small amount of thiol (II*b*) (Scheme II).

A broad, intense IR absorption band at 2650–2900 cm^{-1} supports the existence of the hydrogen-bonded dimer with a significant amount of proton transfer, as postulated for 2-mercaptobenzothiazole (10). Absorption at 2550 cm^{-1} also indicates the presence of thiol. The UV absorption in dimethylformamide was identical to that for 2-mercaptobenzothiazole.

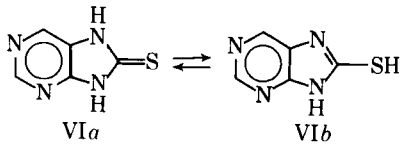
Reaction of 4-amino-3-bromopyridine with carbon disulfide in dimethylformamide failed to give the isomeric pyrido[4,3-*d*]thiazoline-2-thione. Pyrido[2,3-*c*]imidazoline-2-thione (III), however, was synthesized from 2,3-diaminopyridine and carbon disulfide in ethanol; a melting point of "over 300°" had previously been reported for this compound (11). By recrystallizing the product from dimethylformamide and propanol, a melting point of 326–330° dec. was obtained. The IR absorption at 2700–2900 cm^{-1}, as well as the high melting point, again indicated that the hydrogen-bonded dimeric thione was the predominant form of the compound. A small absorption band at 2600 cm^{-1} and solubility in alkali indicated some thiol form (III*b*) (Scheme III).

Condensation of carbon disulfide with 5-amino-4,6-dichloropyrimidine did not give the expected thiazoline-2-thione (IV) but the dithiocarbamate salt (V) instead. Reaction of 4,5-diaminopyrimidine with carbon disulfide, however, gave a 35% yield of purine-8-thiol (VI) (Scheme IV). Previous syntheses of this compound from pyrimidine and thiourea (12) and from purine and sulfur (13) are known. The IR absorption of this compound between 2700 and 2900 cm^{-1} was complex, and its breadth was characteristic of that of the hydrogen-bonded dimeric thione of 2-mercaptobenzothiazole (10). An IR absorption band at 2550 cm^{-1} also indicated the presence of some of the thiol form. Reaction of 2-amino-3-chloropyrazine with carbon disulfide in dimethylformamide at 110° for 22 hr gave only a small amount of orange solid, mp 380° dec., which was not characterized.

Condensations with Cyanomethyl Heterocycles—One attempt to obtain more stable heterocyclic-substituted dimercaptoacrylonitriles involved the use of heterocycles with electron-attracting substituents. 2-Chloro-5-cyanomethylthiophene was prepared from 2-chlorothiophene *via* chloromethylation, and condensation with carbon disulfide was carried out in the presence of sodium hydride. Although the reaction was carried out in anhydrous ether, a hydrated product (VII) was obtained, similar to previous compounds of this type (4). Attempts to prepare the corresponding phenyl and sulfonamido derivatives failed; the electron-withdrawing effects of the substituents apparently deactivated the rings to chloromethylation.

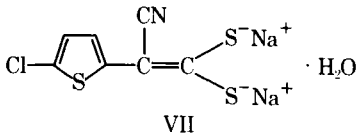


IV V



VIa VIb

Scheme IV



VII

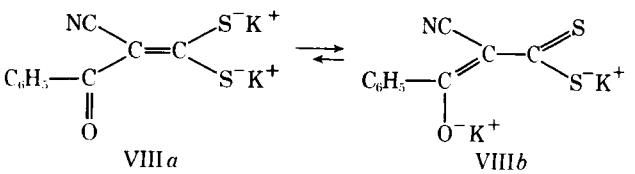
The carbon disulfide condensation product from benzoylacetonitrile (4) was found to be more stable than the condensation products having aromatic rings attached directly to the cyanomethyl group, apparently due to extension of conjugation and shifting of a negative charge from sulfur to the more electronegative oxygen (VIII) (Scheme V). The IR absorption did not show the presence of a carbonyl group, indicating VIIIb to be the predominant form. Condensation of 2-furoylacetonitrile with carbon disulfide and sodium methoxide gave the monothio anion (IX). The IR absorption showed the presence of hydroxy and cyano groups, carbon–carbon unsaturation, and carbon–sulfur unsaturation. When potassium hydroxide was used as the base, an unidentifiable water-soluble red salt was obtained.

Condensation of 2-thenoylacetonitrile with carbon disulfide in the presence of potassium hydroxide, however, gave the dithio dianion (X). The IR absorption showed the presence of the cyano group, carbon–carbon unsaturation, and carbon–sulfur unsaturation but no evidence of a carbonyl group, analogous to the product from benzoylacetonitrile. Neither IX nor X was obtained in a hydrated or solvated form.

Condensations with Sulfonamides—Gompper and Hägele (14) first described the reaction of sulfonamides with carbon disulfide and alkali to give *N*-sulfonylimidodithiocarbonates (XI). These compounds were not isolated but could be converted to derivatives such as mono- or diethers or *N*-sulfonyldithiourethanes (XII) (Scheme VI).

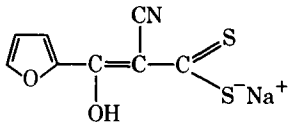
Attempts were made to prepare the *N*-sulfonylimidodithiocarbonates and thioesters of N^4-acetylsulfanilamide, thiazole-2-sulfonamide, benzothiazole-2-sulfonamide, 4-phenylthiazole-2-sulfonamide, and thiophene-2-sulfonamide. Only the thiophene-2-sulfonamide gave products that could be characterized. These compounds were the *N*-sulfonylimidodithiocarbonate (XIII) and the methyl ester of the sulfonyl dithiocarbamate (XIV).

Treatment of sulfanilamide with carbon disulfide and alkali gave 4-sulfamoylphenyldithiocarbamate (XV); both the potassium and triethylammonium salts were isolated. Evidence for the attack of carbon disulfide at the aromatic amine rather than at the sulfonamide nitrogen was found by an IR absorption peak for C=S, the presence of only one cation in the product, and the failure of carbon disulfide to react with N^4-acetylsulfanilamide. Attempts to prepare an *N*-sulfonylimidodithiocarbonate monothioester of XV were also unsuccessful.

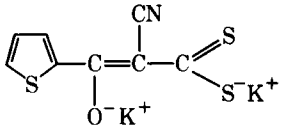


VIIIa VIIIb

Scheme V



IX



X

RESULTS[1]

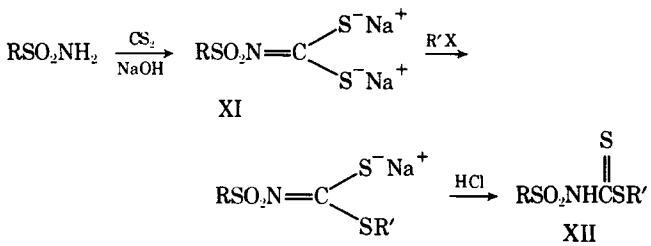
Tests were carried out in mice (groups of six) *versus* 825 rads (X-rays), with an observation period of 30 days. The compounds were administered intraperitoneally in a carboxymethylcellulose–polysorbate vehicle at dose levels of 80–400 mg/kg. Compounds II, VII, and XV and ammonium β-phenethyldithiocarbamate, which had been previously prepared (15), were inactive. Compound XIV provided slight protection (17% survival) in this test at a dose of 400 mg/kg ip.

Several compounds were also screened for antimalarial activity in mice infected with *Plasmodium berghei*, according to the method of Osdene *et al.* (16), and in *Aedes aegypti* infected with *Plasmodium gallinaceum*, according to the method of Gerberg *et al.* (17). In the former test, II, XIV, and XV were listed as inactive; XV, however, showed a test/control survival ratio of 2.2 at a dose of 640 mg/kg. In the latter test, XIV, XV, and ammonium β-phenethyldithiocarbamate were inactive.

On the basis of the radiation-protective test results reported here and in previous papers (4, 18), it may be concluded that compounds with thiol anions or potential thiol anions, capable of rapid hydrogen atom transfer, may be radiation protective without the presence of a strongly basic nitrogen in the molecule. Nevertheless, the presence of an electron-rich heterocyclic ring system or other group capable of binding to DNA or other essential macromolecules appears to be required in the absence of a strongly basic nitrogen. However, by far the most potent of the basic thiol radiation-protective compounds do contain strongly basic amine functions (19).

EXPERIMENTAL[2]

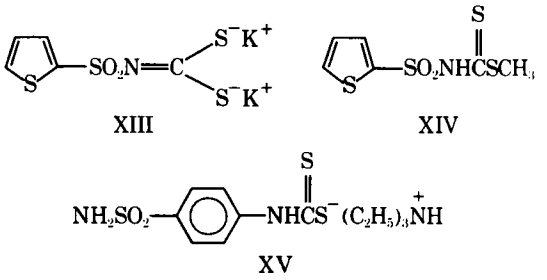
Pyrido[3,2-*d*]thiazoline-2-thione (II)—A mixture of 3-amino-2-chloropyridine[3] (6.43 g, 0.05 mole), carbon disulfide (50 ml), and dimethylformamide (50 ml) was heated at reflux for 47 hr, cooled, and evaporated at 50° and 50 mm pressure to remove excess carbon disulfide. The solution was poured into 300 ml of ice and water with stirring. The precipitate was dried to give 7.2 g of tan powder,



XI

XII

Scheme VI



XIII XIV XV

mp 304–306° dec., and recrystallized from a mixture of 40 ml of dimethylformamide and 100 ml of 1-propanol.

The first crop of tan crystals weighed 5.8 g (69%), mp 310–314° dec. The compound was soluble in acetone and cold dilute sodium hydroxide solution and insoluble in water and hot 8% sulfuric acid. Recrystallization from dimethylformamide–propanol and drying at 100°/0.3 mm gave the analytical sample of off-white spars, mp 312–315° dec. [lit. (7) mp 294°]; IR ν_{max} (KBr): 2650–2900 (NH), 2550 (SH), and 1040 (C=S) cm^{-1}; UV λ_{max} (dimethylformamide): 265 nm.

Anal.—Calc. for $C_6H_4N_2S_2$: C, 42.8; H, 2.4; N, 16.6; S, 38.1. Found: C, 43.0; H, 2.3; N, 16.4; S, 37.8.

Pyrido[2,3-*d*]imidazoline-2-thione (III)—The procedure of Petrow and Saper (11) gave an 89% yield of product, mp 326–330° [lit. (11) mp >300°]. Recrystallization of 9.1 g from a mixture of 40 ml of dimethylformamide and 100 ml of 1-propanol gave 6.9 g (76%), mp 326–330°; IR ν_{max} (KBr): 2700–2900 (NH), 2550 (SH), and 1200 (C=S) cm^{-1}.

Anal.—Calc. for $C_6H_5N_3S$: C, 47.7; H, 3.3; N, 27.8; S, 21.2. Found: C, 48.1; H, 3.4; N, 27.5; S, 21.0.

4,6-Dichloro-5-aminopyrimidinium 4,6-Dichloropyrimidine-5-dithiocarbamate (V)—A mixture of 4,6-dichloro-5-aminopyridine[4] (0.82 g, 0.005 mole), carbon disulfide (5 ml), and dimethyl sulfoxide (5 ml) was heated at reflux for 5 days, freed of carbon disulfide by evaporation, and diluted with 30 ml of water. The yellow powder that separated was dried at 125°/3 mm for 3 hr, giving 0.2 g, mp 239–242° dec.

Anal.—Calc. for $C_9H_6Cl_4N_6S_2$: C, 26.8; H, 1.5; N, 20.8; S, 15.9. Found: C, 27.2; H, 1.4; N, 21.2; S, 16.1.

Purine-8-thiol (VI)—A mixture of 4,5-diaminopyrimidine[5] (4.7 g, 0.043 mole), carbon disulfide (10 ml), and 95% ethanol (50 ml) was heated at reflux for 70 hr. Hydrogen sulfide was liberated, and product separated during the reaction. Addition of 10 ml of water and chilling in an ice bath gave a tan solid. This product was washed with 30% ethanol, dried at 130°/3 mm for 3 hr, and recrystallized from 150 ml of water, yielding 2.3 g (35%) of light-yellow spars, mp 312–318° dec. [lit. (12) mp 268° dec. and (13) mp 295–297° dec.]; IR ν_{max} (KBr): 2700–2900 (NH) and 1200 (C=S) cm^{-1}.

The compound was soluble in cold, dilute sodium hydroxide solution, cold, dilute sulfuric acid, dimethylformamide, and hot 1-propanol.

Anal.—Calc. for $C_5H_4N_4S$: C, 39.4; H, 2.7; N, 36.8; S, 21.0. Found: C, 39.6; H, 3.0; N, 36.4; S, 20.6.

Disodium 2-(5-Chloro-2-thienyl)-3,3-dimercaptoacrylonitrile Hydrate (VII)—Sodium hydride[6] (1.05 g, 0.025 mole, 57% in petroleum ether) was washed three times with anhydrous ether and suspended in ether (12 ml). To this mixture was added carbon disulfide (0.8 ml, 0.013 mole) and 2-chloro-5-cyanomethylthiophene (20) (2 g, 0.013 mole) dropwise during 20 min. The reaction mixture was stirred at room temperature for 7 days.

The precipitate was filtered and washed with anhydrous ether to give a 60% yield of a green powder, mp >360°; IR ν_{max} (KBr): 3450 (OH), 2160 (C≡N), and 1625 (C=C) cm^{-1}.

Anal.—Calc. for $C_7H_2NNa_2S_3 \cdot H_2O$: C, 28.40; H, 1.35; N, 4.75. Found: C, 27.98; H, 1.67; N, 4.50.

Sodium 2-Furoylacetonitrile-2-dithiocarboxylate (IX)—2-Furoylacetonitrile (21) (1 g, 0.0074 mole) and sodium methoxide (0.4 g, 0.0074 mole) were suspended in anhydrous benzene (15 ml) and heated at reflux for 1 hr. Heating was stopped, and carbon di-

[1] Antiradiation screening of several compounds was carried out at the Walter Reed Army Institute of Research. Results were reported through the courtesy of Capt. Gene Withrow.

[2] Melting points were determined in capillaries with a Mel-Temp melting-point block and are corrected. IR absorption spectra were obtained with a Perkin-Elmer model 137 B spectrophotometer with sodium chloride optics. UV absorption spectra were obtained with a Beckman DB-GT spectrophotometer. Elemental analyses were done by Dr. F. B. Strauss, Oxford, England, or by Dr. Carol K. Fitz, Carlisle, Mass. TLC was carried out using silica gel, and products were detected by exposure to iodine vapor.

[3] Aldrich Chemical Co.

[4] Cyclo Chemicals.

[5] K & K Chemicals.

[6] Metal Hydrides, Inc.

sulfide (0.56 g, 0.0074 mole) in methanol (5 ml) was added to the warm mixture. An additional 0.4 g of sodium methoxide was added, and stirring and refluxing were continued for 0.5 hr. The yellow precipitate was filtered, washed with anhydrous ether, and dried *in vacuo*, giving 0.9 g (90%) of yellow powder, mp 275–278°; IR ν_{max} (KBr): 3500 (OH), 2190 (C≡N), 1592 (C=C), and 1015 (C=S) cm^{-1}.

Anal.—Calc. for $C_8H_4NNaO_2S_2$: C, 41.20; H, 1.72; N, 6.01. Found: C, 41.52; H, 2.08; N, 6.29.

Dipotassium 2-(2-Thenoyl)-3,3-dimercaptoacrylonitrile (X)—Potassium hydroxide (0.47 g, 0.007 mole), dissolved in 10 ml of absolute ethanol, was added to a solution of thenoylacetonitrile (21) (0.5 g, 0.0035 mole) and carbon disulfide (0.22 ml, 0.0035 mole) in 10 ml of dry benzene and 5 ml of absolute ethanol. The mixture was stirred at room temperature for 4 days, filtered, and washed with ether, giving a 50% yield of brown powder, mp 208–210°; IR ν_{max} (KBr): 3450 (OH), 2198 (C≡N), 1640 (C=C), and 1000 (C=S) cm^{-1}.

Anal.—Calc. for $C_8H_3K_2NOS_3$: C, 31.70; H, 0.99; N, 4.63; S, 31.70. Found: C, 31.27; H, 1.38; N, 4.75; S, 31.40.

Triethylammonium 4-Sulfamoylphenyldithiocarbamate (XV)—To a solution of 1.72 g (0.01 mole) of sulfanilamide in 20.8 ml of triethylamine were added 10.5 ml (0.15 mole) of carbon disulfide and 60 ml of absolute ethanol. The solution was stirred for 6 hr at room temperature, allowed to stand overnight, and filtered. The product was washed with absolute ethanol and ether, giving 2.65 g (75%) of yellow crystals, mp 138–141°; IR ν_{max} (KBr): 990 (C=S) cm^{-1}.

Anal.—Calc for $C_{13}H_{23}N_3O_2S_3$: C, 44.70; H, 6.59; N, 12.03; S, 27.51. Found: C, 44.67; H, 6.77; N, 12.00; S, 27.91.

Potassium 4-Sulfamoylphenyldithiocarbamate—The potassium salt was prepared from sulfanilamide (1.72 g, 0.01 mole), dimethylformamide (5 ml), absolute ethanol (20 ml), and carbon disulfide (10.5 ml, 0.15 mole) in a manner similar to that described for XV. A yield of 2.25 g (71%) was obtained, mp 219° dec.

Anal.—Calc. for $C_7H_7KN_2O_2S_3$: C, 29.37; H, 2.45; N, 9.79; S, 33.60. Found: C, 29.58; H, 2.71; N, 10.00; S, 33.66.

Dipotassium Thiophene-2-sulfonylimidodithiocarbonate (XIII)—A solution of 1.63 g (0.01 mole) of thiophene-2-sulfonamide (22) in 20 ml of absolute ethanol was mixed with 10.5 ml (0.15 mole) of carbon disulfide and 8 ml of ethanol containing 0.03 mole of potassium hydroxide. The mixture was stirred overnight at room temperature, and the precipitate was washed with absolute ethanol, dry ether, and acetone, giving 1.5 g (47%) of hygroscopic yellow powder, mp 202–204° dec.

Anal.—Calc. for $C_5H_3K_2NO_2S_4$: C, 19.05; H, 0.95; N, 4.44; S, 40.64. Found: C, 18.63; H, 2.06; N, 4.05; S, 41.03.

Methyl *N*-(Thiophene-2-sulfonyl)dithiocarbamate (XIV)—A solution of 3.26 g (0.02 mole) of thiophene-2-sulfonamide (22) in 15 ml of dimethylformamide was treated with 1.2 ml of sodium hydroxide solution (16 g in 20 ml of water) and 0.7 ml (0.01 mole) of carbon disulfide with stirring. After 10 min, 0.6 ml of sodium hydroxide solution and 0.35 ml (0.005 mole) of carbon disulfide were added, and this addition was repeated 10 min later.

After being stirred for an additional 10 min, the solution was placed in an ice bath and treated with 1.1 ml (0.02 mole) of methyl iodide; the resulting solution was stirred for 2 hr and poured into 50 ml of water. Hydrochloric acid (concentrated) was added dropwise to the point of disappearance of turbidity, and the red oil which separated was isolated and shaken with several 50-ml portions of water. The resulting crystals were collected and recrystallized from benzene with a small amount of petroleum ether, giving 1.2 g (24%), mp 104–105°; IR ν_{max} (KBr): 1050 (C=S) cm^{-1}.

Anal.—Calc. for $C_6H_7NO_2S_4$: C, 28.42; H, 2.78; N, 5.53; S, 50.63. Found: C, 28.76; H, 3.01; N, 5.70; S, 51.02.

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Analogs of the Anorexic Mazindol

T. L. LEMKE ˣ, L. A. CATES, M. STEENBERG, and Y. M. CHO

Abstract □ Mazindol, 5-(*p*-chlorophenyl)-2,5-dihydro-3*H*-imidazo[2,1-*a*]isoindol-5-ol, has been shown to be an effective anorexic. To explore the structure–activity relationships, several 1-ethyl-3-substituted-4-aryl-4-hydroxyindeno[1,2-*c*]pyrazoles were prepared and subjected to various animal screens. The 1-ethyl-3-*tert*-butyl-4-aryl-4-hydroxyindeno[1,2-*c*]pyrazoles are capable of significantly depressing forced and spontaneous motor activity in mice but have low LD_{50}'s. Two of these compounds were tested in an Ehrlich ascites tumor screen. The 1-ethyl-3-phenyl-4-aryl-4-hydroxyindeno-[1,2-*c*]pyrazoles depressed forced and spontaneous motor activity at low doses and were relatively nontoxic.

Keyphrases □ Mazindol analogs—synthesis, effect on forced and spontaneous motor activity, mice □ Indeno[1,2-*c*]pyrazoles, 1-ethyl-3-substituted-4-aryl-4-hydroxy (mazindol analogs)—synthesis, effect on forced and spontaneous motor activity, mice

Recently, a new, chemically unique anorexic was introduced for use as an adjunct in the treatment of obesity. Mazindol (I) is a derivative of the imidazo[2,1-*a*]isoindole nucleus. Not only does this nucleus represent a new basic structure with anorexic activity, but the site of action of mazindol is reportedly different from that of previously used agents (1). This drug, in addition to suppressing appetite, has been reported to cause a mild central nervous system (CNS) stimulation in both laboratory animals and humans (2–4).

The relationship between this unique structure and the anorexic effect apparently has not been explored. Because of an interest in novel tricyclic nuclei, an investigation of the indeno[1,2-*c*]pyrazoles, which have structural features in common with the imidazo[2,1-*a*]isoindole nucleus, was undertaken. The synthesis and initial biological screening of these analogs (II) of mazindol are now reported[1].

EXPERIMENTAL[2]

Chemistry—The indeno[1,2-*c*]pyrazol-4-ones were prepared by the procedure of Braun and Mosher (5) as shown in Scheme I. Grignard addition of arylmagnesium bromides to the 1-ethylindeno[1,2-*c*]pyrazol-4-ones (V) gave the appropriate tertiary alcohols (II*a*–II*f*).

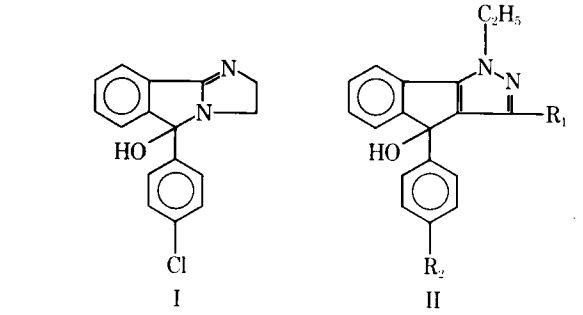
*1-Ethyl-3-*tert*-butylindeno[1,2-c]pyrazole-4(1*H*)-one (V)*—A 9.7-g (0.043-mole) sample of IV (R_1 = *tert*-butyl) (5) was added to 3 liters of 10% sodium hydroxide and heated to boiling. The solution was filtered and cooled, and the resulting solid was collected and dried at 70°, mp > 300°. The solid was added to a solution of 20.0 g (0.18 mole) of ethyl bromide and 150 ml of 100% ethanol and heated at reflux for 16 hr. The solution was cooled to give 9.9 g of material which, after recrystallization from 95% ethanol, gave 7.7 g of product, mp 115–117°; NMR ($CDCl_3$): δ 1.47 (9H, s), 1.63 (3H, t, J = 7 Hz), 4.3 (2H, q, J = 7 Hz), and 7.0–7.5 (4H, m).

Anal.—Calc. for $C_{16}H_{18}N_2O$: C, 75.56; H, 7.13; N, 11.02. Found: C, 75.63; H, 7.24; N, 10.93.

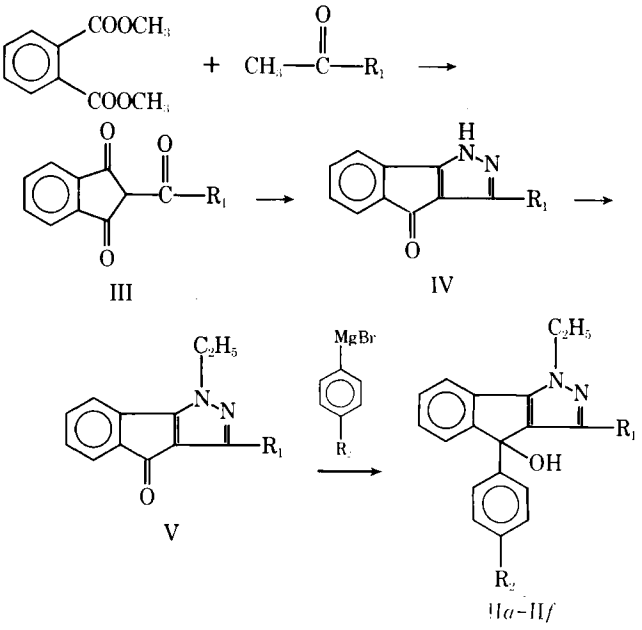
*1-Ethyl-3,4-diphenyl-4-hydroxyindeno[1,2-c]pyrazole (II*a*)*—To 0.96 g (0.04 g-atom) of magnesium in 40 ml of previously distilled tetrahydrofuran was added dropwise 6.28 g (0.04 mole) of bromobenzene in 140 ml of tetrahydrofuran. The reaction was initiated by heating at 70° and stirring under a nitrogen atmosphere. Once the reaction started, addition was at such a rate so as to maintain a gentle reflux. To the Grignard product was added 5.48 g (0.02 mole) of IV (R_1 = C_6H_5) (1) in 80 ml of tetrahydrofuran. The addition took 1.5 hr and was followed by 2 hr of reflux, entirely under a nitrogen atmosphere.

The solvent was removed by distillation, and the residue was added to 200 ml of ice water and allowed to stand overnight. The solid was collected by filtration, dried, and chromatographed on 600 g of silica gel, using methylene chloride as the eluting solvent. The solid coming off the column was recrystallized with ethyl acetate to yield 4.3 g (61%), mp 217–218.5°; NMR ($CDCl_3$–dimethyl sulfoxide-d_6): δ 1.60 (3H, t, J = 7 Hz), 3.07 (1H, b, OH), 4.42 (2H, q, J = 7 Hz), and 6.9–7.8 (14H, m).

Anal.—Calc. for $C_{24}H_{20}N_2O$: C, 81.78; H, 5.72; N, 7.95. Found: C, 81.62; H, 5.76; N, 7.95.



I II



Scheme I

[1] Since this manuscript was submitted, several reports on the chemistry and structure–activity relationship have appeared: P. Aeberli, P. Eden, J. H. Gogerty, W. J. Houlihan, and C. Penberthy, *J. Med. Chem.*, **18**, 177(1975); *ibid.*, **18**, 182(1975); and S. Barcza and W. J. Houlihan, *J. Pharm. Sci.*, **64**, 829(1975).

[2] Melting points were determined with a Thomas-Hoover Unimelt melting-point apparatus and are uncorrected. Chromatography was done on Brinkmann silica gel 60, particle size 0.063–0.2 mm. All products were submitted for NMR analysis on a Varian Associates T-60 or EM-360 and for IR analysis on a Perkin-Elmer 700 spectrophotometer and were consistent with proposed structures. Microanalyses were performed by Atlantic Microlab, Inc., Atlanta, Ga.

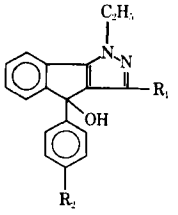


Table I—Acute LD_{50}[a], ED_{50}[b], and Safety Indexes of Mazindol Analogs in Mice

Compound	R_1	R_2	LD_{50}, mg/kg ip	ED_{50}, mg/kg ip	Safety Index, LD_{50}/ED_{50}
IIa	C_6H_5	H	>1000	115	>8.7
IIb	C_6H_5	Cl	>1000	90	>11.1
IIc	C_6H_5	CH_3	>1000	112.0	>8.9
IId	$C(CH_3)_3$	H	172.6	54	3.2
IIe	$C(CH_3)_3$	Cl	43.3	16.8	2.6
IIf	$C(CH_3)_3$	CH_3	485.0	42.5	11.4

[a] The 72-hr LD_{50}. [b] Calculated from Table IV.

1-Ethyl-3-phenyl-4-(p-chlorophenyl)-4-hydroxyindeno[1,2-c]-pyrazole (IIb)—When the same procedure and molar quantities were used as for IIa, 4 g of product was recovered. The solid was chromatographed using chloroform as the eluting solvent to remove the impurity and 5% methanol–chloroform to remove the product. Recrystallization of the product from isopropyl alcohol gave 2.5 g (IIb), mp 230–233°; NMR (dimethyl sulfoxide-d_6): δ 1.57 (3H, t, J = 7 Hz), 4.5 (2H, q, J = 7 Hz), 6.57 (1H, s, OH), and 7.2–8.0 (13H, m).

Anal.—Calc. for $C_{24}H_{19}ClN_2O$: C, 74.51; H, 4.95; Cl, 9.15; N, 7.24. Found: C, 74.36; H, 5.02; Cl, 9.26; N, 7.15.

1-Ethyl-3-phenyl-4-(p-tolyl)-4- hydroxyindeno[1,2-c]pyrazole (IIc)—When the same procedure and molar quantities were used as for IIa, 6.0 g of material was isolated. This material was chromatographed using methylene chloride as the eluting solvent. The product was recrystallized from ethyl acetate to give 4.0 g (IIc), mp 220.5–222.5°; NMR ($CDCl_3$): δ 1.85 (3H, t, J = 7 Hz), 2.22 (3H, s), 3.3 (1H, b, OH), 4.18 (2H, q, J = 7 Hz), and 6.8–7.8 (13H, m).

Anal.—Calc. for $C_{25}H_{22}N_2O$: C, 81.93; H, 6.05; N, 7.65. Found: C, 81.90; H, 6.08; N, 7.63.

1-Ethyl-3-tert-butyl-4-phenyl-4-hydroxyindeno[1,2-c]pyrazole (IId)—The same procedure and molar quantities were used as for IIa, and the concentrated reaction mixture was added to ice water and extracted with ether. The combined ether extract was dried over anhydrous magnesium sulfate. Removal of the solvent followed by chromatography on 600 g of silica gel, using 5% methanol–chloroform as the eluting solvent, resulted in recovery of the product. This product was recrystallized from cyclohexane, mp 142–144°; NMR ($CDCl_3$): δ 1.1 (9H, s), 1.53 (3H, t, J = 7 Hz), 2.43 (1H, b, OH), 4.37 (2H, q, J = 7 Hz), and 7.0–7.5 (9H, m).

Anal.—Calc. for $C_{22}H_{24}N_2O$: C, 79.48; H, 7.28; N, 8.43. Found: C, 79.41; H, 7.29; N, 8.37.

1-Ethyl-3-tert-butyl-4-(p-chlorophenyl)-4-hydroxyindeno[1,2-c]pyrazole (IIe)—When the same procedure and molar quantities were used as for IIa and the ether extraction was performed as for IId, a solid was recovered. This solid was chromatographed using chloroform as the eluting solvent. Recrystallization from cyclohexane gave 3.1 g of IIe, mp 143–148°; NMR ($CDCl_3$): δ 1.07 (9H,s), 1.47 (3H, t, J = 7 Hz), 3.12 (1H, s, OH), 4.27 (2H, q, J = 7 Hz), and 6.9–7.4 (8H, m).

Table II—Survival Study of Swiss Mice Bearing the Ehrlich Ascites Tumor[a]

Group[b]	Drug	Dose, mg/kg × days	Survival Time, days ± SE	Number of Survivors at 60 days
1	IIe	10 × 9	13.6 ± 1.4	0
2	IId	40 × 9	24.0 ± 9.97	1
3	Control	None	14.8 ± 1.6	0

[a] *Cancer Chemother. Rep.*, 3(2) (1972). [b] Five mice per group.

Table III—Effects of Derivatives of Mazindol on Forced Motor Activity in Mice

Compound	Dose, mg/kg	Third Trial, sec ± SE	Fourth Trial, sec ± SE	Percent of Control
IIa	75	201.6 ± 18.4	156.5 ± 22.4	77.6
	100	218.2 ± 13.5	109.9[a] ± 24.3	50.4
	150	203.1 ± 5.7	169.8 ± 23.8	83.6
IIb	50	200.0 ± 25.3	189.0 ± 19.9	94.5
	100	162.2 ± 20.9	139.3 ± 30.5	85.9
	200	203.5 ± 18.7	174.0 ± 12.1	85.5
IIc	75	184.2 ± 18.5	117.3[a] ± 22.2	63.7
	100	164.1 ± 13.7	108.0[a] ± 17.2	65.8
	150	203.2 ± 22.1	122.3[a] ± 21.4	60.2
IId	25	221.1 ± 7.5	199.5 ± 21.2	90.2
	50	259.0 ± 15.2	225.8 ± 14.6	87.2
	100	196.5 ± 6.9	104.5[a] ± 33.3	53.2
IIe	10	223.3 ± 22.0	225.2 ± 18.0	100.9
	14	226.1 ± 26.9	124.0[a] ± 23.2	54.8
	20	187.8 ± 1.5	67.3[a] ± 25.2	35.8
IIf	12.5	180.4 ± 27.1	163.3 ± 25.9	90.5
	25	166.2 ± 24.1	115.1 ± 22.2	69.3
	50	192.7 ± 15.2	107.1[a] ± 20.7	55.6

[a] $p < 0.05$.

Anal.—Calc. for $C_{22}H_{23}ClN_2O$: C, 72.02; H, 6.32; Cl, 9.66; N, 7.64. Found: C, 72.17; H, 6.34; Cl, 9.76; N, 7.57.

1-Ethyl-3-tert-butyl-4-(p-tolyl)-4-hydroxyindeno[1,2-c]pyrazole (IIf)—When the same procedure and molar quantities were used as for IIa and the ether extraction was performed as for IId, a solid was recovered. This solid was chromatographed using methylene chloride as eluting solvent to give 4.0 g of product. Recrystallization from cyclohexane gave 3.3 g of IIf, mp 153–156°; NMR ($CDCl_3$): δ 1.10 (9H, s), 1.55 (3H, t, J = 7 Hz), 2.28 (3H, s), 4.33 (2H, q, J = 7 Hz), and 6.9–7.4 (8H, m).

Anal.—Calc. for $C_{23}H_{26}N_2O$: C, 79.73; H, 7.56; N, 8.09. Found: C, 79.68; H, 7.59; N, 8.09.

Pharmacology—Ascites survival studies were performed on male Swiss albino mice, which were implanted intraperitoneally with 4×10^6 Ehrlich ascites cells. Drug treatment was started 24 hr after implantation and injections were given intraperitoneally once daily for 9 days. Drugs were suspended in 0.5% carboxymethylcellulose–saline.

Male Swiss-Webster mice[3], 20–25 g, were used for forced and spontaneous motor activity tests after being acclimated to laboratory conditions for 3–4 days. Each dose was usually tested in six mice, which were permitted food and water *ad libitum*. The six compounds were suspended in 5% polysorbate 80–95% isotonic saline and administered in doses of 10 ml/kg ip. The injection time was 15 sec. The results that differed from control values at the $p < 0.05$ level (Student t-test) were considered statistically significant.

Method—The acute 72-hr intraperitoneal lethal dose effects were determined in mice using three dose levels, and the LD_{50} was estimated using the method of Litchfield and Wilcoxon (6).

Forced and Spontaneous Motor Activity—The effects of the compounds on forced motor activity of mice were studied using the rotarod. The wood rod rotated at 4 rpm for the first 30 sec, at 6 rpm during the next 30 sec, and at progressively increasing speeds thereafter at 30-sec intervals (maximum 50 rpm) until the mouse fell off. Six animals were tested simultaneously and were given four trials with two spaced 4–6 hr apart on each of 2 consecutive days. The fourth trial was preceded by an interval of 50 min for the administration of vehicle or one selected dose of the experimental compounds. The drug or placebo effect for each animal was computed on a ratio of performance time on the fourth trial divided by performance time on the third trial.

The effects of the compounds on the spontaneous motor activity in mice were measured in three photocell cages[4]. Two animals treated with identical doses of the same compound were placed in each photocell cage 10 min after the initiation of the rotarod test, and a 15-min count was initiated 5 min after the animals were placed in the cages. To negate the differences in sensitivity among

[3] Texas Inbred, Houston, Tex.
[4] Actophotometer, Woodward Research Corp.

Table IV—Effects of Derivatives of Mazindol on Spontaneous Motor Activity in Mice

Compound	Dose, mg/kg	Control ± SE	Drug ± SE	Percent of Control
IIa	75	1199.7 ± 215.7	874.0 ± 79.9	72.9
	100	1199.7 ± 215.7	497.3[a] ± 64.3	41.5
	150	1338.7 ± 46.2	733.0 ± 138.8	54.8
IIb	50	1338.7 ± 46.2	851.3 ± 155.4	63.6
	100	1422.3 ± 89.4	591.7[a] ± 120.5	41.6
	200	1422.3 ± 89.4	743.0[a] ± 56.0	52.2
IIc	75	1214.8 ± 95.8	842.3 ± 174.2	69.3
	100	1214.8 ± 95.8	702.7 ± 286.0	57.8
	150	1249.7 ± 195.1	471.3[a] ± 86.4	42.7
IId	25	860.0 ± 44.1	490.0[a] ± 98.1	57.0
	50	860.0 ± 44.1	437.0[a] ± 65.2	50.8
	100	1201.7 ± 45.9	314.0[a] ± 166.1	26.1
IIe	10	1250.8 ± 180.3	1145.7 ± 92.1	91.6
	14	1201.7 ± 45.9	984.7 ± 124.3	81.9
	20	1250.8 ± 180.3	272.0[a] ± 92.9	21.7
IIf	12.5	938.7 ± 35.6	797.7 ± 27.7	85.0
	25	938.7 ± 35.6	694.3 ± 135.4	74.0
	50	1249.7 ± 195.1	534.0[a] ± 86.4	42.7

[a] $p < 0.05$.

units, each dose was tested in a factorial design in each of the three activity cages. Control animals were tested simultaneously at the same time intervals after administration of an equal volume of vehicle, and the ED_{50} of each compound (defined as the dose that decreased the level of performance to 50% of the control scores) was calculated (6).

The percent of control activity (spontaneous and forced motor activity) at each dose level of the compounds used was plotted in an attempt to evaluate the possible types of activity of each compound.

RESULTS AND DISCUSSION

The products synthesized and investigated are shown in Table I along with their LD_{50}, ED_{50}, and safety index values. Derivatives

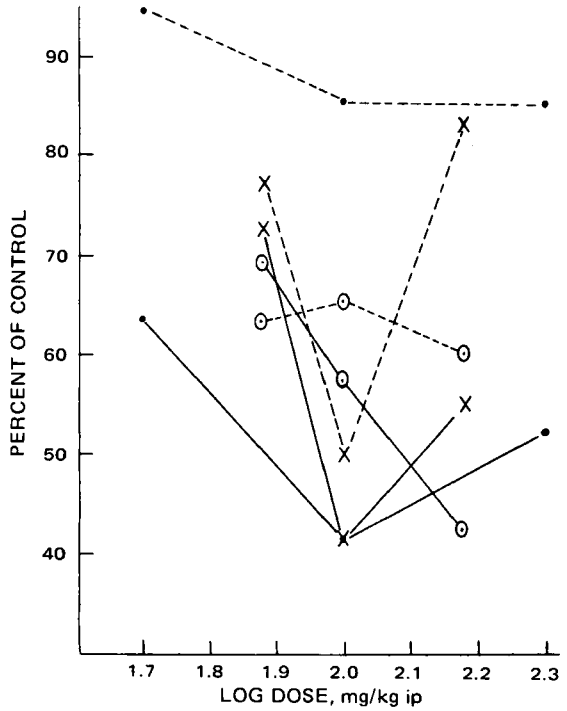


Figure 1—*Decrease in forced and spontaneous motor activity in mice with 1-ethyl-3-phenyl-4-aryl-4-hydroxyindeno[1,2-c]pyrazoles. Key: ——, spontaneous motor activity; - - -, forced motor activity;* ×, *IIa;* ●, *IIb; and* ⊙, *IIc.*

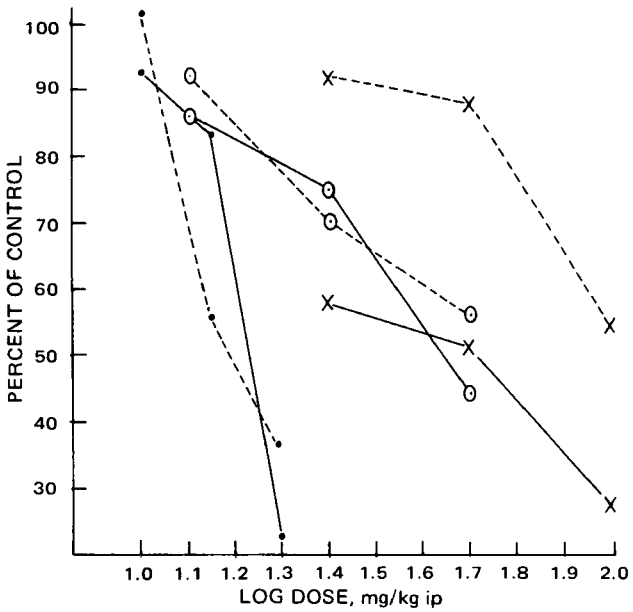


Figure 2—*Decrease in forced and spontaneous motor activity in mice with 1-ethyl-3-*tert*-butyl-4-aryl-4-hydroxyindeno[1,2-c]pyrazoles. Key: ——, spontaneous motor activity; - - -, forced motor activity;* ×, *IId;* ●, *IIe; and* ⊙, *IIf.*

of 1-ethyl-3-phenyl-4-aryl-4-hydroxyindeno[1,2-c]pyrazoles (IIa–IIc) are relatively nontoxic, while the compounds in which a tertiary butyl group has been substituted for the 3-phenyl group are toxic in mice. All compounds, with the exception of IIe, are less toxic than mazindol (LD_{50} 106 mg/kg po).

For all compounds, death was a result of respiratory stimulation and clonic convulsions. Due to a strong interest in potential anticancer agents and, especially, agents possessing the hydrazino moiety (7) such as that found in the pyrazole ring, several of the more toxic agents were submitted to an Ehrlich ascites tumor screen. The results of this preliminary evaluation are shown in Table II.

The compounds were next tested in mice for their effects on forced and spontaneous motor activity, and the results are presented in Tables III and IV, respectively, and in Figs. 1 and 2. Gogerty *et al.* (3) reported that mazindol produced moderate to marked CNS stimulation, appetite suppression, and antidepressant activity in both mice and rats. In studies with the derivatives of mazindol, just the opposite effects were noted, with impressive decreases in spontaneous and forced motor activity. The 1-ethyl-3-*tert*-butyl-4-aryl-4-hydroxyindeno[1,2-*c*]pyrazoles (II*d* and II*e*) were the most potent compounds in reducing both forced and spontaneous motor activity. However, these agents were the more toxic derivatives and for IIe the highest dose used was about 50% of the LD_{50}; the effects may, therefore, be due to a toxic effect. The 1-ethyl-3-phenyl-4-aryl-4-hydroxyindeno[1,2-*c*]pyrazoles (II*a*–IIc) showed a slight decrease in spontaneous and forced motor activity. In two cases (II*a* and II*b*), this decrease was followed by an upward trend in motor activity at higher doses. Perhaps this effect would show an increase at higher doses but the studies were not carried that far.

The antidepressant drugs like imipramine and desipramine when administered in doses of 40 and 60 mg/kg ip to untreated mice, led to ptosis, a decrease in body temperature, and a decrease in both forced and spontaneous motor activity. It is only possible to discover their antidepressant effects by doing a more detailed study of each compound using a battery of tests, including the effects of each compound in reversing the effect of reserpine in mice and rats.

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JOHN T. H. ONG [*] and HARRY B. KOSTENBAUDER [x]

Abstract □ The apparent rate of degradation of penicillin G potassium micellar solutions of 500,000 units/ml, a concentration commonly encountered in vials reconstituted for storage in the refrigerator, was investigated and compared to that of nonmicellar solutions of 8000 units/ml at 25°, ionic strength of 1.1 *M*, and pH range from 5.0 to 9.5. In the micellar solutions the apparent rate of the H^+-catalyzed degradation was increased twofold but that of water- and OH^--catalyzed hydrolysis was decreased two- to threefold. Consequently, the pH–rate profile of the micellar solutions was shifted to higher pH values and the pH of minimum degradation was found to be at 7.0 compared to 6.5 for the nonmicellar solution of the same ionic strength. Compared at their respective pH–rate profile minima, micellar penicillin G is 2.5 times as stable as the nonmicellar solution under the conditions of constant pH and ionic strength.

Keyphrases □ Penicillin G potassium degradation—concentrated aqueous micellar solutions compared to nonmicellar solutions, pH–rate profiles □ Degradation of penicillin G potassium—concentrated micellar aqueous solutions compared to nonmicellar solutions, pH–rate profiles □ Micellar solutions of penicillin G—degradation compared to nonmicellar solutions, pH–rate profiles

The kinetics of penicillin G degradation in aqueous solution have been studied extensively. The effects of pH, temperature, buffers, ionic strength, metal ions, and model catalysts that simulate penicillinase and other enzymes have been investigated (1–6). The effect of surfactants below and above their critical micelle concentration (CMC) was also studied, with the finding that at pH 6.5 all surfactants studied enhanced the rate of penicillin G degradation (7).

The present study was undertaken to assess the kinetics of penicillin G degradation at concentrations above the CMC, particularly at 500,000 units/ml as encountered in hospital usage, and to obtain a comparison of the pH of minimum degradation for solutions above and below the CMC. Instead of buffer, the pH-stat technique was utilized to maintain a constant pH in these studies. The dilution obtained in all studies was negligible due to the very high concentration of titrant (15 *M* KOH) used.

BACKGROUND

In the earlier studies (1–7), relatively low concentrations of penicillin G were used (8000 units/ml or 0.5% w/v). Literature pertaining to the kinetics of penicillin G degradation at high concentration is limited to observations of the percent degraded after a certain time under specific conditions. Although several results tend to indicate that penicillin G degrades faster at higher concentration, no definitive comparison between the degradation rates at the high and low concentrations can be made because the solutions studied were either unbuffered (8, 9) or inadequately and not proportionally buffered (10, 11). Inadequate buffering of concentrated solutions of penicillin G could give an inordinately large apparent rate of degradation due to a marked pH decrease from the production of large quantities of penicilloic acid.

It has been common practice, particularly in hospitals, to batch-reconstitute buffered penicillin G solutions at a concentration of 500,000 units/ml (30% w/v) and store them frozen until needed. Periodically, the frozen solutions are moved into the refrigerator and kept in a liquid state ready to be diluted with intravenous infusion fluid and administered to patients. According to the manufacturer, sterile solutions in the refrigerator may be kept for 1 week without significant loss of potency. Knowledge of the pH of minimum degradation at this high concentration becomes essential.

The formation of micelles of penicillin G in aqueous solution was first observed by Hauser *et al.* (12). Later studies (13, 14) found the CMC to be relatively high, *i.e.*, 0.25 molal, 130,000 units/ml, or 8.26% (w/v). This value was further confirmed using the data of the NMR (100 MHz) chemical shift of the aromatic protons (15). It was indicated that the number of penicillin G ions that come together to form the aggregates is probably not very large.

In recent years, reactions in micellar systems have been increasingly investigated. Extensive reviews of the kinetic and mechanistic implications of micellar catalysis have been presented (16, 17). From the drug product stability point of view, micellization is important because it can either increase or decrease the stability of a drug, depending on the nature of the micelle and the reaction involved. Thus, catalytic effects of micelles have been observed when both reactants are enriched within the micelles for a bimolecular process. A study (18) of the accelerated fading of cationic triphenylmethane dyes in alkaline solutions of the quaternary surfactant cetyltrimethylammonium bromide has become classic. The micelle itself can serve as a substrate for the reaction, *e.g.*, micellization enhances the rate of H^+-catalyzed hydrolysis of sodium lauryl sulfate by 30-fold over that in a single ion state (19). Conversely,

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when one reactant is enriched and the other reactant is excluded from the micelle, a rate decrease in the micellar phase is observed. Thus, sodium lauryl sulfate has been shown to inhibit the OH^--catalyzed hydrolysis of benzocaine (20).

EXPERIMENTAL

Materials—Penicillin G potassium, 1595 units/mg[1], was used. All reagents were of analytical grade. Imidazole[2] was recrystallized twice from benzene and washed with anhydrous ether. Double-distilled water[3] was used in all studies.

Analytical Method—The intact penicillin G concentration was determined according to a spectrophotometric[4] method (21). Molar absorptivity at 325 nm, determined from the standard curve, was 26.3×10^3 which is in excellent agreement with that previously reported (21).

Kinetic Studies—The pH of the penicillin G potassium solution was maintained constant at the desired value with the aid of pH-stat[5]. A 50-ml reaction vessel (V520) was immersed in the thermostated jacket (V525), through which water of 25° was circulated from a constant-temperature bath and circulator[6] (sensitivity ±0.1°). Inserted into the reaction vessel was a glass electrode (G 202C), a saturated calomel reference electrode (K 401), a stirrer, and a delivery tube for titrant (15 *M* KOH). The proportional band of the titrator was set to provide small incremental additions of titrant.

Penicillin G potassium solutions above the CMC were prepared at a concentration of 30% (w/v) in solutions of 0.3 *M* potassium chloride so that the final solutions had an ionic strength of 1.1 *M*. At selected time intervals, 40 μl of sample was withdrawn with a microsyringe[7] and diluted with water to 100 ml in a volumetric flask. Two 1-ml samples of the diluted solution were then pipetted into two separate 10-ml test tubes, and each was assayed separately according to the method described by Bundgaard and Ilver (21).

Solutions of penicillin G potassium below the CMC were prepared at a concentration of 0.5% (w/v) in solutions of 1.1 *M* potassium chloride. The same procedure was followed, except that 1 ml of sample was withdrawn and diluted with water to 50 ml in a volumetric flask.

In both micellar and nonmicellar solutions, the initial rate was determined by following 5–10% of the entire course of the reactions. The observed pseudo-first-order rate constants were then calculated by linear regression analysis of a plot of the logarithm of penicillin G concentration against time.

RESULTS AND DISCUSSION

The concentration of micellar penicillin G used was very high (500,000 units/ml, 0.8 *M*, or 30% w/v). It would not be possible to keep the pH constant using buffer systems, because a very high buffer capacity would be required. Many buffer systems are known to have marked catalytic effects so that a high buffer concentration is not desirable. Although citrate buffer has little catalytic effect at neutral pH (3), the buffer capacity is quite low under these conditions. Large citrate concentrations increase the ionic strength of the solution, which, in turn, enhances the rate of the OH^--catalyzed reaction (3) as well as that at the pH of minimum degradation. Therefore, the concentration of citrate buffer found in commercial products is limited to 1.5% (w/v). The present study was conducted at a constant ionic strength of 1.1 *M* to take into account the contributions of 30% penicillin G potassium and 1.5% citrate buffer. The change in ionic strength due to counterion binding to the penicillin G micelle was assumed to be negligible for these apparently small aggregates.

The results of kinetic studies in nonmicellar and micellar solutions of penicillin G are recorded in Table I. The rates at pH 5 and 5.5 were increased and those at pH 8.5 and 9.0 were decreased twofold in micellar systems relative to nonmicellar solutions.

Table I—Effect of Concentration on the Magnitude of the Observed Pseudo-First-Order Rate Constants and the Corresponding t_{90} at 25° and Ionic Strength of 1.1 *M*

	Nonmicellar (0.5% w/v)		Micellar (30% w/v)	
pH	$10^3 k_{obs}$, hr^{-1}	t_{90}, hr	$10^3 k_{obs}$, hr^{-1}	t_{90}, hr
5.0	10.2	10.3	18.8	5.6
	9.02	11.7	18.2	5.8
5.5	2.88	36.6	6.22	17.0
	—	—	7.57	13.9
6.0	1.49	70.8	2.25	46.9
6.5	1.16	90.8	0.760	138.8
7.0	1.41	74.8	0.466	226.4
	—	—	0.443	238.1
7.5	—	—	0.669	157.7
8.0	2.52	41.9	1.03	102.4
8.5	3.87	27.3	1.77	59.6
	4.49	23.5	2.04	51.7
9.0	11.4	9.2	5.46	19.3
9.5	—	—	16.4	6.4
	—	—	18.7	5.6

Penicillin G is an acid with a pKa of 2.80. The degradation is known to be first order with respect to penicillin G and is catalyzed by acid and base. The pH–rate profile is linear in the pH region of 4–6 and above 8 and has a minimum at pH 6.50–6.75. Above pH 5, penicillin G exists mostly in the anionic form. In the pH region investigated, the principal reactions in both nonmicellar and micellar states are attacks of H^+, water, and OH^- on penicillin G anion. It is also conceivable that in the micelle the molecules could be aligned to facilitate intramolecular or intermolecular attack on the β-lactam carbonyl carbon by the amide carbonyl oxygen (22). The acid-catalyzed reaction of undissociated penicillin G was not significant under the study conditions.

According to Thakkar and Wilham (15), penicillin G ions aggregate primarily by hydrophobic interactions involving the benzyl side chains. The ionized carboxylate groups on the thiazolidine ring form the periphery of the micelle in intimate contact with the surrounding water. Therefore, the micelles formed are of the anionic type. The greater negative charge density of the micelles would exert stronger electrostatic attraction toward hydrogen and sodium ions to be bound as counterions. This would facilitate isomerization to penicillenic acid, since it is known that the formation of penicillenic acid is a result of the reaction of penicillin G ion with hydrogen ions or the kinetically equivalent spontaneous rearrangement of undissociated penicillin (3, 23).

The association between penicillin G ion and hydrogen ions to form the reactive species, undissociated penicillin G, might also be favored by a larger positive entropy change for the micellar reaction. This can be explained in terms of hydration and electrostatic entropy changes. In terms of hydration of ions, the explanation of the entropy factor is that the two oppositely charged ions coming together to form a neutral molecule become partially dehydrated and cause breakdown of the "iceberg" structure of coordinated water molecules around the ions, leading to a decreased orientation of solvent molecules or an increase of entropy (24). The larger charge density of the micelle causes the formation of a more ordered structure, consisting of water molecules and counterions about its outer, polar surface. Thus, there would be a greater overall change in randomness in micellar systems, since the number of electrostatically bound water molecules released in the formation of neutral undissociated penicillin G would be greater for the micelle state than for the single ion state.

In terms of electrostatic theory, the entropy change resulting solely from bringing two ions from infinite separation to the equilibrium distance r is given by (25):

$$\Delta S_{el} = \frac{Z_A Z_B e^2}{Dr}\left(\frac{\partial \ln D}{\partial T}\right)_p \qquad \text{(Eq. 1)}$$

where Z_A and Z_B are the charges of the two reacting ions, and D is the dielectric constant of the medium. The quantity $(\partial \ln D/\partial T)$ is always negative, because thermal motion overcomes the orienta-

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Eastman Kodak Co., Rochester, N.Y.
[3] Corning AG-3 distillator, Corning Glass Works, Parkersburg, W.Va.
[4] Gilford 240 spectrophotometer, Gilford Instrument Labs., Monrovia, Calif.
[5] Radiometer A/S, Copenhagen, Denmark, comprised of a TTT2 automatic titrator, a TTA3 titration assembly, an SBR2c titrigraph, and an ABU11 autoburet (0.25 ml).
[6] Haake F-Junior, Haake Instruments, Rochelle Park, N.J.
[7] Drummond Scientific Co., Broomall, Pa.

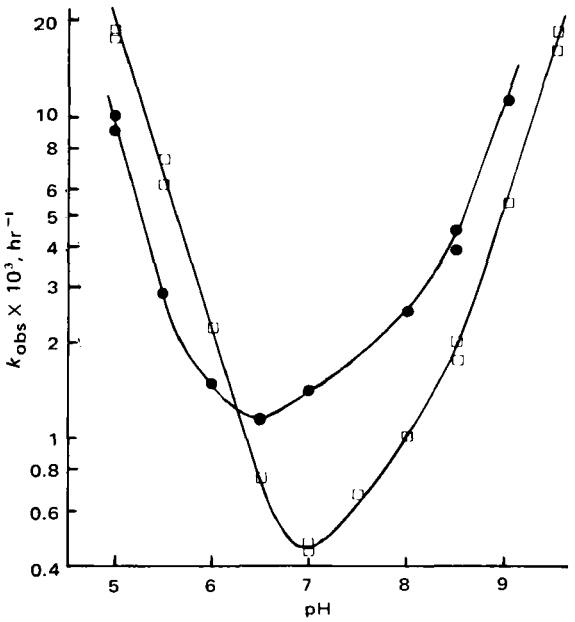

Figure 1—*The pH–rate profiles of penicillin G in 0.5% (w/v) nonmicellar (●) and 30% (w/v) micellar (□) concentrations.*

tion of dipoles in an electric field (26). Thus, for a reaction of oppositely charged penicillin and hydrogen ions, ΔS_{el} has a positive value and is inversely proportional to the dielectric constant of the medium. The surface of micelles appears to be less polar than water itself (27). In the present micellar system, the carboxylate group of the penicillin G ion might be regarded as being attached to a region of lower dielectric constant than water in the bulk of the solution; therefore, ΔS_{el} is larger and more positive than in nonmicellar solution.

At pH around and above neutrality, the main degradation is the water- and OH^--catalyzed hydrolysis of the β-lactam ring to form penicilloic acid. The micellar state somewhat protects the β-lactam ring from nucleophilic attack, because it is located farther inside the micelle surface. Furthermore, the OH^- is excluded from the anionic micelle by electrostatic repulsion. In contrast with the H^+-catalyzed degradation, the OH^- attack on the negatively charged penicillin G ion is not favored by entropy change. The transition state is a more highly charged ion which would be expected to be strongly hydrated, leading to a decrease of entropy. The electrostatic entropy change becomes more negative in the micellar phase than in the bulk of the solution. These unfavorable factors lead to a decrease of rate in the micellar system at pH around and above neutrality.

Figure 1 shows the pH–rate profiles of penicillin G in nonmicellar and micellar concentrations of 0.5 and 30% (w/v), respectively. The profile of micellar penicillin G was shifted to higher pH values. The pH of minimum degradation was obtained at 7.0 for micellar and 6.5 for nonmicellar penicillin G. The present results clearly indicate that micellar solutions of penicillin G degrade slower than nonmicellar solutions above pH 6.25, provided the pH of the solution is held constant. Under the conditions of the previous studies where the penicillin G solutions were either unbuffered (8, 9) or inadequately and not proportionally buffered (10, 11), the faster degradation rate observed at higher concentration can be attributed to a faster decrease of the pH of the solution since penicillin G itself has no buffering activity. The decrease in pH renders the H^+-catalyzed reaction more significant, leading to a faster degradation rate.

Based on the present results, micellar solutions of penicillin G are most stable if they are buffered at pH 7.0, although solutions of concentration less than the CMC (130,000 units/ml or 8.26% w/v) are most stable at pH 6.5. Micellar solutions buffered at pH 7.0 are approximately 2.5-fold more stable than nonmicellar solutions buffered at the pH of the minimum hydrolysis rate.

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Nonisothermal Kinetic Studies III: Rapid Nonisothermal–Isothermal Method for Stability Prediction

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Abstract □ A continuous nonisothermal–isothermal method for stability prediction was developed. The approach yields all necessary parameters for prediction, including reaction order. The experimental procedure involves changing the temperature of the samples being studied until degradation is rapid enough to proceed at a convenient isothermal rate for a sufficient number of half-lives with adequate analytical sensitivity so that the reaction order can be unambiguously determined. The analytical information obtained during the nonisothermal and isothermal portions of the experiment is utilized without curve matching in calculating the activation energy and determining the reaction order, reaction rate, and stability prediction at any desired temperature. Model experiments include the acid-catalyzed hydrolysis of acetylcholine bromide and the inversion of sucrose.

Keyphrases □ Nonisothermal kinetic studies—rapid nonisothermal–isothermal method for stability prediction □ Kinetics—rapid nonisothermal–isothermal method for stability prediction □ Stability prediction—rapid nonisothermal–isothermal kinetic method

Since the primary contribution to the field of nonisothermal kinetics by Borchardt and Daniels (1) in 1957, publications on this subject have appeared at a steady rate (2–8). When compared to isothermal methods, nonisothermal technology generally reduces the time and analytical effort needed to generate kinetic parameters. Although there are limitations (9) to the methods, these are being dealt with wherever possible (8).

Classical isothermal kinetics are often too burdensome to incorporate into predosage form development studies. A need exists for alternative short-term methodology so that the kinetic parameters needed for a knowledge of drug stability are known early in the preformulation phase. The nonisothermal methodology published to date has been subject to limitations (6, 7) of fixed time–temperature relationships, nonlinear curve fitting, and assumptions of reaction order. The method presented offers flexibility of time–temperature relationships, direct calculation of activation energy, and determination of reaction order.

In the proposed method, the investigator searches (through temperature adjustment and analytical determinations) for a temperature to run an isothermal experiment. Whereas this problem would normally be considered a screening study, it has been developed into a total experiment by mathematical treatment of data from the initial probing and subsequent isothermal rate determination.

THEORETICAL

A general method was developed which results in values for the reaction order, activation energy, and reaction rates from a single experiment without time–temperature restrictions or curve matching (5, 6). A time–temperature profile (Fig. 1) is obtained when using this technique, and it consists of two distinct parts: (*a*) a nonisothermal region from t_a to t_b, for which temperature can be any function of time, and (*b*) an isothermal region from t_b to t_c.

The concentration–time profile for the acid-catalyzed hydrolysis of acetylcholine bromide is shown in Fig. 2. The concentration function used is determined by the isothermal part. A function of concentration, $f(c)$, can be chosen that is linear under the isothermal conditions existing from t_b to t_c to describe a concentration, c, at time t when $c = c_b$ at $t = t_b$ (Eq. 1):

$$-[f(c) - f(c_b)] = k_f(t - t_b) \qquad \text{(Eq. 1)}$$

The $f(c) - f(c_b)$ is $c - c_b$ for a zero-order reaction [$f(c) = c$], is 2.303 log c/c_b for a first-order reaction [$f(c) = 2.303 \log c$], and is $1/c_b - 1/c$ for a second-order reaction [$f(c) = 1/c$]. The chosen function must hold for a reaction of a given order at all other temperatures. When the function of concentration, $f(c)$, which is linear against time under isothermal conditions, is plotted against time for the nonisothermal period from times t_a to t_b, it can be considered as a series of sequential isothermal reactions. Although it is relatively easy to describe the change in concentration from t_b to t_c, it is not easy to do so for t_a to t_b where the temperature is changing.

The approach used to resolve this problem is to divide this portion of the curve into a large number of equal segments. If these segments are "short enough," then the concentration function from t_a to t_b can be considered as a series of independent rates, each with its own rate constants (5, 6):

$$-\frac{f_b(c) - f_a(c)}{t_b - t_a} = \frac{k_1 + k_2 + k_3 \ldots k_i \ldots k_n}{n} = \bar{k}_n \qquad \text{(Eq. 2)}$$

According to this equation, the change in the concentration function with time equals the sum of the individual rate constants divided by the total number of constants. This result will equal the average rate constant. The relationship between the rate constant and activation energy, where a^* is the frequency factor, is:

$$k_i = a^* e^{-E_a/RT_i} \qquad \text{(Eq. 3)}$$

These equations can be combined to give Eq. 4, where Δt is the time interval of each short segment:

$$-\frac{f_b(c) - f_a(c)}{n\,\Delta t} = \frac{a^*}{n}\sum_{i=0}^{n-1} e^{-E_a/RT_i} = \bar{k}_n \qquad \text{(Eq. 4)}$$

Replacing the temperature in Eq. 4 by its corresponding time function gives Eq. 5, in which $G(t_i)$ is the time function:

$$\frac{a^*}{n}\sum_{i=0}^{n-1} e^{-E_a/RG(t_i)} = \bar{k}_n = -\frac{f_b(c) - f_a(c)}{n\,\Delta t} \qquad \text{(Eq. 5)}$$

It remains to be determined how large n must be to define the nonisothermal curve. This is accomplished by utilizing the relationship given in Eq. 6:

$$\lim_{n\to\infty}\left[1 + \sum_{i=2}^{n} e^{(E_a/R)(T_i - T_1)/(T_i T_1)}\right]/n = L \qquad \text{(Eq. 6)}$$

The origin of this relationship was described previously (6). It can be shown that the value of this expression approaches a constant value for a specific activation energy as n becomes larger. Therefore, by evaluating the expression for increasing values of n, a

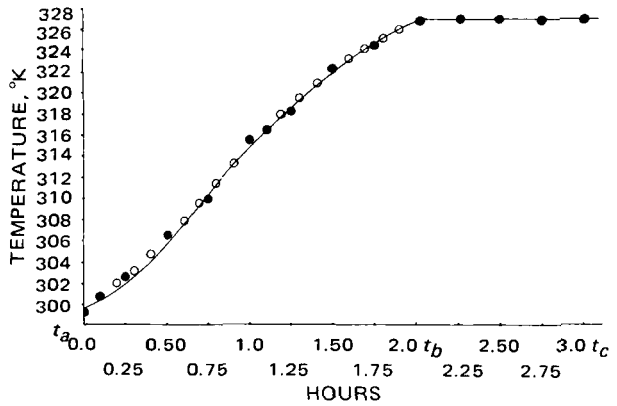

Figure 1—*Time-temperature relationship for the acid-catalyzed hydrolysis of acetylcholine bromide. Key:* O, *mathematical fit; and* ●, *experimental points.*

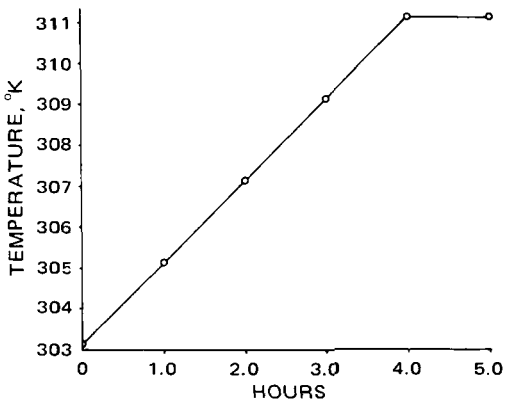

Figure 3—*Time-temperature relationship for the acid-catalyzed inversion of sucrose.*

value of n can be chosen that results in a constant not significantly different from the preceding one.

Finally, an expression can be derived that relates the activation energy to the average rate constant over the nonisothermal region, the rate constant for the isothermal region (k_j), and the length of time of each short segment:

$$-\frac{n[f_b(c) - f_a(c)]}{(t_b - t_a)k_j} = \frac{n\bar{k}_n}{\Delta t k_j} = \sum_{i=0}^{n-1} \exp\left[\frac{E_a}{R}\left(\frac{T_i - T_j}{T_i T_j}\right)\right] = C \quad \text{(Eq. 7)}$$

Making a series expansion of the exponentials results in Eq. 8, in which a, b, c, *etc.*, are the temperature terms and X is E_a/R. This equation can be evaluated by Newton's method to obtain the energy of activation:

$$n + (a + b + c \ldots)X + \frac{(a^2 + b^2 + c^2 \ldots)}{2!}X^2 + \frac{(a^3 + b^3 + c^3 \ldots)}{3!}X^3 + \ldots = C \quad \text{(Eq. 8)}$$

EXPERIMENTAL

Acetylcholine Bromide—A solution of 25 g of acetylcholine bromide (recrystallized from absolute ethanol) in 500 ml of 0.1 *N* HCl was prepared, and 20-ml aliquots were pipetted into 50-ml ampuls which were heat sealed. These ampuls were immersed in a water bath fitted with a thermoregulator[1] and thermometer (0.1° graduations). The thermometer was immersed in an unsealed ampul containing the acetylcholine bromide solution, and the temperature of this solution was allowed to equilibrate before the nonisothermal run was begun. An initial sample was taken, and the nonisothermal run was begun by hand manipulation of the thermoregulator at an appropriate rate of temperature increase. Samples were taken for analysis at convenient times, and the temperature was continuously recorded.

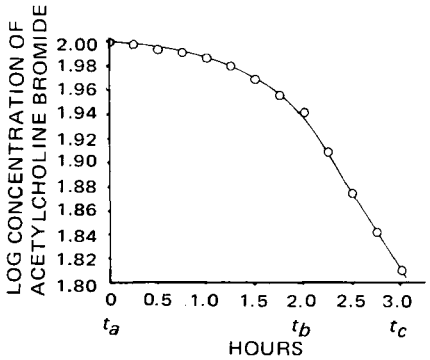

Figure 2—*Log concentration* versus *time relationship for the nonisothermal–isothermal hydrolysis of acetylcholine bromide.*

For analysis, the ampuls were quickly cooled under ice water. Five milliliters of solution was pipetted into a 250-ml erlenmeyer flask containing 50 ml of ice water, and the flask was immersed in ice water. A titration with 0.05 *N* NaOH (three titrations per sample) was performed, using phenolphthalein as the indicator. Correction was made for the initial hydrochloric acid content of the solution, with the remainder of sodium hydroxide consumed being proportional to the amount of acetic acid present. The moles (average of the three titrations) of acetic acid present were subtracted from the acetylcholine bromide initially in solution and used in the first-order calculations.

Sucrose—A 40% (w/v) sucrose solution in distilled water (400 ml) was thoroughly mixed with 0.05 *N* HCl (200 ml) in a 1-liter erlenmeyer flask. The flask was then immersed in a water bath fitted with a thermoregulator[1] and thermometer (0.1° graduations). This mixture was constantly stirred with a magnetic stirrer to prevent a temperature gradient. Sufficient time was allowed for equilibration of the temperature in the reaction solution before the nonisothermal run was begun. Following temperature equilibration, an initial sample was taken and the thermoregulator was fitted with a 2-rpm motor, which increased the temperature of the water bath by 2°/hr. The samples were removed for analysis every 15 min while the temperature was continuously recorded.

For analysis, aliquots (30 ml) were pipetted into 100-ml beakers and immediately diluted with 50 ml of 0.1 *N* NaOH. This solution was read on a polarimeter[2], using the sodium D line. Sucrose concentration is directly proportional to $(\alpha_t - \alpha_\infty)$, where α is the optical rotation. The rotation at time infinity, α_∞, is determined by heating a sample at 90° for 2–3 hr, diluting with 0.1 *N* NaOH, and then reading in the usual manner.

RESULTS AND DISCUSSION

The time-temperature relationship for the nonisothermal segment of Fig. 1 for the acid-catalyzed hydrolysis of acetylcholine bromide may be described by Eq. 9:

$$T = 298.5 + 14.743t + 33.742t^2 - 1.8099t^3 \quad \text{(Eq. 9)}$$

This equation was utilized in testing the convergence of Eq. 6 in order to determine the value of n to be used in Eq. 8. Table I shows that a value for n of 200 is adequate to describe the nonisothermal portion of the curve in Fig. 2. The criterion used to estimate convergence is a variation of less than 1% in Eq. 6 per change in n of 100. The value of n and Eq. 9 allow calculation of the coefficient terms of Eq. 8. The constant C is evaluated for the total experiment by use of the total nonisothermal concentration loss (2.90×10^{-2} hr^{-1}), the isothermal rate (1.38×10^{-1} hr^{-1}), and the value of n.

[1] A 115-v, 60-cycle thermoregulator, Bronwell Scientific Division, Will Corp., Rochester, N.Y.

[2] Model 70, O. C. Rudolph and Sons, Inc., Caldwell, N.J.

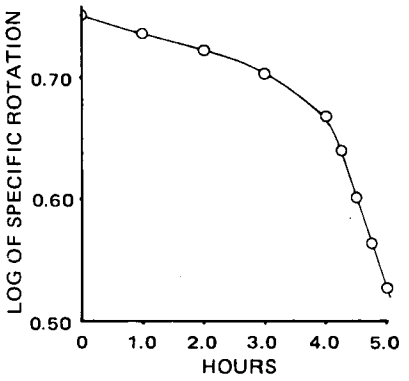

Figure 4—*Log specific rotation* versus *time relationship during a nonisothermal–isothermal acid-catalyzed inversion of sucrose.*

The coefficient terms for Eq. 8 were evaluated for hours 0–2 of the nonisothermal segment, and an equation was formed as follows:

$$157.15 - 0.03668 \times 10^{-1}X + 0.8288 \times 10^{-5}X^2 - 0.2125 \times 10^{-8}X^3 + 0.5867 \times 10^{-12}X^4 - 0.1695 \times 10^{-15}X^5 + 0.5049 \times 10^{-19}X^6 - 0.1537 \times 10^{-22}X^7 = 0 \quad \text{(Eq. 10)}$$

When each set of analytical points was considered and Eq. 8 was revised to include the time segment between these values, the mean activation energy calculated was 17.4 ± 0.9 kcal. The reported value was 17.5 kcal (10). Since the number of analytical points was nine, the number of equations and activation energies considered was $[m!/2(m-2)!]$ or 36 where $m = 9$. The plus or minus value represents 1 *SD* from the mean. For the sucrose experiment (Figs. 3 and 4), the nonisothermal time–temperature relationship is linear and may be expressed by:

$$T = 303.16 + 2t \quad \text{(Eq. 11)}$$

The convergence testing, as shown in Table II, yields a value of $n = 200$. The total loss in drug concentration (Fig. 4) during the nonisothermal portion of the experiment (1.843×10^{-2} hr^{-1}), the isothermal rate (1.539×10^{-1} hr^{-1}), and the value of n are used to calculate a value of 78.24 for C of Eq. 8 for the total experiment. When the coefficient terms of Eq. 8 are calculated, the expression for the total experiment is:

$$78.24 - 0.1278 \times 10^{-1}X + 0.1872 \times 10^{-5}X^2 - 0.2816 \times 10^{-9}X^3 + 0.4343 \times 10^{-13}X^4 - 0.684 \times 10^{-17}X^5 + 0.1097 \times 10^{-20}X^6 - 0.1788 \times 10^{-24}X^7 = 0 \quad \text{(Eq. 12)}$$

Newton's method of solution for this equation yielded a value of 29.2 kcal, which is higher than the literature values of 25.5–27.0 kcal (8). When the five analytical points were used in sets, the 10 resulting equations yielded a mean activation energy of 29.2 kcal (*SD* ± 0.9 kcal). This high value may be due to nonapplicability of the Arrhenius equation over the entire temperature span of the experiment. For each experiment, the linearity of the isothermal segment of Figs. 2 and 4 was assumed to be indicative of reaction order.

The parameters necessary for prediction of stability, that is, activation energy, an isothermal rate, and reaction order were thus obtained from a single continuous experiment. Predictive calculations can easily be made with these parameters by use of the Arrhenius equation and integration of the proper rate equation. It is not necessary to consider all of the mathematically possible sets of nonisothermal analytical values in setting up equations of the type depicted by Eqs. 10 and 12, as was done in this study. Experience in this technique does, however, suggest the use of at least five nonisothermal analytical points and 10 equations to yield 10 activation energies, which then may be considered in determining a mean activation energy.

Table I—Convergence of the Acid-Catalyzed Hydrolysis of Acetylcholine Bromide According to Eq. 6

n	E_a = 10 kcal	E_a = 20 kcal	E_a = 30 kcal
100	2.519	7.361	23.97
200	2.506	7.312	23.76

Table II—Convergence of the Acid-Catalyzed Inversion of Sucrose According to Eq. 6

n	E_a = 10 kcal	E_a = 20 kcal	E_a = 30 kcal
100	1.677	2.981	5.627
200	1.668	2.962	5.584

SUMMARY

This general method for determining activation energy, order of reaction, and rate constants might be very useful for studying reactions that occur slowly at room temperature. The method is subject to limitations imposed by assay precision, the constancy of activation energy, and the applicability of the Arrhenius equation. Equilibrium situations and kinetic salt effects may also present problems in this and other reported nonisothermal methods.

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High-Pressure Liquid Chromatographic Analysis of Penicillin G Potassium and Its Degradation Products

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Abstract □ An anion-exchange high-pressure liquid chromatographic system capable of separating penicillin G potassium from five of its degradation products was developed. The retention times were: penicillin G potassium, 17.5 min; DL-penicillamine, 4.5 min; benzylpenilloic acid, 7.0 and 8.0 min; benzylpenamaldic acid, 13.0 min; benzylpenicilloic acid, 19.5 min; and benzylpenillic acid, 22.0 min. In addition, the system permits quantification using linear calibration curves.

Keyphrases □ Penicillin G potassium and degradation products—high-pressure liquid chromatographic analysis □ High-pressure liquid chromatography—analysis, penicillin G potassium and degradation products

It has been suggested that penicillin degradation products may have a role in eliciting penicillin allergy (1). Since most analytical methods are directed at identifying and quantifying intact penicillin, a method of detection, separation, and quantification is needed to determine the presence of specific degradation products. Such a method would also be useful in pharmacokinetic studies.

Penicilloic acids have been separated from their parent penicillins using low-voltage electrophoresis (2), TLC (3), and solvent extraction (4). TLC has also been used to separate penicillin G potassium and four model decomposition products (5).

Recent reviews (6, 7) indicated that high-pressure liquid chromatography (HPLC) could be useful for the separation and quantification of compounds of pharmaceutical interest. Thus far, limited use has been made of HPLC in analyzing penicillins. The separation of the methylbenzyl ester of penicillin G from its impurities was reported (8), where a stationary polyamide phase was used with a hexane–ethanol mobile phase. An anion-exchange column with a pH 9.1 disodium borate buffer as the mobile phase was used to separate penicillin from additives in a penicillin dosage form[1]. The anion-exchange mode also was used to determine the amount of ampicillin contamination of nitrofurantoin preparations (9). The system separated ampicillin from the nitrofurantoin and other unidentified impurities.

An anion-exchange HPLC method for the separation and quantification of penicillin G potassium and five of its decomposition products was developed and is the subject of this report.

EXPERIMENTAL

Materials—All chemicals used were USP or reagent grade.

Penicillin G potassium[2], 1585 units/mg, and DL-penicillamine[3] were obtained commercially.

Benzylpenillic acid was synthesized following a procedure reported by Cook (10). Penicillin G potassium (0.05 *M*) was allowed to stand in a 0.03 *M* citric acid–0.0067 *M* disodium phosphate buffer, pH 2.70, at 25° for 24 hr. The white crystalline precipitate was collected and dried in a stream of air. Its identity was confirmed by potentiometric titration [pKa 3.25 and 7.7; literature (10) pKa 3.2–3.4 and 7.7], elemental analysis[4], IR spectroscopy, and mass spectrometry.

Benzylpenilloic acid was synthesized by a standard method (11, 12). Two grams of penicillin G potassium was placed in a round-bottom flask with 568 ml of water and 56.2 ml of 0.1 *N* hydrochloric acid (molar equivalent) and refluxed for 3 hr. The product was cooled, frozen, lyophilized[5], and recrystallized from methanol. An NMR spectrum of the product in deuterated chloroform gave signals that agree with the structure for benzylpenilloic acid.

Benzylpenamaldic acid was obtained using HPLC. Penicillin G potassium (0.05 *M*) was dissolved in the pH 2.7 citric acid–disodium phosphate buffer and aged at 37° for 2 hr. Ten-microliter samples were withdrawn and injected onto an anion-exchange[6] column in a high-performance liquid chromatograph[7]. The compounds were eluted with a mobile phase of 15.4 ml of 0.1 *M* citric acid and 7.0 ml of 0.2 *M* disodium phosphate diluted to 650 ml with double-distilled water. The fraction of eluent containing the compound with a retention time of 13 min was collected immediately and then lyophilized. The compound was identified as benzylpenamaldic acid based upon its UV spectrum, which possesses a unique ϵ_{max} in methanol at 282.5 nm, its instability to acid, and its partitioning properties (13).

Benzylpenicilloic acid was prepared using a standard synthesis (11). Exactly 70.6 ml of 0.05 *N* sodium hydroxide was added dropwise over 1 hr to a solution of 1.16 g of penicillin G potassium in 10 ml of double-distilled water. The mixture was allowed to stand at room temperature for 1.5 hr and then was brought to pH 5.6 with the addition of 1.25 ml of 0.1 *N* hydrochloric acid. The resulting solution was lyophilized and the product was shown to be benzylpenicilloic acid by a positive reaction to the arsenomolybdic acid–mercuric chloride test (4).

Instrumentation—The liquid chromatograph[7] was equipped with a UV detector operating at 254 nm and a double-beam refractive index detector. The UV detector was used for the quantitative aspects of all compounds. The double-beam refractive index detector, referenced against a cell filled with mobile phase, was used in the preparation of a calibration curve of benzylpenamaldic acid. The recorder was operated at a chart speed of 2.54 cm/5 min.

The stationary phase was an anion-exchange resin prepacked into a 0.61-m (2-ft) stainless steel column, 2.3 mm i.d.[6]. The mobile phase was a citric acid–disodium phosphate buffer prepared from stock solutions of 0.1 *M* citric acid and 0.2 *M* disodium phosphate. All injections were made on stream with a 25-μl high-pressure syringe[8].

The instrument was operated at ambient temperature at a flow

[1] Waters Associates, Inc., Framingham, Mass., personal communication.

[2] Chas. Pfizer and Co., New York, N.Y.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Midwest Microlab, Ltd., Indianapolis, Ind.
[5] Model USM-15, Virtis Co., Gardiner, N.Y.
[6] Bondapak AX/Corasil, Waters Associates, Inc., Framingham, Mass.
[7] Model ALC 202, Waters Associates, Inc., Framingham, Mass.
[8] Series B-110 Pressure-LOK liquid syringe, Precision Sampling Corp., Baton Rouge, La.

rate of 0.7 ml/min. With a single 0.61-m (2-ft) column, this flow rate was achieved with a pressure of 600–700 psi. When a pair of 0.61-m (2-ft) columns was coupled together, a pressure of 1000–1200 psi was required.

Methods—UV scans were run using a double-beam spectrophotometer[9]. IR[10] spectral samples were prepared as KBr pellets. Mass spectra[11] were obtained at 75 ev. NMR spectra[12] were determined using tetramethylsilane as the internal reference.

The least-squares regression line and standard error of estimate, $S_{y \cdot x}$, calculated using a standard program[13], were used to construct each calibration curve. The standard error of estimate has properties analogous to those of the standard deviation. For example, if lines parallel to the regression line of y on x at respective vertical distances $S_{y \cdot x}$, $2S_{y \cdot x}$, and $3S_{y \cdot x}$ were constructed, there would be included between these lines about 68, 95, and 99.7% of the sample points, respectively.

RESULTS AND DISCUSSION

Development of HPLC Separation System—An assay developed for the separation of penicillin from additives in a penicillin dosage form used an anion-exchange column, with 0.01 *M* disodium borate buffer (pH 9.1) as the mobile phase[1]. The system was the first one investigated for the separation of penicillin degradation products. Intact penicillin G potassium was separated from penicillin degradation products; however, the degradation products were not resolved.

Majors (14) suggested that the pH of the mobile phase for the separation of weakly basic or acidic solutes should be approximately 1.5 pH units above the pKa of the solute. The pKa of penicillin G is 2.76 (12), indicating that a mobile phase having a pH of approximately 4.25 would best achieve separation if the pKa's of the decomposition products were close to that of penicillin G. A 0.135 *M* citric acid–disodium phosphate buffer, which could be adjusted over a pH range of 2.2–7.8 (15), was varied from pH 3.45 to 4.60. Benzylpenicilloic acid, which had been stored at room temperature for 1 year, was used to test the efficiency of the mobile phase. The number of peaks obtained when this sample dissolved in water was injected into the column varied from four to six. The optimum separation was observed when the pH of the mobile phase was 3.58–3.80.

Once the optimum pH for the mobile phase had been established, the length of the column was increased by coupling a pair of 0.61-m (2-ft) columns. The longer column increased the number of peaks obtained from an aged benzylpenilloic acid sample from two to three.

Decreasing the buffer concentration of the mobile phase led to improved separation of the sample peaks. Optimum separation was achieved at pH 3.8 with a 0.0045 *M* mobile phase of a final composition of 15.4 ml of 0.1 *M* citric acid and 7 ml of 0.2 *M* disodium phosphate diluted to 650 ml with double-distilled water. This buffer system was used as the mobile phase with a 1.22-m (4-ft) anion-exchange column for the separations reported. The buffer salts, possibly by displacement of anionic species at the time of sample injection, produced a single peak with a retention time of 4.0 min.

Penicillin G Potassium—Accurately weighed samples of about 2 mg/ml of penicillin G potassium were dissolved in water and injected into the HPLC system. A single peak with a retention time of 17.5 min was eluted. A calibration curve was prepared each day by injecting freshly prepared samples of 1–11 μl and calculating the peak areas by multiplying peak height by the width at half-height. A least-squares linear regression program was used to construct a calibration curve. A typical calibration curve had a slope of 24.6 $mm^2/\mu g$ and an intercept of -2.5 mm^2. The correlation coefficient for the linear region from 2 to 22 μg was 0.999, and the standard error of estimate was 20.9 mm^2.

Benzylpenillic Acid—A similar technique was followed with benzylpenillic acid, except that it was dissolved in 0.05 *M* sodium hydroxide in concentrations ranging from 1 to 2 mg/ml to prepare samples for injection. It was eluted from the column as a single peak with a retention time of 22 min. Linear calibration curves over a range of 1–20 μg were obtained. Benzylpenillic acid is relatively stable in dilute alkali (16), and no chromatographic changes were observed in the freshly prepared samples used to prepare the calibration curves. Values such as 185.2 $mm^2/\mu g$ for the slope and -18.6 mm^2 for the intercept were obtained. The lowest correlation coefficient was 0.982 with a standard error of estimate of 114.4 mm^2.

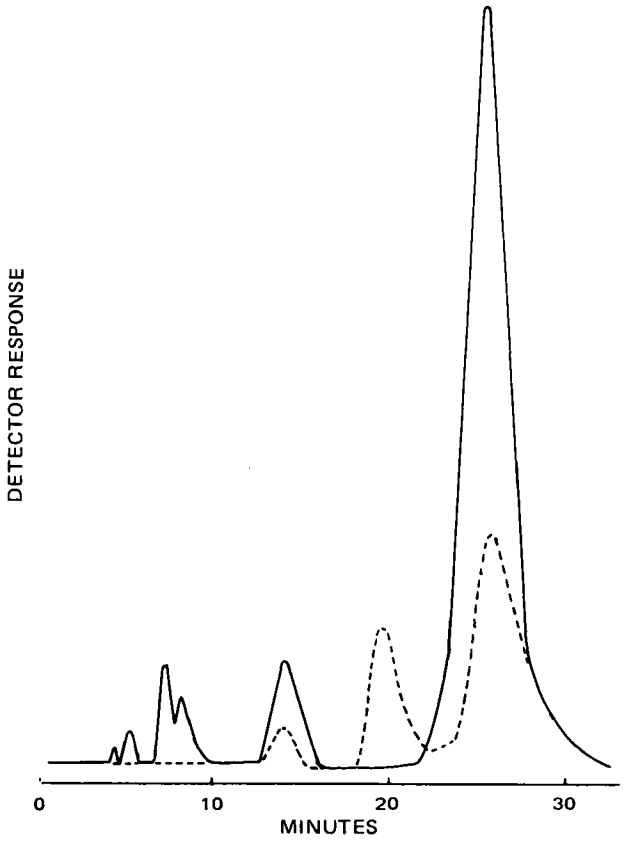

Figure 1—*Liquid chromatogram of a 0.05* M *penicillin G potassium solution aged in a 0.03* M *citric acid–0.0067* M *disodium phosphate buffer, pH 2.7, 37°; UV detector sensitivity, 254 nm; 0.04 auf except 0.08 auf for 31.5-hr benzylpenillic acid peak. Reported retention times are for single-component injections. Key: buffer salts, 4.0 min; penicillamine, 4.5 min; benzylpenilloic acid, 7.0, 8.0 min; benzylpenamaldic acid, 13.0 min; penicillin G potassium, 17.5 min; benzylpenillic acid, 22.0 min; - - - -, aged 7 min at 37°; and —, aged 31.5 hr at 37°.*

Penicillamine—Solutions of DL-penicillamine were prepared in concentrations of 0.49 and 0.90 mg/ml in 0.025 *M* NaOH, and 1–16 μl was injected. A single peak with a retention time of 4.5 min was observed. A linear calibration curve with a slope of 15.8 $mm^2/\mu g$ and an intercept of -12.49 mm^2 was typically obtained. The linear region between 0.9 and 7.8 μg had a correlation coefficient of 0.982. The standard error of estimate was 7.0 mm^2.

Benzylpenamaldic Acid—Benzylpenamaldic acid had a retention time of 13 min. It could not be isolated from the buffer component of the eluent by extraction into acetone, chloroform, octanol, or other organic solvents. Acidification of the eluent caused degradation of benzylpenamaldic acid, as evidenced by the appearance of new peaks when reinjected into the chromatograph.

To construct a calibration curve, the response of the refractive index detector to a blank solution of column eluent, which had been lyophilized and then reconstituted with water, was monitored. The buffer salts produced a single sharp peak 4 min after injection whose height was linear over a range of 50–365 μg of citric acid–disodium phosphate buffer salts. A known weight of the ly-

[9] Cary model 17 spectrophotometer, Cary Instruments, Monrovia, Calif.
[10] *Model IR-33* spectrophotometer, Beckman Instruments Inc., Fullerton, Calif.
[11] Model RMU-6A spectrometer, Hitachi Ltd., Tokyo, Japan. The mass spectra were interpreted with the help of Dr. R. G. Cooks, Director of the Mass Spectrometry Center, Purdue University.
[12] Model T-60 spectrometer, Varian Associates, Palo Alto, Calif.
[13] CAL 360-STAT-4, Wang Laboratories, Inc., Tewksbury, Mass.

ophilized benzylpenamaldic acid–buffer mixture was also redissolved in double-distilled water, and 3–20 μl was injected.

Since the refractive index response for benzylpenamaldic acid came at a retention time of 13 min, the difference between expected and actual responses for the buffer salts at retention time of 4 min was used to calculate the weight of benzylpenamaldic acid that had been injected to produce the corresponding peak from the UV detector. This information was used to construct a calibration curve of UV spectral peak areas *versus* weight of benzylpenamaldic acid. The detector response was linear over a range of 0.45–5.4 μg of benzylpenamaldic acid. A least-squares regression analysis typically gave a line of slope of 94.5 $mm^2/\mu g$, an intercept of 22 mm^2, a correlation coefficient of 0.9976, and a standard error of estimate of 27.4 mm^2.

Benzylpenilloic Acid—Aqueous solutions of benzylpenilloic acid (0.75–2.1 mg/ml) were injected in volumes ranging from 1 to 23 μl and consistently produced a pair of overlapping peaks of approximately equal size at retention times of 7 and 8 min. Florey *et al.* (12) reported that two isomers of benzylpenilloic acid are obtained from the decarboxylation of benzylpenicilloic acid, possibly accounting for the pair of peaks on the chromatograph. Because of the overlapping peaks, the total peak area of the double peak was determined using a planimeter. Linear calibration curves in the 5–27-μg range typically had a slope of 0.0021 planimeter unit/μg, an intercept of −0.0024 planimeter unit, and a correlation coefficient of 0.9950. The standard error of estimate was 0.0021 planimeter unit.

Benzylpenicilloic Acid—Benzylpenicilloic acid was eluted from the column with a retention time of 19.5 min.

CONCLUSIONS

The HPLC system is expected to have great utility in kinetic studies to monitor the occurrence of specific penicillin degradation products. For example, Fig. 1 is a chromatogram of a 0.05 *M* penicillin G potassium solution aged for 7 min and 31.5 hr at 37° in a 0.03 *M* citric acid–0.0067 *M* disodium phosphate buffer, pH 2.70. Peaks suitable for quantification are seen having retention times corresponding to penicillin G potassium, benzylpenillic acid, benzylpenamaldic acid, benzylpenilloic acid, and penicillamine. No other analytical method is available that allows the direct detection and quantification of penicillin degradation products forming during aging.

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Differentiating Nonaqueous Titration of Aspirin, Acetaminophen, and Salicylamide Mixtures

H. J. RHODES, J. J. DeNARDO, D. W. BODE, and M. I. BLAKE *

Abstract □ Mixtures containing aspirin, acetaminophen, and salicylamide were assayed potentiometrically by nonaqueous titration. The difference in pKa values for these weak acids was sufficient to permit successful differentiation. The titrant was tetrabutylammonium hydroxide, and the titration solvent was dimethylformamide. The procedure was applied to commercial dosage forms.

Keyphrases □ Aspirin mixtures with salicylamide or salicylamide/acetaminophen—differentiating nonaqueous titration □ Salicylamide mixtures with aspirin or aspirin/acetaminophen—differentiating nonaqueous titration □ Acetaminophen, aspirin, and salicylamide mixtures—differentiating nonaqueous titration □ Titrimetry, nonaqueous, differentiating—mixtures of aspirin, salicylamide, and acetaminophen

Aspirin, acetaminophen, and salicylamide are frequently used analgesic–antipyretics in tablet dosage forms, individually, in a variety of combinations, and often with other drugs. Suitable analytical procedures are available for estimating the individual components, but generally methods are not available for

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H. J. RHODES, J. J. DeNARDO, D. W. BODE, and M. I. BLAKE *

Abstract □ Mixtures containing aspirin, acetaminophen, and salicylamide were assayed potentiometrically by nonaqueous titration. The difference in pKa values for these weak acids was sufficient to permit successful differentiation. The titrant was tetrabutylammonium hydroxide, and the titration solvent was dimethylformamide. The procedure was applied to commercial dosage forms.

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Aspirin, acetaminophen, and salicylamide are frequently used analgesic–antipyretics in tablet dosage forms, individually, in a variety of combinations, and often with other drugs. Suitable analytical procedures are available for estimating the individual components, but generally methods are not available for

complex mixtures of these components. Methods for the determination of acetaminophen in dosage forms, whether present as the only active ingredient or combined with other drugs, were reviewed previously (1). In that paper, a potentiometric nonaqueous titration procedure was proposed for determining acetaminophen in dosage forms. Sodium methoxide was used as the titrant and dimethylformamide was used as the titration solvent.

Procedures for analyzing mixtures containing acetaminophen and salicylamide in combination with other drugs also were reviewed (2). A differentiating nonaqueous titration method was described for this combination, using tetrabutylammonium hydroxide as the titrant and dimethylformamide as the titration solvent. Differentiating nonaqueous titration methods were reported for dosage forms containing combinations of aspirin and acetaminophen (3), acetaminophen and barbiturates (4), and aspirin and barbiturates (5). Each of these mixtures is composed of weak acids having pKa values sufficiently divergent to permit successful differentiating titrations in the appropriate nonaqueous system. Preliminary extraction and separation of the active components were not necessary, and dosage form excipients apparently did not interfere in the titration.

The present study was an extension of previous work and involved the differentiating nonaqueous titration of mixtures of aspirin (pKa 3.50), acetaminophen (pKa 9.92), and salicylamide (pKa 8.31), using tetrabutylammonium hydroxide as the titrant and dimethylformamide as the titration solvent. The procedure was applied to commercial dosage forms.

EXPERIMENTAL

Apparatus—All titrations were performed potentiometrically with a titrimeter[1] equipped with a glass–calomel electrode system or a platinum–calomel electrode system.

Reagents, Chemicals, and Dosage Forms—Acetaminophen, aspirin, and salicylamide were obtained from commercial sources and were the best quality available. Dosage forms were obtained from commercial sources. All other chemicals and solvents employed were reagent grade and were used without further purification.

Tetrabutylammonium hydroxide, 0.1 *N* in benzene–methanol, was prepared according to the method of Cundiff and Markunas (6) and was standardized potentiometrically against reference standard benzoic acid dissolved in dimethylformamide. The solution was restandardized with each series of titrations.

Titration of Individual Components—Approximately 0.5 mEq of aspirin powder, accurately weighed, was dissolved in 50 ml of dimethylformamide in a 150-ml beaker. The solution, magnetically stirred, was titrated potentiometrically with 0.1 *N* tetrabutylammonium hydroxide. Salicylamide and acetaminophen were titrated similarly as individual components.

Differentiation of Two-Component Mixtures—The differentiation of mixtures containing acetaminophen and salicylamide or aspirin and acetaminophen was reported earlier (2, 3). In the present study, mixtures of aspirin and salicylamide were prepared in the following ratios: 1:1, 1:2, and 2:1. Aliquots of these mixtures, containing at least 0.5 mEq of each component, accurately weighed, were analyzed by dissolving the aliquot in 50 ml of dimethylformamide contained in a 150-ml beaker. The solution, magnetically stirred, was titrated potentiometrically with 0.1 *N* tetrabutylammonium hydroxide.

[1] Fisher model 35.

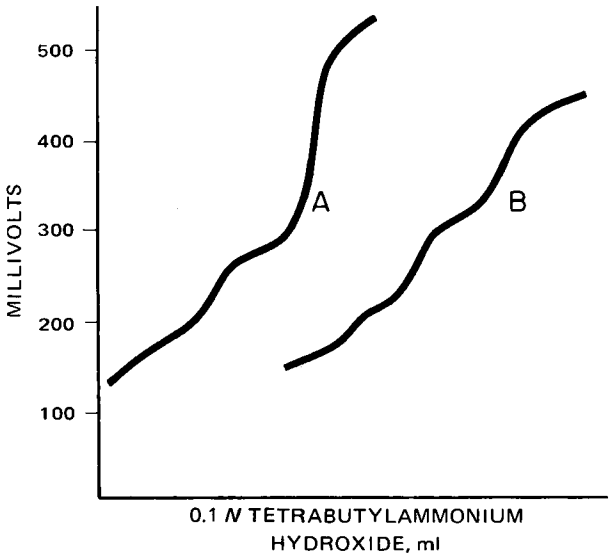

Figure 1—*Titration curves for: (A) binary mixture containing aspirin and salicylamide and (B) ternary mixture containing aspirin, salicylamide, and acetaminophen.*

The equivalence points for the differentiating titrations were determined by plotting $\Delta E/\Delta V$ *versus* V (milliliters). The volume reading where $\Delta E/\Delta V$ was a maximum was taken as an equivalence point. This procedure was not necessary for single-component systems, since the curve obtained by plotting the volume of titrant *versus* millivolt readings permitted the detection of the equivalence point by inspection. A typical titration curve (A) is shown in Fig. 1, and recovery data are presented in Table I.

Titration of Three-Component Mixtures—Mixtures of the three components were prepared so that aspirin–salicylamide–acetaminophen ratios were 1:1:1, 2:1:1, 3:1:1, and 3:2:1. Appropriate amounts of each component were accurately weighed and transferred to a 150-ml beaker. Fifty milliliters of dimethylformamide was added to the beaker, and the resulting solution was stirred magnetically. The solution was titrated potentiometrically with 0.1 *N* tetrabutylammonium hydroxide as the titrant. A typical titration curve (B) is shown in Fig. 1, and recovery data are presented in Table I.

Analysis of Dosage Forms Containing Three-Component Mixture—Twenty tablets from each of two commercial products

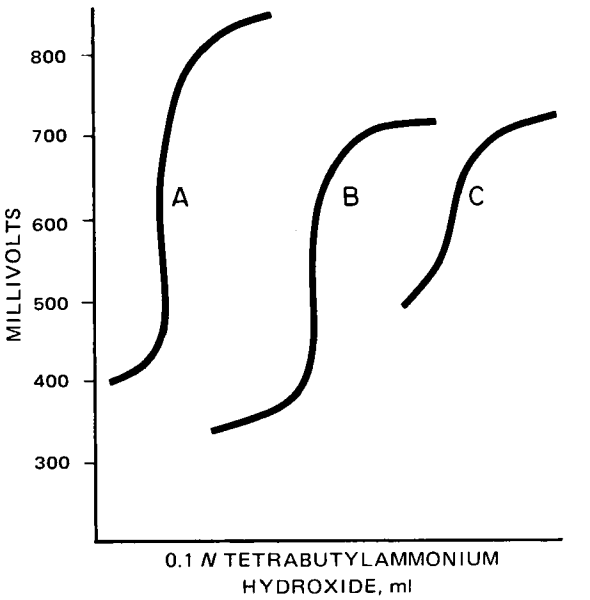

Figure 2—*Typical titration curves: (A) aspirin, (B) salicylamide, and (C) acetaminophen.*

Table I—Analytical Data

Sample	Ratio of Components	Recovery, %		
		Aspirin	Salicylamide	Acetaminophen
Aspirin		99.4 ± 0.6		
Salicylamide			100.9 ± 0.6	
Acetaminophen				101.2 ± 0.4
Aspirin–salicylamide	1:1	95.4 ± 0.4	100.4 ± 1.0	
	2:1	99.7 ± 1.0	95.7 ± 1.1	
	1:2	96.3 ± 5.0	100.3 ± 0.7	
Aspirin–salicylamide–acetaminophen	1:1:1	97.3 ± 1.5	102.3 ± 1.3	100.1 ± 1.1
	2:1:1	95.4 ± 1.9	97.8 ± 1.2	99.3 ± 0.4
	3:1:1	97.8 ± 0.3	97.8 ± 0.4	98.9 ± 2.7
	3:2:1	97.1 ± 0.2	100.6 ± 2.8	96.1 ± 1.1
Tablet dosage form				
A	3:2:1.5	98.5 ± 1.3	96.6 ± 1.7	97.9 ± 2.0
B	3:2:1.5	98.9 ± 0.8	101.5 ± 2.3	100.1 ± 2.0

were accurately weighed and then reduced to a fine powder in a mortar. An aliquot of the powder mass, accurately weighed, was transferred to a 150-ml beaker; then 50 ml of dimethylformamide was added, and the mixture was magnetically stirred for about 10 min. The resulting suspension was filtered through filter paper, which had been wetted with dimethylformamide. The beaker was rinsed with two 10-ml portions of dimethylformamide, which were then passed through the filter. The filtrate containing the active components of the dosage form was titrated potentiometrically as previously described.

RESULTS AND DISCUSSION

Mixtures of weak acids are readily analyzable by differentiating nonaqueous titration if the pKa values of the individual acids are sufficiently divergent and a suitable titrant, titration solvent, and electrode system are selected. This procedure is applicable to pharmaceutical dosage forms if excipients and other active ingredients do not interfere.

Typical two-component weak acid mixtures related to the systems reported in the present study were already mentioned. Other examples of two-component weak acid mixtures that have been successfully titrated differentially include benzoic acid and salicylic acid (6) as synthetic mixtures and in ointment dosage forms, cyclamates and saccharin (7) in liquid dosage forms, and *p*-aminosalicylic acid and its decomposition product *m*-aminophenol (8). The present study reports the analysis of mixtures containing aspirin and salicylamide and three-component mixtures containing acetaminophen in addition to aspirin and salicylamide.

Typical titration curves for the individual components are shown in Fig. 2. Curve A, Fig. 1, is a typical differentiating curve for mixtures containing equimolar amounts of aspirin and salicylamide. The first inflection is for aspirin, and the second end-point is for salicylamide. Curve B, Fig. 1, depicts a typical titration curve for the three-component system in which the components are present in about equimolar concentrations. The third inflection of the curve corresponds to acetaminophen.

Analysis data for the individual components, for the components of the binary and ternary synthetic mixtures, and for some typical tablet dosage forms are listed in Table I. The presence of other components and excipients apparently did not interfere in the titration. Although a preliminary extraction of the active components was performed, separation of the individual compounds was not necessary.

Two electrode systems were used in this study: the glass–calomel and the platinum–calomel pairs. The latter appeared to give sharper end-points, although both systems proved applicable.

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Liquid Chromatography in Pharmaceutical Analysis IV: Determination of Antispasmodic Mixtures

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Abstract □ Parameters associated with the separation of antianxiety–antispasmodic agents were investigated using high-pressure liquid chromatography. Eight widely prescribed drugs were studied. The compounds were chromatographed on reversed-phase octadecyltrichlorosilane (C_{18}) or diphenyldichlorosilane (phenyl) columns, using mixtures of absolute methanol and distilled water buffered with ammonium dihydrogen phosphate, ammonium acid phosphate, or ammonium carbonate. A mixture of phenobarbital–propantheline bromide was selected to demonstrate the utility of the separation and quantification method. The mixture was chromatographed on a phenyl column, using absolute methanol–aqueous 1% ammonium dihydrogen phosphate (60:40) (pH 5.85) at a flow rate of 1.4 ml/min. Each determination can be achieved in approximately 15 min with an accuracy of 1–2%.

Keyphrases □ Antispasmodic mixtures—high-pressure liquid chromatographic analysis, method parameters □ Antianxiety–antispasmodic agents—high-pressure liquid chromatographic analysis, method parameters □ High-pressure liquid chromatography—analysis, antispasmodic mixtures

This paper reports an investigation of the parameters associated with the separation and quantification of antianxiety–antispasmodic agents as a continuation of the study into the use of high-pressure liquid chromatography (HPLC) in the analysis of multicomponent dosage forms. Previous reports (1–3) dealt with the separation, detection, and quantification of cough–cold and diuretic–antihypertensive mixtures by HPLC.

The compounds studied are included in listings of most prescribed drugs used singly or in combination for treatment of functional or organic disorders of the GI tract. The drugs included were scopolamine hydrobromide, hyoscyamine sulfate, isopropamide iodide, prochlorperazine dimaleate, clidinium bromide, chlordiazepoxide hydrochloride, propantheline bromide, and phenobarbital.

Scopolamine and hyoscyamine in dosage forms have been analyzed by many methods including aqueous and nonaqueous titrimetry (4, 5), UV and visible spectrophotometry (6, 7), and TLC and GLC (8–10). Isopropamide iodide was determined by colorimetry (11) and nonaqueous titrimetry (12). GC (13), polarographic (14), and fluorometric (15) methods were reported for prochlorperazine. Clidinium bromide and chlordiazepoxide were determined by nonaqueous titrimetry (16, 17). Other methods for chlordiazepoxide include fluorescence (18), GC (19), and colorimetry (20). Spectrophotometry (21) and nonaqueous titrimetry (22) are used in the analysis of propantheline bromide, while spectrophotometry (23), TLC (24), and GLC (25, 26) are available for the detection and quantification of phenobarbital.

The investigated HPLC operating parameters were limited to solvent composition, pH, and stationary phase polarity. The use of these data can be readily applied to the separation and quantification of selected multidrug dosage forms. The quantification of a typical antispasmodic mixture is described.

EXPERIMENTAL[1]

Reagents and Chemicals—Powdered samples of scopolamine hydrobromide[2], hyoscyamine sulfate[2], isopropamide iodide[3], prochlorperazine dimaleate[3], clidinium bromide[4], and chlordiazepoxide hydrochloride[4] were used. In addition, propantheline bromide[5] and phenobarbital[6] were used in the analytical procedure and in the preparation of standard curves. All other chemicals and solvents were the highest commercially available materials.

Table I—Calibration Data for Standard Drug Solutions

Compound	Final Concentration, mg[a]	D/IS Ratio[b]	Slope	Intercept	$r + s$
Phenobarbital	7.5	0.2397 ± 0.002[c]	0.03390	—0.03075	0.9985 ±0.017
	15.0	0.4534 ± 0.001			
	30.0	0.9944 ± 0.007			
Propantheline bromide	7.5	0.7079 ± 0.040	0.08094	0.13931	0.9985 ±0.041
	15.0	1.4110 ± 0.032			
	30.0	2.5484 ± 0.008			

[a] Total mg/10 ml solution. [b] Data represent three to five replicate injections of standard solutions. D/IS is the ratio of the integrated area of the drug at some concentration divided by the integrated area of *m*-terphenyl at a concentration of 1 mg/10 ml. [c] Confidence limits at $p = 0.05$.

Mobile Phases—The mobile phases consisted of various concentrations of absolute methanol mixed with aqueous solutions containing: *(a)* 1% ammonium dihydrogen phosphate, *(b)* 1% ammonium dihydrogen phosphate and 1% ammonium acid phosphate, or *(c)* 0.5% ammonium carbonate. The solutions were prepared fresh daily.

[1] A Waters Associates liquid chromatograph (model ALC 202), equipped with an M-6000 pump, a UV monitor (254 nm), and an Infotronics integrator (model CRS-204) with digital printout, and Waters packed columns (1.22 m long × 2.3 mm i.d.) was used.

[2] A. H. Robins, Richmond, Va.

[3] Smith Kline and French, Philadelphia, Pa.

[4] Hoffmann-La Roche, Inc., Nutley, N.J.

[5] G. D. Searle and Co., Chicago, Ill.

[6] American Pharmaceutical Co., New York, N.Y.

Table II—Effect of Mobile Phase Composition on Retention Times[a]

Mobile Phase Composition[b]	Chlordiazepoxide Hydrochloride		Scopolamine Hydrobromide		Propantheline Bromide		Hyoscyamine Sulfate		Isopropamide Iodide		Clidinium Bromide		Phenobarbital		Prochlorperazine Dimaleate	
	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl
A	80[a] (24)[c] 100 (48)	80 (24)	83 (24)	84 (42)	111 (48) 194[e] (156)	120 (36)	86 (24)	86 (24)	93 (36)	90 (30)	106 (48)	94 (60)	79 (18)	72 (18)	—[d]	138[e] (66)
B	94 (30) 183 (78)	90 (24)	84 (30)	88 (18)	141 (84) 332[e] (318)	126 (42) 162[e] (72)	88 (30)	82 (24)	96 (48)	122 (42)	113 (60)	114[e] (30)	87 (18)	68[e] (18)	—[d]	256[e] (169)
C	156 (60) 540 (234)	113 (42)	92 (30)	83 (24) 114 (48)	264 (150) 998[e] (792)	153 (54) 208[e] (96)	90 (36)	88 (30) 112 (54)	150[e] (96)	150 (54)	156[e] (108)	89 (42) 136 (54)	92 (30)	68 (18)	—[d]	720[e] (594)
D	118 (42)	78 (24)	82 (30) 108 (48)	88 (30)	66 (30) 80 (36) 135 (72) 414 (396)	91 (30) 146 (60)	120 (60)	122 (36)	114 (90)	112 (30)	90 (30) 156 (108)	139 (54)	72 (24)	74 (24)	—[d]	302[e] (204)
E	104 (24) 216 (78)	79 (30)	100 (36) 196 (84)	87 (30)	84 (24) 123 (66) 207 (186) 318 (156) 954[e] (906)	187 (78)	138 (78)	120 (42)	146[e] (96)	118 (42)	210[e] (186)	134 (48)	84 (30)	74 (24)	—[d]	—[d]
F	144 (56) 524[e] (222)	92 (30)	124 (56)	90 (24)	66 (32) 104 (36) 224 (104) 436[e] (320)	244[e] (120)	172[e] (114)	112 (36)	222[e] (234)	110 (42)	372[e] (366)	135 (66)	86 (24)	72 (24)	—[d]	—[d]
G	82 (30) 99 (42)	78 (24)	86 (24)	78 (18)	74 (24) 156 (54) 276 (216) 916[e] (852)	268[e] (90)	180[e] (108)	180 (72)	130[e] (72)	146 (42)	103 (30) 241[e] (204)	188 (66)	74 (18)	72 (18)	—[d]	336[e] (276)
H	100 (30) 206 (78)	84 (30)	70 (24) 100 (30)	79 (24)	76 (30) 235 (102)	120 (24) 284 (178) 333[e] (198)	72 (30) 396[e] (270)	69 (24) 215[e] (90)	194[e] (162)	150[e] (66)	78 (30) 134 (48) 408[e] (432)	72 (18) 218[e] (54)	73 (18)	66 (18)	—[d]	—[d]

Table II—*Continued*

Mobile Phase Composition[b]	Chlordiazepoxide Hydrochloride		Scopolamine Hydrobromide		Propantheline Bromide		Hyoscyamine Sulfate		Isopropamide Iodide		Clidinium Bromide		Phenobarbital		Prochlorperazine Dimaleate	
	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl	C_{18}	Phenyl
I	70	98	74	84	68	63	52	64	94	162[e]	62	222[e]	82	64	—[d]	—[d]
	(24)	(36)	(24)	(24)	(24)	(12)	(12)	(18)	(36)	(66)	(30)	(90)	(24)	(18)		
	154		144		100	309	86	90	414[e]		78					
	(66)		(54)		(48)	(108)	(90)	(24)	(558)		(30)					
	513				254	372[e]	552[e]	222[e]			250					
	(26)				(144)	(214)	(384)	(90)			(72)					
					1034						898[e]					
					(372)						(918)					

[a] Retention time expressed as seconds measured as elapsed time between injection and attainment of the chromatographic peak maximum. The eluted peaks were monitored using UV detection. [b] Solvent compositions and pH are given in Table III. [c] Base peak width expressed as seconds. [d] Retention time greater than 1800 sec. [e] Significant tailing was observed.

Table III—Solvent Compositions and pH

Mobile Phase	Components	Solvent Ratio	pH
A	Absolute methanol	60	5.85
	1% Ammonium dihydrogen phosphate	40	
B	Absolute methanol	50	5.50
	1% Ammonium dihydrogen phosphate	50	
C	Absolute methanol	40	5.50
	1% Ammonium dihydrogen phosphate	60	
D	Absolute methanol	60	8.20
	1% Ammonium dihydrogen phosphate	20	
	1% Ammonium acid phosphate	20	
E	Absolute methanol	50	7.90
	1% Ammonium dihydrogen phosphate	25	
	1% Ammonium acid phosphate	25	
F	Absolute methanol	40	7.60
	1% Ammonium dihydrogen phosphate	30	
	1% Ammonium acid phosphate	30	
G	Absolute methanol	60	8.65
	0.5% Ammonium carbonate	40	
H	Absolute methanol	50	8.70
	0.5% Ammonium carbonate	50	
I	Absolute methanol	40	8.80
	0.5% Ammonium carbonate	60	

Preparation of Drug Solutions—Separate solutions (150 mg/10 ml) of scopolamine hydrobromide, hyoscyamine sulfate, isopropamide iodide, and clidinium bromide were prepared by dissolving each powder in absolute methanol. Solutions of chlordiazepoxide hydrochloride (3 mg/10 ml), prochlorperazine dimaleate (30 mg/10 ml), and phenobarbital (30 mg/10 ml) were also prepared using methanol.

Internal Standard Solution—The stock internal standard solution (10 mg/10 ml) was prepared by dissolving *m*-terphenyl[7] in absolute methanol. *m*-Terphenyl, mp 85–87°, was purified before use by the method of France *et al.* (27).

Standard Solutions for Calibration Curves—Separate stock solutions (750 mg/100 ml) of phenobarbital and propantheline bromide were prepared in absolute methanol. Accurately pipetted volumes of 1.0, 2.0, and 4.0 ml of each stock solution were placed in 10-ml volumetric flasks. One milliliter of the internal standard stock solution was added to each flask, followed by the addition of absolute methanol to volume.

The three concentrations of each drug were subjected to a linear regression analysis, and the slope and intercept were calculated (Table I).

Chromatographic Separation and Quantification—The degassed mobile phase was pumped through columns containing monomolecular layers of octadecyltrichlorosilane (octadecyl) or diphenyldichlorosilane (phenyl) chemically bonded to a high efficiency pellicular packing, consisting of solid glass cores with a porous silica surface[8], at a flow rate of 1.4 ml/min (2000 psig) at room temperature until a stable baseline was obtained. Replicate 15-μl injections of sample and standard solutions were made using a 25-μl syringe[9]. The chart recorder provided a record of drug elution from the column as peaks on a chromatogram. In all cases, the solute was measured by digital integration of the peak area[1].

RESULTS AND DISCUSSION

The purpose of this study was to determine the operating conditions for HPLC that would optimize the resolution of chemically unrelated therapeutic agents most commonly found in antispasmodic mixtures. Three operating parameters were studied: *(a)* variations in solvent composition containing absolute methanol with changing concentrations of distilled water; *(b)* variations in pH within the 5.5–8.8 range using ammonium acid phosphate, ammonium dihydrogen phosphate, or ammonium carbonate as inorganic buffers; and *(c)* variations in the nonpolar (reversed-phase) stationary phase in which the pellicular packing differed in polarity. The effects of these parameters on retention times and base

[7] Eastman Organic Chemicals, Rochester, N.Y.
[8] Corasil/C_{18} and Corasil/phenyl, 37–50 μm, Waters Associates, Milford, Mass.
[9] Model B-110, Precision Sampling Corp., Baton Rouge, La.

Table IV—Approximate Resolution (*Rs*) Values for Phenobarbital–Propantheline Bromide Mixture on Phenyl Column

Mobile Phase[a]	pH	*Rs* Value[b]
A	5.85	1.78
E	7.90	2.20
F	7.60	2.40
I	8.80	3.60

[a] Letters refer to solvent compositions in Table III. [b] See Footnote 10.

peak widths of the drugs are shown in Table II. In addition, Table II indicates those solvent systems that generate multiplets or tailing of single drug entities.

For the investigated drugs, the following generalizations can be inferred from the data in Table II. Retention time for most drugs on the octadecyl column increases at all pH ranges as the polarity of the mobile phase increases. This effect is also observed with the phenyl column in the acidic pH range. In basic pH ranges on the phenyl column, there is little change in retention time with a change in mobile phase polarity. In most cases, retention time for a drug is longer on the octadecyl column than on the phenyl column.

Changes in solvent polarity have a greater effect on the retention times of the drugs on either the octadecyl or phenyl column than do changes in pH of the mobile phase. However, clidinium bromide and prochlorperazine dimaleate show appreciable changes in retention time with changes in pH of the mobile phase. Drugs containing an ester group give multiple peaks in basic pH ranges, probably because of hydrolysis on the column.

Modifications in the polarity of stationary and mobile phases provided the following observations for the drugs examined. Chlordiazepoxide hydrochloride produces multiple peaks on the octadecyl column in almost all solvent systems while giving only single peaks on the phenyl column. Resolution of the frequently prescribed chlordiazepoxide–clidinium bromide combination can be observed in Solvent Systems B, C, D, E, F, and I (Tables II and III).

The concentration levels of scopolamine and hyoscyamine detected in this study were 100 times higher than the level commonly found in commercial dosage forms. In several cases, the more basic solvents (*i.e.*, G, H, and I) led to tailing and/or multiple peaks for hyoscyamine on the phenyl column. The tailing and/or multiple peaks are probably due to decomposition in alkaline media (28). Neither scopolamine nor hyoscyamine could be detected at therapeutic dose levels with the present instrumentation.

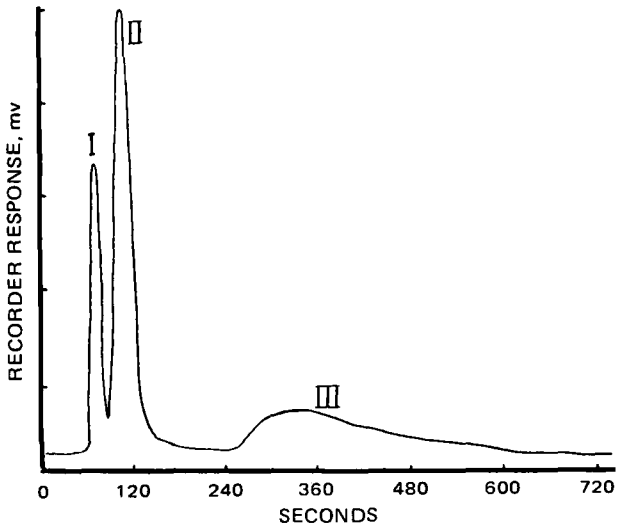

Figure 1—*Chromatogram of a phenobarbital–propantheline bromide mixture on diphenyldichlorosilane (phenyl) column with absolute methanol–1% ammonium dihydrogen phosphate (60:40). Key: I, phenobarbital; II, propantheline bromide; and III, m-terphenyl.*

Table V—Analysis of Phenobarbital–Propantheline Bromide in Known Mixture

Mixture	Milligrams Added	Milligrams Found[a]	Accuracy, %
Phenobarbital	15.0	15.262 ± 0.27[b]	1.75
Propantheline bromide	15.0	15.209 ± 0.20	1.39

[a] Based on five replicate determinations of known mixture. [b] Confidence limits at $p = 0.05$.

A combination of long retention time and basic pH of the mobile phase resulted in multiple peaks for propantheline bromide on the octadecyl column.

A peak associated with iodide ion in isopropamide iodide appeared at the solvent front in all solvent systems and on both columns (not recorded in Table II).

Long retention times in basic solvents (*i.e.*, G, H, and I) led to multiple peaks for clidinium bromide, possibly due to hydrolysis.

Phenobarbital traveled close to the solvent front in all systems, and its retention time could not be distinguished from that of the solvent front.

Prochlorperazine dimaleate exhibited extremely long retention times and considerable tailing in all solvents at all pH's. This compound also produced a peak for maleate near the solvent front (not recorded in Table II).

Satisfactory quantification of a multicomponent dosage form is dependent on adequate resolution of the components. The value *Rs* (29), a theoretical parameter, is a reasonable measurement of the separation of two species on a column. It can be approximated for any two drugs included in this investigation from information contained in Table II[10]. A mixture of phenobarbital–propantheline bromide[11] was selected to exemplify this situation. Table IV shows calculated *Rs* values for the drug combination in various solvent systems and pH ranges on the phenyl column. Mobile phase A was chosen for the quantification since adequate resolution could be achieved in the shortest time.

Figure 1 illustrates a chromatogram of the assayed drugs. Various concentrations of standard solutions of each drug dissolved in the absolute methanol–aqueous 1% ammonium dihydrogen phosphate (60:40) mixture were chromatographed using the phenyl column. *m*-Terphenyl was added to each solution as the internal standard. The area under the curve for each peak on the chromatograms was determined with a digital integrator[1]. The ratio of each peak area to the area of the internal standard was calculated for each chromatogram. A linear regression line of these data at three concentrations of each drug gave the slope, intercept, and correlation coefficient for each calibration curve (Table V).

A known mixture containing a quantity of each drug was chromatographed, and the ratios of drug peak areas/internal standard peak areas (D/IS) were calculated for each drug. The constants (slope and intercept) for the linear regression equation shown in Table I were used to solve for drug concentration [D/IS = slope (concentration) + intercept]. The calculations were performed on a programmable calculator[12].

The data in Table V demonstrate the quantitative results obtained for the simulated dosage form. The utility of HPLC in the analysis of a phenobarbital–propantheline bromide mixture is clearly demonstrated with an accuracy of 1–2%.

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[10] It is possible to calculate the approximate resolution (*Rs*) of two components by the equation: $Rs = 2(t_2 - t_1)/(w_1 + w_2)$, where t_1 and t_2 are retention times and w_1 and w_2 are base peak widths of Compounds 1 and 2, respectively. In past experience, two components with an *Rs* value >1.5 showed satisfactory resolution for quantification by this analytical technique if the peak areas were approximately equal. A significant difference in peak areas for two components may require *Rs* values of 2 or greater.

[11] These two drugs are components of a commercially available dosage form, Pro-Banthine with Phenobarbital (G. D. Searle Co.).

[12] Olivetti-Underwood programma 101.

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Analysis of Individual Thyroid Tablets

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Abstract □ A procedure was developed for the assay of individual thyroid tablets or composite samples equivalent to as little as 0.033 g (0.5 gr) of thyroid. The sample is ignited in a closed atmosphere of oxygen and, after a series of redox reactions, the iodine is determined spectrophotometrically as the triiodide ion. The results agree well with those obtained by the USP thyroid tablet assay.

Keyphrases □ Thyroid—analysis of individual tablets, spectrophotometric method measuring triiodide ion □ Iodine—analysis of individual thyroid tablets □ Spectrophotometry—analysis, individual thyroid tablets, measurement of triiodide ion

The USP thyroid tablet monograph assay (1) is not suitable for individual tablet analysis because the level of iodine in an individual tablet is usually too low to produce an acceptable titration volume. In an effort to provide an individual tablet analysis, the following method was developed. It also provides a satisfactory alternative to the compendial assay method (1).

The proposed method utilizes essentially the same chemical transformations that form the basis of the compendial monograph assay (Scheme I).

$$\text{thyroid} + Na_2CO_3 \longrightarrow I^- + \text{fusion products}$$

$$I^- + 3Br_2 + 3H_2O \xrightarrow{\text{boil}} IO_3^- + 6Br^- + 6H^+$$

$$IO_3^- + 5I^- + 6H^+ \longrightarrow 3I_2 + 3H_2O$$

Scheme I

In contrast to the compendial assay, which requires alkaline fusion of the equivalent of 1 g of thyroid and a final thiosulfate titration, the proposed procedure requires oxygen flask ignition of the equivalent of 30 mg of thyroid and a final spectrophotometric determination. The procedure is applicable to coated or uncoated tablets ranging from 0.033 to 0.4 g (0.5 to 6 gr) of thyroid. Potassium iodate is used as the standard, and the measurement is made on aliquots of the iodate solutions equivalent to 5–35 μg of iodine.

EXPERIMENTAL

Reagents—The standard potassium iodate solution is prepared by quantitatively diluting 0.05 *M* potassium iodate (2) stepwise to 1 in 2500 to obtain a solution equivalent to 2.538 μg I_2/ml.

The bromine–sodium acetate test solution is prepared as directed in Ref. 3.

Acetate buffer, pH 3.9, consists of 1.0 *M* sodium acetate in 5.5 *M* acetic acid.

Buffered potassium iodide is prepared just prior to use by mixing 1.5% (w/v) potassium iodide in pH 3.9 acetate buffer. The solution should remain colorless for several hours.

Assay Preparation—Oxygen ignition[1] was performed using 1-liter conical ignition flasks fitted with all-glass stopper sample holders.

Individual Tablets—Securely fasten one thyroid tablet in a suitable platinum basket in contact with a small strip of black Schöniger ignition paper. Suspend the basket from the sample

[1] Thomas-Ogg oxygen flask ignitor.

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Analysis of Individual Thyroid Tablets

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Abstract □ A procedure was developed for the assay of individual thyroid tablets or composite samples equivalent to as little as 0.033 g (0.5 gr) of thyroid. The sample is ignited in a closed atmosphere of oxygen and, after a series of redox reactions, the iodine is determined spectrophotometrically as the triiodide ion. The results agree well with those obtained by the USP thyroid tablet assay.

Keyphrases □ Thyroid—analysis of individual tablets, spectrophotometric method measuring triiodide ion □ Iodine—analysis of individual thyroid tablets □ Spectrophotometry—analysis, individual thyroid tablets, measurement of triiodide ion

The USP thyroid tablet monograph assay (1) is not suitable for individual tablet analysis because the level of iodine in an individual tablet is usually too low to produce an acceptable titration volume. In an effort to provide an individual tablet analysis, the following method was developed. It also provides a satisfactory alternative to the compendial assay method (1).

The proposed method utilizes essentially the same chemical transformations that form the basis of the compendial monograph assay (Scheme I).

$$\text{thyroid} + Na_2CO_3 \longrightarrow I^- + \text{fusion products}$$

$$I^- + 3Br_2 + 3H_2O \xrightarrow{\text{boil}} IO_3^- + 6Br^- + 6H^+$$

$$IO_3^- + 5I^- + 6H^+ \longrightarrow 3I_2 + 3H_2O$$

Scheme I

In contrast to the compendial assay, which requires alkaline fusion of the equivalent of 1 g of thyroid and a final thiosulfate titration, the proposed procedure requires oxygen flask ignition of the equivalent of 30 mg of thyroid and a final spectrophotometric determination. The procedure is applicable to coated or uncoated tablets ranging from 0.033 to 0.4 g (0.5 to 6 gr) of thyroid. Potassium iodate is used as the standard, and the measurement is made on aliquots of the iodate solutions equivalent to 5–35 μg of iodine.

EXPERIMENTAL

Reagents—The standard potassium iodate solution is prepared by quantitatively diluting 0.05 *M* potassium iodate (2) stepwise to 1 in 2500 to obtain a solution equivalent to 2.538 μg I_2/ml.

The bromine–sodium acetate test solution is prepared as directed in Ref. 3.

Acetate buffer, pH 3.9, consists of 1.0 *M* sodium acetate in 5.5 *M* acetic acid.

Buffered potassium iodide is prepared just prior to use by mixing 1.5% (w/v) potassium iodide in pH 3.9 acetate buffer. The solution should remain colorless for several hours.

Assay Preparation—Oxygen ignition[1] was performed using 1-liter conical ignition flasks fitted with all-glass stopper sample holders.

Individual Tablets—Securely fasten one thyroid tablet in a suitable platinum basket in contact with a small strip of black Schöniger ignition paper. Suspend the basket from the sample

[1] Thomas-Ogg oxygen flask ignitor.

holder into the ignition flask containing 15 ml of water. (For a large coated tablet, use 15 ml of 60% acetic acid.) Flush the flask thoroughly with oxygen, remove the oxygen inlet, and quickly inject 1–2 ml of bromine–sodium acetate test solution into the flask with a medicine dropper. Clamp the stopper securely and ignite the sample.

After the flask has cooled sufficiently for handling, swirl the contents for 4–5 min, allowing the solution to wet *all* internal surfaces repeatedly. Then chill the flask in cold water. In an efficient hood, carefully release the stopper and rinse it and the walls of the flask with water. Add a few silicon carbide boiling chips, and gently boil the solution until its vapors are negative to a moistened potassium iodide–starch test paper and the volume of the solution is conveniently reduced to facilitate its transfer. Do not reduce the volume below 10 ml.

Quantitatively transfer the cooled solution with the aid of water through a funnel, containing a small, tightly packed pledget of fine glass wool, into a volumetric flask of suitable size to obtain a solution concentration in the range equivalent to 0.5–3.5 μg of I_2/ml when diluted to volume.

Sample Composites—Weigh into a No. 0 or 00 gelatin capsule an amount of powdered sample composite equivalent to 65–130 mg of thyroid. Proceed as already described, transferring the solution to a 100-ml volumetric flask, and dilute to volume with water.

Procedure—Pipet 10-ml aliquots of sample, standard, and water to serve as a blank into separate 25-ml volumetric flasks. Add 10 ml buffered potassium iodide solution to each flask, and dilute to volume with water. Mix well and let stand for at least 5 min.

With a suitable spectrophotometer, record the spectrum relative to the blank in 1-cm cells from 460 to 340 nm. Correct the maximum absorbance at about 350 nm by subtraction of the absorbance at 450 nm.

Calculate the percent of iodine per grain of thyroid per tablet by the formula:

$$\%\,I_2 = \frac{(A_u350 - A_u450) \times C \times V_u \times 0.1}{(A_s350 - A_s450) \times D \times 64.8} \qquad \text{(Eq. 1)}$$

where C = concentration of standard (micrograms of I_2 per milliliter), V_u = volume of sample preparation (milliliters), D = declared content of thyroid (grains), and 64.8 = milligrams per grain. When assaying a sample composite, the formula is multiplied by average weight/weight, where average weight = average weight per tablet (milligrams), and weight = sample weight (milligrams).

RESULTS

Various thyroid tablets, coated and uncoated, ranging from 0.033 to 0.4 g (0.5 to 6 gr) in declared thyroid content, were assayed by the developed procedure. However, to emphasize the more significant stages in the evolution of the method, the analytical results obtained at these developmental stages are included in Table I with results using the proposed procedure. The validity of the results was established by comparison with results obtained by the USP thyroid method (1).

DISCUSSION

The primary concerns in the development of this assay of individual thyroid tablets were: *(a)* the suitability and ease of sample preparation and *(b)* the sensitivity and accuracy of the determinative step. This latter concern was adequately dealt with by Graham *et al.* (4) in the development of a procedure for the determination of levothyroxine sodium or liothyronine sodium in tablets. The first concern required a considerable amount of experimentation until a set of conditions was established that gave acceptable results with the various thyroid tablets studied.

In contrast to the free iodoamino acids (4), thyroid-bound iodine cannot be conveniently oxidized directly to iodate with bromine–sodium acetate test solution. No iodate appeared to have been produced by this process in several attempts, thus eliminating this most direct approach to sample preparation.

The remaining immediate options were: *(a)* scale down the USP ignition technique (1) and *(b)* adapt the oxygen flask procedure of Moody *et al.* (5). The first was rejected in favor of the second when the number of operations required to obtain the test solution was considered to be too large.

The results obtained under the conditions used by Moody *et al.* (5) for ignition—*viz.*, 1 liter of oxygen and 30 ml of 0.5 *M* sodium hydroxide as the absorbing solution, are shown in Table I. When the averaged individual tablet analyses were compared with the compendial assay, only Samples B, C, and H gave good agreement. Other anomalies were notable when comparisons were made among the averaged individual tablet assays, composite assays, and compendial assays:

1. Composite and individual tablet assays did not agree (Samples C, D, and F).
2. Composite and compendial assays agreed but individual tablet assays did not (Sample F).
3. Composite assay did not agree with the compendial assay but the individual tablet assay did (Sample C).
4. Composite and individual tablet assays agreed but did not agree with the compendial assay (Samples A and G).

At this point, the observation that some of the large coated tablets burned with a white incandescence while others did not suggested that there may be an insufficiency of oxygen for the combustion of some samples. It was also evident that the degree of subdivision of the sample affected the combustion.

When composite samples were ignited using 2 liters of oxygen with the same absorbing solution, significant changes were noted in Samples C, D, and G (Table I). This modification appeared to be adequate until attention was focused on Sample A. The change in assay was hardly significant, yet there must have been sufficient oxygen for complete combustion of the sample, since ignition of twofold amounts of the sample did not result in decreased assay values.

It was recalled that difficulty was encountered during an attempted determination of the iodine content of iopanoic acid by a similar ignition procedure[2], and it appeared that the same or an analogous situation was occurring with this thyroid sample. When applied to iopanoic acid, the oxygen flask combustion technique produces elemental iodine. The sodium hydroxide solution appeared to have absorbed the iodine during the scrubbing stage; but on the subsequent acidification preliminary to bromine oxidation, iodine vapor was released from solution. This loss was completely circumvented by substituting an aqueous solution of the acidic bromine–sodium acetate reagent in place of the sodium hydroxide solution. Although no iodine was visible during the ignition of the thyroid sample, this scrubbing technique was applied to a composite of Sample A (2 liters of oxygen) with complete success, giving an assay of 0.195%, which agreed well with the compendial assay. The procedure was repeated using a 1-liter flask, which gave an assay of 0.196%.

Since the ignition in 2-liter flasks was cumbersome and reduced the number of samples that could be conveniently handled, all samples were reassayed in 1-liter flasks, using the bromine oxidizing reagent as the scrubbing solution (Table I).

One further modification was made in view of the behavior of coated Samples E–H. When these samples were ignited in 2-liter flasks, the white ash remaining appeared inert when it came in contact with the acidic scrubbing solution. The ash remaining when these same samples were ignited in a 1-liter flask effervesced vigorously when it came in contact with the absorbing solution and the color of the solution was greatly diminished. Since it is desirable to maintain a low pH and an excess of free bromine for the oxidation to iodate, the recommended vehicle for the scrubbing reagent is 60% acetic acid.

The discrepancy remaining in the individual tablet and composite assays of Sample G is attributed to the sampling of this product.

SUMMARY

The developed procedure provides the same information as the USP thyroid and thyroid tablet monograph assays. Neither the compendial procedure nor this proposed procedure provides the highly desirable detailed information concerning the thyroxine and liothyronine content of thyroid samples.

This procedure is recommended for adoption by the USP because of its convenience and comparable accuracy to the compen-

[2] J. H. Graham, unpublished data.

Table I—Comparison of Results of Analysis of Thyroid Tablets by Individual Tablet Assay and Composite Assay (5), Proposed Method, and USP Method (1)

Sample	Individual[a] Tablet Assay, %	Composite[a], %	Composite[b], %	Composite[c], %	Individual[c] Tablet Assay, %	USP Method, %
A						
0.5 gr	0.183, 0.177, 0.182	0.183, 0.180, 0.182	0.183, 0.179, 0.187	0.194, 0.197	0.195, 0.194, 0.190	0.193, 0.193, 0.193,
63.7 mg/tablet	0.181, 0.174, 0.183	0.182, 0.182	0.190		0.195, 0.194, 0.234	0.193, 0.192, 0.193,
					0.189, 0.190, 0.191	0.192, 0.194, 0.196
Average	0.180	0.182	0.185	0.196	0.197	0.193
B						
1 gr	0.224, 0.218, 0.229	0.226, 0.213, 0.224	0.222, 0.220	0.237, 0.232	0.246, 0.246, 0.228	0.223, 0.228
195.8 mg/tablet	0.259, 0.226, 0.228				0.252, 0.266, 0.231	
					0.240, 0.249, 0.233	
					0.236	
Average	0.231	0.221	0.221	0.235	0.243	0.226
C						
2 gr	0.208, 0.202, 0.211	0.173, 0.174, 0.154	0.213	0.212, 0.213	0.217, 0.215, 0.211	0.197, 0.212
358.3 mg/tablet	0.210, 0.210, 0.210	0.155, 0.144, 0.179			0.211, 0.200, 0.201	0.205, 0.206
					0.208, 0.198, 0.195	
					0.207	
Average	0.209	0.163	0.213	0.213	0.206	0.205
D						
2 gr	0.194, 0.193, 0.199	0.164, 0.172, 0.145	0.196	0.200, 0.201	0.202, 0.201, 0.202	0.201, 0.200
	0.196, 0.192, 0.185	0.150, 0.151			0.202, 0.200, 0.197	
					0.193	
Average	0.193	0.156	0.196	0.201	0.200	0.201
E						
5 gr, coated				0.203, 0.207	0.207, 0.166, 0.208	0.203, 0.200
748.2 mg/tablet					0.172, 0.200, 0.196	
					0.202, 0.204, 0.238	
					0.188	
Average				0.205	0.198	0.202
F						
6 gr, coated	0.160, 0.137, 0.119	0.200, 0.194		0.209, 0.203, 0.204	0.168, 0.208, 0.158	0.202, 0.200
781.9 mg/tablet	0.178, 0.194, 0.180			0.201, 0.197	0.181, 0.171, 0.208	
	0.227, 0.159, 0.174				0.207, 0.220, 0.211	
	0.189					
Average	0.172	0.197		0.203	0.192	0.201
G						
6 gr, coated	0.122, 0.125, 0.111	0.120, 0.120, 0.124	0.195, 0.194	0.202, 0.198, 0.195	0.190, 0.183, 0.173	0.200, 0.201
853.0 mg/tablet	0.119, 0.105, 0.127	0.129, 0.121, 0.126		0.194, 0.195	0.213, 0.179, 0.190	
	0.121, 0.175, 0.164				0.185, 0.203, 0.187	
	0.120				0.169, 0.175, 0.200	
					0.181, 0.181	
Average	0.129	0.123	0.195	0.197	0.186	0.201
H						
6 gr, coated	0.187, 0.173, 0.162	0.186, 0.172		0.179, 0.182	0.161, 0.193, 0.152	0.173, 0.177
696.2 mg/tablet	0.182, 0.211, 0.161	0.177			0.160, 0.170, 0.165	
	0.200, 0.167, 0.167				0.188, 0.210, 0.209	
	0.143, 0.188				0.186	
Average	0.177	0.178		0.181	0.179	0.175

[a] One liter of oxygen, sodium hydroxide scrubbing solution. [b] Two liters of oxygen, sodium hydroxide scrubbing solution. [c] Proposed method. One liter of oxygen, bromine scrubbing solution.

dial procedure [the two methods agreed within 2.0% (average of eight sample composites)] in handling small samples, as is needed for individual tablet analysis or when the quantity of sample is too small for assay by the compendial procedure.

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NMR Spectroscopic Analysis of 2-Mercapto-5-methyl-1,3,4-thiadiazole in Cefazolin

D. B. STAIGER, R. J. WARREN ˣ, J. E. ZAREMBO, and A. POST

Abstract □ A rapid, sensitive, and accurate method for the quantitative analysis of 2-mercapto-5-methyl-1,3,4-thiadiazole in cefazolin is presented. The method utilizes NMR spectroscopy and is based on the difference in the chemical shift of the methyl protons on free thiadiazole and the thiadiazole moiety of cefazolin.

Keyphrases □ Cefazolin and cefazolin sodium—NMR analysis of free 2-mercapto-5-methyl-1,3,4-thiadiazole □ 2-Mercapto-5-methyl-1,3,4-thiadiazole—NMR analysis in cefazolin and cefazolin sodium □ NMR spectroscopy—analysis, 2-mercapto-5-methyl-1,3,4-thiadiazole in cefazolin and cefazolin sodium

Free thiadiazole may be found in cefazolin (sodium salt and free acid) as a decomposition product or as unreacted starting material. The contaminant may be determined by high-pressure liquid chromatography[1], but the procedure is tedious and time consuming. The NMR method presented in this paper is specific for free thiadiazole and provides a rapid, sensitive, and accurate quantitative analysis. The method takes advantage of the difference in the chemical shift of the methyl protons on free thiadiazole and the thiadiazole moiety of cefazolin; the NMR signals appear at 2.54 and 2.84 ppm, respectively (Fig. 1).

Quantitative analysis of pharmaceuticals and chemical mixtures by NMR spectroscopy has generally been accomplished with the use of an internal standard such as hexamethylcyclotrisiloxane (1, 2), succinic acid (3), fumaric acid (4), *tert*-butanol (5), and 2,3,5-tribromothiophene (6). However, for the quantitation of free thiadiazole in cefazolin, a calibration curve was derived from spectral data on known mixtures prepared from pure thiadiazole and pure cefazolin (sodium salt or free acid). Considerable time is saved, and potential weighing errors are

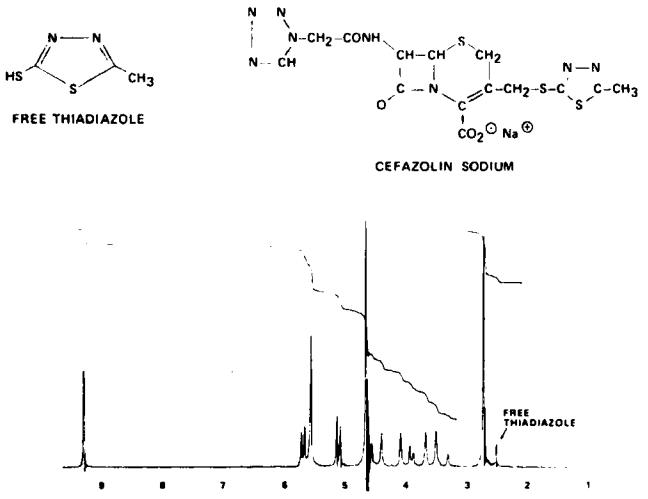

Figure 1—*NMR spectrum of cefazolin sodium containing free thiadiazole.*

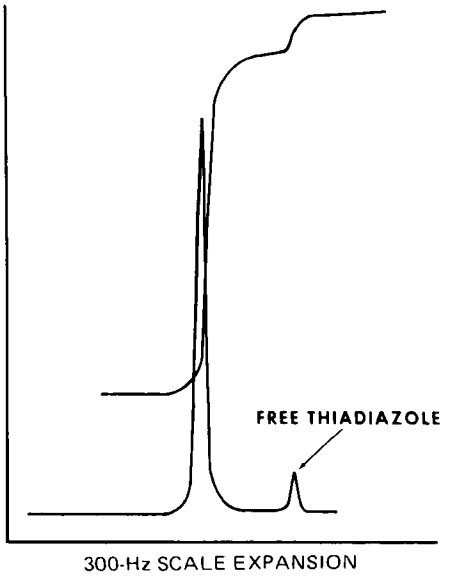

Figure 2—*Quantitative NMR spectrum of free thiadiazole in cefazolin sodium.*

[1] Unpublished data.

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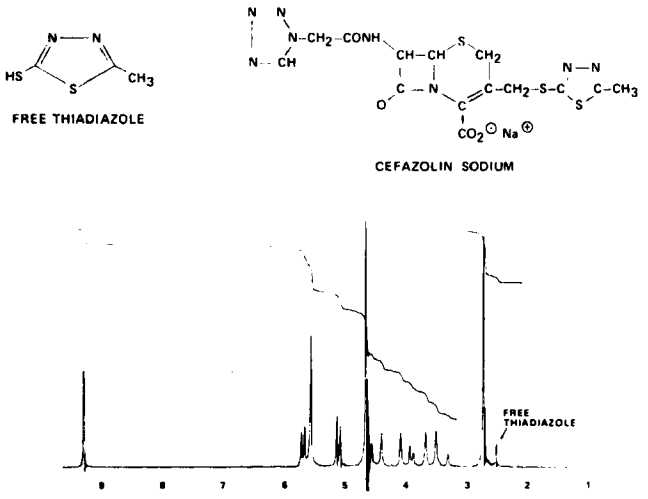
Quantitative analysis of pharmaceuticals and chemical mixtures by NMR spectroscopy has generally been accomplished with the use of an internal standard such as hexamethylcyclotrisiloxane (1, 2), succinic acid (3), fumaric acid (4), *tert*-butanol (5), and 2,3,5-tribromothiophene (6). However, for the quantitation of free thiadiazole in cefazolin, a calibration curve was derived from spectral data on known mixtures prepared from pure thiadiazole and pure cefazolin (sodium salt or free acid). Considerable time is saved, and potential weighing errors are



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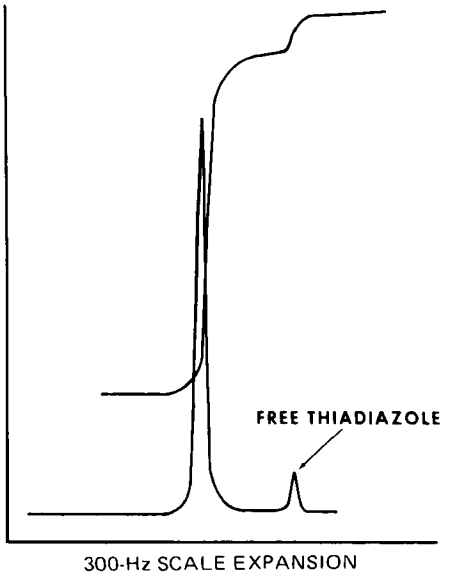

Figure 2—*Quantitative NMR spectrum of free thiadiazole in cefazolin sodium.*

[1] Unpublished data.

Table I—Standard Curve Preparation

Cefazolin Sodium			Cefazolin		
	I_{TD}[a]			I_{TD}[a]	
Thiadiazole, %	$I_{TD} + I_{CS}$	Precision[b]	Thiadiazole, %	$I_{TD} + I_C$	Precision[b]
0.20	Detectable	—	0.50	Detectable	—
0.79	0.039	8.1	1.54	0.039	7.7
1.99	0.083	6.7	2.73	0.075	6.3
3.24	0.148	5.6	7.20	0.190	4.2
4.67	0.201	4.9			

[a] I_{TD} = integration value of thiadiazole, I_C = integration value of cefazolin, and I_{CS} = integration value of cefazolin sodium. [b] Based on five integrations. Precision = (deviation × 100)/average ratio.

avoided by not using an internal standard, particularly when many cefazolin samples are involved.

EXPERIMENTAL

A 90-MHz NMR spectrometer[2] with 5-mm o.d. NMR tubes was used.

The necessary reagents included deuterium oxide[3] (99.7% D minimum isotopic purity) and trifluoroacetic acid[4].

Sample Preparation and NMR Conditions—Approximately 100 mg of cefazolin or cefazolin sodium is transferred to a small glass vial. For cefazolin, trifluoroacetic acid (0.5 ml) is added; for cefazolin sodium, deuterium oxide (0.5 ml) is added. The contents then are agitated to effect complete solution, and the solution is filtered through a cotton plug into an NMR tube.

The NMR spectrum is run on a 10-ppm sweep width and 10-min scan time for identification purposes (Fig. 1). For quantitative purposes, the spectrum is run at 300-Hz sweep width and 10-min scan time in the 135–300-Hz region (Fig. 2). This region is carefully integrated five times.

Preparation of Calibration Curve—Known mixtures, ranging from 0.5 to 8.0% free thiadiazole in cefazolin or cefazolin sodium, were prepared. The NMR spectra were obtained on the mixtures with integrations as already described. The integrations over the methyl resonances provided the data to construct the calibration curve (Fig. 3). Precision was determined for the lowest and the highest levels (Table I).

The ratio *(A)* of the thiadiazole integration to the sum of the thiadiazole and cefazolin or cefazolin sodium integrations was determined. The ratio *(A)* was plotted against the percent of free thiadiazole *(B)* in the mixture (Fig. 3). Sample quantitation followed by determining the ratio *(A)* and comparing it to the standard curve for free thiadiazole content.

Alternatively, a linear regression analysis can be applied to the data pairs to obtain the "best-fit" curve, $(A = mB + C)$, and its slope, m. Since $C = 0$, $A(1/m) = B$. Thus, the thiadiazole content is found by multiplying the factor $(1/m)$ by the ratio *(A)*.

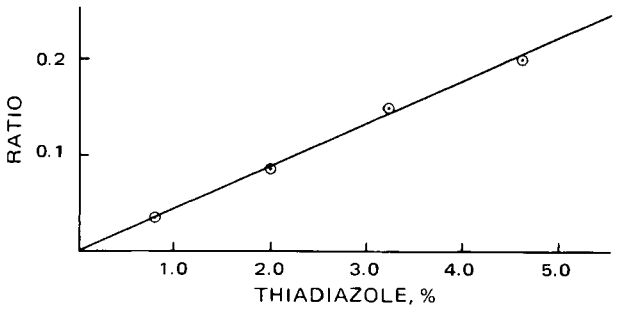

Figure 3—*Calibration curve showing percentages of thiadiazole in cefazolin sodium salt using deuterium oxide as the solvent.*

[2] Perkin-Elmer model R 32.
[3] Merck and Co.
[4] Matheson, Coleman and Bell.

Table II—NMR Analytical Data

Sample	Weight of Cefazolin Sodium, mg	Weight of Thiadiazole, mg	Thiadiazole in Mixture, %	Thiadiazole by NMR, %
1	48.321	1.213	2.45	2.31
2	48.672	1.682	3.34	3.26
3	48.607	2.088	4.12	4.11
4	47.592	2.128	4.28	4.23
5	49.952	1.048	2.05	2.16

RESULTS AND DISCUSSION

The calibration curves are linear for the range of thiadiazole concentrations studied, as indicated by the correlation coefficients of 0.9997 and 0.9952 for cefazolin and cefazolin sodium, respectively. These values were calculated by the method given by Snedecor and Cochran (7).

The sensitivity limit for the trifluoroacetic acid solutions of cefazolin is 0.5%; the sensitivity limit for the deuterium oxide solutions of cefazolin sodium is 0.2%. The sensitivity advantage obtained in deuterium oxide is due to the better resolution achieved with this solvent. Precision values at each concentration for the two solvents are about the same and show a drop in precision as lower concentrations of free thiadiazole are encountered. This finding reflects the limitation in measuring very small integral values. The reproducibility at the 1% level is 8.1%.

Although the NMR spectra of the trifluoroacetic acid solutions were obtained immediately after preparation, it was not of paramount importance to obtain them within this timespan. Studies showed that trifluoroacetic acid solutions of cefazolin are stable for at least 2 hr, and no discernable decomposition was observed from the integrations obtained from repetitive scans during this period.

Five mixtures of thiadiazole in cefazolin sodium were prepared and analyzed by the described NMR method (Table II).

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Unit-Dose Assay of Tropine Alkaloids and Their Synthetic Analogs

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Abstract □ A charge-transfer spectrophotometric method was developed for unit-dose assay of the tropine alkaloids and some of their synthetic analogs. The high molar absorptivities of the charge-transfer bands of the alkaloids with iodine in ethylene dichloride resulted in improved recoveries and good precision, particularly at the low dose levels of pediatric and hypodermic tablets.

Keyphrases □ Tropine alkaloids and synthetic analogs—unit-dose analysis, charge-transfer UV spectrophotometry □ UV spectrophotometry—analysis, tropine alkaloids, and synthetic analogs, unit-dose sensitivity

Quantitative methods for microamounts of the solanaceous alkaloids are few, and those depending on simple and convenient direct measurement in the UV range are lacking. The absorption bands of the tropine alkaloids, which have molar absorptivities of less than 150 (1), are not suitable for analytical purposes. The low sensitivity of the direct UV assay precludes its adoption in unit-dose determinations where amounts of the alkaloids as small as 0.3 mg/single dose are handled (2).

Accordingly, various approaches to the single-dose analysis of the tropine alkaloids were reported (3–13) which do not include direct UV measurement. They include dye–complex methods (3, 4), colorimetry *via* nitration and reduction followed by diazotization and coupling (5, 6), colorimetry as ferric hydroxamates (7), fluorometry (8), indirect spectrophotometry *via* cerimetric oxidation to benzaldehyde (9), TLC with densitometry (10), and GLC procedures (11–13).

With the possible exception of the last approach, most procedures suffer from some drawbacks. Acid–dye methods were reported to yield erratic results (14) owing to extreme sensitivity to pH variations. The colorimetric method (5) is not specific and is quite cumbersome. The ferric hydroxamate procedure requires a prolonged reaction time at low temperature, the colors are not very stable, and, more significant, no great improvement in sensitivity is achieved since reported molar absorptivities are in the 350–600 range (7). Photodensitometry on thin-layer chromatograms gave low recoveries of hyoscyamine (10).

The purpose of the present study was to develop a UV spectrophotometric procedure, for the analysis of the tropine alkaloids and some of their synthetic analogs, of sufficient sensitivity to allow single-dose assay.

Recently, Taha *et al.* (15) reported that about a 50-fold increase in the values of molar absorptivities of the tropine alkaloids could be achieved *via* charge-transfer complex formation with iodine in chlorinated solvents. This method, being of sufficient sensitivity, was adopted in the analytical technique described here.

EXPERIMENTAL[1]

Material—Pharmaceutical grade atropine base, atropine sulfate, scopolamine hydrobromide, hyoscyamine hydrobromide, homatropine hydrobromide, eucatropine hydrochloride, and *N*-butylscopolammonium bromide were utilized as working standards and in the construction of calibration curves.

Atropine sulfate tablets containing 0.3, 0.4, and 0.6 mg alkaloidal salt/100-mg tablet (prepared by direct compression using microcrystalline cellulose[2] as vehicle), atropine sulfate injection[3], tablets of total belladonna alkaloids as malates with phenobarbital[4], atropine sulfate ophthalmic solution[5], and *N*-butylscopolammonium bromide tablets[6] were subjected to the analytical procedure.

Reagents—*Iodine Solution (10^{-3} M)*—Dissolve 25.5 mg resublimed iodine in spectrograde ethylene dichloride in a 100-ml volumetric flask to obtain a solution of 0.255 mg/ml. The solution is stable for 1 week at 4°.

Buffer—Prepare 0.2 *M*, pH 9.0 buffer, standardized against the glass electrode, by dissolving 34.8 g of dibasic potassium phosphate in 900 ml of water. Adjust to pH 9.0 and make to 1 liter with water.

Standard Preparation—*Alkaloidal Salts*—Dissolve an accurately weighed amount of the appropriate working standard in water and dilute the solution quantitatively and stepwise to obtain a concentration of the salt equivalent to 1.0 mg of base/ml. Pipet 1.0 ml of this solution into a 30-ml separator containing 5 ml of buffer. Extract with 10 ml of ethylene dichloride, passing the separated organic layer through 2 g of anhydrous sodium sulfate supported by glass wool in a small funnel. Collect the filtrate in a 10-ml volumetric flask, wash the filter with a few drops of ethylene dichloride, and dilute to volume with the same solvent.

Atropine Base and N-Butylscopolammonium Bromide—Dissolve an accurately weighed amount of the appropriate standard in ethylene dichloride and dilute to obtain a concentration of 0.1 mg/ml.

Assay Preparation—*Atropine Sulfate Tablets*—Place one powdered tablet, or its equivalent from a composite of 20 tablets, in 5 ml of buffer in a 30-ml separator. Extract with two 10-ml fractions of ethylene dichloride, passing the separated organic layers through the same 2 g of anhydrous sodium sulfate suitably supported in a small funnel. Wash the filter with about 3 ml of ethylene dichloride and collect the filtrate and washings in a 25-ml volumetric flask. Dilute to volume with the same solvent.

This procedure is also applicable to tablets of total belladonna alkaloids with phenobarbital.

N-Butylscopolammonium Bromide Tablets—Place one powdered tablet, or its equivalent from a composite of 20 tablets, in a 30-ml beaker. Extract with two 10-ml portions of warm (40–50°) ethylene dichloride. Filter the fractions through a small piece of cotton wool, wash the filter with a small amount of warm solvent, and collect the filtrate and washings in a 25-ml volumetric flask. Cool to room temperature and dilute to volume with ethylene dichloride.

Atropine Sulfate Injection—Proceed as directed under *Atropine Sulfate Tablets*, substituting 1.0 ml, or the measured contents of a single-dose container of the injection, for the tablets.

Atropine Sulfate Ophthalmic Solution—Dilute an appropriate volume of the solution in a volumetric flask so as to represent 0.5 mg/ml. Proceed as directed under *Atropine Sulfate Tablets*, substituting 1.0 ml of the diluted ophthalmic solution for the tablets.

[1] Spectra were made on Spectromom 203 UV-VIS spectrophotometer, Mom, Budapest, Hungary.
[2] Avicel, American Viscose Corp., Marcus Hook, Pa.
[3] El-Nile Co., Cairo, Egypt.
[4] Belladenal, Sandoz, Basel, Switzerland.
[5] Isopto Atropine, Alcon, Fort Worth, Tex.
[6] Buscopan, Boehringer Ingelheim, Germany.

Table I—Peak Position and Intensity in Two Solvents

Compound	Ethylene Dichloride		Chloroform	
	λ_{max}, nm	ϵ_{max}[a]	λ_{max}, nm	ϵ_{max}[a]
Atropine	295	15,900	270	8,200
	375	6,200	410	2,900
Scopolamine	295	8,100	270	1000
	375	4,800	—	—
N-Butylscopolammonium bromide	280	33,500	280	44,200
Homatropine	295	10,500	—[b]	—
	375	4,700		
Eucatropine	295	18,500	—[b]	—
	375	10,200		
Hyoscyamine hydrobromide	—[c]	—	275	856
Scopolamine hydrobromide	—[c]	—	270	613
Homatropine hydrobromide	—[c]	—	275	783

[a] Based on molecular weight of free base. [b] Undetermined. [c] Salts are insoluble in ethylene dichloride.

Procedure—Pipet 1.0 ml of the standard preparation and a volume of the assay preparation equivalent to 0.05–0.15 mg of alkaloid into separate 10-ml volumetric flasks. Add 1.0 ml of iodine reagent solution to each flask and dilute to volume with ethylene dichloride. Determine the absorbance of the assay and standard solutions at the proper λ_{max} (295 nm for atropine, scopolamine, homatropine, and eucatropine and 280 nm for *N*-butylscopolammonium bromide) *versus* a blank prepared from 1.0 ml of iodine reagent solution diluted to 10.0 ml with ethylene dichloride.

Calculate the amount of drug, in milligrams, in the sample taken from the formula: $(f)C(A_u/A_s)$, in which f is a conversion factor derived from the ratio of the formula weight of the alkaloid salt to the molecular weight of its free base, C is the concentration in milligrams per milliliter of the standard preparation, and A_u and A_s are the absorbances of the solutions from the assay preparation and the standard preparation, respectively.

Ferric Hydroxamate Colorimetric Method—The basic procedure of Feldman and Robb (7) was followed.

RESULTS AND DISCUSSION

Peak Position and Intensity—In the preliminary report (15), which included the theoretical background of the method and the determination of the basic thermodynamic constants of the complexes, chloroform and carbon tetrachloride were examined as solvents for charge-transfer complex formation. The present investigation revealed the superior qualities of ethylene dichloride as the assay solvent. It has a favorable boiling point and a low cutoff point. More important, the dilution effects previously reported for chloroform and carbon tetrachloride (15), which resulted in a decrease in peak intensity, were not observed with ethylene dichloride. This finding was probably due to the decreased tendency of ethylene dichloride to form contact charge-transfer pairs (16) with the nitrogen of the alkaloid because of the lower ratio of chlorine to carbon atoms in its molecule.

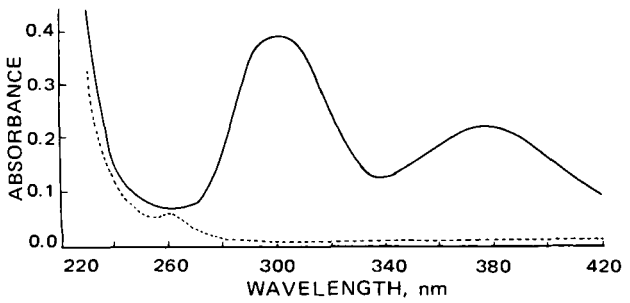

Figure 1—*Spectra of uncomplexed and iodine-complexed atropine in ethylene dichloride. Key: —, atropine (7 μg/ml) with iodine (25.5 μg/ml); and - - - -, atropine (70 μg/ml).*

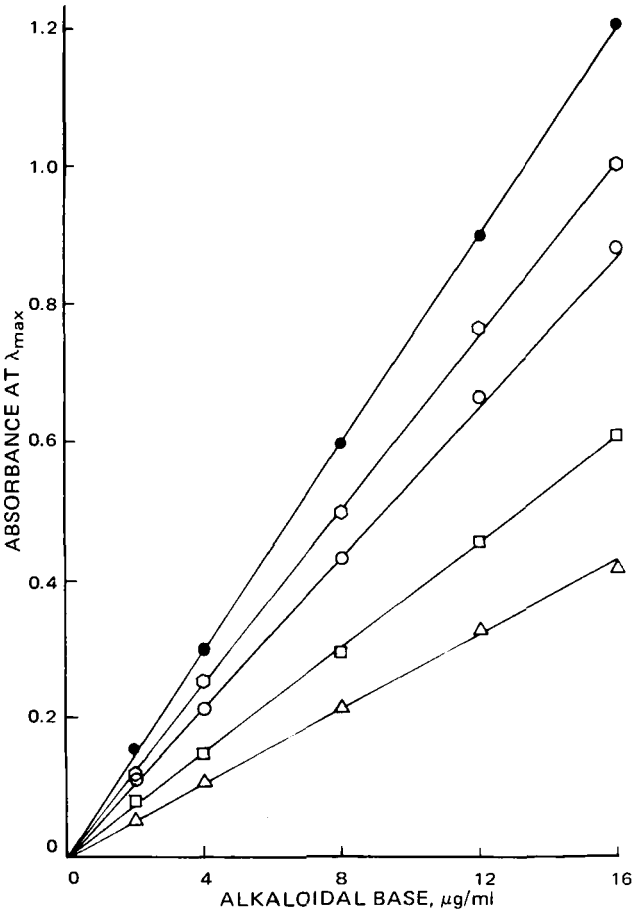

Figure 2—*Calibration curves of* N*-butylscopolammonium bromide (●), eucatropine (○), atropine (○), homatropine (□), and scopolamine (△). The λ_{max} of* N*-butylscopolammonium bromide is at 280 nm; it is at 295 nm for all other compounds.*

Peak positions of the complexes in ethylene dichloride are also different from those in chloroform (Table I). The spectra of complexed and uncomplexed atropine are presented in Fig. 1 as typical examples.

Linearity of Beer's Plots, Accuracy, and Precision—Standard curves for the different compounds were constructed by plotting observed absorbance readings *versus* the concentration of the alkaloid base in micrograms per milliliter of the final dilution. The results are presented in Fig. 2; conformance to Beer's law is evident. The slopes of the curves, determined by the method of least squares, were 0.076, 0.0635, 0.055, 0.0375, and 0.027 for *N*-butylscopolammonium bromide, eucatropine, atropine, homatropine, and scopolamine, respectively (Fig. 2). The slopes may be utilized for computation of unknown alkaloid concentration by applying the relation:

$$\text{concentration of base } (\mu g/ml) = A/\alpha \qquad \text{(Eq. 1)}$$

where A = absorbance of the solution from the assay preparation, and α = slope of the calibration curve of the corresponding alkaloid.

The validity of Eq. 1 was tested by taking 10 standards of atropine and of scopolamine through the procedure. As low as 0.01 mg of atropine and 0.02 mg of scopolamine could be determined, with recoveries of 99.4 and 98.7% and coefficients of variation in the assays of 1.14 and 2.13%, respectively.

Application to Dosage Forms—The charge-transfer method was applied to specially prepared lots of atropine sulfate tablets, conforming to the specifications of the USP XVIII (2), at three dose levels of 0.3, 0.4, and 0.6 mg of alkaloid salt/100-mg tablet. A tablet composite from each lot was assayed by the charge-transfer

Table II—Comparison of Two Assay Methods for Atropine Sulfate

Tablet, mg	Charge Transfer			Ferric Hydroxamate		
	Found[a], mg	Recovery, %	SD, ±%	Found[a], mg	Recovery, %	SD, ±%
0.3	0.308	102.6	2.83	0.263	87.6	7.19
0.4	0.406	101.5	2.24	0.369	92.3	5.30
0.6	0.593	98.8	1.59	0.576	96.0	6.34

[a] Average of six determinations of tablet composite.

and ferric hydroxamate methods (Table II). Placebos were utilized in the preparation of blanks.

Data of Table II suggest that the presented spectrophotometric method could be applied to assay individual dose units of the tropine alkaloids with good accuracy and precision. The poorer recoveries of the colorimetric method reflect differences in molar absorptivities of the absorbing chromogens in the two methods, *e.g.*, ϵ of 15,900 for the charge-transfer band of the atropine–iodine complex compared to ϵ of 420 for the ferric hydroxamate chromogen reported for atropine sulfate (7).

The applicability of the procedure to commercial dosage forms and to content uniformity determination was checked (Table III). Since placebos of commercial products were not available, the method of standard addition was adopted. The results of Table III further confirm the suitability of the charge-transfer spectrophotometric method for control analysis and unit-dose assay of the tropine alkaloids and some of their synthetic analogs.

Comments on Procedure—The charge-transfer method was unaffected by the presence of phenobarbital, commonly prescribed with the belladonna alkaloids. The single partition at the alkaline pH of the procedure effectively separated the acidic barbiturate from the basic alkaloids.

Chlorobutanol and esters of *p*-hydroxybenzoic acid, frequently present as preservatives in ophthalmic solutions, are known to interfere in the ferric hydroxamate method (7, 17). No interference of these compounds could be observed in the presented procedure.

However, the charge-transfer method must be considered nonspecific with regard to differentiation between members of the tropine alkaloids, and it is not directly applicable as a stability-indicating assay since tropine, the major product of degradation of the belladonna alkaloids, was found to give a strong charge-transfer band.

Table III—Assay of Commercial Dosage Forms

Preparation	Label Claim	Found[a], % of Label Claim	Standard	
			Added[b]	Recovered[c], %
Tablets of total alkaloids with phenobarbital	0.25 mg/tablet	104.8 106.5 97.6	0.25 mg/tablet	98.2
Atropine sulfate injection	1.0 mg/ampul	101.6 102.0 101.3	—	—
Atropine sulfate ophthalmic solution	0.50%	97.5[c]	0.25%	101.5
N-Butylscopolammonium bromide tablets	10.0 mg/tablet	100.8 96.6 99.1	5.0 mg/tablet	99.3

[a] Single-dose determination. [b] Added to tablet composite or ophthalmic solution. [c] Average of three determinations.

These shortcomings do not affect the utility of the method in routine analysis and content uniformity determination of singly prescribed alkaloids. The drawbacks may also be overcome by coupling the charge-transfer method to a separation procedure reported for the tropine alkaloids such as paper chromatography (18, 19), partition column chromatography (5, 6, 20), countercurrent distribution (21), or TLC (22–24). The enhanced sensitivity of the method allows for additional handling without fear of increasing error.

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Colorimetric Determination of Peptide Antibiotics: In-Process Assay of Cyclic Octapeptidic Antibiotics in Fermentation Broths

EUGENE IVASHKIV

Abstract □ A spectrophotometric method is presented for monitoring the biosynthesis of a new complex of cyclic octapeptidic antibiotics in fermentation broths. The method is based on the extraction of antibiotic from alkaline broth with butanol. An ion-pair, formed between the octapeptides and bromthymol blue, is extracted into chloroform from a solution buffered to pH 7.5. The absorbance of the colored solution is measured at 420 nm. Results are in good agreement with those obtained by microbiological assay. The method is also applicable to other peptidic antibiotics such as polymyxin B and gramicidin.

Keyphrases □ Octapeptide (cyclic) antibiotic biosynthesis—colorimetric monitoring in fermentation broths, ion-pair formation with bromthymol blue □ Antibiotic biosynthesis, cyclic octapeptide—colorimetric monitoring in fermentation broths, ion-pair formation with bromthymol blue □ Colorimetry—monitoring, in-process cyclic octapeptide biosynthesis, ion-pair formation with bromthymol blue

A new complex of cyclic octapeptidic antibiotics[1] (I) is produced by a strain of *Bacillus circulans*. The antibiotic has broad spectrum antibacterial activity and displays considerable antifungal and antiprotozoal activities (1–3). Proper control of the fermentation of I requires an analytical method that can monitor biosynthesis at any stage and that requires a comparatively short time.

With a microbiological assay[2], one can determine the amount of I in a fermentation broth about 48 hr after sampling. Chemical methods for the determination of polypeptidic antibiotics (4–8) are tedious and time consuming. In these laboratories, a rapid and simple colorimetric method was developed for estimating the amount of I in a fermentation broth. The reaction of bromthymol blue with I to form a chloroform-soluble colored complex is the basis of the method; the amount of colored complex is proportional to the amount of I present.

EXPERIMENTAL

Reagent grade *n*-butanol, chloroform, sodium chloride, sodium hydroxide, and absolute ethanol were used.

Phosphate buffer, pH 7.5, was prepared by dissolving 10 g of dibasic sodium phosphate, 2 g of monobasic potassium phosphate, and 5 ml of 85% phosphoric acid in 900 ml of distilled water. The pH was adjusted to 7.5 with 5 *N* sodium hydroxide, and the solution was diluted to 1 liter with distilled water.

Bromthymol blue, 0.15%, was prepared by dissolving 0.15 g of bromthymol blue and 0.15 g of anhydrous sodium carbonate in distilled water to give 100 ml of reagent.

The I standard, of known purity, was dissolved in distilled water and diluted to a concentration of about 500 μg/ml.

[1] EM49, Squibb Institute for Medical Research, New Brunswick, NJ 08903

[2] T. B. Platt, Squibb Institute for Medical Research, personal communication.

Table I—Effect of pH on Extraction of I—Bromthymol Blue Ion-Pair

pH	Absorbance at 420 nm
2.5	0.000
6.0	0.490
6.5	0.690
7.0	0.780
7.5	0.824
8.0	0.525
9.0	0.419
10.0	0.000
12.0	0.000

PROCEDURE

Extraction—A 5.0-ml sample of fermentation broth and 5.0 ml of standard I solution are pipetted into screw-type test tubes containing 2 g of sodium chloride, 5.0 ml of *n*-butanol, and 2 ml of 5 *N* sodium hydroxide solution. Each tube is covered with a plastic cap and shaken mechanically for 5 min. After centrifugation, 0.2 ml of the butanolic upper layer is pipetted into a screw-type test tube and the solvent is evaporated completely under a stream of air. Immersion of the tube in a water bath at about 70° is used to speed the evaporation of butanol.

Color Development—To each tube, 10.0 ml of pH 7.5 phosphate buffer is added. A reagent blank is prepared by pipetting 10.0 ml of pH 7.5 phosphate buffer into a screw-type test tube. To each tube, 1.0 ml of 0.15% bromthymol blue is added; then the contents are mixed. After the addition of 10.0 ml of chloroform, the tubes are covered with plastic caps and shaken mechanically for 5 min. The tubes are centrifuged at 2500 rpm for 1 min, and the aqueous upper layer is aspirated completely.

A 5.0-ml portion of the chloroform layer is transferred to a test tube containing 0.5 ml of absolute ethanol, and the contents are mixed. The absorbance of the sample and of the standard solutions is measured at 420 nm in a spectrophotometer[3] equipped with 1-cm cells, with the reagent blank in the reference cell. The amount of I is calculated from the concentration and the absorbance of the I standard.

RESULTS AND DISCUSSION

Positively and negatively charged ions can associate as uncharged ion-pairs. These neutral combinations exhibit considerably diminished water solubility and can be readily extracted into organic solvents. The principles of ion-pair formation were reviewed in detail previously (9).

Bromcresol purple, bromphenol blue, bromthymol blue, and methyl orange were tested for possible ion-pair formation with I. Of these indicators, only methyl orange and bromthymol blue formed a precipitate with I. At pH 7.5, both bromthymol blue and methyl orange couple with the positively charged basic molecule of I, but only the yellow I–bromthymol blue ion-pair can be extracted from aqueous solutions into chloroform and other organic solvents such as methylene chloride, ethylene dichloride, and ether. Carbon tetrachloride extracts only 8% of the color, and toluene extracts 25%. Cyclohexane, hexane, and heptane precipitate the ion-pair at the interface of the two phases. Butyl alcohol, isobutyl acetate, and

[3] Beckman DU.

Table II—Recovery of I from Broths

Added I, mg/ml of Broth	Recovery, %
2.0	101.0
1.6	101.0
1.2	99.0
0.8	99.4
0.4	100.0
0.2	101.0
0.1	98.5
0.0	0.0

ethyl acetate extract free bromthymol blue, thus producing very high blanks.

Study of the effect of pH of the aqueous solution on the reaction and extraction of the I–bromthymol blue ion-pair gave the results shown in Table I. Less color was extracted from acidic and alkaline solutions than from a neutral solution. An excessive amount of free bromthymol blue was extracted with chloroform from acidic solutions with pH up to 6.5. For example, the absorbance of the reagent blank at pH 6.0 was 0.730, contrasted with the absorbance of 0.100 from a pH 7.5 buffer. The amount of phosphate used in the preparation of the pH 7.5 buffer proved important. An increase in the phosphate content of the buffer solution led to an increase in the extraction of the ion-pair, but too high a concentration of phosphate in the buffer produced high reagent blanks.

The volume of pH 7.5 buffer used also affects the extraction of the ion-pair with chloroform. The best results were obtained with 10.0 ml of pH 7.5 buffer. For reproducible results, the phosphate buffer should be transferred with a volumetric pipet. The volume of chloroform proved to be unimportant; when the I–bromthymol blue ion-pair was extracted from 10.0 ml of phosphate buffer with 5, 10, 15, or 25 ml of chloroform, the same amount of color was extracted in each case.

Chloroform extracts of the ion-pair are cloudy. They can be clarified by centrifugation, but they become cloudy again on standing. The addition of anhydrous sodium sulfate clarifies the chloroform extract, but the readings of absorbance vary. Reproducible measurements of absorbance are obtained when chloroform extracts are clarified with absolute ethanol.

The I–bromthymol blue ion-pair shows maximum absorbance at 420 nm. Solutions containing up to 200 μg I/ml of chloroform, with an absorbance of 1.16, obey Beer's law.

Various solvents were tried for the extraction of I from aqueous solutions. Isobutyl acetate, ethyl acetate, benzene, isopentyl acetate, chloroform, and ethylene dichloride did not extract I. When the ratio of solvent to the aqueous solution was 1:1, methyl ethyl ketone extracted 30%, isoamyl alcohol extracted 19%, and isobutyl alcohol extracted 53% of the I present. Of the solvents tried, only *n*-butanol extracted about 96% of I. Complete extraction of I with *n*-butanol was obtained when an inorganic salt such as sodium chloride, ammonium chloride, or sodium sulfate was used to salt out the antibiotic. The use of one of these salts also prevented emulsification of the broth during extraction.

The effect of pH on the extraction of I was studied. The pH of solutions was adjusted with 5 *N* sodium hydroxide or 6 *N* hydrochloric acid to points between 1 and 12. Complete extraction of I occurred from solutions adjusted to pH 5 or higher; but at pH 1.0, only 57% of the I was extracted. Extraction of I from highly alkaline broths eliminates blanks from samples.

Table III—Reproducibility of Colorimetric Assay of I in Fermentation Broth

Sample	I, μg/ml of Broth	
	Run 1	Run 2
1	705	718
2	791	784
3	780	795
4	898	876
5	887	887
6	876	889

Table IV—Assay of I in Fermentation Broth by Colorimetric and Microbiological Methods

Duration of Fermentation, hr	Concentration of I, μg/ml of Broth			
	Chemical		Microbiological	
	Run 1	Run 2	Run 1	Run 2
0	0	0	<35	<35
6	56	54	<35	<35
14	89	90	<35	<35
22	488	496	545	474
30	570	560	636	682
38	600	583	682	750
46	660	680	727	818
54	689	710	727	750
62	793	800	727	818
70	902	894	795	886
78	896	900	750	886
86	893	880	886	796
96	901	892	909	886

The ratio of the volume of broth to the volume of butanol did not appreciably affect the efficiency of extraction. Compound I was extracted completely even when the ratio of broth to *n*-butanol was 2:1.

Recovery of I was studied by the addition of the antibiotic to a broth medium. As shown in Table II, complete recovery of I from the medium was obtained in the range tested.

Samples of fermentation broth were analyzed in duplicate. From the data given in Table III, it appears that results can be reproduced within ±2%.

Results from the colorimetric assay were compared with those from the microbiological assay of I. Broth samples were extracted in duplicate from solutions at about pH 12, and portions of the extracts were assayed both colorimetrically and microbiologically. *Bordetella bronchiseptica* (ATCC 4617) was used in the bioassay. As shown in Table IV, the results of the colorimetric assay were more reproducible than those of the microbiological procedure. When mean values for two assays by each method were compared, better correlation of methods was obtained at completion of the fermentation. The greater concentration of I shown by the microbiological assay could be attributable to fermentation products that do not form ion-pairs with bromthymol blue but are used up later in the process.

Broth samples were stable for at least 1 week when refrigerated at 5°. The I standard solutions showed a decrease of about 15% in color intensity after being kept at room temperature for 2 days. The butanolic extracts were stable for at least 1 week at room temperature.

This colorimetric assay of I in fermentation broths can be used to monitor the biosynthesis of other peptide antibiotics such as polymyxin B, gramicidin, and tyrocidine. Gramicidin, a component of the antibiotic tyrothricin, has to be separated from tyrocidine as described (10) before ion-pairs are formed. The $E^{1\%}_{1cm}$ of each polypeptidic antibiotic seems to depend on the number of free amino groups in the compound. The $E^{1\%}_{1cm}$ values of I, polymyxin B, gramicidin[4], and tyrocidine[5] are 772, 775, 193, and 319, respectively.

This new colorimetric assay can also be used for the determination of polymyxin B and gramicidin in pharmaceutical formulations.

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[4] NF reference standard for gramicidin.

[5] Tyrocidine hydrochloride, Mann Research Laboratories, New York, N.Y.

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JAMES T. STEWART ˣ and DAVID A. SETTLE

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Keyphrases □ Isoniazid—colorimetric analysis with 9-chloroacridine □ 9-Chloroacridine—colorimetric reagent for determination of isoniazid □ Colorimetry—analysis, isoniazid with 9-chloroacridine

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Existing analytical procedures for the assay of isoniazid include visible and UV spectrophotometry, polarography, and titrimetry. The colorimetric methods involve the interaction of the drug with 1-chloro- or 1-fluoro-2,4-dinitrobenzene (5, 6) and/or 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (7). Although highly sensitive and specific, these procedures have the disadvantages of instability of color formation and preparation of multireagent solutions. Vanillin has also been used as a chromogenic reagent in the analysis of isoniazid (8).

A procedure involving the hydrolysis of isoniazid to hydrazine, with subsequent color development with *p*-dimethylaminobenzaldehyde, was reported (6). This method is subject to interference by aldehydes and/or ketones. UV spectrophotometry (6) and polarography (6) also have been utilized in the analysis of microgram quantities of isoniazid. The USP XVIII method (9) involves an iodometric titration and is laborious and time consuming.

This paper presents a new colorimetric method for determining microgram quantities of isoniazid with 9-chloroacridine. The method has been successfully applied to the analysis of the drug in pharmaceutical dosage forms. In addition, preliminary investigations suggest that the procedure is useful for the determination of free isoniazid in urine and plasma samples.

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EXPERIMENTAL

Apparatus—Spectra and absorbance measurements were made with spectrophotometers[1,2]. Matched cells with a 1-cm optical path were used.

Reagents and Chemicals—9-Chloroacridine[3] was used as the chromogenic reagent. It was recrystallized before use by the method of Albert (10). Isoniazid[4] was purified by recrystallization from 80% methanol and dried under reduced pressure, mp 172–173°. Isonicotinic acid[5] was used as received. Acetylisoniazid, α-ketoglutaric acid isonicotinoylhydrazone, pyruvic acid isonicotinoylhydra-

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Table II—Determination of Isoniazid in Tablets by the 9-Chloroacridine Method and the USP XVIII Method

Tablet	Claim, mg/Tablet	Found[a], mg/Tablet		Percent of Claim	
		9-Chloroacridine Method	USP XVIII Method	9-Chloroacridine Method	USP XVIII Method
A[b]	100	101.15 ± 0.35[c]	101.46 ± 1.69	101.15	101.46
B[d]	100	100.00 ± 0.93	100.61 ± 0.84	100.00	100.61

[a] Based upon two replicate determinations of each solution. [b] Dosage form contained only isoniazid as active ingredient. [c] Confidence limits at $p = 0.05$. [d] Dosage form also contained pyridoxine hydrochloride (10 mg).

zone, acetylhydrazine, and 1,2-diacetylhydrazine were synthesized by procedures listed by Boxenbaum and Riegelman (8). Isonicotinuric acid was prepared by the method of Gardner *et al.* (11). The melting points of the synthesized compounds were in agreement with literature values. All other chemicals were commercially available and were utilized as received.

Solutions were prepared by dissolving weighed amounts of isoniazid in water or ethanol and the remaining compounds in ethanol. The reagent solution of 9-chloroacridine was prepared by dissolving a weighed amount in tetrahydrofuran[6] and storing in a light-resistant volumetric flask[7].

General Procedure—A quantity of an aqueous or ethanolic solution containing isoniazid was placed in an appropriate volumetric flask. A tetrahydrofuran solution containing an approximate twofold excess of 9-chloroacridine was added to the flask. The solution was acidified with 2 drops of a 5% (v/v) aqueous hydrochloric acid solution and shaken in a constant-temperature water bath–shaker[8] at 50 ± 1° for 30 min. Ethanol was added to volume so that the final concentration of isoniazid in the flask was equal to or greater than 4×10^{-6} *M*. The absorbance was measured at 500 nm, and measurements were corrected for reagent blanks in the procedure.

Analysis of Solid Dosage Form—Tablets were weighed and powdered, and a weighed aliquot was dissolved in distilled water by shaking vigorously for 5–10 min and filtering, if necessary, to give a 10^{-3} *M* solution. Then 1 ml was removed and assayed according to the *General Procedure*.

RESULTS AND DISCUSSION

9-Chloroacridine interacts with isoniazid in the analytical procedure to yield a highly colored orange solution. The absorption curve in the visible spectrum for an analytical solution of isoniazid shows an absorption maximum at 500 nm (Fig. 1).

The interaction between 9-chloroacridine and isoniazid is affected by the temperature and time of heating and the quantity of acridine reagent utilized. Adherence to the Beer–Lambert law was not observed for various concentrations of isoniazid at room temperature for 30–45 min (Fig. 2). However, when the analytical solutions were heated at 40, 50, and/or 60° for 30–45 min, linearity was observed. The results showed that 50° represented the lowest temperature at which both maximum sensitivity and linearity could be obtained. Thus, it was chosen as the desired temperature for the analytical procedure.

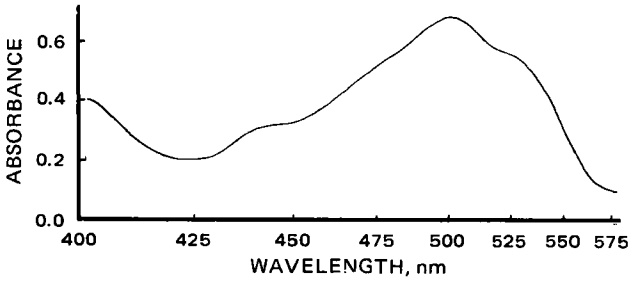


Figure 1—*Absorption spectrum of reaction product at isoniazid concentration of* 4×10^{-5} M.

The minimum heating time was then determined by following the color development. Absorbance values increased through 30 min and then stabilized. A heating period of 30 min at 50 ± 1° was satisfactory for reproducible readings. The color produced appeared to be stable for at least 1 day after the heating. Under these conditions, the reference blank was colorless.

The intensity of the color produced was directly affected by the concentration of 9-chloroacridine employed. Higher absorbance values were obtained up to a twofold excess of the reagent. Quantities of the acridine beyond the twofold excess did not effectively increase the absorbance readings for isoniazid. The stability of the 9-chloroacridine reagent solution was discussed previously (3, 4).

Standard curves can be prepared by plotting observed absorbance readings *versus* volumes taken of equimolar concentrations of isoniazid.

The analysis method is a microprocedure, and sensitivity is in the 0.6–5-μg/ml range (10^{-5} *M*) of isoniazid. Reagent preparation is simple and rapid since 9-chloroacridine is commercially available and easily purified. The time involved in the actual analysis procedure is about 45 min; some existing colorimetric and titrimetric methods involve longer heating and sample preparation times (5–7, 9).

Data from several systems (Table I) reveal that this procedure permits the determination of isoniazid in mixtures containing drugs, preservatives, and isoniazid metabolites which might be present in commercial dosage forms or biological samples containing the drug. Aminosalicylic acid interferes with the analysis method if it is present in final concentrations greater than 2×10^{-5} *M*. Since aminosalicylic acid is normally present in greater quantities than isoniazid in dosage forms and biological fluids where the drugs are given concurrently, it would be desirable before analyzing for isoniazid by this method to separate the two drugs *via* some type of separation technique or to employ the technique of simultaneous spectrophotometric assay. The latter technique should be possible since both aminosalicylic acid and isoniazid interact with 9-chloroacridine to produce maximum absorption at 435 and 500 nm, respectively.

Results obtained by applying the assay procedure to commercially available isoniazid tablets are shown in Table II. The method is comparable to the USP XVIII procedure (9). Extraction of the drug from tablets by shaking the tablet mass with distilled water for 5–10 min at room temperature appears to be as efficient as the USP extraction procedure utilizing heat and methanol. In addition, the colorimetric method is quicker and requires the preparation of only one reagent solution.

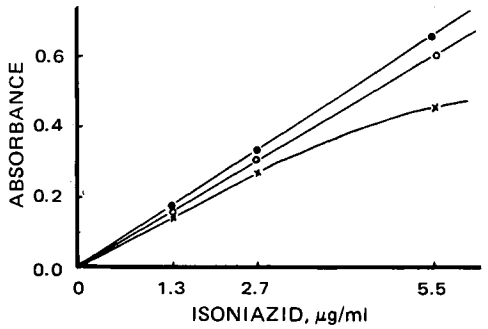


Figure 2—*Effect of temperature on color development and linearity of various isoniazid concentrations. Key:* ×, *30–45 min at room temperature;* ○, *30–45 min at 40°; and* ●, *30–45 min at 50° and also at 60°.*

[6] J. T. Baker Chemical Co., Phillipsburg, N.J.
[7] Low actinic volumetric flask (Corning No. 55640).
[8] Model 2156-1, Warner-Chilcott Instruments Division.

Table III—Analysis of Isoniazid Mixtures in Urine for Isoniazid

Mixture	Components, 4×10^{-5} M	Isoniazid[a] Found, $M \times 10^{-5}$	Isoniazid[a] % of Theory
1	Isoniazid Acetylisoniazid Isonicotinic acid Isonicotinuric acid	4.00 ± 0.04[b]	100.00
2	α-Ketoglutaric acid isonicotinoylhydrazone[c]	4.03 ± 0.18	100.75
3	Pyruvic acid isonicotinoylhydrazone[c]	3.95 ± 0.21	98.75
4	Isoniazid 1,2-Diacetylhydrazine	3.95 ± 0.01	98.75
5	Isoniazid *N*-Acetylhydrazine[d]	3.93 ± 0.04	98.25

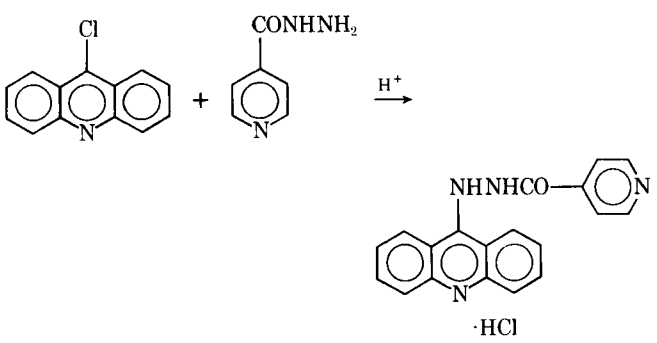
[a] Based upon three replicate determinations of each solution. Percent recovery of isoniazid from urine was 99.38 ± 0.26%. [b] Confidence limits at $p = 0.05$. [c] Hydrazones were hydrolyzed to isoniazid by treatment with 0.2 *N* HCl in a 52° water bath for 60 min; the solution was adjusted to pH 5 with aqueous potassium carbonate followed by the addition of a 9-chloroacridine solution. [d] Final concentration in mixture was 2×10^{-5} *M*.

A preliminary investigation of the analysis of isoniazid in urine and plasma was performed utilizing the 9-chloroacridine method. The biological fluids were spiked with various combinations of isoniazid and its metabolites. For each assay, freshly prepared isoniazid standards in urine and plasma were run concomitantly with the mixtures.

In the determination of isoniazid in urine, isoniazid can be quantitated satisfactorily in the presence of its metabolites (Table III). Since the amounts of α-ketoglutaric acid and pyruvic acid isonicotinoylhydrazones present in the urine are usually expressed in terms of *free* isoniazid (8), urine samples containing each hydrazone were quantitatively hydrolyzed to isoniazid using the procedures of Hughes *et al.* (12) and Short (13). After adjusting the solution pH to 5 with aqueous potassium carbonate, 9-chloroacridine was added and the color developed. Results indicate that the 9-chloroacridine method will detect isoniazid released from hydrolysis of the hydrazones. However, the method will not allow the detection of free isoniazid in the presence of the hydrazones since acid present in the analytical solution promotes hydrolysis of the hydrazones during the color development period.

For plasma samples, the results (Table IV) indicate that isoniazid can be successfully analyzed in mixtures containing its metabolites. The spiked plasma samples were denatured with equal volumes of 5% trichloroacetic acid and centrifuged. A portion of the supernatant liquid was transferred to a volumetric flask, and the pH of the sample was then adjusted to approximately 5–6 with aqueous potassium carbonate before addition of the 9-chloroacridine solution. Any cloudiness in the final solutions was clarified by centrifugation.

The color produced from the interaction of isoniazid with 9-chloroacridine suggested that a free amino function in the molecule is necessary for the reaction. This finding is in agreement with previous results from this laboratory concerning the interaction of primary aromatic amines and/or aromatic hydroxylamines with the acridine (1–4) to form highly colored solutions. The latter functional groups produce maximum absorption around 435 nm compared to isoniazid at 500 nm. Therefore, it should be possible to analyze for isoniazid in the presence of any drugs containing primary aromatic amines and/or aromatic hydroxylamine moieties with the use of the simultaneous spectrophotometric assay.



Scheme I

Table IV—Analysis of Isoniazid Mixtures in Plasma for Isoniazid

Mixture	Components, 4×10^{-5} M	Isoniazid[a] Found, $M \times 10^{-5}$	Isoniazid[a] % of Theory
1	Isoniazid Acetylisoniazid Isonicotinic acid Isonicotinuric acid	3.96 ± 0.04[b]	99.00
2	Isoniazid 1,2-Diacetylhydrazine *N*-Acetylhydrazine	3.93 ± 0.04	98.25

[a] Based upon three replicate determinations of each solution. Percent recovery of isoniazid from plasma was 82.70 ± 1.15%. [b] Confidence limits at $p = 0.05$.

Present studies indicated that amides, phenols, heterocyclic amines, alcohols, carboxylic acids, and hydrazines did not produce any color with 9-chloroacridine under the analysis conditions (Table I). Although one cannot exclude the possibility that these other functional groups react with the acridine in typical nucleophilic displacement reactions, only isoniazid produced any colored product under the conditions of the analytical reaction. Therefore, it is concluded that the reaction between isoniazid and 9-chloroacridine under the experimental conditions likely proceeds as depicted in Scheme I.

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Determination of Polythiazide in Pharmaceutical Dosage Forms by High-Pressure Liquid Chromatography

R. E. MOSKALYK ˣ, R. A. LOCOCK, L. G. CHATTEN, A. M. VELTMAN *, and MONIQUE F. BIELECH

Abstract □ A method is presented for the quantitative analysis of polythiazide tablets by high-pressure liquid chromatography. The powdered tablets are extracted with methanol, containing quinoline as the internal standard, and assayed by comparison of peak heights after liquid chromatography. The chromatographic conditions employed may be adapted for the determination of many other thiazide and nonthiazide diuretics.

Keyphrases □ Polythiazide—high-pressure liquid chromatographic analysis in pharmaceutical dosage forms □ High-pressure liquid chromatography—analysis, polythiazide dosage forms □ Diuretics—high-pressure liquid chromatographic analysis of polythiazide dosage forms

The NF XIII procedure for the analysis of polythiazide tablets requires a time-consuming TLC separation prior to spectrophotometric measurement of the isolated material (1). A similar approach using column chromatography was proposed recently as the official method; however, vanillin commonly added to tablet formulations of this drug interferes with the assay (2). Other available methods include a polarographic procedure (3) and several techniques involving hydrolysis and colorimetric measurement after diazotization and coupling with compounds such as *N*-(1-naphthyl)ethylenediamine dihydrochloride (4), thymol (5), and potassium guaiacolsulfonate (6).

High-pressure liquid chromatography (HPLC) allows for the direct analysis of polythiazide in pharmaceutical dosage forms not only in the presence of the excipient vanillin but also in formulations containing reserpine. The method reported here is simple, rapid, specific, and free of the analytical problems associated with the aforementioned procedures. Furthermore, the proposed assay is sufficiently sensitive that it can be carried out on individual tablets and therefore can serve equally well for the content uniformity determinations of this diuretic in pharmaceutical dosage forms. The application of this technique to the determination of polythiazide and its major metabolite in urine will be the subject of a subsequent publication.

EXPERIMENTAL

Apparatus—A high-pressure liquid chromatograph[1], equipped with a dual detector system, capable of operating at inlet pressures up to 6000 psig was used. The UV detector (254 nm) was also utilized.

Column—The column was purchased from the instrument manufacturer and consisted of a monomolecular layer of diphenyldichlorosilane bonded to a pellicular packing[2] [0.6 m × 0.3 cm (2 ft × 0.125 in.) o.d.].

Chromatographic Conditions—The chromatographic solvent was methanol–water (35:65 v/v). The temperature was ambient, and the solvent flow was approximately 0.4 ml/min (at an inlet pressure of 200 psig). The precision photometer detector (254 nm) was set at a sensitivity of 0.08 absorbance unit full-scale. The injection volume was 5 μl, and the chart speed was 0.5 cm (0.2 in.)/min.

Materials and Reagents—The chromatographic internal standard was quinoline[3]. Polythiazide reference material[4] was used without further purification. The methanol was ACS grade. All other reagents and solvents were of the best grade commercially available and were used without further purification.

Solutions—*Internal Standard Solution*—The quinoline (230 mg) was dissolved in methanol and diluted with methanol to a final volume of 1000 ml.

Polythiazide Standard Solution—Approximately 20 mg of polythiazide reference material was weighed accurately into a 100-ml volumetric flask. The polythiazide was dissolved in the internal standard solution and diluted to 100 ml with this solution.

Preparation of Standard Curve—Two, four, six, and eight milliliters of the polythiazide standard solution were accurately transferred into four 10-ml volumetric flasks and diluted to volume with the internal standard solution. Three injections (5 μl) of each of these solutions and of the undiluted polythiazide standard solution were made into the chromatographic system. The heights of the peaks were measured, and the ratios of the peak height of the polythiazide to that of the internal standard were calculated for each injection. The average peak height ratio for each dilution was plotted against the quantity of polythiazide in the solution.

HPLC Analysis of Polythiazide in Tablets—Twenty tablets were weighed and finely powdered. An accurately weighed portion, equivalent to one tablet weight, was transferred to a 30-ml glass-stoppered centrifuge tube. Ten milliliters of the internal standard solution was added, and the sample was shaken[5] mechanically for 30 min (for the 2-mg tablets, 20 ml of internal standard solution was added). The sample was then centrifuged[6] at 1500 rpm for 15 min, and three 5-μl injections of the clear supernate were made into the chromatographic system. The ratios of the heights of the peaks were calculated for each injection and then averaged. The quantity of polythiazide in the sample then was determined graphically.

NF XIII Analysis of Polythiazide in Tablets—The three different samples of polythiazide tablets were assayed following the compendial procedure (1).

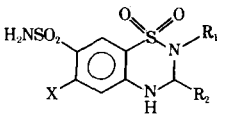
Colorimetric Analysis of Polythiazide in Tablets—The BP 1973 assay for bendrofluazide[7] tablets (7) was adapted for this determination, the method being essentially that described under content uniformity for polythiazide tablets in NF XIII (1).

RESULTS AND DISCUSSION

Column and Mobile Phase Selection—Preliminary experiments indicated that qualitative separation of many thiazide as well as nonthiazide diuretics could be obtained on the diphenyldichlorosilane-bonded column[2], using methanol–water as the mobile phase. The retention times for 12 thiazide and 10 nonthiazide diuretics under the chromatographic conditions described in the *Experimental* section are given in Tables I and II, respectively.

[1] Waters ALC 202/401.
[2] Bondapak phenyl/Corasil, Waters Catalog No. 27283.
[3] Reagent grade, bp 235–237°, Fisher Scientific Co.
[4] Donated by Pfizer Co. Ltd., Montreal, Canada.
[5] Eberbach two-speed utility shaker (set at low speed), Canlab Catalog No. S1105.
[6] International centrifuge, model CS, International Equipment Co.
[7] British Approved Name (BAN) for bendroflumethiazide.

Table I—Chromatographic Behavior[a] of Some Thiazide Diuretics



Compound	X	R_1	R_2	Retention Time, min
Hydrochlorothiazide	Cl	H	H	3.5
Hydroflumethiazide	CF_3	H	H	3.3
Chlorothiazide	Cl	H	($\Delta^{3,4}$)	3.1
Flumethiazide	CF_3	—	($\Delta^{2,3}$)	2.7
Trichlormethiazide	Cl	H	$CHCl_2$	3.5
Methyclothiazide	Cl	CH_3	CH_2Cl	4.3
Cyclopenthiazide	Cl	H		7.5
Cyclothiazide	Cl	H		6.7
Althiazide	Cl	H	$CH_2SCH_2CH{=}CH_2$	5.0
Bendroflumethiazide	CF_3	H	$CH_2C_6H_5$	6.7
Polythiazide	Cl	CH_3	$CH_2SCH_2CF_3$	7.0
Benzthiazide	Cl	H	$CH_2SCH_2C_6H_5(\Delta^{3,4})$	4.9

[a] Chromatographic conditions as described under *Experimental.*

These retention times could be varied considerably by changing the proportions of methanol and water in the mobile phase. Very little resolution was obtained using a similar type of column bonded with octadecyltrichlorosilane[8]. Honigberg *et al.* (8) found that some antihistaminic, antitussive, and analgesic drugs were separated better on the phenyl[2] than on the C_{18}[8] column.

In general, sharp symmetrical peaks were obtained, the only exception being triamterene, which gave a relatively broad peak after a comparatively long retention time. The selection of 35% methanol in water as the mobile phase for this work was dictated by the presence of the interfering excipient vanillin in the commercially available tablet formulations. Maximum separation of the polythiazide and vanillin peaks was obtained by employing this ratio of solvents. Under these chromatographic conditions, vanillin showed a retention time of 3.9 min compared to 7.0 min for polythiazide.

Internal Standard—To minimize injection and apparatus errors, a study was undertaken to find a suitable internal standard. Many compounds in Tables I and II with short retention times could have been selected. For example, by using acetazolamide (3.3 min) as the internal standard, a highly satisfactory quantitative relationship was obtained when analyzing pure polythiazide. However, since vanillin is extracted along with the polythiazide from the powdered tablets and since it is eluted after 3.9 min, a significant overlap of peaks occurs.

After screening a large number of compounds, quinoline (retention time 10.0 min) was found to be an excellent internal standard.

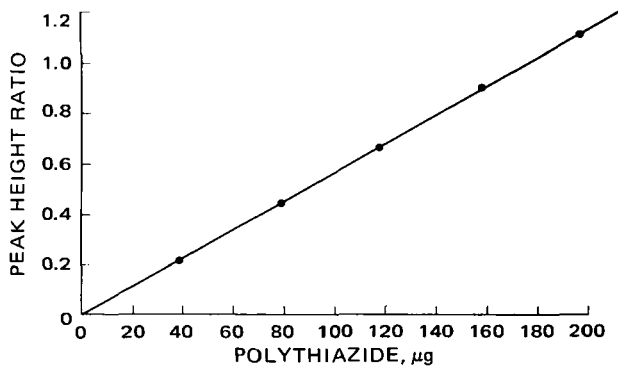


Figure 1—*Standard curve for polythiazide with quinoline as the internal standard.*

[8] Bondapak C_{18}/Corasil, Waters Catalog No. 98159.

Isoquinoline and carbazole, with retention times similar to quinoline, could have been employed, but their peaks were not as well developed.

Response and Linearity—The response *versus* concentration curve for polythiazide demonstrated that sample concentration was linear with peak height and that elution times were reproducible. Peak height rather than peak area was chosen as the method of measurement because previous studies (9, 10) had shown that peak height varies less with fluctuations in inlet pressure (flow rate) than does peak area.

Five different concentrations of polythiazide, ranging from 40 to 200 μg/ml, were injected. Figure 1 shows the linear relationship resulting from plotting varying concentrations of polythiazide against the ratios of the heights of the polythiazide peaks to those of the internal standard.

Analysis of Dosage Forms—Replicate assays were performed, as described under *Experimental,* on three different commercially available polythiazide preparations. Figure 2 illustrates a typical chromatogram obtained from each formulation. As is evident from the chromatograms, vanillin is separated sufficiently from polythiazide and does not interfere in the analysis. The 2-mg tablets (Product II) and the 1-mg tablets with reserpine (Product III) contain a second excipient which is extracted into the methanol, but it is also eluted well before the polythiazide and likewise shows no interference. Reserpine is present in a very low concentration in Product III; this fact, coupled with the limited solubility of reserpine in methanol, likely explains why it is not detected in the chromatogram and, accordingly, why it does not interfere with the analysis of polythiazide.

The assay results for three formulations are given in Table III.

Table II—Chromatographic Behavior[a] of Some Nonthiazide Diuretics

Compound	Retention Time, min
Acetazolamide	3.3
Amisometradine	3.9
Chlorthalidone	4.4
Clopamide	4.3
Dichlorphenamide	3.5
Ethacrynic Acid	3.1
Ethoxzolamide	6.9
Furosemide	3.1
Quinethazone	4.1
Triamterene	11.0[b]

[a] Chromatographic conditions as described under *Experimental.* [b] Very broad peak. Compound is only very slightly soluble in methanol.

Table III—Comparative Analyses of Three Commercially Available Polythiazide Tablet Preparations

Product	Components	Analyses, % of Labeled Claim			
		NF XIII	Colorimetric[a]	Manufacturer's Data	Proposed Method (HPLC)[a]
I[b]	Polythiazide, 1 mg	113.2	106.4	105.0[c]	110.2 ± 0.4
II[b]	Polythiazide, 2 mg	113.0	101.8	105.0[c]	108.1 ± 0.7
III[d]	Polythiazide, 1 mg Reserpine, 0.25 mg	—[e]	—[e]	105.5[f]	112.0 ± 0.6

[a] Average of four determinations. [b] Renese, Pfizer Co. Ltd. [c] TLC method. [d] Renese-R, Pfizer Co. Ltd. [e] Erratic results, reserpine interferes. [f] Spectrophotometric method.

The values obtained by the NF XIII procedure and by a colorimetric method are also given, along with the values received from the manufacturer for these lot numbers; the proposed method gives results that are slightly lower than the NF XIII procedure but somewhat higher than those found by the manufacturer. The standard deviations are quite comparable to those normally obtained with other related analytical techniques.

This procedure was repeated for Product I, using 10 ml of the internal standard solution containing 0.20 mg of added polythiazide reference material. The absolute recovery of the added polythiazide was calculated to be 0.212 ± 0.003 mg based on four replicate determinations.

CONCLUSION

HPLC provides a convenient and efficient method for the quantitative determination of polythiazide in its dosage forms. There was no interference from excipients in the products examined, so no additional extraction or separation procedures are required. The method is rapid and sensitive enough to be used for single-tablet assays.

Although the detailed chromatographic conditions were not explored for the quantitative determination of the other thiazide and nonthiazide diuretics, this method may provide a suitable starting point for the estimation of these diuretics in dosage forms and, possibly, in biological fluids.

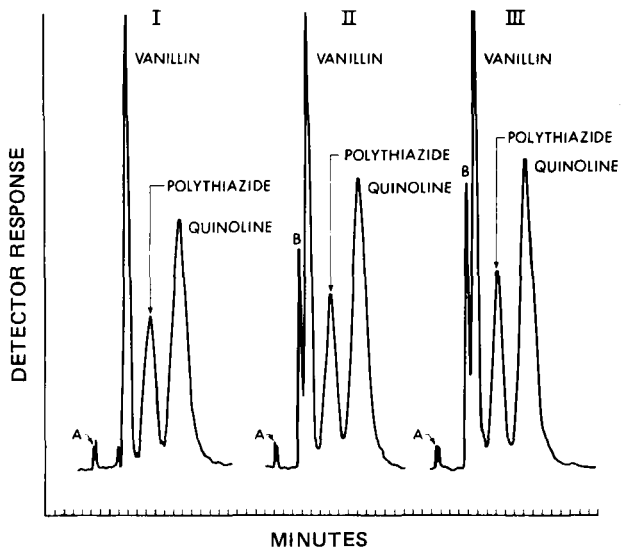


Figure 2—*Typical chromatograms of polythiazide dosage forms. Key: I, polythiazide 1-mg tablets; II, polythiazide 2-mg tablets; III, tablets containing 1 mg of polythiazide and 0.25 mg of reserpine; A, sample injection; and B, unknown excipient. Liquid chromatographic conditions are described under* Experimental.

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Sieve Cuts as Monodisperse Powders in Dissolution Studies

DANA BROOKE

Abstract □ *Dissolution profiles are calculated for sieve cuts of varying width based on a derived equation, which assumes diffusion rate-limited dissolution under sink conditions from spherical particles. It is shown that even the narrowest of sieve cuts may vary from cube root law expectations by as much as 3%, depending on the distribution of particles across the cut. Much smaller deviations from the cube root law occur when the weight distribution of diameters is constant across a sieve cut. It is concluded that when experimental data are treated on the assumption that sieve cuts act like monosized powders, errors generally will be acceptable except in cases of critical tests of dissolution rate theory. In those cases, more information about the powder distribution should be given.*

Keyphrases □ Sieve cuts—dissolution studies, assumption of monodisperse powders □ Dissolution—error introduced from assumption of monodisperse powders in sieve cuts □ Particle size—sieve cuts, monodisperse powders from sieve cuts, introduction of error □ Powder distribution—sieve cuts, assumption of monodisperse powders, introduction of error in dissolution testing

Sieve cuts from powders have often been used as substrates in dissolution experiments. The idea that a narrow distribution of particle sizes can be treated as a monodisperse distribution seems to be fairly well established (1). Some workers have staked quantitative treatments of experimental data on the correctness of the assumption that sieve cuts from powders can be treated like monosized powders.

For example, Niebergall and Goyan (2) calculated dissolution rate constants from data on 20–30-mesh salicylamide and 80–100-mesh benzoic acid by extension of the Hixson–Crowell (3) cube root law. In another paper, Niebergall *et al.* (4) suggested that particles under rapid agitation followed a square root law on the basis of the dissolution behaviors of sieve cuts from various powders. Later, Goyan (5) used some of their data (4) to support a Danckwerts' penetration model for dissolution of particles under rapid agitation. Recently, Pothisiri and Carstensen (6) tested an equation for the nonsink dissolution of monosized particles against experimental data. They used data from their own laboratory and from Hussain (7) on the dissolution of narrow cuts from powders.

Since dissolution rate theory is being proposed or tested on the basis of experimentation with sieve cuts from powders, it seemed worthwhile to examine the validity of the assumption that sieve cuts behave like monodisperse powders. This paper does not consider errors that might arise in dissolution studies from other popular assumptions; for example, when the hydrodynamics of the experimental system do not conform to theory, when dissolution is anisotropic, when particles are not spherical, or when particles agglomerate. This paper seeks to define the limits on errors that might occur in the treatment of dissolution data solely because the particles within a sieve cut are not all the same size.

Dissolution profiles are calculated for narrow cuts of spherical particles wherein the probability frequency, on a weight basis, of diameters is some linear function of particle size. These profiles are compared with that expected for a monodisperse powder in which the diameter is an average of the largest and smallest diameters possible in the sieve cut. Diffusion rate-limited dissolution under sink conditions is assumed.

As will be seen, the assumption that a sieve cut behaves like a monosized distribution should lead to no more than about 3% error, as reflected in a cube root law slope, if the narrowest sieve cuts are used. Wider cuts lead to greater inherent errors. If the frequency of occurrence of diameters on a weight basis is fairly constant across the cut, errors will tend to be much smaller than if the frequency changes sharply, as

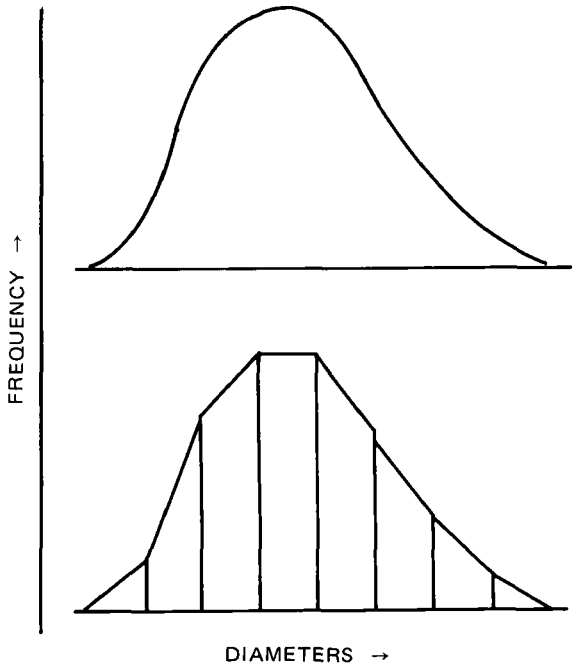

Figure 1—*Approximation of a continuous powder distribution as a series of sieve cuts.*

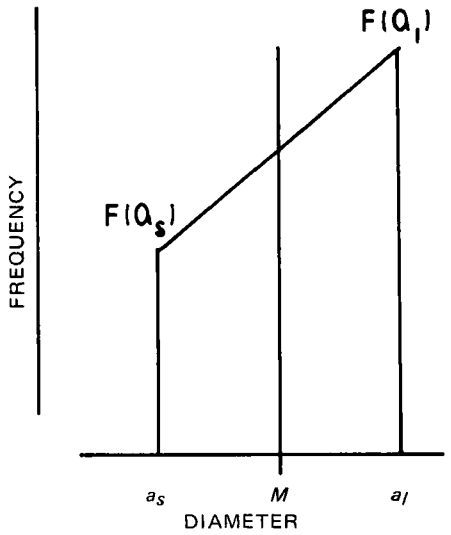

Figure 2—*Representation of a sieve cut from a powder as a separate distribution in which the probability frequency is taken as a linear function of particle size. See text.*

might be the case in a cut from near the tail of a powder.

THEORY AND CALCULATIONS

Consider a powder containing spherical particles of diameters a_0, which are distributed, on a weight basis, according to a continuous probability frequency $f(a_0)$. Let there be a series of cuts approximating the distribution (Fig. 1), and assume that $f(a_0)$ is a linear function of a_0 in each cut (Fig. 2). Once a cut is segregated from the parent distribution, it must be considered a separate distribution with characteristics determined by the original powder.

If the cut is not too wide, it is not unreasonable to consider $f(a_0)$ linear with a_0. However, the derivations of dissolution profiles do not depend on this simplification, since derivations based on considerably more complicated distributions are possible (8, 9). The assumption of linearity does allow a much more general treatment of dissolution profiles than might otherwise be possible.

In Fig. 2, the smallest and largest possible diameters (a_s and a_l, respectively) are averaged to gain a mean diameter M. It is convenient to express[1] a_s as sM and a_l as lM where $s + l = 2$. The probability frequency $f(a_l)$ of particles of diameter a_l is conveniently expressed in terms of $f(a_s)$ by $f(a_l) = Rf(a_s)$. The probability frequency $f(a_0)$ of diameters a_0 can now be derived in terms of a_0, R, s, l, and M based on the recognition that the slope of the linear function $f(a_0)$ is:

$$\text{slope} = \frac{Rf(a_s) - f(a_s)}{lM - sM} \qquad \text{(Eq. 1)}$$

and that the area under the curve is unity so that:

$$\text{area} = 1.0 = (lM - sM)f(a_s) + \frac{1}{2}(lM - sM)[Rf(a_s) - f(a_s)] \qquad \text{(Eq. 2)}$$

Thus, the probability frequency of particle sizes on a weight basis is:

$$f(a_0) = \frac{2[l - Rs + (R - 1)a_0/M]}{(R + 1)(l - s)^2M} \qquad \text{(Eq. 3)}$$

The contribution dw_0 of particles of size a_0 to the total initial weight w_0 can be expressed as:

$$dw_0 = w_0 f(a_0)da_0 \qquad \text{(Eq. 4)}$$

The number dn of particles of size a_0 can be calculated by dividing dw_0 by the weight of a single particle $\pi p a_0^3/6$, where p is particle density:

$$dn = \frac{w_0 f(a_0)da_0}{\pi p a_0^3/6} \qquad \text{(Eq. 5)}$$

Dissolution profiles for sieve cuts can now be derived using the same philosophical approach used in deriving an expression for the dissolution of powders that follow a log-normal distribution (9, 10).

The diameter a_τ of a particle exposed to a medium for some function τ of time can be expressed by:

$$a_\tau = a_0 - \tau \qquad \text{(Eq. 6)}$$

where τ is $2kC_st/p$ and k is a dissolution rate constant, C_s is solubility, and t is time. This notation is essentially that of Carstensen and Musa (11). The equation is based on assumptions of diffusion rate-limited dissolution under sink conditions, isotropic behavior, and a particle-size-independent solubility.

As long as particles that were initially of size a_0 exist (that is, if $\tau < a_0$), the particles will grow smaller but the number of those particles dn will not change. Therefore, the contribution dw_τ to the weight undissolved of particles of size a_τ can be calculated by:

$$dw_\tau = \frac{\pi p a_\tau^3}{6}dn = \frac{\pi p}{6}(a_0 - \tau)^3 dn \qquad \text{(Eq. 7)}$$

Substitution for dn using Eq. 5 and for $f(a_0)$ using Eq. 3 completes the derivation, except for an integration, to achieve w_τ. As far as the integration is concerned, τ is a constant. The integration is over all of the initial particle sizes a_0 of particles remaining because dn is based on initial sizes a_0. Thus, for $\tau \le a_s = sM$, the integration is from $a_s = sM$ to $a_l = lM$. After the smallest particles begin to disappear, the integration is from τ to a_l. That is, after $\tau > a_s$, the smallest nonzero particle remaining at τ was initially of size a_0 such that $a_0 - \tau \to 0$. Thus, it can be shown that the weight fraction undissolved (w_τ/w_0) after some τ is:

$$w_\tau/w_0 = [2/(1 + R)(l - s)^2] \times \left[\begin{array}{l}(l - Rs)(l - t) + (R - 1)(l^2 - t^2)/2 \\ - 3[(R - 1)(l - t) + (l - Rs)\ln(l/t)]\tau/M \\ + 3[(R - 1)\ln(l/t) + (l - Rs)(l - t)/lt]\tau^2/M^2 \\ - [(R - 1)(l - t)/lt + (l - Rs)(l^2 - t^2)/2l^2t^2]\tau^3/M^3\end{array}\right] \qquad \text{(Eq. 8)}$$

where $t = s$ when $\tau \le sM$ and $t = \tau/M$ when $\tau > sM$.

Dissolution profiles for sieve cuts like those shown in Fig. 3 (R =

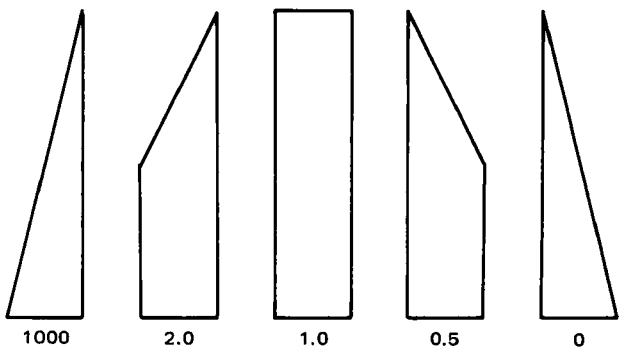

Figure 3—*Representation of various narrow powder distributions for which dissolution profiles are calculated. The plots are similar to the one in Fig. 2, and values for* R *are indicated. See text for additional details.*

[1] It should be obvious that $2 \ge l > s \ge 0$ and that Eq. 1 and others in these derivations are undefined for $s = l$. It further should be recognized that since $2 - l = s$, it is not strictly necessary to use both parameters in all equations.

Table I—Dissolution Profiles[a] for a Monodisperse Powder and for Powders from Sieve Cuts

τ/M	Monodisperse Powder	s = 0.91					s = 0.83					s = 0.75				
		R = 0	0.5	1.0	2.0	1000	0	0.5	1.0	2.0	1000	0	0.5	1.0	2.0	1000
0	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
0.05	85.74	85.29	85.57	85.70	85.84	86.11	84.83	85.35	85.61	85.87	86.39	84.29	85.07	85.46	85.85	86.63
0.10	72.90	72.11	72.60	72.84	73.09	73.57	71.29	72.22	72.69	73.15	74.08	70.35	71.74	72.44	73.13	74.52
0.15	61.41	60.36	61.01	61.34	61.67	62.32	59.29	60.53	61.15	61.77	63.01	58.06	59.92	60.84	61.77	63.61
0.20	51.20	49.97	50.74	51.12	51.51	52.27	48.72	50.19	50.92	51.65	53.11	47.33	49.50	50.59	51.68	53.85
0.25	42.19	40.84	41.69	42.11	42.53	43.38	39.51	41.11	41.92	42.72	44.32	38.03	40.41	41.60	42.79	45.15
0.30	34.30	32.91	33.79	34.23	34.67	35.55	31.55	33.22	34.06	34.89	36.56	30.08	32.55	33.78	35.01	37.47
0.40	21.60	20.27	21.13	21.56	21.99	22.85	19.03	20.65	21.47	22.28	23.90	17.75	20.13	21.32	22.51	24.88
0.50	12.50	11.38	12.13	12.50	12.87	13.62	10.41	11.81	12.51	13.21	14.60	9.49	11.52	12.53	13.54	15.57
0.60	6.40	5.58	6.15	6.44	6.73	7.30	4.95	6.01	6.54	7.08	8.14	4.44	5.96	6.73	7.49	9.01
0.70	2.70	2.21	2.58	2.77	2.96	3.33	1.91	2.60	2.94	3.29	3.98	1.75	2.74	3.23	3.72	4.70
0.80	0.80	0.59	0.78	0.88	0.97	1.16	0.54	0.89	1.07	1.25	1.60	0.57	1.09	1.35	1.61	2.12
β	1.0000	1.0328	1.0126	1.0026	0.9925	0.9725	1.0671	1.0285	1.0093	0.9901	0.9519	1.1073	1.0493	1.0204	0.9917	0.9346

[a] The values in the body of the table were calculated from Eq. 8 or 9 and are $(w_\tau/w_0) \times 100$.

Table II—Sieve Cuts Using Standard Sieves Listed as a Function of s

s = 0.75	s = 0.83	s = 0.91
40/60	20/30	50/60
50/80	30/40	60/70
60/100	40/50	70/80
70/120	50/70	80/100
120/200	60/80	100/120
200/325	70/100	200/230
230/400	80/120	230/270
	200/270	270/325
	230/325	325/400
	270/400	

0, 0.5, 1.0, 2.0, and 1000) were calculated using a computer[2]. Weight fractions undissolved (w_τ/w_0) are given in Table I as a function of τ/M. The choice of a relative time value (τ/M) allows direct comparisons of all dissolution profiles. Also presented in Table I is a dissolution profile for a monosized powder of diameter M, *i.e.*, a powder that exactly follows the cube root law. This profile was calculated using Eq. 9, where the slope β is chosen to be unity (10):

$$(w_\tau/w_0)^{1/3} = 1 - \beta(\tau/M) \qquad \text{(Eq. 9)}$$

The initial slope β for each calculated profile is also given in Table I based on Eq. 9.

The values for s were chosen to be 0.75, 0.83, and 0.91 because these values represent the three narrowest sieve cuts possible when using standard sieves as described in USP XVIII (12). Table II relates these values of s to certain possible sieve cuts.

DISCUSSION

A brief comparison of the dissolution profile for a powder that follows the cube root law, with profiles calculated for various sieve cuts (Table I), will indicate that it is probably acceptable to treat sieve cuts as monosized powders for all but the most critical work. The data also show that the approximation is better for narrower sieve cuts.

The errors that might occur when the approximation is applied to sieve cuts where s = 0.91 are shown graphically in Fig. 4. For distributions that might come from the large or small particle tail of a powder (R = 0 or 1000), the errors are greatest and exceed 10% as the weight fraction remaining falls below 10%. The characteristics of the sieve cut, as reflected by R, influence both the size of the errors expected and their direction (sign). It is clear from Fig. 4 that when the probability frequency is constant (R = 1.0), the dissolution profile for a sieve cut approximates that of a monosized powder excellently. When R = 1.0, the error through 90% dissolution is generally less than 0.2% for the narrowest sieve cut (s = 0.91). A graph of errors when sieve cuts with s = 0.83 are compared to a monodisperse powder would look identical to Fig. 4, except that all errors would be twice as large as those in Fig. 4.

Another way of judging the degree to which a sieve cut approximates the cube root law would be to examine the values for the slope (β) when the data are fitted to Eq. 9. In the ideal case, β will be unity. When the slopes were calculated on the initial portion of the profiles for cuts where s = 0.91, the calculated values ranged from 0.9725 to 1.0328. When all points in the profiles were subjected to least-squares regression, the values for β ranged from 0.9680 to 1.0252.

The exact value calculated for the slope depends on the number of data points and the extent to which dissolution is followed, because all cube root plots based on dissolution of sieve cuts will show some curvature. In some cases the curvature may be too small to identify because of random experimental errors. Nevertheless, it is clear that cuts from the large particle tail of a powder (R = 0) will tend to dissolve faster than might be expected while a cut from the small particle end (R = 1000) will dissolve more slowly than expected.

It can be said that dissolution data based on the narrowest pos-

[2] IBM 1800.

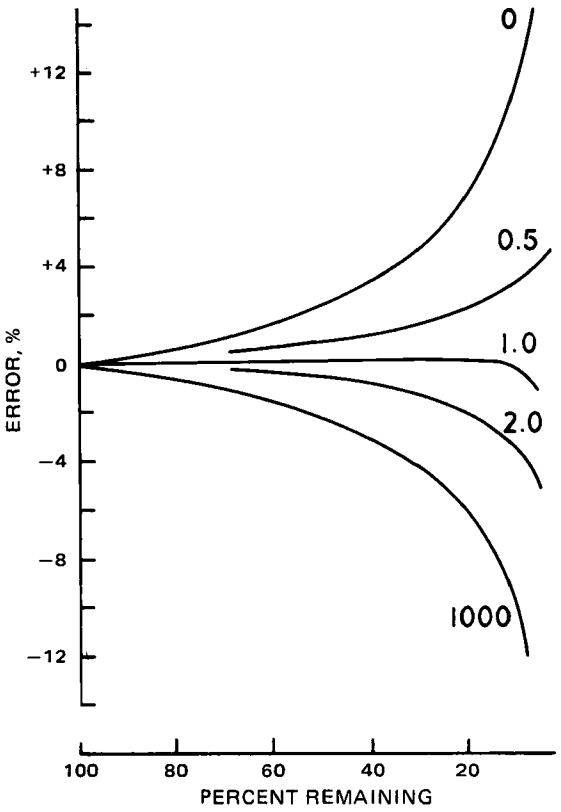

Figure 4—*Deviations between dissolution profiles for an ideal (monodisperse) powder and sieve cuts of* R = *0, 0.5, 1.0, 2.0, or 1000 as a percent of powder undissolved plotted against percent undissolved for sieve cuts having* s = *0.91.*

sible sieve cut (s = 0.91) might contain up to 3.0% error if the treatment of results included the assumption that sieve cuts are equivalent to monosized powders. If, however, sufficient particle-size data are presented with the dissolution data to show that R is close to or equal to 1.0, then the errors could be considered much less than 1.0%. For the next widest sieve cut (s = 0.83), the errors in a cube root law slope could be as great as 6.0%. Errors of 7–10% would be possible if wider sieve cuts (s = 0.75) were treated according to the cube root law. If, on the other hand, the particle sizes within a sieve cut could be described, then an exact equation like Eq. 8 might be useful.

It is concluded that when dissolution data are based on sieve cuts with no further definition of powder sizes and the assumption of a monosized powder is used in the data treatment, then the results may be in error by as much as 3% even for the narrowest sieve cuts. For wider sieve cuts, errors of 6 or even 10% should not be ruled out. These errors would occur solely from the assumption that sieve cuts behave like monosized powders.

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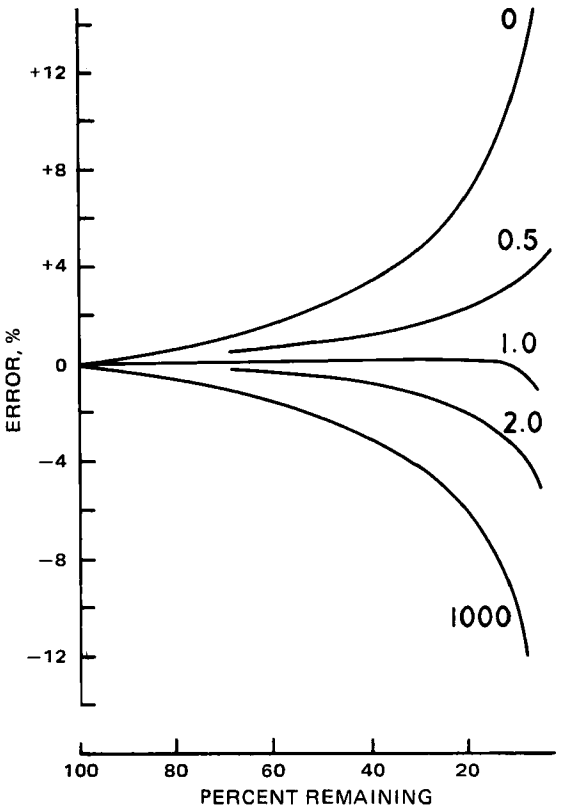

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Table I—Comparison of Methods by Lab-1

Sample	Ethylene Oxide, ppm			
	Head Space	Vacuum	Acetone	Gravimetric
1a	6310	7410	7900	7520
b	6980	5780	8860	8220
c	7330	10900	7450	8870
d	7290	12700	7560	8270
e	6280	7940	7300	7620
f	7230	8740	7140	8490
2a	1240	1930	1640	1800
b	495	818	486	1000
c	520	632	828	570
d	491	601	644	600
e	1860	2280	2000	1700
f	623	703	571	570
3a	488	537	650	530
b	202	217	271	290
c	171	234	382	310
d	472	622	701	210
e	69.1	75.9	140	180
f	728	759	800	1200
4a	40.0	51.5	62.1	ND[a]
b	55.6	63.2	89.1	ND
c	37.1	49.3	64.1	ND
d	81.3	139	137	ND
e	87.0	120	120	ND
f	63.7	76.4	77.9	ND
5a	6.5	7.12	13.0	ND
b	3.61	5.28	8.4	ND
c	5.23	6.45	10.0	ND
d	5.55	8.22	13.5	ND
e	4.41	5.40	8.8	ND
f	4.57	6.09	10.0	ND

[a] ND = not detected.

Table II—Comparison of Methods by Lab-2

Sample	Ethylene Oxide, ppm			
	Head Space	Vacuum	Acetone	Gravimetric
1a	2950	3500	2040	7890
b	2950	46	978	9530
c	3410	839	1154	8180
d	1470	1520	1890	9690
e	5270	1.5	4515	6210
f	2880	475	537	7470
2a	494	ND[a]	408	15900
b	282	4	238	5420
c	631	793	414	5900
d	552	ND	611	40600
e	580	368	442	5880
f	471	ND	658	4290
3a	528	ND	631	
b	232	106	149	4820
c	313	56	439	2150
d	141	101	124	4040
e	258	24	146	4610
f	105	77	126	2520
4a	40	32	31	1080
b	46	23	47	50700
c	44	40	27	—
d	19	11	30	470
e	22	22	57	1560
f	39	25	34	
5a	4.5	1.6	3.3	1910
b	4.8	ND	4.0	4420
c	5.2	2.6	3.2	4840
d	4.0	1.2	2.7	3440
e	4.6	4.3	3.9	4200
f	4.6	0.5	4.2	3100

[a] ND = not detected.

ethylene oxide. Late in 1972, an ad hoc committee was formed within Z79 made up of industrial, regulatory, and academic representatives. One charge to this committee was to establish uniform analytical test procedures for ethylene oxide, ethylene glycol, and 2-chloroethanol in plastic materials. The present report deals with the first part of this objective: the evaluation of methods for residual ethylene oxide analysis.

Methods for residual ethylene oxide analysis fall into four categories: colorimetric (1), titrimetric (2), gravimetric (3), and GC (4–12). A round-robin study was designed by the ad hoc committee to evaluate four of these methods. The major objective of this round robin was not to point out a single method to be used by the entire industry but rather to point out which method or methods could be used to check the validity of an in-house method. Six laboratories participated[1].

EXPERIMENTAL

Methods—Four methods of analysis were studied: a gravimetric method (3) and three GC approaches (9–12). The main difference between the GC methods is sample workup. The vacuum extraction technique (10) vaporizes the ethylene oxide and traps it in a cold coil before injection into the gas chromatograph. The head-space method (11) extracts the ethylene oxide by vaporizing it into the head space of an enclosed vial. An aliquot of the head-space gas is injected into the gas chromatograph. The vial is purged with nitrogen, and the process is repeated until all of the ethylene oxide is extracted from the sample.

Both the vacuum extraction and the head-space methods exhaustively extract the ethylene oxide. In the acetone extraction technique (9, 12), the ethylene oxide in the sample partitions between the sample and the acetone. An aliquot of the acetone solution is injected into the gas chromatograph. This extraction is not generally considered to be exhaustive unless the sample dissolves in the acetone.

Materials—In some cases, analytical results depend on the material being analyzed; *i.e.*, not all of the ethylene oxide may be extracted into acetone due to a highly polar sample. The ad hoc committee discussed this matter at length. The final decision was to use polyvinyl chloride tubing[2]. Specifically, the material was 0.3 cm (0.125 in.) i.d. × 0.6 cm (0.25 in.) o.d. [0.15-cm (0.06-in.) wall]. The material was to contain approximately 5000, 1000, 500, 100, or 10 ppm of ethylene oxide.

The tubing was cut into 2-cm lengths and then into quarters along the length. These quarters weighed about 0.25 g. They were placed into a small glassine envelope and sterilized with pure ethylene oxide gas in a small laboratory sterilizer[3]. At predetermined times during degassing, samples were removed from the sterilizer and the contents of each envelope were placed into 7-ml vials with aluminum-lined septa caps. The vials were then stored in dry ice.

All samples were prepared at one location. Each laboratory received enough material to perform six replicate analyses at each level of ethylene oxide. The samples were shipped by air in insulated dry-ice chests and were received within 24 hr. Samples stored in this manner were shown to be stable for at least 18 days.

The laboratories were instructed to analyze the samples immediately upon opening a vial. All laboratories handled the samples in the same manner as follows. A vial was removed from the dry-ice chest, allowed to thaw for 5 min, and opened. One piece was weighed immediately for the gravimetric method. A second piece was placed in a tared vial of acetone for the acetone extraction method. Another was placed into a tared empty vial for head-space analysis. The final piece was weighed into the stoppered vacuum extraction flask.

The head-space and vacuum extraction methods were run concurrently, followed by the acetone extraction method. The gravimetric samples were stored in an open, dust-free area and weighed

[1] The laboratories and investigators were: Castle Co., Mr. J. Whitbourne and Miss Carolyn Eastman; 3M Co., Mr. G. E. Schwagerl and Mr. W. Peterson; Johnson & Johnson, Mr. A. Heutteman, Dr. G. Kaplan, and Mr. J. Weinberger; Ethicon Inc., Dr. S. J. Romano, Mr. J. A. Renner, Mr. P. J. Rafferty, and Mr. R. McGunnigle; Huntingdon Research Center, Dr. W. F. Stephen, Mr. M. Malik, Mr. R. O'Melia, and Mr. J. Morris; and C. R. Bard Inc., Mr. W. Greif.

[2] Tygon S-50-HL, Norton, Akron, Ohio.

[3] A modified Amsco Cryotherm unit.

Table III—Comparison of Methods by Lab-3

Sample	Ethylene Oxide, ppm			
	Head Space	Vacuum	Acetone	Gravimetric
1a	3732	7433	7801	10022
b	2451	3192	5003	7757
c	3593	5621	7904	12767
d	3570	—	10170	13341
e	2032	3352	5643	4464
f	3763	4604	9416	14895
2a	2421	1204	757	4236
b	2952	1204	1052	3840
c	454	1695	1225	2656
d	3476	1342	892	5219
e	460	1201	967	3328
3a	97	1011	513	2910
b	160	1053	356	2358
c	35	592	240	4459
4a	NR[a]	NR	789	3670
b	NR	NR	83	1702
c	143	349	127	NR
5a	NR	NR	58	3813
b	NR	NR	27	1835

[a] NR = no response.

to constant weight. All of these procedures were required to ensure the four pieces in a vial would be at the same level of ethylene oxide when analyzed. In this way a comparison of the four methods was possible. It was assumed that the vial-to-vial variability would be large since, in effect, these samples are different although at the same nominal level of ethylene oxide. A total of 4 weeks was allowed for the analyses.

RESULTS AND DISCUSSION

Tables I–VI show the raw data reported by the six laboratories. The laboratories are only identified by number, and the order of numbering does not correlate with the order in Footnote 1. Lab-6 only performed the head-space and acetone methods, and each reported result is the average of two determinations. Lab-3 did not perform the required six replicates at the lower levels of ethylene oxide.

Upon examining the data, it is obvious that the original hypothesis of a large laboratory-to-laboratory variability was correct, although all laboratories found the five ethylene oxide levels to be in the same order of magnitude for all methods. Another obvious fact is that the correlation between the GC methods and the gravimetric method breaks down below the highest levels of ethylene oxide studied.

Table IV—Comparison of Methods by Lab-4

Sample	Ethylene Oxide, ppm			
	Head Space	Vacuum	Acetone	Gravimetric
1a	3588	2285	5362	4455
b	3183	3459	5697	8876
c	4291	4525	5198	7789
d	2338	4469	2914	6760
e	1795	2887	3260	3572
f	2447	3238	4661	4930
2a	878	841	532	4756
b	606	1036	472	3594
c	388	558	346	1013
d	1663	1132	985	2823
e	520	446	382	1602
f	691	590	528	1402
3a	211	141	285	3355
b	115	214	114	2287
c	116	77	70	1410
d	147	187	140	1658
e	290	154	248	798
f	160	140	171	1368
4a	21	32	34	155
b	19	14	26	2205
c	15	18	18	1458
d	119	40	96	4301
e	57	24	50	544
f	22	6	255	2952
5a	16	4	81	1975
b	19	3	23	1251
c	9	3	44	1733
d	6	4	24	369
e	4	4	15	1664

Table V—Comparison of Methods by Lab-5

Sample	Ethylene Oxide, ppm			
	Head Space	Vacuum	Acetone	Gravimetric
1a	4431	5552	4661	6990
b	7273	7245	7144	9187
c	4499	5764	5246	6988
d	7052	7712	6945	9090
e	5978	7300	6330	6889
f	5521	6402	6098	2994
2a	709	810	886	969
b	1087	823	609	3479
c	1193	1342	1018	8155
d	859	998	1024	2977
e	1445	1426	1151	—
f	1083	1271	1109	7142
3a	354	125	156	7033
b	261	189	211	6298
c	574	441	583	8230
d	298	298	283	3659
e	323	339	387	1914
f	233	233	219	4762
4a	62	47	56	1875
b	124	97	102	1827
c	118	87	124	984
d	81	116	82	3157
e	77	52	45	
f	106	94	100	
5a	15.9	11.4	8.4	5976
b	14.6	7.3	10.8	—
c	13.3	7.7	10.8	1513
d	10.9	6.8	5.7	—
e	16.4	8.6	7.6	725
f	15.2	21	10.4	4483

Since most statistical treatments depend on equal replication and similar treatments, the data from Lab-6 could not be included in the statistical evaluations. For this same reason, only the two highest levels from Lab-3 were included. Since the noncorrelation of the gravimetric method is obvious, this method was not included in the statistical evaluation. These exclusions somewhat simplified the data, but a monumental statistical problem remained.

Three approaches were taken in an attempt to make some statistically significant statements concerning the data. The data averages were graphed, subjected to analysis of variance, and subjected to a nonparametric ranking treatment.

Graphing of Averages—An accepted method of testing data interactions is the graphing of the averages. The following assumptions were made: (*a*) comparison between methods can only be

Table VI—Comparison of Methods by Lab-6

Sample	Ethylene Oxide, ppm	
	Head Space	Acetone
1a	3860	3150
b	4902	4057
c	4096	3865
d	3865	3380
2a	648	841
b	438	442
c	465	411
d	412	415
3a	95	86
b	245	237
c	171	222
d	182	238
4a	28	31
b	60	70
c	57	59
d	49	57
5a	8	4
b	13	6
c	5	2
d	5	4

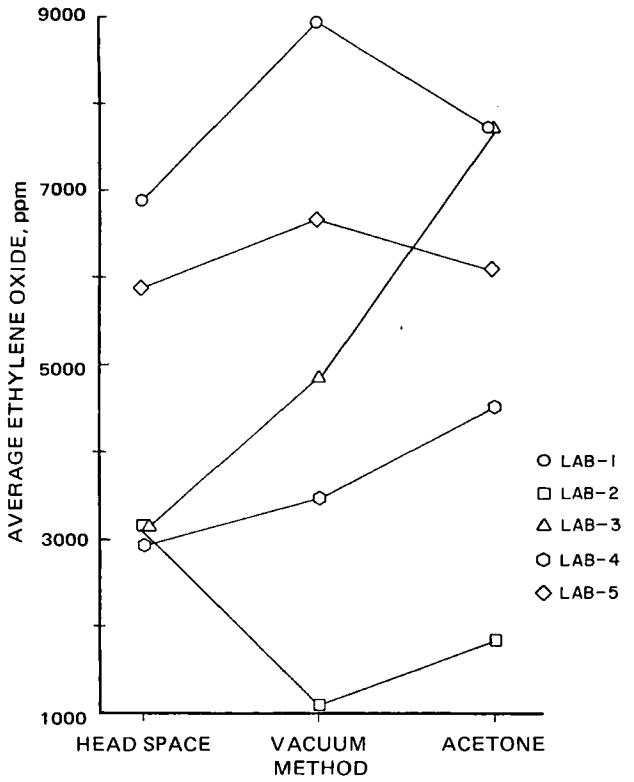

Figure 1—*Level 1 interaction.*

done within an ethylene oxide level, and (*b*) all samples within a level are identical or at least less variable than the variability between methods.

For each set of data, at each level, a method average was computed for each laboratory. These averages were then plotted *versus* the method on an arbitrary scale (Figs. 1–5). There were no obvious trends, *i.e.*, one method was not always low or high. What is obvious is that some laboratories were able to get consistently good correlations between methods while others were not.

The next step was to take the method average for each level of ethylene oxide, regardless of the laboratories, and plot these values *versus* method (Fig. 6). These data show that the methods gave similar results; however, the acetone extraction technique usually gave higher results than the other two methods. This result could be due to the loss of ethylene oxide in both the vacuum and headspace methods. Conversely, acetone impurities or other factors may cause high ethylene oxide results in the acetone method. The important point to note is that sample-to-sample and laboratory-to-laboratory variability was high, so only very general trends can be noted.

Analysis of Variance—Analysis of variance allows one to test statistically the significance of differences between sets of data by computing ratios of variance (*F* ratio test) and comparing these to critical values found in tables. The following qualifications were made in testing the data: (*a*) levels were tested individually, (*b*) all results reported as ND were assumed to be zero, and (*c*) where a data point was missing (only in a few cases), the method average at that level was substituted. With these qualifications, the data were coded and fed into a computer for processing. Table VII gives the results.

A significant difference among methods was found at the very high and very low levels of ethylene oxide. In the area where the industry generally works (1000–50 ppm), no significant difference was found among methods.

A significant difference was found among all laboratories at all levels. This result was expected since samples were "different" and the time of analysis was not the same. However, the possibility that the laboratories made errors in standardization cannot be ruled out. In this case, all results would be internally consistent but a significant difference in the results from the laboratories would occur.

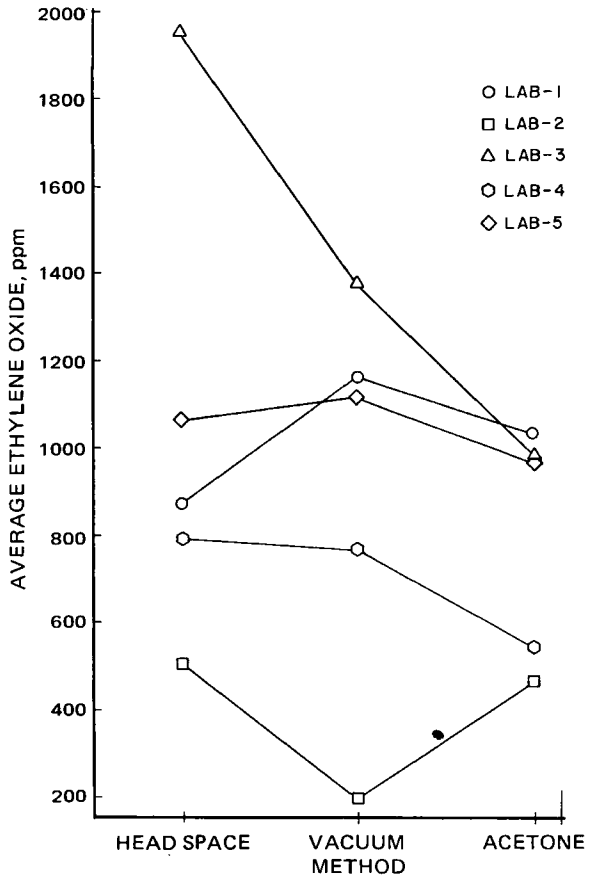

Figure 2—*Level 2 interaction.*

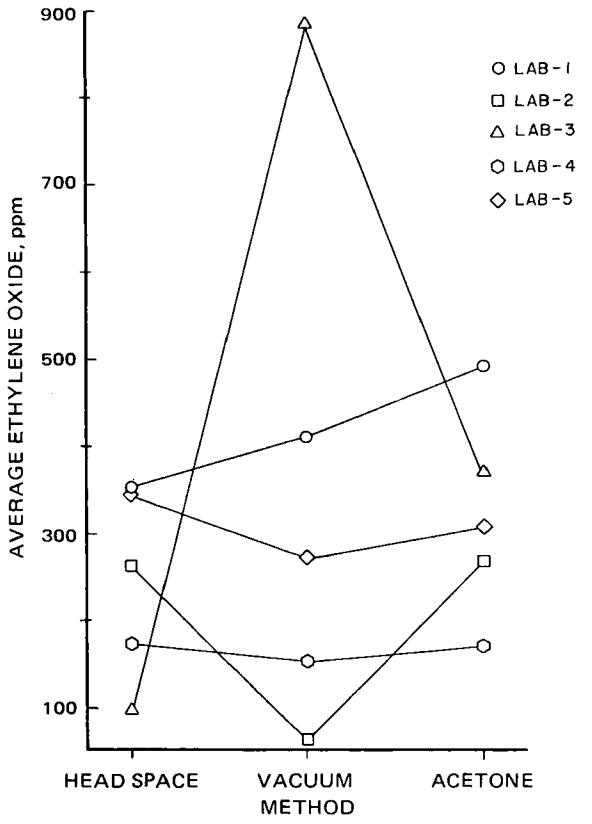

Figure 3—*Level 3 interaction.*

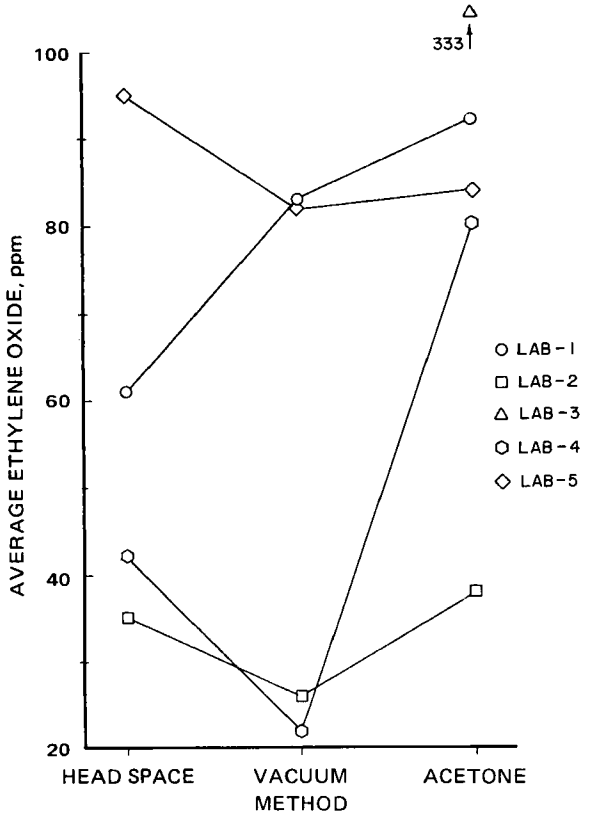

Figure 4—*Level 4 interaction.*

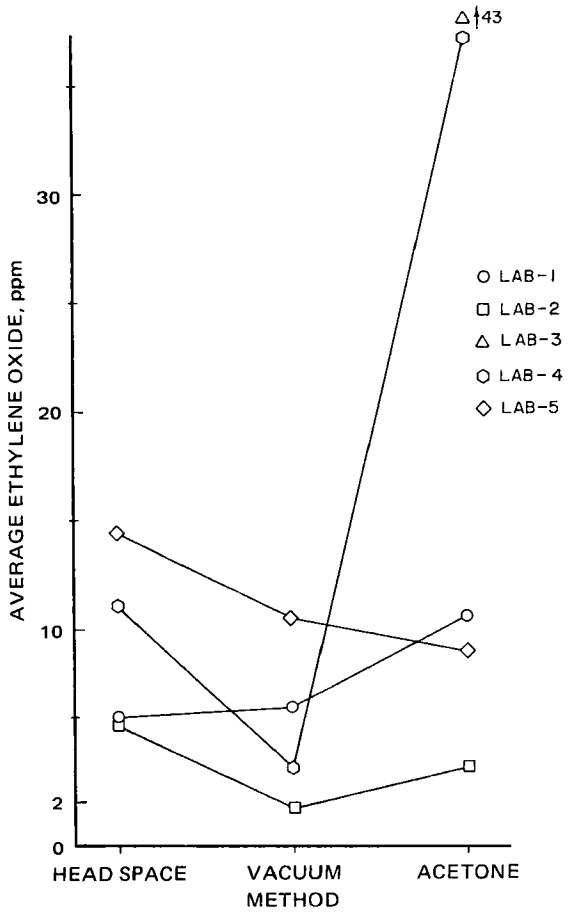

Figure 5—*Level 5 interaction.*

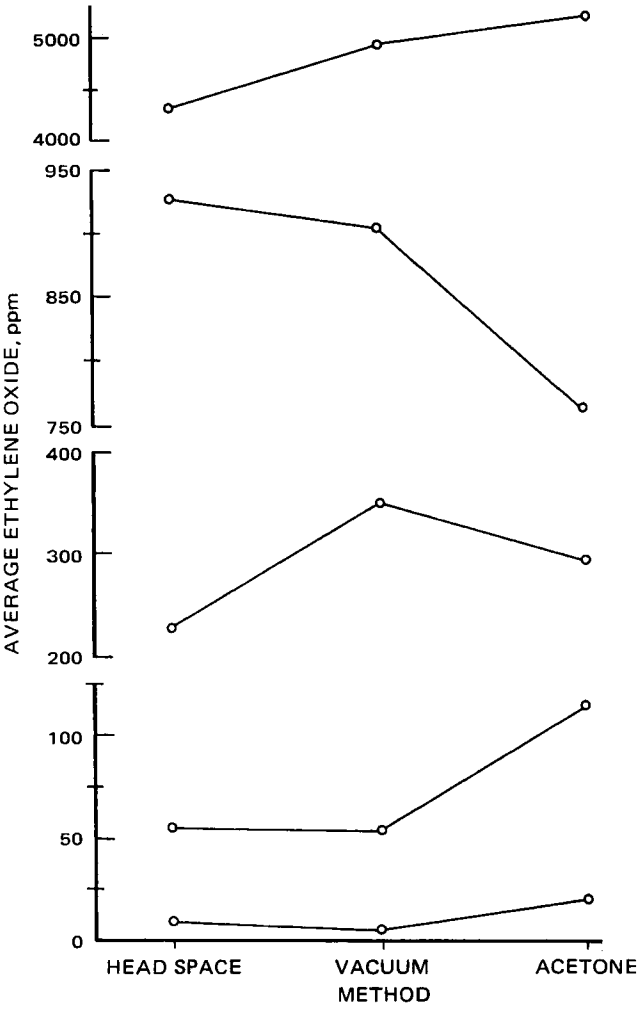

Figure 6—*Method average.*

No significant difference was found among the six replicates within a method in each individual laboratory. This finding is surprising, since there appears to be large differences among replicates. They are, after all, different samples taken from different parts of the sterilizer. This point was conceded at the start of the study, since the only samples thought to be the same are the four samples in the same vial used for the four methods. This data analysis, however, indicates that the differences among samples at a particular level are not significant. The overall interaction showed no significant differences among laboratories, replicates, and methods.

This data analysis indicates the three methods give comparable results and supports the claim that the methods are equivalent in the hands of an experienced operator.

Nonparametric Ranking—Ranking is a rapid statistical procedure based on the assignment of serial numbers, or ranks, to the experimental observations arranged in order of their magnitude. Conclusions are drawn about the significance of differences between categories under study from the sums of the assigned ranks. Such methods are called nonparametric or distribution free. They are simple to compute and require few assumptions about the nature of the sampled population (13).

Results of the ranking of data indicated there was no significant difference among the methods overall. However, some slight differences were detected within each laboratory. For example, Lab-1 found the head-space method to be lower in general than either the vacuum or acetone extraction method (possibly the vials used leaked during heat up), and Lab-2 found the vacuum method gave generally lower results than either of the other two methods (ethylene oxide may have been lost through a warm trapping coil).

These three data treatments are by no means the only statistical

Table VII—Analysis of Variance

Source of Variance	Level of Ethylene Oxide[a] 1	2	3	4	5
Methods	+	—	—	—	+
Laboratories	+	+	+	+	+
Methods and laboratories	+	—	—	—	+
Replicates	—	—	—	—	—
Methods and replicates	—	—	—	—	—
Laboratories and replicates	+ —	—	+	—	—
Methods, laboratories, and replicates	—	—	—	—	—
Grand means	4980	918	272	61.7	9.79

[a] + indicates a significant difference; — indicates no significant difference.

treatments which could be applied to the data, but other treatments probably would draw the same conclusions.

Regardless of the results of this study, one must deal with realistic samples on a daily schedule, and each method has its strong and weak points. The following is a subjective evaluation of each technique studied in the round robin.

The gravimetric method presents the ultimate in simplicity, since it does not use complex apparatus or equipment. However, it is a nonspecific method. Therefore, it also measures other volatile residues such as carbon dioxide or dichlorodifluoromethane[4] from the sterilizing gas and absorbed water as ethylene oxide. The tendency is toward positive errors.

Acetone extraction GC is a relatively simple procedure with which most laboratories have little trouble. However, acetone has impurities which could interfere in the analysis, particularly at low levels. The solvent and plasticizers extracted reduce the life of GC columns. Unless the sample is swollen or dissolves in acetone, one cannot be certain that all of the ethylene oxide has been extracted. Therefore, acetone extraction is not a universal method.

The head-space method is rather simple to perform, and it is very fast and sensitive. The GC columns last indefinitely since no solvents are injected. It is a universal method. Nevertheless, errors may occur due to leaky vials or syringes. Also reabsorption of ethylene oxide on the sample may occur if the sample is allowed to cool.

The vacuum extraction method is a universally applicable method and gives long GC column life and extreme sensitivity since sample size is unlimited. It has the disadvantage of using complex equipment, which could be subject to leaks and, therefore, low results. It also requires more operator time than the other methods.

CONCLUSIONS

In general, it may be concluded from this work that the three GC methods give comparable results in the hands of experienced operators. The correlation between the gravimetric and the GC methods breaks down below the highest levels of ethylene oxide studied. At the lower levels of ethylene oxide, the gravimetric method seems to give very high results overall. At higher ethylene oxide levels than were studied here, *i.e.*, above 0.5% ethylene oxide, the correlation possibly could be better. One should be cautious in its use, however, due to the large errors possible at low levels.

In general, for polyvinyl chloride, the use of whichever GC method feels the most comfortable is recommended. If GC is not available, then the method of choice should be checked against any or all of the GC methods used in this study. Also, since this study pointed out the large variability that could be expected between laboratories and samples, care must be exercised in choosing a statistically significant number of samples when validating an in-house method.

[4] Freon-12.

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[x] To whom inquiries should be directed.

Facile Preparation of 6-Chloro-9-amino-2-hydroxyacridine, a Urinary Metabolite of Quinacrine and Quinacrine Mustard

K. C. TSOU ˣ, S. LEDIS, E. STEIGER, and R. NIETRZEBA

Abstract □ 6-Chloro-9-amino-2-hydroxyacridine was found to be a metabolite of both quinacrine and the antimalarial alkylating agent quinacrine mustard. Its structure was confirmed by a one-step reaction of quinacrine with 48% hydrobromic acid. The presence of this compound as a metabolite of quinacrine mustard suggests a possible *in vivo* activation mechanism for its antitumor activity and a pharmacological basis for its toxicity to the liver. *In vitro* experiments showed that this new compound does react with chromosomes and, therefore, can be both a useful chromosome stain and an intercalating agent.

Keyphrases □ 6-Chloro-9-amino-2-hydroxyacridine—synthesis from quinacrine □ Quinacrine and quinacrine mustard metabolite—synthesis of 6-chloro-9-amino-2-hydroxyacridine from quinacrine

For the investigation of *in vivo* alkylation mechanisms related to cancer chemotherapy, the use of bisbenzimidazole fluorescent alkylating agents was studied (1, 2). Since quinacrine mustard (I*b*) is a known alkylating agent possessing both fluorescence and antitumor activity (2), it was of interest to study its metabolism. Although the design of I*b* was based on the activity of quinacrine (I*a*) in mouse tumors (3), no fluorescence uptake was observed in tumors tested with I*b* or I*a*. In rats treated with I*b*, there was extensive liver damage, resulting in death. Urine samples collected from these rats contained the same fluorescent metabolite, 6-chloro-9-amino-2-hydroxyacridine (II).

TLC and other data supported the identity of the metabolite found in quinacrine-treated rats, and it is now confirmed by a facile synthesis from quinacrine. Its presence was previously suggested (4) in a reaction of I*a* with concentrated hydrochloric acid[1]. Although several *in vivo* studies[2] (5–7) also suggested that this compound could be a metabolite of quinacrine, the antimalarial properties of II are not known.

EXPERIMENTAL

Animal Study—Compound I or II was dissolved in the medium and fed intravenously to Lewis–Wistar rats; urine samples were collected for 24 hr. The urine samples were freeze dried and analyzed by TLC on a silica gel plate [chloroform–methanol (1:1)]. In both samples, a fluorescent green-yellow (R_f 0.58) spot could be seen in addition to other tailing products. Other solvent systems were unsatisfactory. Two groups of experiments were run in triplicate, and no I (R_f 0.45) or II (R_f 0.74) was seen. An authentic synthetic sample produced a spot with the same R_f value.

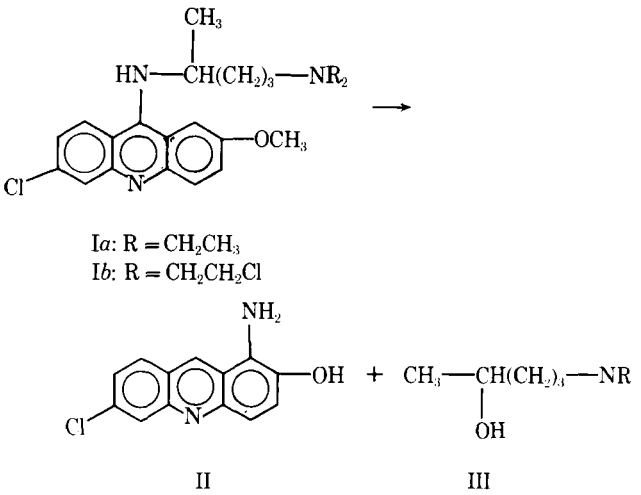
Preparation of 6-Chloro-9-amino-2-hydroxyacridine—A mixture of 5.0 g of quinacrine hydrochloride and 150 ml of 48% hydrobromic acid was refluxed overnight. Upon cooling, the yellowish-orange precipitate was collected and allowed to dry for 3 hr, yielding 3.2 g. Recrystallization from alcohol gave pure II as a light-yellow powder, 2.8 g (88%). The NMR spectra obtained in D_2O showed the loss of the alkyl, methylene, and methoxy protons of I. The IR spectra also showed the loss of methylene protons. The UV spectra showed an absorption at 272 nm, and the fluorescence emission was at 485 when excited at 435 nm.

Reaction of II with Chromosome Preparation—This new derivative was tested in a standard procedure of chromosome staining to compare it with quinacrine or quinacrine mustard. The resulting chromosomes were stained uniformly with no banding. The details will be reported elsewhere (8).

RESULTS AND DISCUSSION

The finding that II is a metabolite of both I*a* and I*b* demonstrated that both I*a* and I*b* could have been degraded *in vivo* to this compound. Therefore, both compounds could probably be biochemically converted *in vivo* by a hydroxylase-mediated mechanism, as shown in Scheme I.

There are ample examples in the literature to support the demethylation of the phenolic compound, even though the degradation of a long chain from an aromatic substituted amine is not known. Therefore, the final support for this mechanism will have to await the isolation of 4-hydroxypentyl nitrogen mustard (III) or another related product. The present data illustrate that the study of alkylation at the biological level is important and that such in-



Scheme I

[1] Reference 4 reported that treatment of atabrine with concentrated hydrochloric acid at 120–125° gave a mixture of 9-amino-6-chloro-2-methoxyacridine and 9-amino-6-chloro-2-hydroxyacridine. However, no proof was given and no attempt was made to separate the mixture.

[2] Based on polarographic evidence, 9-amino-6-chloro-2-hydroxyacridine and methoxy were suggested to be present in urine.

formation can be obtained with fluorescent alkylating agents (1). In addition, the *in vivo* activation mechanism for I*b* may account for the divergent physiological results of numerous other analogs (10).

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PATRICIA M. HUDGINS and JAMES F. STUBBINS [x]

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Keyphrases □ Acetylcholine and acetylcholine mustard—hydrolysis by eel electroplax acetylcholinesterase □ Acetylcholinesterase—hydrolysis of acetylcholine and acetylcholine mustard

The synthesis of acetylcholine mustard [2-(chloroethylmethylamino)ethyl acetate] (I) was first reported by Hanby and Rydon (1). It was demonstrated that acetylcholine mustard can cyclize in buffered aqueous solutions to form an aziridinium ion (II) with alkylating ability (2, 3). This aziridinium ion is a close structural analog of acetylcholine.

In isolated muscle systems, it was found that acetylcholine mustard had about one-fifth the agonist potency of acetylcholine on the muscarinic receptors of the rat jejunum preparation or the nicotinic receptors of the frog rectus abdominis preparation (3). During a 1- or 2-hr exposure of the jejunum segments to either acetylcholine (1.0×10^{-4} *M*) or acetylcholine mustard (1.0×10^{-4} *M*), the contractions slowly declined; after washing, response to freshly applied acetylcholine was inhibited compared to control values. The decline in response was greater in the case of acetylcholine mustard, and the tissue did not fully recover even upon prolonged washing. Although the observed inhibition of response by acetylcholine and some of the effect of acetylcholine mustard could be accounted for by desensitization of receptors, the long-acting inhibition of the mustard was attributed to an irreversible inhibition brought about by alkylation of the receptors by the aziridinium ion (3).

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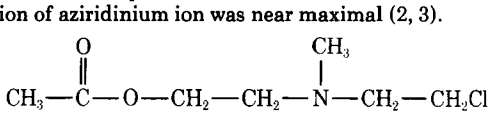
EXPERIMENTAL

In all experiments, acetylcholine mustard was dissolved in buffer solution and allowed to stand for 1 hr at room temperature before use. Previous experiments indicated that at this time the concentration of aziridinium ion was near maximal (2, 3).



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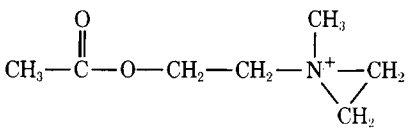

II

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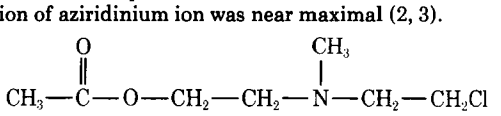

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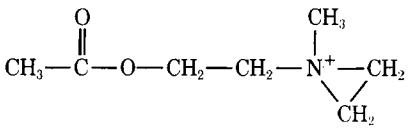

II

Acetylcholinesterase Activity Assay—Acetylcholinesterase, prepared from electric eel[1], was used to catalyze the hydrolysis of acetylcholine or acetylcholine mustard. Disappearance of the substrate was determined by a colorimetric method (4). Briefly, to 2 ml of buffered substrate solution, 5 μl of acetylcholinesterase stock solution was added. The reaction was allowed to proceed for 2 min at 25° before it was stopped by the addition of freshly prepared alkaline hydroxylamine solution. After an additional 1 min, 4 *M* HCl and 0.37 *M* ferric chloride were added and the absorbance was read at 540 nm in a spectrophotometer.

Determination of Kinetic Constants—Estimations of kinetic constants for the hydrolysis of acetylcholine or acetylcholine mustard by acetylcholinesterase were made by determining the velocity of hydrolysis at substrate concentrations between 1 and 10 m*M*. At acetylcholine concentrations above 20 m*M*, substrate inhibition occurred. Kinetic constants were obtained by linear regression analysis of Hofstee plots of experimental data. The *F* test for "goodness of fit" for each curve was significant ($p < 0.05$).

Incubation of Enzymes with Substrates—In separate experiments, acetylcholinesterase was exposed to varying concentrations of either acetylcholine or acetylcholine mustard for 1 hr in buffer solution at 25° and then dialyzed against buffer at 0° for 2 hr to remove the remaining substrate or hydrolysis products. An aliquot of the enzyme solution was then taken for measurement of enzymatic activity as already described.

RESULTS AND DISCUSSION

Nickerson and Gump (5) were the first to demonstrate that the 2-haloethylamine moiety incorporated into α-adrenergic antagonists gave long-lasting activity. Ample evidence has been presented that this persistent antagonism is caused by formation of a covalent bond between the drug molecule in the form of a highly reactive aziridinium ion and a nucleophilic group on or near the receptor site (6–8).

Alkylation of the muscarinic receptor by an antagonist was first shown by Gill and Rang (9). These workers demonstrated that benzilylcholine mustard could produce selective and persistent antagonism of methylfurtrethonium-induced contractions of the isolated guinea pig ileum. Employing some elegant techniques, these workers found that the rate of dissociation of the aziridinium-ion–receptor complex was slow compared to the rate of receptor alkylation. Therefore, the mustard was a highly effective alkylating agent.

The acetylcholine analog, bromoacetylcholine, was shown to be capable of covalent bond formation with nicotinic receptors of eel electroplax (10). In this case, persistent stimulation rather than inhibition resulted. This example was apparently the first one of an irreversible agonist action at a receptor, although the agent was presumably covalently bound to a group just beyond the receptor site rather than within the receptor site proper. The aziridinium ion derived from acetylcholine mustard (II) is also a cholinergic agonist, but alkylation proceeded at a slow rate to yield a prolonged inhibition (3). It was decided to determine whether the acetylcholine mustard could also act as an irreversible inhibitor of acetylcholinesterase.

In some experiments, the effect of prolonged exposure of acetylcholinesterase to acetylcholine or acetylcholine mustard upon subsequent hydrolysis of acetylcholine was measured. In numerous trials, acetylcholinesterase was treated with either substrate as described under *Experimental*. The activity of the enzyme after such treatment proved to be considerably lower than that from controls using freshly prepared enzyme solutions. Although the hydrolysis rate was somewhat lower with enzyme exposed to the mustard than with enzyme exposed to acetylcholine, the difference was not significant.

In the next series of experiments, the initial hydrolysis rates for acetylcholine or acetylcholine mustard were determined at various concentrations of the two substrates. The Michaelis constant (K_m) and maximum velocity (V_m) were obtained by linear regression analysis of data from experiments in which the concentration of substrate was 1–10 m*M*. On the basis of these data (Table I), acetylcholine and the aziridinium ion from acetylcholine mustard appear to have similar affinities for acetylcholinesterase. This finding might be expected because of their close structural similarity. It is also evident from a comparison of the V_m's that acetylcholine mustard is hydrolyzed at a significantly lower maximum velocity than acetylcholine ($p < 0.001$).

Table I—Kinetic Constants for Hydrolysis of Acetylcholine and Acetylcholine Mustard, over a Substrate Concentration Range of 1—10 m*M*, Obtained by Linear Regression Analysis of Hofstee Plots of the Data

Substrate	K_m, m*M* ± *SE*	V_m, μmoles/min/mg ± *SE*	n[a]
Acetylcholine	1.05 ± 0.30	267.4 ± 19.2	166
Acetylcholine mustard	1.06 ± 0.44	135.8 ± 17.2	99

[a] Total number of observations at 1, 2, 3, 4, 5, 6, 8, and 10 m*M*.

In further experiments, the hydrolysis rate as a function of the substrate concentration was studied for acetylcholine, acetylcholine mustard, and a combination of equimolar parts of the two at a concentration range near the K_m. It can be seen in Fig. 1 that the relationship between velocity and substrate concentration is more nearly linear in the case of acetylcholine hydrolysis. A statistical comparison between the velocity of hydrolysis at each substrate concentration revealed acetylcholine was hydrolyzed significantly faster than acetylcholine mustard ($p < 0.001$).

The ratios of the velocities at two substrate concentrations (1.5 and 0.75 m*M*) for the hydrolysis of acetylcholine and acetylcholine mustard were 1.95 and 1.66, respectively. Thus, in the case of acetylcholine, the velocity of hydrolysis was nearly doubled as the substrate concentration was doubled; however, in the case of the mustard, there was a considerably smaller increase in velocity. The ratio of velocities at 1.5 and 0.75 m*M* substrate concentrations for the combination of acetylcholine and acetylcholine mustard was 1.88.

The question arises as to whether the lower V_m (Table I) and the curvature in the rate of hydrolysis at increasing acetylcholine mustard concentrations (Fig. 1) are caused by alkylation of the active site. It was shown (11) that acetylcholinesterase can be inhibited irreversibly by the alkylating agent *N,N*-dimethyl-2-phenylaziridinium chloride. This alkylating agent is not a close structural analog of acetylcholine, and alkylation may have taken place at an anionic site other than that at which the cationic head of acetylcholine normally binds to the enzyme. It is well known that certain quaternary ammonium ions can inhibit cholinesterase at sites different from the acetylcholine binding site (12). Alkylation of such allosteric sites might account for the irreversible inhibition by the aziridinium ion. In fact, O'Brien (13) summarized evidence

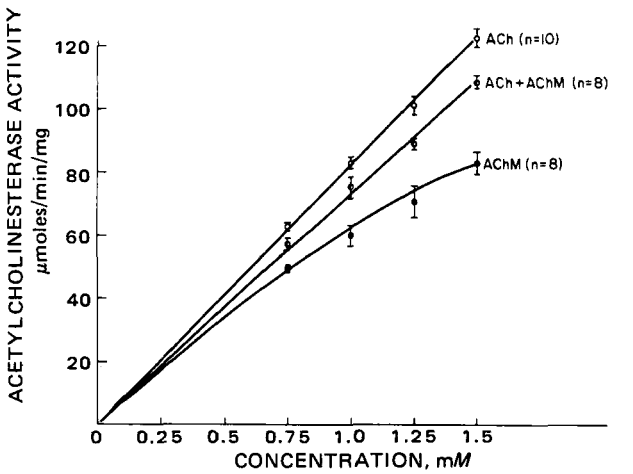

Figure 1—*Hydrolysis of acetylcholine (ACh), acetylcholine mustard (AChM), and an equimolar combination of acetylcholine and acetylcholine mustard (ACh + AChM) by acetylcholinesterase after 2 min of incubation at 25°. Numbers in parentheses refer to the number of separate experiments. Vertical bars represent standard error of the mean.*

[1] Type V, Sigma Chemical Co.

suggesting that the cationic head of acetylcholine may bind to the active site by nonpolar interactions rather than by ionic or polar forces. If such is the case, there may not be any nucleophilic group capable of being alkylated at this position on the enzyme.

Present results do not exclude the possibility that alkylation of the enzyme by the mustard could be occurring at sites other than the acetylcholine binding site. Alkylation by the mustard at the active site or at other sites would tend to lower the velocity of its hydrolysis. This was observed both in the experiments designed to provide kinetic constants (Table I) and in a separate series where each substrate and equimolar concentrations of each substrate were assayed (Fig. 1).

In summary, results of the present study reveal that acetylcholine is hydrolyzed at a significantly faster rate than the mustard, even though their affinities for the enzyme appear to be comparable. In the light of previous results obtained in cholinergic receptor systems, which demonstrated alkylation of a portion of receptors (3), it is possible that the mustard is capable of covalent bond formation with, and subsequent inactivation of, acetylcholinesterase. Due to rapid turnover of enzyme–substrate complexes, conditions for alkylation may not be favorable. In any case, it appears likely that alkylation and enzyme inactivation are occurring during hydrolysis of acetylcholine mustard by acetylcholinesterase. Hydrolysis of mixtures of the substrates would be expected to yield mixed-type inhibition.

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F. C. P. LAW [x], J. C. DRACH, and J. E. SINSHEIMER [x]

Abstract □ Guinea pig lung phenol-*O*-methyltransferase, catechol-*O*-methyltransferase, and benzpyrene hydroxylase activities were examined after nitrogen dioxide and 3-methylcholanthrene treatment. While benzpyrene hydroxylase activity was enhanced by 3-methylcholanthrene, none of the pulmonary enzyme activities was altered after exposure to either 40 or 70 ppm of nitrogen dioxide for 2 hr.

Keyphrases □ Nitrogen dioxide—effects on guinea pig lung phenol-*O*-methyltransferase, catechol-*O*-methyltransferases, and benzpyrene hydroxylase □ 3-Methylcholanthrene—effects on guinea pig lung enzymes □ Enzymes, pulmonary—effects of nitrogen dioxide and 3-methylcholanthrene

A previous report (1) from this laboratory demonstrated that guinea pig lung contains appreciable phenol-*O*-methyltransferase and catechol-*O*-methyltransferases. Furthermore, catechol-*O*-methyltransferases were present both in the supernatant and the microsomal fractions. The presence of these enzymes also has been indicated in rat lungs (2, 3). In addition, benzpyrene hydroxylase activity has been reported in rat lung (4) and is inducible by a wide range of chemicals including cigarette smoke (5), 3-methylcholanthrene (4, 6), flavones (7), and *Cannabis* (6).

The question was raised as to the influence of air pollutants on the primary pulmonary enzyme identi-

Table I—Pulmonary Enzyme Activities after 3-Methylcholanthrene Administration

Lung Enzymes[a]	Untreated Animals	3-Methylcholanthrene-Treated Animals
Benzpyrene hydroxylase	0.22 ± 0.19 (6)[b]	0.43 ± 0.26 (6)[c]
Microsomal phenol-*O*-methyltransferase	6.4 ± 0.72 (3)	7.2 ± 0.92 (3)
Supernatant catechol-*O*-methyltransferase	22.7 ± 1.4 (3)	22.9 ± 0.55 (3)
Microsomal catechol-*O*-methyltransferase	3.0 ± 0.17 (3)	3.6 ± 0.76 (3)

[a] Transmethylation activities are expressed as nanomoles of O-methylated substrates per gram of tissue; hydroxylation activities are expressed as fluorescence units per gram of tissue. [b] Values are given as means ± *SE*, with the number of animals used in parentheses. [c] Values are significantly different ($p < 0.1$) from untreated animals.

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Table II—Pulmonary Enzyme Activities after Nitrogen Dioxide Exposure

Test Group	Benzpyrene Hydroxylase[a]	Microsomal Phenol-*O*-methyltransferase[b]	Supernatant Catechol-*O*-methyltransferase[b]	Microsomal Catechol-*O*-methyltransferase[b]
Control	0.27 ± 0.18 (7)[c]	5.0 ± 0.6 (2)	22.4 ± 0.9 (2)	1.7 ± 0.2
versus				
40 ppm nitrogen dioxide	0.21 ± 0.13 (8)	4.8 ± 1.0 (4)	20.2 ± 0.48 (4)	1.7 ± 0.18 (4)
Control	0.33 ± 0.05 (2)	6.0 ± 0.5 (2)	29.2 ± 1.1 (2)	2.6 ± 0.4 (2)
versus				
70 ppm nitrogen dioxide	0.32 ± 0.028 (4)	6.4 ± 0.92 (4)	29.2 ± 0.99 (4)	2.8 ± 0.46 (4)

[a] Hydroxylation activities are expressed as fluorescence units per gram of tissue. [b] Transmethylation activities are expressed as nanomoles of O-methylated product per gram of tissue. [c] Values are given as means ± *SE*, with the number of animals used in parentheses.

fied to date. Thus, the effects on benzpyrene hydroxylase, phenol-*O*-methyltransferase, and catechol-*O*-methyltransferases were studied in guinea pigs after treatment with nitrogen dioxide by inhalation. Also included in this report, as a control, are the effects of 3-methylcholanthrene subcutaneous injection upon the same lung enzyme systems in guinea pigs.

EXPERIMENTAL

Guinea pigs, 300–500 g, of either sex were used. 3-Methylcholanthrene, dissolved in arachis oil, was administered at a dose of 20 mg/kg ip. Control animals were injected with arachis oil only. Exposures of animals to nitrogen dioxide at 40- and 70-ppm levels for 2 hr were carried out in a dynamic exposure chamber as described previously (8). Chamber concentrations were controlled by flowmeters which were checked periodically during the exposure.

At 24 hr after 3-methylcholanthrene or nitrogen dioxide treatment, the animals were sacrificed. The lungs were removed, rinsed, blotted, and weighed. Preparation of subcellular fractions and assays of *O*-methyltransferase enzyme activities were carried out as previously described (1). The substrates employed were phenol for phenol-*O*-methyltransferase and isoproterenol for catechol-*O*-methyltransferase activities. Benzpyrene was the substrate for the determination of hydroxylase activity using the procedure of Wattenberg *et al.* (9).

The Student *t* test and the U test (10) were used for statistical comparisons. Results confined to the same day and with the same animal shipment were used in these statistical analyses.

RESULTS AND DISCUSSION

3-Methylcholanthrene administration increased guinea pig lung benzpyrene hydroxylase activity about twofold. However, neither microsomal phenol-*O*-methyltransferase nor microsomal and supernatant catechol-*O*-methyltransferase activities were affected (Table I). During these experiments, the basal levels of benzpyrene hydroxylase varied considerably with different shipments of guinea pigs from the supplier. These results are consistent with the findings (4, 6) that pulmonary benzpyrene hydroxylase activity varies with rats and is inducible by 3-methylcholanthrene.

The LD_{50} of nitrogen dioxide exposure for 2–4 hr to guinea pigs was reported to be about 75 ppm (11). The concentrations of 40 and 70 ppm of nitrogen dioxide used in this study were chosen to study the effects of maximally tolerated nitrogen dioxide levels. Lung weights and enzyme activities were not significantly different from experiments carried out on the same day and with the same animal shipment. Table II shows lung enzyme activities from several experiments after nitrogen dioxide treatments. There is no evidence from these studies that nitrogen dioxide affects pulmonary phenol-*O*-methyltransferase, catechol-*O*-methyltransferase, and benzpyrene hydroxylase activities after short-term exposure to nitrogen dioxide even at levels as high as 70 ppm[1].

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x To whom inquiries should be directed.

[1] M. S. Palmer, R. W. Exley, and D. L. Coffin [*Arch. Environ. Health*, **25**, 439(1972)] reported a similar lack of effect with nitrogen dioxide on benzpyrene hydroxylase activity from rat lung.

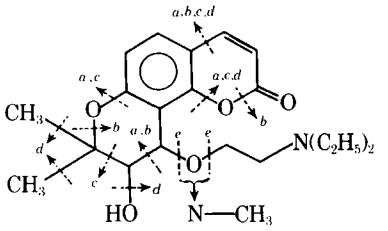
Synthesis and Blood Pressure Lowering Activity of Benzylic Ethers of 2-Diethylaminoethanol and a Related Diamine

SAMIR M. EL-ANTABLY*, TAITO O. SOINE[x], NADIM A. SHAATH, and PREM K. GUPTA ‡

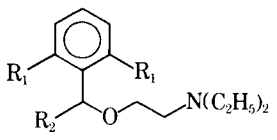
Abstract □ Based upon the unpublished finding that 3′-hydroxy-4′-(β-diethylaminoethoxy)-3′,4′-dihydroseselin possessed a potent blood pressure lowering effect in the cat at a dose of 1 mg/kg, the present study examined the activities of several related compounds. These compounds were derived by dissection of the parent compound to give four benzylic ethers of 2-diethylaminoethanol and a diamine, derived by replacing the ether oxygen of the parent compound with an N—CH_3 function. The simplest compounds were the benzyl and 2,6-dimethoxybenzyl ethers of the aminoalcohol. Closely related to the benzyl compound was a congener with a hydroxymethyl group on the benzylic carbon. The β-diethylaminoethyl ether of 4-chromanol was the most complex of the ethers. The blood pressure measurements were carried out on male cats and compared to papaverine hydrochloride as a standard. In all cases, the most potent blood pressure lowering activity resided in the parent compound, which was not greatly superior to the diamine but substantially more active than the other compounds.

Keyphrases □ 2-Diethylaminoethanol benzylic ethers and related diamine—synthesis, screened for blood pressure lowering activity □ Antihypertensive activity—synthesis and screening of 2-diethylaminoethanol benzylic ethers and related diamine

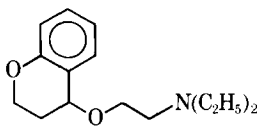
Gupta (1) prepared 3′-hydroxy-4′-(β-diethylaminoethoxy)-3′,4′-dihydroseselin (I*a*) in a search for water-soluble coronary vasodilators, since visnadin (I*b*), the parent compound, together with its closely



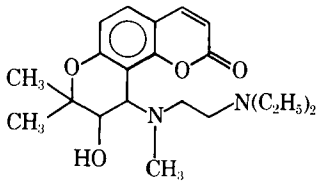
I*a*



II: $R_1 = R_2 = H$ (*a*)
III: $R_1 = OCH_3$; $R_2 = H$ (*b*)
IV: $R_1 = H$; $R_2 = CH_2OH$ (*c*)

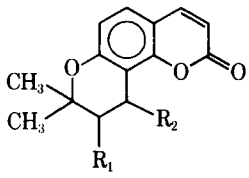


V: *d* to give the indicated structure



VI: *e* with substitution of N—CH_3 for O as indicated in I*a*

Scheme I



I*a*: R_1 = —OH, R_2 = —$OCH_2CH_2N(C_2H_5)_2$
I*b*: R_1 = —$OOCCH(CH_3)CH_2CH_3$, R_2 = —$OOCCH_3$
I*c*: R_1 = —$OOCCH_3$, R_2 = —$OOCC(CH_3)=CH-CH_3$
I*d*: R_1 = —$OOCCH_3$, R_2 = —$OOCCH_2CH(CH_3)_2$

related congeners pteryxin (I*c*) and suksdorfin (I*d*), were reputed (2, 3) to possess this interesting and potentially useful activity. Preliminary observations in these laboratories indicated that I*a* was lethal to rabbits and cats when administered in doses of 3–4 mg/kg iv. Further experimentation indicated that the cause of death was an acute blood pressure drop and that a dose of 1 mg/kg in the cat, while causing a significant blood pressure drop, was not lethal. Because of these observations, some of the simpler forms envisioned within the molecular structure of I*a* were proposed as candidates to determine whether significant activity could be generated in the structural modifications.

DISCUSSION

Consideration of the molecular structure of I*a* immediately suggested that its most important feature was that of being a benzylic ether of 2-diethylaminoethanol, since other compounds in which the ether function was replaced with a variety of acyloxy functions (*e.g.*, α-methylbutyroxy, angeloyloxy, and isovaleroxy) were without significant effect on the blood pressure at comparable dose levels. However, the necessity of the coumarin moiety as an activity-contributing part of I*a* had not been demonstrated, so the dissection of this moiety became the prime focus of this investigation.

The dissections employed are indicated in Scheme I, where the letters *a*, *b*, *etc.*, indicate the points of scission of I*a* and correlate with the numbers of the actual compounds prepared, *i.e.*, II, III, IV, V, and VI.

In II, the molecule has been stripped to the bare essentials of being a benzylic ether; in III, the simple benzylic ether has the oxygen functions of I*a* restored as methoxy groups. In IV, the hydroxy corresponding to that at the 3′-position of I*a* has been retained in a simple benzylic ether. The modification in V restores the benzopyran portion of I*a* (minus the *gem*-dimethyls). Compound VI represents the compound in which all of the elements of I*a* are kept intact except for the introduction of an N—CH_3 group in place of the ether oxygen, although one cannot be sure that the replacing group has the same steric orientation as the C-4′ group in I*a*.

Compound II was prepared by the method of Rericha *et al.* (4). Compound III was readily obtained by reduction of ethyl 2,6-dimethoxybenzoate to the corresponding alcohol with lithium aluminum hydride, followed by treatment of the sodium salt of the alcohol with 2-diethylaminoethyl chloride. 2-Phenyl-2-(β-diethylami-

noethoxy)ethanol (IV) was prepared by the method of Bottari and Macchia (5), which involved the interaction of the sodium salt of 2-diethylaminoethanol with ethyl-α-bromophenyl acetate, followed by lithium aluminum hydride reduction of the resulting product to give IV. The synthesis of V was accomplished by the alkylation of 4-chromanol with 2-diethylaminoethyl chloride. Compound VI was readily accessible from the reaction of Ic with *N,N*-diethyl-*N'*-methylethylenediamine, followed by acid hydrolysis.

Determination of the vasodepressor activity of Ia and the prepared compounds was made using cats as the test animal and papaverine hydrochloride as a standard. All blood pressure drops were compared to the standard drop in pressure achieved with papaverine. The order of vasodepressor activity determined in this manner was: Ia > VI >>> III > II > V > IV. It was clear that Ia and its close structural analog were virtually equivalent in activity and were both substantially superior to the others. Nevertheless, the fact that activity was evident in compounds without the coumarinic lactone feature suggests that additional studies might be desirable.

EXPERIMENTAL[1]

Synthesis—*3'-Hydroxy-4'-(β-diethylaminoethoxy)-3',4'-dihydroseselin (Ia)*—Pteryxin (Ic, 10 g, 0.025 mole) in tetrahydrofuran (40 ml) was treated with sodium amide (1.95 g, 0.05 mole), and the solution was stirred at room temperature for 1 hr. 2-Diethylaminoethanol (5.9 g, 0.05 mole) in tetrahydrofuran (5 ml) was added dropwise and the solution was stirred at room temperature for 2 hr. The contents then were diluted with water (1 liter) and acidified with sulfuric acid.

The aqueous acid solution was washed with ether and made alkaline with 10% aqueous sodium carbonate, and the precipitated base was thoroughly extracted with ether. The ethereal solution was washed with water, dried, and then evaporated to provide a brown oil. On standing in the refrigerator for a few days, the oil crystallized and was then recrystallized from ethanol–water to give white needles, mp 105–108°. The methiodide salt gave a melting point of 191–193°.

Anal.—Calc. for $C_{20}H_{27}NO_5 \cdot H_2O$: C, 63.30; H, 7.65; N, 3.69. Found: C, 63.60; H, 7.32; N, 3.59.

1-Benzyloxy-2-diethylaminoethane (II)—The synthesis was as described by Rericha *et al.* (4), bp 70–74°/0.1 mm [lit. (4) bp 72–76°/0.1 mm].

1-(2',6'-Dimethoxy)benzyloxy-2-diethylaminoethane (III)—2,6-Dimethoxybenzyl alcohol (0.5 g, 0.062 mole), prepared by lithium aluminum hydride reduction of ethyl 2,6-dimethoxybenzoate according to Hejno and Arnold (6), was treated with metallic sodium (1.43 g, 0.062 mole). A solution of 2-diethylaminoethyl chloride (8.5 g, 0.062 mole) in xylene (25 ml) was added dropwise to this solution, and the reaction mixture was refluxed for 12 hr; the contents then were diluted with water and extracted with aqueous 10% hydrochloric acid.

The acid extract was washed with ether, made alkaline with aqueous 10% sodium hydroxide, and extracted with ether to obtain the free base. The ethereal extract was washed with water, dried, filtered, and finally subjected to evaporation. The oily residue was distilled to obtain a product (7.6 g, 45.5%), bp 170–180°/2.5 mm.

The hydrochloride of the base, prepared by the use of ethereal hydrogen chloride, was recrystallized from ethyl acetate–ether to give an analytical sample, mp 98–99°.

Anal.—Calc. for $C_{15}H_{26}ClNO_3$: C, 59.30; H, 8.56; N, 5.26. Found: C, 59.20; H, 8.88; N, 5.59.

2-Phenyl-2-(β-diethylaminoethoxy)ethanol (IV)—Ethyl-α-bromophenyl acetate (19.5 g, 0.08 mole) was prepared according to the method of Narayanan and Martin (7), dissolved in anhydrous toluene (60 ml), and added dropwise to a solution of 2-diethylaminoethanol (10.6 g, 0.09 mole) in anhydrous toluene (25 ml), which had been pretreated with sodium (1.84 g, 0.08 mole) as in the preparation of III. The resultant mixture was refluxed for 2 hr with stirring, cooled, washed with water (50 ml), and extracted thoroughly with aqueous 10% hydrochloric acid. The combined acidic extract was washed with ether (50 ml) and made alkaline with 10% aqueous sodium hydroxide, and the free base was extracted into ether.

The ethereal extract was washed with water, dried, and evaporated to provide 4.6 g (20.7%) of an oil. The oil, after drying overnight in an evacuated desiccator over phosphorus pentoxide, was dissolved in anhydrous ether and added dropwise to a suspension of lithium aluminum hydride (0.7 g) in anhydrous ether. The reaction mixture was refluxed for 0.5 hr, cooled, and decomposed by the cautious addition of water. The organic phase was then dried and evaporated to dryness to yield a yellow oil (2.6 g, 66.6%). This oil was distilled under reduced pressure at bp 130–142°/2 mm [lit. (5) bp 135–140°/1.6 mm].

The hydrochloride salt was prepared by the addition of ethereal hydrogen chloride to a saturated solution of the free base in absolute alcohol. Recrystallization of the salt from acetone–ether yielded colorless crystals, mp 118–119°.

4-(β-Diethylaminoethoxy)chroman (V)—To a stirred suspension of sodium hydride (0.6 g of a 50% suspension in mineral oil) in anhydrous dimethylformamide (15 ml) under nitrogen was added dropwise a solution of 4-chromanol (1.6 g, 0.01 mole) in dimethylformamide (2 ml). The reaction mixture was stirred for 1 hr, and then a solution of 2-diethylaminoethyl chloride (2.7 g, 0.02 mole) in dimethylformamide (2 ml) was added dropwise (25 min). Stirring was continued at room temperature for 21 hr.

The mixture was poured into water and extracted with ether, and the ethereal extract was washed with water before being extracted with 10% aqueous hydrochloric acid (4 × 100 ml). The acidic extract was washed with ether and made alkaline with 20% ammonium hydroxide solution, and the free base was extracted with ether. The ethereal extract was washed with water and dried, and the ether was evaporated to obtain a pale-yellow oil (2 g, 76.9%). This oil was distilled at bp 115–120°/0.2 mm.

The picrolonate salt was prepared by mixing the free base (0.124 g) and picrolonic acid (0.132 g) in absolute ethanol to obtain large brown needles, mp 141–143° dec.

Anal.—Calc. for $C_{25}H_{31}N_5O_7$: C, 58.46; H, 6.08; N, 13.63. Found: C, 58.65; H, 6.27; N, 13.40.

3'-Hydroxy-4'-[methyl(N,N-diethylaminoethyl)amino]-3',4'-dihydroseselin (VI)—A mixture of visnadin (Ib, 19.5 g, 0.05 mole) in anhydrous benzene (250 ml) and *N*-methyl-2-diethylaminoethylenediamine (13 g) was refluxed for 19 hr. The solvent was removed under reduced pressure; the residue was extracted with 10% aqueous hydrochloric acid, alkalinized as before, and extracted with ether; and the ethereal extract was dried and evaporated to give 4 g of an oil. This oil was refluxed with sulfuric acid (20%, 30 ml) for 6 hr; after cooling, the reaction mixture was diluted with water and alkalinized with 20% ammonium hydroxide, and the free base was extracted into ether.

The ethereal extract was dried, concentrated to a small volume, and then treated with ethereal hydrogen chloride. The resulting salt (1.5 g) was chromatographed over neutral alumina (45 g), and

Table I—Blood Pressure Lowering Effects

Compound	Dose, mg/kg	Mean Blood Pressure Drop, mm/Hg	Percent Blood Pressure Drop[a]	Duration, min
Ia	0.5	43.3	72.0	5.0
	1.0	48.2	80.3	6.0
	2.0	74.0	123.0	10.0
II	1.0	17.0	28.3	4.0
	2.0	27.0	45.0	6.0
	3.0	40.0	66.6	9.0
III	1.0	18.0	30.0	3.5
	2.0	28.0	46.6	5.0
	3.0	48.0	80.0	5.5
IV	1.0	9.0	15.0	6.5
	2.0	16.0	26.6	10.0
	3.0	29.0	48.3	12.0
V	0.5	9.0	15.0	1.0
	1.0	15.0	25.0	2.0
VI	0.5	41.0	68.0	4.0
	1.0	48.0	80.0	7.0
	2.0	71.0	118.0	11.0

[a] Papaverine hydrochloride = 100.

[1] Melting points were determined in capillary tubes in a Thomas-Hoover melting-point apparatus and are uncorrected. NMR spectra were determined on a Varian A-60D instrument. Analysis were carried out by the Microanalytical Laboratory, School of Chemistry, University of Minnesota, Minneapolis, Minn., or by Schwarzkopf Laboratories, Woodside, N.Y. Anhydrous sodium sulfate was used to dry solvent extracts unless otherwise specified.

the free base was eluted with ethyl acetate. Reconversion of the free base to the hydrochloride salt with ethereal hydrogen chloride and recrystallization from acetone–ether provided pale-yellow crystals, mp 108–110° dec.

Anal.—Calc. for $C_{21}H_{32}Cl_2N_2O_4$: C, 56.37; H, 7.21; N, 6.26. Found: C, 56.53; H, 7.20; N, 6.11.

Pharmacology—The blood pressure lowering activities of the compounds studied were compared to papaverine hydrochloride, the standard, in three male cats weighing an average of 3 kg. The animals were anesthetized with pentobarbital sodium (30 mg/kg), with additional small doses being given as needed. The blood pressure was monitored continuously by a force transducer[2] connected to a polyethylene catheter placed in the femoral artery and coupled[3] to a recording polygraph[4] at a chart speed of 1 cm/min. The average blood pressures for the cats at the beginning and end of the experiments were 146 and 132 mm/Hg, respectively.

The compounds tested were simply dissolved in normal saline if they were in a salt form. If in the free base form, they were solubilized in the least amount of 0.02 *N* hydrochloric acid and made up to the normal saline concentration with appropriate amounts of sodium chloride and water. The compounds tested were injected as a bolus *via* a femoral venous catheter in doses ranging from 0.5 to 3 mg/kg, followed by a 3-ml saline flush. The results reported (Table I) for each dose are the results of three or more readings. The blood pressure was allowed to return to preinjection levels before additional injections were given.

The protocol for the administration of the several agents being tested was to administer them according to the Latin-square method, whereby each new animal preparation was tested with a different order of the agents. In addition, administration of papaverine hydrochloride at the beginning, during the course of, and at the end of the experiment indicated that the blood pressure was still normally responsive in each cat throughout the experiment. The drop in blood pressure by an average dose of 1.67 mg/kg of papaverine hydrochloride was 60 mm/Hg, and this value was assigned as a 100% drop in the blood pressure because increasing the dose failed to elicit a further drop. All compounds were rated as a percentage against the standard on this basis.

[2] Beckman 215071.
[3] Beckman 9853 coupler.
[4] Beckman R-411 dynograph.

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Cyanogen Condensations as a Route to 3-Amino-2-imino-1,3-benzothiazin-4-ones with CNS Depressant Potential

NED D. HEINDEL ˣ and LEE A. SCHAEFFER

Abstract □ Seven new 3-amino-2-imino-1,3-benzothiazin-4-ones, prepared from the condensation of cyanogen and 2-mercaptobenzhydrazides, were evaluated in a neuropharmacological mouse profile. CNS depression was observed in several members of the class.

Keyphrases □ 3-Amino-2-imino-1,3-benzothiazin-4-ones—synthesized and screened as CNS agents □ CNS agents, potential—synthesis and screening of seven 3-amino-2-imino-1,3-benzothiazin-4-ones □ 1,3-Benzothiazin-4-ones, 3-amino-2-imino—synthesized and screened as CNS agents

Recent interest in the synthesis and pharmacological properties of certain 1,3-benzothiazin-4-ones, particularly as central nervous system (CNS) agents with marked mydriatic activity (1–5), prompted this report on a new synthesis of some uniquely substituted analogs of this class and their biological activities. 2-Mercaptobenzhydrazides (I) and cyanogen (II) were found to undergo facile condensation, in 50–74% yield, to 3-amino-2-imino-3,4-dihydro-2*H*-1,3-benzothiazin-4-ones (III), a reaction that represents cyanogen acting as a one-carbon insertion unit to close a six-membered ring (Scheme I).

Previous examples are known in which cyanogen in heterocyclic syntheses completes a cycle by insertion of a single carbon (6), two carbons (7), or one carbon and a nitrogen (8). An earlier synthetic study with 2-mercaptobenzhydrazide reported its condensation with formaldehyde to form a seven-membered ring, but no biological testing data were provided on the resulting benzo-1,3,4-thiadiazepinone (9).

The benzothiazinone products, III*a*–III*c*, could be structurally distinguished from the equally plausible, and isomeric, seven-membered alternatives, IV*a*–IV*c*, by characteristic chemical reactions. Hydrolysis generated Compound VII, which possessed a free amino group as evidenced by formation of an anil, VIII. Furthermore, condensation of III*a* with phosgene and/or benzoyl chloride produced *s*-triazolo systems, which would have been impossible from the alternative structure, IV. In addition, the pendant amino on III*a* underwent facile anil formation with both *p*-nitro- and *p*-dimethylaminobenzaldehydes.

From a practical viewpoint, solutions of up to 5 *M* cyanogen in anhydrous tetrahydrofuran, stable to

the free base was eluted with ethyl acetate. Reconversion of the free base to the hydrochloride salt with ethereal hydrogen chloride and recrystallization from acetone–ether provided pale-yellow crystals, mp 108–110° dec.

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Abstract □ Seven new 3-amino-2-imino-1,3-benzothiazin-4-ones, prepared from the condensation of cyanogen and 2-mercaptobenzhydrazides, were evaluated in a neuropharmacological mouse profile. CNS depression was observed in several members of the class.

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Recent interest in the synthesis and pharmacological properties of certain 1,3-benzothiazin-4-ones, particularly as central nervous system (CNS) agents with marked mydriatic activity (1–5), prompted this report on a new synthesis of some uniquely substituted analogs of this class and their biological activities. 2-Mercaptobenzhydrazides (I) and cyanogen (II) were found to undergo facile condensation, in 50–74% yield, to 3-amino-2-imino-3,4-dihydro-2*H*-1,3-benzothiazin-4-ones (III), a reaction that represents cyanogen acting as a one-carbon insertion unit to close a six-membered ring (Scheme I).

Previous examples are known in which cyanogen in heterocyclic syntheses completes a cycle by insertion of a single carbon (6), two carbons (7), or one carbon and a nitrogen (8). An earlier synthetic study with 2-mercaptobenzhydrazide reported its condensation with formaldehyde to form a seven-membered ring, but no biological testing data were provided on the resulting benzo-1,3,4-thiadiazepinone (9).

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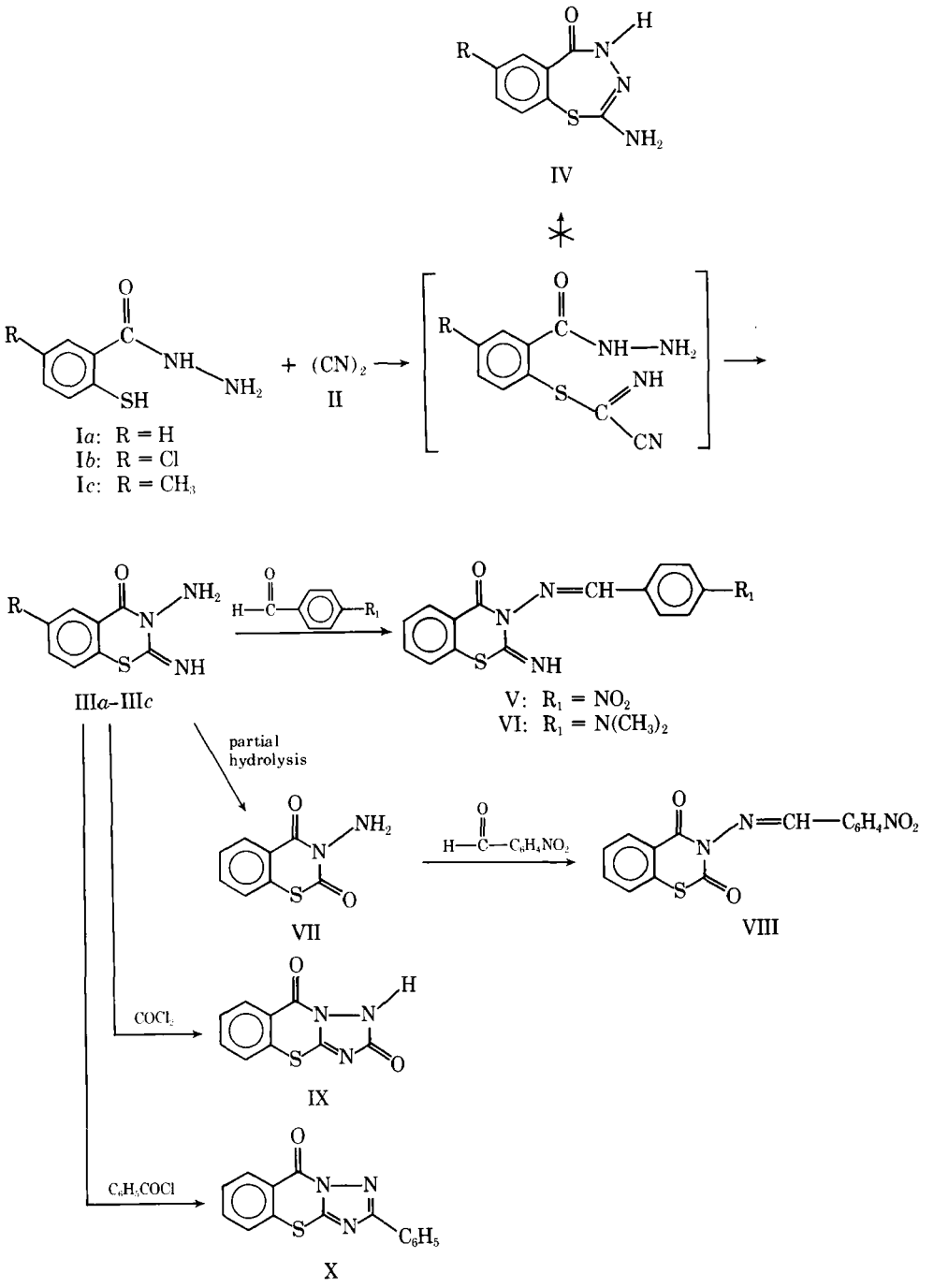
From a practical viewpoint, solutions of up to 5 *M* cyanogen in anhydrous tetrahydrofuran, stable to



Scheme I

prolonged storage, are readily prepared by dissolving measured volumes of the condensed cyanogen into the solvent. The hydrogen cyanide gas evolved during the condensations of cyanogen with *Ia–Ic* is collected in a caustic trap and the heterocyclic products, IIIa–IIIc, precipitate directly in the tetrahydrofuran solution, not as salts but as their free base forms.

RESULTS[1]

Compounds IIIa, IIIb, IIIc, IIIa-HCl, V, VI, and VII were administered intraperitoneally in solution or as a suspension in water–methylcellulose to groups of four mice. Physiological signs were observed and evaluated according to the Irwin method (10). Compound IIIa and its hydrochloride salt, IIIa-HCl, gave virtually identical profiles with the exception of the rapidity of onset of the physiological effects. Both agents effected tonic convulsions and death at any dose above 30 mg/kg with an estimated LD_{50} of 18 mg/kg. Presumably because of accelerated absorption from the peritoneal cavity, IIIa-HCl caused death in less than 3 min post-dosing with 300 mg/kg while IIIa required 10 min. All other signs—hypothermia (4° at 100 mg/kg), salivation, body drop, Staub tail phenomenon, and loss of righting reflexes and of spontaneous motor activity—which were common to both agents, appeared more rapidly in IIIa-HCl.

The methyl analog IIIc gave a similar profile to IIIa but was considerably less toxic (LD_{50} of 178 mg/kg) and less active. At 100 mg/kg, IIIc functioned as a mild CNS depressant sedative with marked reduction in spontaneous motor activity, loss of righting reflexes, and hypothermia (4°). The chloro compound IIIb was

[1] Evaluations were carried out at Pharmakon Laboratories, Inc., Scranton, Pa., under the supervision of Dr. Richard Matthews.

nontoxic and inactive at doses up to 300 mg/kg. Therefore, the nature of the substituent at C-6 appears to influence activity markedly.

The 2-imino moiety apparently is involved in the toxic and depressant effects since there was no activity or toxicity with the hydrolyzed carbonyl counterpart, VII, at the highest dose tested, *i.e.*, 1000 mg/kg. Conversion of the 3-aminobenzylidene to a 4-dimethylaminobenzylidene led to a nontoxic compound, VI, which still evidenced mild catatonia and loss of motor activity at the maximum dose employed of 300 mg/kg. The 4-nitrobenzylidene compound, V, had an LD_{50} of 240 mg/kg; at 100 mg/kg, it too displayed mild CNS depressant effects including abnormal gait, lacrimination, hypothermia, and loss of motor activity. None of the compounds was judged sufficiently active to merit more extensive studies.

EXPERIMENTAL[2]

2-Mercaptobenzhydrazides (Ia–Ic)—The method of Katz *et al.* (9), involving thiolation of the diazonium salt of the corresponding anthranilic acid, esterification of the resulting 2-mercaptobenzoic acid, and its displacement with hydrazine, was employed in the syntheses of the 2-mercaptobenzhydrazides, Ia and Ib. The preparation and properties of Ic have not been reported previously, but by following the exact procedure for conversion of 5-chloroanthranilic acid to Ib, 5-methylanthranilic acid was converted in 26% yield to 2-mercapto-5-methylbenzhydrazide (Ic), mp 146–148°; IR (KBr): 3240 (br) (bonded N—H), 2560 (S—H), and 1610–1580 (C=O) cm^{-1}.

Anal.—Calc. for $C_8H_{10}N_2OS$: C, 52.73; H, 5.53; N, 15.37. Found: C, 52.58; H, 5.57; N, 15.36.

3-Amino-2-imino-3,4-dihydro-2*H*-1,3-benzothiazin-4-ones (IIIa–IIIc)—To a solution of 5.2 g (0.10 mole) of II, 4 drops of triethylamine, and 20 ml of anhydrous tetrahydrofuran at 0° was added dropwise a solution of 1–4 mmoles of the mercaptobenzhydrazide, Ia–Ic, in 65 ml of anhydrous tetrahydrofuran. Crystallization of the product commenced almost immediately, and the mixture was stirred at 5° for 4 hr after the completion of the addition. Dilution of the medium with 100 ml of benzene completed the precipitation of product, which was filtered and recrystallized from benzene. A 74% yield of IIIa, mp 141.5–142.5°, was obtained.

Anal.—Calc. for $C_8H_7N_3OS$: C, 49.73; H, 3.65. Found: C, 49.63; H, 3.54.

A 50% yield of IIIb, mp 144–145°, was obtained.

Anal.—Calc. for $C_8H_6ClN_3OS$: C, 42.20; H, 2.66. Found: C, 42.26; H, 2.62.

A 67% yield of IIIc, mp 154–155°, was obtained.

Anal.—Calc. for $C_9H_9N_3OS$: C, 52.16; H, 4.38. Found: C, 52.01; H, 4.35.

A hydrochloride salt of IIIa was prepared by introducing hydrogen chloride gas into a saturated solution of IIIa in benzene to precipitate IIIa-HCl, mp 206–209°; IR (KBr): 2600 broad (NH salt) cm^{-1}.

Anal.—Calc. for $C_8H_7N_3OS$·HCl: Cl, 15.44. Found: Cl, 15.15.

3-Arylideneimino-2-imino-3,4-dihydro-2*H*-1,3-benzothiazin-4-ones (V and VI)—A solution of 5.0 mmoles of IIIa and 5.0 mmoles of *p*-nitrobenzaldehyde in 50 ml of methanol containing 0.5 ml of acetic acid was refluxed for 2 hr, concentrated *in vacuo* to 20 ml, and filtered. The yellow solid (1.2 g, 76%) was recrystallized from dimethyl sulfoxide to yield the analytical sample of V, mp 307.5–308.5°.

Anal.—Calc. for $C_{15}H_{10}N_4O_3S$: N, 17.17. Found: N, 17.35.

In a similar fashion, 5.0 mmoles of IIIa and 5.0 mmoles of *p*-dimethylaminobenzaldehyde were reacted to yield VI (0.80 g, 48%), mp 264–265°.

Anal.—Calc. for $C_{17}H_{16}N_4OS$: N, 17.27. Found: N, 17.55.

3-Amino-3,4-dihydro-2*H*-1,3-benzothiazin-2,4-dione (VII)—A hydrolysis solution of 2 ml of acetic acid, 35 ml of ethanol, 10 ml of chloroacetaldehyde, and 10 ml of water was employed to dissolve 1.9 g (0.01 mole) of IIIa. The solution was then warmed to 45–55° and held at that temperature for 20 hr, during which time a buff-white solid precipitated. This solid, VII (1.07 g, 55%), was filtered and recrystallized from methanol, mp 199.0–199.5°; IR (KBr): 3310, 3245 (NH), and 1688 (C=O) cm^{-1}.

Anal.—Calc. for $C_8H_6N_2O_2S$: C, 49.48; H, 3.11; N, 14.42. Found: C, 49.85; H, 3.22; N, 14.80.

3-(4-Nitrobenzylideneimino)-3,4-dihydro-2*H*-1,3-benzothiazin-2,4-dione (VIII)—After refluxing equimolar (1.0 mmole) amounts of VII and *p*-nitrobenzaldehyde in 20 ml of methanol containing 5 drops of acetic acid for 1 hr, a 67% yield of VIII was obtained. Recrystallization from methanol gave the analytical sample, white microneedles, mp 220.5–221.5°; IR (KBr): no N—H bands, 1690 (C=O) cm^{-1}.

Anal.—Calc. for $C_{15}H_9N_3O_4S$: C, 55.04; H, 2.77; N, 12.84. Found: C, 54.87; H, 2.81; N, 12.79.

1,2-Dihydro-*s*-triazolo[5,1-*b*]benzo[1,3]-thiazin-2,9-dione (IX)—A well-stirred solution of 5.0 mmoles of IIIa, 15 mmoles of triethylamine, and 50 ml of anhydrous tetrahydrofuran was treated to the dropwise addition over 15 min of 7.5 mmoles of phosgene in 6 ml of benzene. A solid began to precipitate immediately; after 3 hr of stirring, the crystals were removed by filtration, washed with cold methanol, and recrystallized from dioxane to yield 0.56 g (51%) of white solid, IX, mp 310–312°.

Anal.—Calc. for $C_9H_5N_3O_2S$: C. 49.31; H, 2.30; N, 19.17. Found: C, 49.25; H, 2.42; N, 19.12.

2-Phenyl-3-triazolo[5,1-*b*]benzo[1,3]-thiazin-9-one (X)—The reaction in 65 ml of refluxing dioxane of 5.0 mmoles of IIIa and 10 mmoles of benzoyl chloride after 4 hr of reflux produced an insoluble off-white solid. The reaction mixture was filtered hot to separate the product, X, 0.15 g or 11%, from the unreacted starting materials. Recrystallization from dioxane gave the analytical sample, mp 281–282°.

Anal.—Calc. for $C_{15}H_9N_3OS$: C, 64.50; H, 3.25; N, 15.04. Found: C, 64.26; H, 3.64; N, 14.82.

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[x] To whom inquiries should be directed.

[2] Melting points were obtained in capillaries on a Thomas-Hoover apparatus and are uncorrected. IR spectra, in pressed KBr disks, were obtained on a Perkin-Elmer model 257 spectrometer. Combustion analyses were provided by the George I. Robertson Microanalytical Laboratory, Florham Park, N.J.

Fluorinated Quinazolones III: Synthesis and CNS Depressant Activity of Fluorinated Quinazolone Derivatives

KRISHNA C. JOSHI *x, VIRENDRA K. SINGH *, DAULAT S. MEHTA *, RAMESH C. SHARMA ‡, and LALIT GUPTA ‡

Abstract □ The synthesis of 2-allylthio-3-(4′-fluorophenyl)-5,6,7,8-tetrafluoro-4-quinazolone, 2-*n*-propylthio-3-(4′-fluorophenyl)-5,6,7,8-tetrafluoro-4-quinazolone, 3-(4′-bromophenyl)-5,6,7,8-tetrafluoro-2,4-quinazolinedithione, and seven fluorinated 2-alkyl/phenyl-3-aryl-4(3*H*)-quinazolones is described. The synthesized compounds were screened for CNS depressant activity using pentobarbital sleeping time, pentylenetetrazol convulsions, and condition avoidance test as the parameters. Most of the screened compounds exhibited significant depressant activity.

Keyphrases □ Quinazolone derivatives, fluorinated—synthesis and CNS depressant activity □ CNS depressant activity—synthesis and evaluation of fluorinated quinazolone derivatives

Since the introduction of methaqualone [2-methyl-3-(*o*-tolyl)-4(3*H*)-quinazolone] into clinical therapy as a nonbarbiturate hypnotic drug (1, 2), various 4(3*H*)-quinazolones and thioquinazolones have been investigated for pharmacological activity, including analgesic (3, 4), antispasmodic (5, 6), antihistaminic (7), hypotensive (8), muscle relaxant (9), sedative (10–13), anesthetic (10–13), and tranquilizing (10–13) activities.

Recently, the synthesis and pharmacological evaluation of a number of fluorinated 2-alkyl-3-aryl-4(3*H*)-quinazolones, thioquinazolones (14, 15), 2-mercapto-3-alkyl/aryl- and 2-alkylthio-3-aryl-4(3*H*)-quinazolones, and 3-aryl-2,4-quinazolinedithione were reported. Some compounds exhibited pronounced CNS activity. This finding prompted the investigation of the polyfluorinated analogs of the more active fluorinated quinazolone derivatives. This paper reports the preparation and CNS depressant activity of such compounds.

EXPERIMENTAL[1]

Compounds I–III (Table I) were prepared by the condensation of *N*-acyl derivatives of tetrafluoroanthranilic acid with an appropriate aromatic amine in the presence of phosphorus trichloride. Compounds IV–VII were prepared similarly from *N*-acyl/aroyl derivatives of 3- or 4-fluoroanthranilic acids. Compounds VIII and IX were prepared in good yield by refluxing tetrafluoroanthranilic acid and the corresponding phenylisothiocyanate in ethanol. On treatment with allyl chloride and *n*-propyl chloride, VIII was converted into the corresponding allylthio (X) and *n*-propylthio (XI) derivatives, respectively.

The treatment of IX with phosphorus pentasulfide in dry xylene gave 3-(4′-bromophenyl)-5,6,7,8-tetrafluoro-2,4-quinazolinedithione (XII) in quantitative yield.

Synthesis—*2-Methyl-3-(4′-bromophenyl)-5,6,7,8-tetrafluoro-4(3H)-quinazolone (I, Table I)*—To a solution of *N*-acetyl-2,3,4,5-tetrafluoroanthranilic acid (0.01 mole) and *p*-bromoaniline (0.01 mole) in dry toluene (20 ml) was added a solution of phosphorus trichloride (0.46 g, 0.0033 mole) in 5 ml of dry toluene, dropwise with constant stirring over 20 min. The resulting solution was further refluxed for 4 hr with constant stirring. After cooling, the reaction mixture was treated with 20 ml of 10% aqueous sodium carbonate solution and steam distilled to remove the toluene. The precipitated solid mass was removed by filtration, washed well with water, and recrystallized from ethanol using activated charcoal.

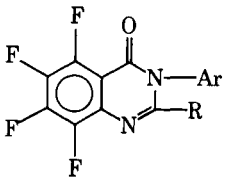
Other quinazolones (II–VII, Table I) were synthesized in a similar manner.

2-Mercapto-3-(4′-fluorophenyl)-5,6,7,8-tetrafluoro-4-quinazolone (VIII)—To a solution of tetrafluoroanthranilic acid (2.09 g, 0.01 mole) in 20 ml of ethanol, 4-fluorophenylisothiocyanate (1.53 g, 0.01 mole) was added. The resulting solution was heated under reflux for 2 hr when a crystalline solid mass was obtained. The solid mass was separated by filtration, washed with cold water, and recrystallized from acetic acid.

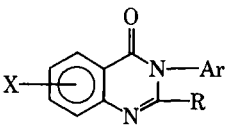
2-Mercapto-3-(4′-bromophenyl)-5,6,7,8-tetrafluoro-4-quinazolone (IX) was prepared in a similar manner.

2-Allylthio-3-(4′-fluorophenyl)-5,6,7,8-tetrafluoro-4-quinazolone (X)—To a solution of sodium hydroxide (0.63 g, 0.019 mole) in 15 ml of 50% ethanol, 2-mercapto-3-(4′-fluorophenyl)-5,6,7,8-tetrafluoro-4-quinazolone (1.77 g, 0.005 mole) was added and the mixture was stirred until a clear solution was obtained. Allyl chloride (0.456 g, 0.006 mole) was then added, and the solution was further stirred for 2 hr at room temperature. After cooling the resulting solution to 0°, the product was removed by filtration, washed with cold water, and recrystallized from aqueous ethanol to give a pure compound.

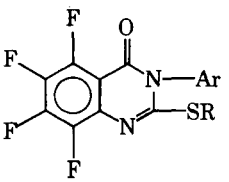
2-*n*-Propylthio-3-(4′-fluorophenyl)-5,6,7,8-tetrafluoro-4-quinazolone (XI) was prepared in a similar manner using *n*-propyl chloride.



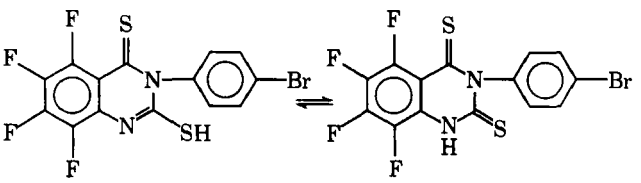
I: R = CH_3, Ar = 4-BrC_6H_4
II: R = CH_3, Ar = 2-$CH_3C_6H_4$
III: R = C_2H_5, Ar = C_6H_5



IV: X = 7-F, R = C_6H_5, Ar = C_6H_5
V: X = 7-F, R = C_6H_5, Ar = 2-$CH_3C_6H_4$
VI: X = 6-F, R = CH_3, Ar = 2-$CH_3C_6H_4$
VII: X = 7-F, R = CH_3, Ar = 2-$CH_3C_6H_4$



VIII: R = H, Ar = 4-FC_6H_4
IX: R = H, Ar = 4-BrC_6H_4
X: R = CH_2=CH—CH_2, Ar = 4-FC_6H_4
XI: R = CH_3—CH_2—CH_2, Ar = 4-FC_6H_4



XII

[1] All melting points are uncorrected. IR spectra were determined (Nujol mull) on a Perkin-Elmer model 337 grating IR spectrophotometer.

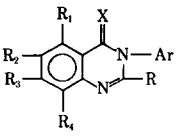


Table I—Fluorinated 2-Alkyl/phenyl-3-aryl-4(3*H*)-quinazolones and 2-Thio/alkylthio-3-aryl-4(3*H*)-quinazolones and Thioquinazolones

Compound	Ar	R	R_1	R_2	R_3	R_4	X	Molecular Formula	Analysis, %	Calc.	Found	Melting Point	Yield, %
I	4-BrC_6H_4	CH_3	F	F	F	F	O	$C_{15}H_7BrF_4N_2O$	N	7.23	7.17	165°	63
II	2-$CH_3C_6H_4$	CH_3	F	F	F	F	O	$C_{16}H_{10}F_4N_2O$	N	8.69	8.61	172°	50
III	C_6H_5	C_2H_5	F	F	F	F	O	$C_{16}H_{10}F_4N_2O$	N	8.69	9.02	98°	60
IV	C_6H_5	C_6H_5	H	H	F	H	O	$C_{20}H_{13}FN_2O$	N	8.84	8.69	153°	58
V	2-$CH_3C_6H_4$	C_6H_5	H	H	F	H	O	$C_{21}H_{15}FN_2O$	N	8.48	8.39	118°	69
VI	2-$CH_3C_6H_4$	CH_3	H	F	H	H	O	$C_{16}H_{13}FN_2O$	N	10.45	10.32	135°	58
VII	2-$CH_3C_6H_4$	CH_3	H	H	F	H	O	$C_{16}H_{13}FN_2O$	N	10.45	10.22	138°	62
VIII	4-FC_6H_4	SH	F	F	F	F	O	$C_{14}H_7F_5N_2OS$	N	8.13	8.01	82–83°	40
IX	4-BrC_6H_4	SH	F	F	F	F	O	$C_{14}H_7BrF_4N_2OS$	N	6.93	7.03	132–134°	35
X	4-FC_6H_4	CH_2=$CHCH_2S$	F	F	F	F	O	$C_{17}H_9F_5N_2OS$	N	7.27	6.94	85–86°	95
XI	4-FC_6H_4	*n*-C_3H_7S	F	F	F	F	O	$C_{17}H_{11}F_5N_2OS$	N	7.22	7.12	69–70°	86
XII	4-BrC_6H_4	SH	F	F	F	F	S	$C_{14}H_7BrF_4N_2S_2$	N	6.65	6.68	170–172°	38

3-(4′-Bromophenyl)-5,6,7,8-tetrafluoro-2,4-quinazolinedithione (XII)—A suspension of 2-mercapto-3-(4′-bromophenyl)-5,6,7,8-tetrafluoro-4-quinazolone (2.02 g, 0.005 mole) and phosphorus pentasulfide (1.21 g, 0.006 mole) in dry xylene (10 ml) was heated under reflux with constant stirring for 4 hr. After cooling, the reaction mixture was treated with sodium carbonate solution and steam distilled to remove the xylene. The precipitated solid was separated and recrystallized from ethanol to afford a pure sample.

The IR spectra of some typical compounds are: I, 1618, 1590, and 1478–1517 cm^{-1}; II, 1610–1620, 1588, and 1480–1520 cm^{-1}; III, 1610, 1560–1590, and 1490–1520 cm^{-1}; IV, 1610, 1560–1595, and 1490–1520 cm^{-1}; and V, 1618, 1570–1590, and 1480 cm^{-1}. These absorption bands correspond to the quinazolone identifying bands (16). Similar quinazolone identifying bands were observed with the other quinazolones reported in this paper.

Biological—All compounds reported in Table I, except VIII and IX, were screened for CNS depressant activity by three tests.

Pentobarbital Sleeping Time—The method followed was similar to one used by Arora *et al.* (17). Albino mice, 24–30 g, were divided into 11 groups of four animals each. The first group received pentobarbital only (40 mg/kg ip) and served as the control group, while the other 10 groups received 10 different drugs at a dose of 100 mg/kg ip. The drugs were given 30 min prior to pentobarbital (40 mg/kg ip).

After pentobarbital administration, the stopwatch was started; as soon as the mice lost the righting reflex, they were kept on their backs separated from each other. The time from the loss of the righting reflex to the regaining of the reflex was noted as the sleeping time.

The results are given in Table II. The most potent compound was XI. Compounds IV and VII were also potent but were not as effective as XI. Compound I exhibited some activity.

Pentylenetetrazol[2] *Convulsions*—The method of Swinyard *et al.* (18) was used for the evaluation of this activity. Albino mice of either sex, 18–25 g, were randomly divided into 11 groups. Each group consisted of five animals. Group I served as the control group and was injected with an equal volume of solvent. The remaining 10 groups received the test compounds at 100 mg/kg ip. The animals were maintained on an adequate diet and were allowed free access to food and water except during the short time when they were removed from their cages for testing. The animals were tested 30 min after drug administration, since the peak effect of these compounds, determined from preliminary experiments, was between 25 and 40 min.

Seizures were induced by a subcutaneous injection of 85 mg/kg of pentylenetetrazol in agar solution. This dose produces maximal convulsions in control mice. The effectiveness of the drugs was studied by their ability to prevent the tonic phase of the seizures. Animals were observed for 30 min only after pentylenetetrazol administration.

The results of the screening data are recorded in Table III. As in the previous test, XI was the most potent anticonvulsant and IV and VII were also potent. Compounds III, IV, and XII increased the initial time for the onset of tonic phase.

Condition Avoidance Tests in Rats—The equipment employed was similar to that used by Cook and Wiedley (19). The uncondi-

[2] Metrazole.

Table II—Comparative Sleeping Times of the Screened Compounds

Compound	Mean Sleeping Time, min ± *SE*	*t*[a]	*p*
Control	21.50 ± 2.40	—	—
I	48.25 ± 5.51	4.40	<0.01
II	27.00 ± 4.20	1.20	>0.1
III	21.50 ± 3.17	0	—
IV	85.50 ± 10.96	6.30	<0.001
V	25.00 ± 2.08	1.10	>0.15
VI	29.50 ± 3.40	1.90	>0.1
VII	73.00 ± 12.87	3.90	<0.01
X	28.25 ± 1.75	2.20	>0.05
XI	100.50 ± 20.19	3.90	<0.01
XII	29.25 ± 4.13	1.60	>0.1

[a] Student *t* test.

Table III—Comparative Anticonvulsant Activity of Screened Compounds by Maximal Pentylenetetrazol Seizure Method

Group	Compound[a]	Protection, %	Mean Time for Onset of Tonic Phase, min ± *SE*	*p* for Onset of Tonic Phase
1	Control	0	11.00 ± 0.872	—
2	I	20	16.25 ± 2.27	>0.05
3	II	20	12.75 ± 1.97	>0.1
4	III	20	19.50 ± 3.03	<0.15
5	IV	40	17.33 ± 1.77	<0.01
6	V	20	13.25 ± 2.04	>0.1
7	VI	20	14.50 ± 1.32	>0.05
8	VII	60	16.00 ± 4.00	>0.1
9	X	20	15.00 ± 2.04	>0.05
10	XI	100	—	—
11	XII	20	15.00 ± 1.58	<0.05

[a] For the control group, the solvent was administered in an equal volume. All other groups received 100 mg/kg ip of the test compound.

Table IV—Comparative Condition of Avoidance Response in Rats with Screened Compounds

Group	Compound[a]	Animals Showing Absence of Conditioned Response, %
1	I	60
2	II	40
3	III	20
4	IV	80
5	V	20
6	VI	40
7	VII	80
8	X	20
9	XI	80
10	XII	40

[a] 100 mg/kg ip.

tioned response was provided to the rats through a grass stimulator (0.1 mamp of 50 cycles ac at 150 v) for 5 sec; a buzzer was used as a conditioning stimulus 12 sec earlier. Rats of either sex, 150–200 g, were trained by an electric shock to climb a pole (unconditioned response) and were then conditioned by the buzzer maintained for 15 sec to climb a pole.

Such conditioned rats were divided into 10 groups of five each. Each group received different drugs at 100 mg/kg ip. Thirty minutes after drug administration, the test was performed for observing the conditioned avoidance response.

The results of screening are recorded in Table IV; VII, XI, and IV were equally potent and I, II, VI, and XII also showed some activity.

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Monoamine Oxidase Inhibiting Activity of a Series of (±)-4-Methoxy-β-hydroxyphenethylamines

GARY G. FERGUSON ˣ and WILLIAM J. KELLER

Abstract □ The synthesis and selected pharmacological testing of (±)-4-methoxy-β-hydroxyphenethylamine [1-(4-methoxyphenyl)-2-aminoethanol] and its *N*-methylated derivatives are presented. Members of this series were found to exert partial prevention of reserpine-induced hypothermia in mice and to inhibit monoamine oxidase in Warburg studies. Activity was essentially dose dependent. The secondary amine was the most active member of the series. The tertiary amine was least active, and the primary amine exhibited intermediate activity.

Keyphrases □ (±)-4-Methoxy-β-hydroxyphenethylamine and *N*-methyl derivatives—synthesis, screened for monoamine oxidase inhibition □ Monoamine oxidase inhibition—synthesis and screening of (±)-4-methoxy-β-hydroxyphenethylamine and *N*-methyl derivatives □ β-Hydroxyphenethylamines, 4-methoxy and *N*-methyl derivatives—synthesis, screened for monoamine oxidase inhibition

The cactus genus *Coryphantha* is known to produce a variety of β-hydroxyphenethylamines (1–7). 4-Methoxy-β-hydroxyphenethylamine [1-(4-methoxyphenyl)-2-aminoethanol], for instance, has been detected in extracts of *Coryphantha cornifera* (DC.) Br. and R. var. *echinus* (Engelm.) L. Benson (1). This compound and its *N*-methylated derivatives have been synthesized and used in this laboratory as chromatographic references when screening extracts of *Coryphantha* species for the presence of alkaloids.

Prior to its detection in a plant system, 4-methoxy-β-hydroxyphenethylamine was reported to be a weak vasoconstrictor and, in large doses, a cardiac depressant (8). The lack of additional reports concerning the pharmacology of this compound and its *N*-methyl homologs prompted a pharmacological investigation. Preliminary studies of hexobarbital sleeping times in mice suggested the possibility of monoamine oxidase inhibition. The present report describes the monoamine oxidase-inhibiting activity of (±)-4-methoxy-β-hydroxyphenethylamine, (±)-*N*-methyl-4-methoxy-β-hydroxyphenethylamine [1-(4-methoxyphenyl)-2-(methylamino)ethanol], and (±)-*N,N*-dimethyl-4-methoxy-β-hydroxyphenethylamine [1-(4-methoxyphenyl)-2-(dimethylamino)ethanol].

EXPERIMENTAL

Synthesis—Synthesis of racemic 4-methoxy-β-hydroxyphenethylamine hydrochloride (mp 168–169°), *N*-methyl-4-methoxy-β-hydroxyphenethylamine hydrochloride (mp 117–118°), and *N,N*-dimethyl-4-methoxy-β-hydroxyphenethylamine hydrochloride (mp 145°) involved a Houben–Hoesch condensation of anisole[1] with aminoacetonitrile hydrochloride[2], *N*-methylaminoacetonitrile hydrochloride[2], and *N,N*-dimethylaminoacetonitrile hydrochloride[2], respectively, followed by a sodium borohydride reduction of the ketone intermediate (9). The UV, IR, and NMR spectra verified the structure of the desired product in each case.

[1] Aldrich Chemical Co.
[2] K and K Laboratories.

Table I—Effects of Compounds in Prevention of Reserpine-Induced Hypothermia

Compound	Control[a]	Experimental[a]	Reference[a] (Iproniazid)
Unsubstituted	7.1 ± 0.6[b]	4.3 ± 0.9[b]	4.1 ± 1.3[b]
N-Methyl substituted	8.1 ± 0.7[b]	4.3 ± 0.7[b]	3.6 ± 0.7[b]
N,N-Dimethyl substituted	6.2 ± 0.5[b]	3.8 ± 1.3[b]	4.1 ± 1.3[b]

[a] Values represent changes in rectal temperature after 6 hr. [b] Mean ± standard error. Differences between control–experimental and control–reference groups were significant at $p \leq 0.05$. Controls received distilled water in a volume equal to that of the drug solution. All animals received reserpine (2.5 mg/kg ip) 1 hr after pretreatment. For details of treatment, see text.

Preliminary Studies—Initial screening for possible monoamine oxidase inhibition was done using the reserpine reversal technique of Pletscher (10). Male, Swiss–Webster albino mice, 21–54 g, were randomly divided into three groups of nine animals each for each study and were injected intraperitoneally as follows:

Group 1 (Control)—Distilled water (same volume as drug solution) 1 hr prior to injection of reserpine (2.5 mg/kg ip).

Group 2 (Experimental)—Phenethylamine derivative (200 mg/kg) 1 hr prior to injection of reserpine (2.5 mg/kg ip).

Group 3 (Reference)—Iproniazid (100 mg/kg) (reference compound) 1 hr prior to injection of reserpine (2.5 mg/kg ip).

Immediately after injection with reserpine, all animals were placed in individual plastic restraining housings. Rectal temperatures were monitored initially and at hourly intervals for 6 hr, using electronic thermometers[3].

Warburg Studies—As a further evaluation of possible monoamine oxidase inhibition, the effects of the compounds on the oxidation of tyramine hydrochloride[4] by rat brain mitochondria were investigated. Brain mitochondria (containing monoamine oxidase) were prepared from albino Wistar female rats, 150–200 g, according to the method of Brody and Bain (11), and monoamine oxidase activity was determined in the presence and absence of the respective compounds by conventional manometric techniques (12)[5].

In the manometric determinations, the main compartment of the Warburg flasks contained the compound studied (or buffer), 1 ml of mitochondrial suspension (representing 500 mg wet weight of original brain tissue), and sufficient 0.01 *M* phosphate buffer (pH 7.4) to make a total volume of 2.7 ml. The side arm of each flask contained 0.3 ml of 0.1 *M* tyramine hydrochloride. After the flasks were allowed to equilibrate for 15 min at 37°, the manometer valves were closed and the side arm contents of each were tipped in.

Manometers were set at 150 mm, and readings were taken at 15-min intervals for 90 min. Values for the 90-min reaction period were obtained for each flask and were multiplied by the respective flask constants after adjusting for changes in the thermobarometer. The total microliters of oxygen uptake for each flask was thus obtained.

Statistical Analysis—The mean ± standard error was determined for each control or treated group. Values thus obtained were compared, using the difference between the means and the standard error of the difference between the means for the respective control-treated pairs of groups. The Student *t* test was applied to the results, and the probability was determined.

[3] Telethermometer model 43TA with probes, model 402, Yellow Springs Instrument Co.
[4] Eastman Kodak Co.
[5] Precision Warburg apparatus, Precision Scientific Co.

Table II—Effects of Compounds on Oxidation of Tyramine Hydrochloride by Rat Brain Mitochondria

Compound	Concentration, *M*	Control[a]	Experimental[a]	Inhibition, %	*t* Value[b]
Unsubstituted	0.003	35.0 ± 1.6	28.5 ± 2.0	19	2.5
	0.006	42.6 ± 1.6	29.5 ± 1.2	31	6.6
	0.012	25.7 ± 1.1	12.0 ± 1.0	53	9.5
	0.024	40.6 ± 1.5	16.5 ± 0.8	59	14.8
N-Methyl substituted	0.003	48.9 ± 3.4	31.9 ± 2.5	35	4.0
	0.006	44.9 ± 3.0	26.0 ± 2.2	42	5.0
	0.012	60.6 ± 2.5	27.9 ± 1.5	54	11.3
	0.024	77.2 ± 5.0	23.5 ± 1.3	70	10.5
N,N-Dimethyl substituted	0.013	30.4 ± 1.4	24.8 ± 1.5	18	2.7
	0.025	27.5 ± 2.0	20.2 ± 1.1	27	3.2
	0.050	32.0 ± 1.7	20.5 ± 1.4	36	5.2
	0.100	33.3 ± 2.3	13.3 ± 1.2	61	7.7

[a] Each value represents the microliters of oxygen uptake (mean ± standard error) for 12 flasks. [b] All differences between means were significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

In studies of reserpine reversal (prevention or partial prevention of reserpine-induced hypothermia), the compounds produced significant ($p \leq 0.05$) effects at a dose of 200 mg/kg (Table I). Iproniazid, a known monoamine oxidase inhibitor, also partially prevented reserpine-induced hypothermia at a dose of 100 mg/kg ($p \leq 0.05$). While these results are not conclusive proof of monoamine oxidase inhibition by the compounds, they do suggest the possibility, since monoamine oxidase inhibitors usually exhibit the reserpine-reversal phenomenon (10).

Direct measurements of inhibition using Warburg studies showed that all three compounds inhibited monoamine oxidase in the concentration ranges employed (Table II). (±)-4-Methoxy-β-hydroxyphenethylamine hydrochloride, in concentrations of 0.003–0.024 *M*, produced significant ($p \leq 0.05$) inhibition of monoamine oxidase when compared to controls. Likewise, (±)-*N*-methyl-4-methoxy-β-hydroxyphenethylamine hydrochloride, in concentrations of 0.003–0.024 *M*, and (±)-*N,N*-dimethyl-4-methoxy-β-hydroxyphenethylamine hydrochloride, in concentrations of 0.013–0.1 *M*, also significantly inhibited monoamine oxidase.

A number of compounds possessing a phenethylamine nucleus are capable of inhibiting monoamine oxidase (13). The phenyl and nitrogen moieties of these compounds are usually unsubstituted. Phenylethanolamine derivatives exhibiting monoamine oxidase inhibition have all been unsubstituted (14). All *para*-methoxylated phenylethanolamines examined in this study inhibited monoamine oxidase. The fact that the secondary amine exhibited the greatest activity, followed by the primary amine, supports previous data. Based on available information, further speculation dealing with competition for the site of action on the enzyme and reversibility of the binding at this site would be difficult since these parameters are dependent on the particular substrate and tissue examined.

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Effect of Plasma Protein Binding on Elimination of Bilirubin

Keyphrases □ Bilirubin—effect of plasma protein binding on elimination □ Protein binding, plasma—effect on elimination of bilirubin

To the Editor:

It is generally appreciated that interindividual differences in plasma protein binding can have a pronounced effect on the elimination kinetics of extensively protein bound drugs, but relatively little is known about the quantitative aspects of this relationship. Levy and Yacobi (1) recently presented an analysis of the pharmacokinetic aspects of plasma protein binding, which led to the prediction of a linear relationship between total plasma (or serum) clearance (P.C.) and the free fraction (f) of a highly protein bound drug in the plasma:

$$\text{P.C.} = k''f \qquad \text{(Eq. 1)}$$

where k'' is an intrinsic clearance constant for the free drug. Experimental support for this hypothesis was presented by demonstrating an excellent linear relationship between the total serum clearance of warfarin and the free fraction of this drug in the serum of individual rats (1).

We now report the results of a study of the relationship between plasma protein binding and total plasma clearance of bilirubin, another highly protein bound substance and one that is eliminated by conjugative pathways (2) rather than by oxidative processes [as is the case with warfarin (3)]. Seven adult male Sprague–Dawley rats, 335–374 g, received an infusion of ^{14}C-bilirubin (4) into the right jugular vein. An infusion rate of 0.8 mg/kg/min was maintained for 150–240 min. Three animals received an initial "loading" infusion of 2 mg/kg/min for 15 min.

Figure 1—*Relationship between total plasma clearance of bilirubin and the fraction of free bilirubin in the plasma of individual rats. The regression line, obtained by the vector least-squares method (7), is described by the equation P.C. = 0.19 + 7880f.*

Blood samples (0.6 ml) were obtained at 2, 3, and 4 hr or at 1, 2, and 2.5 or 3 hr and were assayed for free (5) and total (6) unconjugated bilirubin. The total plasma clearance of bilirubin was calculated by dividing the infusion rate by the steady-state concentration of total bilirubin in plasma.

Essentially constant concentrations of bilirubin in plasma were obtained within 60–120 min after the start of infusion. Steady-state concentrations were determined by averaging the concentrations of the three plasma samples; they differed by less than 6% on the average in individual animals. The steady-state concentrations of bilirubin in the plasma ranged from 11.7 to 20.7 mg/100 ml, and the total plasma clearance ranged from 3.9 to 6.8 ml/kg/min in the seven rats.

The free fraction of bilirubin in plasma, representing the mean of three separate determinations for each animal, ranged from 4.3×10^{-4} to 9.0×10^{-4}, equivalent to from 99.910 to 99.957% protein binding. There was a strong ($r = 0.82$) and statistically highly significant ($p < 0.02$) linear correlation between plasma clearance and free fraction of bilirubin in the plasma of individual rats (Fig. 1).

The results of this investigation are consistent with the theoretical model of Levy and Yacobi (1) and show that the elimination rate of bilirubin is proportional to the concentration of free rather than total (*i.e.*, free and protein bound) bilirubin in plasma.

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REVIEWS

Emulsions and Emulsion Technology, Part I (Volume 6 in Surfactant Science Series). Edited by KENNETH J. LISSANT. Marcel Dekker, 305 E. 45th St., New York, NY 10017, 1974. 440 pp. 15.5 × 23 cm. Price $39.50.

The purpose of the book is to attempt to bring the status of emulsion technology up to date and to introduce the concept of emulsions to a wider group of people.

The first three chapters are introductory. Chapter 1 is entitled Basic Theory (by K. J. Lissant) and is unique in that it deliberately avoids most of the classical mathematical derivations and concentrates on a geometrical approach. This approach was chosen since many emulsion systems behave as though their performance properties were more dependent on their physical and topological configuration than on the chemical properties of their constituents. Thus, emulsions are discussed with regard to low-, medium-, and high-internal-phase-ratio type classifications rather than to classify them according to type of emulsifier employed.

Chapter 2 is entitled Making and Breaking Emulsions (by K. J. Lissant). This chapter also uses the geometric approach and is geared to answer the following questions:

1. Is an emulsion really needed, or would a solution or suspension do a better job?
2. If an emulsion is required, what type would be most suitable?
3. What emulsifiers and what equipment should be used?
4. When dealing with an undesirable emulsion, what techniques should be used for breaking such emulsions?

Chapter 3 is entitled Microemulsions (by L. M. Prince). This still controversial topic is treated well by Prince. Both theoretical and practical aspects are discussed and although some may disagree with the theories presented, it provides a good working basis for practical application.

A reading of the first three chapters should provide enough theoretical basis for the remaining five chapters which deal with applications in specialized areas of interest. These chapters are entitled Agricultural Emulsions (by P. L. Lindner), Food Emulsions (by M. W. Lynch and W. C. Griffin), Medicinal Emulsions (by B. A. Mulley), Emulsion Paints (by G. Allyn), and Asphalt Emulsions (by R. L. Ferm).

Each of these chapters is well written and extensively referenced. While the terminology varies slightly in each area, this does not present a major problem. Part II (not yet available) will deal with additional specialized areas of interest. These include Emulsion Polymerization, Emulsions in the Paper Making Industry, Emulsions in Printing and the Graphic Arts, Hydraulic Fluid Emulsions, and Emulsions in the Cosmetic Industry.

In summary, the book is recommended to all those in industry who must have a working knowledge of emulsions in any of the specialized areas mentioned. Those who want a more fundamental, theoretical basis would be well off first purchasing a text geared to this need such as "Emulsions, Theory and Practice" by Paul Becher.

Reviewed by Norman D. Weiner
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Synthetic Methods of Organic Chemistry, Volume 28, Yearbook 1974. WILLIAM THEILHEIMER, Editor. S. Karger AG, Medical and Scientific Publishers, Arnold-Böcklin-Strasse 25, CH-4011 Basel, Switzerland, 1974. xx + 652 pp. (Albert J. Phiebig, Inc., U.S. Representative of S. Karger, P.O. Box 352, White Plains, NY 10602) 16 × 23 cm. Price $178.75.

Volume 28 of *Synthetic Methods of Organic Chemistry* is the latest volume of a multivolume work which has probably by this time become a standard reference in the field of organic synthesis.

The main portion of the work is a 476-page section devoted to abstracts of descriptions of the methods for the synthesis of specific compounds. The abstracts are of articles published in selected major journals, generally during 1971–1973. Accompanying almost every abstract is a brief structural representation depicting the starting material(s) and the product of the reaction sequence. This affords a rapid visualization of the structural change achieved by the synthetic method. The abstracts are highly but effectively condensed. They present the principal points of information (reactants, solvents, yield, *etc.*) of the method which allow one to judge if that particular synthetic method may or may not be helpful in the formulation of his or her own particular synthetic procedures. If a method appears to have utility, the details can then be found by consulting the original publication for which the literature citation is given after each abstract.

The method of classification of the abstracts is highly systematized. In brief, the major divisions are decided by the bonds formed between elements in the course of the reaction. These divisions are subdivided by the four ways by which the bonds are considered to be formed (addition, rearrangement, exchange, and elimination). A further subdivision is then made based upon the reagent used in the reaction.

The classification system and the abbreviations and symbols it employs, although logical, are by necessity somewhat arbitrary, and so the classification scheme requires some study and practice for mastery and easy usage. If one wishes to avoid using the classification system to find an abstract, he or she may proceed alternatively *via* the extensive subject index (pages 477–612). Here, classes of compounds, methods, and reagents are alphabetically listed along with references to where the appropriate abstracts are found in the main portion of the work. The subject index is cumulative for volumes 26, 27, and 28.

The cross-referencing of this nature contributes greatly to the value of the work by facilitating the location of the abstracts. Also, the abstracts themselves appear to represent a good cross-section of specific organic synthetic conversions, although specialists in certain areas of synthesis might possibly detect that their area of specialization is not treated as thoroughly as they might wish.

Synthetic Methods of Organic Chemistry impresses, then, as a work of considerable value to the medicinal chemist engaged in the synthesis of potentially bioactive compounds. Its value lies principally in the fact that it affords a quick entry into the literature of organic synthesis, especially when one wants to find a specific method for the synthesis of a specific compound. The cost probably precludes its inclusion in a personal library, but it at least ought to be, in this reviewer's opinion, readily accessible in an institutional or company library.

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BIOAVAILABILITY DATA—USEFUL OR USELESS?

We are confused! The "we" here is an editorial "we" and is not intended to reflect upon anyone beyond the writer of this column—although there appears to be ample justification for many of our colleagues and associates to share in our confusion.

Readers of this Journal know that bioavailability has been a leading subject of interest within the pharmaceutical sciences community for at least the past 10 years. Numerous articles have been published on the subject, several conferences have been conducted, various reports have appeared, a number of symposia have been presented, and, in general, reference to drug bioavailability has regularly appeared in speeches, testimony before congressional committees, and filings made to regulatory agencies.

The APhA Academy of Pharmaceutical Sciences was largely responsible for the definition which seems to have won general acceptance; namely, bioavailability means the rate and extent to which the therapeutic moiety is absorbed from a drug product and becomes available to the site of drug action.

Consequently, determination of bioavailability can be an important factor in evaluating the effectiveness and expected performance of a drug product. By the same token, determination of the respective bioavailabilities of two or more different products can be an important means of objectively and critically comparing the effectiveness and expected performance of such products.

On the basis of this approach, or strategy, in comparatively evaluating drug products, APhA launched its "Bioavailability Project." This program was developed with the active assistance of both the scientific and practitioner components of the Association membership. Moreover, the background chapter and individual drug monographs that have been published to date seem to have been well received by all elements of the profession.

More recently, the Food and Drug Administration indicated that it, too, regards this general approach as a viable and valid one in determining the suitability of a drug product and the equivalence of multiple products. This was clearly revealed in the agency's proposed regulations published in late June pertaining specifically to "Bioavailability" and "Bioequivalence." APhA and its Academy of Pharmaceutical Sciences reviewed these proposed regulations and gave them a solid vote of approval.

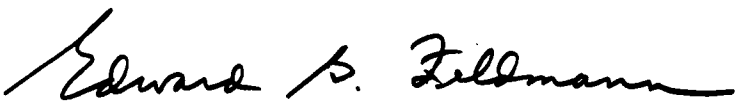
So far, so good. It looked like everyone finally was on the same track and now in agreement on this long-debated, emotionally charged issue.

At least, it looked that way until we saw a recent full-page advertisement proclaiming in big bold print: "Bioavailability Data is Useful . . . as a measure of the absorption of a drug product . . . but not as a measure of equivalence between two or more products."

If bioavailability data are not useful as one of the factors to be considered in comparing, evaluating, and measuring the equivalency between drug products, then we have somehow been misled. And it seems to us like a lot of other people and groups concerned with drug quality have likewise been fooled.

The source of this ad is a major pharmaceutical firm with a large battery of highly qualified scientific personnel.

So we are confused. Does this company really mean to say that bioavailability data aren't useful in comparing product equivalence? Or is this company actually trying to discourage or dissuade pharmacy practitioner readers from utilizing bioavailability data in making product comparisons now that competing firms are releasing such data and inviting objective comparisons based upon hard data rather than catchy slogans?



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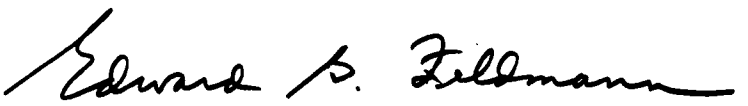

Digoxin Tablets Dissolution Test

The Communication to the Editor from Ylitalo *et al.*[1] in the July 1975 issue and, to a lesser degree, the earlier article by Klink *et al.*[2] appeared to embody a basic misunderstanding of compendial specifications.

Specifically, both of these articles report findings to the effect that certain batches of digoxin tablets failed to pass the dissolution specifications in the USP but yet were found on *in vivo* testing to give satisfactory serum concentrations of the drug.

Although the respective authors did not explicitly so state, the implications were that the USP specification was unsatisfactory or unsuitable and, specifically, that the dissolution requirement did not correlate with *in vivo* performance.

Dissolution specifications, as in the case of all other *in vitro* specifications in the official compendia, are intended to be established at a level, or to be designed in a manner, so that all clinically unsatisfactory products will be excluded. In doing so, it is recognized that, to provide such a level of assurance, at least some otherwise clinically acceptable lots will be excluded also.

For example, typical tablet monographs will specify that the assay show between 95 and 105% of the label amount of the active ingredient to be present. In establishing this tolerance level, it is recognized that a lot assaying 93 or 90%, or perhaps even 85%, may be clinically satisfactory but will nonetheless be rejected on the basis of this *in vitro* specification.

The point is that we do not generally have dose–response information that is so precise, or control over the biological system that is so complete, that a broader range can be safely established. As a consequence, a specification is adopted based upon the biological information that is known, and the tolerances are then drawn sufficiently tight so as to err on the safe side.

Consequently, the batches of digoxin tablets described by the above-mentioned authors were formulated in a manner that fell into a gray zone, where they were not good enough to pass the compendium *in vitro* specification but yet were not quite bad enough to exhibit *in vivo* unsuitability.

In conclusion, the two reports cited here should not be interpreted as reflecting adversely on the USP digoxin tablet specification. On the contrary, these reports demonstrate that the specification is sufficiently stringent that products passing the test can be regarded with confidence as being biologically and, therefore, clinically satisfactory.

Edward G. Feldmann

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[1] P. Ylitalo, G. Wilén, and S. Lundell, *J. Pharm. Sci.*, **64**, 1264(1975).
[2] P. R. Klink, R. I. Poust, J. L. Colaizzi, and R. H. McDonald, Jr., *ibid.*, **63**, 1231(1974).

1975

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Symposium on Assay of Drugs and Other Trace Substances in Biological Fluids, University of Surrey, Guildford, U.K. (Contact Dr. E. Reid, Wolfson Bioanalytical Centre, University of Surrey, Guildford GU2 5XH, U.K.)

October 1–5
American College of Apothecaries, Annual Meeting, Four Seasons Sheraton, Toronto, Ontario, Canada

October 6–8
ASTM Committee E-19 on Chromatography, Annual Meeting, Sheraton-Houston, Houston, Tex. (Contact Mr. Jack Stamm, Instrument Products Division, Photo Products Department, Du Pont Co., Wilmington, DE 19898)

October 6–10
Federation of Analytical Chemistry and Spectroscopy Societies, Annual Meeting, American Chemical Society Division of Analytical Chemistry, Indiana Convention–Exposition Center, Indianapolis, Ind. (Contact I. J. Holcomb, Parke-Davis Co., P.O. Box 118, Detroit, MI 48232)

October 13
Symposium on Cosmetic Analytical Techniques, Marriott Motor Hotel, Twin Bridges, Washington, D.C. (Contact F. F. Kohlhepp, Carter Products, Half Acre Road, Cranbury, NJ 08512)

October 13–16
Association of Official Analytical Chemists, Annual Meeting, Marriott Hotel, Twin Bridges, Washington, D.C. (Contact L. G. Ensminger, Association of Official Analytical Chemists, Box 540 Benjamin Franklin Station, Washington, DC 20044)

October 17
APhA Academy of Pharmaceutical Sciences, Annual Eastern Regional Meeting, Governor Morris Inn, Morristown, N.J. (Contact Dr. Daniel Mufson, USV Pharmaceutical Corp., 1 Scarsdale Road, Tuckahoe, NY 10707)

October 19–21
Pharmaceutical and Analytical Technology 1975 as Related to Current Official Regulations, Society of Pharmacists in Industry Seminar, St. Louis Sheraton Westport Inn, St. Louis, Mo. (Contact Mr. Chris Christensen, Rexall Drug Company, 3901 N. Kingshighway, St. Louis, MO 63115)

October 20–22
Canadian Conference on Medical Devices in Health Protection, Government Conference Centre, Rideau Street, Ottawa, Ontario, Canada (Contact Dr. A. K. DasGupta, Health Protection Branch, Tunney's Pasture, Ottawa, Ontario K1A OL2, Canada)

October 23–24
Symposium on Steroid Hormones: Molecular Structure and Protein Interactions, Buffalo, N.Y. (Contact W. L. Duax, Medical Foundation of Buffalo Research Laboratories, Buffalo, NY 14203)

October 23–24
Technical Conference on New Advances in Separation Technology, Hilton Inn, Tarrytown, N.Y. (Contact W. I. Heneghan, 289 Greenwich Ave., Greenwich, CT 06830)

November 2–6
Society for Neuroscience, Annual Meeting, New York, N.Y. (Contact Neuroscience Meeting Headquarters, Room 200 A, Lee Building, 9650 Rockville Pike, Bethesda, MD 20014)

November 3–6
International Symposium on Advances in Chromatography, Sheraton-Munich Hotel, Munich, Germany (Contact A. Zlatkis, Chemistry Department, University of Houston, Houston, TX 77004)

November 3–7
Reliability Engineering and Management Institute, College of Engineering, Special Professional Education, University of Arizona, Tucson, Ariz. (Contact Roy G. Post, Special Professional Education, University of Arizona, Tucson, AZ 85721)

November 16–20
American Public Health Association, Annual Meeting, Chicago, Ill. (Contact William H. McBeath, APHA, 1015 18th St., N.W., Washington, DC 20036)

November 16–20
APhA Academy of Pharmaceutical Sciences, National Meeting, Marriott Motor Hotel, Atlanta, Ga.

November 21
Eastern Analytical Symposium, Statler-Hilton Hotel, New York, N.Y. (Contact Webster R. Hudgins, Allied Chemical, P.O. Box 1021R CRL, Morristown, NJ 07960)

November 29–December 4
American Medical Association, Clinical Meeting, Honolulu, Hawaii (Contact James H. Sammons, 535 N. Dearborn St., Chicago, IL 60610)

November 30–December 4
International Symposium on Neurotoxicology, Columbia-Presbyterian Medical Center, New York, N.Y. (Contact Dr. Leon Roizin, Psychiatric Institute, 722 W. 168th St., New York, NY 10032)

November 30–December 5
American Chemical Society, National Meeting, Mexico City, Mexico (Contact Meetings Department, 1155 16th St., N.W., Washington, DC 20036)

December 7–11
American Society of Hospital Pharmacists, Midyear Meeting, Washington, D.C.

1976

January 11–14
International Symposium on Quantum Biology and Quantum Pharmacology with Particular Reference to Molecular Diseases, Sanibel Island, Fla. (Contact J. R. Sabin, Williamson Hall, University of Florida, Gainesville, FL 32611)

February 18–24
American Association for the Advancement of Science, Annual Meeting, Boston, Mass. (Contact Ms. Elizabeth Zeutschel, AAAS, 1776 Massachusetts Ave., Washington, DC 20036)

March 1–5
Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Convention Center, Cleveland, Ohio

April 3–8
APhA Annual Meeting, New Orleans, La.

April 4–7
Proprietary Association, Annual Meeting, The Breakers, Palm Beach, Fla.

April 4–9
Centennial American Chemical Society Meeting, New York, N.Y. (Contact A. T. Winstead, American Chemical Society, 1155 16th St., N.W., Washington, DC 20036)

April 10–13
Society for Biomaterials, Annual Meeting, and International Biomaterials Symposium, Philadelphia, Pa. (Contact S. R. Pollack, Department of Metallurgy and Materials Science, University of Pennsylvania, Philadelphia, PA 19174)

April 18–24
Basic Pharmacokinetics and Biopharmaceutics Workshop, American Society of Clinical Pharmacology and Therapeutics, St. Augustine, Fla. (Contact Irene Garrett, Administrative Associate, 1826 N.W. 26th Way, Gainesville, FL 32605)

May 17
APhA Academy of Pharmaceutical Sciences, Annual Midwestern Regional Meeting, Industrial Pharmaceutical Technology and Pharmaceutical Analysis and Control Sections, O'Hare Inn, Chicago, Ill. (Contact Dr. A. Amann, Abbott Laboratories, North Chicago, IL 60064)

Items to be considered for inclusion or corrections should be addressed to:

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REVIEW ARTICLE

Asbestosis: A Reassessment of the Overall Problem

THOMAS J. HALEY

Keyphrases □ Asbestosis—review of chemistry, industrial hygiene, animal and human toxicology, and carcinogenic aspects □ Toxicology, asbestos—review of chemistry, industrial exposure, carcinogenic aspects in animals and humans □ Carcinogenicity, asbestos—review of chemistry, industrial hygiene, animal and human toxicology □ Mesothelioma, asbestos induced—review of asbestos chemistry, industrial exposure, animal and human toxicology, and carcinogenic aspects

It has been established that occupational exposure to asbestos fibers induces mesothelioma of the pleura and peritoneum and carcinoma of the lungs, esophagus, and stomach after a latent period varying from 20 to 40 years (1). The daily dumping, since 1955, of 67,000 short tons of taconite tailings containing amosite asbestos fibers into Lake Superior, the potable water supply for Duluth, has raised the issue of the possibility of an increased cancer risk for the inhabitants of the area. Air pollution by the same type of fibers adds an additional risk. However, legal action against the polluter resulted in the decision that there was no demonstrable hazard from asbestos dumping and that therefore it could be continued (2). At this point in time, it has not been established, either experimentally or in humans, that ingestion of asbestos fibers causes cancer of the GI tract.

The wide use of asbestos and the observation of asbestos bodies in sputum specimens from individuals with no known exposure call for a review and reassessment of the overall problem of asbestosis. It is also essential that problem areas be discussed and that solutions be suggested.

SOURCES AND USES

The generic term "asbestos" describes naturally occurring incombustible mineral silicates that are separated into filaments. The varieties are actinolite [$CaO{\cdot}3(MgFe)O{\cdot}4SiO_2$], amosite [$(FeMg)SiO_3$], anthophyllite [$(MgFe)_7Si_8O_{22}(OH)_2$], chrysotile ($3MgO{\cdot}25{:}O_2{\cdot}2H_2O$), crocidolite [$NaFe(SiO)_2{\cdot}FeSiO_3{\cdot}H_2O$], and tremolite [$Ca_2Mg_2Si_8O_{22}(OH)_2$] (3).

Smither (4) tabulated the amounts and sources of asbestos in current use throughout the world for 1969 (Table I). Asbestos is used in the construction industry, floor tiles, asbestos cements, roofing felts, shingles, insulation materials, cement powders, acoustical products, textiles, brake linings, clutch facings, paper, paints, roof coatings, plastics, and miscellaneous other products. Asbestos filter pads are used in the beverage industry, in processing blood and its fractions, and in clarifying intravenous fluids.

Each and every one of these uses has exposed various segments of the population to the threat of asbestosis; the extent of exposure will be explored industry by industry. It must also be remembered that primary exposure in the insulation industry results in secondary exposure to between 3 and 5 million workers in the construction and shipyard industries (5).

ANALYTICAL CHEMISTRY

The chemical composition of the various forms of asbestos presents a difficult problem of analysis, particularly in cases of asbestosis where the amount of

Table I—Annual World Production of Asbestos (3,000,000 Tons)

Type	Percent Production	Amount[a] and Location
Amosite	3.0	90,000, South Africa
Anthophyllite	0.5	10,000, Finland
Chrysotile	93.0	1,250,000, Canada
		750,000, USSR
		250,000, South Africa
		100,000, United States
		100,000, Europe
		350,000, Others
Crocidolite	3.5	110,000, South Africa

[a] In tons.

offending chemical is small. The situation with air or water samples is much less complicated. Another complication is the contamination of amosite and crocidolite asbestos with benzo[*a*]pyrene and oils (6). Blood serum can elute benzo[*a*]pyrene from crocidolite fibers, but oil is far less efficiently removed (7). Canadian chrysotile asbestos has a very low content of polycyclic aromatic hydrocarbons but becomes contaminated with C_{14}–C_{35} *n*-alkanes during the milling process (8). Storage of the fibers in polyethylene bags results in a reaction which produces a yellow oil with strong absorption at 421 nm. The material has been identified as 3,3′,5,5′-tetra-*tert*-butyldiphenoquinone and is absent from fibers stored in glass (9).

Various asbestos fiber types can be identified with the polarizing microscope using specific refractive index media if the fibers are thicker than 0.5 μm (Table II). Smaller fibers can be identified with the transmission electron microscope, and selected area electron diffraction is more dependable than elemental analysis with the scanning electron microscope (3).

X-ray diffraction has been used for the quantitative determination of amosite, chrysotile, and crocidolite samples by measuring the area under the major diffraction peak and comparing it with a standard. The method is applicable to amosite and crocidolite in the 1–8-mg range and to chrysotile in the 1–10-mg range (10). This method has been applied to bulk and settled dust samples, but they must contain at least 5% chrysotile before the method is usable and more than 20% for accurate results (11). Similar analyses have been made of amosite and crocidolite (12).

X-ray diffraction analysis has been utilized for the accurate analysis of asbestos in lung tissue (13). The technique can be applied to related occupational lung diseases and can quantitatively differentiate between causative agents (14). When amosite, chrysotile, crocidolite, and tremolite are reduced to particles under 10 μm in size by cutting, the accuracy of the X-ray diffraction method decreases and the background increases. Impact reduction does not decrease the fine fibers, and accurate quantitative results are obtained (15).

IR spectrophotometry has been used in the determination of chrysotile asbestos, but difficulties are encountered in preparing disks with a chrysotile content <100 μg. Moreover, at the 2.7-μm absorption band, other serpentine minerals present can invalidate the results (16). A similar situation exists for the other forms of asbestos in that their IR spectra are almost identical. Other problems include reproducible particle size, uniform mixing of the fibers and halide matrix, chemical exchange in the fiber matrix, and chemical alteration of the sample during grinding (17).

The use of atomic absorption spectrophotometric methods in the analysis of asbestos fibers has been confined to the determination of nickel, chromium, cobalt, and manganese, which apparently contaminate the fibers during processing. The actual fibers are destroyed by treatment with hydrofluoric acid; thus atomic absorption spectrophotometric procedures at present cannot directly determine the asbestos content of bulk or environmental samples (18). However, it has been suggested that these trace metals may be associated with the carcinogenic process and that all experimental animal studies should have the asbestos monitored for trace metals (19). Similar studies should be undertaken in industry, *e.g.*, asbestos textiles, because trace metals have been associated with the etiology of asbestosis (20).

It has been suggested that electromotive phenomena are involved in the unexplained exacerbations of asbestos carcinogenesis and that unless trace metal determinations are made the results obtained may exclude important data (21). Neutron activation analysis has been employed to determine the iron, chromium, cobalt, nickel, and scandium content of amosite, anthophyllite, chrysotile, and crocidolite. Milling apparently did not significantly change the trace metal content. The difficulty with the method is the prolonged irradiation time, the time for decay for the short-lived nuclides, and the necessity for chemical separation to improve the accuracy of detection of individual radionuclides (22).

The difficulties in the determination of bulk asbestos is compounded when body tissues are analyzed for their asbestos fiber content. Normal mineral extraction methods including acid, alkali, or peroxide digestion destroy the asbestos fibers, so fiber recovery depends on the fiber type involved and the use of milder methods for tissue destruction. Analysis for tissue fiber types by X-ray diffractometry is of limit-

Table II—Crystallographic Properties and Refractive Indexes of Asbestos

Type	Crystal System	Specific Gravity	Refractive Index	Dispersion Staining Color	
				Parallel	Perpendicular
Amosite	Monoclinic	2.6–3.0	1.66–1.70	Red magenta	Golden yellow
Anthophyllite	Orthorhombic	2.85–3.5	1.60–1.66	Blue green	Golden yellow
Chrysotile	Monoclinic	2.36–2.5	1.49–1.57	Light blue	Magenta
Crocidolite	Monoclinic	3.0–3.45	1.69–1.71	Magenta	Blue magenta

Table III—Dust Limit Values for Asbestos[a]

Country	Mass Concentration	Particle Concentration	
		Limit Value	Measuring Instrument
England	0.24 mg/m³ asbestos, respirable	4 fibers/cm³ > 5 μm (25 years exposure)	Membrane filter
United States	—	12 fibers/cm³ > 5 μm 7.0 particles/cm³	Membrane filter Impinger
USSR and CSSR	2.0 mg/m³ total dust	—	—
East Germany	—	100 particles/cm³ (A > 40 wt. %), 250 particles/cm³ (A > 40 wt. %)	Konimeter
West Germany	Total dust: 1.0 mg/m³ (A^b = 50 wt. %), 1.5 mg/m³ (A = 10–50 wt. %), 2.0 mg/m³ (A = 10 wt. %)	fibers = $\frac{C_s \cdot C_f}{100} \leq 20$ fibers = 20 $C_{max} = \frac{450}{VA_T}$ particles/cm³	Konimeter

[a] Reference 35. [b] A = asbestos component in wt. %; A_T = asbestos component in particle percent.

ed value. The same is true for the IR spectrophotometric method.

Differential thermal analysis also fails as a procedure because residual organic phases produce a series of strong exothermic reactions in the same range as asbestos. Electron beam instruments are the best for identification of asbestos fibers in tissue and allow long-term examination without physical or chemical artifacts interfering (23).

BIOCHEMICAL ASPECTS

Earlier studies suggested that the protein coating of asbestos bodies was collagen or a collagen-like substance (24). It has also been suggested that such bodies adsorb ferritin or a preformed iron–protein complex or that the protein and iron are adsorbed separately to build up the total complex. Histological evidence indicates that the alveolar macrophage cytoplasm contributes to the coating, with the iron being derived from phagocytized erythrocytes (25).

Newer studies on asbestos body coating indicate that the protein is not collagen based upon its hydroxyproline, glycine, leucine, and phenylalanine content (26). A histochemical study suggests that acid mucopolysaccharides are present in asbestos bodies and that such material acts as a matrix for iron deposition (27). This study supports the view that colloidal iron deposits on the acid polysaccharides during the formation of these bodies (28). The iron involved may be derived from hemolyzed erythrocytes (29).

Asbestos also can cause pleural calcification without fibrosis, but the biochemical mechanisms involved have not been elucidated (30). It has been reported that total and free serum amino acids, total protein, and globulins are higher in asbestos workers than in a control population (31). Asbestos workers also show a decrease in prealbumin and albumin and an increase in α-glycoprotein, α_2-lipoprotein, and transferrin and in serum IgG, IgM, and IgA fractions. There was no correlation between the length of asbestos exposure or the degree of asbestosis (32). There was an increase in the antinuclear antibody related to these parameters, but other immunological tests were negative (33).

INDUSTRIAL HYGIENIC ASPECTS

Dust Measurement—Such measurements have been highly controversial, because the instrumentation produced artifacts in the collected particles. Past standards for asbestos dust in the United States were based upon dust collection with an impinger, which collected few fibers and many grains. Moreover, it was not amenable to long personnel sampling, even though such sampling actually best approximated actual worker exposure (34). Therefore, more precise measurements were sought utilizing better instrumentation.

Table III gives the results of such investigations with different types of instruments and the dust limits obtained (35). These values are subject to change as new instrumentation is available and better correlation with epidemiological studies is obtained. It is now possible to evaluate each method in light of its good and bad points.

The British have checked many methods of dust estimation and found that the Owens jet counter is of value for taking snap samples but that efficiency decreases with increasing particle size and increasing air sampled. The konimeter suffers from the same defects and, in addition, the glycerin or petroleum coverslips cannot be ignited to remove combustible contaminants. The thermal precipitator is generally used in the United Kingdom and Europe, but its efficiency decreases for long fibers. The impinger is useful for particles 1 μm and larger but gives low values for asbestos.

The membrane filter method is good for 1-μm and larger fibers. It is consistent and covers the sampling rate between 10 and 500 cc/min. Moreover, samples can be taken for short or long periods, and the apparatus operates either electrically or by hand pump, is

robust, and can be used as a personnel monitor. The gravimetric method is in wide use in the United Kingdom but gives no indication of particle-size or fiber-size distribution. Tyndallometric methods (light scattering) count particles equal to spheres from 0.3 to 10 μm in 15 size ranges covering several counting rates but give a 25% lower value than a membrane filter with 5-μm and larger fibers. Moreover, it is doubtful that light scattering will work with all asbestos fiber types. Other limitations include counting all particles passing the light beam, the large size of equipment, and the necessity of checking by the membrane filter technique (36).

The U.S. Public Health Service has developed a more sophisticated membrane filter technique using counting under phase contrast microscopy and data reduction and statistical analysis by computer. The method is highly suitable for developing basic data for delineating the limits for airborne asbestos dust more accurately (34). Other investigators compared the membrane filter and electrostatic collector methods and found that the former gave excellent results but that the latter was inaccurate in measuring high concentrations of coarse particles because of short circuits and dust adherence to the discharge electrodes (37).

A comparison between the impinger and the membrane filter instruments revealed that the latter was probably better for the evaluation of fibers related to disease induction and, therefore, was more relevant to setting hygienic criteria (38). For measurement of total dust in a given environment or working area, the high volume sampler gives good results but does not, in general, give a direct measurement of individual exposure to dust and fibers. Such fixed-site samplers are used to obtain data on the efficiency of operation of dust control equipment (39).

Specifications for the evaluation of asbestos exposures in the working environment were defined by an international committee (40). Recently, these hygienic standards were extended to include amosite asbestos (41). Plant safety is only one aspect of the problem, because without effluent controls the outside environment can become contaminated and expose nonasbestos workers to the health threat of asbestosis. Moreover, it has been shown that such atmospheric contamination varies with the season, being greater during the winter months (42). The threshold limit for asbestos, 5 million particles/ft^3, or the German MAK value of fibers of 5 μm or more/cm^3 covers only inhalation and not ingestion; ingestion also must be addressed if the total environmental exposure to asbestos is to be realistically assessed (43, 44). Moreover, the threshold limit and MAK values must be modified as better measuring equipment becomes available. The fiberglass industry has recently been evaluated to determine its possible contribution to pneumoconiosis, and the survey indicated that the industry should not be put in the hazardous occupation category (45).

Protective Equipment—Dust in the work environment carries a certain risk, large or small, to health. There is no known absolute zero response because individual responses vary from one person to another and there is often a long latent period in the final development in the disease state (46). Methods for worker protection from airborne dust include: (*a*) enclosed working areas with or without a local exhaust system, (*b*) general room ventilation with large quantities of air, (*c*) water spraying to moisten dust, (*d*) use of a separate building, (*e*) local exhaust system over the work area, (*f*) work during evenings or on weekends to reduce the number of workers exposed, and (*g*) respiratory equipment (47).

All of these methods are used in prevention of asbestos exposure, but only the last, respiratory equipment, is useful in preventing individual exposure to the fibers. Continuous evaluation is necessary to develop performance characteristics of new and, perhaps better, dust respirators for fibrous dusts (48). Spray operators should be protected with a self-powered supplied air respirator or a constant-flow airline respirator with a belt control assembly and a quick-release coupling. For those not subjected to high asbestos dust concentrations, regular approved asbestos dust respirators can be used. All users should be instructed in the proper care and use of the respirator, particularly the necessity of changing filters (49).

The types of respirators required in the United Kingdom for protection against various concentrations of three types of asbestos are listed in Table IV (50). Table V indicates the magnitude of the problem in the English asbestos textile industry and the fact that particles did not include the fibers between 5 and 100 μm. A comparison of the data for 1952 and 1960 shows that the introduction of better hygienic practices reduced the dust exposure in various operations (51). In the dockyard, positive pressure power respirators are used and showering and changing from work clothes have reduced asbestosis cases and prevented further asbestos contamination of the environment (52). Spraying fibrous insulation can produce concentrations with fiber counts of 100 F/cc, requiring the use of air-supplied respirators because conventional filter-type respirators do not provide adequate protection (53). It has been shown that breakdown of the sealant covering sprayed asbestos insulation can introduce potential health hazards to occupants of such areas (54).

From the foregoing review of the industrial hygienic aspects of the asbestos problem, it is evident that

Table IV—Respiratory Protection against Asbestos Dust[a]

Concentration of Chrysotile or Amosite Asbestos, Fibers/cc Air	Concentration of Crocidolite Asbestos, Fibers/cc Air	Respirator
Up to 40	Up to 4	Dust respirator
Up to 200	Up to 20	Positive-pressure dust respirator
Up to 800	Up to 80	Ultrahigh-efficiency dust respirator
Greater than 800	Greater than 80	Positive-pressure airline breathing apparatus

[a] Reference 50.

Table V—Dust Levels at Various Textile Processes, 1952–1966[a]

Department	Process	Yearly Mean Dust Levels: T.P.[b], Particles/cm³ 1952	T.P.[b], Particles/cm³ 1960	LRTP[c] and Membrane, Fibers/cm³ 1961	LRTP[c] and Membrane, Fibers/cm³ 1966
Fiberizing	Mixing floor	500	—	—	—
	Opening	440	Now totally enclosed		
	Bag slitting	—	110	4.5	4
	Mechanical bagging	—	120	4	4.5
Carding	Fine cards	200	200	5.5	5.5
	Medium cards	810	400	7.5	8
	Coarse cards	1140	420	7	7.5
	Electrical sliver cards	490	260	5	2
Spinning	Fine spinning	170	110	4	3.5
	Roving frames	510	150	5.5	5.5
	Intermediate frames	530	100	5.5	5.5
Weaving	Beaming	190	220	8	3.5
	Pirn winding	350	130	3	2.5
	Cloth weaving	180	140	3	2
	Listing weaving	130	110	2	1
Plaiting	Medium plaiting	140	80	4	4

[a] Reference 51. [b] TP = thermal precipitator. [c] LRTP = long-running thermal precipitator.

progress in worker protection has occurred but that problems will constantly arise in other areas.

ANIMAL TOXICOLOGY AND CARCINOGENESIS

All forms of asbestos have been administered to many animal species by various routes except orally.

Intraperitoneal—Heating asbestos powder for 3 hr at 1000° and then cooling produced forsterite (Mg_2SiO_4) and enstatite ($MgSiO_3$) which, when administered intraperitoneally to CF-1 mice, killed 30 of 50 animals within 48 hr. Unheated asbestos did not have this effect. In another experiment with CF-1 mice, intraperitoneal injection of 0.5 ml of a 50% suspension of asbestos and serial sacrifice of the animals over 343 days gave evidence of a progressive, proliferative, granulomatous, invasive fibrosis histologically similar to mesothelioma (55).

Administration of five doses of 20 mg of amosite, anthophyllite, and crocidolite asbestos to female Wistar strain rats showed that the abdominal granulomas produced and the lymph nodes contained fibers of various lengths. Short fibers were intracellular while long fibers were not. Transport of fibers from the site of injection depends upon the length and begins with those measuring 20 μm and increases with decreasing length (56). Injection of chrysotile asbestos in fiber lengths of <5 and <3 μm produced the same percentage of tumors, but the time to tumor was prolonged with the smaller size fibers. The critical size for tumor induction appears to be longer than 2–3 μm. The addition of benzo[a]pyrene did not distinctly influence tumor induction (57).

Intratracheal—When 3.5 mg/ml of an aqueous suspension of chrysotile was administered to rats in total doses of 10.5–14 mg in 3–4 days and the animals were sacrificed at 4 days, the lungs showed a proliferative inflammation of the smaller bronchi and bronchioles. There were polypoid processes of avascular fibroblastic tissue originating from ulcerated areas in the mucosa and bronchial lumen. With time, 12–24 months, this inflammatory tissue converted to collagenous scars, which caused permanent deformities of the bronchi and bronchioles. There was also a macrophage reaction with a minimal stromal participation. It was suggested that the polypoid lesions were artifactual and related to the mode of administration because they did not occur in inhalation studies (58).

Russian chrysotile induced pulmonary carcinomas, sarcomas, premesotheliomas, and mesotheliomas in rats when 2 mg was injected three times monthly. Fiber sizes varied from 5 to 15 μm. When benzo[a]pyrene, 0.14 mg, was adsorbed on the asbestos, the number of lesions increased over the 9–28-month observation period. Moreover, asbestos alone had no effect during the first 6 weeks, but the addition of benzo[a]pyrene produced definite lesions in that time interval (59).

A study of asbestosis in guinea pigs showed that only crystalline chrysotile produced lung lesions. The dose instilled was 60 mg of fibers, 5 and 10 μm in length. The collagen content of the lungs was significantly increased (60). Other studies employing rabbits instilled with 2.5- or 15-μm fibers at a total dose of 100 mg at monthly intervals of 2–19 months showed foreign body reactions in the lungs, nodular reticulinosis, and diffuse interstitial reticulinosis (61).

Intrapleural—Injection of 10 mg of a saline suspension of chrysotile asbestos into the lower right pleural cavity of mice, with sacrifice intervals varying from 1 week to 6 months, resulted in pleural granulomas containing asbestos bodies in 2 weeks. The fibers were coated with acid mucopolysaccharide impregnated with ferritin or hemosiderin. Similar results were obtained with rats injected with a dose of 25 mg of the fibers (62).

Injection of 0.05 ml of a 50% lanolin suspension of asbestos into the right pleural cavity of female Osborne–Mendel rats monthly for 6 months did not produce any neoplasms in the lungs, pleurae, mediastium, or thoracic wall. This lack of carcinogenesis may have been due to the vehicle used, because saline suspensions do induce neoplasms (63). Administration of 20 mg of amosite, chrysotile, crocidolite, or extracted crocidolite intrapleurally in SPF or standard Wistar strain rats of both sexes produced a rapid onset of mesotheliomas. The time to onset was dependent on the type of asbestos, being longer for amosite than for the other varieties, although the fiber lengths (<2–200 μm) were the same. Solvent extraction of the crocidolite did not influence the results (64). A mathematical model was developed to describe the times to occurrence of the mesotheliomas (65).

In another study employing female Sprague–Dawley rats, injection with 66.7 mg of the three asbestos varieties produced mesotheliomas of the pleura and undifferentiated lung carcinomas (66). It has been shown that the carcinogenicity of amosite, chrysotile, and crocidolite asbestos fibers is related to structural

shape rather than to physicochemical properties. In contrast to a previous investigation (64), this study showed that amosite produced a more rapid onset of mesotheliomas (67). Injection of 20 mg of Russian chrysotile produced premesotheliomas and 42.26% mesotheliomas in the animals after 8 months (68).

Because the amount of asbestos required for the induction of mesotheliomas is small, it has been suggested that trace metal contamination may be involved (69). Neutron activation of chrysotile asbestos indicated that trace amounts of scandium, chromium, iron, and cobalt were present and were leached out and excreted and that 90% of the radionuclides were at the injection site at sacrifice. These findings indicate that fiber translocation is a slow process and that trace metal may not be involved in the neoplasm induction (70). Intrapleural injection of 25 mg of chrysotile asbestos into guinea pigs induced pleural granulomas in 14 days (62). This observation was confirmed (71).

Inhalation—Rats were exposed to chrysotile dust (quantity and fiber size not specified) for 18 hr/day for 50 days. The dust was found in the alveolar macrophages, which underwent changes to fibroblasts. There were also small nodular giant cell lesions. Changes occurred in the basement membrane with numerous impocketings in the cytoplasm of both epithelial and endothelial cells (72). Inhalation by rats of chrysotile dust (size range of <1–>20 μm) for 100 hr over 1 month showed that particles <3 μm rapidly produced fibrotic lesions in the lungs. Phagocytic ingestion is required to produce the lesion (73).

Inhalation of 86 mg/m^3 of chrysotile dust by rats for 2 years resulted in lung cancer. The incidence was greater in animals subjected to the additional insult of intratracheal instillation of 0.05 ml of 5% aqueous sodium hydroxide. It was suggested that traces of nickel, cobalt, and chromium might be involved in cancer induction (74). Another study showed that particles <20 μm long were fibrogenic, with this activity limited to groups of alveoli (75). Exposure of guinea pigs to amosite, chrysotile, and impure crocidolite dusts with particle sizes ranging from <0.3 to 2.2 μm produced asbestos bodies and diffuse interstitial fibrosis. Cuboidal metaplasia of the epithelium of the alveoli was also observed, and it had a pseudoadenomatous appearance at the 7th month. Amosite dust induced the asbestosis more rapidly than the other forms of asbestos. The lesions from chrysotile dust were severe and the impure crocidolite caused respiratory infection in the guinea pigs (76). These observations were confirmed for chrysotile fibers (72).

Peribronchiolar fibrosis of the rabbit lung was produced by inhalation of the various types of asbestos fibers (77). Lung lesions appeared earlier and were more severe in rabbits inhaling amosite fibers than in those exposed to chrysotile fibers (76). Rabbits inhaling fibers of amosite (3–5 and 0.2–0.5 μm), chrysotile (6–15 and 0.2 μm), and crocidolite (3–6 and 0.5 μm) in concentrations of 48.2 ± 1.4, 47.4 ± 1.7, and 48.7 ± 2.4 mg/m^3, respectively, developed a pulmonary fibrosis (78).

The selection of the animal species for asbestosis studies is critical because of the differences in the respiratory tract. When asbestos-exposed rats are maintained for more than a year, there is a considerable reduction in the lung dust burden because the alveolar clearance mechanism is more efficient than that of hamsters and guinea pigs (79). Vervet monkeys exposed to amosite, chrysotile, and crocidolite fibers developed the usual signs and symptoms of asbestosis and succumbed more readily to respiratory tract infections (76). Focal areas of interstitial fibrosis were found in a donkey that worked in an amosite mine, in a baboon living in the vicinity, and in rats *(Rattus namaquensis)* whose burrows were near an asbestos mill (80).

Other Routes of Administration—Injection of 58 mg of asbestos in lanolin into the marrow cavity of rats and rabbits resulted in oligocellular lesion but no tumors. Injection of asbestos into the paranasal sinuses of Osborne–Mendel rats gave similar negative results (63). Injection of chrysotile asbestos into the stomachs of rats indicated migration of the fibers *via* the bloodstream into the spleen, omentum, heart, brain, and lungs (81). However, it has been suggested that this mode of administration could have torn stomach blood vessels and that the asbestos suspension under pressure directly entered the bloodstream (82).

***In Vitro* Studies**—Tissue culture studies with guinea pig macrophages and mouse L line fibroblasts in the presence of chrysotile fibers (20–30 or 300 μm long or 10–20 or 100 μm long) were not lethal to, and did not impair glycolytic activity of, the former. The mice fibroblasts were not killed but did produce more collagen than the controls (83). Further studies of guinea pig macrophages indicated that both chrysotile and crocidolite fibers were cytotoxic because there was decreased lactic acid production, a loss of fluorochromasia by the cells, and a marked release of lactate dehydrogenase into the medium. Crocidolite was more toxic than chrysotile and pretreatment of the fibers with chelating agents, edetic acid (EDTA) and polyvinylpyrrolidone *N*-oxide, did not reduce the cytotoxic effect, indicating that the phenomenon was connected with physical and not chemical characteristics of the fibers (84).

Incubation of rat macrophages with amosite, chrysotile, and crocidolite dusts resulted in cell toxicity as measured by changes in acid phosphatase activity, 2,3,5-triphenyl-2*H*-tetrazolium chloride reducing power, and lactic acid production. Acid phosphatase activity was increased by chrysotile but not affected by the others. Chrysotile also slightly decreased the 2,3,5-triphenyl-2*H*-tetrazolium chloride reducing power and inhibited lactic acid production. Only chrysotile hemolyzed rat erythrocytes, and this action was prevented by polyvinylpyrrolidine *N*-oxide (85).

Increased lactic dehydrogenase activity and cell membrane permeability were seen after exposure of guinea pig alveolar and peritoneal macrophages to chrysotile fibers (86). Chrysotile asbestos has a high hemolytic activity whereas amosite, crocidolite, and anthophyllite lack this activity or are only weakly

lytic. This effect was attributed to the adsorptive capacity of chrysotile dust for erythrocyte membrane components (87). Comparison of the hemolytic activity of heated and unheated chrysotile fibers showed that the lytic properties were not identical because the lytic effect of the former was antagonized by carboxymethylcellulose and the latter by polyvinylpyrrolidone *N*-oxide (88). Chrysotile fibers adsorb globulins, and the adsorption bond strength was related to their fibrogenic activity (89).

It was demonstrated that amosite, anthophyllite, chrysotile, and crocidolite dusts absorb both serum IgG and rheumatoid factor, and it was suggested that the amount of the latter in asbestos workers correlated with the intensity of exposure (90). Stimulation of collagen synthesis by crocidolite fibers first elevates and then depresses proline hydroxylase activity; the latter effect occurs as the fibrotic lesion develops (91). Chrysotile produced a marked increase in tritiated thymidine uptake in the rat pleural mesothelium as early as 5–7 days after treatment, indicating a high degree of sensitivity to chrysotile. There was a rapid loss of the label after 7 days (92).

HUMAN TOXICOLOGY AND CARCINOGENESIS

The first case involving asbestos and human disease was recorded in 1907; the first case in the United States was reported in 1930. However, protective standards were not adopted until 1972. The extent of occupational and nonoccupational exposure is unknown, but the use of asbestos in the manufacture of cement pipes, sheets, shingles, floor tiles, millboard, roofing felts, pipe covering, insulation paper, flooring felts, friction and packing material, paints, roof coatings, caulks, sealants, safety clothing, curtains, brake linings, clutch facings, spray insulation, asphalt paving, welding rod coatings, and filter mediums in the pharmaceutical and beverage industries results in widespread exposure of the entire population (93). Such exposures have resulted in lung and pleural carcinoma and mesothelioma (94, 95). To understand the asbestos problem more fully, we shall discuss it industry by industry and point out the salient features of asbestosis from a clinical viewpoint.

Diagnosis—One preliminary sign of exposure to asbestos is the finding of asbestos bodies or asbestos fibers in sputum specimens (96, 97), but this sign does not indicate the presence nor the extent of disease or disability (98). Clinical observation of progressive dyspnea out of proportion to radiological changes in the lung and finger clubbing is indicative of asbestosis. The progression of the disease includes nonspecific interstitial fibrosis of the perihilar region, granular ground-glass-type infiltration, fibrosis extending throughout both lungs and pleura, marked emphysema with blebs and bulba, pleural diaphramatic plaques, and, finally, carcinoma and mesothelioma of the lungs, pleura, and peritoneum (99). Calcification of the parietal pleura and pleural plaques are distinct signs of asbestosis (100).

Definitive diagnosis of asbestosis requires the finding of asbestos fibers or asbestos bodies in affected tissue; but unless a biopsy is performed, such findings usually occur only at necropsy (101). The following diagnostic tests are also useful: a chest X-ray, vital capacity, 1-sec vital capacity, and lung compliance. The latter two tests progressively decrease during asbestos exposure, and the frequency of dyspnea is related to the decreased vital capacity (102).

Pleural calcifications by themselves are of little diagnostic significance (103) but can be helpful when a biopsy specimen shows asbestos bodies (104). "Asbestos pleural effusion" recently were added to the diagnostic signs of asbestosis (105). It also has been suggested that the ventilation perfusion ratio and the determination of blood gases should be included in asbestosis evaluation (106) because this condition is a restrictive disease with decreased diffusion capacity (107). This idea has been disputed, and it has been suggested that radiological changes in the lung coupled with measurements of forced vital capacity and 1-sec forced expiratory volume give a better assessment of the patient's condition (108). Whole body plethysmography and spirography have proven useful in following the progression of asbestosis, but chronic bronchitis can complicate the diagnosis (109).

Major clinical signs of asbestosis include diminished breath sounds, basilar crepitations, limited chest expansion, clubbing, and cyanosis. The latter two signs occur in advanced stages of the disease. Chronic bronchitis and a localized emphysema as well as right ventricular cardiac enlargement are seen. Cor pulmonale is the major complication and cause of death in asbestosis. Pulmonary endarteritis with intimal hyperplasia consistent with pulmonary hypertension and bronchioelectasia are present.

Measurement of pulmonary function is vital for a good diagnosis, because a chest roentgenogram can resemble the picture seen in viral pneumonia, lymphangtic carcinoma, Haman-Rich syndrome, and other diseases (110). Although there is a mixed impairment of the gas transport mechanisms in asbestosis, there is no relation between physical performance or radiological findings and diffusing capacity (111). Examination of 1069 men in the asbestos industry showed that only vital capacity and forced vital capacity could indicate the beginning phases of asbestosis (112).

In asbestosis, pleural reactions may occur in conjunction with mixed fibrotic reactions, making radiographic interpretation difficult. Moreover, the histological changes do not always correlate with the radiological pattern (113). In asbestos workers, vital capacity reduction precedes radiological changes by 10–15 years; the latter occurs after 20 years of exposure. After 30 years of exposure, the incidence of functional and radiographic abnormalities is the same (114). Difficulties in interpretation of radiographic changes in the various pneumoconioses have resulted in an international standard for rating the various degrees of pulmonary damage from dust exposure (115).

Radiocardiographic examination of eight patients with pulmonary asbestosis showed a reduction in

mean pulmonary circulation time and pulmonary blood flow and an increase in total blood volume, right ventricular telediastolic volume, and right ventricular postsystolic residual volume, indicative of a reduced pulmonary vascular bed (116). ECG changes consisting of left axis deviation, partial or complete bundle branch block, and right ventricular hypertrophy correlated with radiological findings in 29 cases of asbestosis (117).

Pulmonary radiological changes in asbestos workers have been related to age and years of employment (118). Examination by radioiodinated albumin scintography of 20 patients with asbestosis revealed pulmonary changes characteristic of fibrosis, with reduced radioelement in the peripheral lung field (119). Mucociliary function, as measured by inhalation and pulmonary clearance of ^{51}Cr or ^{99m}Tc microspheres, was normal in asbestosis (120). Severe asbestosis symptoms can be confused with mitral insufficiency, even when ECG and pulmonary function studies and occupation indicated asbestos was the agent involved in the residual function deficit (121).

From a diagnostic viewpoint, it is necessary not only to define the degree of cardiopulmonary impairment in obstructive pulmonary disease but to do a retrospective analysis of the patient's employment record to arrive at a positive diagnosis of asbestosis.

Pathology—The pathological changes occurring during the development of, and death from exposure to, asbestos have been well documented. It was shown that "folded lung," an extremely rare form of lung pathology, occurs in asbestosis. Folding occurred due to a fibrous membrane on the costal surface of the visceral pleural of the lower lobe. No adhesions were observed (122).

Pathological changes in the lungs in asbestosis include diffuse interstitial fibrosis with collagenization, ground-glass appearance of the parenchymal tissue, thickening of the pleura with calcified plaques, thickening of the alveolar walls and intraalveolar septa, alterations in the capillary network, macrophages containing asbestos fibers, and alveolar dilation with cuboidal metaplasia (123–127).

Asbestos does induce squamous cell carcinoma and adenocarcinomas in the lungs, and cigarette smoking increased the incidence by 8.05 times. Moreover, lung carcinoma exceeded mesothelioma by five to six times (128). In asbestosis, squamous cell carcinoma is usually in the lower lobes where the greatest number of fibers are found (129). Another complication of asbestosis is mesothelioma of the lungs and pleura. One serosal cavity is usually involved, but the underlying viscera are rarely involved. Spread is *via* the local lymph nodes with direct extension to the chest wall and spine. Distant metastases are rare (130). The latent period is usually 20 or more years.

The malignant elements are of both epithelial and mesenchymal character, with a tubular or tubo-papillary pattern containing cuboidal or flattened cells and masses of collagen. Hyaluronic acid is often present (131). The disease is rapidly progressive, averaging 2 years from onset to death (132). Microscopic examination of the malignant mesothelioma cell reveals an epithelial cell, which is large and polygonal with amphophilic cytoplasm and a clear cytoplasmic membrane. The round nucleus occupies one-third of the cell and has a loose vesicular chromatin. There are a large round eosinophilic nucleolus and multiple nucleoli. The nuclear membrane is folded or crenated, and there are many small intracytoplasmic vacuoles. Mitoses are uncommon, but multinucleated tumor cells are seen (133).

Mesotheliomas can be confused with other metastatic tumors if diagnosis is made on histological evidence alone (134). Some confusion has arisen as to the significance of pleural calcification in the development of mesothelioma (135). On the other hand, the association of asbestos exposure, occupational or nonoccupational, with malignant mesothelioma has been confirmed by investigators throughout the world (136–143).

The United Kingdom instituted a mesothelioma register where comparisons are made with carcinoma of the bronchus and lung in cases of asbestosis. Mesotheliomas occur earlier than the other pulmonary cancers. Table VI gives a comparison of mesotheliomas by site and sex for the years 1967–1968 and covers the United Kingdom. It has been suggested that even these data may be an understatement of the prevalence of the disease (144).

Asbestos Bodies in Pulmonary System—Having considered the consequences of asbestos exposure, it is necessary to discuss the actual means used for identification of the fibers themselves in asbestos bodies. The finding of asbestos bodies in the lungs does not indicate that asbestosis or pleural mesothelioma will occur or has occurred, because such bodies have been detected in 50% of the general population in certain areas. Moreover, other fibrous materials produce similar bodies, so asbestos must be positively identified (145). These other bodies may be formed from nonfibrous particulates and carbonaceous fibrous particles. Those with black fibrous core probably are derived from burning leaves, wood, or coal (146). These observations were confirmed (147). Electron micrographs have identified a large variety of dusts and fibers, which become iron coated in the lungs but do not give rise to pulmonary complications like asbestosis (148).

Classification of asbestos *versus* nonasbestos fibers

Table VI—246 "Definite" Mesotheliomas Analyzed by Sex and Site of Tumor[a]

Pathologists	Site of Tumor	Percent of All Mesothelial Tumors		Total
		Males	Females	
UICC[b] panel	Pleural	96	22	118
	Peritoneal	14 (12.8%)	2 (8.3%)	16 (11.9%)
Other pathologists	Pleural	81	17	98
	Peritoneal	10 (10.9%)	4 (19.0%)	14 (12.5%)
All	Pleural	177	39	216
	Peritoneal	24 (12.0%)	6 (13.3%)	30 (12.2%)

[a] Reference 144. [b] Unio Internationale Contra Cancerum.

is difficult, but ultramicroscopic criteria have been developed. At magnifications of 20,000 or 25,000×, asbestos fibers have profiles characterized by steplike interruptions up or down from each other and a surface characterized by parallel longitudinal lines delineating the fibrils. Nonasbestos fibers have uninterrupted linear profiles, and their surface is devoid of longitudinal parallel lines (149). Inhaled fibers become coated with an iron–protein envelope, and the central fiber can be identified as asbestos by electron microscopy.

As the asbestos body matures it changes from a thin yellow headed shape to a shorter, thicker, dark-brown segmentated form with a granulated coating which fragments and is phagocytized. This change in form can make identification difficult (150). Asbestos bodies have been demonstrated in lung smears in 30% of males and 20% of females in 500 autopsies in an urban area, but no pulmonary changes were reported (151). Another study found no correlation between nonoccupationally exposed individuals with asbestos bodies and inflammatory or neoplastic pulmonary disease (152).

An Israeli study could not evaluate the biological and clinical significance of asbestos bodies in the absence of pulmonary carcinoma (153). A New York study, in which 28 cases showed asbestos bodies, suggested that urban dwellers were being subjected to nonoccupational asbestos exposure (154). A Swedish study also reported asbestos bodies in lungs of nonoccupationally exposed individuals (155).

Methods suggested for positive identification of asbestos bodies in lung smears and sections include X-ray diffraction, phase contrast optical microscopy, optical microscopy, and electron microscopy (156–159). The last procedure is the best, particularly when combined with the scanning electron microscope. Isolation of fibers from asbestos bodies requires a digestion procedure that will not dissolve the fibers and still will allow quantitation of the total fiber population in a tissue specimen. Formamide dissolves the fibers while perchloric acid does not, and further solvent removes the excess carbonaceous material, resulting in a more exact determination (160).

Epidemiology—Occupational and environmental exposure to asbestos has been correlated with both pulmonary carcinoma and mesothelioma; but in an area where diagnostic procedures are in a state of flux, an epidemiological study is extremely difficult and unknown factors can make the results of limited value. Cohort studies of individuals in the same geographic areas and in a similar type of industry are required to obtain meaningful information (161). Epidemiological studies of asbestosis point out the long latent period before cancer development and the difficulty in determining the degree of exposure. However, when total deaths for a given locale and age group are compared by the life table technique, it is evident that asbestos does increase both pulmonary and bronchiolar neoplasia (162).

To standardize asbestos epidemiological surveys, the International Union Against Cancer suggested

Table VII—Cause of Death in Insulation Workers, Belfast, Northern Ireland, 1940–1965 (Total: 107; Mean Age: 55.3 ± 11.25 Years)[a]

Cause	Observed	Expected
Malignant:		
Intrathoracic neoplasms	28	5.3
Abdominal neoplasms	15	9.0
Other neoplasms	4	6.7
Nonmalignant:		
Pulmonary disease	31	25.6
Cardiac and circulatory	21	37.1
Other causes	5	21.3
Unknown	3	—

[a] Reference 164.

that detailed information covering social (smoking habits), occupational, environmental, and medical history from early childhood be collected for the individual and family units. As much information as can be obtained should be compiled on cardiopulmonary diseases and various fibroses and carcinoma of the lung, pleura, peritoneum, GI tract, and ovaries. Retrospective, cross-sectional, and prospective epidemiological surveys should be conducted, and one or more control groups should be included. Consultation with statisticians should occur prior to data collection and good experimental design should be employed (163).

The usefulness of a retrospective study is illustrated in Table VII, where the intrathoracic and abdominal neoplasms in insulation workers is excessive compared to the general population. The nonmalignant pulmonary deaths are related to the tuberculosis incidence (164). A cohort study of 1265 white males and 228 white females employed in an asbestos plant in 1938–1939 was analyzed in mid-1964, using Social Security records. Methodology was refined by including both age and duration of employment as well as all causes of death, asbestosis, cardiovascular disease, and malignant neoplasms. Upon this basis, it was possible to relate exposure to asbestos directly to a general increase in mortality rate from all of these causes (165). A further substantiation of the relationship between asbestos exposure and mesothelioma in this same plant was reported in which three more cases of mesothelioma not in the previous cohort were found (166). Further confirmation of the relationship between mesothelioma and slight asbestos exposure 20–30 years previously was shown (167).

Validation of death certificates using the British Registrar General and correlating necropsy reports with histological observations showed that bronchial carcinoma was not underestimated but that mesothelioma was (168). A German epidemiological study associated pleural mesothelioma with asbestos exposure many years previously (169). Material for future epidemiological studies will be obtained from the many industries involved in mining and processing asbestos.

Mining and Milling—The difficulties in associating asbestosis with mining are illustrated by a Bulgarian study of 3325 people living near an asbestos mine. Out of 155 cases of pleural asbestosis, only 23 were involved in mining; the remaining 132 cases

were tobacco workers. Air pollution was not involved, but the farms were composed of rocky soil containing asbestos minerals (170).

An Italian study of 288 cases of asbestosis gave the following distribution: mining, 32; friction products, 161; cement, 60; and insulation, 35; the indication was that finer asbestos dust was more dangerous (171). Exposure to Finnish anthophyllite asbestos in two mines resulted in an excess number of deaths from asbestosis and lung cancer (172, 173). Asbestosis has been reported in Australian miners but no mesotheliomas because the latent period for their development has not been passed (174).

Exposure to South African crocidolite asbestos produced 33 cases of diffuse pleural mesothelioma in the asbestos mining area of the country (175). Thirty-one other cases of asbestosis were examined but no pulmonary carcinomas were observed. This result is probably related to the short-time interval of exposure and the known latency for mesothelioma development (176).

There appears to be a difference between the development of mesotheliomas after exposure to crocidolite and amosite fibers, which suggests that the latter may be the least carcinogenic of the two forms of asbestos or require a longer time interval for tumor induction (177). This difference between the two varieties of asbestos is not evident when fibrosis is considered because they are then equally potent (178).

In a study of mortality in Quebec chrysotile mines covering 9981 individuals, only 97 lung cancers and three mesotheliomas were found. It was suggested that this form of asbestos may be less carcinogenic than the other varieties. Accumulated dust exposure and duration of employment apparently influenced the figures (179). Present dust exposures in these mines and mills are now 10 million particles/ft^3 down from the 75 million particles/ft^3 in 1948; thus estimation of total dust exposure must be adjusted not only for time but for work area and must take into account both fibrous and nonfibrous dust (180). Respiratory symptoms and function tests were related to dust exposure of 1015 individuals in these industries, but the other parameters had little or no influence (181, 182). Mining and milling in other countries do not differ from those reported here, but the types of asbestos do.

Asbestos Friction Materials—One occupation in which exposure to asbestos dust occurs in unsuspecting individuals is routine brake and clutch maintenance of automobiles and trucks. The amount of dust evolved in blowing out the brake drums varies from 1.42 to 3.62 fibers/cm^3; personnel samplers give an average of 0.68 fiber/cm^3 in the breathing area for automobiles, 7.09 fibers/cm^3 for brakes, and 2.25 fibers/cm^3 for trucks. Thus, over a time period, daily exposure could result in the development of asbestosis (183). Most of these figures are above the recommended level of 2 fibers/cm^3 (46).

On the other hand, the temperature generated on braking, 600°, causes the asbestos fibers to be converted into a series of olivine minerals which are unlike asbestos chemically, physically, and physiologically. It was suggested that dust clouds from brake drums are filthy but much less harmful than asbestos (184). Vacuum cleaning reduces the number of escaping particles to a minimum and should be used (185), but a vacuum brush is even better (186).

Asbestos Textiles—All areas of textile production (fiberizing, carding, spinning, plaiting, and weaving) have contributed to the induction of asbestosis, with subsequent development of peritoneal mesothelioma. In one factory, with asbestos exposures ranging from 10 months to 32 years, there were 11 cases of the disease; survival time varied from 20 to 46 years following the initial exposure (187). In the London area, textile industry asbestos exposure produced 83 cases of mesothelioma: 27 peritoneal and 56 pleural tumors. Terminal illness ranged between 16 and 55 years. In nine of these cases, domestic exposure occurred from asbestos dust on work clothes, pointing out one of the many ways the disease may be brought about (188). A factory in Pennsylvania had 68 cases of asbestosis with 21 malignancies; again the time to induction was long, 10–36 years (189). In 42 cases of mesothelioma, 10 persons worked in the factory, eight lived nearby, three were family members, 10 had some degree of asbestos exposure, and 11 had no history of exposure. Thus, environmental exposure was involved to a great extent (190).

Overall mortality from cancer of the respiratory and digestive tracts and cor pulmonale in asbestos textile workers was 21% above the U.S. national average in 1948–1951 (191). German experience indicates a similar type of response to asbestos, complicated with tuberculosis (192). An example of English textile workers dying of pleural mesothelioma is given in Table VIII. Again, long latency is evident (193). In Hamburg, 119 cases of pleural mesothelioma, 79 men and 40 women, were reported for 1958–1968 and 51 cases were definitely linked to asbestos exposure. The average latency for tumor development was 35.2 years (194).

A survey of 1160 asbestos textile workers revealed excess deaths from lung and pleura cancer and cancer at other sites in workers receiving heavy asbestos exposures. Excessive mortality from respiratory disease was also found in this group (195). Examination of smoking records in asbestos workers indicated possible synergism between these lung irritants and the induction of pulmonary fibrosis (196). Study of over 900 female asbestos workers showed that low to moderate exposure produced excess death from cancer; high exposure produced excess death from lung and pleural cancer as well as respiratory disease. Synergism between asbestos and smoking was observed (197).

Asbestos Insulation—The use of asbestos fibers in the insulation of homes and high-rise structures has been a source of asbestosis not only to the appliers but also to those occupying the building and those passing by in the area. Even with a history of prolonged exposure to asbestos, it may be difficult to reach a diagnosis of pleural mesothelioma because so-called benign mesotheliomas do occur and tuberculosis can stimulate the mesothelial cells to give the ap-

Table VIII—Occupational and Pathological Data Relating to Asbestos Workers Dying with Pleural Mesothelioma: All Employees[a]

Year of Death	Sex	Age, Years	Occupation	Period of Exposure	Duration of Exposure, Years			Necropsy		Death Certificate	
					Total	Before Jan. 1, 1933	From Beginning to Death	From End to Death	Performed	Asbestosis Present	Asbestosis Mentioned	Attributed Cause of Death
1936	Male	65	Fiberizer	1913–1936	23	19	23	<1	+	+	+	Endothelioma of pleura
1964	Male	57	Carder, spinner	1922–1923[b]	1 5/12	1 5/12	42	41	+	0	+	Malignant pleural tumor
1966	Female	55	Carder, spinner	1926–1935	9	6	40	31	+	0	0	Carcinoma of lung
1966	Male	59	Weaver	1937–1938[c] 1960–1965	6	0	29	1	+	+	+	Carcinoma of lung
1967	Male	72	Weaver	1914 1919–1920	0 7/12	0 7/12	53	47	+	0	0	Myeloid leukemia (carcinoma of lung associated)

[a] Reference 193. [b] Also employed for 15 years in the rubber department (1929–1944) and 20 years in the warehouse (1944–1964). [c] Also employed in the warehouse (1929–1930).

pearance of neoplasia (198). Examination of 632 building insulation workers revealed 45 deaths from lung or pleural cancer and 39 deaths from cancer of the stomach, colon, or rectum. The relationship of the latter to asbestos exposure could not be definitely proven (199).

The risk for asbestos insulation workers of developing mesothelioma of the pleura or peritoneum is much greater than for the general population, 10 per 307 *versus* 3 per 31,652 (200). Long exposure to asbestos fibers may not be required for the induction of mesothelioma of the lung, because an accountant exposed to asbestos insulation for 1 month developed the disease 6 years later (201).

Asbestosis has also been observed in hardboard and transformer winding workers but the minor exposure, while producing fibrotic changes in the lungs, gave no evidence of pulmonary cancer (202). Studies on 152 asbestos insulation workers revealed 46 deaths with 23 due to malignancy. All workers had 15 years of exposure to asbestos dust, and the range of ages was 33–81 (203). An increase in pleural mesothelioma was projected for Australian insulation workers since increased amounts of asbestos are being used in that country (204). A survey of 370 asbestos insulation workers indicated that those who smoked cigarettes have a 92 time greater risk of developing pulmonary cancer than those who did not smoke (205).

The most hazardous area in the insulation trade is the removal of old asbestos and its replacement, during which pounding of asbestos blocks and mixing finishing muds lead to high environmental fiber levels (206). The high risk of death from asbestos exposure, 300 out of 632 union members, resulted in a modern industrial hygiene program sponsored by the union, industry, government, and medical science. This preventative program should decrease the exposure of insulation workers (207).

Asbestosis has been shown to be a major cause of pulmonary disease in Northern Ireland, Sweden, and Finland (208–211). It had been suggested that insulation workers exposed to amosite might not get cancer, but 25 cases of lung cancer occurred in 230 workers exposed to this form of asbestos (212). A recent report indicates that there is a relationship between asbestosis and cancer of the larynx (213).

Pipe Insulation—The installation and removal of insulation on pipes in ships, power stations, factories, and buildings are very dusty operations. Pipe sections, slabs, powder, and mattresses are composed of 85% magnesia and 15% amosite asbestos. Exposure occurs during sawing and fitting pipe sections, mixing asbestos plaster, and stripping off old lagging (214). A comparison of fiber glass, polyurethane, and asbestos pipe insulation showed that only the latter could be considered hazardous. An evaluation of 33 death certificates gave a 24.2% incidence of malignant neoplasms in the asbestos workers (215). A revision of the threshold limit value for asbestos was suggested when it was discovered that pipe coverers working for 20 or more years had a 38% incidence of asbestosis when exposed to apparently "safe" dust concentrations (216).

Table IX—Ranges of Dust Concentrations in Various Dockyard Areas[a]

Process	Range, Fibers/cm³
Storerooms	0.1–36
Application of amosite sections	9–40
Application and stitching of asbestos cloth	0.05–0.26
Removal of amosite sections (boiler room)	29–1040
Removal of blue sprayed asbestos	112–1906
Removal of asbestos acoustic board	48–683
Bagging asbestos debris	106–3815

[a] Reference 219.

Ship Repairs—Shipboard pipe covering and insulation during overhaul and repairs are extremely hazardous, and it is difficult to prevent massive exposures (217). Diffuse mesothelioma was related to asbestos exposure in 14 shipyard workers in Liverpool (218). The range of fiber counts in various occupations in a dockyard is given in Table IX; removal of asbestos gives the highest counts and the greatest worker exposure (219). Asbestos exposure in English shipyards has been shown to produce pleural fibrosis (28%) but only a few cases of mesothelioma (220, 221).

Dutch investigators found an unusually high incidence of pleural and peritoneal mesotheliomas in shipyard asbestos workers (222). A Scottish retrospective study, covering 1950–1967, discovered 80 cases of malignant mesothelioma in shipyard workers handling asbestos (223). In 1965–1970, 25 cases of pleural mesothelioma were reported in Plymouth shipyard workers (224). A similar observation was made for Italian (225) and Dutch (226) shipyard workers.

In a cohort study, it was found that asbestosis in shipyard workers was 11 times more prevalent than in the controls and prolonged exposure to asbestos, even at low dust concentrations, was hazardous (227, 228). Continuous surveys of British dockyard workers turned up 37 cases of mesothelioma since 1965 and many cases may go undetected (229).

Other Sources of Exposure—The widespread use of asbestos, including asbestos-cement water pipes, beverage filter media, household iron-holders, and asbestos in cigars, is a nonoccupational route of exposure which contributes to the worldwide distribution of cancer (230). The incidence of English males dying from asbestosis and having lung cancer during 1924–1963 is given in Table X. The percent of those with lung cancer has been increasing because the victims have been living longer (231).

Table X—Incidence of Lung Cancer in Males Dying with Asbestosis[a]

Period	Deaths from Asbestosis	Percent with Contaminant Cancer of Lung	Average Age at Death from Uncomplicated Asbestosis, Years
1924–1940	79	16.4	49.3
1941–1950	92	22.8	55.9
1951–1960	144	31.3	58.1
1961–1963	77	54.5	60.4

[a] Reference 231.

Protective asbestos clothing (aprons, gloves, and aluminized asbestos garments) give rise to high fiber counts in the respiratory area (232).

Asphalt–asbestos or vinyl–asbestos tile contains 15–25% asbestos, which becomes airborne during the sanding operation associated with their installation. Mesotheliomas have resulted from inhalation of this dust (233).

Fire-eater's asbestosis has occurred from extinguishing the torch by placing it in the mouth (234).

Coke oven or silo construction requires effective insulation containing asbestos, and workers have developed asbestosis with lung carcinoma and/or mesothelioma (235, 236).

Asbestos cement and wall board present hazards to both the producer and the user. In the latter case, sawing such boards without using a respirator has resulted in the development of asbestosis years later (237, 238).

Asbestosis has also been associated with severe rheumatoid disease with necrobiotic foci similar to those encountered in subcutaneous rheumatoid nodules (239, 240). In 80 cases of asbestosis, antinuclear and rheumatoid factors were found in 28 and 27%, respectively. There was a relationship between these autoantibodies and the degree of radiographic abnormality but not between the duration of asbestos exposure (241).

COMMENT AND CONCLUSIONS

In the controversy concerning amosite fibers in water supplies, it is necessary to identify and establish exact amounts of asbestos per unit volume. It has been established that the Duluth water supply contains 1–30 × 10^6 fibers/liter, equivalent to 1–30 μg/liter (242). This amount would appear to present a hazard to the individuals exposed daily (243). An analysis of cancer mortality in the region indicated no increase in cancer mortality patterns of persons of any age group. However, only 14 years have passed and asbestos-induced cancer requires at least 20 years (244). It was suggested that this lack of carcinogenic effect is related to the inability of particle transmigration through the intestinal mucosa (245). The size of the fibers, less than 5 μm in length, may be incapable of causing fibrosis or cancer (246).

Thus, after reviewing the asbestosis problem, covering chemistry, industrial hygiene, animal and human toxicology, and carcinogenic aspects, it becomes evident that more attention must be paid to analytical chemistry, particle size, and epidemiology. The difficulties encountered in the analysis of asbestos reside in the fact that its chemical composition differs only slightly from one variety to another and, depending upon the method of preparation, it may become contaminated with other metallic ions. Since particle size appears to be the controlling factor in the induction of asbestosis and cancer, accurate sizing must be done to prevent a negative result. Epidemiology should include a continuing follow-up of known cases of asbestosis and the addition of new cases as they become apparent. Cohort studies should

be initiated where possible, and assessment of the degree of exposure should be undertaken to correlate the amount of asbestos inhaled with individual response as related in time.

Experimental studies covering ingestion of various forms of asbestos should be initiated to ascertain the effect of such ingestion on GI penetration and the development of fibrosis and carcinoma. A good statistical design should be employed, utilizing both physical (fiber glass of the same particle-size distribution) and chemical control groups. In the latter case, the chemical should have its major carcinogenic effect on the GI tract. The animal species selected for this experiment should have a low spontaneous incidence of carcinoma of the target tissue and, hopefully, an overall low incidence of tumors in other tissues. The observation of time to tumor should also be included. Diet variations (*e.g.*, ulcerogenic) should be considered as a possible additional aspect of the problem.

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Effects of Normal Alcohols on Intestinal Absorption of Salicylic Acid, Sulfapyridine, and Prednisolone in Rats

WILLIAM L. HAYTON

Abstract □ The rates of intestinal absorption of salicylic acid, sulfapyridine, and prednisolone from solutions containing no alcohol or 0.5% ethanol, *n*-butanol, or *n*-hexanol were determined. At the concentrations used, ethanol did not significantly affect drug absorption. Butanol reduced the rate of absorption of sulfapyridine but did not significantly affect the absorption rates of prednisolone or salicylic acid. Hexanol reduced the rates of absorption of sulfapyridine and salicylic acid and increased the rate of absorption of prednisolone. The absorption-altering effects of the alcohols were concentration dependent and rapidly reversible. Histological studies indicated that the structure of the epithelium was not altered by the alcohols. While the absorption rate of water from the drug solutions was increased by the alcohols, their absorption-altering effects could not be attributed solely to increased water flux. In addition, the absorption-altering effects of the alcohols could not be attributed to formation of drug–alcohol complexes nor to alcohol-induced alterations in the extent of binding of the drugs to nondialyzable materials in the intestinal drug solution.

Keyphrases □ Alcohols, normal—effects on intestinal absorption of salicylic acid, sulfapyridine, and prednisolone, rats □ Salicylic acid—effects of normal alcohols on intestinal absorption, rats □ Sulfapyridine—effects of normal alcohols on intestinal absorption, rats □ Prednisolone—effects of normal alcohols on intestinal absorption, rats □ Absorption, intestinal—salicylic acid, sulfapyridine, and prednisolone, effects of normal alcohols, rats

Several changes in the structure and function of the epithelial lining of the small intestine may occur when it is exposed to aqueous solutions of normal alcohols. For example, ethanol inhibited the active transport of amino acids in the small intestine of humans (1), and several alcohols, including ethanol and *n*-butanol, inhibited the active transport of amino acids and glucose through the everted rat intestine (2). Acute oral administration of aqueous solutions of 5% ethanol to the rat produced hemorrhagic erosions in the intestine and reduced the activities of lactase and thymidine kinase; oxygen consumption by jejunal slices was also reduced by ethanol (3).

Such changes suggest that the capacity of the intestine to absorb drugs may be altered by exposure of the intestine to alcohols. Two percent ethanol in solutions of theophylline recirculated through segments of rat jejunum increased the rate of absorption of theophylline, apparently due to an ethanol-induced increase in the rate of water absorption (4). However, the presence of 2% ethanol in solutions of barbiturates, sulfaguanidine, and promethazine did not significantly alter the rate of drug absorption (5).

The purpose of this study was to determine the effects of low concentrations of ethanol, *n*-butanol, and *n*-hexanol on the rates of intestinal absorption of salicylic acid, sulfapyridine, and prednisolone. These drugs were used because of their diverse physicochemical properties. Salicylic acid was ionized at the pH of these experiments, while sulfapyridine and prednisolone were not. The *n*-butanol–pH 7.0 aqueous buffer partition coefficients of salicylic acid, sulfapyridine, and prednisolone were 0.5, 3, and 40, respectively.

The concentration of alcohol used was 0.5% or less. This concentration of ethanol is readily achieved in the intestinal lumen of humans following oral ingestion of relatively small quantities of ethanol. For example, oral administration of 40 g of ethanol as a 10 or 20% aqueous solution produced ethanol concentrations in the upper jejunum above 1% for 30–60 min (1). While the intestinal mucosa is exposed to a number of aliphatic alcohols following consumption of some alcoholic beverages, exposure to levels of 0.5% butanol or hexanol would only be found after accidental ingestion of preparations containing relatively high concentrations of the alcohols.

EXPERIMENTAL

Materials—Sodium salicylate[1], salicylic acid[1], sodium sulfapyridine monohydrate[2], sulfapyridine[3], prednisolone[4], and ^{3}H-prednisolone[5] were used as received. The labeled radiochemical purity (>95%) of ^{3}H-prednisolone was verified periodically by TLC on glass plates coated with silica gel G and developed with benzene–2-propanol (4:1). Reagent grade absolute ethanol[6], *n*-butanol[7], *n*-pentanol[8], and *n*-hexanol[9], 98%, were used as received. Isopropyl myristate[10] was purified by passing it through a column packed with aluminum oxide. Following purification, no free acid was detected by the USP XVII method (6).

Complex Formation—Apparent association constants for drug–hexanol complexes with assumed 1:1 stoichiometry were determined by the solubility method (7). Fifteen milligrams of prednisolone, 15 mg of sulfapyridine, or 200 mg of salicylic acid was placed in each of a series of 10-ml ampuls; then 5 ml of solutions of hexanol in isopropyl myristate (0.0–0.20 *M*) or water[11] (0.0–0.05

[1] Merck and Co., Rahway, N.J.
[2] City Chemical Corp., New York, N.Y.
[3] Matheson, Coleman and Bell, East Rutherford, N.J.
[4] Sigma Chemical Co., St. Louis, Mo.
[5] Amersham/Searle Corp., Des Plaines, Ill.
[6] U.S. Industrial Chemicals Co., New York, N.Y.
[7] J. T. Baker Chemical Co., Phillipsburg, N.J.
[8] Mallinckrodt Chemical Works, St. Louis, Mo.
[9] Aldrich Chemical Co., Milwaukee, Wis.
[10] Ruger Chemical Co., Irvington, N.J.
[11] 0.1 *M*, pH 6.5 sodium phosphate buffer for sulfapyridine.

M) were added. The ampuls were flame sealed and rotated for 72 hr in a 25 ± 0.1° water bath. After suspended drug particles were allowed to settle, the ampuls were opened, a sample of supernate was removed and filtered through a 0.25 ± 0.03-μm filter[12], and the concentration of drug was determined.

Hexanol did not interfere with the analytical methods used to determine drug concentration; when solutions of the drugs in isopropyl myristate or water were filtered, no change in drug concentration was observed. All phase solubility diagrams were type A_L, and association constants were determined from the slopes and intercepts of lines fitted to the diagrams by least squares (7).

Intestinal Clearance—Male Sprague–Dawley rats, 200–350 g, were housed in wire mesh cages in a room maintained at approximately 20° and lighted automatically 12 hr/day. The clearance of prednisolone, sulfapyridine, and salicylic acid from solutions recirculated at 6 ml/min through a 20-cm segment of *in situ* jejunum was measured by a technique described previously (8). When present, the concentration of alcohol in the recirculating drug solution was maintained constant by infusing the alcohol into the solution at the same rate that it was absorbed by the intestine.

Ethanol and butanol were infused into the recirculating drug solution as aqueous solutions. Due to its low solubility in water, hexanol was infused undiluted. Prednisolone (0.28 mM), containing 0.1 μCi/ml of ^{3}H-prednisolone, was dissolved in 154 mM NaCl solution; sulfapyridine (0.40 mM) and salicylic acid (13 mM) were dissolved in 67 mM sodium phosphate buffer at pH 6.6 and made isotonic with 82 mM NaCl. The amount of sodium chloride in the drug solutions was not reduced when alcohol was present.

The drug solution was sampled periodically and the drug concentration was determined. The drugs were absorbed by apparent first-order kinetics (Fig. 1), and absorption rate constants were determined from the slopes of lines fitted by least squares to plots of drug concentration *versus* time on semilogarithmic coordinates. The initial point was omitted from the least-squares fit to ensure stationary-state conditions; while the initial point was close to the line in the control experiments, it tended to fall above the line when the alcohols were present. This tendency for positive deviation, indicating transient rapid absorption of the drugs, increased with the molecular weight of the alcohol.

The absorption rate constants were converted to clearance per centimeter of intestine by multiplying the rate constant by the volume of perfusate, correcting for drug removed due to sampling, and dividing by the length of the intestine. The absorption rate of water per centimeter of intestine was calculated by dividing the difference between the amount of water added to the drug solution to maintain its volume and alcohol concentration constant and the amount removed as samples by the duration of the experiment and by the length of the intestine. When alcohol was present, its concentration was determined at the beginning, middle, and end of each experiment and the three values were averaged. Not more than 10% of the LD_{50} of any alcohol was absorbed during any experiment.

Drug Binding in Perfusate—Isotonic sodium phosphate buffer, alone or containing 0.3% hexanol, was recirculated through segments of intestine for 1 hr. The perfusate was placed in one chamber of a dialysis cell separated by a cellulose membrane from an equal volume of buffer containing drug and 0.3% hexanol when it was present in the perfusate. The dialysis cell was agitated in a 37° incubator, and the drug concentration on each side of the membrane was measured until successive determinations were the same. The percent of drug bound to nondialyzable substances in the perfusate was found as follows:

$$\% \text{ drug bound} = \frac{C_p - C_b}{C_p} \times 100 \qquad \text{(Eq. 1)}$$

where C_p and C_b refer to the concentration of drug in the perfusate and buffer, respectively, at equilibrium.

Histology—Segments of intestine were perfused with isotonic sodium phosphate buffer, alone or containing 0.5% ethanol, butanol, or hexanol. When present, the concentration of alcohol was maintained by infusion. After 1 hr, each segment was removed and fixed immediately in 10% buffered neutral formalin. Pieces of each segment were embedded in paraffin, and sections of 4–6-μm thickness were cut and stained with hematoxylin and eosin-phloxine (9).

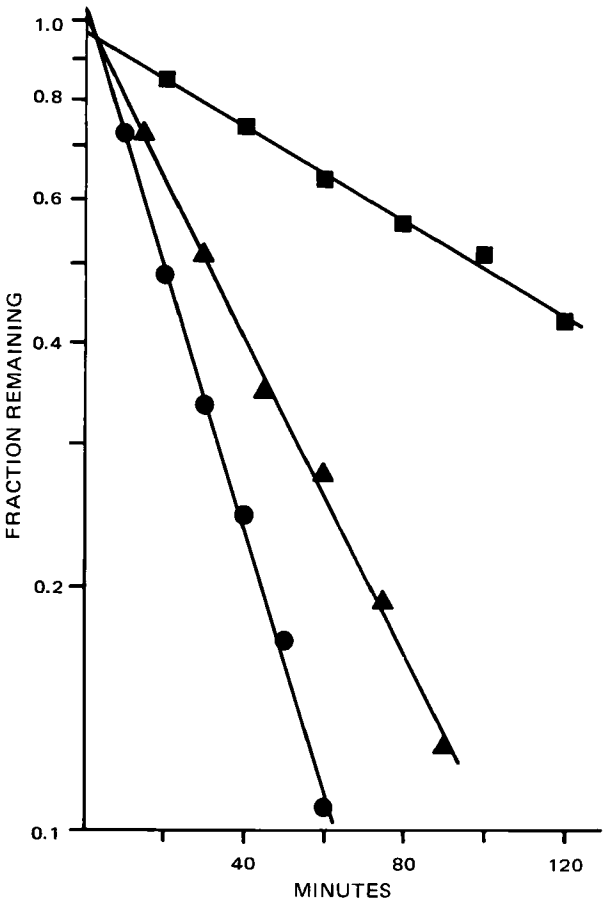

Figure 1—*Fractions of the initial concentrations of prednisolone (■), sulfapyridine (▲), and salicylic acid (●) remaining in the intestinal drug solution as a function of time. Each plot is from a single, representative experiment.*

Analytical Methods—Tritium was determined in 50-μl samples of intestinal perfusate and 1-ml samples of blood plasma by mixing with scintillation fluid[13] and counting in a liquid scintillation spectrometer[14]. For the intestinal perfusate samples, the efficiency of the counting system was constant at approximately 40% and was not affected by the small amounts of alcohol; therefore, correction for quenching was not made routinely. The counting efficiency of the plasma samples was 20–30%, and ^{3}H-toluene was used as the internal standard for quench correction. In studies on complex formation, prednisolone was determined by the USP colorimetric procedure (6). Sulfapyridine and salicylate in samples of intestinal perfusate and in studies on complex formation were determined by colorimetric methods (10, 11). The drugs were extracted from 1-ml samples of isopropyl myristate with 5 ml of 1% Na_2CO_3; 0.5 g of NaCl was added to prevent emulsification.

Ethanol in 0.1-ml samples of intestinal perfusate was determined by a GC method (12). Each sample was placed in a 10-ml serum bottle, which was then sealed with a rubber septum. The sample was incubated for 20 min in a 37° water bath and 0.50 ml of the vapor phase was injected onto a glass column, 4 mm i.d. by 1.8 m (6 ft), packed with 5% Carbowax 1500 on Haloport F (30–60 mesh). The temperatures of the injection port, oven, and flame-ionization detector were 110, 70, and 240°, respectively. The flow rates of helium, hydrogen, and air were 30, 30, and 500 ml/min, respectively. The retention time for ethanol was 3.5 min, and a plot

[12] Millipore type UG.

[13] PCS solubilizer, Amersham/Searle Corp., Des Plaines, Ill.

[14] Packard Tri-Carb model 3320, Packard Instrument Co., Downers Grove, Ill.

Table I—Effect of 0.5% *n*-Hexanol on the Distribution of Radioactivity following 30-min Perfusion of *In Situ* Rat Intestine with Solution of ^{3}H-Prednisolone

	Control[a]	*n*-Hexanol[a]	*p*
Percent of dose absorbed	21.3 (6.24)	33.2 (5.21)	<0.01
Plasma concentration[b]	4.89 (1.78)	8.70 (1.60)	<0.01
Length of intestine perfused, cm	19.4 (1.72)	19.9 (1.46)	>0.50

[a]Mean from seven experiments; *SD* in parentheses. [b](dpm/ml ÷ dpm administered) × 10^4.

of peak height *versus* concentration of ethanol was linear over the 0.1–0.5% range.

Butanol and hexanol in 0.1-ml samples of intestinal perfusate were also determined by GC. The samples were mixed with 0.1 ml aqueous 1-pentanol, which was used as an internal standard; approximately 1 μl was injected onto a stainless steel column, 0.31 cm (0.125 in.) o.d. by 1.8 m (6 ft), packed with Porapak Q, 80–100 mesh. The temperatures of the injection port, oven, and flame-ionization detector were 240, 225, and 230°, respectively. The flow rates of helium, hydrogen, and air were 40, 30, and 500 ml/min, respectively. Plots of the ratios of peak heights of butanol or hexanol to pentanol *versus* concentration were linear over the 0.1–1.0% range. No apparent alcohol was detected in samples of intestinal perfusate without alcohol.

RESULTS AND DISCUSSION

The effects of 0.5% ethanol, butanol, and hexanol on the rates of disappearance of prednisolone, sulfapyridine, and salicylic acid

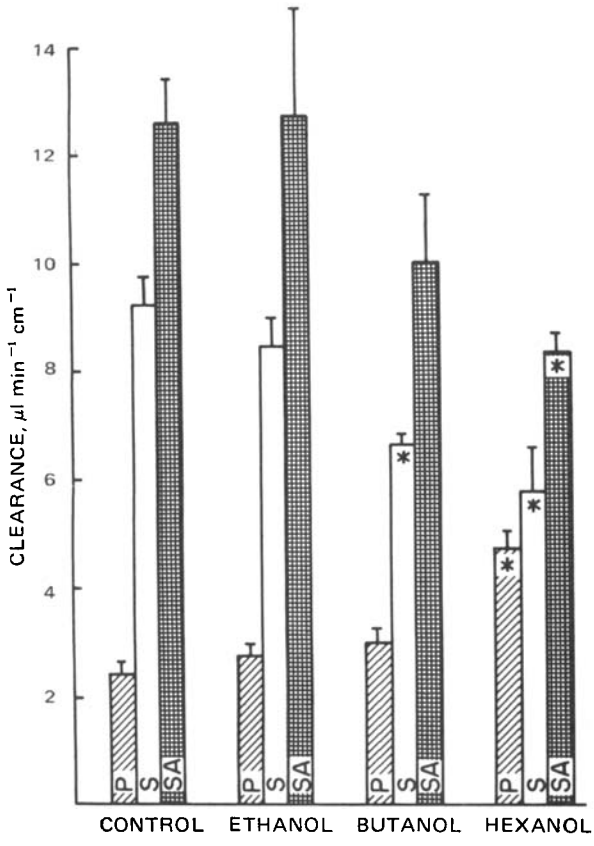

Figure 2—*Effects of constant concentrations of 0.5% ethanol, n-butanol, and n-hexanol on the clearance of prednisolone (P), sulfapyridine (S), and salicylic acid (SA) by the* in situ *rat jejunum. Each bar represents the mean from four or more experiments, lines indicate 1* SE, *and * indicates means that are significantly different (p < 0.05) from the control.*

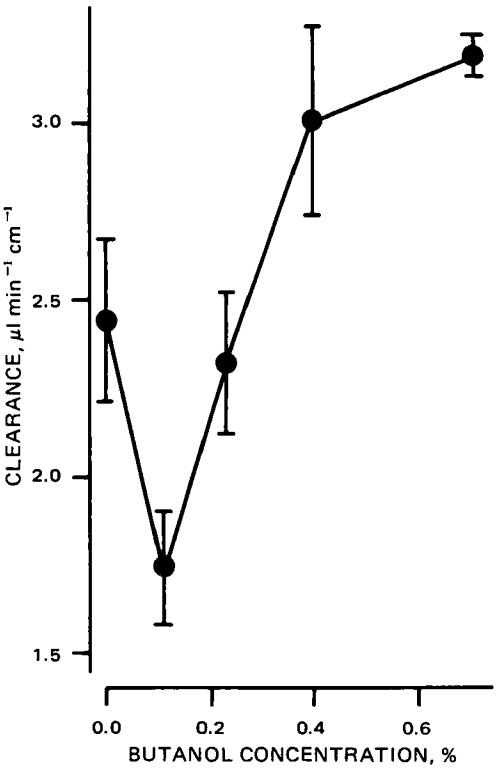

Figure 3—*Effect of various constant concentrations of butanol on the absorption of prednisolone from solutions recirculated through the* in situ *rat jejunum. Each point represents the mean from four or more experiments; bars indicate ±1* SE.

from solutions recirculated through the *in situ* rat jejunum are shown in Fig. 2. Ethanol did not significantly affect the rate of drug disappearance. The rate of disappearance of sulfapyridine was reduced by butanol, while the rates of disappearance of prednisolone and salicylic acid were not significantly altered. Hexanol increased the rate of disappearance of prednisolone and decreased the rates of disappearance of salicylic acid and sulfapyridine. The concentration dependence of the effects of butanol on the rates of disappearance of prednisolone and sulfapyridine is shown in Figs. 3 and 4.

To verify that the alcohol-induced changes in the rates of drug disappearance from the intestinal lumen reflect changes in absorption rates, the tritium concentration in the plasma was measured after perfusing intestinal segments for 30 min with a solution of ^{3}H-prednisolone and 0.5% hexanol. Compared to the control experiment in which no hexanol was present, the tritium concentration in the plasma and the amount of prednisolone absorbed were significantly increased (Table I). The increased rate of prednisolone absorption in the presence of hexanol cannot be attributed to differences in the length of intestine perfused.

A similar experiment with sulfapyridine showed no difference

Table II—Clearance of Prednisolone and Salicylic Acid by Perfused *In Situ* Rat Intestine during Two Consecutive 1-hr Periods

		Clearance[a], μl min^{-1} cm^{-1} of Intestine	
	Period[b], hr	Prednisolone	Salicylic Acid
Control	0–1	2.86 (0.304)	11.5 (2.64)
	1–2	2.60 (0.441)	9.07 (1.53)
Test	0–1	5.24 (0.485)	9.55 (0.861)
	1–2	2.69 (0.278)	6.91 (0.358)

[a]Mean from four experiments or more; *SD* in parentheses. [b]No hexanol was present during either period in the control experiment; in the test experiment, 0.5% hexanol was present only during the 0–1-hr period.

Table III—Effect of 0.5% Alcohols on pH of Solutions of Salicylic Acid and Sulfapyridine following Perfusion of Intestinal Segment

	Final pH[a]
Control	6.1 (0.03)
Ethanol	6.1 (0.07)
Butanol	6.1 (0.05)
Hexanol	6.4 (0.15)

[a]Mean from eight experiments; *SD* in parentheses; initial pH was 6.6.

between the control and hexanol in either the extent of absorption of the drug or its blood plasma concentration. The lack of effect at 30 min was apparently due to the transient rapid absorption[15] of sulfapyridine that occurred in the presence of hexanol. Such behavior illustrates the importance of characterizing the intestinal clearance of drugs by monitoring the drug concentration in the intestinal lumen over a period of time rather than making "single-point" determinations.

The absorption-altering effects of the alcohols may have resulted from damage to the intestinal epithelium exposed to the alcohols. To check this possibility, segments of jejunum were perfused for 60 min with an isotonic sodium phosphate buffer containing no alcohol or 0.5% ethanol, butanol, or hexanol. Thin sections of perfused intestine were prepared and examined by a veterinary pathologist. No differences were detected in the structure of the intestine perfused with 0.5% alcohol compared to the intestine not exposed to alcohol (Fig. 5).

In addition, the absorption-altering effects of hexanol were rapidly reversible. The clearance of prednisolone and salicylic acid was measured during two consecutive 1-hr periods in the same segment of intestine. During the first period, 0.5% hexanol was present. This solution was then removed; during the second period, the intestine was perfused with a solution of drug without hexanol.

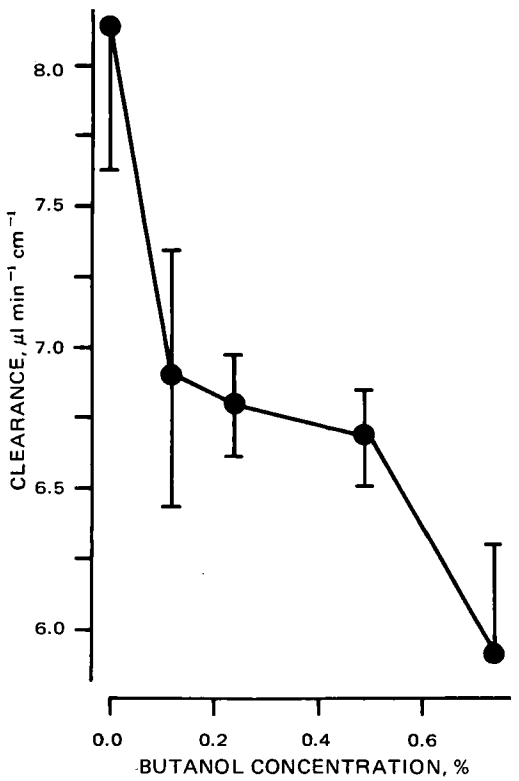

Figure 4—*Effect of various constant concentrations of butanol on the absorption of sulfapyridine from solutions recirculated through the* in situ *rat jejunum. Each point represents the mean from four or more experiments; bars indicate ±1* SE.

[15] Described under *Intestinal Clearance.*

Table IV—Apparent 1:1 Association Constants for Drug–Hexanol Complexes in Isopropyl Myristate and Water at 25°

	$K_{1:1}$[a]	
Drug	Isopropyl Myristate	Water
Salicylic acid	3.5	—[b]
Sulfapyridine	2.7	2.4
Prednisolone	4.2	2.3

[a]Liters per mole. [b]High solubility precluded measurement.

The results of this experiment (Table II) show that the increased clearance of prednisolone produced by hexanol returns immediately to the control value when hexanol is removed from the intestinal perfusate.

The clearance of salicylic acid did not return to the control value during the period following exposure of the intestine to hexanol. However, the control experiment for salicylic acid indicates that the lack of reversibility was due primarily to exposure of the intestine to salicylate rather than hexanol. Reduced absorption of salicylic acid following its repetitive instillation to the stomach in humans (13) and intestine in rats (14) was reported. Thus, it appears that the alcohols do not alter drug absorption rates by damaging the intestinal epithelium.

Salicylic acid and sulfapyridine are weakly acidic, pKa of 3.0 and 8.4, respectively (15), so an alcohol-induced change in the pH of the intestinal drug solution could explain the effects of the alcohols on the rates of drug absorption. The pH of the intestinal drug solution decreased slightly during the absorption experiments when no alcohol was present (Table III), and neither ethanol nor butanol influenced the magnitude of the decrease. Although at the end of the experiment the pH of the drug solution containing hexanol was slightly greater than the control value, it is not likely that this small difference in pH could account for the effect of hexanol on the absorption rates of salicylic acid and sulfapyridine.

The rate of drug absorption may be increased or decreased by formation of molecular complexes, depending on the relative extent of complexation of the drug in the luminal solution and the intestinal epithelium (16). To explore this possibility, the extent of complex formation between the drugs and the alcohols was determined by phase solubility analysis. The apparent 1:1 association constants for sulfapyridine–hexanol and prednisolone–hexanol complexes in water were quite low (Table IV). Not more than 9% of sulfapyridine or prednisolone would be complexed with 0.5% hexanol in the intestinal perfusate. The extent of complexation between the drugs and alcohols in isopropyl myristate was also small, indicating that formation of drug–alcohol complexes in the lipoid barrier of the intestine would be insignificant.

Thus, the lack of appreciable interaction between any of the drugs and alcohols suggests that the absorption-altering effects of the alcohols are not due to formation of drug–alcohol complexes. Another possible mechanism for the absorption-altering effects of the alcohols is an alcohol-induced change in the extent of drug binding to macromolecules released possibly from the intestinal mucosa. Dialysis studies on intestinal perfusates, however, revealed that the extent of drug binding was low and not affected significantly by hexanol (Table V).

The rate of water absorption by the intestine was shown theoretically (17) and experimentally (4, 18) to influence the rate of drug absorption by the intestine. Generally, the rate of drug absorption increases linearly with the rate of water absorption. Al-

Table V—Percent of Drug Bound in the Perfusate to Nondialyzable Material from the Intestinal Mucosa

	Percent Drug Bound[a]	
Drug	Control	Hexanol
Salicylic acid	3.64	2.32
Sulfapyridine	2.96	3.06
Prednisolone	−1.54[b]	2.12

[a]Mean of two determinations. [b]At equilibrium, concentration was lower in perfusate than in buffer.

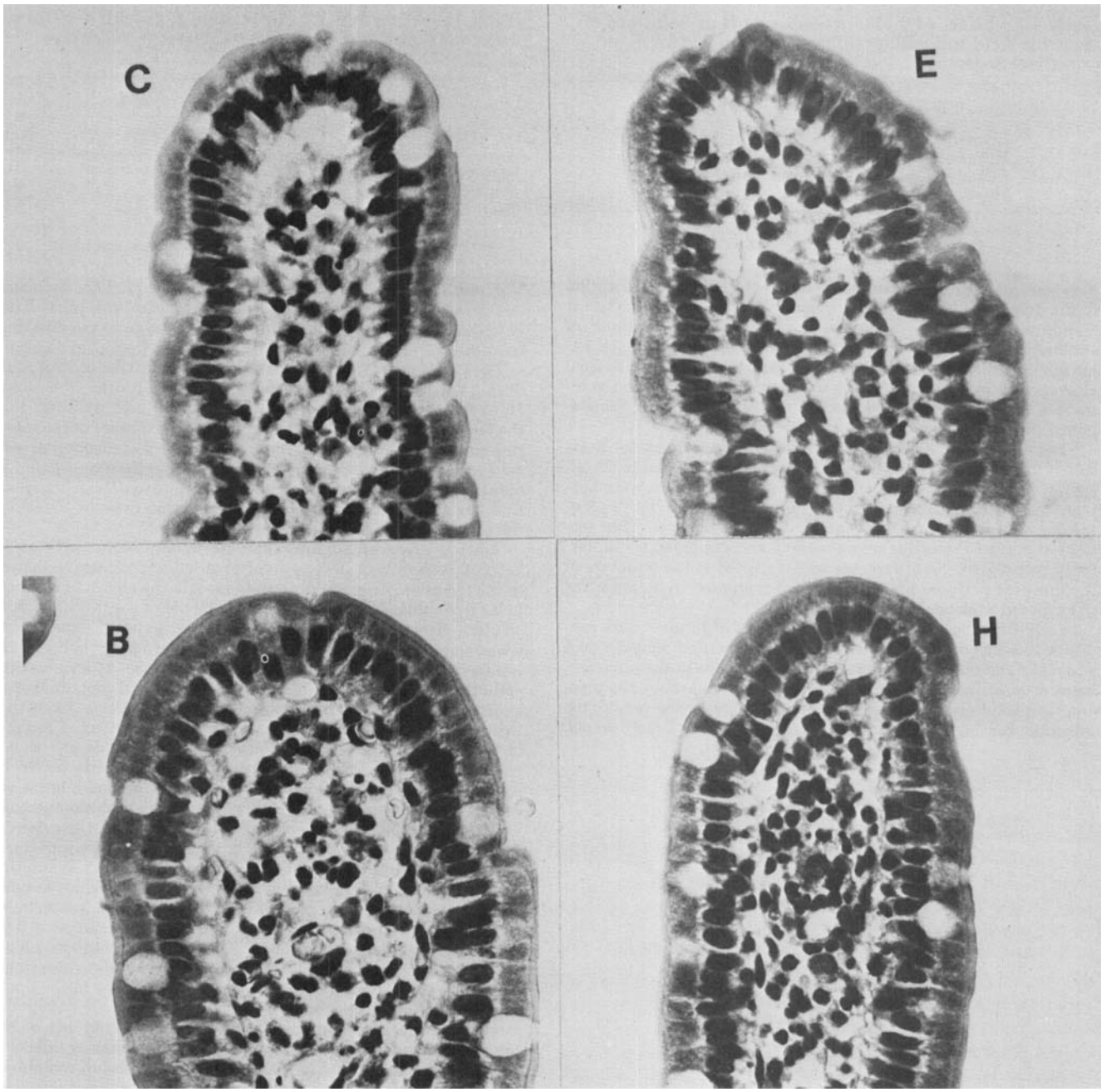

Figure 5—*Representative sections of individual villi from rat jejunum perfused for 1 hr with isotonic sodium phosphate buffer (C) or buffer containing 0.5% ethanol (E), butanol (B), or hexanol (H) (×560).*

though the presence of the alcohols increased the osmotic pressure of the drug solutions, the average rate of water absorption by the intestinal segment was increased significantly by ethanol and butanol compared to the control (Table VI). Hexanol did not appear to alter the rate of water absorption. Ethanol produced a similar increase in water absorption by the rat intestine (4), and butanol increased the permeability coefficient of water in frog eggs (19).

While the rate of water absorption probably influenced the rate of drug absorption by the intestine in this study, there was no significant correlation between drug clearance from solutions con-

Table VI—Absorption Rate of Water from the Rat Intestine Perfused with Drug Solutions Containing No Alcohol or 0.5% Alcohol

	Rate[a], μl min^{-1} cm^{-1} of Intestine			
	Control	Ethanol	Butanol	Hexanol
Salicylic acid	1.19 (0.68)	1.63 (0.45)	2.04 (0.26)	1.32 (0.25)
Sulfapyridine	0.96 (0.29)	1.97 (0.40)	1.86 (0.31)	1.24 (0.56)
Prednisolone	1.85 (0.56)	1.98 (0.24)	2.07 (0.71)	1.98 (0.36)
Weighted mean	1.43	1.88[b]	1.99[b]	1.55
Standard deviation	0.647	0.362	0.439	0.513

[a] Mean from four or more experiments; *SD* in parentheses. [b] Significantly different from control mean at the 95% level of confidence.

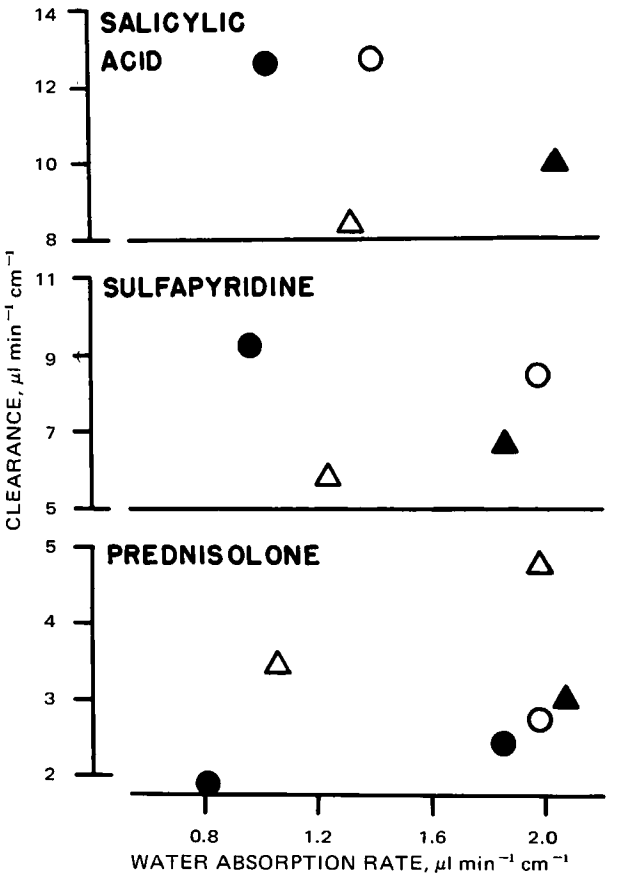

Figure 6—*Clearance of salicylic acid, sulfapyridine, and prednisolone* versus *water absorption rate from solutions containing no alcohol (●) or 0.5% ethanol (○), butanol (▲), or hexanol (△). The clearance of prednisolone from both isotonic sodium chloride and sodium phosphate solutions is shown.*

taining alcohols and the rate of water absorption (Fig. 6). It appears, therefore, that the absorption-altering effects of the alcohols did not result entirely from an alteration in the absorption rate of water. In the control experiment, the rate of water absorption from the solution containing prednisolone was greater than that from the solutions containing salicylic acid or sulfapyridine (Table VI). The latter drugs were in isotonic sodium phosphate buffer, and prednisolone was in isotonic sodium chloride solution.

When the effect of hexanol on the intestinal clearance of prednisolone from the sodium phosphate buffer was examined, hexanol was found to increase the clearance of prednisolone, as it did in the sodium chloride solution (Table VII). Thus, the ionic composition of the perfusate appears to influence the rate of water absorption.

These experiments show that normal alcohols can either increase or decrease the apparent permeability of the rat jejunal mucosa. The magnitude of the change in permeability depends on the concentration and lipophilicity of the alcohol, and the direction of change depends on the drug. The absorption-altering effects of the alcohols cannot be attributed to mucosal damage, pH changes, or complex formation. It is possible that the effects of the alcohols result from a single change in the structure or function of the intestinal mucosa.

For example, the alcohols may displace water from the lipoidal cell membrane, causing a decrease in the polarity of the membrane and thereby increasing the permeability of the membrane to prednisolone, which is relatively nonpolar, and decreasing its permeability to the relatively polar sulfapyridine and salicylate. Alternatively, if the barrier to absorption is structured water, as was suggested (20, 21), the alcohols may increase the degree of structuring of water (22), causing the permeability of the barrier toward nonpolar drugs to increase and toward polar drugs to decrease.

On the other hand, the absorption-altering effects of the alcohols may result from two or more alcohol-induced alterations in the structure or function of the intestinal epithelium. One or more

Table VII—Effect of 0.5% *n*-Hexanol on Clearance[a] of Prednisolone and Absorption Rate[a] of Water from Isotonic Sodium Chloride and Sodium Phosphate Solutions

	Sodium Chloride[b]	Sodium Phosphate[c]	*p*
Control			
Clearance	2.44 (0.613)	1.87 (0.179)	
Water absorption rate	1.85 (0.564)	0.81 (0.084)	<0.01
n-Hexanol			
Clearance	4.76 (0.646)	3.44 (0.269)	
Water absorption rate	1.98 (0.359)	1.06 (0.528)	<0.02

[a] μl min⁻¹ cm⁻¹ of intestine; mean from four or more experiments; *SD* in parentheses. [b] Sodium chloride, 154 mmoles/liter. [c] Sodium phosphate, 67 mmoles/liter; sodium chloride, 82 mmoles/liter.

of the alterations may tend to increase the permeability of the intestine, while the remaining alterations tend to decrease its permeability. The effect observed would then be the net effect of all alterations in the barrier properties of the mucosa. Whether the observed effect of the alcohols is positive or negative would depend on the relative magnitudes of all individual alterations in the intestine as they relate to the particular drug.

Possible structural or functional alterations, which could be caused by the alcohols and which may affect the apparent permeability of the intestinal mucosa, are mucosal blood flow (23), water absorption rate (4), "fluidity" of lipid bilayers (24), thickness of unstirred layers on the luminal side of the absorbing cell (25), dimensions of polar pathways through the epithelium (26), and solute drag (27). Thus, with the limited data available, it is not now possible to establish unequivocally the mechanisms by which normal alcohols alter the permeability of the intestinal epithelium to prednisolone, salicylic acid, and sulfapyridine.

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MICHAEL R. McKAMEY ˣ and LARRY A. SPITZNAGLE

Abstract □ Contrary to earlier literature reports, the impurities in toluidine blue O were shown by column chromatography, TLC, and mass spectrometry to be *N*-methyl homologs of 2-methylthionine rather than *N*-methyl homologs of thionine. Small amounts of 2-methyl-3-amino-7-methylaminophenothiazine and 2-methyl-3,7-diaminophenothiazine were identified in commercial samples of toluidine blue O. However, sample handling and a warm alkaline environment can cause rapid demethylation of the dye.

Keyphrases □ Toluidine blue O and related dyes—chromatography, mass spectra, and visible light absorption □ Dyes—chromatographic, mass spectral, and visible light absorption characteristics of toluidine blue O and related dyes □ Mass spectrometry—impurities, toluidine blue O and related dyes

In recent years there has been increased interest in the medical use of the dye toluidine blue O (3-amino-7-dimethylamino-2-methylphenazathionium chloride, C.I. Basic Blue 17). It recently has been used for the identification of the parathyroids during surgery (1, 2) and as an aid in the diagnosis of small oral cancers (3) and has been suggested as a potential parathyroid and pancreatic scanning agent if labeled with a suitable radionuclide (4).

BACKGROUND

During an investigation into the synthesis of radioactive analogs of toluidine blue O, some problems were encountered in obtaining pure dye samples. Elemental analysis, column chromatography, TLC, and mass spectrometry all indicated the presence of impurities which could not be separated from the dye.

Similar difficulties in analyzing samples of toluidine blue O by TLC were reported (5, 6). The investigators observed three spots for the commercial dye, two blue and one violet. They identified the violet spot as thionine (I) on the basis of its R_f value and visible light absorption spectrum. Extraction of one of the two blue spots and rechromatography again produced two blue spots, which were attributed to different ionic forms of the dye or "solvent effects." In addition, Apgar and Patel (6) reported that visible light absorption experiments indicated that toluidine blue O lost a methyl group (in this case an aryl methyl group) and was converted to azure A (III) upon standing in aqueous solution.

Normann and Normann (7) succeeded in isolating five contaminants from a sample of tritiated toluidine blue O by TLC. The resulting spots were extracted from the thin-layer chromatograms and identified by their absorption maxima (Table I). Their identification of various compounds on the basis of the absorption maxima was not consistent with earlier work (8), which is summarized in Table II.

In 1953, Ball and Jackson reported on chromatographic and spectrophotometric studies of various samples of toluidine blue O and azure A (9). Descending paper chromatograms[1] developed in butanol–acetic acid–water (40:10:50 v/v) indicated that the various samples, supplied by different manufacturers, were indeed not the same dyestuff. Column chromatography on aluminum oxide with varying solvents yielded a number of fractions.

The confusion in the assignment of consistent visible light absorption maxima to the various related dyes was disturbing. In the authors' laboratory, commercial toluidine blue O shows a significant change in absorption characteristics as the pH of the solution is changed. In saline at pH 2, toluidine blue O exhibits a λ_{max} value of 630 nm, while at pH 10 a broad absorption maximum from 550 to 630 nm is observed. Differences in reported λ_{max} values (6, 7) may have been due to sample preparation, since the pH of the solutions studied was not mentioned. In addition, the assignment of the structure of azure A (III) to the degradation product of toluidine blue O implies an aryl-demethylation rather than the more probable *N*-demethylation.

The purposes of the present study were to explain the chromatographic behavior of toluidine blue O and to clarify the interpretation of the visible light absorption data.

EXPERIMENTAL[2]

Column Chromatography—Columns [5.1-cm (2-in.) diameter] were packed with dry silica gel (70–325 mesh) to a depth of 45.7 cm (18 in.). Samples were dissolved in ethanol and applied to the tops of the dry columns. Columns were eluted with ethanol followed by

[1] Whatman No. 1.

[2] The dyes used were obtained from National Aniline and Eastman Kodak and were labeled 80–90% pure. All other chemicals were reagent grade and were used as received. Elemental analyses were performed by Schwarzkopf Microanalytical Laboratory, Woodside, N.Y., and by Strauss Microanalytical Laboratory, Oxford, England.

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Table I—Fractions of ^3H-Labeled Commercial Toluidine Blue O Separated by TLC[a]

Dye	Absorption Maximum, nm	Approximate R_f Values[b]
Thionine (violet)	600	0.81
Toluidine blue O (blue)	616	0.64
Azure A (blue green)	632	0.36
Methylene blue (green)	652	0.17
Methylene blue (gray)	—	0.00

[a] From Ref. 7. [b] R_f values were determined by measurement of a photograph of the chromatogram provided in the text. Values were not reported in Ref. 7.

ethanol–concentrated hydrochloric acid (99:1 v/v). Fractions obtained from the columns were evaporated under reduced pressure.

TLC—Commercially prepared aluminum-backed silica gel plates were used. Developing solvents were: I, methanol–2 *N* HCl (93:7 v/v); and II, ethanol–concentrated hydrochloric acid (99:1 v/v).

Mass Spectrometry—Mass spectra (70 ev) were obtained on a high-resolution mass spectrometer[3] coupled to a computer[4]. All samples were introduced on a direct-insertion probe. Source temperatures varied from 150 to 250°. The computer allowed the spectrometer magnet to decay and recorded multiplier current *versus* time at 85-μsec intervals.

The center of gravity of each peak was determined and compared to perfluorokerosene peaks of known *m/e* values. High-resolution *m/e* values and empirical formulas were calculated with software under control of the operator. Only formulas within ±3 mmass of calculated values are presented here.

Rapid Scan Mass Spectrometry—Rapid, repetitive mass spectra were taken from *m/e* 350 to 180 every 35 sec while under computer control. Scanning commenced when the sample (on the direct-insertion probe) was placed into the source and continued until the monitor current returned to preinsertion levels. Data were stored on magnetic tape and processed like other spectra.

Visible Light Absorption Spectrometry—Visible light absorption spectra were obtained on a recording spectrophotometer[5]. All samples were dissolved in water at pH 3.5. A path length of 1 cm was used.

RESULTS AND DISCUSSION

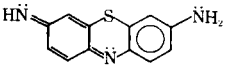
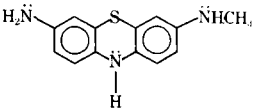
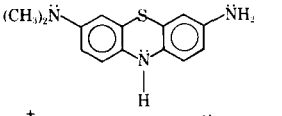
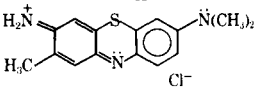
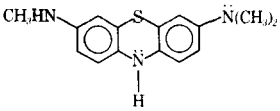
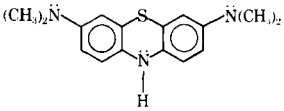
Elemental Analysis of Toluidine Blue O—Elemental analysis of the commercial dye revealed 51.6% C, 5.45% H, 10.46% N, and 9.22% S. This composition yields an empirical formula of $C_{15}H_{19}N_{2.6}S$ (toluidine blue O should be $C_{15}H_{15}N_3S$). Since it was suspected that the depression of nitrogen from the calculated value was due to incomplete burning, a repeat analysis was requested. This analysis included drying the sample to constant weight, burning over vanadium pentoxide, and estimating the chloride ion. This analysis revealed 48.6% C, 4.92% H, 11.86% N, 11.19% S, and 18.24% Cl, which calculates for $C_{11.6}H_{13.9}N_{2.4}SCl_{1.5}$.

TLC of Commercial Toluidine Blue O—TLC (Solvent System I) revealed that the commercial preparation was separable into three colored fractions with R_f values equal to 0.55 (intense blue), 0.69 (blue), and 0.72 (faint violet observable only when rather large amounts of the commercial dye were spotted). Extraction of the intense blue spot (R_f 0.55) with methanol–2 *N* HCl, evaporation of the solvent under reduced pressure at 70–100°, and rechromatography of the blue residue yielded an identical chromatogram to that obtained originally, with the exception that the violet band was not visible.

Treatment of the violet spot at R_f 0.72 in a like manner yielded a chromatogram with only a single spot at R_f 0.72. Other solvent systems were tried with analogous results. Elemental analysis of the fractions was impossible to interpret due to the large amounts of silica gel that dissolved in the solvent and could not be removed from the dye.

[3] AEI MS-9.
[4] PDP-12.
[5] Cary model 14.

Table II—Absorption Maxima for Related Phenothiazine Dyes[a]

Dye	Absorption Maximum, nm	Structure
Thionine (I)	602	
Azure C (II)	611	
Azure A (III)	638	
Toluidine blue O (IV)	635	
Azure B (V)	652	
Methylene blue (VI)	667	

[a] From Ref. 8.

Mass Spectrometry of Toluidine Blue O and Related Dyes—To identify and confirm the presence of contaminants in commercially prepared toluidine blue O, a series of mass spectra (Table III) was obtained for a group of related phenothiazine dyes: toluidine blue O (IV), thionine (I), azure A (III), azure B (V), azure C (II), and methylene blue (VI). For the sake of simplicity, only unit masses are presented in the discussion.

Toluidine Blue O—Inspection of the mass spectrum of commercial toluidine blue O indicates peaks at *m/e* 299 ($C_{17}H_{21}N_3S$), 285 ($C_{16}H_{19}N_3S$), 271 ($C_{15}H_{17}N_3S$), 257 ($C_{14}H_{15}N_3S$), and 243 ($C_{13}H_{13}N_3S$). Since the molecular weight of toluidine blue O is 271, it was apparent that the mass spectrum indicated the presence of contaminants. Since the major fragments were separated by 14 mass units (CH_2), they could not arise by fragmentation, say from the *m/e* 299 peak, because loss of methylene is highly improbable.

It was possible that thermal transfer of methyl groups had occurred in the high temperature source (250°) used to vaporize the samples. Thermal transfer of methyl groups has been reported (10), but data to be presented later indicate that this is probably not the case with toluidine blue O.

Azures A, B, and C—Mass spectra of the azure dyes[6] A (III), B (V), and C (II) are shown in Table III. These spectra are consistent with the presence of contaminating homologs of the parent structure. This finding is easily understood since they are prepared commercially by the oxidative demethylation of methylene blue with chromic acid (11).

Methylene Blue—The methylene blue spectrum (Table III), like the others, indicated that the dye contained a contaminant at *m/e* 299 ($C_{17}H_{21}N_3S$).

Phenothiazine Dyes—In general, phenothiazine systems are characterized by the high abundance of the molecular ion, which is due to the resonance stabilization of the radical ion. Phenothiazine ($C_{12}H_9NS$) fragments primarily to $C_{12}H_9N$ (53%), $C_{12}H_8NS$ (22%), $C_{12}H_8N$ (19%), and $C_{11}H_7S$ (3%) (12). Addition of an exocyclic amine, as in the case of thionine, increases the probability of losing 27 mass units (presumably by the loss of HCN) from the molecular ion.

Addition of *N*-methyl groups favors fragmentation by loss of CH_3; however, the spectra are confusing, due, evidently, to contamination by homologs of the parent with at least one or two ad-

[6] Eastman Kodak.

Table III—Mass Spectra (70 ev) of Some Related Phenothiazine Dyes.

m/e	Empirical Formula	Base Intensity, % Commercial Toluidine Blue O	Thionine	Azure A	Azure B	Azure C	Methylene Blue
299	$C_{17}H_{21}N_3S$	4.	—	—	12.	—	26.
287		1.	—	2.	4.	2.	5.
286		5.	—	6.	17.	6.	22.
285	$C_{16}H_{19}N_3S$	21.	—	29.	92.	37.	100.
284	$C_{16}H_{18}N_3S$	6.	—	7.	25.	8.	42.
283	$C_{16}H_{17}N_3S$	3.	—	—	5.	—	7.
282	$C_{16}H_{16}N_3S$	—	—	—	—	—	3.
273		4.	—	6.	5.	7.	3.
272		17.	—	15.	19.	17.	9.
271	$C_{15}H_{17}N_3S$	100.	—	85.	100.	100.	47.
270	$C_{15}H_{16}N_3S$	22.	—	31.	54.	31.	43.
269	$C_{15}H_{15}N_3S$	8.	—	20.	27.	27.	27.
268	$C_{15}H_{14}N_3S$	—	—	9.	13.	16.	19.
259		—	—	7.	3.	6.	—
258		14.	—	16.	7.	19.	—
257	$C_{14}H_{15}N_3S$	86.	—	100.	41.	100.	9.
256	$C_{14}H_{14}N_3S$	34.	—	50.	44.	54.	17.
255	$C_{14}H_{13}N_3S$	15.	—	36.	18.	50.	11.
254	$C_{14}H_{12}N_3S$	8.	—	19.	15.	33.	11.
253	$C_{14}H_{11}N_3S$	—	—	—	—	6.	4.
252	$C_{14}H_{10}N_3S$	—	—	—	—	8.	5.
245		1.	—	5.	—	2.	—
244		6.	—	10.	2.	9.	—
243	$C_{13}H_{13}N_3S$	39.	—	52.	10.	48.	2.
242	$C_{13}H_{12}N_3S$	34.	—	47.	17.	48.	7.
241	$C_{13}H_{11}N_3S$	6.	—	19.	9.	21.	8.
240	$C_{13}H_{10}N_3S$	—	—	18.	9.	18.	6.
229	$C_{12}H_{11}N_3S$	—	12.	19.	3.	12.	2.
228	$C_{12}H_{10}N_3S$	8.	19.	32.	6.	21.	3.
227	$C_{12}H_9N_3S$	9.	100.	10.	10.	—	5.
226	$C_{12}H_8N_3S$	4.	5.	6.	6.	—	4.
225		3.	—	—	7.	—	—
202		—	4.	—	—	1.	—
201	$C_{11}H_9N_2S$	—	10.	9.	—	5.	—
200	$C_{11}H_8N_2S$	—	78.	6.	—	6.	—
199	$C_{11}H_7N_2S$	—	10.	—	—	—	—
174		—	3.	—	—	—	—
173		—	5.	—	—	—	—
172	$C_{10}H_6NS$	—	7.	—	—	—	—

ditional methyl groups (*i.e.*, differing in empirical formula by one or two CH_2 groups). The loss of HCN (27 mass units) was not observed with the parent structures of azure A, toluidine blue O, and methylene blue.

Rapid Sequence Mass Spectrometry of Commercial Toluidine Blue O—A sample of commercial toluidine blue O was subjected to rapid sequence mass spectral analysis (Fig. 1). The spectra are characterized by the change in the relative abundance of the *m/e* 285 ($C_{16}H_{19}N_3S$), 271 ($C_{15}H_{17}N_3S$), and 257 ($C_{14}H_{15}N_3S$) peaks, which is consistent with a mixture of materials having different volatilities in the source of the mass spectrometer.

Chromatography of Toluidine Blue O—TLC with Solvent System II consistently separated toluidine blue O into four fractions (Fig. 2). A faint violet band at R_f 0.67 (A), a faint blue band at R_f 0.59 (B), an intense blue band at R_f 0.4 (C), and a very faint blue band at R_f 0.17 (D) were routinely observed.

Column chromatography with silica gel and 95% ethanol yielded small amounts of the violet band, A, and somewhat larger quantities of the light-blue fraction, B. The intense blue band, C, was eluted from the column by the addition of hydrochloric acid to the solvent.

The mass spectrum of C is presented in Fig. 3 and seems to be identical to other determinations of the commercial dye. Evidence for all homologs of toluidine blue O was present. Masses at *m/e* 299 ($C_{17}H_{21}N_3S$), 285 ($C_{16}H_{19}N_3S$), 271 ($C_{15}H_{17}N_3S$), and 257 ($C_{14}H_{15}N_3S$) were all present as before.

The mass spectrum of the light-blue fraction, B (Fig. 4), indicated that the mass at *m/e* 257 ($C_{14}H_{15}N_3S$) was the only significant homolog present. This finding was confirmed by rapid sequence

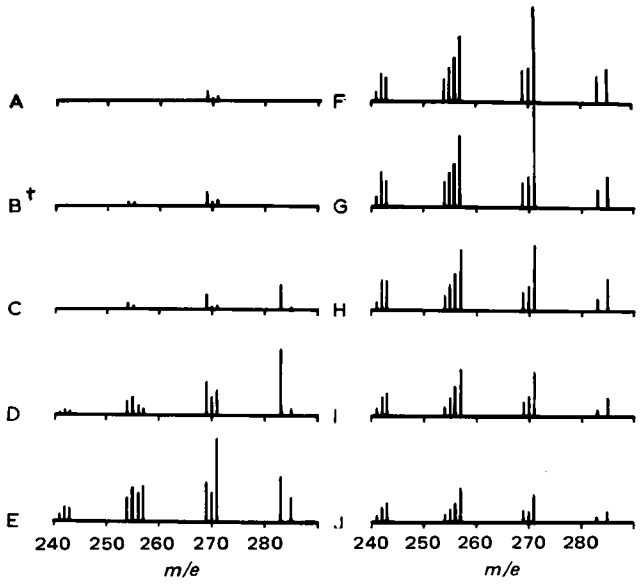


Figure 1—*Rapid sequence mass spectra (70 ev) of commercial toluidine blue O. Peak heights are shown relative to the highest peak seen during an experiment. (†The start of scan B was 35 sec after the beginning of scan A and so on through scan J.)*

mass spectral scans. The scans indicated that the sample did not behave like those in other studies with toluidine blue O; essentially only *m/e* 257 ($C_{14}H_{15}N_3S$) was seen to any extent during the experiment.

Examination of the leading violet band, A, was not successful as the sample could not be volatilized from the direct-insertion probe of the mass spectrometer.

Degradation of Commercial Toluidine Blue O in Aqueous Base—A sample of the light-blue fraction, B, isolated from the commercial dye, previously identified as being $C_{14}H_{15}N_3S$, was ad-

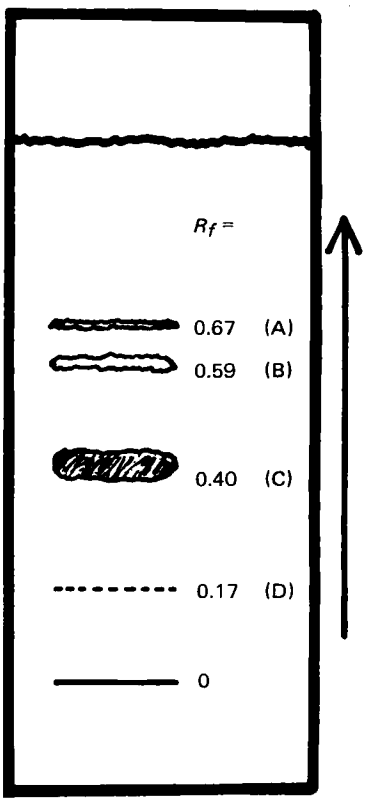


Figure 2—*Thin-layer chromatogram of toluidine blue O [silica gel, ethanol–concentrated hydrochloric acid (99:1 v/v)].*

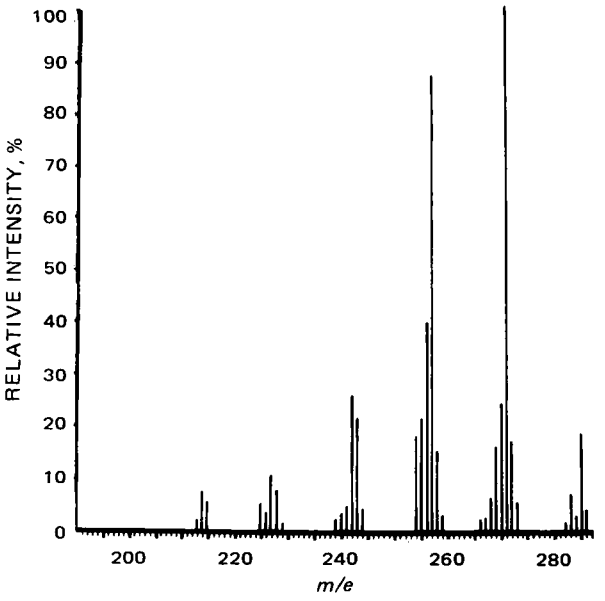


Figure 3—*Mass spectrum (70 ev) of Fraction C isolated by column chromatography from commercial toluidine blue O.*

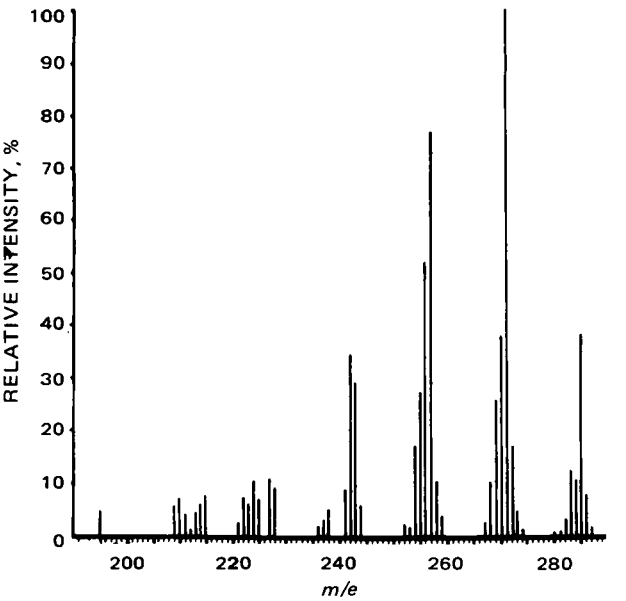


Figure 5—*Mass spectrum (70 ev) of Fraction C isolated from the alkaline degradation of toluidine blue O.*

justed to pH 9 to neutralize the hydrochloric acid used in the chromatography and was evaporated to dryness over a steam bath. Inadvertently, the sample was left on the bath overnight. In the morning the sample was examined and was violet to red in color. Chromatography indicated that B had been converted to the leading violet fraction, A.

Attempts to duplicate this experiment by heating acidic solutions of B and commercial toluidine blue O failed. However, addition of small amounts of base, pH 8–9, showed almost instant degradation of B to A and degradation of toluidine blue O to larger quantities of B and A as well as a red fraction at R_f 0.69. Attempts to isolate large samples of the various impurities from the commercial dye were abandoned in favor of the alkaline degradation of the dye, which did supply large amounts of the principal contaminants for visible light absorption spectroscopy and mass spectrometric studies.

Mass Spectral Analysis of Fractions Isolated from Degraded Commercial Toluidine Blue O—Aqueous solutions of base-treated commercial toluidine blue O were adjusted to pH 1 with hydrochloric acid and were evaporated to dryness under reduced pressure at or below room temperature. The residue was dissolved in a minimum amount of ethanol–concentrated hydrochloric acid, and the fractions were isolated by dry column chromatography. Thin-layer chromatograms indicated that separation was not complete, and the fractions were again chromatographed on silica gel columns.

The fractions were chromatographically (TLC) identical with those isolated directly from the commercial preparation. Mass spectra are presented for the intense blue band, C, the light-blue band, B, and the leading violet band, A (Figs. 5, 6, and 7, respectively). The spectrum of C is consistent with all of the others done on commercial toluidine blue O, with evidence for homologs at *m/e* 257, 271, and 285. However, the spectrum of the light-blue band, B, previously identified as *m/e* 257 ($C_{14}H_{15}N_3S$), contained no higher order homologs.

The spectrum of the leading violet fraction, A (Fig. 7), is similar to the mass spectrum of thionine (Table III). Of note is the +14 mass shift of both the M^+ and M − HCN ions, indicating the addition of a methyl group to the molecule in such a fashion as to provide no additional fragmentation as *N*-methylation would. This observation would be consistent with Structure VII, 2-methylthionine.

By inference, the structure of the light-blue fraction, B, would be VIII, 2-methyl-3-amino-7-methylaminophenothiazine, or IX, 2-methyl-3-methylamino-7-aminophenothiazine.

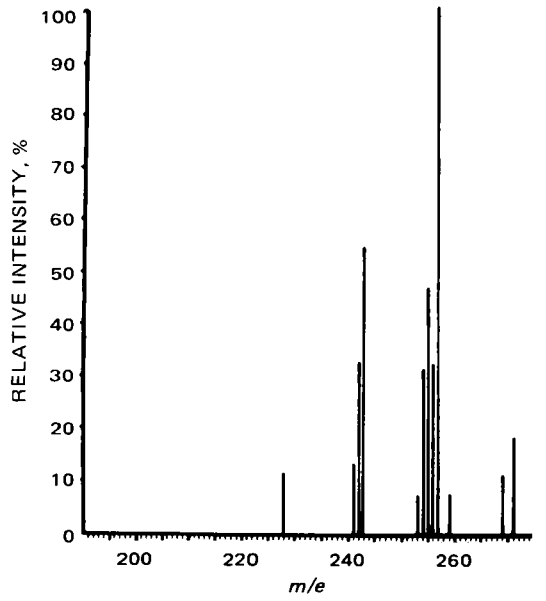


Figure 4—*Mass spectrum (70 ev) of Fraction B isolated by column chromatography from commercial toluidine blue O.*

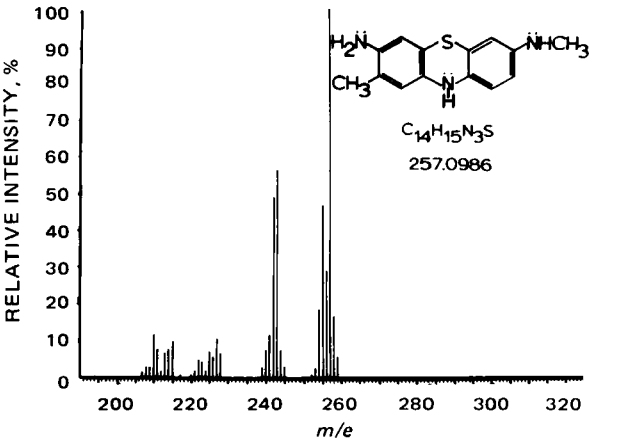


Figure 6—*Mass spectrum (70 ev) of Fraction B isolated from the alkaline degradation of toluidine blue O.*

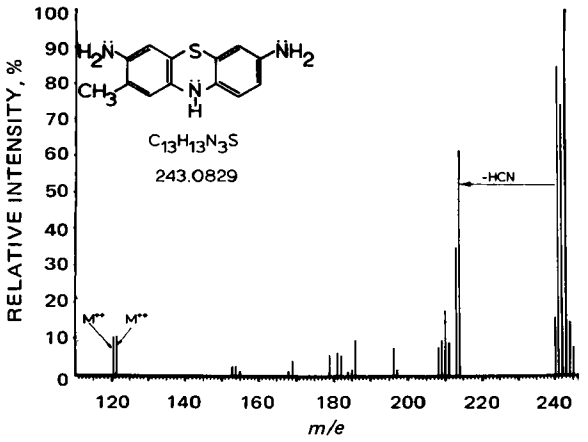


Figure 7—*Mass spectrum (70 ev) of Fraction A isolated from the alkaline degradation of toluidine blue O.*

Mass Spectral Analysis of Red Fraction Isolated from Degradation of Toluidine Blue O in Aqueous Base—In addition to the fractions already mentioned, a fraction with R_f 0.69 was also isolated by TLC with Solvent System II. Mass spectral examination indicated it to be an oxygen-bearing compound of empirical formula $C_{13}H_{10}N_2OS$ (calculated *m/e* 242.0512, found *m/e* 242.0516), potentially of Structure X or XI. Major ions found were $C_{13}H_{10}N_2OS$ (M^+, 100%), $C_{13}H_9N_2OS$ (5%), $C_{12}H_{10}N_2S$ (100%), $C_{12}H_9N_2S$ (38%), and $C_{11}H_8NS$ (48%). Loss of CO to $C_{12}H_{10}N_2S$ would be consistent with the structures drawn.

Visible Light Absorption Spectra of Fractions Isolated from Commercial Toluidine Blue O and Degraded Toluidine Blue O—A summary of the absorption maxima for toluidine blue O, its isolated fractions, and some related dyes is given in Table IV.

Spectra of toluidine blue O at different concentrations are shown in Fig. 8. Of interest is the appearance of an absorption maximum at about 590 nm as the concentration of the dye was increased. Figure 8 is in qualitative agreement with work done by Ohkuma and Furuhata (13). This secondary absorption maximum was reported as being due to dimerization of the dye molecules in solution (14). At the concentrations studied, toluidine blue O apparently does not follow the Lambert–Beer relationship (*i.e.*, the absorbance is not proportional to the concentration of the dye in a linear fashion). Thionine and methylene blue have been reported to not follow the relationship even at concentrations as low as 10^{-7} *M* (15).

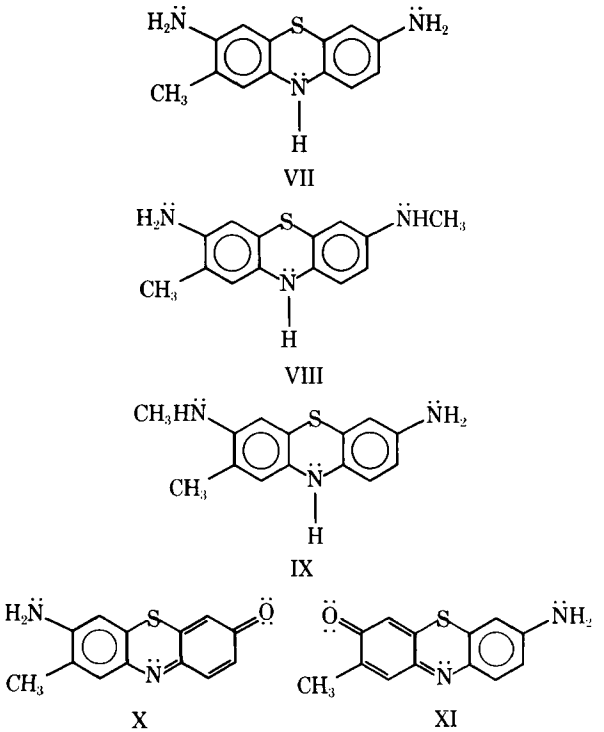
Table IV—Visible Light Absorption Maxima for Toluidine Blue O, Its Isolated Fractions, and Some Related Dyes in Water at pH 3.5

Sample	Absorption Maximum, nm	Concentration, *M*
Commercial methylene blue	652	10^{-4}
Commercial toluidine blue O	634	10^{-5}
Fraction C from commercial dye	638	$\sim 10^{-5}$
Fraction B from commercial dye	618	$\sim 10^{-5}$
Fraction B from degraded dye	613	$\sim 10^{-5}$
Thionine	596	10^{-5}
Fraction A from commercial dye	595	$\sim 10^{-5}$
Fraction A from degraded dye	595	$\sim 10^{-5}$

The mechanism of dimer formation has been discussed previously (15, 16). In dilute aqueous solution, the free energy of dimerization is 4.1 kcal/mole for thionine and 4.9 kcal/mole for methylene blue (15). The association constant has been determined to be about 10^5 for thionine (16).

Interpretation of the Thin-Layer Chromatograms—The data suggest that toluidine blue O and the related dyes are separated on silica gel on the basis of the number of *N*-methyl groups present. Figure 9 gives the chromatographic analysis of the six related dyes. Thionine, which has no *N*-methyl groups and is spectroscopically pure, has the same R_f value as 2-methylthionine (Fraction A) found in toluidine blue O (Fig. 2). 2-Methylthionine also contains no *N*-methyl groups.

Fraction B, isolated from commercial toluidine blue O (R_f 0.59), contains one *N*-methyl group and corresponds (same R_f) to the monomethyl derivative found in azures A and C and, to a lesser extent, azure B. Other bands probably correspond to dimethyl-, trimethyl-, and tetramethylamine derivatives of thionine or 2-methylthionine. The two bands identified as corresponding to dimethylamino derivatives presumably arise from the situation where two methyls are on the same amino group for one compound and one methyl is on each amino group for the other. Since toluidine blue O has both methyls on one amino group, the band at R_f 0.50 probably corresponds to the latter possibility.



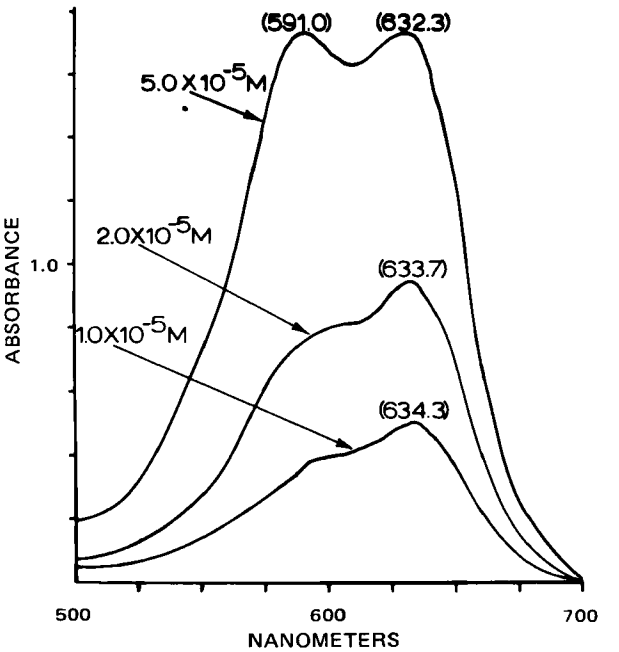


Figure 8—*Visible light absorption spectra of commercial toluidine blue O at various concentrations in water at pH 3.5.*

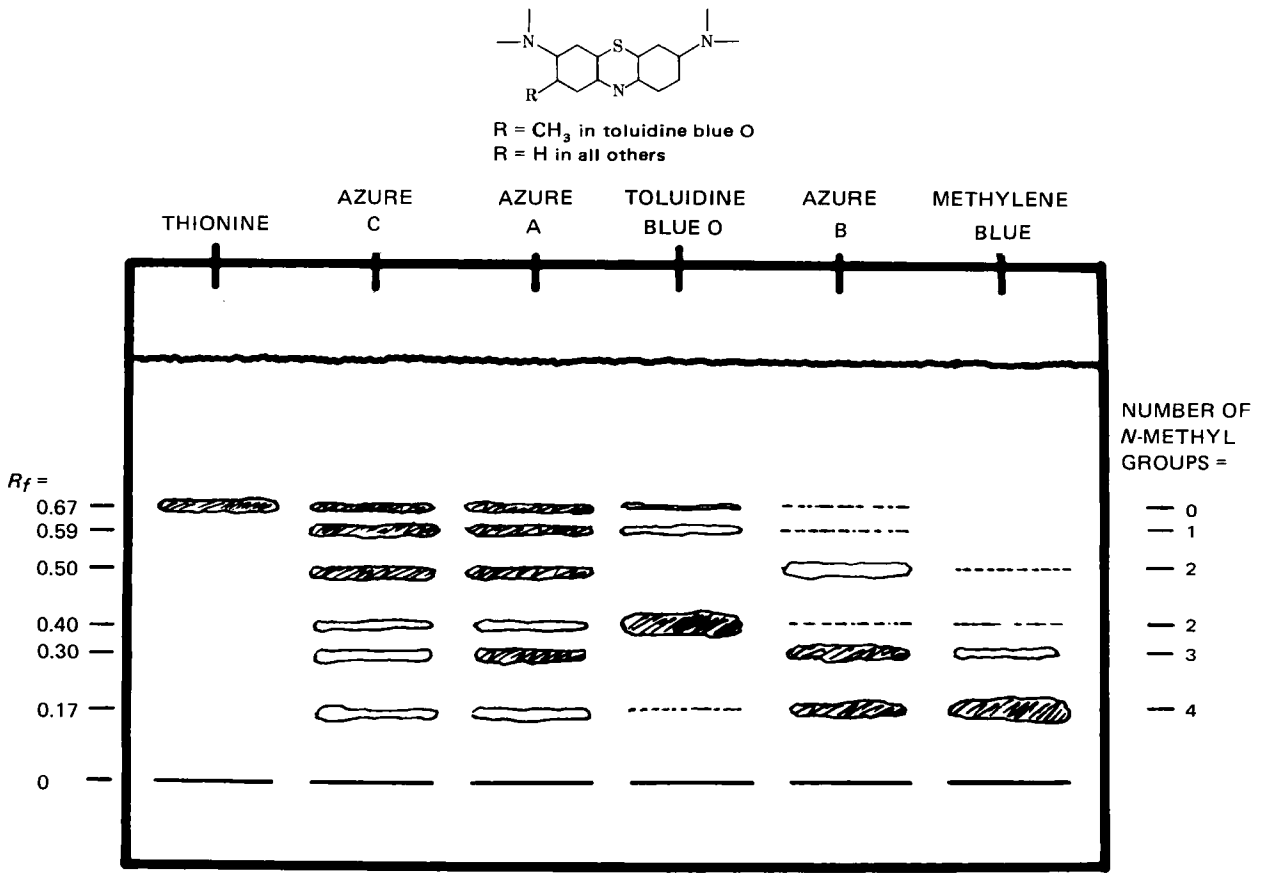


Figure 9—*Thin-layer chromatogram of related phenothiazine dyes [silica gel, ethanol–concentrated hydrochloric acid (99:1 v/v)]. Shaded areas are visibly darker than others. Dotted lines indicate spots that are extremely faint.*

Although the chromatographic correlations mentioned are tentative, they were arrived at by the known purity of thionine, the mass spectral identification of the fractions isolated from commercial toluidine blue O, and the visible light absorption experiments presented here. This system should prove satisfactory for the analysis of the purity of any of the dyes discussed.

Demethylation is known to occur for methylene blue and toluidine blue O by photoreduction (17, 18). It has also been reported that demethylation of methylene blue occurs under basic conditions (19, 20); however, the dye was stable below pH 9.5. Demethylation started between pH 9.5 and 9.8. Azure B was found to undergo demethylation at lower pH values than methylene blue.

As can be seen from Table IV, loss of an *N*-methyl group leads to an approximate 10–20-nm shift in the visible light absorption maximum. The identification of azure A as a contaminant in commercially prepared toluidine blue O-^{3}H at λ_{max} 632 nm by Normann and Normann (7) is undoubtedly an error. The identification of toluidine blue O at λ_{max} 616 nm by Normann and Normann (7) was also consistent with the data presented by Patel (5) and Apgar and Patel (6). Ball and Jackson (9) noted that chromatography of commercial samples of toluidine blue O on aluminum oxide yielded material that gave a rather broad absorption maximum from 590 to 625 nm. These investigators eluted the dye from the column and dried the sample on a water bath at 100°. This experimental technique probably produced a mixture of 2-methylthionine (λ_{max} 595 nm), normethyl-toluidine blue (λ_{max} 613 nm), and toluidine blue O (λ_{max} 638 nm).

Due care must be exercised when one is handling these dyes. The conditions of heat (especially at an alkaline pH) and light must be avoided to assure that demethylation is held to a minimum.

The mass spectral studies of toluidine blue O, isolated from commercial sample by chromatography, still exhibit evidence of a higher homolog at M + 14 (Figs. 4 and 5). Since mass spectral analysis of normethyl-toluidine blue O indicates no higher order homologs (Fig. 6), the possibility of intermolecular methyl transfer taking place in the high temperature source of the mass spectrometer probably is remote. Table III indicates that commercial toluidine blue O contains a small amount of, presumably, a tetramethylated derivative. This fraction could not be separated from the band corresponding to toluidine blue O by column chromatography; consequently, higher order homologs are seen in the mass spectrometer.

SUMMARY

Commercial toluidine blue O has been shown to be a mixture of the normethyl derivative and 2-methylthionine. The synthetic environment used to generate the dye is responsible for a small portion of the contaminants. Heat and alkaline conditions can result in rapid degradation of the parent dye molecule into its demethylated derivatives. Observations made in the literature can be explained on the basis of this demethylation phenomenon. Visible light absorption spectroscopy is a convenient device for estimation of the dyes but is prone to misinterpretation if the purity of the dye is unknown and the deviation from the Lambert–Beer relationship is ignored.

A TLC system has been presented which should prove useful in the estimation of the purity of certain phenothiazine dyes.

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Inhibition of Synaptosomal Accumulation of *l*-Norepinephrine I: *N*-Arylalkyl and *N*-Aryloxyalkyl *dl*-Amphetamines and Related Compounds

JAMES C. SCHAEFFER, ARTHUR K. CHO [x], GLENN T. NAGAMI, and GLENN S. TAKIMOTO

Abstract □ The ability of a group of systematically modified amphetamines to inhibit the accumulation of *l*-norepinephrine by nonstriatal synaptosomes was investigated. *N*-Substitution by the proper bulky hydrophobic groups can be well tolerated. Structure–activity relationships generate a qualitative picture of the inhibitor–carrier interaction site.

Keyphrases □ *l*-Norepinephrine accumulation by nonstriatal synaptosomes—inhibition by *N*-arylalkyl- and *N*-aryloxyalkyl-substituted amphetamines □ Amphetamines, *N*-substituted—inhibition of synaptosomal accumulation of *l*-norepinephrine □ Structure–activity relationships—*N*-substituted amphetamine inhibition of synaptosomal accumulation of *l*-norepinephrine

A catecholamine recycling mechanism located at the presynaptic neuronal membrane is thought to play a major role in the termination of noradrenergic nerve transmission and in the conservation of catecholamines (1–4). Antidepressant and stimulant drugs, such as desipramine (5), cocaine (6), and amphetamine (7), have been shown to inhibit this carrier-mediated transport process *in vitro*. Thus, at least part of their pharmacological action is believed to be derived from their ability to prolong effective catecholamine concentrations in the synaptic cleft and, therefore, at the postsynaptic receptors.

On the basis of kinetic (8) and differential inhibition (5, 9) data, it has been proposed that there are at least two different, anatomically separable, neuronal transport mechanisms for the catecholamines in the central nervous system (CNS). One is associated with the hypothalamus, cerebral cortex, midbrain, and medulla oblongata-pons, where *l*-norepinephrine is the major catecholamine present; the other is found in the corpus striatum, where dopamine is thought to be the important catecholamine transmitter. A wide range of tricyclic, sympathomimetic, anticholinergic, and antihistamine drugs (5, 9–11) have been shown to inhibit the *in vitro* uptake of catecholamines by both systems. However, few of these compounds exhibited a substantial preference for one of the transport processes. Even when selective inhibition was observed, as with desipramine, the less active nonstriatal process was usually the most susceptable to blockade.

In light of this evidence, a study was initiated to elucidate the differences and similarities of the two catecholamine uptake processes in the CNS by attempting to map the topography of the different carrier sites through the use of a series of amphetamine-related inhibitors. Structural features, which take advantage of the topographical variances thus uncovered, would then be integrated into the design of new inhibitors to increase specificity.

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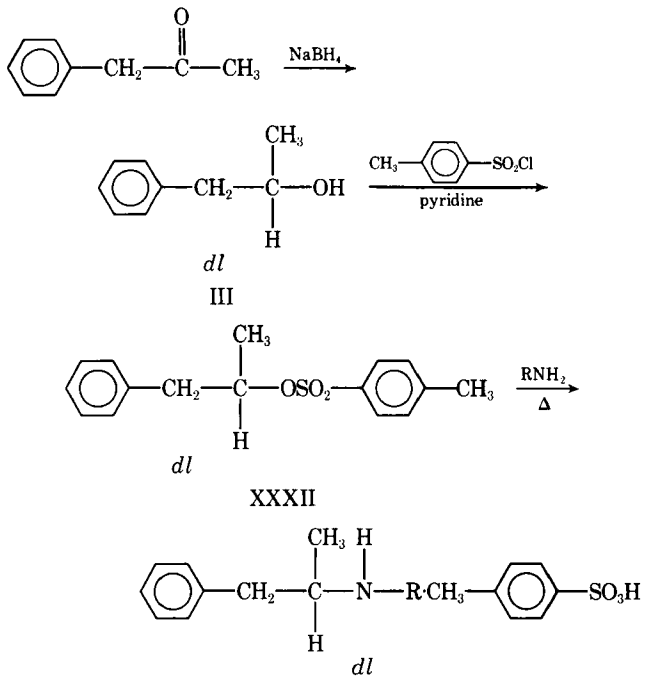
A catecholamine recycling mechanism located at the presynaptic neuronal membrane is thought to play a major role in the termination of noradrenergic nerve transmission and in the conservation of catecholamines (1–4). Antidepressant and stimulant drugs, such as desipramine (5), cocaine (6), and amphetamine (7), have been shown to inhibit this carrier-mediated transport process *in vitro*. Thus, at least part of their pharmacological action is believed to be derived from their ability to prolong effective catecholamine concentrations in the synaptic cleft and, therefore, at the postsynaptic receptors.

On the basis of kinetic (8) and differential inhibition (5, 9) data, it has been proposed that there are at least two different, anatomically separable, neuronal transport mechanisms for the catecholamines in the central nervous system (CNS). One is associated with the hypothalamus, cerebral cortex, midbrain, and medulla oblongata-pons, where *l*-norepinephrine is the major catecholamine present; the other is found in the corpus striatum, where dopamine is thought to be the important catecholamine transmitter. A wide range of tricyclic, sympathomimetic, anticholinergic, and antihistamine drugs (5, 9–11) have been shown to inhibit the *in vitro* uptake of catecholamines by both systems. However, few of these compounds exhibited a substantial preference for one of the transport processes. Even when selective inhibition was observed, as with desipramine, the less active nonstriatal process was usually the most susceptable to blockade.

In light of this evidence, a study was initiated to elucidate the differences and similarities of the two catecholamine uptake processes in the CNS by attempting to map the topography of the different carrier sites through the use of a series of amphetamine-related inhibitors. Structural features, which take advantage of the topographical variances thus uncovered, would then be integrated into the design of new inhibitors to increase specificity.

This report describes the effects of a group of amphetamine-related compounds on the uptake of *l*-norepinephrine by a synaptosome-enriched subcellular fraction from nonstriatal rat brain tissue. First,



Scheme I—Method A

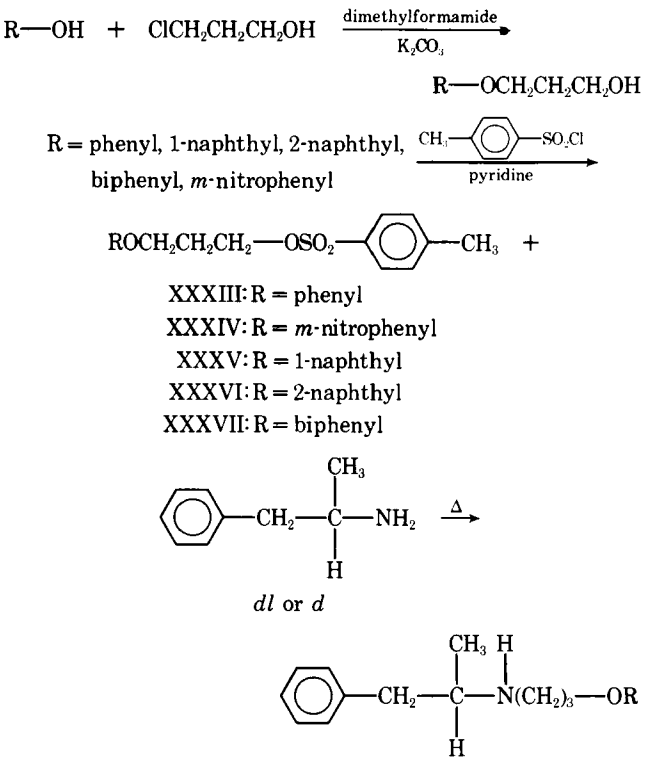
the relative inhibitor potency was investigated in a group of compounds whose structure was systematically modified by small increments to ascertain the important structural and physical–chemical properties connected with the ability of the transport system to recognize its substrate. This series also related the uptake system to previously published results.

Second, following the nonclassical inhibitor rationale of Baker and coworkers (12–14), a series of amphetamines substituted with bulky hydrophobic groups on the nitrogen was synthesized to study possible inhibitor interactions at sites on the carrier adjacent to the normal substrate binding position. The intrinsic affinity of the amphetamine moiety as a substrate mimic was utilized in these compounds to ensure potency, while the bulky substituents provided a means of enhancing selectivity through unique steric effects and/or hydrophobic interactions with adjacent sites on the carrier.

EXPERIMENTAL

Drugs—*S(d)*-Amphetamine[1] (IV), *R(l)*-amphetamine[1] (V), *dl*-amphetamine[1] (II), *dl*-tranylcypromine[1] (IX), *p*-hydroxy-*dl*-amphetamine[1] (VI), *m*-hydroxy-*dl*-amphetamine[1] (VII), phentermine[2] (VIII), *S(d)*-benzphetamine[3] (XVII), *dl*-prenylamine[4] (XXII), and ^{3}H-*l*-norepinephrine[5] (3–8 Ci/mmole) were obtained from commercial sources. The syntheses of other compounds are discussed.

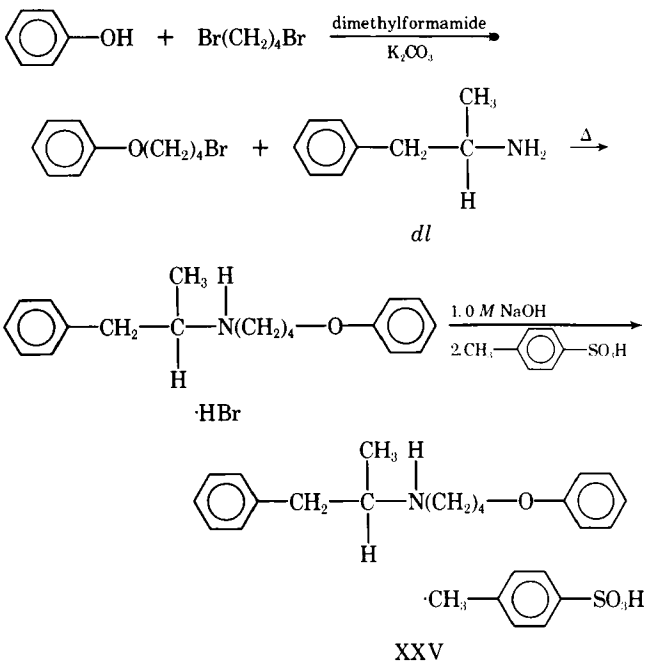
Chemical Methods—The key intermediate in Method A, α-methylphenethyl tosylate (XXXII), was prepared from phenylacetone as shown in Scheme I. This compound was synthesized previously from phenylacetaldehyde (15). Elevated temperatures (100°) and high reactant concentrations (neat conditions) were required to bring about satisfactory tosylate displacement by the



Scheme II—Method B

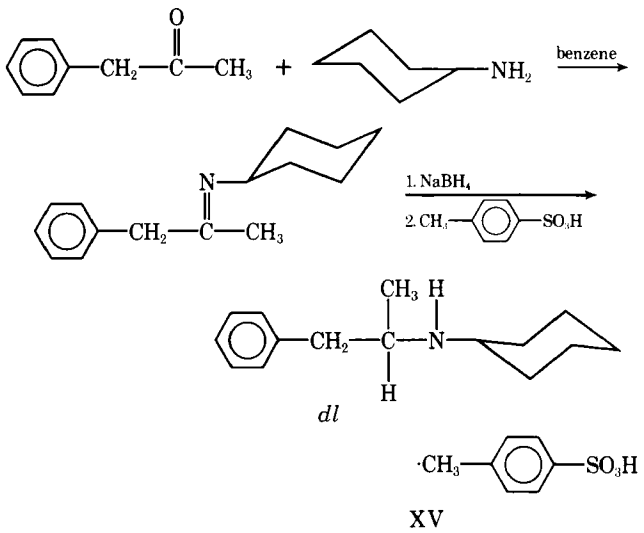
alkyl- or arylalkylamines employed. Equimolar amounts of tosylate (XXXII) and phenylethylamine in acetone at ambient temperature or reflux, or at 65–70° in dimethylformamide, gave only starting material. The reaction of aniline and XXXII at 100° yielded the aniline *p*-toluenesulfonate as the major product. Thus, elimination is apparently favored over substitution when arylamines are used. Compounds XIX–XXIII (Table I) were obtained *via* Method A.

Method B (Scheme II) was employed to synthesize XIV, XXIV, and XXVI–XXX (Table I). The physical properties of the 3-aryloxypropyl tosylate intermediates (XXXIII–XXXVII) are reported in Table II. Compound XXV was prepared from 4-phenoxybutyl bromide and *dl*-amphetamine according to Method C



Scheme III—Method C

[1] Smith Kline and French Laboratories, Philadelphia, Pa.
[2] Pennwalt Corp. Rochester, N.Y.
[3] The Upjohn Co., Kalamazoo, Mich.
[4] Segontin, Hoechst Pharmaceuticals, Somerville, N.J.
[5] New England Nuclear Corp., Boston, Mass.



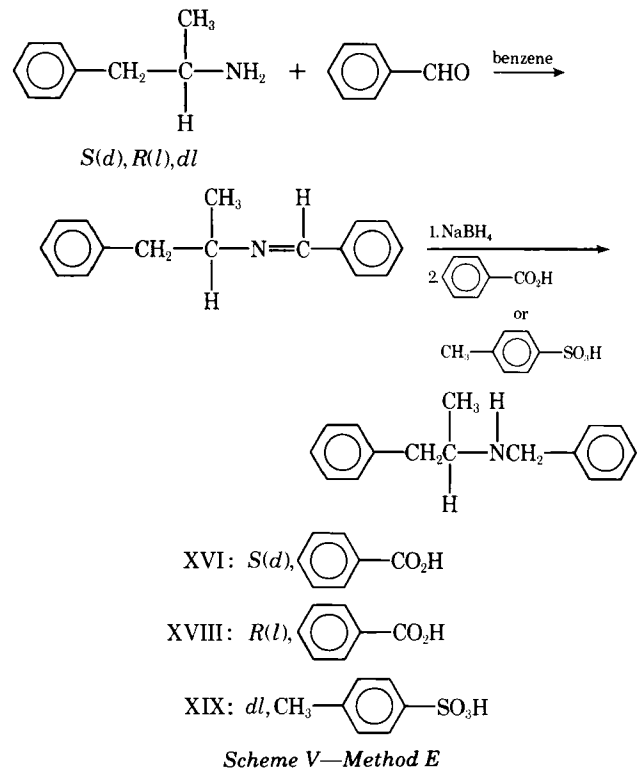
XV

Scheme IV—Method D

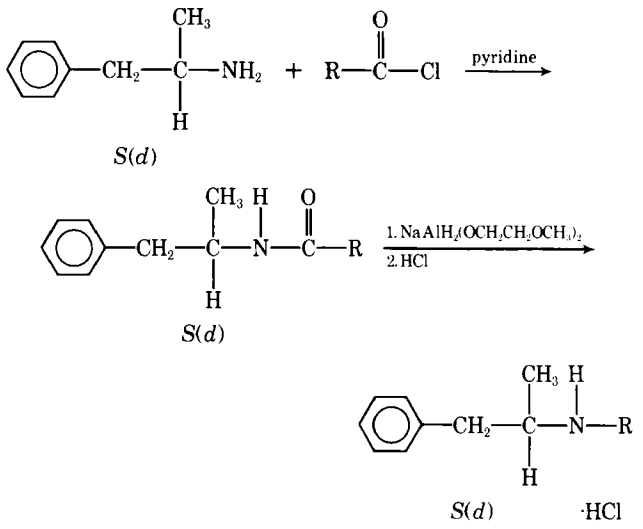
(Scheme III). The reaction of phenylacetone and cyclohexylamine, followed by sodium borohydride reduction, provided XV (Method D, Scheme IV).

To obtain the optical isomers of *N*-benzylamphetamine XVI and XVIII, Method E (Scheme V) was utilized. The benzoic acid salts of these compounds were much more easily recrystallized than the tosylates. The specific rotations of XVI and XVIII were +37.0 and −36.8°, respectively, at 24° (10% w/v, chloroform). The racemic compound, XIX, was also prepared by this method and was identical to that obtained *via* Method A. Method F (Scheme VI) was used to prepare XII and XIII.

Synthetic Procedures[6]—All melting points[7] reported here are uncorrected. The IR spectra were obtained using either neat liquids or mineral oil mulls[8]. At least one compound from each synthetic sequence involving an optically active center was checked on a polarimeter to ensure that the optical outcome of the synthesis was as expected.



Scheme V—Method E



XII: R = $-CH_2CH_3$

XIII: R = $-CH_2CH_2CH_3$

Scheme VI—Method F

General Procedure (Method A)—A mixture of 2 mmoles of *dl*-α-methylphenethyl tosylate (XXXII) and 2 mmoles of the appropriate arylalkylamine was heated at 95–100° for 1–2 hr. After the mixture had cooled to room temperature, the crude tosylate salt was recrystallized from the appropriate solvent and dried under high vacuum over phosphorus pentoxide at 60°. See Table I for additional data.

General Procedure (Method B)—A mixture of 0.1 mole of the appropriate phenolic compound, 0.1 mole of 3-chloro-1-hydroxypropane, 0.11 mole of anhydrous potassium carbonate, and 50 ml of dimethylformamide (dried over 4A molecular sieves) was heated to 85–90° for 18 hr. After the addition of 750 ml of water, the mixture was extracted twice with 150 ml of chloroform. These extracts were combined, backwashed three times with 100 ml of 1 *N* NaOH and three times with 100 ml of water, and dried with magnesium sulfate. The solvent was removed by spin evaporation to give the crude 3-aryloxypropanol. These materials were not rigorously characterized but were sufficiently pure to be used in the next step.

To a solution of 10 mmoles of the appropriate 3-aryloxypropanol in 20 ml of pyridine (dried over potassium hydroxide pellets) was added 2.87 g of freshly recrystallized *p*-toluenesulfonyl chloride (15 mmoles). This mixture was allowed to stand in a refrigerator at 5° for 2 days, during which time pyridine hydrochloride crystallized out. After 400 ml of water was added to the reaction mixture, it was extracted with three 50-ml portions of ether. The ether extract was backwashed with 1 *N* HCl and water, dried with magnesium sulfate, and spin evaporated to the crude tosylate, which was recrystallized from the solvent listed in Table II.

An equimolar mixture of *dl*- or *d*-amphetamine and the appropriate 3-aryloxypropyl tosylate was treated as described in Method A to yield the corresponding *N*-(3-aryloxypropyl)amphetamine. See Table I for further information.

*N-(4-Phenoxybutyl)-*dl*-amphetamine p-Toluenesulfonate (XXV) (Method C)*—A mixture of 9.4 g of phenol (0.1 mole), 100 g of 1,4-dibromobutane (0.46 mole), 15.2 g of potassium carbonate, and 50 ml of dimethylformamide (dried over 4A molecular sieves) was heated at 65° for 6 hr. After the addition of 300 ml of water, the mixture was extracted with 300 ml of heptane. This extract was backwashed once with 100 ml of 1 *N* NaOH and three times with 100 ml of water, dried with magnesium sulfate, and spin evaporated to an oil. This crude mixture was fractionated by high vacuum distillation. The fraction distilling between 113 and 115°/0.5 mm was collected and shown to be 4-phenoxybutyl bromide (5.2 g, 22%) of sufficient purity for use in the next step.

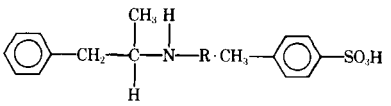
Two hundred and seventy milligrams of *dl*-amphetamine (2

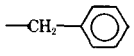
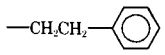
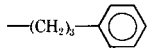
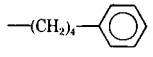
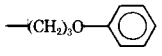
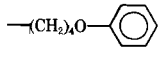
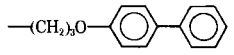
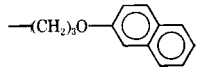
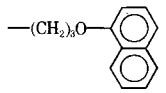
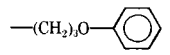
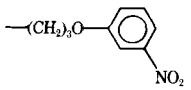
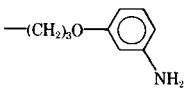
[6] Elemental analyses were performed by H. King, Department of Chemistry, University of California at Los Angeles.

[7] Mel-Temp apparatus.

[8] Infracord, Perkin-Elmer Corp.

Table I—*N*-Alkyl, Arylalkyl, and Aryloxyalkyl *dl*-Amphetamine *p*-Toluenesulfonates[a]



Compound	R	Method	Recrystallization Solvent	Yield, %[b]	Melting Point
XII[c,d,e]	$-CH_2CH_3$	F	Ethanol–acetone (1:5)	10	153–155°
XIII[d,e]	$-CH_2CH_2CH_3$	F	Ethanol	12	183–184°
XIV[f]	$-CH_2CH_2CH_2CH_3$	B[g]	Ether–acetone (5:1)	27	119–121°
XV		D	Ether–acetone (5:1)	29	133–135°
XIX[h]		A, E	Acetone	20	158–161°
XX		A	Acetone	12	160–161°
XXI[i]		A	Acetone	23	156–158°
XXIII		A	Acetone	28	130–132°
XXIV		B	Ethanol	45	164–166°
XXV		C	Acetone	17	132–134°
XXVI		B	Ethanol–acetone (1:2)	39	98–101°
XXVII		B	Acetone–ether (1:2)	26	141–143°
XXVIII		B	Acetone	41	135–137°
XXIX[d]		B	Ethanol–acetone	68	167–169°
XXX[d]		B	Acetone	37	127–130°
XXXI[d,j]		—	Ethanol	40	152–155°

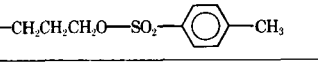
[a] All compounds were uniform by TLC (free base, methanol) and gave satisfactory carbon and hydrogen analyses. [b] Minimum recrystallized yields from last step in synthetic sequence. [c] N. J. Leonard, J. A. Adamick, C. Djerassi, and O. Halpern, *J. Amer. Chem. Soc.*, **80**, 4858 (1958). Apetinil, mp 154–156°. [d] *S(d)*-Isomer. [e] Hydrochloride salt. [f] Hydrochloride salt previously prepared by M. Friefelder and G. R. Stone, U.S. pat. 3,014,966 (1961). [g] Prepared by heating equimolar amounts of *dl*-amphetamine and butyl tosylate at 90–95° for 3 hr. [h] Hydrochloride salt previously prepared by E. H. Woodruff, J. P. Lambody, and W. E. Burt, *J. Amer. Chem. Soc.*, **62**, 922(1940). [i] Hydrochloride salt previously prepared by E. Bumm, British pat. 700,722 (1953). [j] Di-*p*-toluenesulfonate salt. Prepared from the corresponding nitro compound.

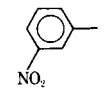
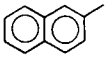
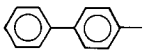
mmoles) and 458 mg of 4-phenoxybutyl bromide (2 mmoles) were heated at 95° for 3 hr. After the crude reaction product was cooled to ambient temperature, 25 ml of 1.0 *N* NaOH was added. This mixture was extracted twice with 25 ml of chloroform, and the extract was dried with magnesium sulfate and spin evaporated to an oil. The oil was dissolved in 8 ml of acetone, and a small amount of insolubles was removed by filtration. Then 380 mg of *p*-toluenesulfonic acid hydrate (2 mmoles) was added to the filtrate. After this mixture stood overnight in a refrigerator, a crystalline precipitate formed. This material was filtered, recrystallized twice from acetone, and pumped dry under high vacuum over phosphorus pentoxide. See Table I for additional data.

N-*Cyclohexyl*-dl-*amphetamine* p-*Toluenesulfonate (XV) (Method D)*—A mixture of 6.7 g of phenylacetone (0.05 mole), 5.54 g of cyclohexylamine (0.055 mole), and 100 ml of benzene was distilled until the vapor temperature of the distillate reached 80°. Benzene was added as necessary. After the reaction had gone to completion, the remaining benzene was removed by spin evaporation. To the residual oil were added 100 ml of ethanol and 6.27 g of sodium borohydride (165 mmoles). After the mixture was refluxed for 4 hr, the ethanol was removed under vacuum and 250 ml of 2 *M* NaOH was added to the residue. This mixture was extracted with 150 ml of ether. The extract was dried with magnesium sulfate and spin evaporated to an oil, which was distilled under high vacuum. The fraction distilling at 111–112°/0.3 mm was collected and shown to be *N*-cyclohexyl-*dl*-amphetamine (3.1 g, 29%). It was converted to its analytically pure tosylate salt by treatment with *p*-toluenesulfonic acid hydrate. See Table I for additional information.

General Procedure (Method E)—The benzene–water azeotrope was distilled from a mixture of 6.75 g of the appropriate amphetamine (50 mmoles), 5.83 g of benzaldehyde, and 100 ml of benzene

Table II—3-Aryloxypropyl Tosylates[a]



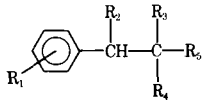
Compound	R	Method	Recrystallization Solvent	Yield, %[b]	Melting Point
XXXIII		B	Ether–hexane (1:1)	57	48–51°
XXXIV		B	Chloroform–ether (1:10)	63	74–75°
XXXV		B	Ether	56	69–71°
XXXVI		B	Ether	55	67–68°
XXXVII		B	Ether	52	96–98°

[a] All compounds moved as single spots on TLC [chloroform–hexane (1:1)] and gave satisfactory carbon and hydrogen analyses. [b] Minimum recrystallized yields.

until the vapor temperature of the distillate reached that of pure benzene. Benzene was added as necessary to maintain a reasonable reaction mixture volume. After the remaining benzene was removed under vacuum, 100 ml of ethanol and 6.27 g of sodium borohydride (165 mmoles) were added. Following a 4-hr reflux, the mixture was spin evaporated to a residue and 250 ml of 2 *M* NaOH was added. The resulting mixture was extracted with 150 ml of ether. The extract was dried with magnesium sulfate and spin evaporated to an oil, which was vacuum distilled.

*N-Benzyl-*d*-amphetamine and N-Benzyl-*l*-amphetamine Benzoates (XVI and XVIII)*—The fraction distilling at 128–130°/0.2 mm was collected (yield of 35–40%). To a solution of 900 mg of either amphetamine isomer (4 mmoles) in 10 ml of acetone was added 488 mg of benzoic acid (4 mmoles). After the solution was cooled overnight, a crystalline precipitate formed; this precipitate was recrystallized from acetone and pumped dry under vacuum over phosphorus pentoxide at 60°. The free bases of both isomers exhibited the same IR spectra, and the benzoate salts both melted at 101–103°. A 10% (w/v) chloroform solution of the free base of the *S(d)*-isomer had a specific rotation of +37.0°, whereas the *R(l)*-isomer had a specific rotation of −36.8°. An elemental analysis of the *R(l)*-isomer gave the following data. Calc. for C, 79.50; H, 7.25. Found: C, 79.29; H, 7.11.

Table III—Inhibition of the Synaptosomal Uptake[a] of *l*-Norepinephrine by Compounds I–IX



Compound	R_1	R_2	R_3	R_4	R_5	Optical Isomer	I_{50}, μM[b]
I	H	H	H	H	NH_2	—	6.0
II	H	H	CH_3	H	NH_2	*dl*	1.3
III[c]	H	H	CH_3	H	OH	*dl*	>>100[d]
IV	H	H	CH_3	H	NH_2	*S(d)*	0.9
V	H	H	CH_3	H	NH_2	*R(l)*	2.3
VI	*p*-OH	H	CH_3	H	NH_2	*dl*	1.1
VII	*m*-OH	H	CH_3	H	NH_2	*dl*	1.8
VIII	H	H	CH_3	CH_3	NH_2	—	1.6
IX	H	—CH_2—		H	NH_2	*dl*	3.7

[a] Substrate concentration was 1 μM *l*-norepinephrine. [b] All values are the average of two or more independent determinations. The variation in each multiple determination was less than 25%. [c] C. Golumbia and D. L. Cottle, *J. Amer. Chem. Soc.*, 61, 996(1939). [d] No inhibition at 100 μM.

*N-Benzyl-*dl*-amphetamine p-Toluenesulfonate (XIX)*—The fraction distilling at 136–137°/0.2 mm was collected (yield of 52%). To a solution of 450 mg of *N*-benzyl-*dl*-amphetamine (2 mmoles) in 40 ml of acetone was added 380 mg of *p*-toluenesulfonic acid hydrate (2 mmoles). After the mixture stood at room temperature overnight, a solid had precipitated. It was filtered, recrystallized from acetone, and dried under high vacuum over phosphorus pentoxide at 60°. The free base of this material did not rotate polarized light. The *p*-toluenesulfonate was identical to that prepared by Method A.

General Procedure (Method F)—To a solution of 6.75 g of *d*-amphetamine (50 mmoles) and 50 ml of pyridine (dried over potassium hydroxide) in an ice bath was added slowly 50 mmoles of the appropriate acid chloride. After this mixture was allowed to stir at room temperature for 25 hr, 750 ml of water was added and the resulting mixture was extracted with three 100-ml portions of chloroform. The extract was backwashed three times with 100 ml of 1 *M* HCl, three times with 100 ml of 2 *M* NaOH, and three times with 100 ml of water; it was then dried with magnesium sulfate and spin evaporated to a residue. The crude amide was dissolved in 50 ml of benzene, and 11.5 g of sodium bis(2-methoxyethoxy) aluminum hydride[9] (40 mmoles) dissolved in 50 ml of benzene was added.

This mixture was refluxed for 27 hr and cooled to 0–4°, and then 300 ml of 1 *N* NaOH was added slowly followed by 100 ml of benzene. The benzene layer was separated, dried with magnesium sulfate, and spin evaporated to an oil. The oil was dissolved in 100 ml of ether, and dry hydrogen chloride gas was bubbled through the solution. The resulting precipitate was filtered, recrystallized, and dried. Further information about XII and XIII, prepared by this method, is presented in Table I.

*N-[3-(3-Aminophenoxy)propyl]-*d*-amphetamine (XXXI)*—A mixture of 974 mg of *N*-[3-(3-nitrophenoxy)propyl]-*d*-amphetamine *p*-toluenesulfonate (XXX) (2 mmoles), 40 ml of ethanol, 5 ml of acetic acid, 5 ml of water, and 1.31 g of zinc powder (20 mmoles) was refluxed for 18 hr. The supernate was decanted from the zinc powder and spin evaporated to a residue. After 150 ml of 1 *N* NaOH was added to the residue, this mixture was extracted with three 50-ml portions of benzene. The extract was dried with magnesium sulfate and evaporated under reduced pressure to an oil. This material was dissolved in 20 ml of ethanol, which contained 760 mg of *p*-toluenesulfonic acid hydrate (4 mmoles). After this solution was cooled, a solid precipitated, which was recrystallized from ethanol and dried under high vacuum over phosphorus pentoxide at 60°. Physical data are provided in Table I.

Filtration Assay Method—*P_2' Preparation*—Male Sprague–Dawley rats, 180–200 g, were killed by decapitation. The brains were immediately removed, and the cerebellum and corpus striatum of each were rapidly dissected and discarded. The remaining brain tissue, henceforth referred to as nonstriatal tissue (1.2 g/brain), was homogenized in nine volumes of ice-cold 0.32 *M* sucrose, using a glass homogenizer with a polytetrafluoroethylene pestle. All subsequent procedures were carried out in ice-cold equipment or at 0–4° in a refrigerated centrifuge[10].

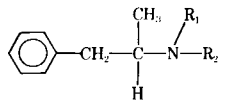
By employing essentially the procedure of Gray and Whittaker (16), a P_2' fraction enriched with synaptosomes was obtained as follows. The brain homogenate was centrifuged for 10 min at 1000×*g* to sediment debris and nuclei. The supernate was carefully separated and centrifuged at 11,000×*g* for 20 min. The resulting pellet (P_2) was uniformly resuspended by hand in 10 ml of 0.32 *M* sucrose/1.2 g of original brain tissue. This suspension was centrifuged for 20 min at 11,000×*g*. The supernate was discarded and the pellet (P_2' or P_2 washed) was resuspended in 2 ml of 0.32 *M* sucrose/1.2 g of original brain weight to give a preparation containing 15–20 mg of protein/ml. All P_2' fractions were used immediately after preparation.

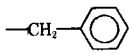
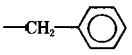
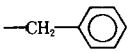
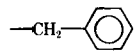
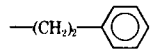
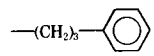
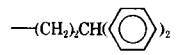
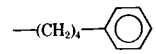
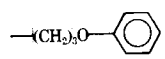
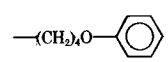
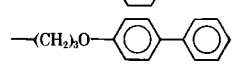
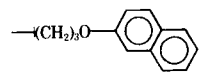
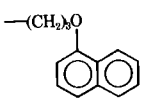
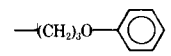
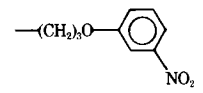
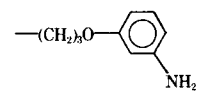
Protein concentrations were measured by the procedure of Lowry *et al.* (17), using bovine serum albumin as the standard.

Assay Procedure—A mixture of 0.53 ml of Krebs–Ringer phosphate buffer (pH 7.4) containing *l*-ascorbate, glucose, and edetic acid, 0.02 ml of 0.19 m*M* nialamide, 0.05 ml of 0.1 *M* NaH_2PO_4–

[9] Red-Al, Aldrich Chemical Co.
[10] Sorvall RC-2B, Dupont Instruments.

Table IV—Inhibition of the Synaptosomal Uptake[a] of *l*-Norepinephrine by Compounds X–XXXI



Compound	R_1	R_2	Optical Isomer	I_{50}, μM[b]	I_{50}, μM[c] (Ionized)
X	H	CH_3	*S(d)*	1.2	1.2
XI[d]	CH_3	CH_3	*S(d)*	7.5	7.5
XII	H	$-CH_2CH_3$	*S(d)*	2.3	2.3
XIII	H	$-(CH_2)_2CH_3$	*S(d)*	8.0	8.0
XIV	H	$-(CH_2)_3CH_3$	*dl*	12.3	12.3
XV	H		*dl*	10.5	10.5
XVI	H		*S(d)*	11.3	6.3
XVII	CH_3		*S(d)*	29.8	3.7
XVIII	H		*R(l)*	4.4	2.5
XIX	H		*dl*	5.0	2.8
XX	H		*dl*	7.3	7.3
XXI	H		*dl*	4.4	4.4
XXII	H		*dl*	5.7	5.7
XXIII	H		*dl*	2.7	2.7
XXIV	H		*dl*	4.4	4.4
XXV	H		*dl*	2.1	2.1
XXVI	H		*dl*	13.3	13.3
XXVII	H		*dl*	5.8	5.8
XXVIII	H		*dl*	5.2	5.2
XXIX	H		*S(d)*	3.7	3.7
XXX	H		*S(d)*	3.8	3.8
XXXI	H		*S(d)*	2.1	2.1

[a] Substrate concentration was 1 μM *l*-norepinephrine. [b] All values are the average of two or more independent determinations. The variation in each multiple measurement was less than 25%. [c] The I_{50} values listed in this column are corrected for the concentration of the cationic species at pH 7.4. All compounds, except for XVI–XIX, have a pKa of 9.5–10.0; therefore, less than 1% is nonionized under normal assay conditions. [d] Prepared by the Eschweiler–Clarke methylation of *d*-amphetamine. S. Senoh and I. Mita, *J. Pharm. Soc. Jap.*, 72, 1096(1952).

Na_2HPO_4 buffer (pH 7.4) with or without inhibitor, and 0.10 ml of the P_2' preparation was preincubated on a metabolic shaker at 37°. After 5 min, 0.05 ml of 15 μM ^{3}H-*l*-norepinephrine was added to give a 1-μM final concentration. The final concentrations of other components of this incubation medium were 123 mM Na^+, 4.2 mM K^+, 2.5 mM Mg^{+2}, 0.9 mM Ca^{+2}, 8.4 mM *d*-glucose, 1.1 mM *l*-ascorbate, 0.064 mM edetic acid, 42.6 mM sucrose, and 12.5 μM nialamide.

After incubation at 37° for 5 min in the presence of 1 μM substrate, the reaction was quenched by the addition of 5 ml of ice-cold 0.9% NaCl solution, which contained 100 μM unlabeled *l*-norepinephrine. This mixture was rapidly filtered through a 0.8-μm cellulose acetate filter[11] (18). The filter was then washed twice with 5-ml aliquots of quench, dissolved in 10 ml of Bray's counting solution (19) by shaking for 30 min, and counted[12]. Blanks for all inhibitor concentrations were obtained by repeating this procedure for 1 min at 0°.

All points were done in triplicate, and the mean value was used to determine picomoles of *l*-norepinephrine uptake in the fol-

[11] Millipore Corp., Bedford, Mass.
[12] All counting was done on an Amersham-Searle Mark II liquid scintillation counter.

lowing manner. The net counts accumulated for each experimental point, $CPM_{37}-CPM_0$ (CPM_{37} represents the radioactivity accumulated at 37°, and CPM_0 represents the radioactivity accumulated at 0°), were divided by the specific activity (counts per minute per picomole) of the ^3H-*l*-norepinephrine stock solution used for each experiment. The specific activity was determined by counting a mixture of 0.05 ml of the radioactive stock solution, 10 ml of Bray's, and a cellulose acetate filter that had gone through the normal incubation and filtration procedure without [^3H]-*l*-norepinephrine. Quenching was shown to be uniform for all samples in each experiment by the standard channels ratio method.

I_{50} Determinations (20)—The accumulation of 1 μM ^3H-*l*-norepinephrine was determined in the presence of three different inhibitor concentrations that reduced control uptake by 30–70%. The ratio of A_0/A_I, where A_0 represents control accumulation (picomoles per milligram of protein) and A_I represents accumulation with inhibitor, was plotted against [I]. These points were fitted by the method of least squares to give the best line. The point at which $A_0/A_I = 2$ provided the I_{50} values.

RESULTS AND DISCUSSION

The compounds in Table III were used as probes to determine the important substrate–carrier interaction points by measuring the relative potency of their inhibitory power. The simple phenethylamine (I) skeleton of *l*-norepinephrine was a good inhibitor. Introduction of an α-methyl group, the conversion of I to *dl*-amphetamine (II), caused a fivefold increase in inhibitor strength. Similar observations have been made using the perfused rat heart (21) and using a crude synaptosomal preparation (S_1) from rat brain hypothalamus (10). The presence of a monoamine oxidase inhibitor (nialamide) in the incubation mixture ruled out the possibility of this result being more a reflection of metabolism than enhanced inhibitor–carrier association. Thus, the introduction of a small hydrophobic substituent at the α-position has a positive effect. Phentermine (VIII) was as good an inhibitor as *dl*-amphetamine, so that addition of a second α-methyl group can also be tolerated. The inclusion of the α-methyl group of amphetamine in a cyclopropyl ring (tranylcypromine, IX), on the other hand, reduced carrier interaction.

The replacement of the basic and polar amine function of II with a neutral and polar hydroxy group (III) led to complete loss of inhibition. This finding indicates the critical role of the positively charged amine function in substrate recognition and appears to rule out the possibility of a strong interaction through hydrogen bond formation. The carrier molecule appears to contain a negatively charged group at its substrate combining site.

Consistent with recently reported results (22, 23), the *S(d)*-isomer of amphetamine (IV) was twice as potent as the *R(l)*-enantiomer (V). Coyle and Synder (7) previously reported a 10-fold difference in the inhibitor potency of these compounds, but they (7, 9) pretreated their animals with 5–2.5 mg/kg of reserpine to deplete catecholamines and inactivate the vesicular storage mechanism so the tissue sources were different.

The substitution of hydroxy groups on the benzene ring (VI and VII) did not alter inhibitor potency to a great extent, so the phenolic hydroxys of *l*-norepinephrine do not appear to play a major role in substrate–carrier binding. The only case in which ring hydroxylation substantially increases inhibition potency is that of tyramine compared to phenylethylamine (10, 11). However, this result may also reflect the well-known releasing effect of tyramine (23). The general structural feature of the substrate required for carrier binding is the phenethylamine structure. The introduction of one or two α-methyl groups enhances inhibitor strength and if one α-methyl is present, the *S(d)*-optical configuration is optimal.

Table IV summarizes investigations into the effect of *N*-substitution on uptake inhibitor strength to see whether large groups at this position could be tolerated by the carrier. Methamphetamine (X) was essentially as good an inhibitor as *S(d)*-amphetamine (IV). However, the addition of a second methyl group (XI) did result in a sixfold loss in inhibitor strength. There are other examples of the drop in potency with increasing *N*-substitution. Inhibition of *l*-norepinephrine uptake into hypothalamic preparations by desipramine is likewise reduced when the compound is converted to imipramine (5). Furthermore, it is well known that successive methylation of amphetamine causes a marked loss of pressor activity (24). The anionic carrier site does not appear to be able to accommodate a tertiary amine as well as a secondary amine, probably due to increased steric hindrance caused by the third ligand.

Since a secondary amine structure can be tolerated, a homologous series of *N*-alkyl amphetamines (XII–XIV) was investigated. Each successive lengthening of the alkyl substituent by one carbon resulted in a corresponding decrease in binding. The bulky cyclohexyl substituent of XV was no worse than the butyl substituent of XIV in decreasing effective carrier interaction. This finding suggests that only one edge of the ring interacts with the carrier surface. Again, steric factors are probably responsible for the observed reductions in inhibitor strength, because there is no correlation between increased molecular hydrophobicity and inhibitor potency.

The addition of a phenyl ring to methamphetamine (XVI) precipitates an apparent 10-fold loss in effectiveness, which could be the result of unfavorable steric requirements. However, the pKa of *N*-benzylamphetamine is 7.5 (25); thus 44% of this compound is present as the neutral form under the assay conditions employed (pH 7.4) in contrast to methamphetamine, which is 99% ionized at pH 7.4. Since a protonated amine is required for strong carrier interaction (II *versus* III), the substantial decrease in inhibitory power could be partially the result of the decreased concentration of the ionized form. Indeed, after the I_{50} value was corrected for ionization, only a fivefold loss could be attributed to steric factors. Similarly, the corrected I_{50} value for benzphetamine (XVII) cation (pKa = 6.55) (25) is 3.7 μM. Thus, in this case, the methylation of a secondary amine does not greatly change the potency of the ionized form (XVI *versus* XVII).

N-Benzyl substitution also reversed the relative inhibitory potency of the optical isomers. The *R(l)*-isomer (XVIII) was at least twice as potent as the *S(d)*-isomer in blocking *l*-norepinephrine accumulation. The sevenfold loss (corrected I_{50} values) in inhibitory power associated with the conversion of *S(d)*-amphetamine (IV) to *N*-benzyl-*S(d)*-amphetamine (XVI) is reduced to almost none for the corresponding *R(l)*-isomers (V and XVIII). The mode of interaction of the *R(l)*-isomer apparently allows greater steric toleration of the bulky benzyl group or may bring this hydrophobic function into contact with a hydrophobic patch on the transport carrier. The racemic *N*-benzyl-*dl*-amphetamine (XIX) had an intermediate I_{50} value as expected and had half the potency of *dl*-amphetamine (II).

N-Phenethyl-*dl*-amphetamine (XX) was three to four times less potent than *N*-ethyl-*d*-amphetamine (XII). Since the pKa values of these compounds are similar, this loss in inhibitor efficiency can probably be best attributed to steric hindrance. A comparison of the *N*-benzyl (XIX) and *N*-phenethyl (XX) compounds shows that the introduction of a second methylene group causes a twofold decrease in binding after correction for pKa differences. With the substitution of a *N*-phenylpropyl group (XXI), a twofold increase in potency was observed over the simple *N*-propyl derivative. This compound also exhibited twofold greater potency than the phenethyl homolog. Increased chain length and flexibility have apparently brought the phenyl ring into position where positive hydrophobic forces come into operation. The addition of a second phenyl substituent (XXII) slightly decreased inhibitor strength.

The placement of a butyl chain between the terminal phenyl ring and the nitrogen of *dl*-amphetamine (XXIII) slightly decreased the I_{50} value when compared to the corresponding propyl derivative (XXI). This compound was five times as potent as *N*-butyl-*dl*-amphetamine (XIV). Once again, strong hydrophobic interaction of the phenyl ring with the carrier site is the best explanation. Neither the substitution of an ether oxygen for a methylene (XXIV) nor the lengthening of the chain to five atoms (XXV) had much effect on inhibitor strength.

The next series of compounds was synthesized and tested to investigate the dimensions and properties of the proposed hydrophobic site adjacent to the normal substrate-combining region on the *l*-norepinephrine carrier. The substitution of a *p*-phenyl ring on XXIV to form the biphenyl derivative (XXVI) resulted in a fourfold loss in binding. The fusion of an aromatic ring to XXIV at the *meta/para*-position (XXVII) or *meta/ortho*-position (XXVIII) gave inhibitors that were only slightly less effective. Thus, the hydrophobic carrier site can tolerate the planar 2-naphthyl (XXVII) or 1-naphthyl (XXVIII) substituent, whereas a large planar biphenyl appears to exceed the dimensions of the hydrophobic pocket.

The possibility that electronic forces play a significant role in

the interaction of the terminal phenyl ring of the *N*-substituent can be ruled out by XXX and XXXI. The substitution of either a strong electron-withdrawing nitro group (XXX) or a strong electron-donating amino group (XXXI) resulted in only minor fluctuations in inhibitor strength. Additionally, the unaltered inhibitor potency after the addition of a polar amino group to XXIV indicates that only part of the terminal phenyl ring is involved in a hydrophobic interaction with the carrier molecule.

Overall, there appears to be no simple correlation between hydrophobicity and inhibitor strength in this series of compounds as has been reported for primary alcohols (26) and certain psychotropic drugs (27). The introduction of the proper bulky groups on the amphetamine nitrogen can be tolerated with little loss in inhibitor potency. Thus, the preparation of specific nonclassical inhibitors with substitution at the nitrogen seems possible.

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Flumizole, a New Nonsteroidal Anti-Inflammatory Agent

E. H. WISEMAN [x], H. M. McILHENNY, and J. W. BETTIS *

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Flumizole[1] is one of a series of substituted diarylimidazoles with anti-inflammatory activity (1, 2). This compound is distinguished from the general class of acidic agents currently used in the management of inflammatory disease by its very weak acidic character (pKa 10.7). This report describes the potent anti-inflammatory activity of flumizole and biopharmaceutical developments undertaken to opti-

[1] 4,5-Bis(*p*-methoxyphenyl)-2-(trifluoromethyl)imidazole, synthesized by Dr. Joseph G. Lombardino, Department of Medicinal Chemistry, Pfizer Central Research, Pfizer, Inc., Groton, CT 06340

the interaction of the terminal phenyl ring of the *N*-substituent can be ruled out by XXX and XXXI. The substitution of either a strong electron-withdrawing nitro group (XXX) or a strong electron-donating amino group (XXXI) resulted in only minor fluctuations in inhibitor strength. Additionally, the unaltered inhibitor potency after the addition of a polar amino group to XXIV indicates that only part of the terminal phenyl ring is involved in a hydrophobic interaction with the carrier molecule.

Overall, there appears to be no simple correlation between hydrophobicity and inhibitor strength in this series of compounds as has been reported for primary alcohols (26) and certain psychotropic drugs (27). The introduction of the proper bulky groups on the amphetamine nitrogen can be tolerated with little loss in inhibitor potency. Thus, the preparation of specific nonclassical inhibitors with substitution at the nitrogen seems possible.

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mize drug absorption during pharmacological and toxicological investigations.

EXPERIMENTAL

Charles River strain rats (male), Hartley strain guinea pigs (male), and beagle dogs (both sexes) were used. Adrenalectomy in the rat was performed through a retroperitoneal incision while the animals were anesthetized with ether. The adrenalectomized rats were maintained on 0.9% saline in place of drinking water and were used 5–7 days postoperatively. Oral studies were conducted with an aqueous solution of drug in polyethylene glycol 300 containing 5% polysorbate 80[2] (I; 0.2 ml in 5 ml water/animal).

Anti-Inflammatory Activity—Inhibitory activity toward the formation of edema in response to the subplantar injection of carrageenan in the hindpaw of the rat (mean weight 170 g) was assessed using the technique of Winter *et al.* (3). Carrageenan (0.05 ml of 1% suspension) was injected 1 hr after oral drug administration, and inhibition of edema formation was assessed after a further 3-hr period. Analogous studies were performed in adrenalectomized rats.

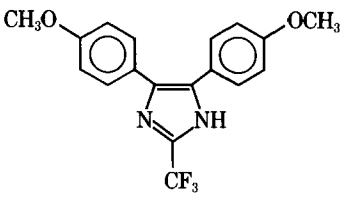
Inhibitory activity toward the formation of granuloma tissue around an implanted irritant was measured by a procedure developed in these laboratories (4). Under ether anesthesia, rats, 140–160 g, were shaved and a 8-cm length of sterile linen string was inserted subcutaneously at the dorsal midline. Drugs were administered orally 4, 24, and 48 hr after implantation. At 72 hr, the animals were sacrificed and weighed and the string together with the granulation tissue was removed and weighed.

Inhibitory activity toward UV irradiation-induced erythema was measured in the guinea pig according to the method of Winder *et al.* (5). Drugs were administered orally in divided doses, 1 hr prior and just subsequent to irradiation. Erythema inhibition was assessed 2 hr after irradiation. Groups of five animals were used, and erythema was scored as follows: no erythema, 0; full circle of erythema 1.0; and intermediate responses, 0.5. Erythema was assessed at three irradiation sites on each animal (maximum possible score = 3.0).

Prostaglandin synthetase was isolated from goat seminal vesicles according to the method of Wlodawer and Samuelsson (6). Labeled (1-^{14}C) and unlabeled arachidonic acid were purified as reported by Flower *et al.* (7). Reaction mixtures contained 1.2 mg/ml of microsomes, 0.36 m*M* arachidonic acid (specific radioactivity 3.45×10^5 cpm/μmole), 10 m*M* L-epinephrine bitartrate, 50 m*M* potassium phosphate buffer (pH 7.2), and 3.0 *M* methanol in a total volume of 1 ml. The range of drug concentrations used were $0.3–4.0 \times 10^{-5}$ *M* indomethacin, $0.2–4.0 \times 10^{-3}$ *M* phenylbutazone, and $0.1–2.0 \times 10^{-6}$ *M* flumizole. The reaction was initiated by the addition of enzyme and was conducted at 37° for 10 min.

Conditions were chosen so that no greater than 30% of the arachidonate was converted to products. The reaction was stopped by the addition of 7 ml of chloroform–methanol (1:1 v/v). Carrier prostaglandin $F_{2\alpha}$ (0.09 μmole), prostaglandin E_2 (0.09 μmole), and arachidonic acid (0.17 μmole) were added. The precipitate was removed by centrifugation, and the radioactive products were extracted and separated by TLC (6). Zones corresponding to prostaglandins $F_{2\alpha}$ and E_2 were visualized with iodine vapors, scraped from the plate, and eluted with 1 ml of methanol. The radioactivity measured was corrected for the amount present in a boiled microsome control. Radiochemical assays were carried out by liquid scintillation[3] with a solvent system of 60 g of naphthalene, 8 g of solute[4], and 100 ml of methanol made to a final volume of 1 liter with dioxane.

Drug Assay in Plasma—To 3–5 ml of plasma was added 2 ml of 1 *M* NaH_2PO_4, and the solution was mechanically extracted for 10 min with freshly distilled benzene (1 × 20 ml). The solvent extract was separated after centrifugation and filtered through a column of silica gel[5], 0.5 g, contained in a 14.6-cm capillary pipet. The filtrate and an additional 3 ml of benzene were rejected. Drug was eluted from the column with 3 ml of benzene–ether (1:1), which was evaporated to 0.2 ml and diluted with 0.5 ml of *n*-hexane. Drug was extracted by mechanical agitation with 1 ml of 1 *M* NaOH; following centrifugation, the aqueous solution was immediately acidified with 1 ml of 2 *M* HCl for spectrophotofluorometric determination[6].



flumizole

Flumizole displayed an excitation maximum at 290 nm with an emission maximum at 460 nm. Response was linear and could be quantitated over a concentration range of 5–1000 ng/ml in plasma. Dilution of the final assay solution permitted quantitative analysis up to 10 μg/ml plasma concentrations. Standardization was achieved by reference to a calibration curve constructed for each assay series by addition of known amounts of flumizole, in duplicate, to untreated animal plasma or normal human serum; these samples were processed in the same manner and at the same time as above. No fluorescent background interferences were present in the plasma of unmedicated rats, dogs, or humans.

Assay precision was determined by repeated analysis, over a 3-week period, of two control samples containing drug. Results showed a reproducibility of 9.5 ± 0.2 ng/ml (seven determinations) and 50.4 ± 4.0 ng/ml (eight determinations). The assay was specific for unchanged drug in plasma as confirmed by fluorescence spectrum, TLC [silica gel GF plates[7], hexane–ethyl acetate–acetic acid (14:6:1)], and GLC (described later) analyses of final assay samples or extracts thereof. Drug recovery from plasma averaged 71 ± 2% over the 10–50-ng/ml concentration range.

Drug Assay in Excreta—Fecal and urinary excretion of flumizole in humans was determined by a GLC procedure. Five milliliters of urine, or 5 ml of 1 *M* NaH_2PO_4 containing 100 mg of lyophilized feces, in a centrifuge tube with 5 ml of pH 4.5 sodium acetate–acetic acid buffer (0.2 *M*) was processed by benzene extraction and silica gel column purification in the manner described for the plasma assay. The urine residue obtained by evaporation of the column eluate to dryness with nitrogen was directly dissolved in 50 μl of methanolic triethylanilinium hydroxide (0.5 *M*) for analysis. The column eluate from the fecal sample was evaporated to 0.2 ml and diluted to 1 ml with *n*-hexane, and drug was extracted into 1 ml of 1 *M* NaOH.

After centrifugation, the aqueous phase was withdrawn, immediately acidified with 1 ml of 2 *M* HCl, and extracted with 10 ml of ethyl acetate. Following centrifugation, the solvent layer was withdrawn, evaporated to dryness, and dissolved in 0.5 *M* triethylanilinium hydroxide, as already described, for analysis. One-microliter aliquots (plus 1 μl of 0.5 *M* triethylanilinium hydroxide solution) were immediately introduced into the injection port (320°) of a gas chromatograph[8] for on-column alkylation to the corresponding N_1-ethylated derivative of flumizole. The derivative was separated on a 0.63 × 183-cm glass column (210°) containing 1% OV-1 on Gas Chrom Q (60–80 mesh) and detected by flame ionization (320°) with a typical retention time, using helium as carrier gas (60 ml/min), of 3.1 min.

Concentration was determined by peak height measurement with reference to a calibration curve constructed from a series of normal human urine or fecal samples fortified with several levels of authentic drug. A linear response over the concentration range of 2.0–10 μg of drug/ml of urine or fecal homogenate permitted quantitative analysis with no background interferences. Recovery of flumizole added to untreated urine or feces was similar to that obtained with plasma, and the derivatization step with triethylanilinium hydroxide was judged 100% complete by GC comparison with the authentic N_1-ethyl analog of flumizole.

[2] Tween 80.
[3] Beckman model LS-250.
[4] Omnifluor, New England Nuclear.
[5] E. Merck AG, 0.05–0.2 mm (70–325 mesh ASTM).
[6] Aminco-Bowman, slit arrangement No. 4.
[7] E. Merck AG 250 μm, Brinkmann Instruments, Inc.
[8] Tractor model MT-220.

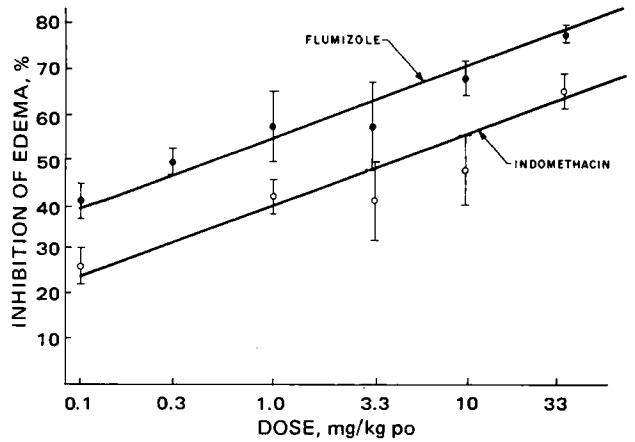


Figure 1—*Dose–response regression lines for the antiedema effects of flumizole and indomethacin in normal rats, six animals at each observation (mean ± SD).*

Estimation of urinary drug excretion in rats and dogs was accomplished by TLC. An aliquot of urine from treated and untreated animals was diluted with an equal volume of 1 *M* NaH_2PO_4, extracted with ethyl acetate (2 × equal volume), and reduced to dryness. The residue obtained directly (or following silica gel column purification as in the plasma assay) was developed on silica gel GF plates[7] with benzene–ethyl acetate–acetic acid (90:5:5) in the presence of authentic flumizole for reference purposes. The presence of drug was revealed by plate exposure to UV light and compared to reference zones of flumizole.

Animal fecal samples were analyzed by fluorometry in aqueous acid (beagles) or by TLC (rats) as already described. Ten percent of administered flumizole would have been detectable in rat feces. Gradual decomposition of flumizole was observed at pH 8 or above in aqueous buffers (the products will be discussed in a forthcoming publication). Drug recoveries averaged 70% (six tests, range of 40–95%) following the incubation of normal dog fecal homogenate with flumizole at concentrations encountered in medicated dog studies. Since a reliable correction factor for potential drug degradation in the gut was not obtainable, fecal assays for flumizole could only be interpreted in a semiquantitative manner and represent, therefore, minimum estimates of actual unabsorbed drug.

Drug Formulation—To prepare the solid dispersion of flumizole for animal bioavailability studies, polyethylene glycol 6000 (II) was melted (approximately 70°) and flumizole was added in small portions with constant stirring. The uniform melt was flash cooled by transferring the beaker to an ice bath. The solidified product was reduced to a fine powder and sieved. Only the powder that passed the 100-mesh but was retained on the 200-mesh sieve was used in the dissolution test.

Analysis was performed by weighing aliquots of the solid dispersions containing approximately 20 mg of flumizole into 100-ml volumetric flasks. After approximately 50 ml of methanol was added, the flasks were shaken mechanically for 30 min and then diluted to volume with methanol. The solutions were filtered[9] to remove undissolved II. A 10-ml aliquot of the filtrate was diluted to 100 ml with methanol. The absorbance of the resulting solution at 267 nm was determined spectrophotometrically[10].

A standard solution was prepared by shaking 20 mg of flumizole and 80 mg of II with methanol, filtering, diluting, and reading as described. A quantity of II equal to that contained in the unknown samples was treated in the same manner as the sample and standard solutions and used as the UV reference solvent. Analysis confirmed drug concentrations in the dispersion within ±5% of theory. Stability of drug in the II melt was established by silica gel GF[7] TLC, using a benzene–5% acetic acid solvent system and UV light irradiation for drug detection.

To determine dissolution rates of the solid dispersion in simulated gastric fluid without pepsin (pH 1.2), an excess of pure flumizole or the solid dispersion beyond its equilibrium solubility was added to 500 ml of simulated gastric fluid maintained at 37 ± 0.5°

[9] Reeve Angel No. 802 paper.
[10] Cary model 15.

Table I—Effects of Flumizole, Indomethacin, and Phenylbutazone on Prostaglandin Synthetase *In Vitro*

Compound	Effective $Dose_{50}$[a] Prostaglandin E_2, μM	Prostaglandin $F_{2\alpha}$, μM	Potency[b]
Flumizole	0.7	0.8	8
Indomethacin	5.6	6.1	1
Phenylbutazone	370	350	0.017

[a] Determined from linear regression analysis of data obtained at arachidonic acid concentrations of 3.6×10^{-4} *M*; a minimum of four concentrations with three replicates was used. [b] Potency relative to indomethacin.

in a 600-ml jacketed beaker. The contents of the dissolution bath were stirred with an overhead T-shaped stirrer rotating at 60 rpm and placed such that the Teflon paddle scraped the bottom of the beaker. Aliquots (10 ml) of the dissolution medium were withdrawn at various times and immediately filtered[11] and diluted to 25 ml with methanolic hydrochloric acid (7 ml of concentrated hydrochloric acid/liter of methanol).

The fluorescence of the resulting solutions was determined[6]. Standard solutions were prepared by dissolving known quantities of flumizole in methanolic hydrochloric acid, adding 10 ml of simulated gastric fluid, and diluting to 25 ml with methanolic hydrochloric acid. There was a linear response over the concentration range studied. The baseline fluorescence of 10 ml of fluid diluted to 25 ml with methanolic hydrochloric acid was subtracted from the readings of all standard and unknown solutions.

For bioavailability studies in animals, the solid dispersion was encapsulated[12]. For clinical investigations, the 100–200-mesh flumizole solid dispersion was mixed with lactose as a bulking agent, followed by the addition of 0.5% fumed silica[13] as a lubricant, to afford a free flowing powder for encapsulation[12]; a 25-mg drug capsule contained 125 mg of solid dispersion and 350 mg of lactose. Dissolution profiles were obtained in simulated gastric fluid according to NF XIII Dissolution Test Method I with minor modifications. Aliquots of the dissolution medium were processed and analyzed spectrophotofluorometrically as previously described.

Biological Availability—Flumizole absorption in the rat was studied after the oral and intraperitoneal routes of administration with the polyethylene glycol 300 vehicle (I) employed in anti-inflammatory tests. Dogs received 10 mg of flumizole/kg, orally as encapsulated unformulated drug or as a solution in I (10 ml) and intravenously in 3 ml of I as a bolus or as a 3-min infusion into a rapid saline drip into the jugular vein.

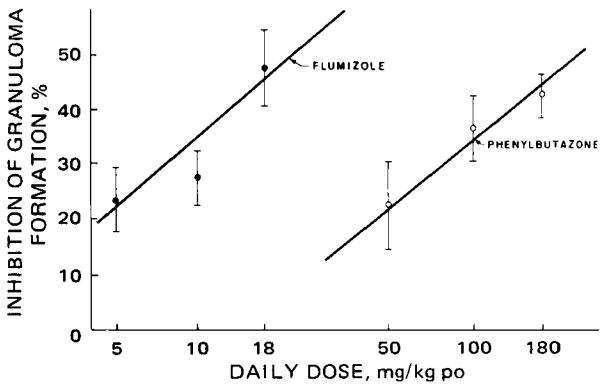


Figure 2—*Dose–response regression lines for the antigranuloma effects of flumizole and phenylbutazone in rats, five animals at each observation (mean ± SD).*

[11] Millipore, 0.22-μm pore.
[12] Parke-Davis No. 2 hard gelatin.
[13] Cab-O-Sil, Cabot Corp.

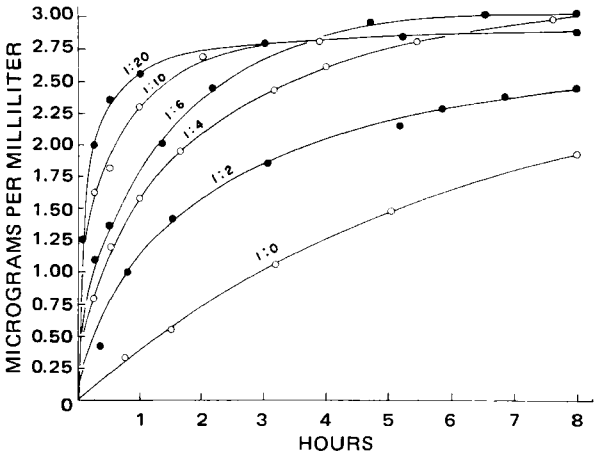


Figure 3—*Dissolution profile of flumizole–polyethylene glycol 6000 (1:x) solid dispersions in simulated gastric fluid (60 rpm, 37 ± 0.5°).*

Comparative dosage form studies with flumizole solid dispersion were conducted in two beagles fasted each night prior to drug administration at weekly intervals. Animals were allowed free access to water after drug ingestion, although food was withheld for an additional 7 hr. Drug analysis was conducted with heparinized whole blood samples drawn at selected intervals from the abdominal aorta of rats maintained under ether anesthesia or from the jugular vein of dogs. Plasma, immediately separated by centrifugation, and total urine and fecal specimens collected in metabolism cages were frozen prior to analysis.

Pharmacokinetic studies were conducted with the solid dispersion capsule formulation in healthy male volunteers during single- (25, 50, and 75 mg administered at weekly intervals) and multiple- (25 mg tid, 10 days) dose tolerance studies. Subjects were fasted overnight prior to single-dose studies and received normal noonday meals 3–4 hr later. During the multiple-dose phase, drug was administered at 4-hr intervals following an initial postprandial morning dose. Serial blood samples were collected into heparinized tubes for plasma drug level determinations.

All samples, including total urine and fecal collections from selected individuals, were frozen upon collection until analysis. Drug level analyses during availability studies were aided by computer-assisted determinations of areas under the plasma concentration–time curves (trapezoidal method). Mean levels based on area ($\bar{c}$) were obtained by dividing area by the number of hours within the time period of interest.

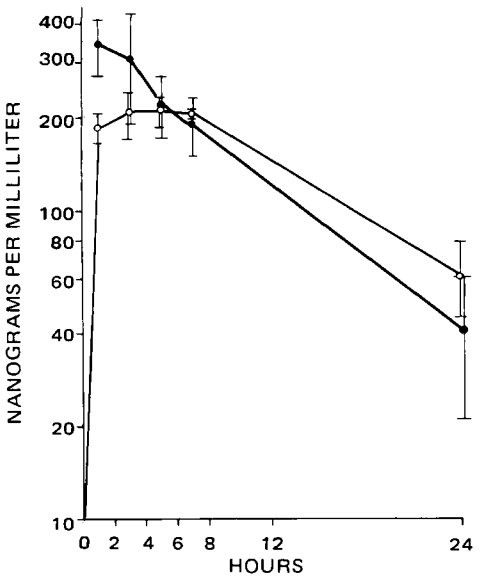


Figure 4—*Mean ± SD plasma levels of flumizole in rats (three per time interval) following intraperitoneal (●) and oral (○) administration of flumizole in polyethylene glycol 300 solution, 3.3 mg/kg.*

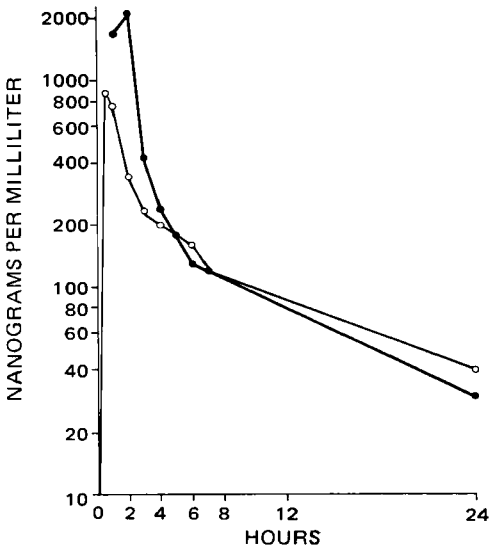


Figure 5—*Plasma levels of flumizole in a female beagle (10.6 kg) following the intravenous bolus (●) and oral (○, 4 weeks later) administration of flumizole in polyethylene glycol 300 solution, 10 mg/kg. Feces (0–48 hr) of this dog contained 43% of the oral dose but none of the intravenous dose as flumizole.*

RESULTS

Antiedema Activity—In the intact Charles River rat, flumizole consistently inhibited edema formation at oral doses of 0.3 mg/kg and above. Some variation was noted, possibly linked to the limited solubility of flumizole, even though administration was performed in the rat in aqueous polyethylene glycol 300 solution. Optimally, however, flumizole was 10 (range of 5–40) times as effective as indomethacin (Fig. 1). The activity of flumizole was not significantly reduced by adrenalectomy; at an oral dose of 1.0 mg/kg, flumizole inhibited edema formation to 54 ± 4% (*cf.*, 57 ± 7% in intact rats).

Antigranuloma Activity—In this test, flumizole possessed 10 times the potency of phenylbutazone (Fig. 2). At the highest doses, both flumizole (18 mg/kg) and phenylbutazone (180 mg/kg) slightly inhibited body weight gain during the 3-day test period.

Antierythema Activity—Flumizole and phenylbutazone both inhibited the erythema caused by UV irradiation in the guinea pig. The largely subjective nature of the evaluation of the end-point of this test (degree of erythema) precluded rigorous estimation of relative potency. Scores for both drugs were 0.8–1.1 at 1.0 mg/kg, 0.5–0.7 at 10 mg/kg, and 0.2–0.4 at 18 mg/kg; concurrently treated control animals scored 3.4–4.0. Therefore, over the 1.0–18-mg/kg dose range, the compounds were approximately equally effective.

Prostaglandin Synthetase Activity—Flumizole inhibited the conversion of arachidonic acid into prostaglandins E_2 and $F_{2\alpha}$ *in vitro* (Table I). The compound was eight times more potent than indomethacin and 470 times more potent than phenylbutazone.

Formulation—Flumizole dissolved in simulated gastric fluid from the solid dispersions containing various amounts of polyethylene glycol 6000 faster than when presented as the compound alone (Fig. 3). Solid dispersions containing increasing amounts of polyethylene glycol 6000 (2, 4, 6, 10, and 20 parts for each part of drug) showed a corresponding increase in the dissolution rate of flumizole.

The addition of lactose and 0.5% fumed silica to the solid dispersion provided a capsule formulation suitable for machine processing. This formulation displayed a dissolution rate profile in simulated gastric fluid comparable to that of the pure solid dispersion. Considerations of capsule size led to the selection of the 1:4 (w/w) formulation for clinical studies.

Biological Availability—Plasma flumizole level determinations in rats following the oral and intraperitoneal administration of flumizole, 3.3 mg/kg in polyethylene glycol 300 solution, indicated that drug was well absorbed at dose levels employed during anti-inflammatory studies (Fig. 4). Peak levels of approximately 200 ng/ml were achieved 3–7 hr after the oral dose. Following in-

Table II—Plasma Levels and Fecal Excretion of Flumizole in Two Beagles following Administration of Flumizole in Different Dosage Forms

Dosage Form	Dose Level, mg/kg	Hours after Dose										0–7-hr Area under Curve, (ng hr)/ml	Fecal Excretion, % of Dose, 0–48 hr
		0	0.25	0.5	1	2	3	5	7	10	24		
		Plasma Levels, ng/ml											
Dog 1 (female, 10.8 kg)													
Intravenous infusion	10	0	4430	—	2740	1610	650	360	190	170	70	7560	0
Oral solution	10	0	70	100	140	180	110	80	40	10	10	700	30
Oral solid drug	10	0	—[a]	0[b]	0	—	0	60	100	—	110	210	16
Oral solid dispersion	10	0	—	0	20	120	120	60	30	—	0	462	32
	50	0	—	0	70	250	220	110	90	—	40	945	68
Dog 2 (male, 17.2 kg)													
Intravenous infusion	10	0	4180	—	4270	1820	560	330	160	90	50	8785	0
Oral solution	10	0	170	360	390	250	170	50	80	10	40	1155	27
Oral solid drug	10	0	—	—	0	—	0	0	0	—	0	0	93
Oral solid dispersion	10	0	—	0	10	150	120	110	40	—	10	595	18
	50	0	—	0	160	310	150	90	0	—	0	833	88

[a] Designates levels not determined. [b] Zero levels represent less than 10 ng/ml, minimum assay detectability during this study.

traperitoneal injection, drug levels declined with an apparent half-life of 7 hr within the first 24-hr period. The absence of detectable unchanged drug in the feces and urine of these animals (oral or intraperitoneal dose) indicated the extensive (>90%) metabolism of flumizole by rats prior to excretion[14].

Preliminary studies in beagles suggested poor absorption of solid unformulated flumizole. Plasma concentrations were low, and a major portion of the dose was excreted unchanged in feces. Oral administration of drug as a solution in polyethylene glycol 300 afforded maximum plasma concentrations within 1 hr; these levels were approximately one-half those obtained following intravenous drug injection in the same animal (Fig. 5). An apparent plasma half-life of 2.5 hr during the 3–7-hr postdose period increased with time, a probable consequence of drug redistribution. The recovery of 43% of the oral dose in feces as flumizole and none following intravenous drug administration indicated that absorption was incomplete at this dose level even when drug was administered in solution. With additional evidence that dog urine (intravenous or oral dose) contained no flumizole, it could be established that absorbed flumizole was completely metabolized in this species.

To support the selection of a flumizole dosage form with optimum biological availability properties, comparative blood level studies were conducted in two beagles at successive weekly intervals (Table II). Baseline data were first obtained following the intravenous infusion and oral ingestion of polyethylene glycol 300 solutions of drug. The solid dispersion afforded maximum plasma drug levels of about 100 ng/ml 2–3 hr after the dose. The availability of flumizole from the dispersion was 50–70% that of oral solutions based on area-under-the-curve analyses. Drug levels following administration of capsules containing solid unformulated drug were appreciably lower or undetectable. At 10 mg/kg, drug in the feces of these animals (Table II) supported the incompleteness with which flumizole is orally absorbed in dogs, regardless of dosage form. A fivefold increase in dose to 50 mg/kg only doubled peak plasma levels and elevated areas under the curve 1.4–2-fold in the beagles to indicate further that limits of the drug absorption processes had been reached.

The availability of flumizole from the solid dispersion capsule formulation was assessed in humans by plasma and fecal drug level determinations following the oral administration of single and multiple doses. In 17 volunteers, drug levels peaked after 1–4 hr and averaged 20, 30, and 48 ng/ml of plasma following single doses of 25, 50, and 75 mg, respectively (Table III and Fig. 6); however, appreciable intersubject plasma level variations were evident. The disappearance of drug from plasma proceeded nonexponentially with an increasing half-life in most subjects. A shoulder, plateau, or occasional secondary peak was observed within the first 6 hr in many individuals; at the 75-mg level, 10 of 17 subjects exhibited this phenomenon, which may reflect an irregular drug absorption pattern[15]. A mean half-life of 4.4 hr was apparent during the first 6 hr in all subjects at the maximum 75-mg dose level. Based on measurable 6- and 24-hr levels at this dose in 11 subjects, a longer secondary half-life phase ranging from 4.5 to 20 hr was indicated (mean of 11 hr).

Plasma flumizole levels during a multiple-dose study were maintained without accumulation (Table III). Levels midway through the 10-day trial, 25 mg tid, averaged about 30 ng/ml. On the morning after the last multiple daily dose, drug levels had declined in all subjects to about 10 ng/ml or less. Analysis following a final single dose on Day 11 revealed the usual pattern of drug clearance with levels of less than 10 ng/ml after 24 hr.

The absence of measurable levels of unchanged drug in feces (<20% of dose) and urine (<5% of dose) from selected volunteers at the 75-mg single-dose level supported good drug absorption and indicates the completeness with which flumizole is metabolized prior to excretion by humans.

DISCUSSION

Flumizole demonstrates potent inhibitory activity in classical animal models of inflammation. The erythema formed in response to UV irradiation was inhibited. Flumizole inhibited carrageenan-induced foot edema with a potency greater than that of indomethacin. Some variation in potency was noted, it could be observed from experiment to experiment and was probably a reflection of the very limited aqueous solubility of flumizole. In addition, the potency varied in the strain of rat, flumizole being a somewhat less effective antiedema agent in the Wistar rat than in the Charles River rat (2). Full antiedema activity was retained in adrenalectomized Charles River rats, revealing that anti-inflammatory activity is not dependent upon adrenal gland stimulation. In a model of more chronic inflammation, flumizole inhibited the formation of granulation tissue around an implanted string irritant (sterile string).

Flumizole also inhibits the activity of prostaglandin synthetase *in vitro*. The drug was severalfold more potent than indomethacin and 470 times more active than phenylbutazone in a system examining prostaglandin production. Thus, despite its different physicochemical properties, flumizole resembles the acidic anti-

[14] Analysis of rat urine revealed the presence of *O*-monodemethylated flumizole by mass spectrometric analysis of material isolated from thin-layer plates. Subsequent confirmation of this finding and the detailed elaboration of flumizole metabolism pathways will be the subjects of a forthcoming paper.

[15] Noonday meals were generally taken during a period coincident with the observed drug level fluctuations. Similar blood level patterns reported with the anti-inflammatory agent indoxole (8) may have been related to food intake and consequent gallbladder evacuation of drug. Although double peaking was observed with flumizole in dogs (three of six experiments, intravenous dose), a biliary fistula beagle that received an oral dose of ^{14}C-flumizole secreted very little (by chromatographic estimation) unchanged drug with bile.

Table III—Mean Plasma Levels of Flumizole in Humans during Single- and Multiple-Dose Tolerance Trials following Administration of Flumizole Solid Dispersion Capsule Formulation

Single-Dose Studies, 17 Subjects

Dose, mg (mg/kg, Mean)	Mean Plasma Drug Levels, ng/ml	
	Peak (Range)	$\bar{c}$, 0—6 hr
25 (0.33)	20 ± 11 (7—36)	11 ± 6
50 (0.67)	30 ± 17 (7—66)	17.5 ± 7
75 (1.0)	48 ± 18 (19—87)	28.5 ± 9

Multiple-Dose Studies, Nine Subjects

	Mean Plasma Drug Levels, ng/ml (Range)[a]								
	Day 1			Day 4		Day 11 (Single am Dose)			
Dose	0 hr	4 hr	6 hr	4 hr	6 hr	0 hr	2 hr	6 hr	24 hr
25 mg tid	5 ±3 (0—8)	22 ±21 (6—74)	25 ±20 (3—68)	26 ±12 (11—46)	31 ±16 (6—56)	8 ±3 (4—13)	26 ±14 (9—54)	17 ±14 (3—50)	3 ±3 (0—7)

[a] Hourly intervals designated refer to time period after the first dose of the day; drug was administered at 8:00 am, noon, and 4:00 pm daily. This 10-day study culminated a 25-day trial, in which the dose increased incrementally from 5 mg bid to 15 mg tid through the first 15-day segment.

inflammatory agents in its inhibition of prostaglandin synthetase. The demonstration of anti-inflammatory activity for flumizole in humans[16] is further support for the hypothesized importance of this activity in the mechanism of action of anti-inflammatory agents (9).

Because the aqueous solubility of flumizole is very low, all pharmacological testing was conducted using a solution of the drug in polyethylene glycol 300 containing polysorbate 80. With the development of sensitive fluorometric assay procedures specific for flumizole, measurement of plasma drug concentrations confirmed the absorption of drug from this vehicle in dogs (10 mg/kg) and rats (3.3 mg/kg). For extended studies in animals and humans, a solid dosage form of flumizole was sought with optimum absorption characteristics.

The dissolution of a poorly water-soluble drug in a melt of the water-soluble carrier polyethylene glycol 6000, followed by rapid cooling, affords a solid in which drug particles are finely dispersed, with resultant improvements in wetting properties of the drug (10). The dissolution rates of griseofulvin (11) and indomethacin (12) and the absorption of griseofulvin in dogs (13) and humans (14) have been improved using polyethylene glycol 6000 dispersion systems. The preparation of a solid dispersion of flumizole with polyethylene glycol 6000 (1:4, w/w) by the melting method provided an oral dosage form with an increased dissolution rate in simulated gastric fluid and improved plasma drug concentration patterns in beagles. Plasma levels were higher and were more rapidly and uniformly achieved than from an unformulated dose; solid dispersion drug levels more closely resembled those following the administration of oral solutions of drug. The amounts of unabsorbed drug in feces generally supported these findings. Therefore, the solid dispersion of flumizole was selected as a superior dosage form for further investigation.

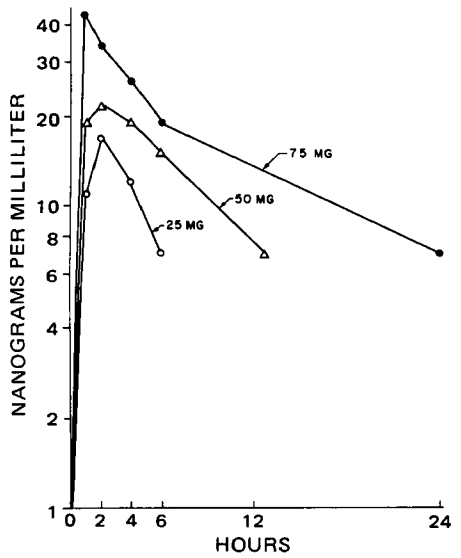


Figure 6—*Mean plasma levels of flumizole in 17 subjects following the administration of single doses of 25 (○), 50 (△), and 75 (●) mg of flumizole solid dispersion capsule formulation.*

Following preclinical safety evaluation in animals[17], bioavailability studies were conducted in humans with the solid dispersion capsule formulation of flumizole. A dose-dependent increase in mean plasma levels of flumizole was observed over the single-dose range of 25–75 mg. Peak plasma levels of flumizole were achieved with 1–4 hr after the dose was received. The magnitude of plasma levels and the absence of appreciable quantities of drug in feces supported good drug absorption during these studies. Similar drug level patterns were observed during multiple-dose regimens of 25 mg tid.

Flumizole was eliminated from the bloodstream of rats (3.3 mg/kg ip) with a plasma half-life of about 7 hr within the first 24-hr period. In dogs (10 mg/kg po and iv) and humans (75 mg po), plasma flumizole levels declined initially with an apparent half-life of 2–4 hr and increased with time, a probable consequence of drug redistribution. Detailed pharmacokinetic properties of flumizole will be discussed in a future publication.

The present studies demonstrate flumizole to be a potent anti-inflammatory agent in standard rodent and prostaglandin synthetase systems. Biological availability of this poorly water-soluble compound is optimized in dogs and humans by administration as a solid dispersion with polyethylene glycol 6000.

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Apparent Absorption Kinetics of Micronized Griseofulvin after Its Oral Administration on Single- and Multiple-Dose Regimens to Rats as a Corn Oil-in-Water Emulsion and Aqueous Suspension

THEODORE R. BATES ˣ and PHILIP J. CARRIGAN

Abstract □ This investigation was designed to quantitate and compare in the rat the oral absorption characteristics of micronized griseofulvin from a corn oil-in-water emulsion dosage form containing suspended drug and a control aqueous suspension after single-dose (50 mg/kg) and multiple-dose (50 mg/kg every 12 hr for five doses) administrations. The time course of intact drug in the plasma of all animals was best described by a one-compartment open model with apparent zero-order absorption. In contrast to that observed with the aqueous suspension, the onset of drug absorption after single-dose administration of the corn oil emulsion was significantly delayed. This difference disappeared upon multiple dosing of the two dosage forms, with the mean onset being quite rapid in both cases. Administration of a single dose of the antibiotic as the corn oil emulsion resulted in considerable increases in the maximum plasma levels of griseofulvin and in the duration, relative extent, and uniformity of drug absorption compared to those observed after administration of the control aqueous suspension. The potentiating effects of the lipid on drug absorption persisted on multiple dosing but at a somewhat reduced level.

Keyphrases □ Griseofulvin, micronized, absorption kinetics—corn oil-in-water emulsion compared to aqueous suspension, single and multiple doses, rats □ Absorption kinetics, micronized griseofulvin—corn oil-in-water emulsion compared to aqueous suspension, single and multiple doses, rats □ Corn oil-in-water emulsion, micronized griseofulvin—absorption kinetics compared to aqueous suspension, single and multiple doses, rats

Several investigators (1–3) suggested that the slow rate and low extent of absorption, as well as the appreciable intersubject differences in the amount of micronized griseofulvin absorbed from conventional dosage forms, are the most common reasons for clinical failure with griseofulvin therapy. In a previous report (4), it was demonstrated, using the rat as an animal model, that the maximum plasma levels, the bioavailability, and, of considerable importance, the uniformity of absorption of micronized griseofulvin were markedly enhanced after oral administration of a single dose of the antibiotic dispersed in a corn oil-in-water emulsion dosage form vehicle.

These findings were recently confirmed in a study conducted in humans in which the absorption characteristics of micronized griseofulvin from the same corn oil-in-water emulsion were compared with those from an aqueous suspension and two different commercial tablet dosage forms (5). The results of this study provided evidence that the maximum plasma levels and bioavailability of micronized griseofulvin can be increased approximately three- to fourfold and twofold, respectively, by administering the drug as a corn oil-in-water emulsion.

It is not without precedence for absorption differences, which appear after *single-dose* drug administration, to diminish or even to disappear upon *multiple* oral dosing of a drug or drug product (6). Hence, the purposes of the present investigation were to assess and to compare the absorption profiles of micronized griseofulvin from a corn oil-in-water emulsion and a control aqueous suspension dosage form after single and repetitive administrations to rats. The resultant plasma antibiotic level–time data were subjected to pharmacokinetic analysis with the intent of developing a mechanistic explanation for the

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enhanced absorption of micronized griseofulvin from lipid-in-water emulsion dosage forms.

EXPERIMENTAL

Dosage Forms—The corn oil-in-water emulsion and control aqueous suspension dosage forms employed in these studies were identical in composition and method of preparation to those previously described (4). Both dosage forms contained suspended micronized griseofulvin[1] (specific surface area of 1.32 m^2/g) and the same quantity of the nonionic surfactant polysorbate 60. Prior to use, they were assayed in triplicate for drug content by the spectrophotometric procedure described earlier (4).

Absorption Studies—Adult male Sprague–Dawley rats[2], 250–325 g, were used. All experiments were initiated between 8:00 and 9:00 am.

Single-Dose Oral Studies (Fasting Animals)—The animals were fasted for 20–24 hr prior to, and during, the absorption experiments, but they were allowed free access to water. The aqueous suspension and corn oil-in-water emulsion dosage forms were administered by oral intubation to 11 or 12 lightly anesthetized (ether) rats at a griseofulvin dosage level of 50 mg/kg and a dosing volume of 5.0 ml/kg. After dosing, the animals were placed in restraining cages.

Approximately 0.5-ml blood samples were collected from the tail artery of each rat into heparinized tubes immediately prior to drug administration and at 1, 2, 4, 6, 8, and 12 hr postadministration. Twenty-four-hour samples were obtained *via* decapitation. The blood samples were then centrifuged, and a 0.2–0.3-ml aliquot of the plasma was accurately measured directly into a culture tube extraction vessel. A 2.0-ml plasma sample was obtained at the 24-hr time period. All plasma samples were immediately frozen solid and assayed for griseofulvin within 48 hr of collection.

Single-Dose Oral Studies (Nonfasting Animals)—The *in vivo* protocol employed in studies designed to establish the effect of food on the oral absorption characteristics of griseofulvin from the corn oil-in-water emulsion was similar to that used for fasting animals. The sole exception was that the animals were allowed free access to food[3] prior to, and during, the absorption experiment.

Multiple-Dose Oral Studies—Six animals were fasted for 20–24 hr prior to oral administration of the first 50-mg/kg dose of griseofulvin as the aqueous suspension or corn oil-in-water emulsion. The animals were then allowed free access to food until 20–24 hr prior to the final dose, at which time they were again fasted for the duration of the absorption experiment. Five 50-mg/kg doses of griseofulvin were administered at a constant dosing interval of 12 hr. Blood samples were collected from the tail artery of each animal immediately prior to, and at 1, 2, 4, 6, 8, and 12 hr after, the administration of the last drug dose.

Plasma samples were assayed for intact griseofulvin content by the spectrofluorometric procedure described earlier (4).

RESULTS AND DISCUSSION

Pharmacokinetic Analysis—Previous studies indicated that the plasma levels of griseofulvin decline in a monoexponential fashion with time after intracardiac (i.c.) administration to rats of a single 5- or 10-mg/kg dose of the antibiotic as a solution in polyethylene glycol 400 (7). There was no evidence of a dose dependency in the disposition kinetics of the drug, and the plasma levels attained in the intracardiac studies were comparable to those observed in the present 50-mg/kg dose oral studies.

Therefore, all plasma griseofulvin concentration–time data obtained following single- or multiple-dose oral administration of the dosage forms were fitted to an appropriate linear pharmacokinetic model, using the digital computer, nonlinear regression analysis program (NONLIN) of Metzler (8), and equal weighting of the data points. The goodness of fit of the data to a one-compartment open model with either apparent first-order (Scheme I and Eqs. 1 and 2) or apparent zero-order (Scheme II and Eqs. 3*a*, 3*c*, 4*a*, and 4*c*) absorption was tested (8–10).

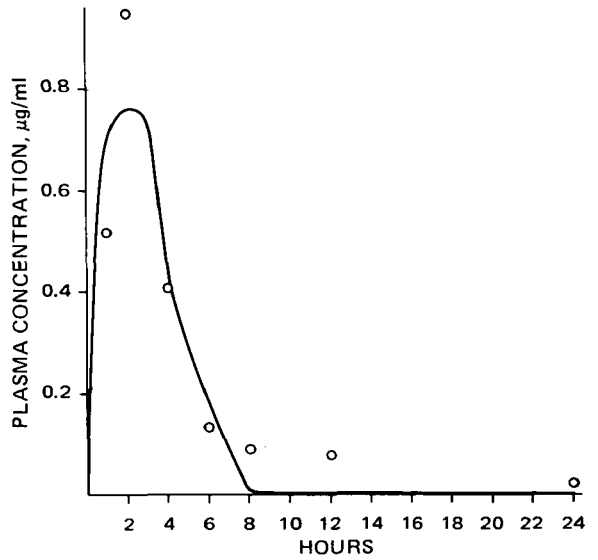

Figure 1—*Plasma concentrations of griseofulvin as a function of time following the oral administration of a 50-mg/kg single dose of micronized griseofulvin as an aqueous suspension to Rat 51. Key:* o, *actual experimental points; and* —, *obtained by computer, nonlinear least-squares regression according to Eq. 1 (CORR = 0.934).*

One-Compartment Open Model with First-Order Absorption—Scheme I shows the simplified model where A_A is the amount of griseofulvin in solution at the absorption site at any time t, k is the apparent first-order absorption rate constant, and K' is the apparent elimination rate constant.

$$A_A \xrightarrow{k} A_B \xrightarrow{K'} A_E$$

Scheme I

For single-dose administration, griseofulvin plasma concentrations (C) at any time t following oral drug administration are given by:

$$C = \left(\frac{FD}{V}\right)\left(\frac{k}{k - K'}\right)[\exp[-K'(t - t_0)] - \exp[-k(t - t_0)]] \quad \text{(Eq. 1)}$$

where F is the fraction of the oral dose absorbed, D is the oral dose, V is the volume of distribution, and t_0 is the lag time before absorption commences or the onset of absorption.

For multiple-dose administration, the plasma concentration of griseofulvin at any time t' following the oral administration of the nth dose (C_n) is given by:

$$C_n = \left(\frac{FD}{V}\right)\left(\frac{k}{k - K'}\right)\left[\left(\frac{1 - \exp - (nK'\tau)}{1 - \exp - (K'\tau)}\right) \times \exp[-K'(t' - t_0)] - \left(\frac{1 - \exp - (nk\tau)}{1 - \exp - (k\tau)}\right) \times \exp[-k(t' - t_0)]\right] \quad \text{(Eq. 2)}$$

where $t' = t - (n - 1)\tau$, n is the number of fixed doses administered, τ is the constant time interval between doses, and C_n is the plasma concentration of griseofulvin at any time t' after the nth dose.

One-Compartment Open Model with Apparent Zero-Order Absorption—Scheme II shows the simplified model where k_0 is the apparent zero-order absorption rate constant.

$$A_A \xrightarrow{k_0} A_B \xrightarrow{K'} A_E$$

Scheme II

[1] Supplied by the Schering Corp., Bloomfield, N.J.
[2] Obtained from Blue Spruce Farms, Altamont, NY 12009
[3] Charles River Rat Formula (low in fat content), Country Foods, Syracuse, N.Y.

For single-dose administration, the plasma concentration of griseofulvin at any time during its absorption over T hours is given by:

$$C = \frac{k_0}{VK'}[1 - \exp[-K'(t - t_0)]] \qquad \text{(Eq. 3a)}$$

with the condition that $t_0 \leq t \leq T$ and where $k_0 = FD/T$, T is the duration of the absorption process, and t is any time after oral administration of griseofulvin.

At the end of the absorption process, the maximum plasma concentration of griseofulvin ($C_{\max,T}$) can be described by the relationship:

$$C_{\max,T} = \frac{k_0}{VK'}[1 - \exp[-K'(T - t_0)]] \qquad \text{(Eq. 3b)}$$

when $t = T$.

After absorption has ceased, the plasma levels of griseofulvin decline monoexponentially with time in accordance with:

$$C = (C_{\max,T})\exp[-K'(t - T)] \qquad \text{(Eq. 3c)}$$

when $t \geq T$.

For multiple-dose administration, at any time t' during the absorption of the nth dose, the plasma levels of griseofulvin are given by:

$$C_n = \frac{k_0}{VK'}\Bigg[1 - \exp - [K'(t' - t_0)] - \left(\frac{1 - \exp - [(n-1)K'\tau]}{1 - \exp - (K'\tau)}\right)(\exp[-K'(t' - t_0 + \tau)] - \exp[-K'(t' - t_0 + \tau - T)])\Bigg] \qquad \text{(Eq. 4a)}$$

with the condition that $t_0 \leq t' \leq T$ and where $t' = t - (n-1)\tau$ = time elapsed after oral administration of the nth dose.

The maximum plasma concentration attained after the nth dose ($C_{n,\max,T}$) is:

$$C_{n,\max,T} = \frac{k_0}{VK'}\Bigg[1 - \exp - [K'(T - t_0)] - \left(\frac{1 - \exp -[(n-1)K'\tau]}{1 - \exp - (K'\tau)}\right) \times (\exp[-K'(T - t_0 + \tau)] - \exp[-K'(\tau - t_0)])\Bigg] \qquad \text{(Eq. 4b)}$$

when $t' = T$.

After the nth dose is absorbed, the plasma concentration decreases with time in accordance with:

$$C_n = (C_{n,\max,T})\exp[-K'(t' - T)] \qquad \text{(Eq. 4c)}$$

when $t' \geq T$.

As can be seen from the representative computer-predicted curves, the use of the apparent first-order absorption model (Scheme I and Eqs. 1 and 2) consistently resulted in poor agreement between the predicted and experimental plasma level–time data obtained from both single- (Figs. 1 and 2) and multiple- (Fig. 3) dose absorption studies. However, when the same experimental data were analyzed by the apparent zero-order absorption model (Scheme II and Eqs. 3a, 3c, 4a, and 4c), excellent computer fits were realized (Figs. 4–6). Therefore, this model was used to interpret the results of all *in vivo* absorption studies performed with griseofulvin.

It is important to note that griseofulvin is *apparently* absorbed in a zero-order fashion from the *test dosage forms* whereas the drug is probably absorbed from *solution* by an apparent first-order process (11, 12). The observed, apparent zero-order absorption profile of griseofulvin can be explained on the basis that a slow, rate-determining dissolution step precedes the true absorption process (13–15). The assumption that the rate-limiting dissolution process is kinetically zero-order appears to be consistent with the unusually low aqueous solubility of griseofulvin (about 15 mg/liter at 37°) and the limited volume of GI fluids available for drug dissolution relative to the oral dose administered to the animals (*i.e.*, 50 mg/kg or approximately 15 mg/animal). A similar hypothesis was proposed (16) to account for the *apparent* zero-order absorption profile of sulfisoxazole from tablet dosage forms in humans.

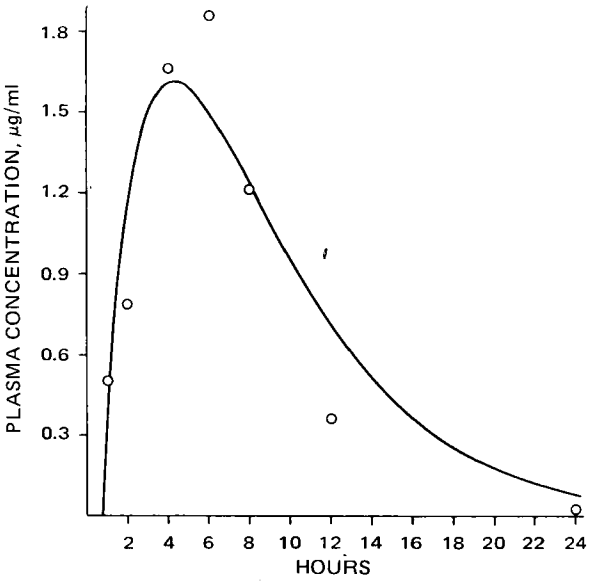

Figure 2—*Plasma concentrations of griseofulvin as a function of time following the oral administration of a 50-mg/kg single dose of micronized griseofulvin as a corn oil-in-water emulsion to Rat 42. Key: O, actual experimental points; and —, obtained by computer, nonlinear least-squares regression according to Eq. 1 (CORR = 0.941).*

The area under the plasma griseofulvin concentration–time curve from time zero to infinity after a single dose of the drug

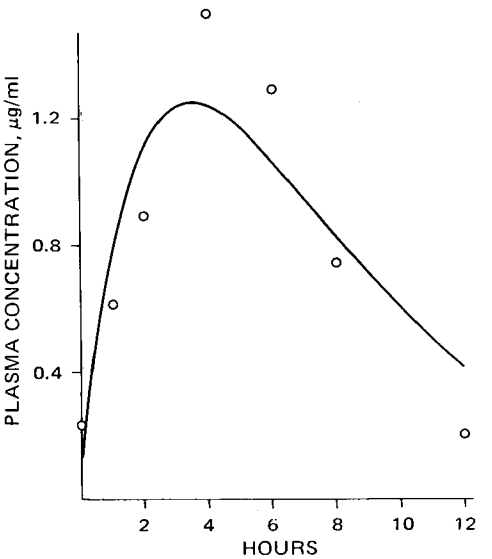

Figure 3—*Plasma concentrations of griseofulvin as a function of time following the oral administration of the fifth 50-mg/kg dose of micronized griseofulvin as a corn oil-in-water emulsion to Rat 38. Key: O, actual experimental points; and —, obtained by computer, nonlinear least-squares regression according to Eq. 2 (CORR = 0.915).*

Table I—Plasma Concentrations of Griseofulvin following Its Oral Administration as a Single 50-mg/kg Dose as an Aqueous Suspension (AS) or a Corn Oil-in-Water Emulsion (COE) to Rats

Hour	Plasma Concentration, μg/ml		
	AS[a] (Fasting Animals, n = 12)	COE[a] (Fasting Animals, n = 11)	COE[a] (Nonfasting Animals, n = 6)
1.0	0.418 (0.06)[b]	0.362 (0.03)	0.279 (0.06)
	0.162–0.822[c]	0.205–0.502	0.164–0.549
2.0	0.715 (0.10)	0.805 (0.07)	0.885 (0.17)
	0.271–1.31	0.606–1.39	0.509–1.58
4.0	0.748 (0.14)	1.76 (0.19)	1.63 (0.20)[d]
	0.154–1.08	0.931–2.96	1.26–2.16
5.0	—	—	2.50[e]
	—	—	1.94; 2.56[f]
6.0	0.400 (0.09)	1.81 (0.20)	1.36 (0.16)[d]
	0.053–0.941	0.756–3.11	1.04–1.65
8.0	0.259 (0.06)	1.29 (0.17)	1.47 (0.56)
	0.056–0.661	0.733–2.60	0.412–1.54
12.0	0.186 (0.04)	0.524 (0.11)	0.307 (0.07)
	0–0.450	0.236–1.40	0.112–0.589
24.0	0.051 (0.01)	0.050 (0.01)	0.025 (0.02)
	0–0.112	0.016–0.100	0.003–0.101

[a] Micronized griseofulvin was in suspension in dosage form. [b] Mean (*SE*). [c] Range. [d] Mean (*SE*) for four animals. [e] Average for two animals. [f] Individual values for two animals.

($AUC_{0-\infty}$) and during the dosing interval after the fifth oral dose (AUC_τ) was determined using the trapezoidal rule (10).

***In Vivo* Absorption Studies**—*Single-Dose Oral Studies (Fasting Animals)*—The mean[4] time course of griseofulvin in the plasma of fasting rats after oral administration of a single 50-mg/kg dose of micronized griseofulvin as the control aqueous suspension or corn oil-in-water emulsion dosage form is presented in Table I. All plasma level–time data were pharmacokinetically interpreted according to a one-compartment open model with apparent zero-order absorption (Eqs. 3*a* and 3*c* and Figs. 4 and 5).

The computer-generated nonlinear least-squares regression estimates of the model parameters[4] are summarized in Table II, together with indications of the goodness of fit of the experimental data to the proposed model. Statistical comparisons of the mean model parameters are presented in Table III. An examination of the mean t_0 values (Table II) reveals that the onset of absorption of griseofulvin from the corn oil emulsion is slightly, but significantly (Table III), delayed relative to that observed from the control aqueous suspension. No statistical difference was noted between the dosage forms with respect to either the relative rate of drug absorption or the apparent elimination rate constant (Table III). However, the duration of absorption of griseofulvin, as reflect-

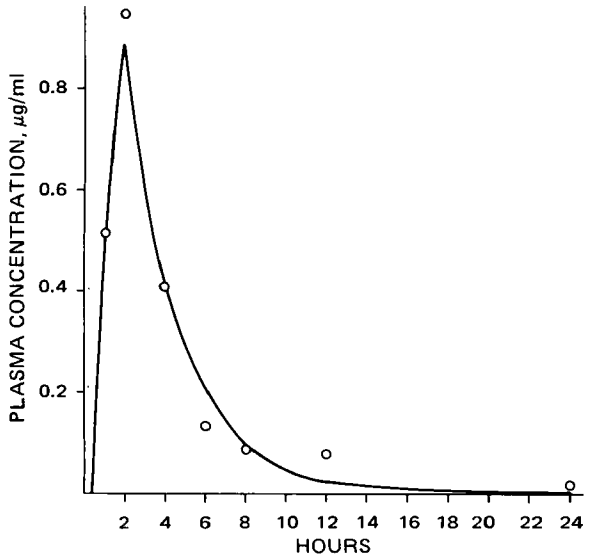

Figure 4—*Plasma concentrations of griseofulvin as a function of time following the oral administration of a 50-mg/kg single dose of micronized griseofulvin as an aqueous suspension to Rat 51. Key: O, actual experimental points; and —, obtained by computer, nonlinear least-squares regression according to Eqs. 3a and 3c (CORR = 0.991).*

[4] Data for each animal are available upon request.

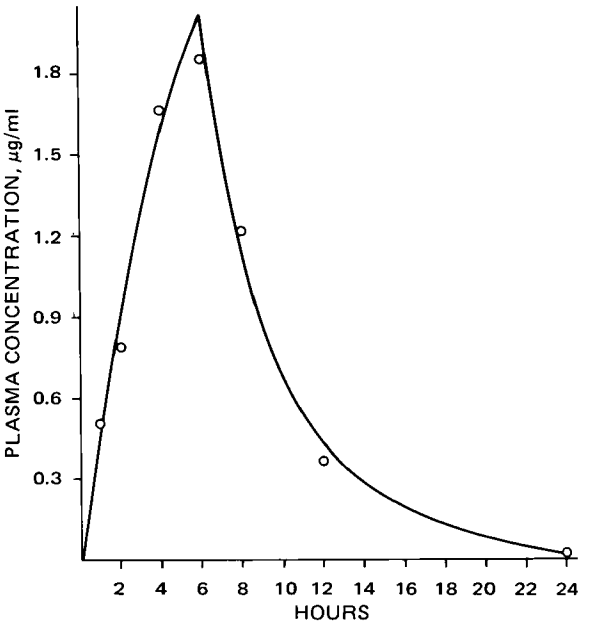

Figure 5—*Plasma concentrations of griseofulvin as a function of time following the oral administration of a 50-mg/kg single dose of micronized griseofulvin as a corn oil-in-water emulsion to Rat 42. Key: O, actual experimental points; and —, obtained by computer, nonlinear least-squares regression according to Eqs. 3a and 3c (CORR = 0.994).*

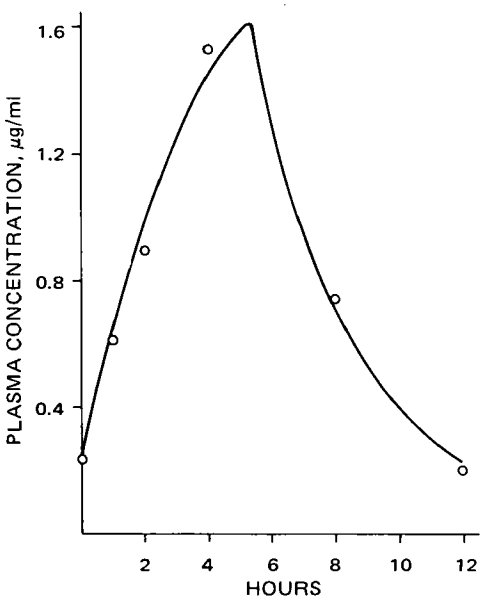

Figure 6—*Plasma concentrations of griseofulvin as a function of time following the oral administration of the fifth 50-mg/kg dose of micronized griseofulvin as a corn oil-in-water emulsion to Rat 38. Key: O, actual experimental points; and —, obtained by computer, nonlinear least-squares regression according to Eqs. 4a and 4c (CORR = 0.994).*

Table II—Pharmacokinetic Parameters Estimated from Plasma Griseofulvin Concentrations following Oral Administration of a Single 50-mg/kg Dose of Micronized Griseofulvin as an Aqueous Suspension (AS) or a Corn Oil-in-Water Emulsion (COE) to Rats

Parameter[a]	AS (Fasting Rats, n = 12)	COE (Fasting Rats, n = 11)	COE (Nonfasting Rats, n = 5)
Apparent onset of absorption (t_0), hr	0.183 (0.05)[b] 0.002–0.431[c] 99[d]	0.516 (0.07) 0.235–0.900 45	0.378 (0.13) 0.071–0.792 78
Apparent duration of absorption (T), hr	2.66 (0.30) 1.23–4.24 39	5.33 (0.37) 3.15–7.16 23	4.90 (0.55) 3.26–6.48 25
Apparent rate of absorption (k_0/V), mg/hr-liter	0.577 (0.09) 0.218–1.41 56	0.769 (0.11) 0.458–1.52 49	0.745 (0.12) 0.457–1.13 36
Apparent elimination rate constant (K'), hr^{-1}	0.255 (0.03) 0.104–0.437 37	0.220 (0.03) 0.106–0.442 50	0.241 (0.02) 0.174–0.287 18
Estimated maximum concentration ($C_{max,T}$)[e], μg/ml	0.944 (0.13) 0.392–1.89 49	2.12 (0.21) 1.11–3.44 32	1.96 (0.28) 1.23–2.70 32
Observed C_{max}, μg/ml	0.875 (0.14) 0.324–2.04 55	1.92 (0.21) 0.931–3.11 36	1.88 (0.18)[f] 1.26–2.56 24
Estimated area under plasma level–time curve [$(AUC)_{0-\infty}$][g], mg-hr/liter	5.72 (1.1) 1.39–14.3 65	17.2 (1.5) 9.82–26.1 29	13.9 (2.2) 8.23–19.9 36
Observed $(AUC)_{0-\infty}$[h], mg-hr/liter	6.42 (1.2) 1.49–14.5 63	17.7 (1.6) 12.0–27.7 30	13.4 (1.9)[f] 8.00–20.8 34
Correlation parameters			
(r^2)[i]	0.990 0.978–0.999	0.990 0.978–0.999	0.985 0.965–0.999
(CORR)[j]	0.987 0.960–0.998	0.985 0.969–0.999	0.988 0.971–0.999

[a] Nonlinear least-squares regression parameters calculated using Eqs. 3*a* and 3*c*. [b] Mean (*SE*). [c] Range. [d] Coefficient of variation, percent. [e] Calculated using Eq. 3*b* and nonlinear least-squares parameters. [f] Mean (*SE*) for six animals. [g] Calculated using the following equation, $(AUC)_{0-\infty} = (k_0/V)(T/K')$, and nonlinear least-squares parameters. [h] Calculated using actual plasma level–time data and the trapezoidal rule. [i] Coefficient of determination. [j] Correlation of observed *versus* predicted plasma levels.

ed by the magnitude of the mean T values listed in Table II, was significantly increased by 2.7 hr (Table III) when the drug was administered to the fasting animals as a suspension in the emulsified corn oil vehicle as compared to the aqueous suspension.

The maximum plasma levels attained following oral administration of griseofulvin in the two dosage forms to fasting animals were calculated with Eq. 3*b* and the computer-generated model parameters; the mean values are listed in Table II. The estimated values compare favorably with those actually observed (Table II). The mean data show that the maximum plasma level of the antibiotic from the corn oil emulsion was approximately two times higher and less variable (lower coefficient of variation) than from the control aqueous suspension. This difference was highly significant (Table III).

The mean estimated and observed areas under the plasma griseofulvin level–time curves for both dosage forms are also listed in Table II, and excellent agreement exists. A statistical comparison of the mean areas (Table III) reveals that there was a highly significant, threefold increase in the amount of drug absorbed from the corn oil emulsion relative to that absorbed from the control aqueous suspension. As reflected by the magnitude of the coefficients of variation associated with this parameter (Table II), the absorption of micronized griseofulvin appears to be more uniform from the corn oil-in-water emulsion.

Multiple-Dose Oral Studies—It was considered important to determine whether the marked improvement in griseofulvin bioavailability observed with the corn oil emulsion dosage form after single-dose administration also occurred upon multiple dosing. Therefore, animals received orally a 50-mg/kg dose of micronized griseofulvin as the control aqueous suspension or corn oil-in-water emulsion dosage form every 12 hr for a total of five doses. The dosing interval employed was designed to yield an average plasma level at steady state (17) of approximately 1 μg/ml and minimal drug accumulation after repetitive oral dosing with the emulsion dosage form.

The mean[4] plasma griseofulvin concentration–time data fol-

Table III—Statistical Comparisons[a] of the Mean Pharmacokinetic Parameters Associated with the Absorption of a Single 50-mg/kg Dose of Micronized Griseofulvin from an Aqueous Suspension (AS) or a Corn Oil-in-Water Emulsion (COE)

Parameter	AS (n = 12) *versus* COE (n = 11), Fasting Animals		COE (Fasting Animals, n = 11) *versus* COE (Nonfasting Animals, n = 5)	
	Student t Value	Level of Significance (p)	Student t Value	Level of Significance (p)
t_0	3.84	<0.001	1.01	>0.3 (N.S.)[b]
T	5.69	<0.001	0.658	>0.5 (N.S.)
k_0/V	1.31	>0.2 (N.S.)[b]	0.129	>0.8 (N.S.)
K'	0.821	>0.4 (N.S.)	0.400	>0.6 (N.S.)
Estimated $C_{max,T}$	4.89	<0.001	0.437	>0.6 (N.S.)
Observed C_{max}	4.24	<0.001	0.120[c]	>0.9 (N.S.)
Estimated $(AUC)_{0-\infty}$	6.33	<0.001	1.22	>0.2 (N.S.)
Observed $(AUC)_{0-\infty}$	5.74	<0.001	1.65[c]	>0.1 (N.S.)

[a] Student t test (20). [b] N.S. = not significant. [c] Value based on the mean of 11 fasting animals and the mean of six nonfasting animals.

Table IV—Plasma Concentrations of Griseofulvin following the Fifth Oral Dose Administered as an Aqueous Suspension (AS) or a Corn Oil-in-Water Emulsion (COE) to Rats (Multiple-Dose Regimen: 50 mg/kg every 12 hr)

Time after Fifth Oral Dose, t', hr	Plasma Concentration, μg/ml	
	AS[a] (n = 6)	COE[a] (n = 6)
0.0	0.207 (0.05)[b]	0.200 (0.02)
	0.106–0.411[c]	0.114–0.264
1.0	0.896 (0.10)	1.13 (0.16)
	0.471–1.15	0.615–1.57
2.0	1.19 (0.15)	1.38 (0.21)
	0.534–1.69	0.895–2.17
4.0	1.14 (0.20)	1.89 (0.13)
	0.541–1.82	1.53–2.36
6.0	0.507 (0.08)	1.38 (0.10)
	0.301–0.805	0.992–1.68
8.0	0.222 (0.05)	0.736 (0.08)
	0.087–0.475	0.559–1.00
12.0	0.106 (0.03)	0.224 (0.03)
	0.022–0.204	0.127–0.378

[a] Micronized griseofulvin was in suspension in dosage form. [b] Mean (SE). [c] Range.

lowing the fifth oral dose of the antibiotic appear in Table IV, and a representative computer fit of the data to Eqs. 4a and 4c is shown in Fig. 6. A statistical comparison of the nonlinear least-squares estimates of the pharmacokinetic parameters associated with the absorption of griseofulvin from the two dosage forms (Tables V and VI) reveals no significant differences in onset and relative rate of drug absorption or in the apparent elimination rate constant of griseofulvin.

Table V—Pharmacokinetic Parameters Estimated from Plasma Griseofulvin Concentrations following the Fifth Oral Dose of Micronized Griseofulvin Administered as an Aqueous Suspension (AS) or a Corn Oil-in-Water Emulsion (COE) to Rats (Multiple-Dose Regimen: 50 mg/kg every 12 hr)

Parameter[a]	AS (n = 6)	COE (n = 6)
Apparent onset of absorption (t_0), hr	0.034 (0.01)[b]	0.025 (0.01)
	0.001–0.070[c]	0.001–0.053
	91[d]	99
Apparent duration of absorption (T), hr	2.81 (0.44)	4.58 (0.40)
	1.54–4.36	3.21–5.31
	39	22
Apparent rate of absorption (k_0/V), mg/hr-liter	0.992 (0.17)	0.945 (0.14)
	0.332–1.53	0.576–1.50
	44	36
Apparent elimination rate constant (K'), hr^{-1}	0.370 (0.09)	0.320 (0.04)
	0.180–0.813	0.236–0.490
	62	28
Estimated maximum concentration ($C_{max,T}$)[e], μg/ml	1.55 (0.09)	2.20 (0.08)
	0.743–2.09	1.62–2.82
	14	8.8
Observed C_{max}, μg/ml	1.32 (0.19)	1.89 (0.13)
	0.541–1.82	1.53–2.36
	35	17
Estimated area under plasma level–time curve, $[(AUC)_T]f$, mg-hr/liter	7.33 (0.81)	12.9 (0.74)
	4.19–9.28	10.6–15.6
	27	14
Observed $[(AUC)_T]f$, mg-hr/liter	6.97 (0.88)	12.5 (0.85)
	3.81–9.01	10.3–16.1
	31	17
Correlation parameters		
$(r^2)g$	0.984	0.982
	0.965–0.999	0.959–0.996
(CORR)[h]	0.978	0.970
	0.934–0.999	0.910–0.994

[a] Nonlinear least-squares regression parameters calculated using Eqs. 4a and 4c. [b] Mean (SE). [c] Range. [d] Coefficient of variation, percent. [e] Calculated using Eq. 4b and nonlinear least-squares parameters. [f] Area under plasma concentration–time curve from t' = 0 to t' = 12 hr as determined with the aid of the trapezoidal rule and computer-generated (estimated value) or actual (observed value) plasma level–time data. [g] Coefficient of determination. [h] Correlation of observed *versus* predicted plasma levels.

Table VI—Statistical Comparison[a] of the Mean Pharmacokinetic Parameters Associated with the Absorption of the Fifth Dose of Micronized Griseofulvin Administered as an Aqueous Suspension or a Corn Oil-in-Water Emulsion (Multiple-Dose Regimen: 50 mg/kg every 12 hr)

Parameter	Student t Value	Level of Significance (p)
t_0	0.567	>0.5 (N.S.)[b]
T	2.94	<0.02
k_0/V	0.206	>0.8 (N.S.)
K'	0.502	>0.6 (N.S.)
Estimated $C_{max,T}$	2.48	<0.05
Observed C_{max}	2.49	<0.05
Estimated $(AUC)_T$	5.03	<0.001
Observed $(AUC)_T$	4.50	<0.005

[a] Student t test (20). [b] N.S. = not significant.

However, in contrast to what was observed after repetitive administration of the aqueous suspension, the presence of emulsified corn oil in the GI tract produced approximately a 1.8-hr increase in the duration of the absorption process, a 1.4-fold increase in the maximum plasma levels attained, and a 1.8-fold increase in the bioavailability of griseofulvin (Table V). All of these differences were highly significant (Table VI).

In addition, the drug was more uniformly absorbed from the emulsion dosage form (*i.e.*, the coefficients of variation were lower, Table V) and plasma levels exceeding 1 μg/ml, the minimum effective level in humans (18) and dogs (15), were maintained for appreciably longer periods (Table IV). The fact that the absorption of micronized griseofulvin from the corn oil emulsion is considerably enhanced after both single and repetitive oral administration demonstrates that the effect of emulsified lipid on griseofulvin absorption is not transient.

Effect of Food on Absorption of a Single Dose of Micronized Griseofulvin from a Corn Oil-in-Water Emulsion—In general, food has an inhibitory effect on the GI absorption of most drugs from pharmaceutical dosage forms (19). To determine whether the presence of food in the GI tract could affect the absorption of griseofulvin from the corn oil-in-water emulsion, a single 50-mg/kg dose of this dosage form was administered orally to another group of rats who had been allowed free access to food both before and after drug administration (nonfasting animals). The mean[4] time course of griseofulvin in the plasma of each nonfasting rat is presented in Table I. The pharmacokinetic analysis of the plasma level data is summarized in Table II.

A statistical comparison of the parameters obtained after oral administration of the corn oil emulsion to nonfasting animals with those obtained in fasting animals (Table II) indicated that food did not affect the apparent onset, duration, or rate of drug absorption from the corn oil emulsion (Table III). In addition, the presence of food did not significantly alter the maximum plasma levels, the bioavailability, or the apparent elimination rate constant of the antibiotic from the corn oil emulsion (Table III).

The mechanism underlying the enhanced absorption of micronized griseofulvin after single- and multiple-dose administration to rats as a lipid-in-water emulsion will be the subject of a subsequent report.

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[x] To whom inquiries should be directed.

Succinylsulfathiazole Crystal Forms II: Effect of Additives on Kinetics of Interconversion

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Abstract □ The effect of various additives on the rate of transformation of the metastable anhydrous succinylsulfathiazole Form I to the water-stable dihydrate Form II in aqueous suspensions was studied. Some structurally related compounds, viscosity-imparting agents, surfactants, and coloring agents were used as possible transformation retardants. The effect of including seeds of Form II in the presence and absence of additives is also discussed. Some additives, *e.g.*, methylcellulose and phthalylsulfathiazole, showed significant transformation-retarding effects. Other additives, *e.g.*, sulfanilamide and glycerin, increased the rate of transformation. Coloring agents had only slight effects. Utilization of the results in the formulation of physically stable aqueous suspensions of succinylsulfathiazole is discussed.

Keyphrases □ Succinylsulfathiazole—crystal forms, interconversion, effect of structurally related compounds, viscosity-imparting agents, surfactants, and coloring agents □ Crystal forms—succinylsulfathiazole, effect of additives on interconversion

Succinylsulfathiazole crystal forms, their preparation, characterization, interconversion, and kinetics of transformation under standard conditions, were previously described (1). It was concluded (1) that the formulation of physically stable aqueous suspensions of succinylsulfathiazole may be achieved in one of two ways. The first involves the use of Form II (the water-stable dihydrate), which does not undergo further transformation in suspension and, consequently, does not change in shape or size distribution, thus producing a stable suspension. The second involves the anhydrous Form I (frequently available commercially), provided that adequate measures are taken to prevent the transformation to Form II which results in physical instability. Stabilization of the metastable form (Form I) in aqueous suspension by the use of some formulation additives is the subject of the present report.

The use of various additives to stabilize drug polymorphs has already been reported (2–6). These additives included structurally related compounds, viscosity-imparting materials (hydrocolloids), surfactants, and coloring agents. The present study is concerned with effects of representative examples of these additives, the suspension concentration of Form I, and seeding with Form II on the rate of transformation of the anhydrous succinylsulfathiazole Form I to the dihydrate Form II in aqueous suspension.

EXPERIMENTAL

Materials and Apparatus—Succinylsulfathiazole[1] Forms I and II were obtained as micronized commercial samples. Sulfanilamide, sulfathiazole, phthalylsulfathiazole, methylcellulose, acacia, glycerin, syrup, polysorbate 80, fluorescein sodium, and amaranth, all of USP, BP, or BPC grade, were used. Yellow No. 4[2] and Bordeaux B (BPC 1949) were also used.

IR measurements were made with a double-beam grating spectrophotometer[3].

[1] Courtesy of Chemical Industries Development, Guiza, Egypt.
[2] Lebensmittel, D.F.G., Germany.
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Succinylsulfathiazole Crystal Forms II: Effect of Additives on Kinetics of Interconversion

A. R. EBIAN, M. A. MOUSTAFA, SAID A. KHALIL [x], and M. M. MOTAWI [†]

Abstract □ The effect of various additives on the rate of transformation of the metastable anhydrous succinylsulfathiazole Form I to the water-stable dihydrate Form II in aqueous suspensions was studied. Some structurally related compounds, viscosity-imparting agents, surfactants, and coloring agents were used as possible transformation retardants. The effect of including seeds of Form II in the presence and absence of additives is also discussed. Some additives, *e.g.*, methylcellulose and phthalylsulfathiazole, showed significant transformation-retarding effects. Other additives, *e.g.*, sulfanilamide and glycerin, increased the rate of transformation. Coloring agents had only slight effects. Utilization of the results in the formulation of physically stable aqueous suspensions of succinylsulfathiazole is discussed.

Keyphrases □ Succinylsulfathiazole—crystal forms, interconversion, effect of structurally related compounds, viscosity-imparting agents, surfactants, and coloring agents □ Crystal forms—succinylsulfathiazole, effect of additives on interconversion

Succinylsulfathiazole crystal forms, their preparation, characterization, interconversion, and kinetics of transformation under standard conditions, were previously described (1). It was concluded (1) that the formulation of physically stable aqueous suspensions of succinylsulfathiazole may be achieved in one of two ways. The first involves the use of Form II (the water-stable dihydrate), which does not undergo further transformation in suspension and, consequently, does not change in shape or size distribution, thus producing a stable suspension. The second involves the anhydrous Form I (frequently available commercially), provided that adequate measures are taken to prevent the transformation to Form II which results in physical instability. Stabilization of the metastable form (Form I) in aqueous suspension by the use of some formulation additives is the subject of the present report.

The use of various additives to stabilize drug polymorphs has already been reported (2–6). These additives included structurally related compounds, viscosity-imparting materials (hydrocolloids), surfactants, and coloring agents. The present study is concerned with effects of representative examples of these additives, the suspension concentration of Form I, and seeding with Form II on the rate of transformation of the anhydrous succinylsulfathiazole Form I to the dihydrate Form II in aqueous suspension.

EXPERIMENTAL

Materials and Apparatus—Succinylsulfathiazole[1] Forms I and II were obtained as micronized commercial samples. Sulfanilamide, sulfathiazole, phthalylsulfathiazole, methylcellulose, acacia, glycerin, syrup, polysorbate 80, fluorescein sodium, and amaranth, all of USP, BP, or BPC grade, were used. Yellow No. 4[2] and Bordeaux B (BPC 1949) were also used.

IR measurements were made with a double-beam grating spectrophotometer[3].

[1] Courtesy of Chemical Industries Development, Guiza, Egypt.
[2] Lebensmittel, D.F.G., Germany.
[3] Perkin-Elmer model 237-B.

Table I—Effect of Additives on the Rate of Transformation of Succinylsulfathiazole Form I in Aqueous Suspension at 40°

Additive	Concentration, % w/v	Lag Time, min	$K \times 10^4$, min^{-1}
Standard	—	0	40.30
Sulfanilamide	0.1[a]	0	78.75
Sulfathiazole	0.1[a]	90	18.59
Phthalylsulfathiazole	0.1[a]	210	12.55
Glycerin	20[b]	0	65.24
Syrup	50[b]	0	40.30
Acacia	2	0	27.18
Polysorbate 80	1.0	0	103.60
Polysorbate 80	0.5	0	85.42
Polysorbate 80	0.05	0	63.49
Polysorbate 80	0.005	0	40.30
Amaranth	0.005	0	42.42
Yellow No. 4	0.005	0	48.48
Fluorescein sodium	0.005	0	54.53
Bordeaux B	0.005	0	50.30

[a]Corresponding to 10% (w/w) of the total solid succinylsulfathiazole in suspension. [b]Percent v/v.

Methods—Aqueous suspensions of succinylsulfathiazole Form I (1% w/v), each containing a known quantity of one additive, were prepared. The suspensions were kept in a thermostated water bath at 40 ± 0.1°. Samples were withdrawn at various time intervals and filtered, and the concentration of Form I in the solid phase was determined as previously described (1). A temperature of 40° was adopted since, as previously shown (1), transformation was rather slow at room temperature.

Acceleration of the transformation of succinylsulfathiazole Form I, in the presence of phthalylsulfathiazole as an additive, was carried out. For this purpose, 1% (w/v) suspensions of this form, containing 10% (w/w) of phthalylsulfathiazole in succinylsulfathiazole, were kept at 55, 70, and 85 ± 0.1°. The suspensions were assayed periodically for Form I as before.

Seeding experiments were carried out to evaluate the effect of including nuclei of the water-stable succinylsulfathiazole Form II on the rate of transformation of Form I. For this purpose, 5% (w/w) of Form II in Form I was used in place of Form I alone in the presence and absence of some additives.

The effect of the suspension concentration on the rate of transformation of succinylsulfathiazole Form I was studied by measuring the rate in suspensions containing 1, 2, 5, and 10% (w/v) of Form I.

RESULTS AND DISCUSSION

The inhibitory effect of the various additives on the rate of transformation of succinylsulfathiazole Form I, as previously described (6), is manifested by the appearance of a lag period during which the transformation is very slow (almost negligible) and/or obvious rate retardation. The lag period may be interpreted (6) as being a measure of the time needed to produce, through transformation of small particles of Form I, an adequate number of nuclei of the water-stable Form II, which is presumably a prerequisite for the transformation to take place under steady-state conditions (when the various factors affecting the system come to an equilibrium state). Table I summarizes the effect of various additives on the rate of transformation of succinylsulfathiazole Form I in aqueous suspension.

The effect of structurally related compounds on the rate of transformation of succinylsulfathiazole Form I is shown in Fig. 1. Sulfathiazole and phthalylsulfathiazole showed lag periods of 90 and 210 min, respectively. These compounds, being similar in structure to succinylsulfathiazole, are effective transformation retardants in a manner which is probably related to the ease of fit of their molecules to developing nuclei of the water-stable form (Form II).

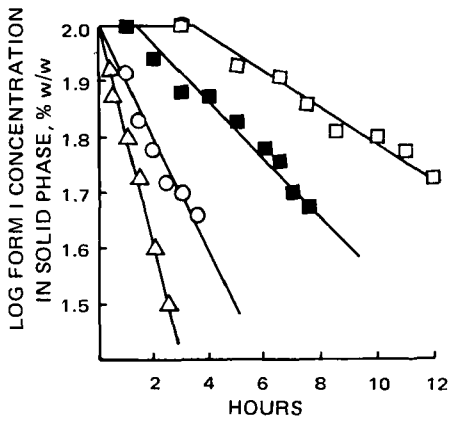

Figure 1—*Effect of structurally related compounds on the rate of transformation of succinylsulfathiazole Form I to Form II at 40°. Key: △, sulfanilamide; ○, standard; ■, sulfathiazole; and □, phthalylsulfathiazole.*

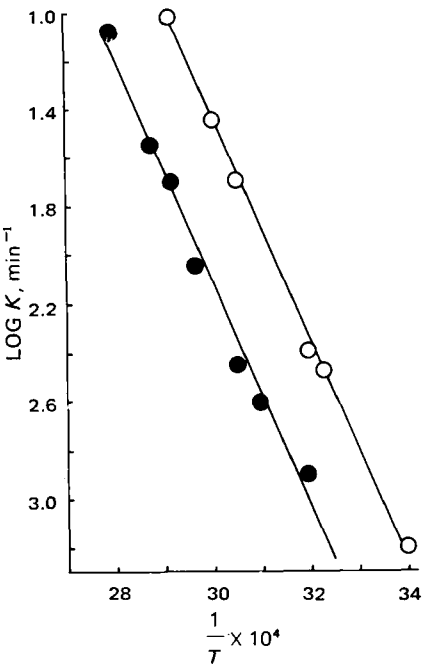

Figure 2—*Arrhenius plot of the transformation of succinylsulfathiazole Form I to Form II. Key: ○, standard; and ●, in the presence of 0.1% (w/v) phthalylsulfathiazole.*

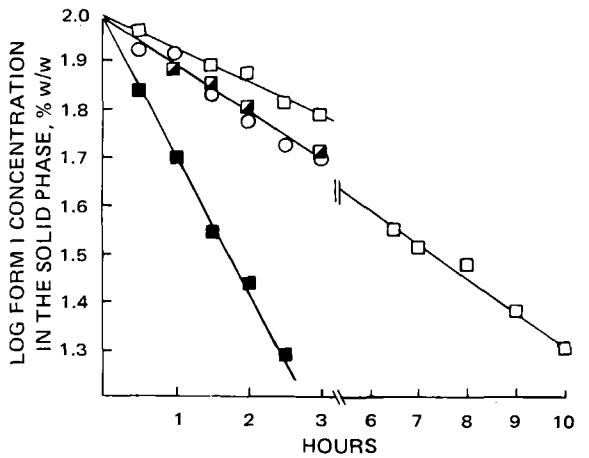

Figure 3—*Effect of viscosity-imparting agents on the rate of transformation of succinylsulfathiazole Form I to Form II at 40°. Key: ■, glycerin, 20% (v/v); ○, standard; ◪, syrup, 50% (v/v); and □, acacia, 2% (w/v).*

Table II—Kinetic Parameters for the Transformation of Succinylsulfathiazole Form I in the Presence and Absence of Phthalylsulfathiazole in Aqueous Suspension

Additive	Rate Constant $K \times 10^4$, min^{-1}										$t_{1/2}$, 25°[a], hr	E_a, kcal/mole
	22°	37°	40°	50°	55°	60°	65°	70°	75°	85°		
Standard[b]	6.3	33.6	40.3	—	203.8	361.8	—	959.4	—	—	13.6	21.1
Phthalylsulfathiazole	—	—	12.6	24.6	35.5	—	87.1	200	282	836	66.4	21.2

[a] Determined by extrapolation. [b] Data from Ref. 1.

Unexpectedly, sulfanilamide increased the rate of transformation of Form I to Form II (Fig. 1). This finding may be interpreted by assuming that sulfanilamide molecules, instead of retarding the transformation rate as with other sulfonamides, probably acts as nuclei for the development of crystals of Form II. The smaller and simpler structure of a sulfanilamide molecule would allow for this behavior. The mechanism of action of structurally related compounds probably involves inhibition of the formation of nuclei of the water-stable form by fitting into developing sites of these nuclei, a mechanism that would require the close similarity of the chemical structure as well as a favorable spatial configuration (7).

Acceleration of the transformation of succinylsulfathiazole Form I to Form II in the presence of phthalylsulfathiazole was carried out at several elevated temperatures, and a comparison of the results with those obtained for a standard suspension of Form I containing no additives is shown in Table II. The Arrhenius plots of the data are shown in Fig. 2. It could be observed from Table II that, although the activation energy calculated from the Arrhenius plot (Fig. 2) of the reaction was not significantly affected by the presence of phthalylsulfathiazole, the half-life at 25° was increased about fivefold and the rate constants for the transformation at all temperatures employed were considerably decreased.

Seeding with nuclei of Form II in aqueous suspensions of succinylsulfathiazole Form I containing sulfathiazole or phthalylsulfathiazole eliminated the lag period, indicating that the lag period is really a measure of the time needed to produce an adequate number of nuclei. However, the transformation rate that followed was not significantly affected (Table III).

The effect of viscosity-imparting materials on retarding the transformation of succinylsulfathiazole Form I is shown in Fig. 3. Methylcellulose, in concentrations ranging from 0.05 to 1% (w/v), inhibited the transformation of Form I for periods of over a year at room temperature. Acacia (2% w/v) retarded the rate of transformation only slightly. Simple syrup (50% v/v) had practically no effect on the rate of transformation. Glycerin (20% v/v), on the other hand, accelerated the transformation rate, probably by improving the wetting and solvation of succinylsulfathiazole Form I in aqueous suspension. The transformation-retarding effect of various viscosity-imparting materials could be related to their suppressive effect on both nuclei formation and solvent-mediated growth of the nuclei formed. This effect is, however, less apparent at higher temperatures.

The effect of polysorbate 80 on the rate of transformation of succinylsulfathiazole Form I to Form II varied with temperature (Fig. 4). At room temperature (≃22°), the rate of transformation decreased about 10 times in the presence of polysorbate 80 (1% w/v). Experiments carried out at a higher temperature (40°), using different concentrations of polysorbate 80 (0.05, 0.5, and 1.0% w/v), showed an increase in the rate of transformation of about twofold. The increase in the transformation rate was found to be directly proportional to the concentration of polysorbate 80.

This behavior might be understood if one considers the opposing effects of a temperature increase and the inclusion of polysorbate 80. Polysorbate 80, at low temperatures, may form an interfacial barrier (6, 8) which retards the transformation rate. However, the increase in the rate of transformation at higher temperatures suggests that the retardation effects are either no longer present or being outweighed by other factors such as a solubilization effect (9, 10). Therefore, it would be expected that higher surfactant concentrations could accelerate the transformation rate at higher temperature conditions.

Coloring agents had a slight transformation-accelerating effect on succinylsulfathiazole Form I (Fig. 5). Coloring agents, because of their slight surface activity, probably improve the wetting of the crystal surfaces, thus accelerating the transformation.

Seeding aqueous suspensions of succinylsulfathiazole Form I with nuclei of the water-stable form (Form II) at various temperatures accelerated the rate of transformation of Form I to Form II. Seeding effects (Table IV and Fig. 6) were more pronounced at lower temperatures. It has already been shown (1) that a temperature increase had a marked transformation-accelerating effect. This entails the formation of a large number of nuclei in a relatively short time. Since, under high temperature conditions, an adequate number of nuclei is available, seeding with more nuclei of the water-stable dihydrate form would only have a slight effect, as shown by the present results.

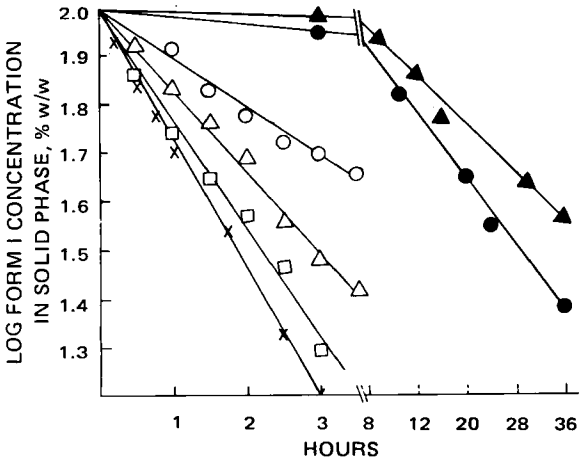

Figure 4—*Effect of polysorbate 80 on the rate of transformation of succinylsulfathiazole Form I to Form II. Key: □, 0.5% (w/v) polysorbate 80 (40°); ○, standard (40°); ●, standard (22°); △, 0.05% (w/v) polysorbate 80 (40°); ×, 1.0% (w/v) polysorbate 80 (40°); and ▲, 1% (w/v) polysorbate 80 (22°).*

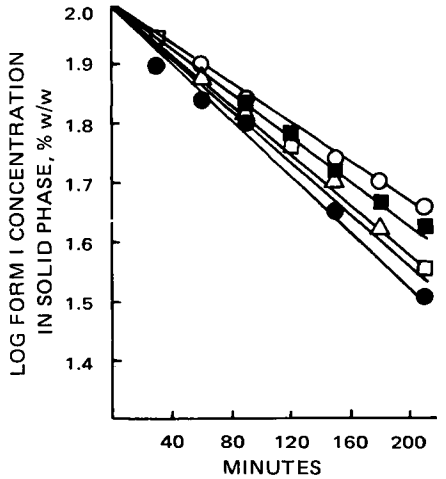

Figure 5—*Effect of coloring agents on the rate of transformation of succinylsulfathiazole Form I to Form II at 40°. Key: ●, fluorescein sodium; □, Bordeaux B; △, Yellow No. 4; ■, amaranth; and ○, standard.*

Table III—Effect of Seeding on the Rate of Transformation of Succinylsulfathiazole Form I to Form II in the Presence of Sulfathiazole and Phthalylsulfathiazole at 40°

Additive	$K \times 10^4$, min^{-1}	
	Unseeded	Seeded
Standard	40.30	154.44
Sulfathiazole	18.59	18.67
Phthalylsulfathiazole	12.55	13.60

Table IV—Effect of Seeding on the Rate of Transformation of Succinylsulfathiazole Form I to Form II in Aqueous Suspension at Various Temperatures

Suspension	$K \times 10^4$, min^{-1}		
	22°	40°	55°
Standard	6.33	40.30	203.82
Seeded	27.59	154.44	460.60

Seeding effects observed under conditions of the present study are not consistent with those previously reported (6) for sulfameter, where the transformation rate was unaffected by seeding. The discrepancy could be attributed to the rapid rate of transformation of the metastable polymorph of sulfameter ($t_{1/2}$ = 15 min at 25°) relative to the rather slow transformation of succinylsulfathiazole Form I ($t_{1/2}$ = 16 hr at 25°). The rapid rate of transformation in the sulfameter system provides, in a short time, an adequate number of nuclei of the water-stable form, so seeding effects are not apparent. The transformation of succinylsulfathiazole Form I, on the other hand, is accelerated when seeds of Form II are introduced, since the formation of nuclei under standard conditions is rather slow.

The effect of suspension concentration of succinylsulfathiazole Form I on the rate of transformation to Form II is shown in Fig. 7.

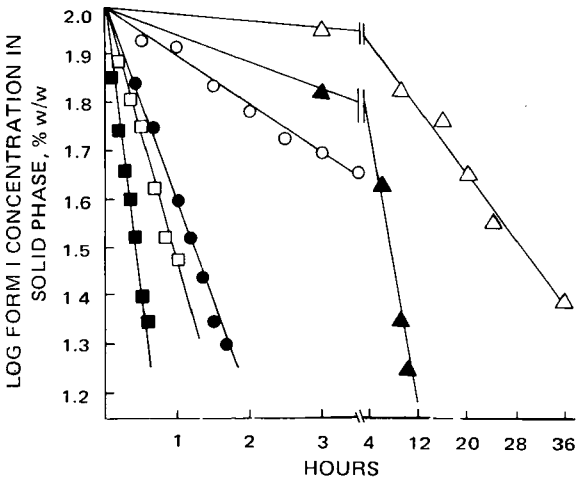

Figure 6—*Effect of seeding with nuclei of Form II on the rate of transformation of succinylsulfathiazole Form I under different temperatures. Key:* □, *55°;* ■, *55° seeded;* ○, *40°;* ●, *40° seeded;* △, *22°; and* ▲, *22° seeded.*

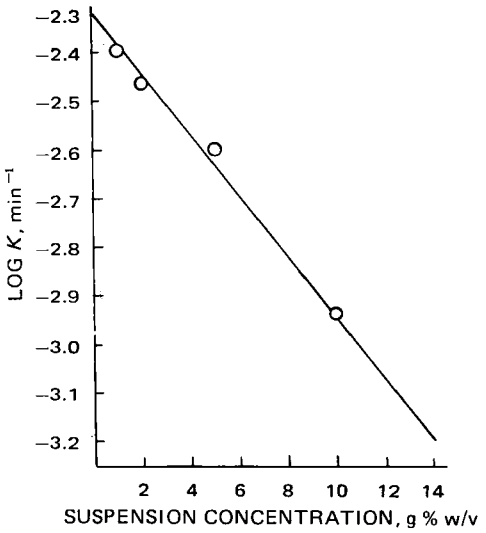

Figure 7—*Effect of suspension concentration of succinylsulfathiazole Form I on the rate of transformation to Form II.*

A linear decrease is observed when the log of the transformation rate constants is plotted against the suspension concentration.

According to the results of the present investigation, assuming the water-stable Form II is not available, the formulation of succinylsulfathiazole Form I in physically stable aqueous pharmaceutical suspensions may be achieved by including a suitable transformation retardant, *e.g.*, methylcellulose. Under these conditions, the suspension would keep its uniformity and ease of resuspension for the expected shelflife of the preparation.

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Succinylsulfathiazole Crystal Forms III: Crystal Growth Studies

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Abstract □ Crystal growth accompanying the transformation of succinylsulfathiazole crystal forms in aqueous suspensions was studied using a projecting microscope. The effects of increase of temperature, agitation, inclusion of seeds of Form II (the water-stable dihydrate), sulfathiazole, methylcellulose, and polysorbate 80 on the particle-size distribution of anhydrous succinylsulfathiazole Form I were examined. Rates of crystal growth, calculated as increase of diameter per unit time, were given under different experimental conditions. Increase of temperature, agitation, and seeding with nuclei of Form II had significant growth-accelerating effects. Sulfathiazole and polysorbate 80 had growth-retarding effects. Methylcellulose inhibited the crystal growth of Form I for over a year. Aqueous suspensions of Form II did not show any change in particle-size distribution. The crystal growth was shown to be a direct consequence of the transformation of the crystal form. Physical conditions and additives which had accelerating or retarding effects on the rate of transformation of Form I had similar effects on the crystal growth rate of succinylsulfathiazole in aqueous suspensions.

Keyphrases □ Succinylsulfathiazole—crystal growth, effect of temperature, agitation, seeding, methylcellulose, and polysorbate 80, particle-size distributions □ Crystals—succinylsulfathiazole, factors affecting growth, particle-size distributions □ Transformations, crystal—succinylsulfathiazole, various factors affecting growth, particle-size distributions

Succinylsulfathiazole crystal forms, their preparation, characterization, and kinetics of interconversions in the presence and absence of some additives, were described previously (1, 2). These reports noted that crystal growth, caking, and the formation of cement-like precipitates in aqueous suspensions of succinylsulfathiazole were consequences of the transformation of metastable crystal forms to a water-stable form.

Changes in particle-size distribution accompanying transformation of crystal forms of various drugs have been described. Studies on methylprednisolone (3), cholesterol (4, 5), cortisone acetate (6, 7), an experimental antihypertensive compound (8), oxyclozanide (9), theophylline (9), and the antiviral compound SK & F-30097 (10) are some examples.

The present work is concerned with a quantitative study of the changes in particle-size distribution accompanying the transformation of anhydrous succinylsulfathiazole Form I to the dihydrate water-stable Form II in aqueous suspension. The effects of some physical factors, *e.g.*, temperature and agitation, on the crystal growth rate are examined. The effects of including various additives, *e.g.*, seeds of Form II, sulfathiazole, methylcellulose, and polysorbate 80, are also investigated.

A correlation of the results of this study with those of the kinetic studies previously reported (1, 2) would help to evaluate the possible use of metastable crystal forms in aqueous pharmaceutical suspensions and its consequences on the physical stability of the suspensions produced.

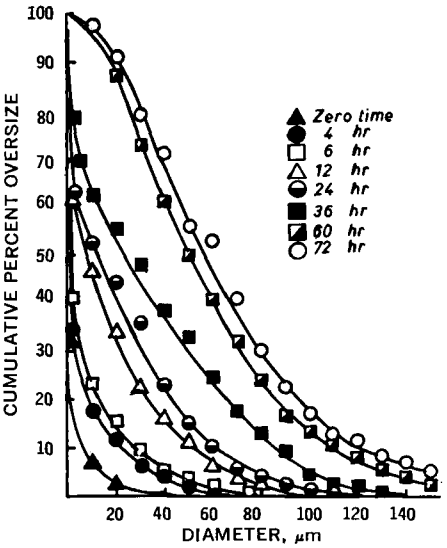

Figure 1—*Change in particle-size distribution with time of aqueous suspension of succinylsulfathiazole Form I at 22°.*

EXPERIMENTAL

Materials and Apparatus—Succinylsulfathiazole Forms I and II were obtained as micronized commercial samples[1], and their identity was checked by IR spectrophotometry (1). Sulfathiazole BPC, methylcellulose, and polysorbate 80 (USP grade) were used.

A projecting microscope[2] was used for particle-size analyses. IR spectra were determined with a double-beam grating spectrophotometer[3].

Methods—Micronized succinylsulfathiazole Form I (0.3 g) was thoroughly mixed with a few drops of distilled water. The smooth paste produced was then gradually diluted with water to produce a 1% (w/v) suspension. The bottle containing the suspension was kept undisturbed in a thermostated water bath maintained at 22 ± 0.1°.

At various time intervals, the suspension was briefly shaken, a drop was transferred to a microscope slide, and the projected diameter was measured for about 100 particles. The particle diameters were classified into 5-μm size range intervals. The procedure was repeated with 5–10 drops of the suspension, taken at the same time. The total counting time varied from 30 to 40 min.

The effect of temperature was examined by repeating this procedure, with the suspension immersed in a thermostated water bath at 37 ± 0.1°.

To investigate the effect of agitation, bottles containing suspensions, prepared as previously described, were rotated at 100 rpm in thermostated water baths at 22 ± 0.1 and 37 ± 0.1° in separate experiments. Sampling was carried out as before.

An experiment was carried out to investigate the effect of seeding aqueous suspensions of succinylsulfathiazole Form I with (10% by weight of the solid phase) Form II on the rate of crystal growth at 22 ± 0.1°.

[1] Courtesy of Chemical Industries Development, Guiza, Egypt.
[2] Reichert, Austria.
[3] Perkin-Elmer model 237-B.

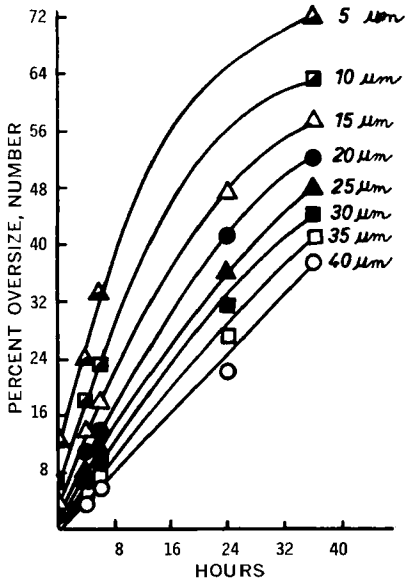

Figure 2—*Change in cumulative count with time of succinylsulfathiazole suspension at 22°.*

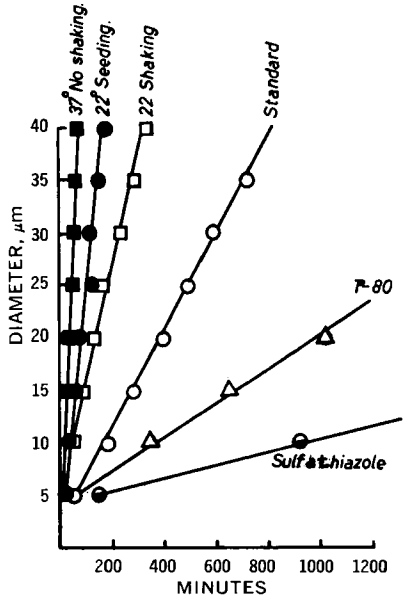

Figure 4—*Comparison of rates of growth above 15% cumulative count.*

The effect of additives, namely sulfathiazole (10% by weight of the solid phase), methylcellulose (0.5% w/v), and polysorbate 80 (1% w/v), on the rate of crystal growth of succinylsulfathiazole Form I in aqueous suspensions at 22 ± 0.1° was examined in separate experiments.

Succinylsulfathiazole Form II was suspended, in place of Form I, in water, and the particle-size distribution was determined as previously described at various time intervals.

IR spectrophotometry (1, 2) was used to follow various stages in the transformation of Form I to Form II in aqueous suspensions under the different experimental conditions adopted.

RESULTS AND DISCUSSION

Particle-size distribution data of anhydrous succinylsulfathiazole Form I in aqueous suspension at 22°, plotted as cumulative oversize frequency percentage *versus* diameter at various time intervals, are shown in Fig. 1. A marked horizontal displacement of the curves to the right with time, indicating crystal growth, was observed. The rate of crystal growth of Form I was derived by following the principles of Edmundson and Lees (11) as applied by Carless *et al.* (6, 7) and Ravin *et al.* (10) in their crystal growth studies.

For this purpose, a plot of the cumulative oversize frequency percentage *versus* time (Fig. 2) was established, from the data in Fig. 1, for various particle diameters. The increase in diameter with time at various cumulative oversize frequency percentages (obtained by following horizontal lines cutting the curves in Fig. 2 at the specified oversize frequency percentages) was then plotted (Fig. 3). The slopes of the lines in Fig. 3 represent the crystal growth rate as the increase of diameter per unit time—*viz.*, in micrometers per hour.

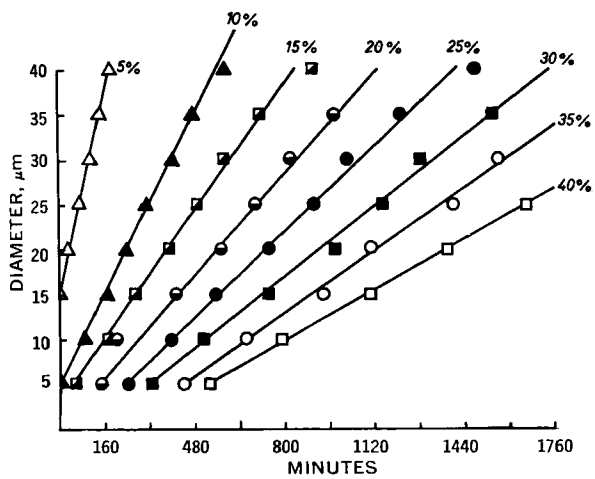

Figure 3—*Rates of growth of aqueous suspension of succinylsulfathiazole Form I at 22°.*

Values of the crystal growth rates at various cumulative oversize frequency percentages are shown in Table I. The rate of crystal growth decreased 5-7 times when the cumulative oversize frequency percentage level at which measurements were made was varied from 5 to 35. IR examination of the solid phase with time showed that a simultaneous transformation of anhydrous succinylsulfathiazole Form I to the dihydrate Form II in aqueous suspension accompanied crystal growth.

Increasing the temperature (37°) was found to increase the rate of crystal growth of succinylsulfathiazole Form I in aqueous suspension about 10 times (Table I). It was previously shown (1, 2) that an increase in temperature increased the rate of transformation of Form I to Form II in aqueous suspension. This would, in turn, lead to the production of the required number of nuclei of Form II necessary for a steady-state transformation (12) in a shorter period.

Since crystal growth was also observed (1) to be a direct consequence of the transformation of crystal form, it is not surprising to find that the rate of crystal growth was increased by an increase of temperature. Furthermore, increased temperature accelerates the diffusion-controlled processes of solution and deposition on the nuclei of the water-stable Form II (13, 14), thus increasing the proportion of large size particles.

Agitation at 100 rpm increased the rate of crystal growth of succinylsulfathiazole Form I in aqueous suspension (Table I). Both nuclei formation and deposition on the nuclei of the water-stable Form II formed involve diffusion-controlled processes (13, 14). Acceleration of the latter processes with agitation is responsible for the crystal growth observed. The effect of agitation becomes negligible at higher temperatures (37°) as the increase in the rate of crystal growth by an increase in temperature largely outweighs the effect of agitation (Table I).

Seeding an aqueous suspension of succinylsulfathiazole Form I with nuclei of the water-stable Form II increased the crystal growth rate (Table I). By increasing the number of nuclei of the water-stable Form II, seeding produced an approximately fourfold increase in the rate of crystal growth.

The presence of sulfathiazole in an aqueous suspension of succinylsulfathiazole Form I decreased the rate of crystal growth approximately 10 times (Table I). This might be explained by the possible fit of sulfathiazole molecules to specific sites in the developing nuclei of succinylsulfathiazole Form II, which results in a re-

Table I—Rate of Crystal Growth as Increase of Diameter per Unit Time at Various Cumulative Oversize Frequency Percentages of Aqueous Suspensions of Succinylsulfathiazole Form I under Different Conditions

	Rate of Growth, μm/hr						
	5%	10%	15%	20%	25%	30%	35%
Standard suspension (22°)	7.89	3.75	2.62	2.16	1.74	1.50	1.27
Standard suspension (37°)	70.80	48.00	31.74	25.09	20.88	16.92	14.46
Seeding (10% Form II, 22°)	30.00	17.50	13.35	11.75	9.85	7.40	6.23
Agitation (100 rpm, 22°)	16.99	9.00	6.76	5.33	4.50	3.67	2.85
Agitation (100 rpm, 37°)	70.80	42.90	27.30	22.20	18.40	15.42	12.49
Polysorbate 80 (1% w/v, 22°)	3.17	1.60	0.80	—	—	—	—
Sulfathiazole (10%, 22°)	0.78	0.45	0.30	—	—	—	—

duction of their number in addition to a decrease in their growth rate.

Polysorbate 80, by imposing an interfacial barrier (3, 15), reduced the rate of transformation of Form I to Form II in aqueous suspension and, consequently, decreased the crystal growth rate (Table I).

Methylcellulose inhibited the transformation of succinylsulfathiazole Form I to Form II in aqueous suspension for over a year (2). Consequently, little change in particle-size distribution was observed in the presence of methylcellulose.

Succinylsulfathiazole Form II, being stable in aqueous suspension (1), did not exhibit any measurable changes of particle-size distribution with time.

A comparison of the effect of various experimental conditions on the rate of crystal growth of succinylsulfathiazole Form I in aqueous suspension is shown in Fig. 4. An increase in temperature had the greatest growth-accelerating effect. Seeding and agitation also had marked, but weaker, growth-accelerating effects. Greater growth-retarding effects could be observed in the presence of sulfathiazole as compared to polysorbate 80. However, methylcellulose was the most effective growth retardant. In all cases, the rate of growth, calculated as an increase in diameter per unit time, was found to be greater the smaller the cumulative oversize frequency percentage level at which measurement was made.

An attempt was made to correlate the rate of crystal growth to the particle diameter of the growing crystals. The plot in Fig. 2 shows that smaller particles grew to bigger size particles at lower cumulative oversize frequency percentage levels than they did at higher levels; *i.e.*, there would be a greater proportion of large size particles at the lower frequency levels. Since rates of growth were found to be greater at lower frequency levels (where larger particles predominate), it follows that the rate of crystal growth is directly proportional to the particle diameter. This result is in agreement with the findings (6, 7) for cortisone acetate and represents an example of the third case of the crystal growth patterns described by Higuchi and Lau (3), for which they found no simple physical model.

The significance of the change in count of various particle diameters was evaluated by plotting absolute frequency percentage *versus* time curves. This type of plot (Fig. 5) was derived from the cumulative oversize frequency percentage *versus* diameter curves (Fig. 1) following the method of Carless *et al.* (6, 7). Figure 5 shows that there were a decrease in count of smaller particles (3.5 μm) with time, an increase followed by a decrease in count of intermediate-size particles (*e.g.*, 7.5 and 12.5 μm), and a progressive increase in count of larger particles (*e.g.*, 22.5 and 72.5 μm).

The decrease in count with time could be attributed to a predominant dissolution of smaller particles (15), whereas the increase in count represents predominant crystal growth. Thus, small particles are the first to go into solution, and larger particles grow at the expense of the dissolving small ones. Particles of intermediate size grow at first, as a result of the dissolution of smaller particles, until they become relatively the smaller particles in the distribution. Then they start to dissolve to give way for the growth of still larger particles. The point of inflection observed in the curves representing intermediate-size particles (*e.g.*, 12.5 μm) might be taken to mean that the two processes of dissolution and crystal growth reach a state of equilibrium.

A comparison of the curves representing the change in frequency percentage of the 12.5-μm diameter particles under various experimental conditions is shown in Fig. 6. The inflection in these curves took place at different time values. In cases where no growth retardants were present, the point of inflection corresponded to about one-third the transformation of Form I to Form II [as determined by IR analysis (2)]. Since the transformation of a crystal form is known to be a prerequisite for crystal growth, it might be assumed that a transformation of about one-third of Form I to Form II would provide the necessary driving force for the predominant crystal growth in aqueous suspensions of Form I.

The transformation-retarding additives, sulfathiazole and polysorbate 80, by interfering at the interface of dissolving and growing sites of both crystal forms, make it necessary for at least a two-thirds transformation of Form I to take place (as determined by IR analysis) before the inflection indicating predominant crystal growth may be observed (Fig. 6).

Another interesting result of the crystal growth studies of succinylsulfathiazole Form I in aqueous suspension was that the physical conditions and additives employed not only affected the crystal growth rate but also the time at which a fixed proportion of the crystal form had been changed, the time at which equilibrium particle size distribution was attained, and the maximum particle size

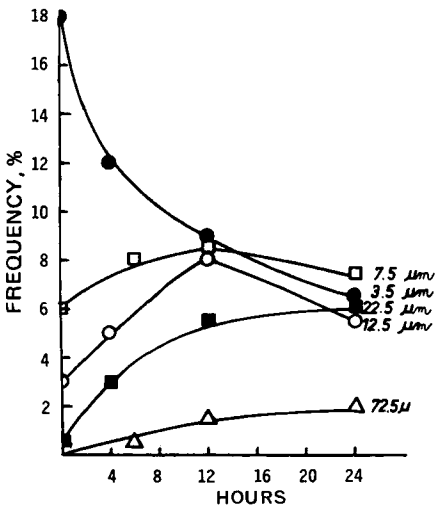

Figure 5—*Change in frequency percentage with time of aqueous suspension of succinylsulfathiazole Form I at 22°.*

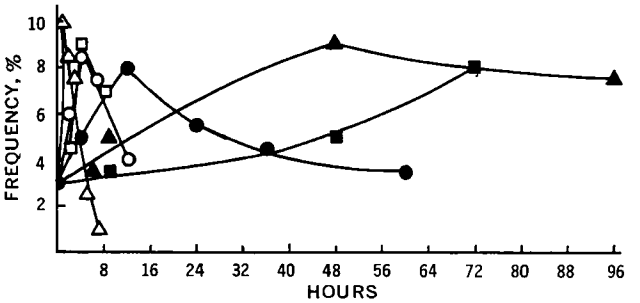

Figure 6—*Change in frequency percentage of 12.5-μm particles with time of aqueous suspensions of succinylsulfathiazole Form I at 22° under different conditions. Key: △, 37°; ▲, polysorbate 80; □, seeding at 22°; ■, sulfathiazole (10% w/w) at 22°; ○, 22° (shaking); and ●, standard at 22°.*

Table II—Effect of Various Experimental Conditions on Transformation of Crystal Form and the Time and Extent of Crystal Growth in Aqueous Suspension of Succinylsulfathiazole Form I

Condition	Time at Inflection in Count of 12.5-μm Particles	Time Required to Attain Equilibrium Particle-Size Distribution	Cumulative Oversize Frequency Percentage (100-μm Diameter)
Standard suspension of Form I (22°)	12	72	17
Standard suspension (37°)	1	7	25
Seeding (10% Form II)	4	10	3.5
Agitation (100 rpm)	4	12	2.5
Polysorbate 80 (1% w/v)	48	72	5
Sulfathiazole (10%)	72	96	1

reached. Table II shows that when the additive had an accelerating effect on the transformation of crystal form, a reduction in the time necessary to achieve the equilibrium particle-size distribution and an increase in the number of relatively larger size particles (100 μm) were also observed and vice versa.

Unexpectedly, seeding and agitation, although increasing the crystal growth rate markedly, did not lead to the production of a high proportion of large particles (100 μm). This finding might be attributed to an increase in the number of nuclei of the water-stable Form II under these conditions. Since the amount of solid material in suspension is roughly constant throughout the experiments, distribution of the solid material on the large number of nuclei present results in the production of only fewer numbers of large particles.

An increase in temperature, on the other hand, accelerated the transformation of crystal form and crystal growth and also produced a relatively high proportion of the 100-μm particles (Table II). It could be assumed that, although an increase in temperature accelerated the transformation of crystal form, the number of nuclei required for a steady-state transformation (2, 12) was not increased under these conditions. These nuclei grew, therefore, to larger size particles.

As a result of the foregoing discussion, the mechanism of crystal growth is thought to consist of two stages. The first involves transformation of the metastable crystal form to the water-stable form. The second stage involves growth of formed nuclei of the latter form. Evidence has been accumulating that the rate-controlling step in the crystal growth process is the change of crystal form. When this was inoperative, *e.g.*, when the water-stable form was suspended in water, no growth was observed. When the transformation of crystal form was retarded, crystal growth was also inhibited (*e.g.*, in the presence of methylcellulose, sulfathiazole, and polysorbate 80).

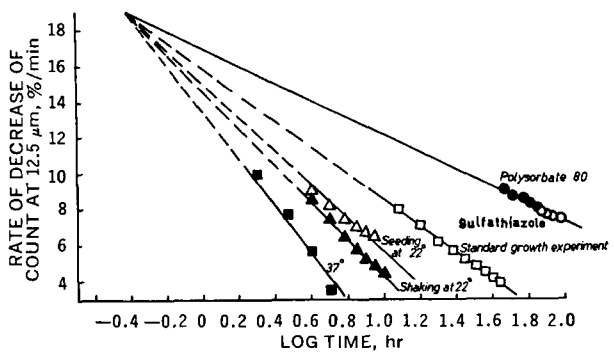

Figure 7—*Comparison of rates of dissolution of succinylsulfathiazole under different conditions.*

Table III—Effect of Experimental Conditions on the Rate of Transformation of Crystal Form and Crystal Growth in Aqueous Suspension of Succinylsulfathiazole Form I

Condition	K[a] Transformation × 10^4, min^{-1}	K Growth (10% Cumulative Oversize Frequency)
Standard suspension (22°)	6.33	3.75
Standard suspension (37°)	33.60	48.00
Seeding (10% Form II)	27.59	17.50
Polysorbate 80 (1% w/v)	4.99	1.60

[a] Data from Ref. 2.

On the other hand, when the transformation of the crystal form was fast or was increased by altering the prevailing physical conditions (*e.g.*, by increasing temperature or agitation), the crystal growth that followed was also quite marked. Seeding effects are also further evidence that the rate-controlling step is the transformation of crystal form. Thus, the inclusion of seeds of the water-stable Form II in aqueous suspensions of Form I increased not only the rate of transformation of crystal form (2) but also the crystal growth rate.

An attempt was made to find out which stage of the growth mechanism is affected by the various experimental conditions and additives employed. For this purpose, the rate of decrease in the count of the 12.5-μm particles, indicating predominant dissolution, *versus* time on a logarithmic scale was plotted (Fig. 7). Figure 7 shows that the rate of decrease in the count (a measure of the dissolution rate) at a very short time after the start of the experiments (about 0.4 hr), obtained by backward extrapolation, was practically the same under various experimental conditions. This finding might be interpreted by assuming that all experimental variables affected the transformation of crystal form and, consequently, the crystal growth of Form II. Form I, existing alone for a very short time, had a high dissolution rate which was not yet affected by the experimental variables. As time went on, transformation of Form I to Form II took place with variable velocities because of the experimental conditions prevailing. Since the developing water-stable Form II is about five times less soluble than Form I (16), the curves in Fig. 7 could be seen to deviate from each other as time progressed.

Table III shows that the rate of crystal growth under different conditions was increased or decreased in the same way as the rate of transformation of crystal form was affected. These arguments are, therefore, supporting evidence of previous conclusions and findings (1, 2) that experimental conditions affect the rate of transformation of Form I to Form II in aqueous suspension and, consequently, the crystal growth rate.

It might be concluded that, in agreement with previous recommendations (1, 2), the formulation of physically stable suspensions of succinylsulfathiazole would best be achieved by using water-stable Form II or, alternatively, including an efficient transformation retardant, *e.g.*, methylcellulose, with Form I. Attention must also be drawn to the incidence of polymorphism among pharmaceuticals as one important factor that could alter the physical stability of pharmaceutical preparations.

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Blood Concentration Profiles of Acetaminophen following Oral Administration of Fatty Acid Esters of Acetaminophen with Pancreatic Lipase to Dogs

CARL T. BAUGUESS ˣ, JULIAN H. FINCHER, FARID SADIK, and CHARLES W. HARTMAN †

Abstract □ Fatty acid esters of acetaminophen were administered orally to dogs, and blood concentrations of acetaminophen were determined at various time intervals. Blood concentrations of acetaminophen following oral administration of a short chain ester, *p*-acetamidophenyl acetate, were not significantly different from those found using acetaminophen. Blood concentrations of acetaminophen following oral administration of intermediate hydrocarbon chain-length compounds were less than those of the control at 1 and 3 hr postdosing. There appears to be a direct relationship between the *in vitro* hydrolysis rates and the blood concentration *in vivo*. Concomitant oral administration of acetaminophen derivatives, pancreatic lipase, and calcium salts resulted in an increase in the blood levels of acetaminophen as compared to administration of the esters alone. Calcium carbonate was included as a source of calcium ion to activate the lipase involved in the hydrolysis of the fatty acid esters. A combination of *p*-acetamidophenyl acetate, *p*-acetamidophenyl dodecanoate, pancreatic lipase, and calcium carbonate was shown to achieve a prolonged release of acetaminophen. *p*-Acetamidophenyl acetate was thought to provide the initial release of acetaminophen; *p*-acetamidophenyl dodecanoate, being hydrolyzed more slowly, provided the prolonged release, which maintained therapeutic blood concentrations for 13 hr following a single dose of the combination in dogs.

Keyphrases □ Acetaminophen—blood concentration profiles after oral administration of *p*-acetamidophenyl acetate and dodecanoate with pancreatic lipase, dogs □ Fatty acid esters of acetaminophen—acetaminophen blood concentration after oral administration with pancreatic lipase, dogs □ *p*-Acetamidophenyl acetate and dodecanoate—acetaminophen blood concentration following oral administration with pancreatic lipase, dogs

Timed release, sustained release, and prolonged action are popular terms for describing oral dosage forms that first release an initial dose and then gradually release a remaining quantity of drug sufficient to maintain a uniform therapeutic effect over an extended period (1).

The unique physiological and morphological features in the GI tract present both a challenge and an opportunity in the design of oral dosage forms. Although the physiological environment is an important factor in designing all dosage forms, many dosage forms are designed to operate in a particular set of biochemical conditions. For example, amide and ester derivatives are prepared frequently to alter the physical and chemical properties of a drug molecule to obtain a more acceptable dosage form. These chemical modifications often lead to pharmacologically inactive compounds; however, the presence of autogenous enzyme systems and/or other suitable conditions found in the various segments of the GI tract facilitate the hydrolysis and release of the active form.

Table I—Blood Concentration (Micrograms per Milliliter) of Acetaminophen following an Oral Dose of 20 mg/kg in Five Dogs

	Minutes				
Subject	30	90	150	240	330
D1	10.27	10.88	22.10	15.03	8.91
D2	28.16	20.74	18.84	14.15	10.68
D3	18.57	22.44	18.16	10.75	11.22
D4	16.46	22.44	24.01	21.56	12.51
D5	13.67	20.40	21.08	15.44	12.58
Average	17.43	19.38	20.84	15.39	11.18
±*SE*	3.02	2.17	1.07	1.75	0.66

Dittert *et al.* (2) found that esters of acetaminophen were substrates for serum lipase. Work in this laboratory indicates that, by controlling the chain length of the esters, it would be theoretically possible to impose a rate-limiting step prior to absorption of the drug from the GI tract, thereby achieving an extended duration of action from acetaminophen (3). Because of the insoluble nature of fatty acid esters of acetaminophen, it was not thought that any appreciable quantity of intact ester would be absorbed.

The objectives of this work were to investigate the feasibility of employing fatty acid ester derivatives of

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Table I—Blood Concentration (Micrograms per Milliliter) of Acetaminophen following an Oral Dose of 20 mg/kg in Five Dogs

	Minutes				
Subject	30	90	150	240	330
D1	10.27	10.88	22.10	15.03	8.91
D2	28.16	20.74	18.84	14.15	10.68
D3	18.57	22.44	18.16	10.75	11.22
D4	16.46	22.44	24.01	21.56	12.51
D5	13.67	20.40	21.08	15.44	12.58
Average	17.43	19.38	20.84	15.39	11.18
±*SE*	3.02	2.17	1.07	1.75	0.66

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The objectives of this work were to investigate the feasibility of employing fatty acid ester derivatives of

Table II—Blood Concentration (Micrograms per Milliliter) for Acetaminophen following an Oral Dose of 40 mg/kg in Six Dogs

Subject	Hours					
	1	3	5	7	10	13
D6	20.13	33.33	17.41	11.83	5.71	4.76
D7	27.21	17.00	6.39	2.99	0.68	0.54
D8	55.36	27.48	9.66	4.08	0.00	0.00
D9	55.90	45.30	20.13	7.21	3.81	0.68
D10	19.32	40.40	17.00	3.67	3.13	2.86
D11	58.22	39.45	22.44	9.39	2.18	0.95
Average	39.36	33.83	15.51	6.53	2.59	1.63
±*SE*	7.75	4.20	2.53	1.45	0.86	0.74

a drug in an enzyme substrate system to control the rate of absorption following oral administration and to establish a possible role for the concomitant administration of pancreatic lipase to provide a more uniform enzyme activity in the GI tract.

EXPERIMENTAL

Acetaminophen Assay in Blood—A 15.1-mg sample of acetaminophen[1], weighed on an analytical balance, was transferred to a 100-ml volumetric flask, and the flask was filled to the mark with distilled water. Aliquots of 0.5, 0.4, 0.3, 0.2, 0.1, 0.050, and 0.025 ml were diluted to 0.5 ml with distilled water and mixed with 0.5 ml of whole blood from pooled samples of blood drawn before the administration of acetaminophen. The removal of serum proteins and red blood cells was accomplished by a method used in the determination of blood sugar (4).

A 2.0-ml portion of 0.12 *N* barium hydroxide solution[2] was added, and the mixture was stirred[3]. After standing for about 2 min, a deep reddish-brown color developed. A 2.0-ml portion of 2% zinc sulfate solution[2] was added with mixing, resulting in the precipitation of red blood cells and serum protein. The samples were centrifuged at about 4000 rpm for 5 min. After filtration of the aqueous portion through a 0.8-μm filter[4], the absorbance of the clear aqueous filtrate of each sample was determined[5] against a reagent blood blank at 245 nm.

Protocol for *In Vivo* Studies—Groups of six to eight healthy, treated line-bred beagle dogs[6] were selected for the blood concentration studies. All food was withheld from the dogs for 24 hr prior to the beginning and during the experiment. The dogs were about 1 year in age and weighed between 9.1 and 13.6 kg (20 and 30 lb). All doses were calculated on a weight basis equivalent to 40 mg of acetaminophen/kg. This amount is comparable to a dose of 2.8 g for a human weighing 70 kg.

Control blood samples for all experiments were drawn from the cephalic vein of either front leg just prior to the administration of the dose. All doses were administered in hard gelatin capsules followed by 10 ml of water to force dogs to swallow the capsules. Water was available to the animals during the experiment. At 1-, 3-, 5-, 7-, 10-, and 13-hr intervals after drug administration, blood samples of about 2 ml were drawn from the cephalic vein(s) with a 2.5-ml disposable syringe, previously flushed with a heparin solution (10,000 USP units/ml)[7], using a 2.54-cm (1-in.), 22-gauge needle.

Immediately following the withdrawal of each blood sample, the needle was removed from the barrel of the syringe and the contents were delivered to clean glass vials. All blood samples were sealed and stored over ice until analyzed. No samples were stored longer than 24 hr, and most samples were prepared for analysis immediately.

Control studies for acetaminophen absorption were performed at two different dose levels to establish blood concentration–time profiles for the model drug compound. Five dogs were administered a dose of 20 mg of acetaminophen/kg. In this control study, blood samples were drawn at 30-, 90-, 150-, 240-, and 330-min intervals after drug administration. Six dogs were administered 40 mg of acetaminophen/kg, and blood samples were taken at 1-, 3-, 5-, 7-, 10-, and 13-hr intervals.

Table III—Blood Concentration (Micrograms per Milliliter) of Acetaminophen following an Oral Dose of *p*-Acetamidophenyl Acetate Equivalent to 40 mg of Acetaminophen/kg in Three Dogs

Subject	Hours					
	1	3	5	7	10	13
D12	36.59	41.76	13.31	5.03	2.45	0.41
D13	22.17	41.22	16.60	2.99	0.68	0.00
D14	36.59	27.21	13.60	4.90	2.31	1.36
Average	31.78	36.73	14.50	4.31	1.81	0.59

Table IV—Blood Concentration (Micrograms per Milliliter) of *p*-Acetamidophenyl Decanoate Equivalent to 40 mg of Acetaminophen/kg in Six Dogs

Subject	Hours					
	1	3	5	7	10	13
D15	2.85	11.56	9.79	4.90	4.35	4.08
D16	0.68	5.44	7.75	7.35	5.85	3.40
D17	1.22	9.52	11.15	5.44	2.45	1.63
D18	0.68	2.58	4.63	5.03	4.76	0.00
D19	5.03	9.66	9.93	6.39	6.94	2.45
D20	0.00	2.72	1.36	0.00	0.68	0.00
Average	1.75	6.91	7.44	4.85	4.17	1.93
±*SE*	0.77	1.58	1.53	1.04	0.93	0.70

The short chain ester derivatives of acetaminophen through the octanoate were administered without pancreatic lipase[8] or other additives. The intermediate length and long chain esters were studied both with and without lipase and other additives. When studied with lipase, these esters were mixed with the enzyme preparation and encapsulated in two or three No. 00 hard gelatin capsules. Lipase preparations were assayed prior to each experiment by the method of Lazo-Wasem (5). Capsules containing the long chain esters and lipase were stored in a refrigerator and/or over ice until administered.

Blood samples were analyzed by the procedure previously described. To 0.5 ml of blood, 0.5 ml of distilled water was added. A 2.0-ml portion of 0.12 *N* barium hydroxide solution was added and

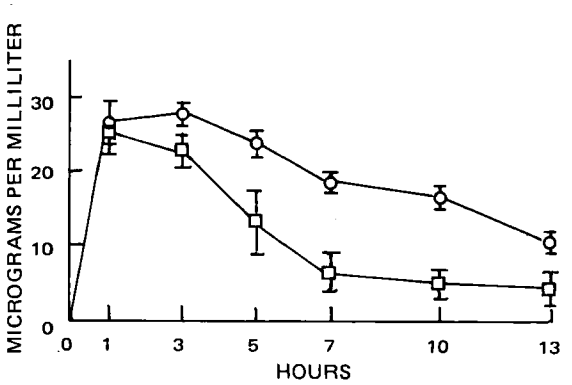

Figure 1—*Blood concentrations of acetaminophen after an oral dose of* p-*acetamidophenyl acetate, equivalent to 20 mg of acetaminophen, or* p-*acetamidophenyl dodecanoate, equivalent to 40 mg of acetaminophen, and 58.9 mg of lipase and 10 mg of calcium carbonate/kg in six dogs. Key:* ○, *average with standard error; and* □, *control average with standard error.*

[1] Eastman Organic Chemicals.
[2] Fisher Scientific Co.
[3] Vortex Jr. mixer, Scientific Products.
[4] Millipore, Millipore Corp.
[5] Beckman DU spectrophotometer, Beckman Instruments.
[6] Supplied by Dr. Joseph Martin, Ripley, Miss.
[7] Hyrex Co.
[8] Supplied by Rheis Chemical Co.

Table V—Blood Concentration (Micrograms per Milliliter) of Acetaminophen following an Oral Dose of *p*-Acetamidophenyl Decanoate Equivalent to 40 mg of Acetaminophen, 54 mg of Lipase, and 10 mg of Calcium Chloride/kg in Seven Dogs

Subject	Hours					
	1	3	5	7	10	13
D26	9.39	20.68	12.65	9.93	9.79	6.80
D27	8.84	22.17	12.24	11.83	5.03	4.35
D28	11.83	12.38	7.21	6.39	11.02	6.94
D29	7.07	20.68	16.05	13.06	8.84	5.17
D30	5.58	18.64	12.79	10.34	5.44	4.90
D31	8.16	19.59	10.07	8.16	7.89	4.63
D32	8.71	13.33	8.57	6.39	5.44	1.36
Average	8.51	18.21	11.37	9.44	7.64	4.88
±*SE*	0.74	1.45	1.11	0.98	0.90	0.70
Level of significance to control (Table IV)	$p < 0.01$	$p < 0.01$	$p < 0.1$	$p < 0.05$	$p < 0.05$	$p < 0.05$

mixed with the blood sample. After standing for 2 min, a 2.0-ml portion of a 2% zinc sulfate solution was added and mixed. The samples were centrifuged and filtered. The absorbance of the filtrates was determined against a blood reagent blank.

RESULTS AND DISCUSSION

Control studies for acetaminophen absorption were performed *in vivo* at two dose levels to establish blood concentration–time profiles. The doses of all animal studies were calculated on a weight basis. Five dogs were given an oral dose of 20 mg/kg as noted in Table I. This dose level produced an average blood concentration peak of 20.84 μg/ml after 2.5 hr. However, some individual animals exhibited a peak concentration level after 1 hr, and it was difficult to assess what happened between samples. After 5.5 hr, the concentration decreased to about half that of the peak concentration.

Since prolonged-release medication required a larger dose, a second control study was conducted at 40 mg of free acetaminophen/kg in six dogs; the results are given in Table II. In this study, a maximum concentration was observed after 1 hr and an exponential decay began at 3 hr and continued through a 10-hr period. A plot of the data on a semilogarithmic graph gave a linear decay, beginning at the 3-hr interval and continuing through the 10-hr interval. A half-life for blood concentration was determined graphically from the data in Table II and was found to be in the order of 1 hr and 40 min.

The availability of acetaminophen after oral administration and administration of its ester derivatives was determined by the following equation (6, 7):

$$\frac{A_T}{V_d} = C_T + K_e\,(AUC) \qquad \begin{matrix} t = T \\ t = 0 \end{matrix} \qquad \text{(Eq. 1)}$$

where A_T is the amount of drug absorbed up to time T, V_d is the distribution volume, C_T is the concentration at time T, K_e is the elimination rate constant, and AUC is the area under the curve. When the data in Table II were treated by this method, the half-life for absorption was found to be 40 min, and acetaminophen was more available when administered as the free drug.

Three dogs were given oral doses of *p*-acetamidophenyl acetate equivalent to 40 mg of acetaminophen/kg. A comparison of blood levels of acetaminophen shown in Table III with the control study presented in Table II indicates a slight increase in the half-life for absorption from 40 to 60 min, although there was only a slight change in the availability of acetaminophen with equivalent doses. Treatment of the data in Table II gave an $(A_T/V_d)_{max}$ of 75.3 μg/ml as compared to 71.5 μg/ml for the data in Table III. These estimates of relative availability most likely underestimate the true value.

From observations on the *in vitro* hydrolysis of acetaminophen derivatives, it was anticipated that intermediate and long chain esters would reduce the availability of acetaminophen (3). The study recorded in Table IV illustrates a reduced availability of aceta-

Table VI—Blood Concentration (Micrograms per Milliliter) of Acetaminophen following an Oral Dose of *p*-Acetamidophenyl Acetate, Equivalent to 20 mg of Acetaminophen, or *p*-Acetamidophenyl Dodecanoate, Equivalent to 40 mg of Acetaminophen, and 58.9 mg of Lipase and 10 mg of Calcium Carbonate/kg in Six Dogs

Subject	Hours					
	1	3	5	7	10	13
D85	19.04	33.33	27.34	17.00	17.00	13.33
D86	23.13	29.92	24.08	15.24	12.79	11.02
D87	28.02	27.75	20.13	16.60	16.46	13.33
D88	38.09	27.75	21.08	19.59	21.36	6.53
D89	27.89	25.17	28.56	17.68	16.60	8.84
D90	29.64	24.21	26.12	25.17	18.23	13.19
Average	27.64	28.02	24.55	18.55	17.07	11.04
±*SE*	2.62	1.35	1.39	1.44	1.18	1.14
	Controls[a]					
D91	23.13	23.81	18.36	8.71	10.07	12.24
D92	23.26	30.47	21.76	8.30	5.85	0.00
D93	30.06	29.79	19.04	15.04	11.97	11.29
D94	34.14	24.08	8.30	7.21	3.81	2.86
D95	21.90	15.37	6.39	0.27	0.00	1.36
D96	23.67	19.86	6.26	1.22	0.54	0.54
Average	26.03	23.90	13.35	6.79	5.37	4.72
±*SE*	2.00	2.36	4.38	2.26	2.00	2.30
Level of significance	$p < 0.70$	$p < 0.20$	$p < 0.01$	$p < 0.005$	$p < 0.001$	$p < 0.05$

[a] Inactive lipase.

minophen following oral administration of equivalent doses of *p*-acetaminophenyl decanoate. The availability of drug from this ester form was 34% of that produced by acetaminophen. Concomitant administration of *p*-acetamidophenyl decanoate, pancreatic lipase (3 Wilson units/mg), and calcium chloride increased the availability from 34 to 73% as calculated from the data reported in Table V.

To investigate the feasibility of using a combination of ester derivatives with pancreatic lipase and calcium ions to achieve a prolonged release of acetaminophen, *p*-acetamidophenyl acetate and *p*-acetamidophenyl dodecanoate, equivalent to 20 and 40 mg/kg of acetaminophen, respectively, were administered with 59 mg/kg of pancreatic lipase and 10 mg/kg of calcium carbonate. The acetate ester was included to provide the initial release, and the dodecanoate ester was selected because of its slower hydrolysis rate. The amounts of pancreatic lipase and calcium carbonate were determined to be adequate from preliminary studies.

The results of this combination using a short chain ester and an intermediate ester are shown in Table VI and in Fig. 1. At the 1-, 3-, and 5-hr intervals, the blood concentrations were somewhat higher than an average of 21 μg/ml; at 7 and 10 hr, they were slightly below. The control used in this study was identical to the experiment, except that the pancreatic lipase used in the dose was inactivated by moisture and heat. No statistical difference at a 90% confidence level was noted at the 1- and 3-hr intervals; however, at 5-, 7-, 10-, and 13-hr intervals, a significant difference was observed. The hydrolysis and subsequent absorption from *p*-acetamidophenyl dodecanoate were facilitated by the inclusion of lipase in the dose. The availability of acetaminophen was increased by a factor of 1.7 when the combination included the active enzyme preparation.

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Correlation of Kinetic Parameters and Thermal Behavior of Segmented Polyurethane Elastomers with Biological Responses

G. W. C. HUNG, L. J. NUNEZ, and J. AUTIAN ˣ

Abstract □ Kinetic studies of thermal degradation of 16 segmented polyether polyurethane samples, containing various amounts of 3,4-diaminotoluene and dibutyltin diacetate as additives, were carried out by thermogravimetry. From a single dynamic thermogravimetric experiment, the temperatures of initiation of degradation, 10, 25, and 50% (w/w) of degradation, as well as the activation energies for degradation, were determined. The activation energies were computed from the thermogravimetric curves using Broido's graphical approximation method, which applies to first-order decomposition kinetics. The results of stepwise multiple linear regression analysis indicate that the biological responses to elastomer samples, such as tissue culture, hemolysis, intramuscular implant, intradermal irritation, systemic toxicity, and histopathological rating, and the cumulative biological response index are highly correlated with thermal stability and kinetic measurements of the materials.

Keyphrases □ Segmented polyurethane elastomers—correlation of kinetic parameters and thermal behavior with biological responses □ Polyurethane elastomers, segmented—correlation of kinetic parameters and thermal behavior with biological responses □ Biomaterials, segmented polyurethane elastomers—correlation of kinetic parameters and thermal behavior with biological responses

Segmented polyether polyurethane elastomers demonstrate superior properties of strength and flex-life (1) and are excellent candidates for biomedical applications (2–9). These polymers consist of at least two major segments in the repeating chain structural unit: a crystalline *hard* segment (urea), melting above 200°, and a *soft* segment (polyether glycol), melting below 50° (10–12). Both segments are polymeric and connected by urethane linkages. The final product is termed a polyether/urethane/urea or a segmented polyurethane.

The chemistry and techniques involved in the production of these polymer materials are well known (13, 14). In addition, several investigations considered the chemical, physical, mechanical, and biological properties (5), the dependence of morphological structure on the chain characteristics and cast conditions (15), the hemolytic activity (16, 17), and the toxicity (18, 19) of the polymeric material relevant to biological applications. However, no systematic studies have been reported that compare and correlate physical properties with biological responses for segmented polyurethane systems containing different kinds and amounts of additives.

The purposes of this paper, therefore, are to consider one system of segmented polyurethanes containing various quantities of additives and to illustrate the correlation between the measured thermal

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The purposes of this paper, therefore, are to consider one system of segmented polyurethanes containing various quantities of additives and to illustrate the correlation between the measured thermal

stability and kinetic parameters with some biological responses[1]. The sample preparations were designed to give a wide range of biological responses and were not related to specific end-use conditions.

The segmented polyurethane elastomers used were prepared and cured from elastomer solution (1). The additives selected were 3,4-diaminotoluene (I) and dibutyltin diacetate (II). The amounts of I and II added to the polyurethane ranged from 0 to 6% (w/w), and the samples were not analyzed for I and II after preparation. Sixteen polyurethane samples containing various amounts of additives were prepared.

Six principal biological response tests, including tissue culture, hemolysis, intramuscular implant, intradermal irritation, systemic toxicity, and histopathological rating of the intramuscular implant site, were carried out in parallel on each preparation. The thermal stability and kinetic parameters of thermal degradation of the polyurethane samples were measured by thermogravimetry, using a dynamic (nonisothermal) degradation procedure under an inert nitrogen atmosphere. The correlations of the six principal biological responses with the overall activation energies for thermal degradation and other physical properties are discussed.

THEORETICAL

During the past few years, several methods using the nonisothermal approach have been developed to allow kinetic analysis of thermogravimetric data (20). The simple and sensitive approximation method proposed by Broido (21) was used in the present work. Broido's integral method applies only to first-order reactions, including the majority of simple pyrolyses (*i.e.*, for situations where the simple Arrhenius equation is applicable).

Modification of Broido's approach by using suitable assumptions and approximations allows a linear equation to be obtained. This equation is presumed to hold for uniform linear heating rates:

$$\log \log Z = -(E_a/2.303R)\,(1/T) + \text{constant} \qquad \text{(Eq. 1)}$$

where:

$Z = \Delta w_{\text{total}}/(\Delta w_{\text{total}} - \Delta w_t)$
Δw_{total} = total change in weight after completion of degradation
Δw_t = change in weight of active material at time, t

Thus, by applying Eq. 1, a plot of log log Z *versus* $1/T$ should yield a straight line if the reaction is first order. Then E_a can be evaluated from the least-squares slope. The calculations were performed by a computer.

EXPERIMENTAL

Materials—The starting material was a segmented polyether polyurethane polymer[2] offered as a 30% (w/w) solution in dimethylacetamide (III). Some physical properties of the elastomer solution are given in Table I.

Compounds I[3] and II[4] and the solvent III[5] were obtained commercially. All other solvents were reagent grade and were used without further purification. Table II lists some physical properties of I–III.

Apparatus and Conditions—The thermogravimetric instrument[6] consisted of a linear temperature programmer[7], a furnace[8], a thermogravimetric accessory[9], an RG electrobalance[10], and a potentiometric recorder[11]. The function of the systems were described previously (22, 23). Briefly, the experimental conditions set up for these studies were as follows: sample size, 10 ± 0.5 mg; electrobalance sensitivity, 0.01 mg; programming rate, 10°/min; recorder span, 10 mg full scale; chart speed, 20.3 cm (8 in.)/min; dynamic inert atmosphere, nitrogen gas, at a flow rate of 200 ml/min; and operational temperature range, room temperature to the completion of degradation (up to about 750°).

Table I—Physical Properties of Elastomer Solution

Physical Property	Description
Solubility	30% (w/w) solution of segmented polyether polyurethane in dimethylacetamide. The solution can be diluted with secondary solvents such as toluene, chlorobenzene, methylene chloride, chloroform, and tetrahydrofuran.
Viscosity	90,000–150,000 cps at 25°. The solution tends to increase in viscosity on standing. However, this viscosity increase can be reversed by stirring.
Density	0.96 g/cc
Flash point	70°
Hygroscopicity	It is extremely hygroscopic and must be protected from contact with atmospheric moisture.

Preparation of Polyurethane Samples—*Without Additives*—The elastomer solution was poured onto a glass plate, and the solvent was allowed to evaporate over a warming plate at 68° for 22 hr.

With Additive II Only—Compound II was added by pipet to the elastomer solution with continuous stirring. After thorough mixing, the mixture was poured onto a warming plate and the solvent was evaporated for 21 hr at 68°.

With Both I and II Additives—Compound II was added by pipet to the elastomer solution with continuous stirring. Compound I was dissolved in the elastomer solvent III and then added to the mixture with continuous stirring. After thorough mixing, the product was poured onto a warming plate and the solvent was evaporated for 20.5 hr at 68°.

With Additive I Only—Compound I was dissolved in III and then added to the elastomer solution with continuous stirring. After thorough mixing, the solution was poured onto a warming plate and the solvent was evaporated for 20.5 hr at 68°.

In general, an evaporation time of 20–22 hr at 68° was sufficient to obtain a firm nonsticky film product.

Measurement of Density of Elastomer Samples—The density of elastomer films was measured at 25 ± 0.1° by a water-displacement method using a pycnometer. The method of calculation is similar to that presented previously (23).

Methanol Extraction (% RW)—Methanol was selected for the extraction of components from elastomer samples. The samples were cut into small pieces and exposed to 150 ml of methanol for 24 hr at room temperature with continuous stirring. The results are reported as percent residue weight (% RW).

Biological Response Tests—The six biological response tests employed for the evaluation of the formulations follow.

Hemolysis Assay (H)—Hemolytic activity was determined by spectrophotometric estimation of hemoglobin release from rabbit erythrocytes in saline solution in the presence and absence of polymer (5 g of polymer/10 ml of saline) at 37° in 1 hr. Percent hemolysis was calculated relative to the positive control (100% hemolysis).

Tissue Culture (TC)—The method of Guess *et al.* (24) was used. The tissue culture response was evaluated in terms of a re-

[1] The data reported here represent part of a study presently being pursued under the auspices of the Food and Drug Administration and directed toward the evaluation of biological test methods for preclinical screening of biomaterials.

[2] Biomer was a gift of Ethnor Medical Products, Division of Ethicon, Inc., Somerville, N.J.

[3] Fisher Scientific Co., St. Louis, Mo.

[4] J. T. Baker Chemical Co., Chattanooga, Tenn.

[5] ICN, K & K Laboratories, Plainview, N.Y.

[6] Fisher TG/DTA Series 100A, Fisher Scientific Co., Pittsburgh, Pa.

[7] Model 360.

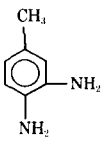
[8] Model 260.

[9] Model 120P.

[10] Cahn Instrument Co., Paramount, Calif.

[11] Servo/riter II, Texas Instruments, Houston, Tex.

Table II—Physical Properties of Solvent and Additives

Compound	Formula Structure	Formula Weight	Melting Point	Boiling Point	Density	Refractive Index (n_d)
Dimethylacetamide (III)	$CH_3CON(CH_3)_2$	87.12	−20.0°	165.0°	0.9366_6^{25}	1.4380^{20}
3,4-Diaminotoluene (I)		122.17	89.0–90.0°	265.0°	—	—
Dibutyltin diacetate (II)	$(C_4H_9)_2Sn(OOCCH_3)_2$	351.00	10.0°	142.–145.0°	1.3200	1.4820

sponse index (RI), reflecting zone size and percent lysis within the zone:

$$RI = ZI/LI \qquad \text{(Eq. 2)}$$

where ZI is the zone index, and LI is the lysis index. The values of ZI and LI are treated as independent values, however, in the calculations that yield the data presented in Table IV. The magnitude of ZI was defined as follows:

0 = no detectable zone around or under the sample
1 = zone limited to area under sample
2 = zone not greater than 0.5 cm in extension from the sample
3 = zone not greater than 1.0 cm in extension from sample
4 = zone greater than 1 cm in extension from sample but not involving the entire plate
5 = zone involving the entire plate

The magnitude of LI was defined as follows:

0 = no observable lysis
1 = less than 20% of zone lysed
2 = less than 40% of zone lysed
3 = less than 60% of zone lysed
4 = less than 80% of zone lysed
5 = greater than 80% lysis within zone

The normalization factors for tissue culture are (ZI + LI) × 10. The numerical values listed in Table III are the sum of four individual tests, including the sample material, cottonseed oil USP extract, polyethylene glycol 400 USP extract, and saline USP extract.

Intradermal Irritation Test (ID)—The USP XVIII method (25) was used. The intensity of gross visible inflammation and necrosis around the implant are primary factors. The intradermal irritation rating scale is as follows:

0 = no detectable irritation
0.5 = minimal irritation detected
1 = very slight but distinct irritation
2, 3, 4, 5 = increasing intensity of inflammation and wheal

A value of 5 represents a response greater than the positive control. A value of 4 for the positive control is normalized to 100%. The intradermal irritation values were calculated by the factor (0–5) × 25. The listed intradermal irritation values are the sum of three individual tests (*i.e.*, extracts of cottonseed oil USP, polyethylene glycol 400 USP, and saline USP).

Intramuscular Implant (IM)—The USP XVIII method (26) was used with the following exceptions: *(a)* the implant period was 7 days, and *(b)* clean, but not necessarily aseptic, technique and samples were employed.

The rating scale for the intramuscular implant test is analogous to that for the intradermal irritation test already presented.

Systemic Toxicity Test (ST)—The USP XVIII method (25) was used. The rating scale (0–5) of the systemic toxicity is the number of deaths after 7 days. The normalization factor is (0–5) × 20. The normalized values reported are the sum of intravenous and intraperitoneal administration.

Histopathological Rating (HR)—The method of Turner *et al.* (27) was used. The histopathological rating values are reported in terms of a 0–4 rating scale and normalized by a factor of (0–4) × 25. The magnitude of histopathological rating was defined as follows:

0 = not different from negative control
1 = minimal response
2, 3, 4 = increasing tissue reaction around implant; degree of necrosis is the primary factor

Cumulative Biological Response Index (CI)—CI values are the sum of the normalized values of response ratings for all tests. CI values represent a broadly based estimate of biological activity.

Molecular Weight Determination—The molecular weight distribution of an elastomer sample containing no additives was determined by gel permeation chromatography[12]. Calibration was based on polystyrene standards, and the sample was run in a dimethylformamide solvent.

Table III—Experimental Results of Biological Response Tests for Elastomer Samples

Sample	Tissue Culture (TC)[a]	Hemolysis (% H)	Intramuscular Implant (IM)	Intradermal Irritation (ID)[b]	Systemic Toxicity (ST)[b]	Histopathological Rating (HR)	Cumulative Biological Index (CI)
1	40	0	0	0	0	0	40
2	140	6	75	100	100	50	471
3	150	8	100	125	100	100	583
4	160	37	100	100	100	100	597
5	210	41	125	225	120	100	821
6	240	44	125	250	200	100	959
7	120	3	0	100	0	50	273
8	190	91	100	225	100	100	806
9	180	100	125	250	160	100	915
10	220	57	125	250	160	100	912
11	160	6	0	50	0	25	241
12	200	12	25	75	0	25	337
13	240	66	125	225	120	100	876
14	260	100	125	275	120	100	980
15	290	100	125	275	160	100	1050
16	180	20	0	0	0	25	225

[a] Sum of tissue culture response (ZI and LI) for material test samples and saline USP, cottonseed oil, and polyethylene glycol 400 extracts. [b] Sum of biological response to saline USP, cottonseed oil, and polyethylene glycol 400 extracts.

RESULTS AND DISCUSSION

Characterization of Segmented Polyether Polyurethane Elastomer—The samples used were prepared from an elastomer solution[13]. The molecular weight and molecular weight distribution were obtained by means of gel permeation chromatography. The molecular weight (based on polystyrene standards) calculated from the assumed mol. wt./size ratio *(Q)* of 25 are $\bar{M}_w$ (weight average) = 115,000, $\bar{M}_n$ (number average) = 51,200, and $\bar{M}_w/\bar{M}_n$ = 2.24.

Thermal Properties of Elastomer Samples—The thermal stability, as indicated by the amount degraded at specific temperatures, and the E_a values for thermal degradation of the 16 samples determined by the thermogravimetric technique are shown in Table IV. The total amount added listed in Table IV represents

[12] DeBell and Richardson, Inc., Enfield, Conn.
[13] According to the manufacturer, the product is based on the original DuPont formula for Lycra.

Table IV—Physical and Thermal Properties of Elastomer Samples

Sample	Amount of I and II Added: I, wt. %	II, wt. %	Total (Moles/10 mg Sample) × 10^7	Density (d), g/cc	Residue Weight of Methanol Extractable Substance (% RW)	T_i	T_{10}	T_{25}	T_{50}	Activation Energy, E_a, kcal/mole
1	0	0	0	1.02	2.44	239.8°	316.2°	349.2°	391.0°	21.07
2	0	0.5	1.39	1.03	2.68	255.0°	311.0°	374.7°	401.4°	20.51
3	0	1.0	2.84	1.04	2.97	253.7°	308.0°	375.3°	399.2°	20.93
4	0	2.0	5.78	1.00	3.78	252.7°	291.0°	369.2°	397.6°	19.19
5	0	4.0	12.90	0.99	4.40	246.3°	276.4°	360.8°	395.6°	18.96
6	0	6.0	17.00	0.99	6.55	238.1°	289.8°	368.5°	396.5°	18.99
7	0.5	0	4.03	1.04	2.66	238.3°	316.9°	357.2°	377.0°	23.10
8	0.5	2.0	9.74	0.94	3.81	235.9°	289.3°	363.5°	389.2°	17.73
9	0.5	4.0	15.23	0.93	5.07	237.4°	298.6°	370.8°	385.6°	19.02
10	0.5	6.0	20.95	0.94	5.54	236.9°	289.0°	366.4°	381.5°	19.54
11	1.0	0	8.20	1.04	3.07	257.0°	322.8°	365.6°	387.8°	24.55
12	2.0	0	16.30	1.02	3.79	250.9°	320.1°	368.3°	390.8°	25.25
13	2.0	2.0	22.10	0.99	5.31	243.3°	287.5°	361.4°	391.0°	17.90
14	2.0	4.0	27.60	1.02	6.84	242.8°	283.2°	366.9°	393.2°	16.97
15	2.0	6.0	32.80	1.03	7.76	241.6°	281.0°	354.2°	392.2°	15.74
16	4.0	0	33.10	1.04	6.11	246.8°	318.4°	367.2°	390.5°	25.01

the sum of I and II in terms of number of moles/10 mg of sample. The four degradation temperatures are T_i, T_{10}, T_{25}, and T_{50}; T_i is the temperature of initiation of degradation, and T_{10}, T_{25}, and T_{50} represent the temperatures at which 10, 25, and 50% (w/w) of degradation occur.

The E_a values were evaluated from the slope of the plot of log log Z *versus* $1/T$ using Eq. 1. Figure 1 shows some typical examples (Samples 1, 6, 15, and 16) of such plots. The thermal degradation data of the elastomer samples follow the linear form of Eq. 1 very nicely; the average relative precision of the determinations is about 3.5%. The E_a values range from 16 to 25 kcal/mole, depending upon the kinds and quantities of additives in the sample.

For example, the E_a value for Sample 16, which contains the highest amount of I (4.0% added), is the largest (25 kcal/mole), while Sample 15, which consists of the highest quantity of II (6.0% added) and a medium amount of I (2.0% added), is the smallest (16 kcal/mole). Apparently, I and II affect the structural properties that contribute to activation energy but are not simply additive.

Physical Properties of Elastomer Samples—Density *(d)* data and the residue weight of methanol extractable substances (% RW) are also listed in Table IV.

In general, the % RW is directly related to the amounts of I and II added. However, it should be pointed out that the nonzero percent extracts (2.44%) observed for Sample 1 suggest that some unknown components other than the materials added are leached out of the polymers by methanol.

Biological Response Tests on Elastomer Materials—The experimental results of biological response tests are listed in Table III.

The results of no hemolytic activity and no other biological responses for the sample without additives (Sample 1) are consistent with the findings reported by other investigators (6, 16, 17). Generally speaking, % H is higher for those samples containing higher amounts of II added (*e.g.*, Samples 8, 9, 14, and 15). Samples 2, 3, 7, 11, and 12, which have the smallest quantities of either I or II added, have the lowest % H.

Multiple Correlations of Physical Properties and Biological Responses—To determine which variables best predict the biological responses, a stepwise multiple linear regression analysis was carried out. This analysis included 10 properties: percent 3,4-diaminotoluene (I) added, percent dibutyltin diacetate (II) added, total amount of I and II added, E_a, T_i, T_{10}, T_{25}, T_{50}, density *(d)* and residue weight of methanol extractable substance (% RW) as independent variables, and the seven biological responses as dependent variables. The results are given below.

$$(TC) = 31.93\,(RW) + 43.03 \qquad (R = 0.844) \quad \text{(Eq. 3)}$$

$$(\%\ H) = -7.20\,(E_a) + 1.54\,(\text{total added}) - 315.70\,(d) + 483.53 \qquad (R = 0.936) \quad \text{(Eq. 4)}$$

$$(IM) = -7.37\,(E_a) - 1.99\,(T_{10}) + 2.80\,(T_{25}) - 195.90 \qquad (R = 0.975) \quad \text{(Eq. 5)}$$

$$(ID) = 24.54\,(\text{dibutyltin diacetate}) - 13.62\,(E_a) + 376.46 \qquad (R = 0.913) \quad \text{(Eq. 6)}$$

$$(ST) = 24.11\,(\text{dibutyltin diacetate}) + 3.65\,(T_{50}) - 1401.08 \qquad (R = 0.920) \quad \text{(Eq. 7)}$$

$$(HR) = -2.10\,(T_{10}) + 1.79\,(T_{25}) + 47.43 \qquad (R = 0.930) \quad \text{(Eq. 8)}$$

$$(CI) = 54.96\,(\text{dibutyltin diacetate}) - 12.14\,(T_{10}) + 9.68\,(T_{25}) + 606.85 \qquad (R = 0.956) \quad \text{(Eq. 9)}$$

Each coefficient of the independent variables in Eqs. 3–9 is significant at the $\alpha = 0.05$ level or better. Equation 3 indicates that tissue culture response is correlated with the residue weight (percent) of methanol extractable substance. In other words, the data suggest that the tissue culture reaction results directly from leachable materials existing in the polymer matrix. The multiple correlation coefficient is 0.844.

For percent hemolysis, Eq. 4, a multiple correlation coefficient of 0.936 is obtained. Since the percent hemolysis determination is performed at 37° and the total exposure time of the polymer to aqueous solutions is no greater than 90 min, it may be somewhat surprising that E_a is so highly correlated to percent hemolysis assay. However, the E_a value may be considered as a measure of thermal stability; the greater the E_a value, the more stable is the material. A simple correlation coefficient of −0.788 is obtained for the E_a–% H relationship, showing that the easier it is for the plastic to decompose, the greater is the hemolysis. Furthermore, evaporation of additives from the polymer in the process of thermal analysis is not distinguishable from decomposition of the polymer so that the E_a value calculated from the thermogravimetric curve is certainly influenced by the presence of additives.

Correlation of percent hemolysis assay with the total amount of additives is to be expected, since at the conditions of hemolysis it would appear that the additives could be easily leached out and contribute to cellular destruction.

Density appears as the third variable in Eq. 4. The simple correlation coefficient of d–% H is −0.587. There is little or no simple correlation between density and the total amount of additives or between density and E_a ($r = 0.427$).

The multiple correlation coefficient corresponding to Eq. 5 for intramuscular implant is 0.975, the highest value observed for all seven dependent variables analyzed. The simple correlation coefficients for IM–E_a and IM–T_{10} are −0.858 and −0.899, respectively. These results strongly suggest that the intramuscular implant response is highly dependent on the thermal stability of elastomer samples. The easier the material is degraded, the higher is the tissue reaction around the implant.

Equation 6 indicates that intradermal irritation is a function of percent II added and the E_a values. The simple correlation coefficient between the percent II added and the E_a value is −0.736, indicating that E_a is inversely related to the percent II added; the simple correlation coefficients for ID–% II and ID–E_a are 0.873

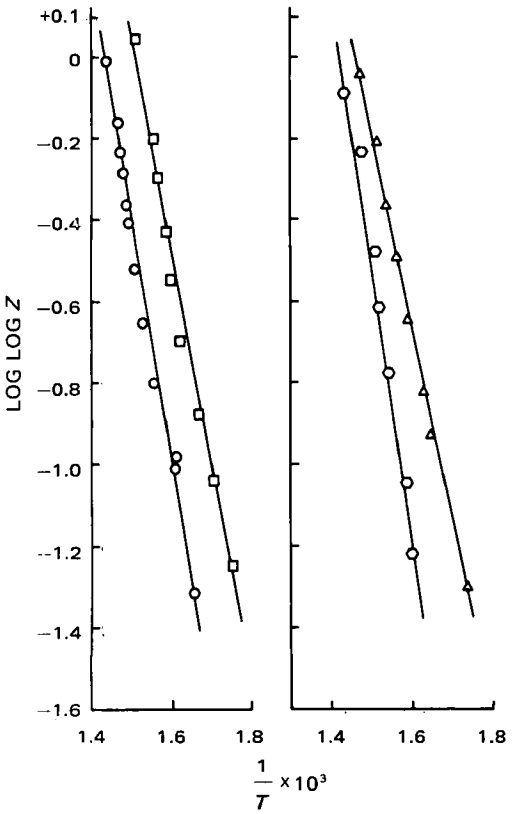


Figure 1—*Typical examples showing the determination of activation energies for thermal degradation of segmented polyether polyurethane elastomers by Broido's (21) method. Key:* ○, *Sample 1, no additives;* □, *Sample 15, 2% 3,4-diaminotoluene and 6% dibutyltin diacetate;* ○, *Sample 16, 4% 3,4-diaminotoluene only; and* △, *Sample 6, 6% dibutyltin diacetate.*

and −0.825, respectively. The higher the percent II added, the lower is the E_a required for degradation (hence the less stable the material) and the higher is the intradermal irritation response. Therefore, it is obvious that intradermal irritation is also directly related to the thermal stability of the elastomer samples. The multiple correlation coefficient is 0.913.

The systemic toxicity is correlated with the percent II added and T_{50} (Eq. 7, R = 0.920). However, the simple correlation coefficient for ST–% II is 0.851; but for ST–T_{50}, it is only 0.358.

The multiple correlation coefficient corresponding to Eq. 8 for histopathological rating is 0.930. But only two variables, T_{10} and T_{25}, are found in Eq. 8. A simple correlation coefficient of −0.866 was obtained for the T_{10}–HR relationship, indicating that T_{10} is negatively correlated to histopathological rating.

In general, T_{10} is considered as a measure of the degradation of active material free from the influence of contamination or sorption of volatile components or solvent on the material. It is, therefore, a better measurement of the thermal stability of the material than T_i (T_i is very sensitive to impurities). The stability of the material may indeed play an important role in the histopathological rating response, since implantation was continued for 1 week. Thus, the lower the value of T_{10}, the easier the material can decompose to products that increase the histopathological rating value.

Furthermore, since the histopathological rating and total amount of additives have a simple correlation coefficient of only 0.254, the additives apparently are relatively ineffective in producing the histopathological response. The data suggest that the additives may strongly influence the thermal stability of the material and thereby indirectly affect the histopathological rating response through the leaching process in the body of the animal.

Equation 9 expresses the multiple correlation of cumulative biological index to the amount of II added, T_{10} and T_{25} (R = 0.956). Since the cumulative biological index is a composite of six major tests, it may be viewed as an overall response. The amount of II added strongly contributes to this overall response. The simple correlation coefficient between II and the cumulative biological index is 0.88. The simple correlation coefficient relating percent I added to the cumulative biological index is only −0.07, a negligible value. Thus, it appears that the amount of II added rather than the amount of I added is largely responsible for the cumulative biological response. However, the average amount of II added for all samples was larger by a factor of two than the amount of I added, and this may in part account for its presence in Eq. 9. The terms T_{10} and T_{25} are related to the cumulative biological response index in a manner similar to the histopathological rating, as already discussed.

Stepwise multiple linear regression analysis has shown that the magnitude of various biological responses is highly correlated to the thermal and physical properties of the polymer system as well as to the amounts of dibutyltin diacetate added to the polymer system in arbitrarily excessive quantities. Since the segmented polyether polyurethane elastomer *per se* showed very low biological response and was present in the same absolute amount in each preparation, it was concluded that its contribution to biological response in the presence of additives was limited to its influence on the leachability of the additives, the biological response being highly correlated with the total amount of additives and the thermal properties of the complex polymer system.

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Decomposition of Amitriptyline Hydrochloride in Aqueous Solution: Identification of Decomposition Products

R. P. ENEVER, A. LI WAN PO, B. J. MILLARD, and E. SHOTTON [x]

Abstract □ The decomposition of amitriptyline hydrochloride upon autoclaving in a buffered solution (pH 6.8) was investigated. Three major decomposition products [3-(propa-1,3-dienyl)-1,2:4,5-dibenzocyclohepta-1,4-diene, dibenzosuberone, and 3-(2-oxoethylidene)-1,2:4,5-dibenzocyclohepta-1,4-diene] were detected and identified by chromatographic and spectroscopic techniques. Evidence is presented that the latter two compounds are formed by further oxidation of 3-(propa-1,3-dienyl)-1,2:4,5-dibenzocyclohepta-1,4-diene, and a possible decomposition pathway is outlined.

Keyphrases □ Amitriptyline hydrochloride—identification of decomposition products, aqueous solution, mechanisms □ Decomposition—amitriptyline hydrochloride in aqueous solution, identification of products, mechanisms □ Antidepressants—decomposition of amitriptyline hydrochloride in aqueous solution, identification of products, mechanisms

The antidepressant drug amitriptyline hydrochloride may be formulated as a parenteral solution and sterilized by filtration according to BP 1974. Although Henwood (1) showed that amitriptyline base breaks down into dibenzosuberone and Bouche (2) indicated that anthraquinone was formed when the base was boiled in alkaline potassium permanganate solution, there are little published data concerning the stability of the drug.

A project was initiated to assess the drug's instability in aqueous solution and to identify the decomposition products produced during autoclaving and long-term storage. Preliminary experiments showed that decomposition occurred when solutions of amitriptyline hydrochloride in water or phosphate buffers were autoclaved for 30 min at 115–116° in the presence of excess oxygen. The purpose of this paper is to describe the isolation of the decomposition products by TLC and GLC and their identification using mass spectrometry, NMR, and other spectroscopic techniques.

EXPERIMENTAL

Materials—The following chemicals were used: amitriptyline hydrochloride[1], dibenzosuberone[2], ethyl iodide[3], potassium chloride[4], potassium dihydrogen phosphate[4], ether[4], *n*-propanol[4], carbon tetrachloride[4], ethylene dichloride[4], benzene[4], hexane[4], chloroform[4], ethanol[4], methanol[4], ethyl acetate[4], and acetone[4].

Degradation of Amitriptyline Hydrochloride Solution—A 0.5% (w/v) amitriptyline hydrochloride solution was prepared in 0.2 *M* phosphate buffer solution (pH 6.8); 2-ml quantities were distributed in 10-ml ampuls to ensure excess oxygen and sealed. The ampuls were autoclaved at 115–116° for up to 6 hr to produce sufficiently large quantities of the degradation products for subsequent identification by various physicochemical techniques.

TLC—The plates (20 × 20 cm) were coated with silica gel G[5], 250 μm thick, and activated by heating at 110° for 2 hr. They were stored in a desiccator prior to use.

The contents of a heated ampul were extracted with 200 μl of ether, and 50 μl was spotted on a plate in 10-μl portions. Ascending development was carried out over 15 cm with benzene–carbon tetrachloride (7:3 v/v). The spots were detected visually in transmitted light and also by spraying with 4% (v/v) formaldehyde solution BP in concentrated sulfuric acid and viewing under UV light. Dragendorff reagent and 0.4% (w/v) 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid were also used as spray reagents to detect nitrogen- and carbonyl-containing compounds, respectively. The R_f values were determined for the various degradation products (Table I).

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[2] Authentic specimen, British Pharmacopoeia Commission.
[3] Koch Light Ltd.
[4] Analar grade reagent, B.D.H. Ltd.
[5] Type 60, E. Merck, Darmstadt, West Germany.

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Table I—TLC Data for Amitriptyline Hydrochloride Degradation Products and Reference Compounds

Compound	R_f Value	Color Reaction: 4% Formaldehyde Solution BP in Concentrated Sulfuric Acid, Daylight	Color Reaction: 4% Formaldehyde Solution BP in Concentrated Sulfuric Acid, UV Light	Color Reaction: 2,4-Dinitrophenylhydrazine Reagent, Daylight
A	0.70	Brown	—	—
B	0.33	Yellow[a]	Yellow fluorescence	Light orange
C	0.17	Brown	—	Orange
Synthesized I	0.70	Brown	—	—
Dibenzosuberone	0.33	Yellow[a]	Yellow fluorescence	Light orange

[a]The yellow color was not evident at low concentrations, although even low concentrations fluoresce under UV light.

TLC was also carried out in a similar manner using the solvent systems listed in Table II.

To obtain sufficient quantities of the degradation products, 20 ml of autoclaved solution was extracted with two 5-ml quantities of ether. The volume of ether was reduced by evaporation under nitrogen to 2 ml, and this sample was streaked on five plates and developed with benzene–carbon tetrachloride (7:3 v/v). Subsequently, each plate was partially covered by a screen so that only approximately 1 cm was exposed. This area was sprayed to reveal the position of the degradation products, and the nonsprayed areas were removed and eluted separately with methanol. These solutions were evaporated under nitrogen to obtain the degradation products.

GLC—The contents of a heated ampul were extracted with 1 ml of ether, and 5 μl was analyzed using a chromatograph equipped with a flame-ionization detector[6]. A 1.5-m (5-ft) × 0.6-cm (0.25-in.) i.d. glass column, packed with 3% (w/w) OV-25 on 80–100-mesh Chromosorb W AW/DCMS, was used at 220°. The flow rates of hydrogen, air, and nitrogen were 83, 450, and 28.5 ml/min, respectively. Peak assignment was achieved by injecting ethereal solutions of each degradation product separated by TLC.

Spectroscopic Analysis—The UV spectra[7] of solutions of the isolated degradation products in methanol were obtained using 1-cm path length silica cells.

To obtain IR spectra[8], samples were presented in the form of liquid films or dilute solutions in carbon tetrachloride.

NMR spectra[9] were obtained by preparing solutions of the products in deuterated chloroform containing tetramethylsilane as the internal marker.

Mass spectra[10] were obtained using a beam energy of 70 ev with the sample probe maintained at 210°.

Synthesis of 3-(Propa-1,3-dienyl)-1,2:4,5-dibenzocyclohepta-1,4-diene (I)—Amitriptyline hydrochloride (500 mg) was made alkaline with 1 *N* sodium hydroxide and extracted with ether. The extract was dried over anhydrous sodium sulfate and filtered. The filtrate was evaporated to dryness *in vacuo*, and 10-ml volumes of ethyl iodide and ether were added. The mixture was refluxed on a steam bath for 20 min, and the unreacted reagents were removed by distillation. Then the residue was dried in a stream of dry nitrogen and dissolved in a minimum of methanol, and excess silver oxide was added.

The mixture was shaken and filtered, and the filtrate was sealed in ampuls under nitrogen and autoclaved for 5 hr. The solvent was removed, and the residue was dissolved in 10 ml of ether and extracted with 5 ml of 5 *N* hydrochloric acid. The ethereal layer was washed with a further two 5-ml volumes of acid, dried over anhydrous sodium sulfate, and filtered, and the product was isolated by vacuum evaporation. The purity of the compound was checked by TLC.

The physicochemical data on the compound were as follows: UV (methanol): λ_{max} 268 nm; IR (liquid): 3060 (aromatic CH stretch), 2920 (CH_2 stretch), 1650 (diene), 910 and 990 (—CH=CH_2), and 755 and 765 (aromatic hydrogen) cm^{-1}; NMR: δ 3.2 (unresolved multiplet, protons on seven-membered ring), 5–5.5 (multiplet, methylene protons), 6.2–6.7 (multiplet, methine protons), and 7.2 (multiplet, aromatic protons) ppm.

The mass spectrum showed a molecular ion at *m/e* 232 (100%) corresponding to $C_{18}H_{16}$ and significant peaks at 231 (34), 229 (8), 218 (11), 217 (65), 216 (26), 215 (52), 204 (18), 203 (23), 202 (36), 191 (10), 189 (16), 151 (8), 142 (17), 116 (24), 113 (13), 106 (17), 105 (13), 104 (8), 92 (8), 88 (7), and 49 (7).

Anal.—Calc. for $C_{18}H_{16}$: C, 93.11; H, 6.89. Found: C, 92.85; H, 7.14.

RESULTS AND DISCUSSION

TLC of degraded amitriptyline hydrochloride, using benzene–carbon tetrachloride (7:3 v/v), indicated the presence of three major decomposition products (A, B, and C). Table I gives their R_f values and color reactions. Also included in Table I are the data for the synthesized I and dibenzosuberone (II). The chromatographic behavior of A and B and the two reference compounds in a range of developing solvent systems is shown in Table II.

GLC produced peaks that correspond to amitriptyline, A, and B. Product C could not be detected in the extract, but a peak was obtained when injecting the eluate from thin-layer plates. Table III shows the retention times of these products together with those of the reference compounds.

From the chromatographic results in Tables I–III, it is apparent that Product A was I. This finding was confirmed by the similarity of their UV, IR, NMR, and mass spectra.

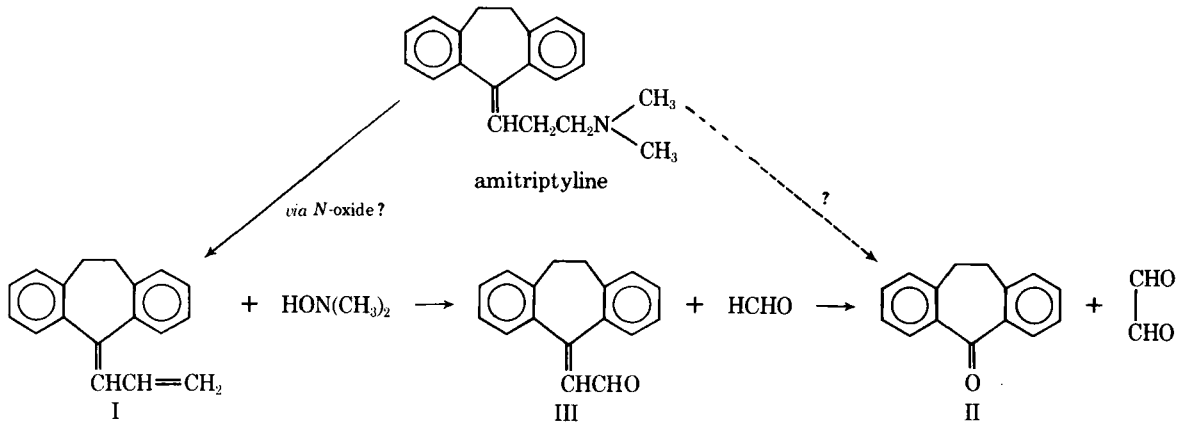
When chromatograms of A, produced by development with benzene–carbon tetrachloride (7:3 v/v), were exposed to air for 12 hr and then developed at right angles to the direction of initial development with chloroform–benzene–diethylamine (5:4:1 v/v), the decomposition product was broken down into B and C.

The identity of B was established by comparing its physicochemical data with those of dibenzosuberone. As shown in Tables I–III, their chromatographic data were identical, as were their UV spectra (λ_{max} 270 nm). Final confirmation that B was dibenzosuberone was obtained by comparison of their mass spectra. These spectra were identical, with a molecular ion at *m/e* 208 (100%) corresponding to $C_{15}H_{12}O$ and significant peaks at 207 (43), 192 (7), 181 (7), 180 (45), 179 (48), 178 (36), 177 (5), 166 (7), 165 (24), 151 (7), 150 (5), 90 (7), 88 (24), 77 (7), 76 (2), 75 (17), 62 (10), 50 (7), 49 (5), and 39 (7).

Table II—Chromatographic Behavior of A, B, Dibenzosuberone, and Synthesized I

Solvent System	R_f Value: Product A	Synthesized I	Product B	Dibenzosuberone
Benzene–hexane (3:2 v/v)	0.77	0.77	0.27	0.27
Ethylene dichloride–hexane (1:1 v/v)	0.83	0.83	0.37	0.37
Chloroform–benzene (1:4 v/v)	0.77	0.77	0.48	0.48
Ethyl acetate–carbon tetrachloride (3:2 v/v)	0.73	0.73	0.69	0.69
n-Propyl alcohol–benzene–carbon tetrachloride (3:3:4 v/v)	0.93	0.93	0.91	0.91
Ethanol–water (3:1 v/v)	0.75	0.75	0.75	0.75

[6] Pye 105 gas chromatograph.
[7] Pye-Unicam SP1800 spectrophotometer.
[8] Perkin-Elmer 157G spectrophotometer.
[9] Varian A60 spectrometer.
[10] A.E.I. MS 902 mass spectrometer.



Scheme I—Proposed decomposition pathway for amitriptyline in aqueous solution

Table I shows that C reacted with 2,4-dinitrophenylhydrazine, and this finding indicated the presence of a carbonyl function. The physicochemical data for the compound were as follows: UV (methanol): λ_{max} 280 nm; IR (liquid): 3020 (aromatic CH stretch), 2920 and 2860 (CH_2 stretch), 1675 (—C=O in conjugated system =CH—CHO), and 767 and 757 (aromatic hydrogen) cm^{-1}; NMR: δ 3.3 (unresolved multiplet, protons on seven-membered ring), 4.4–4.6 (triplet, methine protons), 7.3 (multiplet, aromatic protons), and 9.6–9.7 (aldehydic proton) ppm.

The mass spectrum showed a molecular ion at *m/e* 234 (100%) corresponding to $C_{17}H_{14}O$ and significant peaks at 233 (69), 232 (7), 219 (17), 206 (20), 205 (23), 204 (10), 203 (20), 202 (23), 193 (7), 192 (7), 191 (20), 190 (7), 189 (13), 178 (13), 177 (7), 164 (10), 151 (10), 128 (10), 115 (10), 109 (10), 91 (7), 69 (17), 57 (7), 55 (7), 43 (7), and 41 (7).

From these data, it was concluded that C was 3-(2-oxoethylidene)-1,2:4,5-dibenzocyclohepta-1,4-diene (III).

The formation of dibenzosuberone upon autoclaving amitriptyline hydrochloride could be explained by direct oxidation of the olefinic double bond of the drug. However, such a mechanism does not account for the production of I and III, since only portions of the side chain have been lost. Cope *et al.* (3) showed that loss of dialkyl amino function can proceed *via* formation of *N*-oxide. Although it has not yet been possible to isolate an *N*-oxide in the degraded sample, it seems that *N*-oxidation, followed by loss of dimethylhydroxylamine, is the most probable route of decomposition.

Table III—GLC Behavior of Degradation Products of Amitriptyline Hydrochloride and Reference Compounds

Compound	Retention Time, min
A	3.5
B	4.5
C	8.0
Dibenzosuberone	4.5
Synthesized I	3.5
Amitriptyline	7.0

The aldehyde (III) has been shown to result from the further oxidation of I. The formation of formaldehyde was also detected in this step. A quantity of the isolated allylic compound (I) was placed in a conical flask having a sealed vapor trap (containing chromotropic acid in sulfuric acid) connected to the neck. Upon storage, the chromotropic acid solution developed a violet color, indicating the presence of formaldehyde.

During the subsequent oxidation of III to dibenzosuberone, glyoxal was formed. It was detected by adding excess *o*-aminophenol and calcium oxide to an ethanolic solution of aldehyde, which had been allowed to stand overnight. A positive brick-red color, indicative of glyoxal, was produced.

It is evident that oxidation of the olefinic double bond of amitriptyline hydrochloride is not necessarily the first step in the degradation of the drug; Scheme I shows the proposed decomposition pathway.

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(2) R. Bouche, *J. Pharm. Sci.*, **61**, 987(1972).

(3) A. C. Cope, T. T. Foster, and P. H. Towle, *J. Amer. Chem. Soc.*, **71**, 3929(1949).

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Effect of Polysorbate 60 on Interphase Transport of Cholesterol

P. B. McNULTY

Abstract □ Interphase cholesterol transport was investigated at 24 ± 1° in a stirred diffusion cell and in various oil-in-water emulsions. Cholesterol uptake by vegetable oil from a cholesterol–surfactant-rich aqueous phase was extremely slow in the stirred cell; no measurable transport had occurred after 500 hr. Cholesterol transport in oil-in-water emulsions following dilution with a cholesterol–surfactant-rich aqueous phase was much faster due to the greatly increased interfacial area available for mass transfer. Equilibration half-lives, $t(50)$, varied from 2.02 to 28.1 hr. Variations in the $t(50)$ were due to: *(a)* differences in the mean oil droplet diameter among various emulsions, and *(b)* differences in cholesterol–polysorbate 60 micelle sizes among various dilution media. When polysorbate 60 was omitted from the dilution medium, transport occurred in a two-stage process. In the first stage, transport was extremely rapid, with the $t(50)$ less than 30 sec; in the second stage, transport was comparable to previous emulsion rates, with the $t(50)$ varying from 7.9 to 8.1 hr. The significance of this two-stage transport to mechanisms of interphase cholesterol transport is briefly discussed.

Keyphrases □ Polysorbate 60—effect on interphase transport of cholesterol, mechanism □ Cholesterol—effect of polysorbate 60 on interphase transport, mechanisms and equations □ Transport, interphase—cholesterol, effect of polysorbate 60, stirred diffusion cell, mechanism □ Emulsions—vegetable oil–surfactant–water, effect of polysorbate 60 on interphase transport of cholesterol

Much research has been devoted to investigating the transport of various solute molecules in aqueous media across biomembranes, using both *in vivo* and *in vitro* techniques. Such studies have enhanced the understanding of the mechanisms of absorption, distribution, and metabolism of biologically important compounds in living systems.

Recent studies (1, 2) attempted to elucidate the mechanism (or mechanisms) of *in vivo* transport of cholesterol and related steroids by investigating interphase transport in liquid hexadecane oil-in-water emulsions stabilized by various surfactants. The results of these studies led to the postulation of two possible mechanisms of transport: "free drug delivery" and "micelle delivery." The term "drug" is used because the investigators regard the steroids as model drug compounds. The term "free drug" denotes a steroid dissolved in monomeric form in aqueous solution in contrast to the aggregated or micellar form consisting of steroid–surfactant micelles dispersed throughout the aqueous medium.

The free drug delivery model is based on that proposed by Sylvén and Borgström (3) for absorption and digestion of fat, in which monomeric or free cholesterol diffuses from an oil droplet into the intestinal aqueous phase, where it associates with the bile salts and the polar split products of the oil phase formed by the action of lipase. This micelle transports cholesterol to the "vicinity" of the cell membrane, where the micelle dissociates and monomeric cholesterol is absorbed by the cell while the bile salts return to the bulk aqueous phase. According to Surpuriya and Higuchi (2), the micelle may not take part in the transfer of solute across the hexadecane–water interface and only the free drug is involved in the rate-determining step.

The micelle delivery model proposes that only the micelle takes part in the transfer of drug across the hexadecane–water interface, although free drug is present in solution. Surpuriya and Higuchi (2) suggested that the micelle: "interacts with the hexadecane–water interface and unloads the solute, which passes into the oil phase, and the micelle returns to the aqueous phase."

The objective of this investigation is to present data from a related study (4), which suggest an alternative interpretation of interphase cholesterol transport.

EXPERIMENTAL

Stirred Diffusion Cell Experiment—Cholesterol[1] uptake by vegetable oil[2] containing 1.5% (w/v) sorbitan monostearate[3] from an aqueous phase containing 5% isopropanol[4] and 1.1% polysorbate 60[5] was studied at 24 ± 1° in a 1-liter aspirator bottle[6]. The initial aqueous cholesterol concentration, C_{wi}, was 500 μg/ml. The water was double distilled and deionized.

The upper oil phase (200 ml) was stirred at 12.5 rpm by an umbrella stirrer[7] driven by a variable speed motor[8]. The lower aqueous phase (200 ml) was stirred at 52 rpm by a 25.4 × 7.9-mm octagonal magnetic stirring bar powered by a stirrer[9]. Both stirrers were hooked to variable autotransformers[10] to provide sensitive stirring speed control.

Transport was measured periodically by withdrawing 0.5 ml of the aqueous phase (through the stirred cell side arm sealed with a serum cap) in a 0.5-ml tuberculin syringe fitted with a 50.8-mm 22G hypodermic needle. The samples were mixed with 2 ml of isopropanol and analyzed by colorimetry[11] at 550 nm using a standard procedure (5). Full experimental details were described elsewhere (4).

Emulsion Experiments—*Emulsion Preparation*—Liquid oil-in-water emulsions were prepared using vegetable oil and the sorbitan monostearate–polysorbate 60 emulsifier system (4). The emulsifier hydrophilic–lipophilic balance (HLB) was 11.8, and the concentration was 2.67% (w/v) of oil plus emulsifier. Emulsification was by the agent-in-oil method (6), and the initial oil volume fraction, ϕ_i, was 0.5.

The oil droplet size was varied by using emulsification treatments of increasing severity: *(a)* propeller mixing in a blender[12] at an autotransformer setting of 30 v, *(b)* propeller mixing in the

[1] Eastman grade, Eastman Organic Chemicals, Rochester, N.Y.
[2] Vegetable oil (Wiley mp <0°, ~80% soybean and 20% cottonseed oil) was obtained from Hunt-Wesson Foods Inc., Fullerton, Calif.
[3] Span 60 (HLB = 4.7), ICI America Inc., Wilmington, Del.
[4] Reagent grade, Merck & Co., Rahway, N.J.
[5] Polyoxyethylene (20) sorbitan monostearate (Tween 60, HLB = 14.9), ICI America Inc., Wilmington, Del.
[6] No. 1220, Corning Glass Works, Corning, N.Y.
[7] No. 14-514, Fisher Scientific Co., Medford, Mass.
[8] No. 59880-10, Matheson Scientific Co., Stoneham, Mass.
[9] No. 69302, Precision Scientific Co., Chicago, Ill.
[10] No. 116B, Superior Electric Co., Bristol. Conn.
[11] AutoAnalyzer, No. AA1, Technicon Inc., Tarrytown, N.Y.
[12] No. 1001, Waring Products Co., Winsted, Conn.

blender at an autotransformer setting of 100 v, and *(c)* treatment *(b)* followed by piston homogenization at 140 kg/cm^2 (1 pass) in a single-stage laboratory homogenizer[13]. Particle size, d_{vs}, and distribution, d_m/d_n (*i.e.*, polydispersity), were measured[14] (4), where d_{vs} is the volume to surface mean droplet diameter, and d_m and d_n are the mass and number average diameters, respectively.

Preparation of Aqueous Dilution Media—Cholesterol-rich aqueous phases were prepared using different solvents—*viz.*, ethanol[15], isopropanol, and polysorbate 60 (4) (Table I). The cholesterol–polysorbate 60 micelle size was estimated by filtration through 25-mm diameter filters[16] with pore sizes of 0.025, 0.05, 0.1, 0.22, 0.3, and 0.45 μm. The filters were contained in a microsyringe filter holder[17] attached to a 5-ml hypodermic luer-lok syringe. Samples in the syringe were forced under manual pressure through the filter by the syringe plunger. Approximately 1 ml of filtrate was collected in a 3-ml vial and was analyzed for cholesterol concentration as described previously.

Measurement of Cholesterol Transport—Ninety-five milliliters of the appropriate aqueous dilution medium at 24 ± 1° was weighed into a 125-ml erlenmeyer flask, which was placed on a rotary shaker. Then 5 ml of emulsion (ϕ_i = 0.5) at 24 ± 1° in a 5-ml graduated cylinder was rapidly poured into the dilution medium at zero time. (This procedure gave an emulsion dilution factor, DF_{em}, of 20.) The shaker was immediately switched on at 150 rpm.

The sampling procedure involved: *(a)* withdrawing ~3 ml of diluted emulsion in a 5-ml syringe attached to a 102-mm 15G stainless hypodermic needle, *(b)* inverting the syringe and detaching the needle, *(c)* attaching the syringe to the filter holder, and *(d)* filtering the sample through 0.22-μm pore size filters. The earliest separation time was 30 sec. Aqueous filtrates of unlabeled cholesterol were analyzed as described previously. Cholesterol-4-^{14}C[18] was analyzed by liquid scintillation counting[19] after 0.5 ml of aqueous filtrate was pipetted into 10 ml of scintillation cocktail[20] in a 20-ml scintillation vial.

RESULTS AND DISCUSSION

Stirred Cell Experiment—Transport rate may be evaluated from Eq. 1, which applies to one-dimensional unsteady-state mass transfer across an oil–water interface in cylindrical stirred diffusion cells (4, 7):

$$\ln \frac{C_{we} - C_w}{C_{we} - C_{wi}} = -\frac{K_w A}{V_w}\left[\frac{V_w + V_o K_p}{V_o K_p}\right]t = -S_{sc}t \quad \text{(Eq. 1)}$$

where $(C_{we} - C_w)/(C_{we} - C_{wi})$ = fraction of unattained equilibrium; C_{we} and C_{wi} = equilibrium and initial aqueous concentrations, respectively; K_w = overall mass transfer coefficient accounting for the diffusional resistance in each phase expressed in terms of the aqueous concentration gradient; A = interfacial area; V_o and V_w = oil and aqueous volumes, respectively; S_{sc} = stirred cell transport rate; and t = time. The partition coefficient, K_p, is defined as follows:

$$K_p = C_{oe}/C_{we} \quad \text{(Eq. 2)}$$

where C_{oe} and C_{we} = concentrations in the oil and aqueous phases at equilibrium, respectively.

Figure 1 shows that no perceptible uptake of cholesterol by vegetable oil from an aqueous phase containing 5% isopropanol and 1.1% polysorbate 60 was detected over 500 hr. The extremely slow uptake rate was surprising, particularly since transport rates equivalent to equilibration half-lives of 2.0–13.5 hr were reported for normal alcohols (C_3–C_8) in a similar system (4, 7) and the estimated diffusion coefficients of cholesterol in vegetable oil and water were only slightly less than *n*-octanol diffusivities (4).

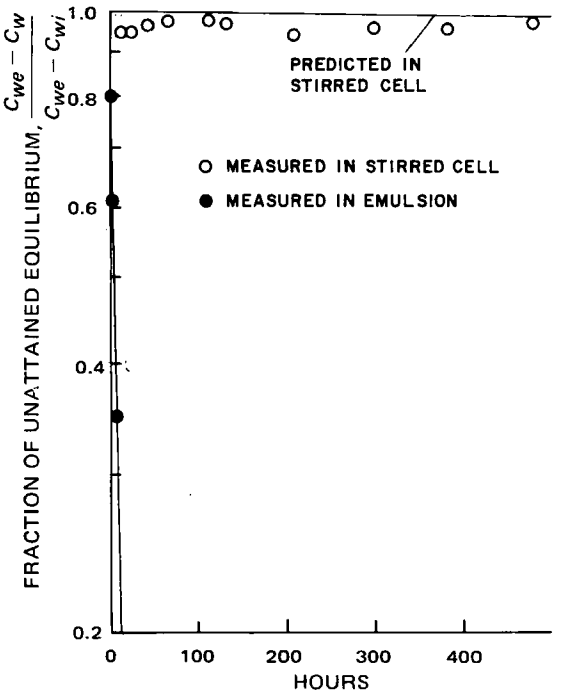

Figure 1—*Comparison of cholesterol transport rates in the stirred diffusion cell (experimental and predicted) and in a vegetable oil-in-water emulsion (Experiment 4) at 24°.*

Prediction of Cholesterol Transport in Emulsions—A scale-up model was developed (4, 8) on the assumption that Eq. 1 also applies to three-dimensional diffusion to spherical oil droplets in oil-in-water emulsions following instantaneous aqueous dilution, *i.e.*:

$$\psi = S_{em}/S_{sc} = t_{sc}(50)/t_{em}(50) \quad \text{(Eq. 3)}$$

where ψ is the scale-up factor (*i.e.*, transport rate ratio) and $t(50)$ is the time for the attainment of 50% equilibration. Equation 1 can be written in a form more suitable for mass transfer in diluted emulsions when the following relationships are used:

$$DF_{em} = \phi_i/\phi \quad \text{(Eq. 4)}$$

$$V_w = (1 - \phi)V_{ow} \quad \text{(Eq. 5)}$$

$$d_{vs} = 6\phi V_{ow}/A \quad \text{(Eq. 6)}$$

where DF_{em} = emulsion dilution factor; ϕ_i and ϕ = initial and diluted oil volume fractions, respectively; and V_{ow} = emulsion volume. Rewriting Eq. 1 gives:

$$\ln \frac{C_{we} - C_w}{C_{we} - C_{wi}} = -\frac{K_w}{d_{vs}}\left[\frac{6\phi_i/DF_{em}}{1 - \phi_i/DF_{em}}\right] \times \left[\frac{K_p - 1 + DF_{em}/\phi_i}{K_p}\right]t = -S_{em}t \quad \text{(Eq. 7)}$$

If $K_{p,em} = K_{p,sc}$ and $K_{w,em} = K_{w,sc}$, an isothermal scale-up factor, ψ, can be expressed in measurable stirred cell and emulsion parameters by substituting Eqs. 1 and 7 into Eq. 3 as follows:

$$\psi = \left[\frac{V_{ow}(1 - \phi)}{A}\right]_{sc}\left[\frac{6\phi_i/DF_{em}}{d_{vs}(1 - \phi_i/DF_{em})}\right]_{em} \times \left[\frac{K_p - 1 + DF_{em}/\phi_i}{K_p - 1 + 1/\phi_{sc}}\right] \quad \text{(Eq. 8)}$$

If 50% equilibration has been attained in the diluted emulsion, Eqs. 3 and 7 can be combined to give:

$$t_{em}(50) = 0.693/\psi S_{sc} = 0.693/S_{em} \quad \text{(Eq. 9)}$$

[13] No. 15M-8TBA, Manton-Gaulin Manufacturing Co., Everett, Mass.
[14] Coulter counter, model B, No. 3303, Coulter Electronics Inc., Hialeah, Fla.
[15] Reagent grade, U.S. Industrial Chemical Co., New York, N.Y.
[16] Millipore Corp., Bedford, Mass.
[17] No. XX30-025-00, Millipore Corp. Bedford, Mass.
[18] 0.344 mg (0.05 mCi) was obtained in 2.5 ml of benzene from New England Nuclear Corp., Boston, Mass.
[19] No. LS 250, Beckman Instruments Inc., Fullerton, Calif.
[20] The cocktail consisted of 2,5-diphenyloxazole (7 g), naphthalene (100 g), and dioxane (*q.s.* 1000 ml).

Table I—Interphase Cholesterol Transport in Oil-in-Water Emulsions at 24°

Experiment	Aqueous Dilution Medium, % Polysorbate 60	Isopropanol	Ethanol	C_{wi}, μg/ml	$\frac{C_{wd}}{C_{we}}$	K_p	d_{vs}, μm	$\frac{d_m}{d_n}$	$S_{em} \times 10^4$, 1/sec	t_{soem}, hr
1	0.106	5.0	0	500	NM[a]	NM	5.78	9.27	NM	NM
2	1.06	5.0	0	500	3.12	82.6	5.78	9.27	0.0685	28.1
3	1.06	5.0	0	500	3.33	90.8	3.28	3.14	0.178	10.8
4	1.06	5.0	0	500	2.84	71.4	1.56	1.94	0.447	4.31
5	5.0	0	0	500	1.20	7.8	1.56	1.94	0.956	2.02
6	1.1	0	0.15	0.134	2.12	43.6	1.56	1.94	0.725	2.66
7	0	0	0.15	0.134	5.40	172	1.56	1.94	NM	(*a*) <30 sec (*b*) 8.1 hr
8	0	0	0.15	0.134	5.61	180	1.56	1.94	NM	(*a*) <30 sec (*b*) 7.9 hr

[a] NM = not measured.

Hence the time for the attainment of 50% equilibration in the emulsion can be predicted from Eq. 9 once ψ is evaluated from Eq. 8, provided K_p and S_{sc} are known. However, due to the slow cholesterol transport in the stirred diffusion cell, S_{sc} and K_p could not be evaluated from Eqs. 1 and 2 because of insufficient data. Therefore, emulsion transport rates could not be predicted.

Emulsion Experiments—Filtration of the aqueous dilution media used in Experiments 1–5 (Table I) revealed that some cholesterol was retained by the filters and that the extent of cholesterol retention increased as both filter pore size and polysorbate 60 concentration decreased (Fig. 2). These results were expected, since micelle size in the micellar solutions increased sharply as the polysorbate 60 concentration decreased, as was indicated by an increase in turbidity. It is clear from Fig. 2 that micelle size in the case of the 0.106% polysorbate 60 (P60) concentration level was extremely large; for example, approximately 485 out of 500 μg/ml cholesterol was retained on a 0.1-μm (1000 Å) filter. When the polysorbate 60 concentration is further reduced, cholesterol precipitates out of solution (4).

The extent of cholesterol uptake by emulsion oil droplets was evaluated using Eq. 10, derived from the model of McNulty and Karel (9):

$$\frac{C_{wd}}{C_{we}} = \frac{\phi_i(K_p - 1) + DF_{em}}{DF_{em} - \phi_i} \qquad \text{(Eq. 10)}$$

where C_{wd} = aqueous concentration immediately after emulsion dilution. If K_p is not known, C_{wd} and C_{we} may be estimated by extrapolating C_w *versus* t plots to zero and infinite times, respectively. Then K_p may be evaluated using Eq. 10.

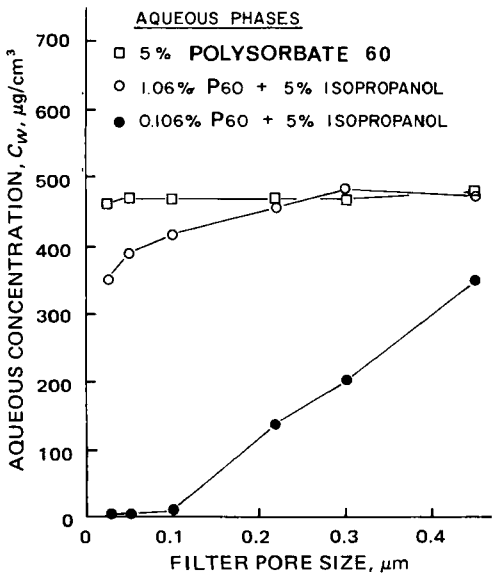

Figure 2—*Effect of aqueous phase composition on the size of cholesterol–polysorbate 60 (P60) micelles* (C_{wi} = *500 μg/ml) at 24°.*

Results of emulsion experiments revealed that the extent of cholesterol transport, C_{wd}/C_{we}, increased as the concentration of polysorbate 60 in the dilution media decreased (Table I). This finding was expected, since the cholesterol equilibrium will increasingly favor the oil phase (*i.e.*, K_p increases) as the aqueous polysorbate 60 concentration decreases.

In Experiment 1, it was difficult to distinguish between the cholesterol retained on the filter and that taken up by the oil due to the large cholesterol–polysorbate 60 micelle size (Table I, Fig. 2). Consequently, the transport rate could not be measured.

In Experiments 2–4, transport rates increased as expected with a decrease in the mean droplet diameter, d_{vs}, due to the increase in interfacial area available for mass transfer. The relative increases in transport rate (1.0:2.6:6.5) were less than the corresponding decreases in d_{vs} (3.7:2.1:1.0), probably due to the corresponding decreases in polydispersity, d_m/d_n. For example, when an emulsion is highly polydisperse (*e.g.*, Experiment 2), a large volume of the dispersed phase exists in a relatively small number of large droplets. Consequently, the transport rate should be slower than that in a less polydisperse emulsion of equivalent d_{vs} due to the relatively slow penetration of diffusing solute into large, as opposed to small, droplets.

Equilibration half-lives for transport of both labeled and unlabeled cholesterol varied from 2.02 to 28.1 hr (Table I). These values are similar to those (*i.e.*, 1.4–4.2 hr) estimated from the data of Bikhazi and Higuchi (10) for cholesterol-4-^{14}C transport in liquid hexadecane oil-in-water emulsions stabilized by polysorbate 80. These rates are extremely slow when compared to equilibration half-lives of normal alcohols (C_3–C_8), $t(50) < 15$ sec, in a similar system (4, 8).

Although emulsion rates could not be predicted, it was possible to use emulsion data and the scale-up model to predict the uptake rate in the stirred cell. Initial experimental results qualitatively confirmed the prediction (Fig. 1).

The effect of surfactant on the uptake rate of cholesterol-4-^{14}C was studied by omitting polysorbate 60 from the dilution medium and using a concentration level in the range of its solubility[21] (*i.e.*, approximately 0.1 μg/ml). The results in Fig. 3 suggest that when polysorbate 60 is omitted, cholesterol transport occurs in a two-stage process, probably due to the following scheme of events:

1. When the emulsion is diluted with aqueous cholesterol, the excess polysorbate 60 in the aqueous phase of the emulsion competes with the oil droplets for the monomeric cholesterol. A fraction of monomeric cholesterol is taken up by the oil droplets very rapidly, as shown in Fig. 3.
2. The remaining cholesterol is rapidly taken up by the polysorbate 60 micelles, and the uptake of surfactant-associated cholesterol is a slow process occurring at approximately the same rate as that in the earlier experiments where polysorbate 60 was included

[21] Aqueous cholesterol solubility was reported as approximately 0.025 μg/ml (11). The approximation in this study is larger, presumably because 0.15% ethanol increases the aqueous solubility.

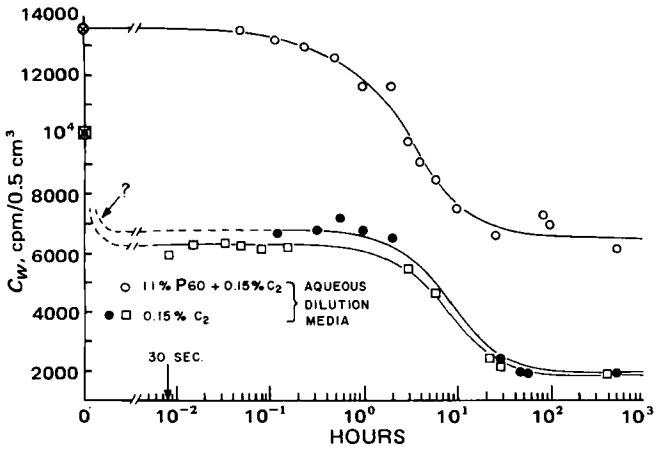

Figure 3—*Effect of the dilution medium composition on the transport rate of cholesterol-4-^{14}C in vegetable oil-in-water emulsions at 24° (Experiments 7 and 8; P60 = polysorbate 60; C_2 = ethanol).*

in the dilution medium. The slow transport is probably due to the opposing driving forces to which cholesterol is subjected, *i.e., (a)* an overall interphase gradient of chemical potential which drives cholesterol out of the micelle, through the water, and into the oil; and *(b)* a local gradient of chemical potential, which maintains the hydrophobic cholesterol in the micelle and out of the water.

It is suggested that, in ternary systems, cholesterol equilibration between oil and water should be rapid as indicated by the rapid monomeric cholesterol transport observed in this study. Furthermore, cholesterol equilibration between water and aqueous surfactant micelles should also be rapid. For example, Nakagawa (12) indicated that "solubilization is a phenomenon proceeding spontaneously." On the other hand, interphase cholesterol transport in quaternary (or multicomponent) emulsion systems proceeds very slowly.

As mentioned previously, the free drug delivery and micelle delivery models (1, 2) describe free drug transfer and micelle association or dissociation as the respective rate-determining steps in interphase cholesterol transport. In the absence of an opposing driving force, as in the described ternary systems, such equilibrations should be rapid. Therefore, the opposing driving force model proposed here appears to be more conceptually consistent.

It is interesting to speculate on the reported transport rates of related steroids (1). For example, in hexadecane oil-in-water emulsions stabilized by polysorbate 80, interfacial permeabilities for cholesterol, demosterol, β-sitosterol, and vitamin D_3 varied from 2.5 to 60 × 10^{-8} cm/sec. For 20α-hydroxycholesterol, progesterone, and testosterone, they varied from 2.0 to 30 × 10^{-5} cm/sec. No explanation was offered for the large difference in permeability between the two groups.

McNulty (4) observed that the steroids of lower permeability are sterols containing one hydroxy group. In contrast, the steroids of higher permeability possess an additional hydrophilic group on the opposite side of the steroid backbone; *i.e.*, 20α-hydroxycholesterol has two hydroxy groups, progesterone has two carbonyl groups, and testosterone has one carbonyl and one hydroxy group. The presence of an additional hydrophilic group will increase aqueous solubility and may produce a more randomly organized steroid–surfactant micelle, since both steroid hydrophilic groups will tend to orient themselves toward the hydrophilic exterior of the micelle. These two effects should reduce the magnitude of the opposing driving forces and thus increase permeability.

In conclusion, the equilibria and kinetics of steroid transport should be quantitatively established in steroid–oil–water and steroid–surfactant–water. [For the latter case, investigators should note the procedure of Short and Rhodes (13) who proposed a thermodynamic description of the effect of surfactants on diffusion of steroids across cellulose acetate membranes.] Characterization of such ternary systems should then permit a more quantitative interpretation of steroid transport in quaternary (or multicomponent) emulsion systems and also help to establish unambiguously the mechanism (or mechanisms) of interphase steroid transport.

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Effect of Carbonated Beverages and of an Antiemetic Containing Carbohydrate and Phosphoric Acid on Riboflavin Bioavailability and Salicylamide Biotransformation in Humans

J. BRIAN HOUSTON and GERHARD LEVY ˣ

Abstract □ Two carbohydrate–phosphoric acid solutions, one a widely used beverage (Solution C) and the other a pharmaceutical product used as an antiemetic (Solution E), administered together with riboflavin-5′-phosphate or salicylamide to healthy human adults, significantly increased the bioavailability of riboflavin and appreciably altered the metabolic fate of salicylamide (increased conversion to the sulfate and decreased formation of the glucuronide). A beverage containing phosphoric acid but no carbohydrates (Solution T) also increased the bioavailability of riboflavin but not as much as Solution C. These effects are attributed to a decrease of the gastric emptying rate caused by carbohydrates and phosphoric acid, consistent with the empirical use of Solution C syrup and Solution E as antinauseants and antiemetics. The results demonstrate also that the choice of beverage to be taken with medication can affect the bioavailability and/or metabolic fate of medicinals with saturable absorption and/or biotransformation characteristics.

Keyphrases □ Phosphoric acid and phosphoric acid–carbohydrate solutions—effect on riboflavin bioavailability and salicylamide biotransformation in humans □ Carbohydrate–phosphoric acid solutions—effect on riboflavin bioavailability and salicylamide biotransformation in humans □ Gastric emptying rates—effects of phosphoric acid and phosphoric acid–carbohydrate solutions, riboflavin bioavailability and salicylamide biotransformation in humans □ Antiemetic effect—carbohydrate–phosphoric acid solutions

A recent study in humans showed that caffeine contained in a proprietary carbonated beverage (Solution C[1]) is absorbed much more slowly from this beverage than from coffee or tea (1). Solution C contains sucrose and phosphoric acid, both of which inhibit gastric emptying (2, 3). A proprietary antinauseant and antiemetic product (Solution E[2]) also contains sugars and phosphoric acid (and no other active ingredients) and has been found to reduce the amplitude and rate of contraction of isolated strips of rabbit GI smooth muscle (4). Solution C syrup is widely used as an antiemetic, apparently on an empirical basis. Because of these findings, it was considered likely that Solution C (and other widely used beverages with similar ingredients) can inhibit significantly the rate of gastric emptying in humans and that the antiemetic properties of Solution C syrup and Solution E may be due to an inhibition of gastric motility produced by the carbohydrates and phosphoric acid.

The primary purposes of this investigation were to determine if Solution C can modify the absorption of concomitantly administered medicinal agents and to assess the relative contribution of carbohydrates and phosphoric acid to such an effect. The availability of a carbohydrate-free cola beverage, Solution T[3], otherwise practically identical to Solution C in composition (Table I), facilitated such an assessment. A secondary purpose was to obtain some indication of the mechanism whereby Solutions E and C exert an antiemetic effect; the remarkable similarity in the composition of these products (Table I) suggests that they act by an identical mechanism.

The medicinal agents utilized, riboflavin and salicylamide, were chosen because the bioavailability of the former and the metabolic fate of the latter are strongly affected by their absorption rate. Riboflavin is absorbed by a specialized, saturable process, apparently located in the proximal region of the small intestine (5–7). Large doses are almost completely recovered in the urine following parenteral injection (8, 9) and are only partially absorbed upon oral administration (5–7). Food, viscous solutions, hypothyroidism, and anticholinergic drugs increase the absorption of riboflavin, apparently by decreasing the rate of gastric emptying and thereby prolonging the residence time of riboflavin at specialized absorption sites in the proximal region of the small intestine (10–13).

Salicylamide is eliminated in humans by biotransformation to the sulfate and glucuronide and by formation of gentisamide (14). The formation of the sulfate conjugate is a saturable process of very low capacity; the fraction of a dose of salicylamide converted to salicylamide sulfate increases, therefore, with the decreasing rate of absorption (14). These pronounced nonlinear pharmacokinetic characteristics make riboflavin and salicylamide ideal agents for a safe, noninvasive, and convenient indirect determination of possible changes of the gastric emptying rate in humans. If a beverage such as Solution C can modify the bioavailability of riboflavin and the metabolic fate of salicylamide in humans, it is likely to have a similar effect on other medicinal agents with saturable absorption or biotransformation characteristics.

EXPERIMENTAL

Five healthy male volunteers, 26–38 years old, capable by education and experience to give their informed consent (research assistants and associates), participated in the study. They did not take any vitamins or drugs for 1 week before and during the experiments. They were given 41 mg of riboflavin-5′-phosphate (equivalent to 30 mg of riboflavin) in the morning on an empty stomach and did not eat for the next 4 hr. The vitamin was dissolved in 60 ml of water, 450 ml of water, 60 ml of Solution E, or 450 ml of So-

[1] Coca-Cola.
[2] Emetrol, W. H. Rorer Co., Fort Washington, Pa.
[3] Tab.

Table I—Composition of Solutions C, T, and E

Solution C[a]		Solution T[a]		Solution E[b]	
Sucrose	48.0 g	Saccharin	0.147 g	Invert sugars syrup	45.0 g
Phosphoric acid	0.25 g	Phosphoric acid	0.25 g	Phosphoric acid	0.18 g
Caffeine	0.056 g	Caffeine	0.064 g	Glycerin	7.5 g
Flavor and color		Flavor and color		Flavor, color, and preservative	
Water *q.s.*	450 ml	Water *q.s.*	450 ml	Water *q.s.*	60 ml
pH 2.5		pH 2.8		pH 1.5–1.6	

[a] Coca-Cola USA, Atlanta, Ga., personal communication. [b] Reference 4.

Table II—Effect of Solutions C and T on Riboflavin[a] Bioavailability

	Percent of Dose Excreted in Urine								
	4 hr			24 hr			36 hr		
Subject	Water	Solution C	Solution T	Water	Solution C	Solution T	Water	Solution C	Solution T
H	14.8	28.6	16.6	20.5	38.9	25.3	23.4	40.5	30.3
L	6.9	22.6	—	10.6	34.1	—	12.4	36.0	—
O	8.5	23.0	12.1	16.0	32.9	25.2	18.4	34.5	28.5
S	7.6	12.8	—	16.6	27.2	—	18.9	30.9	—
Y	4.8	21.9	11.1	10.5	32.8	19.5	11.5	35.4	23.5
Mean	8.5	21.8	13.3	14.8	33.2	23.3	16.9	35.5	27.4
SD	3.8	5.7	2.9	4.3	4.2	3.3	4.9	3.4	3.5
Statistical significance of difference from control (*p* value)[b]		<0.005	<0.05		<0.001	<0.05		<0.005	<0.025

[a] Riboflavin-5′-phosphate, 41 mg, administered orally in 450 ml of water, Solution C, or Solution T. [b] Paired *t* test.

lution C according to a modified Latin-square experimental design. The solutions were at room temperature and the container was rinsed with 10 ml of water, which was also ingested. Three of the subjects also took riboflavin in 450 ml of Solution T, the sugar-free equivalent of Solution C.

In the second part of this investigation, the five subjects were given 1 g of salicylamide in four hard gelatin capsules with 450 ml of Solution C or water, in crossover fashion, in the morning on an empty stomach. The subjects did not eat for 4 hr and collected urine according to the same protocol as was used in the riboflavin study. All experiments were carried out at 1-week intervals.

Each subject emptied his bladder immediately before vitamin or drug administration and then collected urine every 30 min for 4 hr, every hour for the next 4 hr, every 2 hr until bedtime, and then at desired intervals for up to 36 hr (riboflavin study) or 24 hr (salicylamide study). The subjects were allowed to drink 50–100 ml of water after each urine collection to facilitate urine flow, except during the first 2 hr of the Solution C and T studies and during the 1st hr of the Solution E study when no fluids were permitted.

Urine containing riboflavin was collected in brown polyethylene bottles, and 5 ml of 25% acetic acid was added. All urine samples were stored in a refrigerator. They were assayed fluorometrically for riboflavin (6, 7) and for total salicylamide, salicylamide glucuronide, salicylamide sulfate, and gentisamide as described previously (14). All data were corrected for physiological excretion rates of riboflavin (based on a 12-hr urine collection) and for apparent salicylamide blank values (based on −1 to 0-hr urine samples). A separate experiment with Solution C alone established that ingestion of this beverage did not increase the blank values of urine.

RESULTS

The results of the study of the effects of Solutions C and T on the bioavailability of riboflavin are summarized in Table II. An example of the time course of the urinary excretion rates of riboflavin in the three experiments in an individual subject is presented in Fig. 1. The bioavailability of riboflavin was increased by 110%, from 16.9 to 35.5% of the dose on the average, by administration with Solution C. Solution T caused an average increase in bioavailability of 60%, *i.e.*, from 17.8 to 27.4% of the dose.

The data for Solution E are summarized in Table III, and an individual example of the time course of riboflavin excretion rates with and without Solution E is presented in Fig. 2. The bioavailability of riboflavin was increased by 70%, from 18.0 to 30.7% of the dose. The one subject (O) who did not show an increase in bioavailability with Solution E experienced nausea after taking the solution.

Because of the difference in the volume of Solutions C and E, different controls (riboflavin-5′-phosphate in 60 and 450 ml of water, respectively) were used in the two studies. There was no statistically significant difference in bioavailability of riboflavin from the two aqueous solutions.

There was a strong ($r = -0.938$) and statistically highly significant ($p < 0.001$) negative correlation between the bioavailability of riboflavin in control experiments and the increase in bioavailability obtained with Solutions C and E. Both sets of data could be fitted to the same regression line (Fig. 3). A similar negative relationship was observed for Solution T but with a much different slope,

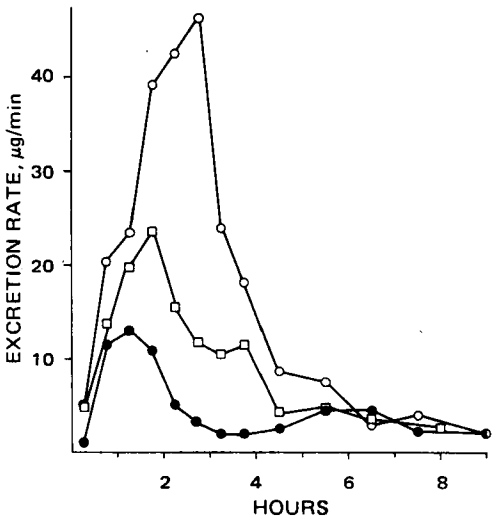

Figure 1—*Time course of urinary excretion rate of riboflavin after oral administration of 41 mg of riboflavin-5′-phosphate in 450 ml of water (●), Solution T (□), or Solution C (○). Data are for Subject Y.*

Table III—Effect of Solution E on Riboflavin[a] Bioavailability

Subject	Percent of Dose Excreted in Urine					
	4 hr		24 hr		36 hr	
	Water	Solution E	Water	Solution E	Water	Solution E
H	9.1	22.3	15.6	31.8	17.8	34.0
L	4.3	20.5	8.3	33.8	11.4	37.3
O	9.2	13.3	17.9	17.9	20.2	20.1
S	7.3	19.0	19.4	29.2	21.6	31.6
Y	10.4	17.8	16.9	26.6	19.0	30.3
Mean	8.1	18.6	15.6	27.9	18.0	30.7
SD	2.4	3.4	4.3	6.2	3.9	6.5
Statistical significance of difference from control (p value)[b]		<0.01		<0.05		<0.05

[a] Riboflavin-5′-phosphate, 41 mg, administered orally in 60 ml of water or Solution E. [b] Paired t test.

consistent with the smaller effect of this carbohydrate-free solution on riboflavin availability.

The results of the study of salicylamide biotransformation are summarized in Table IV. The total recovery of salicylamide metabolites was essentially quantitative and identical in the control and Solution C experiments. Administration of salicylamide with Solution C caused a statistically significant increase in the formation of the sulfate conjugate and a decrease in the glucuronide fraction. The change in the gentisamide fraction was not statistically significant. The maximum urinary excretion rate of salicylamide sulfate increased from 1.25 ± 0.45 mg of salicylamide equivalent/min in the control experiments to 2.11 ± 0.29 mg/min in experiments with Solution C ($p < 0.001$). The time of occurrence of maximum excretion rates of salicylamide sulfate was 1.05 ± 0.27 hr in the controls, and it was 1.65 ● 0.42 hr in experiments with Solution C ($p < 0.05$).

There was a strong ($r = -0.934$) and statistically significant ($p < 0.025$) negative correlation between the maximum excretion rate of salicylamide sulfate in control experiments and the increase in that rate with Solution C (Fig. 4). A similar negative correlation was found with respect to the fraction of the dose excreted as salicylamide sulfate ($r = -0.768$), but it was not statistically significant because of one unusual value. There was no apparent correlation between the relative increase in riboflavin bioavailability and in the fraction of salicylamide sulfate produced by concomitant administration of Solution C in the individual subjects ($r = 0.177$).

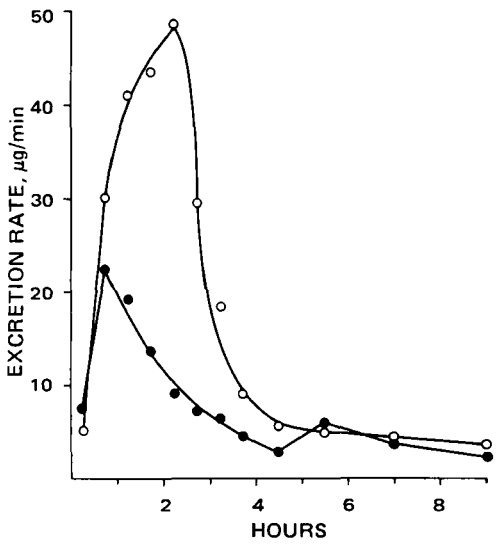

Figure 2—*Time course of urinary excretion rate of riboflavin after oral administration of 41 mg of riboflavin-5′-phosphate in 60 ml of water (●) or Solution E (○). Data are for Subject H.*

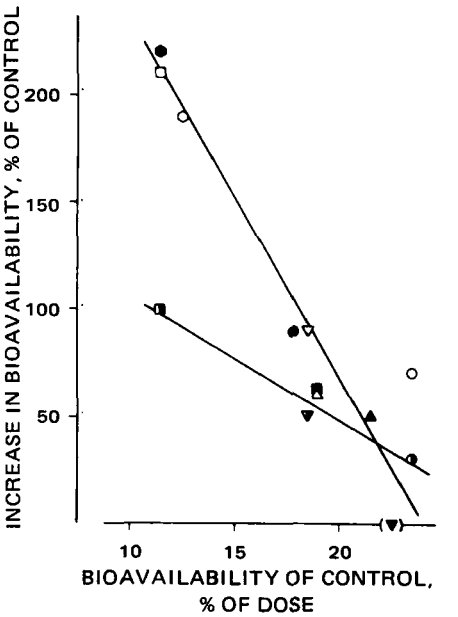

Figure 3—*Relationship between the bioavailability of riboflavin in control experiments (60 or 450 ml of water) and the increase in bioavailability upon administration of the vitamin in Solution C (open symbols), Solution E (solid symbols), and Solution T (half-open symbols). Each subject is represented by a differently shaped symbol. The symbol in parentheses was excluded in the regression analysis.*

DISCUSSION

Gastric emptying can be a major determinant of the drug absorption rate, and individual variations in the rate of drug absorption from any one dosage form may be due largely to differences in the rate of gastric emptying (15). The very slow absorption of caffeine from Solution C, relative to its absorption from coffee and tea (1), suggested that this beverage may have a pronounced inhibitory effect on gastric emptying. This supposition was strengthened by the fact that two major ingredients of Solution C, sugar and phosphoric acid, are known to inhibit gastric emptying in hu-

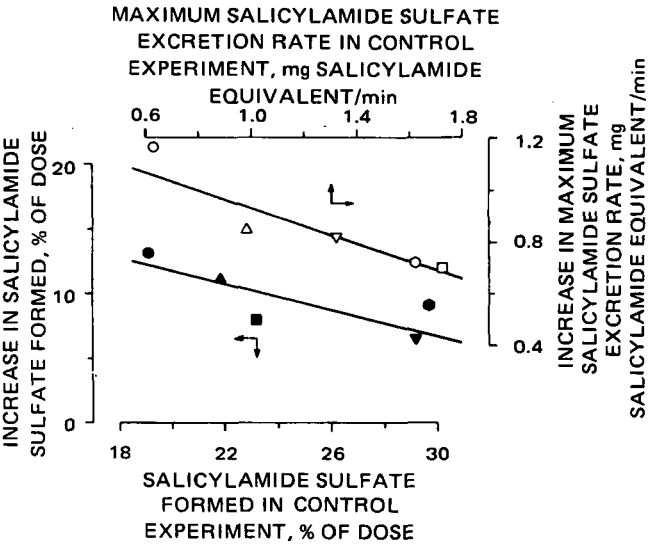

Figure 4—*Relationship between* (a) *the percent of the dose of salicylamide converted to the sulfate in control experiments and the increase in this value in the experiments with Solution C (solid symbols), and* (b) *the maximum excretion rate of salicylamide sulfate in control experiments and the increase of this rate in the experiments with Solution C (open symbols). Each subject is represented by a differently shaped symbol.*

Table IV—Effect of Solution C on the Metabolic Fate of Salicylamide[a]

	Urinary Recovery, % of Dose							
	Total Metabolites		Salicylamide Glucuronide		Salicylamide Sulfate		Gentisamide	
Subject	Control	Solution C	Control	Solution C	Control	Solution C	Control	Solution C
H	98.1	93.4	69.0	52.2	19.2	32.1	10.0	9.0
L	98.7	101.6	59.4	57.6	29.4	39.6	9.8	4.4
O	96.9	97.2	56.3	53.4	28.4	34.9	12.2	8.9
S	99.2	98.1	71.4	57.3	21.8	32.9	5.9	7.9
Y	90.6	94.9	61.3	55.6	23.2	31.1	6.1	8.1
Mean	96.7	97.0	63.5	55.2	24.4	34.1	8.8	7.7
SD	3.1	3.1	6.5	2.3	4.4	3.4	2.7	1.9
Statistical significance of difference from control (p value)[b]		N.S.		<0.05		<0.001		N.S.

[a] Salicylamide, 1 g, in gelatin capsules, followed by 450 ml of water or Solution C. [b] Paired t test.

mans (2, 3). The similarity of the composition of Solutions C and E, an antiemetic product, provided additional indirect support for this theory and suggested that the mechanism of the antiemetic effect of both products is an inhibition of gastric motility.

A change in the gastric emptying rate can, by modifying the rate of absorption, alter the time course of drug concentrations in the body. If drug biotransformation involves one or more saturable processes, changes in drug absorption kinetics can alter the metabolic fate of a drug (16). Examples of such absorption rate-dependent quantitative changes in biotransformation are the conjugation of salicylamide with sulfate (14) and the acetylation of p-aminobenzoic acid (17) and p-aminosalicylic acid (18). It is appropriate, therefore, to assess possible effects of beverages, diseases, drugs, and other modalities on the gastric emptying rate in terms of their pharmacokinetically most relevant aspects: the magnitude of change in the absorption and biotransformation of drugs with saturable absorption or biotransformation characteristics. This purpose was served by riboflavin and salicylamide in this investigation.

The results of this investigation demonstrate that the type of beverage taken with a medicinal agent may modify appreciably its absorption. This can obviously affect the onset, intensity, and time course of drug action. An additional implication, although speculative, is that the beverage taken with drug products in bioavailability studies may affect the results obtained *relative* to the standard product. It is known, for example, that a decrease in GI motility can bring about an increase in the bioavailability of incompletely absorbed digoxin tablets while having no effect on the bioavailability of a digoxin solution (19). Small differences in pH or agitation intensity can have a pronounced effect on the relative dissolution rate of a drug in different tablet formulations (20, 21). Changes in the gastric emptying rate will affect the length of time of exposure of tablets to the low pH and mild agitation conditions of the gastric environment and the much higher pH and more intensive agitation in the small intestine.

Finally, the results of this study show that both Solutions C and E inhibit gastric motility and suggest that this may be the mechanism of their antiemetic effect. The effectiveness of Solution T, which contains phosphoric acid but no sugars, and the greater effectiveness of Solution C show that both sugar and phosphoric acid contribute significantly to the inhibition of gastric emptying observed in this investigation.

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Preliminary Investigations of Intrinsic and Extrinsic Optical Activity as Purity Criterion for Human Serum Albumins

J. H. PERRIN * and J. J. VALLNER ‡x

Abstract □ Induced circular dichroism measurements were made to follow the binding of four acidic drugs to two lots of crystalline and two lots of fraction V human serum albumins. The magnitude of the induced circular dichroism varied with all lots of albumin, suggesting a strong sensitivity of the phenomenon to small changes in purity or secondary structure of the albumins. The circular dichroism of the albumins themselves showed much less variation. The more classical analytical techniques of UV absorption, measurement of absorption following methyl orange binding, gel electrophoresis, and ultracentrifugation were also performed on the albumins for comparison.

Keyphrases □ Albumins, human serum—intrinsic and extrinsic optical activity as a purity criterion, circular dichroism compared to other techniques □ Optical activity, intrinsic and extrinsic—purity criterion for human serum albumins, circular dichroism studies □ Circular dichroism—intrinsic and extrinsic optical activity as a purity criterion for human serum albumins, binding of four acidic drugs

Dicumarol has been shown to become optically active on binding to human serum albumin (1, 2). During a quantitative investigation of this binding, it was found that the shape and magnitude of the induced circular dichroism (CD) curves varied with the lot of albumin (2). Therefore, it was decided to investigate the curves generated by four planar and acidic drugs, dicumarol, sulfaethidole, phenylbutazone, and flufenamic acid, following the binding of two new lots of crystalline and two new lots of fraction V human serum albumin to see whether the phenomenon is purity related.

The CD curves of the albumins themselves were also investigated. More classical analytical techniques such as ultracentrifugation, gel electrophoresis, UV absorption, and absorption following methyl orange binding were performed on these albumins for comparison.

EXPERIMENTAL

Materials—Dicumarol[1] USP, flufenamic acid[2], and phenylbutazone[3] were used as supplied. Sulfaethidole[4] was recrystallized twice from water, mp 185–186°. All other materials were reagent grade, and deionized water was used throughout. Two lots of crystalline and two lots of fraction V human serum albumins were obtained from leading manufacturers. They were stored, refrigerated, and desiccated under identical conditions and were used as supplied by the manufacturers.

Methods—All CD spectra were obtained in a 6003 CD attachment to a spectropolarimeter[5] with a slit programmed for a half bandwidth of 15 Å. All measurements were made in 0.054 *M* sodium phosphate buffer, pH 7.4, adjusted to isotonicity with sodium chloride. All solutions were scanned in 10- or 20-mm cells from regions of high wavelength having no ellipticity to the lower limit of the system.

All albumin samples were subjected to sodium lauryl sulfate gel electrophoresis, similar to that described by Weber and Osborn (3). The samples were dissolved in 0.01 *M* sodium phosphate buffer, pH 7.0, containing sodium lauryl sulfate and 1% β-mercaptoethanol, and were heated at 37° for 2 hr. Electrophoresis was carried out at 3 mamp/gel for 18 hr with the anode in the lower chamber. Gels were then stained for 4 hr with 0.25% Coomassie brilliant in 50% methanol and 9.2% acetic acid; destaining was accomplished by diffusion in 5% methanol and 9.2% acetic acid.

Sedimentation velocity experiments were carried out at 20° in an analytical ultracentrifuge[6]. A Kel F cell with a sapphire window was used in these runs, carried out at 68,000 rpm on 0.9% albumin solutions. The schlieren optical system was used to record the rate of change in the refractive index of the sample as a function of distance from the rotor's axis of rotation.

Spectrophotometric[7] measurements were made in silica cells of 10-mm path length. For the determination of the albumins by methyl orange, the original method of Bracken and Klotz (4) was modified in that twice as much methyl orange was used and measurements were made at 535 and 550 nm.

Table I—$E^{1\%}_{1\,cm}$ of pH 7.4 Solutions of the Albumins

Sample	Absorbance, nm	
	279	210
A, fraction V	5.85	157
B, fraction V	6.13	168
C, crystalline	6.36	190
D, crystalline	3.85	136

Table II—Percentage Purity of Albumins Using the Methyl Orange Method of Bracken and Klotz (4), Taking Sample C as the Standard

Sample	Purity, %	
	550 nm	534 nm
A	96	98
B	99	98
C	100	100
D	72	72

RESULTS AND DISCUSSION

UV Characteristics—The absorbances of 1% solutions of the albumins in 1-cm cells at 279 and 210 nm are shown in Table I. Sample D gave a significantly lower absorption than the others, and this crystalline sample repeatedly behaved as if it contained 25% of a nonabsorbing impurity, perhaps an inorganic salt used in the purification procedures. Even if Sample D is omitted, there was still almost a 10% fluctuation in the absorbances at 279 nm.

Most literature reports give a figure of $E^{1\%}_{1\,cm}$ for "pure" serum albumin of 5.30–5.80 (5–7). The absorption in this region is primarily due to the aromatic amino acid residues. Therefore, contamination due to globulins, which contain more aromatic amino

[1] Abbott Laboratories, North Chicago, Ill.
[2] Parke-Davis, Detroit, Mich.
[3] Geigy Research, Ardsley, N.Y.
[4] Smith Kline and French, Philadelphia, Pa.
[5] Cary 60, Cary Instruments, Monrovia, Calif.
[6] Beckman model E, Beckman Instruments, Palo Alto, Calif.
[7] Cary 14, Cary Instruments, Monrovia, Calif.

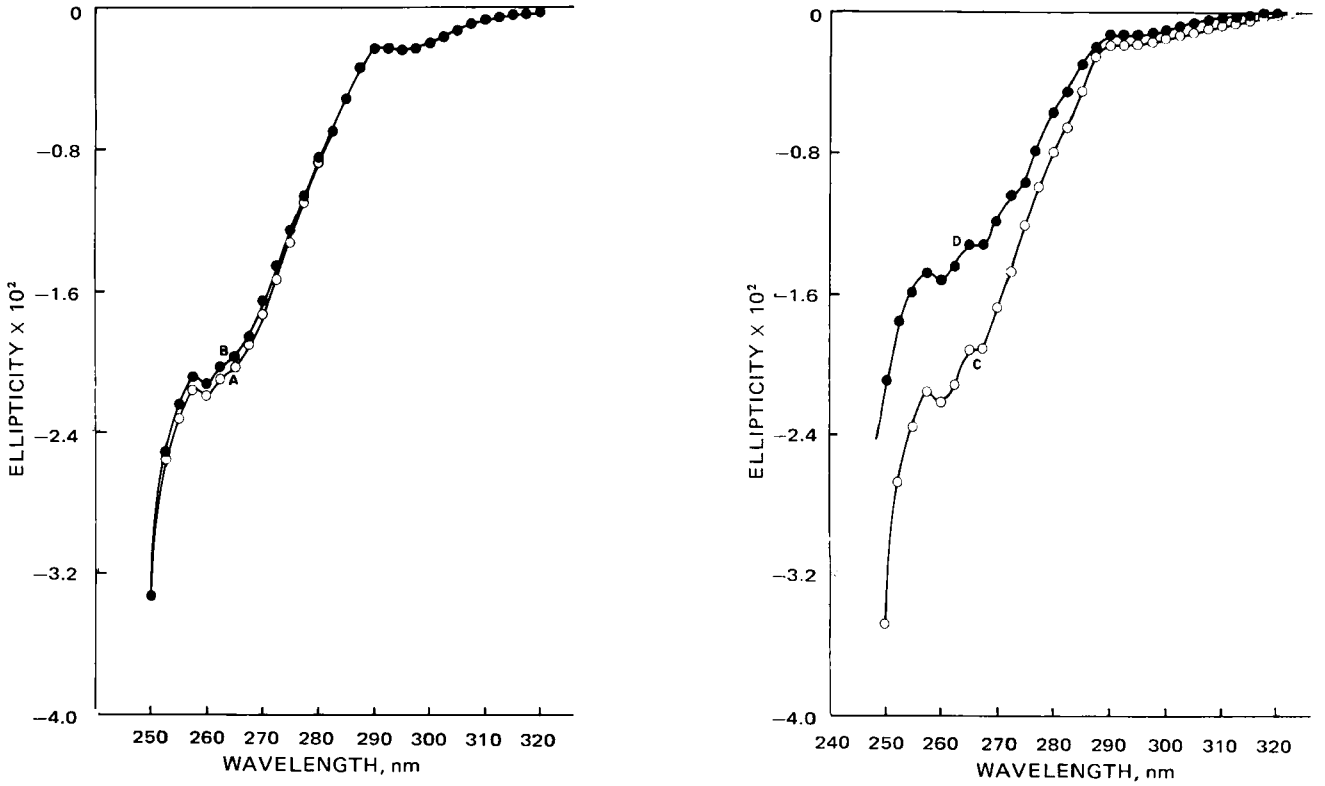

Figure 1—*CD curves of albumin samples alone in the 250–300-nm region. Human serum albumin concentration is 0.2% (2.9 × 10^{-5} M) in 10-mm cells at pH 7.4. (See Table I.)*

acids than do albumins, results in an increased absorbance at this wavelength. Any aromatic impurity acquired during the extraction or purification process or added as a stabilizer also contributes to the absorbance in this region.

Globulins and albumins have been reported to have similar UV absorbances at 210 nm, and a pure sample of albumin was reported to have an $E^{1\%}_{1\,cm}$ of 190 (5). This value was obtained with the crystalline Sample C. The fraction V samples gave significantly lower values than 190. The UV spectrum at 210 nm resulted from aromatic residues and contributions from peptide linkages. The latter contributions are from both the primary and secondary structures of the protein.

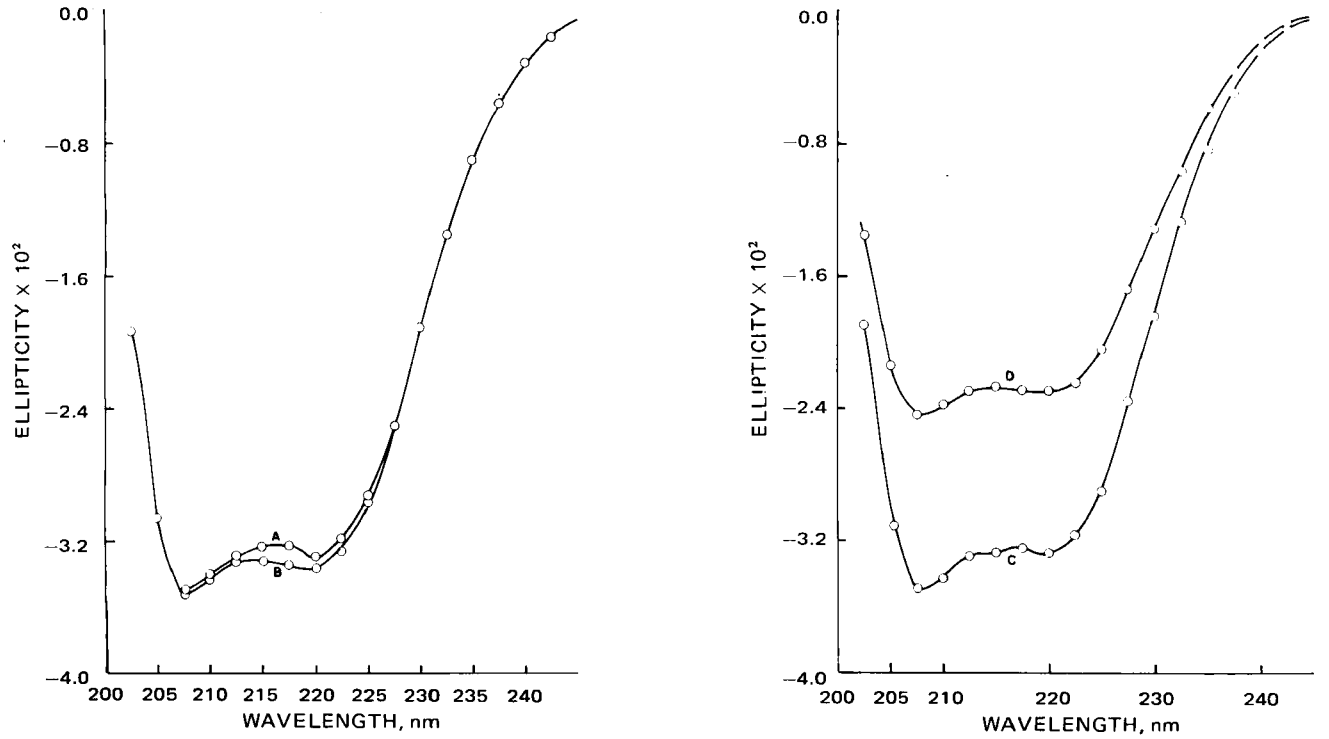

Figure 2—*CD curves for the four albumins in the 200–250-nm region. Human serum albumin concentration is 0.001% (1.45 × 10^{-7} M) in 20-mm cells at pH 7.4. (See Table I.)*

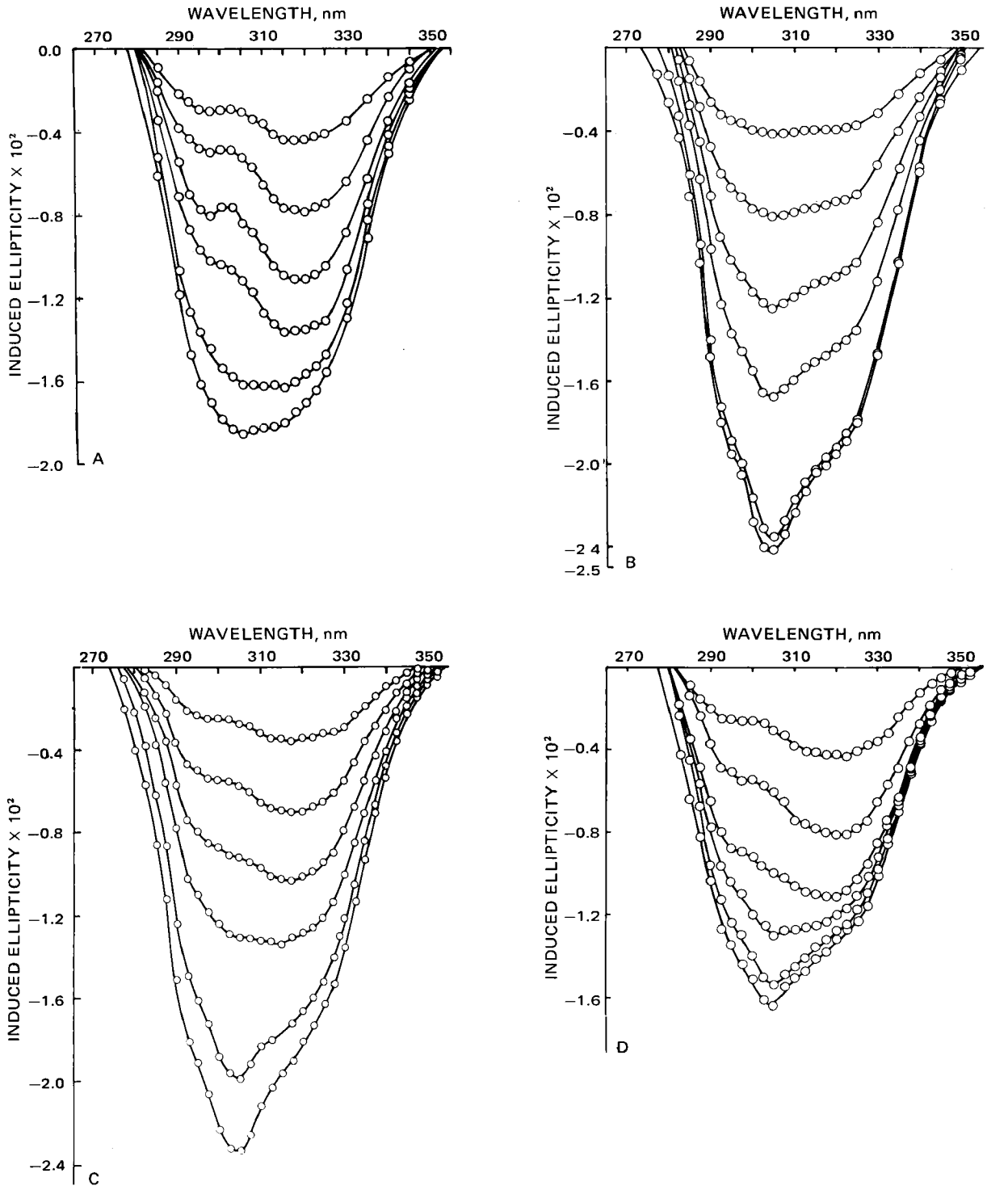

Figure 3—*Induced CD of dicumarol with the four albumin samples. (See Table I.) Human serum albumin concentration is 1.45 × 10⁻⁵ M in 10-mm cells at pH 7.4. Dicumarol concentrations are 0.743, 1.48, 2.23, 2.96, 4.46, and 5.92 × 10⁻⁵ M.*

The decreased absorbance following reaction with methyl orange was measured at 550 and 535 nm. The percentages are expressed taking Sample C as the standard, because this sample gave the largest decrease in absorbance (Table II). Methyl orange does not react with globulins (5). This method, involving the reaction with dyes, is greatly superior to the UV measurement of the albumins, but it demands a correct choice of an albumin standard and it may not be sensitive to changes in the secondary structure of the albumins.

CD Characteristics—CD in the 250–300-nm region is due to the aromatic residues in the primary structure of albumin. As evident from Fig. 1, there is considerable fine structure present in this region due to contributions from various electronic transitions associated with phenylalanine, tryptophan, tyrosine, and histidine residues. Samples A, B, and C were virtually superimposable over this wavelength region, suggesting that they had similar concentrations of albumin. However, Sample D, as expected, showed decreased ellipticity of approximately 30%.

The 200–250-nm region is frequently regarded as being due to the secondary structure of the protein. However, it contains contributions from the aromatic residues as well. The spectra of Samples A, B, and C in this region were again virtually superimposable, having peaks near 220 and 208 nm (Fig. 2). Greenfield and Fasman (8) suggested that the percentage of α-helix in a protein, using

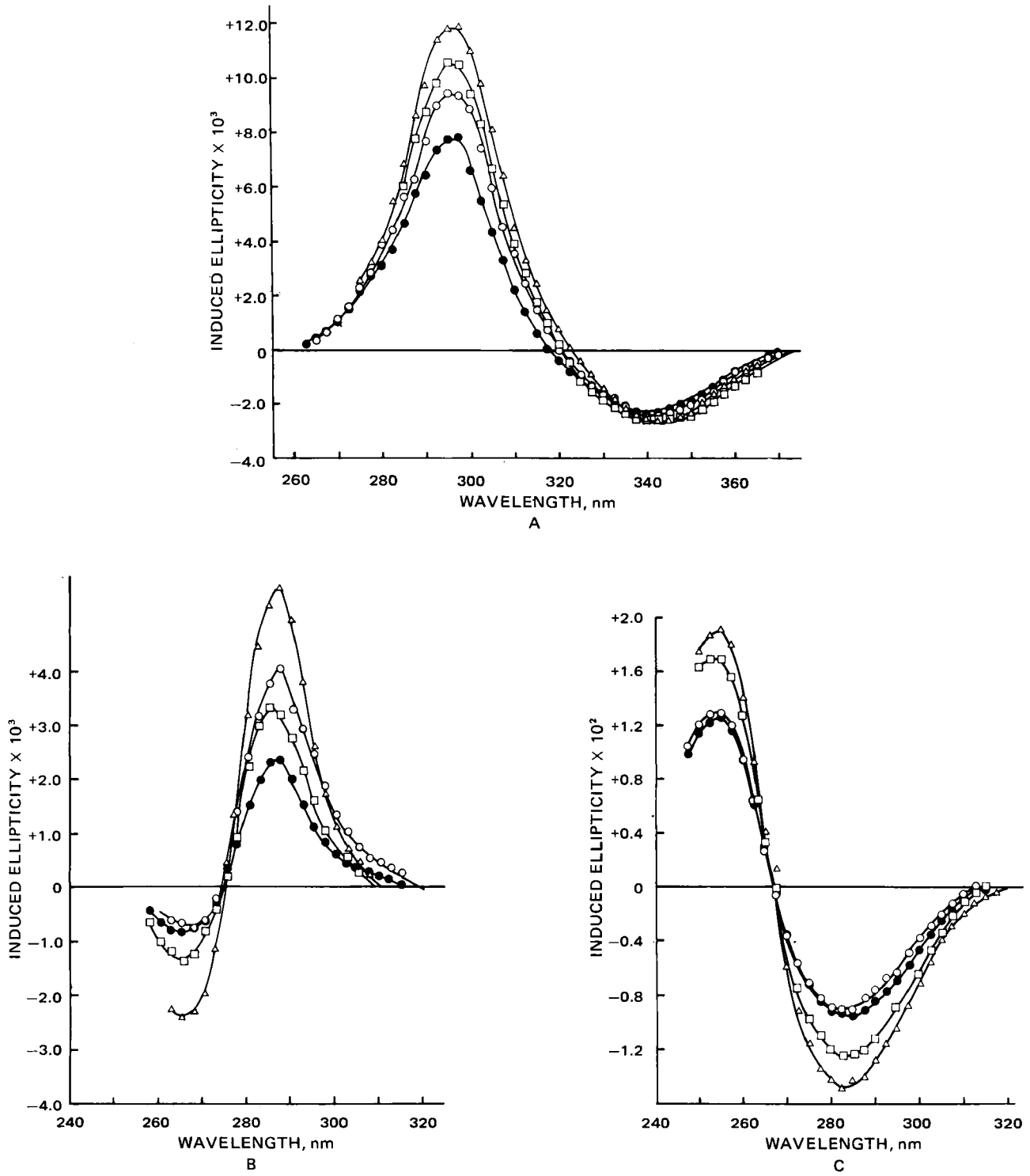

Figure 4—*Induced CD of flufenamic acid (A, 5 × 10^{-5} M), phenylbutazone (B, 2.59 × 10^{-5} M), and sulfaethidole (C, 1.27 × 10^{-5} M) with the four albumin samples. Human serum albumin concentration is 1.45 × 10^{-5} M in 10-mm cells at pH 7.4. Key: ●, albumin A; ○, albumin B; △, albumin C; and □, albumin D.*

poly-L-lysine as a model, is given by:

$$\% \ \alpha\text{-helix} = \frac{-[\theta]_{208} - 4000}{33{,}000 - 4000} \times 100 \qquad \text{(Eq. 1)}$$

Using a mean residue molecular weight of 113 for human serum albumin and substituting in this formula give the data shown in Table III. Samples A, B, and C had essentially the same helical content. The shape of the CD spectra was consistent with the remainder being primarily in the random coil conformation rather than being the β-structure (7). Sample D had the same shape CD curve, but it was of greatly reduced intensity due to the presence of nonabsorbing and nonoptically active impurities. This finding suggests that there had been no macrodenaturation of the albumins during their preparation.

Induced CD—Figures 3 and 4 show the induced CD of the four drugs following their binding to the various albumin samples. The resultant binding curves were apparently sensitive to impurities or a localized denaturation of the albumin. The four sets of dicumarol–albumin curves showed no new peaks; however, the shape of

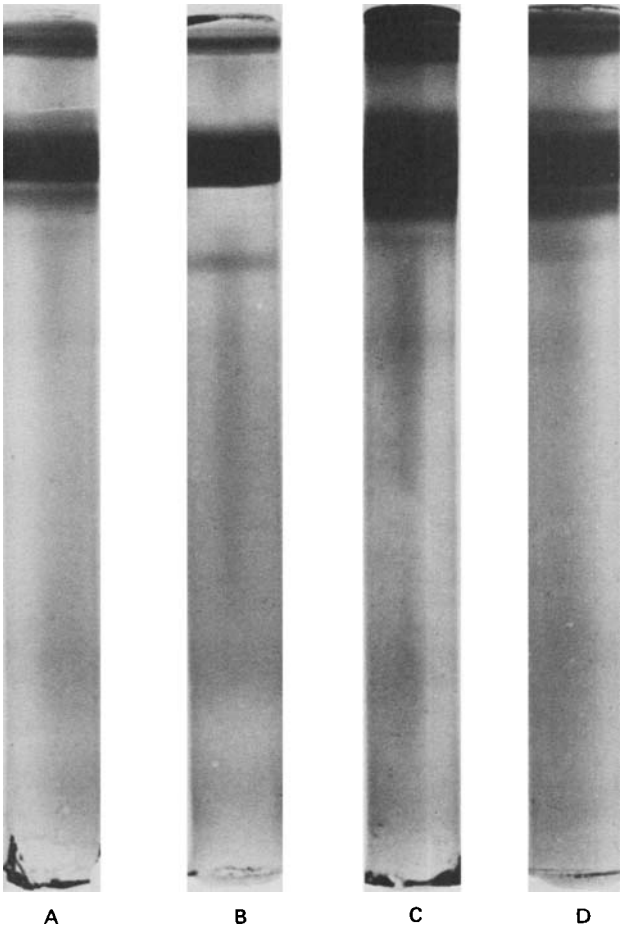

Figure 5—*Sodium lauryl sulfate gel electrophoresis. The letters refer to the albumin samples.*

the induced curves was different, as were their concentration dependencies. The curves of albumin Sample A–dicumarol gave characteristics similar to the fraction V sample used in earlier work (2). The widely different concentration dependence of the peak heights is immediately apparent on comparison of Samples A and B. The two crystalline samples (C and D) gave similar shaped curves when corrections were made for the impurity present in Sample D.

Figure 4 shows the induced CD of flufenamic acid, sulfaethidole, and phenylbutazone when bound to the various albumin samples. Curves obtained with Sample D were continually lower in peak height, and this problem appears to be entirely a concentration effect. The curves obtained with sulfaethidole (Fig. 4C) were similar for both fraction V samples, but the small differences between the two were reproducible; however, both curves were much smaller than the curves seen with the crystalline albumins.

The shape of the sulfaethidole-induced CD was concentration independent, and only one class of site was capable of inducing optical activity[8]. When sulfaethidole was bound to crystalline and fraction V samples of bovine serum albumin, the induced curves were similar in size and the binding parameters were of about equal value (9). The binding curves of flufenamic acid and phenylbutazone, which were very concentration dependent, emphasize the differences in the fraction V samples. Some small conformational change may, by slightly altering the nature of the binding process, account for the observed differences in the curves.

Gel Electrophoresis—The result of the sodium lauryl sulfate gel electrophoresis examination is shown in Fig. 5. The protein became negatively charged by the sodium lauryl sulfate and migrated toward the bottom. The gels were overloaded with protein to make apparent obvious inconsistencies between samples. It was immediately apparent that Sample B, a fraction V albumin, was of higher quality than the others tested. Samples A, C, and D had a small amount of low molecular weight material, probably about 50,000, and all samples contained a higher molecular weight material of molecular weight around 150,000. Since mercaptoethanol treatment in the presence of sodium lauryl sulfate destroys disulfide bonds (10), the dimer probably was eliminated; therefore, this high molecular weight material must have been another protein impurity.

[8] J. H. Perrin, unpublished observations.

Table III—Percentage of α-Helical Content of the Four Albumin Samples[a]

Sample	Observed Ellipticity at 208 nm × 10^2	Calculated α-Helix, %
A	3.51	55
B	3.49	54
C	3.50	54
D	2.41	33

[a] All solutions were 1.45×10^{-5} *M*; measurements were made in 20-mm cells using physiological phosphate buffer of pH 7.4.

Ultracentrifugation—Schlieren patterns developed during ultracentrifugation of the albumin samples are shown in Fig. 6. It can be seen from the areas of the peaks that Sample D contained less total protein than the other samples. Sample B showed a negligible amount of high molecular weight impurity; however, Samples A, C, and D all showed a small peak due to a high molecular weight material, possibly some albumin dimer or γ-globulin (γ-immunoglobulin G). The percentages of this high molecular weight material in the samples are given in Table IV.

CONCLUSION

The samples of albumin were assumed to be essentially free of globulins due to their method of preparation. Nevertheless, gel electrophoresis suggests the presence of a very small quantity of high molecular weight material which should not be albumin polymers. Globulins contain more aromatic residues than albumins,

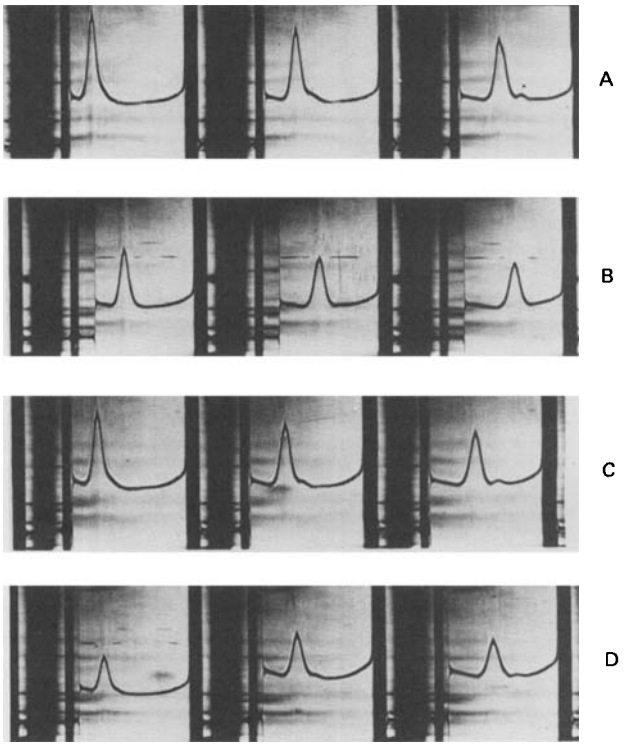

Figure 6—*Results of analytical ultracentrifugation analysis at 68,000 rpm for 2 hr. The letters correspond to the albumin samples.*

Table IV—Analysis of Impurity in Albumin Samples by Ultracentrifugation for Approximately 100 min at 68,000 rpm

Sample	Impurity, %
A	7.6
B	<1.0
C	6.5
D	7.7

and their presence would complicate the UV absorbances, particularly at 280 nm. Aromatic impurities can also arise from stabilizers and fractionating aids or as impurities in extracting solvents. The absorbance at 210 nm, although more difficult to measure, is a better analytical tool for total protein content because the main contribution at this wavelength is from the peptide bond.

There does not appear to be extensive literature on the binding of anionic drugs to other components of human serum such as globulins or lipoproteins. The anionic dye, methyl orange, does not appear to bind to γ-globulin (4, 11), but it has been reported to bind to β-lipoprotein as strongly as it does to albumin (12). It has also been reported that molecular aggregation of albumin in solution has no effect on its methyl orange binding capacity (5) whereas heat denaturation does (13).

The induced CD curves seem to be very sensitive to either impurities (perhaps lipoproteins, globulins, or albumin dimers) or minor changes in conformation. The effect of a high molecular weight impurity can best be seen when curves B and C are compared (Fig. 3). These albumins gave the largest induced CD with dicumarol, but C contained much more impurity than B. Therefore, the difference was probably not due to the impurity. Significant differences in shape and concentration dependence are evident in all CD curves of the dicumarol–albumin complexes. With the other drugs investigated, all albumins gave similarly shaped curves, but they exhibited variations in the magnitude of the induced signals.

CD curves of Samples A, B, and C in the 250–300-nm region were similar and showed considerable fine structure due to aromatic moiety contributions. These findings suggest that these albumin samples are of similar total albumin concentration. In the lower wavelength region, where the induced CD is primarily from secondary structure, the curves were again similar and suggest a helical content of about 55%; however, the small and reproducible differences shown may well be significant.

Small but locally significant conformational changes cannot be easily examined by CD since the technique measures the average contribution over the whole albumin molecule. Such small changes in the binding site region could well explain the differences observed and could be the results of variations in solvent or possibly heat treatment during the albumin extraction process. It is apparent that during any binding study, particularly if of a quantitative nature, a single lot of albumin should be used. Moreover, established analytical techniques such as reaction with biuret (14) or phosphotungstophosphomolybdate (15) or spectrophotometric color determination with bromcresol green (16), bilirubin (5), or methyl orange (4) should be used to check for gross contamination as was found with Sample D.

CD of albumins alone or in conjunction with a molecule giving extrinsic Cotton effects may be an extremely sensitive technique for assessing the quantity and quality of albumin samples. In any quantitative investigations using crystalline or fraction V albumins, an investigator should use a single lot of albumin.

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Synthesis of *dl*-4ξ-(4-Carboxybutyl)-5-carbethoxy-*cis*-hexahydropyrrolo-[3,4-*d*]imidazol-2-one (*N*-Carbethoxyazabiotin)

HENRY C. WORMSER ˣ and HANLEY N. ABRAMSON

Abstract □ The total synthesis of *dl*-4ξ-(4-carboxybutyl)-5-carbethoxy-*cis*-hexahydropyrrolo[3,4-*d*]imidazol-2-one (*N*-carbethoxyazabiotin) by a 16-step sequence, starting from 2-bromo-6-methoxyhexanoic acid, has been accomplished.

Keyphrases □ *dl*-4ξ-(4-Carboxybutyl)-5-carbethoxy-*cis*-hexahydropyrrolo[3,4-*d*]imidazol-2-one—16-step synthesis from 2-bromo-6-methoxyhexanoic acid □ *N*-Carbethoxyazabiotin—16-step synthesis from 2-bromo-6-methoxyhexanoic acid □ Biotin derivatives—16-step synthesis of *N*-carbethoxyazabiotin □ 2-Bromo-6-methoxyhexanoic acid—starting material for 16-step synthesis of *N*-carbethoxyazabiotin

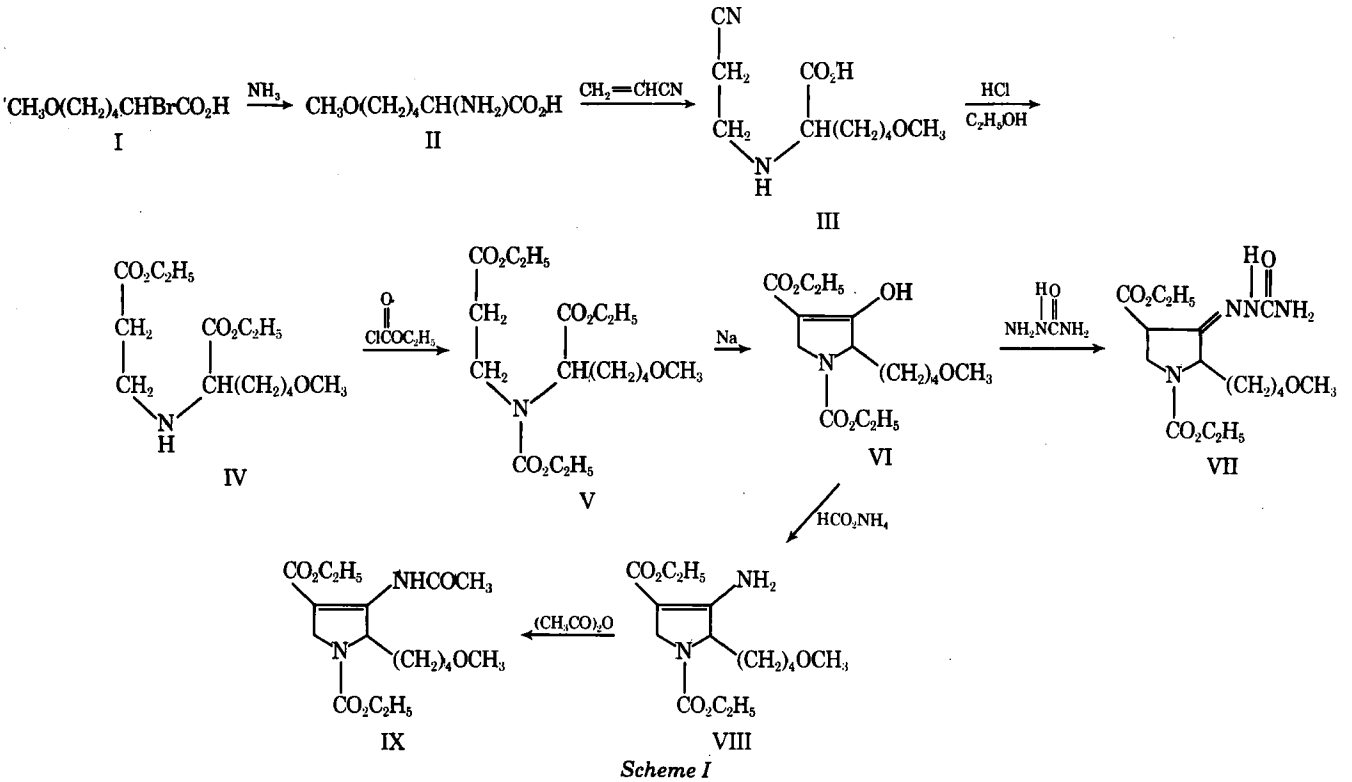
As part of a program for the development of new agonists and antagonists of biotin (1–3), a close analog of the vitamin has been synthesized in which the sulfur atom has been replaced by a nitrogen. It is yet unclear whether the sulfur atom is indeed essential for optimum activity of the cofactor, since compounds such as oxybiotin (4–6), carbobiotin (3), and desthiobiotin (7) exhibit some degree of activity as growth promoters in microorganisms.

In previous articles (1, 2), a scheme was devised for the synthesis of substituted pyrrolo[3,4-*d*]imidazole compounds. The method now has been applied in the synthesis of the title compound, *dl*-4ξ-(4-carboxybutyl)-5-carbethoxy-*cis*-hexahydropyrrolo[3,4-*d*] imidazol-2-one, hereafter referred to as *N*-carbethoxyazabiotin (XIX) (Schemes I–III).

DISCUSSION

The synthesis of XIX starts with 2-bromo-6-methoxyhexanoic acid (I), prepared by the method of Schmid (8) from 1,4-dibromobutane. The α-bromo acid was first converted to the corresponding α-amino acid (II) by treatment with concentrated ammonium hydroxide. Monocyanoethylation with acrylonitrile in aqueous base followed by ethanolysis–esterification gave the aminodiester (IV) in good yield. The amino group was protected with a carbethoxy group by treating IV with ethyl chloroformate, and a Dieckmann condensation afforded an oily pyrroline (VI) in 93% yield.

A crystalline semicarbazone (VII) of the β-keto ester (VI) was prepared to secure an analytical sample since attempts to distill the Dieckmann product only led to decomposition. Condensation of VI with ammonium formate in ethanol afforded a 96% yield of the enamine (VIII). Following acetylation with acetic anhydride at 90°, the oily pyrroline was reduced at 1000 psi of hydrogen over freshly prepared W-2 Raney nickel. Reduction at lower pressures (Parr apparatus) was very slow and incomplete, and reduction utilizing platinum as the catalyst failed completely. A crystalline 3,4-*cis*-pyrrolidine (X) was obtained in 40% yield from the Raney nickel reduction.



Scheme I

Hydrazinolysis of X under relatively mild conditions afforded a mixture of two products, consisting primarily of the 3,4-*cis*-hydrazide (XII*a*) and a small amount of the 3,4-*trans*-epimer (XII*b*). This latter product could also be obtained by first epimerizing X in ethanol containing catalytic amounts of ethoxide and subsequent treatment of the 3,4-*trans*-pyrrolidine (XI) with hydrazine hydrate. Because of the relatively small amount of the 3,4-*trans*-epimer by-product in the crude hydrazide product, no attempt was made to purify the mixture for the next step and it was found suitable as such.

Conversion to the azide, followed by Curtius rearrangement, gave the pyrrolo[3,4-*d*]imidazole (XIII) as a dark oil. Cleavage of the side-chain methoxy group without additional damage to the ring system proved somewhat problematic. Attempts with 48% hydrobromic acid in acetic acid, concentrated hydrochloric acid, and the mixed anhydride acetyl *p*-toluenesulfonate (9) failed to afford a suitable product either by giving unresolvable resinous mixtures or by failing to alter the starting material at all. A solution of XIII in acetic anhydride containing boron trifluoride etherate finally afforded the triacetyl compound (XIV) along with small quantities of the *N,N*-diacetyl ether (XIV*a*).

Room temperature hydrolysis of XIV in 48% hydrobromic acid gave the carbinol (XV), which was converted to the homologous nitrile (XVII) *via* the mesylate (XVI). Ethanolysis of the nitrile to the ester (XVIII) followed by base hydrolysis gave the title compound (XIX). Although XIII–XVIII were obtained as noncrystalline, nondistillable oils, spectroscopic data for these compounds were in agreement with the assigned structures.

EXPERIMENTAL[1]

2-Amino-6-methoxyhexanoic Acid (II)—2-Bromo-6-methoxyhexanoic acid (I) (8) (225 g, 1.0 mole) was treated with 3 liters of concentrated ammonium hydroxide and allowed to stand at room temperature for 24 hr. The solution was concentrated to about one-quarter volume, and the precipitated crystals were collected by suction filtration and gave 125.0 g, mp 269–271°. An additional 21.0 g, mp 269–271°, was obtained from the mother liquor. Recrystallization from ethanol gave 129.5 g (80%), mp 272–274°. IR: λ_{max} (mineral oil) 3.75, 3.87, and 6.05 μm.

Anal.—Calc. for $C_7H_{15}NO_3$: C, 52.17; H, 9.38; N, 8.69. Found: C, 52.29; H, 9.25; N, 8.78.

2-(2-Cyanoethylamino)-6-methoxyhexanoic Acid (III)—Compound II (129.0 g, 0.80 mole) was dissolved in 600 ml of water and treated with a solution of 32.0 g (0.80 mole) of sodium hydroxide in 200 ml of water, followed by 42.5 g (0.80 mole) of acrylonitrile, while stirring magnetically in an ice bath. Dropwise treatment with 46 ml (0.80 mole) of acetic acid gave a white precipitate. The product was filtered and washed with ether–ethanol followed by ether. The mother liquor was concentrated, filtered, and similarly washed. The total yield amounted to 140.0 g (82% yield), mp 218–224°. Two recrystallizations from ethanol–water gave an analytical sample, mp 220–222°; the melt resolidified at 222–223° and remelted at 249–252° dec.; IR: λ_{max} (mineral oil) 4.44 μm.

Anal.—Calc. for $C_{10}H_{18}N_2O_3$: C, 56.05; H, 8.47; N, 13.08. Found: C, 56.11; H, 8.41; N, 13.13.

Ethyl 2-(2-Carbethoxyethylamino)-6-methoxyhexanoate (IV)—A solution of 140.0 g (0.65 mole) of III in 500 ml of absolute ethanol was treated with dry hydrogen chloride gas for 8 hr while stirring in an ice bath. After refluxing the reaction mixture for 1 hr, the ethanol was removed *in vacuo*. The residue was dissolved in water and cooled in an ice bath. The cold solution was treated with 10% ammonium hydroxide to pH 9. The oil was extracted into ether. The ether extract was washed with saturated sodium chloride, dried (sodium sulfate), and concentrated to give 149.0 g (79%) of colorless liquid; IR: λ_{max} (neat) 2.91, 3.00, 5.82, and 8.50 μm. The product was considered suitable for conversion to the carbamate (V).

Ethyl 2-[*N*-(2-Carbethoxyethyl)-*N*-carbethoxy]amino-6-methoxyhexanoate (V)—To a solution of 149.0 g (0.52 mole) of IV in 400 ml of chloroform, cooled in an ice bath, was added dropwise 66.5 g (0.62 mole) of ethyl chloroformate followed by a solution of 28.0 g (0.26 mole) of sodium carbonate in 100 ml of water. After stirring in the cold for 2 hr, the reaction mixture was heated at 60° for 1 hr. The chloroform layer was separated, washed with water, dried (magnesium sulfate), and concentrated. Distillation gave 159.0 g (85%) of a colorless liquid, bp 147–150° (0.12 mm); IR: λ_{max} (neat) 5.81 and 5.92 μm; NMR: δ 1.26 (9H, t, $-OCH_2CH_3$, J = 7 Hz), 2.67 (1H, t, $-CH_2CHN(C{=}O)-$, J = 7 Hz), 3.36 (3H, s, OCH_3), 3.46 (2H, t, $-OCH_2CH_2-$, J = 6 Hz), and 4.20 (9H, q, $-COOCH_2CH_3$, J = 7 Hz).

Anal.—Calc. for $C_{17}H_{31}NO_7$: C, 56.50; H, 8.65; N, 3.88. Found: C, 56.36; H, 8.74; N, 3.96.

1,4-Dicarbethoxy-2-(4-methoxybutyl)-3-hydroxy-3-pyrroline (VI)—A suspension of 12.2 g (0.53 mole) of sodium in 500 ml of toluene was heated at reflux and treated with a solution of 159.0 g (0.44 mole) of V in 600 ml of toluene. When the addition was complete, the reaction mixture was refluxed overnight. While cooling in an ice bath, the mixture was treated dropwise with a solution of 65 ml of acetic acid and 250 ml of water. The mixture was extracted with chloroform. The organic layer was washed with water, dried, and concentrated to yield 129.0 g (93%) of a brown oil; IR: λ_{max} (chloroform) 5.73, 5.85 (sh), 5.90 (sh), 6.00, and 6.15 (sh) μm; UV: λ_{max} (ethanol) 250 and 280 nm; addition of base gave λ_{max} 278 nm. The copper chelate had a melting point of 61–65° after recrystallization from ether.

The pyrroline (VI) was converted to its semicarbazone (VII), mp (analytical sample) 158–161° (from ethanol–water).

Anal.—Calc. for $C_{16}H_{28}N_4O_6$: C, 51.60; H, 7.58; N, 15.04. Found: C, 51.79; H, 7.54; N, 15.01.

1,4-Dicarbethoxy-2-(4-methoxybutyl)-3-amino-3-pyrroline (VIII)—A solution of 125.0 g (0.40 mole) of VI and 50.5 g (0.80 mole) of ammonium formate in 2 liters of absolute ethanol was refluxed for 4 hr. The solvent was removed *in vacuo*, and the residue was partitioned between water and chloroform. The organic layer was washed with water, dried (magnesium sulfate), and concentrated to yield 120.0 g (96%) of an oil; IR: λ_{max} (chloroform) 2.88, 3.00, 5.91, and 6.09 μm; UV: λ_{max} (ethanol) 275.5 nm; NMR: δ 1.30 (6H, t, $-OCH_2CH_3$, J = 7 Hz), 3.32 (1H, s, $-OCH_3$), 3.32 (2H, t, $-OCH_2CH_2$, J = 6 Hz), 4.21 (4H, q, $-OCH_2CH_3$, J = 7 Hz), and 5.78 (2H, s, $-NH_2$).

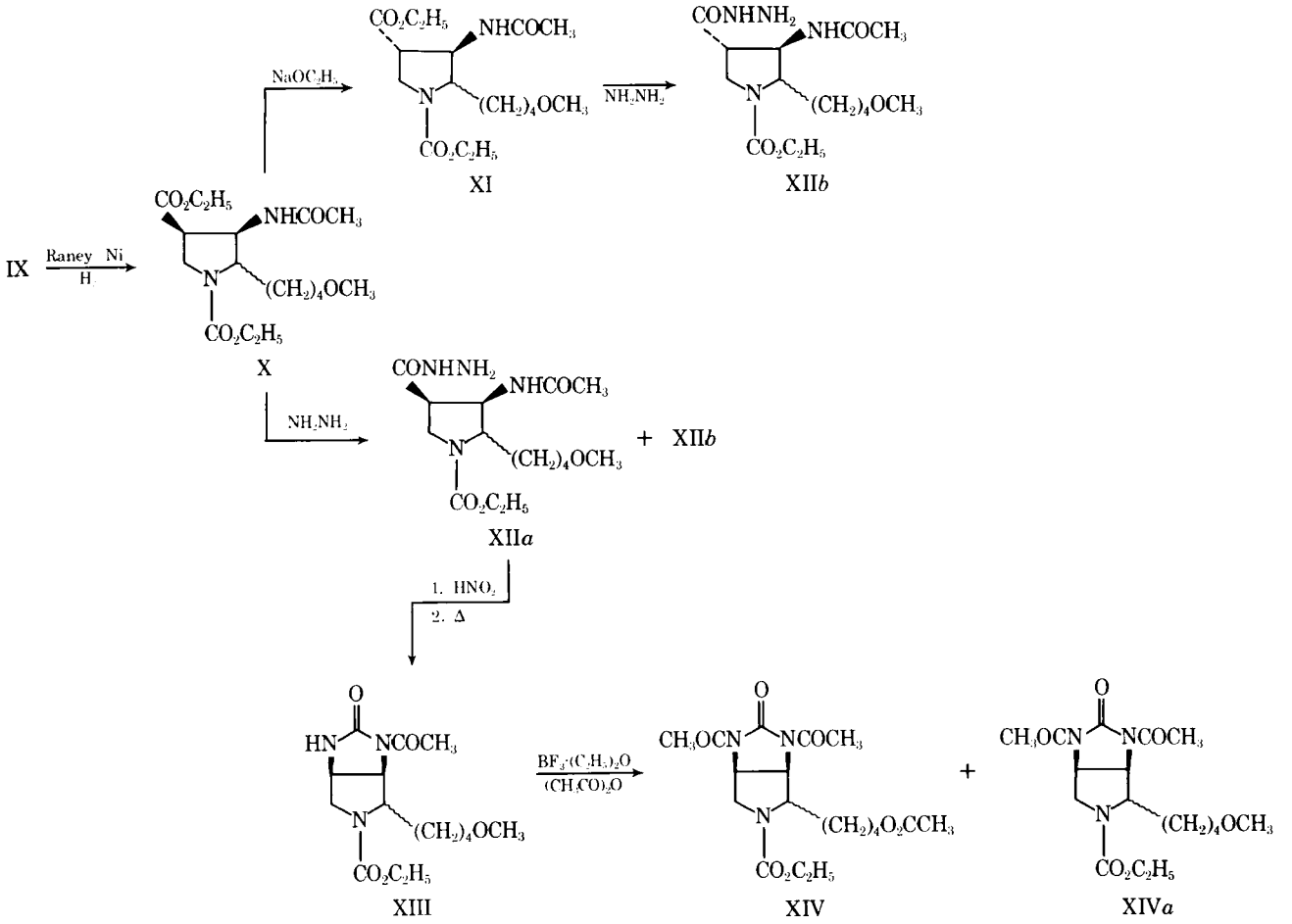
1,4-Dicarbethoxy-2-(4-methoxybutyl)-3-acetamido-3-pyrroline (IX)—A solution of 120.0 g (0.38 mole) of VIII in 300 ml of acetic anhydride was heated on an oil bath at 90–100° for 36 hr. The excess acetic anhydride was removed under high vacuum to give 135.0 g (100%) of a dark-red oil; IR: λ_{max} (chloroform) 3.10, 6.03, and 6.20 μm; UV: λ_{max} (ethanol) 275 nm; NMR: δ 1.30 (6H, t, $-OCH_2CH_3$, J = 7 Hz), 2.17 (3H, s, $-NCOCH_3$), 3.32 (3H, s, $-OCH_3$), 3.32 (2H, t, $-CH_2CH_2O$, J = 6 Hz), 4.24 (4H, q, $-OCH_2CH_3$, J = 7 Hz), and 5.67 (1H, s, —NH).

1,4-Dicarbethoxy- 2ξ- (4-methoxybutyl)-3-acetamido-3,4-*cis*-pyrrolidine (X)—A solution of 45.0 g (0.126 mole) of IX in 150 ml of absolute ethanol was treated with 25 teaspoonsful of W-2 Raney nickel catalyst and hydrogenated at 1000 psi and room temperature for 15 hr. The catalyst was filtered off, and the ethanol was evaporated *in vacuo*. The oily residue was scratched with isopropyl ether. The crystals that appeared were removed by filtration and washed with cold isopropyl ether to yield 18.5 g of 1,4-dicarbethoxy-2ξ-(4-methoxybutyl)-3-acetamido-3,4-*cis*-pyrrolidine (X), mp 95–98°. Two recrystallizations from isopropyl ether gave an analytical sample, mp 98–99°; IR: λ_{max} (chloroform) 2.91, 5.82 (sh), and 5.99 μm; NMR: δ 1.25 (6H, t, $-OCH_2CH_3$, J = 7 Hz), 2.02 (3H, s, $-NCOCH_3$), 3.36 (3H, s, $-OCH_3$), 3.36 (2H, t, OCH_2CH_2-, J = 6 Hz), 4.27 (4H, q, $-OCH_2CH_3$, J = 7 Hz), and 6.90 (1H, d, —NH, J = 5 Hz).

Anal.—Calc. for $C_{17}H_{30}N_2O_6$: C, 56.96; H, 8.44; N, 7.82. Found: C, 56.93; H, 8.40; N, 7.48.

1,4-Dicarbethoxy- 2ξ- (4-methoxybutyl)-3-acetamido-3,4-*trans*-pyrrolidine (XI)—A sample of 358 mg (1.0 mmole) of X was refluxed with 5 ml of a freshly prepared 1% sodium ethoxide

[1] Melting points were taken on a Fisher-Johns melting-point stage and a Thomas-Hoover melting-point apparatus and are uncorrected. UV absorption spectra were determined in 95% ethanol on a Beckman (model DK2A) recording spectrophotometer. IR absorption spectra were recorded on Beckman (models 8 and 33) recording spectrophotometers. NMR spectra were determined in deuterochloroform, using tetramethylsilane as reference standard, on Varian T-60 and Varian EM 360 spectrometers. Microanalyses were carried out by Spang Microanalytical Laboratory, Ann Arbor, Mich. TLC was carried out with silica gel G, silica gel $HF_{254+366}$, and silica gel $PF_{254+366}$.



Scheme II

solution for 30 min. The solvent was evaporated, and the residue was partitioned between chloroform and water. The organic layer was washed with water, dried (magnesium sulfate), and concentrated to give 325 mg (91%) of crystals of XI (the 4-epimer of X), mp 75–79°. Recrystallization from ether gave the analytical sample, mp 78–80°; IR: λ_{max} (chloroform) 2.90, 3.00 (sh), 5.82 (sh), and 5.99 μm; NMR: δ 1.26 (6H, t, $-OCH_2CH_3$, J = 7 Hz), 2.03 (3H, s, $-NCOCH_3$), 3.36 (3H, s, $-OCH_3$), 4.24 (4H, q, $-OCH_2CH_3$, J = 7 Hz), and 6.5 (1 H, broad s, NH).

Anal.—Calc. for $C_{17}H_{30}N_2O_6$: C, 56.96; H, 8.44; N, 7.82. Found: C, 56.88; H, 8.36; N, 7.76.

1-Carbethoxy-2ξ-(4-methoxybutyl)-3-acetamidopyrrolidine-4-carboxylic Acid Hydrazide (XII)—A solution of 25.0 g (0.07 mole) of X in 250 ml of absolute ethanol was treated with 50 ml of hydrazine hydrate. The mixture was heated at 45–50° for 4 hr, when TLC analysis showed the absence of starting material. The alcohol and excess hydrazine hydrate were removed *in vacuo*. The resulting product (actually a mixture of epimers consisting primarily of XII*a* with small amounts of XII*b*) weighed 24.0 g and was used directly in the preparation of XIII.

A 430-mg sample of XII was subjected to preparative TLC, utilizing 25% methanol in chloroform as the eluent. The higher R_f band gave 250 mg of a crystalline substance, mp 137–138°. Three recrystallizations from methanol–ether gave an analytical sample, mp 142–144°; IR: λ_{max} (mineral oil) 2.98, 3.02, 6.00, and 6.14 μm; NMR: δ 1.26 (3H, t, $-OCH_2CH_3$, J = 7 Hz), 2.00 (3H, s, $-NCOCH_3$), 3.33 (3H, s, $-OCH_3$), 3.33 (2H, t, OCH_2CH_2-, J = 6 Hz), 4.15 (2H, q, $-OCH_2CH_3$, J = 7 Hz), and 7.01 (3H, m, —NH). This compound was designated as the *cis*-isomer (XII*a*).

Anal.—Calc. for $C_{15}H_{28}N_4O_5$: C, 52.31; H, 8.19; N, 16.27. Found: C, 52.23; H, 8.07; N, 16.09.

The lower R_f band yielded 72 mg of an oil, which solidified on long standing in a desiccator. The solid was recrystallized from methanol–ether, mp 125–127°; IR: λ_{max} (chloroform) 3.01 and 6.01 μm; NMR: δ 1.26 (3H, t, $-OCH_2CH_3$, J = 7 Hz), 2.01 (3H, s, $-NCOCH_3$), 3.33 (3H, s, $-OCH_3$), 3.33 (2H, t, $-OCH_2CH_2$, J = 6 Hz), and 4.15 (2H, q, $-OCH_2CH_3$, J = 7 Hz).

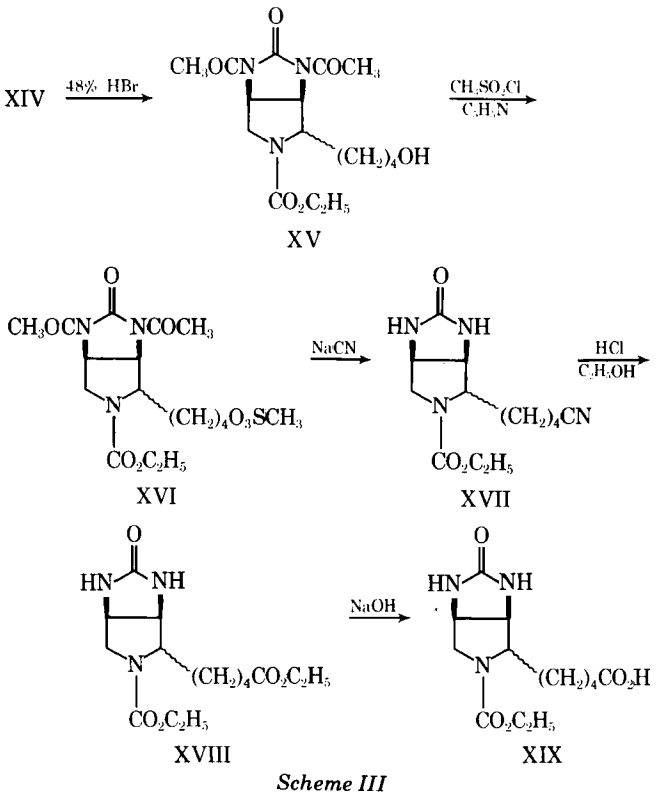
This compound was designated as the *trans*-isomer (XII*b*), since it could be readily prepared by treatment of XI with hydrazine hydrate in ethanol at room temperature.

Anal.—Calc. for $C_{15}H_{28}N_4O_5$: C, 52.31; H, 8.19; N, 16.27. Found: C, 52.13; H, 8.16; N, 16.09.

3-Acetyl-4ξ-(4-methoxybutyl)-5-carbethoxy-*cis*-hexahydropyrrolo[3,4-*d*]imidazol-2-one (XIII)—A solution of 24.0 g (0.07 mole) of a mixture of XII*a* and XII*b* in 300 ml of 1 *N* hydrochloric acid was cooled in an ice–salt bath and treated with a solution of 20 g of sodium nitrite in 100 ml of water. Near the end of the addition, a flocculent white precipitate formed in the reaction flask. The product was extracted with ethyl acetate several times. The extract was washed with water, dried (magnesium sulfate), and concentrated to give 20.2 g of solid acyl azide; IR: λ_{max} (chloroform) 4.65 μm. This solid was redissolved in 150 ml of ethyl acetate and refluxed on a steam bath for 3 hr. The solvent was evaporated to give 18.0 g of a dark oil; IR: λ_{max} (chloroform) 2.90, 3.05, and 6.05 μm; NMR: δ 1.26 (3H, t, $-OCH_2CH_3$, J = 7 Hz), 2.57 (3H, s, $-NCOCH_3$), 3.34 (3H, s, $-OCH_3$), 3.34 (2H, t, $-OCH_2CH_2$, J = 6 Hz), 4.18 (2H, q, $-OCH_2CH_3$, J = 7 Hz), and 6.57 (1H, s, NH).

1,3-Diacetyl-4ξ-(4-acetoxybutyl)-5-carbethoxy-*cis*-hexahydropyrrolo[3,4-*d*]imidazol-2-one (XIV)—A solution of 29.0 g (0.09 mole) of XIII in 100 ml of acetic anhydride was treated with 15 ml of freshly distilled boron trifluoride etherate. The mixture was heated at 50° for 3.5 hr and then concentrated under high vacuum. The residue was chromatographed on a silica gel column, using increasing concentrations of chloroform in benzene. Elution with chloroform–benzene (3:2) gave 9.4 g of XIV as an uncrystallizable oil; IR: λ_{max} (chloroform) 5.70–5.95 μm (strong, broad); NMR: δ 1.26 (3H, t, $-OCH_2CH_3$, J = 7 Hz), 2.04 (3H, s, $-OCOCH_3$), 2.58 (6H, s, $-NCOCH_3$), and 4.18 (2H, q, $-OCH_2CH_3$, J = 7 Hz).

Further elution of the column with chloroform–benzene (3:2)



Scheme III

gave 1,3-diacetyl-4ξ-(4-methoxybutyl)-5-carbethoxy-*cis*-hexahydropyrrolo[3,4-*d*]imidazol-2-one (XIV*a*) as an uncrystallizable oil; IR: λ_{max} (chloroform) 5.70–5.95 μm (strong, broad); NMR: δ 1.26 (3H, t, $-OCH_2CH_3$, J = 7 Hz), 2.58 (6H, s, $-NCOCH_3$), 3.34 (3H, s, $-OCH_3$), and 4.18 (2H, q, $-OCH_2CH_3$, J = 7 Hz).

1,3- Diacetyl- 4ξ- (4-hydroxybutyl) -5 - carbethoxy- *cis*-hexahydropyrrolo[3,4-*d*]imidazol-2-one (XV)—A solution of 9.4 g (0.024 mole) of XIV in 50 ml of 48% hydrobromic acid was stirred at room temperature for 16 hr. The water and hydrobromic acid were removed under high vacuum. The residue was chromatographed on a silica gel column, using increasing concentrations of ether in benzene. Elution with ether–benzene (1:4) gave 3.3 g of XV as an uncrystallizable oil; IR: λ_{max} (chloroform) 2.80, 2.90, 5.70, and 5.90 μm; NMR: δ 1.26 (3H, t, $-OCH_2CH_3$, J = 7 Hz), 2.58 (6H, s, $-NCOCH_3$), 3.30 (1H, s, CH_2OH), 3.60 (2H, m, CH_2CH_2OH), and 4.15 (2H, q, $-OCH_2CH_3$, J = 7 Hz).

1,3-Diacetyl- 4ξ- (4-methanesulfonylbutyl)-5-carbethoxy-*cis*-hexahydropyrrolo[3,4-*d*]imidazol-2-one (XVI)—A solution of 1.63 g (0.0046 mole) of XV in 10 ml of pyridine was treated with 3 ml of freshly distilled methanesulfonyl chloride. The solution was allowed to stand in the refrigerator for 16 hr and the pyridine and excess methanesulfonyl chloride were removed under high vacuum. The residue was dissolved in chloroform, washed with water, dried (magnesium sulfate), and concentrated to give 1.90 g (96%) of an uncrystallizable oil; IR: λ_{max} (chloroform) 5.70, 5.92, 7.05, and 8.53 μm; NMR: δ 1.26 (3H, t, $-OCH_2CH_3$, J = 7 Hz), 2.59 (3H, s, $-NCOCH_3$), 3.02 (3H, s, $-SO_3CH_3$), and 4.16 (2H, q, $-OCH_2CH_3$, J = 7 Hz).

4ξ- (4-Cyanobutyl)-5- carbethoxy-*cis*- hexahydropyrrolo-3,4-*d*]imidazol-2-one (XVII)—Compound XVI (1.68 g, 0.0039 mole) was dissolved in 5 ml of methanol. To this solution was added a solution of 1.62 g of sodium cyanide in 5 ml of water. The reaction mixture was diluted with 5 ml of methanol to remove the cloudiness and was then refluxed for 1 hr. After concentration to dryness *in vacuo*, the residue was partitioned between water and ethyl acetate. The ethyl acetate layer was dried (magnesium sulfate) and concentrated to an uncrystallizable oil (620 mg); IR: λ_{max} (chloroform) 2.83, 3.00, 4.40, and 5.70–6.00 μm; NMR: δ 1.26 (3H, t, $-OCH_2CH_3$, J = 7 Hz), 4.18 (2H, q, $-OCH_2CH_3$, J = 7 Hz), 5.98 (1H, broad s, NH), and 6.50 (1H, broad s, NH).

4ξ-(4-Carbethoxybutyl)-5-carbethoxy-*cis*-hexahydropyrrolo[3,4-*d*]imidazol-2-one (XVIII)—A cold solution of nitrile XVII (0.985 g, 3.51 mmoles) in 20 ml of absolute ethanol was treated with dry hydrogen chloride gas for 2 hr while keeping the reaction flask in a salt–ice bath. The reaction mixture was allowed to reach room temperature and was refluxed for 1 hr. The precipitated ammonium chloride was filtered, and the filtrate was concentrated to dryness. The residue was dissolved in chloroform, and the extract was washed with water, dried (magnesium sulfate), and concentrated under reduced pressure to give a pale-brown oil (0.920 g, 80%); IR: λ_{max} (chloroform) 2.88, 3.08, and 5.88–6.00 μm; NMR: δ 1.27 (6H, t, $-OCH_2CH_3$, J = 7 Hz), 4.17 (4H, q, $-COOCH_2CH_3$, J = 7 Hz), 6.25 (1H, broad s, NH), and 6.40 (1H, broad s, NH).

4ξ- (4-Carboxybutyl)-5- carbethoxy-*cis*- hexahydropyrrolo[3,4-*d*]imidazol-2-one (XIX)—A 1% solution of sodium hydroxide (2 ml) was added to 100 mg (0.31 mmole) of ester XVIII. After 20 min of stirring at room temperature, dissolution of the oily ester was complete. The cooled yellow solution was acidified with concentrated hydrochloric acid, and the mixture was concentrated to dryness. Two successive recrystallizations from 95% ethanol afforded colorless crystals (35 mg, 38%), mp 192–195°; IR: λ_{max} (mineral oil) 3.07, 5.85, and 6.00 μm.

Anal.—Calc. for $C_{13}H_{21}N_3O_5$: C, 52.17; H, 7.07; N, 14.04. Found: C, 52.19; H, 6.93; N, 14.13.

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[x] To whom inquiries should be directed.

Evaluation of a Convective Diffusion Drug Dissolution Rate Model

ASHOK C. SHAH [x] **and KENNETH G. NELSON** *

Abstract □ A recently introduced drug dissolution rate model based on convective diffusion was evaluated by experimentally determining dissolution rates. Alkyl *p*-aminobenzoates were used as the test compounds in a dissolution cell which promoted laminar flow of the liquid past the dissolving surface. The parameters evaluated were diffusivity, solubility, rate of shear, dissolving surface shape, and orientation of the surface relative to flow. The agreement between theory and experiment was quite satisfactory with respect to the functional dependence of the rate on these parameters as well as the actual magnitude of the rates.

Keyphrases □ Dissolution—evaluation of a convective diffusion drug dissolution rate model, evaluation of diffusivity, solubility, rate of shear, dissolving surface shape, and orientation of surface relative to flow □ Diffusion—evaluation of a convective diffusion drug dissolution rate model, alkyl *p*-aminobenzoates, discussion of various parameters □ Convective diffusion—drug dissolution rate model, experimental evaluation using dissolution rates of alkyl *p*-aminobenzoates

A convective diffusion model was introduced recently (1) to describe the phenomenon of drug dissolution rate. The equations given for the rate of dissolution, R, were:

$$R = 0.808D^{2/3}C_0\alpha^{1/3}bL^{2/3} \quad \text{(Eq. 1)}$$

for a rectangular surface of width b and length L (in the direction of flow), and:

$$R = 2.157D^{2/3}C_0\alpha^{1/3}r^{5/3} \quad \text{(Eq. 2)}$$

for a circular surface of radius r. In these equations, D is the diffusivity, C_0 is the solubility, and α is the rate of shear over the dissolving surface.

The experimental apparatus used to evaluate the applicability of the model, however, was not ideal with respect to one principle upon which the model was based. In particular, the tangential annular flow system did not facilitate the development of a well-behaved hydrodynamic boundary layer. The present report describes experimental studies on drug dissolution rates utilizing a flow system designed to produce a laminar layer over the dissolving solid. The data are evaluated in terms of the functional dependence of the experimental rate on the various parameters in Eqs. 1 and 2.

EXPERIMENTAL

A schematic diagram of the dissolution cell employed is shown in Fig. 1. It consists of two rectangular transparent plastic[1] plates, which are fastened together by bolts and wing-nuts. The top plate has a liquid entry port on one end, which opens into a rectangular channel cut into the underside of the plate. The bottom plate is recessed to accommodate the die employed in the previous study (1). When installed, the surface of the die is coplanar with the surface of the bottom plate, and the die may be oriented with either the long or short axis of the rectangular dissolving surface parallel t the direction of liquid flow. For exposing circular surfaces, th plastic die employed in the previous study was used. The bottor plate also has a vertical duct, which serves as an outlet for th flowing liquid.

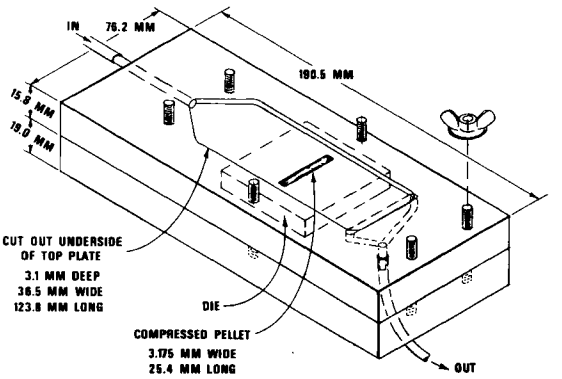

Figure 1—*Schematic diagram of the dissolution cell.*

As in the previous study (1), a compressed pellet of an alky ester of *p*-aminobenzoic acid was prepared in the metal or plasti die. The die was then placed in the recess of the lower plate, an the dissolution cell was assembled. Silicone grease was applied a the points of contact between the plates to ensure a watertigh seal. The cell was positioned on a leveled, vibration-free platform and distilled water (25°) was pumped through the cell at a set flo rate with an infusion syringe pump[2]. The effluent liquid was a sayed spectrophotometrically[3] at 285 nm for the ester. The esti concentration, together with the flow rate, permitted calculation o the experimental dissolution rate.

The solubilities of the methyl through pentyl esters of *p*-amino benzoic acid were determined by equilibrating an excess amount o each with distilled water for 2 days at 25°. After filtration and ap propriate dilution, the concentration was determined spectrophotometrically.

RESULTS AND DISCUSSION

The dissolution cell employed was designed with a relative long liquid flow channel to promote the development of lamin liquid flow in the region of the dissolving solid. To verify that su was the case, the flow visualization technique used before (1), i volving a water-soluble dye dispersed in polyethylene glycol 60C was employed. The dissolved dye displayed a wedge-shaped pa tern, which flowed over the dissolving surface as desired. The li uid flow appeared to be uniform and homogeneous.

The dependence of the experimental dissolution rate on solubi ty was studied by determining the rates for the methyl throu pentyl esters of *p*-aminobenzoic acid. The rates were determin with the long axis of the dissolving surface perpendicular to the d rection of liquid flow and with a flow rate of 5.5 ml/min. The da are shown as a log–log plot of dissolution rate *versus* solubility Fig. 2; this plot yields a least-squares slope of 0.978 and a correl tion coefficient of 0.999. This slope corresponds satisfactorily wi the slope of unity predicted by Eq. 1, assuming the diffusivities be essentially constant.

[1] Plexiglas.

[2] Model 220, Sage Instruments, Cambridge, MA 02139

[3] Zeiss recording spectrophotometer DMR 21, Oberkochen/Wuertt, W Germany.

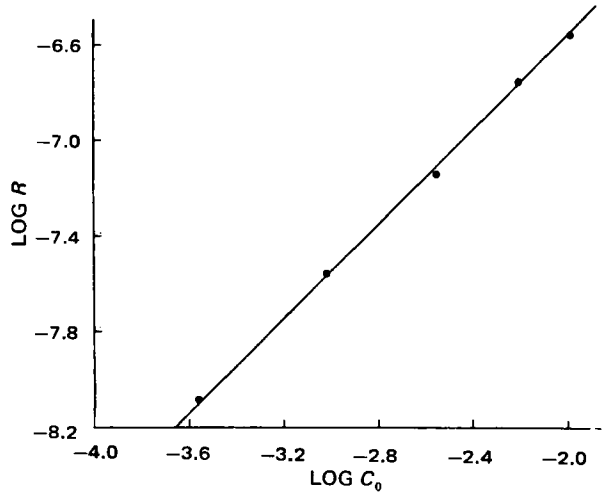

Figure 2—*Log–log plot of dissolution rate as a function of solubility. Log* C_0 *for the methyl ester is −1.98; log* C_0 *values decrease sequentially from the methyl to the pentyl ester.*

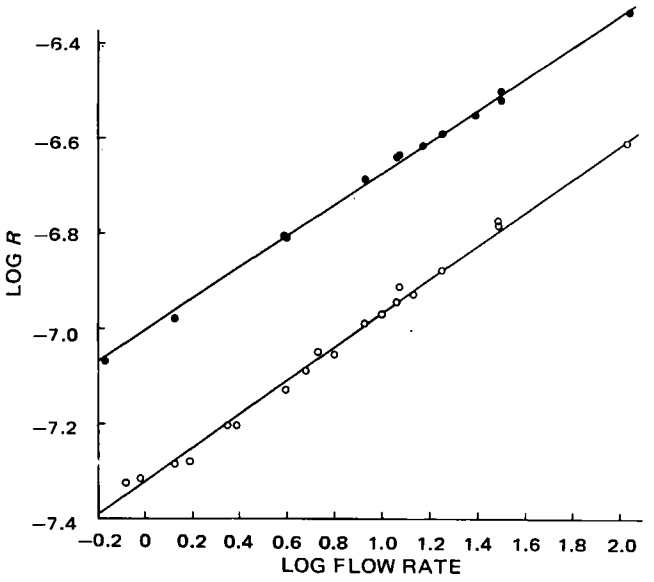

Figure 3—*Log–log plot of dissolution rate as a function of fluid flow rate.*

The effect of the rate of shear can be demonstrated by measuring the dissolution rate at various rates of flow. If the edge and end effects are neglected, the velocity profile for flow in the dissolution cell, *i.e.*, flow between two parallel planes, is parabolic in nature (2). If the limiting slope of the velocity profile at either plane is taken to be the rate of shear, then it can be shown that (see *Appendix*):

$$\alpha = \frac{6Q}{H^2W} \qquad \text{(Eq. 3)}$$

where Q is the volumetric flow rate, and H and W are the height and width, respectively, of the channel in the dissolution cell. Inasmuch as α is directly proportional to Q in Eq. 3, a plot of log R *versus* log Q should give a straight line with a slope of ~0.33 according to Eq. 1. Such a plot is shown in Fig. 3 for ethyl *p*-aminobenzoate with the long axis of the dissolving surface perpendicular to (upper curve) and parallel with (lower curve) the direction of flow. The least-squares slope of the upper curve is 0.336 with a correlation coefficient of 0.999, and the corresponding values for the lower curve are 0.359 and 0.997. The agreement between theory and experiment with respect to the functional dependence of the dissolution rate on the rate of shear is quite satisfactory.

The convective diffusion equations are based on the fluid having a uniform density; *i.e.*, natural convection is not considered. To determine whether density gradients in the vicinity of the dissolving surface might be present and influence the experimental rates in the present studies, the experiments at different flow rates were repeated with the dissolution cell inverted. The data from these experiments are shown in Fig. 4. It can be seen that natural convection has an influence on the dissolution rate at relatively slow flow rates and when the long axis of the dissolving surface is parallel to the direction of flow (lower curve). The slopes and correlation coefficients of the upper and lower lines in Fig. 4 are 0.342 and 0.998 and 0.331 and 0.994, respectively, and are in good agreement with the theoretical slope of 0.333. The first four points from the lower set of data were omitted in the statistical calculations.

The relative position of the two lines in each set of data (Figs. 3 and 4) is due to the orientation of the rectangular dissolving surface with respect to the direction of flow. The die was designed with a length-to-width ratio of the dissolving surface of 8:1. As such, the convective diffusion theory (Eq. 1) would predict a twofold increase in dissolution rate in going from an orientation having the long axis of the dissolving surface parallel to the direction of flow to that having the long axis perpendicular. The distance between the lines at about the midrange of the data (log flow rate = 1.0) is 0.294 in Fig. 3 and 0.272 in Fig. 4. This correlation between theory and experiment is quite satisfactory inasmuch as Eq. 1 predicts the difference between the lines on these plots of 0.301 (*i.e.*, log of 2). This effect emphasizes the importance of including convection along with diffusion when considering drug dissolution phenomena. To consider the dissolution rate simply as proportional to area is inadequate.

A further evaluation of the relationship between the geometry of the dissolving surface and the dissolution rate was accomplished by determining the dissolution rates from the surface of a series of circular disks of ethyl *p*-aminobenzoate. Equation 2 indicates that a log–log plot of dissolution rate *versus* radius would be linear with a slope of ~1.67. Data from this series of experiments with a flow rate of 24 ml/min is shown in Fig. 5; the plot yields a least-squares slope of 1.619 and a correlation coefficient of 0.999. Additional data (not shown) having a flow rate of 14.7 ml/min gave a slope of 1.656 with a correlation coefficient of 0.998. The functional dependence of the dissolution rate on the radius as predicted by Eq. 2 is thus borne out by experiment.

The convective diffusion equations indicate that the dissolution rate is a function of the diffusivity to the two-thirds power. A convenient method of experimentally studying the dependence on the diffusion coefficient is to carry out experiments using a solubilized system (3). In this method, it is assumed that the free and solubilized solutes are in equilibrium in any small volume so that the sol-

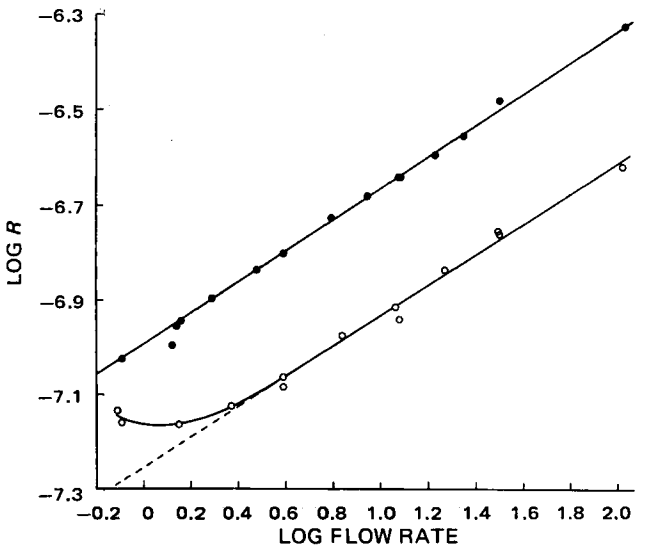

Figure 4—*Log–log plot of dissolution rate as a function of fluid flow rate with the dissolution cell inverted.*

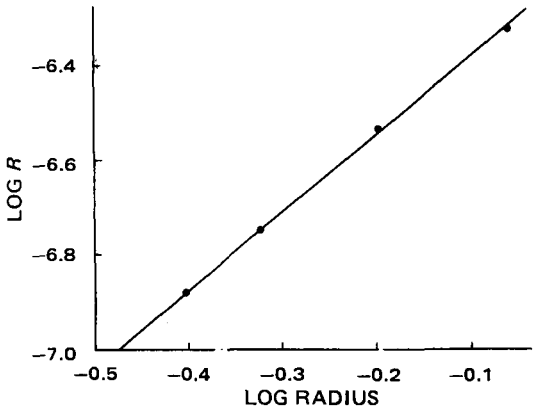

Figure 5—*Log-log plot of dissolution rate as a function of tablet radius.*

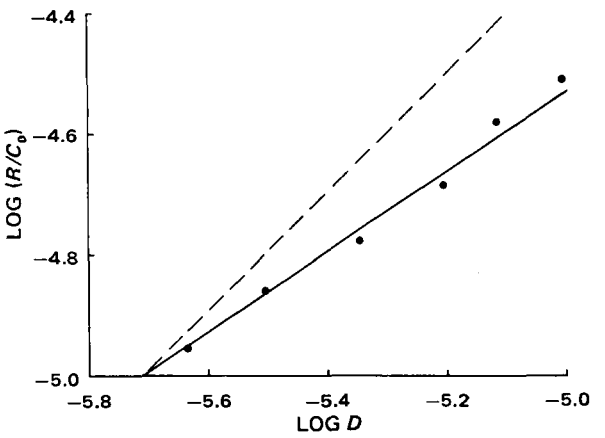

Figure 6—*Log-log plot of (dissolution rate/solubility) as a function of diffusivity.*

ubility and diffusivity measured on a macroscopic level may be employed as the effective parameters in a theoretical treatment.

Dissolution rate experiments were carried out with disks (r = 0.476 cm) of ethyl *p*-aminobenzoate at 37° and a flow rate of 14.9 ml/min. The liquid medium was water containing various concentrations (0–6%) of polysorbate 80, a system for which the effective solubility and effective diffusion coefficient of ethyl *p*-aminobenzoate have been determined (3). The data are shown in Fig. 6 as a plot of log R/C_0 *versus* log D, which yields a least-squares slope of 0.706 and a correlation coefficient of 0.990. These values are in satisfactory agreement with convective diffusion theory in that Eq. 2 indicates a slope of 0.667.

A dashed line of slope 1 is included in Fig. 6 to permit visual comparison of the experimental data with the expected relationship according to the commonly employed stagnant diffusion layer model (1). It is apparent that the convective diffusion model more accurately describes the role of diffusion in the process of dissolution into a moving liquid.

These considerations have focused primarily on the functional dependence of the dissolution rate on solubility, diffusivity, rate of shear, and geometry and orientation of the dissolving surface. To complete this evaluation, the actual value of a dissolution rate is calculated, based on independently determined parameters, and compared with the corresponding experimental dissolution rate. For this purpose, the rate corresponding to the first point (no polysorbate) on Fig. 6 is used. The diffusivity and solubility were reported (3) to be 9.86×10^{-6} cm^2/sec and 7.27×10^{-6} mole/cm^3, respectively. The rate of shear is calculated to be 4.25 sec^{-1} with Eq. 3 by substituting in the dimensions of the dissolution cell and the flow rate (14.9 cm^3/min). These values, together with the dissolving surface radius of 0.476 cm, are substituted into Eq. 2 to give a theoretical dissolution rate of 2.03×10^{-7} mole/min. The experimentally determined dissolution rate for these conditions is 2.25×10^{-7} mole/min. The ability of the model to predict the dissolution rate to within 10% of the actual value inspires considerable confidence in the convective diffusion approach to describing drug dissolution rate.

The convective diffusion approach enables one to describe drug dissolution rates in terms of the fundamental processes of convection and diffusion. As discussed in Ref. 1, the model currently utilized to describe drug dissolution is the Noyes–Whitney equation, which includes the Nernst unstirred diffusion layer. This model is useful, but the general approach cannot account for such factors as agitation and the orientation of the surface relative to flow because convection is not included in the mathematical model.

The convective diffusion model considered in this report is also somewhat limited in that it describes dissolution under rather idealized conditions. Under these conditions, however, the model can quantitatively describe the effects of solubility, diffusivity, rate of shear (agitation), and geometry and orientation of the surface relative to flow. Any inability of the model to describe drug dissolution from tablets or other solid dosage forms will be primarily due to experimental conditions that differ from those assumed in the model, *e.g.*, flow over a curved surface such that the boundary layer leaves the surface. The constant rate of shear was assumed in the model to retain a high degree of generality. It is recognized that the rate of shear over a surface can be more complex because of such conditions as end effects, distance downstream, curved surfaces, and natural convection.

APPENDIX

The flow pattern between two parallel planes a distance H apart (in the Z direction) is described by a parabolic function of Z. If the edge and end effects are ignored, the velocity function, v_x, for flow in a cell of width W and the length l is given by:

$$v_x = \frac{\Delta P H^2}{8 \mu l}\left[1 - \left(\frac{2Z - H}{H}\right)^2\right] \qquad \text{(Eq. A1)}$$

where ΔP is the pressure difference over l, and μ is the viscosity (2). The volumetric flow rate, Q, is given by (2):

$$Q = \frac{\Delta P H^3 W}{12 \mu l} \qquad \text{(Eq. A2)}$$

Differentiating Eq. A1 with respect to Z yields:

$$\frac{dv_x}{dz} = \frac{\Delta P(H - 2Z)}{2 \mu l} \qquad \text{(Eq. A3)}$$

This differential is the rate of shear. In the vicinity of the dissolving surface, the rate of shear, α, may be considered to be constant and is thus obtained by substituting $Z = 0$ into Eq. A3. If this result is combined with Eq. A2, an equation relating α with the volumetric flow rate results:

$$\alpha = \frac{6Q}{H^2 W} \qquad \text{(Eq. A4)}$$

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Comparative Bioavailabilities from Truncated Blood Level Curves

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Abstract □ The period of time after administration over which blood level measurements are required to obtain a reliable bioavailability comparison of two or more formulations of the same drug was considered by the analysis of bioavailability data taken from the literature. The drugs examined, selected to represent a range of absorption and elimination half-lives, were acetaminophen, aminosalicylic acid, chloramphenicol, chlordiazepoxide, digoxin, isoniazid, phenylbutazone, sulfamethizole, tetracycline, and warfarin. For most drugs, ratios of areas under the curve changed little between the end of the absorption period and the time when blood sampling was terminated. Reliable bioavailability comparisons among different brands of the drugs apparently could have been made by blood sampling over 24 hr or less.

Keyphrases □ Bioavailability—length of time for blood level measurements, analysis of literature data, 10 drugs considered, truncated blood level curves □ Blood level curves, truncated—comparative bioavailabilities of 10 drugs, analysis of literature data, determination of length of time necessary for blood level measurements □ Sampling, blood—time necessary for bioavailability studies, analysis of literature data, truncated blood level curves

Bioavailability is an important parameter in the comparison of commercial drug formulations. It was defined by Riegelman (1) as the relative rate and extent at which an administered dose reaches the general circulation. However, it is commonly interpreted as only the relative extent of absorption and is expressed as the percent ratio of test to reference formulations absorbed. This ratio is estimated either by the appropriate ratio of total areas under the curves of drug concentration in the blood following administration of the doses (2) or by the ratio of the total cumulative amounts of test and reference drugs excreted in the urine.

Ideally, the areas under the blood concentration curves *(AUC)* should be calculated to infinite time; but in practice, it is usually suggested that areas calculated over three elimination half-lives are sufficient (3). The extrapolation to infinity can frequently be made but often makes no appreciable difference to the final bioavailability estimate. For many commercial formulations, it was observed that the ratios of areas of test to reference formulations approach a limiting value many hours prior to the complete elimination of the drug. In this study, 10 drugs of widely differing kinetic properties were examined to determine how long blood sampling should continue after drug administration to estimate adequately the ultimate test to reference *AUC* ratio.

EXPERIMENTAL

The data used came from both the literature and internal unpublished investigations. Bioavailability studies comparing different formulations of the same drug commonly report blood levels at each sampling time averaged (arithmetic means) over all subjects.

Table I—*AUC* Ratios (Percent) of Acetaminophen Formulations[a]

Formulation	Blood Sampling Time, hr						
	0.33	0.67	1.00	1.50	2.00	4.00	6.00
K_1	120	88	83	86	88	92	93
K_2	139	105	93	87	86	86	87
K_3	110	84	78	78	79	78	79
N	121	81	69	71	76	83	85
O	161	113	97	94	96	97	97
P	153	122	111	108	108	106	102
Q	88	75	74	80	86	93	89
S	197	131	109	101	100	97	97

[a] Calculated from McGilveray *et al.* (5).

By using these average blood levels, the *AUC* at each sampling time was determined by the trapezoidal rule for the test and reference formulations, and *AUC* ratios were calculated at these sampling times.

Occasionally, blood levels are reported for individual subjects. In these cases, areas from the time of administration to each sampling time were calculated by the trapezoidal rule for each subject. Geometric means of the *AUC*'s at each sampling time were determined for each formulation in the study, and *AUC* ratios of test to reference formulations were calculated. Graphs of *AUC* ratios *versus* time were tested for parallelism, height, and slope, using the method of profile analysis (4).

RESULTS

Acetaminophen—*AUC* ratios at each sampling time from 20 min to 6 hr were calculated from the individual blood level data of McGilveray *et al.* (5). Tablet R (5) was used as the reference formulation. The curves constructed from the bioavailability–time data (Table I) were found to be approximately parallel ($p > 0.9$) and at the same level ($p > 0.3$). *AUC* ratios did not change significantly after 2 hr postadministration ($p > 0.2$).

Aminosalicylic Acid—The individual blood level data reported by Schirmer *et al.* (6) were not in a suitable form for profile analysis. Mean blood level curves were used to calculate the *AUC* ratios at 2, 4, 6, 8, and 12 hr postadministration (Table II). This study included uncoated and enteric-coated tablets, and uncoated Tablet A was used as the reference formulation. Plasma levels of the drug from some enteric-coated tablets were low and erratic and were not included in the analysis. There was little change in the *AUC* ratios of uncoated Tablets B, C, and D after 6 hr. Enteric-coated Tablet E1 showed slowly increasing *AUC* ratios to 12 hr.

Wagner *et al.* (7) recently reported blood level curves of aminosalicylic acid obtained from solutions of the sodium salt, suspension, compressed tablets, and enteric-coated tablets. In all cases the absorptive phases were complete within 3 hr.

Chloramphenicol—Mean plasma levels determined colorimetrically by Glazko *et al.* (8) were used to calculate *AUC* ratios of four brands of chloramphenicol capsules (Table III). Formulation A was used as the reference. Six hours after administration, the *AUC* ratios approached a constant value for Capsules B and C. The *AUC* ratio of Capsule D appeared to increase slightly between 6 and 24 hr but remained very low compared to B and C.

Chlordiazepoxide—*AUC* ratios determined in this laboratory[1] are given in Table IV for from 1 to 54 hr. The ratios were calculat-

[1] I. J. McGilveray and G. L. Mattok, unpublished work.

Table II—*AUC* Ratios (Percent) of Aminosalicylic Acid Formulations[a]

Formulation	Blood Sampling Time, hr				
	2	4	6	8	12
B	92	84	78	78	78
C	80	83	85	85	85
D	97	91	85	83	83
E2	—	100	100	100	100
E1	—	78	86	94	98

[a] Calculated from Schirmer *et al.* (6).

Table III—*AUC* Ratios (Percent) of Chloramphenicol Formulations[a]

Formulation	Blood Sampling Time, hr							
	0.5	1.0	2.0	4.0	6.0	8.0	12.0	24.0
B	19	22	32	45	52	54	55	53
C	24	32	45	57	62	63	64	62
D	10	12	16	22	26	29	32	35

[a] Calculated from Glazko *et al.* (8).

ed on the basis of total drug, *i.e.*, free chlordiazepoxide and metabolite. With the exception of Formulation 43, the bioavailabilities calculated at 7 hr were within 12% of those calculated at 54 hr. The bioavailability of Formulation 43 increased from 1 to 54 hr, although the increase after 24 hr was only about 10%. *AUC* ratios calculated from the data of Foldes *et al.* (9) varied erratically between 4 and 24 hr.

Digoxin—Table V gives the *AUC* ratios of a digoxin formulation calculated from the data of Wagner *et al.* (10); Formulation A was used as the reference. The ratios remain unchanged, within a few percent, from 1.5 to 96 hr. More than half the area under the curve to 96 hr was in the 24–96-hr interval.

After 24 hr, the plasma levels were low and their determination was subject to greater error than when the plasma level was high. Thus, measurement of plasma levels over a period of days apparently gives no additional information about relative availability and, in fact, may increase experimental error.

Isoniazid—*AUC* ratios were calculated from the individual blood level data of Gelber *et al.* (11), using Formulation 4 as the reference (Table VI). Results of profile analysis indicated that curves of *AUC* ratios *versus* time were approximately parallel ($p > 0.7$) and at the same level ($p > 0.8$). For each curve, the analysis showed that the slope was not significantly different ($p > 0.1$) from zero in the time interval from 2 to 8 hr.

Phenylbutazone—*AUC* ratios of nine tablet formulations were calculated from the mean plasma level data of Van Petten *et al.* (12), taking a solution of the drug as the reference formulation.

Table IV—*AUC* Ratios (Percent) of Chlordiazepoxide Formulations[a]

Formulation	Blood Sampling Time, hr								
	1	3	5	7	24	27	31	48	54
31	100	103	102	102	105	105	103	101	100
32	78	94	102	104	103	103	102	100	100
33	76	95	102	102	101	100	99	96	95
34	99	103	107	107	104	104	102	96	95
35	84	88	90	92	96	96	95	93	92
41	109	104	107	110	118	117	117	116	116
42	88	85	89	92	98	98	98	97	96
43	36	41	49	55	68	68	70	76	77
44	63	74	86	90	95	95	96	99	100
45	47	63	79	85	93	93	92	92	92
51	110	109	105	104	100	99	99	100	100
52	111	107	105	106	104	104	104	106	106
53	120	108	103	104	106	106	105	103	102
54	108	112	113	112	108	108	108	107	107
55	94	93	95	96	94	93	93	94	94

[a] Calculated from unpublished data of I. J. McGilveray and G. L. Mattok of this laboratory.

Table V—*AUC* Ratios of a Digoxin Formulation[a]

t, hr	*AUC* Ratio, %
0.25	27
0.50	36
0.75	43
1.00	49
1.50	54
3.00	54
5.00	52
12.00	51
24.00	54
48.00	54
72.00	57
96.00	57

[a] Calculated from Wagner *et al.* (10).

Table VI—*AUC* Ratios (Percent) of Isoniazid Formulations[a]

Formulation	Blood Sampling Time, hr								
	0.25	0.50	0.75	1.00	1.50	2.00	4.00	6.00	8.00
1	31	34	54	71	90	100	109	109	108
2	47	77	94	104	111	114	116	115	113
3	73	75	83	91	102	109	112	111	112
5	40	48	73	83	94	100	110	111	112
6	59	84	91	97	105	107	105	103	102

[a] Calculated from Gelber *et al.* (11).

Van Petten *et al.* divided their study into two groups (Table VII). Except for Formulation D, the ratios varied little after 12 hr but varied considerably before that time. The extended time required to reach constant *AUC* ratios may be due to the fact that many phenylbutazone tablets are coated.

Sulfamethizole—*AUC* ratios were calculated from the individual blood level data of Mattok and McGilveray (13), using Tablet B as the reference (Table VIII). The *AUC* ratio curves were approximately parallel ($p > 0.7$), at the same level ($p > 0.6$), and constant in slope ($p > 0.3$) over the profile.

Table VII—*AUC* Ratios (Percent) of Phenylbutazone Formulations[a]

Formulation	Blood Sampling Time, hr						
	2	4	6	8	12	24	48
			Group I				
D	24	34	45	42	61	72	79
C	87	83	83	84	86	87	87
A	104	100	99	99	99	99	99
F	58	63	68	73	78	82	84
E	18	28	37	44	50	57	62
			Group II				
H	61	70	79	85	89	95	101
J	152	131	120	115	111	108	108
I	104	98	95	95	95	98	98
G	152	131	121	118	113	107	104

[a] Calculated from Van Petten *et al.* (12).

Table VIII—*AUC* Ratios (Percent) of Sulfamethizole Formulations[a]

Formulation	Blood Sampling Time, hr				
	1.5	2.5	4.0	6.0	8.0
C	92	95	105	112	114
D	61	80	90	93	93
E	58	65	85	98	100

[a] Calculated from Mattok and McGilveray (13).

Table IX—*AUC* Ratios (Percent) of Tetracycline Formulations[a]

Formulation	Blood Sampling Time, hr					
	2	3	4	6	9	24
A	68	74	78	81	81	78
B	68	71	73	74	74	72
C	81	84	83	82	83	82
D	86	90	92	93	94	95
E	81	79	78	78	77	71
F	29	32	35	36	34	29
G	26	27	27	26	25	22
H	71	76	79	81	81	78
K	76	79	81	83	83	83

[a] Calculated from Lovering *et al.* (14).

Table X—*AUC* Ratios (Percent) of Warfarin Formulations[a]

Formulation	Blood Sampling Time, hr							
	1	4	8	12	24	48	72	96
C	125	112	108	108	107	105	104	103
D	82	95	98	98	97	95	93	92

[a] Calculated from Wagner *et al.* (15).

Tetracycline—Lovering *et al.* (14) reported individual blood level data for nine tablet formulations, and these data were used to calculate *AUC* ratios relative to a reference solution. Results of the profile analysis (Table IX) showed that the *AUC* ratios of Tablets F and G differed significantly ($p > 0.05$) from the other tablets. The mean *AUC* ratio of F and G decreased between 6 and 24 hr, but the mean ratio for the remaining formulations was level from 6 to 9 hr and then decreased to 24 hr. The decrease for both groups was slight.

Warfarin—Individual plasma level data from Wagner *et al.* (15) were analyzed for three 5-mg tablets, using Formulation A as the reference (Table X). Results of profile analysis indicated that curves were approximately parallel ($p > 0.8$) and at approximately the same level ($p > 0.07$). The hypothesis of equal *AUC* ratios across all sampling times was not rejected ($p > 0.6$).

Based on a one-compartment model, approximate first-order absorption and elimination rate constants, k_1 and k_2, respectively, were taken from the papers cited or were calculated from:

$$C_t = \frac{fD}{V}\frac{k_1}{k_1 - k_2}(e^{-k_2 t} - e^{-k_1 t}) \qquad \text{(Eq. 1)}$$

where C_t is the concentration of drug in the blood at time t; f is the fraction of dose, D, absorbed; and V is the apparent volume of distribution (16) (Table XI). Elimination rate constants were calculated using the final points on the mean blood level–time curves. Absorption rate constants were calculated from the first derivative of Eq. 1 and the mean peak blood level time, t^*, when $dC_t/dt = 0$:

$$t^* k_2 - \ln k_2 = k_1 t^* - \ln k_1 \qquad \text{(Eq. 2)}$$

The time to 99% absorption was calculated by assuming the exponential disappearance of drug from the GI tract at a rate controlled by k_1 (Table XI).

DISCUSSION

Ten formulations (Tables I–X) exhibited changes in *AUC* ratios of more than 15% after the estimated absorption period. The *AUC* ratios of an enteric-coated aminosalicylic acid tablet measured against an uncoated reference tablet increased from 78 to 98% between 4 and 12 hr. The increase may be the result of slow drug release from the enteric-coated tablet. The *AUC* ratio of chlordiazepoxide Formulation 43 increased 57% between 5 and 54 hr, but the increase was only 10% after 24 hr. Formulations 44 and 45 showed some increase between 5 and 24 hr but were relatively constant thereafter.

Three phenylbutazone formulations showed *AUC* ratio changes of more than 15% between 6 and 48 hr, but only slight changes occurred after 24 hr. The results may indicate that the time for complete absorption lies between 6 and 24 hr for a number of phenylbutazone formulations. Chloramphenicol Formulations B and D (Table III) and Formulation F (Table IX) showed large changes in the *AUC* ratio in the postabsorption period, but these formulations were of low bioavailability.

The constancy of the *AUC* ratios in these studies, a few hours after administration of the drug, suggests that it may not be necessary to follow blood levels to complete elimination of the drug, or even over two to three elimination half-lives, to obtain *AUC* ratios that are approximately equal to the bioavailability (3). The time over which samples should be taken depends upon the relative values of the test and reference absorption rate constants, k_1 and k_1^*, respectively, and the elimination rate constant, k_2.

The *AUC* ratios also depend upon the time available for absorption. Formulation comparison studies are usually carried out in starved, healthy subjects. Under these conditions, the rate at which the drug, whether in solution or not, flows through those regions of the GI tract that favor dissolution and absorption may be uniform; for certain regions of the GI tract, the rate may be rapid.

Consider a one-compartment model with first-order absorption and elimination, in which absorption is allowed to proceed from time zero to time T, where T is the end of the absorption period. The concentration of drug in the blood at time t is given by Eq. 1 if $t \leq T$. If $t > T$:

$$C_t = C_T e^{-k_2(t-T)} \qquad \text{(Eq. 3)}$$

where C_T is the concentration of drug in the blood at time T. If $t \leq T$ (16), the corresponding *AUC*'s to time t are:

$$A_t = \frac{fD}{V}\frac{1}{k_2(k_1 - k_2)}(k_2 e^{-k_1 t} - k_1 e^{-k_2 t} + k_1 - k_2) \qquad \text{(Eq. 4a)}$$

If $t > T$:

$$A_t = A_T + \frac{C_T}{k_2}(1 - e^{-k_2(t-T)}) \qquad \text{(Eq. 4b)}$$

where A is the *AUC* at time T.

The *AUC* ratios of test to reference formulations follow. Quantities marked by an asterisk refer to the reference formulation. If it

Table XI—Approximate First-Order Appearance and Elimination Rate Parameters

Drug	Reference	Appearance Constant (k_1), hr^{-1}	Elimination Constant (k_2), hr^{-1}	Time to Peak, hr	Time to 99% Absorption, hr
Acetaminophen	5	1.80	0.23	1.3	2.5
Aminosalicylic acid	6	2.00	0.80	2.0	2.3
Chloramphenicol	8	1.20	0.30	2.0	3.7
Chlordiazepoxide	—[a]	1.70	0.02	3.0	3.3
Digoxin	10	4.00	0.07	1.0	1.2
Isoniazid	11	2.90	0.20	1.0	1.6
Phenylbutazone	12	0.75	0.01	5.0	6.0
Sulfamethizole	13	0.90	0.56	1.5	5.0
Tetracycline	14	0.85	0.09	3.0	5.0
Warfarin	15	1.40	0.02	<4.0	3.3

[a] Unpublished data of I. J. McGilveray and G. L. Mattok.

Table XII—Calculated *AUC* Ratios for $k_2 = 0.1\ hr^{-1}$

	for $k_1^* = 0.5\ hr^{-1}$ and $k_1 = 0.125\ hr^{-1}$						for $k_1^* = 4.0\ hr^{-1}$ and $k_1 = 1.0\ hr^{-1}$							
T, hr	2 hr	4 hr	8 hr	12 hr	24 hr	∞	0.5 hr	1.0 hr	2.0 hr	4.0 hr	8.0 hr	12.0 hr	24.0 hr	∞
0.5	0.27	0.27	0.27	0.27	0.27	0.27	0.38	0.43	0.44	0.45	0.45	0.45	0.45	0.46
1.0	0.29	0.30	0.30	0.30	0.30	0.30	0.38	0.49	0.58	0.62	0.63	0.64	0.64	0.64
2.0	0.31	0.34	0.35	0.35	0.35	0.35	0.38	0.49	0.66	0.78	0.83	0.85	0.86	0.87
4.0	0.31	0.38	0.43	0.44	0.45	0.45	0.38	0.49	0.66	0.82	0.92	0.95	0.97	0.98
8.0	0.31	0.38	0.51	0.58	0.63	0.64	0.38	0.49	0.66	0.82	0.92	0.96	0.99	1.00
24	0.31	0.38	0.51	0.62	0.84	0.95	0.38	0.49	0.66	0.82	0.93	0.96	0.99	1.00

Table XIII—Calculated *AUC* Ratios for $k_2 = 0.01\ hr^{-1}$

	for $k_1^* = 0.5\ hr^{-1}$ and $k_1 = 0.125\ hr^{-1}$							for $k_1^* = 4.0\ hr^{-1}$ and $k_1 = 1.0\ hr^{-1}$							
T, hr	2 hr	4 hr	6 hr	8 hr	12 hr	24 hr	∞	0.5 hr	1.0 hr	2.0 hr	4.0 hr	8.0 hr	12 hr	24 hr	∞
0.5	0.27	0.27	0.27	0.27	0.27	0.27	0.27	0.38	0.42	0.44	0.45	0.45	0.45	0.45	0.45
1.0	0.29	0.30	0.30	0.30	0.30	0.30	0.30	0.38	0.49	0.58	0.61	0.62	0.63	0.64	0.64
2.0	0.31	0.34	0.34	0.34	0.35	0.35	0.35	0.38	0.49	0.65	0.77	0.82	0.83	0.85	0.87
4.0	0.31	0.38	0.41	0.42	0.44	0.45	0.45	0.38	0.49	0.65	0.81	0.90	0.93	0.96	0.98
8.0	0.31	0.38	0.44	0.49	0.55	0.61	0.64	0.38	0.49	0.65	0.81	0.91	0.94	0.97	1.00
24	0.31	0.38	0.44	0.49	0.58	0.76	0.95	0.38	0.49	0.65	0.81	0.91	0.94	0.97	1.00

is assumed that k_2, D, and V are the same for the test and reference formulations administered to a given subject, then, if $t \leq T$:

$$\frac{A_t}{A_t^*} = \frac{f}{f^*}\frac{(k_1^* - k_2)(k_2e^{-k_1t} - k_1e^{-k_2t} + k_1 - k_2)}{(k_1 - k_2)(k_2e^{-k_1^*t} - k_1^*e^{-k_2t} + k_1^* - k_2)} \quad \text{(Eq. 5a)}$$

If $t > T$:

$$\frac{A_t}{A_t^*} = \frac{A_T + \frac{C_T}{k_2}(1 - e^{-k_2(t-T)})}{A_T^* + \frac{C_T^*}{k_2}(1 - e^{-k_2(t-T)})} \quad \text{(Eq. 5b)}$$

Tables of *AUC* ratios were constructed from Eqs. 1 and 3, taking typical values of k_1^* and k_2 from Table XI. The value of k_1 was taken as $0.25k_1^*$ throughout to represent formulations from which drug is released slowly. The time to which absorption was allowed to proceed, T, varied from 0.5 to 24 hr, and the *AUC* ratio was calculated over appropriate time periods. The *AUC*'s in Tables XII and XIII at the end of the period available for absorption, $t = T$, are usually within 10–20% of the *AUC* ratio when t is infinite. The *AUC* ratios at $t = 2T$ are, in most cases, within a few percentage points of the *AUC* ratio at infinite time and experimentally indistinguishable from it. Thus, for slowly eliminated drugs, a limited period during which absorption occurs can account for the approach to constant values of *AUC* ratios long before two or three elimination half-lives have elapsed.

The duration of the absorption period may vary considerably among drugs. If a drug dissolves only at gastric pH and is administered with water on an empty stomach, the dissolution period will be fixed by the gastric emptying time and may be less than 1 hr. If absorption occurs only over a short proximal segment of the GI tract, the absorption period also may be very short. In any case, barring adhesion of drug crystals to the intestinal mucus, the absorption period is probably limited by the time required for the intestinal contents to reach solid matter in the lower intestine and certainly by the total intestinal transit time (about 30 hr).

In conclusion, analysis of blood level profiles obtained in several bioavailability studies indicated that "partial" *AUC* ratios at the end of the absorption period often agree with the total area ratios. Careful consideration of the rate constants and the general behavior observed during experimental work, which must precede any bioavailability trial, may permit shorter blood sampling schedules.

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Configuration of Dienestrol

THOMAS D. DOYLE *x, JAMES McDONALD STEWART ‡, NICOLAE FILIPESCU §, and WALTER R. BENSON *

Abstract □ Uncertainty concerning the configuration of dienestrol was resolved by a detailed spectrochemical investigation, including single-crystal X-ray diffraction, of the active drug and its stereoisomers. A symmetric structure in which the phenyl and methyl groups are *cis* about each double bond is unambiguously assigned.

Keyphrases □ Dienestrol—determination of configuration, single-crystal X-ray diffraction □ Estrogens—determination of configuration of dienestrol, X-ray diffraction □ X-ray diffraction—determination of dienestrol configuration

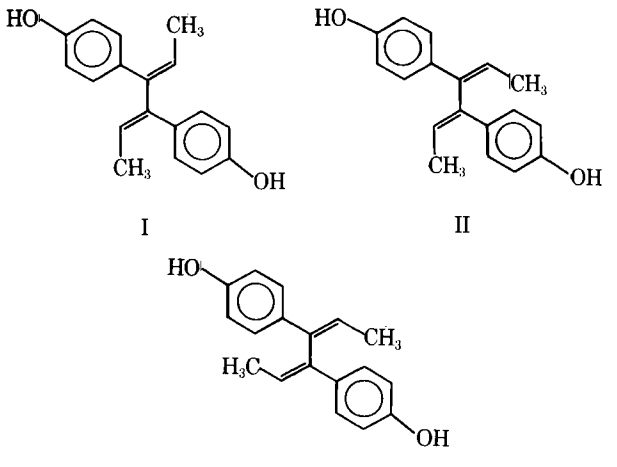
The synthetic estrogen dienestrol [3,4-bis(*p*-hydroxyphenyl)-2,4-hexadiene] was originally prepared in 1939 (1). Its exact structure, however, has remained a controversial subject (2–5); the configuration remains unspecified in NF XIV (6).

Theoretically, there are three possible stereoisomers, I–III. Koch and coworkers (2, 3) suggested configuration I for the isomer available to them. Later, presumed authentic samples of each stereoisomer were synthesized (4, 5); their corresponding diacetates were prepared by dehydration of mixtures of *meso*- and racemic 3,4-bis(*p*-acetoxyphenyl)-3,4-hexanediols. On the basis of the IR absorption of the diacetates, Lane and Spialter (5) concluded that the drug dienestrol has steric configuration III.

In view of the authors' interest in the stereochemistry and photoreactivity of synthetic estrogens (7), the question of the structure of dienestrol was reexamined from five distinct points of view: NMR spectroscopy, UV spectrophotometry, molecular models, chemical reactivity, and X-ray diffraction. Results prove that dienestrol has Structure I.

EXPERIMENTAL

Dienestrol was used as received from the supplier[1] without further purification, except that the specimen for X-ray crystallography was recrystallized from absolute alcohol, mp 227–228°.



[1] K&K Laboratories, Plainview, N.Y.

NMR spectra[2] were obtained in acetone-d_6 with tetramethylsilane internal standard.

Electronic absorption spectra were obtained in 95% ethyl alcohol on a recording spectrophotometer[3]; 1-cm silica cells were used.

X-ray crystallography was performed on an automated single-crystal X-ray diffractometer[4]. Preliminary investigation of the space group and cell dimensions were made by means of oscillation and Weissenberg photographic techniques. The accurate cell dimensions and intensities were collected on the X-ray diffractometer by the θ–2θ scan technique. All calculations for the preliminary data treatment, solution by direct methods, and refinement by full matrix least squares of the structure and the calculations of bond lengths and angles were carried out using XRAY72 (8) on a computer[5]. Figure 1 was produced using the ORTEP (9) crystallographic plotting program.

The structure determination was carried out using the NORMSF, SINGEN, PHASE, and FOURR links of the XRAY72 system of crystallographic programs. The E map produced revealed the molecule, including hydrogen atoms lying on a center of symmetry at ½, ½, ½. The coordinates from the E map were used as input to the LOADAT, CRYLSQ, BONOLA, and LSQPL links of the XRAY72 system. The result of the refinement is displayed in the figures. The final conventional R value ($\Sigma 1\Delta F1/1F_0 1$) is 0.048. The parameters refined were the scale factor, the secondary extinction factor, and the positional and thermal factors. The equation of a least-squares plane through C(1), C(2), C(3), and C(4) (see Fig. 2) is:

$$7.753X - 5.509Y - 4.454Z + 1.138 = 0 \qquad \text{(Eq. 1)}$$

These four atoms are all within 0.01 Å of this plane.

The phenyl ring system, C(4) + C(9), is also planar, with an average deviation of 0.004 Å. These two planes are found to be at an angle of 87.6° to one another[6].

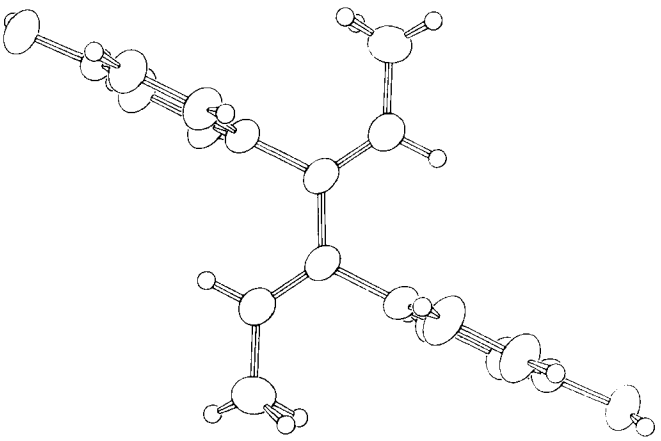


Figure 1—*The ORTEP computer plot of the whole molecule of dienestrol, showing the projection normal to the plane of C(1), C(2), C(3), and C(4).*

[2] Varian A-60 NMR spectrophotometer.
[3] Cary model 118, Applied Physics Corp., Monrovia, Calif.
[4] Picker FACS-1, Cleveland, Ohio.
[5] UNIVAC 1108.
[6] The structure factor lists were deposited with the Division of Drug Chemistry, Food and Drug Administration, Washington, DC 20204, and are available upon request to the authors.

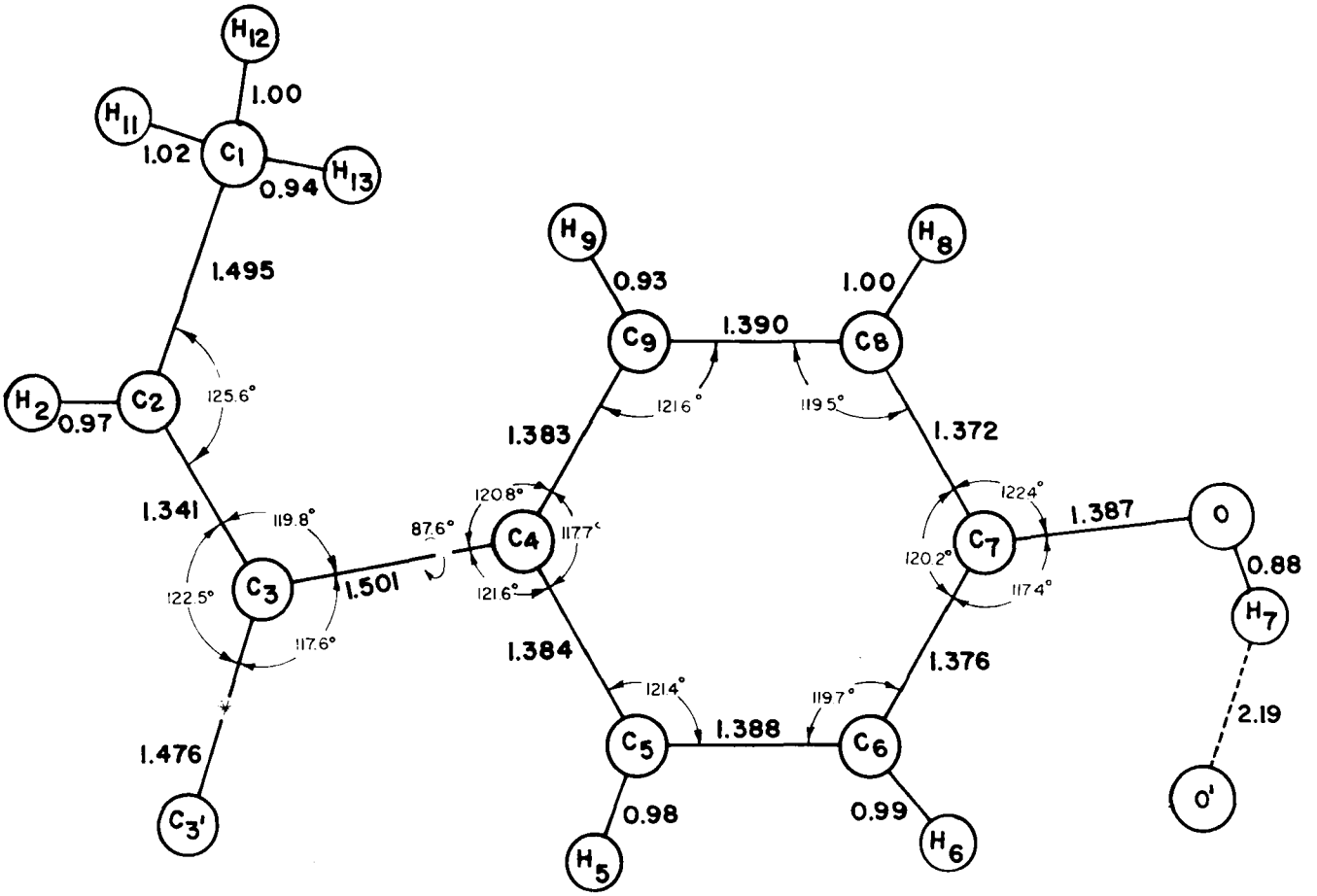


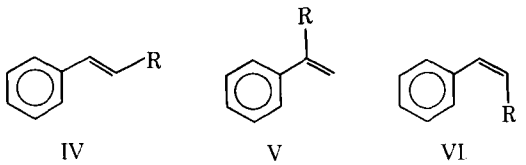
Figure 2—*Bond lengths in angstroms and angles in degrees for the refined structure of dienestrol. Since the molecule has a center of symmetry (denoted by the asterisk), only one-half of it is shown. For clarity, bond angles involving H atoms are omitted. The average estimated error in the bond angles shown is 0.15°.*

RESULTS AND DISCUSSION

The NMR spectrum of the therapeutic agent dienestrol, identical with the substance of Dodds *et al.* (1), showed that the two methyl groups have an equivalent molecular environment and identical chemical shifts, δ 1.50 ppm (doublet, J = 6.5 Hz, =CH—C*H*$_3$, 6H), and that the two vinyl protons are equivalent: δ 5.37 ppm (quartet, J = 6.5 Hz, =C*H*—CH$_3$, 2H). The only other signals in the NMR spectrum were caused by the aromatic protons at δ 6.65–7.30 ppm (multiplet, 8H) and the hydroxylic protons at δ 8.45 ppm (singlet, 2H). These findings eliminate the low symmetry Structure II as a possible dienestrol configuration.

The remaining two alternatives, I and III, could be differentiated by analysis of the UV absorption spectra of dienestrol and diacetoxy derivatives of stereoisomers I–III. Dienestrol diacetate was separately prepared from dienestrol by treatment with acetic anhydride in pyridine and recrystallization from ethanol–water, mp 113–114°. The near-UV absorption of dienestrol and its diacetate is compared to those of the other two isomeric diacetates[7] in Fig. 3. The absorption maxima of the pharmaceutical form, its diacetylated derivative, and the other diacetates are displayed at 228, 221, and 247 and 252 nm, respectively.

Evaluation of these absorption curves in terms of steric interference of substituents with normal styrene chromophore absorption allows configuration I to be assigned to dienestrol. Unsubstituted styrene, simple *trans*-β-alkylstyrenes (IV), and simple α-alkylstyrenes (V) exhibit absorption maxima in the 243–255-nm spectral region (10). On the other hand, *cis*-β-alkylstyrenes (VI) lack the characteristic styrene chromophore absorption because of out-



IV V VI

Table I—Physical Constants of Dienestrol

Molecular formula	$C_{18}H_{18}O_2$
Molecular weight	266.32 amu
Habit	Prismatic rectangular rods
Crystal size	0.25 mm along *a*, 0.4 mm along *b*, and 0.5 mm along *c*
Space group	*Pbca*
a	18.9969 (29) A
b	14.2930 (18) A
c	5.3816 (7) A
v	1461.23 A^3
ρ calculated	1.210 g/cm^3
ρ observed[a]	1.215 g/cm^3
Radiation MoKα from HOG monochromator	0.71069 A
Number of independent reflections	1457
Molecules per unit cell	4
Linear absorption coefficient, μ at MoKα	0.67 cm^{-1}
Number of parameters	128
Number of atoms in asymmetric set	19

[a] Determined by the flotation method by suspension in aqueous sodium bromide.

[7] Obtained from White Laboratories. The same source was utilized by Lane and Spialter (5).

Table II—Positional and Thermal Parameters of Atoms

				× 10^2					
	x	y	z	U_{11} or U	U_{22}	U_{33}	U_{12}	U_{13}	U_{23}
O	0.22572 (6)	0.30130 (8)	0.2933 (3)	3.78 (6)	5.24 (7)	7.06 (9)	−1.31 (5)	−0.01 (6)	−0.62 (6)
C(1)	0.42099 (12)	0.65507 (14)	0.1776 (5)	5.97 (12)	5.29 (10)	8.17 (15)	0.48 (9)	−1.99 (11)	0.93 (9)
C(2)	0.47565 (9)	0.60830 (11)	0.3341 (3)	3.76 (8)	4.56 (8)	5.22 (10)	−0.28 (6)	−0.34 (7)	−0.14 (6)
C(3)	0.47118 (7)	0.52165 (10)	0.4283 (3)	3.01 (7)	4.43 (8)	3.76 (7)	−0.25 (5)	−0.08 (6)	−0.43 (6)
C(4)	0.40574 (7)	0.46486 (10)	0.3878 (3)	3.03 (7)	4.37 (7)	3.77 (8)	−0.25 (6)	−0.36 (6)	−0.03 (6)
C(5)	0.34834 (8)	0.47213 (11)	0.5451 (3)	3.65 (7)	4.78 (8)	4.92 (9)	−0.15 (6)	0.40 (6)	−1.08 (7)
C(6)	0.28820 (8)	0.41879 (11)	0.5085 (4)	3.39 (8)	5.14 (8)	5.75 (10)	−0.29 (6)	0.94 (7)	−0.59 (7)
C(7)	0.28535 (7)	0.35675 (9)	0.3132 (3)	2.99 (7)	3.87 (7)	4.98 (9)	−0.24 (5)	−0.43 (6)	0.46 (6)
C(8)	0.34105 (10)	0.34920 (13)	0.1522 (3)	4.71 (9)	6.29 (10)	4.50 (9)	−1.37 (7)	0.23 (7)	−1.56 (8)
C(9)	0.40093 (9)	0.40311 (14)	0.1910 (4)	4.00 (9)	7.49 (11)	4.36 (9)	−1.48 (8)	0.77 (7)	−1.63 (8)
H(11)	0.403	0.616 (4)	0.031 (13)	18 (2)					
H(12)	0.372 (3)	0.636 (3)	0.225 (9)	16 (1)					
H(13)	0.419 (2)	0.720 (2)	0.202 (7)	11 (1)					
H(2)	0.518 (1)	0.645 (1)	0.363 (4)	4.8 (5)					
H(5)	0.348 (1)	0.515 (1)	0.687 (4)	6.5 (6)					
H(6)	0.248 (1)	0.421 (1)	0.622 (4)	4.9 (5)					
H(7)	0.225 (2)	0.273 (2)	0.149 (6)	9.2 (9)					
H(8)	0.337 (1)	0.304 (2)	0.010 (5)	8.3 (7)					
H(9)	0.439 (1)	0.398 (2)	0.083 (5)	7.3 (6)					

isotropic temperature factors are of the form:

$$\exp[-8\pi^2 U(\sin\theta/\lambda)^2]$$

anisotropic temperature factors are of the form:

$$\exp[-2\pi^2(h^2a^{*2}U_{11} + \ldots + 2klb^*c^*U_{23})]$$

of-plane distortions of the vinyl group with respect to the aryl π-system brought about by the steric hindrance between the alkyl substituent and the hydrogen at the *ortho*-position (11). Consequently, the λ_{max} at 221 nm displayed by dienestrol diacetate in Fig. 3 is incompatible with structural types IV or V, which leaves by elimination the *cis*-β-alkylstyrenic Structure I, analogous with VI. The fact that the other two stereoisomeric diacetates exhibit near-UV absorption consistent with the preservation of the styrene chromophore confirms their concordance with Structures II and III.

Conclusions consistent with these interpretations were also obtained from examination of scale molecular models[8] of the geometric isomers. The models suggest that the two olefinic bonds in stereoisomer I readily attain coplanarity with each other, without strain, outside the planes of the aryl nuclei, whereas prohibitive tension would be required to reach double bond conjugation in Structures II and III.

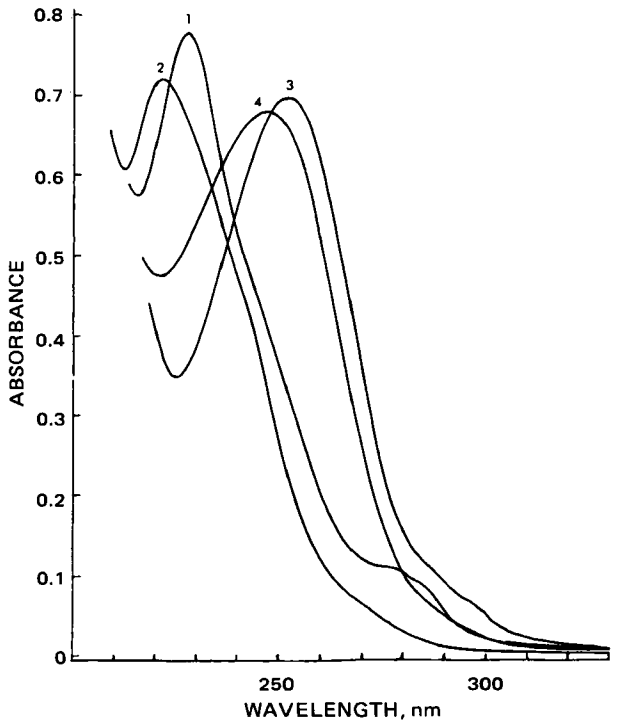


Figure 3—*Electronic absorption spectra of: (1) the active drug dienestrol, (2) the diacetate of I, (3) β-dienestrol diacetate, and (4) γ-dienestrol diacetate. The concentrations are: (1) 2.68 × 10^{-5} M (ε = 29,100), (2) 2.78 × 10^{-5} M (ε = 25,900), (3) 3.11 × 10^{-5} M (ε = 22,600), and (4) 3.50 × 10^{-5} M (ε = 19,600).*

[8] Corey–Pauling–Koltun models, Schwarz BioResearch, Orangeburg, N.Y.

The conjugation of the two vinyl groups in I offers compensatory stabilization energy for the lost styrene π-delocalization and retains a butadiene chromophore characterized by an absorption maximum at 217 nm. This may be interpreted as additional evidence for the assignment of configuration I to dienestrol, since of the four compounds in Fig. 3, only the drug and its diacetate have absorption maxima compatible with the presence of an isolated butadiene chromophore.

These deductions are in accord with the known chemical reactivity of dienestrol and its diacetate toward adduct formation with Diels–Alder dienophiles[9]. This evidence is consistent with configuration I but would not be expected with III, since planarity of the butadiene system is required for this reaction; hindrance from the methyl groups in III would prevent this planar *cis*-conformation.

Although these lines of evidence were compelling, they nevertheless were based on indirect inferences. Unambiguous configurational assignment in complete accord with the preliminary deductions was obtained by single-crystal X-ray diffraction. Table I summarizes the physical constants obtained, and Table II gives the coordinates and temperature parameters of the asymmetric atoms of the unit cell.

Figure 1 is a computer-produced view of the molecule perpendicular to the plane of the hexadiene chain, as determined by the crystallographic analysis. Assignments of bond lengths and bond angles in the crystalline state are shown in Fig. 2. The structure of dienestrol is I, in agreement with the postulation of Koch and coworkers (2, 3) but contrary to the conclusions of Lane and Spialter (5).

A least-squares plane calculated from the coordinates of C(1), C(2), C(3), C(3′), C(2′), and C(1′) shows that these atoms of the hexadiene chain are planar within 0.02 Å. In contrast, the phenyl rings lie virtually perpendicular (87.6°) to the hexadiene system and are thus out of conjugation with the remainder of the molecule. These findings for the crystalline state buttress the interpretation of the electronic spectrum of dienestrol in solution and support arguments based on molecular models and chemical reactivity.

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Stabilizing Effect of Inorganic Phosphate Salts on Antibiotic–Steroid Ophthalmic Preparations

JAMES W. McGINITY ˣ and ROBERT L. BROWN

Abstract □ Drocinonide phosphate potassium forms an insoluble complex with neomycin sulfate in aqueous solution. Dibasic sodium phosphate can be employed in an ophthalmic formulation to prevent the formation of this precipitate without affecting the stability of the steroid or the bioactivity of the antibiotic. Other phosphate steroid salts behaved in a like manner.

Keyphrases □ Ophthalmic formulations—antibiotic–steroid preparations, stabilizing effect of inorganic phosphate salts, drocinonide phosphate potassium–neomycin sulfate complex studied □ Steroid–antibiotic ophthalmic preparations—stabilizing effect of inorganic phosphate salts □ Antibiotic–steroid ophthalmic preparations—stabilizing effect of inorganic phosphate salts □ Drocinonide phosphate potassium—complex with neomycin sulfate, prevention of precipitation by use of inorganic phosphate salts □ Inorganic salts—as stabilizers of antibiotic–steroid ophthalmic preparations

Neomycin sulfate is a polybasic antibiotic which ionizes in aqueous solution to give a positively charged species of neomycin. A wide range of anionic compounds were studied to assess their compatibility with the antibiotic (1). Approximately 50% of the high molecular weight anionic compounds tested precipitated with neomycin sulfate as an addition complex of low solubility. The stability of neomycin was investigated in several pharmaceutical preparations, and it was found that the antibiotic was relatively stable in solution over pH 2.0–9.0 (2). All products studied showed a minimum stability of 1 year at room temperature; tablets, troches, and ointment bases were stable for 2 years.

The present report describes a method for overcoming the incompatibility of neomycin sulfate with corticosteroid drugs in aqueous ophthalmic preparations. As a model, the soluble steroid chosen for investigation was drocinonide phosphate potassium[1]. To formulate this steroid with neomycin sulfate in an ophthalmic solution, the incompatibility of the two drugs must be overcome to produce a clear, particle-free solution in which the drug remains stable and active.

EXPERIMENTAL

Materials—The following were used: neomycin sulfate[2], drocinonide phosphate potassium[1], anhydrous disodium hydrogen phospate[3], anhydrous dipotassium hydrogen phosphate[3], sodium borate[3], sodium bisulfite[3], dexamethasone sodium phosphate[4], prednisolone sodium phosphate[4], polyvinylpyrrolidone[5], sodium formaldehyde sulfoxylate[6], sorbitol[7], polymyxin B sulfate[8], and thimerosal[9].

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Microbiological Assay of Neomycin Sulfate—The microbiological activity of neomycin sulfate was determined by the cylinder-plate method described in USP XVIII (5).

[1] Also known as tetrahydrotriamcinolone acetonide dipotassium phosphate.
[2] Squibb, New Brunswick, N.J.
[3] Mallinckrodt, St. Louis, Mo.
[4] Merck, Rahway, N.J.
[5] Plasdone C-30, GAF Corp., New York, N.Y.
[6] Fine Organics, Lodi, N.J.
[7] Atlas, Wilmington, Del.
[8] Pfizer, Groton, Conn.
[9] Lilly, Indianapolis, Ind.
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Myron Smith is thanked for the preparation of Fig. 2.

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Stabilizing Effect of Inorganic Phosphate Salts on Antibiotic–Steroid Ophthalmic Preparations

JAMES W. McGINITY ˣ and ROBERT L. BROWN

Abstract □ Drocinonide phosphate potassium forms an insoluble complex with neomycin sulfate in aqueous solution. Dibasic sodium phosphate can be employed in an ophthalmic formulation to prevent the formation of this precipitate without affecting the stability of the steroid or the bioactivity of the antibiotic. Other phosphate steroid salts behaved in a like manner.

Keyphrases □ Ophthalmic formulations—antibiotic–steroid preparations, stabilizing effect of inorganic phosphate salts, drocinonide phosphate potassium–neomycin sulfate complex studied □ Steroid–antibiotic ophthalmic preparations—stabilizing effect of inorganic phosphate salts □ Antibiotic–steroid ophthalmic preparations—stabilizing effect of inorganic phosphate salts □ Drocinonide phosphate potassium—complex with neomycin sulfate, prevention of precipitation by use of inorganic phosphate salts □ Inorganic salts—as stabilizers of antibiotic–steroid ophthalmic preparations

Neomycin sulfate is a polybasic antibiotic which ionizes in aqueous solution to give a positively charged species of neomycin. A wide range of anionic compounds were studied to assess their compatibility with the antibiotic (1). Approximately 50% of the high molecular weight anionic compounds tested precipitated with neomycin sulfate as an addition complex of low solubility. The stability of neomycin was investigated in several pharmaceutical preparations, and it was found that the antibiotic was relatively stable in solution over pH 2.0–9.0 (2). All products studied showed a minimum stability of 1 year at room temperature; tablets, troches, and ointment bases were stable for 2 years.

The present report describes a method for overcoming the incompatibility of neomycin sulfate with corticosteroid drugs in aqueous ophthalmic preparations. As a model, the soluble steroid chosen for investigation was drocinonide phosphate potassium[1]. To formulate this steroid with neomycin sulfate in an ophthalmic solution, the incompatibility of the two drugs must be overcome to produce a clear, particle-free solution in which the drug remains stable and active.

EXPERIMENTAL

Materials—The following were used: neomycin sulfate[2], drocinonide phosphate potassium[1], anhydrous disodium hydrogen phospate[3], anhydrous dipotassium hydrogen phosphate[3], sodium borate[3], sodium bisulfite[3], dexamethasone sodium phosphate[4], prednisolone sodium phosphate[4], polyvinylpyrrolidone[5], sodium formaldehyde sulfoxylate[6], sorbitol[7], polymyxin B sulfate[8], and thimerosal[9].

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[8] Pfizer, Groton, Conn.
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Table I—Composition of Formula I

Ingredient	Amount, mg
Neomycin sulfate	3.26
Drocinonide phosphate potassium	3.4
Disodium hydrogen phosphate	6.0
Polyvinylpyrrolidone[a]	8.0
Sodium formaldehyde sulfoxylate	2.0
Thimerosal	0.02
Sorbitol, 70% (w/v)	0.01 ml
Purified water, *q.s.*	1.0 ml

[a] PVP-C-30.

Preparation of Formulas I–IV—These formulas appear in Tables I and II. The neomycin sulfate was dissolved in 60% of the purified water. The soluble phosphate buffer agent was dissolved in 15% of the water and was then added to the solution of neomycin sulfate. The steroid salt plus other additives was dissolved in 15% of the water and added slowly to the neomycin sulfate–phosphate buffer solution. Then the solution was adjusted to volume.

Stoichiometry of Antibiotic–Steroid Complex—To determine the stoichiometry of the complex, 0–10 ml of a 0.05 *M* solution of neomycin sulfate was pipetted in increments of 0.5 or 1 ml into 20-ml vials. Enough 0.05 *M* steroid solution was added until the volume of each vial totaled 10 ml. The mixtures were swirled and permitted to equilibrate at 25°; after 24 hr, the contents of each container were filtered. The precipitated complex was dried and weighed. The data obtained are plotted in Fig. 1, according to the method of Job (6). Data points represent the average of duplicate experiments.

RESULTS AND DISCUSSION

The ideal agent used to overcome the incompatibility of drocinonide phosphate potassium and neomycin sulfate in an ophthalmic solution should exhibit little or no physiological activity and be effective in very small concentrations. Various materials were studied for their effectiveness in solubilizing the precipitate or in preventing the formation of the low solubility complex.

Preliminary screening studies showed that the solubility of the antibiotic–steroid complex was dependent on pH and that the addition to the mixture of strongly acidic or basic amino acid salts produced clear solutions. However, the addition of amino acid salts closer to neutrality was unsuccessful. Sodium borate, which has been used to solubilize a number of drugs (*e.g.*, riboflavin), proved reasonably effective when used in a great concentration (20 mg/ml) at pH 9.2. However, when the preparation was buffered to a pH range desirable for ophthalmic solutions (pH 6–8.5), a precipitate slowly appeared.

An agent successful in overcoming the incompatibility of drocinonide phosphate potassium and neomycin sulfate was disodium hydrogen phosphate. Singh (7), who studied the interaction of various antibiotics with condensed phosphates, found that neomycin possessed the strongest binding capacity for these higher phosphates. Disodium hydrogen phosphate proved an ideal agent for overcoming the incompatibility of drocinonide phosphate potassium and neomycin sulfate, because it was a good buffer agent and was effective in small concentrations. The pH of a solution of disodium hydrogen phosphate (7.8–8.0) may be lowered by the addition of sodium dihydrogen phosphate or dilute phosphoric acid to produce the monobasic salt *in situ*.

Table I shows the composition of a prototype formulation containing the two active ingredients in therapeutic amounts. This product was packaged in 30-ml amber-glass containers and stored at various temperatures to assess the stability of the steroid. Soluble steroid salts have been shown to undergo hydrolysis in aqueous solution, the process being accelerated at temperatures above room temperature. Saccharin sodium (8), creatinine, and niacinamide (9) have been shown to slow this hydrolytic process.

Preliminary studies[11] of stability had shown that polyvinylpyrrolidone was effective in preventing the hydrolysis of drocinonide phosphate potassium, and it was included in Formula I as a stabilizing agent. The data in Table III show that the stability of the steroid was maintained at 33° after 6 months but had declined at 40° after the same period.

[11] Unpublished data.

Table II—Formula Assayed Microbiologically for Neomycin Activity

Ingredient	Formula II	Formula III	Formula IV
Neomycin sulfate	3.26 mg	—	3.26 mg
Drocinonide phosphate potassium	3.4 mg	3.4 mg	—
Disodium hydrogen phosphate	6.0 mg	6.0 mg	6.0 mg
Purified water, *q.s.*	1.0 ml	1.0 ml	1.0 ml

To assess the influence of the steroid on the bioactivity of neomycin, three formulations were prepared (Table II). To eliminate interference with the neomycin assay, the other additives listed in Table I were omitted from the formulation. The data in Table IV show that the steroid did not interfere with the antimicrobial activity of neomycin and that the steroid itself possessed no antimicrobial activity.

To characterize the complex further, its stoichiometry was determined, as outlined under *Experimental*. From Fig. 1, it can be seen that maximum interaction between the two drugs occurred when the mole fraction of neomycin (X_A) to steroid (X_B) was 1:3. The level of disodium hydrogen phosphate required to maintain a clear solution proved to be dependent on the concentration of neomycin sulfate and not on that of drocinonide phosphate potassium.

For a concentration of neomycin sulfate equivalent to 2.5 mg/ml as neomycin base, the minimum recommended level of disodium hydrogen phosphate was 6 mg/ml. At lesser concentrations of buffer (*e.g.*, 4 mg/ml), it was also possible to obtain a crystal-free solution if both reactants were well diluted. However, when both solutions had been frozen at −20° for 12 hr, the preparation containing disodium hydrogen phosphate at 6 mg/ml produced a clear solution after being shaken at room temperature, whereas the preparation containing buffer at 4 mg/ml contained crystals. These crystals did not dissolve at room temperature, but their solubility increased as the temperature was raised; all crystals were in solution at 50°.

NMR spectroscopy was employed to investigate the system further. The primary amino groups on the neomycin molecule were suspected to be the sites of interaction with the steroid. The buffer agent and the two active ingredients were each lyophilized from deuterated water solutions to ensure the deuteration of the water of hydration. The NMR spectra obtained from deuterated water solutions of neomycin sulfate in the presence of the disodium hydrogen phosphate, with and without the steroid, indicated no interaction among the species. This fact, plus the fact that neomycin was bioactive in the presence of the steroid, suggested that the two active constituents were present as individual moieties and would be bioavailable to exert the desired therapeutic effect, but this assumption has yet to be proven *in vivo*.

Disodium hydrogen phosphate was also investigated for its ability to overcome the incompatibility that occurred when polymyxin

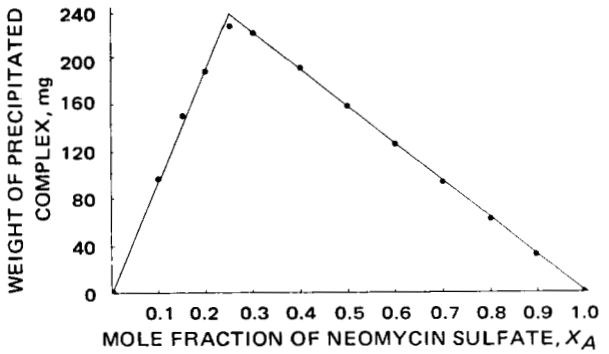

Figure 1—*Job (6) plot showing stoichiometric relationship between neomycin sulfate and drocinonide phosphate potassium. (See text for details.)*

Table III—Stability of Drocinonide Phosphate Potassium in Formula I[a]

Temperature	1 Month	2 Months	3 Months	6 Months
5°	97.0	99.4	97.7	96.4
25°	95.2	96.2	100.3	93.5
33°	94.7	95.3	99.7	97.9
40°	94.1	95.9	95.6	80.0

[a] Expressed as percent undegraded steroid.

B sulfate was added to the drocinonide phosphate potassium–neomycin sulfate system to increase the antibacterial activity of a combination ophthalmic preparation. Increasing the buffer concentration an additional 25% and adding the steroid slowly to the disodium hydrogen phosphate–antibiotic mixture in dilute solution made it possible to prepare a solution of the three drugs in concentrations considered desirable for therapeutic effect.

A very slight haze in the product developed immediately after preparation. Filtration yielded a crystal-clear solution that remained clear. The haze also appeared in the product formulated without neomycin sulfate. (The haze might be due to a small amount of impurity in polymyxin that was not protected by disodium hydrogen phosphate and was, therefore, precipitated by the steroid.) The bioactivity of polymyxin in these systems has not been investigated and certainly warrants further investigation.

The ability of disodium hydrogen phosphate to counteract the incompatibility of neomycin sulfate with other steroids, such as dexamethasone sodium phosphate and prednisolone sodium phosphate, was also investigated. As with drocinonide phosphate potassium, solutions of these steroids in the presence of neomycin sulfate were clear and free of crystals.

A currently marketed ophthalmic solution of dexamethasone sodium phosphate and neomycin sulfate contains sodium bisulfite as an antioxidant to help overcome the incompatibility between the steroid and the antibiotic. Studies in these laboratories have shown sodium bisulfite to be effective in the drocinonide phosphate potassium–neomycin sulfate system; small concentrations of bisulfite produced a crystal-free solution. When this solution was purged with oxygen, the bisulfite was oxidized to bisulfate, and rosette crystals began to grow on the walls of the container. The advantage to using disodium hydrogen phosphate rather than bisulfite was the stability of phosphate in the presence of oxygen, which permitted packaging of the product in a plastic squeeze-bottle container.

Dipotassium hydrogen phosphate exhibited properties similar to those of the disodium salt. No studies have been made of the stability or bioactivity of drocinonide phosphate potassium and neomycin sulfate in the presence of the dipotassium salt, but no marked difference is expected between the two salts.

SUMMARY AND CONCLUSIONS

Dibasic phosphate salts can be satisfactorily utilized to overcome the chemical incompatibility between neomycin sulfate and soluble corticosteroid phosphate salts.

Of the many mechanisms that could play a role in this "salting-in" process, two are: (*a*) phosphate ions from disodium hydrogen phosphate exert a common-ion effect, *via* ion-pair formation, or a cosolute effect (10); and (*b*) phosphate ions from disodium hydrogen phosphate interact with reactive sites on neomycin and sterically hinder the chemical reaction between the antibiotic and the steroid salt. By this latter mechanism, the bonding of phosphate ions to the reactive site(s) on neomycin would probably be *via* weak electrostatic bonds, since NMR spectra suggested that both neomycin sulfate and drocinonide phosphate potassium are present as separate moieties. The stoichiometry of the antibiotic–steroid complex (1:3) seems to indicate that the complex may not be formed by a simple ionic interaction between the two high molecular weight species. Further studies of the salting-in mechanism are currently underway.

The stability and bioactivity of drocinonide phosphate potassium and neomycin sulfate in a disodium hydrogen phosphate solution were not adversely influenced by the presence of the other drug. The use of disodium hydrogen phosphate as a simple and efficient way to overcome the chemical incompatibility between neomycin sulfate and soluble phosphate salts of corticosteroids is strongly recommended for ophthalmic formulations where some combination of these drugs is desirable.

Table IV—Bioactivity[a] of Neomycin in Formulas II, III, and IV Stored at Various Temperatures

Time	Temperature	Formula II	Formula III	Formula IV
Initial	25°	99.5	0	96.3
1 month	5°	99.1	0	98.0
	25°	97.3	0	98.0
	33°	98.0	0	97.3
	40°	96.4	0	96.4
3 months	5°	99.5	0	97.7
	25°	99.1	0	96.8
	33°	99.5	0	97.7
	40°	96.8	0	95.9
6 months	5°	97.7	0	97.2
	25°	97.3	0	97.2
	33°	100.0	0	96.3
	40°	90.9	0	90.0

[a] Expressed as a percent of the predetermined potency of the neomycin sulfate used in the formulation. Confidence limits of less than 4% (2 days) were used for all assay values.

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GLC Determination of 7-Chloro-5,11-dihydrodibenz[*b,e*][1,4]-oxazepine-5-carboxamide in Serum or Plasma

A. O. NIEDERMAYER ˣ **and R. G. KOSKI**

Abstract □ This report describes the isolation, derivative formation, GLC, and quantitation of unmetabolized 7-chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepine-5-carboxamide (I) in blood serum or plasma. Carbamazepine is used as the internal standard to compensate for losses of I during extraction and handling. Essentially complete recovery (100 ± 6%) was demonstrated over a concentration range of 1–30 μg of I/ml of serum.

Keyphrases □ 7-Chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepine-5-carboxamide—GLC, analysis in serum and plasma as 5-nitrile derivative □ GLC—analysis, 7-chloro-5,11-dihydrodibenz[*b,e*]-[1,4]oxazepine-5-carboxamide in serum and plasma as 5-nitrile derivative

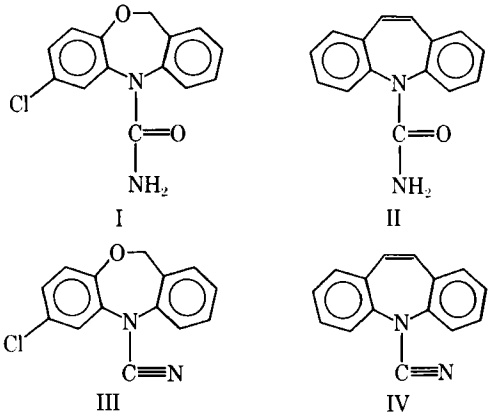
7-Chloro-5,11-dihydrodibenz[*b,e*][1,4]oxazepine-5-carboxamide (I) (1) exhibits antidepressant properties related to those of imipramine and anticonvulsant properties like those of carbamazepine (II). The pharmacological evaluation of I and a comparison of it with carbamazepine both require determination of the blood concentration of I.

The structural similarity of I and II suggested that a procedure for isolating carbamazepine from serum (2) might be useful in isolating I. It also appeared that carbamazepine might be used as an internal standard in any quantitative assay for I. Both premises proved to be correct.

The search for derivatives of I and II that are easier to chromatograph and assay than the parent compounds led to the synthesis of nitriles III and IV of I and II, respectively. These compounds were characterized by mass spectrometry, NMR spectroscopy, and IR spectrophotometry.

The flame-ionization response of the 5-nitrile derivative (III) was used successfully in the GLC determination of unmetabolized I in plasma and serum.

This methodology will prove useful in comparative bioavailability studies[1] in sheep, dogs, and humans, as well as for ascending-dose oral safety and short-term, multiple-dose tolerance studies[1] in humans.



EXPERIMENTAL

Reagents—Chloroform, methanol, *n*-hexane, and benzene, all chromatographic quality, were used without further purification. Trimethylamine[2] (0.2–0.5 *M*) in benzene was prepared by bubbling the amine through chilled benzene (dried for at least 24 hr over molecular sieve) at a slow rate until the desired concentration, as determined by perchloric acid titration, was achieved. The refrigerated reagent was found to deteriorate slowly, and its concentration was checked monthly by titration.

Ammonium hydroxide (5%) was prepared by diluting 17 ml of the concentrated reagent to 100 ml with water. Heptafluorobutyric anhydride[3], obtained in 1-ml ampuls, was stored refrigerated in a glass-stoppered tube after opening and was used for not more than 2 weeks. Compound I[4] and carbamazepine[5] were prepared as methanolic solutions with concentrations near 340 and 240 μg/ml, respectively. Phosphate buffer (pH 7.2) was prepared according to USP XVIII.

Hydrochloric acid (0.25 *N*) was prepared by diluting 21.4 ml of the concentrated reagent to 1 liter with distilled water. Tribasic potassium phosphate (0.2 *M*) was made by dissolving 4.2 g of K_3PO_4 in sufficient distilled water to yield 100 ml of solution. The 1% OV-17 on 80–100-mesh Chromosorb G (HP)[6] was used as purchased.

Apparatus—The gas chromatograph[7] was equipped with a 1.7-m (5.5-ft) × 0.3-cm (0.125-in.) o.d. glass column filled with OV-17 packing and had both flame-ionization and ^{63}Ni electron-capture detectors. A multistation evaporator[8] was used for solvent stripping during isolation and purification. The centrifuge was capable of handling 50-ml tubes and of attaining speeds of at least 1500 rpm. The typical laboratory glassware used included 2.5-, 13-, 15-, and 35-ml centrifuge tubes, Pasteur 14.6-cm (5.75-in.) disposable pipets, and 10-, 50-, and 250-μl syringes[9].

GLC Conditions—Operating temperatures for the inlet, column, and flame-ionization detector were 240, 210, and 270°, respectively. Helium at 30 psig was applied at the column head. The flow of air and hydrogen for the flame detector was optimized. The electrometer input sensitivity was adjusted to $\sim 2 \times 10^{-10}$ Å, and output attenuation was applied as necessary. The samples injected never exceeded 1 μl. Under the stated conditions, the retention times for the nitriles corresponding to carbamazepine and I were ~9 and 12 min, respectively (Fig. 1).

Newly prepared columns were conditioned for at least 16 hr under operating conditions. In this time, a stable baseline was achieved and the subsequent chromatograms appeared completely satisfactory.

The ^{63}Ni electron-capture detector was operated in dc and pulsed modes, using nitrogen and argon with 5% methane as the respective carrier gases.

Standard Preparation—Exactly 40 μl each of internal standard solution (carbamazepine) and standard solution (I) were transferred by syringe into 2.5-ml glass-stoppered centrifuge tubes. The solvent was removed by placing the tube in a 55° water bath[8] and flushing its head space with a gentle stream of nitrogen gas to remove the solvent vapor.

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[5] Geigy Pharmaceuticals, Ardsley, N.Y.
[6] Regis Chemical Co., Chicago, Ill.
[7] Model 900, Perkin-Elmer Corp., Norwalk, Conn.
[8] "N-EVAP," Organomation Associates Inc., Shrewsbury, Mass.
[9] Hamilton Co., Reno, Nev.

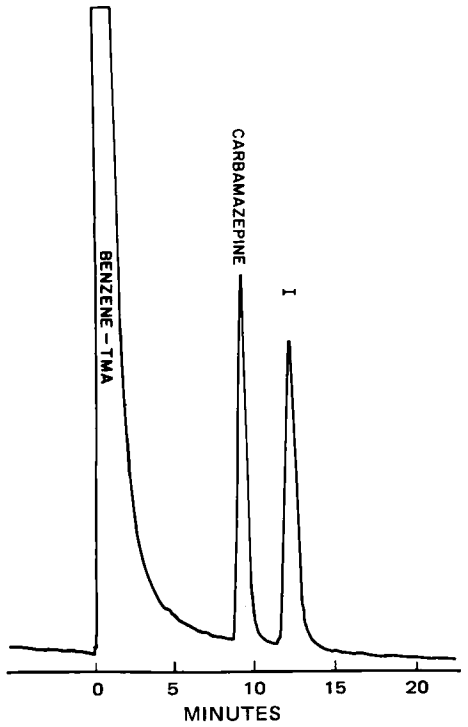


Figure 1—*Chromatogram of nitrile derivatives of I and carbamazepine.*

Sample Preparation (Extraction and Cleanup)—Exactly 5 ml of cell-free serum or plasma, obtained by standard techniques, or fraction thereof diluted to 5 ml with water was pipetted into a 35-ml centrifuge tube. Then 40 μl of internal standard solution was added by microsyringe, followed successively by 1 ml of pH 7.2 phosphate buffer and 13 ml of chloroform. The tube was shaken gently for 15 min but vigorously enough to ensure thorough mixing of the phases and was then centrifuged for 5 min at 1500 rpm. Occasionally, an emulsion formed but it was dispersed by stirring with a glass rod and recentrifugation.

The entire lower solvent phase was transferred by pipet into a 15-ml glass-stoppered centrifuge tube to effect complete removal of solvent (55° water bath and stream of nitrogen). To the resulting residue was added 3 ml of methanol, 2 ml of 0.25 *N* HCl, and 5 ml of *n*-hexane. After 5-min applications of shaking and centrifugation of the tube, the upper solvent phase was discarded. The wash cycle was repeated once with fresh hexane.

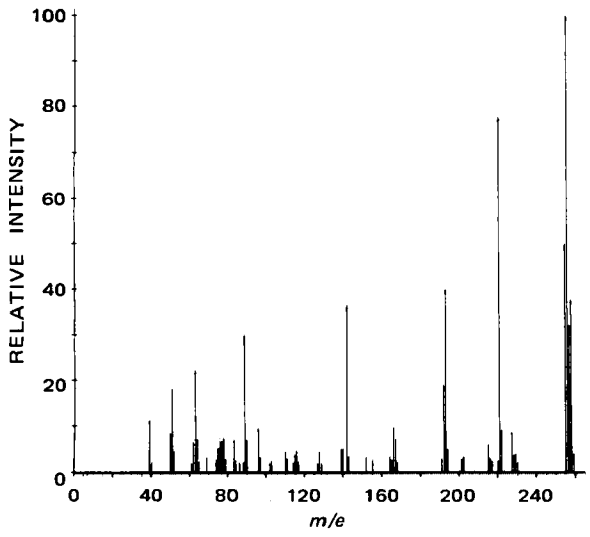


Figure 2—*Mass spectra of III.*

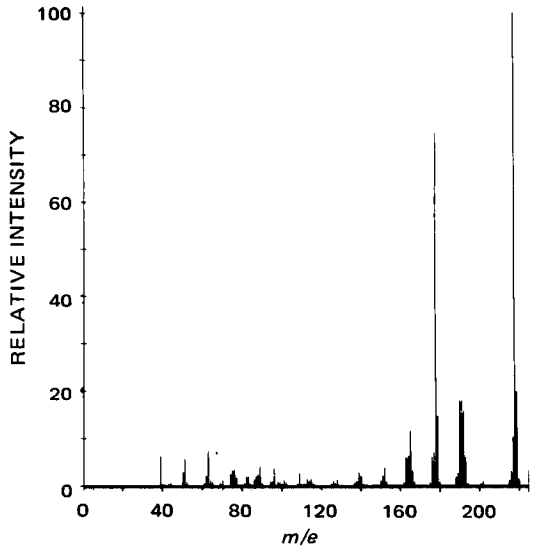


Figure 3—*Mass spectra of carbamazepine reaction product.*

The lower aqueous methanolic hydrochloric acid solution was transferred with a disposable pipet into a clean 13-ml centrifuge tube and extracted with 5 ml of chloroform for 5 min. After 3 min of centrifugation, the upper aqueous phase was discarded. The remaining chloroform solution was washed for 3 min with 5 ml of 0.2 *M* tribasic potassium phosphate and was then centrifuged. After centrifugation, the aqueous phase was discarded.

The washed chloroform extract was transferred in three aliquots into a 2.5-ml glass-stoppered centrifuge tube with intermittent solvent stripping (55° water bath and stream of nitrogen). Finally, the inside of the tube was twice alternately rinsed with a 1-ml aliquot of chloroform and restripped to concentrate the residue in the tube bottom.

Derivative Formation (Standards and Samples)—The residue of standard or sample contained in the 2.5-ml glass-stoppered centrifuge tube was dissolved in 100 μl of trimethylamine–benzene reagent. Five microliters of heptafluorobutyric anhydride was added, and the solutions were thoroughly mixed. The tube was tightly stoppered, and the solutions were allowed to react at 55° for 10 min.

After the reaction mixture had been cooled to room temperature, 0.5 ml of water was added and the two-phase system was thoroughly shaken. Following the addition of 0.5 ml of 5% NH_4OH, the tube was shaken again. After centrifugation, the lower aqueous phase was withdrawn almost completely by means of a capillary pipet; the upper solvent phase was lowered into the constricted part of the tube to facilitate sampling for chromatography.

Quantitation—After the peaks attributed to carbamazepine and I were identified by their retention times, baselines were interpolated and either peak height or area under the peak was measured. Then:

$$R_s = \frac{H_s}{H_{is}} \text{ and } R_u = \frac{H_u}{H_{is}} \qquad \text{(Eq. 1)}$$

where R_s is the ratio of standard peak height (H_s) to internal standard peak height (H_{is}), and R_u is the ratio of unknown peak height (H_u) to internal standard peak height (H_{is}). Finally:

$$\begin{matrix}\text{micrograms of I}\\ \text{per milliliter of}\\ \text{serum}\end{matrix} = \frac{R_u}{R_s} \times \frac{\text{micrograms of I in standard}}{\text{milliliters of serum taken}} \qquad \text{(Eq. 2)}$$

RESULTS AND DISCUSSION

Selection of Extraction and Cleanup Procedure—The structural similarity of carbamazepine and I made Kupferberg's extraction methodology (2) appear to be applicable to the extraction of I from serum. When ^{14}C-labeled I was added to human serum and its recovery from various stages of Kupferberg's extraction pro-

Table I—Recovery of I from Spiked Serum

I Added, μg	I Recovered, μg	Recovery, %
5	4.5	90
13.9	13.9	100
20.1	19.1	95
26.0	25.4	98
39.0	37.1	95
40.2	39.3	98
52.0	51.3	99
93.8	82.2	98
157.5	159.4	101

cedure was monitored by scintillation counting, the recovery of I was essentially quantitative.

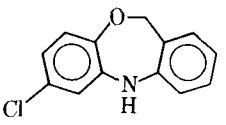
Selection of Internal Standard—The structural similarity of carbamazepine and I is the prime reason for the choice of carbamazepine as the internal standard. The comparable chemical and physical properties of the two compounds ensured similar behavior during isolation. When added to the serum early in the assay, the internal standard compensates for I losses due to partitioning and handling. As a result, the need for accuracy in measuring aliquots for chromatographic injection is reduced. The chromatographic system chosen produced an adequate separation of the pertinent components. The cleanup procedure removed all interferences.

Derivative Formation—Several chromatographic procedures were studied and rejected. Chromatography of underivatized I suffered from problems observed (3) with carbamazepine—*viz.*, absorption, decomposition, and chromatographic peaks unsuitable for quantitation. Formation of the trimethylsilyl derivative by standard techniques was rapid, but the derivatives exhibited poor stability. Furthermore, conflicting peaks were evident in serum extracts, precluding quantitation of I.

The anticipated need for high sensitivity led to consideration of electron-capture detection and the feasibility of preparing an electron-capturing derivative of I. Heptafluorobutyramides exhibit great electron affinity and have been used as amine derivatives (4). Reaction of heptafluorobutyric anhydride with amides was unreported.

The unanticipated reaction products produced by the attempted heptafluorobutyrylation of I and carbamazepine chromatographed well (Fig. 1). However, it was quickly established that they had little electron-capturing capability. Neither dc nor pulsed-mode operation of the detector yielded responses comparable in sensitivity to those of flame ionization. Mass spectrometry of the I derivative (Fig. 2) failed to reveal the expected molecular ion at *m/e* 470. This failure is consistent with the lack of electron-capturing capability of the compound. The molecular ion at *m/e* 256 is 18 mass units less than the molecular weight of I, possibly the result of dehydration. As expected for a chlorine-containing molecule, a second ion was evident at *m/e* 258 with one-third the intensity. The analogous derivative of carbamazepine revealed a molecular ion at *m/e* 218, also indicative of the corresponding 5-nitrile (Fig. 3).

Both derivatives exhibited IR absorption at 2220 cm^{-1}, indicative of a nitrile, probably the result of dehydration. NMR indicated the absence of exchangeable NH protons, along with a methylene singlet at δ 5.25 and an aromatic proton multiplet between δ 6.80 and 7.60 to support the formation of the 5-nitrile. The use of acetic anhydride as a substitute for heptafluorobutyric anhydride in the dehydration reaction failed to produce the expected products. Trifluoroacetic anhydride, however, produced the nitriles in yields comparable to those obtained with heptafluorobutyric anhydride, but the use of heptafluorobutyric anhydride was continued for consistency.



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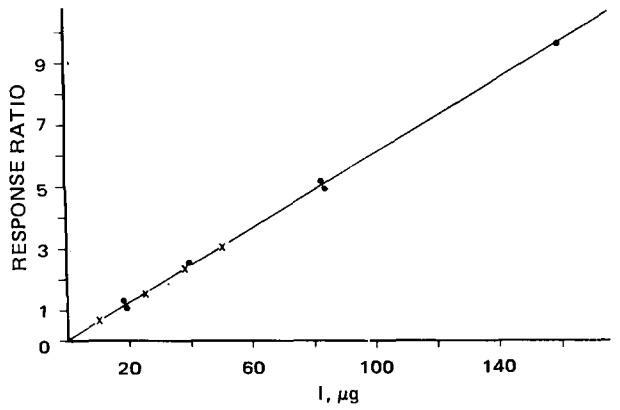

Figure 4—*Calibration curve of I.*

Linearity and Sensitivity—The linearity of the assay was first demonstrated for concentrations of I from 0 to 60 μg and then extended through 160 μg. The composite calibration curve is shown in Fig. 4. The extended linear range permits the assay of higher concentrations of I through signal attenuation rather than by rerunning a reduced size sample.

The response ratio (Fig. 4) represents the corrected I peak height response, for attenuation through ×8, related to the peak height of the internal standard. The concentrations are expressed as micrograms of I per sample (1–5 ml) of serum. The curve slope is ~16.5 (response per microgram of I), and deviation from the origin is negligible. A change in the concentration of internal standard obviously yields a different slope. In practice, the same internal standard solution is used for both standard and sample, and rigid control of its concentration is not necessary.

The limit of detection can be visualized on the calibration curve as 1–2 μg of I. When using 5 ml of serum, 0.2–0.4 μg/ml should be quantifiable. In practice, 0.2 μg/ml was readily achieved, since the sample background was negligible.

Specificity and Interference—Serum and plasma, known to be free of I or carbamazepine, were carried through the entire analytical procedure to demonstrate the absence of any normally occurring interference.

The precursor for I, 2-chloro-6,11-dihydrobenz[*b,e*][1,4]oxazepine (V), was tested for possible chromatographic interference during the quantitation of I. The nitrile derivative of I was unaffected by V. There was, however, chromatographic interference with the derivative of the internal standard, carbamazepine, since V is situated on its tail. The degree of interference was related to the condition and efficiency of the chromatographic column. The presence of V inflates the response of the carbamazepine, thus depressing the response ratio for I and leading to low biased assays. It is unlikely that significant quantities of the precursor V will ever be present during therapy, since it apparently is not a degradation product of I.

The anticoagulants potassium oxalate and edetic acid were examined for their effects on the assay of I. Both substances are commonly used to prevent coagulation of blood samples drawn by vein. At anticoagulant concentrations up to 2 mg/ml of whole blood (the concentration of anticoagulant usually employed), the recovery of I was essentially quantitative. There was no chromatographic evidence of either anticoagulant.

Recovery from Serum—The validity of any assay method for the blood level of a substance is the recovery of such material from fortified serum. This validation procedure was applied to the entire analytical range (Table I).

Stability of I in Frozen Serum—When 5-ml serum samples, containing 12.7 μg of I each, were stored frozen for 1, 3, 8, and 102 weeks, recoveries were 102, 106, 104, and 97%, respectively. Storage of serum for more than 8 weeks is not anticipated. The data for the 102-week period demonstrate that I can be recovered from serum stored that long.

Recoverability of I from Urine—The described methodology was also applied to urine spiked with I. The recovery of I from frozen urine stored for 8 weeks was 97%. No drug could be found in the urine of several subjects given I orally, suggesting that I had been excreted as a metabolite or conjugate.

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JOEL L. ZATZ [x] and GARY W. CLEARY [*]

Abstract □ The molecular arrangement of dipalmitoyl lecithin and cholesterol in mixed monolayers was investigated with the aid of a physical model. The two lipids are miscible at the surface, but there is no indication of a specific interaction. In equimolar mixed monolayers at 25 and 37°, the lipids are in tail contact. Lecithin molecules are able to remain hydrated in the mixed monolayers at high values of surface pressure.

Keyphrases □ Monolayers—cholesterol and dipalmitoyl lecithin, molecular arrangement, force–area isotherms, model □ Cholesterol–dipalmitoyl lecithin monolayers—molecular arrangement, force–area isotherms, model □ Dipalmitoyl lecithin–cholesterol monolayers—molecular arrangement, force–area isotherms, model □ Films—molecular arrangement in cholesterol–dipalmitoyl lecithin mixed monolayers, model

The notion that monomolecular films might serve as a physical model for cellular membranes was first suggested by Langmuir (1) in 1917. Like a natural membrane, the monolayer is a coherent, organized, interfacial structure. Consequently, it is a particularly useful model system for obtaining information on the orientation and arrangement of membrane components.

Special significance has been ascribed to the behavior of monolayers containing both cholesterol and a phospholipid, such as lecithin, since these materials are known to be important constituents of biological membranes. Usually, investigators have reported the mean area per molecule (at constant surface pressure) for the mixture as a function of monolayer composition (2–7). In some cases, the mean molecular area has been a linear function of composition, indicating that each component occupied essentially the same molecular area in the mixed system as in a monolayer of the pure material.

However, deviation from linearity has been observed. The mean molecular area in such systems is less than that expected on the basis of the monolayer properties of the pure materials (2–7). These deviations have been interpreted in various ways. One view is that they are evidence of a significant interaction between the two lipids as a result of van der Waals' forces, configurational entropy effects, and alteration in water structure (3), leading to the conclusion that cholesterol must strengthen or stabilize the monolayer. The implication is that cholesterol also stabilizes biological membranes.

Another explanation of the reduction in mean area per molecule was put forth by Shah and Schulman (4), who attributed the effect to the formation of cavities between phospholipid molecules as a result of thermal motion of the fatty acyl chains. Cholesterol molecules were believed to be accommodated in the cavities. These authors noted that monolayers of pure dipalmitoyl lecithin at a surface pressure of 35 dynes/cm underwent a transition to the gel state and that compression to 40 dynes/cm resulted in the formation of a two-dimensional solid. The transitions were not observed in mixed monolayers containing cholesterol, which remained fluid at all values of surface pressure. It was concluded that cholesterol decreases monolayer cohesion and, by inference, that it acts as a "biological plasticizer" in natural membranes (4).

Gershfeld and Pagano (8) reviewed these theories and, based on the results of adsorption experiments, concluded that dipalmitoyl lecithin is immiscible with cholesterol at the surface and that a rational interpretation of the mixed monolayer experiments is not possible.

A physical model (the "heads-or-tails" model), which should help clarify the role of cholesterol, was recently suggested (9). It is based on the assumptions that lipids with long hydrocarbon chains are essentially vertically oriented and that the surface area of each molecule in a coherent monolayer conforms either to the area of the hydrocarbon tails (tail contact) or the hydrated polar head (head contact). According to this model, phospholipids in tail contact (*i.e.*, in a condensed surface state) do not exhibit a reduction in the mean molecular area in the presence of cholesterol, a result in accord with published findings (4, 5). But if the phospholipid molecules are extensively hydrated, cholesterol molecules may find free space between the hydrocarbon tails and thus require little

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Table I—Calculation of Mean Molecular Area in Mixed Monolayers of Cholesterol and Dipalmitoyl Lecithin Using the Heads-or-Tails Model[a]

Case	Criterion	A_m =		
1	$(A_2 - A_{HC}) \approx 0$	$A_1X_1 + A_{HC}X_2$		
		for $X_2 \leq 0.5$		for $X_2 \geq 0.5$
2	$A_1 > (A_2 - A_{HC}) > 0$	$A_1X_1 + A_{HC}X_2$		$(A_1 + A_{HC})X_1 + A_2(X_2 - X_1)$
3	$(A_2 - A_{HC}) = A_1$	$A_1X_1 + A_{HC}X_2$		A_2X_2
		for $X_2 \leq 0.333$	for $X_2 \geq 0.333$	
4	$2A_1 > (A_2 - A_{HC}) > A_1$	$A_1X_1 + A_{HC}X_2$	$A_2(2X_2 - X_1) + (2A_1 + A_{HC}) \cdot (X_1 - X_2)$	A_2X_2

[a] A_m = mean per area per molecule, X_1 = mole fraction of cholesterol, A_1 = area per molecule in cholesterol monolayer, X_2 = mole fraction of lecithin, A_{HC} = area per molecule of lecithin hydrocarbon tail, and A_2 = area per molecule in lecithin monolayer.

additional area. Therefore, in the presence of cholesterol, the effective area of a phospholipid molecule is the area of its hydrophobic tail rather than that of the bulkier polar portion, provided that the monolayer components are miscible.

An advantage of the proposed model is that it permits the derivation of quantitative relations for the mean area per molecule in a mixed monolayer as a function of the surface properties of the pure components, the composition of the mixed monolayer, and the area of the hydrocarbon tail of the phospholipid. This report deals with results obtained with mixtures of cholesterol and dipalmitoyl lecithin at two temperatures.

EXPERIMENTAL

Cholesterol[1] and dipalmitoyl lecithin[1] were reportedly 99% pure. Organic solvents were spectrograde, and sodium chloride was reagent grade. Water was double distilled; the final distillation took place in an all-glass still. The apparatus used was described previously (10).

The subphase contained 0.9% sodium chloride. Dipalmitoyl lecithin and lecithin–cholesterol mixtures were spread from solution in hexane–ethanol (4:1), and cholesterol was spread from solution in hexane. A thermostat[2] was used to maintain the subphase temperature constant within 0.1°. Surface pressure (π) was measured by the Wilhelmy plate method.

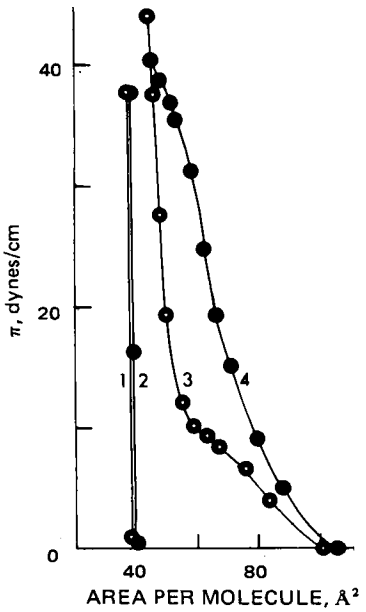

Figure 1—*Surface pressure as a function of available surface area of pure lipids. Key: curve 1, cholesterol at 25°; curve 2, cholesterol at 37°; curve 3, dipalmitoyl lecithin at 25°; and curve 4, dipalmitoyl lecithin at 37°.*

RESULTS AND DISCUSSION

Force–area isotherms for cholesterol and dipalmitoyl lecithin monolayers at 25 and 37° are plotted in Fig. 1. The cholesterol monolayer at 25° is in a highly condensed state at surface pressure values above 1 dyne/cm. An increase in the subphase temperature to 37° has no effect on the state of the monolayer. The entire isotherm is displaced to slightly higher values of surface area per molecule. Based on its low compressibility and the correspondence of the experimental surface area per molecule with the cross-sectional area of the hydrocarbon portion of cholesterol (11), it was concluded that the hydrocarbon tail determines the surface area required for each cholesterol molecule (9).

The π–A curve for dipalmitoyl lecithin at 25° (Fig. 1) is of the expanded type below a surface pressure of about 20 dynes/cm. In terms of the model, the molecules are described as being in head contact. In other words, the area occupied by each molecule is that of the hydrated polar portion. With increasing surface pressure, a change of state takes place and the area per molecule approaches that of the hydrocarbon tails (11). In contrast to the behavior of cholesterol, dipalmitoyl lecithin monolayers undergo marked changes in surface state as a function of temperature. At 37° (Fig. 1), the monolayer is considerably more expanded than at 25°. A state of tail contact is not realized until the surface pressure reaches 40 dynes/cm.

The equations previously derived (9) permit calculation of A_m, the mean area per molecule at a given surface pressure of the

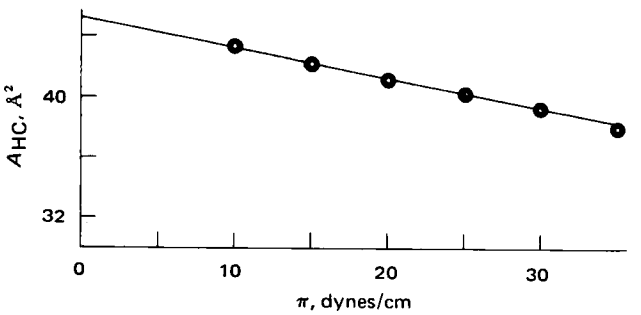

Figure 2—*Area per molecule of the hydrocarbon tail of dipalmitoyl lecithin in the 1:1 mixed monolayer with cholesterol as a function of surface pressure at 25°.*

[1] Schwartz-Mann, Orangeburg, N.Y.

[2] Lauda K-2.

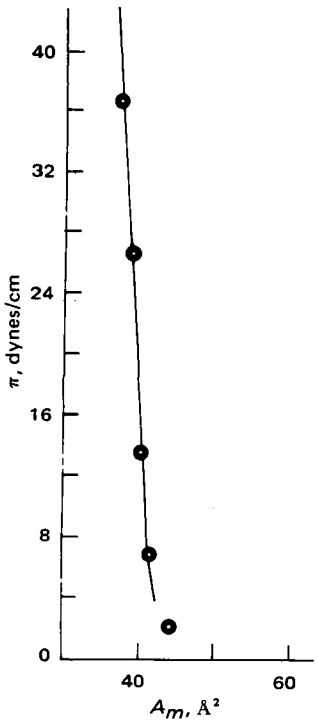

Figure 3—*The π–A curve for mixed monolayers of cholesterol–dipalmitoyl lecithin (1:1) at 25°. Key: —, calculated from heads-or-tails model; and O, experimental points.*

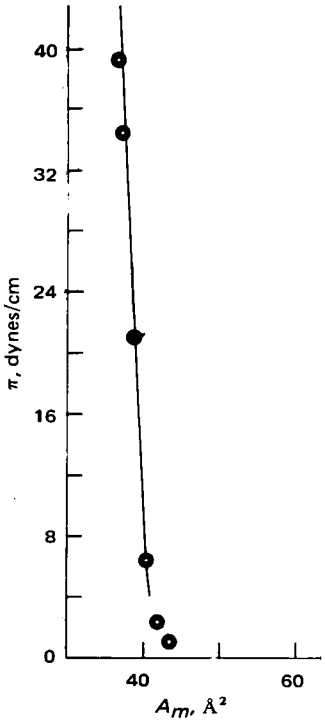

Figure 4—*The π–A curve for mixed monolayers of cholesterol–dipalmitoyl lecithin (2:1) at 25°. Key: —, calculated from heads-or-tails model; and O, experimental points.*

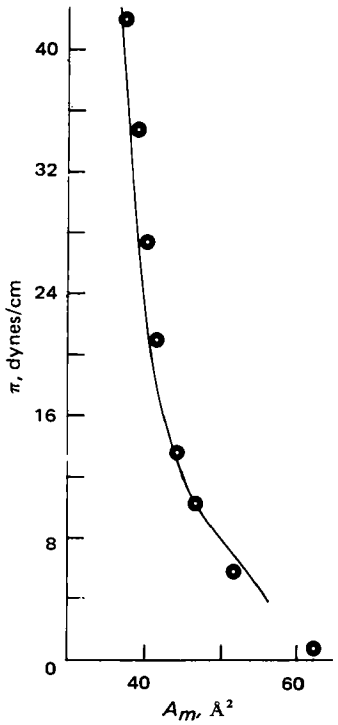

Figure 5—*The π–A curve for mixed monolayers of cholesterol–dipalmitoyl lecithin (1:2) at 25°. Key: —, calculated from heads-or-tails model; and O, experimental points.*

mixed monolayer, from the surface properties of the pure components. These relations are summarized in Table I. The only parameter needed for the calculation that may not be obtained directly from experiment is A_{HC}, the area of the hydrocarbon tail of the phospholipid. It is possible to estimate A_{HC} from molecular models and data from π–A curves of simple lipids. However, to obtain a more accurate approximation and also to see the effect of surface pressure on A_{HC}, another method was chosen.

The 1:1 mixed monolayer at 25° represents a Case 2 situation (above a surface pressure of about 5 dynes/cm), so Eq. 1 applied:

$$A_m = A_1X_1 + A_{HC}X_2 \qquad \text{(Eq. 1)}$$

where X_1 and X_2 are the mole fractions of cholesterol and lecithin, respectively; and A_1 is the area per molecule in a monolayer of pure cholesterol at the same surface pressure. This equation may be rearranged to:

$$A_{HC} = \frac{A_m - A_1X_1}{X_2} \qquad \text{(Eq. 2)}$$

where A_1, X_1, and X_2 are known. By using the experimental values of A_m at each surface pressure for the 1:1 mixed monolayer, A_{HC} can be calculated. As shown in Fig. 2, A_{HC} is a linear function of π, obeying the relationship:

$$A_{HC} = 45.2 - 0.2\pi \qquad \text{(Eq. 3)}$$

The compressibility of the hydrocarbon tails corresponds to what one would expect for a liquid condensed monolayer (12) and thus supports the notion that the tails are vertically oriented.

The π–A data for the pure monolayers at 25° and values of A_{HC} from Eq. 3 were used to calculate A_m at various values of surface pressure for each mixed monolayer at that temperature. For each system, the theoretical points were joined to produce a continuous curve. These curves and the corresponding experimental data are presented in Figs. 3–5. It is not surprising that the fit to experimental data is so good for the 1:1 monolayer (Fig. 3), since the values for A_{HC} were derived from this system. However, the correspondence between theory and experiment is also quite good for the other mixtures (Figs. 4 and 5).

The values of A_m for a 1:1 mixed monolayer at 37° were also calculated. However, since A_{HC} is expected to vary with temperature, the values at 25° from Eq. 3 could not be used directly at the higher temperature. The effect of temperature on A_{HC} was estimated

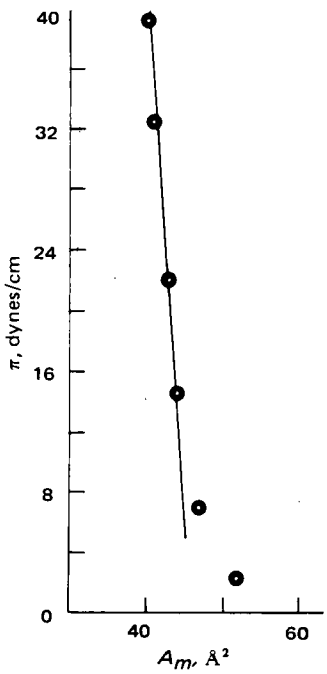

Figure 6—*The π–A curve for mixed monolayers of cholesterol–dipalmitoyl lecithin (1:1) at 37°. Key: —, calculated from heads-or-tails model; and O, experimental points.*

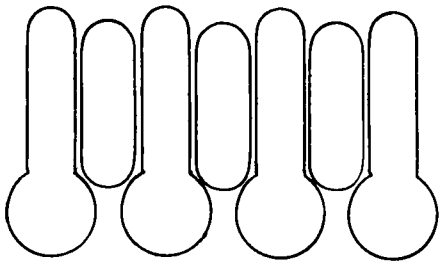

Figure 7—*Representation of molecular arrangement in equimolar mixed monolayers of cholesterol (short rod) and dipalmitoyl lecithin at surface pressure values above 5 dynes/cm.*

from the surface properties of palmitic acid, since its hydrocarbon chain is saturated and contains 16 carbon atoms, as do each of the hydrocarbon chains of our lecithin. An adjustment was made based on the observation that a comparable increase in temperature causes an expansion of 2.8 Å^2/molecule of palmitic acid in the condensed state (13).

Therefore, A_{HC} of dipalmitoyl lecithin at 37° was assumed to be 2.8 Å^2/hydrocarbon chain, or 5.6 Å^2/molecule, higher than at 25° at the same surface pressure. The values of A_m were calculated for various surface pressures, and the theoretical points were joined to produce a continuous curve. The curve and corresponding experimental data are compared in Fig. 6.

The good agreement found in these experiments between calculations based on the heads-or-tails model and the experimental results strongly supports the validity of the assumptions of the model, at least with respect to mixtures of dipalmitoyl lecithin and cholesterol. Therefore, it is concluded that these substances are miscible in monomolecular films (and presumably in biological membranes). Furthermore, there is no indication of a specific interaction between the two lipids. Shah and Schulman (4) arrived at the same conclusion, based on surface potential measurements.

In the mixed monolayers, lecithin molecules are in tail contact with cholesterol molecules (Fig. 7). Since the hydrocarbon portions of the molecules are area determining, lecithin molecules in the mixed monolayers may remain hydrated even at high values of surface pressure. In contrast, compression of pure lecithin monolayers to surface pressures above 20 dynes/cm severely restricts the space available to the molecules (Fig. 1), thereby limiting the ability of the polar groups to surround themselves with water molecules. This difference in the extent of hydration may explain the liquefaction of monolayers of dipalmitoyl lecithin by cholesterol.

The mechanical properties of surface films depend not only on the size and shape of the hydrocarbon chains but also on interactions among polar groups and between polar groups and the subphase (14). In fact, the high values of surface viscosity found for monolayers of long chain alcohols and amides have been ascribed to the existence of two-dimensional networks as a result of intermolecular hydrogen bonding (15). If the formation of similar networks is responsible for the rheological changes that occur in monolayers of dipalmitoyl lecithin at 25° (4), then the effect of cholesterol in permitting interactions of lecithin molecules with the subphase rather than with each other can account for the lower viscosity of monolayers containing cholesterol.

The force–area isotherms for the equimolar mixed monolayers at the two temperatures studied are quite similar (Figs. 3 and 6) and, as shown, differences between them are accounted for by the dependence of the hydrocarbon tail area on temperature. The arrangement of the lipids in the 1:1 mixed monolayers is practically independent of temperature, while the packing of dipalmitoyl lecithin in monolayers of the pure material is a function of temperature. This observation may have some significance in the extrapolation of work with model membranes containing dipalmitoyl lecithin at room temperature to a biological system at some other temperature.

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Fluorocarbon Aerosol Propellants VI: Interspecies Differences in Solubilities in Blood and Plasma and Their Possible Implications in Toxicity Studies

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Abstract □ Solubilities of the three most commonly used fluorocarbon aerosol propellants were determined in the blood and plasma of humans, monkeys, dogs, rats, and mice. Differences as large as ~fourfold in blood and more than 33-fold in plasma were found in different species. The partitioning of fluorocarbons between blood cells and plasma showed even greater differences between species. An indirect method was suggested for the calculation of plasma concentrations from total blood concentrations. A comparative evaluation was reported for the nonprotein bound fractions of fluorocarbons in the blood samples of various species. A correlation was drawn for the pharmacokinetic properties of the fluorocarbons within and between species, and a rationale was provided for the extrapolation of toxicity data from animals to humans.

Keyphrases □ Fluorocarbon aerosol propellants—interspecies differences in blood and plasma solubilities, toxicity implications □ Aerosol fluorocarbon propellants—interspecies differences in blood and plasma solubilities, toxicity implications □ Trichloromonofluoromethane—interspecies differences in blood and plasma solubilities, toxicity implications □ Dichlorodifluoromethane—interspecies differences in blood and plasma solubilities, toxicity implications □ Dichlorotetrafluoroethane—interspecies differences in blood and plasma solubilities, toxicity implications

Due to the wide use of fluorocarbon aerosol propellants in various household, cosmetic, and pharmaceutical pressurized packages, the possible adverse effects or toxicities of these compounds have been extensively studied in recent years. These studies have concerned the effects on the cardiovascular system (1–10), enzyme activities (7, 11–13), mutation (14), and ozone concentrations in the stratosphere (15, 16).

The prediction of acute toxicity of the propellants in humans has often been made by extrapolating the toxicity data from various animal species to humans (2, 10, 17, 18). However, the possibility of interspecies differences may render such extrapolations less important. One means of evaluating such differences is the study of absorption, distribution, metabolism, and elimination of these compounds in various species and of possible differences in the acute and chronic accumulation following accidental or normal exposures to these compounds.

The three most commonly used fluorocarbon aerosol propellants, trichloromonofluoromethane, dichlorodifluoromethane, and dichlorotetrafluoroethane, exist in gaseous form at 37°. Therefore, the absorption, distribution, elimination, and accumulation of these compounds are likely to be subjected to parameters that have been shown to be important for gaseous anesthetics (19, 20). Two such parameters are the solubilities of the gaseous compound in the blood and plasma of various species.

For gaseous or volatile compounds, the term "solubility" is commonly defined as the ratio of concentration of the compound between the liquid phase and the gaseous phase at the equilibrium state (19, 20). Such a ratio is, in fact, identical to the partition coefficient, P, between the two phases, as used conventionally for nonvolatile compounds between organic and aqueous phases. The purpose of this paper is to report the results of a study on the solubilities of the three most commonly used fluorocarbon propellants in the blood and plasma of various species. The possible correlation between the solubilities and the pharmacokinetic properties and a rationale for extrapolation of animal toxicity data to humans also will be discussed.

EXPERIMENTAL

Propellants and GC Analyses—The three pure propellants were supplied by the manufacturer[1]. These compounds were analyzed using a dual-column gas chromatograph equipped with a tritium electron-capture detector. The exact procedure followed was reported previously (21).

Blood and Plasma—Blood samples were freshly drawn and were used within 2 hr. Plasma samples were prepared by centrifuging either pooled or individual blood samples for 20 min in a clinical centrifuge and were also used within 2 hr after the withdrawal of blood samples. Blood samples were collected in 7.0-ml vacuum tubes[2], each containing 60 mg of ethylenediaminetetraacetate (humans, monkeys, dogs, and rats) or heparin (mice) as the anticoagulant.

Humans—About 15 ml of blood was collected from each of four healthy male volunteers, 24–35 years of age and weighing 50–70 kg.

Dogs—About 15 ml of blood was collected from four male German pointer dogs, weighing 12–16 kg.

Monkeys—About 20 ml of blood was collected from each of four rhesus monkeys, weighing 6–8 kg.

Rats—Four Sprague–Dawley-strain male rats, weighing ~300 g, were anesthetized with 65 mg/kg ip of pentobarbital sodium. The chest was opened with a quick incision, and blood samples were drawn directly from the ventricles of the heart.

Mice—Twenty BDF_1-strain male mice, weighing ~25 g, were given a deep incision in the throat, and the oozing blood was collected in a tube containing heparin. Only initial samples were collected, however, to avoid contamination with clotting factors. From 0.5 to 1 ml of blood was obtained from each mouse, and all blood samples were pooled together. There was no visible precipitation or coagulation in the pooled blood sample.

All plasma prepared from each individual was pooled, although the blood samples were studied individually except in the case of mice.

Partition Coefficient Determination—The partition coefficient or solubility, S, was determined based on the head-space method (21, 22) and can be expressed as:

$$S = \frac{A_t - C_h(V_t - V_l)}{V_l C_h} \qquad \text{(Eq. 1)}$$

where A_t = total amount of fluorocarbon added to a sealed serum bottle, V_h = volume of the head space, C_h = concentration of fluo-

[1] E. I. duPont de Nemours & Co., Wilmington, Del.
[2] Vacutainer, Scientific Products, McGraw, Ill.

Table I—Solubilities of Fluorocarbon Aerosol Propellants in the Blood and Plasma of Various Species[a]

Liquid Phase	Dichlorodifluoromethane	Dichlorotetrafluoroethane	Trichloromonofluoromethane
Human:			
Blood[b]	0.282 ± 0.009 (1)	0.264 ± 0.011 (1)	0.940 ± 0.021 (1)
Plasma[c]	0.349 ± 0.007 (1)	0.474 ± 0.018 (1)	0.924 ± 0.006 (1)
Dog:			
Blood[b]	0.449 ± 0.011 (1.59)	0.285 ± 0.017 (1.08)[d]	2.330 ± 0.107 (2.48)
Plasma[c]	0.126 ± 0.013 (0.36)	0.172 ± 0.007 (0.36)	0.877 ± 0.049 (0.95)[d]
Monkey:			
Blood[b]	0.339 ± 0.015 (1.20)	0.140 ± 0.015 (0.53)	0.993 ± 0.032 (1.06)[d]
Plasma[c]	0.162 ± 0.018 (0.46)	0.150 ± 0.014 (0.31)	0.770 ± 0.007 (0.83)
Mouse[e]:			
Blood	0.660 ± 0.017 (2.34)	0.303 ± 0.014 (1.15)	3.501 ± 0.016 (3.72)
Plasma	0.170 ± 0.004 (0.48)	0.121 ± 0.019 (0.25)	0.602 ± 0.023 (0.65)
Rat:			
Blood[b]	0.288 ± 0.014 (1.02)[d]	0.123 ± 0.008 (0.47)	1.024 ± 0.028 (1.09)[d]
Plasma[c]	0.035 ± 0.001 (0.10)	0.017 ± 0.003 (0.03)	0.399 ± 0.004 (0.43)

[a] Values in parentheses are for comparison purposes; the solubility value in human blood or plasma was considered unity. [b] Mean ± *SE* (n = 8). [c] Mean ± *SE* (n = 4). [d] Values not statistically significant compared to humans at p = 0.05. [e] Mean ± range (n = 2).

Table II—Partition Coefficients of Fluorocarbon Propellants between Blood Cells and Plasma of Various Species[a]

Species[b]	Dichlorodifluoromethane	Dichlorotetrafluoroethane	Trichloromonofluoromethane
Human	0.591 (1.00)	0.057 (1.00)	1.036 (1.00)
Dog	6.634 (11.22)	2.444 (42.87)	4.641 (4.47)
Monkey	3.601 (6.09)	0.841 (14.75)	1.689 (1.63)
Mouse	7.946 (13.44)	4.625 (81.14)	12.604 (12.16)
Rat	16.714 (28.28)	14.555 (255.35)	4.405 (4.25)

[a] Values in parentheses are for comparison purposes; the partition coefficient in humans was considered unity. [b] Hematocrit values used in calculations were: human, 47%; dog, 45.5%; monkey, 42%; mouse, 41.5%; and rat, 46%.

rocarbon in the head space, V_t = internal volume of the sealed bottle, and V_l = volume of liquid phase in the sealed bottle.

The partition coefficients were determined at two concentrations, and the equilibrium concentration in the liquid phase, C_l, was determined using the following mass balance equation:

$$C_l = \frac{A_t}{V_l + V_h/S} \qquad \text{(Eq. 2)}$$

The effect of ethylenediaminetetraacetate and heparin on the partition coefficient was studied by comparing the solubility of the three fluorocarbon propellants in normal saline with or without heparin (1%) or ethylenediaminetetraacetate (1%).

RESULTS

The solubility values of the three fluorocarbon propellants in blood and plasma of five species are summarized in Table I. The solubility in human blood and plasma is also compared to that in other species.

The partition coefficient between blood cells and plasma ($P_{bc/plasma}$) was calculated using the information in Table I through the following mass balance equations:

$$P_{blood/air} = (\text{hematocrit})(P_{bc/air}) + (1 - \text{hematocrit})(P_{plasma/air}) \qquad \text{(Eq. 3)}$$

Rearranging Eq. 3 gives:

$$P_{bc/air} = \frac{[P_{blood/air} - (1 - \text{hematocrit})(P_{plasma/air})]}{\text{hematocrit}} \qquad \text{(Eq. 4)}$$

The partition coefficient between blood cells and plasma can be expressed as:

$$P_{bc/plasma} = \frac{P_{bc/air}}{P_{plasma/air}} \qquad \text{(Eq. 5)}$$

Substituting Eq. 4 into Eq. 5 gives:

$$P_{bc/plasma} = \frac{P_{blood/air}}{(\text{hematocrit})(P_{plasma/air})} - \frac{(1 - \text{hematocrit})}{\text{hematocrit}} \qquad \text{(Eq. 6)}$$

Table II shows the partition coefficient values of the three fluorocarbon propellants between blood cells and plasma along with the comparison of the values in humans to other species. The hematocrit values for various species were obtained from the literature (23).

The concentration of fluorocarbons in the plasma could be calculated using the following relationship:

$$\frac{C_{plasma}}{C_{blood}} = \frac{C_{plasma}/C_{air}}{C_{blood}/C_{air}} = \frac{P_{plasma/air}}{P_{blood/air}} = \text{constant} \qquad \text{(Eq. 7)}$$

and, hence:

$$C_{plasma} = (C_{blood})(\text{constant}) \qquad \text{(Eq. 8)}$$

Table III shows the comparative concentrations in the plasma for an arbitrary concentration of 10 μg/ml in the blood of the five species. Table IV shows the head-space concentration if the plasma obtained from a 10-μg/ml blood sample is equilibrated to obtain estimates of nonprotein bound fractions as discussed later.

The solubility values determined at two concentration levels (50 and 100 ng/ml for dichlorodifluoromethane and 30 and 60 ng/ml for trichloromonofluoromethane and dichlorotetrafluoroethane) did not show any dependence on the equilibrium concentration. Similarly, no effect was observed due to the presence of heparin and ethylenediaminetetraacetate.

DISCUSSION

Table I shows the solubilities of the three fluorocarbons in the blood and plasma of five species. These values are higher than the solubilities of these fluorocarbons in water (22), but the order of the solubility is the same as that found in water, *i.e.*, trichloromonofluoromethane > dichlorodifluoromethane > dichlorotetrafluoroethane. Marked differences were seen, however, in the solubilities in the blood and plasma of the five species studied. For example, an almost fourfold difference was seen in the solubility of trichloromonofluoromethane in the blood of five species (Table I). Similarly, an almost 30-fold difference was noted for the solubility of dichlorotetrafluoroethane in the plasma of the five species. Such large differences can only be attributed to differences in the interaction of these fluorocarbons with the components of the blood. In general, the higher solubility of fluorocarbons in the plasma compared to water can be attributed to binding to plasma proteins.

Table III—Concentrations (Micrograms per Milliliter) of Fluorocarbon Propellants in Plasma for an Arbitrary Propellant Concentration of 10 μg/ml in Whole Blood[a]

Species	Dichlorodifluoromethane	Dichlorotetrafluoroethane	Trichloromonofluoromethane
Human	12.37 (1.00)	17.95 (1.00)	9.82 (1.00)
Dog	2.80 (0.23)	6.03 (0.33)	3.76 (0.38)
Monkey	4.77 (0.38)	10.71 (0.60)	7.75 (0.79)
Mouse	2.57 (0.21)	3.99 (0.22)	1.71 (0.17)
Rat	1.21 (0.10)	1.38 (0.08)	3.89 (0.40)

[a] Values in parentheses are for comparison purposes; the concentration in human plasma was considered unity.

Table IV—Gas Phase Concentrations[a] (Micrograms per Milliliter) of Equilibrated Plasma Obtained from Blood Samples with an Arbitrary Concentration of 10 μg/ml[b]

Species	Dichlorodifluoromethane	Dichlorotetrafluoroethane	Trichloromonofluoromethane
Human	35.44 (1.00)	37.87 (1.00)	10.63 (1.00)
Dog	22.22 (0.63)	35.06 (0.92)	4.29 (0.40)
Monkey	29.44 (0.83)	71.40 (1.88)	10.06 (0.95)
Mouse	15.12 (0.43)	32.97 (0.87)	2.84 (0.27)
Rat	34.57 (0.97)	81.18 (2.14)	9.75 (0.92)

[a] These values are proportional to nonprotein bound fractions in the blood. [b] Values in parentheses are for comparison purposes; the concentration for humans was considered unity.

This observation recently was confirmed in several reports (24–27) where the binding of these fluorocarbons with human and bovine albumins was demonstrated. Not all proteins show specific interactions resulting in an increase in the solubility, and it was recently demonstrated in this laboratory that bovine globulin in fact caused a decrease in the solubility of fluorocarbons compared to water. The net solubility in the plasma, therefore, reflects the effect of the presence of several proteins showing varying degrees of interactions with the fluorocarbons.

The solubility of fluorocarbons in the blood is contributed to not only by specific interactions with plasma proteins but also to blood cells as shown in Table II, where the partition coefficients between blood cells and plasma were calculated based on Eq. 6. Differences much larger than those reported for the solubility in blood and plasma (Table I) were found for partitioning between blood cells and plasma. A 255-fold difference was seen for dichlorotetrafluoroethane between human and rat blood, the latter showing a greater degree of partitioning.

In general, dichlorotetrafluoroethane showed greater partitioning into blood cells compared to the other two fluorocarbons in all species studied. In all cases, however, the blood cells possessed a higher concentration than in the plasma, indicating the possibility of specific interactions of fluorocarbons with various structures of the blood cells. It is obvious, however, that the blood cells show a higher magnitude of interaction than the net interaction of fluorocarbons in the plasma. The postulation of the interaction of fluorocarbons with cellular components, as already mentioned, was demonstrated recently (12); trichloromonofluoromethane was shown to bind with the phospholipid of a microsomal preparation and also to another site that appeared to be similar to the carbon monoxide binding site.

The solubility values reported here form an important basis for a comparative evaluation of the absorption, distribution, elimination, and toxicity of the fluorocarbon in various species.

The effect of solubility of volatile compounds in blood on their uptake and distribution has been emphasized (20, 28, 29). The elimination of fluorocarbon propellants takes place mainly through the lungs. In a recent study (30), almost 97% of a single dose of trichloromonofluoromethane was expired unchanged from Wistar rats. The possibility of the metabolism of trichloromonofluoromethane was also studied. No possible metabolites were, however, detected following incubation with microsomal preparations from chickens, rats, mice, guinea pigs, and hamsters pretreated with phenobarbital. The resistance of fluorocarbon propellants against metabolism is mainly due to the higher stability of C—F bonds as compared to C—Cl bonds (31). Since the elimination of fluorocarbon takes place mainly through the lungs, it is believed that the observations that have been made regarding the uptake and elimination of many volatile anesthetics also eliminated through the lungs could be applied to the fluorocarbon propellants.

Stoelting and Eger (32) studied the effect of solubility on recovery from anesthesia from various gaseous anesthetics. They showed that the solubility in blood of the three anesthetics, methoxyflurane, halothane, and nitrous oxide, was directly proportional to their recovery time. Recovery time was defined as the time required for the alveolar concentration to drop to a certain fraction following various degrees of equilibration of these anesthetics with body tissues. They also showed that compounds with high solubility such as methoxyflurane (solubility = 13) and halothane (solubility = 2.3) showed a significant distribution to the tissues, which was reflected in the longer recovery times following equilibration for longer periods as compared to nitrous oxide (solubility = 0.47). Consequently, higher solubility compounds also will have larger uptakes and longer biological half-lives if they are absorbed and eliminated primarily from the lungs.

For example, it was shown (33) that upon inhalation in humans of a mixture of four fluorocarbons, including the three in this report, the most soluble, trichloromonofluoromethane, was absorbed in the blood to a greater extent than dichloromonofluoromethane and dichlorotetrafluoroethane, which have lower solubility in the blood (Table I). Trichloromonofluoromethane also showed a comparatively slower elimination from the body.

Detailed pharmacokinetics of these fluorocarbons recently were studied (34, 35) in dogs following intravenous dosing, and it was found that the biological half-lives of dichlorotetrafluoroethane and dichlorodifluoromethane ranged from 40 to 60 min but the half-life of trichloromonofluoromethane was significantly longer (90–100 min). This difference corresponds very well to the differences in solubility values (Table I) in the dog blood. The differences in the solubility of the three fluorocarbons in the dog blood were very well represented in their elimination behavior. For example, the pulmonary clearance of the high solubility fluorocarbon trichloromonofluoromethane was 0.9 liter/min compared to 2.05 and 2.53 liters/min for dichloromonofluoromethane and dichlorotetrafluoroethane, respectively (34).

The distribution of these fluorocarbons from the central (blood) compartment to the tissue compartments followed the general principle that compounds having low solubility in the blood are distributed faster to the tissue. For example, it took 5 min to obtain peak concentration in one tissue compartment and 10 min for the other tissue compartment for both dichloromonofluoromethane and dichlorotetrafluoroethane, but the corresponding time periods were 10 and 30 min for the two compartments for trichloromonofluoromethane in dogs (34).

These observations suggest that solubility values indeed offer an important index for the evaluation of absorption, distribution, and elimination and can be used to study toxicity correlations and differences between species. Since these fluorocarbons are primarily eliminated from the lungs, interspecies variations of several properties can be anticipated. For example, an almost fourfold difference in the blood solubility of trichloromonofluoromethane be-

tween humans and mice is most likely to result in a significant difference in the uptake and disposition characteristics between the two species; any extrapolation of possible toxicity based on these properties is probably less justified than between monkeys and humans where the solubility of this fluorocarbon is in good agreement. On the other hand, placing great confidence in solubility values to anticipate uptake and disposition patterns is also not justified in view of the differences in the pulmonary ventilation and cardiac output both within and between species (23) since these factors can seriously affect the uptake and disposition patterns.

It is generally agreed that the plasma concentrations of the drugs correlate better with various pharmacological and toxicological properties than the dose administered. In some instances, the nonprotein bound fraction of the drug in a blood sample correlates even better. However, it is not very practical to measure directly the plasma concentrations of fluorocarbon propellants by first centrifuging the blood samples, since the high volatility of these compounds can result in a significant loss to the atmosphere, especially at room temperature during handling. An indirect method can be easily employed if the partition coefficients between blood cells and plasma are available, as described earlier (Eq. 6). The plasma concentration can then simply be calculated using Eq. 8.

Table III shows the plasma concentrations of the three fluorocarbons in the five species for an arbitrary whole blood concentration equal to 10 μg/ml. Differences as large as 13-fold existed in the plasma concentrations between various species; in all instances, the human plasma showed the highest concentration at equal blood concentrations. These differences are attributable to the differences in the partitioning between blood cells and plasma. This observation can have an important bearing on comparative evaluations of toxic blood concentrations in various species if the plasma concentrations correlate better with the possible toxic manifestations. Nevertheless, this conclusion can be misleading if due consideration is not given to the nonprotein bound fraction in the plasma. Although no such measurements were made directly, an indirect comparison can be made easily by dividing the plasma concentration by the partition coefficient between plasma and air to obtain the concentration in the gaseous phase equilibrating with the plasma. In such a hypothetical equilibration, the concentration in the gaseous phase will be directly proportional to the unbound fraction in the plasma. These values are shown in Table IV.

Although the relative nonprotein bound concentration was generally higher for humans, except for dichlorotetrafluoroethane, the maximum difference decreased to about 3.5-fold compared to a 13-fold difference in the plasma (Table III).

In conclusion, the large differences in the solubilities of fluorocarbon aerosol propellants can result in significant differences in the pharmacokinetics of these fluorocarbons. Thus, any interspecies extrapolation of toxicity data based on parameters such as dose, toxic blood concentration, accumulation, and uptake should take into account the various solubility parameters described here.

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High-Performance Liquid Chromatographic Analysis of Ampicillin

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Abstract □ A high-pressure liquid chromatographic method for the analysis of ampicillin is described. The method uses a 1-m long stainless steel column packed with anionic exchange resin, with a mobile phase of 0.02 *M* $NaNO_3$ in 0.01 *M* pH 9.15 borate buffer at a flow rate of 0.45 ml/min. The degradation products of ampicillin, penicillenic and penicilloic acids of ampicillin, can be separated and quantitated in less than 12 min of chromatographic time. The relative standard deviation for the analysis of ampicillin is less than 1%, and the method is sensitive to approximately 20 ng of ampicillin/sample injected. The method was applied to the analysis of various pharmaceutical preparations of ampicillin. It is also applicable, with a slight modification, for the analysis of penicillins G and V.

Keyphrases □ Ampicillin—high-pressure liquid chromatographic analysis □ Penicillins G and V—high-pressure liquid chromatographic analysis □ High-pressure liquid chromatography—analysis, ampicillin and penicillins G and V

Numerous chemical and physical methods and several automated methods are available for the determination of penicillins (1–13). Of these methods, the iodometric and hydroxylamine methods have been officially recognized by the Food and Drug Administration for "potency" determination (1). Although the speed of the automated iodometric and hydroxylamine methods (9–13) is fast, 20–60 determinations/hr, they are incapable of differentiating the intact penicillin molecules from the degradation compounds, since they determine the degraded penicillins, penicillanic and penicilloic acids.

Several GLC methods for penicillin have been reported (14–17); however, the methods determine penicillanic acid and have not been used for the analysis of ampicillin due mostly to difficulties in solubilization and derivatization of ampicillin. Chromatographic separation using a cross-linked dextran gel[1] column (18, 19) is slow and, to the knowledge of the authors, no high-performance liquid chromatographic (HPLC) method has been used to quantitate ampicillin, although an HPLC method was reported for the detection of ampicillin in nitrofurantoin (20) and for the determination of cephalosporin C (21).

EXPERIMENTAL

Apparatus—A modular liquid chromatograph equipped with a 254-nm UV monitor[2], a minipump[3], and a pulse dampener[4] were used.

An empty stainless steel column[5], 2.1 × 1000 mm, was first rinsed with tetrahydrofuran. Then the inside of the tubing was vigorously cleaned with a cotton string, presoaked with tetrahydrofuran, to remove loose metal particles. Chloroform was then drawn through the column, and the column was dried under a stream of dry nitrogen.

A 10-μm pore size stainless steel frit was fitted into the inlet end of the column, and a hex nut[6] with a stainless steel front, back lock ferrules, and cap[7] were attached to the column. Anionic exchange resin[8] was dry packed into the open end of the column by adding a small amount of the resin at a time and lightly tapping on the floor. After the column was tightly packed, a 2-μm pore size stainless steel frit was inserted into the outlet end of the column. The packed column was attached to a septumless injector valve[9] and the 254-nm UV monitor. The theoretical plates of the column, thus prepared, were approximately 555/m for the ampicillin peak.

Reagents—For the mobile phase, 0.02 *M* sodium nitrate in 0.01 *M* sodium borate, pH 9.15, was used.

Preparation of Ampicillin Standard Solution—The USP ampicillin reference standard was used "as is" at 867 μg/mg. Approximately 10 mg of the reference standard was accurately weighed[10] and placed in a 10-ml volumetric flask.

Prior to analysis, the standard was dissolved and diluted to the volume with the mobile phase.

Preparation of Samples—*Ampicillin Bulk Powder*—Approximately 10 mg of ampicillin bulk powder was accurately weighed and placed in a 10-ml volumetric flask. Prior to analysis, the sample was dissolved and diluted to volume with the mobile phase.

Hard-Filled Capsule—To minimize capsule weight variation, 10 capsules were emptied and their contents were accurately weighed. A portion of the powder equivalent to 1000 mg ampicillin was accurately weighed and placed in a 1000-ml volumetric flask. The contents were then diluted to volume with double-distilled water and shaken for 5 min to dissolve the ampicillin. About 25 ml of the suspension was placed into a 50-ml conical bottom centrifuge tube and then centrifuged at 2000 rpm for 20 min.

Sterile Injectables—Each vial containing 250 mg, 500 mg, or 1 g of product was reconstituted with the recommended volume of double-distilled water. An ultrasonicator[11] was used to dissolve each sample quickly.

From each of four 250-mg vials, 0.1 ml was removed with a 100-μl micropipet[12] with a disposable tip and placed in a 100-ml volumetric flask. The sample was then diluted to volume with the mobile phase.

From each of two 500-mg vials, 0.1 ml was removed and placed in a 100-ml volumetric flask. The sample was then diluted to volume with the mobile phase.

From one 1-g vial, 0.1 ml was removed and placed in a 100-ml volumetric flask. The sample was then diluted to volume with the mobile phase.

[1] Sephadex.
[2] Model 1285, Laboratory Data Control, Riviera Beach, Fla.
[3] Milton Roy, Laboratory Data Control, Riviera Beach, Fla.
[4] Model 709, Laboratory Data Control, Riviera Beach, Fla.
[5] DuPont, Wilmington, Del.
[6] No. 820349, DuPont.
[7] No. 201724, DuPont.
[8] VYDAC P150, AX, Applied Science Laboratories, Inc., State College, Pa.
[9] Micromeritics, Inc., Norcross, Ga.
[10] Cahn electrobalance, model G, Cahn Instrument Corp., Paramount, Calif.
[11] Ultrasonics, Inc., Plainview, Long Island, N.Y.
[12] Eppendorf, Brinkmann Instruments, Westbury, N.Y.

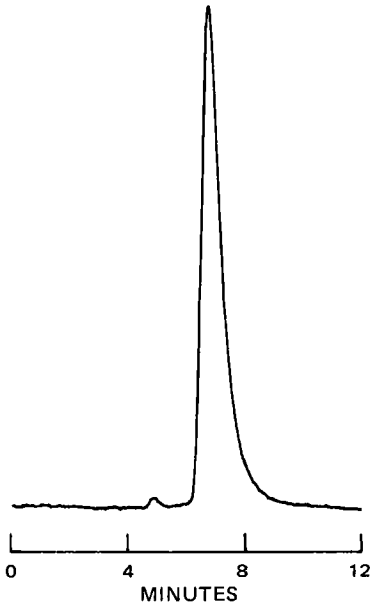

Figure 1—*HPLC analysis of ampicillin using a 1-m long anion-exchange column (room temperature) at a flow rate of 0.45 ml/min. Mobile phase was 0.02* M *$NaNO_3$ in 0.01* M *pH 9.15 borate buffer.*

Oral Formulation—Each bottle containing 125 or 250 mg of oral preparation was reconstituted with the recommended volume of double-distilled water. From one 125-mg bottle, 5 ml was pipetted into a 50-ml round-bottom centrifuge tube. Then 0.1 ml of concentrated hydrochloric acid was added and mixed, followed by 30 ml of chloroform. The centrifuge tube was capped and shaken vigorously for 5 min, using a reciprocal shaker[13], and then centrifuged for 2 min at 2000 rpm. Four milliliters of the upper aqueous layer was pipetted into a 100-ml volumetric flask and diluted to volume with 0.02 *M* pH 6.0 phosphate buffer.

From one 250-mg bottle, 5 ml was pipetted into a 50-ml round-bottom centrifuge tube. Then 0.1 ml of concentrated hydrochloric acid was added and mixed, followed by 30 ml of chloroform. The centrifuge tube was capped and shaken vigorously for 5 min, using the reciprocal shaker, and then centrifuged for 2 min at 2000 rpm. Two milliliters of the upper aqueous layer was pipetted into a 100-ml volumetric flask and diluted to volume with 0.02 *M* pH 6.0 phosphate buffer.

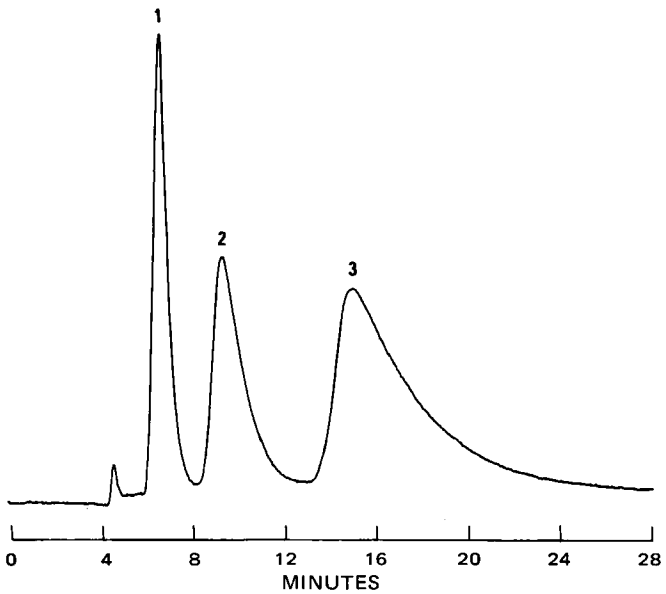

Figure 2—*HPLC separation of (1) ampicillin, (2) penicillin G, and (3) penicillin V using a 1-m long anion-exchange column (room temperature) at a flow rate of 0.45 ml/min. Mobile phase was 0.02* M *$NaNO_3$ in 0.01* M *pH 9.15 borate buffer.*

[13] Eberbach.

Table I—Precision of the HPLC Analysis for Ampicillin

Weight, mg	Peak Area	Area to Weight Ratio	Peak Height	Peak Height to Weight Ratio
10.036	289.3	28.83	25.15	2.506
10.000	280.7	28.07	25.30	2.530
10.062	304.1	30.22	26.00	2.584
9.976	279.2	27.99	25.25	2.531
10.110	283.6	28.05	25.45	2.517
10.034	284.3	28.33	25.70	2.561
10.158	280.0	27.56	25.75	2.535
10.000	294.7	29.47	25.70	2.570
10.032	283.0	28.21	25.60	2.562
10.000	263.2	26.32	25.80	2.580
10.036	280.4	27.94	25.65	2.556
10.120	285.7	28.23	25.65	2.535
	RSD	3.40%	*RSD*	0.98%

Chromatographic Conditions—The flow rate of the mobile phase was 0.45 ml/min, with a column pressure of about 34 atm (500 psi). The column temperature was ambient, and the chart speed was 0.6 cm (0.25 in.)/min. A 2.0-μl quantity of sample was quantitatively injected into the column, using a septumless injector valve at an electrometer range setting of 0.02 full scale.

RESULTS AND DISCUSSION

A typical chromatogram of ampicillin is shown in Fig. 1. The HPLC method takes less than 12 min of chromatographic time to analyze one sample.

Penicillins G and V can also be separated and quantitated by the HPLC method (Fig. 2). For the quantitation of penicillin G samples, a slight increase in the amount of sodium nitrate in the mobile phase to 0.03 *M* optimizes the retention time of penicillin G and speeds up the analysis. The relationship between the concentration of sodium nitrate and the retention time of various compounds was described by Nelson (22). Since procaine elutes much slower than penicillin G (relative retention of 11) and interferes with subsequent, multiple analysis of samples, procaine must be removed prior to analysis. For the quantitative analysis of penicillin V, the addition of methanol to 10% in the mobile phase speeds up the analysis.

Detection of Degradation Compounds—Commercially produced ampicillin powder was degraded in an alkaline solution for 1 week, and the degraded ampicillin sample was then chromatographed. As shown in Fig. 3, the HPLC method is capable of dif-

Table II—Analysis of Ampicillin Trihydrate Bulk Powder

Lot	Ampicillin, μg/ml	
	HPLC	Iodometric
1	883	893
2	871	886
3	842	863
4	849	847
5	859	863
6	853	859
7	853	853
8	860	880
9	871	888
10	895	880

Table III—Analysis of Ampicillin Products

Label Content	Lot	Ampicillin, mg/Dosage Unit HPLC	Iodometric
Sterile injectable			
250 mg	a	285, 281	277
	b	287, 287	285
500 mg	c	500, 480	527
	d	507, 502	556
1 g	e	943	1100
Hard-filled capsule			
250 mg	a	265	264
	b	244	246
	c	251	265
	d	246	256
500 mg	e	513	516
Oral formulation			
125 mg/5 ml	a	130	122
	b	124	121
	c	125	135
	d	124	122
250 mg/5 ml	e	257	259
	f	248	266
	g	248	262

ferentiating penicillenic and penicilloic acids of ampicillin from ampicillin. The presence of a small amount of penicillin G was also detected in this sample. These peaks were identified by matching the relative retention times with those of relatively pure penicillenic and penicilloic acids of ampicillin. Penicillenic acid was prepared by forming a mercuric mercaptide of ampicillin followed by decomposition with hydrogen sulfide (23). The purity of penicillenic acid thus prepared was approximately 84%. The penicilloic acid sample was obtained from a commercial source[14]. Since penicillanic acid has no UV absorption, its presence may not be detected by this method.

Since a slight degradation, less than 5%, can occur when ampicillin is left in the mobile phase for more than 1 hr, the ampicillin sample should be diluted in the mobile phase just prior to the analysis.

The minimum quantity of ampicillin detection was approximately 20 ng/sample when injected at an electrometer range setting of 0.01 full scale. Since the method is capable of detecting degradation compounds and since penicillenic acid, a degradation compound, was reported to have "allergenic equivalence" with penicillin G (24, 25), the HPLC method is of value for penicillin cross-contamination studies.

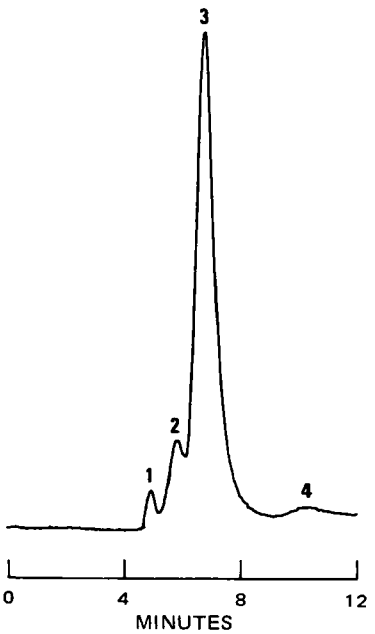

Figure 3—*HPLC analysis of ampicillin degraded in an alkaline solution indicating separation of (1) penicillenic acid, (2) penicilloic acid of ampicillin, (3) ampicillin, and (4) penicillin G.*

[14] Bristol Laboratories.

Table IV—Analysis of Ampicillin Trihydrate Bulk Powder Stored for 1 Month at 55°

Lot	Initial HPLC	Initial Iodometric	Initial Microbiological	Aged HPLC	Aged Iodometric	Aged Microbiological
	Ampicillin, μg/mg					
1	883	893	879, 863	823	862	830, 826
2	842	863	825, 894	770	840	777, 808
3	849	847	—	743	818	732, 757

Quantitative Determination of Ampicillin—Various amounts of the USP ampicillin reference standard, ranging from 0.1 to 15 mg/ml, were prepared, and a 2.0-μl quantity of each was quantitatively injected into the column using a septumless injector valve. The calibration curve was linear (correlation coefficient of 1.000) over the entire range, with a linear regression of $y = 3.33x - 0.036$.

The precision of the HPLC method was determined by comparing 12 replicate preparations of the USP ampicillin reference standard at approximately 1.0 mg/ml. Table I indicates that the relative standard deviation of the method is 3.4% when the peak area to weight ratio is used for the calculation and less than 1% when the peak height to weight ratio is used. Thus, good precision can be obtained by use of the septumless injector valve without an internal standard.

Ten current lots of ampicillin trihydrate bulk powder were analyzed (Table II). The potency was calculated using the ampicillin reference standard at 867 μg/mg. The drug contents thus calculated were compared to those of the iodometric method. The values obtained by the HPLC method showed no statistically significant difference from those of the iodometric assay method.

Table III shows the results of analysis of pharmaceutical products containing ampicillin. The potencies calculated from the HPLC method showed no statistical difference from those of the iodometric method.

Three lots of ampicillin trihydrate powder were aged for 1 month at 55°. Data obtained by the HPLC method showed 6.8–12.5% reductions in potencies, indicating that the powders underwent degradation by the heat treatment (Table IV). As expected, no loss in potencies was detected by the iodometric method. Microbiological assay (1) values of these samples indicated degradation and agreed very well with those of the HPLC method.

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Determination of Major Impurity in Chlordiazepoxide Formulations and Drug Substance

W. N. FRENCH, F. F. MATSUI, and S. J. SMITH [x]

Abstract □ Procedures for quantitating the lactam impurity, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide, which can be present in chlordiazepoxide formulations, is presented. The method consists of trapping chlordiazepoxide in sulfuric acid in kieselguhr, eluting the impurity with ether, and quantitating by UV spectrophotometry in absolute alcohol at 312 nm.

Keyphrases □ Chlordiazepoxide and chlordiazepoxide hydrochloride—UV analysis for lactam impurity (7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide) □ 7-Chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide—UV analysis in chlordiazepoxide and chlordiazepoxide hydrochloride □ UV spectrophotometry—analysis, 7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide in chlordiazepoxide formulations

Pharmacopeial monographs for the widely used tranquilizer chlordiazepoxide [7-chloro-2-(methylamino)-5-phenyl-3*H*-1,4-benzodiazepine 4-oxide] and the hydrochloride salt include limit tests for the lactam (7-chloro-1,3-dihydro-5-phenyl-2*H*-1,4-benzodiazepin-2-one 4-oxide, I) and phenone (2-amino-5-chlorobenzophenone, II) impurities (1–3). These specifications allow a maximum of 0.1% of I and 0.01 (1, 2) or 0.05% (3) of II in the chlordiazepoxide drug substance. Monographs for capsules prepared with the hydrochloride salt permit a maximum of 3.0 and 0.1% of the two impurities (1, 3), respectively, while tablets prepared with chlordiazepoxide base may contain a maximum of 4% of I and 0.1% of II (2).

While limit tests are effective in monitoring impurities in chlordiazepoxide drug compounds and formulations, quality assurance procedures often require that the levels of impurities present in a drug substance or formulation be known precisely. This article describes methodology employing a "trap" column suitable for the quantitation of the impurity I present in the drug substance and formulations.

EXPERIMENTAL

Apparatus and Materials—Glass columns (2.5 × 20 cm), 0.2 *N* sulfuric acid, water-washed ether (ethyl), and absolute ethanol were used.

For purified kieselguhr, soxhlet extract kieselguhr[1] with methanol (ACS grade) for 24 hr. Thoroughly dry the support material and store in a well-closed bottle.

For purified absorbent cotton, soxhlet extract absorbent cotton with methanol (ACS grade) for 24 hr. Dry thoroughly and store in well-stoppered bottles.

For the standard solution of I, dissolve sufficient I in ether so that each milliliter contains 150 μg.

Procedure—Place a pledget of purified absorbent cotton at the bottom of the column. Then place purified kieselguhr (3 g), which has been triturated with 0.2 *N* sulfuric acid (3 ml), over the cotton. Tamp the layer lightly but evenly. Triturate a second 3-g portion of purified kieselguhr with 3 ml of 0.2 *N* sulfuric acid, add an accurately weighed portion of powdered tablet or capsule material or drug substance equivalent to about 25 mg of chlordiazepoxide (base or salt) or 5.0 ml of standard solution of I, and mix thoroughly. Transfer this material to the prepared column and tamp into a uniform second layer. Wipe the beaker and mixing rod with purified absorbent cotton and place the cotton on top of the prepared kieselguhr column.

Elute the column with water-washed ether. Collect at least 25 ml of eluate in a 50-ml volumetric flask and evaporate the eluate to dryness, employing a stream of dry nitrogen. Dissolve the resultant residue in ethanol and make to volume with this solvent. Measure the absorbances of standard and sample solutions at 312 nm against a similarly prepared blank containing no sample or standard.

Calculate the percent of I as follows:

$$\%\,\mathrm{I} = \frac{\text{weight of I in standard } (\mu g)}{} \times \frac{A_{spl}}{A_{std}} \times \frac{100}{\text{weight of chlordiazepoxide taken } (\mu g)} \quad \text{(Eq. 1)}$$

[1] E. M. Chemicals Inc., Elmsford, NY 10523

(15) C. Highta, D. L. Mays, and M. Garofalo, *Anal. Chem.*, **43**, 1530(1971).
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Elute the column with water-washed ether. Collect at least 25 ml of eluate in a 50-ml volumetric flask and evaporate the eluate to dryness, employing a stream of dry nitrogen. Dissolve the resultant residue in ethanol and make to volume with this solvent. Measure the absorbances of standard and sample solutions at 312 nm against a similarly prepared blank containing no sample or standard.

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[1] E. M. Chemicals Inc., Elmsford, NY 10523

Table I—Trap Chromatography of Chlordiazepoxide and Compound I

Sample	Chlordiazepoxide Hydrochloride Introduced to Column[a], mg	Compound I Introduced to Column[b], μg	Absorbance of Eluant at 312 nm
1	25.60	—	0.002
2	30.73	—	0.009
3	50.00	264	0.160
4	40.62	264	0.164
5	30.88	264	0.161
6	—	264	0.158
7	—	264	0.158
8	—	264	0.160

[a] Actual quantities of chlordiazepoxide weighed and added to acidic kieselguhr layer. [b] Where indicated, an ethereal solution containing 264 μg of I was added to the acidic kieselguhr layer.

where A_{spl} is the absorbance at 312 nm of the sample solution, and A_{std} is the absorbance at 312 nm of the standard solution.

DISCUSSION

When I is present in chlordiazepoxide, it cannot be quantitated by direct UV spectrophotometry because of the similar maxima exhibited by the two compounds. GLC also is unsuitable since preliminary studies indicated, as reported previously (4), that chlordiazepoxide decomposed during chromatography. Thus it was decided to take advantage of the basic nature of chlordiazepoxide and the acidic character of I and to separate the compounds by employing a trap chromatography procedure (5–7).

Since the procedure was required specifically for the quantitation of I, experimental conditions were chosen so that chlordiazepoxide would be retained on the column, thus allowing the isolation and subsequent quantitation of the I present. Sulfuric acid in the trap layer was used to ensure that all parent drug was converted to the salt form, which is insoluble in ether. The presence of the acid also ensured that no basic material, which might be present in the sample, could cause retention of I. Ether was chosen as the eluant because of its known poor solvolytic power for chlordiazepoxide and salts but good affinity for I. In addition, this solvent did not elute sulfuric acid from the trap layer under the experimental conditions.

Samples of chlordiazepoxide, of I, and of mixtures were chromatographed through trap columns, employing the procedure described. Results (Table I) indicated that chlordiazepoxide was quantitatively retained while I was recovered from eluates. Weak absorbances observed with chlordiazepoxide samples (Samples 1 and 2) and slightly higher absorbances observed with drug–I mixtures (Samples 3–5) compared to pure I (Samples 6–9) were probably due to a combination of traces of I present in the drug compound, solvent residues (discussed later), and instrumental variation. High-pressure liquid chromatography (HPLC) of trap column eluates from the mixtures (Samples 3–5) did not detect the presence of chlordiazepoxide.

Table II—Recovery of Lactam Impurity I through the Trap Column

Theoretical Concentration in Final Ethanol Solution, g/ml	Absorptivity at 312 nm Obtained with No Column Trap	Absorptivity at 312 nm after Trap Chromatography	Recovery through Column, %
30.76	36.4	35.5	97.5
21.53	35.2	35.4	100.5
15.38	35.8	35.3	98.6
6.152	35.7	36.0	100.8
4.306	34.8	35.0	100.5
3.076	35.1	34.4	98.0
Mean value	35.5[a]	35.3	99.6
Coefficient of variation	1.6	1.5	1.2

[a] Absorptivity of ethanolic solutions of impurity I measured at 312 nm was 35.6 (two determinations).

Table III—Trap Column Analysis of I in a Triturate and a Commercial Capsule Formulation

Triturate[a]			
Triturate Taken, mg	I Determined, %	Recovery, %	Capsule Composite[b], I Found, %
42.47	1.58	103.9	2.93
49.64	1.55	101.9	2.91
61.02	1.47	96.9	2.85
81.41	1.44	94.7	2.88
50.64	1.59	104.3	2.83
51.11	1.55	101.8	—
Mean recovery		100.6	2.88
Coefficient of variation		3.9	1.5

[a] Content of I, 1.52%, was determined by tumbling seven weighed aliquots of triturate with ether (25 ml) for 1 hr, filtering the mixture, washing the filter and residue with ether (3 × 5 ml), evaporating the ether solution to dryness with dry nitrogen, dissolving the residue in 50.0 ml ethanol, and measuring the absorbance of the final solution. [b] Various weights of the composite were taken for analysis.

To determine whether any I was retained by the trap chromatography column, 5-ml aliquots of various concentrations of I in ether were treated as described in *Procedure* and the absorptivities of eluants were determined. These data were compared with absorptivities obtained when similar aliquots of I were diluted to 25 ml with ether, the ether solution was evaporated to dryness with dry nitrogen, and the residue was made to 50.0 ml with ethanol. The data (Table II) indicate that I was not retained on the trap column.

The data also show that the absorbance of solutions of I is linear over the range indicated with a mean recovery of 99.68% [coeffi-

Table IV—Comparative Analysis of Compound I in Chlordiazepoxide Formulations

Sample and Form of Chlordiazepoxide	Percent I Relative to Chlordiazepoxide Found by: Trap Chromatography Method	TLC[a]	HPLC[b]
A, capsule, hydrochloride salt	5.52	5	—
B, capsule, hydrochloride salt	0.31	<0.3	0.24
C, capsule, hydrochloride salt	4.25	4	4.01
D, capsule, hydrochloride salt	4.55	5.5	—
E, capsule, hydrochloride salt	2.56	2–2.5	—
F, capsule, hydrochloride salt	3.15	3.5	—
G, capsule, hydrochloride salt	5.92	5	5.57
H, tablet, free base	0.45	0.4	0.38
I, tablet, free base[c]	8.56	8	8.49

[a] TLC was carried out on silica gel F60-precoated layers (Merck) in ethyl acetate–ethanol (9:1) (8). Visualization was effected by spraying with Bratton–Marshall reagent (1), and estimation was made by comparison with standard spots. [b] HPLC was performed in a 5-μm microparticulate silica gel column with 5% ammoniacal ethanol (3% v/v), 30% tetrahydrofuran, and 65% *n*-hexane as solvent in a Varian model 4100 HLSL liquid chromatograph with a 254-nm UV detector. The internal standard was nitrazepam. [c] A pink material in the tablet coating interfered with the trap column method. Consequently, powdered tablet containing an equivalent of 25 mg of drug was extracted with acetone, the extract was taken to dryness, the residue was taken up in the kieselguhr containing sulfuric acid, and the analysis was concluded in the manner described.

cient of variation (*CV*) 1.2%]. Manipulation of samples does not lead to the loss of material, since absorptivity measurements of a directly prepared 5.1-μg/ml solution of I in ethanol afforded an absorptivity of 35.6.

The similar absorptivity values obtained between solutions of I prepared directly in ethanol and those prepared through an ether stage indicate that any residue from ether that could be present does not appear to make a significant absorbance contribution at 312 nm. To confirm this finding, 25-ml portions of ether were evaporated to dryness with a stream of dry nitrogen, any residue present was dissolved in 50.0 ml of ethanol, and the absorbance was measured. Values of 0.01 or lower were obtained.

Results from TLC examination of chlordiazepoxide formulations carried out over an extended period showed that all formulations contained well under 0.1% 2-amino-5-chlorobenzophenone (II). Thus, normally encountered levels of II in chlordiazepoxide formulations, while present in column eluates, do not interfere significantly with the absorbance of I at 312 nm. The absorbance maximums of a solution of I in ethanol (equivalent to 3% I using procedures described under *Experimental*) and a I–II mixture (equivalent to 3 and 1%, respectively) were nearly the same, 0.531 and 0.538, respectively. The level of II utilized was 10 times higher than pharmacopeial specifications (1–3) and was present in a much greater proportion relative to I than is ever encountered in the practical situation. Thus, it can be concluded that the absorbance contribution due to II, if present, will be well within experimental variation.

The accuracy of the proposed method for quantitating I in chlordiazepoxide was determined by analyzing aliquots of lactose triturate of I. The data obtained from this study (Table III) indicated a through-column recovery of 100.58% I (*CV* 3.9%). The precision of the proposed method was determined by analyzing replicate samples of a commerical capsule formulation, which indicated a coefficient of variation of 1.5% (Table III). This coefficient of variation of 1.5% compared very closely with coefficient of variation values obtained from ethereal solutions of I measured directly (1.6) and put through the trap column (1.5) (Table II) and with results obtained when mixtures of chlordiazepoxide and I were passed through the trap column (Table I, Samples 3–5, coefficient of variation of absorbance at 312 nm, 1.28%). In comparison, it would appear that the higher coefficient of variation values obtained from I–lactose triturate samples (Table III) reflect problems encountered in attempting to obtain a homogeneous triturate of a low level of I rather than the imprecision of the method.

Nine samples of chlordiazepoxide tablets and capsules, from a number of suppliers, were analyzed for I, employing the trap column method. The results from duplicate analyses were compared with values for I obtained from TLC and/or HPLC. Data obtained (Table IV) confirm the accuracy of the method of analysis and underline the suitability of the trap column procedure for the quantitation of decomposition product I in chlordiazepoxide.

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FOTIOS M. PLAKOGIANNIS [x] and AHMED M. SAAD

Abstract □ A rapid method for the routine determination of acetaminophen and dichloralantipyrine in capsules is reported. The determination of acetaminophen is based on the ability of its hydrolytic product, *p*-aminophenol, to produce an intensive yellow color with vanillin. The determination of dichloralantipyrine is based on the fact that it, as well as its major metabolite chloral hydrate, produces a blue color with quinaldine ethiodide. No interferences were encountered, and good recovery and precision data were obtained.

Keyphrases □ Acetaminophen–dichloralantipyrine—spectrophotometric analysis of capsule formulation □ Dichloralantipyrine–acetaminophen—spectrophotometric analysis of capsule formulation □ UV spectrophotometry—analysis, acetaminophen and dichloralantipyrine in capsules

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Archer and Haugar (7) found out that the addition compound, dichloralantipyrine, produces, upon the addition of quinaldine ethiodide, a blue color in proportion to its chloral hydrate content.

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Keyphrases □ Acetaminophen–dichloralantipyrine—spectrophotometric analysis of capsule formulation □ Dichloralantipyrine–acetaminophen—spectrophotometric analysis of capsule formulation □ UV spectrophotometry—analysis, acetaminophen and dichloralantipyrine in capsules

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Archer and Haugar (7) found out that the addition compound, dichloralantipyrine, produces, upon the addition of quinaldine ethiodide, a blue color in proportion to its chloral hydrate content.

Table I—Recovery of Acetaminophen and Dichloralantipyrine Added

Acetaminophen, mg		Dichloralantipyrine, mg	
Added	Found	Added	Found
150	149.6	75.3	77
	151.9		75.5
	152.4		75.1
	148.1		75.1
	151.6		74.3
	149.7		74.9
Mean	150.5	Mean	75.3

This report describes a quantitative UV spectrophotometric technique for the determination of acetaminophen and dichloralantipyrine in capsules containing 325 mg of acetaminophen, 100 mg of dichloralantipyrine, and 65 mg of isometheptene mucate.

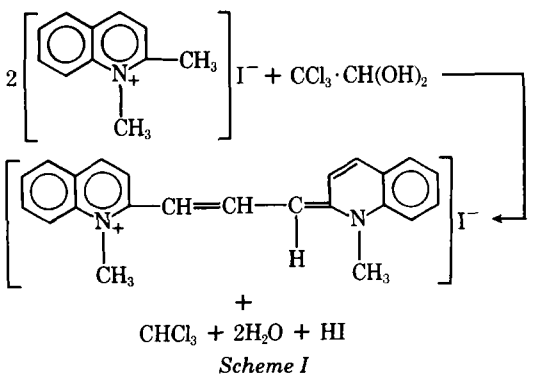
EXPERIMENTAL[1]

Materials and Reagents—The following were used (all of analytical grade): acetaminophen–dichloralantipyrine–isometheptene mucate capsules[2], 5% vanillin[3] in isopropanol, acetaminophen[4], dichloralantipyrine[5], 1.5% (w/v) quinaldine ethiodide[6] solution (dissolve 1.5 g of quinaldine ethiodide in 100 ml of water and filter if necessary), and 0.1 *N* ethanolamine[3] (dissolve 6.1 g of ethanolamine in water and dilute to 1 liter).

Acetaminophen Assay—The colorimetric curve is prepared as follows. Accurately weigh about 120 mg of acetaminophen in 100 ml of 4 *N* HCl. Transfer by pipet 0.5, 1.0, 1.5, 2.0, and 2.5 ml of the standard solution to five test tubes. Add 5 ml of 4 *N* HCl and place the tubes in a beaker of boiling water for 1 hr. Allow the tubes to cool and transfer the contents quantitatively to 50-ml volumetric flasks.

Add 10 ml of vanillin reagent to each flask and dilute to volume. Mix well and measure the absorption, after appropriate dilution, at 395 nm against a blank (6 ml of 4 *N* HCl, 10 ml of vanillin reagent, and enough water to make 50 ml). The absorbances obtained plot into a straight line.

Acetaminophen Sample Preparation and Assay—Weigh and finely powder the sample to correspond to 120 mg of acetaminophen based on label claim. Transfer it to a suitable container and add about 50 ml of 4 *N* HCl. Agitate with mechanical shaker for 20 min, filter through paper into a 100-ml volumetric flask, and dilute to volume with 4 *N* HCl. Mix well and transfer 1 ml to a test tube. Add 5 ml of 4 *N* HCl and heat for 1 hr in boiling water. Then proceed as directed under *Acetaminophen Assay* beginning with: "Allow the tubes to cool and . . . ." Calculate the amount of *p*-aminophenol from the calibration curve and multiply it by the factor 1.385 and the dilutions used.



Scheme I

Dichloralantipyrine Assay—The calibration curve is prepared as follows. Dissolve 250 mg of chloral hydrate in water and dilute to 500 ml. Dilute 10 ml of this solution to 100 ml with water, so that the standard chloral hydrate solution contains 50 μg/ml. Transfer by pipet 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, and 1.6 ml of the standard solution to seven test tubes and pipet 1 ml of water into an eighth test tube to serve as a blank. Add to each tube 1 ml of quinaldine ethiodide solution and 6 ml of isopropanol, mix well, add 0.5 ml of 0.1 *N* ethanolamine, dilute to 10 ml with water, and mix. Then place the tubes in a water bath at 60° for 1 hr. Remove the tubes from the water bath, cool, and measure the absorption at 605 nm after appropriate dilutions. The absorbances obtained plot into a straight line.

Dichloralantipyrine Sample Preparation and Assay—Weigh an aliquot from the previously finely powdered capsules to correspond to 75 mg of dichloralantipyrine based on label claim. Transfer it to a suitable container so that it can be diluted with water to contain 60 μg/ml. Transfer by pipet 1 ml to a test tube and proceed as described under *Dichloralantipyrine Assay*, beginning with: "Add to each tube 1 ml of quinaldine ethiodide solution . . . ." Calculate the amount of chloral hydrate from the calibration curve and multiply it by the factor 1.63 and the dilutions used.

Table II—Arithmetic Mean of Determination of an Accurately Weighed Capsule Mixture

Acetaminophen			Dichloralantipyrine		
Reported, mg	Found, mg	%	Reported, mg	Found, mg	%
120	120.0	100.0	60	58.8	99.7
	119.5	99.6		59.7	99.5
	118.8	99.0		60.3	100.5
	120.7	100.6		59.3	98.8
	119.6	99.7		59.0	98.3
	119.9	99.9		58.5	99.2
Mean	119.7	99.8		59.6	99.3

RESULTS AND DISCUSSION

Vaughn (6) reported that complete hydrolysis of acetaminophen with 1 *N* HCl is not attained even after 45 min, so he chose 10 min in boiling water for convenience. The present assay uses 4 *N* HCl and 1 hr in boiling water to obtain as complete an hydrolysis as possible. However, studies in which the duration of time in boiling water was reduced to 20 min gave identical results. The hydrolytic product, *p*-aminophenol, produced a stable, intense yellow color upon the addition of vanillin reagent, with a maximum absorption peak at 395 nm. The amount of acetaminophen in each capsule was determined by taking into account that the ratio of acetaminophen to *p*-aminophenol is 1:1.385.

Archer and Haugar (7) showed that quinaldine ethiodide reacts with the addition compound, dichloralantipyrine, to produce a blue color in proportion to its chloral hydrate content (Scheme I). This reaction is specific for dichloralantipyrine and chloral hydrate, and substances such as trichloroacetic acid, acetic acid, chloroform, bromoform, and glucose do not produce a blue color.

The quantity of dichloralantipyrine was based on the fact that each mole of dichloralantipyrine produces 2 moles of chloral hydrate, so the ratio of dichloralantipyrine to chloral hydrate is 1:1.63. Isometheptene mucate did not interfere with either assay, and the results of adding known amounts of acetaminophen and dichloralantipyrine are shown in Table I. The assay of the capsules is reported in Table II.

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[3] Matheson, Coleman and Bell.
[4] Lot 1122, McNeil.
[5] Lot M0944, Delmar Chem.
[6] K & L Laboratories.

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High-Speed Liquid Chromatographic Analysis of Sulfasalazine (Salicylazosulfapyridine)

LYLE D. BIGHLEY *x and J. PATRICK McDONNELL ‡

Abstract □ A high-speed liquid chromatographic method for analysis of sulfasalazine (salicylazosulfapyridine) in bulk powder and tablet dosage form is presented. Analysis is accomplished with a reverse-phase partition column and 10% 2-propanol in pH 7.7 phosphate buffer as the mobile phase. The method of analysis utilizes a simple, one-step, solubilization procedure with dimethylformamide, addition of an internal standard, and chromatography. The method is specific for sulfasalazine in the presence of starting materials, degradation products, or by-products from its manufacture.

Keyphrases □ Sulfasalazine—high-speed liquid chromatographic analysis, bulk powder and tablet dosage form □ Salicylazosulfapyridine—high-speed liquid chromatographic analysis, bulk powder and tablet dosage form □ High-speed liquid chromatography—analysis, sulfasalazine bulk and tablet forms

Several analytical methods have been reported for the determination of sulfasalazine {salicylazosulfapyridine, 5-[*p*-(2-pyridylsulfamoyl)phenylazo]salicylic acid} (I). These methods include spectrophotometry (1), titration with titanium trichloride (1), polarography (2), and nonaqueous potentiometric titration (2). However, most of these methods fail to determine this compound selectively in the presence of its by-products of synthesis or degradation products. Conversely, a chromatographic method would be expected to provide simple, rapid, and specific separation and quantitation of this compound in the presence of its impurities.

Although GC has not been investigated, its use for the underivatized I probably would be unsuccessful since this compound was thermally labile when subjected to differential thermal analysis[1]. TLC, although specific, has the disadvantage of potentially long analysis times and requires a number of manipulative steps which may adversely affect precision. For example, a relative standard deviation of 2–6% resulted just from spotting and chromatography (3). (Quantitation in these experiments was effected directly on the plate with a densitometer.) Consequently, even larger errors would be expected in the overall assay if the compound is removed from the TLC plate and eluted from the adsorbent prior to quantitation.

Table I—Column Packing Materials

Column	Packing	Column Dimensions
a	Neutral alumina[a], 100–200 mesh	1 m × 0.3 cm o.d.
b	Ether stationary phase chemically bonded on a controlled porous surface[b]	1 m × 0.3 cm o.d.
c	Strong anion-exchange resin coated on a controlled porous surface[c]	0.6 m × 0.3 cm o.d.
d	Spherical siliceous particles with a controlled porous surface[d]	0.6 m × 0.3 cm o.d.
e	Diphenyldichlorosilane stationary phase chemically bonded on a controlled porous surface[e]	0.6 m × 0.3 cm o.d.
f	Octadecyltrichlorosilane stationary phase chemically bonded on a controlled porous surface[f]	1.2 m × 0.3 cm o.d.

[a] AG-7, Bio Rad. [b] Permaphase ETH, duPont. [c] Zipax SAX, duPont. [d] Corasil II, Waters Associates. [e] Corasil/phenyl, Waters Associates. [f] Corasil/C_{18}, Waters Associates.

The present study was undertaken because high-speed liquid chromatography (HSLC) appears to offer several advantages (4) for the analysis of I that other methods do not possess. For example, it can be used for thermally labile compounds, the precision is good, and the method is specific and rapid. This technique has been successfully utilized to assay I in tablet dosage forms as well as the bulk chemical.

EXPERIMENTAL

Apparatus—A liquid chromatograph[2] equipped with a UV photometric detector (254-nm radiation using a low pressure mercury source) was used. The UV detector can operate at a sensitivity of 0.02 absorbance unit full scale (aufs).

Columns—The packing materials shown in Table I were used in precision-bore stainless steel columns. With the exception of Columns d, e, and f, which were purchased commercially, the columns

[1] Unpublished data.

[2] Model ALC 202, Waters Associates.

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d	Spherical siliceous particles with a controlled porous surface[d]	0.6 m × 0.3 cm o.d.
e	Diphenyldichlorosilane stationary phase chemically bonded on a controlled porous surface[e]	0.6 m × 0.3 cm o.d.
f	Octadecyltrichlorosilane stationary phase chemically bonded on a controlled porous surface[f]	1.2 m × 0.3 cm o.d.

[a] AG-7, Bio Rad. [b] Permaphase ETH, duPont. [c] Zipax SAX, duPont. [d] Corasil II, Waters Associates. [e] Corasil/phenyl, Waters Associates. [f] Corasil/C_{18}, Waters Associates.

The present study was undertaken because high-speed liquid chromatography (HSLC) appears to offer several advantages (4) for the analysis of I that other methods do not possess. For example, it can be used for thermally labile compounds, the precision is good, and the method is specific and rapid. This technique has been successfully utilized to assay I in tablet dosage forms as well as the bulk chemical.

EXPERIMENTAL

Apparatus—A liquid chromatograph[2] equipped with a UV photometric detector (254-nm radiation using a low pressure mercury source) was used. The UV detector can operate at a sensitivity of 0.02 absorbance unit full scale (aufs).

Columns—The packing materials shown in Table I were used in precision-bore stainless steel columns. With the exception of Columns d, e, and f, which were purchased commercially, the columns

[1] Unpublished data.

[2] Model ALC 202, Waters Associates.

Table II—Chromatographic Behavior of Sulfasalazine

Column Packing[a]	Mobile Phase	Adjusted Retention[b] Time, min	Flow Rate, ml/min
a	Heptane	>15.0[c]	1.0
b	5% CH_3CN in water	0.6	2.0
b	7% CH_3CN in water	0.6	2.0
c	pH 9.2 borate buffer + 0.1 *M* $NaNO_3$	>28.4[c]	1.0
c	pH 7.7 phosphate buffer	>24.1[c]	1.5
c	pH 4.1 citrate buffer + 0.1 *M* $NaNO_3$	>15.3[c]	1.0
d	$CHCl_3$	>16.3[c]	1.0
d	$CHCl_3$–CH_3CN–*n*-butanol (60:15:15)	>10.3[c]	1.0
e	10% 2-propanol in pH 7.7 phosphate buffer	2.8	1.0
e	5% CH_3CN in water	0.0	1.0
e	19% 2-propanol in pH 7.7 phosphate buffer	0.0	1.0
e	15% 2-propanol in pH 7.7 phosphate buffer	1.3	1.0
f	5% CH_3CN in water	24.1	2.0
f	10% CH_3CN in water	3.4	1.5
f	5% 2-propanol in pH 7.7 phosphate buffer	31.9	1.5
f	15% 2-propanol in pH 7.7 phosphate buffer	0.6	1.5
f	10% 2-propanol in pH 6.5 phosphate buffer	3.1	1.5
f	10% 2-propanol in water	0.0	1.5
f	10% CH_3OH in pH 7.7 phosphate buffer	>16.3[c]	1.5
f	5% CH_3OH in water + 0.1 *M* ammonium carbonate	>15.6[c]	1.0
f	10% 2-propanol in pH 7.7 phosphate buffer	3.2	1.5
f	5% CH_3OH in water	>23.4[c]	1.0

[a] See Table I. [b] Times measured from the leading edge of solvent peak to the peak maximum. [c] Retention times were greater than these values, but the exact times were not determined.

were dry packed using the tap fill method of Kirkland (5). All columns were operated at ambient room temperature.

Mobile Phases—The mobile phases employed are listed in Table II. The phosphate and citrate buffers were prepared by the addition of 2.5 *N* sodium hydroxide to 0.01 *M* monobasic sodium phosphate and citric acid (monohydrate), respectively. The pH 9.2 borate buffer (0.01 *M*) was prepared by dissolving sodium tetraborate decahydrate in distilled water.

Materials—The following were used: sulfasalazine[3], salicylic acid[3], sulfapyridine[3], propylparaben[4], monobasic sodium phosphate[5], heptane[5], *n*-butyl alcohol[5], methanol[6], acetonitrile[6], sodium nitrate[6], sodium tetraborate decahydrate[6], ammonium carbonate[6], 2-propanol[6], sulfanilic acid[7], dimethylformamide[7], chloroform[7], 2-aminopyridine[8], citric acid USP (monohydrate)[9], and commercially available I tablets[10,11].

Analysis of Bulk Chemical—*Internal Standard Solution*—Weigh accurately approximately 125 mg of propylparaben and quantitatively transfer to a 25-ml volumetric flask with the aid of dimethylformamide. Dilute to volume with dimethylformamide and mix well.

Sample Solution—Accurately weigh approximately 50 mg of I and quantitatively transfer to a 50-ml volumetric flask with the aid of dimethylformamide. Add 10 ml of the internal standard solution, dilute to volume with dimethylformamide, and mix well.

Standard Solution—Prepare concurrently a standard solution, containing an amount of internal standard identical to the sample solution, and a reference lot of I in approximately the same quantity as the sample solution.

[3] Salsbury Laboratories.
[4] White Label, Eastman.
[5] Analytical reagent, Mallinckrodt.
[6] Certified ACS, Fisher.
[7] C. P., J. T. Baker.
[8] Reilly Tar and Chemical.
[9] Mallinckrodt.
[10] Rowell Laboratories.
[11] Pharmacia Laboratories.

Table III—Individual Retention Times for Several Possible Impurities of Sulfasalazine

Compound	Adjusted Retention Time[a], min
Impurity 1[b]	0.0
Impurity 2[b]	0.0
2-Aminopyridine	1.8
Sulfapyridine	0.0
Impurity 3[b]	8.9
Sulfanilic acid	0.0
Impurity 4[b]	4.7
Salicylic acid	0.0

[a] Time measured from the leading edge of solvent peak to the peak maximum. [b] Isolated but unidentified compounds.

Liquid Chromatography—Inject alternately 5 μl of the sample and standard solutions with a 10-μl syringe[12], using the stop-flow technique. Operational parameters for the liquid chromatographic separations are listed with the appropriate figures.

Quantitation—Quantitation was accomplished either by measurement of peak heights or of peak areas with a planimeter. The

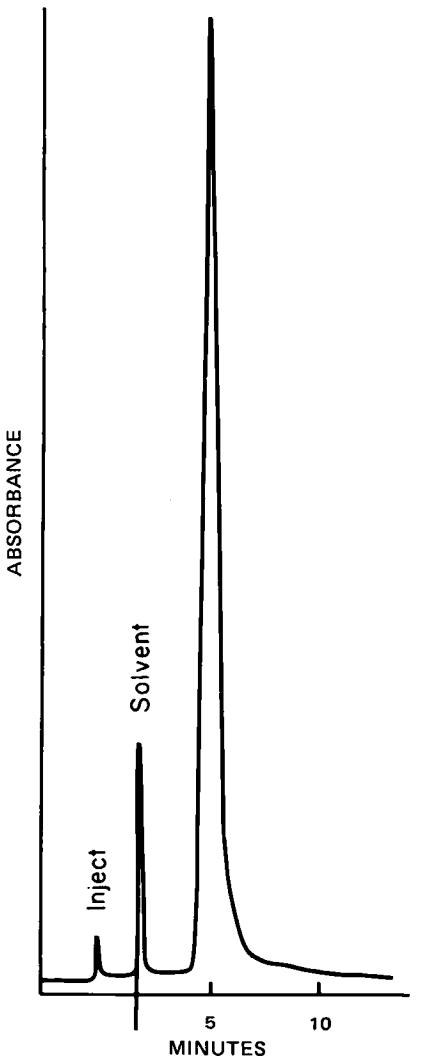

Figure 1—*Typical chromatogram of sulfasalazine. Chromatography conditions were: Column f; solvent, 10% 2-propanol in pH 7.7 phosphate buffer; chart speed, 30.48 cm (12 in.)/hr; flow, 1.5 ml/min; detector sensitivity, 0.16 aufs; and sample size, 5 μl.*

[12] Hamilton 701 NWG.

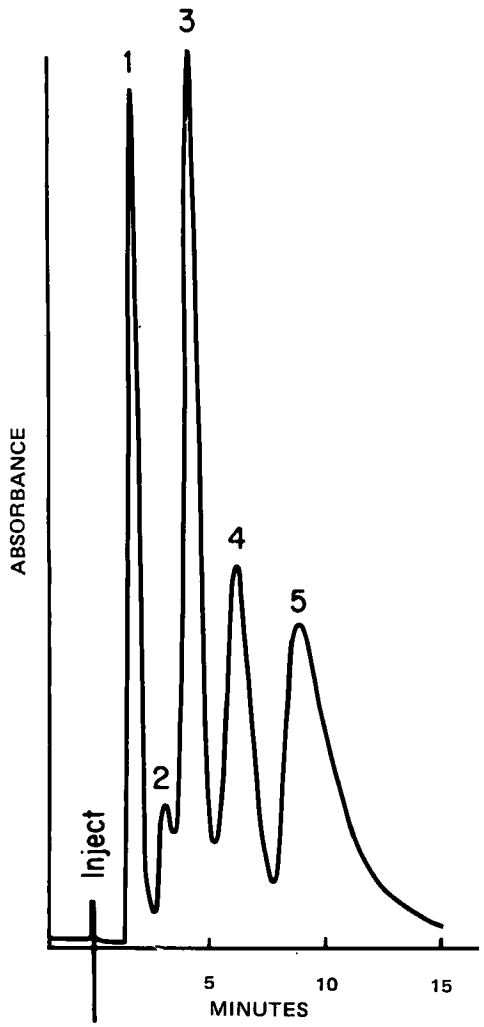

Figure 2—*Separation of a synthetic mixture containing sulfasalazine and some possible impurities. Key: peak 1, impurities 1 and 2, sulfapyridine, sulfanilic acid, and salicylic acid; peak 2, 2-aminopyridine; peak 3, sulfasalazine; peak 4, impurity 4; and peak 5, impurity 3. Chromatography conditions were the same as in Fig. 1.*

percentage of I in the sample was determined by:

$$\% \text{ I in sample} = \frac{(P_1W_2)/(P_2W_1)}{(P_3W_4)/(P_4W_3)} \times 100 \qquad \text{(Eq. 1)}$$

where P = peak height or peak area, W = weight, subscript 1 = I in sample, subscript 2 = internal standard in sample, subscript 3 = I in standard, and subscript 4 = internal standard in standard.

Since the weight of the internal standard is identical in the sample and standard solutions, this term can be deleted from Eq. 1. In addition, this equation can be simplified by using the ratio of the peak area (or peak height) of I/peak area (or peak height) of propylparaben for both standard and sample, $R_{sample} = P_1/P_2$ and $R_{std} = P_3/P_4$. Then it can be shown that:

$$\% \text{ I in sample} = \frac{R_{sample}W_3}{R_{std}W_1} \times 100 \qquad \text{(Eq. 2)}$$

Analysis of Tablets—Place one tablet (label claim 500 mg of I) in a beaker, add approximately 20 ml of dimethylformamide, and crush the tablet with a stirring rod. Quantitatively transfer the contents of the beaker to a 100-ml volumetric flask and rinse the beaker with three additional portions of dimethylformamide. Dilute to volume with dimethylformamide and mix well.

Pipet 10 ml of this solution into a 50-ml volumetric flask, add 10 ml of the internal standard solution previously described, and dilute to volume with dimethylformamide. Alternate 5-μl injections of the standard solution with the tablet formulation solution. The standard solution is prepared as described under *Analysis of Bulk Chemical.*

Quantitation is done as described under *Analysis of Bulk Chemical.* Since the weight of internal standard is identical for the tablet formulation and the standard solution, the equation is:

$$\text{I in tablet} = \frac{R_{sample}W_3D}{R_{std}} \qquad \text{(Eq. 3)}$$

where D = dilution factor, *i.e.*, 10 with this dilution; subscript 1 = I in tablet; subscript 2 = internal standard in tablet solution; subscript 3 = I in standard; subscript 4 = internal standard in standard; $R_{sample} = P_1/P_2$; $R_{std} = P_3/P_4$; and the other symbols have the same meaning as before.

RESULTS AND DISCUSSION

Initially, the chromatographic behavior of I was studied as a function of several column packing and mobile phase combinations; the adjusted retention times relative to the dimethylformamide peak for these systems are presented in Table II. Studies were not continued with those packing and mobile phase combinations giving long retention times. With the other systems, it became evident that an adjusted retention time for I of approximate-

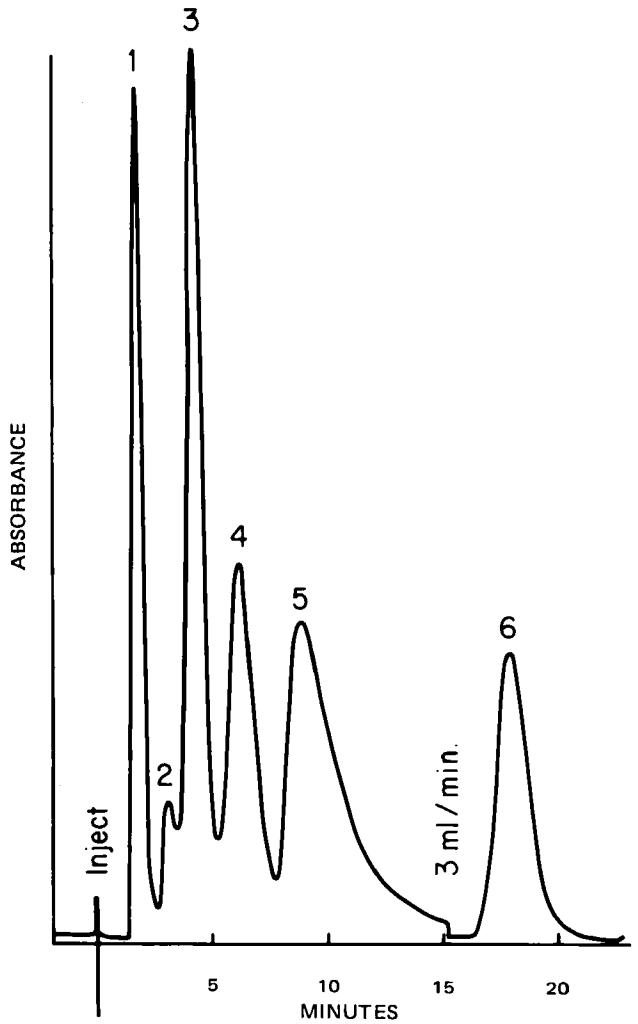

Figure 3—*Separation of a synthetic mixture containing sulfasalazine, some possible impurities, and an internal standard. Key: peak 1, impurities 1 and 2, sulfapyridine, sulfanilic acid, and salicylic acid; peak 2, 2-aminopyridine; peak 3, sulfasalazine; peak 4, impurity 4; peak 5, impurity 3; and peak 6, propylparaben. Chromatography conditions were the same as in Fig. 1, except the flow rate was increased to 3 ml/min after 15 min.*

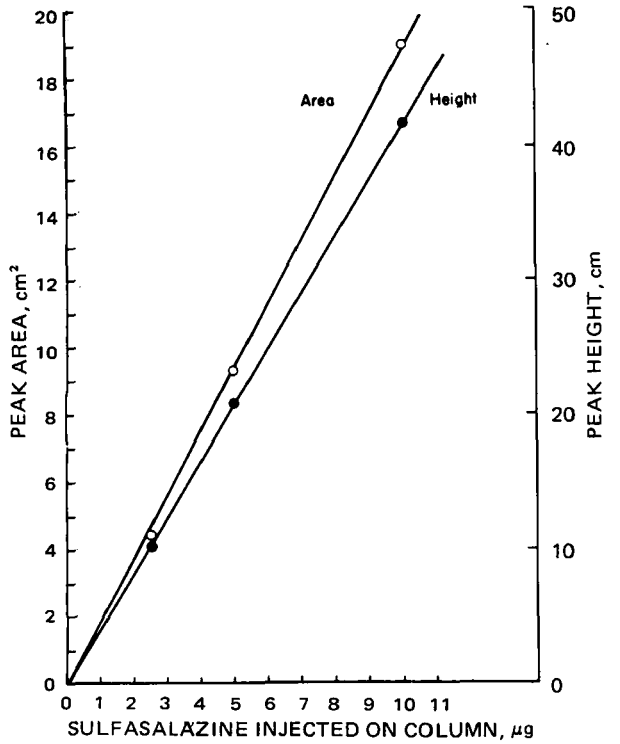

Figure 4—*Linearity of detector response to sulfasalazine.*

ly 3 min was necessary, since certain known impurities which may be present in I elute at, or immediately after, the solvent front. Consequently, only four column packing and mobile phase combinations met this requirement: Column e or f with 10% 2-propanol in pH 7.7 phosphate buffer, and Column f with either 10% acetonitrile in water or 10% 2-propanol in pH 6.5 phosphate buffer (Table I).

Of these four systems, only Column f with 10% 2-propanol in pH 7.7 phosphate buffer was satisfactory. When this system was used with the conditions shown in Fig. 1, I has an adjusted retention time of approximately 3.2 min. The other three systems were undesirable for various reasons. For example, Column f with 10% acetonitrile in water was unacceptable because certain by-products from synthesis had the same retention time as I or were not eluted. When 10% 2-propanol in pH 6.5 phosphate buffer was used with this packing, the peak width broadened slightly. Similarly, Column e in combination with 10% 2-propanol in pH 7.7 phosphate buffer resulted in a broadening of the I peak.

Further studies were undertaken with Column f and 10% 2-propanol in pH 7.7 phosphate buffer to determine if this system would resolve I from various structurally related compounds which could be present as impurities under normal circumstances. Adjusted individual retention times for several possible degradation products, starting materials, and by-products of synthesis were found to be sufficiently different from the retention time of I (Table III). Consequently, a synthetic mixture was prepared by dissolving approximately equal amounts of I and these compounds

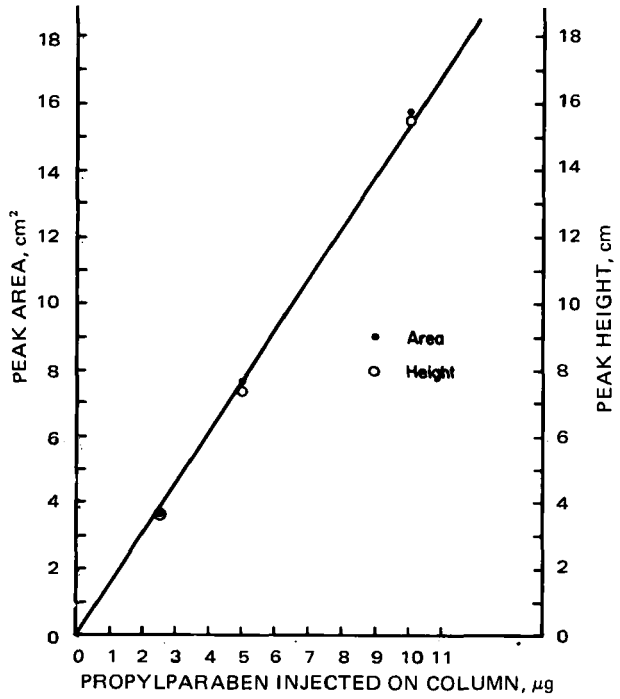

Figure 5—*Linearity of detector response to propylparaben.*

Table IV—Precision Study for Sulfasalazine Using Peak Height Measurements

Sample	Individual Assays, %			Average of Duplicate Assays, %
1	A	93.3		
	B	94.9		
	Average			94.1
2	A	99.1		
	B	97.4		
	Average			98.3
3	A	92.8		
	B	98.7		
	Average			95.8
4	A	95.1		
	B	95.9		
	Average			95.5
5	A	97.6		
	B	96.5		
	Average			97.0
6	A	92.1		
	B	92.5		
	Average			92.3
Mean individual assay, %		95.49	Mean duplicate assays, %	95.49
SD, %		2.44	*SD*, %	2.11
RSD, %		2.55	*RSD*, %	2.21

Table V—Precision Study for Sulfasalazine Using Peak Area Measurements

Sample	Individual Assays, %			Average of Duplicate Assays, %
1	A	93.2		
	B	94.7		
	Average			94.0
2	A	95.6		
	B	89.3[a]		
	Average			92.5
3	A	93.9		
	B	92.7		
	Average			93.3
4	A	95.1		
	B	97.1		
	Average			96.1
5	A	96.6		
	B	93.9		
	Average			95.3
6	A	96.8		
	B	97.8		
	Average			97.3
Mean individual assay, %		94.7	Mean duplicate assays, %	94.8
SD, %		2.36	*SD*, %	1.81
RSD, %		2.49	*RSD*, %	1.91

[a]The mean individual assay, standard deviation, and relative standard deviation calculated with Sample 2B removed (Z score = −2.29, $p < 0.02$) are 95.2, 1.70, and 1.79%, respectively.

Table VI—Analysis of Commercial Tablet Formulations of Sulfasalazine[a]

Using Peak Areas	Found, mg
Product A	498.5
Product B	496

[a] Label claim: 500 mg of sulfasalazine.

in dimethylformamide. A chromatogram of this mixture is shown in Fig. 2. Although baseline separation was not achieved, separation of I was adequate to permit quantitation even in the presence of these large amounts of impurities.

The specificity of this HSLC system was further confirmed by repeatedly injecting a sample of approximately 85% pure I and collecting the effluent when the peak with an adjusted retention time of 3.2 min appeared. When this effluent was subjected to TLC, using the solvent system of Kiger and Kiger (6), only I was found in the trapped samples. Plates that were sprayed with a solution containing 4-dimethylaminocinnamaldehyde and titanium trichloride (7) also only showed one spot.

Quantitative analysis of I samples was accomplished by the internal standard technique, using propylparaben as the internal standard. This compound was selected because it could be separated from the impurities shown in Fig. 2. Since these impurities elute immediately before and after the I peak, the compound selected as the internal standard had to have a relatively long retention time; and propylparaben was the only compound tested that met this requirement. Figure 3 shows the synthetic mixture containing propylparaben. The flow rate was increased from 1.5 to 3 ml/min after 15 min to hasten the elution of the propylparaben. The change in flow rate at this time permits elution of the impurities without overlapping either the I or propylparaben.

The UV detector response to I and propylparaben is linear over the concentration ranges used in this study. Plots of absorbance peak heights or areas *versus* concentration are shown in Figs. 4 and 5.

The precision of the HSLC method was determined by comparing the results obtained on six subsamples of I taken from one lot of bulk chemical. Peak heights are shown in Table IV, and peak areas are shown in Table V. Values for each injection and the average of duplicate injections are shown for each analysis. The standard deviation and relative standard deviation were calculated for individual and duplicate injections and are consistent with values reported for the liquid chromatographic analysis of other compounds (8–11).

The precision of measurement of peak areas probably could have been further improved in this study if integration were done with either a mechanical or electronic integrator. However, since a planimeter was used, precision of area measurements was maximized by increasing the recorder chart speed so that the peak area could be determined more precisely.

It is evident from the precision studies that either peak height or area can be used for quantitative analysis. However, if peak heights are used, the time at which the flow rate is increased from 1.5 to 3 ml/min must be closely regulated to prevent band spreading, which results from increased retention time. Use of peak areas would not require such close attention with respect to the time at which the flow rate was changed because the area of the propylparaben should remain the same even if some band spreading does occur, provided resolution remains adequate for accurate quantitation. Since the propylparaben is well resolved from I and other possible impurities, slight band spreading would not be a problem.

Two commercially available tablet formulations were examined for their I content to determine the feasibility of this method for tablet dosage forms. Preliminary results (Table VI) indicate that this procedure is applicable. Tablet excipients did not interfere with the analysis and need not be separated from I prior to liquid chromatography.

Since dimethylformamide was used as the solvent for I in the assay procedure and since solutions were occasionally prepared several hours prior to injection, it was desirable to determine whether this solvent degrades I. Therefore, a sample of I was dissolved in dimethylformamide and subjected to thermal stress at 80° for 196 hr. No degradation was observed when this solution was analyzed by TLC and HSLC.

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Effect of Surface Roughness and Coating Solvent on Film Adhesion to Tablets

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Abstract □ The adhesion of a polymer film to selected tablet substrates was studied. The effect of tablet surface roughness and film-coating solvent on the adhesion of the film to the tablet was determined. A film-coating solvent having a solubility parameter close to that of the polymer was found to produce a stronger adhesional bond than a solvent having a lower surface tension. An increase in tablet surface roughness also increased film adhesion to the tablet. The adhesion was measured as the force required to peel a section of film from the tablet with a stress–strain analyzer.

Keyphrases □ Films—adhesion to tablets, effect of surface roughness and coating solvent □ Tablets—effect of surface roughness and coating solvent on film adhesion □ Adhesion—polymer films to tablet substrates, peel strength, effect of surface roughness and coating solvent □ Solvents, coating—effect on polymer film adhesion to tablet substrates

Adhesion to their substrates is a major prerequisite of pharmaceutical film coatings. Assessment of coating adhesion in fundamental terms is very difficult and is undoubtedly a factor that has limited the research in this field. The method of choice for studying coat bonding to substrates appears to be the measurement of the force required to peel the film from the substrate.

In a recent adhesion study involving pharmaceutical systems (1), the adhesion of film coating to tablets was quantified by measuring the force required to pull films from tablet surfaces. A correlation was found between peeling force and the critical surface tension of the tablet surface but not between surface tension of the coating solution and peeling force. As pointed out by Wood and Harder (1), this latter finding was contrary to theory.

The solvent from which a polymer film is cast can significantly affect the film's adhesion to the substrate (2). Engel and Fitzwater (3) found that the nature of the solvent used in film formation significantly affected the peel strength of methyl methacrylate films. They also observed a good correlation between the peel strength and the cohesive energy density of the solvents employed using a tin substrate. Peel strengths of film prepared from polymer solutions containing 25% solvent showed an increase in numerical value with an increase in solvent cohesive energy density. The solvent showing the maximum peel strength was considered the most efficient for a particular polymeric system.

While studying the effect of fillers on peel strength, Brantley (4) noticed that the addition of higher concentrations of talc decreased the peel strength. He attributed this effect to embedded filler particles between the polymer film and the substrate, which interfered with the polar groups of the film or shielded them from the substrate.

The effect of polymer molecular weight on adhesion has been studied (5, 6), and the peel strength was observed to be independent of molecular weight in one case (5). Vakula *et al.* (6), studying the adhesion of copolymers of acrylonitrile and butadiene, found an initial increase in adhesion with an increase in molecular weight up to 350,000. Beyond this point, the adhesion was independent of molecular weight. The work was carried out on a polyamide substrate.

The effect of the percentage of polar groups of cellulosics on adhesion to nonferrous metals was studied, and an increase in adhesion with increased nitration in cellulose and increased ethoxy content in ethylcellulose was found (7). McLaren (8) demonstrated the increase in adhesion of poly(alkyl methacrylate) as the alkyl group was changed from ethyl through *n*-butyl. This increase in peel strength was thought to be due to a change in crystallinity of the film material resulting in an increase in adhesion.

The objective of this research was to investigate the effect of the coating solvent on film adhesion to tablet substrates; in addition, the effect of surface roughness on film adhesion is also reported.

EXPERIMENTAL

Preparation of Substrates—Three tablet substrates (Table I) were prepared by compressing the powder mixtures into disks with a laboratory press[1]. Each 2.54-cm (1-in.) diameter, flat-face disk weighed approximately 2 g and was prepared at a compressional load of 9091 kg (20,000 lb), held for 15 sec. For surface roughness studies, disks were also compressed at 4545 kg (10,000 lb) and 6818 kg (15,000 lb). The compressed disks were stored in a desiccator prior to use.

Preparation of Polymeric Coating Solutions—The polymer used was poly(methyl vinyl ether/maleic anhydride), molecular weight 250,000[2]. Eight percent (w/v) solutions of the polymer were prepared in acetone, methyl acetate, methyl ethyl ketone, and tetrahydrofuran. Each solution also contained 6% glyceryl triacetate[3]. All solvents were reagent grade.

Coating of Compressed Substrate Disks—To facilitate film coating, maintain individual tablet identity, control coating thickness, and facilitate subsequent quantitative testing, compressed substrate disks were coated using an immersion coating method.

[1] Carver model B, Fred S. Carver, Inc., Summit, N.J.
[2] Courtesy of General Aniline and Film Corp., New York, N.Y.
[3] Eastman Organic Chemicals, Rochester, N.Y.

Table I—Tablets Employed in Film Adhesion Studies

Formula for 50 Tablets	Tablet Substrate I[a], g	Tablet Substrate II[a], g	Tablet Substrate III[b], g
Microcrystalline cellulose[c]	100	—	—
Acacia	—	100	—
Magnesium oxide granulation	—	—	100
Talc	0.5	1.0	0.25
Magnesium stearate	1.0	2.0	0.25

[a] Substrates I and II were prepared by hand sieving all ingredients through a 40-mesh sieve, mixing for 20 min in a planetary mixer, and compressing. [b] Substrate III was prepared by wet massing the magnesium oxide with 7.5% starch paste followed by hand granulating through a 30-mesh sieve. The resulting granules were dried overnight at 48.8° (120°F), sized through a 16-mesh sieve, and compressed. [c] Avicel.

Each compressed substrate disk was brushed with a camel-hair brush to remove dust and was measured for thickness with a micrometer gauge[4]. A penciled rectangle of 0.5 × 0.75 cm was marked on the substrate disks to provide a reference for subsequent film peeling in evaluating adhesion properties of polymeric films.

The tablets were coated individually by maintaining the disk at the end of a small glass tube with suction applied by a vacuum pump. One side of the flat tablet was coated by immersing it into the coating solution 12 times. The resulting film thickness varied from 0.12 to 0.16 mm as measured by a micrometer[4]. Each coat was allowed to air dry for 3–5 min before the next coat was applied. Coated disks were stored at 40° and 52% relative humidity for 12 hr before testing in the peeling studies.

Contact Angle Measurements of Polymeric Solutions on Compressed Substrate Disks—The environmental chamber of a goniometer[5] was saturated for 24 hr with vapors of the solvent used in the coating solution prior to the contact angle measurements. This operation was carried out to minimize the recession of the contact angle during the measurement. The substrate disks were individually placed on the platform of the goniometer chamber. One small drop of the polymeric coating solution was then placed on the disks with a capillary pipet and the chamber was covered immediately. Measurement of the wetting angles was taken within 60 sec.

Surface Tension Measurement—The surface tension of the polymer solutions and of the pure solvents was determined[6] at 25°. Correction factors were calculated according to the procedure in the manufacturer's instruction manual. Density determinations necessary for the correction factor calculation were determined using a 25.00-ml pycnometer.

Surface Roughness Measurements of Compressed Substrate Disks—To evaluate the effect of substrate surface morphology upon polymeric film peeling strength, surface roughness characteristics of the substrates were determined. The surface profile measurements were made using a surface analyzer[7]. This instrument is used in the measurement of the surface profiles of thin films and roughness characteristics of solid substrates. The specifications used in the experimental studies were as follows: sensitivity, 500,000 and 100,000 Å full scale; minimum detectable step, 5000 and 1000 Å; scan speed, 0.1–10 cm/min; stylus diameter, 0.0005 cm; and stylus tracking force (preset), 15 mg.

Surface roughness measurements were carried out by the method described by Schwartz and Brown (9). The surface peak-to-valley distance was used to characterize surface roughness, and an average of six such readings was reported as the average peak-to-valley distance.

Peel Strength Determination—The peel adhesion of applied polymeric films was evaluated using a stress analyzer[8]. The tension load cell was calibrated at the lowest range of 10 g for full-chart deflection. This calibration enabled the use of all ranges of amplification (1–50). The cross-head speed of 1.27 cm/min (0.5 in./min) was chosen to take into account a gradual and slow peeling of the film and reduce elastic deformation of the film. A preset chart speed of 50.8 cm/min (20 in./min) gave a total amplification of deflection by 40×. The gauge and return dials were set to reproduce a predetermined distance of 2.54 cm (1 in.) of peeling of the film from the substrate.

The samples of coated substrate disks previously marked (rectangle of 0.5 × 0.75 cm) were tightly clamped to the platform. One end of the rectangular outline of the film, representing 25% or less of the total gauge length, was cut with a small razor knife and pulled free for insertion into the jaws of the grip. The dimensions of the peeled film was such that when the sample was clamped and inserted into the jaws of the grip, a small initial distance was allowed for weakening of the film contact to the substrate surface. This distance was taken into account prior to recording the actual peeling by zero adjustment. All measurements were carried out using 20× amplifier sensitivity to enable the load cell to record to a maximum of 200 g of force.

The angle of peel was almost constant (90°) during the peeling of films. Due to the very small distance over which the peel adhesion was studied, no attempt was made to slide the platform to maintain a constant angle of peel. Measurements were carried out at 25° and 52% relative humidity.

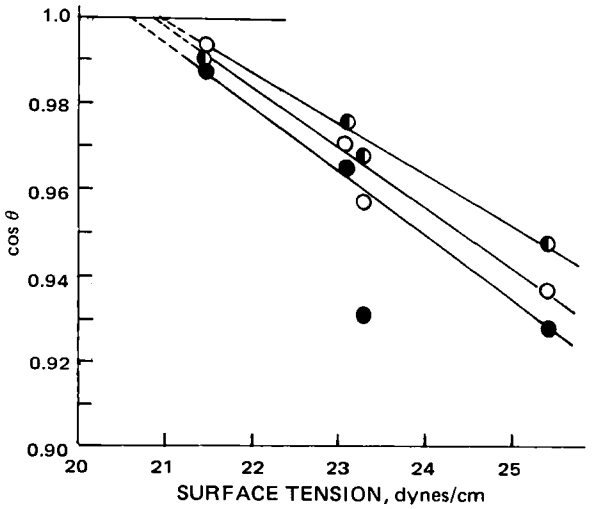

Figure 1—*Zisman plot of cos θ versus surface tension. The intercept at cos θ = 1 represents the critical surface tension of the solid. Key: ◐, Tablet I; ○, Tablet II; and ●, Tablet III.*

RESULTS AND DISCUSSION

Relationship between Contact Angles and Film Adhesion—Contact angle measurements of film-coating solutions on the three tablet formulations were made, and the adhesion of the corresponding polymer film to the three tablets was measured as the peel strength of the films from the tablets (Table II). As expected, the film adhesion, as reflected by the force required to peel the film from the tablet, increased with a decrease in the contact angle. Thus, the better the wetting of the tablet by the film solution, the better was the film adhesion.

However, an unusual phenomenon, previously reported (1), was observed with respect to the relationship between film adhesion and the surface tension of the film solution. Instead of an increase in adhesional force with a decrease in solution surface tension, an increase in adhesional force was observed with an increase in solution surface tension. In other words, the contact angle of the solution on the tablet increased as the surface tension of the film solution decreased.

To try to discover the reasons for the observed deviations from theory, the contact angles of the pure solvents used to make the film solutions were determined on the three tablet formulations. The data are given in Table III and are shown in Fig. 1 as a Zisman plot, with the extrapolation intercept at cos θ = 1 (θ = 0) pro-

[4] Ames Co., Waltham, Mass.
[5] Rame-Hart, Inc., Mountain Lakes, N.Y.
[6] Model 20 Fisher surface tensiometer, Fisher Scientific, Pittsburgh, Pa.
[7] Dektat, Sloan Technology Corp., Santa Barbara, CA 93103
[8] Instron universal testing instrument, floor model (TT), Instron Co., Canton, Mass.

Table II—Contact Angle and Peel Strength Data for Films Applied to the Tablets as Affected by Film-Coating Solvent

Tablet	Solvent[a]	Contact Angle ± 1°	Surface Tension of Polymer Solution, dynes/cm	Peel Strength, g/cm	Solubility Parameter of Solvent
I	Acetone	21	22.7	12.7	9.9
	Methyl acetate	18	23.1	19.4	9.6
	Methyl ethyl ketone	18	23.3	24.1	9.3
	Tetrahydrofuran	15	26.1	32.5	9.1
II	Acetone	28	22.7	11.4	9.9
	Methyl acetate	25	23.1	17.0	9.6
	Methyl ethyl ketone	22	23.3	22.0	9.3
	Tetrahydrofuran	19	26.1	29.5	9.1
III	Acetone	32	22.7	8.1	9.9
	Methyl acetate	28	23.1	15.4	9.6
	Methyl ethyl ketone	28	23.3	22.0	9.3
	Tetrahydrofuran	25	26.1	24.5	9.1

[a] Each solution contained 8% poly(methyl vinyl ether/maleic anhydride), molecular weight 250,000, and 6% glyceryl triacetate.

viding the apparent critical surface tension of the solid. The contact angle–surface tension relationship for the pure solvent–tablet system is as the Zisman theory would predict. Thus, the theory does hold when considering the pure solvent, which was used to prepare each of the four coating solutions, in contact with the tablet.

To better understand the observed adhesional phenomenon, the polymer solution must be considered further. Since it is the polymer that is adhering to the tablet and not the solvent, it is the wetting of the tablet by the polymer that is important. The adhesional bond that forms between polymer and tablet depends on both the duration of the polymer solution–tablet contact and the intimacy of the contact between the polymer, while in solution, and the tablet. The length of polymer solution contact with the tablet is usually very short because the solvent evaporates very rapidly, producing an immobilized polymer in a viscoelastic film. Yet the wetting of the tablet by the polymer must occur in that short time period while the polymer is in solution and prior to the formation of the viscoelastic film. Therefore, it is important that the polymer solution provide for maximum polymer tablet interaction immediately on contact. It would be expected that for a given polymer the greater the extent of polymer solvation by the solvent, the greater the polymer tablet interaction as the solvent initially contacts and penetrates the tablet surface.

One way of approaching the problem is to use the solubility parameter of the solvent as a qualitative measure of the extent of polymer solvation and to analyze its relationship to the observed contact angles. Table II shows the relationship between the solubility parameters of the four solvents and the observed contact angles. Tetrahydrofuran (δ = 9.1) would be expected to be the most efficient solvent for the polymer (δ = 8.7) since its solubility parameter is closest to that of the polymer. The tetrahydrofuran solvent did produce the lowest contact angle and highest adhesional strength, although it had the highest surface tension. It is certainly apparent that the choice of a film-coating solvent having a solubility parameter close to that of the polymer is important in achieving good film adhesion, and it is even more important than selecting a solvent solely on the basis of surface tension considerations.

Table III—Contact Angles of Pure Solvents on Tablet Substrates

Tablet	Solvent	Surface Tension, dynes/cm	Contact Angle ± 1°	cos θ
I	Acetone	21.5	8.0	0.9903
	Methyl acetate	23.1	12.5	0.9763
	Methyl ethyl ketone	23.3	15.0	0.9659
	Tetrahydrofuran	25.4	17.5	0.9537
II	Acetone	21.5	7.0	0.9925
	Methyl acetate	23.1	14.0	0.9700
	Methyl ethyl ketone	23.3	17.0	0.9563
	Tetrahydrofuran	25.4	20.5	0.9367
III	Acetone	21.5	9.0	0.9877
	Methyl acetate	23.1	15.0	0.9659
	Methyl ethyl ketone	23.3	21.5	0.9304
	Tetrahydrofuran	25.4	22.0	0.9272

The nature of the polymer itself may influence the adhesional bond formed between the film and the tablet substrate. Additional studies are in progress to determine the effect of alkyl ether substitution on the film–tablet adhesional bond.

Wetting and Adhesion as Related to Tablet Surface Roughness—Two of the tablet formulations were compressed at various compressional loads to obtain different surface roughness characteristics. The tablets were compressed at 4545, 6818, and 9091 kg, and the surface roughness characteristics of these tablets were measured (Table IV). For both formulations, the average peak-to-valley height was the greatest for the tablets compressed at 4545 kg and decreased with increasing compressional load; the tablets were becoming smoother with increasing compressional load.

The adhesion of a polymer film to the tablets with differing surface roughness characteristics was determined as the peel strength of the film from the tablet (Table IV). Only one solvent, acetone, was used to deposit the polymer on the tablet formulations, so the surface tension of the coating solution remained constant. From Table IV it is apparent that the peel strength correlates with the roughness data; the rougher the tablet, the higher the peel strength. The differences in peel strength are quite significant and must result from the increased surface area of the rougher tablet which provides for greater interfacial contact between polymer solution and tablet.

A relationship between the contact angle and surface roughness was proposed by Wenzel (10):

$$r = \frac{\cos\theta'}{\cos\theta} \qquad \text{(Eq. 1)}$$

where θ' is the apparent contact angle on a rough surface, θ is the contact angle on an ideal smooth surface, and r is the roughness factor and is defined as:

$$r = \frac{\text{true surface area}}{\text{apparent plane area}} \qquad \text{(Eq. 2)}$$

Since r is always greater than unity, the apparent contact angle, θ', is less than the true contact angle θ when θ is less than 90°. Thus, the effect of roughening the tablet surface is to make the apparent contact angle between the coating solution and the tablet less than the true contact angle. In other words, the polymer solu-

Table IV—Wetting Angle and Adhesion Peel Strength of Films Applied to Substrates as Related to Substrate Surface Roughness

Tablet	Compressional Load, kg	Roughness, Average Peak-to-Valley Distance, A	Wetting Angle	Peel Strength, g/cm
I	4545	17,330	14°	29.1
	6818	16,000	19°	17.7
	9091	7,666	22°	12.7
II	4545	12,400	17°	24.1
	6818	12,000	21°	16.5
	9091	6,833	28°	11.4

tion appears to spread more when the the tablet surface is rough, making the exact determination of the critical surface tension, γc, of a compressed tablet very difficult.

In most cases ($\theta \leq 90°$), the true critical surface tension is greater than that determined experimentally. However, from a film-coating standpoint, the influence of surface effects on film adhesion may well be minimal, with the correlation of solvent–polymer solubility parameters being the major factor in determining film adhesion. Regardless of the tablet surface characteristics, the adhesional bond between tablet and polymer must form at a faster rate than the cohesional forces in the polymer film. When this occurs, a strong adhesional bond is established between the tablet and the film.

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Laboratory Automation of High-Pressure Liquid Chromatography

WILLIAM F. BEYER *‡ and DAVID D. GLEASON ‡

Abstract □ An automated system for high-pressure liquid chromatography was developed. The system is built around commercial modules wherever possible, modified to varying degrees. An automatic sampler, a sample pump, a high-pressure sampling valve, a recorder with an integrator, and a high-pressure liquid chromatograph comprise the commercial instruments. Relays, solenoid valves, and timers control chromatographic events, *i.e.*, duration of sampling and rinse, mobile phase pump refill, sample injection, and chromatographic time. The automated system is dependable over long periods of unattended operation. With the 40-sample capacity of the sample tray and the last sample stop capability, the automated system produces, for example, 40 20-min chromatograms in approximately 13 hr of unattended operation. Data demonstrate the reliability and utility of the system.

Keyphrases □ High-pressure liquid chromatography—automated equipment developed and discussed □ Automated analysis—high-pressure liquid chromatographic equipment developed and discussed

High-pressure liquid chromatography (HPLC), with its widespread use over the last 5 years, has undergone dramatic growth from the standpoint of methodology and instrumentation. Michaelis *et al.* (1) made it apparent that HPLC is of major importance in quantitative analysis and identification of various pharmaceutical agents. An HPLC equipment review (2) covers a large number of domestic and foreign manufacturers.

The application of HPLC for stability-indicating assays because of specificity, sensitivity, and relative ease of sample preparation is clearly indicated, particularly for labile compounds. Specific applications of HPLC were reported for various pharmaceutical products containing various active compounds (3) and for steroid formulations (4–7). The increased use of HPLC for stability-indicating assays of steroid formulations, for example, has produced the need for an automated HPLC system. Specific applications of the automation of particular aspects of HPLC have been reported. Among these are the use of short columns and low pressures (8) and the determination of the antibiotic tetracycline (9). More recent work (10) covers the use of a dedicated small computer to study the precision in HPLC.

Although automated HPLC systems are obtainable from commercial manufacturers[1,2], they are not readily available. A symposium presentation (11) covered an automated HPLC system developed at Lederle Laboratories. To increase the output of HPLC assays, an automated HPLC system (patent applied for) was developed from commercially available instruments, relays, solenoid valves, and timers and is the subject of this report.

EXPERIMENTAL

The commercial instruments comprising a portion of the automated HPLC system, modified to varying degrees, follow.

Instruments and Modifications—A liquid chromatograph[3]

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[2] Altex, Berkley, Calif.
[3] Model 820, DuPont.

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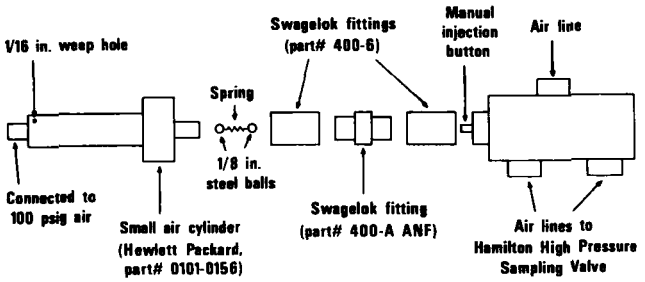

Figure 1—*Schematic drawing showing the modification of the high-pressure sampling valve to make it operate automatically.*

with 316 stainless steel columns, 100 cm long × 2.1 mm i.d., and permanently bonded, reverse-phase column packings[4,5] were used. The chromatograph was equipped with a 254-nm UV detector set at a sensitivity of 0.04 absorbance unit full scale.

A 1-mv recorder[6] was equipped with an integrator[7]. Chart speeds were 0.25 and 0.51 cm/min.

The high-pressure sampling valve[8] provides plug injection of sample at liquid pressures up to 5000 psig. It is mounted in the column oven and is actuated by 30–100 psig of air, supplied from the pneumatic section of the mobile phase pumping system of the chromatograph. A 5.0-μl sample valve was used. The manual injection switch of the high-pressure sampling valve was modified to operate automatically from the 30–100-psig air *via* an air cylinder[9] and a solenoid valve[10]. Figure 1 gives a schematic drawing of the details.

The holdup volume of the sample pump[11] was reduced with Teflon inserts in the entrance and exit chambers. The inserts have openings approximately 0.13 cm (0.050 in.) in diameter for fluid transport. The power plug was changed to a pin type, dedicating it to the automated system.

The standard timing cams were eliminated in the automatic sampler[12]. Sampling time is controlled by an automatic timer[13] located in a separate unit. A metal disk was constructed and placed under the sample tray to act as a support for 45 × 14-mm o.d. glass vials holding approximately 3 ml. A glass cover plate replaced the plastic one provided by the manufacturer. Retaining clips were added to the glass plate and to the sample tray to orient and anchor the cover plate.

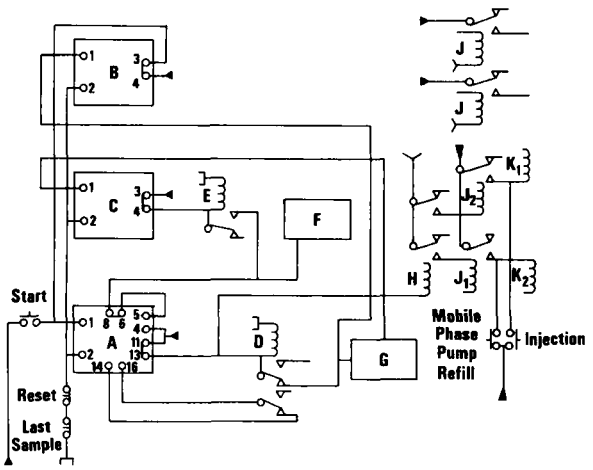

Figure 2—*Schematic drawing showing relative position and wiring of modules comprising the automated HPLC system. Key: ▲, 115 v ac; △, 24 v ac; >, 24 v dc; and], neutral. See text for details.*

[4] DuPont ODS.
[5] Waters C-18.
[6] Electronik 19, Honeywell, Philadelphia, Pa.
[7] Disc Instruments, Santa Ana, Calif.
[8] Hamilton, supplied by DuPont.
[9] Hewlett-Packard, Palo Alto, Calif.
[10] Automatic Switch Co., Florham Park, N.J.
[11] miniPump, Milton Roy, St. Petersburg, Calif.
[12] Sampler II, Technicon, Tarrytown, N.Y.
[13] Model 335, Automatic Timing Controls Co., King of Prussia, Pa.

Table I—Replicate Automated HPLC Injections of 5 μl of a Solution Containing 0.075 mg/ml of Fluorometholone and 0.05 mg/ml of Norethindrone as the Internal Standard

Injection	Peak Height Ratio	Injection	Peak Height Ratio
1	1.3571	18	1.3621
2	1.3571	19	1.3576
3	1.3571	20	1.3702
4	1.3571	21	1.3576
5	1.3571	22	1.3676
6	1.3620	23	1.3624
7	1.3701	24	1.3699
8	1.3527	25	1.3424
9	1.3592	26	1.3564
10	1.3460	27	1.3691
11	1.3484	28	1.3704
12	1.3675	29	1.3684
13	1.3519	30	1.3677
14	1.3599	31	1.3579
15	1.3460	32	1.3484
16	1.3482	33	1.3586
17	1.3519	34	1.3542
		Average =	1.3583
		RSD =	0.61%

Approximately 0.10-cm (0.040-in.) i.d. polyethylene tubing connects the automatic sampler and the sample pump. Stainless steel tubing, 0.03 cm (0.010 in.) i.d., connects this pump to the sampling valve. The same dimension tubing connects the high-pressure sampling valve to the column.

Wiring and Functions of Electronic Components—To control and to coordinate the sequence of events necessary for the flexible operation of the automated HPLC sampling system, appropriate timers, relays, and solenoid valves were installed. Timers were contained in a separate control unit; relays and solenoids were mounted in the chromatograph.

As shown in Fig. 2, three separate timers[13] establish sample, rinse, and chromatographic times. The pump timer (A) is wired for momentary start and resets by the time delay relay (D) contact opening (adjustable 3–30 sec, normally set to 4 sec). The chromatographic timer (B) is wired for momentary start and automatic reset. Depression of the start switch located in the control unit causes the sample pump timer (A) to start timing. This allows the sample pump (F) to run for the preset time.

At the end of the sampling time, No. 8 contacts open, stopping the sample pump; No. 13 contacts close and apply power to the time relay (D), the automatic sampler (G), and the standard 115 v ac relay (H). The sampler (G) starts, and the sample probe is allowed to cycle to the wash position. The time delay relay (D) then times out to keep the sample probe from coming out of the rinse solution. When in the rinse position, the sample pump (F) is re-

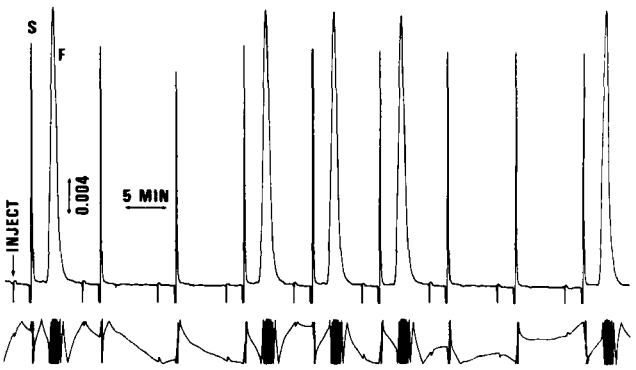

Figure 3—*Eight-minute chromatography from the automated HPLC sampling system showing the absence of carryover from fluorometholone, 0.15 mg/ml (F), or 75% 3A alcohol (S). The sequence of injections, from left to right, was F, S, S, F, F, F, S, S, and F. Chromatographic conditions were: ODS column packing, 6% aqueous acetonitrile with 0.0075 M borate buffer at pH 11.0 as the mobile phase, 500 psig column pressure (0.67 ml/min), and column temperature at 35°.*

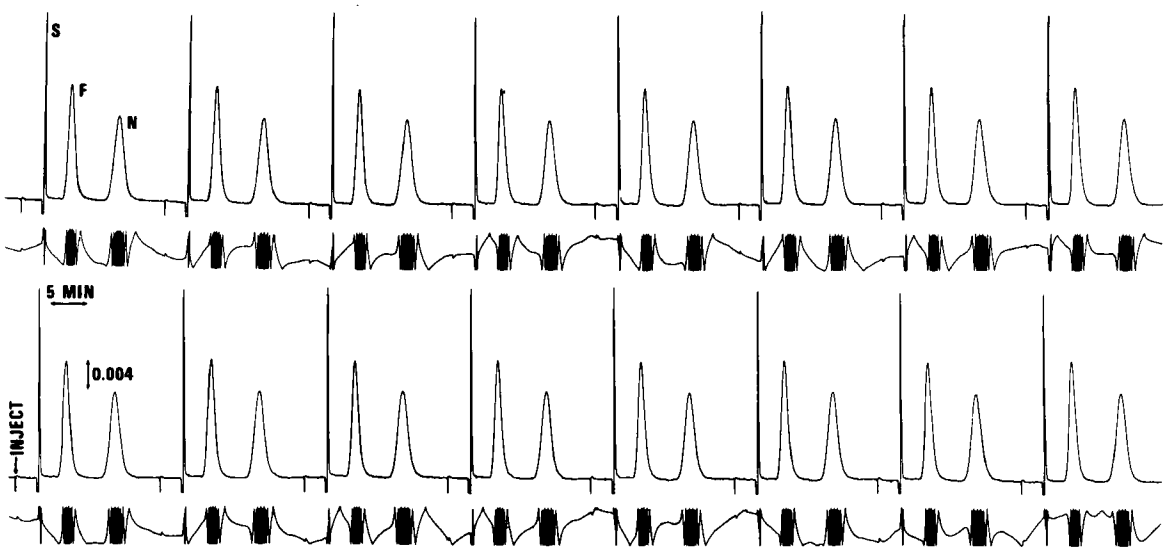

Figure 4—*Twenty-minute chromatograms of automatically sampled fluorometholone, 0.075 mg/ml (F), and internal standard norethindrone, 0.050 mg/ml (N), in 75% 3A alcohol (S). Chromatographic conditions were: ODS column packing, 6% aqueous acetonitrile with 0.0075 M borate buffer at pH 11.0 as the mobile phase, 300 psig column pressure (0.4 ml/min), and column temperature at 35°.*

started *via* a standard 115 v ac relay (E), mounted in the sampler and energized by the rinse timer (C). The timer (C), started by the sampler, operates for the programmed rinse time. After timing out, the sampler probe moves to the sampling position and rests until the next sampling and injection cycle.

The 115 v ac relay (H) closing allows the 24 v dc supply to pull in the mobile phase pump refill relay (J_1) and the sample injection relay (J_2) which, in turn, supplies 24 v ac current to two solenoid valves[10] (K_1 and K_2). K_2 supplies air to refill the mobile phase pump, and K_1 supplies air to the air cylinder, which pulses the injector valve and causes the sample from the flowing stream to be injected. The spring, steel balls, and weep hole ensure return of the plunger after injection to the normally off position.

The two solenoid valves can also be activated *via* front panel push button switches in the manner provided by the chromatograph manufacturer. The time delay relay (D) times out, resets sample pump timer (A), which, in turn, removes 115 v ac from contact 13, removing power from the solenoid valve *via* the relay (H). The chromatograph timer (B), on timing out, restarts the sample pump timer (A) *via* contact 3. The chromatograph time can be stopped at any desired time by manually pushing a reset button or by an automatic stop activated by the last sample in the sample tray. The last sample stop also removes power from the recorder and the mobile phase pump. The UV detector remains on to ensure its stability.

Each of the four functions—control of sample pump, control of automatic sampler, mobile phase pump refill, and sample injection—has a separate relay (J), which can be activated by a 24 v dc signal. This system was used so that the automated HPLC sampling system can be computer operated, independent of the three timers now used. The three timers were wired in the following manner: pump timer, wired for momentary start and separate reset switch; rinse timer, wired for sustained start; and chromatograph timer, wired for momentary start and automatic reset at end of programmed time.

Procedure—The sample probe is placed in a vial of liquid (preferably sample solvent), and the sample pump is primed. The sampling rate is selected from the vernier of the sample pump; sampling and rinse times are set on their appropriate timers. The length of chromatographic time is set, with the exact time determined from a test chromatogram. This time is calculated as the elapsed time from one sample injection to the next sample injection. Sampling time occurs for a preset period prior to injection. Sampling, therefore, can be programmed to begin during the elution of the last peak, depending on what effect, if any, the initiation of sampling signal has on the chromatogram.

The glass sample vials or plastic sample cups can be closed with aluminum foil, if necessary, to prevent solvent evaporation prior to placement in the sample tray. For many applications, however, the glass cover plate is satisfactory, particularly if internal standards are used.

Table II—Replicate Automated HPLC Injection of 5 μl of a 0.25-mg/ml Solution of 9α-Fluoroprednisolone Acetate

Injection	Peak Height[a]	Injection	Peak Height[a]
1	57.3	11	58.0
2	56.9	12	58.7
3	57.7	13	58.1
4	57.5	14	58.3
5	57.7	15	58.0
6	57.9	16	58.0
7	57.5	17	58.9
8	56.4	18	58.5
9	57.7	19	57.4
10	58.1	20	58.0
		Average =	57.8
		RSD =	1.0%

[a]Percent of full chart.

RESULTS AND DISCUSSION

Selections of the sampling rate by vernier adjustment of the sample pump and sampling time with a timer control the sample

Table III—Automated HPLC Analysis of 9α-Fluoroprednisolone Acetate in a Commercial Veterinary Product with and without an Internal Standard

	9α-Fluoroprednisolone Acetate, mg/ml	
Sample	With Internal Standard	Without Internal Standard
1	1.91	2.00
2	2.06	2.00
3	2.01	1.99
4	2.03	1.97
5	2.00	2.00
6	1.98	1.94
7	2.00	1.95
8	2.00	1.99
9	2.02	2.03
10	2.02	2.03
11	2.04	2.01
12	2.01	2.01
Average =	2.01	1.99
RSD =	1.84%	1.41%

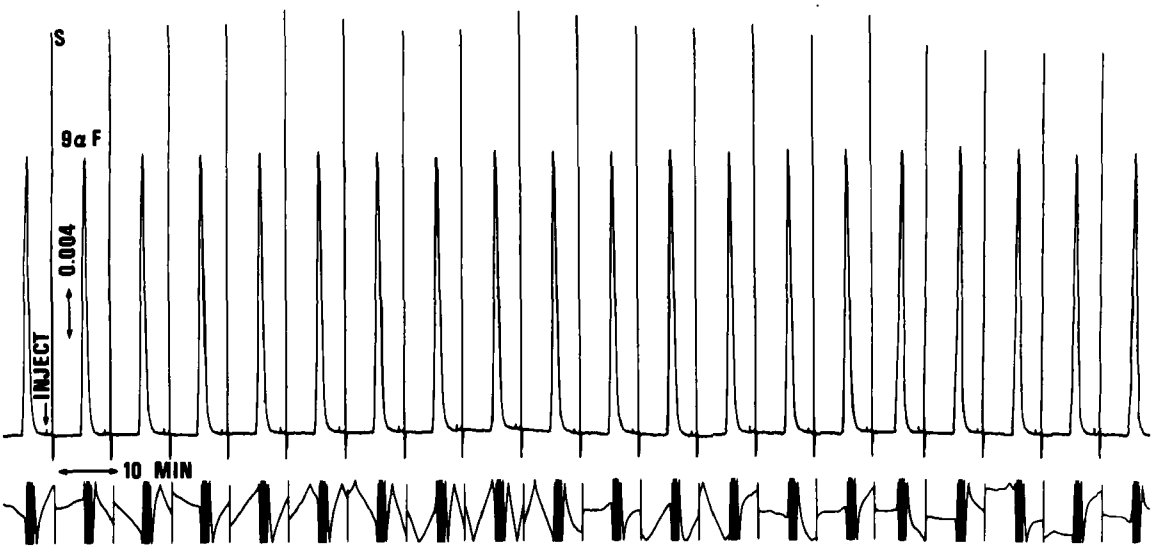

Figure 5—*Ten-minute chromatograms of automatically sampled 9α-fluoroprednisolone acetate, 0.25 mg/ml (9αF), in 75% 3A alcohol (S). Chromatographic conditions were: C-18 column packing, 24% aqueous acetonitrile mobile phase at 1000 psig (1.5 ml/min), and column at ambient temperature.*

volume desired to flush the automated injection system. The volume of sample available for injection was usually 10 ml or more; therefore, volume was no problem. Approximately 3 ml of sample and 3 ml of rinse were used routinely. Settings of 50 on the sample pump vernier and of 2 min on sampling and rinse timers gave these volumes. Figure 3 gives chromatograms showing the absence of carryover when going from a steroid sample, producing a peak approximately 77% of full scale, to 75% 3A alcohol solvent. For these chromatograms, an ODS column at 35° was used with a 6% aqueous acetonitrile mobile phase containing 0.0075 *M* borate buffer at pH 11.0. The flow rate was 0.67 ml/min, and pressure was 500 psig.

These conditions are similar to those used for the assay of fluorometholone in a topical steroid cream[14]. Either 5 μl of fluorometholone at 0.15 mg/ml in 75% 3A alcohol or 75% 3A alcohol was automatically injected. Chromatographic time was set for 8 min. Under the same chromatographic conditions, except for a pressure of 300 psig (0.4 ml/min), fluorometholone (0.075 mg/ml) and norethindrone internal standard (0.05 mg/ml) were automatically injected over approximately 11.5 hr, yielding 34 separate chromatograms, each of 20-min duration. The peak height ratios with a relative standard deviation of 0.61% are shown in Table I. A number of the chromatograms are shown in Fig. 4.

Additional data using the automated HPLC sampling system were obtained using a C-18 column with a mobile phase of 24% aqueous acetonitrile. Column pressure was 1000 psig with a flow rate of 1.5 ml/min at ambient temperature. The compound chromatographed was 9α-fluoroprednisolone acetate in a veterinary product[15] at approximately 0.24 mg/ml in 75% 3A alcohol. The relative standard deviation for 20 separate sample injections was 1.0% (Table II). A reproduction of 20 chromatograms, each of 10 min duration, is given in Fig. 5.

HPLC assays of 9α-fluoroprednisolone acetate, prepared for assay with and without the internal standard, were made using the automated HPLC sampling system. Twelve separate samples were prepared for assay using 2.0 ml of the steroid product and 15.0 ml of the internal standard solution (norethindrone at 0.20 mg/ml in 75% 3A alcohol). These samples were assayed against a 9α-fluoroprednisolone acetate standard (0.25 mg/ml) dissolved in the internal standard solution. Assay samples without the internal standard were prepared by adding 2.0 ml of the steroid formulation to 15.0 ml of 75% 3A alcohol. These samples were assayed against 9α-fluoroprednisolone acetate standard with a placebo vehicle. The standard solution was prepared by adding 2.0 ml of placebo vehicle to 15.0 ml of a 0.25-mg/ml 9α-fluoroprednisolone acetate solution in 75% 3A alcohol. The placebo vehicle was added to the standard solution to compensate for the volume change of the sample preparation. Table III shows that nearly identical results were obtained, with the steroid content of the 12 samples near the labeled amount (2.0 mg/ml).

The data show that the automated HPLC system described is capable of supplying uniform data over a long, unattended period. The data also suggest that the application of automated HPLC assays without the use of internal standards is possible, thus reducing analysis time as well as development time. Peak areas were also available for the data of this report; however, peak heights gave better precision and, therefore, were used in the calculations.

[14] Oxylone, topical cream, 0.025%, The Upjohn Co., Kalamazoo, Mich.

[15] Predef 2X, 2 mg/ml sterile aqueous suspension, The Upjohn Co., Kalamazoo, Mich.

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Relationship of *In Vitro* Hydrolysis of 17-Chloroacetylajmaline and 17-Acetylajmaline in Different Animal Species

MARIO SALMONA ˣ, KATERINA LAKSZNER *, ROBERTO FANELLI, CARLO SARONIO, ROBERTO BIANCHI, and EMILIO MUSSINI

Abstract □ 17-Chloroacetylajmaline and 17-acetylajmaline are reported to have *in vivo* antiarrhythmic activity and are metabolized by hydrolysis. Since the hydrolysis product, ajmaline, may be the actual antiarrhythmic agent, the hydrolysis of these derivatives by various tissues of the guinea pig, rat, and mouse was determined *in vitro* by a titrimetric method and compared to hydrolysis by α-naphthylacetate. The heart is the most active tissue in the guinea pig for hydrolyzing 17-chloroacetylajmaline. The hydrolyzing activity is greater in the guinea pig than in rat or mouse heart, corresponding with the more significant pharmacological activity in the guinea pig. 17-Chloroacetylajmaline has a significantly lower K_m value than 17-acetylajmaline, which is in agreement with the *in vivo* activity.

Keyphrases □ 17-Chloroacetylajmaline—*in vitro* hydrolysis, relationship to hydrolysis of 17-acetylajmaline, different animal species □ 17-Acetylajmaline—*in vitro* hydrolysis, relationship to hydrolysis of 17-chloroacetylajmaline, different animal species □ Antiarrhythmic activity—*in vitro* hydrolysis of 17-chloroacetylajmaline and 17-acetylajmaline, different animal species

The presence of several esterases in the blood and body tissues may be important in the distribution and metabolism of drugs possessing an ester bond (1). The pharmacological effects also may be influenced, depending upon whether an active or an inactive moiety of the particular drug is released with this esterase activity.

This problem should be considered not only for the blood but also for the tissues, with particular reference to the target organ of drug action. Recently, a new ester of ajmaline (2–4), 17-chloroacetylajmaline, was proposed as a therapeutic agent for the treatment of arrhythmias[1]. Preliminary data showed that this new compound is more effective in guinea pigs than in other animal species such as rats and mice. Therefore, it was of interest to investigate comparatively the capacity of blood and tissues, with particular reference to the heart, of these animal species to hydrolyze 17-chloroacetylajmaline.

This study may show whether 17-chloroacetylajmaline is active *per se* or through formation of ajmaline. It may also give an explanation of the outstanding action seen in guinea pigs.

EXPERIMENTAL

Animals—Male Sprague–Dawley rats (200–250 g), male albino Swiss mice (20–25 g), and male albino guinea pigs (300–350 g) were used in all experiments and were fed *ad libitum*.

Substrates—17-Chloroacetylajmaline[2] (I) and 17-acetylajmaline[2] (II) are odorless, white, microcrystalline powders. They do not have a significant melting point but begin decomposing above 220 and 230°, respectively. Their purity was checked by TLC utilizing silica gel[3] precoated aluminum sheets with a layer thickness of 0.25 mm and F_{254} UV indicator.

The plates were activated at 60° for 1 hr. After the application of about 20 ng of each compound, the plates were developed with

Table I—Distribution of 17-Chloroacetylajmaline, Acetylajmaline, and α-Naphthylacetate Hydrolyzing Esterases in Guinea Pigs

Tissue	Activity[a], μmoles/min/g Protein		
	17-Chloroacetylajmaline	Acetylajmaline	α-Naphthylacetate
Plasma	0.043 ± 0.024	0.044 ± 0.020	0.537 ± 0.029
Liver	1.000 ± 0.029	0.172 ± 0.019	13.698 ± 0.108
Brain	1.080 ± 0.042	0.539 ± 0.023	1.150 ± 0.054
Heart	1.708 ± 0.090	0.449 ± 0.068	2.151 ± 0.102
Leg muscle	0.359 ± 0.017	0.247 ± 0.015	1.855 ± 0.036

[a] Each value represents the mean ± *SE* of the enzymatic activity of three enzyme preparations from different animals.

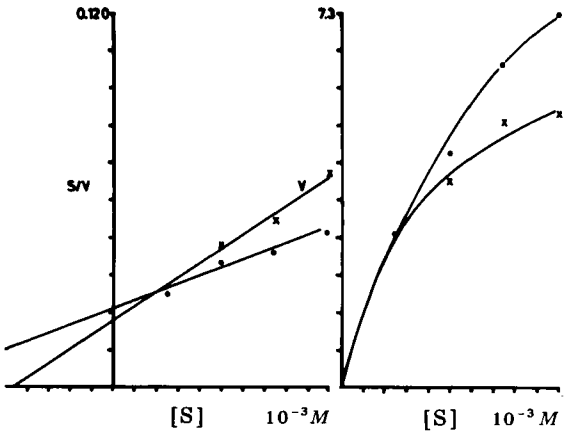

Figure 1—*Woolf plot for the hydrolysis of 17-chloroacetylajmaline in the guinea pig heart. The* K_m *and* V_{max} *values in the atria (×—×) are 4.5 × 10⁻⁴ M and 10 eu, respectively. The* K_m *and* V_{max} *values in the ventricles (●—●) are 9.8 × 10⁻⁴ M and 9.7 eu, respectively. V = initial velocity expressed as micromoles per minute per milligram of protein; S = substrate concentration (molar).*

[1] C. Capra, Laboratorio Farmacologico Inverni & Della Beffa, S.p.A., Milan, Italy, personal communication.

[2] Supplied by Inverni & Della Beffa, S.p.A., Milan, Italy.

[3] Silica gel F_{254}, Merck, Darmstadt, Germany.

Table II—Distribution of 17-Chloroacetylajmaline, Acetylajmaline, and α-Naphthylacetate Hydrolyzing Esterases in Mice

Tissue	Activity[a], μmoles/min/mg Protein		
	17-Chloro-acetylajmaline	Acetylajmaline	α-Naphthyl-acetate
Plasma	0.141 ± 0.050	0.085 ± 0.010	0.354 ± 0.012
Liver	0.818 ± 0.103	0.262 ± 0.051	2.375 ± 0.054
Brain	1.085 ± 0.023	0.087 ± 0.019	1.910 ± 0.070
Heart	0.424 ± 0.090	0.424 ± 0.082	0.169 ± 0.063
Leg muscle	0.066 ± 0.022	0.060 ± 0.020	1.615 ± 0.031

[a] Each figure represents the mean ± *SE* of the enzymatic activity of three enzyme preparations from different animals.

Table III—Distribution of 17-Chloroacetylajmaline, Acetylajmaline, and α-Naphthylacetate Hydrolyzing Esterases in Rats

Tissue	Activity[a], μmoles/min/mg Protein		
	17-Chloro-acetylajmaline	Acetylajmaline	α-Naphthyl-acetate
Plasma	Absent	Absent	0.372 ± 0.010
Liver	0.694 ± 0.141	0.297 ± 0.125	3.766 ± 0.056
Brain	0.556 ± 0.030	0.556 ± 0.028	2.450 ± 0.025
Heart	0.339 ± 0.112	0.339 ± 0.103	0.745 ± 0.093
Leg muscle	0.246 ± 0.020	0.246 ± 0.022	3.033 ± 0.031

[a] Each figure represents the mean ± *SE* of the enzymatic activity of three enzyme preparations from different animals.

methanol. Compounds I and II presented one spot each with R_f values of 0.63 and 0.55, respectively.

α-Naphthylacetate (III) was synthesized as previously described (5).

Preparation of Biological Samples—Tissue and plasma samples were prepared as previously described (5) and were immediately assayed for their enzymatic activity.

Protein Analysis—Protein concentration in plasma and tissues was determined according to Lowry *et al.* (6).

Determination of Esterase Activity—The esterase activity of tissues were determined by a titrimetric method[4] according to Schwark and Ecobichon (7). All substrates were dissolved in 0.05 *M* phosphate buffer at the desired pH value. The volume of the reaction mixture was always 16 ml. A volume of 0.020 ml of appropriate supernate was added to the reaction vessel. The final concentration of the substrates in the vessel was 10^{-3} *M*.

Sodium hydroxide, at a concentration of 0.01 *M*, was used for the titrations, which were carried out at 37° for 6 min. In fact, the reaction was linear for at least 20 min. The pH of the reaction was 7.0 instead of 7.6, which is described to be the optimum for many esterases, because I and II are highly unstable at pH values above 7.2. However, with these experimental conditions in the absence of the enzymatic preparations, the spontaneous hydrolysis of I and II was negligible.

The initial rate of hydrolysis was determined from the titration curves as micromoles of sodium hydroxide utilized per minute per milligram of protein of extract (eu).

RESULTS AND DISCUSSION

Enzyme Distribution in Tissue—To determine the distribution of the enzyme hydrolyzing I and II, a substrate of reference α-naphthylacetate was used; this ester is known to be hydrolyzed, although at a different rate, by all esterases (8–10). Tables I, II, and III show the enzyme distribution in plasma and tissues of the guinea pig, mouse, and rat, respectively.

Compound I was hydrolyzed more rapidly in the heart than in other tissues of the guinea pig, and I was hydrolyzed fourfold faster than II. In rat and mouse hearts, I and II were hydrolyzed at equal rates but this activity was less than in the brain and liver. Since work performed *in vivo* (2) showed that I was much more active than II in the guinea pig only, this finding may suggest that the antiarrhythmic activity is due to the hydrolysis product ajmaline, at the site of action, namely the heart muscle.

It was evident from the high hydrolysis of I in the guinea pig that a large amount of ajmaline was released. Even though ajmaline was reported to be an active compound, if ajmaline itself was administered, it was less effective than 17-chloroacetylajmaline. The reported esterase activity for I present in the heart could have therapeutic significance because it may permit sustained release of ajmaline, the active moiety, at the effected site. It is remarkable that the hydrolysis of 17-chloroacetylajmaline was several fold higher in the guinea pig than in the rat and mouse, in agreement with the more significant pharmacological activity in the former animal species as compared to the two latter species (2).

Kinetic Parameters—Since I acts on the heart, it was of interest to determine the kinetic parameters of the esterase activity in the atria and ventricles of the guinea pig. This species was selected because it showed the highest esterase activity with respect to I among the three species tested.

Figures 1–3 show Woolf plots for I in comparison to II and III in the guinea pig heart. The K_m values in the atria are, for the three substrates, 4.5×10^{-4}, 4.3×10^{-3}, and 5.0×10^{-5} *M*; the V_{max} values are 10.00, 6.14, and 17.09 eu, respectively. In ventricles, the K_m values are calculated to be 9.8×10^{-4}, 7.8×10^{-3}, and 5.0×10^{-5} *M*; the V_{max} values are 9.70, 8.00, and 17.09 eu, respectively.

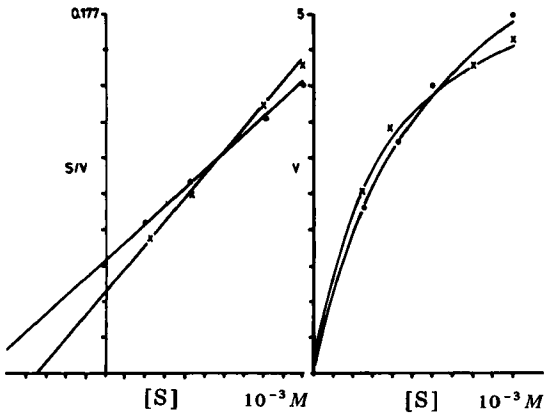

Figure 2—*Woolf plot for the hydrolysis of acetylajmaline in the guinea pig heart. The* K_m *and* V_{max} *values in the atria (×—×) are 4.3 × 10⁻³ M and 6.14 eu, respectively. The* K_m *and* V_{max} *values in the ventricles (●—●) are 7.8 × 10⁻³ M and 8.0 eu, respectively. V = initial velocity expressed as micromoles per minute per milligram of protein; S = substrate concentration (molar).*

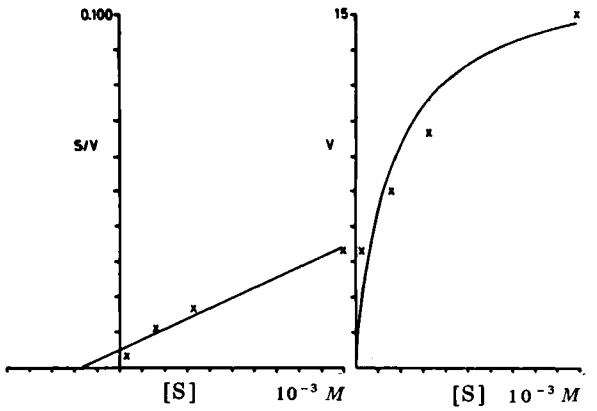

Figure 3—*Woolf plot for the hydrolysis of α-naphthylacetate in the guinea pig heart. The atria and ventricles show the same* K_m *and* V_{max} *values, 5 × 10⁻⁵ M and 17.09 eu, respectively. V = initial velocity expressed as micromoles per minute per milligram of protein; S = substrate concentration (molar).*

[4] pH-Stat, TTT lc Apparatus, Radiometer, Copenhagen, Denmark.

From these results, it is evident that the chloroacetyl ester of ajmaline (I) is more effectively hydrolyzed than the acetyl ester (II) by both the atria and ventricles. Also, the apparent affinity for the enzyme is at least 10 times higher for the chloroacetyl than for the acetyl ester, thus substantiating its *in vivo* effect. Further *in vivo* work is being conducted to determine: *(a)* the levels of ajmaline produced at the site of action after administration of I and II and *(b)* the levels reaching the site of action after administration of ajmaline itself.

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Synthesis and *In Vitro* Evaluation of 8-Hydroxyquinolines as Dental Plaque Inhibitors

VICTOR D. WARNER *ˣ, JOSEPH D. MUSTO *, SAMUEL S. TURESKY ‡, and BARBARA SOLOWAY ‡

Abstract □ Some 4- and 5-substituted 8-hydroxyquinolines, with predicted log *P* values in the range of 1.48–2.90, were synthesized by modified Skraup reactions or thermal cyclization. These hydroxyquinolines include the 5-methyl, 4,5-dimethyl, 4-methyl, 5-hydroxy-4-methyl, 5-methoxy, 5-methoxy-4-methyl, 4-hydroxy, 4-chloro, 4-amino, and 5-amino analogs. Partition coefficients, antibacterial activity, and antiplaque activity were determined. Four analogs showed *in vitro* antiplaque activity. None of the derivatives with ionizable functions was active.

Keyphrases □ 8-Hydroxyquinoline analogs—synthesis and antibacterial and antiplaque activities □ Antibacterial activity—8-hydroxyquinoline analogs □ Antiplaque activity—8-hydroxyquinoline analogs

If dental plaque is to be controlled, it must be actively removed or its formation must be prevented. Mechanical cleansing is the principal means of removing plaque and its effectiveness is limited. Since dental plaque consists primarily of bacteria and their metabolites, consideration has been given to controlling plaque formation by use of antibacterial agents administered in dentifrices, mouthwashes, troches, or other appropriate vehicles. The present level of knowledge indicates that bacterial plaque is the direct cause of gingivitis and marginal periodontal disease and that without bacterial plaque there will be a great reduction in the occurrence of caries and peridontitis.

Previous research (1) showed that 8-hydroxyquinoline and some of its analogs inhibit *in vitro* plaque formation. It has been suggested that the antibacterial activity of the 8-hydroxyquinolines and their ability to chelate with trace ions on the surface of teeth, with subsequent slow release, should result in good long-term inhibition of dental plaque. Plaque inhibition by 8-hydroxyquinoline has been correlated favorably with the lipid–water partition coefficients. Analogs with log *P* values in the 1–4 range have been shown to have good antiplaque activity.

In an attempt to optimize antiplaque activity and to investigate other physicochemical factors that influence activity in addition to the partition coefficient, the following compounds with calculated log *P* values in the 1–4 range were synthesized and tested: 5-methyl- (I), 4,5-dimethyl- (II), 4-methyl- (III), 5-hydroxy-4-methyl- (IV), 5-methoxy- (VIII), 5-methoxy-4-methyl- (IX), 4-hydroxy- (XIV), 4-chloro- (XV), 4-amino- (XVII), and 5-amino-8-hydroxyquinoline (XVIII).

Compounds I–III, XV, XVII, and XVIII were prepared by standard methods (Table I). Compounds IV, VIII, and IX are unreported derivatives of 8-hydroxyquinoline, while XIV was previously prepared (10) but by a different method than is presented here. Intermediates VI and VII are known compounds (11, 12), but a different method for their preparation is presented in Scheme I.

Antiplaque activity as displayed by 8-hydroxyquinoline requires that a compound be an antibacterial agent. Therefore, the analogs were first evaluated for their *in vitro* antibacterial activity against *Streptococcus mutans* (No. 6715), a pure strain of plaque-forming bacteria. The antiplaque activity of the analogs was evaluated using an *in vitro* screening procedure (2).

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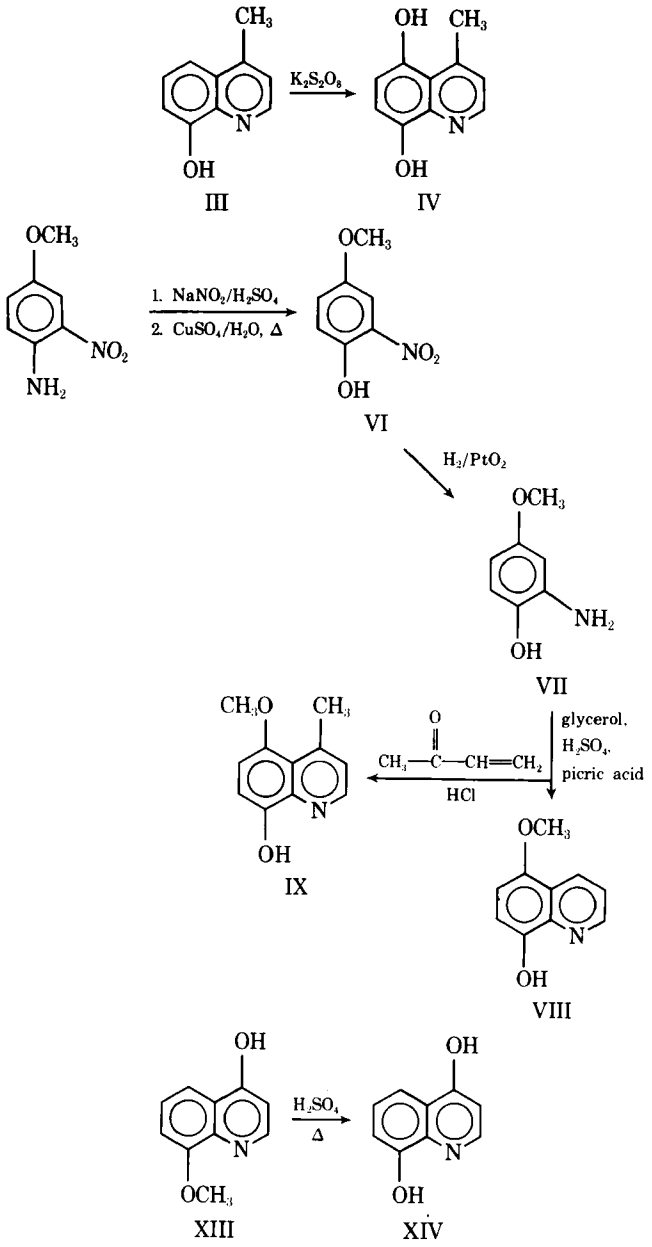

Scheme I

EXPERIMENTAL[1]

5,8-Dihydroxy-4-methylquinoline (IV)—4-Methyl-8-hydroxyquinoline (3.18 g, 0.02 mole), sodium hydroxide (4.00 g), and water (120 ml) were stirred and treated dropwise with a solution of potassium persulfate (5.40 g, 0.02 mole) in 100 ml of water. The temperature was maintained between 5 and 10° during the addition. The reaction mixture was stirred for 30 min, refrigerated for 4 days, adjusted to pH 6 with 10% HCl, and filtered. After filtration, the filtrate was extracted with ether (3 × 30 ml).

The aqueous solution was treated with 50 ml of concentrated hydrochloric acid and allowed to stand refrigerated for 4 days. The red crystals which formed were filtered and heated with 30 ml of concentrated hydrochloric acid on a steam bath for 1 hr and then cooled to give 0.4 g (9.5%) of the IV-HCl. Isolation of the free base (neutralization with 20% NaOH) and crystallization from benzene gave the pure compound (IV), mp 294–296°.

4-Methoxy-2-nitrophenol (VI)—A suspension of 22.00 g (0.13 mole) of 4-methoxy-2-nitroaniline in 150 ml of 50% H_2SO_4 was cooled, and 150 ml of ice was added. After a homogeneous mixture and a temperature between 0 and 5° were obtained, a solution of sodium nitrite (26 g + 60 ml of water) was added to the bottom of the mixture while the temperature was maintained. The presence of excess nitrous acid was determined using starch iodide paper. Stirring was continued for 10 min after the completion of the reaction, and the product (V) was used immediately.

A solution containing 350 g of cupric sulfate pentahydrate in 1 liter of water was heated to boiling. The diazonium solution (V) was added slowly as the phenolic product was removed *via* steam distillation. The distillate was filtered, and the precipitate was dissolved in 5% NaOH, filtered, and extracted with ether. The aqueous layer was then acidified with 5% HCl, and the precipitate was recrystallized from petroleum ether, yielding 4.88 g (22.1%) (VI), mp 81–82° [lit. (11) mp 80°].

4-Methoxy-2-aminophenol (VII)—A solution of 5.00 g (0.03 mole) of VI in 150 ml of 95% ethanol and 150 mg of platinum oxide were hydrogenated (120 psi of H_2) for 30 min. The catalyst was filtered off, and the solvent was evaporated to give 3.60 g (95%) of a dark crystalline material (VII), mp 129–132° [lit. (12) mp 135°]. This compound was used without further purification.

5-Methoxy-8-hydroxyquinoline (VIII)—To 4.30 g (0.046 mole) of glycerol were added 2.0 g (0.01 mole) of VII and 0.45 g (0.002 mole) of picric acid. The temperature was raised to 110–120° as 3.50 g (0.04 mole) of concentrated sulfuric acid was added dropwise. The reaction mixture was then stirred and heated to 120–140° for 4 hr. After cooling, 72 ml of water was added and the pH was adjusted to 3 with 5% NaOH.

The product was obtained by steam distillation of the reaction mixture, followed by neutralization with 5 *N* ammonium hydroxide and extraction with ether. Recrystallization from hexane yielded 0.70 g (28%) of VIII, mp 83–85°.

4-Methyl-5-methoxy-8-hydroxyquinoline (IX)—To 100 ml (3.33 mole) of concentrated hydrochloric acid was added 28.0 g (0.20 mole) of VII. The temperature was raised to 100°, and 35.0 g (0.50 mole) of methyl vinyl ketone was added slowly. The reaction mixture was then stirred and heated for 5 hr at 100–120°. After cooling, the pH was made slightly alkaline with 10% NaOH and the mixture was steam distilled. The product was recrystallized from hexane to yield 5.40 g (14.3%) of IX, mp 130–132°.

4,8-Dihydroxyquinoline (XIV)—A solution of 4-hydroxy-8-methoxyquinoline (XIII) (1.50 g, 0.009 mole), 9.80 ml of concentrated sulfuric acid, and 5.8 ml of water was refluxed for 5.5 hr. Then the solution was poured over crushed ice and neutralized with concentrated ammonium hydroxide to precipitate the product. Recrystallization from water yielded 0.80 g (58%) of XIV, mp 316–318° [lit. (10) mp 315–319°].

Partition Coefficients[2]—The compounds were partitioned between 1-octanol saturated with water and distilled water saturated with octanol. Usually 50–150-ml portions of octanol and water were used. In partitioning these compounds, gentle shaking for 90 min was carried out at room temperature (25 ± 5°). The volume ratio of the two phases and the amount of sample were chosen so that the absorbance of the sample from the water layer after partitioning usually had a value between 0.2 and 0.9 using a 1-cm cell. Only the concentration of the sample in the water layer was determined; that in the octanol was obtained by difference.

Analyses of the concentrations of the partitioned substances were made by spectrophotometry[3]. The partition coefficient was calculated as $P = C_{octanol}/C_{water}$. Each determination was done in at least triplicate, using different amounts of sample; the average value for log P has been reported. The partition coefficient of XVIII could not be determined since the free base was not stable.

Antibacterial Activity—To 7.85 ml of sterile trypticase broth, 1 ml of an aqueous solution of the hydrochloride salt of the test compound and 1 ml of 50% sterile sucrose solution were added. The medium was inoculated with 0.15 ml of a 24-hr culture of *S. mutans*[4] (No. 6715), a pure strain of plaque-forming bacteria. This

[1] Melting points were determined on a Thomas-Hoover capillary melting-point apparatus and are uncorrected. Microanalyses were performed by Midwest Microlab Ltd., Indianapolis, Ind. Where analyses are indicated only by symbols of the elements, analytical results obtained for those elements were within ±0.4% of the theoretical values.

[2] In calculating partition coefficients, a least-squares computer program was used both to produce the equation for the standards and to determine concentrations from experimental values.

[3] Beckman DB-G spectrophotometer.

[4] Isolated at, and made available by, the National Institute of Dental Research.

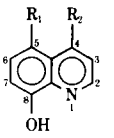

Table I—Physical Data for the 8-Hydroxyquinoline Analogs

Compound	R_1	R_2	Yield, %	Melting Point	Formula	Analysis, % Calc.	Analysis, % Found	Reference
I	CH_3	H	62.5	118–120°	$C_{10}H_9NO$	C 75.38 H 5.65 N 8.79	75.27 5.79 8.79	3
II	CH_3	CH_3	12.8	153–154°	$C_{11}H_{11}NO$	C 76.27 H 6.40 N 8.09	75.98 6.53 8.03	4
III	H	CH_3	10.4	137–139°	$C_{10}H_9NO$	C 75.38 H 5.65 N 8.79	75.24 5.73 8.70	5
IV	OH	CH_3	9.5	294–296°	$C_{10}H_9NO_2$	C 68.56 H 5.18 N 7.99	68.40 5.06 7.69	—
VIII	OCH_3	H	28.0	83–85°	$C_{10}H_9NO_2$	C 68.56 H 5.18 N 8.00	68.29 5.31 8.14	—
IX	OCH_3	CH_3	14.3	131–133°	$C_{11}H_{11}NO_2$	C 69.82 H 5.86 N 7.40	69.92 5.72 7.18	—
XIV	H	OH	58.0	316–318°	$C_9H_7NO_2$	C 67.07 H 4.37 N 8.69	66.93 4.50 8.70	6, 10
XV	H	Cl	64.0	142–143°	C_9H_8ClNO	C 60.18 H 3.37 N 7.80	60.38 3.45 7.87	6, 7, 8
XVII	H	NH_2	67.0	207–208°	$C_9H_8N_2O$	C 67.06 H 5.00 N 17.38	67.17 5.07 17.09	9
XVIII	NH_2	H	87.0	245–265°	$C_9H_8N_2O \cdot 2HCl$	C 46.36 H 4.32 N 12.02	45.39 4.47 11.46	—[a]

[a] J. N. Sane, Northeastern University, Department of Medicinal Chemistry, Boston, Mass., personal communication.

Table II—Partition Coefficient and Antibacterial and Antiplaque Activities for 4- and 5-Substituted 8-Hydroxyquinolines

Compound	log *P*[a]	Inhibition, % Antibacterial[b] 10^{-4} *M* 24 hr	10^{-4} *M* 48 hr	10^{-5} *M* 24 hr	10^{-5} *M* 48 hr	Antiplaque[c], 10^{-1} *M* 24 hr	Antiplaque[c], 10^{-1} *M* 48 hr
8-Hydroxyquinoline	1.94 (0.034)[d]	100	100	100	100	100	100
I	2.37 (0.020)	100	100	20	0	80	40
II	2.71 (0.045)	100	100	80	40	100	60
III	2.36 (0.058)	100	100	100	70	80	40
IV	1.59 (0.103)	0	0	—	—	0	0
VIII	2.06 (0.081)	100	100	0	0	0	0
IX	2.75 (0.168)	100	0	—	—	100	80
XIV	0.62 (0.038)	0	0	—	—	0	0
XV	2.67 (0.050)	0	0	—	—	0	0
XVII	−0.11 (0.037)	0	0	—	—	0	0
XVIII	—	0	0	—	—	0	0

[a] All compounds were evaluated in triplicate. [b] All compounds were evaluated as their hydrochloride salts in aqueous solutions. [c] All compounds were evaluated as free bases (except XVIII) in dimethyl sulfoxide solution on five teeth. Values show percentage of teeth that did not show plaque formation after stated incubation period. [d] All determinations were done in triplicate; values in parentheses indicate standard deviations.

mixture was incubated under anaerobic conditions[5] at 37°. Bacterial growth was determined after 24 and 48 hr.

Assay for *In Vitro* Antiplaque Activity—Sterilized extracted human teeth were immersed in dimethyl sulfoxide solutions of the test compounds for two 1-min periods, each of which was followed by a 1-min exposure to air. These treated teeth were washed with 250 ml of distilled water for 5 min and then incubated at 37° under anaerobic conditions in a 5% sucrose-containing trypticase broth with *S. mutans*. The teeth were suspended in test tubes on orthodontic wire[6] (0.71 mm in diameter) threaded through a hole in the root so that the entire tooth was completely immersed.

After 24 and 48 hr, subjective estimates were made of adherent microbial growth on the test tube walls, wire, and teeth and of nonadherent growth in the broth using a scale of 0 (no growth) to 4 (maximum growth). The total microbial accumulation was considered as *in vitro* plaque. For the results, growth ratings of 0 or 1

[5] BBL-Gaspak, Division of Bioquest, Cockeyville, Md.

[6] Rocky Mount.

were scored as plaque inhibition. Each compound was evaluated on five teeth, with percent inhibition values being reported. This method indicates the percentage of teeth that did not show plaque formation after the stated incubation period. The solvent served as the control.

RESULTS AND CONCLUSIONS

In the group of compounds tested, I–III and VIII showed maximal activity at 10^{-4} *M* after 24 and 48 hr while IX showed a shorter duration of activity. Only III showed equivalent activity to that of 8-hydroxyquinoline at 10^{-5} *M* after 24 hr. At 10^{-5} *M*, all of the active, new agents showed decreased duration of action or decreased activity. The antiplaque studies showed that II and IX had 24-hr activity equal to 8-hydroxyquinoline at 10^{-1} *M* while I and III had only 80% of the activity of the parent compound. Compounds IV, XIV, XVI, XVII, and XVIII showed neither antibacterial nor antiplaque activity.

Analogs with ionizable functions in the 4- or 5-position of 8-hydroxyquinoline showed no antibacterial or antiplaque activity. The inactivity may be due to either the test compounds inability to cross the bacterial membrane or their inability to chelate properly as required for biological activity. These data, as well as previous studies in this laboratory (1), indicate that a single parameter such as log *P* is not adequate to predict antiplaque activity accurately.

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Abstract □ Langmuir isotherms for the adsorption of neomycin sulfate to clays such as attapulgite, bentonite, and magnesium aluminum silicate were constructed. Monovalent and divalent cations were investigated for their influence on the formation of neomycin–clay adsorbates and the resulting equilibrium concentration in a neomycin solution. Divalent magnesium ions were more effective in displacing the antibiotic from each clay than were monovalent sodium ions. Ions present in the GI fluid might increase the bioavailability of neomycin from such neomycin–clay adsorbates.

Keyphrases □ Neomycin sulfate—sorption to attapulgite and montmorillonite clays, influence of monovalent and divalent electrolytes, Langmuir isotherms □ Attapulgite clay—sorption of neomycin sulfate, influence of monovalent and divalent electrolytes, Langmuir isotherms □ Montmorillonite clay—sorption of neomycin sulfate, influence of monovalent and divalent electrolytes, Langmuir isotherms

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Wai and Banker (10) studied the adsorption of cationic drugs to montmorillonite clays and concluded that the mechanism of binding was ion exchange. This mechanism was verified in a recent investigation[1]. These studies, which also encompassed the influence of mono- and divalent electrolytes on the adsorption of amphetamine sulfate to montmorillonite, showed that the electrolytic cations had a significant effect on displacing the drug from the clay.

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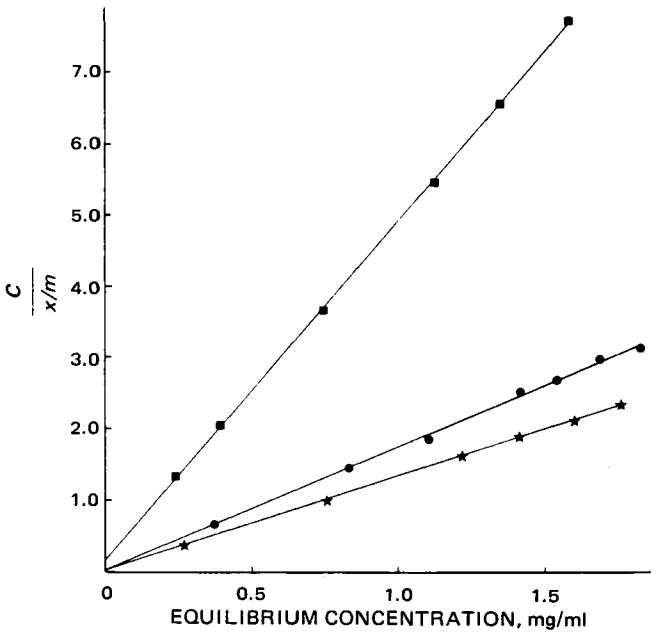

Figure 1—*Langmuir adsorption isotherms for neomycin sulfate and various clays. Key: ■, attapulgite; ●, bentonite; and ★, magnesium aluminum silicate.*

vide data as a basis for speculating on the probable bioavailability of neomycin sulfate in antidiarrheal suspensions containing these clays.

EXPERIMENTAL

Reagents—The following were used: magnesium aluminum silicate[2], bentonite[3], attapulgite[4], amaranth[5], potassium hydrogen phthalate[6], neomycin sulfate[7], sodium chloride[6], and magnesium chloride[6].

Neomycin Sulfate Assay—The assay[8] for the antibiotic is based on a colorimetric reaction between amaranth and neomycin sulfate, buffered to pH 4.0 with potassium hydrogen phthalate. The absorbance of the uncomplexed dye was read spectrophotometrically[9] at 520 nm. A linear relationship existed between the absorbance of the dye and the concentration of the antibiotic; the amount of free amaranth was inversely related to the concentration of neomycin sulfate present. Excellent agreement exists between the results of this chemical assay and those obtained by an assay of microbiological activity.

Sample Preparation for Langmuir Studies—Neomycin sulfate, 200 mg, was dissolved in 10 ml of purified water and added to weighed amounts of each clay. Clay samples had previously been passed through a 100-mesh screen and hydrated in 80 ml of 60° water. Each suspension was adjusted to 100 ml with room temperature purified water and then agitated in a constant-temperature water bath. After centrifugation, the supernatant liquid was assayed colorimetrically for neomycin content.

Sample Preparation for Electrolyte Studies—The quantity of each clay to adsorb 80% of the neomycin was calculated from the Langmuir studies. These concentrations (w/v) were: magnesium aluminum silicate, 0.8%; bentonite, 1.2%; and attapulgite, 3.2%. Neomycin sulfate, 200 mg, was dissolved in 10 ml of purified water and added to the hydrated clay (prepared as described) with stirring.

After agitation and equilibration for 24 hr at 24°, the electrolyte solution was added with stirring and the resulting mixture was adjusted to 100 ml. After an additional 4 hr of equilibration, the mixture was centrifuged and the supernatant liquid was assayed for neomycin.

[2] Veegum Regular, R. T. Vanderbilt Co., New York, N.Y.
[3] White Bentonite, Whittaker, Clark and Daniels, Inc., South Plainfield, N.J.
[4] Pharmasorb Colloidal, Engelhard, Edison, N.J.
[5] H. Kohnstamm & Co., New York, N.Y.
[6] Mallinckrodt, St. Louis, Mo.
[7] E. R. Squibb & Sons, Princeton, N.J.
[8] Developed by J. Hill and coworkers, unpublished data.
[9] Spectronic 20.

Table I—Adsorptive Capacities of Attapulgite and Montmorillonite Clays for Neomycin Sulfate

Clay	Limiting Adsorptive Capacity, b^a, mg/g
Attapulgite	215
Bentonite	604
Magnesium aluminum silicate	772

[a] b appears in the Langmuir equation and denotes the theoretical maximum adsorptive capacity of an adsorbent for a particular drug.

RESULTS AND DISCUSSION

Data obtained from adsorption experiments fit the linear form of the Langmuir equation (11):

$$\frac{C}{x/m} = \frac{1}{ab} + \frac{1}{b}(C) \qquad \text{(Eq. 1)}$$

where x/m is the weight of neomycin in milligrams adsorbed per gram of adsorbent, C is the concentration of neomycin in milligrams per milliliter at equilibrium, and a and b are constants. Constant a is related to the forces involved in binding the drug to the clay, and constant b is the maximum amount of neomycin that can be adsorbed per gram of clay.

Langmuir isotherms for the adsorption of neomycin sulfate to magnesium aluminum silicate, bentonite, and attapulgite appear in Fig. 1. From the reciprocal of the slope for each linear curve, the limiting adsorptive capacity of each clay for neomycin was calculated (Table I). These experimental values were calculated from the regression lines in Fig. 1, using the method of least squares. The b values, particularly for the montmorillonite clays, demonstrated the strong binding capacity of these clays for neomycin.

The influence of sodium chloride and magnesium chloride on the adsorption of neomycin to magnesium aluminum silicate, bentonite, and attapulgite is demonstrated in Figs. 2–4. These studies showed that divalent magnesium cations were more efficient than

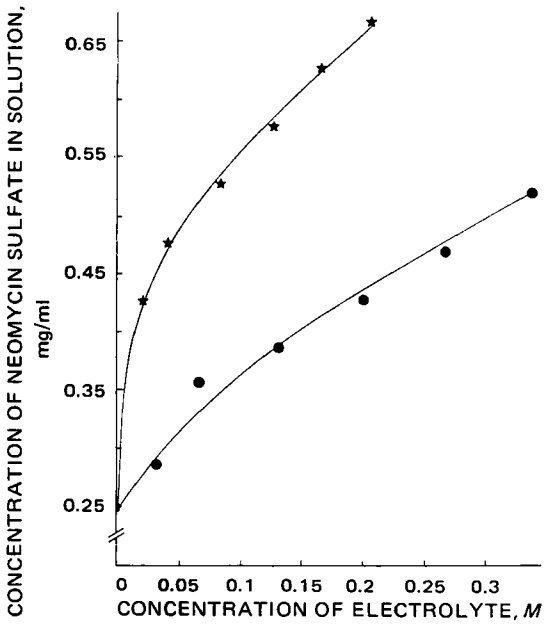

Figure 2—*Effect of electrolyte concentration and valency on the desorption of neomycin from a neomycin–magnesium aluminum silicate adsorbate. Key: ●, sodium chloride; and ★, magnesium chloride.*

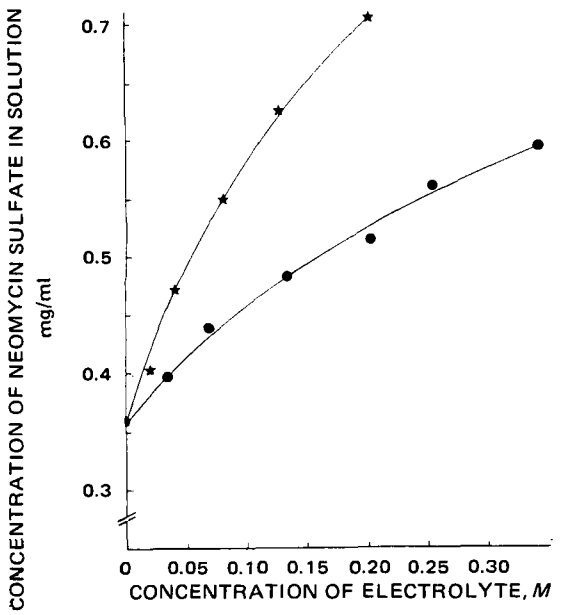

Figure 3—*Effect of electrolyte concentration and valency on the desorption of neomycin from a neomycin–bentonite adsorbate. Key: ●, sodium chloride; and ★, magnesium chloride.*

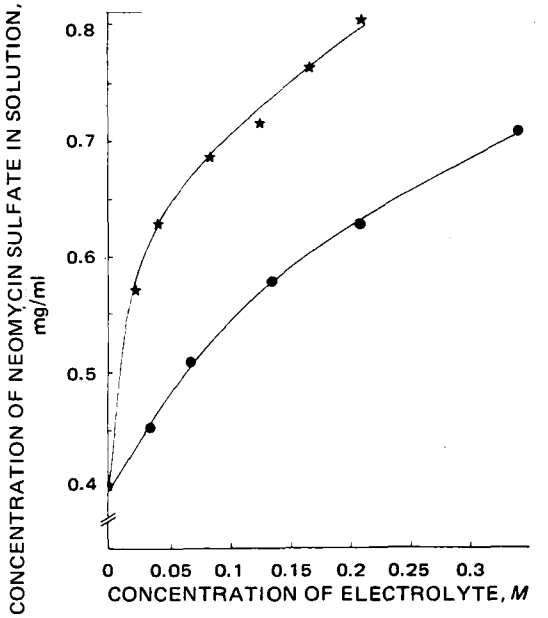

Figure 4—*Effect of electrolyte concentration and valency on the desorption of neomycin from a neomycin–attapulgite adsorbate. Key: ●, sodium chloride; and ★, magnesium chloride.*

monovalent sodium ions in displacing neomycin from negatively charged clays. These data suggest that any cations secreted in the GI fluids could influence the displacement of the antibiotic from these clays.

Sorby (5) suggested earlier that the presence of electrolytes and dilution by various fluids within the GI tract could hasten the release of promazine from activated attapulgite. The test doses that Sorby administered to human subjects had 75% of the drug adsorbed to the clay. However, under the conditions of the Sorby study, the clay slowed the rate of adsorption but had no significant effect on the total availability of promazine.

The average concentrations for sodium ions and potassium ions in GI fluids were reported (12) as 140 and 15 mEq/liter, respectively, but the concentrations of these ions are greater in the lower portion of the intestinal tract, precisely where the local action of neomycin is desired. On the other hand, divalent calcium and magnesium ions appear in negligible quantities in the luminal fluids. However, calcium from an external source (*e.g.*, milk), administered along with the antidiarrheal suspension, may act synergistically with secreted monovalent cations in releasing more free neomycin from the adsorbate. Supplemental oral therapy to replace sodium or potassium ions lost by the patient suffering diarrhea should also increase the desorption of neomycin and, consequently, improve the bioavailability of the antibiotic. During passage through the GI tract, the neomycin–clay adsorbate is continually exposed to freshly secreted fluids, so the desorption process is continuous.

Data presented in Figs. 2–4 were generated in a closed system in an attempt to establish rapidly an equilibrium between adsorbed drug and drug in solution. This equilibrium prevented excessive desorption of neomycin by the electrolyte *in vitro*. However, since equilibrium conditions are never established in the body, it can be argued that electrolytes would exert a greater influence *in vivo* than is suggested by the present *in vitro* studies.

Results of these studies also imply that the poor bioavailability of neomycin from similar adsorbates seen in *in vitro* studies (2, 3, 9) may not have accurately reflected the actual concentration of drug in the luminal fluids under the sink conditions of the body. Besides the electrolytic effect, other factors, such as the presence of bile salts, may influence the process of desorption, making it more difficult still to extrapolate from data for these neomycin–clay systems obtained *in vitro* to the actual situation *in vivo*.

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Deviation of Dielectric Constant from Ideality for Certain Binary Solvent Systems

A. K. AMIRJAHED ˣ and MARTIN I. BLAKE

Abstract □ An equation was derived which relates the dielectric constant of mixtures of a binary solvent system to the mole fraction of the polar and nonpolar components of the system. The coefficients of the formula are readily calculated from the dielectric constant and the molar volume of the pure components of the binary solvent system. These calculated values are employed in graphical representations of the theoretical relationship of the dielectric constant of the binary solvent system and the mole fraction of the polar component of the solvent system. Experimental measurements are employed to demonstrate deviations from ideality.

Keyphrases □ Dielectric constant—binary solvent systems, deviation from ideality, equation developed □ Binary solvent systems—deviation of dielectric constant from ideality, equation developed □ Solvents—binary systems, deviations from ideality, equation developed relating the dielectric constant to mole fraction

For solutions of polar solutes in nonpolar solvents, the following equation applies (1):

$$P_M = [(D - 1)/(D + 2)](M/\rho) \qquad \text{(Eq. 1)}$$

By using this equation, the dielectric constant of a binary solvent mixture (D_m) was related to the mole fractions of the nonpolar solvent (X_1) and the polar solvent (X_2):

$$D_m = (aX_1 + bX_2)/(cX_1 + dX_2) \qquad \text{(Eq. 2)}$$

The coefficients a and b are summations of the molar volume and molar polarization of pure nonpolar and polar solvents, respectively; c and d represent the difference between the molar volume and molar polarization of the nonpolar and the polar solvents, respectively (2). These coefficients can be derived and represented as:

$$a = \bar{V}_1 + 2[(D_1 - 1)/(D_1 + 2)]\bar{V}_1 = \bar{V}_1[(3D_1)/(D_1 + 2)] \qquad \text{(Eq. 3)}$$

$$b = \bar{V}_2 + 2[(D_2 - 1)/(D_2 + 2)]\bar{V}_2 = \bar{V}_2[(3D_2)/(D_2 + 2)] \qquad \text{(Eq. 4)}$$

$$c = \bar{V}_1 - [(D_1 - 1)/(D_1 + 2)]\bar{V}_1 = \bar{V}_1[(3)/(D_1 + 2)] \qquad \text{(Eq. 5)}$$

$$d = \bar{V}_2 - [(D_2 - 1)/(D_2 + 2)]\bar{V}_2 = \bar{V}_2[(3)/(D_2 + 2)] \qquad \text{(Eq. 6)}$$

where $\bar{V}_1$ and $\bar{V}_2$ are the molar volumes and D_1 and D_2 are the dielectric constants of the nonpolar and polar components of the binary solvent system, respectively.

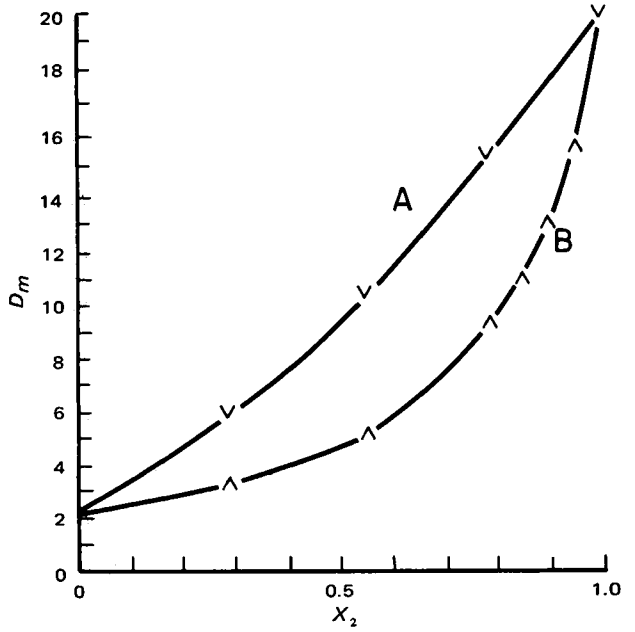

Figure 1—*Relationship of the dielectric constant* (D_m) *and the mole fraction of acetone* (X_2) *for the benzene–acetone binary solvent system. A is the experimental curve, and B is the theoretical curve.*

Table I—Theoretical Values of a, b, c, and d for Benzene–Acetone and Benzene–Methyl Isobutyl Ketone Binary Solvent Systems

Coefficient	Benzene–Acetone	Benzene–Methyl Isobutyl Ketone
a	142.40	142.40
b	201.80	325.50
c	61.90	61.90
d	9.64	25.25

EXPERIMENTAL

Benzene was selected as the nonpolar component of the nonaqueous binary solvent system. The polar component was methyl isobutyl ketone in one system and acetone in the other. These reagents, of the highest purity grades available, were used without further purification.

The benzene–methyl isobutyl ketone solvent system had a dielectric constant range of 2.29 for pure benzene to 12.80 for pure methyl isobutyl ketone (3). The dielectric constant range for the benzene–acetone solvent system was 2.29 for pure benzene to 20.97 for pure acetone. The Heterodyne-Beat apparatus and procedure (2) were employed in determining the dielectric constants of the different mixtures of the two binary solvent systems. All measurements were performed at 25°.

RESULTS AND DISCUSSION

The theoretical values for the coefficients of Eq. 2 are calculated from the molar volumes ($\bar{V}_1$ and $\bar{V}_2$) and dielectric constants (D_1 and D_2) of the pure solvents. The $\bar{V}_1$ and $\bar{V}_2$ values are readily determined from the molecular weight and the density of the pure solvents. The D_1 and D_2 values were determined experimentally and approximated those reported in the literature (3).

The theoretical values of a, b, c, and d for benzene–acetone and benzene–methyl isobutyl ketone are reported in Table I. By using these values and Eq. 2, the theoretical dielectric constant of any mixture at any given mole fraction of the two components can be calculated. These values are reported in Table II.

Mixtures of definite composition were prepared, and their dielectric constants were measured. The results for benzene–acetone and benzene–methyl isobutyl ketone are also reported in Table II. The theoretical and experimental values for the benzene–acetone binary solvent system are plotted in Fig. 1. The experimental curve shows definite deviations from the theoretical (the same is observed when the results for the benzene–methyl isobutyl ketone

Table II—Theoretical, Experimental, and Computer-Calculated Values of D_m for Benzene–Acetone and Benzene–Methyl Isobutyl Ketone Binary Systems

	Benzene–Acetone D_m				Benzene–Methyl Isobutyl Ketone D_m		
X_2	Theoretical	Experimental	Computer Calculated[a]	X_2	Theoretical	Experimental	Computer Calculated
0.000	2.30	2.30	2.19	0.000	2.30	2.30	2.27
0.284	3.39	5.83	5.85	0.190	3.22	4.28	4.23
0.549	5.24	10.42	10.20	0.420	4.72	6.66	6.63
0.784	9.37	15.65	15.14	0.690	7.33	9.59	9.50
0.850	11.02	—	—	0.800	8.88	—	—
0.900	13.18	—	—	0.900	10.62	—	—
0.950	16.19	—	—	0.950	11.67	—	—
1.000	20.90	20.90	20.98	1.000	12.89	12.90	12.86

[a] Based on computer-determined values of the coefficients. (See Ref. 2.)

system are plotted), because the ideal conditions under which the relationship was derived were not satisfied in real mixtures.

For example, the molar volumes of the two solvents change upon mixing. The electrical forces of the surrounding molecules exert an appreciable effect on each other. Consequently, the molar polarizations of the solvents also change upon mixing. Hence, the D_m values calculated from theoretical values of a, b, c, and d are significantly different from the experimentally determined D_m values. Therefore, it becomes necessary to measure the D_m values experimentally and subject them, along with the X_1 and X_2 values, to computer analysis to determine the values of the coefficients that will produce the best fit of the relationship to the experimental data.

The best values of the a, b, c, and d coefficients were determined by computer analysis (2) based on a series of laboratory experiments. The dielectric constant for any mixture of the benzene–acetone or the benzene–methyl isobutyl ketone binary solvent system can thus be predicted with the use of these values and Eq. 2. As reported in Table II, the predicted values (computer calculated) for D_m compare favorably with the experimentally determined values. This finding supports the usefulness of Eq. 2 for predicting D_m values of the binary solvent systems of the type studied here.

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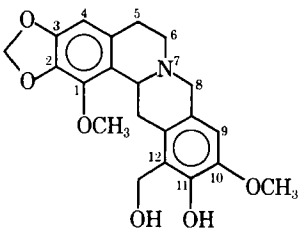
Alkaloids of *Papaver orientale* and *Papaver pseudo-orientale*

A. SHAFIEE [x], I. LALEZARI, P. NASSERI-NOURI *, and R. ASGHARIAN *

Abstract □ Dry latex of *Papaver orientale* was shown to contain 20% oripavine and 9% thebaine. Dry latex of *Papaver pseudo-orientale* contained two major alkaloids, isothebaine (11.7%) and orientalidine (0.5%), and the minor alkaloids bracteolin, salutaridine, Or_1, Or_2, PO-4, alborine (PO-5), and a novel alkaloid aryapavine.

Keyphrases □ *Papaver* alkaloids—isolation and identification from *P. orientale* and *P. pseudo-orientale* □ Aryapavine—isolation, identification from *Papaver pseudo-orientale*

As part of a broad study of the *Papaver* genus in Iran (1–3), the chemotaxonomy of *Papaver bracteatum*[1], *Papaver orientale*[1], and *Papaver pseudo-orientale*[1] has been studied.



I

Contradictory results exist in the literature with regard to the alkaloids of *P. bracteatum* and *P. orientale*. In addition to thebaine, other alkaloids have been reported in *P. bracteatum* (4–7). However, the present authors and others have demonstrated the existence of only thebaine (1, 3, 8) or a small amount of alpinigenine in addition to thebaine (2, 9–11) in *P. bracteatum*. Similar contradictory results have been reported for *P. orientale*. In addition to isothebaine,

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Table II—Theoretical, Experimental, and Computer-Calculated Values of D_m for Benzene–Acetone and Benzene–Methyl Isobutyl Ketone Binary Systems

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A. SHAFIEE [x], I. LALEZARI, P. NASSERI-NOURI *, and R. ASGHARIAN *

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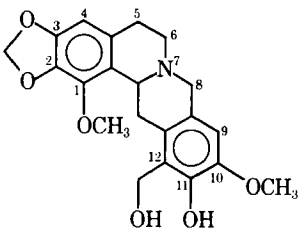

I

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thebaine has been reported to exist in *P. orientale* (12). Some workers found oripavine in *P. orientale* (13, 14), while others reported the same alkaloid in *P. bracteatum* (4). These results suggest that the previous alkaloid accounts are unreliable with respect to species determination.

With the objective of clarifying present uncertainties, the authors undertook the study of the alkaloids of these species, keeping in mind the taxonomic studies of these plants.

Three species of section oxytona were classified according to their haploid chromosome number: *P. bracteatum*, $n = 7$; *P. orientale*, $n = 14$; and *P. pseudo-orientale*, $n = 21$[2].

In the present work, the alkaloid content of the dry latex of *P. orientale* and *P. pseudo-orientale* is reported. These wild plants grow in the mountains in north and northwest Iran.

EXPERIMENTAL[3]

Extraction of Alkaloids from *P. orientale*.—Dried latex (2 g), obtained by incision of the capsules of the plant growing in Takab (northwest Iran), was treated with dilute ammonia to obtain a soft paste and extracted by trituration with chloroform (3 × 20 ml). The extract was filtered and evaporated under reduced pressure. The residue (0.64 g) was chromatographed (TLC, silica gel), using ethyl acetate–methanol–ammonia (85:10:5) as the eluting solvent. The fractions were worked up and crystallized from appropriate solvents.

Extraction of Alkaloids from *P. pseudo-orientale*.—Dried latex (100 g), naturally excreted (as flakes) from capsules during maturation, was worked up as described for *P. orientale*. The residue obtained from the evaporation of chloroform was subjected to column chromatography on silica gel using chloroform–petroleum ether (50:50) as the eluting solvent at first and then gradually increasing the chloroform proportion. Finally, a mixture of chloroform–methanol (95:5) was used, and the proportion of methanol was gradually increased up to 50%. The fractions were further purified by TLC [ether–acetone–diethylamine (85:8:7)] and crystallized.

RESULTS AND DISCUSSION

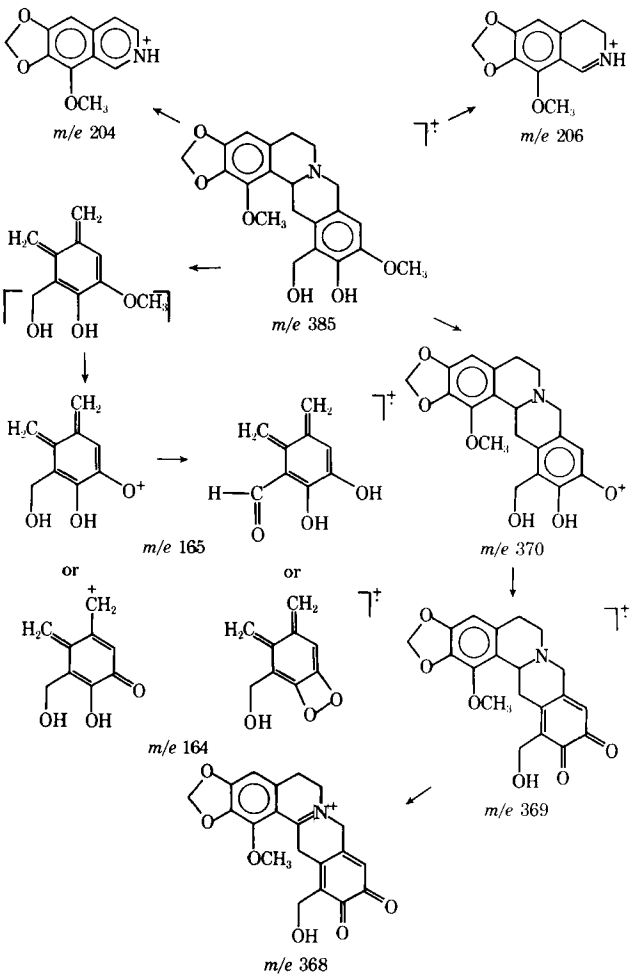
Alkaloids of *P. orientale*.—Two alkaloids were isolated and crystallized:

1. Oripavine (0.45 g, 20%), mp 201–203° (ethanol) [lit. (14) mp 200–201°]. The UV, IR, and NMR spectral properties of the separated alkaloid were identical with those of the authentic sample.
2. Thebaine (0.18 g, 9%), mp 193° (ethanol) [lit. (1–3) mp 193°].

Other samples of the plants collected from different regions of northwest Iran also had oripavine and thebaine; however, in one case, plants collected from Khalkhal (northwest Iran) had trace amounts of isothebaine in addition to oripavine and thebaine.

Alkaloids of *P. pseudo-orientale*.—The following alkaloids were characterized from the extensive studies of the dried latex:

1. Orientalidine, crystallized from ether (0.5 g, 0.5%), mp 192–195° [lit. (15, 16) mp 192–195°].
2. Isothebaine, crystallized from ethanol (11.7 g, 11.7%), mp 203–205° [lit. (15) mp 203–204°].
3. Bracteolin, crystallized from ether, mp 228–230° [lit. (15) mp 226–229°].
4. Salutaridine, crystallized from ether, mp 197–199° [lit. (15) mp 196–198°].
5. Or_1, crystallized from petroleum ether, mp 106–107° [lit. (15) mp 109°].



Scheme I

6. Or_2, crystallized from petroleum ether, mp 120–121° [lit. (15) mp 124–127°]; UV λ_{max} (ethanol): 285 (log ϵ 3.27) and 226 (log ϵ 3.12) nm; NMR ($CDCl_3$): δ 6.63 (s, 1H, aromatic), 3.98 (s, 3H, OCH_3), 3.43 (s, 3H, OCH_3), and 2.47 (s, 3H, NCH_3) ppm. The mass spectrum had a molecular ion at 333.
7. PO-4 (16), crystallized from acetone–ether, mp 300° [lit. (17) mp 300°].
8. Alborine (PO-5) (16), crystallized from methanol–ether, mp 300° [lit. (17) mp 300°].

The spectral data (UV, IR, NMR, and mass) of these alkaloids were identical with those reported in the literature (1–3, 15–17).

In addition to these alkaloids, a new alkaloid was also isolated, 11-demethylmecambridine (aryapavine) (I), mp 156–157° (acetone); UV λ_{max} (ethanol): 287 nm (log ϵ 3.87); NMR ($CDCl_3$): δ 6.62 (s, 1H, H_4), 6.43 (s, 1H, H_9), 5.97 (s, 2H, OCH_2O), 4.80 (s, 2H, CH_2O), 4.07 (s, 3H, OCH_3), 3.93 (s, 3H, OCH_3), and 3.90–2.20 (m, 9H, aliphatic) ppm; IR (KBr): 3400 cm^{-1} (broad, hydrogen bonded hydroxy). The mass spectrum had peaks at *m/e* (%) 385 (M, 70), 370 (77), 369 (85), 368 (63), 206 (100), 204 (77), 165 (55), and 164 (77). Its empirical formula was $C_{21}H_{23}NO_6$. These data were in agreement with Structure I for this alkaloid.

Anal.—Calc. for $C_{21}H_{23}NO_6$: C, 65.45; H, 5.97; N, 3.64. Found: C, 65.58; H, 6.02; N, 3.78.

The electron fragmentation pattern of this compound (Scheme I) was in agreement with the suggested structure. This pattern was similar to that of mecambridine (18). The spectral data of this alkaloid (I) were identical with those of demethylmecambridine as synthesized recently (19).

[2] P. Goldblat, Missouri Botanical Garden, St. Louis, Mo., personal communication.

[3] Melting points were taken with a Koffler hot-stage microscope and are uncorrected. UV spectra were recorded on a Varian Techtron 635 instrument. NMR spectra were taken with a Varian A60A instrument, using tetramethylsilane as the internal standard. Mass spectra were recorded on a CH5 spectrometer. IR spectra were obtained with a Leitz model III spectrograph.

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* Research assistant on a grant supported by the Iranian Ministry of Sciences and the U.S. Department of Agriculture.

ˣ To whom inquiries should be directed.

Antitumor Agents XV: Deoxyelephantopin, an Antitumor Principle from *Elephantopus carolinianus* Willd.

KUO-HSIUNG LEE ˣ, CAROLE M. COWHERD *, and MacARTHUR T. WOLO

Abstract □ The active principle responsible for the significant inhibitory activity against the Walker 256 carcinosarcoma (ascites) in rats, isolated from the chloroform extract of the whole plant of *Elephantopus carolinianus* Willd., was characterized as deoxyelephantopin, a sesquiterpene lactone.

Keyphrases □ *Elephantopus carolinianus* Willd.—isolation and structure identification of deoxyelephantopin, antitumor activity □ Deoxyelephantopin—isolation from *Elephantopus carolinianus* Willd., structure identification, antitumor activity □ Antitumor agents, potential—deoxyelephantopin, major constituent of *Elephantopus carolinianus* Willd. □ Structure–activity relationships—sesquiterpene lactones (deoxyelephantopin) as antitumor agents

As a result of the continuing search for *Elephantopus* principles having new and novel potential antitumor agents (1, 2)[1], the chloroform extract of the whole plant of *Elephantopus carolinianus* Willd. (Compositae) was found to show significant inhibitory activity against the Walker 256 carcinosarcoma (ascites) in rats[2]. The extraction of the active principles was carried out according to an exact procedure described in the literature (4). A preliminary examination of the chloroform extract revealed that the antitumor activity was associated with the presence of the terpene-like constituents. Fractionation, characterization, and screening[2] of the isolated compound led to the conclusion that this major active principle was the germacranolide dilactone, deoxyelephantopin (I).

DISCUSSION

Deoxyelephantopin (I), the antitumor principle, was isolated in 0.15% yield as colorless needles from the chloroform eluate. Deoxyelephantopin, mp >320° (sintered at 198–200°)[3], has the empirical formula $C_{19}H_{20}O_6$ as determined by high-resolution mass spectrometry[4]. It shows IR bands in chloroform[5] at 1760 (double strength) and 1650 cm^{-1} and a pair of low field doublets in the NMR spectrum ($CDCl_3$)[6] at δ 6.22 (1H, d, J = 3.5 Hz, H-13) and 5.64 (1H, d, J = 3.5 Hz, H-13)[7], characteristic of an α-methylene-γ-lactone ring system.

An IR band at 1710 cm^{-1} and a characteristic base peak in the mass spectrum of I at m/e 69 suggested the presence of a methacrylate ester side chain, and the NMR signals at δ 1.93 (3H, t, J = 1.0 Hz, H-18), 5.66 (1H, t, J = 1.0 Hz, H-19)[7], and 6.14 (1H, t, J = 1.0 Hz, H-19), which are comparable with those found in molephantin (1) and phantomolin (2), established the presence of this structural feature. The NMR spectrum of I showed, in addition to the well-defined one-proton doublet of doublets (δ 5.13, J = 10.0

[1] See K. H. Lee, T. Ibuka, H. C. Huang, and D. L. Harris, *J. Pharm. Sci.*, **64**, 1077(1975).

[2] *In vivo* antitumor activity was assayed by Dr. I. H. Hall, Department of Medicinal Chemistry, School of Pharmacy, University of North Carolina at Chapel Hill, using a literature method (3). Deoxyelephantopin showed significant inhibitory activity against the Walker 256 (ascites) carcinosarcoma in rats at 226% T/C at the 2.5-mg/kg level. A compound is active if it exhibits a T/C of ≥125% (3).

[3] Reference 5 reported mp 198–200°.

[4] The authors thank Dr. David Rosenthal and Mr. Fred Williams of the Research Triangle Center for Mass Spectrometry for mass spectral data.

[5] This IR absorption band shifted to 1755 and 1738 cm^{-1} in mineral oil. Reference 5 reported 1766 and 1747 cm^{-1} in KBr.

[6] The authors thank Dr. David L. Harris of the Department of Chemistry, University of North Carolina at Chapel Hill, for NMR measurements. The model XL-100 used for the NMR measurements was purchased from grants from the National Science Foundation and the National Institutes of Health to the Department of Chemistry, University of North Carolina at Chapel Hill.

[7] Partially overlapped with H-19. Reference 5 reported H-13 and H-19 as a multiplet at δ 5.64.

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* Research assistant on a grant supported by the Iranian Ministry of Sciences and the U.S. Department of Agriculture.

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Antitumor Agents XV: Deoxyelephantopin, an Antitumor Principle from *Elephantopus carolinianus* Willd.

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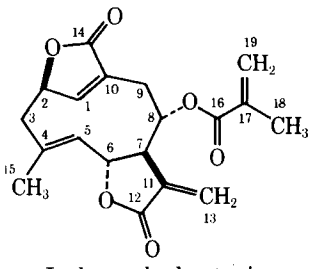
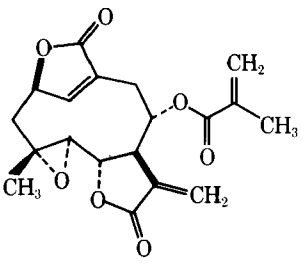


I: deoxyelephantopin



II: elephantopin

and 8.0 Hz) for the proton at C-6 (showing the *trans*-disposition of the lactone fusion) (6), a second vinyl methyl group, seen as a doublet, slightly split by allylic coupling (J = 1.0 Hz) at δ 1.93 (3H). These observations are indicative of the relationship of the protons at C-15, C-5, C-6, and C-7 of I.

The absence of the hydroxy groups and the presence of a strong carbonyl absorption band at 1760 cm^{-1} in the IR spectrum, coupled with the fact that the NMR spectrum of I showed a broad, low field, one-proton singlet at δ 7.13 corresponding to a vinyl proton beta to carbonyl, indicates that the remaining two oxygens must form a second α,β-unsaturated γ-lactone ring.

After treatment with *m*-chloroperbenzoic acid in chloroform, deoxyelephantopin gave a 4,5-epoxide (II: $C_{19}H_{20}O_7$; mp 263–265°[8]) whose spectral data were all in accord with the assigned Structure II. The identity of this compound with elephantopin, a tumor inhibitor isolated from *Elephantopus elatus* (7), was established by comparative TLC and NMR (dimethyl sulfoxide-d_6) spectral analyses[9].

The foregoing evidence led to the conclusion that I was deoxyelephantopin, a germacranolide dilactone which had been isolated from *Elephantopus scaber* (5). To establish the identity of the germacranolide beyond doubt[10], the IR (KBr), NMR, and mass spectra of I were compared with those of deoxyelephantopin. Studies on the structure–activity relationships among deoxyelephantopin-related sesquiterpene lactones are currently in progress.

EXPERIMENTAL[11]

Extraction of *E. carolinianus* Willd.—The *E. carolinianus* (Compositae) used was from a collection made on Oct. 13, 1972, along Flat River at Rt. 1005 in Durham County, N.C.[12]. The ground, air-dried, whole plant material (735 g) was exhaustively extracted with chloroform and worked up in the usual manner (4, 8), affording 18.4 g of a dark-brown syrup.

Isolation of Deoxyelephantopin (I)—The syrup was chromatographed on silica gel (4 × 65 cm) with elution with benzene, chloroform, and acetone. Twenty-four fractions of about 100 ml each were collected and examined by TLC. The first benzene eluate (fractions 1–6) contained only traces of low melting waxes. The subsequent chloroform eluate (fractions 8–16) contained mainly material giving a single spot on TLC. Upon removal of the chloroform solvent, these fractions gave an oily residue which crystallized upon addition of anhydrous ether. One recrystallization from chloroform yielded 974 mg of I as colorless needles, mp > 320° (sintered at 198–200°)[3]. The identity of this compound with deoxyelephantopin was established by their equivalent IR, NMR, and mass spectral data as already described.

Anal.—Calc. for $C_{19}H_{20}O_6$: m/e 344.1260. Found: m/e 344.1256.

The acetone eluate (372 mg) obtained from fractions 17–24 afforded a mixture which, upon rechromatography on silica gel[13] (70–325 mesh, 1.5 × 15 cm, eluted with chloroform), yielded mainly additional deoxyelephantopin (100 mg) (total yield 0.15%) and very small amounts of polar substances whose structures are now under investigation.

Epoxidation of Deoxyelephantopin (I) to Yield Elephantopin (II)—A solution of I (40 mg) in chloroform (1 ml) was treated with a solution of *m*-chloroperbenzoic acid (50 mg) in chloroform (1 ml), and the mixture was allowed to stand at room temperature overnight. Then the solution was washed with 10% sodium sulfite, 5% sodium bicarbonate, and water, dried, and evaporated *in vacuo* to give a crystalline residue. Silica gel column chromatography (0.6 × 10 cm, eluted with chloroform), followed by recrystallization from methylene chloride–absolute ethanol, yielded II as colorless needle tufts, mp 263–265°[14]. The identity of this compound with an authentic sample of elephantopin was confirmed by TLC and superimposable NMR spectra (in dimethyl sulfoxide-d_6).

Anal.—Calc. for $C_{19}H_{20}O_7$: m/e 360.1209. Found: m/e 360.1200.

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ˣ To whom inquiries should be directed.

[8] Reference 7 reported mp 262–264°.

[9] The authors thank Professor S. M. Kupchan, Department of Chemistry, University of Virginia, Charlottesville, Va., for providing the specimen as well as a copy of the NMR spectrum of elephantopin for comparison.

[10] The authors thank Professor Tadashi Kurokawa of the Department of Bacteriology, School of Medicine, Tohoku University, Sendai, Japan, for providing copies of the IR, NMR, and mass spectra of deoxyelephantopin for comparison.

[11] Unless otherwise specified, melting points were determined on a Thomas-Hoover melting-point apparatus and are corrected. IR spectra were determined in chloroform with a Perkin-Elmer 710 A grating IR spectrophotometer. NMR spectra were measured in $CDCl_3$ with a Varian XL-100 NMR spectrometer using tetramethylsilane as an internal standard. Mass spectra were determined on an A.E.I. MS-902 instrument at 70 ev using a direct inlet system. Silica gel for column chromatography refers to Baker A.R. No. 3405, and silica gel for TLC refers to Merck silica gel G developed with chloroform–acetone (3:1) and visualized by spraying with concentrated sulfuric acid and heating.

[12] The authors thank Mr. S. W. Leonard, Costal Zone Resources Corp., Wilmington, N.C., for collecting and identifying the plant material. A voucher specimen has been placed in the herbarium of the Department of Botany, University of North Carolina at Chapel Hill.

[13] Merck.

[14] Reference 7 reported mp 262–264°.

Reduction of Doxorubicin (Adriamycin) Bone Marrow Toxicity

PHILLIP A. HARRIS ˣ, AGNES S. GARAI, and MARIA A. VALENZUELA

Abstract □ Doxorubicin (adriamycin), an antineoplastic antibiotic, is a potent suppressant of bone marrow. Previous studies on doxorubicin disposition indicated that its diversion from bone marrow in the first few minutes after administration should result in a marked decrease in total exposure to the drug (concentration × time) with a concomitant reduction in concentration–time-dependent toxicity. To test this hypothesis, the descending aorta of rabbits was occluded just proximal to the iliac bifurcation for 30 min to deprive bone marrow of blood flow. Both these rabbits and the control rabbits were given 5 mg/kg of doxorubicin intravenously, and the total white cell count in peripheral blood was monitored periodically for 15 days. The decrease in toxicity produced by the occlusion was quite evident by comparison of white cell counts and deaths in all groups. A possible mechanism of this effect was shown to be a decreased doxorubicin exposure of bone marrow tissue in the occluded animals as judged by relative doxorubicin concentration–time curves in rabbits with and without the aortic occlusion.

Keyphrases □ Doxorubicin (adriamycin)—reduction of bone marrow toxicity, aortic occlusion, rabbits □ Adriamycin—reduction of bone marrow toxicity, aortic occlusion, rabbits □ Toxicity, bone marrow—doxorubicin, reduction by aortic occlusion technique, rabbits □ Antineoplastic agents—reduction of doxorubicin bone marrow toxicity, aortic occlusion, rabbits

Doxorubicin (adriamycin) is a valuable chemotherapeutic drug in the treatment of solid neoplasms (1, 2). The serious toxicity of doxorubicin includes depression of proliferative elements of bone marrow as well as a mucositis and a cumulative dose-dependent delayed myocardiopathy (1–3). In short-term therapy with doxorubicin, the bone marrow suppression with the resultant leukopenia is the major dose-limiting toxicity. Recent pharmacokinetic investigations in these laboratories[1] led to the conclusion that the bone marrow toxicity of doxorubicin may be decreased by a clinically feasible method, albeit not yet proven acceptable or desirable.

This paper concerns the reduction of doxorubicin bone marrow toxicity and its probable mechanism by a temporary restriction of bone marrow blood flow.

EXPERIMENTAL

Female New Zealand white rabbits, 2.0–3.0 kg, were anesthetized with pentobarbital sodium USP[2], 25 mg/kg iv. A lower midline laparotomy was performed, and the aorta was occluded just proximal to the iliac bifurcation with a bulldog clamp. Doxorubicin hydrochloride[3] was administered in a dose of 5 mg/kg into the marginal ear vein immediately after the aorta was occluded. The clamp was removed after 30 min, and the incision was closed. Three hundred thousand units of penicillin G benzathine suspension[4] was administered intramuscularly to prevent possible infection due to surgery.

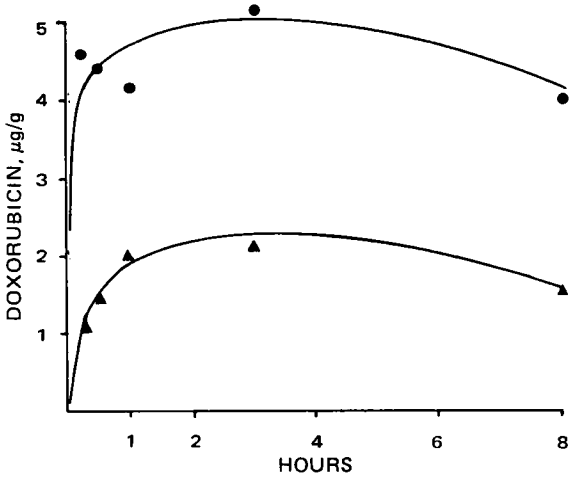

Figure 1—*Femur bone marrow concentrations after doxorubicin given intravenously, 3 mg/kg, in rabbits with (▲) and without (●) aortic occlusion. Each point represents the mean of duplicate determinations in one rabbit.*

Two control groups of animals were treated in the following manner. One group underwent surgery as described with aortic occlusion and penicillin therapy, but doxorubicin was not administered. The second group underwent the surgery, identical handling, trauma, and penicillin therapy as before and received 5 mg/kg of doxorubicin but the aorta was not occluded.

Blood samples were taken from all rabbits prior to and periodically after surgery, and the total white blood cell count was monitored until death or up to 15 days after surgery. The blood counts were performed[5] on a blind basis.

For studies of doxorubicin uptake into bone marrow, the aorta of each of five rabbits was occluded for 30 min as already described and doxorubicin was given intravenously into the marginal ear vein in a dose of 3 mg/kg immediately after the clamp was applied. Rabbits were killed at 15, 30, 60, 180, and 480 min after injection; bone marrow was removed from the femur and analyzed for doxorubicin levels according to a previously published method (4). Five control rabbits were treated similarly, except the aorta was not occluded.

RESULTS AND DISCUSSION

The effect of temporary reduction of blood flow to a major blood cell-producing tissue on doxorubicin toxicity was tested. Occlusion of all blood flow through the aorta at the level of the iliac bifurcation for 30 min was performed to accomplish a reduction or a near complete halt in blood flow to the pelvis and hindlimbs.

A group of five rabbits received 5 mg/kg of doxorubicin immediately after the aorta was occluded. For controls, three rabbits underwent aortic occlusion without administration of doxorubicin and another five rabbits that had no aortic occlusion received 5 mg/kg of doxorubicin. The total white cell count was monitored in all animals (Table I).

Aortic occlusion with no doxorubicin administration had no significant effect on the white blood cell count of the rabbits. When doxorubicin was administered in a dose of 5 mg/kg with no aortic occlusion, four out of the five rabbits died. As can be seen from the

[1] *Cancer Chemother. Rep.*, in press.
[2] Obtained from Western Medical Supply, Inc., Arcadia, Calif.
[3] Supplied by Dr. Harry Wood, Drug Development Branch, National Cancer Institute, Bethesda, Md.
[4] Bicillin L-A, Wyeth Laboratories, Philadelphia, Pa.
[5] By the clinical laboratory of the University of Southern California Medical School Vivarium.

Table I—Total White Cell Counts in Rabbits

Day Post-surgery	Rabbit Number 1	2	3	4	5	6	7	8	9	10	11	12	13
	5 mg/kg Doxorubicin with Aortic Occlusion					5 mg/kg Doxorubicin without Aortic Occlusion					No Doxorubicin with Aortic Occlusion		
0	7,800	7,800	8,400	10,800	11,500	10,100	8,900	9,200	10,500	9,300	9,600	8,400	8,300
1	11,400	7,800	8,400	10,700	11,300	7,800	7,400	8,100	9,600	12,500	—	—	—
2	6,400	5,500	7,700	10,300	11,300	4,600	3,600	6,700	6,600	5,200	11,900	8,500	7,700
3	6,300	4,800	6,800	9,900	9,500	2,700	2,900	3,300	3,200	3,000	—	—	—
4	5,200	4,600	4,500	9,500	7,800	1,600	2,100	2,200	1,800	2,200	—	—	—
5	4,900	3,800	2,600	8,800	6,000	500	900	3,800	Died	1,200	8,000	7,900	6,800
6	5,200	4,200	3,200	8,200	6,200	Died	Died	3,900	—	Died	—	—	—
7	5,200	5,400	4,200	8,200	6,200	—	—	4,400	—	—	—	—	—
8	4,800	5,600	3,800	7,800	6,400	—	—	3,400	—	—	9,400	6,600	8,400
9	5,400	5,700	5,400	8,100	6,600	—	—	4,800	—	—	—	—	—
10	6,200	6,400	6,200	8,200	6,600	—	—	5,600	—	—	—	—	—
11	—	—	6,000	8,000	6,600	—	—	—	—	—	8,800	7,000	9,200
12	6,800	6,900	6,600	8,300	7,900	—	—	6,500	—	—	—	—	—
13	—	—	—	—	—	—	—	—	—	—	—	—	—
14	—	—	—	—	—	—	—	—	—	—	—	—	—
15	7,600	7,400	7,800	9,200	9,200	—	—	6,800	—	—	8,900	7,500	9,200

total white cell count, death was probably due to severe bone marrow depression. The fifth rabbit experienced a moderate depression in white cell count. The rabbits that underwent aortic occlusion and received 5 mg/kg of doxorubicin had only mild or moderate bone marrow depression. None of these animals died.

The rabbits were all in excellent health when experiments were commenced. A transient body weight loss of 100–200 g was observed after surgery. No other changes in their clinical condition were observed except for the sudden death of four out of the five rabbits without aortic occlusion. Although the exact cause of death was not determined, a fatal septicemia was strongly suspected because of the leukopenic pattern noted before death.

The results of experiments to determine the effect of 30 min of occlusion on femur marrow uptake of doxorubicin are shown in Fig. 1. Data from rabbits without occlusion that received 3 mg/kg of doxorubicin are also shown. The rabbits with occlusion had an area under the concentration–time curve up to 8 hr of 923 μg min/ml compared to 2235 μg min/ml for those without occlusion. Both areas correlate to the respective smooth curves that were estimated by sight.

Doxorubicin uptake into tissues occurs rapidly (5–8), and the binding to tissues is extremely tenacious[1]. The pelvic and hindlimb bone marrow which normally receive blood flow distal to the occlusion do not receive a normal supply of doxorubicin for 30 min after injection because of blood flow reduction. During this time the doxorubicin becomes "fixed" to other tissues.

When the clamp is released and more normal blood flow resumes, the doxorubicin plasma concentration is very low. Hence, the marrow concentrations of doxorubicin do not reach values encountered without occlusion up to 8 hr after drug administration. The exposure of the marrow based on relative concentration–time areas is only 41% that without occlusion. There is little reason to expect this relationship to change significantly after 8 hr since the marrow concentrations are well past their maximums and the drug should decay from this tissue at similar rates in both cases.

Close examination of marrow levels in the occluded animals indicates that blood flow to the femur marrow was not completely halted during occlusion. The 15- and 30-min points had significant doxorubicin concentrations. There would not, of course, be any doxorubicin in this tissue at these times if flow were stopped completely. Perhaps the clamp on the aorta was not well placed, thus allowing some aortic flow to proceed distally to the femur marrow. It is also possible that some collateral blood vessels supply the femur marrow and receive flow from a point higher than the clamp was placed.

The dose levels selected were based on experiments in this laboratory. A 3-mg/kg doxorubicin dose in rabbits may be given every 7–10 days generally without fatality. The white cell counts are variably depressed at this dose. This level and frequency of doxorubicin would be a likely treatment regimen in rabbits. Therefore, this dose was selected for the uptake studies.

A single 5-mg/kg dose produces a much more profound and less variable drop in white cell count than does a 3-mg/kg dose. For this reason, a 5-mg/kg dose was used for studying the effect of aortic occlusion on white cell count.

Occlusion of the abdominal aorta in humans was used previously to increase tumor exposure to nitrogen mustard (mechlorethamine hydrochloride) (9, 10). The bone marrow of the lower part of the body of rats and rabbits was demonstrated to be protected by temporary occlusions of the abdominal aorta after nitrogen mustard administration (11). Nitrogen mustard has a very short $T_{1/2}$ in the body since it is so reactive. A significant proportion of nitrogen mustard was irreversibly eliminated from the blood during the period the occlusion was applied. Hence, the tissues distal to the occlusion receive protection.

Temporary occlusion of the abdominal aorta is used to control hemorrhage in emergency treatment or during elective aortic surgery. The occlusion may be accomplished by directly clamping the aorta after entering the abdominal cavity or by using external abdominal tourniquets or compression. The use of catheters equipped with inflatable sleeves or balloons for obstructing the aorta was described and evaluated (12–14). These catheters may be introduced by peripheral arterial catheterization.

The metabolic and hemodynamic sequel to abdominal aortic occlusion and declamping also was described (15–17). The main problems appear to be the frequent occurrence of acidosis and severe hypotension and occasionally ventricular fibrillation or cardiac arrest after release of the aortic blood flow. The control and prevention of the shock may be accomplished by fluid therapy (17, 18) or use of vasopressor drugs regionally (19).

Clearly, the results of the total white cell count and doxorubicin bone marrow uptake experiments reported here demonstrate that a significant reduction in the drug exposure of bone marrow and in leukopenic response after doxorubicin administration may be accomplished by only 30 min of aortic occlusion at the iliac bifurcation. These results may have clinical significance in the use of doxorubicin since leukopenia often delays doxorubicin therapy. Also, combination therapy with other marrow toxic drugs or radiation may be more flexible using this approach.

A more complete study should be performed to determine the clinical feasibility, safety, and mechanism of the reduction of doxorubicin toxicity by temporary blood flow restriction.

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COMMUNICATIONS

Relative Bioavailability of Cyclobarbital Calcium from Aqueous Solution Compared to Tablets

Keyphrases □ Cyclobarbital calcium—relative bioavailability from aqueous solution and tablets □ Bioavailability—cyclobarbital calcium, aqueous solution and tablets

To the Editor:

For biopharmaceutical studies, the aqueous drug solution is generally recommended as the reference dosage form if no comparison can be made with intravenous data (1). The highest relative bioavailability of a drug is expected from the solution since dissolution of the drug in the GI tract seems to be bypassed. The present report on cyclobarbital calcium shows that this supposition is not always true.

The pharmacokinetics and relative bioavailability of cyclobarbital calcium were studied in six healthy volunteers (21–26 years of age). Three preparations were used:

1. Tablets[1], containing 200 mg of cyclobarbital calcium, 60 mg of potato starch, 27 mg of lactose, 7 mg of talc, 3 mg of magnesium stearate, and gelatin *q.s.*
2. Tablets[2], containing 200 mg of cyclobarbital calcium.
3. Aqueous solution of cyclobarbital calcium (300 mg/150 ml), which was prepared within 15 min prior to administration.

After fasting overnight, at 9 am the volunteers were given 300 mg of cyclobarbital calcium incorporated in one of the preparations together with 150 ml of water. Additional water was not supplied when cyclobarbital calcium was given in solution. The volunteers remained in an upright position for 15 min after intake of the drug and then rested supine for at least 3.5 hr.

Blood samples were taken at 0.15 or 0.33, 0.66, 1.0, 1.5, 2.0, 2.5, 3.0, 5.0, 8.0, 12, 24, and 32 hr following drug administration. Each volunteer received the three preparations in a random sequence, with an interval of at least 1 week. Cyclobarbital plasma concentrations were determined after a single extraction step by GC with nitrogen selective detection, following on-column methylation with trimethylanilinium hydroxide. The use of a nitrogen detector for the sensitive assay of barbiturates in biological fluids was described previously for hexobarbital (3).

In Table I the cyclobarbital plasma peak concentrations and times (mean values) are given, as well as the bioavailability relative to Preparation 1. Relative bioavailability was estimated for each individual by comparing the areas under the experimental plasma concentration curves and correcting for the undetermined area to infinity. The elimination of cyclobarbital followed first-order kinetics with an average half-life of 11.6 hr (range of 8–17 hr). The half-lives were fairly constant within the individuals during the three trials.

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Table I—Plasma Peak Concentrations and Relative Bioavailability of Cyclobarbital following Oral Administration of the Three Cyclobarbital Calcium Preparations

Preparation	Mean t_{max} (Range), hr	Mean C_{max} (Range), mg/liter	Mean F_{rel} (Range), %
1. Tablets	1.2 (0.33—3.0)	8.3 (7.3—10.8)	100
2. Tablets	1.2 (0.66—2.5)	8.7 (7.4—10.3)	101 (89—136)
3. Aqueous solution	0.7 (0.25—1.5)	8.3 (5.8—11.7)	78 (64—100)

Although substantial variations in absorption rate and relative bioavailability were observed between individuals (see range values), the rank order of these values for the three preparations was generally the same for each volunteer. For one subject, absorption was comparatively slow (t_{max} of 3.0, 2.5, and 1.5 hr for Preparations 1, 2, and 3, respectively), but still the drug was absorbed more rapidly and to a lesser extent when given in aqueous solution.

The present findings clearly indicate that the extent of bioavailability of cyclobarbital in humans is lower when its calcium salt is administered in aqueous solution. This finding is unexpected, whereas the highest absorption rate is in accordance with the usual behavior of an oral aqueous drug solution. It may be argued that after oral administration of barbiturate salts, precipitation of the poorly soluble free acid will occur as soon as the acidic medium of the stomach is reached. This precipitation, however, will also occur for the calcium salt incorporated in the tablets. The precipitate formed after administration of the aqueous solution may have unfavorable redissolution properties in comparison to the precipitate formed after tablet administration. If so, this situation only holds for part of the precipitate, since the rapid absorption of cyclobarbital calcium solution also suggests that rapid (re)dissolution occurs.

A similar observation was made in a separate study when comparing absorption of heptabarbital as free acid with heptabarbital sodium, both from solid dosage forms. The salt showed the highest absorption rate; however, bioavailability relative to the free acid was approximately 20% lower (4). Higuchi *et al.* (5) showed that, in general, salts show higher dissolution rates than the corresponding nonionic drug at any pH, even though the final equilibrium solubility of the drug and its salt is the same. Additional *in vitro* experiments with cyclobarbital calcium are required to study the influence of precipitate formation on the redissolution characteristics of cyclobarbital under acid and weak alkaline conditions.

Although barbiturates are weak acids, their major site of absorption is the intestine (6, 7); therefore, another factor involved in the present findings may be the rate of drug transfer from the stomach to the intestine. However, it is hard to see how this factor could explain the discrepancy between the rate and the extent of cyclobarbital calcium bioavailability from the aqueous solution. Its rapid rate of absorption suggests that rapid drug transfer takes place, which, in principle, favors high bioavailability.

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Fluorocarbon Aerosol Propellants VII: Interaction Studies with Human and Bovine Globulins Using Partition Coefficient Method

Keyphrases □ Fluorocarbon aerosol propellants—interaction with human and bovine globulins, partition coefficient method □ Globulins, human and bovine—interaction with fluorocarbon aerosol propellants, partition coefficient method □ Plasma protein binding—interaction of fluorocarbon aerosol propellants with human and bovine globulins, partition coefficient method

To the Editor:

The partition coefficients of some commonly employed propellants between the aqueous phase and the head space have been shown to be higher in plasma–air systems for humans (1, 2) and other species (2) than in the water (or normal saline)–air system. This finding was postulated to be due to the binding or complexation of these propellants to certain constituent(s) in plasma, as was subsequently confirmed by the partition coefficient method using purified human and bovine albumins (3, 4).

The ability of trichloromonofluoromethane to bind to bovine albumin also was recently demonstrated using a fluorescent probe technique, and these results will be published later. The binding or complexation with other tissue components was also hypothesized to account partially for the high apparent volume of distribution of these neutral propellants in dogs (5).

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Table I—Average Partition Coefficients ± *SD* of the Three Propellants in Solutions of Plain Buffer and 5% (w/v) Bovine and Human γ-Globulins

Propellant	Plain Buffer	Bovine γ-Globulin	Human γ-Globulin
Trichloromonofluoromethane	0.298 ± 0.0084	0.273 ± 0.0053 ($p < 0.01$)	0.308 ± 0.0085 ($p < 0.1$)
Dichlorodifluoromethane	0.0916 ± 0.0008	0.0887 ± 0.0017 ($p < 0.05$)	0.0882 ± 0.0013 ($p < 0.02$)
Dichlorotetrafluoroethane	0.0296 ± 0.0009	0.0294 ± 0.0013 ($p < 0.8$)	0.0275 ± 0.0010 ($p < 0.05$)

Since it is important to identify various components of plasma that may interact with these propellants and since γ-globulin has the second highest concentration among various plasma proteins, a preliminary study was conducted on the possible interaction between the three most widely used propellants and human and bovine globulins using the partition coefficient method (3, 4).

The crystallized and lyophilized bovine and human γ-globulins were obtained commercially[1] and were used without further purification. Protein solutions (5% w/v) were freshly prepared in pH 7.4 phosphate buffer (3). The equilibrium study was conducted in a water bath maintained at 21.5–22°. The equilibrium concentrations of the propellants in the aqueous phase were about 0.05, 0.08, and 0.015 mg/ml for trichloromonofluoromethane, dichlorodifluoromethane, and dichlorotetrafluoroethane, respectively.

All propellants were studied individually, and at least four runs were performed on each propellant. The propellant concentrations in both the aqueous phase and the head space in sealed serum bottles were analyzed by a GC method using an electron-capture detector (6). The enhancing effect of *n*-hexane used in the extraction on peak heights in the GC analysis for each propellant was corrected in the calculations (7).

The results of this preliminary study are summarized in Table I. Contrary to the albumin study, all of the partition coefficients in globulin–air systems are only slightly different from those in the plain buffer–air system, indicating an insignificant degree of interaction in spite of statistically significant differences in some systems. The slight decrease of the partition coefficients of some propellants in the globulin solutions is most likely due to the salting-out effect of globulin.

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[x] To whom inquiries should be directed.

Novel, Low Cost Approach to Digitizing Tablet Hardness Values

Keyphrases □ Tablet hardness tester—modified to give digitized values □ Digitized tablet hardness values—equipment described

To the Editor:

When formulating tablets with deliberately low hardness values, *i.e.*, <2 kg, it is difficult to determine these values repeatedly and accurately to 0.1 kg on the meter scale of a hardness tester[1]. Shown in Fig. 1 is an inexpensive addition to the hardness tester which will give a digitized reading of these hardness values and print them on an optional printer if desired.

The transducer used for sensing the force applied to break the tablet is a Hall generator[2]. The output signal from a Hall generator is a voltage that is proportional to the current flowing through it and the magnetic flux density (1).

As shown in Fig. 1, a Hall generator is attached to the movable counterweight shaft and a small magnet is attached to the case of the instrument approximately 5 mm from the Hall device. As force is applied to the tablet, the shaft moves, causing the Hall device to move away from the magnet. This change in magnetic flux density is sensed by the Hall generator; its

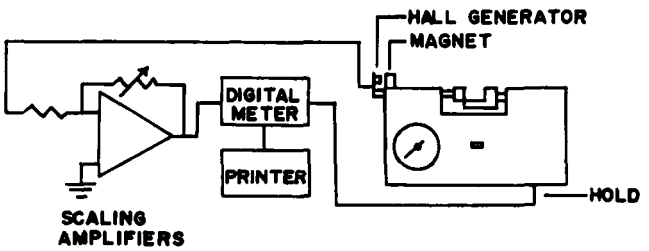

Figure 1—*Schematic diagram of instrument for digitizing tablet hardness.*

[1] Heberlein tester, Cherry-Burell, Park Ridge, Ill.
[2] Hall effect magnetic field sensor, model 63SS2-1, Micro Switch, Freeport, IL 61032

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The crystallized and lyophilized bovine and human γ-globulins were obtained commercially[1] and were used without further purification. Protein solutions (5% w/v) were freshly prepared in pH 7.4 phosphate buffer (3). The equilibrium study was conducted in a water bath maintained at 21.5–22°. The equilibrium concentrations of the propellants in the aqueous phase were about 0.05, 0.08, and 0.015 mg/ml for trichloromonofluoromethane, dichlorodifluoromethane, and dichlorotetrafluoroethane, respectively.

All propellants were studied individually, and at least four runs were performed on each propellant. The propellant concentrations in both the aqueous phase and the head space in sealed serum bottles were analyzed by a GC method using an electron-capture detector (6). The enhancing effect of *n*-hexane used in the extraction on peak heights in the GC analysis for each propellant was corrected in the calculations (7).

The results of this preliminary study are summarized in Table I. Contrary to the albumin study, all of the partition coefficients in globulin–air systems are only slightly different from those in the plain buffer–air system, indicating an insignificant degree of interaction in spite of statistically significant differences in some systems. The slight decrease of the partition coefficients of some propellants in the globulin solutions is most likely due to the salting-out effect of globulin.

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Novel, Low Cost Approach to Digitizing Tablet Hardness Values

Keyphrases □ Tablet hardness tester—modified to give digitized values □ Digitized tablet hardness values—equipment described

To the Editor:

When formulating tablets with deliberately low hardness values, *i.e.*, <2 kg, it is difficult to determine these values repeatedly and accurately to 0.1 kg on the meter scale of a hardness tester[1]. Shown in Fig. 1 is an inexpensive addition to the hardness tester which will give a digitized reading of these hardness values and print them on an optional printer if desired.

The transducer used for sensing the force applied to break the tablet is a Hall generator[2]. The output signal from a Hall generator is a voltage that is proportional to the current flowing through it and the magnetic flux density (1).

As shown in Fig. 1, a Hall generator is attached to the movable counterweight shaft and a small magnet is attached to the case of the instrument approximately 5 mm from the Hall device. As force is applied to the tablet, the shaft moves, causing the Hall device to move away from the magnet. This change in magnetic flux density is sensed by the Hall generator; its

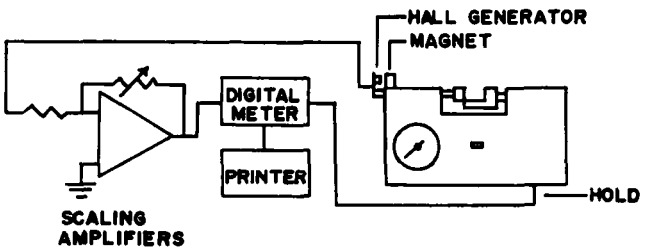

Figure 1—*Schematic diagram of instrument for digitizing tablet hardness.*

[1] Heberlein tester, Cherry-Burell, Park Ridge, Ill.
[2] Hall effect magnetic field sensor, model 63SS2-1, Micro Switch, Freeport, IL 61032

output voltage, which is proportional to force, is amplified, scaled, and displayed on a digital voltmeter calibrated directly in kilograms. The display on the digital panel meter is held at the maximum force value by utilizing the tablet breakage point sensing circuitry in the hardness tester.

Calibration and linearity of the digital readings can be checked by displacing the anvil on the hardness tester in small increments while noting the reading on the meter incorporated in the hardness tester. Magnet placement, Hall device power supply voltage, and scaling amplifiers are adjusted for the proper reading.

A method for calibrating the hardness tester was mentioned previously (2).

Advantages of the digital approach include: *(a)* accuracy, *(b)* low cost of the transducer and system, *(c)* ease of construction, *(d)* no change in operating procedures, *(e)* automatic printout if desired, and *(f)* hardness tester not affected by operation of the transducer.

(1) "The Hall Effect and Its Application," 2nd ed., F. W. Bell, Inc., Columbus, Ohio, 1969.

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Drug–Nitrite Interactions: Formation of *N*-Nitroso, C-Nitroso, and Nitro Compounds from Sodium Nitrite and Various Drugs under Physiological Conditions

Keyphrases □ Nitrite–drug interactions—formation of *N*-nitroso, C-nitroso, and nitro compounds from sodium nitrite and 20 drugs under physiological conditions □ Nitroso compounds—formation from sodium nitrite and 20 orally administered drugs under physiological conditions □ Interactions—20 orally administered drugs with sodium nitrite, formation of *N*-nitroso, C-nitroso, and nitro compounds under physiological conditions

To the Editor:

Many alkyl, aryl, and cyclic *N*-nitroso compounds are known to be carcinogenic (1, 2). Recently, several drugs containing secondary and tertiary amino groups were found to react with sodium nitrite *in vitro* and *in vivo* to yield *N*-nitroso compounds (3–8). Coadministration of aminopyrine and sodium nitrite by the oral route produced liver tumors in rats identical to those produced by *N*-nitrosodimethylamine, a product of drug–nitrite interaction in the stomach (9, 10). Similarly, concurrent administration of piperazine and sodium nitrite to mice induced lung adenomas attributable to *N*-nitrosopiperazine formed *in vivo* (11).

Since many orally administered drugs contain secondary and tertiary amino groups which could form potentially toxic *N*-nitroso compounds in the stomach, where suitable acidic conditions and significant concentrations of nitrite through dietary intake may prevail, the drug–nitrite interaction has been implicated in the etiology of human cancer (2, 12–15).

In the present communication, the isolation and mass spectrometric identification of *N*-nitroso, C-nitroso, and nitro compounds that arise from the reaction of sodium nitrite with 20 drugs (Table I) are described. These drugs were selected because they are commonly administered by the oral route and represent a wide variety of chemical structures. These studies were initiated to gain a better understanding of drug–nitrite interactions and to delineate the structural features of the drugs most likely to yield potentially toxic nitroso compounds.

The nitrosative cleavage of tertiary amines was studied previously (16–18). We observed that, depending upon the structure of drugs, five types of nitroso compounds may be formed as a result of drug–nitrite interactions (Table I). For the reactions at pH 1–2, 100 mg of drug was incubated with 200 mg of sodium nitrite in 10 ml of 10% hydrochloric acid at 37° for 4 hr; the reactions at pH 3–4 were carried out in 10 ml of 10% acetic acid. The reaction mixtures were adjusted to pH 11 with solid sodium hydroxide and extracted twice with 8 ml of methylene chloride and twice with 8 ml of ethyl acetate.

The volatile *N*-nitroso compounds in the methylene chloride extracts were identified by GC–mass spectrometry and quantitated by GC (19). The nonvolatile nitroso and nitro compounds in the ethyl acetate extract were purified by TLC on silica gel, using a solvent system of ethyl acetate–methanol (1:1), and characterized by mass spectrometry. The yields were calculated on the basis of the isolation of homogeneous products (8, 20). The methylene chloride extracts contained the products listed in Groups 1 and 2, while the ethyl acetate extracts contained the products in Groups 3–5.

The mass spectra of all *N*-nitroso compounds in Groups 1 and 2 (Table I) were consistent with those reported in the literature (19, 21). The *N*-nitroso derivatives in Groups 3 and 4 exhibited fragmentation patterns typical of *N*-nitroso compounds (the characteristic loss of NO from the parent ion) as well as those consistent with the drug structure (8, 20). The mass spectra of synthetic 4-nitrosoantipyrine and 4-nitroantipyrine (22) were identical to the spectra of the respective products from the reaction of antipyrine (XX) with nitrite.

The following comments may be made regarding the formation of nitroso and nitro compounds from drug–nitrite interactions.

Group 1 Products (*N*-Nitrosodialkylamines)—

output voltage, which is proportional to force, is amplified, scaled, and displayed on a digital voltmeter calibrated directly in kilograms. The display on the digital panel meter is held at the maximum force value by utilizing the tablet breakage point sensing circuitry in the hardness tester.

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The following comments may be made regarding the formation of nitroso and nitro compounds from drug–nitrite interactions.

Group 1 Products (*N*-Nitrosodialkylamines)—

Table I—Nitroso and Nitro Compounds from the Reaction of Sodium Nitrite with Orally Administered Drugs

Number	Drug	Nitroso or Nitro Compound Formed	Yield[a], % Reaction at pH 1–2	Yield[a], % Reaction at pH 3–4
		Group 1 Products		
I	Aminopyrine[b]	*N*-Nitrosodimethylamine	31.4	71.4
II	Captodiame	*N*-Nitrosodimethylamine	0	0.7
III	Chlophedianol	*N*-Nitrosodimethylamine	0	0.3
IV	Chlorpromazine[b]	*N*-Nitrosodimethylamine	0	0.6
V	Fonazine	*N*-Nitrosodimethylamine	0	0.8
VI	Oxytetracycline[b]	*N*-Nitrosodimethylamine	0.6	63.2
VII	Diethazine	*N*-Nitrosodiethylamine	0	0.4
VIII	Lucanthone[b]	*N*-Nitrosodiethylamine	0	0.5
IX	Thiphenamil	*N*-Nitrosodiethylamine	0	0.3
		Group 2 Products		
X	Clemizole	*N*-Nitrosopyrrolidine	0	1.4
XI	Cycrimine	*N*-Nitrosopiperidine	0	1.8
XII	Hexadiphane	*N*-Nitrosohexamethyleneimine	0	2.1
XIII	Phenadoxone	*N*-Nitrosomorpholine	0	2.5
XIV	Butaperazine	*N*,*N*′-Dinitrosopiperazine	0	2.8
		Group 3 Products		
IV	Chlorpromazine	*N*-Nitrosodesmethylchlorpromazine	0.4	1.7
XV	Imipramine	*N*-Nitrosodesipramine	0.3	1.2
XVI	Tripelennamine	*N*-Nitrosodesmethyltripelennamine[c]	0.7	5.8
		Group 4 Products		
XVII	Desipramine	*N*-Nitrosodesipramine	10.9	2.8
XVIII	Piperazine[b]	*N*-Nitrosopiperazine	35.0[d]	54.6[d]
		N,*N*′-Dinitrosopiperazine	78.4	84.6
XIX	Protriptyline	*N*-Nitrosoprotriptyline	11.6	3.4
		Group 5 Products[e]		
XX	Antipyrine	4-Nitrosoantipyrine	0[f]	60.8[f]
		4-Nitroantipyrine	89.3	83.7

[a] Percentage of theoretical yield from the reaction of drug with sodium nitrite at 37° for 4 hr. [b] Reaction of this drug with nitrite was reported earlier (3, 5, 24, 31). [c] Formation of this *N*-nitroso derivative was described recently (8). [d] Yield of the mononitroso derivative when the reaction was run for 15 min. [e] Aromatic mononitro and dinitro derivatives of IV, XV, XVII, and XIX were formed (yield of 1–5%) during the reaction of these drugs with nitrite at pH 1–2. Studies are in progress to deduce the structure of these nitro compounds. [f] Yield of the nitroso compound from the reaction at room temperature. Nearly quantitative yield of 4-nitrosoantipyrine can be achieved if the reaction is run in 10% HCl at 0° (22).

The nitrosative cleavage of tertiary amino groups from the dialkylaminoalkyl side chain in Compounds II–V and VII–IX appeared to be the least favored reaction and was completely inhibited at pH 1–2. However, such cleavage of tertiary amino groups attached to ring structures (I and VI) occurred quite readily; indeed the yield of *N*-nitrosodimethylamine formed from aminopyrine was quite high (31.4%) even at pH 1–2.

Group 2 Products (*N*-Nitroso Cyclic Amines)—The yield of cyclic *N*-nitroso compounds from the drug–nitrite reaction at pH 3–4 was between 1.4 and 2.8% with Compounds X–XIV, which contain pyrrolidine, piperidine, hexamethyleneimine, morpholine, and piperazine moieties. Group 2 products were not found when the reactions were carried out at pH 1–2.

Group 3 Products (*N*-Nitroso Derivatives of *N*-Dealkylated Drugs)—Between the two pH ranges examined, higher yields of *N*-nitroso derivatives were obtained at pH 3–4 with all three drugs (IV, XV, and XVI) that gave Group 3 products with nitrite.

Group 4 Products (*N*-Nitroso Derivatives of Drugs)—The reaction of nitrite with drugs containing secondary amino groups yielded substantial quantities of *N*-nitroso derivatives at both pH ranges examined.

Group 5 Products (C-Nitroso and Nitro Derivatives of Drugs)—The highly reactive antipyrine (XX) formed the 4-nitro derivative quite readily in both pH ranges studied. The formation of the C-nitroso derivative of XX was observed only when the reaction was run in 10% acetic acid at room temperature. Aromatic mononitro and dinitro derivatives of drugs containing aromatic rings (IV, XV, XVII, and XIX) were present (yield of 1–5%) in the ethyl acetate extracts of the drug–nitrite reaction mixtures (pH 1–2) as determined from their mass spectra. The location of the nitro groups in the aromatic rings remains to be assigned.

All *N*-nitroso compounds listed under Groups 1 and 2 are known carcinogens (1, 2, 12–14), but the toxicity of the products listed in Groups 3–5 remains unknown. In preliminary studies with tripelennamine (XVI), *N*-nitrosation did not enhance the toxic potential of the drug in rats and the *N*-nitroso derivative was devoid of both central nervous system and antihistaminic activities of the parent drug (8). There is evidence that nitrite has a short half-life in the stomach (14, 23) and that ascorbic acid and other dietary constituents are inhibitors of drug–nitrite reactions (24–27). Nevertheless, the formation of *N*-nitrosamines in the human stomach has been demonstrated (28–30).

Further studies are needed to assess the potentially adverse effects of drug–nitrite interactions in humans, especially those on chronic medication where readily nitrosatable drugs and high dietary intake of nitrite may be involved.

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Application of Mixed Electron-Impact–Chemical Ionization High-Resolution Mass Spectrometry to a Medicinal Agent

Keyphrases □ Structure determination—application of mixed electron-impact–chemical ionization high-resolution mass spectrometry □ Mass spectrometry, mixed electron impact–chemical ionization—structure determination

To the Editor:

Chemical ionization mass spectrometry has proven to be a useful technique for molecular structure determination of various classes of medicinal agents, especially for compounds that contain no molecular ion in the electron-impact spectra. Successful identification of drug metabolites and multicomponent drug mixtures, such as those received by forensic laboratories, is an excellent example of the utility of chemical ionization mass spectrometry. Several comprehensive listings of chemical ionization and electron-impact mass spectra are available for comparison with spectra of unknown drugs (1–3). Therefore, it is useful to obtain both types of spectra for structure elucidation of medicinal agents.

Chemical ionization mass spectra are produced *via* gaseous ion–molecule reactions occurring in the ion source of the mass spectrometer. For an ion–molecule reaction to take place, it is necessary to raise the low pressure (10^{-6} torr) which is characteristic of the conventional electron-impact source. "Closing" the source allows the pressure to increase to approximately 0.5 torr when a reagent gas such as isobutane is added. However, this modification, which facilitates ion–molecule reactions in the chemical ionization mode, may precipitate reactions in the source between sample ions and sample molecules in the electron-impact mode (*i.e.*, self-ionization).

Many investigators have noted the occurrence of collision-induced ions in the electron-impact spectra obtained from closed source instruments (4), particularly MH^+ and fragments generally associated with chemical ionization spectra. Collision-induced ions together with those ions resulting directly from electron impact constitute what is referred to here as a mixed electron-impact–chemical ionization spectra. The ratio of collision ions to electron-impact ions contained in a mixed electron-impact–chemical ion-

Table I—Mixed Electron-Impact–Chemical Ionization High-Resolution Mass Spectral Data of Meprobamate

Elemental Composition	Theoretical	Found	Relative Intensity, %
$C_9H_{19}N_2O_4$	219.1344	219.1335	79
$C_8H_{16}NO_2$	158.1180	158.1177	100
$C_7H_{14}NO_2$	144.1024	144.1007	81
$C_7H_{14}O$	114.1044	114.1032	15
C_7H_{12}	96.0938	96.0931	22
C_6H_{11}	83.0860	83.0844	90
CH_4NO_2	62.0241	62.0242	35
C_4H_7	55.0546	55.0550	68
CH_2NO	44.0136	44.0125	51

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Application of Mixed Electron-Impact–Chemical Ionization High-Resolution Mass Spectrometry to a Medicinal Agent

Keyphrases □ Structure determination—application of mixed electron-impact–chemical ionization high-resolution mass spectrometry □ Mass spectrometry, mixed electron impact–chemical ionization—structure determination

To the Editor:

Chemical ionization mass spectrometry has proven to be a useful technique for molecular structure determination of various classes of medicinal agents, especially for compounds that contain no molecular ion in the electron-impact spectra. Successful identification of drug metabolites and multicomponent drug mixtures, such as those received by forensic laboratories, is an excellent example of the utility of chemical ionization mass spectrometry. Several comprehensive listings of chemical ionization and electron-impact mass spectra are available for comparison with spectra of unknown drugs (1–3). Therefore, it is useful to obtain both types of spectra for structure elucidation of medicinal agents.

Chemical ionization mass spectra are produced *via* gaseous ion–molecule reactions occurring in the ion source of the mass spectrometer. For an ion–molecule reaction to take place, it is necessary to raise the low pressure (10^{-6} torr) which is characteristic of the conventional electron-impact source. "Closing" the source allows the pressure to increase to approximately 0.5 torr when a reagent gas such as isobutane is added. However, this modification, which facilitates ion–molecule reactions in the chemical ionization mode, may precipitate reactions in the source between sample ions and sample molecules in the electron-impact mode (*i.e.*, self-ionization).

Many investigators have noted the occurrence of collision-induced ions in the electron-impact spectra obtained from closed source instruments (4), particularly MH^+ and fragments generally associated with chemical ionization spectra. Collision-induced ions together with those ions resulting directly from electron impact constitute what is referred to here as a mixed electron-impact–chemical ionization spectra. The ratio of collision ions to electron-impact ions contained in a mixed electron-impact–chemical ion-

Table I—Mixed Electron-Impact–Chemical Ionization High-Resolution Mass Spectral Data of Meprobamate

Elemental Composition	Theoretical	Found	Relative Intensity, %
$C_9H_{19}N_2O_4$	219.1344	219.1335	79
$C_8H_{16}NO_2$	158.1180	158.1177	100
$C_7H_{14}NO_2$	144.1024	144.1007	81
$C_7H_{14}O$	114.1044	114.1032	15
C_7H_{12}	96.0938	96.0931	22
C_6H_{11}	83.0860	83.0844	90
CH_4NO_2	62.0241	62.0242	35
C_4H_7	55.0546	55.0550	68
CH_2NO	44.0136	44.0125	51

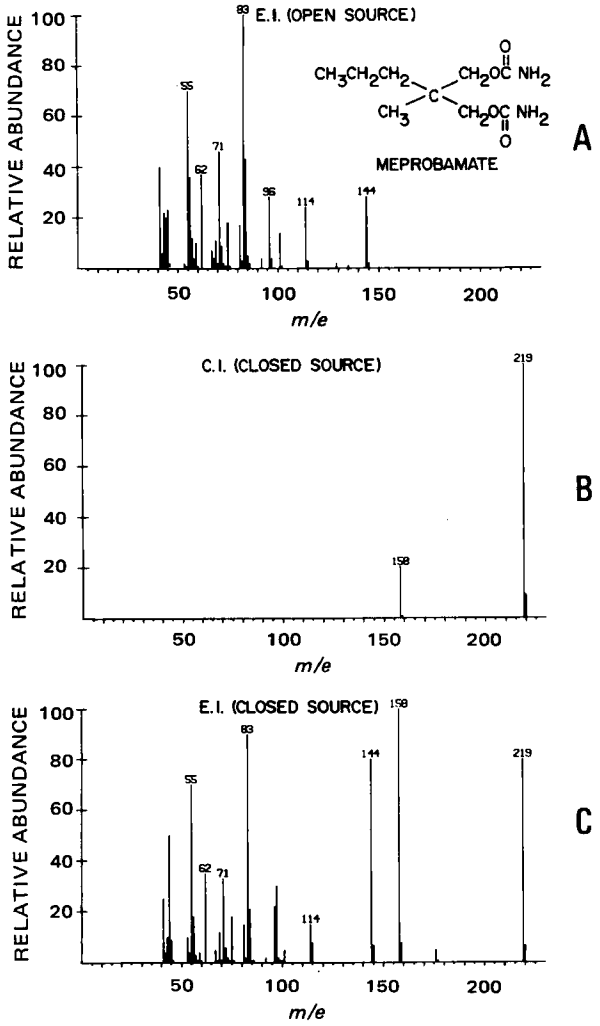

Figure 1—*(A) Mass spectrum of meprobamate obtained prior to chemical ionization modification of the instrument. (B) Chemical ionization (CI) spectrum of meprobamate after installation of a dual electron-impact (EI)–chemical ionization source. (C) Mixed electron-impact–chemical ionization spectrum of meprobamate.*

ization spectra can be altered by varying the pressure in the closed ion source. Higher pressure can easily be attained by either increasing the sample size or reducing the dimensions of the electron entrance and ion exit slit.

Mixed electron-impact–chemical ionization spectra have been particularly useful for compounds, such as meprobamate, that show no molecular ion in the electron-impact spectra (open source). Dicarbamates and many other classes of medicinal agents readily give mixed electron-impact–chemical ionization spectra because of their large cross section for proton capture (5).

All of the mass spectra shown here were obtained on the same high-resolution mass spectrometer coupled with a data processor[1]. Samples were introduced into the source *via* a direct insertion probe. Spectra were obtained at a source temperature of 90°. Figure 1A is a mass spectrum of meprobamate obtained prior to chemical ionization modification of the instrument (open source). Figure 1B shows the chemical ionization spectrum of the same compound after installation of a dual electron-impact–chemical ionization source (closed source). Isobutane was used as the reagent gas. Figure 1C is an illustration of a mixed electron-impact–chemical ionization spectrum obtained from a dual electron-impact–chemical ionization source instrument. This closed source spectrum was obtained in the electron-impact mode by merely increasing the sample pressure. No reagent gas was added.

By adding perfluorokerosene and carefully adjusting the pressure in the source, it is possible to obtain a mixed electron-impact–chemical ionization spectrum suitable for high-resolution mass measurements. While fast scanning the mixed electron-impact–chemical ionization high-resolution spectrum, we were able to measure both electron-impact and collision-induced ions.

Meprobamate shows no molecular ion in the electron-impact spectrum of an unmodified instrument (6, 7). However, additional information was available in the mixed electron-impact–chemical ionization spectra. An MH^+ ion was observed at *m/e* 219, and high-resolution mass spectrometry established its elemental composition as $C_9H_{19}N_2O_4$. The base peak at *m/e* 158 was derived from the MH^+ ion through the loss of carbamic acid. In addition, high-resolution printout data were obtained on all abundant fragment ions usually contained in the electron-impact spectra. In Table I, the exact measured mass values of several major ions are listed and compared with the theoretical mass values.

Simple inspection of the electron-impact spectra obtained from a dual electron-impact–chemical ionization source is usually sufficient to distinguish ions produced by electron impact from collision-induced ions. Mixed electron-impact–chemical ionization spectra cannot be compared with standard electron-impact spectra due to the presence of collision-induced ions. If comparison is desired, however, electron-impact spectra corresponding to a standard can be readily obtained by a simple reduction of the sample pressure in the source.

In conclusion, the mixed electron-impact–chemical ionization high-resolution operation is a simple technique and provides both electron-impact and chemical ionization information on the same spectrum. This technique has proven very useful for the structure determination of medicinal agents in our laboratory.

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Methodological Differences in Correlating Digoxin Dissolution with Bioavailability

Keyphrases □ Digoxin—correlating dissolution and bioavailability, differences in methods, paddle-water method and rotating-basket apparatus □ Dissolution—digoxin, methods of determination, correlation with bioavailability □ Bioavailability—digoxin, correlation with dissolution, methods of dissolution determination

To the Editor:

A recent report by Klink *et al.* (1) indicated that the "paddle-water" method for determining dissolution rates for digoxin tablets failed to reflect the comparative bioavailability properties of two commercially available digoxin products. Specifically, large differences in dissolution rates were observed which failed to reflect the similar bioavailabilities of the two brands studied.

A number of reports have appeared indicating successful *in vivo–in vitro* correlation employing different methods (2–8), including the method recently adopted by the USP (9). Thus, the purpose of this communication is to report the results of recent dissolution rate studies carried out on the same batches of tablets employed by Klink *et al.* (1) using the USP dissolution rate test method.

The dissolution properties of six tablets each from Treatment I[1] and Treatment II[2] tablets were determined using the USP rotating-basket apparatus and employing the conditions specified in the USP (9). Samples were withdrawn and assayed by a fluorometric method (10) at 15, 30, 60, and 120 min after commencement of the studies.

The results of these studies are summarized in Table I, which lists the amount of digoxin in solution at various times for both the USP method and the paddle-water method (1). As indicated by these results, the two brands showed fairly similar dissolution properties, especially at the 60- and 120-min sampling times, when the USP method was employed. This finding is in contrast to the large differences observed at all sampling times when the paddle-water method was employed; Treatment I tablets dissolved much more rapidly than did Treatment II tablets.

These results are of significance when the *in vivo* performance of the two brands is considered. As reported by Klink *et al.* (1), the bioavailabilities of Treatment I and Treatment II tablets, as determined by the area under the serum level–time curves from 0 to 48 hr relative to similar areas obtained from digoxin in elixir data, were 106.38 ± 10.27 and 100.75 ± 24.09%, respectively. Similar relationships between the two brands were observed when the 0–5-, 0–12-, and 0–24-hr areas were compared.

Thus, from the dissolution data presented, it appears that, in the case of the specific lots of the two brands of digoxin tablets studied, the USP method for determining dissolution rates is of greater reliability than is the paddle-water method in predicting bioavailability. Based on these findings and the findings of others (2–8), it appears that much work remains to be done before the acceptance of a single *in vitro* dissolution test for the prediction of digoxin bioavailability from compressed tablets.

Table I—Mean Percent (±Standard Error) of Labeled Digoxin in Solution at Various Times following *In Vitro* Dissolution by Two Different Methods

	USP Method[a]		Paddle-Water Method[b]	
Minutes	Treatment I	Treatment II	Treatment I	Treatment II
15	65.1 ± 2.4	29.6 ± 2.7	—[c]	—
30	76.6 ± 1.8	60.0 ± 4.1	34.9 ± 4.3	6.0 ± 1.2
60	83.7 ± 3.0	77.0 ± 1.4	46.9 ± 6.1	8.0 ± 0.8
120	92.2 ± 0.9	86.7 ± 0.9	59.1 ± 3.9	19.4 ± 3.5

[a] Mean of six tablets. [b] Data from Ref. 1; mean of five tablets. [c] Samples not taken.

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The chapter on Pharmaceutical Product Stability by C. J. Lintner provides a general condensed review on stability of pharmaceuticals. Information is provided in this chapter on regulations applicable to stability, types of degradation (hydrolysis, oxidation–reduction, *etc.*,) physical stability of different dosage forms, preservatives, antioxidants, chelating agents, isothermal *versus* nonisothermal reactions, containers and closures, and computer programs for treating and storing stability data; there are also short synopses on stability attributes of specific drug compounds. Because of the considerable amount of information the author provides, no one area of his presentation could be given in depth. The author of this chapter has provided an excellent comprehensive bibliography on his subject.

The chapter on Quality Control of Veterinary Biologics by G. R. Sharpless is concerned for the most part with potency testing of bacterial, viral, sera, toxoid and bacterin–toxoid, and diagnostic products. It also provides a brief review of the tests and procedures outlined by the Animal and Plant Health Inspection Service of the U.S. Department of Agriculture, Veterinary Services Branch, which is responsible for the government regulation of veterinary biologics.

The chapter on the Control of Sterilization Procedures by R. R. Ernst does not discuss sterility tests or testing methods, but instead gives an in-depth presentation of the various factors involved in steam and gaseous sterilization and the types of equipment utilizing these two methods of sterilization. Sterilization by radiation, filtration, and dry heat is treated superficially.

The chapter on Quality Control for Pharmaceutical and Cosmetic Aerosol Products by J. J. Sciarra has only a minor portion related to the quality control of aerosol products and components. The major portion of the chapter is concerned with the general philosophy and principles of quality control and assurance in the pharmaceutical industry.

The chapter on The Test Program of the National Center for Drug Analysis on the Quality Control of Drug Products by A. W. Steers discusses the FDA regulations pertaining to drug quality, the organization of the National Center for Drug Analysis (NCDA), the Retail-Based Drug Monitoring Program, and the Formulator-Oriented Rx Drug Studies (FORDS) Program. Data are presented on the drug products evaluated from the retail-based and formulator-based studies at the NCDA.

Certain chapters of this volume provide the pharmaceutical scientist, quality control chemist, and biologist with pertinent information not readily found in other books which should be of use in their daily work.

Reviewed by Leon Lachman
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Analytical Profiles of Drug Substances, Vol. 4. Edited by KLAUS FLOREY. Academic, 111 Fifth Ave., New York, NY 10003, 1975. 16 × 23.5 cm. 526 pp. Price $26.50.

Volume 4 of this unique series provides coverage of 20 additional drugs including cefazolin, estradiol valerate, methaqualone, norethindrone, and tybamate. Utilizing a monograph format, these volumes provide frequently difficult to locate information on physical and chemical data, methods of synthesis, pathways of physical and biological degradation, metabolism, and methods of analysis.

Each monograph is amply illustrated and well referenced. Considerable information concerning the drugs is compiled and presented in an easy-to-use format. These volumes should be available to everyone working in pharmaceutical research.

Staff Review

Journal of Pharmaceutical Sciences

OCTOBER 1975

VOLUME 64 NUMBER 10

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HARMLESS, BUT INEFFECTIVE, REMEDIES

Once they disappear, problems are quickly forgotten. If they are not completely forgotten, certainly our once strong feelings concerning their seriousness are greatly dulled and dampened.

Communicable diseases constitute a classic example of this changing pattern of interest on the part of society. Another striking example is that of quack medicines.

It is only within the last few years that we have reached the point that physicians, pharmacists, and patients could rely upon drug products to be effective for the uses, conditions, and treatments claimed for them in their labeling and advertising. But it was not always so.

A century ago, quackery was blatant, with medicine men traveling by horse-drawn wagons across the country hawking the latest concoction. These "cure-alls" generally either tasted terrible or were heavily laced with alcohol. Neither of these considerations made the product harmful but added considerably to the perceived effectiveness—what we today refer to as the "placebo" factor.

Quackery became more sophisticated during the last generation or so—but there still was a lively market for liver pills, Hadacol, honey and vinegar, copper bracelets, electronic devices, and a myriad of other products of highly dubious therapeutic value.

The medical and pharmacy professions campaigned vigorously in an effort to stamp out such quackery, but it wasn't until the Kefauver–Harris Drug Amendments of 1962 were enacted that the Food and Drug Administration finally had an effective weapon to employ in the battle to rid the market of such products. This was because the 1962 amendments, for the first time, required that an article must be demonstrated as *effective* if it is to be promoted as having some therapeutic or medicinal usefulness.

The quack medicines were generally all harmless and nontoxic; safety *per se* was not a problem. However, the health professions—and the public through their elected representatives—decided this was not enough. If a drug is to be on the market, it must have more than just a placebo effect. Skilled health care practitioners must be able to rely upon their therapeutic tools. Moreover, the public must not be bilked by slick con-artists who are eager to take advantage of the hopes and desperation of sick persons and their families by selling useless products for hard-earned money.

If public protection is needed by any group, it is the victims of cancer, arthritis, and similar diseases where hope is little and despair is great. It is this unfortunate group which is most vulnerable to the siren-song of the quacks.

Normally we think of the *New York Times* as a model newspaper in that its editorial views and opinions are well-considered and show care and judgment in their formulation. We were disappointed, therefore, by an editorial appearing this summer entitled "Illegal Laetrile."

The *Times* editorial correctly explained that current law prohibits the marketing of any new drug until it is proven to be both effective and safe. The editorial further explained that there appears to be no concern regarding the safety of laetrile (amygdalin), but that no evidence has been presented to FDA that the drug is useful in the treatment of cancer. In fact, the editorial admits that even animal experiments have been almost uniformly negative except for some curious initial results at one institution which that same institution now regards as spurious.

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In our view, the same argument can be made—and in many cases, has been made—for every quack medicine ever offered to the public. Indeed, some of these at least had some indication of value, if not proven evidence. Based upon present information, there is no more justification for the FDA to permit the marketing of laetrile than there would be for FDA to permit the bottling of sugar water by some huckster who would then sell it at a fancy price as a guaranteed safe cancer cure!

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Edward G. Feldmann

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OCTOBER 1975

VOLUME 64 NUMBER 10

REVIEW ARTICLE

Inclusion Compounds

SYLVAN G. FRANK

Keyphrases □ Inclusion compounds—polymolecular, monomolecular, blue-iodine reaction products, and macromolecular, review □ Complexes—review of various types of inclusion compounds □ Polymolecular inclusion compounds—urea and thiourea, choleic acids, 4,4′-dinitrobiphenyl, hydroquinone, water, phenol, Dianin's reagent, cycloveratril, and tri-*o*-thymotide, review □ Monomolecular inclusion compounds—cyclodextrins, review □ Blue-iodine reaction inclusion compounds—review □ Macromolecular inclusion compounds—zeolites, dextrans, and silica gels, review □ Urea and thiourea inclusion compounds—review □ Choleic acid inclusion compounds—review □ Water inclusion compounds—review □ Cyclodextrin inclusion compounds—review

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An inclusion compound is a unique form of chemical complex in which one molecule is enclosed within another molecule or structure of molecules. This combination is characterized by the absence of ordinary chemical bonds; the essential criterion is simply that the enclosed molecule or "guest" be of a suitable size and shape to fit into a cavity within a solid structure formed by "host" molecules (1–13). The hollow space formed by the host may be in the form of a channel, cage, or layer.

The stereochemistry and, possibly, the polarity of both the host and the guest molecules determine whether inclusion can occur. The resulting close fit of the two components produces a combination of significant strength due to the total dispersion forces between the interacting components. This type of spatial complex formation does not occur by means of ionic, covalent, or coordinate covalent bonds but rather is dependent upon dispersion forces and, possibly, highly oriented dipoles for stability, contrasting markedly with the usual concept of chemical complexation (14).

Inclusion compounds were first observed by Mylius (15) in 1886 as unusual complexations occurring between hydroquinone and several volatile com-

pounds. He proposed that the two components were interacting without chemical bonding and suggested that one molecule was enclosing the other. These observations were confirmed many years later by X-ray analysis; it was determined that one molecule of gas or liquid formed an insoluble inclusion compound with three molecules of hydroquinone, the host component, which formed a cage-like structure around the guest molecule (16). The presence of the guest component in inclusion compounds of this type could not be detected by odor; however, when the inclusion compound was heated or dissolved in water, the guest molecule was released.

The general title for this class of complexes, "inclusion compounds," was first used by Schlenk (17). Other terms that have been used to describe these complexes are "occlusion compounds," "adducts," and "clathrates" (18). Barrer (19) divided inclusion compounds into three categories, based on the varying concept of the host crystal, as *(a)* those that are stable both in the presence and in the absence of the guest molecule; *(b)* those in which the amount of guest may be changed but which have a critical concentration of guest molecules, below which the host structure becomes metastable and recrystallizes; and *(c)* those in which the host framework continuously readjusts itself as the content of guest molecules fluctuates.

A more convenient and workable classification, utilized in this review, is based upon the organization of inclusion compounds by their structure and properties as follows:

1. Polymolecular inclusion compounds
   —Channel-like spaces
   —Cage-like spaces
2. Monomolecular inclusion compounds
3. Products of the blue-iodine reaction
4. Macromolecular inclusion compounds

This classification differs from that of Baron (20), in that the products of the blue-iodine reaction follow the monomolecular inclusion compounds instead of appearing as the last category. This modification allows them to follow the classification to which they are more closely related.

Polymolecular inclusion compounds consist of a host structure composed of several molecules oriented in a loosely arranged lattice. The individual molecules of the host lattice are joined by hydrogen bonds to form a channel- or cage-like structure enclosing the guest molecule. However, they are not arranged in a whole number ratio to guest molecules as would be expected by the coordination theory (21). The inclusion cavity of specific dimensions forms only in the presence of the guest molecule but not necessarily using the guest as a template. In the absence of a guest molecule, compounds in this category form denser crystal structures without cavities.

As will be pointed out subsequently, similar molecules belonging to the same chemical class will crystallize to form cavities of different sizes and shape, depending on their atomic structure. Polymolecular inclusion compounds that form channel-like spaces include urea, thiourea, and various choleic acids; the latter complexes are among the earliest documented inclusion compounds (22).

A special category of the channel-like inclusion compounds consists of those formed by diphenyl derivatives in which the host molecules are "stacked" on one another in such a way as to leave channels in which an appropriate sized guest can be accommodated (23, 24). The interesting aspect of this arrangement is that no localized bonding is thought to exist.

The term "clathrate," derived from the Latin "clathratus," meaning "enclosed by the bars of a grating," (18) has been used to describe the cage-like structure of the hydroquinone inclusion compounds. The clathrates make up the second group of polymolecular inclusion compounds. In addition to hydroquinone, this group consists of: water or gas hydrates; tetraethylammonium hydrates; phenols; Dianin's compound, a product of the condensation of phenol and mesityl oxide; cycloveratril; and a number of complexes in which the cage-like structure is inorganic and the guest is organic (10). Although *Chemical Abstracts* and several other reference sources use the term "clathrates" as a general descriptor for inclusion compounds, this term more appropriately describes only the cage-like polymolecular inclusion compounds.

Probably the most versatile of the polymolecular inclusion compounds is tri-*o*-thymotide, which can form either channel- or cage-like void spaces, depending on the size and shape of the guest molecule (25).

The second major classification of inclusion compounds includes the monomolecular inclusion compounds. Although the polymolecular inclusion compounds constitute the largest group of inclusion compounds, the monomolecular inclusion compounds are of increasing interest and utility, particularly with reference to biological and pharmaceutical systems. Monomolecular inclusion compounds interact generally on a 1:1 basis with the guest molecule, which is enclosed within a cavity in the host molecule. The cyclodextrins, bis-*N,N'*-alkylenebenzidine compounds, antibiotics, and certain proteins are included in this category. The conclusion that certain proteins may behave as monomolecular inclusion compounds appears to offer a viable explanation for understanding antigen–antibody interactions, enzymatic reactions, and other shape-dependent processes.

Related to both the polymolecular and the monomolecular inclusion compounds are the products of the blue-iodine reaction. Iodine interacts with starch, cyclodextrins, flavones, coumarin, benzophenone, benzamide, cellulose, and barbituric acids to give a blue addition compound. This phenomenon has been attributed to the polymerization of iodine within unique channels formed by these compounds (26, 27).

The terms macromolecular inclusion compounds and "molecular sieves" have been used to characterize the fourth group of complexes. These compounds have been investigated extensively and have wide use in industrial and laboratory processes (19, 28, 29). Of

this group, the numerous zeolites are the most commonly known; however, the modified dextrans, polyacrylamide and agarose gels, silica gels, and other substances are also included.

The basic structure of the zeolites is a crystalline framework of silicon–oxygen or aluminum–oxygen tetrahedra which form a three-dimensional array with many cavities and interconnecting channels. Depending on the size and shape of these void spaces, various guest molecules can enter and be enclosed within the network. Although some macromolecular inclusion compounds occur naturally, it is also possible to custom tailor them to accept a specific guest by using the guest as a template during crystallization of the host.

Graphite is also generally classified as a macromolecular inclusion compound. However, it is more accurately classified as an intercalation compound, in which the guest is entrapped between layers of the graphite crystal (30, 31).

Inclusion compounds have been studied by a variety of conventional analytical methods including microscopy, IR and UV spectrophotometry, differential thermal analysis, NMR, and X-ray analysis[1].

Although a wide range of inclusion compounds is discussed here, emphasis is placed on those areas most apropos to the pharmaceutical sciences. Other areas are mentioned to present a comprehensive overview of this class of complexes. Previous reviews provided more extensive treatment of these latter areas (1–13).

POLYMOLECULAR INCLUSION COMPOUNDS

Compounds Forming Channel-Like Void Spaces—These inclusion compounds are classified as channel or canal complexes. Urea, thiourea, deoxycholic acid, and several other substances form long, tube-shaped cavities in which the guest molecules are oriented end to end.

Urea and Thiourea—Urea inclusion compounds were first reported by Bengen (32) from experiments on the effect of the addition of urea to pasteurized milk. Bengen was able to separate quantitatively the butterfat; however, in an attempt to control the formation of an emulsion at the oil–water interface, he added a small amount of *n*-octanol. After a short period, crystals were observed at the interface. These crystals later proved to be a urea–*n*-octanol inclusion compound. Bengen and Schlenk (21) were then prompted to study inclusion compound formation by urea and many aliphatic straight-chain hydrocarbons, leading eventually to the development of the use of urea inclusion compound formation as a method for separating hydrocarbons (33, 34).

In the presence of straight-chain hydrocarbons, urea generally forms a hexagonal structure surrounding a channel-like void space (Fig. 1) (17, 35–37), although the formation of a rhombohedral structure has been reported for some long chain hydrocarbons

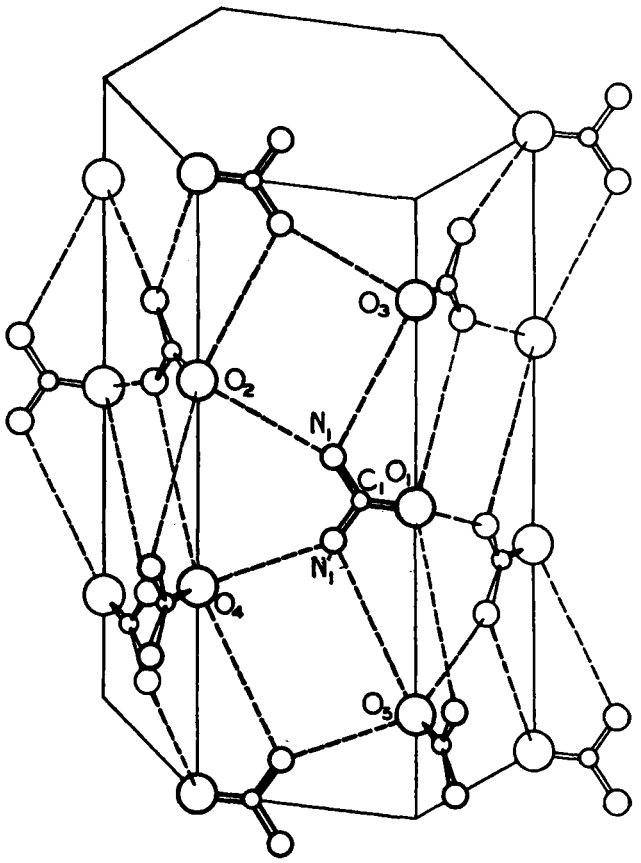

Figure 1—*Arrangement of hydrogen bonding in urea–n-hydrocarbon complexes. (Reprinted, with permission from Van Nostrand Reinhold Co., from M. Hagan, "Clathrate Inclusion Compounds," Litton Educational Publishing, 1962, and, with permission, from Ref. 36.)*

(38). The overall structure of the crystal is that of parallel tubes in which the hydrocarbon molecules are enclosed. The molecules of urea are held together in a loose network of hydrogen bonds between the nitrogen and oxygen atoms (36). The cavities have a diameter of approximately 5 Å, which is sufficient to accommodate straight-chain hydrocarbons and fatty acids or their derivatives. Nonwhole number molal ratios of urea to guest are found such as 6.3 moles of urea/mole of *n*-octane, 3.78 moles of urea/mole of methyl ethyl ketone, and 12.2 moles of urea/mole of oleic acid (39).

At room temperature, substances of a chain length of six or more carbon atoms form complexes with urea, while certain shorter chain-length compounds form urea inclusion compounds only at lower temperatures. In the absence of a guest component, urea crystallizes in a tetragonal arrangement. The structure is relatively open with low van der Waals interactions between the molecules (36). In the presence of a suitable size guest molecule, urea assumes the hexagonal arrangement around the guest, the combination producing a denser structure than that of the tetragonal arrangement. The inclusion compound is thus energetically favored. Schlenk (40) indicated an energy of formation of about 2.8 kcal/methylene group of the guest.

Several studies (34, 41) provided evidence for the

[1] These techniques were discussed in the review article by J. K. Haleblian, *J. Pharm. Sci.*, 64, 1269(1975).

solubilization of cetane, stearic acid, and *n*-valeric acid in saturated solutions of urea; this solubilization was not found for molecules that did not form urea inclusion compounds. The isomer of *n*-valeric acid, α-methylbutyric acid, does not form an inclusion compound and is soluble to the same extent in saturated urea solution as in water. The limited solubility of the isomer also indicates that the carboxyl group does not interact with the urea.

Many other substances form inclusion compounds with urea, including amino acids (42), epoxyesters (42), trioxane (43), aliphatic dinitriles (44), acrylic acid (45), and 1,2-bromotricosane (46). Urea inclusion compounds also have been used to separate racemic mixtures into optical antipodes (42, 47); the guest molecule may be collected by thermal or solvent decomposition of the inclusion compound (10, 42, 48). Fetterly (41) suggested that fatty acids dimerize in a urea inclusion compound, increasing the effective bonding by replacing a van der Waals bond by two hydrogen bonds. Thus, fatty acid–urea inclusion compounds are more stable in water solutions than the corresponding *n*-aliphatic hydrocarbon–urea complex.

As indicated earlier, the urea channel has a diameter of approximately 5 Å, limiting entrance to unbranched hydrocarbons. However, the sulfur analog of urea, thiourea, which also forms channel-like complexes, has a channel diameter of approximately 7 Å due to the larger size of the sulfur atom (36). A broad range of compounds can enter the larger thiourea cavity, including cyclic paraffins and polymethylated and polychlorinated hydrocarbons (49–51). Straight-chain hydrocarbons cannot form inclusion compounds with thiourea because the host framework is not stable and collapses. Since thiourea inclusion compounds do not give continuous layer lines in an X-ray pattern, it has been concluded that the guest molecule is fixed preferentially at certain planes in the channel.

The ability of urea and thiourea to differentiate between unbranched and branched molecules has led to significant industrial applications of these inclusion compounds (52–56). Numerous patents have been issued on processes utilizing the ability of the urea and thiourea inclusion compounds to separate mixtures effectively by removing molecules of a particular size and shape. Most of these applications are in the petroleum industry and involve the separation of hydrocarbon mixtures.

An example of such a process is the removal of high melting-point paraffins, which tend to solidify at low temperatures, from various oils. The straight-chain hydrocarbons are removed from the petroleum fraction by mixing the oil with urea and allowing the formation of the urea channel compounds. The inclusion compound is filtered or allowed to settle, and the supernate is decanted. Similar improvements can be made in other petroleum fractions where it is desired to remove naphthenes, terpenes, or other cyclic hydrocarbons. Both batch and fluidized-bed methods have been developed for these processes (34). The rate of inclusion compound formation is subject to the presence of inhibitors or activators (49, 57, 58).

The free fatty acids in acid-cracked lanolin can be removed as urea complexes, leaving neutral lanolin (59). The fatty acids can be collected by thermally decomposing the inclusion compound and then utilized for other purposes. The selective affinities of urea and thiourea can be used to improve ozokerites and other hydrocarbons of cosmetic and pharmaceutical interest (60).

One interesting aspect of urea and thiourea inclusion compounds is their use to increase the stability of easily oxidized substances. Vitamin A (61), unsaturated fatty acids (62), *d-trans*-oxocamphor (63), turmerone (63), decalin (64), and terpenes of orange oil and lemon oil and related compounds (65) have been stored as channel complexes. As long as the guest remains within the channel, it is protected from attack by atmospheric oxygen or from hydrolysis.

A novel solution to the handling of viscous, adhesive materials and potent or dangerous compounds is the formation of a urea or thiourea inclusion compound with appropriate substances in these categories. Liquid nonionic polyoxyethylene surfactants have been complexed with urea to form a solid powder which is easily handled (66–68). Straight-chain alkyl ethers, esters, and fatty acid derivatives of the polyoxyethylene surfactants form inclusion compounds, whereas sorbitan alkylate and alkyl aryl ethers of the polyoxyethylene series do not due to the large size of the sorbitan moiety and of the benzene ring. Likewise, the handling of potent insecticides such as chlordane is facilitated if the chlordane is in the form of a solid thiourea–chlordane inclusion complex (69, 70).

Cadwallader and Islam (71) prepared tablets of a dry, solid urea–benzalkonium chloride inclusion compound by direct compression.

Polymerization within inclusion compounds was first reported by Clasen (72) in 1956, who carried out stereospecific polymerization reactions of guest molecules within urea and thiourea host crystals. Brown and White (73) and White (74) irradiated monomers such as vinyl chloride and butadiene within their urea hosts and 2,3-dimethylbutadiene, 2,3-dichlorobutadiene, and 1,3-cyclohexadiene in a thiourea complex to form their respective polymers. The channels acted as smooth, straight templates holding the guest monomers, producing 1,4-*trans* addition polymers. The polymers obtained were crystalline and had higher melting points than polymers obtained by irradiation of the liquid monomers. Ivanov *et al.* (75) polymerized piperylene in the channels of urea and reported that the channel polymer had a higher degree of unsaturation (95–98%) than did polymers formed by other means (88%).

The formation of radicals during γ-irradiation of hydrocarbons (76), alkyl halides (77), and fatty acids (78) was recently reported.

The major limitation to this polymerization process is the limited number of molecules of proper size and shape able to reside in the urea or thiourea channels. The use of the channel as the template for the polymerization requires a reasonably precise fit of

the monomer within the channel.

Choleic Acids—The second group of polymolecular inclusion compounds forming channel-like void spaces are the choleic acids. Deoxycholic acid, a bile acid, forms channel-like inclusion compounds with numerous compounds including carboxylic acids, esters, alcohols, ethers, phenols, and hydrocarbons (79, 80). Apocholic acid (8,14-dehydrodeoxycholic acid) also forms similar inclusion compounds; however, the other naturally occurring bile acids are not known to form these complexes, apparently due to the absence of hydroxy groups at C-3 and C-12 (81).

Wieland and Sorge (22) first reported the composition of a choleic acid. Two products were evolved by thermal decomposition of the choleic acid *in vacuo:* deoxycholic acid (92–94%) and a waxy mixture of stearic and palmitic acids (6–8%). Excess palmitic, stearic, and oleic acids can be added to hot alcoholic solutions of deoxycholic acid to precipitate choleic acids of the same solubility and melting point, which can be distinguished only by crystallographic analysis (82). Fourier analysis of the structure of the deoxycholic acid–palmitic acid inclusion compound indicated that the deoxycholic acid molecules formed a framework around the axis of the guest molecules in which the host molecules were linked by hydrogen bonds (82); the host and guest molecules interacted *via* van der Waals forces. Similar results were found by Fourier analyses of choleic acids formed with other fatty acids. Since the choleic acids of C_4–C_{26} normal fatty acids had identical unit cells, they could not be coordination compounds.

Cramer (83) later indicated that a choleic acid is an inclusion compound with fatty acid guest molecules oriented in an end-to-end arrangement within the open channels of the deoxycholic acid crystal. An interesting aspect of the combination of these two unreactive components is that the nature of the molar ratio of the components differs from that in the urea–hydrocarbon inclusion compounds. Nonwhole number ratios were determined for the latter complexes, whereas the choleic acids are characterized by whole number ratios such as four molecules of deoxycholic acid to one of C_4–C_8 acid, six to one of C_9–C_{14} acid, and eight to one of C_{15}–C_{29} acid.

The preparation of choleic acids is not a difficult process. Both host and guest are dissolved in an appropriate hot solvent, *e.g.,* absolute methanol or ethanol, and then slowly cooled to permit crystallization of the choleic acid. The crystals can then be collected and washed, using small amounts of solvent to avoid dissociating the complex. It must be kept in mind that the choleic acids are readily dissociated in water.

The properties of certain substances are modified when they form choleic acids (81). Not only is the resistance to degradation increased, but other properties are influenced as well. For example, ethyl acetoacetate in liquid form or in solution contains 8–13% of the enol form, whereas it is 100% enolized in its choleic acid form. Likewise, acetylacetone is 70% enol in solution and 100% when complexed with deoxycholic acid. When the choleic acid crystals are dissolved, they dissociate and the liberated enol is ketonized at a measurable rate until tautomeric equilibrium is achieved. Racemic mixtures also have been separated by selective choleic acid formation; partial separations have also been accomplished for camphor, *sec*-butylpicramide, methylethylacetic acid, and α-terpineol (84, 85).

The stabilization of autoxidizable compounds by formation of a choleic acid has proved to be a fruitful area, producing inclusion compounds that are highly resistant to degradation not only by oxidation but also by heat and by chemical attack. Choleic acids of vitamins K_1 and K_3 were prepared (86), and complexes of vitamins D_2 and D_3 with deoxycholic acid and apocholic acid were patented (87). Crystalline choleic acids were formed that had vitamin activity equal to that of the uncomplexed vitamins but were significantly more stable with time. Likewise, stable choleic acids with high vitamin activity were prepared from vitamin A and vitamin A palmitate (66, 88–90).

One such crystalline choleic acid, "gallesterol," prepared by treating an egg-yolk preparation with deoxycholic acid, contained approximately 16.8% of highly stable vitamin A (91). The stoichiometry of the vitamin A choleic acids has been variously reported as one molecule of vitamin A to five (91) or eight (92) molecules of deoxycholic acid. By contrast, choleic acids of β-carotene, a precursor to vitamin A, have been reported (93) to be four molecules of deoxycholic acid per molecule of β-carotene. Other stable choleic acids include those prepared from deoxycholic acid and linoleic acid (88), linolenic acid (88), methyl linolenate (88), cinnamaldehyde (88), pentaerythritol tetranitrate (94), 2-methyl-1,4-naphthoquinone (95), and various aromatic solvents (96).

The earliest pharmaceutical use of choleic acid complexes was the preparation of stable choleic acid inclusion compounds of camphor with deoxycholic acid ("codechol") and apocholic acid ("camphochol") (97). The complexes were determined to have a guest–host ratio of either 1:1 or 1:2. "Cholosulin," the choleic acid of insulin and deoxycholic acid, was prepared and investigated (98); the absence of a pharmacological effect of the orally administered complex was reported. There is no direct evidence of the bioavailability of the choleic acids, although it has been indicated that choleic acids of streptomycin sulfate and of dihydrostreptomycin sulfate and its base could be formulated in solution, parenteral, emulsion, powder, tablet, and ointment dosage forms (99). One does not necessarily question the stability of the complex and of the guest molecule in the including structure; however, in the absence of evidence to the contrary, the bioavailability of the drug from such preparations must remain questionable and would be contingent upon the rate of dissociability of the complex and the site and conditions under which it occurred.

Indeed, such evidence was provided on the retardation of salicylic acid excretion after the oral administration to humans of a suspension of a deoxycholic acid–salicylic acid inclusion compound (100). The bioavailability of salicylic acid from the choleic acid

was less than that of salicylic acid from the administration of an equivalent dose of salicylic acid in solution. The difference in bioavailability was presumably due to the acid stability of the choleic acid. Micronization of the choleic acid increased the bioavailability of the salicylic acid. The stability of choleic acids also was studied under tablet manufacturing conditions (101).

Deoxycholic acid is limited in aqueous solubility, but its salts are highly water soluble. The salts are surface active, and micellar solubilization of lipids by these compounds is considered to be part of the mechanism by which fats are absorbed from the GI tract. It would be expected that a choleic acid would be reasonably stable at gastric pH but unstable as pH increased. The guest molecule would then be released in the intestine at a pH-dependent rate. Depending upon the site at which this release occurs, the guest may or may not be well absorbed, if it is absorbed at all. Since the bile salts, including deoxycholate salts, are believed to form cylindrical (channel-like) micelles containing solubilized lipids (102), in the broadest sense, micellar solubilization may be considered to be a form of polymolecular inclusion in solution, albeit that the nature of the interaction of the solubilizate, the guest component, with the surfactant host molecules does not necessarily fit the generalized "no bond" mechanism of the host–guest interaction characteristic of inclusion compounds.

Except for lamellar structures, micelles do reach a limiting size and shape, dependent upon the nature of the solubilizate, surfactant, and solvent. The surfactant monomers within the micelle are joined by van der Waals forces and by electrostatic bonds to form an essentially two-dimensional liquid crystalline structure enclosing (enclathrating?) the solubilizate. Thus, in the case of the choleic acids, one form of an inclusion compound may be exchanged for another, depending on pH and other conditions.

Several other steroids are thought to form inclusion compounds. Mesley (103) reported the inclusion of chloroform within lattices formed by hydrocortisone, dexamethasone acetate, and prednisone.

4,4′-Dinitrobiphenyl—These polymolecular inclusion compounds are formed by 4,4′-dinitrobiphenyl with certain 4,4′-substituted biphenyl derivatives (104). 4,4′-Dinitrobiphenyl molecules were described as being stacked in a face-centered arrangement so as to form channels that enclose the guest molecule (105, 106) (Fig. 2). The abundance of π-electrons electrostatically influences the guest molecules, which are generally nucleophilic and include benzidine, biphenyl, 4-bromobiphenyl, 4-iodobiphenyl, 4-hydroxybiphenyl, 4-aminobiphenyl, *N,N,N′,N′*-tetramethylbenzidine, 4,4′-dimethoxybiphenyl, 4-acetoxybiphenyl, and 4,4′-diacetoxybiphenyl.

Compounds Forming Cage-Like Void Spaces—Inclusion complexes formed by compounds in this category consist of a cage-like structure entrapping a guest molecule. The individual host molecules link together to form a cage or a cup-shaped structure, the open end of which joins with the open end of a second similar structure to form the cage. Because of the characteristic structure of these inclusion compounds, Powell (18) named them clathrates.

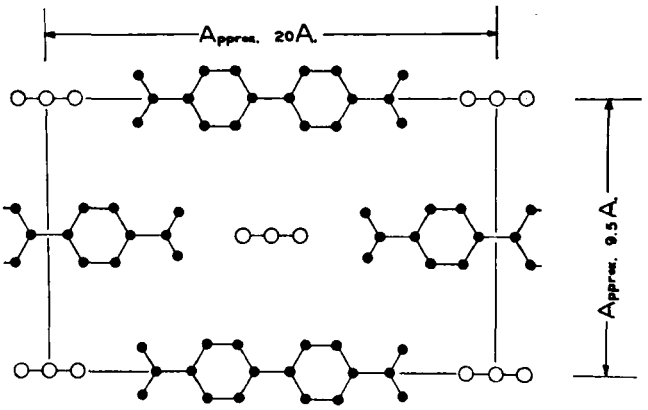

Figure 2—*Cross section of an idealized structure of the dinitrobiphenyl adducts. (Reprinted, with permission from the Eastman Kodak Co., from Ref. 24.)*

Hydroquinone—The hydroquinone–hydrogen sulfide complex was first described in 1849 (107). Mylius (15) later determined that whatever bonding was occurring in the hydroquinone–formic acid complexes he was investigating, it was not of the conventional types. He concluded that one molecule was enclosing another in a relatively stable structure. Examples of this class of inclusion compounds are those formed between hydroquinone and methanol, sulfur dioxide, hydrogen sulfide, hydrogen chloride, or other substances of a suitable size and shape.

Powell (18, 108–112) extensively studied the formation and properties of these complexes. He determined that six hydroquinone molecules are linked by hydrogen bonding to form a large open structure, which may be visualized as consisting of two interpenetrating "cups," each cup formed by three hydroquinone molecules. The hydroquinone molecules in each cup are hydrogen bonded at one end, with the molecules tilted at an angle outward from a plane perpendicular to the hexagonal plane of the hydrogen-bonded hydroxy groups (Fig. 3). The open ends of two such cups interpenetrate to form the cage. Alternate hydroquinone molecules incline upward or downward from a plane hexagon formed by the six oxygen atoms of the six hydroquinone molecules.

Hagan (10) and Brown (11) published excellent diagrams of these structures. The individual hydroquinone structures are not chemically bonded to each other but form an infinite framework. The cage-like framework encloses void spaces approximately 4 Å in diameter, from which the guest molecules cannot escape. Hydroquinone clathrates are formed only by molecules that can fit inside the cavity; molecules small enough to slip out of the cage do not form a clathrate.

Hydroquinone and similar clathrates are prepared by saturating a solution of the host component with the guest. The framework gradually develops and the clathrate forms as a crystalline solid. Some systems are more difficult to prepare and require high gas pressures and/or temperature adjustments to ensure

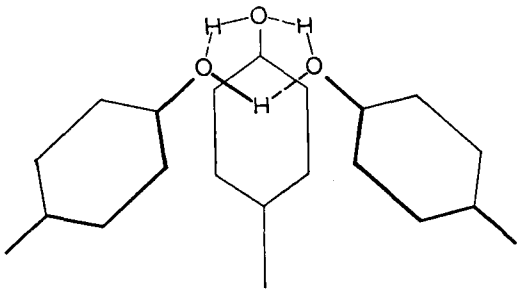

Figure 3—*"Cup" of three quinol molecules formed by hydrogen bonds. (Reprinted, with permission, from Ref. 6.)*

the desired ratio of host to guest to initiate crystallization. A less than stoichiometric ratio may result, indicating that not all of the void spaces are occupied. For a clathrate to form, the guest must be in place when the host framework forms. It is also possible that the solvent may be included if of a suitable molecular size. Solvent clathrates form in the absence of any other guest compound or, alternatively, the solvent molecule may compete with a suitable guest for inclusion.

The crystals that form are very stable; odors, if any, of guest gas molecules cannot be detected. However, the clathrate may be dissociated by heating, by dissolution in water, or by crushing or grinding the crystals (113–115).

Numerous compounds form clathrates with hydroquinone including gases, thiophene, benzene, furan, pyrroles, phenol, hydrochloric acid, and methane (6, 10, 12). The important criterion is the magnitude of the molecular volume of the guest component relative to the volume of the cage; that is, the clathration process is essentially physical in nature, not chemical.

Studies of the thermodynamics of the formation of hydroquinone clathrates of methanol and sulfur dioxide have indicated that the heat of formation of these clathrates is zero and that the free energy differences between the clathrates and the pure components is entropic in nature (116). Aside from crystal lattice interactions, the interactions of the guest and host molecule in hydroquinone clathrates are the only significant forces (117). Evans and Richards (118) suggested that such clathrates thermodynamically resemble dilute solutions.

Van der Waals and Platteeuw (119) proposed a statistical mechanical treatment for the clathrate system, consisting of three phases in equilibrium: clathrate, nonclathrate modification of host (polymorphic changes), and pure nonpolar gas, assuming only one guest molecule per host void space. The guest molecule can move within the cage, but the potential energy of the guest does not entirely depend on the sum of the two terms depending on the potential and orientation coordinates but also on the vibrational state of the molecule moving in its cage. The rotational contribution to the free energy depends on the vibrational state; the molecule loses orientational freedom when enclathrated, and this effect is greater for polyatomic molecules than for nonatomic gases.

Hydroquinone clathrates have found use in extractive crystallization by selective clathration. Rare gases are good examples. Argon may be separated from neon by adjustment of the conditions under which the clathrate forms to conditions specific for one gas or the other, and krypton may be separated from xenon (120). Selective clathration offers great potential for the removal of suitable solutes from solution and for the recovery of impurities or trace compounds.

The good storage capability and ease of handling of the hydroquinone clathrate crystals were utilized in a novel method for the handling of radioactive krypton, ^{85}Kr (121). The dry, crystalline hydroquinone–^{85}Kr clathrate was easy to handle when compared to the use of the heavy, bulky cylinders normally used to store this gas under pressure. The clathrate crystals produced radiation approximately 25 times as intense as that of an equal volume of gas at atmospheric pressure. The clathrate forms an almost leakproof container for the ^{85}Kr gas, which can be powdered and compacted with a minimal loss of radioactivity. These stable radioactive sources find a number of applications wherever ^{85}Kr is used to eliminate static and measure thickness and in other uses of β-radiation such as those of Schuler *et al.* (122) who measured acute changes in regional blood flow by intramuscular administration of ^{85}Kr clathrate.

Water—Under certain circumstances, water molecules form a crystalline clathrate framework containing cavities in which gases or low boiling-point liquids can be entrapped. Such clathrates are termed "gas hydrates" or "liquid hydrates." Compounds with chemically bound water of crystallization are also called hydrates but differ from clathrates in the nature of the bonds holding the water and in the structural arrangement of associated water molecules.

Davy first reported the chlorine hydrate in 1811. Subsequently, Faraday, Lowing, and others analyzed this complex and others, but much controversy raged concerning the exact nature of the inclusion complexes (123). It was not until X-ray diffraction studies of the gas hydrates that the water clathrates were determined to be solid solutions of a gas or a gas contained in a metastable water lattice.

The water clathrate framework resembles the ice lattice in that each oxygen atom is hydrogen bonded to four other oxygen atoms. The hydrogen bonds in ice have a length of 2.76 Å, virtually the same as in the clathrate (2.75 Å). X-ray diffraction studies have shown that water molecules in the clathrate framework are joined mostly in rings of five molecules rather than of six as in ice (124–134). The pentagons are oriented to form dodecahedrons, which are the "building blocks" of the clathrate structure. Since the dodecahedrons cannot be perfectly packed, void spaces are formed; it is in these spaces that the guest molecules are held (Fig. 4). Ordinary ice also has void spaces, but they are not of sufficient capacity to hold molecules larger than helium or hydrogen.

There are three classes of water clathrates, but the first two are most often referred to in the literature

Table I—Polyhedral Hydrates[a]

Class I	Class II	Class III
Guest Molecules		
Ar, Kr, Cl_2, H_2S, PH_3, SO_2, $C_2H_5NH_2$, $(CH_3)_2NH$, CH_2CH_2O, $CH_2CH_2CH_2O$, CH_4, C_2H_2, C_2H_4, C_2H_6, CH_3Cl, CH_3SH, CH_3CHF_2, CH_2CHF	$CHCl_3$, CH_3CHCl_2, $(CH_3)_2O$, C_3H_8, $(CH_3)_3CH$, C_3H_7Br, $(CH_3)_2CO$, C_6H_6, $CH_2CH_2CH_2CH_2O$, cyclo-C_6H_{12}, $CH_2CH_2OCH_2CH_2O$	$(n\text{-}C_4H_9)_4N^+F^-$, $(n\text{-}C_4H_9)_4N^+{}^-O_2CC_6H_5$, $[(n\text{-}C_4H_9)_4N^+]_2WO_4^{2-}$, $(\text{iso-}C_5H_{11})_4N^+F^-$, $(n\text{-}C_4H_9)_3S^+F^-$, $(n\text{-}C_4H_9)_4P^+Cl^-$, $(CH_3)_3N$, $(CH_2)_6N_4$, $n\text{-}C_3H_7NH_2$, $(CH_3)_4N^+OH^-$, $\text{iso-}C_3H_7NH_2$, $(C_2H_5)_2NH$, $(CH_3)_3C\text{-}NH_2$, C_4H_9OH
Stoichiometry		
$M\cdot5\frac{3}{4}H_2O$, $M\cdot7\frac{2}{3}H_2O$	$M\cdot17H_2O$, $M\cdot M'_2\cdot17H_2O$	$M\cdot(5\text{–}40)H_2O$
Unit Cell		
$46H_2O$	$136H_2O$	Variable number of H_2O
Polyhedra	Polyhedra	Polyhedra
2 H_{12}(5 Å)	16 H_{12}(5 Å)	H_8, H_{12}, H_{14}, H_{15}
6 H_{14} (6 Å)	8 H_{16}(7 Å)	H_{16}, H_{17}, H_{18}, H_{60}, *etc.*
Faces	Faces	Faces
Pentagons	Pentagons	Quadrilaterals
Hexagons	Hexagons	Hexagons
		Heptagons
$M_2\cdot M_6\cdot46H_2O$	$M_8\cdot M'_{16}\cdot136H_2O$	

[a] Reprinted, with permission, from Ref. 135, p. 109. (H_n symbolizes a polyhedron with n faces.)

(Table I). Water clathrates consisting of 46 water molecules associated with eight guest molecules in each unit cell are in the first classification. The unit cell has eight cavities; two are dodecahedrons with a diameter of 5 Å, while the remaining six are tetrakaidecahedrons with a diameter of 6 Å (Fig. 4). As indicated in Table I, many small molecules can form clathrates with water. If only the larger cavities are occupied, the stoichiometry is $M_6\cdot46H_2O$ (where M is an inert gas molecule); however, mixed hydrates are possible, with the stoichiometric formula of $M_2\cdot M_6\cdot46H_2O$ (135).

The second class of water clathrates forms with larger guest molecules (Table I). The unit cell consists of 136 water molecules oriented as dodecahedra enclosing 24 interstitial spaces, 16 5 Å in diameter and eight 7 Å in diameter (Fig. 5). The molecules listed in Table I would occupy the larger cavities, although mixed hydrates form with smaller molecules occupying the 16 smaller cavities. In the third class of water hydrates, the cage may be formed of portions of the simpler polyhedra (136, 137). There is no specified unit cell to describe the third class of clathrates, because each guest molecule requires a specific surrounding cage.

The water clathrates are stabilized by the energy of the hydrogen bonds of the water framework, al-

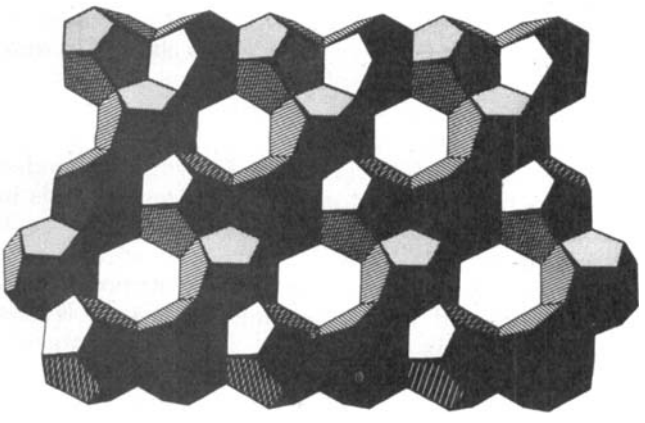

Figure 4—*Structure of the type I hydrate. The tetrakaidecahedral cavities (12 pentagonal plus two hexagonal faces) are formed when layers of dodecahedra, arranged as shown, are stacked on top of each other so that the hexagonal openings line up perpendicular to the plane of the drawing. (Reprinted, with permission, from J. F. Catchpool, in "Structural Chemistry and Molecular Biology," A. Rich and N. Davidson, Eds., Freeman, San Francisco, Calif., 1968, p. 345.)*

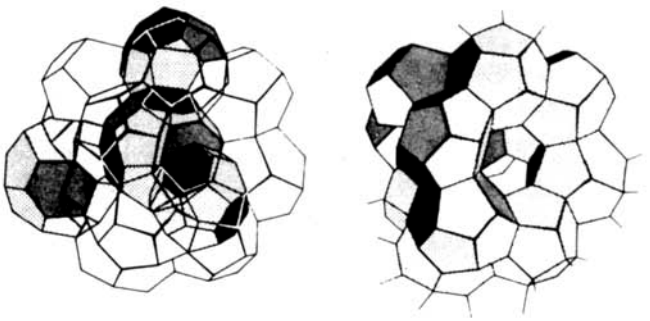

Figure 5—*Two drawings of a folded-paper model of the hexakaidecahedral cavity of the type II hydrate structure. The 16-sided cavities are formed between 16 dodecahedra framing four hexagonal faces. The plane of each hexagonal face lies on the side of a regular tetrahedron. Each hexagonal face is shared by two hexakaidecahedra. (Reprinted, with permission, from J. F. Catchpool, in "Structural Chemistry and Molecular Biology," A. Rich and N. Davidson, Eds., Freeman, San Francisco, Calif., 1968, p. 345.)*

though the hydrogen bond stabilization of the clathrate is less than that in ice due to the more open clathrate structure (138). Van der Waals interactions between the guest and host molecules contribute, however, to the high degree of the overall stability of the clathrate.

A statistical mechanical model for several gas hydrates based on the Lennard–Jones and Devonshire model has been described (139) and the dissociation pressures, compositions, and heats of dissociation were calculated. Daee *et al.* (140) determined that the Helmholtz free energy of formation of the water clathrate was not a smoothly increasing function of the number of molecules but instead showed a minimum corresponding to closed cages. Phase diagrams for several water clathrate systems have been reported (141–143). Calculations of water vapor pressure over a crystalline gas hydrate indicated the formation of the clathrate structure (144). Phase equilibrium studies indicated the existence of mixed hydrates and of multiple hydrates where more than one guest can occupy a cavity (145). Multiple occupancy is limited to small guest molecules such as neon.

The stable clathrate crystals form when water and a gas or volatile liquid are brought together under high pressures and low temperatures. This process can be used, under proper conditions, for the separation of gas (146) or liquid (147) mixtures. Argon can be separated from krypton, krypton from xenon, and short chain (C_3 and C_4) aliphatic olefinic hydrocarbons from mixtures of higher molecular weight compounds. One interesting and novel approach to the desalination of water depends upon the formation of a propane or halogenated propane clathrate in sea water under reduced pressure and temperature (148). The water clathrate that forms can be separated from the salt water and decomposed by heating to 7.2° (45° F) under pressure to give propane and salt-free water. However, water clathrates do not form in highly concentrated salt solutions (149). The melting temperature of clathrates with a high proportion of empty cavities decreases with increasing pressure while the reverse holds for clathrates with a small proportion of empty cavities (150).

Clathrates are formed not only by inert gases but by hydrocarbons and their derivatives (151, 152), inorganic gases, and a number of polar compounds (Table I). Some guests interact with, or are joined to, the water lattice while others rotate freely (153, 154). Pauling (138) proposed an intriguing mechanism for the action of anesthetic gases based upon the formation of minute water clathrate crystals in the brain. He suggested that the anesthetic actions of certain gases of low chemical reactivity (*e.g.*, chloroform and cyclopropane) may result from clathrate formation in the encephalonic fluid. According to this theory, the clathrates would trap ions and electrically charged side chains of protein molecules in such a way as to decrease the energy of electric oscillations in the brain. (Figure 6 shows a diagram of a similar system.) This process would tend to produce a loss of consciousness due to increased electrical impedence of the neural network to electrical waves.

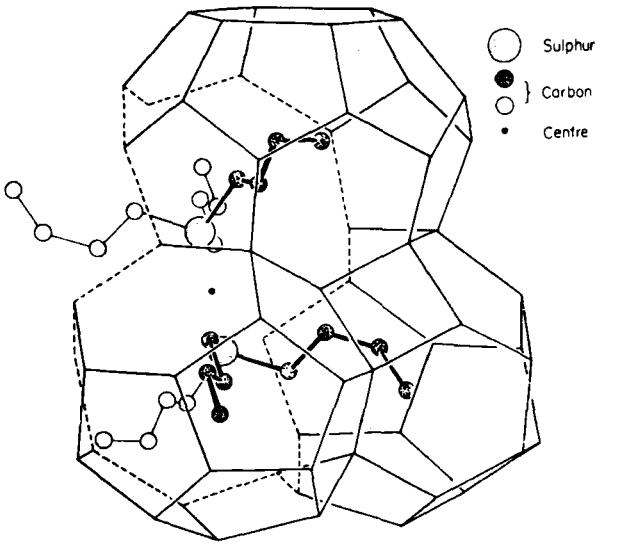

Figure 6—*Projection of clathrate cavity containing a pair of $(C_4H_9)_3S^+$ ions. Only one-half of the cavity is shown, that into which the shaded carbon chains project. The second half surrounds the open-circle carbon chains. The complete polyhedron is constituted of 60 faces. (Reprinted, with permission, from Ref. 135, p. 111.)*

In support of his theory, Pauling determined that a correlation exists between the narcotizing partial pressure of the anesthetic gases and the partial pressure necessary to cause the formation of hydrate crystals. As Pauling indicated, however, approximately the same correlation would be found between the anesthetizing partial pressure of the nonhydrogen-bonding anesthetic agents and any property based upon the van der Waals attraction of the agents for other molecules, *e.g.*, the lipid solubility of the anesthetic gases or the oil–water partition coefficient. The energy of the intermolecular interactions is nearly proportional to the molecular polarizability of the anesthetic agents. If water clathrates do indeed form in the brain and do have a biological function, then it would be a logical extension of this phenomenon to expect their occurrence in other body fluids. This interesting prospect is worthy of further investigation.

Abundant information attests to the formation of hydrate "icebergs" around many ions and proteins in aqueous systems. Included in this category are the trialkylsulfonium and tetraalkylammonium salts. Although the trialkylsulfonium salts and tetraalkylammonium salts are univalent electrolytes, they show a behavior similar to that of the water clathrates (11, 155). Typical formulas for each of these compounds are $2(n\text{-}C_4H_9)_3S^+F^-\cdot 40H_2O$ and $2(\text{iso-}C_5H_{11})_4N^+F^-\cdot 76H_2O$, respectively.

Dyadin *et al.* studied the influence of cation size on clathrate formation in water–quaternary ammonium salt systems (156) and determined the phase diagram for the water–tetra-*n*-butylammonium bromide system (157). Wen (158) reviewed the formation of clathrates of tetraalkylammonium salts.

The crystal lattice of the tri-*n*-butylsulfonium compound resembles the Class I water clathrate with

46 water molecules in the unit cell, except that six of the water molecules are replaced by the two sulfur ions and by the two fluorine ions. The remaining 40 water molecules form the clathrate framework. The interstitial cavities are occupied by the six *n*-butyl hydrocarbon chains rather than by gas molecules. The tri-*n*-butylsulfonium compound is considered to be structurally similar to the chlorine hydrate. McMullan and Jeffrey (155) established the structural arrangement of tetraalkylammonium salts to be similar to the water clathrates. An interesting aspect of the clathrate is that it is highly stable. Although the inclusion complex is 68% water, it remains stable as an ice up to 31.1° (88° F).

The proteins are also highly hydrated crystals with thousands of water molecules per molecule of protein, comprising as much as 90% of the crystal; as such, a water clathrate structure may be expected to exist around the protein molecule. The presence or absence of the water clathrate surrounding the protein molecules may influence the biological function (or lack of function) of the protein. In addition, protein molecules themselves appear to resemble host compounds in that the disulfide bridges between protein layers create spaces between the layers that are capable of enclosing certain hydrocarbons (159).

Phenol—Investigation of the solubility of components of petroleum in liquid hydrogen sulfide led to the discovery of the phenol–hydrogen sulfide clathrate (160). Phenol forms a clathrate with various compounds, including xenon; hydrogen selenide, chloride, bromide, iodide, and sulfide; carbon dioxide, carbon disulfide, and carbon disulfide plus air; carbonyl sulfide; methyl bromide; methylene chloride; tetrafluoroethylene; and 1,1-difluoroethane (161, 162). Lahr and Williams (163) also reported the formation of phenol clathrates with argon and krypton. Some derivatives of phenol also formed clathrates (12, 164). The phenol clathrates were prepared by dissolving phenol in solutions of the guest component or, in the case of gases, dissolution of the gas under pressure. The phenol clathrates separated readily from the solution and were stable at room temperature, but there may be a slight diffusional loss of gas with time.

As described by Mandelcorn (9), the structure of the phenol clathrate depends upon hydrogen-bonded O—O linkages formed by hydroxy groups of six phenol molecules, the linked molecules forming a planar hexagon with the phenyl nuclei oriented alternately above and below the hexagon. A cage is formed when a pair of the six-membered groups are oriented with the hexagons opposite and parallel to one another. The unit cell is rhombohedral, with each corner occupied by the cage arrangement of the 12 phenol molecules, giving an elongated, rather large cage. The unit cell of the phenol clathrate consists of 12 phenol molecules, one large cavity, and a small cavity. Mandelcorn also described the maximum stoichiometry of the phenol clathrates as a function of the size of the guest component.

Dianin's Compound—Dianin's compound (4-*p*-hydroxyphenyl-2,2,4-trimethylchroman) is the condensation product of two molecules of phenol and one of mesityl oxide; upon alkaline fusion, it gives salicylic acid as a product. Dianin (165) observed that this compound retained fixed amounts of the solvent from which it was recrystallized. Several studies showed that Dianin's compound formed clathrates with sulfur dioxide, iodine, argon, and nearly 40 other organic compounds (166, 167). Other guest compounds include heptanol (168), di-*tert*-butyl nitroxide (169), and carbohydrates (170).

A complex structure was proposed in which six molecules of Dianin's compound formed a clathrate cavity (166). The structure has been described as being similar in shape to an hourglass from which the globes have been cut off in the middle. A hexagon of hydrogen-bonded hydroxy groups forms the constriction at the midpoint, and six molecules of Dianin's compound alternately point up or down to form the cups above and below the planar hexagons. These structures are stacked linearly upon each other in the crystal with their hexagons parallel to and above each other. The open end of two cups face each other to form a cavity when the adjacent molecules are held together by strong van der Waals attractions.

Detailed structural analysis by X-ray diffraction has been reported for crystalline inclusion compounds of Dianin's compound with ethanol or chloroform (171). Only one hydroxy group in Dianin's compound is available for hydrogen bonding, so an infinite network of cages does not occur. However, the stacking arrangement in the crystal gives separate columns of individual cages. The hourglass-shaped cage of Dianin's compound is large, approximately 11 Å long and 6.2 Å at its point of maximum width, or about twice as long as the hydroquinone cage.

Mandelcorn (9) compiled a list of the three maximum composition formulas for the Dianin's compound clathrates, depending upon the size of the guest component. Although there are no reported pharmaceutical uses of Dianin's compound clathrates, many simple synthetic precursors and solvents form complexes with Dianin's compound, suggesting that these inclusion compounds might be useful in drug synthesis or purification (172).

Cycloveratril—Formaldehyde and 1,2-dimethoxybenzene (veratrole) combine to form cycloveratril (Fig. 7), which forms numerous clathrates with organic compounds. Caglioti *et al.* (173) prepared clathrates of cycloveratril and benzene, chlorobenzene, chloroform, toluene, acetone, thiophene, carbon disulfide, butyric acid, acetic acid, and decalin. These workers proposed that the benzene rings of the cycloveratril molecule are tilted, giving a nonplanar configuration to the molecule. Strong repulsions between hydrogen atoms of adjacent benzene rings, through rotations of the aliphatic to aromatic carbons, are responsible for the tilting of the benzene rings. Packing of the nonplanar molecules in the crystal leaves small cavities in which guests of small molecular volume can be accommodated.

Miscellaneous Compounds—Over 75 years ago, Hofmann and Küspert (174) reported that a crystalline precipitate formed when benzene was added to a

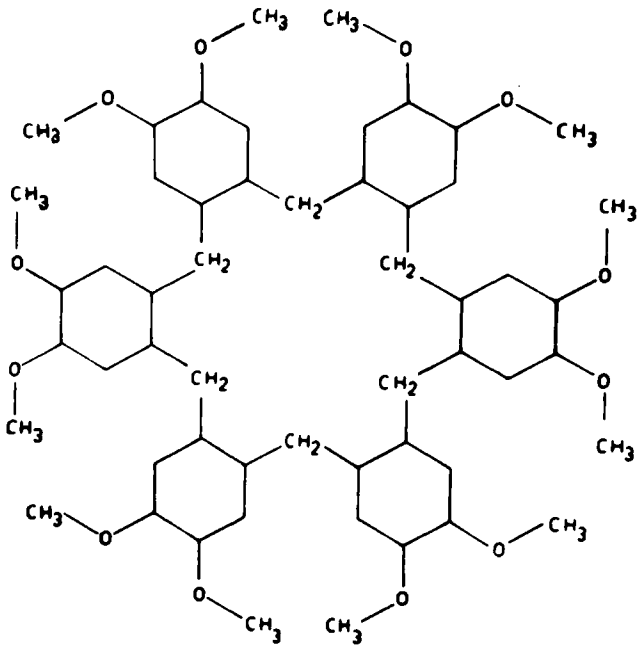

Figure 7—*Structural formula of cycloveratril. (Reprinted, with permission from Pergamon Press, from Ref. 173.)*

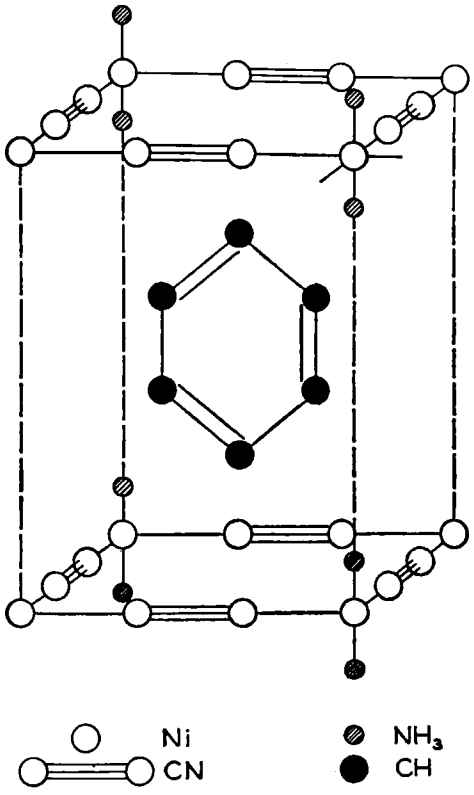

Figure 8—*Schematic representation of the clathrate compound, $Ni(CN)_2NH_3$–C_6H_6. (Reprinted, with permission, from Ref. 175.)*

solution of nickel cyanide in aqueous ammonia solution containing acetic acid. This report was the first on the formation of a clathrate of an organic molecule in an inorganic cage. Numerous organic compounds form clathrates with the nickel cyanide–ammonia complex (12), whose maximum composition formula is $Ni(NH_3)^{+2}2CN^-$·M, where M is the guest molecule. The guest molecules are enclosed in a cavity formed horizontally by two planes of nickel cyanide and vertically by parallel pairs of ammonia molecules. The ammonia molecules are bonded to the nickel atoms and project above and below each plane, giving rise to the cavity when the complex crystallizes (175) (Fig. 8).

The nickel cyanide–ammonia clathrates and similar clathrates are used for the separation of benzene from hydrocarbon mixtures by selective clathration (176). High purity benzene can be recovered from the clathrate under vacuum or by steam decomposition. Clathrates of this type are frequently called "Hofmann's benzene clathrate" if benzene is the guest component or "Hofmann-type clathrates" for other enclathrated guests.

Related to the monoamminonickel (II) cyanide clathrates are clathrates where certain Werner complexes or hexamethylisocyanidoferrous chloride act as the host component. The Werner complexes differ from the nickel cyanide–ammonia complex in that there are usually four neutral molecules and some anions coordinated with the metal ion. An example of a Werner complex forming clathrate compounds is tetra(4-methylpyridine)nickel (II) dithiocyanate. A number of transition elements forming Werner complexes also form clathrates including cobalt, manganese, iron, copper, and zinc. The basic nitrogen compound and the anion also may be varied; the latter may be mono- or polyatomic.

The clathrate compounds of Werner complexes are used industrially for separating and resolving organic mixtures (177–179) and for separating aromatic isomers in liquid phase chromatography (180).

The last of the cage-forming compounds is hexamethylisocyanidoferrous chloride. Water is the guest component in the clathrate, and the overall formula is $Fe(CNCH_3)_6^{+2}2Cl^-·3H_2O$. The $CNCH_3$ groups project octahedrally around each ferrous ion, the unit cell being composed of two hexagonal layers of the ferrous ion (181). No other clathrates of this compound are known.

Bhatnagar (12) discussed these clathrates extensively and there are numerous references in the literature.

Compounds Forming Either Channel or Cage Void Spaces—Tri-*o*-thymotide (182), a versatile host molecule, forms clathrate complexes with many organic compounds (25). The clathrates of tri-*o*-thymotide are unique in that they are arranged in trigonal spirals. The spirals can enlarge in all directions without any constraints, unlike those formed in the hydrogen-bonded hydroquinone framework in which an increase in size in one direction causes a decrease in size in the other.

The general formula is $2C_{33}H_{36}O_6$·M, when the guest molecule (M) has a length not exceeding 9.5 Å. However, very small molecules (*e.g.*, carbon dioxide) have a low probability of clathration. The structure of the clathrate is that of an elongated cage. For molecules longer than 9.5 Å, a channel-like structure is produced and the formula becomes $C_{33}H_{36}O_6$·*x*M,

where x decreases as the chain length of the guest increases.

The tri-*o*-thymotide clathrates are stable; certain complexes can be heated to 100° above the boiling point of the guest compound without loss of the guest. In addition, Lawton and Powell (25) observed spontaneous optical resolution with tri-*o*-thymotide clathrates. If tri-*o*-thymotide is crystallized from a racemic solvent, the cavities of any one crystal will enclose either the dextro- or levo-form of the solvent molecule. If the two different crystals are separated, the solvent can be likewise separated.

MONOMOLECULAR INCLUSION COMPOUNDS

By definition, monomolecular inclusion compounds represent the complexation of a single host molecule and a single guest molecule. The host molecule is characterized by the presence of a cavity or hole into which the guest molecule is inserted.

Cyclodextrins—The most thoroughly investigated monomolecular inclusion compounds are those formed by the cyclodextrins, which are also known as cycloamyloses and as Schardinger dextrins.

The cyclodextrins are water-soluble, nonreducing, macrocyclic polymers, containing glucose molecules joined by α-1,4-linkages (Fig. 9). The most common of these compounds are the α-, β-, and γ-cyclodextrins formed by six, seven, and eight glucose units, respectively. The ring-shaped molecule encloses a cavity of about 6, 8, and 10 Å in diameter for the α-, β-, and γ-cyclodextrins, respectively.

The cyclodextrins are produced by the degradation and cyclization of starch by an enzyme produced by *Bacillus macerans*. The biosynthesis and properties of the cyclodextrins have been discussed in detail (183–188).

Molecules of a suitable size and shape can be held within the cavity of a particular cyclodextrin by van der Waals forces. Advantage of this property is taken in the selective precipitation of the α-, β-, and γ-cyclodextrins from the crude mixture of cyclodextrins produced by the enzymatic process. Cyclohexane or trichloroethylene forms an insoluble inclusion compound with α-cyclodextrin, fluorobenzene or toluene forms an insoluble inclusion compound with β-cyclodextrin, and bromobenzene or anthracene forms one with the γ-cyclodextrin (184). The cyclodextrins readily form inclusion compounds with a great number of organic compounds, including many derivatives of benzene.

The inclusion compounds formed with apolar molecules are only slightly soluble in water (184), whereas those formed with polar molecules are moderately soluble (189, 190). As indicated by Lammers *et al.* (191), the structures of the cyclodextrin inclusion compound in solution and in the crystal differ significantly. In solution, the guest molecule occupies the cavity in the cyclodextrin host and the entire complex is surrounded and solvated by water molecules. In the crystal, however, the guest may be enclosed in a void space of a lattice and not necessarily by individual cyclodextrin molecules. This arrangement may result in the formation of nonstoichiometric inclusion compounds. Exceptions to this behavior are certain cyclodextrin derivatives that do not form lattice-structured inclusion compounds, *e.g.*, cyclodextrin–epichlorohydrin resins (192).

The cyclodextrin cavity was described in the earlier literature as being hydrocarbon in nature, based upon the assumption of a boat configuration of the glucose monomers. Later NMR and X-ray studies confirmed the C-1 chair conformation for the glucose molecules (193). The cyclodextrin molecule would then be arranged with primary and secondary hydroxy groups around the opening of the cavity on opposite ends of the torus with H-3, H-5, and H-6 located within the cavity and H-1, H-2, and H-4 located on the exterior. Cramer and Dietsche (194) proposed that the interior of the cavity behaves as a Lewis base due to its high electron density. Broser and Lautsch (195) suggested that the cyclodextrin was electrophilic due to the increased stability of an inclusion compound in solution with increased basicity of the guest component.

Inclusion of a guest molecule within the cyclodextrin cavity can be utilized to increase the solubility or stability of the guest species. As indicated earlier, many organic compounds form inclusion compounds with the cyclodextrins, including various molecules of pharmaceutical interest. Vitamin A and several unsaturated fatty acids have been stabilized by cyclodextrin inclusion, protecting them from exposure to oxygen and thereby retarding oxidation (88). Cohen and Lach (196) found that α- and β-cyclodextrins formed complexes with the hydroxybenzoic acids and *p*-hydroxybenzoates. Hydrogen bonding was determined to play a role in the formation of the complex, particularly with the hydroxybenzoic acid complex in aqueous solution.

These studies were later extended to include various other drug molecules including sulfadiazine, tetracycline, morphine, aspirin, benzocaine, ephedrine, and sorbic acid (197). In general, the smallest drug molecules showed the greatest complexing activity with the cyclodextrins, and those compounds with the lowest water solubility showed the greatest percent increase in solubility as a function of the concentration of cyclodextrin. Most compounds investigated were too large to fit in the cyclodextrin cavity, leading to the conclusion that the observed interaction was a combination of attractive forces (hydrogen bonding) and inclusion formation.

Pauli and Lach (198) reported that unsaturated phenyl-substituted carboxylic acids were less reactive with β-cyclodextrin than were the corresponding saturated acids. As the chain length of the saturated acids increased, giving greater separation of the phenyl ring and the carboxyl group, the interaction became stronger.

A large number of drug molecules such as reserpine, cortisone acetate, adiphenine, tripelennamine, and testosterone form inclusion compounds with β-cyclodextrin, even though they are too large to be completely included within the cyclodextrin cavity (199). The smaller the guest molecule, the greater is

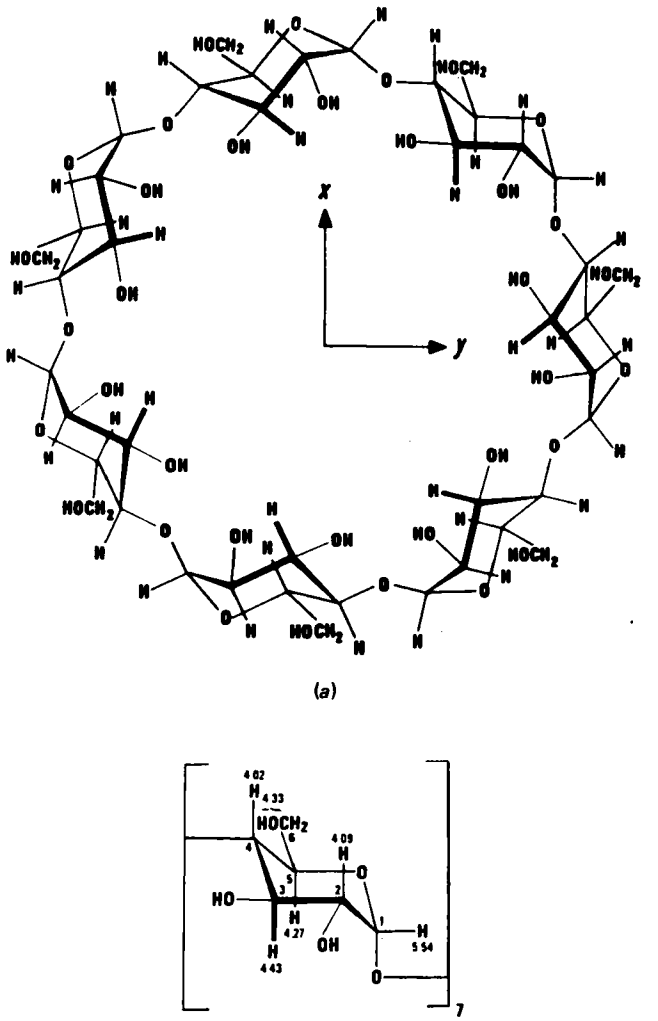

Figure 9—*(a) Cycloheptaamylose molecule. The z-axis is parallel to the cycloheptaamylose cavity, i.e., normal to plane of the page. (b) Recorded chemical shifts in parts per million for various cycloheptaamylose protons. (Reprinted, with permission, from Ref. 203.)*

its complexing activity. Likewise, the degree of activity for the large molecules is dependent on the presence of a suitable group or ring capable of entering the cavity. The ratio of the drug to β-cyclodextrin in the inclusion compounds was 1:1 (adiphenine and tripelennamine) or 2:1 (cortisone acetate and testosterone). The formation constants were relatively high, indicating stable complexes.

The interactions of β-cyclodextrin with barbiturates (200), copper II (201), and several prostaglandins (202) also have been reported. The insoluble inclusion compound of the prostaglandins was collected, dried, and reported to be formulated into suspension, capsule, tablet, suppository, topical, and parenteral dosage forms (202).

Investigations of the apparent formation of inclusion compounds between cyclodextrins and various drug molecules have made significant contributions toward resolving the stoichiometry and mechanism of formation of these complexes. But these studies did not provide conclusive, direct proof of the formation of an inclusion compound. Such evidence has resulted from proton magnetic resonance studies of the inclusion compounds formed between β-cyclodextrin and substituted benzoic acids, aspirin, tetracycline, *d*-phenylalanine, substituted phenols, and other compounds (203). Protons H-3 and H-5, located in the interior of the β-cyclodextrin torus, were shielded by guest protons upon the formation of an inclusion compound; increasingly larger chemical shifts of H-3 and H-5 were detected with increasing concentrations of *p*-hydroxybenzoic acid up to saturation concentration. This study and a subsequent study (204) demonstrated that large molecules can form inclusion compounds if an aromatic moiety or other group of a suitable size and shape can fit into the β-cyclodextrin cavity.

Thakkar *et al.* (205) reported correlation between the formation constants measured by phase solubility analysis for β-cyclodextrin–barbiturate complexes with those obtained by quantitative treatment of induced Cotton effects in the circular dichroism spectra of these inclusion compounds. Circular dichroism was used earlier to study the interaction of α- and β-cyclodextrins with azo dyes (206) and similar complexes with methyl orange and benzoic acid (207).

Cramer *et al.* (208) described the formation of α-cyclodextrin inclusion compounds with azo dyes as consisting of six steps:

1. Approach of the guest or substrate molecule to the cyclodextrin molecule.
2. Loss of the water structure within the cyclodextrin cavity with removal of some water molecules.
3. Breakdown of the water structure around the portion of the substrate that will be included and transport of some water molecules into the solution.
4. Interaction of substituent groups of the substrate with groups on the rim or inside the cyclodextrin ring.
5. Possible formation of hydrogen bonds between the substrate and the cyclodextrin.
6. Reestablishment of the water structure around the exposed parts of the substrate after inclusion has occurred.

These authors (208) pointed out that the rate of formation of the complex should be dependent on the steric factors of Steps 1, 4, and 5. Within the same class of guest molecules, Steps 1, 2, and 6 should not be rate determining. If kinetic specificity with regard to substituent groups is found, Step 3 or 4 and, possibly, Step 5 must be considered. The influence of the breakdown of the water structure and the removal of water molecules from within the ring must also be considered, particularly for the azo dyes. It was determined that the rates of recombination of nitrophenol, its anion, and an azo dye were diffusion controlled; Step 1 was the rate-determining step.

Hoffman and Bock (209) studied the interaction of cyclodextrins with nucleic acids. They determined that only adenosine and inosine compounds (adenosine monophosphate and inosine monophosphate) interacted with β-cyclodextrin and that none interacted with α-cyclodextrin. The primary factors determining the formation of the inclusion complex in ad-

dition to the degree and position of phosphorylation, pH, and polymerization of the nucleotide were the size of the base and its ability to be accommodated in the cyclodextrin cavity. Bases in single-stranded stacked conformation interact to form a complex. Formoso (210) reported the binding constant of 5′-adenosine monophosphate to β-cyclodextrin as well as the interaction of β-cyclodextrin with other nucleic acid monomer units. Hydrophobic interactions did not apparently contribute significantly to the stability of the complexes.

The spatial requirements for the formation of a cyclodextrin inclusion compound in part mimic the "lock and key" mechanism of enzyme catalysis which occurs if the substrate or guest molecule is oriented properly with respect to the active centers of the host (211). It is also possible to use the cyclodextrin ring to include or hold a molecule partially, blocking some reactive sites on the guest but exposing others. Breslow and Campbell (212) produced *p*-chloroanisole by forming the α-cyclodextrin–anisole inclusion compound in which the hydroxy groups rimming the cyclodextrin cavity were converted to intracomplex hypochlorite groups by reaction with hypochlorous acid. The hypochlorite groups react catalytically to chlorinate the exposed *para*-position of the partially included anisole molecule.

Lach and Chin (213) reported the inhibitory effect of β-cyclodextrin on the hydrolysis of ethyl *p*-aminobenzoate (benzocaine). They attributed this effect to the complete inclusion of the ester in the β-cyclodextrin cavity, protecting the ester linkage from attack. Their results indicated that the degradation of benzocaine was dependent on the uncomplexed drug in solution and not on the total amount present.

The results of this work, however, differed from those reported by Bender *et al.* (214, 215) and Van Etten *et al.* (216, 217), which indicated that the cyclodextrins accelerated ester hydrolysis, primarily by nucleophilic attack of the alkoxide ion of the cyclodextrin on the ester within the cyclodextrin cavity. Chin *et al.* (218) explained the apparent difference by observing that the compounds that form an inclusion compound in which the entire molecule (*e.g.*, benzocaine) or the susceptible portion are located in the cyclodextrin cavity (*e.g.*, atropine) exhibited a deceleration in the rate of hydrolysis; the rate of hydrolysis was dependent on the free ester in solution. Conversely, compounds that undergo partial inclusion but leave the active center fixed in close proximity to the hydroxy group of the cyclodextrin (*e.g.*, aspirin) underwent acceleration of hydrolysis. In this case, the rate of hydrolysis is dependent not only on the free ester in solution from dissociation of the complex but also on the nucleophilic attack of the alkoxide ion of the cyclodextrin on the ester linkage. An ester linkage that could be included within the β-cyclodextrin cavity, but only partially within the α-cyclodextrin cavity, would show deceleration of hydrolysis in the former and acceleration in the latter.

Cramer and Kampe (219) reported catalysis of the decarboxylation of methylphenylcyanoacetic acids with α- and β-cyclodextrins in aqueous solution. The rate of reaction was accelerated up to a factor of 14.8, depending on the size of the substituent groups and the nature of the inclusion compound formed. Additional information concerning the relationship between enzyme and inclusion catalysis was reported by Hennrich and Cramer (220), who studied the stereospecific catalysis of the fission of pyrophosphates by cyclodextrin. Cyclodextrins accelerated the cleavage of symmetrical diesters of pyrophosphate with phenols, which are stable in neutral or alkaline solution. β-Cyclodextrin was the most efficient catalyst and the assumption was made that a single phenyl group entered the cyclodextrin cavity, enclosing the pyrophosphate on one side only. Reaction mechanisms were proposed in which the cyclodextrin functions as a neighboring group and/or acts by a push–pull polarization of the pyrophosphate, the result being that the internal phosphate residue of the guest is transferred to the cyclodextrin host.

The cyclodextrins have been used to carry out many other stereospecific catalyses (194, 211). In one case, α-hydroxy ketones were converted from their keto to enol forms upon inclusion in the α-cyclodextrin cavity. The cyclodextrin catalyzed the reaction, which occurs only when the compound is in the enol form.

Cramer and Mackensen (221) synthesized amorphous, nonstoichiometric cyclodextrin–imidazole compounds by reacting cyclodextrins and cyclodextrin derivatives with 4(5)-chloromethylimidazole or 4(5)-aminoalkyl imidazoles. These compounds accelerated the hydrolysis of *p*-nitrophenol acetate about 300-fold. The cyclodextrin–imidazole compounds appear to be models for the complexation of a hydroxy group of the amino acid serine and an imidazole residue of histidine in the active site of chymotrypsin.

Solms and Egli (222, 223) prepared insoluble cyclodextrin–epichlorohydrin resins from mixed cyclodextrins. These compounds were useful in gel chromatographic separations of *o*- and *p*-nitrophenol and of phenylalanine and tryptophan. Wiedenhof and coworkers (192, 224, 225) prepared the α- and β-cyclodextrin–epichlorohydrin resins and determined that they have affinity for aromatic compounds. The isothermal inclusion of undissociated benzoic acid could be described by a Langmuir isotherm; however, the inclusion of *m*-chlorobenzoic acid could be described only by a Freundlich isotherm. Lammers *et al.* (191) also prepared soluble epichlorohydrin resins of α- and β-cyclodextrins. The water-soluble derivatives of α- and β-cyclodextrins form soluble inclusion compounds with nonpolar guest molecules, in contrast to the very slightly soluble complexes the same guests form with the cyclodextrins themselves. These derivatives should prove useful in studying the nature of the cyclodextrin inclusion compounds in solution.

Hoffman (226) separated various nucleotide and nucleoside mixtures, particularly those containing adenine, on stable, cross-linked β-cyclodextrin gels, similar to the mixed cyclodextrin gels described by Solms and Egli (222). The column chromatographic separations were believed to occur by the formation of an inclusion compound. A process for using cyclo-

dextrin gels was patented for the separation of vitamins, perfumes, and aromatic amino acids (227).

The partial resolution of racemic mixtures has been accomplished with modest success by selective inclusion with α- and β-cyclodextrins. β-Cyclodextrin has been particularly effective in differentiating between *d*- and *l*-forms; Cramer (83) partially resolved a number of esters using this host molecule. The resulting resolutions ranged from 0.84 to 11.33%. Mikolajczyk *et al.* (228) partially resolved sulfoxides with β-cyclodextrin, achieving resolutions ranging from 4.4 to 14.5%.

Miscellaneous Compounds—Aside from the cyclodextrins, other substances appear to fit into the classification of monomolecular inclusion compounds. Many large molecules have cavities or other structural arrangements capable of partially or completely enclosing a guest molecule (6). Examples of such compounds are the bis-*N,N'*-alkylenebenzidines which, when crystallized, can enclose a molecule of benzene or of dioxane in an intramolecular ring (229).

Cramer (6) suggested that proteins, minerals, cellulose, and graphite form monomolecular inclusion compounds. The specificity of reactions between various proteins and guest molecules such as those of dyes and other compounds with serum albumin, chromoproteins, and hemoglobin suggests a possible inclusion process; however, normal salt formation could account for this complexation and may negate this concept. Another strong possibility for the consideration of proteins as monomolecular inclusion compounds are the highly specific antigen–antibody interactions, which are dependent upon shape-matching factors. The hapten or nonproteineous portion of the antigen fits closely and specifically within the antibody and actually determines the antigen-to-antibody specificity. Hydrogen bonds and electrostatic attractions play a role in leading to a stable, tightly bound complex, in addition to van der Waals forces resulting from the close spatial proximity of the two interacting species.

Further biological ramifications come from suggestions that deoxyribonucleic acid (DNA) may have a function analogous to that of a host molecule (6). Protamines combine with DNA by fitting into a groove running through the helical macromolecule in a screw line (230). The biological activity of the nucleic acids may therefore be related to an inclusion process. The template function of ribonucleic acid (RNA) may also be a specific inclusion process as well as the interaction of gene mutations with DNA.

The antibiotic gramicidin A has a channel along its helix axis which forms a transmembrane channel through the lipid portion of a membrane when gramicidin A interacts with the membrane (231, 232). Although this does not represent an inclusion process *per se*, the presence of a cavity or channel in this compound and in another antibiotic, valinomycin (233), suggests that these compounds may act as host structures. A specific inclusion possibly could occur that might block the transmembrane channel or otherwise alter the biological activity of these compounds, perhaps leading to the synthesis of molecules with channels specific for a particular guest. The concept of inclusion compound formation as a component of the complex biochemistry of the living organism is exciting and should open new vistas in research for a complete understanding of those vital processes in health and disease.

PRODUCTS OF THE BLUE-IODINE REACTION

The blue-black color resulting from the reaction between iodine and starch has intrigued scientists for over a century and a half. In 1939, Freudenberg *et al.* (234) proposed that this behavior was the result of inclusion compound formation. Subsequent work by Cramer (6, 235, 236) indicated that a soluble, channel-like inclusion compound was formed by the polymerization of iodine atoms into a linear "template" around which the starch molecules formed a helix. This type of helical structure enclosing the iodine polymer was stable only as the inclusion compound. Instead of the characteristic red-brown color of iodine solutions, the inclusion compound was blue-black; hence the common name for this phenomenon is the "blue-iodine reaction."

The blue-black color of the cyclodextrin–iodine inclusion compound occurs by a similar process to that of starch (6). When the cyclodextrin–iodine complex crystallizes from solution, the individual cyclodextrin molecules align themselves to form a long, channel-like structure with the cyclodextrin rings lying side by side and the polyiodide chain occupying the now continuous channel. One turn of the starch helix contains six glucose molecules, the same as that of α-cyclodextrin. Both α- and β-cyclodextrins form inclusion compounds with iodine; α-cyclodextrin can also complex with chlorine and bromine. However, the β-compound can complex only with bromine, because the chlorine molecule is too small to stay in the cavity (6). γ-Cyclodextrin forms a loose complex only with iodine.

The linear iodine polymers are also stabilized by inclusion compound formation with many other compounds including benzonitrile, coumarins, cholic acid, cortisone, flavones, benzamide, barbituric acid, quinine and other alkaloids, and benzophenone (6).

The most likely mechanism to explain the nature of the polyiodide chain is that the host molecule acts as an electron donor, forming a loose charge-transfer complex with the iodine (237, 238) (Scheme I). This mechanism is substantiated by the fact that all host molecules forming blue-iodine compounds have such donor groups, *e.g.*, the electron pairs of the oxygen atoms of the cyclodextrins and the carbonyl groups of the flavones and coumarins.

$$\text{donor} + I_2 \rightleftharpoons (\text{donor}, I)^+ + I^-$$

Scheme I

Two other examples of the blue-iodine reaction should be mentioned. The first example is that of swollen cellulose which interacts with iodine to give a blue-black color (6), leading to the concept that the process of dyeing cellulose and other materials is an inclusion process. The second example is the polariz-

ing filter (239). These filters are prepared by treating a sheet of polyvinyl alcohol polymer with iodine, followed by stretching the sheet to orient the linear polyiodide chains within the polyvinyl alcohol hosts. The electrons along the polyiodide chain resemble those of a metal because they can move along the chain. However, they cannot move perpendicular to it, accounting for the very high extinction coefficient of the polyiodide chains in the inclusion compounds. This property of the electrons of the chain allows the absorbance of light polarized parallel to the direction of the stretched orientation of the polyvinyl alcohol–iodine complex, and only the light polarized at right angles passes through. Bolewski and Uchman (240) reported that the clathrating ability of polyvinyl alcohol decreased with an increase in the degree of branching.

MACROMOLECULAR INCLUSION COMPOUNDS

The last category of inclusion compounds includes those of a macromolecular size. In many cases the differentiation between monomolecular and macromolecular inclusion compounds is not clearly defined so considerable overlap results. Some large molecules such as certain minerals, cellulose, and proteins can be discussed in either category.

A detailed discussion of the macromolecular inclusion compounds is unfortunately beyond the scope of this review. Well over a 100 or more publications appear annually concerned with the preparation, properties, and applications of these useful compounds.

Zeolites—The major category of macromolecular inclusion compounds is commonly known as molecular sieves which are widely used in pharmaceutical processes and other chemical and biological applications due to their unique ability to fractionate molecules according to size. Molecular sieves are three-dimensional inorganic crystalline lattices with a myriad of interconnected cavities, capable of retaining or sorbing water or other molecules, depending on the size of the channels and cavities within the crystal. Of the molecular sieves, the most widely used are the many zeolites which occur naturally and are also synthesized for specific purposes.

The zeolites are hydrated aluminum silicates and may contain sodium, potassium, calcium, and barium (241–244). The void spaces of the zeolites normally contain water, but the water can be removed by heating to give a uniformly porous lattice with empty spaces which can be refilled with gases, vapors, or molecules from solution. Although the zeolites give off water vapor when heated, unlike most other water-bearing crystals, they do not then collapse or disintegrate. After cooling, the lost water vapor is replaced or another gas is adsorbed.

X-ray diffraction studies have indicated that the zeolites contain a precise arrangement of cavities linked by channels (242). Water and exchangeable ions can pass through the interconnecting channels and occupy the cavities, facilitating the reversible processes of water removal and ion exchange. The structure of any zeolite crystal is based on a tetrahedral arrangement of oxygen atoms around a silicon or aluminum ion. Barrer and Meier (241) and Barrer and Stuart (242) described the structure of the common zeolite, chabazite, as consisting of two tight hexagons, each with six silicon and aluminum atoms, along with their associated oxygen atoms, facing each other and forming a flat prism. Eight such prisms are joined together to form an oval cavity of approximately 11.0 Å. The cavities are linked by six channels about 3.9 Å in diameter. Twenty-four water molecules and four calcium ions can occupy each chabazite cavity, of which there are 5×10^{21} cavities/in.^{3} of crystal (245). By contrast, the feldspars have the same silicon–oxygen and aluminum–oxygen tetrahedra, but they are arranged in a way that does not allow free spaces capable of enclosing guest molecules.

The zeolites are capable of sorbing, separating, or exchanging similar molecules (243–245). The molecule to be sorbed or exchanged must have a shortest dimension less than the shortest distance between chains in the zeolite lattice. The zeolites sorb only those molecules that are small enough to enter the channels of the dehydrated mineral lattice. Kipling (246) described the selective separation of mixtures of various organic compounds strictly by molecular size. For example, a particular molecular sieve adsorbs hexane while rejecting benzene, but the larger pore size molecular sieves accept benzene. Mixtures of compounds of a suitable size can be sorbed simultaneously; if the zeolite is sequentially exposed to the individual components, the first substance sorbed will be difficult to displace, resulting in very low sorption of a subsequent component (247).

The zeolites are also capable of ion exchange (243, 244); zeolites containing sodium exchange cations with a solution containing calcium ions, a property utilized commercially in the removal of calcium from hard water. The structure of the zeolites is essentially a silicate tetrahedra with partial replacement by aluminum, requiring additional exchangeable cations for electrical neutrality.

Benson and King (248) indicated that the molecular discrimination in adsorption by zeolites is largely due to differences in the polarizability of the adsorbate. Large molecules would be expected to undergo weak adsorption due to their bulkiness, which prevents strong interaction with adsorption sites. There appears to be a good correlation between the strength of adsorption and the polarizability of the adsorbate. Electrostatic bonding rather than dispersion forces is indicated as the mechanism of bonding by the function of certain zeolites which can discriminate between *ortho*- and *para*-hydrogen. Adsorption isotherms are approximately Langmuir in type, and multilayer adsorption does not generally occur above the critical temperature of the adsorbate. Below the critical temperature, multilayer adsorption generally occurs, and capillary condensation may result from the pores or channels limiting the extent of multilayer formation.

Synthetic zeolites can be prepared for specific separations by crystallization under controlled condi-

tions of temperature and the presence of specific ions to determine the channel size (243, 245, 249, 250). Adjustment of temperature affects the vibrations of the atoms in the zeolite crystal, which, in turn, affect the effective diameter of the channels. The essential criterion in the function of these molecular sieves is that the guest molecule be able to pass through the channel. An example of a synthetic zeolite is a sodium-bearing zeolite that will not sieve *n*-octane from isooctane. In fact, neither compound can enter its channels. However, a calcium-bearing zeolite has channels sufficiently large for only the *n*-octane to enter, giving rise to industrial processes used to improve gasolines. Recently synthesized zeolites specifically adsorb cyclohexane, carbon tetrachloride, *n*-hexane, or water (249).

A major use of the zeolites is in the dehydration of solvents or other materials due to their high affinity for water. The sorption capacity of the zeolites also makes them useful for gas storage, for storage and recovery of radioactive material, as carriers for catalysts in chemical reactions, and for many other applications in adsorption separations, hydrocarbon catalysis, and purifications (249).

Dextrans—Other materials capable of selective inclusion include the modified dextrans and certain support media that are used in column chromatography.

The modified dextrans are cross-linked dextran macromolecules that form a three-dimensional network of polysaccharide chains capable of functioning as a molecular sieve and of fractionating molecules according to size (251). Most modified dextrans are hydrophilic and swell in water or electrolyte solution to form a gel, hence the name "gel filtration" for the fractionating ability of these dextrans. There is also a chemical modification of dextran that is lipophilic and swells in many polar organic solvents as well as in water.

The dextran gel matrix has a porosity determined by the degree of cross-linkage in the polysaccharide network. Molecules larger than the maximum pore size of a particular modified dextran do not enter the matrix, whereas smaller molecules diffuse into the matrix to different degrees, depending on their size. Aqueous mixtures separate as a solution is passed through the gel column, the smallest molecules being the last to be eluted from the column. In addition to their molecular sieve capabilities, the modified dextrans are also used as gel matrixes for ion exchange in a manner similar to that of the zeolites. Other related materials used for affinity chromatography include agarose and the polyacrylamide gels.

Silica Gels—The last group of macromolecular inclusion compounds to be discussed is the silica gels. Like the zeolites and modified dextrans, the silica gels are molecular sieves with a high sorptive capacity. The silica gels are also capable of very high selectivity, perhaps greater than that of the other macromolecular inclusion compounds.

Silica gels are composed of an irregular three-dimensional network of silicon atoms connected by oxygen atoms in the same spatial relationship as the hydrogen-bonded oxygen atoms of water. The silica gels are formed by crystallization of the silicon–oxygen network around a guest molecule. After the inclusion structure has formed, the guest can be removed by heating or degassing. Since the guest acts as a template, the silica gel has strong sorptive properties for that particular molecular species. For example, a silica gel made in the presence of butyl orange as the guest will sorb that molecule to a greater extent than it will adsorb propyl orange (243), although it will accept other molecules of a suitable size and shape. The tailoring of silica gel adsorptive properties is a most unique capability that can be of significant importance when it is necessary to have such a highly specific separation.

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Keyphrases □ 8-(Methylthio)cyclic 3′,5′-adenosine monophosphate—metabolism, dogs and rats after oral and intravenous doses, *in vitro* metabolism by subcellular dog liver preparations □ Adenosine monophosphate—activity mimicked by 8-(methylthio)cyclic 3′,5′-adenosine monophosphate, metabolism of analog *in vivo* and *in vitro* □ Metabolism—8-(methylthio)cyclic 3′,5′-adenosine monophosphate in dogs and rats after oral and intravenous doses and *in vitro* by subcellular dog liver preparations

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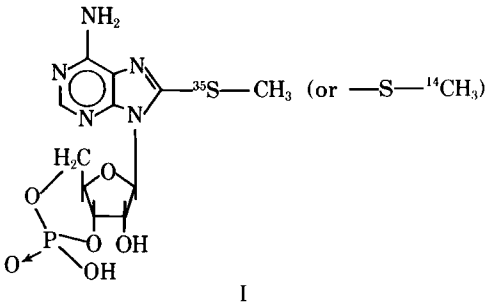
Ample documentation now exists that the biological effects of cyclic 3′,5′-adenosine monophosphate can be mimicked by certain of its analogs (1, 2). Such biologically active analogs might exhibit greater po-

tency, penetrability, tissue specificity, or stability than is the case with cyclic 3′,5′-adenosine monophosphate (2). 8-(Methylthio)cyclic 3′,5′-adenosine monophosphate (I) is one such analog that has exhibited activity in the stimulation of a purified cyclic 3′,5′-adenosine monophosphate-dependent protein kinase (3), the inhibition of cyclic nucleotide phosphodiesterase from cat heart and rat brain (4, 5), and the activation of tyrosine transaminase from hepatoma cells grown in culture (6).

The metabolism of I given to rats and dogs by oral and intravenous routes was studied to determine whether the 8-(methylthio) substituent altered its disposition as compared with that of cyclic 3′,5′-adenosine monophosphate. The results show that I was converted to 8-(methylthio)adenosine and other unidentified metabolites. In addition, I was metabolized *in vitro* more slowly than cyclic 3′,5′-adenosine monophosphate.



I

EXPERIMENTAL

Animals—Albino rats (Sprague–Dawley or Charles River CD) and purebred beagles were used. Animals were fasted overnight before the administration of drug and were housed in metabolism cages which permitted the separate collection of urine and feces.

Purity and Specific Activity—Compound I-^{35}S had an initial specific activity of 12.1 μCi/mg; no radiochemical impurities were detectable. Compound I-^{14}C had a specific activity of 2.76 μCi/mg; no radiochemical impurities were detectable.

Surgical Preparation of Dogs—Dogs were anesthetized with 30 mg/kg of pentobarbital sodium administered intravenously. A catheter was inserted into the bladder for the collection of urine. The radial vein was cannulated and infusion of the following solution, at the rate of 3 ml/min, was begun: mannitol, 100 g; KH_2PO_4, 200 mg; K_2HPO_4, 900 mg; pentobarbital sodium, 25.5 mg/kg; and sufficient water to make 2 liters. Mannitol was given to ensure an adequate flow of urine.

A midline incision was made, and the entrance to the gallbladder was clamped just above its point of entry into the common bile duct. A polyethylene catheter (No. 100) was then inserted about 1.3 cm (0.5 in.) into the common bile duct. The catheter was tied in place, and the midline incision was closed with wound clips.

Analysis of Urine and Bile—Samples of urine and bile were counted directly in 15 ml of Bray's scintillation fluid (7).

Analysis of Blood for ^{35}S—A 0.2-ml sample of heparinized blood was digested in 0.5 ml of 1.0 *N* NaOH by heating overnight at 80°. The sample was then bleached with 30% hydrogen peroxide, followed by neutralization with 0.2 ml of 2-ethylhexanoic acid. The sample was counted with 15 ml of Bray's scintillation fluid.

Analysis of Blood for ^{14}C—Samples of blood (0.2 ml) were combusted with a biological material oxidizer[1].

Analysis of Plasma—A 0.4-ml sample of plasma was dissolved in 2 ml of solubilizer[2]. The sample was counted with 15 ml of Bray's or toluene scintillation fluid. The toluene scintillation fluid contained, per liter, 5 g of 2,5-diphenyloxazole and 300 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

Analysis of Feces for ^{35}S—Fecal samples were homogenized in about three volumes of methanol. A 100-mg sample of the homogenate was digested in 1 ml of solubilizer for 16 hr with shaking and then bleached with 30% hydrogen peroxide. The sample was counted with 15 ml of Bray's scintillation fluid.

Analysis of Feces for ^{14}C—Samples of feces were homogenized with two to three volumes of methanol. About 400 mg of homogenate was combusted with the biological material oxidizer.

Analysis of Tissue for ^{35}S—Samples of brain were homogenized in water, using an all-glass homogenizer[3]; about 200 mg of homogenate was digested in 2 ml of solubilizer. Certain portions (about 200 mg) of the eye (lens, cornea, aqueous and vitreous humors, and combined retina, choroid, and sclera) or representative portions of the pancreas, renal and omental fat, skin (without hair), adrenal, thyroid and pituitary glands, and ovary were each placed in 2 ml of solubilizer. Samples of tissue from the heart, kidneys, liver, lungs, thigh muscle, and testes or the entire rat carcass were analyzed by first grinding the tissue in a meat grinder and then digesting about 200 mg of the well-mixed sample in 2 ml of solubilizer. All samples were counted in 15 ml of Bray's scintillation fluid.

Analysis of Tissue for ^{14}C—Samples of brain were homogenized in water, using an all-glass homogenizer; about 400 mg of homogenate was combusted with the biological material oxidizer. Certain portions of the eye (lens, cornea, aqueous and vitreous humors, and combined retina, choroid, and sclera) and representative portions of the pancreas, renal and omental fat, skin (without hair), adrenal, thyroid and pituitary glands, and ovary were combusted. Samples of tissue from the heart, kidneys, liver, lungs, thigh muscle, and testes or the entire rat carcass were analyzed by first grinding the tissue in a meat grinder and then combusting about 400 mg of the well-mixed sample.

Measurement of ^{14}C-Carbon Dioxide—To measure the $^{14}CO_2$ evolved, each rat was placed in a plastic metabolism cage[4] enclosed in a plastic bag. Air was drawn from the cages through two carbon dioxide-absorbing traps, connected in series with a vacuum line and containing monoethanolamine–2-methoxyethanol (1:3). The amount of radioactivity in the expired air was measured by adding a 0.5-ml sample of the absorbing solution to 2.0 ml of solubilizer, followed by 15 ml of toluene scintillation fluid.

Binding of Drug to Plasma Proteins—Binding measurements were made on 3-ml samples of plasma which were transferred to ultrafiltration membrane filter cones[5]; they were centrifuged at 25–30° for 30 min at 2000 rpm. After centrifugation, an aliquot of the ultrafiltrate and the plasma sample prior to centrifugation were solubilized in 2.0 ml of solubilizer and counted with 15 ml of toluene scintillation fluid.

Extraction Procedure for Feces—Samples of feces were homogenized with two to three volumes of methanol and then centrifuged; the supernatant fluid was recovered and saved. Chloroform–methanol (1:1), equal in volume to the supernatant fluid recovered, was added to the fecal residue, and the mixture was shaken for 30 min and centrifuged; the supernatant fluid was saved. This last step was repeated one more time. The supernates were combined and spotted directly for chromatography or were first concentrated to a suitable volume at 40–50° under reduced pressure. This extraction procedure removed an average of 59.8% of the radioactivity present in the fecal homogenates of dogs.

Chromatography—Samples of urine, bile, and extracts of feces and plasma were spotted on cellulose Q2-F[6] plates and developed in one or more of the following solvent systems: 1, isopropanol–28% ammonia–water (7:1:2); 2, *n*-butanol–pyridine–acetic acid–water (30:20:6:24); and 3, 95% ethanol–buffer (70:30). (The buffer was prepared by mixing 1 *M* ammonium acetate and 3×10^{-3} *M* ethylenediaminetetraacetate and adjusting the pH to 5.0 with acetic acid.)

Counting of Samples—The radioactivity in each sample was

[1] R. J. Harvey Instrument Corp., Hillsdale, N.J.
[2] NCS Solubilizer, Amersham/Searle Corp., Des Plaines, Ill.
[3] Potter-Elvehjem, Fisher Scientific Co., Springfield, N.J.
[4] Microchemical Specialties Co., Berkeley, Calif.
[5] Centriflo, Amicon Corp., Lexington, Mass.
[6] Quantum Industries, Fairfield, N.J.

Table I—Plasma Half-Lives of Radioactivity after the Oral or Intravenous Administration of I to Dogs

Dog[a]	Sex	Route[b, c]	Dose, mg/kg	Time to Maximum Plasma Concentration, hr	Plasma Half-Life, hr	Time Interval, hr
Q2-168	M	Intravenous	0.25	—	3.7	1–12
Q1-349	F	Intravenous	0.25	—	2.9	1–12
Q2-56	M	Intravenous	2.5	—	3.0	1–12
Q2-80	F	Intravenous	2.5	—	2.9	1–12
Q2-240	M	Intravenous	50	—	4.8	1–24
Q3-53	F	Intravenous	50	—	4.3	1–24
Q3-39	M	Oral	2.5	3	2.3	6–12
Q0-297	F	Oral	2.5	3	2.7	6–12
Q3-10	M	Oral	2.5	2	3.0	3–12
Q2-198	F	Oral	2.5	4	2.8	4–12
Q3-10	M	Oral	50	8	9.6	8–48
Q2-198	F	Oral	50	4	11.9	12–48

[a] The type of radioactivity given to these dogs is indicated in Table V. [b] Intravenous doses were dissolved in 0.1 *M* Na_2CO_3. [c] Oral doses were administered in capsules. Tepid water (100 ml) was given to each dog immediately after the capsule.

determined with a liquid scintillation spectrometer[7]. Counting efficiency was determined with automatic external standardization and the use of previously prepared quench curves. Chromatograms were scanned for the presence of radioactivity with a scanner[8]. Areas of the various radioactive peaks were integrated by the use of a planimeter.

Calculation of Biological Half-Lives and Absorption and Elimination Rate Constants—Biological half-lives in the plasma were estimated graphically from linear portions of the appropriate semilogarithmic plots. Absorption rate constants were calculated as described by Rescigno and Segre (8).

Determination of Glucose Concentrations in Plasma—These analyses were performed automatically[9] by the method of Kornberg and Horecker (9).

Determination of Insulin Concentrations in Plasma—These analyses were performed employing an insulin radioimmunoassay kit[10]. The results were calculated from standard curves prepared from standardized human insulin.

Preparation of Subcellular Fractions from Dog Liver—Male beagle dogs (10–12 kg) were anesthetized intravenously (thiopental sodium, 30 mg/kg) and killed by exsanguination; the livers were removed. Samples of liver from different lobes were homogenized immediately in three volumes of 1.15% KCl, using a homogenizer[3]. The whole homogenate was centrifuged at 9000×*g* for 20 min, and the supernatant fraction was decanted through a single layer of gauze to remove some fat droplets; this fraction was designated the "postmitochondrial supernate."

The postmitochondrial supernate was centrifuged at 105,000×*g* for 60 min. The supernatant material, filtered through a single layer of gauze, was designated the "soluble fraction," and the pellet was resuspended in 6 ml of 1.15% KCl. This suspension was designated the "microsomal fraction," which was centrifuged again at 105,000×*g* for 60 min. After removal of the supernatant fluid, the pellet was resuspended again in 6 ml of 1.15% KCl and was designated the "washed microsomal fraction."

As controls, the postmitochondrial supernate or the soluble fraction was inactivated in a boiling water bath for 10 min. The coagulated protein was removed by filtration through a thin plug of glass wool, and the clear filtrate was designated the "boiled postmitochondrial supernate" or the "boiled soluble fraction."

Incubation of I and Cyclic 3′,5′-Adenosine Monophosphate *In Vitro*—Each incubation contained 0.2 ml of I, 5 m*M*; 0.2 ml of tromethamine buffer, 0.35 *M*, pH 7.4; 0.02 ml of $MgCl_2$, 250 m*M*; 0.02 ml of glucose-6-phosphate, 500 m*M*; 0.02 ml of NADP, 15 m*M*; and 0.02 ml of glucose-6-phosphate dehydrogenase[11] (two units). To each incubation was added 0.2 ml of the whole homogenate, postmitochondrial supernate, microsomal, washed microsomal, soluble, or boiled postmitochondrial supernatant fraction. Distilled water (0.32 ml) was added to provide a final volume of 1.0 ml. The incubation was carried out, with shaking, at 37° for 60 min; then the tubes were frozen immediately in an acetone–dry ice bath and stored in the freezer.

For the study of the metabolism of I-^{14}C (1 m*M*) or cyclic 3′,5′-adenosine-8-^{14}C monophosphate[12] (1 m*M*) as a function of time, the incubation mixtures were scaled up by increasing the amount of each ingredient by 3.5. Only the soluble fraction was used as the enzyme preparation, and the boiled soluble fraction served as the control. Aliquots (0.5 ml) were removed at different time intervals, as indicated in each experiment. Each aliquot was frozen immediately in an acetone–dry ice bath and then stored in the freezer. Each sample was chromatographed, as described under *Chromatography*. For the studies with cyclic 3′,5′-adenosine monophosphate, nonradioactive cyclic 3′,5′-adenosine monophosphate, adenosine-5′-monophosphate, and adenosine were used as reference compounds.

RESULTS

Absorption—In separate experiments, two dogs were given the ^{35}S-labeled compound, either 0.25 or 2.5 mg/kg iv, or the ^{14}C-labeled compound, 50 mg/kg iv. The concentrations of radioactivity found in the plasma of dogs after each dose during the first 12 hr are shown in Fig. 1. Shortly after dosing, concentrations of radioactivity in plasma reflected the amount of compound administered. After the distribution of radioactivity into tissues, the plasma concentrations of radioactivity decreased according to first-order kinetics.

Four dogs were given 2.5-mg/kg doses of I as a finely divided powder in capsules. Figure 2 shows the average concentrations of radioactivity in the plasma of these dogs during the first 12 hr after dosing. The compound was absorbed slowly, attaining an average maximum plasma concentration 3 hr after dosing, which was similar (about 1–2 μg/ml) to that found after a comparable intravenous dose. An average of 16.9 ± 6.4% (±*SE*) of the radioactivity was bound to the plasma proteins of these dogs 2 hr after they had been dosed.

Figure 3 shows similar data for two dogs that received an oral dose of 50 mg/kg. Plasma concentrations found at this higher dose reflect: *(a)* the greater dose administered, *(b)* the greater time required to absorb this larger dose, and *(c)* the greater persistence of radioactivity in the plasma, since the time scale for these two dogs extends to 48 hr.

Table I summarizes plasma half-lives for each dog after the oral and intravenous administration of I. The plasma half-lives were determined by a linear regression analysis for as long as the curves were linear and still significantly above background. Plasma half-lives after intravenous doses were about 3 hr with either the small-

[7] Packard Tri-Carb, model 3380, Packard Instrument Co., Downers Grove, Ill.
[8] Nuclear-Chicago Actigraph III, Amersham/Searle Corp., Des Plaines, Ill.
[9] Autoanalyzer, model DSA-560, Beckman Instruments, Fullerton, Calif.
[10] Amersham/Searle Corp., Des Plaines, Ill.
[11] Sigma Chemical Co., St. Louis, Mo.
[12] New England Nuclear, Boston, Mass.

Table II—Concentrations of Glucose in the Plasma of Dogs as a Function of Time after the Intravenous Administration of I

Time	0.25 mg/kg[a]		2.5 mg/kg[b]		50 mg/kg[c]	
	Dog Q2-168	Dog Q1-349	Dog Q2-56	Dog Q2-80	Dog Q2-240	Dog Q3-53
	Percent Change					
0 min	(0)	(0)	(0)	(0)	(0)	(0)
5 min	25	73	48	41	103	135
15 min	33	58	49	20	211	294
30 min	−4	13	1	5	231	564
1 hr	0	8	14	−7	188	496
1.5 hr	−1	5	4	15	−13	197
2 hr	−8	−20	−9	2	−55	96
3 hr	−2	−6	−18	1	−4	−47
4 hr	−7	2	−10	−14	26	−51
6 hr	—	—	—	—	8	−38

[a] Average glucose concentration in the plasma of these dogs at zero time was 84 ± 1 mg %. [b] Average glucose concentration in the plasma of these dogs at zero time was 83 ± 4 mg %. [c] Average glucose concentration in the plasma of these dogs at zero time was 76 ± 4 mg %.

est or the intermediate dose and about 5 hr with the largest dose. After oral doses of 2.5 mg/kg, it took 2–4 hr to attain maximum plasma concentrations of radioactivity; after oral doses of 50 mg/kg, 4–8 hr was required.

Plasma half-lives after oral doses of 2.5 mg/kg ranged from 2.3–3.0 hr for the indicated time intervals, which were essentially the same as those found after a comparable intravenous dose. After oral doses of 50 mg/kg, plasma half-lives ranged from 9.6 to 11.9 hr and were about twice as long as those cited previously for the same intravenous doses. Absorption half-times, which were 1.1 hr with the smaller dose and 3.2 hr with the larger dose, support the slower absorption of the compound at the larger dose.

Studies of Glucose and Insulin—Compound I, like cyclic 3′,5′-adenosine monophosphate, is capable of producing a hyperglycemic effect. The data of Table II show plasma glucose concentrations as a function of time after intravenous doses of 0.25 or 2.5 mg/kg; they are expressed as the percent change from control values for each individual dog. Within 5 min, intravenous doses of 0.25 or 2.5 mg/kg of I produced significant elevations of plasma glucose; these levels returned to control values within 30–60 min after dosing. In these particular dogs, the larger intravenous dose did not produce a greater elevation of glucose concentration than did the smaller dose.

After an intravenous dose of 50 mg/kg, a marked elevation in glucose concentrations was seen within 5 min; these levels persisted for 1–2 hr and then returned toward control values. The elevations in plasma glucose seen after an intravenous dose of 50 mg/kg were considerably greater than after either of the two smaller doses. In fact, the response was so marked that both dogs seemed to exhibit an overshoot phenomenon in which plasma glucose concentrations were transiently depressed to approximately one-half the control values.

Changes in plasma glucose concentrations as a function of time after oral doses of 2.5 or 50 mg/kg are shown in Table III. At the smaller dose, average elevations of glucose were slight because one dog showed a good response, one dog showed a mild response, and two dogs showed no response. However, with the larger dose, both dogs showed hyperglycemic responses, beginning between 30 and 60 min in one case and between 1 and 1.5 hr in the other. This response was more delayed than that seen after intravenous dosing and correlated with the slow absorption of the compound. The extent of the response after oral dosing was also much less than that seen after comparable intravenous doses.

Plasma insulin concentrations were measured as a function of time in dogs that had received intravenous doses of 0.25 or 2.5 mg/kg of I (Table IV). Insulin concentrations in plasma generally followed a time course similar to that observed for glucose concentrations; they were steeply elevated within 5 min after dosing and returned to control values within 30–60 min. However, the elevations found for insulin concentrations, rising more than an order of magnitude for two dogs, were generally much greater than those found for glucose.

Excretion Studies—Excretion of radioactivity by rats after in-

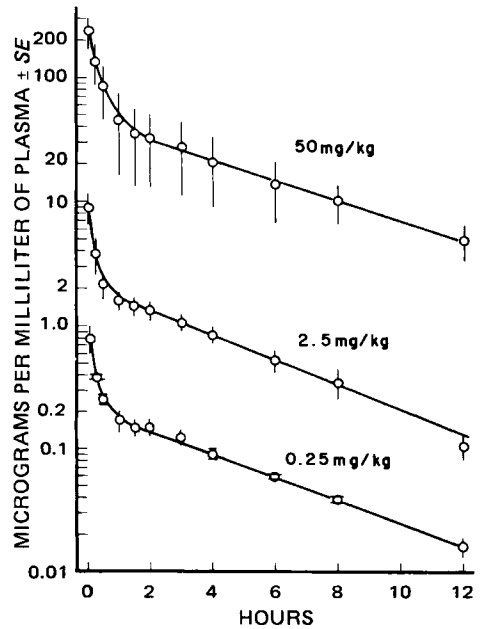


Figure 1—*Concentrations of radioactivity in the plasma of dogs after the intravenous administration of I. A male and a female dog were given the compound at each dose.*

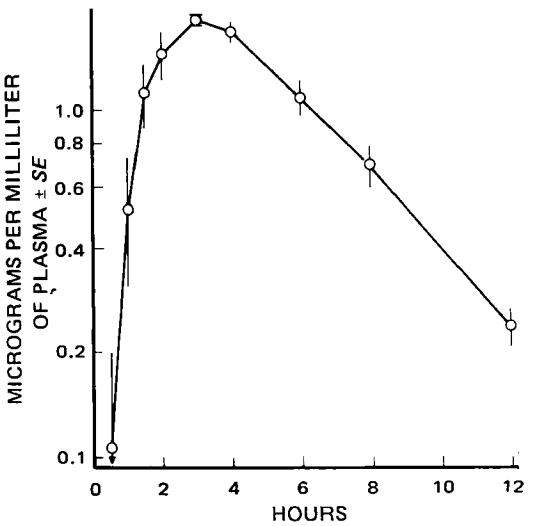


Figure 2—*Concentrations of radioactivity in the plasma of dogs after the oral administration of 2.5 mg/kg of I. Two male and two female dogs were given the compound in capsules.*

Table III—Concentrations of Glucose in the Plasma of Dogs as a Function of Time after the Oral Administration of I

Hours	2.5 mg/kg[a]	50 mg/kg[b] Dog Q3-10	50 mg/kg[b] Dog Q2-198
	Percent Change		
0	(0)	(0)	(0)
0.5	2 ± 6[c]	−1	15
1	5 ± 3	4	51
1.5	12 ± 14	40	80
2	21 ± 15	61	61
3	5 ± 10	4	27
4	4 ± 6	1	28
6	5 ± 6	22	30

[a] Four dogs, two of each sex, were used. Average glucose concentration in the plasma of these dogs at zero time was 110 ± 6 mg %. [b] Average glucose concentration in the plasma of these dogs at zero time was 80 ± 6 mg %. [c] Standard error.

travenous doses of 5 mg/kg of I bearing either the ^{35}S or ^{14}C label is shown in Table V. After administration of either radioactive compound, 74–87% of the dose was excreted in the urine and 8–14% in the feces. Dogs given intravenous doses of the ^{35}S-labeled compound at 0.25 and 2.5 mg/kg excreted radioactivity in a manner quantitatively similar to that seen for rats.

A single dog, prepared surgically for the collection of bile and given a 2.5-mg/kg dose of I intravenously, excreted 64% of the dose in the urine and 19% in the bile in 7 hr. One hour after it had been dosed, this dog had 8.8% of the radioactivity in the plasma bound to the plasma proteins. After an intravenous dose of 50 mg/kg, excretion of the ^{14}C-labeled compound was quantitatively similar to that of the ^{35}S-labeled compound when the latter had been given at 0.25 and 2.5 mg/kg.

After oral doses of 2.5 mg/kg, the excretion of radioactivity by dogs over a 5-day period was similar to that found after 50 mg/kg (Table V). From 76 to 82% of the dose was excreted in the urine and the remainder was excreted in the feces. Most of this radioactivity was excreted during the first 24 hr. These data are very similar to those found after comparable intravenous doses and indicate that at least four-fifths of an oral dose of I was absorbed by dogs.

Table VI shows the rate of excretion by rats of a 5-mg/kg iv dose of I-^{14}C. Of the 89% of the dose recovered in 4 days, 81% was excreted during the 1st day. Only 0.22% of the dose was excreted by these rats as $^{14}CO_2$, indicating that the methylthio side chain is not removed from the molecule to any appreciable extent.

Tissue Distribution—The distribution of radioactivity was studied in dogs 5 and 60 min after receiving 2.5 mg/kg iv of I (Table VII). One dog of each sex was studied at each time. Five minutes after dosing, when compared with the values found in the blood, radioactivity was highly localized in the excretory organs and widely distributed to most of the other tissues including the skin, pancreas, and other endocrine structures. Radioactivity was present in much smaller concentrations in the portions of the brain

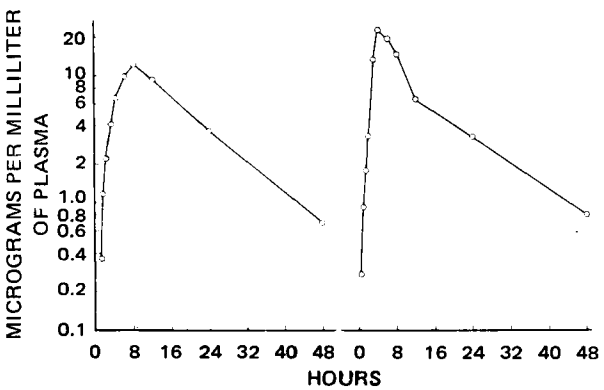


Figure 3—*Concentrations of radioactivity in the plasma of dogs after the oral administration of 50 mg/kg of I. A male (left) and a female (right) dog were given the compound in capsules.*

Table IV—Concentrations (Microunits per Milliliter) of Insulin in the Plasma of Dogs as a Function of Time after the Intravenous Administration of I

Minutes	0.25 mg/kg Dog Q2-168	0.25 mg/kg Dog Q1-349	2.5 mg/kg Dog Q2-56	2.5 mg/kg Dog Q2-80
0	Lost	17	15	22
5	58	145	135	214
15	96	90	278	235
30	17	18	30	95
60	27	14	13	18

examined and in the various portions of the eye. Sixty minutes after dosing, the distribution of radioactivity in the various organs was much more uniform, but the amounts present in the brain were still only about one-tenth those present in the blood.

Biotransformation *In Vivo*—Urine that had been collected from rats and dogs during the first 24 hr after dosing with either 5 or 2.5 mg/kg, respectively, was chromatographed on thin-layer plates coated with cellulose and developed in Solvent System 1. The chromatographic reference compounds used were the parent compound, I, 8-(methylthio)adenosine-5′-monophosphate, 8-(methylthio)adenine, and 8-(methylthio)adenosine.

Four radioactive components were present in the urine of both rats and dogs (not shown), although the amounts varied between the species, particularly for the slowest moving component. Chromatography in System 1, as well as in the other two systems, indicated that the fastest moving component corresponded to 8-(methylthio)adenosine. No 8-(methylthio)adenosine-5′-monophosphate appeared to be present in these samples. Solvent System 2 clearly separates 8-(methylthio)adenosine from its adenine analog, and none of the latter was found in these samples of urine.

Figure 4 illustrates clearly the time-dependent transformation of I. These chromatograms are of urine from a dog in which the bile duct had been externalized; the urine was collected at hourly intervals. The sample collected during the 1st hr showed the presence primarily of unchanged I and of some 8-(methylthio)-

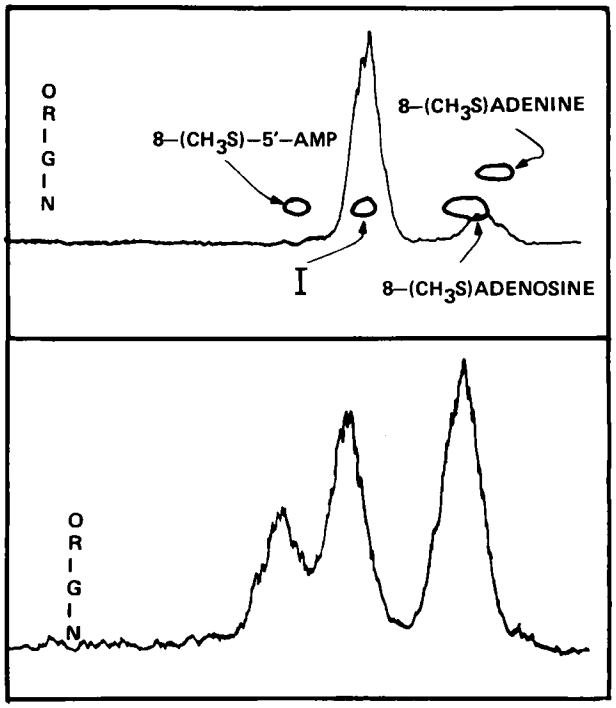


Figure 4—*Chromatograms of urine collected during the 1st (upper) and 3rd (lower) hr after the intravenous administration to a dog of 2.5 mg/kg of I. Urine was collected continuously from this dog for 7 hr. Solvent System 3 was employed. 8-(CH$_3$S)-5′-AMP is 8-(methylthio)adenosine-5′-monophosphate.*

Table V—Excretion of Radioactivity by Rats and Dogs after the Oral or Intravenous Administration of I

Species	Radio-activity	Route[a, b]	Dose, mg/kg	Average Percent of Dose ± SE: Urine	Feces or (Bile)	Total
4 Rats	^{35}S	Intravenous	5	86.66 ± 5.89	8.31 ± 2.00	94.97 ± 4.27[c]
4 Rats	^{14}C	Intravenous	5	74.15 ± 8.82	14.29 ± 5.75	88.44 ± 7.40[c]
2 Dogs	^{35}S	Intravenous	0.25	82.39 ± 1.10	22.77 ± 1.95	105.16 ± 3.05[d]
2 Dogs	^{35}S	Intravenous	2.5	80.00 ± 1.31	25.62 ± 3.04	105.62 ± 1.73[d]
1 Dog	^{14}C	Intravenous	2.5	63.87	(19.30)	83.17[e]
2 Dogs	^{14}C	Intravenous	50	77.53 ± 8.20	22.24 ± 6.10	99.77 ± 2.10[d]
4 Dogs	^{14}C	Oral	2.5	76.35 ± 11.84	16.25 ± 2.94	92.60 ± 10.98[d]
2 Dogs	^{14}C	Oral	50	82.30 ± 2.27	17.03 ± 0.46	99.33 ± 1.81[d]

[a] Intravenous doses were dissolved in 0.1 *M* Na_2CO_3. [b] Oral doses were administered in capsules. Tepid water (100 ml) was given to each dog immediately after the capsule. [c] Collections were made for 4 days from two rats of each sex. [d] Collections were made for 5 days from one or two dogs of each sex. [e] Seven-hour experiment.

Table VI—Excretion of Radioactivity by Rats after the Intravenous Administration of I

Days	Percent of Dose[a] ± SE: Urine	Feces	$^{14}CO_2$	Total[b]
1	70.16 ± 9.30	10.93 ± 4.81	0.22 ± 0.02	81.31 ± 7.41
2	3.12 ± 0.84	2.22 ± 0.95	0.00	5.34 ± 1.77
3	0.61 ± 0.24	0.84 ± 0.32	—	1.44 ± 0.56
4	0.20 ± 0.09	0.30 ± 0.22	—	0.50 ± 0.29
Cage rinse	0.07 ± 0.03	—	—	0.07 ± 0.03
	74.16 ± 8.82	14.29 ± 5.75	0.22 ± 0.02	88.66 ± 7.41

[a] Using 5 mg/kg. [b] After 4 days, the carcasses contained an average of 0.083 ± 0.044% (±*SE*) of the dose.

adenosine, based on chromatography in the three solvent systems. A sample of urine similarly analyzed during the 3rd hr after dosing showed the formation of an additional metabolite that traveled more slowly than I but was not 8-(methylthio)adenosine-5′-monophosphate and the increasing prominence of 8-(methylthio)adenosine. Studies with bile from this same animal (not shown) showed a similar time-dependent metabolic transformation.

Figure 5 shows chromatograms of urine collected from dogs during the first 24 hr after oral or intravenous doses of 50 mg/kg. Based on chromatography in the three solvent systems, these samples contained some I[13] and a good deal of 8-(methylthio)adenosine but no 8-(methylthio)adenosine-5′-monophosphate or 8-(methylthio)adenine. 8-(Methylthio)adenosine was also the principal metabolite in the feces of dogs that had been dosed orally with 2.5 mg/kg of I, as well as in the plasma of dogs that had been dosed intravenously with 2.5 or 50 mg/kg.

Table VIII shows some of these aspects quantitatively for the individual dogs. Chromatograms of urine of a dog shown in Fig. 5 indicate that only about 27% of the parent compound was present 5 hr after dosing, whereas 43% was then accounted for as 8-(methylthio)adenosine. In the urine of other dogs that had been given 50 mg/kg, from 33 to 59% was present as I in a 24-hr collection, and a large portion of the balance was accounted for as 8-(methylthio)-adenosine. After oral doses of 2.5 mg/kg, about 30% of the radioactivity in urine was I, with a good deal of the remainder being represented by the adenosine metabolite. After 50 mg/kg po, somewhat less of the radioactivity on the average was the parent compound, with a correspondingly greater amount as the adenosine metabolite.

Biotransformation *In Vitro*—The metabolism of I *in vitro* was studied with subcellular fractions of dog liver for two reasons: *(a)* to determine whether the cleavage of the 3′-bond occurred, since no 8-(methylthio)adenosine-5′-monophosphate was detectable *in vivo;* and *(b)* to determine the comparative rates of metabolism of I and cyclic 3′,5′-adenosine monophosphate *in vitro* under identical conditions. Incubation of I and appropriate cofactors with the whole liver homogenate, postmitochondrial supernate, and the soluble or microsomal fractions indicated that the soluble fraction contained all of the enzymatic activities involved in converting I to its metabolites.

[13] Presence of I was confirmed by mass spectrometry; unpublished data.

Figure 6 shows the results obtained by incubating I with the soluble fraction of dog liver. When the samples were developed in the three solvent systems, three components were consistently obtained. The slowest one corresponded in each case to 8-(methylthio)adenosine-5′-monophosphate, the component consistently found in the middle represented the unchanged parent compound, and the fastest moving component consistently corresponded to 8-(methylthio)adenosine.

Table VII—Concentrations of Radioactivity in Selected Tissues of Dogs after the Intravenous Administration (2.5 mg/kg) of I[a]

Tissue	After 5 min, μg/g ± SE	After 60 min, μg/g ± SE
Adrenals	3.00 ± 0.02	1.43 ± 0.08
Brain: cerebellum	0.11 ± 0.02	0.14 ± 0.00
Brain: cortex	0.16 ± 0.02	0.14 ± 0.01
Brain: dorsal and hypothalamus	0.099 ± 0.032	0.10 ± 0.02
Brain: stem	0.094 ± 0.007	0.11 ± 0.00
Brain: subcortex	0.052 ± 0.004	0.096 ± 0.025
Brain: sagittal section	0.086 ± 0.008	0.11 ± 0.00
Cornea	0.22 ± 0.01	0.57 ± 0.07
Combined retina, choroid, and sclera	0.69 ± 0.07	1.35 ± 0.09
Aqueous humor	0.14 ± 0.03	0.34 ± 0.13
Vitreous humor	0.30 ± 0.02	0.69 ± 0.06
Heart	1.97 ± 0.15	1.44 ± 0.39
Kidneys	56.18 ± 25.84	4.45 ± 0.80
Lens	0.034 ± 0.013	0.22 ± 0.01
Liver	30.23 ± 0.43	6.75 ± 0.90
Lungs	3.88 ± 0.15	1.36 ± 0.05
Omental fat	0.38 ± 0.08	0.69 ± 0.02
Pancreas	2.50 ± 0.13	2.09 ± 0.72
Pituitary	3.10 ± 0.41	1.55 ± 0.21
Portal vein	3.05 ± 2.05	1.35 ± 0.35
Skeletal muscle	1.64 ± 0.02	1.34 ± 0.01
Skin	1.90 ± 0.33	1.48 ± 0.31
Testes	1.41[b]	1.36[b]
Ovaries	5.28[b]	1.75[b]
Thyroid gland	2.94 ± 0.11	1.27 ± 0.13
Blood (μg/ml)	3.19 ± 0.27	1.26 ± 0.07

[a] One dog of each sex was sacrificed at each time. [b] Single value.

Table VIII—Amounts of I and 8-(Methylthio)adenosine in the Urine of Dogs after the Oral and Intravenous Administration of I

Dog	Route	Dose, mg/kg	Collection Interval, hr	I, %	8-(Methylthio)-adenosine, %
Q2-56[a]	Intravenous	2.5	0–1	81.2	18.8
Q2-56[a]	Intravenous	2.5	2–3	36.0	43.6
Q2-56[a]	Intravenous	2.5	4–5	26.8	43.4
Q2-240	Intravenous	50	0–24	58.5	29.6
Q3-53	Intravenous	50	0–24	32.8	40.9
Q3-10	Oral	2.5	0–24	33.1	29.2
Q2-198	Oral	2.5	0–24	30.1	41.8
Q3-10	Oral	50	0–24	27.5	38.4
Q2-198	Oral	50	0–24	19.9	50.1

[a] Urine was collected continuously.

Table IX shows the conversion of I to the adenosine-5′-monophosphate and adenosine metabolites as a function of time. 8-(Methylthio)adenosine-5′-monophosphate appeared to be formed first from I, since little of the adenosine metabolite was present after 20 min. After 3 hr, there were approximately equal amounts of I and 8-(methylthio)adenosine-5′-monophosphate and about 14% of the adenosine metabolite.

Table X shows the results of having incubated I-^{14}C and cyclic 3′,5′-adenosine monophosphate with the soluble fraction of dog liver as a function of time. Cyclic 3′,5′-adenosine monophosphate was converted very rapidly to adenosine-5′-monophosphate. After 30 min, only 20% of the original amount of cyclic 3′,5′-adenosine monophosphate remained, whereas 80% of the original amount of I was still present. Two hours after incubation with the soluble fraction of dog liver, 67% of the I still remained, but *none* of the cyclic 3′,5′-adenosine monophosphate was found at this time.

DISCUSSION

The metabolism of cyclic 3′,5′-adenosine monophosphate has been studied in a number of laboratories. Verbert and Cacan (10) showed the formation of cyclic 3′,5′-adenosine monophosphate, adenosine-5′-monophosphate, adenosine, and a trace of adenine after adenosine-5′-triphosphate-^{3}H had been incubated with rat liver plasma membranes containing adenyl cyclase. Pull and McIlwain (11) studied the metabolism of adenine-^{14}C by superfused and electrically stimulated cerebral tissues of guinea pigs. The compounds released from brain tissue to superfusion fluids included adenine, adenosine, inosine, and hypoxanthine and small amounts of cyclic 3′,5′-adenosine monophosphate, adenosine-5′-monophosphate, adenosine-5′-diphosphate, and adenosine-5′-triphosphate. The output of all these compounds, except adenine, increased upon electrical stimulation. When adenosine-5′-triphosphate-^{14}C was added at low concentrations to fluids superfusing the cerebral tissue, adenosine-^{14}C was the main product.

Rutten *et al.* (12), employing homogenates of rat pancreas to study the assay of cyclic 3′,5′-adenosine monophosphate phospho-

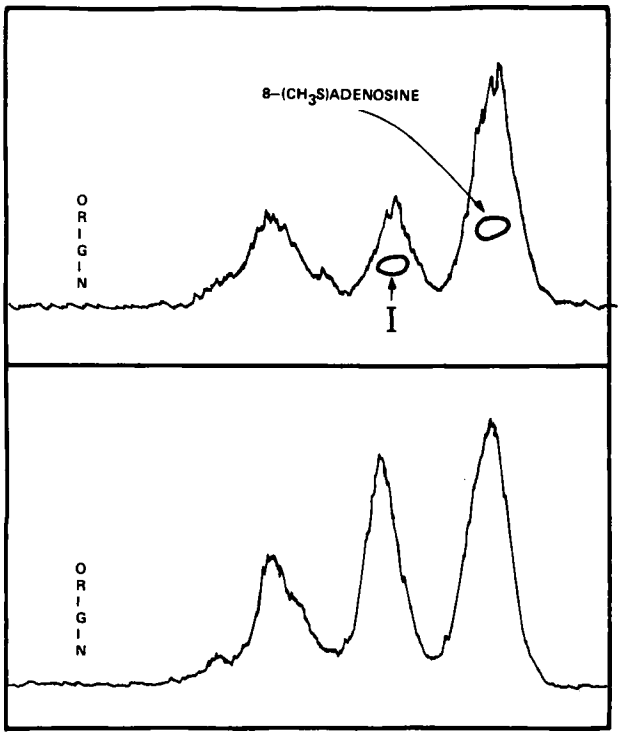


Figure 5—*Chromatograms of urine collected during the first 24 hr after the oral (upper) and intravenous (lower) administration to dogs of 50 mg/kg of I. Solvent System 2 was employed.*

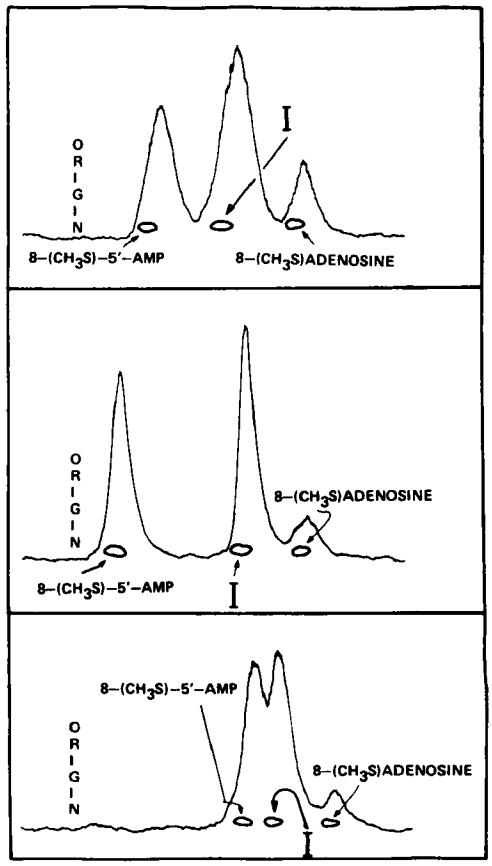


Figure 6—*Chromatograms of the incubation of I with the soluble fraction of dog liver. The samples were incubated at 37° for 150 min and developed in Solvent Systems 3 (upper), 1 (middle), and 2 (lower). 8-(CH_3S)-5′-AMP is 8-(methylthio)adenosine-5′-monophosphate.*

Table IX—Metabolism of I *In Vitro* by the Soluble Fraction of Dog Liver as a Function of Time

Incubation Time, min	I[a], %	8-(Methylthio)adenosine-5′-monophosphate[a], %	8-(Methylthio)adenosine[a], %
20	76	22	2
45	64	31	5
90	55	37	8
120	54	36	10
150	50	34	15
180	45	41	14

[a] The amount of each component was determined based on chromatography in Solvent System 3.

Table X—Metabolism of I and Cyclic 3′,5′-Adenosine Monophosphate by the Soluble Fraction of Dog Liver

Incubation Time, min	I[a], %	8-(Methylthio)adenosine-5′-monophosphate[a], %	8-(Methylthio)adenosine[a], %
30	80	17	3
120	67	16	17
Incubation Time, min	Cyclic 3′,5′-Adenosine Monophosphate[a], %	Adenosine-5′-monophosphate[a], %	Adenosine[a], %
5	33	67	0
30	20	79	1
120	0	91	9

[a] The amount of each component was determined based on chromatography in Solvent System 3.

diesterase, concluded that the hydrolysis of cyclic 3′,5′-adenosine monophosphate to adenosine *via* adenosine-5′-monophosphate was the predominant pathway but that the conversion of cyclic 3′,5′-adenosine monophosphate to cyclic 3′,5′-inosine monophosphate also occurred to some extent. In another study, Lee and Dubos (13) examined the metabolism of cyclic 3′,5′-adenosine monophosphate in mice. Nine hours after the injection of cyclic 3′,5′-adenosine-8-^{14}C monophosphate into mice, the plasma contained some adenosine-5′-monophosphate, large amounts of adenosine, small amounts of adenine, and a small amount of an unidentified metabolite.

Compound I appears to be metabolized in a manner similar to that of cyclic 3′,5′-adenosine monophosphate. After incubation with the soluble fraction of dog liver *in vitro*, both cyclic 3′,5′-adenosine monophosphate and I are metabolized by the action of a phosphodiesterase to adenosine-5′-monophosphate or its 8-(methylthio) derivative and, subsequently, by the action of a nucleotidase to adenosine or its 8-(methylthio) derivative. *In vivo*, I appears to be excreted primarily as 8-(methylthio)adenosine, but other metabolites, which have not yet been identified, are also excreted.

The hyperglycemic response found after oral or intravenous doses of I would seem to be mediated by I, rather than by some of its potential metabolites, since neither 8-(methylthio)adenosine nor 8-(methylthio)adenine produced any increases in serum glucose after intravenous administration to dogs. Comparable studies with 8-(methylthio)adenosine-5′-monophosphate resulted in only marginal increases in serum glucose[14]. Compound I appears to be absorbed at least partly as such after oral dosing, since increases in glucose concentrations in the plasma were found in each of two dogs after 50 mg/kg and in one of four dogs after 2.5 mg/kg.

In these studies, 8-(methylthio)adenosine was found to be the major metabolite of I. Adenosine, the corresponding metabolite of cyclic 3′,5′-adenosine monophosphate, has been reported to have biological activity *in vitro* in a variety of tests. McKenzie and Bar (14) studied the mechanism of the inhibition of adenylate cyclase by adenosine. They found that adenylate cyclase activities from various sources were inhibited by adenosine but not by inosine. The inhibition of cyclic 3′,5′-adenosine monophosphate-dependent protein kinases by adenosine also has been reported (15, 16). Melnykovych and Bishop (17) studied glycogen concentrations in cultured HeLa cells. In this system, the presence of adenosine decreased the degradation of glycogen, thereby resulting in an increase of the glycogen concentrations.

In an unrelated study, Ortiz *et al.* (18) found that adenosine produced morphological alterations of iris epithelial cells *in vitro*. Adenosine has also been proposed as a regulator of lipolysis in isolated fat cells. Thus, Ebert and Schwabe (19) reported that lipolysis was increased by adenosine in a dose-dependent manner, and Fain (20) reported that the accumulation of cyclic 3′,5′-adenosine monophosphate in fat cells was inhibited by adenosine.

Adenine is toxic when administered intravenously to dogs for 3 weeks because of the formation and deposition of 2,8-dihydroxyadenine in the kidneys (21). Earlier studies (22, 23) with rats and mice that had been given adenine orally and intraperitoneally demonstrated its oxidation *in vivo* to 2,8-dihydroxyadenine and the deposition of the latter in the renal tubules. In the present studies on the metabolism of I in rats and dogs, the formation of 8-(methylthio)adenine did not appear to be a significant pathway, if it occurred at all. Moreover, the 8-position of the purine ring of I could not have been hydroxylated, since it already had a methylthio substituent.

Recently, Merits and Anderson (24) reported on the metabolic fate of the noncyclic nucleoside derivative, adenosine-5′-carboxylic acid ethyl ester-8-^{14}C, in rats, mice, and dogs. Their studies indicated that at least 94% of an oral dose of the compound was absorbed by dogs and that the compound did not traverse the blood–brain barrier to any great degree. Adenosine-5′-carboxylic acid ethyl ester was metabolized by rats, mice, and dogs, predominantly to adenosine-5′-carboxylic acid and, to a much smaller extent, inosine-5′-carboxylic acid. However, the dog eliminated only 71–82% of an oral dose of the radioactive compound within 3 days; most of the remainder was excreted slowly in the urine over the next 21 days. More detailed studies by Merits (25) of this phenomenon of prolonged elimination revealed that radioactivity from a dose of adenosine-5′-carboxylic acid ethyl ester was incorporated into the nucleic acids of mice and dogs. Most of the radioactivity seemed to be associated with ribosomal ribonucleic acid rather than with soluble ribonucleic acid or deoxyribonucleic acid. Merits (25) attributed the greater incorporation of radioactivity into the nucleic acids of dogs than those of mice to the more efficient mechanism of adenine "salvage" in dogs. The present studies of the metabolism of I in dogs indicate that there was no significant incorporation, if any, of radioactivity into nucleic acids, since the type of delayed excretion reported by Merits and Anderson (24) for adenosine-5′-carboxylic acid ethyl ester was not observed.

[14] Unpublished data, Department of Toxicology, Squibb Institute.

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Microbial Kinetics of Drug Action against Gram-Positive and Gram-Negative Organisms II: Effect of Clindamycin on *Staphylococcus aureus* and *Escherichia coli*

SAMUEL M. HEMAN-ACKAH

Abstract □ Clindamycin-affected *Staphylococcus aureus* cultures show biphasic steady-state generation curves. An initial (phase I) generation of the clindamycin-affected *Staph. aureus* is followed by an ultimate (phase II) generation at the same dose level. The phase I apparent generation rate constant is greater than the phase II apparent generation rate constant and suggests the development of resistant *Staph. aureus* mutants to clindamycin action after a finite period of drug–bacteria contact at any subcompletely inhibitory concentration level. It is rationalized that the increased resistance to drug action in mutant strains is due to a comparatively reduced ribosomal binding affinity for clindamycin. In contrast, clindamycin-affected *Escherichia coli* cultures show monophasic steady-state generation curves at all concentration levels; *E. coli* cultures do not develop resistance to clindamycin action. The dependence of the apparent generation rate constant on drug concentration yields a sigmoidal curve, which is coincident by a potency factor for the phase I and phase II generations of clindamycin-affected *Staph. aureus* and suggests a common mechanism of action for both generation phases. That of clindamycin-affected *E. coli* yields an asymptote curve, which indicates a different mechanism of action. Clindamycin possesses both a bacteriostatic and a bactericidal action on initial and mutant resistant strains of *Staph. aureus*, whereas its action on *E. coli* is only bacteriostatic. Consequently, clindamycin has a minimum inhibitory concentration (MIC) against *E. coli* that is about 1000 times the MIC value against *Staph. aureus* at 37.5°. The effect of pH changes in broth media on generation inhibition of both *Staph. aureus* and *E. coli* by clindamycin action indicates that the unprotonated fraction of drug concentration contributes to the activity, possibly because of its ready penetration through cell membranes.

Keyphrases □ Microbial kinetics—drug action on Gram-positive and Gram-negative organisms, comparison of clindamycin action on *Staphylococcus aureus* and *Escherichia coli* □ Clindamycin—comparison of effects on *Staphylococcus aureus* and *Escherichia coli*, microbial kinetics □ *Staphylococcus aureus*—effects of clindamycin, compared to *Escherichia coli* □ *Escherichia coli*—effects of clindamycin, compared to *Staphylococcus aureus* □ Antimicrobial activity—comparison of clindamycin on *Staphylococcus aureus* and *Escherichia coli*

Clindamycin is one of the 7*(S)*-chloro analogs of lincomycin (1, 2) that is claimed to be more than four times as active as the parent antibiotic against a variety of Gram-positive and Gram-negative organisms (3). Like lincomycin, it interferes with ribosomal functioning in the synthesis of cell proteins (4, 5) to inhibit microbial growth and generation.

Comparative studies on the action of lincomycin and its 7*(S)*-chloro analogs against *Escherichia coli* by microbial kinetics (6) confirmed the enhanced potency of clindamycin action relative to that of lincomycin. These studies also revealed that clindamycin has only one of two mechanisms of lincomycin action against *E. coli*. Recently, it was shown (7) that lincomycin also possesses one mechanism of action against *Staphylococcus aureus*. However, *Staph. aureus* cultures, unlike *E. coli*. cultures, develop resistence to lincomycin after a finite period of organism contact with subcompletely inhibitory concentrations of the drug.

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It was of interest to study the kinetics and dependencies related to modes of clindamycin action

against *Staph. aureus* and to compare the data with those obtained for *E. coli*. The results of such studies are presented in this paper.

EXPERIMENTAL

Materials and Methods—*Staph. aureus* (ATCC 6538) and *E. coli* (ATCC 12407) were used in all of the experiments. They were cultivated in broth medium[1] and used for determining antibiotic–bacteria reaction in the same manner as previously described (7). The antibiotic was an assayed sample of clindamycin hydrochloride[2] (860 μg base equivalent/mg). A particle-size counter[3] and the poured plate method, using sandwiched agar plates, were employed in the determination of total and viable counts, respectively, on drug-free and drug-affected *Staph. aureus* cultures as previously described (7).

Effect of Antibiotic Concentrations on Generation Rates—Aliquots (0.5 ml) of aqueous solutions of clindamycin were aseptically added to replicate 50-ml samples of cultures to yield the desired antibiotic concentrations. The solutions were added to the cultures in an exponential phase of generation at 37.5°. The cell concentration at the time of drug addition was 2.0×10^6/ml. Culture samples were withdrawn every 30 min, and the organism population was determined by the appropriate method.

Generation curves were obtained by the total count method for *Staph. aureus* and *E. coli* cultures affected with a range of clindamycin concentrations (0–0.1 and 0–120 μg/ml, respectively). Generation curves for *Staph. aureus* cultures affected with selected clindamycin concentrations (0, 0.04, 0.05, 0.07, 0.08, 0.16, and 0.32 μg/ml) and for *E. coli* cultures affected with selected clindamycin concentrations (0, 25, 50, 100, and 200 μg/ml) were also obtained by both total and viable count methods.

Effect of Broth Composition on Drug-Affected Generation Rates—Three different types of broth, containing half, single, and double concentrations of the ingredients as specified by the manufacturer, were prepared and adjusted to pH 7.05 ± 0.05 where necessary. They were used for determining the generation curves at 37.5° of replicate *Staph. aureus* cultures affected with clindamycin concentrations of 0, 0.03, 0.05, 0.07, and 0.09 μg/ml. The organism concentration at the time of drug addition was 2×10^6 cells/ml. Total counts were obtained on samples withdrawn every 30 min.

Effect of Organism Population on Drug-Affected Generation Rates—Three sets of single strength broth were prepared and used for determining the generation curves of replicate *Staph. aureus* cultures affected with clindamycin concentrations of 0, 0.03, 0.05, 0.07, and 0.09 μg/ml. The organism concentrations at the time of drug addition were 5.0×10^5, 2.0×10^6, and 2.0×10^7 cells/ml in the respective sets of broth. Total counts were obtained on samples withdrawn every 30 min.

Reversibility of Drug Action—An aliquot (0.5 ml) of an aqueous solution of clindamycin was aseptically added to a 50-ml sample of *Staph. aureus* culture in the exponential phase of generation at 37.5° to yield a final concentration of 0.03 μg/ml of clindamycin. The cell concentration at the time of drug addition was 2.0×10^6 cells/ml. When the drug-affected culture settled to a steady-state generation phase I, an aliquot (0.5 ml) was added to 49.5 ml of fresh broth so that both the organism and drug concentrations were diluted 100-fold. At the same time, an aliquot (0.5 ml) of the culture was diluted 100-fold in broth containing enough clindamycin so that the drug concentration was restored to 0.03 μg/ml.

A drug-free culture was likewise diluted 100-fold as a control. When the sample of the drug-affected culture, which was diluted in fresh broth, emerged from a lag phase into steady-state generation, an aliquot (0.5 ml) of clindamycin solution was added to its replicate culture to reestablish the drug concentration at 0.03 μg/ml.

The entire dilution steps were repeated on the initial drug-affected culture when it entered into a steady-state generation phase II. Total counts were obtained on samples withdrawn every 30 min.

The experiment then was performed on another 50-ml sample of *Staph. aureus* culture affected with a higher concentration of the drug, *i.e.*, 0.06 μg/ml of clindamycin.

Effect of pH on Drug-Affected Generation Rates—Sufficient amounts of 2.0 *N* HCl and 2.0 *N* NaOH were added to the broth media to obtain pH values of 5.9, 6.3, 6.5, 7.0, 7.3, 7.6, 7.8, and 8.2. Six replicate 50-ml volumes of each broth, inoculated with *Staph. aureus*, were maintained at 37.5° until the organism population reached 2.0×10^6 cells/ml. Aliquots (0.5 ml) of clindamycin solutions were added to five replicates to achieve the desired concentrations of antibiotic. The sixth replicate in each set contained no drug. Total counts were obtained from samples withdrawn every 30 min.

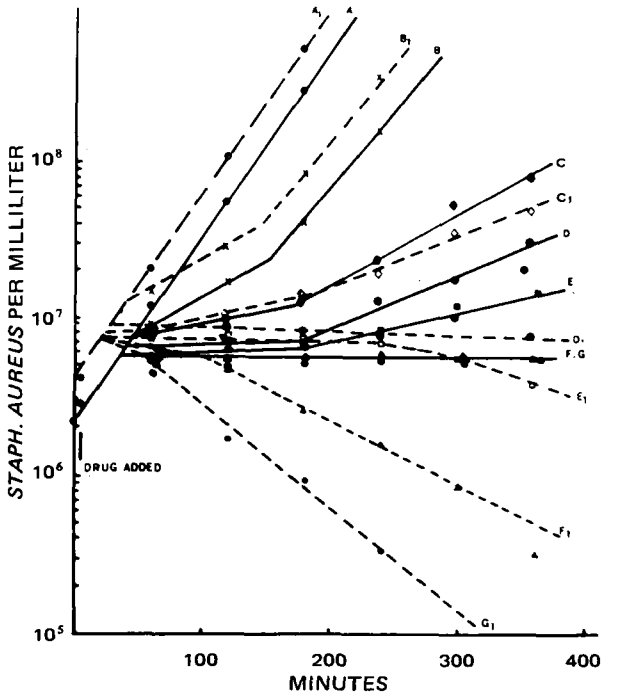


Figure 1—*Generation curves of* Staph. aureus *at pH 7.05 and 37.5° in the absence and presence of clindamycin obtained by total (——) and viable (- - -) counts. The curves and respective drug concentrations (micrograms per milliliter) are: A and A_1, 0.0; B and B_1, 0.04; C and C_1, 0.05; D and D_1, 0.07; E and E_1, 0.08; F and F_1, 0.16; and G and G_1, 0.32.*

RESULTS

Shape of Generation Curves for Drug-Affected Cultures—Semilogarithmic plots of total and viable counts *versus* time are shown in Fig. 1 for the action of selected clindamycin concentrations of 0, 0.04, 0.05, 0.07, 0.08, 0.16, and 0.32 μg/ml on *Staph. aureus*. Figure 2 shows these plots for the action of clindamycin concentrations of 0, 25, 50, 100, and 200 μg/ml on *E. coli*. The addition of graded concentrations of clindamycin to balanced cultures of *Staph. aureus* (Fig. 1) and *E. coli.* (Fig. 2) in the exponential phase of generation at 37.5° and pH 7.05 decreased the generation rates after a lag period approximating 20 min. With the *Staph. aureus* cultures, a newly established steady-state phase of generation (phase 1) was followed after one to two generations by an ultimate steady-state phase of generation (phase II). Clindamycin-affected *E. coli* cultures exhibited one steady-state phase of generation.

Apparent generation rate constants of the drug-affected cultures are obtained from the slopes of the linear portions of semilogarithmic plots of the counts *versus* time in accordance with the expression:

$$\ln N = \ln N_0 + k_{app}t \qquad \text{(Eq. 1)}$$

where N is the number of organisms per unit volume of broth medium of any time, t; N_0 is the number of organisms per unit volume of broth medium at some initial time, 0, in the generation phase; and k_{app} (in seconds^{-1}) is the apparent generation rate con-

[1] Antibiotic Medium 3, Difco Laboratories, Detroit, Mich.
[2] Courtesy of Dr. J. B. Whitfield, Jr., The Upjohn Co., Kalamazoo, Mich.
[3] Model ZB1, Coulter Electronics Co., Hialeah, Fla.

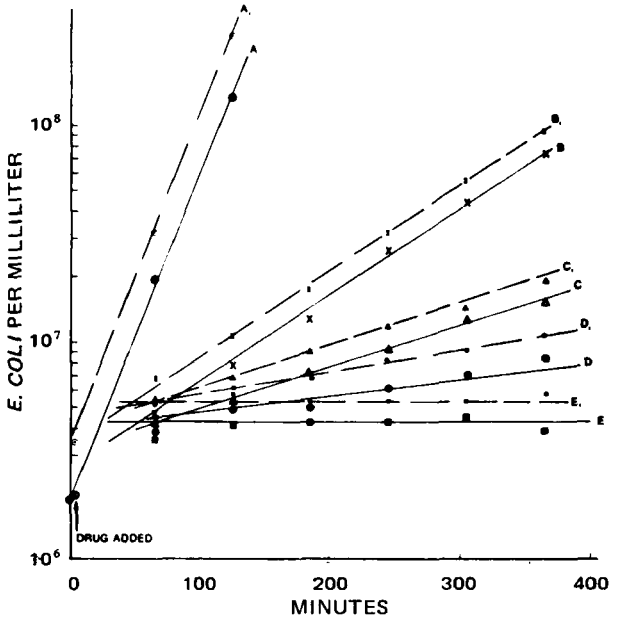


Figure 2—*Generation curves of* E. coli *at pH 7.05 and 37.5° in the absence and presence of clindamycin obtained by total (——) and viable (- - -) counts. The curves and respective drug concentrations (micrograms per milliliter) are: A and A_1, 0.0; B and B_1, 25.0; C and C_1, 50.0; D and D_1, 100.0; and E and E_1, 200.0.*

stant. In the absence of drug, $k_{app} = k_0$, which is the generation rate constant for the drug-free or control culture.

The k_{app} for phase I and phase II generations of clindamycin-affected *Staph. aureus* is designated k_{app_I} and $k_{app_{II}}$, respectively. The k_{app_I} value is less than the $k_{app_{II}}$ value for the action of subcompletely inhibitory concentrations of clindamycin determined by the total count method.

Generation Curves of Drug-Affected Cultures Determined by Total and Viable Count Methods—Parallel generation curves are obtained from plots of the total and viable counts for *Staph. aureus* cultures (Fig. 1) affected with clindamycin in the 0–0.04-μg/ml concentration range. The total count was 50% of the viable count at each concentration level (curves A_1, A and B_1, B). The cultures affected with 0.05–0.08 μg/ml of clindamycin showed

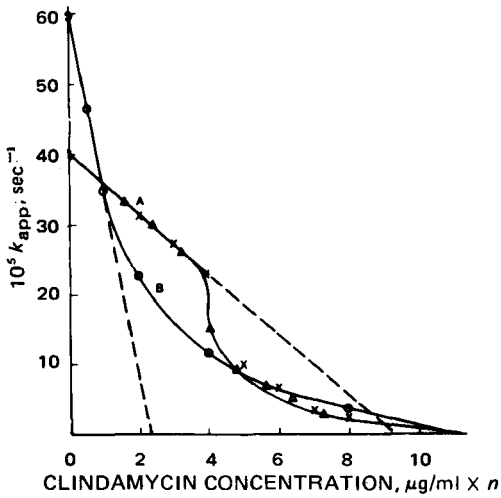


Figure 3—*Functional dependency of apparent generation rate constants,* k_{app} *in seconds*$^{-1}$*, for drug-affected* Staph. aureus *and drug-affected* E. coli *on concentrations of clindamycin (micrograms per milliliter) at pH 7.05 and 37.5°. Actual concentrations are multiplied by a factor,* n, *where* n = *100 in curve A for* Staph. aureus *phase I generation (×),* n = *80 in coincident curve for* Staph. aureus *phase II generation (▲), and* n = *0.1 in curve B for* E. coli *(○).*

complete generation inhibition for 200 min when the total count also remained 80–90% of the viable count. Thereafter, there was a drop in the viable count whereas there was a progressively small increase in the total count with time (curves C_1, C, D_1, D, and E_1, E).

Cultures affected with concentrations of clindamycin greater than 0.08 μg/ml showed coincidence of plots for the total counts, which remained constant for the entire experiment (curves F and G). The corresponding plots for the viables showed a rapid drop of the count with time as a function of the drug concentration (curves F_1 and G_1). This finding clearly indicates that a bactericidal or lytic phenomenon is superimposed on generation inhibition by the action of clindamycin at concentrations of 0.04 μg/ml.

Parallel generation curves are obtained from plots of the total and viable counts for *E. coli* cultures (Fig. 2) affected with clindamycin in the subcompletely inhibitory concentration range (curves A_1, A, B_1, B, C_1, C, and D_1, D) and also above the bacteriostatic concentration (curves E_1 and E). The total count was 50–80% of the viable count at each concentration level. There was no evidence of a bactericidal or lytic phenomenon superimposed on generation inhibition by the action of clindamycin in the concentration range studied.

Functional Dependency of Generation Rates on Drug Concentrations—Figure 3 shows the plot of k_{app} *versus* clindamycin concentration for *Staph. aureus*-affected cultures (curve A) and for *E. coli*-affected cultures (curve B). Coincidence of plots was obtained when the actual concentrations for the phase I and phase II clindamycin action on *Staph. aureus* were multiplied by a factor of 1.0 and 0.80, respectively (curve A). The extent of generation inhibition was directly proportional to the clindamycin concentration, C, in the 0–0.04-μg/ml range for the phase I action and in the 0–0.05-μg/ml range for the phase II action on *Staph. aureus* or in the 0–10.0-μg/ml range for the action on *E. coli*, in accordance with the expression:

$$k_{app} = k_0 - k_c C \qquad \text{(Eq. 2)}$$

where k_c is the specific inhibitory rate constant.

At higher drug concentrations, the k_{app} was not a linear function of increasing drug concentrations, but it asymptotically approached zero. There was a striking difference between the k_{app} dependency on drug concentration for *Staph. aureus* cultures affected with clindamycin at concentrations greater than 0.04 μg/ml and that for *E. coli* cultures affected with clindamycin at concentrations greater than 10.0 μg/ml. The rate of decrease of k_{app} with an increase in drug concentration was greater than would be obtained by extrapolation of the initial linear portion of the regression for the *Staph. aureus* cultures (curve A) but was less than would be predicted by such an extrapolation for *E. coli* cultures (curve B). Consequently, sigmoidal curves were obtained from plots of the data for *Staph. aureus* cultures. This finding may indicate that there is a change in the bioavailability or binding affinity of the clindamycin for bioreceptors which permits enhanced inhibition and/or kill of the *Staph. aureus* cultures but reduces the extent of generation inhibition of *E. coli* cultures.

Applicability of Saturation Kinetics to Action of Clindamycin on *Staph. aureus* and *E. coli*—The plot of $C/(k_0 - k_{app})$ *versus* C is given in Fig. 4. The plot is in accordance with a previously (8) derived, saturable receptor site model:

$$C/(k_0 - k_{app}) = C(k_b/k_a) + 1/k_a \qquad \text{(Eq. 3)}$$

where $k_b = K_1K_2$ and $k_a = qK_1K_2$. The term K_1 is the drug partition constant through cell membranes, K_2 is the drug affinity constant for bioreceptors, and q is a constant of proportionality related to the metabolic activity of the microbial cell.

Adherence to Eq. 3 is observed from linear plots obtained for concentrations of clindamycin greater than 0.04 μg/ml in phase I action (curve A) and greater than 0.05 μg/ml in phase II action (curve B) against *Staph. aureus* or greater than 10.00 μg/ml (curve C) against *E. coli*. Deviations from linearity of the plot occurred at concentrations of clindamycin less than 10.00 μg/ml against *E. coli* or less than 0.04 μg/ml in phase I and less than 0.05 μg/ml in phase II against *Staph. aureus*. These findings demonstrate a nonadherence to a saturable process at the lower concentration ranges. Additionally, the plots for *Staph. aureus* show a complete break and displacement of the saturable from the nonsaturable level, which may indicate a change in drug partitioning and/or binding properties in the biophase.

Table I—Kinetic Parameters of Clindamycin Action on *Staph. aureus* and *E. coli*

Kinetic Parameter[a]	*Staph. aureus* Phase I	*Staph. aureus* Phase II	*E. coli*
$10^5 k_c$, ml/μg sec[b]	416.67	322.50	2.56
$10^5 k_a$, ml/μg sec[c]	1333.33	1111.11	3.70
$10^5 k_a/k_b$, sec^{-1}[c]	54.17	54.17	70.00
$(k_a/k_b)/k_0$[c]	1.35	1.35	1.11
$10^2 k_b$, ml/μg[c]	2461.53	2051.27	5.28
MIC, μg/ml[d]	0.11	0.14	113.20

[a] Derived from data of Fig. 3. [b] Calculated from the slope of the plot of k_{app} *versus* concentration from 0 to 0.04 μg/ml of clindamycin in phase I action and from 0 to 0.25 μg/ml of clindamycin in phase II action on *Staph. aureus* or from 0 to 10 μg/ml of clindamycin action on *E. coli*, in accordance with Eq. 2. [c] The k_a, k_a/k_b, $(k_a/k_b)/k_0$, and k_b values are estimated from the slopes and intercepts of plots of C $(k_0 - k_{app})$ *versus* C from Eq. 3 for clindamycin concentrations greater than 0.04 μg/ml in phase I action and greater than 0.05 μg/ml in phase II action on *Staph. aureus* or greater than 10 μg/ml on *E. coli*. [d] Calculated from Eq. 4, where clindamycin is at the MIC.

The values of k_a and k_b calculated from the slopes and intercepts of such plots are given in Table I. The minimum inhibitory concentration (MIC) of clindamycin in Table I is calculated from the expression (7):

$$C_m = k_0/(k_a - k_0 k_b) \qquad \text{(Eq. 4)}$$

where C_m is the MIC. It is estimated that clindamycin has an MIC value against *E. coli* that is about 1000 times the MIC value against *Staph. aureus*.

Effect of Broth Media of Different Compositions—Table II gives the apparent generation rate constants of drug-free and drug-affected *Staph. aureus* cultures in three broth media of different compositions. Variations in generation rate constants among the different media at each concentration level of clindamycin were not significantly different from those observed in the daily variations observed in organism generation rates, as previously was found for *E. coli* (6).

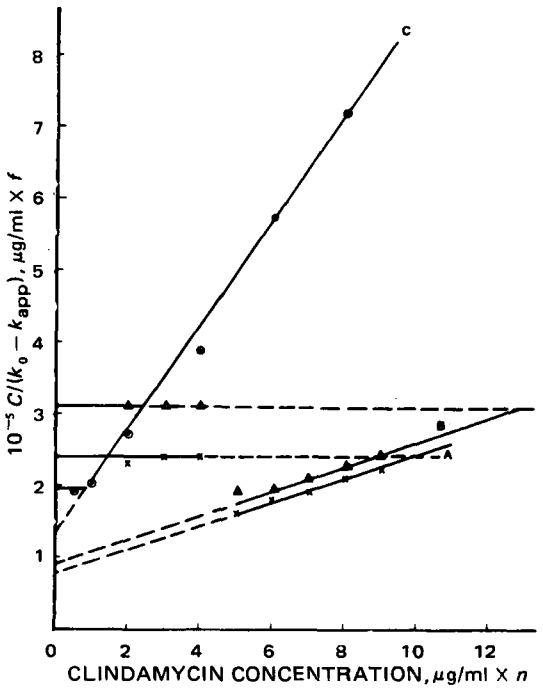


Figure 4—*Applicability of saturation kinetics to the action of clindamycin on* Staph. aureus *and* E. coli *at pH 7.05 and 37.5°. Curve A is for clindamycin-affected* Staph. aureus *in phase I generation (×), curve B is for clindamycin-affected* Staph. aureus *in phase II (▲), and curve C is for clindamycin-affected* E. coli *(⊙). Curves are plotted in accordance with Eq. 3. Actual values of* $C/(k_0 - k_{app})$ *are multiplied by a factor,* f, *where* f = *1000 for curves A and B and* f = *5 for curve C. Actual clindamycin concentrations are also multiplied by a factor,* n, *where* n = *100 for curves A and B and* n = *0.1 for curve C.*

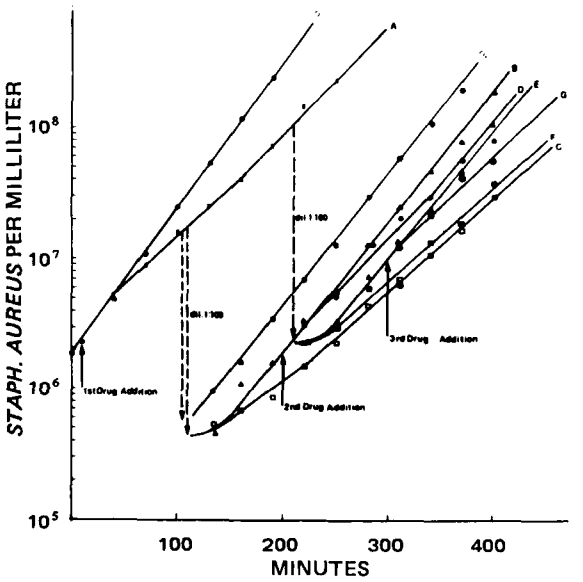


Figure 5—*Semilogarithmic plots of reversibility studies of* Staph. aureus *with time of addition of clindamycin and dilution of the cultures with broth. Curve O is without drug. Curve A is after addition of clindamycin to the culture of curve O to a final concentration of 0.03 μg/ml; curve O_1 is for the drug-free culture of curve O diluted 100-fold with broth. Curves B and E are for the drug-affected culture of curve A diluted 100-fold with broth during phase I and phase II generations, respectively, so that the final clindamycin concentration is reduced to 0.0003 μg/ml. Curves C and F are for the drug-affected culture of curve A diluted 100-fold with broth containing a sufficient amount of clindamycin during phase I and phase II generations, respectively, so that the final clindamycin concentration is reestablished at 0.03 μg/ml. Curve D is for the diluted culture of curve B that has emerged from lag phase and clindamycin has been added to restore the concentration to 0.03 μg/ml. Curve G is for the diluted culture of curve E that has similarly emerged from lag phase and clindamycin has been added to restore the concentration to 0.03 μg/ml.*

Effect of Inoculum Size at Time of Drug Addition—The apparent generation rate constants of drug-free and drug-affected *Staph. aureus* cultures, which varied in organism population at the time of drug addition, are also given in Table II.

There were no significant variations among the generation rate constants for the different organism populations at each concentration level of the clindamycin studied, as previously was found for *E. coli* (6).

Reversibility of Clindamycin Action—The results obtained for reversibility of clindamycin action on *Staph. aureus* at concentration levels of 0.03 and 0.06 μg/ml are shown in Figs. 5 and 6, respectively.

The equilibration of clindamycin between the broth medium and the biophase in *Staph. aureus* was readily achieved. It took 20–25 min after drug addition for cultures affected with 0.03 μg/ml (curve A in Fig. 5) and 0.06 μg/ml (curve A in Fig. 6) to attain a new steady-state phase of generation.

The steady-state generation of drug-free culture (curve O in Figs. 5 and 6), when diluted 100-fold, reverted to a new steady state with a predictable rate constant (curve O_1 in Figs. 5 and 6). The diluted culture showed an apparent initial lag phase period of 5–10 min before reverting to the new steady state. This lag may be attributed to a possible need for cell rejuvenation before reestablishment of the new steady state or to a consequence of the shock of dilution.

Table II—Effect of Variations in Broth Composition and Inoculum Size on Phase I and Phase II Generation Rates of Clindamycin-Affected *Staph. aureus*

Clindamycin Concentration, μg/ml	$k_{app} \times 10^{-5}$ sec^{-1}											
	Phase I			Phase II			Phase I			Phase II		
	B_1[a]	B_2[b]	B_3[c]	B_1	B_2	B_3	I_2[d]	I_2[e]	I_3[f]	I_1	I_2	I_3
0	37.96	41.00	42.90	—	—	—	41.98	40.13	38.50	—	—	—
0.03	28.94	27.64	25.90	28.67	31.33	29.03	28.44	27.53	27.08	30.17	30.40	29.61
0.05	8.60	8.26	7.52	14.98	12.67	15.75	7.07	8.78	8.79	18.21	14.73	12.35
0.07	3.20	3.49	2.76	6.66	7.45	7.96	3.40	3.61	2.97	6.70	6.68	7.77
0.09	0	0	0	2.30	2.24	3.62	0	0	0	2.89	2.65	3.18

[a] Half strength broth (B_1). [b] Normal strength broth (B_2). [c] Double strength broth (B_3). [d] Inoculum size of 5.0×10^5 cells/ml (I_1). [e] Inoculum size of 2.0×10^6 cells/ml (I_2). [f] Inoculum size of 2.0×10^7 cells/ml (I_3).

The generation pattern of drug-affected *Staph. aureus*, when diluted 100-fold, depended on the concentration level of the clindamycin employed. For the culture affected with the 0.03-μg/ml clindamycin concentration, dilution of the culture in phase I (curve B in Fig. 5) or phase II (curve E in Fig. 5) generation resulted in a short lag phase of approximately 20–25 min. Thereafter, a new steady generation was established at a rate similar to that of the control culture (curve O in Fig. 5). This finding shows that the drug action is nullified by high dilution in broth. It also indicates that the rate of dissociation of the drug–receptor complex in the biophase is the same as the rate of formation of complex and/or the rate of diffusion of drug from the broth medium into the biophase through cell membranes.

The addition of clindamycin to the diluted cultures of curves B and E (Fig. 5), which had emerged from the lag phase, to restore the concentration to 0.03 μg/ml resulted in a short period of equilibration, *i.e.*, 5–10 min, followed by a new steady-state generation (curves D and G, respectively) with predictable rate constants. Likewise, a 1:100 dilution of the drug-affected culture of curve A (Fig. 5) during phase I (curve C in Fig. 5) or phase II (curve F in Fig. 5) so as to reestablish the concentration to 0.03 μg/ml resulted in a new steady-state generation with predictable rate constants. The new steady-state generations were preceded by a short lag period of 20–25 min. These observations indicate that clindamycin action on *Staph. aureus* at a concentration level of 0.03 μg/ml is rapidly reversible and is similar to that reported (6) for *E. coli* at a

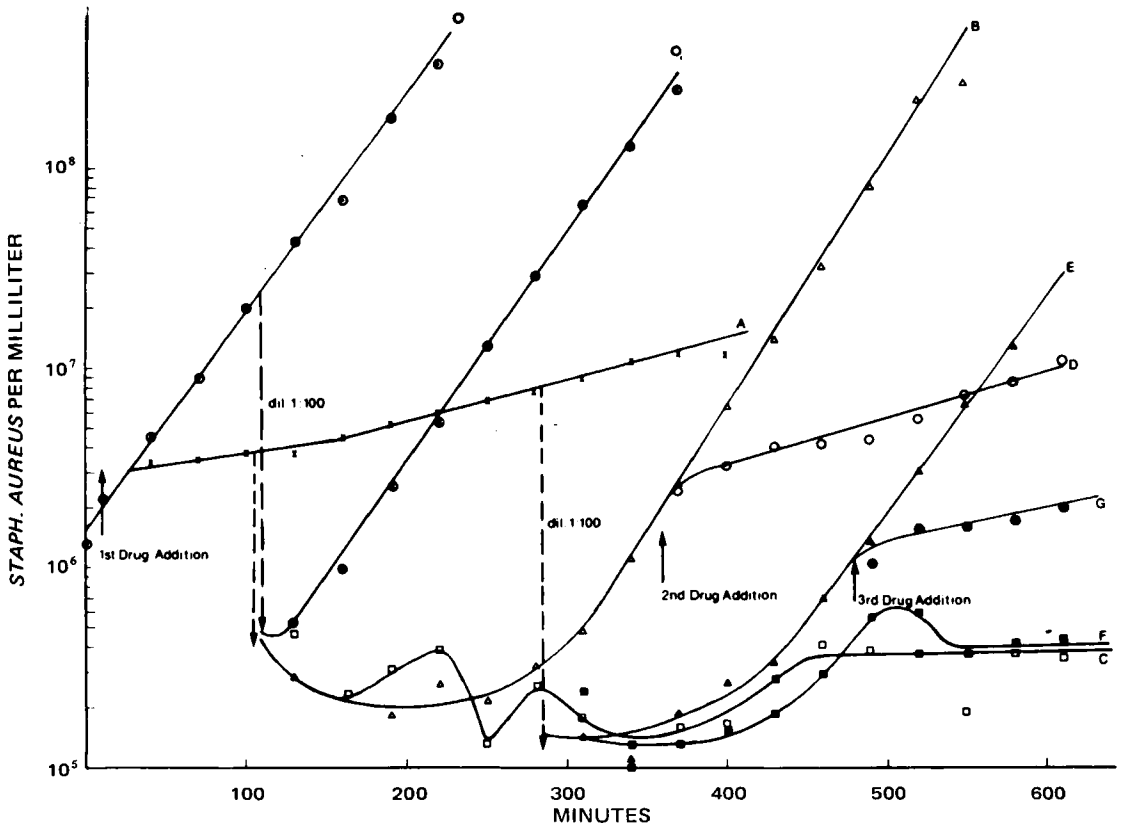


Figure 6—*Semilogarithmic plots of reversibility studies of* Staph. aureus *with time of addition of clindamycin and dilution of the cultures with broth. Curve O is without drug. Curve A is after addition of clindamycin to the culture of curve O to a final concentration of 0.06 μg/ml; curve O_1 is for the drug-free culture of curve O diluted 100-fold with broth. Curves B and E are for the drug-affected culture of curve A diluted 100-fold with broth during phase I and phase II generations, respectively, so that the final clindamycin concentration is reduced to 0.0006 μg/ml. Curves C and F are for the drug-affected culture of curve A diluted 100-fold with broth containing a sufficient amount of clindamycin during phase I and phase II generations, respectively, so that the final clindamycin concentration is reestablished at 0.06 μg/ml. Curve D is for the diluted culture of curve B that has emerged from lag phase and clindamycin has been added to restore the concentration to 0.06 μg/ml. Curve G is for the diluted culture of curve E that has similarly emerged from lag phase and clindamycin has been added to restore the concentration to 0.06 μg/ml.*

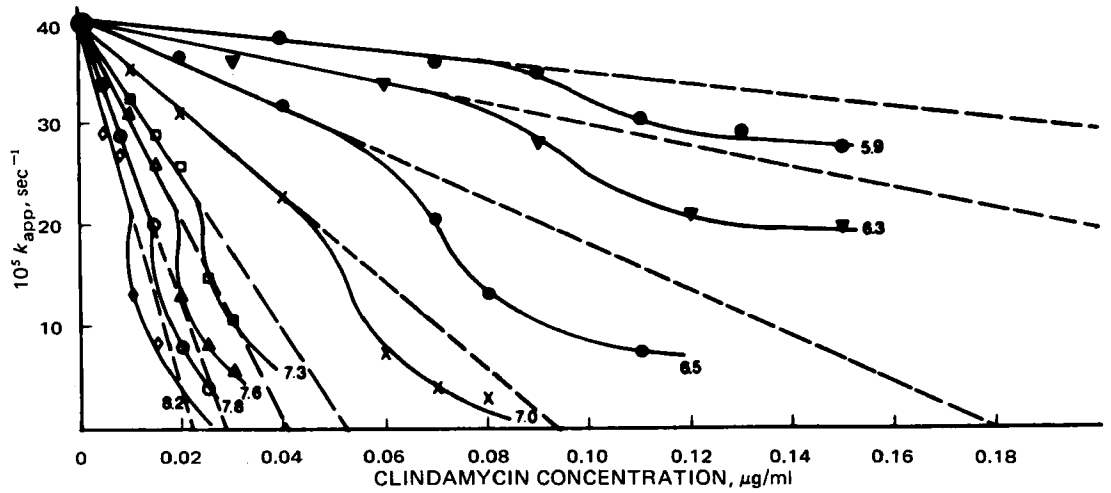


Figure 7—*Dependence of apparent generation rate constant,* k_{app} *in seconds*$^{-1}$*, of clindamycin-affected* Staph. aureus *cultures in phase I generation on the pH of the broth medium. Curves are labeled with the pH values.*

concentration level of 33.34 μg/ml (6).

On the other hand, a 1:100 dilution of the culture affected with clindamycin at a concentration of 0.06 μg/ml during phase I (curve B in Fig. 6) or phase II (curve E in Fig. 6) generation resulted in a prolonged lag period of approximately 150–200 min. Subsequently, a new steady-state generation was established at a rate similar to that of the control culture (curve O in Fig. 6). This finding indicates that the drug action is nullified by the high dilution of the culture. However, even if the time for rejuvenation of the cells or the consequence of shock on dilution is considered, the prolonged lag period observed for the diluted culture to attain a new steady-state generation suggests that the rate of dissociation of the drug–receptor complex in the biophase is less than the rate of drug diffusion from the broth medium into the biophase through cell membranes. Alternatively, it may suggest that there is a kill of the greater proportion of cells by a bactericidal or lytic phenomenon, which leaves relatively few survivors whose lag phase becomes unduly prolonged.

The addition of clindamycin to the diluted cultures of curves B and E (Fig. 6), which had emerged from the lag phase, to restore the concentration to 0.06 μg/ml resulted in a short period of equilibration, *i.e.*, 20–25 min. Subsequently, a new steady-state generation was established with predictable rate constants (curves D and G in Fig. 6). However, a 1:100 dilution of the drug-affected culture of curve A (Fig. 6) during phase I (curve C in Fig. 6) or phase II (curve F in Fig. 6) generation to reestablish the concentration of clindamycin in the broth to 0.06 μg/ml produced an initial phase of erratic generation curves followed by a stationary phase. This finding may indicate a type of generation inhibition in which an increase in the number of survivor cells is offset by a decrease in the number of cells killed by a bactericidal or lytic phenomenon. Since the dilution did not result in changes in thermal or osmotic conditions of the environment, it is concluded that lysis of the cells could occur from permanent damage or injury of cell membranes and/or cell walls resulting in leakage of cell contents or as a consequence of autolysis of cells killed by bactericidal action of clindamycin at a concentration level of 0.06 μg/ml.

The action of clindamycin at the high concentration level (0.06 μg/ml) clearly was not as readily and rapidly reversible as at the low concentration level (0.03 μg/ml). Furthermore, a bactericidal or lytic phenomenon could be superimposed on normal generation inhibition at the high concentration level.

Effect of pH on Drug-Affected Generation Rates—Apparent generation rate constants obtained for *Staph. aureus* in broth at pH 5.9–8.2, in the absence and presence of graded concentrations of clindamycin, are plotted in Fig. 7. The generation rate constants for drug-free cultures were invariant with pH, but those for drug-affected cultures were significantly decreased as the pH was increased at the same clindamycin concentration. The sigmoidal shape of the curve obtained from plots of the generation rate constant *versus* drug concentration at pH 7.05 (Fig. 3) was reproduced at all other pH values studied.

The calculated values of k_c, obtained from slopes of plots of k_{app_I} *versus* clindamycin concentration, *C*, in accordance with Eq. 2, are plotted as a function of pH (curve C) in Fig. 8. The calculated values k_a, obtained from intercepts of plots of $C/(k_0 - k_{app_I})$ *versus C* in accordance with Eq. 3, are also plotted as a function of pH (curve B in Fig. 8). The values for k_c and k_a increased about 10-fold per unit increase in pH over the 5.9–7.0 range, but the slopes of the plots tended to lessen, in both cases, as the pH approached the pKa value of clindamycin. The plots of k_a *versus* pH obtained from the data (6) of clindamycin action on *E. coli* (curve A in Fig. 8) show similar evidence.

DISCUSSION

The suitability of a particle-size counter for monitoring the organism population in an estimate of apparent generation rate constants of drug-affected *E. coli* cultures has been well established (8). Its applicability for similar determinations on drug-affected *Staph. aureus* cultures recently was reported (7). The present work also confirms that the method is applicable for determining

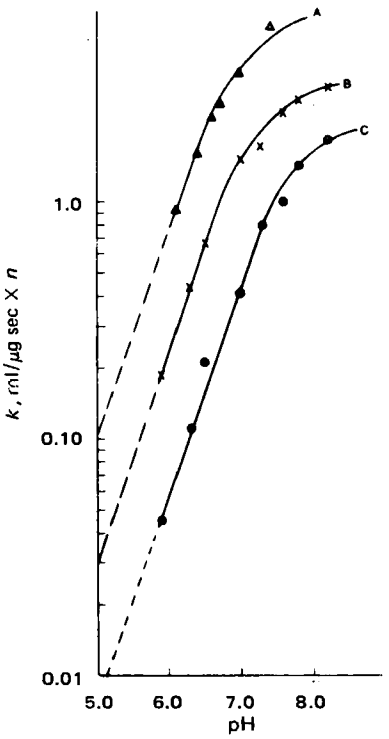


Figure 8—*Semilogarithmic plots of apparent inhibitory rate constant,* k *(in milliliters per microgram seconds), for the action of clindamycin on* E. coli *(curve A) and* Staph. aureus *(curves B and C). In curves A and B,* k *values are the* k_a *values derived from the intercepts of plots of* $C/(k_0 - k_{app})$ versus C *in accordance with Eq. 3; k values in curve C are the* k_c *values derived from the slopes of plots of* k_{app} versus C *in accordance with Eq. 2.*

the generation rates of *Staph. aureus* and *E. coli* cultures affected with subcompletely inhibitory concentrations of clindamycin.

At the low clindamycin concentration level (0–0.04 μg/ml), the total count on *Staph. aureus*-affected cultures was 50% of the viable count (Fig. 1). This finding is attributed (7) to the dissociation of cell aggregates by the high dilution steps and/or subsequent generation of the culture samples withdrawn for the viable count procedure, which result in relatively high colony counts. However, the generation curves obtained from the total count remained parallel to those of the viable count. Reversibility studies (Fig. 5) demonstrated a rapidly reversible action of the clindamycin on *Staph. aureus* cells. The conclusion is that there is no kill or death of the organisms superimposed on normal generation inhibition by the action of clindamycin in this low concentration range.

At the high clindamycin concentration level (> 0.04 < 0.1 μg/ml), the total count on the drug-affected *Staph. aureus* in phase I generation was increased to 80–90% of the viable count (Fig. 1). This finding suggests a condition of "dynamic bacteriostasis" (9), in which the rate of kill of the organisms is balanced by the rate of generation of survivor cells in the culture. Consequently, the total count remains virtually the same as the viable count. During the phase II generation of the cultures, however, there was an apparent increase in the rate of generation obtained by the total count. This result may be attributed to an increased rate of generation of mutant-resistant survivor cells that are counted indiscriminately with dead cells by the particle counter. The corresponding viable counts, on the other hand, showed that the rate of generation was: *(a)* increased to a relatively less extent than the increase obtained by total count for the culture affected with a subcompletely inhibitory clindamycin concentration of 0.05 μg/ml, *(b)* unchanged for the culture affected with an inhibitory clindamycin concentration of 0.07 μg/ml, or *(c)* slightly decreased for the culture affected with the clindamycin concentration of 0.08 μg/ml.

These observations indicate that there is a kill and/or poor recovery of a large percentage of cells damaged or injured by a bactericidal or lytic phenomenon as a function of drug concentration and drug–bacteria contact time. Furthermore, the clindamycin action in this subcompletely inhibitory concentration range is not readily and rapidly reversible (Fig. 6). The inference is that a bactericidal or lytic phenomenon may be superimposed on generation inhibition of the *Staph. aureus* cultures by the action of clindamycin. At very high clindamycin concentrations (> 0.1 μg/ml), there was a rapid drop in the viable count with time as a function of drug concentration whereas the total count remained constant with time and was invariant with drug concentration.

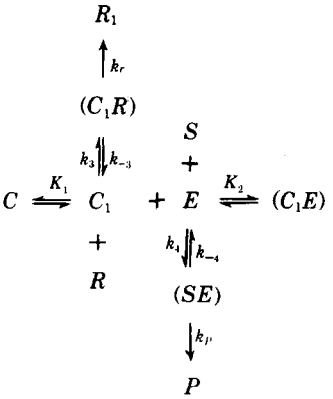
These findings demonstrate unequivocally that clindamycin exerts a bactericidal effect on the cultures in this concentration range. In contrast, the viable count of clindamycin-affected *E. coli* was 50–80% of the total count (Fig. 2), and the generation curves obtained from the viable count remained parallel to those obtained from the total count at any concentration level. There was no evidence of a bactericidal or lytic phenomenon superimposed on generation inhibition by the action of clindamycin on *E. coli* in the subcompletely inhibitory concentration range of 0–100 μg/ml or above the inhibitory concentration levels, *e.g.*, 200 μg/ml. It is, therefore, quite evident that clindamycin exerts a "true" bacteriostatic action (9) on *E. coli*.

Staph. aureus cultures affected with subcompletely inhibitory concentrations of clindamycin exhibited biphasic steady-state generation curves (Fig. 1). The apparent generation rate constant (k_{app_I}) of the initial (phase I) generation was less than the apparent generation rate constant ($k_{app_{II}}$) of the subsequent (phase II) generation of the clindamycin-affected *Staph. aureus*, suggesting organism adaptation or development of resistance to drug action after a finite period of drug–bacteria contact. On the other hand, *E. coli* cultures affected with subcompletely inhibitory concentrations of clindamycin exhibited monophasic steady-state generation curves (Fig. 2). There was no evidence of organism adaptation or development of resistance to drug action.

The functional dependency of the apparent generation rate constant, k_{app}, on drug concentration for clindamycin-affected *Staph. aureus* in phase I was the same as that of the phase II generation (Fig. 3), suggesting a common mechanism of action in both generation phases. Therefore, the plots of k_{app} *versus* drug concentration for phase I and phase II generations are superimposable by a potency factor of 0.80 over the entire concentration range studied. This finding indicates that only 80% of the available drug concentration in broth is effective against the resistant mutants of *Staph. aureus* in phase II generation.

The curve for the dependence of the k_{app} on drug concentration for the clindamycin-affected *Staph. aureus* has a sigmoidal shape similar to that recently reported for the action of fusidate sodium (10) and quinacrine (11) on *E. coli*. It has been shown from theoretical considerations (10) that this shape can be attributed to either: *(a)* a saturable enzymatic degradation of the drug in the biophase of the microorganism or *(b)* drug binding to saturable binding sites in the medium. Table II shows no evidence of interference in clindamycin action on *Staph. aureus* to suggest that degradation of the drug occurs from microbial metabolic activities as a function of organism numbers or that a depletion of free or unbound drug in the broth medium is produced by binding, absorption, or inactivation processes as a result of variations in broth composition. On the other hand, there is demonstrated evidence of a bactericidal phenomenon which operates concurrently with generation inhibition at subcompletely inhibitory concentrations of clindamycin (>0.04 μg/ml). It is possible, therefore, that perturbations in the kinetics of the drug inhibitory action produced by the onset of a bactericidal action may account for the sigmoidal shape of the curve for the dependence of k_{app} on drug concentration.

A model consistent with the dual mechanism of clindamycin action on *Staph. aureus* is shown in Scheme I.



Scheme I

In this model, C is the clindamycin concentration in the broth medium, which is in equilibrium with C_1, the clindamycin concentration within the cell or biophase that reversibly binds with enzyme, E, to form the drug–enzyme complex, C_1E; S is the normal substrate (or metabolite) synthesized by the enzyme into the protein product, P, from the substrate–enzyme complex, SE, and is utilized in cell development and generation. The clindamycin concentration in the biophase also reacts irreversibly with a "target" site (or vital structure), R, to form a damaged (or injured) site or a toxic product, R_1, from a reaction complex, C_1R. The target site may be a sensitive compound of the cytoplasmic membrane, protoplasmic or nuclear material, or a metabolic or transport agent, which is essential for organism survival (12–16). The following equations apply if it is postulated that:

1. The rate of increase in microbial numbers, dN/dt, is proportional to the net rate of microbial generation, dG/dt, above microbial death, dD/dt, and to the number, N, of organisms present.
2. The rate of microbial generation, dG/dt, is proportional to the net rate of protein synthesis, dP_1/dt, above a certain minimum, Δp, required for life-sustaining processes and to the number, N, of organisms present.
3. The rate of microbial death, dD/dt, is proportional to the rate of formation of the damaged (or injured) site or the toxic product and to the number, N, of organisms present.

The equations are:

$$dN/dt = k_n(dG/dt - dD/dt)N = k_{app}N \qquad \text{(Eq. 5)}$$

where k_n is a constant of proportionality and k_{app} is the apparent generation rate constant of the drug-affected culture, and:

$$dG/dt = k_g(dP_1/dt)N = k_g(dP/dt - \Delta p)N \qquad \text{(Eq. 6)}$$

where k_g is a constant of proportionality and dP/dt is the overall

rate of protein synthesis. Moreover:

$$dD/dt = k_d(dR_1/dt)N \quad \text{(Eq. 7)}$$

Combination of Eqs. 5–7 yields:

$$k_{app} = k_n\{k_g(dP/dt - \Delta p) - k_d(dR_1/dt)\} \quad \text{(Eq. 8)}$$

On the basis of Scheme I, it can be stated that:

$$dP/dt = (k_pk_4/k_p + k_{-4})(S)(E) = k_p{}^1E = k_p{}^1(1 - \theta)E_t \quad \text{(Eq. 9)}$$

where $k_p{}^1 = (k_pk_4/k_p + k_{-4})S$, S is assumed invariant, and $\theta = C_1E/E_T$ is a fraction of total enzyme, E_t, bound to the drug.

The value of θ is explicitly defined by a previously derived equation (1, 6) as follows:

$$\theta = K_1K_2C/(1 + K_1K_2C) \quad \text{(Eq. 10)}$$

where K_1 is the drug partition constant through cell membranes and K_2 is the drug affinity constant for the enzyme, E.

Combination of Eqs. 9 and 10 yields:

$$dP/dt = k_m\{1 - [K_1K_2C/(1 + K_1K_2C)]\} \quad \text{(Eq. 11)}$$

where $k_m = k_p{}^1E_t$.

Also, under steady-state conditions:

$$dR_1/dt = (k_rk_3/k_r + k_{-3})(C_1)(R) \quad \text{(Eq. 12)}$$

and:

$$C_1 = K_1C \quad \text{(Eq. 13)}$$

where K_1 is as defined in Eq. 10.

Combination of Eqs. 12 and 13 yields:

$$dR_1/dt = (k_rk_3/k_r + k_{-3})(K_1)(C)(R) \quad \text{(Eq. 14}a\text{)}$$

$$dR_1/dt = k_eC \quad \text{(Eq. 14}b\text{)}$$

where $k_e = (k_rk_3/k_r + k_{-3})(K_1)(R)$, and R is assumed to be comprised of an infinitely large number of molecules (12) so that the rate of formation of R_1 becomes only dependent on drug concentration. Substitution of Eq. 14*b* into Eq. 7 yields:

$$dD/dt = (k_dk_eC)N = (k_uC)N = -dN/dt \quad \text{(Eq. 15)}$$

where $k_u = k_dk_e$ is the death rate constant, and $-dN/dt$ is the rate of decrease in microbial numbers. Equation 15 is the typical equation of the "mechanistic theory" that attempts to explain the bactericidal process of many compounds (12–20).

Combining Eqs. 8, 11, and 14*b* and simplifying give:

$$k_{app} = k_nk_g(k_m - \Delta p) - [k_nk_gk_mK_1K_2C/(1 + K_1K_2C) + k_dk_eC] \quad \text{(Eq. 16}a\text{)}$$

$$k_{app} = k_0 - \{[k_{a_1}C/(1 + k_bC)] + k_uC\} \quad \text{(Eq. 16}b\text{)}$$

where k_0 is the generation rate constant of the drug-free culture, $k_b = K_1K_2$, $k_{a_1} = k_nk_gk_mK_1K_2$, and k_u is as defined in Eq. 15. Therefore:

$$k_a/k_b = k_nk_gk_m > k_0 \quad \text{(Eq. 17)}$$

Equation 16*b* describes the functional dependencies of k_{app} on the drug concentrations for clindamycin-affected *Staph. aureus* in phase I and phase II generations (Fig. 3). At low clindamycin concentrations in phase I action (0–0.04 μg/ml) or in phase II action (0–0.05 μg/ml), when the death rate is exceedingly small so that $k_uC \rightarrow 0$ and when few enzyme sites are possibly interacted with drug to inhibit protein synthesis so that $k_bC \ll 1$, Eq. 16 simplifies to Eq. 2, which is the expression for the observed linear dependence of k_{app} on drug concentration in these concentration ranges (Fig. 3).

At high drug concentrations, it is possible that the extent of generation inhibition is decreased with an increase in drug concentration because of the saturation of enzyme sites by the drug, *i.e.*, $k_bC > 1$. Under such conditions, the k_{app} should decrease asymptotically to zero with an increase in drug concentration similar to that observed for lincomycin action on *Staph. aureus* (7). However, an onset of a bactericidal action, when $k_uC \gg 0 < 1$, causes a reduction in the numbers of viable cells in the active phase of multiplication to alter the functional dependency of the k_{app}. The overall interaction of bacterial death from a bactericidal action, with generation inhibition as a consequence of decreased rate of protein synthesis by the clindamycin action, results ultimately in a sigmoidal shape of the curve for the dependence of k_{app} on drug concentrations, which is described by Eq. 16*b*.

Transformation of Eq. 16*b* yields:

$$C/(k_0 - k_{app}) = Ck_b/[(k_{a_1} + k_u) + k_bk_uC] + 1/[(k_{a_1} + k_u) + k_bk_uC] \quad \text{(Eq. 18)}$$

If k_uk_bC values are extremely small compared to exceedingly large $k_{a_1} + k_u$ values, then Eq. 18 simplifies to Eq. 3 as follows:

$$C/(k_0 - k_{app}) = k_bC/(k_{a_1} + k_u) + 1/(k_{a_1} + k_u) = k_bC/k_a + 1/k_a \quad \text{(Eq. 19)}$$

where $k_a = k_{a_1} + k_u$.

Equation 19 is adhered to by the data of Fig. 3 from linear plots of $C/(k_0 - k_{app})$ *versus* C for clindamycin concentrations greater than 0.04 μg/ml in phase I action (curve A in Fig. 4) or greater than 0.05 μg/ml in phase II action (curve B in Fig. 4) and may indicate a saturable mechanism of the clindamycin action at high concentration levels. The plots are, however, completely displaced from those of clindamycin action in the low concentration range, *i.e.*, 0–0.04 μg/ml in phase I or 0–0.05 μg/ml in phase II action, which do not adhere to the saturable receptor site model. This finding confirms that a different mechanism of action (*i.e.*, the bactericidal action) is superimposed on generation inhibition at the saturable level.

Table II shows kinetic parameters derived from plots of the data of Fig. 3 in accordance with Eqs. 12 and 19. The k_a/k_b value for *Staph. aureus* is 1.35 times the k_0 value, which is in agreement with theoretical predictions of Eq. 17. The calculated value of k_b in Eq. 19 for the phase I generation is 1.20 times the value for the phase II generation. Since k_b is derived from K_1 and K_2 as defined in Eq. 10, it can be rationalized for clindamycin–*Staph. aureus* interactions, as in the case of lincomycin–*Staph. aureus* interactions (7), that K_2 is decreased in the phase II generation of the drug-affected cultures. The resistant mutant strains in the phase II generation may have ribosomal components whose binding affinity for clindamycin is less than that of the initial strains in the phase I generation. Therefore, the observation that 80% of the total drug concentration in broth is kinetically active against the drug-affected organism in phase II generation is attributed to this phenomenon.

The dependence of the generation rate constant, k_{app}, on drug concentration, C, for clindamycin-affected *E. coli* is linear at low drug concentrations (0–10 μg/ml) but is curvilinear at high drug concentrations (>10 μg/ml) when it also decreases asymptotically to zero with an increase in drug concentration (Fig. 3). An operative kinetic model (6), which defined the action of lincomycin and its 7(*S*)-chloro analogs, is applicable. Clindamycin action on *E. coli* is explained as competitive binding of a saturable receptor site, which is also the binding locus for a metabolite normally utilized by the receptor site in the synthesis of protein for microbial generation. The functional dependency of k_{app} on clindamycin (curve B in Fig. 3) is explicitly defined by the equation:

$$k_{app} = k_0 - k_aC/(1 + k_bC) \quad \text{(Eq. 20)}$$

where k_0, k_a, and k_b are as defined in Eq. 10. Adherence to a saturable receptor site model is observed from linear plots of $C/(k_0 - k_{app})$ *versus* C (Fig. 4), obtained from the data of Fig. 3 in accordance with the expression in Eq. 3 for clindamycin concentrations greater than 10 μg/ml. Deviations occur at low concentrations (0–10 μg/ml), when only few receptors can possibly be interacted with drug.

The calculated value of k_b (Table II) for clindamycin-affected *E. coli* is 0.21% of the value for clindamycin-affected *Staph. aureus* in phase I or 0.25% of the value for clindamycin-affected *Staph. aureus* in phase II generation. Since $k_b = K_1K_2$ as defined in Eq. 10, it is inferred that clindamycin is far more readily partitioned through cell membranes into the biophase and/or has exceedingly higher affinity for receptors in *Staph. aureus* than in *E. coli*, due possibly to differences in gross morphology and physiology of the organisms.

At the MIC, C_m, when $k_{app} = 0$, Eq. 3 simplifies to Eq. 7 and the calculated values of C_m (Table II) for clindamycin action against

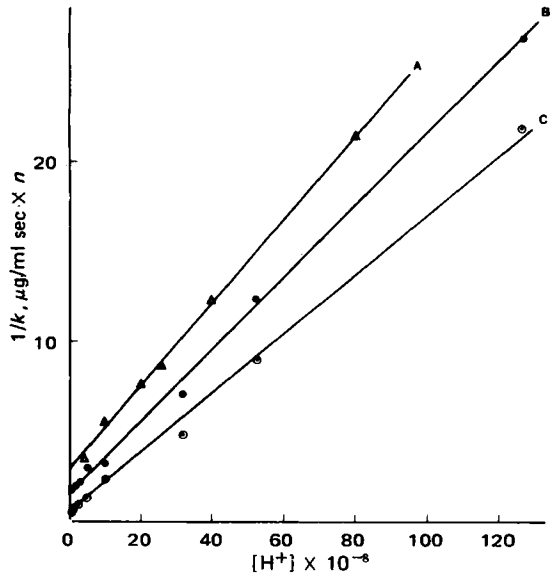


Figure 9—*Linearity of plots for 1/k (micrograms per milliliter second)* versus *[H+] in accordance with Eq. 22 for the action of clindamycin on* E. coli *(curve A) and* Staph. aureus *(curves B and C). In curves A and B,* k *values are the* k_a *values derived from the intercepts of plots of* $C/(k_0 - k_{app})$ versus C *in accordance with Eq. 3;* k *values in curve C are the* k_c *values derived from the slopes of plots of* k_{app} versus C *in accordance with Eq. 2.*

E. coli are 1000 times that against *Staph. aureus*, which is attributed to differences in the modes of clindamycin action and the nature of the respective organisms.

The pH of the broth medium influences the activity of clindamycin on *Staph. aureus* (Fig. 7) in a manner similar to what was observed for its action on *E. coli* (6). The k_{app} values of drug-affected cultures decrease with an increase in the pH at any specified concentration. Therefore, progressively larger quantities of the drug are needed to produce the same generation inhibition as the pH is decreased. The sigmoidal shape of curve for the k_{app} dependence on drug concentration is observed at all other pH values studied. The constant k_c (milliliters per microgram second), as defined in Eq. 2, and the constant k_a (milliliters per microgram second), as defined in Eq. 3, for various pH values from the data of drug-affected *Staph. aureus* in Fig. 7, or the constant k_a (milliliters per microgram second), as defined in Eq. 3, for various pH values obtained from previous data (6) of drug-affected *E. coli* adhere to the expression:

$$k = k^* f K_a/(K_a + [H^+]) \qquad \text{(Eq. 21)}$$

where k is either k_c or k_a, k^* is the intrinsic value of k for the unprotonated drug molecule, f is the fraction of the unprotonated drug, K_a is the dissociation constant of the unprotonated base, and $[H^+]$ is the hydrogen-ion concentration. The plots of log k *versus* pH (Fig. 8) approach a slope of unity when $[H^+] > K_a$ but have a null slope when $K_a > [H^+]$, suggesting that the unprotonated drug molecule is the active species. The linear plots of $1/k$ *versus* $[H^+]$ in Fig. 9 are obtained from the data of Fig. 8 in accordance with an expression derived from Eq. 21:

$$1/k = [H^+]/(k^* K_a) + 1/k^* \qquad \text{(Eq. 22)}$$

The pKa and k^* values of clindamycin, derived from the slopes and intercepts of the plots for drug-affected *Staph. aureus* (curves B and C in Fig. 9) and *E. coli* (curve A in Fig. 9) are given in Table III. The kinetically derived pKa values are in reasonably good agreement with the 7.0 value (6) obtained by potentiometric titration. It is concluded that Eq. 21 holds and that the unprotonated form of the clindamycin molecule contributes to activity because of its ready penetrability through cell membranes of Gram-positive and Gram-negative organisms.

Table III—Values of pKa and Intrinsic Inhibitory Rate Constants (k^*) for Clindamycin Action on *Staph. aureus* and *E. coli*

Organism	$10^5 k_c^{*a}$	$10^5 k_a^{*b}$	Kinetically[c] Determined pKa	Potentiometrically[d] Determined pKa
Staph. aureus	—	3333.34	7.2	7.0
Staph. aureus	1666.67	—	7.4	7.0
E. coli	—	6.67	6.9	7.0

[a] As determined from the intercept of the plot of $1/k_c$ *versus* $[H^+]$ in accordance with Eq. 22, where k_c is obtained from the data of Fig. 7 and in accordance with Eq. 2. [b] As determined from the intercept of the plot of $1/k_a$ *versus* $[H^+]$ in accordance with Eq. 22, where k_a is obtained from the data of Fig. 7 for *Staph. aureus* or from the data of Ref. 6 for *E. coli* and in accordance with Eq. 3. [c] Calculated from the quotient of the slope and intercept of the plots in Footnote *a* or *b*, whichever is applicable. [d] Reference 6.

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Microbial Kinetics of Drug Action against Gram-Positive and Gram-Negative Organisms III: Effect of Lincomycin and Clindamycin Combinations on *Staphylococcus aureus* and *Escherichia coli*

SAMUEL M. HEMAN-ACKAH

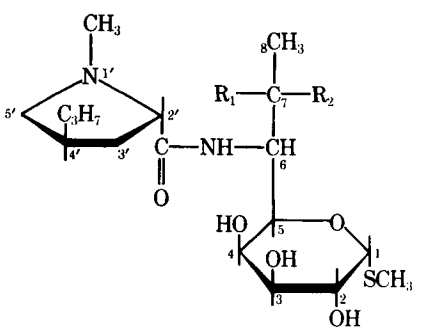
Abstract □ The functional dependencies of apparent first-order generation rate constants, k_{app}, of drug-affected cultures on drug concentrations indicate that lincomycin and clindamycin possess the same mechanism of action, which is bacteriostatic, against *Staphylococcus aureus*. Clindamycin also possesses another mechanism of action, which is bactericidal, at high concentration levels. However, clindamycin possesses only one of the two mechanisms of lincomycin action, which is bacteriostatic, against *Escherichia coli*. The relative potency of action of a clindamycin–lincomycin combination against *Staph. aureus* is variable, and the effective ratio ranges between 5:1 and 9:1; the effective ratio against *E. coli* is fixed at 6:1 over a wide concentration range. This difference is attributed to differences in bioavailability and/or binding characteristics of the drugs for bioreceptors, as a consequence of structural modifications in the drug molecules, and to differences in modes of action in the respective organisms. Mixtures containing equipotent fractions of clindamycin and lincomycin show "equivalence" or "indifference" of effects on *Staph. aureus*. The combined action of the mixtures can be quantitatively predicted from the separate dose–response curves of either component drug alone. Therefore, it is concluded that clindamycin and lincomycin may bind to the same receptor site that is engaged in microbial protein synthesis to inhibit the generation of *Staph. aureus*. However, combinations of clindamycin and lincomycin are less active than the *a priori* equipotent concentration of either drug alone in their action against *E. coli*, demonstrating unequivocally an antagonism of effects. Furthermore, the degree of antagonism is dependent on the order of addition of the drugs, which is attributed to the possibility that clindamycin and lincomycin bind differently on active and allosteric loci of the same receptor site functionally engaged in protein synthesis in *E. coli*. A rational approach to the quantification and prediction of combined antibiotic action must, therefore, be based not only on the kinetics and mechanisms of action as well as on the dose–response relationship over a wide concentration range for the separate antibiotics but also on the strain and species of the test organism.

Keyphrases □ Microbial kinetics—effect of lincomycin and clindamycin combinations on *Staph. aureus* and *E. coli*, drug–bacteria reactions, generation curves □ Lincomycin and clindamycin combinations—comparison of effect on *Staph. aureus* and *E. coli*, microbial kinetics, drug–bacteria reactions, generation curves □ Clindamycin and lincomycin combinations—comparison of effect on *Staph. aureus* and *E. coli*, microbial kinetics, drug–bacteria reactions, generation curves □ *Staphylococcus aureus*—effect of lincomycin and clindamycin combinations, compared to effects on *E. coli*, drug–bacteria reactions, generation curves, microbial kinetics □ *Escherichia coli*—effect of lincomycin and clindamycin combinations, compared to effects on *Staph. aureus*, drug–bacteria reactions, generation curves, microbial kinetics □ Antibiotic combinations—effect of lincomycin and clindamycin combinations on *Staph. aureus* and *E. coli*

Since the discovery of lincomycin (I), an antibiotic produced by *Streptomyces lincolnensis* (1, 2), different structurally modified analogs have been prepared with a view to understanding structure–activity relationships (3). Halogen substitution at the 7(*S*)-configuration of the lincomycin molecule is claimed to potentiate antibacterial effects. Clindamycin (II), which is the 7-deoxy-7(*S*)-chloro analog of lincomycin, is reported to be more than four times more active than the parent antibiotic against a variety of Gram-positive and Gram-negative organisms (4). The similarity in chemical structure would imply that clindamycin should have the same mechanism of action as lincomycin but possess different intrinsic biological activities, so the combined action of clindamycin with lincomycin should produce "additive" or "synergistic" effects (5) on microorganisms.

Comparative studies on the action of lincomycin and its 7(*S*)-chloro analogs, such as the 7-deoxy-7(*S*)-chloro-*N*-demethyl-4-pentyl analog (III) and clindamycin, against *Escherichia coli* by microbial kinetics (6) revealed that combinations of II and III yielded "indifference" of effects (5) but that combinations of I and II or I and III yielded "antagonism" of effects (5). The latter evidence indicates incompatibility in the use of combinations of lincomycin and clindamycin as recommended in current chemotherapy. However, these antibiotics are normally employed for the treatment of Gram-positive infections, which are highly sensitive to the action of the antibiotics (7), and not for Gram-negative infections, which show little or no sensitivity. Therefore, data obtained for *E. coli*, a typical Gram-negative organism, may not be readily extrapolated to Gram-positive organisms without experimental evidence.

Recently, microbial kinetic studies were applied to the action of lincomycin (8) and clindamycin (9) against *Staphylococcus aureus*, a representative Gram-positive coccus, and relative differences in their modes of action against *E. coli* were established. However, the effects of lincomycin and clindamycin combinations on *Staph. aureus* remain to be evaluated and quantified experimentally. These were the objectives of the study reported here.



I: R_1 = OH, R_2 = H
II: R_1 = H, R_2 = Cl

EXPERIMENTAL

Antibiotics—The antibiotics[1] were assayed samples of lincomycin hydrochloride (860 μg of base equivalent/mg) and clindamycin (860 μg of base equivalent/mg). Stock solutions of the antibiotics were prepared by membrane filtration and stored at 4°.

Bacteria and Growth Conditions—*Staph. aureus* (ATCC 6538) and *E. coli* (ATCC 12407) were cultivated in broth medium[2] and used for the experiments. An overnight (12–18 hr) culture of the respective organism was grown aerobically at 37.5° in a shaker water bath[3] to a cell density of 2.0×10^7 cells/ml. It was diluted 100-fold into 50-ml replicates of fresh broth medium and brought to the exponential phase of generation. Aliquots (0.5 ml) of the suitably diluted antibiotic solutions or mixtures were then added to the replicate exponential cultures at a cell density of about 10^6 cells/ml.

Total Count Method—Total counts of drug-free and drug-affected cultures were determined by a particle-size counter[4] as previously described (10). The suitability of the total count method for determining the generation rates of drug-affected cultures previously was established by a demonstrated correlation between the total count and viable (colony) count for lincomycin-affected *Staph. aureus* (8) and *E. coli* (11) and for clindamycin-affected *Staph. aureus* (9) and *E. coli* (9).

RESULTS

Effect of Drug Concentrations on Generation Rates—Semilogarithmic plots of the total count *versus* time yield generation curves of the drug-affected cultures as previously described (8). Lincomycin-affected or clindamycin-affected *Staph. aureus* cultures show two phases of steady-state generation. An initial (phase I) generation expressed as:

$$\ln N = \ln N_0 + k_{app_I} t \qquad \text{(Eq. 1)}$$

is followed by an ultimate (phase II) generation:

$$\ln N = \ln N_0 + k_{app_{II}} t \qquad \text{(Eq. 2)}$$

where k_{app_I} and $k_{app_{II}}$ are the apparent generation rate constants of the respective generation phases, N_0 is the number of organisms at some initial time (0), and N is the number of organisms at any time (t). The $k_{app_{II}}$ is greater than k_{app_I} due to *Staph. aureus* adaptation or development of resistance to lincomycin (8) or clindamycin (9) action after a finite period of drug–bacteria contact.

Lincomycin-affected *E. coli* cultures also show two phases of steady-state generation whose characteristics are different from those of lincomycin-affected and clindamycin-affected *Staph. aureus* cultures. The k_{app_I} is greater than $k_{app_{II}}$, which is attributed (6) to dual mechanisms of the lincomycin action which become "synergistic" (5) in the subsequent generation phase of the culture. On the other hand, clindamycin-affected *E. coli* cultures show one phase of steady-state generation (6):

$$\ln N = \ln N_0 + k_{app} t \qquad \text{(Eq. 3)}$$

where k_{app} is the apparent generation rate constant.

Figure 1 shows the plots of k_{app} *versus* drug concentration for the lincomycin-affected *Staph. aureus* cultures (curve A), in which the phase I and phase II generation curves are superimposable by a potency factor. Likewise, curve B shows superimposable curves of the k_{app} dependence on drug concentration for phase I and phase II generations of clindamycin-affected *Staph. aureus*. The curve for lincomycin-affected *Staph. aureus* (curve A) is linear at low drug concentrations: 0–0.35 μg/ml (in the phase I action) or 0–0.55 μg/ml (in the phase II action). At concentrations greater than 0.35 μg/ml in the phase I action or greater than 0.55 μg/ml in the phase II action, the k_{app} is not a linear function of the increasing drug concentration. The plot for clindamycin-affected *Staph. aureus* (curve B) is a complex function of the drug concentration and yields a sigmoidal shape of curve for the phase I and phase II generations, which are also superimposable by a potency factor.

[1] Courtesy of Dr. J. B. Whitfield, Jr., The Upjohn Co., Kalamazoo, Mich.
[2] Antibiotic Medium 3, Difco Laboratories, Detroit, Mich.
[3] Model RW 65, New Brunswick Scientific Co., New Brunswick, N.J.
[4] Coulter counter, model 2BI, Coulter Electronics Co., Hialeah, Fla.

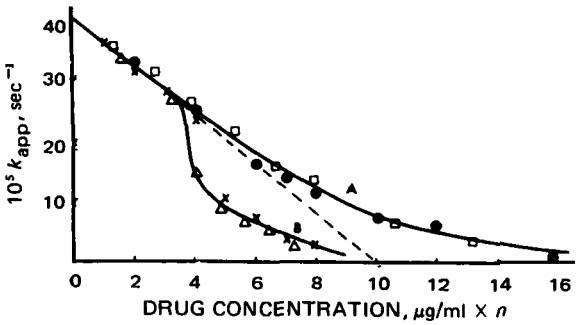


Figure 1—*Dependence of apparent generation rate constants,* k_{app} *in seconds*$^{-1}$*, for drug-affected* Staph. aureus *on drug concentrations (micrograms per milliliter) at pH 7.05 and 37.5°. Actual concentrations are multiplied by a factor,* n, *where* n = *20 for lincomycin phase I action (●) and* n = *13.20 for lincomycin phase II action (□) on* Staph. aureus *in curve A and* n = *100 for clindamycin phase I action (×) and* n = *80 for clindamycin phase II action (△) on* Staph. aureus *in curve B.*

Figure 2 shows plots of k_{app} *versus* drug concentration for the phase I lincomycin action on *E. coli* (curve A), which is coincident with that of clindamycin action on *E. coli* when normalized by a potency factor. The curve for the phase II lincomycin action on *E. coli* (curve B) is different from that of its phase I action (curve A) and is, therefore, not superimposable.

Effect of Order of Addition of Antibiotics on Microbial Generation—*Action of Equipotent Lincomycin Added to Clindamycin-Affected* Staph. aureus—The generation curves for the drug-free *Staph. aureus* cultures are shown as curves A in Figs. 3 and 4. In Fig. 3, the resultant generation curve for the action of 0.04 μg/ml of clindamycin on the *Staph. aureus* culture of curve A is shown as curve B; the generation curve for the culture affected with a mixture of equipotent concentrations of clindamycin (0.04 μg/ml) and lincomycin (0.3 μg/ml) is shown as curve E and is coincident with the curve for the culture affected with an *a priori* equipotent concentration of clindamycin (0.07 μg/ml).

Furthermore, Fig. 3 shows that equipotency of action is demonstrated by coincident or parallel generation curves of the drug-affected cultures having the same generation rate constant, k_{app}. The addition of an equipotent concentration of lincomycin (0.3 μg/ml) to the steady-state phase I generation of the clindamycin-affected culture of curve B produces an ultimate generation curve D, which shows two generation phases parallel to the respective phase I and phase II generations of the drug-affected cultures of curve E. Likewise, the addition of an equipotent concentration of

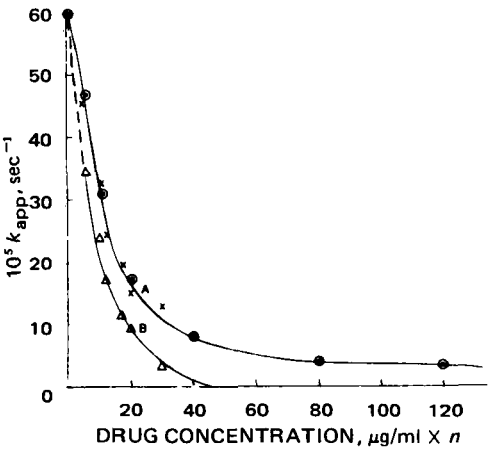


Figure 2—*Dependence of apparent generation rate constants,* k_{app} *in seconds*$^{-1}$*, for drug-affected* E. coli *on drug concentrations (micrograms per milliliter) at pH 7.05 and 37.5°. Actual concentrations are multiplied by a factor,* n, *where* n = *0.15 for lincomycin phase I action (×) and* n = *1.0 for clindamycin action (⊙) on* E. coli *in curve A and* n = *0.15 for lincomycin phase II action (△) on* E. coli *in curve B.*

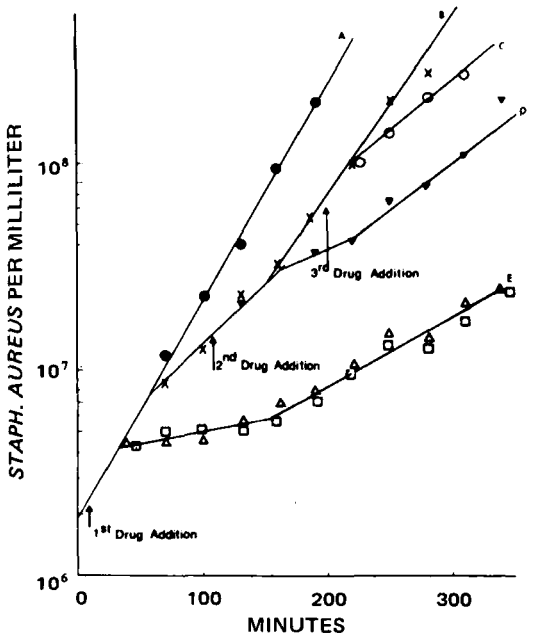


Figure 3—*Effects of addition of an equipotent concentration of lincomycin to clindamycin-affected* Staph. aureus. *Curve A is for the generation of drug-free culture (⊙). Curve B is for the generation of culture affected with 0.04 μg/ml of clindamycin (×). Curve E is for the generation of culture affected with either 0.07 μg/ml of clindamycin (△) or a mixture of equipotent 0.04 μg/ml of clindamycin and 0.3 μg/ml of lincomycin (□). Curve D is generated when an equipotent concentration of lincomycin (0.3 μg/ml) is added to the phase I clindamycin-affected culture of curve B (▼), and curve C is generated when an equipotent concentration of lincomycin (0.3 μg/ml) is added to the phase II clindamycin-affected culture of curve B (○).*

lincomycin (0.3 μg/ml) to the phase II generation of the clindamycin-affected culture of curve B yields a generation curve C, which is parallel only to the phase II generation of the drug-affected cultures of curve E.

Action of Equipotent Clindamycin Added to Lincomycin-Affected Staph. aureus—In Fig. 4, the generation curve produced by the action of 0.3 μg/ml of lincomycin on the *Staph. aureus* culture of curve A is shown as curve B; the generation curve for the culture of curve A affected with a mixture of equipotent concentrations of lincomycin (0.3 μg/ml) and clindamycin (0.04 μg/ml) is shown as curve E and is coincident with the curve obtained from the culture affected with an *a priori* equipotent concentration of lincomycin (0.6 μg/ml).

Furthermore, Fig. 4 shows that the addition of an equipotent concentration of clindamycin (0.04 μg/ml) to the steady-state phase I generation of the lincomycin-affected culture of curve B produces an ultimate generation curve D, which is parallel to that for the culture of curve E and shows typical phase I and phase II generations. The addition of an equipotent concentration of clindamycin (0.04 μg/ml) to the phase II generation of the lincomycin-affected culture of curve B results in a generation curve C, which is parallel only to the phase II generation curve of the drug-affected cultures of curve E.

These observations indicate that the order of addition of equipotent concentrations of lincomycin and clindamycin on *Staph. aureus* produces an ultimate generation inhibition that is not significantly different from that of the *a priori* equipotent concentration of either antibiotic alone.

Effect of Order of Addition of Clindamycin and Lincomycin on Generation of E. coli—In Fig. 5, the generation curve for the drug-free *E. coli* culture is shown as curve A; the generation curve for the action of clindamycin (15 μg/ml) on the *E. coli* culture of curve A is shown as curve B and is coincident with the phase I action of lincomycin (100 μg/ml) in curve C, which indicates equipotency of action. The generation curve produced by the action of clindamycin (30 μg/ml) in curve G is also coincident with the phase I action of lincomycin (200 μg/ml) in curve H.

The addition of a mixture of equipotent concentrations of clindamycin (15 μg/ml) and lincomycin (100 μg/ml) to the *E. coli* culture of curve A yields an ultimate generation phase (curve F), which has a slope higher than the curve for the action of the *a priori* equipotent concentration of clindamycin (30 μg/ml) in curve G or the phase I lincomycin (200 μg/ml) in curve H. This finding indicates decreased effectiveness or "antagonism" (5) of effects for the clindamycin and lincomycin mixture. The addition of an equipotent amount of lincomycin (100 μg/ml) to the steady-state phase generation of the clindamycin-affected *E. coli* culture of curve B results in an ultimate generation curve that is coincident with that of the phase II generation of the lincomycin-affected culture of curve C. This finding suggests that the added equipotent amount of lincomycin virtually nullifies the initial action of clindamycin on the culture.

However, the addition of an equipotent amount of clindamycin (15 μg/ml) to the steady-state phase I and phase II generations of the lincomycin-affected *E. coli* culture of curve C produces generation curves E and D, respectively, whose slopes are the same as that of the drug-affected culture of curve F. This finding indicates that the added equipotent amount of the clindamycin does not have an effect on the initial action of lincomycin on the culture but that clindamycin yields a relatively reduced effect on the ultimate generation inhibition.

These observations demonstrate that the order of addition of clindamycin and lincomycin to *E. coli* cultures produces significant effects on the ultimate generation inhibition and suggests antagonism of effects as observed previously (6).

Effect of Equipotent Mixtures of Different Fractions of Lincomycin and Clindamycin on Generation Rates—The gen-

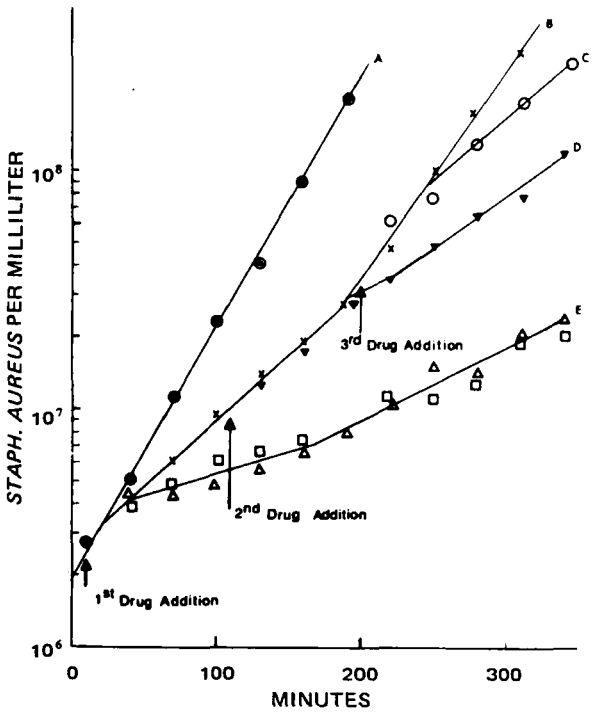


Figure 4—*Effects of addition of an equipotent concentration of clindamycin to lincomycin-affected* Staph. aureus. *Curve A is for the generation of drug-free culture (⊙). Curve B is for the generation of culture affected with 0.3 μg/ml of lincomycin (×). Curve E is for the generation of a culture affected with either 0.6 μg/ml of lincomycin (△) or a mixture of equipotent 0.3 μg/ml of lincomycin and 0.04 μg/ml of clindamycin (□). Curve D is generated when an equipotent concentration of clindamycin (0.04 μg/ml) is added to the phase I lincomycin-affected culture of curve B (▼), and curve C is generated when an equipotent concentration of clindamycin (0.04 μg/ml) is added to the phase II lincomycin-affected culture of curve B (○).*

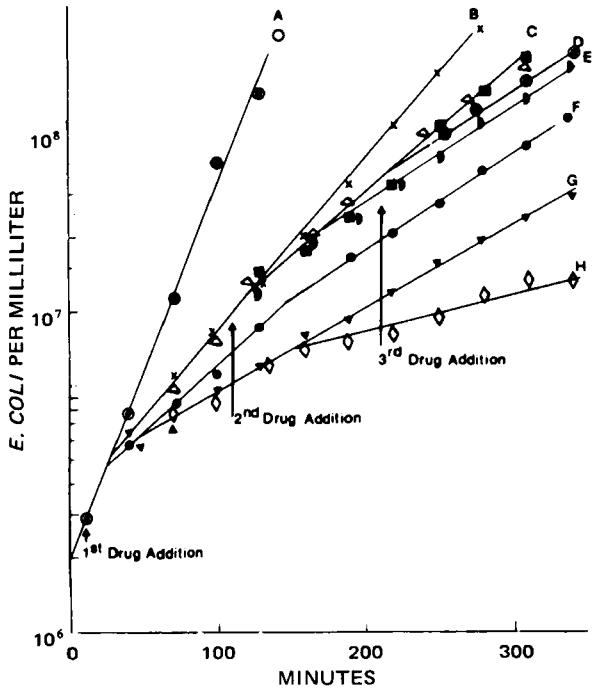


Figure 5—*Effects of the order of addition of equipotent concentrations of clindamycin and lincomycin on the generation rates of* E. coli. *Curve A is for the generation of drug-free culture. Curve B is for the generation of culture affected with 15.0 μg/ml of clindamycin (×). Curve C is generated by a culture affected with an equipotent 100-μg/ml concentration of lincomycin (△), and its coincident curve is generated when an equipotent 100-μg/ml concentration of lincomycin is added to the clindamycin-affected culture of curve B (■). Curve E is generated when an equipotent 15-μg/ml concentration of clindamycin is added to the phase I lincomycin-affected culture of curve C (◗). Curve D is generated when an equipotent 15-μg/ml concentration of clindamycin is added to the phase II lincomycin-affected culture of curve C (⊙). Curve F is generated when a mixture of equipotent concentrations of clindamycin (15 μg/ml) and lincomycin (100 μg/ml) is added to the culture of curve A (●). Curve G is generated when 30 μg/ml of clindamycin is added to the culture of curve A (▼), and curve H is generated when an equipotent 200-μg/ml concentration of lincomycin is added to the culture of curve A (◇).*

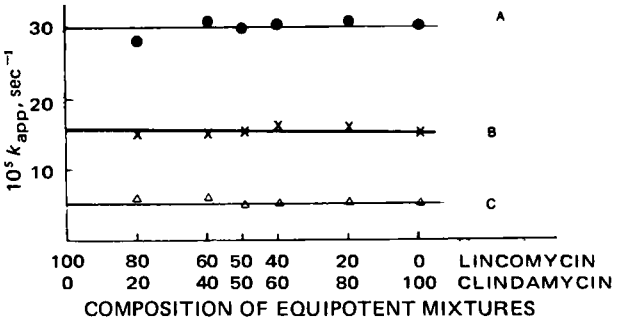


Figure 6—*Effect of varied clindamycin and lincomycin fractions in equipotent mixtures at three different levels of activity on the apparent generation rate constants,* k_{app} *in seconds*$^{-1}$, *of* Staph. aureus *at pH 7.05 and 37.5°. A, B, and C indicate the activities of* a priori *equipotent concentrations: 0.02 μg/ml of clindamycin = 0.11 μg/ml of lincomycin, 0.04 μg/ml of clindamycin = 0.36 μg/ml of lincomycin, and 0.05 μg/ml of clindamycin = 0.50 μg/ml of lincomycin, respectively.*

eration rate constant, k_{app}, for the steady-state phase I generation of *Staph. aureus* cultures affected with equipotent mixtures of clindamycin and lincomycin are shown in Fig. 6. The mixtures consisted of 100, 80, 60, 50, 40, 20, and 0% lincomycin and the residual percentage of an equipotent concentration of clindamycin. The mixtures were prepared so as to be *a priori* equipotent in their combined action on *Staph. aureus* generation in accordance with the dependence of k_{app} on drug concentration (Fig. 1). The null slopes for the plots of k_{app} for all the *a priori* equipotent mixtures at three different levels of activity demonstrate "indifference" or "equivalence" of effects (5).

The k_{app} values of *E. coli* cultures similarly affected with equipotent mixtures of clindamycin and lincomycin are shown in Fig. 7. The equipotent mixtures show lessened activity, as indicated by higher k_{app} values than when the *a priori* equipotent antibiotic was used alone at all levels of activity. These values demonstrate unequivocally an antagonism of effects for the combined action of clindamycin and lincomycin on *E. coli* cultures and confirm previous observations (6).

DISCUSSION

The data reported in this paper show (Fig. 1) that phase I and phase II generations of lincomycin-affected *Staph. aureus* (curve A) or clindamycin-affected *Staph. aureus* (curve B) have similar functional dependencies of generation rate constants, k_{app}, on drug concentrations. This finding suggests the same mechanism of drug action for phase I and phase II generations of drug-affected cultures. The ratio of biological potency for the lincomycin action on phase I and phase II generations of *Staph. aureus* is 1:0.67. The corresponding ratio for the clindamycin action is 1:0.80. These apparent differences are attributable to adaptation or development of initial strains of *Staph. aureus* in phase I generation into resistant mutants in phase II generation whose ribosomal components have reduced binding affinity for the drugs (8).

The dependence of k_{app} on drug concentration for the lincomycin-affected *Staph. aureus* is similar to that of clindamycin-affected *Staph. aureus* in the low concentration range and indicates a common mechanism (9) of action. However, the dependencies are different at the high concentration levels, which may indicate variations in modes of action. It has been explained that both lincomycin (8) and clindamycin (9) inhibit microbial generation by competitive binding on a receptor site, which is also the binding site for a substrate (or metabolite) utilized in microbial protein synthesis. At low drug concentrations, when possibly few receptors are interacted with drug, there is a linear dependence of the k_{app} on drug concentration, as shown by the coincident portion of the regression for lincomycin-affected *Staph. aureus* and clindamycin-affected *Staph. aureus* (Fig. 1). At high drug concentrations, the already complexed receptor sites reduce the availability of remaining sites by steric effects, protective colloid action, or other mechanisms (11). Therefore, it takes progressively greater drug concentrations to bind the remaining sites, and the k_{app} is a nonlinear function of drug concentration but follows saturation kinetics (6, 8, 11).

In the case of clindamycin, it has been demonstrated (9) that a bactericidal phenomenon is also superimposed on generation inhibition by the action of clindamycin at the saturable level. Therefore, perturbations in the kinetics of generation inhibition result in a complex function for the dependence of the k_{app} on drug concentration. The overall effect yields a sigmoidal shape for curves of plots of k_{app} *versus* clindamycin concentration. The potency ratio for the action of lincomycin and clindamycin on *Staph. aureus* was not constant over the concentration range studied (Table I). Equipotent lincomycin and clindamycin concentrations are, therefore, estimated from the separate dose–response curves of either drug alone.

Equipotent mixtures comprised of different fractions of lincomycin and clindamycin are quantifiable as kinetically "equivalent" or "indifferent" (5) to the *a priori* equipotent concentration of either drug alone in the inhibition of *Staph. aureus* phase I generation at different levels of activity (Fig. 6). This is not inconsistent with the same mechanism (10) of action for the drugs. Furthermore, the order of addition of equipotent amounts of the drugs (Figs. 3 and 4) does not affect the ultimate generation inhibition of *Staph. aureus* cultures, which indicates a common locus of action (6).

There is an observable difference in the potency of action for

Table I—Potency Ratio for the Action of Clindamycin and Lincomycin against *Staph. aureus*[a]

Clindamycin Concentration, μg/ml	Lincomycin Concentration, μg/ml	k_{app} for Phase I of Drug-Affected Cultures, sec^{-1}	Potency Ratio of Clindamycin–Lincomycin
0.020	0.10	32.5	5.0
0.038	0.20	24.5	5.6
0.040	0.30	17.5	7.5
0.044	0.40	12.0	9.1
0.055	0.50	7.5	9.1
0.070	0.60	5.0	8.6

[a] Obtained from the data of Fig. 1.

lincomycin and clindamycin, but this difference can be attributed to relative differences in the drug partition through cell membranes and/or affinity for bioreceptors. Another factor is the bactericidal action of clindamycin, which causes a reduction in the number of survivor cells in the active state of growth and multiplication with a consequent decrease in the apparent generation rate of the drug-affected cultures. Of course, the high activity of clindamycin may be related to halogen substitution at the 7(*S*)-configuration. An increase in the lipophilicity of the molecule in the order I > Br > Cl, which correlates with enhanced antimicrobial activity, was reported for the 7(*S*)-halogen analogs of lincomycin (3). The enhanced activity may be the result of an increased drug availability in the biophase and/or modification of drug–receptor interactions. Additionally, halogen substitution in a homologous series of compounds was reported (12, 13) to alter antibacterial properties of compounds toward bactericidal activities.

The k_{app} dependence on drug concentration for clindamycin-affected *E. coli* is the same as for lincomycin-affected *E. coli* in phase I generation (Fig. 2) and suggests the same mechanism (10) of action, which is different from that for lincomycin-affected *E. coli* in phase II generation. On this presumption, the ratio of 1:6 for the biological potencies of lincomycin (phase I)–clindamycin confirms previous observations (6). However, equipotent mixtures of lincomycin (phase I) and clindamycin demonstrate unequivocally an antagonism (5) of effects (Fig. 7). The mixtures are less effective on the generation of *E. coli* cultures than the *a priori* equipotent concentrations of either drug alone at different levels of activity. This observation confirms a previous report (6) and suggests the possibility that lincomycin (phase I) and clindamycin do not have a common locus of action. Therefore, the similarity in the functional dependency of the k_{app} on drug concentration can be attributed to the binding of clindamycin and lincomycin at different loci of a receptor site that are functionally linked.

Steric configuration possibly plays an important role in the selective binding of the drugs at the sites, as was found for a number of compounds (14). In the case of lincomycin analogs, it was observed (3) that replacement of the 7(*R*)-hydroxy group by a 7(*R*)-halogen substituent has little or no effect on *in vitro* antibacterial activity. However, a 7(*S*)-halogen substituent increases the activity 4–32-fold. This type of evidence suggests that the 7(*R*)- and 7(*S*)-epimers bind differently on the same receptor site. On this premise, it was postulated (6) that two binding sites—a "catalytically active" site and an "allosterically modifying site" (14–16)—possibly exist on the same receptor surface in *E. coli* for the lincosaminide antibiotics. This conceivably may not be the case for *Staph. aureus*, which is different in gross morphology and physiology from *E. coli* (17).

The high activity of clindamycin relative to lincomycin phase I action against *E. coli* suggests that the former binds to the active site while the latter binds to the allosteric site. The binding of lincomycin (phase I) to the allosteric site possibly produces an extensive conformational change (15) which reduces the binding affinity of the active site for clindamycin (or the normal substrate) as a consequence of an unfavorable configuration. The effects of the order of addition of the drugs (Fig. 5) on the ultimate generation inhibition of *E. coli* cultures provide evidence in support of this hypothesis. Pretreatment of the cultures with lincomycin reduces the effect of an added equipotent concentration of clindamycin but not vice versa. It was also implied that, in this type of allosteric

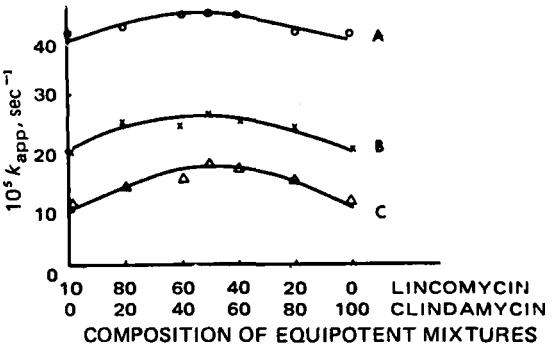


Figure 7—*Effect of varied clindamycin and lincomycin fractions in equipotent mixtures of three different levels of activity on the apparent generation rate constants, k_{app} in seconds^{-1}, of* E. coli *at pH 7.05 and 37.5°. A, B, and C indicate the activities of* a priori *equipotent concentrations: 10.0 μg/ml of clindamycin = 67.0 μg/ml of lincomycin, 20.0 μg/ml of clindamycin = 130.0 μg/ml of lincomycin, and 40.0 μg/ml of clindamycin = 260.0 μg/ml of lincomycin, respectively.*

interaction, only a noncompetitive type of antagonism (18), which is insurmountable by the addition of an excessive amount of one drug in the presence of the other, can result from the combined action of the drugs, as shown by the effects of mixtures of equipotent concentrations of lincomycin and clindamycin (Fig. 7).

The lincomycin phase II action on *E. coli* was attributed (6, 11, 19, 20) to a different mechanism, which is interdependent with the lincomycin phase I action but becomes rate limiting on microbial protein synthesis to enhance generation inhibition after a finite period of drug–bacteria contact. The lincomycin phase II action is the result of lincomycin binding at a receptor site that is different from the binding receptor site for the lincomycin phase I or clindamycin action. The separate sites are engaged in "sequential" metabolic pathways (21) leading to protein synthesis. Therefore, the sequential blocking (22) of the metabolic pathway by lincomycin results in "synergism" (5) of the phase I action by the onset of the phase II action, as evidenced by the effects of lincomycin alone or in combination with clindamycin on *E. coli* (Fig. 5). Although clindamycin action is antagonized by the lincomycin phase I action, it is kinetically equivalent (5) to the lincomycin phase II action, as was observed (20) for erythromycin and the lincomycin phase II action on *E. coli*.

The present work confirms earlier observations (6, 19, 20) that the kinetics and mechanisms of action, as well as the dose–response relationship, of the separate antibiotics must be considered in the quantification and prediction of combined antibiotic action. Moreover, the effect of antibiotic combinations is dependent on the strain and species of the test organism.

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Synthesis of Cyclopropyl Analogs of Stilbene and Stilbenediol as Possible Antiestrogens

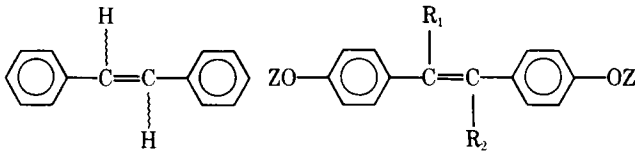
ROBERT A. MAGARIAN ˣ **and ERIC JOEL BENJAMIN** *

Abstract □ Conformationally rigid analogs of stilbene and stilbenediol were prepared *via gem*-dichlorocyclopropyl precursors utilizing two different synthetic methods: a two-phase catalytic method and an organomercurial method. These precursors were reduced to the corresponding cyclopropyl analogs using sodium and methanol. All compounds are being tested to discriminate between estrogenic and antiestrogenic activity, to determine estrogen binding ability, and to evaluate tissue culture anticancer activity.

Keyphrases □ Cyclopropyl analogs of stilbene and stilbenediol—synthesis as potential antiestrogens □ Stilbene and stilbenediol cyclopropyl analogs—synthesis as potential antiestrogens □ Antiestrogens, potential—synthesis of cyclopropyl analogs of stilbene and stilbenediol

A previous publication (1) discussed the cyclopropane moiety in connection with work on estrogen receptor elucidation. The preparation of *gem*-dichlorocyclopropyl analogs of the stilbene (I) and stilbenediol (II) series (Table I), using two different synthetic methods, and their subsequent reduction to the corresponding cyclopropyl analogs (Table II) are reported here.

Antiestrogens that arrest epithelial proliferation caused by estrogens in the uterus or vagina could have some utility in a particular uterine or vaginal cancer. Furthermore, certain antiestrogens may prove effective against other hormonal-dependent cancers such as breast cancer. Effective chemotherapeutic agents against such solid, hormone-sensitive tumors have not been fully developed and the duration of chemotherapeutic remissions is not as long lasting as those induced by endocrine manipulation.



I II

Currently, the compounds reported in Tables I and II are being tested to discriminate between their estrogenic and antiestrogenic activities and for their estrogen binding ability, and they are being evaluated in a tissue culture anticancer assay[1].

DISCUSSION

A two-phase catalytic method (2) and an organomercurial method (3–7) have proven effective as applied to olefins of low reactivity (*e.g.*, *trans*-stilbene) toward dichlorocarbenes generated by other procedures. Of practical significance is the fact that yields are usually high by these two routes.

The *gem*-dichlorocyclopropyl analogs of stilbene were prepared according to both methods, while the stilbenediols were subjected only to the latter mercurial method since the phenolic groups were sensitive to strong base and some stilbenediols were poorly soluble in chloroform. The *gem*-dichlorocyclopropane precursors listed in Table I were subsequently reduced with sodium metal and methanol (8, 9).

The general reaction developed by Simmons and coworkers (10–12) for the stereospecific synthesis of cyclopropane derivatives involves the treatment of olefins with diiodomethane and zinc–copper couple. This convenient method could have allowed omission of the reductive step; however, in spite of various modifications (13–17), this method does not consistently produce respectable yields when the olefins are weak nucleophiles. Olefins in which the nucleophilic character of the double bond has been reduced by electron-withdrawing groups should react less readily with carbenes than those in which the nucleophilic character has been increased by electron-donating groups (18–20).

Method A—All reports of the syntheses of *gem*-dihalocyclopropane derivatives have stressed the necessity of operating under strictly anhydrous conditions because of the rapid hydrolysis of dichlorocarbene. On the other hand, the method established by Makosza and Wawrzyniewicz (2) and improved by Dehmlow and Schonefeld (21, 22) (Scheme I) generates dichlorocarbene in the reaction among chloroform, a concentrated solution (50%) of sodium hydroxide, and a catalytic amount of triethylbenzylammonium chloride, thus allowing the preparation of *gem*-dichlorocyclopro-

[1] Tests are being performed by the Cancer Section, Oklahoma Medical Research Foundation, Oklahoma City, and the College of Pharmacy, University of Oklahoma, Norman, Okla.

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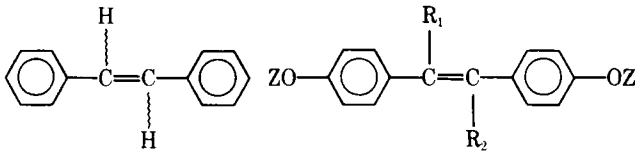

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Table I—*gem*-Dichlorocyclopropanes

Compound	R_1	R_2	R_3	R_4	Melting Point	Yield, % Method A	Yield, % Method B	Crystallization Solvent	Formula	Analysis[a], %	Calc.	Found
III	H	C_6H_5	C_6H_5	H	38–40°[b]	86	86	—[c]	$C_{15}H_{12}Cl_2$		—	—
IV	H	C_6H_5	H	Ph	51–52°	69	86	—[d]	$C_{15}H_{12}Cl_2$	C	68.45	68.32
										H	4.59	4.71
										Cl	26.94	26.66
V	H	C_6H_4—OCH_3	C_6H_4—OCH_3	H	88–89°	—[e]	52	Ethanol	$C_{17}H_{16}Cl_2O_2$	C	63.17	63.46
										H	4.99	5.13
										Cl	21.93	21.98
VI	CH_2CH_3	C_6H_4—OCH_3	C_6H_4—OCH_3	CH_2CH_3	111–112°	74.7	59[f]	Methanol	$C_{21}H_{24}Cl_2O_2$	C	66.49	66.21
										H	6.38	6.31
										Cl	18.69	18.78
VII	CH_2CH_3	C_6H_4—$OCOCH_3$	C_6H_4—$OCOCH_3$	CH_2CH_3	137–137.5°[g]	—[h]	72[f]	Ethanol	$C_{23}H_{24}Cl_2O_4$	C	63.45	63.28
										H	5.56	5.58
										Cl	16.29	16.43
VIII	CH_2CH_3	C_6H_4—OH	C_6H_4—OH	CH_2CH_3	147.5–148°		84[i]	Benzene	$C_{19}H_{20}Cl_2O_2$	C	64.96	64.81
										H	5.74	5.87
										Cl	20.19	20.01

[a] Elemental analyses were performed by Midwest Microlab. Ltd., Indianapolis, Ind. [b] Lit. (5, 6) mp 39–40.5°, 90%; lit. (21, 22) mp 39°, 96%. [c] Distillation. [d] Sublimation. [e] Method A was not used because of the insolubility of the olefin in chloroform. [f] Product was obtained by a double run; yields are based on the olefin. [g] See *Experimental* for melting-point behavior. [h] No reaction. [i] *gem*-Dichlorocyclopropane was obtained from the hydrolysis of VII.

pane derivatives with retention of configuration by the addition of dichlorocarbene to olefins.

Although the reaction is carried out in an aqueous medium, only a small fraction of the generated dichlorocarbene is hydrolyzed. This result may be explained by the catalytic mode of action of the quaternary ammonium compound (2). In concentrated sodium hydroxide solution, triethylbenzylammonium chloride is transformed into the hydroxide, which is insoluble in the reaction medium. Thus, the quaternary ammonium hydroxide migrates to the boundary between the aqueous and organic phases, where it reacts with chloroform to yield the quaternary ammonium derivative of the trichloromethyl anion. After diffusion into the organic phase, the derivative is transformed into dichlorocarbene and the ammonium chloride (Scheme II).

The dichlorocarbene reacts rapidly with the olefin present in the organic layer, whereas the quaternary ammonium chloride passes to the phase boundary and reenters the transformation cycle. Thus, dichlorocarbene is only slightly hydrolyzed. On the other hand, the high degree of dichlorocarbene utilization, despite the use of stoichiometric amounts of olefin and chloroform, is due to the fact that the quantity of dichlorocarbene that can be formed at one time is small and does not exceed the amount of the catalyst, *i.e.*, 1%. Consequently, dichlorocarbene is always confronted with a large excess of olefin.

This catalytic method seems to be the simplest procedure used so far, and the isolation of the resulting products appears to present no difficulties. The yields of the products obtained by this method are high, and no excess of the olefin is required; the ratio of olefin to chloroform is 2:1 to 1:2, depending on the availability of the olefin.

Excellent yields were obtained by this method for olefins that gave either no product or poor yields by other methods (8, 23–26). For example, the *gem*-dichlorocyclopropyl analog of *trans*-stilbene was not isolated using the Doering and Hoffman procedure (8); it was produced in 90% yield by the Seyferth *et al.* method (5, 6) and in 96% yield by the Dehmlow and Schonefeld method (21, 22). In most cases, high yields (74–86%) were obtained by this method when commercially available olefins were used.

This basic method, however, was not suited for diphenolic olefins such as diethylstilbestrol (II, $R_1 = R_2 = CH_2CH_3$, Z = H) due to salt formation from the concentrated sodium hydroxide solution. The enormous decrease in solubility of diethylstilbestrol in chloroform posed a problem. The diacetate ester of diethylstilbestrol, which was soluble in chloroform, was tried at room temperature, but it too was hydrolyzed to the salt and thrown out of organic phase and thus was unavailable for reaction with the dichlorocarbene.

Method B—Seyferth and coworkers (3–7) reported that phenyl(trihalomethyl)mercury compounds were very useful and versatile reagents as sources for dihalocarbenes; they reacted with olefins to give *gem*-dihalocyclopropanes in high yields. It was found that phenyl(bromodichloromethyl)mercury is far superior to phenyl(trichloromethyl)mercury in its reaction with olefins to produce exclusively 1,1-dichlorocyclopropanes (3). This mercurial route does not involve basic reaction conditions and does not proceed *via* nucleophilic intermediates (such as trichloroacetate or trichloro-

Scheme I

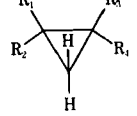


Table II—Cyclopropyl Analogs of Stilbene and Stilbenediol

Compound	R_1	R_2	R_3	R_4	Boiling Point/ Melting Point	Yield, %	Crystallization Solvent	Formula	Analysis[a], %	
									Calc.	Found
IX*a*	H	C_6H_5	C_6H_5	H	90° (0.01 mm)[b]	69[c]	—	$C_{15}H_{14}$	C 92.74 H 7.26	92.62 7.29
IX*b*	H	C_6H_5	H	C_6H_5	38–38.5°[d]	68[c]	Sublimation	$C_{15}H_{14}$	C 92.74 H 7.26	92.45 7.35
X	CH_2CH_3	C_6H_4—OH	C_6H_4—OH	CH_2CH_3	139–141°	70[e]	Benzene	$C_{19}H_{22}O_2$	C 80.81 H 7.85	81.00 7.66
XI	H	C_6H_4—OCH_3	C_6H_4—OCH_3	H	70.5–71.5°[f]	67[c]	Ethanol	$C_{17}H_{18}O_2$	C 80.28 H 7.13	80.01 7.19
XII	CH_2CH_3	C_6H_4—OCH_3	C_6H_4—OCH_3	CH_2CH_3	75–76°	77[c]	Methanol	$C_{21}H_{26}O_2$	C 81.25 H 8.44	81.41 8.45

[a] Elemental analyses were performed by Midwest Microlab. Ltd., Indianapolis, Ind. [b] Lit. (37–40) bp 144–145.3° (3.8 mm). [c] Obtained by sodium–methanol reduction of *gem*-dichlorocyclopropane. [d] Lit. (37–40) mp 38.0–38.5°. [e] Obtained by demethylation of dimethyl ether XII with boron tribromide. [f] Lit. (41) mp 70.5–71.5°.

methide ion); high yields at lower temperatures are obtained. The conversion of *cis*- and *trans*-olefins to *gem*-dichlorocyclopropanes proceeds with retention of configuration, as illustrated in the preparation of 1,1-dichloro-*trans*-2,3-diphenylcyclopropane in 90% yield from *trans*-stilbene (Scheme III).

The preparation of the Seyferth reagent, phenyl(bromodichloromethyl)mercury, is time consuming and tedious, involving anhydrous conditions. A special high-speed stirring apparatus is needed since both the phenylmercuric halide and the potassium *tert*-butoxide (in the form of its monosolvate) have very low solubility in the solvent system, and a large excess of the haloform (bromodichloromethane) is needed (3, 4). The monosolvate was found to produce high yields. Seyferth and Lambert (4) succeeded in eliminating the need for preparing potassium *tert*-butoxide–*tert*-butyl alcohol monosolvate by modifying the original procedure so that commercial potassium *tert*-butoxide could be substituted; however, the modified procedure resulted in 5% yields in this laboratory. Consequently, freshly prepared potassium *tert*-butoxide as the monosolvate with *tert*-butyl alcohol was used and yields increased to 69%. During the crystallization of this reagent, the first crop of crystals was contaminated with unchanged phenylmercuric chloride. Hence, the procedure was modified in this laboratory to prevent the contamination.

This was the method of choice in preparing the *gem*-dichlorocyclopropane derivatives of 4,4′-dimethoxystilbene (II, $R_1 = R_2 = H$, $Z = CH_3$), dimestrol (II, $R_1 = R_2 = CH_2CH_3$, $Z = CH_3$), and diethylstilbestrol diacetate (II, $R_1 = R_2 = CH_2CH_3$, $Z = COCH_3$). In the reactions using dimestrol or diethylstilbestrol diacetate, the PMR spectrum of the product indicated the presence of a considerable amount of unchanged olefin. Despite a twofold quantity of reagent, the yield did not improve. Since all attempts to separate the mixture failed, it was necessary to remove the precipitated phenylmercuric bromide and rerun the reaction using the original mixture (unused olefin and product) with additional amounts of the Seyferth reagent to induce the reaction to go to completion. Using this variation, Compounds VI and VII were obtained in 59 and 72% yields, respectively.

Minor limitations in the Seyferth method were reported (27). Phenyl(bromodichloromethyl)mercury reacted with carboxylic acids in benzene at 60–80° to give dichloromethyl esters in high yields, and the mercurial reagent reacted with alcohols to form dichloromethyl ethers and a mixture of products. Refluxing diethylstilbestrol with phenyl(bromodichloromethyl)mercury in benzene at 80° yielded a dark liquid from which no product was isolated. This promoted the use of dimestrol and diethylstilbestrol diacetate as substrates (Scheme IV).

$$CHCl_3 + (CH_3CH_2)_3\overset{+}{N}CH_2C_6H_5\ \overset{-}{O}H \rightleftharpoons \overset{-}{C}Cl_3(CH_3CH_2)_3\overset{+}{N}CH_2C_6H_5 + H_2O$$

$$\overset{-}{C}Cl_3(CH_3CH_2)_3\overset{+}{N}CH_2C_6H_5 \longrightarrow :CCl_2 + (CH_3CH_2)_3\overset{+}{N}CH_2C_6H_5\overset{-}{C}l$$

Scheme II

Reduction of aliphatic *gem*-dihalocyclopropyl compounds by sodium in wet methanol was originated by Doering and Hoffman (8). All *gem*-dichlorocyclopropanes involved in this study were reduced by this method (Scheme V) except VII and VIII. The 2,3-dialkyl derivatives were difficult to reduce, but increasing the equivalent amount of sodium metal and the quantity of methanol brought about good yields (Table II).

Being unable to reduce VII and VIII completely to the corresponding cyclopropyl analogs because of hydrolysis, it was of interest to search for a method that would bring about demethylation of XII to the analog X. Demethylation of aryl methyl ethers can be effected by a variety of reagents (28, 29), but usually it is necessary to employ high temperatures and acidic reagents. These conditions are too drastic for the cyclopropyl ring. Aromatic ethers have been cleaved at room temperature or lower using boron tribromide (30–32) (Scheme VI). The reactants are mixed in methylene chloride or *n*-pentane at −80°, and the mixture is allowed to warm to room temperature with continuous stirring for 15–20 hr. Methylene chloride is reported to have the most powerful solvent action and is preferred.

Even though cyclopropanes are sensitive to Lewis acids, boron tribromide was used successfully as a demethylating agent on XII.

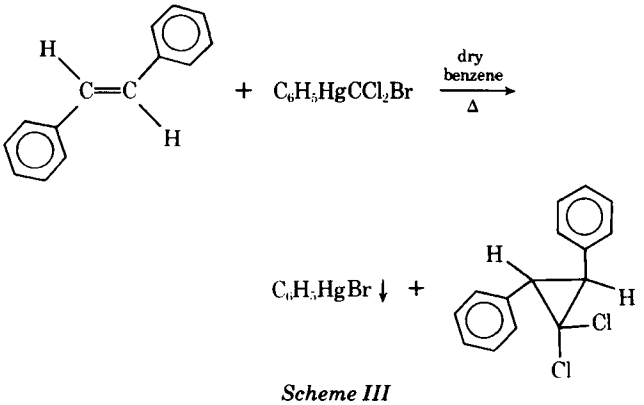
EXPERIMENTAL[2]

Phenyl(bromodichloromethyl)mercury—The procedure described by Seyferth and Lambert (4) was modified. Phenylmercuric chloride[3] (31.25 g, 0.1 mole) and bromodichloromethane[3] (25 g, 0.15 mole) were dissolved in 600 ml of tetrahydrofuran (dried with calcium hydride and distilled from lithium aluminum hydride[4]) in a flame-dried, 1-liter, three-necked flask. The flask was equipped with a mechanical stirrer, a nitrogen inlet tube, and an air condenser fitted with a mercury trap. The mixture was stirred and cooled in a dry ice–acetone bath. To a 500-ml conical flask in a dry box under anhydrous nitrogen was added 24 g (0.13 mole) of potassium *tert*-butoxide–*tert*-butyl alcohol monosolvate (33).

[2] Methods A and B are representative for the preparation of most compounds listed in Table I. Analyses and physical properties for all compounds are reported in Tables I and II. All IR spectra were recorded on a Beckman IR-20A, using polystyrene film as a standard to ascertain reproducibility. The NMR spectroscopic analyses were performed on a Varian T-60 MHz instrument. Most spectra were reported in either $CDCl_3$ or CD_3OD with tetramethylsilane as the internal standard. Mass spectra were taken with a Hitachi-RMU-6E mass spectrometer. Melting points were taken on a Hoover capillary melting-point apparatus and are uncorrected. Yields are based on pure samples. Research chemicals were of the purest grade and, in all cases, were used without further purification. Solvents were reagent grade and most were purchased from Eastman Chemical Co., Rochester, NY 14650

[3] Aldrich Chemical Co.

[4] Pflatz and Bauer, Flushing, NY 11368



Scheme III

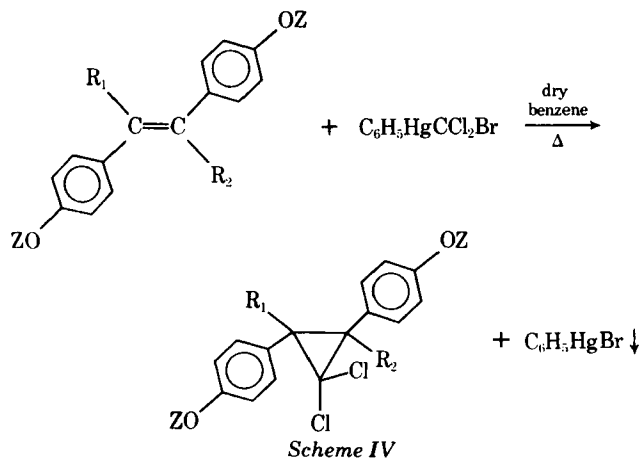
The conical flask was connected to the 1-liter three-necked flask by 2.54-cm (1-in.) Tygon tubing, and the contents were transferred to the reaction mixture slowly over 30 min. After addition, the mixture was stirred for 15 min at −55° and transferred to a 2-liter single-necked flask. The solvent was removed *in vacuo* (3 mm) through a dry ice–acetone trap without heating the flask until most of the solvent had evaporated. The remaining solvent was removed on a water bath at 20°, yielding a gray solid to which was added 500 ml of benzene.

The mixture was agitated until a partial solution was effected. Then 100 ml of water was added, the mixture was shaken vigorously, and 8.0 g of gray solid was collected. The layers were separated, and the aqueous phase was extracted with three 100-ml portions of benzene. All of the organic layers were collected, and benzene was removed *in vacuo*. To the white solid was added 500 ml of hot hexane–chloroform (3:1) solution in small portions with swirling and occasional heating on a steam bath until most of the solid dissolved. The flask was then placed at 0°, and 4.0 g of unreacted phenylmercuric chloride was collected on filter paper.

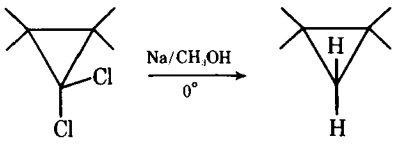
The clear filtrate was placed in the refrigerator at 0°. Crystals formed and were collected, yielding 22.74 g of white needles, mp 108–110° dec. Concentrating the mother liquor and placing the residue into the refrigerator yielded another 7.4 g of the mercurial reagent; the total yield was 30.14 g (68.5%).

1,1-Dichloro-*trans*-2,3-diphenylcyclopropane (III) (Method A)—Following the method reported (21, 22), 16.0 g (0.09 mole) of *trans*-stilbene[3] and 2.0 g (0.008 mole) of triethylbenzylammonium chloride[3] were dissolved in 300 g of chloroform contained in a 1-liter three-necked flask fitted with a mechanical stirrer, condenser, and dropping funnel. The flask was cooled in an ice water bath, and 200 g of ice-cold 50% sodium hydroxide solution was added dropwise. The mixture was stirred at 10–20° for 6 hr and then at room temperature for 2 days. The dark-brown mixture was diluted with 100 ml of water and filtered. Two layers were separated, and the aqueous layer was washed with three 50-ml portions of methylene chloride.

All organic extracts were combined, washed with water, and dried over anhydrous magnesium sulfate. The drying agent was filtered, and the chloroform was evaporated under reduced pressure,



Scheme IV



Scheme V

yielding 28 g of a dark-brown oil. Distillation of the crude oil at 140° (0.5 mm) gave 20 g (86%) of a light-yellow liquid, which solidified after several days at 5°, mp 38–40° [lit. (5, 6) mp 39–40.5°]. In subsequent reactions the distilled compound was passed through neutral alumina (activity I)[5], using petroleum ether (bp 30–60°) as the eluent to remove a small amount of the decomposed products; ν_{max} (mineral oil mull): 3095 (weak), 3060 (medium), 3025 (medium), 1610 (medium), 1503 (strong), 1220 (weak), 1175 (medium), 1108 (weak), 1095 (strong), 1060 (medium), 1035 (medium), 915 (weak), 865 (strong), 755 (strong), 743 (weak), and 695 (strong) cm^{-1}; $\delta_{60\ MHz}$ ($CDCl_3$): 3.15 (s, 2H) and 7.37 (s, 10H).

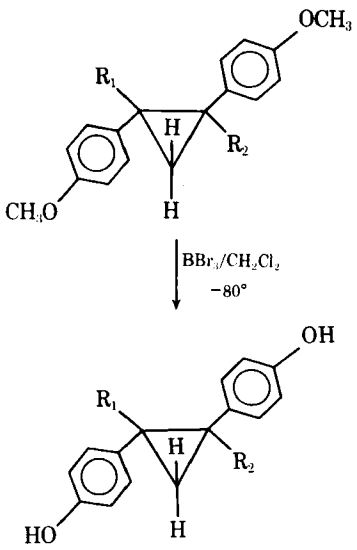
1,1-Dichloro-*cis*-2,3-diphenylcyclopropane (IV)—*Method A*—To a cold solution of 0.50 g (0.002 mole) of triethylbenzylammonium chloride and 110 g of chloroform in a three-necked flask was added 6.0 g (0.33 mole) of *cis*-stilbene[3], and the solution was stirred to dissolve the stilbene. A 50% sodium hydroxide solution (75 g) was added carefully through a dropping funnel; it was stirred at 10–20° for 6 hr and then at room temperature for 24 hr by means of a magnetic stirrer. The mixture was diluted with 100 ml of water, and a dark-brown chloroform layer separated. The aqueous phase was extracted with three 50-ml portions of methylene chloride, and the organic layers were collected and dried over anhydrous magnesium sulfate. Evaporation of the solvent *in vacuo* yielded 7.5 g of dark-brown oil.

The crude oil was purified by placing it on a 1.3 × 33-cm column of alumina (activity I)[5] and eluting with purified petroleum ether (bp 30–60°). A cream-colored oil, 6.0 g (69%), solidified at 0°, mp 48–49°.

The IR and NMR spectra were identical to those obtained using Method B.

Method B—According to the literature method (5, 6), 3.6 g (0.02 mole) of *cis*-stilbene[3] was added to 10.0 g (0.022 mole) of phenyl(bromodichloromethyl)mercury in benzene. After the resulting solution was refluxed with stirring under dry nitrogen and maintained at 82–88° in an oil bath for 1.5 hr with stirring, phenyl mercuric bromide precipitated (7.2 g, 92%) and the reaction mixture turned yellow. The relative proton absorption in the NMR spectrum showed small quantities of the unchanged olefin.

The mixture then was refluxed with stirring for an additional hour. The NMR spectrum of this mixture indicated that the olefin



Scheme VI

[5] E. Merck ag Darmstadt.

had reacted. Benzene was removed on a flash evaporator, yielding 6.5 g of crude product, which was dissolved in petroleum ether (bp 30–60°) and filtered to remove a small amount of white precipitate, mp 175° dec. The crude product was purified through a 2 × 18-cm column of neutral alumina (activity I)[5] using purified petroleum ether (bp 30–60°). A cream-colored oil was eluted, which solidified after standing in a refrigerator overnight. The solid had a melting-point range of 49–51° and weighed 4.5 g (86%).

An analytical sample was obtained by sublimation. The sublimator was kept at 45° (0.03 mm) in an oil bath while the inner cold finger was maintained at −5° by a cold water pump. The white needles melted at 50–51.5°; ν_{max} (neat): 3065 (medium), 3050 (strong), 1960 (weak), 1890 (weak), 1810 (weak), 1609 (medium), 1587 (weak), 1500 (strong), 1453 (strong), 1386 (weak), 1250 (weak), 1160 (weak), 1080 (medium), 1050 (medium), 1035 (medium), 1005 (weak), 958 (weak), 927 (medium), 840 (weak), 813 (strong), 754 (strong), 721 (weak), 700 (strong), 648 (weak), and 626 (weak) cm^{-1}; $\delta_{60\ MHz}$ ($CDCl_3$): 3.28 (s, 2H) and 7.18 (m, 10H).

1,1-Dichloro-*trans*-2,3-diethyl-2,3-(4,4′-dimethoxyphenyl)-cyclopropane (VI)—*Method A*—In 150 g of chloroform were dissolved 9.0 g (0.03 mole) of dimestrol[3] (34) and 0.75 g (0.0032 mole) of triethylbenzylammonium chloride in a three-necked flask, fitted with a condenser and a dropping funnel. The mixture was cooled in an ice water bath, and 75 g of a 50% sodium hydroxide solution was added dropwise while the mixture was agitated with a magnetic stirrer. Then the mixture was stirred at 10–20° for 6 hr and at room temperature for an additional 40 hr.

To the dark mixture was added 100 ml of water, and the solution was transferred to a separator. A dark-brown chloroform layer was separated after the phases were allowed to separate. The aqueous layer was neutralized with dilute hydrochloric acid and extracted with two 50-ml portions of chloroform. The chloroform extracts were combined and dried over anhydrous magnesium sulfate, and the drying agent was filtered. Evaporation of chloroform at reduced pressure gave 13.0 g of a dark-brown oil, which solidified on cooling.

This dark-brown solid was refluxed with 200 ml of petroleum ether (bp 30–60°) for 15–20 min, and the hot mixture was filtered from 2.5 g of a dark-brown solid, mp 170° dec. The light-brown solution was concentrated to about 40 ml at reduced pressure until crystallization occurred. After cooling and stirring, 8.0 g of a yellow solid was collected, mp 109–110°. Another 2.0 g was isolated from further concentration of the filtrate, bringing the total yield to 10 g.

This crude product was crystallized from 200 ml of hot methanol, yielding 8.5 g (74.7%) of light-yellow crystals, mp 110–111°. In a subsequent reaction, the product was purified using a neutral alumina column (activity I)[5] and *n*-hexane as the eluent.

Repeated crystallization from hot methanol gave an analytical sample, mp 111–112°; ν_{max} (CCl_4): 3038 (weak), 2975 (strong), 2940 (strong), 2920 (weak), 2880 (weak), 2835 (medium), 1613 (strong), 1580 (weak), 1508 (strong), 1463 (strong), 1445 (medium), 1415 (weak), 1375 (medium), 1333 (weak), 1293 (strong), 1245 (strong), 1175 (strong), 1110 (medium), 1090 (weak), 1035 (strong), 960 (weak), 925 (weak), 885 (weak), 845 (strong), 830 (strong), and 655 (medium) cm^{-1}; $\delta_{60\ MHz}$ ($CDCl_3$): 0.8 (t, 6H), 1.5 (m, 4H), 3.8 (s, 6H), and 7.12 (m, 8H).

Method B—A mixture of 3.4 g (0.011 mole) of dimestrol (34) and 5.5 g (0.013 mole) of phenyl(bromodichloromethyl)mercury in 35 ml of dry benzene was stirred and refluxed at 85–87° (oil bath) under dry nitrogen for 2.0 hr. During refluxing, phenylmercuric bromide precipitated and the reaction mixture turned yellow. The NMR spectrum showed a considerable amount of unchanged olefin. The mixture was stirred and refluxed for an additional 2 hr, but a considerable amount of unchanged olefin was still present as ascertained by NMR spectroscopy.

From the mixture was filtered 3.8 g (87%) of phenylmercuric bromide. Benzene was evaporated under reduced pressure, and a cream-colored solid (3.0 g) was stirred and refluxed under dry nitrogen with 3.0 g of phenyl(bromodichloromethyl)mercury in benzene for 2.0 hr. At this time no unchanged olefin was present in the NMR spectrum. The cream-colored solid (2.8 g) was dissolved in cold chloroform and filtered to remove a small amount of white precipitate. Evaporation of the chloroform and recrystallization of the cream-colored solid from hot methanol gave 2.5 g (59%) of white crystals, mp 110–112°. The NMR and IR spectra were identical to those obtained using Method A.

1,1-Dichloro-*trans*-2,3-diethyl-2,3-(4,4′-diacetoxyphenyl)-cyclopropane (VII) (Method B)—A mixture of 6.0 g (0.016 mole) of diethylstilbestrol[6] diacetate (35, 36) and phenyl(bromodichloromethyl)mercury (8.8 g, 0.02 mole) in 30 ml of dry benzene was refluxed for 5 hr (oil bath) with stirring, using a magnetic stirrer under dry nitrogen. The solution turned yellow and phenylmercuric bromide (6.65 g, 93%) precipitated. An NMR spectrum of the mixture indicated the presence of a small amount of unreacted olefin.

The benzene was evaporated *in vacuo*, and the solid collected was dissolved in 30 ml of dry benzene and refluxed with 4.4 g of phenyl(bromodichloromethyl)mercury under dry nitrogen at 87–90° for 3 hr. At this time, the NMR spectrum showed no unchanged olefin, and the mixture was warmed; 3.65 g (91%) of phenylmercuric bromide was collected. The solvent was removed under vacuum, yielding 6.5 g of a yellow solid.

The crude yellow compound was stirred with cold ethanol to dissolve the unreacted mercurial reagent. A light-yellow solid (5.5 g) was collected, mp 134–180° (melted with partial decomposition at 134° and another melt at 180°). Recrystallization from hot ethanol yielded 5.0 g (72%) of cream-colored crystals, mp 135–137°.

An analytical sample, mp 137–137.5°, was obtained from additional recrystallizations from hot ethanol; ν_{max} (CCl_4): 3050 (weak), 2290 (strong), 2948 (medium), 2795 (weak), 1775 (very strong), 1610 (weak), 1508 (strong), 1465 (medium), 1448 (weak), 1418 (weak), 1377 (strong), 1340 (weak), 1312 (weak), 1205 (very strong), 1170 (strong), 1115 (weak), 1092 (weak), 1022 (strong), 948 (medium), 915 (strong), 852 (strong), 705 (weak), 658 (weak), 618 (medium), and 565 (weak) cm^{-1}; $\delta_{60\ MHz}$ ($CDCl_3$): 0.8 (t, 6H), 1.53 (m, 4H), 2.3 (s, 6H), and 7.28 (q, 8H).

1,1-Dichloro-*trans*-2,3-diethyl-2,3-(4,4′-dihydroxyphenyl)-cyclopropane (VIII)—In 50 ml of methanol on a steam bath was dissolved 4.35 g (0.01 mole) of VII with stirring. After complete solution was obtained, 10 g of 15% sodium hydroxide solution was added dropwise to the warm solution with stirring for 10 min. Then the solution was poured over an ice water bath with vigorous stirring. This mixture was neutralized carefully with 7 ml of 36% HCl. The gum which appeared was collected on a filter paper, dissolved in 80 ml of hot benzene, and left at 37° for crystallization. White needles, mp[7] 143–145° dec, 2.95 g (84%), were obtained.

An analytically pure sample, mp 147.5–148° dec.[7] (with melting) was obtained by a second recrystallization from hot benzene; ν_{max} (KBr): 3400 (strong and broad), 3040 (weak), 2980 (medium), 2940 (weak), 2880 (weak), 1610 (strong), 1600 (strong), 1505 (strong), 1435 (strong), 1380 (strong), 1333 (strong), 1295 (weak), 1230 (strong), 1170 (strong), 1110 (medium), 1088 (weak), 1030 (weak), 1015 (medium), 965 (weak), 934 (medium), 895 (weak), 835 (strong), 789 (weak), 750 (weak), 670 (medium), 630 (weak), 560 (medium), and 498 (weak) cm^{-1}; $\delta_{60\ MHz}$ ($CDCl_3$ + CD_3OD): 0.80 (t, 6H), 1.50 (q, 4H), 3.80 (s, 2H), and 7.03 (m, 8H).

1,1-Dichloro-*trans*-2,3-(4,4′-dimethoxyphenyl)cyclopropane (V) (Method B)—To 150 ml of dry benzene (distilled with calcium hydride) under nitrogen was dissolved 2.4 g (0.01 mole) of 4,4′-dimethoxystilbene (light brown in color, mp 210°) with stirring (magnetic bar). The solution was refluxed using an oil bath and cooled to 85°. Then 6.0 g (0.014 mole) of phenyl(bromodichloromethyl)mercury was added, and the mixture was refluxed with stirring under nitrogen for 2.5 hr. The mixture turned yellow-brown with a large quantity of precipitate.

From the warm solution was filtered 3.3 g (68%) of light-brown phenylmercuric bromide. Benzene was removed under vacuum, yielding a waxy, dark-blue substance, which solidified on standing. The solid was stirred with 20 ml of chloroform to effect a partial solution, which was allowed to stand at 0° for 3 hr. The starting olefin was collected, 1.0 g (41%), and the filtrate was transferred to a 100-ml flask. The chloroform was evaporated *in vacuo*, leaving a yellow oil, which solidified.

To the solid was added 50 ml of hot ethanol; this mixture was heated on a steam bath to effect a partial solution and was filtered. The yellow filtrate was cooled slowly, and 1.7 g (52%) of light-yellow crystals, mp 86–88°, was collected. The crystals were dissolved in hot ethanol and treated with activated charcoal. After filtering

[6] Nutritional Biochemical Corp., Cleveland, OH 44128

[7] To obtain an accurate melting point, the bath was first heated to 135° and the capillary was introduced.

and cooling, 1.5 g (45%) of cream-colored needles, mp 88–89.5°, was obtained. An analytical sample was prepared from hot ethanol; ν_{max} (CCl_4): 3045 (weak), 3000 (medium), 2960 (medium), 2940 (weak), 2910 (weak), 2840 (medium), 1610 (strong), 1585 (weak), 1505 (strong), 1465 (medium), 1440 (medium), 1300 (medium), 1250 (very strong), 1175 (strong), 1105 (medium), 1070 (weak), 1045 (strong), 870 (strong), and 825 (medium) cm^{-1}; $\delta_{60\ MHz}$ (CCl_4): 2.95 (s, 2H), 3.7 (s, 6H), and 6.96 (q, 8H).

***trans*-1,2-Diphenylcyclopropane (IX*a*)**—The reductive method of Dale and Swartzentruber (9) was used. To a 250-ml three-necked flask, fitted with a dry ice–acetone condenser and a dropping funnel, were added 3.3 g (0.012 mole) of 1,1-dichloro-*trans*-2,3-diphenylcyclopropane (III) and 40 ml of ether. This solution was stirred (magnetic stirrer) and cooled in an ice water bath. Sodium metal (5.5 g, 0.25 g-atom) was added in small pieces as 60 ml of methanol–water (100:3.3 ml) was added dropwise. The sodium reacted after 2 hr, and a white solid was observed. When all of the sodium had reacted, 20 ml of water was added and the aqueous layer was extracted with two 30-ml portions of ether.

The aqueous phase was neutralized slowly and carefully on an ice–salt bath with concentrated hydrochloric acid and extracted with two 20-ml portions of ether. The ether extracts were dried over anhydrous magnesium sulfate and filtered. The solvent was evaporated at reduced pressure, and the light-yellow liquid was distilled and collected in three fractions at 0.01 mm.

Fraction 1 (bp 38–52°) was kerosene (used for storing sodium), fraction 2 (bp 67°) was a mixture, and fraction 3 (bp 90–92°) yielded 1.6 g (69%) of 1,2-diphenylcyclopropane.

An analytical sample was obtained by refractionation, bp 90° (0.01 mm) [lit. (37–40) mp 15.3°, bp 144–145.3°(3.8 mm)]; ν_{max} (neat): 3065 (medium), 3030 (strong), 2938 (medium), 2860 (weak), 1948 (medium), 1875 (weak), 1795 (weak), 1605 (very strong), 1585 (medium), 1495 (very strong), 1450 (strong), 1280 (weak), 1212 (strong), 1183 (medium), 1158 (weak), 1118 (strong), 1072 (strong), 1030 (strong), 1002 (weak), 989 (weak), 940 (medium), 905 (strong), 840 (weak), 775 (medium), 750 (strong), and 695 (very strong) cm^{-1}; $\delta_{60\ MHz}$ ($CDCl_3$): 1.4 (m, 2H), 2.15 (m, 2H), and 7.18 (s, 10H).

***cis*-1,2-Diphenylcyclopropane (IX*b*)**—A solution of 2.0 g (0.008 mole) of IV and 30 ml of ether in a 250-ml three-necked flask, fitted with a dry ice–acetone condenser, was stirred (magnetic stirrer) in an ice water bath. Sodium metal (6.9 g, 0.3 g-atom) was added in small pieces over 1.5 hr, and 60 ml of wet methanol (2 ml of water) was introduced slowly through a dropping funnel with stirring (magnetic stirrer). After all of the sodium metal reacted, 20 ml of water was added and the aqueous layer was extracted with two 30-ml portions of ether.

The aqueous phase was neutralized slowly with concentrated hydrochloric acid on an ice–salt bath and extracted with two 30-ml portions of ether. The ether extracts were dried over anhydrous magnesium sulfate and filtered. Evaporation of ether at 30° under vacuum yielded 1.5 g of a yellow oil, which solidified when left at 0°. Sublimation at 40–50° (0.04–0.05 mm) with the cold finger at −40° yielded 1.0 g (68%) of white needles, mp 38–38.5° [lit. (37–40) mp 38–38.5°]; ν_{max} (neat): 3015 (strong), 1950 (medium), 1880 (weak), 1805 (weak), 1603 (strong), 1580 (weak), 1493 (strong), 1443 (strong), 1380 (weak), 1362 (medium), 1200 (medium), 1160 (medium), 1110 (weak), 1073 (strong), 1030 (strong), 915 (medium), 833 (medium), 775 (medium), 760 (medium), 720 (medium), 690 (medium), and 625 (medium) cm^{-1}; $\delta_{60\ MH_z}$ ($CDCl_3$): 1.42 (m, 2H), 2.5 (t, 2H), and 7.0 (s, 10H).

1,2-Diethyl-*trans*-1,2-(4,4′-dimethoxyphenyl)cyclopropane (XII)—To 150 ml of ether in a 1-liter three-necked flask, fitted with a dry ice–acetone condenser, dropping funnel, and mechanical stirrer, was added 8.34 g (0.02 mole) of VI. To this stirred mixture was added 40 g (1.7 g-atom) of sodium metal in small portions over 3.5 hr, along with 300 ml of wet methanol (10 ml of water), which was introduced slowly through a dropping funnel.

After all of the sodium metal had reacted, 100 ml of water was added slowly and carefully and the aqueous layer was extracted with two 50-ml portions of ether. The aqueous phase was neutralized with concentrated hydrochloric acid slowly on an ice–salt bath and extracted twice with 50 ml of ether. Ether extracts were collected and dried over anhydrous magnesium sulfate and filtered. Evaporation of ether *in vacuo* yielded 8.4 g of a yellow-brown oil, which solidified on standing at 0°. The crude compound was dissolved in hot methanol, treated with activated charcoal, filtered, and allowed to remain at 37° until crystals formed. Light-yellow crystals were obtained in four different crops[8], totaling 5.25 g (77%), mp 75–78°.

An analytical sample was obtained through sublimation at 70° (0.03 mm), mp 75–76°; ν_{max} (CCl_4): 3030 (weak), 2985 (medium), 2955 (strong), 2935 (medium), 2870 (weak), 2835 (weak), 1605 (strong), 1575 (weak), 1503 (strong), 1455 (medium), 1435 (medium), 1368 (weak), 1300 (weak), 1285 (medium), 1235 (very strong), 1165 (strong), 1103 (medium), 1063 (weak), 1033 (strong), 825 (strong), 692 (weak), and 608 (medium) cm^{-1}; $\delta_{60\ MHz}$ ($CDCl_3$): 0.6 (m, 6H), 0.95 (s, 2H), 1.48 (m, 4H), 3.78 (s, 6H), and 7.03 (q, 4H); mass spectrometry M^+: *m/e* 310.

1,2-Diethyl-*trans*-1,2-(4,4′-dihydroxyphenyl)cyclopropane (X)—A reported demethylation procedure (30–32) was followed; 3.1 g (0.01 mole) of XII was dissolved in 60 ml of anhydrous methylene chloride (dried and distilled from anhydrous calcium hydride) contained in a 250-ml, flame-dried, single-necked flask fitted with a dropping funnel with a calcium chloride drying tube. The single-necked flask was placed in a dry ice–acetone bath (−80°); then 4.7 g (0.019 mole) of boron tribromide[3], which was weighed in the hood and quickly diluted with 60 ml of dry methylene chloride and transferred to the dropping funnel, was added dropwise to the dimethyl ether solution over 10–15 min while the mixture was stirred (magnetic stirrer).

After 15 min, the cooling bath was removed and the reddish mixture was stirred at room temperature for 20 hr. The resulting pale-brown mixture was placed in an ice water bath and decomposed slowly with 100 ml of water. The organic material was extracted with two 100-ml portions of ether, and the combined ether layers were extracted with 150 ml of 2 *N* sodium hydroxide solution.

The alkaline solution was neutralized with dilute hydrochloric acid and extracted with ether. The ether was washed with water, dried over anhydrous magnesium sulfate, and filtered. Evaporation of the ether *in vacuo* yielded a pale-brown oil, which was dissolved in hot chloroform, treated with activated charcoal, filtered, and cooled, yielding 1.98 g (70%) of white crystals, mp 138–140°.

Recrystallization from hot benzene provided an analytical sample, mp 139–141°; ν_{max} (KBr): 3300 (broad), 3043 (weak), 2960 (medium), 2872 (weak), 2792 (weak), 2074 (weak), 1890 (medium), 1764 (weak), 1607 (strong), 1592 (strong), 1507 (strong), 1432 (strong), 1347 (strong), 1307 (weak), 1292 (weak), 1222 (strong), 1164 (weak), 1097 (medium), 1067 (medium), 1034 (medium), 1007 (medium), 963 (weak), 952 (weak), 922 (weak), 892 (weak), 860 (weak), 827 (strong), 764 (weak), 734 (weak), 672 (weak), 612 (weak), and 522 (weak) cm^{-1}; $\delta_{60\ MHz}$ (CCl_4–CD_3OD): 0.6 (m, 6H), 0.95 (s, 2H), 1.55 (m, 4H), 2.55 (s, 2H), and 6.98 (q, 8H); mass spectrometry M^+: *m/e* 282.

***trans*-1,2-Bis(*p*-methoxyphenyl)cyclopropane (XI)**—The method reported by Doering and Hoffman (8) was followed; 1.6 g (0.005 mole) of V and 6.0 g (0.26 g-atom) of sodium metal were added to 60 ml of wet methanol in 30 ml of ether. A light-yellow oil (1.2 g), which solidified when absolute ethanol was added, was obtained. The mixture was heated on a steam bath and filtered. The solution was allowed to cool slowly and was stored at 0° overnight. A white solid (0.42 g), mp 66–68°, was collected. The filtrate was concentrated by evaporation and placed into a refrigerator. Another 0.6 g of white crystals, mp 68–70°, brought the total yield to 1.02 g (78%).

Sublimation at 70–75° (0.05 mm) with the cold finger at −5° (ammonium chloride–sodium nitrate–sodium chloride–ice–water mixture) yielded 0.86 g (67%) of a white solid, mp 70.5–71.5° [lit. (41) mp 70.5–71.5°]; $\delta_{60\ MHz}$ ($CDCl_3$): 1.3 (M= 2H), 2.05 (m, 2H), 3.78 (s, 6H), and 6.95 (q, 8H).

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[8] Cooling of the concentrated solution at 0° caused separation of oil instead of crystals; therefore, it was necessary to crystallize first at room temperature and then at 10°. The concentration of the mother liquor gave an additional crop of crystals.

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Effect of pKb on Lipophilic Binding of Disopyramide Derivatives to Human Plasma

YIE W. CHIEN ˣ, MICHAEL J. AKERS *, and PETER K. YONAN

Abstract □ The extent of plasma binding, the partition coefficient, and the pKb of 13 disopyramide derivatives were determined. The structural variation on the diisopropylaminoethyl group of disopyramide molecules influenced these physical parameters to varying degrees. Results demonstrated that the extent of interaction between drugs and human plasma was a linear function of their lipophilicity and inversely proportional to the magnitude of the pKb value.

Keyphrases □ Disopyramide derivatives—determination of plasma binding, partition coefficient and pKb, relationship between pKb and binding □ Lipophilic binding of 13 disopyramide derivatives to human plasma—relationship to pKb □ Plasma binding, 13 disopyramide derivatives—determination, relationship to pKb □ Partition coefficients, 13 disopyramide derivatives—determination

Previously, it was reported (1) that the extent of plasma binding for 20 disopyramide derivatives was linearly related to their lipophilicity, log (p.c.), as defined by the following relationship:

$$\log (D_b/D_f) = \log (D_b/D_f)_0 + \log (\text{p.c.}) \qquad \text{(Eq. 1)}$$

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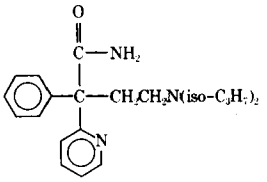
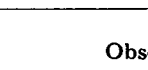
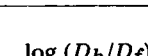
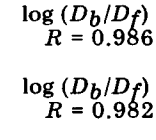
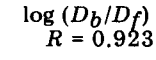
Table I—Linear Relationship between the Extent of Plasma Binding and the Lipophilicity of Disopyramide Derivatives with the Structural Variation of their Amide, Pyridyl, or Phenyl Moieties



Structural Variation on	n^a	Observed Relationships[b]
	10	$\log (D_b/D_f) = -0.303 + 0.473 \log$ (p.c.) $R = 0.986$ $s^2 = 0.0034$
	7	$\log (D_b/D_f) = -0.369 + 0.641 \log$ (p.c.) $R = 0.982$ $s^2 = 0.0059$
	6	$\log (D_b/D_f) = -0.476 + 0.734 \log$ (p.c.) $R = 0.923$ $s^2 = 0.0303$

[a] n = number of derivatives used in the analysis. [b] R = correlation coefficient; s^2 = residual variance.

Table II—Disopyramide Derivatives Series (I)

Compound[a]	R	$\log (D_b/D_f) + \log (K_b + [H^+])$	log (p.c.)	pKb
I	$-N(iso-C_3H_7)_2$	1.219	−0.181	8.36
II	$-N(n-C_3H_7)_2$	0.826	0.905	9.50
III		0.747	−0.842	9.92
IV		2.680	−0.553	5.40
V		0.999	0.560	8.50
VI		1.190	0.689	8.86
VII		0.495	−0.373	9.95

[a] SC-13957, SC-13268, SC-24566, SC-13251, SC-13212, SC-13482, and SC-27829, respectively.

where log (D_b/D_f) was theoretically derived and related to the equilibrium constant for drug–plasma interactions; D_b and D_f are the concentrations of drug bound to plasma protein and freely existing in the solution phase, respectively; and log $(D_b/D_f)_0$ is the intercept of log (D_b/D_f) *versus* log (p.c.) plots.

It was observed that the structural variation of the amide, pyridyl, or phenyl moieties resulted in varying degrees of influence on the extent of plasma binding and the lipophilicity of disopyramide derivatives (Table I). It also was found that the omission of the diisopropylaminoethyl group produced no change in the degree of plasma binding while the lipophilicity was enhanced more than 10-fold (fraction of drug bound remained at 27.3% although the partition coefficient in the *n*-octanol–phosphate buffer system was increased from 0.66 to 6.87). This observation was rationalized by the fact that the diisopropylaminoethyl group (with a pKb of 8.36) was protonated at the physiological pH of 7.4 and did not interact with the plasma protein itself.

For a better understanding of the behavior of the diisopropylaminoethyl group during the interaction of the other three moieties of disopyramide molecules with plasma protein, the studies were extended to cover a new series of disopyramide derivatives with structural variation on the diisopropylaminoethyl moiety only while the other three functional groups were held constant. These results are reported in this paper.

EXPERIMENTAL

Binding of disopyramide derivatives to human plasma and partitioning studies in the system of *n*-octanol–pH 7.4 phosphate buffer were carried out in the same manner as described earlier (1).

Potentiometric pH titration[1] was employed for the determination of pK values. This method is highly reproducible (pK ± 0.03 unit) for acids or bases having pK values between 2.5 and 11 (2). Apparent pK's were determined by the Parke and Davis method (3) instead of by the conventional half-neutralization method because the solubility of the compounds was low and the inflection of the titration curves was not very sharp.

An aqueous solution containing 2.5×10^{-4} *M* compound was titrated with standardized 0.1 *N* hydrochloric acid. An identical solution containing no compound was subsequently titrated, and the difference between these two pH *versus* titrant volume titration curves was calculated. The apparent pKb value was then measured as the pH at the point of inflection of the "pH-different titrant volume curve." Determinations of pKb values were run in duplicate for each compound. No pKb was observed for the compound without the diisopropylaminoethyl group.

RESULTS AND DISCUSSION

In solution, disopyramide derivative molecules (D) exist as an equilibrium mixture of neutral and protonated species (Scheme I):

$$D + H_3O^+ \rightleftharpoons HD^+ + H_2O$$

Scheme I

The degree of protonation (σ) of the diisopropylaminoethyl group varies according to its basicity (K_b) in a series of disopyramide derivatives and the pH profile of the drug solution:

$$\sigma = \frac{[H^+]}{K_b + [H^+]} \qquad \text{(Eq. 2)}$$

If drug molecules are bound to plasma protein only as the neutral form, the equilibrium constant (K_n) for the interaction (4) between the neutral species of disopyramide derivatives and plasma protein is related to the overall equilibrium binding constant (K) as follows:

$$K_n = \frac{K}{1 - \sigma} = \frac{K}{K_b/K_b + [H^+]} \qquad \text{(Eq. 3)}$$

or:

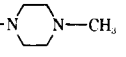
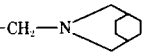
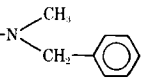
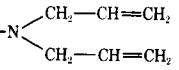
$$\log K_n = \log K - \log K_b + \log (K_b + [H^+]) \qquad \text{(Eq. 4)}$$

As analyzed previously (1), the equilibrium constant, K_n, for the interaction between the neutral species of drug and plasma protein was linearly related to its lipophilicity [as represented by log (p.c.)]:

$$\log K_n = \frac{\mu_0^\circ - \mu_p^\circ}{2.303RT} + \log \text{(p.c.)} \qquad \text{(Eq. 5)}$$

[1] Copenhagen Radiometer, London Co., Westlake, Ohio.

Table III—Disopyramide Derivatives Series (II)

Compound[a]	R	log (D_b/D_f) + log $(K_b$ + $[H^+])$	log (p.c.)	pKb
VIII		1.203	0.083	6.86
IX		0.808	0.501	7.81
X		3.894	0.874	4.74
XI		4.234	0.956	4.68
XII		3.079	1.122	6.90
XIII		1.617	0.877	8.11

[a] SC-13127, SC-13173, SC-13209, SC-13733, SC-13489, and SC-13486, respectively.

where $\mu_0°$ and $\mu_p°$ are the standard chemical potentials for a drug species existing in the organic phase and bound to a protein molecule, respectively.

Substituting Eq. 5 for the log K_n term in Eq. 4 gives:

$$\log K + \log (K_b + [H^+]) = \frac{\mu_0° - \mu_p°}{2.303RT} + \log (\text{p.c.}) - \text{pKb} \quad \text{(Eq. 6)}$$

The Scatchard relationship (5) may be expressed alternatively as:

$$K = \frac{D_b}{D_f} \times \frac{1}{\bar{n}p} \quad \text{(Eq. 7)}$$

since $\bar{n} \gg \bar{v}$. Therefore:

$$\log (D_b/D_f) + \log (K_b + [H^+]) = \log (D_b/D_f)_0 + \log (\text{p.c.}) - \text{pKb} \quad \text{(Eq. 8)}$$

where:

$$\log (D_b/D_f)_0 = \frac{\mu_0° - \mu_p°}{2.303RT} + \log \bar{n}p \quad \text{(Eq. 9)}$$

The terms $\bar{n}$ and p were defined earlier (1) as the total number of binding sites on a protein molecule and the total concentration of plasma protein in the bloodstream, respectively.

Equation 8 predicts that the extent of plasma binding is dependent on both the lipophilicity, log (p.c.), and the pKb of disopyramide derivatives. When the pH of a drug–plasma mixture is maintained at the physiological pH of 7.4, the higher the lipophilicity of drug the greater is the extent of the drug–plasma interaction. On the other hand, as the magnitude of pKb value of diisopropylaminoethyl group increases, the degree of plasma binding decreases.

Table IV—Comparison between the Observed and the Calculated Fraction of Disopyramide Derivatives Bound to Plasma Protein in Table II

Compound[a]	Fraction of Drug Bound, %[b]		
	Observed	Calculated	
		Eq. 10	Eq. 12
I	27.30	30.74	32.89
II	14.35	13.11	9.56
III	12.26	8.91	5.48
IV	10.43	8.67	27.04
V	18.81	22.16	29.10
VI	27.4	28.22	20.47
VII	7.25	9.33	5.26

[a] SC-13957, SC-13268, SC-24566, SC-13251, SC-13212, SC-13482, and SC-27829, respectively. [b] Fraction of drug bound (%) = $[D_b/(D_b + D_f)] \times 100$.

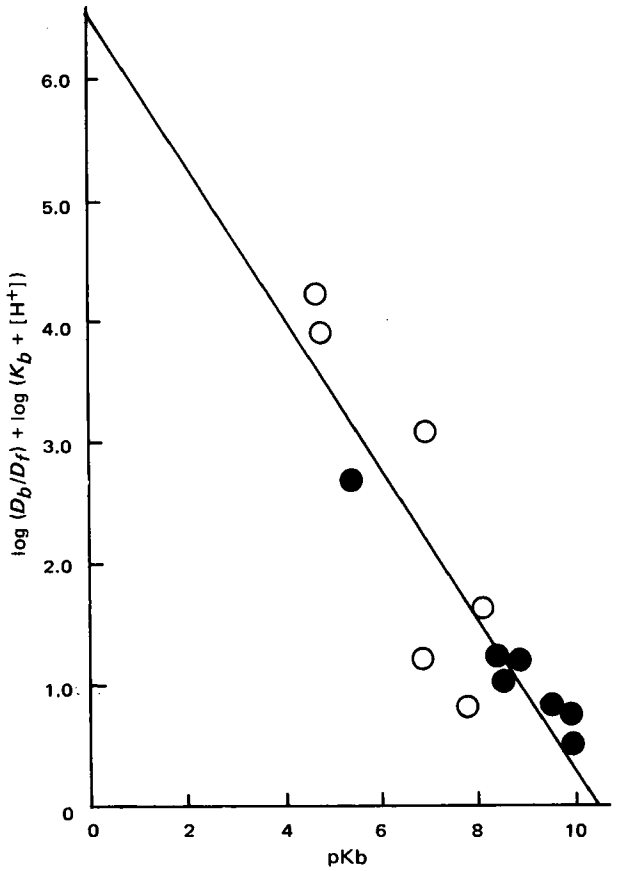


Figure 1—*Linear plot between log* (D_b/D_f) + log $(K_b + [H^+])$ *and pKb as defined by Eq. 12. All terms are defined in the text. The open circles are the data points from Table III, and the solid circles are the data points from Table II.*

The values of log (D_b/D_f) + log $(K_b + [H^+])$, log (p.c.), and pKb tabulated in Tables II and III were submitted separately to multiple regression analysis. Two relationships were obtained:

$$\log (D_b/D_f) + \log (K_b + [H^+]) = 5.035 - 0.448(\pm 0.039)\text{pKb} \quad \text{(Eq. 10)}$$

n	R	s^2
7	0.982	0.016

$$\log (D_b/D_f) + \log (K_b + [H^+]) = 5.835 + 1.857(\pm 0.29) \log (\text{p.c.}) - 0.726(\pm 0.074)\text{pKb} \quad \text{(Eq. 11)}$$

n	R	s^2
6	0.992	0.028

Both the multiple correlation coefficient (R) and the residual variance (s^2) demonstrate that the degree of plasma binding of disopyramide derivatives is highly related to their pKb values and/or their lipophilicity. An F test indicates that both Eq. 10 [$F_{1,5}$ = 134.4; $F_{1,5}$ (when α = 0.01) is 16.26] and Eq. 11 [$F_{2,3}$ = 94.16; $F_{2,3}$ (when α = 0.01) is 30.80] are highly significant statistically.

By using Eqs. 10 and 11, estimated β values (fraction of drug bound) for the disopyramide derivatives were computed and tabulated in the third column of Tables IV and V together with the observed β values (second column).

Equations 10 and 11 demonstrated that structural variation on the diisopropylaminoethyl group resulted in two subgroups whose

Table V—Comparison between the Observed and the Calculated Fraction of Disopyramide Derivatives Bound to Plasma Protein in Table III

Compound[a]	Fraction of Drug Bound, %[b]		
	Observed	Calculated	
		Eq. 11	Eq. 12
VIII	8.23	5.46	50.92
IX	10.48	18.59	46.31
X	30.03	36.41	17.35
XI	45.03	43.91	16.61
XII	87.85	83.12	51.20
XIII	46.35	44.17	39.15

[a] SC-13127, SC-13173, SC-13209, SC-13733, SC-13489, and SC-13486, respectively. [b] Fraction of drug bound (%) = $[D_b/(D_b + D_f)] \times 100$.

plasma binding was dependent, in a different degree, on the magnitude of pKb and lipophilicity as demonstrated by the difference in their slopes (−0.448 and −0.726, respectively).

If the influence of lipophilicity, log (p.c.), is neglected and only the pKb values are related to the extent of plasma binding for these two subgroups of disopyramide derivatives, the importance of pKb can be clearly shown. The linear relationship (Fig. 1) is defined by the following expression:

$$\log (D_b/D_f) + \log (K_b + [H^+]) = 6.528 - 0.621(\pm 0.090)\text{pKb} \quad \text{(Eq. 12)}$$

n	R	s^2
13	0.901	0.279

Because of the neglect of the lipophilicity term in Eq. 12, the data points from Table III were noticeably displaced from the slope (Fig. 1). The deviation of the calculated from the observed values was significantly greater for the chemicals in Table III (comparing column 2 with column 4 in Table V) than for the chemicals in Table II (comparing column 2 with column 4 in Table IV). The results demonstrate that the series of disopyramide derivatives in Table II will be better expressed by Eq. 10 and that the extent of plasma binding is linearly correlated with the magnitude of pKb with a slope of −0.448. On the other hand, the extent of plasma binding for the disopyramide derivatives in Table III is a linear function of their lipophilicity and inversely proportional to the magnitudes of pKb. This relationship is best defined by Eq. 11.

In conclusion, the extent of interaction between plasma protein and disopyramide derivatives is significantly influenced by the magnitude of the pKb of the diisopropylaminoethyl group.

REFERENCES

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* Present address: Alcon Laboratories, Fort Worth, Tex.

x To whom inquiries should be directed.

Preparation, Isolation, and Identification of 4-Dedimethylamino-11-methoxyanhydrotetracycline

L. J. STOEL *, E. C. NEWMAN ‡, G. L. ASLESON, and C. W. FRANK x

Abstract □ The reaction of diazomethane with 4-dedimethylaminoanhydrotetracycline produced a number of mono-, di-, and trimethylated products. The isolation of one monomethylated product (4-dedimethylamino-11-methoxyanhydrotetracycline) and its subsequent identification utilizing mass spectral and NMR data are described.

Keyphrases □ 4-Dedimethylamino-11-methoxyanhydrotetracycline—isolation and identification after diazomethane methylation of 4-dedimethylaminoanhydrotetracycline □ Methylation of 4-dedimethylaminoanhydrotetracycline with diazomethane—isolation and identification of one reaction product □ Mass spectrometry—identification, 4-dedimethylamino-11-methoxyanhydrotetracycline

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EXPERIMENTAL

NMR spectra were obtained using high-resolution NMR spectrometers[1]. The chemical shift data were measured relative to tetramethylsilane as an internal standard and reported as parts per million. All NMR spectra were recorded using d_6-dimethyl sulfoxide as the solvent. The mass spectra were obtained on a medium

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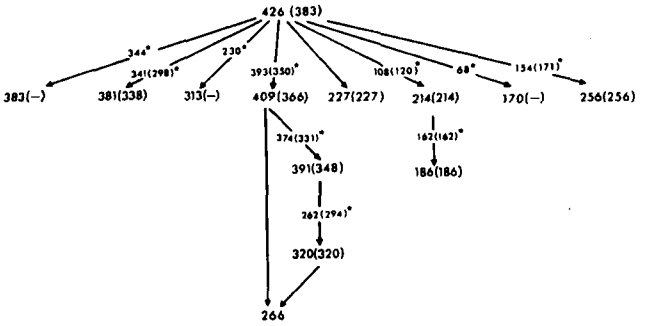


*Scheme I—Mass spectral fragmentation pathways of anhydrotetracycline. The fragmentation pathway of I is indicated in parentheses. * = metastable; (–) = not observed in I.*

resolution mass spectrometer[2] utilizing the direct inlet at approximately 250°. An ionization energy of 70 ev was used for all spectra reported.

Significant methylation of the C-4 dimethylamine group was reported for the reaction of diazomethane with tetracycline (1). Therefore, this functional group was first removed from the starting material (tetracycline hydrochloride[3]).

Preparation of 4-Dedimethylaminotetracycline—The synthesis of 4-dedimethylaminotetracycline from tetracycline with tetracycline methiodide as an intermediate product, as reported by McCormick *et al.* (2), was used for this preparation. TLC on silica gel H, using acetone–ethyl acetate–water (20:10:3) as the eluting solution revealed a long streaking spot of R_f 0.33. This spot turned pink when exposed to air for about 15–20 min. Nonaqueous titration of the sample in dimethylformamide resulted in an equivalent weight of 411 ± 15 (calc. 401). Mass spectral analysis revealed a parent ion at *m/e* 401.

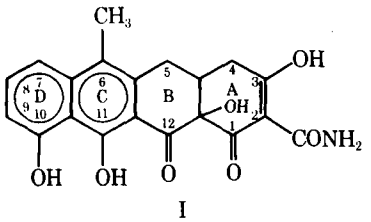
Preparation of 4-Dedimethylaminoanhydrotetracycline—4-Dedimethylaminotetracycline (0.5096 g) was dissolved in 150 ml of a 50% chloroform–isopropanol mixture to which was added 25 ml of concentrated hydrochloric acid. The resulting solution was refluxed for 2 hr and filtered. The volume of the solution was decreased by vacuum, and the remainder was cooled to 0°. The resulting 4-dedimethylaminoanhydrotetracycline (3, 4) was filtered, washed with distilled water, and dried at 40° for 12 hr in a vacuum oven.

The product (0.4314 g), mp 235° dec, had a mass spectral parent ion at *m/e* 383. Nonaqueous titration in dimethylformamide resulted in an equivalent weight of 382 ± 3 (calc. 383).

Anal.—Calc. for $C_{20}H_{17}NO_7$: C, 62.66; H, 4.47; N, 3.65. Found: C, 63.06; H, 4.43; N, 3.65.

Preparation and Separation of Methyl Ethers—An ethereal solution of diazomethane was prepared, using *N*-methyl-*N*-nitroso-*p*-toluenesulfonamide as the precursor, by the methods outlined by the manufacturer[4]. A solution of 4-dedimethylaminoanhydrotetracycline dissolved in a minimum amount of chloroform was treated with the ethereal diazomethane solution. After standing at room temperature for 2 hr, the sample was evaporated to dryness under vacuum and stored under nitrogen.

The methylated mixture was redissolved in chloroform and separated on preparative silica gel H TLC plates. Acetone–ethyl acetate–water (20:10:3) was used as the eluting solvent. Bands were observed at R_f values of 0.47, 0.54, 0.64, and 0.81. The band at R_f 0.54 was extracted into chloroform and subsequently purified by



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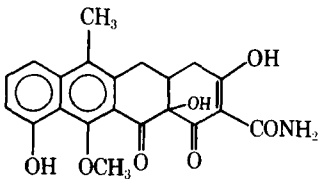


II

separation on 10% $CaCl_2$-treated silica gel preparative TLC plates using the previously mentioned eluting solution. The isolated band was extracted from the silica gel by treatment with a heterogeneous mixture of 0.1 *N* HCl and chloroform. The chloroform layer was dried with anhydrous sodium sulfate and evaporated to dryness under vacuum. The sample was dried at 60° in a vacuum oven, mp 210° dec.

The band at R_f 0.47 was identified as 4-dedimethylaminoanhydrotetracycline by mass spectrometry and NMR spectroscopy. Isolation and examination of the other TLC bands indicated that di-, tri-, and, possibly, tetramethylated products are present.

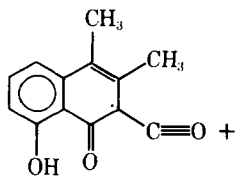
DISCUSSION

The structure of the methylated product of 4-dedimethylaminoanhydrotetracycline (R_f 0.54) was determined from the mass spectral and NMR data. The 70-ev mass spectral fragmentation patterns of some tetracyclines were reported, and several common fragments were assigned in these spectra (5). As in steroid systems (6), the dimethylamino group was observed to have a major effect on the fragmentation pattern of tetracyclines. In cases where the dimethylamino group had been removed, the carbonyl groups exhibited a dominating influence on the fragmentation pathways. In all cases, the initial fragmentation involved the loss of water to form a corresponding anhydro analog. The subsequent fragmentation patterns were reported to be the same as those of the corresponding 5a,6-anhydrotetracycline (5).

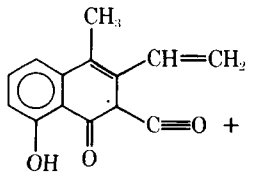
A fragmentation pathway involving the loss of NH_3 (*m/e* 17) and H_2O (*m/e* 18) and the subsequent single-step loss of CH_3NCH_2 and CO (combined *m/e* 71) was observed in the mass spectrum of anhydrotetracycline (5). This fragmentation sequence was confirmed by metastable peaks (*m/e* 393, 374, and 262, respectively) and is outlined in Scheme I. A similar fragmentation pattern was observed for 4-dedimethylaminoanhydrotetracycline (I).

A fragment corresponding to the loss of $CONH_3$ (*m/e* 45) from the parent ion was observed at *m/e* 381 in the mass spectrum of anhydrotetracycline and at *m/e* 338 in the mass spectrum of 4-dedimethylaminoanhydrotetracycline. Metastable peaks at *m/e* 341 and 298, respectively, confirmed these fragmentations. These resulting fragments retained the tetracyclic structure of the parent compound.

Somewhat similar fragmentation patterns were observed in the mass spectrum of the methylated product of 4-dedimethylaminoanhydrotetracycline; however, possibly as a result of methyl substitution, ring cleavage was the favored fragmentation pathway for this compound. The mass spectrum of the methylated 4-dedimethylaminoanhydrotetracycline (Fig. 1) contained a parent ion peak at *m/e* 397. The loss of NH_3 (*m/e* 17) from the parent ion resulted in a peak at *m/e* 380 and was confirmed by an observed metastable peak at *m/e* 364. Peaks at *m/e* 362 and 334 indicated the subsequent loss of H_2O and CO, respectively, as was previously observed



III: *m/e* 214



IV: *m/e* 227

[2] Hitachi Perkin-Elmer RMU 6E.
[3] Pfizer, Inc.
[4] Aldrich Chemical Co.

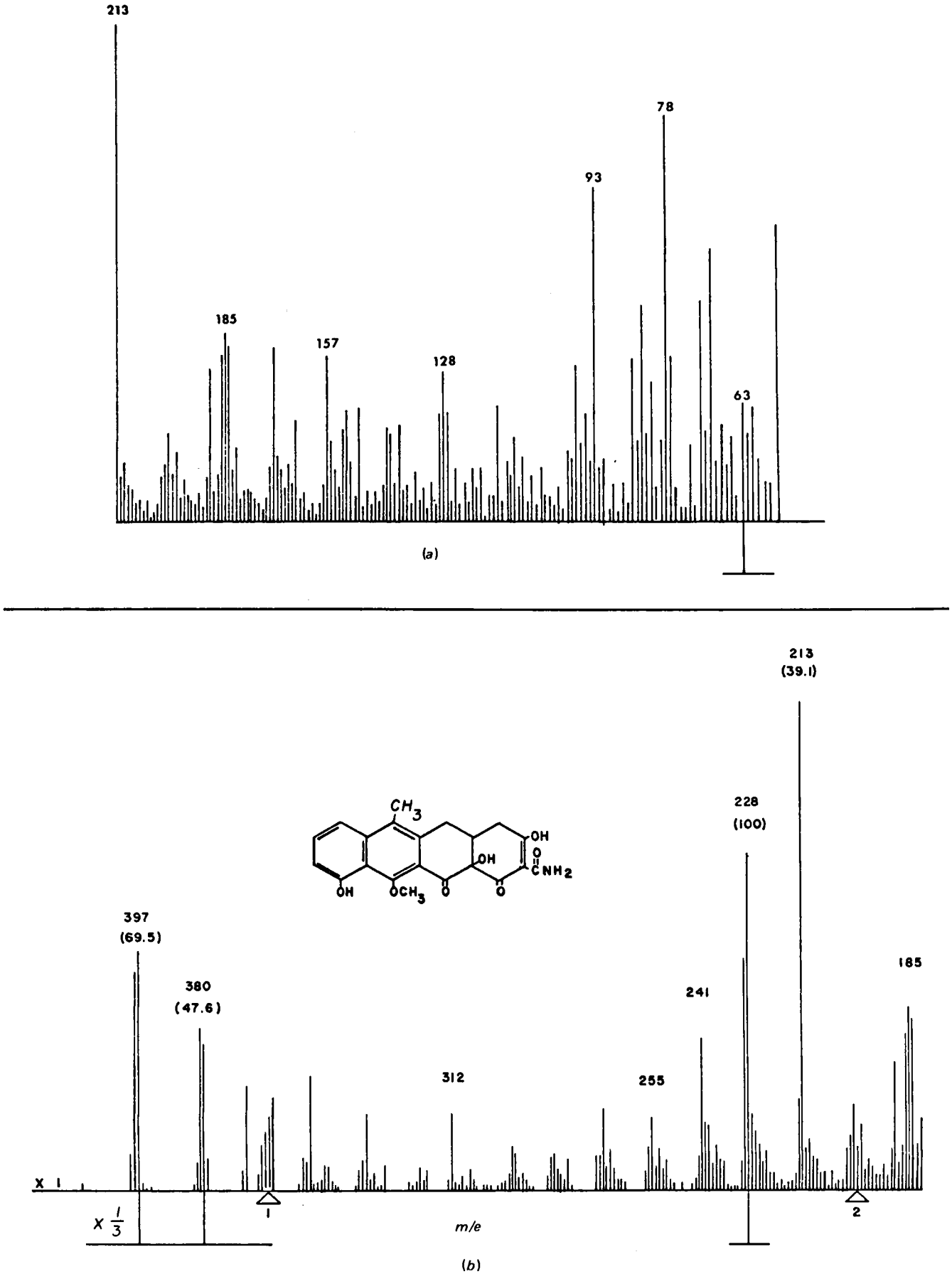
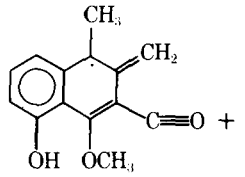


Figure 1—*Mass spectrum of 4-dedimethylamino-11-methoxyanhydrotetracycline* (a) *below* m/e *185 and* (b) *above* m/e *185. The* Δ_1 *and* Δ_2 *designate metastable ions at* m/e *341 and 199, respectively.*



V: *m/e* 228

for 4-dedimethylaminoanhydrotetracycline. A peak at *m/e* 352 was also observed for the loss of $CONH_3$ from the parent ion. The presence of these peaks indicated that only one methyl ether group was present in the methylated product and was retained on these fragments.

Because the C and D rings are aromatic, most ring cleavages of anhydrotetracyclines were reported to occur in the A or B rings. Peaks observed at *m/e* 313, 270, and 256 in the mass spectrum of anhydrotetracycline were reported to be a result of A ring fragmentation (5). Fragmentation of the A ring of 4-dedimethylaminoanhydrotetracycline was observed at *m/e* 298, 270, 266, 256, and 241. Corresponding peaks were observed at *m/e* 312, 284, 279, 269, and 255 in the mass spectrum of the monomethyl ether of 4-dedimethylaminoanhydrotetracycline (II) and indicated that the methyl ether was not on the A ring of this tetracycline.

Cleavage of the B ring was observed by fragments at *m/e* 227 and 214 in the mass spectra of both anhydrotetracycline and 4-dedimethylaminoanhydrotetracycline. Possible structures (III and IV) for these fragments are related to the intact C and D rings (5).

The 70-ev mass spectrum of the methylated product contained peaks at *m/e* 241 and 228; the *m/e* 228 fragment (V) was the dominant peak. A possible structure for this fragment, analogous to III, was derived from the intact C and D rings, since both can be generated by a similar fragmentation process. The presence of these fragments indicated that the methyl ether function was located on the C or D ring.

The *m/e* 228 fragment subsequently decomposed, losing CH_3 (*m/e* 15) and CO (*m/e* 28). This stepwise loss of C_2H_3O was observed and reported for the mass spectra of a number of aryl methyl ethers (7, 8). A metastable peak was observed at *m/e* 199, confirming the decomposition path.

The addition of methylene to the C-10 or C-11 hydroxy of 4-dedimethylaminoanhydrotetracycline was confirmed by the use of deuterodiazomethane (CD_2N_2). The 70-ev mass spectrum of the isolated product contained a dominant peak at *m/e* 230, and the subsequent decomposition of this fragment to the *m/e* 213 fragment was noted. This stepwise loss of CD_2H and CO was the same as was previously observed for the monomethyl ether of 4-dedimethylaminoanhydrotetracycline and indicated that the CH_3 fragment was from the methyl ether functional group. The loss of this methyl group made further assignment of the smaller fragments very difficult; however, it was concluded that the methyl ether function was located at either the C-10 or C-11 position.

The differentiation between methylation at the C-10 or C-11 position was accomplished using NMR spectroscopy. A number of exchangeable protons were observed downfield from 8 ppm in the NMR spectra of tetracyclines in d_6-dimethyl sulfoxide (9). Based on observations from model compounds, these signals were assigned to the hydrogen-bonded hydroxy protons of the C-10, C-11, and C-3 positions and the amide protons. The NMR spectrum of 4-dedimethylaminoanhydrotetracycline contained the signals of five exchangeable protons downfield from 8 ppm. The signals at 8.9 and 9.0 ppm were assigned to the amide protons of the carboxamido group, while the signals at 9.9, 15.3, and 18.2 ppm were assigned to the hydroxy protons of the C-10, C-11, and C-3 positions, respectively (9).

The NMR spectrum of methylated 4-dedimethylaminoanhydrotetracycline contained signals at 8.9, 9.1, 10.1, and 18.3 ppm. The absence of a signal in the 15-ppm region indicated that the C-11 hydroxy proton had been replaced by a methyl group. An additional singlet at 3.95 ppm, corresponding to three protons, was observed for these methoxy protons.

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Bioavailability Assessment under Quasi- and Nonsteady-State Conditions I: Theoretical Considerations

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Abstract □ A strategy was devised to permit bioavailability estimations at quasi- and nonsteady states. The proposed method retains most attributes of a steady-state comparison without being burdened by its protractiveness. The only necessary requirements are that drug disposition obeys linear kinetics and that succeeding doses are administered during the log-linear phase free from the influence of continuing absorption.

Keyphrases □ Bioavailability—estimations under quasi- and nonsteady states, theoretical considerations and equations □ Drug accumulation under quasi- and nonsteady-state conditions—bioavailability estimations, theory and equations

An accepted method of assessing the relative bioavailability of dosage forms is to compare their mean plasma concentrations with those of a reference over a comparable dosage interval at steady state. The theoretical basis for this kind of comparison is analogous to that of comparing total areas under the plasma concentration curve in single-dose studies (1). In either case, the underlying assumptions are that plasma clearance is not concentration dependent and is constant between treatments.

Implicit in the choice of steady-state measurements is that there will be drug accumulation for the specified dosage regimen. Relative to single-dose studies, the steady-state approach has several obvious advantages. First, fewer data points are required because the time course of change in plasma concentrations is less precipitous and sampling times are bounded by the dosage interval. Second, plasma concentrations are higher. Third, the method is model independent so long as drug disposition obeys linear kinetics. The main disadvantage is that the clinical aspects are much more difficult to control and execute. It may take days or even weeks to achieve steady state. In any protracted study, the potential for lapses in subject compliance increases with time.

Unfortunately, many factors that lend support to steady-state measurements also prolong the clinical phase. Plasma half-life, $t_{1/2}$, is by far the most important parameter in drug accumulation. With increasing $t_{1/2}$, the extent of accumulation increases for a given dosage regimen, the differences between peak and trough plasma concentrations are diminished, and the plasma time course is less sensitive to the effect of variations in absorption rates.

Provided that one is not attempting to demonstrate timed-release properties, a long half-life tends to make steady-state studies more attractive. On the other hand, the study becomes more unwieldy because the time to achieve steady state also increases with increasing $t_{1/2}$. Additionally, prudent planning dictates that the longest $t_{1/2}$ among individuals should be accommodated, which simply means that the best designed studies must also be the most time consuming.

Thus, even though drug accumulation can be anticipated, the steady-state option may not always be a practical alternative to single-dose comparisons. The purpose of this article is to propose an alternative strategy to utilize the obvious advantages of drug accumulation in bioavailability assessment.

THEORETICAL

The overall strategy is to effect sufficient drug accumulation to facilitate an assessment of bioavailability without unduly prolonging the clinical phase of the study.

For a drug obeying one-compartment model kinetics, it has been shown (1–3) that the mean plasma level (the first *noncentral moment* over τ) following the administration of a fixed dose at equal time intervals is estimated by:

$$\bar{C}p^{(ss)} = \frac{FD}{\tau k_e V_d} \qquad \text{(Eq. 1)}$$

and:

$$\bar{C}p^{(i)} = \frac{k_a FD}{\tau V_d (k_e - k_a)}\left[\frac{(1 - e^{-ik_a\tau})}{k_a} - \frac{(1 - e^{-ik_e\tau})}{k_e}\right] \qquad \text{(Eq. 2)}$$

where $\bar{C}p^{(ss)}$ and $\bar{C}p^{(i)}$ are the mean plasma concentration at steady state and at the ith dose, respectively, after repeated administration of the drug at interval τ; k_a and k_e are first-order rate constants for absorption and elimination; V_d is the apparent volume of distribution; and F is the fraction of the dose, D, absorbed.

The extent and rate of drug accumulation may be calculated by:

$$\bar{C}p^{(ss)} = \frac{(k_e - k_a)\bar{C}p^{(i)}}{k_e(1 - e^{-ik_a\tau}) - k_a(1 - e^{-ik_e\tau})} \qquad \text{(Eq. 3)}$$

and:

$$\left[1 - \frac{\bar{C}p^{(i)}}{\bar{C}p^{(ss)}}\right] = \frac{k_e}{k_e - k_a}e^{-ik_a\tau} - \frac{k_a}{k_e - k_a}e^{-ik_e\tau} \qquad \text{(Eq. 4)}$$

When $k_a \gg k_e$, Eqs. 3 and 4 may be further simplified to:

$$\bar{C}p^{(ss)} \cong \frac{\bar{C}p^{(i)}}{1 - e^{-ik_e\tau}} \qquad \text{(Eq. 5)}$$

and:

$$\ln\left[1 - \frac{\bar{C}p^{(i)}}{\bar{C}p^{(ss)}}\right] \cong -k_e(i\tau) \qquad \text{(Eq. 6)}$$

Defining the half-time for accumulation, $(i\tau)_{1/2}$, as the time required to reach 50% of steady state, *i.e.*, when $\bar{C}p^{(i)} = \bar{C}p^{(ss)}/2$, one obtains:

$$(i\tau)_{1/2} \cong \frac{\ln 2}{k_e} = (t_{1/2})_e \qquad \text{(Eq. 7)}$$

Thus, the half-time for accumulation is approximately equal to the half-life for elimination. Equation 7 represents the limiting case described by van Rossum (3).

Analogous expressions for a general N-compartment mammilla-

ry model with first-order absorption are:

$$\bar{C}p^{(i)} = \frac{k_a F D}{\tau V_1}\left[\frac{(1-e^{-ik_a\tau})\prod_{j=2}^{N}(E_j-k_a)}{k_a(\alpha-k_a)(\beta-k_a)\ldots(\psi-k_a)} + \frac{(1-e^{-i\alpha\tau})\prod_{j=2}^{N}(E_j-\alpha)}{\alpha(k_a-\alpha)(\beta-\alpha)\ldots(\psi-\alpha)} + \cdots + \frac{(1-e^{-i\psi\tau})\prod_{j=2}^{N}(E_j-\psi)}{\psi(k_a-\psi)(\alpha-\psi)\ldots(\chi-\psi)}\right] \quad \text{(Eq. 8)}$$

$$\bar{C}p^{(ss)} = \frac{FD\Pi_j E_j}{\tau V_1 \alpha\beta\ldots\psi} \quad \text{(Eq. 9)}$$

where E_j is the sum of exit rate constants from compartment $j = 2, 3, \ldots, N$; $\alpha, \beta, \ldots, \psi$ are the hybrid rate constants, of which there are N in number; and V_1 is the apparent volume of distribution for the central compartment. Their derivation is given in the *Appendix*. It can be seen that Eqs. 1–7 are for the special case where $N = 1$.

One can define ω as the smallest plasma decay constant and V_0 as an operational constant such that ωV_0 is an estimate of body drug clearance. If ω is much smaller (say, one-fifth or less) than any exit rate constant, E_j, and the next larger hybrid constant, including k_a, then:

$$\bar{C}p^{(ss)} \cong \frac{\bar{C}p^{(i)}}{1-e^{-i\omega\tau}} \quad \text{(Eq. 10)}$$

$$\ln\left[1-\frac{\bar{C}p^{(i)}}{\bar{C}p^{(ss)}}\right] \cong -\omega(i\tau) \quad \text{(Eq. 11)}$$

$$(i\tau)_{1/2} \cong \frac{\ln 2}{\omega} = (t_{1/2})_\omega \quad \text{(Eq. 12)}$$

and combining Eqs. 9 and 10:

$$\bar{C}p^{(i)} = \frac{FD\Pi_j E_j}{\tau V_1 \alpha\beta\ldots\omega}(1-e^{-i\omega\tau}) = \frac{FD}{\tau\omega V_0}(1-e^{-i\omega\tau}) \quad \text{(Eq. 13)}$$

providing τ is chosen such that succeeding doses are administered during the log-linear phase (see *Appendix*).

From the foregoing, it is evident that the most important parameter in drug accumulation is the slowest rate constant for plasma decay, *i.e.*, the terminal slope.

Suppose two treatments of the same drug were to be compared by administering sequentially l doses of formulation x followed immediately by m doses of formulation y according to the same dosage regimen. If conditions for Eq. 10 are met, then:

$$\bar{C}p^{(l)} = \frac{F_x D}{\tau\omega V_0}(1-e^{-l\omega\tau}) \quad \text{(Eq. 14)}$$

and:

$$\bar{C}p^{(m+l)} = \frac{D}{\tau\omega V_0}\left[F_x e^{-m\omega\tau}(1-e^{-l\omega\tau}) + F_y(1-e^{-m\omega\tau})\right] \quad \text{(Eq. 15)}$$

where $\bar{C}p^{(l)}$ and $\bar{C}p^{(m+l)}$ are the observed mean plasma levels during the lth and $(m+l)$th dosage intervals, respectively; and F_x and F_y are the fractions of dose absorbed from formulations x and y, respectively. Implicit in Eqs. 14 and 15 are that ω remains constant, but k_a and F may differ between treatments. (See *Appendix* for derivation.)

The relative bioavailability of formulation y with respect to formulation x can be estimated by solving Eqs. 14 and 15:

$$\frac{F_y}{F_x} = \left[\frac{\bar{C}p^{(m+l)}}{\bar{C}p^{(l)}} - e^{-m\omega\tau}\right]\frac{(1-e^{-l\omega\tau})}{(1-e^{-m\omega\tau})} \quad \text{(Eq. 16)}$$

which collapses to the usual expression for relative bioavailability at steady state; *i.e.*, when $l \to \infty$ and $m \to \infty$:

$$\frac{F_y}{F_x} = \frac{[\bar{C}p^{(ss)}]_y}{[\bar{C}p^{(ss)}]_x} \quad \text{(Eq. 17)}$$

These considerations can be easily extended to accommodate three or more treatments. For example, if treatment y were followed immediately by n doses of treatment z, then:

$$\frac{F_z}{F_x} = \left[\frac{\bar{C}p^{(n+m+l)}}{\bar{C}p^{(l)}} - \frac{\bar{C}p^{(m+l)}e^{-n\omega\tau}}{\bar{C}p^{(l)}}\right]\frac{(1-e^{-l\omega\tau})}{(1-e^{-n\omega\tau})} \quad \text{(Eq. 18)}$$

In the interest of clarity, Eqs. 13, 16, and 18 have been derived with the assumption that the following simplifying conditions prevail: *(a)* the same dosage is administered repetitively for a given treatment, *(b)* the same dosage regimen is followed for all treatments, *(c)* the time intervals between successive doses are uniform throughout, *(d)* there are no discontinuities (extended "washout" periods) between treatments, *(e)* the terminal half-life for a given subject remains constant, *(f)* body drug clearance remains constant, and *(g)* succeeding doses are administered during the log-linear phase which is not influenced by continuing absorption.

Subsequent discussion will attempt to show that most, if not all, of these conditions can be modified, verified, or circumvented through appropriate experimental design and/or data analysis.

DISCUSSION

Experimental strategies for bioavailability estimation have been almost exclusively limited to comparisons following single doses or at steady state. In general, multiple-dose studies are considered only when useful accumulations can be realized through some convenient dosage regimen. However, the attainment of steady state may require an unacceptably long time. As already illustrated, most advantages of multiple-dose comparisons can be incorporated into bioavailability studies without the need to achieve steady state after each treatment. Basically, the proposed method evolves from the observation that in a linear system the incremental change in drug accumulation is largest, and therefore most useful, in the initial, nonsteady-state region.

Implicit in all bioavailability comparisons is the assumption that the observed data following a given dose are a measure of the expected performance of the administered dosage form. The task is one of acquiring a sufficient number of random samples to permit conclusions within a prescribed degree of confidence. Variability in treatment performance is generally regarded to be greater between than within subjects; hence, crossover comparisons are preferred for precision and economy. But even in crossover studies, within subject comparisons of plasma concentrations and urinary recoveries *per se* may be influenced by variations in drug disposition between treatments.

It is evident that experimental designs should include provisions to compensate for possible changes in drug distribution and elimination. Several possibilities for verifying internal consistency in single-dose comparisons were suggested (4). In multiple-dose comparisons, the observed changes in distribution and/or elimination may be considered as random samples of their mean values over the entire time course of the respective treatments. Adjustments, if necessary, are construed to be applied to their respective means in the same sense that the resultant estimates of bioavailability are measures of mean performances.

Experimentally, the direct application of Eqs. 16 and 18 calls for estimates of mean plasma concentration over the last dosage interval for each test preparation and an estimate of ω. Conceptually, what is being attempted is simply a comparison among $\bar{C}p^{(ss)}$ values, each projected from an observed mean plasma concentration at quasi- or nonsteady state, $\bar{C}p^{(i)}$. In other words, bioavailability estimation by the proposed method is analogous to comparing $\bar{C}p^{(ss)}$. For example, $[\bar{C}p^{(ss)}]_x$ for the first treatment is project-

ed from $\bar{C}p^{(l)}$ by Eq. 10, $[\bar{C}p^{(ss)}]_y$ for the second treatment is calculated from $\bar{C}p^{(m+l)}$ by first correcting for the residual contribution due to $\bar{C}p^{(l)}$, and so on. Therefore, the validity of the method is dependent on the accuracy to which $\bar{C}p^{(ss)}$ can be estimated from $\bar{C}p^{(i)}$.

All of the dosage regimen constraints embodied in the derivation of Eqs. 16 and 18 are nonsubstantive, provided that there is an accurate accounting of the dosing sequence. That is to say, the dose and the dosage intervals for the various treatments need not be uniform throughout. Once having estimated F/V_0 through $\bar{C}p^{(ss)}$ for some treatment, it can be compared with that projected from $\bar{C}p^{(i)}$ for all other treatments regardless of how they are given. The sampling periods need not be confined to the last dose of each treatment nor to a single estimate of $\bar{C}p^{(i)}$ per treatment. Dosages may be skipped to permit more extensive sampling during the log-linear phase.

Unlike the extent of drug accumulation at steady state, the predicted time course of accumulation is more or less model dependent. The rate of accumulation approaches model independence when: *(a)* ω is much smaller than E_j, *(b)* ω is much smaller than the next larger eigenvalue, *(c)* succeeding doses are administered during the log-linear phase, and *(d)* the terminal slope is not k_a. From the prior knowledge of drug disposition and from the nature of the dosage forms, it should be possible to discern whether any of these cited conditions is likely to be satisfied. The first is most ambiguous in that E_j's are model-dependent parameters which can assume a wide range of values, albeit always greater than ω, depending on how the model is depicted. However, the degree of uncertainty is no worse than that incurred in any other kinetic method of bioavailability assessment where the model and the disposition parameters are assumed to be constant between treatments in a given subject.

The second and third conditions can be easily verified and accommodated in the experimental design. If absorption is, or is suspected to be, prolonged, then the log-linear phase would be manifestly delayed. In the event that the terminal slope should represent k_a rather than the slowest disposition constant, precautions should be taken to allow for its change among treatments. Therefore, the application of simplified Eqs. 13, 16, and 18 demands that absorption is not continuing when the next dose is administered.

The importance of these constraints diminishes rapidly as steady state is approached. Accordingly, any given treatment suspected to be aberrant may be studied at times as close to steady state as is required for the specified level of accuracy.

Various checks may be included in the experimental design. The constancy of the terminal slope can be verified by judicious sampling during the log-linear phase throughout the study. Alternatively, urine samples may be included so that constancy in plasma clearance may be inferred through estimates of renal clearance (5). Observed changes in ω or $\bar{V}_{cl,r}$ can be incorporated into the analysis by appropriate modifications in Eqs. 14–18. The combined use of plasma and urinary data also permits the identification of data points free from the influence of absorption (4). Finally, most of the simplifying assumptions can be circumvented by pharmacokinetic modeling. This approach would entail the inclusion of an intravenous dosage and the application of Eq. 8 *et sequelae*.

In summary, an experimental strategy was devised for bioavailability estimation at quasi- or nonsteady state. The proposed method may be applied to most situations where steady-state comparisons are favored. The necessary requirements are that drug disposition obeys linear kinetics and that successive doses are administered when absorption is no longer operative.

The method seeks to optimize the obvious advantages and disadvantages of steady-state comparisons. The positive aspects of high drug concentration, limited sampling, and model independence (with minimal constraints) are retained with a concomitant reduction in study duration Experimentally, the nominal requirements consist of estimates of a mean plasma concentration over a specified dosage interval for each treatment and an estimate of the terminal half-life. Additional safeguards may be incorporated into the experimental design for verification.

APPENDIX

The plasma concentration in the central compartment was derived by Benet (6) for any linear N-compartment mammillary model with first-order absorption:

$$Cp = \frac{k_a F D}{V_1}\left[\frac{[\Pi_j(E_j - k_a)]e^{-k_a t}}{(\alpha - k_a)(\beta - k_a)\cdots(\chi - k_a)(\psi - k_a)} + \frac{[\Pi_j(E_j - \alpha)]e^{-\alpha t}}{(k_a - \alpha)(\beta - \alpha)\cdots(\psi - \alpha)} + \cdots + \frac{[\Pi_j(E_j - \psi)]e^{-\psi t}}{(k_a - \psi)(\alpha - \psi)\cdots(\chi - \psi)}\right] \quad \text{(Eq. A1)}$$

where:

- k_a = first-order absorption rate constant
- F = fraction of dose D absorbed
- V_1 = volume of distribution of the sampleable central compartment (compartment 1)
- Π_j = continuous product of terms with index j ranging from 2 to N; the term is defined as 1 when $N = 1$
- N = number of compartments
- j = running index of compartments having exit rate constant into compartment 1
- E_j = sum of exit rate constants from compartment j
- $\alpha, \beta, \ldots, \chi, \psi$ = hybrid rate constants; they are the roots of Laplace parameters, obtained by method of partial fractions such that $\alpha > \beta > \ldots > \chi > \psi$

As examples of Eq. A1, consider the one-compartment open model where $N = 1$ and $\alpha = k_e$:

$$Cp = \frac{k_a F D}{V_1}\left[\frac{e^{-k_a t}}{(k_e - k_a)} + \frac{e^{-k_e t}}{(k_a - k_e)}\right] \quad \text{(Eq. A2)}$$

For a three-compartment model where the three roots are α, β, and γ:

$$Cp = \frac{k_a F D}{V_1}\left[\frac{(E_2 - k_a)(E_3 - k_a)}{(\alpha - k_a)(\beta - k_a)(\gamma - k_a)}e^{-k_a t} + \frac{(E_2 - \alpha)(E_3 - \alpha)}{(k_a - \alpha)(\beta - \alpha)(\gamma - \alpha)}e^{-\alpha t} + \frac{(E_2 - \beta)(E_3 - \beta)}{(k_a - \beta)(\alpha - \beta)(\gamma - \beta)}e^{-\beta t} + \frac{(E_2 - \gamma)(E_3 - \gamma)}{(k_a - \gamma)(\alpha - \gamma)(\beta - \gamma)}e^{-\gamma t}\right] \quad \text{(Eq. A3)}$$

If the drug is repetitively given for i doses with dosage interval τ, the plasma levels during the ith interval become:

$$Cp^{(i)} = \frac{k_a F D}{V_1}\left[\frac{(1 - e^{-ik_a\tau})[\Pi_j(E_j - k_a)]}{(\alpha - k_a)(\beta - k_a)\ldots(\psi - k_a)(1 - e^{-k_a\tau})}e^{-k_a t'} + \frac{(1 - e^{-i\alpha\tau})[\Pi_j(E_j - \alpha)]}{(k_a - \alpha)(\beta - \alpha)\ldots(\psi - \alpha)(1 - e^{-\alpha\tau})}e^{-\alpha t'} + \cdots + \frac{(1 - e^{-i\psi\tau})[\Pi_j(E_j - \psi)]}{(k_a - \psi)(\alpha - \psi)\ldots(\chi - \psi)(1 - e^{-\psi\tau})}e^{-\psi t'}\right] \quad \text{(Eq. A4)}$$

where t' = time from the last dose. The average plasma level during that period is defined as:

$$\bar{C}p^{(i)} = \frac{1}{\tau}\int_0^\tau Cp^{(i)}\,dt' = \frac{k_a F D}{\tau V_1}[Q - P] \quad \text{(Eq. A5)}$$

where:

$$Q = \frac{[\Pi_j(E_j - k_a)]}{k_a(\alpha - k_a)\ldots(\psi - k_a)} + \frac{[\Pi_j(E_j - \alpha)]}{\alpha(k_a - \alpha)\ldots(\psi - \alpha)} + \cdots + \frac{[\Pi_j(E_j - \psi)]}{\psi(k_a - \psi)\ldots(\chi - \psi)} \quad \text{(Eq. A6)}$$

and:

$$P = \frac{[\Pi_j(E_j - k_a)]e^{-ik_a\tau}}{k_a(\alpha - k_a)\cdots(\psi - k_a)} + \frac{[\Pi_j(E_j - \alpha)]e^{-i\alpha\tau}}{\alpha(k_a - \alpha)\cdots(\psi - \alpha)} + \cdots + \frac{[\Pi_j(E_j - \psi)]e^{-i\psi\tau}}{\psi(k_a - \psi)\cdots(\chi - \psi)} \quad \text{(Eq. A7)}$$

At steady state where $i\tau \to \infty$ and $P \to 0$:

$$\bar{C}p^{(ss)} = \frac{k_a FD}{\tau V_1} Q \quad \text{(Eq. A8)}$$

By induction, the quantity Q can be proved to be identical to:

$$Q = \frac{\Pi_j(E_j)}{k_a\alpha\beta\cdots\chi\psi} \quad \text{(Eq. A9)}$$

An operational constant, V_0, is defined such that:

$$V_0 = \frac{\alpha\beta\cdots\chi\psi}{\Pi_j(E_j)}\cdot(V_1/\omega) \quad \text{(Eq. A10)}$$

where ω = terminal slope (slowest disposition constant ψ or k_a, whichever is smaller). Substituting Eqs. A9 and A10 into Eq. A8:

$$\bar{C}p^{(ss)} = \frac{FD}{\tau V_0\omega} \quad \text{(Eq. A11)}$$

Equation A10 is the general expression for the constant V_0, which has a dimension of volume. For example, in a two-compartment open model in which elimination takes place solely from the central compartment, *i.e.*, when $E_2 = k_{21}$:

$$V_0 = V_{d,\text{area}} = \frac{\alpha\beta}{k_{21}}\left(\frac{V_1}{\beta}\right) = \frac{\alpha}{k_{21}}V_1 = \frac{k_{10}}{\beta}V_1 \quad \text{(Eq. A12)}$$

Equation A11 shows that the average plasma levels at steady state are inversely proportional to the dosage interval τ and to the terminal slope ω.

If τ is chosen so that successive doses are given in the log-linear region of the plasma curve with the slope ω (*i.e.*, k_a or ψ, whichever is smaller), the right-hand side of Eq. A7 may be reduced to one single exponential term:

$$P \simeq \frac{[\Pi_j(E_j - \omega)]}{\omega(k_a - \omega)\cdots(\chi - \omega)} e^{-i\omega\tau} \quad \text{when } \omega = \psi \quad \text{(Eq. A13}a\text{)}$$

or:

$$P \simeq \frac{[\Pi_j(E_j - \omega)]}{\omega(\alpha - \omega)\cdots(\psi - \omega)} e^{-i\omega\tau} \quad \text{when } \omega = k_a \quad \text{(Eq. A13}b\text{)}$$

If, furthermore, ω is much smaller than the next hybrid rate constant and is also smaller than any composite exit rate constant, Eqs. A13*a* and A13*b* may be further reduced to their counterparts:

$$P \simeq \frac{\Pi_j(E_j)}{\omega k_a\alpha\cdots\chi} e^{-i\omega\tau} \quad \text{(Eq. A14}a\text{)}$$

or:

$$P \simeq \frac{\Pi_j(E_j)}{\omega\alpha\beta\cdots\psi} e^{-i\omega\tau} \quad \text{(Eq. A14}b\text{)}$$

By substituting Eqs. A9, A10, and A14 into Eq. A5:

$$\bar{C}p^{(i)} \simeq \frac{FD}{\tau V_0\omega}(1 - e^{-i\omega\tau}) \quad \text{(Eq. A15)}$$

Comparison of Eqs. A11 and A15 shows that:

$$\frac{\bar{C}p^{(i)}}{\bar{C}p^{(ss)}} = (1 - e^{-i\omega\tau}) \quad \text{(Eq. A16)}$$

which is identical to Eq. 10 in the text.

Equation 15 of the text was derived as follows. When l doses of formulation x is given with dosage interval τ and followed immediately by additional m doses of formulation y, the average plasma level during the $(m + l)$th period is given by:

$$\bar{C}p^{(m+l)} = \bar{C}p^x + \bar{C}p^y \quad \text{(Eq. A17)}$$

where $\bar{C}p^x$ is the contribution from formulation x and $\bar{C}p^y$ is that from formulation y. The expression for $\bar{C}p^x$ is:

$$\bar{C}p^x = \frac{1}{\tau}\int_{m\tau}^{(m+1)\tau} Cp^{(l)}\,dt' = \frac{k_a{}^x F_x D}{\tau V_1}\left[\frac{(1 - e^{-lk_a{}^x\tau})\,[\Pi_j(E_j - k_a{}^x)]}{k_a{}^x(\alpha - k_a{}^x)\cdots(x - k_a{}^x)(\psi - k_a{}^x)} e^{-mk_a{}^x\tau} + \cdots + \frac{(1 - e^{-l\psi\tau})\,[\Pi_j(E_j - \psi)]}{\psi(k_a{}^x - \psi)\cdots(\chi - \psi)} e^{-m\psi\tau}\right] \quad \text{(Eq. A18)}$$

Again, if the conditions to Eqs. A14*a* and A14*b* are satisfied, then:

$$\bar{C}p^x = \frac{k_a{}^x F_x D}{\tau V_1} Q_x(1 - e^{-l\omega\tau})e^{-m\omega\tau} = \frac{F_x D}{\tau V_0\omega}(1 - e^{-l\omega\tau})e^{-m\omega\tau} \quad \text{(Eq. A19)}$$

$$\bar{C}p^y = \frac{k_a{}^y F_y D}{\tau V_1} Q_y(1 - e^{-m\omega\tau}) = \frac{F_y D}{\tau V_0\omega}(1 - e^{-m\omega\tau}) \quad \text{(Eq. A20)}$$

Therefore:

$$\bar{C}p^{(m+l)} = \frac{D}{\tau V_0\omega}[F_x e^{-m\omega\tau}(1 - e^{-l\omega\tau}) + F_y(1 - e^{-m\omega\tau})] \quad \text{(Eq. A21)}$$

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Solution–Solubility Dependency of Controlled Release of Drug from Polymer Matrix: Mathematical Analysis

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Abstract □ Two types of drug release mechanisms—matrix controlled ($Q - t^{1/2}$ relationship) and partition controlled ($Q - t$ relationship)—were reported previously. The dependency of the transition between these two processes on the solution solubility of drug is now analyzed. Mathematical expressions are derived to treat the systemic effects of the addition of varying volume fractions of the cosolvent system on the concentration gradients across both the drug depletion zone and hydrodynamic diffusion layer as well as on the partitioning behavior at the polymer–solution interface. The results are in excellent agreement with reported experimental observations.

Keyphrases □ Drug release from polymer matrix, controlled—solution–solubility dependency, equations □ Polymer matrix—controlled drug release, solution–solubility dependency, equations

A rapid *in vitro* drug elution system, which permits direct characterization of drug release mechanisms and rates, was recently reported (1). Two types of release mechanisms—matrix controlled and partition controlled—were observed when the release profiles of ethynodiol diacetate from silicone vaginal devices were followed daily in this system (2). In the region of the partition-controlled process, steady-state drug release profiles were defined by a linear $Q - t$ relationship and found to be linearly dependent on the partition coefficient of drug (K) from polymer toward solution.

In the region of the matrix-controlled process, steady-state drug release profiles were defined by a $Q - t^{1/2}$ linearity and found to be independent of the variation in the magnitude of K. The transition between the matrix-controlled and the partition-controlled processes was observed to be dependent on the solution solubility of drug in the elution medium. This article provides a mathematical analysis of the solution–solubility dependency of the controlled release of drug from a drug-dispersed polymer matrix.

MATHEMATICAL DERIVATION AND EXPERIMENTAL OBSERVATION

For such a system, one can draw a concentration profile, which may exist after a finite time following drug elution, on a unit section of a polymeric device (Fig. 1). In this model, the drug is homogeneously dispersed as solid crystals in the polymer matrix. It is visualized that the solid drug on the surface layer of the device is first eluted; when this layer becomes exhausted, the next layer is then depleted. A depletion zone, with a thickness of δ_m, therefore occurs. The interface of the drug dispersion zone–drug depletion zone moves further into the core of the device for another differential thickness, $d(\delta_m)$, when more solid drug is released.

Microscopically, a thin layer of solution, the hydrodynamic diffusion layer, adheres to the immediate surface of the device. The thickness (δ_D) of this diffusion layer may be controlled at a finite value (3) by a constant angular rotation. In the present study, this diffusion layer thickness ($\leq 70 \times 10^{-4}$ cm) is much smaller than the surface area of the device (33.75 cm^2) available for the diffusion of drug molecules. Therefore, the diffusion of drug molecules to and from the polymeric device across the hydrodynamic diffusion layer may be treated simply as one-dimensional diffusion to a plane surface (4). The flux of diffusional, J_D, across the plane surface of a unit area is defined by Fick's first law of diffusion (5):

$$J_D = -D\frac{dC}{d\chi} \qquad \text{(Eq. 1)}$$

where $dC/d\chi$ is the concentration gradient across the diffusion path of $d\chi$, and D is the diffusivity of a drug species. With the assumptions that: (*a*) diffusion is the primary mode of drug release, (*b*) the diffusion coefficient is constant irrespective of the distance traveled and the difference in concentration, and (*c*) a pseudosteady state exists, Fick's arguments may be extended to describe the mechanisms of drug release from a polymeric device.

Under diffusion laws, the rate of drug diffusion from a point at the drug dispersion zone–depletion zone interface to the polymer–solution interface may be expressed as:

$$\frac{dQ_m}{dt} = \frac{D_m}{\delta_m}(C_p - C_m) \qquad \text{(Eq. 2)}$$

where D_m is the effective diffusivity of drug in the polymer matrix, C_p is the drug concentration at the interface of the drug dispersion zone–depletion zone (that is, the polymer solubility of the drug), and C_m is the drug concentration at the polymer–solution interface.

The rate of drug diffusion across the hydrodynamic diffusion layer (with a thickness of δ_D) at the immediate surface of the device may be described by:

$$\frac{dQ_d}{dt} = \frac{D_s}{\delta_D}[C_d - C_b(t)] \qquad \text{(Eq. 3)}$$

where D_s is the diffusivity of the drug in the elution solution, C_d is the drug concentration at the solution–polymer interface, and $C_b(t)$ is the drug concentration in the bulk of the elution medium at a given time.

When a steady state is established, the rate of drug diffusion across the depletion zone, dQ_m/dt, should be equilibrated with the rate of drug diffusion across the hydrodynamic diffusion layer, dQ_d/dt. That is:

$$\frac{D_m}{\delta_m}(C_p - C_m) = \frac{D_s}{\delta_D}[C_d - C_b(t)] \qquad \text{(Eq. 4)}$$

Rearrangement of Eq. 4 gives:

$$\frac{C_p}{C_m} = 1 + \frac{D_s\delta_m}{D_m\delta_D}\left[\frac{C_d}{C_m} - \frac{C_b(t)}{C_m}\right] \qquad \text{(Eq. 5)}$$

According to Eq. 5, if $C_b(t)/C_m$ is equal to C_d/C_m in their magnitudes, the ratio of C_p over C_m is unity. Under such a circumstance, there is no concentration gradient across the depletion zone and no release of drug is detected. In other words, if the magnitude of $C_b(t)/C_m$ is approaching the value of C_d/C_m, the difference between C_p and C_m is becoming smaller and the rate of drug release (dQ_m/dt in Eq. 2) is reduced (since $dQ_d/dt \rightarrow 0$ in Eq. 3). This

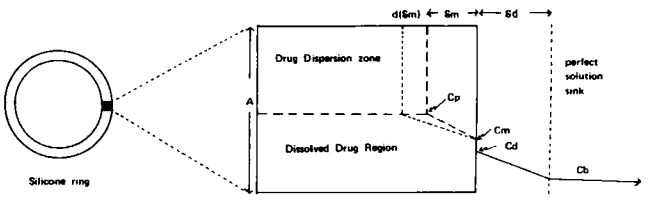

Figure 1—*Theoretical concentration profile existing in a drug-dispersed polymeric device in contact with a perfect solution sink. A is the initial amount of drug impregnated in a unit volume of device. See text for definitions of* C_p, C_m, C_d, $C_b(t)$, δ_m, and δ_D.

Table I—Effect of Polyethylene Glycol 400 on the Solution Solubility (C_s) and Bulk Concentration [$C_b(t)$] of Ethynodiol Diacetate

Volume Fraction of Polyethylene Glycol 400[a], % v/v	C_s, μg/ml	$C_b(t)$[b], μg/ml
0	13.7	—
20	37	6.77
30	64.6	10.66
40	99.7	14.56
50	156	24.18
55	196	36.65
60	437	47.63
62.5	647.5	75.11
65	780	92.0
70	1390	152.9
75	2430	316.7
80	4450	395.2
85	8000	425.8

[a] In distilled water. [b] For maintaining a sink condition, the bulk drug concentration (C_b) in drug elution cells was monitored and the elution solution was renewed daily (Ref. 1). Each value represents the average of the $C_b(t)$ values measured during a 10-day experiment (Ref. 2).

suggests that the magnitude of the $C_b(t)/C_m$ term (Eq. 5) has to be maintained at a level much lower than the value of the C_d/C_m term.

This sink condition may be satisfied either by setting $C_b(t)$ very close to zero or by making C_d much greater than $C_b(t)$. The former approach has been applied in many conventional dissolution and elution studies and is best represented by the use of a large volume (60 liters/day) of distilled water as the elution medium for release studies of medroxyprogesterone acetate (6) (aqueous solubility of 3.25 μg/ml). The other approach was demonstrated by the application of a small volume (150 ml) of cosolvent–water combinations to enhance remarkably the aqueous solubility (13.7 μg/ml) of ethynodiol diacetate by from three- to 584-fold (1, 2), depending on the volume fraction (20–85% v/v) of cosolvent added.

Apparently, the magnitude of $C_b(t)$ in the latter case is a finite value and cannot be approximated to zero as in the former approach. This fact is illustrated in Table I, where $C_b(t)$ has various finite values in various elution media, although they are much smaller than their corresponding C_s (solution solubility) values.

The magnitude of $C_b(t)$ is enhanced by the addition of polyethylene glycol 400 in a manner that seems proportional to the increase in the magnitude of the C_s term. The correlation of $C_b(t)$ with C_s becomes quite apparent when one plots $C_b(t)$ values against their corresponding C_s values (Fig. 2). The use of a log–log scale in Fig. 2 allows the complete coverage of a wide range of both

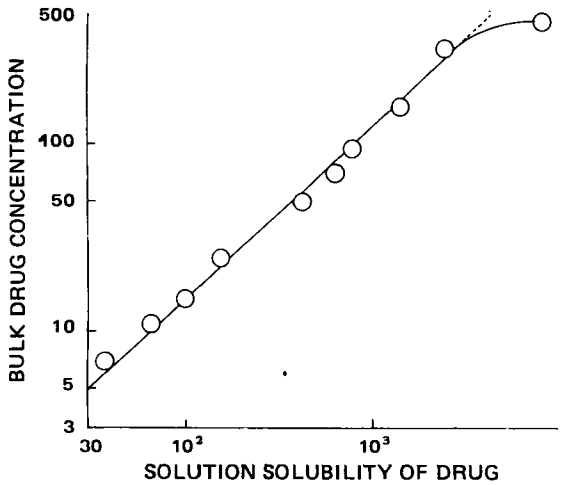

Figure 2—*Logarithmic relationship between the bulk concentration (C_b) of drug in the sink elution medium and the solution solubility of drug (C_s). Slope = 0.933.*

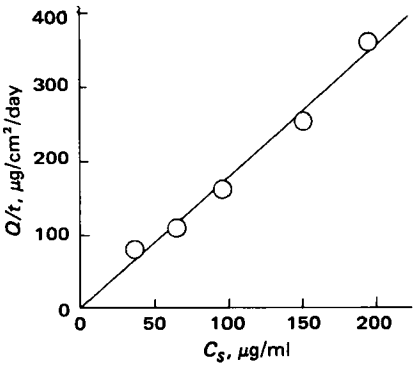

Figure 3—*Linear relationship of the drug release rate (Q/t) with the solution solubility (C_s) of drug following Eq. 24. Slope (kD_s/δ_D) = 1.77 cm/day.*

$C_b(t)$ and C_s values. As expected, a linear relationship with a slope (0.93) close to unity was obtained (Eq. 6a):

$$C_b(t) = \alpha C_s^{(1)} \qquad \text{(Eq. 6a)}$$

Since:

$$C_m = \beta C_p \qquad (\beta \leq 1) \qquad \text{(Eq. 6b)}$$

then:

$$\frac{C_b(t)}{C_m} = \frac{\alpha C_s}{\beta C_p} = k'K \qquad \text{(Eq. 7)}$$

where the partition coefficient, K, for the partitioning of drug molecules from the polymer surface toward the elution solution (6) is expressed as:

$$\frac{C_s}{C_p} = K = \frac{C_d}{C_m} \qquad \text{(Eq. 8)}$$

and:

$$k' = \frac{\alpha}{\beta} \qquad \text{(Eq. 9)}$$

Substituting Eqs. 7 and 8 into Eq. 5 results in:

$$\frac{C_p}{C_m} = 1 + \frac{D_s\delta_m}{D_m\delta_D}[1 - k']K \qquad \text{(Eq. 10a)}$$

or:

$$\frac{C_p}{C_m} = 1 + \frac{D_s\delta_m kK}{D_m\delta_D} \qquad \text{(Eq. 10b)}$$

where:

$$k = 1 - k' \qquad \text{(Eq. 11)}$$

Rearrangement of Eq. 10b gives:

$$C_m = \frac{D_m\delta_D C_p}{D_m\delta_D + D_s\delta_m kK} \qquad \text{(Eq. 12)}$$

If the dissolution of the drug particles into the surrounding polymer matrix in the drug dispersion zone (Fig. 1) is the first step in drug release dynamics (prior to the diffusion of solvated drug molecules across the depletion zone), then the cumulative amounts (Q and Q') of drug released from a unit surface area of device with a thickness of δ_m or ($\delta_m + d\delta_m$) are expressed, respectively, by:

$$Q = \left(A - \frac{C_p + C_m}{2}\right)\delta_m \qquad \text{(Eq. 13a)}$$

$$Q' = \left(A - \frac{C_p + C_m}{2}\right)(\delta_m + d\delta_m) \qquad \text{(Eq. 13b)}$$

The differential cumulative amount (dQ) of drug released due to the formation of a differential zone ($d\delta_m$) of depletion can be calculated from the difference between Q' and Q as follows:

$$dQ = Q' - Q \qquad \text{(Eq. 14a)}$$

$$dQ = \left(A - \frac{C_p + C_m}{2}\right)d\delta_m \qquad \text{(Eq. 14b)}$$

The rate of expansion, dQ/dt, of this differential zone of depletion is then described by:

Table II—Calculation of k

Volume Fraction of Polyethylene Glycol 400, % v/v	D_S/δ_D, cm/day	k[a]
20	56.66	0.031
30	36.82	0.048
40	23.87	0.074
50	15.39	0.114
55	14.30	0.123
60	9.92	0.177
62.5	8.77	0.201
65	8.03	0.219
70	6.44	0.273
75	5.27	0.334
80	4.12	0.427
85	3.38	0.521

[a] Calculated from the relation of $k = 1.77/(D_S/\delta_D)$ (Eq. 24).

$$\frac{dQ}{dt} = \left(A - \frac{C_p + C_m}{2}\right)\frac{d\delta_m}{dt} \qquad \text{(Eq. 14c)}$$

where A is the initial amount of solid drug impregnated homogeneously in a unit volume of device.

The theoretical model shown in Fig. 1 indicates that the rate of expansion (dQ/dt) of the depletion zone will have a finite value only if the surface concentration of drug, C_m, on the polymeric device is maintained at a constantly smaller level than C_p (the polymer solubility of drug) throughout an observation.

Substituting Eq. 12 for the C_m term in Eqs. 2 and 14c and then equating them at a steady state result in:

$$\frac{D_m}{\delta_m}\left[C_p - \frac{D_m\delta_D C_p}{D_m\delta_D + D_s\delta_m kK}\right] = \left[A - \frac{C_p}{2} - \frac{D_m\delta_D C_p}{2(D_m\delta_D + D_s\delta_m kK)}\right]\frac{d\delta_m}{dt} \qquad \text{(Eq. 15a)}$$

Rearrangement of Eq. 15a and integration yield:

$$C_m D_s D_m kK \int_0^t dt = (A - C_p)D_m\delta_D \int_0^{\delta_m} d\delta_m + \left(A - \frac{C_p}{2}\right)D_s kK \int_0^{\delta_m} \delta_m d\delta_m \qquad \text{(Eq. 15b)}$$

and then:

$$\delta_m{}^2 + \frac{2(A - C_p)D_m\delta_D\delta_m}{(A - C_p/2)D_s kK} = \frac{2C_p D_m}{(A - C_p/2)}t \qquad \text{(Eq. 16)}$$

Experimentally, the system was designed so that the initial amount of solid drug (A) dispersed homogeneously in a unit volume of polymer matrix was much larger than the polymer solubility (C_p) of the drug ($A \geq 98.8$ g/10^3 cm^3 and $C_p = 1.4791$ g/10^3 ml). Therefore, $2A \gg (C_p + C_m)$ may be established (since $C_m = \beta C_p$ and $\beta \leq 1$) and Eq. 13a may be approximated:

$$Q \simeq \left(A - \frac{C_p}{2}\right)\delta_m \qquad \text{(Eq. 13c)}$$

or:

$$Q \simeq A\delta_m \qquad \text{(Eq. 13d)}$$

without any significant error involved as long as the condition $2A \gg (C_p + C_m)$ is satisfied.

The value of δ_m is a time-dependent variable and may be estimated by measuring the size of the formed depletion zone by microscopy after sectioning a device (6). The δ_m term in Eqs. 13a, 13c, and 13d also may be calculated from the time-independent parameters, *e.g.*, A, C_p, D_s, D_m, δ_D, and K. These release rate-limiting physicochemical parameters can be measured easily from a diffusion experiment.

Table III—Comparison of k[a] with K[b] for Partition-Controlled Processes

Volume Fraction of Polyethylene glycol 400, % v/v	k	K	k/K
20	0.031	0.036	0.86
30	0.048	0.044	1.10
40	0.074	0.061	1.21
50	0.114	0.099	1.15
55	0.123	0.133	0.93
		Mean ($\pm SD$)	1.05 (0.15)

[a] Defined in Eq. 11. [b] Defined in Eq. 8.

Table IV—Calculated *versus* Observed Rates of Drug Release (Q/t)

Volume Fraction of Polyethylene Glycol 400, % v/v	Q/t, μg/cm²/day	
	Calculated[a]	Observed
20	65.5	82.6
30	114.2	109.8
40	176.1	141.3
50	273.7	245.7
55	344.7	360.0

[a] Calculated from Eq. 24. The C_S data are from Table I, and the D_S/δ_D and k data are from Table II.

In these studies, the magnitude of K was a variable depending on the volume fraction of cosolvent in the elution medium. The following two cases were observed as the value of K was varied to make the magnitude of the $\delta_m{}^2$ term (Eq. 16) either much greater or much smaller than the second term in Eq. 16.

Case 1—For a matrix-controlled process, K is very large; therefore:

$$\delta_m{}^2 \gg \frac{2(A - C_p)D_m\delta_D}{(A - C_p/2)D_s kK}\delta_m \qquad \text{(Eq. 17)}$$

and Eq. 16 is reduced to a $t^{1/2}$-dependent δ_m:

$$\delta_m = \sqrt{4C_p D_m t/(2A - C_p)} \qquad \text{(Eq. 18)}$$

Substituting Eq. 18 for the δ_m term in Eq. 13c results in:

$$Q = \sqrt{D_m(2A - C_p)C_p t} \qquad \text{(Eq. 19)}$$

which is the equation developed earlier by Higuchi (7). Equation 19 is slightly different from Eq. 3 of Ref. 1. In the earlier derivation, the contribution due to the penetration of elution solution in the microscopic channels (formed after the depletion of drug particles) was considered. In the present derivation of Eq. 19, this contribution was considered to be very small in comparison with the contribution from the diffusion across the polymer structure. The difference in the magnitudes of the resultant $Q/t^{1/2}$ values was

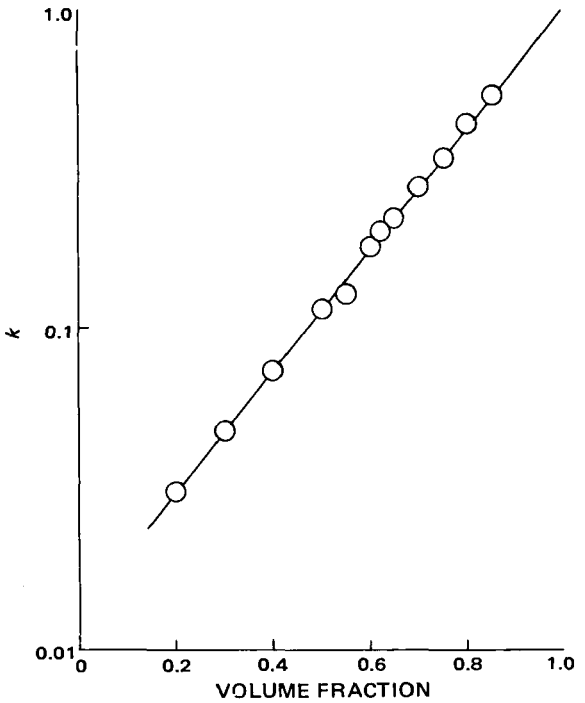

Figure 4—*Semilogarithmic relationship between* k *and the volume fraction of polyethylene glycol 400. Slope* (m) = *1.87.*

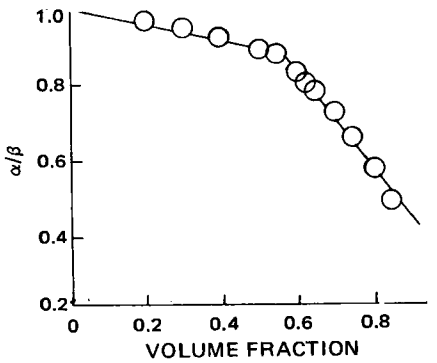

Figure 5—*Biphasic relationship of α/β (relative concentration gradient across diffusion layer over that across depletion zone) with volume fraction of polyethylene glycol 400. The slopes were 0.22 [volume fraction ≤55% (v/v)] and 1.30 [volume fraction ≥60% (v/v)], respectively.*

negligibly small between Eq. 3 ($Q/t^{1/2}$ = 3.03–3.09 mg/cm^2/day$^{1/2}$) and Eq. 19 ($Q/t^{1/2}$ = 3.08 mg/cm^2/day$^{1/2}$).

Case 2—For a partition-controlled process, K is very small; therefore:

$$\delta_m^2 \ll \frac{2(A - C_p)D_m\delta_D}{(A - C_p/2)D_sK}\,\delta_m \qquad \text{(Eq. 20)}$$

and Eq. 16 is reduced to:

$$\delta_m = \frac{kKD_sC_p}{(A - C_p)\delta_D}\,t \qquad \text{(Eq. 21}a\text{)}$$

or:

$$\delta_m \simeq \frac{kKD_sC_p}{A\delta_D}\,t \qquad \text{(Eq. 21}b\text{)}$$

Substituting Eq. 21b for the δ_m term in Eq. 13d gives the following relationship:

$$Q = \frac{kKD_sC_p}{\delta_D}\,t \qquad \text{(Eq. 22)}$$

From Eq. 7, $KC_p = C_s$. Therefore, Eq. 22 is expressed alternatively as:

$$Q = \frac{kD_sC_s}{\delta_D}\,t \qquad \text{(Eq. 23)}$$

The experimental observations on the linear $Q - t$ relationship (Eq. 23) were presented previously (2).

The rate of drug release (Q/t) for a partition-controlled process may be calculated from the slope of the linear Q *versus* t plots and is defined as:

$$\frac{Q}{t} = \frac{kD_s}{\delta_D}C_s \qquad \text{(Eq. 24)}$$

According to Eq. 24, the rate of drug release (Q/t) should be linearly proportional to the magnitude of drug solubility (C_s) in the elution medium used. This linearity is demonstrated in Fig. 3. The slope (kD_s/δ_D) of this linear plot was estimated to be 1.77 cm/day.

Following the addition of polyethylene glycol 400 as a cosolvent, the viscosity of the elution medium was increased. This increase in solution viscosity resulted in an increase in the thickness of the diffusion layer (δ_D) and a decrease in the magnitude of the solution diffusivity (D_s). Therefore, k should be a variable while the overall kD_s/δ_D term is constant (1.77 cm/day) experimentally.

The magnitude of k at various combinations of D_s/δ_D values may be easily calculated (Table II). As expected, the magnitude of k increases as the ratio of D_s over δ_D decreases. Furthermore, a proportionality appears to exist between the values of k and the volume fraction of cosolvent used in the elution medium. This proportionality is plotted semilogarithmically in Fig. 4. Attention is called to the result that k = 1 was obtained at 100% (v/v) of polyethylene glycol 400 (volume fraction = 1). The linear relationship of k with the volume fraction of polyethylene glycol 400 may be expressed as:

$$\log k = m\,(\text{volume fraction of polyethylene glycol 400}) + \text{constant} \qquad \text{(Eq. 25)}$$

with a slope (m = 1.87) very close to 2.

Table V—Calculation of α, β, and C_m

Volume Fraction of Polyethylene Glycol 400, % v/v	α[a]	β[b]	C_m[c], μg/ml	C_s, μg/ml
20	0.183	0.189	279.3	37.0
30	0.165	0.173	256.4	64.6
40	0.146	0.158	233.2	99.7
50	0.155	0.173	258.8	156
55	0.187	0.213	315.4	196
60	0.109	0.132	195.9	437
62.5	0.116	0.145	214.7	647.5
65	0.118	0.151	223.5	780
70	0.110	0.151	223.8	1390
75	0.130	0.195	288.7	2430
80	0.089	0.155	229.7	4450
85	0.053	0.111	163.7	8000
$\bar{x}$ (±SD)(n = 12)		0.162 (0.028)	240.3 (±41.9)	

[a] Calculated from Eq. 6a; $\alpha = C_b(t)/C_s$. [b] Calculated from Eq. 26; $\beta = \alpha/(1 - k)$. [c] Calculated from Eq. 8; $C_m = \beta C_p$; C_p = 1.4791 × 10^3 μg/ml.

The magnitude of k for the partition-controlled process was essentially equal to that of K, the partition coefficient of drug from the polymer surface toward the elution medium (Table III). The coincidence of k with K ($k/K \simeq 1$) may provide an explanation for previous observations on the linear relationship between the rate of drug release (Q/t) and the partition coefficient for the partition-controlled process. The validity of Eq. 24 is evident when one compares the rates of drug release in various elution solutions with those calculated from the values of C_s, D_s, δ_D, and k (Table IV).

The following relationship may be derived from Eqs. 9 and 11:

$$\beta = \frac{\alpha}{1 - k} \qquad \text{(Eq. 26)}$$

From Eq. 26, the proportionality (β) between the drug concentration (C_m) at the polymer–solution interface and that (C_p) at the interface of the drug dispersion–drug depletion zone (Eq. 8) may be calculated (Table V). A mean value of 0.162 ± 0.028 was obtained. As predicted, the magnitude of β is considerably smaller than unity ($\beta \leq 1$ in Eq. 8). This result indicates that an effective concentration gradient ($C_p - C_m$) was maintained across the depletion zone (Eq. 2) for all volume fractions of polyethylene glycol 400 used.

The drug concentration (C_m) at the polymer–solution interface can be calculated from Eq. 8, using an experimentally predetermined C_p value (1.4791 × 10^3 μg/cm^3) (Table V), and the C_m values found are substantially smaller than C_p. Unlike the behavior demonstrated by both C_s and $C_b(t)$, the magnitude of C_m is quite independent of the variation in the volume fraction of cosolvent added (a mean of 240.3 ± 41.9 μg/ml). The value of C_m is greater than the value of C_s (Table V) for the partition-controlled process [volume fraction of polyethylene glycol ≤55% (v/v)] but smaller than the value of C_s in the region of the matrix-controlled process [volume fraction of polyethylene glycol ≥60% (v/v)].

The effect of cosolvent on the concentration gradient (α) across the diffusion layer relative to that (β) across the depletion zone is significant (Fig. 5). The effect of cosolvent is considerably smaller when its volume fraction is equal to or smaller than 55% (v/v) (slope = 0.22). The magnitude of this effect is enhanced sixfold (from 0.22 to 1.30) when the volume fraction of cosolvent used is increased above 60% (v/v). These observations may provide some insights into the mechanism of the transition between matrix-controlled and partition-controlled processes reported previously (2).

From the enclosed analysis, it was concluded that the variation in the magnitude of solution solubility (C_s) dictates the mechanism and rate of drug release from a drug dispersed in a polymer matrix.

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Effects of Selected Drugs on an Auditory or Thalamic Conditioned Stimulus Eliciting Recruitment in the Cat

B. DUBINSKY [x], W. J. KINNARD, Jr. [*], and J. P. BUCKLEY [‡]

Abstract □ Minimally effective oral doses of chlorpromazine, imipramine, and pentobarbital necessary to block a discrete trial (bar-press) conditioned avoidance response were compared in cats chronically implanted with electrodes over the cerebral cortex and in the nucleus centralis medialis of the thalamus. Three conditioned stimulus contingencies consisting of tone and low or high voltage thalamic stimulation were presented. Minimal conditioned response blocking doses of these agents produced only slight qualitative changes in cortically recorded recruitment. Drug treatment affected the conditioned stimulus contingencies differentially, and the rank order in terms of ease of disruption of the conditioned avoidance response was high voltage thalamic conditioned stimulus > low voltage thalamic conditioned stimulus > auditory conditioned stimulus. The differential effect of these drugs might have been due to the additive inhibition of these agents and the thalamic conditioned stimulus on performance. With the exception of chlorpromazine, the behavioral effects of these drugs and their effects on recruitment were dissociated.

Keyphrases □ Chlorpromazine—effects on conditioned avoidance response, conscious cat □ Imipramine—effects on conditioned avoidance response, conscious cat □ Pentobarbital—effects on conditioned avoidance response, conscious cat □ Thalamic recruitment—effects of chlorpromazine, imipramine, and pentobarbital, conditioned avoidance response, conscious cat

Electrical stimulation of midline thalamic nuclei and other subcortical sites evokes potentials which, when recorded cortically, resemble spontaneous spindle bursts. Since these potentials increase in amplitude with time, apparently through the recruitment of additional responsive neurons, the recorded event has come to be known as the recruiting response. Several studies (1–3) defined these properties and examined the sites of origin of the recruiting response. Since these classic studies, many investigators have studied the behavioral effects of electrical stimulation of regions of the brain from which cortical recruitment may be elicited.

Midline thalamic stimulation has been shown to act as a conditioned stimulus for the formation of a conditioned avoidance response in cats (4–6), dogs (7), and squirrel monkeys (8). Changes in the amplitude of recruitment during learning and performance have been variously interpreted as reflecting behavioral facilitatory or inhibitory processes (6). However, Pecci-Saavedra *et al.* (8) attributed these changes to alterations in the level of arousal associated with learning.

The effects of psychotropic drugs which modify learned behavior on the recruiting system have been extensively tested; however, the relationship between the effects of these agents on recruitment and their effects on behavior is unknown since all studies have been done in paralyzed or anesthetized preparations (9–14). The present study was designed to bridge the gap between these studies and, thus, to examine the mutual alterations in behavior induced by stimulation of the recruiting system and the action of psychotropic drugs. The effects of selected drugs on avoidance behavior conditioned by either tone or midline thalamic stimulation were studied to compare directly the behavioral effects of these agents to their effects on recruitment.

EXPERIMENTAL

Adult female cats, 2.5–3.5 kg, were chronically implanted with cortical and subcortical electrodes under aseptic conditions. Stainless steel rivets (3-mm diameter heads) insulated with epoxy varnish (except at their tips) were positioned on the dura through burr holes in the skull, and an uninsulated rivet was cemented in the bone overlying the frontal sinus as a connection to ground. A bipolar concentric electrode, consisting of an outer 23-gauge stainless steel cannula and an inner insulated stainless steel wire [0.01 cm (0.005 in.) diameter], was placed in the region of the nucleus centralis medialis of the thalamus according to reported stereotaxic coordinates (anterior, 9.5 mm; lateral, 0.0 mm; horizontal, 1.0 mm) (15). The electrode was insulated with epoxy varnish, and the tips were bared 0.5 mm and separated by 1.0 mm.

The position of this electrode was considered satisfactory if, during surgery, recruiting potentials could be recorded from the cortical leads upon stimulation of the medial thalamus. The parameters of stimulation[1] are given under *Results*. The electrodes were wired to a connector[2], and the assembly was secured to the skull with dental acrylate.

Experiments were initiated no sooner than 2 weeks following

[1] Grass model S-4 stimulator and SIU-4 isolation unit were used.
[2] Amphenol No. 57-40140.

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[2] Amphenol No. 57-40140.

Table I—Thalamic Conditioned Stimulus (CS) Parameters

Cat	Low Voltage CS, v	High Voltage CS, v	Duration, msec	Frequency, Hz
P-102	4.0	4.5	0.2	10
C-103	4.0	4.5	0.5	8
M-104	5.0	6.4	1.0	8

electrode placement. Cats were trained and tested in a chamber (55.8 × 58.4 × 58.4 cm internal dimensions) which was electrically shielded and grounded. A discrete-trial conditional avoidance–escape schedule with variable intertrial intervals was automatically programmed by conventional timers and relays. One trial consisted of presentation of the conditioned stimulus for 12 sec. If a bar-press response was not made during this time, the conditioned stimulus was terminated and 3 mamp of randomly scrambled foot shock was delivered through a grid floor. After animals had learned to avoid shock by responding during delivery of tone (auditory conditioned stimulus), they were subsequently trained to bar press during presentation of thalamic stimulation which elicited recruitment (thalamic conditioned stimulus).

In drug studies, an experimental session consisted of 10 discrete trials of each of three randomly presented conditioned stimulus contingencies: the auditory conditioned stimulus and two differential voltage levels of the thalamic conditioned stimulus. Selection of these voltages of stimulation is described under *Results*.

The effects of chlorpromazine hydrochloride[3], imipramine hydrochloride[4], and pentobarbital sodium at selected oral doses, administered in gelatin capsules, on these contingencies were studied. The animals were tested 1 hr following drug administration on the day following a control run (lactose); thus, each cat served as its own control for a given experiment. Drugs were given no more frequently than once weekly to avoid dose interaction, and one replicate of each dose was obtained. The cats were observed remotely with the aid of a closed-circuit television system[5], and the effects of these drugs on recruitment and on the spontaneous electrocorticogram (ECoG) were recorded by means of an electroencephalograph[6].

Difference between the conditioned response rate under control and drug conditions was the measure used in an analysis of variance, and differences between means for drug days and preceding control sessions were determined by a paired t-test. Since the subjects were trained to at least a 90% avoidance rate under each contingency, it was expected that drug effects would be unidirectional, and a one-tailed test of statistical significance ($p < 0.05$) was used. Individual animal performance was considered changed if the response rate differed by more than 2 *SD* of the mean response of all previous control days. Mean performance in control sessions under each contingency was compared using the Student t test (two tailed).

RESULTS

The subjects of this experiment learned to avoid shock in 98.5 ± 1.2% of control trials (historical value for 18 sessions) when tone was used. The performance rates in control sessions were 95.6 ± 1.8 and 91.3 ± 2.6% using the low and high voltage thalamic conditioned stimulus, respectively. Compared to tone, the performance rate for either the low ($p < 0.4$) or the high ($p < 0.1$) voltage thalamic conditioned stimulus trials was not significantly less. The parameters of stimulation used in each animal are shown in Table I.

The intensity of the low voltage thalamic conditioned stimulus was selected to be threshold for eliciting recruitment waves that waxed and waned, and the amplitudes of these evoked waves were as high as 500 μv. In preliminary experiments, pairing of the thalamic stimulation with the auditory conditioned stimulus was chosen to be subthreshold for behavioral inhibition. It caused recruiting wave amplitudes as high as 500 μv to be more consistently recorded.

Although no attempt was made to determine the voltage threshold for blockade of performance in each subject, recruitment could be elicited without concurrent conditioned avoidance response disruption by reducing the stimulating voltage to the levels shown in Table I. Thus, stimulation of the thalamus affected performance in a voltage-dependent manner.

The effects of chlorpromazine, imipramine, and pentobarbital on each conditioned stimulus continency are shown in Tables II–IV. Drug effects on duplicate tests at each of three dose levels in three subjects were compared by an analysis of variance. In spite of the variability of the subjects in their sensitivity to the drugs ($F = 6.2$, $df = 2/52$, $p < 0.05$), a highly significant dose–response relationship was obtained ($F = 42.2$, $df = 2/52$, $p < 0.01$). Drug treatment affected the three contingencies differentially ($F = 11.9$, $df = 2/52$, $p < 0.01$), and the rank order in terms of ease of response disruption was high voltage thalamic conditioned stimulus > low voltage thalamic conditioned stimulus > tone.

The lowest dose of chlorpromazine that produced a significant attenuation of performance for the group ($n = 3$) compared to control sessions was 1 mg/kg for either the low ($p < 0.05$) or high ($p < 0.01$) voltage thalamic conditioned stimulus, and it was 2 mg/kg ($p < 0.05$) for the auditory conditioned stimulus (Table II). Ataxia and motor incoordination in these subjects were slight or moderate following doses of 1 and 2 mg/kg, respectively. A dose as low as 1 mg/kg altered the spontaneous ECoG in all animals, but presentation of tone abolished high voltage slow wave activity.

The ECoG representing recruitment evoked by thalamic stimulation in Cat P-102 is shown in Fig. 1. This subject and Cat M-104 terminated the thalamic conditioned stimulus in control trials and in chlorpromazine (0.5 mg/kg) trials following a secondary increase in the negativity of the recruiting response. Chlorpromazine generally increased recruitment amplitude, but the administration of 1 and 2 mg/kg to two animals (P-102 and M-104) prevented the secondary increase in recruitment negativity subsequent to the initial burst in trials in which the avoidance response was disrupted (Fig. 1).

Compared to control sessions, the lowest dose of imipramine studied (2 mg/kg) interfered with the action of the high-voltage thalamic conditioned stimulus ($p < 0.05$); however, performance conditioned to either tone or low voltage thalamic stimulation was not altered significantly in the group ($n = 3$) following 2, 4, or 8 mg/kg (Table III). However, based upon comparison with performance on 18 control days, three out of three subjects in tone trials were affected by the 8-mg/kg dose. Performance was affected in as many as two out of three subjects following 4 mg/kg in low voltage trials. ECoG synchrony was observed following all doses, and presentation of tone usually activated the record. Drug treatment increased the negativity of recruitment elicited by the thalamic conditioned stimuli and increased the tendency for recruitment amplitude to wax and wane during stimulus presentation. In Cat P-102 (Fig. 2), the recruiting response change from control trials was less following 2 mg/kg than following either 4 or 8 mg/kg.

The lowest dose of pentobarbital that produced significant attenuation of performance for the group ($n = 3$) compared to control sessions (Table IV) was 10 mg/kg for tone ($p < 0.01$) and either the low ($p < 0.01$) or high ($p < 0.05$) voltage thalamic conditioned stimulus. Moderate motor incoordination was produced by each dose, and the subjects appeared to doze intermittently following the 10-mg/kg dose. High voltage slow waves and occasional spindle burst activity were recorded in all subjects following 5 mg/kg, and higher doses (7.5 and 10 mg/kg) increased the occurrence of spindle activity that was incompletely blocked by presentation of tone. Recruitment amplitudes were slightly increased; but as shown in Figs. 2 and 3, imipramine and pentobarbital produced essentially similar changes. The general appearance of the recruiting response seemed to be similar following doses of pentobarbital which either did or did not disrupt performance.

The results reported were obtained from subjects in which placement of the electrode within the nucleus centralis medialis of the thalamus was histologically confirmed.

DISCUSSION

Sufficient evidence has been accumulated from several studies (16, 17) to confirm the observation that recruitment occurs in parallel to behavioral inhibition. However, elicitation of the recruiting

[3] Supplied by Smith Kline and French, Philadelphia, Pa.
[4] Supplied by Geigy, Ardsley, N.Y.
[5] Motorola, Inc.
[6] Model D, Medcraft.

Table II—Effects of Chlorpromazine on Three Conditioned Stimulus (CS) Contingencies

Dose, mg/kg po	Cat	Percent Conditioned Avoidance: Auditory CS, Tone Control	Auditory CS, Tone Treatment	n/N[a]	Thalamic CS: Low Voltage Control	Low Voltage Treatment	n/N[a]	High Voltage Control	High Voltage Treatment	n/N[a]
0.5	P-102	95	90		90	85		100	85	
	C-103	100	95		90	80		100	35	
	M-104	100	95		100	75		90	0	
Mean ± *SEM*		98.3 ± 1.7	93.3 ± 1.7	1/3	93.3 ± 3.3	80.0 ± 2.9	3/3	96.7 ± 3.3	40.0 ± 24.7	2/3
1.0	P-102	95	65		100	70		95	35	
	C-103	100	15		95	10		100	5	
	M-104	100	80		100	40		80	5	
Mean ± *SEM*		98.3 ± 1.7	53.3 ± 19.6	3/3	98.3 ± 1.7	40.0 ± 17.3[b]	3/3	91.7 ± 6.0	15.0 ± 10.0[c]	3/3
2.0	P-102	95	25		90	15		95	0	
	C-103	95	5		90	0		85	0	
	M-104	100	15		100	0		75	0	
Mean ± *SEM*		96.7 ± 1.7	15.0 ± 5.8[c]	3/3	93.3 ± 3.3	5.0 ± 5.0[c]	3/3	85.0 ± 5.8	0.0 ± 0.0[c]	3/3

[a] Number of cats showing a significant change on drug day in percent conditioned avoidance/response number of cats in group; based upon comparison with performance on 18 control days. [b] $p < 0.05$ (Student t test, one tailed). [c] $p < 0.01$ (Student t test, one tailed).

Table III—Effects of Imipramine on Three Conditioned Stimulus (CS) Contingencies

Dose, mg/kg po	Cat	Percent Conditioned Avoidance: Auditory CS, Tone Control	Auditory CS, Tone Treatment	n/N[a]	Thalamic CS: Low Voltage Control	Low Voltage Treatment	n/N[a]	High Voltage Control	High Voltage Treatment	n/N[a]
2.0	P-102	90	90		85	90		100	75	
	C-103	100	100		100	50		85	30	
	M-104	100	100		100	100		95	15	
Mean ± *SEM*		96.7 ± 3.3	96.7 ± 3.3	1/3	95.0 ± 5.0	80.0 ± 15.3	1/3	93.3 ± 4.4	40.0 ± 18.0[b]	3/3
4.0	P-102	95	60		85	55		90	10	
	C-103	100	95		95	15		95	0	
	M-104	100	95		95	100		100	5	
Mean ± *SEM*		98.3 ± 1.7'	83.3 ± 11.7	1/3	91.7 ± 3.3	56.7 ± 24.6	2/3	95.0 ± 2.9	5.0 ± 2.9[c]	3/3
8.0	P-102	100	75		100	70		70	0	
	C-103	100	0		100	0		95	0	
	M-104	100	90		95	90		90	0	
Mean ± *SEM*		100.0 ± 0.0	55.0 ± 27.8	3/3	98.3 ± 1.7	53.3 ± 27.3	2/3	85.0 ± 7.6	0.0 ± 0.0[c]	3/3

[a] Number of cats showing a significant change on drug day in percent conditioned avoidance response/number of cats in group; based upon comparison with performance on 18 control days. [b] $p < 0.05$ (Student t test, one tailed). [c] $p < 0.01$ (Student t test, one tailed).

Table IV—Effects of Pentobarbital on Three Conditioned Stimulus (CS) Contingencies

Dose, mg/kg po	Cat	Percent Conditioned Avoidance: Auditory CS, Tone Control	Auditory CS, Tone Treatment	n/N[a]	Thalamic CS: Low Voltage Control	Low Voltage Treatment	n/N[a]	High Voltage Control	High Voltage Treatment	n/N[a]
5.0	P-102	100	75		90	95		75	55	
	C-103	100	100		100	100		100	100	
	M-104	100	100		100	100		95	80	
Mean ± *SEM*		100.0 ± 0.0	91.7 ± 8.3	1/3	96.7 ± 3.3	98.3 ± 1.7	0/3	90.0 ± 7.6	78.3 ± 13.0	2/3
7.5	P-102	100	45		95	35		90	10	
	C-103	100	90		95	25		100	5	
	M-104	100	80		95	95		100	100	
Mean ± *SEM*		100.0 ± 0.0	71.7 ± 13.6	3/3	95.0 ± 0.0	51.7 ± 21.8	2/3	96.7 ± 3.3	38.3 ± 30.9	2/3
10.0	P-102	95	0		95	0		65	0	
	C-103	100	0		100	0		100	0	
	M-104	100	15		100	10		100	25	
Mean ± *SEM*		98.3 ± 1.7	5.0 ± 5.0[b]	3/3	98.3 ± 1.7	3.3 ± 3.3[b]	3/3	88.3 ± 11.7	8.3 ± 8.3[c]	3/3

[a] Number of cats showing a significant change on drug day in percent conditioned avoidance response/number of cats in group; based upon comparison with performance on 18 control days. [b] $p < 0.05$ (Student t test, one tailed). [c] $p < 0.01$ (Student t test, one tailed).

response by thalamic stimulation was not exclusively associated with behavioral inhibition and, as shown in the present investigation and as reported by others (4, 5, 7, 8), it was possible to establish avoidance behavior conditioned to thalamic stimulation eliciting recruitment.

Even though the avoidance rate in control trials using the auditory conditioned stimulus was not significantly different from rates obtained using either voltage of the thalamic conditioned stimulus, higher intensities of thalamic stimulation presented concurrent to tone prevented ongoing performance in these subjects in

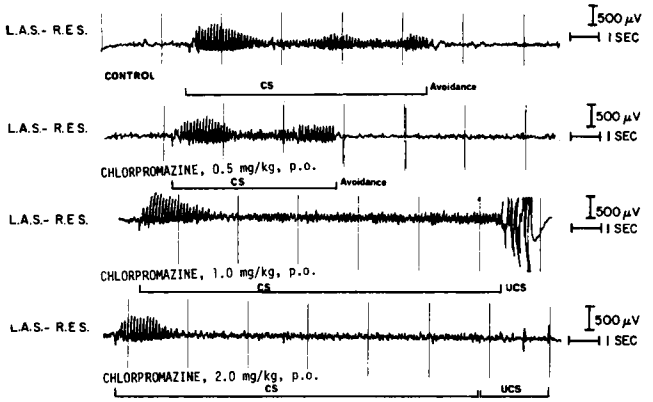

Figure 1—*Effects of chlorpromazine hydrochloride on the ECoG of the recruiting response evoked by the high-voltage thalamic conditioned stimulus (CS) in the cat (P-102). The duration of the conditioned stimulus (CS) (4.5 v, 10 Hz, 0.2-msec stimulation of the nucleus centralis medialis) is shown by the bracketed area beneath each trace here and in Figs. 2 and 3. The unconditioned stimulus (UCS) was an escapable 3-mamp shock applied through a grid floor; the abbreviation L.A.S.-R.E.S. represents a bipolar lead between the left anterior sigmoid gyrus and right ectosylvian gyrus.*

preliminary experiments. Similar cessation of lever pressing in cats by low frequency stimulation of nonspecific thalamic nuclei was previously reported for negatively reinforced behavior (4) and positively reinforced behavior (16). The results of the present investigation suggest that the nonspecific inhibitory effect is voltage dependent; *i.e.*, higher voltages of stimulation produce greater inhibition of behavior.

Conditioned responses are abolished by doses of chlorpromazine that do not seriously affect motor abilities (18); however, much higher doses of imipramine are required to suppress the conditioned avoidance response (19). The barbiturates block conditioned responding only at doses that produce motor incoordination. While similar results were obtained in this study, it was shown that a lower dose of chlorpromazine or imipramine was required to disrupt significantly responses conditioned to thalamic stimulation than to tone. The differential effects of these agents might be explained by the summation of their inhibitory effects on conditioned performance and their facilitation of the diffuse thalamic system (12), which would increase the nonspecific inhibitory effect and decrease response probability to a greater degree than expected.

The fact that pentobarbital did not differentially affect any contingency is in agreement with the results of Rutledge and Doty (20), who compared drug effects on behavior in cats conditioned to either tone or high frequency (50 Hz) cortical stimulation. While the frequencies and sites of brain stimulation in the two studies

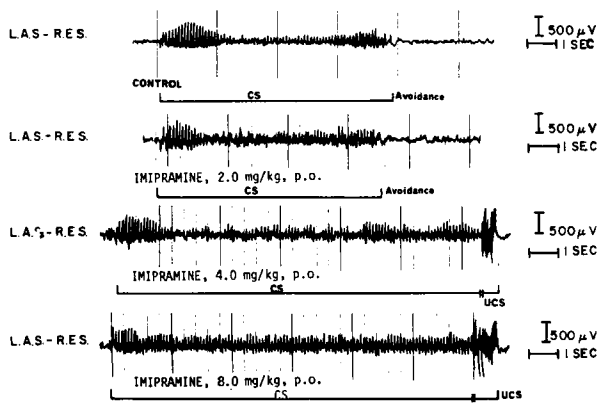

Figure 2—*Effects of imipramine hydrochloride on the ECoG of the recruiting response evoked by the high-voltage thalamic conditioned stimulus (CS) in the cat (P-102).*

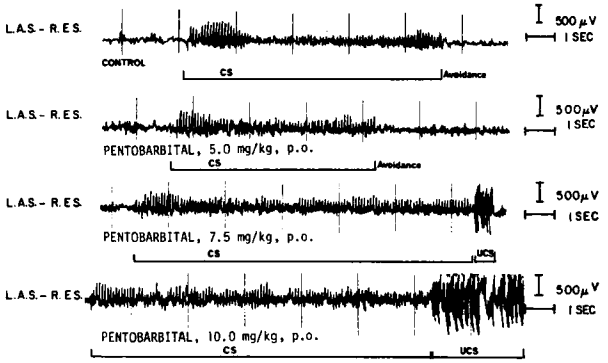

Figure 3—*Effects of pentobarbital sodium on the ECoG of the recruiting response evoked by the high-voltage thalamic conditioned stimulus (CS) in the cat (P-102).*

differed, lower doses of chlorpromazine were required to block responses conditioned to tone than to cortical stimulation. The finding in the present study that chlorpromazine produced reverse differential effects on behavior conditioned to either tone or thalamic stimulation suggests that, compared to cortical stimulation, the motivational effect of thalamic stimulation might be inferior and might provide additional evidence of drug-induced enhancement of a nonspecific inhibitory effect at the thalamic level.

The subjects of this study never bar pressed during the initial recruitment envelope in either control or drug trials, a finding that supports the conclusion of Angyan *et al.* (4) that recruitment is associated with behavioral inhibition. However, in the present experiment, thalamic stimulation was presented for 12 sec rather than for 5 sec as in the former study, allowing an avoidance response to be made following the initial interval of inhibition. The secondary increase of recruiting response negativity observed prior to an avoidance response may be analogous to surface-negative potentials described by Skinner (21) as associated with expectancy or attention or both. Since these increases in recruitment negativity did not appear in trials where chlorpromazine blocked the behavioral response, this type of recruitment change following chlorpromazine may represent the effect of the drug on either expectancy or attention rather than on the recruitment mechanism itself. Chlorpromazine slightly increased recruitment amplitude elicited by the thalamic conditioned stimulus, perhaps through a direct or indirect facilitation of the diffuse thalamic system. There is evidence that chlorpromazine suppresses the cortex and the brain stem reticular formation but stimulates the diffuse thalamic system (12).

The increase in recruitment amplitude produced by imipramine, especially following the 8-mg/kg dose, is in accord with the results of Monnier and Krupp (12), who reported an increased recruiting response in the rabbit cortex by a similar dose. However, Sigg (13) found that imipramine (5–10 mg/kg) did not alter the threshold voltage required to induce recruitment in the cat cortex, and others (14) reported that imipramine decreased both its surface-positive and surface-negative components. Nevertheless, the present results show that imipramine blocks performance conditioned to thalamic stimulation independent of its effects on recruitment.

Domino (9) suggested that the enhancement of recruitment by low doses of pentobarbital was a release phenomenon due to depression of the midbrain reticular formation rather than to direct stimulation of the diffuse projection system; this would account for the small increase in its amplitude observed in the present study. Like imipramine, the effects of pentobarbital on recruitment appear to be independent of its effects on conditioned behavior, and, as noted previously, the barbiturate generally slightly enhanced recruitment following doses that either did or did not prevent responding conditioned to thalamic stimulation. These results suggest that, with the exception of chlorpromazine, the effects of these behavior-modifying drugs upon the characteristics of the cortically recorded recruiting response are not related to their effects on learned behavior.

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Nonsink Dissolution Rate Equations

MAHENDRA PATEL and J. T. CARSTENSEN ˣ

Abstract □ In spite of the fact that film theory is based on severe assumptions, it is shown to be a good working model. The Niebergall–Goyan equation, the Short–Sharkey–Rhodes equation, and the Pothisiri–Carstensen equation—all based on simple film theory—are shown to hold through 80–90% of the dissolution process for *p*-hydroxybenzoic acid and sodium chloride, both at values below and above the amount necessary to saturate the dissolution medium. Deviations are attributed to experimental difficulties and to improper definition of monodisperseness rather than to the assumptions made in the theory.

Keyphrases □ Nonsink dissolution of monodisperse powders—various rate equations examined □ Dissolution of monodisperse powders—various rate equations examined □ Powders, monodisperse—various nonsink dissolution rate equations examined

Dissolution of monodisperse powders has been studied and reported by several investigators. The intents of this study are to consolidate a variety of equations, to point out their shortcomings and strengths, and to show that film theory is a good working model.

BACKGROUND

The dissolution rate equations dealt with here are based on the Noyes–Whitney equation (1):

$$-dW/dt = kO(S - C) \qquad \text{(Eq. 1)}$$

where W is the weight remaining, k is the intrinsic dissolution rate constant, O is the surface area, S is the saturation concentration, and C is the concentration at time t.

The assumptions made are: *(a)* a film [the so-called Nernst–Brunner layer (2–4)] surrounds each particle; *(b)* the film need not be hydrodynamically stagnant but behaves so that there is a linear concentration gradient in it; and *(c)* the concentration gradient imparts a concentration, C, at the boundary between the film and the bulk solution. The thickness of this boundary is assumed not to change during dissolution; *i.e.*, it is assumed to be independent of particle size. Niebergall *et al.* (5) studied this point, but it will not be discussed in this report.

Another assumption is that the particles are isotropic and isometric, although Carstensen and Patel (6) showed that this condition need not be a prerequisite. The Ostwald–Freundlich effect (7, 8), where a smaller particle is more soluble than a larger particle, is also neglected.

A dissolution rate equation for monodisperse powders was first suggested by Wilhelm *et al.* (9); they employed graphical integration to solve exact dissolution rate equations. Hixson and Crowell (10) arrived at the cube root law for dissolution of monodisperse powders under sink conditions (*i.e.*, when $C \ll S$):

$$W_o^{1/3} - W^{1/3} = \frac{W_o^{1/3} 2kS}{\rho d} t \qquad \text{(Eq. 2)}$$

where d is the diameter of the particle (assumed spherical), and ρ is the particle density. For Fick's law (11) to apply to film theory, it is required that:

$$k = D/h \qquad \text{(Eq. 3)}$$

where D is the diffusion coefficient of the solute in solution, and h is the thickness of the Nernst–Brunner layer. Since D can be determined experimentally or obtained by the Stokes–Einstein equation (12) or the Wilke equation (13), an estimate of h can be made from dissolution rate studies.

Niebergall and Goyan (14) extended the Hixson–Crowell equation to the situation where the amount studied (W_o) equals exactly

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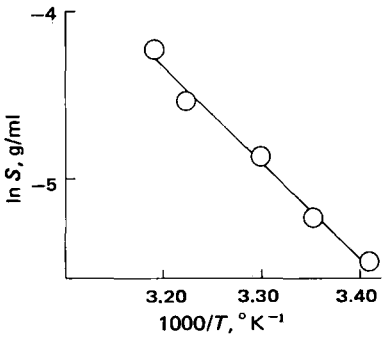

Figure 1—*Temperature dependence of the solubility of* p-*hydroxybenzoic acid.*

the amount needed to saturate the solution (W_s). As shown in the *Appendix*, they found the negative two-thirds law as expressed in Eq. 4:

$$W^{-2/3} - W_o^{-2/3} = \beta t \qquad \text{(Eq. 4)}$$

where the proportionality constant β is given by: $\beta = kN^{1/3}\Gamma/V$, in which N is the number of particles and Γ is a shape factor given by: $\Gamma = [6\sqrt{\pi}/\rho]^{2/3}$. Niebergall and Goyan (14), employing salicylamide and benzoic acid as test substances, showed Eq. 4 to hold but only to about 30% of saturation.

Short *et al.* (15) solved the dissolution rate equation under nonsink conditions, where W_s is different from W_o. The correct form of their equation with the nomenclature used here is:

$$\psi(W) = -[(k\sqrt[3]{N})(\Gamma/V)]t + \psi(W_o) \qquad \text{(Eq. 5)}$$

where:

$$\psi(W) = 0.5F^{-2}\{\ln[(u+F)^2/(F^2 - uF + u^2)]\} + \sqrt{3}F^{-2}\arctan[(2u - F)/(F\sqrt{3})] \qquad \text{(Eq. 6)}$$

and where $F^3 = W_s - W_o$ and $W = u^3$. The derivation of Eq. 5 is shown in the *Appendix*. Short *et al.* (15) tested the equation on the dissolution of hydrocortisone and found it *not* to hold.

Pothisiri and Carstensen (16) developed a similar equation under nonsink conditions; the correct form of their equation with the nomenclature used here is[1]:

$$\psi(r) = \psi(r_o) - \frac{k}{\rho}t \qquad \text{(Eq. 7)}$$

where:

$$\psi(r) = \frac{q}{6\beta}\ln\left[\frac{(r+q)^2}{(r^2 - qr + q^2)}\right] + \frac{q}{\beta\sqrt{3}}\arctan\left[\frac{2r - q}{q\sqrt{3}}\right] \qquad \text{(Eq. 8)}$$

where r is the "radius" of the particle; $\alpha = N\rho 4\pi/(3V)$, where V is the volume of the dissolving medium; $\beta = S - (W_o/V)$; and $q^3 = \beta/\alpha$. All derived equations are based on film theory. Pothisiri and Carstensen (16) showed Eq. 8 to hold up to 65% of saturation. Although simple film theory should fail on many counts (17, 18), it, amazingly, gives excellent correlation with experimental values.

EXPERIMENTAL

p-Hydroxybenzoic acid dissolution was tested in a 1000-ml jacketed beaker[2]. The *p*-hydroxybenzoic acid was recrystallized in a nonisothermal fashion (6) from alcohol USP, air dried, and sieved through a nest of sieves. The fraction retained by a 60-mesh screen and passing a 40-mesh screen was used in most experiments. One particular experiment was conducted with a 16–20-mesh cut; but unless otherwise stated, the experiments refer to the 40–60-mesh cut.

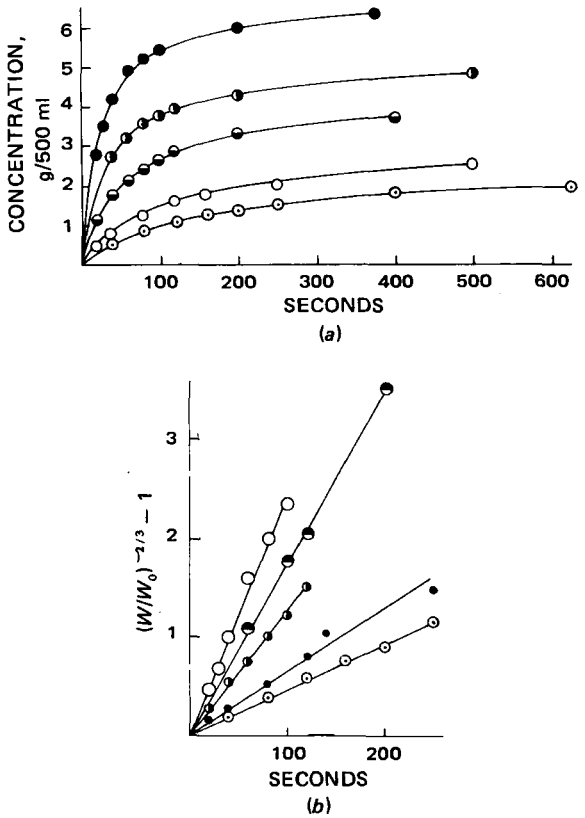

Figure 2—*(a) Concentration* versus *time profile for* p-*hydroxybenzoic acid at different temperatures in 0.1* N *HCl (initial weight* $W_o = W_s$*). Key: ⊙, 20°; ○, 25°; ⊖, 30°; ◑, 35°; and ●, 40°.* (b) *Plots of data from Fig. 2a plotted according to Eq. 4. Key: ⊙, 20°; ●, 25°; ◑, 30°; ⊖, 35°; and ○, 40°. The slopes of the lines are denoted* B.

Several batches were made in this fashion, and the various cuts were pooled to obtain adequate yields for the dissolution rate studies. The dimensions of the 1000-ml jacketed beaker were 8 cm in diameter and 18 cm in height; it was agitated by a Teflon-coated magnetic stirring bar, 8 mm in diameter and 36 mm in length, operating at 120 rpm. The jacket was connected with Tygon tubing *via a pump*[3] *to a thermostated bath, so the jacket temperature* could be maintained to ±0.3°. An amount of 0.1 *N* HCl slightly in excess of 500 ml was placed in a 500-ml volumetric flask, the flask was placed in the water bath, and the temperature was equilibrated.

The amount in excess of 500 ml was removed by pipet, and the 500 ml was transferred to the jacketed beaker. An excess of *p*-hydroxybenzoic acid (*i.e.*, an amount in excess of the solubility) was added to the solution, and the beaker was then covered with aluminum foil. The concentration of *p*-hydroxybenzoic acid in the supernate was determined from time to time; when a steady level had been reached (24 hr), the concentration was assumed to be the saturation concentration. The temperature of the contents was determined periodically and found to vary by less than 0.6° at the extremes, *i.e.*, ±0.3°. The experiment was carried out at 20, 25, 30, 35, and 40°. The assay method used was the spectrophotometric method described by Pothisiri and Carstensen (16). The solubility as a function of temperature is shown in Fig. 1.

The experiment was then repeated except that exactly the amount of *p*-hydroxybenzoic acid necessary to saturate the 500 ml of 0.1 *N* HCl at the test temperature (W_s) was added, and (approximately) 1-ml samples were removed through glass wool with a tared hypodermic syringe which was then weighed. The contents were transferred quantitatively to a volumetric flask, assayed, and

[1] The last fraction in Eq. 11 of the paper by Pothisiri and Carstensen (16) is inverted, and the equation should read as shown in Eq. 8 here. The solubility reported in Ref. 16 is higher (10 mg/ml corresponding to $W_o = 0.82W_s$) than reported here (6.7 mg/ml corresponding to $W_o = 1.27W_s$). Regardless of the solubility figure used, the data of Ref. 16 plot well according to Eq. 7 (as shown in Fig. 7) and Eq. 5 (as shown in Fig. 8).

[2] Jacketed beaker, ACE Glass Corp., Vineland, N.J.

[3] LAB Vibrostatic pump, Lab Apparatus Co., Cleveland, OH 44128

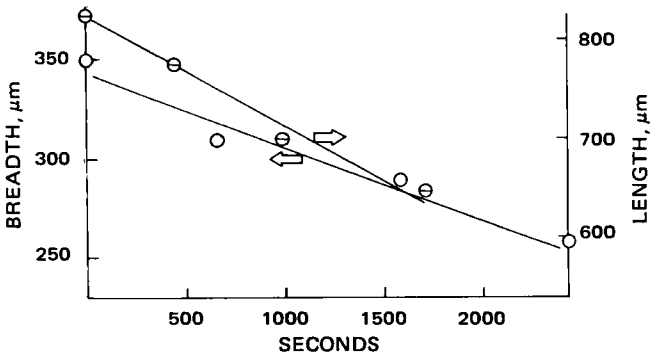

Figure 3—*Dissolution rate experiment conducted with single crystal of* p-*hydroxybenzoic acid in 0.1* N *HCl on microscope slide.*

converted to grams of *p*-hydroxybenzoic acid per 500 ml at the testing temperature by knowledge of the density of *p*-hydroxybenzoic acid solutions (at various concentrations) at the testing temperature. The results of these studies are shown in Fig. 2*a*. No more than five samples were removed during a test; if more points were necessary, the experiment was repeated at the different time points desired.

An experiment was also performed using an amount of *p*-hydroxybenzoic acid corresponding to 1.15 $W_s = W_o$, and an experiment was performed with a 16–20 sieve cut with 1.5 $W_s = W_o$. A dissolution rate experiment on a crystal of *p*-hydroxybenzoic acid was performed on a microscope slide in 3 drops of 0.1 *N* HCl by the method described by Carstensen and Patel (6); the length *(b)* and the breadth *(a)* decrease linearly in time as shown in Fig. 3.

A microscopic determination of the dimensions of 60 particles was performed (Fig. 4). *p*-Hydroxybenzoic acid is actually monoclinic, but the crystals approximate a rectangular parallelepiped with sides *a*, *a* and *b*; Fig. 4 shows that the ratio of *b* to *a* is about 2.4. Since, as seen in Fig. 3, the rate constant for the breadth is $k_a \sim 0.04$ cm/sec and the rate constant for the length is $k_b \sim 0.1$ cm/sec, $k_b/k_a \sim 2.5$, which is close to the ratio of the sides (= 2.4). Since, therefore, the sides decrease in magnitude with rates approximately proportional to the lengths themselves, the dissolution is considered fairly isotropic.

The volumes ($V = a^2b$) of the particles in Fig. 4 were calculated based on the assumption that the particles are parallelepipeds. The cube length equivalents ($\sqrt[3]{V}$) calculated in this fashion are shown in Table I. If the sieve cut were from a single crystallization, it would be expected to have a monotonely decreasing frequency function with increasing particle size (with the assumption that the sieve cut is above the number based mean). Since it is a blend of several batches, this, of course, cannot be expected. In fact, the distribution appears to be a square distribution (*i.e.*, frequency is equal for each size within the cut). The data in Table I fail to show a significant difference from such a distribution ($\chi^2 = 12$, which is smaller than the critical χ^2-value for 11 degrees of freedom and a

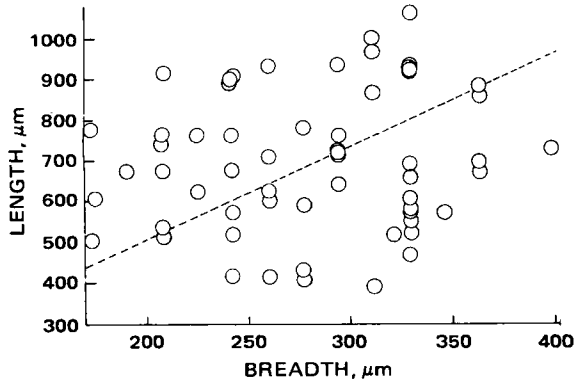

Figure 4—*Distribution of dimensions of 60 crystals of* p-*hydroxybenzoic acid of a 40–60 sieve cut.*

Table I—Distribution of Crystals from Fig. 4

10^{-3} $V^{1/3}$ Interval, μm	Logarithm of Lower Number	Number of Crystals in Interval	Percent Crystals (Cumulative)
1.1–1.2	0.041	2	3.3
1.2–1.3	0.079	2	6.3
1.3–1.4	0.114	5	14.6
1.4–1.5	0.146	9	29.6
1.5–1.6	0.176	4	36.3
1.6–1.7	0.204	5	44.6
1.7–1.8	0.230	8	57.9
1.8–1.9	0.255	8	71.2
1.9–2.0	0.279	2	74.5
2.0–2.1	0.301	5	82.8
2.1–2.2	0.322	6	92.8
2.2–2.3	—	4	100

probability of 0.05, $\chi^2_{crit} = 19.7$). Brooke (19) recently discussed this type of phenomenon in depth.

DISCUSSION

Although the data by Niebergall and Goyan (14) suggest that the negative two-thirds relation (Eq. 4) holds to 30% of saturation only, data from other literature sources suggest that it may have wider applicability. Figure 5 shows the data from Wilhelm *et al.* (9) for $W_o = W_s$; the test substance was sodium chloride, and the data adhere to Eq. 4 to better than 80%. Deviations above 80% are due to the fact that, in this range, the difference is a small difference between large numbers.

The data from the dissolution of *p*-hydroxybenzoic acid shown in Fig. 2*a* are shown plotted according to Eq. 4 in Fig. 2*b*. Here, again, the data adhere to Eq. 4 to better than 80% of solubility. The deviations from adherance to Eq. 4 are negative and are attributed to the fact that (aside from the poor precision in the higher range) the 40–60-mesh material is not quite monodisperse, so loss of the smallest particles occurs in this range.

As shown in the *Appendix* (Eq. A8), Eq. 4 can be written:

$$(W/W_o)^{-2/3} - 1 = S^{2/3}2kN^{1/3}\Gamma/(3V^{1/3})t = Bt \quad \text{(Eq. 9}a\text{)}$$

or:

$$(W/W_o)^{-2/3} - 1 = [4kS/(\rho d_o)]t \quad \text{(Eq. 9}b\text{)}$$

It follows that the slope, *B*, adheres to:

$$\ln (B/T) = \ln S + \ln (k/T) + \ln[4/(\rho d_o)] \quad \text{(Eq. 10)}$$

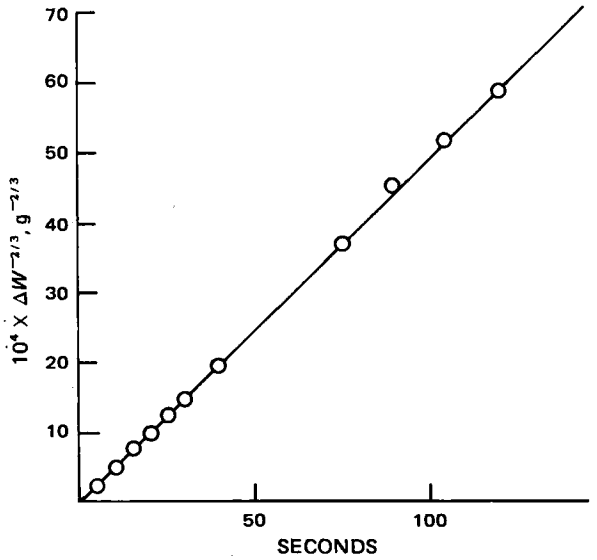

Figure 5—*Data from Ref. 9 plotted according to Eq. 4 when* $W_o = W_s$.

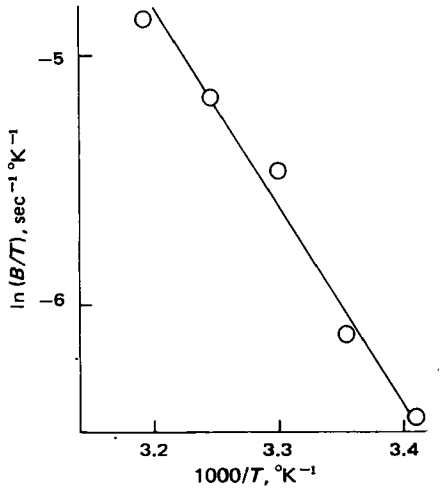

Figure 6—*The slopes,* B, *from Fig. 5 plotted as ln* (B/T) *as a function of reciprocal absolute temperature (1000/T °K^{-1}).*

Carstensen and Patel (6) showed that ln (k/T) is linear with $1/T$ with a slope of between E/R and $2E/R$, where E (<0) is the activation energy for the viscosity of the dissolution medium, and the $2E/R$ term holds with higher agitation intensities such as are reported here. The data in Fig. 1 give ln $S = [-(\Delta H/R)(1/T)] + q$, where ΔH is the heat of solution and q is a constant. From the data in Fig. 1, it is seen that $(\Delta H/R) = 4850$ cal/mole.

Therefore, a plot of ln (B/T) *versus* $1/T$ should be linear and have a slope of $[(2E/R) - \Delta H]$. The linearity is demonstrated in Fig. 6; the slope is −8000 cal/mole. Therefore, $(2E/R) - 4850 = -8000$; *i.e.*, E is between −3150 and −6300 cal/mole, close to E for water (−4700 cal/mole). An equation derived from the film theory (Eq. 4) apparently shows consistency in temperature dependence and the attained slopes are of the expected magnitude.

In spite of several assumptions in the film theory (*viz.*, isotropicity, isometricness, constant film thickness, neglect of convection, and size-independent solubility) and some experimental approximations (*viz.*, geometric surface areas and the assumption of well-defined particle size), the data developed from the theory adhere well to experimental values. This statement is not an attempt to minimize the theoretical importance of the assumptions but simply notes that the effects of the assumptions either cancel out or fall below experimental scrutiny in many cases.

Other equations (15, 16) are also based on simple film theory with the same assumptions. They basically do not differ, and it is apparent from the *Appendix* that data following one should also follow the other. Literature data (9, 16) are shown plotted according to Eq. 7 in Fig. 7 and according to Eq. 5 in Fig. 8, and the data hold up to about 70% of saturation.

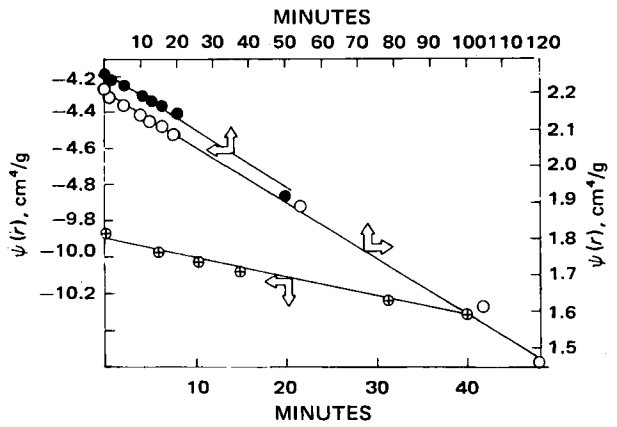

Figure 7—*Data plotted according to Eq. 7. Key:* ⊕, *data from Ref. 9 where initial weight* $W_o = 1.2W_s$; ○, *data from Ref. 16 where initial weight* $W_o = 0.82W_s$; *and* ●, *data from Ref. 16 where initial weight* $W_o = 1.27W_s$.

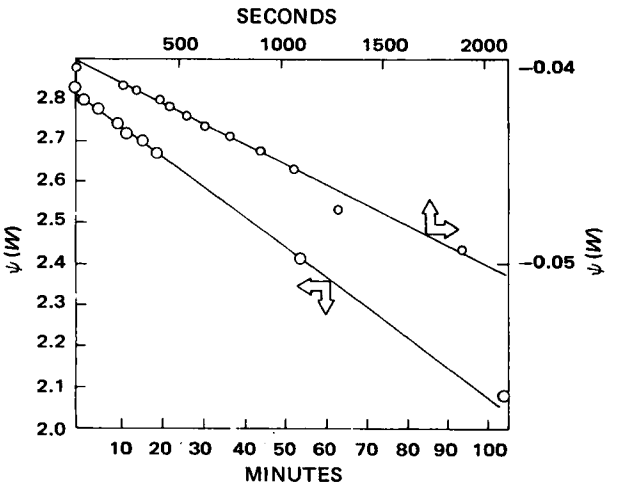

Figure 8—*Data plotted according to Eq. 5. Key:* ○, *sodium chloride data from Ref. 9 where initial weight* $W_o = 1.2W_s$; *and* ○, p-*hydroxybenzoic acid data from Ref. 16 where initial weight* $W_o = 0.82W_s$.

The derivations of Eqs. 5 and 7 require that $W_o < W_s$ or $W_s < W_o$. A dissolution test using p-hydroxybenzoic acid at $W_o = 1.15W_s$ is shown in Fig. 9, and the data, again, adhere to both Eqs. 7 and 5 to about 70% of saturation. In all cases, the deviation is in the direction of smaller k values. Neither the concept that film thickness decreases with the size of the particle nor the concept that solubility increases with a decrease in particle size would explain this result, since the former, in decreasing h, would *increase* k $(=D/h)$ and the latter would increase the concentration gradient and, hence, the dissolution rate. It may, however, be fallacious to consider a sieve fraction comparable to monodisperse powder.

The point here is that, essentially, one is equating the surface area of the "mean" particle size with the surface area of the sieve cut. If particles were isotropic, then the most proper mean to use would be of the volume–surface type; using the cube length equivalent, it is possible to define $\bar{x}_{sv} = \Sigma nx^3/(\Sigma nx^2) = [\int f(x)x^3\,dx]/[\int f(x)x^2\,dx]$, where $f(x)$ is the frequency of the occurrence of the length x. However, the use of the number average mean here gives better results from a surface area point of view.

If one considers the data in Table I as representing a square distribution, then the frequency function, $f(x)$, would be independent of particle length, *i.e.*:

$$f(x) = A \qquad \text{(Eq. 11)}$$

where A is a constant. Normalizing this over the interval of 110–230 μm yields:

$$\int_{110}^{230} f(x)\,dx = 120A = 1 \qquad \text{(Eq. 12}a\text{)}$$

so that:

$$A = 1/120\ (\mu m)^{-1} \qquad \text{(Eq. 12}b\text{)}$$

The surface area (per one particle equivalent) of a sieve fraction

Table II—Errors in Assuming a Sieve Cut of 40–60 Mesh to be Monodisperse Assuming Linear Length Reduction during Dissolution

x_1, μm	x_2, μm	$\bar{x}$, μm	A/A^*	Percent Dissolved	Volume[a], μm^3
110	230	170	1.04	0	$5.5\ 10^6$
80	200	140	1.06	43	$3.23\ 10^6$
55	175	115	1.09	65	$1.93\ 10^6$
20	140	80	1.19	85	$0.80\ 10^6$
0	120	60	1.33	97	$0.24\ 10^6$

[a] Normalized to the equivalent of one particle.

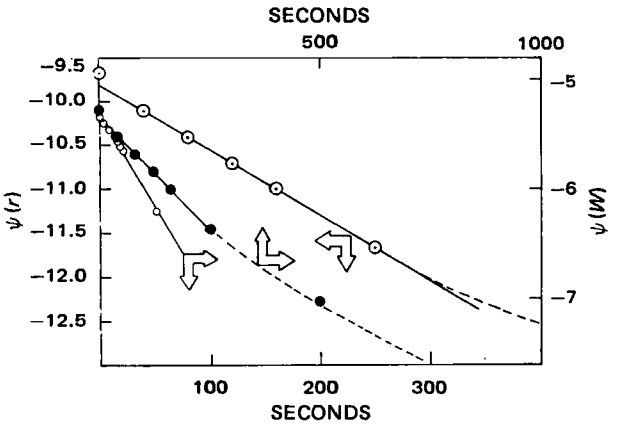

Figure 9—*Data from this study plotted according to Eqs. 5 and 7. Key:* ⊙, *p-hydroxybenzoic acid at 25°, 40–60 mesh, using Eq. 7 with initial weight* $W_o = 1.15W_s$; ●, *p-hydroxybenzoic acid at 25°, 40–60 mesh, using Eq. 5 with initial weight* $W_o = 1.15W_s$; *and* ○, *p-hydroxybenzoic acid at 25°, using Eq. 7 with initial weight* $W_o = 1.27W_s$ *from Ref. 16.*

between x_1 μm and x_2 μm is given by:

$$A = (1/120)\int_{x_1}^{x_2} 6x^2\,dx = (1/60)\,(x_2^3 - x_1^3) \quad \text{(Eq. 13)}$$

The volume (per one particle equivalent) of a sieve fraction between x_1 μm and x_2 μm is given by:

$$A = (1/120)\int_{x_1}^{x_2} x^3\,dx = (1/480)\,(x_2^4 - x_1^4) \quad \text{(Eq. 14)}$$

If one assumes that x_1 decreases at the same rate as x_2, then one can calculate the area of the sieve fraction A as opposed to the area (A^*) it would have had if it had been monodisperse with the arithmetic mean diameter. Data computed in this fashion are shown in Table II. The area estimate is relatively close up to about 65% dissolved, at which point it is 10% high. The A/A^* increases (almost linearly) up to 65% dissolved, after which it rises drastically; this increase would impart linearity to plots *via* Eq. 5 or 7, but the slopes would differ somewhat from those given by the equations.

It is noted that $x_{sv} = 138$ μm; if this value (rather than 170 μm) had been used in Table II, deviations would have been greater. (This would not be so for example with a true sphere.) The assumption that x_1 and x_2 decrease with the same rate is not correct, and actual figures would be less favorable than those shown in Table II.

In a semiquantitative way, the foregoing arguments imply that,

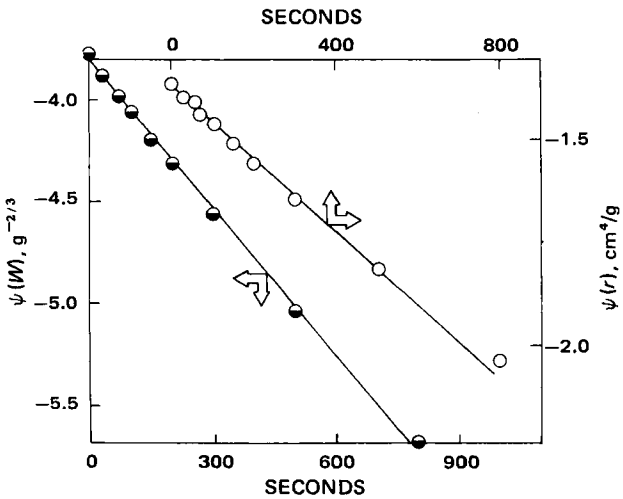

Figure 10—*Data from this study plotted according to Eq. 5 (⊖) and Eq. 7 (○) with p-hydroxybenzoic acid at 25°, 16–20 mesh, and initial weight* $W_o = 1.5W_s$.

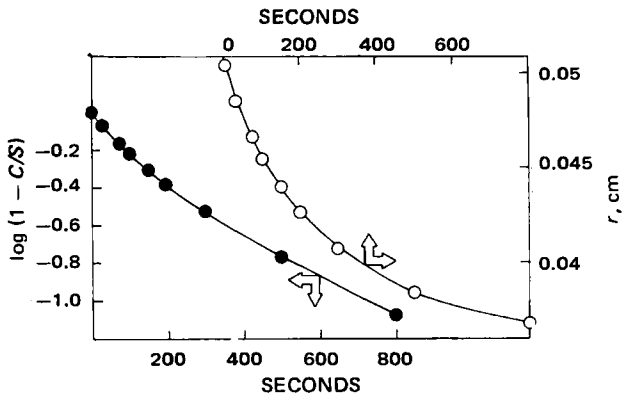

Figure 11—*Data from Fig. 10 plotted (●) according to Eq. 15 (which assumes constant surface area). Curve to the right (○) shows the change in radius (assuming spherical particles) as a function of time.*

in the case of the 40–60 cut, adherance to predicted equations could be expected to only about 65% of complete dissolution of the available solid when $W_o < W_s$ (and to more than 65% of the complete dissolution process when $W_o > W_s$). In Figs. 2*b* and 7–9, linearity prevails to better than 70% of complete dissolution or of the dissolution process; the use of sieve cuts apparently is an explanation for nonadherance beyond this point. The relatively larger A value has the effect of a larger r value in Eq. 7, which gives a higher value of $\psi(r)$ (smaller value of k). This trend is shown in Fig. 9.

Higher W_o's and larger particle sizes should reduce these effects, and the data in Fig. 10 ($W_o = 1.5W_s$, 16–20-mesh fraction) show that this coarser sample in higher amounts adheres to Eqs. 5 and 7 through the whole dissolution process. At very high amounts of material used, the point will be reached where the surface area will stay practically constant during the dissolution process and should follow (11):

$$\ln[1 - (C/S)] = -kOt/V \quad \text{(Eq. 15)}$$

It is seen from Fig. 11 that $W_o = 1.5W_s$ is not a sufficiently large excess of powder to make Eq. 15 apply. Larger excesses are difficult to test, since dissolution becomes rapid with the increased surface areas.

A secondary observation makes the previous considerations pertinent to many actual samples of recrystallized powders. Many crystallizers are built on the principle of nonisothermal crystallization followed by fines destruction (through a raise in temperature at the end of the process). Blending of batches is also a practice, and it would, therefore, be expected that distributions other than log normal would result and that distributions such as the one shown in Table I might be common. As shown in Fig. 12, a cumulative plotting of this type resembles a log-normal distribution, and this may be one reason (other than the reasons stated in the literature) that crystallized materials appear to be log normal.

In conclusion, it should be mentioned once again that a series of

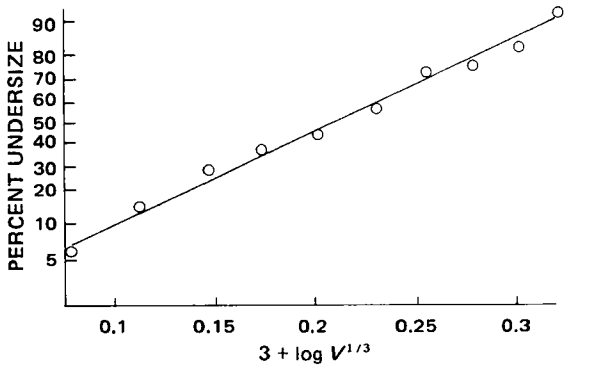

Figure 12—*Data from Fig. 4 shown as a log-normal distribution.*

assumptions is made in the basic film theory of dissolution which cannot be disregarded in dissolution work. However, in studying dissolution behaviors of powder samples, experimental techniques at present do not suffice to detect the effect of the assumptions because of the difficulty in producing monodisperse samples of crystalline samples. In general, film theory has produced equations through a large range of W_o values [Eqs. 5 and 7, Eq. 2 (the cube root law), Eq. 4 (the negative two-thirds law), and Eq. 15], which are workable models for dissolution of powders.

APPENDIX

The Noyes–Whitney equation (1) in the nomenclature used here is:

$$-dW/dt = kO(S - C) \qquad \text{(Eq. A1)}$$

where O is surface area at time t. The concentration, C, may be expressed as $(W_o - W)/V$, and saturation concentration, S, may be expressed as W_s/V, so that Eq. A1 becomes:

$$-V(dW/dt) = kO[W_s - (W_o - W)] \qquad \text{(Eq. A2)}$$

For N particles, the surface area and weights are given by:

$$O = N\pi d^2 \qquad \text{(Eq. A3)}$$

$$W = N\pi d^3\rho/6 \qquad \text{(Eq. A4)}$$

so that:

$$OW^{-2/3} = N^{1/3}[6/(\pi\rho)]^{2/3}\pi = N^{1/3}[6\sqrt{\pi}/\rho]^{2/3} = N^{1/3}\Gamma \qquad \text{(Eq. A5)}$$

Inserting Eq. A5 in Eq. A2 then gives:

$$-V(dW/dt) = kN^{1/3}\Gamma W^{2/3}[W_s - W_o + W] \qquad \text{(Eq. A6)}$$

If $W_s = W_o$, then this becomes:

$$-dW/(W^{5/3}) = (kN^{1/3}\Gamma/V)\,dt \qquad \text{(Eq. A7)}$$

which can be integrated and subjected to initial conditions to give:

$$W^{-2/3} - W_o^{-2/3} = \frac{2kN^{1/3}\Gamma}{3V}t \qquad \text{(Eq. A8)}$$

which is equivalent to the Niebergall equation (Eq. 4).

In general, Eq. A6 can be simplified by introduction of the following substitutions:

$$W_s - W_o = F^3 \qquad \text{(Eq. A9)}$$

$$W = u^3 \qquad \text{(Eq. A10)}$$

in which case it becomes:

$$du/(F^3 + u^3) = (kN^{1/3}\Gamma/3V)dt \qquad \text{(Eq. A11)}$$

which, after integration and use of initial conditions, gives the Short–Sharkey–Rhodes equation (Eq. 5)[4].

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[x] To whom inquiries should be directed.

[4] The equation differs slightly from that reported by Short *et al.* (15) due to a printing error in Ref. 15.

High-Pressure Liquid Chromatographic Determination of Sulfamethazine Residue in Bovine Tissue

KARL L. JOHNSON, DELBERT T. JETER ˣ, and ROBERT C. CLAIBORNE

Abstract □ A sensitive high-pressure liquid chromatographic assay procedure is described for residues of sulfamethazine in bovine kidney, liver, muscle, and fat tissue at a sensitivity of 0.04 ppm. Studies were conducted using sulfamethazine-negative bovine tissue to ensure that the peak eluting at the indicated retention volume was sulfamethazine and that the recoveries from spiked tissues were consistent. Data are presented showing the levels of sulfamethazine in the tissue of 12 treated animals found by this technique. The method is more specific than the frequently used colorimetric methods and does not require the tedious separations involved in TLC assays.

Keyphrases □ Sulfamethazine—residues, high-pressure liquid chromatographic determination, bovine tissue □ High-pressure liquid chromatography—residue analysis, sulfamethazine, bovine tissue

Sulfamethazine is frequently used in intravenous injections, oral solutions, tablets, and bolus forms for the treatment of bovine diseases. These veterinary preparations are utilized for mastitis, metritis, calf pneumonia, calf diphtheria, and calf scours (1) caused by pathogens sensitive to sulfamethazine.

Previous analytical methods relied upon diazotization of the aryl amine followed by either a potentiometric titration (2) or a colorimetric assay (3). These methods suffer from a lack of specificity since they react with all aryl amines and, to some extent, with primary and secondary alkyl amines (4) to yield diazotization products and colored addition compounds which interfere with the assay. More recently, TLC methods (2) have been used to separate sulfonamides and their various degradation products but such methods are tedious to perform.

An alternative approach was sought which would allow for the separation from bovine tissue and the quantitation of residues of sulfamethazine remaining in bovine tissue after cessation of treatment. The method outlined in this paper separates sulfamethazine from other tissue components; therefore, it is more specific than existing colorimetric assays. Furthermore, it is sensitive enough to detect levels as low as 0.04 ppm of sulfamethazine present in the tissue. The technique is more specific than most existing assays but does not suffer from decreased sensitivity.

EXPERIMENTAL

Animals—Twelve calves[1] were divided into four groups of three each and administered sulfamethazine sodium, 214.5 mg/kg po for 1 day followed by 107.2 mg/kg/day for 4 days. Group I was slaughtered at 0–6 hr, Group II at 5 days, Group III at 8 days, and Group IV at 10 days posttreatment. Samples of muscle, fat, liver, and kidney were taken for assay purposes.

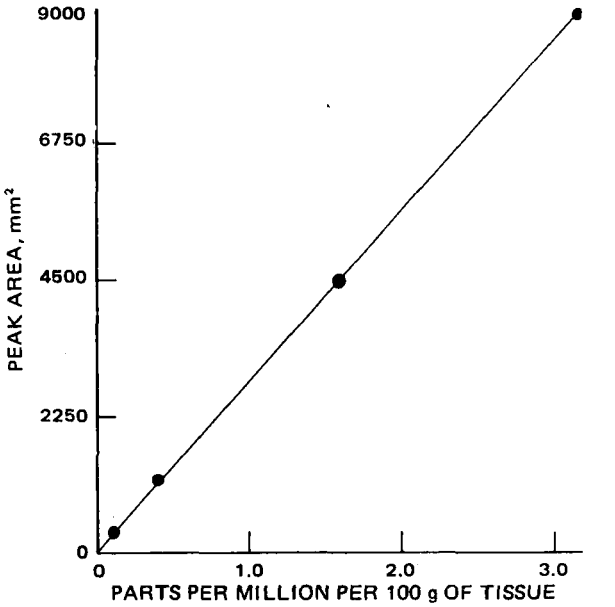

Figure 1—*Calibration curve prepared with sulfamethazine standards (see text).*

Materials—All reagents were ACS or analytical reagent grade, except the *n*-hexane[2] which was technical grade. The *n*-hexane and chloroform were freshly distilled from an all-glass system; the first and last 100 ml from each 2-liter distillation were discarded.

Liquid Chromatography—A 50-μl aliquot of solution was injected by syringe[3] into a high-speed liquid chromatograph[4] equipped with a 254-nm UV detector. The detector signal was fed to a 2-mv strip-chart recorder[5] operating at a chart speed of 0.5 cm/min. A 0.61-m (2-ft) × 2-mm i.d. column, packed with a permanently bonded octadecyl–porous silica[6] material, was purged for at least 2 hr with the mobile phase prior to use. All purging and assay work was done at a constant flow of 0.6 ml/min.

Mobile Phase—The mobile phase, pH 7.70 buffer–2.5% (v/v) isopropyl alcohol, was prepared as follows. Potassium phosphate monobasic crystals, 6.89 g, and 8.79 g of potassium phosphate dibasic (anhydrous) were dissolved in 800 ml of distilled water. The pH was adjusted to 7.70 with 1.0 *M* sodium hydroxide. The solution was transferred to a 1-liter volumetric flask containing 25 ml of isopropyl alcohol and diluted to volume with distilled water.

This solution was poured into a 2-liter round-bottom flask and refluxed for 45 min under a 400-mm Allihn-type water-cooled condenser. The solution was allowed to cool to room temperature with the condenser attached and then was poured gently into the reservoir of the liquid chromatograph. The reservoir was designed to minimize the evaporation of isopropyl alcohol.

Standards—Sulfamethazine USP, 100 mg, was accurately weighed into a 100-ml volumetric flask, dissolved in 0.1 *M* hydrochloric acid, diluted to volume, and mixed. Aliquots of 1.0, 4.0, 16.0, and 32.0 ml were transferred into separate 100-ml volumetric flasks and diluted to volume with distilled water. (These solutions

[1] All animals were located at the Philips Roxane Clinical Research Farm, Cosby, Mo., under identical conditions and were fed antibiotic- and sulfonamide-free rations.

[2] Mallinckrodt Chemical Co., St. Louis, Mo.

[3] Precision Sampling model B-110, Precision Sampling Corp., Baton Rouge, La.

[4] Waters Associates ALC-202, Waters Associates, Inc., Milford, Mass.

[5] Linear Instruments model 252, Linear Instruments Corp., Irvine, Calif.

[6] Porasil B/C_{18} Bondapak, 37–50 μm, Waters Associates, Milford, Mass.

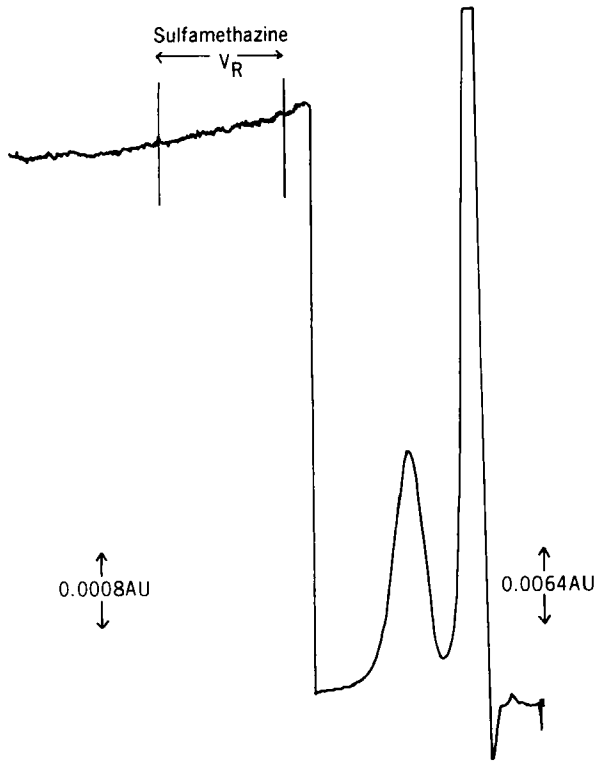

Figure 2—*Chromatogram of control liver extract.*

corresponded to 0.01, 0.04, 0.16, and 0.32 mg/ml of sulfamethazine, respectively, and were retained to prepare recovery samples.)

One-milliliter aliquots of each of the four standards were transferred into 50-ml glass-stoppered graduated cylinders, the volumes were adjusted to 30 ml with 1 *M* hydrochloric acid, and the solutions were mixed. (These solutions contained 10, 40, 160, and 320 μg and corresponded to 0.1, 0.4, 1.6, and 3.2 ppm of sulfamethazine

Table I—Sulfamethazine Residue Results

Animal Number	Slaughter Time Post-treatment	Sulfamethazine Residue, ppm			
		Fat	Muscle	Kidney	Liver
132	0–6 hr	325	54.1	88.6	42.5
177	0–6 hr	150	45.3	62.4	28.0
178	0–6 hr	115	42.1	95.6	33.2
138	5 days	0.06	<0.04	<0.04	0.04
168	5 days	0.27	<0.04	0.08	0.09
169	5 days	0.36	0.17	0.37	0.31
174	8 days	0.04	<0.04	<0.04	<0.04
175	8 days	0.06	<0.04	<0.04	<0.04
176	8 days	0.12	<0.04	<0.04	<0.04
170	10 days	0.06	<0.04	<0.04	<0.04
171	10 days	<0.04	<0.04	<0.04	<0.04
172	10 days	0.06	<0.04	<0.04	<0.04

present, respectively, in a 30-ml extract of 100 g of tissue.) Fifty microliters of each of the last four standards was injected into the liquid chromatograph, and the area of the peak eluting at a retention volume (V_r) of 12 ml was determined.

Since the concentration ranges were so great, the detector attenuation was varied between 0.04 and 0.32 absorbance unit full scale (aufs). The equivalent area at 0.04 aufs was then calculated using:

$$\text{corrected area (0.04 aufs)} = \frac{\text{area sulfamethazine peak} \times \text{aufs used}}{0.04} \quad \text{(Eq. 1)}$$

A calibration curve was prepared (Fig. 1) by plotting the corrected area (0.04 aufs) against the concentration (parts per million) present in 100 g of tissue.

Procedure—The tissue was sliced in squares (2 × 2 × 1 mm thick), and 100 g was weighed into a wide mouth glass jar. Then 150 ml of acetone–chloroform (1:1 v/v) was added, and the mixture was shaken vigorously for 2 min and allowed to set for 15–20 min. The samples were again shaken vigorously for 2 min. The solvent was decanted into a funnel containing an 18.5-cm phase-separating

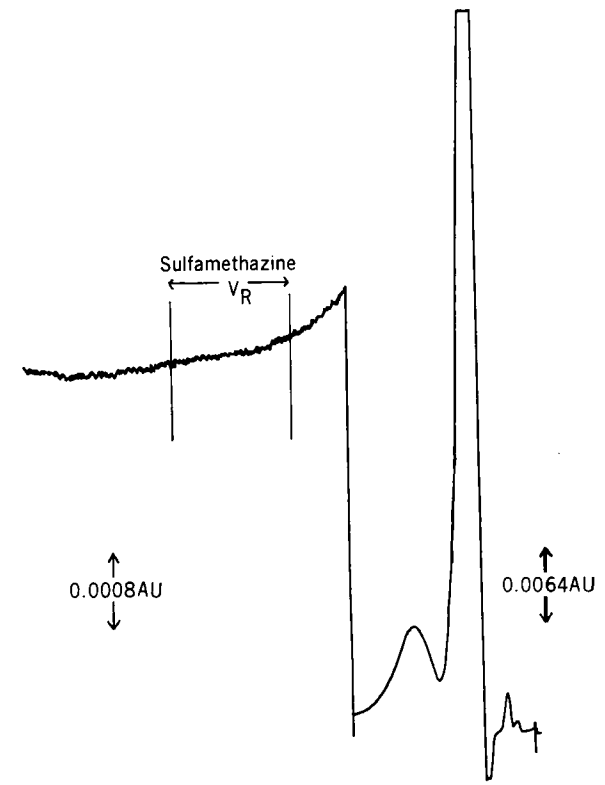

Figure 3—*Chromatogram of control kidney extract.*

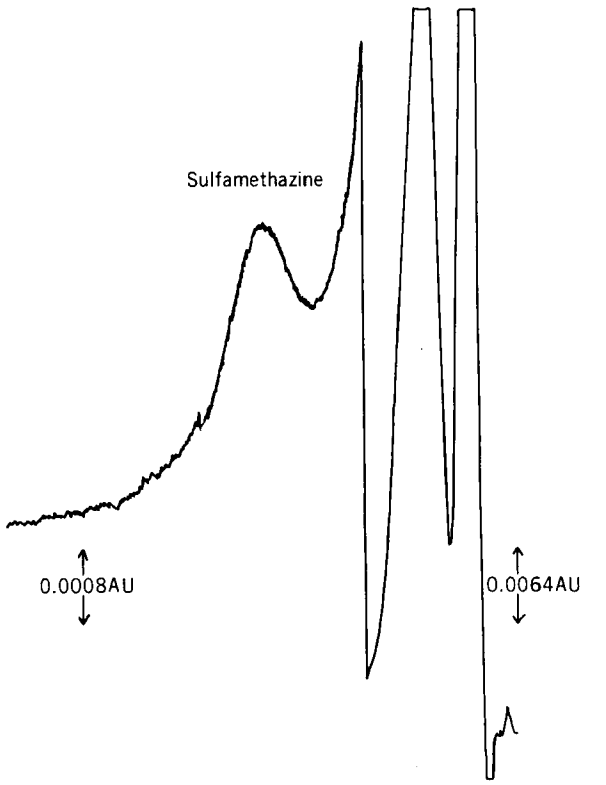

Figure 4—*Chromatogram of bovine liver containing 0.31 ppm of sulfamethazine.*

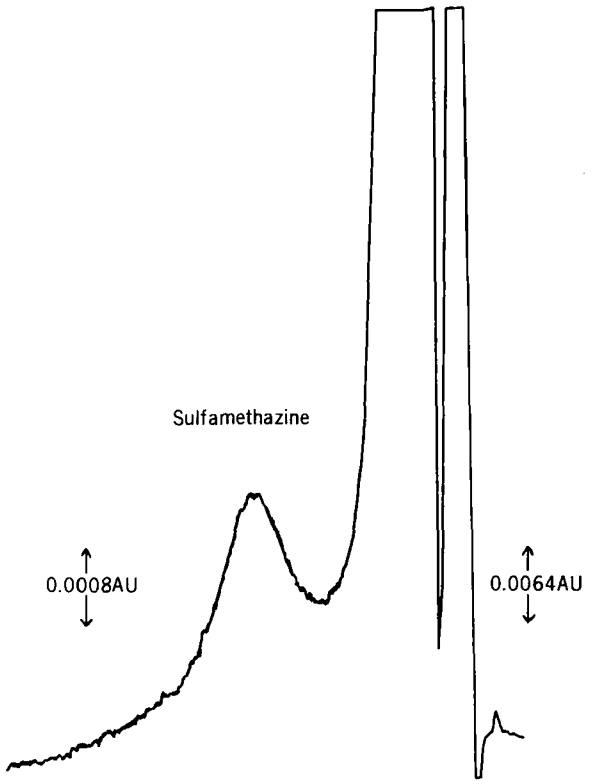

Figure 5—*Chromatogram of bovine kidney containing 0.37 ppm of sulfamethazine.*

paper[7], and the filtrate was collected in a 1-liter round-bottom flask. Two additional 2-min extractions of the tissue were made using 100-ml portions of acetone–chloroform (1:1) and filtering each extract. The filter was rinsed with 100 ml of the acetone–chloroform (1:1). (If water from the muscle tissue seeped through the filter, the filtrate was refiltered through fresh paper.)

The filtrate was evaporated to an oily residue, using a hot water bath at 60° and a rotary thin-film vacuum evaporator[8], and was transferred to a 250-ml separator. The following rinses were used: (*a*) 4 × 25 ml of *n*-hexane, (*b*) 1 × 3 ml of acetone, (*c*) 2 × 10 ml of 3.5 *M* hydrochloric acid (the flask was heated in hot water during this rinse), (*d*) 1 × 3 ml of acetone, and (*e*) 2 × 25 ml of *n*-hexane.

The mixture was shaken gently for 2 min. The emulsion, if present, was allowed to separate. The aqueous layer, including the emulsion, was drained into a 125-ml separator. The organic layer was washed with 3 × 10-ml portions of 1 *M* hydrochloric acid, shaking more vigorously on each successive wash, and the washes were combined in the 125-ml separator. The combined hydrochloric acid extracts were washed gently with 5 ml of chloroform, and the chloroform was discarded. The chloroform wash was repeated twice, shaking more vigorously on each succeeding wash.

The pH of the aqueous phase was adjusted to 8.5–9.0 using 14 *M* ammonium hydroxide. The ammoniacal solution was then washed with 3 × 5-ml portions of chloroform, and the washes were discarded. The aqueous phase was transferred to a 250-ml wide mouth erlenmeyer flask, and the separator was rinsed with a small amount of water. The solution was evaporated on a hot plate until the salts present began to precipitate. Then the mixture was cooled and transferred to a 50-ml glass-stoppered graduated cylinder using 1 *M* hydrochloric acid and diluted to 30 ml with 1 *M* hydrochloric acid. A portion was filtered through a 0.45-μm membrane filter[9] and transferred to a screw-capped test tube. Fifty microliters was injected into the liquid chromatograph under the same conditions as the standard calibration curve.

The corrected area (0.04 aufs) was determined using Eq. 1, and the amount of sulfamethazine present in the tissue was determined from the standard calibration curve. The results obtained from this study are shown in Table I. Representative chromatograms of actual tissue samples are shown in Figs. 2–5.

[7] Whatman PS-1 phase-separating paper.
[8] Rinco Inc.
[9] Nalge 245, Sybron Corp., Rochester, N.Y.

Table II—Sulfamethazine Recovery Data

Sample Number	Parts per Million Added	Peak Area	Corrected Area	Parts per Million Recovered	Percent
		Bovine Fat			
1	0.1	125	127	0.042	42
2	0.4	363	725	0.246	61
3	0.4	296	592	0.200	50
4	0.4	336	672	0.228	57
5	1.6	361	2886	0.978	61
6	1.6	433	3465	1.17	73
				Average recovery	57
		Bovine Muscle			
1	0.1	240	240	0.081	81
2	0.1	228	228	0.077	77
3	0.1	317	317	0.107	107
4	0.4	352	704	0.240	60
5	0.4	975	975	0.330	83
6	0.4	876	876	0.296	74
7	1.6	418	3344	1.14	71
8	1.6	415	3323	1.13	71
9	1.6	535	4282	1.45	91
				Average recovery	79
		Bovine Liver			
1	0.1	172	172	0.058	58
2	0.1	170	170	0.058	58
3	0.4	383	767	0.260	65
4	0.4	320	640	0.217	54
5	0.4	362	724	0.245	61
6	1.6	420	3360	1.14	71
7	1.6	432	3456	1.17	73
8	1.6	378	3024	1.02	64
9	1.6	387	3096	1.05	66
				Average recovery	63
		Bovine Kidney			
1	0.1	149	149	0.050	50
2	0.1	188	188	0.064	64
3	0.4	371	741	0.251	63
4	0.4	564	1128	0.382	96
5	1.6	383	3066	1.04	65
6	1.6	375	3000	1.02	64
7	1.6	337	2700	0.91	57
				Average recovery	66

Recoveries—Sulfamethazine-negative muscle, kidney, fat, and liver tissue were obtained from bovines with a negative antibiotic and sulfonamide treatment history for at least 60 days. Eight control samples (two of each tissue) were then assayed according to the procedure outlined here. Seven were found to be negative; one muscle control was positive but was calculated to be <0.02 ppm.

Six to nine samples of each kidney, fat, muscle, and liver tissue were individually "spiked" with the water dilutions of sulfamethazine prepared in the *Standards* section. Each 1 ml of the 0.01-, 0.04-, 0.16-, and 0.32-mg/ml standard added to 100 g of sample (1–2-mm slices) and mixed well corresponds to 0.1, 0.4, 1.6, and 3.2 ppm of sulfamethazine in the tissue, respectively. These recoveries were assayed according to the procedure, and results are shown in Table II.

DISCUSSION

Several publications (3, 5–7) reported the recoveries of sulfonamides utilizing the colorimetric assays, and all reported values of 75–86%. Only Houston and Umstead (7) reported on sulfamethazine, and these results were a combination of sulfamethazine–sulfathiazole residues in milk.

The major points critical to the sensitivity of this method are the success of the "cleanup" steps and the "noise level" of the liquid chromatograph.

The tissue component eluting just before the sulfamethazine must be reduced to a level so that its tail is not so steep that it ren-

ders quantitation of small sulfamethazine peaks impractical. The chloroform wash of the basic aqueous mixture seems to affect this component most. Overwashing at this point, however, causes a loss of sulfamethazine.

Homogenizing or grinding the tissue prior to extraction tends to improve recovery somewhat but usually generates cleanup difficulties which negate the improved recovery.

Techniques critical to reducing the noise level of the liquid chromatograph include the following:

1. The solvent should be degassed to minimize the effect of small bubbles which are not visible but give detector response. This procedure also helps prevent buildup of air in the pump head, which would result in pulsations.
2. All fittings should be very tight to eliminate even the smallest leaks.
3. Detector electronics and cell windows should be dry, and any air or gas used to purge the reference cell should be scrupulously clean and dry.
4. In some instances, an arrangement to equilibrate the solvent thermally to the temperature of the detector might be helpful.
5. The electrical line to which the chromatograph is connected must be free of interference and large fluctuations. Both the chromatograph and the recorder should be well grounded.
6. The final aqueous solution must be adjusted to pH 8.5–9.0 before evaporation to reduce the volume. If the solution is acidic at this point, the sulfamethazine can be hydrolyzed to sulfanilamide and sulfanilic acid which elute with tissue components.

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(2) "The United States Pharmacopeia," 18th rev., Mack Publishing Co., Easton, Pa., 1970, p. 695.

(3) F. Tishler, J. L. Sutter, J. N. Bathish, and H. E. Hagman, *J. Agr. Food Chem.*, **16**, 50(1968).

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[x] To whom inquiries should be directed.

Comparative Pharmacokinetics of Coumarin Anticoagulants XIV: Relationship between Protein Binding, Distribution, and Elimination Kinetics of Warfarin in Rats

AVRAHAM YACOBI and GERHARD LEVY [x]

Abstract □ The relationships between the protein binding, distribution in the body, and kinetics of elimination of warfarin were studied. Individual rats eliminated warfarin by apparent first-order kinetics, with a biological half-life of 5.9–41 hr and a total plasma clearance of 2.4–22 ml kg^{-1} hr^{-1}. There is a strong positive correlation between the apparent volume of distribution (V_d) and the elimination rate constant (k_{el}). There was no apparent concentration dependence of warfarin binding to serum proteins over a wide concentration range, but there were pronounced intersubject variations in protein binding, with the free fraction of drug (f) in serum ranging from 0.172×10^{-2} to 1.53×10^{-2}. There are strong positive correlations between f and k_{el}, f and V_d, and f and the kidney–serum concentration ratio of warfarin. Consistent with theory, there is an excellent positive linear correlation between f and total plasma clearance of the drug. The intersubject variation in f is not related to variations in serum albumin or total protein concentration. There is a strong correlation between values of f for serum and liver homogenate in individual animals, consistent with the lack of correlation between f in serum and the liver–serum concentration ratio of warfarin. These results show that the pronounced intersubject variation in the elimination of warfarin observed in this investigation was related to interindividual differences in plasma protein binding of the drug. The differences in protein binding cannot be ascribed to differences in plasma protein concentrations and may reflect configurational differences of proteins or the presence of an endogenous displacing agent at different concentrations.

Keyphrases □ Coumarin anticoagulants—relationship between protein binding, distribution, and elimination kinetics of warfarin in rats □ Warfarin—relationship between protein binding, distribution, and elimination kinetics in rats □ Plasma protein binding, warfarin—relationship to distribution and elimination, rats

The anticoagulant warfarin is a highly plasma protein bound drug with pronounced intersubject variability in its elimination kinetics in humans and animals (1–7). In this investigation, the relationships between the protein binding, distribution in the body, and kinetics of elimination of warfarin were studied in individual rats. A theoretical basis for a relationship between plasma protein binding and the total clearance of warfarin by the body was presented in a preliminary report (8).

EXPERIMENTAL

The studies were carried out on adult male Sprague–Dawley rats, weighing 400–450 g. They were selected from a larger number of animals on the basis of the results of a dicumarol screening test (9, 10) in order to obtain a group of animals with a wide difference

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The studies were carried out on adult male Sprague–Dawley rats, weighing 400–450 g. They were selected from a larger number of animals on the basis of the results of a dicumarol screening test (9, 10) in order to obtain a group of animals with a wide difference

Table I—Biological Half-Life, Apparent Volume of Distribution, and Total Clearance of Warfarin in Individual Rats

Rat	Half-Life, hr	Apparent Volume of Distribution, ml/kg	Total Clearance, ml kg^{-1} hr^{-1}
1	5.91	184	21.6
2	8.13	189	16.1
3	8.80	206	16.2
4	8.93	198	15.4
5	9.24	202	15.2
6	10.3	189	12.7
7	10.3	204	13.7
8	16.7	150	6.2
9	17.7	145	5.7
10	18.6	151	5.6
11	21.1	137	4.5
12	21.9	141	4.5
13	41.3	140	2.4

in warfarin elimination kinetics. The rats received an intravenous injection of ^{14}C-warfarin, 0.6 mg/kg, and blood samples were obtained at frequent intervals.

The rats were killed at a time after injection when the warfarin concentration in plasma was expected to have decreased to about 0.4 μg/ml. They were killed by removing all blood from the aorta under ether anesthesia. Liver and kidneys were removed rapidly, each was cut into five or six slices, and the slices were compressed slightly between paper tissue to remove any remaining blood. The organs were weighed and homogenized with two (liver) or five (kidneys) volumes of ice-cold 0.9% sodium chloride solution, using a homogenizer[1] with a 50-ml Teflon-coated stainless steel sealed container at a rheostat setting of 70 for about 1 min. The blood samples were kept at room temperature for 15 min, and then the serum was separated by centrifugation at 1000×*g* for 10 min.

Warfarin concentrations in serum and plasma were determined by scintillation spectrometry following extraction and TLC, as previously described (7). Warfarin concentrations in liver and kidney homogenates were determined similarly after extracting a mixture of 1 ml of homogenate and 1 ml of 0.3 *N* hydrochloric acid with 10 ml of ethylene dichloride. Recovery of added ^{14}C-warfarin from these homogenates was 85 ± 5% (mean ± *SD*, n = 12) and was independent of concentration in the 0.05–2.5-μg/ml range.

Serum protein binding determinations were carried out by equilibrium dialysis at 25° and subsequently with serum from 12 additional rats at both 25 and 37°. Undiluted serum obtained from the individual animals at the end of the experiment, with no added drug (*i.e.*, with only the concentrations of warfarin and metabolites from the injection of warfarin), was dialyzed for 20 hr against isotonic 0.15 *M* pH 7.4 phosphate buffer in Plexiglas cells separated

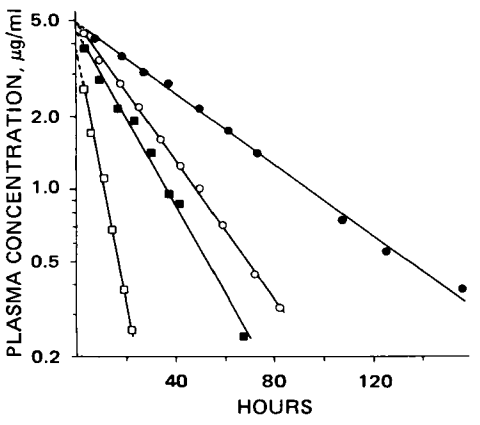

Figure 1—*Concentration of warfarin in the plasma of four rats as a function of time after intravenous injection of ^{14}C-warfarin, 0.6 mg/kg. Key: □, Rat 1; ■, Rat 8; ○, Rat 11; and ●, Rat 13.*

[1] Lourdes Multi-Mix, Lourdes Instrument Corp., Brooklyn, N.Y.

Table II—Concentrations of Warfarin in Liver, Kidneys, and Serum of Individual Rats at Approximately Similar Serum Concentrations

Rat	Warfarin Concentration, μg/g or ml			Concentration Ratio	
	Liver	Kidneys	Serum	Liver–Serum	Kidney–Serum
1	0.977	—[a]	0.288	3.39	—[a]
2	0.976	—[a]	0.590	1.65	—[a]
3	0.764	0.229	0.244	3.13	0.94
4	1.31	—[a]	0.517	2.53	—[a]
5	0.863	—[a]	0.494	1.75	—[a]
6	0.933	—[a]	0.408	2.29	—[a]
7	0.895	0.293	0.313	2.86	0.94
8	0.897	0.218	0.297	3.02	0.73
9	0.851	—[a]	0.491	1.73	—[a]
10	1.07	0.254	0.369	2.89	0.69
11	0.710	0.214	0.380	1.87	0.56
12	1.13	0.225	0.393	2.88	0.57
13	0.942	0.267	0.375	2.51	0.71
Mean	0.948	0.243	0.397	2.50	0.73
SD	0.156	0.028	0.101	0.59	0.16

[a] Not determined.

by a cellophane membrane[2]. Liver binding was determined similarly, using liver homogenate diluted with 0.15 *M* pH 7.4 phosphate buffer to yield a final concentration of 1 g of liver/10 ml. The liver homogenates were spiked with ^{14}C-warfarin, 0.3 μg/ml of homogenate.

Total protein concentrations in serum were determined by the method of Gornall *et al.* (11), using rat albumin as the standard. The fraction of albumin and other serum proteins was determined by electrophoresis[3], and concentrations of these proteins were determined from the product of the fraction and total protein concentration. The coefficient of variation of the serum albumin concentration determinations averaged 3.7% in six determinations on each of five samples.

For the correlation studies between certain pharmacokinetic parameters and serum protein binding, additional data from control experiments of other studies[4] were included. The fraction of warfarin *(F)* in the liver or kidneys relative to the amount of warfarin (free and protein bound) in the entire body was calculated from Eq. 1:

$$F = W_t C_s{}^0 C_t (C_s \times \text{dose})^{-1} \qquad \text{(Eq. 1)}$$

where W_t is the weight of the organ, C_t is the drug concentration

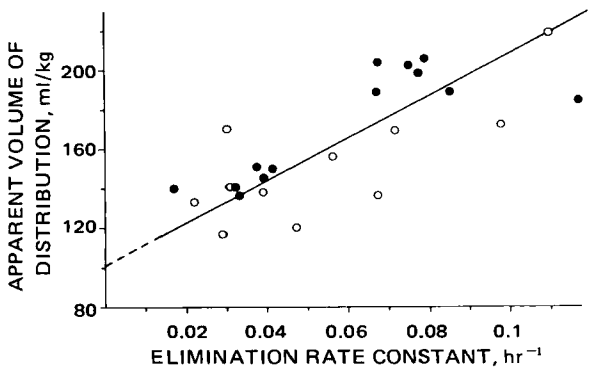

Figure 2—*Relationship between the apparent volume of distribution and the apparent first-order elimination rate constant of warfarin in individual rats that received an intravenous injection of ^{14}C-warfarin, 0.6 mg/kg (r = 0.761, p < 0.001). Key: ●, data from this study; and ○, data from another study (to be published).*

[2] Cut from dialysis tubing, average pore radius 24 Å, VWR Scientific, Rochester, N.Y.

[3] Gelman serum protein electrophoresis system, Gelman Instrument Co., Ann Arbor, Mich.

[4] To be published.

Table III—Effect of Concentration on Protein Binding of Warfarin in Rat Serum[a]

Rat A		Rat B	
Concentration, μg/ml	Free Fraction (f) × 100	Concentration, μg/ml	Free Fraction (f) × 100
0.325	0.364	0.285	1.06
0.620	0.328	0.588	1.20
1.22	0.347	1.25	1.13
3.70	0.387	2.55	1.14

[a] Serum from unmedicated animals was spiked with ^{14}C-warfarin.

in the organ, C_s^0 is the zero-time drug concentration in plasma, and C_s is the drug concentration in the serum when the liver and kidneys were removed. Other experimental details and the methods of pharmacokinetic analysis were described previously (7).

RESULTS

The biological half-life, the apparent volume of distribution, and the total plasma clearance of warfarin in 13 rats are listed in Table I. Figure 1 shows the time course of plasma concentrations in the animals with the shortest and longest half-lives of warfarin and in two animals with intermediate half-life values. There is a positive and highly statistically significant correlation between the apparent volume of distribution and the apparent first-order elimination rate constant for warfarin in individual rats (Fig. 2).

The average serum warfarin concentration when the animals were killed was about 0.4 μg/ml (Table II). The concentrations of warfarin in the liver were about 2.5 times higher on the average, while the concentrations in the renal tissues were 56–94% of the serum concentrations (Table II). The livers contained about 44% of the total amount of warfarin in the body on the average (F_{liver} = 0.44); the kidneys contained only 2.7% on the average. There is no significant correlation between F_{liver} and the elimination rate constant of warfarin.

The extent of serum protein binding of warfarin, expressed as the free fraction (f_{serum}), varied widely between animals, with f_{serum} values ranging from 0.00172 to 0.0153 at 25°, equivalent to 98.47–99.83% protein binding. The f_{serum} value in any one animal was essentially constant over a wide concentration range, irrespective of whether the animal was one with a relatively high or low f_{serum} value (Table III). There is an excellent correlation between f_{serum} values at 25 and 37° for the serum from any one animal (Fig. 3); the average ratio of f_{serum} values, 37°/25°, was 1.32.

There are highly statistically significant positive correlations between f_{serum} and the apparent volume of distribution (Fig. 4) and between f_{serum} and the elimination rate constant of warfarin (Fig. 5) in individual rats. The f_{serum} values obtained at 25° were multiplied by 1.32 to obtain equivalent values at 37° since f_{serum} determinations in subsequent studies were done at the higher temperature.

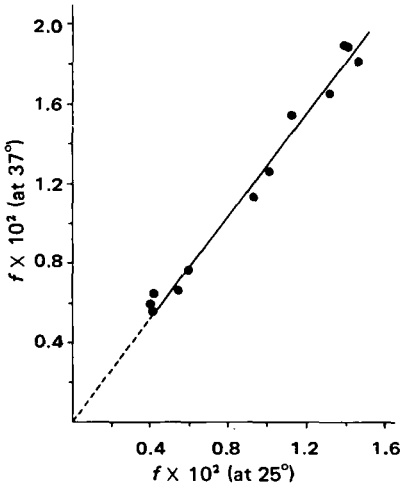

Figure 3—*Effect of temperature on serum protein binding of warfarin. The graph shows the relationship between the free fraction of warfarin at 25 and 37° in the same samples of serum, with a warfarin concentration of 0.3–2.1 μg/ml (r = 0.989, p < 0.001). The average ratio of the free fraction at 37 and 25° is 1.32.*

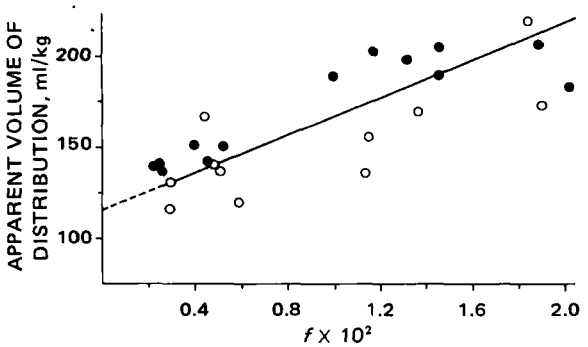

Figure 4—*Relationship between the apparent volume of distribution of warfarin and the fraction of free drug in serum of individual rats (r = 0.787, p < 0.001). Symbols are as given in Fig. 2. The ● values in this figure and in Figs. 5–7 are the experimental values multiplied by 1.32 to obtain f at 37°.*

A theoretical pharmacokinetic equation was formulated recently which predicts a linear correlation between the total plasma or serum clearance of warfarin and f_{serum}, with a zero intercept (8). The experimental data are in excellent agreement with this theory, showing a strong and statistically highly significant positive linear correlation between total clearance and f_{serum}, with the intercept of the regression line close to the origin (Fig. 6).

There is no relationship between individual f_{serum} values and either the albumin or total protein concentration in the serum of a total of 42 rats (Fig. 7). Binding studies carried out with diluted, ^{14}C-warfarin-spiked liver homogenates from seven rats yielded f_{liver} values that correlate strongly and highly statistically significantly (r = 0.946, $p < 0.005$) with f_{serum} values in the same animals (Table IV). To rule out the possibility that this correlation is an artifact due to retained blood in the liver, f_{serum} values in 1:100 diluted serum were determined (Table V). The significance of these data will be considered under *Discussion*.

There is no correlation between the liver–serum concentration ratio of warfarin (Table II) and f_{serum}, but there is a strong and highly statistically significant correlation between the kidney–serum concentration ratio and f_{serum} in individual rats (r = 0.918, $p < 0.005$).

DISCUSSION

The results of this investigation indicate that plasma protein binding is the major determinant of the pronounced interindividual differences in warfarin elimination kinetics in the rats studied.

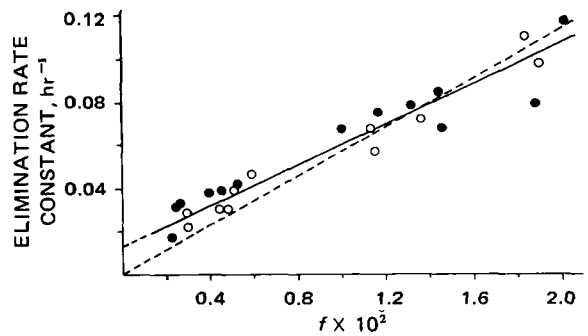

Figure 5—*Relationship between the apparent first-order elimination rate constant of warfarin and the fraction of free drug in the serum of individual rats (r = 0.970, p < 0.001). Symbols are as given in Fig. 2. The continuous line was fitted to the data by the double regression method (13); the stippled line was forced through the origin.*

Table IV—Protein Binding of Warfarin in Serum and Liver Homogenate of Individual Rats

Rat[a]	Free Fraction[b] × 100	
	Serum	Liver[c]
3	1.43	18.0
7	1.11	20.8
8	0.396	9.80
10	0.300	9.04
11	0.198	9.22
12	0.191	7.26
13	0.172	7.46

[a] Determinations of free fraction in liver homogenate of Rats 1, 2, 4, 5, 6, and 9 were not made. [b] At 25°. [c] A 1:10 homogenate of liver containing about 0.1 μg/ml (final concentration) of added ^{14}C-warfarin.

It was suggested earlier (8) that:

$$\text{total plasma clearance} = k''f_{serum} \qquad \text{(Eq. 2)}$$

where k'' is an intrinsic clearance constant equal to the product of k' (an intrinsic elimination rate constant) and V_w (a physiological space consisting of plasma water and other water exclusive of dissolved macromolecules capable of binding warfarin). The results of this investigation are in excellent agreement with this prediction (Fig. 6).

These results imply that the activity of the warfarin-metabolizing enzyme system(s) did not vary significantly between different rats under the experimental conditions. A subsequent study[4], in which the activity of the warfarin-metabolizing enzyme system(s) was altered by pretreatment of the animals with an enzyme inhibitor, did show a change in k'' value (*i.e.*, a decrease in the slope of the total clearance *versus* f_{serum} plot) as a result of such treatment.

The experimental data shown in Figs. 4 and 5 yield the empirical relationships:

$$V_d = a + bf_{serum} \qquad \text{(Eq. 3)}$$

$$k_{el} = a' + b'f_{serum} \qquad \text{(Eq. 4)}$$

The experimental value of a' in Eq. 4 does not differ significantly from zero, as should be the case if the rate of biotransformation is a function of only the free drug concentration. Combination of Eqs. 3 and 4 yields:

$$V_d k_{el} = aa' + (ab' + a'b)f_{serum} + bb'f_{serum}^2 \qquad \text{(Eq. 5}a\text{)}$$

or:

$$\text{total clearance} = k_1 + k_2 f_{serum} + k_3 f_{serum}^2 \qquad \text{(Eq. 5}b\text{)}$$

Equation 5*b* suggests a parabolic rather than a linear relationship between total clearance of warfarin and f_{serum}. The curvature of the parabola is, however, very slight over the range of the f_{serum} values observed in this study (Fig. 8). It is not feasible, therefore, to distinguish on the basis of the experimental data between the linear relationship represented by Eq. 2 (which is based on theoretical considerations) and the parabolic relationship represented by Eq. 5*b* (which was obtained empirically by assuming that Eqs. 3 and 4 describe the experimental data in Figs. 4 and 5). The apparently linear relationship between V_d and k_{el} (Fig. 2) is consistent with the reasonably good fit of the experimental data to Eqs. 3 and 4. When a' is assumed to be near zero, combination of the two equations by eliminating f_{serum} yields:

$$V_d = a + \frac{b}{b'}k_{el} \qquad \text{(Eq. 6)}$$

which describes the experimental data in Fig. 2 quite well.

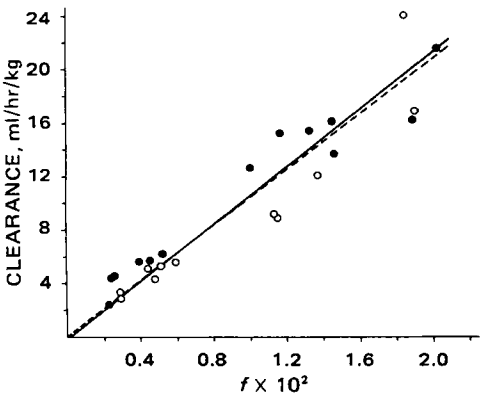

Figure 6—*Relationship between total plasma clearance of warfarin and free fraction of drug in the serum of individual rats (r = 0.947, p < 0.001). Symbols are as given in Fig. 2; regression lines are as given in Fig. 5.*

Table V—Effect of Dilution on Protein Binding of Warfarin in Rat Serum

Rat	Free Fraction[a] × 100	
	Undiluted Serum	Serum Diluted 1:100
1-V	0.560	26.7
4-V	0.660	33.3
9-V	1.65	57.9
12-V	1.89	58.3

[a] Undiluted serum contained 0.3–0.7 μg of ^{14}C-warfarin/ml; diluted serum contained 0.6–0.7 μg/ml. Binding studies were carried out at 37°.

Surprisingly, the striking interindividual differences in the serum protein binding of warfarin are not due to differences in the concentration of serum proteins (Fig. 7). The serum concentrations of albumin and total protein observed in this study are in good agreement with the concentrations reported by other investigators (12). The correlation between the extent of warfarin binding to serum proteins and liver in any one animal is another very significant observation. It is not an artifact due to retention of blood in the excised livers. Even if the excised livers retained as much as 10% of their weight of blood, the diluted liver homogenate (one part of liver in 10 parts of diluted homogenate) would contain only about 0.5% plasma.

The free fraction values obtained with a 1% aqueous solution of serum are much higher than the f_{liver} values obtained with the di-

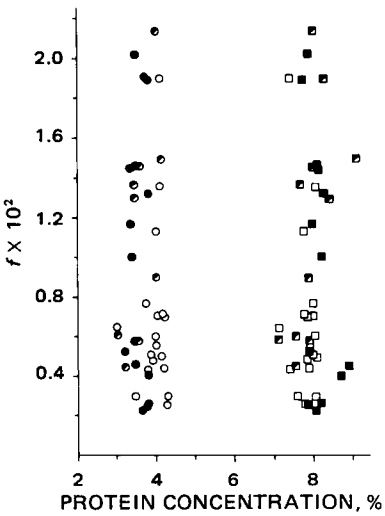

Figure 7—*Relationship between serum protein binding of warfarin and the concentrations of albumin (circles) and total protein (squares) in the serum of individual rats. Solid symbols represent data obtained in this investigation; open and half-open symbols represent data from two other studies carried out in this laboratory (to be published).*

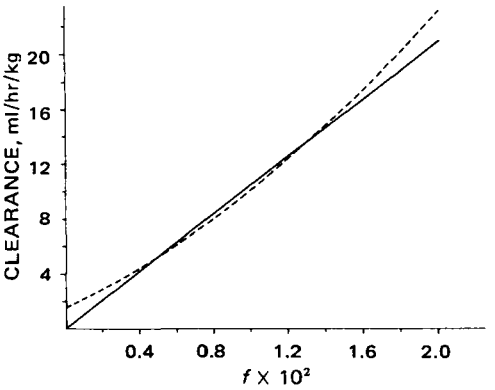

Figure 8—*Comparison of the directly determined relationship between total plasma clearance of warfarin and free fraction in serum (continuous line from Fig. 6) and the relationship as calculated from the regression equations for the data in Figs. 4 and 5 (stippled line).*

luted liver homogenates (Tables IV and V), showing that the liver tissues are responsible for the warfarin binding in liver homogenates. The fact that the liver–serum concentration ratio of warfarin is 2.5 (Table II), *i.e.*, considerably above unity, is consistent with this conclusion. Also consistent is the observation that the liver–serum concentration ratio of warfarin shows no correlation with f_{serum}; a parallel change in f_{serum} and f_{liver} would minimize any changes in the liver–serum concentration ratio as a function of f_{serum}. No direct studies of warfarin binding to kidney tissues were performed, but the fact that the kidney–serum concentration ratios correlate strongly with f_{serum} suggests that the f_{kidney} values do not change in parallel with the f_{serum} values in individual rats.

Since there is no correlation between f_{serum} and serum protein concentrations, one or more other factors must be responsible for the pronounced differences in the serum protein binding of warfarin. All animals were of the same strain and were obtained from the same supplier. They received identical food and housing. Whatever accounts for any one animal's f_{serum} value is unlikely to vary appreciably with time since the kinetics of warfarin elimination in any given animal are highly reproducible with time (9). Among the reasons potentially responsible for interindividual differences in protein binding of warfarin are *(a)* differences in the structure of certain plasma and liver proteins, and *(b)* the presence of an endogenous displacing agent at different concentrations in different animals. These and other mechanisms, as well as possible genetic influences on them, are being explored.

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Photolytic Degradation of α-[(Dibutylamino)methyl]-6,8-dichloro-2-(3′,4′-dichlorophenyl)-4-quinoline Methanol: An Experimental Antimalarial

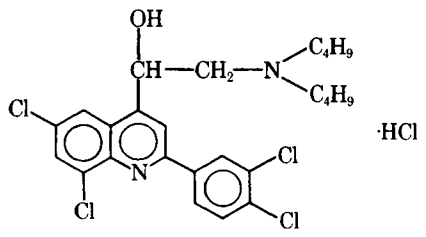
H. OKADA *§, V. STELLA *x, J. HASLAM ‡, and N. YATA *¶

Abstract □ A study of the effects of various storage conditions on the rate and products of degradation of the quinoline methanol antimalarial agent, α-[(dibutylamino)methyl]-6,8-dichloro-2-(3′,4′-dichlorophenyl)-4-quinoline methanol, was undertaken. The degradation was followed by high-pressure liquid chromatography and TLC in oxygenated and deoxygenated methanol, ethanol, chloroform, and chloroform–heptane mixtures under UV and laboratory fluorescent lighting irradiation, as well as in the absence of light. The kinetics of degradation confirmed the major catalyzing factor to be UV irradiation. The compound was stable in the absence of light and reasonably stable under fluorescent lighting both in the presence and absence of oxygen. The degradation resulted in a major product, 6,8-dichloro-2-(3′,4′-dichlorophenyl)-4-quinolinecarboxaldehyde, whose structure was confirmed by elemental analysis and IR, NMR, and mass spectral data.

Keyphrases □ α-[(Dibutylamino)methyl]-6,8-dichloro-2-(3′,4′-dichlorophenyl)-4-quinoline methanol—photolytic degradation □ Photolysis—degradation of α-[(dibutylamino)methyl]-6,8-dichloro-2-(3′,4′-dichlorophenyl)-4-quinoline methanol

The antimalarial agent α-[(dibutylamino)methyl]-6,8-dichloro-2-(3′,4′-dichlorophenyl)-4-quinoline methanol monohydrochloride[1] (I) has been shown to be useful in the treatment of resistant strains of *Plasmodium vivax* and *Plasmodium falciparum*. However, it has been shown to cause photosensitivity in some experimental animals and humans, although this effect is minor when compared to a number of other quinoline methanol antimalarials.

In the process of accumulating data on the physical and chemical properties of I and the free base form (II) in various organic solvents, it was noted that chemical degradation was occurring, particularly in standard solutions of II dissolved in chloroform and blood extractant solutions of II that were allowed to stand in the presence of laboratory lighting. Therefore, a study of the effects of various solvents and storage conditions on the rate and products of degradation of II was undertaken. The products of such degradation were of interest from a mechanistic viewpoint as well as from a toxicity viewpoint. That is, could the products of photolysis of II be the agents causing the phototoxicity reaction in animals and humans.



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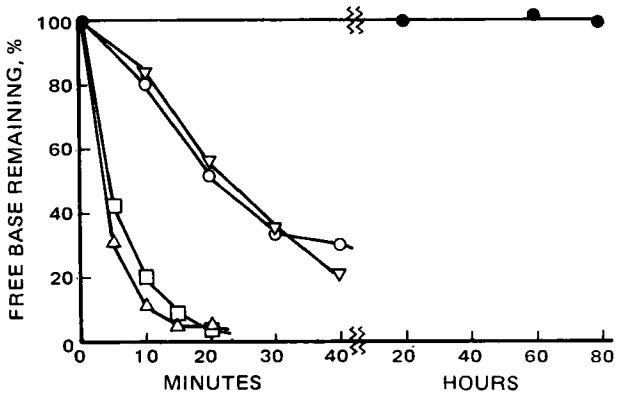


Figure 1—*Plot of percent of II remaining in various organic solvents as a function of time. Open points represent solutions exposed to direct sunlight; closed points represent a solution protected from direct irradiation or exposed only to laboratory fluorescent lighting. Key:* □, *methanol (0.001% II);* △, *20% methanol–ethanol (0.001% II);* ○, *chloroform (0.005% II); and* ▽, *20% chloroform–heptane (0.005% II).*

Most of the work was done on II and not I because preliminary data indicate that II is the predominant form of I at physiological pH[2].

RESULTS AND DISCUSSION

Degradation of II, which was followed quantitatively by high-pressure liquid chromatography (HPLC) and qualitatively by TLC, was found to be strongly catalyzed by UV irradiation, but solutions of II in various solvents were stable when protected from light. Exposure to normal laboratory fluorescent lighting catalyzed the breakdown of II but only after 4–5 days of exposure. Typical plots of the loss of II from UV-irradiated solutions in various solvents, concentrations, and UV energy sources, as a function of time, are shown in Figs. 1 and 2.

Solutions of II in chloroform exposed to UV irradiation, with and without oxygen-free nitrogen bubbled through the solution, showed no quantitative or qualitative differences in their degradation rates or products.

Factors other than the presence of UV irradiation affected the rate of photolytic decomposition of II. These factors included:

1. The concentration of II dissolved in the organic solvent. The higher the concentration of II in the solvent, the smaller was the percent loss of II per unit time. This result is consistent with the degradation rate passing from a rate-determining loss of II to an apparent pseudo-zero-order degradation dependent on the amount of incident UV energy (1).

[1] This compound, abbreviated WR-30,090, is currently under testing by the Walter Reed Research Institute. WR-30,090 itself is the hydrochloride salt. Most work in organic solvent and extraction systems requires the use of the free base because of the poor solubility of the hydrochloride salt in organic solvents.

[2] The pKa of I in aqueous solvents is suspected to be unpredictably low. The loss of I and II from aqueous solution and their unusual physical and chemical properties in aqueous solutions will be discussed later.

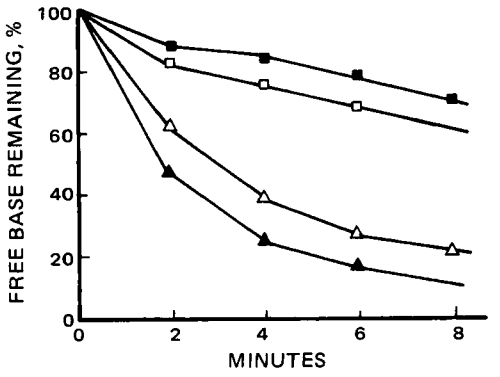


Figure 2—*Plot of percent of II remaining in various organic solvents containing various concentrations of II exposed to UV irradiation in a photochemical reactor at 3000 Å as a function of time. Key: ■, chloroform (0.005% II); □, chloroform (0.001% II); △, methanol (0.001% II); and ▲, methanol (0.0002% II).*

2. At the same concentration, the degradation of II was solvent dependent. UV-irradiated solutions of II degraded faster in methanol and methanol–ethanol than in chloroform and chloroform–heptane solvents.

3. Compound I, *i.e.*, the hydrochloride salt, was stable to short-term exposure to UV irradiation. Long-term exposure (>1 day) to irradiation at 2537 and 3000 Å in a photochemical reactor produced degradation, but the degradation products were numerous and not the same as the degradation products of II.

4. The degradation of II was quenched by the removal of the UV irradiation source; *i.e.*, the reaction was UV irradiation catalyzed and not just UV irradiation initiated. This result is adequately demonstrated in Fig. 3.

5. The UV-catalyzed degradation of I or II in aqueous and aqueous methanol solutions could not be studied because of apparent adsorption of II onto glass and its very poor aqueous solubility.

There did not appear to be any quantitative differences in degradation rates between solutions irradiated at 2537 or 3000 Å. However, corrections for light intensities, *etc.*, were not made.

Isolation of the major degradation product using column chromatography resulted in a yellow product, mp 195.5–197°, which gave methanol or ethanol solvates when recrystallized from methanol or ethanol solutions. The solvent-free compound gave a characteristic IR aldehyde carbonyl band at 1700 cm^{-1} and an aldehydic C—H stretch at 2860 cm^{-1}. Mass spectral data showed a peak at 368 and peaks at 370, 372, 374, and 376, consistent with P − 1, P + 1, P + 3, P + 5, and P + 7 peaks, respectively, of 6,8-dichloro-2-(3′,4′-dichloro)phenyl-4-quinolinecarboxaldehyde (III). A molecular weight of 35 was assumed as the base for chlorine. This structure was confirmed by NMR spectroscopy, and elemental analysis confirmed an empirical formula of $C_{16}H_7Cl_4NO$, the empirical formula for III (see *Experimental* for details).

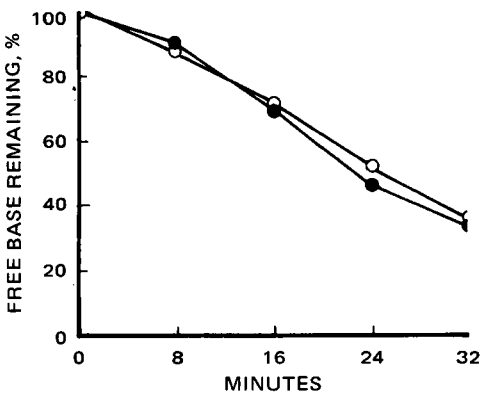
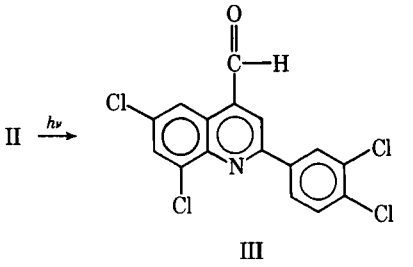


Figure 3—*Plot showing the effect of sunlight on the stability of II, 0.005% in chloroform. Key: ○, samples analyzed immediately upon sampling of the reaction mixture; and ●, samples taken at the same time but analyzed after 24 hr (these samples were protected from light during this period).*



III

Scheme I

A minor degradation product (as yet unidentified), mp 214–215.5°, was also isolated and is currently under investigation. By TLC, the reaction products did not appear to be solvent dependent. Isolation of the major degradation product from chloroform and methanol was accomplished. The characteristically yellow product, III (Scheme I), appeared to be the major degradation product in each case.

Davidson and Orton (2) recently showed that the fragmentation of 2-aminoethanols occurs in the presence of UV irradiation and suitable sensitizer molecules. Their results suggest that the mechanism illustrated in Scheme II may be involved in the fragmentation of 2-aminoethanols, where Y* is a sensitizer molecule such as anthraquinone, perylene, 2,3-diphenylquinoxaline, or acenaphtho[1,2-*b*]quinoxaline.

In the present study, it was initially felt that this sensitizer may have been the photoinduced free radicals in chloroform such as $Cl_3C\cdot$. Because the reactions proceeded just as fast if not faster in methanol and methanol–ethanol, it was felt that II, being a heterocyclic polychlorinated molecule, may be quite capable of acting as a sensitizer, catalyzing its own degradation *via* an autosensitizing photolytic decomposition.

The results of the present study concerning the decomposition of II to III in various organic solvents in the presence of UV irradiation appear consistent with the results of Davidson and Orton (2).

Because of the possible photoinduced fragmentation of other 2-amino alcohol antimalarial agents by UV irradiation to reactive aldehydes, a study of the susceptibility of these agents to photolysis and a correlation to their photosensitization properties might be desirable.

EXPERIMENTAL

Kinetic Studies—Solutions of II at various concentrations in a number of oxygenated and deoxygenated organic solvents were prepared. Methanol, methanol–ethanol, ethanol, chloroform, and chloroform–heptane were the various solvents[3] studied. Concentrations of II in the various solvents from 0.001 to 0.005% were studied. These solutions were exposed to sunlight, exposed to laboratory fluorescent lighting, protected from light by wrapping containers in aluminum foil, or photolyzed in a photochemical reactor[4] at 2537 or 3000 Å.

The degradation of II in the presence and absence of UV irradiation at ambient room temperature was followed by withdrawal of 5-μl samples from the quartz sample tubes at various predetermined times. Analysis of II was accomplished by HPLC[5]. The eluting solvent of choice was determined to be degassed and dried heptane–dioxane–methanol (80:20:5). An external standard of II dissolved in 20% chloroform–heptane protected from light was used. That this solution remained undegraded was confirmed by TLC. After isolation of one minor and one major degradation product, subsequent injections of these products on the HPLC column showed that neither interfered with the assay of II under the conditions described.

TLC using silica gel plates[6] with an eluting solvent of acetic

[3] All solvents were reagent grade quality from various sources.

[4] Rayonet Reactor, Southern New England Ultraviolet Co., Middletown, Conn. Two RPR 2537 Å lamps were used for the 2537-Å study, and two RPR 3000-Å lamps were used for the 3000-Å study. Ten-centimeter long quartz sample tubes, 1 cm in diameter, containing 5 or 15 ml of reaction solution provided the reaction vessel.

[5] Varian model 4000 liquid chromatograph equipped with a 280-nm detector and a Corasil II column (Waters Associates).

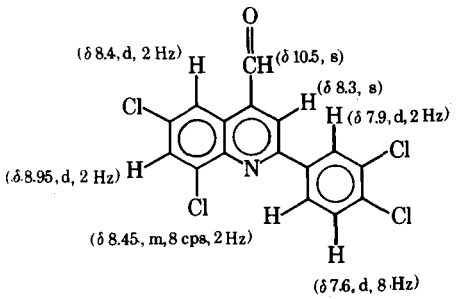
[6] Baker-Flex prepared silica gel plates.

acid–methanol–water (3:40:5) separated II satisfactorily from its apparent degradation products.

Preparation, Isolation, and Identification of Major Degradation Product—Eighty-seven milligrams of II dissolved in 50 ml of chloroform (or methanol) was exposed to direct UV irradiation in a photochemical reactor at 2537 Å for 2.5 hr. The photolyzed sample was evaporated to dryness, reconstituted in a minimum amount of chloroform, and added to a packed column[7]. The eluting solvent was chloroform. Fractionation resulted in the isolation of a small amount of unreacted II and approximately 50 mg of a yellow crystalline material, III, mp 195.5–197° (II, mp 129.5–130°; I, mp 215–218°).

Compound III was recrystallized from methanol or ethanol, either of which formed solvates with III, and dried overnight in a vacuum oven. TLC of III, using the previously described systems, showed a single spot with an R_f value of 0.70 (II, R_f 0.91), confirming it to be the major degradation material.

The IR spectrum[8] (KBr dispersion) of III gave a characteristic >C=O stretch at 1700 cm^{-1} and a C—H aldehyde stretch at 2860 cm^{-1}. NMR[9] of III, dissolved in $CDCl_3$ with tetramethylsilane as an internal standard, gave a spectrum consistent with the postulated aldehyde, III. The following assignments were tentatively made:

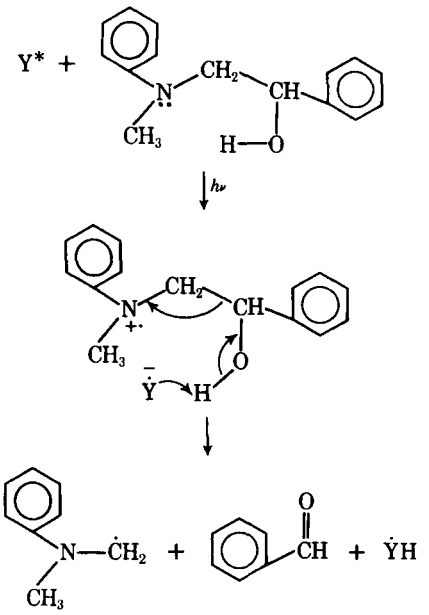


In the parentheses the first numbers refer to chemical shifts, in parts per million, from an internal standard of tetramethylsilane; s = singlet, d = doublet, and m = multiplet (also partially buried); and the last numbers are approximate spin-coupling constants. Integration was consistent with this structure when the single aldehydic proton was used as an internal standard.

If the molecular weight for the chlorine atom is assumed to be 35, the molecular weight of III is 369. A mass spectrum[10] of III showed a peak at 368 (P − 1), corresponding to $R—C{\equiv}O^+$, and a smaller P peak. The P + 1, P + 3, P + 5, and P + 7 peaks at 370, 372, 374, and 376 (as well as smaller P, P + 2, P + 4, P + 6, and P + 8 peaks), respectively, of magnitude relative to the P − 1 peak, 1.28:0.66:0.125:~0.01, confirm the presence of the four chlorine atoms in III. These relative peaks sizes are very close to the expected ratio of 1.31:0.64:0.14:0.01 (3). A peak corresponding to 369 − R′, where R′ = mass of —CHO at 340, is also consistent with the postulated structure of III.

Elemental analysis of III gave: C, 51.97; H, 2.08; N, 3.57. Calculated values for III, assuming the postulated structure, were: C, 51.75; H, 1.89; N, 3.77.

[7] Column containing 50 ml of Florisil, 100–200 mesh, packing material (Fisher Scientific Co.).
[8] Beckman IR-33 IR spectrophotometer.
[9] Varian T60 NMR spectrometer.
[10] Varian CH-5 mass spectrometer.



Scheme II

CONCLUSION

The degradation of the 2-aminoethanol (II), the free base form of I, in various organic solvents was found to be photoinduced, with the major degradation product being III. Compound II can be successfully extracted from blood or stored as standard solutions in various organic solvents as long as direct sunlight and long-term exposure to fluorescent lighting are excluded.

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GLC Determination of Plasma Drug Levels after Oral Administration of Clorazepate Potassium Salts

D. J. HOFFMAN and A. H. C. CHUN ˣ

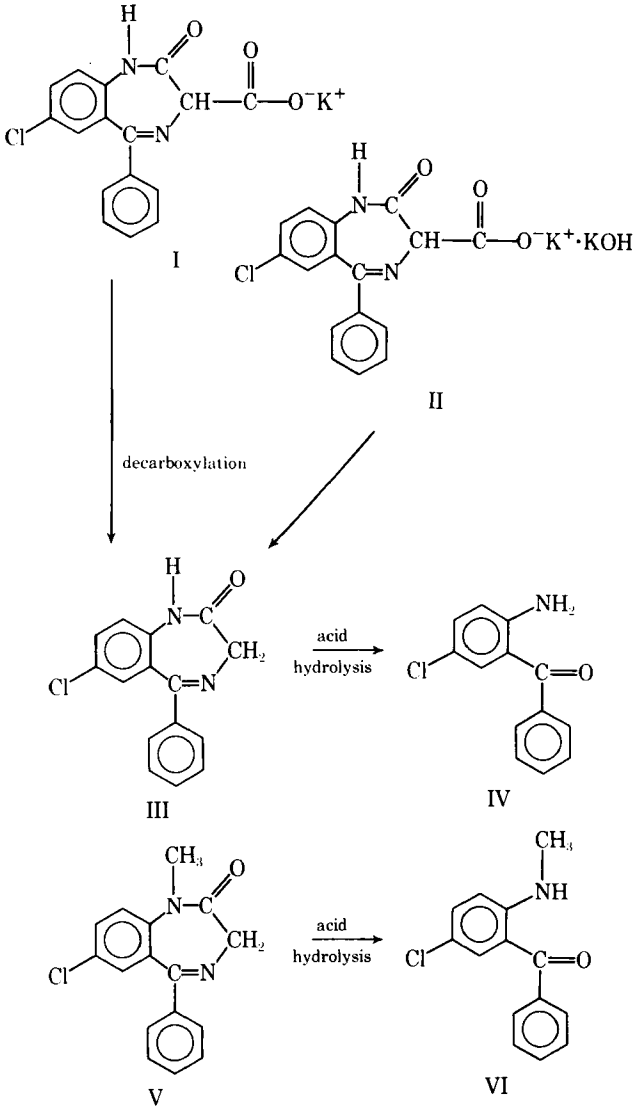
Abstract □ Plasma nordiazepam levels resulting from the oral administration of clorazepate potassium salts were determined by a sensitive GLC assay. Nordiazepam and the internal standard (diazepam) were selectively extracted into ether at pH 9.2, hydrolyzed to their respective benzophenones, and quantified by electron-capture detection. The assay was used in a comparative bioavailability study of single equimolar oral doses of monopotassium and dipotassium salts of clorazepate in dogs. Both clorazepate salts were rapidly absorbed and exhibited mean peak total drug levels after 1 hr. Clorazepate levels accounted for about 50% of the total drug levels present. No statistical difference in the plasma drug levels of clorazepate mono- and dipotassium salts and the metabolite was found in dogs.

Keyphrases □ Clorazepate monopotassium and dipotassium salts—determination of plasma levels after oral administration, dogs, GLC analysis of nordiazepam metabolite □ Nordiazepam—GLC analysis in plasma as clorazepate metabolite, dogs □ GLC—analysis, nordiazepam as clorazepate metabolite after oral administration, determination of clorazepate plasma levels

Clorazepate potassium salts (I and II) are new 1,4-benzodiazepine antianxiety agents (1–4). These salts are highly water soluble and rapidly decarboxylate (Scheme I) to nordiazepam (III) at a low pH. Decarboxylation also occurs at physiological pH but at a much slower rate[1].

This paper describes an assay procedure for nordiazepam (III) in plasma. The procedure is a modification of the GLC method of de Silva *et al.* (5), which converts III to 2-amino-5-chlorobenzophenone (IV) with subsequent quantification by GLC using electron-capture detection (Scheme I). Diazepam (V), which is converted by hydrolysis to 2-methylamino-5-chlorobenzophenone (VI), is used as the internal standard.

Plasma clorazepate levels were calculated from the difference between the total measured nordiazepam levels, by converting the residual parent drug (clorazepate) to nordiazepam, and the nordiazepam levels measured after immediate extraction. The method was applied to the determination of the bioequivalence of a single equimolar oral dose of clorazepate mono- and dipotassium salts in dogs.



Scheme I

EXPERIMENTAL

Drug Administration to Dogs—Twelve female beagle dogs of approximately equal weights were used in this crossover study to determine the bioequivalency of these two compounds after oral administration. Six dogs were randomly assigned 6.5-mg doses of the monopotassium salt or 7.5-mg doses of the dipotassium salt during the first period of the study. The administration of formulations to the dogs was reversed during the second period 1 week later. These doses were equivalent on a molar basis to 5.8 mg of the free acid. The capsule formulations were administered to the dogs in the morning after an overnight fast, and 30 ml of water then was given to each dog.

Blood samples were taken in 5-ml heparinized vacuum tubes before dosing and at 0.5, 1, 1.5, 2, 3, 4, 6, 8, 12, and 24 hr after dosing. Samples (3 ml) were cooled and centrifuged within 3 min after collection. One-half milliliter of the 0.5-, 1-, 1.5-, 2-, 3-, and 4-hr plasma samples was placed into tubes containing buffer and ether, and these samples were immediately extracted after collection to minimize conversion of clorazepate to nordiazepam (Scheme I). The remaining portion of the plasma samples was frozen until they were assayed.

Instrumental Conditions—Analysis was performed with a gas chromatograph[2] equipped with a ^{63}Ni electron-capture detector, containing a 2 mCi ^{63}Ni β-ionization source, and an electronic inte-

[1] R. C. Sonders and D. J. Anderson, Abbott Laboratories, North Chicago, IL 60064, 1974, unpublished data.

[2] Hewlett-Packard model 7620A.

Table I—Reproducibility of Plasma Nordiazepam Assay

Sample (0.15 μg/ml)	Peak Area IV	Peak Area VI	Peak Area Ratio
1	8491	6673	0.786
2	11320	8680	0.767
3	12150	9842	0.810
4	11760	9633	0.819
5	12750	9983	0.783
6	13450	11650	0.866
7	10570	9042	0.855
8	12910	10700	0.823
		Mean ± *SD*	0.813 ± 0.035
		Coefficient of variation = 4.31%	

grator[3]. Argon–methane[4] (95:5) was used as the carrier gas, and the column head pressure was preset at 40 psig. A 1.56-m (6-ft), 2-mm i.d. borosilicate helical glass column was packed with 3% Poly A-103 on Gas Chrom Q[5] and conditioned for 15 hr at 270° with the carrier gas flowing at 25 ml/min. Column life was 2–3 months when the carrier gas was passed through an oxygen trap[6].

The temperature settings were as follows: oven, 250°; injection port, 265°; and detector, 325°. The electrometer[7] range was set at 10^3, the output attenuation of the integrator was set at 2, and the recorder[8] chart speed was 0.6 cm (0.25 in.)/min. The pulse interval for the electron-capture detector was 150 μsec. Under these conditions, IV (hydrolysis product of III) and VI (5) (hydrolysis product of V; internal standard) had retention times of 5.0 and 3.9 min, respectively. By using the following procedure, the minimum measurable amount of III is 0.01 μg/ml of plasma.

Assay Procedure—*Preparation of Plasma Standards*—Prepare a nordiazepam primary standard of 100 μg/ml in ethanol. Use

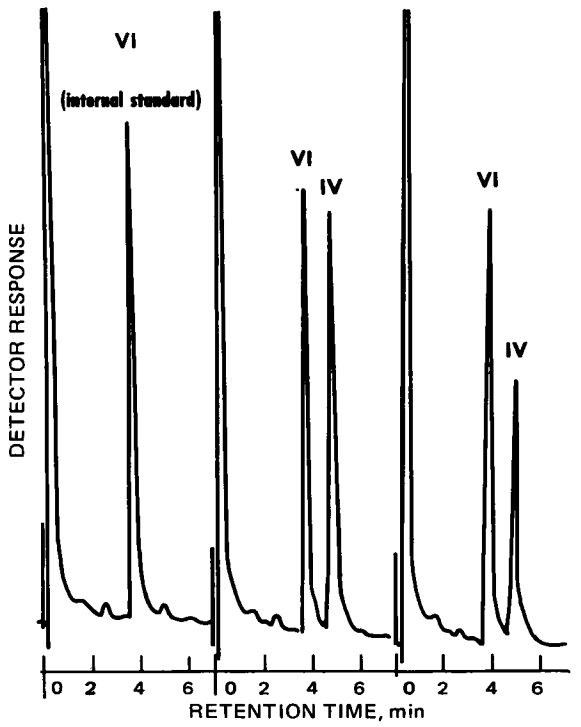


Figure 1—*Gas–liquid chromatograms (electron-capture detector) of plasma extracts: left, normal plasma specimen with internal standard; middle, plasma specimen after a single oral dose of a clorazepate salt; and right, plasma standard containing 0.15 μg/ml of nordiazepam.*

[3] Hewlett-Packard model 3370A.
[4] Matheson, oil pumped and dry.
[5] Applied Science.
[6] Altech Associates.
[7] Model 7650A.
[8] Model 7128A.

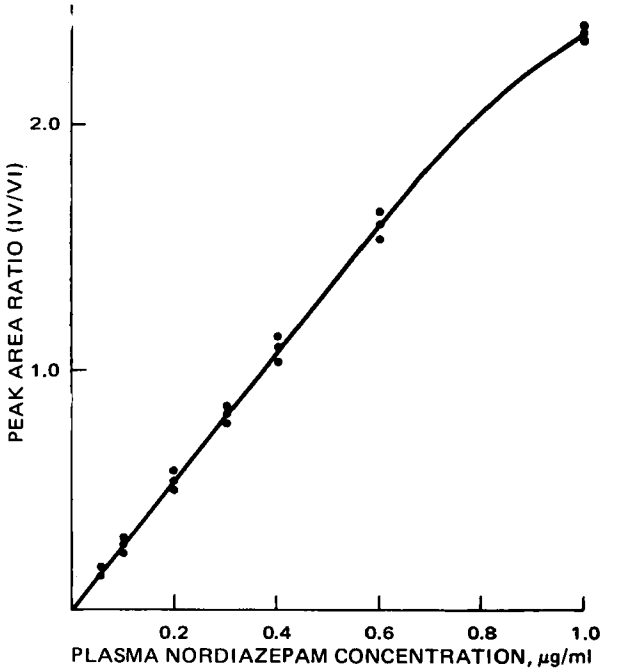


Figure 2—*Electron-capture detector calibration curve using the peak area ratio of IV/VI* versus *the micrograms per milliliter of nordiazepam in the plasma.*

1.0 ml of the ethanolic standard to prepare 100 ml of plasma standard at a concentration of 1.00 μg/ml. Further dilute the plasma to 0.50, 0.25, and 0.125 μg/ml with plasma.

Determination of Nordiazepam—Immediately centrifuge (within 3 min) freshly collected heparinized blood in a refrigerated centrifuge set at 2°. Transfer 0.5 ml of plasma to a 20-ml screw-capped test tube containing 5 ml of cold (5°) 0.1 *M* K_2HPO_4 buffer (pH 9.2) containing 0.10 μg of diazepam/ml (freshly prepared). Add ether[9] (7 ml) immediately, shake the tube on a reciprocal shaker at 250 cpm for 10 min, and then separate by centrifugation for 10 min at 2000 rpm. Transfer 5 ml of ether to a 20-ml screw-capped tube containing 5 ml of 6 *N* HCl. After shaking and centrifuging as already described, discard the ether layer by aspiration. Also aspirate off a small amount of the acid layer to assure complete removal of ether.

Place the samples in a water bath and heat to boiling with occasional shaking of the samples. Cap the tubes tightly and leave them in the boiling water bath for 1 hr to convert completely III to IV and V to VI. After cooling samples in an ice bath, add 5.5 ml of

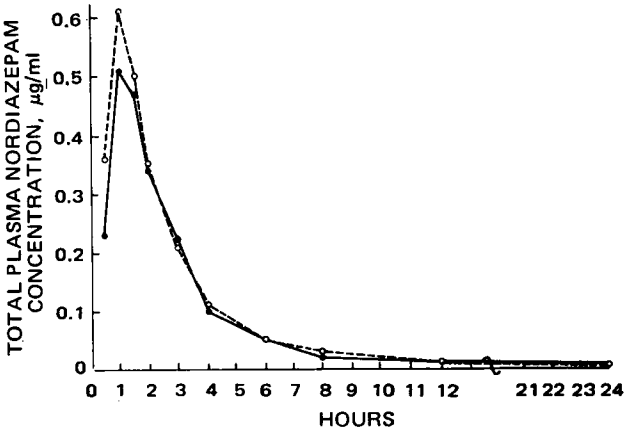


Figure 3—*Mean total plasma nordiazepam level curves in dogs (12 dogs/compound) following the oral administration of clorazepate salts. Key: ●, 6.5-mg dose of I; and ○, 7.5-mg dose of II.*

[9] Mallinckrodt, anesthetic grade.

Table II—Mean Plasma Nordiazepam (Immediate Extraction) Levels after Oral Administration of 6.5 mg of Clorazepate Monopotassium and 7.5 mg of Clorazepate Dipotassium to Dogs (12 Dogs/Compound)

Compound	Plasma Nordiazepam[a], μg/ml							Area under Curve[a], (0–4 hr)
	0 hr	0.5 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	
Clorazepate monopotassium								
Mean	0	0.10	0.29	0.26	0.19	0.11	0.06	0.61
SD		±0.12	±0.15	±0.09	±0.06	±0.06	±0.04	±0.22
Range		0.00–0.39	0.04–0.53	0.04–0.41	0.03–0.26	0.02–0.19	0.01–0.11	0.10–0.88
Clorazepate dipotassium								
Mean	0	0.14	0.31	0.24	0.19	0.12	0.07	0.64
SD		±0.12	±0.13	±0.11	±0.08	±0.08	±0.06	±0.30
Range		0.01–0.36	0.08–0.48	0.06–0.41	0.05–0.34	0.05–0.28	0.01–0.20	0.22–1.22

[a] No significant differences were found at the 0.05 level.

Table III—Mean Calculated Plasma Clorazepate Levels after Oral Administration of 6.5 mg of Clorazepate Monopotassium and 7.5 mg of Clorazepate Dipotassium to Dogs (12 Dogs/Compound)

Compound	Calculated Plasma Clorazepate[a], μg/ml							Area under Curve[a] (0–4 hr)
	0 hr	0.5 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	
Clorazepate monopotassium								
Mean	0	0.14	0.22	0.21	0.14	0.11	0.04	0.52
SD		±0.18	±0.25	±0.21	±0.14	±0.08	±0.04	
Clorazepate dipotassium								
Mean	0	0.22	0.30	0.25	0.16	0.09	0.04	0.62
SD		±0.13	±0.21	±0.19	±0.13	±0.06	±0.04	

[a] No significant differences were found at the 0.05 level.

6 *N* NaOH and 6 ml of ether[9]. Shake and centrifuge the samples as previously described.

Transfer 5 ml of the ether phase to a 15-ml conical screw-capped tube and evaporate to dryness at 50° with a stream of air. Dissolve the residue in 1.0 ml of benzene and inject 2 μl into the GLC. This procedure results in GLC tracings essentially free of coextracted impurities (Fig. 1).

Determination of Clorazepate—Plasma clorazepate concentrations[10] were determined by subtracting immediately extracted nordiazepam levels from the total drug levels obtained after hydrolysis and multiplying the difference by 1.16 to correct for the molecular weight difference.

Plasma nordiazepam concentrations were determined after immediate extraction under conditions that minimized *in vitro* conversion of clorazepate to nordiazepam. Another aliquot of the plasma sample was incubated at 40° overnight and extracted as previously described to determine the total drug concentration (nordiazepam and clorazepate). Incubation converts essentially all (>99%) of the clorazepate to nordiazepam[11].

Calculations—The peak area ratios of IV to VI are plotted against known standards of nordiazepam, expressed as micrograms per milliliter of plasma. Values for unknown concentrations of nordiazepam in plasma are read directly from the calibration curve (Fig. 2).

RESULTS AND DISCUSSION

Assay Reproducibility, Sensitivity, and Specificity—The analysis of eight plasma samples at 0.15 μg/ml gave a coefficient of variation of 4.31% (Table I). Comparable reproducibility was found with samples at other concentrations. Under the described assay conditions, the lower limit of the quantitative detection of nordiazepam in plasma is 0.01 μg/ml. This value is based on a sample signal equivalent to about 2% of full-scale response. A linear relationship between peak area ratios and concentration was obtained over the 0.01–0.80-μg/ml range. Quantification from a calibration curve was used (Fig. 2).

The method is not specific for nordiazepam. If significant levels of other 1,4-benzodiazepines, such as oxazepam, are present, they also are hydrolyzed to IV (6). Oxazepam, a metabolite of nordiazepam, is found almost exclusively in urine (7, 8) with small but insignificant amounts in plasma (9).

Serum or plasma samples for human subjects receiving clorazepate salts do not require incubation since little, if any, clorazepate is present[12].

Plasma Nordiazepam Levels in Dogs—Mean (±*SD*) nordiazepam peak levels of 0.29 ± 0.15 and 0.31 ± 0.13 μg/ml occurred 1 hr after oral administration of equimolar doses of the monopotassium and dipotassium salts, respectively. These levels declined to about 0.07 μg/ml at 4 hr (Table II). There were no significant differences by crossover analysis of variance ($p < 0.05$) in the nordiazepam levels at all sampling times and in the 0–4-hr area under the plasma level curve between the two compounds.

Plasma Clorazepate Levels—Plasma clorazepate levels were calculated based on the difference between total nordiazepam levels (parent drug plus metabolite) and nordiazepam levels (Table III). About one-half of the total nordiazepam level was present as clorazepate, although large variations (10–90% clorazepate) existed between dogs. However, no significant differences in clorazepate levels were obtained with both salts (Table IV).

Total Drug[13] Plasma Levels—Total plasma drug levels expressed as nordiazepam were determined after converting plasma clorazepate to nordiazepam and measuring the combined concentrations as nordiazepam. The mean total nordiazepam levels are given in Table IV. Both clorazepate salts were rapidly absorbed, with mean (±*SD*) peak total plasma nordiazepam levels of 0.51 ± 0.24 μg/ml for the monopotassium salt and 0.61 ± 0.24 μg/ml for the dipotassium salt (Fig. 3). Total levels declined to about 0.01 μg/ml at 12 hr and were undetectable after 24 hr.

There were no significant differences ($p < 0.05$) in plasma levels between the two clorazepate salts at all sampling times. Also, no significant differences were found between the areas under the plasma level curve for various time intervals (Table III). The total levels of combined parent drug and metabolite levels are more representative than either species alone in the assessment of bioavailability of these two potassium salts of clorazepate.

[10] Clorazepate concentrations are based on the free ionized form since both potassium salts would exist as such at a physiological pH.

[11] Clorazepate has an *in vitro* half-life of 70 min in dog plasma at 38°. This value was determined by the rate of formation of ether-soluble radioactivity from ^{14}C-clorazepate.

[12] A. H. Chun, Abbott Laboratories, North Chicago, IL 60064, 1974, unpublished data.

[13] "Total drug levels" is the term used for the combined levels of parent drug and metabolite measured as nordiazepam.

Table IV—Mean Total Plasma Drug Levels (Expressed as Nordiazepam) after Oral Administration of 6.5 mg of Clorazepate Monopotassium (I) and 7.5 mg of Clorazepate Dipotassium (II) to Dogs (12 Dogs/Compound)

Compound	Plasma Nordiazepam[a], μg/ml											Area under Curve[a]	
	0 hr	0.5 hr	1 hr	1.5 hr	2 hr	3 hr	4 hr	6 hr	8 hr	12 hr	24 hr	0–4 hr	0–12 hr
I													
Mean	0	0.23	0.51	0.47	0.34	0.22	0.10	0.05	0.02	0.01	0	1.12	1.41
SD		±0.21	±0.24	±0.19	±0.13	±0.09	±0.05	±0.03	±0.02	±0.02		±0.43	±0.56
Range		(0.00 −0.69)	(0.24 −1.16)	(0.28 −0.94)	(0.17 −0.58)	(0.07 −0.36)	(0.02 −0.20)	(0.01 −0.10)	(0.01 −0.07)	(0.00 −0.05	0.00 −0.01	0.60 −2.10	0.71 −2.80
II													
Mean	0	0.36	0.61	0.50	0.35	0.21	0.11	0.05	0.03	0.01	0	1.26	1.56
SD		±0.15	±0.14	±0.17	±0.13	±0.09	±0.06	±0.03	±0.02	±0.01		±0.34	±0.53
Range		(0.10 −0.62)	(0.42 −0.85)	(0.25 −0.84)	(0.16 −0.60)	(0.08 −0.36)	(0.02 −0.21)	(0.01 −0.11)	(0.00 −0.08)	(0.00 −0.04)	0.00 −0.00	0.64 −1.82	0.68 −2.34

[a] No significant differences were found at the 0.05 level.

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Hansch Analysis of Interaction of Haptens with Benzylpenicilloyl Antibodies

A. E. BIRD

Abstract □ Hansch analysis is applied to inhibition data obtained from the interaction of penicilloic and penilloic acid haptens with benzylpenicilloyl specific antibodies. Significant regressions with partition coefficient and molar volume parameters indicate the importance of hydrophobic and steric effects in these interactions.

Keyphrases □ Benzylpenicilloyl antibodies—interaction with penicilloic and penilloic acid haptens, Hansch analysis of inhibition data, hydrophobic and steric effects □ Penicilloic and penilloic acid haptens—interaction with benzylpenicilloyl specific antibodies, Hansch analysis of inhibition data □ Hansch analysis—interaction of haptens with benzylpenicilloyl antibodies □ Structure–activity relationships—interaction of penicilloic and penilloic acid haptens with benzylpenicilloyl specific antibodies

Munro *et al.*[1] studied the cross-reaction with benzylpenicilloyl specific antibodies of the penicilloic (I) and penilloic (II) acids obtained from several penicillins (III). They determined the extent of this interaction by measuring the inhibition by the haptens of agglutination of sensitized erythrocytes caused by the antibodies.

[1] A. C. Munro, M. G. Chainey, and S. R. Woroniecki, to be published.

In this paper the data so obtained are subjected to Hansch quantitative structure–activity analysis to evaluate the role of various physicochemical properties of the haptens in their interaction with the antibodies. Published applications of Hansch analysis to hapten antibody interactions appear to be confined to a single paper in which Kutter and Hansch (1) showed the importance of steric factors in such interactions.

EXPERIMENTAL

The term C in log $1/C$ in Table I is the molar concentration of hapten required to give 50% inhibition of hemagglutination by benzylpenicilloyl specific antibodies.

Hydrophobic effects are modeled by log P, where P is the octanol–water partition coefficient of the parent penicillins in their unionized form. Values of P are not available for the penilloic or penicilloic acids, but the variation of P with changes in the side chain is expected to be the same for these compounds as for the parent penicillins. Log P for penicillin G (benzylpenicillin), cloxacillin, methicillin, phenethicillin, penicillin V (phenoxymethyl penicillin), and propicillin are measured values from Bird and Marshall (2). The log P values for ampicillin and carbenicillin are calculated from those for penicillin G with the appropriate hydrophobic frag-

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II													
Mean	0	0.36	0.61	0.50	0.35	0.21	0.11	0.05	0.03	0.01	0	1.26	1.56
SD		±0.15	±0.14	±0.17	±0.13	±0.09	±0.06	±0.03	±0.02	±0.01		±0.34	±0.53
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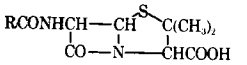
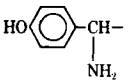
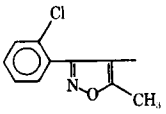
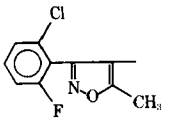
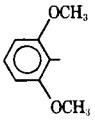
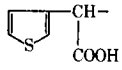
Table I—Inhibition Data and Physicochemical Parameters for Inhibition of Erythrocyte Agglutination by Penilloic and Penicilloic Acids



Parent Penicillin	R	log P[a]	MV[a]	pKa[a]	Penicilloic Acids log 1/C Obs.[b]	Penicilloic Acids log 1/C Calc.[c]	Penicilloic Acids Δ log 1/C	Penilloic Acids log 1/C Obs.[b]	Penilloic Acids log 1/C Calc.[d]	Penilloic Acids Δ log 1/C
Amoxicillin		0.04	134.3	1.9	3.11	3.15	−0.04	—	—	—
Ampicillin		0.90	128.7	1.8	3.71	3.77	−0.06	—	—	—
Carbenicillin		1.26	145.0	5.0	4.43	3.74	0.69	3.41	2.98	0.43
Cloxacillin		2.44	193.4	3.8	3.84	3.73	0.11	2.63	2.77	−0.14
Floxacillin		2.30	204.8	3.8	3.41	3.33	0.08	2.81	2.51	0.30
Methicillin		1.13	152.9	3.7	3.41	3.44	−0.03	2.63	2.80	−0.17
Penicillin G	$C_6H_5CH_2$–	1.76	114.5	4.3	5.22	4.95	0.27	4.14	3.74	0.40
Penicillin V	$C_6H_5OCH_2$–	2.01	125.0	3.2	4.92	4.94	−0.02	3.53	3.69	−0.16
Phenethicillin		2.19	147.2	3.2	4.43	4.59	−0.16	3.23	3.41	−0.18
Propicillin		2.58	169.4	3.2	4.14	4.44	−0.30	3.11	3.24	−0.13
Ticarcillin		0.94	136.5	5.0	3.11	3.63	−0.52	2.63	2.96	−0.33

[a] See text for sources of these constants. [b] From A. C. Munro, M. G. Chainey, and S. R. Woroniecki, to be published. [c] Calculated from Eq. 2. [d] Calculated from Eq. 4.

mental constants and proximity effect (3). For example:

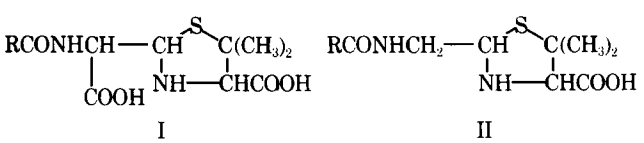
$$\log P(\text{ampicillin}) = \log P(\text{penicillin G}) - f(CH_2) + f(NH_2) + f(CH) + \text{p.e.} \quad \text{(Eq. 1}a\text{)}$$

$$\log P(\text{ampicillin}) = 1.76 - 0.52 + (-1.38) + 0.24 + 0.8 = 0.9 \quad \text{(Eq. 1}b\text{)}$$

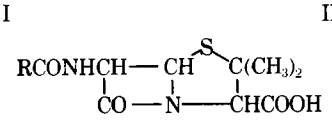
The proximity effect (p.e.) is used because the amino or carboxy group is separated by one carbon from the side-chain amide function. Log P for amoxicillin was calculated as for ampicillin plus a Hansch π term for the hydroxy group [−0.50, from substituted phenylacetic acids (4)]. Log P for floxacillin was calculated from that for cloxacillin plus a π term for the fluoro group (0.14, from substituted nitrobenzenes[2]). Log P for ticarcillin was calculated as for carbenicillin minus 0.32, which is the difference in log P between benzene and thiophene (5).

The molar volume of the side chain was used as a parameter, giving a measure of variations in size of the haptens. These values were calculated from the atomic volumes of Le Bas given by Partington (6). Where a range of values was given (*e.g.*, ring sulfur 21.6–22.5), the average was used. An average nitrogen value of 12.9 was used for the nitrogen of the isoxazole ring. The fluorine value used for floxacillin was from Exner (7) because there is no value in Le Bas' list. Exner's values for chlorine and bromine are very similar to those of Le Bas, so the fluorine value should be consistent.

The pKa values of the side-chain acids (R—COOH) were used as a measure of the variation in electronic effects with a change of side chain. The values for phenylacetic, phenoxyacetic, and phenylmalonic acids are from the literature (8). Phenylmalonic acid has pKa values of 2.6 and 5.0. The relevant value for carbenicillin was taken as that at which one carboxy group is ionized, *i.e.*, 5.0, because the side-chain carboxy of carbenicillin is ionized at the pH of the inhibition experiments. The pKa values for the side chains of amoxicillin, ampicillin, methicillin, phenethicillin, and cloxacillin are approximate values measured in these laboratories. The values for the side-chain acids of floxacillin, propicillin, and ticarcillin



I II



III

[2] A. E. Bird and A. C. Marshall, unpublished data.

were assumed to be the same as those for cloxacillin, phenethicillin, and carbenicillin, respectively.

Regression equations were obtained in the usual way by computer calculation using the method of least squares. Initially the penicilloic and penilloic acids were treated separately, but a final equation was calculated for all data.

RESULTS AND DISCUSSION

Penicilloic Acids—Linear regressions with log P, pKa, and molar volume (MV) had correlation coefficients (r) of 0.458, 0.189, and 0.371, respectively, and the t test showed that they were not statistically significant at the 90% level. Two-parameter equations in log P and pKa and in pKa and MV were also not significant, with correlation coefficients of 0.469 and 0.441, respectively; log P and MV gave:

$$\log(1/C) = \underset{(\pm 0.425)}{0.977 \log P} - \underset{(\pm 0.011)}{0.024\ \mathrm{MV}} + 5.961 \quad \begin{matrix} n & r & s \\ 11 & 0.899 & 0.350 \end{matrix} \quad \text{(Eq. 2)}$$

where n is the number of data points, s is the standard deviation from the regression, and the figures in parentheses are the 95% confidence limits on the regression coefficients. The t test shows that both terms in Eq. 2 are significant at the 99.5% level.

Addition of a pKa term to Eq. 2 gave:

$$\log(1/C) = \underset{(\pm 0.456)}{0.956 \log P} + \underset{(\pm 0.263)}{0.079\ \mathrm{pKa}} - \underset{(\pm 0.012)}{0.024\ \mathrm{MV}} + 5.725 \quad \begin{matrix} n & r & s \\ 11 & 0.906 & 0.362 \end{matrix} \quad \text{(Eq. 3)}$$

This equation has a higher standard deviation than Eq. 2, and the pKa term is not statistically significant.

Penilloic Acids—Linear regressions were not statistically significant, with correlation coefficients of 0.080, 0.001, and 0.658 for log P, pKa, and MV, respectively. Two-parameter equations in log P and pKa and in pKa and MV were also not significant, with correlation coefficients of 0.115 and 0.675, respectively; log P and MV gave:

$$\log(1/C) = \underset{(\pm 0.562)}{0.506 \log P} - \underset{(\pm 0.011)}{0.017\ \mathrm{MV}} + 4.757 \quad \begin{matrix} n & r & s \\ 9 & 0.828 & 0.333 \end{matrix} \quad \text{(Eq. 4)}$$

The t test shows that log P is significant at 90% and MV at 98%. Addition of a pKa term to Eq. 4 gave:

$$\log(1/C) = \underset{(\pm 0.785)}{0.837 \log P} + \underset{(\pm 0.575)}{0.334\ \mathrm{pKa}} - \underset{(\pm 0.011)}{0.018\ \mathrm{MV}} + 3.101 \quad \begin{matrix} n & r & s \\ 9 & 0.884 & 0.304 \end{matrix} \quad \text{(Eq. 5)}$$

Although this equation has a slightly lower s value than Eq. 4, the pKa term is not statistically justified (t found 1.488; t 90%, 5 df 2.015).

Penicilloic and Penilloic Acids—A regression for the combined data was calculated with log P, MV, and a dummy parameter assigned a value of zero for the penicilloic and of 1 for the penilloic acids; this parameter was introduced to allow for the inherent difference in activity of the two sets of acids:

$$\log(1/C) = \underset{(\pm 0.316)}{0.801 \log P} - \underset{(\pm 0.341)}{0.930\ (0/1)} - \underset{(\pm 0.007)}{0.021\ \mathrm{MV}} + 5.780 \quad \begin{matrix} n & r & s \\ 20 & 0.903 & 0.353 \end{matrix} \quad \text{(Eq. 6)}$$

The t test shows that all three terms are significant at the 99.9% level.

Intuitively, one would not expect $(\log P)^2$ to be a significant factor in describing this type of biological activity, because the inhibition apparently depends on a single-step binding of hapten to antibody. Therefore, multiple interactions in a "random walk" to the active site, which can produce (9) parabolic dependence of activity on the partition coefficient, do not occur. However, $(\log P)^2$ terms were tried in some regressions. For penilloic acids, the two-parameter equation in log P and $(\log P)^2$ had both terms significant at the 95% level, but the r and s values of 0.751 and 0.393, respectively, were poor. In other cases, particularly when $(\log P)^2$ was added to the log P and MV regressions, $(\log P)^2$ terms were not significant.

Hansch (10) pointed out that partition coefficient and molar volume are not independent variables for simple substituents. The collinearity of these variables for the 11 compounds studied here was 33% ($r^2 = 0.33$). This value shows that although the variables are not completely independent, they are not very highly correlated and their use in the same equation to model different aspects of the binding process seems to be justifiable (11).

Thus, Eqs. 2, 4, and 6 are the best regressions obtained. Although they are statistically significant, the standard deviations of these regressions are quite high, due in part perhaps to experimental error in the inhibition data. A twofold dilution method was used to obtain these data, so the experimental error may be quite large and the regressions may be as good as can be expected with data of this accuracy. It is also possible that the effect of the large variations in the side chain of the compounds studied cannot be fully explained by the simple models used. For example, Kutter and Hansch (1) showed that separate parameters are required in regression equations for *ortho*-, *meta*-, and *para*-substituents in benzoic and arsonic acid haptens. This indicated a fine degree of dependence of the interaction with antibodies on the steric properties of the haptens. It is not possible to analyze the present data in such detail. However, some useful conclusions can be drawn from the regression analysis.

The nonsignificance of pKa indicates that electronic effects induced by variation of the side chain can play only a minor part in the binding of hapten to antibody.

The positive and negative coefficients for log P and MV, respectively, show that inhibitory activity increases with an increase in log P and a decrease in MV. This finding is consistent with an increase in hydrophobic binding of hapten to antibody as log P increases and with a decrease in binding due to steric or bulk effects as MV increases. Corresponding regression coefficients in Eqs. 2 and 4 are not significantly different (the 95% confidence limits overlap), indicating that the dependence of activity on log P and MV is similar for penilloic and penicilloic acids, although the data are not sufficiently precise to be certain of this dependence. However, the good fit of all data obtained with Eq. 6 encourages the view that the interaction of both sets of haptens with antibody depends in the same way on log P and MV.

The cause of the near 10-fold difference in the inhibitory activity of corresponding penilloic and penicilloic acids is of interest. The log P and MV values for the penicilloic acid whole molecule (not just the side chain) are less and greater, respectively, than the values for the corresponding penilloic acid. Since inhibitory activity is promoted by an increase in log P and a decrease in MV, penilloic acid would be expected to be more active than the corresponding penicilloic acid if these were the only factors involved in the interaction of the haptens and antibody. Thus, it is clear that some overriding factor (presumably binding *via* the C_6 carboxy group) promotes the activity of penicilloic acids relative to that of penilloic acids.

Examination of the differences between observed and calculated values of log $(1/C)$ in Table I shows particularly high differences for carbenicillin and ticarcillin in the penicilloic acid series and for carbenicillin in the penilloic acid series. The estimated log P values for these compounds may be open to question. However, a systematic error in log P or MV cannot explain the high differences because it would affect both compounds in the same way and the sign of the difference is positive for carbenicillin and negative for ticarcillin. The high differences for these compounds and for benzylpenilloic acid may be due to random errors in the data.

The fragmental constant of the carboxy group used to calculate log P for carbenicillin and ticarcillin relates to an unionized carboxy group (3), so that log P relates to the unionized penicillin. At the pH of the inhibition experiments (7.2), the side-chain carboxy group will be ionized, so log P for the molecule with this group ionized might be more appropriate. Hansch (12) gave a difference in π value between neutral and ionized carboxy groups of 4.1, which gives log P values of −2.84 and −3.16 for carbenicillin and ticarcillin, respectively, with the carboxy group ionized.

Use of these values in log P and MV regressions gave nonsignificant log P terms. Omission of carbenicillin and ticarcillin from the regressions had only slight effects on the regression coefficients and the r and s values given in Eqs. 2 and 4. Thus, it is clear that the activity of the carboxy compounds fits with that of the other

penicillin derivatives when log P for the unionized form is used rather than log P of the apparently more appropriate ionized form. Similar behavior was found in relating log P to the extent of binding of carbenicillin and ticarcillin to serum proteins[2]. Possibly this effect is coincidental due to ionic binding by the side-chain carboxy group to an extent approximately sufficient to offset the low hydrophobic binding due to the ionic nature of this group.

Nishida *et al.* (13) reported binding constants (k_{rel}) relative to benzylpenicilloic acid for binding to benzylpenicilloyl specific antibodies of some penicilloic acids not studied in this work. Included were the penicilloic acids of *o*-, *m*-, and *p*-chloro- and *o*-, *m*-, and *p*-nitrobenzylpenicillins and of methyl- and ethylpenicillins. Equation 2 was used to calculate values of log (1/C) (and of k_{rel} using the experimental C value for benzylpenicilloic acid) for these compounds. This equation cannot distinguish the effect of varying the substituent position in the benzene ring, but values were calculated for chloro- and nitrobenzylpenicilloic acids using the appropriate MV values (131.1 and 143.7, respectively) and log P values (2.47 and 1.48, respectively) calculated from those of penicillin G with Hansch π values of 0.71 and −0.28 for chloro and nitro, respectively (14). The k_{rel} values obtained were 1.02 and 0.06 for the chloro and nitro compounds, respectively. These values are reasonably close to the experimental values of 1.36–5.17 for the three chloro compounds and 0.115–0.33 for the three nitro compounds, especially when it is recalled that Nishida *et al.* (13) used a fivefold dilution technique for their determinations so their results may not be very precise.

The log P value for methylpenicillin was calculated in two ways: (*a*) from log P penicillin G minus π phenyl, 1.76 − 1.89 = −0.13; and (*b*) from log P heptylpenicillin (15) minus 6 π methylene, 3.32 − 3.0 = 0.32. The mean value of 0.22 is used, and log P of ethylpenicillin then becomes 0.72 by addition of π methylene. Substitution of these values with MV values of 25.9 and 48.1 in Eq. 2 leads to k_{rel} values of 2.16 and 1.96 for methyl- and ethylpenicilloic acids, respectively.

These values are very different from Nishida *et al.*'s experimental values of 0.009 and 0.016. Thus, penicilloic acids with such small side chains are outside the range of applicability of Eq. 2. Intuitively, it seems probable that Eq. 2 will be applicable only to a limited range of penicilloic acids, presumably with log P and MV values centered about those of benzylpenicilloic acid because the antibodies were raised to a benzylpenicilloyl antigen. Experiments with antibodies raised to other penicilloyl antigens would be interesting in this context.

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Cytotoxic Principles of *Parquetina nigrescens* (Afzel.) Bullock (Asclepiadaceae)

W. H. MARKS, H. H. S. FONG ˣ, M. TIN-WA, and N. R. FARNSWORTH

Abstract □ Systematic fractionation of the cytotoxic extracts of the roots of *Parquetina nigrescens* (Afzel.) Bullock (Asclepiadaceae) on silica gel column chromatography led to the isolation of three cardenolides: cymarin, strophanthidin, and a strophanthidin glycoside (XS-89). Also isolated during the investigation were β-sitosterol-β-D-glucoside, a mixture of α- and β-amyrins, a mixture of alkanols, and a mixture of plant sterols.

Keyphrases □ *Parquetina nigrescens* (Afzel.) Bullock (Asclepiadaceae)—isolation and identification of cytotoxic principles □ Cytotoxicity—isolation and identification of cymarin, strophanthidin, and strophanthidin glycoside from *Parquetina nigrescens* □ Cardenolides—isolation and identification from *Parquetina nigrescens*

Parquetina nigrescens (Asclepiadaceae), a woody shrub native to Africa, is sometimes referred to as *Periploca nigrescens* (1). Previous studies (2–5) showed it to contain cardiac glycosides of the strophanthidin type. Strophanthidiol-β-glucoside acetates, strophanthidin, strophanthidiol, 16-hydrostrophanthidin, convallatoxin, 17α-strophanthidin, 16-acetoxystrophanthidin, 16-acetoxystrophanthidin rhamnoside, 16-dehydrostrophanthidin, 16-dehydrostrophanthidol, nigrescigenin, tetra-*O*-acetylstrophanthidin-β-D-glucoside, and several incompletely identified cardenolides have been isolated from this plant.

Other species of the genus have also been investi-

penicillin derivatives when log P for the unionized form is used rather than log P of the apparently more appropriate ionized form. Similar behavior was found in relating log P to the extent of binding of carbenicillin and ticarcillin to serum proteins[2]. Possibly this effect is coincidental due to ionic binding by the side-chain carboxy group to an extent approximately sufficient to offset the low hydrophobic binding due to the ionic nature of this group.

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W. H. MARKS, H. H. S. FONG ˣ, M. TIN-WA, and N. R. FARNSWORTH

Abstract □ Systematic fractionation of the cytotoxic extracts of the roots of *Parquetina nigrescens* (Afzel.) Bullock (Asclepiadaceae) on silica gel column chromatography led to the isolation of three cardenolides: cymarin, strophanthidin, and a strophanthidin glycoside (XS-89). Also isolated during the investigation were β-sitosterol-β-D-glucoside, a mixture of α- and β-amyrins, a mixture of alkanols, and a mixture of plant sterols.

Keyphrases □ *Parquetina nigrescens* (Afzel.) Bullock (Asclepiadaceae)—isolation and identification of cytotoxic principles □ Cytotoxicity—isolation and identification of cymarin, strophanthidin, and strophanthidin glycoside from *Parquetina nigrescens* □ Cardenolides—isolation and identification from *Parquetina nigrescens*

Parquetina nigrescens (Asclepiadaceae), a woody shrub native to Africa, is sometimes referred to as *Periploca nigrescens* (1). Previous studies (2–5) showed it to contain cardiac glycosides of the strophanthidin type. Strophanthidiol-β-glucoside acetates, strophanthidin, strophanthidiol, 16-hydrostrophanthidin, convallatoxin, 17α-strophanthidin, 16-acetoxystrophanthidin, 16-acetoxystrophanthidin rhamnoside, 16-dehydrostrophanthidin, 16-dehydrostrophanthidol, nigrescigenin, tetra-*O*-acetylstrophanthidin-β-D-glucoside, and several incompletely identified cardenolides have been isolated from this plant.

Other species of the genus have also been investi-

gated for the presence of cardenolides (6–8). Periplogenin and cymarose have been isolated from *P. calophylla* (6) and *P. sepium*. Periplogenin and periplocymarin (7) as well as periplocin (8) were found in *P. graeca*.

P. graeca has folkloric uses as a herbal or arrow poison (9), and *P. sepium* has been an ingredient of a cure-all drug in Chinese herbal medicine (2, 10). Pharmacological studies on various species of the genus have centered on their cardiotonic effects (11–13).

DISCUSSION

A 50% alcoholic extract of the roots of *P. nigrescens* was found to be cytotoxic[1] ($ED_{50} < 8.8 \times 10^{-1}$ μg/ml). However, this extract and fractions from subsequent workups of the plant material were inactive when tested in the P-388 leukemia system in mice.

TLC of the alcoholic extract indicated the presence of several cardenolides (positive reactions to the Kedde spray reagent).

Partition of a 95% alcoholic extract between water and chloroform resulted in the isolation and identification of β-sitosterol-β-D-glucoside and cymarin from the chloroform-soluble fraction (C). The isolation of cymarin represents the first reported occurrence of this cardenolide in *P. nigrescens*.

Chromatographic separation of a 50% alcoholic extract of the marc resulted in the isolation of strophanthidin and a novel cardenolide, which was partially characterized as a strophanthidin glycoside.

The petroleum ether solubles (Fraction A) yielded mixtures of alkanols, phytosterols (campesterol, stigmasterol, and β-sitosterol), and triterpenes (α- and β-amyrins).

Biological evaluation of the isolates demonstrated that the cytotoxic activity of this plant was due, at least in part, to the three cardenolides present.

EXPERIMENTAL[2]

Plant Material—The plant material[3] was collected in Ghana during March 1971. The roots were collected, air dried, and ground to a coarse powder.

Extraction and Initial Fractionation—A 4.5-kg sample of the plant material was defatted with petroleum ether (bp 30–60°), and the extractive was concentrated *in vacuo* to yield 119 g of Fraction A. The defatted marc was air dried and then continuously extracted with 95% ethanol to give Fraction B (199 g).

A 100-g portion of Fraction B was taken to a syrupy consistency and partitioned between chloroform and water to give Fractions C (24 g) and D (70 g), respectively.

The 95% ethanol-exhausted marc was air dried and continuously extracted with 50% ethanol to yield Fraction E (64 g).

Separation of Fraction C—Fraction C was subjected to column chromatography on 600 g of silica gel PF-254[4], using benzene–95% ethanol (3:1) as the eluent. Then 120 15-ml fractions were collected, and fractions were combined on the basis of TLC monitoring.

Isolation and Identification of β-Sitosterol Glucoside—Fractions 33–44 were combined, filtered, and taken to dryness. The residue was dissolved in hot dioxane and, upon cooling, yielded a copious white amorphous precipitate. The substance had a melting point of 260–270°, gave a transparent UV spectrum, and showed IR absorptions at 3425 (s, OH), 2980 (s, CH_3 stretch), 2890 (w), 1470 (w), 1380 (w), 1180 (s), and 1025 (s) cm^{-1}.

The mass spectrum was superimposable with that of β-sitosterol, but the sample did not vaporize until 270° *in vacuo*. The latter point, plus the compound's different TLC, IR, and solubility properties as compared to reference β-sitosterol, suggested that the isolate was a glycoside. A comparison of the IR spectrum with that of reference β-sitosterol-β-D-glucoside[5] showed them to be superimposable. Furthermore, a mixed melting-point determination did not lower the melting-point range of the mixture. Therefore, the isolate was identified as β-sitosterol-β-D-glucoside.

Isolation and Identification of Cymarin—Fractions 47–50 were combined and dried *in vacuo* to a residue (500 mg), which was rechromatographed on a column of 75 g silica gel PF-254 and eluted with benzene–absolute ethanol (3:1). Forty-one 10-ml fractions were collected, and fractions 33–37 were combined on the basis of their TLC patterns. The dried, pooled fraction (200 mg) was dissolved in methanol, and ether was added until a cloudy appearance was observed.

The flask was stoppered and clear crystalline needles (72 mg) were obtained after 4 hr. These needles gave a Kedde positive reaction and had a melting point of 138–141°. Mass spectra of the isolate were run at 15 and 70 ev and showed fragmentation patterns consistent with those of cymarin (14). In addition, the NMR, UV, and IR spectra were superimposable with those of reference cymarin[6], and a mixed melting-point determination showed no depression.

Separation of Fraction E—Fraction E, the 50% alcoholic extract of the 95% ethanol-exhausted marc, was partitioned between chloroform and water. The chloroform layer was shown to be devoid of Kedde-positive material. The aqueous layer was evaporated *in vacuo*, and the residue (250 mg) was chromatographed on a 75-g silica gel PF-254 column, eluting with benzene–methanol (8:1). Forty 10-ml fractions were collected.

Isolation of Strophanthidin—Column fractions 15–20 were combined on the basis of their TLC patterns and taken to dryness. The residue (70 mg) was dissolved in hot methanol, ether was added to slight turbidity, and the flask was stoppered and allowed to stand overnight. The resulting colorless crystals (50 mg) were recrystallized from methanol–ether to afford an analytical sample which gave both positive Kedde and Liebermann–Burchard reactions.

The product had a melting point of 140–142° and exhibited NMR, UV, and IR spectra superimposable with those obtained for reference strophanthidin[7]. The mass spectrum of the isolate was consistent with that of strophanthidin (14). That this isolate was strophanthidin was further supported by an undepressed mixed melting-point determination.

Isolation and Characterization of the Strophanthidin Glycoside (XS-89)—Column fractions 21–25 were combined and evaporated, and the residue (80 mg) was dissolved in methanol. Water was added to the methanolic solution until it became cloudy, and the flask was stoppered and set aside in the refrigerator. The crystalline needles that formed were collected and dried *in vacuo* to give a total weight of 62 mg. The mother liquor was concentrated by slow evaporation at room temperature, and a second crop was harvested (8 mg). This compound was subsequently determined to be a new cardenolide possessing cytotoxic activity.

The isolate had a melting point of 163–168° and exhibited a UV spectrum at λ_{max} 218 nm ($\log \epsilon^{1\%}_{1cm}$ 2.47). IR spectral absorptions were observed at 3500 (s, OH), 2980 (s, CH_3 stretch), 2910 (w), 1750 (s, α,β-unsaturated lactone), 1725 (w, aldehyde), 1500 (m),

[1] The extracts were tested through the auspices of the Drug Research and Development branch of the National Cancer Institute. An extract is considered cytotoxic if it exhibits an $ED_{50} \leq 20$ μg/ml, and an isolate is considered active it it shows an $ED_{50} \leq 4$ μg/ml in the 9KB cell culture *in vitro* (17).

[2] All melting points reported were determined using a Kofler hot-stage melting-point apparatus and are uncorrected. UV spectra were recorded using a Beckman model DB-G spectrophotometer with methanol as the solvent. IR spectra in KBr were recorded using a Beckman model 18-A spectrophotometer with polystyrene calibration at 1601 cm^{-1}. Absorption bands are recorded in wave numbers ($centimeters^{-1}$) and intensities as s (strong), m (medium), and w (weak). NMR spectra were recorded in deuterochloroform on Varian Associates models A-60 and T-60 instruments using tetramethylsilane as the internal standard. Chemical shifts are reported in parts per million (δ, ppm). Mass spectra were determined using a Hitachi Perkin-Elmer model RMU-60 spectrometer; samples were introduced as solids. GLC analysis was carried out using a Perkin-Elmer model 881 gas chromatograph with helium as the carrier gas. The glass column was coiled, 1.8 m, 0.6 cm (6 ft, 0.125 in.) o.d., packed with a 5% OV-101 stationary phase on Gas Chrom Q, 100–120 mesh.

[3] The plant material was collected and identified by Mr. O. B. Dokosi, University of Ghana. Herbarium specimens are on file in the Department of Pharmacognosy and Pharmacology, College of Pharmacy, University of Illinois at the Medical Center, Chicago, IL 60612

[4] E. Merck, Darmstadt, Germany.

[5] Kindly supplied by Dr. C. R. Mitra, Assistant Director, National Botanic Gardens, Lucknow, India.

[6] Kindly supplied by Professor R. Hänsel, Director Freie Universität Berlin, Institut für Pharmakognosie, Berlin, Germany.

[7] Kindly supplied by Dr. J. Trojanék, Pharmaceutical and Biochemical Research Institute, U Elektry 8, Praha 9, Czechoslovakia.

and 1080 (w) cm^{-1}. The NMR spectrum in deuterochloroform showed major signals as singlets at δ 0.80 (3H, angular methyl), 4.83 (2H, methylene protons of lactone ring), 5.90 (1H, olefinic proton of lactone ring), and 10.00 (1H, aldehyde).

The mass spectrum showed the expected fragmentation pattern for a strophanthidin-type cardenolide (14), with significant fragments being recorded at *m/e* 368 (8.3), 358 (4.1), 340 (45.8), 322 (31.6), 308 (4.1), 215 (4.1), 197 (7.5), 188 (20.8), 160 (15.0), 147 (100.0), 145 (12.0), and 131 (40.0).

A sample of the isolate (2 mg) was hydrolyzed by refluxing overnight in 20 ml of a mixture of 95% ethanol–water–concentrated hydrochloric acid (85:15:10). The solution was taken to dryness *in vacuo* and extracted with a small volume of water. The aqueous solution was filtered and mixed with an ion-exchange resin[8] (OH-form) until it was neutral to pH paper. The neutralized solution was filtered, dried *in vacuo*, and trimethylsilylated[9].

GLC of the silylated glycone portion demonstrated the presence of glucose. Several other peaks were also present, but their identity could not be discerned. However, fructose, digitoxose, galactose, mannose, arabinose, and rhamnose were absent.

TLC of the water-insoluble residue remaining from the hydrolysis demonstrated the presence of a component which was Kedde test positive and identical with reference strophanthidin in two solvent systems [1-butanol–acetic acid–water (4:4:1) and chloroform–methanol (8:1)]. These results, coupled with the observed mass spectral fragmentation pattern of the parent glycoside, indicated the aglycone to be strophanthidin. Attempts are underway to elucidate the structure of this compound.

Separation of Fraction A—Ten grams of the petroleum ether-soluble extract was dissolved in boiling acetone. Upon cooling, a copious, white, plate-like precipitate formed, which was collected and set aside. It exhibited a low broad melting-point range (64–72°). GLC analysis indicated the presence of several compounds which were not identified.

The mother liquor was evaporated, and the residue (6.24 g) was chromatographed on silica gel PF-254 (300 g), using benzene–ethyl acetate–acetone (10:1:1) as the eluent. A total of 38 fractions of 15 ml each was collected.

Isolation of Alkanol Mixture—Column fractions 20–24 were combined on the basis of TLC and evaporated. The residue (2.5 g) was dissolved in benzene, and acetone was added. The resulting white, plate-like precipitate, mp 60–68°, exhibited a transparent UV spectrum and the IR spectrum was typical of an alkanol (15). On comparison with reference alkanols, GLC analysis indicated the presence of 1-docosanol, 1-tetracosanol, and 1-hexacosanol.

Isolation of Triterpene Mixture—Column fractions 28–32 were combined and evaporated. This residue (1.9 g) was dissolved in chloroform, and a mixture of methanol–water (9:1) was added until a cloudy solution persisted. The microcrystalline precipitate that formed was collected and dried. The isolate (1.2 g), mp 125–130°, had a transparent UV spectrum, and it gave a positive Liebermann–Burchard test for triterpenes.

The mass spectrum showed a molecular ion at *m/e* 426 and a base peak, indicative of the amyrin series of triterpenes (16), at *m/e* 218. GLC analysis of this sample with reference standards showed the isolate to be a mixture of 70% α-amyrin and 30% β-amyrin.

Isolation of Phytosterols—Column fractions 36–38 were combined and dried *in vacuo*. On dissolving the residue (0.23 g) in 95% ethanol, small needle-like crystals (50 mg), mp 132–137°, were obtained. The isolate gave a positive Liebermann–Burchard test and exhibited a transparent UV spectrum. The IR spectrum (KBr) was identical to that of reference β-sitosterol[10]. The mass spectrum showed a molecular ion at *m/e* 414 and a base peak at *m/e* 218.

GLC of the sample at 260°, however, indicated the isolate to be composed of three constituents. These were tentatively identified as campesterol (20%), stigmasterol (7%), and β-sitosterol (73%) by comparison with chromatographically pure reference sterols, using cholestane as the internal standard.

[8] Amberlite IRA-410.
[9] SIL-PREP, Applied Science Laboratories, State College, Pa.
[10] ICN Nutritional Biochemicals Corp., Cleveland, OH 44128

Table I—Cytotoxic Results of Isolates from *P. nigrescens*

Isolate	9KB Cell Culture Test[a], μg/ml
β-Sitosterol-β-D-glucoside	$>1 \times 10^{1}$
Cymarin	$<1 \times 10^{-1}$
Strophanthidin	$<1 \times 10^{0}$
Unidentified strophanthidin glycoside (XS-89)	$<2.5 \times 10^{-1}$
Mixture of alkanols	$>1 \times 10^{2}$
Mixture of α- and β-amyrins	Not tested
Phytosterol mixture	$>4 \times 10^{1}$

[a] A compound is deemed active if it exhibits an $ED_{50} \leqq 4$ μg/ml.

Biological Activity—The results of biological evaluation indicated that the three cardenolides isolated, cymarin, strophanthidin, and the strophanthidin glycoside (XS-89), are cytotoxic but inactive in the P-388 lymphocytic leukemia (3PS) system (Table I). All other isolates tested were inactive in both systems.

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Fluorometric Determination of Drug–Protein Association Constants: Binding of Pamaquine by Bovine Serum Albumin

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Abstract □ The binding of pamaquine to bovine serum albumin is accompanied by the enhancement of the fluorescence efficiency of the former but without shifting its fluorescence energy. This phenomenon was used to evaluate the stoichiometry and strength of the binding. The results indicate that three singly protonated pamaquine molecules are bound by each bovine serum albumin molecule. The individual binding constants were calculated by using the Bjerrum technique. The average values of the three constants were $K_1 = 6.4 \times 10^7$, $K_2 = 3.1 \times 10^6$, and $K_3 = 1.9 \times 10^5$, indicating that, compared to anionic drugs and fluorescent probes, pamaquine is very strongly bound by the protein.

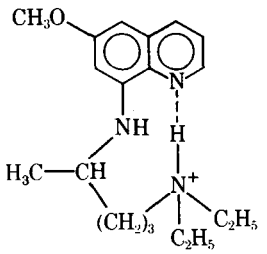
Keyphrases □ Pamaquine—binding by bovine serum albumin, fluorometric determination of drug–protein association constants □ Bovine serum albumin—pamaquine binding, fluorometric determination of drug–protein association constants □ Binding—pamaquine to bovine serum albumin, fluorometric determination of association constants □ Fluorometry—determination, drug–protein association constants, binding of pamaquine by bovine serum albumin

Recently, it was observed in this laboratory that the fluorescence intensity of the antimalarial pamaquine was enhanced in the presence of bovine serum albumin at pH 7.4. Consequently, it was felt that by systematically studying the variation of the quantum yield of fluorescence of pamaquine as a function of relative protein concentration, the parameters of the binding of the drug to the protein could be quickly and reasonably accurately established.

EXPERIMENTAL

Purified pamaquine phosphate[1] and crystalline bovine serum albumin of A grade[2] were used. The concentrations of bovine serum albumin solutions were calculated from the weight of the sample and then checked by determining absorbance at 280 nm. The average molecular weight of 69,000 and the molar absorptivity of 4.55×10^4 at 280 nm were used in the calculations. The bovine serum albumin concentrations determined directly by weighing were in excellent agreement with the concentrations determined photometrically.

All bovine serum albumin and pamaquine solutions were prepared 2 hr prior to fluorometric titration from distilled, deionized water buffered to pH 7.4 with a total phosphate ($[H_2PO_4^-] + [HPO_4^{-2}]$) concentration of 0.1 M. The fluorometric titrations were carried out as follows: 2.0 ml of the protein solution of appropriate concentration in a 1-cm silica cell was titrated by successive additions of 1-μl volumes of a 1×10^{-3} M solution of pamaquine, delivered from an air- and liquidtight microsyringe[3]. The temperature during the titrations was maintained at 25°. The fluorescent intensity measurements were made on an uncorrected fluorescence spectrophotometer[4] with excitation at 367 nm and fluorescence monitored at 518 nm.



I

DISCUSSION

Pamaquine (I) exists in its singly protonated form at pH 7.4, and an intramolecular hydrogen bond is present between the protonated diethylamino group and the ring nitrogen (1).

It has been suggested (1) that in the thermally equilibrated, excited state, the proton would be covalently bonded to the ring nitrogen and electrostatically hydrogen bonded to the diethylamino group. When pamaquine is bound to bovine serum albumin at pH 7.4, the fluorescence band does not shift but the fluorescence intensity is enhanced. The ratio of the quantum yields of the bound pamaquine to that of the pamaquine is 2.5. The fact that the fluorescence band of pamaquine does not shift on binding indicates that the intramolecular hydrogen bond of pamaquine is not broken in the process of binding to the protein, because any change in the electron density around the ring nitrogen would result in shifting of the fluorescence band.

For substrates like pamaquine, which have some fluorescence in the free form and whose fluorescence is enhanced without a change in the emission wavelength when bound to a protein, the fraction of substrate bound, α, can be determined by using Eq. 1 (see *Appendix* for derivation):

$$\alpha = \frac{F_p - F_f}{F_b - F_f} \qquad \text{(Eq. 1)}$$

where F_p and F_f are the fluorescence intensities of a given concentration of substrate in solutions of low protein concentration and in solutions without any protein, respectively; and F_b is the fluorescence intensity of the same concentration of the fully bound substrate. The latter is taken to be the fluorescence intensity of the substrate in the presence of excess protein. Such a treatment will yield values of α, provided the fluorescence intensities of the bound pamaquine and of the free pamaquine are linear functions of the respective concentrations.

The value of F is calculated using:

$$F = \phi I_0 \left[2.3A - \frac{(2.3A)^2}{2!} + \frac{(2.3A)^3}{3!} - \frac{(2.3A)^4}{4!} + \cdots \right] \qquad \text{(Eq. 2)}$$

where ϕ is the quantum yield of the emitting species, I_0 is the intensity of the exciting radiation, and A is the absorbance. The linearity of F with the concentration of the emitting species can only be taken for granted at very low absorbance (<0.02) at the exciting wavelength, in which case the higher power terms in Eq. 2 become negligible by comparison with the first term. For example, when $A > 0.02$, the deviation from linearity is greater than 2%. At absorbances of $0.02 < A < 0.15$, the second term in Eq. 2 results in $115 \times A$% deviation from linearity at any point in the fluorometric titration. At higher absorbances ($A > 0.15$), the third term in Eq. 2 generally also becomes large enough to have to be considered.

Several fluorometric titrations were carried out by increasing

[1] Control No. N432UE, Sterling-Winthrop Research Institute, Rensselaer, N.Y.

[2] Lot No. 300597 (Pentex brand product of Miles Laboratory), Calbiochem, San Diego, Calif.

[3] Unimetrics.

[4] Model MPF-2A, Perkin-Elmer, Norwalk, Conn.

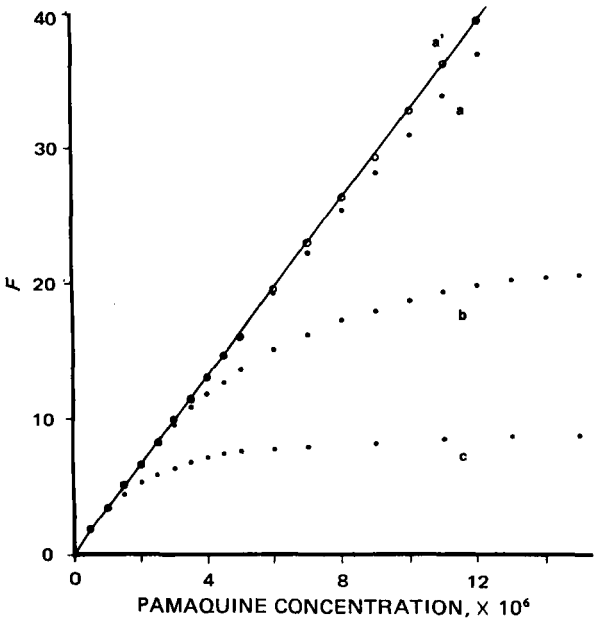


Figure 1—*Plots of relative fluorescence intensities,* F, *as a function of total pamaquine concentration for the bovine serum albumin–pamaquine titrations with a constant amount of the protein* (P_T). *Key: curve a,* $P_T = 2.5 \times 10^{-5}$ *and* 5×10^{-5} M; *curve b,* $P_T = 2.5 \times 10^{-6}$ M; *and curve c,* $P_T = 1 \times 10^{-6}$ M. *The straight line a′ is obtained after correcting the fluorescence intensities in curve a for the absorbance effect (see text).*

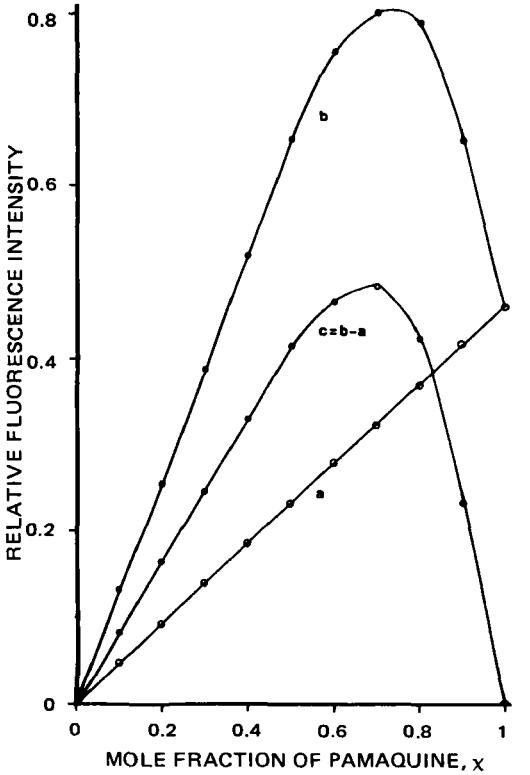


Figure 2—*Job's plot for the bovine serum albumin–pamaquine binding. Line a is the variation of the fluorescence intensity with the concentration of pamaquine in the 0–1* $\times 10^{-5}$ M *range. Curve b is the total observed fluorescence intensity as a function of the mole fraction (χ) of pamaquine. Curve c is the apparent enhancement in the fluorescence intensity as a function of χ. The total concentration of the protein plus pamaquine was kept constant at 1* $\times 10^{-5}$ M.

the initial protein concentration. The titrations with the protein concentrations of 2.5×10^{-5} and 5.0×10^{-5} *M* both gave exactly the same net enhancement in fluorescence intensity for a given amount of pamaquine, indicating that at these concentrations of bovine serum albumin the added pamaquine was essentially in the bound form. At the excitation wavelength, 367 nm, the molar absorptivity for the pamaquine–bovine serum albumin system was determined to be 5.2×10^3 liters $mole^{-1}$ (pamaquine) cm^{-1}. Hence, at pamaquine concentrations greater than 3.85×10^{-6} *M* ($A = 0.02$), the deviation from linearity will be greater than 2%, and appropriate corrections to the observed intensities should be applied to compensate for the second term in Eq. 2.

For the two titrations with the high protein concentrations (2.5×10^{-5} and 5.0×10^{-5} *M*) and for the titration with no protein, the observed fluorescence intensities (F_b and F_f, respectively) at every point during the titration were corrected by applying the correction factor 1.15*A*. Curve a in Fig. 1 was plotted using the observed values of $F_b - F_f$. After applying the correction to the observed fluorescence intensities, line a′ was obtained, verifying that the deviation from linearity of curve a was indeed due to the absorbance effect.

When the titrations are carried out at low protein concentration so that the substrate is only partially bound, the fluorescence intensity, F_p, is the sum of the fluorescence intensities of both the bound and unbound forms of pamaquine; therefore, F_p cannot simply be corrected for the second term in Eq. 2 unless the concentration of bound (or free) pamaquine is known in advance. To circumvent this problem, the concentration of bound pamaquine was estimated from Eq. 1 using the uncorrected F_p values and these approximate values were then used to correct the F_p values for the absorbance effect. Curves b and c in Fig. 1 are the plots of corrected $F_p - F_f$ values *versus* total concentration of pamaquine for bovine serum albumin concentrations of 2.5×10^{-6} and 1.0×10^{-6} *M*, respectively. The binding parameters were calculated by using the corrected values of F_f, F_p, and F_b.

The maximum number of binding sites, *N*, was determined from Job's plot (2, 3) as follows. First, the fluorescence intensity of pamaquine in pH 7.4 buffer was measured for solutions having concentrations from 0 to 1.0×10^{-5} *M* (line a in Fig. 2). The total fluorescence intensities of several pamaquine–bovine serum albumin solutions, made by varying the mole fraction (χ) of pamaquine and keeping the total concentration of pamaquine plus bovine serum albumin at 1.0×10^{-5} *M* (curve b in Fig. 2), were then measured. The apparent enhancement of fluorescence intensity due to the binding at different mole fractions of pamaquine is the difference between curve b and line a in Fig. 2. Curve c is plotted with the apparent enhancement of fluorescence intensity as a function of mole fraction of pamaquine. The point of inflection is at $\chi = 0.74$, which corresponds to a ratio of pamaquine to bovine serum albumin of 2.85. This value indicates that the maximum number of binding sites on the protein molecule for pamaquine is three.

In the evaluation of protein binding equilibria, the commonly used equation is the Scatchard equation (4):

$$\frac{\bar{n}}{[D]} = Nk - \bar{n}k \qquad \text{(Eq. 3)}$$

where $\bar{n}$ represents the ratio of moles of bound substrate per mole of protein, *[D]* is the concentration of unbound substrate, *N* is the maximum number of binding sites on a protein molecule, and *k* is the binding constant. The value of $\bar{n}$ can be calculated from the fraction of pamaquine bound by using the relationship:

$$\bar{n} = \alpha \frac{D_T}{P_T} \qquad \text{(Eq. 4)}$$

where D_T and P_T are the formal concentrations of pamaquine and bovine serum albumin, respectively. The concentration of unbound pamaquine was obtained by multiplying D_T by $(1 - \alpha)$. When the *N* binding sites are noninteracting and have the same binding affinities, a plot of $\bar{n}/[D]$ *versus* $\bar{n}$ (known as the Scatchard plot) gives a straight line with the *x*-axis intercept at $\bar{n} = N$, the *y*-axis intercept at $\bar{n}/[D] = Nk$, and the slope of $-k$. However, if the individual binding sites have different binding affinities, due either to the distinct nature of the sites or to the interaction be-

Table I—Equilibrium Constants for the Binding of Pamaquine to Bovine Serum Albumin at 25°[a]

	$P_T = 2.5 \times 10^{-6}$ M		$P_T = 1.0 \times 10^{-6}$ M		
	Scatchard Treatment	Bjerrum Treatment	Scatchard Treatment	Bjerrum Treatment	Average
$\log k_1$	7.81	7.82	7.80	7.78	7.80
$\log k_2$	6.55	6.37	6.60	6.43	6.49
$\log k_3$	5.36	5.15	5.45	5.21	5.29

[a] P_T is the total concentration of the protein.

tween the bound substrates at different sites, the Scatchard plot gives a downward curve instead of a straight line.

If the binding strength of the individual binding sites are significantly different from each other, the Scatchard plot may still be used to estimate binding parameters by appropriately analyzing the curve in relation to the following modified form of Eq. 3, where k_i is the binding constant for the ith site. Figure 3 shows the Scatchard plot for the bovine serum albumin–pamaquine system at the protein concentration of 2.5×10^{-6} M.

The curve obtained indicates that the three binding sites on bovine serum albumin for pamaquine have different binding strengths. Graphical analysis of this plot according to:

$$\bar{n} = \sum_{i=1}^{i=N} \frac{k_i[D]}{1 + k_i[D]} \qquad \text{(Eq. 5)}$$

yielded binding constants for the three sites. The values are listed in Table I along with the values obtained for another titration at $P_T = 1.0 \times 10^{-6}$.

Multiple equilibria involving a small number of ligands can be best treated by the Bjerrum technique (5). This approach is applicable to any system in thermodynamic equilibrium and makes no presupposition concerning the equivalence of binding sites or the relative magnitude of successive equilibrium constants. In the case of the bovine serum albumin–pamaquine system, the three successive equilibria (Scheme I, P = protein and D = pamaquine) are characterized by equilibrium constants k_1, k_2, and k_3 such that:

$$P + D \overset{k_1}{\rightleftharpoons} PD_1$$
$$PD + D \overset{k_2}{\rightleftharpoons} PD_2$$
$$PD_2 + D \overset{k_3}{\rightleftharpoons} PD_3$$

Scheme I

$$k_1 = \frac{[PD]}{[P][D]} \qquad \text{(Eq. 6)}$$

$$k_2 = \frac{[PD_2]}{[PD][D]} \qquad \text{(Eq. 7)}$$

$$k_3 = \frac{[PD_3]}{[PD_2][D]} \qquad \text{(Eq. 8)}$$

The average number of bound pamaquine molecules per mole of protein can now be expressed as:

$$\bar{n} = \frac{[PD] + 2[PD_2] + 3[PD_3]}{[P] + [PD] + [PD_2] + [PD_3]} \qquad \text{(Eq. 9)}$$

which, when combined with Eqs. 6–8, gives:

$$\bar{n} = \frac{k_1[D] + 2k_1k_2[D]^2 + 3k_1k_2k_3[D]^3}{1 + k_1[D] + k_1k_2[D]^2 + k_1k_2k_3[D]^3} \qquad \text{(Eq. 10)}$$

Equation 10 predicts that if k_1, k_2, and k_3 are widely different from each other so that essentially only one equilibrium is experimentally detectable at any point in the titration, a plot of $-\log [D]$ *versus* $\bar{n}$ (known as the Bjerrum plot) would show (for the present system) three distinct inflection regions corresponding to $-\log [D] = \log K_1$ when $\bar{n} = 0.5$, $-\log [D] = \log K_2$ when $\bar{n} = 1.5$, and $-\log [D] = \log K_3$ when $\bar{n} = 2.5$.

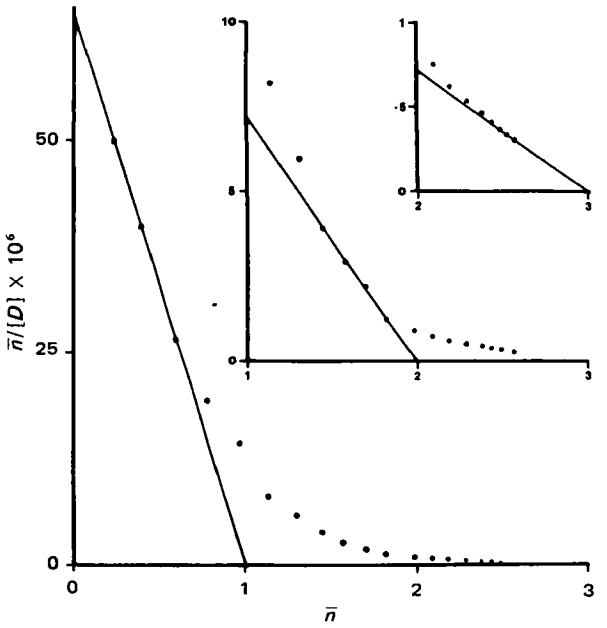


Figure 3—*Scatchard plot of* $\bar{n}$/[D] versus $\bar{n}$ *for the bovine serum albumin–pamaquine titration with the protein concentration of* 2.5×10^{-6} M. *Insets are the plots with an expanded* y*-axis.*

Figure 4 shows the Bjerrum plot of $-\log [D]$ *versus* $\bar{n}$ for the bovine serum albumin–pamaquine titration ($P_T = 2.5 \times 10^{-6}$ M). The absence of sharp inflection regions is indicative of overlapping successive equilibria. However, the plot does show shallow inflection regions at the expected values of $\bar{n}$; therefore, $-\log [D]$ values at $\bar{n} = 0.5$, 1.5, and 2.5 can be taken as a fair estimate of $\log k_1$, $\log k_2$, and $\log k_3$, respectively. The results obtained from this Bjerrum plot are given in Table I along with the results obtained for another titration at $P_T = 1.0 \times 10^{-6}$ M. The binding constants thus obtained are in good agreement with those estimated from the Scatchard treatment.

The overall magnitude of the binding constants obtained for the bovine serum albumin–pamaquine system is quite large compared, for example, to the binding of negatively charged 8-anilino-1-naphthalenesulfonate (6), indicating that pamaquine binds strongly to bovine serum albumin. Because of the positively charged nature of pamaquine, it is probable that the binding site on the protein for pamaquine is negatively charged. The most common anionic sites on bovine serum albumin consist of ionized carboxyl groups of various amino acids. There are about 37 free glutamic

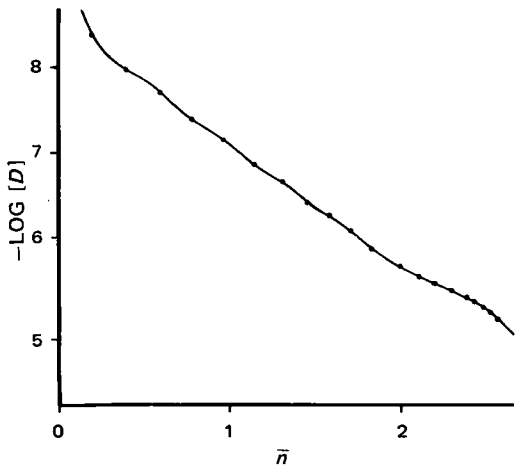


Figure 4—*Bjerrum plot of* $-\log$ [D] *as a function of* $\bar{n}$ *for the bovine serum albumin–pamaquine titration with the protein concentration of* 2.5×10^{-6} M.

acid residues and 56 free aspartyl acid groups (7).

However, in spite of the presence of such a large number of negative centers on bovine serum albumin, only three pamaquine molecules bind to it. Apparently, not all negative centers are available for the binding of pamaquine. Clearly, many are buried in the interior of the protein. Moreover, it is possible that the binding sites of pamaquine are ionized carboxyl groups on the outside of the protein with a suitable juxtaposition of other substituents or side chains so as to make the pamaquine–bovine serum albumin interaction energetically favorable. Even though pamaquine is positively charged, the quinoline ring, the alkyl side chain, and the ethyl groups on the terminal amino nitrogen could make its interaction with water less favorable than with a site on the protein which is negatively charged and has nonpolar side groups.

APPENDIX

The following illustrates the derivation of the equation to calculate the fraction of substrate bound when bound and free substrate both fluoresce, but with different intensities, at the analytical emission wavelength.

If F_p is the observed fluorescence intensity of a solution containing a total protein concentration of P and a total substrate concentration of S, then:

$$F_p = F_U + F_B \qquad \text{(Eq. A1)}$$

where F_U and F_B are the fluorescence intensities due to the unbound and bound fractions of substrate, respectively.

Assuming that the absorbance, A, by the emitting species at the wavelength of excitation is less than 0.02, the fluorescence intensity is related to the concentration of emitting species as:

$$F = 2.303 I_0 \phi \epsilon c l \qquad \text{(Eq. A2)}$$

where I_0 = intensity of incident radiation, ϕ = quantum yield of the emitter, ϵ = molar absorptivity, c = concentration of the emitter, and l = path length.

Since $I_0 l$ is an instrumental constant, Eq. A2 can be rewritten as:

$$F = k\phi\epsilon c \qquad \text{(Eq. A3)}$$

Substituting Eq. A3 into Eq. A1 yields:

$$F_p = k(\phi_U \epsilon_U [U] + \phi_B \epsilon_B [B]) \qquad \text{(Eq. A4)}$$

where subscripts U and B stand for unbound and bound fractions of substrate, respectively. But since $S = [U] + [B]$, Eq. A4 can be rewritten as:

$$F_p = k[\phi_U \epsilon_U (S - [B]) + \phi_B \epsilon_B [B]] \qquad \text{(Eq. A5)}$$

Similarly, when all of the substrate is free, *i.e.*, in the absence of protein, the fluorescence intensity F_f will be given by:

$$F_f = k\phi_U \epsilon_U S \qquad \text{(Eq. A6)}$$

and when all of the substrate is bound to protein, *i.e.*, in presence of excess protein, the fluorescence intensity F_b will be given by:

$$F_b = k\phi_B \epsilon_B S \qquad \text{(Eq. A7)}$$

Taking the ratio of Eqs. A6 and A7 gives:

$$\phi_U \epsilon_U = \frac{F_f}{F_b} \times \phi_B \epsilon_B \qquad \text{(Eq. A8)}$$

Substituting Eq. A8 into Eq. A5 yields:

$$F_p = k\left[\frac{F_f}{F_b}\phi_B \epsilon_B (S - [B]) + \phi_B \epsilon_B [B]\right] \qquad \text{(Eq. A9a)}$$

$$F_p = k\phi_B \epsilon_B \left\{\frac{F_f}{F_b} S - \frac{F_f}{F_b}[B] + [B]\right\} \qquad \text{(Eq. A9b)}$$

Dividing Eq. A9 by Eq. A7 gives:

$$\frac{F_p}{F_b} = \frac{\frac{F_f}{F_b} S - \frac{F_f}{F_b}[B] + [B]}{S} \qquad \text{(Eq. A10)}$$

Dividing throughout by S gives:

$$\frac{F_p}{F_b} = \frac{F_f}{F_b} - \frac{F_f [B]}{F_b S} + \frac{[B]}{S} \qquad \text{(Eq. A11)}$$

But $[B]/S = \alpha$, the fraction of substrate bound, and Eq. A11 becomes:

$$\frac{F_p}{F_b} = \frac{F_f}{F_b} - \frac{F_f}{F_b}\alpha + \alpha \qquad \text{(Eq. A12)}$$

Solving for α yields:

$$\alpha = \frac{F_p - F_f}{F_b - F_f} \qquad \text{(Eq. A13)}$$

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Separation and Quantitative Determination of Impurities in Tetracycline

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Abstract □ Anhydrotetracycline, epianhydrotetracycline, epitetracycline, and chlortetracycline are generally found as impurities in tetracycline. A new thin-layer partition chromatographic method is described in which the impurities are separated on a kieselguhr layer impregnated with ethylene glycol–water–acetone–ethyl acetate (2:2:15:15). Compared to previous methods, this determination is easier to perform and more versatile and it prevents rapid epimerization. The quantitative determination of the tetracyclines utilizes spectrophotometry or direct TLC fluorometry.

Keyphrases □ Tetracycline—separation and analysis of impurities □ TLC—separation, tetracycline impurities

The quantitative determination of tetracycline antibiotics by titrimetry (1), polarography (2), colorimetry (3), paper chromatography (4), and column chromatography (5–8) has been reported, but TLC has proved most useful. The identification of three tetracyclines using a kieselguhr layer impregnated with glycerin was reported (9), and several tetracyclines were separated from their epimers using a similar system (10). Three tetracyclines were separated using kieselguhr previously washed several times with 6 *N* HCl to remove the binder (11). Anhydrotetracycline (I), epianhydrotetracycline (II), and epitetracycline (III) were separated from tetracycline (IV) and were quantitated by spectrophotometry (12).

Dijkhuis and Brommet (13) determined I and chlortetracycline (V) in IV on a layer impregnated with a citrate–phosphate buffer containing 10% glycerin. Van den Bulcke (14) studied the influence of the binder and polyalcohols in the stationary phase and of the solvent systems both on the separation of tetracyclines and the detection of their impurities. Microcrystalline cellulose was used to separate I and other impurities from IV (15–18).

Gyanchandani *et al.* (19) first impregnated the plates with the solvent system, a process that conditioned them so that they could be stored for several weeks. Finally, van Hoeck *et al.* (20) separated I, II, and III from IV on a solvent-impregnated plate and determined IV by direct fluorometry. The methods described in Refs. 12–14, 19, and 20 seem to be the most reliable, although they are subject to important criticism.

The use of acid-washed kieselguhr is undesirable (14) because the washing process is lengthy and does not result in reproducible separations. Microcrystalline cellulose is also unsatisfactory because the support, as described by Simmons *et al.* (15–17), is difficult to prepare and does not allow a good separation while the use of citrate and/or phosphate buffers may markedly increase the epimerization of the tetracyclines (19, 21, 22). For the same reason, the temperature should be controlled during the development (21). Most investigators used solutions of IV at concentrations ranging from 0.05% (20) to 0.2% (12, 19), but their procedures failed when higher concentrations of 0.5–1% were used to increase the sensitivity of detection of the impurities to an acceptable level. Failure resulted from the tailing of the IV spot.

Table I—TLC of I–V

Compound	Concentration, μg/μl	$R_f \times 100$ I[a]	II[b]	III[c]
I	0.025	98	86	95
V	0.025	21	32	72
II	0.025	16	27	30
IV	5.0	10	20	50
III	0.025	4	10	28

[a] With 10% VI plates. [b] With 3.7% VI plates used immediately after impregnation. [c] With 3.7% VI plates used 24 hr after impregnation.

The purpose of this research was to develop a TLC procedure allowing separation of traces of I–III and V as impurities in a 0.5% (w/v) solution of IV and their quantitation by means of spectrophotometry or direct TLC fluorometry.

EXPERIMENTAL

Reagents—Disodium ethylenediaminetetraacetate (VI), ethylene glycol, acetone, ethyl acetate, and sodium hydroxide were analytical grade.

Kieselguhr—Both MN-kieselguhr G[1] and kieselguhr G[2] were used. The former was preferred since the slurry was easier to handle and provided a coating with a more homogeneous appearance; it also gave a higher resolution.

Preparation of Plates—Two different supports were prepared using: *(a)* a slurry of 20 g of kieselguhr and 47 ml of 10% VI solution adjusted to pH 8.5 with 20% NaOH, and *(b)* a slurry of 20 g of kieselguhr and 47 ml of 3.7% VI solution. Plates (20 × 20 cm), four at a time, were prepared[3] with a layer thickness of 0.25–0.30 mm. The plates were dried at room temperature (25°) and at a relative humidity of 30–50% for 1 hr. Prior to use, they were developed with the solvent system for 1–2 hr and dried at room temperature for 1 hr.

Solvent System—The system was made up of ethylene glycol–water–acetone–ethyl acetate (2:2:15:15 v/v).

Preparation of Solutions—The substances were dissolved in methanol or a mixture of methanol and 0.1 *N* HCl (9:1). For identification of I–III and V, a concentration of 0.025 μg/μl was used. The solution of IV was used at 5 μg/μl. Calibration curves for di-

[1] Macherey, Nagel & Co, D-516 Düren, Germany.
[2] E. Merck, Darmstadt, Germany.
[3] Desaga applicator, Heidelberg, Germany.

Table II—Absorptivities of I–V Determined on 10% VI Plates

Compound	Wavelength, nm	a [a]	SD	a_w [a]	SD	Recovery, %
I	430	19.32	0.498	17.70	0.796	91
II	430	16.89	0.591	15.16	0.923	90
III	355	32.10	0.169	29.59	0.608	92.5
IV	355	30.79	0.165	27.78	1.454	90.5
V	367.5	20.60	0.500	18.90	1.020	92
	265	35.60	0.670	33.50	0.840	94

[a] Each result is the mean of five determinations.

rect TLC fluorometry were prepared using appropriate concentrations.

Apparatus—A TLC chromatotank (20 × 20 × 8 cm) was lined with filter paper, and 120 ml of solvent was added. The tank was allowed to equilibrate for at least 1 hr. A spectrophotometer[4], a TLC spectrophotometer[5], an electrobalance[6], and a recorder[7] were also used.

Application of Solutions—Volumes of 50 or 100 μl were spotted by syringe[8], and volumes of 1 μl were spotted by disposable pipets[9].

Qualitative Chromatography—One-microliter volumes were applied as spots on a line 2.5 cm above the lower edge of the plate. The plate was placed in the tank and developed for 40 min at 25°. After drying the plate, the spots were observed under UV light (366 nm) and fluorescence was increased by exposure to ammonia vapor. The R_f values observed are listed in Table I. Typical TLC separations on 10% VI plates and 3.7% VI plates are shown in Figs. 1 and 2.

Quantitative Analysis—*Spectrophotometric Assay*—A suitable volume (50–100 μl) of a solution of the test mixture was applied as a 15-cm band on a line 2.5 cm above the lower edge of a 10% VI plate and developed until the solvent front had moved 13–14 cm. After the plate was dried, the fluorescing zones were quickly marked under UV light, removed separately, and extracted with 0.1 *N* HCl. Since centrifuging did not produce a clear solution, the kieselguhr was removed using a porcelain filter A_1, which was washed with 0.1 *N* HCl; the filtrates were combined and adjusted to volume. Further dilutions were prepared using 0.1 *N* HCl.

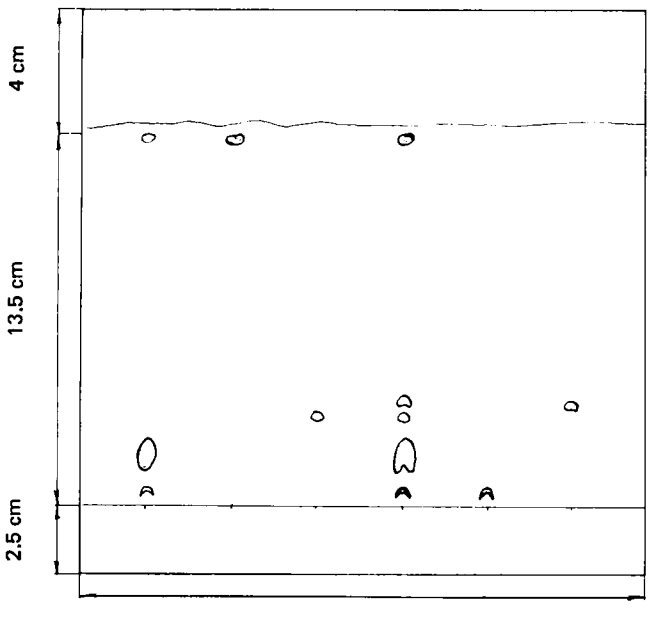

Figure 1—*Example of a typical TLC separation of tetracycline impurities on kieselguhr with 10% VI at pH 8.5. Key (left to right): 5 μg of IV, 0.025 μg of I, 0.025 μg of II, mixture (I–V), 0.025 μg of III, and 0.025 μg of V.*

Compounds I and II were determined using the absorbance at 430 nm, Compounds III and IV were determined at 355 nm, and Compound V was determined at 265 or 367.5 nm against a blank prepared in the same way as the test solutions. The absorbance of the blank at 430 nm was 0.002–0.008 for 20 cm^2 of the kieselguhr layer; at 355 nm, it was 0.050; and at 265 and 367.5 nm, it was 0.240 and 0.038, respectively.

The amount of substance in the mixture was calculated as follows:

$$\% = \frac{(A)(f)100}{(a)(p-l)} \qquad \text{(Eq. 1)}$$

where p is the concentration of sample in grams per liter, l is the loss on drying, f is the dilution factor, a is the absorptivity of the substance being examined, and A is the absorbance measured.

The absorptivities *(a)* were determined for the pure reference substances (Table II). The values a_w were obtained from measurements on the eluates after chromatographing the pure reference substances.

Spectrophotometric determination of the impurities was difficult, because rather large quantities were necessary to produce reliable measurements, *i.e.*, absorbances between 0.150 and 0.750. The final concentration of the extracts from the kieselguhr ranged from 10 to 40 μg/μl. At lower concentrations, the errors increased significantly.

Direct TLC Fluorometry—An irregular distribution of the substances within the spot is unimportant when direct TLC fluorometric evaluation is used. Spots with a regular form are measured with a circular aperture, and the recorded value is proportional to the total fluorescence and, thus, to the concentration of the sample. When using a circular aperture, a slit width of approximately 1.5 mm is used.

With spots of an irregular shape, a rectangular aperture is used. In this case, the area between the curve and the abscissa should be determined on the recorded graph. Disturbing effects such as background, front line fluorescence, and, particularly, dust particles may influence the measurement and are to be avoided. For drawing a calibration curve, the internal standard method is used.

Assay—For the evaluation of II, III, and V, 10% VI plates were used; for the evaluation of I, 3.7% VI plates were used. For each impurity, five solutions with concentrations in geometric progression were prepared; 1 μl of each was spotted at 2.5-cm intervals on the baseline together with 1 μl of the solution of IV. One microliter of the test solution of IV was also applied separately. Two or more impurities were evaluated on a single plate by preparing a combined standard solution of these impurities in a definite proportion and diluting it in a geometric progression.

After separation, the plate was allowed to dry at room temperature for 10 min. The TLC spectrophotometer was adjusted to the spot with the highest concentration, and the transmission scale was adjusted to 100 units. A rectangular aperture was used for all measurements; the other conditions used for each impurity are listed in Table III.

The fluorescence was excited by the 365-nm Hg line and measured by scanning the accurately positioned spots in the x axis or, if necessary, in the y axis direction with a scanning speed of 3 cm/min. The recorded speed was kept at 5 cm/min. The results obtained are reported in Table IV.

Each fluorescent spot was recorded as a peak and was quantitated by measuring the peak area either by triangulation (results by

[4] Zeiss PMQ II.
[5] Zeiss.
[6] Cahn.
[7] Varian A 25.
[8] Hamilton-Bonanduz Schweiz.
[9] Vitrex, Chr. Bardram Birkerod, Denmark.

Table III—Conditions for TLC Spectrophotometric Determination of Each Impurity

Compound	Wavelength, nm	Slit Width, mm	Slit Height, mm	Amplification[a] 2 μg	0.5 μg	0.125 μg	0.0312 μg	0.0078 μg
I	514	0.6	8	0.8/1/II/F	4.1/1/II/F	7.8/1/II/F	1/10/II/F	—
II	533	0.6	6	1.2/1/II/F	4.4/1/II/F	9/1/II/F	3.2/10/II/F	—
III	523	0.6	6	—	5.5/1/II/F	7.5/1/II/F	1.8/10/II/F	6.1/10/II/F
V	528	0.6	6	—	2.2/1/II/F	7/1/II/F	1.6/10/II/F	5.7/10/II/F

[a] Amplification of the TLC spectrophotometric response corresponding to different concentrations of the substances in the spot.

this procedure were unreliable and variable) or, preferably, by cutting out and weighing the peak areas. These weights are a measure of the areas and were used as such in the calculation of the regression line (23) as follows. Let y be the weight of an area corresponding to a concentration x of a spot on the chromatogram and $\tilde{y}$ is the true value of y at any given value of x. Then the equation of the true regression line will be:

$$\tilde{y} = a + bx \qquad \text{(Eq. 2)}$$

where b is the slope and a is the intercept on the y axis at $x = 0$. This equation can be rearranged to:

$$\tilde{y} = \bar{y} + b(x - \bar{x}) \qquad \text{(Eq. 3)}$$

since the point $(\bar{x},\bar{y})$ lies on the estimated regression line. The slope b is given by the following equation:

$$b = \frac{\Sigma xy - \dfrac{(\Sigma x)(\Sigma y)}{n}}{\Sigma x^2 - \dfrac{(\Sigma x)^2}{n}} \qquad \text{(Eq. 4)}$$

where n = sample size.

To draw the estimated regression line, first $(\bar{x},\bar{y})$ is plotted. A convenient amount is added to $\bar{x}$ and b times this amount to $\bar{y}$. Thus, a second point can be plotted. These two points determine the regression line. The standard deviation of the individual y values about the regression line is designated $s_{y.x}$ and is computed by the square root of the following expression:

$$s^2_{y.x} = \frac{1}{n-2}\left\{\Sigma y^2 - \frac{(\Sigma y)^2}{n} - b\left[\Sigma xy - \frac{(\Sigma x)(\Sigma y)}{n}\right]\right\} \qquad \text{(Eq. 5)}$$

The regression was calculated in this way for each impurity and is illustrated for II and V in Fig. 3.

It has been verified that the assay procedure gives a valid estimate. Therefore, the recovery of the concentration of the impurity added in geometrical progression to the spot of the unknown mixture was calculated. The mean of each series of these values was plotted against the corresponding amount of impurity added. An example of the correlation between I added and I recovered is shown in Fig. 4.

RESULTS AND DISCUSSION

Separation of Tetracyclines—Rapid and accurate evaluation of the tetracycline impurities requires a very sensitive separation method. Both the 10% VI method and the 3.7% VI method were suitable for qualitative and quantitative purposes. Figures 1 and 2 show that III, IV, II, V, and I separated in that order. Epichlortetracycline was also separated, with an R_f value between that of III and IV.

Both methods allowed a spectrophotometric determination of the separated substances. However, the 10% VI method gave the best separation; the spots were round and regular in shape with a good resolution appropriate for direct TLC fluorometry. This procedure was adopted for most experiments (Table IV). The spot of I moved so close to the solvent front in this procedure that it could not be evaluated by direct fluorometry and the 3.7% VI method had to be used to estimate this impurity. Before use, the plates were impregnated with the solvent system for at least 1 hr. However, the optimum impregnation time, resolution of the spots, and R_f values varied slightly with the ambient atmospheric conditions such as temperature and relative humidity. Activation of the plates prior to impregnation did not improve the results but resulted in tailing of the spots.

Care was taken to dry layers slowly, preferably at 30–50% relative humidity and a temperature of 25°. Unimpregnated plates prepared in this way could be stored for an unlimited time. The solvent used for impregnation could be used two or three times. Test and standard solutions, when kept at 0° in the dark, were stable for at least 1 week.

Sensitivity—Mixtures of IV with impurities ranging from 4 to 16 mg/ml were adequately resolved by these methods. It was possible to resolve a 16-mg/ml solution of IV containing impurities as follows: IV, 99.78%; I and III, 0.1%; II, 0.07%; and V, 0.5% (Table IV).

Since high concentrations of IV increased tailing, it was preferable to apply the samples as 4–8-mg/ml solutions, using a 1-μl micropipet to prevent overloading of the layer and to keep the spot size small enough to allow estimation by direct fluorometry.

Spectrophotometric Analysis—Spectrophotometry, although an accurate procedure, was less sensitive. If, for instance, 100 μl of a 16-mg/ml tetracycline solution was streaked onto the plate over a distance of 15 cm, the impurities had to be present in quantities of at least 3% of total tetracycline to be detected. Indeed, spectrophotometric determination required a minimum concentration in the eluate of 10 μg/ml of the substance concerned. An impurity percentage less than 3% could be estimated by spotting a greater volume or by increasing the concentration of the solution, but both procedures gave a poor separation.

The absorptivities of I–V are listed in Table II. Each result is the mean of five determinations and the relative standard deviation was less than 3.5% in all instances. The a_w values, however, showed a relative standard deviation up to 6%. Compound V

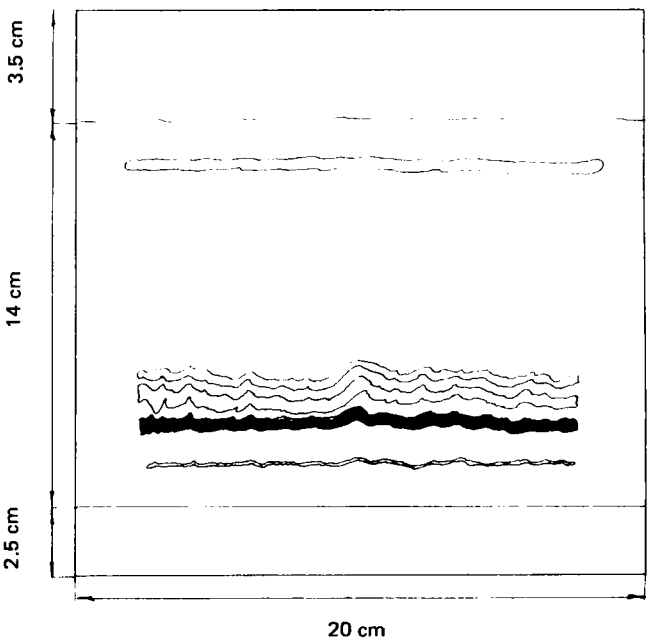

Figure 2—*Example of a typical TLC separation of impurities of tetracycline on kieselguhr with 3.7% VI for spectrophotometric purposes. Bands in ascending order are: 100 μg of III, 5000 μg of IV, 100 μg of II, 100 μg of V, and 100 μg of I.*

Table IV—Recovery of I–III and V Added to Different Amounts of IV by Direct TLC Fluorometry

Compound	Spotted Quantity, μg	Composition, %	Recovery[a], μg	Confidence Limits[a] Maximum	Confidence Limits[a] Minimum	Recovery, %
IV	4.0170	89.60				
I	0.1230	3.06	0.1196	0.1228	0.1165	103
II	0.2219	5.52	0.2163	0.2217	0.2109	97.5
III	0.0458	1.14	0.0454	0.0472	0.0435	99
V	0.0275	0.68	0.0275	0.0283	0.0268	100
IV	4.0170	94.80				
I	0.0615	1.53	0.0691	0.0779	0.0604	112
II	0.1109	2.76	0.1217	0.1319	0.1114	109.5
III	0.0229	0.57	0.0228	0.0260	0.0197	99.5
V	0.0137	0.34	0.0135	0.0148	0.0121	98.5
IV	4.0170	97.41				
I	0.0307	0.76	0.0298	0.0360	0.0236	97
II	0.0555	1.38	0.0588	0.0697	0.0479	106
III	0.0114	0.28	0.0137	0.0178	0.0097	120
V	0.00685	0.17	0.0071	0.0078	0.0064	103.5
IV	4.0170	98.70				
I	0.0154	0.38	0.0144	0.0173	0.0114	93.5
II	0.02775	0.69	0.0227	0.0289	0.0165	82
III	0.0057	0.14	0.0047	0.0072	0.0022	82.5
V	0.0034	0.085	0.0035	0.0043	0.0027	103
IV	4.0170	99.36				
I	0.0077	0.19	0.0061	0.0072	0.0051	79
II	0.0139	0.34	0.0137	0.0164	0.0111	98.5
III	0.00285	0.07	0.0023	0.0035	0.0011	80.5
V	0.0017	0.04	0.0016	0.0021	0.0011	94
IV	8.8658	95.12				
I	0.0825	0.93	0.0819	0.0865	0.0773	99
II	0.1970	2.23	0.1966	0.2020	0.1911	100
III	0.1202	1.36	0.1202	0.1222	0.1200	100
V	0.03216	0.36	0.0323	0.0325	0.0321	100.5
IV	8.8658	97.56				
I	0.0412	0.46	0.0395	0.0432	0.0357	96
II	0.0985	1.12	0.0947	0.1114	0.0781	96
III	0.0601	0.68	0.0591	0.0637	0.0546	98
V	0.01608	0.18	0.0158	0.0166	0.0150	98
IV	8.8658	98.78				
I	0.0206	0.23	0.0199	0.0249	0.0149	96.5
II	0.04925	0.56	0.0525	0.0598	0.0452	106.5
III	0.03005	0.34	0.0324	0.0357	0.0290	108
V	0.00804	0.09	0.0079	0.0087	0.0070	98
IV	8.8658	99.39				
I	0.0103	0.12	0.0086	0.0113	0.0058	83.5
II	0.02462	0.28	0.0248	0.0374	0.0153	101
III	0.01502	0.17	0.0145	0.0158	0.0132	96.5
V	0.00402	0.045	0.0043	0.0052	0.0033	107
IV	8.8658	99.69				
I	0.00515	0.06	0.0107	0.0176	0.0039	208
II	0.01231	0.14	0.0054	0.0083	0.0024	44
III	0.00751	0.09	0.0078	0.0090	0.0066	104
V	0.00201	0.022	0.0021	0.0029	0.0013	105
IV	16.0020	99.346				
I	0.0360	0.225	0.0368	0.0382	0.0354	102
II	0.0220	0.137	0.0221	0.0248	0.0195	100.5
III	0.0330	0.206	0.0333	0.0381	0.0284	101
V	0.0138	0.086	0.0141	0.0149	0.0134	102
IV	16.0020	99.672				
I	0.0180	0.113	0.0158	0.0185	0.0130	88
II	0.0110	0.069	0.0101	0.0139	0.0063	92
III	0.0165	0.103	0.0152	0.0197	0.0107	92
V	0.0069	0.043	0.0064	0.0083	0.0046	92.5
IV	16.0020	99.837				
I	0.0090	0.056	0.0082	0.0098	0.0066	91
II	0.0055	0.035	0.0053	0.0080	0.0026	96
III	0.0082	0.051	0.0073	0.0125	0.0021	89
V	0.0034	0.021	0.0029	0.0037	0.0021	85
IV	16.0020	99.918				
I	0.0045	0.028	0.0063	0.0079	0.0046	140
II	0.0028	0.017	0.0031	0.0048	0.0015	111
III	0.0042	0.026	0.0041	0.0050	0.0032	97.5
V	0.0017	0.011	0.0015	0.0018	0.0012	88
IV	16.0020	99.959				
I	0.0022	0.014	0.0027	0.0040	0.0013	123
II	0.0014	0.009	0.0108	0.0196	0.0020	771
III	0.0021	0.013	0.0065	0.0131	0.0000	310
V	0.00085	0.005	0.0014	0.0017	0.0013	165

[a] Each result is the mean of five determinations. [b] $p = 0.05$, 4 *df*, $t^* = 2.78$.

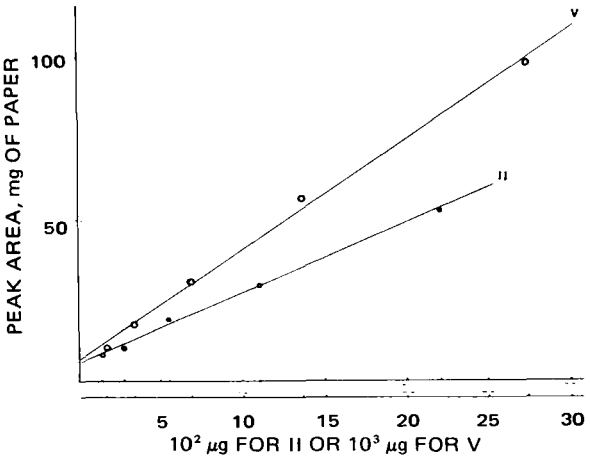

Figure 3—*Example of a calibration graph for II and V separated from a 4-mg/ml tetracycline solution on a 10% VI layer.*

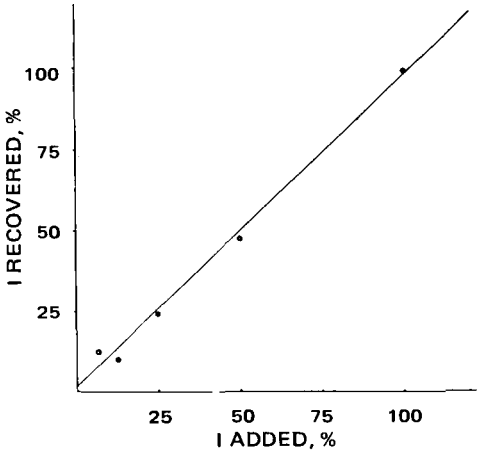

Figure 4—*Example of the correlation between I added and I recovered from an 8.8-mg/ml tetracycline mixture on a 3.7% VI layer by direct TLC fluorometry.*

should be measured at 367.5 nm because the great absorbance of the blank at 265 nm could interfere with an exact evaluation.

Direct TLC Fluorometry—As mentioned previously, a rectangular aperture was used for the measurements since the slit width was much easier to handle and to adjust to the spot concerned; it also allowed a three to fourfold amplification. Since the slit height could not be altered continuously, poor accuracy was obtained where the spots were not completely resolved. Before making quantitative measurements, plates were always inspected under UV light to allow removal of fluorescing dust particles. A chart speed of 5 cm/min was desirable to allow the cutting out of the peak areas. The computation of results by estimation of a regression line and its confidence limits seems somewhat tedious. In cases of routine tetracycline analysis, however, when impurities will occur in quantities of 1% or more and great accuracy is not needed, sufficiently accurate evaluations can be achieved graphically.

The individual regression lines calculated for each impurity separated from 4- and 8-mg/ml tetracycline solutions showed good linearity with a significant correlation coefficient (lowest value 0.9529). Consequently, the mean results of the correlation between the impurity added (%) and the impurity recovered (%) showed good linearity. Compounds I, with a linear equation of $y = 0.27 + 0.99x$ (correlation coefficient 0.9958), and V, with a linear equation of $y = 0.19 + 0.997x$ (correlation coefficient 0.9998), separated from a 4-mg/ml mixture had especially good linearity; $y = 1.68 + 0.962x$ (0.9956) and $y = 0.09 + 0.998x$ (0.998) were obtained, respectively, from an 8.8-mg/ml solution of tetracycline.

The confidence limits (95% confidence interval) of the means are given by $\bar{x} \pm (\sigma/\sqrt{n} \times t^*)$, where $\sigma/\sqrt{n}$ is the standard error and t^* is 2.78 (df 4 and $p = 0.05$)[10]. These values are listed in Table IV and indicate the acceptable accuracy of the direct fluorometric method; each result is the mean of five determinations. For fewer determinations, the critical t^* value becomes much larger and it is better to express the confidence interval as $\bar{x} \pm (1.96 \times \sigma_x)$, where σ_x is the standard deviation. The results, using a 16-mg/ml solution of tetracycline, were not very reliable. Compounds II and III gave regression lines for which the slopes were not significant, although the recoveries were very close to the expected values. For these reasons, the separation of II and III from a 16-mg/ml solution has only a qualitative use. For quantitative purposes, 4–8-mg/ml solutions should be used.

CONCLUSIONS

The TLC method described allows a separation of anhydrotetracycline, epianhydrotetracycline, epitetracycline, and chlortetracycline from a 16-mg/ml solution of tetracycline. Effective measurements of epichlortetracycline at low concentration is impeded by the luminous background left by the spot of tetracycline. A good separation probably can be achieved with other impurities such as doxycycline, rolitetracycline, methacycline, and oxytetracycline.

Direct TLC fluorometry is a very sensitive means of evaluating the impurities in tetracycline, allowing the determination down to a concentration of 0.5% (w/w) in tetracycline. The limit of detection is 50–100 times greater than when spectrophotometry is used.

[10] Table III of Fisher and Yates, "Statistical Tables for Biological, Agricultural and Medical Research."

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GLC Analysis of Caffeine and Codeine Phosphate in Pharmaceutical Preparations

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Abstract □ A procedure for the determination of caffeine and codeine phosphate in pharmaceutical preparations was developed. It depends upon a one-step extraction followed by GLC analysis of the concentrated extract.

Keyphrases □ Caffeine and codeine phosphate—GLC analysis in pharmaceutical products □ Codeine phosphate and caffeine—GLC analysis in pharmaceutical products □ GLC—analysis, caffeine and codeine phosphate

Caffeine and codeine phosphate are used singly or in combination with other drugs in various pharmaceutical preparations. Quality control requirements (1) have made it mandatory to determine both the caffeine and codeine phosphate content uniformity. James (2) reported a procedure for the determination of the codeine and caffeine contents of individual aspirin, phenacetin, caffeine, and codeine phosphate tablets based upon a fluorometric–UV spectroscopic assay. While accurate, this method and other spectroscopic assays (3, 4) involve time-consuming multiple extractions which are liable to inherent errors.

The reported GC methods are either not amenable to single-tablet assay, do not include codeine, or are rather involved (2, 5).

The developed GLC procedure is simple, and the sample preparation involves only two extractions per tablet. This process represents a 77% reduction in the extraction requirements when compared with the compendial method (1) with no loss in accuracy. Caffeine, codeine phosphate, and phenacetin are extracted from the tablet matrix into a common solvent, and no further separation is needed.

EXPERIMENTAL

Equipment—A gas chromatograph[1] equipped with a flame-ionization detector was used. The detector signal was fed into a 1-mv recorder[2] operated with a chart speed of 1.3 cm (0.5 in.)/min. One-microliter samples were injected with a 10-μl syringe[3].

Materials—High purity helium was the carrier gas. Purified hydrogen and compressed air were used in the detector. The stationary phase was 3%, OV-17 on Chromasorb W[4] (80–100 mesh), acid washed and silanized, packed in a 1-m × 0.64-cm o.d. glass column. All chemicals employed were spectrograde. All sample and standard materials were of NF or USP grade.

Operating Conditions—The column was operated isothermally at 240°. Both the detector and the injector port were held at 250°. The carrier gas flow rate was 28 ml/min at an inlet pressure of 40 psig. The initial electrometer range and attenuation settings were 10^2 and 32, respectively. After the elution of the solvent and the first two components, the range setting was 10 and the attenuation setting was 8 for the duration of the run.

Preparation of Standard Solutions—Two standard solutions were prepared, an internal standard and the working standard.

Internal Standard—Three hundred milligrams of nortriptyline hydrochloride was dissolved in 20 ml of 15% sodium chloride solution in a 125-ml separator. The solution was made basic by the addition of 5 ml of 2 *N* NaOH and extracted with 2 × 15 ml of chloroform. The extracts were filtered through filter paper[5] containing 5 g of anhydrous sodium sulfate, and the extracts were combined in a 50-ml volumetric flask. The filter was washed with 15 ml of chloroform, and the washings were added to the combined extracts. Chloroform was used to bring the contents to volume.

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Table I—GLC Data

Compound	Retention Time, min	Relative Retention Time, min
Phenacetin	1.2	0.276
Caffeine	1.95	0.448
Nortriptyline	4.35	1.000
Codeine	8.60	1.970

Table II—Standard Samples

Ingredient	Mixture 1, mg	Found, mg	Mixture 2, mg	Found, mg
Aspirin	234	—	234.7	—
Phenacetin	151	—	150.8	—
Caffeine	31.6	31.0	30.6	30.3
Codeine phosphate	15.0	14.9	30.4	30.2

[1] Hewlett-Packard model 402.
[2] Hewlett-Packard model 7127-A.
[3] Hamilton No. 701.
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Table III—Analysis of Caffeine and Codeine Phosphate in Prepared Aspirin, Phenacetin, Caffeine, and Codeine Phosphate Tablets

Tablet Number	Codeine Phosphate, mg		Caffeine, mg	
	Claim	Found	Claim	Found
A-1	30	33.4	30	30.0
A-2	30	28.9	30	27.3
A-3	30	32.9	30	30.7
B-1	60	60.4	30	31.9
B-2	60	60.4	30	31.9
B-3	60	60.5	30	30.9
C-1	15	15.0	30	30.5
C-2	15	16.0	30	29.7
C-3	15	14.8	30	31.3

washings were collected in a 50-ml erlenmeyer flask and evaporated to dryness on a steam bath under nitrogen flow. The dried extract was redissolved in 3 ml of the internal standard solution prior to use.

Preparation of Sample Solutions—One tablet, or one tablet weight, of aspirin, phenacetin, caffeine, and codeine phosphate was put into a 125-ml separator. Twenty milliliters of 15% sodium chloride solution was added, and the contents were shaken until the tablet disintegrated. The mixture was then treated exactly as described for the working standard beginning with: "The mixture was treated in . . . ."

Calculations—For the standard chromatogram, the ratio, R-1, of the areas of the nortriptyline/caffeine peaks and the ratio, R-2, of the areas of the nortriptyline/codeine peaks were obtained. For the sample chromatogram, the ratio, R-3, of the caffeine/nortriptyline peak areas and the ratio, R-4, of the codeine/nortriptyline peak areas were obtained. Then:

$$(R\text{-}1) \times (R\text{-}3) \times Cf = \text{mg caffeine in sample} \quad \text{(Eq. 1)}$$

where Cf is the milligrams of caffeine in the working standard, and:

$$(R\text{-}2) \times (R\text{-}4) \times Cc = \text{mg codeine phosphate in sample} \quad \text{(Eq. 2)}$$

where Cc is the milligrams of codeine phosphate in the working standard.

RESULTS AND DISCUSSION

Nortriptyline hydrochloride served as the internal standard in the compendial procedure for the analysis of codeine phosphate (1). In this study, it was equally satisfactory for the quantitative evaluation of both caffeine and codeine phosphate. Figure 1 illustrates a typical chromatogram. Area ratios were used rather than peak height ratios, since the area ratios yielded analytical data without repetitive chromatograms. As the figure shows, isothermal operation of the column at the temperature and carrier gas flow rate employed resulted in excellent resolution of the compounds. Table I presents the associated GLC data.

The accuracy of the method is evident from the data given in Table II. Each of the two simulated preparations contained the four active ingredients in amounts similar to the sample tablet preparations. Each mixture was subjected to the entire analytical procedure. Use of an integrator to measure the peak areas would enhance the accuracy further. The present error is estimated to be about 2%.

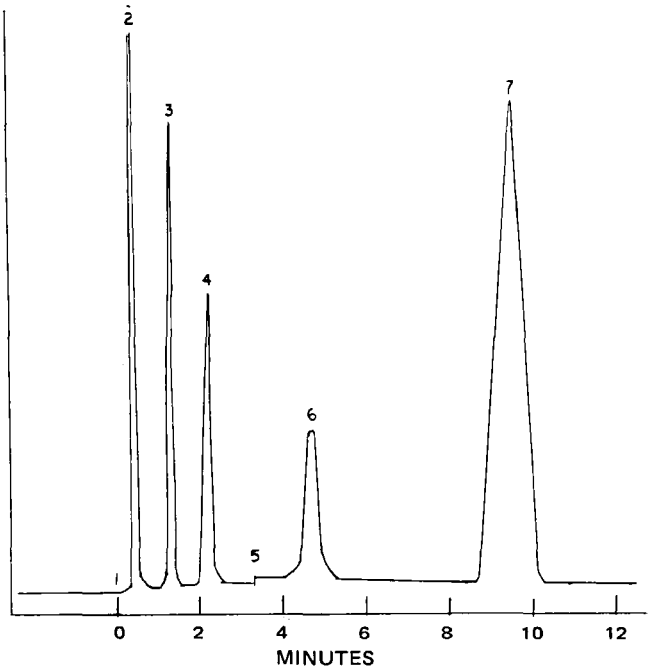

Figure 1—*Gas chromatogram of a sample extract with internal standard. Key: 1, inject; 2, solvent; 3, phenacetin; 4, caffeine; 5, attenuation change; 6, nortriptyline (internal standard); and 7, codeine.*

Application of the procedure to the analysis of tablet preparations is given in Table III. By first extracting the mixture with chloroform, then making the solution basic, and then extracting again with chloroform, all four ingredients are present in the combined extracts. In the present case, the aspirin remains in the aqueous phase as the sodium salt. Temperature programming would allow all active ingredients to be analyzed, but now only phenacetin, caffeine, and codeine phosphate can be analyzed.

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Use of Factorial Design to Evaluate Granulations Prepared by Spheronization

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Abstract □ This study was designed to demonstrate that properties of a granulation with a given composition, prepared by the spheronizing technique, could be altered by slight changes in process variables alone to satisfy the requirements of the formulator. A complete factorial experimental design was found satisfactory for demonstrating the range of properties to be expected and for showing statistically significant main effects and any linear interactions between selected variables. Results using two levels of five variables showed that initial water content and spheronizer speed had significant main effects on all primary granulation properties studied. While only one formulation was studied, the data suggest that the factorial design can have utility in predicting the properties of granulations prepared at conditions within the limits imposed by the equipment or formulation.

Keyphrases □ Spheronization—process variables, effect on granulation properties □ Tablet granulation—spheronization method utilized to control granule properties □ Factorial experimental design—utility for optimizing pharmaceutical process conditions

Extruded particles processed to exhibit a fairly uniform spherical shape with a narrow size distribution were reported (1, 2) to offer advantages in pharmaceutical formulations for tableting. The selection of spheronization process variables had a significant effect on tablet hardness and dissolution when acetaminophen tablets were prepared by this unique granulation procedure (3). Studies of tablet granulations prepared with an instrumented single-screw extruder also demonstrated the importance of solvent selection on the final characteristics of the particles (4).

These studies, plus the popularity of pellet-type pharmaceuticals, particularly for capsule fills, suggest the need for detailed information describing the effects of processing variables on the properties of uniform, free-flowing particles in the sieve ranges employed for pharmaceutical products, no matter what process is used for their preparation. In a previous report, a factorial experimental design was recommended to identify significant variables and to predict the effects of changes in processing conditions on selected tablet properties (3). The work presented here summarizes the effect of selected variables on the properties of extruded spheronized granulations utilizing this procedure.

EXPERIMENTAL

Materials—Microcrystalline cellulose, containing 11% sodium carboxymethylcellulose[1], and acetaminophen NF[2] were used. A sieve analysis of both materials appears in Table I.

Processing—The equipment for preparing granulations was described previously (3) and consisted of an extruder[3] and a spheronizer[4] unit. Processing conditions (3) were carried out on a batch composed of 1 kg of materials plus the required quantity of purified water.

Testing Procedures—The temperature of the extruder chamber was monitored by a thermocouple[5] attached to the screen surfaces. Percent loss on drying of the extrudate was determined during extrusion and ranged between 17.6 and 25%. Analysis showed that the extrudate moisture content was determined only by the amount of water added prior to extrusion.

Percent loss on drying was recorded for each dried product, employing a 5.5-w lamp setting at 3.8 cm for 15 min on an IR balance[6]. Bulk density, expressed as grams per milliliter, was determined by measuring the volume occupied by 50.0 g of granulation in a 100.0-ml graduated cylinder.

A sieve analysis of each product was performed using 200-g samples passed through U.S. Standard sieves, from 8- to 60-mesh sizes, with the aid of a sieve shaker[7] at the number 6 setting for 5 min.

Flow rate was determined for each granulation using a conical stainless steel funnel, 23.5 cm high with a top 22.9 cm in diameter and a lower orifice 1.27 cm in diameter. The time required for 500 g of granulation to flow through the orifice was measured. Tests were run in triplicate; the mean value, expressed as grams per second, was used in calculations. Granule friability was evaluated by a procedure described previously (3).

Experimental Design—Details of the experimental design and mathematical treatment of the data as used to evaluate tablet properties were reported previously (3). The results reported here were obtained from the same series of experiments using data generated by various tests on the granulations. The methods described by Yates and Anderson (5) were used to identify main effects (ME) and to indicate first- and second-order interactions between the five variables chosen: water content (A), 250 or 325 ml; extruder speed (B), 39 or 59 rpm; screen size (C), 0.8 or 1.5 mm; spheronizer speed (D), 700 or 1010 rpm; and spheronizer residence time (E), 1.0 or 3.0 min. The ranges were selected to give a wide variation of granule properties within the limitations of the equipment and the requirements of the various tests.

RESULTS AND DISCUSSION

Test values for the various parameters are given in Tables II and III, and the ranges observed for each are included in Table IV. The data on percent fines and maximum amount on one screen are in Table V. An example of the analysis of variance results for flow rate is given in Table VI. Discussion of results will include significant main effects and first-order interactions (those between two variables). A summary of all significant main effects is given in Table VII.

Three parameters—extruder screen temperature, escape, and extrudate water content—were measured during the extrusion step of the spheronization process and could only be affected by water content, extruder speed, and screen size.

[1] Avicel RC 581, FMC Corp., Marcus Hook, Pa.
[2] Special powder, S. B. Penick & Co., New York, N.Y.
[3] Model EXDS-60, Elanco Products Co., Division of Eli Lilly & Co., Indianapolis, Ind.
[4] Marumerizer model Q-230, Elanco Products Co., Division of Eli Lilly & Co., Indianapolis, Ind.
[5] Model 42 SC, Yellow Springs Instrument Co., Yellow Springs, Ohio.
[6] Model 6010, Ohaus Scale Corp., Union, N.J.
[7] Cenco-Meinzer, Central Scientific Co., Chicago, Ill.

Table I—Particle-Size Distribution of Starting Materials[a]

U.S. Standard Sieve No.	Microcrystalline Cellulose	Acetaminophen
20	—	1.1
40	—	9.5
60	—	13.6
80	0.3	34.4
100	0.8	3.0
120	2.6	10.0
<120	96.3	28.4

[a] Weight percent.

Maximum Extruder Screen Temperature—Analysis of variance indicated that water content and extruder speed were significant main effects. No significant interactions were found. An increased water level decreased the maximum extruder screen temperature whereas an increased extruder speed increased the maximum extruder screen temperature. With heat-labile materials, it would be desirable to maintain the temperature at the extruder screens at a minimum. This could be accomplished at high water content and also at low extruder speed.

Escape—Material that passed down the overflow chute during extrusion was collected, weighed, and corrected for moisture content to arrive at a value for the weight of dry material that was not acceptable for spheronization. Water content was the only significant main effect, and the interaction of water content–screen size was the only significant interaction. An increased water content decreased the weight of escape. The significant first-order interaction is shown in Table VIII.

An increased water content resulted in a greater decrease in escape at the low screen size. An increased screen size resulted in a decrease in escape at the low water content whereas escape increased at the high water content. To arrive at maximum product yield, escape should be kept at a minimum. This condition would result from a high water content and a low screen size.

Extrudate Water Content—Analysis of variance of water content of the wet, extruded material indicated only the obvious main effect; namely, an increased water content resulted in an increased water content of the extrudate. The results are presented only to indicate that neither speed nor screen size affected the extrudate water content. Theoretical values would be 20.0% at the low water content and 24.5% at the high water content.

Table II—Granulation Data

Experiment	Maximum Extruder Screen Temperature[a]	Escape[a], g	Extrudate Moisture Content[a], %	Loss on Drying, %
1	42°	86	24.5	1.2
2	41°	92	23.8	1.1
3	40°	55	25.0	1.1
4	52°	96	19.5	1.0
5	43°	132	23.5	1.2
6	60°	196	18.5	0.7
7	40°	143	24.0	1.2
8	63°	262	18.9	1.0
9	58°	120	18.7	0.8
10	61°	236	18.4	0.5
11	43°	142	23.7	1.0
12	70°	288	17.6	1.0
13	41°	133	23.9	1.0
14	40°	87	23.7	1.0
15	52°	132	20.0	1.2
16	60°	146	17.9	1.2
17	64°	170	19.3	1.3
18	61°	148	19.1	1.2
19	43°	102	24.3	0.8
20	65°	186	17.7	0.8
21	59°	160	18.2	1.3
22	50°	125	23.7	1.0
23	43°	82	24.3	1.2
24	62°	186	19.3	1.2
25	55°	143	24.6	0.9
26	51°	159	22.9	0.8
27	56°	184	19.3	1.0
28	46°	122	24.0	1.3
29	66°	198	19.3	1.0
30	57°	19	23.3	1.5
31	45°	141	24.5	1.1
32	61°	170	19.4	1.3

[a] Dependent on three variables only.

Table III—Granulation Data Parameters Subjected to Full Factorial Analysis

Experiment	Flow Rate[a], g/sec	Bulk Density[a], g/ml	Granule Friability[a]	Mean Particle Size, μm
1	19.4	0.690	1.9	611
2	19.7	0.694	0.8	687
3	18.1	0.670	1.3	882
4	18.8	0.670	3.2	697
5	22.0	0.735	0.6	681
6	17.3	0.602	6.0	498
7	20.1	0.694	0.6	795
8	18.1	0.625	3.5	543
9	21.6	0.725	1.5	598
10	18.5	0.667	2.0	490
11	17.9	0.714	0.5	774
12	17.6	0.667	3.1	531
13	19.0	0.735	0.3	963
14	17.9	0.676	0.9	603
15	15.9	0.625	4.4	683
16	17.6	0.694	1.6	616
17	21.1	0.725	0.6	531
18	16.9	0.667	3.8	732
19	17.7	0.704	0.5	630
20	19.2	0.719	1.1	506
21	18.7	0.667	2.4	527
22	18.9	0.709	0.6	634
23	18.7	0.704	0.7	620
24	20.5	0.714	2.1	626
25	20.8	0.746	0.2	850
26	18.8	0.714	0.7	779
27	17.3	0.654	4.2	560
28	17.5	0.667	2.0	869
29	15.9	0.625	5.3	731
30	20.2	0.725	0.5	650
31	20.9	0.735	0.3	751
32	20.2	0.667	2.0	507

[a] Mean of three values.

Intermediate Level Experiment (No. 33)—An experiment was run at mean levels of the five variables studied in the factorial experiment (Table IV). The variable levels were: water content, 290 ml; extruder speed, 50 rpm; screen size, 1.0 mm; spheronizer speed, 85 rpm; and spheronizer time, 2.0 min. In all cases, the value of the parameter for Experiment 33 fell within the range of the original experiments. This finding indicated that it was possible to use the results of this study to modify the characteristics of a given formulation to be spheronized. Details of the complete factorial analyses of the primary granulation properties are included here.

Flow Rate—The significant main effects indicate that an increased water content, spheronizer speed, or spheronizer time resulted in granulations with an increased flow rate.

The first-order interaction, spheronizer speed–spheronizer time, is shown in Table IX. As spheronizer speed increased, a greater in-

Table IV—Comparison of Results Obtained with Intermediate Level Experiment and Original 32 Experiments

Parameter	Intermediate Level Experiment	Range of Original 32 Experiments
Escape[a], g	74	19–288
Extruder screen temperature[a]	48°	40–70°
Flow rate, g/sec	20.4	15.9–22.0
Bulk density, g/ml	0.685	0.602–0.746
Granule friability	1.4	0.2–6.0
Mean particle size, μm	655	490–963
Fines, <0.25 mm, %	0.18	0.09–8.44
Maximum percent on one sieve	71.6	34.1–74.1

[a] These results relate only to variables A, B, and C.

Table V—Sieve Analyses: Percent of Each Mesh Size

Experiment	Mesh Size >8	8–16	16–20	20–30	30–40	40–60	<60
1	0.0	0.0	0.7	70.9	26.7	1.6	0.1
2	0.0	0.1	3.8	73.0	20.9	2.2	0.0
3	0.0	5.6	50.4	33.3	6.4	3.7	0.6
4	0.0	1.2	25.2	43.6	17.1	9.9	3.0
5	0.0	0.1	4.3	67.8	25.2	2.5	0.0
6	0.0	0.0	0.1	24.5	58.4	14.2	2.9
7	0.0	3.6	37.1	44.6	10.1	4.1	0.6
8	0.3	0.3	0.3	33.0	55.4	8.8	1.8
9	0.0	0.9	10.5	40.5	27.3	17.3	3.4
10	0.0	0.0	0.1	19.4	60.9	17.1	2.6
11	0.4	7.6	34.1	33.1	13.4	9.3	2.1
12	0.0	0.1	0.2	35.5	52.3	10.5	1.4
13	0.0	10.2	58.9	26.5	3.5	0.9	0.0
14	0.0	0.0	0.7	67.7	29.3	2.1	0.2
15	0.0	0.7	21.9	48.2	16.2	9.2	3.8
16	0.1	1.3	18.0	36.9	20.0	15.4	8.4
17	0.1	0.2	0.3	30.7	53.4	14.1	1.3
18	0.1	1.1	29.6	44.8	15.3	7.3	1.8
19	0.0	0.1	2.7	66.9	27.8	2.5	0.1
20	0.0	0.2	0.3	20.5	53.7	23.6	1.8
21	0.0	0.2	0.3	27.9	57.9	12.1	1.6
22	0.2	0.1	1.7	74.1	22.6	1.4	0.1
23	0.0	0.1	2.4	64.2	30.0	3.3	0.2
24	0.0	0.9	16.4	38.5	23.7	17.5	3.1
25	0.7	6.3	35.1	47.0	9.4	1.4	0.0
26	1.9	2.6	34.9	44.7	12.0	3.7	0.2
27	0.0	0.6	8.6	38.1	26.9	17.6	8.2
28	0.0	2.1	50.9	39.8	5.6	1.4	0.2
29	0.0	0.9	29.4	46.6	12.9	7.0	3.3
30	0.6	0.9	3.6	69.0	23.4	2.4	0.1
31	0.2	2.1	28.1	53.7	12.7	3.1	0.1
32	0.0	0.0	0.1	28.3	57.0	12.8	1.8

crease in flow rate was noted at the high spheronizer time. An increased spheronizer time resulted in a greater increase in flow rate at the high level of spheronizer speed. The maximum flow rate would be obtained at high levels of water content, spheronizer speed, and spheronizer time. If it were necessary to use larger extruder screens, improved flow rates would result with an increased spheronizer time at either level of water content.

Bulk Density—The significant main effects indicated that an increased water content, spheronizer speed, or spheronizer time resulted in granulations with an increased bulk density. As spheronizer speed was increased, a greater increase in the value for bulk density was observed at the low water content, although values for bulk density were higher overall at the high water content (Table X). An increased water content resulted in similar effects, that is, a greater increase at the low spheronizer speed but with higher values for bulk density overall at the high spheronizer speed.

If it were desirable to have high values for bulk density, a high water content, spheronizer speed, and residence time in the spheronizer would be required. Experiments run at the high level of either water content or spheronizer speed resulted in nearly identical bulk density values. When both of these variables were at the high level, even greater values for bulk density were obtained.

Mean Particle Size—Calculated mean particle sizes were obtained from plots of cumulative percent undersize on the probability scale *versus* particle size on the arithmetic scale. Sieve analysis data appear in Table V. The significant main effects indicated that an increased water content or screen size resulted in granulations with an increased mean particle size whereas an increased spheronizer speed resulted in a decreased mean particle size.

As screen size increased, a greater increase in mean particle size was observed at the low spheronizer speed. As spheronizer speed increased, no change in mean particle size was observed at the low screen size whereas mean particle size decreased at the high screen size (Table XI). The mean particle size resulting from a given extruder screen size is considerably smaller than the screen size itself. The average of mean particle sizes for 16 experiments using screens with 0.8-mm openings was 0.578 mm; when using screens with 1.5-mm openings, the average of mean particle sizes was 0.744 mm. A low water content, small screen size, and high spheronizer speed would result in smaller particles in the final granulation. If it were necessary to use larger screens, a smaller mean particle size would be obtained at a high spheronizer speed. With small screens, no change in mean particle size was obtained by increasing spheronizer speed.

Table VI—Analysis of Variance: Flow Rate

Source of Variation	Sum of Squares	Degrees of Freedom	Variance	$F_{1,6}$
Water content (A)	4.805	1	4.805	11.10[a]
Extruder speed (B)	1.620	1	1.620	3.74
Screen size (C)	1.805	1	1.805	4.17
Spheronizer speed (D)	20.801	1	20.801	48.04[b]
Spheronizer time (E)	22.111	1	22.111	51.07[b]
AB	2.205	1	2.205	5.09
AC	0.720	1	0.720	1.66
AD	1.901	1	1.901	4.39
AE	0.011	1	0.011	0.03
BC	0.405	1	0.405	0.94
BD	0.211	1	0.211	0.49
BE	0.151	1	0.151	0.35
CD	1.051	1	1.051	2.43
CE	1.711	1	1.711	3.95
DE	3.125	1	3.125	7.22[a]
ABC	2.205	1	2.205	5.09
ABD	1.711	1	1.711	3.95
ABE	1.901	1	1.901	4.39
ACD	0.061	1	0.061	0.04
ACE	3.001	1	3.001	6.93[a]
ADE	0.125	1	0.125	0.29
BCD	0.551	1	0.551	1.27
BCE	0.061	1	0.061	0.14
BDE	0.180	1	0.180	0.42
CDE	0.005	1	0.005	0.01
Higher order	2.598	6	0.433	

[a] Significant at $p < 0.05$. [b] Significant at $p < 0.001$.

Percent Fines—For this study, fines were particles passing a 60-mesh sieve. The amounts present are listed in Table V. The significant main effects indicated that an increased screen size or spheronizer speed resulted in granulations with increased fines whereas an increased water content or spheronizer time resulted in decreased fines.

Significant first-order interactions are shown in Table XII. The water content–screen size interaction may be explained by observing that little increase in percent fines occurred at the high water content as screen size was increased. At the low water content, an increased screen size resulted in a large increase in percent fines.

The water content–spheronizer speed interaction may be explained in the same manner. At the high water content, an increased spheronizer speed resulted in little change in percent fines. At the low spheronizer speed, an increased water content resulted in a smaller decrease in fines than at the high spheronizer speed, both percent fines values being equivalent at the high water content.

The water content–spheronizer time interaction may be described by noting that, at the low water content, the percent of fines was decreased by increasing spheronizer time; at the high

Table VII—Summary of Significant Main Effects[a]

	Water Content	Extruder Speed	Screen Size	Spheronizer Speed	Spheronizer Time
Extruder screen temperature	***	**	—	—	—
Escape	***	—	—	—	—
Extrudate water content	***	—	—	—	—
Flow rate	*	—	—	***	***
Bulk density	***	—	—	***	***
Mean particle size	***	*	***	**	—
Percent fines	***	—	***	**	***
Granule friability	***	—	—	**	*

[a] * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$.

Table VIII—Values for Escape (Grams)[a]

Screen Size, mm	Water Content, ml	
	250	325
0.8	208	91
1.5	151	130

[a] Mean of eight experiments.

Table IX—Mean Flow Rate (Grams per Second) of Eight Experiments

Spheronizer Time, min	Spheronizer Speed, rpm	
	700	1010
1.0	17.5	18.5
3.0	18.6	20.8

water content, a much smaller decrease in percent fines occurred since few fines were present. A greater decrease in percent fines occurred at the low spheronizer time as water content was increased, although fewer fines were present throughout at the high spheronizer time. The screen size–spheronizer speed interaction was explained by noting that no change in percent fines was observed as spheronizer speed was increased at the low screen size but a large increase in percent fines occurred at the high screen size. As screen size increased, a much greater increase in percent fines was noted at the high spheronizer speed.

The screen size–spheronizer time interaction was explained by noting that, as spheronizer time was increased, a greater decrease in percent fines was apparent at the high screen size, although fewer fines were present throughout at the low screen size. As screen size increased, a greater increase in percent fines occurred at the low spheronizer time. The spheronizer speed–spheronizer time interaction was explained by noting that, as spheronizer time increased, a greater decrease in percent fines occurred at the high spheronizer speed, the values at both levels of spheronizer speed being nearly equal at the high spheronizer time. As spheronizer speed increased, a greater increase in percent fines occurred at the low spheronizer time.

Lower levels of fines may be obtained at high water content and spheronizer time but low screen size and spheronizer speed. If it were necessary to use screens with relatively large openings, low percent fines could be obtained at high water content. Even lower percent fines would be obtained under these conditions at low spheronizer speed or high spheronizer time. When using screens with small openings, changing spheronizer speed or spheronizer time had little effect on percent fines.

Maximum Percent on One Sieve Fraction—From sieve analysis data, the maximum percent of granules on a single sieve fraction was obtained. When using 0.8-mm screens in the extruder, this maximum occurred in the 20–30-mesh fraction for the eight experiments run at the high water content and in the 30–40-mesh fraction for the eight experiments run at the low water content.

Table X—Mean Bulk Density (Grams per Milliliter) of Eight Experiments

Spheronizer Speed, rpm	Water Content, ml	
	250	325
700	0.644	0.694
1010	0.696	0.720

Table XI—Mean Particle Size (Micrometers)[a]

Spheronizer Speed, rpm	Screen Size, mm	
	0.8	1.5
700	579	792
1010	577	696

[a] Mean of eight experiments.

Table XII—Mean Percent Fines of Eight Experiments

Screen Size, mm	Water Content, ml	
	250	325
0.8	1.9	0.1
1.5	4.4	0.5
Spheronizer Speed, rpm	**Water Content, ml**	
	250	325
700	2.5	0.2
1010	3.8	0.4
Spheronizer Time, min	**Water Content, ml**	
	250	325
1.0	4.1	0.5
3.0	2.2	0.1
Spheronizer Speed, rpm	**Screen Size, mm**	
	0.8	1.5
700	1.0	1.6
1010	1.0	3.2
Spheronizer Time, min	**Screen Size, mm**	
	0.8	1.5
1.0	1.2	3.4
3.0	0.8	1.5
Spheronizer Time, min	**Spheronizer Speed, rpm**	
	700	1010
1.0	1.6	3.0
3.0	1.0	1.2

When using 1.5-mm screens in the extruder, the maximum occurred 12 times in the 20–30-mesh fraction and four times in the 16–20-mesh fraction, the latter all occurring at the high water content.

Granule Friability—A value of 10.0 is maximum and would indicate 100% friability of a 10.0-g sample. The significant main effect indicated that an increased water content, spheronizer speed, or spheronizer time resulted in granulations with a decreased granule friability. The significant first-order interaction is shown in Table XIII. An increased spheronizer speed increased granule friability much more at the low water content. A decreased water content resulted in a greater increase in granule friability at the low spheronizer speed.

If, as indicated in this study, slight changes in process variables can significantly affect granulation properties, the spheronizing system might prove to be a useful research tool in addition to its production-oriented applications. Certainly, if granule properties can be controlled, correlation of other observed data should be facilitated.

While only one system was studied, experiments with other substances give reason to expect that most formulations could be modified to provide soft granulations for tablet compression or harder, more dense particles suitable for coating once the effects of process variables have been characterized.

Properties of the granules may change on aging, and Reier and Shangraw (6) reported effects of aging related to storage of tablets prepared with microcrystalline cellulose as the inert ingredient. The friability test used here plus a granule strength test such as was proposed by Ganderton and Hunter (7) could be of value in specific formulations where aging effects might be expected.

Table XIII—Mean Granule Friability of Eight Experiments

Spheronizer Speed, rpm	Water Content, ml	
	250	325
700	4.0	1.1
1010	1.9	0.5

The advantages provided by pellet-type pharmaceuticals have led to a renewed interest in several techniques for agglomerating powders, including the subjecting of wet powders to a vibrating surface or a cascading motion inside a tilted rotating drum (8). This agglomerating process has been called balling, glomulation, or pelletizing. The term spheronizing has been used (9), and it seems to be suited to the process performed by this system, particularly since the particulate product usually has a spherical shape, narrow size distribution, and increased density (10).

SUMMARY

The use of a complete factorial analysis to define the significant main effects and interactions between the variables of water content, extruder speed, screen size, spheronizer speed, and spheronizer residence time on various properties of pelletized granulations of acetaminophen produced by the spheronizing process was studied. Water content and spheronizer speed had a significant effect on all primary granulation properties studied and were involved in most of the first-order interactions noted.

The utility of factorial analysis as a tool for modifying product properties *via* changes in process conditions was given further support by a final experiment using intermediate values for the variables. All data from tests on the final batch fell within the ranges exhibited by the original series.

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Dissolution Studies with a Multichannel Continuous-Flow Apparatus

C. CAKIRYILDIZ, P. J. MEHTA, W. RAHMEN, and D. SCHOENLEBER ˣ

Abstract □ A multichannel continuous-flow apparatus for dissolution rate measurements is described. Typical data are presented to demonstrate its utility for studies with bulk drug powders as well as with tablets and capsules without any change of setup. Procedures are given for the preparation of powder samples for dissolution studies and for a simple method of changing pH for "retard" tablets. The precision in dissolution rates obtained with this apparatus and method is 1–10% mean *RSD*. The advantages of the method are flexibility, reproducibility, and ability to obtain data in integral or differential form.

Keyphrases □ Dissolution rate measurements—design and evaluation of multichannel continuous-flow apparatus, application to powders, tablets, and capsules □ Tablet dissolution rates—design and evaluation of multichannel continuous-flow apparatus, also applied to powders and capsules □ Capsule dissolution rates—design and evaluation of multichannel continuous-flow apparatus, also applied to powders and tablets □ Drug powder dissolution rates—design and evaluation of multichannel continuous-flow apparatus, also applied to tablets and capsules

During the past several years, several dissolution rate testing methods have been developed. The beaker method (1, 2), the rotating-basket method (3–5), the rotating-flask method (6), the pressure change method (7), continuous-flow methods with or without cumulating reservoirs (8–22), and others (23–25) are important.

The continuous-flow (7, 8) and pressure change (7) methods have been stated to possess several advantages over the other methods. These advantages include flexibility, reproducibility, the possibility of obtaining data in differential or integral form, the ability to discriminate between similar formulations of the same drug, and the maintenance of sink conditions in the system.

Although a modified beaker method is both simple and adaptable for dissolution rate testing with solid dosage forms, this method gave poorly reproducible dissolution measurements with micronized and difficult-to-wet substances in bulk form. Therefore, a continuous-flow apparatus suitable for both drug substances (powders) and solid dosage forms was developed. The cell is designed to obtain a free flow of the dissolution medium even with large tablets, and

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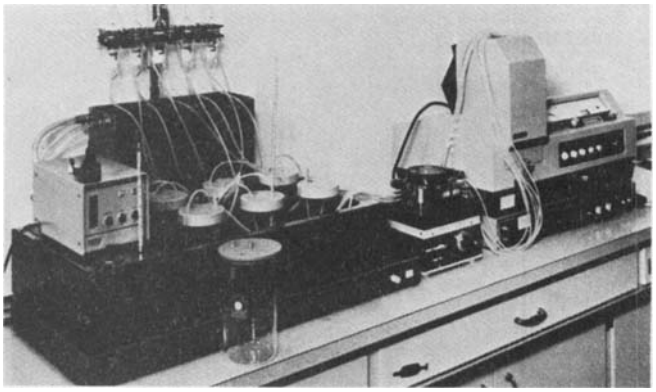

Figure 1—*Multichannel continuous-flow apparatus.*

it is leakproof under the pressure generated when using membrane filters of a very small pore size.

The apparatus is multiple channel, so up to five samples can be studied at one time, and it is capable of being automated. Several automated dissolution devices are described in the literature where spectrophotometry (26–35), potentiometry (4, 36), conductometry (37, 38), and other methods (39, 40) are employed for the measurement of drug in solution. Since most drug substances possess a chromophore in the UV region, spectrophotometry is widely used.

Examples of the determination of the dissolution rate of compressed powders (41, 42) and pellets (43) are known. Some trials have been reported concerning bulk drug powders, but a satisfactory and reproducible method has not been described (7, 8, 10–16, 24), especially for difficult-to-wet powders.

This report deals with a six-channel apparatus for the determination of the dissolution rate of drug powders as well as of tablets and capsules. The special treatment of powder samples is described in detail.

EXPERIMENTAL

Apparatus—The dissolution system (Fig. 1) consists of a six-channel peristaltic pump[1], a thermostatted water bath[2] with a magnetic stirring plate for six beakers, an additional smaller water bath[2], a spectrophotometer[3] equipped with an automated cell changer[4] and a recorder[4], and five identical independent dissolution units with a sixth one for circulation of a reference solution.

The dissolution cell (sample compartment: height 60 mm, i.d. 25 mm) is shown in an exploded view in Fig. 2. A filter[5] is placed between the upper and the lower flanges to restrict the dissolution process to the cell by preventing any undissolved particles from being carried over to the reservoir containing the dissolution medium. A stainless steel sieve is placed above the filter to provide mechanical strength against pressure which may develop from the use of membrane filters. Strong clamps are used to make the system leakproof.

From the reservoir, the dissolution medium goes (*via* the pump) to the flow-through quartz cell, to the bottom of the dissolution cell, and, from its top, returns to the reservoir. All connections are made by silicone tubing (3 mm i.d., 5 mm o.d.).

[1] Model MP-6, Ismatec Ltd., Zürich, Switzerland.
[2] No. 01 T 643 TB and No. TD 6/1000, Type K, Heto Ltd., Birkerød, Denmark.
[3] Beckman DB-GT or DB-G.
[4] W & W Electronics Ltd., Basle, Switzerland.
[5] Cellulose fleece filter, Schleicher and Schuell Ltd., Feldmeilen, Switzerland (for tablets). Membrane filters (0.47 μm), Millipore or Sartorius (for powders and capsules).

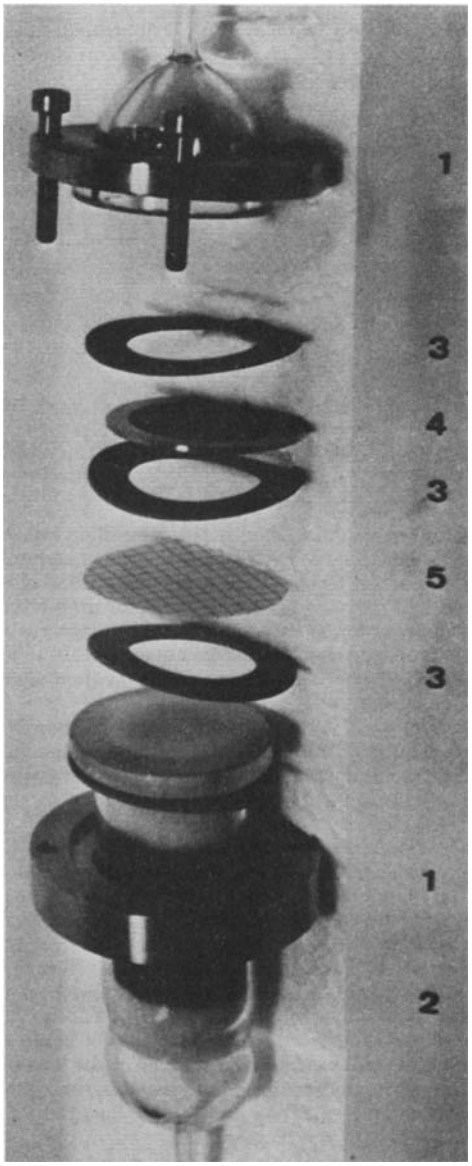

Figure 2—*Dissolution cell filled with drug powder–glass beads mix (exploded view). Key: 1, stainless steel clamp; 2, upper and lower parts of dissolution cell (sample compartment in lower part: height 60 mm, i.d. 25 mm); 3, rubber gaskets; 4, stainless steel sieve (18 mesh U.S.); and 5, filter.*

Preparation of Drug Powder Samples—About 35 g of glass beads (0.2 mm diameter) are divided into two portions. The first portion is placed in a special mixing flask (Fig. 3), and a calculated amount of drug powder is weighed onto these beads. Then the second portion is poured on top of the powder. The flask is rotated at the lowest speed on a rotary evaporator[6] without vacuum for 5 min. The mixture is then quantitatively transferred over a 1-cm bed of glass beads in the lower part of the dissolution cell. The remaining space is filled with more glass beads to the ground joint. A membrane filter (0.47 μm) and a stainless steel sieve are placed between rubber gaskets, the upper portion of the dissolution cell is placed in position, and the flanges are securely clamped (Fig. 2).

General Procedure—*Capsule Dissolution Studies*—One capsule, weighted by a ring of stainless steel wire slipped carefully over it, is placed on a bed of glass beads (0.2 mm, 20 g) in the dissolution cell. A membrane filter (0.47 μm) is used. The cell is closed as described previously.

Tablet Dissolution Studies—One tablet is placed on top of a

[6] W. Büchi Ltd., Flawil, Switzerland.

Table I—Dissolution Data of Bulk Drug Substances

Minutes	Drug Substance A, a Modified Ergot Alkaloid		Drug Substance B, a Neuroleptic Drug
	Nonmicronized	Micronized	
	Percent Dissolved		
2.5	14	35	61
5	33	68	93
7	49	83	99
10	58	91	102
15	67	94	
20	79	95	
25	86	96	
30	92	97	
Mean *RSD*	7.4% (8 runs)	4.7% (10 runs)	1.6% (10 runs)
$t_{63.2\%}$	12.5 min	4.7 min	2.6 min

bed of glass beads (0.2 mm, 20 g) in the dissolution cell. A cellulose fleece filter is used. The cell is closed as described earlier.

Measurement of Drug Dissolution—One liter of simulated gastric fluid USP (without pepsin) is used for drug powders. Less liquid is employed for capsules and tablets so that the maximum concentration of drug in solution does not exceed 10–15% of its saturation solubility. The temperature of the medium is maintained at 37°.

A flow rate of 8.0 ± 0.2 ml/min is used for drug powder, and the same rate or 16.0 ± 0.5 ml/min is used for capsule dissolution studies. These rates minimize a buildup of pressure at the membrane filter and provide enough points of measurement to characterize the dissolution curve.

For tablet dissolution studies, a flow rate of 33.0 ± 1.0 ml/min (about 2 liters/hr) and a cellulose fleece filter are used. The cellulose fibers are randomly arranged, and the pores are small enough to prevent particles formed by tablet disintegration from passing through the filter.

Changing pH of Dissolution Medium for "Retard" Tablets and Capsules—Simulated gastric fluid USP (without pepsin) is used for the first 2 hr. To change the pH, 22.50 g of trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$) is added per liter of fluid.

The circulating pumps should be stopped at the time of addition and restarted when all trisodium phosphate has gone into solution. After circulation of the medium for 3–5 min, the pH is 7.5 ± 0.1.

RESULTS AND DISCUSSION

Apparatus—The apparatus as described has been used successfully during the past 3 years for various dissolution studies. Before that time, several trials using a Millipore cell[7] had been made. Experience showed that the inside of the cell was easily scratched by the glass beads. A more serious drawback was the low recovery observed in the powder studies, due to the retention of the undissolved drug in scratches on the inner wall of the cell and the adhesion of powder particles to the polycarbonate material. Tingstad *et al.* (11, 44) recently employed the same cell for tablets (but without using glass beads).

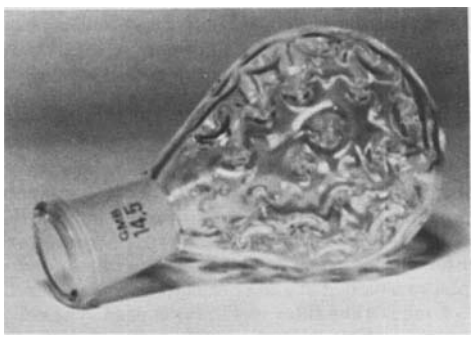

Figure 3—*Mixing flask for powders.*

[7] Part XX 4202500, Millipore Corp.

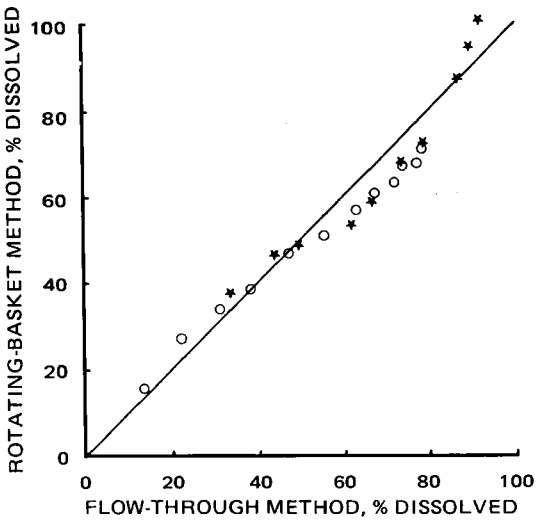

Figure 4—*Correlation plot of flow-through method at 33.0 ml/min* versus *rotating-basket method at 60 rpm. Percent dissolved values of equal sampling time are plotted against each other. Compression-coated tablets were Type I (★) and Type II (○). Straight line shows 100% correlation.*

Drug Powder Dissolution Studies—Measurement of dissolution of drug powders in media of high surface tension (such as water or simulated gastric fluid) is frequently difficult because of irregular wetting of the powder by the medium. Even weak agglomerates, which are present in every fine powder or are formed when the substance is poured, dissolve very slowly (at a rate comparable to the dissolution of similarly sized large single crystals), thus yielding unrealistic dissolution times. In some cases there was no complete solution even after 24 hr.

This effect is especially observed with particles carrying small electrostatic charges, *e.g.*, very fine and micronized powders. When such powders are mixed with small glass beads (average weight about 10 μg) as described under *Experimental*, the effective surface for dissolution is considerably increased. The majority of clusters and single substance agglomerates is destroyed by the mixing process, and the buildup of new ones is substantially reduced. Sim-

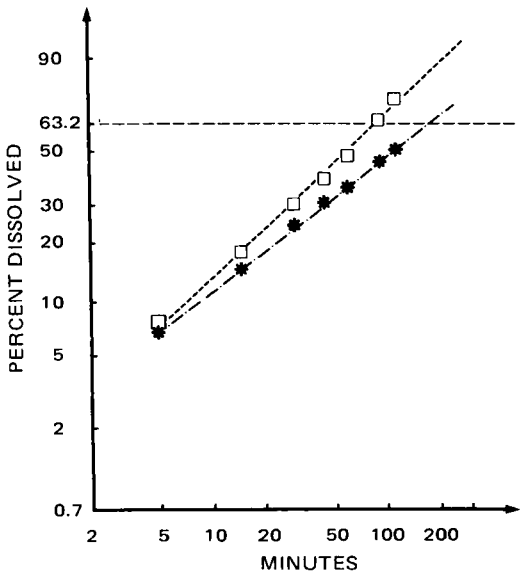

Figure 5—*Dissolution of nicotinic acid from equally composed retard tablets manufactured on different tablet machines (RRS diagram). Key: □, Tablet A, excentric machine, $t_{63.2\%}$ = 86 min; and *, Tablet B, rotary machine, $t_{63.2\%}$ = 170 min (extrapolated).*

Table II—Dissolution Experiments with Aspirin of Different Particle Sizes and at Differing Flow Rates

Particle Size	Flow, ml/min	Percent Dissolved at 5 min	7 min	10 min	15 min	20 min	30 min	$t_{63.2\%}$, min
Micronized	8	6.4	37	71	85	91	97	9.6
	14	60	81	89	96	98	100	5.0
	20	82	91	96	99	100		2.9
	30	93	98	99	100			1.5
Sieve fraction 297–420 μm	8	2	13	40	75	93	100	14.5
	14	21	41	67	92	99	100	9.8
	20	34	55	78	97	100		7.9
Sieve fraction 420–595 μm	8	1	7	22	55	74	96	18.7
	14	11	26	47	70	87	98	13.9
	20	14	33	57	81	95	100	11.6

ilar phenomena take place during dry powder mixing for a solid formulation.

At this point, checking by microscope showed no instance of any breakdown of primary particles during the mixing step. Therefore, the increase of the dissolution-affecting surface area of a powder is exclusively due to the disintegration of agglomerates and not to a size diminution of primary particles. So the mixing procedure helps to create better defined starting conditions (and more similarity to a formulation mix), thus providing a more reproducible drug powder dissolution process than without pretreatment.

Table I presents results obtained from the same relatively insoluble drug substance (Drug Substance A) in micronized and unmicronized form after mixing with 0.2-mm glass beads. As expected, the micronized powder dissolved more rapidly than the nonmicronized powder; without pretreatment, more than 24 hr was required for the dissolution of both forms due to cluster formation. This trial and other experience led to the conclusion that the more homogeneous the glass bead mix is, the smaller the relative standard deviation of the measurement will be. Drug Substance A has a strong agglomeration tendency, so a mean relative standard deviation of only 4.7% compared to the nonmicronized value of 7.4% was achieved. Fairly soluble and easily wettable drug powders usually yield more reproducible values, with relative standard deviations of about 2% as shown for Drug Substance B in Table I.

Capsule and Tablet Dissolution Studies—In contrast to the procedure used for powder dissolution studies, capsules and tablets have to be placed on top of the glass beads to ensure an unimpeded decay or swelling of the pharmaceutical form. Capsules are weighted by a coil of stainless steel wire so that they will not float in the dissolution medium.

Tablets usually float in the liquid at the beginning of the dissolution rate measurement for about 10 sec. They then become wetted and sink onto the glass beads where they remain. Since this movement of the tablets only occurs in the first few seconds, the dissolution process is not influenced.

The dissolution profile of a drug substance as well as that of formulations is very strongly affected by changes in the liquid flow rate. With aspirin as an example, the dissolution behavior as a function of flow rate is shown in Table II for samples of differing particle size. Due to this interdependence, a highly constant flow rate of the dissolution medium is necessary; this point was considered in establishing the rather tight specifications.

The 8.0-ml/min flow rate was chosen as a standard for powders since—in contrast to higher rates—it permitted the ready recognition of differences in the first, steep range of the dissolution profile. The data in Table II also confirm the well-known findings of the reduction of dissolution rate with increasing particle size (45).

The 33.0-ml/min (2 liters/hr) rate for tablets was chosen because this flow showed an optimum dissolution rate correlation with the USP rotating-basket method, corresponding to the findings described by Tingstad *et al.* (12). Figure 4 gives an example of a typical correlation plot for two kinds of compression-coated tablets tested with both methods.

The reproducibilities of the flow-through method and the USP method were compared measuring the same batch of tablets. The results are listed in Table III for four different tablet series. Obviously, the method described here is capable of providing more reproducible data than the official rotating-basket method, a fact that allows better discrimination between different species.

Even slight variations in manufacturing or in the composition of a formulation can be detected by measuring the dissolution rate. Figure 5 depicts the dissolution profiles of two tablet series having exactly the same composition but manufactured on different tablet machines. The results are plotted by the Rosin–Rammler–Sperling (RRS) method (46, 47). The $t_{63.2\%}$ corresponds to the time for dissolution of 63.2% of the drug. As can be seen from the plot, the variation of dissolution rate from one tablet to the other is significant.

Another example of the discriminating capability of this flow-through method is illustrated in Fig. 6. Here two tablets, differing only in the quality of a single excipient (cellulose acetate phthal-

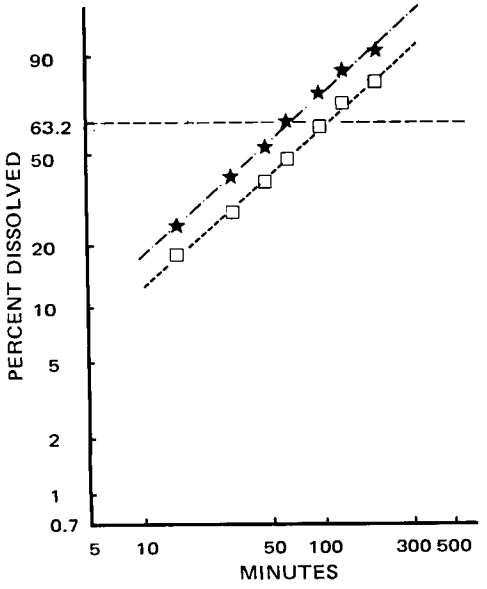

Figure 6—*Dissolution profile of equally composed and identically manufactured retard tablets, differing only in quality of a single excipient (cellulose acetate phthalate) (RRS diagram). Key: ★, Tablet A, cellulose acetate phthalate quality I, $t_{63.2\%}$ = 63 min; and □, Tablet B, cellulose acetate phthalate quality II, $t_{63.2\%}$ = 101 min.*

Table III—Experimental Comparison of Reproducibilities of Rotating-Basket and Flow-Through Methods Established with Four Completely Different Tablets

Tablet Series	Mean *RSD*, out of Eight Values, %: USP Rotating-Basket Method, 60 rpm	Flow-Through Method, 33.0 ml/min
1	2.9	2.7
2	6.5	5.1
3	4.5	2.4
4	12.2	6.8

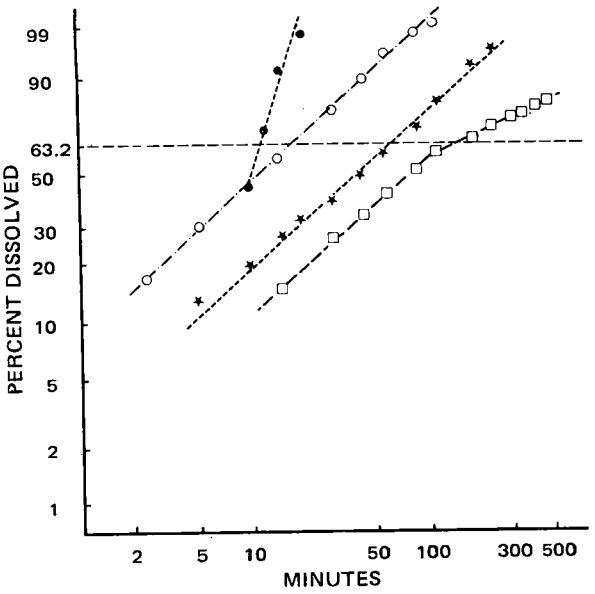

Figure 7—*Dissolution rate data. Key:* ●, *tetracycline capsule;* ○, *Tablet 1, antiobesity drug;* ★, *Tablet 2, neuroleptic drug; and* □, *Tablet 3, retard formulation.*

ate) but manufactured in the same way in every respect, were measured. The plot exhibits a significantly different dissolution behavior.

In Fig. 7, applications are presented of the flow-through method to a hard gelatin capsule, a tablet, a sugar-coated tablet, and a retard tablet.

Dissolution from hard gelatin capsules of tetracycline hydrochloride was carried out using a 16.0-ml/min flow rate. The capsules needed more than 5 min to open, but drug dissolution was rapid thereafter.

The dissolution data of an antiobesity drug (Tablet 1) showed good tablet-to-tablet uniformity (s_{mean} from six values was less than 1%). The slope of the straight line in the RRS plot is about 0.95, indicating an apparent first-order dissolution process. The $t_{63.2\%}$ is 17 min.

The profile for a neuroleptic drug from a sugar-coated tablet (Tablet 2) yielded a slope of 0.9 and a $t_{63.2\%}$ of 70 min in the RRS plot (Fig. 7). The tablet-to-tablet uniformity was not as good as in the previous case (s_{mean} from eight values was about 6%).

Release of phenylpropanolamine from an experimental retard formulation (Tablet 3) is also shown in Fig. 7. The pH of the dissolution medium was changed to 7.5 after 2 hr, as described under *Experimental.* At this point, a decrease in slope indicates a slowdown of the dissolution process at higher pH.

The procedure for the change of pH described here involves a simple addition of solid trisodium phosphate. An increase of about 2% in the volume of the dissolution medium takes place. The resulting pH of 7.5 is equal to that of simulated intestinal fluid USP; the ionic strength is, however, 0.248, almost twice as much as that of the USP solution. This procedure is suitable in all cases where drug dissolution is not affected by ionic strength in the 0.12–0.25 range, and it is applicable to the USP–NF dissolution tests as well as to continuous-flow methods with cumulating reservoirs. For the latter procedure, the well-known half-change method can be used.

Unfortunately, many modern drugs are used in low doses and, therefore, yield only low absorbance values. For the determination of the dissolution rate of such drugs from their retard dosage forms, the described procedure for change of pH is very useful because there is no impediment to the analytical assay by the dilution. Tromethamine has been used to change pH when working with phosphate-incompatible drugs.

CONCLUSION

The described apparatus is versatile enough to determine the dissolution patterns of drug powders and of tablet and capsule formulations without any change of the experimental setup. The novel preparation of drug powder samples for dissolution studies is given in detail as well as a facile procedure to change pH during dissolution of retard preparations. In 3 years' experience with the apparatus, it has been simple to use and has yielded reproducible dissolution data. In addition to being capable of showing formulation differences, the apparatus is sensitive enough to reveal process changes.

The criteria for a dissolution apparatus proposed by Wagner (48) are satisfied. Although the apparatus has been used with a cumulating reservoir to yield integral dissolution data, it is quite suitable for obtaining data in the differential form. Finally, the multichannel capability allows the determination of up to five dissolution rates concomitantly.

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Enhancing Fluidity of Concentrated Antacid Suspensions

ALLEN HEYD [x] and DADI J. DHABHAR

Abstract □ Highly concentrated antacid suspensions can be fluidized by adding a colloidal polyelectrolyte to alter the charge on antacid particles from positive to negative. A deflocculated state is assumed to exist in such preparations, as supported by electrophoretic and viscometric analyses. When viscosity is directly correlated with the ζ-potential, viscometry becomes particularly useful in confirming that the suspension has been maximally fluidized.

Keyphrases □ Antacid suspensions, concentrated—fluidized by addition of colloidal polyelectrolyte, viscosity correlated with ζ-potential, electrophoretic and viscometric measurements □ Fluidized antacid suspensions—preparation using colloidal polyelectrolyte, viscosity correlated with ζ-potential, electrophoretic and viscometric measurements

Primary goals in formulating stable suspensions of coarse particles are to prevent caking and, thus, to maintain a stable shelflife. Obtaining these objectives becomes particularly difficult when preparations are highly concentrated because the classical techniques of flocculation no longer apply.

Flocculation appears to be the preferred method for preparing coarse suspensions which can be redispersed easily by shaking (1–3). In the phenomenon of flocculation, the force of attraction between particles predominates, causing the particles to form loose aggregates. Should repulsion forces prevail, however, the particles separate or deflocculate, settling slowly. A suspending and/or gelling agent is required to stabilize such a preparation to prevent caking.

In a study of the surface characteristics of particles and the intensity of particle interaction related to the caking of insoluble particles in aqueous dispersion, flocculation was controlled by adding an electrolyte; the resulting product did not cake (4). Martin (5), in reviewing the physicochemical principles applicable to caking and flocculation, emphasized that maximal flocculation occurred within a range of low positive to low negative ζ-potential. Deflocculation and caking occurred at either extreme, *e.g.*, high positive or high negative (Fig. 1). Haines and Martin (6), in studying the electrokinetic behavior of insoluble drug particles, correlated the magnitude of the ζ-potential, as calculated from electrophoresis data, with the tendency to cake.

Table I—Physical Properties of Antacids

Property	Dried Aluminum Hydroxide Gel USP[a]	Magnesium Hydroxide NF[b]	Calcium Carbonate USP[c]
Particle size[d], μm	2.34 (0.28–6.42)[e]	8.12 (1.4–42)	4.39 (0.28–9.2)
Shape	Asymmetric round	Asymmetric round	Barrel shaped
Bulk density, lb/ft³	19–23	17–25	17

[a] J. T. Baker Chemical Co., Phillipsburg, N.J. [b] Mallinckrodt Chemical Works, Jersey City, N.J. [c] Pfizer Mineral, Pigments and Metals Division, Clifton, N.J. [d] Mean volume diameter. [e] Particle-size range.

Flocculation technology evolved from research performed on suspensions containing no more than 10% solids. Thus, current flocculation technology did not seem applicable to the preparation of coarse suspensions containing, for example, 40% solids. Antacid preparations containing 40% solids do not contain enough free water to achieve a low enough viscosity and easy flow. Furthermore, concentrated suspensions cannot be flocculated to a maximal extent since the potential maximum sedimentation volume on dilution would greatly exceed the original total volume (7).

An alternative to flocculation is fluidization. An elegant system was described for a superior barium sulfate suspension used as a diagnostic contrast medium (8). The use of radiographic techniques to evaluate the *in vivo* performance of the system was also described (8). In addition, Brown (9) referred to increased fluidity in the preparation of highly concentrated, gelating, antacid systems.

In an investigation of this system (8, 9), the fluidization technique was evaluated. Highly concentrated antacid suspensions of high fluidity (*e.g.*, marked flowability and low viscosity) can be successfully pre-

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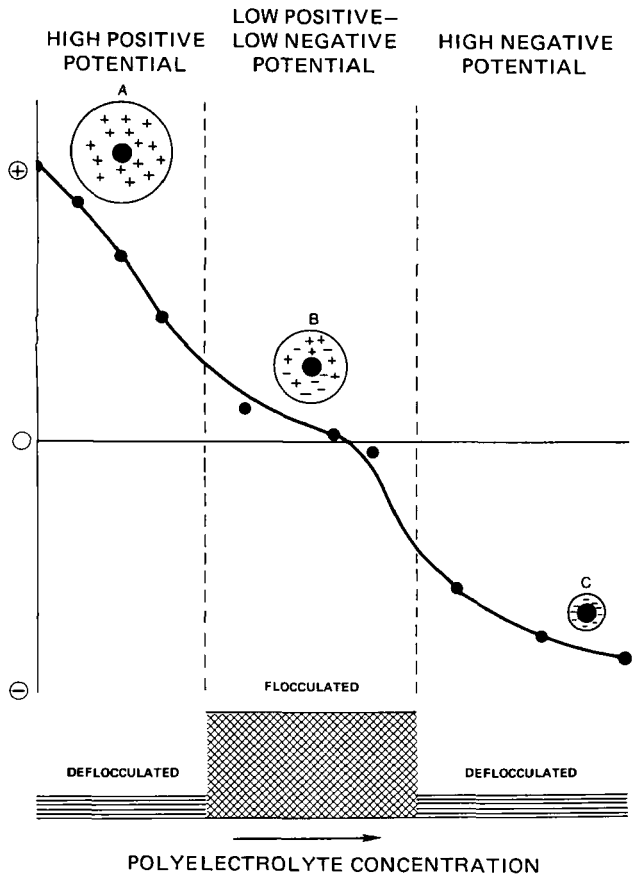

Figure 1—*Schematic diagram depicting deflocculation, which occurs at a high positive or high negative ζ-potential; flocculation occurs at a low positive or low negative ζ-potential. Antacid particle hydration is shown as a function of the ζ-potential. Key: A, high positive (high hydration); B, low positive–low negative (medium hydration); and C, high negative (low hydration).*

pared by this technique using a low molecular weight, colloidal polyelectrolyte as a "fluidizing" agent.

Theoretically, in this system (8, 10) the negatively charged colloidal polyelectrolyte would selectively adsorb onto the surfaces of insoluble particles, such as antacid particles, and deflocculate them once the ζ-potential exceeds the critical value. The purposes of this study were: (*a*) to determine whether such concentrated suspensions can, in fact, be fluidized; (*b*) to determine whether electrophoresis can be utilized to follow the degree of fluidization; and (*c*) to correlate ζ-potential results with viscosity data.

EXPERIMENTAL

Materials—The physical characteristics of the substances used in preparing two antacid suspensions are listed in Table I. Carrageenan sodium[1] of low molecular weight (8000–10,000) served as the negatively charged colloidal polyelectrolyte in each instance.

Aluminum Hydroxide Suspension (25%)—For viscosity measurements, a 25% dispersion of aluminum hydroxide was prepared. The concentration of carrageenan sodium was increased stepwise from 0.2 to 3.2% in seven replicate samples intended for viscosity determinations. Because its small particle size produces a good suspension and allows unhindered movement in the electrophoresis cell, a 0.025% dispersion of aluminum hydroxide served as a

[1] Marine Colloids Inc., Springfield, N.J.

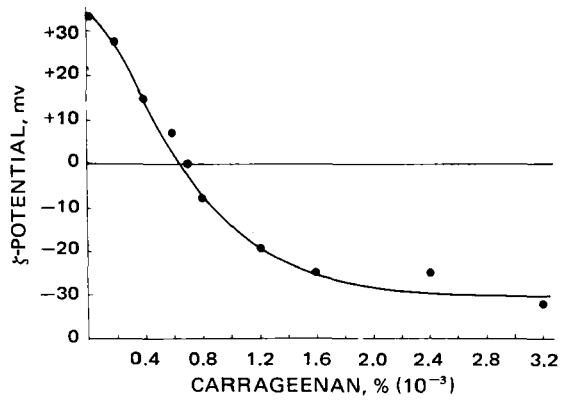

Figure 2—*ζ-Potential of aluminum hydroxide as a function of carrageenan concentration.*

model for the initial measurements of the ζ-potential. It was assumed that this dilution from a 25% dispersion would not alter the equilibrium between the electrolyte and solids and thereby affect the results from a practical standpoint. Concentrations of carrageenan sodium added to the 0.025% suspension were increased stepwise from 0.0002 to 0.0032% in seven replicate samples to determine the effect of a negatively charged colloidal polyelectrolyte upon the ζ-potential of aluminum hydroxide.

Antacid Mixture (38%)—A prototype antacid mixture (38% suspension) was prepared consisting of magnesium hydroxide, dried aluminum hydroxide gel, and calcium carbonate (1:2:6). The content of carrageenan sodium was increased stepwise from 0.2 to 3.2% in eight replicate samples intended for viscosity determinations. The relatively large size of the magnesium hydroxide and calcium hydroxide particles did not permit accurate electrophoretic measurements so the ζ-potential could not be calculated.

Measuring ζ-Potential—Samples of the aluminum hydroxide preparation containing graded amounts of polyelectrolyte were placed in an electrophoresis cell[2] having a molybdenum anode and a platinum cathode. The setting was 150 v. Particles were tracked on a stereoscope[3] using an 8× objective. The ζ-potential was calculated from electrophoretic mobility (EM) data using a standard conversion formula (9):

$$\text{absolute EM} = \frac{(\mu\text{m/sec})}{(\text{v/cm})} = \frac{(\mu\text{m})(\text{cm})}{(\text{v})(\text{sec})} \qquad \text{(Eq. 1)}$$

The mobility of charged particles (expressed in micrometers) was determined from their tracking length as seen in a full-scale 8× ocular micrometer. The time required to traverse 1 full-scale μm division was measured in seconds, v represented the voltage applied, and the distance between electrophoresis cell ports was measured in centimeters.

Measuring Viscosity—Viscosity was determined with a viscometer[4] mounted on a helipath stand. T-bar spindles were used in obtaining measurements of the 25% aluminum hydroxide suspension, while standard round spindles were employed in testing the 38% antacid mixture. The effect upon viscosity resulting from controlling the surface potential of suspended particles with carrageenan could not be determined in the presence of a suspending or gelling agent because the latter "dampened out" the fluidizing action of a polyelectrolyte. However, carrageenan sodium in the low molecular weight range used did not contribute to viscosity.

RESULTS AND DISCUSSION

In the aluminum hydroxide suspension, a change in the ζ-potential was associated with increasing concentrations of carrageenan sodium (Fig. 2). This negatively charged, hydrophilic, colloidal polyelectrolyte reduced the positive charge, causing a rapid fall in the ζ-potential. A negative ζ-potential was reached at 0.7×10^{-3}% carrageenan concentration.

Maximum fluidization was achieved at approximately 1.6 ×

[2] Riddick Type II UVA, Riddick Zeta Meter Inc., New York, N.Y.
[3] Zeta Meter.
[4] Model LVT-5x, Brookfield Engineering Laboratories, Stoughton, Mass.

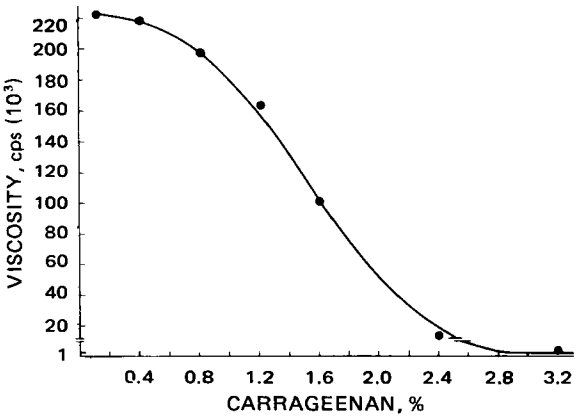

Figure 3—*Viscosity of aluminum hydroxide in water as a function of carrageenan concentration.*

10^{-3}% colloid, as evidenced by a plateau in the ζ-potential and as confirmed with viscosity data. Higher concentrations produced no further change. The ζ-potential curve indicates that the aluminum hydroxide particles acquired a negative charge, corresponding to a negative ζ-potential, which was great enough to surpass the critical value and thus become deflocculated (5). If deflocculation occurs, then it is the mechanism by which fluidization is achieved.

Viscosity of the 25% aluminum hydroxide suspension decreased as the carrageenan concentration increased (Fig. 3). A plateau in viscosity occurred at about 2.4% colloid. Initially, the viscosity of the 25% suspension was approximately 220,000 cps; this preparation had a light paste-like consistency and did not pour. After 3.2% carrageenan was added, the viscosity decreased to approximately 1000 cps; the suspension poured easily from a narrow-necked bottle. An inverse correlation is apparent between colloid concentration and viscosity (Fig. 3) as well as ζ-potential (Fig. 2).

Similarly, the viscosity of the 38% antacid mixture decreased with increasing amounts of carrageenan (Fig. 4). Initially, the viscosity of the mixture was about 1900 cps; it had a thick, lotion-like consistency. Viscosity began to approach the plateau at a concentration of 1.2% carrageenan; and at a concentration of 2.0% carrageenan, the viscosity reached a plateau. This preparation was a thin, smooth liquid which poured easily from a narrow-necked bottle. This change correlated closely with the change in the ζ-potential (Fig. 2).

When comparing viscosity data (Figs. 3 and 4) with ζ-potential values (Fig. 2), it appears that in both antacid systems the decrease in viscosity following the addition of a colloidal polyelectrolyte is correlated with a change from a positive to a negative ζ-potential. Viscometry, therefore, becomes a simple and practical replacement for ζ-potential determinations in this and similar systems. Viscometry can be particularly important when establishing a condition of maximal or optimal fluidization during the large-scale manufacture of antacid suspensions.

These data demonstrate that fluidity of a concentrated antacid mixture can be achieved by using a colloidal polyelectrolyte (fluidizer) such as low molecular weight carrageenan sodium. The mechanism appears to be that the fluidizing agent—electronegatively

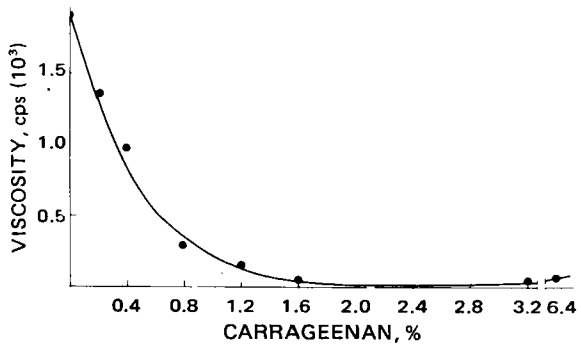

Figure 4—*Viscosity of an antacid mixture in water as a function of carrageenan concentration.*

charged—selectively adsorbs onto the positively charged antacid particles, deflocculating them (Fig. 1). Such adsorption imparts an electronegative charge (as measured by the ζ-potential) onto the antacid particles, decreasing the demand for water. In effect, particles become *dehydrated* relative to their earlier electropositive hydrated state (11).

The Derjaguin–Landau–Verwey–Overbeek theory, which describes the role of electrolytes in flocculation, is another possible explanation for the fluidization phenomenon. This theory has been reviewed frequently (1–4) and can be used to describe the concept of less water being associated with the antacid particles. As a result, a greater number of particles may be suspended in a given volume of water, thus enhancing the fluidity of the preparation. This fluidized suspension of low viscosity can then be made physically stable by incorporating suitable suspending and/or gelling agents.

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Synthesis of *d*-Pilocarpine-*N*-$^{14}CH_3$

JOSEPH I. DeGRAW *x, JOHN S. ENGSTROM *, and EDWARD WILLIS ‡

Abstract □ The total synthesis of *d*-pilocarpine specifically labeled as *N*-$^{14}CH_3$ is reported. *dl*-Homopilopic acid was prepared as a key intermediate and resolved into the *d*-enantiomer *via* the α-methylbenzylamine salt. The penultimate intermediate, 2-mercaptopilocarpine, was desulfurized by oxidation with dilute hydrogen peroxide, minimizing isomerization to isopilocarpine. Procedures for the analysis of pilocarpine–isopilocarpine mixtures by *high-pressure* liquid chromatography are also described.

Keyphrases □ Pilocarpine-*N*-$^{14}CH_3$—synthesis *via dl*-homopilopic acid □ High-pressure liquid chromatography—analysis, pilocarpine–isopilocarpine mixtures

Comparative studies of pilocarpine bioavailability and pharmacokinetics, when administered by membrane-controlled drug delivery systems or eyedrops, have required the preparation of natural pilocarpine specifically labeled with ^{14}C. Accordingly, a total synthesis of pilocarpine-*N*-$^{14}CH_3$ was undertaken. At the terminal stage of the work, it was necessary to establish the purity of the pilocarpine, especially with regard to contamination by isopilocarpine. Chromatographic techniques such as GLC and TLC were not useful for detecting 1–5% of the isomeric impurity, but high-pressure liquid chromatography (HPLC) was quite effective.

DISCUSSION

Methods for the total synthesis of pilocarpine starting from furfural were reported previously (1, 2). The method used in this study is shown in Scheme I. The key intermediate, homopilopic acid (I), was prepared by a previously reported procedure (1). Optical resolution of the racemic acid so obtained was achieved *via* salt formation with (+)-α-methylbenzylamine to afford the required *d*-homopilopic acid. The *d*-acid was converted to the acid chloride, which was used to acylate di-*tert*-butylacetamidomalonate.

Acid hydrolysis of the ester (II) caused cleavage of the *tert*-butyl groups and subsequent decarboxylation to afford the *d*-homopilopyl aminomethyl ketone (III) as the hydrochloride salt. Condensation of III with methyl isothiocyanate yielded 2-mercaptopilocarpine (IV). The ring closure reaction was intensively investigated in cold runs, and a consistent yield of 38% was obtained regardless of changes in solvents, temperature, or ratio of reactants.

Removal of the 2-mercapto group by desulfurization with nickel produced unacceptable amounts of isopilocarpine, probably because of isomerization catalyzed by residual alkali in the nickel. In fact, even stirring pilocarpine hydrochloride with silver oxide in ethanol at room temperature to prepare the free base caused substantial isomerization. However, treatment of IV with aqueous hydrogen peroxide, followed by neutralization with sodium bicarbonate to pH 8 and subsequent extraction into methylene chloride, gave high yields of pilocarpine (V) containing about 5% isopilocarpine.

Methyl isothiocyanate-$^{14}CH_3$ was prepared from ^{14}C-methylamine hydrochloride by the procedure of Moore and Crossley (3). Condensation with III gave *d*-2-mercaptopilocarpine-*N*-$^{14}CH_3$ (IV), which was desulfurized with peroxide to afford *d*-pilocarpine-*N*-$^{14}CH_3$ (V). Thick-plate chromatography, followed by dilution with four parts of natural pilocarpine and recrystallization of the nitrate salt, yielded material containing 2% isopilocarpine as the only significant contaminant.

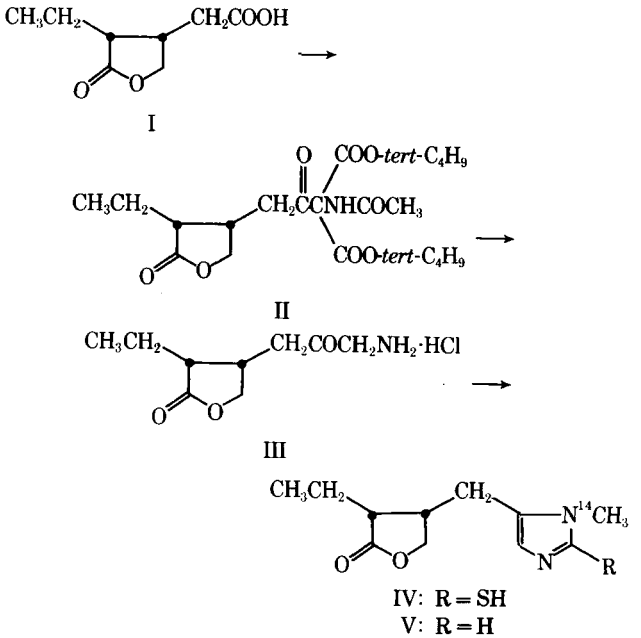
EXPERIMENTAL

Aliquots of ^{14}C-pilocarpine were subjected to HPLC[1] calibrated to separate pilocarpine, isopilocarpine, and pilocarpic acid (Fig. 1). The system used a cation-exchange resin[2] in a 10 × 0.6-cm i.d. column. The mobile phase was 0.2 *M* tromethamine buffer containing 5% isopropanol, pH 9.2. The flow was 0.4 ml/min at a pressure of 200–300 psi.

The locations of pilocarpine and isopilocarpine were verified by UV detection of unlabeled standards run simultaneously with the radioactive sample. Two-minute fractions were collected from the high-pressure column directly into counting vials. The fractions were counted in scintillator fluid[3], and a previously constructed quench curve was used for converting counts per minute to disintegrations per minute.

***d*-Homopilopic Acid (I)**—*dl*-Homopilopic acid was prepared by the procedure of DeGraw (1). A solution of 16.0 g (0.093 mole) of the *dl*-acid in 140 ml of warm absolute ethanol was treated with 11.3 g (0.093 mole) of *d*-α-methylbenzylamine followed by 490 ml of ether. After 2 hr, the crystals were collected and dried to leave 8.9 g, mp 106–119°. Four recrystallizations from ethyl acetate (30 ml/g) afforded 3.7 g of white crystals, mp 113–121°.

The salt was partitioned between 3 *N* hydrochloric acid and ether. The ether was dried over magnesium sulfate and evaporated to leave the theoretical weight of *d*-homopilopic acid as a clear



Scheme I

[1] *Chromatronix.*
[2] Aminex A-7.
[3] Aquasol, New England Nuclear Corp., Boston, Mass.

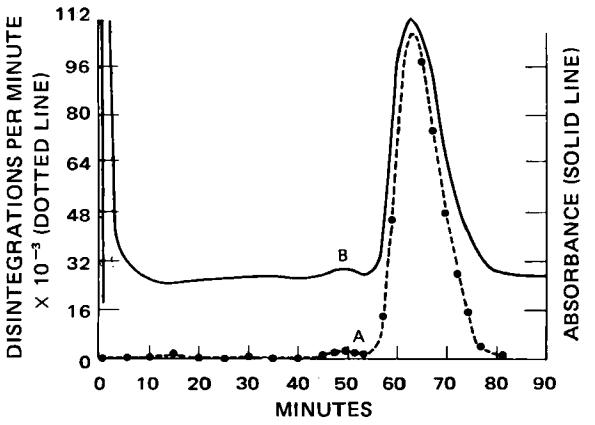


Figure 1—*High-pressure liquid chromatogram of pilocarpine-N-$^{14}CH_3$, detected by (A) radioactivity (disintegrations per minute) and (B) UV absorbance.*

syrup, $[\alpha]_D^{23}$ + 82.2° (1% in ether). The melting point of the salt was not a reliable index of optical purity.

^{14}C-Methyl Isothiocyanate—This labeled reagent was prepared essentially by the procedure of Moore and Crossley (3). ^{14}C-Methylamine hydrochloride (387 mg, 5.73 mmoles) was dissolved in 0.68 ml of water, cooled to 0–5°, and treated with 0.38 ml (6.3 mmoles) of carbon disulfide followed by 515 mg (12.9 mmoles) of sodium hydroxide in 1.1 ml of water. After 30 min, the ice bath was removed and the mixture was stirred at ambient temperature for 60 hr. Ethyl chloroformate (0.61 ml, 6.40 mmoles) was added dropwise and stirring was continued for 1 hr.

The mixture was extracted with six 1-ml portions of methylene chloride, and the combined extracts were dried over magnesium sulfate. The solution was concentrated to one-half volume on a rotary evaporator and then transferred to a microdistillation assembly with an ice-cooled receiver. The remaining solvent was removed (bp 30–35°), and the product fraction was collected from 70 to 100° to afford 200 mg (48%) of yellow semisolid distillate. Yields of 65–70% were obtained in larger scale cold runs.

***d*-2-Mercaptopilocarpine-*N*-$^{14}CH_3$ (IV)**—*d*-Homopilopyl aminomethyl ketone hydrochloride (III) was prepared in 51% yield from I by the reported procedure (1), proceeding through the acyl di-*tert*-butylacetamidomalonate (II). To a stirred mixture of 200 mg (2.7 mmoles) of ^{14}C-methyl isothiocyanate, 277 mg (2.0 mmoles) of potassium carbonate, and 6.6 ml of 70% tetrahydrofuran was added 670 mg (2.7 mmoles) of *d*-amino ketone hydrochloride (III); stirring was continued for 14 hr. The upper layer was removed by pipet, and the lower aqueous phase was twice extracted with 1-ml portions of ether.

The combined organic extracts were evaporated *in vacuo*, and the residue was successively triturated with 2-ml portions of water and warm isopropanol. The residual solid was washed with ether and dried to leave 210 mg (32%) of pale-yellow crystals. TLC [silica gel, chloroform–acetone (1:1)] showed a single spot, R_f 0.75, identical with authentic IV as detected by UV and iodine vapor; radioautography also showed a minor spot at the origin.

***d*-Pilocarpine-*N*-$^{14}CH_3$ (V)**—A mixture of 210 mg (0.87 mmole) of IV and 1.35 ml (4.0 mmoles) of 8% hydrogen peroxide was stirred for 2 hr at ambient temperature, and then 3.2 ml of saturated sodium bicarbonate was added (pH 7–8). The solution was extracted with three 5-ml portions of methylene chloride, and the extracts were dried over magnesium sulfate and evaporated *in vacuo* to leave 190 mg of a clear gum.

The crude material was subjected to preparative chromatography [silica gel, chloroform–methanol (9:1)]. The chromatogram showed an elongated single spot at R_f 0.30–0.45. The material from R_f 0.30–0.35 was scratched out and extracted with methanol to afford 11 mg of gum; HPLC indicated 97% of pilocarpine, 2% of isopilocarpine, and approximately 1% of an unidentified substance near the origin; $[\alpha]_D^{23}$ + 106° (1.1% in methylene chloride) [lit. (4) $[\alpha]^{18}$ + 106°]. The material at R_f 0.35–0.45 was similarly handled to afford 109 mg; HPLC showed 85% of pilocarpine, 9% of isopilocarpine, and 6% at the origin.

A 30-mg portion of this latter fraction (specific activity 38 mCi/mmole) was diluted with 119 mg of natural pilocarpine and treated with an equivalent of 0.5 *N* nitric acid. The water was removed *in vacuo*, and the residual nitrate salt was twice recrystallized from ethanol to afford 150 mg (77%) of white crystals. The salt was dissolved in 5 ml of water and treated with 2 ml of saturated sodium bicarbonate. This solution was extracted with three 2-ml portions of methylene chloride which, after drying and evaporation, yielded 118 mg; HPLC showed 97% of pilocarpine, approximately 2% of isopilocarpine, and less than 1% at the origin.

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Paramethadione and Metabolite Serum Levels in Humans after a Single Oral Paramethadione Dose

D. J. HOFFMAN and A. H. C. CHUN ˣ

Abstract □ A GLC method was developed to determine quantitatively paramethadione and its major metabolite, 5-ethyl-5-methyl-2,4-oxazolidinedione, in serum. The method was reproducible and sensitive to 0.2 μg/ml. After administering a single 300-mg oral dose to human subjects, the average paramethadione serum levels of 6.0 μg/ml occurred at 1 hr and decreased to 0.3 μg/ml after 48 hr. Metabolite serum levels gradually increased to 8.4 μg/ml at 32 hr and were still at this level at 48 hr, which was the last sampling point.

Keyphrases □ Paramethadione and metabolite—serum levels, single oral dose, humans, GLC analysis □ 5-Ethyl-5-methyl-2,4-oxazolidinedione—paramethadione metabolite, GLC determination of serum levels, single oral dose, humans □ GLC—analysis, paramethadione and metabolite serum levels, humans

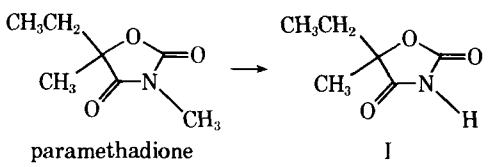
Several GLC methods (1, 2) are available for the quantitative determination of anticonvulsants in biological fluids. However, these methods lack the sensitivity needed to determine serum levels after a single oral dose. Butler (3) studied the metabolism of paramethadione in humans given multiple doses of paramethadione. A UV spectrometric method (4) was used to determine metabolite, 5-ethyl-5-methyl-2,4-oxazolidinedione (I), serum levels. Paramethadione levels were not determined by this method.

The metabolism of paramethadione to I is shown in Scheme I. A method is presented that can quantitatively determine paramethadione and its metabolite in serum after administration of a single oral 300-mg dose of paramethadione to humans.

EXPERIMENTAL

Subjects—Three normal, healthy, adult male subjects were fasted 8 hr prior to dosing (only water was allowed). Paramethadione capsules[1] were administered with 120 ml (4 oz) of water. A standard breakfast was provided to the subjects after the 2-hr blood sample was withdrawn. Twenty milliliters of blood was taken before dosing and at 0.5, 1, 2, 4, 8, 24, 32, and 48 hr after dosing. The serum was separated and frozen until assayed.

GLC—Chromatographic separations were performed with a gas chromatograph[2] equipped with an electronic integrator[3], automatic sampler, and dual flame-ionization detectors. Two 1.85-m × 2-mm (i.d.) glass columns were treated overnight with a 5% dimethylchlorosilane solution in toluene and then packed with 3% OV-17 coated on 100–120-mesh Gas Chrom Q[4]. All glass wool was also treated with the 5% dimethylchlorosilane solution. Before the columns were connected to the detectors, they were further silated by injecting 5 μl of *N,O*-bis(trimethylsilyl)acetamide three times at 10-min intervals and were conditioned overnight at 260°.



paramethadione I

Scheme I

The flow of helium carrier gas was 45 ml/min. The hydrogen flow rate was 30 ml/min, and the air flow rate was 250 ml/min. The temperature of the injection port was 172° and that of the detector was 190°. The oven temperature was programmed as follows: 2 min at 90°, then to 130° at 8°/min, and hold at 130° for 2 min. The electrometer range was 10 with an attenuation of ×4.

Isolation and Assay Procedures (1)—To 4 ml of serum in a 15-ml conical centrifuge tube was added 3 ml of 1 *N* H_2SO_4 and 3

Table I—Precision of Serum Paramethadione and Metabolite Assay at Various Concentrations

Parameth-adione Concentration, μg/ml	Coefficient of Variation, %[a]	Metabolite I Concentration, μg/ml	Coefficient of Variation, %[a]
0.5	10.2	0.5	8.4
1.0	8.4	1.0	6.1
2.0	6.2	2.0	5.8
4.0	5.1	4.0	4.2
8.0	5.5	8.0	3.7

[a] Three samples at each concentration.

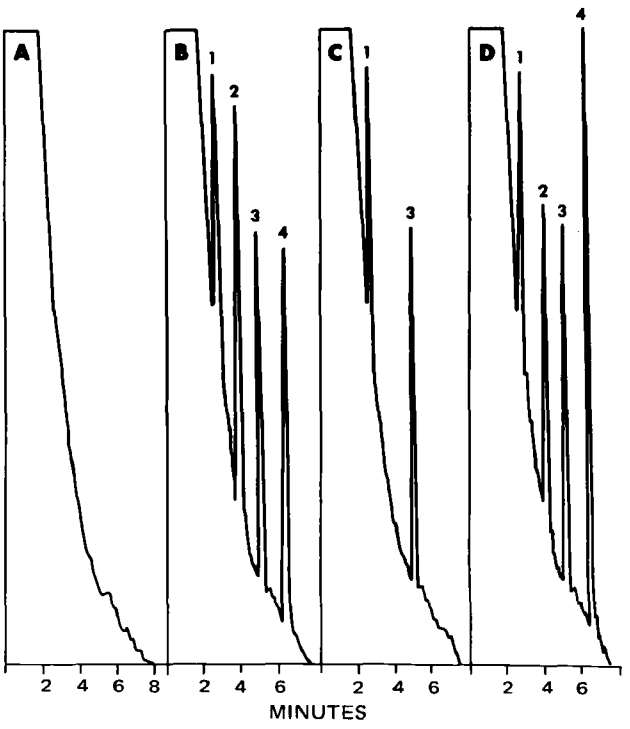


Figure 1—*Gas-liquid chromatograms of serum extracts. Key: A, serum blank without internal standards; B, serum standard containing paramethadione and I at 2.5 μg/ml; C, serum sample from Subject H.J. before dosing with paramethadione; D, serum sample from Subject H.J. 4 hr after a 300-mg oral dose of paramethadione; 1, trimethadione (internal standard); 2, paramethadione; 3, 5-dimethyl-2,4-oxazolidinedione (internal standard); and 4, Metabolite I.*

[1] Paradione Capsules, 300 mg, List 3868, Lot 08-078-AF, Abbott Laboratories, North Chicago, IL 60064
[2] Hewlett Packard model 7620A.
[3] Hewlett Packard model 3370A.
[4] Applied Science Laboratories.

Table II—Serum Paramethadione and Metabolite Levels after Oral Administration of 300 mg of Paramethadione

Subject	Age	Height, m	Weight, kg	Compound	Serum Concentration, μg/ml 0.5 hr	1 hr	2 hr	4 hr	8 hr	24 hr	32 hr	48 hr
H.J.	30	1.78 (5 ft 10 in.)	73.9 (163 lb)	Paramethadione	7.6	6.8	3.7	1.1	1.6	0.6	1.0	0
				Metabolite I	0.8	1.6	2.4	3.8	5.3	7.4	8.1	9.2
K.L.	30	1.75 (5 ft 9 in.)	74.8 (165 lb)	Paramethadione	0	5.4	2 3	2.7	2.0	—[a]	1.2	0.5
				Metabolite I	0	1.7	2.5	3.2	4.9	—[a]	8.6	7.4
J.M.	41	1.85 (6 ft 1 in.)	85.7 (189 lb)	Paramethadione	0.5	5.8	2.6	2.4	2.0	0.9	0.8	0.3
				Metabolite I	0	1.1	2.6	3.5	5.3	7.7	8.6	8.7
			Mean	Paramethadione	2.7	6.0	2.9	2.1	1.9	0.8	1.0	0.3
			Mean	Metabolite I	0.3	1.5	2.5	3.5	5.2	7.6	8.4	8.4

[a] Sample lost.

ml of 10% sodium tungstate dropwise, and the solution was mixed. After standing for 15 min, the samples were centrifuged at 3000 rpm for 15 min. The supernate (6 ml) was transferred to a 20-ml screw-capped tube containing 0.2 ml of 1 *N* H_2SO_4. Three grams each of anhydrous sodium sulfate and crystalline magnesium sulfate (0.7 μg/ml) was added with mixing. Chloroform (7 ml) containing the internal standards trimethadione (0.3 μg/ml) and 5-dimethyl-2,4-oxazolidinedione (0.7 μg/ml) was added, and the sample was shaken for 10 min and centrifuged at 2500 rpm for 10 min.

The aqueous layer was discarded by aspiration, and 5 ml of the chloroform layer was evaporated to about 0.1 ml at 30° with a gentle stream of air. Chloroform (0.1 ml) was added with mixing and then transferred to a microvial[5] and capped. The sample (10 μl) was automatically injected into the gas chromatograph, and the temperature program was initiated.

Standard serum solutions of both paramethadione and I were prepared in duplicate at concentrations of 0, 0.5, 1.0, 2.0, 4.0, and 8.0 μg/ml. Standard curves were constructed by plotting concentration *versus* paramethadione–trimethadione and I–5-dimethyl-2,4-oxazolidinedione peak area ratios.

RESULTS AND DISCUSSION

This GLC method has sufficient sensitivity to determine quantitatively paramethadione and its metabolite in serum after a single oral dose of paramethadione. The sensitivity of the method is 0.2 μg/ml. This value is based on a sample signal equivalent to about 2% of full-scale deflection. The precision of the assay is shown in Table I. Variance, as expected, was greater for the low concentrations. Coefficients of variation for paramethadione ranged from 5.5% at 8 μg/ml to 10.2% at 0.5 μg/ml. For I, they ranged from 3.7 to 8.4% at the same concentrations. Standard curves of serum paramethadione or metabolite concentrations *versus* peak area ratios were linear to at least 8 μg/ml. As shown in Fig. 1, GLC tracings were free of interfering peaks.

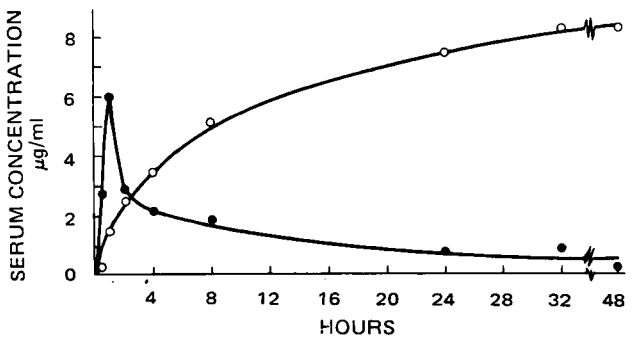


Figure 2—*Mean serum paramethadione and metabolite levels after oral administration of 300 mg of paramethadione in humans. Key: ●, paramethadione; and ○, Metabolite I.*

Individual paramethadione and metabolite serum levels are shown in Table II. The mean serum levels are plotted in Fig. 2. Paramethadione is rapidly absorbed with peak serum levels of 5.4–7.6 μg/ml occurring between 0.5 and 1 hr after a single 300-mg oral dose of paramethadione. A biphasic decline of serum paramethadione was observed, with serum levels decreasing to 0.3 μg/ml at 48 hr.

There appeared to be a slight rise in levels at 32 hr. The average serum half-life of the β-phase determined graphically was estimated to be 16 hr for the three subjects. This value is comparable to a serum half-life of 17 hr for trimethadione[6].

Metabolite serum levels were detectable within 1 hr after drug administration and gradually increased to 8.4 μg/ml in 32 hr; they remained at this level up to the last sampling time (48 hr) (Fig. 2). These results suggest that paramethadione is rapidly metabolized to I and that this metabolite has a long serum half-life, as evidenced by the apparent plateau of serum levels after 48 hr. This view is in accord with the data of Butler (3), from which a serum half-life of 14 days was estimated.

5-Dimethyl-2,4-oxazolidinedione, the metabolite of trimethadione, has a half-life of about 13 days in humans (4) and appears to play a considerable role in the anticonvulsant effect of trimethadione (5). The long serum half-life of 5-dimethyl-2,4-oxazolidinedione is attributed to its extremely slow, pH-dependent, renal excretion (6). The structural similarity of 5-dimethyl-2,4-oxazolidinedione and I and the apparently long serum half-life suggest that the latter metabolite also has a slow renal excretion rate.

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[x] To whom inquiries should be directed.

[5] Hewlett Packard, 200-μl volume microvial.

[6] Dr. H. E. Booker, University of Wisconsin Medical Center, Madison, Wis., personal communication.

IR Studies on Carrageenan of *Ahnfeltia concinna*, a Marine Red Alga

GERTRUDES A. SANTOS and MAXWELL S. DOTY ˣ

Abstract □ The principal polysaccharide of *Ahnfeltia concinna* gave IR absorptions at 1240, 930, 845–850, and 805 cm^{-1}, all typical of a carrageenan. The polysaccharide was separated into soluble and insoluble fractions with potassium chloride.

Keyphrases □ Carrageenan—isolation from *Ahnfeltia concinna*, IR identification □ *Ahnfeltia concinna*—isolation of carrageenan, IR identification □ IR spectrophotometry—identification, carrageenan isolated from *Ahnfeltia concinna* □ Alga—IR identification of carrageenan isolated from *Ahnfeltia concinna*

Carrageenan is a red seaweed polysaccharide extensively used as a food additive, especially in dairy products, and as a suspending agent in the cosmetic and pharmaceutical industries. Carrageenan is prepared from different species of *Chondrus, Gigartina, Eucheuma, Hypnea,* and *Iridaea.* These genera and the genus *Ahnfeltia* belong to the order Gigartinales. Although *Ahnfeltia plicata* has long been been known as the major source of agar in Russia, *Ahnfeltia durvilliae* of Peru was reported to contain carrageenan (1).

There are three main groups of carrageenans: the κ-, λ-, and ι-carrageenan groups. The three types differ only in their 3,6-anhydrogalactose content and in their content and position of ester sulfate groups in the molecule. Scheme I shows the idealized structure of κ-, ι-, and λ-carrageenans proposed previously (2). The μ- and ν-carrageenans are the precursors of κ- and ι-carrageenans, respectively.

From the report of Lawson *et al.* (3), *A. durvilliae* contains a "deviant" ι-carrageenan. Methylation studies showed that the polysaccharide consists of β-D-galactopyranose 4-sulfate (45%), β-D-galactopyranose (5%), 3,6-anhydro-α-D-galactopyranose (24%), 3,6-anhydro-α-D-galactopyranose 2-sulfate (12%), 4-linked α-D-galactopyranose 2,6-disulfate (7%), and 4-linked α-D-galactopyranose (7%) (4).

A. concinna is a marine red alga widely distributed in the Hawaiian Islands. It forms a yellow-brown to dark red-brown band in the intertidal region on basalt rocks. In a preliminary report on the polysaccharide of this alga, the name ahnfeltan was given to the principal carbohydrate (5). The purpose of this investigation was to show whether the polysaccharide of *A. concinna* is definitely a carrageenan or at least not an agar.

EXPERIMENTAL

The sample used was collected from north of Makapuu Point on the island of Oahu, Hawaii. The seaweed was quite clean and devoid of epiphytes. The fresh live seaweed gave as much as 27% clean dry weed.

Extraction of Polysaccharide—Clean dry weed (20 g) was heated in water (700 ml) adjusted to pH 9 by addition of 2% sodium hydroxide solution in a boiling water bath for 1 hr. The seaweed was then blended to a paste and heated for a further 3 hr. A filter aid[1] (50 g) was added, and the mixture was stirred continuously for 30 min and then filtered with pressure. The filtrate, about 700 ml–1 liter, was about 1% carrageenan.

A sodium chloride solution (10%, 50 ml) was added, and the mixture was poured into twice its volume of 85% isopropanol with rapid stirring. The precipitate was strained through nylon cloth, washed twice with 600 ml of the 85% isopropanol, and dried at 60°. The dried polysaccharide was ground to a powder, yielding 11.92 g (59.6%); SO_3Na, 31.86%; 3,6-anhydrogalactose ($C_6H_{10}O_4$).

The modified carrageenan was prepared by treating the dry seaweed (20 g) in water (700 ml) with lime (4 g) instead of a 2% sodium hydroxide solution and heating and filtering as already described. The filtrate was adjusted to pH 8.5–9.0 with dilute hydrochloric acid. Addition of 10% sodium chloride was not necessary before precipitation of the polysaccharide with 85% isopropanol. The yield was 11.86 g (59.3%); SO_3Na, 26.29%; 3,6-anhydrogalactose.

Fractionation of Carrageenan—The fractionation method used was that of Stancioff and Stanley (1). The powdered carrageenan (8.00 g) was dispersed by continuous stirring for 1 hr at room temperature in 2.5% potassium chloride solution, and the mixture was allowed to sit overnight. A filter aid (40 g) was stirred into the mixture for 1 hr, the mixture was filtered with suction, the filter cake was washed with potassium chloride solution (2.5%, 100 ml), and the filtrate and washing were combined and evaporated to 600 ml and precipitated with 2.5 volumes of 85% isopropanol. The precipitate was washed twice with 300-ml portions of the solvent, dried at 60° for 5 hr, and ground into a powder, yielding 1.13 g (15.76%); SO_3Na, 31.12%; 3,6-anhydrogalactose, 11.00%.

The filter cake was then suspended in hot water (800 ml), the mixture was stirred for 1 hr at 85–100° and filtered with pressure, and the cake was washed with hot water (100 ml). The filtrate and washing were combined and heated to 70°, and sodium chloride solution (10%, 50 ml) was added with stirring. The hot mixture was poured into twice its volume of 85% isopropanol with vigorous stirring. The coagulum was squeezed, washed twice with 500-ml por-

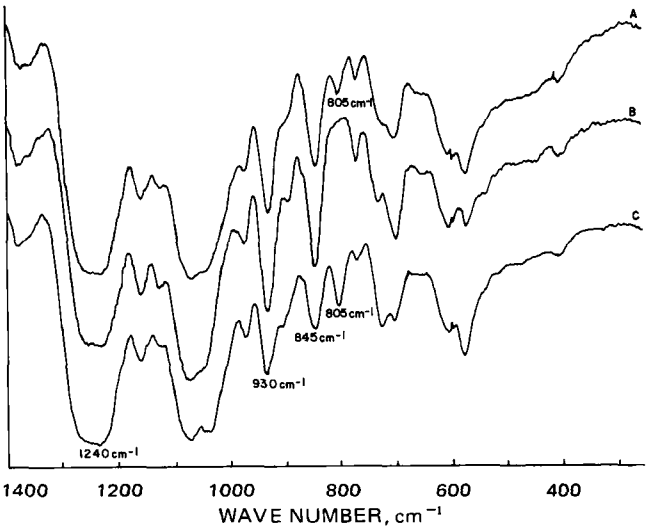
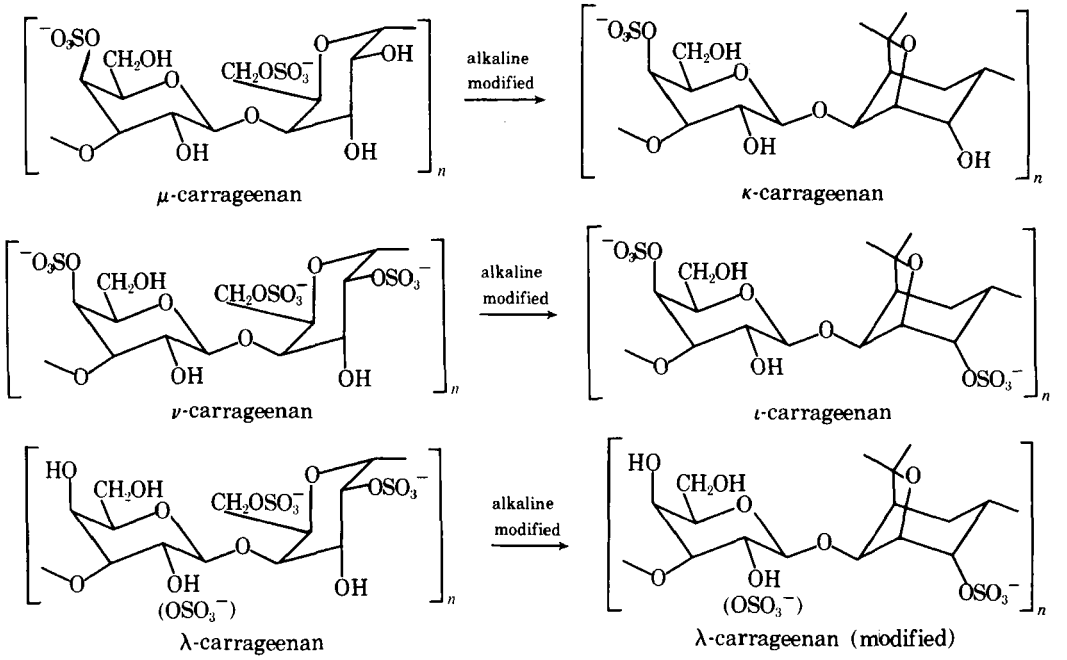


Figure 1—*IR spectra of: A,* A. concinna *carrageenan; B, κ-carrageenan from* Eucheuma cottonii; *and C, ι-carrageenan from* Eucheuma spinosum.

[1] Diatomite.



Scheme I

tions of isopropanol, and dried in a 60° oven for 5 hr, yielding 6.04 g (84.24%); SO_3Na, 33.13%; 3,6-anhydrogalactose, 27.03%.

Sodium Borohydride Treatment of Potassium Chloride-Soluble Fraction—The Rees method (6) was used in which the polysaccharide (1.6 g) was dissolved in water (600 ml), sodium borohydride was added (0.2 g), and the solution was allowed to sit overnight at room temperature. More sodium borohydride (7.2 g) and sodium hydroxide (24 g) were added to the solution with cooling. After the rapid evolution of gas had subsided, the solution was heated on a water bath at 80° for 2 hr. The solution was neutralized with acetic acid and then poured, with stirring, into 2.5 times its volume of 85% isopropanol. The coagulum was squeezed, washed twice with isopropanol, and dried at 60° for 5 hr, yielding 1.46 g (91.25%); SO_3Na, 24.37%; 3,6-anhydrogalactose, 23.63%.

IR Determination—The polysaccharide (30 mg) was dissolved in distilled water (15 ml), and 3 ml of the solution was poured onto mercury (4 ml) contained in a 15-ml porcelain crucible. The solution was dried to a thin film in a heated vacuum desiccator (70°). The mercury was poured off, the film was mounted between two 15 × 5-cm pieces of cardboard provided with 2.5 × 1.3-cm windows, and the spectra were determined[2].

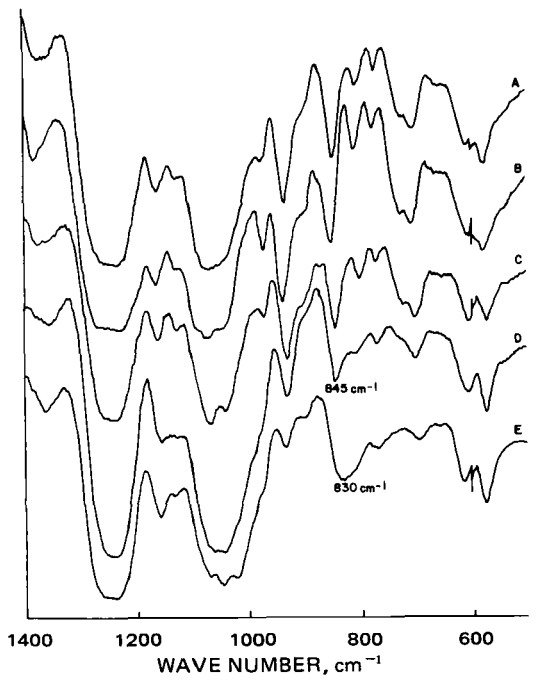


Figure 2—*IR spectra of: A,* A. concinna *carrageenan; B, the modified* A. concinna *carrageenan; C, the potassium chloride-insoluble fraction; D, the potassium chloride-soluble fraction; and E, λ-carrageenan.*

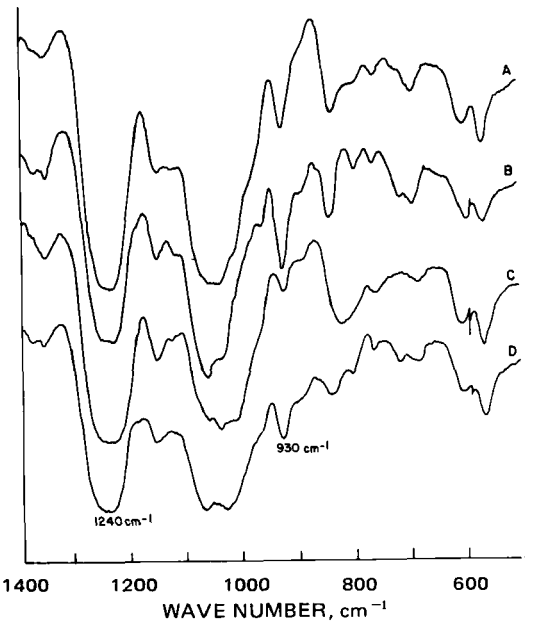


Figure 3—*IR spectra of: A, potassium chloride-soluble fraction of* A. concinna *carrageenan; B, modified potassium chloride-soluble fraction; C, λ-carrageenan; and D, modified λ-carrageenan.*

[2] Beckman IR 467 spectrophotometer.

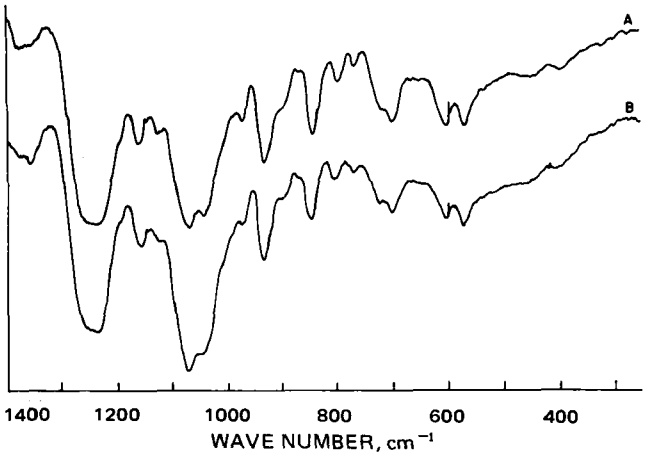


Figure 4—*IR spectra of: A, potassium chloride-insoluble fraction of* A. concinna *carrageenan; and B, modified potassium chloride-soluble fraction.*

RESULTS AND DISCUSSION

The IR spectra of the polysaccharide from *A. concinna* showed absorption bands characteristic of a carrageenan with: 1240 cm^{-1}, S—O stretching vibration; 930 cm^{-1}, typical of the presence of 3,6-anhydrogalactose; and 845–850 cm^{-1}, due to the secondary axial 4-sulfate absorption. But it is neither a κ- nor an ι-carrageenan as shown in Fig. 1. As shown in Fig. 2, the broadening at the base of the 850- and 805-cm^{-1} absorptions was possibly due to the presence of the primary equatorial 6-sulfate absorption at 820 cm^{-1}, which disappeared upon alkaline modification with the elimination of the primary 6-sulfate and the formation of the 3,6-anhydrogalactose (1, 2, 7–16).

Fractionation of the polysaccharide with 2.5% aqueous potassium chloride yielded the potassium chloride-insoluble and potassium chloride-soluble fractions. The IR spectrum (Fig. 2) of the potassium chloride-insoluble fraction is very similar to that of modified carrageenan. The potassium chloride-soluble fraction could not be λ-carrageenan, because the broad absorption at 850–800 cm^{-1} has a maximum at 850 cm^{-1} while that of the λ-carrageenan is at 830 cm^{-1}. The broad absorption band at 850–800 cm^{-1} of the potassium chloride-soluble fraction was resolved into two sharp peaks (Fig. 3) at 850 and 805 cm^{-1} upon modification with sodium borohydride, and the IR spectrum of the product (Fig. 4) was very similar to that of the potassium chloride-insoluble fraction. However, the potassium chloride-soluble fraction of the *A. concinna* carrageenan cannot possibly be the precursor of the potassium chloride-insoluble fraction because of the low sulfate and 3,6-anhydrogalactose content of the modified carrageenan.

There is a possibility that the structure of the carrageenan of *A. concinna* could be identical or closely related to that of *A. durvilliae*, which Lawson *et al.* (3) investigated and classified as a deviant ι-carrageenan.

According to the physical properties and IR studies, the polysaccharide of *A. concinna* is definitely a carrageenan; however, further studies are still needed to determine the different monosaccharide units in the molecule and to clarify the chemical structure.

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Analysis of *N*-Glycosyl Bond Length in Crystal Structures of Nucleosides and Nucleotides

ALBERT LO *, ELI SHEFTER *x, and TODD G. COCHRAN ‡

Abstract □ A relationship was found between the *N*-glycosyl bond length in β-nucleosides and nucleotides and the spatial relationship of this bond with respect to the lone-pair orbitals on the furanose ring oxygen. This relationship may be relevant to the geometry involved in the hydrolysis of the glycosidic bond.

Keyphrases □ Nucleosides and nucleotides—analysis of *N*-glycosyl bond length in crystal structures □ Glycosyl bonds—analysis of bond length in crystal structures of nucleosides and nucleotides □ Hydrolysis—glycosyl bonds, analysis of bond length in crystal structures of nucleosides and nucleotides

The *N*-glycosyl bonds of nucleic acids are readily hydrolyzed by either enzymes (specific nucleosidases) or acid-catalyzed reactions. Although the mechanism of enzymatic cleavage of these glycosidic bonds has been poorly characterized, numerous kinetic studies on the acid-catalyzed hydrolysis of these bonds in nucleosides have produced some definitive ideas on the hydrolytic mechanism (1–3).

Two schemes have been proposed for the acid-catalyzed route. One involves the opening of the protonated furanose ring at the C_1'—O_1' bond, with subsequent water attack and the formation of the base and sugar products. In the other scheme, the glycosidic bond of the base-protonated species (mono- or dication) is directly cleaved. Evidence for the latter scheme appears to be strongest for most nucleosides (3). Although mechanisms have been developed, there is a dearth of any detailed structural information concerning the geometry of the minimum energy pathway followed by the reaction, *i.e.*, reaction coordinates.

Recently, crystal structure data were used to analyze the geometry of some nucleophilic addition reactions (4, 5). The general approach was to examine the atomic coordinates of a large number of molecules possessing similar chemical subunits for structural variations that might correspond to existing knowledge about these subunits in a reaction scheme. The geometrical parameters observed for any molecule in a crystal are a complicated function of inter- and intramolecular forces. Therefore, one would expect to find differing geometries for a particular subunit in a series of molecular structures.

The hypothesis behind the use of these data is that the interplay of forces that displaces the subunit of interest from its geometrical minimum energy configuration in the crystal can provide a clue as to the path that may occur in the chemical breakdown of the entity. This hypothesis assumes that the initial stages of the chemical breakdown and the geometrical distortions in the crystal structures both follow similar minimum energy routes.

It was deemed worthwhile to utilize this approach to analyze the *N*-glycosyl linkage of nucleotides and nucleosides. For this purpose, published crystal structures whose accuracy was sufficient for such a correlation were selected. The structures examined are listed in Table I together with some pertinent parameters describing the glycosidic bond.

DISCUSSION

A scrutiny of the parameters in the table shows that there is a significant variation in the C_1'—N bond length among the tabulated structures. Since the range of C—N bond lengths is reflective of distortions that could occur at the initial stages of the hydrolytic sequence, one might glean some clues from them as to the beginning of the hydrolytic reaction.

The change in the glycosidic bond length is not accompanied by any significant alteration in bonding parameters about C_1', *i.e.*, bond lengths and angles. However, some subtle trends in these parameters are worth noting even though they are not *highly* significant. As the glycosidic bond lengthens, the N—C_1'—C_2' angle decreases slightly and the furanose ring angle (O_1'—C_1'—C_2') tends to enlarge.

The torsion angle about the C_1'—O_1' bond [τ(N—C_1'—O_1'—C_4')] provides an indication of the spatial relationship of the lone pair of orbitals on O_1' with regard to the glycosidic bond (Fig. 1). Geometric constraints imposed by the furanose ring limit the values obtained for the C_1'—O_1' torsion angle to those between synclinal (60°) and antiperiplanar (180°). The manner by which

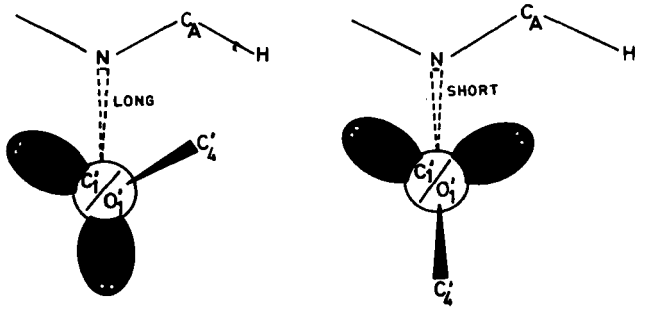

Figure 1—*Schematic drawing of the N—C_1'—O_1'—O_4' torsion angle. Key: left, synclinal; and right, antiperiplanar.*

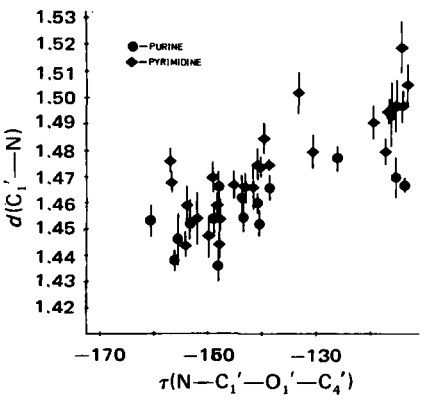

Figure 2—*Relationship between τ(N—C_1'—O_1'—C_4') and the glycosidic bond length.*

Table I—Intramolecular Parameters about C_1' in a Number of Nucleosides and Nucleotides

Compound[a]	Bond Length, Å					Bond Angles, degrees						Torsion Angles, degrees		σ_d[b], Å	σ_a[b], degrees
	C_1'—N	C_1'—O_1'	C_1'—C_2'	C_1'—H	O_1'—C_4'	N—C_1'—O_1'	N—C_1'—C_2'	N—C_1'—H	O_1'—C_1'—H	O_1'—C_1'—C_2'	C_2'—C_1'—H	C_A—N—C_1'—O_1'	N—C_1'—O_1'—C_4'		
I Py	1.436	1.409	1.528	1.07	1.456	110.1	114.0	103	116	105.7	108	−135.0	−148.2	0.006	0.4
II Pu	1.438	1.422	1.520	1.02	1.451	109.8	114.7	101	116	104.1	111	73.0	−156.2	0.004	0.2
III Py	1.444	1.420	1.510	0.95	1.461	109.2	116.1	111	104	103.9	112	65.4	−154.2	0.005	0.5
III Py	1.445	1.415	1.516	1.04	1.441	109.2	115.3	111	99	105.4	115	57.8	−148.0		
IV Pu	1.446	1.412	1.499	0.97	1.457	108.4	117.3	100	114	103.5	114	54.6	−155.6	0.01	0.7
V Py	1.448	1.431	1.560	1.01	1.436	108.2	116.0	108	107	108.4	109	58.4	−149.9	0.008	0.7
VI Pu	1.452	1.412	1.522	0.98	1.455	108.3	114.6	105	112	103.7	114	46.6	−160.6	0.006	
VI Pu	1.454	1.413	1.528	0.99	1.447	108.4	113.0	107	110	105.8	113	122.5	−143.5		
VII Py	1.452	1.426	1.517	0.89	1.435	106.8	114.2	109	110	107.0	109	52.8	−152.0	0.01	1.0
VIII Py	1.454	1.413	1.521	0.98	1.417	109.8	114.1	102	113	105.3	113	57.1	−148.0	0.008	0.4
VIII Py	1.459	1.420	1.507	0.98	1.450	108.4	115.6	102	116	103.5	112	65.5	−153.9		
IX Pu	1.452	1.410	1.516	1.01	1.450	107.3	114.4	104	113	105.1	113	120.1	−153.4	0.009	0.5
IX Pu	1.460	1.416	1.525	1.01	1.452	107.6	113.4	109	107	105.1	114	49.1	−143.7		
X Py	1.459	1.404	1.535	0.99	1.442	108.9	112.3	111	110	105.6	109	45.1	−148.4	0.005	0.3
XI Py	1.467	1.402	1.524	0.95	1.446	108.6	113.9	107	112	105.8	110	52.3	−145.2	0.006	0.3
XII Py	1.466	1.413	1.525	1.05	1.459	109.0	113.8	105	114	105.9	109	49.7	−143.1	0.006	0.3
XIII Py	1.467	1.416	1.531	0.97	1.460	108.6	114.4	106	113	103.9	112	42.1	−156.7	0.004	0.2
XIV Py	1.466	1.413	1.535	0.99	1.434	108.8	119.2	98	110	107.6	113	−96.6	−141.7	0.009	0.8
I Pu	1.468	1.410	1.510	1.20	1.445	108.4	113.6	98	118	106.1	113	−143.7	−148.1	0.006	0.4
XV Pu	1.466	1.416	1.524	0.96	1.455	108.6	113.9	109	112	106.3	107	−103.9	−138.5	0.005	0.3
XVI Pu	1.454	1.413	1.529	1.06	1.457	108.2	114.2	104	117	105.3	108	−132.9	−149.1	0.006	0.3
XVII Pu	1.452	1.430	1.545	1.01	1.439	107.9	115.8	104	111	108.5	110	−125.7	−140.6	0.005	0.3
XVIII Pu	1.460	1.415	1.528	0.98	1.454	107.2	113.0	105	114	105.6	112	43.1	−140.8	0.004	0.3
XIX Pu	1.467	1.411	1.530	1.01	1.450	109.3	111.6	108	110	107.3	111	9.9	−113.7	0.003	0.2
XX Pu	1.470	1.390	1.528	1.19	1.472	108.1	114.7	105	115	107.8	107	28.9	−115.2	0.008	0.4
XXI Py	1.470	1.403	1.536	1.03	1.463	106.9	114.3	109	110	105.9	110	34.0	−149.0	0.006	0.4
XXII Py	1.474	1.410	1.525	0.95	1.448	107.8	113.6	106	112	106.6	111	51.4	−140.2	0.004	0.3
XXIII Py	1.475	1.418	1.513	0.91	1.458	107.7	112.9	100	119	105.9	112	41.8	−140.8	0.006	0.5
XXIV Py	1.475	1.407	1.540	0.90	1.444	108.5	112.1	106	109	106.5	114	26.7	−138.7	0.002	0.2
XXV Pu	1.477	1.409	1.526	1.01	1.441	107.2	113.5	106	112	108.6	109	3.7	−126.0	0.005	0.3
XXVI Py	1.476	1.420	1.512	0.95	1.447	108.2	114.5	104	117	104.4	109	41.8	−156.9	0.005	0.3
XXVII Py	1.480	1.434	1.515	0.96	1.460	109.2	114.5	106	113	106.5	109	39.1	−130.6	0.006	0.4
XXVIII Py	1.486	1.418	1.525	1.03	1.433	108.0	113.1	104	107	106.7	118	54.1	−139.7	0.006	0.5
XXIX Py	1.492	1.423	1.539	1.06	1.463	108.8	113.9	108	108	107.1	111	19.6	−119.4	0.006	0.4
XXX Pu	1.494	1.444	1.508	1.12	1.475	107.3	112.6	100	126	106.6	104	25.7	−116.1	0.012	0.7
XXXI Py	1.494	1.405	1.536	1.00	1.449	108.7	112.2	109	107	110.2	102	20.3	−116.6	0.005	0.3
XXXII Py	1.496	1.410	1.543	1.00	1.458	108.4	112.5	108	104	107.7	116	18.1	−120.0	0.004	0.2
XXXIII Py	1.497	1.412	1.527	1.04	1.445	108.7	111.4	110	112	106.9	108	18.3	−114.1	0.006	0.4
XXXIV Py	1.502	1.431	1.538	1.20	1.437	106.4	114.3	102	113	108.3	112	57.4	−133.2	0.008	0.4
XXXIV Py	1.505	1.387	1.528	0.97	1.463	108.6	112.4	100	106	107.7	121	27.8	−113.27	0.008	0.4
XXXV Py	1.508	1.404	1.508	0.99	1.460	108.2	112.3	106	109	107.2	114	−0.09	−111.55	0.003	0.2
XXXVI Py	1.510	1.404	1.528	0.96	1.454	107.7	113.6	105	102	108.1	120	−6.2	−114.36	0.003	0.2
XXXVI Py	1.519	1.397	1.534	1.05	1.456	108.3	112.2	110	102	108.6	115	−6.1	−114.26	0.01	0.6
Average	1.471	1.414	1.527	1.01	1.450	108.6	113.9	106	111	106.3	112	16.0	−137.2		
(*SD*)	(0.02)	(−)	(0.01)	(0.07)	(−)	(1.6)	(1.6)	(5)	(5)	(1.4)	(4)	(62.9)	(15.6)		

[a] Name and reference are listed below. Pu and Py denote purine and pyrimidine analog, respectively. If more than one crystallographically unique molecule appears in a crystal, they are listed with the same identifying number.

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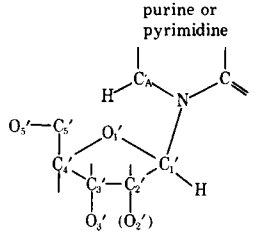

[b] σ_d and σ_a are the average standard deviations reported for bond lengths and angles, respectively, of parameters not involving hydrogen. Those for hydrogen are much greater.

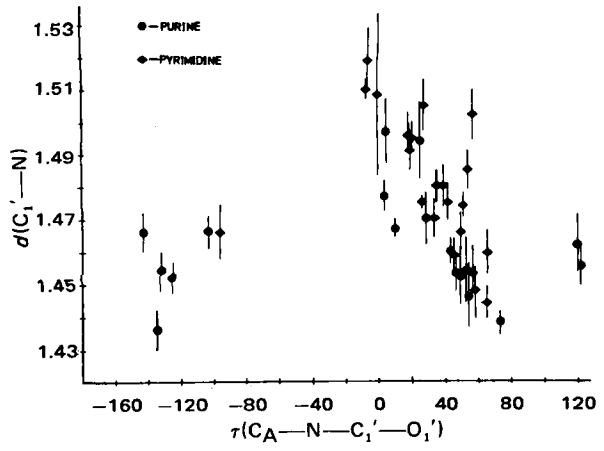

Figure 3—*Correlation of $\tau(C_A—N—C_1'—O_1')$ and glycosidic bond length.*

the furanose ring puckers influences the value of this torsion angle.

A relationship between τ(N—C_1'—O_1'—C_4') and the glycosidic bond length appears to exist (Fig. 2). The *N*-glycosyl linkage is shortest when this constellation of atoms is antiperiplanar and longest when synclinal. The orientation of a lone-pair orbital on the oxygen becomes antiperiplanar as this angle tends toward a synclinal value. Thus, the *N*-glycosyl bond length appears to be longest when it is antiperiplanar to a lone-pair orbital on the oxygen.

The following regression equation (r = 0.80) was derived for the relationship shown in Fig. 2:

$$d(C_1'—N) = 1.625 - 0.00112\tau(N—C_1'—O_1'—C_4') \quad \text{(Eq. 1)}$$

This equation predicts the shortest value possible for the glycosidic bond (occurring at τ = 180°) to be 1.43 Å. This length is much shorter than an N(sp^2)—C(sp^2) single bond (1.485 Å) (6) or the N—C bonds of alkylated nucleic acid bases (7).

In a structural analysis of tetrahedral dioxo compounds and orthoesters, a similar type of relationship was found (5). The C—O′ bond in these compounds was a function of the O′—C—O″—H torsion angle. The C—O′ bond length was longest when a lone-pair orbital on O″ was antiperiplanar to it. This observation is supported by studies carried out on the decomposition of conformationally rigid hemi-orthoesters (8) and hemi-amide acetals (9), where the bond most readily cleaved appeared to be the one with two lone-pair orbitals antiperiplanar to it.

Jeffrey *et al.* (10), using nonempirical molecular orbital calculations, found a similar relationship between bond length and conformation for methylenediol. The difference in bond length for the synclinal and antiperiplanar arrangements is also reflected in the stretching force constants (11). The calculated values are 5.8 and 6.0 mdynes/Å for the longer and shorter bonds, respectively. Therefore, the reaction that cleaves the longer bond (antiperiplanar conformation with respect to a lone-pair orbital) would be energetically favored, at least in the initial phase.

Other investigators (12, 13) indicated that the observed differences in glycosidic bonds could be attributed to nonbonded interactions between the base and the sugar. The conformation of the base relative to the sugar can be described by the torsion angle, $\tau(C_A—N—C_1'—O_1')$. This angle was correlated by these investigators to the glycosidic bond length. With the tabulated data, the correlation appears to break down (Fig. 3). However, this correlation is valid if no differentiation is made between the sides of the purine or pyrimidine rings, *i.e.*, between the *syn*- and *anti*-conformations (14). If 180° is added to the $C_A—N—C_1'—O_1'$ torsion angle of the six nucleosides with a *syn*-conformation that show a marked deviation from the linear trend in Fig. 3, a linear correlation is observed (r = 0.65).

Since the torsion angles about the $C_1'—O_1'$ and $C_1'—N$ bonds can both be related to the glycosidic bond length, the conformations about these bonds appear to be dependent upon one another. Regression analysis of the tabulated values for these two torsion angles is suggestive of this, showing a linear correlation (r = 0.67) for the 43 nucleosides and nucleotides in Table I. This result is not unexpected in light of work (15) showing that the freedom of rotation about the glycosidic bond in nucleosides and nucleotides is closely related to the puckering in the furanose ring.

In terms of reaction coordinates, the tabulated data show that a conformation in which the $N—C_1'—O_1'—C_4'$ torsion angle tends toward a synclinal value would be favorable for a direct cleavage mechanism of hydrolysis, since this conformation is associated with an increasing glycosidic bond length and an antiperiplanar orientation between the glycosidic bond and a lone-pair orbital on the oxygen. While this cannot be construed as direct evidence that such a geometry occurs during hydrolysis, it is easily envisioned that a catalytic species (nucleosidase) could induce such a conformational change in a nucleoside or nucleotide, thus facilitating the hydrolysis.

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Plasma Protein Binding of Drugs as a Function of Age in Adult Human Subjects

A. DOUGLAS BENDER *x, ALEX POST *, JOSEPH P. MEIER *, JOHN E. HIGSON *, and GEORGE REICHARD, Jr. ‡

Abstract □ The intent of this study was to determine what influence, if any, increasing age has on the binding of drugs by plasma proteins. Plasma from healthy subjects ranging in age from 21 to 94 years was used. The binding of phenytoin (diphenylhydantoin) (acid), penicillin G potassium (benzylpenicillin potassium), and phenobarbituric acid was determined by equilibrium dialysis of ^{14}C-labeled compounds. No differences were found in total protein concentration; however, albumin was reduced in subjects over 50 years of age. Plasma binding of each drug studied was not related to age; this finding suggests that age *per se* is not a factor in the binding of drugs by plasma proteins.

Keyphrases □ Plasma protein binding—drugs, effect of age, adult human subjects ■ Binding—drugs to plasma protein, effect of age, adult human subjects □ Age—effect on binding of drugs to plasma proteins, human subjects

Several years ago, Ehrnebo *et al.* (1) reported that human fetal and neonatal plasma has a lower binding capacity for drugs than plasma from young healthy adult subjects. The investigators attributed the lower binding capacity to lower total protein and albumin concentrations in fetal and newborn plasma. They also implied that qualitative differences in protein binding may have contributed to the decrease in binding capacity.

From a practical viewpoint, the decreased drug binding capacity in the newborn means that more free drug is available at a given total drug concentration in the plasma. Therefore, there exists the potential for an increase in drug activity and the incidence of undesirable effects.

Elderly patients exhibit an increased sensitivity to drugs (2–4), which in some cases has been shown to be related to higher and more prolonged blood drug levels brought about by a slower rate of drug metabolism and elimination in older patients (5–7). The objectives of the present study were to assess the influence of increasing age on the binding of drugs by plasma proteins and, thus, to determine whether or not altered drug binding could potentially contribute to the enhanced effect of drugs in geriatric patients.

Table I—Concentrations and Specific Activities of Drugs Used in Determining Drug Binding Capacity of Human Plasma

Drug	Concentration, μg/ml	Specific Activity, μCi/mg
Phenytoin (acid)		
Day 1	16.87	0.82
Day 2[a]	12.93	2.92
Phenobarbituric acid		
Day 1[b]	20.21	4.14
Penicillin G potassium		
Day 1[b]	13.78	2.43

[a] A fresh solution was prepared for the analysis of a second batch of human plasma. [b] The same solution was used with succeeding batches of human plasma on different days. The solution was stored at −10° when not in use.

Table II—Total Protein, Globulin, and Albumin Concentrations, A/G Ratio, and Other Laboratory Parameters of Plasma from Normal Subjects

	Subjects Less than 50 Years of Age (n = 14, Mean = 29 Years)	Subjects Greater than 50 Years of Age (n = 10, Mean = 63.5 Years)
Total protein, g/100 ml	7.1 ± 0.10[a]	7.0 ± 0.10
Globulin, g/100 ml	3.1 ± 0.08	3.5 ± 0.16[b]
Albumin, g/100 ml	4.0 ± 0.06	3.4 ± 0.09[c]
A/G ratio	1.3 ± 0.04	1.0 ± 0.06[c]
Blood urea nitrogen, mg/100 ml	14.5 ± 0.44	16.5 ± 1.16
Serum glutamic pyruvic transaminase, I.U.[d]	13.5 ± 1.03	11.7 ± 1.25
Serum glutamic oxaloacetic transaminase, I.U.	16.5 ± 1.21	15.0 ± 1.68
Alkaline phosphatase, I.U.	54.0 ± 2.84	58.0 ± 4.30

[a] Mean and standard error. [b] Significant difference at $p < 0.05$. [c] Significant difference at $p < 0.01$. [d] I.U. = international units.

EXPERIMENTAL

Plasma Samples—Blood was obtained by venipuncture from 25 healthy subjects ranging in age from 21–94 years. The subjects had not received any drug for at least 1 week prior to the time blood was withdrawn. All blood samples were heparinized and centrifuged, and the red cell-free plasma was stored at −10°. Prior to analysis, the plasma was thawed at 4° and then allowed to reach room temperature. Ehrnebo *et al.* (1) showed that freezing and thawing plasma samples do not affect the drug binding capacity of plasma.

Clinical Chemistry—Total protein[1], serum glutamic oxaloacetic transaminase[1], serum glutamic pyruvic transaminase[1], alkaline phosphatase[1], globulin[2], and blood urea nitrogen[2] levels were determined using commercially available procedures. Plasma albumin concentration was calculated as the difference between the total protein and globulin concentrations.

Radioactive Compounds—5,5-Diphenylhydantoin-4-^{14}C as the acid[3] (specific activity, 5.21 mCi/mmole), phenobarbituric acid-2-^{14}C[3] (specific activity, 3.34 mCi/mmole), and potassium 6-phenyl-1-^{14}C-acetamidopenicillinate[4] (specific activity, 24.7 mCi/mmole) were obtained commercially. All radiochemical purities were reported by the suppliers to be greater than 98%.

Because of the high specific activities of these compounds, it was necessary to dilute them with their respective nonradioactive carriers. Both the labeled and nonlabeled compounds were diluted in pH 7.38 buffer (1) to a suitable concentration that was amenable

[1] Smith Kline Instruments Inc., Palo Alto, Calif.
[2] Dow Chemical, Midland, Mich.
[3] New England Nuclear Corp., Boston, Mass.
[4] Amersham-Searle, Arlington Heights, Ill.

Table III—Drug Binding, Total Protein Concentration, and Albumin Concentration of Plasma from Normal Subjects[a]

Drug	Subjects Less than 50 Years of Age				Subjects Greater than 50 Years of Age			
	Patients	Total Protein, g/100 ml	Albumin, g/100 ml	Percent Drug Bound	Patients	Total Protein, g/100 ml	Albumin, g/100 ml	Percent Drug Bound
Phenytoin (acid)	6	7.0 ± 0.08[b]	4.0 ± 0.07	82.4 ± 0.84	3	7.3 ± 0.18	3.4 ± 0.18	83.6 ± 0.35
Phenobarbituric acid	5	7.3 ± 0.23	4.1 ± 0.09	41.8 ± 1.33	3	6.8 ± 0.12	3.4 ± 0.22	41.9 ± 2.07
Penicillin G potassium	5	7.0 ± 0.16	3.9 ± 0.13	42.4 ± 3.00	4	6.9 ± 0.15	3.5 ± 0.15	45.1 ± 3.62

[a] With one exception (plasma from 21-year-old subject), plasma samples were subjected to a single drug binding experiment. Not included are data from a 94-year-old subject with a serum albumin of 1.3 g/100 ml, elevated serum glutamic oxaloacetic transaminase, and alkaline phosphatase levels. These data are referred to in the text. [b] Mean and standard error.

to the drug binding experiment. The concentrations and specific activities are listed in Table I.

Equilibrium Dialysis—Equilibrium dialysis was carried out in two-compartment Lucite cells[5]. The compartments on each side of the dialysis membrane measured 17.46 mm in diameter and 3.98 mm in depth. The volume of each compartment was approximately 0.87 ml. The viscose membrane[6] had a thickness of 0.203 mm with an average pore size of 4.8 mμ. This type of membrane retains components in aqueous solutions with molecular weights of 12,000 and higher. Prior to its use, the membrane was soaked overnight in deionized water and the excess water was removed before placing it into the apparatus.

A plasma sample, 0.87 ml, was placed into one compartment of the cell and the labeled drug dissolved in the buffer was placed into the other. The cell was sealed with tape and attached to an apparatus described in USP XVIII (8). This device raised and lowered the dialysis cell 30 times/min in a 37 ± 1° water bath for 6 hr. This time period[7] was the same as was used previously (1).

Assay of Drugs—Liquid scintillation counting of the radioactivity was used to determine the amount of labeled drug in the plasma and buffer solutions. Exactly 0.300 ml of each solution was removed, and 0.6 ml of a 25% aqueous tetramethylammonium hydroxide solution was added to solubilize the protein in the plasma aliquot. The solutions were allowed to digest overnight at room temperature; then 15 ml of a liquid scintillator solution[8] was added, shaken, and counted[9] for 10 min. The absolute disintegration rate was obtained by determining the quench effect by conventional internal standardization technique with hexadecane-^{14}C.

Calculation of Binding Capacity—Equation 1 (1) was used to determine the percentage of bound drug:

$$\% \text{ bound} = \frac{[C_{plasma} - C_{buffer}] \times 100}{C_{plasma}} \qquad \text{(Eq. 1)}$$

where C_{plasma} = concentration of drug in the plasma compartment after dialysis, and C_{buffer} = concentration of drug in the buffer compartment after dialysis.

RESULTS AND DISCUSSION

With the exception of elevated serum glutamic oxaloacetic transaminase and alkaline phosphatase levels in a 94-year-old subject, results of the clinical laboratory studies reveal no abnormal or significant findings (Table II) and thus confirm that the subjects were healthy and free of complicating disease. Data for this subject are not included in the statistical analysis but are discussed in reference to the relationship of drug binding capacity to plasma albumin levels.

In Table II, mean values are given for total protein, globulin (G), and albumin (A) concentrations along with the A/G ratio for subjects of different ages. These data show that plasma total protein is not altered by increasing age, while plasma albumin is significantly reduced in patients over 50 years of age with a corresponding increase in plasma globulin. The calculated A/G ratio is decreased from 1.3 for patients under 50 years of age to 1.0 for patients 50 years and over. These results are consistent with data reported by Libow (9). In this latter study, a total protein concentration of 7.2 g/100 ml was reported for healthy subjects 18–36 years of age and a value of 6.9 g/100 ml was given for healthy subjects 65–92 years of age. This difference was not statistically significant. Serum albumin, however, was significantly reduced in the older subjects. In the young group, the albumin concentration was 3.8 g/100 ml; in older subjects, the value was 3.3 g/100 ml.

The plasma binding values reported here are lower than those reported previously (1); however, the significance or explanation of this difference is difficult to judge since the specifications for the membrane used in the earlier investigation were not given. It has been pointed out that it is difficult to interpret the results of binding studies in which a single drug concentration was used, as was the case in this study. However, Shah *et al.* (10) showed that the percentage of phenytoin (diphenylhydantoin) bound to plasma protein is constant for a wide range of concentrations (1–40 μg/ml). Nevertheless, these issues, although of interest certainly to future studies in this area, do not affect the interpretation of the current results since the interest here was to establish the influence of age on the *relative*, not necessarily the *absolute*, drug binding capacity of plasma.

In Table III, the binding of each drug is compared for subjects under 50 years of age and those over 50 years of age. This comparison was made because after age 50 there was a significant reduction in serum albumin levels. Despite a reduction in plasma albumin concentration, no changes were noted in plasma binding capacity. These data suggest that age *per se* does not influence the binding of drugs to plasma proteins. More specifically, they indicate that increasing age is not associated with: (*a*) a defect in drug binding proteins or (*b*) the production of an endogenous substance which competes with a drug for binding sites on the protein molecule. With a reduction in serum albumin and no change in drug binding, the amount of drug bound for a given amount of albumin is increased with age.

While the data from the normal subjects in this study show no change in plasma binding capacity, this finding does not rule out the potential for a reduction in binding capacity in elderly patients, as with any other patients whose plasma albumin and/or total protein may be lower than that encountered in this study. For example, Ehrnebo *et al.* (1) reported a reduction in drug binding capacity in the newborn associated with a significant reduction in both plasma albumin and total protein. Andreasen (11) found a reduced binding capacity associated with lower concentrations of albumin in surgical patients with acute renal failure.

Indeed, in the present study, in a single subject of 94 years of age (not included in Table III) with a plasma albumin level of 1.3 g/100 ml, the percentage of phenobarbital bound to plasma protein was reduced to 12.1%, a figure far below the mean value for all patients regardless of age. In two other 90-year-old subjects with plasma albumin levels above 3.0 g/100 ml, plasma protein binding of phenytoin and phenobarbital was the same as that found for younger subjects.

The results of a recent study (12) are relevant to this issue. The incidence of unwanted central nervous system depression with diazepam was significantly greater in patients with a plasma albumin level of less than 3.0 g/100 ml than in patients with a value of greater than 4.0 g/100 ml. Interestingly, age was not a factor.

[5] Fabricated at the Smith Kline & French Laboratories.
[6] Catalog No. EDR3787-D22, A. H. Thomas Co., Philadelphia, Pa.
[7] In other studies in these laboratories with other drugs, no statistical difference in binding determined after 6 and 12 hr of dialysis was observed.
[8] Omnifluor–Cabosil–naphthalene–absolute ethanol–dioxane (9.6 g:50 g:100 g:100 ml:1000 ml).
[9] Packard Tri-Carb model 3003.

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Activity of Local Anesthetic Agents in Goldfish

STUART FELDMAN ˣ, MARY DeFRANCISCO, and PETER J. CASCELLA

Abstract □ The activity of procaine hydrochloride, lidocaine hydrochloride, tetracaine hydrochloride, and dibucaine hydrochloride in producing overturn in goldfish was measured in pH 8.0 buffer. Calculation of the apparent minimum effective concentration of local anesthetic necessary to result in overturn of the goldfish showed that the activity of these agents increased in the following order: procaine hydrochloride < lidocaine hydrochloride < tetracaine hydrochloride < dibucaine hydrochloride. The effect of these agents on goldfish can be correlated with previous work on the minimum concentration necessary to block conduction in isolated nerve and muscle fibers. The activity of the local anesthetic agents could be explained, in part, by the relationship between the chloroform–pH 8 buffer partition coefficient and the minimum effective concentration in goldfish. Experimental results indicate that the unionized drug molecule is responsible for the observed effects.

Keyphrases □ Anesthetics, local—activity of procaine hydrochloride, lidocaine hydrochloride, tetracaine hydrochloride, and dibucaine hydrochloride in producing goldfish overturn, structure–activity relationships □ Structure–activity relationships—local anesthetics, goldfish overturn □ Goldfish—model for determining structure–activity relationships of local anesthetics

Recent reports (1–3) indicated that goldfish may serve as a biological test model in the assessment of structure–activity relationships of drugs. The goldfish was an adequate test system for discerning structure–toxicity relationships of substituted phenothiazine derivatives (1), and the goldfish was used as a model to study the effects of alkyl chain length on the alkyl *p*-aminobenzoate-induced narcosis in the fish (2). The use of this simple and inexpensive biological test system to study the correlation between pharmacological and toxicological activity with physical–chemical properties has many advantages (1). The present report concerns preliminary findings as to the relationships that may exist between structure and activity of local anesthetic agents in goldfish.

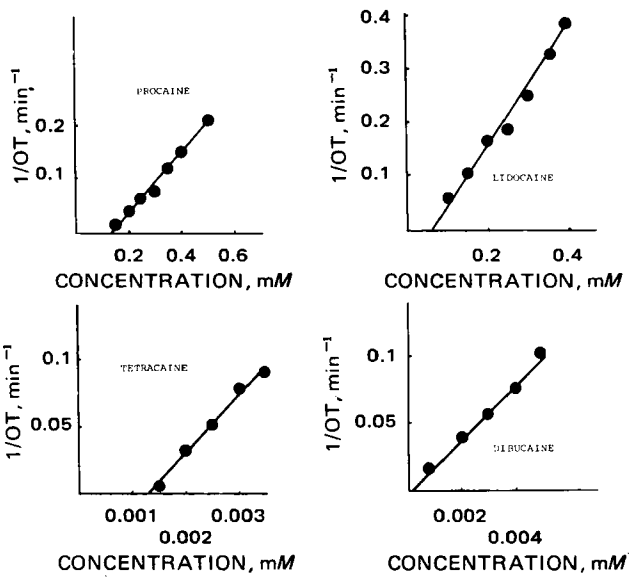

Figure 1—*Plot of the reciprocal of overturn time of local anesthetics in goldfish* versus *concentration of local anesthetic agent.*

EXPERIMENTAL

Goldfish, common variety (*Carassius auratus*), weighing 3–4 g were purchased locally. The overturn time (4) of individual goldfish in 100 ml of various concentrations of procaine hydrochloride[1], lidocaine hydrochloride[2], tetracaine hydrochloride[1], and dibucaine

[1] Amend Drug and Chemical Co., Livingston, N.J.

[2] Astra Pharmaceuticals, Worcester, Mass.

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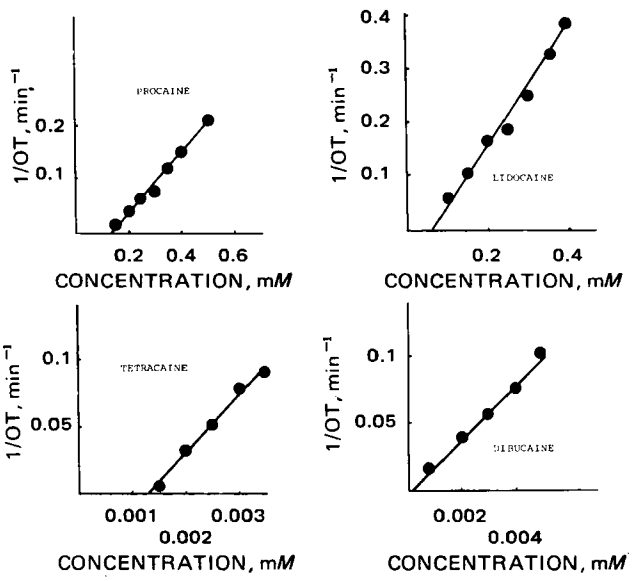

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Table I—Average Overturn Times Produced by Various Concentrations of Local Anesthetic Agents in pH 8.0 Buffer

Agent	Concentration, m*M*	Number of Fish	Mean Overturn Time, min	Standard Deviation	Mean Reciprocal of Overturn Time, min^{-1}
Procaine hydrochloride	1.5×10^{-1}	5	63.1	15.5	0.016
	2.0×10^{-1}	5	24.2	8.00	0.041
	2.5×10^{-1}	5	16.0	0.38	0.063
	3.0×10^{-1}	5	13.5	2.08	0.074
	3.5×10^{-1}	5	8.57	0.80	0.117
	4.0×10^{-1}	5	6.65	0.98	0.150
	5.0×10^{-1}	5	4.69	1.36	0.213
Lidocaine hydrochloride	1.0×10^{-1}	5	15.2	5.76	0.066
	1.5×10^{-1}	5	9.28	0.84	0.108
	2.0×10^{-1}	5	5.78	1.08	0.173
	2.5×10^{-1}	5	5.41	0.46	0.185
	3.0×10^{-1}	5	4.01	0.41	0.249
	3.5×10^{-1}	5	3.01	0.62	0.332
	4.0×10^{-1}	5	2.58	0.52	0.388
Tetracaine hydrochloride	1.5×10^{-3}	5	120.	28.3	0.008
	2.0×10^{-3}	5	30.3	8.57	0.033
	2.5×10^{-3}	5	20.5	2.58	0.049
	3.0×10^{-3}	5	12.8	1.91	0.078
	3.5×10^{-3}	5	11.1	2.10	0.090
Dibucaine hydrochloride	1.0×10^{-3}	5	45.1	9.04	0.022
	2.0×10^{-3}	5	24.1	4.13	0.041
	2.5×10^{-3}	5	21.8	2.95	0.046
	3.0×10^{-3}	5	17.9	2.04	0.056
	4.0×10^{-3}	5	13.0	3.81	0.077
	5.0×10^{-3}	5	8.65	3.01	0.116

hydrochloride[1] in pH 8.0 phosphate buffer (0.01 *M*) was determined. The solutions were freshly prepared before each experiment. At least five goldfish were used at each local anesthetic drug concentration. The goldfish tank and test solutions were maintained at 22 ± 2°.

Partition coefficients of each local anesthetic agent were determined between chloroform and pH 8.0 phosphate buffer (0.01 *M*). Fifteen milliliters of organic phase and 15 ml of aqueous phase containing the local anesthetics were placed in glass bottles and shaken[3] until equilibrium was established by repetitive sampling. Assay for drug in the aqueous phase after appropriate dilution was done spectrophotometrically[4] at 288 nm for procaine, 262 nm for lidocaine, 310 nm for tetracaine, and 328 nm for dibucaine. All determinations were made at room temperature (22–24°).

RESULTS AND DISCUSSION

Inspection of Table 1 and Fig. 1 reveals that good agreement exists with the four local anesthetic agents tested between the reciprocal of overturn time (1/OT) and drug concentration (4). The data were analyzed by linear regression, and the intercept on the *x*-axis was calculated for each local anesthetic. This value was taken as the apparent minimum effective concentration (MEC) necessary to produce overturn in the goldfish. While it has been shown (5) that the relationship between 1/OT and concentration is not linear at concentrations of drug near the MEC but is hyperbolic, it was felt that this extrapolation would not lead to a significant error in the determination of the MEC. The apparent MEC values for the local anesthetic agents investigated in the present study are listed in Table II.

According to the values listed in Table II, the potency of the local anesthetics in producing overturn in goldfish increases in the following order: procaine < lidocaine < tetracaine < dibucaine. This order of activity is similar to previous results reported for these anesthetics on isolated nerve preparations (6) and in clinical evaluations in human subjects (7).

Agin *et al.* (6), in a report on the action of anesthetic agents on excitable membranes, tabulated the minimum blocking concentration (MBC) of the anesthetic agents necessary to produce conduction blockage on isolated nerve or muscle fibers. These values for

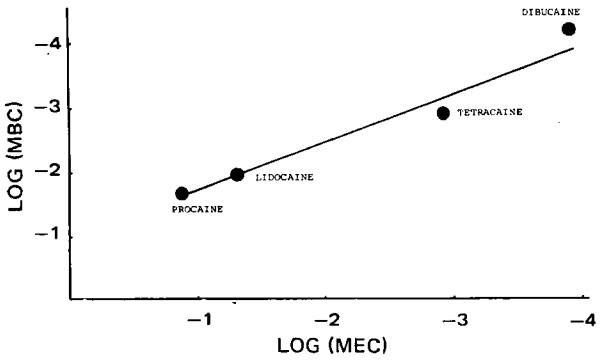

Figure 2—*Plot of the logarithm of the minimum blocking concentration (MBC) of local anesthetics on isolated nerve and muscle fibers* versus *the logarithm of the minimum effective concentration (MEC) of these agents in goldfish. (Data for MBC were obtained from Ref. 6.)*

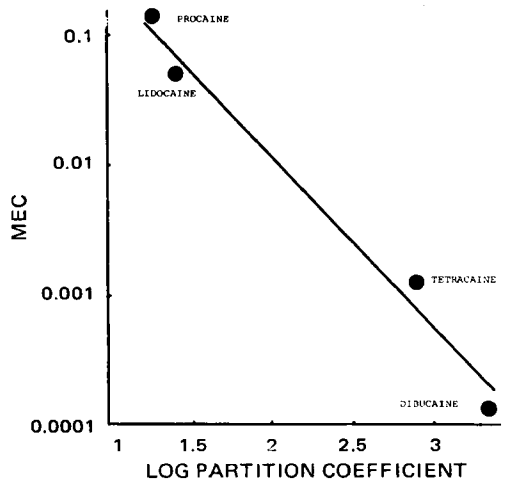

Figure 3—*Plot of the logarithm of the minimum effective concentration (MEC) of local anesthetics in goldfish* versus *the logarithm of the partition coefficient.*

[3] Precision water bath shaker model 25, Precision Scientific Co.
[4] Beckman DB-GT, Beckman Instruments.

Table II—Minimum Effective Concentrations (MEC) in Goldfish, Minimum Blocking Concentrations (MBC) in Isolated Fibers, and Partition Coefficients of Local Anesthetics

Local Anesthetic	MEC, m*M*	Log MBC, m*M*[a]	Partition Coefficient[b]
Procaine hydrochloride	0.136	−1.67	19
Lidocaine hydrochloride	0.049	−1.96	41
Tetracaine hydrochloride	0.0012	−2.9	800
Dibucaine hydrochloride	0.00013	−4.2	2200

[a] Data obtained from Ref. 6. [b] Between chloroform and pH 8 buffer.

procaine, lidocaine, tetracaine, and dibucaine are listed in Table II. A comparison of the results of the present study with those of Agin *et al.* (6) are presented in Fig. 2; the agreement between the two sets of data is good, and the results of present experiments utilizing the goldfish would give a good prediction as to the effect of the local anesthetics on isolated nerve and muscle fibers.

The correlation of biological activity with physical–chemical properties of the drug has been well documented (8). To determine the role of lipid solubility on the effect of the local anesthetic agent on overturn time in goldfish, partition coefficients between chloroform and pH 8 buffer were measured (Table II). The order of increasing lipid solubility of the agents between chloroform and pH 8 buffer is as follows: procaine < lidocaine < tetracaine < dibucaine. The relationship between partition coefficient and the apparent MEC is illustrated in Fig. 3. The fit is rather good and demonstrates that the activity of the local anesthetics in producing overturn in goldfish is due, in part, to the lipophilicity of the drug molecule.

Experimental results also appear to indicate that the nonionized drug molecule is responsible for the observed effect of the anesthetics on overturn time. In an experiment with lidocaine where the pH of the buffer solution was varied, the mean overturn time in goldfish for a 0.4 m*M* solution was 2.6 min at pH 8 and 16.8 min at pH 7; there was no response at pH 6. Lidocaine, with a pKa of approximately 7.9 (9), is 55.7% unionized at pH 8, 11.2% unionized at pH 7, and 1.2% unionized at pH 6. Thus, with decreasing pH and the subsequent decrease in in the concentration of unionized drug and the ability of the drug to penetrate biological membranes, there is a reduction in the pharmacological activity of lidocaine in the goldfish. A similar effect of pH on the overturn of goldfish in the presence of the other anesthetics investigated was noted.

The results of the present study illustrated the potential usefulness of goldfish as a model for the prediction of drug activity. Investigations are currently underway concerning the structure–activity relationships involved in producing overturn and death in goldfish for a series of lidocaine derivatives.

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Keyphrases □ Poison oak—isolation and characterization of urushiol components □ *Toxicodendron diversilobum*—isolation and characterization of urushiol components □ Urushiol components of poison oak (*Toxicodendron diversilobum*)—isolation and characterization

Plants of the genus *Toxicodendron* (Anacardiaceae) have long been known for their ability to produce contact dermatitis in susceptible individuals. The best known species of this genus in the United States are *Toxicodendron radicans* (poison ivy), *Toxicodendron diversilobum* (western poison oak), and *Toxicodendron vernix* (poison sumac). The systematics of this genus have been well characterized (1).

The composition of the allergenic urushiol components (2–4) of poison oak (*T. diversilobum*) was studied. A prior study of the urushiol content of poison

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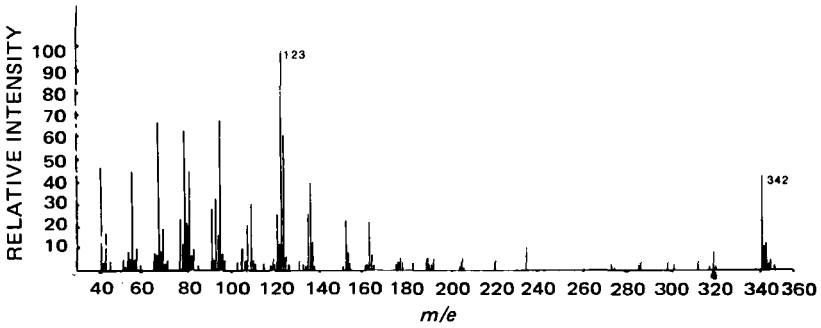


Figure 1—*Mass spectrum of isolated urushiol fraction. Molecular ions at 342, 344, 346, and 348 are for a series of unsaturated C_{17} catechol congeners.*

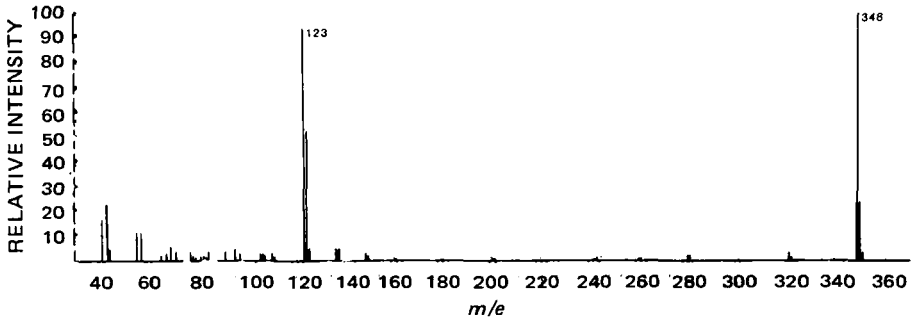


Figure 2—*Mass spectrum of hydrogenated urushiol extract. Molecular ion of heptadecylcatechol is at* m/e *348.*

ivy (*T. radicans*) indicated that it consists of four components (5, 6) having the carbon skeleton of 3-pentadecylcatechol (I), with the components differing from each other only in the degree of unsaturation in the C_{15} side chain. An early investigation (4) of the active component of poison oak indicated the presence of a catechol nucleus; however, a complete structural identification was not made.

This report demonstrates that the urushiol of poison oak is composed principally of a similar series of catechol components with a C_{17} side chain (II). The structural characterization of poison oak urushiol was made by mass spectrometry and UV and NMR analyses.

DISCUSSION

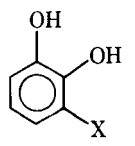
Pure urushiol was isolated as 0.18% of the fresh weight of *T. diversilobum*, employing two chromatographic steps. Additional urushiol fractions, totaling 0.10% of the fresh weight, were contaminated by nonphenolic materials and were not used for characterization of poison oak urushiol. The pure urushiol was a pale-amber-colored oil which solidified below 0°. The double bond number showed that most of the urushiol is the triunsaturated component.

The UV absorption spectrum of the hydrogenated urushiol was virtually identical with that of the urushiol etract with regard to the position and magnitude of their absorption maxima and minima. This observation parallels that reported for the poison ivy analysis (6) and thus precludes the existence of any conjugation in either the diolefinic or triolefinic component. In addition, the maximum absorption peak at 275 nm compares favorably with that of free catechol (λ_{max} 274 nm), which also indicates that there is no interaction between the centers of unsaturation in the side chain and the aromatic ring.

A direct probe mass spectral analysis of the isolated urushiol fraction shows a series of intense ions characteristic of an olefinic homologous series, ranging in mass from *m/e* 342 to 348 (Fig. 1). Although the relative intensities of these ions vary slightly with probe and source temperature, the ion at *m/e* 342 is always the most abundant; this ion is due to the molecular species of the triolefin. The most intense fragment ion occurs at *m/e* 123 and is probably a dihydroxytropylium ion, as might be expected from alkyl–aromatic compounds of this type.

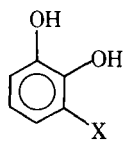
To confirm that the spectrum represents a homologous series of molecular ions having a varying number of double bonds, a sample of the extract was reduced by hydrogenation and again analyzed by mass spectrometry. The results in Fig. 2 show a single intense peak in the molecular ion region at *m/e* 348. A compound of this molecular weight would be the product of a reduction reaction involving mono-, di-, and triolefinic precursors. The most intense fragment ion again occurs at *m/e* 123, which is consistent with the structure previously postulated since the aromatic nucleus is not reduced by the mild hydrogenation process.

Confirmation of the molecular formula of these species was made by high-resolution mass spectrometry (Table I). A weak intensity ion at *m/e* 320 was detected in the hydrogenated sample;



I

Ia: X = $C_{15}H_{25}$
Ib: X = $C_{15}H_{27}$
Ic: X = $C_{15}H_{29}$
Id: X = $C_{15}H_{31}$



II

IIa: X = $C_{17}H_{29}$
IIb: X = $C_{17}H_{31}$
IIc: X = $C_{17}H_{33}$
IId: X = $C_{17}H_{35}$

Table I—High-Resolution Analysis of Molecular Ions

Ion	Theoretical Mass	Experimental Mass	Formula	Difference
320	320.2715	320.2704	$C_{21}H_{36}O_2$	0.0011
342	342.2559	342.2547	$C_{23}H_{34}O_2$	0.0012
348	348.3028	348.3016	$C_{23}H_{40}O_2$	0.0012
123	123.0446	123.0452	$C_7H_7O_2$	0.0006

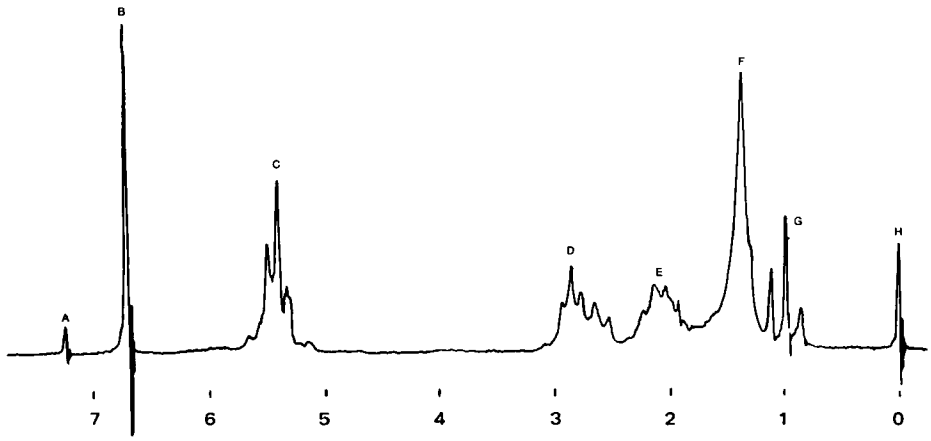


Figure 3—*NMR spectrum of isolated urushiol fraction. Key: A, chloroform; B, phenyl; C, vinyl; D, benzyl; E, allyl; F, methylene; G, methyl; and H, internal standard.*

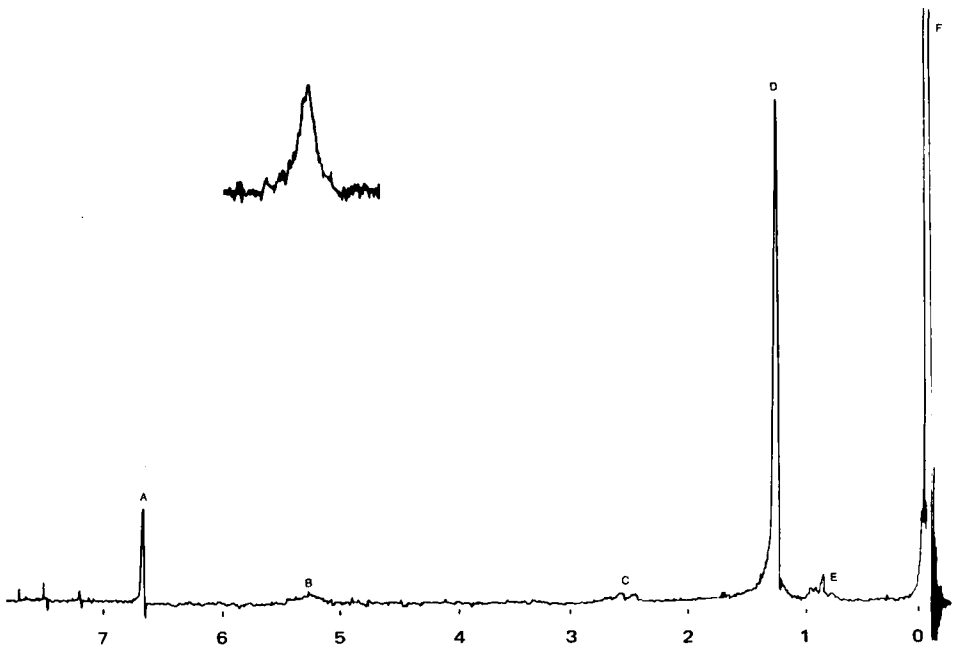


Figure 4—*NMR spectrum of hydrogenated urushiol extract. Key: A, phenyl; B, phenol (also expanded scale); C, benzyl; D, methylene; E, methyl; and F, internal standard.*

high-resolution mass spectrometry showed it to be the molecular ion of pentadecylcatechol, which is the saturated urushiol component reported in poison ivy. Subsequent analysis of the poison oak sample by mass spectrometry indicated that some of the C_{15} isomer is present in the poison oak urushiol, although the dominant component is clearly a series of C_{17} unsaturated congeners of catechol.

The complexity of the mixture and the uncertainty as to the relative rates of volatilization of each component precluded any effort to quantitate accurately the various homologs by direct insertion probe mass spectrometry. However, an approximate visual analysis indicated the triolefin to be the most abundant species and the saturated heptadecylcatechol to be the least. Use of direct probe mass spectrometry allowed for the incorporation of the molecular distillation technique which, in turn, provided sufficient control of sample volatility for analysis.

Efforts to resolve the sample components by GC so that a GC–mass spectrometric analysis could be made have been unsuccessful. The underivatized catechols or their trimethylsilyl derivatives have not been completely resolved under the GC conditions used. Efforts are underway to determine techniques to separate these olefinic catechols so that a quantitative result can be obtained. Furthermore, the double bond positions cannot be unambiguously assigned until the pure components can be analyzed. Structural information of this type was obtained from the mass spectra of various polyunsaturated fatty acids (7) and epoxy-derivatized olefins (8).

The NMR spectra of the urushiol components support the structure of a 3-*n*-alkylcatechol (Fig. 3). The benzylic, phenolic, aromatic, allylic, vinylic, methylene, and terminal methyl protons are all clearly identified. A spectrum of the reduced product (Fig. 4) retains only the aromatic, phenolic, benzylic, methylene, and methyl protons. The integrated NMR spectrum of the hydrogenated sample is easier to analyze than is the extract because of the unresolved nature and unsaturation of the urushiol prior to reduction. The integration of the methylene–methyl region (0.5–1.5) accounts for 33 protons. Two benzylic protons are observed at 2.6, and the aromatic and phenolic groups account for the remaining protons expected for this structure.

The conclusion to be drawn from the presented data is that the urushiol sample is a long chain C_{17} unbranched hydrocarbon, present as part of a catechol molecular structure. Thus, while poison ivy urushiol consists of the linear C_{15} side-chain components (9), poison oak urushiol is primarily composed of catechols with

Table II—Identification of Urushiol Fractions by TLC

Fraction	Weight, g	Purity (TLC)	R_f Solvent I	R_f Solvent II	Urushiol Test ($FeCl_3$)
A	0.3	Two major spots	0.50	0.60	— —
			0.35	0.30	+ +
B	0.9	Single spot	0.35	0.30	+ +
C	0.2	One major spot plus origin material	0.35	0.30	+ +

linear C_{17} side chains. These components, isolated from poison oak (*T. diversilobum*), are shown to differ from those reportedly found in poison ivy (*T. radicans*) by a $-CH_2CH_2-$ unit in the unbranched alkyl side chain.

EXPERIMENTAL[1]

Isolation and Purification of Urushiol—Fresh leaves, berries, and green stems of *T. diversilobum*[2] were collected in northern California, packed in dry ice, and shipped air freight. The material was processed immediately upon receipt. All extractions, evaporations, and chromatography were performed under a nitrogen atmosphere to retard air oxidation of the urushiol.

Fresh plant material (500 g) was covered with 4 liters of ethanol and allowed to stand for 4 days at 5°. Then the ethanol was decanted and evaporated *in vacuo* (40°) to give a black syrupy residue. This residue was partitioned between 500 ml of benzene and 200 ml of 5% NaCl solution and evaporated *in vacuo* to give 5.2 g of a dark-green oil. All of the oil was chromatographed on 100 g of silicic acid[3] packed in a 1.5-cm i.d. column. Elution with pure benzene and combination of all fractions producing a positive phenol test with 2% ethanolic ferric chloride gave 2.3 g of a dark-brown oil after evaporation *in vacuo*. Final purification of the urushiol was achieved by use of dry column chromatography (10).

The silicic acid[3] (100 g) was deactivated by treatment with 5 ml of water and was then treated with 10 ml of 10% ether–hexane. The treated silicic acid was packed in a 1.5-cm i.d. glass column in portions with the aid of a mechanical vibrator and 4.5 kg (10 lb) gauge of nitrogen pressure. The crude urushiol was dissolved in 2 ml of 10% ether–hexane and applied to the column. Development of the column with 10% ether–hexane was assisted by the application of 2.6 kg (5 lb) of nitrogen pressure.

Recovery of materials was made by complete elution from the column, as is occasionally done with dry columns (11). Thirty 15-ml fractions were collected and analyzed by TLC on silica gel plates[4] with the following solvents: I, ethyl acetate–hexane (3:10); and II, methylene chloride. Visualization of separate plates was made with 1% ferric chloride in 0.1 *N* HCl in methanol and with 10% sodium dichromate in 30% H_2SO_4. Combination of similar fractions gave three urushiol fractions of varying purity (Table II).

Fraction B, a pale-amber-colored oil, was considered to be pure urushiol; n_D^{25} 1.5201 ± 0.0002; double bond number based on a molecular weight of 348 ($C_{23}H_{40}O_2$): 2.8. UV λ_{max} (methanol): 275 nm; IR (neat): 3450 (medium, broad), 3025 (sh 3080, medium), 2980 (medium), 2950 (strong), 2875 (medium), 1630 (weak), 1605 (weak), 1490 (strong), 730, 775, and 830 (medium) cm^{-1}.

Mass spectral data are shown in Fig. 1 and NMR data are given in Fig. 3.

Hydrogenation of Urushiol—A 100-mg sample of the urushiol was hydrogenated[5] in 20 ml of methanol, with 10 mg of 10% palladium-on-charcoal as the catalyst. After filtration and evaporation of the solvent, the residue was recrystallized (methanol) to give 70 mg of a white amorphous solid, mp 63–65°; UV λ_{max} (methanol): 277 nm.

Mass spectral data are shown in Fig. 2 and NMR data are given in Fig. 4.

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[1] Mass spectra were obtained on a high-resolution DuPont 21-492, using direct probe analysis. NMR spectra were obtained at ambient probe conditions with a Jeol C-60HL instrument; chemical shift data were measured relative to tetramethylsilane as an internal standard, and all values are presented as parts per million. Deuterated chloroform was used as the solvent in all cases. UV spectra were measured with a Beckman Acta-III in methanol solutions. IR spectra were obtained neat with a Beckman IR-33. Microhydrogenations were obtained from Schwarzkopf Laboratories, Woodside, N.Y. All solvents used in the purification were ACS reagent grade.

[2] Supplied in July 1972 by Hollister-Stier Laboratories, Spokane, Wash. The samples were authenticated by Dr. Maynard W. Quimby, and voucher specimens are stored in the drug plant herbarium at the University of Mississippi.

[3] Analytical reagent, 100 mesh, Mallinckrodt, St. Louis, Mo.

[4] GF-254, E. Merck.

[5] Parr shaker.

Distinction of Synthetic Cannabidiol, Cannabichromene, and Cannabivarin by GLC Using On-Column Methylation

KEITH BAILEY ˣ and DENISE GAGNÉ

Abstract □ The on-column flash methylation of synthetic cannabidiol, cannabichromene, and cannabivarin by trimethylanilinium hydroxide offers a rapid, simple GLC method for their distinction. The phenolic groups of other cannabinoids are methylated by the procedure described.

Keyphrases □ Cannabidiol, cannabichromene, and cannabivarin synthetic mixtures—GLC separation and analysis, on-column methylation □ Cannabichromene, cannabidiol, and cannabivarin synthetic mixtures—GLC separation and analysis, on-column methylation □ Cannabivarin, cannabidiol, and cannabichromene synthetic mixtures—GLC separation and analysis, on-column methylation □ Cannabinoid synthetic mixtures (cannabidiol, cannabichromene, and cannabivarin)—GLC separation and analysis □ GLC—separation and analysis, cannabidiol, cannabichromene, and cannabivarin synthetic mixtures, on-column methylation

Work in these laboratories has confirmed reports (1–3) that it is difficult to distinguish mixtures of synthetic cannabidiol (I), cannabichromene (II), and cannabivarin[1] (III) (4) by GLC analysis. Turner and Hadley (3) recently showed that trimethylsilylation of cannabidiol and cannabichromene gave the corresponding ethers, which were clearly separated by GLC on an OV-17 column, but cannabivarin was not examined in their study.

These three cannabinoids have phenolic groups which were expected to be amenable to on-column flash methylation using trimethylanilinium hydroxide (5, 6). The present study showed that the flash methylation technique offers a rapid and simple GLC method for the unequivocal distinction of the three pure compounds and for their separation in synthetic mixtures.

EXPERIMENTAL

GLC analyses were performed on two gas chromatographs[2], both equipped with flame-ionization detectors and fitted with 0.6-cm (0.25-in.) by 1.8-m (6-ft) glass columns packed with 5% OV-7[3] on 80–100-mesh Chromosorb W. Both the injection port and flame-ionization detector temperatures were 275°, and the nitrogen flow rate was 30 ml/min. Retention times were measured at several oven temperatures (Table I), and relative retention times were compared to the internal standard, 4-androstene-3,17-dione[4].

For methylation, 10 μl of a 0.5% cannabinoid solution in methanol (~0.16 μmole) was treated with 6–10 μl of 0.2 *M* trimethylanilinium hydroxide in methanol[5] (1.2–2 μmoles), and ~0.2 μl of the mixture was injected into the gas chromatograph. Emergent materials from the gas chromatograph was collected by extinguishing the detector flame and placing an open-ended glass capillary tube of the melting-point type over the jet at the predetermined time of peak emergence. The materials were examined by mass spectrometry[6] at an ionizing voltage of 70 ev and a probe temperature of 200°.

RESULTS AND DISCUSSION

Complete methylation of I, II, and III was shown to take place under the described conditions by the appearance of a single GLC peak and by examination of the emergent material by mass spectrometry. Thus, the mass spectrum of cannabidiol dimethyl ether had abundant ions at *m/e* (relative percent) 342 (14), 275 (36), 274 (100), 259 (11), 244 (21), 243 (91), 235 (19), 234 (17), 221 (67), 174 (13), 173 (57), and 158 (16). Cannabichromene methyl ether had abundant ions at *m/e* (relative percent) 328 (6), 313 (5), 246 (21), 245 (100), 231 (6), and 188 (8). Cannabivarin methyl ether had abundant ions at *m/e* (relative percent) 296 (6), 295 (21), 281 (35), 280 (100), 266 (9), 252 (9), 245 (9), 238 (21), and 209 (11).

Insufficient quantities of the trimethylanilinium hydroxide reagent resulted in the formation of the monomethyl ether of I, which had approximately the same retention time as the methyl ether of II. The mass spectrum of cannabidiol monomethyl ether had ions at *m/e* (relative percent) 328 (5), 327 (16), 313 (8), 286 (6),

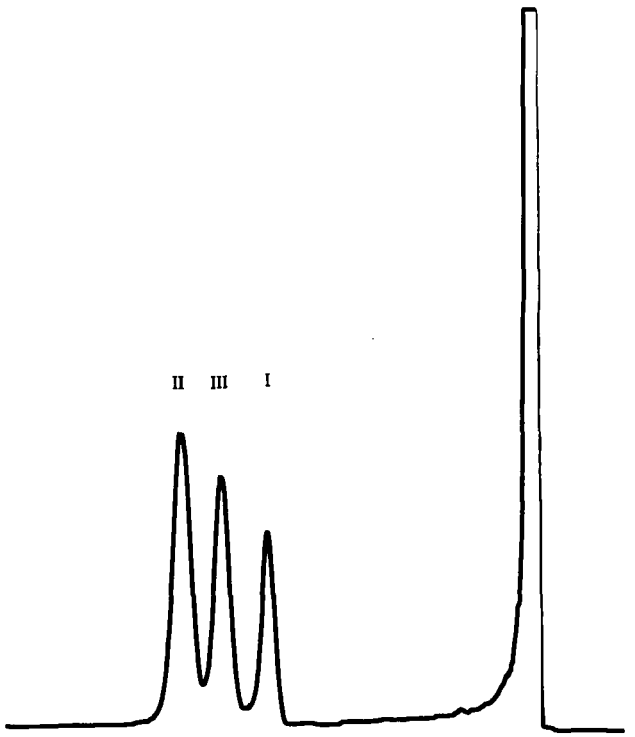


Figure 1—*Chromatogram obtained at 225° of a mixture of the dimethyl ether of cannabidiol (I) and the methyl ethers of cannabivarin (III) and cannabichromene (II).*

[1] Cannabivarin (the propyl homolog of cannabinol) and the other cannabinoids used in this study were prepared in these laboratories by published procedures; their identities were confirmed by proton magnetic resonance and mass spectral analyses.

[2] Varian Aerograph model A-700 and Hydro-Flow Systems Series 3000.

[3] A polar phenylmethyldimethylsilicone from Chromatographic Specialties, Ltd., Brockville, Ontario, Canada.

[4] See Ref. 3.

[5] Prepared and donated by Dr. K. K. Midha of these laboratories.

[6] Hitachi Perkin-Elmer RMU-6L.

Table I—GLC Retention Times and Relative Retention Times of Cannabinoids

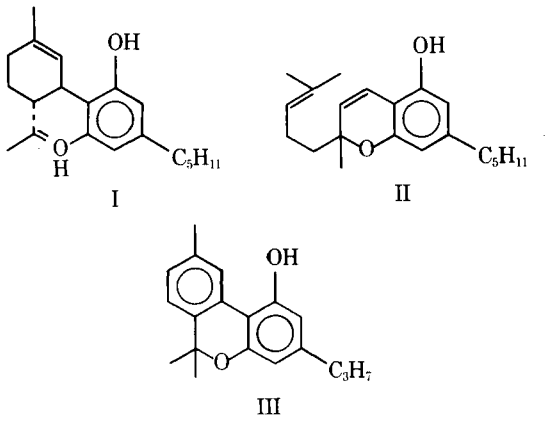
Cannabinoid	Retention Time, min				Relative Retention Time	
	Column Temperature[a]		Column Temperature[b]		Column Temperature[b]	
	225°	250°	190°	215°	190°	215°
Cannabivarin (III)	15.7	6.2	15.3	6.6	0.40	0.43
Cannabichromene (II)	15.7	6.2	16.6	7.0	0.44	0.45
Cannabidiol (I)	15.7	6.2	16.8	7.1	0.44	0.46
Δ^8-Tetrahydrocannabinol (IV)	19.8	7.8	21.0	8.5	0.55	0.55
Δ^9-Tetrahydrocannabinol (V)	21.5	8.2	22.8	9.2	0.60	0.59
Cannabigerol (VI)	24.9	9.3	26.2	10.0	0.69	0.65
Cannabinol (VII)	26.3	10.3	27.6	11.0	0.73	0.71
I dimethyl ether	8.5	3.8	9.3	4.3	0.24	0.28
III methyl ether	9.9	4.3	10.6	4.9	0.28	0.32
II methyl ether	11.1	4.6	11.7	5.2	0.31	0.34
I monomethyl ether	11.2	4.6	12.2	5.3	0.32	0.34
IV methyl ether	12.6	5.3	13.7	5.9	0.36	0.38
VI dimethyl ether	14.1	5.6	15.0	6.1	0.39	0.39
V methyl ether	14.4	5.8	15.6	6.4	0.41	0.41
VI monomethyl ether	16.8	6.9	18.0	7.6	0.47	0.49
VII methyl ether	17.6	7.1	19.1	7.8	0.50	0.50
4-Androstene-3,17-dione			38.0	15.5	1.00	1.00

[a] Varian Aerograph model A-700. [b] Hydro-Flow Systems Series 3000.

261 (10), 260 (41), 246 (27), 245 (100), 243 (9), 229 (8), 220 (9), and 207 (30).

Methylation of Δ^8-tetrahydrocannabinol (IV), Δ^9-tetrahydrocannabinol (V), cannabigerol (VI), and cannabinol (VII) also took place under these conditions; relative retention time and retention time data measured at two column temperatures on both instruments[2] are presented in Table I.

Synthetic cannabidiol, cannabichromene, and cannabivarin are easily distinguished by the simple methylation procedure described here. A chromatogram of a methylated mixture of the three is shown in Fig. 1. The applicability of the method to the determination of the cannabinoid content of *Cannabis* preparations, important when pharmacological studies are undertaken (7), remains to be investigated.



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Rapid Methods for Bioavailability Determination Utilizing Urinary Excretion Data

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Abstract □ Two equations were developed which enable urinary excretion data to be utilized for estimating drug bioavailability within 12 hr starting between one and two half-lives of the drug, depending upon the relative rates of absorption, distribution, and elimination. Both equations were examined using simulated data for both the one- and two-compartment open models. One equation was tested using literature data with excellent results.

Keyphrases □ Urinary excretion data—equations for rapid estimation of bioavailability □ Bioavailability—equations for rapid estimation using urinary excretion data

The most widely used methods for estimating bioavailability for drugs following linear compartmental pharmacokinetics are based upon the measurement of unchanged drug in blood or urine after the administration of a single dose of drug. While the former readily yields estimates of peak blood level and time to reach peak blood level, as well as the extent of bioavailability, it does contain a number of rather serious pitfalls (1). Among these are:

1. The total area under the curve must be estimated, involving a model-dependent correction for the period between the cessation of sampling and time infinity.
2. The correction term requires a knowledge of the rate constant for the elimination phase.
3. Depending upon the model, parameters such as renal clearance, various rate constants, and volumes of distribution must be assumed to be constant either inter- or intrasubject.
4. The assay procedure must be sensitive enough to determine drug levels approximately one-tenth to one-twentieth that of the peak level.

Measurement of intact drug in the urine has certain advantages. The method is model independent as long as the drug does not exhibit nonlinear pharmacokinetics; samples are easier and less costly to obtain; urinary excretion data are more meaningful in instances where drugs are used to treat urinary tract infections or where there is no correlation between blood levels and pharmacological activity; and the only major assumption is that the average fraction of drug reaching the circulation that is excreted in the urine for a given panel is the same for two or more treatments.

The most serious disadvantage of using urinary excretion data for estimating the extent of bioavailability is that urine samples should be collected for at least seven to 10 half-lives of the drug (1). This requirement can be quite a problem with drugs having relatively long half-lives. This report presents two methods for analyzing urinary excretion data which demonstrate that, provided care is taken during the sampling procedure, the extent of bioavailability can be estimated with urine samples taken over 12 hr starting at one or two half-lives of a drug.

THEORY

When the absorption phase for drugs conforming to the one-compartment open model and both the absorptive and distributive phases for the two-compartment open model have been completed, the equations (2) describing the cumulative amount of drug excreted in the urine as a function of time for the one-compartment open model become:

$$U = U_\infty - \frac{U_\infty k_a}{k_a - k_e} e^{-k_e t} = U_\infty - P_1 e^{-k_e t} \qquad \text{(Eq. 1)}$$

and for the two-compartment open model:

$$U = U_\infty - \frac{U_\infty(k_{tc} - \beta)}{\beta(k_a - \beta)(\alpha - \beta)} e^{-\beta t} \doteq U_\infty - P_2 e^{-\beta t} \qquad \text{(Eq. 2)}$$

in which U is the cumulative amount of drug excreted unchanged in the urine up to time t, U_∞ is the total amount of drug excreted unchanged in the urine, k_a is the absorption rate constant, k_e is the overall elimination rate constant, k_{tc} is the rate constant for the transfer of drug from the tissue to the central compartment, and both α and β are the usual collections of constants representing the distributive and elimination phases, respectively.

In the interest of brevity, since both Eqs. 1 and 2 have the same general form, all further developments will be given using Eq. 1 as the example. If urine samples are collected at uniform time intervals, Δ, the cumulative amount, U', of drug excreted up to time $t + \Delta$ is:

$$U' = U_\infty - P_1 e^{-k_e(t+\Delta)} \qquad \text{(Eq. 3)}$$

Equations 1 and 3 can be rearranged to:

$$U - U_\infty = -P_1 e^{-k_e t} \qquad \text{(Eq. 4)}$$

and:

$$U' - U_\infty = -P_1 e^{-k_e t} e^{-k_e \Delta} \qquad \text{(Eq. 5)}$$

Dividing Eq. 4 by Eq. 5 and rearranging give (3):

$$U' = U(e^{-k_e \Delta}) + U_\infty(1 - e^{-k_e \Delta}) \qquad \text{(Eq. 6)}$$

Thus, a plot of U' *versus* U should give a straight line with a slope equal to $e^{-k_e\Delta}$ and an intercept equal to $U_\infty(1 - e^{-k_e\Delta})$. The intercept divided by 1 minus the slope yields U_∞, and the natural logarithm of the slope divided by the collection time interval (Δ) yields $-k_e$. The final equation for the two-compartment open model is the same as Eq. 6, with the exception that β replaces k_e.

RESULTS

To determine whether the equations describing the cumulative amount of drug excreted in the urine as a function of time would simplify to the forms given by Eqs. 1 and 2 within a reasonable period of time, data were simulated for both pharmacokinetic models. For the one-compartment open model, the fraction of the dose (400 mg) absorbed was assumed to be 0.80, the fraction excreted in the urine unchanged was 0.65, and the volume of distribution was 10 liters.

Data were generated for drugs having half-lives ($T_{1/2}$) of 12, 18, 24, and 48 hr. In each instance, the absorption rate constant was varied, being equal to 2, 5, 10, and 20 times the elimination rate constant, respectively. Constants for the two-compartment open

Table I—Percent of Actual Bioavailability from Data Evaluated over a 12-hr Period Starting at n Half-Lives Using Eq. 6

	R^a			
n	20	10	5	2
1	100.0	100.1	102.7	141.0
2	100.0	100.0	100.1	104.4
3	100.0	100.0	100.0	100.8

[a] R is the ratio of the absorption rate constant to the elimination rate constant.

model were taken from the literature for nortriptyline (4), having a $T_{1/2}$ of approximately 34 hr, assuming that 80% of a given dose would be absorbed and 65% of the dose would be excreted in the urine unchanged.

The data for all simulations were evaluated over 12 or 16 hr starting at one, two, and three half-lives. Thus, when $T_{1/2}$ was equal to 12 hr, the cumulative amount of drug excreted up to 14 hr was plotted *versus* the cumulative amount excreted up to 12 hr, the amount excreted up to 16 hr was plotted *versus* the amount excreted up to 14 hr, and the amount excreted up to 18 hr was plotted *versus* the amount excreted up to 16 hr. This procedure was continued for a 12-hr interval if the collection interval (Δ) was equal to 2 hr; it was continued for a 16-hr interval if the collection interval was equal to 4 hr. The procedure was repeated starting at 24 hr and again at 36 hr.

Except for round-off error in the calculations (percent relative bioavailability was calculated to three significant figures), all data for the one-compartment open model yielded the same results regardless of the $T_{1/2}$ or the collection interval. Therefore, the results obtained for the drug with $T_{1/2}$ equal to 12 hr and a collection interval of 2 hr are representative of the results obtained in the entire study (Table I). It can be seen from Table I that acceptable estimates of bioavailability should be obtainable from urine collections starting at approximately one half-life if the absorption rate constant is equal to at least five times the elimination rate constant. Acceptable values can be obtained after two half-lives if the absorption rate constant is at least equal to twice the elimination rate constant. The data for the two-compartment open model ($T_{1/2}$ = 34 hr) were evaluated over the period of 68–80 hr with a constant time interval of 2 hr. The bioavailability was estimated to within 99.9% of the correct value.

Unfortunately, studies involving the urinary excretion of drugs are not necessarily designed for adequate testing of Eq. 6. In one study (5), urine specimens were collected at constant time intervals starting at approximately two half-lives. The drug, phosphonomycin, has a $T_{1/2}$ of approximately 2.3 hr. The data were evaluated (5) for the pharmacokinetic parameters of a two-compartment open model, and predicted as well as observed cumulative urinary excretion values of the drug were given at various times up to, and including, 36 hr (about 16 half-lives). The data for the 4–8- and the 8–12-hr collection periods were analyzed according to Eq. 6 (Table II). The agreement between U_∞ predicted from Eq. 6 and the actual value is remarkable, considering the fact that only two collection periods could be utilized.

Table II—Evaluation of Literature[a] Data for Phosphonomycin Using Eq. 6

		U_∞, mg	
Subject Number	Eq. 7	Literature Predicted	Literature Actual
9	479.5 (0.99)[b]	479.5 (0.99)	484.3
10	376.1 (1.70)	375.4 (1.51)	369.8
11	460.4 (0.09)	461.4 (0.30)	460.0
12	597.4 (0.20)	598.0 (0.10)	598.6
13	438.4 (0.90)	438.0 (0.99)	442.4
14	407.6 (1.32)	408.2 (1.47)	402.3

[a] Reference 5. [b] The relative error for each prediction is given in parenthesis ($T_{1/2}$ = 2.3 hr).

Since times corresponding to one or two half-lives might not be convenient, a reasonable method would be to give the drug in the morning and start timed urine collections at 24, 48, 72, *etc.*, hr, whichever is equal to at least one half-life. The drug could also be given in the evening and timed urine collections started at 12, 36, 60, *etc.*, hr.

In some instances, it might not be convenient to collect urine specimens at constant time intervals. In such situations, urinary excretion rates can be utilized to predict U_∞. Differentiating Eq. 1 with respect to time gives:

$$dU/dt = \dot{U} = P_1 k_e e^{-k_e t} \qquad \text{(Eq. 7)}$$

Substituting the value for $P_1 e^{-k_e t}$ from Eq. 1 into Eq. 7 and rearranging give:

$$U = U_\infty - (\dot{U}/k_e) \qquad \text{(Eq. 8)}$$

A plot of U *versus* $\dot{U}$ should be linear with the intercept equal to U_∞. Equation 2 can be developed in a similar manner to yield an equation resembling Eq. 8, with the exception that β would replace k_e. All data were analyzed according to Eq. 8 with results identical to those already shown, within round-off error.

SUMMARY

Although technical problems do exist, urine specimens can be collected at short time intervals during the initial phase of a bioavailability study and the data can be analyzed (6) to yield an estimate of the absorption rate constant. Specimens can then be collected at times convenient to each test subject. At 24, 48, *etc.*, hr, whichever is closest to one or two half-lives (depending upon the estimated ratio of the absorption rate constant to the elimination rate constant), all initial samples should be pooled. Samples should then be collected at exactly 2-hr intervals for 12 hr. The data for the 12-hr period should then be analyzed utilizing Eq. 6. If the collection intervals cannot be kept constant during the 12 hr, the data should be analyzed according to Eq. 8.

The data in Tables I and II indicate that the methods presented are valid. Whether useful estimates of bioavailability are obtained in practice will depend upon the care with which the data are collected. The methods presented probably are more sensitive to incomplete bladder emptying than the method in which specimens are collected over seven to 10 half-lives of a drug. However, forcing fluids should dilute the urine enough that the magnitude of this problem can be reduced. Patient variability in bladder emptying can be further reduced through the use of a crossover study. Finally, shortening the collection period from seven to 10 half-lives to approximately one or two half-lives should help with subject compliance with the study protocol.

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In Vitro and *In Vivo* Availability of Commercial Prednisone Tablets

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Abstract □ A three-way crossover bioavailability study was performed using nine adult male volunteers with three different commercial prednisone tablets. Plasma samples were assayed for prednisolone, the active metabolite of prednisone, by a radioimmunoassay method. Statistical analysis showed significant differences in the rate of appearance of prednisolone in plasma but not in the amount converted to prednisolone. The results suggest that differences in *in vivo* rates of appearance of prednisolone in plasma correlate with *in vitro* rates of dissolution.

Keyphrases □ Prednisone—*in vitro* and *in vivo* bioavailability of commercial tablets □ Bioavailability—commercial prednisone tablets

Previous studies (1, 2) demonstrated that the clinical effectiveness of prednisone tablets can be correlated with *in vitro* dissolution rates. Two generic prednisone tablets that were inactive clinically dissolved much slower than a clinically active brand of prednisone tablet. Based at least in part on this discovery, USP XVIII included a dissolution test for prednisone tablets (3).

It is well documented that orally administered prednisone is rapidly metabolized to prednisolone, which is believed to be the bioactive form (4–6). With the development of a radioimmunoassay for prednisolone (6), it is now possible to measure the plasma levels of prednisolone following oral administration of single, low doses of prednisone.

This paper reports the results of a three-way crossover bioavailability study with commercially available prednisone tablets.

EXPERIMENTAL

The *in vitro* dissolution studies and content uniformity tests were performed in the laboratories of the Food and Drug Administration (FDA) according to USP XVIII specifications.

A three-way crossover bioavailability study was performed using nine healthy adult male volunteers between the ages of 24 and 31 years and weighing between 67.6 and 93.4 kg. The criteria for subject selection were previously reported (7). In each phase of the crossover study, each subject ingested 10 mg of prednisone as two 5-mg tablets of Tablet A, B, or C[1]. The treatment schedule is shown in Table I. Treatments were separated by 1 week.

The evening before each treatment period, 1.0 mg of dexamethasone was administered orally to each subject to suppress endogenous secretion of cortisol. The suppression was maintained by administration of 0.5 mg of dexamethasone 8 hr after the administration of prednisone.

Whole blood samples were taken from a forearm vein at 0, 0.25, 0.5, 1, 2, 3, 4, 6, 8, 12, and 24 hr postdosing with prednisone. Each sample was centrifuged as soon as possible after collection; the plasma was placed in a stoppered vial, quick-frozen, and kept frozen until just prior to assay.

[1] Tablet A was Meticorten, 5 mg (Schering Corp.), Lot No. 2ABB804; Tablet B was prednisone, 5 mg (Marshall Pharmacal Corp., South Hackensack, N.J.), Lot No. 7165; and Tablet C was prednisone, 5 mg (Heather Drug Co., Cherry Hill, N.J.), Lot No. 090156.

Table I—Treatment Schedule

Subjects	Group	Phase I (Week 1)	Phase II (Week 2)	Phase III (Week 3)
1, 2, 3	I	A	B	C
4, 5, 6	II	B	C	A
7, 8, 9	III	C	A	B

Table II—Results of USP Tests on Tablets Used in Human Study

Tablet	Content Uniformity, % of Label/Tablet		Dissolution Test, % Dissolved/20 min	
A	Range 91.3–100.7, average 96.8		110.8	116.4
			111.6	117.8
			115.0	114.3
			108.0	111.4
			108.8	111.4
			110.6	112.2
			Average	112.4[a]
			SD[b]	2.96
B	90.8	87.4	15.9[c]	10.5[c]
	92.3	84.8	12.2[c]	7.6[c]
	88.4	92.1	15.3[c]	12.4[c]
	121.7[c]	95.4		
	93.5	96.6	Average	12.3
			SD	3.08
	Average	94.2		
	SD	10.3		
C	99.7	105.5	8.9[c]	12.6[c]
	98.6	100.2	10.5[c]	14.0[c]
	101.9	100.0	13.0[c]	9.5[c]
	99.0	97.6		
	96.2	96.7	Average	11.4
			SD	2.07
	Average	99.6		
	SD	2.78		

[a] The higher than label values are most probably caused by nonsteroidal components which absorb at the wavelength used in the tablet dissolution test. [b] Standard deviation. [c] Not in compliance with USP specifications.

All plasma samples were assayed for prednisolone using a modification of the procedure of Colburn and Buller (6), described previously (7).

All calibration curves were fitted essentially perfectly to a biexponential equation using the program NONLIN and a computer[2]. The amount of drug in the experimental plasma samples was then determined with an iterative program using an electronic calculator[3].

RESULTS AND DISCUSSION

In Vitro—Results of the USP content uniformity and tablet dissolution tests are summarized in Table II. Tablet A passed both the content uniformity and the tablet dissolution tests as well as the assay test (101.0% of label). Tablets B and C failed the tablet dissolution test since they gave values of 12.3 and 11.4% dissolved, respectively; the USP requires at least 60% of the labeled amount of prednisone to be dissolved in 20 min.

[2] IBM 360/67.
[3] Hewlett-Packard 9100.

Table III—Results of Nine Subject Three-Way Crossover Prednisone Study of Prednisolone Plasma Concentrations

Hours	Average Plasma Concentrations, ng/ml[a] Tablet A	Tablet B	Tablet C	Significance Level[b]	Tukey's Multiple Comparison Test at $p = 0.05$ Level[c] Tablet A *versus* B	Tablet A *versus* C
0.25	76.9 (110)	5.67 (161)	9.89 (225)	$0.01 < p < 0.025$	Sig.	Sig.
0.50	198 (63.3)	27.7 (63.4)	69.1 (60.0)	$p < 0.001$	Sig.	Sig.
1	254 (31.4)	111 (30.2)	185 (41.2)	$0.001 < p < 0.005$	Sig.	N.S.
2	250 (16.0)	164 (21.3)	228 (12.3)	$p < 0.001$	Sig.	N.S.
3	202 (18.3)	184 (15.7)	240 (14.9)	$0.001 < p < 0.005$	N.S.	Sig.
4	154 (14.9)	178 (15.7)	190 (18.6)	$0.01 < p < 0.025$	N.S.	Sig.
6	83.2 (30.5)	103 (19.2)	105 (39.9)	N.S. $(0.10 < p < 0.25)$	N.S.	N.S.
8	39.6 (33.2)	48.3 (36.2)	48.6 (49.2)	N.S. $(p > 0.25)$	N.S.	N.S.
12	19.3 (30.6)	20.0 (59.2)	22.6 (61.0)	N.S. $(p > 0.25)$	N.S.	N.S.
24	3.46 (178)	10.7 (162)	4.23 (156)	N.S. $(p > 0.25)$	N.S.	N.S.

[a] Averages are followed by percent coefficient of variation (standard deviation/mean × 100) in parenthesis. [b] Based on treatment mean square from the analysis of variance for crossover design. [c] Sig. = significant, and N.S. = not significant.

Table IV—Summary of Results of Nine Subject Three-Way Crossover Prednisone Study—Some Pharmacokinetic Parameters

Parameter	Parameter Value[a] Tablet A	Tablet B	Tablet C	Significance Level[b]	Tukey's Multiple Comparison Test at $p = 0.05$ Level[c] Tablet A *versus* B	Tablet A *versus* C
Area 0→12 hr, ng/ml × hr	1290 (15.9)	1100 (15.4)	1320 (22.2)	N.S. $(0.05 < p < 0.10)$	N.S.	N.S.
ln (area 0→12)	7.15	6.99	7.16	N.S. $(0.10 < p < 0.20)$		
Area 0→24 hr, ng/ml × hr	1430 (16.7)	1290 (20.2)	1430 (21.5)	N.S. $(p > 0.25)$	N.S.	N.S.
Peak concentration[d], ng/ml	285 (19.4)	192 (15.1)	255 (12.9)	$p < 0.001$	Sig.	N.S.
Peak time, hr[d]	1.28	3.00	2.11	$p < 0.001$	Sig.	Sig.
Half-life, hr	2.58 (20.2)	2.43 (25.7)	2.38 (27.6)	N.S. $(p > 0.25)$	N.S.	N.S.
Area $(0\rightarrow\infty)$/ mg/kg dose × half-life	4190	3860	4620	N.S. $(p > 0.25)$		

[a,b,c] See footnotes to Table III. [d] From observed peak plasma levels of individual subjects.

Tablet B failed the initial phase of the content uniformity test since one of the 10 tablets tested was out of compliance, but the results of the second phase of this test were not available. Tablet C passed the content uniformity test. These results were of interest because all three lots of tablets were purchased on the open market by the FDA.

In Vivo—One lot of generic tablets (Tablet B or C) had a history of clinical failure, and the following is a statement written by the FDA concerning this lot (designated 078-692F by the FDA):

> *The FDA documented therapeutic failures with the prednisone tablets in two patients afflicted with congenital adrenal hyperplasia in December, 1971. Immediate follow-up by FDA's Newark District Office ascertained that the subject lot of tablets failed the USP dissolution test. Further investigation by the FDA revealed that the product was no longer in the channels of distribution and thus was not available for recall or seizure. A "Consent Decree of Permanent Injunction" was signed by the firm on May 1, 1972 in the U.S. District Court of New Jersey which stipulated that the firm will not manufacture and introduce products into interstate commerce which fail to meet appropriate standards.*

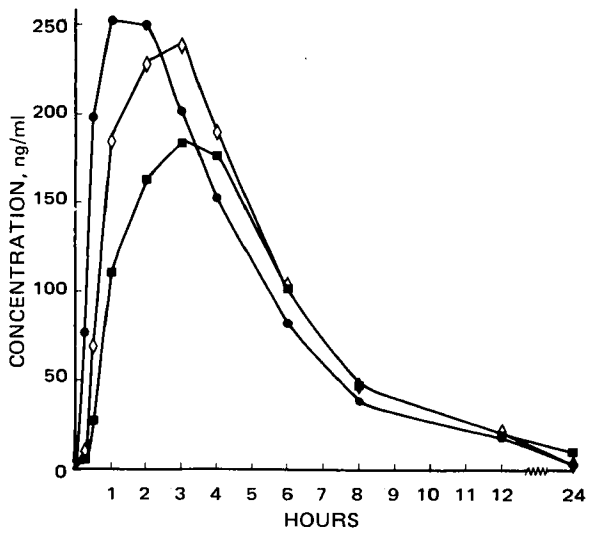

Figure 1—*Average plasma concentrations of prednisolone following oral administration of prednisone as Tablet A (●), Tablet B (■), or Tablet C (◊).*

In the human bioavailability study, it was important to consider the cross-reactivity of prednisolone and cortisol with the antiserum (7). To obtain a specific assay for prednisolone, it was necessary to suppress endogenous secretion of cortisol. This suppression was accomplished by the administration of dexamethasone (8). Any effect of dexamethasone on the conversion of prednisone to prednisolone is unknown, but it would be expected to be minimal and unimportant under the study conditions because of the time interval between doses and the relative magnitudes of the doses.

Dexamethasone, even in high concentrations, does not interfere with the binding of prednisolone to the antibody. Prednisone has also been shown not to interact significantly with the antibody (6).

Tables III and IV summarize the results of the bioavailability study and the statistical analysis of the data. It is apparent from Table III that there are significant differences in the rates of appearance of prednisolone in plasma. Analyses of variance of plas-

ma concentrations indicated highly significant differences among average plasma concentrations from 0.25 to 4 hr, inclusive. Tukey's Multiple Comparison Test (9) was used to identify significant differences between treatment pairs. The test indicated significant differences during the absorptive phase when Tablet A was compared to either Tablet B or C. The particular patterns of significant differences between treatments can be rationalized by considering the average plasma level curves for each (Fig. 1).

Differences in peak plasma concentrations and time of the peak were also seen (Table IV). The average peaks and rates of appearance were in the order: A > B or C. Since Tablet A was the innovator's product and considered the "standard" preparation, comparisons of only Tablet A *versus* B and Tablet A *versus* C were made. No statistical comparison of Tablet B *versus* C should be inferred (Tables III and IV).

After the 4-hr sampling time, the differences among average plasma prednisolone concentrations were not significant ($p > 0.10$). At the 24-hr sampling time, 11 out of the total 27 samples produced measurable levels of steroid, which in some cases were as high or higher than those of the 12-hr sample. Ten of these incidents occurred within the same four subjects. It seems reasonable to conclude that these levels may be due to the recovery of normal adrenal function rather than to the presence of prednisolone in the sample.

There were no significant differences among treatment average areas, either 0–12 hr or 0–24 hr (Table IV). This finding suggested that the efficiencies of absorption of prednisone, as indicated by the presence of prednisolone, did not differ significantly among treatments. The logarithm of area from 0 to 12 hr and the ratio of area divided by the dose in milligrams per kilogram times the half-life were investigated as additional measures of efficiencies of absorption, but the differences among these average parameters were not significant. The rationale for analyzing the logarithm of area was proposed by Westlake (10).

The differences among mean half-lives of prednisolone were not significant. The half-life ranged from 1.51 to 3.65 hr. The overall average half-life was 2.47 hr. None of the 24-hr points was used to calculate the half-life; the 4–12-hr points generally gave a straight line on semilogarithmic paper. The half-lives estimated are similar to those reported when prednisolone itself was administered orally (7).

CONCLUSIONS

The data presented indicate that prednisone Tablets A, B, and C differ only in the rate of appearance of prednisolone in the plasma and not in the amount of prednisone that reaches the circulation. The results document that differences in *in vivo* rates of appearance of prednisolone in humans correlate with *in vitro* rates of dissolution of prednisone and with a reported clinical failure in the use of prednisone.

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Intestinal pH as a Factor in Selection of Animal Models for Bioavailability Testing

WILLIAM G. CROUTHAMEL[x], CRAIG R. ABOLIN*, JOAN HSIEH, and JAMES K. LIM

Abstract □ Previous *in situ* studies showed that intestinal drug absorption is strongly influenced by intestinal pH. Three animal species considered for bioavailability testing were studied *in situ* to determine intestinal pH. Results from the rat and dog correlated well with results in humans, while results from the rabbit did not. The rabbit appears to be a poor candidate for attempted animal–human bioavailability correlations.

Keyphrases □ Intestinal pH, rats, dogs, and rabbits—*in situ* determination, factor in selection of animal model for bioavailability testing □ Bioavailability correlations, animal–human—intestinal pH of rats, dogs, and rabbits as a factor in model selection □ Animal models for bioavailability testing—intestinal pH as factor for model selection, *in situ* determination in rats, dogs, and rabbits

New drug entities are routinely tested in animal models. Following initial and toxicological tests, candidate dosage forms are frequently tested in animals to determine the best dosage forms for further clinical testing. Although it is recognized that there are many differences between animals and humans and between animal species, little information is available concerning differences in absorption between these groups. With the recent increase in emphasis on bioavailability testing, the search for better animal models for these tests has intensified.

The Food and Drug Administration (1) recently suggested that animal models be developed that correlate bioavailability in animals with similar results in humans. However, before this plan can be accomplished on a widespread basis, more information must be available on the similarities and dissimilarities of these animal models. Dogs have occasionally been used in these studies, but other animals have also been suggested (2).

Previous studies (3) in animals showed that the absorption of drugs from the intestine is highly dependent on intestinal pH. This study was undertaken to determine intestinal pH in several animal species suggested for bioavailability studies.

EXPERIMENTAL

The *in situ* method of Doluisio *et al.* (4) was used in these experiments. Briefly, in each animal model the intestine was exposed by midline incision. The intestinal section to be studied was prepared by making a small slit in the intestinal wall at each end of the segment, inserting a small polyethylene cannula in each slit, and ligating these cannulas into place to form a cannulated intestinal segment. Care was taken to keep trauma to the intestine to a minimum and to maintain an intact blood supply.

A plastic syringe barrel was then attached to the exposed end of each cannula, and the intestinal solution was placed in the intestinal loop. The intestinal solutions used were isotonic saline in the rat, isotonic phosphate buffer (0.055 *M*) in the rabbit, and isotonic phosphate buffer (0.095 *M*) in the dog. All solutions were checked for isotonicity and adjusted to pH 6.0 prior to instillation. At time intervals of 10–20 min, the solution was gently forced into one syringe barrel by air pressure from the other, and the pH was determined by a micro-pH electrode. The solution was then returned to the intestine.

This procedure ensured that the contents of the intestine were thoroughly mixed during the experiment. Although the solutions used differed in buffer capacity, the intestine has a large intrinsic ability to buffer its contents. The equilibrium pH values reported in Table I were recorded at 50, 60, and 100 min in the dog, rat, and rabbit, respectively.

In the rat studies, the duodenal section consisted of approximately 10 cm of duodenum measured from the pylorus to 4 cm distal to the ligament of Trietz. The bile duct was either left intact (normal bile) or ligated (no bile). The rat upper jejunal section consisted of 45 cm of jejunum beginning 4 cm distal to the ligament of Trietz, and the lower jejunal section consisted of an equal length of jejunum measured proximally beginning 5 cm proximal to the cecum.

In the rabbit studies, the duodenal section consisted of 25 cm of duodenum measured distally beginning 1 cm below the bile duct, and the jejunal section consisted of an equal length measured distally beginning 45 cm below the pylorus. In the dog experiments, the jejunal section consisted of 26 cm of jejunum measured distally beginning 26 cm below the ligament of Trietz.

Although the upper jejunal sections used are not exactly comparable in the three species due to experimental limitations, intestinal pH in the dog upper jejunum was found constant in four different sections studied.

RESULTS AND DISCUSSION

The pH values reported in Table I indicate that there is little difference in pH between the duodenum and upper jejunum and between the presence and absence of bile flow in the rat. In the rabbit, duodenal and jejunal pH values are also quite similar but are considerably higher than those found in either the rat or dog. Human intestinal pH values in normal subjects are reported to be (5): duodenum, 4.7–6.5, upper jejunum, 6.2–6.7, and lower jejunum, 6.2–7.3. Thus, pH values reported in humans correspond well with those found in the rat and dog but not with those found in the rabbit. Based on pH considerations alone, the rabbit does not appear to be a good animal model for attempting human–animal correlations.

The pH values reported here are equilibrium values in the bulk intraluminal fluid and may not necessarily be representative of the pH found in the "unstirred layer," the thin aqueous film located directly next to the absorbing membrane (6). However, previous studies (3) in the *in situ* model showed a good correlation between the rate of drug absorption and intraluminal pH over a wide pH range. Therefore, it is not unreasonable to speculate that drug ab-

Table I—Intestinal pH Values *In Situ*

Intestinal Section	pH Values, Mean ± *SD*		
	Rat	Rabbit	Dog
Duodenum (no bile flow)	6.6 ± 0.2 (6)[a]	7.8 ± 0.1 (15)	—
Duodenum (normal bile flow)	6.5 ± 0.3 (6)	—	—
Upper jejunum	6.4 ± 0.1 (4)[b]	7.5 ± 0.1 (19)	6.2 ± 0.1 (10)
Lower jejunum	7.2 ± 0.1 (5)	—	—

[a] Number in parentheses indicates number of experiments. [b] At 120 min.

sorption may be influenced by the differences in pH described here.

Under any circumstances, the intraluminal pH will have an important effect on the disintegration and dissolution characteristics of dosage forms administered to intact animals. This is particularly true of special dosage forms such as suspensions, coated tablets, and timed-release products where pH is an inherent part of the product design. Just as differences in biliary recycling between species can influence the pharmacokinetics of drugs administered in intact animals, intraspecies differences in intestinal pH can also be expected to influence the site and extent of absorption as well as the intestinal contribution to the volume of distribution (7).

When selecting an animal model for pharmacokinetic or bioavailability studies, intestinal pH should be considered as a potential determinant of the outcome. Of course, some drugs are potentially too toxic (*e.g.*, methotrexate) to be tested for bioavailability in normal human subjects, so for these drugs the development of suitable animal models is especially important.

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COMMUNICATIONS

Effect of an Antacid on Absorption of Digoxin in Dogs

Keyphrases □ Digoxin—effect of antacids on absorption, dogs □ Absorption—digoxin, effect of antacid, dogs □ Antacids—effect on absorption of digoxin, dogs

To the Editor:

Recently, Khalil demonstrated that the *in vitro* dissolution of digoxin tablets[1] was suppressed by a commercial antacid containing aluminum hydroxide (0.31 g/5 ml) and magnesium trisilicate (0.60 g/5 ml). The present study examined the effect of this commercial antacid[2] on the absorption of digoxin in dogs after oral administration.

Following overnight fasting, each of four mongrel dogs was given either two digoxin tablets[3] (1.0 mg) or 20 ml of the commercial antacid plus two tablets at weekly intervals in accordance with a crossover design. The antacid was given by stomach intubation, and 50 ml of water was given to each dog following administration of the dosage formulation.

Blood (4 ml) was withdrawn from the radial vein at 0, 0.5, 1, 1.5, 2, 2.5, 3, 4, 7, and 24 hr following drug administration (Table I). The plasma samples were analyzed using a commercial digoxin assay kit[4]. A standard curve was obtained for each animal using its blank plasma.

Analysis of variance on each data point indicated that there was no significant difference ($p \leq 0.01$) between concentrations at any time point following the

Table I—Mean Plasma Digoxin Concentrations (Nanograms per Milliliter) following Digoxin or Commercial Antacid plus Digoxin Administrations

	0.5 hr	1.0 hr	1.5 hr	2.0 hr	2.5 hr	3.0 hr	4.0 hr	7.0 hr	24.0 hr
				Antacid plus Digoxin Tablets					
Mean	2.13	6.43	6.52	5.81	5.25	5.45	5.00	2.93	0.99
SD	3.14	2.43	1.75	1.83	1.59	2.57	1.38	0.82	0.10
				Digoxin Tablets					
Mean	1.51	4.23	5.33	6.64	5.50	5.24	4.98	3.52	1.26
SD	2.03	2.59	3.45	1.82	2.07	2.54	1.69	0.76	0.39

[1] Lanoxin tablets BP.

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crossover administrations. The results of this study ran contrary to a possible implication (*i.e.*, impairment of digoxin bioavailability by the antacid) of Khalil's *in vitro* findings in which the dissolution of digoxin tablets was virtually completely suppressed by the antacid (1).

Further experiments in humans are necessary to substantiate these findings in dogs.

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Blood Levels from a Sustained-Release Dosage Form

Keyphrases □ Dosage forms—sustained release, blood levels, fast and slow release drug components, equations □ Timed-release dosage forms—blood levels, fast and slow release components, equations □ Blood levels—sustained-release dosage form, fast and slow release components, equations

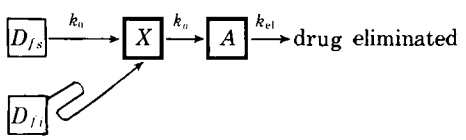
To the Editor:

The ideal oral dosage form for obtaining a desired plateau level of drug rapidly in the body is one that releases part of the dose instantaneously and the balance by a slow zero-order process. Pharmacokinetic approaches to calculate the desired proportion of fast and zero-order release components have been described (1, 2).

One method (1) gives the desired steady-state drug level at the peak time, t_p, for the fast release component and also later during the sustained-release period. Between these times, however, drug levels tend to be higher, producing a hump in the overall blood drug level *versus* time profile.

Another method (2) provides a constant plateau level of drug C_{ss}, which is reached slightly later than time t_p. The constancy of the drug levels and the somewhat simpler calculations probably make it the method of choice in most situations.

The objective of this communication is to describe a method of calculating fast and slow release drug components. While similar to the method of Robinson and Eriksen (2), it facilitates more rapid calculation of the drug fractions and permits considerable simplification of the rather lengthy equations used



Scheme I

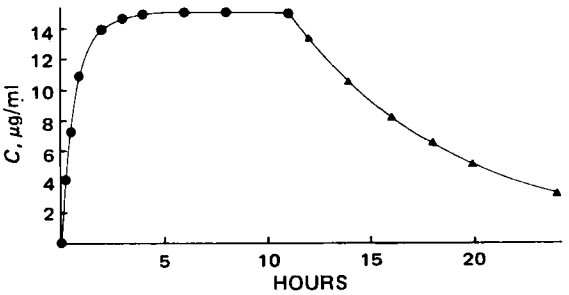


Figure 1—*Simulated blood level curve using data from Table I and Eqs. 6 (●) and 7 (▲).*

hitherto to describe this model. The overall process is described in Scheme I.

In the model, D_{fs} and D_{fi} are the sustained-release and fast release components of the total dose D, respectively; X is the amount of drug in solution and available for absorption in the gut; A is the amount of drug distributed in a single homogeneous volume, V, in the body to give a concentration, C; k_0 is the zero-order rate constant for release of D_{fs}; and k_a and k_{el} are first-order rate constants for drug absorption into the body and elimination by all routes, respectively. The crooked arrow represents instantaneous release of D_{fi} into X.

The assumptions in this model are as stated previously (1): all drug in the body is homogeneously distributed in one apparent volume, V, and the rate of drug absorption is invariant throughout the GI tract. The sustained-release component, D_{fs}, releases drug from time zero to time T, so $D_{fs} = k_0 T$ and both D_{fi} and D_{fs} contribute to the blood level beginning at time zero.

Integrated expressions to describe the amounts of drug D_{fs}, X, and A at any time during drug release are given by Eqs. 1–3:

$$D_{fs}(t) = D_{fs} - k_0 t \qquad \text{(Eq. 1)}$$

$$X = \frac{k_0}{k_a}[1 - e^{-k_a t}] + D_{fi}e^{-k_a t} \qquad \text{(Eq. 2)}$$

$$A = \frac{k_0}{k_{el}}(1 - e^{-k_{el}t}) + \left(\frac{k_a D_{fi} - k_0}{k_{el} - k_a}\right)(e^{-k_a t} - e^{-k_{el}t}) \qquad \text{(Eq. 3)}$$

To calculate D_{fi}, Rowland and Beckett (1) set A equal to A_{ss} at the single time t_p. This method, as previously stated, results in a hump in the drug blood level profile for an intermediate time following t_p. An alternative approach is based on k_0 being equal to $(k_{el})(A)$ at the steady state. Then substituting for k_0 in Eq. 3 and rearranging give Eq. 4, where $A = A_{ss}$:

$$D_{fi} = \left(\frac{1}{k_a} + \frac{(k_{el} - k_a)e^{-k_{el}t}}{k_{el}k_a(e^{-k_a t} - e^{-k_{el}t})}\right)(k_{el})(A_{ss}) \qquad \text{(Eq. 4)}$$

To avoid a hump in the blood level profile, A must be maintained at A_{ss}, which requires that Eq. 4 be time independent. Since $k_a > k_{el}$ in the usual case, time independency occurs only when $e^{-k_a t}$ becomes insignificant relative to $e^{-k_{el}t}$. Then Eq. 4 simplifies to:

$$D_{fi} = A_{ss} = \frac{k_0}{k_{el}} \qquad \text{(Eq. 5)}$$

crossover administrations. The results of this study ran contrary to a possible implication (*i.e.*, impairment of digoxin bioavailability by the antacid) of Khalil's *in vitro* findings in which the dissolution of digoxin tablets was virtually completely suppressed by the antacid (1).

Further experiments in humans are necessary to substantiate these findings in dogs.

(1) S. A. H. Khalil, *J. Pharm. Pharmacol.*, **26**, 961(1974).

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Blood Levels from a Sustained-Release Dosage Form

Keyphrases □ Dosage forms—sustained release, blood levels, fast and slow release drug components, equations □ Timed-release dosage forms—blood levels, fast and slow release components, equations □ Blood levels—sustained-release dosage form, fast and slow release components, equations

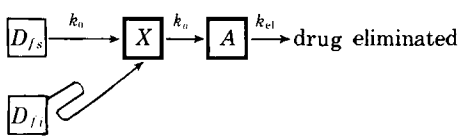
To the Editor:

The ideal oral dosage form for obtaining a desired plateau level of drug rapidly in the body is one that releases part of the dose instantaneously and the balance by a slow zero-order process. Pharmacokinetic approaches to calculate the desired proportion of fast and zero-order release components have been described (1, 2).

One method (1) gives the desired steady-state drug level at the peak time, t_p, for the fast release component and also later during the sustained-release period. Between these times, however, drug levels tend to be higher, producing a hump in the overall blood drug level *versus* time profile.

Another method (2) provides a constant plateau level of drug C_{ss}, which is reached slightly later than time t_p. The constancy of the drug levels and the somewhat simpler calculations probably make it the method of choice in most situations.

The objective of this communication is to describe a method of calculating fast and slow release drug components. While similar to the method of Robinson and Eriksen (2), it facilitates more rapid calculation of the drug fractions and permits considerable simplification of the rather lengthy equations used



Scheme I

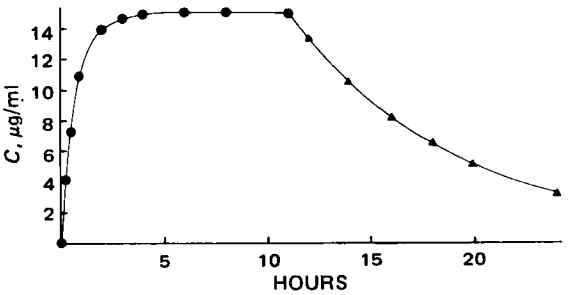


Figure 1—*Simulated blood level curve using data from Table I and Eqs. 6 (●) and 7 (▲).*

hitherto to describe this model. The overall process is described in Scheme I.

In the model, D_{fs} and D_{fi} are the sustained-release and fast release components of the total dose D, respectively; X is the amount of drug in solution and available for absorption in the gut; A is the amount of drug distributed in a single homogeneous volume, V, in the body to give a concentration, C; k_0 is the zero-order rate constant for release of D_{fs}; and k_a and k_{el} are first-order rate constants for drug absorption into the body and elimination by all routes, respectively. The crooked arrow represents instantaneous release of D_{fi} into X.

The assumptions in this model are as stated previously (1): all drug in the body is homogeneously distributed in one apparent volume, V, and the rate of drug absorption is invariant throughout the GI tract. The sustained-release component, D_{fs}, releases drug from time zero to time T, so $D_{fs} = k_0 T$ and both D_{fi} and D_{fs} contribute to the blood level beginning at time zero.

Integrated expressions to describe the amounts of drug D_{fs}, X, and A at any time during drug release are given by Eqs. 1–3:

$$D_{fs}(t) = D_{fs} - k_0 t \qquad \text{(Eq. 1)}$$

$$X = \frac{k_0}{k_a}[1 - e^{-k_a t}] + D_{fi}e^{-k_a t} \qquad \text{(Eq. 2)}$$

$$A = \frac{k_0}{k_{el}}(1 - e^{-k_{el}t}) + \left(\frac{k_a D_{fi} - k_0}{k_{el} - k_a}\right)(e^{-k_a t} - e^{-k_{el}t}) \qquad \text{(Eq. 3)}$$

To calculate D_{fi}, Rowland and Beckett (1) set A equal to A_{ss} at the single time t_p. This method, as previously stated, results in a hump in the drug blood level profile for an intermediate time following t_p. An alternative approach is based on k_0 being equal to $(k_{el})(A)$ at the steady state. Then substituting for k_0 in Eq. 3 and rearranging give Eq. 4, where $A = A_{ss}$:

$$D_{fi} = \left(\frac{1}{k_a} + \frac{(k_{el} - k_a)e^{-k_{el}t}}{k_{el}k_a(e^{-k_a t} - e^{-k_{el}t})}\right)(k_{el})(A_{ss}) \qquad \text{(Eq. 4)}$$

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$$D_{fi} = A_{ss} = \frac{k_0}{k_{el}} \qquad \text{(Eq. 5)}$$

Table I—Calculation of Sustained-Release Parameters for Theophylline

Parameter	Value	Method of Calculation
A_{ss}	480 mg	$A_{ss} = C_{ss}V$
D_{fi}	480 mg	Eq. 5
k_0	57.6 mg/hr	$k_0 = (k_{el})(A_{ss})$
D_{fs}	633.6 mg	$D_{fs} = k_0 T^b$
D^a	1113.6 mg	$D_{fs} + D_{fi}$
D_{fi}/D_{fs}	0.76	

[a] D may be contained in two or more dosage units, provided k_0 is 57.6 mg/hr and D_{fi}/D_{fs} is constant. [b] T = 11 hr.

Substitution from Eq. 5 into Eq. 3 gives Eq. 6*a* or 6*b*:

$$A = D_{fi}[1 - e^{-k_a t}] \qquad \text{(Eq. 6a)}$$

or:

$$C = \frac{A_{ss}}{V}[1 - e^{-k_a t}] \qquad \text{(Eq. 6b)}$$

which provides a simple expression to describe blood levels from this type of formulation for all values of $t \leq T$. After this time, the first-order decline in blood levels is given by:

$$C = C_{ss}e^{-k_{el}(t-T)} \qquad \text{(Eq. 7)}$$

where $t \geq T$.

The value of D_{fi} is independent of k_a and t_p. However, the lag time between t_p and the time taken for A to approach A_{ss} increases as k_a decreases. Since the blood levels of a drug administered as a zero-order release dosage form with no fast release component are given by:

$$C = \frac{k_0}{k_{el}}[1 - e^{-k_{el}t}] + \frac{k_0}{k_a - k_{el}}[e^{-k_a t} - e^{-k_{el}t}] \qquad \text{(Eq. 8)}$$

the reduction in the time taken to reach plateau blood levels with the introduction of a fast release component is clearly a function only of the relative values of k_a and k_{el}.

The method may be demonstrated using theophylline as an example. A recent study in normal human subjects yielded average values for k_a, k_{el}, and V of 1.3 hr^{-1}, 0.12 hr^{-1}, and 32 liters, respectively (3). With the assumption that a desirable therapeutic blood level C_{ss} is 15 μg/ml (4), values for a suitable sustained-release formulation are given in Table I.

The blood level resulting from these calculations is given in Fig. 1. The t_p value resulting from the fast release component is 2.0 hr (5), and the time taken for blood levels to reach 95% of their asymptotic value is only slightly greater at 2.3 hr (Eq. 6*b*).

This approach is not meant to imply that the ideal sustained-release blood profile can be obtained in all patients. Each individual patient will have different rates of absorption and elimination, as exemplified in Ref. 3. However, this method provides a simple approach which is of general application for dosage calculation with this type of sustained-release formulation.

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Effect of Propantheline on Nitrofurantoin Absorption

Keyphrases □ Propantheline—effect on nitrofurantoin absorption □ Nitrofurantoin—absorption, effect of propantheline □ Absorption—nitrofurantoin, effect of propantheline

To the Editor:

Since an adverse reaction to nitrofurantoin therapy is gastric upset, the manufacturers recommend that the drug be given with food or milk to minimize this effect (1). However, concurrent administration of food with nitrofurantoin apparently not only reduces the possibility of GI reaction but also significantly increases the bioavailability of the drug, especially in relation to the macrocrystalline form (2). The increase in absorption has been attributed to food causing an increase in the gastric emptying time, which would also increase the residence time of the drug in the gastric fluids, thereby allowing for a greater amount of nitrofurantoin to be dissolved prior to its passage into the duodenum where absorption is optimal.

Since other studies showed that increases in the serum level of digoxin (3) and the urinary excretion of riboflavin (4) occur when these drugs are administered with propantheline due to its effect of reducing gastric motility, it was felt that concurrent administration of nitrofurantoin with propantheline would be a good means of confirming whether increased gastric emptying time modifies nitrofurantoin absorption, as postulated by Bates *et al.* (2).

Six healthy subjects (three males and three females) were administered a single 100-mg oral dose of nitrofurantoin macrocrystals[1] with 100 ml of water. A crossover design was employed; three subjects received 30 mg of propantheline[2] 45 min prior to nitrofurantoin administration while the other three subjects received only nitrofurantoin. One week later the experimental conditions were re-

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Table I—Cumulative Excretion (Milligrams) of Nitrofurantoin for Six Subjects under Propantheline and Control Conditions

Subject	Control (Nitrofurantoin Only)	Nitrofurantoin and Propantheline
1	17.2	30.2
2	29.0	34.7
3	12.3	19.5
4	15.2	31.8
5	13.0	33.6
6	16.0	22.3
Mean ± *SE*	17.1 ± 2.49	28.7 ± 2.57

versed. All subjects fasted overnight and for 4 hr after drug administration. Total urine specimens were collected for 24 hr. Nitrofurantoin in the urine was determined by a spectrophotometric method (5) specific for unchanged drug.

The results (Table I) indicate a statistically significant increase in nitrofurantoin excretion when propantheline was coadministered as compared with the control condition ($p < 0.01$ as determined by paired t-test). No statistical significance was observed for differences in urinary volume or urinary pH between the two conditions. Thus, the results are consistent with the hypothesis that a delay in gastric emptying possibly will increase the bioavailability of nitrofurantoin and indicate that this effect might explain why food produces similar effects.

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Simple Method for Monitoring Flow Rate during Gravity Perfusion

Keyphrases □ Flow rates—apparatus for continuous monitoring during perfusion studies □ Perfusion studies—apparatus for continuous monitoring of flow rate □ Equipment—apparatus for monitoring flow rate during gravity perfusion

To the Editor:

The apparatus described here fills the need for an inexpensive, easily constructed device for continuously monitoring flow rates during perfusion studies. Although extremely simple in design, it can be employed to advantage with modern electronic recording equipment.

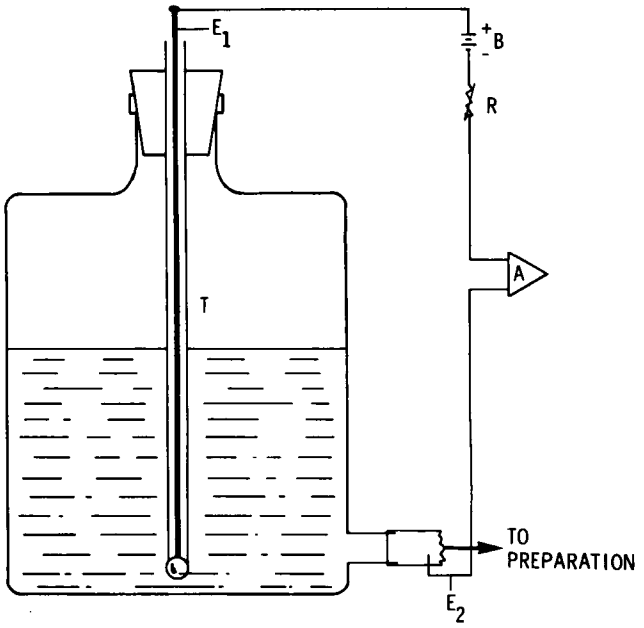

Figure 1—*Components of device for continuously monitoring flow rates during perfusion studies.*

The device makes use of the familiar air bubbles which emerge from the pressure regulating tube (T) of the well-known Marriotte bottle. The last 1 or 2 cm of this tube is coated on both the inner and outer surfaces, most simply by dipping the tubing in melted paraffin. One electrode (E_1)—essentially a length of stainless steel wire insulated except at the tip—is passed down the glass tubing (Fig. 1). A second electrode (E_2), which can be much shorter, is placed in the perfusion solution, usually through a piece of rubber tubing leading from the outlet of the reservoir.

Leads from these two electrodes are connected to the input of the recorder amplifier (A) with an ordinary 1.5-v flashlight battery (B) and variable resistor (R) placed in the circuit as indicated. As the perfusion proceeds, the column of air in the glass tubing (T) descends and "breaks off" in the form of air bubbles. These bubbles serve to break the circuit, and this breakage is registered on the recorder. Changes in flow caused by interventions that alter the perfusion rate are rapidly indicated on the chart. This system avoids the time-consuming process of collecting and measuring the effluent to determine whether a change in flow has actually occurred. The amount of change can thus be measured, either by collecting the effluent or counting the marks on the chart recorder over a specific length of time.

By its nature, a tracing provides a stronger visual impact than does a number, and this advantage is particularly noticeable when other parameters are being recorded simultaneously on the same strip chart. The system has been very valuable also for demonstrations to large classes, using closed circuit T.V. with the camera focused on the chart. The apparatus described here has been employed extensively

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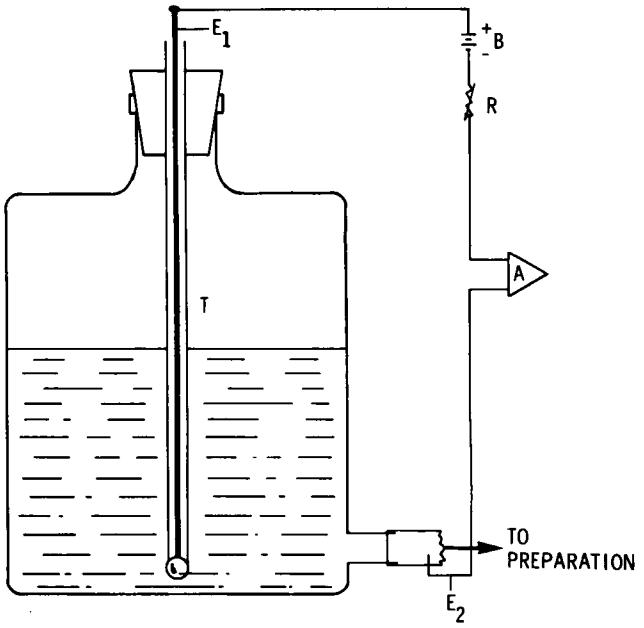

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to study the effects of drugs or procedures on isolated perfused hearts, lungs, and blood vessels. The very small current employed (approximately 0.2 mamp) has no observable effects on either the preparation or the actions of the drugs studied.

In short, this apparatus provides a method for rapidly detecting changes in flow by using the Marriotte bottle to provide graphic information as well as to serve simply as a reservoir for the perfusion solution. Ease of construction and low cost make it particularly useful for teaching purposes, since each group of students can be supplied with one of these devices.

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Controlled Release from Matrix Systems

Keyphrases □ Matrix systems—controlled release of ethynodiol diacetate from silicone rubber devices, comments □ Silicone rubber matrix—release of ethynodiol diacetate, comments on controlled release □ Release, controlled—ethynodiol diacetate from silicone rubber devices, comments

To the Editor:

Two publications by Chien and coworkers (1, 2) described the release of ethynodiol diacetate from silicone rubber devices. These authors treated two cases for the matrix release process: matrix-controlled and partition-controlled drug release mechanisms. The equations describing the diffusional process were previously presented by Higuchi (3) and Roseman and Higuchi (4). With the assumptions that (*a*) the matrix acts as the diffusion medium, (*b*) a pseudo-steady-state condition exists during the release process, and (*c*) the drug particles are uniformly distributed throughout the matrix and are quite small relative to the average distance of diffusion, Higuchi (3) derived the following relationship for the release of drug from a planar homogeneous matrix:

$$Q = [D_s C_s (2A - C_s)t]^{1/2} \quad \text{(Eq. 1)}$$

where Q = amount of drug released per unit area, D_s = diffusion coefficient of drug in the homogeneous matrix phase, C_s = solubility of drug in the matrix phase, t = time, and A = total amount of drug present per unit volume of matrix.

When $A \gg C_s$, Eq. 1 reduces to:

$$Q = (2AD_s C_s t)^{1/2} \quad \text{(Eq. 2)}$$

Chien *et al.* (1), however, incorrectly quoted the Higuchi equation as:

$$Q = [D_s C_s (2A - C_a)t]^{1/2} \quad \text{(Eq. 3)}$$

where C_a is the solubility of drug in the elution medium[1]. Although the $(2A - C_a)$ term does not appear to have any physical significance (except, of course, when $C_a = C_s$), Eq. 3 does yield Eq. 2 when $2A$ is much greater than C_a.

The equations derived by Roseman and Higuchi (4) are an extension of the concepts discussed by Higuchi (3) for the specific case where diffusion from the surface of the device is considered in series with the diffusional step through the matrix. In this instance, the release of drug from a planar matrix is given by the following expressions, when $A \gg C_s$:

$$Q = Al \quad \text{(Eq. 4)}$$

$$l^2 + \frac{2D_e h_a l}{D_a K} = \frac{2D_e C_s t}{A} \quad \text{(Eq. 5)}$$

where[1] l = diffusional distance in the matrix (depleted zone); K = partition coefficient (C_a/C_s); h_a = diffusional distance in the boundary diffusion layer; $D_e = D_s\epsilon/\tau$ where ϵ and τ are the volume fraction and tortuosity of the matrix,respectively; and D_a = diffusion coefficient of drug in the elution medium. The other terms were defined previously.

Except for consideration of the boundary diffusion layer, the basic assumptions in the derivation of these equations are the same as those used to derive Eq. 2. Equations 4 and 5 describe a general case for matrix release, which was termed the matrix-boundary diffusion layer model (4). This nomenclature corresponds to the partition-controlled and matrix-controlled cases subsequently used by Chien and Lambert (2) for the two limiting cases. When $l \gg 2D_e h_a/D_a K$, Eqs. 4 and 5 yield Eq. 2 (matrix-controlled case)[2]. Conversely, when $l \ll 2D_e h_a/D_a K$, Eqs. 4 and 5 yield:

$$Q = \frac{D_a C_a t}{h_a} \quad \text{(Eq. 6)}$$

which is boundary layer controlled release (partition-controlled release).

In the publication by Chien and Lambert (2), the Higuchi equation is again misquoted as the authors derived a series of equations following the theoretical treatment resulting in Eqs. 4 and 5. For the limiting condition that yields Eq. 6, Chien and Lambert (2) presented the following expressions[1]:

$$Q = \frac{KD_a C_s t}{h_a} \quad \text{(Eq. 7)}$$

$$Q = \frac{KD_a C_a t}{h_a} \quad \text{(Eq. 8)}$$

Equation 7 is correct since it reduces to Eq. 6 (note that $K = C_a/C_s$). However, Eq. 8 is only valid for the trivial case when $C_s = C_a$. But the data on ethynodiol diacetate indicate that C_s does not equal C_a; therefore, analysis of the data does not support the contention that Eq. 8 is valid.

I hope this communication clarifies the discrepancies between the original equations reported in Refs.

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[2] Equation 2 was derived for a homogeneous matrix; therefore, ϵ and τ were unity.

to study the effects of drugs or procedures on isolated perfused hearts, lungs, and blood vessels. The very small current employed (approximately 0.2 mamp) has no observable effects on either the preparation or the actions of the drugs studied.

In short, this apparatus provides a method for rapidly detecting changes in flow by using the Marriotte bottle to provide graphic information as well as to serve simply as a reservoir for the perfusion solution. Ease of construction and low cost make it particularly useful for teaching purposes, since each group of students can be supplied with one of these devices.

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Controlled Release from Matrix Systems

Keyphrases □ Matrix systems—controlled release of ethynodiol diacetate from silicone rubber devices, comments □ Silicone rubber matrix—release of ethynodiol diacetate, comments on controlled release □ Release, controlled—ethynodiol diacetate from silicone rubber devices, comments

To the Editor:

Two publications by Chien and coworkers (1, 2) described the release of ethynodiol diacetate from silicone rubber devices. These authors treated two cases for the matrix release process: matrix-controlled and partition-controlled drug release mechanisms. The equations describing the diffusional process were previously presented by Higuchi (3) and Roseman and Higuchi (4). With the assumptions that (*a*) the matrix acts as the diffusion medium, (*b*) a pseudo-steady-state condition exists during the release process, and (*c*) the drug particles are uniformly distributed throughout the matrix and are quite small relative to the average distance of diffusion, Higuchi (3) derived the following relationship for the release of drug from a planar homogeneous matrix:

$$Q = [D_s C_s (2A - C_s)t]^{1/2} \quad \text{(Eq. 1)}$$

where Q = amount of drug released per unit area, D_s = diffusion coefficient of drug in the homogeneous matrix phase, C_s = solubility of drug in the matrix phase, t = time, and A = total amount of drug present per unit volume of matrix.

When $A \gg C_s$, Eq. 1 reduces to:

$$Q = (2AD_s C_s t)^{1/2} \quad \text{(Eq. 2)}$$

Chien *et al.* (1), however, incorrectly quoted the Higuchi equation as:

$$Q = [D_s C_s (2A - C_a)t]^{1/2} \quad \text{(Eq. 3)}$$

where C_a is the solubility of drug in the elution medium[1]. Although the $(2A - C_a)$ term does not appear to have any physical significance (except, of course, when $C_a = C_s$), Eq. 3 does yield Eq. 2 when $2A$ is much greater than C_a.

The equations derived by Roseman and Higuchi (4) are an extension of the concepts discussed by Higuchi (3) for the specific case where diffusion from the surface of the device is considered in series with the diffusional step through the matrix. In this instance, the release of drug from a planar matrix is given by the following expressions, when $A \gg C_s$:

$$Q = Al \quad \text{(Eq. 4)}$$

$$l^2 + \frac{2D_e h_a l}{D_a K} = \frac{2D_e C_s t}{A} \quad \text{(Eq. 5)}$$

where[1] l = diffusional distance in the matrix (depleted zone); K = partition coefficient (C_a/C_s); h_a = diffusional distance in the boundary diffusion layer; $D_e = D_s\epsilon/\tau$ where ϵ and τ are the volume fraction and tortuosity of the matrix,respectively; and D_a = diffusion coefficient of drug in the elution medium. The other terms were defined previously.

Except for consideration of the boundary diffusion layer, the basic assumptions in the derivation of these equations are the same as those used to derive Eq. 2. Equations 4 and 5 describe a general case for matrix release, which was termed the matrix-boundary diffusion layer model (4). This nomenclature corresponds to the partition-controlled and matrix-controlled cases subsequently used by Chien and Lambert (2) for the two limiting cases. When $l \gg 2D_e h_a/D_a K$, Eqs. 4 and 5 yield Eq. 2 (matrix-controlled case)[2]. Conversely, when $l \ll 2D_e h_a/D_a K$, Eqs. 4 and 5 yield:

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Metabolic Fate of Flurazepam II: A New Potent Metabolite Obtained by *In Vitro* Liver Drug-Metabolizing Enzyme System

Keyphrases □ Flurazepam—isolation of new metabolite using *in vitro* liver drug-metabolizing enzyme system □ Metabolism—flurazepam, isolation of potent metabolite, *in vitro* liver drug-metabolizing enzyme system

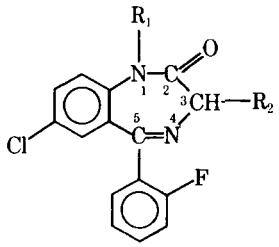
To the Editor:

Although a few investigations on the metabolism of flurazepam (I), 7-chloro-1-[2-(diethylamino)ethyl]-5- (*o*-fluorophenyl) -1,3-dihydro-2*H*- 1,4-benzodiazepin-2-one, in humans (1,2) and experimental animals (1, 3, 4) have been reported, its detailed *in vitro* metabolism is still unknown. This communication describes a new potent metabolite found using an *in vitro* liver drug-metabolizing enzyme system[1].

Reaction mixtures contained the following: 0.5 ml of male DDY mouse or Wister rat liver 9000×*g* supernate or the microsomal fraction (150 mg as fresh liver), NADPH generating system (1 μmole of NADP, 30 μmoles of glucose-6-phosphate, 25 μmoles of nicotinamide, 37.5 μmoles of magnesium chloride, 1.4 units of glucose-6-phosphate dehydrogenase), 5 μmoles of substrate, and 225 μmoles of tromethamine–hydrochloric acid buffer in 2.75 ml of total volume. After incubation at 37° for 90 min, the reaction mixtures were adjusted to pH 9.0 and were extracted once with 10 ml of ethyl acetate. Organic phases were concentrated *in vacuo*, and aliquots of the concentrated organic phases were applied to TLC plates [Kieselgel GF_{254}, benzene–methanol–acetic acid (90:10:10)].

Besides some of the known major metabolites, *e.g.*, II–IV, a minor unknown spot was detected under UV light when I was used as the substrate. Since this metabolite (V) was the predominant metabolite of IV, which is one of the known metabolites of I, 90 mg of authentic IV was then metabolized using mice liver enzyme for the preparation of V.

The metabolite was purified by preparative TLC (Kieselgel GF_{254}) followed by alumina (Woelm, neutral) column chromatography to yield 11.7 mg of oily material. Purified V gave a single spot with several TLC solvent systems, and its chromatographic behavior differed from that of the known metabolites. The IR spectrum of the metabolite showed the increased strength of the OH stretching band at 3400 cm^{-1} and the appearance of a CO stretching band at 1130 cm^{-1}, suggesting that the introduction of the secondary OH to the parent compound (IV) had occurred.



I: $R_1 = CH_2CH_2N(C_2H_5)_2$, $R_2 = H$
II: $R_1 = CH_2CH_2NHC_2H_5$, $R_2 = H$
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V: $R_1 = CH_2CH_2OH$, $R_2 = OH$

The mass spectrum (25 ev) of V showed a weak, but apparent, molecular ion at *m/e* 348 (11%) followed by other prominent fragments at *m/e* 320 (21), 319 (100), 301 (33), 287 (14), 275 (28), and 260 (13). This increment of molecular weight by 16 mass units from that of IV also indicated that one oxygen atom had been introduced to the substrate (IV), but the lack of an M − 16 ion suggested that it might not be any oxide form. The molecular ion of acetylated V (*m/e* 432) and the successive split of two ketenes, giving fragments at *m/e* 390 and 348, were additional supports for the idea.

The proton NMR spectrum of V run in $CDCl_3$,

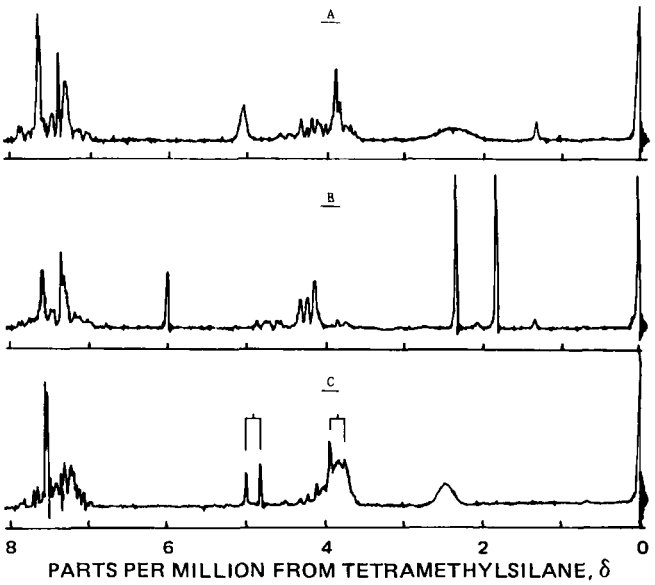

Figure 1—*Proton NMR spectra of V, V-acetate, and authentic parent compound IV in $CDCl_3$: Key: A, V; B, V-acetate; and C, authentic IV (two methylene protons of C_3 at 3.84 and 4.90 ppm are specified).*

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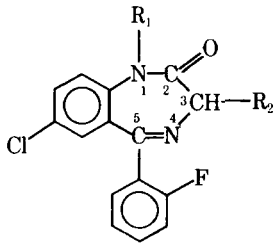

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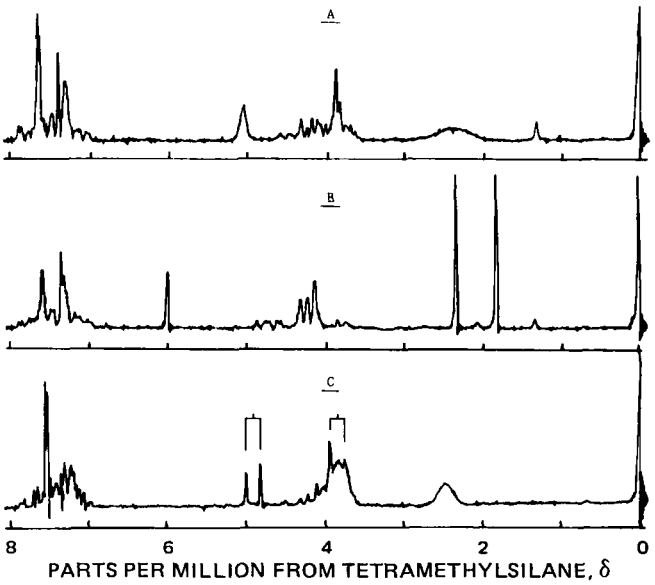

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This compound was then synthesized in a separate experiment, and its psychotropic activity was studied. Its CNS-depressant activity in DDY strain male mice was at the 50-mg/kg dose level (oral), which was approximately the same potency as unchanged I. It is of interest that V was found to be one of the metabolites in humans, and details of this work will be reported.

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Pithed Rat Blood Pressure in the Assay of Norepinephrine

Keyphrases □ Norepinephrine—analysis in plasma and cerebrospinal fluid of rats by pithed blood pressure method □ Sympathomimetics—norepinephrine, assay, pithed rat blood pressure

To the Editor:

Measurement of rat blood pressure is a standard method in biological estimations of acetylcholine (1–5), angiotensin (6), norepinephrine (6, 7), epinephrine (6, 7), and vasopressin (8). Preparations reported for norepinephrine assay show a sensitivity of 1–2 × 10^{-7} g/ml. The pithed rat blood pressure in 250 preparations, with some modifications used in our study for the estimation of norepinephrine in plasma (9) and cerebrospinal fluid (10), was sensitive from 1 × 10^{-9} to 5 × 10^{-10} g/ml. The changes followed in these preparations are described here since a highly sensitized preparation could be mounted with the additional operations.

Albino Wistar rats of either sex, 200–350 g, were used. The rats, after being fasted overnight, were injected intraperitoneally with 0.4 ml (0.4% in 0.85% saline) of atropine sulfate[1] 20 min before anesthetization. Anesthesia was induced by spraying ethyl chloride into the glass funnel initially and was maintained with anesthetic ether until the rat became unconscious.

The anesthetized rat was opened for tracheotomy, and a polyethylene cannula with a side opening was immediately introduced into the trachea and tied in position. The rat was then turned on its back to one side and pithed as described previously (6). Once the brain and spinal cord were destroyed, as seen by spasticity of contralateral muscles, the rat was immediately ventilated mechanically by connecting the tracheal cannula to a mini-respiratory pump. The regulator was adjusted to ensure sufficient ventilation so that inflation and deflation of the chest and heart rate were 30–40 strokes/min.

Both carotid arteries were ligated at the central end. Then a laparotomy was done, and the viscera were removed from the esophagus to the rectum, including the spleen; the liver was left *in situ* as per the method of Venkatakrishna-Bhatt and Haranath (3), and the bleeding points were carefully ligated. The left carotid artery and the right femoral vein were cannulated and connected to a manometer[2] and saline stand, respectively. Heparin[3], 0.5 mg, was injected into the femoral vein to prevent clotting. Then 1–3 ml of saline was injected to compensate for the loss of blood volume during evisceration.

Initially, the response with a low concentration of norepinephrine[4] (10^{-11} g/ml) was recorded on a kymograph. Sensitivity increased on repeated injections of standard norepinephrine into the femoral vein at regular intervals. Thus, when the blood pressure reached a steady level, usually after 10–15 trials, the test solution was injected. The volume of fluid injected was always kept constant, usually being 0.1 ml followed by 0.1 ml of saline. Care was taken to prevent leakage or passing of air bubbles from the syringe or leaking points.

This preparation was frequently found to be sensitive to a concentration of 10^{-9}–10^{-10} g/ml of norepinephrine when prepared in saline with 5 × 10^{-6} g/ml of ascorbic acid. Ascorbic acid in saline as a control, when injected in a way similar to the test and standard norepinephrine solutions, did not elicit a peak response as did the test solutions. Although skill and precision are essential for handling the preparation,

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Estimation of Hepatic First-Pass Effect of Acetaminophen in Humans after Oral Administration

Keyphrases □ Acetaminophen—estimation of hepatic first-pass effect, oral administration, humans □ Metabolism—acetaminophen, estimation of hepatic first-pass effect, oral administration, humans □ First-pass effect, hepatic—acetaminophen, estimation, oral administration, humans

To the Editor:

Acetaminophen is one of the most frequently used analgesics and antipyretics today. It is primarily metabolized in humans after oral administration. In one study, only about 3–4% of the dose was recovered in urine as intact compound after dosing (1). Recently, Cohen *et al.* (2) reported that its availability to the systemic circulation after intraperitoneal administration to rats was only 34% of that after intravenous administration, indicating an extensive hepatic first-pass metabolic effect. This finding prompted them to speculate (2) that a marked variation in peak acetaminophen plasma concentrations in humans with different stomach emptying rates (3) might be caused to a varying degree by the hepatic first-pass effect.

The purpose of this communication is to estimate theoretically the extent of the hepatic first-pass effect of acetaminophen in humans, using an equation derived recently (4, 5). The following assumptions were made in the derivation: (*a*) the metabolism takes place only in the liver; (*b*) the excretion of intact drug occurs only from the kidney and/or lung; (*c*) the liver, kidneys, and lungs are part of the central compartment; (*d*) the elimination and distribution processes between the central and other peripheral compartments are all first-order processes; and (*e*) the administered drug is all absorbed after oral or intraperitoneal administration.

The fraction of the dose metabolized during the first passage to liver, f_m, can be estimated by the following equation:

$$f_m = \frac{(F_m)(\text{dose})}{\text{dose} + (HFR)(AUC)} \qquad \text{(Eq. 1)}$$

where F_m = fraction of the administered dose metabolized at infinite time, HFR = hepatic blood flow rate, and AUC = total area under blood concentration *versus* time curve in infinite time.

A search and evaluation of past studies related to acetaminophen oral absorption in humans revealed that some work reported by McGilveray *et al.* (1) was suitable for this pharmacokinetic prediction analysis. That article reported the acetaminophen blood level data up to 6 hr after oral administration of 1 g of the drug dissolved in 200 ml of water to 10 normal subjects 1.5 hr after a light breakfast. Based on the urinary excretion data and the solution dosage form administered, one could reasonably assume that all of the dose was completely absorbed (1, 6, 7).

An AUC of 5626 (min) (μg/ml) was obtained by the summation of the area from 0 to 6 hr calculated by the trapezoidal rule and of the area from 6 hr to infinity calculated by the extrapolation method (5, 7). An average biological half-life of 3.26 hr (1) was used in the calculation for the extrapolated area. This half-life is similar to (7), but different than (8), the values from studies reported by other workers, indicating the possibility of intersubject variation. The F_m was assumed to be 0.96. By substituting a value of 1500 ml/min for HFR (4, 5) and other appropriate data into Eq. 1, one obtains an f_m value of 0.102. This value is obviously much lower than those observed (f_m = 0.66) and predicted (f_m = 0.60) for rats (2).

These results indicate that there is a marked interspecies variation in the extent of the first-pass effect. Although absorption of several drugs from intraperitoneal administration has been shown to occur predominantly *via* the portal circulation in dogs and rats (9), this has not been proven for acetaminophen.

The f_m value estimated by Eq. 1 generally represents a maximum value. If the hepatic metabolic process deviates from pseudo first order as the absorption rate of drug increases (based on Michaelis–Menten enzyme kinetics), the observed f_m value would be lower than that predicted by Eq. 1. In other words, the maximum fraction of the orally administered acetaminophen dose metabolized in the liver during the first pass is only about 0.10, and the effect of the

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absorption rate on the extent of the first-pass effect should be clinically insignificant.

This contention is also supported by a study that showed that although food could significantly reduce the absorption rate and peak blood levels of acetaminophen in humans, it had no significant effect on total bioavailability, as indicated by similar areas under blood concentration curves of acetaminophen (10). From this finding, it is also reasonable to suggest that differences in the contribution of the first-pass effect for rectally and orally administered acetaminophen would not be of clinical significance.

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BOOKS

REVIEWS

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ment of analytical procedures. One chapter deals with the radioimmunoassay for digoxin. The successful growth and vitality of a clinical pharmacokinetics program depend on the investigators' ability to measure drug levels in biological specimens. Therefore, innovators of diversified analytical methodology must be an integral part of such a group.

Several of the chapters deal with the pharmacokinetic control of the clinical therapy of different drugs or drug classes. These chapters provide the greatest insight into the contributions that clinical pharmacokinetics can provide for rational drug therapy. Although dealing with specific drugs, the chapters contain much in the way of providing the basis for other investigators to monitor drug levels and/or design studies for other drugs which should be monitored as outlined in the final chapter.

Two chapters deal with the use of a clinical pharmacokinetics laboratory for bioavailability assessment and discuss the design and evaluation of bioavailability studies. Another chapter discusses the role of the clinical pharmacist as an interface with the physician. However, the book lacks input from the clinical pharmacologist involved in such studies.

Overall, the book represents a useful contribution which provides a valuable insight into the potential of clinical pharmacokinetics in rational drug therapy.

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Principles of Medicinal Chemistry. Edited by WILLIAM O. FOYE. Lea & Febiger, Philadelphia, Pa., 1974. 857 pp. 19 × 27 cm. Price $29.50.

This text, as the editor notes in the preface, is aimed primarily at undergraduate students, and it has been written by 38 teachers and researchers in medicinal chemistry. The book demonstrates that extensive literature has been taken into consideration in developing an account of the various classes of drugs and types of drug action. The reviewer agrees with the editor that the major objective of introducing the appropriate fundamental chemical principles to beginning students is to provide the basis for the understanding of drug action. Moreover, the reviewer feels that the understanding of chemical principles of drug action is essential to the study of therapeutics, biopharmaceutics, and clinical pharmacy; hence this book should be useful not only to students in medicinal chemistry, but also to students in pharmacology, clinical pharmacy, and pharmaceutics.

Foye excellently edited a total of 37 chapters as a review of the vast area of medicinal chemistry. The first six chapters cover general topics which are usually found in contemporary medicinal chemistry and pharmacology textbooks: historical milestones in the development of drug therapy; physicochemical properties and bioactivity; modern approaches to rational drug design; the relationship between stereochemistry and pharmacological properties; drug metabolism; and drug–receptor interactions. These preliminary chapters provide an effective introduction to the rest of the book and to the fields of medicinal chemistry and pharmacology.

The treatment of the major classes of medicinal agents begins with those affecting the central nervous system—an effective approach to the study of the chemistry of drug action relating to neuropharmacology. Subsequently, agents acting on the peripheral nervous system are surveyed. Cardiovascular drugs, anticoagulants, coagulants, plasma expanders, and diuretics are included in three chapters by the same author, thus providing continuity that makes for easy reading. Anti-allergenic agents are well covered with emphasis on the relevant biochemical principles and discussion of the major structural classes.

The medicinal chemistry of steroid endocrinology is introduced with sections on cholesterol metabolism and antilipidemic agents. For more effective utilization by undergraduates, it seems that the chapter (24) on amino acids, peptides, and proteins should precede or immediately follow the consideration of steroid hormones to relate the hypothalamic and pituitary peptides to their effects on steroid hormone production; then, the chapter on insulin and other agents affecting carbohydrate metabolism would follow logically with the thyroid agents.

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Most principal chemotherapeutic agents are structurally characterized and their biochemical actions are related to therapeutic significance.

Finally, miscellaneous medicinals affecting the respiratory system (*e.g.*, nasal decongestants, antitussives, *etc.*,) and diagnostic agents are presented in two chapters.

Perhaps other educators agree with the reviewer that for undergraduate teaching a combination of texts (*e.g.*, Foye's; Burger's *Medicinal Chemistry;* and Wilson, Gisvold, and Doerge's *Textbook of Organic Medicinal and Pharmaceutical Chemistry*) together with appropriate journal articles serve the best purpose in presenting a well-balanced complementation of lecture material in medicinal chemistry, thus providing students with a good introduction to the literature *via* selected bibliographies. In this capacity, Foye's text will be a contribution to the pharmaceutical sciences and pharmaceutical education in general. This book can also serve as a useful review for pharmaceutical scientists who feel a need for updating in elementary medicinal chemistry. Furthermore, pharmacy and medical practitioners will find this text important for refamiliarization with the chemical basis of modern drug therapy.

Reviewed by Jaime N. Delgado
College of Pharmacy
University of Texas
Austin, TX 78712

ment of analytical procedures. One chapter deals with the radioimmunoassay for digoxin. The successful growth and vitality of a clinical pharmacokinetics program depend on the investigators' ability to measure drug levels in biological specimens. Therefore, innovators of diversified analytical methodology must be an integral part of such a group.

Several of the chapters deal with the pharmacokinetic control of the clinical therapy of different drugs or drug classes. These chapters provide the greatest insight into the contributions that clinical pharmacokinetics can provide for rational drug therapy. Although dealing with specific drugs, the chapters contain much in the way of providing the basis for other investigators to monitor drug levels and/or design studies for other drugs which should be monitored as outlined in the final chapter.

Two chapters deal with the use of a clinical pharmacokinetics laboratory for bioavailability assessment and discuss the design and evaluation of bioavailability studies. Another chapter discusses the role of the clinical pharmacist as an interface with the physician. However, the book lacks input from the clinical pharmacologist involved in such studies.

Overall, the book represents a useful contribution which provides a valuable insight into the potential of clinical pharmacokinetics in rational drug therapy.

Reviewed by Stanley A. Kaplan
Research Division
Hoffmann–La Roche, Inc.
Nutley, NJ 07110

Principles of Medicinal Chemistry. Edited by WILLIAM O. FOYE. Lea & Febiger, Philadelphia, Pa., 1974. 857 pp. 19 × 27 cm. Price $29.50.

This text, as the editor notes in the preface, is aimed primarily at undergraduate students, and it has been written by 38 teachers and researchers in medicinal chemistry. The book demonstrates that extensive literature has been taken into consideration in developing an account of the various classes of drugs and types of drug action. The reviewer agrees with the editor that the major objective of introducing the appropriate fundamental chemical principles to beginning students is to provide the basis for the understanding of drug action. Moreover, the reviewer feels that the understanding of chemical principles of drug action is essential to the study of therapeutics, biopharmaceutics, and clinical pharmacy; hence this book should be useful not only to students in medicinal chemistry, but also to students in pharmacology, clinical pharmacy, and pharmaceutics.

Foye excellently edited a total of 37 chapters as a review of the vast area of medicinal chemistry. The first six chapters cover general topics which are usually found in contemporary medicinal chemistry and pharmacology textbooks: historical milestones in the development of drug therapy; physicochemical properties and bioactivity; modern approaches to rational drug design; the relationship between stereochemistry and pharmacological properties; drug metabolism; and drug–receptor interactions. These preliminary chapters provide an effective introduction to the rest of the book and to the fields of medicinal chemistry and pharmacology.

The treatment of the major classes of medicinal agents begins with those affecting the central nervous system—an effective approach to the study of the chemistry of drug action relating to neuropharmacology. Subsequently, agents acting on the peripheral nervous system are surveyed. Cardiovascular drugs, anticoagulants, coagulants, plasma expanders, and diuretics are included in three chapters by the same author, thus providing continuity that makes for easy reading. Anti-allergenic agents are well covered with emphasis on the relevant biochemical principles and discussion of the major structural classes.

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Journal of Pharmaceutical Sciences

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VOLUME 64 NUMBER 11

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NATIONAL RESEARCH POLICY

A frequently heard criticism relates to the absence of any clearly defined national science policy on the part of the United States government. Inasmuch as research is a key element of science—and in the minds of many is synonymous with it—it is evident that what is equally lacking is a clear federal policy concerning research *per se*.

Naturally, what is of greatest interest to us and to the majority of our readers is that biomedical research constitutes one facet of this great policy wasteland.

We were heartened, therefore, when U.S. President Ford—at the urging of Congress—appointed a group of knowledgeable scientists to review, study, and report on the subject of biomedical and behavioral research. The group, designated "The President's Biomedical Research Panel," is to come up with specific recommendations based upon its year-long analysis.

As part of the fact-finding and opinion-soliciting phase of operation, the panel identified a number of organizations in the health-care field and invited comments from them. Included among these organizations was the American Pharmaceutical Association. APhA accepted the invitation to offer its views and, in turn, solicited input from its two academies—the Academy of Pharmaceutical Sciences and the Academy of Pharmacy Practice.

Ultimately, a rather comprehensive paper was prepared and submitted by APhA based upon the collective input provided from its academies, its officers, and its staff. Reflected in this paper is a broad spectrum of views and thinking which hopefully will have a positive impact on the report prepared by the President's panel. And, subsequently, we trust that the panel's report will, in fact, result in the establishment of the long-needed federal biomedical research policy. If, as we hope and anticipate, the panel prepares a report at least compatible with—if not patterned after—the views expressed by APhA, then it is our further wish that the President will respond favorably, with the result that the panel's report forms the basis of the national research policy that is established.

In light of the fact that many of our readers have a basic interest in this subject, we are publishing in full, on pages iii–vi in this issue of the *Journal of Pharmaceutical Sciences*, the comments approved by the APhA Board of Trustees and submitted by the Association. We respectfully suggest that these comments be read in their entirety by everyone having an interest in the subject of Biomedical Research.

Edward G. Feldmann

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Edward G. Feldmann

1975

November 16–20
American Public Health Association, Annual Meeting, Chicago, Ill. (Contact William H. McBeath, APHA, 1015 18th St., N.W., Washington, DC 20036)

November 16–20
APhA Academy of Pharmaceutical Sciences, National Meeting, Marriott Motor Hotel, Atlanta, Ga.

November 21
Eastern Analytical Symposium, Statler-Hilton Hotel, New York, N.Y. (Contact Webster R. Hudgins, Allied Chemical, P.O. Box 1021R CRL, Morristown, NJ 07960)

November 29–December 4
American Medical Association, Clinical Meeting, Honolulu, Hawaii (Contact James H. Sammons, 535 N. Dearborn St., Chicago, IL 60610)

November 30–December 4
International Symposium on Neurotoxicology, Columbia-Presbyterian Medical Center, New York, N.Y. (Contact Dr. Leon Roizin, Psychiatric Institute, 722 W. 168th St., New York, NY 10032)

November 30–December 5
American Chemical Society, National Meeting, Mexico City, Mexico (Contact Meetings Department, 1155 16th St., N.W., Washington, DC 20036)

December 2–3
Food and Drug Law Institute, Annual Meeting, Washington, D.C.

December 7–11
American Society of Hospital Pharmacists, Midyear Meeting, Washington, D.C.

1976

January 11–14
International Symposium on Quantum Biology and Quantum Pharmacology with Particular Reference to Molecular Diseases, Sanibel Island, Fla. (Contact J. R. Sabin, Williamson Hall, University of Florida, Gainesville, FL 32611)

January 18–23
Arden House Conference, Columbia University College of Pharmaceutical Sciences and APhA Academy of Pharmaceutical Sciences Industrial Pharmaceutical Technology Section, Arden House, N.Y.

February 18–24
American Association for the Advancement of Science, Annual Meeting, Boston, Mass. (Contact Ms. Elizabeth Zeutschel, AAAS, 1776 Massachusetts Ave., Washington, DC 20036)

February 28–29
Western State Drug Conference, Marriott Hotel, Los Angeles, Calif.

March 1–5
Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, Convention Center, Cleveland, Ohio

March 25
Safety and Toxicity of Pharmaceuticals and Consumer Products, Annual Symposium, Chemical Institute of Canada, Holiday Inn, Don Valley-Eglinton, Toronto, Canada (Contact T. J. Carrothers, Bristol-Myers Products Canada Ltd., 99 Vanderhoof Ave., Toronto, Canada M4G 2H6)

April 3–8
APhA Annual Meeting, New Orleans, La.

April 4–7
Proprietary Association, Annual Meeting, The Breakers, Palm Beach, Fla.

April 4–9
Centennial American Chemical Society Meeting, New York, N.Y. (Contact A. T. Winstead, American Chemical Society, 1155 16th St., N.W., Washington, DC 20036)

April 10–13
Society for Biomaterials, Annual Meeting, and International Biomaterials Symposium, Philadelphia, Pa. (Contact S. R. Pollack, Department of Metallurgy and Materials Science, University of Pennsylvania, Philadelphia, PA 19174)

April 18–24
Basic Pharmacokinetics and Biopharmaceutics Workshop, American Society of Clinical Pharmacology and Therapeutics, St. Augustine, Fla. (Contact Irene Garrett, Administrative Associate, 1826 N.W. 26th Way, Gainesville, FL 32605)

April 26–May 1
Third International Symposium on Detection and Prevention of Cancer, Americana Hotel, New York, N.Y. (Contact Dr. Herbert E. Nieburgs, Mount Sinai School of Medicine, City University of New York, Fifth Ave. and 100th St., New York, NY 10029)

May 10–12
Pharmaceutical Manufacturers Association, Annual Meeting, The Greenbrier, White Sulphur Springs, W. Va.

May 17
APhA Academy of Pharmaceutical Sciences, Annual Midwestern Regional Meeting, Industrial Pharmaceutical Technology and Pharmaceutical Analysis and Control Sections, O'Hare Inn, Chicago, Ill. (Contact Dr. A. Amann, Abbott Laboratories, North Chicago, IL 60064)

May 19–21
Belgian Chemical Society, International Meeting on Drug Receptor and Drug Enzyme Interactions, Namur, Belgium (Contact F. Durant, Department of Chemistry, Facultes Universitaires de Namur, 61, Rue de Bruxelles, B-5000 Namur, Belgium)

June 3–5
Annual Summer Symposium, American Chemical Society Division of Analytical Chemistry, Tempe, Ariz. (Contact R. S. Juvet, Arizona State University, Tempe, AZ 85281)

June 20–24
National Medicinal Chemistry Symposium, American Chemical Society Division of Medicinal Chemistry, University of Utah, Salt Lake City, Utah (Contact L. B. Townsend, Department of Biopharmaceutical Sciences, University of Utah, Salt Lake City, UT 84112)

June 27–July 1
American Medical Association, Annual Meeting, Dallas, Tex.

August 29–September 2
American Chemical Society, National Meeting, San Francisco, Calif. (Contact A. T. Winstead, American Chemical Society, 1155 16th St., N.W., Washington, DC 20036)

October 7–10
American College of Apothecaries, Annual Meeting, Bellevue Stratford, Philadelphia, Pa.

October 17–21
American Public Health Association, Annual Meeting, Miami Beach, Fla.

November 14–18
APhA Academy of Pharmaceutical Sciences, National Meeting, Carleton House Hotel, Orlando, Fla.

November 14–18
American Dental Association, Annual Meeting, Las Vegas, Nev.

December 4–8
American Medical Association, Clinical Meeting, Philadelphia, Pa.

December 5–10
American Society of Hospital Pharmacists, Midyear Meeting, Anaheim, Calif.

December 14–15
Food and Drug Law Institute, Annual Meeting, Washington Hilton, Washington, D.C.

Items to be considered for inclusion or corrections should be addressed to:

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Journal of Pharmaceutical Sciences

NOVEMBER 1975
VOLUME 64 NUMBER 11

REVIEW ARTICLE

Microbial Models of Mammalian Metabolism

ROBERT V. SMITH *x **and JOHN P. ROSAZZA** ‡

Keyphrases □ Drug metabolism—mammals, microorganisms, mechanisms, C-hydroxylation, *N*-dealkylation, *O*-dealkylation, *N*-oxidation, S-oxidation, alcohol dehydrogenation, keto reduction, nitro reduction, azo reduction, review □ Mammalian biotransformations—parallel microbial transformations, review □ Microbial transformations—parallel mammalian metabolism, review

An understanding of the metabolism of drugs and other foreign chemicals in mammals has been pursued actively during the past 15 years. Metabolism can include the phenomena of absorption, distribution, and excretion (1). However, in this paper, it will be used synonymously with the more strictly defined term biotransformation. Study of mammalian biotransformations has been stimulated by the fact that useful information on the mechanism of action and/or toxicity of certain compounds can often be obtained.

While there is considerable impetus to perform metabolism studies, several practical problems are encountered including: (*a*) development of analytical methods suitable for the determination of drugs and their metabolites in biological media; (*b*) qualitative and quantitative metabolic differences in various species of animals, especially when attempting to compare lower animals to humans; and (*c*) procurement of sufficient quantities of metabolites for structure elucidation and biological testing. Considerable effort has been expended in solving the first two problems. The refinement of radiochemical techniques (2) and the development of GC (3) and mass spectral (4, 5) methodology have aided significantly in the first case, while recent emphasis on comparative metabolism should ultimately provide solutions to the second difficulty (6, 7).

Until recently, however, there have been relatively few solutions to the problem of preparative synthesis of metabolites. Although direct structural information is often obtainable (through mass spectrometry) on the mere microgram quantities of metabolites usually available from biological specimens, there is no substitute for having larger (gram) quantities for complete structure elucidation and biological evaluation. While organic synthetic methods provide an obvious solution for metabolites of relatively simple drugs, preparation of metabolites of more complex molecules may require vast expenditures in time and effort. Although mammalian tissue preparations (*e.g.*, liver microsomes) occasionally may be utilized in synthesizing preparative quantities of metabolites, there are difficulties related to the stability of such systems (8).

Recently, Smith and Rosazza (9) suggested that it might be possible to define microbial transformation systems that could mimic many biotransformations observed in mammals. As a descriptive name, these systems are called "microbial models of mammalian metabolism." It is important to realize that a microbial transformation system is viewed as a selected group of microorganisms that could be used together to conduct parallel mammalian and microbial xenobiotic metabolism studies. It is unlikely, although not impossible, that a single microorganism can mimic all of the biotransformations conducted by mammalian systems (*e.g.*, liver microsomal preparations). Where metabolites common to both the microbial transfor-

mation system and mammalian systems are observed, the microbial system would be used to obtain sizable quantities (grams) of metabolites *via* routine fermentation scale-up techniques. By this process, difficult-to-synthesize metabolites would be readily obtained for structure elucidation and biological testing. Further advantages of the microbial transformation system, especially with polyfunctional molecules, center about the selectivity and mild conditions with which biotransformations occur.

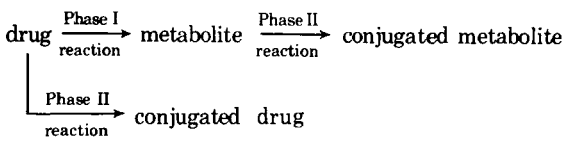
The literature abounds with reports on the metabolism of many types of compounds by microorganisms. These include steroids (10–13), alkaloids (11, 14), antibiotics (15, 16), amino acids (17, 18), and insecticides (19, 20). The general approach taken is one of revealing biogenetic pathways by which compounds are metabolized by one or more microorganisms. The fields of microbial conversions with steroids and antibiotics represent some of the most serious attempts at harnessing the synthetic potential of microorganisms.

Although the literature reveals many types of reactions by microorganisms, many of these are low yielding; few systematic studies on the use of microorganisms for the preparation of metabolites (except steroids and antibiotics) have been made (10–13, 15, 16). The fundamental bases for the selection and use of these microbial systems and their potentialities are the subjects of this paper.

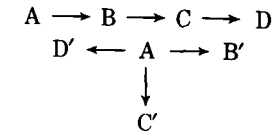
PRINCIPLES OF MAMMALIAN BIOTRANSFORMATIONS

Most drugs or other foreign chemicals entering the mammalian organism are chemically altered. In many instances, changes are effected in a biphasic process. Phase I consists of oxidation, reduction, and hydrolysis reactions, while Phase II involves conjugative reactions (sometimes known as synthetic reactions) between the drug (or metabolite) and common body constituents. These processes are diagrammatically represented in Scheme I. Through biotransformations, drugs are generally converted into more polar compounds which can be readily excreted (primarily *via* the kidney) from the organism. Thus, biotransformation reactions may act as mechanisms for terminating the pharmacological activity of drug substances. Indeed, it has been suggested that in lieu of metabolic transformations, certain drug entities might have biological lifetimes far exceeding those of most animal organisms (21).

In general, biotransformations lead to chemical entities possessing less biological activity and/or toxicity; however, there are exceptions. Particularly with Phase I reactions, cases are now well documented wherein metabolites possessing enhanced activities are produced.



Scheme I—Biotransformations of drugs



Scheme II—Parallel versus *sequential biotransformations of drugs*

Study of the metabolic fate of foreign compounds is complicated by the fact that parallel or sequential biotransformations may occur (Scheme II). Consequently, for some complex molecules (*e.g.*, phenothiazine tranquilizers), tens of metabolites can be formed in mammals (22).

OXIDATIVE TRANSFORMATIONS

Of the three Phase I reactions, oxidative biotransformations are quantitatively of greatest importance. A tremendous explosion has occurred over the past decade regarding the understanding of the mechanisms of oxidation of all types of compounds. In 1962, Hayaishi (23) paid great attention to the gross aspects of oxygen metabolism. Since that time, an understanding of the basic mechanisms of oxygen activation and of the physiological function of the oxygenases has increased (24). New classes of oxygen-activating enzymes appeared during this period, including cytochrome P-450 enzyme systems, flavoprotein oxygenases, and α-ketoglutarate-requiring oxygenases. A greater understanding of superoxide anion, superoxide dismutase, and superoxide-using enzymes also has been gained.

Most oxidative Phase I reactions are catalyzed by cytochrome P-450-linked monooxygenases (Table I), located primarily in the liver of mammals but also occurring in the kidneys, lungs, small intestine, spleen, and steroidogenic organs such as the adrenal cortex, testes, ovary, and placenta (8). Considerable effort has gone into unraveling the electron-transport chain associated with these rather nonspecific enzyme systems (8, 25, 26). Briefly, the oxidized form of cytochrome P-450 (Fe III) complexes with substrate, apparently at a site near the iron atom of the heme protein. The resulting complex undergoes rapid reduction *via* cytochrome P-450 reductase (Fe III reduced to Fe II). This reduction is followed by formation of a ternary reduced cytochrome–substrate–molecular oxygen complex.

After reduction of the oxygen by the cytochrome iron atom (Fe II → Fe III) and further reduction by another electron from an unknown source, the resulting complex internally disproportionates with the transfer of one oxygen to bound substrate and acceptance of two protons by the second oxygen atom. Finally, the complex dissociates into water, oxidized substrate, and oxidized cytochrome P-450. This mechanism (Scheme III) is important because of its apparent similarity to monooxygenases occurring in microorganisms (*vide infra*).

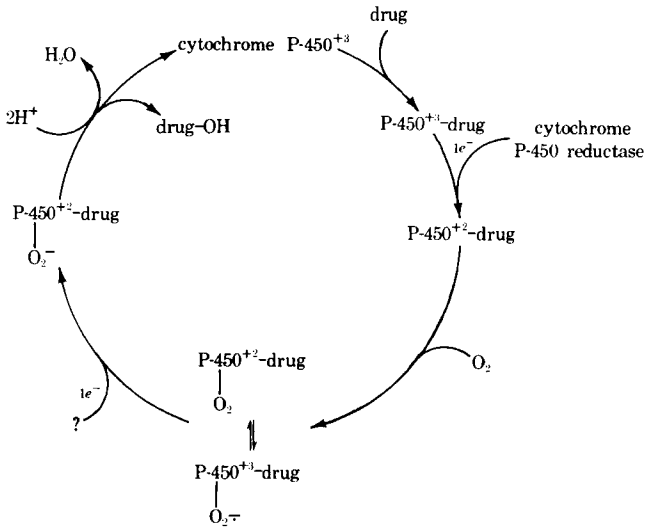
Cytochrome P-450 monooxygenases are believed to mediate aliphatic and aromatic hydroxylations and *N*-, *O*-, and S-dealkylations in mammals. In general, they can be visualized as mediating the attack of sub-

Table I—Mammalian Oxidative Biotransformations

Reaction Type	Substrate	Intermediate	Product
Hydroxylation	Aliphatic and alicyclic compounds	—	Alcohols
	Aromatic compounds	Arene oxides	Phenols
N-Dealkylation	Secondary and tertiary amines	$HOCHRNR_2'$	Aldehyde plus primary or secondary amine
O-Dealkylation	Alkyl–alkyl and alkyl–aryl ethers	HOCHROR'	Aldehyde plus alcohol or phenol
S-Dealkylation	Alkyl–alkyl and alkyl–aryl sulfides	HOCHRSR'	Aldehyde plus thiol or thiophenol
S-Oxidation	Sulfides	—	Sulfoxide
	Sulfoxide	—	Sulfone
N-Oxidation	Primary and secondary amines and amides	—	Hydroxylamine and *N*-hydroxyamides
	Tertiary amines	—	*N*-Oxides
Deamination	Primary and secondary amines	RCHNHR' OH	Ammonia and primary amine plus aldehyde
Dehydrogenation	Primary alcohol	—	Aldehyde
	Secondary alcohol	—	Ketone
	Aldehyde	—	Carboxylic acid
	trans-Dihydrodiol	—	Catechol

strate by an electron-deficient oxygen atom; in most instances, this results in the formation of unstable intermediates that rearrange to the metabolites indicated in Table I. In the case of aliphatic hydroxylations, this process can be thought of as an insertion reaction; in aromatic hydroxylations, arene oxides primarily occur as intermediates (27, 28). In most cases, the hydroxyalkyl intermediates formed during *N*-, *O*-, and S-dealkylations are so unstable that they spontaneously decompose to their corresponding dealkylated metabolites.

S-Oxidation, *N*-hydroxylation (primary and secondary amines), *N*-oxide formation (tertiary amines), and deaminations (primary amines) are mammalian biotransformations (Table I) that occur in a manner similar to that already described. However, these reactions probably involve enzymes other than cytochrome P-450 monooxygenases. All of the oxidative reactions, except oxidative deamination, that occur in mammalian tissues can be duplicated *in vitro* with so-called microsomal fractions (1). The latter arise as microglobular bodies, formed (as artifacts) during disruption of the endoplasmic reticulum of cells.



Scheme III—Oxidation of drugs via cytochrome P-450-linked monooxygenase

Alcohols are oxidized to aldehydes and ketones by a number of oxidoreductases or alcohol dehydrogenases. In this regard, the enzyme of principal importance is (the soluble) liver alcohol dehydrogenase. The coenzyme for this transformation is NAD; the stoichiometry of the reversible reaction involved is shown in Scheme IV.

$$NAD + \text{alcohol} \rightleftharpoons NADH + \text{ketone or aldehyde} + H^+$$

Scheme IV

Because of proton formation, the carbonyl product is favored at higher pH's (optimum = 10). *In vivo*, the conversion of alcohols to aldehydes is favored due to subsequent facile conversion to readily excreted carboxylic acids. Aldehydes are converted to carboxylic acids by a series of soluble enzymes, including aldehyde dehydrogenases, aldehyde oxidase, and xanthine oxidase.

Oxidative deamination of naturally occurring amines is catalyzed by mitochondrial amine oxidases (principally monoamine oxidase). Monoamine oxidase also attacks various exogenous amines.

Enzymes associated with oxidative transformation of xenobiotics appear to exhibit a low degree of substrate specificity; that is, practically all classes of compounds serve as substrates. Within classes of compounds, however, certain directional influences seem to play a major role in determining what position becomes oxidized. For instance, with a given aromatic hydrocarbon, a preponderance of one phenolic metabolite over another is often observed. Such observations pose a dilemma in that it is difficult to discern whether specificities regarding sites of oxidation are qualitative (involving different enzymes) or quantitative (involving different amounts of enzymes) manifestations of enzyme activity. Although considerable research is required to answer such questions, certain generalizations may be noted for given types of oxidative biotransformations.

As a convenient means of representing parallels between microbial and mammalian biotransformations, various reactions that appear to be important

in the metabolism of drug substances will be discussed.

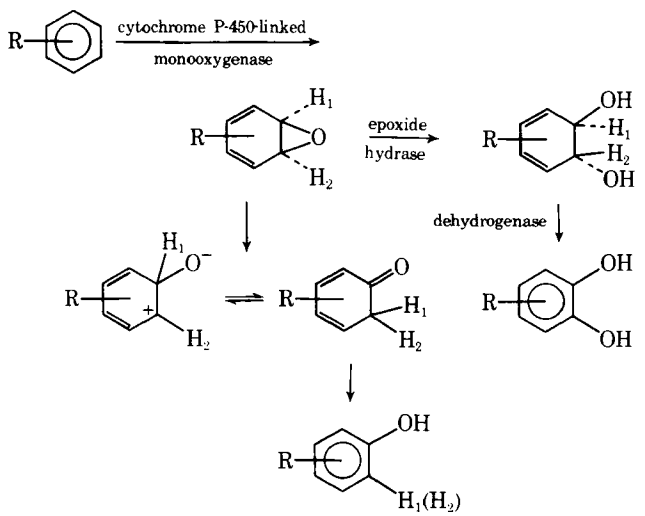
Aromatic Hydroxylations—Aromatic compounds are often converted to phenols in mammalian systems. Patterns of hydroxylations frequently follow the rules of electrophilic substitution reactions. Activated (electron-rich) aromatic rings are readily hydroxylated, whereas deactivated (electron-deficient) aromatic rings are hydroxylated slowly or not at all. Furthermore, hydroxylation occurs at positions predicted by concepts of electrophilic substitution; activated rings [*e.g.*, aniline and anisole (27–30)] are hydroxylated in *ortho*- and *para*-positions, while certain deactivated rings [*e.g.*, benzoic acid and benzamide (30)] are hydroxylated in the *meta*-position. The proportions of *ortho*- to *para*-hydroxylated products generally favor the latter; as expected, in di- and trisubstituted systems, hydroxylation is directed to positions predicted by a summation of substituent effects (29, 31).

Aromatic hydroxylation can be viewed as proceeding by a number of different mechanisms. However, most data are consistent with an oxenoid mechanism involving the intermediacy of an arene oxide and the formation of phenolic metabolites *via* nonenzymatic rearrangement (32). This process is usually accompanied by the so-called NIH shift (27, 28, 33–35) or retention of a substituent at the site of hydroxylation by rearrangement to an adjacent carbon (Scheme V).

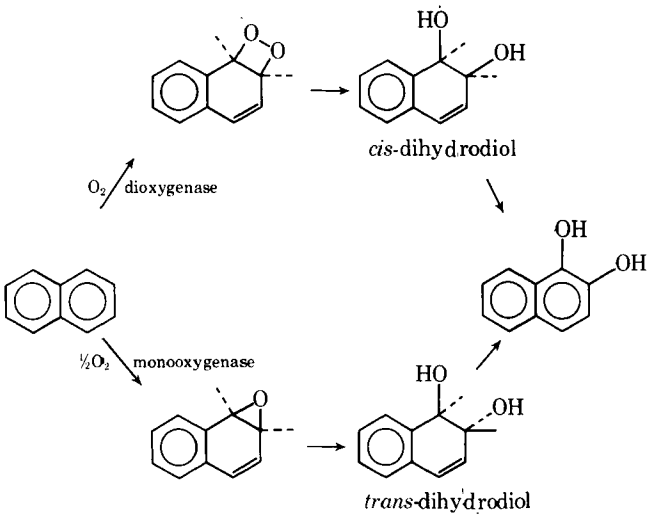
The same enzymes that form arene oxides from aromatic compounds seem to be capable of forming epoxides from isolated and conjugated olefins (32).

Catechols and their conjugates are important metabolites of certain organic compounds [*e.g.*, chlorobenzene (36)]. These metabolites appear to be formed through the action of soluble hepatic dehydrogenases on dihydrodiols rather than by hydroxylation of monophenols (37).

In many biological systems, cytochrome P-450 monooxygenases appear to be linked to epoxyhydrases. Thus, where more stable arene oxides are formed [*e.g.*, naphthalene-1,2-oxide (33, 38)], these may be converted to *trans*-dihydrodiols. When "stable"



Scheme V—Mammalian oxidative biotransformations of aromatic compounds



Scheme VI—Effect of dioxygenase versus *monooxygenase enzymes on naphthalene*

arene oxides are formed, they may participate in alkylation reactions with cellular components. Such processes may be important with respect to the toxicity and/or carcinogenicity of certain aromatic compounds (27, 28).

Lack of a primary isotope effect in enzymatic phenol formation (*e.g.*, deuterated aromatic substrates), demonstration of the NIH shift, and *trans*-dihydrodiol formation are becoming recognized as *prima facie* evidence for the presence of oxenoid monooxygenase activity (27, 28, 39). Such evidence has been useful in differentiating monooxygenases from dioxygenases in microorganisms.

In bacteria, aromatic compounds were traditionally thought to be degraded solely by dioxygenase pathways (40–43) (Scheme VI). A substrate such as naphthalene is converted through the hypothetical cyclic peroxide intermediate to a dihydrodiol and, ultimately, to the catechol derivative. These catechols are further degraded to carbon dioxide and water in bacteria. Jerina *et al.* (44) demonstrated that the reaction was dioxygenase mediated by isolating the *cis*-dihydrodiol of naphthalene, which was characterized by NMR spectral analysis. Prior to this time, a *trans*-dihydrodiol intermediate had been suggested as being predominantly involved in the degradation of naphthalene and other aromatic compounds in microorganisms (44–49). In contrast to this mechanism in microbiological systems, mammalian systems form *trans*-dihydrodiol intermediates *via* a monooxygenase oxidation step; the resulting arene oxide is opened by enzymatic hydration (33, 50, 51).

More recent data indicate that microorganisms may oxidize aromatic compounds like naphthalene through the formation of arene oxide intermediates similar to those obtained in the mammalian system. Cytochrome P-450 enzyme systems have been characterized in *Pseudomonas* species (52), *Claviceps purpurea* (53), and yeasts (54). Additionally, reactions that apparently operate through arene oxides formed in cytochrome P-450 monooxygenase systems from liver occur in fungi (55, 56).

In 1971, Auret *et al.* (55) reported the first occur-

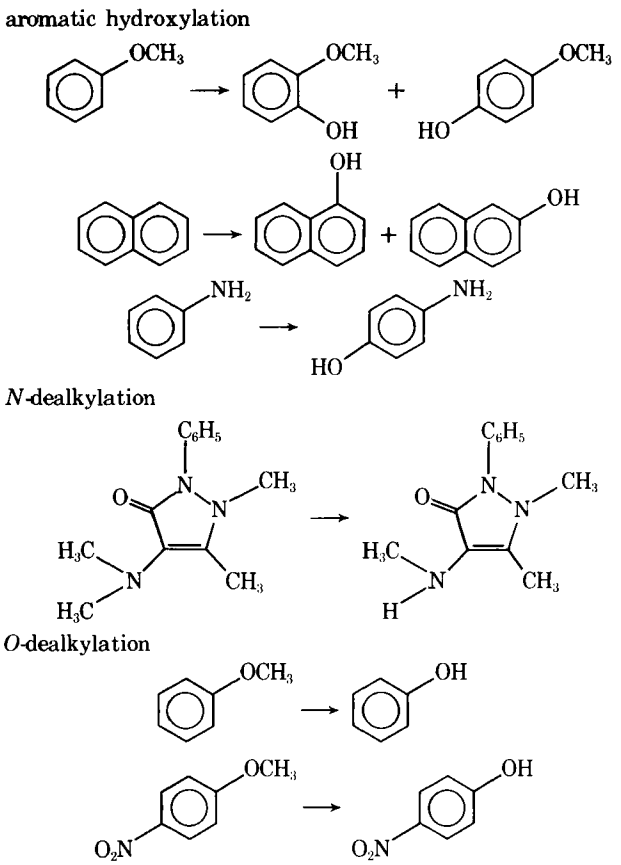
rences of the NIH shift in the hydroxylation of aromatic compounds by fungi. Both 4-D- and 2-D-anisoles were added as substrates to cultures of *Aspergillus niger, Sporotrichum sulfurescens, Cunninghamella elegans, Rhizopus arrhizus, Helicostylum piriforme, Mucor parasiticus, Rhizopus stolonifer, Curvularia falcata,* and *Cunninghamella bainieri,* which displayed the NIH shift during aromatic hydroxylations (55, 56). For *p*-anisole, 69–72% migration and retention of deuterium were observed in the 4-hydroxylated product; 35–57% migration and retention were observed with 2-hydroxylation. The magnitude of the NIH shift observed was consistent with that observed in hydroxylations of anisole by hepatic microsomes (57, 58).

Hydroxylations of these anisole derivatives and other substrates were rationalized on the basis of the involvement of arene oxide intermediates which ultimately gave the phenolic products. Several interesting observations were made on the data obtained in these early experiments:

1. Aryl oxidase enzymes of fungi appear to be similar to monooxygenases of hepatic microsomes, since both show the NIH shift and both have broad substrate specificities.
2. *ortho*-Hydroxylation appears to be predominant in fungi, while *para*-hydroxylation occurs to a greater extent in hepatic systems.
3. Polar substrates seem to oxidize more readily than nonpolar materials in fungi, while the reverse appears to be true in hepatic systems.
4. Arene oxide isomerases similar to those found in plant systems apparently are absent in fungal species.

The importance of this work has been underlined through studies by Ferris *et al.* (56) on the monooxygenase enzyme system in *C. bainieri.* When resting cells were used, monooxygenase activity nearly paralleling that seen in hepatic monooxygenase systems was observed. Naphthalene was converted into a *trans*-dihydrodiol, demonstrating the presence of the enzyme epoxide hydrase. Furthermore, a mixture of α- and β-naphthols was obtained in the ratio of 9:1; this result was very close to that obtained with *in vivo* and *in vitro* mammalian systems (33). Both 2- and 4-hydroxylations of anisole as well as *O*-dealkylation, were observed with this microorganism; however, the ratio of these products was different than that reported in mammalian systems (29). Other transformations reported included *N*-demethylation of aminopyrine, *O*-dealkylation of *p*-nitroanisole, and 4-hydroxylation of aniline (Scheme VII). Ferris *et al.* (56) observed that no other fungal or bacterial system had ever exhibited such a close metabolic parallelism to the drug-metabolizing system of liver microsomes.

In this work, 4-hydroxylation greatly exceeded 2-hydroxylation of anisole. Thus, the conclusion reached by Auret *et al.* (55) that *ortho*-hydroxylation predominates in fungal systems may be faulty, or differences in results obtained by the two groups of workers (55, 56) may be related to the experimental conditions employed by each.



Scheme VII—Transformations affected by C. bainieri (*Ref. 56*)

All previous results that pointed to the similarities in microbial and mammalian enzyme systems represented a foundation for establishing microbial models of mammalian metabolism. Our attention was initially focused on aromatic hydroxylation to test the feasibility of this proposal for three reasons: (*a*) it represents a commonly occurring mammalian biotransformation; (*b*) aryl hydroxylase activity similar to that displayed by mammalian cytochrome P-450 had been demonstrated in several microbial species (53, 55, 56); and (*c*) phenol formation, vis-à-vis arene oxide intermediates, had been implicated in mechanisms of toxicity of certain aromatic compounds (27, 28, 60).

A literature survey indicated that a number of microorganisms possessed aromatic hydroxylating activity (18, 55, 56); 11 microorganisms [*A. niger* (ATCC[1] 9142), *Penicillium chrysogenum* (ATCC[1] 10002), *Cunninghamella blakesleeana* (ATCC[1] 8688a), *Aspergillus ochraceous* (ATCC[1] 1008), *Gliocladium deliquescens* (1086), *Streptomyces* species (1158w), *R. stolonifer* (NRRL[2] 1477), *Curvularia lunata* (NRRL[2] 2178), *Streptomyces rimosus* (ATCC[1] 23955), *C. bainieri* (ATCC[1] 9244), and *H. piriforme* (QM[3] 6945)] were selected as microbial models of mammalian aromatic hydroxylation (9). A selection

[1] ATCC = American Type Culture Collection, Rockville, Md.
[2] NRRL = Northern Regional Research Laboratories, ARS, U.S. Department of Agriculture, Peoria, Ill.
[3] QM = Quartermaster Culture Collection, U.S. Army Laboratories, Natick, Mass.

Table II—Metabolites Formed by Microorganisms

Substrate	Metabolite	Organism(s) Causing Transformation
Acetanilide	2-Hydroxyacetanilide	*A. ochraceous*
	Aniline	*A. niger, Pen. chrysogenum, A. ochraceous, G. deliquescens,* and *S. rimosus*
Acronycine	9-Hydroxy-, 11-hydroxy-, and 9,11-dihydroxy-acronycines	*A. niger, C. blakesleeana, S. rimosus,* and *C. bainieri*
Aniline	Acetanilide	*C. blakesleeana* and *C. bainieri*
	2-Hydroxyacetanilide	*C. blakesleeana, A. ochraceous,* and *C. bainieri*
	4-Hydroxyaniline	*C. bainieri*
Anisole	2-Hydroxyanisole	*C. blakesleeana, A. ochraceous, G. deliquescens, Streptomyces* sp., *R. stolonifer, C. bainieri,* and *H. piriforme*
	4-Hydroxyanisole	*Pen. chrysogenum, C. blakesleeana, A. ochraceous,* and *C. bainieri*
	Phenol	*A. niger, C. blakesleeana, A. ochraceous, Streptomyces* sp., *R. stolonifer, C. bainieri,* and *H. piriforme*
	Hydroquinone	*A. ochraceous*
Benzene	Phenol	*Pen. chrysogenum, C. blakesleeana, G. deliquescens, Streptomyces* sp., and *C. bainieri*
Benzoic acid	2-Hydroxybenzoic acid	*A. niger* and *C. bainieri*
	4-Hydroxybenzoic acid	*Cur. lunata* and *C. bainieri*
	3,4-Dihydroxybenzoic acid	*A. ochraceous*
Biphenyl	2-Hydroxybiphenyl	*A. niger, S. rimosus,* and *H. piriforme*
	4-Hydroxybiphenyl	*A. niger, C. blakesleeana, Streptomyces* sp., *Cur. lunata, C. bainieri,* and *H. piriforme*
	4,4′-Dihydroxybiphenyl	*C. blakesleeana* and *C. bainieri*
Chlorobenzene	2-Hydroxychlorobenzene	*A. ochraceous, G. deliquescens, Streptomyces* sp., *R. stolonifer,* and *C. bainieri*
	4-Hydroxychlorobenzene	*A. ochraceous* and *C. bainieri*
Coumarin	7-Hydroxycoumarin	*G. deliquescens, Streptomyces* sp., *C. bainieri,* and *H. piriforme*
Naphthalene	1-Hydroxynaphthalene	*A. niger, C. blakesleeana, A. ochraceous, Streptomyces* sp. *stolonifer, C. bainieri,* and *H. piriforme*
	2-Hydroxynaphthalene	*A. niger, C. bainieri,* and *H. piriforme*
trans-Stilbene	*trans*-4-Hydroxystilbene	*A. niger, C. blakesleeana, A. ochraceous, Streptomyces* sp., *R. stolonifer,* and *C. bainieri*
	trans-4,4′-Dihydroxy-stilbene	*Streptomyces* sp. and *C. bainieri*
Toluene	2-Hydroxytoluene	*Pen. chrysogenum, A. ochraceous, Streptomyces* sp., *R. stolonifer, C. bainieri,* and *H. piriforme*
	4-Hydroxytoluene	*Pen. chrysogenum, R. stolonifer,* and *C. bainieri*

of aromatic compounds was chosen to be incubated with growing cultures of each microorganism. These included acetanilide, acronycine, aniline, anisole, benzene, benzoic acid, biphenyl, chlorobenzene, coumarin, naphthalene, nitrobenzene, *trans*-stilbene, and toluene. Mammalian systems have been shown to hydroxylate each of these compounds, and this permitted a comparison of mammalian and microbial aromatic hydroxylase activities. Furthermore, these compounds represent the aromatic portions of numerous chemical entities of environmental importance and of drugs.

All of these compounds were incubated with growing cultures of the 11 microorganisms. The results of TLC analyses (61, 62) of the fermentations are summarized in Table II.

The microbial system demonstrated substantial capacity to hydroxylate simple substituted benzenoid compounds. 2- and 4-Hydroxylations were observed in all cases, except with acetanilide where the 2-hydroxyacetanilide product was obtained exclusively. In earlier reported work, Theriault and Longfield (63) showed a *Streptomyces* species to be capable of hydroxylating acetanilide exclusively in the 4-position, while one *Basidiomycete* hydroxylated acetanilide in the 2-position. Hydroxylation at either the 2- or 4-position almost exclusively was observed for microorganisms with anisole, benzoic acid, aniline, and chlorobenzene (9). The microbial metabolites obtained are compared to the mammalian metabolites reported from *in vitro* and *in vivo* metabolism studies in Table III. In general, a good correlation was obtained between the microbial and mammalian metabolizing systems. Nitrobenzene was the only simple substituted benzenoid compound that failed to yield hydroxylated products with the microbial system. The 2-, 3-, and 4-hydroxynitrobenzenes are obtained with mammalian systems.

Additional biotransformations occurred with these substrates. Five microorganisms demonstrated ami-

Table III—Comparison of Metabolites Formed by Microorganisms and Mammalian Systems

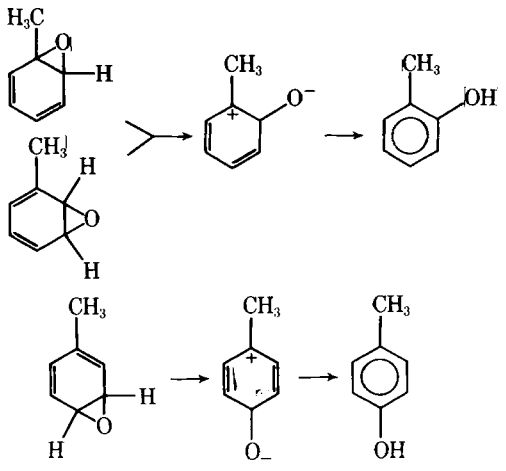
Substrate	Microbial Metabolite[a]	Mammalian Metabolite	
		In Vitro (Reference)	*In Vivo* (Reference)
Acetanilide	2-Hydroxyacetanilide and aniline	2-Hydroxyacetanilide, 4-hydroxyacetanilide, and aniline (57, 64)	4-Hydroxyacetanilide and aniline (65)
Acronycine	9-Hydroxy-, 11-hydroxy-, and 9,11-dihydroxyacronycines	Not reported	9-Hydroxy-, 11-hydroxy-, and 9,11-dihydroxyacronycines (66)
Aniline	Acetanilide, 2-hydroxyacetanilide, and 4-hydroxyaniline	4-Hydroxyaniline (57)	Acetanilide and 2-hydroxy-, 3-hydroxy-, and 4-hydroxyanilines (67)
Anisole	2- and 4-Hydroxyanisoles and phenol	2- and 4-hydroxyanisoles and phenol (64)	2- and 4-Hydroxyanisoles (68)
Benzene	Phenol	Phenol (57)	Phenol (69)
Benzoic acid	2- and 4-Hydroxybenzoic acids and 3,4-dihydroxybenzoic acid	3-Hydroxybenzoic acid (57)	2-, 3-, and 4-Hydroxybenzoic acids (71)
Biphenyl	2- and 4-Hydroxybiphenyls and 4,4′-dihydroxybiphenyl	2- and 4-Hydroxybiphenyls (57, 70)	4-Hydroxy-, 3,4-dihydroxy-, and 4,4′-dihydroxybiphenyls (72)
Chlorobenzene	2- and 4-Hydroxychlorobenzenes	2-, 3-, and 4-Hydroxychlorobenzenes (57)	2-, 3-, and 4-Hydroxychlorobenzenes (73)
Coumarin	7-Hydroxycoumarin	7-Hydroxycoumarin (74)	3- and 7-Hydroxycoumarins [4-, 5-, 6-, and 8-hydroxycoumarins[b] (75, 76)]
Naphthalene	1- and 2-Hydroxynaphthalenes	1- and 2-Hydroxynaphthalenes (33, 59)	1- and 2-Hydroxynaphthalenes (77)
Nitrobenzene	—	4-Hydroxynitrobenzene (57)	2-, 3-, and 4-Hydroxynitrobenzenes and 2-, 3-, and 4-hydroxyanilines (78, 79)
trans-Stilbene	*trans*-4-Hydroxy- and *trans*-4,4′-dihydroxystilbenes	*trans*-4-Hydroxystilbene and *trans*-4,4′-dihydroxystilbene (80)	*trans*-4-Hydroxy-, *trans*-3,4-dihydroxy-[c], and *trans*-4,4′-dihydroxystilbenes (80)
Toluene	2- and 4-Hydroxytoluenes	2- and 4-Hydroxytoluenes and benzyl alcohol (57)	Benzoic acid and conjugates (67)

[a] See Ref. 9. [b] Formed in trace quantities. [c] Presumed metabolite based on characterization of mono-*O*-methyl ethers.

dase activity in removing the acetyl group from acetanilide, a minor mammalian biotransformation. Acetylase activity was observed in four microorganisms with aniline; this pathway is common in mammals. *A. ochraceous* gave good yields of 2-hydroxyacetanilide when incubated with acetanilide as the substrate. In this microorganism, acetylation apparently must precede hydroxylation. This requirement for an amide functional group in the hydroxylation of various substrates was reported by Johnson *et al.* (81) and Fonken *et al.* (82), who have observed that in an extensive series of carbocyclic amines and nitrogen heterocycles an acyl substituent is required on the nitrogen atom for hydroxylation to occur.

With anisole, seven microorganisms caused *O*-demethylation. This reaction was common and occurred with cultures that caused 2-hydroxylation as well. Our results were consistent with those observed by Auret *et al.* (55). *O*-Demethylase activity was reported before, particularly with certain *Pseudomonads* (83, 84) and with *A. niger* (85). With our culture of *A. niger* (9), *O*-demethylase activity was dominant; phenol was the only metabolite of anisole detected. With *A. ochraceous*, greater than 50% conversion of benzoic acid to 3,4-dihydroxybenzoic acid was obtained (9). Microorganisms convert benzoic acid into various hydroxylated products (85–87); in fungi, 3,4-dihydroxybenzoic acid is commonly produced with 3- or 4-hydroxybenzoate serving as intermediates (86, 87).

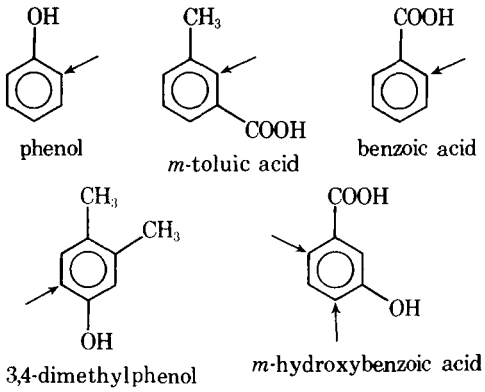
Another interesting, albeit somewhat hidden, result was obtained with the microbial system of Smith and Rosazza (9). Although the number of microorganisms in the study was insufficient to establish a complete trend, ring-activating groups generally resulted in a greater number of microorganisms giving hydroxylated products and provided higher yields. As expected, the products obtained were hydroxylated *ortho* and *para* to ring substituents, a result that may be predicted by considering a composite of the various cationic intermediate forms of possible epoxide ring openings as shown in Scheme VIII (27). The relative reactivities of simple benzenoid compounds (R—C_6H_5) toward microbial hydroxylases follow the order R = OCH_3 > NH_2, CH_3 > H, Cl, $NHCOCH_3$ > NO_2 (9). The highest yields and inci-



Scheme VIII—Conceptualized cationic intermediates associated with ring openings of arene oxides

dences of microbial hydroxylation occurred with anisole, while no hydroxylation was obtained with nitrobenzene as the substrate. Benzoic acid was eliminated from the sequence because it is a more natural substrate for microorganisms.

Omori and Yamada (88) related the position of hydroxylation of several simple aromatic compounds to their electronic structures. The π-electron distributions of various aromatic compounds selected from the literature were calculated using the assumption that microbiological hydroxylation occurs *via* a radial type of mechanism. With phenol, *m*-toluic acid, benzoic acid, 3,4-dimethylphenol, and *m*-hydroxybenzoic acid, good correlation was obtained between the position of hydroxylation [shown by arrows (88)] and the



points of highest calculated frontier electron densities. This work represented a purely theoretical approach to the problem of predicting sites of hydroxylation since no experimental verification of the results was reported. Furthermore, the simple compounds studied render themselves quite readily to simple first-order predictions regarding sites of oxygenation. A more vigorous application of this approach would involve complicated substrates which, after predicting sites of hydroxylation, could be incubated with selected microorganisms.

Excellent correlation between the metabolites obtained in the microbial system of Smith and Rosazza (9) and mammalian *in vitro* and *in vivo* systems was observed with more complicated aromatic compounds such as acronycine, biphenyl, coumarin, naphthalene, and *trans*-stilbene (Table III). A somewhat stronger parallel existed between the *in vitro* mammalian studies and the microbial model system with biphenyl, chlorobenzene, coumarin, and *trans*-stilbene. Table III shows some of the discrepancies in results obtained in *in vitro* and *in vivo* metabolic studies. These findings indicate that intercomparison between *in vivo* and *in vitro* metabolic systems is not always valid and, in fact, may be no more valid than comparisons of metabolic profiles of microorganisms and liver systems.

The production of 9-hydroxyacronycine as a microbial metabolite by *Cunninghamella echinulata* serves as an excellent example of the potential for obtaining gram quantities of difficult-to-synthesize compounds *via* microbial aromatic hydroxylation (61). Acronycine is an antitumor compound, and its metabolism has been studied in humans, rats, pigs, dogs, and mice (66). The major metabolite produced in mammalian systems is 9-hydroxyacronycine, although minor amounts of other metabolites are also observed. In the microbial system (9), acronycine was hydroxylated by four microorganisms in the 9-position. Other metabolites also were detected in small-scale fermentation screening studies (9, 61). In a scaled-up fermentation, 4 g of acronycine was added as the substrate to 7.5 liters of a fully grown culture of *C. echinulata;* a 31% yield of 9-hydroxyacronycine was isolated and fully characterized (61).

Other examples of the use of microbial transformations for the production of hydroxylated metabolites are 5-anilino-1,2,3,4-thiatriazoles (89), fenclozic acid (90), 5-hydroxytryptophol (17), α-methylfluorene-2-acetic acid (91), and levodopa (18).

Mammalian *N*-, *O*-, and S-Dealkylations—Hydroxylation of sp^3 carbon atoms alpha to a nitrogen, oxygen, or sulfur atom generally leads to dealkylation due to the instability and subsequent rearrangement of the C—OH intermediates. The *N*-, *O*-, and S-dealkylations are important biotransformations, because they can significantly alter the lipid solubility and biological potency of drugs.

N-Dealkylation of numerous amines, amides, and imides has been described. Tertiary amines containing dimethylamino functions are often rapidly *N*-demethylated to their corresponding secondary amines, while the latter are much more slowly transformed into primary amines (92). This finding is in accord with the report that *N*-demethylation rates are directly proportional to lipid solubility and inversely proportional to pKa values (93).

The rate of *N*-dealkylation generally seems to decrease as the size and/or complexity of the *N*-alkyl groups increase (94–97). However, Abdel-Monem and Portoghese (98) found that the degree of *N*-dealkylation increased in a series of *N*-substituted normeperidines, going from methyl to ethyl to *n*-propyl, and then progressively decreased with homologs from *n*-butyl to *n*-nonyl. Because of the lack of an α-hydrogen, *N*-*tert*-butyl groups should be resistant to *N*-dealkylation. However, Kamm and coworkers (99, 100) showed that, by a sequence of oxidation reactions (beginning with hydroxylation of the C-methyl group), this alkyl function can be removed *in vivo* and *in vitro*. *N*-De-*tert*-butylation also emphasizes the fact that C-oxidation probably becomes more important as the size of the *N*-alkyl function increases.

A significant degree of stereospecificity has been shown in the *N*-dealkylation of several enantiomeric pairs of drugs. In light of the fact that biological activity often resides in one enantiomer, this observation deserves further attention (101).

Axelrod (102) first reported that *O*-alkyl functions were cleaved in a similar fashion to *N*-alkyl groups. In addition, *para*-substituted ethers are more readily cleaved than their corresponding *ortho*- or *meta*-isomers (102). In 22 *p*-nitrophenyl ethers, the rate of *O*-dealkylation decreased as the size and/or complexity of the *O*-alkyl function increased (103). Creaven *et al.* (104) found a similar relationship for a homologous series of alkoxy biphenyls, although they noted

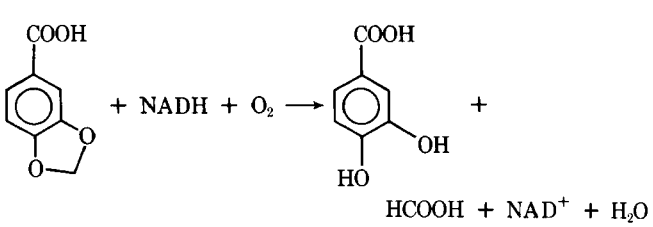
an increase in *O*-dealkylation from methyl to ethyl. Tsukamoto *et al.* (105) reported that *n*-butyl-*p*-nitrophenyl ethers were slowly *O*-dealkylated while ω-1 hydroxylation of the *n*-butyl function predominated in rabbits. The dimethylaminoethyl ether function, often incorporated into drug substances, appears to be quite resistant to *O*-dealkylation *in vitro* and *in vivo* in rats (103).

S-Dealkylation has not been extensively studied, primarily because alkyl–aryl and alkyl–alkyl thioethers are not widely used in drugs. Sulfides embodied in heteroaromatic systems are fairly prevalent among drug entities but are more prone to S-oxidation. The few reported studies indicate that S-demethylation proceeds by a similar mechanism to *N*- and *O*-dealkylation in intact animals and with hepatic microsomal systems (92).

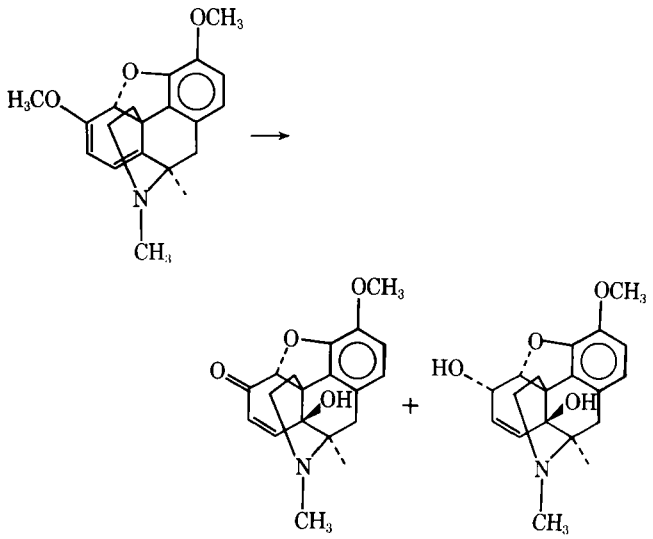
Microbial *O*-Dealkylations—The biodegradation of *O*-alkyl aromatic ethers in bacteria is a monooxygenase-mediated process requiring pyridine nucleotides and oxygen (84). In general, an active form of oxygen attacks the α-carbon of alkyl ethers; the former alkyl group, which exists as an unstable hemiacetal, is easily removed.

Bernhardt *et al.* (84) prepared a cell-free extract and a purified enzyme system from *Pseudomonas putida* capable of *O*-demethylating 4-methoxybenzoate. The purified enzyme system consisted of an iron-flavoprotein and an iron-sulfur protein and was obtained from *P. putida* cells grown on 4-methoxybenzoic acid as the sole carbon source. NADH and oxygen represented cofactor requirements for this enzyme. The purified system acted on many different substrates, but the greatest affinity was observed for 4-methoxybenzoate itself. *para*-Substituted benzoic acid derivatives were the best substrates, while *m*- and *o*-benzoic acids were only slowly demethylated. In this microbial enzyme system, the carboxyl group was an absolute requirement for *O*-demethylation.

Methylenedioxy compounds, which are represented in many natural products, are also converted to their respective phenolic products by *Pseudomonas* species (83). Demethylenation has been reported to occur in NADPH-dependent monooxygenases of rat liver (106). The essential features of the reaction are shown in Scheme IX. Each reaction product was characterized. The catabolism of piperonylic acid by *Pseudomonas fluorescens* appears to be an example of cooxidation in that the methylenedioxy compound did not serve as the sole carbon and energy source and was only degraded when 4-methoxybenzoate (anisic acid) was present. *O*-Dealkylation of various simple methoxylated compounds such as 3-hydroxy-



Scheme IX—Microbial O*-demethylenation of 3,4-methylenedioxybenzoic acid by* Pseudomonas *species (Ref. 83)*



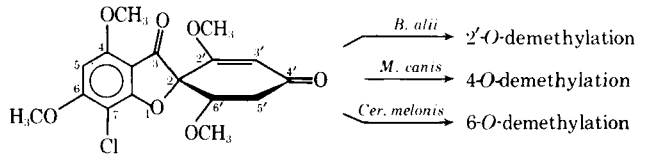
Scheme X—Microbial transformations of thebaine (Refs. 110 and 111)

4-methoxybenzoate (107), anisole (9, 56, 108), and methyl anisate (109) also has been reported in fungi.

Several more complicated examples of *O*-dealkylation have been reported. The alkaloid thebaine undergoes a series of reactions in which the methoxy group is lost (Scheme X). The mechanism by which this reaction occurs is obscure and may involve concerted elimination of the methyl group as a result of hydroxylation (110, 111). The alkaloid aconitine obtained from *Aconitum napellus* (Wolfbane) is also *O*-demethylated by *Streptomyces paucisporogenes* to a compound that is less toxic than the parent alkaloid (112). Boothroyd *et al.* (113) studied the demethylation of griseofulvin by fungi. Several microorganisms were screened for *O*-dealkylase activity; four of these, *Microsporum canis, Alternaria solani, Botrytis alii,* and *Cercosporum melonis,* could metabolize griseofulvin to phenolic products. Each of the three methoxy groups on the griseofulvin molecule could be selectively removed by a different microorganism (Scheme XI). Such selective *O*-demethylase activity has great synthetic utility.

An unusual example of the reverse reaction, *O*-alkylation, was reported (114). With *Lentinus lepideus* surface cultures, *p*-hydroxycinnamic acid was converted into 4-methoxycinnamate in the presence of methionine. This result was confirmed by using ^{14}C-labeled methionine. The methoxy group formed was cleaved by the same fungus when prolonged incubations were conducted.

We have conducted several experiments on microbial *O*-dealkylations, predominantly with methoxylated aporphine and benzylisoquinoline derivatives

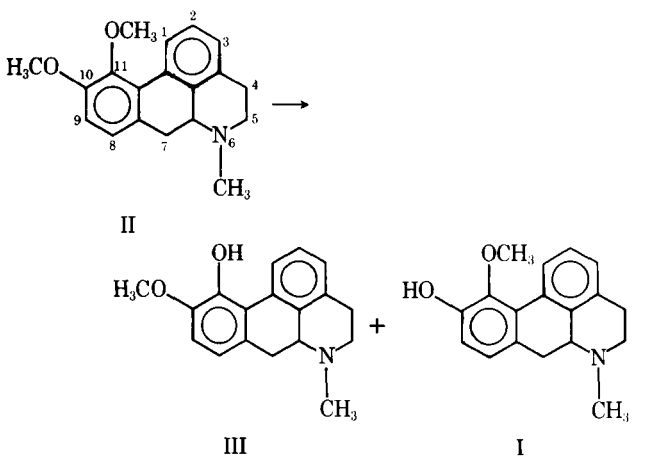


Scheme XI—Microbial O*-demethylation of griseofulvin (Ref. 113)*

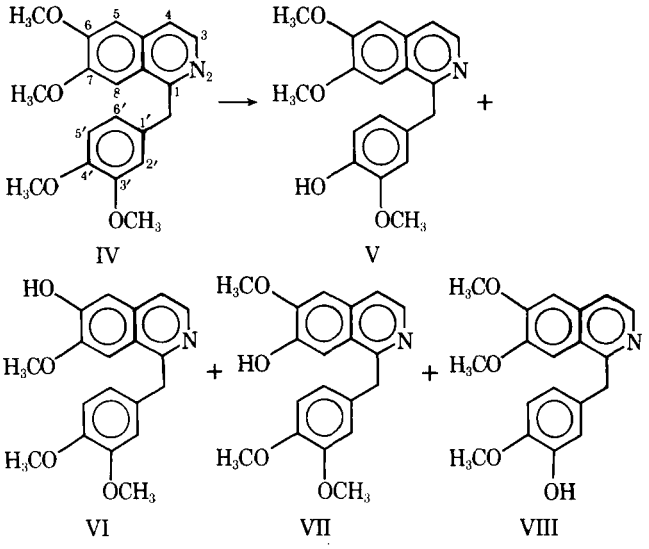
(115). Cannon *et al.* (116) studied the synthesis of the isomeric monomethyl metabolites of apomorphine and metabolism of apomorphine in a catechol-*O*-methyltransferase liver preparation. Considerable difficulty was encountered in the synthesis of one of the possible monomethyl ether isomeric metabolites, isoapocodeine (I). In preliminary experiments, we demonstrated that a *Streptomyces* species converts 10,11-dimethoxyaporphine (II) into an equal mixture of apocodeine (III) and I in about 50% estimated yield (Scheme XII). This process occurs within 24 hr after the substrate is added to growing cultures of the microorganisms.

Two cultures of the microorganisms, *C. blakesleeana* and *C. echinulata,* yielded I as the predominant metabolite, with only traces of III being formed. These results are interesting in view of the results obtained by Cannon *et al.* (116), where the ratio of apocodeine–isoapocodeine obtained in the mammalian liver system was about 75:1. It was rationalized that the formation of isoapocodeine was prohibited due to the sterically hindered hydroxy group at position 11 (Scheme XII). One might expect the reverse reaction (*i.e.,* *O*-dealkylation at position 10) also to be influenced so that isoapocodeine would predominate. Our findings with *C. blakesleeana* and *C. echinulata* (115), which produced mostly isoapocodeine, support the result of Boothroyd *et al.* (113) that selective *O*-dealkylation may be achieved with different microorganisms.

We have also been studying the microbial metabolism of the drug papaverine (IV) (115). Papaverine has been used in treating cerebral and peripheral ischemia associated with arterial spasm (117). The metabolic disposition of papaverine in humans and other mammals has been studied (118). Negligible amounts of the drug are excreted unchanged in urine, indicating that it must be nearly completely metabolized. Only one compound, 4′-*O*-desmethylpapaverine (V), was chemically characterized in the mammalian studies (118). We are developing microbial systems capable of providing gram quantities of all possible *O*-dealkylated isomers, which will be used for structure elucidation and biological testing.



Scheme XII—Microbial O-demethylation of 10,11-dimethoxyaporphine



Scheme XIII—Microbial O-demethylation of papaverine

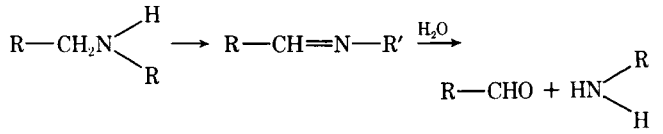
C. echinulata is capable of producing four phenolic metabolites of papaverine (115); two of these have been chromatographically identified as 6-hydroxy-7-methoxy-(3′,4′-dimethoxybenzyl)isoquinoline (VI) and 7-hydroxy-6-methoxy-(3′,4′-dimethoxybenzyl)-isoquinoline (VII) (Scheme XIII). The 3′-hydroxy-4′-methoxybenzyl-6,7-dimethoxyisoquinoline product (VIII) [or its 4′-hydroxy-3′-methoxy isomer (V)] is believed to be the sole product with *C. blakesleeana* (115). Again, this finding demonstrates the selectivity in *O*-dealkylation that may be achieved with microorganisms. Screening of microorganisms is being continued to detect other cultures that might selectively *O*-dealkylate similar systems.

Microbial *N*-Dealkylations—According to Large (119), three major types of pathways are involved in the *N*-dealkylation of amines by microorganisms (Scheme XIV). The oxidases may involve the intermediacy of hydrogen peroxide, while the oxygen of the aldehyde produced comes from water in both the oxidases and dehydrogenases. The third type of reaction is mediated by monooxygenases where molecular oxygen is the source of the oxygen in the aldehyde product.

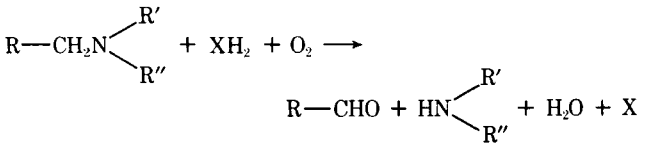
Secondary and tertiary amines are degraded by more than one pathway by bacterial monooxygenases. In *Pseudomonas aminovorans,* trimethylamine is converted into trimethylamine *N*-oxide by an NADPH-dependent tertiary amine monooxygenase enzyme (Scheme XV). The trimethylamine *N*-oxide is then nonoxidatively demethylated by trimethylamine *N*-oxide demethylase to formaldehyde and dimethylamine.

This latter enzyme has been isolated in two bacteria, *P. aminovorans* and a *Bacillus* species (120). This type of *N*-demethylation of an *N*-oxide is apparently not restricted to microbial systems but has been described in the metabolism of tertiary amines by liver microsomal systems (121). Similarities and differences between microsomal and bacterial amine monooxygenases have been compiled (119). The similarities include secondary amines converted to pri-

oxidases and dehydrogenases



monooxygenases

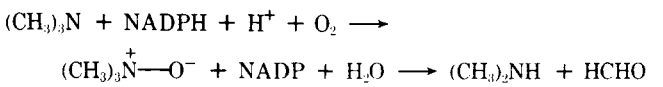


Scheme XIV—Types of microbial N*-dealkylations*

mary amines plus the appropriate aldehyde; tertiary amines converted to amine oxides; tertiary amine oxidation not inhibited by carbon monoxide, cyanide, or proadifen hydrochloride[4]; amine oxide enzymatically converted to secondary amine plus aldehyde; and NADH and NADPH serving as active electron donors.

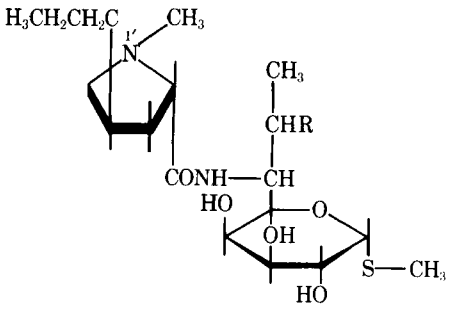
However, in the microsomal system, identical enzymes *N*-dealkylate secondary and tertiary amines, a very wide range of compounds are oxidized, and amine oxide demethylase is inhibited by carbon monoxide, proadifen hydrochloride, and pyridine but not cyanide. In contrast, in the bacterial system, different enzymes affect *N*-dealkylation of secondary and tertiary amines; active substrates must possess one of these structures: CH_3NHR, CH_3CH_2NHR, $(CH_3)_2NR$, or $(CH_3CH_2)_2NR$, where R = C_1 to C_4; and amine oxide demethylase of *P. aminovorans* is inhibited by cyanide but not carbon monoxide, proadiphen hydrochloride, or pyridine. Most importantly, substrate specificity of bacterial *N*-dealkylases appears to be narrower than those of mammalian systems. However, this has not yet been fully explored.

Microbial *N*-dealkylations have been observed in more complicated structures than those already described, although few studies have been conducted concerning the mechanism(s) of these reactions. Clindamycin (IX) and lincomycin (X) are related antibiotics metabolized by various *Streptomyces* species (122, 123). Argoudelis *et al.* (122) produced the 1′-demethylclindamycin compound when clindamycin was added to fermentations of *Streptomyces punipalus*. However, when the compound was metabolized by *Streptomyces armentosus*, primarily the clindamycin sulfoxide was obtained. Other species of microorganisms could convert clindamycin to either the sulfoxide or the 1′-demethylated product or a mixture of both (122). These transformations are significant because the latter two compounds are mammalian metabolites of clindamycin (124), while the *N*-demethylated product is about twice as active as the



Scheme XV—Dealkylation of trimethylamine by P. aminovorans

[4] SK&F 525-A.



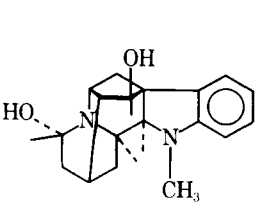
IX: R = Cl
X: R = OH

parent antibiotic. Lincomycin, a structurally related antibiotic, also undergoes *N*-demethylation and sulfoxidation (123). The product in the latter case, however, is approximately one-twentieth as active as the parent antibiotic.

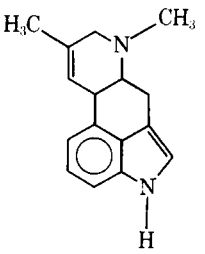
Various compounds that are either drugs or structurally related to classes of drugs are *N*-dealkylated by microorganisms. Bellet and Van Thuong (125) reported the *N*-dealkylation of the dihydroindole alkaloid ajmaline (XI) in 40% yields with a culture of *Streptomyces platensis*. *N*-Demethylation of agroclavine (XII) (126) and certain 6,14-*endo*-ethenotetrahydrothebaine alkaloids (such as XIII) (127) by *C. echinulata* has been reported. Mitscher *et al.* (127) commented on the parallel between the latter *N*-dealkylation and a similar biotransformation observed in mammalian systems.

Tropine (XIV), the basic nucleus of a number of anticholinergic drugs, is *N*-demethylated by *Arthrobacter atropini;* the tranquilizer diazepam (XV) is similarly transformed by *Pellicularia filamentosa* and in 12% yields by *Sp. sulfurescens* (12). This latter transformation again represents a parallel to mammalian metabolism.

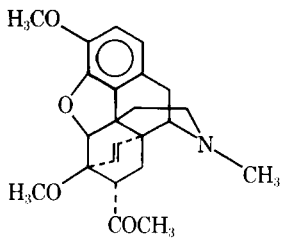
Methamphetamine is *N*-demethylated by the intestinal microflora of guinea pigs. Observing differences in recoveries of metabolites of methamphetamine (XVI) fed to animals, Caldwell and Hawksworth (128) concluded that microflora of the intestine could play a role in degrading the drug. The



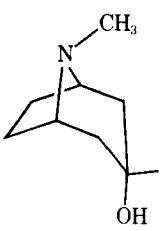
XI



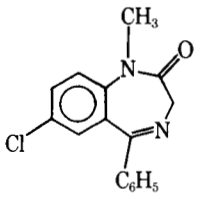
XII



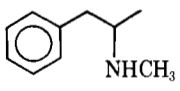
XIII



XIV



XV



XVI

cecal contents of Dunkin–Hartley guinea pigs were removed and incubated with (±)-2-methylamino-1-phenyl-1-^{14}C-propane hydrochloride. Twenty-nine percent of the dose was recovered unchanged while the *N*-dealkylated product, amphetamine, was recovered in 47% yield. An unknown product was also observed. Several genera of human gut bacteria were isolated and examined for their potential to metabolize methamphetamine. In particular, *Lactobacilli, Enterococci,* and *Clostridia* showed the ability to metabolize methamphetamine.

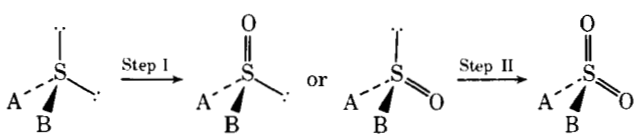
Little is known concerning microbial S-dealkylations. The reported conversion of the thioether group of lincomycin (X) to a hydroxy function by *Streptomyces lincolnensis* (123) might involve S-dealkylation.

S-Oxidations—Sulfides are metabolized to sulfoxides and sulfones in mammals (67) (Table I). No systematic studies have been performed on these biotransformations. The fact that certain sulfide-containing drugs could be converted to optically active sulfoxides (129) appears to have been overlooked. From work with model compounds, there is some reason to believe that low stereoselectivity will be observed with mammalian sulfoxidations (*vide infra*).

Microbiological sulfur oxidations recently have been studied. In general, the oxidation of thioethers (sulfides) proceeds stepwise through the sulfoxide (Step I) to the sulfone derivatives (Step II), as shown in Scheme XVI. In microorganisms, it is possible to stop the reaction at the sulfoxide level, obtaining only small amounts of the sulfone derivative. Two primary advantages exist in using microbiological systems in the oxidation of sulfur-containing compounds:

1. Selective oxidations are possible when other labile groups are found on the substrate molecules.
2. Stereospecificity is obtained to a variable extent, sometimes resulting in sulfoxide products with high degrees of optical purity (12).

One early example of microbial production of optically active sulfoxides was in the isolation of very low yields of (−)-biotin sulfoxide formed as a metabolite of biotin by *A. niger* (130). More recently, Auret *et al.* (131–133) conducted extensive studies related to the microbiological formation of optically pure sulfoxides. *A. niger* cultures and acetone powders obtained from this microorganism produce optically active sulfoxides when unsymmetrical thioethers are added as substrates to fermentations (131). Stereoselectivities ranging from 4 to 100% were observed, depending on the thioethers used as substrates. In the case of *tert*-butyl-*p*-tolyl sulfide, the *R*-(+)-sulfoxide was obtained with a higher optical rotation than ever before recorded.



Scheme XVI—Microbiological oxidation of sulfides

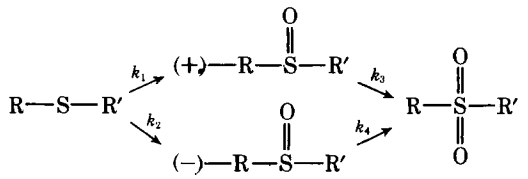
As indicated in Scheme XVI, the absolute configuration of products reflects a demand of various groups for either position A or B. A *p*-tolyl group, for example, prefers position A, while a *tert*-butyl prefers position B. When both substituents were present on the same substrate molecule, optical purities of 94% were obtained (24% yield). Interestingly, with *tert*-butyl-*p*-tolyl sulfide, acetone powders of *A. niger* cells yielded 98–100% optically pure sulfoxide in about 25% yield. With *p*-tolyl sulfides, stereoselectivity in forming sulfoxides depended on the degree of substitution of the α-carbon atom where it was found that alkyl = *tert*-butyl (99%), isopropyl (70%), and *n*-butyl (32%) (average optical purities in percent).

Auret *et al.* (132) extended this work by describing a process whereby optically pure sulfoxides could be obtained from racemic sulfoxide mixtures. When growing cultures of *A. niger* were used, both sulfoxide and sulfone products are obtained, whereas acetone powders seem to be capable of stopping the reaction at the sulfoxide stage. With growing cultures, it was suggested that optical activities observed with sulfoxides could arise partly by further selective oxidation of one or the other sulfoxide intermediate. This proposal is illustrated in Scheme XVII.

To check this hypothesis, racemic mixtures of various sulfoxides were added to growing cultures of *A. niger.* In some instances, the recovered sulfoxides were optically active (132). In addition to substituent effects and selective oxidation of sulfoxides, the influence of species and strain of microorganisms on stereoselectivity of sulfur oxidations was studied (133). Strains originating from the same parent culture yielded sulfoxide products of different configuration and in different yields. This result suggests the importance in reproducing in detail the experimental conditions reported in the literature with this and possibly other biotransformations.

Although a similarity in the pattern of products formed by the hydroxylation of aromatic compounds and other type of reactions by microorganisms and mammalian systems has been noted, stereoselectivity in the oxidation of thioethers to sulfoxide apparently is lower in animals (rat liver microsomes) than in fungal processes. Auret *et al.* (133) incubated *p*-tolyl benzyl sulfide with a rat liver microsomal preparation and obtained a sulfoxide only slightly enriched (1.3% optical purity) in the *R*-enantiomer.

Other examples of the microbial sulfoxidation of drug substances can be cited. Both lincomycin (X) and clindamycin (IX) may be converted into their respective sulfoxide derivatives. *S. lincolnensis* yields lincomycin sulfoxide and 1-demethylthio-1-hydroxylincomycin (123), while *S. armentosus* produces primarily clindamycin sulfoxide (122). *Calonectria de-*



Scheme XVII—Enantiomeric enrichment of sulfoxide racemates; optical purity enhanced when $k_3 > k_4$ *or* $k_4 > k_3$ *(Ref. 132)*

cora converts the steroidal thioether (XVII) into its optically active sulfoxide (134).

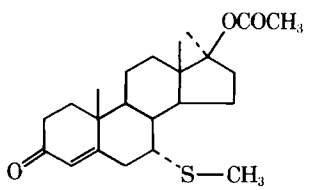
N-Oxidations—Since the *N*-oxidation products of numerous therapeutic agents possess enhanced toxicities, there is considerable interest in this biotransformation (135, 136). The instability and/or low incidence of *N*-oxidized metabolites, however, have hampered such investigation.

In the mammalian system, *N*-oxidation of primary and secondary amines leads to hydroxylamines, while the oxidation of tertiary amines results in *N*-oxides (Table I). These two types of metabolites possess considerably different physicochemical properties. Because certain arylamines are believed to cause methemoglobinemia or tumor formations *via* hydroxylamine metabolites, there has been particular interest in the *N*-oxidation of this class of compounds.

The extent of *N*-oxidation is affected by the nature of the substituent groups on the nitrogen atom. *N*-Methyl- and *N*-ethylaniline are *N*-hydroxylated much faster than aniline. As the alkyl chain length increases, however, the degree of *N*-hydroxylation decreases and the amount of *para*-hydroxylation increases (137). *N*-Hydroxylation is also enhanced in *para*-substituted anilines (136). *ortho*-Substitution seems to inhibit *N*-hydroxylation of *N*-arylamines (135).

N-Hydroxylated metabolites have been reported for *N*-acylated aromatic amines (135), a variety of alicyclic and aliphatic amines (138–141), and the simple carbamate urethan (136). Furthermore, Beckett (140) reported significant stereochemical influences on *N*-hydroxylation *versus* *N*-dealkylation in *N*-benzylamphetamines.

N-Oxides are generally formed as minor metabolites of tertiary amines. Yet, there continues to be considerable interest in these biotransformation products because of their suspected roles in biological activity and/or toxicity of certain drugs (142). *N*-Oxides have been implicated as intermediates in the *N*-dealkylation of tertiary amines, although evidence has been provided to contradict this implication (121, 135, 142). Correlations between structures of tertiary amines and their potential for *N*-oxide formation apparently have not been studied in mammalian systems.

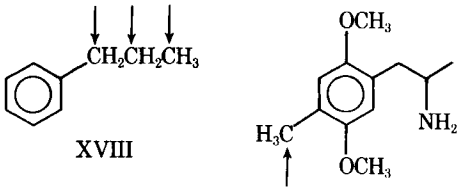


XVII

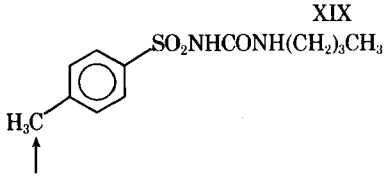
In microorganisms, several examples may be cited in which primary amines are metabolized to their respective nitro analogs. The reaction conducted by *Streptomyces thioluteus* was studied with a series of aromatic amines such as *p*-aminobenzoic acid (143–145). Rates of conversion were slower with *p*-aminophenylacetic acid and *p*-amino-L-phenylalanine, indicating a rather narrow substrate specificity for this reaction. Mechanistically, it was proposed that the primary amino group was converted into a hydroxylamine by the action of an oxygenase and molecular oxygen; this intermediate is subsequently oxidized to the corresponding nitro derivative. Other examples of the amino to nitro oxidation include various aminoimidazoles (146, 147) and the conversion of a chloramphenicol amine precursor to the antibiotic (148).

The production of *N*-oxides by microorganisms has not been widely described, because chemical conversions of tertiary amines to their *N*-oxides may be accomplished with relative ease. Microorganisms were used to make the *N*-oxides of hydroxycodeinone analogs (111) and strychnine and brucine (149). The formation of trimethylamine *N*-oxide by *P. aminovorans* was described earlier (119). Enzymes involved in this microbial oxidation were not fully characterized with regard to substrate selectivity.

Hydroxylation of Aliphatic Compounds—In mammals, acyclic aliphatic compounds are typically hydroxylated at ω and ω-1 positions. Microorganisms also attack terminal and subterminal carbons in series of alkanes (150, 151). When an aliphatic chain is attached to an aromatic ring, the resulting benzylic position becomes prone to hydroxylation by mammalian (152) and microbial (153) systems. Substrates typically hydroxylated by mammals at aliphatic carbons include *n*-propylbenzene (XVIII) (152), the psychotomimetic 1-(2,5-dimethoxy-4-methylphenyl)-2-aminopropane (XIX) (154), and tolbutamide (XX) (155, 156) (site of hydroxylation indicated by arrows).



XVIII XIX



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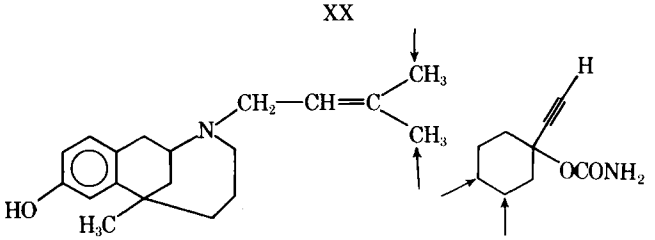
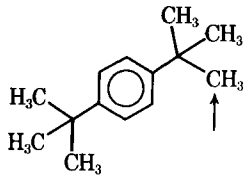
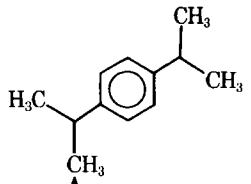


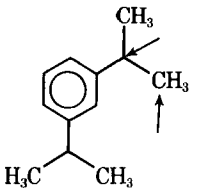
XXIV XXV



XXI



XXII



XXIII

The microbial oxidation of several branched aliphatic substrates (XXI–XXIII) has been systematically studied (153) (site of hydroxylation indicated by arrows). Products obtained with XXII were optically active. By shifting the isopropyl function to a 1,3-orientation on the benzene ring, mixtures of products, some exhibiting optical activity, were obtained (153). In mammalian systems (32), isotopic labeling experiments suggest that alkyl side-chain oxygenations occur by direct insertion of oxygen atoms.

Compounds with allylic carbon atoms, such as pentazocine (XXIV), are hydroxylated in mammalian systems (157). Microbial oxidations of allylic and benzylic positions have been adequately described (12). Ring positions of alicyclic compounds are often oxidized; ethinamate (XXV) (158), for example, is hydroxylated at positions 3 and 4 of the ring. This hydroxylation pattern possesses similarities to the hydroxylation of alicyclic amides by the mold *Sp. sulfurescens* (82).

Oxidative Deamination—Compounds containing the basic structural unit R—CH_2NH_2 are the best substrates for monoamine oxidase. Substitution at the methylene carbon reduces reactivity, while increasing the chain length of an alkyl R group or the complexity of R can reduce reactivity of a substrate with this enzyme (159, 160). On the other hand, monoamine oxidase can accommodate compounds with a variety of aryl—R groups (159). If R is a phenyl group, substitution in the *ortho*-position can substantially decrease deamination (161). In contrast, substitution with electron-withdrawing groups improves substrate susceptibility to enzymatic transformation (161, 162).

Secondary amines can be deaminated by monoamine oxidase, although the reaction rates are substantially slower than with primary amines. Microbial deamination of amine substrates has been demonstrated with tryptamine (163, 164). This type of reaction is much less common in microorganisms than the usual deamination of amino acids, which may occur *via* pyridoxal-mediated transaminases or typical oxidative deamination enzymes.

Oxidation of Alcohols and Aldehydes—Foreign primary alcohols, except methanol, are readily oxidized to aldehydes and finally to carboxylic acids in mammals. Liver alcohol dehydrogenase, the enzyme principally responsible for the initial oxidation reaction, has been extensively studied (165–168). The rate of oxidation increases in the order ethanol < *n*-propanol < *n*-butanol but then decreases. However, higher molecular weight alcohols (*e.g.*, benzyl and furfuryl) are still good substrates for liver alcohol dehydrogenase (166).

With the apparent exception of cyclohexanol (166, 167), secondary alcohols are oxidized substantially slower than primary alcohols. Liver alcohol dehydrogenase appears to have no effect on the following secondary alcohols: 2-propanol, cyclopentanol, methyl phenyl carbinol, 1-indanol, and 1-tetralol (169). McMahon (169) surveyed other mammalian alcohol oxidoreductases that may play a role in the oxidation of xenobiotic alcohols. Most of these, however, seem to have considerably narrower substrate specificities than liver alcohol dehydrogenase and are probably of lesser significance.

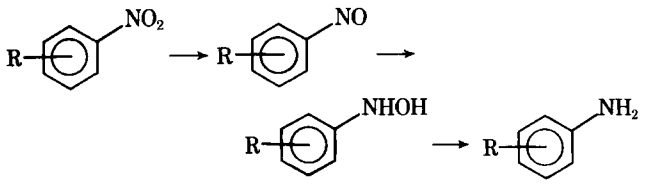
Mammalian aldehyde dehydrogenases oxidize a wide range of aldehydes. While formaldehyde is oxidized at only half the rate of acetaldehyde by liver aldehyde dehydrogenase, higher homologs, including propionaldehyde, butyraldehyde, and isovaleraldehyde, are only slightly poorer substrates than acetaldehyde (170). Substituted benzaldehydes serve as substrates for mammalian aldehyde dehydrogenase (170, 171). Benzaldehyde is the best substrate, while substitution with electron-withdrawing or electron-donating groups decreases activity. A good correlation was observed between log k and Hammett sigma values for this biphasic phenomenon (171).

Several aldehydes have been shown to be substrates for hepatic aldehyde oxidase (172, 173). This enzyme has been described as a hydroxylase (160) rather than a dehydrogenase. Aldehyde oxidase also attacks the α-carbon of a wide variety of *N*-heteroaromatic compounds (*e.g.*, quinoline), causing conversions to their corresponding α-lactam derivatives (173). Xanthine oxidase appears to have similar specificities to those of aldehyde oxidase (169).

Microorganisms commonly oxidize alcohols and aldehydes into their next higher oxidation states (10, 12, 13, 126, 174). These reactions often occur sequentially when a methyl group is first hydroxylated and then converted through the aldehyde to a carboxylic acid derivative. Polyol, terpene, and steroidal alcohol oxidations have been described (13). Various aldehydes are oxidized to carboxylic acids by an aldehyde dehydrogenase from *P. fluorescens* (175).

REDUCTIVE TRANSFORMATIONS

Nitro and Azo Reductions—A number of aromatic nitro compounds are converted to their nitroso, hydroxylamino, or amino derivatives in mammals (Scheme XVIII). This reaction is of considerable importance because of the potential toxicities of these metabolites. It is now generally believed that four to five enzyme systems affect nitro reductions: microsomal enzymes including cytochrome P-450 (176), NADPH-cytochrome c (177), and cytochrome b_5



Scheme XVIII—Bioreduction pathway for aromatic nitro compounds

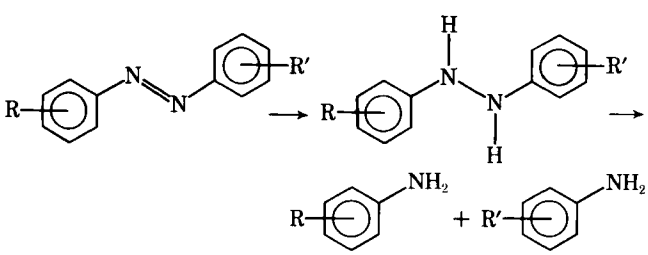
(178) reductases; an unidentified soluble "*p*-nitrobenzoate" reductase (179); and xanthine oxidase (180). All of these enzymes occur in the liver, although unspecified nitroreductase activity has also been detected in kidney, lung, heart, and brain tissue preparations (181).

The extent to which these enzymes reduce nitro compounds *in vivo* can be seriously questioned, because of their dependence on anaerobic conditions and the likely oxidation of proposed intermediates under the oxygen tension in the liver. For these reasons and because of the anaerobic conditions in the gut, bacterial involvement has been implicated in the reduction of aromatic nitro compounds (182). Zachariah and Juchau (183) reported approximately four times greater nitroreductase activity in rat cecal contents than in rat liver preparations.

Numerous aryl nitro compounds are reduced by mammalian and/or bacterial systems (182, 184). However, no systematic study of possible structural correlations has been reported.

The findings with azoreductases in mammals parallel those of nitroreductases. While hepatic azoreductases have been described (184), it seems highly probable that most azo reductions are mediated *in vivo* by gut microorganisms (182, 184, 185). Azo reductions are believed to proceed through hydrazo intermediates (184, 186) and result in the formation of corresponding aromatic amines (Scheme XIX). Because of the production of potentially toxic aromatic amines and the presence of azo linkages in numerous dyestuffs, there is considerable interest in azoreductases. Little seems to be known about possible influences of structural features on the facility with which azo compounds are reduced.

Reductions of Aldehydes and Ketones—Several enzyme systems are responsible for aldehyde and ketone reductions in mammals. Principal among these is liver alcohol dehydrogenase, which reversibly catalyzes reactions with equilibria favoring alcohol formation at physiological pH's (*vide supra*). Simple aliphatic aldehydes are reduced to primary alcohols by liver alcohol dehydrogenase; the activity increases from acetaldehyde through propionaldehyde and is



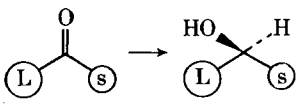
Scheme XIX—Bioreduction pathway for azo compounds

maximum for butyraldehyde (166). As indicated earlier, however, this reaction is of minor importance *in vivo* because of the facile conversion of aldehydes to readily excretable carboxylic acids.

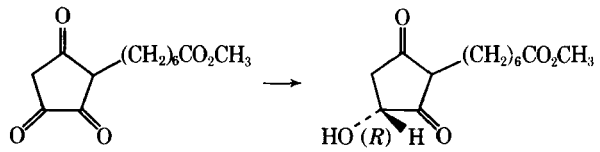
In contrast to the case with aldehydes, reduction of ketones to alcohols by liver alcohol dehydrogenase is an important *in vivo* biotransformation (169). The reduction of cyclic ketones by liver alcohol dehydrogenase has been extensively studied (187, 188). Considering the highly favorable substrate character of cyclohexanone [relative rate of reaction (RRR) = 100], the reduction rates of substituted derivatives are substantially dependent upon stereochemical features. For example, both enantiomers of 2-methylcyclohexanone are poor substrates (RRR = 1–2), while (−)-3-methylcyclohexanone has excellent activity (RRR_{calc} = 102) compared to its (+)-enantiomer (RRR = 0.6). 4-Methylcyclohexanone is a good substrate (RRR = 41), but activity drops dramatically in its more bulky homologs, 4-isopropylcyclohexanone (RRR = 21) and 4-*tert*-butylcyclohexanone (RRR = 2.5). Interesting differences were similarly noted in the reduction of *cis*- and *trans*-2-decalones. Reductions of cyclic ketones are stereoselective, depending on both the position and the absolute configuration of substituents (188). While many six-membered ketones are good substrates for liver alcohol dehydrogenase, cyclopentanone is without activity (169), and adamantanone is essentially inactive (189).

The reduction of ketones by alcohol dehydrogenase from microorganisms leads to the formation of (*S*)-carbinols when the carbonyl is dissymmetrically substituted by a bulky group and a small group (187, 188, 190) (Scheme XX). Presumably, the attack of the carbonyl by the NADH coenzyme in the microorganism is favored on one side only, as has been demonstrated (191) with numerous bi- and tricyclic ketone substrates using a reductase from *Cur. falcata*. On the other hand, alcohols of *R* absolute configuration have been produced by the reduction of compounds such as the triketone (XXVI) in completely stereospecific fashion (192) by *Dipodascus uninucleatus* (Scheme XXI) (193). It has been suggested that the extent of stereospecificity of the reaction product not only depends on interactions of the enzyme–substrate but also on the chirality of the alcohol dehydrogenase of a particular organism (193). Thus, it may be expected that microbial alcohol dehydrogenases capable of producing (*S*)- or (*R*)-alcohol products will be discovered.

In vitro experiments with liver alcohol dehydrogenase are not wholly satisfactory in predicting reductions of aldehydes and ketones in intact animals, because *in vivo* interconversions involving patterns of reduction → oxidation → reduction may occur and additional reductases also may be important in aldehyde and ketone metabolism. The first suggestion is



Scheme XX—Formation of (S)*-carbinol by alcohol dehydrogenase*



Scheme XXI—Formation of (R)-*carbinol by* D. uninucleatus (*Ref. 192*)

demonstrated by the work of Elliott *et al.* (190, 194–196), who showed that cyclohexanones and decalones are converted to their more thermodynamically stable equatorial alcohols in rabbits. This finding is in contrast to the substantial number of axial alcohols produced *in vitro* with liver alcohol dehydrogenase preparations (187, 188).

A number of other aldehyde–ketone reductases have been isolated from mammalian tissues. Similar NADPH-dependent aromatic aldehyde–ketone reductases (A–K reductase) have been isolated from rabbit liver and kidney preparations (169, 197). These enzymes are true reductases in that they do not catalyze the dehydrogenation of carbinols. A relationship similar to that derived for the reduction of benzaldehydes by liver alcohol dehydrogenase (198) was observed for substituted acetophenones and benzaldehydes (197, 199). In addition, a pentanone and adamananone were reduced by kidney A–K reductase (197).

It has been suggested that liver A–K reductase may be the same as the pig liver reductase isolated by Prelog (187). Fraser *et al.* (200) reported the isolation of an α,β-unsaturated ketone reductase from human liver. This enzyme mediates the reduction of either the double bond or carbonyl groups of an α,β-unsaturated ketone and is similar to A–K reductase (197, 199) in that it does not catalyze the reverse reaction (oxidation).

HYDROLYSES

Ester Hydrolyses—Esterases capable of hydrolyzing foreign esters have been found primarily in mammalian plasma, erythrocytes, liver, and kidneys (201). These enzymes include cholinesterase (pseudocholinesterase), carbonic anhydrase, arylesterases, and acetylesterases, which can collectively hydrolyze a vast array of esters. Because of the multiplicity of esterases and significant variation (both qualitative and quantitative) among animal species (67, 201), an understanding of the exact role that ester hydrolyses play in drug metabolism and drug action has been elusive. Some common ester-containing drugs susceptible to ester hydrolysis *in vivo* have been reviewed (201).

Scheline (182) noted that ester hydrolysis may be mediated by intestinal microorganisms. However, in humans this would only be of importance for poorly absorbed drugs (*e.g.*, acetyldigitoxin) capable of reaching the lower intestine. Numerous esterases have been characterized and used in the microbial transformation of steroids (202), and the application of esterases as a synthetic tool in the field of prostaglandin synthesis has been recorded (203). In the latter case, a racemic mixture of esters was stereoselectively hydrolyzed to yield a mixture of the free (*R*)-alcohol and the (*S*)-ester, which could be easily separated.

Amide Hydrolyses—Some amides may serve as substrates, albeit poor ones, of plasma or tissue esterases (67). The prolonged activity of procainamide *versus* procaine can be rationalized in this way. Krisch (204, 205) isolated a microsomal esterase from hog liver which cleaves several amides including acetanilide and lidocaine. In addition, a number of mammalian arylacylamide amidohydrolases have been described as occurring in liver and kidney tissue. These enzymes have been shown to deacylate some acetamido compounds and appear to be especially important in the mechanism of action of sulfonamides (206).

Parke (67) reported that primary amides (*e.g.*, benzamides), hydroxamic acids, hydrazides, and carbamates undergo hydrolysis in mammals, although it is unclear whether all of these are hydrolyzed as the result of enzymatic catalysis. Amide hydrolysis also may be catalyzed by intestinal microorganisms (207). Interestingly, the biotransformation of the sulfamate derivative, cyclamate, to the toxic metabolite, cyclohexylamine, in humans appears to be exclusively mediated by an intestinal microorganism (probably *Enterococci* sp.) (208). Smith and Rosazza (9) observed the hydrolysis of acetanilide in their microbial systems, and amide hydrolyses have been recorded in other microbial systems as well. Particularly well studied is the hydrolysis of amides in several antibiotics by many microorganisms (16).

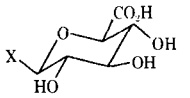
CONJUGATIONS

The major types of Phase II reactions are indicated in Table IV. Most of these biotransformations lead to conjugates of drugs and their metabolites that are more water soluble (and thus more readily excreted). These conjugates often possess less biological activity than the parent drugs. Thus, attempts are rarely made to characterize fully Phase II metabolites. In lieu of typical structure elucidation approaches, structures of conjugates are often inferred from selective hydrolysis reactions (*e.g.*, glucuronidase–sulfatase and chemical cleavages) and characterization of metabolites is done on the "drug-derived" portion of the conjugate.

Two Phase II reactions are exceptional with respect to these generalizations. *N*-Acetylation of amines and methylation of phenols result in more lipid-soluble metabolites that merit evaluation of biological activity. Foreign aliphatic and aromatic primary amines and hydrazides are substrates for a number of *N*-acetyltransferases which occur widely in mammalian species (206, 209–211). These enzymes are usually dependent on acetylcoenzyme A and are distributed in various tissues (67). The influence of substrate structure *versus* formation of *N*-acetyl metabolites is not well understood.

Monomethylation of catechols by the *S*-adenosylmethionine-dependent enzyme, catechol-*O*-methyltransferase, is an important biotransformation of cat-

Table IV—Phase II Biotransformations

Reaction Type	Substrate	Product
Glucuronidation		
	Alcohol	X = R—O
	Phenol	X = aryl—O
	Enol	X = $R'_2C{=}CR{-}O$
	Hydroxylamine	X = $R_2C{=}N{-}O$
	Carboxylic acid	X = RCO_2-
	Primary and secondary amines	X = R_2N-
	Amide	X = RCONR
	Thiol	X = RS
Sulfate conjugation		X′—OSO_3H
	Alcohol	—
	Phenol	Sulfate ester
	Enol	—
	Amine	Sulfamate
Glycine conjugates	Carboxylic acid	$RCONHCH_2CO_2H$
Acetylation	Primary amine	$RNHCOCH_3$
	Hydrazine	$RNHNHCOCH_3$
	Hydrazide	$RCONHNHCOCH_3$
	Sulfonamide	$RSO_2NHCOCH_3$
N-Methylation	Primary, secondary, and tertiary amines	Corresponding secondary, tertiary, and quaternary amines
O-Methylation	Catechol	Monomethyl catechol
	Phenol	Methyl aryl ether

echolamine neurotransmitters and their respective metabolites (212, 213). Catechol-*O*-methyltransferase principally occurs in the liver but is found in kidney, spleen, small intestine, lung, and brain tissue (213, 214). In most instances, the oxygen atoms of a catechol are nonequivalent in terms of electronic and/or steric characteristics. Preferential methylation of one group *versus* another was at one time thought to be a function of relative nucleophilicities (215). However, recent studies indicated that the steric and electronic properties of substituents, as well as the relative lipophilicity of substrate, are equally or more important factors affecting relative isomer formation (*e.g., meta–para* ratios) (216–219). Recently, evidence for mammalian enzymes that methylate monophenols and monomethyl catechols has been presented (220–222). The importance of these biotransformations *in vivo,* however, is still unclear.

An enzyme that occurs in the soluble portion of rabbit lung homogenates has been found to *N*-methylate a variety of foreign primary and secondary aliphatic amines (223, 224). An apparently similar enzyme also has been discovered in rat brain (225). The importance of these enzymes with *in vivo* drug biotransformations has not been established.

Interesting microbial parallels to a number of Phase II reactions have been observed, including the phosphorylation of clindamycin (226) by *Streptomyces coelicolor* and the formation of ribonucleotides of this antibiotic (227). *N*-Acetylation occurs commonly with amines such as tryptamine (and with tryptophan) (17), aniline (9), and other compounds (13, 126). Lysine (228), serine (229, 230), and glycine (231) conjugates of 2,3-dihydroxybenzoic acid are produced by microorganisms. A microbial parallel to mammalian *O*-methylation was previously described in this review in the O-*Dealkylation* section (114).

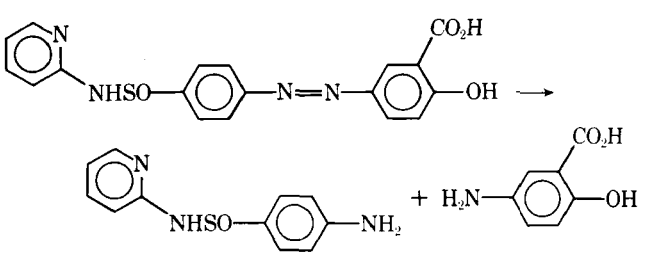
Transformations by Intestinal Microorganisms—Significant interest has been demonstrated recently in the metabolism of drugs by intestinal microorganisms (167, 232, 233). Orally administered drugs, particularly those with low solubility or delayed absorption, pass to the lower portion of the intestinal tract and may be susceptible to biotransformation by the intestinal flora. Additionally, compounds excreted *via* the intestinal route may be susceptible to such biochemical changes. A variety of microorganisms may be found in the intestinal tract.

In a very important study (because of its thoroughness), 113 distinct types of organisms were identified in fecal samples of Japanese–Hawaiians (234). Most fecal organisms are bacteria; however, the nature of the flora may be quite different, depending on such factors as diet, age, health, and use of drugs such as antibiotics, which may destroy the normal GI tract flora. Many types of microbial conversions have been attributed to the intestinal flora, some of which have been described already including *N*-demethylation of methamphetamine (128), reduction of nitro and azo compounds (182), and hydrolysis of esters (182) and amides (208).

Type-reactions carried out by intestinal microorganisms (233) are: hydrolysis of glucuronides, glycosides, ethereal sulfates, amides, glycine conjugates, *N*-acetyl compounds, esters, and sulfamates; C- and *N*-dehydroxylation; decarboxylation; *O*- and *N*-demethylation; dehalogenation; heterocyclic ring fission; reduction of double bonds, nitro groups, azo groups, aldehydes, ketones, alcohols, *N*-oxides, and arsonic acids; aromatization; deamination; nitrosamine formation; acetylation; and esterification.

The mode of action of a drug may be influenced by intestinal flora metabolism (233). For example, it was suggested that when sulfasalazine (salicylazosulfapyridine) (XXVII) (Scheme XXII) (used in ulcerative colitis and other inflammatory diseases of the bowel) is metabolized to its respective components (XXVIII and XXIX), the metabolites were really responsible for the action of the drug. Drug action was ultimately related to the presence of 5-aminosalicylate (salt of XXVIII) in the intestines. When 5-aminosalicylic acid is given orally or parenterally, little of the compound appears in the colon. Thus, sulfasalazine serves as a prodrug and becomes activated only after a microbial conversion step in the intestine.

Other toxicological implications regarding the bio-



Scheme XXII—Microbial transformation of sulfasalazine

transformations of compounds in the GI tract involve the hydrolysis of glucuronides and other conjugates. Many drugs and foreign organic compounds or their metabolites are excreted as glucuronide conjugates and are poorly reabsorbed from the intestines. Bacterial β-glucuronidase activity is high in the intestine; aglycones produced as a result of hydrolytic activity may be reabsorbed and passed again through the liver. For example, glucuronide hydrolysis and reabsorption have been reported with diethylstilbestrol (235) and indomethacin (236).

Microbiological Methodology—Few researchers engaged in mammalian metabolism studies are familiar with microbial transformation experiments, and vice versa. This brief section will provide a simple introduction to some methodology involved in microbial transformation studies. Of course, a review of this scope cannot provide sufficient experimental detail or background to enable the reader to conduct such experiments immediately. Rather, years of experience are required before it becomes possible to deal instinctively with any biological system. Perhaps the ideal situation is one involving the simultaneous efforts of an interdisciplinary team which may include microbiologists (pharmacognosists), analytical medicinal chemists, and pharmacologists.

Microorganisms are convenient sources of the enzymes that mediate reactions like those outlined in the text. Although microbes are capable of conducting single, selective reactions on even the most complex molecule, a host of reactions also can occur simultaneously. It is possible, however, to adjust conditions of microbial experiments to favor formation of a single product or a single type of reaction to increase the yield of a desired metabolite. Typical fermentation conditions that are frequently varied include organic and inorganic medium components, pH, degree of aeration of cultures (usually attained by shaking at different rates), temperature of incubation, and substrate concentration.

Variations in pH, medium composition, and temperature are known to influence the outcome of fermentation experiments. The importance of dissolved oxygen levels for the production of enzymes (237) and on the microbial transformations of steroids (238) has been noted. The time and mode of addition of substrates to fermentations can also influence microbial metabolic profiles (238–244). Several general references detail many aspects of the fermentation process (12, 13, 242, 245). Others dealing with specific types of microbial transformations were cited earlier in this review.

The selection of microorganisms capable of performing a desired chemical transformation may be accomplished in a number of ways. Cultures may be isolated from the soil or from sewage treatment facilities, or they may be obtained in pure form from culture collections. A compilation of sources of microorganisms was published (246), and smaller lists of useful culture collections have been described (10, 12, 13). Microbiologists are often willing to share cultures from the culture collections they maintain. Because cultures from some culture collections are expensive, most workers in the field of microbial transformations maintain collections in their own laboratories (perhaps 100–400 cultures).

Once cultures are on hand, they may be screened for their potential to perform, for example, hydroxylation of aromatic substrates. It is possible to be more discriminating in the choice of microorganisms to conduct a specific type of reaction by taking advantage of the rapidly growing literature in microbial transformations. For success in obtaining active cultures that perform a desired transformation, it is often necessary to combine the "literature" approach and the "random screening" approach.

Several types of microorganisms are used in microbial transformation experiments. These are the bacteria (including the Actinomycetes), yeasts, and filamentous fungi. Growth habits, medium requirements, and culture conditions are generally quite different for each type of microorganism. Some microorganisms are pathogenic and must be handled with extreme care. Most, however, are relatively easy to work with and may be cultivated with little difficulty. Detailed descriptions and taxonomic classifications of various microorganisms have been published (247, 248). Whatever the source of the microorganism, it must be properly identified (*e.g.*, culture collection acquisition number and authentication by suitable authority) and precautions should be taken to prevent contamination with other microorganisms.

To accommodate the diverse growth requirements of different microorganisms, various media have been employed. A large listing of media cross-indexed with various microorganisms is available (249). Media and glassware employed in microbial transformation experiments are usually sterilized in an autoclave (121° for 15 min). After sterilization, solid (agar-containing) medium is caused to solidify into slants in sealed culture tubes. These slants are usually inoculated with various cultures and are incubated for approximately 1 week to obtain fresh and vigorous cultures. The resulting cultures may be maintained in a viable state for long periods (6 months or longer) in a refrigerator at 3–4°. Culture vigor is maintained by periodically transferring stored cultures to fresh slants. Other methods used in the maintenance of culture collections include low temperature storage (from −70 to −198° liquid nitrogen) and lyophilization.

Microbial transformation experiments *per se* are conducted in liquid medium contained in vessels ranging in size from small test tubes to fermentors with several hundred or even thousand gallon capacities. The usual laboratory-scale incubations are carried out in cotton-plugged erlenmeyer flasks containing about one-fifth of their volumes of medium. Table V shows a selection of media used in these kinds of experiments. Note the differences in medium pH used for the cultivation of bacteria and fungi. In general, bacteria grow in media with initial pH values of 7.0 while fungi and yeasts grow well under slightly acidic (pH 4–6) conditions. As a result of the consumption of nutrients and primary metabolic activity, the pH of the medium of most fermentations changes as the cultures grow.

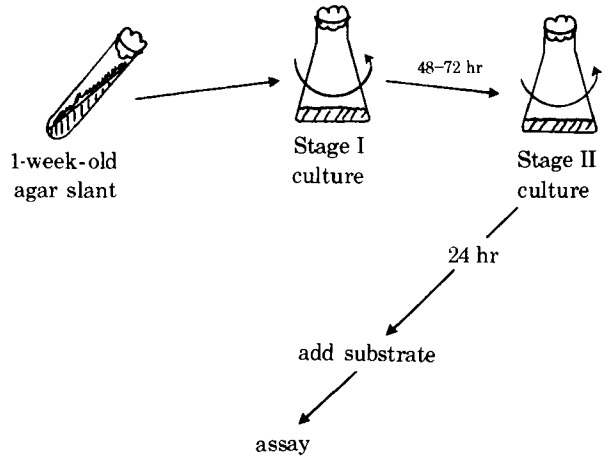
Table V—Various Media Employed in Microbial Transformation Experiments

Research Group	Media Used
Jones *et al.* (250)	Dextrose ($1H_2O$), 30 g; ammonium tartrate, 7.5 g; KH_2PO_4, 2 g; $MgSO_4 \cdot 7H_2O$, 0.5 g; trace elements, 0.1 g; yeast extract, 1.0 g; and distilled water, 1 liter
	For fungi and streptomycetes, use at pH 5.5; for bacteria, use at pH 7.0
Smith and Rosazza (9) and Betts *et al.* (61)	Dextrose, 20 g; yeast extract, 5 g; soybean meal, 5 g; NaCl, 5 g; K_2HPO_4, 5 g; and distilled water, 1 liter
	Adjust to pH 7.0 with 5 *N* HCl; use for yeasts, fungi, and actinomycetes
Kluepfel and Vezina (251)	Dextrose, 50 g; edamine, 20 g; corn steep liquid, 3 g; and distilled water, 1 liter
	For fungi, use at pH 5.8
	Dextrose, 1.5 g; yeast extract, 1.5 g; peptone, 5.0 g; K_2HPO_4, 3.5 g; and distilled water, 1 liter
	For bacteria, use at pH 7.0
Goodman *et al.* (252)	Dextrose, 20 g; peptone, 5 g; tryptone, 5 g; $CaCO_3$, 2.5 g; and distilled water, 1 liter
	Use for various bacteria

Fermentations are conducted in many ways in the laboratory. Small-scale screening-type fermentations are conducted in 50–125-ml erlenmeyer flasks containing 10–25 ml of medium and are usually incubated with shaking to ensure a rich supply of oxygen for the medium. Larger scale incubations are conducted in large carboys or in stirred fermentors agitated by impellers and aerated by sparging air through the medium. Typical laboratory-scale incubations using stirred fermentors range from 1.5 to 10 liters in volume.

A fermentation scheme is outlined in Scheme XXIII. The description of the fermentation provided is applicable to small- and large-scale experiments and has been generally applied successfully in our laboratory.

Cultures are maintained on Sabouraud–maltose agar slants and are stored in a refrigerator at 4° prior to use.



Scheme XXIII—Typical conditions for conducting microbial transformations (Ref. 9)

Cultures are grown in a soybean meal–glucose medium (Table V).

The surface growth from slants of microorganisms is suspended in 5-ml volumes of sterile soybean meal–glucose medium. This suspension is used to inoculate 25 ml of the soybean meal–glucose medium held in cotton-plugged 125-ml erlenmeyer flasks (Stage I). The Stage I flasks are incubated at 27° on a rotary shaker operating at 250 rpm for 48–72 hr, at which time most cultures are late in the log phase or in the stationary phase of growth. Approximately 3-ml volumes of the actively growing Stage I cultures are transferred to 25 ml of fresh medium held in cotton-plugged 125-ml erlenmeyer flasks (Stage II). After 24 hr of incubation on a rotary shaker, 12.5 mg of substrate dissolved in 0.1 ml of dimethylformamide is added to Stage II flasks. The substrate-containing flasks are incubated for an additional 24–72 hr before being terminated.

The time of addition of substrate to a fermentation, as well as its concentration in the medium, may be critical in microbial transformation experiments. Substrates may be added in concentrated solutions of dimethylformamide, dimethyl sulfoxide, ethanol, acetone, or other solvents to facilitate dispersion in aqueous media. Compounds may be added in water-soluble form, as hydrochloride salts of alkaloids or amino acids, or as sodium or potassium salts of carboxylic acids. In the usual screening experiment, a nominal level of substrate of 0.1–0.5 mg/ml of Stage II fermentation has been successfully employed in our laboratory. Limited success in microbial transformation experiments has been obtained when substrates were added too soon to Stage II cultures. Growth is inhibited, and there is evidence that an optimum time for substrate addition exists with microorganisms.

In screening experiments, analyses are usually performed at two times during the fermentation, 24 and 72 hr after substrate addition. Some microorganisms may completely degrade a substrate early in a fermentation, whereas others may possess inducible enzymes which will only produce metabolites after being in contact with substrates for a period of time. This arbitrary selection of two time periods has worked well in our hands.

Liquid–liquid extraction methods are most widely used in the analysis and isolation of microbial metabolites. Occasionally, for difficult to extract solutes, culture samples have been frozen and lyophilized to facilitate the isolation process. Freeze-dried samples may then be extracted with polar solvents such as alcohols or acetone. In general, it is recommended that the whole culture be examined for metabolites rather than culture filtrates alone because some substrates (and their metabolites) may bind to cellular material. We have observed this to be true both with steroids and with alkaloids such as acronycine. In fact, acronycine and its metabolite, 9-hydroxyacronycine, are easily removed from fermentations by centrifugation of the solids from fermentations of *C. echinulata*

(61). Only traces of acronycine and its metabolite are left in supernates, and the centrifuged solids may be simply extracted with hot ethanol to isolate the relatively insoluble 9-hydroxyacronycine.

CONCLUSIONS

The metabolic fate of drugs and other xenobiotics in mammalian organisms represents an area of intense contemporary interest. Typically, biological systems used to study biotransformations are capable of yielding only minute quantities of metabolites. Recent developments in comparative biochemistry have made it possible to link diverse metabolic systems through similarities in the pathways by which they alter foreign organic compounds. Thus, the potential exists for utilizing microbial metabolic systems to study and possibly predict the metabolic fate of a drug or other foreign compounds in mammals. Furthermore, it should be possible to use such systems to produce preparative quantities of drug metabolites for structure elucidation studies and biological testing.

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RESEARCH ARTICLES

Solvolytic Reactions of Cyclic Anhydrides in Anhydrous Acetic Acid

M. J. HADDADIN *, T. HIGUCHI ˣ, and V. STELLA

Abstract □ The reversible reactions of several cyclic anhydrides with acetic acid to form acetic anhydride and the corresponding dicarboxylic acid, catalyzed by perchloric acid at 25°, were studied. The equilibrium constants, calculated from spectral data, were 4.85×10^{-4}, 1.08×10^{-1}, and 4.6×10^{-1} *M* for succinic, *trans*-1,2-cyclohexanedicarboxylic, and glutaric anhydrides, respectively. Maleic, phthalic, and *cis*-1,2-cyclohexanedicarboxylic anhydrides did not undergo any detectable reaction with acetic acid under these conditions, suggesting still higher stability. The reverse rate constants were found to be relatively independent of the structure of the attacking diacid, while the forward rate constants were found to parallel the equilibrium constants. The rate-determining step for the forward reaction appears to be the breakdown of the tetrahedral intermediate formed by the attack of an acetic acid molecule on the protonated cyclic anhydride.

Keyphrases □ Anhydrides, cyclic—solvolytic reactions in anhydrous acetic acid, rate constants, mechanisms □ Solvolysis of cyclic anhydrides in anhydrous acetic acid—rate constants, mechanisms □ Perchloric acid—catalyst for solvolytic reactions of cyclic anhydrides in anhydrous acetic acid □ Acylation—reversible reactions of cyclic anhydrides in anhydrous acetic acid, rate constants, mechanisms

The chemistry of cyclic and linear anhydrides is of major interest to those concerned with transacylation reactions in both *in vitro* and *in vivo* systems. There is very little doubt that these active chemical species play significant roles in many acyl transfer reactions and in cleavage and formation of polycarboxylic acid derivatives. The present report is concerned with some data obtained on the solvolytic behavior of a series of cyclic anhydrides of varying stability in anhydrous acetic acid. The results indicate relatively strong structural dependency on the ring opening and very little sensitivity to the reverse process.

Anhydrides of dicarboxylic acids have been used in the preparation of hemiesters of water-insoluble

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Keyphrases □ Anhydrides, cyclic—solvolytic reactions in anhydrous acetic acid, rate constants, mechanisms □ Solvolysis of cyclic anhydrides in anhydrous acetic acid—rate constants, mechanisms □ Perchloric acid—catalyst for solvolytic reactions of cyclic anhydrides in anhydrous acetic acid □ Acylation—reversible reactions of cyclic anhydrides in anhydrous acetic acid, rate constants, mechanisms

The chemistry of cyclic and linear anhydrides is of major interest to those concerned with transacylation reactions in both *in vitro* and *in vivo* systems. There is very little doubt that these active chemical species play significant roles in many acyl transfer reactions and in cleavage and formation of polycarboxylic acid derivatives. The present report is concerned with some data obtained on the solvolytic behavior of a series of cyclic anhydrides of varying stability in anhydrous acetic acid. The results indicate relatively strong structural dependency on the ring opening and very little sensitivity to the reverse process.

Anhydrides of dicarboxylic acids have been used in the preparation of hemiesters of water-insoluble

Table I—Wavelengths and Species Monitored for All Reactions Studied

Reaction	Wavelength, nm	Species Monitored[a]
Succinic anhydride + acetic acid	252	Acetic anhydride (A)
Succinic acid + acetic anhydride	252	Acetic anhydride (D)
Glutaric anhydride + acetic acid	268	Acetic anhydride (A)
Glutaric acid + acetic anhydride	255	Acetic anhydride (D)
Phthalic acid + acetic anhydride	285	Phthalic acid (D)
Maleic acid + acetic anhydride	280	Maleic acid (D)
Phthalic anhydride + acetic acid	302	No change
Maleic anhydride + acetic acid	265	No change
cis-1,2-Cyclohexanedicarboxylic anhydride + acetic acid	265	No change
cis-1,2-Cyclohexanedicarboxylic acid + acetic anhydride	255	Acetic anhydride (D)
trans-1,2-Cyclohexanedicarboxylic anhydride + acetic acid	259	Acetic anhydride (A)

[a] Disappearance and appearance are represented by D and A, respectively.

drugs such as hydrocortisone[1] (1), methylprednisolone[1] (1), and chloramphenicol (2). The popularity of these hemiesters as prodrugs stems from the enhanced aqueous solubility of their salt forms compared to the parent drugs. The ease with which they release the parent drug *in vivo* also has been noted.

The closure of these hemiesters and corresponding acids to their respective anhydrides is often taken as a simple model of intramolecular catalysis. The demonstration of participation of anhydrides in the hydrolysis and formation of amides (3, 4) and in the hydrolysis of hemiesters of dicarboxylic acids (5) strongly supports analogous mechanistic pathways for other related reactions. Transacylations in solid dosage forms, such as the reaction of amines and phenolic compounds with high energy acylators like acetylsalicylic acid, can be simulated by the reactivity of anhydrides (6, 7).

The solvolysis of cyclic anhydrides in aqueous solutions has been studied (8), and the equilibrium constants for the cyclic anhydride–diacid systems in water were largely determined by the formation of the cyclic anhydride.

The possible roles of anhydrides as high energy compounds in biological systems recently received some attention. For example, succinic (9) and acetic (10) anhydrides have been postulated in the biosynthesis of acetyl and succinyl coenzymes. Anhydrides also have been postulated as intermediates in model enzymatic reactions (11).

Attempts in these laboratories to react various drug molecules with succinic anhydride to form hemisuccinate esters using anhydrous acetic acid as a solvent resulted in the formation of acetate esters rather than the hemisuccinate. The results prompted a study of the reactions of several cyclic anhydrides in anhydrous acetic acid in an attempt to understand the quantitative aspects of acetic anhydride formation in anhydrous acetic acid in the presence of cyclic anhydrides and perchloric acid. The objectives were to relate this formation of acetic anhydride to the structure of the cyclic anhydride and to understand the mechanism of this transacylation reaction.

[1] Products marketed as Solu-Cortef and Solu-Medro by The Upjohn Co., Kalamazoo, Mich.

Table II—Absorptivities[a] of Anhydrides Used in Calculations

Anhydride	$\lambda^{25°}_{\text{acetic acid}}$	ϵ
Succinic	252	3.4
Glutaric	268	1.6
trans-1,2-Cyclohexanedicarboxylic	272	0.36
Acetic	252	38.2
	268	5.6
	272	3.15

[a] Absorptivities were obtained from Beer's law plots.

EXPERIMENTAL

Materials—All chemicals used were generally of reagent or analytical reagent grade and were purified before use.

Anhydrous acetic acid was prepared from analytical reagent grade acetic acid[2] by titration with freshly distilled acetic anhydride in the presence of catalytic quantities of perchloric acid. The reaction was monitored either thermometrically or spectrophotometrically. The thermometric technique involved the incremental addition of acetic anhydride until no further heat evolution could be detected. The spectrophotometric technique involved the addition of a slight excess of acetic anhydride. The excess amount of unreacted anhydride was determined at 252 nm and subsequently neutralized with an equivalent amount of water.

Acetic anhydride[2] was distilled over magnesium turnings; the middle fraction distilling at 137.5° was collected [lit. (12) bp 139°].

Acetous perchloric acid was prepared from 70% reagent grade perchloric acid[3] by reaction in an ice bath with a calculated amount of distilled acetic anhydride added slowly to the perchloric acid. The last 2 ml of acetic anhydride was used to titrate the remaining water thermometrically. The acetous perchloric acid was standardized against NBS potassium acid phthalate, using gentian violet as an indicator (2% in nitrobenzene).

Succinic anhydride[3], mp 118° [lit. (12) mp 119.6°], glutaric anhydride[4], mp 55° [lit. (13) mp 56°], phthalic anhydride[5], mp 130° [lit. (12) mp 130.8°], and maleic anhydride[6], mp 52° [lit. (13) mp 52.8°], were purified by refluxing with excess acetic anhydride to remove any diacids. They then were precipitated and washed with petroleum ether and dried in a vacuum oven. *cis*-1,2-Cyclohexanedicarboxylic anhydride[4] was dissolved in benzene, filtered, precipitated with petroleum ether, and dried in a vacuum oven, mp 33–34° [lit. (13) mp 32°]. *trans*-1,2-Cyclohexanedicarboxylic anhydride[4] was used without further purification, mp 145–146° [lit. (13) mp 145–146°].

Succinic acid[4] was recrystallized from benzene, mp 185° [lit. (12) mp 185–187°], while maleic acid, mp 130° [lit. (12) mp 130–131°], and glutaric acid, mp 97° [lit. (12) mp 97.5–98°], were recrystallized from chloroform. Both *cis*-1,2-cyclohexanedicarboxylic acid[4], mp 190° [lit. (13) mp 192°], and *trans*-1,2-cyclohexanedicarboxylic acid[3], mp 221° [lit. (13) mp 221°], were purified by dissolving in hot sodium hydroxide solution, decolorizing with charcoal, and precipitating by the dropwise addition of hydrochloric acid while still hot.

Kinetic and Equilibrium Measurements—The reactions between the various cyclic anhydrides and acetic acid were monitored spectrophotometrically[7] at 25°. Table I gives the wave-

[2] Mallinckrodt Chemical Works, St. Louis, Mo.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] Aldrich Chemical Co., Milwaukee, Wis.
[5] Matheson, Coleman and Bell, Norwood, Ohio.
[6] Eastman Kodak Co., Rochester, N.Y.
[7] Cary model 14 UV recording spectrophotometer with a thermostated cell compartment.

Table III—Conditions for Spectrophotometric Evaluation of k_{-I} for Scheme II

Acid	Excess Reactant	k_{-I} Evaluated from	Cell Path Length, cm
Succinic	Succinic acid	Slope of k_{obs} *versus* [acid]	5
Glutaric	Glutaric acid	$(k_I + k'_{-I}) - k_I$; k_I obtained from initial slopes or Scheme I[a]	1
Phthalic	Acetic anhydride	Slope of k_{obs} *versus* $[(CH_3CO)_2O]$	1
Maleic	Acetic anhydride	Slope of k_{obs} *versus* $[(CH_3CO)_2O]$	1
cis-1,2-Cyclohexanedicarboxylic	*cis*-1,2-Cyclohexanedicarboxylic acid	Slope of k_{obs} *versus* [acid]	1
trans-1,2-Cyclohexanedicarboxylic	*trans*-1,2-Cyclohexanedicarboxylic acid	Slope of k_{obs} *versus* [acid]	1

[a] $k'_{-I} = k_{-I}$[glutaric acid].

lengths used and the species monitored. An accurately known quantity of the cyclic anhydride was quickly dissolved in a known volume of solution of perchloric acid (about 1×10^{-3} *M*) in anhydrous acetic acid in a 1-cm cylindrical cell.

The rate constant for the forward reaction, k'_I, as shown in Scheme I:

$$A \underset{k'_{-I}}{\overset{k'_I}{\rightleftharpoons}} B + C$$

Scheme I

where *A* is the cyclic anhydride, *B* is acetic anhydride, and *C* is the dicarboxylic acid, is obtained by plotting the integrated equation (14). For Scheme I, this equation is:

$$\ln \frac{a_0(x_e - x)}{a_0 x_e + x(a_0 - x_e)} = -\frac{2a_0 - x_e}{x_e} k'_I t \qquad \text{(Eq. 1)}$$

where a_0 is the initial concentration of the cyclic anhydride, x_e is the concentration of acetic anhydride at equilibrium, and x is the concentration of acetic anhydride at any time t. The rate constant k'_I is equal to k_I [$HClO_4$]. The acetic anhydride concentration x, at any time t, was calculated from known absorptivity constants (Table II) and measured absorbance readings using Eq. 2:

$$x = \frac{\Delta A}{\Delta \epsilon} \qquad \text{(Eq. 2)}$$

where ΔA is the change in absorbance from $t = 0$ to any time t, and $\Delta\epsilon$ is the difference in absorptivities of acetic and cyclic anhydrides at the wavelength used. Because the reaction between the cyclic anhydride and the solvent, acetic acid, proceeded slowly in the absence of the catalyst, perchloric acid, evaluation of the UV absorptivities of the various cyclic anhydrides could readily be accomplished.

Table III shows the conditions for the evaluation of the reverse rate constant k_{-I}. The unidirectional nature of the reactions was assured by the use of excess reactants, and the rate constant k_{obs} was obtained from normal semilogarithmic plotting procedures.

The equilibrium constant *K*, defined by Eq. 3, was evaluated spectrophotometrically. This was possible because the cyclic anhydride and the formed acetic anhydride were the only absorbing species at the selected wavelength:

$$K = \frac{[(CH_3CO)_2O]_{eq}[\text{diacid}]_{eq}}{[\text{cyclic anhydride}]_{eq}} \qquad \text{(Eq. 3)}$$

$$K = \frac{(\Delta A/\Delta\epsilon)^2}{a_0 - (\Delta A/\Delta\epsilon)} \qquad \text{(Eq. 4)}$$

$$K = \frac{(\Delta A/\Delta\epsilon)(\Delta A/\Delta\epsilon + [\text{diacid}])}{[\text{cyclic anhydride}]_0 - \Delta A/\Delta\epsilon} \qquad \text{(Eq. 5)}$$

where [diacid] is the amount of dicarboxylic acid added to the system, $\Delta A/\Delta\epsilon$ (as defined earlier) is equal to the amount of acetic anhydride formed, and $[\text{cyclic anhydride}]_0$ is the initial concentration of the cyclic anhydride. The subscript eq refers to equilibrium concentration. Equation 4 could be used when no added diacid was needed to observe the equilibrium formation, whereas Eq. 5 was used for systems where free dicarboxylic acid was added along with the cyclic anhydride. The value of *K* was also calculated from the ratio k_I/k_{-I}.

RESULTS

The course of the reaction between cyclic anhydrides with acetic acid in anhydrous acetic acid as a solvent, catalyzed by perchloric acid, to produce acetic anhydride and the corresponding dicarboxylic acid can be readily followed spectrophotometrically and can be described by Scheme II:

$$CH_3COOH \text{ (excess)} + \text{cyclic anhydride} + H^+ \underset{k_{-I}}{\overset{k_I}{\rightleftharpoons}} (CH_3CO)_2O + \text{dicarboxylic acid} + H^+$$

Scheme II

The spectral observations were possible because of differences in absorptivities between acetic anhydride and the cyclic anhydrides at given wavelengths (Tables I and II). The observed change in UV absorbance at 268 nm for a solution containing 0.25 *M* glutaric anhydride in acetic acid in the presence of 1×10^{-3} *M* $HClO_4$ and a similar plot for 0.07 *M* *trans*-1,2-cyclohexanedicarboxylic anhydride in the presence of 3.73×10^{-3} *M* $HClO_4$ at 259 nm as a function of time is shown in Fig. 1.

The reversibility of Scheme II was established spectrophotometrically. Also, when starting with the cyclic anhydride, the dicarboxylic acid could occasionally be isolated. Similarly, when starting with the dicarboxylic acid and an excess of acetic anhydride, the cyclic anhydride could be isolated. The change in UV absorbance of the reaction between succinic anhydride and acetic acid was shown to be a function of added succinic acid. Table IV shows the decrease in absorbance as the added concentration of succinic acid was increased for a constant initial concentration of succinic anhydride. The reverse reaction is applied commercially to prepare succinic anhydride (12). Succinic anhydride was isolated from a reaction mixture of succinic acid and acetic anhydride (in the presence of perchloric acid) in acetic acid and identified by its melting point of 118°.

The same trend in UV absorbance was observed for *trans*-1,2-cyclohexanedicarboxylic anhydride when acetic anhydride was added to the system. When more than 0.35 *M* of the anhydride was dissolved in acetic acid, *trans*-1,2-cyclohexanedicarboxylic acid precipitated out. It was identified by its melting point of 221°.

Table IV—Change in UV Absorbance as a Function of Added Succinic Acid for the Reaction Shown in Scheme II[a]

Added Succinic Acid	Change in UV Absorbance[b]
0	0.45
0.0075	0.33
0.03	0.15
0.075	0.08

[a] Succinic anhydride initial concentration was 0.40 *M* in all cases, and [$HClO_4$] was of the order of 1×10^{-3} *M*. [b] Added succinic acid forced the equilibrium to the left in Scheme II, suppressing the formation of acetic anhydride, the major absorbing species, at 252 nm.

Table V—Equilibrium Constant in Acetic Acid at 25° and Equilibrium Concentrations of the Species in Scheme II

$[\text{Succinic Anhydride}]_o$, M	$[\text{Succinic Acid}]_o$, M	$[\text{Succinic Anhydride}]_{eq}$, M	$[\text{Acetic Anhydride}]_{eq}$, M	$[\text{Succinic Acid}]_{eq}$, M	K^a, M	Average, M
0.40	0	0.39	1.42×10^{-2}	1.42×10^{-2}	5.06×10^{-4}	
0.30	0	0.29	1.23	1.23	5.04	
0.40	0.03	0.4	0.50	3.5	4.90	4.85×10^{-4}
0.40	0.075	0.4	0.21	7.71	4.43	

$[\text{Glutaric Anhydride}]_o$, M	$[\text{Acetic Anhydride}]_{eq}$, M	$[\text{Glutaric Anhydride}]_{eq}$, M	$[\text{Glutaric Acid}]$, M	K, M	Average, M
0.501	0.301	0.200	0.301	0.453	
0.402	0.261	0.139	0.261	0.489	0.460
0.305	0.204	0.096	0.204	0.433	
0.200	0.150	0.050	0.150	0.450	

H^+ + *trans*-1,2-Cyclohexanedicarboxylic anhydride + CH_3COOH (excess) $\overset{K}{\rightleftharpoons}$ H^+ + $(CH_3CO)_2O$ + *trans*-1,2-Cyclohexanedicarboxylic acid

$[\text{Anhydride}]_o$, M	$[\text{Acetic Anhydride}]_{eq}$, M	$[\text{Anhydride}]_{eq}$, M	K, M	Average, M
0.3169	0.1392	0.1767	0.105	
0.2129	0.0978	0.1141	0.095	0.108
0.1786	0.0911	0.875	0.083	
0.1038	0.0712	0.0326	0.150	

[a] $K = k_I/k_{-I}$.

Table V shows the equilibrium constants calculated from spectral data for succinic, *trans*-1,2-cyclohexanedicarboxylic, and glutaric anhydrides based on Eqs. 4 and 5. These were the only anhydrides of those studied that showed an observable reaction under the conditions employed. Spectra of phthalic, *cis*-1,2-cyclohexanedicarboxylic, and maleic anhydrides in acetous perchloric acid did not show any noticeable change. The concentrations of the cyclic anhydrides used in these measurements ranged from 0.1 to 0.5 M, except for phthalic anhydride which was about 1×10^{-4} M. The equilibrium constants reflect the relative stability of cyclic anhydrides which were 1000:5:1 for succinic, *trans*-1,2-cyclohexanedicarboxylic, and glutaric anhydrides, respectively.

The other anhydrides, maleic, *cis*-1,2-cyclohexanedicarboxylic, and phthalic, were much more stable, *i.e.*, $K < 1 \times 10^{-4}$ M.

The effect of moisture on the precision of the equilibrium constant determinations should be noted. Acetic acid is very hygroscopic; it has been estimated (15) that the water content of almost "dry" acetic acid, when exposed to the atmosphere for 0.5 hr, increased by 5×10^{-2} M. Although utmost care was taken to avoid moisture, errors of up to 10% were introduced into the equilibrium constant determinations by moisture. The relatively concentrated solutions of the anhydrides (not less than 0.1 M) used helped reduce the error due to moisture.

The forward rate constant k_I at 25° for the system defined by Scheme II was evaluated from plots of Eq. 1. Figure 2 is a representative plot for the loss of glutaric anhydride from solution. Although all cyclic anhydrides were studied, only k_I values for succinic, glutaric, and *trans*-1,2-cyclohexanedicarboxylic anhydrides could be determined. The concentration range of cyclic anhydrides used in the rate evaluations was 0.1–0.5 M; as mentioned earlier, these relatively high concentrations were employed to help reduce the effect of extraneous moisture. The excellent fit of all data to Scheme I and Eq. 1 suggests that the reaction can be described by Scheme II.

Values of k_I for the three cyclic anhydrides studied are shown in Table VI.

Table VII shows the third-order rate constant k_{-I}, *i.e.*, the rate constant of the reaction of the dicarboxylic acids with acetic anhydride catalyzed by perchloric acid. These rate constants were obtained under pseudo-first-order conditions, *i.e.*, where perchloric acid was maintained constant and an excess of one of the other reactants was used. For example, acetic anhydride was used in excess with acids having good chromophores such as phthalic and maleic acids. The disappearance of the acids followed pseudo-first-order kinetics. The third-order rate constant k_{-I} was then obtained from plots of k_{obs} *versus* the excess reagent. In the case of succinic and *cis*-1,2-cyclohexanedicarboxylic acids, the acids were used in excess and the disappearance of acetic anhydride was followed.

Two typical examples of these plotting techniques are shown in

Table VI—Forward Rate Constants, k_I (Scheme II), at 25° in Acetic Acid

	Initial Cyclic Anhydride Concentration, M	$[\text{Acetic Anhydride}]_{eq}$, M	[Perchloric Acid], M		k_I, M^{-1} min^{-1}
Succinic anhydride	0.4	0.0164	0.007		0.143
	0.3	0.0150	0.007		0.149
	0.2	0.0105	0.007		0.178
				Average	0.157
trans-1,2-Cyclohexanedicarboxylic anhydride	0.317	0.1392	0.007		21
	0.213	0.0978	0.007		20
	0.179	0.0911	0.007		20
	0.104	0.0712	0.007		18
				Average	19
Glutaric anhydride	0.2	0.15	0.0075		74
	0.3	0.204			78
	0.4	0.276			70
				Average	74[a]

[a] When using the initial slopes, a value of 81 M^{-1} min^{-1} was obtained for this case.

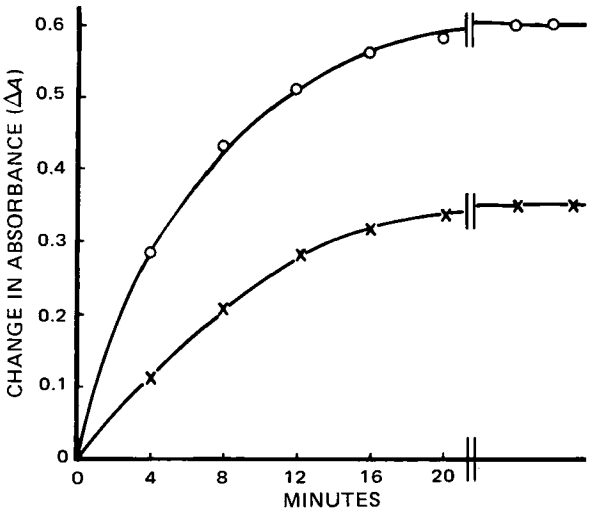


Figure 1—*Increase in absorbance due to production of acetic anhydride as a function of time for the reaction of the cyclic anhydride with acetic acid catalyzed by perchloric acid in acetic acid. The plots show the change in absorbance* versus *time for: 0.25* M *glutaric anhydride (O) in the presence of 1.0 × 10^{-3}* M *$HClO_4$ at 268 nm and 25°, and for 0.07* M trans-*1,2-cyclohexanedicarboxylic anhydride (×) in the presence of 3.73 × 10^{-3}* M *$HClO_4$ at 259 nm and 25°.*

Figs. 3 and 4. The rate constant k_{-1} for glutaric acid cyclization was obtained from the slope of the semilogarithmic plot of absorbance *versus* time where glutaric acid was in excess. The slope of such a line has the value of $k_1 + k_{-1}$ [glutaric acid]. Because k_1 was already estimated, k_{-1} could be calculated. The constant k_{-1} for *trans*-1,2-cyclohexanedicarboxylic acid was obtained from K/k_1 because of the insolubility problems with the dicarboxylic acid and its difficulty of purification.

Table VIII shows that the value of K as obtained from the k_1/k_{-1} ratio is in good agreement with the value of K determined independently by direct spectroscopic measurements.

DISCUSSION

A detailed scheme describing the interaction of the cyclic anhydrides with acetic acid is shown in Scheme III. The part of the reaction scheme played by perchloric acid has not been included, Compound I is used to illustrate a general cyclic anhydride, and Compound V is used to illustrate its equivalent diacid.

Figure 5 shows the good log–log correlation between K and k_1 and the smaller inverse dependency of K on k_{-1}. As can be seen from Table VII, the rate of reaction of the diacids with acetic anhydride is relatively independent of structure, although subtle differences do appear to exist. When referring to Scheme III and, in particular, the reverse reaction pathways, reactions represented by the constants k_{-2} and k_{-1} would be expected to be rather fast relative to the steps represented by the rate constants k_{-3} and k_{-4}. The constant k_{-2} is expected to be quite large because it represents an intramolecular attack by a carboxyl group at an anhydride linkage. Intramolecular reactions are known to be many thousand times faster than their intermolecular equivalents (16).

The subtle differences in k_{-1} possibly suggest that the rate-determining step in the reaction of diacids with acetic anhydride is represented by the attack of the diacids on acetic anhydride. In the presence of perchloric acid, acetic anhydride is probably protonated. Maleic acid and phthalic acid [pKa 2.00 (17) and 2.95 (17), respectively], the two strongest acids in aqueous solution, have the fastest rates when reacting with acetic anhydride whereas the weakest acid, glutaric acid [pKa 4.34 (17)], has the slowest reaction rate. This finding does not imply that the same order of acidity exists in acetic acid; however, it may suggest that the nucleophilicity of the reactant dicarboxylic acids is important. This finding further supports the possibility that the rate-determining step in the

Table VII—Values of k_{-1} at 25° for Scheme II

Dicarboxylic Acid	k_{-1}, M^{-2} min^{-1}
Glutaric	156
trans-1,2-Cyclohexanedicarboxylic	220
Succinic	400
cis-1,2-Cyclohexanedicarboxylic	445
Phthalic	585
Maleic	1000

Table VIII—Equilibrium Constants for Reactions (Scheme II) of Glutaric Anhydride and Succinic Anhydride with Acetic Acid

Anhydride	Constants Calculated from Equilibrium Data[a], *M*	Constants Calculated from Rate Data[b], *M*
Succinic	4.85 × 10^{-4}	4.5 × 10^{-4}
Glutaric	0.49	0.47

[a] Determined from the UV absorbance of the reaction system at equilibrium and absorptivities of the species involved. [b] Determined from the rate constants $K = (k_1/k_{-1})$. It is recognized that the calculation of the k_1 values utilized equilibrium concentrations and were influenced to a degree by equilibrium data.

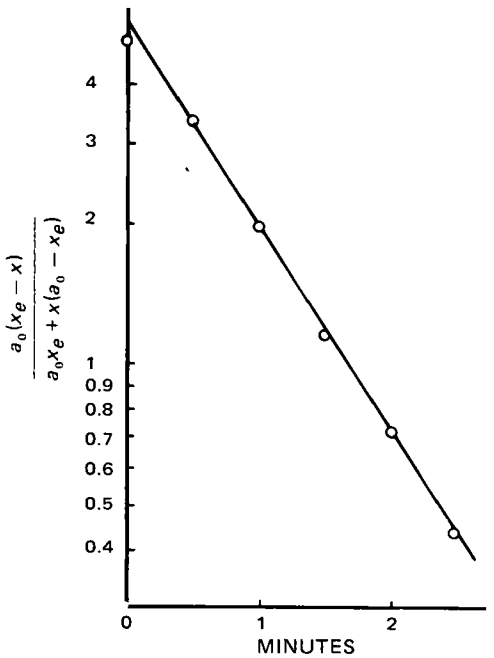


Figure 2—*Perchloric acid-catalyzed reaction of glutaric anhydride with acetic acid. The figure shows a semilog plot of* $a_0(x_e - x)/[a_0x_e + x(a_0 - x_e)]$ *against time of the reaction of glutaric anhydride with acetic acid catalyzed by perchloric acid at 25° as described by:*

$$log\ \frac{a_0(x_e - x)}{a_0x_e + x(a_0 - x)} = -\frac{2a_0 - x_e[HClO_4]k_1t}{x_e 2.303}$$

and:

$$k_1 = \frac{-slope \times 2.303}{[(2a_0 - x_e)/x_e]}[HClO_4]$$

where a_0 *is the initial glutaric anhydride concentration. The concentrations of acetic anhydride at any time and at equilibrium are represented by* x *and* x_e, *respectively;* $a_0 = 0.40$ M; $[HClO_4] = 7.5 \times 10^{-3}$ M; $x_e = 0.242$ M; *and* k_1 *is the pseudo-second-order rate constant.*

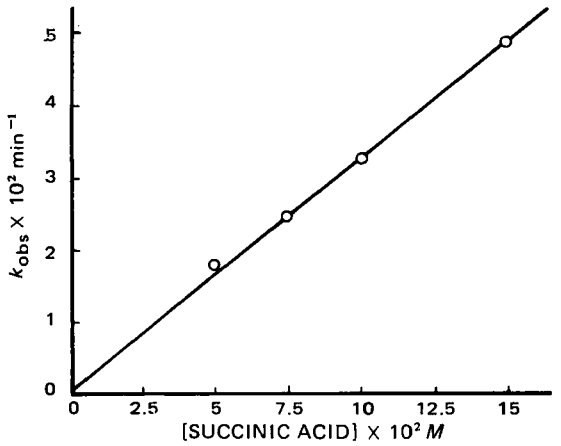


Figure 3—*Perchloric acid-catalyzed reaction of succinic acid with acetic anhydride. The figure shows a plot of* k_{obs} *as a function of succinic acid for the perchloric acid-catalyzed reaction of acetic anhydride with succinic acid in acetic acid at 25°;* k_I = *slope/*$[HClO_4]$, *and* $[HClO_4] = 5.6 \times 10^{-3}$ M.

determination of k_{-1} is the formation of the tetrahedral intermediate IV.

As seen in Table VI, the values of k_1 appear to be a strong function of the molecular structure of the anhydrides. Since the steric and electrophilic factors of acetic anhydride and the mixed anhydride, III, would be considered to be similar, it seems reasonable to assume that the attack of acetic acid on the mixed anhydride and the subsequent breakdown of the intermediate IV would not be strongly influenced by the molecular structure of the diacid part of the mixed anhydride.

Similarly, because the hydrolytic behavior of all anhydrides, including acetic anhydride, is so similar (18) and the rate-determining step in the hydrolysis of the anhydrides is considered to be the formation of the tetrahedral intermediate, it follows that k_1 is probably not sufficiently perturbed by structure to result in the differences in reactivity seen with the various cyclic anhydrides. Therefore, the probable slow step in the reaction of the cyclic anhydrides with acetic acid is the breakdown of the tetrahedral intermediate II.

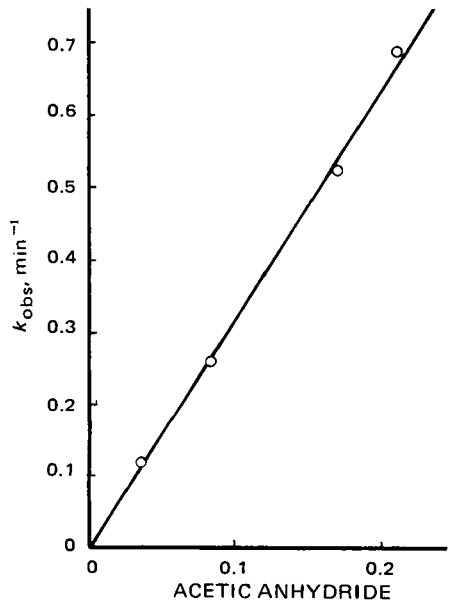


Figure 4—*Perchloric acid-catalyzed reaction of phthalic acid with acetic anhydride as a function of acetic anhydride concentration,* $[(CH_3CO)_2O]$. *The figure shows a plot of* k_{obs} *against acetic anhydride for the perchloric acid-catalyzed reaction of phthalic acid and acetic anhydride in acetic acid at 25°;* k_{-1} = *slope/*$[HClO_4]$, *and* $[HClO_4] = 1.0 \times 10^{-3}$ M.

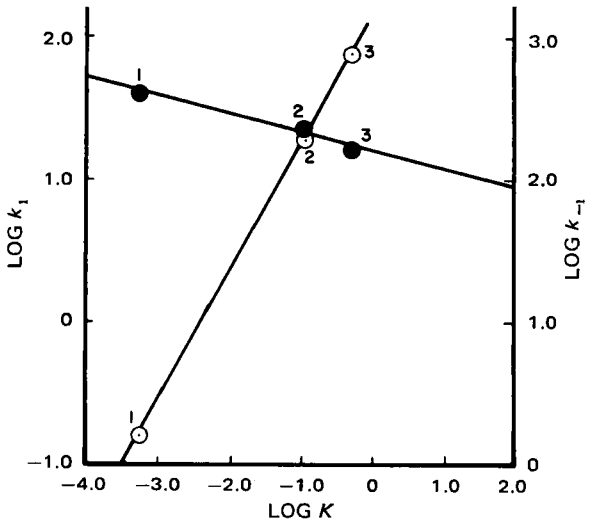


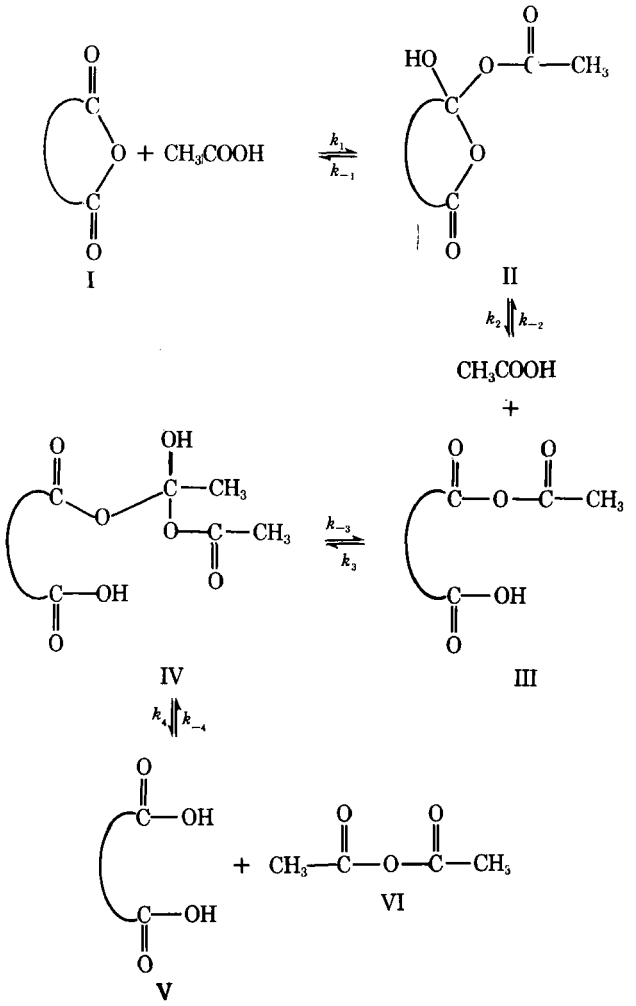
Figure 5—*Plot showing the log–log correlation of* k_I *and* k_{-I} *to* K *for Scheme II. The open circles represent the log* k_I versus *log* K *relationship, and the closed circles represent the log* k_{-I} versus *log* K *relationship. Compound 1 is succinic anhydride, Compound 2 is* trans-*1,2-cyclohexanedicarboxylic anhydride, and Compound 3 is glutaric anhydride.*

The slowness of this step is rationalized on the basis that the transition state for the step requires the expulsion of the neighboring carboxyl group. The reverse of this step, represented by the constant k_{-2}, involves an intramolecular reaction. Increased rates of intramolecular reactions have been observed due to backbone alkyl substitution. Other means by which intramolecular reaction rates have been increased are by "locking" the reaction center "in line" with the nucleophile by constraining rotation within a ring system by: (*a*) decreasing ring size, *e.g.*, glutaric to succinic; (*b*) placement of a double bond in the ring system to prevent free rotation, *e.g.*, succinic to maleic and succinic to phthalic; or (c) placing the reaction centers near each other using favorable rigid analogs, *e.g.*, *cis*-1,2-cyclohexanedicarboxylic acid compared to *trans*-1,2-cyclohexanedicarboxylic acid. A complete argument on the subtleties of these various points can be found in Refs. 4, 5, 7, 8, and 19–28.

On the basis of the Hammond principle (29), the reverse of such an intramolecular reaction, or the ease with which the neighboring carboxyl group leaves, should be inverse to the ease with which the intramolecular reaction takes place. The order of reactivity of the cyclic anhydrides in the present study follows this pattern. The dependency of K on the constant k_1 and, subsequently, k_2 of Scheme III shows that the thermodynamic stability of the cyclic anhydrides is closely related to their molecular structure and can be rationalized on the basis of the known relative reactivity of intramolecular reactions compared to intermolecular reactions.

By the theory of microscopic reversibility, it follows that k_{-4} cannot be the rate-determining step for the reverse reaction and k_2 be the rate-determining step for the forward reaction. It was stated earlier that k_{-2} should be large relative to other rate constants for the reverse reaction because it involves an intramolecular reaction. Even though k_{-1} is not greatly perturbed by the structure of the cyclic anhydrides (Table VII), the order of reactivity of the dicarboxylic acids with acetic anhydride is consistent with those species most likely to undergo an intramolecular reaction, *i.e.*, maleic > phthalic > succinic > glutaric. It appears, therefore, that the transition state for the reaction of II in equilibrium with III probably represents the species of greatest free energy for the equilibrium formation of acetic anhydride from cyclic anhydrides dissolved in acetic acid.

In summary, the transacylation reaction presented in this study, *i.e.*, the equilibrium formation of acetic anhydride in anhydrous acetic acid in the presence of a high energy acylator such as a cyclic anhydride, could be rationalized on the basis of the known chemical reactivity of the anhydrides. These results provide additional insight into the chemistry of the energy-rich tetrahedral interme-



Scheme III

diates and cyclic anhydrides, which have been postulated in numerous pharmaceutically important reactions. The intermediacy of anhydrides in the hydrolysis of certain hemiesters has been established (5), and the acylating capacity of high energy pharmaceutical esters and amides such as aspirin and penicillin has been noted.

The present study provides a further quantitative picture of the effect of molecular structure on the partitioning of the tetrahedral intermediate between the open high energy configuration and the cyclic anhydride.

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Acylation of Phenol by Cyclic and Acyclic Anhydrides in Anhydrous Acetic Acid

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Abstract □ Acylation of phenol with succinic, glutaric, *trans*-1,2-cyclohexanedicarboxylic, maleic, phthalic, and *cis*-1,2-cyclohexanedicarboxylic anhydrides in anhydrous acetic acid generally resulted in phenyl acetate as the major product. The formation of phenyl acetate as the major reaction product could be rationalized as being due to the reactivity of the cyclic anhydrides with acetic acid to form acetic anhydride as well as the greater reactivity of phenol with formed acetic anhydride than with the cyclic anhydride.

Keyphrases □ Phenol—acylation by cyclic and acyclic anhydrides in anhydrous acetic acid, rate constants, mechanisms □ Acylation—phenol by cyclic and acyclic anhydrides in anhydrous acetic acid, rate constants, mechanisms □ Perchloric acid—catalyst for acylation of phenol in anhydrous acetic acid, rate constants, mechanisms □ Anhydrides, cyclic and acyclic—acylation of phenol in anhydrous acetic acid, rate constants, mechanisms

Haddadin *et al.* (1) recently quantitated the equilibrium formation of acetic anhydride from the reaction of cyclic anhydrides with anhydrous acetic acid catalyzed by perchloric acid. Mechanistically, the rate-determining step for the forward reaction was postulated to be the breakdown of the tetrahedral intermediate formed by the attack of acetic acid on the protonated cyclic anhydride. The hydrolysis of cyclic and acyclic anhydrides has been extensively studied (2), and the results suggest that the rate-determining step usually is the formation of the tetrahedral intermediate adduct. The present study concerns the reactivity of a model nucleophile, phenol, with cyclic and acyclic anhydrides in anhydrous acetic acid. The main objective was to gain an understanding of the mechanisms involved in transacylation reactions taking place in nonaqueous media. This study provides a quantitative picture of the effects of molecular structure and the partitioning of tetrahedral intermediate formation and breakdown on leaving group tendencies, especially when cyclic and acyclic anhydrides are the substrates and phenol is the nucleophile. The reactivity of energy-rich tetrahedral intermediates as well as cyclic and acyclic anhydrides will be discussed as models for high energy acylators, as postulated in numerous pharmaceutically important reactions. The pharmaceutical implications of transacylation reactions have been well documented (3–8).

EXPERIMENTAL

Materials—All materials were as described previously (1). Phenyl acetate was vacuum distilled at 211° and 0.7 mm Hg. The refractive index was 1.5008 [lit. (9) 1.5017] at 25°. Phenyl hemisuccinate was synthesized and purified according to the method of Baddar and Assal (10); phenol[1] was purified by sublimation, mp 42° [lit. (9) mp 43°].

p-Methylphenol[2] was sublimed and dried under vacuum, mp 36° [lit. (9) mp 34°]. *p*-Methoxyphenol[3], mp 56–57° [lit. (9) mp 57°], *p*-iodophenol[3], mp 93–94° [lit. (9) mp 93–94°], and *p*-fluorophenol, mp 46–47° [lit. (9) mp 48°], were recrystallized from benzene–petroleum ether.

Apparatus[4]—For the thermometric measurements, a simple dewar calorimeter was assembled from a wheatstone bridge circuit, a 1-mv recorder, a 2-K thermister probe, a dewar flask of convenient size, a thermometer[5], two variable resistance boxes of 111.1 K, and a mercury cell of 1.35 v (11, 12). The recorder was calibrated to read a difference of 0.4° at ambient temperature per full-scale deflection.

All spectroscopic measurements were carried out in the thermostated cell compartment of a UV spectrophotometer[6].

Procedures—The reactions between acetic anhydride and phenol in anhydrous acetic acid, catalyzed by perchloric acid, were followed both spectrophotometrically and thermometrically.

For the spectrophotometric method, a few microliters of approximately 1×10^{-3} *M* phenol in anhydrous acetic acid was injected directly into a thermostated 1-cm cylindrical cell containing 0.1–0.5 *M* acetic anhydride and 1×10^{-3} *M* perchloric acid in anhydrous acetic acid. The disappearance of phenol was conveniently followed at 278 nm.

For the thermometric method, a few milliliters of 1×10^{-2} *M* phenol, dissolved in anhydrous acetic acid, was injected into an anhydrous acetic acid solution containing 0.1–0.5 *M* acetic anhydride and 1×10^{-4} *M* perchloric acid. All experiments were run at approximately 25°. The reaction was monitored by following the small change in the temperature of the solution. In all cases, this change was <0.09°.

The rate constants were obtained under pseudo-first-order conditions in which acetic anhydride was always in excess. The con-

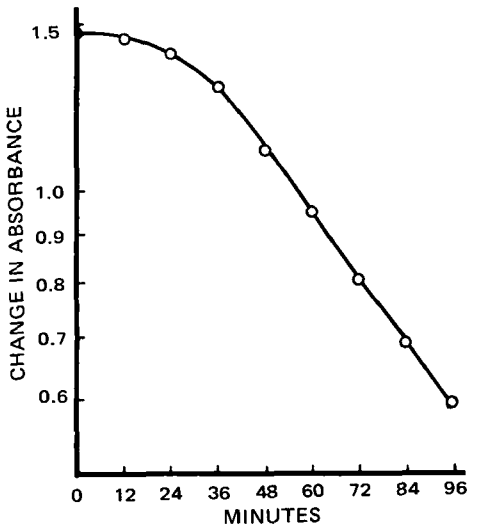


Figure 1—*Semilog plot of change in absorbance against time, demonstrating the lag time for the perchloric acid-catalyzed reaction of phenol with a fresh solution of succinic anhydride in acetic acid at 25°. The initial concentration of succinic anhydride was 0.30* M, *and* $[HClO_4] = 1.244 \times 10^{-2}$ M.

[1] J. T. Baker Chemical Co., Phillipsburg, N.J.
[2] Unknown origin. Structure was confirmed by NMR and elemental analyses.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] NMR spectra were obtained on a Varian T60 NMR spectrometer.
[5] Beckman Instruments Inc., Fullerton, Calif.
[6] Cary model 14 with a thermostated cell compartment maintained at 25°.

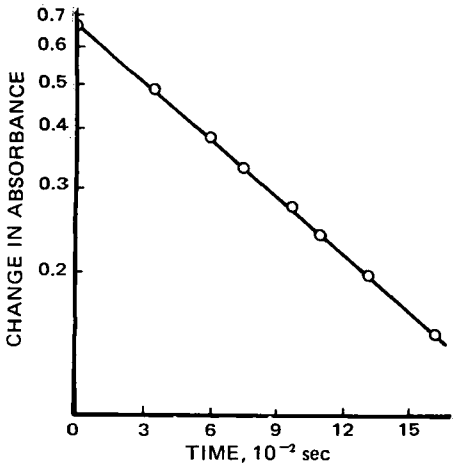


Figure 2—*Semilog plot of change in absorbance against time, demonstrating first-order behavior in phenol when it was reacted with an equilibrated succinic anhydride solution, 0.51* M *in acetic acid, in the presence of* 1.244×10^{-3} M $HClO_4$ *at 25°.*

centrations of acetic anhydride and the catalyst, perchloric acid, were varied independently. The slopes of the observed rate constant, k_{obs}, *versus* acetic anhydride concentrations and perchloric acid concentrations, [$HClO_4$], were used to calculate the third-order rate constant for the reaction of phenol with acetic anhydride.

The reaction of *para*-substituted phenols was followed under the same conditions previously described, except that a convenient wavelength for following the disappearance of the phenol was used.

Reactions between phenol and succinic, glutaric, phthalic, maleic, *cis*-1,2-cyclohexanedicarboxylic, and *trans*-1,2-cyclohexanedicarboxylic anhydrides were studied by adding dilute solutions of phenol in anhydrous acetic acid to preequilibrated solutions of the cyclic anhydride in the anhydrous acetic acid in the presence of perchloric acid. The disappearance of phenol was followed at 278 nm and 25°. The concentrations of the cyclic anhydrides ranged from 0.1 to 0.5 *M*, and the final concentrations of phenol and perchloric acid were 1×10^{-4} and 1×10^{-3} *M*, respectively.

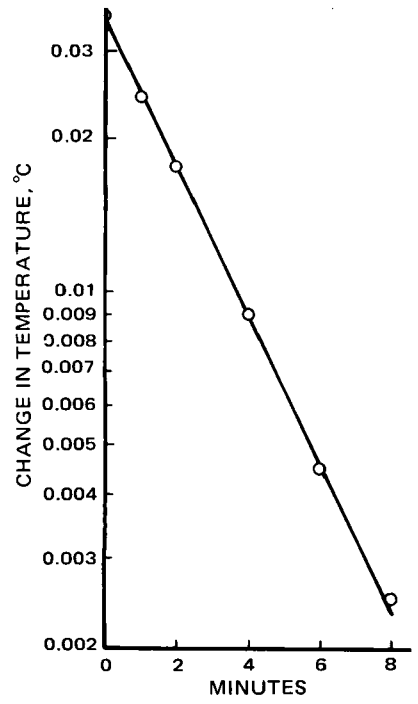
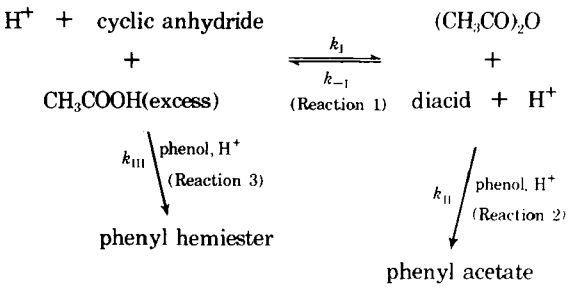


Figure 3—*Semilog plot of change in temperature against time, demonstrating first-order behavior with respect to phenol for the perchloric acid-catalyzed reaction of phenol with acetic anhydride in acetic acid at 25°;* $[(CH_3CO)_2O] = 0.21$ M, *and* $[HClO_4] = 5.0 \times 10^{-3}$ M. *The reaction was monitored thermometrically.*



Scheme I

To study the effects of added succinic acid on the reaction of phenol in the succinic anhydride–acetic acid system, succinic anhydride was maintained at 0.4 *M* while the succinic acid concentration was varied from 0.015 to 0.1 *M*. Similar studies with the corresponding acids of the other cyclic anhydrides were not attempted.

Determination of Reaction Products—The quantitative determination of the phenol ester products found in the phenol–cyclic anhydride–acetic acid reaction mixture was accomplished using the following procedure. Phenyl acetate was estimated by neutralizing a pipetted aliquot with ice-cold sodium carbonate solution to pH 9 and extracting with ether or carbon tetrachloride. The extracts were made up to a convenient volume. The UV absorbance of the solution at 266 and 278 nm was used to calculate the amount of phenyl acetate formed. The absence of phenol in the reaction mixture was verified by absorbance measurements at 278 nm. The shape of the absorbance *versus* wavelength scan corresponded to that of phenyl acetate. The concentration of phenyl acetate extracted was estimated by comparison with the absorbance of a standardized sample. The concentration of the phenol hemiester was calculated from the difference between the initial phenol concentration and the phenyl acetate concentration found.

To check if phenyl hemisuccinate underwent a reversible reaction in the perchloric acid–acetic acid solution, a standardized phenyl hemisuccinate solution was compared. Repetitive UV scanning and TLC showed that phenyl hemisuccinate was stable, *i.e.*, did not undergo a transacylation reaction.

TLC was employed to identify the phenol esters and to verify the spectrophotometric results. A few microliters of the reaction mixtures was spotted along with authentic samples of the various reactants and possible products on dry silica gel TLC plates (preheated at 120° for 30 min). The mobile phase was 3% butyl ether in pentane; the spots were developed in iodine vapor.

RESULTS AND DISCUSSION

When phenol was added to a freshly prepared solution of succinic anhydride in acetic acid with perchloric acid present as a catalyst, the disappearance of the phenol showed a lag time of about 30 min followed by an apparent first-order disappearance (Fig. 1). If the reaction mixture of succinic anhydride and acetic acid was allowed to equilibrate before the addition of phenol, no lag time was observed and the disappearance of phenol followed first-order kinetics (Fig. 2). Similar results were obtained with glutaric anhydride and *trans*-1,2-cyclohexanedicarboxylic anhydride.

The presence of a lag time in the freshly prepared solution was postulated to be due to the formation of a more reactive intermediate, *i.e.*, acetic anhydride, which would then react with phenol as shown in Scheme I.

To test Scheme I, the reactivity of phenol with acetic anhydride in anhydrous acetic acid and in the presence of perchloric acid was studied. The products resulting from the reaction of phenol with the cyclic anhydride in acetic acid were determined as described under *Experimental*.

In the reaction of phenol with succinic anhydride in anhydrous acetic acid, for example, TLC revealed only one spot corresponding to phenyl acetate. When exogenous succinic acid was included in the system before adding phenol, both the rate of formation and the amount of phenyl acetate formed decreased. Furthermore, a

Table I—Rate Constants of the Acetic Anhydride–Phenol Reactions at 25° in Acetic Acid with Perchloric Acid Present as a Catalyst

Phenol	k_{II}, M^{-2} min^{-1}
p-Methoxyphenol	884
p-Methylphenol	482
Phenol	300[a]
	287[b]
p-Fluorophenol	271
p-Iodophenol	96

[a] From spectrophotometric method. [b] From thermometric method.

second spot corresponding to phenyl hemisuccinate was observed. This finding is consistent with Scheme I because exogenous succinic acid would be expected to force Reaction 1 to the left, thereby reducing the equilibrium concentration of acetic anhydride. Similar results were obtained for glutaric and *trans*-1,2-cyclohexanedicarboxylic anhydrides.

The third-order rate constant, k_{II}, for the reaction of phenol with acetic anhydride catalyzed by perchloric acid was determined independently, *i.e.*, in the absence of the cyclic anhydrides. Pseudo-first-order plots for the disappearance of phenol were obtained when perchloric acid was maintained constant and acetic anhydride was used in excess of the phenol. The disappearance of phenol monitored thermometrically and spectrophotometrically is shown in Figs. 3 and 4, respectively. The first-order dependency in acetic anhydride is shown in Fig. 5, and the first-order dependency on perchloric acid is shown in Fig. 6. The importance of perchloric acid can be seen in the near zero intercept.

The relative reactivity of various *para*-substituted phenols is shown in Table I. Included in this table are the values obtained for phenol. The objective of this study was to gain insight into the mechanism of acetylation of phenol under the reaction conditions. A Hammett plot of log k_{II} for the various phenols *versus* σ *para* is shown in Fig. 7. These data support a mechanism involving nucleophilic attack of the phenol on the protonated acetic anhydride as the rate-determining step.

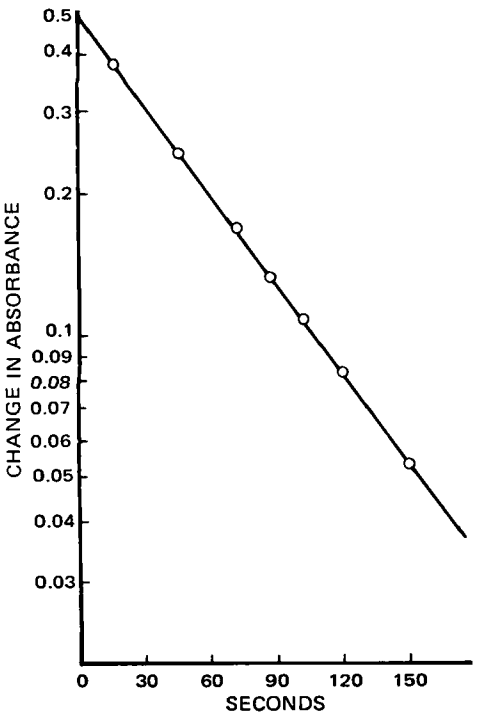


Figure 4—*Semilog plot of change in absorbance against time, demonstrating first-order behavior with respect to phenol for the perchloric acid-catalyzed reaction of acetic anhydride with phenol in acetic acid at 25°; $[(CH_3CO)_2O] = 0.501$ M, and $[HClO_4] = 5.0 \times 10^{-3}$ M.*

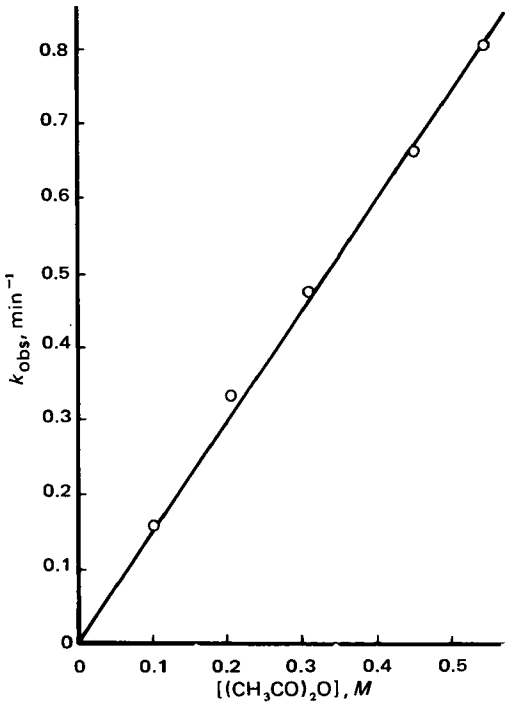


Figure 5—*Plot of k_{obs} against $[(CH_3CO)_2O]$, showing first-order dependency with respect to acetic anhydride in its perchloric acid-catalyzed reaction with phenol; $[HClO_4] = 5.0 \times 10^{-3}$ M. The reactions were monitored thermometrically.*

If a steady-state assumption is made for acetic anhydride in Scheme I, the following equation can be derived:

$$k_{obs} = k'_{III}\,[\text{cyclic anhydride}]_{eq} + k'_{II}\,[(CH_3CO)_2O] \quad \text{(Eq. 1)}$$

It follows that:

$$\frac{k_{obs}}{[\text{cyclic anhydride}]_{eq}} = k'_{III} + \frac{Kk'_{II}}{[\text{diacid}]_{eq}} \quad \text{(Eq. 2)}$$

where k_{obs} is the observed pseudo-first-order rate constant for the disappearance of phenol, k'_{II} is equal to k_{II} $]HClO_4]$, k'_{III} is equal to k_{III} $[HClO_4)$, $[\text{cyclic anhydride}]_{eq}$ is the concentration of the cyclic anhydride in the equilibrated solution, $[\text{diacid}]_{eq}$ is the concentration of the diacid at equilibrium, and $K = k_I/k_{-I}$.

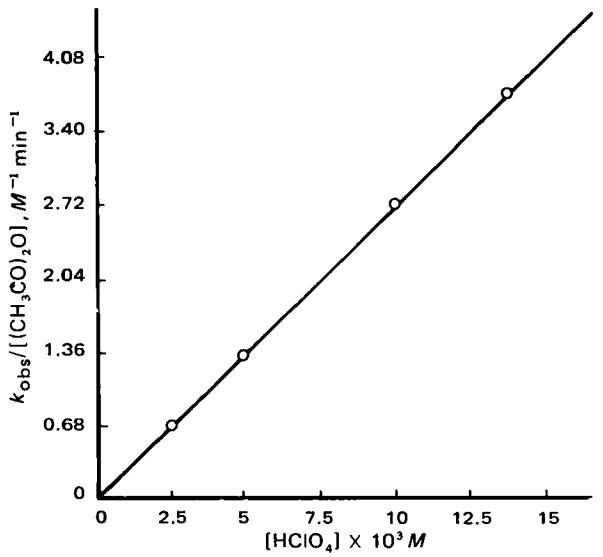


Figure 6—*Plot of $k_{obs}/[(CH_3CO)_2O]$ against the perchloric acid concentration, demonstrating first-order behavior with respect to perchloric acid, the catalyst, in the reaction of acetic anhydride with phenol; $[(CH_3CO)_2O] = 0.53$ M.*

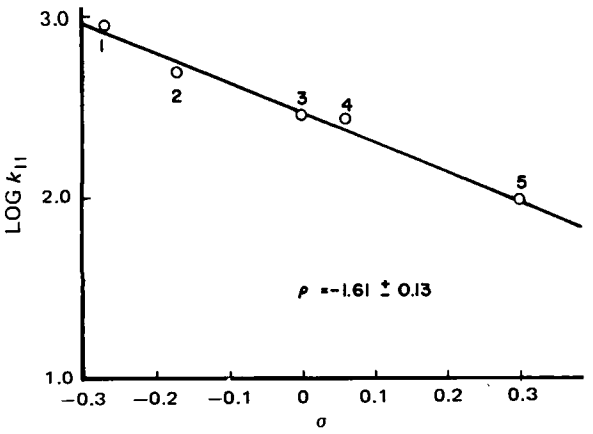


Figure 7—*Semilog plot of* k_{II} *(a third-order rate constant) as a function of the Hammett* σ para *(23). Key: Compound 1,* p-*methoxyphenol; Compound 2,* p-*methylphenol; Compound 3, phenol; Compound 4,* p-*fluorophenol; and Compound 5,* p-*iodophenol.*

The equilibrium concentration of the cyclic anhydride and the corresponding diacid under the experimental conditions described were previously determined (1). If the reaction shown in Scheme I and a steady state for acetic anhydride are assumed, then Eq. 2 predicts that a plot of k_{obs}/[cyclic anhydride]$_{eq}$ *versus* 1/[diacid]$_{eq}$ should be linear with slopes equal to Kk'_{II} and an intercept of k_{III}. Such plots were found to be linear, and a representative one is shown in Fig. 8. The third-order constant k_{III} was calculated to be 1.5, 37, and 48 M^{-2} min^{-1} for succinic, glutaric, and *trans*-1,2-cyclohexanedicarboxylic anhydrides, respectively.

The validity of these intercepts was checked for succinic and *trans*-1,2-cyclohexanedicarboxylic anhydrides by comparing the expected ratios of the phenyl hemiester of the appropriate dicarboxylic acid to phenyl acetate from kinetic data to those found by the quantitative recovery of the products. With 0.2 *M* succinic anhydride in the presence of 0.1 *M* succinic acid and 1×10^{-3} *M* perchloric acid, the ratio of phenyl hemisuccinate to phenyl acetate was expected to be 1:1 but experimentally the recovery was 0.8:1. With 0.2 *M trans*-1,2-cyclohexanedicarboxylic anhydride in the presence of 1×10^{-3} *M* perchloric acid, the kinetically expected ratio of phenyl hemiester of *trans*-1,2-cyclohexanedicarboxylic acid to phenyl acetate was 1:8 whereas the experimental recovery was 1:8.6.

Both from the kinetic and TLC studies, no reaction between phenol and maleic, *cis*-1,2-cyclohexanedicarboxylic, and phthalic anhydrides to form the hemiesters could be detected.

The relative reactivity toward phenol of the various cyclic anhydrides, when compared to the reaction of phenol with acetic anhydride, is not consistent with the relative hydrolytic behavior of these anhydrides (2). Eberson and Landström (2) showed that acetic, succinic, and glutaric anhydrides at 20° and pH 5.20 have almost identical hydrolysis rates, and they stated that there does not seem to be any mechanistic difference between the hydrolysis of open chain and cyclic anhydrides. Recent studies (13) with the slowly hydrolyzing tetramethylsuccinic anhydride suggest that the hydrolysis of this anhydride involves the breakdown of the tetrahedral intermediate whereas the spontaneous hydrolysis of other cyclic and acyclic anhydrides involves rate-determining formation of the tetrahedral intermediate adduct.

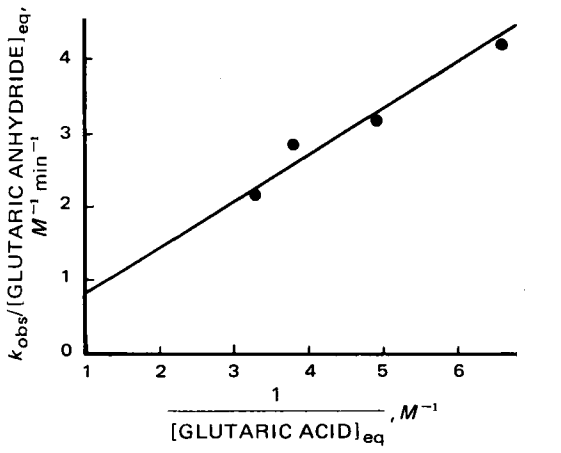
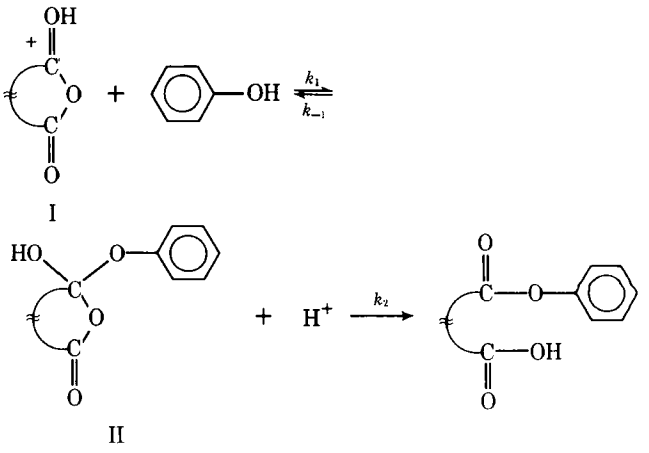


Figure 8—*Plot of* k_{obs}/*[glutaric anhydride]*$_{eq}$ versus *1/[glutaric acid]*$_{eq}$ *for the perchloric acid-catalyzed reaction of phenol with the glutaric anhydride–acetic acid system, varying the initial glutaric anhydride concentration. Slope =* $k_{III}[HClO_4]$*, where* $[HClO_4] = 5.6 \times 10^{-3}$ M.



Scheme II

The relative reactivity of the *para*-substituted phenols toward acetic anhydride in anhydrous acetic acid in the presence of perchloric acid suggests that nucleophilicity of the phenol is important. This is consistent with the rate-determining step being the attack of the phenol on the protonated, positively charged, acetic anhydride. The relative reactivity of phenol toward succinic anhydride compared to acetic anhydride suggests that a change in the rate-determining step has occurred. Such a mechanism is shown in Scheme II, where I represents a cyclic or acyclic anhydride.

The ability of hemiesters and amides of cyclic acids to undergo a facile intramolecular reaction to form the corresponding anhydride has been well recognized (14–22). The relative reactivity of cyclic anhydrides to undergo solvolytic reactions with acetic acid was rationalized on the basis of the difficulty of expulsion of a carboxyl group from the tetrahedral intermediate formed by the attack of acetic acid on the protonated anhydride (reaction represented by k_1 in Scheme I of Ref. 1). By the same argument, it appears that the rate-determining step in the reaction of phenol with succinic anhydride under the present conditions may be k_2, *i.e.*, the breakdown of the tetrahedral intermediate II, a conclusion based on the large difference between k_{II} and k_{III}.

The large negative entropy of activation changes and the large solvent isotope effects seen in the spontaneous hydrolytic reactions of various cyclic and acyclic anhydrides suggest that the transition state energy was lowered by hydrogen bonding with solvent molecules (2). The reduced ability to solvate a polar transition state by specific interaction in a lower dielectric medium like acetic acid will contribute to differences in the transition state for nucleophilic reactions in water *versus* those in acetic acid.

The relative reactivity of phenol with the cyclic anhydrides studied in the present case (including those anhydrides for which no measurable reactivity could be determined, *i.e.*, $k_{III} \ll 1\ M^{-2}$ min^{-1}) is inverse to the ability of the hemiester and amides of the corresponding dicarboxylic acid to undergo intramolecular reactions (14–22). This finding suggests that the difficulty in expelling the neighboring carboxyl group, *i.e.*, k_2 of Scheme II, is probably the rate-determining step.

SUMMARY

The formation of phenyl hemiesters of dicarboxylic acids from the reaction of phenol with a dicarboxylic anhydride in anhydrous acetic acid catalyzed by perchloric acid is a function of two interre-

lated factors: (*a*) the reactivity of phenol toward the cyclic anhydride *versus* the reactivity of phenol toward the *in situ* formed acetic anhydride, and (*b*) the equilibrium concentration of acetic anhydride resulting from the solvolytic reaction of acetic acid with the cyclic anhydride. The more reactive the cyclic anhydride, the more readily it reacts with both phenol and acetic acid.

The present study provides a further quantitative picture of the effect of molecular structure on the partitioning between rate-determining formation and breakdown of the energy-rich tetrahedral intermediate for reaction at an acyl function. The poor leaving group tendency of the constrained neighboring carboxyl group in the reaction of phenol with cyclic anhydrides, due to the inclination of the neighboring carboxyl to undergo the reverse intramolecular reaction, resulted in tetrahedral intermediate breakdown being the rate-determining step. It is postulated that the good leaving group tendency of the unconstrained acetic acid in the reaction of phenol with the acyclic anhydride, acetic anhydride, results in tetrahedral intermediate formation being the rate-determining step. These differences in mechanism between the reactivity of cyclic and acyclic anhydrides in acetic acid compared to their hydrolytic reactivity must be due to the reduced ability of acetic acid, when compared to water, to solvate polar transition states.

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Dissolution Patterns of Polydisperse Powders: Oxalic Acid Dihydrate

J. T. CARSTENSEN ˣ and MAHENDRA PATEL

Abstract □ The dissolution of oxalic acid dihydrate crystals of log-normal particle-size distribution in 0.1 *N* HCl was studied. A biphasic cube root dependence was found; the slopes of the initial cube root plots were consistent with theory based on dissolution of isometric, isotropic particles where assumptions were made of: (*a*) sink conditions, (*b*) particle-size-independent solubility, and (*c*) particle-size-independent film thickness of adsorbed liquid layers.

Keyphrases □ Dissolution of polydisperse powders (oxalic acid dihydrate)—dissolution patterns, equations □ Oxalic acid dihydrate (polydisperse powder)—dissolution patterns, equations □ Powders, polydisperse—dissolution, oxalic acid dihydrate, equations

The rates of dissolution and the mechanisms involved in the dissolution process have been the subjects of many publications in the last decade. However, most dissolution work done on powders has been restricted to monodisperse powders (1–4).

BACKGROUND

Hixson and Crowell (5), basing their derivation on the Noyes–Whitney equation (6), arrived at the so-called cube root dissolution rate law:

$$\sqrt[3]{W_0} - \sqrt[3]{W} = Kt \qquad \text{(Eq. 1)}$$

where W_0 is the initial weight of the powder, W is the weight of undissolved powder at time t, and K is an apparent dissolution rate constant having units of $(\text{weight})^{1/3}$ per unit time. The assumptions made in the derivation of Eq. 1 are: (*a*) all particles dissolve isotropically, (*b*) the particles are isometric, (*c*) the thickness of the diffusion barrier around each particle (the "film thickness,"

lated factors: (*a*) the reactivity of phenol toward the cyclic anhydride *versus* the reactivity of phenol toward the *in situ* formed acetic anhydride, and (*b*) the equilibrium concentration of acetic anhydride resulting from the solvolytic reaction of acetic acid with the cyclic anhydride. The more reactive the cyclic anhydride, the more readily it reacts with both phenol and acetic acid.

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Dissolution Patterns of Polydisperse Powders: Oxalic Acid Dihydrate

J. T. CARSTENSEN ˣ and MAHENDRA PATEL

Abstract □ The dissolution of oxalic acid dihydrate crystals of log-normal particle-size distribution in 0.1 *N* HCl was studied. A biphasic cube root dependence was found; the slopes of the initial cube root plots were consistent with theory based on dissolution of isometric, isotropic particles where assumptions were made of: (*a*) sink conditions, (*b*) particle-size-independent solubility, and (*c*) particle-size-independent film thickness of adsorbed liquid layers.

Keyphrases □ Dissolution of polydisperse powders (oxalic acid dihydrate)—dissolution patterns, equations □ Oxalic acid dihydrate (polydisperse powder)—dissolution patterns, equations □ Powders, polydisperse—dissolution, oxalic acid dihydrate, equations

The rates of dissolution and the mechanisms involved in the dissolution process have been the subjects of many publications in the last decade. However, most dissolution work done on powders has been restricted to monodisperse powders (1–4).

BACKGROUND

Hixson and Crowell (5), basing their derivation on the Noyes–Whitney equation (6), arrived at the so-called cube root dissolution rate law:

$$\sqrt[3]{W_0} - \sqrt[3]{W} = Kt \qquad \text{(Eq. 1)}$$

where W_0 is the initial weight of the powder, W is the weight of undissolved powder at time t, and K is an apparent dissolution rate constant having units of $(\text{weight})^{1/3}$ per unit time. The assumptions made in the derivation of Eq. 1 are: (*a*) all particles dissolve isotropically, (*b*) the particles are isometric, (*c*) the thickness of the diffusion barrier around each particle (the "film thickness,"

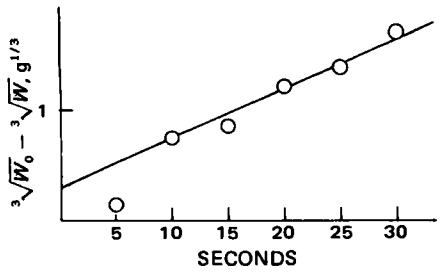

Figure 1—*Dissolution pattern of a "fine" powder of isothermally recrystallized oxalic acid dihydrate.*

h) is constant, (d) there are no particle-size solubility effects, and (e) sink conditions exist.

Most systems in pharmaceutical dosage forms are polydisperse systems; many investigators (7–12) have shown that particles prepared by procedures such as milling and precipitation produce skewed distribution functions which closely resemble log-normal particle-size distributions.

The dissolution of polydisperse powders of log-normal distribution was first studied by Higuchi and Hiestand (13) and Higuchi *et al.* (14). In their treatment, they approximated the log-normal frequency function:

$$f(\log x) = \frac{1}{\sqrt{2\pi}(\sigma)} \exp[(\log x - \log M)^2/2\sigma^2] \qquad \text{(Eq. 2)}$$

when expressed as a cumulative oversize distribution by the cumulative function:

$$g(x) = K/x^4 \qquad \text{(Eq. 3)}$$

where x is the particle diameter, M is the geometric mean diameter, and σ is the standard deviation of the distribution. The nomenclature employed in Eq. 2 is adhered to in this study. Good agreement with experimental values was found previously using the approximation in Eq. 3 (13, 14).

Carstensen and Musa (15) generated log-normal distributions by computer and followed the dissolution under assumptions (a)–(e); they found that the dissolution patterns could be approximated by a cube root law up to a critical time, t_c, when the smallest particle of diameter a_0 would be completely dissolved. Beyond this point, the cube root plot changes slope. The equation that held prior to the critical time was:

$$\sqrt[3]{W_0} - \sqrt[3]{W} = \frac{1.84kC_s \sqrt[3]{W_0}}{\sigma^{0.02}M\rho} t \qquad \text{(Eq. 4)}$$

where C_s is the saturation concentration, and k is the dissolution rate constant in centimeters per second. The distributions generated by Carstensen and Musa were all truncated (at + and -3σ). Equation 4 was found to hold in the limited range of σ values tested; aside from the limitation of the σ range tested, the equation[1] suffers from having the slope, α, tend toward infinity when σ tends toward zero.

Brooke (16, 17) arrived at an exact expression of the dissolution rate profile of a nontruncated, log-normal, particle-size distributed powder with the previously mentioned assumptions (a)–(e). The treatment was expanded to truncated distributions by envoking the Hatch–Choate (11) equation; this equation is applicable when $\sigma < 0.14$ (18, 19). Brooke (17) also showed that a cube root relationship could be explained by the theoretically rigorous treatment and suggested the following equation:

$$\sqrt[3]{W_0} - \sqrt[3]{W} = \exp[-2.54\sigma_D^2] \frac{2kC_s \sqrt[3]{W_0}}{\rho M} t \qquad \text{(Eq. 5)}$$

where $\sigma_D = 2.3\sigma$. Brooke's equation has the same dependence on density, mean diameter, rate constant, initial weight, and solubility as that of Carstensen and Musa (15), but the dependence on standard deviation in Eq. 5 is different; it does not suffer from the limitations with which Eq. 4 is afflicted.

[1] The equation reported by Carstensen and Musa (15) contains an error in the exponent to σ and in the numerical factor; the equation given here is correct in relation to the data reported in Ref. 15.

One main purpose of this study was to test whether real powders that do not contain spherical particles and do not (necessarily) adhere to assumptions (a)–(e) follow a cube root law and, if so, whether Eqs. 4 and 5 are applicable to the dissolution profiles. Benet (20) pointed out that in spite of the theoretical work published to date, no experimental work other than that of Higuchi *et al.* (14) has been published.

The information obtained from dissolution of powders can be valuable regarding the bioavailability of oral dosage forms in solid or suspension form. Carstensen (4), using data from Prescott *et al.* (21), showed the relationship between the mean diameter of an orally administered powder and the fraction of drug absorbed.

EXPERIMENTAL

The test substance used was oxalic acid dihydrate[2]. It was recrystallized by dissolving 100 g of oxalic acid in 200 ml of distilled water at 70° and cooling the solution at a rate of about 1°/10 min. This temperature was measured by a thermometer placed in the center of the beaker containing the solution. The temperature distribution by this procedure is very uneven (differing by as much as 15° between extreme points), giving rise to distributions that are somewhat off the log-normal distribution.

The suspension was allowed to stand for 24 hr at room temperature, and the crystals were then filtered off and air dried. The method produces crystals coarser than 70 mesh and as coarse as 4 mesh. The dried crystals were segregated into mesh fractions with USP sieves. To avoid vitrification[3], the fractions were stored in desiccators over saturated sodium chloride solution with excess sodium chloride (33% relative humidity). Oxalic acid dihydrate crystals of various mesh sizes were then blended in ratios that were log normal on a number basis.

The individual particle weights were obtained by weighing counted numbers of particles in each mesh fraction. A microscopic particle-size distribution was carried out on a random sample of particles from a −20/+30-mesh fraction. The density of oxalic acid was determined pycnometrically, using saturated oxalic acid solution as the liquid vehicle.

The general dissolution method was as follows. A 4-liter beaker was filled with 4 liters of 0.1 N HCl and placed on a magnetic stirrer setup[4] equipped with a Teflon-coated stirring bar, 36 mm long and 8 mm in diameter; the solution was stirred at 400 rpm. This agitation condition allows the crystals to be suspended, whereas lower speeds do not accomplish good dispersion of the solid after it is added. The liquid in the beaker was equilibrated in a water bath at 25.8°[5] in a room at 25 ± 0.2°. Immediately prior to the start of the dissolution experiment, the beaker was transferred to the magnetic stirrer; 80 g of oxalic acid dihydrate powder was then added and the timing was started.

Approximately 6-ml samples were removed by hypodermic syringe at various time points. After removal, the sample was emptied into a test tube, exactly 5 ml was removed, and the oxalic acid content was determined by titration with 0.05 N sodium hydroxide. In dissolution studies carried out at temperatures other than room temperature, the 0.1 N HCl was measured out to 4 liters at room temperature and its temperature was then adjusted to the test temperature. Approximately 6-ml samples were taken as before, but 5 ml was not removed until the sample reached room temperature.

The solubility of oxalic acid dihydrate in 0.1 N HCl was determined at various temperatures, and the determinations were made using a thermostated shaker bath[6].

The dissolution of a single crystal of oxalic acid was determined by placing it on a hemocytometer slide, adding 3 drops of 0.1 N HCl, and observing the length, L, and breadth of the crystal microscopically as a function of time.

[2] Mallinckrodt analytical reagent oxalic acid dihydrate, Mallinckrodt Chemical Works, St. Louis, Mo.

[3] The water vapor pressure over the salt pair oxalic acid + oxalic acid dihydrate is 2.65 torr at 25° (22, 23), and the water vapor pressure over saturated oxalic acid solution is 23 torr (97% relative humidity) at 25° (24).

[4] Model 4812, solid-state magnetic 6 × 6 stirrer with thermostatically controlled hot plate, Cole Parmer, Chicago, IL 60648

[5] With the agitation conditions used, addition of 80 g of oxalic acid caused a temperature drop of 0.8°.

[6] Aquaterm water bath shaker model R-86, New Brunswick Scientific Co., New Brunswick, N.J.

Table I—Oxalic Acid Particles Used in a Particle-Size Distribution with Mean by Number of M = 1500 μm and SD = 0.108

Mesh Fraction	Oversize Diameter, μm	Weight of Particles Used/80 g of Sample	Percent Particles by Number (Calculated)	Number of Particles/10^8 Particles
−5/+6	3360	1.33	0.065	54,000
−6/+10	2000	31.13	12.44	12,439,000
−10/+20	840	47.38	86.40	86,450,000
−20/+30	590	0.16	1.09	1,057,000

RESULTS AND DISCUSSION

It was mentioned previously (1) that substances, when crystallized from solution, tend to have particle sizes with a truncated log-normal distribution. Oxalic acid, when recrystallized isothermally from water, indeed shows a log-normal particle-size distribution. By varying the temperature at which the crystals are allowed to grow, it is possible to alter the mean particle size and standard deviation. However, the magnitudes of the standard deviations are all close so that if it were desired to study the effect of the standard deviation of a powder sample on its dissolution pattern, then isothermally recrystallized powders do not offer an approach that will allow a sufficiently wide variation in the variable to permit data analysis. Such powders, however, dissolve in a cube root fashion as predicted previously (15–17). The dissolution of a sample of isothermally recrystallized oxalic acid with a log-normal distribution, with a mean of 250 μm and a standard deviation of σ = 0.13, is shown in Fig. 1.

It was predicted by computer simulation (15) that when σ is not excessively large, a cube root plot of amounts undissolved *versus* time should give a biphasic linear plot. The point where the two lines intersect occurs at the critical time, t_c, corresponding to the point in time where the smallest particle of the original powder sample has just dissolved completely. It is noted in Fig. 1 that the cube root plot is quite linear but does not appear to be biphasic; on the other hand, it does not intercept at the origin but rather at a positive y value. This result is due to the particles being too small (and the sample, in a relative sense, having too large a standard deviation) to allow detection of the initial phase.

Since attempts to grow particles of substantially larger mean diameters by isothermal recrystallization were not successful, larger crystals were grown nonisothermally. Log-normal populations were then obtained by sieving these coarser crystals and reblending the obtained fractions in ratios that were log normal on a number basis.

The data of numbers of particles from various mesh fractions used to produce these distributions are shown in Table I. The oxalic acid dihydrate crystals are not altogether regular in shape but appear microscopically to be parallelepipeds of length L and breadth B (Fig. 2).

A microscopic count from a −20/+30-mesh fraction is shown in Fig. 3. On the average, the length L is 2.7 times the breadth B. The volume, Q, is $LB^2 = 2.7B^3$, and the dimension is $\sqrt[3]{Q} = (2.7B^3)^{1/3} = 1.39B$ (Table II). The dimensions correspond well with the mesh

Table II—Number Distribution of Crystal Volumes in a −20/+30 Fraction of Nonisothermally Crystallized Oxalic Acid Dihydrate Crystals

Volume, (ml) × 10^{-3}	Dimension, $(Q/2.7)^{1/3}$, cm	Cumulative Percent	SD
0.3	0.048	10	−1.28
0.5	0.057	20	−0.84
0.7	0.064	35	−0.38
1.1	0.074	46	−0.12
1.3	0.078	65	+0.38
1.5	0.082	75	+0.68
1.9	0.089	85	+1.04
2.3	0.095	95	+1.65

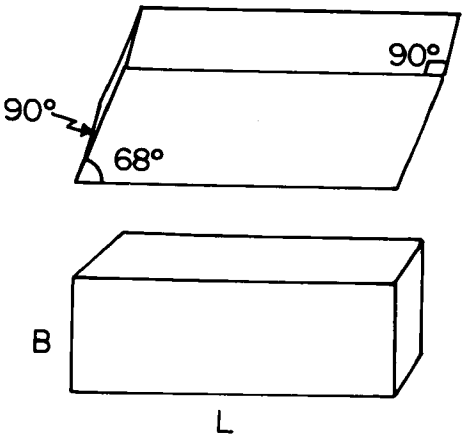

Figure 2—*Actual (top) and idealized (bottom) crystals of oxalic acid dihydrate.*

average (0.070 *versus* 0.0715 cm) and the mesh limits (0.048–0.095 cm). Furthermore, the average volume in Table II (0.73 × 10^{-3} ml) multiplied by the density of oxalic acid dihydrate (1.6 g/ml) gives a result fairly close to the correct weight per particle (1.2 *versus* 1.0 mg).

It is seen from Fig. 3 that the particles within the mesh fraction of a nonisothermally crystallized powder are fairly *normally* distributed[7]. Since the log-normally distributed powders are made by mixing mesh fractions in log-normal ratios, the powders can only be claimed to be approximately log normal, since the distribution within the fractions themselves is normal[7]. Brooke (25) discussed the ramifications of the use of mean mesh size diameters on the results of cube root dissolution.

One assumption made in deriving both Eqs. 4 and 5 is that the particles dissolving are isometric; *i.e.*, the surface area, O, can be related to the volume, Q, by:

$$O = \gamma(Q)^{2/3} \qquad \text{(Eq. 6)}$$

If, as seen in Fig. 2, the volume $Q = LB^2$ and the surface $O = 2B^2 + 4BL$, then Q and O cannot be placed in a relationship as dictated by Eq. 6 without involving B and L. For an isometric shape, *e.g.*, a sphere, the relationship in Eq. 6 is obtained without involvement of dimensions [the factor γ being $(36\pi)^{1/3}$ for a sphere].

It was found microscopically that $L = 2.7B$ on the average. If this is the case throughout the dissolution, then $Q = 2.7B^3$ and $O = 2B^2 + 10.8B^2 = 12.8B^2$. This would lead to $O/[Q^{2/3}] = 10.3$, independent of B or L, so that the particles can be considered isometric only if the ratio stays constant at 2.7 during the dissolution.

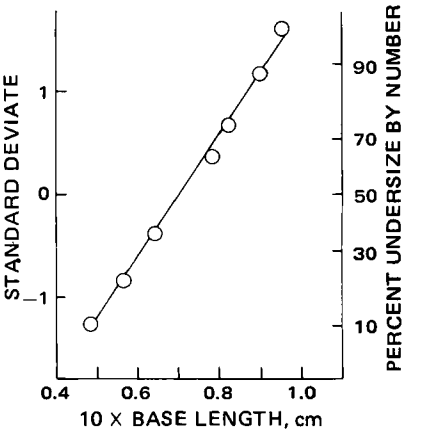

Figure 3—*Distribution of* B *values of oxalic acid dihydrate crystal of a −20/+30-mesh fraction.*

[7] This result is due to the fact that the −20/+30 cut is close to the "mean" of the nonisothermally recrystallized sample. Other cuts may be of a triangular distribution.

Table III—Results from Three Different Runs under Identical Experimental Conditions (Mean = 1050 μm, σ = 0.21)

Seconds	$-\Delta\sqrt[3]{W}$, g$^{1/3}$, Run 1	$-\Delta\sqrt[3]{W}$, g$^{1/3}$, Run 2	$-\Delta\sqrt[3]{W}$, g$^{1/3}$, Run 3	$-\Delta\sqrt[3]{W}$, g$^{1/3}$, Average	Letter Designation in Fig. 5
15	0.43	0.40	0.49	0.44	A
30	1.02	0.93	0.96	0.97	B
40	1.31	1.24	1.21	1.25	C
50	1.59	1.50	1.53	1.54	D
60	1.83	1.72	1.72	1.76	E
70	2.11	2.00	1.95	2.03	F
80	2.30	2.15	2.13	2.20	G
90	2.48	2.33	2.30	2.37	I
105	2.70	2.54	2.55	2.60	H
120	2.91	2.85	2.79	2.85	J

It was stated previously (15, 16) that the particles should dissolve isotropically (*i.e.*, the dissolution rate constants should be independent of the face of the crystal). This assumption is, however, not rigid, as will be shown. As noted in Fig. 2, there are four surfaces with an area of size LB^2. A decrease in B is due to dissolution from the sides of area LB, so the dissolution rate constant for these faces is denoted k_B. Similarly, there are two sides with areas of size B^2 and a dissolution rate constant of k_L, so that dissolution from the crystal occurs with the rate:

$$-dm/dt = [4k_BLB + 2k_LB^2]C_s \qquad \text{(Eq. 7)}$$

If the length-to-breadth ratio is now denoted as $f = L/B$, then Eq. 7 may be written:

$$-dm/dt = B^2[4k_Bf + 2k_L]C_s \qquad \text{(Eq. 8)}$$

Since $Q = LB^2 = fB^3$, it follows that $-(dm/dt) = -\rho(dQ/dt) = -3\rho fB^2(dB/dt)$, so the equation becomes:

$$-dB/dt = [4k_Bf + k_L]/(3\rho f) \qquad \text{(Eq. 9)}$$

For Eqs. 4 and 5 to be structurally correct, dB/dt must be constant, which it is if f is time independent, *i.e.*, if the particle is isometric throughout the dissolution. It is granted that this isometry is related to it being isotropic, but it is fairly easy to check the constancy of f by conducting a dissolution rate experiment on a single crystal under the microscope. The results of such experiments are shown in Fig. 4; the dimensions decrease linearly with time, implying that $[4k_Bf + 2k_L]/(3\rho f)$ is time independent. The original f value is 0.12/0.045 = 2.7; after 100 sec (when 67% of the crystal is dissolved), the ratio is 0.09/0.03 = 3, *i.e.*, not much different.

The first dissolution rate experiment was carried out at one particular standard deviation and one particular mean. It was carried out three times to check reproducibility and to allow statistical evaluation of the critical time (Table III and Fig. 5). Visual examination of Fig. 5 supports the predicted biphasic nature of the cube root plots and of the existence of critical times. To show this result in a more quantitative manner, the three sets of experiments in

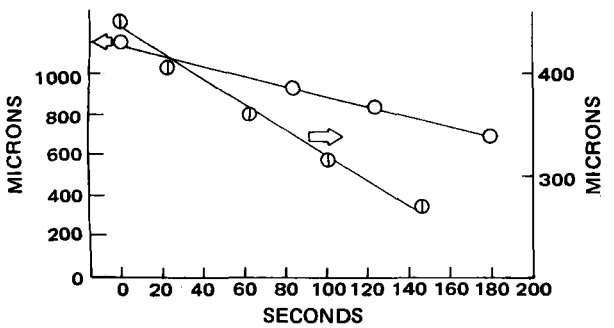

Figure 4—*Dissolution of single crystal of oxalic acid dihydrate at 25° in 0.2 ml of 0.1* N *HCl as determined microscopically. Left axis is length,* L, *of crystal; right axis is breadth,* B, *of crystal.*

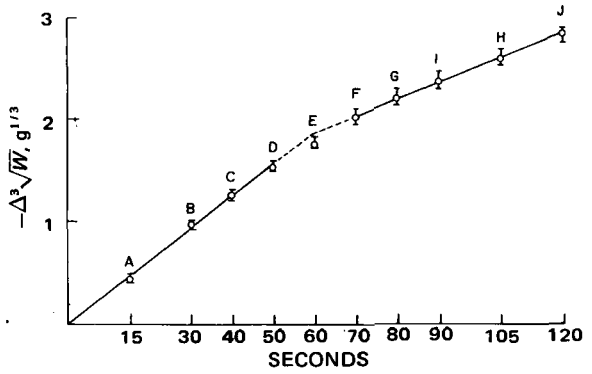

Figure 5—*Results from three different runs under identical experimental conditions; mean = 1050 μm and* SD = *0.21.*

Table III were subjected to statistical analysis. A linear regression analysis with nonzero intercept was carried out on the first four points (A, B, C, and D), and it was shown that the intercept was not significantly different from zero. The points were then fitted by least-squares fitting, and the following equation ensued:

$$-\Delta\sqrt[3]{W} = (0.0308 \pm 0.0014)t \qquad \text{(Eq. 10)}$$

The data points F, G, H, I, and J were then fitted by least-squares fitting, and the observed equation was:

$$-\Delta\sqrt[3]{W} = 0.0163t + 0.894 \qquad \text{(Eq. 11)}$$

By equating Eq. 11 with Eq. 10, one obtains: (*a*) the lower limit for t_c = 56 sec, (*b*) the average value for t_c = 62 sec, and (*c*) the upper limit for t_c = 68 sec.

To show that the curve *is* biphasic, an alternative fit to the set of data was considered. This fit was a monophasic smooth curve, namely a power function of the type $y = ax^n$. The data were fitted to this equation by the method of least squares (Table IV), and the least-squares fit was found to be:

$$\tilde{y} = -\Delta\sqrt[3]{W} = 0.0455t^{0.882} \qquad \text{(Eq. 12)}$$

If this curve is a proper type with which to fit the data, then the deviations from the actual points (y values) to those of Eq. 12 should alternate in sign in fairly random fashion as shown by Durbin and Watson (26). It can be seen from Table IV that this is not the case; six negative deviations occur followed by three positive. A similar argument holds for other smooth curves tested ($[1/y] = a[1/x] + b$ and $y = a[1 - \exp(bx)]$).

In general, in the following, t_c values were obtained in this fashion; the cutoff points between the "first" and "last" points were always determined visually at first and then justified by inclusion of an extra point and demonstration of an increase in the residual sum of squares divided by $n - 2$ (s_{yx}^2). The t_c values obtained in this fashion are ±10%.

Equation 5 may be written:

$$1 - \sqrt[3]{(W/W_0)} = \exp(c\sigma_D^2)\frac{2kC_s}{\rho M}t \qquad \text{(Eq. 13)}$$

This equation will be called a "reduced" form in the following discussion. Equation 4, of course, can be written in reduced form as

Table IV—Points in Fig. 5 Plotted as a Power Function: $\tilde{y} = -\Delta\sqrt[3]{W} = 0.0455t^{0.882}$

Seconds	$\tilde{y}$	y	$\Delta = \tilde{y} - y$
15	0.496	0.44	+0.056
30	0.914	0.97	−0.056
40	1.178	1.25	−0.072
50	1.434	1.54	−0.106
60	1.684	1.76	−0.076
70	1.929	2.03	−0.101
80	2.170	2.20	−0.029
90	2.408	2.37	+0.038
105	2.759	2.60	+0.159
120	3.103	2.85	+0.253

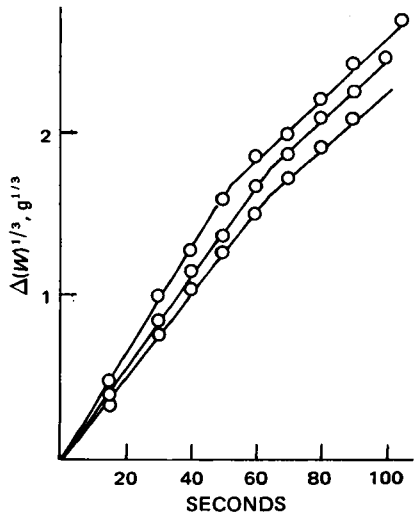

Figure 6—*Dissolution pattern of oxalic acid dihydrate of geometric mean 1500 μm and* SD *0.108 (top), 0.129 (middle), and 0.155 (bottom).*

well. According to this form, lines drawn according to the reduced equations should have slopes independent of the initial weight. To test this assumption, dissolution experiments were carried out with different amounts of oxalic acid dihydrate. The results are listed in Table V; the calculated rate constants are quite independent of the initial amount of solid.

Dissolution rate tests were performed at 25° on powders of identical mean diameter but with different standard deviations (Fig. 6). The slopes of the lines, after division by $\sqrt[3]{W_0}$ (=4.3), should, according to Eq. 13, follow the equation:

$$\ln [\text{slope}/\sqrt[3]{W_0}] = c\sigma_D^2 + \ln (2kC_s/[\rho M]) \quad \text{(Eq. 14)}$$

The plot of the data in Fig. 6 according to Eq. 14 are shown in Fig. 7; the linearity is obvious, and the least-squares fit line of Fig. 7 is:

$$\ln [\text{slope}/\sqrt[3]{W_0}] = -3.07\sigma_D^2 - 4.744 \quad \text{(Eq. 15)}$$

According to Brooke (16, 17), $c = -2.54$, so that both the linearity of Fig. 7 and the numerical value (2.54 being close to 3.07) support his equation. Knowledge of the values of C_s, ρ, and M allows calculation of k from the intercept of Fig. 7; *i.e.:*

$$\ln [2kC_s/(M\rho)] = -4.744 \quad \text{(Eq. 16)}$$

Inserting the values $\rho = 1.60$ g/ml, $C_s = 0.157$ g/ml, and $M = 0.15$ cm gives:

$$k = 6.7 \times 10^{-3} \text{ cm/sec} \quad \text{(Eq. 17)}$$

Equation 14 may be written:

$$\ln [\text{slope}/\sqrt[3]{W_0}] = \ln (1/M) + c\sigma_D^2 + \ln (2kC_s/\rho) \quad \text{(Eq. 18)}$$

To test this equation, dissolution rate studies were conducted at 25° with samples of oxalic acid dihydrate having the same standard deviation and different means (Fig. 8). This plot is a straight

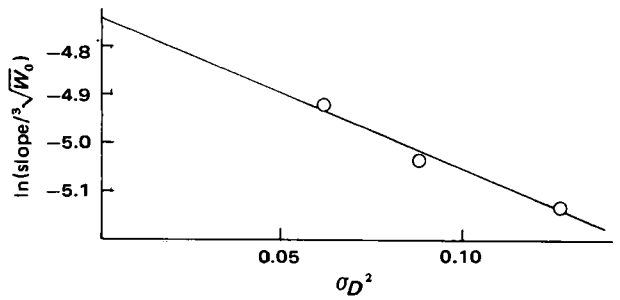

Figure 7—*Plot of weight-independent cube root slope* versus *variance of particle population.*

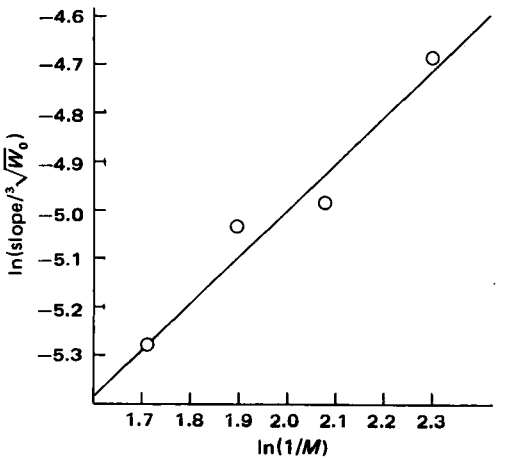

Figure 8—*Plot of weight-independent cube root slope* versus *reciprocal of geometric mean diameter.*

line as predicted by Eq. 18; it has a slope of 0.94 and should have a slope of unity.

Using the value of c from Fig. 7 ($c = -3.07$) and the value of the intercept from Fig. 8 (−6.8752), one can now calculate k, since:

$$c\sigma_D^2 + \ln (2kC_s/\rho) = -6.8752 \quad \text{(Eq. 19)}$$

Inserting the values for c, C_s, ρ, and $\sigma_D = 0.3569$, one obtains:

$$k = 7.8 \times 10^{-3} \text{ cm/sec} \quad \text{(Eq. 20)}$$

Table V shows that the average weight-independent slopes of powders with a mean diameter of 1500 μm and a variance of $\sigma_D^2 = 0.0618$ have a value of 7.16×10^{-3} sec^{-1}. Use of Eq. 14 and $c = -3.07$ gives a value of k of:

$$k = 6.7 \times 10^{-3} \text{ cm/sec} \quad \text{(Eq. 21)}$$

Although they are not identical, the three k values in Eqs. 17, 20, and 21 are of the same order of magnitude. This result supports the views stated in this report.

According to Eq. 5, the slope, α, of a cube root plot is given by:

$$\alpha = \{\exp[c\sigma_D^2]\}\sqrt[3]{W_0}\,2kC_s/(\rho M) \quad \text{(Eq. 22)}$$

Inserting $c = -3.07$, $\sigma_D^2 = 0.0618$, $M = 0.15$ cm, $\rho = 1.60$ g/ml, and $\sqrt[3]{W_0} = 4.3$ g$^{1/3}$, one gets the simpler expression:

$$\alpha = 29.64kC_s \quad \text{(Eq. 23)}$$

The effect of temperature on dissolution is shown in Fig. 9 (and Fig. 4 for 25°). To examine the temperature dependence of α, it is

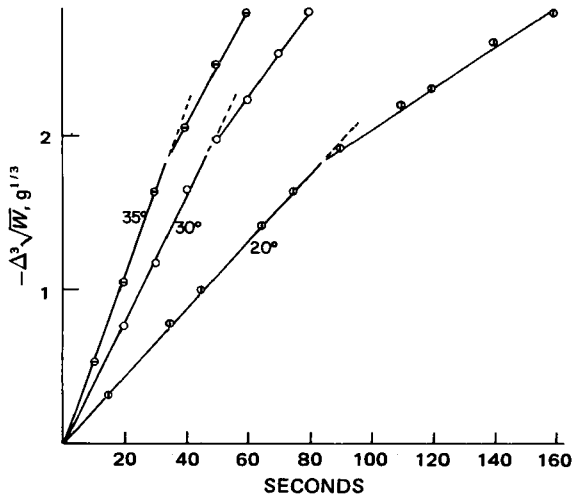

Figure 9—*Cube root plots as a function of temperature; mean = 1500 μm and* SD *= 0.108. The 25° data are shown in Fig. 4.*

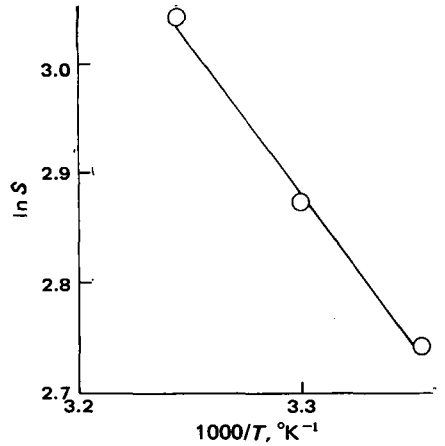

Figure 10—*Solubility–temperature plot of oxalic acid dihydrate.*

necessary to know the temperature dependence of C_s and k; C_s was determined experimentally at various temperatures (Fig. 10). The least-squares fit of the line is:

$$\ln C_s = -\frac{2751}{T} + 7.355 \qquad \text{(Eq. 24)}$$

where C_s is expressed as grams per milliliter. If the viscosity of the dissolving medium (here assumed to be equal to the viscosity of water) is denoted η and r is the radius of the dissolved molecule, then the Stokes–Einstein equation requires that:

$$k = \frac{1.38 \times 10^{-16} T}{hr6\pi\eta} \qquad \text{(Eq. 25)}$$

From Eqs. 23 and 25, the following expression is obtained:

$$\alpha = [(29.64)(1.38 \times 10^{-16})/6\pi]\, C_s T/(hr\eta) = 2.17 \times 10^{-16} C_s T/(hr\eta) \qquad \text{(Eq. 26)}$$

It is noted that (hr) is an unknown quantity. If it is assumed to be temperature independent over the temperature range studied, then:

$$\ln(\alpha/T) = -\ln(\eta) + \ln C_s + \ln[2.17 \times 10^{-16}/(hr)] = -\ln(\eta) + \ln C_s - \ln(hr) - 36.07 \qquad \text{(Eq. 27)}$$

The temperature dependence of the viscosity of water is:

$$\eta = 1.01 \times 10^{-5} \exp(2002/T) \qquad \text{(Eq. 28)}$$

or:

$$\ln(\eta) = 2002\,(1/T) - 11.434 \qquad \text{(Eq. 29)}$$

Inserting Eqs. 24 and 29 into Eq. 27 gives:

$$\ln(\alpha/T) = -\frac{4737}{T} - 17.29 - \ln(hr) \qquad \text{(Eq. 30)}$$

The data from Fig. 9 are plotted according to Eq. 30 in Fig. 11 and the linearity is obvious. The least-squares fit is:

$$\ln(\alpha/T) = -\frac{4760}{T} + 6.756 \qquad \text{(Eq. 31)}$$

That the slopes are identical is evident, although the proximity of the two values in Eqs. 30 and 31 is accidental. Experimental error is such that agreement to within 10% is considered adequate.

Table V—Dissolution with Various Amounts of Initial Powder, W_0, of a Powder with Mean Diameter of 1500 μm and *SD* 0.108 in 0.1 *N* HCl at 25°

W_0, g	$W_0^{1/3}$, $g^{1/3}$	Slope, $g^{1/3}$/sec	Slope/$(W_0^{1/3})$, sec^{-1}
80	4.3	0.0313	$7.28\ 10^{-3}$
70	4.12	0.0281	$6.84\ 10^{-3}$
60	3.91	0.0286	$7.31\ 10^{-3}$
50	3.68	0.0263	$7.15\ 10^{-3}$

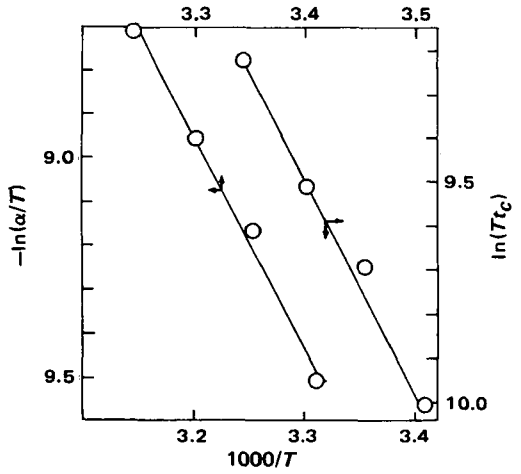

Figure 11—*Arrhenius plots of slope divided by absolute temperature (left) and critical time multiplied by absolute temperature (right).*

The intercept from Eq. 31 equals that from Eq. 30, so:

$$6.76 = 17.29 - \ln(hr) \qquad \text{(Eq. 32)}$$

i.e., $\ln(hr) = -24.05$, so that:

$$hr = \exp(-24.05) = 4 \times 10^{-11} \qquad \text{(Eq. 33)}$$

Since r is of the order of 10^{-8}–10^{-7} cm, h would be of the order of 40–4 μm, which is of the expected order of magnitude (3).

Carstensen and Musa (15) showed that the critical time is given in terms of the smallest diameter, a_0, by:

$$t_c = a_0\rho/(2kC_s) \qquad \text{(Eq. 34)}$$

so that Arrhenius plots of t_cT should have the same numerical slope with opposite sign as Arrhenius plots of α/T. This is the case, as demonstrated in Fig. 11. The definition of a_0 for an actual crystal is vague, and the fact that allows comparison is that all of the powders in Fig. 11 have the same particle distribution and, hence, the same a_0 by whatever definition.

SUMMARY

1. The experimental data presented here demonstrate that initially a powder with log-normal distributed dimensions will dissolve under sink conditions *via* a cube root law as predicted previously (15–17). The plots eventually are biphasic.

2. The dependence of the slopes of the cube root plots on the mean diameter and on weight is as described previously (15).

3. The dependence of the slopes of the cube root plots on the standard deviation of the powder distribution is as described previously (17).

4. The temperature dependence of the slopes of the cube root and of the critical times was derived theoretically and substantiated by experiment.

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Controlled Drug Release from Polymeric Delivery Devices III: *In Vitro–In Vivo* Correlation for Intravaginal Release of Ethynodiol Diacetate from Silicone Devices in Rabbits

YIE W. CHIEN [*x], STANLEY E. MARES [‡], JOHN BERG [‡], SUSAN HUBER [‡], HOWARD J. LAMBERT [*], and KENNETH F. KING [§]

Abstract □ Forty female rabbits were implanted with silicone vaginal devices containing ethynodiol diacetate for up to 8 weeks. As predicted from *in vitro* studies, a $Q - t^{1/2}$ (matrix-controlled) release profile was observed *in vivo*. The *in vivo* drug release profile was compared with *in vitro* data measured at three hydrodynamic conditions, and the diffusional resistance across the vaginal wall was estimated. Drug released from silicone devices yielded a prolonged plasma level when compared with data following intravaginal or intravenous administration of a solution dose. The rate constant for elimination was unchanged. The plasma concentration of the drug was related to the intravaginal drug release profile both theoretically and experimentally and was above the concentration required to inhibit fertilization.

Keyphrases □ Drug delivery systems—controlled release, polymeric devices, ethynodiol diacetate in silicone matrix, *in vitro–in vivo* correlation of intravaginal release, rabbits □ Ethynodiol diacetate—intravaginal release from silicone matrix, *in vitro–in vivo* correlation, rabbits □ Silicone matrix—ethynodiol diacetate delivery system, *in vitro–in vivo* correlation for intravaginal release, rabbits □ Vaginal devices—controlled release of ethynodiol diacetate from silicone matrix, *in vitro–in vivo* correlation, rabbits

An *in vitro* drug release system, which allows a direct and rapid characterization of a drug release profile and mechanism, was recently reported from this laboratory (1). Two types of drug release mechanisms, matrix controlled and partition controlled, were observed when the drug release profiles of ethynodiol diacetate from silicone devices were followed daily in this system (2).

To develop a device with a desirable, long-acting, drug release profile, it is necessary to examine intravaginal drug release mechanisms in animals to establish an *in vitro–in vivo* correlation. This paper reports results of drug release studies with silicone devices containing ethynodiol diacetate placed in the vaginal tracts of 40 rabbits for up to 8 weeks.

EXPERIMENTAL

***In Vitro* Release Studies**—The apparatus for *in vitro* drug release studies and the assay of drug samples were essentially the same as those reported previously (1). The ring-shaped silicone device, containing 112.6 mg/cm^3 of ethynodiol diacetate[1], was mounted in the arms of a Plexiglas holder and then rotated at 81 or 30 rpm or held stationary (to simulate more closely the status of implants in the vaginal lumen) in 150 ml of a 75% polyethylene glycol 400 solution as the elution medium at 37°. The solution was mixed well prior to sampling, and the drug concentration in the medium was assayed daily (1, 2). The reproducibility of the *in vitro* $Q/t^{1/2}$ profiles measured at various dates within 1 year was excellent (Table I).

***In Vivo* Release Studies**—The same silicone devices as those used in the *in vitro* drug release studies were cut into sections of 1 cm in length. One segment was inserted into the anterior vagina of each of 40 young adult New Zealand white female rabbits *via* a midventral laparoelytrotomy. It was anchored with a single polyethylene suture knotted on one side of the implant (through the implant perpendicular to the long axis) and then drawn through the vaginal wall and knotted to a 1-cm section of medical-grade tubing[2].

[1] SC-11800.
[2] Silastic, Dow Corning Corp., Midland, Mich.

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[2] Silastic, Dow Corning Corp., Midland, Mich.

Table I—Reproducibility of *In Vitro* Release Profiles ($Q/t^{1/2}$) of Ethynodiol Diacetate from Silicone Vaginal Devices[a]

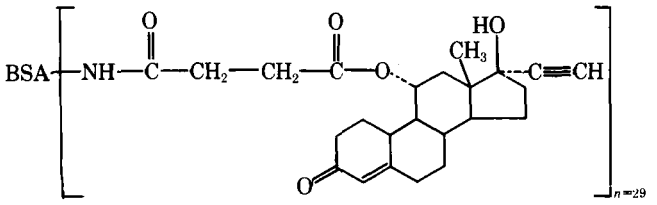
Experiments	Age of Devices, days	$Q/t^{1/2}$, mg/cm²/day$^{1/2}$
121	Initial	3.40
122	312	3.135
123	312	2.985
124	312	2.966
127	331	3.34
Mean (±*SD*) = 3.1652 (±0.185)		

[a] Intravaginal devices were rotated at constant 81 rpm in 150 ml of 75% polyethylene glycol 400 at 37°.

At various intervals, three to six rabbits were sacrificed. The implants were removed, cut into thin slices, and extracted by constant shaking for 2 days in 25 ml of spectral grade methanol. Drug content in the methanolic solution was assayed spectrophotometrically as described earlier (1, 2), and residual drug content was calculated. The results on the residual drug content and the amount of drug released per unit area are shown in Table II.

Radioimmunoassay—Tokuda *et al.* (3) reported that the stepwise hydrolysis of the acetate moieties of ethynodiol diacetate gives its 3β,17β-diol metabolite. This metabolite is rapidly transformed to the 3-keto derivative, norethindrone (Table III), the major metabolite of ethynodiol diacetate.

Preparation of 11α-Hydroxynorethindrone–Bovine Serum Albumin Conjugate—The hemisuccinate of 11α-hydroxynorethindrone was prepared first and then conjugated to bovine serum albumin (BSA). Analysis of the conjugate by UV spectroscopy and nitrogen analysis indicated that each conjugate molecule was composed of 29 steroid molecules to one bovine serum albumin molecule with the following chemical structure:



Preparation of Antiserums—New Zealand rabbits were immunized with norethindrone–bovine serum albumin conjugate by the technique described by Vaitukaitus *et al.* (4). The resultant antiserums were characterized, divided into several small aliquots, and stored at −80°.

Immunoassay Procedure—The assay was carried out in glass tubes (12 × 75 mm), and 0.1% gelatin–phosphate buffer (0.05 *M* phosphate and 0.15 *M* NaCl at pH 7.5) was utilized as the diluent for all dilution preparations of antiserums, standards, and samples. To each tube, 10,000 dpm of 1,2-^{3}H-norethindrone (20 Ci/mmole), 200 μl of diluted antiserum (capable of binding 40% of the labeled antigen added), and 20 μl of sample serum (to calibrate the standard curve, 20 μl of control rabbit serum and 10–1000 pg of crystalline norethindrone were used instead) were added and mixed (vortex).

Duplicate sets of the binding mixture were prepared and incubated at 4° for 12 hr. Antibody-bound antigen was then separated from the unbound antigen by the addition of 0.5 ml/tube of dextran-coated charcoal (prepared from 50 mg of dextran[3] and 500 mg of charcoal in 50 ml of phosphate buffer). The mixtures were immediately mixed and then centrifuged at 2000×*g* and 4° for 10 min. The resultant supernate was mixed with 15 ml of scintillator[4] in glass vials of low background. The radioactivity of each sample was then determined[5].

Conversion of counts per minute to disintegrations per minute was accomplished by the external standard channel ratio method. Disintegrations per minute data were evaluated and converted to the corresponding drug concentration by a computer program utilizing a logit–log transformation of standards.

[3] Pharmacia T-70.
[4] PCS, Amersham/Searle Corp., Arlington Heights, Ill.
[5] Nuclear Chicago Mark II scintillation counter.

Table II—Intravaginal Release of Ethynodiol Diacetate from Silicone Devices Implanted in Rabbits

Days[a]	Number of Rabbits	Amount of Drug Remaining in Devices, mg (±*SD*)	Cumulative Amount of Drug[b] Released per Square Centimeter, mg/cm² (±*SD*)
Initial	—	30.428 (0.83)	0.0
7	3	25.948 (2.40)	2.239 (0.97)
14	3	17.660 (0.78)	5.210 (0.32)
25	5	13.964 (3.80)	6.720 (1.60)
42	6	4.997 (6.70)	10.380 (2.70)
49	4	0.083 (0.09)	12.386 (0.04)
52	4	1.238 (0.38)	11.914 (0.15)

[a] Five rabbits were implanted with devices (containing no ethynodiol diacetate) and sacrificed at various dates as the references. [b] Total surface area of the device used is 2.45 cm² each.

A measure of the accuracy of the immunoassay technique is illustrated in Fig. 1. Crystalline norethindrone was added to control rabbit serum and assayed as an unknown. A linear relationship was observed between added and recovered norethindrone, with a tendency for overestimation at high concentrations.

The specificity of the anti-11-hydroxynorethindrone antiserum was demonstrated by examining its cross-reactivity with ethynodiol diacetate and its seven major metabolites. As shown in Table III, this antiserum specifically reacted only with norethindrone.

The cross-reactivity of 11-hydroxynorethindrone antiserum to progesterone, 20α-hydroxyprogesterone, estradiol, corticosterone, hydrocortisone, and testosterone was also examined. These common steroids showed no cross-reactivity except for testosterone, which has a cross-reactivity of only 0.15%.

RESULTS AND DISCUSSION

***In Vivo* Drug Release Studies**—The intravaginal drug release data (Table II) indicate that the longer the period of implantation, the lower the drug content in the device and the greater the quantity of drug released. If the release of ethynodiol diacetate from the silicone vaginal devices implanted in the vaginal tract of rabbits is assumed to be predominately matrix controlled (1) and not partition controlled (2), then the cumulative amount of drug released (Q) in a rabbit's vagina should be defined by:

$$Q = \sqrt{D_m(2A - C_s)C_p t} \qquad \text{(Eq. 1)}$$

where Q is the cumulative amount of drug released from a unit surface area of device (grams per square centimeter); A is the initial amount of solid drug impregnated in a unit volume of device

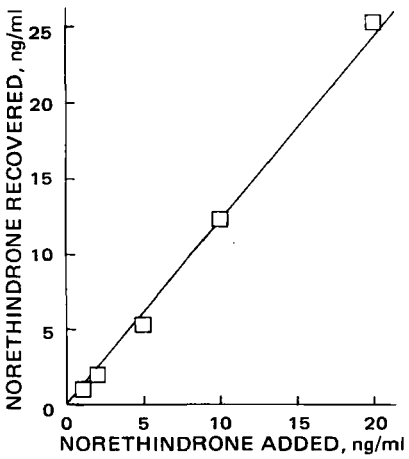
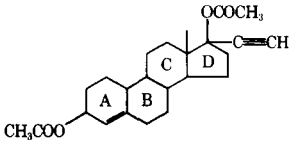
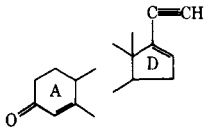
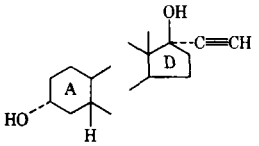
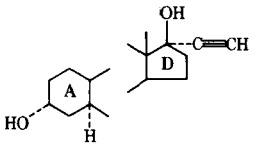
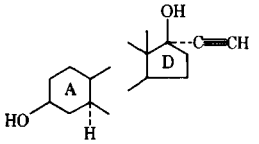
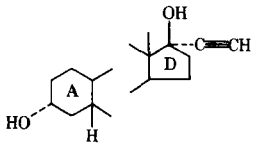
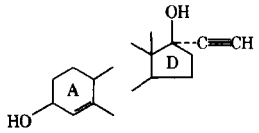
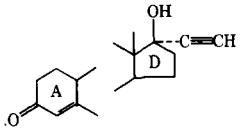


Figure 1—*Linear relationship between norethindrone added to control serum* versus *norethindrone determined by radioimmunoassay. Each data point is a mean of eight determinations.*

Table III—Cross-Reactivity of Anti-11-hydroxynorethindrone Antiserum[a] to Ethynodiol Diacetate and Its Metabolites[b]

Ethynodiol Diacetate Metabolite	Molecular Structure	Reactivity[c], %
Ethynodiol diacetate		N.R.[d]
Metabolites:		
I		0.58
II		1.22
III		1.50
IV		1.63
V		2.50
VI		6.00
VII[e]		100.00

[a] 2-4-12-73-ws; a dilution of 1:10,000 was used. [b] Ref. 9. [c] Based on 50% inhibition of binding assay. [d] No reactivity could be detected. [e] Norethindrone.

(grams per cubic centimeter); D_m is the effective diffusivity of the drug species in the matrix; C_s and C_p are the solubilities of the drug in the elution medium and in the silicone polymer phase, respectively; and t is time.

According to Eq. 1, the cumulative amount (Q) of ethynodiol diacetate released from a unit surface area of device should be linearly proportional to the square root of the implantation time ($t^{1/2}$) as shown in Fig. 2. By using Eq. 2:

$$Q/t^{1/2} = \sqrt{D_m(2A - C_s)C_p} \qquad \text{(Eq. 2)}$$

the intravaginal $Q/t^{1/2}$ profile was estimated from the slope of the Q *versus* $t^{1/2}$ plot to be 2.10 mg/cm^2/day$^{1/2}$.

The drug molecules released from the silicone vaginal device are presumably dissolved in the vaginal fluid surrounding the implanted device. They are then transported across the vaginal membrane

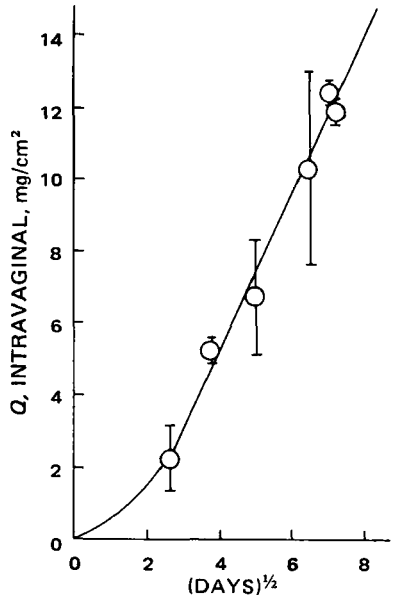


Figure 2—*Linear relationship between the cumulative amount of drug released* (Q) *in the rabbit's vagina and the square root of time* ($t^{1/2}$) *following Eq. 1. The data in Table II (fourth column) were plotted. The* $Q/t^{1/2}$ *value for this intravaginal device was calculated from the slope, following Eq. 2, as 2.10 mg/cm*^{2}*/day*$^{1/2}$.

into a biological sink maintained by hemoperfusion. In such a case, the rate of drug permeation (dQ'/dt) across the vaginal membrane (with an aqueous diffusion layer on each side of the membrane) should be directly proportional to the drug concentration (C_v) in the vaginal fluid (as the driving force for drug permeation) and inversely proportional to the total diffusional resistance (ΣR) encountered by the drug molecules along the way across the vaginal wall:

$$\frac{dQ'}{dt} = \frac{C_v}{\Sigma R} \qquad \text{(Eq. 3)}$$

The total diffusional resistance (5) is the sum of the diffusional resistances as follows:

$$\Sigma R = R_l + R_v + R_c \qquad \text{(Eq. 4}a\text{)}$$

where R_l and R_c are the diffusional resistances across the aqueous diffusion layer on the luminal side and on the circulation side of the vaginal membrane, respectively; and R_v is the diffusional resistance across the vaginal membrane itself. Equation 4*a* is equivalent to:

$$\Sigma R = \frac{\delta_l}{D_l K_{l/l}} + \frac{\delta_v}{D_v K_{v/l}} + \frac{\delta_c}{D_c K_{c/v}} \qquad \text{(Eq. 4}b\text{)}$$

where δ, D, and K represent the thickness, diffusivity, and partition coefficient across two contacting phases, respectively; and the subscripts v, l, and c stand for vaginal membrane and the aqueous diffusion layers on the luminal and the circulatory hemoperfusion sides of the vaginal barrier, respectively.

The instantaneous rate of drug release at time t may be derived by differentiating Eq. 1 with respect to time:

$$\frac{dQ}{dt} = \frac{1}{2}\sqrt{D_m(2A - C_s)C_p/t} \qquad \text{(Eq. 5)}$$

At steady state, the rate of drug release (dQ/dt) should be equal to the rate of drug permeation (dQ'/dt). Equating Eq. 3 with Eq. 5 yields Eq. 6:

$$\Sigma R[D_m(2A - C_s)C_p]^{1/2} = 2C_v(t)^{1/2} \qquad \text{(Eq. 6)}$$

Equation 6 indicates that the drug concentration (C_v) in the vaginal fluid decreases with the square root of time ($t^{1/2}$) as the drug molecules diffuse actively to a sink; that is, under a matrix-controlled process, the magnitude of $2C_v(t)^{1/2}$ is a constant since both ΣR and $[D_m(2A - C_s)C_p]^{1/2}$ are constants under a controlled con-

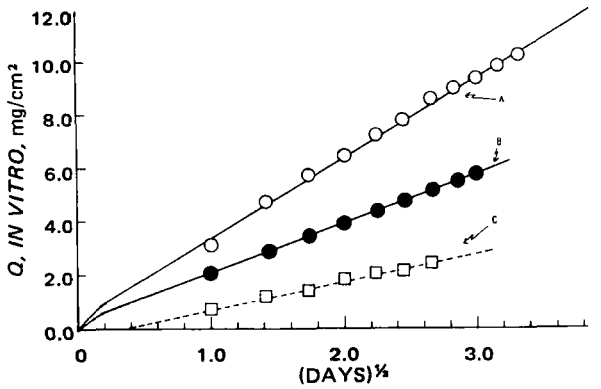


Figure 3—*Linear relationship between the cumulative amount of drug release (Q) and the square root of time ($t^{1/2}$) obtained from the* in vitro *drug release studies conducted at the rotation speed of 81 (A), 30 (B), and stationary state (0) (C) rpm. Each data point represents an average of three determinations. The slope ($Q/t^{1/2}$) was estimated as 3.1652 (A), 1.862 (B), and 1.065 (C) mg/cm²/day$^{1/2}$, respectively.*

dition. Equation 6 also states that the value of C_v is directly proportional to the magnitude of the total diffusional resistance (ΣR) when the same silicone vaginal device is implanted in a different animal with a different value of ΣR. Therefore, the greater the magnitude of ΣR, the higher the value of C_v since the intravaginal permeation of the drug is inhibited. The validity of Eq. 6 will be further analyzed later.

***In Vitro* Drug Release Studies**—A series of parallel *in vitro* drug release studies using similar devices was conducted at low rotation speed (81 or 30 rpm) and at a stationary state to simulate more closely the status of implants in the vaginal lumen. The *in vitro* release of ethynodiol diacetate followed a linear $Q - t^{1/2}$ relationship in all three cases (Fig. 3). The magnitude of the $Q/t^{1/2}$ values calculated for the rotating devices (Table IV, fourth column) are 2.97 (81 rpm) or 1.75 (30 rpm) times higher than that for a stationary one. Since D_m, A, C_s, and C_p were the same in all three *in vitro* studies, the 1.75–2.97-fold difference in the magnitudes of the $Q/t^{1/2}$ value between the rotating and the stationary devices indicates the existence of a rate-limiting parameter, *i.e.*, a hydrodynamic diffusion layer on the immediate surface of the devices.

When a device is immersed stationary in a solution, a diffusion layer is established on the immediate surface of the device. The effective thickness of this diffusion layer (6) varies with the square root of time as follows:

$$(\delta_l)s = \sqrt{D_l \pi t} \qquad \text{(Eq. 7)}$$

If this device is rotated at a constant speed, convection results. The convective transport of the drug to and from the device surface is much faster than when natural diffusion is operating on a stationary device, because the concentration gradient of drug extends over a thinner diffusion layer (7). The effective thickness of the diffusion layer on the rotating device becomes time independent and may now be defined by the Levich equation:

$$(\delta_l)r = 1.62 D_l^{1/3} v^{1/6} w^{-1/2} \qquad \text{(Eq. 8)}$$

where v and w represent the kinematic viscosity of the elution medium and the angular rotation speed of the silicone device, respectively; and the subscripts s and r stand for stationary and rotation states, respectively. This equation was developed originally for the relationship of the thickness of the convective diffusion layer at a rotating disk and also was successfully applied to the case of a polymeric membrane-covered rotating disk (8). Using Eq. 8, the magnitude of $(\delta_l)r$ on a rotation device can be calculated if D_l, v, and w are known or predetermined. The $(\delta_l)r$ values were calculated and are given in Table IV.

In the *in vitro* elution studies, the elution medium was maintained at sink conditions throughout the experiment. In this case, only the thickness of the hydrodynamic diffusion layer (δ_l) exists between the surface of the device and the bulk of the elution medium. Both the R_v and R_c terms in Eq. 4 may be dropped, and Eq. 4 may be reduced to:

$$\Sigma R = R_l = \frac{\delta_l}{D_l} \qquad \text{(Eq. 9)}$$

where $k_{l/l} = 1$ and Eq. 6 becomes:

$$\delta_l[D_m(2A - C_s)C_p]^{1/2} = 2D_l C_v(t)^{1/2} \qquad \text{(Eq. 10)}$$

Equation 10 indicates that at a given value of $2D_lC_v(t)^{1/2}$, the magnitude of $[D_m(2A - C_s)C_p]^{1/2}$ should be inversely proportional to the effective thickness, δ_l, of the diffusion path on the immediate surface of the silicone device. In other words, the larger the value of δ_l on a device, the less is the amount of drug released at a given time (the lower the magnitude of $Q/t^{1/2}$). This relationship is shown by the data of Table IV. For instance, the magnitude of $Q/t^{1/2}$ for the device rotating at 81 rpm (3.165 mg/cm²/day$^{1/2}$) is 1.7 times higher than that for the device rotating at 30 rpm (1.862 mg/cm²/day$^{1/2}$).

This difference in the magnitudes of $Q/t^{1/2}$ may be explained by comparing the value of δ_l for the device rotating at 81 rpm (154.7 μm) to that for the device rotating at 30 rpm (254.0 μm). The δ_l value for the higher rotating device (81 rpm) is 1.64-fold lower than that for the lower rotating one (30 rpm). When all other experimental conditions are the same, the thickness of the diffusion layer (δ_l) becomes the rate-determining parameter in the process of drug release (Eq. 10). The increase in the thickness of the diffusion layer, due to the reduction in the angular rotation speed of the device, tends to decrease proportionally the magnitude of $Q/t^{1/2}$.

***In Vitro–In Vivo* Correlation**—Analysis of the intravaginal drug release data (Fig. 2) yields an *in vivo* $Q/t^{1/2}$ value of 2.10 mg/cm²/day$^{1/2}$. This value is between those observed for the two *in vitro* rotation experiments (Table IV). Since the smooth muscle of the vaginal lumen shows a periodic constriction and dilatation, it may be assumed that the implanted silicone device was moved

Table IV—Comparison of *In Vivo* and *In Vitro* Data on $Q/t^{1/2}$

Conditions	n, rps	δ_l[a], cm × 10⁴	$Q/t^{1/2}$ Observed, mg/cm²/day$^{1/2}$
In Vitro			
81 rpm	1.328	154.7	3.165
30 rpm	0.500	254.0	1.862
Stationary	0.0	—	1.065
In Vivo, intravaginal			2.10

[a] Calculated from $\delta_l = 1.62 D^{1/3} v^{1/6} W^{-1/2} = 4.5027 \times 10^{-4} \times W^{-1/2}$, where $W = 2n\pi$.

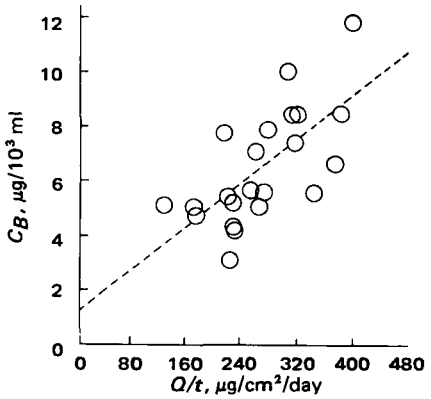
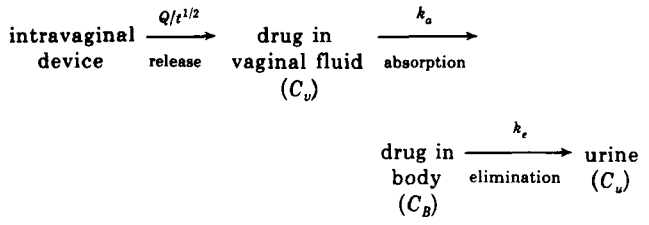


Figure 4—*Correlation between the plasma concentration (C_B) of norethindrone, the major metabolite of ethynodiol diacetate, in each rabbit and the flux (Q/t) of ethynodiol diacetate released from a silicone vaginal device at a given time. The slope = 1.96 (±0.49) × 10⁻⁵ day/cm, and the intercept = 1.199 × 10⁻³ μg/ml; F = 16.583 [F1, 20 (α = 0.01) = 8.10].*



Scheme I

slightly in a periodic fashion. Therefore, a diffusion layer (δ_l) (on the lumen side of the vaginal wall) with an effective thickness smaller than 254 μm (slow rotation state) and larger than 154.7 μm (high rotation state) may be expected. Literature data do not allow the calculation of the total diffusional resistance term (ΣR) of Eq. 4. The absence of a known ΣR makes the prediction of *in vivo* release from *in vitro* data impossible. However, the data may be utilized to establish a proportionality between *in vivo* and *in vitro* $Q/t^{1/2}$ profiles. This proportionality should be equivalent to the ratio of *in vitro* diffusional resistance (R_l) over *in vivo* total diffusional resistance (ΣR) as follows:

$$\frac{R_l}{\Sigma R} = \frac{(Q/t^{1/2})\ in\ vivo}{(Q/t^{1/2})\ in\ vitro,\ 30\ rpm} = \frac{2.10}{1.862} = 1.13 \quad \text{(Eq. 11)}$$

Such a proportionality may be useful for predicting the magnitude of $(Q/t^{1/2})$ *in vivo* from the *in vitro* drug release profiles measured under controlled conditions (when R_l is constant).

Pharmacokinetics of Intravaginal Absorption—If the absorption, distribution, and elimination of ethynodiol diacetate after its release from an intravaginal device follow Scheme I, then the instantaneous rate of change in the body concentrations of drug may be expressed as:

$$\frac{d(C_B)}{dt} = k_a C_v - k_e C_B \quad \text{(Eq. 12)}$$

where k_a and k_e are the respective rate constants for absorption and elimination, and C_v, C_B, and C_u are the drug concentrations in the vagina, body (including blood, tissues and related compartments), and urine, respectively. Equation 12 may be transformed to Eq. 13 by substituting Eq. 2 into Eq. 6 and then for the C_v term in Eq. 12:

$$\frac{d(C_B)}{dt} = \left(\frac{k_a \Sigma R}{2}\right)\left(\frac{Q}{t}\right) - k_e C_B \quad \text{(Eq. 13)}$$

At steady state, the change in the body concentration of the drug will be very small, that is, $dC_B/dt \simeq 0$, and the body concentration of norethindrone (C_B), the major metabolite of ethynodiol diacetate (9), may be related to the amount of ethynodiol diacetate (Q) released at time (t) as follows:

$$C_B = \left(\frac{k_a \Sigma R}{2k_e}\right)\left(\frac{Q}{t}\right) \quad \text{(Eq. 14)}$$

Equation 14 states that the plasma concentration of norethindrone in each rabbit should be directly proportional to the flux of ethynodiol diacetate released from the vaginal devices (Q/t) at a given time. The result is illustrated in Fig. 4. When the correlation of C_B with Q/t was submitted to an F test, the analysis resulted in an F value of 16.583, indicating that the correlation was statistically significant at the 99% confidence level ($F1, 20\ [\alpha = 0.01] = 8.10$).

To inhibit fertilization effectively, a rabbit required a subcuta-

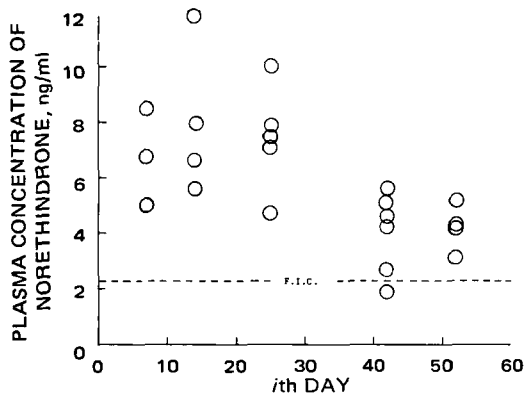


Figure 5—*Plasma concentration of norethindrone from each rabbit measured by radioimmunoassay at the time of sacrifice and removal of the intravaginal device. The fertilization inhibitory concentration (FIC) of 2.27 ng/ml, resulting from the daily subcutaneous injection of 30–50 μg of ethynodiol diacetate per rabbit for 6 days prior to mating and 1 day after reduced fertilization, is also shown. Most data resulting from the intravaginal absorption of ethynodiol diacetate in silicone devices are higher than the FIC required.*

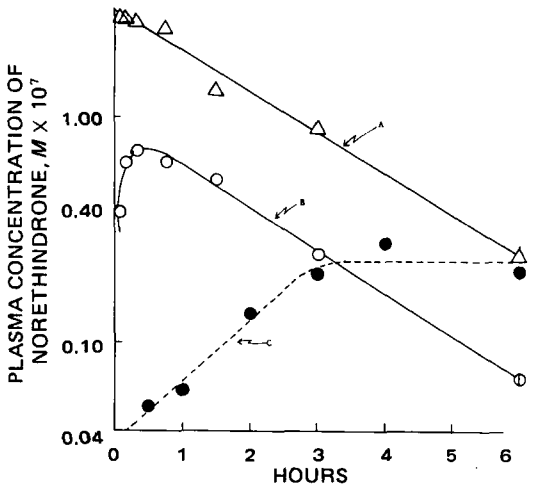


Figure 6—*Time course of plasma norethindrone concentration after the administration of a single solution dose (1 mg/0.2 ml) of norethindrone via intravenous injection (A) and ethynodiol diacetate via intravaginal absorption (B), and intravaginal absorption of ethynodiol diacetate from a silicone vaginal device (C) (A = 0.1126 g/cm³). The rate constants for absorption (k_a) are: 10.6 (B) and 0.244 (C) hr⁻¹. The rate constants for elimination (k_e) are 0.40 (A) and 0.42 (B) hr⁻¹.*

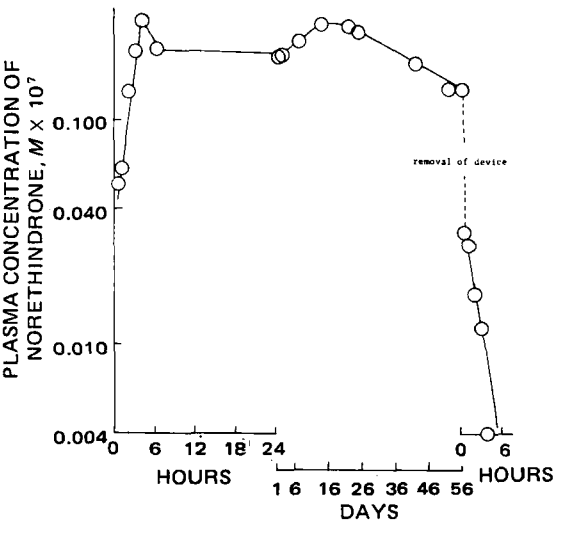


Figure 7—*Overall plasma norethindrone concentration profile after the intravaginal implantation of silicone devices containing ethynodiol diacetate in rabbits for up to 56 days. The k_a and k_e values are 0.244 and 0.511 hr⁻¹, respectively. The k_e value after the removal of the devices (elimination of the residual drug content in the body) is 0.436 hr⁻¹.*

Table V—Comparison of Calculated and Observed Plasma Concentrations of Norethindrone

Days	Plasma Concentration of Norethindrone, ng/ml: Calculated[a]	Observed[b]: Range	Observed[b]: Mean (±SD)	Observed/Calculated
22	8.77	6.15–9.41	7.78 (1.63)	0.89
56	5.49	2.32–7.42	4.12 (1.80)	0.75

[a] Calculated from $C_B = (k_a \Sigma R)/2k_e \times (1.13) \times (Q/t)$ *in vitro* = 22.11 (Q/t) *in vitro* (ng/ml). [b] Data of four to six rabbits for each scheduled interval.

neous dose of 30–50 μg/day of ethynodiol diacetate for 6 days prior to and 1 day after mating. These doses normally resulted in a maximum plasma concentration of 2–3 ng/ml of norethindrone in a 4-kg rabbit. The latter concentration is referred to as the fertilization inhibitory concentration (FIC). As shown in Fig. 5, the release of ethynodiol diacetate from implanted silicone devices resulted in a plasma concentration of norethindrone well above the required FIC. This concentration was maintained for up to 8 weeks.

The plasma norethindrone profile after the intravaginal absorption of ethynodiol diacetate from a solution dose of 1 mg/0.2 ml is compared to the profile after an intravenous injection of an equivalent dose of norethindrone in Fig. 6. As illustrated, the intravaginal dose of ethynodiol diacetate (curve B) was rapidly absorbed ($k_a = 10.6\ hr^{-1}$) and showed a peak plasma level of norethindrone within 20 min after administration. The observed elimination rate constant ($k_e = 0.42\ hr^{-1}$) agreed with the value of $0.40\ hr^{-1}$ found for the intravenous dose of norethindrone (curve A) in the same animal. From the ratio of the area under the curve (AUC) for intravaginal absorption over that for the intravenous dose, the availability of norethindrone from an intravaginal dose of ethynodiol diacetate was calculated to be 34.76%.

The plasma level of norethindrone following intravaginal absorption from a vaginal device containing ethynodiol diacetate is also shown in Fig. 6 (curve C). The rate of appearance of drug in plasma was sustained (C in Fig. 6), and the plasma level of norethindrone was significantly prolonged (Fig. 7). After removal of the device, the residual drug content in the body (Fig. 7) was eliminated with a rate constant ($0.436\ hr^{-1}$) comparable to those seen after the intravenous and intravaginal administrations.

Figure 7 illustrates the overall plasma norethindrone concentration profile after the implantation and removal of the silicone vaginal devices. A prolonged drug level was maintained for up to 56 days. After the removal of the device, the residual plasma drug content was eliminated as fast as after a solution dosage.

The rate constants for absorption ($k_a = 10.6\ hr^{-1}$) and for elimination ($k_e = 0.42\ hr^{-1}$), determined earlier from the intravaginal absorption study of a solution dose of ethynodiol diacetate, may be applied into the slope ($k_a \Sigma R/2k_e$) of the C_B *versus* Q/t profile to estimate the total diffusional resistance (ΣR) across the vaginal membrane of the rabbit:

$$\Sigma R = \frac{\delta_l}{D_l k_{l/l}} + \frac{\delta_c}{D_c k_{v/l}} + \frac{\delta_c}{D_c k_{c/v}} \quad \text{(Eq. 15a)}$$

$$= \frac{2ke \times 1.96 \times 10^{-5}}{k_a} \quad \text{(Eq. 15b)}$$

$$= 1.55 \times 10^{-6}\ \text{day/cm} \quad \text{(Eq. 15c)}$$

The resultant ΣR value will be useful as a working factor for predicting plasma norethindrone levels (C_B) from the *in vivo* release profile (Q/t) of ethynodiol diacetate (using Eq. 14). The *in vivo* $Q/t^{1/2}$ value may be estimated from *in vitro* drug release data after a correction factor of 1.13 is made (Eq. 11). This information should facilitate the future development of a suitable long-acting delivery system, directly from short-term *in vitro* drug release experiments, for the vaginal administration of ethynodiol diacetate. For instance, based on a 1-week *in vitro* experiment, it was predicted that a plasma concentration of norethindrone at 8.77 and 5.49 ng/ml will be found after 22- and 56 days of implantation, respectively (Table V). Intravaginal implantation of such devices in 10 rabbits for 22 and 56 days resulted in a plasma concentration of 7.78 (±1.63) and 4.12 (±1.80) ng/ml, respectively (Table V).

In conclusion, in developing a long-acting delivery system for a given drug, a study should be performed to evaluate the *in vitro–in vivo* correlation of both the mechanisms and the rates of drug release. This study should also measure the pharmacokinetic parameters for the absorption, distribution, and elimination of the drug *via* a given route of administration. The information so gained can then be applied to additional studies to develop a device that delivers the drug at a programmed rate, thereby maintaining constant plasma drug levels for an optimum duration of treatment.

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Kinetics and Mechanisms of Monolayer Interactions III: Models to Explain Time-Dependent Effects of Injected Cetrimonium Bromide

EDWARD R. GARRETT and FEDERICO A. VILALLONGA ˣ

Abstract □ Two kinetic models are derived to explain the time-dependent effects of cetrimonium ions injected beneath an air–aqueous solution interface without any previously adsorbed or spread monolayer at different ionic strengths. The data show excellent fit and consistency with a multicompartmental kinetic model which postulates a barrier or intermediate compartment between the ionic surfactant in the subphase and the sites on the surface. Such a barrier inhibits free diffusion of the surfactant from the bulk of the solution to these sites. The experimental data are not inconsistent with a model that postulates a time-dependent binding process and the absence of a diffusion-limiting subinterface since, within the limits of the error, the derived rate constants conform to the model prediction that they should be proportional to the surfactant-ion concentration.

Keyphrases □ Monolayer interactions—kinetics and mechanisms for time-dependent effects of cetrimonium bromide injected beneath air–aqueous solution interface without previously adsorbed or spread monolayer at different ionic strengths □ Cetrimonium bromide—kinetics and mechanisms for time-dependent effects after injection beneath air–aqueous solution interface without previously adsorbed or spread monolayer at different ionic strengths

Recent studies on the kinetics and mechanisms of the interaction of phospholipid monolayers at the air–water interface with long hydrocarbon ionic surfactants injected in the subphase demonstrated a time-dependent process for achievement of a final value of surface pressure (1, 2).

The present study describes changes of the surface tension with time after the injection of surfactant ions beneath an air–water interface without any previously adsorbed or spread monolayer and the effect of ionic strength on the adsorption kinetics and final equilibrium surface pressure.

EXPERIMENTAL

Materials—Cetrimonium bromide[1] was the same sample used in previous studies (1, 2). No detectable minimum in the plots of surface tension against the logarithm of the molar concentration was observed. Analytical reagent sodium chloride and potassium chloride were roasted at 650–700° for 6 hr prior to preparation of the aqueous solutions to remove surface-active organic impurities. Deionized, triple-distilled water was used throughout the study. Its pH, after air equilibration, was consistently between 5.6 and 6.0, and its surface tension (γ_0) was within 99.8–100.2% of the value calculated from the Harkin equation (3). The air–water interfacial potential (V_0) was −500 mv (±20 mv).

Instruments and Methods—A 9-cm diameter Teflon dish, provided with two identical micropipets[2] and a Teflon-coated magnetic stirring bar (1.25 × 0.8 cm), was used as a trough in a closed system described previously (1). The final liquid volume in the trough was always 45 ml, corresponding to a liquid depth of 1.58 cm.

Surface tension was measured with a Wilhelmy platinum plate (2.5 × 1.25 × 0.01 cm) attached to an electrobalance[3]. Surface potential was measured with an americium 241 air electrode attached to an electrometer[4]. The outputs of both instruments were fed into a dual-pen recorder[5].

With the platinum plate, the electrodes, and the micropipets in position, water (or the saline solution) was added slowly (42–43 ml) so that it just made contact with the lower edge of the platinum plate (4). After 1 min the surface tension and the interfacial potential were measured and recorded. The liquid volume was adjusted to 45 ml, and the surface tension (γ_0) and interfacial potential (V_0) were measured again. These control values were used for the calculation of the surface pressure ($\Delta\pi$) and surface potential (ΔV) of the final surfactant solution: $\Delta\pi = \gamma_0 - \gamma_s$ and $\Delta V = V_s - V_0$. The subscript s indicates the values that correspond to the surface tension and interfacial potential, respectively, of the final surfactant solution.

A given volume, equal to the volume of the concentrated surfactant solution to be injected, was withdrawn from the dish using the appropriate micropipet. With the other micropipet filled previously with the concentrated surfactant solution, the injection was then started simultaneously with the magnetic stirrer. Within the limits of the experimental error, no significant differences were observed in the $\Delta\pi$ values by stirring 1, 3, 5, or 10 min for a given final concentration. Consequently, after 1 min of stirring, the recording of the surface tension (γ_s) and interfacial potential (V_s) was started. The time necessary for the injection of the desired amount of concentrated surfactant solution was between 10 and 35 sec. The criteria for equilibrium were the constancy of the surface tension (±0.2 dyne cm^{-1}) and the constancy of the interfacial potential (±20 mv) during 30 min. The experiments were performed at 21 ± 1°.

The concentration of the concentrated surfactant solution to be injected was kept below the critical micelle concentration (CMC), and the volumes injected were such as to obtain a final concentration in the 45 ml in the Teflon dish of 8, 4, or 2 μM; at these concentrations, cetrimonium bromide is completely dissociated (5).

At least three different experiments were performed for each final surfactant concentration. The reproducibility was within ±0.5 dyne cm^{-1} for the variation of the surface pressure and ±25 mv for the variation of the surface potential.

RESULTS

The effects of the injection of cetrimonium bromide on the surface pressure ($\Delta\pi$) are compared in Fig. 1 in the absence and in the presence of different concentrations of sodium chloride. In the absence of sodium chloride, the effective changes in the surface pressures were essentially instantaneous processes under the experimental conditions. In the presence of sodium chloride, the equilibrium surface pressure ($\Delta\pi_{eq}$) varied and was a function of the sodium chloride concentration. The rate of achievement of the equilibrium surface pressure was primarily a function of the surfactant-ion concentration in these latter cases.

In all cases, the plot of the reciprocal of the equilibrium surface pressure ($1/\Delta\pi_{eq}$) against the reciprocal ($1/n$) of the final surfactant concentration (n), expressed in molecules per milliliter, gave reasonably straight lines (Fig. 2) within the concentration limits studied.

The substitution of potassium chloride for sodium chloride gave

[1] Eastman Kodak, Rochester, N.Y.
[2] Manostat, New York, N.Y.
[3] Cahn Division, Ventrom Instruments Corp., Paramount, Calif.
[4] Keithley Instruments (610c), Cleveland, Ohio.
[5] Speedomax w/1, Leeds and Northrup, North Wales, Pa.

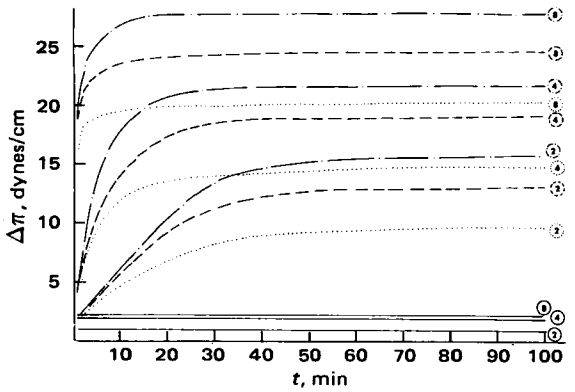


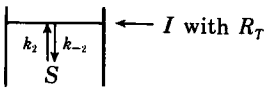
Figure 1—*Effect of the injection of cetrimonium bromide on the surface pressure ($\Delta\pi$) of air–aqueous solution interfaces without any previously adsorbed or spread monolayer. Key: —, water; . . ., 0.15 M NaCl; - - -, 0.30 M NaCl; and - . -., 0.45 M NaCl. Curves are labeled as to micromolar final concentrations of the injected cetrimonium ion.*

similar results. The various $\Delta\pi_{eq}$ values of the several potassium chloride and sodium chloride concentrations are given in Table I.

DISCUSSION

Although it was proposed early (6) that the time dependence of surface tension of surfactant solutions was diffusion dependent, the kinetics of the adsorption processes in many cases could not be explained in terms of simple diffusion alone (7–13) and the concept of a surface potential energy barrier had to be introduced (14–23). These time-dependent processes of adsorption can be quantified by applying the principles of compartmental analysis to a system that is composed of several kinetically distinct compartments.

Model I—Model I postulates a rate-determining process of binding of surfactant ions (S) to a finite number of sites at the interface (I) (Scheme I).



Scheme I

The process can be described by Scheme II:

$$S + R \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (SR)$$

Scheme II

where an amount, S, of surfactant is homogeneously dispersed in a volume, V_s, of solution. The total number of receptor sites at the interface, R_T, is the sum of the amount bound, (SR), and free, R:

$$R_T = R + (SR) \qquad \text{(Eq. 1)}$$

The rate of change of bound sites with time can be formulated as:

$$d(SR)/dt = k_2S(R) - k_{-2}(SR) = k_2S[R_T - (SR)] - k_{-2}(SR) \qquad \text{(Eq. 2)}$$

which can be put into integral form as:

$$\int_0^{(SR)} \frac{d(SR)}{k_2SR_T - (k_2S + k_{-2})(SR)} = \int_{t_0}^{t} dt \qquad \text{(Eq. 3)}$$

If it is postulated that the total number of sites is so small that even when completely saturated, *i.e.*, when $(SR) = (SR_T)$, there is no significant depletion of the surfactant amount in bulk solution so that $S = S_{eq}$, a constant, Eq. 3 can be integrated to:

$$\ln\left[\frac{k_2S_{eq}R_T - [k_2S_{eq} + k_{-2}](SR)}{k_2S_{eq}R_T}\right] = -(k_2S_{eq} + k_{-2})(t - t_0) \qquad \text{(Eq. 4)}$$

and:

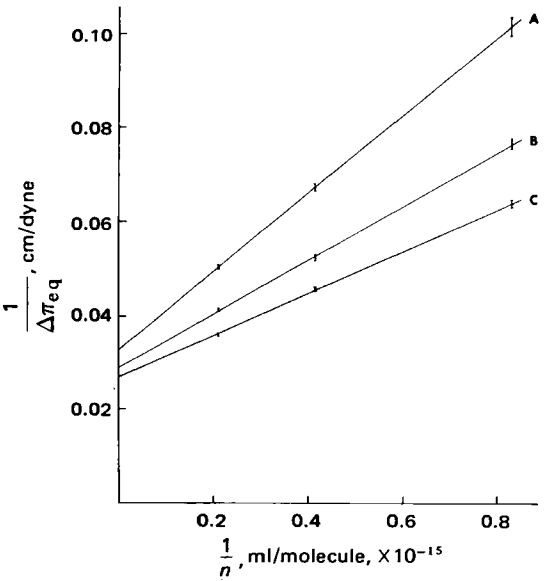


Figure 2—*Typical plots of $1/\Delta\pi_{eq}$ against 1/n for the adsorption of cetrimonium bromide at the air–aqueous solution interfaces, where n is the final surfactant concentration in molecules per milliliter and $\Delta\pi_{eq}$ is the equilibrium surface pressure. Key: A, 0.15 M NaCl; B, 0.30 M NaCl; and C, 0.45 M NaCl.*

$$(SR) = \frac{k_2S_{eq}R_T}{k_2S_{eq} + k_{-2}}[1 - e^{-(k_2S_{eq} + k_{-2})(t - t_0)}] \qquad \text{(Eq. 5)}$$

When final equilibrium is achieved:

$$\lim_{t \to \infty}(SR) = \frac{k_2S_{eq}R_T}{k_2S_{eq} + k_{-2}} = (SR)_{eq} \qquad \text{(Eq. 6)}$$

and:

$$(SR) = (SR)_{eq}[1 - e^{-(k_2S_{eq} + k_{-2})(t - t_0)}] \qquad \text{(Eq. 7)}$$

If the surface pressure ($\Delta\pi$) is proportional to the number of bound sites at the interface, as was postulated previously (24):

$$\Delta\pi = \alpha(SR) \qquad \text{(Eq. 8)}$$

and:

$$(\Delta\pi)_{eq} = \alpha(SR)_{eq} \qquad \text{(Eq. 9)}$$

Equation 7 can be expressed in terms of experimental values as:

$$\Delta\pi = \Delta\pi_{eq}[1 - e^{-(k_2S_{eq} + k_{-2})(t - t_0)}] \qquad \text{(Eq. 10)}$$

or in logarithmic form as:

$$\log(\Delta\pi_{eq} - \Delta\pi) = -\frac{k_2S_{eq} + k_{-2}}{2.303}(t - t_0) + \log \Delta\pi_{eq} \qquad \text{(Eq. 11)}$$

so that a plot of the logarithm of the difference between the final value of the surface pressure ($\Delta\pi_{eq}$) and any value of the surface pressure at a time t should be a straight line of negative slope, m, and intercept $\log \Delta\pi_{eq}$ (Fig. 3*a*) where:

$$-2.303m = k_2S_{eq} + k_{-2} \qquad \text{(Eq. 12)}$$

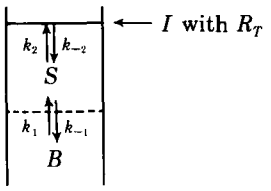
It follows that m should be a linear function of the amount of surfactant added to a constant volume, V_s, of solution. When the obtained $-m$ values are plotted against surfactant concentration $[S]$:

$$-m = \frac{k_2}{2.303}[S]V_S + \frac{k_{-2}}{2.303} \qquad \text{(Eq. 13)}$$

a straight line should result of slope $V_sk_2/2.303$ and intercept $k_{-2}/2.303$

The plots of $-m$ against the bulk surfactant concentration (Fig. 4) are not inconsistent with Eq. 13 within the limits of concentration studied based on the principles underlying Model I.

Model II—Model II postulates a rate-determining process of diffusion from the bulk solution (B) into the subsurface (S) with



Scheme III

an instantaneous equilibrium with receptor sites (Scheme III). There is strong evidence to postulate the structural ordering of water adjacent to any interface, solid–water, liquid–water, or air–water (24). Particularly in the case of solid–water interfaces (25), anomalous values of thermal expansion, electro- and thermal conductivity, and degeneration of the degree of molecule rotation demand a high degree of ordering for molecules at the interface to produce a new interfacial quasisolid structure.

This model presumes that a compartment exists between the interface (*I*) and the surfactant that is dissolved in the bulk solution. This compartmental model postulates the following compartments.

The Interface (I)—The boundary between the aqueous and the gaseous phases that contains the "active sites" postulated by Langmuir (26).

The Subinterface (S)—A thin region with a thickness of $10–10^3$ water molecule diameters immediately beneath the interface, postulated previously by Ward and Tordai (9). It is further assumed that water molecules are oriented in a given array in this region (25, 27), so that the surfactant must diffuse from a normal homogeneously dispersed solution through this array before it can reach the sites on the interface.

The Bulk Aqueous Phase (B)—The region below the subinterface. Water and solute molecules are distributed at random in this region.

The process can be described by Scheme IV:

$$[B] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [S] + R \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} (SR)$$

Scheme IV

in which $[B]$ and $[S]$ represent the surfactant-ion concentration in the bulk phase and subinterface, respectively; R is the number of active sites per unit of surface area of the interface (I); and (SR) is the number of surfactant ions attached per unit area.

If it is postulated that the interaction of molecules of the surfactant in the subinterface (S) with sites in the interface (I) is instantaneous so that the interaction between S and R in Scheme III is in equilibrium:

$$K_2 = \frac{k_2}{k_{-2}} = \frac{(SR)}{[R][S]} \qquad \text{(Eq. 14)}$$

The fraction, θ, of the total number of sites, R_T, occupied by surfactant molecules is:

$$\theta = \frac{(SR)}{R_T} = \frac{K_2[S]}{1 + K_2[S]} \qquad \text{(Eq. 15)}$$

so that if the surface pressure ($\Delta\pi$) is proportional to the number of bound sites at the interface:

$$\Delta\pi = \alpha(SR) = \alpha\theta R_T = \frac{\alpha R_T K_2[S]}{1 + K_2[S]} = \frac{\Delta\pi_\infty K_2[S]}{1 + K_2[S]} \qquad \text{(Eq. 16)}$$

in which:

$$\Delta\pi_\infty = \alpha R_T \qquad \text{(Eq. 17)}$$

is the maximum value of $\Delta\pi$ that can be achieved with any concentration $[S]$.

If the total number of interface sites, R_T, is relatively small so that $[S]$ is not significantly depleted by binding of the surfactant ions to those sites, the rate of change of surfactant concentration with time from Scheme III is:

$$\frac{d[S]}{dt} = k_1[B] - k_{-1}[S] \qquad \text{(Eq. 18)}$$

As the volume of the subinterface, S, is small with respect to the bulk solution, B, the rise in concentration in the subinterface will not significantly deplete the concentration in the bulk solution; then, for all apparent purposes, $k_1[B]$ is a constant and Eq. 18 may be integrated to give:

$$[S] = \frac{k_1[B]}{k_{-1}}[1 - e^{-k_{-1}(t - t_0)}] \qquad \text{(Eq. 19)}$$

The substitution of this value for $[S]$ in Eq. 16 results in:

$$\Delta\pi = \frac{\frac{\Delta\pi_\infty K_2 k_1[B]}{k_{-1}}[1 - e^{-k_{-1}(t-t_0)}]}{1 + \frac{K_2 k_1[B]}{k_{-1}}[1 - e^{-k_{-1}(t-t_0)}]} = \frac{\Delta\pi_\infty[1 - e^{-k_{-1}(t-t_0)}]}{k_{-1}/K_2 k_1[B] + [1 - e^{-k_{-1}(t-t_0)}]} \qquad \text{(Eq. 20)}$$

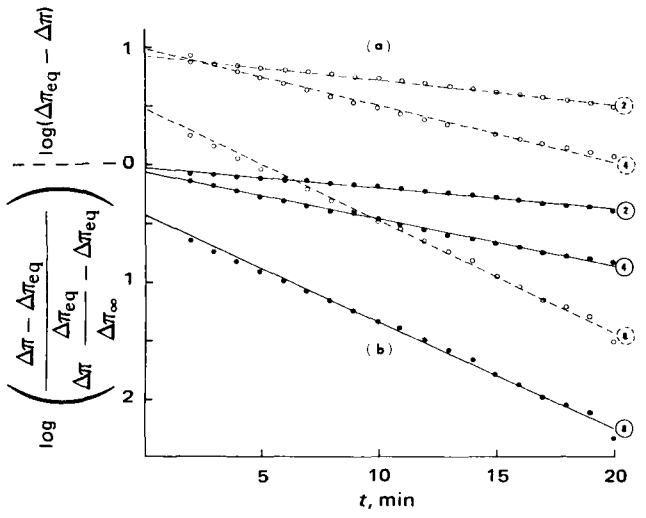


Figure 3—*Plots of log ($\Delta\pi_{eq} - \Delta\pi$) (Model I) and log $\{(\Delta\pi - \Delta\pi_{eq})/[(\Delta\pi)(\Delta\pi_{eq}/\Delta\pi_\infty) - \Delta\pi_{eq}]\}$ (Model II) against time for the adsorption of cetrimonium ions at the air–0.15 M NaCl interface. Lines are labeled as to micromolar final concentration of the injected cetrimonium ions.*

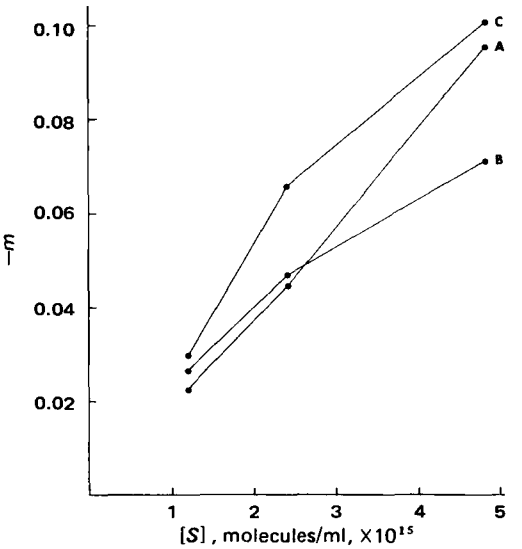


Figure 4—*Plots of −m against the final concentration of cetrimonium ions [S] in molecules per milliliter. Key: A, 0.15 M NaCl; B, 0.30 M NaCl; and C, 0.45 M NaCl.*

Table I—Evaluated Parameters for Adsorption of Various Solution Concentrations of Cetrimonium Ion at the Air–Water Interface for Various Concentrations of Sodium Chloride and Potassium Chloride

		Cetrimonium Ion, Final Concentration					
		2 μM		4 μM		8 μM	
Concentration, M	$\Delta\pi_\infty$, dynes/cm	$\Delta\pi_{eq}$, dynes/cm	k_{-1}, min^{-1}	$\Delta\pi_{eq}$, dynes/cm	k_{-1}, min^{-1}	$\Delta\pi_{eq}$, dynes/cm	k_{-1}, min^{-1}
Sodium chloride							
0.15	30.4	9.9	0.050	14.9	0.086	19.9	0.201
0.30	34.5	13.2	0.050	19.2	0.086	24.2	0.145
0.45	37.1	15.8	0.069	21.8	0.124	27.7	0.218
Potassium chloride							
0.15	30.1	9.8	0.069	14.8	0.108	19.9	0.185
0.30	34.0	13.1	0.066	18.9	0.124	24.1	0.157
0.45	36.6	15.6	0.079	21.7	0.129	27.5	0.248

so that:

$$\lim_{t\to\infty} \Delta\pi = \frac{\Delta\pi_\infty}{1 + k_{-1}/k_1[B]K_2} = \Delta\pi_{eq} \qquad \text{(Eq. 21)}$$

and:

$$\Delta\pi_\infty = \Delta\pi_{eq}\left(1 + \frac{k_{-1}}{k_1[B]K_2}\right) \qquad \text{(Eq. 22)}$$

$$\frac{1}{\Delta\pi_{eq}} = \frac{1}{\Delta\pi_\infty} + \frac{k_{-1}}{k_1K_2\Delta\pi_\infty}\left(\frac{1}{[B]}\right) \qquad \text{(Eq. 23)}$$

so that a plot of the reciprocal of the equilibrium surface pressure ($1/\Delta\pi_{eq}$) against the reciprocal of a particular concentration ($1/[B]$) should be a straight line of positive slope equal to k_{-1}/k_1K_2 ($\Delta\pi_\infty$) and intercept $1/\Delta\pi_\infty$.

When $[1 + (k_{-1}/k_1K_2[B])]$ of Eq. 22 is substituted in Eq. 20:

$$\Delta\pi = \frac{\Delta\pi_\infty(1 - e^{-k_{-1}(t-t_0)})}{\Delta\pi_\infty/\Delta\pi_{eq} - e^{-k_{-1}(t-t_0)}} \qquad \text{(Eq. 24)}$$

and:

$$\frac{\Delta\pi - \Delta\pi_{eq}}{(\Delta\pi)\dfrac{\Delta\pi_{eq}}{\Delta\pi_\infty} - \Delta\pi_{eq}} = e^{-k_{-1}(t-t_0)} \qquad \text{(Eq. 25)}$$

or:

$$\log\left[\frac{\Delta\pi - \Delta\pi_{eq}}{(\Delta\pi)\dfrac{\Delta\pi_{eq}}{\Delta\pi_\infty} - \Delta\pi_{eq}}\right] = \frac{k_{-1}}{2.303}(t - t_0) \qquad \text{(Eq. 26)}$$

so that the logarithmic function for a change in surface pressure from $\Delta\pi$ to its final equilibrium value, $\Delta\pi_{eq}$, when plotted against time should be linear (Fig. 3b) of slope $-k_{-1}/2.303$, where k_{-1} is the first-order rate constant for the return of surfactant from the subinterface to the bulk solution.

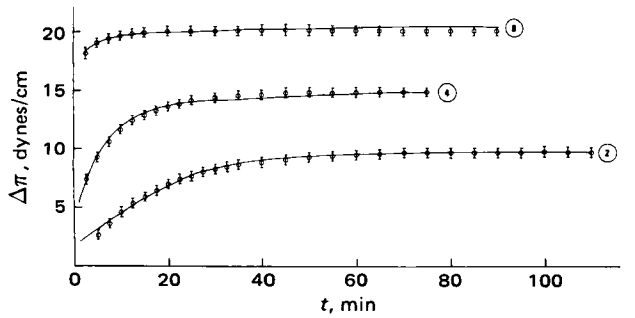


Figure 5—*Plots of surface pressure ($\Delta\pi$) against time (t) for the adsorption of cetrimonium ions at the air–0.15 M NaCl interface; Φ = surface pressures calculated with Eq. 25 (Model II). Curves are labeled as to micromolar final concentrations of the injected surfactant ion.*

The fact that the data conform to Eq. 26 is demonstrated by the linearity of the plots given in Fig. 3b. It was not expected that the data at initial times would conform to Eq. 26, since the agitation of the experimental procedure close to time zero would definitely perturb and accelerate the kinetic processes in the initial moments. When the values of k_{-1} (Table I) obtained from the slopes of the plots are substituted into Eq. 24, the resultant $\Delta\pi$ values fit the $\Delta\pi$–t experimental curves (Fig. 5) excellently within the limits of the experimental error for all saline concentrations studied. Thus, the kinetics of the adsorption of cetrimonium ions at the air–aqueous solution interfaces can be described by the postulation of an intermediate compartment between the interface and the bulk phase.

The product of certain important constants can be estimated from the ratio of the intercept and slope of Eq. 23:

$$\frac{\text{intercept}}{\text{slope}} = \frac{\dfrac{1}{\Delta\pi_\infty}}{\dfrac{k_{-1}}{k_1K_2\Delta\pi_\infty}} = \frac{k_1K_2}{k_{-1}} = K_1K_2 \qquad \text{(Eq. 27)}$$

where $K_1 = k_1/k_{-1}$ and $K_2 = k_2/k_{-2}$ (Scheme IV) are the equilibrium constants of the distributive and surface interaction processes, respectively.

It was shown previously (1, 2) that the energy of activation (ψ) for the process of the interaction of injected cetrimonium ions with a monomolecular layer spread at the air–water interface may be estimated on the assumption that each molecule forming the monolayer constitutes one active site. Thus, the value of (Ns/A) (Eq. 7 of Ref. 1) that represents the number of active sites per square centimeter was given by the known number of molecules necessary to form the monolayer at a given surface pressure.

In the case of an air–water interface without any previously adsorbed or spread monolayer, the term (Ns/A) may be estimated on the basis of the available space per adsorbed surfactant molecule. Molecular models show that the cross-sectional area of the cetrimonium ion is 26.3×10^{-16} cm^2/ion, so that the reciprocal 3.80×10^{14} ions/cm^2 is the maximum number of cetrimonium ions that may exist per square centimeter at the interface at the condition defined by $\Delta\pi = \Delta\pi_\infty$. It is reasonably assumed that this number is

Table II—Estimated Equilibrium Constants for Receptor Site Binding at Interface (K_2) and Partition across a Diffusional Barrier (K_1) and Their Associated Free Energies

Concentration, M	K_1, $\times 10^{-3}$	ΔG_1, kcal/mole	K_2, $\times 10^7$	$\Delta G_2 = \psi$, kcal/mole
Sodium chloride				
0.15	5.96	3.0	3.73	−10.2
0.30	5.91	3.0	4.99	−10.4
0.45	5.83	3.0	6.02	−10.5
Potassium chloride				
0.15	5.95	3.0	3.99	−10.2
0.30	5.93	3.0	5.07	−10.4
0.45	5.88	3.0	6.12	−10.5

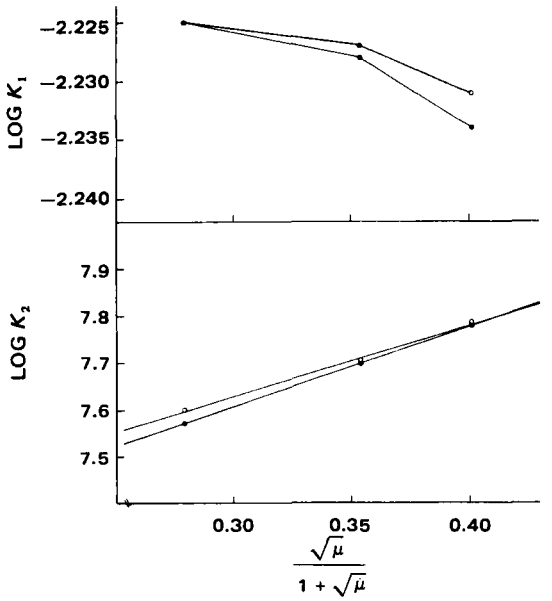


Figure 6—*Plots of log* K_1 *and log* K_2 *against* $[\sqrt{\mu}/(1+\sqrt{\mu})]$ *of the bulk solution. Key:* ●, *sodium chloride; and* ○, *potassium chloride.*

physically equivalent to the number of active sites.

Equation 8 of Ref. 1 can be rewritten in the form:

$$\frac{1}{\Delta\pi_{eq}} = \frac{1}{\Delta\pi_{\infty}} + \frac{(Ns/A)(2\pi m/kT)^{1/2}\nu e^{-\psi/kT}}{\Delta\pi_{\infty}}\left(\frac{1}{n}\right) \quad \text{(Eq. 28)}$$

in which $\Delta\pi_{eq}$ is the equilibrium surface pressure obtained for a final concentration of injected surfactant ion, n (molecules per milliliter); $\Delta\pi_{\infty}$ is the maximum value of $\Delta\pi$ that can be achieved with any final concentration of surfactant ion; (Ns/A) is the number of active sites per square centimeter; π is 3.14 . . . ; m is the absolute mass of the cetrimonium ion; k and T are the Boltzmann constant and the absolute temperature, respectively; ν is the classical frequency of a harmonic oscillator at room temperature (10^{12}/sec); and ψ is the energy of activation of the process of the interaction of cetrimonium ions with active sites at the interface.

The values of ψ obtained for different concentrations of sodium and potassium chlorides on the assumption of $(Ns/A) = 3.80 \times 10^{14}$ active sites/cm^2 are listed in Table II.

The free energy of adsorption per methylene group of a hydrocarbon chain that adsorbs at a clean air–water interface was estimated to be between −0.600 and −0.625 kcal/mole (23, 26, 28, 29). Thus, the value estimated for the hexadecyl hydrocarbon chain of the cetrimonium ion is between −9.6 and −10.0 kcal/mole and compares reasonably with the absolute values of ψ listed in Table II.

The van't Hoff equation:

$$\psi = \Delta G = -RT \ln K \quad \text{(Eq. 29)}$$

permits estimation of the numerical values of K_2 (Table II). It can be observed that these values are a function of the ionic strength of the saline solution in the bulk phase.

Thus, the numerical value of the equilibrium constant K_1 can be estimated from the K_1K_2 value (Eq. 27) and the estimated value of K_2 (Eq. 29). The corresponding free energy values can be calculated from Eq. 29 (Table II). These values do not appear to depend on the ionic strength of the bulk solution.

Effect of Ionic Strength—The experimental values of $\Delta\pi_{\infty}$, $\Delta\pi_{eq}$, and, less consistently, k_{-1} increase with increasing ionic strength (Table I). These facts are reflected in increases of the estimated K_1 and K_2 values with ionic strength (Table II). Log K_1 and log K_2 are plotted against $\sqrt{\mu}/(1+\sqrt{\mu})$ (30), where μ is the ionic strength (Fig. 6).

Such plots of log K_2 were linear with correlation coefficients of 0.99999 and 0.99833 for sodium and potassium chlorides, respectively, whereas the plots of log K_1 showed only small, nonlinear dependence on the ionic strength of the bulk solution. It is reasonable that in this last case the effect of the ionic strength of the bulk solution is counterbalanced by a proportional increase in the ionic strength of the subinterface.

In summary, the following facts are consistent with Model II:

1. The plots of $1/\Delta\pi_{eq}$ against $1/[n]$ are linear as predicted by Eq. 23.
2. The values of k_{-1} estimated from the plot of the logarithmic function of $(\Delta\pi - \Delta\pi_{eq})/[(\Delta\pi)(\Delta\pi_{eq}/\Delta\pi_{\infty}) - \Delta\pi_{eq}]$ against time (Eq. 26) when substituted in Eq. 24 give $\Delta\pi$ values that fit the $\Delta\pi$–t experimental curves excellently between the limits of the experimental error in all saline concentrations studied.
3. The free energy of adsorption estimated from the equilibrium constant K_2 and the van't Hoff equation coincide with those expected from the adsorption of a hexadecyl hydrocarbon chain at the air–water interface.
4. The effect of the ionic strength of the bulk solution on the $\Delta\pi_{\infty}$ and $\Delta\pi_{eq}$ values is consistent with the existence of a subinterface compartment.

The application of this model to the results already obtained with the injection of cetrimonium ions beneath phospholipid monolayers spread at the air–aqueous solution interfaces will be discussed in later papers.

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Characterization of Hydrogen Bonding between Selected Barbiturates and Polyethylene Glycol 4000 by IR Spectral Analysis

B. L. CHANG, N. O. NUESSLE, and W. G. HANEY, Jr. [x]

Abstract □ Several barbiturates and primidone were equilibrated with polyethylene glycol 4000 in pyridine. IR spectral properties of these samples indicate that seven disubstituted barbiturates complex with polyethylene glycol 4000 while five disubstituted barbiturates and two trisubstituted barbiturates as well as primidone do not. Forces responsible for complexation of barbiturates with polyethylene glycol 4000, as inferred from spectral data, consist of hydrogen bonds formed between N^1 and N^3 hydrogens of the barbiturate ring and two oxygen atoms of the —O—CH_2CH_2—O— moiety. Also, there appear to be three configurations of intermolecular hydrogen bonding sites between disubstituted barbiturates. Several factors affect the barbiturate–polyethylene glycol 4000 interaction, including the nature of the solvent, C_5 substituents, the number of hydrogen bonds formed between reactants, and the 2-carbonyl group of the barbiturate ring. Complexes of polyethylene glycol 4000 with phenobarbital, butabarbital, and cyclobarbital are stable in water at 26° or below, but complexes of polyethylene glycol 4000 with butethal, cyclopentenyl allylbarbituric acid, pentobarbital, and probarbital are not.

Keyphrases □ Barbiturates—IR characterization of hydrogen bonding with polyethylene glycol 4000 □ Primidone—interaction with polyethylene glycol 4000 □ Hydrogen bonding of barbiturates and primidone with polyethylene glycol—characterized by IR spectrophotometry □ Complexes, barbiturates and polyethylene glycol 4000—IR characterization □ IR spectrophotometry—characterization, hydrogen bonding between barbiturates and polyethylene glycol 4000

Table I—N—H Absorption Bands in Spectra of Barbiturates and Their Complexes

Compound	N—H Absorption Bands, cm^{-1}				
Phenobarbital	3310	3200			3090
Phenobarbital complex	3220			3110	
Pentobarbital		3210	3170		3090
Pentobarbital complex	3240			3110	
Probarbital		3220			3100
Probarbital complex	3270			3120	
Butabarbital		3220			3095
Butabarbital complex	3260			3120	
Cyclobarbital		3220			3100
Cyclobarbital complex	3280			3120	
Butethal		3200			3090
Butethal complex	3220			3100	
Cyclopentenyl allylbarbituric acid		3210	3160		3080
Cyclopentenyl allylbarbituric acid complex		3210			3080

Barbiturates may interact with various organic molecules by hydrogen bonding to form soluble or insoluble complexes such as phenobarbital–theophylline (1), phenobarbital–polyvinylpyrrolidone (2), phenobarbital–caffeine (3), barbital–aminopyrine (4), and phenobarbital–poly-*N*-vinyl-5-methyl-2-oxazolidinone (5). Application of such complexation to pharmaceutics has been discussed in the literature (1–7).

In 1968, Kyogoku *et al.* (8) found that barbiturates also form specific and strong hydrogen bonds to adenine derivatives. Since adenine derivatives exist in a number of biochemicals, *e.g.*, coenzymes and adenosine triphosphate, this interaction formed the basis of a hypothesis explaining the molecular mechanism underlying the pharmacological mode of action of barbiturates. Kyogoku and Yu (9–11) extended the research from ethyladenine to nicotinamide adenine dinucleotide (NAD) and flavine adenine dinucleotide (FAD) and postulated that barbiturates inhibit biological oxidation by interaction with the adenine moiety of NAD and FAD. Thus, an understanding of hydrogen bonding properties is important in barbiturate dosage form development and may allow explanation of the mechanism by which barbiturates inhibit biological oxidation. It is of interest, therefore, to study hydrogen bonding properties of the barbiturate ring in detail.

It was shown (8) that phenobarbital–9-ethyladenine has an association constant of 1200 M^{-1}, whereas barbital–9-ethyladenine and pentobarbital–9-ethyladenine have association constants of 1000 M^{-1}. The larger association constant of phenobarbital–9-ethyladenine was attributed to the lower pKa of phenobarbital (7.3) as compared to that of barbital (7.8) and pentobarbital (8.0). However, since pentobarbital–9-ethyladenine has the same association constant

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Butabarbital		3220			3095
Butabarbital complex	3260			3120	
Cyclobarbital		3220			3100
Cyclobarbital complex	3280			3120	
Butethal		3200			3090
Butethal complex	3220			3100	
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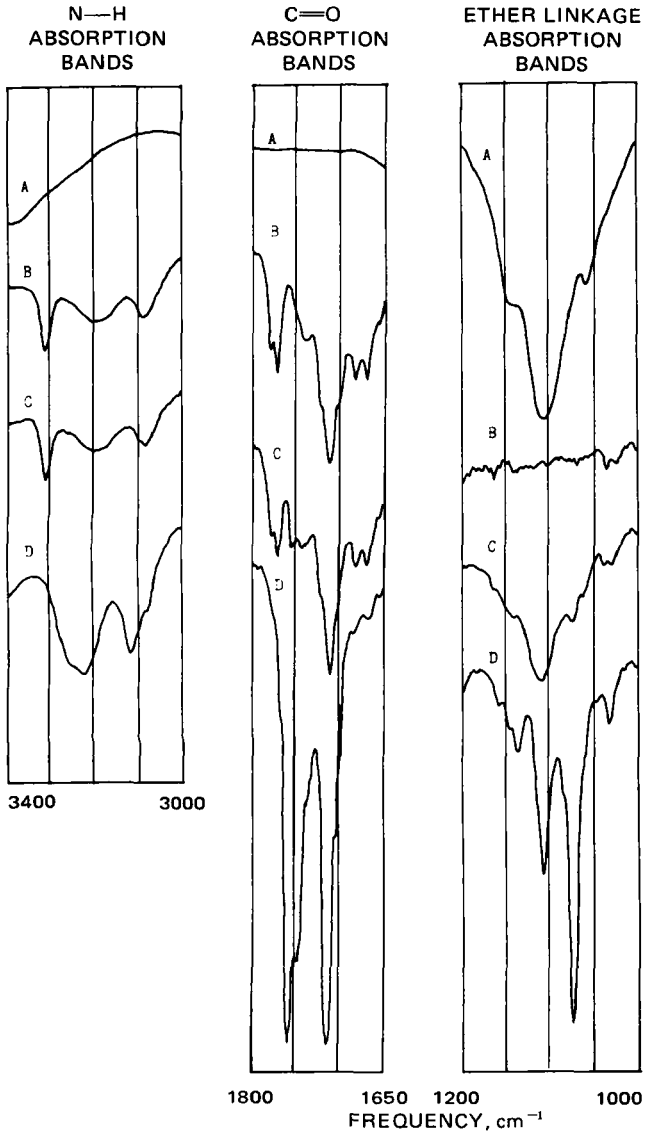


Figure 1—*IR spectra of phenobarbital–polyethylene glycol 4000 from water. Key: A, polyethylene glycol 4000; B, phenobarbital; C, physical mixture of phenobarbital and polyethylene glycol 4000; and D, phenobarbital–polyethylene glycol 4000 complex from water.*

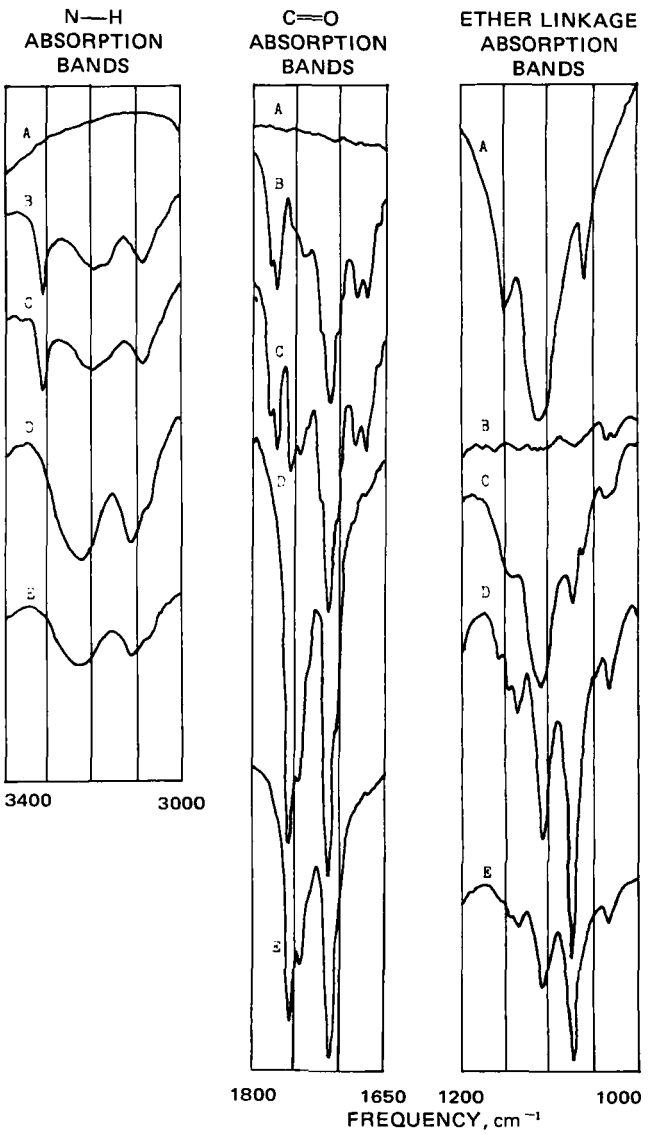


Figure 2—*IR spectra of phenobarbital–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, phenobarbital; C, physical mixture of phenobarbital and polyethylene glycol 4000; D, equilibrated phenobarbital–polyethylene glycol 4000; and E, aqueous-washed equilibrated phenobarbital–polyethylene glycol 4000 shown in D.*

as barbital–9-ethyladenine, other factors (*e.g.*, the steric effect of the C_5 substituent) must contribute to the strength of hydrogen bonds formed between barbiturates and 9-ethyladenine.

A particularly interesting observation (7) related to hydrogen bonding properties of barbiturates is that phenobarbital interacts with polyethylene glycol 4000 to form a water-insoluble complex with a 1:2 stoichiometric ratio while barbital and pentobarbital do not form this complex. To account for this observation, it was suggested that the interaction mechanism is influenced primarily by the dipole–dipole interaction of reactants and secondarily by the "squeezing-out" effect of water. However, barbital and, especially, the lipophilic pentobarbital are not as favorably affected by the squeezing-out effect as is phenobarbital. Negative results obtained with barbital and pentobarbital were attributed in part to a stereochemical effect and in part to the absence of the aromatic ring, which participates more readily in complexation. It is of interest, therefore, to identify the stereochemical factors and the mechanism by which the phenyl substituent favorably affects the interaction.

This investigation was undertaken to examine the interaction between barbiturates and polyethylene glycol 4000 and to elucidate factors contributing to this interaction using IR spectral analysis. In barbiturates, the hydrogen bond donor is the =N—H group and the hydrogen bond acceptor is the >C=O group. In polyethylene glycol 4000, the oxygen atom of the ether linkage is the acceptor and the contribution of terminal hydroxy groups, present in low proportion, may be neglected. These functional groups have characteristic absorption bands in the IR region.

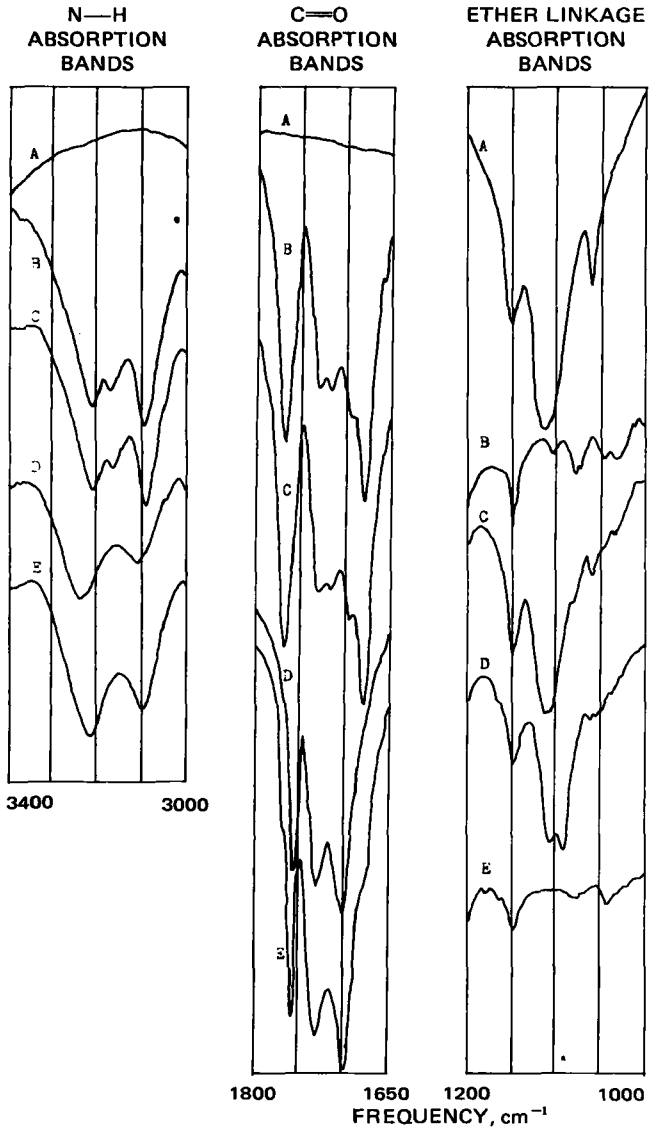


Figure 3—*IR spectra of pentobarbital–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, pentobarbital; C, physical mixture of pentobarbital and polyethylene glycol 4000; D, equilibrated pentobarbital–polyethylene glycol 4000; and E, aqueous-washed equilibrated pentobarbital–polyethylene glycol 4000 shown in D.*

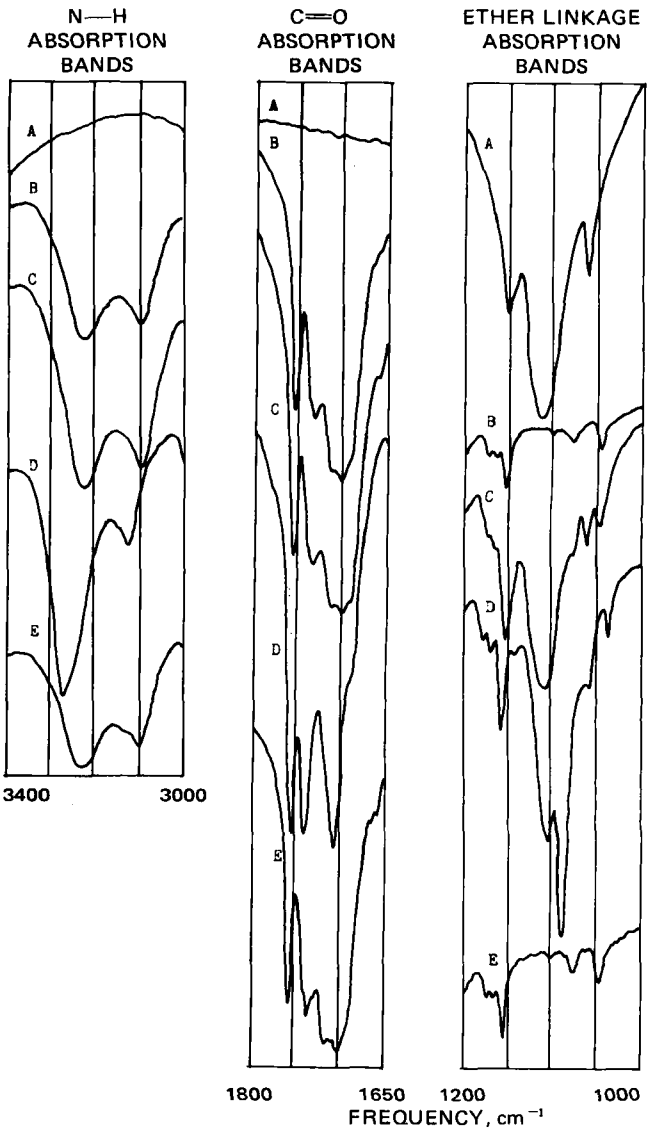


Figure 4—*IR spectra of probarbital–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, probarbital; C, physical mixture of probarbital and polyethylene glycol 4000; D, equilibrated probarbital–polyethylene glycol 4000; and E, aqueous-washed equilibrated probarbital–polyethylene glycol 4000 shown in D.*

Stretching and bending force constants for NH, C=O, and ether linkages are changed after hydrogen bond formation, resulting in a shift of the characteristic absorption bands (12). Based on this principle, characteristic C=O, NH, and ether linkage bands in the IR spectra of the barbiturate, polyethylene glycol 4000, and barbiturate–polyethylene glycol 4000 equilibrated sample were examined for shifts. These variations were, in turn, used to characterize the interaction between barbiturates and polyethylene glycol 4000.

EXPERIMENTAL[1]

Reagents—The reagents included phenobarbital, barbital, butabarbital sodium, amobarbital, probarbital sodium, pentobarbital sodium, cyclobarbital, talbutal[2], hexobarbital sodium[2], butethal, vinbarbital, cyclopentenyl allylbarbituric acid[3], allobarbital, primidone, mephobarbital, polyethylene glycol 4000, and pyridine[4].

Preparation of Free Barbiturates from Salts—Butabarbital, hexobarbital, and pentobarbital were obtained as sodium salts. To prepare the free barbituric acid derivative, an aqueous solution of the salt was acidified with 10% hydrochloric acid, and the mixture was cooled and filtered. The white crystalline precipitate was washed with water until the washings were free from chloride ion, and the solid was dried at 105°. The butabarbital so obtained had a melting range of 165–169°, the hexobarbital had a melting range of 145–147°, and the pentobarbital had a melting range of 127–133°.

Probarbital and cyclobarbital were obtained as calcium salts. The calcium salt was suspended in 10% hydrochloric acid, and the

[1] IR spectra were obtained on a Perkin-Elmer 457 grating spectrophotometer.

[2] Sterling-Winthrop Research Institute, Rensselaer, N.Y.
[3] Cyclopal, The Upjohn Co., Kalamazoo, Mich.
[4] J. T. Baker Co., Phillipsburg, N.J.

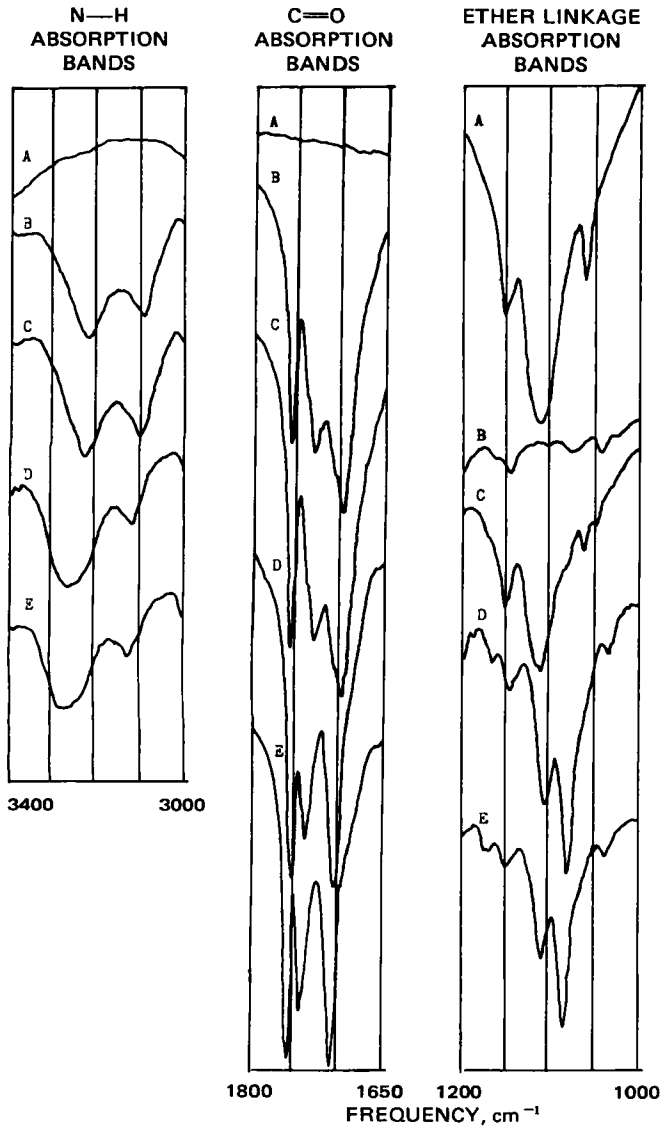


Figure 5—*IR spectra of butabarbital–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, butabarbital; C, physical mixture of butabarbital and polyethylene glycol 4000; D, equilibrated butabarbital–polyethylene glycol 4000; and E, aqueous-washed equilibrated butabarbital–polyethylene glycol 4000 shown in D.*

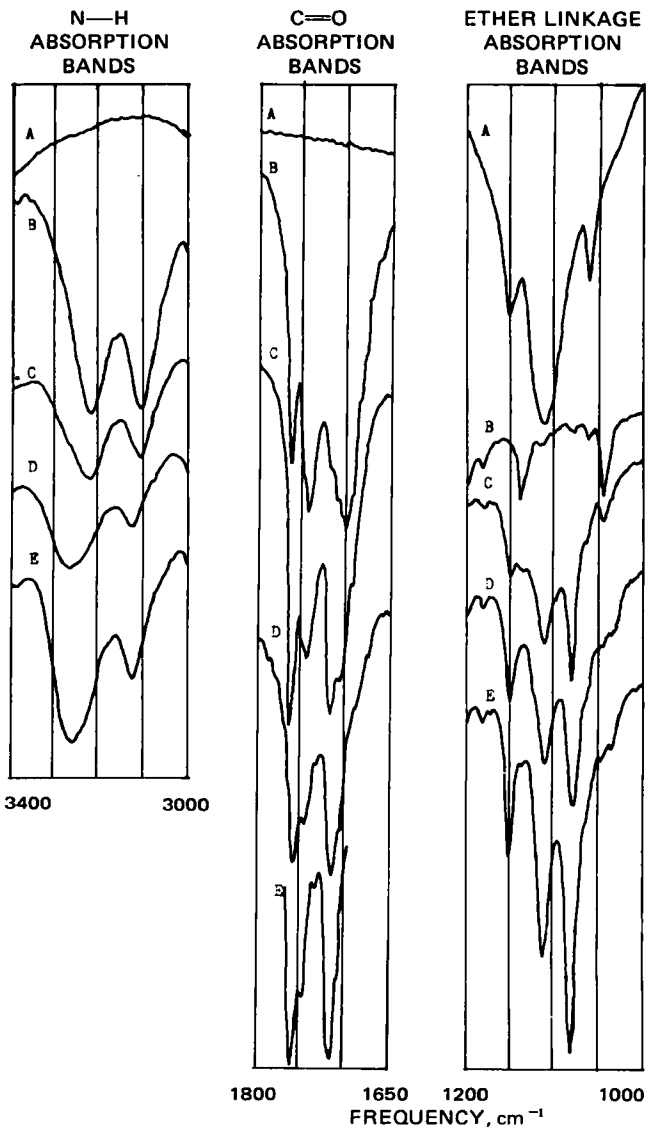


Figure 6—*IR spectra of cyclobarbital–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, cyclobarbital; C, physical mixture of cyclobarbital and polyethylene glycol 4000; D, equilibrated cyclobarbital–polyethylene glycol 4000; and E, aqueous-washed equilibrated cyclobarbital–polyethylene glycol 4000 shown in D.*

mixture was stirred for 10 min. The mixture was extracted three times with ether, and the combined ether extracts were washed with water and evaporated to dryness in a current of air. The residue was dried at 105°. Probarbital so obtained had a melting range of 200–202°, and cyclobarbital had a melting range of 170–174°.

Solvent Selection for Complexation Studies—Initially, a procedure was required for the preparation of barbiturate–polyethylene glycol 4000 complexes. The phenobarbital–polyethylene glycol 4000 complexation in water appears to be a special case, since other barbiturates do not undergo this interaction. Complexes might, however, be prepared from other solvents.

Since the primary mechanism of complexation appears to involve hydrogen bond formation, the ideal solvent should possess only weak hydrogen bond acceptor and donor properties but still dissolve the barbiturate and polyethylene glycol 4000. In addition, IR spectral characteristics of the phenobarbital–polyethylene glycol 4000 complex prepared from this solvent must be the same as those of the complex prepared from water (Fig. 1, curve D). Finally, the solvent should be easily evaporated at room temperature under reduced pressure.

Pyridine was found to be useful for the preparation of barbiturate–polyethylene glycol 4000 complexes. It dissolves both phenobarbital and polyethylene glycol 4000, and after evaporation the complex has an IR spectrum (Fig. 2, curve D) identical to that of the complex obtained from water.

Preparation of Barbiturate–Polyethylene Glycol 4000 Equilibrated Samples from Pyridine—One millimole of the barbiturate and 2 mEq of polyethylene glycol 4000 were dissolved in 3.0 ml of pyridine. The solvent was evaporated in a current of air, and the residue was placed in a reduced-pressure desiccator for 24 hr. The residue was ground and passed through a No. 80 sieve.

Crystallization of Reactants from Pyridine—Polymorphism of barbiturates is well known and has been extensively investigated (13–16). An important contributor to the formation of polymorphs is the solvent of crystallization. Cleverley and Williams (13) and Mesley (16) prepared a series of polymorphs for each barbiturate using different solvents and found that each form exhibited different IR absorption bands. The presence of different polymorphic forms would increase the difficulty of properly interpreting IR spectral changes. Therefore, all constituents of the study

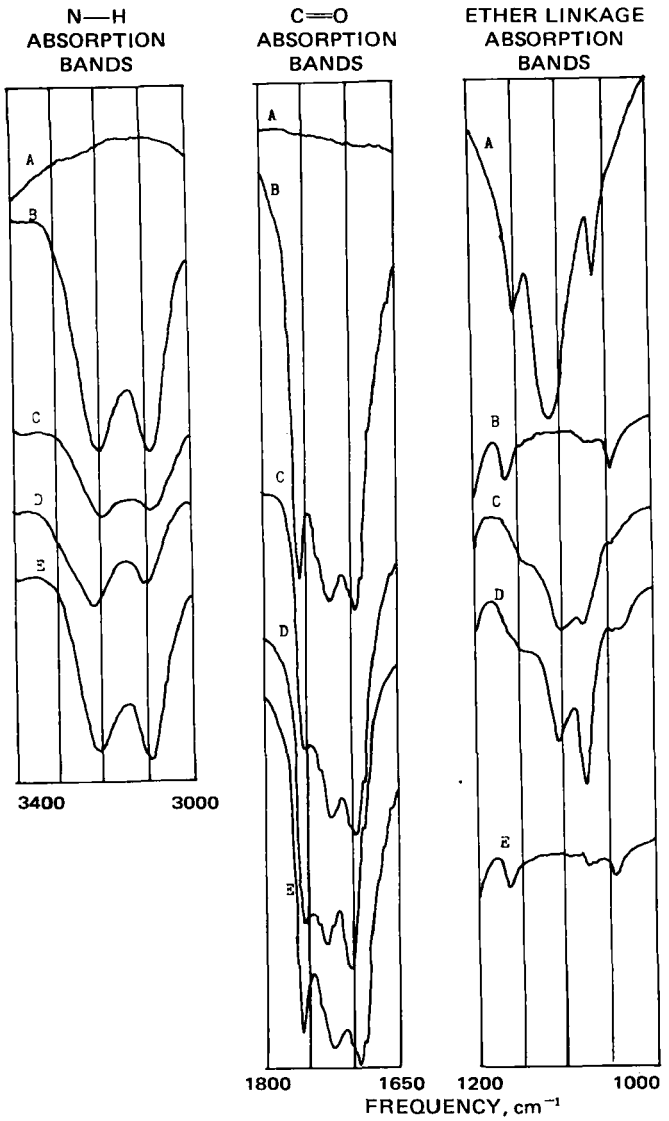


Figure 7—*IR spectra of butethal–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, butethal; C, physical mixture of butethal and polyethylene glycol 4000; D, equilibrated butethal–polyethylene glycol 4000; and E, aqueous-washed equilibrated butethal–polyethylene glycol 4000 shown in D.*

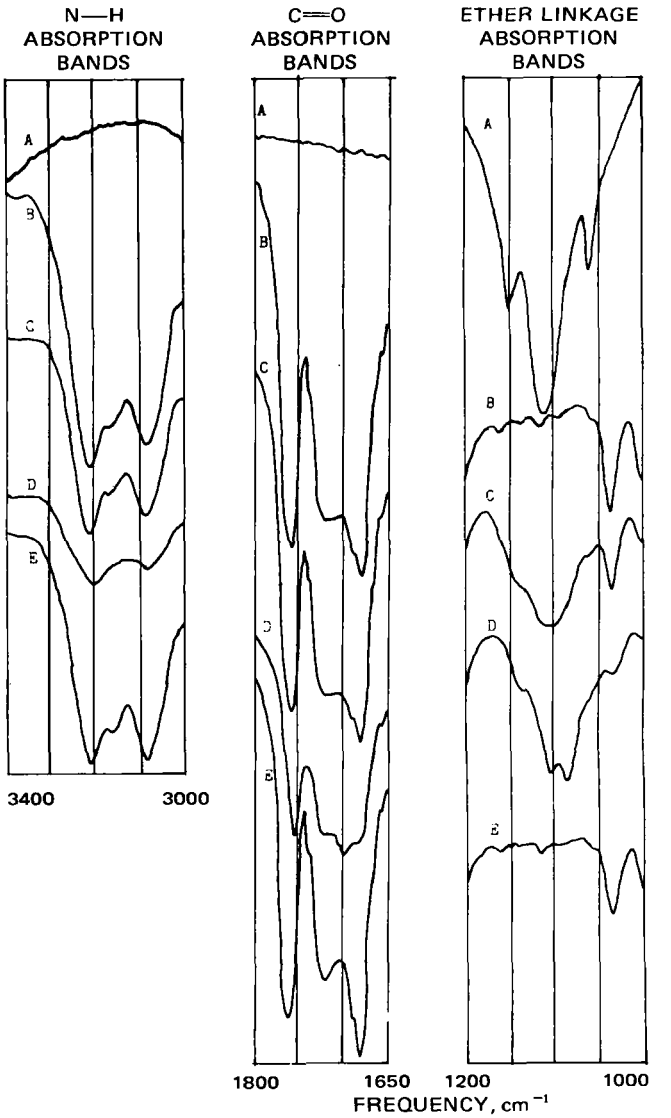


Figure 8—*IR spectra of cyclopentenyl allylbarbituric acid–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, cyclopentenyl allylbarbituric acid; C, physical mixture of cyclopentenyl allylbarbituric acid and polyethylene glycol 4000; D, equilibrated cyclopentenyl allylbarbituric acid–polyethylene glycol 4000; and E, aqueous-washed equilibrated cyclopentenyl allylbarbituric acid–polyethylene glycol 4000 shown in D.*

were recrystallized from a single solvent (pyridine) prior to IR spectral analysis.

Determination of Aqueous Stability of Complexes—A 0.5-g quantity of the complex was suspended in 10.0 ml of water in a screw-capped vial. The vial was fastened in a horizontal position on an agitator, and the shaking speed was adjusted to agitate the vial contents actively. After shaking for 2 hr at 26°, the mixture was filtered and the solids were washed with 20.0 ml of water. The solid was dried at room temperature in a vacuum desiccator for 3 days, and the IR spectrum was taken. Differences between the spectra of the complex before and after treatment with water indicate the stability of the complex.

Preparation of Potassium Bromide Dispersion—The sample and potassium bromide were obtained in proportions by weight of 1:200. The mixture was triturated in a small mortar and pressed in a die under 24,000 psi.

RESULTS AND DISCUSSION

Discussion will focus on the regions from 3400 to 3000 [NH stretching bands (16–19)], from 1800 to 1650 [C=O stretching bands (16, 20)], and from 1200 to 1000 [aliphatic ether bands (21)] cm^{-1}. The 3000–2700-cm^{-1} region will not be discussed since it appears to be of no analytical significance (17). No bands occur between 2700 and 1800 cm^{-1} in any of the tested samples. In the areas from 1650 to 1200 and from 1000 to 250 cm^{-1}, each barbiturate has a characteristic series of bands. Accurate assignment of these bands is difficult with currently available information (16), particularly after they are coupled with absorption bands of polyethylene glycol 4000 in these regions.

In Figs. 1 and 2, N—H stretching absorption bands of the physical mixture of phenobarbital and polyethylene glycol 4000 (curve C) are the same as those of phenobarbital alone (curve B). There are three absorption bands: 3310, 3200, and 3090 cm^{-1}. According to Levi and Hubley (17) and Mesley (16), 3310 cm^{-1} is assigned as a free N—H stretching absorption band while the remaining bands are intermolecular hydrogen-bonded N—H stretching absorption bands. The intermolecular hydrogen bond is between the N—H and C=O of different barbiturate molecules (16).

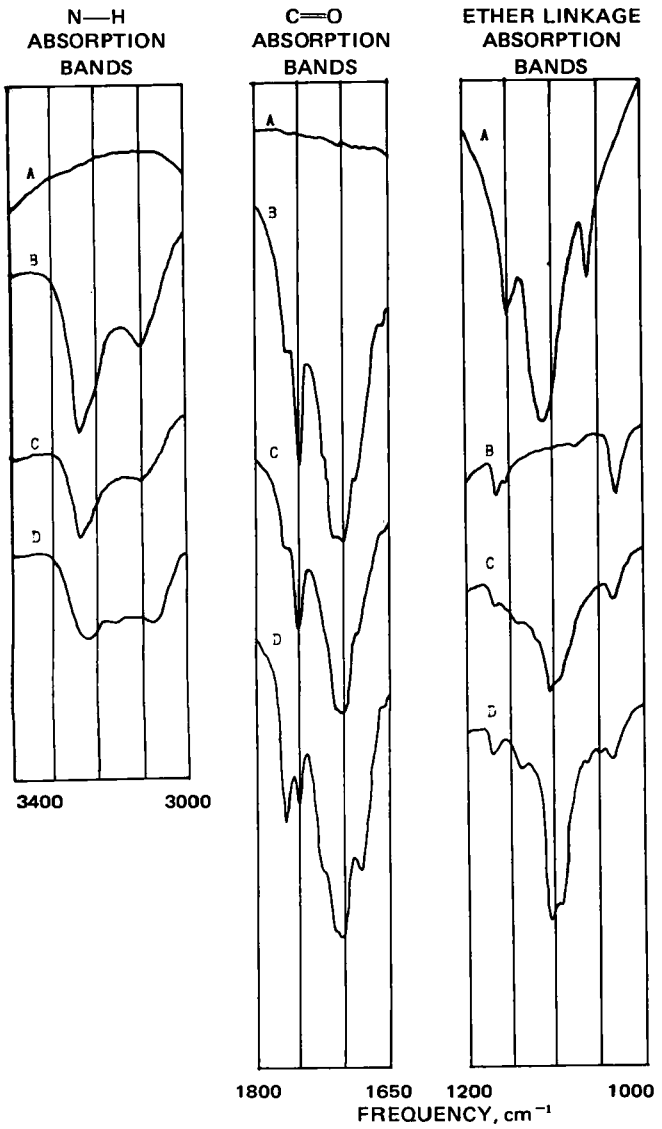


Figure 9—*IR spectra of barbital–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, barbital; C, physical mixture of barbital and polyethylene glycol 4000; and D, equilibrated barbital–polyethylene glycol 4000.*

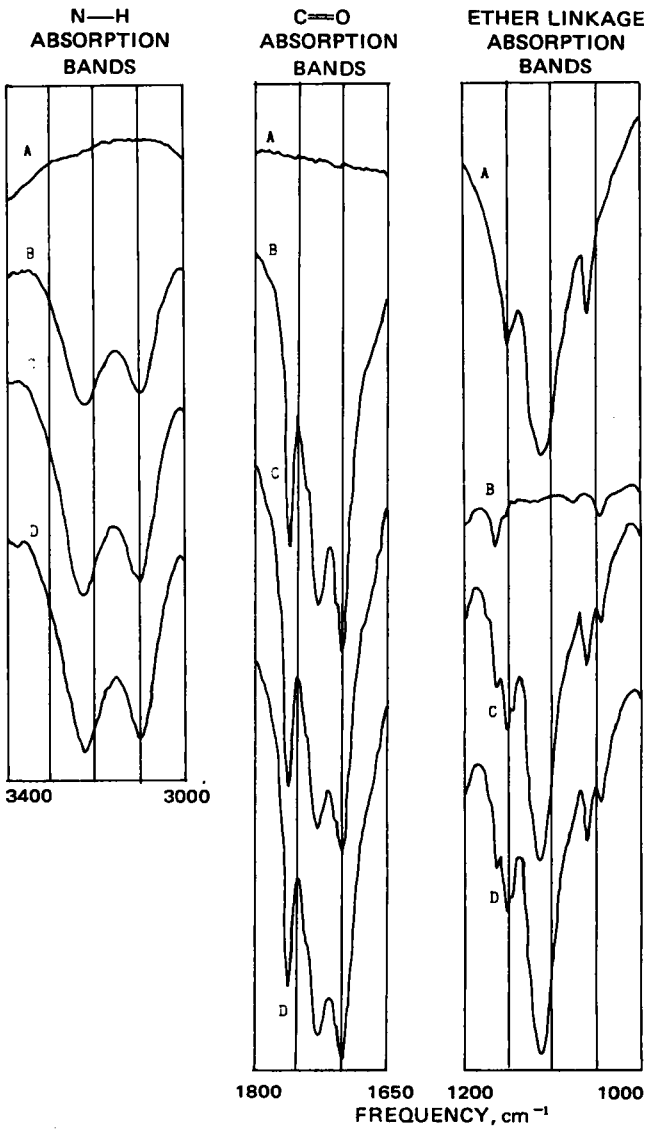


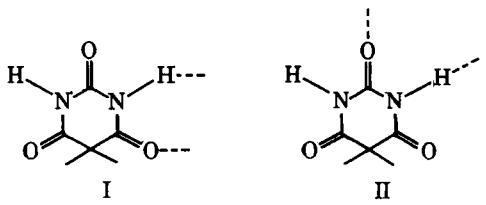
Figure 10—*IR spectra of amobarbital–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, amobarbital; C, physical mixture of amobarbital and polyethylene glycol 4000; and D, equilibrated amobarbital–polyethylene glycol 4000.*

The N—H stretching absorption regions of the phenobarbital–polyethylene glycol 4000 complex from water (Fig. 1, curve D) and equilibrated phenobarbital–polyethylene glycol 4000 from pyridine (Fig. 2, curve D) differ from that of phenobarbital alone (Figs. 1 and 2, curves B). In the spectrum of the complex, the band at 3220 cm^{-1} is stronger and broader than the one at 3110 cm^{-1}. Loss of the free N—H stretching absorption band (present in the spectrum of phenobarbital) in the spectra of the complex from water and the equilibrated samples from pyridine can be attributed to hydrogen bond formation. The 3220- and 3110-cm^{-1} N—H bands of the complex and equilibrated sample have higher frequencies compared to the 3200- and 3090-cm^{-1} N—H bands of phenobarbital. This shifting can be attributed to hydrogen bonds formed between phenobarbital and polyethylene glycol 4000, which displace hydrogen bonds formed between phenobarbital molecules. Since the acceptor molecules are different, the N—H stretching vibration frequency is changed in the complex and equilibrated sample.

In Figs. 3–13, the N—H stretching absorption region of each barbiturate shows the same pattern: two absorption bands, one in the range from 3270 to 3200 cm^{-1} and the other in the range from 3100 to 3090 cm^{-1}. These two bands are assigned as intermolecular hydrogen-bonded N—H stretching bands (16). In Figs. 3–8, the higher frequency band of the equilibrated system is more intense than the lower frequency band, and both bands occur at a higher frequency than those of the barbiturate alone.

The N—H absorption bands of equilibrated samples and the corresponding barbiturates are summarized in Table I. The observed spectral differences indicate that pentobarbital, butabarbital, cyclobarbital, butethal, and cyclopentenyl allylbarbituric acid interact with polyethylene glycol 4000. Mesley (16) already noted the possible existence of intermolecular hydrogen bonds in barbiturate crystals, and Craven and coworkers (22–24) proved it by X-ray diffraction analysis.

Since the phenobarbital spectrum has both free and hydrogen-



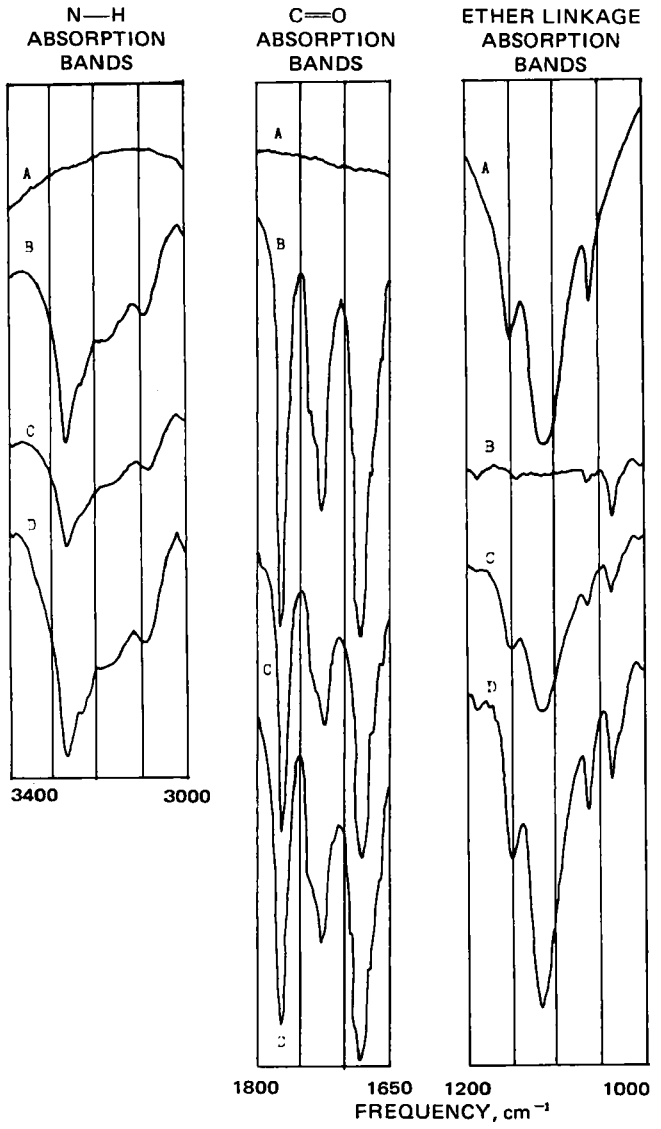


Figure 11—*IR spectra of vinbarbital–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, vinbarbital; C, physical mixture of vinbarbital and polyethylene glycol 4000; and D, equilibrated vinbarbital–polyethylene glycol 4000.*

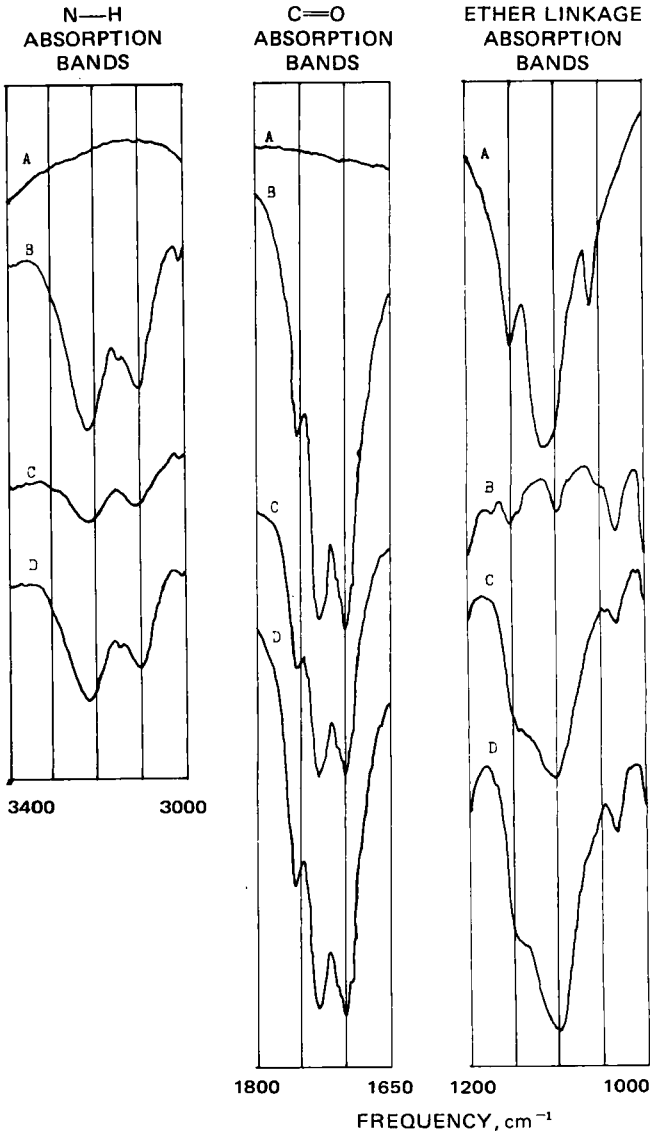
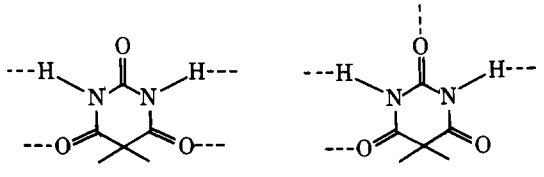


Figure 12—*IR spectra of talbutal–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, talbutal; C, physical mixture of talbutal and polyethylene glycol 4000; and D, equilibrated talbutal–polyethylene glycol 4000.*

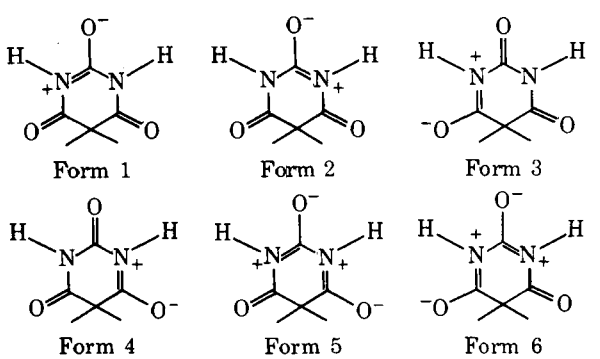
bonded N—H stretching absorption bands, the conformation of hydrogen bonding sites in the phenobarbital crystal would be as in I or II. The lack of free N—H stretching absorption bands in the spectra of the remaining barbiturates implies a conformation of hydrogen bonding sites as in III or IV. Upon complexation, phenobarbital also assumes a hydrogen bonding arrangement as in III or IV, as indicated by the loss of free N—H stretching.

The N—H stretching absorption bands of the barbital–polyethylene glycol 4000 equilibrated sample (Fig. 9) occur at a lower frequency than those of barbital. If hydrogen bond formation between barbiturates and polyethylene glycol 4000 shifts N—H stretching to a higher frequency than intermolecular hydrogen bond formation (as previously discussed), then shifting (Fig. 9) to a lower frequency must not be due to hydrogen bond formation between barbital and polyethylene glycol 4000. Instead, the change may be attributed to an alteration in the configuration of hydrogen bonding sites upon crystallization of barbital from pyridine in the presence of polyethylene glycol 4000.

Hydrogen bonding increases the stability of resonance forms contributing to the resonance stabilization of barbital by decreas-



III IV



Form 1 Form 2 Form 3

Form 4 Form 5 Form 6

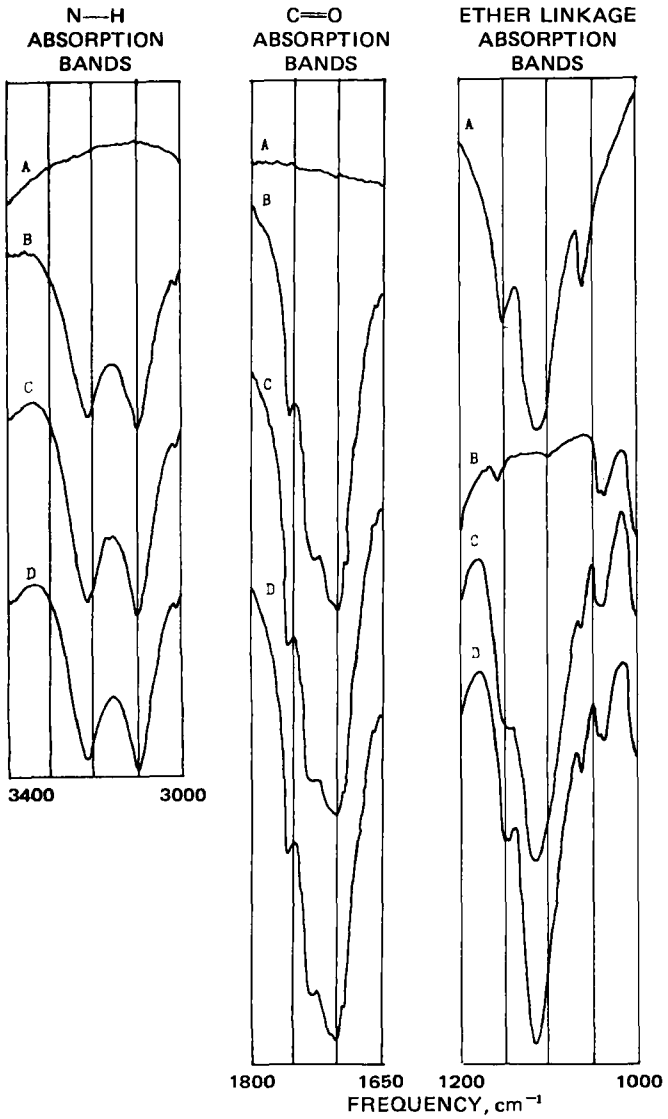


Figure 13—*IR spectra of allobarbital–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, allobarbital; C, physical mixture of allobarbital and polyethylene glycol 4000; and D, equilibrated allobarbital–polyethylene glycol 4000.*

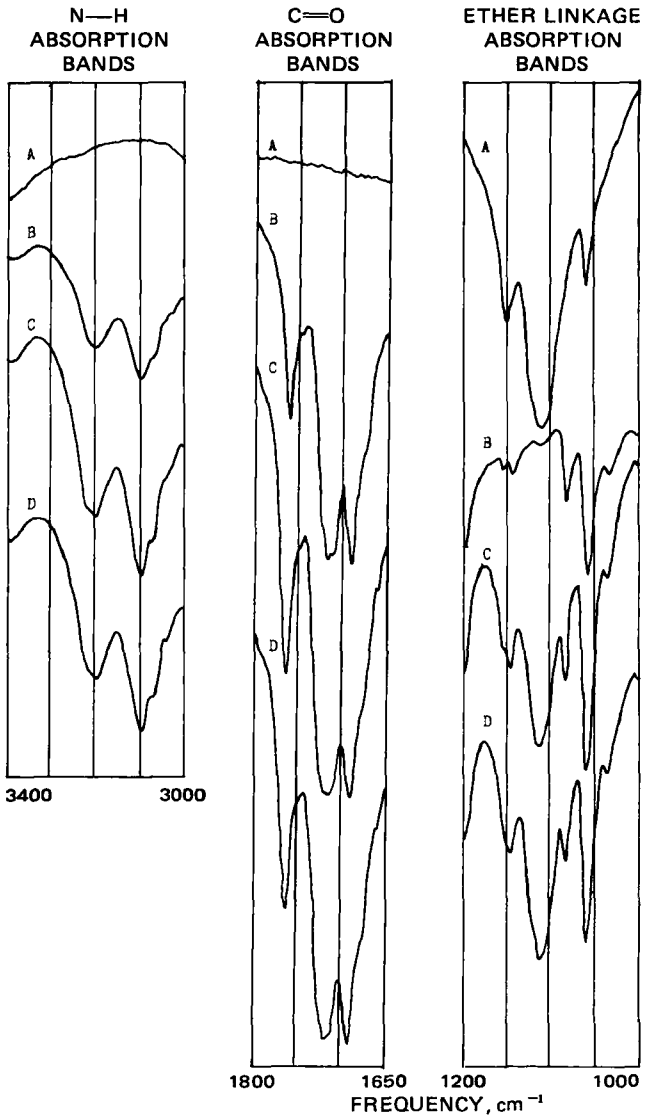
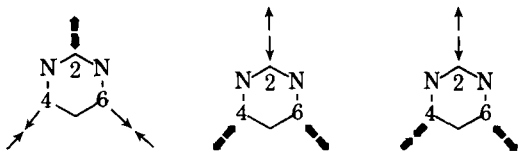


Figure 14—*IR spectra of mephobarbital–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, mephobarbital; C, physical mixture of mephobarbital and polyethylene glycol 4000; and D, equilibrated mephobarbital–polyethylene glycol 4000.*

ing the negative charge on the oxygen and the positive charge on the nitrogen. Thus, those canonical forms stabilized by hydrogen bonding in III (Forms 3 and 4) would contribute most to the stabilization energy of barbital molecules hydrogen bonding as in III, while forms stabilized by hydrogen bonding in IV (Forms 1, 2, 5, and 6) contribute most to molecules having bonding sites arranged as in IV. As a result, the N—H stretching of barbital molecules hydrogen bonding as in IV is at a higher frequency due to the more positive character of the nitrogens in these molecules. Based on this rationale and the fact that the N—H absorption of the equilibrated sample is at a lower frequency than barbital, barbital crystallized from pyridine has hydrogen bonding sites as in III, and barbital crystallized from pyridine with polyethylene glycol 4000 hydrogen bonds as in IV.

The N—H stretching absorption bands of amobarbital, vinbarbital, talbutal, allobarbital, mephobarbital, hexobarbital, and primidone (Figs. 10–16) show no evidence of pattern change when equilibrated with polyethylene glycol 4000, indicating that no interaction occurs.

In the carbonyl stretching absorption area of Figs. 1 and 2, the IR spectrum of phenobarbital (curve B) shows three absorption bands: 1780–1770, 1720–1710, and 1680–1670 cm^{-1}. According to Price *et al.* (20), the three carbonyl bonds act as coupled oscillators, giving rise to the following vibration modes:



Vibration 1 (associated mainly with 2-carbonyl vibration) was assigned as the low frequency band because the 2-carbonyl has two neighboring nitrogens to participate in tautomerism and thus the most single bond (C—O^-) character.

Vibration 2 (associated mainly with 4- and 6-carbonyl symmetric vibration) has a higher frequency since the associated carbonyls have only one nitrogen that participates in tautomeric resonance.

Vibration 3 (associated with 4- and 6-carbonyl asymmetric stretching) has a higher frequency than Vibration 2 because the combined force of carbonyl unsymmetric stretching is perpendicular to the 2-carbonyl stretching, and the stretching force of the 2-carbonyl has little influence on it. In contrast, 2-carbonyl stretch-

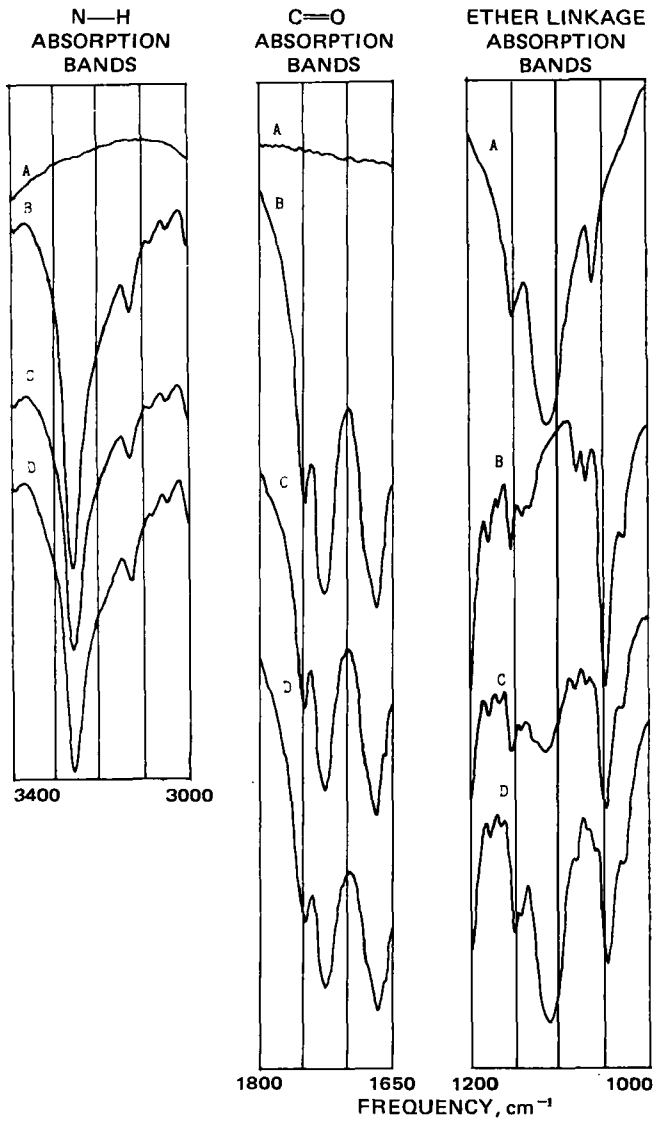


Figure 15—*IR spectra of hexobarbital–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, hexobarbital; C, physical mixture of hexobarbital and polyethylene glycol 4000; and D, equilibrated hexobarbital–polyethylene glycol 4000.*

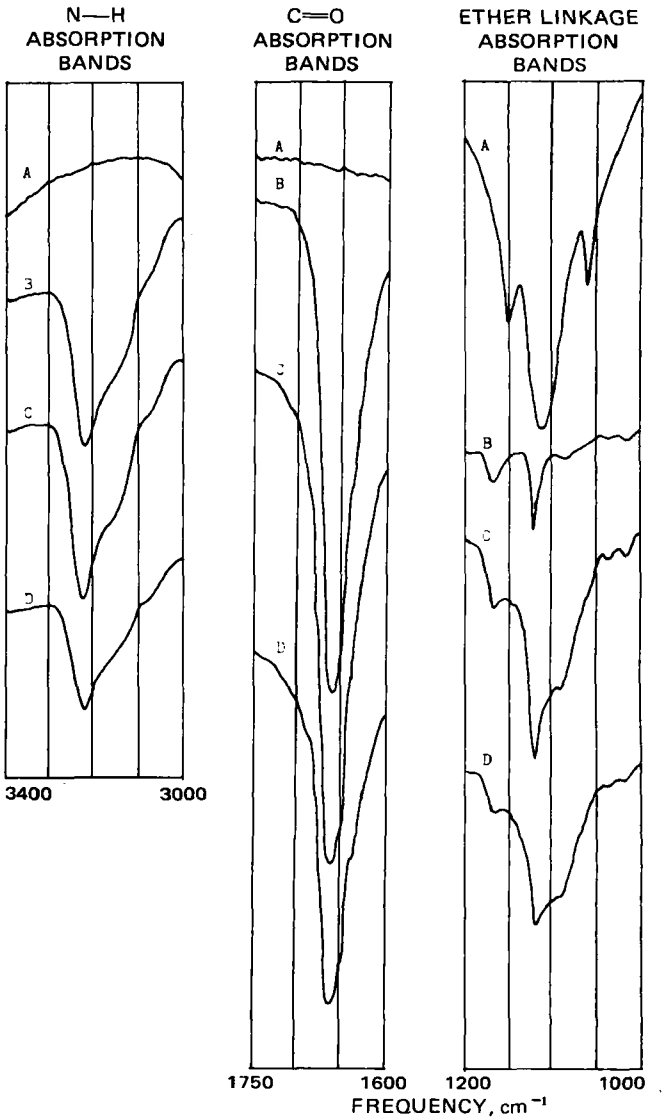


Figure 16—*IR spectra of primidone–polyethylene glycol 4000 from pyridine. Key: A, polyethylene glycol 4000; B, primidone; C, physical mixture of primidone and polyethylene glycol 4000; and D, equilibrated primidone–polyethylene glycol 4000.*

ing in Vibration 2 opposes the combined force of 4- and 6-carbonyl stretching. However, Goenechea (25) assigned the high frequency band to the 2-carbonyl vibration (Vibration 1) because the high frequency band disappears in the 2-thio analog.

Mesley (16) contrasted the frequency of carbonyl absorption bands of barbiturates as determined in ether and in the solid state and showed average changes of 25, 22, and 8 cm^{-1} for the low, middle, and high frequency bands, respectively. Since the 2-carbonyl is free both in the solid state and in ether, the frequency change should be small. The 4- and 6-carbonyl groups, hydrogen bonded in the solid state but free in ether solution, should show much greater frequency changes, leading to an assignment in agreement with that of Goenechea (25).

Although assignment of the high frequency band to the 2-carbonyl is strongly supported by Goenechea's data and in conflict with the assignment of Price *et al.* (20), the coupling oscillator theory stated by the latter is still a powerful method for analyzing force factors of barbiturate vibrations resulting in carbonyl stretching absorption bands. If the assignment of the high frequency band to 2-carbonyl stretching (Vibration 1, or 2-, 4-, and 6-carbonyl unsymmetric in-phase stretching) is accepted, then the middle frequency band must be assigned to 4- and 6-carbonyl out-of-phase stretching (Vibration 3) and the low frequency band must be assigned to 4- and 6-carbonyl symmetric in-phase stretching (Vibration 2), since the latter has the lowest frequency according to this interpretation (20).

The absorption frequencies of carbonyl bands of the barbiturates and their complexes are summarized in Table II. These data indicate that the 2-carbonyl stretching band is shifted to a lower frequency while the 4- and 6-carbonyl out-of-phase stretching and symmetric in-phase stretching are shifted to a higher frequency upon complexation. The 2-carbonyl band of phenobarbital undergoes a large shift upon complexation, indicating a large change in the electronic environment of the 2-carbonyl upon complexation. The direction of the shift indicates that the 2-carbonyl is free in the phenobarbital crystal (I) but hydrogen bonded in the complex (IV).

The shift of 4- and 6-carbonyl out-of-phase stretching and symmetric in-phase stretching to a higher frequency is explicable if the 4- or 6-carbonyl is hydrogen bonded in the barbiturate crystal (III) but free in the complex (IV). Electron density on the free carbonyl is greater than on the hydrogen-bonded carbonyl, giving more double bond character to the carbon–oxygen bond. Thus, both 4- and 6-carbonyl in-phase and out-of-phase stretching are shifted to higher frequencies.

Figures 3–8 have the same pattern of carbonyl absorption bands

Table II—Carbonyl Absorption Bands in Spectra of Barbiturates and Their Complexes

Compound	2-Carbonyl Stretching Out-of-Phase, cm^{-1}		2-, 4-, and 6-Carbonyl Symmetric Stretching In-Phase, cm^{-1}		2-, 4-, and 6-Carbonyl Unsymmetric Stretching In-Phase, cm^{-1}	
Phenobarbital	1780			1710	1680–1670	
Phenobarbital complex		1765	1748		1712	
Pentobarbital	1770		1730–1720			1680
Pentobarbital complex		1760	1730		1700	
Probarbital	1755			1730		1700
Probarbital complex		1752	1740		1705	
Butabarbital	1755			1730		1697
Butabarbital complex		1750	1740		1705	
Cyclobarbital	1762			1742		1700
Cyclobarbital complex		1758	1748		1712	
Butethal	1758			1724		1695
Butethal complex		1752	1730		1700	
Cyclopentenyl allylbarbituric acid	1755			1720		1680
Cyclopentenyl allylbarbituric acid complex		1752	1720		1700	

when absorption of the equilibrated sample (curve D) is compared to that of the free barbiturate (curve B).

This discussion indicates that the phenobarbital crystal may be represented as I while pentobarbital, probarbital, butethal, and cyclopentenyl allylbarbituric acid have crystal forms such as III. Upon complexation, each barbiturate should be represented as IV.

A comparison of the carbonyl absorption areas in Figs. 10–16 of the barbiturate alone (curve B), the physical mixture (curve C), and the equilibrated sample (curve D) indicates that amobarbital, vinbarbital, allobarbital, talbutal, mephobarbital, and hexobarbital do not interact with polyethylene glycol. Changes observed in the absorption patterns of the barbital system (Fig. 9) may again be rationalized on the basis of a change in hydrogen bond configuration upon crystallization with polyethylene glycol 4000, and complexation need not be implicated.

The ether linkage of polyethylene glycol has a characteristic absorption band at 1110 cm^{-1} (Figs. 1 and 2). Upon complexation, however, a new band is formed at a lower frequency (Figs. 1–8, curves D). The decreased frequency is attributable to the formation of a hydrogen bond upon complexation, in which the oxygen of the ether linkage serves as a hydrogen acceptor.

The aqueous stability of the seven barbiturates found to interact with polyethylene glycol was determined (Figs. 2–8, curves E). The differences between the spectra before (curve D) and after (curve E) water washing are summarized in Table III.

Barbiturates that complex with polyethylene glycol can be classified into three groups on the basis of the water stability of the complexes:

1. Water-stable complexes in which the hydrogen bond between the constituents of the complex is stronger than that between water and the constituents of the complex. Phenobarbital, butabarbital, and cyclobarbital give such complexes.
2. Water-unstable complexes with the barbiturate returning to the original crystal state. Probarbital, butethal, and cyclopentenyl allylbarbituric acid give such complexes, as evidenced by the similarity between curves E and A.
3. Water-unstable complexes with the barbiturate having a different crystal state. Pentobarbital gives such a complex, and the similarity between curves D and E indicates that the hydrogen bonding configuration in the complex is carried over into that of the barbiturate (IV).

SUMMARY

Several factors affecting the barbiturate–polyethylene glycol 4000 interaction have been identified. Since only phenobarbital interacts with polyethylene glycol in water while phenobarbital, butabarbital, butethal, cyclobarbital, cyclopentenyl allylbarbituric acid, pentobarbital, and probarbital interact in pyridine, the nature of the solvent must play an important role. Seven disubstituted barbiturates interact while five disubstituted barbiturates do not, so the nature of the C_5 substituents is critical. Trisubstituted barbiturates do not interact, so two hydrogen bonds are required for complexation. Finally, the fact that primidone does not interact indicates that the 2-carbonyl has an important role in complexation.

Evidence from this study suggests that phenobarbital crystallized from pyridine has hydrogen bonding sites arranged as in I, whereas other barbiturates that complex have bonding sites as in III. All barbiturates, upon complexation, are represented by IV.

Of the seven barbiturate complexes studied, probarbital, butethal, cyclopentenyl allylbarbituric acid, and pentobarbital complexes were unstable in water at 26°. Pentobarbital retains the IV arrangement, but the other three barbiturates revert to III after water washing.

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Table III—Variation of Absorption Bands of Barbiturate Complexes after Water Washing

Equilibrated Sample	N—H Absorption	C=O Absorption	Ether Linkage Absorption
Phenobarbital–polyethylene glycol 4000	No change	No change	No change
Butabarbital–polyethylene glycol 4000	No change	No change	No change
Cyclobarbital–polyethylene glycol 4000	No change	No change	No change
Pentobarbital–polyethylene glycol 4000	No change	No change	Loss of ether absorption band
Probarbital–polyethylene glycol 4000	Bands same as probarbital alone	Bands same as probarbital alone	Loss of ether absorption band
Butethal–polyethylene glycol 4000	Bands same as butethal alone	Bands same as butethal alone	Loss of ether absorption band
Cyclopentenyl allylbarbituric acid–polyethylene glycol 4000	Bands same as cyclopentenyl allylbarbituric acid alone	Bands same as cyclopentenyl allylbarbituric acid alone	Loss of ether absorption band

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Binding of Metronidazole and Its Derivatives to Plasma Proteins: An Assessment of Drug Binding Phenomenon

DILIP R. SANVORDEKER [x], YIE W. CHIEN, TENG K. LIN, and HOWARD J. LAMBERT

Abstract □ Metronidazole and four derivatives were studied *in vitro* to investigate the differences in the extent of their binding to plasma proteins. Modification at the terminal portion of the alkyl side chain resulted in wide differences in the extent of binding. Molecular orbital calculations were performed by the CNDO and MINDO/2 methods to estimate the frontier electron density on the hetero atom at the 3′-position of the alkyl side chain. A linear correlation between the protein binding parameter ($\log_e P$) and the frontier electron density (q_r) was observed for the binding of this group of trichomonicidal drugs. NMR spectroscopy was used to demonstrate that the alkyl side chain participated in the binding of these compounds to plasma proteins.

Keyphrases □ Metronidazole and four derivatives—plasma protein binding, correlation with frontier electron density □ Plasma protein binding—metronidazole and four derivatives, correlation with frontier electron density □ Electron density, frontier—metronidazole and four derivatives, correlation with plasma protein binding parameters

Several reports provided information on the theoretical, biological, and clinical aspects of drug–protein binding phenomena (1–6). The majority of these investigations was concerned with the drug–serum albumin interaction, since it is the predominant phenomenon occurring in humans. Quantitative correlation has been found between physicochemical parameters such as lipophilicity, pKa, and electronic charge density and the strength of drug binding to serum albumin (7–10).

It is well recognized that the therapeutic activity of a drug is primarily dependent upon the availability of its free (unbound) permeable species at the effective receptor sites located either in the vascular or extravascular compartments of the body (1, 11–14). Recent investigations demonstrated that any structural modification in a functional group can influence significantly both the activity and the extent of protein binding of disopyramide phosphate and its derivatives (14–16).

Metronidazole[1] has been shown to be an effective antiprotozoal agent with a broad spectrum of activity against anaerobes (17–21). In this study, it was observed that the extent of plasma protein binding of

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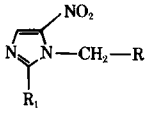
Table I—Structural Formulas and Other Parameters for Metronidazole and Its Derivatives



Compound	R	R_1	Partition[a] Coefficient
I	$—CH_2OH$	CH_3	0.778 ± 0.029
II	$—CH_2SSO_3Na$	CH_3	0.027 ± 0.01
III	$—CH_2NHCOCH_3$	CH_3	0.339 ± 0.008
IV	—COOH	H	0.030 ± 0.001
V	$—CH_2OCOCH_3$	CH_3	2.00 ± 0.07

[a] Determined in the system of *n*-octanol–pH 7.4 phosphate buffer.

Table II—Binding of Metronidazole and Its Derivatives to Human Plasma Proteins

Total Drug Concentration, $M \times 10^5$	Fraction of Drug Bound, %				
	Metronidazole Derivatives				
	I	II	III	IV	V
40.0	0.43	—	—	4.45	—
24.0	1.06	—	—	2.72	—
8.0	—	—	—	—	1.51
6.4	3.07	66.37	11.0	—	2.67
4.8	4.22	67.12	11.36	4.45	2.36
3.2	3.74	68.33	10.85	1.86	1.36
1.6	—	71.18	—	5.93	2.86

metronidazole and its derivatives is significantly dependent upon the nature of their alkyl side chains. Molecular conformation analysis, coupled with frontier electron density (q_r) calculations by the CNDO[2] and MINDO/2[3] methods, was performed to support and rationalize these observations. NMR spectroscopy was also used to confirm the participation of the alkyl side chain in the binding process. This article reports the results and interpretations of these investigations.

EXPERIMENTAL

Materials—The following were used: metronidazole[4] (I), 1β-thiosulfate sodium methyl-2-methyl-5-nitroimidazole[5] (II), 1β-aminoacetylethyl-2-methyl-5-nitroimidazole[6] (III), 1β-carboxymethyl-5-nitroimidazole[7] (IV), and metronidazole acetate (V). All compounds were tested for structure identity and purity prior to use.

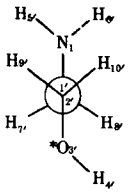
Plasma Protein Binding Study—The procedure reported previously (16) was utilized.

CNDO and MINDO/2 Study—These studies were conducted on the basis of established procedures (22–24). All calculations were performed with a high-speed memory computer[8].

NMR Study—Lyophilized normal human serum[9], 40% NaOD in D_2O[10], and 38% DCl in D_2O[11] were used to prepare appropriate stock solutions in 99.7% pure D_2O[11]. An appropriate amount of the drug was accurately weighed and transferred to a 10-ml volumetric flask, followed by the addition of 3–6 ml of D_2O to dissolve the drug. The pD of the solution was adjusted to a near neutral region (6–8) with the addition of a few drops of dilute NaOD or DCl solution.

Aliquots of a freshly prepared stock solution of human serum (containing 4.6% serum albumin) in D_2O were then added to the solution and the pD was readjusted to 7. The final volume of the solution was made up with D_2O to obtain a 0.0467 *M* solution of the drug without albumin and with human serum containing 1.15 and 2.5% albumin. All NMR spectra were obtained on a high-resolution (60 MHz) NMR spectrometer[12]. A 1% tetramethylsilane[11] solution in $CDCl_3$ was used as an external standard to calibrate the instrument for all measurements.

Table III—Coordinate System and Minimized Energy of $H_2NCH_2CH_2OH$ in Bohr Units[a]



Atom	*x*	*y*	*z*
N_1	0.02975	−0.05078	0
C_1'	2.72334	0.13006	0
C_2'	3.54814	2.88035	0
$*O_3'$	6.02929	3.22673	0
H_4'	7.98232	3.94783	0
H_5'	−1.26634	1.61069	0
H_6'	−0.95916	−1.90575	0
H_7'	3.47664	−1.09985	1.80593
H_8'	3.47664	−1.09985	−1.80593
H_9'	2.42889	3.94561	1.78834
H_{10}'	2.42889	3.94561	−1.78834

[a] Total energy = −32.64253 a.u. (MINDO/2). Frontier electron density at 3′-position = 0.1255 (CNDO).

RESULTS AND DISCUSSION

Table I illustrates the structural formulas and the effect of alkyl side-chain modification on the lipophilic character of metronidazole and its derivatives. In these five nitroimidazole derivatives, the functional group on the *N*-1 position of the imidazole nucleus was varied chemically to assess its effects on the extent of their

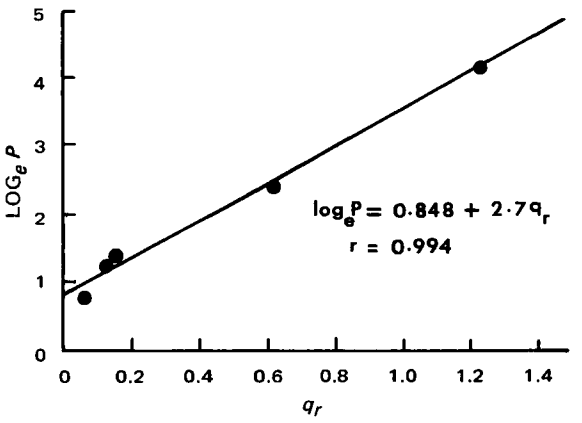


Figure 1—*Linear relationship between the protein binding parameter ($\log_e$ P) and frontier electron density (q_r) for I and its derivatives.*

[2] QCPE-141 computer program by P. Dobosh, Carnegie-Mellon University, Pittsburgh, Pa.

[3] QCPE-217 computer program by A. Komornicki and J. McIver, Department of Chemistry, State University of New York, Buffalo, N.Y.

[4] SC-10295; marketed as Flagyl by G. D. Searle.

[5] SC-21253.

[6] SC-24231.

[7] SC-28905.

[8] A 600/6000 series, Honeywell Corp., Minneapolis, Minn.

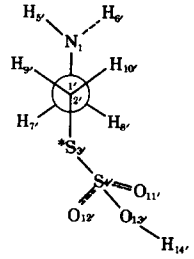
[9] Metrix, Lot 5466, Armour Pharmaceutical Co., Chicago, Ill.

[10] Aldrich Chemical Co., Milwaukee, Wis.

[11] Merck & Co., Rahway, N.J.

[12] Model A60-D, Varian Associates, Palo Alto, Calif.

Table IV—Coordinate System and Minimized Energy of $H_2NCH_2CH_2S_2O_3H$ in Bohr Units[a]



Atom	x	y	z
N_1	0	0	0
C_1'	2.64567	0	0
C_2'	3.61712	2.74331	0
$*S_3'$	6.45177	2.74331	0
S_4'	6.51443	6.33331	0
H_5'	−1.00157	1.73478	0
H_6'	−1.00157	−1.73478	0
H_7'	3.33956	−0.97975	1.69698
H_8'	3.33956	−0.97975	−1.69698
H_9'	2.92519	3.72445	1.69698
H_{10}'	2.92519	3.72445	−1.69698
O_{11}'	5.12476	7.35440	−2.43748
O_{12}'	5.12476	7.35440	2.43748
O_{13}'	9.38180	7.29272	0
H_{14}'	9.43127	9.18183	0

[a] Total energy = −108.48450 a.u. (CNDO). Frontier electron density at the 3'(*)-position = 1.2487 (CNDO).

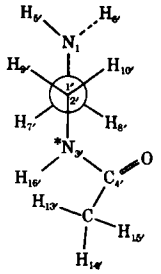
binding to plasma proteins (Table II). The concentration range studied for each drug was based on realistic values equivalent to or above the minimum inhibitory concentration (MIC) required against *Trichomonas vaginalis*.

The extent of plasma protein binding (Table II) and the lipophilic character (Table I) of this group of compounds do not show any linear correlation. This finding is demonstrated by the fact that metronidazole acetate (V), despite its high lipophilicity, shows poor protein binding (Tables I and II), whereas II, with poor lipophilic character, exhibits extensive binding to plasma proteins. Thus, a substitution of the hydroxy group with a thiosulfonyl group on II resulted in an approximately 16-fold increase in the binding of II over metronidazole. On the other hand, substitution with an acetamido group in III resulted in a slight increase in the extent of binding of that compound, despite a 10-fold increase in its lipophilicity over that of II.

CNDO and MINDO/2 calculations were performed to investigate conformational similarity and to assess whether the frontier electron density (q_r) on the hetero-atom at the terminal portion of the alkyl side chain contributed to these observed differences in the extent of protein binding. It is reasonable to assume that the contributions of the nitroimidazole moiety common to all these compounds is approximately the same with reference to the plasma protein binding phenomenon. This assumption implies that the observed differences in the protein binding of these compounds are due only to the variation of the functional group on the *N*-1 position of the imidazole ring. Under this condition, electron densities on the terminal molecular groups, namely H_2N—CH_2CH_2OH, H_2N—$CH_2CH_2SSO_3H$, H_2N—$CH_2CH_2NHCOCH_3$, H_2N—CH_2COOH, and H_2N—$CH_2CH_2OCOCH_3$, were computed on the basis that these parts of the molecule form localized molecular orbitals. Consequently, certain physicochemical properties of these functional groups can be considered nearly independent of their associated nitroimidazole nucleus.

The computations were performed in the following manner. At first, molecular conformation was roughly optimized by the CNDO (Complete Neglect of Differential Overlap) method (22). Second, the molecular energy was extensively minimized by the MINDO/2 (Modified Intermediate Neglect of Differential Overlap) method (23). These two methods belong to the classical semiempirical

Table V—Coordinate System and Minimized Energy of $H_2NCH_2NHCOCH_3$ in Bohr Units[a]



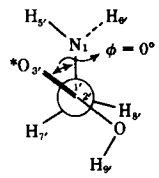
Atom	x	y	z
N_1	0.072210	0.01824	0.00000
C_1'	2.77920	0.059677	0.00000
C_2'	3.80939	2.718158	0.00000
$*N_3'$	6.55198	2.86855	−0.00000
C_4'	7.86141	5.17065	0.00000
H_5'	−1.07487	1.79479	0.00000
H_6'	−1.03062	−1.78361	−0.00000
H_7'	3.53119	−1.11542	−1.85245
H_8'	3.53119	−1.115421	1.85245
H_9'	2.98725	3.82200	−1.85647
H_{10}'	2.98726	3.82200	−1.856471
O_{11}'	6.71896	7.14799	−0.00000
C_{12}'	10.65700	5.12682	−0.00000
H_{13}'	11.62171	7.21036	0.00000
H_{14}'	11.58767	4.08591	−1.81875
H_{15}'	11.58767	4.08591	1.81875
H_{16}'	7.5661	0.99440	0.00000

[a] Total energy = −51.36125 a.u. (MINDO/2). Frontier electron density at the 3'(*)-position = −0.6374 (CNDO).

SCF-MO (Self-Consistent Field Molecular Orbital) method. Although both methods are not quantitatively as accurate as *ab initio* SCF-MO calculations (24), they reflect a quantitative estimation of physicochemical properties, such as frontier electron density of relatively large molecules.

Whereas the MINDO/2 method provides satisfactory results in terms of molecular conformation, the CNDO method provides computation of the frontier electron density (q_r) because its differ-

Table VI—Coordinate System and Minimized Energy of H_2NCH_2COOH in Bohr Units[a]



Atom	x	y	z
N_1	−0.04768	−0.08112	0
C_1'	2.62060	0.30440	0
C_2'	3.35046	3.05365	0
$*O_3'$	1.68097	4.66750	0
O_4'	5.77752	3.51890	0
H_5'	7.60299	4.54614	0
H_6'	−1.48876	1.45411	0
H_7'	−0.89224	−2.00850	0
H_8'	3.49451	−0.87853	1.78392
H_9'	3.49451	−0.87853	−1.78392

[a] Total energy = −42.98751 a.u. (MINDO/2). Frontier electron density at the 3'(*)-position = 0.1554 (CNDO).

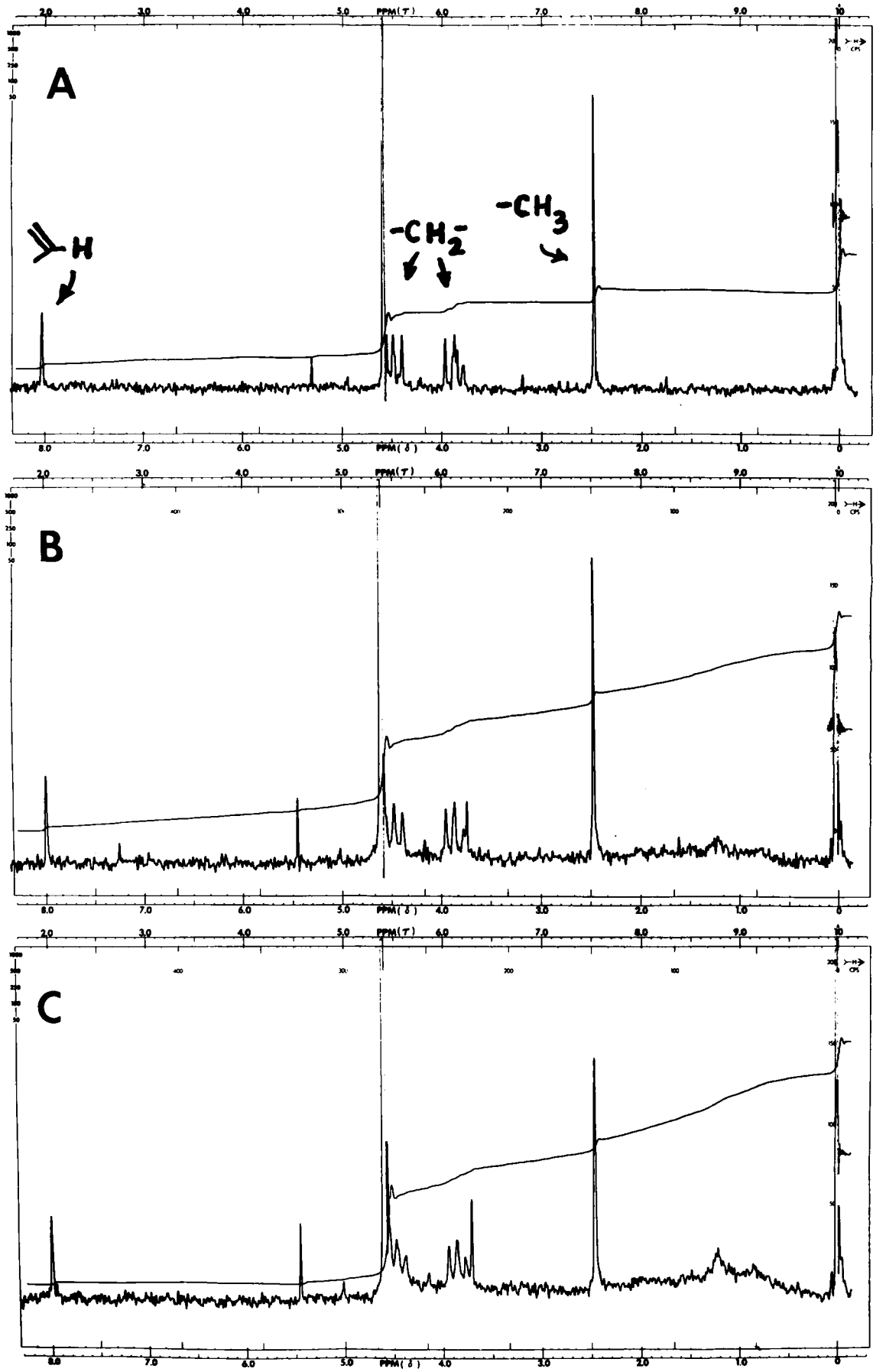


Figure 2—*NMR spectra illustrating the effect of human serum albumin on the alkyl protons of I at pD 7. Key: A, no protein; B, with 1.15% human serum albumin; and C, with 2.3% human serum albumin.*

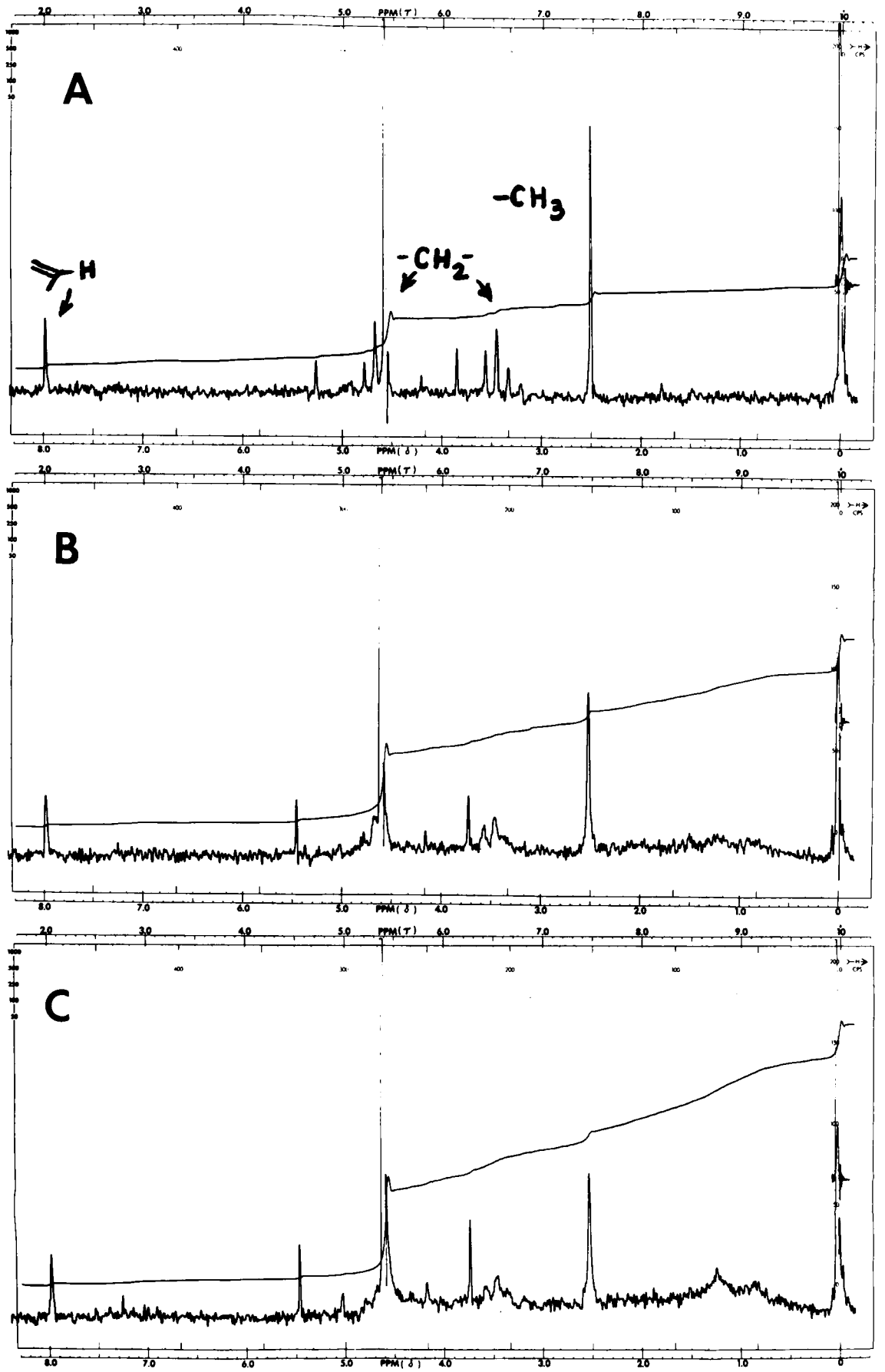


Figure 3—*NMR spectra illustrating the effect of human serum albumin on the alkyl protons of II at pD 7. Key: A, no protein; B, with 1.15% human serum albumin; and C, with 2.3% human serum albumin.*

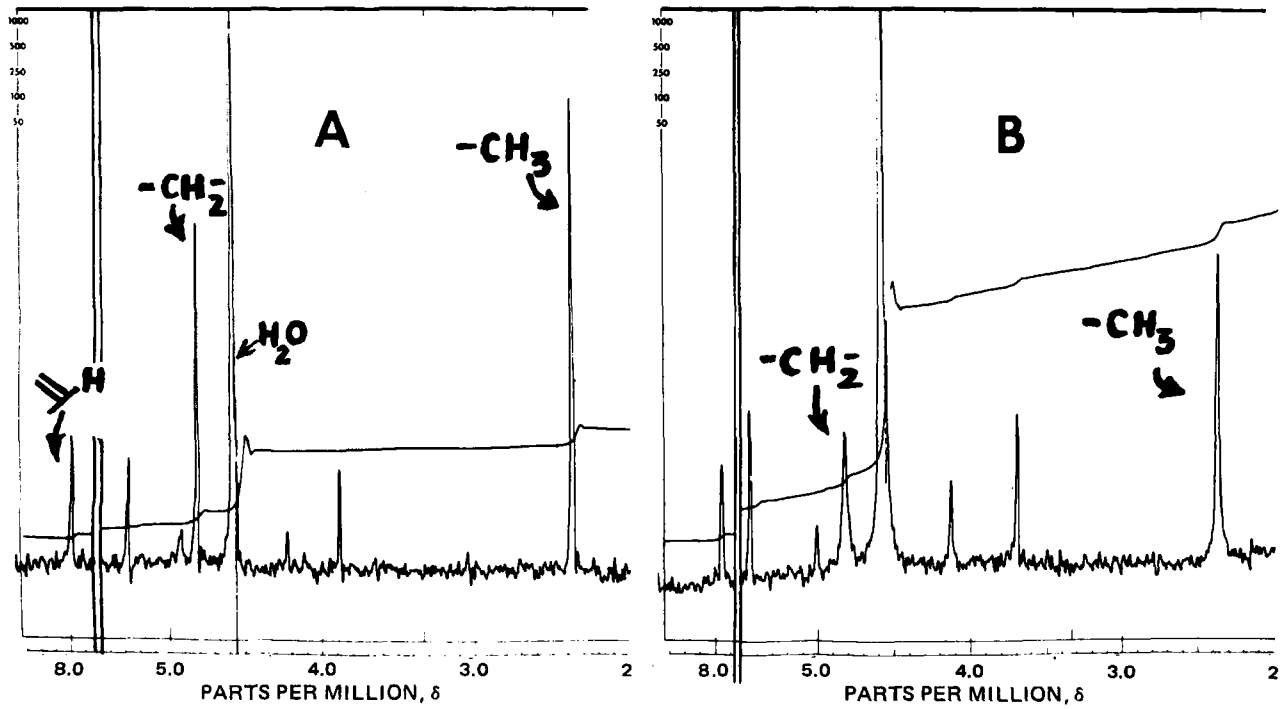
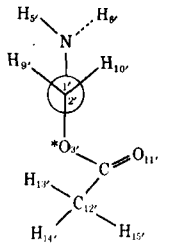


Figure 4—*NMR spectra illustrating the effect of human serum albumin on the alkyl protons of IV at pD 7. Key: A, no protein; and B, with 2.3% human serum albumin.*

ential overlaps are completely neglected. The frontier electron density (q_r) can thus be assembled from the parts of the HOMO (Highest Occupied Molecular Orbital) wave function, which can be expressed as follows:

$$q_r = \sum_i 2C_i^2(\eta) \qquad \text{(Eq. 1)}$$

Table VII—Coordinate System and Minimized Energy of $H_2NCH_2CH_2OCOCH_3$ in Bohr Units[a]



Atom	x	y	z
N_1	0.20424	0.00424	0.00000
$C_{1'}$	2.91006	−0.08213	0.00001
$C_{2'}$	3.69591	2.67055	−0.00000
$*O_{3'}$	6.13957	3.44112	−0.00002
$C_{4'}$	7.32873	5.64244	−0.00000
$H_{5'}$	−1.00271	1.73960	0.00000
$H_{6'}$	−0.97005	−1.73433	0.00000
$H_{7'}$	3.49477	−1.38893	−1.80857
$H_{8'}$	3.49478	−1.38893	1.80857
$H_{9'}$	2.49923	3.55327	−1.83180
$H_{10'}$	2.49927	3.55326	1.83182
$O_{11'}$	6.28668	7.71015	−0.00000
$C_{12'}$	10.15115	5.69633	0.00000
$H_{13'}$	10.93365	7.85597	0.00000
$H_{14'}$	11.27841	4.84339	−1.80702
$H_{15'}$	11.27840	4.84340	1.80703

[a] Total energy = −1.24652 a.u. (MINDO/2). Frontier electron density at the 3′ position = 0.06552 (CNDO).

where C_i (η)'s are HOMO eigenvectors of the CNDO at the ηth position, and Σ_i's constitute the sum of s, p_x, p_y, and p_z atomic orbitals of the atom at the ηth position on the functional group of each compound.

Tables III–VII illustrate the optimized coordinates system and minimized energy computed on the basis of these methods. The possibility of intramolecular interaction, such as hydrogen bonding, was also examined by the CNDO method. Except for IV, which showed a gain of 1.272 kcal/mole (possibly through hydrogen bonding), none of these compounds exhibited any intramolecular interaction.

It is recognized that the binding of drugs to plasma proteins, such as serum albumin, proceeds with the interaction at the primary binding site(s) on the protein, followed by a saturation phase involving any secondary and ternary binding sites available. If it is assumed that the drug–serum albumin interaction occurs only at the primary binding sites at a low drug concentration range, then the plasma protein binding index [$\log_e P_{(mean)}$] (defined as the natural logarithm of the mean percent fraction of the total drug in the bound state) becomes important in determining the availability of the free drug to diffuse into the extravascular compartment. This $\log_e P$ value for each compound at and below 6.4×10^{-5} M concentration was observed to be virtually constant (Table II).

In performing molecular orbital calculations, it was observed that the frontier electron density (q_r) at the 3′-position was the highest for all compounds in this series. Table VIII lists the experi-

Table VIII—Relationship between the Protein Binding Parameter ($\log_e P$) and the Frontier Electron Density (q_r) at the 3′-Position on the Alkyl Side Chain of Metronidazole and Its Derivatives

Compound	Fraction of Drug Bound, %, Mean ± SD	Frontier Electron Density, q_r	Protein Binding Parameter, $\log_e P$	
			Experimental	Calculated
I	3.68 ± 0.58	0.1255	1.30	1.18
II	68.25 ± 0.21	1.2487	4.22	4.18
III	11.07 ± 0.26	0.6374	2.40	2.52
IV	4.08 ± 2.06	0.1554	1.41	1.26
V	2.31 ± 0.21	0.0655	0.84	1.02

mental and calculated $\log_e P_{(mean)}$ values and the frontier electron density at the 3′(*)-position of the N_1-alkyl side chain of metronidazole and its derivatives. The calculated values were determined from Eq. 2. A linear correlation between the $\log_e P_{(mean)}$ and the frontier electron density at the 3′-position (calculated by the CNDO and MINDO/2 methods) was observed for this group of compounds. Such a relationship, as illustrated in Fig. 1, is defined by:

$$\log_e P_{(mean)} = 0.848 + 2.668q_r \qquad \text{(Eq. 2)}$$

$$n = 5 \qquad r = 0.994 \qquad s = 0.131$$

where n is the number of compounds, r is the correlation coefficient, and s is the standard deviation.

A correlation coefficient of 0.994 for the regression line reflects excellent fit for the protein binding data with the computed frontier electron density at the 3′-position of the N_1-alkyl side chain. These results suggest that the plasma protein binding of metronidazole and its derivatives is nonspecific in nature and is dependent upon the frontier electron density at the 3′-position. That is, the drug–protein (mostly serum albumin) interaction occurs through a combination of van der Waals and electrostatic forces.

Evidence that the alkyl side chain and the imidazole moiety do participate in the drug binding process was obtained using NMR spectroscopy. This technique has been used to identify the functional groups on a drug molecule that participate in the drug–protein binding process (25–27). Figures 2–4 illustrate the NMR spectra obtained on solutions of metronidazole and its derivatives with and without human serum albumin at two different concentrations. Due to solubility limitations, these studies could not be performed with III and V (metronidazole acetate).

Whereas the methylene (—CH_2—) and methyl (—CH_3) protons of the metronidazole showed very little change in the presence of serum albumin (Fig. 2), the same protons on II (Fig. 3) showed extensive relaxation proportional to the serum albumin concentration. The magnitude of the relaxation effect observed for II as compared to that observed for metronidazole (I) is consistent with the quantitative results on the extent of binding of these two compounds (Table II). The specific relaxation of the methyl protons at the C-2 position and the methylene protons of the alkyl side chain is indicative of the participation of these functional moieties in the overall binding process.

Similar proton relaxation effect for the alkyl and methyl protons of IV in the presence of plasma proteins is illustrated in Fig. 4. The magnitude of methylene proton relaxation observed for this compound is relatively small compared to that observed for II. Such an observation is consistent with the protein binding data (Table II) and strongly suggests a stabilization effect due to the alkyl side chain and the imidazole moiety.

In conclusion, this study demonstrated that modifications of a functional group on the basic metronidazole molecule caused significant differences in its protein binding characteristics. The degree of binding of this group of nitroimidazoles was shown to be primarily dependent upon the frontier electron density on the terminal portion of the alkyl side chain. The greater the frontier electron density, the greater was the degree of drug binding to plasma proteins. On the basis of these observations, it appears that the frontier electron density parameter with reference to a defined position on a drug molecule can provide useful information concerning the drug–protein interaction of a group of drugs with structural similarities.

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Utilization of Model Compounds to Evaluate Effects of Slight Chemical Modifications on Their Distribution Pharmacokinetic Parameters in Rats and Mechanisms Inferred for Their Transmembrane Transport

YASHWANT M. AMIN * and JANARDAN B. NAGWEKAR ˣ

Abstract □ The pharmacokinetics of each of the model compounds benzoylformic acid (I), *p*-methylbenzoylformic acid (II), *p*-ethylbenzoylformic acid (III), D-(−)-mandelic acid (IV), D-(−)-*p*-methylmandelic acid (V), D-(−)-*p*-ethylmandelic acid (VI), and D-(−)-*p*-isopropylmandelic acid (VII) were studied in rats to determine the influence of slight chemical modifications of the compounds on their distribution pharmacokinetic parameters in rats. The effects of the specific chemical modifications considered were those of the >CHOH group of IV against the >C=O group of I, the *para*-alkylation of I and IV, and the branched alkyl group (isopropyl) against the straight chain alkyl groups of the homologs of IV. While the disappearance of I from the blood was describable by the three-compartment open model, that of IV was describable by the two-compartment open model. The apparent volume of distribution of the central compartment (V_1) for IV was smaller than that for I, but the volume of the peripheral compartment (V_2) for IV was greater than that ($V_2 + V_3$) for I. The disappearance of V, VI, and VII from the blood was also describable by the two-compartment open model, but the apparent V_1 and V_2 for these compounds were lower than those for the parent compound, IV. However, the disappearance of II and III from the blood was describable by a one-compartment open model. Evaluation of the appropriate distribution pharmacokinetic parameters suggested that the peripheral compartment for the anions of these compounds consisted of moderately perfused tissues and that the transmembrane transport of these organic anions between the central and peripheral compartments occurs by diffusion mainly through the aqueous membrane pores, which are lined with polar portions of the membrane proteins and/or phospholipids. The possible increased hydrophobic bonding between the alkyl groups of these compounds and the hydrophobic groups of the proteins and/or phospholipids of the membrane pores is implicated to decrease the distribution of the *para*-alkylated homologs into the peripheral compartments and, consequently, diminish the volumes of their peripheral compartments. The heteroporosity of the membranes of the tissues of the central compartment is proposed as the reason for the diminished volume of the central compartment for V and VI as compared to that of IV or VII.

Keyphrases □ Pharmacokinetics—distribution parameters, use of model compounds to evaluate effects of chemical modifications, mechanisms of transmembrane transport, rats □ Distribution—model compounds, effects of chemical modifications, pharmacokinetic parameters, transport mechanisms, rats □ Transport—model compounds, effects of chemical modifications, distribution pharmacokinetic parameters, rats

It is well recognized that the extent of pharmacological response of a drug depends not only on the drug–receptor interaction but also on the concentration of the drug at the site of action containing the receptor(s). Therefore, it is common practice to synthesize and use the analogs or homologs of drugs to modify their biological activities. Although theories have been suggested concerning the drug structure–activity relationship on the basis of biological effects, such theories are not complete without considering the influence on the pharmacological actions of the rates of distribution of drugs between plasma and tissues where the site(s) of action may reside. However, the rates of distribution of drugs at the site(s) of action are governed by several processes, namely, binding of drugs to plasma proteins, metabolism of drugs, renal and nonrenal excretion, and renal tubular secretion of drugs, all of which occur simultaneously.

In recent years, studies have been performed to compare the effects of substituent groups of analogs or homologs of drugs on their pharmacokinetic parameters to determine the effect of specific substituent groups on the specific pharmacokinetic parameters of the compounds. However, such efforts do not appear to be too successful, mainly because the determination of the pharmacokinetic parameters is complicated due to the differences of the homologs or analogs in their extent of binding to plasma (1–6), pKa values, rates of metabolism (7), and partition coefficients between aqueous and lipid phases within the body (4, 7).

According to current concepts, biological membranes are composed mainly of proteins and phospholipids; the proportions of these building blocks are not identical for the membranes of all organs. Several mechanisms for the transmembrane transport of solutes (mainly inorganic electrolytes and organic nonelectrolytes) have been proposed, including diffusion of permeant molecules by dissolving in the membrane; diffusion of permeant molecules through the aqueous pores, which are lined with polar portions of the proteins and/or phospholipids as carrier(s); and active transport, which may involve enzymes and adenosine triphosphatase (8). Therefore, depending upon the organ of permeation and mechanism of transport of solutes through the membrane of such organ, the differences in the rates of diffusion of the compounds can be expected.

In view of these various mechanisms for transmembrane transport of solutes and their interaction with components of the membrane through various forces (8) (electrostatic interactions, hydrogen bonding, and hydrophobic bonding), it is apparent that the effects of substituent groups of homologs or analogs of drugs on the distribution pharmacokinetic parameters cannot be determined unless the effects of the various processes (protein binding, metabolism, and renal tubular reabsorption) to which the drug is subjected simultaneously in the body are minimal. Consequently, studies with several drugs and their

homologs and/or analogs were unable to determine exclusively the effects of substituents on the distribution pharmacokinetic parameters (1–7).

In recent years, D-(−)-mandelic acid and several of its *para*-alkylated homologs have been shown to possess ideal properties by virtue of which they are neither metabolized nor reabsorbed from the renal tubules of rats and are negligibly bound to plasma proteins (9–12). Since these compounds have low pKa values, they remain completely ionized in the body fluids and serve as model organic anions.

Therefore, the purposes of this project were to study the pharmacokinetics of benzoylformic acid (I) and its *para*-alkylated homologs *p*-methylbenzoylformic acid (II) and *p*-ethylbenzoylformic acid (III) as well as D-(−)-mandelic acid (IV) and its *para*-alkylated homologs D-(−)-*p*-methylmandelic acid (V), D-(−)-*p*-ethylmandelic acid (VI), and D-(−)-*p*-isopropylmandelic acid (VII) from blood level data in rats and to determine the effects of slight chemical variations on their distribution pharmacokinetic parameters. The effects of the specific chemical modifications considered were those of the >CHOH group of IV against the >C=O group of I, the *para*-alkylation of I and IV, and the branched alkyl group (isopropyl) of VII against the straight chain alkyl groups of V, VI, and D-(−)-*p*-*n*-propylmandelic acid (13).

An additional purpose of this project was to utilize the difference observed in the pharmacokinetic parameters of the compounds to reveal the possible mechanisms for their transmembrane transport between central and peripheral compartments of the body at a molecular level by considering the possible electrostatic, hydrogen bonding, and hydrophobic interactions between the permeant molecules and certain components of the membranes.

EXPERIMENTAL

Materials—The following were used: D-(−)-mandelic acid[1], mp 132–133°, $[\alpha]_D^{25}$ −154°; D-(−)-*p*-methylmandelic acid[2], mp 130–131°, $[\alpha]_D^{25}$ −159°; D-(−)-*p*-ethylmandelic acid[2], mp 119–120°, $[\alpha]_D^{25}$ −120°; D-(−)-*p*-isopropylmandelic acid[3], mp 159–160°, $[\alpha]_D^{25}$ −129°; benzoylformic acid[1], mp 62–64°; *p*-methylbenzoylformic acid, mp 97–99°, synthesized by the method of Kindler *et al.* (14); *p*-ethylbenzoylformic acid, mp 65–67°, also synthesized by the method of Kindler *et al.* (14); and DL-tropic acid[1], mp 118–119°.

Apparatus—Compounds I, IV, and their respective *para*-alkylated homologs present in rat blood were analyzed by GLC[4] using a hydrogen-flame detector.

Methodology—Approximately 250 Sprague–Dawley male rats, weighing between 170 and 230 g (a majority weighed ~200 g), were used. Blood levels of the compounds were determined in eight to 10 rats on a given day. Food was withheld from the rats 12–14 hr prior to their use in the study as well as during the study.

Five milliliters of normal saline was injected intraperitoneally into each rat 20 min prior to intravenous administration of the compound(s) in order to be consistent with the procedure followed previously (9–13) in urinary excretion studies so that the pharmacokinetic parameters obtained from the blood level studies can be compared with those obtained from the urinary excretion studies. The rat was anesthetized with ether for less than 1 min for intravenous injection of a fixed dose of the compound contained in 2 ml of isotonic solution. Each injection time was accurately noted.

Only one blood sample was obtained from a given rat upon its decapitation at an appropriate time following administration of the intravenous dose of the compound. The blood samples, which were obtained over 1–100 min for benzoylformic acid and over 1–65 min for the rest of the compounds, were collected in 50-ml beakers previously coated with heparin to prevent coagulation of blood. Each beaker was coated with 0.1 ml (10 units) of heparin. The blood samples were analyzed for the compound on the same day they were collected. The pharmacokinetics of I were studied at the three intravenous dose levels (166.65[5], 249.80, and 333.33 μmoles/kg). The pharmacokinetics of II (152.45 μmoles/kg)[5], III (140.5 μmoles/kg)[5], IV (165.35 μmoles/kg)[5], V (149.6 μmoles/kg)[5], VI (138.25 μmoles/kg)[5], or VII (128.1 μmoles/kg)[5] were studied at a single intravenous dose level. Each compound was injected as its sodium salt.

GLC Analysis—Depending on the anticipated concentration of the compound in a blood sample, an appropriate volume of blood (1–5 ml) was transferred to a 150-ml beaker. The proteins and blood cells were precipitated with a dropwise addition of 5 *N* HCl, with continuous stirring for 10 min with a magnetic stirrer. To extract the compounds from this blood sample into ether, 25 ml of ether was added to the blood sample and stirring was continued for another 15 min. The ethereal layer was then quantitatively transferred to a 60-ml separator, and it was extracted with 15–20 ml of 5 *N* sodium hydroxide solution to recover completely the compound in the alkaline aqueous phase as its sodium salt; the ethereal layer was discarded.

The residue of each precipitated blood sample remaining in the 150-ml beaker after the first ether extraction was further extracted with two additional 20-ml volumes of ether for I and its *para*-alkylated homologs and with three additional 20-ml volumes of ether for IV and its *para*-alkylated homologs. The compound was recovered from these ethereal phases using the alkaline solution employed in the first extraction. The alkaline solution was then acidified with 5 *N* HCl and extracted twice with ether for I and its *para*-alkylated homologs and three times for IV and its *para*-alkylated homologs, using 30 ml of ether each time.

The ethereal layer was collected in a 100-ml beaker and the ether was evaporated by transferring the ethereal layer in portions to a 20-ml graduated tube. The ether was evaporated from the test tube on a water bath maintained at 42°. The residue of the compound thus obtained in the test tube was treated with sufficient amount of ethereal diazomethane to convert the compound to its methyl ester. Excess ether and diazomethane were removed from the tube on a water bath at 42°. A sufficient quantity of absolute methanol was added to the residue to dissolve the compound and to adjust the volume to 1 ml. The methanolic solution thus prepared was ready for GLC analysis.

An appropriate volume (generally 20 μl) of the methanolic solution was injected onto the GLC column. The conditions employed for the GLC analysis and the column used are described in Table I.

To determine accurately the amount of a compound (*i.e.*, I or one of its homologs or IV or one of its homologs) present in the blood samples, a calibration curve for the compound was prepared in the following manner. The solutions of the compound were prepared in the blood obtained from generally three rats, which were not previously treated with any compound. To each 2-ml volume of blood, a known quantity of the compound was added. Then each solution was treated to obtain the methyl ester of the compound according to the procedure described previously. The known quantities of the compound added to 2-ml blood volumes were those obtained from the stock solution prepared on the same day for injecting the rats.

The calibration curve for the compound was also prepared in this manner by using 5 ml of blood, instead of 2 ml, to determine the influence of blood volume on the quantitative recovery of the compound (Fig. 1). The area under each chromatogram was calculated by the trapezoidal rule and plotted against the corresponding amount of the compound (Fig. 1). With reference to such a calibration curve, prepared separately for each study, the amount of the compound present in each blood sample was calculated.

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] Previously synthesized (Ref. 11).
[3] Previously synthesized (Ref. 12).
[4] F&M 810R gas chromatograph.

[5] These doses represent 25 mg/kg.

Table I—Conditions Employed for the GLC Analysis and Retention Time Observed for the Methyl Esters of the Acids

Compound	Column Used	Oven Temperature	Detector and Injection Port Temperature	Helium Flow Rate, ml/min	Retention Time, min
I	3% E.G.S.[a]	150°	200°	40	5.2
II	3% E.G.S.[a]	150°	200°	40	8.6
III	3% E.G.S.[a]	160°	210°	40	6.0
IV	5% E.G.S.[a]	165°	210°	60	5.8
V	15% GE-SE 30[b]	180°	210°	40	5.8
VI	15% GE-SE 30[b]	180°	210°	40	7.2
VII	5% E.G.S.[a]	165°	210°	40	12.2

[a] Ethylene glycol succinate on Diatoport S (80–100 mesh) packed in a 1.2-m (4-ft) long and 0.6-cm (0.25-in.) diameter copper column. [b] GE-SE 30 silicone gum rubber on Diatoport S (80–100 mesh) packed in a 1.2-m (4-ft) long and 0.6-cm (0.25-in.) diameter copper column.

Determination of Dissociation Constants and Apparent Partition Coefficients of Compounds and Their Binding to Whole Rat Blood as well as Plasma Proteins—These determinations for I and its *para*-alkylated homologs were carried out by the methods previously described for IV and its several homologs (9–12).

RESULTS

Selection of Compounds—Selection of the compounds for the present study was based on the following ideal properties:

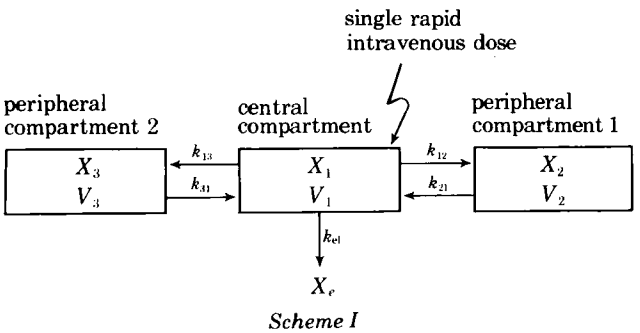
1. From the overall urinary recovery (Table II) of the administered intravenous doses of I–III, it was demonstrated that virtually 100% of the administered dose of each compound was recovered in the unchanged form, indicating negligible metabolism in rats. The fact that the intravenous dose of IV–VII is also completely recovered in the urine in the unchanged form was already reported (9–12).

2. The pKa values determined for I, II, and III are 3.2, 3.3, and 3.3, respectively; those reported for IV, V, VI, and VII are 3.3, 3.4, 3.4, and 3.6, respectively. Therefore, these compounds should remain in the ionized form in the biological fluids.

3. The partition coefficients ($C_{organic}/C_{buffer}$) determined for these compounds, according to the procedure previously described (9), were zero. Therefore, it was assumed that the reabsorption of the compounds in the renal tubules is negligible.

4. From the equilibrium dialysis studies, it was noted that at the equilibrium concentrations of 1.07 mg/4 ml of I, 0.51 mg/4 ml of II, and 1.03 mg/4 ml of III in the blood as well as in plasma, the extent of binding observed was 13.5, 4, and 4%, respectively. If, as generally believed (15), the plasma protein–drug complex is assumed to dissociate rapidly at the secretion site, the effect of 13.5% binding of I to plasma proteins can be expected to exert no pronounced effect on the biological half-life of the compound. Therefore, for practical purposes, this extent of binding of I as well as of II and III was considered negligible. The binding of IV–VII to plasma proteins also is negligible (9–12).

Determination of Pharmacokinetic Parameters—*Pharmacokinetics of I–III*—The pharmacokinetics of I were studied at three intravenous doses to determine if there were any apparent dose-dependent kinetics in the 166.65–333.33-μmole/kg dosage range. The typical blood level data obtained are presented in Fig. 2. Feathering of the semilogarithmic plots of the data obtained at each dose indicated that the decline in blood levels of the compound can best be described to occur triexponentially rather than biexponentially (Fig. 3). Therefore, these data were analyzed according to the three-compartment open model (Scheme I) with elimination occurring from the central compartment. The elimination process consisted of only urinary excretion, as demonstrated from the overall urinary excretion studies.



Scheme I

Table II—Overall Urinary Excretion Recovery Observed following Intravenous Administration of I–III to Rats

Compound	Dose per Rat, mg	Number of Studies	Intact Compound Recovered, mg
I	5	6	4.79–4.92
	10	4	9.63–10.00
II	5	3	4.92–4.96
	10	3	8.76–9.85
III	1	1	1.02
	3	1	2.73
	5	1	4.85
	10	3	8.90–9.85

In Scheme I, X_1, X_2, and X_3 are the amounts of the compound in the central compartment, peripheral compartment 1, and peripheral compartment 2, respectively, at time t; V_1, V_2, and V_3 are the apparent volumes of the central compartment, peripheral compartment 1, and peripheral compartment 2, respectively; X_e is the cumulative amount of the compound excreted in the urine up to time t; k_{el} is the apparent first-order rate constant of elimination of the compound; and k_{12}, k_{21}, k_{13}, and k_{31} are the apparent first-order rate constants for the transfer of the compound from one compartment to the other.

The general solution for the three-compartment open model was described (16) as:

$$C_b = C_1e^{-\alpha t} + C_2e^{-\beta t} + C_3e^{-\gamma t} \qquad \text{(Eq. 1)}$$

where C_b is the concentration of the compound in the blood at time t. The blood data were plotted on semilogarithmic paper (Fig. 2), and preliminary estimates for the intercepts (C_1, C_2, and C_3) and slopes ($-\alpha/2.303$, $-\beta/2.303$, and $-\gamma/2.303$) were obtained by using appropriate equations (16).

By using these initial estimates, the blood level data were fitted to a three-compartment open model according to the NONLIN least-squares program (17). The excellent fit of the data was indicated by the high coefficient of determination and the correlation

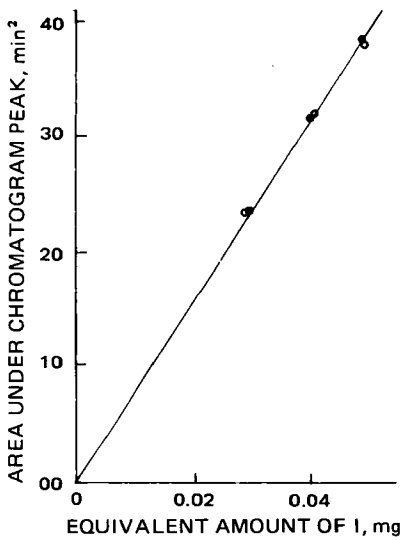


Figure 1—*Standard curve prepared for I contained in 2 (●) and 5 (○) ml of blood, indicating negligible influence of blood volume on the recovery of the compound from the blood.*

Table III—Pharmacokinetic Parameters Estimated and Derived for the Three-Compartment Open Model by Nonlinear Least-Squares Fitting (NONLIN) of Whole Blood Concentration Data Obtained for I in Rats

Pharmacokinetic Parameters	Intravenous Dose (166.65 μmoles/kg)	Intravenous Dose (249.80 μmoles/kg)	Intravenous Dose (333.33 μmoles/kg)	Average for Three Studies
Estimated Parameters				
k_{12}, min^{-1}	0.0518 (0.199)[a]	0.0785 (0.03)	0.0432 (0.076)	0.0578
k_{21}, min^{-1}	0.1707 (0.177)	0.1108 (0.038)	0.1762 (0.175)	0.1525
k_{13}, min^{-1}	0.1126 (0.156)	0.0770 (0.023)	0.1313 (0.066)	0.1069
k_{31}, min^{-1}	0.0617 (0.090)	0.0383 (0.021)	0.0551 (0.020)	0.0618
k_{el}, min^{-1}	0.1037 (0.025)	0.1003 (0.014)	0.1035 (0.005)	0.1025
α, min^{-1}	0.3436	0.3103	0.3471	0.3336
β, min^{-1}	0.1328	0.0769	0.1416	0.1171
γ, min^{-1}	0.0239	0.0180	0.0205	0.0205
V_1, ml/kg	242.95 (10.30)	220.85 (7.20)	218.45 (9.50)	227.40
Derived Parameters				
V_2, ml/kg	85.60	186.80	60.65	110.00
V_3, ml/kg	723.75	825.45	829.10	792.75
C_b^0, μmoles/ml	0.6859	1.1310	1.4280	
f_c	0.2290	0.1800	0.1980	0.2020
k_{12}/k_{21}	0.3030	0.7080	0.2450	0.4187
k_{13}/k_{31}	1.8270	1.9930	2.3810	2.0670
$t_{1/2\gamma}$, min	29.00	38.50	33.80	33.78
Measures of Fit				
Coefficient of determination (r^2)	0.996	0.998	0.991	
Correlation coefficient	0.995	0.998	0.992	

[a] The number in parentheses refers to the standard deviation of the corresponding parameter.

coefficient (Table III). The volumes of the central compartment, peripheral compartment 1, and peripheral compartment 2 were calculated, using appropriate equations. The volume of the central compartment (V_1) refers to the volume of the blood and interstitial fluids that are readily accessible to the compound, and V_2 and V_3 refer to the volumes constituted by different tissue fluids.

To determine if I shows any apparent dose-dependent kinetics in the dosage range used, the blood level data of each dose were treated according to the superposition method (18). Accordingly, plots of C_b/intravenous dose *versus* time were prepared for the blood level data obtained at each dosage level. As can be seen in Fig. 3, no apparent dose-dependent kinetics are observed. Therefore, to facilitate the interpretation of the data in the later sections, the appropriate pharmacokinetic parameters obtained at these three dosage levels were averaged (Table III).

The pharmacokinetics of II and III each were studied at only one intravenous dose level (25 mg/kg); there was no specific need to determine their dose-dependent kinetics for this study. Examination of the blood level data obtained for these compounds (Fig. 4) indicated that their disappearance from the blood occurred monoexponentially. Therefore, these data were analyzed according to the one-compartment open model (Scheme II). The elimination process of the compound consisted of urinary excretion only, as demonstrated from the overall urinary excretion studies (Table II).

In Scheme II, X_1 is the amount of the compound in the body at time t, X_e is the cumulative amount of the compound excreted in the urine up to time t, k_{el} is an apparent first-order rate constant for elimination of the compound, and V_1 is an apparent volume of

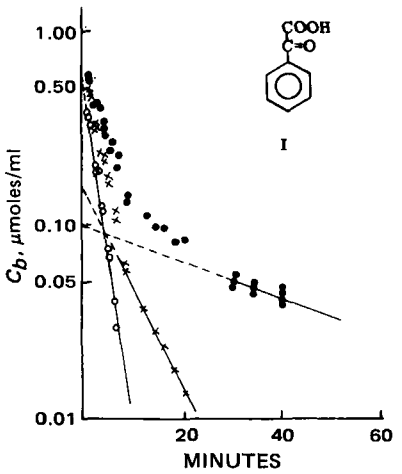


Figure 2—*Plot showing triexponential decline of blood levels of I following administration of a 166.65-μmoles/kg iv dose per rat. (Each data point represents the concentration of the compound noted in a given rat, as explained in the text.) Key: ●, experimental data points; and ×, ○, data points obtained upon feathering.*

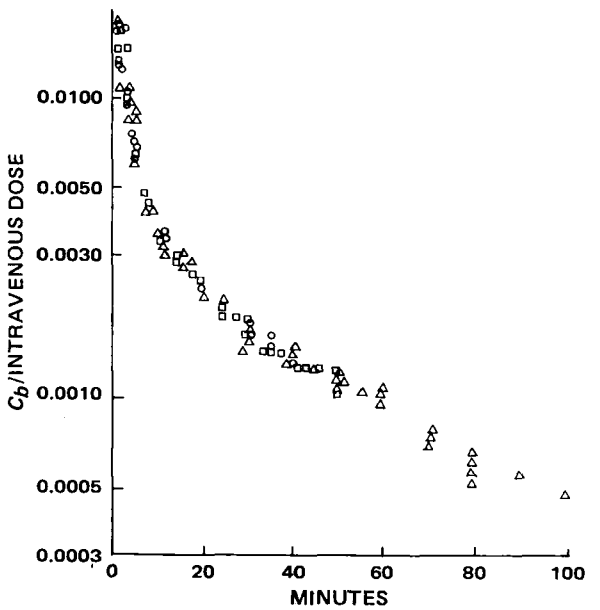
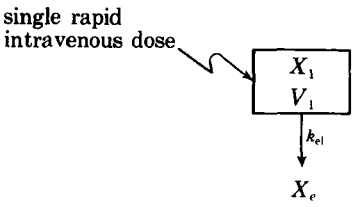


Figure 3—*Superposition plot of C_b/intravenous dose versus time, indicating the apparent absence of dose-dependent kinetics for I in the dosage range of 166.65–333.33 μmoles/kg in rats. Key: ○, 166.65-μmoles/kg iv dose; □, 249.80-μmoles/kg iv dose; and △, 333.33-μmoles/kg iv dose.*



Scheme II

distribution for the compound in the body. The blood level data were treated according to the following equation:

$$\log C_b = \log C_b^0 - k_{el}t/2.303 \qquad \text{(Eq. 2)}$$

where C_b is the concentration of the compound at time t, and C_b^0 is the concentration of the compound at zero time. The values of C_b^0 for these compounds were determined from the intercepts obtained by extrapolating the respective least-squares straight lines to zero time (Fig. 4). The volumes of distribution and the biological half-lives were calculated according to the equations V_d = intravenous dose/C_b^0 and $t_{1/2} = 0.693/k_{el}$. The pharmacokinetic parameters determined are recorded in Table IV.

Pharmacokinetics of IV–VII—Examination of the data obtained for IV (Fig. 5) indicated that the disappearance of the compound from the blood occurs biexponentially. Therefore, these data were treated according to the two-compartment open model (Scheme III), with elimination occurring from the central compartment. The elimination process for the compound consisted of urinary excretion only, as demonstrated by Randinitis *et al.* (9).

In Scheme III, X_1 and X_2 are the amounts of the compounds in the central compartment and the peripheral compartment, respectively, at time t; X_e is the cumulative amount of the compound excreted in the urine up to time t; V_1 and V_2 are the apparent volumes of the central compartment and the peripheral compartment, respectively; k_{el} is the apparent first-order rate constant of elimination of the compound; and k_{12} and k_{21} are the apparent first-order rate constants for the transfer of the compound from one compartment into another. The general solution of this system gives the following equation:

$$C_b = C_1e^{-\alpha t} + C_2e^{-\beta t} \qquad \text{(Eq. 3)}$$

where C_b is the concentration of the compound in the blood at time t. Preliminary estimates for the intercepts (C_1 and C_2) and slopes ($-\alpha/2.303$ and $-\beta/2.303$) were obtained by feathering the semilogarithmic plot (Fig. 5). The initial estimates for k_{12}, k_{21}, and k_{el} were obtained using the usual equations (19). By using these initial estimates, the blood level data were fitted to a two-compartment open model according to the NONLIN least-squares program (17). The estimated and derived parameters are presented in Table V. The excellent fit of the data is indicated by the high coefficient of determination and the correlation coefficient (Table V). The volumes of the central compartment (V_1) and the peripheral compartment (V_2) were calculated from V_1 = intravenous dose/C_b^0 and $V_2 = (k_{12}/k_{21})V_1$, respectively (19). The fraction of the dose of the compounds, $f_c = \beta/k_{el}$, and the $t_{1/2\beta} = 0.693/\beta$ values were calculated.

Table IV—Pharmacokinetic Parameters Determined for II (152.45 μmoles/kg iv) and III (140.50 μmoles/kg iv) from Blood Level Data Obtained in Rats

	II	III
Pharmacokinetic Parameters		
C_b^0, μmoles/ml	0.6194	1.137
k_{el}, min^{-1}	0.0410	0.133
$t_{1/2}$, min	14.10	5.19
V_d, ml/kg	246.10	123.50
Measures of Fit		
Coefficient of determination (r^2)	0.997	0.996
Correlation coefficient	0.998	0.998

The data obtained for V–VII are presented in Fig. 6. Examination of the blood level data for each compound revealed that the decline in the blood levels of the compounds also occurs biexponentially, as was noted for IV. Therefore, these data were analyzed according to the two-compartment open model system and the initial estimates were determined. By using the initial estimates, the blood level data were fitted to a two-compartment open model according to the NONLIN least-squares program (17). The estimated and derived parameters are presented in Table V. The excellent fit of the data is indicated by the high coefficient of determination and the correlation coefficient (Table V).

DISCUSSION

Effect of Substitution of Alkyl Groups in the *para*-Position of IV on Their Distribution Pharmacokinetic Parameters—To determine the effect of alkyl group substitution on the distribution pharmacokinetic parameters of IV and its *para*-alkylated homologs, the parameters appropriate for evaluation are V_1, V_2, and k_{12}/k_{21}. Examination of the data presented in Table V reveals a certain trend in the magnitudes of these parameters which relate to the influence of the alkyl groups.

Comparison of V_1 *and* V_2—In general, the volume of the central compartment (V_1) for IV appears to be greater than that for its *para*-alkylated homologs, although the V_1 for VII may be considered very similar to that for IV. The apparent volume of the peripheral compartment (V_2) for IV is greater than that for each of its homologs. Substitution of a methyl group or an ethyl group resulted in a sharp decrease (by a factor of almost 4.5–5) in the volume of distribution of the peripheral compartment, whereas sub-

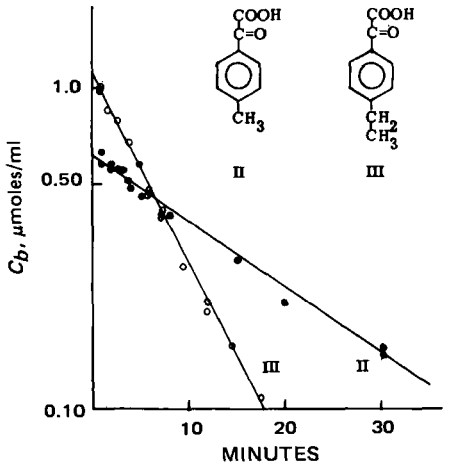


Figure 4—*Plot showing monoexponential decline of blood levels of II (●) and III (○) following administration of a 152.45- and 140.50-μmoles/kg iv dose per rat, respectively. (Each data point represents the concentration of the compound noted in a given rat, as explained in the text.)*

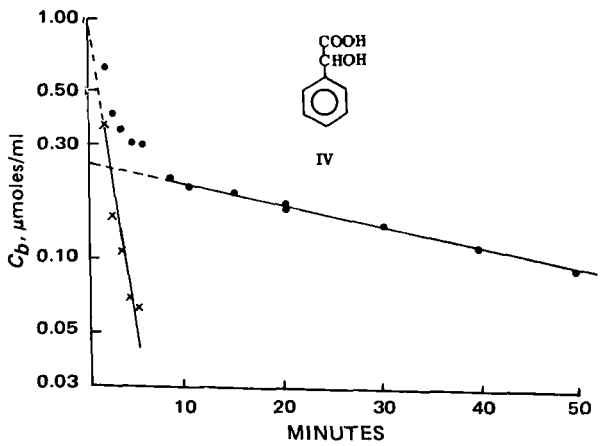


Figure 5—*Plot showing biexponential decline of blood levels of IV following administration of a 164.35-μmoles/kg iv dose per rat. (Each data point represents the concentration of the compound noted in a given rat, as explained in the text.) Key: ●, experimental data points; and ×, data points obtained upon feathering.*

Table V—Pharmacokinetic Parameters Estimated and Derived for the Two-Compartment Open Model by Nonlinear Least-Squares Fitting (NONLIN) of Whole Blood Concentration Data Obtained for IV and Its *para*-Alkylated Homologs in Rats

Pharmacokinetic Parameters	IV (164.35 μmoles/kg)	V (149.60 μmoles/kg)	VI (138.25 μmoles/kg)	VII (128.10 μmoles/kg)
Estimated Parameters				
C_b^0, μmoles/ml	0.8537	0.9070	0.9213	0.6806
	(0.049)[a]	(0.015)	(0.090)	(0.038)
k_{12}, min^{-1}	0.3377	0.2107	0.2495	0.1633
	(0.036)	(0.008)	(0.052)	(0.028)
k_{21}, min^{-1}	0.0580	0.1597	0.1319	0.0718
	(0.003)	(0.002)	(0.016)	(0.006)
k_{el}, min^{-1}	0.1891	0.2102	0.2035	0.1817
	(0.016)	(0.008)	(0.061)	(0.038)
α, min^{-1}	0.5667	0.5154	0.5347	0.3826
β, min^{-1}	0.0200	0.0651	0.0502	0.0340
Derived Parameters				
V_1, ml/kg	192.50	164.95	149.90	188.20
V_2, ml/kg	1121.20	217.75	283.60	428.90
k_{12}/k_{21}	5.82	1.32	1.89	2.28
f_c	0.106	0.310	0.247	0.187
$t_{1/2\beta}$, min	34.70	10.60	13.80	20.40
Measures of Fit				
Coefficient of determination (r^2)	0.997	1.000	0.998	0.981
Correlation coefficient	0.997	1.000	0.999	0.993

[a] The number in parentheses refers to the standard deviation of the corresponding parameter.

stitution of an isopropyl group resulted in a decrease in the volume of the peripheral compartment by a factor of 2.

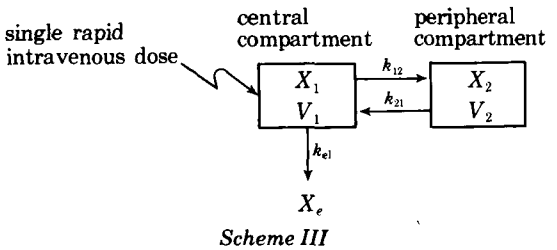
It is generally believed that the peripheral compartment consists of less moderately or poorly perfused tissues such as fatty tissues. Therefore, it is argued that if the peripheral compartment in which these compounds are distributed does consist of poorly perfused fatty tissues, then the *para*-alkylated compounds, because of their lipophilic alkyl groups, should distribute into the peripheral compartment to a greater extent than does IV, and each should consequently exhibit higher values of V_2 than those observed for IV. This, however, was not the case in the present study.

Furthermore, since IV and its *para*-alkylated homologs have pKa values in the 3.3–3.8 range, all of these compounds should remain in the systemic body fluids in the anionic form and are, consequently, least soluble in the fatty tissues or poorly perfused tissues. Therefore, it is believed that, for the compounds under discussion, the peripheral compartment consists of moderately perfused tissues and the rate of diffusion of each *para*-alkylated homolog into such tissues is significantly lower than that of IV.

Comparison of Ratios of k_{12}/k_{21}—Although the individual distribution rate constants, k_{12} and k_{21}, are calculated in the present study, it is considered desirable to compare the ratios of these rate constants (k_{12}/k_{21}), rather than the individual rate constants, to determine the effect of alkyl groups of the homologs of IV on their distribution characteristics. The reason for this approach is that one cannot be certain if the values of the forward or backward distribution rate constants are absolute since these rate constants are the result of various diffusion steps involved in a given compartment and these rate constants are calculated from the hybrid parameters of α and β.

The appropriateness of this approach is illustrated by the fact that when the distribution rate constants k_{12} = 0.993 hr^{-1} and k_{21} = 1.99 hr^{-1} determined by Wagner *et al.* (20) for spectinomycin were recalculated for the data by Pfeffer (21), using a different computer program, the values obtained were k_{12} = 0.160 hr^{-1} and k_{21} = 0.345 hr^{-1}. Although the values of k_{12} and k_{21} determined by Wagner *et al.* (20) did not agree with those determined by Pfeffer (21), their ratios of k_{12}/k_{21} were very similar. Likewise, while the values of the distribution rate constants reported for ^{3}H-alphaprodine (22) in the same dog are k_{12} = 0.097 min^{-1} and k_{21} = 0.042 min^{-1} at a 1-mg/kg iv dose, the values reported for these constants are k_{12} = 0.059 min^{-1} and k_{21} = 0.028 min^{-1} at a 2-mg/kg iv dose, indicating the possible differences that can exist in the individual values of the distribution rate constants determined at different doses. However, when the ratios of k_{12}/k_{21} are considered for these two doses, the values are very similar. As seen in Table V, the ratio k_{12}/k_{21} obtained for IV in the present study is almost 3–4 times greater than the ratios for its homologs.

To account for (*a*) the lower values of V_2 noted for V–VII than for IV, (*b*) the slightly lower values of V_1 noted for V and VI than for IV or VII, and (*c*) the lower k_{12}/k_{21} ratios noted for V–VII than for IV, it is desirable that the general structure of biological membranes and the mechanisms for transmembrane transport of solutes be considered. Although the biological membranes are composed mainly of phospholipids and proteins, the proportions of these building blocks are not identical for membranes of all organs.



Scheme III

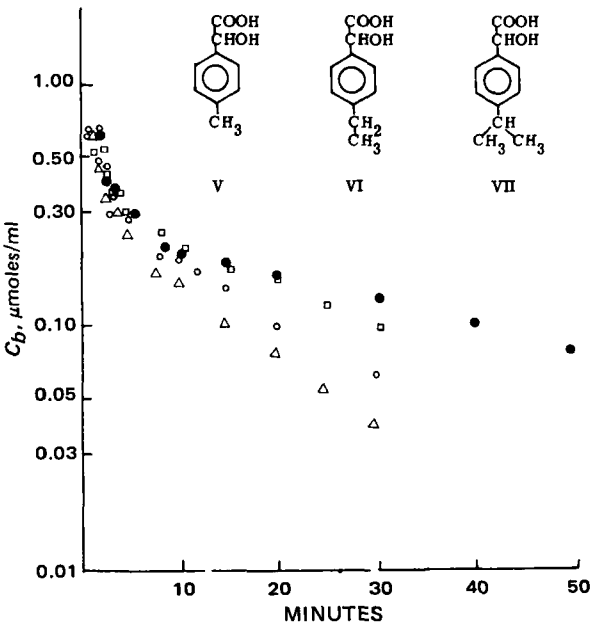


Figure 6—*Plot showing biexponential decline of blood levels for* para*-alkylated homologs of IV. Key:* ●, *IV;* △, *V;* ○, *VI; and* □, *VII.*

For instance, Aschworth and Green (23) showed that the amounts of phospholipids in the red blood cell ghost, liver plasma, muscle sarcolema, and mitochondrial membrane of liver and mitochondrial membranes of muscles of rats are approximately 61, 42, 28, and 11 mg/100 mg of proteins, respectively. Furthermore, they reported that the membranes of the microsomal liver fractions I, II, and III of rats contain 30, 80, and 35 mg of phospholipids/100 mg of protein, respectively.

For transmembrane transport of solutes, several mechanisms (8) have been proposed including: (*a*) diffusion of permeant molecules by dissolution in the membrane; (*b*) diffusion of permeant molecules through aqueous pores lined with polar portions of the proteins and possibly phospholipids; (*c*) carrier-mediated transport, which may include protein and phospholipid as carriers; and (*d*) active transport, which may involve enzymes and adenosine triphosphatase. Therefore, depending upon the organ of permeation and the mechanism of transport of solute through the membrane of the organ, differences in the rates of diffusion of the compounds of a homologous series can be expected.

Although several studies described the transport of organic nonelectrolytes and the ions of inorganic electrolytes through the membranes of many body tissues or organs, the transmembrane transport of organic electrolytes (ionized forms of acids and bases) has been studied mainly through the renal tubular membrane. Previous studies (9–13) showed that a carrier mechanism is involved in the renal tubular transport of IV and its homologs and that the affinity of VI and D-(−)-*p*-*n*-propylmandelic acid for the carrier is greater than that of IV.

In the present study, however, the transport of V–VII from the central compartment into the peripheral compartment decreased with an increase in the chain length of the homologs, as evidenced by the decrease in the k_{12}/k_{21} ratios. Therefore, it may be inferred that the transport of these compounds from the central compartment into the peripheral compartment does not involve a carrier mechanism. Furthermore, since the molecules of IV and its homologs are completely ionized in the systemic body fluids, the solubilities of the compounds in the lipoidal membranes can be considered negligible.

It is generally believed that the ions dissolve in the membrane by interacting with the oppositely charged constituents of the membrane, and if they are transported across the membrane, they do so because the membrane constituent(s) serves as carrier(s). Although the carrier mechanism for the transmembrane transport for IV and its homologs is discounted, as discussed here, it seems possible to explain the transport of these compounds between the central and peripheral compartments by considering the aqueous pore mechanism, especially in view of the magnitudes of the ratios of their distribution rate constants. The diffusion of organic nonelectrolytes through plasma membranes was shown (24, 25) to be governed by their ability to hydrogen bond with membrane components in addition to their lipid solubilities and molecular size. The larger molecular size and greater extent of hydrogen bonding of the permeant molecules decrease their rate of diffusion through the aqueous pores. Since the lining of these aqueous pores is composed of proteins and phospholipids, the diffusion rates of IV and its homologs, with their polar or ionic groups exposed to the aqueous phase of the pores, from one compartment into another may be influenced by their interaction with the ionic or polar portions of the proteins or phospholipids of the pores through electrostatic interaction, hydrogen bonding, and hydrophobic bonding.

In view of these considerations, it seems possible to explain why the volume of the peripheral compartment and the rates of diffusion of V–VII through the aqueous pores are lower than those of IV. It is reasonable to expect that, because of the presence of alkyl groups, neither the charge densities of V–VII nor the hydrogen bonding ability of the hydroxy groups present in their side chains is going to be different from those of IV. Therefore, the lower k_{12}/k_{21} ratios of distribution constants observed for V–VII, as compared to the ratio for IV, are attributed to the presence of alkyl groups. The lower rates of diffusion of the homologs from the central compartment to the peripheral compartment need not be attributed to the molecular size of these molecules, since the homologs of nonelectrolytes, with molecular weights above 140, have increased rates of membrane permeability with an increase in their molecular weights (24).

It is fairly well established that the structure of water within the membrane, including that in the aqueous pore, is much different than the bulk of water, since icebergs are formed by the water molecules present within the membrane in association with the nonpolar portions of phospholipids and/or protein (26, 27). Therefore, if the interactions between the permeant molecules and the protein or phospholipid molecules of the aqueous pores occur through electrostatic forces and/or hydrogen bonding, the nonpolar portions of the permeant molecules and the stationary receptor site(s) of the aqueous pores (proteins and/or phospholipids) may interact through hydrophobic bonding, thus releasing the water molecules associated with the nonpolar portions of these interactants and giving rise to increases in entropy. The association of these reactants then would be stronger, as shown previously in model copolymer system studies (28, 29). This would then result in a decrease in the rate of diffusion of V–VII and, consequently, in a decrease in the volume of the peripheral compartment.

The difference observed between V_2 values of V and VI cannot be ascertained in view of how these values were calculated in the present study; therefore, these values may be considered to be similar. However, the difference between the V_2 values of V and VII appears to be substantial. In view of the mechanism suggested for the transmembrane transport of these organic anions and the role that hydrophobic bonding (between the permeant organic anions and the constituents of the lining of the aqueous membrane pores) plays in reducing the rate of transmembrane transport, the higher V_2 observed for VII (higher even than for V) can be attributed to its branched group, which apparently may not form as strong a hydrophobic bond as does V, since the isopropyl group may not allow close contact of the phenyl group with the hydrophobic portion of the receptor protein or phospholipid of the aqueous pore lining.

As pointed out previously, the V_1 noted for V and VI is somewhat (about 15–20%) lower than that for IV. In view of the fact (discussed in Ref. 30) that, in the presence of a renal tubular inhibitor (DL-tropic acid), the V_1 for IV or VII decreased but that for V or VI remained unchanged, it seems possible to explain the lower V_1 observed for V or VI in terms of the heteroporosity of the membranes. It is known that the membrane pores of a given organ are heteroporous, with their pores ranging in size from 8 to 320 Å; the majority of the pores have a pore diameter of 70–120 Å (31–33). When considering the diameter of the molecule of IV and its homologs, especially when they are hydrated, it seems reasonable to expect that only one molecule of any of these compounds could pass at a time through those pores, which are about 8 Å in diameter. The percentage of small size (8 Å diameter) pores in a membrane of a given organ is apparently not reported in the literature; but if it is assumed that the pores of this size constitute a significant population of the total pores, it is conceivable that molecules showing greater intermolecular interactions with constituents of the pore lining would not be able to occupy the same volume of distribution in a given organ as would similar size molecules exhibiting less intermolecular interactions. Therefore, it is believed that V and VI probably show greater interactions with the aqueous pore lining through hydrophobic bonding than do IV and VII. Consequently, the V_1 for V or VI can be expected to be smaller than that for IV or VII.

Comparison of Effect of Carbonyl Group of I with Effect of Alcoholic Hydroxy Group of IV—The striking effect of the carbonyl group of I, as compared to that of the hydroxy group of IV, becomes apparent from the fact that the disappearance of I from the rat blood is describable by a three-compartment open model whereas that of IV is describable by a two-compartment open model, even though the biological half-lives of the compounds determined from their respective predominantly elimination phases are very similar. One possible explanation for this difference follows.

The rate of diffusion of the molecules of IV through the aqueous pores of tissue membranes, which constitute the peripheral compartment, can be expected to be different due to the differences in the chemical composition of the lining of the pores of different tissues. Apparently, however, such differences in the rates of diffusion do not exist so as to distinguish the various tissues of the peripheral compartment into more than one peripheral compartment. However, the differences in the rates of diffusion of the molecules of I through the aqueous pores of the peripheral compartment tissues may vary widely because they have less ability to hydrogen bond, as compared to those of IV; therefore, the various tissues of the peripheral compartment become kinetically distinguishable as two peripheral compartments. The reason for expect-

ing the molecules of I to show these differences is that the chemical composition of proteins and phospholipids of the membranes of all tissue organs (34) and, therefore, of their aqueous pore lining is not identical. It is conceivable, therefore, that the molecules of I, which have one less potential or a weaker site for hydrogen bonding than the molecules of IV, could show relatively greater hydrogen bonding with the constituents of the lining of pores of the one tissue organ than with the constituents of the lining of pores of other tissue organs of the peripheral compartments.

On this basis, those organs whose aqueous pores exhibit stronger hydrogen bonding with the molecules of I would constitute the peripheral compartment that would have a smaller apparent volume of distribution; organs whose aqueous pores exhibit weaker hydrogen bonding with the molecules of I would constitute the peripheral compartment that would have a larger apparent volume of distribution. The former peripheral compartment may be designated as peripheral compartment 1 with volume V_2, and the latter peripheral compartment may be designated as peripheral compartment 2 with volume V_3. As stated previously, if the differences in the extent of hydrogen bonding of IV with the constituents of the aqueous pores of these peripheral compartments are not sufficiently large, these peripheral compartments would then be kinetically describable as a single peripheral compartment for IV.

It is observed that the volume of the central compartment for I is larger than that for IV, and the volume of its peripheral compartments ($V_2 + V_3$) is smaller than that for the molecules of IV. In connection with the transport of these molecules through the aqueous pores, it seems possible that I is able to equilibrate into part of the compartment that serves as a peripheral compartment of IV somewhat more readily than does IV, thus resulting in a larger volume of the central compartment. Also, since the volume of the peripheral compartment is in no sense an absolute value of the actual volume but rather a mathematically manipulated value, the lower volume of the peripheral compartments ($V_2 + V_3$) for I may possibly be explained on the basis that I probably interacts with the biopolymers of the intracellular fluids of the tissue cells of the peripheral compartments, thereby restricting its diffusion into only a limited region of the tissues of the peripheral compartments. The reason for speculating about the interaction of I with the intracellular biopolymers is based on the observation that I is bound to plasma protein to the extent of 13–15% while IV shows no binding to plasma proteins (9). The binding of drugs to tissue proteins, such as nucleic acids and DNA, is reported in the literature (34).

Effect of Substitution of Alkyl Groups in the *para*-Position of I—Since the blood level data for I can be described by a three-compartment open model, the revelation that the blood level data for II and III can be described by a one-compartment open model demonstrated a dramatic effect of alkyl groups on the distribution of these compounds in rats.

Among IV–VII, although the effect of *para*-alkyl groups is not seen in terms of reducing the two-compartment open model for IV to a one-compartment open model for V–VII, the dramatic effect of the alkyl groups in terms of a substantial decrease in the volume of the peripheral compartment is observed (Table V). However, as pointed out later, blood level data for D-(−)-*p*-*n*-propylmandelic acid are indeed describable by a one-compartment open model (13), thereby demonstrating the effect of the propyl group in reducing the volume of distribution of the peripheral compartment to zero, as observed for II and III. Clearly, for the *para*-alkylated homologs of I and IV, the apparent effect of the alkyl groups is to diminish the volume of the peripheral compartment, but the disappearance of a kinetically describable peripheral compartment remains a function of the size of the peripheral compartment for the parent compounds.

Accordingly, if the volume of the peripheral compartment is relatively large for the parent compound, the peripheral compartment may not become kinetically indistinguishable for the short alkyl chain *para*-alkylated homologs as seen for V–VII. However, if the volume of the peripheral compartment is relatively small for the parent compound, it may become kinetically indistinguishable, even for the short alkyl chain *para*-alkylated homologs as noted for II and III. As explained in the case of the homologs of IV, the effect of alkyl groups in restricting the distribution of the compound to the central compartment is a consequence of the decreased rate of diffusion of the compounds due to the strengthened interaction (through hydrophobic bonding) of the permeant molecules with the proteins and/or phospholipids of the lining of the aqueous pores of the tissue organs.

The volume of the central compartment noted for II is slightly greater than that for I. This slightly higher volume may be due to incorporation of that volume of the peripheral compartment that is not kinetically distinguishable because of its highly reduced size. It is believed that, since the rate of diffusion of II from the central compartment into the peripheral compartment is considerably reduced, the amount of the compound distributed into the peripheral compartment is relatively small, and the system cannot be kinetically described either as a two-compartment open model or a three-compartment open model.

If the pattern observed for I and its homologs with respect to their volumes of the central compartment is considered, the volume of the central compartment for III would be anticipated at 225–250 ml, which is the range of the volume of the central compartment observed for I and II. However, the volume of the central compartment noted for III is only about 125 ml. A study is under consideration to determine the possible reason for this finding.

Comparison of Effect of Branched Alkyl Group (Isopropyl) against Straight Chain Alkyl Group (Propyl)—The kinetics of disappearance of D-(−)-*p*-*n*-propylmandelic acid (VIII), which also possesses the "ideal properties," from the blood has been studied in rats (13). The pharmacokinetics of this compound are describable by a one-compartment open model, and its apparent volume of distribution is 220 ml. The dramatic effect of hydrophobic bonding in reducing the rate of transmembrane transport through aqueous membrane pores is readily seen with VIII, as with II and III. Clearly, the propyl group of this compound is able to form a substantially stronger hydrophobic bond with constituents of the membrane pores, thus restricting the compound mainly in the central compartment of distribution.

Although the total volume of distribution (one-compartment open model) observed for VIII is 20% larger than that observed for the central compartment (two-compartment open model) of VII, the volume of distribution of the central compartment for VIII may not be larger than that observed for VII or for that matter for IV–VI. It is believed that, since the rate of diffusion of VIII from the central compartment into the peripheral compartment is considerably reduced, the amount of the compound distributed into the peripheral compartment is relatively small and the system cannot be kinetically described as a two-compartment open model.

The influence of the geometrical factor relating to the extent of surface contact area between the permeating molecule and the membrane was illustrated by Diamond and Wright (25). They showed that the branched nonelectrolytes such as isobutyramide and isovaleramide exhibited less permeability in several epithelia than did their corresponding straight chain isomers butyramide and valeramide. This observation is considered to be very striking because the partition coefficients of the straight chain isomers are noted to be lower than those of the branched isomers and, in water, the diffusion coefficients of butyramide and isobutyramide are similar (35). The possible explanation for this observation is that the area of close contact with surrounding molecules decreases with increased branching, so the van der Waals forces between the permeating molecule and the membrane are weakened. This explanation suggests that the selectivity is determined by the types of interactions in the membrane interior which, in the case of these molecules, implies that the rate-limiting step is diffusion through the membrane and not transition from aqueous solution to the membrane phase (35).

In the case of the isomers of organic nonelectrolytes, the isomers containing branched alkyl groups showed less permeability into the epithelial membrane than did the corresponding isomers containing straight chain alkyl groups (25); in the case of organic electrolytes of the present study, the isomer containing a branched alkyl group (VII) showed a greater rate of permeability through the aqueous pores of membranes than did the corresponding isomer containing a straight chain alkyl group (VIII). If, indeed, the major pathway for the transfer of VII and VIII anions from the central body compartment into the peripheral body compartment was through the membrane (possibly *via* a "carrier" mechanism), the pharmacokinetics of VIII would probably have been describable according to the two-compartment open model, with V_2 greater than that noted for VII and possibly IV. Therefore, this observation is supportive of the aqueous pore mechanism suggested for transmembrane transport of I, IV, and some of its *para*-alkylated

homologs between central and peripheral body compartments.

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Mechanistic Evaluation of Modifications of Distribution Pharmacokinetic Parameters of Model Organic Anions in Presence of a Model Renal Tubular Secretion Inhibitor in Rats

YASHWANT M. AMIN * and JANARDAN B. NAGWEKAR ˣ

Abstract □ The effects of DL-tropic acid (VIII) on the distribution pharmacokinetic parameters of the model compounds benzoylformic acid (I), *p*-methylbenzoylformic acid (II), *p*-ethylbenzoylformic acid (III), D-(−)-mandelic acid (IV), D-(−)-*p*-methylmandelic acid (V), D-(−)-*p*-ethylmandelic acid (VI), and D-(−)-*p*-isopropylmandelic acid (VII) were studied in rats. Since VIII is a competitive inhibitor of renal tubular secretion of I–VII and since all of these compounds (I–VIII) are negligibly bound to plasma proteins and are neither metabolized nor reabsorbed from the renal tubules, they were considered as model compounds. Therefore, changes observed in the values of the distribution pharmacokinetic parameters of I–VII were attributed to the influence of VIII on the transmembrane transport of the compounds between body compartments in rats. The decrease in the apparent volumes of the central compartments for I, IV, and VII, the increase in the apparent volumes of the peripheral compartments for IV–VII, the absence of change in the volumes of the central or peripheral compartments for the other compounds, and the increase in the ratios of the rate constants of the transfer of compounds from one compartment into another for I and IV–VII were explained in terms of the "aqueous pore" mechanism for the transmembrane transport of the anions of the compounds as well as the heteroporosity of the tissue membranes.

Keyphrases □ Pharmacokinetics—distribution parameters, mechanistic evaluation of modifications, model organic anions in presence of model renal tubular secretion inhibitor, rats □ Distribution—pharmacokinetic parameters, mechanistic evaluation, model organic anions in presence of model renal tubular secretion inhibitor, rats □ Transport—mechanistic evaluation of modifications of distribution pharmacokinetic parameters, model organic anions in presence of model renal tubular secretion inhibitor, rats

It was recently shown (1, 2) that a renal tubular secretion inhibitor such as probenecid increases the blood levels of penicillins not only by depressing their renal tubular secretion but also by decreasing the volumes of body central compartments for the drugs. The reason suggested (2) to account for the decrease in the volume of distribution was that the presence of probenecid caused an increase in the plasma protein binding of these antibiotics and/or limited their penetration or distribution into certain body compartments. It was further speculated that the higher rate constant of metabolism noted for the antibiotics in the presence of probenecid was due to the decrease in the apparent volume of distribution, which resulted in presenting a larger fraction of the drug at the site of metabolism.

If a drug is subjected simultaneously to metabolism, protein binding, and renal tubular secretion, it is difficult to isolate the effect of the renal tubular secretion inhibitor on its distribution pharmacokinetic parameters, especially if the renal tubular inhibitor can displace the drug from its protein binding sites or interfere with its metabolism. Therefore, if one intends to determine specifically the effect of the renal tubular secretion inhibitor on the distribution pharmacokinetic parameters of substrate compounds, it is advantageous to have compounds and their renal tubular secretion inhibitor that are neither bound to plasma proteins, metabolized, nor reabsorbed from the renal tubules. In addition, they should be completely recovered in the urine.

Since benzoylformic acid, D-(−)-mandelic acid, and their respective *para*-alkylated homologs possess such desirable properties (3–7) and DL-tropic acid, which is also a homolog of D-(−)-mandelic acid and possesses these ideal properties, is a competitive renal tubular secretion inhibitor of D-(−)-mandelic acid and its *para*-alkylated homologs (3–6) and of benzoylformic acid and its *para*-alkylated homologs (8), the purpose of this study was to determine in rats the effect of DL-tropic acid (VIII) on the distribution pharmacokinetic parameters of benzoylformic acid (I), *p*-methylbenzoylformic acid (II), *p*-ethylbenzoyl-

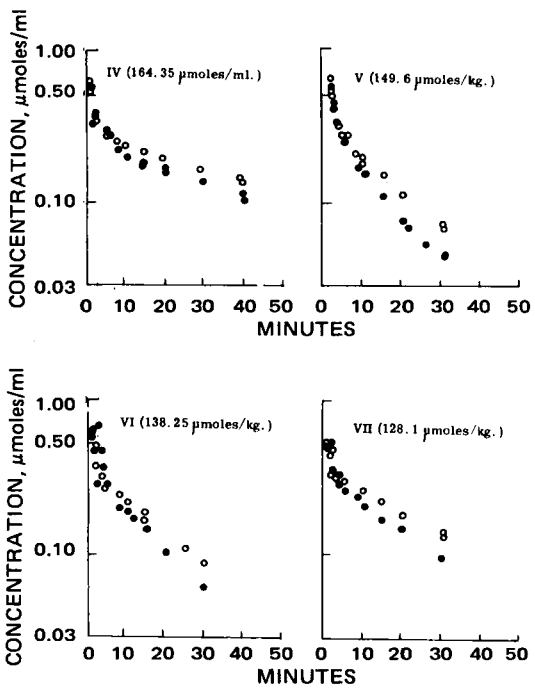

Figure 1—*Comparative semilogarithmic plots showing biexponential decline of blood levels of IV–VII in rats in the absence* (●) *and in the presence* (○) *of a 3012-μmoles/kg iv dose of VIII. The number in each parentheses refers to the intravenous dose of the respective compound. Each data point represents the concentration of the compound noted in a given rat, as explained in the text.*

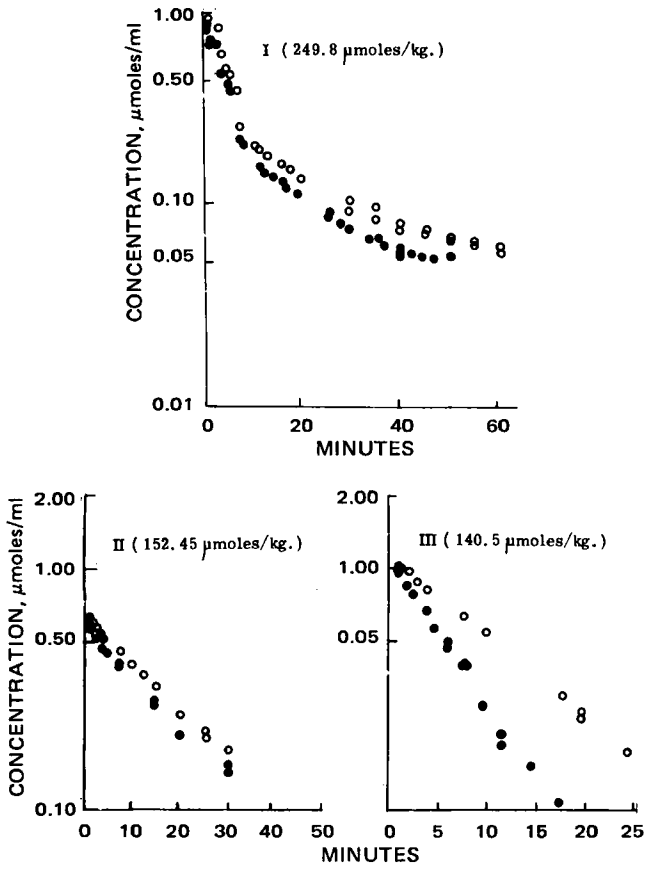

Figure 2—*Comparative semilogarithmic plots showing triexponential decline in blood levels of I and monoexponential decline in blood levels of II and III in rats in the absence (●) and in the presence (○) of a 3012-μmoles/kg iv dose of VIII. The number in parentheses refers to the intravenous dose of the respective compound. Each data point represents the concentration of the compound noted in a given rat, as explained in the text.*

formic acid (III), D-(−)-mandelic acid (IV), D-(−)-*p*-methylmandelic acid (V), D-(−)-*p*-ethylmandelic acid (VI), and D-(−)-*p*-isopropylmandelic acid (VII).

Furthermore, since the pKa values of these compounds are in the 3.2–4.2 range, these compounds are expected to remain in the systemic body fluids only in the anionic form. Consequently, any changes observed in the values of the distribution pharmacokinetic parameters of these anions in the presence of VIII (anionic) can be attributed to the influence of VIII on the transmembrane transport of these compounds between the body compartments in rats. As discussed previously (7), the distribution pharmacokinetic parameters considered for the compounds exhibiting a multicompartment open-model system are the apparent volumes of the central compartment (V_1), the apparent volumes of the peripheral compartments (V_2 and V_3), and the ratios of the apparent first-order rate constants of transport of compounds from one compartment into another. For those compounds that follow the one-compartment open model, the apparent volume of distribution (V_d) is considered.

It is anticipated that, in light of the mechanism suggested (7) for reversible transmembrane transport between the central and peripheral compartments, it

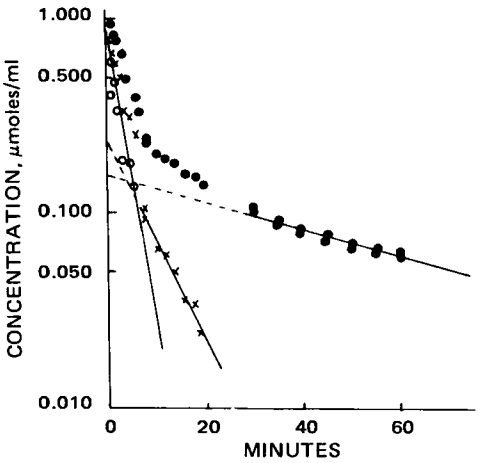

Figure 3—*Plot showing triexponential decline of blood levels of I following the administration of a 249.8-μmoles/kg iv dose per rat in the presence of a simultaneously administered 3012-μmoles/kg dose of VIII. Each data point represents the concentration of the compound noted in a given rat, as explained in the text. Key: ●, experimental data points; and ×, ○, data points obtained upon feathering.*

is possible to rationalize mechanistically the effect(s) of VIII that may be observed on the distribution pharmacokinetic parameters of I–VII.

EXPERIMENTAL

Materials—Compounds I–VII were the same as described previously (7). In addition, DL-tropic acid[1], mp 117–119°, was used.

Methodology—The methodology followed was described previously (7), except that 2 ml of the intravenous solution, containing an appropriate dose of one of the seven substrate compounds (I–VII), also contained 3012 μmoles/kg of VIII. Compound VIII served as a renal tubular secretion inhibitor of the substrate compounds.

The intravenous doses of the substrate compounds administered were 165.65[2], 249.8, and 333.33 μmoles/kg for I; 152.45 μmoles/kg[2] for II; 140.5 μmoles/kg[2] for III; 164.35 μmoles/kg[2] for IV; 149.6 μmoles/kg[2] for V; 138.25 μmoles/kg[2] for VI; and 128.1 μmoles/kg[2] for VII. Each compound (I–VIII) was injected as its sodium salt. The fact that a 3012-μmoles/kg iv dose[3] of VIII completely inhibits renal tubular secretion of I–III (7) and IV–VII (3–6) has been demonstrated already.

GLC Analysis—The equipment and procedure used to analyze I–VIII gas chromatographically in blood were the same as those described previously (7).

Binding of I to Whole Rat Blood and Plasma Proteins in Presence of VIII—The equilibrium dialysis procedure used for determining the binding of I to whole rat blood and plasma proteins was the same as that described previously (3–6), except that, along with 2 mg of I, the 4 ml of blood or plasma contained 40 mg of VIII. Since the binding of the other model compounds was considered negligible, no binding of those compounds was studied in the presence of VIII.

RESULTS AND DISCUSSION

Effect of VIII on Distribution Pharmacokinetic Parameters of I and IV and Their Respective Homologs—The blood level data obtained for the compounds in this study are presented in Figs. 1 and 2, along with those reported previously (7) for the same compounds in the absence of VIII. As illustrated in Figs. 3 and 4 for I and IV, respectively, the observed kinetic pattern of disappearance of each compound from the blood was the same as that noted in the absence of VIII. Initial estimates for the phar-

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] These doses represent 25 mg/kg.
[3] This dose represents 500 mg/kg.

Table I—Pharmacokinetic Parameters Estimated and Derived for the Three-Compartment Open Model by Nonlinear Least-Squares Fitting (NONLIN) of Whole Blood Concentration Data Obtained for I in Rats in the Presence of a Simultaneously Administered 3012-μmoles/kg iv Dose of VIII

Pharmacokinetic Parameters[a]	Average[a] of Three Studies in the 166.65–333.33-μmoles/kg Dosage Range
Estimated Parameters	
k_{12}, min^{-1}	0.0750
k_{21}, min^{-1}	0.1177
k_{13}, min^{-1}	0.1101
k_{31}, min^{-1}	0.0467
k_{el}, min^{-1}	0.0944
α, min^{-1}	0.3455
β, min^{-1}	0.0821
γ, min^{-1}	0.0163
V_1, ml/kg	181.45
Derived Parameters	
V_2, ml/kg	180.05
V_3, ml/kg	704.95
f_c	0.172
k_{12}/k_{21}	0.709
k_{13}/k_{31}	2.500
$t_{½\gamma}$, min	43.57

[a] Average of pharmacokinetic parameters determined for the data obtained at each dosage level study. The coefficients of determination (r^2) for the data at 166.65-, 249.80-, and 333.33-μmoles/kg doses were 0.997, 0.996, and 0.993, respectively; the correlation coefficients were 0.997, 0.997, and 0.994, respectively.

macokinetic parameters were obtained by use of appropriate equations as discussed previously. The data were found to fit very well to a three-compartment open model for I, to a two-compartment open model for IV and its *para*-alkylated homologs, and to a one-compartment open model for II and III, as indicated by the coefficients of determinations and correlation coefficients (Tables I–III).

The comparative plots (Figs. 1 and 2) of concentration *versus* time for these compounds showed that VIII elevated the blood levels of each substrate compound, as expected. The comparative data concerning the distribution pharmacokinetic parameters for I–VII, both in the absence and presence of VIII, are presented in Tables IV and V. As noted (7) in the absence of VIII, no apparent dose-dependent pharmacokinetics were observed for I in the intravenous dosage range of 165.65–333.33 μmoles/kg in the presence of VIII (Fig. 5).

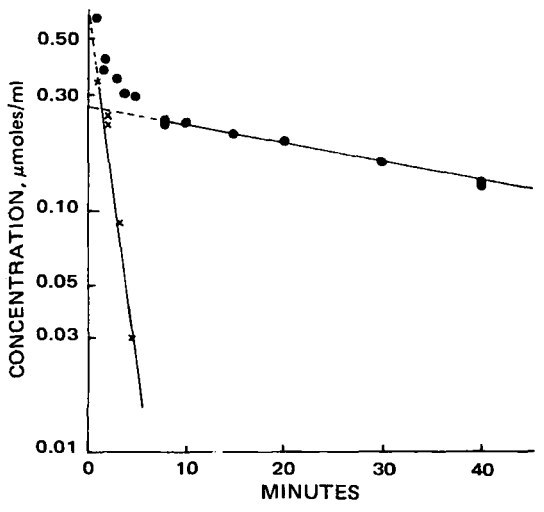

Figure 4—*Plot showing biexponential decline of blood levels of IV following the administration of a 164.35-μmoles/kg iv dose per rat in the presence of a simultaneously administered 3012-μmoles/kg dose of VIII. Each data point represents the concentration of the compound noted in a given rat, as explained in the text. Key:* ●, *experimental data points; and* ×, *data points obtained upon feathering.*

Table II—Pharmacokinetic Parameters Determined for II (152.45 μmoles/kg iv) and III (140.5 μmoles/kg iv) from Blood Level Data Obtained in Rats in the Presence of a Simultaneously Administered 3012.0-μmoles/kg iv Dose of VIII

	II	III
Pharmacokinetic Parameters		
C_b^0, μmoles/ml	0.6152	1.13
k_{el}, min^{-1}	0.0410	0.0737
$t_{½}$, min	16.9	9.4
V_d, ml/kg	246.80	125.20
Measures of Fit		
Coefficient of determination (r^2)	0.986	0.999
Correlation coefficient	0.993	0.999

Effect on IV and Its *para*-Alkylated Homologs—A comparison of the distribution pharmacokinetic parameter data (Table IV) for IV and its *para*-alkylated homologs shows that the volumes of the peripheral compartments and the ratios of k_{12}/k_{21} increased in the presence of a large dose of VIII. Furthermore, while the apparent volume of the central compartment slightly decreased (about 15–20%) for IV and VII, it remained practically unchanged for V and VI. These effects of VIII may be explained in terms of the aqueous pore diffusion mechanism previously advocated (7) for the transmembrane transport of these compounds.

It is reasonable to expect that VIII (pKa = 4.2), which is also a homolog of IV, should follow the same pathway for its transmembrane transport between the body compartments as do IV and its *para*-alkylated homologs. To facilitate comparison, the pharmacokinetics of VIII were studied following administration of 150.45 μmoles/kg iv. The blood level data obtained are presented in Fig. 6; the disappearance of the compound from the blood is also describable by a two-compartment open model. The initial estimates of the appropriate pharmacokinetic parameters of the compound were obtained and used to refine the values after fitting the data according to the two-compartment open model, using the NONLIN least-squares program (Table VI).

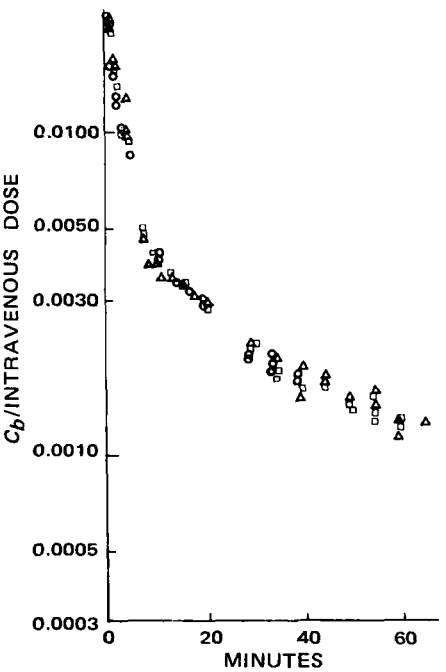

Figure 5—*Superposition plot of* C_b*/intravenous dose* versus *time indicating apparent absence of dose-dependent pharmacokinetics for I in the 166.65–333.33-μmoles/kg dosage range when simultaneously administered to rats in the presence of a 3012-μmoles/kg dose of VIII. Key:* ○, *166.65-μmoles/kg iv dose;* □, *249.80-μmoles/kg iv dose; and* △, *333.33-μmoles/kg iv dose.*

Table III—Pharmacokinetic Parameters Estimated and Derived for the Two-Compartment Open Model by Nonlinear Least-Squares Fitting (NONLIN) of Whole Blood Concentration Data Obtained for IV and Its *para*-Alkylated Homologs in the Presence of a Simultaneously Administered 3012-μmoles/kg iv Dose of VIII

Pharmacokinetic Parameters	IV (164.35 μmoles/kg)	V (149.60 μmoles/kg)	VI (138.25 μmoles/kg)	VII (128.10 μmoles/kg)
Estimated Parameters				
C_b^0, μmoles/ml	1.0300	0.8994	0.8623	0.8521
	(0.036)[a]	(0.028)	(0.025)	(0.028)
k_{12}, min^{-1}	0.5282	0.2601	0.2506	0.5133
	(0.027)	(0.020)	(0.018)	(0.030)
k_{21}, min^{-1}	0.0648	0.1217	0.1028	0.0707
	(0.002)	(0.004)	(0.004)	(0.005)
k_{el}, min^{-1}	0.2417	0.2071	0.2090	0.4203
	(0.011)	(0.018)	(0.018)	(0.028)
α, min^{-1}	0.8155	0.5423	0.5212	0.9737
β, min^{-1}	0.0192	0.0465	0.0412	0.0305
Derived Parameters				
V_1, ml/kg	159.55	166.30	160.15	150.35
V_2, ml/kg	1301.40	355.45	390.45	1092.20
k_{12}/k_{21}	8.15	2.14	2.44	7.27
f_c	0.079	0.224	0.197	0.072
$t_{1/2\beta}$, min	36.10	14.90	16.80	22.70
Measures of Fit				
Coefficient of determination (r^2)	0.997	0.998	0.998	0.999
Correlation coefficient	0.999	0.997	0.996	0.996

[a] The number in parentheses refers to the standard deviation of the corresponding parameter.

Table IV—Distribution Pharmacokinetic Parameters Obtained for IV–VII in Rats in the Absence and Presence of VIII

	IV		V		VI		VII	
Pharmacokinetic Parameters	VIII Absent[a]	VIII Present	VIII Absent[a]	VIII Present	VIII Absent[a]	VIII Present	VIII Absent[a]	VIII Present
V_1, ml/kg	192.5	159.5	164.9	166.3	149.9	160.1	188.2	150.3
V_2, ml/kg	1121.2	1301.4	217.7	355.4	283.6	390.4	428.9	1092.2
k_{12}/k_{21}	5.8	8.1	1.3	2.1	1.8	2.4	2.2	7.2

[a] Data obtained from Ref. 7.

Since the molecules of VIII are expected to follow the same pathway for their transport from the central compartment to the peripheral compartment, it is conceivable that these molecules would compete for the same sites of adsorption in the aqueous pore lining as do IV and its *para*-alkylated homologs, especially when a large dose of VIII is administered along with a much smaller dose of each substrate compound. In this event, it is not unlikely that the substrate molecules of IV or any of its homologs would find less resistance for their transport through the aqueous pores and, consequently, exhibit higher values not only of the volumes of peripheral compartments but also of the ratios of the distribution rate constants (k_{12}/k_{21}). Another factor that may be expected to contribute to the increased volume of the peripheral compartment is the increase in the $t_{1/2\beta}$ (Table III), which would tend to favor an increase in time to allow further distribution or penetration of the compound into the peripheral compartment.

The decrease in the volume of the central compartment for IV and VII in the presence of VIII may be explained on the basis of the heteroporosity of the membrane of the tissues of the central compartment (7). As pointed out previously (7), a substantial percentage of the total pores of these membranes may be about 8 Å in diameter, which probably would allow passage of only one molecule at a time through a pore. Therefore, when a large excess of the molecules of VIII are present in the central compartment, these molecules would predominantly occupy the small size pores and prevent access to the substrate molecules of IV or any of its *para*-alkylated homologs, thus diminishing the volume of the central compartment for the substrate compound. Since the molecules of V and VI are indicated to have little or no access through the small

Table V—Distribution Pharmacokinetic Parameters Obtained for I in Rats in the Absence and Presence of VIII

Pharmacokinetic Parameters	VIII Absent[a]	VIII Present
V_1, ml/kg	227.40	181.55
V_2, ml/kg	110.00	180.10
V_3, ml/kg	792.80	705.00
$V_2 + V_3$, ml/kg	902.80	885.00
k_{12}/k_{21}	0.418	0.709
k_{13}/k_{31}	2.067	2.500

[a] Data obtained from Ref. 7.

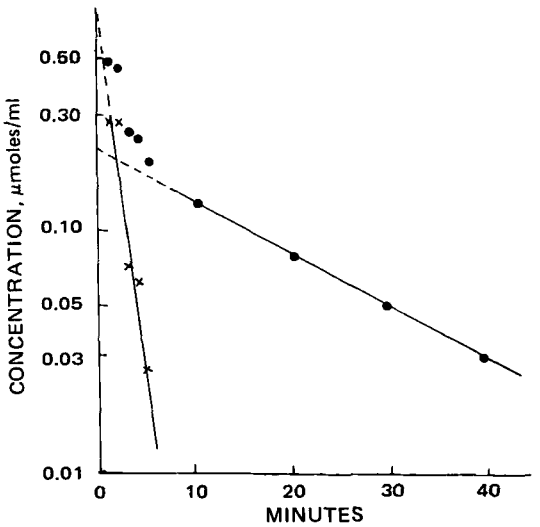

Figure 6—*Plot showing biexponential decline of blood levels of VIII following the administration of a 150.45-μmoles/kg iv dose per rat. Each data point represents the concentration of the compound noted in a given rat, as explained in the text. Key:* ●, *experimental points; and* ×, *data points obtained upon feathering.*

Table VI—Pharmacokinetic Parameters Estimated and Derived for the Two-Compartment Open Model by Nonlinear Least-Squares Fitting (NONLIN) of Whole Blood Concentrations Data Obtained for VIII in Rats

Pharmacokinetic Parameters	Dose (150.45 μmoles/kg iv)
Estimated Parameters	
C_b^0, μmoles/ml	0.7422 (0.067)[a]
k_{12}, min^{-1}	0.2508 (0.026)
k_{21}, min^{-1}	0.1396 (0.021)
k_{el}, min^{-1}	0.1691 (0.040)
α, min^{-1}	0.5135
β, min^{-1}	0.0460
Derived Parameters	
V_1, ml/kg	202.7
V_2, ml/kg	364.0
k_{12}/k_{21}	1.8
f_c	0.272
$t_{½\beta}$, min	15.1
Measures of Fit	
Coefficient of determination (r^2)	0.982
Correlation coefficient	0.987

[a] The number in parentheses refers to the standard deviation of the corresponding parameter.

size pores previously mentioned, the volume of the central compartment for these compounds in the presence of an excessively large number of molecules of VIII can be expected to remain unchanged.

Effect on I—Comparison of the distribution pharmacokinetic parameter data (Table V) for I in the presence of VIII shows that the volume of the central compartment, the volume of peripheral compartment 1 (V_2), and the volume of peripheral compartment 2 (V_3) decreased, increased, and practically remained unchanged, respectively. The decrease in the volume of the central compartment for I can be explained on the same basis (*i.e.*, heteroporosity of the membranes of the tissues of the central compartment) as the decrease in the volume of the central compartment for IV and VII. Accordingly, when present in excessively large numbers in the central compartment, the molecules of VIII would predominantly occupy the small size pores of the membranes of the tissues of the central compartment and prevent access to the molecules of the substrate compound, I, thereby diminishing V_1 for I. The decrease in the volume of the central compartment in the case of I (20%) is similar to that noted in the case of IV (18%).

Although, in the presence of VIII, V_2 for I substantially increased and V_3 slightly decreased, the total volume ($V_2 + V_3$) of the peripheral compartments remained practically unchanged (Table V). The rationalization for these observations follows. In the preceding paper (7), it was indicated that the binding of I with the biopolymers of the intracellular fluids of the tissues of the peripheral compartments restricts its diffusion into only limited regions of the tissues of the peripheral compartments as compared to that of IV. Even when the concentration of VIII was several times greater than that of I, it did not affect the binding of I to plasma proteins, as determined from *in vitro* equilibrium dialysis studies, thus leading to the assumption that VIII does not interfere *in vivo* with the binding of I to the biopolymers of intracellular fluid of the tissues of the peripheral compartments. Therefore, it may not be too surprising that the total volume ($V_2 + V_3$) of the peripheral compartments for I remained practically unchanged in the presence of VIII.

However, in accordance with the other idea proposed previously (7), that the two peripheral compartments are identified for I because of the differences in the extent of hydrogen bonding of its molecules with the constituents (proteins and/or phospholipids) of the aqueous pores of membranes of the tissues of the peripheral compartments, the diffusion of molecules of I through the aqueous pores may be affected to different degrees in the presence of the molecules of VIII to reflect a substantial increase in V_2 and a very slight decrease (which may even be discounted as being within experimental error) in V_3 for I, as noted in this study. The apparent increase in V_2 may also be attributed to the increased $t_{1/2\gamma}$ of I in the presence of VIII.

The fact that the ratios of the distribution rate constants (k_{12}/k_{21} and k_{13}/k_{31}) observed for I increased in the presence of VIII also supports the interpretation (made earlier in connection with the increase in k_{12}/k_{21} value of IV) that the substrate molecules of I find less resistance for their transport through the larger aqueous pores in the presence of a large excess of the molecules of VIII.

Table VII—Distribution Pharmacokinetic Parameters Obtained for I in Rats in the Absence and Presence of VIII

	II		III	
Pharmacokinetic Parameter	VIII Absent[a]	VIII Present	VIII Absent[a]	VIII Present
V_d, ml/kg	247.8	248.0	123.5	124.2

[a] Data obtained from Ref. 7.

Effect on II and III—The volume of distribution of II as well as III remained unchanged in the presence of VIII (Table VII). This finding suggested that the diffusion of these *para*-alkylated compounds through the small size aqueous membrane pores is not involved, as was inferred in the case of V and VI, the *para*-alkylated (methyl and ethyl) homologs of IV. This finding also confirmed that the V_d of III is indeed as low as 125 ml/kg, as noted previously (7).

The increase in the biological half-lives of these compounds did not tend to increase their V_d, as was implicated with the compounds following a two- or three-compartment open model. This finding suggests that, in the case of compounds (organic anions) whose distribution is primarily restricted to well-perfused tissues, the increase in their residence time in the body (because of their increased biological half-lives) brought about by the renal tubular secretion inhibitor would probably not increase their V_d.

CONCLUSIONS

It is recognized that a majority of drugs do not possess the ideal properties described here for the model compounds; most drugs are metabolized, bound to plasma proteins, may or may not be secreted or reabsorbed in the renal tubules, or may remain in the systemic body fluids partially or completely ionized or unionized. It is also recognized that the renal tubular secretion inhibitor of substrate drugs may or may not interfere with these processes of the substrate drugs. However, in the event that differences are observed in the distribution pharmacokinetic parameters of a drug in the presence of its renal tubular secretion inhibitor, it is desirable to know what factors are primarily responsible for such differences. Unless one is cognizant of all possible effects each factor can produce, the task of proposing mechanisms to account for the changes observed becomes difficult and highly speculative.

The literature survey indicated that either no deliberate efforts have been made to determine the effect of renal tubular secretion inhibitors on the distribution pharmacokinetic parameters of the substrate compounds or, if such efforts have been made, the reasons offered have remained highly speculative. Therefore, it is extremely desirable to build up a body of knowledge, based on model studies where the effect of each factor is exclusively determined, which will allow more realistic speculation. Although the compounds employed in the study are not used as drugs, except for D-(−)-mandelic acid, they served as model compounds to reveal not only features involved in the transmembrane transport process of organic anions but also to illustrate the influence of a renal tubular secretion inhibitor on the distribution pharmacokinetic parameters of these substrate organic anions.

The essential features revealed for the compounds exhibiting multicompartment open-model systems are that: (*a*) the organic anions most probably diffuse through the aqueous pores of the membranes; (*b*) the permeating molecules are subjected to electrostatic forces, hydrogen bonding, and hydrophobic bonding in the aqueous pores; and (*c*) the heteroporosity of the membranes may influence the volume of a given body compartment. The feature revealed by the compounds that followed a one-compartment open model was that the volume of distribution of the substrate organic anions would not necessarily change in the presence of their renal tubular secretion inhibitor.

It is hoped that the insight gained into the pharmacokinetic be-

havior of the model organic anions employed in this study will provide further understanding, not only of the design and modification of drug molecules but also of the effects of the competitive renal tubular secretion inhibitor on the distribution pharmacokinetic parameters of the substrate compounds.

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Behavior of Erythrocytes in Ternary Solvent Systems

SHIAO-HUAN KU and DONALD E. CADWALLADER ˣ

Abstract □ The effect of ternary solvent systems on erythrocytes was investigated. Hemolysis experiments were run at 37° in solutions containing various amounts of water, two nonaqueous solvents, and 0.9% sodium chloride. The nonaqueous solvents were propylene glycol, polyethylene glycol 400, dimethyl sulfoxide, dimethylformamide, and tetramethylurea. Ternary diagrams based on the critical hemolytic compositions of the various ternary systems are presented.

Keyphrases □ Erythrocytes—behavior in ternary solvent systems, ternary diagrams, critical hemolytic compositions □ Solvents—effect of ternary solvent systems on erythrocytes, ternary diagrams, critical hemolytic compositions □ Blood—behavior of erythrocytes in ternary solvent systems, ternary diagrams, critical hemolytic compositions □ Intravenous preparations—behavior of erythrocytes in ternary solvent systems

To prepare a safe and efficacious injection, it is sometimes necessary to employ a mixed solvent system consisting of water and a nonaqueous solvent. The behavior of rabbit and human erythrocytes in aqueous solutions of propylene glycol and liquid polyethylene glycol (1–3) and the behavior of human erythrocytes in aqueous solutions of dimethyl sulfoxide, liquid amides, tetramethylurea systems, and monohydric alcohols (4–7) were reported in previous papers in this series.

Binary solvent systems are not the only systems that can be used for intravenous preparations. Since aqueous solutions containing two or more nonaqueous solvents might be necessary to prepare a suitable solution, the effects of some ternary solvent systems on red blood cells were studied. Experiments were conducted to determine the behavior of human erythrocytes in systems containing two nonaqueous solvents in 0.9% aqueous saline.

The nonaqueous solvents selected were propylene glycol, polyethylene glycol 400, dimethyl sulfoxide, dimethylformamide, and tetramethylurea. Critical hemolytic concentrations were determined for the various ternary solvent systems, and the data were used to construct ternary diagrams depicting the various concentrations of water and two nonaqueous solvents, mixed together as ternary solvent blends, which are compatible with human red cells.

EXPERIMENTAL

Materials—Propylene glycol[1] USP, polyethylene glycol 400[2] (average mol. wt. 380–420), tetramethylurea[3], and reagent grades of dimethylformamide[4] and sodium chloride[2] were used.

Preparation of Solutions—All solvent systems were volume-

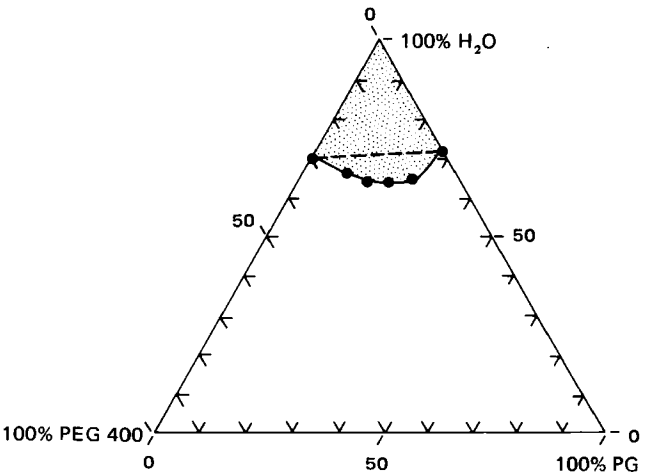

Figure 1—*Ternary diagram for aqueous propylene glycol (PG)–polyethylene glycol 400 (PEG 400) solutions containing 0.9% sodium chloride at 37°.*

[1] Fisher Scientific Co.
[2] J. T. Baker Chemical Co.
[3] Aldrich Chemical Co.
[4] Mallinckrodt Chemical Works.

havior of the model organic anions employed in this study will provide further understanding, not only of the design and modification of drug molecules but also of the effects of the competitive renal tubular secretion inhibitor on the distribution pharmacokinetic parameters of the substrate compounds.

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Behavior of Erythrocytes in Ternary Solvent Systems

SHIAO-HUAN KU and DONALD E. CADWALLADER ˣ

Abstract □ The effect of ternary solvent systems on erythrocytes was investigated. Hemolysis experiments were run at 37° in solutions containing various amounts of water, two nonaqueous solvents, and 0.9% sodium chloride. The nonaqueous solvents were propylene glycol, polyethylene glycol 400, dimethyl sulfoxide, dimethylformamide, and tetramethylurea. Ternary diagrams based on the critical hemolytic compositions of the various ternary systems are presented.

Keyphrases □ Erythrocytes—behavior in ternary solvent systems, ternary diagrams, critical hemolytic compositions □ Solvents—effect of ternary solvent systems on erythrocytes, ternary diagrams, critical hemolytic compositions □ Blood—behavior of erythrocytes in ternary solvent systems, ternary diagrams, critical hemolytic compositions □ Intravenous preparations—behavior of erythrocytes in ternary solvent systems

To prepare a safe and efficacious injection, it is sometimes necessary to employ a mixed solvent system consisting of water and a nonaqueous solvent. The behavior of rabbit and human erythrocytes in aqueous solutions of propylene glycol and liquid polyethylene glycol (1–3) and the behavior of human erythrocytes in aqueous solutions of dimethyl sulfoxide, liquid amides, tetramethylurea systems, and monohydric alcohols (4–7) were reported in previous papers in this series.

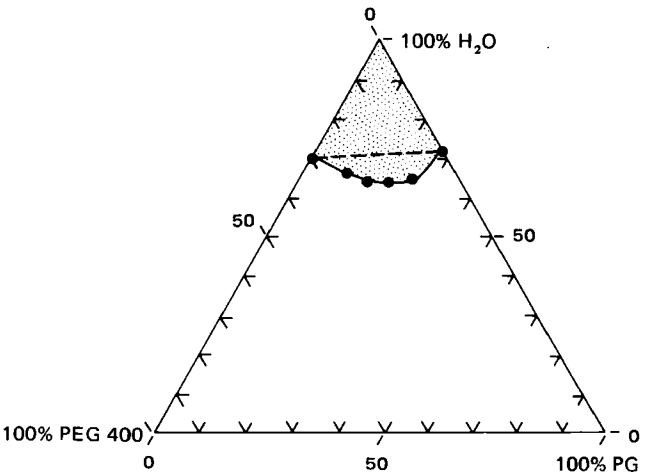
Binary solvent systems are not the only systems that can be used for intravenous preparations. Since aqueous solutions containing two or more nonaqueous solvents might be necessary to prepare a suitable solution, the effects of some ternary solvent systems on red blood cells were studied. Experiments were conducted to determine the behavior of human erythrocytes in systems containing two nonaqueous solvents in 0.9% aqueous saline.

The nonaqueous solvents selected were propylene glycol, polyethylene glycol 400, dimethyl sulfoxide, dimethylformamide, and tetramethylurea. Critical hemolytic concentrations were determined for the various ternary solvent systems, and the data were used to construct ternary diagrams depicting the various concentrations of water and two nonaqueous solvents, mixed together as ternary solvent blends, which are compatible with human red cells.

EXPERIMENTAL

Materials—Propylene glycol[1] USP, polyethylene glycol 400[2] (average mol. wt. 380–420), tetramethylurea[3], and reagent grades of dimethylformamide[4] and sodium chloride[2] were used.

Preparation of Solutions—All solvent systems were volume-



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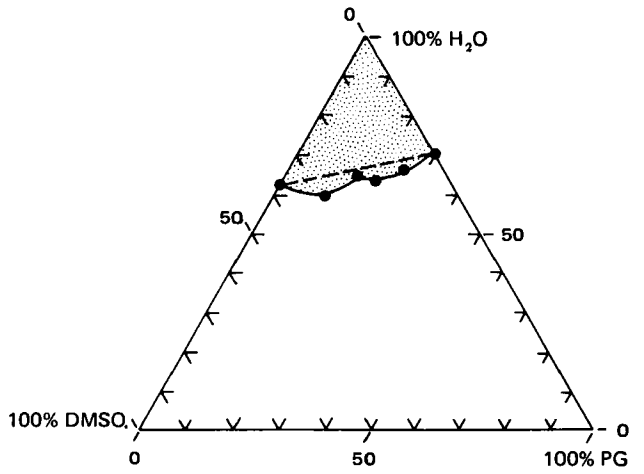

Figure 2—*Ternary diagram for aqueous propylene glycol (PG)–dimethyl sulfoxide (DMSO) solutions containing 0.9% sodium chloride at 37°.*

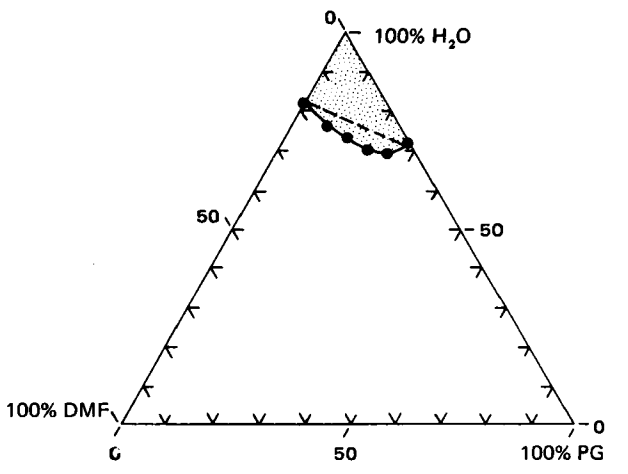

Figure 4—*Ternary diagram for aqueous propylene glycol (PG)–dimethylformamide (DMF) solutions containing 0.9% sodium chloride at 37°.*

in-volume percentage preparations, and each ternary system contained 0.9% (w/v) sodium chloride (0.15 *M*). Necessary dilutions were made from stock solutions, and distilled water was used to prepare all solutions.

Collection of Blood—Blood samples were obtained from the forearm veins of several 20–25-year-old Caucasian and Oriental subjects. Fresh blood samples were used. Approximately 10 ml of blood was obtained from each donor and placed in a 50-ml round-bottom flask containing 10–15 glass beads. The flask was rotated gently for about 5 min to separate the fibrin, and then the blood was decanted into a 50-ml conical flask and aerated by swirling the flask gently for about 5 min.

Quantitative Determination of Percent Hemolysis—In each experiment, the hemolytic method was used to determine the degree of hemolysis of erythrocytes in the various solvent systems. This quantitative method is based on the fact that a hypotonic solution liberates oxyhemoglobin in direct proportion to the number of cells hemolyzed.

Into each of two test tubes was transferred 5 ml of the standard sodium chloride solution (0.32, 0.34, . . ., 0.46, and 0.48%) or 5 ml of the mixed solvent systems. The standard sodium chloride solutions were used to ensure the osmotic normalcy of each blood sample. After the test tubes were brought to a constant temperature in a water bath (37 ± 0.5°), 0.05 ml of blood was pipetted into each tube. Each tube was shaken on a vibratory mixer to ensure thorough mixing and allowed to remain at 37° for 45 min. The tubes were centrifuged at approximately 2500 rpm, and the light absorbance of the supernatant liquid was measured using a photoelectric colorimeter[5] equipped with a No. 54 filter.

To find the percent hemolysis, these absorbance readings were divided by the absorbance readings for 0.05 ml of blood in 5 ml of distilled water (standard for 100% hemolysis) and multiplied by 100. A blank, made by placing 0.05 ml of blood in 5 ml of 0.9% sodium chloride solution, was used to cancel any light absorbance inherent in the blood sample. Both the standard and the blank were subjected to the same conditions of standing at 37° for 45 min followed by centrifuging (2500 rpm for 5–10 min).

Construction of Ternary Diagram—A ternary phase diagram can be constructed when three liquid components are involved in a solution at a fixed temperature. In the current studies, one apex of the triangle represents 100% of water, and each of the other two apexes represents 100% of a different nonaqueous component. The side of the triangle opposite each apex represents zero percentage of that particular component.

The points in the ternary diagram are the experimental values, and each point represents the average of at least two experiments using different blood samples. The points were connected to give an experimental curve for the particular ternary solvent system. The region within each curve (shaded area) defines the compositions of ternary solvent systems in which human red cells do not hemolyze (<5% hemolysis) under the experimental conditions. The region outside the curve describes the solvent compositions

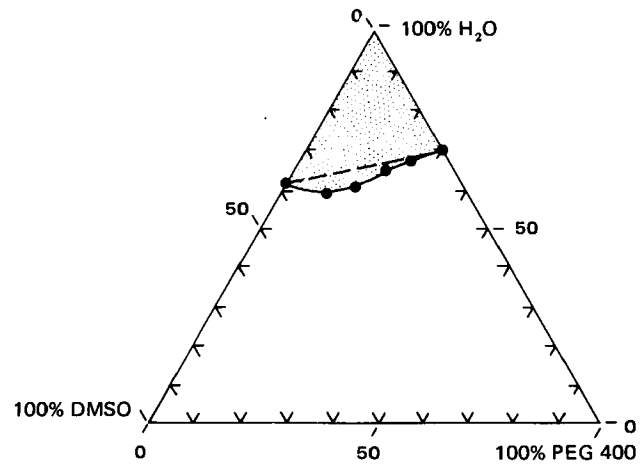

Figure 3—*Ternary diagram for aqueous polyethylene glycol 400 (PEG 400)–dimethyl sulfoxide (DMSO) solutions containing 0.9% sodium chloride at 37°.*

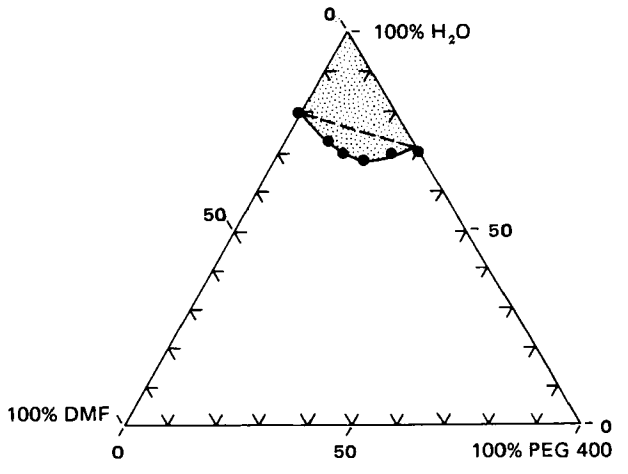

Figure 5—*Ternary diagram for aqueous polyethylene glycol 400 (PEG 400)–dimethylformamide (DMF) solutions containing 0.9% sodium chloride at 37°.*

[5] Klett–Summerson.

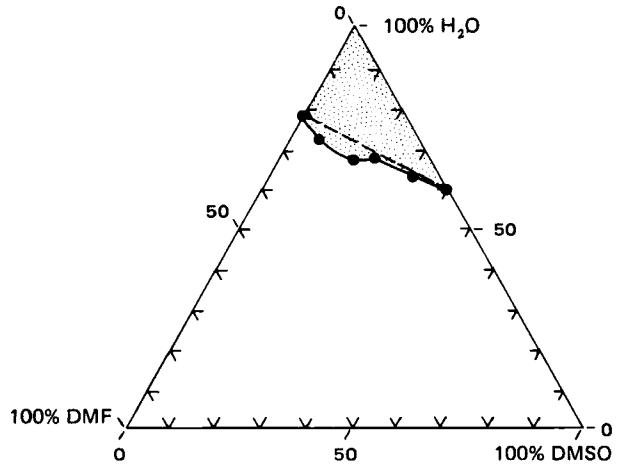

Figure 6—*Ternary diagram for aqueous dimethyl sulfoxide (DMSO)–dimethylformamide (DMF) solutions containing 0.9% sodium chloride at 37°.*

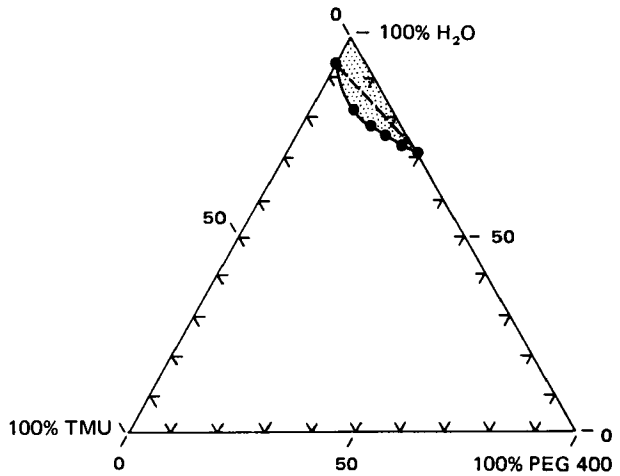

Figure 8—*Ternary diagram for aqueous polyethylene glycol 400 (PEG 400)–tetramethylurea (TMU) solutions containing 0.9% sodium chloride at 37°.*

where hemolysis and/or discoloration and precipitation were not prevented.

Each ternary diagram has a tie-line (dashed line) connecting the critical concentrations of the two nonaqueous solvents. If the hemolytic effect of each individual component is strictly an additive property of the solvent, this tie-line should represent the theoretical curve delineating nonhemolytic from hemolytic compositions.

RESULTS AND DISCUSSION

Binary Solvent Systems—Experiments were carried out to determine the critical hemolytic concentration of the individual nonaqueous solvents in 0.9% saline solution. These data were utilized for the baseline points in the construction of the individual ternary diagrams. The critical hemolytic concentrations (% v/v) of the solvents were: propylene glycol, 30%; polyethylene glycol 400, 31%; dimethyl sulfoxide, 38%; dimethylformamide, 20%; and tetramethylurea, 8%. Each critical concentration is the average of four experiments with different blood samples and is in good agreement with the data reported previously (2–6).

Ternary Solvent Systems—To determine the effect of ternary solvent systems on erythrocytes, hemolysis experiments were run at 37° in solutions containing various amounts of water, two nonaqueous solvents, and 0.9% sodium chloride. Ternary diagrams showing the critical concentrations of each ternary solvent system are shown in Figs. 1–10. These curves were constructed as described under *Experimental*, utilizing the experimental data. In each diagram, the experimental curve separates the nonhemolytic from the hemolytic compositions of the ternary systems. The transition from nonhemolytic to hemolytic concentrations was very abrupt.

All ternary solvent systems had experimental curves that were convex with reference to the theoretical tie-lines and penetrated into higher nonaqueous solvent concentration areas. This finding indicated that one or both of the nonaqueous components in the solvent system contributed somewhat to the tonicity of the extracellular solvent. Since there was no radical departure of the experimental curves from the theoretical tie-lines, the hemolytic effect of the nonaqueous combinations apparently was essentially dependent on their total concentration in solution and did not depend on one solvent being at or near its critical concentration.

The most convex curves were obtained for ternary systems containing polyethylene glycol 400 (Figs. 1, 3, 5, and 8), while the least deviation from the theoretical tie-line was observed for the aqueous dimethylformamide–tetramethylurea system (Fig. 10). The results indicated that the hemolytic effect of the aqueous dimethylformamide–tetramethylurea system was additive, since the experimental curve was very similar to the theoretical tie-line. The relatively greater convex experimental curves of the ternary systems containing polyethylene glycol 400 indicated that these systems offered more protection to red cells than did solvent systems without polyethylene glycol 400.

Previous workers (3) reported that polyethylene glycol 400 has the ability to contribute to the tonicity of extracellular solutions, and they calculated a hemolytic i value (isotonic coefficient) of 0.6

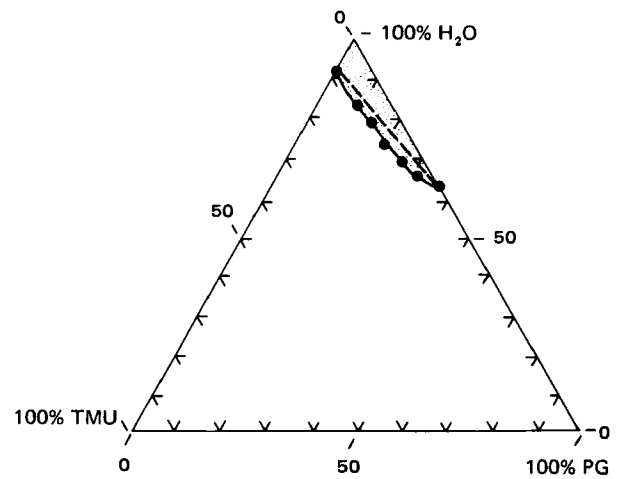

Figure 7—*Ternary diagram for aqueous propylene glycol (PG)–tetramethylurea (TMU) solutions containing 0.9% sodium chloride at 37°.*

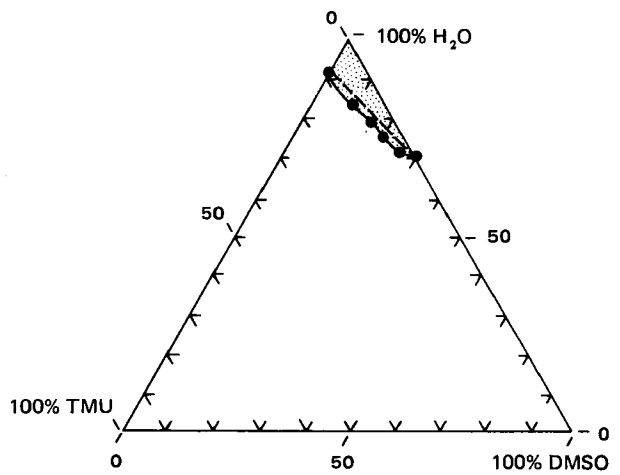

Figure 9—*Ternary diagram for aqueous dimethyl sulfoxide (DMSO)–tetramethylurea (TMU) solutions containing 0.9% sodium chloride at 37°.*

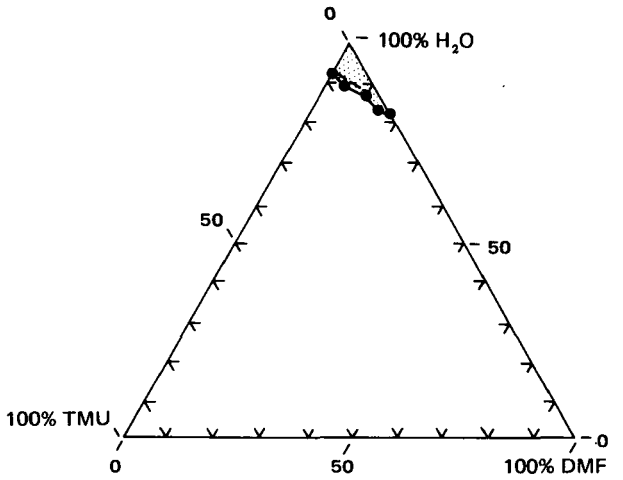

Figure 10—*Ternary diagram for aqueous dimethylformamide (DMF)–tetramethylurea (TMU) solutions containing 0.9% sodium chloride at 37°.*

for this compound. All other solvents have been reported to offer very little protection to red blood cells from hemolysis at all concentrations (1, 4–6). Therefore, the convex deviation from the theoretical tie-line can be explained by the fact that polyethylene glycol 400 contributed to the tonicity of the ternary solvent by virtue of its intrinsic property of incomplete penetration into the red blood cell.

The components of ternary solvent systems not containing polyethylene glycol 400 do contribute to the tonicity of the system but to a lesser degree than the polyethylene glycol 400 systems. Hemolytic isotonic coefficients (i values) were reported previously for sodium chloride in all solvents used in this study (1, 4–6). These i values for sodium chloride in aqueous solutions containing approximately one-half the critical hemolytic concentrations of the solvents were 1.71 for 4% tetramethylurea (6), 1.90 for 10% dimethylformamide (5), 1.95 for 15% propylene glycol (1), and 2.10 for 20% dimethyl sulfoxide (4).

When the isotonic coefficients are used as an indicator of the individual solvent's contribution to the tonicity of extracellular solutions, tetramethylurea would have the least contribution and dimethyl sulfoxide the most. Therefore, according to these hemolytic i values, the aqueous tetramethylurea–dimethylformamide system (average i value 1.80) should contribute very little, if at all, to the extracellular tonicity and should display little deviation from the theoretical tie-line. The experimental data (Fig. 10) are in agreement with this explanation.

The experimental data for the other systems also concur with this explanation, since the rank order of the average sodium chloride i values of the two nonaqueous components in a particular ternary system was approximately the same as the rank order of the degree of deviation of the experimental curves from their theoretical tie-lines. The rank order for the amount of deviation from the theoretical tie-line was propylene glycol–dimethyl sulfoxide (i = 2.03) > dimethylformamide–dimethyl sulfoxide (i = 2.00) > dimethylformamide–propylene glycol (i = 1.93) = tetramethylurea–dimethyl sulfoxide (i = 1.92) > tetramethylurea–propylene glycol (i = 1.83) > tetramethylurea–dimethylformamide (i = 1.80).

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W. J. GRIFFIN [x], H. P. BRAND, and J. G. DARE

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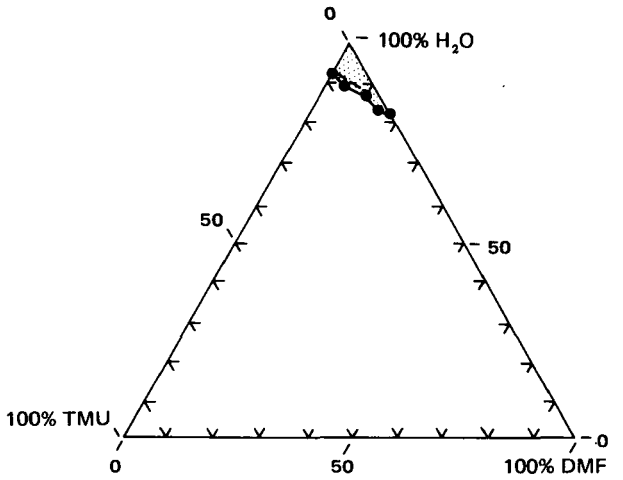
Keyphrases □ *Duboisia myoporoides* and *Duboisia leichhardtii*—GLC analysis for scopolamine and hyoscyamine, slope ratio method □ Scopolamine and hyoscyamine—GLC determination in *Duboisia* samples, slope ratio method □ Phenylacetyltropine—internal standard in GLC analysis of scopolamine and hyoscyamine in *Duboisia* samples

Two species of *Duboisia, Duboisia leichhardtii* and *Duboisia myoporoides,* are commercial sources of the mydriatic tropane alkaloid scopolamine, 1000 tons being grown annually. They are cultivated together with their hybrids, and it was essential that an accurate analytical method for the major alkaloids, scopolamine and hyoscyamine, be established for cultivation trials. The assay is complicated by the wide spectrum of tropane alkaloids the genus offers (Table I). Coulson and Griffin (1) found that aerial parts of



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D. myoporoides contain 12 different tropane alkaloids. The complex nature is complemented by the reported presence of the pyridine alkaloids, nicotine and anabasine (2).

Various analytical procedures for the assessment of scopolamine and hyoscyamine have been published. Solomon *et al.* (3) were the first to apply GLC to the quantitation of scopolamine and atropine in plant powders. A standard linear regression line was established of quantity *versus* peak area. For the nine results quoted, the amounts estimated differed from the actual amounts by as much as 27.5%, although five differed by less than 4%. On-column decomposition of the alkaloids to their apo forms also occurred.

An assay procedure was described for unit dose quantities of scopolamine, using homatropine as an internal standard (4, 5). The amounts estimated differed from the stated actual amount by as much as 14%. The use of tetraphenylethylene as an internal standard was described (6).

This paper reports the use of phenylacetyltropine as an internal standard and gives a critical appraisal of various assays. Where estimations from a known regression were made, predicted inverse tolerance limits were obtained according to Williams (7).

EXPERIMENTAL[1]

Materials—The carrier gas was helium. Dual columns of Pyrex glass (150 cm × 4 mm i.d.) were packed with acid-washed, silanized Chromosorb W (80–100 mesh) containing 1.5% SE-30. The silanized support material was coated with a trace of polyethylene glycol 4000 and then with SE-30 according to the method of Street (8). The packing was held by a small plug of silanized glass wool.

All solvents were fractionally distilled, and the chemicals used were of analytical reagent grade. Plant material was dried at 50° and then milled[2] to a moderately coarse powder (BP). Hyoscyamine base[3], scopolamine hydrobromide[3], homatropine[3], hexamethyldisilazane[4], *N,O*-bis(trimethylsilyl)acetamide[5], and tetraphenylethylene[6] were obtained commercially. Phenylacetyltropine was prepared by chemical synthesis.

Slope Ratio Assay—The column temperature was 194° with an injection port heater set at 244°. The gas inlet pressure was 28 psi and gave a flow rate of 190 ml/min. The output was attenuated ×2000 to a 10-mv recorder with a chart speed of 20 mm/min. Peak areas were measured by the peak height–peak width method (9).

Solutions were prepared by dissolving scopolamine in pyridine containing 5% hexamethyldisilazane on the day before use. By forming the trimethylsilyl derivative, on-column decomposition to the apo form was avoided and symmetrical peaks with no tailing were obtained. Both the standard and unknown preparations were injected on the column in five replicates using two separate volumes, 10 and 20 μl, to simplify the statistical evaluation.

Several assays (A–C) were performed in which the exact milligrams per milliliter content of scopolamine was known in both the standard and the unknown but in which the strength of the unknown solution was not disclosed to the analyst until after the assay was completed. A commercial sample of *Duboisia* (D), previously assayed by a titrimetric method (10), was analyzed. Other samples of *Duboisia* (E–H) fortified with increasing amounts of scopolamine were assayed similarly (Table II).

A 10-g sample of coarsely powdered *D. leichhardtii*[7] leaves, mixed with 1 g of calcium hydroxide, was moistened with 10 ml of distilled water (or, in later experiments, water containing a known quantity of scopolamine hydrobromide). After 30 min to allow a thorough penetration of alkali, the mixture was exhaustively extracted by repeated maceration with cold ether (3 × 150 ml). The combined ether extracts, after concentration to 100 ml, were extracted with 0.5 *N* hydrochloric acid (2 × 100 ml), and the combined acidic extracts were basified with excess ammonium hydroxide solution (wt./ml 0.88).

The alkaloid bases were extracted with chloroform (3 × 150 ml), and the combined chloroform extracts were evaporated under reduced pressure. The residue was taken up in pyridine containing 5% hexamethyldisilazane. The solution was quantitatively transferred to a 50-ml volumetric flask, and the volume was adjusted with more solvent.

Internal Standard Assay—The column temperature was 235° with an injection port heater set at 285°. The gas inlet pressure was 15 psi and gave a flow rate of 60 ml/min. The output was attenuated ×5000 to a 10-mv integrating recorder.

Regression Line Investigation for Phenylacetyltropine and Tetraphenylethylene—Standard solutions of scopolamine hydrobromide (0.125 g/50 ml of water), phenylacetyltropine picrate (0.125 g/50 ml of chloroform), hyoscyamine (0.075 g/50 ml of chloroform), and tetraphenylethylene (0.125 g/50 ml of chloroform) were prepared. One milliliter of the phenylacetyltropine solution was added to 16 separators using a buret[8]. To every separator were added measured volumes of the scopolamine and hyoscyamine solutions (4 × 0.5, 4 × 1, 4 × 1.5, and 4 × 2 ml), so that each separator contained identical volumes of the two solutions.

The volumes of organic and aqueous phases in each separator were adjusted to 3 and 2 ml, respectively, by appropriate addition, if necessary, of chloroform and water. The extraction then was continued as follows. Approximately 1 ml of ammonium hydroxide solution (wt./ml 0.88) was added, and the two phases were shaken together. The chloroform layer was run off into a stoppered test tube, and the aqueous layer was reextracted with chloroform (2 × 2 ml). To the combined chloroform extracts was added 1 ml of the tetraphenylethylene standard solution, and the extract was evaporated under a stream of nitrogen. The residue was taken up in 1 ml of anhydrous pyridine, and 0.30 ml of hexamethyldisilazane was added. After 8 hr, 10 μl of the silanized extract was subjected to GLC analysis.

Regression Line Investigation for Homatropine—Standard solutions of scopolamine hydrobromide (0.254 g/100 ml of water), hyoscyamine (0.156 g/100 ml of chloroform), and homatropine (0.200 g/100 ml of chloroform) were prepared. Extracts were prepared as described previously with the omission of the standard solutions of phenylacetyltropine and tetraphenylethylene, the inclusion of homatropine as the internal standard, and the replacement of hexamethyldisilazane by *N,O*-bis(trimethylsilyl)acetamide as the silanizing agent.

Table I—GLC Retention Times of *Duboisia* Alkaloids[a]

Alkaloids	A[b]	B[c]
Tetraphenylethylene[d]	1.27	1.30
Scopolamine	1.0	1.0
Hyoscyamine	0.73	0.69
Norhyoscyamine	0.74	0.69
Homatropine[d]	0.53	0.47
Meteloidine[e]	0.37	0.31
Phenylacetyltropine[d]	0.36	0.27
Valeroidine	0.18	0.13
Tigloidine	0.15	0.10
Valtropine	0.12	0.07
Butropine	0.09	0.05
Anabasine	0.09	0.05
Nicotine	0.07	0.03
Acetyltropine	0.06	0.03

[a] Actual retention time = (A) × 15 min and (B) × 32.8 min. All alkaloids were treated with hexamethyldisilazane. [b] Column temperature was 233°, column pressure was 15 psi, and flow rate was 60 ml/min. [c] Column temperature was 194°, column pressure was 28 psi, and flow rate was 190 ml/min. [d] Internal standards. [e] A tropane alkaloid not isolated from *Duboisia*.

[1] A Pye series 104 gas chromatograph fitted with dual flame-ionization detectors and a Rikadenki 10-mv integrating recorder were used.
[2] The mill was supplied by Christy and Norris Ltd., Chelmsford, U.K.
[3] Macfarlan Smith Ltd., Edinburgh, U.K.
[4] British Drug House Chemicals Ltd., Poole, U.K.
[5] Supelco, Inc., Bellefonte, Pa.
[6] Koch Light Laboratories, Colnbrook, U.K.
[7] Supplied by E. Carter and Son, cultivators of *Duboisia*.
[8] Metrohm.

Table II—Results of Slope Ratio Assays

Assay	Amount Found	Confidence Limits, $p = 0.99$		Confidence Limits, $p = 0.99$, as Percentages	Actual Amount	Percentage Error from Actual
		Lower	Upper			
		Analysis of Known Solutions of Scopolamine				
A	3.5546 mg/ml	3.4945 mg/ml	3.6147 mg/ml	±1.68	3.5768 mg/ml	−0.62
B	3.6412 mg/ml	3.5953 mg/ml	3.6870 mg/ml	±1.46	3.5768 mg/ml	+1.80
C	3.5343 mg/ml	3.4705 mg/ml	3.5880 mg/ml	±1.50	3.5768 mg/ml	−0.91
Mean	3.5767 mg/ml				3.5768 mg/ml	
		Analysis of Samples of *D. leichhardtii* Leaves				
D	0.435%	0.430%	0.442%		0.44%	
E	65.747 mg	64.747 mg	66.858 mg	±1.68	65.747 mg	
F[a]	76.378 mg	73.180 mg	79.570 mg	±4.17	76.336 mg	+0.05
G[b]	82.032 mg	79.500 mg	84.500 mg	±3.00	81.181 mg	+1.04
H[c]	83.103 mg	80.210 mg	85.990 mg	±3.47	84.849 mg	+2.05

[a] E + 10.589 mg of scopolamine. [b] E + 15.434 mg of scopolamine. [c] E + 19.102 mg of scopolamine.

Table III—Regression Equations for Internal Standard Assays[a]

Internal Standard	Scopolamine	Hyoscyamine
Phenylacetyltropine (0.0025 g picrate)	$y = 0.817529x - 0.153925$	$y = 0.80385x - 0.130925$
Tetraphenylethylene (0.0025 g)	$y = 0.267702x - 0.0722498$	$y = 0.29095x - 0.082875$
Homatropine (0.0020 g)	$y = 0.834662x - 0.057589$	$y = 0.71885x - 0.026988$

[a] x = weight of scopolamine or hyoscyamine expressed in milligrams; y = ratio of integrated areas of scopolamine or hyoscyamine *versus* internal standard.

Extraction and Assay—A 10-g sample of coarsely powdered *D. leichhardtii* leaves, mixed with 1 g of calcium hydroxide, was moistened with 10 ml of distilled water. After 30 min to allow a thorough penetration of alkali, the mixture was exhaustively extracted by repeated maceration with cold ether (3 × 150 ml). On evaporation, the combined ether extracts gave a residue which was taken up in chloroform and the volume was adjusted to 25 ml. A suitable quantity (1, 2, or 4 ml) was pipetted into a separator, and 1 ml of either homatropine or phenylacetyltropine solution was added. The volume was adjusted to 5 ml if necessary with chloroform.

The chloroform extract was shaken with 0.5 *N* hydrochloric acid (1 × 5 and 1 × 3 ml), and 1 ml of ammonium hydroxide solution (wt./ml 0.88) was added to the combined acidic extract, which was extracted with chloroform (1 × 5, 1 × 3, and 1 × 2 ml). Then the chloroform extracts were combined. One milliliter of tetraphenylethylene was added if required as an internal standard. The chloroform was removed under a stream of nitrogen, the residue was dissolved in 1 ml of anhydrous pyridine, and 0.3 ml of either hexamethyldisilazane or *N,O*-bis(trimethylsilyl)acetamide was added according to the internal standard employed. After 8 hr, a 10-μl sample was subjected to GLC analysis.

Phenylacetyltropine—Atropine sulfate (5 g) was refluxed with barium hydroxide (6 g) in distilled water (200 ml) for 2 hr. After cooling, excess 2 *N* sulfuric acid was added and the barium sulfate so formed was filtered off. The filtrate was extracted with ether (2 × 150 ml) to remove tropic acid. The aqueous fraction was made alkaline with excess ammonium hydroxide solution (wt./ml 0.88) and extracted with chloroform (4 × 100 ml).

The chloroform extracts were combined and gave tropine (2 g, 95% yield) after evaporation under reduced pressure. Tropine (1 g) was added to phenylacetyl chloride (20 ml), and the mixture was heated for 2 hr on a water bath. The reaction mixture was diluted with 80 ml of distilled water and made acidic with 2 *N* sulfuric acid. Phenylacetic acid was removed by ether extraction (2 × 100 ml). The aqueous residue was made alkaline with the addition of excess ammonium hydroxide solution (wt./ml 0.88) and extracted with chloroform (3 × 100 ml). Evaporation of the combined chloroform extracts gave phenylacetyltropine which, when neutralized with 0.2 *N* sulfuric acid and treated with a saturated aqueous solution of sodium picrate, gave phenylacetyltropine picrate (2.5 g, 72% yield), mp 166°.

Anal.—Calc. for $C_{16}H_{21}NO_2 \cdot C_6H_3N_3O_7$: C, 54.1; H, 4.9; N, 11.5. Found: C, 54.4; H, 5.1; N, 11.2.

Sampling from a Bale of Duboisia *Leaf*—One bale (175 × 150 × 100 cm, approximately 175 kg) of commercial *D. myoporoides* leaves[9] was subdivided into 11 vertical layers (A–K). Then each layer was divided into 16 lots, distributed horizontally in a 4 × 4 square so that the horizontal position of each lot could be identified. From these, four lots were selected at random, namely E5, F9, G2, and J12. Each lot was subdivided into 16 samples. Four samples were selected randomly from each lot and annotated such that E5/15 indicates Layer E, Lot 5, Sample 15. Each sample was analyzed with four replicates, using phenylacetyltropine as the internal standard.

RESULTS AND DISCUSSION

Slope Ratio Assay—Two injection volumes of a standard solution, *S* (10 and 20 μl), and two identical volumes of an unknown solution, *U*, are injected into the gas chromatograph. If the two regression lines of the responses to *S* and *U* intersect at the origin, then the ratio of the concentrations of the two solutions is equal to the ratio of the slopes of the regression lines. By using the appropriate experimental design and tests of significance, an estimate of confidence limits may be calculated from internal evidence in the assay. The statistical methods used were fully discussed by Finney (11).

For Assays A, B, and C, the analyst was presented with two solutions, labeled standard and unknown. The three standards were of different concentrations, but in each case the unknown was actually a sample of one solution. Thus, the analyst was unknowingly assaying the same preparation; but since the standards differed for each assay, the slope ratios in each case were different. Individual assays showed some error, varying from −0.62 to +1.8% (Table II). Each assay provided its own limits of precision, but in one instance the true concentration was just outside the limits. Confidence limits in Assays E, F, G, and H, in which varying amounts of scopolamine were added as the hydrobromide to powdered *D. leichhardtii*

[9] The bale was supplied by G. R. Davies, Hornsby, N.S.W. A voucher specimen, annotated BRI 179202, was lodged with the Queensland Herbarium.

Table IV—Predicted x Values and Tolerance Limits Determined from Regression Equations of the Internal Standard Assay Using y Values at the Mean, Upper, and Lower Parts of the Regression Line

Internal Standard	Assay for	y Value Observed	Mean of	Predicted x Value	Limits, $p = 0.95$
Phenylacetyltropine	Scopolamine	4.09	1	5.19	4.61–5.77 (±0.58, ±11.2%)
		4.09	4	5.19	4.85–5.50 (±0.31, ±6%)
		1.30	1	1.78	1.16–2.38 (±0.60, ±33%)
		1.30	4	1.78	1.31–2.25 (±0.47, ±25%)
		7.0	1	8.75	8.15–9.37 (±0.62, ±7.1%)
		7.0	4	8.75	8.40–9.12 (±0.37, ±4.2%)
Phenylacetyltropine	Hyoscyamine	3.48	1	4.5	3.97–5.02 (±0.52, ±11.2%)
		3.48	4	4.5	4.22–4.77 (±0.27, ±6.2%)
		1.1	1	1.53	0.97–2.06 (±0.53, ±35%)
		1.1	4	1.53	1.20–1.84 (±0.31, ±20%)
		6.0	1	7.63	7.10–8.18 (±0.55, ±7.3%)
		6.0	4	7.63	7.31–7.96 (±0.33, ±4.4%)
Tetraphenylethylene	Scopolamine	1.085	1	4.32	4.03–4.62 (±0.30, ±6.1%)
		1.085	4	4.32	4.16–4.48 (±0.16, ±3.6%)
		0.4	1	1.76	1.45–2.06 (±0.30, ±17%)
		0.4	4	1.76	1.57–1.94 (±0.18, ±13%)
		1.8	1	7	6.68–7.30 (±0.30, ±4.4%)
		1.8	4	7	8.81–7.18 (±0.18, ±2.7%)
Tetraphenylethylene	Hyoscyamine	1.01	1	3.75	3.35–4.15 (±0.40, ±10%)
		1.01	4	3.75	3.53–3.96 (±0.21, ±5.8%)
		0.4	1	1.66	1.23–2.07 (±0.41, ±24%)
		0.4	4	1.66	1.40–1.90 (±0.24, ±11%)
		1.8	1	6.47	6.04–6.91 (±0.44, ±6.8%)
		1.8	4	6.47	6.21–6.74 (±0.27, ±4.3%)
Homatropine	Scopolamine	0.98	1	1.25	1.03–1.46 (±0.21, ±17%)
		0.98	4	1.25	1.13–1.36 (±0.11, ±9.2%)
		0.4	1	0.55	0.32–0.76 (±0.21, ±39%)
		0.4	4	0.55	0.41–0.67 (±0.12, ±23%)
		1.6	1	1.98	1.77–2.21 (±0.23, ±11%)
		1.6	4	1.98	1.85–2.10 (±0.12, ±7.0%)
Homatropine	Hyoscyamine	0.87	1	1.25	1.10–1.39 (±0.14, ±11%)
		0.87	4	1.25	1.17–1.32 (±0.07, ±6.2%)
		0.30	1	0.45	0.30–0.60 (±0.15, ±33%)
		0.30	4	0.45	0.35–0.54 (±0.09, ±19%)
		1.50	1	2.12	1.97–2.28 (±0.16, ±7.3%)
		1.50	4	2.12	2.03–2.22 (±0.10, ±4.6%)

leaves, were greater than in previous assays because they included errors incurred in the additional extraction stage.

If Assay E is assumed to be exact, errors varied from +0.05 to +2.05%. Thus, the slope ratio method appears to provide a valuable and accurate quantitative assay for scopolamine.

Internal Standard Assay—Analysis of the results gave six regression equations for values of x (milligrams of scopolamine or hyoscyamine) and of y (ratio of integrated areas of alkaloid *versus* internal standard) (Table III). Silanization of the alkaloid mixture was essential to prevent dehydration to the apo derivatives. Of the internal standards employed, homatropine suffered the disadvantage that it underwent silanization with difficulty. Hexamethyldisilazane would not cause the silanization to go to completion, and a more active agent was necessary [*N,O*-bis(trimethylsilyl)-acetamide]. Internal standards such as homatropine and phenylacetyltropine are superior to tetraphenylethylene, since they are introduced into the assay during the extraction stage so that any losses during extraction are reflected in the confidence limits. The results demonstrate that of the three internal standards, phenylacetyltropine and tetraphenylethylene are the standards of choice.

If it is important that the confidence limits be close, for example to deduce whether two samples are different or if one is superior to the other, then replicate determinations will vastly narrow them. Such an occasion may arise in the selection of trees for cultivation trials. In the results (Table IV), x values are quoted at the mean of y and also at values of y at the lower and upper limits of the regression line. Greater accuracy can be achieved by adjusting the experiment so that the regression line in and around the mean is employed during the assay.

The assay for hyoscyamine also includes norhyoscyamine since both alkaloids have similar retention times. Norhyoscyamine has been isolated as a minor alkaloidal component of *D. myoporoides* (1) and *D. leichhardtii* (12). This does not detract from the value of the assay since samples of *Duboisia* for commercial purposes are estimated on their scopolamine content alone.

Commercial *Duboisia* consignments are obtained from both cultivated and natural sources. In the past, *D. leichhardtii* was the most cultivated of the two species. Cultivation of the genus was hampered by the poor germination of *Duboisia* seed, and most plantations were derived from naturally set seedlings. Hills *et al.* (13) found considerable variations in alkaloid type and yield in natural stands of *Duboisia*, suggesting the existence of chemical varieties. Thus, consignments of *Duboisia*, whether cultivated or naturally obtained, are unlikely to be homogeneous in alkaloid content.

Samples were selected randomly from a bale of *D. myoporoides*, which formed part of a commercial consignment obtained from natural sources. There was a threefold variation in the scopola-

Table V—Analysis of Samples of *D. myoporoides* Leaves Obtained from a Commercial Bale

	Percentage Scopolamine			Percentage Hyoscyamine		
		Confidence Limits, $p = 0.95$			Confidence Limits, $p = 0.95$	
Sample	Amount Found	Upper	Lower	Amount Found	Upper	Lower
E5/5	0.209	0.223	0.194	0.244	0.257	0.231
E5/15	0.487	0.507	0.468	0.213	0.225	0.201
E5/10	0.415	0.432	0.398	0.163	0.175	0.151
E5/13	0.552	0.575	0.531	0.121	0.134	0.109
F9/5	0.267	0.280	0.254	0.239	0.251	0.227
F9/15	0.351	0.367	0.333	0.169	0.180	0.157
F9/12	0.223	0.236	0.210	0.103	0.115	0.090
F9/3	0.256	0.269	0.243	0.165	0.176	0.153
G2/6	0.283	0.300	0.265	0.093	0.110	0.075
G2/16	0.414	0.431	0.398	0.078	0.091	0.065
G2/8	0.218	0.231	0.205	0.067	0.080	0.054
G2/11	0.372	0.387	0.357	0.104	0.116	0.092
J12/5	0.573	0.596	0.551	0.073	0.084	0.066
J12/3	0.586	0.611	0.564	0.088	0.100	0.075
J12/10	0.569	0.592	0.547	0.086	0.099	0.073
J12/14	0.587	0.611	0.565	0.220	0.208	0.232

mine content, depending on sample position within the bale (Table V). This finding demonstrates the difficulty in obtaining a representative sample from a consignment.

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Solubilization and Stabilization of the Cytotoxic Agent Coralyne

M. J. CHO [*§], A. J. REPTA [*x], C. C. CHENG [‡], K. Y. ZEE-CHENG [‡], T. HIGUCHI [*], and I. H. PITMAN [*]

Abstract □ Kinetic studies were carried out on the ring opening of the quaternary nitrogen cation, coralynium ion (I), to yield 6′-acetylpapaverine (III), on the cyclization of III to yield I, and on a photochemical reaction undergone by I in aqueous solutions exposed to visible light. From the results, it was concluded that: (*a*) I and III are in facile equilibrium in aqueous solution but appreciable amounts of III do not exist in dilute solutions with pH values below 10; (*b*) the photochemical reaction of I in water (presumably a photohydration) can be reversed by lyophilization, by heating, and by increasing the pH of solutions to values greater than 12; (*c*) the photochemical reaction of I can be inhibited by protecting the aqueous solutions from visible light, and the rate in the presence of light can be reduced by increasing the concentration of I in the solution; and (*d*) although the chloride and sulfoacetate salts of I react identically and have similar solubilities in water, it is possible to prepare more concentrated and, hence, more stable solutions of the sulfoacetate salt by including sodium hydroxide in the solvent. The solubility of coralyne chloride remains about the same in dilute sodium hydroxide as in water.

Keyphrases □ Coralyne salts—photohydration, ring cleavage, pH dependence of solubility, stabilization, formation of 6′-acetylpapaverine □ Cytotoxic agents—coralyne salts, solubilization and stabilization, photohydration, pH dependence, formation of 6′-acetylpapaverine □ 6′-Acetylpapaverine—kinetics of formation from coralynium ion, cyclization □ Solubility—coralyne chloride and sulfoacetate, pH dependence

Coralyne chloride (I*a*) is a berbinium salt possessing antileukemic activity against both the P-388 and L-1210 strains (1, 2). However, the clinical evaluation of I*a* has been hampered by the relatively high expected dose of 1-2 g[1] together with the rather low aqueous solubility of about 5 mg/ml and a report[2] that solutions of the drug stored under different conditions or for varying times exhibited altered spectral characteristics relative to freshly prepared solutions.

This study was undertaken to determine the stability of coralynium ion (I) in aqueous solution with respect to chemical transformation. Previous reports led to the expectation that the most likely degradation pathways would be a ring opening to yield 6′-acetylpapaverine (III) (3) and a covalent hydration reaction (4). Although these reactions were postulated, no quantitative information about reaction rates was reported.

An investigation into the chemical stability and solubility of coralyne sulfoacetate (I*b*), isolated as the initial product in a general synthesis of I (1), suggests that I*b* may be a better coralynium salt to use in liquid dosage forms than I*a*.

EXPERIMENTAL

Materials—Coralyne chloride[3] and coralyne sulfoacetate[4] were provided by the National Cancer Institute and used without further purification. The IR spectrum of coralyne chloride used was identical to the reported spectrum[2]. Coralyne sulfoacetate has an extra absorption band at 1720 cm^{-1} due to the carbonyl group in the anionic part. 6′-Acetylpapaverine was prepared, following the

[1] J. P. Davignon, National Cancer Institute, personal communication.

[2] P. Lim and S. Stone, Stanford Research Institute Report 9180 to National Cancer Institute, June 28, 1972.

[3] NSC 96349, lot pH 9-29-1, mol. wt. 399.9, mp 246–250° dec. [lit. (1) 248–250° dec.].

[4] NSC 154890, lot pH 5-113-1, mol. wt. 503.5, mp 268–274° dec. [lit. (1) 278–280° dec.].

mine content, depending on sample position within the bale (Table V). This finding demonstrates the difficulty in obtaining a representative sample from a consignment.

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Solubilization and Stabilization of the Cytotoxic Agent Coralyne

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Abstract □ Kinetic studies were carried out on the ring opening of the quaternary nitrogen cation, coralynium ion (I), to yield 6′-acetylpapaverine (III), on the cyclization of III to yield I, and on a photochemical reaction undergone by I in aqueous solutions exposed to visible light. From the results, it was concluded that: (*a*) I and III are in facile equilibrium in aqueous solution but appreciable amounts of III do not exist in dilute solutions with pH values below 10; (*b*) the photochemical reaction of I in water (presumably a photohydration) can be reversed by lyophilization, by heating, and by increasing the pH of solutions to values greater than 12; (*c*) the photochemical reaction of I can be inhibited by protecting the aqueous solutions from visible light, and the rate in the presence of light can be reduced by increasing the concentration of I in the solution; and (*d*) although the chloride and sulfoacetate salts of I react identically and have similar solubilities in water, it is possible to prepare more concentrated and, hence, more stable solutions of the sulfoacetate salt by including sodium hydroxide in the solvent. The solubility of coralyne chloride remains about the same in dilute sodium hydroxide as in water.

Keyphrases □ Coralyne salts—photohydration, ring cleavage, pH dependence of solubility, stabilization, formation of 6′-acetylpapaverine □ Cytotoxic agents—coralyne salts, solubilization and stabilization, photohydration, pH dependence, formation of 6′-acetylpapaverine □ 6′-Acetylpapaverine—kinetics of formation from coralynium ion, cyclization □ Solubility—coralyne chloride and sulfoacetate, pH dependence

Coralyne chloride (I*a*) is a berbinium salt possessing antileukemic activity against both the P-388 and L-1210 strains (1, 2). However, the clinical evaluation of I*a* has been hampered by the relatively high expected dose of 1-2 g[1] together with the rather low aqueous solubility of about 5 mg/ml and a report[2] that solutions of the drug stored under different conditions or for varying times exhibited altered spectral characteristics relative to freshly prepared solutions.

This study was undertaken to determine the stability of coralynium ion (I) in aqueous solution with respect to chemical transformation. Previous reports led to the expectation that the most likely degradation pathways would be a ring opening to yield 6′-acetylpapaverine (III) (3) and a covalent hydration reaction (4). Although these reactions were postulated, no quantitative information about reaction rates was reported.

An investigation into the chemical stability and solubility of coralyne sulfoacetate (I*b*), isolated as the initial product in a general synthesis of I (1), suggests that I*b* may be a better coralynium salt to use in liquid dosage forms than I*a*.

EXPERIMENTAL

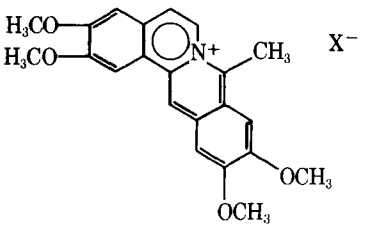
Materials—Coralyne chloride[3] and coralyne sulfoacetate[4] were provided by the National Cancer Institute and used without further purification. The IR spectrum of coralyne chloride used was identical to the reported spectrum[2]. Coralyne sulfoacetate has an extra absorption band at 1720 cm^{-1} due to the carbonyl group in the anionic part. 6′-Acetylpapaverine was prepared, following the

[1] J. P. Davignon, National Cancer Institute, personal communication.

[2] P. Lim and S. Stone, Stanford Research Institute Report 9180 to National Cancer Institute, June 28, 1972.

[3] NSC 96349, lot pH 9-29-1, mol. wt. 399.9, mp 246–250° dec. [lit. (1) 248–250° dec.].

[4] NSC 154890, lot pH 5-113-1, mol. wt. 503.5, mp 268–274° dec. [lit. (1) 278–280° dec.].



I: coralynium ion
Ia: X = Cl
Ib: X = $HO_2C—CH_2—SO_3$

procedure given by Schneider and Schroeter (3), from coralyne chloride.

Water was double distilled, with the second distillation being from acid–permanganate solution in an all-glass still. Indoor light was from an ordinary fluorescent bulb[5], and the photohydration was carried out about 1.5 m (5 ft) from such lights. Deoxygenated water was obtained by bubbling double-distilled water with nitrogen gas passed through a pyrogallol solution in concentrated sodium hydroxide. Commercially available borosilicate glass containers were used except where otherwise specified.

Kinetic Experiments—Formation of 6′-acetylpapaverine from coralyne was followed by the absorbance change at 297 nm on a UV spectrophotometer[6]. The reaction was carried out in such a way that, after rapid mixing with sodium hydroxide solution in an optical cell, a coralyne chloride concentration of 1.64×10^{-5} *M* and a coralyne sulfoacetate concentration of 1.82×10^{-5} *M* were obtained. Whenever the half-life of the reaction was shorter than 10 sec, a stopped-flow spectrophotometer[7] was used. In this case, a 1:1 volume ratio of coralyne solution to sodium hydroxide solution was adopted. A temperature of 25.0 ± 0.5° was maintained during the reaction. Ionic strength was not controlled, since no significant primary salt effect was observed when the final ionic strength of the solution, which was 0.5 *M* in sodium hydroxide, was adjusted to 1.0 *M* with sodium sulfate.

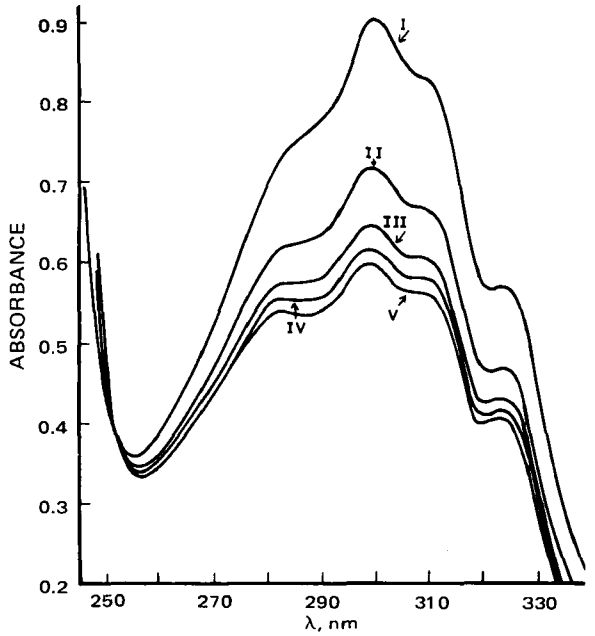
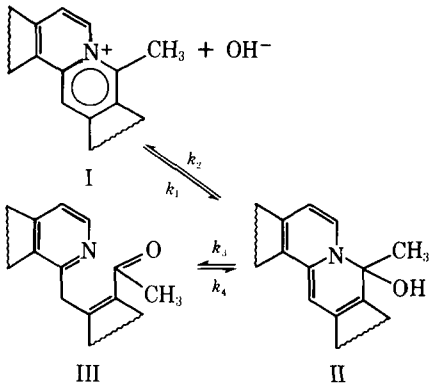


Figure 1—*Spectral changes observed at various times for a solution of* Ia *(~2 × 10⁻⁵ M) in aqueous 0.1 M NaOH at 25°. (Identical spectral changes were observed for* Ib *under the same conditions.) The spectra were recorded at various times after preparation of the solution. Key: I, 38 sec; II, 218 sec; III, 218 sec; IV, 308 sec; and V, 647 sec.*

[5] F40CW, Lifeline, Sylvania.
[6] Cary model 16, Varian Instruments.
[7] Durrum-Gibson.



Scheme I

The aromatization reaction of 6′-acetylpapaverine was followed in a similar manner. A concentrated solution, freshly prepared in chloroform, was used as a stock solution (~6 mg/ml). After about 3.5 μl of this stock solution was directly transferred into an optical cell through a microsyringe, the solvent was evaporated by nitrogen gas, the residue was rapidly dissolved in the appropriate buffer solutions, and the absorbance change at 297 nm was followed on a spectrophotometer. Since the reaction was extremely slow in 0.1 *N* HCl (Table I), sometimes a concentrated stock solution (~6 mg/ml) prepared in 0.1 *N* HCl was used in the kinetic experiments. Temperature and the final ionic strength were maintained at 25.0 ± 0.1° and 0.1 *M*, respectively.

Solubility Studies—The solubility of Ia and Ib in aqueous media was determined as follows. About 100 mg of either Ia or Ib was placed into separate 4-dram screw-capped vials. To each vial was added about 5 ml of distilled water. The tightly closed vials were wrapped in aluminum foil and attached to a rotating shaft in a water bath thermostated at 25 ± 0.1°. The samples were allowed to equilibrate for 7 days.

The solubility of Ib in various concentrations of sodium hydroxide was determined similarly. To a series of vials were added successively increasing amounts of Ib and 5 ml of increasingly concentrated aqueous sodium hydroxide solution. In a typical series of vials, the composition of the contents of each vial relative to the added amounts of Ib and the concentration of the sodium hydroxide solution, respectively, were as follows: vial I, ~125 mg, 0.02 *M*; vial II, ~200 mg, 0.04 *M*; vial III, 275 mg, 0.06 *M*; and vial IV, 350 mg, 0.08 *M*.

The vials were tightly closed and equilibrated as already described. Equilibration was complete after about 30 hr. Following equilibration, samples of the various solutions were withdrawn using a filter[8] and the filtrate was analyzed.

Analysis of Coralyne Chloride and Coralyne Sulfoacetate in Solution—The determination of the concentration of the coralyne salts in solution was carried out spectrophotometrically. The UV spectra of both compounds were qualitatively identical in solutions of anhydrous methanol and in 1% water–methanol. Beer's law plots were constructed for each salt in anhydrous methanol at λ_{max} = 312 nm. The molar absorptivity of the two compounds differed slightly. The experimental values determined from Beer's law plots were $\epsilon_{312} = 5.3 \times 10^4$ liters/mole cm for Ia and $\epsilon_{312} = 5.85 \times 10^{-4}$ liter/mole cm for Ib. This difference in absorptivities may be due to the presence of greater amounts of water in Ia (up to 10%)[9] as compared to Ib (0–2% water)[10].

Filtered aliquots of the aqueous coralyne solutions were suitably diluted (100-fold or more) with anhydrous methanol. The absorbance was then measured at 312 nm in a 1-cm cell, and the concentration was calculated utilizing the molar absorptivity value obtained for the particular salt being analyzed.

Methanol was used as the solvent for the analysis to prevent photohydration, which would have occurred rapidly in dilute aqueous solutions.

[8] Metricell, Scientific Products Co., Kansas City.
[9] P. Lim and S. Stone, Stanford Research Institute Report 1274 to National Cancer Institute, July 27, 1972.
[10] P. Lim, A. Cheung, and B. Kauanaugh, Stanford Research Institute Report 1307 to National Cancer Institute, Jan. 25, 1973.

RESULTS AND DISCUSSION

Stability of Ia and Ib in Solutions Protected from Light—The UV spectra of aqueous solutions of *Ia* and *Ib* that had pH values below 10 and were completely protected from light (*e.g.*, stored in stoppered flasks wrapped in aluminum foil) did not change more than ±2% at any wavelength during 48 hr. The spectra of these two solutions were identical and were similar to the spectrum of *Ia* in methanol.

When the pH values of solutions of *Ia* and *Ib* were above 12, spectral changes occurred in a manner suggesting that coralynium ion was being converted into one or more products. An isosbestic point was maintained at 253 nm during these changes. Figure 1 displays the spectra measured at several times after the mixing of 0.6 ml of a 1×10^{-4} *M* solution of *Ia* in acetonitrile (a solvent in which *Ia* appeared to be stable for several days) with 3.0 ml of 1×10^{-1} *M* NaOH.

It was reported previously (3) that coralynium ion (I) is converted into 6′-acetylpapaverine (III) in alkaline solutions. Hence, it is believed that the spectral changes observed in solutions of *Ia* and *Ib* at pH > 12 were caused by the conversion of I to III. The results of studies (5) on the ring opening of other ring systems containing a quaternary nitrogen atom led to the conclusion that the reactions shown in Scheme I are probably involved in the conversion of I to III.

Compound III, formed by the reactions of *Ia* and *Ib* in solutions at pH ≥ 12, reverted to coralynium ion when the pH value of the solutions was reduced to values below 11. The spectral changes observed during the reaction for the conversion of III to I were precisely the reverse of those shown in Fig. 1, and the isosbestic point maintained during the closure reaction was at the same wavelength (253 nm) as that observed during the conversion of I to III in solutions with pH > 12. These spectral data represent evidence that the reactions observed at pH < 11 were the reverse of the reactions of *Ia* in more alkaline solutions.

Furthermore, a precipitate obtained 24 hr after III (5 mg/ml) was dissolved in 0.1 *N* HCl had an identical IR spectrum (measured in a KBr disk) and NMR spectrum (when dissolved in trifluoroethanol) to those of an authentic sample of *Ia*. It has recently been observed (2) that the pharmacological activities of *Ia* and III are similar. This observation is consistent with the postulates that I and III are rapidly interconvertable in solution and that III would be rapidly converted to I at physiological pH.

When the initial concentration of sodium hydroxide, $[NaOH]_0$, was at least five times greater than the initial concentration of *Ia* or *Ib*, the conversion of I to III was a first-order process. Pseudo-first-order rate constants, k^0_{obs}, for this reaction sequence were calculated from the slopes of linear plots of log $(A_t - A_\infty)$ against time. Values of k^0_{obs} are shown plotted against $[NaOH]_0$ in Fig. 2.

The conversion of III to I in aqueous buffers was also a first-order process, and values of a pseudo-first-order rate constant, k^c_{obs}, were calculated from the slopes of linear plots of log $(A_t - A_\infty)$ against time. Values of k^c_{obs} at several different pH values, together with the buffer systems, are listed in Table I. No attempt

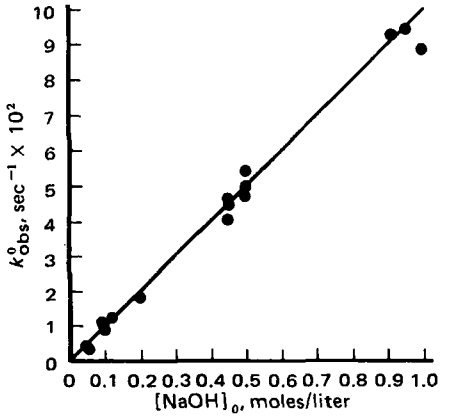


Figure 2—*Plot of the observed pseudo-first-order rate constants for the conversion of I to III as a function of the sodium hydroxide concentration in aqueous solution at 25°.*

Table I—Observed First-Order Rate Constant for the Conversion of III to I as a Function of pH[a]

pH (Buffer System)	$k^c_{obs} \times 10^4$ sec^{-1}
3.07 (monochloroacetate)	1.20
4.81 (acetate)	9.81
6.05 (succinate)	27.7
7.05 (phosphate)	67.3
8.41 (tromethamine)	19.4
9.87 (carbonate)	8.32
10.57 (carbonate)	3.54

[a] Ionic strength and temperature were kept at 0.1 *M* and 25°, respectively.

was made to evaluate buffer-independent rate constants or general-acid and general-base catalysis constants.

The facts that pure first-order kinetics were observed during both the conversion of I to III and the reverse reactions and that the same isosbestic point was maintained during the forward and reverse reactions strongly suggest that no significant concentrations of an intermediate (*i.e.*, the carbinolamine, II) accumulated during either the forward or the reverse reactions. Therefore, an identity relating k^0_{obs} and k^c_{obs} values to the microscopic rate constants in Scheme I can be arrived at by using a steady-state treatment. An identity that should apply at any pH value where species III exists primarily as a neutral molecule is:

$$k^0_{obs} = k^c_{obs} = \frac{k_1 k_3}{(k_2 + k_3)}[OH^-] + \frac{k_2 k_4}{(k_2 + k_3)} \qquad \text{(Eq. 1)}$$

Values of the slope and intercept of the plot of k^0_{obs} against $[NaOH]_0$ indicate that $k_1k_3/(k_2 + k_3) = 0.1\ M^{-1}$ sec^{-1} and $k_2k_4/(k_2 + k_3) \ll 10^{-2}$ sec^{-1}. Values of k^c_{obs} calculated from experiments in which the pH values were varied between 6 and 11 (*i.e.*, where $[H^+]$ and $[OH^-]$ varied by 10^5) only differed from one another by a factor of about 20. Hence, it appears that under these conditions the term $k_1k_3[OH^-]/(k_2 + k_3) \ll k_2k_4/(k_2 + k_3)$.

The relatively small change observed in k^c_{obs} values was perhaps caused by the different general acids and general bases in each reaction system. If this conclusion is correct, the value of the buffer-independent term, $k_2k_4/(k_2 + k_3)$, is likely to be no greater than $\sim 3.5 \times 10^{-4}$ unit, which is the smallest value of k^c_{obs} observed throughout this pH range (Table I). The decrease in the value of k^c_{obs} that occurred when the pH value was reduced from 6 to 3 was most likely caused by protonation of the nitrogen atom of 6′-acetylpapaverine. The conjugate acid of III would be expected to have a pKa value similar to that of isoquinoline, 5.4 (6). Protonation of III would be expected to reduce its rate of cyclization to yield II and would thus reduce the apparent value of k_4.

It can be concluded that $k_1k_3/k_2k_4 = [III]/[I][OH^-] \simeq 10^{-1}/3 \times 10^{-4} \simeq 3 \times 10^2\ M^{-1}$. Hence, at pH < 11, [III]/[I] < 0.3; at pH ~ 6, [III]/[I] would be of the order of 10^{-5}. Thus, the interconversion of I and III occurs relatively rapidly in solution and only an insignificant fraction of the total alkaloid persists as III in solutions with pH values ≤10.

Photochemical Reactions of Ia and Ib—It has been suggested (4) that I becomes covalently hydrated in aqueous solution. This conclusion was based on similarities between the UV spectra of freshly prepared aqueous coralyne solutions and solutions of coralyne salts in anhydrous solvents, such as acetonitrile and methanol, and the differences between these spectra and that of an aged aqueous solution. The reversibility of the hydration reaction was established from the fact that lyophilization of aged solutions of *Ia* or *Ib* yielded residues which, on redissolution in methanol, produced solutions with UV and NMR spectra identical to those obtained from freshly prepared solutions of the authentic salts.

In the present study, the reactions that occur in aqueous solutions of *Ia* and *Ib* at pH values where no appreciable amounts of III are formed were investigated and the following additional information is reported.

1. The reaction of I that has been referred to as a covalent hydration reaction is a photochemical reaction and appeared to occur as a result of exposure of solutions of I to visible light. Hence, whereas the UV spectra of solutions of *Ia* and *Ib* in water do not change appreciably during 24 hr if the solutions are completely

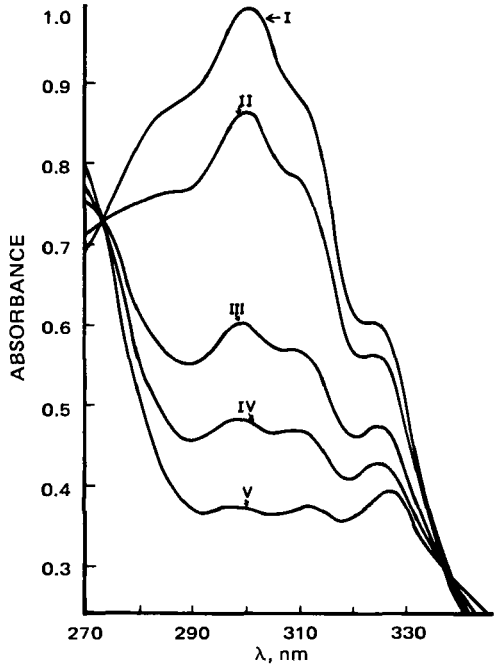


Figure 3—*Spectral changes for an aqueous solution of Ia (~2 × 10^{-5} M) exposed to fluorescent laboratory light for various periods. Key: I, 0 min (freshly prepared solution); II, 15 min; III, 60 min; IV, 180 min; and V, 2 days (no further spectral changes occurred).*

protected from light, they do change rapidly upon exposure to sunlight or normal laboratory illumination in either open or closed glass vessels.

Figure 3 displays the spectrum of a freshly prepared solution of I*a* in water and the spectra measured after various periods of exposure of the solutions to normal laboratory illumination. If a solution that had been exposed to illumination for any length of time was then protected from light (*e.g.*, by storing it in an amber glass container or in a container painted black or wrapped with aluminum foil), its spectrum underwent no further changes during the protection period.

The rate and nature of spectral changes upon illumination were the same in solutions of I*a* and I*b* stored in either borosilicate glass or quartz containers. Because the materials of the former type of container absorb UV light whereas those of the latter type transmit UV light, it seems highly probable that the visible range of radiant energy is involved in the photochemical reaction. Thus, it is

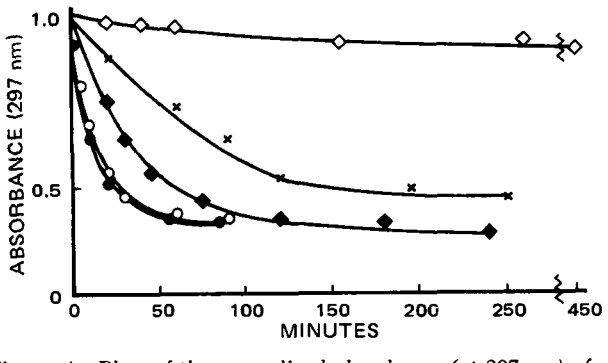


Figure 4—*Plots of the normalized absorbance (at 297 nm) of solutions of I*b versus *the time of exposure to normal laboratory light. The studies were conducted at room temperature (about 19–22°). The initial concentrations of I*b *in the solutions were: ◇, 2.15 × 10^{-3} M; ×, 2.15 × 10^{-4} M; ♦, 7.54 × 10^{-5} M; ○, 1.08 × 10^{-5} M; and ●, 7.10 × 10^{-6} M. Normalization was achieved by multiplying measured absorbances at 297 nm by a factor that took into account differences in the initial concentration and the path length of the cell. The initial absorbance of a 2.15 × 10^{-5} M solution of I*b *in a 1-cm cell was 0.984.*

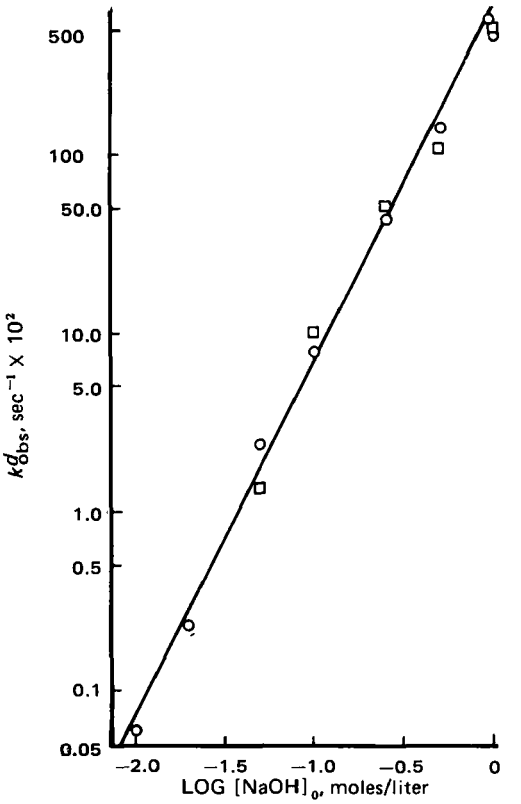


Figure 5—*Logarithmic plot of observed first-order rate constants for the conversion of the postulated photohydrate to I as a function of sodium hydroxide concentration. T = 25°. Identical results were obtained with I*a *(○) and I*b *(□).*

necessary to exclude light to prevent the reaction from occurring.

2. The rate of spectral changes during illumination was not affected by the presence of dissolved oxygen in the solutions. This fact was established by comparing the rates of spectral changes in illuminated solutions of I*a* in water that had been saturated with either oxygen or nitrogen. It can be concluded from this result that oxygen is not involved in the photochemical reaction. Furthermore, oxygen is a quencher of many photochemical reactions involving triplet-state excited molecules (T^1). Hence, the fact that the rate of the photochemical reaction of I is insensitive to oxygen concentrations suggests that it involves single-state (S^1) rather than triplet-state (T^1) excited molecules. A similar conclusion was drawn (7) about the photohydration of uracil, because the rate of this reaction was also insensitive to oxygen concentrations.

3. The rate of photohydration reaction was the same in 0.1 *N* HCl as it was in water. This result suggests that the rate-determining step involves a reaction between an excited form of the coralynium ion (presumable S^1 species) and undissociated water molecules rather than hydroxide ions or hydrogen ions.

4. The apparent rate of the photohydration reaction decreased as the initial concentration of I in the solution was increased. This result can be seen in Fig. 4, where plots of the absorbance (at 297 nm) of different solutions of I*b* against time of exposure to laboratory illumination are displayed.

In a typical experiment, 10–12 identical volumetric flasks (25 ml), wrapped with aluminum foil, were filled with a solution of I*b* in water. This operation was performed in a photographic darkroom where no appreciable photochemical reaction occurred. The flasks were then placed on an illuminated laboratory bench and the aluminum foil was removed. At various time intervals, a flask was selected at random and the UV absorbance of its solution at 297 nm was measured in a 0.2-, 1.0-, or 5.0-cm cell or an appropriate dilution was made in a darkroom and the absorbance then was measured. The absorbance data were normalized so that they could be conveniently presented on a single graph.

No simple rate law could be derived to account for the absorbance changes as a function of time. This observation suggests that the distribution of light energy is not uniform throughout the reac-

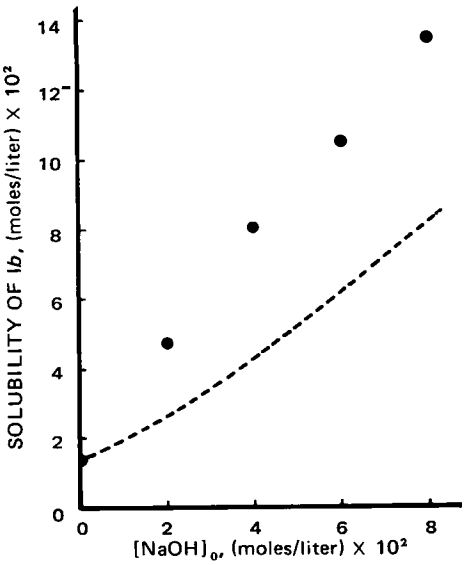


Figure 6—*Plot of aqueous solubility of Ib as a function of added sodium hydroxide, [NaOH]₀. The points represent the experimental results. The dashed line represents the curve calculated using Eq. 6 and a value of* $K_{sp}^{Ib} = 1.72 \times 10^{-4}$ M²; T = 25°.

tion solutions, a phenomenon that is commonly encountered in photochemical reactions where the excited-state molecules have only a very short lifetime (8). However, the results clearly indicate that the apparent rate of the photochemical reaction decreases as the concentration of I increases. Further confirmation of this fact comes from the observation that rapid dilution with water of a saturated solution of Ia, which had been stored in a flask on a laboratory bench for 2 months, yielded a solution that had a UV spectrum identical to that of a freshly prepared solution of Ia.

It was reported (4) that the presumed hydration of I can be reversed by lyophilization of solutions or by dilution of solutions of the hydrate with methanol. Two other methods of causing a reversal of the hydration reaction have become apparent as a result of this work. In the first case, when a 10^{-4} *M* solution of Ia, which had been illuminated with laboratory light for several weeks, was refluxed for 1 hr and then cooled in the dark, its spectrum was almost the same as that of a freshly prepared solution of Ia.

In the second case, if a solution of Ia in water was exposed to light for several weeks and was then made alkaline to pH > 12, its spectrum rapidly changed from that of the hydrate to one that was very similar to the spectrum of Ia. This spectrum then changed in the manner that has been attributed to the conversion of I to III. Since an isosbestic point is maintained at 253 nm during the conversion of I to III and the absorbance of I and its hydrate at 253 nm differ significantly, the kinetics of conversion of the hydrate to I could be studied by measuring absorbance changes at this wavelength. The reaction appeared to be first order in the concentration of the hydrate, and pseudo-first-order rate constants, k_{obs}^{d}, were calculated from the slopes of linear plots of log $(A_t - A_\infty)$ against time.

As can be seen from the logarithmic plot of k_{obs}^{d} against $[NaOH]_0$ in Fig. 5, the rate of this reaction had a second-order dependence on hydroxide-ion concentrations. An explanation of this result cannot be offered at this time. Dehydration reactions of photohydrates of molecules such as uracil have been reported (9) to be first order with respect to hydroxide-ion concentration, but we are unaware of any dehydration reactions that have been shown to be second order with respect to this reactant. This result may cast some doubt on whether the product of the photochemical reaction is indeed a photohydrate.

This study did not yield additional evidence as to the structure of the product; its characterization rests on the observations that it only forms in water and not in anhydrous methanol or acetonitrile and that it is readily converted into I by lyophilization, by heating, or by making the solution alkaline.

Irrespective of the exact nature of the product of the photochemical reaction of I, the reaction can be prevented by completely protecting the aqueous solutions from visible light and the rate in the presence of light can be greatly reduced by increasing the concentration of I in the solution. These facts, together with the ability to reverse the photochemical reaction through lyophilization, are extremely important in the ultimate development of formulations for parenteral solutions of I.

Solubility of Ia and Ib—It was desirable to identify a method for obtaining more concentrated solutions of I to facilitate the intravenous administration of the cytotoxic agent, especially in view of the data in Fig. 4 indicating the apparent increase in chemical stability achieved through increased concentration.

The solubility of Ia and Ib in water at 25° was determined to be about 4.5 and 6.5 mg/ml, respectively. It had been shown[1] that the solubility of Ia was no greater in weakly alkaline aqueous media than in distilled water, as expected for such a compound. The solubility of Ib as a function of added sodium hydroxide is shown in Fig. 6. It is obvious that the apparent solubility of Ib is greatly increased by the presence of added sodium hydroxide. Qualitatively, this solubility behavior of Ib was expected, since the sulfoacetate anion (in contrast to Cl^-) is an acid and would be ionized by the added hydroxide ion. This behavior can be expressed mathematically as follows.

The apparent solubility product (K_{sp}) for Ib is given by:

$$K_{sp}^{Ib} = [I][(HO_2C—CH_2—SO_3)^-] \quad \text{(Eq. 2)}$$

Because $(HO_2C—CH_2—SO_3)^-$ is a weak acid, it would react with each added hydroxide ion according to Scheme II:

$$(HO_2C—CH_2—SO_3)^- + (OH)^- \rightarrow (O_2C—CH_2—SO_3)^{-2} + H_2O$$

Scheme II

and:

$$[NaOH]_0 \simeq [(O_2C—CH_2—SO_3)^{-2}] \quad \text{(Eq. 3)}$$

As long as the only solid phase in the system is Ib, the following identities must be obeyed:

$$[I] = [(HO_2—C—CH_2—SO_3)^-] + [(O_2C—CH_2—SO_3)^{-2}] \quad \text{(Eq. 4)}$$

$$[I] = [(HO_2C—CH_2—SO_3)^-] + [NaOH]_0 \quad \text{(Eq. 5)}$$

Substituting Eq. 5 into Eq. 2 and solving for [I] lead to:

$$[I] = \{[NaOH]_0 + ([NaOH]_0^2 + 4K_{sp}^{Ib})^{1/2}\}/2 \quad \text{(Eq. 6)}$$

The dashed line in Fig. 6 was calculated using Eq. 6 and a K_{sp}^{Ib} value that was calculated from the identity:

$$K_{sp}^{Ib} = [I]_{sat}^2 \quad \text{(Eq. 7)}$$

where $[I]_{sat}$ ($= 1.31 \times 10^{-2}$ *M*) is the solubility of Ib in water at 25°.

There are at least two possible explanations for the fact that the actual solubility of Ib was higher in aqueous sodium hydroxide than was predicted using Eq. 6. First, no allowance was made for the fact that the K_{sp}^{Ib} value would change as the ionic strength of the solution increased. The solubility of Ib and the concentration of the dianionic species $(O_2C—CH_2—SO_3)^{-2}$ obviously increased as the initial concentration of sodium hydroxide was increased. Therefore, the ionic strength of the solution increased, and such an increase probably would be expected to increase the value of K_{sp}^{Ib} (10).

Second, there is a strong possibility that I dimerizes or forms higher order complexes in solution. The occurrence of this phenomenon might also be expected to lead to an increase in the apparent solubility of the salt. Although this latter phenomenon was not investigated further, related quaternary nitrogen-containing ring systems such as acridines do form dimers and higher order complexes in aqueous solution (11).

The important result of this solubility study is that much more concentrated solutions of I can be formed by dissolving Ib (or, presumably, other salts of I and the anions of polyprotic acids) *in* aqueous alkaline solutions than can be prepared using Ia. Moreover, the salt Ib is the initial product obtained by a general synthesis of I (1).

Application of Results—These results have been useful in the development of a formulation of I which obviates the problems that had previously limited the clinical evaluation of the cytotoxic

coralyne salts. This formulation is presently being utilized and is prepared by dissolving Ib in sufficient aqueous sodium hydroxide to yield a final concentration of 25 mg/ml (calculated on the basis of Ib) at pH ~ 6.5. The solution obtained is sterile filtered, frozen, and lyophilized. The lyophilized product is stable, and reconstitution as a solution is easily accomplished upon the addition of water. Such a formulation affords good solubility and stability with respect to I and appears to be completely suitable for intravenous use.

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Micelle Formation and Its Relationship to Solubility Behavior of 2-Butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl Ketone Hydrochloride

LOUIS J. RAVIN ˣ, ELIE G. SHAMI ‡, and ELISABETH S. RATTIE

Abstract □ Micelle formation by 2-butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone hydrochloride was studied by conductance measurements. The CMC was approximately 0.05% and was independent of temperature between 20 and 50°. The heat of formation for the micelle was calculated to be 6.9 kcal/mole. The unusual solubility behavior of the compound was attributed to its ability to form micelles. Ultracentrifuge studies indicate the molecular weight of the micelle to be approximately 100,000. Anions such as chloride, sulfate, acetate, tartrate, and citrate significantly affect the equilibrium solubility of the compound. NMR spectroscopic data indicate that the solubility behavior, in part, is related to an effect on the CMC of the compound by the anionic environment.

Keyphrases □ 2-Butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone hydrochloride—micelle formation, relationship to solubility □ Solubility—2-butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone hydrochloride, micelle formation □ Micelles—2-butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone hydrochloride, relationship to solubility

It was reported previously (1, 2) that 2-butyl-3-benzofuranyl-4-[2-(diethylamino)ethoxy]-3,5-diiodophenyl ketone hydrochloride[1] (I) was capable of existing in the micellar state and that surfactants such as polysorbate 80, sodium lauryl sulfate, and cetyldimethylbenzylammonium chloride had a pronounced effect on its equilibrium solubility. This paper reports the results of studies on the micellar behavior of I, its relationship to the solubility characteristics, and the effect of various anions on its equilibrium solubility.

EXPERIMENTAL

Materials—Ultrapure water[2] was used. All other chemicals were of analytical reagent grade quality.

Equipment—A recording spectrophotometer[3] was used for UV absorption measurements. A conductivity bridge[4] and a conductivity cell[5] were used in the conductance experiments.

Equilibrium Solubility Studies—*Effect of Temperature*—Approximately 200 mg of I was added to screw-capped vials containing 15 ml of water. The vials were rotated for 24 hr at 20, 25, 30, 35, 40, 45, and 60°. The contents of the vials were filtered through syringes fitted with Swinney filter adapters containing a

[1] SK&F 33134-A, marketed as Cordarone by Labaz Laboratories in several European countries.

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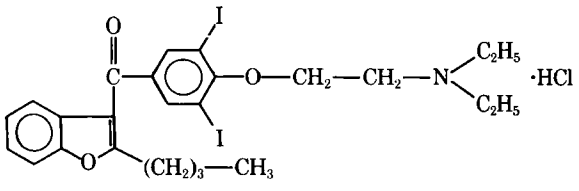

I

0.45-μm filter disk[6]. The resulting clear solutions were diluted with anhydrous methanol and assayed for I using a recording spectrophotometer.

Effect of Various Salt Solutions—Approximately 200 mg of I was added to screw-capped vials containing 15 ml of salt solutions of known concentrations. The vials were rotated for 24 hr at 30°. The contents of the vials were filtered as previously stated. The resulting solutions were diluted with anhydrous methanol and assayed for I.

Determination of Critical Micelle Concentration (CMC)—The CMC for I was determined by conductance measurements. A series of solutions of various concentrations was prepared and equilibrated at 20, 25, 30, 37, 45, and 50°. Conductance measurements were made on the solutions. The CMC was determined from a plot of equivalent conductance *versus* square root of concentration, using the point of intersection of the straight-line segments of the curve as the CMC value.

Ultracentrifuge[7] Studies—The ultracentrifuge studies were conducted using the sedimentation equilibrium method (3). Samples containing 4.6, 3.5, and 1.5% of I were prepared. To effect solution, it was necessary to dissolve the drug at temperatures in excess of 60°. When cooled, I remained in solution. The samples were centrifuged at 38,000 rpm. Photographs were taken at intervals of 0, 15, 30, 47, 64, and 147 min to follow the movement of the front.

NMR Studies—The NMR spectrum of a solution of I (0.15% in 0.02% sodium sulfate solution) was run in deuterium oxide at 25 and 60°.

RESULTS

CMC—The CMC for I in aqueous solution was approximately 0.05%. Figure 1 shows a plot of the equivalent conductance *versus* the square root of concentration. The CMC was independent of temperature between 20 and 50°.

Effect of Temperature on Equilibrium Solubility—The equilibrium solubility of I in water at 25° was approximately 5.1×10^{-4} mole/liter, and showed a dramatic temperature dependency. Figure 2 shows a typical van't Hoff plot, illustrating the temperature dependency on solubility. The solubility increased gradually up to about 35°; the heat of formation for the micelle was calculated to be 6.9 kcal/mole. At 35°, there was a sharp increase in the solubility, apparently due to the appearance of a new species in solution. The concentration of I in solution at the inflection point corresponded to the CMC value determined by conductance measurements. The calculated apparent heat of formation for the micelle is high when compared to reported values (4). In the 35–60° range, the solubility remained temperature dependent. A sample

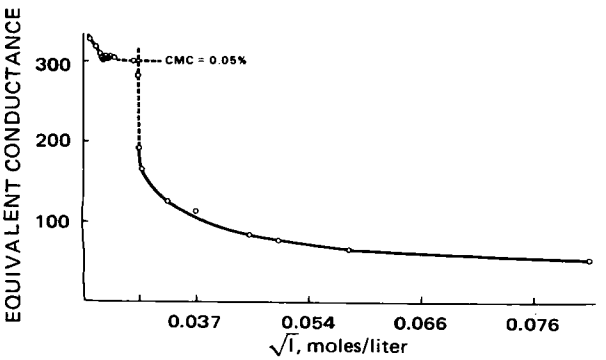

Figure 1—*Plot showing the CMC for I.*

[6] Millipore.
[7] Model E, Spinco Division, Beckman Instruments, Bellmont, California.

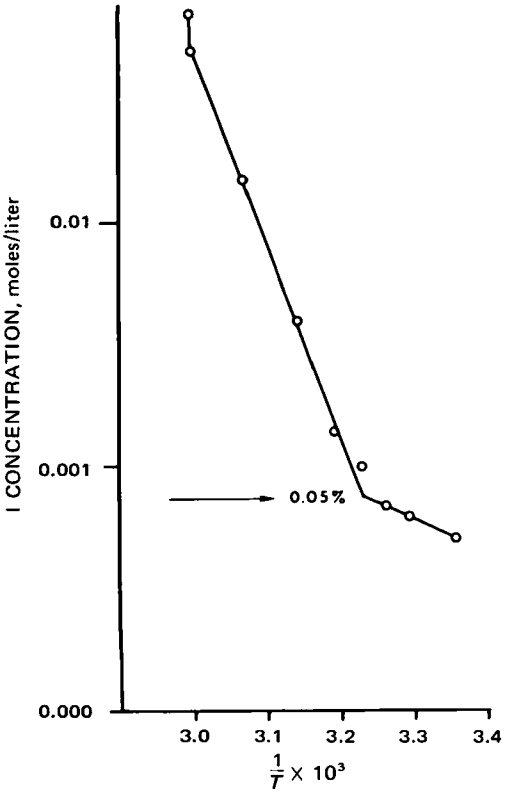

Figure 2—*Typical van't Hoff-type plot illustrating the effect of temperature on the aqueous solubility of I.*

equilibrated at 40° for 48 hr had a solubility of 1.4×10^{-3} mole/liter; however, when this solution was brought to 25° and equilibrated for 48 hr, the solubility approximated the value previously determined. Thus, in this temperature range, the solubility was reversible. However, above 60°, complete solution of the excess compound was achieved.

This solution remained clear after shock-cooling and crystal seeding to induce crystallization and formed a metastable micellar solution. Subsequent dilutions of this metastable solution remained clear until the effective concentration on dilution approached the CMC. Below this concentration, the compound precipitated from solution.

Determination of Micelle Molecular Weight—To characterize the system further, sedimentation equilibrium studies were conducted to determine the approximate size and molecular weight of the micelle. Figure 3 shows a series of ultracentrifuge patterns, illustrating the approach to sedimentation equilibrium for a 4.6% solution of I. The character of the curves suggests that the system is heterogeneous. The distance the front moved with time was determined, and a plot of the log of this distance against time is shown in Fig. 4. The slope of the line was determined and

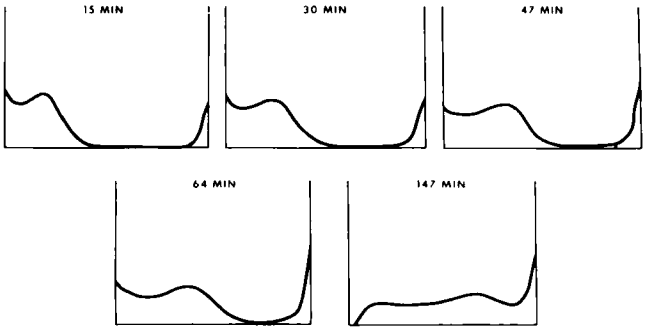

Figure 3—*Representative ultracentrifuge patterns illustrating the approach to sedimentation equilibrium for a micellar solution of I.*

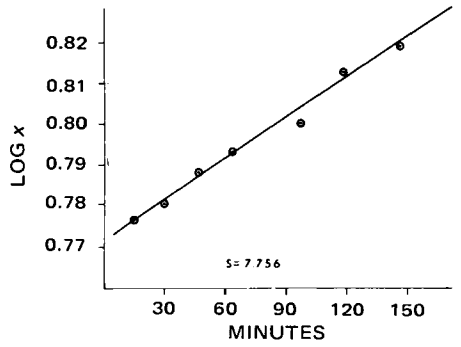

Figure 4—*Determination of sedimentation coefficient from a plot of log* x (*distance of boundary to axis of rotation*) versus *time for a micellar solution of I.*

used in the following equation to calculate the apparent sedimentation coefficient:

$$S = \frac{2.303\ d\ \log x/dt}{60(\omega)} \qquad \text{(Eq. 1)}$$

where S = sedimentation coefficient, and ω = angular velocity.

A sedimentation coefficient of 7.756 was calculated for the system. When utilizing this value and extrapolating from a plot for protein compounds in the literature (5), it appears that the micelle is composed of approximately 150 monomeric units having a molecular weight in excess of 100,000.

Effect of Various Anions—Previously it was reported that chloride ion depressed the solubility of I (2); therefore, it was decided to investigate the effect of some commonly used buffer salts on the equilibrium solubility. Figure 5 illustrates the effect of citrate and tartrate ions on the equilibrium solubility of I. The solubility increases rapidly and then suddenly decreases to a constant value below the equilibrium solubility previously determined.

The pH of these solutions ranged from 4.3 to 5.4. To eliminate the possible effect of pH with increasing salt concentrations, several neutral sulfate salts were studied. The pH of these solutions remained relatively constant at pH 4.3. The effect of these salts on the solubility of I is shown in Fig. 6. Again there are a sharp increase and the sudden drop to a constant value.

Another anion, acetate, was utilized in the solubility studies. The pH of these solutions ranged from 4.0 to 4.7. The effect (Fig. 7) was similar to that previously observed with the chloride ion (2). The solubility was depressed by the addition of increasing amounts of acetate ions. Apparently, the effect on solubility was not due to an ionic strength effect. When solubility was plotted as a function of increasing normality, these data were superimposable.

The possibility of interconversion of the hydrochloride salt to

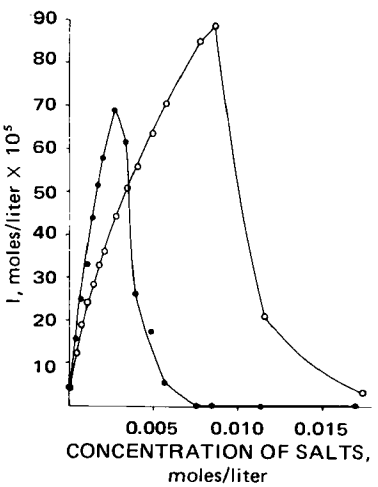

Figure 5—*Plot showing the effect of sodium citrate* (●) *and sodium tartrate* (○) *on the equilibrium solubility of I.*

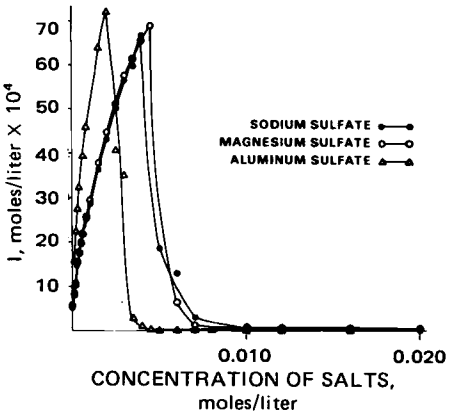

Figure 6—*Plot showing the effect of various sulfate salts on the equilibrium solubility of I.*

the citrate salt being responsible for the solubility behavior was considered. The citrate salt of I was prepared. The equilibrium solubility of this salt at 25° was 9.1×10^{-5} mole/liter, which is not in agreement with the solubility values achieved during the studies with the citrate ion.

NMR Spectroscopy—It was previously reported that the NMR spectrum of I in micellar solution had broad resolution bands at 25°; however, when the temperature was increased, the resolution became sharp and reached a maximum at 60° (1). The conclusions were that the micellar system was completely disrupted at 60° and that the resulting spectrum was of the monomer of I.

To explain the dramatic increase in solubility, a solution isolated from the sulfate solubility studies was investigated using this technique. This solution showed behavior similar to that of the micellar solution. At 25°, there was no apparent resolution. However, when the temperature was raised to 60°, the resolution bands became sharper (Fig. 8). Apparently, the various ions exert an effect on the equilibrium solubility of I by affecting its CMC. The NMR data confirm this observation. This finding is not surprising since ionic environment has been shown to affect CMC values (6, 7).

CONCLUSION

The pharmaceutical importance of this study is apparent, and the implications of equilibrium solubility and dissolution rate to drug availability in a dosage form are obvious. When formulating this drug, the development pharmacist must exercise extreme care in the selection of the proper excipients and/or buffer systems and also in the concentration of these ingredients. A study of this type points out that a thorough understanding of the physical–chemical behavior of complex drug molecules is extremely important.

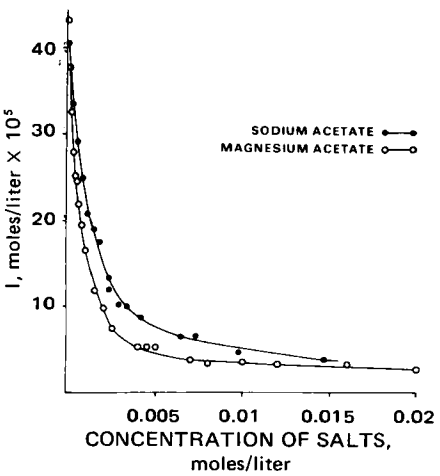

Figure 7—*Plot illustrating the effect of acetate salts on the equilibrium solubility of I.*

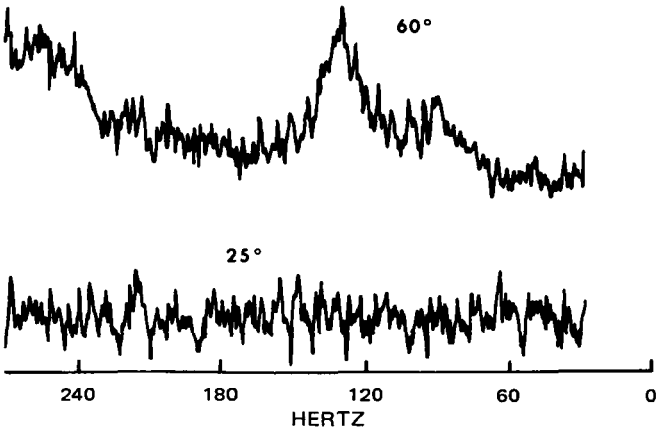

Figure 8—*NMR spectra for I (0.15% in 0.02% sodium sulfate solution) at 25 and 60°.*

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Solvolysis of a Substituted Imidazoline, Mazindol

H. V. MAULDING[x], P. TURI, D. GROSSMAN*, and J. P. NAZARENO

Abstract □ Hydrolysis of mazindol to form 2-(2-aminoethyl)-3-(*p*-chlorophenyl)-3-hydroxyphthalimidine was followed spectrophotometrically in aqueous solutions at temperatures between 37 and 70°, pH values up to 7.6, and an ionic strength of 0.2. The effects of acetate, formate, and phosphate buffers as well as ionic strength on the observed rate constants were investigated. An interesting nonlinear dependency of the k_{obs} with buffer concentration was noted. The velocity constants declined with increasing hydrogen-ion concentration; the log k–pH profile and rate law are given along with other relevant data.

Keyphrases □ Solvolysis—mazindol, a substituted imidazoline, pH values up to 7.6 □ Mazindol—solvolysis in aqueous solutions, pH up to 7.6

The chemical stability of organic medicinal agents from the viewpoint of the finished dosage forms and the kinetic aspects of the pertinent drugs are of great interest.

The transformation of the nonphenethylamine anorexigenic, mazindol[1] [5-(*p*-chlorophenyl)-2,5-dihydro-3*H*-imidazo[2,1-*a*]isoindol-5-ol] (I), to its hydrolysis product, 2-(2-aminoethyl)-3-(*p*-cholorophenyl)-3-hydroxyphthalimidine (II), was practically quantitative under the conditions employed as monitored by spectrophotometric and TLC methods. The process is somewhat analogous to the hydrolysis of 1,3-diphenyl-2-imidazolinium chloride recently reported (1).

EXPERIMENTAL

Kinetic Studies—A stock solution was prepared by dissolving 90 mg (3.16×10^{-4} mole) of I in 5 ml of 0.1 *N* HCl, and water was added to 50 ml. Two-milliliter aliquots were added to 200-ml volumetric flasks, equilibrated at various temperatures, containing 198 ml of the acidic or buffer solution. Ten-milliliter samples were withdrawn periodically and scanned[2] from 210 to 350 nm. The absorbance readings at 272 ± 1 nm were used to evaluate the rate of conversion of I to II.

A stock solution of II containing 95.5 mg (3.16×10^{-4} mole) in 50 ml of 0.01 *N* HCl was prepared. Two-milliliter aliquots were added to 198 ml of acidic or buffer solution previously equilibrated at various temperatures. Samples (10 ml) were taken periodically, and their UV spectra were scanned from 210 to 350 nm to monitor possible degradation of II.

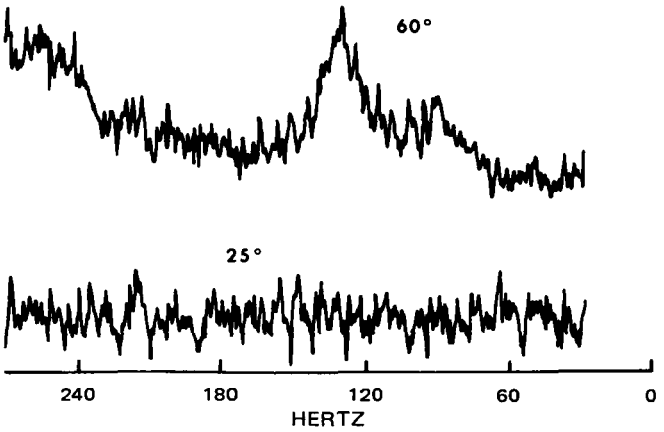
Preparation of II from I—Two grams of I was refluxed in 100 ml of 48% aqueous ethanol for 2 hr until the solution became almost clear. The solvent was removed *in vacuo*. The resultant white solid dissolved in 50 ml of benzene with heating. Petroleum ether was added until the point of slight turbidity, and the compound was allowed to crystallize overnight at room temperature. The yield after two recrystallizations was 1.5 g (71%) of II, mp 170–171°.

Determination of pKa Values—A stock solution of 6.3×10^{-3} *M* I was prepared and added to 0.01 *M* tromethamine buffers (pH 7.8, 8.0, 8.2, and 8.4) along with 0.1 *N* HCl and 0.09 *N* NaOH to give a final concentration of 6.3×10^{-5} *M*. The samples were quickly read[2] at 272 nm. The pKa was calculated to be 8.55 ± 0.05 by the procedure of Albert and Sergeant (2).

Compound II (151.4 mg, 0.005 *M*) was dissolved in 95 ml of boiled water with the aid of 5 ml of 0.1 *N* HCl. The solution was titrated with 0.5-ml increments of 0.1 *N* KOH, giving a pKa value of 8.53 ± 0.05 at 25° (2).

[1] Sandoz Pharmaceuticals, East Hanover, N.J.

[2] Cary model 14 recording spectrophotometer.



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Solvolysis of a Substituted Imidazoline, Mazindol

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Abstract □ Hydrolysis of mazindol to form 2-(2-aminoethyl)-3-(*p*-chlorophenyl)-3-hydroxyphthalimidine was followed spectrophotometrically in aqueous solutions at temperatures between 37 and 70°, pH values up to 7.6, and an ionic strength of 0.2. The effects of acetate, formate, and phosphate buffers as well as ionic strength on the observed rate constants were investigated. An interesting nonlinear dependency of the k_{obs} with buffer concentration was noted. The velocity constants declined with increasing hydrogen-ion concentration; the log k–pH profile and rate law are given along with other relevant data.

Keyphrases □ Solvolysis—mazindol, a substituted imidazoline, pH values up to 7.6 □ Mazindol—solvolysis in aqueous solutions, pH up to 7.6

The chemical stability of organic medicinal agents from the viewpoint of the finished dosage forms and the kinetic aspects of the pertinent drugs are of great interest.

The transformation of the nonphenethylamine anorexigenic, mazindol[1] [5-(*p*-chlorophenyl)-2,5-dihydro-3*H*-imidazo[2,1-*a*]isoindol-5-ol] (I), to its hydrolysis product, 2-(2-aminoethyl)-3-(*p*-cholorophenyl)-3-hydroxyphthalimidine (II), was practically quantitative under the conditions employed as monitored by spectrophotometric and TLC methods. The process is somewhat analogous to the hydrolysis of 1,3-diphenyl-2-imidazolinium chloride recently reported (1).

EXPERIMENTAL

Kinetic Studies—A stock solution was prepared by dissolving 90 mg (3.16×10^{-4} mole) of I in 5 ml of 0.1 *N* HCl, and water was added to 50 ml. Two-milliliter aliquots were added to 200-ml volumetric flasks, equilibrated at various temperatures, containing 198 ml of the acidic or buffer solution. Ten-milliliter samples were withdrawn periodically and scanned[2] from 210 to 350 nm. The absorbance readings at 272 ± 1 nm were used to evaluate the rate of conversion of I to II.

A stock solution of II containing 95.5 mg (3.16×10^{-4} mole) in 50 ml of 0.01 *N* HCl was prepared. Two-milliliter aliquots were added to 198 ml of acidic or buffer solution previously equilibrated at various temperatures. Samples (10 ml) were taken periodically, and their UV spectra were scanned from 210 to 350 nm to monitor possible degradation of II.

Preparation of II from I—Two grams of I was refluxed in 100 ml of 48% aqueous ethanol for 2 hr until the solution became almost clear. The solvent was removed *in vacuo*. The resultant white solid dissolved in 50 ml of benzene with heating. Petroleum ether was added until the point of slight turbidity, and the compound was allowed to crystallize overnight at room temperature. The yield after two recrystallizations was 1.5 g (71%) of II, mp 170–171°.

Determination of pKa Values—A stock solution of 6.3×10^{-3} *M* I was prepared and added to 0.01 *M* tromethamine buffers (pH 7.8, 8.0, 8.2, and 8.4) along with 0.1 *N* HCl and 0.09 *N* NaOH to give a final concentration of 6.3×10^{-5} *M*. The samples were quickly read[2] at 272 nm. The pKa was calculated to be 8.55 ± 0.05 by the procedure of Albert and Sergeant (2).

Compound II (151.4 mg, 0.005 *M*) was dissolved in 95 ml of boiled water with the aid of 5 ml of 0.1 *N* HCl. The solution was titrated with 0.5-ml increments of 0.1 *N* KOH, giving a pKa value of 8.53 ± 0.05 at 25° (2).

[1] Sandoz Pharmaceuticals, East Hanover, N.J.

[2] Cary model 14 recording spectrophotometer.

Table I—Observed First-Order Rate Constants, k_{obs} in hours^{-1}, for Decomposition of Mazindol in Aqueous Solution (6.32×10^{-5} *M*)[a]

Buffer	Buffer pH	Observed Rate Constants[b,c]			
		37°	45°	55°	70°
Phosphate buffer[d]	7.6	1.07	1.87	—	—
Phosphate buffer	7.15	0.41	0.88	2.03	—
Phosphate buffer	6.7	0.134	0.31	1.29	—
Phosphate buffer	6.35	—	0.20	0.48	—
Phosphate buffer	6.0	0.055	0.097	0.23	0.90
Phosphate buffer	5.7	0.023	0.056	0.14	0.56
Acetate buffer[e]	5.4	—	0.038	0.092	0.46
Acetate buffer	5.0	0.0136	0.033	0.073	0.25
Acetate buffer	4.5	0.0122	0.027	0.057	0.21
Acetate buffer	4.0	0.0103	0.023	0.051	0.17
Formate buffer[e]	3.75	0.0073	0.0156	0.038	0.134
Formate buffer	3.35	0.0054	0.0128	0.031	—
Formate buffer	3.00	0.0043	0.0119	0.024	0.088
Formate buffer	2.75	0.0032	0.0070	0.0177	0.065

[a] The reaction was followed by loss of absorbance at 272 nm and studied for at least two half-lives. Rate constants were reproducible within 15%. The pH values were measured at the specified temperatures (ionic strength 0.2). [b] Supplementary values for rate constants listed are as follows [pH and k (hr^{-1})]: At 37°: 7.62, 0.75; 7.1, 0.33; 6.65, 0.124; and 6.0, 0.051. At 55°: 6.67, 0.88; 6.64, 0.59; 6.5, 0.35; 6.0, 0.22; 5.85, 0.150; 6.67, 0.80; 6.61, 0.77; 6.5, 0.68; and 6.4, 0.51. At 60.5°: 6.8, 1.66. At 70° (run at formate-ion concentration of 0.08 *M* and ionic strength 0.2): 5.0, 0.22; 4.50, 0.15, 0.16; and 4.0, 0.14. [c] Some k_{obs} values deviate somewhat from those listed in Fig. 3. This is likely a consequence of slight pH deviations in the pH 6–7.2 region, which result in large changes in the k_{obs} at these pH values where the slope of the log k_{obs}–pH profile is +1. These experiments were run by different operators in different months, *etc.*, so other factors may also come into play. [d] Phosphate buffers were prepared according to Ref. 8, except the pH 6.00 buffer which was made from 0.115 *M* NaH_2PO_4 and 0.028 *M* Na_2HPO_4 and the pH 6.35 buffer which was adjusted with sodium hydroxide. [e] Acetate and formate buffers were produced from 8 g of sodium hydroxide, sufficient acid, and dilution with water to give the appropriate pH in 1 liter of solution.

All pH measurements were carried out on a pH meter[3] standardized with phthalate and borate buffers at the specified temperatures (2).

TLC—The solvent system utilized was chloroform–ethanol–ammonia (80:20:1). Silica gel GF plates were spotted with 20 μl of solution containing 25–50 μg of I and/or II. The walls of the tank were lined with filter paper saturated with solvent. The product (II) and reactant (I) showed R_f values of 0.8 and 0.9, respectively, by this method when the plates were dried and visualized under shortwave UV light.

RESULTS AND DISCUSSION

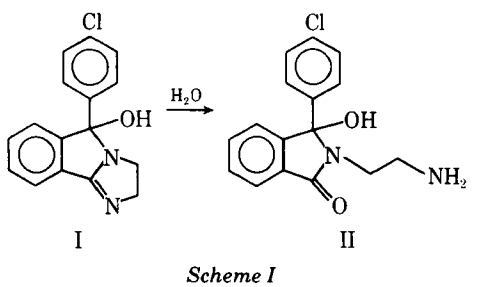
The solvolysis of I was studied between pH 0.3 and 7.2. The phthalimidine reaction product (II) was preparatively made from I (Scheme I) and was compared with an authentic sample of II[4]; they were found to be identical by NMR, mass, IR, and UV spectra, microanalysis, and melting-point determination.

Compound II was produced in almost quantitative yields from I by a first-order process. At higher acidities (below pH 2.6), the reaction was followed for at least 300 hr and exhibited pseudo-first-order kinetics for that time. Only I and II were discernible by TLC and UV spectrophotometry under the conditions examined.

The observed first-order rate constants were calculated from the expression:

$$\log (A_t - A_\infty) = \log (A_0 - A_\infty) - k_{obs}t/2.303 \qquad \text{(Eq. 1)}$$

where A_t, A_0, and A_∞ are absorbances at times t, zero, and infinity, respectively; k_{obs} is the observed or pseudo-first-order rate constant in hours^{-1}; and t is time in hours. Absorbance disappearance was followed at 272 ± 1 nm, with the hydrolysis product (II) and reactant (I) possessing molar absorptivities of 2.37×10^3 and 14.00×10^3 liters/mole cm, respectively. The absorbances for a given concentration of I and II were practically invariant over the pH scale studied (up to about pH 7.6).



Scheme I

Compound II did not degrade further under the conditions employed except after 500 hr in 0.1 *N* HCl at 70°; a slight change in UV spectra was seen along with a third trace spot on the TLC plates. Figure 1 illustrates data obtained for the decomposition of I at various pH values at 55°. Table I shows typical results from which the rate–pH profile was constructed (Fig. 2).

Hydroxide-Ion Catalysis—The right-hand portion of the rate–pH profile in Fig. 2, above pH 6, has a slope of about 1. This result is partially the consequence of hydroxide-ion attack on the imidaz-

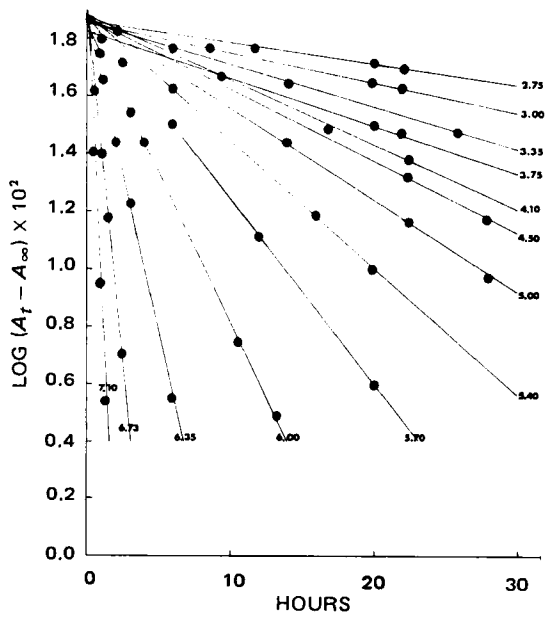


Figure 1—*Typical observed first-order plots for hydrolysis of mazindol at various pH values and 55°. The reaction was followed by loss of absorbance at 272 nm, with final attainment of residual absorbance, A_∞, characteristic of the reaction product (II).*

[3] Metrohm model E-300.

[4] Obtained from Dr. W. Houlihan, Sandoz Pharmaceuticals, East Hanover, N.J.

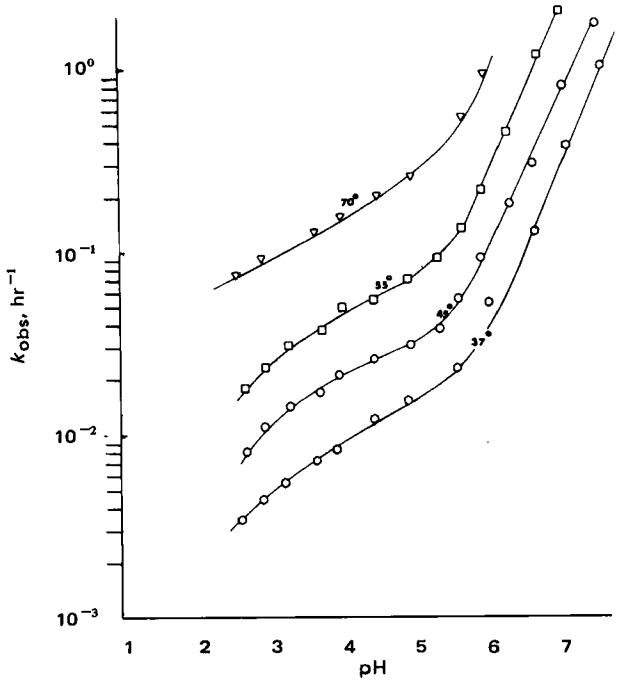


Figure 2—*Rate–pH profiles for the solvolysis of mazindol producing II at various temperatures. The reaction was monitored by absorbance loss at 272 nm (ionic strength 0.2).*

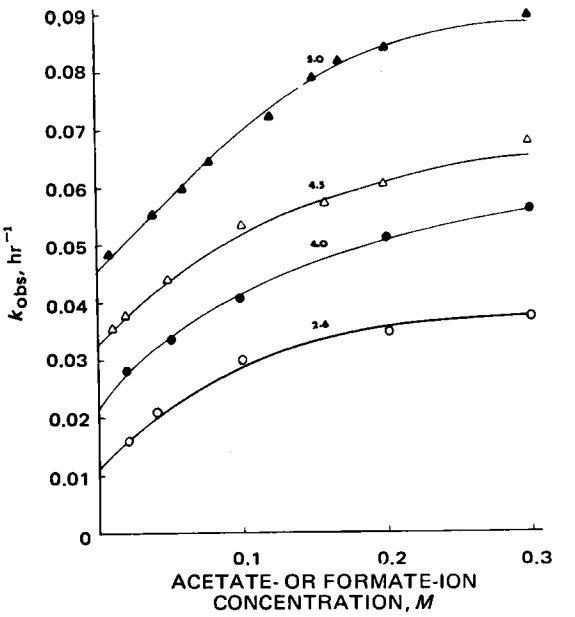


Figure 4—*Plot of observed first-order rate constants for hydrolysis of mazindol* versus *acetate- and formate-ion concentration at 55° (ionic strength constant) at various pH values. The pH 2.6 is formate buffer and the remainder are acetate systems.*

oline (I) in solution and may be written:

$$-d(I)_{tot}/dt = k_{obs}I_{tot} = k'_{OH^-}[OH^-][IH^+] + k_{OH^-}[OH^-][I] \quad \text{(Eq. 2)}$$

or:

$$k_{obs} = k'_{OH^-}[OH^-]f_{IH^+} + k_{OH^-}[OH^-]f_I \quad \text{(Eq. 3)}$$

where I plus IH^+ is the total compound in solution as neutral and protonated species, and k'_{OH^-} and k_{OH^-} are bimolecular rate constants for attack of hydroxide ion on the protonated fraction, f_{IH^+}, and the neutral fraction, f_I, respectively. The expression may be given as:

$$k_{obs} = k'_{OH^-}[OH^-]\frac{[H^+]}{[H^+] + K_{a_1}} + k_{OH^-}[OH^-]\frac{K_{a_1}}{K_a + [H^+]} \quad \text{(Eq. 4)}$$

At a pH above 7.6, the neutral molecule precipitated from aqueous solution. This precipitation, as well as a high reaction velocity, precluded study in these ranges. In the absence of other catalytic agents, *i.e.*, buffers, the k_{obs} of Eqs. 1–6 is actually the k_0 in Eq. 7.

The second term in the right-hand side of Eq. 4 may be omitted when $[H^+] \gg K_a$; $K_a/(K_a + [H^+]) \sim 0$. The resultant equation is written in the logarithmic form when $[H^+] \gg K_a$ in the usual manner:

$$\log k_{obs} = \log k'_{OH^-} + \log K_w - \log [H^+] \quad \text{(Eq. 5)}$$

or:

$$\log k_{obs} = \log k'_{OH^-} - pKw + pH \quad \text{(Eq. 6)}$$

In the range below 7.25 (about 1.30 units below the pKa), a plot of the log of the observed rate constant *versus* pH should give a straight line with a slope of 1 and an intercept of $\log k'_{OH^-} - pKw$.

Buffer Effects—Equations 4–6 hold in the absence of other catalytic species; however, a specific increment of the observed rate constant may be assigned to phosphate buffer catalysis at and above pH 5.7 (Fig. 2). The catalytic constants for $H_2PO_4^-$ and HPO_4^{-2} may be defined in the following manner, as mentioned by Notari and Caiola (3):

$$k_{obs} = k'_{H_2PO_4^-}[H_2PO_4^-] + k_{HPO_4^{-2}}[HPO_4^{-2}] + k_0 \quad \text{(Eq. 7)}$$

The k_0 is composed primarily of $k'_{OH^-}[OH^-]$ as stated in Eq. 4. Equation 7 may be written as:

$$k_{obs} = \{k_{H_2PO_4^-}(R) + k_{HPO_4^{-2}}[HPO_4^{-2}] + k_0 \quad \text{(Eq. 8)}$$

where $R = [H_2PO_4^-]/[HPO_4^{-2}]$ and is invariant at a given pH. Thus, a plot of k_{obs} *versus* $[HPO_4^{-2}]$ with the pH constant gives:

$$\text{slope} = k_{H_2PO_4^-}(R) + k_{HPO_4^{-2}} \quad \text{(Eq. 9)}$$

A plot of the slopes of Eq. 8 at three or more pH values against the R values should yield a straight line with slope $k_{H_2PO_4^-}$ and a y intercept, $k_{HPO_4^{-2}}$, as expressed in Eq. 9.

The value for $k_{HPO_4^{-2}}$ at 55° was calculated using Eqs. 8 and 9 and found to be 6.8 liters/mole hr, with $k_{H_2PO_4^-}$ being negligible. The constants for $k_{HPO_4^{-2}}$ catalysis (liters per mole hour) at other

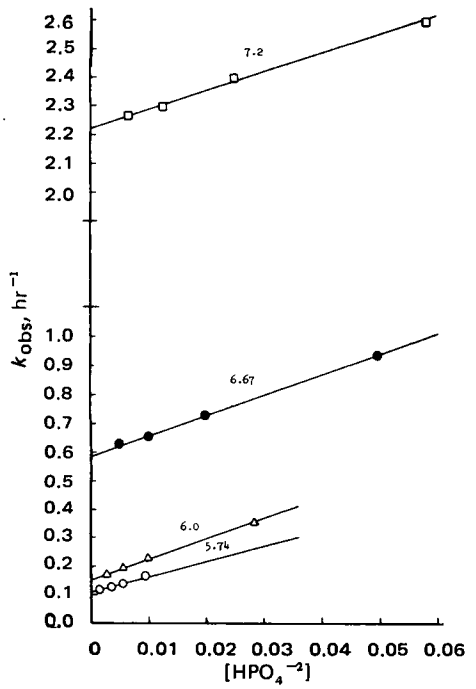


Figure 3—*Observed first-order rate constants for hydrolysis of mazindol at 55° and ionic strength 0.2, as a function of monohydrogen phosphate concentration at various pH values.*

Table II—Catalytic Constants[a] for Solvolysis of Mazindol in Aqueous Solution at Various Temperatures[b]

Constant	70°	55°	45°	37°
k'_{OH^-}	4.6×10^6	2.1×10^6	1.1×10^6	8.1×10^5
k''_{OH^-}	9×10^8	4.2×10^8	$(2.45 \times 10^8)^c$	$(1.55 \times 10^8)^c$
k''_{H_2O}	1.5×10^{-4}	6.5×10^{-5}	$(3.6 \times 10^{-5})^d$	$(2.2 \times 10^{-5})^d$
$k_{HPO_4^{-2}}$	18.0	6.8	2.6	1.5
$k_{CH_3COO^-}[CH_3COO^-]$	—	0.03	—	—

[a] Constants were calculated in liters per mole hour, except $k_{CH_3COO^-}[CH_3COO^-]$ which was estimated from data in Fig. 4 and k''_{H_2O} which was estimated in hours^{-1}. [b] Other pertinent values are: pKa_1, 8.55 (spectrometric determination at 25°), and pKa'_2, 2.9 (kinetic determination at 55°). [c] Determined by extrapolation of the Arrhenius equation. [d] Estimated from Eq. 12.

temperatures were: 37°, 1.5; 45°, 2.6; and 70°, 18.0 (Table II). Figure 3 shows an example of the utilization of Eq. 8 where the observed first-order constant is graphed on the ordinate and the $[HPO_4^{-2}]$ is graphed on the abscissa.

It is evident that the log k–pH profile, where $f_{IH^+} = 1$, in the phosphate buffer region may be adequately described in the following terms:

$$k_{obs} = k'_{OH^-}[OH^-] + k_{HPO_4^{-2}}[HPO_4^{-2}] \qquad \text{(Eq. 10)}$$

The k'_{OH^-} may be determined from Eq. 10 and/or the y intercepts in Fig. 3 by division of that value by the hydroxide-ion concentration at that temperature. The k'_{OH^-} values (liters per mole hour) were: 70°, 4.6×10^6; 55°, 2.1×10^6; 45°, 1.1×10^6; and 37°, 8.1×10^5 (Table II).

In the acetate and formate buffer range (pH 2.7–5.4) a nonlinear dependency of rate constants on conjugate base existed contrary to the expected straight lines (Fig. 4). This phenomenon was encountered by Martin *et al.* (4) in the *S*- to *N*-acetyl transfer of *S*-acetyl-β-mercaptoethylamine and was further investigated by Barnett and Jencks (5). These investigators predicted a sequence containing a catalyzed rate-determining step at low acetate and formate concentrations, followed by an uncatalyzed rate-determining step at higher buffer concentrations. Notari *et al.* (6) observed the same occurrence in the general-acid-catalyzed deamination of arabinosylcytosine.

With the imidazoline (I), the increments attributable to acetate and formate catalysis exhibited this nonlinear dependency. No attempt was made to dissect the function into constants. The amount contributed by hydroxide ion in the region, pH 2.6–5.0, may be estimated by extrapolation of acetate- and formate-ion concentrations to zero. These amounts at 55° (Fig. 4) were 0.012, 0.022, 0.033, and 0.046 hr^{-1} for pH values 2.6, 4.0, 4.5, and 5.0, respectively, and were the result of specific base (hydroxide-ion) catalysis. These values should be composed of two components: (*a*) k'_{OH^-}, and (*b*) k''_{OH^-}, which is indicative of hydroxide-ion reaction with a diprotonated species.

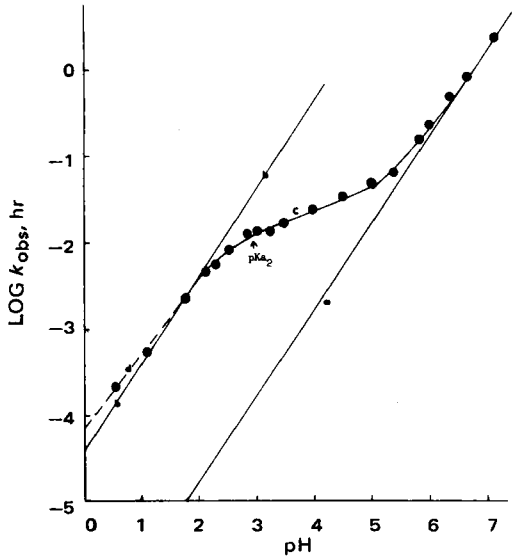


Figure 5—*Synthesis of the log* k–*pH profile for the solvolysis of mazindol at 55° (ionic strength 0.2). Line a represents hydroxide-ion attack on monoprotonated mazindol,* $k'_{OH^-}[OH^-]$, *while line b describes hydroxide-ion reaction with diprotonated mazindol,* $k''_{OH^-}[OH^-]$. *Line c is a summation of the two functions, where* $K_{a2} = 1.26 \times 10^{-3}$ M. *Line d is a summation of line b plus the increment added by water hydrolysis of the substrate as given in Eq. 11. The solid dots represent the experimental data points extrapolated to zero buffer concentration.*

Log *k*–pH Profile—Table III lists rate constants and half-life times for hydrolysis of I under acidic conditions.

The curvature of the real data points in Fig. 5 indicate the likelihood of a second pKa. The predisposition of the velocity constants to increase as the pH rises point toward specific hydroxide-ion catalysis of both mono- and diprotonated species. Due to the prevalence of buffer catalysis in the acidic region, reaction with water might be considered since the two frequently occur concomitantly.

At low pH values, pH < 2, one may write an equation of the type:

$$k_{obs} \approx k_0 = (k''_{OH^-}[OH^-] + k''_{H_2O})f_{IH_2^{+2}} \qquad \text{(Eq. 11)}$$

which simplifies when $f_{IH_2^{+2}} \approx 1$ to:

$$k_{obs} = k''_{OH^-}[OH^-] + k''_{H_2O} \qquad \text{(Eq. 12)}$$

explaining the results of Fig. 5. Thus, a plot of k_{obs} *versus* $[OH^-]$ at low pH values, *i.e.*, between 0 and 2, should yield a straight line of slope k''_{OH^-} and intercept k''_{H_2O}. The k''_{OH^-} values are listed in Table II along with results at 37 and 45° obtained by extrapolation of the Arrhenius plot.

Line b of Fig. 5 was derived from the function $k''_{OH^-}[OH^-]$, while line d is a combination of the function plus the increment attributable to k''_{H_2O}. The k''_{H_2O} values of 1.5×10^{-4} (70°) and 6.5×10^{-5} (55°) in hours^{-1} (Table II) were calculated from Eq. 12.

These findings support the thought that two principal kinetic

Table III—Observed First-Order Rate Constants for Hydrolysis of Mazindol in Acidic Solution (6.32×10^{-5} *M*)[a]

	Observed Rate Constants, hr^{-1}		$t_{1/2}$, hr	
HCl	55°	70°	55°	70°
0.005 *N*	0.0058	—	120.0	—
0.010 *N*	0.0047	—	143.8	—
0.02 *N*	0.0020	0.0069	346.7	100.3
0.10 *N*	0.0006	0.0025	1045.5	278.2
0.20 *N*	—	0.0017	—	401.2
0.50 *N*	0.00018	0.0011	3850.0	605.3
0.75 *N*	—	0.00086	—	802.3
1.00 *N*	—	0.00076	—	903.1
H_2PO_4–NaH_2PO_4[b]				
pH 2.50	0.0086	—	79.9	—
pH 2.75	0.0115	—	70.0	—
pH 3.00	0.0144	—	47.9	—
pH 3.25	0.0153	—	44.9	—

[a] Reactions were followed by loss of absorbance at 272 nm and monitored for at least 300 hr. Ionic strength was made to 0.2 with sodium chloride where possible. [b] Solutions were made from 0.05 *M* $NaH_2PO_4 \cdot H_2O$ with phosphoric acid added to give the proper pH. No evidence of phosphoric acid or phosphorus acid catalysis was noted.

Table IV—Effect of Ionic Strength (μ) on Observed First-Order Velocity Constants for Hydrolysis of Mazindol in Aqueous Solution (6.32×10^{-5} *M*) in Acetate and Phosphate Buffers at 55°

Buffer[a]	pH[b]	$\mu^{1/2}$	μ	k, hr^{-1}
Phosphate	6.67	0.10	0.01	0.862
Phosphate	6.67	0.224	0.05	0.802
Phosphate	6.67	0.447	0.20	0.715
Phosphate	6.67	0.707	0.50	0.675
Phosphate	6.67	1.0	1.0	0.595
Acetate	4.5	0.10	0.01	0.045
Acetate	4.5	0.224	0.05	0.040
Acetate	4.5	0.447	0.20	0.036
Acetate	4.5	0.707	0.50	0.029
Acetate	4.5	1.0	1.0	0.024

[a] Reaction solutions (200 ml) were prepared by taking 10 ml of acetate or phosphate buffer (μ = 0.2) with addition of sodium chloride to bring the solutions up to the stated ionic strengths. Phosphate buffers were prepared according to the specifications of Ref. 8. Acetate buffers were made by adding acetic acid to 8 g of sodium hydroxide to give the proper pH and dilution to 1 liter with water. [b] Due to the extreme effect of pH on rate constants of pH 6.67 phosphate buffer, a small amount of 1 *N* NaOH had to be added to the solutions containing larger amounts of sodium chloride. The most added was 0.2 ml of 1.0 *N* NaOH to adjust pH to 6.67.

factors work at pH < 2: (*a*) spontaneous decomposition, and (*b*) hydroxide-ion attack on some polyprotonated form of I.

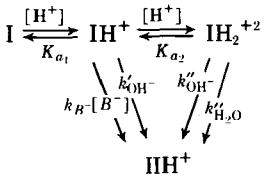
Figure 5 contains a second diagonal line of slope 1, line a, which signifies hydroxide-ion reaction with the monoprotonated molecule, $k'_{OH^-}[OH^-]$, from Eq. 4 (Table II). Once the bimolecular rate constants for attack of hydroxide ion on monoprotonated and diprotonated imidazoline were determined, the K_{a_2} could be estimated (Eq. 13) by means of data fitting to meet experimental results. The intermediate area between lines a and b, line c, is a combination of the two functions $k'_{OH^-}[OH^-]$ and $k''_{OH^-}[OH^-]$, with K_a 1.26×10^{-3}, pKa 2.9, being used for analysis of the data. A small contribution was made by the k''_{H_2O}, but it may be neglected above pH2.0.

The k''_{OH^-} values of 9×10^8 (70°) and 4.2×10^8 (55°) liters/mole hr were evaluated from the data in Table III by means of Eqs. 11 and 12. These values are more than two orders of magnitude above the k'_{OH^-} values for similar temperatures, which were 4.6×10^6 and 2.1×10^6 liters/mole hr, respectively. Results of this type may be expected for the reaction of hydroxide ion with IH_2^{+2}, because the constants should be considerably larger than those for the reaction of OH^- with IH^+.

Figure 5 illustrates the theoretical log k_0–pH profile, using the relationship:

$$k_0 = k'_{OH^-}[OH^-]\frac{[H^+]}{[H^+]+K_{a_1}} + (k''_{OH^-}[OH^-] + k''_{H_2O})\frac{[H^+]}{[H^+]+K_{a_2}} \quad \text{(Eq. 13)}$$

Ionic Strength Effects—The perturbation of the observed first-order velocity constants by addition of neutral salts at constant pH values are shown in Table IV. These results imply reaction of a negative and positive ion at pH 6.67 and 4.5. This finding



Scheme II—Schematic diagram for the solvolysis of mazindol and its protonated forms, IH^+ and IH_2^{+2}, to produce 2-(2-aminoethyl)-3-(p-chlorophenyl)-3-hydroxyphthalimidine (II) in its protonated form, IIH^+. (The possibility exists of diprotonated II, but it was not noted by the experimental methods utilized.) Both molecules primarily protonated (95+%) under the conditions investigated.

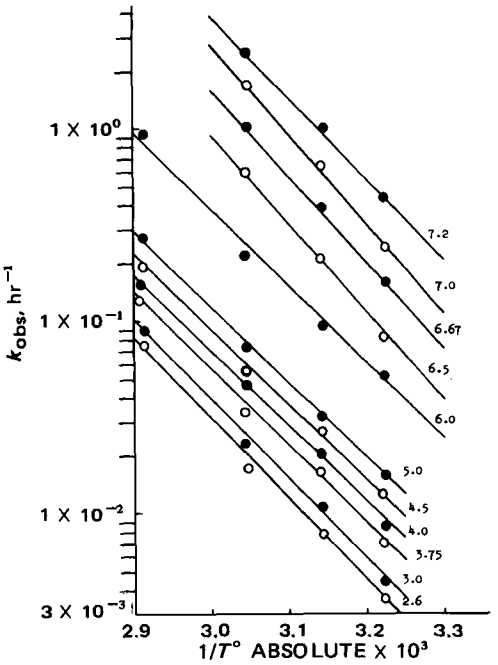


Figure 6—*Typical Arrhenius plots for hydrolysis of mazindol in aqueous media at the indicated pH values.*

cannot be considered conclusive since the Debye–Hückel theory and Brønsted–Bjerrum equation are valid for solutions below 0.01 *M* of 1:1 electrolytes (7).

Dependency of Reaction Rate on Temperature—The Arrhenius parameters for solvolysis of I were determined from the slopes and intercepts of plots of log k_{obs} *versus* the reciprocal of the absolute temperature in accordance with the equation:

$$\log k_{obs} = \log P - E_a/2.303RT \quad \text{(Eq. 14)}$$

with R being 1.987 cal deg^{-1} $mole^{-1}$. The results for several pH values are illustrated in Fig. 6. The apparent energies of activation between pH 6 and 7.2 were 20.9–22.3 kcal/mole, while those in the acetate and formate buffers varied between 17.9 and 21.1 kcal/mole. The k'_{OH^-} values give a E_a equal to 11.35 kcal/mole; $k_{HPO_4^{-2}}$ was found to have 20.9 kcal/mole as the activation energy.

SUMMARY

Mazindol (I) hydrolyzes in aqueous solution to yield a phthalimidine product (II) in practically quantitative amounts.

The solvolysis possibly proceeds through a tetrahedral intermediate similar to that suggested by Robinson (1). The intermediate should show a diminution of absorbance if it has an appreciable lifetime. This phenomenon was not encountered under the conditions studied.

The process is a relatively facile transformation at moderate temperatures in neutral media, which leads to II as a simple hydrolytic product. This transformation is an instance where the velocity constants vary directly with pH, signifying hydroxide-ion catalysis over the entire range. Negligible acid catalysis was encountered, but both general-base and water catalysis were manifest. The observed or apparent rate constant may be reasonably well described between pH 7.6 and zero by the following relationship:

$$k_{obs} = k'_{OH^-}[OH^-]\frac{[H^+]}{[H^+]+K_{a_1}} + (k''_{OH^-}[OH^-] + k''_{H_2O})\frac{[H^+]}{[H^+]+K_{a_2}} + k_B[B^-][I_{tot}] \quad \text{(Eq. 15)}$$

with $k_B[B^-]$ indicative of species in HPO_4^{-2}, acetate, and formate. Acetate and formate must be estimated due to a nonlinear dependency of the rate constant on the concentration of conjugate base. At higher acidities, the reaction rate is mainly dependent upon hydroxide-ion concentration and water attack on the diprotonated molecule in solution.

Arrhenius parameters are given and may allow prediction of shelflife as well as stability in biological media at various temperatures and conditions. Salt effects are also discussed and point toward reaction between two oppositely charged ions. A diagram of the entire process is shown in Scheme II.

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Isolation and Identification of Three New Flavones from *Achillea millefolium* L.

A. J. FALK *§, S. J. SMOLENSKI ‡, L. BAUER *, and C. L. BELL *ˣ

Abstract □ Column chromatography on silica gel of a petroleum ether extract of the flowering heads of *Achillea millefolium* L. allowed three flavones to be separated and identified. Spectral studies (PMR, mass spectrometry, and UV) and a comparison with data for compounds reported in the literature established the flavones as 5-hydroxy-3,6,7,4′-tetramethoxyflavone, artemetin, and casticin. These compounds have not been reported previously as constituents of *A. millefolium*.

Keyphrases □ Flavones—chromatographic separation and spectral identification from *Achillea millefolium* □ *Achillea millefolium*—chromatographic isolation and spectral identification of flavone components □ Medicinal plants—*Achillea millefolium*, isolation and identification of flavone components

Achillea millefolium L. (Compositae), commonly known as yarrow, grows abundantly throughout America and Europe (1). The plant has been a popular medicinal remedy, having been known to Dioscorides (2), and has been recommended for use as a tonic, a stomachic, a hemostatic, an antispasmodic, an antihemorrhoidal, an emmenagogue, an antiseptic, and an anthelmintic (3).

Chemical investigations of the plant have been numerous, extending back for more than 200 years (4, 5). One class of compounds that has received considerable attention is the flavonoids; they have been proposed as the cause of the spasmolytic activity of *A. millefolium*. Horhammer (6) examined a number of flavones and extracts of different plants for antispasmotic activity and showed *A. millefolium* to be the most active plant. He found that the plant contained apigenin and luteolin-7-glycosides. These flavones, as well as caffeic acid, were found also by Michaluk (7), and the presence of caffeic acid was more recently verified (8). In 1964, luteolin-7-glucoside was isolated from *A. millefolium* and synthesized (9). More recently, the presence of isorhamnetin was reported (10).

The present work yielded three polymethoxy flavones not previously reported as constituents of this plant.

EXPERIMENTAL

Plant Material—The flowering heads of *A. millefolium* were harvested in June 1970 at the University of Illinois Drug and Horticultural Experimental Station, Downers Grove, Ill. The seeds of wild growing *A. millefolium* were collected in the Bemis Woods, McHenry County, Ill., and were planted in a greenhouse at the Station. Shoots were transplanted from one generation to the next to assure that material of the same species was maintained. Only the inflorescence of the plant was used and was harvested at the time of opening of the flowers. The plant was identified as *A. millefolium* by chromosome count[1].

Extraction—One kilogram, 500 g at a time, of air-dried, coarsely ground plant material was extracted in a soxhlet extractor. Each 500-g portion was extracted for 24 hr with 3400 ml of petroleum ether, the extract was removed, 2500 ml of fresh solvent was added, and the extraction was continued for an additional 48 hr. All solvents were combined, and an insoluble precipitate, F1, was filtered off and set aside. The filtrate was condensed by evaporation to 1500 ml and again filtered, yielding another precipitate, F2.

The precipitate F1 was crystallized from ligroin, and the filtrate was combined with F2. The crystals were triturated with ether, and the filtrate again was added to F2. Finally, the crystals were recrystallized from chloroform–ether to yield 3.75 g of achillin, a sesquiterpene lactone previously isolated from this plant (11), and this filtrate was combined with F2.

[1] Performed by Dr. Edward D. Garbner, Department of Botany, University of Chicago.

Arrhenius parameters are given and may allow prediction of shelflife as well as stability in biological media at various temperatures and conditions. Salt effects are also discussed and point toward reaction between two oppositely charged ions. A diagram of the entire process is shown in Scheme II.

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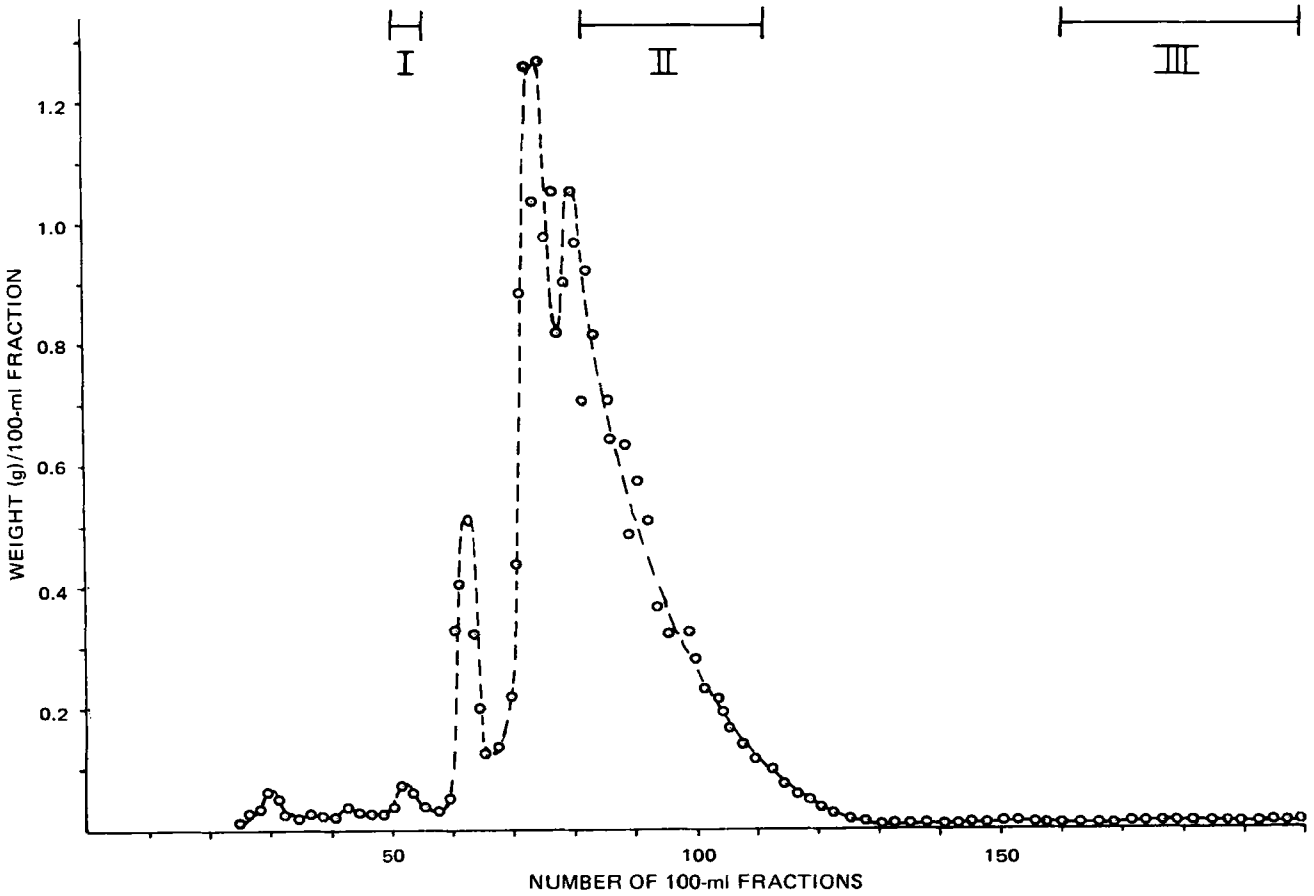


Figure 1—*Residue weights* versus *column fraction number for chromatographic separation on silica gel.*

Fraction F2, containing the flavonoids, was evaporated to dryness *in vacuo* (36.4 g). The fraction was chromatographed on a silica gel[2] column prepared by first activating the silica (1800 g) at 120° for 24 hr and then deactivating with 10% distilled water before preparing a 30% slurry in benzene–ethyl acetate (9:1). The column was allowed to settle for 24 hr before being charged, resulting in a column having the dimensions of 80 × 8 cm.

The fraction was dissolved in a minimum of eluent, benzene–ethyl acetate (9:1), and chromatographed. Two hundred column fractions of 100 ml each were collected, each was evaporated to dryness, and the residue weight was determined. A plot of the residue weight *versus* fraction number is shown in Fig. 1.

Isolation of 5-Hydroxy-3,6,7,4′-tetramethoxyflavone (I)—Column fractions 50–56 (Fig. 1) were combined and evaporated to dryness *in vacuo* (0.31 g). The residue was crystallized from ether, yielding the flavone I as pale-yellow crystals (0.142 g), mp 168–170° [lit. (12) mp 169–171°]; PMR[3] (deuterochloroform with tetramethylsilane as the internal reference), ppm (δ): 3.87 (s, 3), 3.90 (s, 3), 3.92 (s, 3), 3.95 (s, 3), 6.49 (s, 1), 7.01–8.07 (m[A_2B_2], 4), and 12.60 (s, 1). UV[4] spectra in methanol and with various reagents are given in Table I. The mass spectrum[5] of I is given in Fig. 2.

Isolation of Artemetin (II)—As indicated in Fig. 1, fractions 78–109 were combined and evaporated to dryness *in vacuo*, yielding 11.84 g of residue. This residue consisted primarily of achillin, as shown by TLC and spectral data, with one minor component (<5%). This mixture was rechromatographed, using 300 g of basic alumina (deactivated with 5% water) on a 3 × 31-cm column, and eluted with 875 ml of chloroform–acetone (1:1) to remove the achillin. The column was then eluted with 625 ml of 1-butanol–acetic acid–water (4:1:1) to remove the flavone.

After evaporation to dryness *in vacuo*, a powdery residue of the compound and alumina remained. To remove the alumina, 200 ml of benzene was added and the mixture was extracted twice with 200-ml portions of 0.1 *N* HCl. The aqueous layer was extracted twice with 200-ml portions of benzene, and the benzene layer was finally separated and evaporated to dryness *in vacuo*, leaving an orange residue. Recrystallization was from benzene–petroleum ether to yield a yellow-orange solid (0.070 g), mp 159–160° [lit. (13) mp 161.5°].

Table I—UV Spectral Data in Methanol with Various Reagents

Reagent	λ_{max}, nm (log ϵ)	
	Compound I	Compound III
None	213 (4.58)	212 (4.64)
	274 (4.41)	258 (4.36)
	338 (4.46)	270 (4.32)
	—	351 (4.38)
Sodium methoxide	223 (4.57)	214 (4.75)
	294 (4.52)	273 (4.45)
	323 (sh)	385 (4.15)
Aluminum chloride	213 (4.46)	214 (4.67)
	238 (4.18)	270 (4.39)
	282 (4.24)	383 (4.39)
	305 (4.18)	—
	370 (4.43)	—
Aluminum chloride–hydrochloric acid	212 (4.43)	213 (4.61)
	236 (4.16)	267 (4.37)
	288 (4.26)	282 (4.35)
	358 (4.41)	373 (4.34)
Sodium acetate	225 (4.49)	225 (4.64)
	273 (4.40)	258 (4.36)
	338 (4.49)	270 (4.32)
	—	351 (4.38)
Sodium acetate–boric acid	226 (4.49)	227 (4.59)
	274 (4.42)	258 (4.36)
	338 (4.49)	270 (4.32)
	—	351 (4.38)

[2] PF-254, E. Merck, Darmstadt, Germany.
[3] Varian Associates models A60 and T60A spectrometers.
[4] Perkin-Elmer model 202 recording spectrophotometer.
[5] Hitachi-Perkin-Elmer RMU-6D operating at 70 ev.

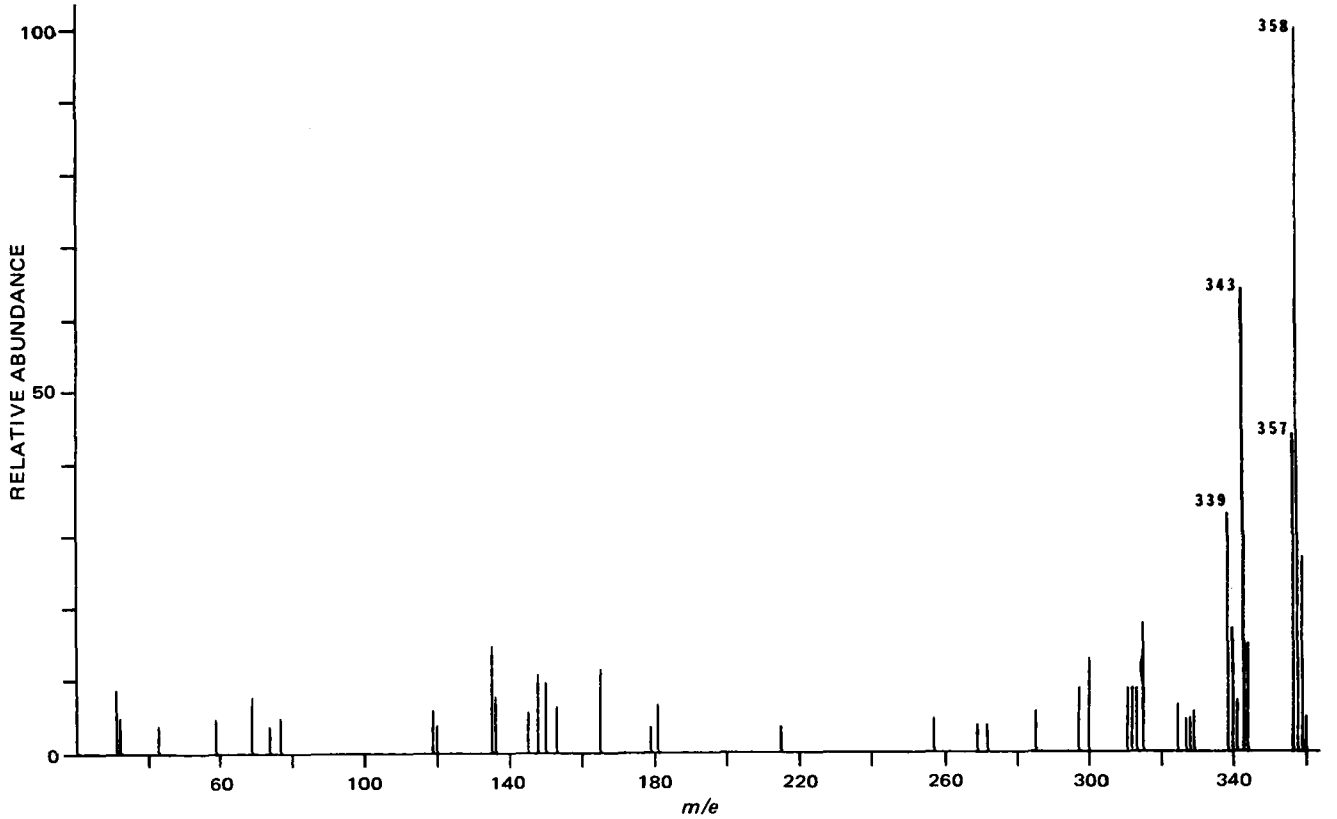


Figure 2—*Mass spectrum of 5-hydroxy-3,6,7,4′-tetramethoxyflavone at 70 ev.*

The mass spectrum of II is given in Fig. 3. Other spectral data already appear in the literature (13, 14).

Isolation of Casticin (III)—Column fractions 157–200 (Fig. 1) were combined and evaporated to dryness *in vacuo* to yield a yellow solid (0.25 g). This solid was dissolved in 50 ml of chloroform, extracted twice with 20-ml portions of 5% NaOH, and separated. The pH of the aqueous phase was adjusted to 8 by bubbling carbon dioxide gas through the solution, causing a yellow-green precipitate to form.

The solid was separated by centrifugation and dried (0.090 g), mp 183–185° [lit. (15, 16) mp 186–187°]; PMR[3] (deuterochloroform with tetramethylsilane as the internal reference), ppm (δ):

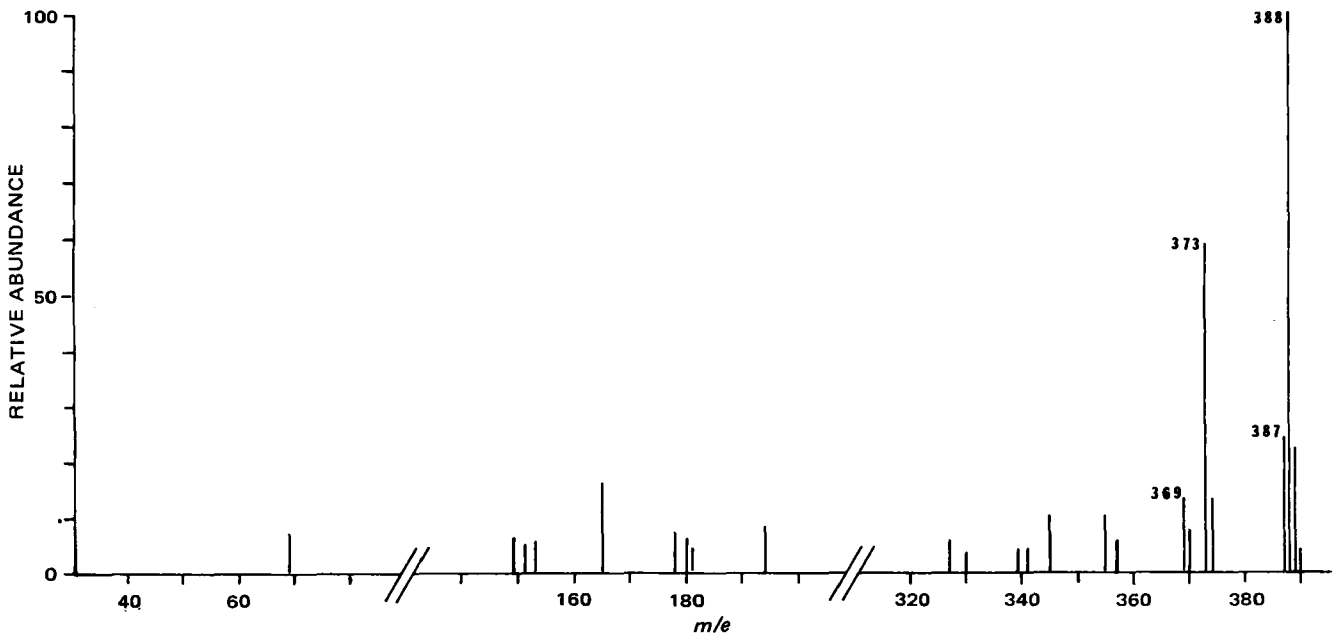


Figure 3—*Mass spectrum of artemetin at 70 ev.*

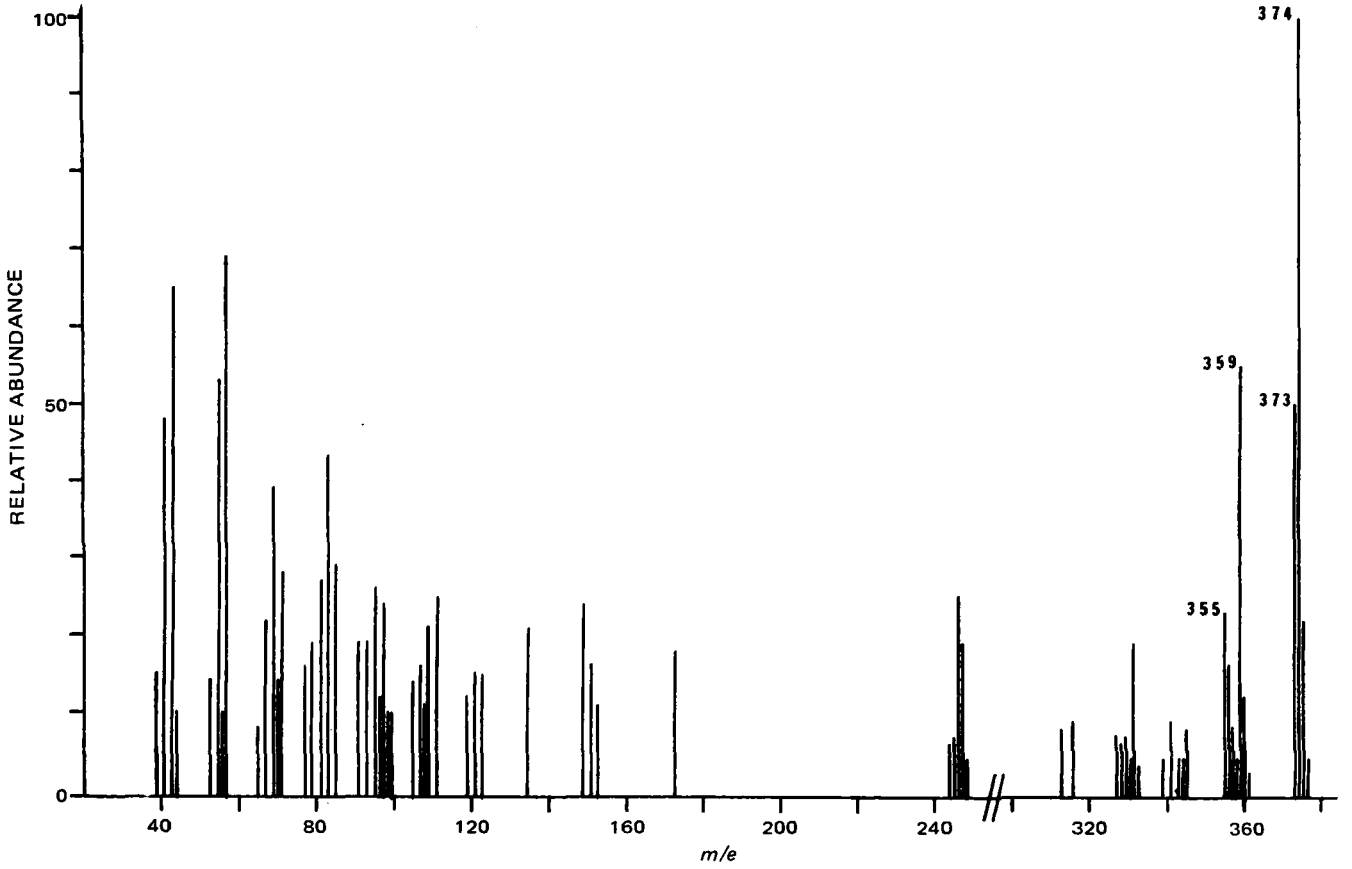


Figure 4—*Mass spectrum of casticin at 70 ev.*

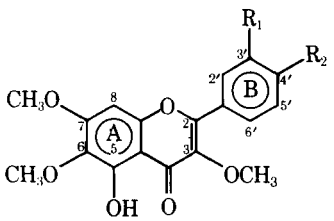
3.87 (s, 3), 3.92 (s, 3), 3.95 (s, 3), 3.98 (s, 3), 6.49 (s, 1), 6.95 (d, 1, J = 9.6 Hz), 7.65 (d, 1), 7.69 (dd, 1, J = 9.6 Hz, J = 2.0 Hz), 12.58 (s, 1), and 5.76 (s, 1). UV[4] spectra in methanol and with various reagents are given in Table I. The mass spectrum[5] of III is shown in Fig. 4.

RESULTS AND DISCUSSION

Three flavonoids were isolated from the petroleum ether extract of the inflorescence of *A. millefolium* and proved to be 5-hydroxy-3,6,7,4′-tetramethoxyflavone (I), artemetin (II), and casticin (III).

Compound I was purified by column chromatography and identified by its physical and spectral properties. The mass spectrum showed both a parent peak at *m/e* 358 (base peak) and a large P − 1 fragment ion, characteristic of flavones, at *m/e* 357.

A prominent ion at *m/e* 343, representing a methyl loss from the parent ion, and a peak at *m/e* 339, attributed to the loss of water from the P − 1 fragment, also were observed. An expected ion in flavone spectra arising from a retro Diels–Alder fragmentation of the heterocyclic ring was not observed, and its absence was explained by the substitution at the 3-position on the flavone nucleus (17). This conclusion was corroborated by the similarity in the mass spectra of the other isolated flavones, which also bear a substituent at the 3-position. Stabilization of the heterocyclic ring by the loss of a methyl radical to produce an oxonium ion (IV) probably occurred in each instance to produce a stable even-electron ion.



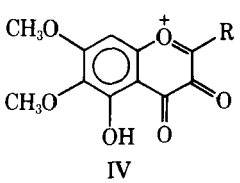
I: $R_1 = H$, $R_2 = OCH_3$
II: $R_1 = R_2 = OCH_3$
III: $R_1 = OH$, $R_2 = OCH_3$

The IR spectrum[6] revealed two strong absorptions at 1650 and 1600 cm^{-1}, indicating an α,β-unsaturated ketone with extended conjugation (18).

The 60-MHz PMR spectrum contained four singlets (3H each), indicating four methoxy groups at δ 3.87, 3.90, 3.92, and 3.95. The singlet at δ 6.49 (1H) represented an isolated aromatic proton (8-position), and the A_2B_2 pattern at δ 7.01 (2H) and 8.07 (2H) indicated a *para*-substituted phenyl group. The remaining downfield singlet (1H) at δ 12.60 was highly characteristic of a C-5 phenolic hydroxy proton hydrogen bonded to the pyrone carbonyl (19).

From these spectral data, a monohydroxy tetramethoxyflavone with a molecular formula of $C_{19}H_{18}O_7$ was concluded. While the PMR spectrum gave some information on the substitution pattern on the flavone nucleus, the precise arrangement of substituents was verified by the method of Mabry *et al.* (14) by observing the effect of a series of reagents on the UV spectrum in methanol.

The UV maximum observed in methanol was indicative of a flavone with oxygen substitution in the A ring. The bathochromic shift noted upon addition of sodium methoxide indicated that a hydroxy group was present but not on the 3- or 4′-position. The spectrum in the presence of aluminum chloride fixed the hydroxy substituent at the 5-position, and the addition of hydrochloric acid



IV

[6] Perkin-Elmer models 337 and 700 spectrophotometers. Solution spectra were obtained in deuterochloroform, using 0.15-mm KBr cells.

to the previous solution caused a shift of 20 nm, considered diagnostic of a 5-hydroxyflavone having a methoxy group on the adjacent 6-position.

These deductions, coupled with the PMR pattern reported, fixed the position of all but two methoxy groups with permissible ring sites limited to the 3-, 7-, and 8-positions. Chemical shift data fixed the substituents at the 3- and 7-positions, since the singlet at δ 6.49 was characteristic of a C-8 proton with a nonhydroxy substituent at the 7-position (14). Furthermore, the chemical shifts observed for the B ring protons were consistent with an oxygenated 3-position (14, 20).

The structure assignment of 5-hydroxy-3,6,7,4′-tetramethoxyflavone was verified by a comparison with literature data, since the compound had been isolated from *Dodonaea labulata* (20) and synthesized by Quijano *et al.* (12).

The flavone II was more readily identified, since its PMR and UV spectra appeared in the literature (13, 14). The mass spectrum (Fig. 3) exhibited a molecular ion at *m/e* 388 and the same lossess of 1 and 15 mass units observed for I. In addition, a peak at *m/e* 369 corresponded to the water loss from the P − 1 fragment ion mentioned previously. A 220-MHz PMR spectrum[7] and the series of six UV spectra (using the reagents shown in Table I) were identical with those published for artemetin (5-hydroxy-3,6,7,3′,4′-pentamethoxyflavone) (13, 14).

The third flavonoid, III, exhibited spectral characteristics similar to I and II. The 60-MHz PMR spectrum revealed four methoxy groups and signals due to four aromatic protons. The aromatic proton region of the spectrum was virtually identical (chemical shift difference <0.04 ppm) to that obtained for II. A somewhat broadened singlet at δ 5.76 (1H) could be assigned to another hydroxy group in addition to the distinctive C-5 hydroxy proton with the characteristic low-field resonance at δ 12.58.

A comparison of the mass spectra of II and III indicated a mass difference equivalent to replacing one methoxy group in II by a hydroxy group to give a molecular ion at *m/e* 374. The characteristic fragment ions were the same as observed for the other two flavones. Based on the mass spectra and PMR data, III was concluded to be a dihydroxytetramethoxyflavone.

In the manner previously described, the UV spectra in methanol with various reagents were used to determine the positions of substituent groups (Table I). The methanol spectrum exhibited a maximum at 351 nm, well within the region expected for a flavone with a methoxy group at the 3-position, and two maxima in the 250–275-nm region, indicative of 3′,4′-oxygenated flavones. Addition of sodium methoxide to the methanol solution resulted in a 34-nm bathochromic shift from 351 to 385 nm and a decrease in intensity. These results eliminated the possibility of a hydroxy group at the 4′-position (14).

The addition of sodium acetate or sodium acetate–boric acid produced no changes in the UV spectrum from that obtained in methanol alone and indicated the absence of a hydroxy group at the 3-, 7-, or 4′-position. The aluminum chloride and aluminum chloride–hydrochloric acid treatments verified, as before, a C-5 hydroxy group with an adjacent methoxy group at C-6.

The similarities in the PMR spectrum with that of artemetin and the UV spectral behavior described indicated that III was 5,3′-dihydroxy-3,6,7,4′-tetramethoxyflavone (casticin). Physical properties verified the assignment, since casticin was isolated previously from *Vitex agnus castus* (15) and was synthesized by Horhammer *et al.* (16). The isolation of these three flavones, new to *A. millefolium*, was repeated, following the same procedure, on a larger scale (4 kg of plant material) to obtain sufficient material for biological testing.

[7] Varian Associates HR 220 spectrometer.

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Quantitative GLC Determination of Pentylenetetrazol in Biological Fluids

H. W. JUN ˣ, W. B. ITURRIAN, J. T. STEWART, and B. H. LEE

Abstract □ A quantitative, sensitive, and specific GLC method was developed for the determination of pentylenetetrazol in water, plasma, and urine. The assay involves a single extraction of the sample into chloroform followed by centrifugation, evaporation, and chromatography. The method for pentylenetetrazol is reproducible, and the sensitivity limit of the assay is 0.5 μg of pentylenetetrazol/ml of biological fluid using a 2-ml sample. This method has a sensitivity sufficient to detect human plasma levels after therapeutic clinical doses and was successfully applied to monitor complete plasma level profiles of this drug in dogs. The data indicate that this drug is very rapidly absorbed following an oral dose, and the half-life of the drug in plasma is approximately 1 hr.

Keyphrases □ Pentylenetetrazol—GLC analysis in plasma and urine □ CNS stimulants—pentylenetetrazol, GLC analysis in biological fluids □ GLC—analysis, pentylenetetrazol in plasma and urine

Pentylenetetrazol[1] is being used for various clinical purposes in humans and animals. The main use in humans is as a central nervous system (CNS) stimulant for the therapeutic management of chronic mental depression in geriatric patients. Although several GC methods (1–3) and other techniques (4, 5) have been described for the determination of pentylenetetrazol in biological samples, complete blood level profiles of this drug have not been reported. One reported GC method (3) involves extraction of pentylenetetrazol from aqueous samples with ether or benzene. However, its usefulness in quantitative determinations of the drug in plasma samples was limited by low extraction recoveries and inadequate reproducibility.

To evaluate the absorption and elimination of this drug in the body, a sensitive and specific method of drug analysis was needed. This report describes a sensitive and specific GLC method for the quantitative determination of pentylenetetrazol in biological fluids.

EXPERIMENTAL

Chemicals—Pentylenetetrazol[1] and the calibration marker, hexamethylenamine[2], were used as received. Spectrophotometric grade chloroform[3] and absolute ethanol[4] USP were employed.

Instrumentation—Chromatographic determinations were made with a gas chromatograph[5] equipped with a dual flame-ionization detector and a recorder[6]. The chromatographic column was a 0.3-cm (0.125-in.) o.d. stainless steel column, 1.8 m (6 ft) in length, packed with 10% UC-W98 on Diatoport S (80–100 mesh)[7].

[1] Metrazol, Knoll Pharmaceutical Co., Orange, N.J.
[2] Wm. P. Poythress & Co., Richmond, Va.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] U.S. Industrial Chemicals Co., New York, N.Y.
[5] Varian Aerograph model 1800.
[6] Bristol's Co., Waterbury, Conn.
[7] Hewlett-Packard, Avondale, Pa.

Table I—Partition Coefficients of Pentylenetetrazol between Water and Various Organic Solvents at 22°

Organic Solvents	Partition Coefficient[a], Solvent Drug Concentration/ Water Drug Concentration
Ether	0.11
Carbon tetrachloride	0.25
n-Octanol	1.37
Benzene	2.25
Chloroform	23.4

[a] Each value is an average of three experiments.

The column was conditioned for 24 hr at the operating settings after each chromatographic determination. The operating conditions were: column oven temperature, 195°; injection tube temperature, 250°; and detector block temperature, 255°. Compressed nitrogen was used as a carrier gas at the flow rate of 60 ml/min. The flow rates of hydrogen and compressed air were maintained for optimal results.

Extraction Procedure—Place a 2-ml plasma or urine sample in a Teflon-lined screw-capped centrifuge tube (15 ml) and add 12 ml of water-saturated chloroform. When more than a 2-ml sample is used, the volume of chloroform is at least six times greater than the volume of the sample. The sample is then extracted by mixing for at least 5 hr in a rotating apparatus[8], followed by centrifugation at 2000 rpm for 10 min.

About 8 ml of the filtered chloroform layer is transferred to a centrifuge tube and evaporated to dryness in a water bath at 80°. The wall of the centrifuge tube is washed down with 0.5 ml of anhydrous ether, followed by complete evaporation at room temperature. The residue is then redissolved in 10–20 μl of ethanol containing hexamethylenamine (1-μg/μl solution). One microliter of ethanol, containing an unknown amount of pentylenetetrazol and 1 μg of hexamethylenamine, is injected into the chromatograph. Hexamethylenamine is used as a calibration marker due to its stability, solubility, and adequate retention time.

Standard Calibration Curve for Pentylenetetrazol Standards—Known quantities of pentylenetetrazol are dissolved in ethanol containing hexamethylenamine (1 μg/μl) to obtain pentylenetetrazol concentrations over a 0.2–4.0-μg/μl range. When 1-μl aliquots of these standard solutions are injected into the column, the samples contain varying amounts of pentylenetetrazol per microgram of the calibration marker. The calibration curve is then obtained by plotting the known concentrations of pentylenetetrazol against the corresponding peak height ratios. The peak height ratios are obtained by dividing the peak height due to the hexamethylenamine calibration marker into the peak height of the pentylenetetrazol standard.

Calibration Curve of Pentylenetetrazol Standards Analyzed in Human Plasma—Known amounts of pentylenetetrazol (5–30 μg) are added to 2-ml aliquots of control human plasma, and the drug is then extracted in the same manner as described in *Extraction Procedure*. After evaporation of the solvent extracts, the residues are dissolved in 10-μl aliquots of ethanol containing 1 μg/μl of hexamethylenamine. Following the injection of 1 μl of each sample into the chromatograph, a calibration curve for pentylenetetrazol standards analyzed in plasma is made as previously described.

[8] Menold, Lester, Pa.

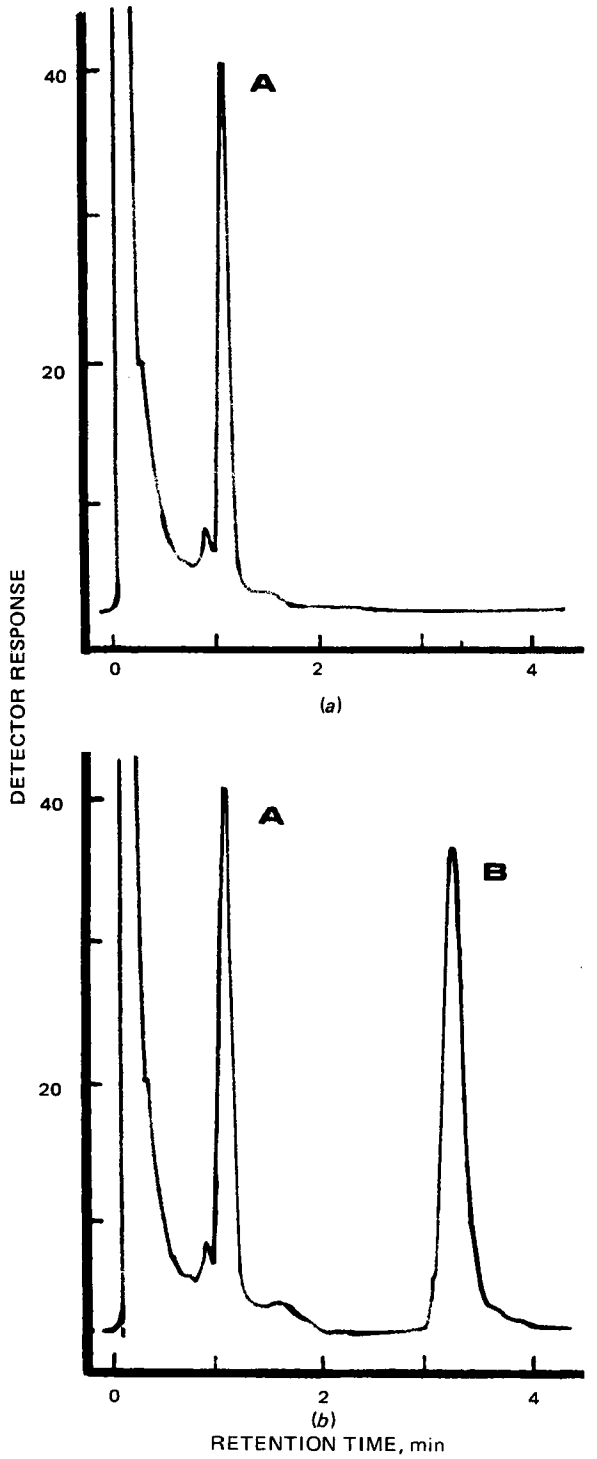

Figure 1—*Gas–liquid chromatograms of:* (a) *dog plasma extracts of control plasma and* (b) *plasma extract containing pentylenetetrazol. Key: A, hexamethylenamine; and B, pentylenetetrazol.*

Drug Recovery Studies—Known quantities of pentylenetetrazol (2–30 μg) were added to 2 ml of dog control plasma. The samples were thoroughly mixed and processed in the same manner as the unknown samples. The pentylenetetrazol in each sample was determined chromatographically by injecting a 1-μl aliquot of the ethanol containing the dissolved residue into the column and measuring the peak height ratios.

Calculations—The concentration of pentylenetetrazol (micrograms per milliliter) in unknown plasma or urine samples is determined as follows:

$$\text{drug concentration (micrograms per milliliter)} = \frac{\text{slope of calibration curve} \times \text{peak height ratio} \times \text{microliters of ethanolic solution} \times \text{milliliters of chloroform used for extraction}}{\text{milliliters of chloroform taken for evaporation} \times \text{milliliters of plasma or urine sample}} \quad \text{(Eq. 1)}$$

Drug Partition Studies—To determine the best solvent for the extraction of pentylenetetrazol from biological samples, the partition properties of this drug between water and various organic solvents were determined at room temperature. Ten milliliters of pentylenetetrazol in water (10 mg/ml) and an equal volume of a water-saturated organic solvent were mixed in a centrifuge tube with a Teflon-lined screw-cap by a rotating apparatus for 20 hr. After equilibrium, 5 mg of hexamethylenamine was dissolved in 5 ml of the aqueous layer, and 1 μl of this solution was directly injected into the chromatograph. Peak height ratios were measured for the determination of pentylenetetrazol in each water layer. The partition coefficients are expressed as the ratio of the drug concentration in the organic layer to the drug concentration in the water.

Drug Plasma Level Studies—To demonstrate the utility of this method, the pentylenetetrazol dose (15 mg/kg) was intravenously administered to one dog (11 kg) and orally administered to another dog (9 kg). Blood samples (4 ml) were withdrawn from the cephalic vein of the front leg using a heparinized in-dwelling catheter at various time intervals after dosing. Blood samples were centrifuged, and the plasma was transferred for extraction. During the experiment, dogs were anesthetized with pentobarbital sodium. Urine samples were collected for the chromatographic examination.

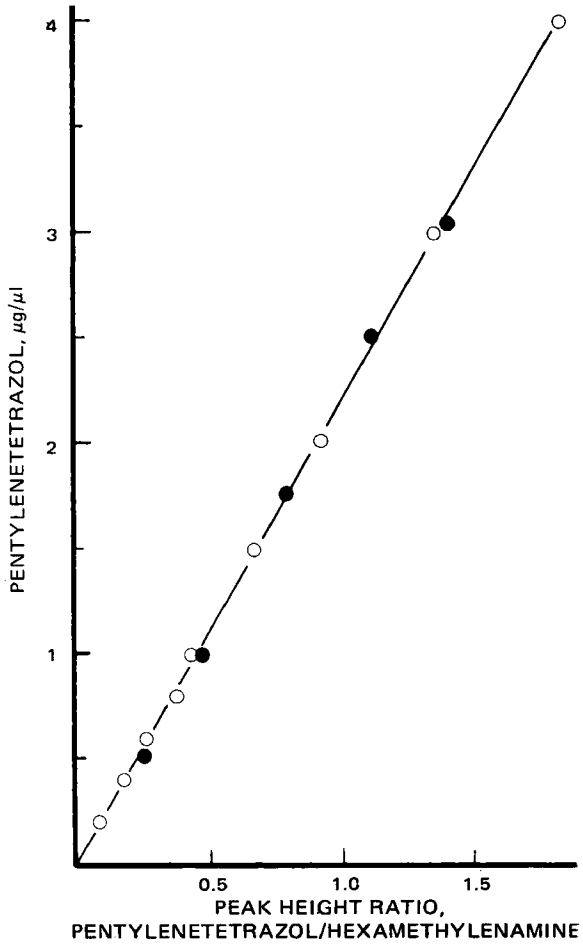

Figure 2—*Standard calibration curve for pentylenetetrazol to the calibration marker in ethanol (○), and for pentylenetetrazol extracted from human plasma (●).*

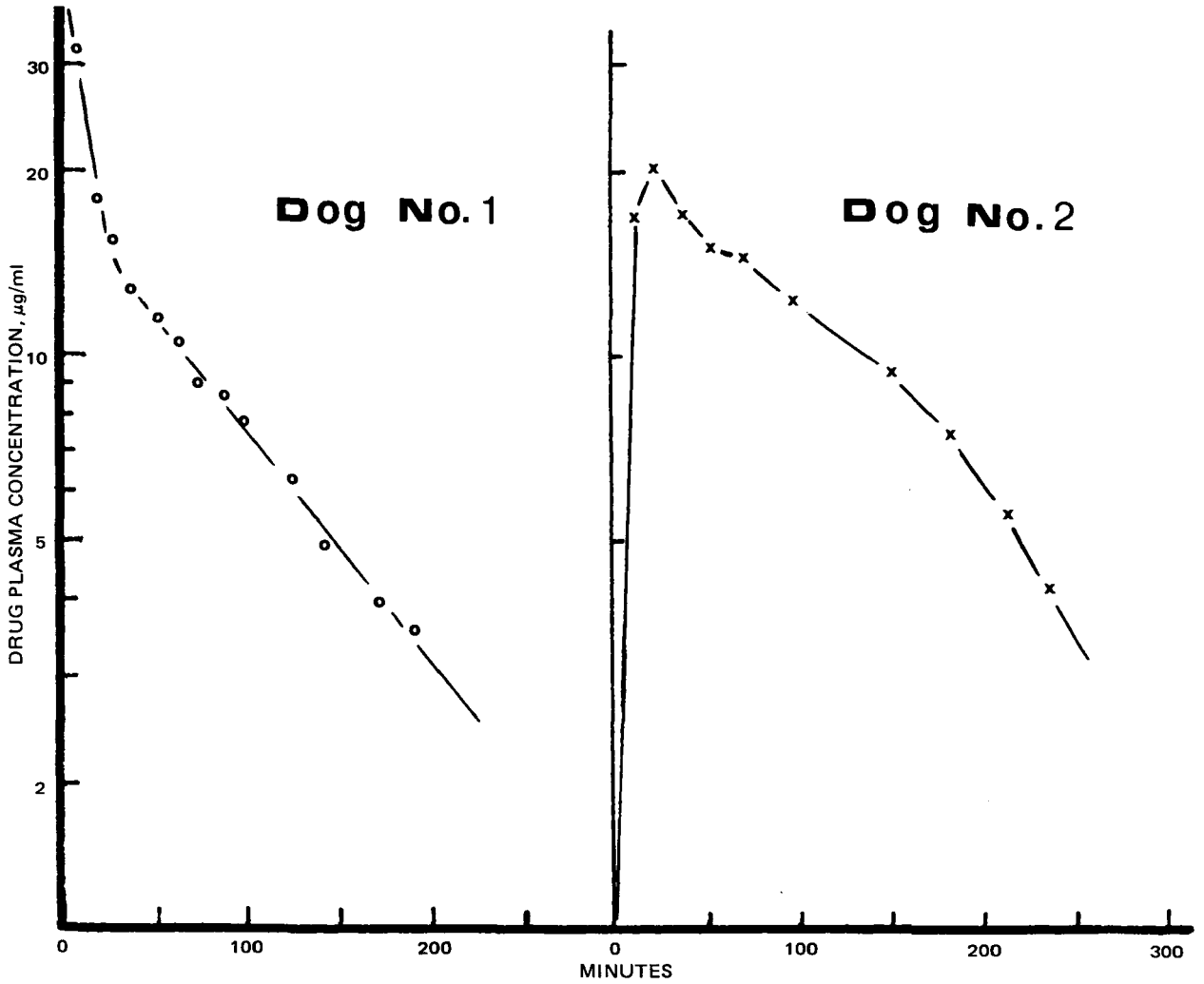

Figure 3—*Plasma concentrations of pentylenetrazol following the rapid intravenous administration of a 15-mg/kg dose in Dog 1 (11 kg) and following the oral administration of a 15-mg/kg dose in Dog 2 (9 kg).*

Human blood samples were obtained from three patients (57–68 years of age) regularly taking pentylenetetrazol tablets. This experiment was performed to test the sensitivity of the proposed technique for detecting drug plasma levels in patients after subconvulsive therapeutic doses.

RESULTS AND DISCUSSION

Drug Partition Studies—Since pentylenetetrazol has a high solubility in water as well as in most organic solvents, it was necessary to determine the partition properties of this compound (Table I). The large partition coefficient of pentylenetetrazol in the chloroform layer led to the use of chloroform in the extraction. The partition coefficient of this drug in chloroform is about 200 times greater than in ether, although these two solvents have similar dielectric constants (6). This difference may be due to the formation of a molecular interaction between pentylenetetrazol and chloroform. After equilibrium, the concentration of pentylenetetrazol in the water layer was determined by direct injection of an aliquot of the aqueous phase into the chromatograph and determination of the peak height ratio of the drug to the calibration marker.

Table II—Recoveries of Pentylenetetrazol Standards Added to Control Biological Fluids of the Dog

Pentylenetetrazol Added, μg/ml		Recovery of Pentylenetetrazol[a], %	
		Plasma	Urine
1		105.0	96.0
2		105.0	—
3		96.7	103.0
5		98.0	—
7		107.1	102.5
9		105.6	98.0
11		98.2	—
13		—	96.5
15		93.4	95.1
	Mean	101.1	98.5
	± *SD*	5.1	3.4

[a] Each percent recovery of pentylenetetrazol represents the results from a single sample subjected to the complete pentylenetetrazol analytical method.

Chromatography—Figure 1 shows typical chromatograms for a control plasma extract in which the calibration marker was added and for a pentylenetetrazol standard added to a control plasma sample and analyzed by this procedure using the calibration marker. Both peaks are sharp and symmetrical, and the chromatographic time for each determination is relatively short. Analysis of many control samples of plasma showed no interfering peaks with retention times similar to pentylenetetrazol (3.2 min) or hexamethylenamine (1.1 min).

Calibration Curves—As shown in Fig. 2, a linear relationship exists for known concentrations of pentylenetetrazol over a 0.2–4-μg/μl range when plotted against the corresponding peak ratios. An identical linear line is also obtained for the analysis of drug standards added to control samples by plotting the concentrations of pentylenetetrazol added to control plasma samples against the peak height ratio as described under *Experimental*. This correlation indicates a high efficiency of extraction and reproducibility of the analytical method for pentylenetetrazol.

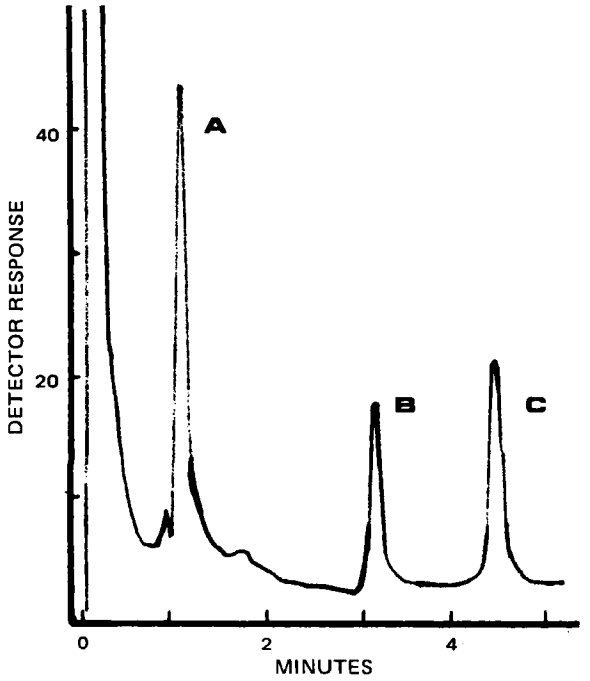

Figure 4—*Gas–liquid chromatogram of dog plasma extracts. Key: A, calibration marker; B, pentylenetetrazol; and C, unidentified metabolite.*

Drug Recovery—Results for recovery experiments of pentylenetetrazol standards added to control dog plasma and urine are shown in Table II. The data indicate that recovery of the drug from dog plasma is essentially quantitative in the 1–15-μg/ml range using a 2-ml plasma sample (mean ± *SD* = 101.1 ± 5.1%). Actual concentrations of the drug in dog plasma samples were also in this concentration range.

The average recovery of the drug added to control dog urine (2 ml) was 98.5 ± 3.4%. Because of its high partition property into chloroform, almost complete recovery of the drug was achieved after a single extraction process. The mean recoveries of the drug from control human plasma (95.8 ± 4.4%) and urine samples (97.0 ± 3.9%) were also quantitative.

Drug Plasma Level Studies—The described analytical method for pentylenetetrazol was used in the determination of plasma levels of pentylenetetrazol in dogs following rapid intravenous and oral administrations of a 15-mg/kg dose. Plasma profiles after the rapid intravenous injection (Fig. 3) indicate the applicability of the two-compartment pharmacokinetic model proposed previously (7, 8). The half-life of the drug in the plasma of one dog (male, 11 kg) was about 1 hr.

The drug plasma levels after oral administration of the aqueous solution (135 mg in 20 ml of water) to the other dog (female, 9 kg) indicate that GI absorption of this drug was very rapid and efficient. After the maximal drug peak, which occurred in the plasma at about 25 min (Fig. 3), the plasma levels declined slowly and then more rapidly. The initial slow decline was due to the simultaneous absorption and elimination process.

Table III—Plasma Concentrations of Pentylenetetrazol in Human Plasma Samples

Subject	Dose, mg	Sample Time, hr Postdose	Concentration, μg/ml
A	100	3	1.86
B	100	5	1.45
C	100	1.25	3.1

Detection of Pentylenetetrazol in Human Plasma—Human plasma samples were obtained from three patients given therapeutic oral doses of the drug. These plasma samples (4 ml) were then analyzed by the described method (Table III). The chromatographic results indicate that the method is sufficiently sensitive to detect the plasma levels of pentylenetetrazol after therapeutic doses of the drug.

Detection of Unidentified Metabolite—During a chromatographic analysis of plasma extracts from drug-treated dogs, a new peak due to a drug metabolite with a retention time of 4.5 min was consistently observed (Fig. 4). The size of the peak due to the metabolite in the plasma reached a maximum after about 1 hr and decreased slowly thereafter. Analysis of urine samples for the drug also showed the peak due to the metabolite at the identical retention time (4.5 min). No peak with this retention time was seen in the control plasma or urine samples. Extensive metabolism of this drug was previously reported (4, 9).

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Enterohepatic Circulation of Tetracycline in Rats

JOSEPH ADIR

Abstract □ The absorption of tetracycline hydrochloride excreted in the bile of rats was evaluated using the *in situ* intestinal preparation. For comparative purposes, the absorption of the drug from an aqueous solution having the same pH as that of the bile was also determined. After 4 hr, the amounts of tetracycline absorbed from the bile and aqueous solutions were 72.92 and 77.34%, respectively. There was no significant difference in the amount of drug accumulated in the gut tissue. The disappearance of the drug from the intestinal lumen was biexponential, and the kinetic parameters appeared to be similar. It was concluded that tetracycline excreted in the bile is readily absorbed from the rat intestine. Accordingly, biliary excretion does not seem to account for a significant elimination of this antibiotic from the body.

Keyphrases □ Tetracycline—intestinal absorption from bile and aqueous solutions, elimination, rats □ Absorption—intestinal, tetracycline from bile and aqueous solutions, rats □ Excretion, biliary—role in tetracycline elimination, rats □ Biliary elimination—tetracycline, rats

Studies on the disposition of tetracycline in humans and animals indicate that 50–70% of the dose is eliminated unchanged in the urine after both intravenous and oral administrations (1–5). Since no significant metabolism of tetracycline has been observed (6), excretion *via* the bile is presumed to be the other route of elimination (7–10). The observed high levels of tetracycline in the bile (11–13) and the detection of high percentages of the administered dose in feces (3, 4, 14) supported this notion.

Recently, von Wittenau and Twomey (8) reported that doxycycline excreted in dog feces could not be totally extracted by the same solvent system employed to extract the unchanged drug from other fecal excreta. Accordingly, it was speculated that the positively charged doxycycline may form a complex with the negatively charged bile salts and that this complex is unavailable for reabsorption from the intestine (8).

The purposes of this investigation were to determine whether tetracycline excreted in the bile is reabsorbed from the small intestine and, eventually, to delineate the role of biliary excretion in the overall elimination of this drug from the body.

EXPERIMENTAL

Chemicals—^{3}H-7-Tetracycline hydrochloride powder was obtained commercially in a sealed borosilicate glass container[1]. The radioactive material (specific activity of 2.9 mCi/mg) was dissolved in methanol and stored at −20°. Nonlabeled tetracycline hydrochloride was used as obtained[2]. Anhydrotetracycline was synthesized according to the method of McCormick *et al.* (15), and 4-epitetracycline was synthesized according to the method of Simmons *et al.* (16).

Purity of Samples—The radiolabeled tetracycline was claimed by the manufacturer to be 98% pure according to TLC analysis on silica gel developed in 10% citric acid solution saturated with *n*-butanol and by paper chromatography on Whatman P20 cellulose phosphate cation paper in 0.1% NH_4Cl. To ascertain this purity, TLC purity and UV absorption of both labeled and nonlabeled materials were determined. Glass plates, 20 × 20 cm, were coated with a 0.25-mm layer of a slurry composed of 50 g of Kieselguhr G[3] and a mixture of 95 ml of 0.1 *M* edetate sodium in distilled water and 5 ml of 20% (v/v) polyethylene glycol 400 in glycerol (17). After air drying, the plates were developed in methyl ethyl ketone saturated with citric acid–phosphate buffer at pH 4.7 (17).

Twenty-five microliters of the methanolic solution of the labeled tetracycline was spotted and developed. After drying in air, the different spots were located by fluorescence under long wavelength UV light. The spots were identified by comparing their R_f values with those observed with methanolic solutions of tetracycline, anhydrotetracycline, and 4-epitetracycline. The plates were then radioscanned, and the area under the peaks was determined by the cut-and-weigh method.

UV absorption wavelengths were determined by scanning[4] a methanolic solution of each compound. The concentration of anhydrotetracycline was determined by absorption at 391 nm. Tetracycline was quantitated by the difference in absorption at 357 and 391 nm, using an equation reported previously (18). 4-Epitetracycline could not be quantitated by UV absorption.

Biliary Excretion Studies—Male Sprague–Dawley rats, 380–510 g, were anesthetized with urethan (1.3 g/kg im). A midline abdominal incision was made, and the common bile duct was cannulated with polyethylene (PE 10) tubing. After a steady flow of bile was observed, a 10-mg/kg dose of tetracycline (specific activity of 34.7 μCi/mg) was injected in the femoral vein over 5 min. The injectable dose was freshly prepared by evaporating the methanol from the tritiated tetracycline solution and adding nonlabeled aqueous drug solution to yield the desired specific activity.

The exteriorized bile was collected under a gentle stream of nitrogen in covered dark vials to avoid decomposition of its constituents. After 8–15 hr of collection, the bile solution was frozen and then mixed with bile collected from other rats treated in a similar way (total of six rats). The pH of the pooled bile from rats was determined, and an aliquot was used for qualitative and quantitative analyses. No attempt was made to calculate the percent of dose excreted in the bile of individual rats.

***In Situ* Absorption Studies**—Overnight fasted, male Sprague–Dawley rats, 280–380 g, were used. After urethan anesthesia, an abdominal midline incision was made. An 80–100-cm segment of the small intestine was isolated and cannulated with Tygon tubing about 2 cm distal to the common bile duct junction and proximal to the cecal end. The loop was washed thoroughly with 0.9% NaCl at 37° to remove any residual food and flushed with a mixture of 95% oxygen and 5% carbon dioxide to expel excess saline solution. Each cannula was attached to a 20-ml syringe to allow sampling of the loop content.

Five milliliters of the pooled bile solution was introduced, and a 0.1-ml aliquot was removed at 10-min intervals for the 1st hr, every 15 min for the 2nd hr, and every 30 min for the next 2 hr. Sampling was alternated between the two syringes. The volume loss due to water absorption from the drug solution was replenished with distilled water, adjusted to the same pH as the bile solution (8.75). At the end of the experiment, the intestinal segment was excised and its length was measured. It then was homogenized and incubated with a tissue solubilizer[5], and an aliquot was used for quantitative analysis. The *in situ* intestinal preparation was similar to that reported previously (19).

For comparative purposes, the absorption of tetracycline from

[1] Batch 10, Amersham/Searle Corp., Arlington, Ill.
[2] Lederle Laboratories, batch 48355-177, American Cyanamid Co., Pearl River, N.Y.
[3] E. Merck AG., Darmstadt, Germany.
[4] Beckman 25, Beckman Instruments Corp., Fullerton, Calif.
[5] Soluene TH 100, Packard Instrument Co., Downers Grove, Ill.

Table I—Quantitative Analysis of Tetracycline in the Bile and Aqueous Solutions

Solution	R_f[a]	Composition, % Labeled: Radioscanning	Labeled: UV Absorption	Nonlabeled, UV Absorption
Aqueous				
Unknown	0.09 (0.0)	0.9	N.D.[b]	N.D.
4-Epitetracycline	0.24 (0.26)	2.5	N.D.	N.D.
Tetracycline	0.56 (0.62)	89.1	75.6[c]	84.4[c]
Anhydrotetracycline	0.98 (1.00)	7.2	10.4[d]	N.P.[e]
Bile				
Unknown[f]	0.03	94.9	N.D.	N.D.
Anhydrotetracycline	0.98	5.1	N.D.	N.D.

[a] R_f values given in parentheses were reported by Lanman *et al.* (20). [b] N.D. = not determined. [c] Calculated by the equation of Pernarowski *et al.* (18). [d] Determined from Beer's law plot of anhydrotetracycline solutions. [e] N.P. = not present. [f] See text for explanation.

an aqueous solution having a pH and specific activity similar to the bile solution was determined as already described.

Partition Coefficient Studies—The partitioning characteristics of the bile and aqueous solutions of tetracycline were determined by mixing equal volumes of either solution and isoamyl alcohol in a shaker[6] at 37°. The partition coefficient is defined as the equilibrium ratio of radioactivity of the isoamyl alcohol phase to that of the test solution.

Assay—The level of radioactivity in the bile and aqueous and tissue homogenate samples was quantitated in 15 ml of a scintillation counting solution consisting of 0.1 g of 1,4-bis[2-(5-phenyloxazolyl)]benzene, 5.5 g of 2,5-diphenyloxazole, and 250 ml of octoxynol[7] and diluted to 1 liter with toluene. The counting[8] efficiency of each sample was determined by the internal standard method using tritiated toluene.

RESULTS

Table I shows the composition of the tetracycline samples. In spite of the presence of some degradation products, the composition of the labeled and nonlabeled samples was similar. About 85–90% of the sample was present as pure tetracycline according to radioscanning and UV absorption analyses. However, analysis of the bile solution indicated that the major portion of radioactivity (95%) was attributed to an unknown species which remained at the origin of the thin-layer chromatogram. Further disparity between the properties of the bile and aqueous solutions is indicated by the partition coefficients. The partition coefficient of the bile solution (0.573) was more than twice that of the aqueous solution (0.213), as indicated by the equilibrium ratio of the radioactivity of the isoamyl alcohol phase to that of the test solution.

The results of the *in situ* absorption studies of tetracycline from the bile and aqueous solutions are shown in Table II. A negligible percent of the dose initially introduced in the intestinal loop accumulated in the gut wall. Furthermore, there was no statistical difference in the amount of drug found in the gut walls between the bile (2.24%) and aqueous (2.44%) solutions. Accordingly, the disappearance of the drug from the intestinal lumen could be construed to indicate its absorption into the systemic circulation. On the average, 72.92% of the drug in the bile solution was absorbed compared with 77.34% absorbed from the aqueous solution. The difference in the percent absorbed was statistically significant ($p < 0.05$).

Figure 1 shows the time course of the disappearance of tetracycline from the rat intestinal lumen expressed as the ratio of the amount of drug present at a given time to the amount introduced at zero time. Only the values obtained at and beyond the 75-min sample were statistically greater ($p < 0.05$) for the bile than for the aqueous tetracycline solutions. However, the disappearance of the drug from both solutions was rapid initially. On the average, 40–50% of the initial dose disappeared from the lumen during the first 0.5-hr.

[6] Dubnoff incubator, Precision Scientific Co., Chicago, Ill.
[7] Triton X-100.
[8] Packard Tri-Carb 2425 liquid scintillation spectrometer, Packard Instrument Co., Downers Grove, Ill.

Table II—Absorption of Tetracycline in the Bile and Aqueous Solutions from the Rat Intestine[a]

Solution	Loop, Length, cm	Percent Absorbed[a]	Percent in Tissue
Aqueous			
Mean (five rats)	87.8	77.34	2.44
SD	10.8	2.73	0.52
Bile			
Mean (five rats)	86.2	72.92	2.24
SD	8.2	3.20	0.27
p[b]	>0.8	<0.05	>0.6

[a] Obtained by subtracting the amount of drug found in the lumen and tissue at 240 min from the initial amount placed in the lumen. [b] Unpaired t test.

DISCUSSION

The report by Lanman *et al.* (20) on the purity of tritiated tetracycline hydrochloride samples available commercially instigated a thorough examination of the purity of the samples used in the present study. These workers found that two commercially available radioactive samples contained only 50–60% pure tetracycline hydrochloride; the rest consisted of the degradation products, anhydrotetracycline and 4-epitetracycline. Another marketed sample consisted mainly of anhydrotetracycline (20).

It is obvious that the presence of substantial quantities of the degradation products of tracers could lead to serious misinterpretations. Usually, these tracers are mixed with nonlabeled, chemically pure materials which may behave differently in the biological system. Therefore, it was essential to verify the purity of the labeled tetracycline, although the sample employed in the present study was obtained from a different source than the samples tested by Lanman *et al.* (20). The findings of the present study indicate that both the labeled and nonlabeled samples contained 85–90% pure tetracycline.

Analysis of the tetracycline bile solution showed that except for one spot corresponding to anhydrotetracycline, 95% of the radioactivity remained at the origin. Therefore, the tetracycline compounds existed in a polar form which could not migrate in the solvent system employed in the development of the thin-layer plates. It is possible that this polar form is a complexation product of the charged tetracycline species and bile constituents. Similar inference was made by von Wittenau and Twomey (8) with respect to the extraction of oxytetracycline from dog fecal excreta. On the other hand, this complex appears to possess a greater lipid solubility than the uncomplexed drug, as indicated by its greater tendency to partition into the isoamyl alcohol from the test solution. Further investigations are needed to characterize more fully the properties of this complex.

Although the difference between the amount of tetracycline absorbed from the bile and aqueous solutions is statistically signifi-

Table III—Kinetic Parameters of the Disappearance of Tetracycline in the Bile and Aqueous Solutions from the Rat Intestine[a]

Solution	A	α, min^{-1}	B	β, min^{-1}
Aqueous				
Mean (five rats)	0.648	0.062	0.361	0.0025
SD	0.004	0.013	0.040	0.0003
Bile				
Mean (five rats)	0.447	0.061	0.443	0.0025
SD	0.117	0.028	0.092	0.0010
p[b]	<0.01	>0.95	>0.2	>0.95

[a] The ratios of the amount of tetracycline at a given time to the amount initially introduced were fitted to Eq. 1 (see text). [b] Unpaired t test.

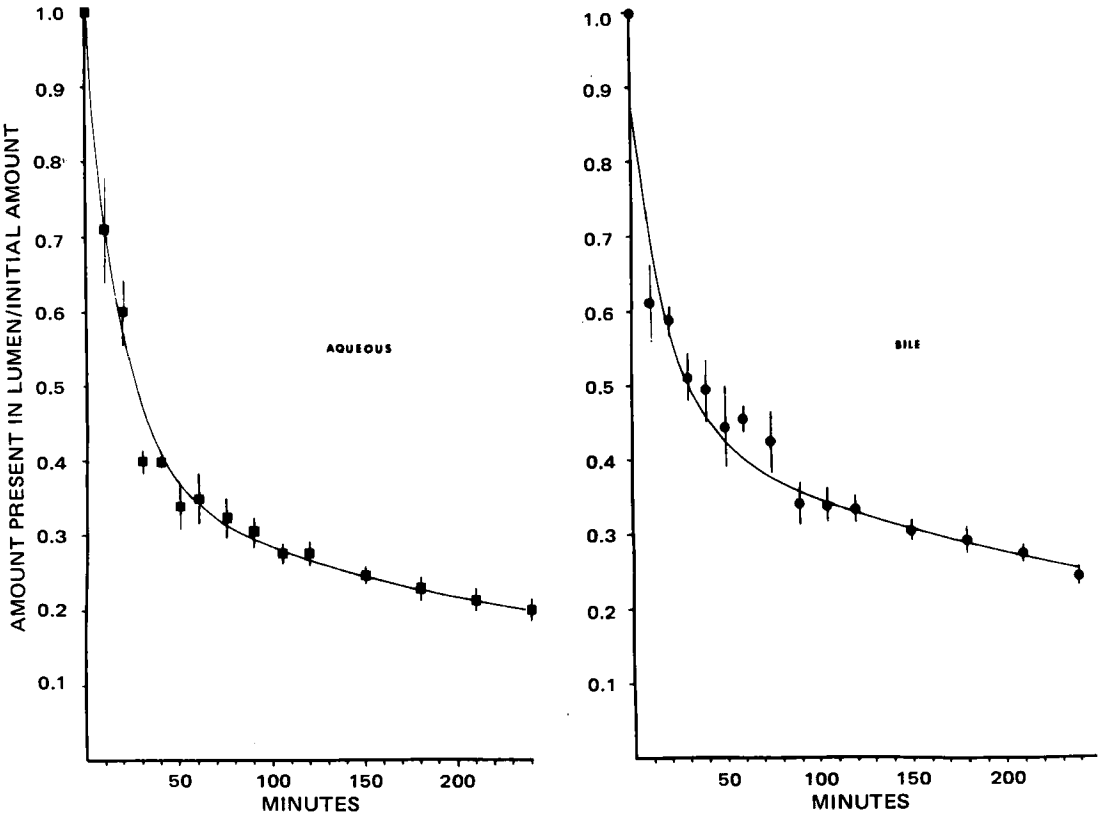

Figure 1—*Disappearance of tetracycline hydrochloride in the bile and aqueous solutions from the rat intestine as a function of time. The ordinates represent the ratio of the amount of the drug present at a given time to the amount introduced initially. Each point represents the mean of five rats, and vertical lines indicate the standard error of the mean. The continuous curves denote computer-fitted lines to the mean data.*

cant (Table II), it may not be biologically important unless it is accompanied by a similar difference in the rate of absorption of the drug from the two solutions. The time course of the disappearance of tetracycline from the rat intestinal lumen was plotted on semilogarithmic graph paper and found to be biphasic. Accordingly, data of each rat were fitted to the following biexponential equation using the SAAM 25 digital program (21):

$$R = Ae^{-\alpha t} + Be^{-\beta t} \qquad \text{(Eq. 1)}$$

where R is the ratio of amount of radioactivity present at a given time, t, to the amount initially introduced; α and β are the rate constants (minutes^{-1}) characterizing the rapid and more slowly declining phases, respectively; and A and B are constant coefficients, the sum of which represents the value of R at $t = 0$. The derived kinetic parameters are listed in Table III. It is clear that there are no statistical differences between the kinetic parameters B, α, and β whereas the value of A is significantly less for the bile solution than for the aqueous solution ($p < 0.01$). This may be explained by the greater tendency of the former to partition onto the gut wall, as indicated by its higher partition coefficient compared to the aqueous solution.

The findings of this investigation raise questions regarding the other routes of elimination of tetracycline in addition to excretion in urine. In a well-designed study, von Wittenau *et al.* (22) demonstrated that diffusion of doxycycline from the blood into the lumen of the small intestine and its ultimate excretion in feces constitute the other major route of elimination of this antibiotic in the rat. Other investigators detected tetracycline in the lower intestinal lumen of mice at various times after the intravenous administration of the drug (23, 24). Pindell *et al.* (25) demonstrated that tetracycline is rapidly absorbed from the duodenum but that negligible absorption takes place from the colon.

It is quite possible, therefore, that because of the poor absorption from the lower intestine and colon, tetracycline excreted into these areas is eventually eliminated in the feces. In fact, the biphasic disappearance of tetracycline from the isolated loop observed in the present study may be explained by the back-diffusion of drug absorbed from the duodenum and jejunum into the lower small intestine. The intestinal segment used in this investigation was sufficiently long to include the major portion of the ileum.

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Complexation in Formulation of Parenteral Solutions: Solubilization of the Cytotoxic Agent Hexamethylmelamine by Complexation with Gentisic Acid Species

BO KREILGÅRD *, T. HIGUCHI ‡, and A. J. REPTA ˣ

Abstract □ The apparent solubility of hexamethylmelamine in aqueous solutions suitable for intravenous use was increased by complexation with gentisic acid. Studies were carried out in the pH 0–8 range. Unprotonated hexamethylmelamine did not form complexes with the gentisate ion, while the hexamethylmelammonium ion appeared to form several different complexes with both the gentisate ion and gentisic acid. Two different solid complexes were isolated and characterized. The solubility increases observed at pH 3.5–5.0 are described by mathematical relationships involving the stability constants of some postulated complex species. From these results, suitable formulations for use as parenteral solutions are proposed. The increase in the apparent aqueous solubility of hexamethylmelamine in such formulations may range from five- to 90-fold, depending upon the pH and total gentisate-ion concentrations.

Keyphrases □ Complexation—hexamethylmelamine–gentisic acid species, pH 0–8, effect on solubility □ Solubilization—hexamethylmelamine by complexation with gentisic acid species, pH 0–8, intravenous formulations □ Cytotoxic agents—hexamethylmelamine, solubilization by complexation with gentisic acid species, intravenous formulations □ Hexamethylmelamine—solubilization by complexation with gentisic acid species, intravenous formulations □ Triazine derivatives—hexamethylmelamine, solubilization by complexation with gentisic acid species, intravenous formulations

Hexamethylmelamine[1], a cytotoxic triazine, was evaluated in extensive phase I and II clinical trials (1). Although the efficacy is not particularly striking in any tumor type, clinical interest in hexamethylmelamine continues in view of its consistent, although low, response rate in several tumors including bronchogenic carcinoma (2, 3).

In phase I and II trials, hexamethylmelamine was given by the oral route (1), and GI side effects such as nausea and vomiting were encountered in such studies (4, 5). To avoid such problems, an intravenous dosage form was developed. Because of the weakly basic nature of the drug and its low aqueous solubility (<0.1 mg/ml), the only parenteral formulation used was an aqueous hydrochloric acid solution (pH ~2) of the drug[2]. The clinical use of this solution has been restricted by the occurrence of thrombophlebitis and local irritation. To determine whether these untoward reactions at the injection site are inherently due to hexamethylmelamine or to the very acidic formulation, a less acidic (pH > 3) formulation of hexamethylmelamine at a concentration of 2–5 mg/ml was desired[2].

Previous experience with the complexation tendencies of various hydroxybenzoic acids with substances exhibiting characteristics akin to those of hexamethylmelamine (6–9) suggested that the apparent solubility of this drug probably could be increased through complexation. Such complexation necessitated the identification and use of a suitable ligand. Preliminary studies with gentisic acid as the ligand demonstrated a significant increase in the apparent solubility of hexamethylmelamine.

This report describes the qualitative and quantitative nature of the interactions of hexamethylmelamine with gentisic acid species, with particular attention being paid to those pH and concentration condi-

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tions that might normally be encountered in the formulation and use of an intravenous solution.

EXPERIMENTAL

Chemicals—Hexamethylmelamine was used as received[3], mp 171–173° [lit. (10) mp 172–174°]. Titrations with perchloric acid in acetic acid showed the molecular weight to be 210 ± 0.5 (theoretical 210.3). TLC [silica gel F-254 and benzene–acetone–formic acid (8:4:1)] indicated that the material was homogeneous when 100 μg was spotted and visualized by iodine vapor and UV light at 254 nm.

Gentisic acid[4], mp 204–205° [lit. (9, 11) mp 205°] was also found to be homogeneous by TLC using the same conditions. Analysis of gentisic acid, using the USP (12) assay for benzoic acid, indicated a purity >99.5%. All other chemicals were of analytical reagent grade and were used without further purification. The water used was distilled from aqueous acidified potassium permanganate solutions and stored in Pyrex glass containers.

Solubility Studies—The experimental method used was described in detail previously (13). In the studies at pH 3.5–5.0, equal volumes of a stock solution of the ligand were pipetted into the 15-ml screw-capped vials containing 210 mg of hexamethylmelamine (1.00×10^{-3} mole). Then different amounts of a solution of either 0.1 or 1 *N* hydrochloric acid were added, and the solution in each vial was brought to a constant final volume of 10 ml with water.

Ligand stock solutions of the desired concentration and pH were prepared by dissolving the necessary quantity of gentisic acid and sodium bicarbonate in water. In the preparation of these stock solutions, ~0.1% sodium bisulfite was included to minimize oxidation of the ligand. The addition of sodium bisulfite did not appear to influence the complexation reaction quantitatively compared to studies in which the solutions were deoxygenated by flushing with nitrogen gas.

Solubility equilibrium was obtained by fixing the sealed vials to a rotating shaft in a water bath thermostated at 25.0 ± 0.1°. The contents of the vials were allowed to equilibrate until no further changes in the concentration of the species in solution were detectable. After 20 hr or more, samples corresponding to the ascending portion of the phase diagram were at equilibrium; samples corresponding to the remaining portion of the phase diagrams achieved equilibrium after 42 hr or more. To avoid oxidation of gentisate, no samples were allowed to equilibrate for more than 72 hr.

Following equilibration, the contents of the vials were filtered through sintered-glass filters. The pH of each solution was measured[5] at 25°, and the total concentrations of the hexamethylmelamine and gentisic acid species were determined.

Since the system was highly pH dependent and since changes in pH occurred when precipitation of the complex took place, it was necessary to run a series of experiments covering the desired pH range (pH 3.5–5) for each concentration of ligand used. As an example, Table I shows experimental results at a gentisate-ion concentration of 0.44 *M*. In Group A samples, the increasing amounts of hydrochloric acid added caused a decrease in pH and an increase in dissolved hexamethylmelamine. This relationship was typical and to be expected when no precipitation of complex took place. However, when a solid complex precipitated, no significant changes in pH occurred with the incremental increase in the volume of hydrochloric acid solution added (Group B). During this precipitation of the complex, the concentration of hexamethylmelamine appeared to be constant while the concentration of gentisic acid species gradually decreased.

With the Group C samples, further addition of hydrochloric acid caused a decrease in pH as well as in the concentrations of substrate and ligand because the supply of excess solid substrate had been exhausted by the precipitation of the complex (in those samples represented by Group B).

The composition of all solutions was plotted against pH, and the concentrations of substrate and ligand at each particular pH value were read. Table I illustrates that no definite value for the concentration of hexamethylmelamine could be obtained at pH 4.5 in this particular experiment. Similar studies were carried out to obtain phase solubility diagrams in the pH 3.5–5.0 range.

Table I—Experimental Results Obtained in Phase Solubility Studies of Hexamethylmelamine at an Initial Gentisate-Ion Concentration of 0.44 *M* at 25°[a]

Group	1 *N* HCl Added, ml	pH	Hexamethylmelamine in Solution, $M \times 10^3$	Gentisic Acid Species in Solution, *M*
A	0.0	5.01	29.0	0.431
	0.1	4.88	39.1	0.439
	0.2	4.76	46.5	0.436
	0.3	4.69	54.8	0.431
	0.4	4.55	68.0	0.439
B	0.5	4.40	47.1	0.421
	0.6	4.38	49.9	0.413
	0.7	4.35	48.5	0.408
	0.8	4.36	46.0	0.377
	0.9	4.33	46.2	0.381
	1.0	4.37	45.4	0.368
	1.1	4.23	39.1	0.353
	1.2	4.41	48.5	0.342
C	1.4	4.28	32.1	0.304
	1.6	4.05	18.9	0.286
	1.8	3.77	11.0	0.269
	2.0	3.54	8.1	0.263
	2.2	3.34	6.5	0.267

[a] Hexamethylmelamine (210 mg, 1.0×10^{-3} mole) was added to each sample, and the total volume of the aqueous phase was 10 ml. See *Experimental* section for detailed explanation.

In studies done at pH > 7, increments of the stock solution of the ligand were pipetted into the vials, sodium bicarbonate (1 *N*) was added to bring the pH in the final solution to pH > 7, and water was added to a final volume of 10 ml. Otherwise, the procedure was as already described.

For studies done in highly acidic solutions, gentisic acid was used as the substrate due to the high solubility of hexamethylmelamine in strongly acidic media. The gentisic acid (231 mg, 1.5×10^{-3} mole) was combined with aliquots of a solution of hexamethylmelamine in 1 *N* hydrochloric acid, and the final liquid volume was adjusted to 10 ml with 1 *N* hydrochloric acid. The remainder of the procedure was as already described.

The influence of ionic strength on the aqueous solubility of hexamethylmelamine was determined at 25°. Distilled water was adjusted to pH 9–10 with aqueous sodium bicarbonate (1 *N*), and the ionic strength was adjusted to the desired value with sodium chloride. Such solutions were equilibrated with excess solid hexamethylmelamine for 20 hr, after which the concentration of dissolved hexamethylmelamine remained constant. Some results are shown in Table II, and the solubility of hexamethylmelamine at zero ionic strength was estimated by extrapolation to be 4.15×10^{-4} *M* at 25.0°.

Analytical Procedure—Hexamethylmelamine and gentisate in aqueous solutions were analyzed by measuring the absorbances[6], at 228 and 322 nm, of solutions diluted to the required extent with sodium borate (10^{-2} *M*) in methanol–water (1:4). Since hexamethylmelamine did not absorb at 322 nm, the concentration of gentisate could be calculated directly from the absorbance of the solution at 322 nm and an independently determined value of $\epsilon_{322} = 3850\ M^{-1}\ cm^{-1}$ for gentisate in the described solvent system.

The calculation of hexamethylmelamine was not quite as straightforward as anticipated due to a small and varying degree of oxidation of gentisate, which occurred during the preparation of the gentisate stock solutions (involving the neutralization of gentisic acid by addition of alkali). This oxidation caused the absorbance values for gentisate at 228 nm to vary by about 2–5% from the value predicted using the molar absorptivity value of $\epsilon_{228} = 7240\ M^{-1}\ cm^{-1}$. This value was obtained using solutions prepared by dissolving gentisic acid directly into the sodium borate–methanol–water system already described.

[3] National Cancer Institute.
[4] 2,5-Dihydroxybenzoic acid, Aldrich Chemical Co.
[5] Radiometer pH-meter, model 26, equipped with Corning glass and calomel electrodes.
[6] Cary model 14 or 15 spectrophotometers.

Table II—Influence of Ionic Strength (μ) on the Acid Dissociation Constants of Hexamethylmelamine, pKa_M, and Gentisic Acid, pKa_G, and on the Solubility of Hexamethylmelamine Base, $[HM]_0$ ($T = 25°$)

μ	pKa_M	pKa_G	$[HM]_0$, $M \times 10^4$
0	4.92[a]	2.95[a]	4.15[b]
0.05	4.97	2.87	4.09
0.10	5.02	2.82	3.94
0.50	5.15	2.66	2.86
1.00	5.20	2.57	1.89

[a] Obtained by calculations as described in the *Experimental* section. [b] Value at zero ionic strength was obtained by extrapolation.

Such oxidation of gentisate did not affect the absorption at 322 nm and, once prepared, the absorbance spectrum of any particular stock solution did not change over a period of more than a week. Therefore, it was possible to obviate the problem by measuring the absorbance values at 322 (A'_{322}) and 228 (A'_{228}) nm for the particular gentisate stock solution used in the samples to be analyzed. A value R ($= A'_{228}/A'_{322}$) was then calculated and used in estimating the concentration of hexamethylmelamine in the samples according to the expression:

$$[\text{hexamethylmelamine}] = [A_{228} - (RA_{322})]/\epsilon_{228,HM} \quad \text{(Eq. 1)}$$

where A_{228} and A_{322} are the values of the absorbances in the sample being analyzed, and $\epsilon_{228,HM}$ (=50,700 M^{-1} cm^{-1}) is the molar absorptivity of hexamethylmelamine at 228 nm.

All reported absorptivity values were obtained from appropriate Beer–Lambert-type plots.

Ionization Constants—The acid dissociation constant, pKa_M, for the hexamethylmelammonium ion was determined at various ionic strengths by a solubility technique which involved measuring the apparent solubility of hexamethylmelamine at several pH values (14) (Table II). When utilizing the Debye–Hückel equation (15), correction of the results obtained at $\mu \leq 0.1$ yielded the value $pKa_M = 4.92$ at zero ionic strength. The corresponding constant for gentisic acid, pKa_G, was determined by potentiometric titrations (14) at 25.0° (Table II). Again using the Debye–Hückel equation (15), the value of pKa_G at zero ionic strength was determined to be 2.95, which agrees with the literature value of 2.97 (16).

Isolation and Characterization of Complexes—Complexes were isolated during the solubility studies at pH 3.5 and 4.0. Immediately after separation by filtration, the crystalline complexes were washed twice with 5 ml of cold water and then dried *in vacuo* at room temperature. Samples of the dry solid were dissolved in methanol, and the ratio of total hexamethylmelamine and gentisate species was determined by UV spectrophotometry as already described. The alkaline equivalent weights of these solids was obtained by nonaqueous potentiometric titration with perchloric acid (0.01 N) in acetic acid. The acidic equivalent weights were determined by titration with aqueous sodium hydroxide (0.5 N) (Table III).

At pH < 0.5, the solid complex was isolated during the solubility studies from solutions with an initial concentration of 0.08 M hexamethylmelammonium chloride. Since the complex dissolved very rapidly when washed with water, it could not be washed free of hydrochloric acid. UV spectrophotometric determination showed it to contain 0.68 mole of hexamethylmelammonium ion/mole of gentisic acid, which closely agrees with a stoichiometry of 3:2.

RESULTS AND DISCUSSION

Phase Solubility Studies—The complexation of hexamethylmelamine and gentisic acid was studied at 25° by the solubility method in the pH 0–8 range. The apparent solubility of hexamethylmelamine at a pH between 7 and 8 did not change measurably with increasing amounts of gentisate ion (0–0.5 M). This finding indicated that the hexamethylmelamine base and gentisate ion did not appreciably associate.

In hydrochloric acid (1 N) solutions at 25°, gentisic acid and hexamethylmelammonium ion did interact appreciably. As shown in Fig. 1, the solubility of the substrate (gentisic acid) was increased by addition of the ligand at concentrations up to about 0.01 M. The slope of the ascending part of the diagram is approximately 2, indicating formation of a soluble 2:1 (or higher order) gentisic acid–hexamethylmelammonium-ion complex (13). At concentrations of ligand >0.01 M, a solid complex precipitated. Based on the length of the plateau region (13), the stoichiometry of the solid gentisic acid–hexamethylmelammonium-ion complex was calculated to be 3:2. This stoichiometric value was supported both by the data obtained from analysis of the isolated solid complex and by calculations utilizing the data for the descending part of the phase solubility diagram (13), which corresponds to the precipitation of the complex.

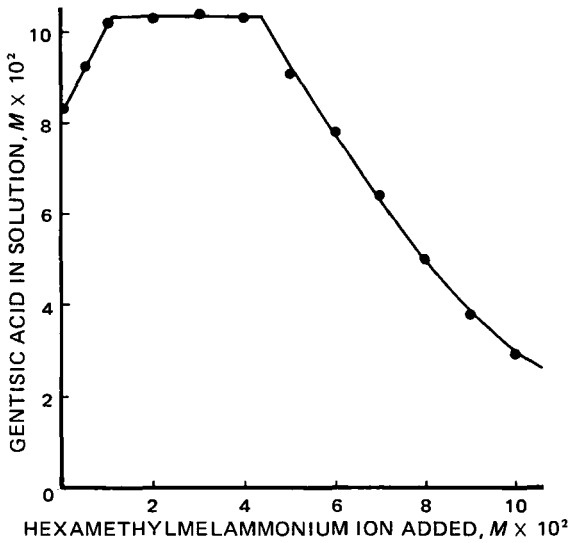

Figure 1—*Solubility of gentisic acid in 1 N hydrochloric acid at 25° as a function of hexamethylmelammonium ion added.*

Similar solubility studies were carried out at 25° and pH values between 3.5 and 5.0, where both the charged and uncharged species of the compounds are present. In this range, control of pH was difficult since the substrate and ligand had electrolytic character and the association as soluble or insoluble complexes caused changes in pH. Therefore, experiments were done covering the whole pH range at each ligand concentration. In such studies, hexamethylmelamine was used as the substrate.

The results at pH 3.5 and 4.0 are shown in Fig. 2, and those obtained at 4.5 and 5.0 are presented in Fig. 3. In all four phase diagrams, the solubility of total hexamethylmelamine increased as increasing amounts of ligand were added. At pH 3.5 and 4.0, a solid complex was precipitated, as indicated by the plateau regions in Fig. 2; at pH 5.0, no complex precipitated. At pH 4.5 and a ligand concentration >0.425 M, a precipitate was also observed, but the concentration data obtained from the solution phase in such samples appeared to be quite scattered and were not reproducible. The cause and nature of these results were not examined further.

Inspection of the data in Fig. 2 demonstrated some unusually sharp changes in concentration of substrate as a function of added ligand at both pH 3.5 and 4.0. To determine whether such abrupt changes were due to supersaturation, 10-ml samples (pH 3.5) of 210 mg of hexamethylmelamine (10^{-3} M) and gentisate concentrations of 0.04–0.07 M were allowed to equilibrate for 1 day. Then, as an aid to nucleation, glass wool and some crystals of the complex isolated at pH 3.5 were introduced and the samples were again kept at 25°.

Supersaturation appeared to be responsible for high substrate concentrations in solution only at ligand concentrations above ~0.065 M. At ligand concentrations below ~0.065 M, the concentration of hexamethylmelamine species did not change as a function of time from ~1 to 7 days, although the substrate concentrations were significantly higher than those at the plateau region. While these studies at pH 3.5 did not elucidate the causes for the sharp changes in substrate concentrations observed at pH 3.5 and 4.0, such variations may be due to small changes in the pH values of the solutions resulting from the precipitation of the complex (as exemplified in Table I and discussed in *Experimental*).

Analytical data for the solid precipitates isolated at pH 3.5 and 4.0 (Table III) strongly indicated that a 1:1:1 gentisic acid–hexamethylmelammonium-ion–gentisate-ion complex was the solid phase in both cases. A plot of the concentration of the ligand added *versus* the ligand in solution (17) verified the 1:2 ratio between gentisic acid species and hexamethylmelamine species.

An interesting observation was made during isolation of this 1:1:1 complex. When the complex was isolated from a sample containing 0.1% sodium bisulfite, the solid complex had a pronounced yellow color. However, if retardation of oxidation was accomplished by expelling the molecular oxygen by bubbling nitrogen gas through the solution, the solid complex obtained was almost white. In both cases, the solutions themselves were colorless. Analysis of the complexes obtained under the different conditions did not show any significant differences in the chemical composition, and the reason for difference in color of the solids has not been explained (Table III).

Estimation of Stability Constants—To use the data obtained for descriptive and predictive purposes, attempts were made to define the formation of complexes and the corresponding increase in the apparent solubility of hexamethylmelamine at different pH values in terms of stability constants.

Since no complexation appeared to occur between free hexamethylmelamine and gentisate ion, as indicated by the studies at pH ≥ 7, only complexes between the hexamethylmelammonium ion and gentisic acid and/or gentisate were considered. At pH 5.0, only a very small fraction of the total gentisate added was present as gentisic acid. The distinct curvature of the solubility diagram indicates formation of complexes of higher order with respect to the gentisate ion. The assumption was made that the increase in apparent solubility of hexamethylmelamine was due mainly to the formation of two complexes, ($HMH^+ \cdot G^-$) and [$HMH^+ \cdot (G^-)_2$][7]. The stability constants for each complex, expressed in terms of the concentration of the various species, are given by Eqs. 2 and 3:

$$K_1 = \frac{[HMH^+ \cdot G^-]}{[HMH^+][G^-]} \qquad \text{(Eq. 2)}$$

$$K_2 = \frac{[HMH^+ \cdot (G^-)_2]}{[HMH^+ \cdot G^-][G^-]} \qquad \text{(Eq. 3)}$$

Calculations (8) made on the basis of these equations did not adequately describe the results obtained in the pH 3.5–5.0 range. Although not isolated at pH 4.5 and 5.0, the solid complex found at pH 3.5 and 4.0 having the composition ($GH \cdot HMH^+ \cdot G^-$) was expected also to be involved in the equilibrium at pH 4.5 and 5.0. Therefore, a stability constant, K_3, was included and may be defined as:

$$K_3 = \frac{[GH \cdot HMH^+ \cdot G^-]}{[HMH^+ \cdot G^-][GH]} \qquad \text{(Eq. 4)}$$

However, calculations based on the involvement of the three complexes defined in terms of K_1, K_2, and K_3 still did not correlate well with the experimental data. Therefore, it was necessary to consider other equilibria to fit the data.

If it is assumed that in highly acidic solvents, the increased solubility seen at low concentrations of ligands (Fig. 1) was primarily due to formation of a 2:1 complex, [$(GH)_2 \cdot HMH^+$], and if it is also assumed that the 2:1 complex forms by association of a 1:1 complex and excess ligand, it is necessary to consider two additional complexes, ($HMH^+ \cdot GH$) and [$HMH^+ \cdot (GH)_2$]. The stability constants for these species may be defined as:

$$K_4 = \frac{[HMH^+ \cdot GH]}{[HMH^+][GH]} \qquad \text{(Eq. 5)}$$

and:

$$K_5 = \frac{[HMH^+ \cdot (GH)_2]}{[HMH^+ \cdot GH][GH]} \qquad \text{(Eq. 6)}$$

The mass balance equations are:

$$[HM]_t = [HM] + [HMH^+] + [HMH^+ \cdot G^-] + [HMH^+ \cdot (G^-)_2] + [GH \cdot HMH^+ \cdot G^-] + [HMH^+ \cdot GH] + [HMH^+ \cdot (GH)_2] \qquad \text{(Eq. 7)}$$

[7] HM = hexamethylmelamine, HMH^+ = hexamethylmelammonium ion, GH = gentisic acid, and G^- = gentisate ion.

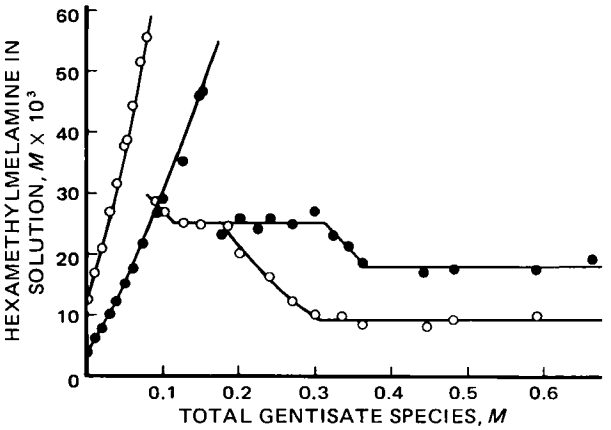

Figure 2—*Solubility of hexamethylmelamine in aqueous solution at pH 3.5 (○) and pH 4.0 (●) at 25° as a function of gentisic acid species added. The ascending part of the curves are calculated from Eq. 7.*

and:

$$[GH]_t = [GH] + [G^-] + [HMH^+ \cdot G^-] + 2[HMH^+ \cdot (G^-)_2] + 2[GH \cdot HMH^+ \cdot G^-] + [HMH^+ \cdot GH] + 2[HMH^+ \cdot (GH)_2] \qquad \text{(Eq. 8)}$$

where $[HM]_t$ is the total concentration of hexamethylmelamine species, and $[GH]_t$ is the total concentration of gentisate species added.

The concentration of uncomplexed hexamethylmelamine species is calculated (18) from:

$$[HM] + [HMH^+] = [HM]_0\left(1 + \frac{(H^+)}{K_{aM}}\right) \qquad \text{(Eq. 9)}$$

where $[HM]_0$ is the solubility of hexamethylmelamine at each particular ionic strength (Table II).

In all calculations, corrections were made for the effect of ionic strength on the solubility of hexamethylmelamine and the values of pK_{aM} and pK_{aG}.

The reportedly (11) weak binding between gentisic acid and gentisate ion was not taken into account, since calculations showed it to have no significant influence (≤5%) on the stability constants reported here.

Estimates of the values of K_1 and K_2 were obtained based on the reasonable assumption that only negligible amounts of gentisic

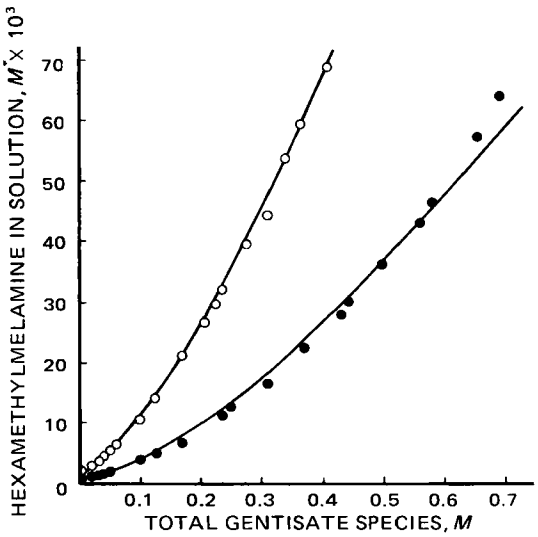

Figure 3—*Solubility of hexamethylmelamine in aqueous solution at pH 4.5 (○) and pH 5.0 (●) at 25° as a function of gentisic acid species added. The solid curves are calculated from Eq. 7.*

Table III—Results of Analysis of Some Solid Hexamethylmelamine–Gentisic Acid Complexes

Complex Isolated at	Melting Point	Ratio HM/GH[a]	Acidic Equivalent Weight[b]	Alkaline Equivalent Weight[c]	Elemental Analysis, %		
					C	H	N
pH 3.5[d]	134–135°	1:2.02	258	515	53.21	5.87	16.36
pH 4.0[d]	134–135°	1:2.09	259	512	53.43	5.77	16.54
pH 4.0[e]	130–134°	1:2.09	257	512	53.63	5.88	16.53
Theoretical[f]	—	1:2.00	259	518	53.58	5.83	16.21

[a] The ratio of hexamethylmelamine (HM) species to total gentisic acid (GH) species was determined by UV spectrophotometry. [b] Determined by titration with 0.5 *N* sodium hydroxide. [c] Determined by titration with 0.01 *N* perchloric acid in acetic acid. [d] Solution contained 0.1% sodium bisulfite. [e] Solution was flushed with nitrogen. [f] Calculated for a 1:1:1 gentisic acid–hexamethylmelammonium ion–gentisate-ion complex.

acid were present at pH 5.0. The values obtained for K_1 and K_2, as calculated by a previously described method (8), were 48 and 7.3 M^{-1}, respectively.

To calculate $[HM]_t$ from Eq. 6, the free ligand concentration, $[G^-]$, must be known. By combining Eqs. 2–6 and 8 and solving for $[G^-]$, Eq. 10 was obtained:

$$[G^-] = [-\alpha + (\alpha^2 + 8[GH]_t\beta)^{1/2}]/4\beta \quad \text{(Eq. 10)}$$

where:

$$\alpha = 1 + K_1[HMH^+] + [H^+](1 + K_4[HMH^+])/K_{aG} \quad \text{(Eq. 11)}$$

and:

$$\beta = K_1K_2[HMH^+] + [H^+][HMH^+](K_1K_3 + K_4K_5[H^+]/K_{aG})/K_{aG} \quad \text{(Eq. 12)}$$

By using the values of K_1 and K_2 already mentioned and varying the values of K_3, K_4, and K_5, values of $[HM]_t$ that best fit the experimental results were calculated[8]. Values of $K_3 = 150\ M^{-1}$, $K_4 = 200\ M^{-1}$, and $K_5 = 50\ M^{-1}$ gave a good correlation between the experimental points and the calculated curve (solid line) shown in Figs. 2 and 3. Only the curve related to pH 5.0 shows a significant deviation between the calculated curve and the experimental results, particularly at ligand concentrations above 0.6 *M*. The deviation probably was due to formation of other soluble complexes of higher order in gentisic acid or gentisate ion than those considered.

The relatively large number of stability constants considered here should be regarded only as empirical parameters that adequately describe the observed increase in the apparent solubility of hexamethylmelamine in the presence of gentisic acid in the pH 3.5–5.0 range. They should not be interpreted as evidence that all such postulated associations actually occur or that other complexes do not exist.

The fact that the hexamethylmelammonium ion associated strongly with the gentisate ion while the corresponding base apparently does not appear to associate is attributed to the difference in resonance structure occurring when the triazine ring is protonated. In the case of gentisic acid and gentisate ion, however, the charge on the gentisate ion is not conjugated with the aromatic ring and no great change in the charge distribution of the aromatic binding site would be expected. Therefore, it seems reasonable that both gentisic acid and gentisate ion would form complexes with hexamethylmelammonium ion as observed.

The importance of a charge transfer reaction between hexamethylmelamine cation and gentisate anion is probably negligible, since such interactions may occur only to a very small extent between organic species in aqueous solution (19).

If plane-to-plane orientation occurs, as has been suggested in the case of 1:1 complexes (19, 20), it seems reasonable to assume that the solid complex isolated at pH 3.5 and 4.0 has a gentisate-ion molecule and a gentisic acid molecule on either side of the hexamethylmelammonium ion. Since this complex is electrically neutral, it is not unreasonable that the solubility of this particular species would be relatively low and precipitation could be expected.

Formulation—From the data discussed, it is apparent that the interactions between the various gentisic acid and hexamethylmelamine species are somewhat complex and highly dependent on pH and system composition. However, the use of gentisic acid species as a ligand clearly resulted in increases in the apparent solubility of hexamethylmelamine. Such increases range from approximately fivefold at pH 3.5 to at least approximately 90-fold at pH 5.0. Thus, physically stable solutions with concentrations of ≥5 mg of hexamethylmelamine/ml can be prepared at pH ≥ 3.5.

Solutions containing 5 mg/ml of drug (pH 3.5; gentisic acid, 6 mg/ml) and 10 mg/ml of drug (pH 4.5; gentisic acid, 54 mg/ml) were prepared and lyophilized[9]. Such preparations were readily reconstituted and appeared to be suitable for use in the clinical testing of hexamethylmelamine. Lyophilization of the solutions was considered desirable to avoid the possible oxidation of gentisic acid species in aqueous solution. In the pH 3.5 preparation, about one-half of the amount of hexamethylmelamine was present as a complex; in the pH 4.5 preparation, more than 95% of the drug was complexed.

The systems described in Figs. 2 and 3 allow for appreciable choice in the composition of a formulation. However, if one chooses a high pH value, the formulation will necessarily require greater concentrations of gentisate species to obtain a given dose of hexamethylmelamine.

Upon administration of such described parenteral solutions, a very rapid release of hexamethylmelamine will occur due to dilution of the sample as well as change in pH to a value of ~7.4 at which no complexation occurs.

SUMMARY AND CONCLUSIONS

The present study demonstrated the practical use of complexation in the formulation of the cytotoxic agent, hexamethylmelamine, as a product potentially suitable for intravenous use. Where the desired objectives of the formulation can be realized through the use of complexation, it is a very useful and attractive method, particularly where a reversible alteration of the physical and/or chemical properties of a drug substance is desired to improve drug delivery. Since complexation is a reversible phenomenon and the equilibrium is shifted by concentration changes, it is obvious that upon dissolution of a solid complex or dilution of a solution containing complexed species, the complexed molecules will dissociate and provide rapid release of the free drug. In view of these rather unusual drug delivery advantages, complexation may well be included when defining prodrug approaches.

However, the application of complexation to the solubilization of drugs is somewhat limited, since complexation between organic molecules generally results in apparent solubility increases of only an order of magnitude or less and this increase is often not sufficient. Another important problem is that it is often difficult to identify suitable ligands which will associate appreciably with the given substrate.

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[8] Hewlett-Packard 9810A calculator.

[9] Virtis model 10-800 lyophilizer attached to a Cenco Hyrac 14 vacuum pump. Precautions had to be taken to avoid sublimation of hexamethylmelamine during the secondary drying.

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Amino Acid Analogs III: New Syntheses of Monomethyl- and Monophenylglutamic Acids

HERMAN GERSHON ˣ, RAULO PARMEGIANI, VINCENT R. GIANNASIO, and IRA S. KRULL

Abstract □ Glutamic acid analogs containing 3- and 4-methyl and 2-, 3-, and 4-phenyl substituents were prepared. The 3- and 4-methyl- and 3- and 4-phenylglutamic acids did not inhibit *Plasmodium berghei* and were nontoxic to the host (mice) at 640 mg/kg. The five analogs in addition to 2-methylglutamic acid were inactive against *Lactobacillus casei* at 1000 μg/ml in a defined medium; against *Escherichia coli*, only 2-methylglutamic acid caused 27% inhibition at 10,000 μg/ml. All six analogs failed to inhibit *Aspergillus niger, Aspergillus oryzae, Trichoderma viride*, and *Myrothecium verrucaria* in a defined medium below 10,000 μg/ml.

Keyphrases □ Glutamic acid analogs—syntheses, inhibition of plasmodia, bacteria, and fungi, toxicity in mice, potential antimalarial activity □ Antimalarial agents, potential—syntheses of methyl- and phenylglutamic acids

As a result of an interest in synthesizing potential antimalarial agents, it became necessary to prepare quantities of 3- and 4-methyl- and 3- and 4-phenylglutamic acids. The four glutamic acid analogs were reported previously (1–4).

DISCUSSION

3-Methylglutamic acid (IIIa) was obtained by reacting crotonaldehyde with ethyl acetamidomalonate by means of a Michael condensation, followed by oxidation of the aldehyde function (I) with permanganate and hydrolysis of the oxidation product (II) with acid. The preparation of 4-methylglutamic acid (IIIb) was based on condensing methacrolein with ethyl acetamidomalonate, oxidizing, and hydrolyzing the products, as for 3-methylglutamic acid. 3-Phenylglutamic acid (IIIc) was obtained in a similar manner, by a Michael condensation of cinnamaldehyde with ethyl acetamidomalonate followed by oxidation and hydrolysis.

For the preparation of 4-phenylglutamic acid (V), tropic acid was made into the acid chloride by means of thionyl chloride which, in turn, was esterified with ethanol. Ethyl tropate was subsequently converted to ethyl 3-bromo-2-phenylpropionate with phosphorus tribromide, and the bromo compound was condensed with ethyl acetamidomalonate. Upon hydrolysis of the condensation product with acid, 4-phenylglutamic acid was obtained. Scheme I indicates the preparation of IIIa–IIIc, and Scheme II indicates the preparation of V.

Since it was desired to carry out microbiological and other testing on the three monomethyl- and three monophenylglutamic acids, 2-methyl- and 2-phenylglutamic acids were also required. The synthesis of 2-phenylglutamic acid was reported previously, but it cyclized spontaneously to 2-phenylpyroglutamic acid (5). For the present study, benzoylpropionic acid was treated with ammonium carbonate and potassium cyanide, according to the method of Henze and Speer (6), to form the hydantoin (VI). Compound VI was hydrolyzed to 2-phenylglutamic acid (VII) by successive treatments with sodium hydroxide and hydrochloric acid (Scheme III). This preparation of 2-phenylglutamic acid did not cyclize spontaneously at room temperature. 2-Methylglutamic acid was commercially available. IR spectra of the six glutamic acid analogs appear in Figs. 1 and 2.

3-Methyl-, 4-methyl-, 3-phenyl-, and 4-phenylglutamic acids were screened against *Plasmodium berghei* in mice[1]. These com-

[1] Testing done at the Rane Laboratory, University of Miami, Miami, Fla.

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Abstract □ Glutamic acid analogs containing 3- and 4-methyl and 2-, 3-, and 4-phenyl substituents were prepared. The 3- and 4-methyl- and 3- and 4-phenylglutamic acids did not inhibit *Plasmodium berghei* and were nontoxic to the host (mice) at 640 mg/kg. The five analogs in addition to 2-methylglutamic acid were inactive against *Lactobacillus casei* at 1000 μg/ml in a defined medium; against *Escherichia coli*, only 2-methylglutamic acid caused 27% inhibition at 10,000 μg/ml. All six analogs failed to inhibit *Aspergillus niger, Aspergillus oryzae, Trichoderma viride*, and *Myrothecium verrucaria* in a defined medium below 10,000 μg/ml.

Keyphrases □ Glutamic acid analogs—syntheses, inhibition of plasmodia, bacteria, and fungi, toxicity in mice, potential antimalarial activity □ Antimalarial agents, potential—syntheses of methyl- and phenylglutamic acids

As a result of an interest in synthesizing potential antimalarial agents, it became necessary to prepare quantities of 3- and 4-methyl- and 3- and 4-phenylglutamic acids. The four glutamic acid analogs were reported previously (1–4).

DISCUSSION

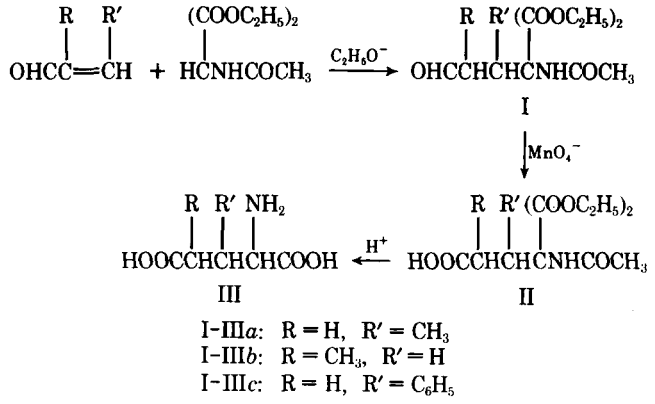
3-Methylglutamic acid (IIIa) was obtained by reacting crotonaldehyde with ethyl acetamidomalonate by means of a Michael condensation, followed by oxidation of the aldehyde function (I) with permanganate and hydrolysis of the oxidation product (II) with acid. The preparation of 4-methylglutamic acid (IIIb) was based on condensing methacrolein with ethyl acetamidomalonate, oxidizing, and hydrolyzing the products, as for 3-methylglutamic acid. 3-Phenylglutamic acid (IIIc) was obtained in a similar manner, by a Michael condensation of cinnamaldehyde with ethyl acetamidomalonate followed by oxidation and hydrolysis.

For the preparation of 4-phenylglutamic acid (V), tropic acid was made into the acid chloride by means of thionyl chloride which, in turn, was esterified with ethanol. Ethyl tropate was subsequently converted to ethyl 3-bromo-2-phenylpropionate with phosphorus tribromide, and the bromo compound was condensed with ethyl acetamidomalonate. Upon hydrolysis of the condensation product with acid, 4-phenylglutamic acid was obtained. Scheme I indicates the preparation of IIIa–IIIc, and Scheme II indicates the preparation of V.

Since it was desired to carry out microbiological and other testing on the three monomethyl- and three monophenylglutamic acids, 2-methyl- and 2-phenylglutamic acids were also required. The synthesis of 2-phenylglutamic acid was reported previously, but it cyclized spontaneously to 2-phenylpyroglutamic acid (5). For the present study, benzoylpropionic acid was treated with ammonium carbonate and potassium cyanide, according to the method of Henze and Speer (6), to form the hydantoin (VI). Compound VI was hydrolyzed to 2-phenylglutamic acid (VII) by successive treatments with sodium hydroxide and hydrochloric acid (Scheme III). This preparation of 2-phenylglutamic acid did not cyclize spontaneously at room temperature. 2-Methylglutamic acid was commercially available. IR spectra of the six glutamic acid analogs appear in Figs. 1 and 2.

3-Methyl-, 4-methyl-, 3-phenyl-, and 4-phenylglutamic acids were screened against *Plasmodium berghei* in mice[1]. These com-

[1] Testing done at the Rane Laboratory, University of Miami, Miami, Fla.



Scheme I

pounds at 640 mg/kg, the highest level administered, were both inactive to the *Plasmodium* and nontoxic to the host.

Since the bacterial cell wall of many species is known to contain D-glutamic acid (7–9), it was of interest to determine whether the substituted glutamic acids would inhibit bacterial growth, presumably by preventing the incorporation of D-glutamic acid into the bacterial wall. It was previously reported that when *Lactobacillus plantarum*, a glutamic acid-requiring mutant, was grown in a medium containing sufficient L-glutamic acid to afford half-maximal growth and the three monomethylglutamic acids were incorporated into the medium individually up to a molar ratio of 186:1, only 4-methylglutamic acid caused any inhibition of growth (10%) (10).

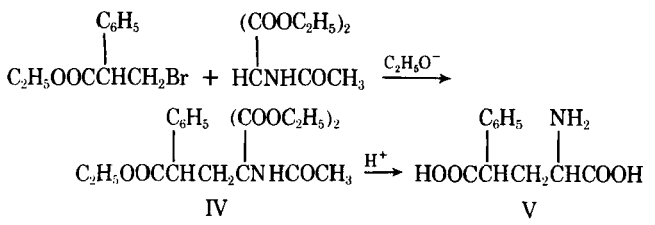
A test of the compounds against *Lactobacillus casei* (ATCC 9595), a second glutamic acid-requiring mutant, was carried out. Glutamic acid assay medium[2] enriched with 20 μg/ml of L-glutamic acid hydrochloride, allowed 85% of the optimal growth. No inhibition of the organism was observed when 1000 μg/ml of each of the six glutamic acid analogs was added to the assay medium. All six glutamic acid analogs were tested against *Escherichia coli* (ATCC 9723) in the medium of Davis and Mingioli (11). Only 2-methylglutamic acid inhibited the growth of the organism (27%) at a level of 10,000 μg/ml. All of these glutamic acid analogs also failed to inhibit four fungi, *Aspergillus niger* (ATCC 6275), *Aspergillus oryzae* (ATCC 1101), *Trichoderma viride* (ATCC 8678), and *Myrothecium verrucaria* (ATCC 9095), in Czapek–Dox agar[2] at pH 5.6 and 7.0 at 10,000 μg/ml, the highest level tested.

EXPERIMENTAL[3]

Ethyl 2-Acetamido-2-carbethoxy-3-methyl-5-oxovalerate (Ia)—This compound was prepared as reported previously (12), yielding 95%, mp 91–92°. An analytical sample was crystallized from ethanol, mp 95° [lit. (12) mp 88–89°].

Anal.—Calc. for $C_{13}H_{21}NO_6$: C, 54.35; H, 7.37; N, 4.88. Found: C, 54.15; H, 7.42; N, 4.90.

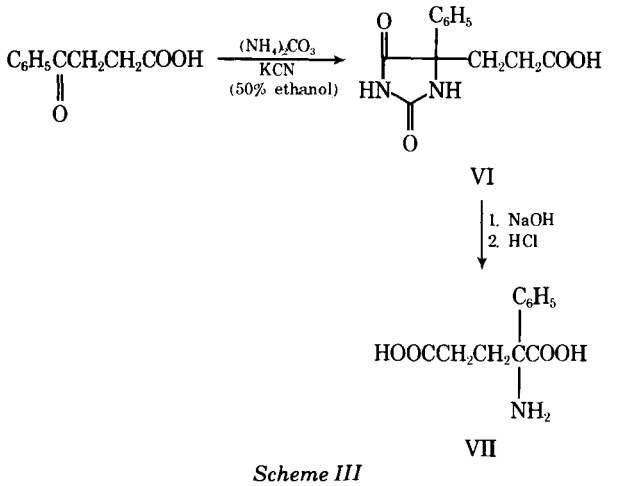
Ethyl 2-Acetamido-2-carbethoxy-3-methylglutarate (IIa)—Compound Ia (28.8 g, 0.1 mole) was dissolved in 380 ml of water at 70–75°. Potassium permanganate (22.5 g, 0.14 mole), dissolved in 450 ml of water, was added over 2 hr. The mixture was stirred for an additional 2 hr at 70–75°. The manganese dioxide was removed



Scheme II

[2] Difco.

[3] Melting points were taken in a Thomas-Hoover melting-point apparatus and are uncorrected. IR spectra were obtained with a Perkin-Elmer model 221 spectrophotometer. 3-Benzoylpropionic acid, tropic acid, and 2-methylglutamic acid were obtained commercially



Scheme III

by filtration, and the filtrate was decolorized with a small quantity of sodium thiosulfate and refiltered. After acidification with dilute sulfuric acid, the solution was extracted with 5 × 100 ml of methylene chloride. The combined washings were backwashed with 50 ml of water and dried (sodium sulfate). The solid obtained upon evaporation of the solvent was crystallized from ether, yielding 15.5 g (56%), mp 100–103°. An analytical sample was crystallized from ether, mp 102–103°.

Anal.—Calc. for $C_{13}H_{21}NO_7$: C, 51.48; H, 6.98; N, 4.62. Found: C, 51.65; H, 7.12; N, 4.53.

DL-3-Methyl-DL-glutamic Acid (IIIa)—Compound IIa (28.4 g, 0.1 mole) was heated under reflux with 285 ml of concentrated hydrochloric acid overnight. The excess acid was removed by flash evaporation, and the syrupy residue was diluted to 50 ml with water and adjusted to pH 3.2 with pyridine. Three volumes of ethanol was added to the solution, and the mixture was refrigerated at −10° overnight. The product was removed by filtration, washed with ethanol, and dried at 70° overnight, yielding 12.1 g (75%), mp 164° dec. [lit. (1) mp 164° dec.].

Ethyl 2-Acetamido-2-carbethoxy-4-methyl-5-oxovalerate (Ib)—Compound Ib was prepared from methacrolein (technical grade contained 90% methacrolein and 10% acetic acid) and ethyl acetamidomalonate (12), and additional sodium was used to neutralize the acetic acid. The ethanol solution was evaporated to near dryness in a rotary evaporator, and the residue was dissolved in chloroform, washed free of salts (water), dried (sodium sulfate), and taken to dryness in the evaporator. The product was obtained as a syrup in 98% yield (12). An analytical sample was prepared by drying under vacuum overnight.

Anal.—Calc. for $C_{13}H_{21}NO_6$: C, 54.35; H, 7.37; N, 4.88. Found: C, 54.06; H, 7.31; N, 4.95.

Ethyl 2-Acetamido-2-carbethoxy-4-methylglutarate (IIb)—Compound IIb was prepared from the aldehyde by oxidation with potassium permanganate in the same manner as IIa. The yield was 43%, mp 121.5–122.5°. An analytical sample was crystallized from ether, mp 125.5–126°.

Anal.—Calc. for $C_{13}H_{21}NO_7$: C, 51.48; H, 6.98; N, 4.62. Found: C, 51.68; H, 6.75; N, 4.72.

DL-4-Methyl-DL-glutamic Acid (IIIb)—Compound IIIb was prepared by hydrolysis of IIb with concentrated hydrochloric acid in the same manner as IIIa. The yield was 66%, mp 152° dec. An analytical sample was crystallized from aqueous acetone, mp 160.5° dec. [lit. (13) mp 154–155°].

Anal.—Calc. for $C_6H_{11}NO_4$: C, 44.72; H, 6.88; N, 8.69. Found: C, 45.06; H, 6.83; N, 8.56.

Ethyl 2-Acetamido-2-carbethoxy-5-oxo-3-phenylvalerate (Ic)—Compound Ic was prepared from cinnamaldehyde and ethyl acetamidomalonate in the same manner as Ia. The yield was 84%, mp 95–96°. An analytical sample was crystallized from ethanol, mp 96.5–97.5°.

Anal.—Calc. for $C_{18}H_{23}NO_6$: C, 61.88; H, 6.64; N, 4.01. Found: C, 62.02; H, 6.62; N, 4.00.

Ethyl 2-Acetamido-2-carbethoxy-3-phenylglutarate (IIc)—Compound Ic was oxidized to IIc with potassium permanganate in the same manner as Ia, except that the product crystallized from the filtrate upon acidification. It was obtained by filtration,

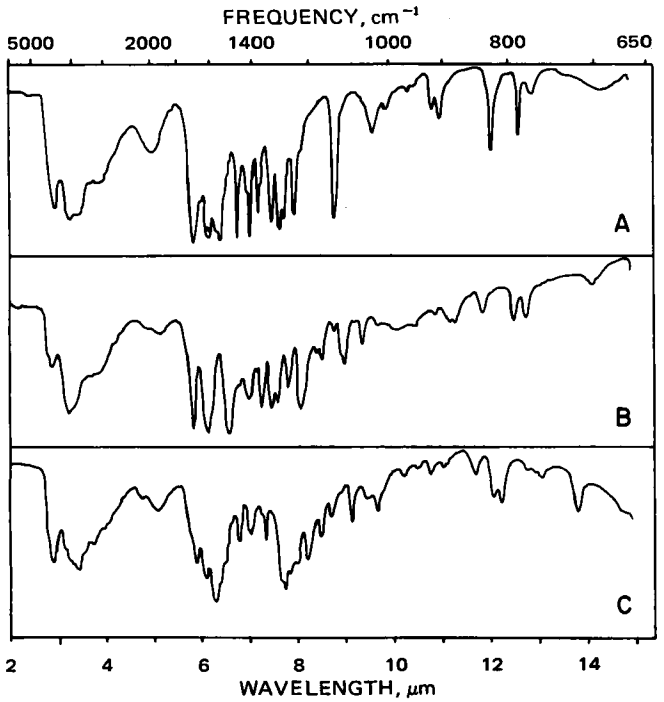


Figure 1—*IR spectra in KBr of DL-2-methylglutamic acid (A), DL-3-methyl-DL-glutamic acid (B), and DL-4-methyl-DL-glutamic acid (C).*

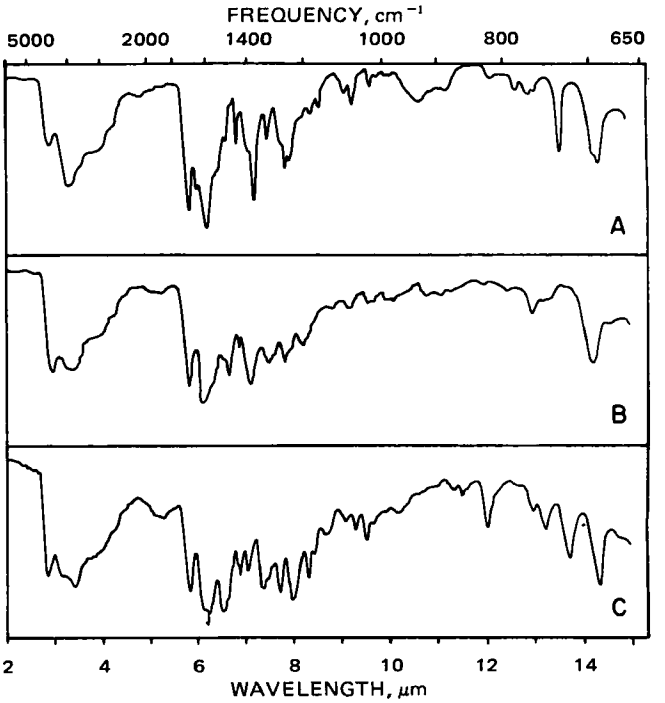


Figure 2—*IR spectra in KBr of DL-2-phenylglutamic acid (A), DL-3-phenyl-DL-glutamic acid (B), and DL-4-phenyl-DL-glutamic acid (C).*

washed free of salts with water, and dried at 70° overnight, mp 179–180° (56%). An analytical sample was crystallized from water, mp 180–180.5°.

Anal.—Calc. for $C_{18}H_{23}NO_7$: C, 59.17; H, 6.34; N, 3.83. Found: C, 59.33; H, 6.29; N, 3.87.

DL-3-Phenyl-DL-glutamic Acid (IIIc)—Compound IIIc was prepared by hydrolysis of IIc with concentrated hydrochloric acid in the same manner as IIIa. The yield was 89%, mp 179–180° dec. An analytical sample was crystallized from aqueous acetone, mp 187–189° dec. [lit. (3) mp 175° dec.].

Anal.—Calc. for $C_{11}H_{13}NO_4$: C, 59.19; H, 5.87; N, 6.27. Found: C, 59.59; H, 6.03; N, 6.01.

Ethyl Tropate—Tropic acid (500 g, 3.0 moles) was heated with thionyl chloride (1300 ml) under reflux with stirring and exclusion of moisture for 4 hr. The excess thionyl chloride was removed by flash evaporation, and the residue was heated with 2 liters of ethanol under reflux with stirring overnight. The alcohol was removed by flash evaporation, and the residue was distilled under vacuum. The yield of product was 524 g (90%), bp 120–124° (2 mm) [lit. (4) bp 152–154° (12 mm)].

Ethyl 3-Bromo-2-phenylpropionate—To ethyl tropate (194 g, 1.0 mole) in 200 ml of dry chloroform was added 100 g (0.36 mole) of phosphorus tribromide dropwise with stirring; the temperature was kept between −5 and 10°. The reaction mixture was stirred overnight and allowed to come to room temperature. It was again cooled to 0°, added slowly with stirring to a slurry of ice and water, extracted with chloroform, and washed twice with sodium bicarbonate followed by water. The chloroform layer was separated, dried (sodium sulfate), and freed of solvent by flash evaporation. The residue was distilled under vacuum, yielding 206 g (80%), bp 97–100° (0.5 mm) [lit. (4) bp 102° (0.5 mm)].

Ethyl 2-Acetamido-2-carbethoxy-4-phenylglutarate (IV)—To a solution of 4.5 g (0.196 g-atom) of sodium in 500 ml of anhydrous ethanol was added ethyl acetamidomalonate (42.5 g, 0.196 mole). A solution of ethyl 3-bromo-2-phenylpropionate (47.6 g, 0.196 mole) in 100 ml of dry ethanol was added dropwise with agitation. Upon completion of addition of the bromo compound, the mixture was kept under reflux with stirring overnight. The salts were removed by filtration, and the solvent was flash evaporated. The residue was dissolved in chloroform, washed twice (water), and dried (sodium sulfate). After evaporation of the solvent, the residue was dissolved in isopropanol and kept at −15° for several days. A yield of 67 g (85%) was obtained, mp 68–70°. An analytical sample was crystallized from isopropanol, mp 68.5–70° [lit. (4) mp 95°].

Anal.—Calc. for $C_{20}H_{27}NO_7$: C, 61.06; H, 6.92; N, 3.56. Found: C, 61.18; H, 6.91; N, 3.53.

DL-4-Phenyl-DL-glutamic Acid (V)—Compound IV (32.0 g, 0.081 mole) was heated under reflux in 320 ml of concentrated hydrochloric acid overnight, and excess acid was removed in a rotary evaporator. The residue was brought to pH 3.2 with pyridine and diluted with two volumes of ethanol. After refrigeration for several hours, the product was obtained by filtration, washed free of chloride with ethanol, and dried at 70° overnight. The yield of compound was 14.8 g (81%), mp 170–171° dec.

After recrystallization from aqueous acetone followed by vacuum drying at room temperature, the compound melted at 177–179° dec. Upon further heating, the material solidified and remelted at 203–204° (pyroacid). Additional recrystallizations yielded products of higher melting points. All possessed the same retention time by GC (trimethylsilyl derivative on 1% Apiezon column) [lit. (4) no melting point reported].

Anal.—Calc. for $C_{11}H_{13}NO_4$: C, 59.19; H, 5.87; N, 6.27. Found: C, 59.35; H, 5.61; N, 6.20.

5-Phenyl-5-(2-carboxyethyl)hydantoin (VI)—A solution of 3-benzoylpropionic acid (100 g, 0.56 mole), ammonium carbonate (215 g, 2.24 moles), and potassium cyanide (72.8 g, 1.12 moles) in 1400 ml of 50% aqueous ethanol was kept at 60° for 2 hr. After the solution was cooled in an ice bath for several hours, the product crystallized and was removed by filtration, washed with a small volume of cold water, and dried at 100° under vacuum overnight. The yield was 131 g (94%), mp 214–215°. An analytical sample was crystallized from water, mp 215.5–216.5°.

Anal.—Calc. for $C_{12}H_{12}N_2O_4$: C, 58.06; H, 4.87; N, 11.28; O, 25.78. Found: C, 57.98; H, 5.05; N, 11.12; O, 25.98.

DL-2-Phenylglutamic Acid (VII)—A solution of 10 g (0.04 mole) of VI and 2.5 g (0.06 mole) of sodium hydroxide in 100 ml of water was kept at 160° in a stainless steel bomb for 16 hr. The bomb was cooled, and the solution was removed and boiled for 5 min to remove free ammonia, brought to pH 4 with acetic acid, and again boiled for 5 min to remove carbon dioxide. It was then passed through an ion-exchange resin[4] column (acidic) to remove sodium ions.

[4] Amberlite IR-120.

The column was washed with 1500 ml of hot water, the effluent was brought to near dryness by flash evaporation, and the residue was heated under reflux for 2.5 hr with 50 ml of concentrated hydrochloric acid. The hydrochloric acid was removed in a rotary evaporator, and the residual solution was dissolved in a small volume of water, decolorized with carbon, and adjusted to pH 3.1 with pyridine; then three volumes of ethanol was added.

After cooling at −15° overnight, the crystalline product was removed by filtration, washed with ethanol followed by ether, and dried at 70° overnight. The yield was 4.3 g (48%), mp 162–164°. On further heating, the melt solidified and remelted at 202–203° (2-phenylpyroglutamic acid). The analytical sample was crystallized from aqueous ethanol, mp 166–167° [lit. (5) mp 170°]; with further heating, the melting point was 205–206° [lit. (5) mp 207°].

Anal.—Calc. for $C_{11}H_{14}NO_4$: C, 59.19; H, 5.87; N, 6.27; O, 28.67. Found: C, 59.36; H, 5.95; N, 6.14; O, 28.89.

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[x] To whom inquiries should be directed.

Inhibitory Effect of Dioctyl Sodium Sulfosuccinate on Trypsin Activity

GURCHARAN S. JODHKA, M. WAFIK GOUDA [x], RUSTEM S. MEDORA, and SAID A. KHALIL [x]

Abstract □ The inhibitory effect of dioctyl sodium sulfosuccinate on the proteolytic activity of trypsin was investigated over the pH 6–8 range. The antitryptic activity was determined using two different substrates: casein and *N*,α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride. The mechanistic studies revealed the substrate–inhibitor interaction to be the overall major mechanism of inhibition. This interaction was shown to involve substrate depletion, probably involving some primary sites of the natural substrate casein. Some inhibition was also shown to be due to an interaction between the enzyme and the inhibitor molecules. The interactions of the inhibitor with the enzyme and the substrate were irreversible. The possible therapeutic significance of the inhibitory effect of the surfactant is discussed.

Keyphrases □ Dioctyl sodium sulfosuccinate—trypsin inhibition, pH 6–8, two different substrates, therapeutic significance □ Trypsin—inhibition by dioctyl sodium sulfosuccinate, pH 6–8, two different substrates, therapeutic significance □ Enzyme inhibition—trypsin by dioctyl sodium sulfosuccinate, pH 6–8, two different substrates, therapeutic significance

The theories on the role of proteolytic enzymes in physiological and pathological states in humans and animals have been the topic of great controversy. The old belief that these enzymes catalyze protein synthesis no longer exists, yet it would be equally unjustified to restrict the role of body proteinases to a few processes such as cleavage of food proteins, mobilization of tissue proteins, and protein degradation usually associated with physiological wear and various traumas. In fact, it is now evident that these enzymes play a causative or adjunctive role in some disease processes such as inflammation, thromboembolic disorders, complement-dependent immune reactions, peptic and duodenal ulcers, and pancreatitis and even in some syndromes of malignant carcinomas (1).

Trypsin and kallikrein, which are present in the body tissues and fluids, are capable of releasing pharmacologically active plasma kinins (2). These proteolytic enzymes play an indirect but significant role in such important conditions as hypertension, shock, certain pains, changed capillary permeability, edema, and leucocyte migration. In the light of this knowledge, the proteinase inhibitors, both synthetic and natural, have gained new theoretical and practical importance (3). Recent attempts have been made to synthesize some active-site-oriented inhibitors as well as to improve their inhibitory activity (4–6). Also, it is reported that proteolytic enzymes are inhibited by agents such as sodium lauryl sulfate (7) and some sulfated polysaccharides (8).

Dioctyl sodium sulfosuccinate is an anionic surfactant, widely used medicinally as a fecal softener. The effect of this surfactant on drug absorption through membranes of varying complexities has been re-

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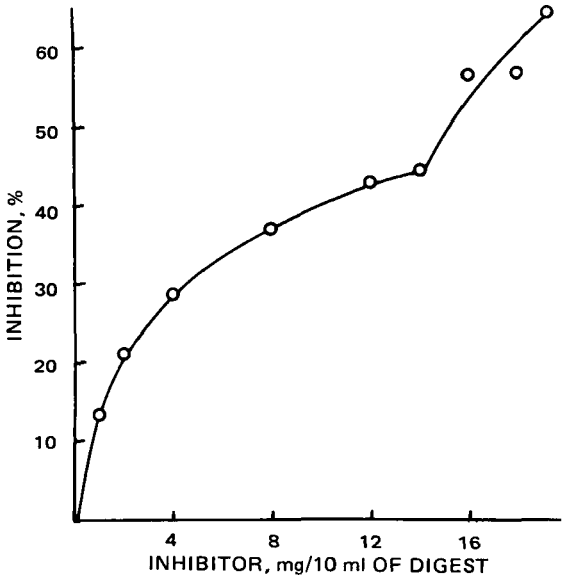

Figure 1—*Effect of various inhibitor concentrations on the percentage inhibition at pH 7.6. Ten milliliters of digest contained 10 μg of enzyme and 50 mg of substrate.*

ported (9–11), as has the absorption of dioctyl sodium sulfosuccinate into the systemic circulation (12). These findings and the similarity of the chemical nature of this surfactant to some proteolytic inhibitors such as sodium lauryl sulfate and sulfated polysaccharides led to this investigation of the inhibitory effect of dioctyl sodium sulfosuccinate on tryptic activity.

EXPERIMENTAL

Materials—Trypsin[1], casein[2], *N*,α-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride[3], dioctyl sodium sulfosuccinate[4], and tromethamine[5] were obtained commercially. All other chemicals used were either USP or reagent grade.

Methods for Antitryptic Activity—*Method I*—Sørensen phosphate buffers (0.1 *M*) of various pH values were used. A fresh 1% casein (I) solution was prepared by suspending 1.0 g of casein in 100 ml of buffer and heating the suspension in a boiling water bath for 15 min. Enzyme (II) solutions of various concentrations, ranging between 10 and 50 μg/ml, were prepared by first dissolving 10 mg of trypsin in 10 ml of 0.001 *M* HCl and then diluting with the appropriate buffers. Various inhibitor (III) solutions were prepared by dissolving required amounts of the inhibitor in the buffers at the appropriate pH.

The assay was carried out using a modification of Kunitz's procedure (13). All solutions were freshly prepared and mixed at room temperature before incubation. The final volume of the digestion mixture was kept at 10 ml in all cases. In this method, two different mixing procedures were followed.

In one mixing procedure, inhibitor was added to the enzyme before digestion. One milliliter of enzyme solution was pipetted into flasks followed by 1 ml of the inhibitor solution. Five milliliters of the substrate solution was then added to each flask at 30-sec intervals. All flasks were incubated in a water bath metabolic shaker[6] maintained at 37° for 1 hr. Samples were shaken at 200 rpm throughout the digestion period. At the end of the incubation period, 3 ml of 10% trichloroacetic acid was added to each flask, in the same order, and the flasks were allowed to stand at room temperature for 10 min. Blanks were prepared in the same way as samples except that the casein solution was added after the addition of trichloroacetic acid.

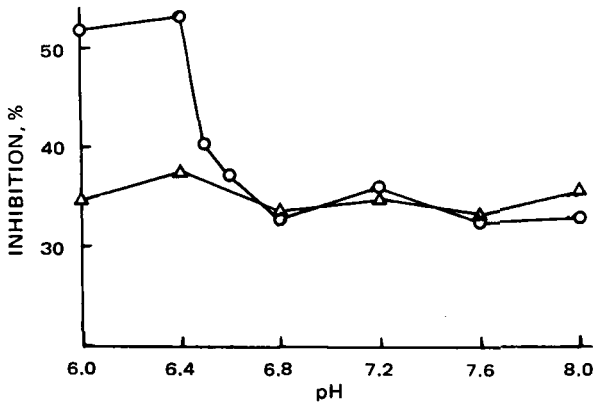

Figure 2—*Effect of pH and mixing orders on percentage inhibition. Ten milliliters of digest contained 20 μg of enzyme, 50 mg of substrate, and 10 mg of inhibitor. Key: O, inhibitor added to enzyme first; and Δ, inhibitor added to substrate first.*

Both samples and blanks were then centrifuged[7] at 8000 rpm for 20 min, and the supernates were filtered[8]. The absorbance of each filtrate was read[9] at 280 nm against the respective blank. Controls were run simultaneously for every inhibition experiment. The controls differed from the samples in having inhibitor solution replaced by equivalent amounts of buffers.

The absorbance values were used as measures of reaction velocities in the kinetic studies. The inhibition (i) equals $[1 - (v_i/v)]$, where v_i and v represent digestion velocities with and without the inhibitor, respectively. Percentage inhibition values were calculated from i values.

The other mixing procedure for digestion was the same as already described, except that the inhibitor solution was added to the substrate first and mixed, followed by addition of enzyme.

Method II—*N*,α-Benzoyl-DL-arginine-*p*-nitroanilide hydrochloride solution (10^{-3} *M*) was prepared as described by Erlanger *et al.* (14). Various concentrations of enzyme and inhibitor solu-

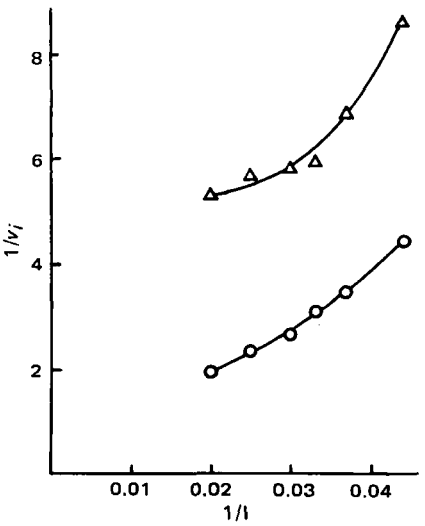

Figure 3—*Plots of $1/v_i$ against $1/I$ for dioctyl sodium sulfosuccinate at two pH values. Ten milliliters of digest contained 20 μg of enzyme, 10 mg of inhibitor, and 23–50 mg of substrate. Key: Δ, pH 6.4; and O, pH 7.6.*

[1] Twice crystallized, salt-free, and lyophilized beef pancreas, Nutritional Biochemicals Corp., Cleveland, Ohio.
[2] Hammersten type, Nutritional Biochemicals Corp., Cleveland, Ohio.
[3] Schwarz/Mann, Dickinson and Co., Van Nuys, Calif.
[4] Aerosol O.T. 100%, Sargent-Welch Scientific Co., Skokie, Ill.
[5] Tris(hydroxymethyl)aminomethane, Sigma Chemical Co., St. Louis, Mo.
[6] Model G-77, New Brunswick Scientific Co., New Brunswick, N.J.
[7] Sorvall SS-4 superspeed centrifuge, Ivan Sorvall Inc., Norwalk, Conn.
[8] Whatman No. 2 filter paper.
[9] Spectronic-600 spectrophotometer, Analytical Instruments, Bausch & Lomb, Rochester, N.Y.

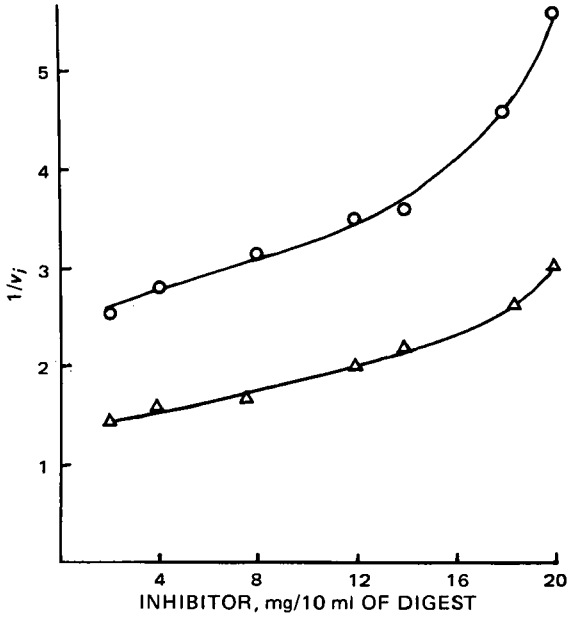

Figure 4—*Plots of $1/v_i$ against III for dioctyl sodium sulfosuccinate at pH 7.6. Key: O, 10 ml of digest contained 10 μg of enzyme and 50 mg of substrate; and Δ, 10 ml of digest contained 20 μg of enzyme and 50 mg of substrate.*

tions were prepared as described in Method I, except that the buffers used were 0.05 *M* tromethamine at various pH values. An amidase assay procedure was used with a few modifications (14). The final volume of the reaction mixture was 8 ml in each case. Incubation of the samples was done at 37° for 300 sec, and the reaction was stopped by adding 1 ml of 30% acetic acid to each flask.

All incubation mixtures were centrifuged and then filtered as described in Method I. Blanks and controls were prepared in the same manner as described in Method I. The absorbance of the filtrates of both samples and blanks was recorded[10] at 410 nm against distilled water. Differences between the absorbance of samples and blanks represent the proteolytic activity. Inhibition values (i) and the percentage inhibitions were calculated as already described. Different orders of mixing were carried out according to the two procedures used in Method I.

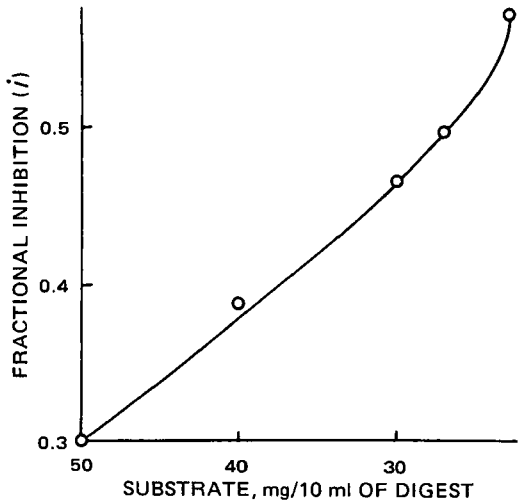

Figure 5—*Plot of* i *against I for dioctyl sodium sulfosuccinate at pH 7.6. Ten milliliters of digest contained 20 μg of enzyme and 10 mg of inhibitor.*

[10] Beckman Acta CIII spectrophotometer, Beckman Instruments, Inc., Fullerton, Calif.

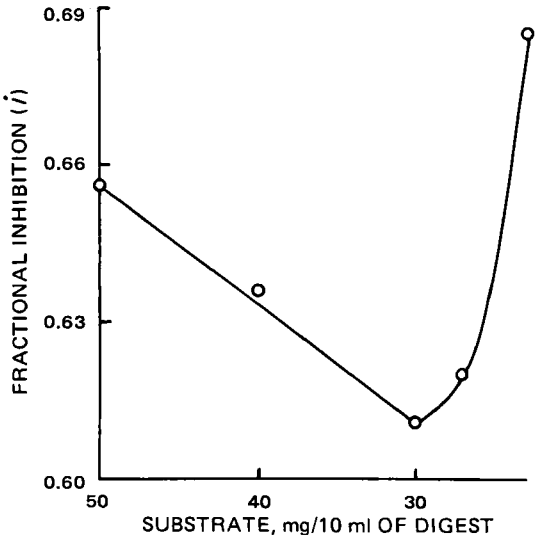

Figure 6—*Plot of* i *against I for dioctyl sodium sulfosuccinate at pH 6.4. Ten milliliters of digest contained 20 μg of enzyme and 10 mg of inhibitor.*

Preincubation Studies—In the preincubation studies the inhibitor was allowed to react for an additional 15 min either with the enzyme or with the substrate before digestion was started. The controls were also preincubated for the same period. Digestion was carried out according to both procedures described under *Method I*.

Dialysis Studies—To find out whether the interaction of inhibitor toward substrate and enzyme is reversible or irreversible, dialysis studies were carried out at pH 6.4 and 7.6. Regenerated cellulose dialysis tubings[11] were cut into pieces of 15–23 cm (6–9 in.) and thoroughly washed with distilled water. The tubings were then immersed in 500 ml of 10 m*M* edetate sodium solution and heated at 70–80° for 2 hr. Then the tubings were allowed to cool, washed again thoroughly with double-distilled water, and stored in distilled water at 2–4°. Prior to use, the tubings were soaked in an appropriate buffer for 18–24 hr.

The inhibitor was added to the enzyme or substrate, and the reaction was allowed to proceed at 37° for 60 min in a metabolic shaker at 200 rpm. A portion of the mixture was then assayed for tryptic activity using Method I, and the rest of the mixture was put in a preconditioned dialysis tubing sac. The sac was immersed in 1000 or 2000 ml of 0.1 *M* phosphate buffer of appropriate pH, and dialysis was carried out at 4° for 96 hr. The external buffer was replaced every 24 hr during the dialysis period, at the end of which the contents were again assayed for their tryptic activity. A control dialysis experiment was run simultaneously in the same way, except that the inhibitor solution was replaced by an equivalent amount of buffer solution. Percentage inhibition values before and after dialysis were calculated as already described.

All experiments were performed at least in duplicate.

RESULTS AND DISCUSSION

The inhibitory effect of dioctyl sodium sulfosuccinate on tryptic activity was studied at various inhibitor concentrations using Method I. Percentage inhibition was plotted against inhibitor concentration (Fig. 1). In all studies, the inhibitor was added to the enzyme first, except for the data of Fig. 2 in which case both mixing procedures were used. The biphasic plot in Fig. 1 can be explained as due to the saturation of either the enzyme or the substrate with the inhibitor at the point of inflection.

The effect of pH and mixing orders on the percentage inhibition was also studied using Method I (Fig. 2). Figure 2 shows that when the inhibitor was added to the substrate first, the percentage inhi-

[11] No. 20, 1.6 cm (0.62 in.) in flat width, for trypsin dialysis and No. 36, 2.85 cm (1.1 in.) in diameter, for casein dialysis were used. Average pore size was 24 Å. Van-Waters and Rogers, Inc., San Francisco, Calif.

Table I—Irreversible Binding of the Inhibitor to Enzyme and Substrate[a]

pH	Component Bound to the Inhibitor before Dialysis	Amount of the Components in 10 ml of Digestion Mixture			Inhibition, %	
		Enzyme, μg	Substrate, mg	Inhibitor, mg	Before Dialysis	After Dialysis
6.4	Trypsin	10	50	1.0	100.0	100.0
	Trypsin	10	50	0.1	65.0	57.5
	Trypsin	20	50	0.1	94.4	95.0
	Casein	20	50	10.0	42.0	38.0
7.6	Trypsin	10	50	1.0	65.6	67.6
	Casein	20	50	10.0	36.7	34.3

[a] Casein.

bition remained reasonably constant throughout the pH range studied. When the inhibitor was added to the enzyme first, the resulting percentage inhibition was significantly higher ($p < 0.001$ as determined by the Student t test for unpaired data) at pH <6.5. This marked difference in inhibition pattern below pH 6.5 will be discussed later.

Using casein as the substrate, double reciprocal plots in the presence of the inhibitor at both pH 6.4 and 7.6 (Fig. 3) and plots of $1/v_i$ against III at pH 7.6 (Fig. 4) indicate the substrate–inhibitor interaction as the major mechanism of inhibition (15). When using the data of Figs. 3 and 4, plots of I/v_i against I, v_i against v_i/I, and $1/i$ against 1/III also yielded curves typical of substrate–inhibitor interaction (16).

Plots of fractional inhibition (i) against I at pH 7.6 (Fig. 5) and 6.4 (Fig. 6), with the data of Fig. 3, suggest that the enzyme–inhibitor interaction as well as the substrate–inhibitor interaction also contributes to the overall inhibition. This contribution seems to be least at pH 7.6 (Fig. 5) and most at pH 6.4 (Fig. 6). This apparent increased involvement of the enzyme with the inhibitor as a consequence of a change in pH can be well explained by the fact that dioctyl sodium sulfosuccinate is a molecule carrying a net negative charge and that trypsin possesses an isoelectric point in the vicinity of pH 7 (17). Therefore, increased amount of inhibition below pH 6.5 is expected. This explains the increased inhibition below pH 6.5 observed in Fig. 2. Also, the biphasic curve of percentage inhibition against the inhibitor concentration (Fig. 1) can be explained on the basis of a dual mechanism of inhibition involving both the substrate and the enzyme.

A double reciprocal plot (Fig. 7) also was made using a synthetic substrate (Method II), and the mechanism of inhibition seems to be similar to the one observed when casein was used as the substrate. This similarity in mechanisms when two different substrates were used indicates that the interaction between the inhibitor and casein molecules involves some primary sites of the natural protein.

A general inhibitory mechanism involving a substrate can be further distinguished into three basic mechanisms by plotting III against $i/(1 - i)$ (18). These mechanisms are: (a) substrate depletion, (b) inhibition of the enzyme by a substrate–inhibitor complex, and (c) combination of both mechanisms. The shapes of such plots (Fig. 8) for dioctyl sodium sulfosuccinate at pH 7.6, using casein as a substrate, are typical of substrate depletion. Similar plots showing substrate depletion as the basic mechanism were also obtained using different substrate and enzyme concentrations. The results of the dialysis studies (Table I) indicate that dioctyl sodium sulfosuccinate binds both casein and trypsin irreversibly at both pH 6.4 and 7.6.

Preincubation studies (Table II) show that preincubation of the substrate with the inhibitor has no significant effect on the inhibition. Preincubation of the enzyme with the inhibitor prior to digestion seems to increase the percentage inhibition at almost all substrate–inhibitor ratios. These results suggest that, besides the substrate–inhibitor interaction, the enzyme–inhibitor interaction is a part of the overall mechanism of inhibition at pH 7.6. This finding is in good agreement with what has already been concluded from the mechanistic studies.

The effect of various mixing orders on percentage inhibition was also studied using the synthetic substrate. Results were similar to those obtained with casein.

The effect of various shaking rates was investigated using casein as the substrate. The degree of inhibition was unaltered at various shaking speeds ranging between 0 and 200 rpm.

Pancreatitis can be regarded as a chemical autolytic process, the pathogenesis of which is well documented from postmortem, operative, and experimental studies (19). The exact mechanism of the pathological process has yet to be established. However, evidence supports the central role of trypsin in the pathogenesis of the disease (1, 3, 20). It is then apparent that means of controlling the undesirable hypertryptic activity at the site of disease could serve as an approach to alleviate the disease. The present inhibitory studies with dioctyl sodium sulfosuccinate and the recent finding that this compound is absorbed into the systemic circulation (12) suggest a possible use of this medicinal surfactant in pancreatitis. The interaction of dioctyl sodium sulfosuccinate with casein

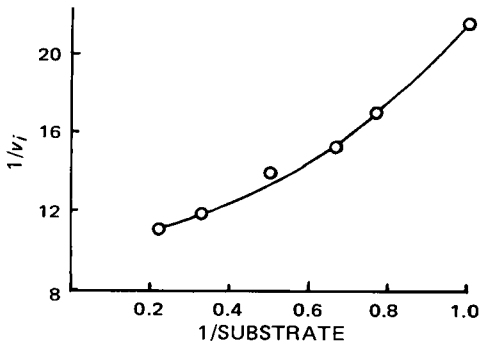

Figure 7—*Plot of* $1/v_i$ *against 1/substrate for dioctyl sodium sulfosuccinate at pH 8.2. Eight milliliters of the incubation mixture contained 40 μg of enzyme, 0.25 mg of inhibitor, and* $1–5 \times 10^{-6}$ *mole of substrate. Substrate used was* N,α-benzoyl-DL-arginine-p-nitroanilide hydrochloride.

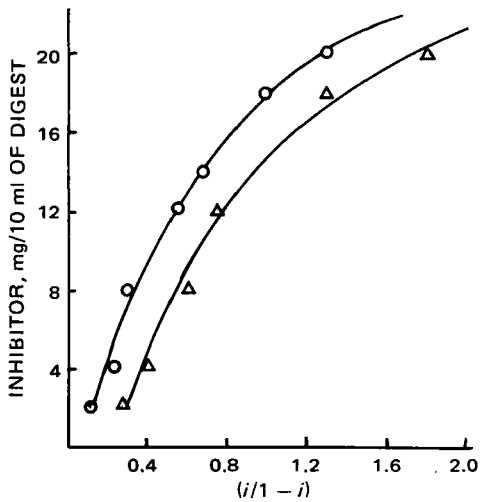

Figure 8—*Plots of III against* (i/1 − i) *for dioctyl sodium sulfosuccinate at pH 7.6. Key:* O, *10 ml of digest contained 20 μg of enzyme and 50 mg of substrate; and* Δ, *10 ml of digest contained 10 μg of enzyme and 50 mg of substrate.*

Table II—Effect of Preincubation on Antitryptic Activity of Dioctyl Sodium Sulfosuccinate at pH 7.6

Substrate[a], mg	Inhibitor, mg	Substrate/Inhibitor	Percentage Inhibition[b]		
			A	B	C
40	20	2	71.0	66.5	88.6
40	10	4	38.5	39.2	66.4
40	5	8	25.0	25.0	45.5
40	2.5	16	19.5	21.8	36.7
40	1.25	32	11.2	10.0	23.4

[a] Casein. [b] Amount of enzyme used was 30 μg. A, no preincubation; B, the inhibitor was preincubated with casein for 15 min; and C, the inhibitor was preincubated with trypsin for 15 min.

suggests the possibility of a similar interaction between the inhibitor molecule and the polysaccharide proteins at the site of inflammation. Such an interaction *in vivo* may prove to be useful in protecting the site from necrosis and erosion (21).

These inhibitory studies with dioctyl sodium sulfosuccinate also suggest that possible drug interactions may arise with this compound in situations and conditions where concomitant use of proteolytic enzymes such as trypsin and chymotrypsin is desirable. Drug interactions are also possible with drug esters such as penamecillin and chloramphenicol palmitate and certain *N*-acylated drugs when given orally along with dioctyl sodium sulfosuccinate. Such drugs are usually acted upon by proteinases in the GI tract resulting in changed chemical and/or pharmacological activity. These drug interactions may be difficult to recognize, particularly when dioctyl sodium sulfosuccinate is used as an excipient for the formulation of any drug being used simultaneously.

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Determination of Mercury-Containing Pharmaceuticals by Vapor Phase Atomic Absorption Spectroscopy

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Abstract □ A procedure was developed for the determination of mercurials of pharmaceutical interest. Protic acid cleavage of the compound was followed by reduction of the resulting mercuric ion and vapor phase atomic absorption spectroscopy. This procedure was applied to 11 different mercurial compounds in various pharmaceutical preparations and offers excellent sensitivity with respect to presently used compendial assays. Comparative analytical data between this procedure and compendial methodology are presented.

Keyphrases □ Mercurials—determination by vapor phase atomic absorption spectroscopy, compared to compendial methods □ Atomic absorption spectroscopy, vapor phase—determination, mercury-containing pharmaceuticals, compared to compendial methods

Pharmaceutical compounds containing the element mercury, either organically combined or as the inorganic form, have found widespread medicinal use as diuretics, anti-infectives, and bacteriostatic agents. Mercury-containing pharmaceuticals have been analyzed by numerous analytical approaches. Many presently used procedures (1–3) involve conversion of mercurial mercury to mercuric ion followed by classical thiocyanate titrimetry or precipitation of the insoluble mercuric sulfide and gravimetric measurement. Theimer and Arnow (4) applied the thiocyanate titration procedure to injectable organomercurial diuretics.

A titration procedure similar in sensitivity to the official methods involves the acetolysis of the compound in the presence of methylamine hydrochloride followed by nonaqueous titration of the liberated strong base with perchloric acid (5). A more sensitive and convenient approach, utilizing coulometric titration of mercuric ion with electrogenerated sulfhydryl reagent, was developed (6) and applied to marketed dosage forms. Mercaptomerin sodium, which contains a mercury–sulfur bond, could not be analyzed by this method. However, the determination can be carried out in the presence of chloride ion, a species incompatible with both thiocyanate titration and acetolysis procedures.

Direct current polarography has been used to analyze thimerosal (7, 8). Unsubstituted phenylmercuric compounds and mersalyl and meralluride were investigated also (8).

More recently, several sensitive instrumental approaches have been used in mercurial compound analysis. The principle of isotopic exchange utilizing ^{203}Hg was employed in the determination of 13 different mercurials of pharmaceutical or agricultural interest, including three commercial preparations (9). Margosis and Tanner (10) applied neutron activation analysis to the determination of seven different organomercurial compounds, three in pharmaceutical dosage forms. Their results, obtained from the ^{197}Hg- and ^{203}Hg-induced radioactivity, were compared to those obtained by X-ray fluorescence and atomic absorption techniques. The X-ray fluorescence results generally were lower than those achieved with the neutron activation procedure; the atomic absorption results obtained by direct and vapor phase techniques, although incomplete, generally were higher. The poor correlation obtained with the vapor phase technique was attributed to the possible presence of volatile aromatic-type compounds.

The introduction of atomic absorption spectroscopy as a sensitive and specific means for metal analysis has resulted in numerous applications for the determination of mercury, primarily from the environmental impact of the presence of this element (11–13). To date, only a few studies have employed this approach in the analysis of mercury-containing pharmaceuticals. Leaton (14) described a direct atomic absorption procedure for mercuric iodide in ointments, and similar methodology for the direct analysis of phenylmercuric nitrate in ophthalmic preparations was proposed (15). More recently, vapor phase atomic absorption was applied to the analysis of mercurial bacteriostatic agents (16, 17), including phenylmercuric acetate, phenylmercuric nitrate, and thimerosal (17).

The purpose of this study was to determine the feasibility of vapor phase atomic absorption spectroscopy for the analysis of mercurial compounds of pharmaceutical interest and their commercial preparations. The described procedure is convenient, sensitive, and accurate with respect to the official methods.

EXPERIMENTAL

The developed procedure is based on "protodemercuration" of the mercurial compound with hydrochloric acid or a hydrochloric acid–nitric acid mixture under various heating conditions followed by reduction of the resulting mercuric ion to elemental mercury with subsequent detection and quantitation by vapor phase (*e.g.*, flameless, cold vapor) atomic absorption spectroscopy. The final determinative step is in accordance with the reduction procedure of Hatch and Ott (18) as modified by Munns and Holland (19).

Table I—Analysis of Mercurial Compounds[a]

Compound	Heating Condition[b]	Atomic Absorption Method		Official Method	
		Recovery, %	Range, %	Recovery, %	Range, %
Thimerosal	C	100.1	0.2	100.0[c]	0.0
Mersalyl	A	103.1	0.2	103.5[d]	0.4
Meralluride	A	100.0	0.9	99.8[e]	0.1
Mercaptomerin sodium	C	100.9	0.6	99.5[e]	0.1
Phenylmercuric acetate	B	100.4	0.3	100.2[c]	0.1
Chlormerodrin	A	100.5	0.6	100.1[c]	0.0
Merbromin	A	94.6	0.1	94.8[f]	0.6
Nitromersol	B	99.5	1.0	99.8[c]	0.2
Phenylmercuric nitrate	B	99.2	1.5	98.4[c]	0.7
Mercuric oxide, yellow	D	100.8	0.5	100.0[c]	0.1
Ammoniated mercury	D	99.4	0.0	98.8[e]	0.0

[a] Mean of duplicate determinations. [b] See *Experimental*. [c] NF XIII. [d] NF XI. [e] USP XVIII. [f] NF XII.

Eleven mercurial compounds and the respective commercial preparations representing six different types of products were analyzed using the proposed methodology.

Apparatus—An atomic absorption spectrophotometer[1] equipped with a mercury hollow cathode lamp[2], a hydrogen continuum lamp[2] to provide background correction capability, and a cylindrical flow-through cell (2.1-cm i.d. × 10.0-cm) with quartz windows was used. Absorbance measurements were monitored with a 0–100-mv strip-chart recorder[3]. Mercury analysis was performed under the following conditions: wavelength, 253.7 nm; hollow cathode lamp current, 3 mamp; scale, 0.5; circulating pump flow rate, 4.8 liters/min; slit width, 160 μm; and chart speed, 2.5 cm (1 in.)/min.

The reduction apparatus was essentially as described previously (19) with the following modifications. The reduction vessel consisted of a 250-ml two-necked distilling flask with a vertical side neck and ⅋ 24/40 joints. The reducing solution was added *via* a 100-ml cylindrical separator attached to the side neck of the reduction vessel. All connections within the system were of Tygon tubing (5.0-mm i.d.).

Glassware was thoroughly rinsed with hot nitric acid (50% v/v) followed by distilled water prior to use. Dilutions of samples, pure compounds, and mercury standards were carried out in the presence of hydrochloric acid to minimize adsorption of mercuric ion on glassware surfaces.

Materials and Reagents—Powdered portions of thimerosal[4], mersalyl[5], meralluride[6], mercaptomerin sodium[7], phenylmercuric acetate[8], chlormerodrin[6], merbromin[9], nitromersol[10], phenylmercuric nitrate[11], yellow mercuric oxide[12], and ammoniated mercury[12] (mercury amide chloride) were used. Pharmaceutical preparations containing each mercurial compound were obtained through commercial sources. Crystalline mercuric chloride[12] prepared in an acidic medium was employed as the standard mercury solution. All other chemicals and reagents were reagent grade, commercially available materials and were used without further purification.

Standard Mercury Solution—A standard stock solution was prepared by dissolving 0.1354 g of crystalline mercuric chloride in 1 *N* HCl and diluting to 100.0 ml with the same solvent. This solution was prepared fresh on a biweekly basis. Suitable dilutions were prepared in 0.05 *N* HCl to provide a working standard solution having a concentration of 0.25 μg of mercury/ml. These dilutions were carried out daily and just prior to the quantitative reduction step.

General Procedure—Accurately weighed portions of solid materials or measured aliquots of the liquid forms representing the various mercurial compounds or respective products were treated under one of the following heating conditions with occasional swirling of the mixture:

A. Heated for 1 hr on a steam bath with 75 ml of concentrated hydrochloric acid in a 1-liter volumetric flask.

B. Boiled gently for 25 min on a hotplate with 75 ml of concentrated hydrochloric acid in a 300-ml erlenmeyer flask containing five glass beads and fitted with a 6.5-cm diameter powder funnel. Care was taken to prevent the solution from going to dryness by addition of 5–10 ml of the acid when necessary.

C. Heated for 1 hr on a steam bath with 75 ml of diluted hydrochloric acid–nitric acid mixture[13] in a 1-liter volumetric flask.

D. Heated for 15 min on a steam bath with 80 ml of dilute hydrochloric acid (10% v/v) in a 1-liter volumetric flask.

The required heating condition for each mercurial compound and the respective commercial preparation is noted in Table I. Upon completion of the heating step, the 1-liter flask and contents were cooled under tap water to room temperature and the acidic solution was diluted to volume with distilled water. When heating condition B was employed, the cooled acidic solution was quantitatively transferred to a 1-liter volumetric flask with distilled water and diluted to volume.

Further dilutions were prepared with 0.05 *N* HCl to provide an assay solution having a final concentration of approximately 0.25 μg of mercury/ml. Three milliliters of this assay solution was transferred to the reduction vessel, and the published procedure (19) was followed. The system was normally equilibrated with several standard determinations prior to the reduction of sample aliquots. A reagent blank consisting of the appropriate acid solution was carried through the entire procedure in a similar manner to the samples. Quantitation was accomplished by direct comparison of the sample absorbance value to the absorbance obtained with 3.0 ml of the working standard solution of mercury.

Bulk Drug Substances or Reference Compounds—An accurately weighed portion of the powdered material, equivalent to 60 mg of mercury, was taken for analysis. After completion of the appropriate heating step and dilution of the acid solution to 1 liter with distilled water, an assay solution was prepared by further dilution of 2.0 ml to 500.0 ml with 0.05 *N* HCl. A 3.0-ml aliquot of the assay solution was transferred to the reduction vessel for quantitative purposes.

Commercial Preparations—The quantities of mercury taken for the analysis of the various products are given in Table II. The amount of mercury present in the sample portions ranged from 2.5 to 82.5 mg, depending on the particular formulation with the exception of the product containing phenylmercuric nitrate[14]. Therefore, specified dilutions are not given due to the diversity in concentrations of mercury in the preparations.

Tablets and Gels—An accurately weighed portion of a powdered tablet composite or well-mixed gel preparation was taken for the analysis. The general procedure was followed.

Solutions and Tinctures—An accurately measured aliquot was evaporated to dryness under a current of air on a steam bath, the

[1] Model 353, Instrumentation Laboratory, Lexington, Mass.
[2] Instrumentation Laboratory, Lexington, Mass.
[3] Model 194, Honeywell Electronik.
[4] Eli Lilly and Co., Indianapolis, Ind., and NF reference standard.
[5] Winthrop Laboratories, Rensselaer, N.Y.
[6] Lakeside Laboratories, Milwaukee, Wis.
[7] Wyeth Laboratories, Philadelphia, Pa.
[8] Holland-Rantos Co., Trenton, N.J.
[9] Hynson, Westcott and Dunning, Baltimore, Md.
[10] Abbott Laboratories, North Chicago, Ill.
[11] Eastman Kodak Co., Rochester, N.Y.
[12] Mallinckrodt Chemical Works, St. Louis, Mo.

[13] Prepared by mixing 10 parts of water, eight parts of hydrochloric acid, and four parts of nitric acid by volume just prior to use.
[14] A 4.0-g sample portion equivalent to 0.24 mg of mercury was taken for the analysis.

Table II—Analysis of Commercial Products

Mercurial Compound	Type of Product	Declared Amount	Sample Size, Milligrams of Mercury Taken for Atomic Absorption Analysis	Percent of Declared Amount Found[a]			
				Atomic Absorption Method	Range	Official Method[b]	Range
Thimerosal	Tincture	1:1000	2.5	101.4	0.8	104.0	0.0
Mersalyl with theophylline	Injection	100 mg/ml	80	101.7	0.0	102.2	0.2
Meralluride	Injection	39 mg/ml[c]	78	100.2	0.5	100.8	0.1
Mercaptomerin sodium	Injection	125 mg/ml	82.5	100.7	0.0	99.0	0.0
Phenylmercuric acetate	Solution	1:500	6	110.2	0.5	107.2[d]	1.5
Chlormerodrin	Tablet	18.3 mg/tablet	10	97.8	0.0	102.4	0.7
Merbromin	Solution	2%	26.7	90.7	0.5	89.5	1.0
Nitromersol	Tincture	1:200	14.2	89.2	0.2	87.0	0.4
Phenylmercuric nitrate	Gel	0.01%	0.24	77.4	0.9	—[e]	—
Mercuric oxide, yellow	Ointment	2%[f]	32.4	100.5	1.0	102.0	0.0
Ammoniated mercury	Ointment	10%[g]	80	103.6	0.6	102.5	0.0

[a] Mean of duplicate determinations. [b] See Table I. [c] Label claim: 39 mg of mercury in organic combination and 48 mg of theophylline/ml. [d] Product assayed by the NF XIII procedure for phenylmercuric acetate; 250.0 ml was evaporated to dryness on a steambath and the NF XIII procedure was followed beginning with "add 15 ml. water, and 5 ml. of formic acid." [e] Official method not available. [f] Twice the NF strength. [g] Twice the USP strength.

required acid was added, and heating condition A, B, or C was followed (*General Procedure*).

Injectables—An accurately measured aliquot was taken directly for the analysis as described under *General Procedure*.

Ointments—An accurately weighed portion of the well-mixed product was transferred to a 125-ml separator and dispersed by shaking with 50 ml of ether. The ethereal dispersion was extracted four times with 20-ml volumes of dilute hydrochloric acid (10% v/v), and the acidic extracts were drained into a 1-liter volumetric flask. The general procedure was followed, beginning with heating condition D. This condition was employed to facilitate the removal of the residual ether present.

Calculations—The amount in milligrams of the mercurial compound present in the weighed portion or aliquot was calculated in the following manner:

$$\text{mg} = \frac{A_u}{A_s} \times C \times D \times \frac{E}{200.59} \times \frac{1\text{ mg}}{1000\ \mu\text{g}} \qquad \text{(Eq. 1)}$$

where A_u and A_s represent the absorbance values obtained for the reduced sample and standard aliquots, respectively (corrected for any reagent blank absorbance); C is the concentration in micrograms of mercury per milliliter in the final diluted mercuric chloride standard solution; D is the appropriate dilution factor; E is the molecular weight of the mercurial compound; and the value[15] 200.59 represents the atomic weight of mercury. Calculation of the quantity of the mercurial compound per dosage unit or labeled amount was achieved by introduction of the appropriate factors into Eq. 1.

Linearity—Under the assay conditions described, a linear relationship between absorbance and micrograms of mercury was obtained over a 0–1.4-μg range. A quantity of mercury equal to 0.75 μg was selected for the reduction step of the general procedure.

Official Methods—For comparative purposes, each pure compound and pharmaceutical preparation, except the product containing phenylmercuric nitrate, was assayed in duplicate by compendial methodology (1, 2).

RESULTS AND DISCUSSION

Preliminary experiments were conducted to determine the most suitable acid medium for the protolysis of the mercurial compounds in this study, since several different chemical bonds associated with mercury were apparent. Adaptation of a sulfuric acid–nitric acid digestion medium utilized for marine products (19) proved to be both time consuming and cumbersome. Modification of similar procedures (11, 12) under nonreflux conditions with respect to time and temperature was also investigated, but the results obtained were erratic due to the possible loss of mercury from volatilization. Evidence regarding volatility losses of mercury employing such digestion procedures for the total destruction of organic material was reported previously (11, 20).

It was concluded from these initial experiments that a heating step under acidic conditions was necessary to effect cleavage of mercury from the compounds in most cases. The inorganic mercurials, yellow mercuric oxide and ammoniated mercury, were easily cleaved at room temperature; compounds such as phenylmercuric acetate and mercaptomerin sodium containing the mercury–carbon and mercury–sulfur bond, respectively, required more drastic reaction conditions. Organomercurial compounds such as mersalyl and meralluride containing the mercury–nitrogen linkage were intermediate with respect to ease of cleavage. These observations were in agreement with the relative stabilities of such bonds discussed by Makarova and Nesmeyanov (21).

The use of hydrochloric acid to facilitate the cleavage of mercury–carbon bonds in addition to mercury in combination with oxygen or nitrogen has been described (6). The importance of bond type associated with mercury in analytical studies has also been noted (5). A study of the protolysis of the mercury–carbon bond with concentrated hydrochloric acid has been carried out (22). Based on these reported observations, hydrochloric acid was investigated for use as a protolytic solvent under varying time and temperature conditions to cleave mercury from the compounds considered in this study. Many difficulties associated with the total digestion procedures were overcome with hydrochloric acid. Moreover, total mercury could be successfully determined using hydrochloric acid for all compounds with the exception of mercaptomerin sodium and thimerosal, both of which contain the mercury–sulfur bond. The mercury present in these two compounds was effectively cleaved by a hydrochloric acid–nitric acid medium.

The results obtained for the bulk drug substances and reference compounds by both the described atomic absorption procedure and official methodology are summarized in Table I. The comparative data based on the amount of mercury found illustrate that the correlation between these two procedures is very good.

The comparative results for the analysis of the mercurial compounds in the pharmaceutical preparations by the atomic absorption and official procedures are presented in Table II. The atomic absorption procedure produced slightly different results for the product containing chlormerodrin, in which the mean percent of declared was 4.6% lower with the atomic absorption procedure.

[15] Twice this value was employed for the calculation of phenylmercuric nitrate, an equimolar combination of phenylmercuric nitrate and phenylmercuric hydroxide having an assigned molecular weight of 634.40.

The reason for this difference is not readily apparent from the limited analytical data available. In addition, mean differences of 2.6 and 3.0% of the percent of the declared amount were obtained for the products containing thimerosal and phenylmercuric acetate, respectively.

The product containing phenylmercuric nitrate, which gave a mean value of 77.4% of the declared amount by the atomic absorption procedure, could not be analyzed by adaptation of official methodology due to the low concentration of this ingredient. Additional atomic absorption analysis of this product utilizing heating condition C gave a mean value of 78.5% of the declared amount. Approximately 13 months after the initial analyses were performed, the product was reassayed by both the proposed atomic absorption method and a total digestion procedure (19). The results obtained from single determinations were 60.7 and 60.8% of the declared amount, respectively. These observations provide corroborative evidence that adsorption of this compound by the container material (polyethylene in this case) may occur over an extended period (16). A simulated preparation of the product was formulated in this laboratory and subjected to atomic absorption analysis utilizing heating condition B. The mean recovery for the phenylmercuric nitrate based on duplicate results was 99.6% with a range of 0.8%.

An orange precipitate was formed during the protolysis of the merbromin reference standard, which, upon isolation and total digestion under reflux conditions (19), revealed the absence of mercury. The product containing merbromin showed a similar precipitate during this stage of the analysis.

Recovery experiments employing heating condition B (hotplate) with mercuric chloride standards representing quantities of mercury in the range encountered with the commercial preparations yielded values between 98.7 and 100.4%. This finding indicated that loss of mercury due to volatilization was negligible.

At the sensitivity level employed in the atomic absorption procedure, the use of the background correction mode indicated the absence of interferences due to nonspecific absorption.

In general, the results obtained indicate the overall applicability of the described atomic absorption procedure or modifications thereof to the analysis of various mercurial compounds present in bulk form, as reference compounds, or in pharmaceutical mixtures. The method also should be adaptable to products containing *o*-hydroxyphenylmercuric chloride and various other preparations containing thimerosal.

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SALVATORE MARDENTE [x] and FRANCO De MARCHI

Abstract □ A specific, rapid, and sensitive GLC method for purity control of hexadiphane and its determination in pharmaceutical preparations is described. The method utilizes an extraction of the free base, followed by GLC on a 0.5% OV-17 column at isothermal temperature for 6 min and then the temperature was programmed. Results from this method and from a titrimetric method were compared, and no significant differences were found.

Keyphrases □ Hexadiphane—GLC analysis in pharmaceutical formulations □ GLC—analysis, hexadiphane in pharmaceutical formulations

Hexadiphane, 1,1-diphenyl-3-hexamethyleneiminopropane (I), is a papaverine-like compound with weak anticholinergic effects. It is widely employed as an antispasmodic (1–5).

Quantitative determination of hexadiphane is essentially based on the chemistry of the imine moiety. The methods employed are those used for basic nitrogen compounds, primarily titration in nonaqueous

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Quantitative determination of hexadiphane is essentially based on the chemistry of the imine moiety. The methods employed are those used for basic nitrogen compounds, primarily titration in nonaqueous

Table I—Assay Results of the Liquid Commercial Preparation of Hexadiphane Hydrochloride (Label Claim, 2 mg/10 ml)

Analysis Number	Titrimetric Method		GLC Method	
	Found, mg	Recovered, %	Found, mg	Recovered, %
1	2.03	101.5	2.03	101.5
2	2.01	100.5	1.98	99.0
3	2.03	101.5	1.98	99.0
4	2.04	102.0	1.99	99.5
5	2.04	102.0	2.03	101.5
6	2.05	102.5	2.04	102.0
Mean	2.033		2.008	
SD	0.0136		0.0277	
RSD, %	0.669		1.379	
$F = 4.16$	$t = 1.96$			

solvents or with anionic surfactants and determination with picric acid (trinitrophenol), silicotungstic acid, tetraphenylboron, reineckate, and acid dyes (6). Such methods offer accuracy and precision and many are listed in the current pharmacopeias. However, they lack specificity and often require long and complicated separations.

Several GLC methods have been proposed for the determination of basic nitrogen compounds. Greenwood and Guppy (7) extensively reviewed the relevant literature. The GLC technique offers greater specificity, rapidity, and simplicity than do "classical" methods, with no loss of precision or accuracy.

The need for specificity and rapidity in the hexadiphane determination is great because the compound, which is obtained by decyanation of 1,1-diphenyl-3-hexamethyleneiminobutyronitrile (II), must be checked during the reaction to make sure that a quantitative reaction has occurred.

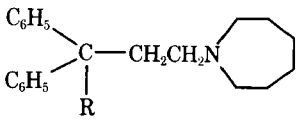
This paper describes a rapid quantitative GLC method for the determination of hexadiphane in pharmaceutical preparations. The preparations examined included a liquid form for oral use[1], a combination with oxazepam hemisuccinate in hard gelatin capsules[2], and a combination with digestive enzymes and dimethylpolysiloxane in coated tablets[3]. With the two solid forms, the analysis was carried out on specially prepared mixtures containing all components of the preparations.

The results were compared with those observed following titration with an anionic surfactant (0.01 *M* sodium lauryl sulfate).

EXPERIMENTAL

Equipment and Materials—The following were used: a gas chromatograph[4], dual column, equipped with flame-ionization detectors; a recorder[5]; a stainless steel column, 2 m × 2 mm i.d., packed with 0.5% methyl phenyl silicone (OV-17) on Gas Chrom Q, 80–100 mesh; a 10-μl syringe[6]; hexadiphane hydrochloride[7], hexadiphane maleate[7], and diphenylpyraline hydrochloride, pure to GLC analysis and at least 99.5% pure to titration in nonaqueous

[1] Commercially available.
[2] Spasmo Nulans Schiapparelli.
[3] Prandium Schiapparelli.
[4] Fractovap model D, C. Erba.
[5] Speedomax G, Leeds and Northrup Co.
[6] Hamilton.
[7] Synthesized in these laboratories.



I: R = H
II: R = CN

solvents; daily-distilled ether; carbon disulfide, analytical grade; ether-washed anhydrous sodium sulfate; 0.1 *N* hydrochloric acid; and 1 *N* sodium hydroxide.

Operating Conditions—The column was conditioned by heating at 250° for 1 hr with carrier gas flow, then at 300° for 4 hr without carrier gas flow, and finally at 250° for 18 hr with carrier gas flow.

The operational parameters were: column temperature, 190°, isothermally for 6 min and then programmed to 250° at a heating rate of 22°/min; injection port temperature, 300°; detector temperature, 280°; carrier gas, nitrogen at 35 ml/min; hydrogen, 25 ml/min; air, 0.5 kg/cm^2; and attenuation, 10 × 128.

Hexadiphane Standard Solution—About 25 mg of hexadiphane hydrochloride, accurately weighed, was dissolved in water and diluted to 100 ml in a volumetric flask.

Internal Standard Solution—About 16 mg of diphenylpyraline hydrochloride, accurately weighed, was dissolved in water and diluted to 100 ml in a volumetric flask.

Correction Factor and Calibration Curve—Two milliliters of the hexadiphane standard solution and 2 ml of the internal standard solution were pipetted into a 30-ml stoppered centrifuge tube, made basic with 1–2 drops of 1 *N* sodium hydroxide, and extracted with two portions of ether (10 and 5 ml) with shaking for 5 min. After decantation or centrifugation, as necessary, the ether solution was removed by aspiration and filtered on anhydrous sodium sulfate into a conical bottom 20-ml tube.

The ether was evaporated in a current of nitrogen, and the residue was dissolved in 0.2 ml of carbon disulfide. About 2 μl of the solution was injected into the gas chromatograph. This operation was repeated at least three times. The correction factor was calculated as follows:

$$f = \frac{H_d C_h}{H_h C_d} \qquad \text{(Eq. 1)}$$

where f = correction factor, H_d = height of the diphenylpyraline peak, H_h = height of the hexadiphane peak, C_d = diphenylpyraline hydrochloride concentration, and C_h = hexadiphane hydrochloride concentration.

The linearity of the response was checked by keeping constant the concentration of the internal standard (0.16 mg/ml) and varying the hexadiphane concentration (0.05–0.6 mg/ml). The calibration curve was made by plotting the hexadiphane/diphenylpyraline peak height ratio against the amount of hexadiphane.

Procedure—For the liquid preparation, virtually the same procedure as described for the determination of f was followed. A solution equivalent to 400 μg of hexadiphane hydrochloride and 2 ml of the internal standard solution were pipetted into a 30-ml stoppered centrifuge tube and extracted with two portions of ether (10 and 5 ml). The ether was then discarded. This extraction was

Table II—Assay Results of the Hexadiphane–Oxazepam Hemisuccinate Combination (Hexadiphane Maleate, 3.2 mg/Capsule)

Analysis Number	Titrimetric Method		GLC Method	
	Found, mg	Recovered, %	Found, mg	Recovered, %
1	3.26	101.9	3.24	101.2
2	3.22	100.6	3.12	97.5
3	3.28	102.5	3.20	100.0
4	3.22	100.6	3.16	98.7
5	3.25	101.5	3.26	101.9
6	3.24	101.2	3.19	99.7
Mean	3.245		3.195	
SD	0.0235		0.051	
RSD, %	0.724		1.596	
$F = 4.78$	$t = 2.18$			

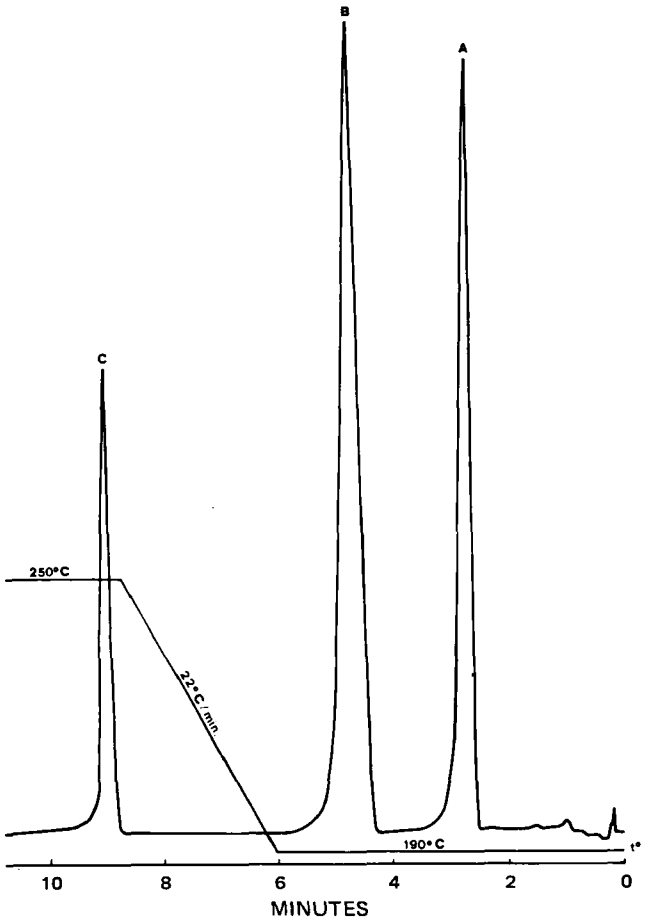


Figure 1—*Typical chromatogram. Key: A, diphenylpyraline; B, hexadiphane; and C, 1,1-diphenyl-3-hexamethyleneiminobutyronitrile.*

adopted to remove ether-soluble substances which might interfere in the analysis. After alkalinization with 1 *N* sodium hydroxide, the procedure was the same as that employed for determining *f*.

The solid products were finely pulverized. An accurately weighed quantity of powder equivalent to about 4 mg of hexadiphane hydrochloride or 6 mg of hexadiphane maleate was transferred into a 50-ml volumetric flask, and about 40 ml of 0.1 *N* hydrochloric acid was added. After heating at 70–80° and shaking for 30 min, the solution was allowed to cool. It then was brought to volume with 0.1 *N* hydrochloric acid, thoroughly mixed, and centrifuged. Five milliliters of this solution and 2 ml of the internal standard solution were pipetted into a 30-ml stoppered centrifuge tube, and the procedure described for the liquid preparation was then followed. The amount of hexadiphane hydrochloride in the sample analyzed was determined by:

$$\text{hexadiphane hydrochloride} = \frac{H_h C_d}{H_d} f \qquad \text{(Eq. 2)}$$

The value was multiplied by 1.241 to obtain the corresponding hexadiphane maleate content.

Table III—Assay Results of the Hexadiphane–Digestive Enzymes–Dimethylpolysiloxane Combination (Hexadiphane Hydrochloride, 2 mg/Tablet)

	GLC Method	
Analysis Number	Found, mg	Recovered, %
1	2.03	101.5
2	1.94	97.0
3	1.97	98.5
4	1.98	99.0
5	2.01	100.5
6	2.02	101.0
Mean	1.991	
SD	0.0375	
RSD, %	1.883	

RESULTS AND DISCUSSION

The liquid preparation and the combination with oxazepam hemisuccinate were assayed by titration with sodium lauryl sulfate and GLC. Since there was no interference from other basic nitrogen compounds, the titration was possible without additional separations.

Direct titration could not be done on the combination with digestive enzymes because of the complexity of the formulation. Only GLC analysis was performed.

The GLC results (Tables I–III) show that the method is sufficiently accurate and precise for use in the analysis of pharmaceutical preparations. Although the standard deviation of the GLC results is higher than that of the titrimetric results, the *t* test for the means and the *F* test for the variances (Tables I and II) indicate no significant difference at the 5% level.

GLC analysis, however, is preferable because of its simplicity, rapidity, and selectivity; as shown in Fig. 1, there is sharp separation of hexadiphane from its precursor II. In the absence of additional separations, the presence of this precursor would not have been revealed by titration and other chemical methods.

These features make the GLC method suitable for routine checks and those carried out during synthesis and manufacture as well as for purity checks.

Carbon disulfide was chosen as the solvent because of its relatively low response with the flame-ionization detector.

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Diffusion Model for Fluidized-Bed Drying

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Abstract □ A sucrose–lactose–starch granulation was used to study particulate motion and attrition in a fluid bed dryer. There is some classification of material in the dryer as drying proceeds; fine particles are dried faster and become less dense, and the less dry but denser large particles show some (although not great) accumulation tendencies in the lower central area. Unlike countercurrent rotary drying, fluid bed drying cannot be accounted for by water diffusion inside the granule as the rate-limiting step. In its place, a model of external water vapor diffusion is proposed and is supported by vapor–concentration curves and by the linear dependence of the rate constants on the linear air velocities. The dried granulation exhibits the same trend as does countercurrent dried material in that larger particles have higher moisture contents than do smaller particles. Quantitative relationships between content of moisture and size were developed and are supported by experimental data. The granulation, upon storage, does not equilibrate, indicating that this type of water distribution is a problem in batch process granulations as well as in the earlier reported case of granulations for continuous production.

Keyphrases □ Drying—fluid bed dryer, diffusion model, sucrose–lactose–starch granulation, rate-limiting step, relationships between moisture and size □ Diffusion—model for fluidized-bed drying, moisture and size relationships, rate-limiting step □ Fluid bed drying—diffusion model, moisture and size relationships, rate-limiting step, equations

Drying of granulations has traditionally been performed by tray drying, and the factors involved have been classified as a dynamic balance between the rate of moisture movement from inside the solid to the surface and the evaporation of moisture from the surface (1, 2). It has been shown (3) that the drying rate is controlled by the film thickness of the vapor above the bed and that there is no difference fundamentally between the heat balances involved in fluidized drying and those involved in tray drying (4). In countercurrent rotary dryers, the rate-determining step is diffusion of moisture *in* the granule (5). An investigation of the *basic* mass transfer balance in a fluidized bed at fluidizing air velocities was the primary purpose of the study reported here.

The general phenomena involved in fluid bed drying are described in various texts; the text by Vanecek *et al.* (6) will be referred to frequently. Fluidization occurs between an incipient fluidizing velocity and is limited at the higher end by an entrainment velocity beyond which conveyance takes place. The linear velocity of the air in the bed is different from that in the ducts. The velocity between the particles is referred to as interstitial velocity; the velocity in the free cross section, *i.e.*, in the ducts, is denoted as superficial velocity.

One purpose of this study was to determine whether particulate fluidization or aggregative fluidization takes place. In the former case, the bed is uniform throughout; in the latter case, it is not. The solids motion in the bed is somehow controlled by the fact that bubbles are created at the distributor. Not only the bubbles but also their wakes contribute to the solids motion (7). Bubbles do not mix in a horizontal plane, but they coalesce whenever a bubble is less than one bubble diameter behind another in a vertical path (8). Therefore, bubble size is less above the distributor than higher up in the bed.

Several "freezing" techniques were described in investigations of mixing, such as the one by Abrahami and Resnick (9). Essentially, it is shown that mixing will occur beyond the incipient fluidization velocity. The actual mixing patterns in a fluid bed dryer were described by Schugerl (10).

In a mixture of two particle sizes, it was shown that when one fraction (f_1) is abundant there is a tendency for accumulation of the coarse fraction above the distributor (11).

The temperature of the material being dried is frequently uniform throughout the bed, except for a small range immediately above the distributor (12). It is not possible to obtain a countercurrent arrangement of the streams of the material and drying medium in a fluid bed dryer (6). Therefore, one would not expect that the drying mechanism reported in countercurrent drying (5) would apply to fluid bed drying.

Although attempts to quantitate the stream lines in a fluidized bed have been made (6), no previous studies reported particle-size distributions and classifications (or lack of classifications) in fluid bed dryers. It was observed in continuous rotary drying (5) that the moisture content of various screen fractions of a continuously dried granulation differs in the sense that the coarse particles contain more moisture than the fines. It was speculated that this fact would be of no consequence in batch operations, since the particles would, presumably, equilibrate. This latter point was investigated in this study.

EXPERIMENTAL

Granulations were made of the following formula: 17.55 kg of lactose USP, 11.43 kg of sucrose (containing 3% starch), 4.20 kg of starch USP, 0.0093 kg of yellow FD&C No. 6, and 0.0045 kg of red FD&C No. 2. The lactose, sucrose, and 4.05 kg of starch were mixed for 5 min in a mixer[1] at a speed of 140 rpm. A starch suspension was made of 150 g of starch in 300 ml of water. The dyes were dissolved in 2700 ml of water and heated to boiling; then the starch suspension was added to make approximately 3 liters of dye starch

[1] Littleford-Lodige model FM-100, 3.3 ft³ capacity, Littleford Brothers, Inc., Cincinnati, OH 45202

paste. One-fourth of this paste was added to the powder blend in the mixer, and blending was carried out for 30 sec; the next one-fourth was then added and so on until all of the paste had been added.

The wet granules (5.0 kg) were screened through a No. 4 stainless steel hand screen and placed in the basket of a fluid bed dryer[2]. The fluid bed dryer was equipped with a velocity meter[3] of the Pitot-tube type with wet and dry bulb thermometers in inlet and outlet ducts. The position of the Pitot-tube was (as recommended by the manufacturer) positioned on linear portions of the duct work. The velocity could be varied by a conventional damper system. The ranges of velocity were such that no fluidization took place below 396 m (1300 ft)/min air velocity; above 823 m (2700 ft)/min air velocity, air conveyance would start. The range used was 396–815 m (1300–2675 ft)/min. The inlet temperature was 43°, and the exit temperature was 30°.

The heat capacity of the granulation was determined by calorimetric means. A Dewar flask, equipped with a rubber stopper and a Beckmann thermometer, was placed in a 20° constant-temperature bath along with 250 ml of heptane. When the temperature was equilibrated, the heptane was transferred to the Dewar flask. Then 100 g of granulation was heated to 30° by equilibrating it for 6 hr in a glass container in a 30° constant-temperature bath. It was then transferred to the Dewar flask, and the temperature increase was recorded. The calorimetric constant (80 cal/degree) was determined by water calibration. The heat capacity is 0.45 cal/g/degree of dry weight.

THEORETICAL

It will be assumed that the drying chamber is cylindrical (although in actuality it is slightly conical) with a cross section of A cm^2 and height of l cm. When fluidized, the bed has a void fraction (in the conventional sense) of ϵ and a solid fraction of $(1 - \epsilon)$. The free cross-sectional area (7) is then, on the average, $A\epsilon$. The granulation is polydisperse, containing fraction f_1 of particles with "diameter" a_1, f_2 with "diameter" a_2, and so on. The assumption is made that the particle is spherical.

In a small section of the cylinder of length dx, the total solids volume is $A\,dx(1 - \epsilon)$. The volume of particles of diameter a_i in the section is $A\,dx(1 - \epsilon)f_i$. There are, hence, $A\,dx(1 - \epsilon)f_i/(\pi a_i^3/6)$ particles of diameter a_i, each having a surface of πa_i^2, so that the total solids area in the section of length dx is then given by:

$$O = A\,dx(1 - \epsilon)\sum_{i=1}^{n} f_i \frac{6}{a_i} \qquad \text{(Eq. 1)}$$

The drying, as shown in Fig. 1, is governed by diffusion of water vapor from the solid surface (where the gaseous concentration is q^* in grams of water per cubic centimeter of dry air) to the bulk of the air stream where the water content at position x is q_x in grams of water per cubic centimeter of dry air. The interface where the water vapor concentration has just dropped to q_x is h cm from the granule surface and is referred to here as the "film." By way of Fick's first law applied to the length of this film, when a linear concentration gradient is assumed (8), the rate of moisture given off at time t and position x from the solids to the air stream is:

$$\frac{D}{h}(q^* - q_x)A(1 - \epsilon)\sum_{i=1}^{n}(f_i 6/a_i)\,dx = \gamma(q^* - q_x)A\,dx \qquad \text{(Eq. 2)}$$

where:

$$\gamma = \frac{D}{h}(1 - \epsilon)\sum_{i=1}^{n}(f_i 6/a_i) \qquad \text{(Eq. 3)}$$

and D is the diffusion coefficient for the water vapor in the gas stream.

The total moisture content of the solids is denoted m_0 and m at times zero and t sec, respectively. The moisture loss rate of the total solids mass is obtained by integrating Eq. 2 between the limits zero and l, *i.e.*:

$$-\frac{dm}{dt} = \gamma A\int_0^l (q^* - q_x)\,dx \qquad \text{(Eq. 4)}$$

If the relative humidity ($100q_x/q^*$) gradient is constant, then:

$$q_x = q_0 + \frac{q_l - q_0}{l}x \qquad \text{(Eq. 5)}$$

where $100q_l/q^* = \text{RH}$ is the relative humidity of the exit air. The average water vapor concentration, q, is:

$$q = (q_l + q_0)/2 \qquad \text{(Eq. 6)}$$

Combining Eqs. 5 and 6 with Eq. 4 yields:

$$-\frac{dm}{dt} = \gamma A\{(q^* - q_0)l - [\tfrac{1}{2}(q_l - q_0)/l]\,l^2\} =$$

$$\gamma Al[q^* - \tfrac{1}{2}(q_l + q_0)] = \gamma Al(q^* - q) \qquad \text{(Eq. 7)}$$

When the system is considered to be a suspension with a solids volume of $lA(1 - \epsilon)$ and an air volume of $lA\epsilon$, the increase in average concentration of water vapor in the gas is the amount of water given up by the solids divided by the gas volume $[-(dm/dt)/(\epsilon lA)]$ minus the amount lost in the gas stream, *i.e.*, the difference between incoming and outgoing moisture quantities[4] $[vA\epsilon(q_l - q_0)]$ divided by the gas volume, *i.e.*:

$$\frac{dq}{dt} = \frac{1}{\epsilon lA}\frac{dm}{dt} - \frac{vA\epsilon}{\epsilon lA}(q_l - q_0) \qquad \text{(Eq. 8)}$$

where v is the linear velocity of the air. Inserting Eqs. 7 and 6 into Eq. 8 now yields:

$$\frac{dq}{dt} = \frac{\gamma}{\epsilon}(q^* - q) - \frac{2v}{l}(q_l - q_0) = \alpha(q - \beta q^* - \sigma q_0) \qquad \text{(Eq. 9a)}$$

where:

$$\alpha = -\left(\frac{\gamma}{\epsilon} + \frac{2v}{l}\right) < 0 \qquad \text{(Eq. 9b)}$$

$$\beta = \frac{(\gamma/\epsilon)}{(\gamma/\epsilon) + (2v/l)} \qquad \text{(Eq. 9c)}$$

$$\sigma = \frac{(2v/l)}{(\gamma/\epsilon) + (2v/l)} \qquad \text{(Eq. 9d)}$$

It is noted that $\alpha + \sigma = 1$. Integration of Eq. 9a yields:

$$\ln(q - \beta q^* - \sigma q_0) = \alpha t + I \qquad \text{(Eq. 10)}$$

where I is an integration constant to be determined by initial conditions. Boundary conditions dictate that when t becomes infinite, the term under the logarithm sign approaches zero; *i.e.*, since q then approaches q_0:

$$q_0 - \beta q^* - \sigma q_0 = 0 \qquad \beta = (1 - \sigma)q_0/q^* \qquad \text{(Eq. 11a)}$$

Inserting Eq. 11a into Eq. 10 then gives:

$$\ln(q - q_0) = \alpha t + I \qquad \text{(Eq. 11b)}$$

where I is an integration constant. The value of I can be obtained from initial conditions; here the inlet air has a moisture content of q_0 and a (saturated) content of q^* at the outlet, so that initially $q = (q_0 + q^*)/2$, which, inserted in Eq. 11b, yields:

$$\ln[(q_0 + q^*)/2 - q_0] = 0 + I \qquad \text{(Eq. 11c)}$$

so that $I = \ln[(q^* - q_0)/2]$. The complete equation may be written:

$$\ln[(q_l - q_0)/2 - q_0)] = \alpha t + \ln[(q^* - q_0)/2] \qquad \text{(Eq. 12a)}$$

or:

$$\log(\text{RH} - \text{RH}_0) = (\alpha/2.3)t + \log(100 - \text{RH}_0) \qquad \text{(Eq. 12b)}$$

where $\alpha < 0$. Data plotted according to Eq. 12b (Fig. 2) show that this equation is obeyed at all air velocities tested. It is noted that:

$$-\alpha = \frac{\gamma}{\epsilon} + \frac{2v}{l} \qquad \text{(Eq. 13)}$$

so that the negative slopes in Fig. 2 multiplied by 2.3 should be linearly related to the linear velocity, as demonstrated in Fig. 3.

[2] Aeromatic AG model STR-5, Aeromatic Ltd., Basel, Switzerland.
[3] Catalog No. 102AV, Dwyer Instruments Inc., Michigan City, IN 46360

[4] It is noted that q_0 and RH_0 are equal to the values in the outlet at infinite time.

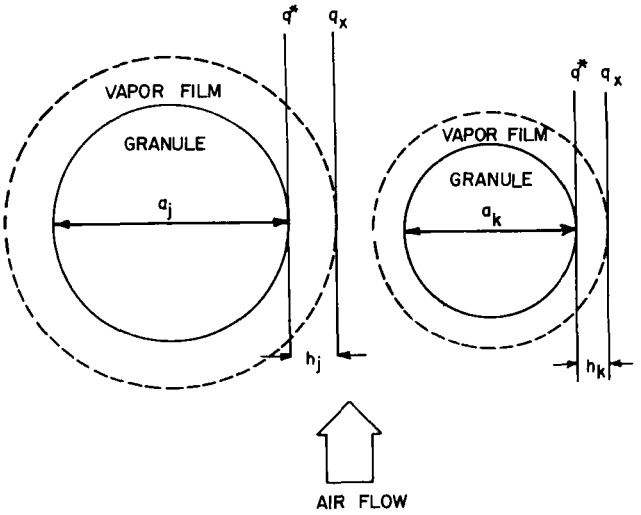

Figure 1—*Particles of diameter* a_j *and* a_k *and film thicknesses* h_j *and* h_k.

If it is assumed that all sieve fractions start with the same moisture content and level off at about 0.04 g of water/g of dry solid, then the moisture loss rates may be estimated from Table I. In particular, at any given time, according to Eqs. 3 and 7:

$$(dm_i/dt)_{t=t'} = D(1-\epsilon)(6f_i/a_ih_i)Al(q^* - q_{t'}) \quad \text{(Eq. 14)}$$

Data from $t = 1.25$ min calculated from Table I are shown graphically in Fig. 4.

According to hydrodynamic theory (8), diffusion in turbulent flow occurs through a film of thickness h, which is related to the Reynolds number (Re) by an exponential relation, *i.e.*, $h = (\omega)Re^{-(\zeta-1)}$. Here, $Re = v(a/\nu)$, where v is the velocity of the stream flowing past the body, a is the characteristic dimension of the body, and ν is the kinematic viscosity of the stream. Therefore, it follows that Eq. 14 will take the form:

$$\log[-(dm_i/dt)_{t=t'}] = (\zeta - 2)\log a_i + \log\left[D(1-\epsilon)(6f_i)Al(q^* - q_{t'})\left(\frac{1}{\omega}\right)\right] + (\zeta - 1)\log(v/\nu) \quad \text{(Eq. 15)}$$

Figure 4 shows that Eq. 15 is obeyed, the value of $(\zeta - 2)$ being -0.40. Hence, $\zeta = 1.6$ and $h = (\omega)Re^{-0.6}$. This result is close to the usual hydrodynamic postulate that the sorbed "film" of fluid (or gas) is inversely proportional to the square root of the velocity (13). This should not be construed as implying quantitative proof of the proposed model, but the adherence to Eq. 15 lends credence to the proposed view.

RESULTS AND DISCUSSION

The granulation from a 15-min drying experiment, conducted in the manner described, was sampled from the top (2–3 cm deep) in a zone at the center in a circle with radius 7.6 cm (3 in.), then in a zone 7.6–15.4 cm (3–6 in.) from the center, and then in a zone more than 15.4 cm (6 in.) from the center. The samples were subjected to a sieve analysis through a nest of standard U.S. screens, and the various screen fractions were subjected to a Karl Fischer moisture analysis (Table II).

The screen analysis is fairly independent of the zone, and the moisture analysis shows that the coarse material is somewhat moister than the fine material. Apparently, there is a slight tendency for a larger proportion of the coarse fraction to be positioned at the center of the distributor; this so-called immobile zone has a minimal effect on the general particle distribution. Initially, the wet granules (prior to drying) contain 97.5% (w/w) of granules coarser than 60 mesh; these granules contain 9.5% moisture. The 2.5% material finer than 60 mesh is slightly drier (6.4%).

The next experiment utilized a 5-min drying time. The dried granulation was mixed in a large plastic bag, and the material was subjected to sieve analysis. The experiment was then repeated for 15, 30, and 45 min (Table III). Table III shows that the particle-size distribution stays fairly constant throughout the drying and that the formula is resistant to the possible abrasion in the fluidizing motion.

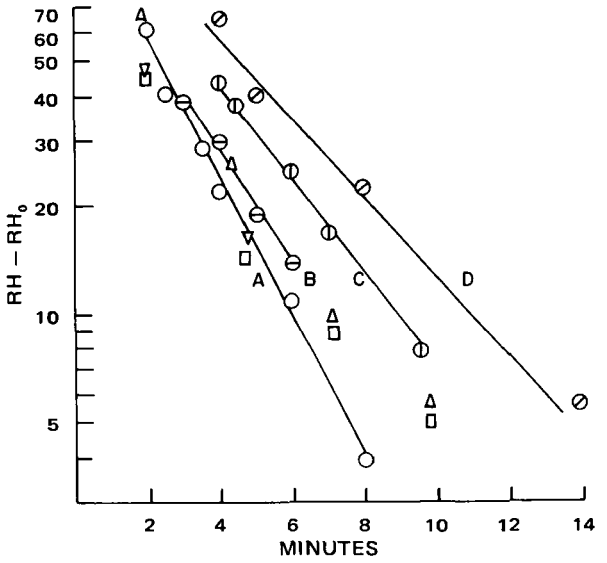

Figure 2—*Relative humidity of outlet air minus relative humidity of ambient air* ($RH - RH_0$) *plotted in semilogarithmic fashion versus time. Least-squares fit slopes are: A (470 cm/sec),* -0.186 min^{-1}; *B (460 cm/sec),* -0.155 min^{-1}; *C (407 cm/sec),* -0.130 min^{-1}; *and D (274 cm/sec),* -0.114 min^{-1}. *Key:* △, *37° (460 cm/sec);* ▽, *30° (460 cm/sec); and* □, *25° (460 cm/sec).*

Moisture assays by Karl Fischer titration were performed on various fractions from the experiments (Table I). Table I illustrates the increased drying time necessary to bring the larger granules to moisture levels comparable to those achieved rapidly by the smaller graules. A sample was dried for 2.5 min and blended and screened as described. Samples of the various fractions were assayed for moisture; the remainder of the batch was reblended and placed in a plastic bag in a sealed fiber drum for 2 days. Screening, sampling, assay, blending, and restorage were repeated twice. Inspection of Table IV shows that equilibration of the tested formula does *not* occur subsequent to drying and that the previous observation (5) that the uneven distribution of moisture could be a problem in continuous processes is apparently also true for batch processing.

A series of runs was then performed in which the linear velocity of the air was varied; the temperatures of the incoming air and the temperatures of the exit air were monitored to permit calculation of the relative humidities of inlet and outlet streams at all times (Fig. 2).

The process that dominates continuous rotary drying, as found by Pitkin and Carstensen (5), dictates (as detailed in the *Appen-*

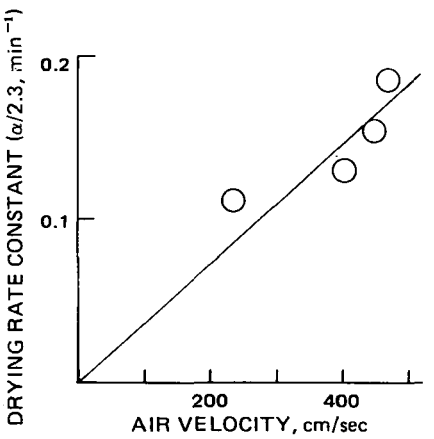

Figure 3—*Negative slopes from Fig. 2 plotted versus linear velocities.*

Table I—Karl Fischer Moisture Contents of Various Mesh Cuts as a Function of Drying Time

	20 Mesh		40 Mesh		60 Mesh		80 Mesh		100 Mesh		Pan	
Minutes	K.F.[a]	D.B.[b]	K.F.[a]	D.B.[b]	K.F.[a]	D.B.[b]	K.F.[a]	D.B.[b]	K.F.[a]	D.B.[b]	K.F.[a]	D.B.[b]
0	8.52	9.31	8.52	9.31	8.52	9.31	8.52	9.31	8.52	9.31	8.53	9.31
2.5	8.50	9.29	7.20	7.76	6.25	6.67	5.45	5.76	4.63	4.85	4.03	4.20
5	8.27	9.02	6.53	6.98	5.84	6.21	4.92	5.17	4.29	4.48	4.18	4.36
15	5.64	5.98	4.67	4.90	4.37	4.57	4.00	4.21	3.94	4.10	3.86	4.01
30	5.26	5.55	4.15	4.33	4.05	4.22	3.88	4.04	3.94	4.10	3.85	4.00
45	5.15	5.43	4.11	4.29	4.05	4.22	4.15	4.33	4.15	4.33	3.89	4.05

[a] K.F. = Karl Fischer moisture value. [b] D.B. = 100 × g of moisture/g of dry weight.

Table II—Particle-Size Distribution in the Top Zones of the Basket and Moisture Contents of Granulation Dried in a Fluid Bed Drier

	Percent on Screen			Moisture Content, %		
Screen Fraction	0–7.6 cm[a]	7.6–15.4 cm	>15.4 cm	0–7.6 cm	7.6–15.4 cm	>15.4 cm
+20	13	12	8	4.5	4.8	4.8
−20/+40	4	4	6	4.3	4.1	4.0
−40/+60	19	21	21	3.8	3.9	3.9
−60/+80	29	30	30	3.8	3.9	3.8
−80/+100	14	15	15	3.8	3.9	3.8
−100/pan	21	18	21	3.8	3.9	3.9

[a] Zone of basket.

dix) that the drying curve be of the form:

$$\log(RH - RH_0) = (\alpha'/2.3)t + \beta' \qquad \text{(Eq. 16)}$$

where RH is the relative humidity in the outlet air, RH_0 is the relative humidity of the inlet air, and α' is independent of air velocity. As seen in Fig. 3, α (Eq. 12*b*) *is* dependent on air velocity and the rate-limiting step here cannot be moisture movement within the granule.

SUMMARY

Fluid bed drying is governed by external diffusion because:

1. Equation 12*b* predicts a found dependency on interstitial velocity but no temperature dependence. The lack of temperature dependence is shown in Fig. 2.
2. Data demonstrate a dependence on particle size predicted by external diffusion (Eq. 15) but not internal diffusion (Eq. A7).
3. The dependence of drying on velocity is identical to that expected from hydrodynamic film theory.

With dried granules, the larger the moisture content the larger is the granule. The granules do *not* equilibrate on storage.

APPENDIX

If, as shown schematically in Fig. 1, the air flow is Q cm³ of dry air/sec and the dry bulb temperature, T, of the stream is constant, and if the air contains, on entry, q_0 g of water/cm³ of dry air, then the relative humidity (RH) of the incoming air will be $(q_0/q^*)100$, where q^* denotes the saturation amount of moisture per cubic centimeter of dry air at $T_0°$. The air mass "gains" $-dm/dt$ g of water/sec, where m is the moisture content of the granulation and where m_0 is the initial moisture content of the granulation. In time element dt, therefore, $Q\,dt$ cm³ of air gain $(-dm)$ g of water, so that, at time t:

$$RH/100 = [q_0 - (dm/Q\,dt)]/q^* = (RH_0/100) - (1/Qq^*)\,dm/dt \qquad \text{(Eq. A1)}$$

Pitkin and Carstensen (5) showed that for a monodisperse fraction of a granulation:

$$m = m_0(6/\pi^2)\sum_{j=1}^{\infty}(1/j^2)\exp(-j^2t/k) \qquad \text{(Eq. A2)}$$

where $k = a^2/(4\pi^2D)$.

As before, if there is a fraction f_1 of particles with diameter a_1, f_2 with diameter a_2, *etc.*, then:

$$m = \sum_{k=1}^{\infty}\sum_{j=1}^{\infty} f_k m_0(6/\pi^2)(1/j^2)\exp[(j^2 4\pi^2 Dt)/a_k{}^2] \qquad \text{(Eq. A3)}$$

Differentiating yields:

$$-dm/dt = -m_0(6/\pi^2)\sum_{k=1}^{\infty}\sum_{j=1}^{\infty} f_k 4\pi^2 D/(a_k{}^2) \times \exp(-j^2 4\pi^2\, Dt/a_k{}^2) \qquad \text{(Eq. A4)}$$

This equation, inserted in Eq. A1, yields:

$$(RH - RH_0)/100 = (m_0/Qq^*)\sum_{k=1}^{\infty}\sum_{j=1}^{\infty} f_k 24D/(a_k{}^2) \times \exp(-j^2\pi^2\, Dt/a_k{}^2) \qquad \text{(Eq. A5)}$$

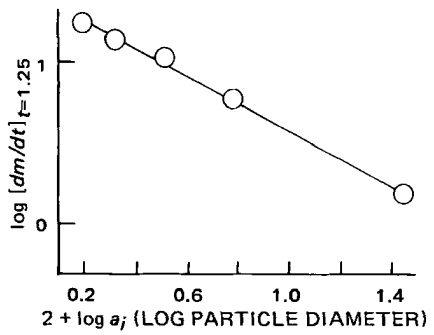

Figure 4—*Negative slope estimated from Table I at time* t = *1.25 min, plotted logarithmically* versus *the logarithm of the mean diameter of the screen cut.*

Table III—Particle-Size Distribution *versus* Time of Drying

Screen Fraction	5 min	15 min	30 min	45 min
+20	67, 60	59, 60	60, 60	58, 59
−20/+40	17, 18	16, 16	17, 17	18, 17
−40/+60	10, 13	14, 14	14, 14	15, 14
−60/+80	3, 4	5, 5	4, 4	5, 5
−80/+100	1, 2	2, 2	1.5, 1.5	1.5, 1.5
−100/pan	2, 3	4, 3	3.5, 3.5	3.5, 3.5

Table IV—Screen Analysis and Moisture Contents of Drum-Stored Granulation

Screen Fraction	Percent (w/w)				Moisture Content, %			
	Initial	2 Days	3 Days	5 Days	Initial	2 Days	3 Days	5 Days
+20	59	57	57	57	8.9	8.5	8.4	8.2
−20/+40	12	13	12	12	6.9	7.7	6.9	7.4
−40/+60	10	15	13	19	6.4	6.6	5.7	6.6
−60/+80	7	10	12	9	5.4	5.6	5.8	5.8
−80/+100	4	3	4	1	5.0	4.1	4.4	4.0
−100/pan	8	2	2	2	4.5	3.6	4.1	3.9

If there is a predominance of a fine fraction (size a_1, fraction f_1) where $j = 1$ and $k = 1$, then at reasonably high t Eq. A5 has one predominant term. It then simplifies to:

$$(RH - RH_0)/100 = (f_1 m_0/Qq^*)(24D/a_1^2) \exp(-4\pi^2 Dt/a_1^2) \quad \text{(Eq. A6)}$$

and hence:

$$\log(RH - RH_0) = -[4\pi^2 D/(2.3a_1^2)]t + \log(100C_0 24D)/(Qq^* a_1^2) f_1 \quad \text{(Eq. A7)}$$

RH_0 is the relative humidity of the incoming air (*not* RH at time zero). According to Eq. A7, $t \to \infty$ implies that $RH \to RH_0$ in conformity with the physical picture presented. Hence, at conditions *other* than times strictly close to time zero, Eq. A7 predicts that the log of the term $(RH - RH_0)$ is linear with time. Figure 2 shows this to be so. However, the slope in Eq. A7 is independent of air velocity.

The efficiency of the dryer is calculated conventionally (6) as follows. Five kilograms of 10.22% initial moisture and 4% final moisture loses 511 − 187 = 324 g water, so that 324 × 0.582 = 199 kcal is employed for evaporation. The bed in the process is heated 5°, *i.e.*, 0.45 × 4.489 × 5 = 10 kcal is used. Drying time is 15 min. The amount of heat given up is enthalpy of air at entry minus enthalpy at exit. The entry air is 43°, 10% RH; the exit air is 30° and the RH (Fig. 2) is estimated as follows. From Fig. 2 (slope A), $RH(t)$ from 0 to 15 min was measured and found to be 516 RH-minutes so that the average RH is:

$$RH = \frac{1}{15}\int_0^{15} RH(t)\,dt = \frac{516}{15} = 35\%\ RH \quad \text{(Eq. A8)}$$

The enthalpy of the outgoing air is found from a psychrometric chart to be 13 + 0.35(43 − 13) = 23.5 Btu/lb dry air, and the enthalpy of the incoming air is 18 + 0.10(82 − 18) = 24.4 Btu/lb dry air so the difference is 0.9 Btu/lb dry air. One pound of dry air at 110°F is found from the psychrometric chart to occupy 14.5 ft^3, so the enthalpy difference is 0.9/14.5 = 0.062 Btu/ft^3. The superficial air flow is 2500 ft/min through a 0.166-ft^2 duct for 115 min; *i.e.*, 2500 × 0.166 × 15 = 6225 ft^3. Hence, the heat expended is 6225 × 0.062 = 386 Btu = 386 × 0.817 = 315 kcal, so the efficiency of the dryer is (199 + 10) 0.100/315 = 64%.

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Spectrofluorometric Determination of the Antibiotic Lasalocid in Blood

M. A. BROOKS *x, L. D'ARCONTE *, J. A. F. de SILVA *, G. CHEN ‡, and C. CROWLEY ‡

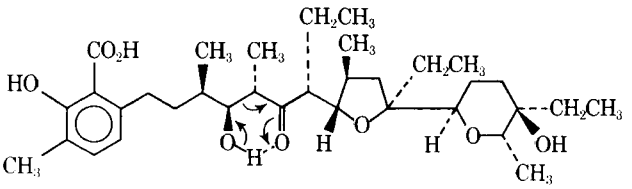
Abstract □ A spectrofluorometric assay was developed for the determination of the antibiotic lasalocid in dog blood, based on the intrinsic fluorescence of the compound in ethyl acetate. The assay can measure "total" levels of drug and any metabolites present. The specificity of the assay was verified by TLC separation of the dog blood extract, which indicated the presence of only intact drug. The overall recovery (±*SD*) of lasalocid was 62.0 ± 3.6% in the concentration range of 1.0–10 μg of compound/ml of dog blood. The sensitivity of the assay is 0.5 μg/ml. The assay was applied to the determination of blood levels of lasalocid in the dog following the intravenous administration of a 5-mg/kg dose.

Keyphrases □ Lasalocid—spectrophotofluorometric analysis in blood after intravenous administration, dogs □ Spectrophotofluorometry—analysis, lasalocid in dog blood

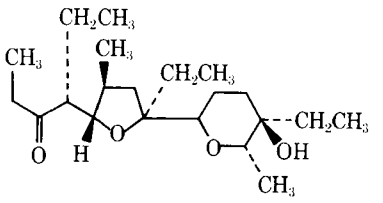
The isolation of the antibiotic lasalocid[1] (I) was first reported in 1951 (1), and it is presently under investigation as an anticoccidial agent in chicks (2). Studies pertaining to the structure elucidation (3–5) and the biosynthesis (6–8) of the compound were reported. Other investigations have dealt with chemical transformations and their effects on antibacterial activity (9), nitration (10), and pyrolytic cleavage (11).

Analytical investigations of lasalocid include GLC determination of the compound in fermentation broths (12), in which the compound is pyrolyzed in the injection port to two naphthols (III[2] and IV[3]) and a retroaldol ketone (II[4]). The ketone is used for quantitation employing a flame-ionization detector. Bioautographical procedures also were reported for the measurement of the antibiotic in tissues of chickens following administration at the 0.0075% level (13).

The compound shows strong optical activity in the presence of several amines and monovalent and divalent metals (14, 15). This activity was reported in terms of circular dichroic absorption (14, 15) and fluorescence emission (15) of the complexed compound. A spectrofluorometric assay to measure lasalocid in finished feeds and premixes also was reported (16). The assay involves extraction of the drug into ethyl acetate from the premixes and finished feeds, followed by a differential spectrofluorometric measurement of lasalocid.



I



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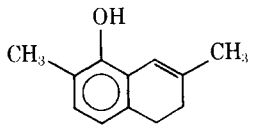
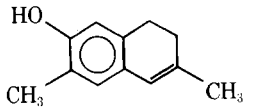


III



IV

The present work employs several modifications of the previously reported spectrofluorometric assay (16) in order to measure lasalocid in blood. The assay is based on the intrinsic fluorescence of the compound in ethyl acetate and has a sensitivity limit of 500 ng/ml. The assay measures "total" levels of the drug and any metabolites present. The specificity of the assay was verified by TLC separation of the dog blood extract, which indicated the presence of only intact drug. The method was applied to the determination of blood levels of the drug in the dog following intravenous administration of a 5-mg/kg dose.

EXPERIMENTAL

Instrument—A spectrofluorophotometer[5] equipped with a selected high energy output dc xenon arc energy source and a high sensitivity (>10 lumen rating) photomultiplier[6] was used. The 10-nm slit arrangement was used in all four positions, and the instrument was adjusted for maximum energy output using a Pyrex reference rod. Spectra were obtained on an *X–Y* recorder[7].

Standard Solutions—Prepare a stock solution (Solution A) of lasalocid (I) ($C_{34}H_{53}NaO_8$, mol. wt. = 612.78, pKa 5.13), by dissolving 10 mg of I in 100 ml of methanol. Make a 1:10 dilution of this

[1] 6-[7(*R*)-[5(*S*)- Ethyl-5-[5(*R*)-ethyltetrahydro-5-hydroxy-6(*S*)- methyl-2*H*- pyran-2(*R*)-yl]tetrahydro -3(*S*)-methyl- 2(*S*)- furanyl]- 4(*S*)-hydroxy-3(*R*),5(*S*)-dimethyl-6-oxononyl]-2-hydroxy-3-methylbenzoic acid. Lasalocid is also designated as X-537A in the literature.

[2] 5,6-Dihydro-2,7-dimethyl-1-naphthol.

[3] 7,8-Dihydro-3,6-dimethyl-2-naphthol.

[4] 4-[5-Ethyl-3-methyl-5-(ethyl-5-hydroxy-6-methyl-2-tetrahydropyranyl)-2-tetrahydrofuryl]-3-hexanone.

[5] Model Mark I, Farrand Optical Co., Mount Vernon, N.Y.

[6] RCA 1P 28 or equivalent photomultiplier tube.

[7] Model 2200-3-3, Houston Instruments, Bellaire, Tex.

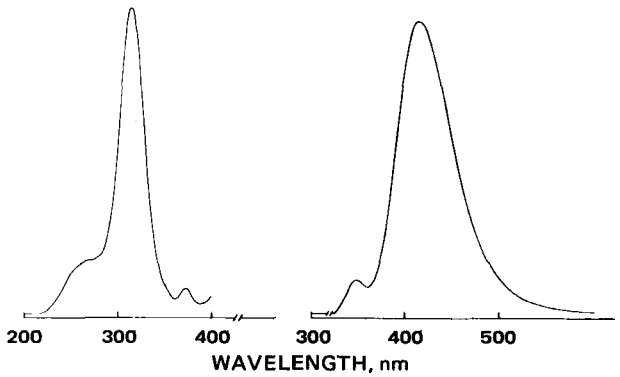


Figure 1—*Excitation–emission spectra of 1 μg of lasalocid in 5 ml of ethyl acetate. Left: excitation (λ_{max} 310 nm). Right: emission (λ_{max} 415 nm). Attenuation = 3×.*

stock solution in methanol to yield a working solution (Solution B) containing 10 μg/ml; suitable aliquots of Solution B are added to blood as internal standards.

Reagents—The reagents used were of analytical reagent grade (ACS) purity and included saturated tribasic sodium phosphate (Na_3PO_4) (pH 12.3), ethanol–0.1 *N* NaOH (80:20, spray reagent), anhydrous ether[8], benzene[9], methanol[9], and ethyl acetate[10].

Analysis in Blood—Into a 50-ml centrifuge tube, add 1 ml of sample and then mix[11] for about 5 sec. Add 1 ml of ethanol, stopper, and mix well again for about 5 sec, making sure that all of the sample has been in contact with the alcohol; then place in a water bath at about 70° for 5 min for protein precipitation.

Allow the samples to cool to room temperature. Add 5 ml of saturated tribasic sodium phosphate solution (pH 12.3), and mix[11] well to ensure that the coagulated sample is uniformly suspended in the mixture. Add 20 ml of ether, mix again for 5 sec, and place in a reciprocating shaker for 20 min. Centrifuge at 2400–2600 rpm for about 5 min.

Transfer an 18-ml aliquot of the ether extract into a clean 50-ml tube and evaporate to dryness. Reconstitute by the addition of 200 μl of ether, stopper, and mix[11] well. To the ether solution, add 10 ml of ethyl acetate (spectral grade); then mix well and measure the fluorescence of the solution in a 1-cm path quartz cell at 415 nm with excitation at 310 nm. Along with the unknowns, run internal standards of 0.5, 1.0, 2.0, 5.0, and 10.0 μg of I (0.05, 0.10, 0.20, 0.50, and 1.0 ml of Solution B, respectively, evaporated to dryness under nitrogen) in blood taken preferably from the same dog prior to medication or from a pooled control source.

TLC Analysis of Blood Extracts—The specificity of the fluorometric assay in blood can be verified as follows. Following fluorometric analysis, evaporate the ethyl acetate solution to dryness and dissolve the residue in 100 μl of ether. Transfer the extract quantitatively onto a 20 × 20-cm 60-μm silica gel chromatoplate[12]. Rinse each tube with an additional 50 μl of ether and transfer to the TLC plate to effect a quantitative transfer.

Develop the plate for 15 cm ascending in a vapor-saturated tank using benzene–methanol (90:10). Air dry the plate, spray it lightly with a mixture of ethanol–0.1 *N* sodium hydroxide (80:20), and heat in an oven at 100° for 15 min. The parent drug (I, $R_f \simeq 0.32$) is identified by reference to the R_f of 10 μg of authentic standard run alongside the sample extract as a blue fluorescent spot seen under short- and longwave UV irradiation characteristic of salicylate-like fluorescence. These zones turn yellow upon standing and exposure to the atmosphere.

Calculations—All fluorescence readings [(*TM*) = transmittance (*T*) × meter multiplier factor (*M*)] are corrected for blank readings and for any dilutions made. Unknown concentrations are interpolated from the internal standard curve or from direct comparison with one of the internal standards. The percent recovery of the assay is calculated based on the comparison of (*TM*/μg/ml) values of the internal and external standards (corrected for aliquots taken).

The percentage recovery should be calculated routinely as a check on analytical precision and reproducibility.

Recovery and Sensitivity Limits—The assay has an overall recovery (±*SD*) of 62.0 ± 3.6% in the range of 1.0–10 μg of I/ml of dog blood. The sensitivity limit of the assay is 0.5 μg of I/ml of blood. At the 0.5-μg/ml level, the recovery is approximately 15% lower than that in the 1.0–10-μg/ml range (Table I).

Table I—Recovery of Lasalocid from Dog Blood

Amount of Lasalocid Added to 1.0 ml of Control Blood, μg	Amount Recovered, μg	Recovery[a], %
0		
0		
0.5	0.20	44.4 } 44.4
0.5	0.20	44.4 }
1.0	0.56	62.2 } 62.2
1.0	0.56	62.2 }
2.0	1.18	65.4 } 65.5
2.0	1.18	65.4 }
5.0	3.12	69.3 } 69.5
5.0	3.14	69.7 }
10.0	5.82	64.6 } 68.4
10.0	6.50	72.2 }
		62.0 ± 3.6% (*SD*)[b]

[a] Corrected for dilution factor of 1.11. [b] Excluding the 0.5-μg samples.

RESULTS AND DISCUSSION

The UV absorption spectrum of lasalocid shows a major band at 305 nm with an absorbance (*A*/μg/ml) ≃0.008 in ethyl acetate, indicating a potential sensitivity limit of about 10–20 μg/ml of blood. Because of this poor sensitivity, a spectrophotometric assay was not attempted and the development of a spectrofluorometric assay based on a reported method (16) was undertaken.

The compound exhibits moderate fluorescence emission at 415 nm (*TM*/μg/ml = 498) in ethyl acetate with an excitation maximum at 310 nm (Fig. 1). The fluorescence of the compound is linear with concentration over the range of 0.04–20 μg of I/ml of ethyl

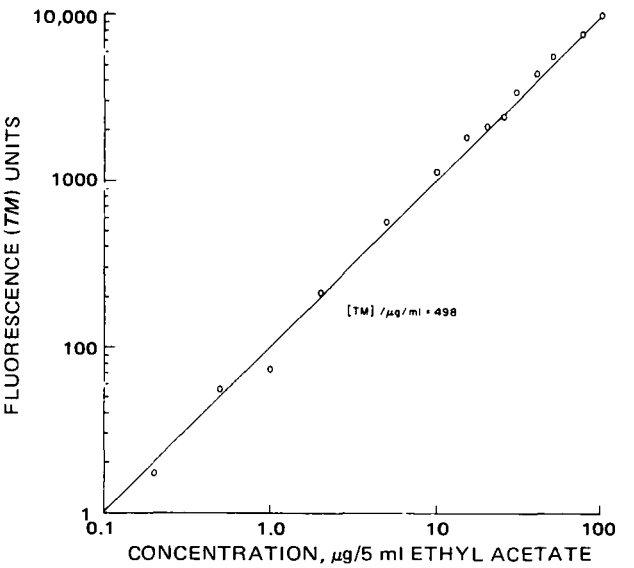


Figure 2—*Linear dynamic range of fluorescence* versus *concentration of lasalocid in ethyl acetate.*

[8] Mallinckrodt, St. Louis, MO 63160
[9] Fisher, Springfield, NJ 07081
[10] Spectroquality grade, Matheson, Coleman and Bell, East Rutherford, NJ 07073
[11] Vortex action supermixer, Catalog No. 1290, Lab Line Instruments, Melrose Park, Ill.
[12] Chromatoplates were manufactured by E. Merck (F_{254}), Darmstadt, Germany, and marketed by Brinkmann & Co., Westbury, NY.

Table II—Blood Levels of Lasalocid in a Dog following a 5-mg/kg iv Dose

Hours	Micrograms per Milliliter of Blood	
	Spectrofluorometric	Bioautography
0.0083	15.8	14.1
0.25	8.8	11.8
0.50	7.8	7.3
1	5.8	5.1
3	3.9	4.9
5	2.7	3.8
24	0.4	0.4

acetate (Fig. 2). The overall sensitivity limit of the assay, 0.5 μg of I/ml of sample, is restricted by interferences from the sample extract of the dog blood.

The ether residues of the sample extracts contain a yellow-colored coextracted biological impurity, which necessitates the addition of 200 μl of ether to effect its solution in ethyl acetate. This impurity imparts a distinct yellow color to the ethyl acetate solution used for spectrofluorometric measurement and produces fluorescence quenching to the extent of about 10%. Therefore, the samples and internal standards must be measured in the same volume of ethyl acetate.

Back-extraction of the samples, using a variety of acidic and basic aqueous solutions to effect better cleanup, was unsuccessful. Contact with solutions below pH 11 resulted in a shift of the excitation and emission maxima to 320 and 370 nm, respectively, with a corresponding decrease in overall fluorescence yield. Washing with strongly alkaline solutions also decreased the overall sensitivity.

Application of the pyrolysis flame-ionization GLC (12) was not successful due to the presence of many coextracted impurities that interfered with the measurement of the analytical retroaldol ketone (II) peak.

The spectrofluorometric assay presented is more rapid than the bioautographical assay (13), but it is not as sensitive. In the bioautographical assay, larger volumes of samples can be extracted. This same assay procedure is applicable to urine samples with recoveries ($\pm SD$) of 80.5 ± 4.2% in the concentration range of 1.0–10.0 μg/ml.

APPLICATIONS

Blood levels were determined in a male dog by the spectrofluorometric assay following intravenous administration of 5 mg of lasalocid/kg (Table II). A peak blood level of 15.8 μg of I/ml was obtained at 5 min and declined to 0.4 μg of I/ml at 24 hr.

These same samples were also analyzed by the bioautographical assay (13), and satisfactory correlation was found between the two assays. Examination of the ethyl acetate solutions by TLC following fluorometric analysis did not reveal the presence of other fluorescent zones due to possible metabolites (after spraying with ethanol–sodium hydroxide) that may interfere with the specificity or accuracy of the fluorometric assay. This finding does not, however, preclude the presence of nonfluorescent UV-absorbing metabolites that are extractable under the same conditions.

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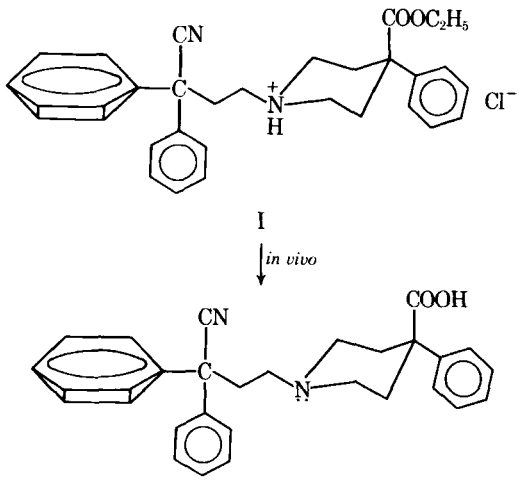
In Vitro Adsorption of Diphenoxylate Hydrochloride on Activated Charcoal and Its Relationship to Pharmacological Effects of Drug *In Vivo* I

DILIP R. SANVORDEKER ˣ **and ESAM Z. DAJANI**

Abstract □ The adsorption of diphenoxylate hydrochloride, a potent antidiarrheal agent, on activated charcoal powder was studied *in vitro*. Langmuir adsorption isotherms were established at pH 4 and 7, and the maximum adsorption capacity of charcoal for this drug was estimated using these values. Activated charcoal modified the bioavailability of diphenoxylate hydrochloride *in vivo*. The antipropulsive action of diphenoxylate in the mouse was strongly inhibited in the presence of activated charcoal. A comparative evaluation of charcoal and chromium oxide used as inert, nonabsorbable markers revealed that chromium oxide may be the marker of choice in GI transit studies in laboratory animals since it does not influence the bioavailability of diphenoxylate hydrochloride.

Keyphrases □ Diphenoxylate hydrochloride—adsorption on activated charcoal and chromium oxide, effect on bioavailability □ Bioavailability—diphenoxylate hydrochloride, effect of activated charcoal and chromium oxide □ Adsorption—diphenoxylate hydrochloride on charcoal and chromium oxide, pH 4 and 7 Langmuir isotherms

It has been established that the presence of a solid adsorbent, such as activated charcoal, interferes with the drug absorption process, resulting in a decrease in bioavailability of some drugs (1–5). Such an interference in the systemic availability of a drug is brought about by its adsorption on the activated surface of the solid adsorbent, thus preventing the adsorbed fraction of the drug from permeating through the GI mucosa into the bloodstream. In general, under *in vitro* experimental conditions the drug adsorption phenomenon exhibits Langmuir adsorption isotherm characteristics (4–8). Indeed, this principle of surface adsorption has found clinical usefulness in the management of acute toxicity in drug overdosing of pediatric as well as adult patients (9).



Scheme I

Diphenoxylate hydrochloride (I), a potent antidiarrheal agent, has been shown to be clinically effective for the management of severe diarrhea (10). In pediatric use, however, there is a persistent concern about the overdosage of this drug given in combination with atropine sulfate (11, 12). One aspect of this problem is the rapid absorption of the drug as manifested by possible enterohepatic circulation of diphenoxylic acid (II), the major metabolite (Scheme I), from the lower intestinal tract (13).

It was of interest, therefore, to explore the possibility of altering the *in vitro* availability of diphenoxylate hydrochloride (I) in the presence of activated charcoal powder and to assess its relationship to the pharmacological activity of this drug *in vivo*. This report presents the results of these investigations and their clinical implications in the management of overdosage problems related to this drug.

EXPERIMENTAL

Materials—All reagents used were analytical grade. Diphenoxylate hydrochloride[1] was tested for its structure identity and purity prior to use. Activated charcoal powder[2], chromium oxide[3], methylcellulose[4], and absolute alcohol[5] were used as supplied.

Methods—All *in vitro* adsorption studies were conducted at 25°. Studies with activated charcoal were conducted at pH 2, 4, and 7 with a 0.2% solution of diphenoxylate hydrochloride in hydroalcoholic mixtures (50:50 v/v). Accurately weighed amounts of activated charcoal powder, 0.02–1 g, were transferred to 25-ml test tubes with screw caps. After 25 ml of a 0.2% solution of diphenoxylate hydrochloride was added to each test tube, the charcoal suspensions were continuously mixed[6] for 20 hr. The suspensions were then rapidly centrifuged, and the clear supernatant liquid was assayed spectrophotometrically at 258 nm[7].

Appropriate control solutions were also run and assayed to ensure control over experimental conditions. The amount of diphenoxylate hydrochloride adsorbed on the charcoal was determined on the basis of a previously established standard curve. Studies with chromium oxide were conducted in a similar manner with the adsorbent at 0.5 and 1.0 g in suspension with a 0.2% solution of diphenoxylate hydrochloride and the blank solvent. The following *in vivo* studies were performed.

GI Transit Time in Mice—The method used was adapted from previous studies (14, 15). In these experiments, the extent of GI propulsion was measured with the aid of nonabsorbable and visually identifiable markers such as charcoal. Six male Charles River mice, 20–25 g, were fasted in screen-bottom cages with water supplied *ad libitum* for 24 hr prior to the test. The animals were orally pretreated with logarithmically graded doses (0.3–18 mg/kg) of diphenoxylate hydrochloride in a 0.5% solution of methylcellulose in water. The total volume of the suspension administered to

[1] Lot D538049, Searle Laboratories, Chicago, IL 60680
[2] Mallinckrodt Chemical Co., St. Louis, Mo.
[3] Matheson, Coleman and Bell, Norwood, Ohio.
[4] Dow Chemical Co., Midland, Mich.
[5] Union Carbide Co., South Charleston, W. Va.
[6] Model 150V rotating mixer, Scientific Industries, Springfield, Mass.
[7] Model 124D dual-beam spectrophotometer, Perkin-Elmer Corp., Maywood, Ill.

Table I—Effect of Charcoal on the Antipropulsive Activity of Diphenoxylate Hydrochloride in the Mouse

Treatment Condition	$ED_{50} \pm SE$, mg/kg po	Relative Potency
Diphenoxylate hydrochloride administered 30 min prior to charcoal	3.12 ± 1.29	1.0
Diphenoxylate hydrochloride and charcoal administered simultaneously	14.77 ± 6.59	0.22 ± 0.14[a]

[a] Statistically significant at $p = 0.02$ level.

these animals was kept constant at 10 ml/kg. The control group received the same volume of 0.5% methylcellulose solution.

Thirty minutes after drug administration, the animals were given a single 0.2-ml dose of a 10% suspension of activated charcoal powder in 1% methylcellulose solution. The animals were sacrified 3.5 hr later, and the cecum was examined for the presence or absence of charcoal on an all-or-none basis. In 95% of all control animals, charcoal was present in the cecum. Antidiarrheal drugs, such as diphenoxylate hydrochloride, interfere with the GI propulsive activity, so the presence or absence of charcoal in the cecum provides a measure for the pharmacological response to these drugs. Consequently, the median effective dose (ED_{50}) can then be calculated by using the logistic method of Berkson (16).

In a second experiment, logarithmically graded doses (4.6–46.0 mg/kg) of diphenoxylate hydrochloride mixed with charcoal powder in methylcellulose solutions were administered to the animals; the extent of charcoal translocation also was evaluated in a similar manner. The relative potencies for the two experiments were calculated using the logistic method of Berkson (16).

Effect of Chromium Oxide as a Marker on Antipropulsive Activity of Diphenoxylate Hydrochloride in Mice—In this study, the antipropulsive activity of diphenoxylate hydrochloride was evaluated using a second nonabsorbable and visually identifiable marker, chromium oxide. The experimental design was essentially the same as described previously. Aqueous solutions of methylcellulose (0.5%) were used to prepare 10% suspensions of charcoal or chromium oxide. Thirty minutes after the administration of diphenoxylate hydrochloride as a suspension in 0.5% methylcellulose, the animals were given a single 0.2-ml dose of the marker preparation. The animals were sacrificed 3.5 hr later, and the presence or absence of the marker in the cecum was evaluated on an all-or-none basis.

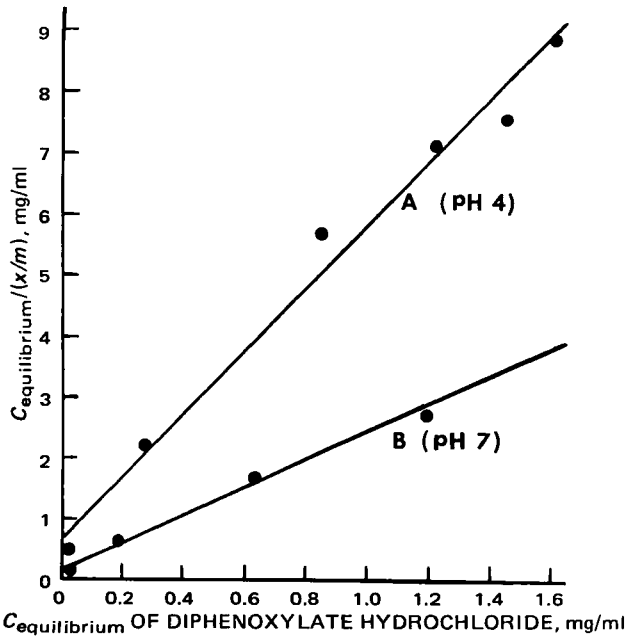


Figure 1—*Langmuir isotherm for the adsorption of diphenoxylate hydrochloride on activated charcoal at pH 4 (A) and pH 7 (B).*

Table II—Effect of Chromium Oxide and Charcoal on the *In Vivo* Antipropulsive Effect of Diphenoxylate Hydrochloride in the Mouse

Marker	$ED_{50} \pm SE$, mg/kg po	Relative Potency ± *SE*
Charcoal	3.12 ± 1.29	1.0
Chromium oxide	0.68 ± 0.18	6.07 ± 2.97[a]

[a] Statistically significant at $p < 0.05$.

RESULTS AND DISCUSSION

Figure 1 illustrates the Langmuir adsorption isotherm for the uptake of diphenoxylate hydrochloride by activated charcoal powder suspended in pH 4 and 7 solutions. The reciprocal of the slope of the two lines (A and B) gives the maximum binding capacity of charcoal at the given pH. A twofold increase in the binding capacity was observed for diphenoxylate hydrochloride at pH 7 as compared to pH 4 solutions. No significant difference in the extent of drug adsorption was observed between pH 2 and 4 solutions.

These data indicate that the nonionized species of diphenoxylate hydrochloride (pKa = 4.4) is more preferably adsorbed than its ionized species in solution. The lipophilic moieties (phenyl and piperidyl) of this drug probably promote drug adsorption through hydrophobic interaction with the activated surface of the charcoal particles. Such a possibility is consistent with previous findings on the nature of the activated carbon surface (7, 17). Furthermore, the lipophilic nature of diphenoxylate hydrochloride, as evidenced by its partition coefficient[8] ($PC_{CHCl_3-H_2O} \geq 68.5$) supports the occurrence of such an interaction between diphenoxylate hydrochloride and activated charcoal. Under given experimental conditions, the maximum adsorption capacity of charcoal was estimated at 200 and 416 mg/g of charcoal in suspension at pH 4 and 7, respectively.

To assess whether charcoal modifies the pharmacological activity of diphenoxylate hydrochloride *in vivo*, GI transit studies of the drug with and without activated charcoal were carried out in the mice. Table I illustrates the effect of charcoal on the antipropulsive activity of diphenoxylate hydrochloride in the mouse; coadministration of the two compounds significantly affected the potency of diphenoxylate hydrochloride as a constipating agent. In the presence of activated charcoal, there was a fourfold decrease in the relative potency of diphenoxylate hydrochloride. These results strongly suggest that the adsorption of diphenoxylate to activated charcoal can occur *in vivo*, resulting in a modification of the antipropulsive activity of this drug.

In view of these findings, it was of interest to examine whether a different marker, such as chromium oxide, which shares with charcoal the property of being pharmacologically inert, can affect the observed antipropulsive activity of diphenoxylate hydrochloride. Furthermore, investigations on sterol balance in humans (18) have provided ample evidence that chromium oxide exhibits superior performance as an inert, nonabsorbable marker for GI transit studies. *In vitro* studies showed that a 2–4% suspension of chromium oxide adsorbs diphenoxylate hydrochloride only to the extent of 10–18%, whereas activated charcoal at the same level in suspension adsorbs virtually all drug in solution.

As evident from Table II, the quantitative response of diphenoxylate hydrochloride was strongly influenced by the choice of the marker in the mouse studies. With chromium oxide, diphenoxylate hydrochloride showed a sixfold increase in its relative potency as compared to charcoal used conventionally to screen for antipropulsive activity of antidiarrheal agents. The important implication of these findings is that charcoal may not provide the desired "inert" nature as a marker since it can modify the apparent pharmacological activity of antidiarrheal agents such as diphenoxylate hydrochloride.

The clinical implications of these adsorption studies are obvious when one considers the maximum adsorption capacity of activated charcoal for diphenoxylate hydrochloride. Although the use of activated charcoal for the management of accidental overdosage of

[8] The partition coefficient was estimated on the basis of saturation solubility of this drug in the two solvents.

diphenoxylate hydrochloride has been suggested previously, the present studies provide *in vitro* as well as *in vivo* evidence to support the possibility that activated charcoal may significantly modify the bioavailability of this drug.

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Electron-Capture GLC Determination of Timolol in Human Plasma and Urine

D. J. TOCCO [x], F. A. deLUNA, and A. E. W. DUNCAN

Abstract □ A GLC procedure was developed for measuring nanogram quantities of timolol in plasma and urine. The unchanged drug was extracted into heptane–4% isoamyl alcohol from alkalinized plasma or urine, together with a homolog of timolol which served as the internal standard. The compounds were subsequently back-extracted into 0.1 *N* HCl and then into chloroform following adjustment of the acid phase to an alkaline pH. The compounds in the chloroform extract were derivatized with heptafluorobutyrylimidazole to form the diheptafluorobutyryl derivatives; these were quantitated by electron-capture GLC. Recovery of timolol added to normal plasma and urine was quantitative and reproducible, and no interfering substances were observed in normal biological samples. The method is capable of measuring concentrations as low as 2 ng/ml in plasma or 20 ng/ml in urine. After a 10-mg oral dose of ^{14}C-timolol, peak plasma levels of approximately 30 ng/ml were observed in 1–2 hr.

Keyphrases □ Timolol—extraction, derivatization, GLC analysis, biological fluids □ Adrenergics—timolol, extraction, derivatization, GLC analysis, biological fluids □ GLC—analysis, timolol, biological fluids

Timolol maleate, (−)-1-(*tert*-butylamino)-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]-2-propanol maleate, is a new β-adrenergic receptor blocking agent (1). More recently, it was shown to reduce the frequency of anginal episodes and thus have a place in the treatment of angina pectoris (2). When administered orally to rats and dogs, the compound is several times more potent than propranolol in blocking isoproterenol-induced cardiac acceleration (3). Its activity in humans also has been demonstrated (4, 5).

This report is concerned with the determination of timolol in biological fluids. In view of the small therapeutic doses used (generally 5–15 mg po), a sensitive as well as a specific analytical method is required. The procedure involves extraction of the compound from biological fluids followed by derivatization to form the diheptafluorobutyryl analog and determination by electron-capture GLC. The procedure is capable of quantitating timolol in concentrations as little as 2 ng/ml in plasma and 20 ng/ml in urine. Accordingly, the method should prove useful in clinical applications where quantitation and diagnostic confirmation are necessary.

EXPERIMENTAL

Reagents—Pesticide quality *n*-heptane[1] was used without further purification. Reagent grade isoamyl alcohol[2] was glass distilled prior to use. Methylene chloride[3], reagent grade, was washed successively with 1 *N* HCl, 1 *N* NaOH, and three times with water and then glass distilled. Distilled, deionized water was used in the preparation of 0.1 *N* HCl and 2.0 *N* NaOH. Heptafluorobutyrylimidazole[4], in 1-ml ampuls stored under nitrogen, was diluted 1:10 with pesticide quality ethyl acetate[5] immediately before use. Timolol (I) was used as the maleate salt, and desmethyltimolol, 1-isopropylamino-3-[(4-morpholino-1,2,5-thiadiazol-3-yl)oxy]-2-propanol hydrochloride (II) served as the internal standard. All concentrations were expressed in terms of the free base.

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Table I—Recovery of Timolol from Human Plasma

Timolol Added, ng/ml	Found, ng/ml	Percent Recovery, Mean ± *SD*
50	49.0 ± 4.0	98 ± 7
40	38.0 ± 5.0	95 ± 12
20	18.0 ± 2.0	90 ± 9
10	8.5 ± 2.0	85 ± 18
5	5.7 ± 0.9	120 ± 18
2	1.9 ± 0.8	95 ± 40

equipped with a ^{63}Ni (2 mCi) electron-capture detector and a 1.83-m × 0.64-cm (6-ft × 0.25-in.) column packed with 1% OV-17 on Gas Chrom Q[7] (80–100 mesh). The instrument was operated isothermally with oven, detector, and injection port temperatures maintained at 185, 300, and 250°, respectively; the carrier gas (helium) and the purge gas (10% methane in argon) were each maintained at 75 ml/min. The pulse mode of voltage was used with a pulse interval of 50 μsec. Gas lines were fitted with filters containing molecular sieve 4A.

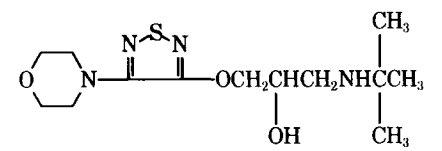
Procedure—In a 30-ml glass-stoppered centrifuge tube were placed 1.0 ml of plasma or 0.1 ml of urine, 0.1 ml of desmethyltimolol internal standard (stock solution, 500 ng/ml of water), 0.1 ml of 1.0 *N* NaOH, and 10 ml of heptane–4% isoamyl alcohol. The mixture was shaken mechanically for 10 min and centrifuged. In a clean 13-ml glass-stoppered centrifuge tube were placed 1.0 ml of 0.1 *N* HCl and 8.0 ml of the organic phase.

After the tube was shaken for 10 min and centrifuged, the organic phase was discarded by aspiration and the aqueous phase was washed three times with 10 ml of *n*-heptane to remove traces of the isoamyl alcohol. To the aqueous phase were added 0.2 ml of 2 *N* NaOH and 5.0 ml of methylene chloride. The samples were shaken for 10 min and centrifuged. The aqueous phase was carefully aspirated, and 4.0 ml of the organic phase was added to a clean 13-ml glass-stoppered tube. The contents were evaporated to dryness in a 60° water bath under a stream of nitrogen.

The residue was dissolved in 0.1 ml of the heptafluorobutyrylimidazole–ethyl acetate reagent, and the stoppered tube was immersed in a boiling water bath for 1 hr. The tubes were cooled to room temperature, 3.0 ml of *n*-heptane plus 1.0 ml of 0.1 *N* HCl was added, and the tubes were then shaken for 10 min. After centrifugation, 2.0 ml of the *n*-heptane phase was transferred to a clean 13-ml conical centrifuge tube and the contents were evaporated to dryness at 60° using a stream of nitrogen.

The residue was dissolved in 0.1 ml of *n*-heptane, and 1–5 μl was chromatographed. The retention times of derivatized timolol and derivatized internal standard were 7.6 and 6.0 min, respectively. GC–mass spectrometric methods showed that the products of acylation of these compounds are the diheptafluorobutyryl derivatives[8].

Peak heights for derivatized timolol and internal standard were measured to the nearest millimeter. A standard curve was constructed by analysis of samples of control plasma containing known quantities of timolol and internal standard. The ratio obtained by dividing the timolol peak height by the internal standard peak height was plotted against the concentration of timolol in



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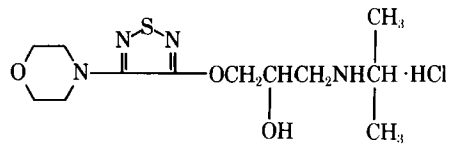

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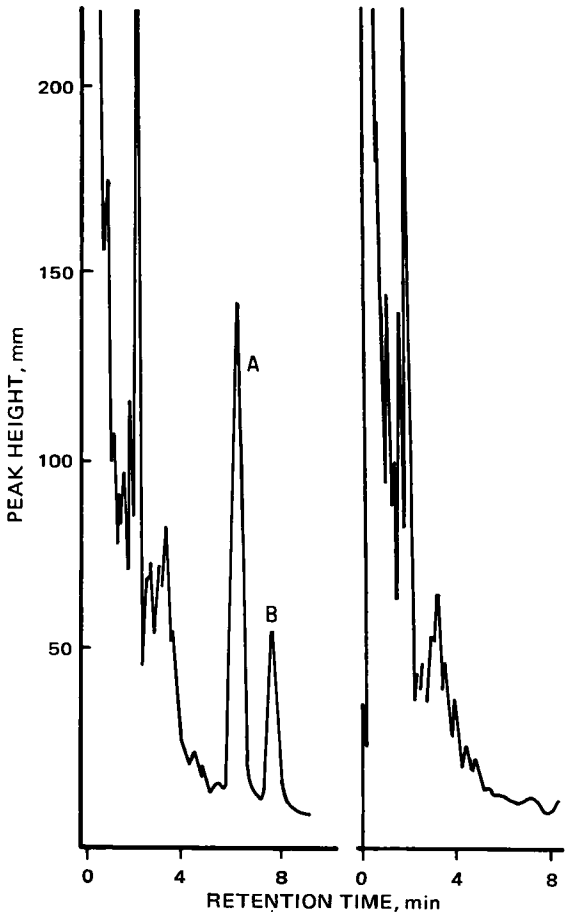

Figure 1—*Gas–liquid chromatograms (electron-capture detector) of human plasma extracts. Left: 50 ng of desmethyltimolol (A) and 20 ng of timolol (B) added to 1.0 ml of plasma. Right: control human plasma.*

nanograms per milliliter of plasma or urine. The line that best fit the data was drawn through the experimental points and the origin. Concentrations of timolol in biological samples were determined by interpolation from the standard curve.

Timolol in Human Plasma—Three healthy human subjects were given a single oral dose (either a tablet or solution) containing 10 mg of ^{14}C-timolol in which both carbons of the thiadiazole ring were labeled. Fourteen days later, the same subjects were given a second 10-mg dose in a simple crossover design. Blood was collected in heparinized containers at intervals during 24 hr following each dose, and plasma was separated by centrifugation and frozen until assayed.

"Apparent timolol" in plasma was determined by measuring the radioactivity in the heptane–4% isoamyl alcohol extract of plasma. Aliquots (0.5 ml) were evaporated to dryness in counting vials. The samples were counted in 16 ml of a toluene medium containing 2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,3,4-oxadiazole (8 g/liter), 2-(4-biphenylyl)-6-phenylbenzoxazole (0.5 g/liter), and solubilizer[9] (125 ml/liter).

At least 5000 total counts were made on all samples[10]. The counting efficiency was determined by external standardization and corrected for a background counting rate of approximately 40 cpm.

RESULTS AND DISCUSSION

Figure 1 shows a typical chromatogram obtained when plasma was assayed by the electron-capture procedure. In this sample, 50 ng of internal standard (peak A) and 20 ng of timolol (peak B)

[7] Applied Science Laboratories.
[8] W. J. A. VandenHeuvel, unpublished data.
[9] Beckman Biosolve, Beckman Instruments.
[10] Beckman LS-100 liquid scintillation spectrometer.

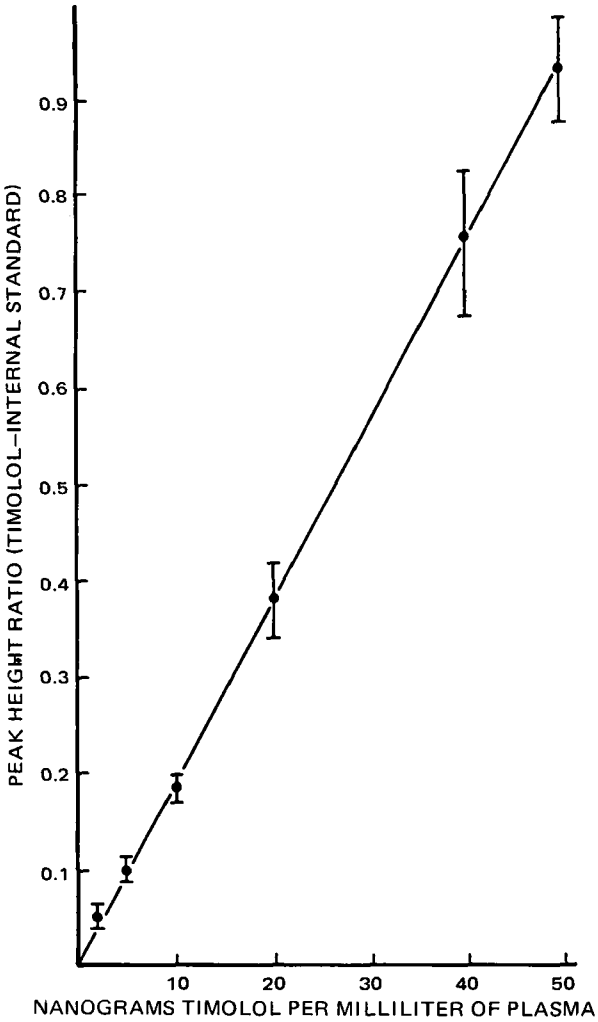

Figure 2—*Calibration curve showing the relationship between the peak height ratio of the diheptafluorobutyryl derivatives of timolol and desmethyltimolol and the amount of timolol in plasma. Each point represents the mean ± SD for four determinations.*

were added to 1.0 ml of human plasma and carried through the procedure. This concentration of timolol gave a peak height of approximately 50 mm (32 pamp) at a range setting of 10 and an attenuation of 16. Blank plasma samples assayed in the same manner gave no interfering peaks on the chromatogram.

Quadruplicate samples, containing 2.0–50 ng of timolol/ml of plasma, were adequately recovered (Table I). The concentrations found were equal to 98 ± 22% (*SD*) of the actual values. A calibration curve prepared from these data indicates that the peak height ratios of timolol–internal standard were linear when plotted against the concentration of timolol, and the curve passed through the origin (Fig. 2). The lower limit of sensitivity was 2.0 ng/ml, and the upper limit of linearity was at least 50 ng/ml.

Plasma levels of timolol were determined in six human subjects over 24 hr following a single oral 10-mg dose of ^{14}C-timolol (Table II). Apparent timolol, *i.e.*, radioactive equivalents of timolol that were extracted by heptane–4% isoamyl alcohol, showed reasonably

Table II—Mean Plasma Levels of Timolol in Six Human Subjects after a Single Oral 10-mg Dose of ^{14}C-Timolol[a]

Time after Dose, hr	Timolol in Plasma, ng/ml ± *SD*		Ratio of Apparent Timolol–Timolol
	"Apparent Timolol"[b]	Timolol	
0.5	15.0 ± 9.6	16.2 ± 10.8	0.93 ± 0.15
1.0	30.0 ± 11.7	30.3 ± 11.2	1.00 ± 0.16
2.0	34.3 ± 12.6	31.2 ± 12.5	1.09 ± 0.08
3.0	34.0 ± 17.2	28.4 ± 11.2	1.20 ± 0.20
4.0	27.3 ± 16.4	25.0 ± 13.6	1.09 ± 0.14
5.0	22.0 ± 14.2	21.2 ± 14.7	1.04 ± 0.14
6.0	16.2 ± 11.4	13.5 ± 10.6	1.20 ± 0.27
8.0	11.3 ± 9.0	10.3 ± 9.2	1.10 ± 0.37
24.0	0	0	0.98 ± 0.05

[a] Timolol labeled with ^{14}C in both carbons of the thiadiazole ring had a specific activity of 3996 dpm/μg. [b] The radioactive equivalents of timolol, expressed as nanograms per milliliter, that were extracted from plasma during the analytical procedure for timolol.

good agreement with the timolol concentration as measured by the electron-capture procedure. The ratio of apparent timolol–timolol was 1.11 ± 0.24 (mean ± *SD*). Peak plasma levels of 31 ng/ml occurred at 2 hr after drug administration. The half-life of the drug in plasma was approximately 3 hr.

The electron-capture method was applied to numerous samples of urine as well as plasma. Electrophoretograms of human urine have shown that timolol is the only drug-related base in the urine[11]. Hence, the method is presumed to be specific for determining the intact compound in plasma and urine. In humans, 10–20% of an oral 10-mg dose of timolol was excreted unchanged. Frequently the concentration of drug in the urine was between 500 and 2000 ng/ml. Therefore, to avoid unnecessary dilution at the end of the assay, a smaller aliquot (0.1 ml of urine) should be processed at the beginning. As with plasma, blank urine samples gave no interfering peaks on the chromatogram.

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[x] To whom inquiries should be directed.

[11] Unpublished observations.

Synthesis of *dl*-4ξ-(2-Carboxyethyl)-*cis*-hexahydropyrrolo-[3,4-*d*]imidazol-2-one (Bisnorazabiotin)

HENRY C. WORMSER ˣ **and HANLEY N. ABRAMSON**

Abstract □ Bisnorazabiotin was synthesized in a six-step sequence from 1,5-dioxo-2-carbethoxypyrrolizidine. It is anticipated that the molecule will serve as a cofactor for biotin-requiring enzymes.

Keyphrases □ Biotin analog—synthesis of *dl*-4ξ-(2-carboxyethyl)-*cis*-hexahydropyrrolo[3,4-*d*]imidazol-2-one (bisnorazabiotin), IR, UV, and NMR spectra □ Bisnorazabiotin—synthesis of biotin analog *dl*-4ξ-(2-carboxyethyl)-*cis*-hexahydropyrrolo[3,4-*d*]imidazol-2-one, IR, UV, and NMR spectral analyses

Previous articles (1–3) reported the synthesis of several analogs of biotin (I) prepared as potential cofactors for biotin-dependent enzymes. The synthesis of bisnorazabiotin, utilizing a slightly modified synthetic approach, is reported here.

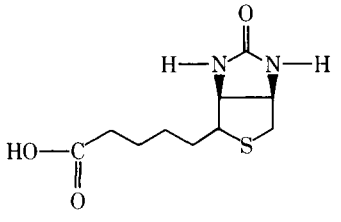
DISCUSSION

The starting point in this sequence (Scheme I) is 1,5-dioxo-2-carbethoxypyrrolizidine (II), prepared in high yield by a modification of the procedure of Gensler and Wu (4). Conversion of II to the enamine (III) was accomplished with ammonium formate in hot isopropanol. When methanol or ethanol was the solvent for this amination, the yield of enamine was considerably lower and TLC of the crude mixtures revealed the presence of an additional product of lesser polarity than either II or III.

Column chromatography of the product obtained from the amination of II in ethanol gave a 30% yield of a compound characterized as ethyl *N*-(2-carbethoxyethyl)-5-oxopyrrolidine-2-carboxylate (IV*b*) (5). The latter actually represents a reverse Dieckmann product of II. Since each stage of the Dieckmann condensation is completely reversible, the reverse reaction has been frequently used to convert β-ketoesters to open chain dicarboxylates (6–10). Under normal circumstances, a small amount of alkoxide in alcoholic solvent is sufficient to bring about the transformation. Although ammonium formate in alcohol is not a particularly basic medium, a minute amount of alkoxide might conceivably form, due to the ionization of the salt, catalyzing the reverse Dieckmann reaction.

When methanol was used as solvent, a 40% yield of methyl *N*-(2-carboxyethyl)-5-oxo-2-pyrrolidinecarboxylate (IV*a*) was obtained. A close examination of the same reaction in isopropanol failed to reveal a reverse Dieckmann product, probably indicating steric hindrance to the approach of isopropoxide anion. Treatment of III with acetic anhydride gave a crystalline enamine acetate (V) in 89% yield. Compound V should be used soon after its preparation or stored under an inert atmosphere, since exposure to air leads to the formation of the pyrrole (VI) (1, 3).

Reduction of V to *cis*-VII was effected catalytically in the presence of platinum in a Parr shaker. Conversion of VII to the tricyclic IX was carried out through the intermediacy of a hydrazide and the Curtius rearrangement of the corresponding acyl azide. Hydrolysis of the lactam ring of IX with aqueous base gave the desired amino acid (X).



I

EXPERIMENTAL[1]

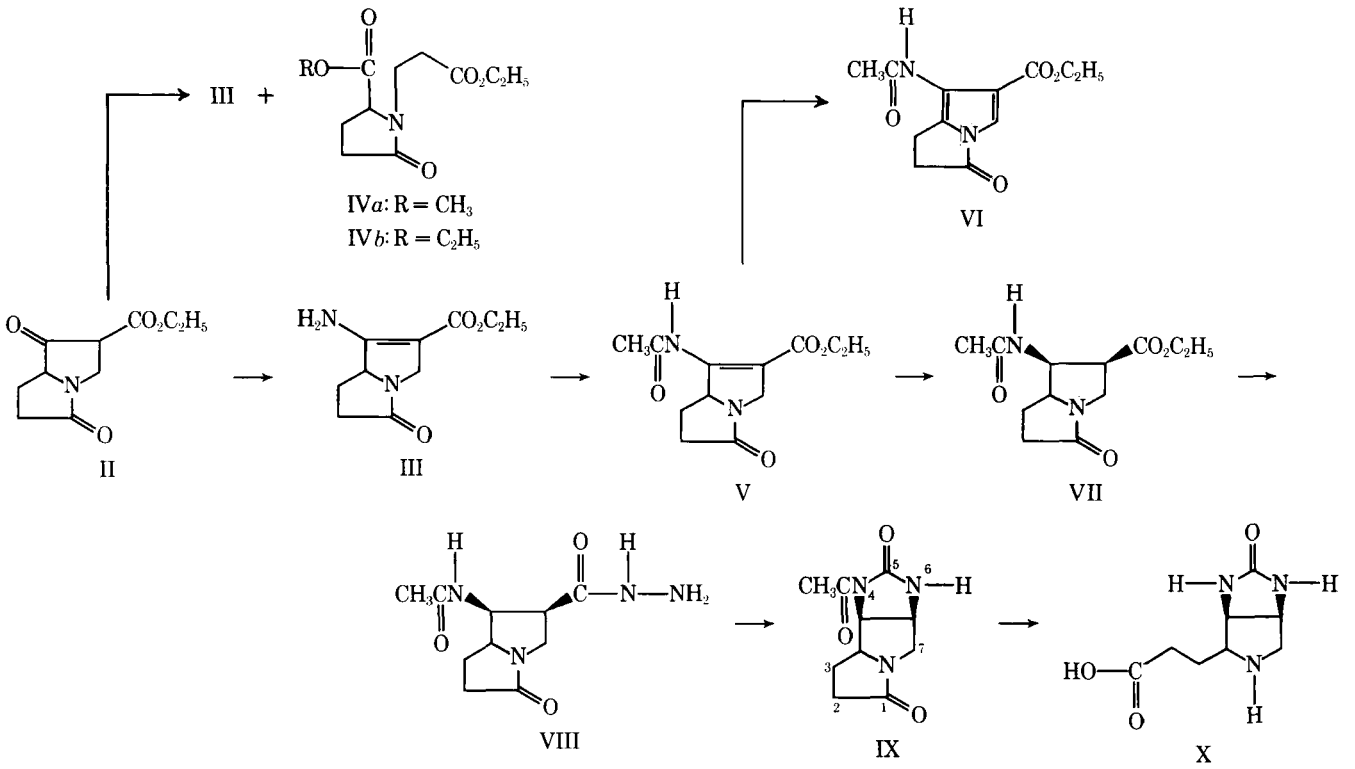
1,5-Dioxo-2-carbethoxypyrrolizidine (II) (4)—A solution of ethyl *N*-(2-carbethoxyethyl)-5-oxopyrrolidine-2-carboxylate (5) (51.46 g, 0.20 mole) in 250 ml of dry benzene was treated with sodium (4.8 g, 0.21 mole) and refluxed for 30 hr. The glassy, brown sodium enolate was filtered and dried (48.32 g, 100%). The free enol ester was released by adding the powdered enolate salt to a stirring slurry of chipped ice, concentrated hydrochloric acid, and chloroform. The chloroform solution was separated, and the aqueous phase was extracted with several portions of fresh chloroform. The combined chloroform extracts were washed with water and dried (magnesium sulfate). Evaporation of the solvent afforded 41.3 g of oily product, which showed one spot on TLC (30% methanol in ether) and gave a purple color with ferric chloride solution; IR: λ_{max} (chloroform) 5.63, 5.76, 5.87, and 6.13 μm.

Reaction of II with Ammonium Formate—*In Isopropanol*—A solution of 21.12 g (0.10 mole) of II and 12.6 g (0.20 mole) of ammonium formate in 150 ml of isopropanol was refluxed for 4 hr. The solvent was removed *in vacuo*, and the residue was partitioned between water and chloroform. The organic layer was washed with water, dried (magnesium sulfate), and concentrated to yield 20.6 g (98%) of 1-amino-5-oxo-2-carbethoxypyrrolizid-1-ene (III) as a light-brown oil. TLC on silica gel GF_{254} (10% methanol in ether) showed primarily one spot. Preparative TLC on silica gel $PF_{254+366}$, as well as column chromatography on silica gel, failed to afford a crystalline product; IR: λ_{max} (chloroform) 2.84, 2.95, 5.93, and 6.12 μm; UV: λ_{max} (ethanol) 274.5 nm; NMR: δ 1.28 (3H, t, $-OCH_2CH_3$, J = 7 Hz), 2.00–2.80 (4H, m, $-CH_2CH_2$), 4.22 (2H, q, $-OCH_2CH_3$, J = 7 Hz), and 5.97 (2H, s, $-NH_2$).

In Methanol—A mixture of 4.23 g (0.02 mole) of II and 2.5 g (0.04 mole) of ammonium formate in 50 ml of methanol was stirred at room temperature for 48 hr and then refluxed for 1 hr. The separation, as described for isopropanol, gave 3.66 g of a dark-brown oil. TLC (10% methanol in ether) showed primarily two spots, with the lower R_f compound corresponding to III. The crude oil was chromatographed on 50 g of silica gel, eluting with progressively increasing concentrations of chloroform in benzene (200-ml fractions). Fractions 6–13 afforded a yellow oil (1.96 g, 40%), which proved homogeneous by TLC on silica gel G (2% methanol in ether). Fractions 17–19 afforded 1.46 g (34.7%) of a light-brown oil; IR, UV, and NMR data were identical to those of the product obtained using isopropanol (III). The less polar oil was distilled and afforded a pale-yellow oil (bp 148–149° at 0.23 mm); IR: λ_{max} (chloroform) 5.75 and 5.90 μm; NMR: δ 1.27 (3H, t, $-OCH_2CH_3$, J = 7 Hz), 2.62 [2H, t, ring $-CH_2C(=O)-$, J = 7 Hz], and 4.17 (4H, q, $-OCH_2CH_3$, J = 7 Hz); $[\alpha]_D^{25}$ 0°.

Anal.—Calc. for $C_{11}H_{17}NO_5$: C, 54.31; H, 7.04; N, 5.76. Found: C, 54.36; H, 7.06; N, 5.80.

[1] Melting points were taken on a Fisher-Johns melting-point stage and a Thomas-Hoover melting-point apparatus and are uncorrected. UV absorption spectra were determined in 95% ethanol on a Beckman recording spectrophotometer (model DK2A). IR absorption spectra were recorded on Beckman recording spectrophotometers (models 8 and 33). NMR spectra were determined in deuteriochloroform, using tetramethylsilane as the reference standard, on a Varian EM 360 spectrometer. Microanalyses were carried out by Spang Microanalytical Laboratory, Ann Arbor, Mich. TLC was carried out with silica gel G, silica gel GF_{254}, or silica gel $PF_{254+366}$. Column chromatography was performed with silica gel 60 (particle size 0.063–0.200 mm).



Scheme I

On this basis, the less polar oily product was identified as methyl *N*-(2-carbethoxyethyl)-5-oxo-2-pyrrolidine-2-carboxylate (IV*a*).

In Ethanol—A mixture of 6.24 g (0.03 mole) of II, 3.5 g (0.056 mole) of ammonium formate, and 95% ethanol (100 ml) was stirred for 48 hr at room temperature. The reaction mixture was refluxed for 1 hr, and the usual workup afforded 6.10 g of a dark-brown oil. TLC of this material was quite similar to that of the crude product obtained using methanol. The crude oil was chromatographed on 75 g of silica gel using the same elution scheme as described for the methanol method. Fractions 8–10 gave a yellow oil (2.3 g, 30%), homogeneous on TLC (2% methanol in ether; 5% methanol in ether). Distillation gave a colorless oil (bp 143–145° at 0.2 mm); IR: λ_{max} (chloroform) 5.77 and 5.92 μm; NMR: δ 1.26 (3H, t, —OCH_2CH_3, J = 7 Hz), 1.32 (3H, t, —OCH_2CH_3, J = 7 Hz), 2.61 [2H, t, ring —$CH_2C(=O)$—, J = 7 Hz], 4.16 (2H, q, —OCH_2CH_3, J = 7 Hz), and 4.26 (2H, q, —OCH_2CH_3, J = 7 Hz). This product was characterized as ethyl *N*-(2-carbethoxyethyl)-5-oxopyrrolidine-2-carboxylate (IV*b*) on the basis of superimposability of spectral data. Fractions 15–17 by TLC gave homogeneous III as a yellow oil (2.85 g, 45%).

1-Acetamido-5-oxo-2-carbethoxypyrrolizid-1-ene (V)—A solution of 12.6 g (0.05 mole) of III in 150 ml of acetic anhydride was heated on an oil bath at 90–100° for 3 hr. The excess acetic anhydride was removed under vacuum to give 13.4 g of a dark-brown oil, which crystallized upon standing at room temperature for several days. Recrystallization from ether gave colorless crystals (11.2 g, 89%), mp 123–126°. Two additional recrystallizations from ether gave the analytical sample, mp 127–129°; IR: λ_{max} (chloroform) 3.01, 5.87, 5.95, and 6.13 μm; UV: λ_{max} (ethanol) 274 nm (ε = 23,300); NMR: δ 1.31 (3H, t, —OCH_2CH_3, J = 7 Hz), 2.18 [3H, s, N—(C=O)CH_3], 2.00–2.90 (4H, m, —CH_2CH_2—), 4.26 (2H, q, —OCH_2CH_3, J = 7 Hz), and 5.25 (1H, s, NH).

Anal.—Calc. for $C_{12}H_{16}N_2O_4$: C, 57.13; H, 6.39; N, 11.10. Found: C, 57.10; H, 6.40; N, 11.06.

1-Acetamido-5-oxo-2-carbethoxy-6,7-dihydro-5*H*-pyrrolizine (VI)—A thin film of V (0.25 g, 1.0 mmole) on a watchglass was allowed to remain at room temperature while exposed to the air for 5 weeks. TLC on silica gel GF_{254} (10% methanol in ether) showed the appearance of a new spot with a higher R_f than V and very little remaining starting material. Crystallization from ether gave colorless crystals, mp 108–110°; IR: λ_{max} (chloroform) 2.96, 3.14, 5.68, and 5.92 μm; UV: λ_{max} (ethanol) 240 nm (ε = 31,300); NMR: δ 1.37 (3H, t, —OCH_2CH_3, J = 7 Hz), 2.17 [3H, s, NC(=O)CH_3], 4.37 (2H, q, —OCH_2CH_3, J = 7 Hz), 7.54 (1H, s, =CH), and 9.06 (1H, s, NH).

Anal.—Calc. for $C_{12}H_{14}N_2O_4$: C, 57.59; H, 5.64; N, 11.19. Found: C, 57.53; H, 5.79; N, 11.21.

1-Acetamido-5-oxo-2-carbethoxypyrrolizidine (VII)—Compound V (0.85 g, 3.37 mmoles), dissolved in 50 ml of absolute ethanol, was hydrogenated at 60 psi in a Parr shaker over platinum (100 mg of platinum oxide). The catalyst was filtered, and the ethanol was evaporated *in vacuo*, affording a colorless viscous oil (0.84 g, 98%). TLC on silica gel G (10% methanol in ether) showed disappearance of V and a new spot of lower R_f; IR: λ_{max} (chloroform) 2.91, 5.85, and 5.97 μm; NMR: δ 1.30 (3H, t, —OCH_2CH_3, J = 7 Hz), 2.02 [3H, s, NC(=O)CH_3], 4.24 (2H, q, —OCH_2CH_3, J = 7 Hz), and 6.85 (1H, d, NH, J = 7 Hz). All attempts to crystallize the compound failed.

6-Acetyldecahydroimidazo[4,5-*a*]pyrrolizine-1,5-dione (IX)—Compound VII (5.4 g, 0.021 mole), dissolved in 100 ml of absolute ethanol, was treated with 10 ml of hydrazine hydrate and kept at room temperature for 6 hr. The excess reagent and solvent were removed *in vacuo*, and the oily residue was subjected to vacuum for an additional 24 hr. A foamy, hygroscopic residue was obtained (5.14 g). This hydrazide, dissolved in 70 ml of 20% hydrochloric acid, was cooled to 3° and treated dropwise with sodium nitrite (2.53 g, 0.037 mole) in 10 ml of water, keeping the temperature near 0°. The reaction mixture was extracted with ethyl acetate (5 × 50 ml), the combined fractions were dried (magnesium sulfate), and the ethyl acetate solution was refluxed for 3 hr. Evaporation of the solvent gave a yellow oil, which crystallized upon the addition of 95% ethanol (3.2 g, 68%), mp 250–254°. Two recrystallizations from ethanol gave the analytical sample, mp 253–254°; IR: λ_{max} (mineral oil) 3.01, 3.13, 5.75, 5.93, and 6.01 μm.

Anal.—Calc. for $C_{10}H_{13}N_3O_3$: C, 53.81; H, 5.87; N, 18.82. Found: C, 53.75; H, 5.80; N, 18.79.

***dl*-4ξ-(2-Carboxyethyl)-*cis*-hexahydropyrrolo[3,4-*d*]imidazol-2-one (X)**—A suspension of IX (0.095 g, 0.43 mmole) in 3 ml of 10% sodium hydroxide was heated at 120° in a sealed tube. The colorless solution was evaporated to dryness, and the residual powder was neutralized with ethanolic hydrogen chloride. The crystalline product obtained (mp 174–176°, resolidifies at 177°, and then mp 252–254°) was recrystallized from aqueous ethanol. TLC on silica gel G in the following systems showed the product to be ho-

mogeneous: *n*-butanol–acetic acid–water (3:1:1), 95% ethanol–water (7:3), *n*-propanol–34% ammonia (7:3), 95% ethanol–34% ammonia (7:3), and *n*-propanol–water (1:1). One additional recrystallization from aqueous ethanol gave the analytical sample (mp 178–180°, resolidifies, and then mp 253–255°); IR: λ_{max} (mineral oil) 2.86, 2.91, and 5.92 μm.

Anal.—Calc. for $C_8H_{13}N_3O_3 \cdot H_2O$: C, 44.23; H, 6.96; N, 19.34. Found: C, 44.30; H, 7.00; N, 19.44.

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Sodium Chloride Equivalents, Cryoscopic Properties, and Hemolytic Effects of Certain Medicinals in Aqueous Solution III: Supplemental Values

CURTIS SAPP [*], MICHAEL LORD [*], and E. ROY HAMMARLUND [x]

Abstract □ A supplemental table of sodium chloride equivalents and freezing-point depressions at various concentrations for 44 different substances in aqueous solution is presented. Also given in the table is the isosmotic concentration of each material that can form such a solution. The degree of hemolysis of human erythrocytes was determined in 24 different isosmotic solutions, and the data are presented in a table to supplement the previously published values. Eleven isosmotic solutions prevented hemolysis, and 13 others failed to prevent hemolysis.

Keyphrases □ Sodium chloride equivalents—data for 44 drugs □ Cryoscopic properties—data for 44 drugs □ Hemolytic effects—data for 44 drugs □ Drug substances—sodium chloride equivalents, cryoscopic properties, and hemolytic effects determined for 44 drugs

The sodium chloride equivalents and freezing-point depressions for 456 substances in aqueous solution were determined experimentally and reported previously (1–4). Furthermore, the degree of hemolysis of fresh human erythrocytes in certain aqueous isosmotic solutions was determined using the hemolytic method (3–5).

The objectives of the current investigation were to study, using the same methods, some additional available substances not included in the earlier cryoscopic and hemolytic investigations and to present these data in suitable tables to supplement the previous data.

EXPERIMENTAL

Cryoscopic Measurements—The method used for the measurements of the freezing points of the solutions was the same as that already reported; all freezing-point data were obtained with a cryoscopic osmometer (4).

The freezing-point measurements were corrected for the amount of disengaged ice, and −0.52° was used as the comparative freezing point for aqueous 0.9% reagent grade sodium chloride solution, which is isotonic and isosmotic with blood and tears. The materials studied were of the official grade of purity or better, and the grade of purity of the donated specialty preparations complied with the manufacturer's specifications.

Hemolysis of Human Erythrocytes—Colorimetric hemoglobin determinations were made to indicate the degree of hemolysis for solutions that could be made isosmotic. The method, utilizing a 45-min incubation period of erythrocytes in the isosmotic solution followed by centrifugation of the erythrocytes and ghosts and determination of absorbance *versus* a standard at 520 nm in a colorimeter, was reported previously in detail (3–5).

RESULTS AND DISCUSSION

Table I lists the sodium chloride equivalents and freezing-point depressions at various concentrations for the 44 currently studied substances. To use these data, one should employ the sodium chloride equivalent that represents the concentration nearest to the desired final concentration of medicinal substance used. Because of general interest in the colligative properties of medicinal solutions, the freezing-point depressions and sodium chloride equivalents are included for several substances that are not necessarily used as isotonic or isosmotic solutions. The sodium chloride equivalents and isosmotic concentrations are reported to the nearest 0.01.

The percent of hemolysis found for the 24 compounds studied is listed in Table II in addition to the isosmotic concentration used for each and the solution's approximate pH. For solutions that developed a color or cloudiness upon the addition of blood, the proportional decrease in the volume of the packed, nonhemolyzed centrifuged erythrocytes was estimated visually. Any noticeable change in appearance of the erythrocytes or the solution is referred to in the footnotes for Table II. Of the compounds studied, 11 isosmotic solutions prevented hemolysis of human erythrocytes and 13 failed to prevent hemolysis.

A compilation of the 293 substances whose isosmotic solutions were studied using the same hemolytic method in this laboratory

mogeneous: *n*-butanol–acetic acid–water (3:1:1), 95% ethanol–water (7:3), *n*-propanol–34% ammonia (7:3), 95% ethanol–34% ammonia (7:3), and *n*-propanol–water (1:1). One additional recrystallization from aqueous ethanol gave the analytical sample (mp 178–180°, resolidifies, and then mp 253–255°); IR: λ_{max} (mineral oil) 2.86, 2.91, and 5.92 μm.

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Table I—Sodium Chloride Equivalents and Freezing-Point Depressions[a]

Chemical	Concentration of Solution, Sodium Chloride Equivalents						
	0.5%	1%	2%	3%	5%	At Isosmotic Concentration	
Acetylcysteine	0.20	0.20	0.20	0.20	—	0.20	4.58%[b]
	0.055°	0.113°	0.227°	0.341°	—	0.52°	4.58%
Alphaprodine hydrochloride	0.19	0.19	0.18	0.18	—	0.18	4.98%
	0.053°	0.105°	0.212°	0.315°	—	0.52°	4.98%
Aminohippuric acid	0.13	0.13	—	—	—	—	—
	0.035°	0.075°	—	—	—	—	—
Anileridine hydrochloride	0.19	0.19	0.19	0.18	0.18	0.18	5.13%
	0.052°	0.104°	0.212°	0.316°	0.509°	0.52°	5.13%
Arginine glutamate	0.17	0.17	0.17	0.17	0.17	0.17	5.37%
	0.048°	0.097°	0.195°	0.292°	0.487°	0.52°	5.37%
Bupivacaine hydrochloride	0.17	0.17	0.17	0.17	0.17	0.17	5.38%
	0.048°	0.096°	0.193°	0.290°	0.484°	0.52°	5.38%
Butabarbital sodium	0.27	0.27	0.27	0.27	—	0.27	3.33%
	0.078°	0.155°	0.313°	0.470	—	0.52°	3.33%
Capreomycin sulfate	0.04	0.04	0.04	0.04	0.04	—	—
	0.011°	0.020°	0.042°	0.063°	0.106°	—	—
Carbenicillin disodium	0.20	0.20	0.20	0.20	—	0.20	4.40%
	0.059°	0.118°	0.236°	0.355°	—	0.52°	4.40%
Carboxymethylcellulose sodium	0.03	0.03	—	—	—	—	—
	0.007°	0.017°	—	—	—	—	—
Chloroquine phosphate	0.14	0.14	0.14	0.14	0.13	0.13	7.15%
	0.039°	0.082°	0.162°	0.242°	0.379°	0.52°	7.15%
Chlortetracycline hydrochloride	0.10	0.10	0.10	—	—	—	—
	0.030°	0.061°	0.121°	—	—	—	—
Clindamycin phosphate	0.08	0.08	0.08	0.08	0.08	0.08	10.73%
	0.022°	0.046°	0.095°	0.144°	0.242°	0.52°	10.73%
Colistimethate sodium	0.15	0.15	0.15	0.15	0.14	0.13	6.73%
	0.045°	0.085°	0.170°	0.253	0.411°	0.52°	6.73%
Cytarabine	0.11	0.11	0.11	0.11	0.11	0.10	8.92%
	0.034°	0.066°	0.134°	0.198°	0.317°	0.52°	8.92%
Deferoxamine mesylate	0.09	0.09	0.09	0.09	0.09	—	—
	0.023°	0.047°	0.093°	0.142°	0.241°	—	—
Dicloxacillin sodium (monohydrate)[c]	0.10	0.10	0.10	0.10	—	—	—
	0.030°	0.061°	0.122°	0.182	—	—	—
Doxycycline hyclate	0.12	0.12	0.12	0.11	0.09	—	—
	0.035°	0.072°	0.134°	0.186°	0.264°	—	—
Dyphylline	0.10	0.10	0.09	0.09	0.08	—	—
	0.025°	0.052°	0.104°	0.155°	0.245°	—	—
Floxuridine	0.14	0.13	0.13	0.12	0.12	0.12	8.47%
	0.040°	0.076°	0.147°	0.213°	0.335°	0.52°	8.47%
Gentamicin sulfate	0.05	0.05	0.05	0.05	0.05	—	—
	0.015°	0.030°	0.060°	0.093°	0.153°	—	—
Glycine	0.41	0.41	0.41	—	—	0.41	2.19%
	0.118°	0.235°	0.470°	—	—	0.52°	2.19%
Hetacillin potassium	0.17	0.17	0.17	0.17	0.17	0.17	5.50%
	0.048°	0.095°	0.190°	0.284	0.474°	0.52°	5.50%
Indigotindisulfonate sodium	0.30	0.30	—	—	—	—	—
	0.085°	0.172°	—	—	—	—	—
Isometheptene mucate	0.18	0.18	0.18	0.18	—	0.18	4.95%
	0.048°	0.095°	0.196°	0.302°	—	0.52°	4.95%
Isoproterenol sulfate	0.14	0.14	0.14	0.14	0.14	0.14	6.65%
	0.039°	0.078°	0.156°	0.234°	0.389°	0.52°	6.65%
Levallorphan tartrate	0.13	0.13	0.13	0.12	0.12	0.10	9.40%
	0.036°	0.073°	0.143°	0.210°	0.329°	0.52°	9.40%
Levorphanol tartrate	0.12	0.12	0.12	0.12	—	—	—
	0.033°	0.067°	0.136°	0.203°	—	—	—
Mesoridazine besylate	0.10	0.07	0.05	0.04	0.03	—	—
	0.024°	0.040°	0.058°	0.071°	0.087°	—	—
Methocarbamol	0.10	0.10	—	—	—	—	—
	0.030°	0.060°	—	—	—	—	—
Methylergonovine maleate	0.10	0.10	—	—	—	—	—
	0.028°	0.056°	—	—	—	—	—
Methylprednisolone sodium succinate	0.10	0.09	0.09	0.08	0.07	—	—
	0.025°	0.051°	0.102°	0.143°	0.200°	—	—
Minocycline hydrochloride	0.10	0.10	0.09	0.08	—	—	—
	0.030°	0.058°	0.107°	0.146°	—	—	—
Naloxone hydrochloride	0.14	0.14	0.14	0.13	0.13	0.11	8.07%
	0.042°	0.083°	0.158°	0.230°	0.367°	0.52°	8.07%
Novobiocin sodium	0.12	0.10	0.07	—	—	—	—
	0.033°	0.057°	0.073°	—	—	—	—
Orphenadrine citrate	0.13	0.13	0.13	0.12	0.10	—	—
	0.037°	0.074°	0.144°	0.204°	0.285°	—	—
Paraldehyde	0.25	0.25	0.25	0.25	—	0.25	3.65%
	0.071°	0.142°	0.288°	0.430°	—	0.52°	3.65%
Pyridostigmine bromide	0.22	0.22	0.22	0.22	—	0.22	4.13%
	0.062°	0.125°	0.250°	0.377	—	0.52°	4.13%

(*continued*)

Table I—*Continued*

Chemical	Concentration of Solution, Sodium Chloride Equivalents						
	0.5%	1%	2%	3%	5%	At Isosmotic Concentration	
Rolitetracycline	0.11	0.11	0.10	0.09	0.07	—	—
	0.032°	0.064°	0.113°	0.158	0.204°	—	—
Spectinomycin hydrochloride	0.16	0.16	0.16	0.16	0.16	0.16	5.66%
	0.045°	0.092°	0.185°	0.280	0.460°	0.52°	5.66%
Thiotepa	0.16	0.16	0.16	0.16	0.16	0.16	5.67%
	0.045°	0.090°	0.182°	0.278	0.460°	0.52°	5.67%
Tridihexethyl chloride	0.16	0.16	0.16	0.16	0.16	0.16	5.62%
	0.047°	0.096°	0.191°	0.280	0.463°	0.52°	5.62%
Triflupromazine hydrochloride[c]	0.10	0.09	0.05	0.04	0.03	—	—
	0.031°	0.051°	0.061°	0.073	0.092°	—	—
Tromethamine	0.26	0.26	0.26	0.26	—	0.26	3.45%
	0.074°	0.150°	0.300°	0.450	—	0.52°	3.45%

[a] Top value is sodium chloride equivalent, and bottom value is freezing-point depression. [b] Isosmotic concentration, % w/v. [c] Solution foams readily.

shows that 136 failed to prevent hemolysis while 157 prevented hemolysis completely. One must be careful not to equate isotonicity and isosmoticity without knowledge of the corresponding data whenever a biological membrane is being utilized. This aspect was discussed in detail previously (2, 4–8).

Earlier reports in the series contained data on substances that foamed in aqueous solution and apparently aggregated above certain critical concentrations. These critical concentrations can be determined readily from the abrupt change in their cryoscopic graphs (3, 4, 9). The following behaved in this manner: benzethonium chloride, chlorpheniramine maleate, chlorpromazine hydrochloride, bromodiphenhydramine hydrochloride, dexchlorpheniramine maleate, dibucaine hydrochloride, fluphenazine hydrochloride, hydroxyzine hydrochloride, methotrimeprazine hydrochloride, pramoxine hydrochloride, pyrilamine maleate, nafcillin sodium, tetracaine hydrochloride, and valethamate bromide. Later, Altwood (10) studied micelle formation of some antihistamines in aqueous solution with a light-scattering photometer and reported their critical micelle concentrations (CMC), micellar weights, and aggregation numbers, which agreed well with our cryoscopic data.

Because of interest in the apparent aggregation of some substances in aqueous solution, the authors were especially looking for this characteristic discontinuity in the cryoscopic graphs from any of the 44 new compounds in this study, but none behaved in this manner. Triflupromazine hydrochloride was the only one whose graph even slightly suggested the possibility of any aggregation in solution. Its graph showed a slight discontinuity at a concentration of 0.019 *M* and approximately −0.05°, which corresponded to an apparent aggregation number of six. However, the discontinuity in the graph was not as distinct as it was with all of the before-mentioned substances.

A sufficient number of compounds demonstrated that they tend to aggregate in solution to suggest that this interfacial phenomenon may play a role in drug action by affecting biological activity. Associations taking place in the complex biological medium undoubtedly affect the thermodynamic activity of a given drug at the molecular level and, therefore, warrant increased study.

Table II—Hemolysis of Erythrocytes in Isosmotic Solutions

Substances	Isosmotic Concentration, % w/v	Hemolysis, %	Approximate pH
Acetylcysteine	4.58	100[a]	2.0
Alphaprodine hydrochloride	4.98	100	5.3
Anileridine hydrochloride	5.13	12	2.6
Arginine glutamate	5.37	0	6.9
Bupivacaine hydrochloride	5.38	83	6.8
Butabarbital sodium	3.33	0	6.8
Carbenicillin disodium	4.40	0	6.6
Chloroquine phosphate	7.15	0	4.3
Clindamycin phosphate	10.73	58[b]	6.8
Colistimethate sodium	6.73	0	7.6
Cytarabine	8.92	0	8.0
Floxuridine	8.47	3[c]	4.5
Glycine	2.19	0[d]	6.2
Hetacillin potassium	5.50	0	6.3
Isometheptene mucate	4.95	0	6.2
Isoproterenol sulfate	6.65	Trace	4.5
Levallorphan tartrate	9.40	59[b]	6.9
Naloxone hydrochloride	8.07	35	5.2
Paraldehyde	3.65	97	5.3
Pyridostigmine bromide	4.13	0	7.2
Spectinomycin hydrochloride	5.66	3	4.4
Thiotepa	5.67	10[e]	8.2
Tridihexethyl chloride	5.62	97	5.4
Tromethamine	3.45	0	10.2

[a] Solution turned dark green-brown. [b] Solution and cells turned brown. [c] Cells clumped. [d] Cell volume increased slightly, darkened in color, and clumped. [e] Solution became turbid.

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Binding of Bile Acids to Cholestyramine at Gastric pH Conditions

SIEGFRIED LINDENBAUM ˣ and TAKERU HIGUCHI

Abstract □ The binding of bile salts to cholestyramine was studied under varying conditions of pH and added electrolyte. The taurine-conjugated bile salts were strongly absorbed by the anion-exchange resin at low pH and in the presence of chloride anions. Glycocholic acid binding was very weak at low pH but increased strongly with increasing pH. The presence of chloride ions strongly decreased the amount of glycocholate bound by the anion-exchange resin.

Keyphrases □ Bile acids—binding to cholestyramine at gastric pH □ Cholestyramine—binding of bile acids at gastric pH □ Binding—bile acids to cholestyramine at gastric pH

Recently published evidence shows that the reflux of the duodenal contents into the stomach is frequently the cause of gastric ulcers (1) and gastritis (2). It has been shown that bile salts disrupt the mucosal barrier and render it permeable to hydrogen ions (3, 4). The name "bile reflux gastritis" has been applied to this condition (2).

Cholestyramine resin has been used in recent clinical trials to reduce the intragastric bile salt content (1, 2, 5).

It is not clear, however, from these studies whether the cholestryamine significantly binds bile salts in the stomach or whether the anion-exchange resin acts chiefly to bind bile acids in the intestine, thereby generally reducing the circulating bile acid pool.

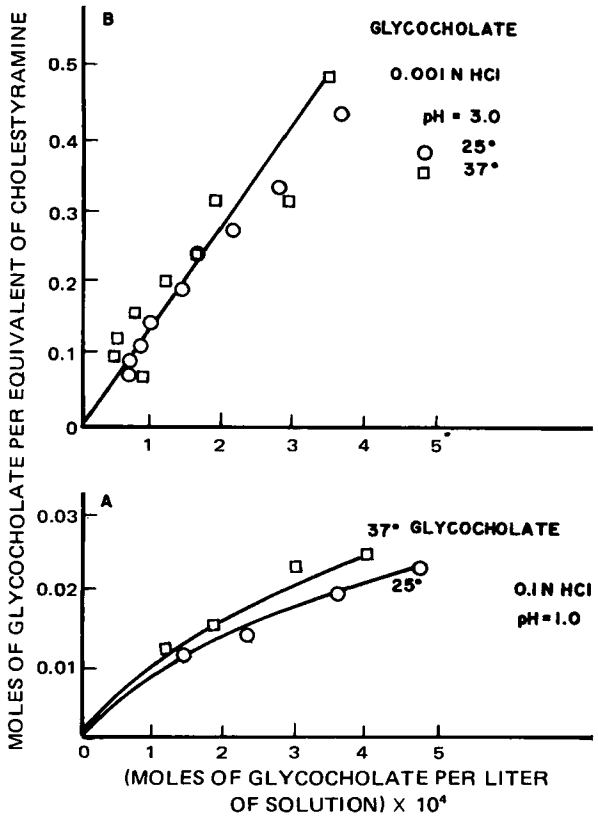

Figure 1—*Isotherms for the binding of glycocholate to cholestyramine at pH 1.0 (A) and 3.0 (B).*

Table I—Binding of Bile Salts by Cholestyramine

Bile Salt	pH	Ionic Strength	Millimoles per Gram of Resin	Grams Bound per Gram of Resin[a]
Glycocholic acid	1.0	0.1	0.08	0.039
	3.0	0.001	2.1	1.00
	3.1	0.1	0.1	0.048
	4.2	0.1	0.26	0.125
Taurocholic acid	1.0	0.1	0.50	0.269
Taurodeoxycholic acid	1.0	0.1	3.63	1.89

[a] The equilibrium concentration of bile salt in the aqueous solution is 4×10^{-4} *M* in each case.

Cholestyramine resin has been shown to be effective in lowering serum cholesterol levels by removing bile salts from the intestine and increasing their fecal elimination (6, 7). The increased fecal loss of bile salts due to binding by the anion-exchange resin leads to increased metabolism of cholesterol to replace the lost bile acids.

Under the relatively high pH conditions prevailing in the small intestine, both *in vitro* (8, 9) and *in vivo* (6, 7) studies showed that bile acids are bound by cholestyramine resin. Since the glycine-conjugated bile acids are weak acids (glycocholic acid, pKa = 3.95; glycodeoxycholic acid, pKa = 4.89) (10), they will be only slightly ionized at the low pH of the stomach and their binding by the anion-exchange resin consequently decreased. The taurine-conjugated bile acids are much stronger acids; even at low gastric pH, a significant fraction of the bile acid will be in the ionized form.

The pH of the stomach will, however, significantly determine the extent of bile acid binding since approximately 75% of the bile acid in adult human bile is glycine conjugated (11).

The objective of this study was to determine quantitatively the binding of bile acids by cholestyramine at acid pH conditions.

EXPERIMENTAL

Glycocholic acid[1], sodium taurodeoxycholate[2], sodium taurocholate, and cholestyramine resin[3] USP were used as received. All other chemicals were reagent grade. The ion-exchange capacity of the resin was determined by argentometric titration of the chloride ions released from the resin in the presence of excess potassium ni-

[1] Sigma Chemical Co., St. Louis, Mo.
[2] Calbiochem, Los Angeles, Calif.
[3] Supplied by Dr. L. Schroeter, Merck Sharp and Dohme, West Point, Pa.

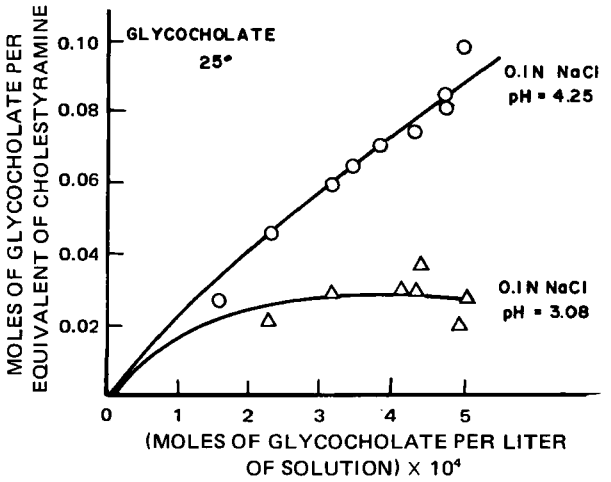

Figure 2—*Isotherms for the binding of glycocholate to cholestyramine in the presence of 0.1* N *NaCl at pH 3.08 and 4.25.*

trate in aqueous solution. The capacity was 3.59 mEq/g. Bile acid analyses were performed spectrophotometrically using the method of Eriksson and Sjovall (12).

Ion-exchange adsorption isotherms were measured by shaking the aqueous bile acid solutions with samples of cholestyramine resin in a constant-temperature bath. Preliminary experiments indicated that overnight shaking was sufficient to attain equilibrium. The solutions were filtered, and the bile acid content of the supernatant liquid was determined. The amount of bile acid absorbed by the ion-exchange resin was calculated as the difference between the amount initially in solution and the amount in solution after equilibration.

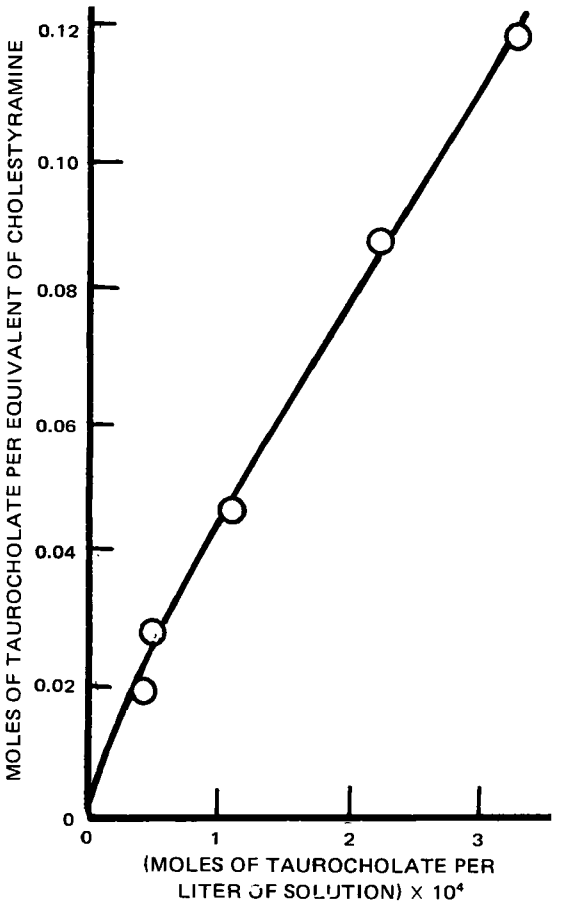

Figure 3—*Isotherms for the binding of taurocholate to cholestyramine at pH 1.0 and 25° in 0.1* N *HCl.*

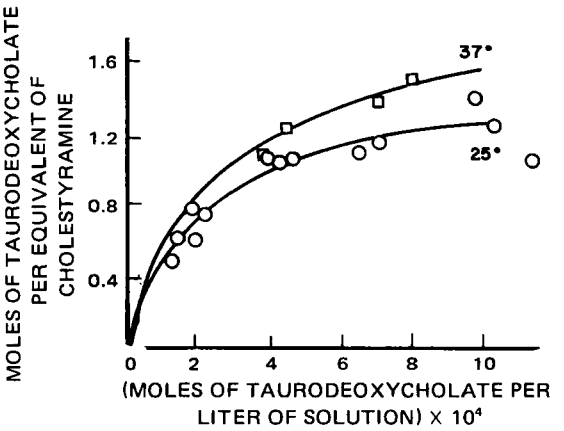

Figure 4—*Isotherms for the binding of taurodeoxycholate to cholestyramine at pH 1.0 in 0.1* N *HCl.*

RESULTS AND DISCUSSION

Ion-exchange isotherms for the distribution of bile salts between cholestyramine resin and aqueous bile salt solution are given in Figs. 1–4. Glycocholate (Fig. 1A) is only slightly absorbed from 0.1 *N* HCl. The binding is weak because the glycocholic acid is only slightly ionized at pH 1.0 and there is competition for the exchange sites by chloride ions. Previous studies of anion-exchange binding of bile salts at higher pH showed that chloride ions compete strongly for the resin anion-exchange sites (9). The isotherms also show that binding is stronger at higher temperature. This result indicates that the enthalpy change for the reaction shown in Scheme I:

$$RCl + A^- \rightleftharpoons RA + Cl^-$$

Scheme I

(where RCl represents cholestyramine resin in the chloride form) is positive, suggesting that the binding of glycocholate ion to ion-exchange resin is caused partly by a hydrophobic interaction between the glycocholate ion and the resin matrix. The interaction between the resin and the glycocholate ion is facilitated by the release of "structured" water surrounding the bile acid anion (13, 14). The temperature effect also may possibly be due in part to a decrease in pKa with increasing temperature.

Much stronger binding of glycocholic acid by the resin is found at pH 3.0 and reduced ionic strength (Fig. 1B). At this pH, more glycocholic acid is in the ionized form and the main interaction with the cholestyramine is probably coulombic. No difference is obtained in the adsorption isotherm between 25 and 37°, suggesting that the hydrophobic contribution is small.

The effect of pH alone on the ion-exchange binding of glycocholic acid is demonstrated better with the data given in Fig. 2. These isotherms at constant ionic strength show that there is a large increase in ion-exchange binding when the pH is increased from 3 to 4. This result suggests that the binding of glycine-conjugated bile acids may be considerably improved by increasing the pH in the gastric lumen.

Taurocholate (Fig. 3) is strongly bound by the resin even at low pH and in competition with 0.1 *M* chloride-ion concentration. Binding of taurodeoxycholate is much stronger and also shows a strong positive temperature dependence. Since taurodeoxycholic acid is a dihydroxy bile acid, it is considerably less polar (more hydrophobic) than the trihydroxy acids and is, therefore, more subject to hydrophobic binding forces.

Further evidence that hydrophobic binding predominates in this case is that the binding capacity for taurodeoxycholic acid, as seen from the ordinate of Fig. 4, exceeds 1.0. Therefore, more than 1 mole of bile acid anion is adsorbed per equivalent of exchange site, suggesting that the hydrophobic resin matrix plays an important role in the binding process.

The effects of pH and added electrolyte on the binding of bile salts by cholestyramine are summarized in Table I. In calculating the amount of bile salt bound per gram of resin, it is assumed that the equilibrium aqueous concentration of bile salt is 4×10^{-4} *M*.

The taurine-conjugated acids are strongly bound even at low pH and in the presence of 0.1 *M* chloride. The glycocholic acid binding is strongly pH dependent, and effective binding is only obtained at low pH in the absence of competing chloride ion. However, previous work (9) showed that much more glycocholate is bound by cholestyramine at higher pH (5.4–6.8), even in the presence of 0.15 *M* sodium chloride or sodium carbonate. Under these conditions, the glycocholate is present almost entirely in the dissociated anionic form.

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Tumor-Inhibitory Agent from *Montezuma speciosissima* (Malvaceae)

S. D. JOLAD, R. M. WIEDHOPF, and J. R. COLEˣ

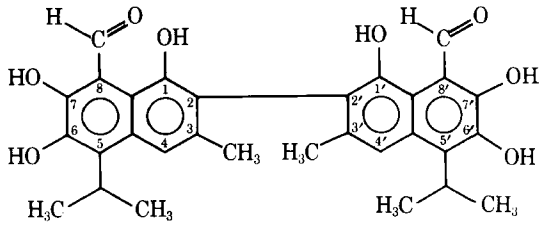
Abstract □ The petroleum ether extract of *Montezuma speciosissima* Sesse and Moc. demonstrated tumor-inhibiting properties in the P-388 lymphocytic leukemia test system (3PS). The constituent responsible for this activity was shown to be a symmetrically substituted 2,2′-dinaphthol, identified as gossypol ($C_{30}H_{30}O_8$).

Keyphrases □ *Montezuma speciosissima*—extract, PMR, UV, IR, and mass spectra, gossypol isolated □ Gossypol—isolated from *Montezuma speciosissima*, PMR, UV, IR, and mass spectra, tumor inhibitory activity □ Tumor inhibition—gossypol, isolated from *Montezuma speciosissima*, PMR, UV, IR, and mass spectra

As a result of the continuing search for plants yielding tumor-inhibiting constituents, it was found that the petroleum ether extract of the woody stems and stem barks of *Montezuma speciosissima* Sesse and Moc. (Malvaceae)[1] showed inhibitory activity toward the P-388 lymphocytic leukemia test system (3PS)[2].

DISCUSSION

The ether-soluble portion of the petroleum ether extract, after mild alkali extraction followed by careful acidification, gave a product which, when subjected to silica gel column chromatography, yielded a brilliant golden-yellow compound. This compound was characterized as 1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl[2,2′-binaphthalene]-8,8′-dicarboxaldehyde, identical to gossypol (I) in all respects. This identity was demonstrated by



I

means of its melting point, mixed melting point, elemental analysis, PMR, mass spectrometry, and comparison of UV and IR spectra with authentic sample spectra. Gossypol, the principal yellow pigment of cottonseed, was isolated first by Marchlewski (1) and then synthesized by Edwards (2). The structure of gossypol has been extensively studied by various workers (3).

The petroleum ether extract demonstrated activity of 131% test/control (T/C) at 50 mg/kg, 136% T/C at 25 mg/kg, and 125% T/C at 16 mg/kg. Gossypol demonstrated activity of 150% T/C at 10 mg/kg and 129% T/C at 5 mg/kg in the 3PS test system. Activity in the 3PS system is defined as an increase in the survival of treated animals over that of controls resulting in a T/C ≥ 125% (4).

EXPERIMENTAL[3]

The woody stems and stem barks (10 kg) of *M. speciosissima* were ground and extracted exhaustively in a Lloyd-type extractor with petroleum ether. After removal of the solvent in air, the residue (69 g) was repeatedly extracted with ether and filtered. The

[1] Identification was confirmed by Dr. Robert E. Perdue, Medicinal Plant Resource Laboratory, Plant Genetics and Germ Plasm Institute, Beltsville, Md. A reference specimen was deposited in that herbarium. The plant was collected in Puerto Rico in February 1972.

[2] Of the Division of Cancer Treatment, National Cancer institute, National Institutes of Health, Bethesda, Md.

[3] Carbon and hydrogen analyses were performed by Chemalytics, Inc., Tempe, Ariz. PMR, IR, and mass spectra were determined using a Varian T-60 spectrometer, a Beckman IR-33, and a Hitachi Perkin-Elmer double-focusing spectrometer (model RMU-6E), respectively. The melting points were determined on a Kofler hot-stage apparatus and are uncorrected.

The taurine-conjugated acids are strongly bound even at low pH and in the presence of 0.1 *M* chloride. The glycocholic acid binding is strongly pH dependent, and effective binding is only obtained at low pH in the absence of competing chloride ion. However, previous work (9) showed that much more glycocholate is bound by cholestyramine at higher pH (5.4–6.8), even in the presence of 0.15 *M* sodium chloride or sodium carbonate. Under these conditions, the glycocholate is present almost entirely in the dissociated anionic form.

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Tumor-Inhibitory Agent from *Montezuma speciosissima* (Malvaceae)

S. D. JOLAD, R. M. WIEDHOPF, and J. R. COLEˣ

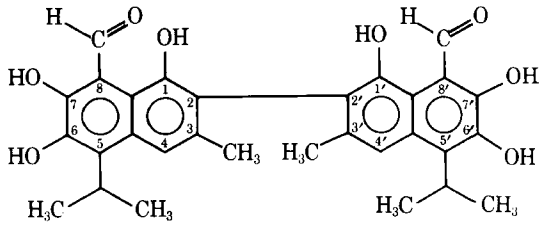
Abstract □ The petroleum ether extract of *Montezuma speciosissima* Sesse and Moc. demonstrated tumor-inhibiting properties in the P-388 lymphocytic leukemia test system (3PS). The constituent responsible for this activity was shown to be a symmetrically substituted 2,2′-dinaphthol, identified as gossypol ($C_{30}H_{30}O_8$).

Keyphrases □ *Montezuma speciosissima*—extract, PMR, UV, IR, and mass spectra, gossypol isolated □ Gossypol—isolated from *Montezuma speciosissima*, PMR, UV, IR, and mass spectra, tumor inhibitory activity □ Tumor inhibition—gossypol, isolated from *Montezuma speciosissima*, PMR, UV, IR, and mass spectra

As a result of the continuing search for plants yielding tumor-inhibiting constituents, it was found that the petroleum ether extract of the woody stems and stem barks of *Montezuma speciosissima* Sesse and Moc. (Malvaceae)[1] showed inhibitory activity toward the P-388 lymphocytic leukemia test system (3PS)[2].

DISCUSSION

The ether-soluble portion of the petroleum ether extract, after mild alkali extraction followed by careful acidification, gave a product which, when subjected to silica gel column chromatography, yielded a brilliant golden-yellow compound. This compound was characterized as 1,1′,6,6′,7,7′-hexahydroxy-5,5′-diisopropyl-3,3′-dimethyl[2,2′-binaphthalene]-8,8′-dicarboxaldehyde, identical to gossypol (I) in all respects. This identity was demonstrated by



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means of its melting point, mixed melting point, elemental analysis, PMR, mass spectrometry, and comparison of UV and IR spectra with authentic sample spectra. Gossypol, the principal yellow pigment of cottonseed, was isolated first by Marchlewski (1) and then synthesized by Edwards (2). The structure of gossypol has been extensively studied by various workers (3).

The petroleum ether extract demonstrated activity of 131% test/control (T/C) at 50 mg/kg, 136% T/C at 25 mg/kg, and 125% T/C at 16 mg/kg. Gossypol demonstrated activity of 150% T/C at 10 mg/kg and 129% T/C at 5 mg/kg in the 3PS test system. Activity in the 3PS system is defined as an increase in the survival of treated animals over that of controls resulting in a T/C ≥ 125% (4).

EXPERIMENTAL[3]

The woody stems and stem barks (10 kg) of *M. speciosissima* were ground and extracted exhaustively in a Lloyd-type extractor with petroleum ether. After removal of the solvent in air, the residue (69 g) was repeatedly extracted with ether and filtered. The

[1] Identification was confirmed by Dr. Robert E. Perdue, Medicinal Plant Resource Laboratory, Plant Genetics and Germ Plasm Institute, Beltsville, Md. A reference specimen was deposited in that herbarium. The plant was collected in Puerto Rico in February 1972.

[2] Of the Division of Cancer Treatment, National Cancer institute, National Institutes of Health, Bethesda, Md.

[3] Carbon and hydrogen analyses were performed by Chemalytics, Inc., Tempe, Ariz. PMR, IR, and mass spectra were determined using a Varian T-60 spectrometer, a Beckman IR-33, and a Hitachi Perkin-Elmer double-focusing spectrometer (model RMU-6E), respectively. The melting points were determined on a Kofler hot-stage apparatus and are uncorrected.

combined ether solution (about 2.5 liters) was subjected to mild alkali extraction (5% aqueous sodium carbonate). The alkaline phase was carefully neutralized with 10% aqueous hydrochloric acid, extracted with ether, and dried, and the solvent was removed *in vacuo*.

The resulting product (26 g) was then subjected to silica gel (130 g) column chromatography. The column was eluted with hexane–ether (4:1) followed by ether, acetone, and methanol. Fractions from the hexane–ether eluate, which showed two spots on TLC, were combined, and the solvent was removed *in vacuo*. The residue was treated with an excess of acetone, stirred, and filtered. The filtrate, on concentration *in vacuo*, gave a buff-colored precipitate, which was removed by filtration. On further concentration followed by cooling, the filtrate gave tiny yellow needles of gossypol, which were collected by filtration. More crystals were obtained from the mother liquor by repeating this step.

Crystallization from methylene chloride–petroleum ether yielded pure gossypol in long brilliant golden-yellow needles, mp 178–180°; this melting point was not depressed by mixing with an authentic sample[4]. Several melting points have been reported for gossypol: 184° (5), 199° (6), and 214° (7); gossypol exists in three crystalline modifications, each with a characteristic melting point.

All spectra obtained by UV, IR, PMR, and mass spectrometry agreed with literature data (8–10).

[4] The authors are grateful to the Division of Drug Research and Development, National Cancer Institute, Bethesda, Md., for providing an authentic sample.

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COMMUNICATIONS

Isolation of Lysergide (LSD) with Agarose-Bound Antibodies to Lysergic Acid

Keyphrases □ Lysergide—isolation, agarose-bound antibodies to lysergic acid, column chromatography □ Antibodies—lysergide binding, coupled to agarose, isolation of lysergide

To the Editor:

Several research groups (1–3) have obtained antiserums specific to lysergide (D-lysergic acid diethylamide, LSD). These antiserums have been useful in the development of sensitive and specific radioimmunoassay procedures for lysergide in plasma and urine (2, 3).

We have now succeeded in purifying lysergide-binding antibodies from sheep antiserum, utilizing affinity chromatographic (4) columns prepared by covalent binding of D-lysergic acid to a suitable derivative of beaded agarose. Purified specific antibodies are themselves coupled to beaded agarose and subsequently utilized for the selective absorption of lysergide from solution. Elution of lysergide from the column is readily achieved.

D-Lysergic acid (I) was readily coupled to the long chain hydrazide derivative of beaded agarose (II) (5) (Scheme I). To 10 g of II was added 40 mg of D-lysergic acid dissolved in 32 ml of saline–phosphate buffer (0.15 *N* NaCl and 0.01 *M* phosphate, pH 7.4) containing 5% ethanol. Then 50 mg of water-soluble carbodiimide reagent (III) was added, and the pH was adjusted to 5.50. After stirring for 24 hr, the agarose was washed thoroughly with buffer and made up to a final volume of 20 ml. The washings were analyzed for uncoupled D-lysergic acid by UV absorbance at 308 nm and the resultant affinity adsorbent (IV) was calculated to contain 2.5 μmoles (0.67 mg) of D-lysergic acid/g.

To a column of IV (1.5 ml, 0.5 × 15 cm, packed in pH 7.4 buffer) was added 1.0 ml of undiluted lysergide antiserum obtained from sheep after immunization with a lysergic acid–human serum albumin conjugate (3). [This antiserum (10 μl) showed the ability to bind 50% of 1 ng of tritium-labeled lysergide at a dilution of 1:1000 (3).] One hour was allowed for equilibration prior to elution from the column. Both protein absorbance (280 nm) and ability to bind tritiated lysergide in the radioimmunoassay system were monitored.

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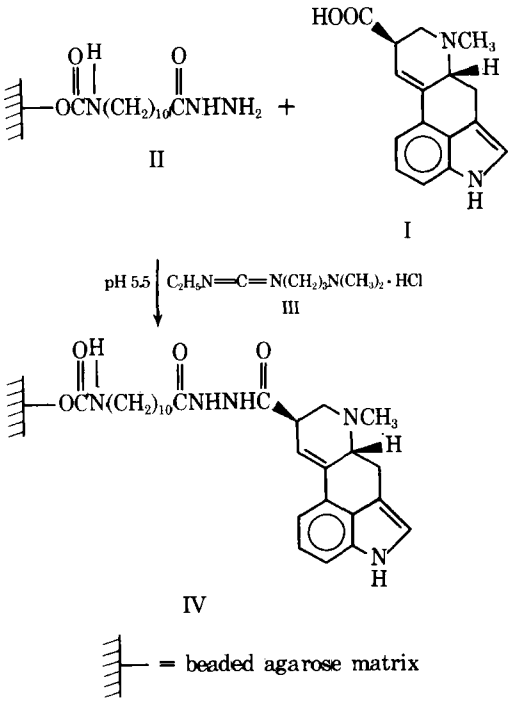

Scheme I—Coupling of D-lysergic acid (I) to long chain hydrazide derivative of agarose (II)

The results (Fig. 1) show that the lysergide antibody was indeed retained; it could be eluted by the use of 0.1 *M* acetic acid, 0.01 *N* HCl, 6 *M* guanidine, lysergic acid, or lysergide. Fractions eluted with acid were neutralized with sodium hydroxide to pH 7.4 (phenol red) immediately upon collection. Other protein components of the serum, with no lysergide-binding ability, passed through the column in early fractions. In several experiments, it was determined that the protein content of the lysergide binding effluent was approximately 0.9 mg of protein/ml of original antiserum (6.0 nmoles/ml). As shown in Fig. 1, about two-thirds of this amount was eluted from the column with dilute hydrochloric acid; the remaining third required the use of 6 *M* guanidine hydrochloride (later removed by dialysis).

Microzone electrophoresis studies on material from fraction 11 (Fig. 1) indicated the presence of a faint band in the γ-globulin region and the absence of other protein bands characteristic of the whole antiserum components. This material was employed in subsequent studies.

For coupling of the agarose–hydrazide derivative (II) to the specific lysergide antibody, the hydrazide was first activated by conversion to the corresponding azide with nitrous acid (5). Three grams of the cold, freshly activated, azide derivative was then added to a cold solution of a mixture of 0.3 mg of antilysergide and 5 mg of bovine serum albumin carrier in phosphate buffer (pH 7.4). After 20 hr, the mixture was filtered and washed well with buffer. As a control preparation, 5 mg of bovine serum albumin alone was coupled to II under identical conditions. From subsequent calculations, it was determined that the coupled antilysergide content of the agarose was about 4.7×10^{-5} μmole/ml of agarose (sufficient

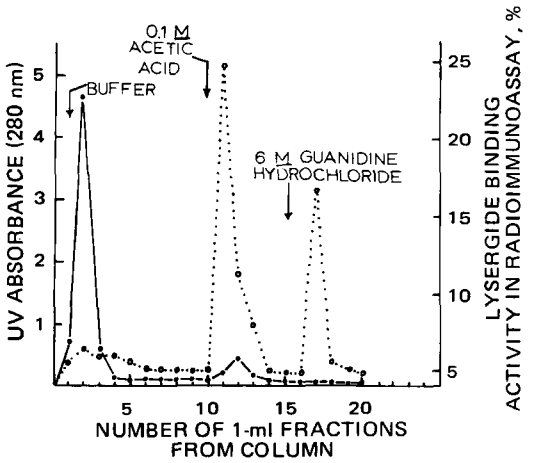

Figure 1—*Selective adsorption of specific lysergide antibodies from sheep antiserum by means of D-lysergic acid immobilized on beaded agarose (IV). Curve A (solid line) indicates protein retention by the affinity column as measured by UV spectral analysis of effluent fractions. About 8% of the protein was specifically retained. Curve B (dotted line) indicates that the major portion of the immunochemical binding ability of the effluent was contained in the fractions eluted with acid or guanidine hydrochloride. Bovine serum albumin (0.5%) was added to the elution buffer for stabilization of the antibody activity.*

to bind 30 ng of lysergide, with each divalent antibody binding 2 moles of lysergide).

To determine the selective retention of lysergide by the column, tritiated lysergide of specific activity 1.9 Ci/mmole was employed[1]. The radioactive lysergide (1 or 5 ng in a typical run) was placed in 1.0 ml of buffer on the column of adsorbent (1.5 ml as before) and allowed to equilibrate for 1 hr. Elution was accomplished with buffer or 0.01 *N* hydrochloric acid (Fig. 2). One-milliliter effluent fractions were counted *via* scintillation counting techniques, and the quantities of lysergide in each fraction were calculated.

Under these conditions, typical results indicated 64% retention of 1 ng of lysergide and 79% retention

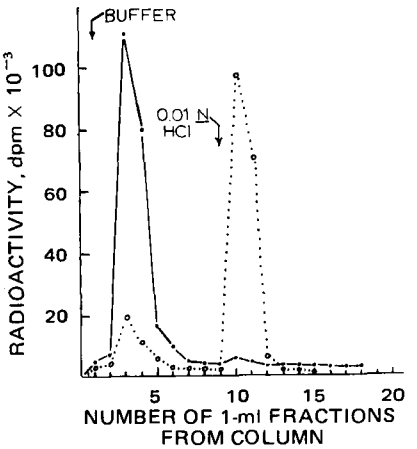

Figure 2—*Selective removal of tritiated lysergide from buffer by insolubilized antibodies. Curve A (solid line) indicates bovine serum albumin coupled to the matrix. Curve B (dotted line) indicates bovine serum albumin plus antibody coupled to the matrix.*

[1] New England Nuclear Corp., Boston, Mass.

of a 5-ng sample. Retained material was eluted from the column with 0.01 N hydrochloric acid. Under identical conditions, the column composed of coupled bovine serum albumin alone retained less than 1% of the radioactive material. Essentially identical results were obtained when ^{3}H-lysergide was added initially to the columns in pooled human plasma or human urine rather than buffer. Columns appeared to be equally effective upon at least one reuse after washing thoroughly with 0.01 N hydrochloric acid and then buffer.

Upon further quantitation and development, and perhaps with the use of a more specific antibody of higher titer, the described techniques might be utilized to advantage in conjunction with quantitative radioimmunoassay determinations of lysergide, either to isolate selectively very small quantities of lysergide far below the current sensitivity capabilities or to separate lysergide from tissue homogenates which may contain materials that render present assay methods inexact. Such methodology may be of importance in the study of lysergide levels in the brain and other organs.

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Determination of Protein Binding Parameters in Systems Involving Interaction between Sites

Keyphrases □ Protein binding—determination of parameters in systems involving interaction between sites, evaluation of method □ Binding, protein—parameters, interaction between sites, evaluation of method □ 1-Anilinonaphthalene-8-sulfonate—discussion of binding parameters

To the Editor:

A recent report by Ma *et al.* (1) utilized a log Q *versus* $\bar{\upsilon}$ plot to determine the protein binding parameters of 1-anilinonaphthalene-8-sulfonate in systems where interactions between binding sites were assumed to be occurring. As a result of this study, it is our feeling that the basic concepts underlying such systems and the application of the log Q *versus* $\bar{\upsilon}$ plot (2) to them should be reviewed. The intent of this communication is to ensure the proper use of the log Q *versus* $\bar{\upsilon}$ treatment by investigators encountering binding behavior similar to that reported by Ma *et al.* (1) for the interaction of 1-anilinonaphthalene-8-sulfonate with serum albumin.

The log Q *versus* $\bar{\upsilon}$ method applies to systems involving interactions between binding sites when n (number of) binding sites are initially all equivalent insofar as the binding of a drug molecule is concerned. As soon as one such molecule is bound, however, the affinity constant for a subsequent molecule's binding to a neighboring site is altered. The binding of successive molecules further alters the affinity constant for a remaining unoccupied site. Thus, in the case of n sites, the intrinsic binding constants $K_1, K_2, \ldots, K_n$ are not equal but may progressively increase or decrease. To calculate affinity constants under such conditions, a function Q is used:

$$Q = \bar{\upsilon}/(n - \bar{\upsilon})[D] \qquad \text{(Eq. 1)}$$

where $\bar{\upsilon}$ is the number of moles of drug bound per mole of protein at a free drug concentration $[D]$, with n representing the number of binding sites possible (2). A plot of log Q *versus* $\bar{\upsilon}$ is made, and the limiting values at $[D] \rightarrow 0$ and $[D] \rightarrow \infty$ give K_1 and K_n, respectively. Also, the limiting slopes, $d \ln Q/d\bar{\upsilon}$, at $\bar{\upsilon} \rightarrow 0$ and $\bar{\upsilon} \rightarrow n$ give $(n - 1)(K_2 - K_1)/nK_1$ and $(n - 1)(K_n - K_{n-1})/nK_{n-1}$, respectively (2). The log Q *versus* $\bar{\upsilon}$ plot is generally appropriate when K values are close (ratio of largest to smallest much less than 1000).

In using the log Q *versus* $\bar{\upsilon}$ plot, it should be noted that Eq. 1 implies that the value of n must be known or at least inferred with confidence in order to make a determination of the intrinsic binding constants. Ma *et al.* (1) suggested that n can be determined as that assumed value that best provides a linear log Q *versus* $\bar{\upsilon}$ plot. This usage is not proper, because the semilogarithmic plot need not approach linearity even when the value for n is known. As indicated by Eq. 1, the relationship between log Q and $\bar{\upsilon}$ is strictly linear only when the n binding constants are independent and equivalent. In such a situation, Q obviously is independent of free drug concentration and, indeed, is equal to the intrinsic binding constant. Thus, with independent and equivalent sites, a plot of log Q *versus* $\bar{\upsilon}$ is linear with a slope of zero.

When the binding sites are not independent, *i.e.*, when there is enhanced or inhibited binding of a small molecule to an available site, two situations may arise. In one case, the ratio of intrinsic binding constants, $K_{(s+1)}/K_{(s)}$, is a constant independent of s for all values of s from 1 to n (2). The constant ratio may be greater or less than unity, depending on whether interactions result in facilitated or inhibited binding. In the other system, the ratio of binding constants, $K_{(s+1)}/K_{(s)}$, is not constant for all values of s from 1 to n. Both systems will be considered.

In the first case, where a constant ratio of binding constants is present, a log Q *versus* $\bar{\upsilon}$ plot may have a region of linearity but the plot would not be expected

of a 5-ng sample. Retained material was eluted from the column with 0.01 N hydrochloric acid. Under identical conditions, the column composed of coupled bovine serum albumin alone retained less than 1% of the radioactive material. Essentially identical results were obtained when ^{3}H-lysergide was added initially to the columns in pooled human plasma or human urine rather than buffer. Columns appeared to be equally effective upon at least one reuse after washing thoroughly with 0.01 N hydrochloric acid and then buffer.

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The log Q *versus* $\bar{\upsilon}$ method applies to systems involving interactions between binding sites when n (number of) binding sites are initially all equivalent insofar as the binding of a drug molecule is concerned. As soon as one such molecule is bound, however, the affinity constant for a subsequent molecule's binding to a neighboring site is altered. The binding of successive molecules further alters the affinity constant for a remaining unoccupied site. Thus, in the case of n sites, the intrinsic binding constants $K_1, K_2, \ldots, K_n$ are not equal but may progressively increase or decrease. To calculate affinity constants under such conditions, a function Q is used:

$$Q = \bar{\upsilon}/(n - \bar{\upsilon})[D] \qquad \text{(Eq. 1)}$$

where $\bar{\upsilon}$ is the number of moles of drug bound per mole of protein at a free drug concentration $[D]$, with n representing the number of binding sites possible (2). A plot of log Q *versus* $\bar{\upsilon}$ is made, and the limiting values at $[D] \rightarrow 0$ and $[D] \rightarrow \infty$ give K_1 and K_n, respectively. Also, the limiting slopes, $d \ln Q/d\bar{\upsilon}$, at $\bar{\upsilon} \rightarrow 0$ and $\bar{\upsilon} \rightarrow n$ give $(n - 1)(K_2 - K_1)/nK_1$ and $(n - 1)(K_n - K_{n-1})/nK_{n-1}$, respectively (2). The log Q *versus* $\bar{\upsilon}$ plot is generally appropriate when K values are close (ratio of largest to smallest much less than 1000).

In using the log Q *versus* $\bar{\upsilon}$ plot, it should be noted that Eq. 1 implies that the value of n must be known or at least inferred with confidence in order to make a determination of the intrinsic binding constants. Ma *et al.* (1) suggested that n can be determined as that assumed value that best provides a linear log Q *versus* $\bar{\upsilon}$ plot. This usage is not proper, because the semilogarithmic plot need not approach linearity even when the value for n is known. As indicated by Eq. 1, the relationship between log Q and $\bar{\upsilon}$ is strictly linear only when the n binding constants are independent and equivalent. In such a situation, Q obviously is independent of free drug concentration and, indeed, is equal to the intrinsic binding constant. Thus, with independent and equivalent sites, a plot of log Q *versus* $\bar{\upsilon}$ is linear with a slope of zero.

When the binding sites are not independent, *i.e.*, when there is enhanced or inhibited binding of a small molecule to an available site, two situations may arise. In one case, the ratio of intrinsic binding constants, $K_{(s+1)}/K_{(s)}$, is a constant independent of s for all values of s from 1 to n (2). The constant ratio may be greater or less than unity, depending on whether interactions result in facilitated or inhibited binding. In the other system, the ratio of binding constants, $K_{(s+1)}/K_{(s)}$, is not constant for all values of s from 1 to n. Both systems will be considered.

In the first case, where a constant ratio of binding constants is present, a log Q *versus* $\bar{\upsilon}$ plot may have a region of linearity but the plot would not be expected

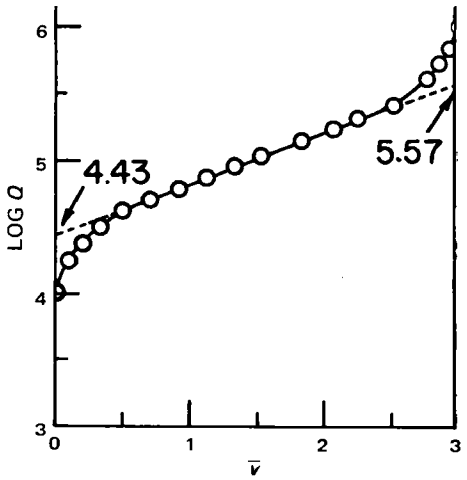

Figure 1—*Relationship between log* Q *and* $\bar{v}$ *for the binding of a hypothetical drug where* n = 3 *with increasing affinity constants of* $K_1 = 10^4$, $K_2 = 10^5$, *and* $K_3 = 10^6$.

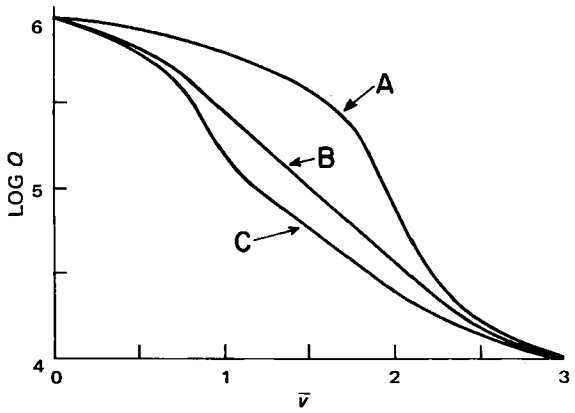

Figure 2—*Relationship between log* Q *and* $\bar{v}$ *for the binding of hypothetical drugs where* n = 3 *in all cases. The affinity constants used were: in A,* $K_1 = 10^6$, $K_2 = 5 \times 10^5$, *and* $K_3 = 10^4$; *in B,* $K_1 = 10^6$, $K_2 = 10^5$, *and* $K_3 = 10^4$; *and in C,* $K_1 = 10^6$, $K_2 = 5 \times 10^4$, *and* $K_3 = 10^4$.

to be linear over the entire $\bar{v}$ range. As discussed by Edsall *et al.* (2) and as depicted by the hypothetical data in Fig. 1, Eq. 1 does not predict a linear semilogarithmic relationship.

The data in Fig. 1 were generated by assuming three dependent sites where a constant ratio of binding constants of 10 occurs. The constants used were $K_1 = 10^4$, $K_2 = 10^5$, and $K_3 = 10^6$. Figure 1 does show, however, that linearity may be found over a large range of $\bar{v}$ (0.5–2.5 in this hypothetical case). It is evident that, in accordance with Eq. 1, curvature in a log Q *versus* $\bar{v}$ plot should not be used to indicate that an erroneous selection of the n value was made. When linearity is found over a large range of $\bar{v}$ values, the interpretation should be that the linearity is a consequence of the unique and constant ratio among the intrinsic binding constants.

In a system where the ratio of affinity constants, $K_{(s+1)}/K_{(s)}$, is not constant for all values of s from 1 to n, curvature and even breaks in a log Q *versus* $\bar{v}$ plot are to be expected, depending on the relationship and magnitude of the differences between the intrinsic binding constants. As an example, Fig. 2 shows the relationship between log Q and $\bar{v}$ for three hypothetical systems. In these systems, $n = 3$ and, as opposed to Fig. 1, inhibited binding is assumed upon successive site occupation. For curve B of Fig. 2, a constant ratio of affinity constants was used ($K_1 = 10^6$, $K_2 = 10^5$, and $K_3 = 10^4$). This curve is included only for comparison. In curve A, where the assumed constants were $K_1 = 10^6$, $K_2 = 5 \times 10^5$, and $K_3 = 10^4$, the ratio K_2/K_1 is 25 times that of K_3/K_2. An expected marked curvature is found in this case. In curve C, where $K_1 = 10^6$, $K_2 = 5 \times 10^4$, and $K_3 = 10^4$, the ratio K_3/K_2 is four times larger than the ratio K_2/K_1. Deviations from linearity are apparent in this hypothetical system also, but it might be interpreted as approximately linear for the $\bar{v}$ region between 1 and 2.

In each curve in Fig. 2, nonlinearity is again a consequence of the relationship between the intrinsic binding constants and not solely dependent on the value for n. Thus, the use of the log Q *versus* $\bar{v}$ treatment of binding data depends on knowing or reasonably estimating the number of binding sites. Figure 2 demonstrates that linearity in a log Q *versus* $\bar{v}$ plot should not be used to determine n as was suggested by Ma *et al.* (1).

One other consideration should be pointed out with respect to analyzing a system where a constant ratio of binding constants is found. It is not correct to extrapolate the linear portion of a log Q *versus* $\bar{v}$ plot to $\bar{v} = 0$ and $\bar{v} = n$, where $[D] \rightarrow 0$ and $[D] \rightarrow \infty$, to determine K_1 and K_n, respectively, as was done by Ma *et al.* (1). At best, such extrapolated values should be used as gross first estimates of the binding constants. Significant errors in affinity constants may be introduced by simply extrapolating the linear region of the relationship as opposed to extrapolating limiting data. Theory dictates that the extrapolated limiting data determine K_1 and K_n.

To use the method properly, data collected in these extremes must be accurate. Figure 1 illustrates the inaccuracy introduced when an extrapolation of the linear portion of the log Q *versus* $\bar{v}$ plot is used to determine K_1 and K_n. Hypothetical data are used in this figure where the ratios K_2/K_1 and K_3/K_2 are equal. The experimental data collected in regions where limiting slopes and intercepts are characterized are subject to large experimental error, regardless of whether or not a constant ratio of affinity constants is operative. Therefore, Edsall and Wyman (3) recommended that extrapolated values using limiting data be used as first estimates of affinity constants which may be used to calculate an expected curve. If the calculated curve significantly differs from the observed, new affinity constants are estimated and the curve fitting is continued until reliable constants are found.

Protein binding systems analogous to those of 1-anilinonaphthalene-8-sulfonate are certainly possible, and it may be proper to interpret and analyze them by assuming that interactions of the type invoked by Ma *et al.* (1) do take place. It is our hope that this communication will clarify the factors that make such an analysis correct and complete.

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Group Contribution Data Obtained by Ion-Pair Extraction of Prostaglandin B_2 with Aliphatic Amines

Keyphrases □ Ion-pair extraction—prostaglandin B_2 with aliphatic amines, methylene group contribution, partition coefficients □ Partition coefficients—ion-pair extraction of prostaglandin B_2 with aliphatic amines, methylene group contribution □ Prostaglandin B_2—ion-pair extraction with aliphatic amines, methylene group contribution, partition coefficients

To the Editor:

The concept of ion-pairs (1) is significant in various areas including chemistry, organic synthesis (2), solvolytic reactions (3), partition chromatography (4–6), pharmaceutical analysis (7–10), and facilitated absorption of quaternary ammonium compounds in the GI lumen (11). Also, systematic studies on the ion-pair extraction successfully yielded functional group contributions to the thermodynamic parameters involved in transferring a given molecule from one phase to another (12, 13). Although our original study (14) was initiated to explore the practical utility of ion-pair formation of prostaglandins in all of these aspects, the present communication is specifically concerned with the free energy of transferring a methylene group from water to chloroform as determined by the ion-pair extraction process.

The importance of the lipophilicity of a drug molecule in the structure–activity relationship has been recognized and, consequently, great effort has been directed toward developing some means by which the partition coefficient of a drug molecule between oil (or lipophilic biological membrane) and aqueous systems can be predicted accurately (13, 15–17). Presently, the most popular way to achieve this goal appears to be the group contribution approach, in which the partition coefficient of a molecule is considered as the sum of the contribution of each functional group present in the molecule. Experimentally, an accurate measurement of the partition coefficient becomes very difficult as the value becomes either too large or too small.

Since the apparent partition coefficient of acidic or basic compounds depends not only upon the pH of the aqueous layer but also upon the type and the concentration of the counterions, by adjusting these factors it is possible to control the partition coefficient so that it is experimentally measurable. For a given charged solute, if the structural changes in a series of counterions are far removed from the electrostatically interacting charged portions of the molecules, it can be assumed that the change in the partition coefficient observed is independent of the electrostatic interaction and that the change solely reflects the structural modification of the counterions.

We determined the ion-pair extraction coefficient (K) of prostaglandin B_2 (I) with 10 simple aliphatic amines ranging from n-propyl- to n-dodecylamine at 25°:

$$K = \frac{\{A^-BH^+\}}{[A^-]\{BH^+\}} \qquad \text{(Eq. 1)}$$

The partition system consisted of chloroform presaturated with water and tromethamine buffer (pH 8 and ionic strength 0.1 M) also presaturated with chloroform. Throughout the paper, prostaglandin B_2, amines, and ion-pair are represented as AH, B, and A^-BH^+, respectively. The braces { } and brackets [ ] indicate the molar concentrations of a given species in chloroform and aqueous buffer, respectively. Since, in most cases, the system is very dilute with respect to prostaglandin B_2 and amines, the molar concentrations are assumed to be equal to thermodynamic activities.

Simple algebraic manipulation of Eq. 1 yields:

$$P_{obs} = K[BH^+] + P_{app} \qquad \text{(Eq. 2)}$$

where:

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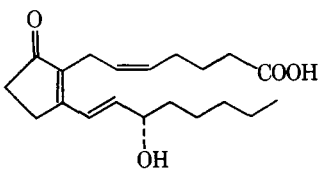
In deriving Eq. 2, it was assumed that ion-pairs do not exist in aqueous solutions, not only because the dielectric constant of water is extremely high but also because the concentrations of prostaglandin B_2 and amines in aqueous solutions are low. The dissociation constant of ion-pairs in an organic solvent of low dielectric constant is usually in the order of from 10^{-4} to 10^{-7} M (12, 18). Therefore, in our study, it was assumed that no significant dissociation takes place in chloroform. Moreover, at pH 8 the concentrations of neutral species of our acid and bases (*i.e.*, AH and B) are in the order of 0.1% of the total concentrations, because the pKa values of prostaglandin B_2 and



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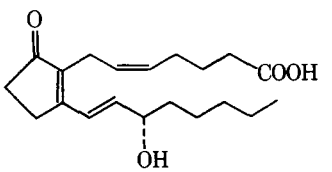

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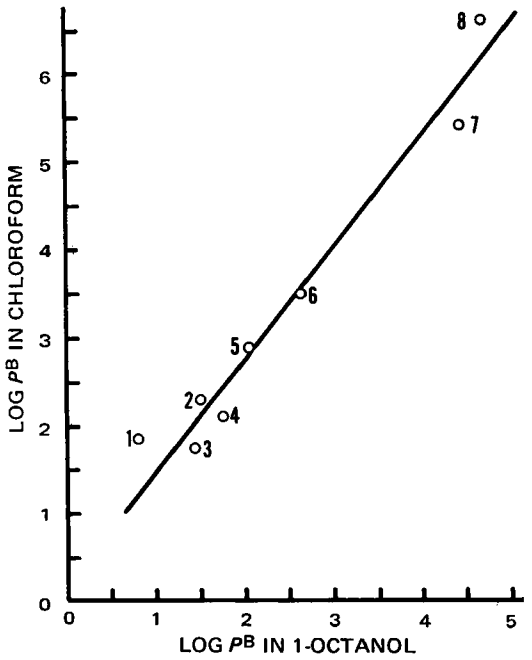

Figure 1—*Relationship between the intrinsic partition coefficient* (P^B) *of aliphatic amines between chloroform and water and that of the same amines between 1-octanol and water at 25°. The straight line is log* $P^B_{CHCl_3}$ = *1.276 log* P^B_{oct} + *0.171. Amines are: 1,* n-*butyl; 2,* n-*hexyl; 3, triethyl; 4, dipropyl; 5,* n-*heptyl; 6, dibutyl; 7, tributyl; and 8, dihexyl.*

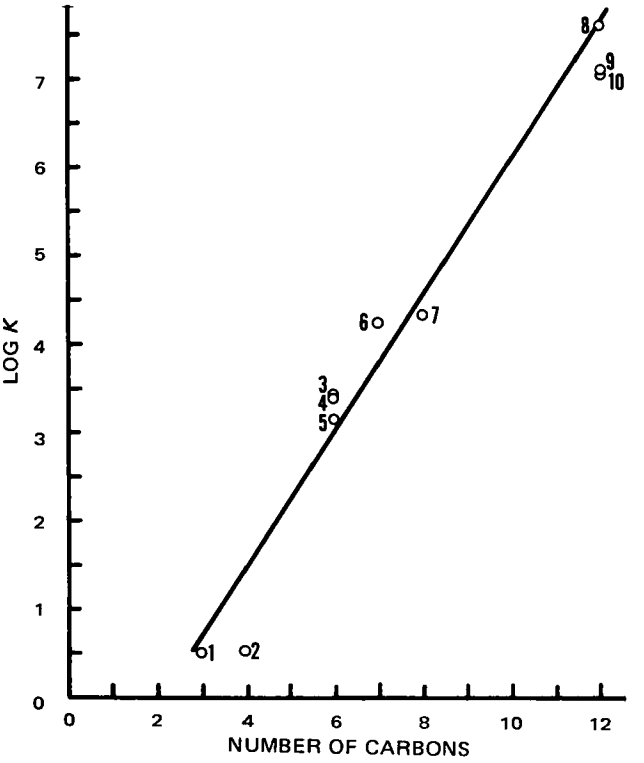

Figure 2—*Logarithm of the ion-pair extraction coefficient* (K) *for prostaglandin* B_2*–amine pairs at 25° as a function of carbon numbers in the amines. The slope of the straight line is 0.77. Amines are: 1,* n-*propyl; 2,* n-*butyl; 3, triethyl; 4, dipropyl; 5,* n-*hexyl; 6,* n-*heptyl; 7, dibutyl; 8,* n-*dodecyl; 9, dihexyl; and 10, tributyl.*

amines employed are approximately 5 (14) and 11, respectively. Equation 2 predicts a linear relationship between P_{obs} and [BH^+].

The values of P_{obs} were determined by spectrophotometrically measuring the total concentrations of prostaglandin B_2 ($\epsilon_{280} \sim 2.50 \times 10^4$ in water) both in chloroform and aqueous layers at distribution equilibrium for at least five different amine concentrations. To reduce the experimental errors in determining P_{obs}, from 1:1 to 1:20 phase volume ratios were employed. We found it very difficult to measure [BH^+] accurately at equilibrium, because in most cases [BH^+] was extremely low. Therefore, we calculated [BH^+] from the independently determined apparent partition coefficients of amines at pH 8, the initial concentrations of amines, [A^-] measured at equilibrium in the presence of amines, P_{obs}, and the known phase volume ratio. The entire calculation procedure was described elsewhere (14).

The apparent partition coefficients of amines at pH 8 were obtained by dividing {B} at equilibrium by the amine concentrations in the aqueous layer. The former was determined by nonaqueous titration (19), and the latter was obtained by subtracting {B} from the known initial concentration of the amine in chloroform. Experimental data, when plotted following Eq. 2, invariably showed negative deviations from the linearity as [BH^+] increased, presumably due to the premicellar aggregation of the amines in the aqueous layers. Therefore, the initial slopes were measured as the values of K.

Determination of the apparent partition coefficient of amines at pH 8 not only allowed us to calculate [BH^+] in Eq. 2 at distribution equilibrium but also resulted in an independent measurement of methylene group contribution. The intrinsic partition coefficients of the amines were calculated from the apparent partition coefficients determined at pH 8 and the known pKa values of amines (20). The pKa values at ionic strength 0.1 M were obtained from the so-called thermodynamic pKa values (pKa°) by correcting the activity coefficient of ionic species using the Davies equation (21); pKa at ionic strength 0.1 M = pKa° + 0.224. The pKa° values of dihexyl- and n-dodecylamine, which were not available in the literature, were assumed to be the same as those of dibutyl- and n-heptylamine, respectively.

The intrinsic partition coefficients of amines thus obtained were linearly correlated with those for the 1-octanol–water system (15), as shown in Fig. 1. The slope, intercept, and correlation coefficient are 1.27, 0.20, and 0.978, respectively, in excellent agreement with the relationship reported in the literature (22). Since the methylene group contribution to the partition coefficient for a 1-octanol–water system is 0.50 in terms of a logarithmic scale (*i.e.*, π_{CH_2} = 0.50) (15), the linear relationship shown in Fig. 1 strongly implies that the methylene group contribution to the partition coefficient for a chloroform–water system is 0.5 × 1.27 = 0.64. This value is again in good agreement with the value reported in the literature (13, 23, 24).

When the logarithms of the intrinsic partition coefficients of amines we obtained were plotted

against the carbon number, a slope of 0.64 was obtained for a series of primary amines. As expected, secondary and tertiary amines showed somewhat smaller partition coefficients than the primary amines of the same carbon number.

On the other hand, when the logarithms of K were plotted against the carbon number of the amines (Fig. 2), a slope of 0.77 was obtained. The discrepancy between the methylene group contribution obtained by the ion-pair extraction studies (0.77) and that obtained by the measurement of the intrinsic partition coefficient (0.64) was small but significant and deserves further comment. The ion-pair extraction coefficient defined by Eq. 1 is an overall equilibrium constant for the formation as well as the partition of ion-pairs:

$$K_1 = \frac{[A^-BH^+]}{[A^-][BH^+]} \qquad \text{(Eq. 5)}$$

$$K_2 = \frac{\{A^-BH^+\}}{[A^-BH^+]} \qquad \text{(Eq. 6)}$$

$$K = K_1K_2 \quad \text{or} \quad \log K = \log K_1 + \log K_2 \qquad \text{(Eq. 7)}$$

According to Eq. 5, ion-pairs are formed in the aqueous phase. In reality, this formation most likely would occur at the interface. No matter where the ion-pairs are formed, the separation of K into K_1 and K_2 shown in Eq. 7 should be valid. As shown in Eq. 7, for the slope shown in Fig. 2 truly to represent K_2 and ultimately the methylene group contribution, K_1 must be constant regardless of the amine employed. The fact that we obtained 0.77 for the methylene group contribution from ion-pair extraction studies and 0.64 from ordinary partition experiments strongly suggests that, at least in certain cases, the group contribution data obtained from ion-pair studies may not be generally the same as obtained in straightforward partition experiments.

In the present study, as the amine becomes more hydrophobic, the equilibrium constant for the ion-pair formation (K_1) appears to increase at a rate of $\Delta \log K_1/CH_2 = 0.13$. It is to be seen in the future whether such a deviation, intrinsically derived from the assumption that the change in the ion-pair extraction coefficient (ΔK) is independent of the electrostatic interaction, is universal or not.

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REVIEWS

Plastic Containers for Pharmaceuticals—Testing and Control. By JACK COOPER. World Health Organization, Geneva 27, Switzerland (WHO Offset Publication No. 4), 1974. 204 pp. 22 × 28 cm. Price SW. fr. 32, $12.80.

Packaging of pharmaceuticals in plastic containers has increased at an unprecedented rate. Testing and control procedures are needed for the pharmaceutical industry. During revision of the second edition of the *International Pharmacopoeia*, the World Health Organization recognized the importance of general monographs on this subject and it was at their request that Mr. Cooper prepared this comprehensive report. Mr. Cooper's experiences with the sorption of preservatives by rubber closures are well documented. As Chairman of a Commission for the Standardization of Regulatory Requirements for Plastic Containers set up by Fédération Internationale Pharmaceutique, he is cognizant of the potential problems in the use of plastics in pharmacy. He has done an excellent job of compiling his information.

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ACHIEVING THE FULL VIEWPOINT

As this is written, a young girl lies in a hospital in New Jersey, her very existence being sustained by a battery of complex machines and instruments. We use the word "existence," rather than "life," because depending upon one's definition of life, it can be argued that medical technology is just maintaining isolated physiological processes and that, in fact, she is already dead.

The controversy that swirls about this girl is both heartrending and complex. Some physicians agree that her brain function has been irreversibly destroyed, but nonetheless contend that on legal, liability, or ethical grounds they are prohibited from unplugging the apparatus that maintains her artificial existence. Even the theologians appear to be split, with opinions divided as to where the limit lies concerning our obligation to maintain human life.

Our purpose here is not to offer any opinion on this most unfortunate case. Hopefully, it will have been resolved long before this journal reaches our readers.

We do, however, feel that it drives home the need for broad-based input in reaching decisions on many matters and issues facing society. We recently heard a clergyman comment upon the case mentioned above, and he pointed out that this question could not have even arisen twenty years ago because the technology did not exist at that time. We now have technology available that presents us with options never before known. This is true whether the subject is heart transplants, or environmental protection, or child-proof containers.

Each of these questions involves a multiplicity of issues and of viewpoints. If a complex medical decision arises, it is no longer adequate or satisfactory to create a panel consisting solely of physicians for purposes of reaching a decision. Other health-care practitioners must be included, as well as persons who reflect other considerations such as the moral and legal aspects.

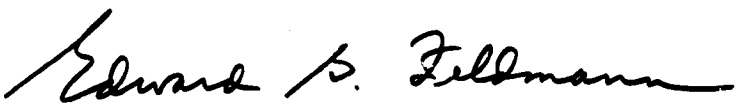
Many corporations are beginning to recognize this fact and are broadening their boards of directors. Formerly, banks were run exclusively by bankers, industries by industrialists, and so on. Slowly, but surely, this is changing. And contrary to initial expectations, inclusion of these nonspecialists appears to have been beneficial to the dynamic growth and financial health of the corporations involved.

Presently, there is a movement toward expansion or reorganization of the state boards which regulate the respective professions. The intent again is to broaden the makeup of these groups in order to provide a cross section of backgrounds and input. If we subscribe to the belief that a profession exists to serve the public, then it would seem to follow that the public should have at least some voice in how that profession is regulated and, most importantly, should be in a position to ascertain that the particular regulatory body is regulating on behalf of the public rather than in the self-interests of that profession.

Even the Food and Drug Administration has been routinely including a consumer representative and an industry representative on each of its recently appointed advisory panels.

And finally, scientists, too, need to recognize that their actions and decisions are subject to reasonable review, scrutiny, and question. "Scientific freedom" is limited by the same constraints as the exercise of other "freedoms." And nowhere in science is the need for broadened input of viewpoint more evident than in the matter of grant review and approval.

Two years ago, our column was titled "Peer Review under Fire" and discussed the unhappiness then prevalent with the National Institutes of Health and related federal government grant programs. Rather than improve, this situation has deteriorated further during the interim. In our opinion, the prognosis for peer review as it presently operates remains dismal at best, unless there is a prompt reorganization of the system to involve participation by nonscientists.



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REVIEW ARTICLE

Pharmacokinetics and Clinical Use of Cephalosporin Antibiotics

CHARLES H. NIGHTINGALE *x, DOUGLAS S. GREENE *, and RICHARD QUINTILIANI ‡

Keyphrases □ Cephalosporins—11 antibiotic derivatives, pharmacokinetics, absorption, metabolism, excretion, clinical considerations, review □ Antibiotics—11 cephalosporin derivatives, pharmacokinetics, absorption, metabolism, excretion, clinical considerations, review □ Pharmacokinetics—11 cephalosporin antibiotic derivatives, absorption, metabolism, excretion, clinical considerations, review □ Absorption—11 cephalosporin antibiotic derivatives, pharmacokinetics, clinical considerations, review □ Metabolism—11 cephalosporin antibiotic derivatives, pharmacokinetics, clinical considerations, review □ Excretion—11 cephalosporin antibiotic derivatives, pharmacokinetics, clinical considerations, review

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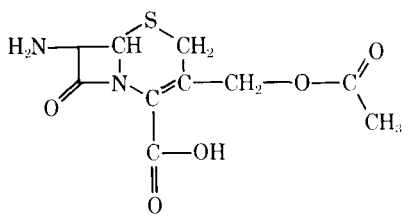
Cephalosporins are a series of compounds containing the 7-aminocephalosporanic acid [7-amino-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid acetate] nucleus (I). Although cephalosporin C was one of the original and active members of the series, being derived from the fermentation products of *Cephalosporium acremonium*, it has not proved to be useful clinically. However, I has been used as the basis for the synthesis of various derivatives (II–XII). An excellent review of the history, isolation, synthesis, analysis, and physicochemical properties of cephalosporins was published (1). The purpose of this work is to review the pharmacokinetics and clinical use of cephalosporin antibiotics.

PHARMACOLOGY AND PHARMACOKINETICS

Cephalexin—Cephalexin, 7-(D-2-amino-2-phenylacetamido) - 3 - methyl - 8 -oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylic acid (II), is an orally active semisynthetic derivative of I with a spectrum of activity against both Gram-positive and Gram-negative bacteria. It has relatively low solubility in water; *i.e.*, 1–2 mg was dissolved in 1 ml of water at 37° (2). At pH 2 and 8, the solubility was increased to approximately 120 and 100 mg/ml, respectively (2). The increase in solubility as a function of pH is due to the zwitterionic nature of the drug. Cephalexin is very unstable at physiological pH (3), showing the presence of decomposition products within 20 min of solution in water. It shows less deterioration at low pH. However, the drug is most stable at pH 4.5, its isoelectric point, and may be kept frozen when dissolved in biological fluids for long periods ($t_{1/2}$ > 90 days) (2).

Studies of the protein binding of cephalexin showed that, in concentrations usually found in therapy, less than 10% of the total drug was bound to human plasma proteins (2). At a total cephalexin concentration of 3.2 μg/ml, 6% of the total drug was in the protein-bound state (2). Cephalexin is removed from the body solely by the processes of glomerular filtration and excretion in the kidney tubules.

Cephalexin is advocated for the treatment of infections of the upper and lower respiratory tract, genitourinary system, skin and soft tissue, and bones and joints and for certain other infections due to susceptible organisms. An excellent review of its antibacterial, pharmacological, and therapeutic properties was presented (4). Blood concentrations of the drug, after the usual therapeutic dose, are high enough to be bactericidal against most susceptible organisms (5).



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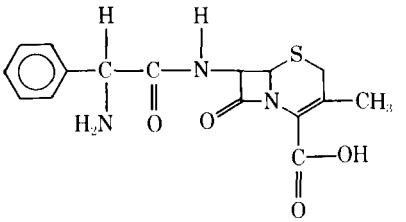


II: cephalexin

Absorption—The oral and intramuscular absorption of cephalexin in normal human subjects has been studied (2, 5–19). After oral administration, absorption was rapid and complete, with peak levels being reached within 1 hr (2, 14). The time to reach peak levels may vary between individuals after administration of the drug in capsule form. This finding can be attributed to a lag time in the absorption process due to dosage form effects. Following a 500-mg dose given as a 5% suspension, cephalexin appeared in the serum in 9 min compared with 38 min when given in capsule form (14).

The peak height is dose dependent, usually reaching a level of 18 μg/ml after a 500-mg dose (14, 20). Single doses of 125, 250, 500, and 1000 mg in 12 fasting subjects produced average peak levels of 4.5, 9, 18, and 32 μg/ml, respectively, at 1 hr (20). Some investigators (6, 10, 17, 19, 21) found peak blood levels that were slightly lower than those reported by others (5, 7, 8, 13, 20, 22). Lower peak values may be related to the time food is ingested.

When cephalexin is administered with food, there is a delay in the onset of absorption, a lower peak, and a prolongation of blood levels (14, 20). A total of 82% of a 500-mg dose was found in the urine when the patients were fasting compared to 73% when the dose was taken with food, indicating that the total amount of drug absorbed was not appreciably altered (20). Similarly, a 1-g dose of cephalexin resulted in a peak serum level of approximately 31 μg/ml in fasted subjects but only 20.9 μg/ml after a standard meal (11). The peak time was increased to 2 hr after administration. Similar results were also reported (23). Apparently, small amounts of food do not significantly affect the absorption process (17, 24).

Absorption and excretion of cephalexin are delayed and peak serum levels are less in newborns and infants in the first 6 months of life. Peak serum levels occurred after 3 hr in these patients, 2 hr in infants 9–12 months in age, and 1 hr in children older than 1 year (25–29). Food, *i.e.*, milk, had the same effect in the infant as did food in the adult; *i.e.*, lower peak levels occurred at later times (26).

The influence of disease on cephalexin absorption is variable. In children (18–20 months) with a malabsorption syndrome, a reduction was reported in the excretion of unchanged cephalexin (28). With a dose of 50 mg/kg, however, effective blood levels were

reached but did not peak until 4 hr postadministration. In another study, absorption was significantly impaired in one of three patients with pernicious anemia and obstructive jaundice (30). Reports of impaired absorption due to infectious diseases are lacking with the exception of the report of decreased plasma levels with a prolonged peak time in patients with severely impaired kidney function (creatinine clearance of 0–2.5 ml/min) (18).

Distribution—Cephalexin is widely distributed in body tissues. The only tissues in which concentrations of the drug were higher than in serum were the kidneys and liver (31, 32). Significant levels of the drug in animals also were demonstrated in other tissues, including lung, spleen, fat, heart muscle, and bone. In humans, levels in the appendix, stomach, gallbladder, omentum, peritoneum, and vein and tumor tissues were lower than in serum following an oral dose of 0.5 or 1.0 g (33). Bile levels were lower than serum levels after a dose of 0.5 or 1.0 g of cephalexin (34, 35). Peak levels of 0.3–32.0 μg/ml were observed at 2–3 hr. Probenecid administration significantly increased biliary levels of the antibiotic, probably due to interference with renal excretion producing higher blood levels. Low tissue levels (<1 μg/ml) were found in the gallbladder wall suspension of two patients.

Low levels of the drug in saliva were reported (36, 37). Detectable levels in the aqueous humor at 1 hr and peak levels of 0.75–2.0 μg/ml at 2–3 hr after an oral dose of 2 g were reported (38). These concentrations occurred whenever the serum level exceeded 10 μg/ml.

Cephalexin does not appear to penetrate into the cerebrospinal fluid in significant amounts in the absence of meningeal inflammation (28, 30, 39).

The appearance of cephalexin in the serum and exudate of normal and leukemic patients was studied (40). After a 500-mg oral dose, peak serum levels (12 μg/ml) were obtained at approximately 1.5 hr. Exudate levels reached a peak of 5 μg/ml within the same period and appeared to decline at a slow rate. After 4 hr, the level declined to approximately 3.5 μg/ml, illustrating that cephalexin rapidly appears in skin exudates, with a half-life of elimination from that fluid of approximately 4 hr.

In leukemic patients, distribution into skin exudate was decreased. The ratio of the mean exudate level, expressed as a percent of the serum level, between normal and leukemic patients was 210, 17, and 3.2 after 1, 2, and 3 hr postadministration, respectively. The data suggest that the volume of distribution of the antibiotic may be changed in the leukemic patient. It is not clear if this result is due to the disease state or to the influence of antineoplastic drug therapy.

Metabolism and Excretion—Cephalexin is not metabolized in the body and is rapidly excreted unchanged, principally by the kidney, although small amounts are also excreted in the bile. Studies with labeled ^{14}C-cephalexin in rats and mice showed that the drug is not metabolized and is eliminated unchanged *via* the kidney (41). In rats, 84% of an orally administered dose appeared in the urine after 24 hr, with 77% appearing in the first 4 hr. Fifteen percent was recovered in the feces and was attributed to biliary excretion. In humans, cephalexin does not undergo enzymatic metabolism (42). Excretion of cephalexin in the urine occurs partly through glomerular filtration (66%) and partly by tubular secretion (33%) (24).

The renal clearance of cephalexin was calculated to be 210 ml/min/1.73 m^2, with 72.2% of the total plasma clearance of the drug being accounted for by renal clearance (43). The ratio between the renal and plasma clearances was 1.69, indicating a significant (28%) nonrenal excretion of cephalexin. Since no metabolite of cephalexin has been found (2), it is difficult to account for the apparent nonrenal excretion. Kirby and Regamey (44) reported values of 252 ± 5 and 248 ± 11 ml/min/1.73 m^2 for renal and serum clearances, respectively. When these values are considered, it can be seen that the entire dose can be accounted for through renal excretion. Other values reported for the plasma clearance of cephalexin are 260 and 376 ml/min (8, 11).

The administration of probenecid shortly before or simultaneously with cephalexin enhances both the peak serum concentration and the duration of activity of the antibiotic in the serum (6, 11, 17, 20). The administration of 500 mg of probenecid with 1 g of cephalexin caused an increase in the mean peak serum level of 79% over the mean peak serum level achieved with a similar dose without probenecid (*i.e.*, an increase from 20.9 to 36.7 μg/ml) (11). Levels at 6 hr were also increased, ranging from 4.5 to 9.7 μg/ml (mean of 7.1 μg/ml). Similar findings also were reported (7, 20, 45).

In subjects with normal renal function, cephalexin had a serum half-life of approximately 0.6–2.1 hr after oral administration (2, 7, 8, 10, 12, 14, 16, 18, 19, 39, 42–47), with a mean and standard deviation of 1.2 ± 0.47 hr. In fasting subjects, the mean serum half-life was 49.5 min, but this value increased to 76.5 min in subjects who received cephalexin with food (11). This latter value probably was due to the half-life being calculated during the time when both absorption and elimination were occurring simultaneously. The serum half-life following oral administration is prolonged in neonates and infants. A half-life of approximately 5 hr in newborn infants and of 2.5 hr in infants 3–12 months of age was demonstrated (27, 29).

Due to its extremely short half-life and long dosing interval (4–6 hr), little accumulation of cephalexin in the serum of patients with normal renal function has been observed (24, 48). Blood level curves and urinary excretion values following doses of 250 or 500 mg of cephalexin every 6 hr were unchanged after 1, 3, and 5 days (20). Similarly, the blood level curves and mean urinary recovery values in six subjects after 1-g doses every 6 hr were not significantly different from those following a single dose (11). Some accumulation was noted by O'Callaghan *et al.* (14), who found that both peak concentration and the length of time the serum levels exceeded 12.5 μg/ml

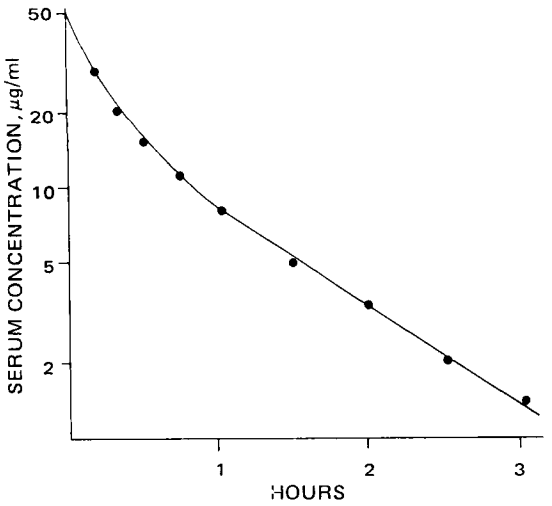


Figure 1—*Serum levels for cephalexin administered intravenously (rate constants for data generation from Table I).*

were significantly increased after the fifth 1-g dose (every 6 hr). These increases did not occur after doses of 500 mg, which is to be expected for a drug with a short half-life and relatively long dosing interval.

Between 82 and 100% of an orally administered dose appeared in the urine after 250- and 500-mg doses of cephalexin (13, 50). However, urinary recoveries of 29–89% were reported in patients with urinary tract infections (49). Urinary levels of the antibiotic are, therefore, high, and peak levels in the urine may be more than 100 times higher than in the serum. Thornhill *et al.* (17) found mean urinary concentrations in a 6-hr period of 830 μg/ml and 1.1 mg/ml after 250 and 500 mg of cephalexin, respectively. Mean urinary concentrations of 2.3 and 5.0 mg/ml were observed in the first 1–2-hr period following administration of 0.5 and 1.0 g of cephalexin, respectively (24). Similar results also were observed (6, 20).

The clearance of cephalexin is prolonged in the presence of renal dysfunction, resulting in higher and more sustained serum levels; *i.e.*, an increase in the serum half-life occurs as renal function decreases (18, 19, 39, 51–55). Serum half-lives of 7.7, 10.8, and 13.9 hr were observed in subjects with creatinine clearances of 13.5, 9.2, and 4 ml/min, respectively (19). No significant increase was found in the serum half-life in patients with creatinine clearances of 45 ml/min; but when kidney function fell below one-fourth of normal, *i.e.*, creatinine clearance of less than 30 ml/min, the half-life increased significantly (55). Similarly, only moderate increases were noted in plasma half-lives when the creatinine clearance was between 10 and 40 ml/min, but a rapid increase was noted thereafter when the value fell below 10 ml/min (52).

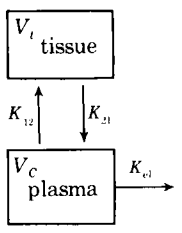
Peak serum levels after single oral doses of 500 mg of cephalexin were reached in 1–2 hr, with appreciable levels still present at 12–24 hr (53). In patients with a creatinine clearance of less than 3 ml/min, however, the peak serum levels occurred at 4–8 hr, and levels of 5 and 6 μg/ml were still present at 48 and 60 hr, respectively. Detectable levels were present for 72–90 hr. A delay in the time to reach the peak serum level occurred in patients with severe renal failure (18, 51). Repeated administration of the drug in patients with renal impairment led to accumulation (18, 19).

Urinary excretion of cephalexin is inversely related to the serum half-life and directly related to the creatinine clearance. Less than 5% of a single dose of cephalexin was excreted within 24 hr in patients with creatinine clearances of less than 2.5 ml/min (18). The urinary excretion of cephalexin at 6, 24, and 48 hr after a 500-mg dose was 7.5, 23, and 37%, respectively (51). Satisfactory antibacterial levels were attained. Satisfactory urine levels were found in patients with creatinine clearances of 3 ml/min (56); however, in another report (19), acceptable levels were not achieved if the creatinine clearance was less than 9 ml/min.

Hemodialysis resulted in a fourfold decrease in serum levels within 7 hr (54). Similarly, a fivefold reduction in half-life was reported when hemodialysis was employed for 12 hr (18, 53). Peritoneal dialysis resulted in a twofold decrease in serum levels after 7 hr (54). Bennett *et al.* (57) described an adjusted dosage regimen as a guide in treating patients with various degrees of renal failure.

Pharmacokinetic Considerations—When the available literature blood level data after intravenous administration of cephalexin are carefully examined (Fig. 1), it appears that the drug follows a two-compartment open model (Scheme I). All investigators analyzed their data using a one-compartment open model. This model may lead to errors in the calculation of the volume of distribution and the elimination and absorption rate constants (58, 59). A reevaluation of selected literature data is shown in Tables I and II. The half-lives reported using a one-compartment open model analysis are equivalent to the half-life of the β-phase. The value of the half-life of the distributive (α) phase was 0.15 hr. Therefore, to describe completely the time course of the drug in plasma, it is necessary to remove at least two or three blood samples within the first 45 min after administration.

Cephalexin, which is only 6% protein bound in the therapeutic range, is distributed widely within plasma water and some extracellular water. The volume of the central compartment using a two-compartment open model analysis is 10.8 liters. In contrast, the volume of distribution of cefazolin (V), another cephalosporin antibiotic, is approximately 4 liters. Cefazolin, being 90% protein bound, is distributed mainly in the plasma water. When the volume of distribution found for cephalexin is compared to that of



Scheme I—Two-compartment open model

Table I—Pharmacokinetic Parameters for Cephalexin Administered Intravenously (Literature Data)[a]

Parameter	Literature Reference 12	14	45	47	Mean ± *SD*
α, hr^{-1}	4.327	4.645	4.382	3.710	4.270 ± 0.400
β, hr^{-1}	0.976	0.773	0.671	0.646	0.766 ± 0.151
K_{12}, hr^{-1}	1.151	1.682	1.546	1.211	1.384 ± 0.238
K_{21}, hr^{-1}	2.369	1.899	1.791	1.846	1.976 ± 0.266
K_{el}, hr^{-1}	1.783	1.889	1.716	1.299	1.671 ± 0.260
$T_{1/2}\alpha$, hr	0.160	0.149	0.159	0.187	0.163 ± 0.016
$T_{1/2}\beta$, hr	0.710	0.897	1.095	1.072	0.943 ± 0.179
V_c, liters	14.544	9.018	8.902	10.681	10.786 ± 2.633
Vd_{ss}, liters	21.612	16.748	17.355	17.687	18.350 ± 2.208
Vd_β, liters	26.568	22.050	23.301	21.462	23.345 ± 2.281
Vd_{ext}, liters	34.997	30.989	33.760	27.271	31.754 ± 3.426
$t_{1/2}$ reported by author, hr	0.7	0.8	1.1	0.61	—

[a] Analysis of data using a two-compartment open model (Scheme I).

cefazolin, the effect of protein binding on the volume of distribution of a drug is evident.

The volume of the tissue compartment into which cephalexin distributes can be found by using Eq. 1:

$$V_t = \frac{K_{12}}{K_{21}} V_c \qquad \text{(Eq. 1)}$$

where K_{12}, K_{21}, V_c, and V_t are defined in Scheme I. The volume of the tissue compartment using this formula is 7.6 liters. This value gives a total volume of distribution within the body of 18.4 liters, a value that is usually reported as Vd_{ss}, the volume of distribution at steady state.

The current literature contains only two references to values of the volume of distribution of cephalexin. A value of 15 ± 2.3 liters was found (43) after a 3-hr intravenous infusion. The formula used was:

$$Vd = \frac{i}{(p)(K_{el} \times 10^3)} \qquad \text{(Eq. 2)}$$

where i is the infusion rate, p is the concentration of cephalexin in the plasma at steady state, and K_{el} is the elimination rate constant for cephalexin. The value of K_{el} was determined by using a log-linear regression on the postdistribution portion of the blood level *versus* time curve after infusion. The value of K_{el} obtained in this manner is analogous to the value of beta obtained from a two-compartment open model analysis. Therefore, the value determined by the use of Eq. 2 is equivalent to the value of Vd_β, the volume that can be determined during the β-phase in a two-compartment open model.

The value of Vd_β shown in Tables I and II when the data were analyzed with a two-compartment open model is approximately 23 liters, which is significantly greater than the reported value of 15 liters (43). The reason for the difference becomes clear when Eq. 2 is examined. If the value of K_{el} were too large, it would cause the value of the volume of distribution calculated by this formula to be too small. One factor that could cause the value of K_{el} to be very large would be the method by which it was obtained. Since it has been shown that cephalexin follows two-compartment open model kinetics, it follows that after infusion is terminated, the resultant curve will have an α- (distributive) phase and a β- (disposition or elimination) phase. Therefore, use of the entire curve after infusion instead of the late postinfusion curve results in an overestimation of the value of K_{el}.

The effect of using this method of analysis of two-compartment open model data is apparent if Fig. 2 is examined. A shortening of the half-life with an increased value of the rate constant is the result of the incorrect analysis of serum level data. deMaine and Kirby (43) reported a relatively short half-life (0.61 hr) for cephalexin. It is difficult, however, to determine if α-phase data were utilized in their determination.

A value of 37 liters (range of 26–53 liters) was reported for cephalexin after intramuscular administration (8). This volume of distribution was determined using:

$$Vd = \frac{\text{dose}}{(AUC)(K_{el})} \qquad \text{(Eq. 3)}$$

where AUC represents the area under the plasma level *versus* time curve from time zero to time infinity. Since the value of K_{el} is analogous to the value of beta used in the two-compartment open model, the volume found by the use of this equation is equal to the value of Vd_β.

The value of 37 liters (8) is much larger than the value of Vd_β determined using the two-compartment open model. The overestimation (8) is attributable to

Table II—Pharmacokinetic Parameters for Cephalexin Administered Orally (Literature Data)[a]

Parameter	Literature Reference 12	13	14	Mean ± *SD*
α, hr^{-1}	3.609	4.247	4.463	4.106 ± 0.444
β, hr^{-1}	0.661	0.989	0.936	0.862 ± 0.176
K_a, hr^{-1}	2.388	1.493	1.889	1.923 ± 0.448
K_{12}, hr^{-1}	1.173	1.000	1.203	1.125 ± 0.110
K_{21}, hr^{-1}	1.652	2.496	2.572	2.240 ± 0.511
K_{el}, hr^{-1}	1.445	1.740	1.624	1.603 ± 0.149
Lag time, hr	0.422	0.280	0.234	0.312 ± 0.098
$T_{1/2}\alpha$, hr	0.192	0.167	0.155	0.171 ± 0.019
$T_{1/2}\beta$, hr	1.048	0.724	0.741	0.837 ± 0.182
V_c, liters	16.800	10.442	9.919	12.387 ± 3.831
Vd_{ss}, liters	28.734	15.213	14.558	19.501 ± 8.002
Vd_β, liters	36.702	19.030	17.211	24.314 ± 10.767
Vd_{ext}, liters	49.989	26.023	21.382	32.464 ± 15.353
$t_{1/2}$ reported by author, hr	1.1	—	0.8	—

[a] Analysis of data using a two-compartment open model (Scheme I).

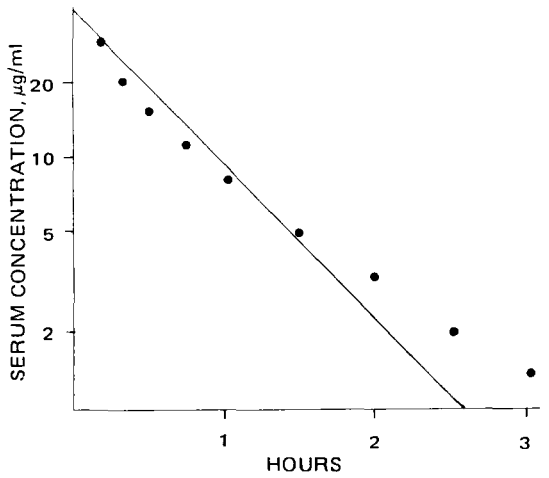
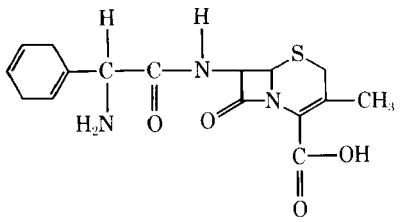


Figure 2—*Log-linear regression on biphasic data.*

an underestimation of the value of K_{el}. It appears that these investigators overestimated the half-life of cephalexin (1.1–2.3 hr) due to the fact that absorption and elimination occurred during the entire sampling time. In studies where the absorptive processes are allowed to vary due to improper fasting or precipitation at the site of muscular injection, the apparent absorption rate constant might be smaller and the $t_{1/2}$ for elimination might appear longer than expected. These phenomena could explain the wide range of elimination half-lives reported in the oral studies.

Use of the one-compartment open model to fit cephalexin data results in errors in the volume of distribution term. Since the therapeutic range of the drug is high, the use of the larger *Vd* (extrapolated) term as a one-compartment open model volume of distribution will not compromise the efficacy of cephalexin therapy since the normal dose results in blood levels that are usually well above the minimum inhibitory concentration (MIC). Since the half-life of cephalexin is short compared to the normal dosing interval (6 hr), drug accumulation is negligible and errors in calculation of the absorption rate constant are not significant. It appears that routine pharmacokinetic analysis of cephalexin could be accomplished using the one-compartment open model treatment without introducing serious error, provided the $t_{1/2}$ was determined correctly from intravenous data, *i.e.*, after the distributive phase and the volume terms are properly interpreted.

Cephradine—Cephradine, 7-[D-2-amino-2-(1,4-cyclohexadien-1-yl)acetamido]-3-methyl-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid monohydrate (III), is a semisynthetic cephalosporin



III: cephradine

used orally, intramuscularly, and intravenously. The structure of cephradine is similar to that of cephalexin, the only difference being in the six-membered ring. Cephalexin has three double bonds forming an aromatic system while cephradine has two double bonds in the same ring. The antibacterial activity of cephradine is similar to that of cephalexin (60).

Cephradine is a white crystalline powder with a molecular weight of 349.4 (61). The synthesis of cephradine has been discussed (62). Cephradine is freely soluble in aqueous solvents. It is a zwitterion, containing both an alkaline amino group and an acidic carboxyl group. In the pH range of 3–7, cephradine exists as an internal salt (63). Cephradine is stable for 24 hr at 25° within the pH range of 2–8. Since it is stable in acidic media, there is little loss of activity in the gastric fluid; losses of less than 7% have been reported (64).

Cephradine is weakly bound to human serum proteins. In one study (63), the drug was less than 20% bound to the serum proteins. At a serum concentration of 10–12 μg/ml, 6% of the total drug was in the protein-bound complex. Another study (65) found that at a total concentration of 10 μg/ml, 28% of the drug was in the protein-bound state; at a total concentration of 100 μg/ml, 30% of the drug was in the protein-bound state. This study also showed that the addition of serum to cephradine decreased antibiotic activity. Another study (61) showed that the protein binding of cephradine varied from 8 to 20%, depending on the concentration of the drug. However, a study by Gadebusch *et al.* (64) found no change in the MIC of cephradine toward either *Staphylococcus aureus* or *Escherichia coli* after the addition of human serum.

Absorption—Cephradine has been administered orally, intramuscularly, and intravenously and is well absorbed using these routes. This large degree of absorption is evidenced by recoveries of approximately 100% of the administered dose in the urine (63, 66–69). In one study (63), tritiated cephradine was administered orally to human subjects in the form of a 250-mg capsule. Urine and feces were collected and assayed for the drug; 92% of the drug was present in the urine at the end of 24 hr, while minimal amounts were present in the feces at the end of 72 hr. These findings are evidence for the claim that cephradine is well absorbed orally and apparently not excreted in the bile.

After oral administration of cephradine, the time of peak serum concentration was at 1 hr with peak concentration in the serum ranging from 6 to 7 μg/ml after a 250-mg capsule (63, 68, 69). After the administration of 500 mg of cephradine in the form of two 250-mg capsules, peak serum levels were in the range of 11–18 μg/ml (60, 63, 66–68). After the administration of 500 mg of cephradine as an oral suspension, a peak serum concentration of 19.5 μg/ml was found at 0.5 hr (68). When human subjects were given 1 g of cephradine, the peak levels ranged from 15 to 24 μg/ml (60, 68).

After the administration of 1 g im, a peak level of 10.4 μg/ml at 2 hr was attained (63). While the peak

concentration of cephradine after intramuscular injection was lower than that after oral administration, the areas under the curve were identical. One advantage of the intramuscular route over the oral route was that the concentration of the antibiotic in the serum was below 1 μg/ml at 4 hr after oral administration while the concentration after intramuscular administration was 6.8 μg/ml (63).

One study (66) found that the rate of absorption after oral administration was influenced by the presence of food but that the extent of absorption was not affected. Serum levels at 30 min after administration were 7.9 μg/ml for nonfasting volunteers and 15.8 μg/ml for fasted volunteers. However, peak levels were not significantly different; values of 19.2 and 18.3 μg/ml were found for nonfasting and fasted volunteers, respectively.

After intravenous administration of cephradine, the peak levels are reached immediately. In one study, a serum concentration of 56 μg/ml at 7 min after injection was attained after administration of 1 g (70). In another study (63), the administration of 1 and 0.5 g of cephradine intravenously gave levels of 86.3 and 46.0 μg/ml, respectively, 5 min after injection. When cephradine was given as an intravenous constant infusion (0.166 g/hr), a steady-state level of 4.8 μg/ml was attained after 3 hr (70).

Distribution—In a study performed with mice (71), cephradine was widely distributed throughout the body. In this study, 50 mg/kg of tritiated cephradine was administered orally to mice and tissue levels were determined as a function of time. The levels in the stomach, small intestine, and kidneys were all above 100 μg/g of tissue. The liver had a level of more than 50 μg/g of tissue at 1 hr after administration. Almost all other body tissues had levels above 1 μg/g of tissue. The levels found in the brain were 0.8–2.2 μg/g of tissue in the 24-hr period of the study.

The tissue levels of cephradine in humans after oral dosing were examined (67). The level in lung tissue 6 hr after administration of 500 mg po was 0.46 μg/g of tissue (serum level was 0.58 μg/ml). Three hours after dosing, adipose tissue levels of 0.46–0.56 μg/g of tissue were found (serum level was 0.31–0.60 μg/ml). Cephradine levels for various other tissues were also given (67). The volume of distribution of cephradine was reported to be 21 liters/1.73 m^2 (72).

Metabolism and Excretion—A study in mice and dogs using paper chromatographic techniques showed that no metabolites of cephradine could be found in the urine (71, 73). A study in humans also could not find any metabolites of cephradine (63).

When 500 mg of cephradine was administered orally to subjects having cholecystolithiasis (67), the levels of cephradine in the bile ranged from 2.2 to 41.0 μg/ml at 3–7.5 hr after administration.

The major route for the elimination of cephradine is renal excretion. Cephradine is removed from the body by the processes of glomerular filtration and tubular secretion (63). A study (61) in which probenecid was coadministered with cephradine resulted in a prolonged half-life and elevated serum levels.

The recovery of unchanged cephradine in the urine ranged from 78.3 to 95.9% (63, 66–68). The urinary concentration of cephradine in the first 2 hr after administration of 500 mg was between 1.1 and 3.2 mg/ml (63, 66, 68).

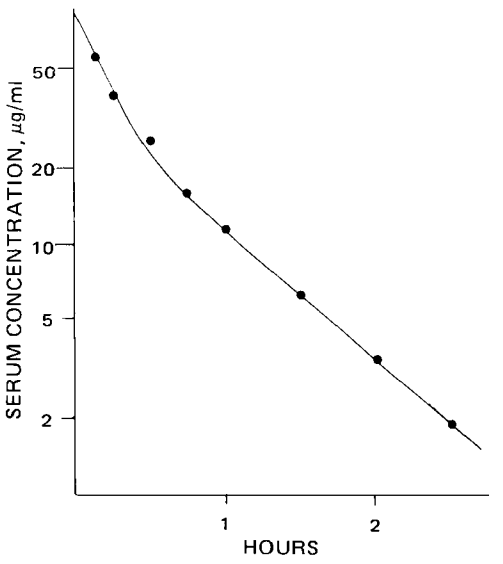


Figure 3—*Serum levels for cephradine administered intravenously (rate constants for data generation from Table III).*

The half-lives of elimination of cephradine were 32 min (70) after intravenous administration, 40–50 min after intramuscular administration (63), and 42 min after oral administration (68). These studies were performed in patients having normal kidney function. During a constant infusion, patients having a creatinine clearance of 125 ml/min had serum and renal clearances of cephradine of 435 and 367 ml/min, respectively (72).

In another study (74), 500 mg of cephradine was administered orally to patients with impaired kidney function. The time required to excrete 90% of the administered cephradine was 24 hr in normal patients. Individuals having impaired kidney function required much longer to excrete a similar amount of the drug.

Pharmacokinetic Considerations—Little work has been performed on the pharmacokinetics of cephradine in subjects with normal and impaired kidney function. Further study is necessary to elucidate these parameters.

A plot of cephradine serum concentration *versus* time (Fig. 3) indicates a biexponential decay. Therefore, fitting of the data for cephradine should be performed using a two-compartment open model. The effects of using a one-compartment open model analysis when a two-compartment open model is indicated were discussed previously.

When the data for intravenous cephradine were fit to a two-compartment open model, the half-life was approximately 45 min. The values of the parameters obtained are listed in Table III.

The volume of distribution was 22 liters, compared to 21 liters/1.73 m^2 reported previously (72). A value of 17 ± 3.9 liters/1.73 m^2 also was reported (61).

The pharmacokinetics of cephradine probably will

Table III—Pharmacokinetic Parameters for Cephradine Administered Intravenously (Literature Data)[a]

Parameter	Literature Reference 63	70	70[b]	Mean ± SD
α, hr^{-1}	5.411	7.369	9.20	6.390 ± 1.385
β, hr^{-1}	0.726	1.262	1.31	0.994 ± 0.379
K_{12}, hr^{-1}	2.167	2.349	3.25	2.258 ± 0.129
K_{21}, hr^{-1}	1.720	3.893	4.69	2.807 ± 1.537
K_{el}, hr^{-1}	2.250	2.389	2.58	2.320 ± 0.098
$T_{1/2}\alpha$, hr	0.130	0.094	0.075	0.112 ± 0.025
$T_{1/2}\beta$, hr	0.969	0.549	0.529	0.759 ± 0.297
V_c, liters	7.906	10.562	9.624	9.234 ± 1.878
Vd_{ss}, liters	17.679	16.936	—	17.308 ± 0.525
Vd_{β}, liters	24.651	19.993	—	22.322 ± 3.294
Vd_{ext}, liters	36.802	24.516	—	30.659 ± 8.678

[a] Analysis of data using a two-compartment open model (Scheme I). [b] Literature values.

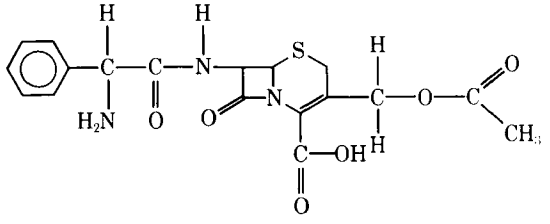
be similar to those found for cephalexin due to the similarity of their structures. The protein binding of these two compounds is not significantly different, so the volume of distributions should be similar, as was found in the reanalysis of literature data (Table III).

Cephaloglycin—Cephaloglycin, 7-(2-amino-2-phenylacetamido)-3-(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid, acetate (ester) (IV), is a zwitterion of 7-(D-α-aminophenylacetamido)cephalosporanic acid and exhibits an antibacterial spectrum similar to that of other cephalosporins. It is water soluble [8 mg/ml at 25° (75)] and orally absorbed, and it is indicated for use in treating bacterial infections of the urinary tract (76). The stability of cephaloglycin is dependent on pH, degrading very rapidly in alkaline media and remaining stable under acid conditions (77, 78). The decomposition rate is 6–8%/hr in aqueous solution at pH 7.0, but at pH 4.5 the rate is less than 1%/hr (75).

Cephaloglycin exhibits moderate protein binding. Wick (79) found a decrease of 24% in antibacterial activity when the drug was in the presence of human serum as compared to buffer.

Absorption—Several investigators reported mean peak blood serum activity after a 500-mg dose in the range of 0.9–2.0 μg/ml (48, 80–85). Peak concentrations of 0.6–0.8 μg/ml were reported (83, 86), but in another study no serum levels could be detected (87). A concentration of 2.2–3.0 μg/ml was found after a 500-mg dose (48). The activity in serum is due in part to the parent compound but largely to the deacetylated metabolite (78).

Food was reported not to interfere with cephaloglycin absorption (84); however, another study (6) found decreased peak levels after a meal. Urinary recovery was also decreased. Prolonged serum activity was reported when the drug was administered with



IV: cephaloglycin

food as compared to the fasting state (84). Peak concentrations occurred 2 hr after administration (84, 86). Peak concentrations were found 1 hr after administration to fasting patients and 2 hr after administration to nonfasting individuals (6). Similar results were obtained in children 1–15 years old (88).

Peak heights were compared after oral and intramuscular doses of the drug (48). Only 25% of the orally administered dose was assumed to be absorbed.

Distribution—The antibacterial activity in the bile of rats and mice exceeded that in the circulating blood, but only 0.04–0.23% of a 200-mg/kg dose was recovered in the bile during a 24-hr period. The antibacterial activity in the tissues of mice was determined 4 hr after an oral dose of 200 mg/kg (89). The concentrations of drug in liver and kidney were 1.9 and 3.2 times greater than in the serum, respectively. Other tissues such as lung, spleen, and heart and skeletal muscle had approximately 40% of the activity of the serum. Rat liver levels were similar to blood levels after administration of 100 mg/kg of cephaloglycin, but lung and spleen levels were slightly lower and kidney levels were much higher (90).

Metabolism—Esterases can remove the acetyl group from the C-3 methyl position of I to yield desacetyl cephalosporins (91). Carefully controlled studies, by microbiological assay procedures and chromatographic techniques, showed that 2–10% of the antibiotic activity of serum and urine specimens from humans receiving cephaloglycin is due to the parent compound. The remainder of the biological activity (90–98%) is due to the active metabolite desacetylcephaloglycin (78, 92–94). The metabolite had approximately equal activity against staphylococci but was about fourfold less active against other Gram-positive cocci and Gram-negative bacilli (94).

Excretion—Cephaloglycin and its metabolite are excreted in the urine. A half-life of 2.2 μg/ml/hr was reported (85), but it is not clear how this value was obtained. Other data (6) suggest a half-life for microbiological activity of approximately 1.5 hr.

Several studies (6, 48, 82) found 25% of the orally administered activity in the urine. Stein *et al.* (85) recovered 18% of a 500-mg dose. Most recovered activity was due to the desacetyl metabolite (48, 78, 93, 94). Urine concentrations expressed as antibacterial activity after a 500-mg dose varied from 76 to 330 μg/ml. Somewhat lower (18–255 μg/ml) results were reported (86), as were ranges of 18–750 μg/ml (83) and 70–900 μg/ml (95). It was reported that probenecid increased the serum concentration of the antibacterial activity (6). However, other studies reported no increase in serum concentrations with probenecid, but the duration of assayable activity was prolonged (82, 84).

Disease, except decreasing kidney function, does not appear to affect the pharmacokinetics of this drug. An increase in the half-life of the drug was observed with decreasing renal function (85). Significant prolongation of serum half-life occurred when creatinine clearances fell below 20 ml/min. Adequate urine concentrations in all but anuric patients were observed after a 500-mg dose (85). Hemodialysis did

not appreciably affect the elimination of the drug in anuric patients (85). Peritoneal dialysis caused a 40% decrease in blood levels within 6 hr (83).

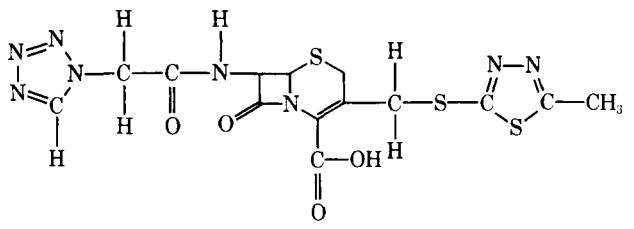
Pharmacokinetic Considerations—The data reported do not allow an accurate estimation of the pharmacokinetic parameters of this drug. Human metabolism of cephaloglycin has not been extensively studied, and blood level data obtained *via* microbiological assay do not yield accurate pharmacokinetic constants; instead, apparent constants are obtained based upon the total activity of the drug and its metabolite.

Since this drug yields poorer blood levels, has a similar spectrum, and does not yield superior urinary levels as compared to cephalexin, no clear advantage for its use is evident. From a pharmacokinetic view, cephaloglycin has many disadvantages compared to cephalexin and should be abandoned in favor of the latter drug. For practical purposes, cephaloglycin is an uninteresting drug, and further pharmacokinetic analysis is not warranted.

Cefazolin—Cefazolin, 3-{[(5-methyl-1,3,4-thiadiazol-2-yl)thio]methyl}-8-oxo-7-[2-(1*H*-tetrazol-1-yl)-acetamido]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (V), is a semisynthetic cephalosporin used parenterally. Its synthesis was described previously (96). Cefazolin is a broad spectrum antibiotic, active against most strains of Gram-positive and Gram-negative bacteria (97). It is active against *S. aureus, E. coli, Klebsiella pneumoniae,* and *Proteus mirabilis.*

The free acid form of cefazolin is insoluble in water, becomes more soluble as the pH increases, and reaches a maximum solubility between pH 5 and 6 (98). Cefazolin is available commercially as the sodium salt with a molecular weight of 476.5 (99). In the sodium salt form, cefazolin is highly soluble in aqueous solvents, slightly soluble in methanol, and practically insoluble in acetone, benzene, chloroform, and ethanol (96, 100). The pH of a reconstituted solution and region of maximum stability of cefazolin sodium lies between 4.6 and 6.0 when in an aqueous medium. In a more acidic or alkaline medium, cefazolin is hydrolyzed to products that possess little or no antibacterial activity (98, 99, 101). The pKa of cefazolin in an aqueous solution (23.8 mg of cefazolin sodium in 5.0 ml of water) was 2.3 (99).

The solid lyophilized powder of cefazolin was stable for 1 year when stored at 0–15°, with 92–95% of the original activity remaining when assayed microbiologically (96). The product information brochure (98) states that the lyophilized powder is stable for 2 years at room temperature. Various reports of the



V: cefazolin

stability of the reconstituted aqueous solutions of cefazolin sodium have been published (96, 97, 101, 102). These studies found that the drug is stable at 25° and below, with approximately 90% of the original activity remaining after 72–120 hr (96, 97). Cefazolin is less stable at 37°, and a net loss of 20% of the original activity was found after 72 hr (97). A complete survey of the stability of cefazolin, in which the effects of various diluents and temperatures were studied, is available (101).

Cefazolin is highly bound to human plasma proteins. Investigations using the equilibrium dialysis technique found values of 73% (103), 81% (104), and 84% (105, 106) of the total drug being bound to plasma proteins in normal patients. Values of 74% (107) and 86% (44, 108) were found when the ultrafiltration technique was used to analyze the plasma samples. In one study, the effect of total drug concentration on serum protein binding was studied (105). The binding to serum protein varied from 80 to 90% in the normal therapeutic range of cefazolin. The effect of protein binding on antibiotic activity is unclear, but one study (102) showed that the presence of human serum caused a decrease in antimicrobial activity of 41–48% as compared to a cefazolin solution made in buffer.

Absorption—Cefazolin is not absorbed orally, so it is only administered intramuscularly and intravenously. Cefazolin is well absorbed when administered intramuscularly, as evidenced by urinary recoveries of approximately 100% of the administered dose. The time of peak concentration after intramuscular administration was usually within 45 min to 1 hr after administration (44, 103–126). However, in several cases, the time of peak serum concentration appeared as late as 2 hr after administration (127, 128).

The peak concentration of cefazolin after an intramuscular injection of 250 mg ranged from 12.3 to 30.0 μg/ml (104, 117, 119). After an intramuscular injection of 500 mg, peak levels ranged from 11.0 to 70.0 μg/ml (44, 103, 104, 106–109, 111, 112, 114, 115, 117, 119–121, 124–126, 128, 129); after administration of 1 g of cefazolin, peak levels of 38.8–75.7 μg/ml were found. In one study where 2 g of cefazolin was administered, levels of 111.0 μg/ml were attained; 3 g of cefazolin and 1 g of probenecid resulted in a level of 200.0 μg/ml (123).

Distribution—Rats were injected intramuscularly with ^{14}C-labeled cefazolin, and the tissue concentrations were determined using radioactive and microbiological assays (98, 117, 120, 126, 130, 131). The concentration of cefazolin was very high in the kidneys, lungs, and liver. Peak levels reported (120) for these organs were 68.64, 19.52, and 22.41 μg/g of tissue, respectively. The concentration of cefazolin found in the serum at the time of peak tissue levels was 77.60 μg/ml; levels of 14.42 μg/g of heart tissue, 1.05 μg/g of brain tissue, and 5.10 μg/g of spleen tissue were also found.

In one study where synovial fluid levels of cefazolin were measured in humans (113), the levels of cefazolin found were similar to the levels of cefazolin found in the blood. After an intramuscular injection of 1.0

g, peak serum levels of 65 μg/ml were attained. The synovial fluid level at that time was approximately 75 μg/ml. Cerebrospinal fluid concentrations in human volunteers also were measured (120). In this study, intravenous doses of 10 mg/kg were given and the cerebrospinal fluid and serum levels were determined as a function of time. The serum levels varied from 40 μg/ml at 30 min after injection to 9 μg/ml at 4 hr after injection. However, the cerebrospinal fluid levels remained constant at 4 μg/ml throughout the entire study.

Studies performed on mucous membranes (132) in patients with chronic sinusitus found that the levels of cefazolin were 1.5 and 3.5 μg/g of tissue after a 250- and 500-mg intramuscular dose, respectively. The tissue used was the mucus membrane removed from the maxillary sinus. In other studies performed by these same authors, doses of 250 and 500 mg of cefazolin were administered to patients with tonsillar hypertrophy. The tissue levels attained were 3.4 and 7.6 μg/g of tissue, respectively.

Metabolism and Excretion—The metabolism of cefazolin is a very insignificant process in the elimination of the drug. Various studies using chromatographic separation techniques found that no microbiologically active metabolites were present in the urine of rats or humans (102, 117, 131). In studies where ^{14}C-cefazolin was injected intramuscularly into rats, two metabolites of cefazolin were present in the bile and one metabolite was present in the urine (122). These metabolites accounted for a small fraction of the original dose. Some metabolites were recovered in the urine and the bile (131), but the amounts recovered were very small. Another similar study found no cefazolin metabolites (120).

The bile of normal patients and patients with gallbladder and bile duct impairments was examined for cefazolin content after systemic administration of the drug (113, 121, 133). In one study (113), cefazolin was infused intravenously at a rate of 500 mg/hr for 1 hr and then at a rate of 250 mg/hr for 2 hr. During the steady state, the serum concentration of cefazolin was 50 μg/ml and the concentration of cefazolin in the bile was 12.2 μg/ml. Cefazolin biliary excretion was 0.104 ml/min/1.73 m^2. Other studies performed using patients with normal bile duct and gallbladder function found a biliary concentration twice that of the serum concentration of cefazolin (121); bile levels seven times those found in serum were observed in one study (133).

In diseased patients having a gallbladder or bile duct dysfunction, without obstruction of the bile duct, the levels in the bile were at least twice, and sometimes as high as seven times, those of the serum. These patients had cholelithiasis, cholecystitis, and a nonobstructed cystic duct. The levels found in the gallbladder tissue were similar to those found in the bile. In patients with an obstructed cystic duct, the levels of cefazolin in the bile were below those found in the serum (133).

The recovery of cefazolin in the bile of rats given 20-mg/kg im or sc doses of cefazolin was as high as 20% of the original administered dose (117). Thus, considering the high levels of drug appearing in the bile, cefazolin may be effectively used to treat infections in the bile duct or gallbladder.

Cefazolin is eliminated from the body mainly through renal excretion. Urinary recovery studies (44, 70, 98, 99, 104–109, 111, 113–118, 121, 127, 134) showed that essentially 100% of the administered dose can be accounted for as unchanged drug in the urine. The serum and renal clearances for cefazolin were reported to be 62 ± 6 and 64 ± 6 ml/min, respectively (44). Since these values are not significantly different, it can be concluded that the renal route of elimination is responsible for removal of a major percentage of the drug.

The mechanisms responsible for the elimination of cefazolin are glomerular filtration and tubular secretion. This elimination was first postulated by Regamey *et al.* (108) by comparing the creatinine clearance, a measure of glomerular filtration, to the clearance of cefazolin. The mechanisms involved in the elimination of cefazolin were further elucidated when probenecid was administered concurrently with the antibiotic (44, 114, 123). In all of these studies, a prolonged half-life and elevated serum levels characteristic of the competitive inhibition of the tubular secretion of cefazolin were found.

Numerous pharmacokinetic studies have been performed using patients with normal kidney function (44, 70, 98, 99, 103–122, 127, 128, 134, 135) and impaired kidney function (98, 99, 103, 105, 106, 109, 111, 114, 122, 128, 136). The range of half-lives reported for cefazolin after intravenous administration is 69–108 min (44, 70, 99, 108, 127, 134), with an average of 88 min. After intramuscular administration, the values of the reported half-lives range from 96 to 153 min (44, 99, 104, 105, 109, 114, 127, 134), with an average of 121 min.

In one study where multiple dosing of cefazolin was investigated, a half-life of 124 min was found after the first dose and a value of 88 min was found after a series of multiple doses (106). The reason for this change of half-life after a series of intramuscular multiple doses is unclear, but the explanation reported was that cefazolin-metabolizing enzymes were produced (106). However, since cefazolin has not been shown to be appreciably metabolized, this explanation does not seem plausible.

The renal and total body clearances of cefazolin have been studied (44, 70, 105, 106, 108, 113, 114, 128, 130). The plasma clearance found in one study was 61.5 ± 6.1 ml/min and the renal clearance was 64.6 ± 6.0 ml/min after either intravenous or intramuscular administration (44). Values of 50 ml/min (106, 128), 61.2 ml/min (105), 78.0 ml/min (130), 81.8 ml/min (113), and 83.6 ml/min (70) have been reported for renal clearance.

When probenecid was administered concurrently with cefazolin, higher levels and a prolonged half-life were found as compared to the administration of cefazolin without probenecid (123). Cefazolin was administered at a dose of 2 g intramuscularly to human volunteers. When cefazolin was administered with probenecid, peak levels of about 140 μg/ml were at-

tained. When cefazolin was administered alone, the peak level attained was about 110 μg/ml. There was a significant difference in the serum levels attained at the end of 12 hr when the two levels were compared. At that time, the cefazolin serum levels were less than 40 μg/ml with, and 10 μg/ml without, the coadministration of probenecid. Therefore, concurrent administration of cefazolin and probenecid resulted in levels above the MIC of many susceptible bacterial organisms for a prolonged period. In this study, equal cefazolin serum levels were attained when 3 g of the drug was administered concurrently with probenecid compared to the administration of 2 g of cefazolin and 1 g of probenecid. Increased serum levels and prolonged half-lives were obtained in other studies (44, 114).

Since cefazolin is eliminated from the body primarily by renal excretion, a longer half-life and a higher serum level result in a patient with impaired kidney function. The effect of kidney function on the pharmacokinetics of cefazolin has been investigated (98, 99, 103, 105, 106, 109, 111, 114, 122, 128, 136). In one study (114), normal volunteers and patients with varying degrees of renal impairment (creatinine clearance of 0–47 ml/min) were given 500-mg intramuscular doses of cefazolin. The peak levels achieved in the patients with normal kidney function ranged from 44.0 to 70.0 μg/ml at 30–60 min after injection. The peak levels attained in the patients with kidney failure ranged from 42.0 to 81.5 μg/ml at 30–120 min after drug administration. The half-life of cefazolin in the normal patients ranged from 1.37 to 1.96 hr, with a mean value of 1.60 hr. In the patients with renal impairment, the half-life ranged from 4.84 hr in a patient with a renal clearance of 47.2 ml/min to 69.2 hr in an anuric patient.

A similar study (128) found that as creatinine clearance decreased, the half-life increased. Czerwinski *et al.* (128) stated that: "in patients with serum creatinine concentrations less than 2.5 mg/dl or creatinine clearances greater than 25 ml/min modification of cefazolin dosage is not necessary since urinary excretion is rapid and exponentially related to plasma concentration. In patients with more severe renal impairment, modification of dosage is necessary to prevent accumulation." This observation was based on the fact that the plasma half-life of cefazolin does not significantly change until the creatinine clearance falls below 25 ml/min. Below 25 ml/min, the half-life of cefazolin increased rapidly (128).

In this study (128), the effect of renal function on the volume of distribution, total serum protein, and serum albumin was examined. No significant differences in total serum protein existed in normal patients as compared to patients with varying degrees of renal dysfunction. However, the volume of distribution in normal patients was much less than that of patients having severe renal impairment. In patients having normal kidney function, the volume of distribution was 13.52 ± 3.70% of body weight. Values of 13.62 ± 4.06 and 16.53 ± 2.62% of body weight were found in patients with moderate kidney function and severe impairment, respectively.

In a study in which cefazolin was administered to patients with varying degrees of renal dysfunction, patients with lower creatinine clearances generally bound cefazolin to plasma proteins to a lower extent than patients with higher creatinine clearance (105). Since there is a lower degree of protein binding in the patients with poor kidney function, this increase in the volume of distribution is expected.

In patients with renal impairment, the reported urinary recovery of cefazolin was less than that of normal patients. Patients with normal kidney function excreted 81.47 ± 6.80% of the dose in 24 hr, with almost 55% of the dose excreted in the first 6 hr (128). In patients with moderate impairment (creatinine clearance of 47–69 ml/min), 36% was excreted in the first 6 hr, with a total 24-hr cumulative excretion of 68.14 ± 6.08% of the original dose. In patients with severe renal impairment (creatinine clearance of 5–21 ml/min), 5.9% of the dose was excreted in the first 6 hr, with a total of 22.08 ± 14.13% of the dose excreted in 24 hr. Urinary concentrations remained relatively high in patients having normal kidney function and only moderate impairment; urinary concentrations of 698 ± 216 and 638 ± 245 μg/ml were reported for the first 6 hr, respectively. However, in patients with severe impairment, the urinary concentration was 58 ± 85 μg/ml in the first 6 hr.

In another study (109), levels in the urine somewhat higher than those previously reported (128) were found. In patients having normal kidney function, levels of 1.5 ± 0.2 mg/ml were attained in the first 4 hr. In patients having moderately impaired kidney function (creatinine clearance of 56–80 ml/min), levels of 1.9 ± 0.6 mg/ml were reached. In patients with severe kidney impairment, a mean value of 368 ± 199 μg/ml was attained. However, even in patients having severely impaired kidney function, the urinary concentrations were above the MIC for many organisms. Cefazolin is known to be an effective antibiotic for the treatment of urinary infections (129).

The effect of hemodialysis on the pharmacokinetics of cefazolin has been investigated (105, 109, 122, 128, 136). Four patients received 500 mg of cefazolin intramuscularly about 2 hr before undergoing hemodialysis (128). The half-life of cefazolin during dialysis was 9.3 ± 2.5 hr, which represents a 33% decrease in the half-life (39.14 ± 18.07 hr) between dialysis periods.

In another study (122), 1 g of cefazolin was injected intravenously before dialysis was started. Between dialyses, the serum cefazolin levels were 89.8 ± 28.5 μg/ml at 15 min after administration and 61.6 ± 25.3 μg/ml after 8 hr. The half-life was 32.7 ± 9.3 hr. During dialysis, the serum concentration of cefazolin was 84.3 ± 20.7 μg/ml at 15 min after administration and fell to 31.6 ± 6.0 μg/ml at 8 hr. The half-life was reduced to 6.9 ± 2.3 hr during the dialysis period. The hemodialysis extraction percentage was 23% (122).

During dialysis, little difference existed between the concentration of cefazolin in the arterial plasma and venous plasma (136). In one study where the effect of peritoneal dialysis was investigated (109), it

Table IV—Pharmacokinetic Parameters for Cefazolin Administered Intravenously (Literature Data)[a]

Parameter	Literature Reference								Mean ± *SD*
	44	70	70[b]	110	118	122	127	139[b]	
α, hr^{-1}	3.604	8.743	7.80	4.766	1.494	1.487	2.873	4.832	3.971 ± 2.510
β, hr^{-1}	0.346	0.447	0.44	0.433	0.419	0.299	0.315	0.573	0.404 ± 0.095
K_{12}, hr^{-1}	1.687	4.660	2.41	1.817	0.265	0.447	1.279	1.961	1.730 ± 1.452
K_{21}, hr^{-1}	0.946	3.370	4.46	2.583	1.054	0.735	0.889	2.148	1.674 ± 1.028
K_{el}, hr^{-1}	1.317	1.160	1.44	0.799	0.594	0.605	1.020	1.299	0.970 ± 0.309
$T_{1/2}\alpha$, hr	0.192	0.080	0.08	0.145	0.464	0.466	0.241	0.167	0.240 ± 0.169
$T_{1/2}\beta$, hr	2.005	1.550	1.56	1.600	1.652	2.321	2.197	1.248	1.796 ± 0.387
V_c, liters	2.371	4.269	1.091	4.308	5.461	6.881	4.330	3.482	4.442 ± 1.429
Vd_{ss}, liters	6.598	10.171	—	7.338	6.831	11.065	10.566	6.791	8.479 ± 2.013
Vd_{β}, liters	9.003	11.074	—	7.948	7.736	13.930	13.999	8.145	10.266 ± 2.763
Vd_{ext}, liters	12.869	12.115	—	8.681	9.240	18.756	19.323	10.133	13.013 ± 4.378

[a] Analysis of data using a two-compartment open model (Scheme I). [b] Literature values.

was found that this procedure had a less dramatic effect on decreasing the half-life of cefazolin. The half-life of the drug before the dialysis was not given, but the half-lives found after dialysis were 20.3 and 46 hr.

Use in Children and Infants—The use of cefazolin in pediatrics has been discussed (110, 124, 135, 137, 138). In one study (124) in which 25 mg/kg of cefazolin was injected into a neonate, peak levels of 35 μg/ml were attained in the serum. At the end of 12 hr, the serum level was 10.7 μg/ml. It was reported that 41.5% of the dose was excreted in the urine within the first 24 hr.

After a 6.25-mg/kg dose of cefazolin in children (ages not given), the concentration of cefazolin in the urine was 850 μg/ml (135). After a dose of 10.0–12.5 mg/kg, the concentration in the urine was 1425 μg/ml. Another study (137) found similar results with newborn babies; however, in premature infants, the 7-hr urinary recovery was 61.5% as compared to the 7-hr urinary recovery of 41.4% in newborn babies.

The concentration of cefazolin in the mothers' milk was reported to be very low (124, 275). The levels in the umbilical cord after an injection of 500 mg into the mother before delivery was between 5 and 7 μg/ml 15 min after injection (124). Levels in newborns were not reported.

Pharmacokinetic Considerations—All studies of the pharmacokinetics of cefazolin, except two (70, 139), were performed using a one-compartment open model. As can be seen in Fig. 4, the resultant plot appears to be biexponential and the two-compartment open model analysis (70, 139) appears to describe more appropriately the pharmacokinetics of this drug than do other published studies. Use of the two-compartment open model analysis of cephalosporin data was discussed in the section on cephalexin.

The pharmacokinetic parameters obtained when the literature data were analyzed using a two-compartment open model appear in Table IV. Other reported values (70, 139) also appear in this table for comparison.

The half-life of cefazolin when analyzed by a two-compartment open model is approximately 1.8 hr. This value is not significantly different from the reported values of 1.5 and 1.95 hr for cefazolin after intravenous and intramuscular administration, respectively.

Cefazolin, being over 80% bound at therapeutic concentrations, has a relatively small volume of distribution. The volume of the central compartment, found when literature data were analyzed using a two-compartment open model, was 4.4 liters. This value approximates the volume of the plasma water. In one report (70), the volume of distribution (15 liters) was found by using a constant-infusion one-compartment open model. This volume represents a Vd_{β} of a two-compartment open model, and its significance was discussed under *Cephalexin*.

A volume of distribution of 10 liters after an intravenous infusion was found by using Eq. 3 and is analogous to Vd_{β} (44). It is not significantly different from the term shown in Table IV.

The volume of distribution after intramuscular administration of cefazolin was approximately 12.2% of body weight (105); thus the volume of distribution would be 8.5 liters in a 70-kg human. This value was calculated by fitting the blood levels of cefazolin to a one-compartment open model. Therefore, the volume found in this analysis is analogous to the two-compartment Vd_{ext}. This number is not appreciably different from the two-compartment Vd_{ext} (Table IV).

A value of 13.2% of body weight was reported for

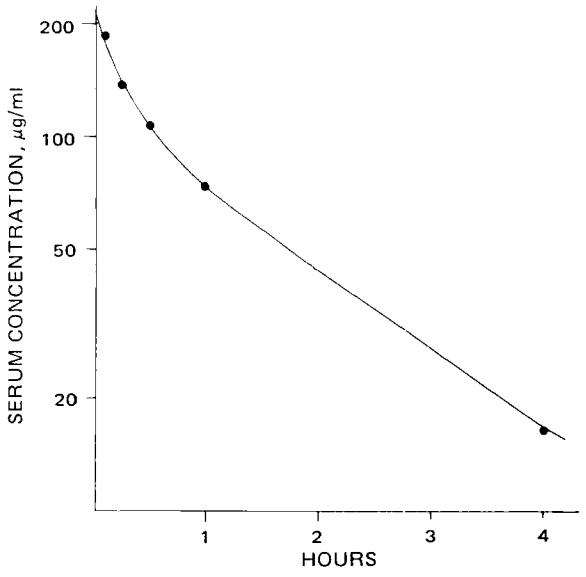


Figure 4—*Serum levels for cefazolin administered intravenously (rate constants for data generation from Table IV).*

the volume of distribution of cefazolin after intramuscular administration (128). The method used was to extrapolate the log-linear portion of the serum level curve to the y axis. The y intercept would erroneously be called $C_p{}^0$, and the volume of distribution is then dose/$C_p{}^0$. However, this intercept is actually $(C_p{}^0k_a)/(k_a - K)$, where K and k_a are the overall one-compartment open model elimination and absorption rate constants, respectively. Therefore, the value of this intercept was not correctly evaluated, and the volume obtained is incorrect.

The value for the renal clearance of cefazolin when analyzed by a two-compartment open model is 71.8 ml/min. This value is similar to the values reported with a one-compartment open model analysis. Reported values range from 61.5 to 81.8 ml/min (108, 113).

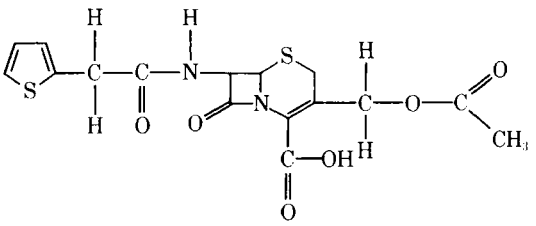
The normal dosage for patients with normal kidney function is 0.5–1.0 g every 12 hr (99). Since 12 hr is approximately six half-lives, over 98% of the drug has left the body. Since cefazolin has a wide therapeutic range and since accumulation does not take place due to the short half-life and long dosing interval, the use of one-compartment open model kinetics for this drug as well as for cephalexin will not seriously affect data analysis.

Cephalothin—Cephalothin, 3-(hydroxymethyl)-8-oxo-7- [2-(2-thienyl)acetamido]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate acetate (ester) (VI), is a semisynthetic cephalosporin and is available as the sodium salt for intravenous or intramuscular administration. The molecular weight of cephalothin sodium is 418.4. The synthesis of cephalothin has been discussed (139). It is moderately soluble in aqueous media, *i.e.*, up to 300 mg of cephalothin sodium may be dissolved in 1 ml of water. The pH of an aqueous solution of cephalothin sodium is 5.2; however, the activity of cephalothin is not altered by a variation of pH from 4 to 7.5 (153). It is active against both Gram-negative and Gram-positive bacteria, including such strains as *Diplococcus pneumoniae, S. aureus,* and *E. coli* (140).

In one study of the stability of cephalothin (141), the drug was stable for 12 days when stored at −15° in either serum or pH 6 phosphate buffer. When stored at 4°, 10% of the original activity was lost in 48 hr and 40% was lost by the end of the 12th day. When cephalothin was stored in serum at room temperature, only 13% of the original activity remained after 2 days. However, the drug in the phosphate buffer lost only 15% of its original activity after 12 days when stored at room temperature. Other studies of cephalothin stability have been reported (142, 143).

With the ultrafiltration technique, cephalothin was found to be 65% protein bound to human serum proteins (44, 105, 108, 144). In one study (141), cephalothin was about 60% less active against a test organism when in serum as compared to phosphate buffer.

Absorption—Cephalothin is not absorbed when administered orally (140). When 500 mg of cephalothin was administered orally, there was no detectable antibacterial activity in the serum between 1 and 4 hr (140). The recovery of cephalothin in the urine



VI: cephalothin

ranged from 4.3 to 11.3% of the administered dose. These results were confirmed in another study (145).

Cephalothin is well absorbed following intramuscular administration. After an intramuscular dose of 500 mg, peak levels of 6.3–8.0 μg/ml were attained. When the dose was increased to 1 g, peak levels of 15.0–20.0 μg/ml were attained (140, 141, 145–148). Peak levels were obtained approximately 0.5–1 hr after administration. When probenecid was administered concurrently with intramuscular cephalothin, peak levels of 20.2 and 33.0 μg/ml were attained after 500-mg and 1.0-g doses, respectively.

Peak levels after intravenous administration of 1 g of cephalothin as a single intravenous dose ranged from 30 to 60 μg/ml at 15 min after injection (149–153). After an intravenous infusion of 1 g over 15 min, levels of 10.22 μg/ml were attained (149). When cephalothin was administered intraperitoneally, a serum level of 4.45 μg/ml was attained 1 hr after dosing (149).

Sheng *et al.* (160) studied the serum concentrations of cephalothin after intramuscular administration of 12.5 mg/kg to infants and children. In newborn infants (1.5 hr–8 days old), peak levels of 25 μg/ml were attained 30 min after injection. In older infants and children, levels of 14.72 μg/ml were attained 0.5 hr after injection.

Distribution—When cephalothin was administered on a dosage schedule of 2 g every 4 hr intramuscularly, the levels of cephalothin were high in the heart, liver, and kidneys. A concentration of 640 μg/ml was found in the heart blood, with a corresponding level of 201 μg/g of tissue in the myocardium of the heart (153). The level of the drug in the kidneys was 548 μg/g of tissue in the renal cortex, and it was 63 μg/g of tissue in the liver (153). Brain cortex levels were reported to be 11 μg/g of tissue.

In a study of the penetration of cephalothin into the spinal fluid, levels of 0.16 and 0.31 μg/ml were attained at 30–60 min after an intravenous injection of 2 g to patients with meningitis or nervous system inflammation (155). In patients not having meningitis, the concentrations of cephalothin were below the limits of the assay (0.16 μg/ml) and could not be detected. A spinal fluid level of 11.7 μg/ml (serum concentration of 15.9 μg/ml) was found 2 hr after administration of 1 g intramuscularly (140).

At 1 hr after injection of 1 g of cephalothin intravenously, the level of cephalothin in pleural fluid was 70.6 μg/ml; at 2 hr, the level was 40.8 μg/ml (140). A level of 12.6 μg/ml in synovial fluid 20 min after injection of 1 g of cephalothin intravenously also was reported (150). Distribution of the drug into the

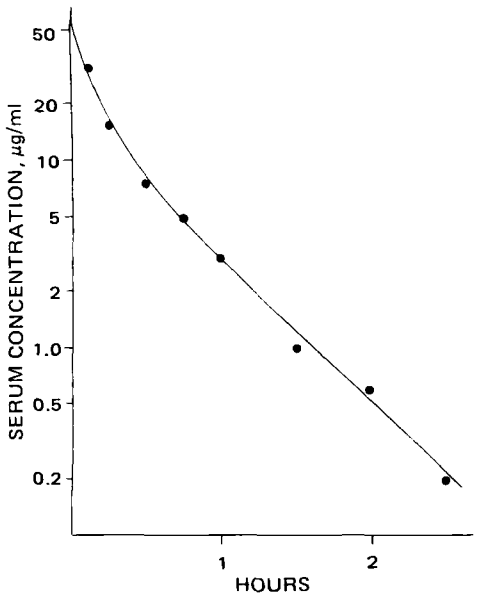


Figure 5—*Serum levels for cephalothin administered intravenously (rate constants for data generation from Table V).*

aqueous humor of the eye was reported (152). In this study, 1 g of cephalothin was administered intravenously and an aqueous humor level of 0.48 μg/ml 0.5 hr after administration was found. Ascitic fluid levels of 2.6–4.0 μg/ml after intravenous administration of 1 g of cephalothin were reported (149).

Many studies have appeared concerning the placental transfer of cephalothin (156–160, 275). Cephalothin is transferred rapidly from the maternal blood supply to the fetus (156, 157, 275). Concentrations of 30.2 and 12.5 μg/ml were found in the mother and fetus after an intravenous injection of cephalothin (156). The drug appeared in the amniotic fluid within 15 min after administration. The concentration of cephalothin in the mother's milk was reported to be very low (275).

Metabolism—Cephalothin is rapidly metabolized in the body to desacetylcephalothin. The site of the desacetylation of cephalothin is speculated to be within the liver and kidneys (161). It is possible to separate the unchanged drug from its metabolite by use of chromatographic techniques (162). One study found that 33% of the recovered urinary antibiotic activity is due to the metabolite (161).

Wick (163) studied the antibacterial activity of desacetylcephalothin against various strains of bacteria. He found that while the metabolite was active against many forms of bacteria, it was less effective than the unchanged drug. The levels of the metabolite in the urine of mice were almost twice those of the unchanged drug.

The time course of desacetylcephalothin appearance was studied in a patient with chronic renal failure after a 1-g iv dose (42). Appreciable metabolite production did not occur until approximately 2 hr after dosing. A differential microbiological assay was apparently used, but the procedure was not discussed in detail. Examination of the data indicates that the metabolite was assayed in the presence of greater than 100 times the concentration of the parent compound. Due to the lack of experimental detail and the technical difficulties of such an analysis, it is not possible to assess the meaning of these findings.

Excretion—The biliary excretion of cephalothin was studied in humans by injecting 1 g of cephalothin intravenously and collecting bile and serum (164). The peak levels of cephalothin in the bile ranged from 0.4 to 42.0 μg/ml. The time of the peak concentration was between 1 and 3 hr. In the first 3 hr after administration, the levels in the bile were generally above 1 μg/ml. In duodenal juice collected by intubation from patients with normal kidney function, the levels of cephalothin ranged from 4.6 to 6.5 μg/ml. In patients with renal insufficiency, the levels in duodenal juice were slightly elevated, with maximum levels between 10 and 16 μg/ml. This increase in biliary levels was due to the elevated serum concentration attributed to poor kidney function. Therapeutic levels were found in only 2.4% of the subjects in one study (276).

The half-life of cephalothin in patients with normal kidney function was reported to be between 0.47 and 0.85 hr (44, 105, 108, 141, 165–167). After an intramuscular dose of 1 g, levels of 800 and 2500 μg/ml were found in the urine (48, 140, 145, 168).

Cephalothin is excreted through the processes of tubular secretion and glomerular filtration, as is apparent by the increase in plasma levels after the coadministration of probenecid (140, 146). Serum levels of 33.0 μg/ml, almost double those in individuals not given probenecid, were reported after administration of 1.0 g of cephalothin and probenecid (140). The reported renal clearance was 274 ml/min, and the total body clearance of cephalothin was 472 ml/min (108). Therefore, 58% was removed from the body by renal processes. The rest was removed by metabolism.

Since cephalothin is metabolized *in vivo*, the total recovery of unchanged drug does not equal the amount of administered drug. In one study (131), approximately 52% of the initial dose was recovered in the urine after 24 hr; other recoveries ranged from 50 to 70% (140, 141).

The half-life of cephalothin is increased in patients with impaired renal function (169). The half-life of cephalothin increased to 18 hr in patients with creatinine clearance less than 5 ml/min (165). The half-life of cephalothin increased from 0.5 hr in a normal patient to 2.9 hr in a patient with a creatinine clearance of 5 ml/min (166). The serum half-life of cephalothin only increased moderately even in a patient with severe kidney dysfunction (103). In another study (42), a uremic patient was given 1 g of cephalothin intravenously. The half-life of cephalothin was about 2 hr. The levels of the metabolite, desacetylcephalothin, increased over the first 12 hr and then declined, with the half-life of the metabolite being about 8 hr.

Cephalothin is removed from the body during both hemodialysis and peritoneal dialysis (148, 170). The half-life of cephalothin was reported to be 3.3 hr during dialysis (170). The half-life of cephalothin during the nondialysis periods was not given. Perkins *et al.* (148) found that 24% of cephalothin was removed

Table V—"Apparent" Pharmacokinetic Parameters for Cephalothin Administered Intravenously (Literature Data)[a]

Parameter	Literature Reference					Mean ± *SD*
	70	70[b]	98	164	246	
α, hr^{-1}	10.850	6.4	6.620	4.550	7.090	7.277 ± 2.625
β, hr^{-1}	1.750	1.53	1.740	0.610	2.140	1.560 ± 0.660
K_{12}, hr^{-1}	3.680	1.45	1.390	1.520	1.430	2.005 ± 1.118
K_{21}, hr^{-1}	3.520	2.37	2.680	1.080	3.650	2.732 ± 1.183
K_{el}, hr^{-1}	5.400	4.20	4.280	2.550	4.140	4.092 ± 1.173
$T_{1/2}\alpha$, hr	0.063	1.08	0.105	0.152	0.098	0.104 ± 0.037
$T_{1/2}\beta$, hr	0.396	0.45	0.399	1.139	0.324	0.564 ± 0.385
V_C, liters	11.637	—	4.681	5.660	9.081	7.764 ± 3.198
Vd_{ss}, liters	23.818	—	7.101	13.598	12.628	14.286 ± 6.969
Vd_β, liters	35.890	—	11.549	23.738	17.622	22.199 ± 10.395
Vd_{ext}, liters	59.927	—	24.168	46.831	29.650	40.144 ± 16.345

[a] Analysis of data using a two-compartment open model (Scheme I). [b] Literature values.

from the body during peritoneal dialysis.

Pharmacokinetic Considerations—If the serum levels of cephalothin after intravenous administration are plotted on a semilogarithmic axis (Fig. 5), the resultant plot appears to be biexponential. However, since cephalothin is metabolized, it is possible that the two-compartment open model is not appropriate and the kinetics should be described by a model with a provision for a saturable metabolic process.

A complicating factor in the analysis of cephalothin is that the reported serum concentrations of the drug do not represent actual concentrations of cephalothin but the total combined antibiotic activities of cephalothin and desacetylcephalothin. In addition, the standard curves used in microbiological analysis are constructed using pure drug and a particular microorganism. The sensitivity of these microorganisms to the parent compound and the metabolite are dissimilar.

As drug blood levels decline, a change in the proportion of free drug to metabolite occurs as a function of time. The measured zone sizes, therefore, do not represent a simple log-linear relationship with concentration since the sensitivity of the microorganism to the combined "antimicrobial agents" is apparently changing as a consequence of an altered parent compound to metabolite ratio. In addition, if one uses a different microorganism with different sensitivity to these drugs, one would expect the assay results to yield somewhat altered "apparent concentrations."

It is, therefore, not possible to analyze the published data pharmacokinetically, since the calculated rate constants are artifactual. Since this drug is widely used and considerable blood level data have been published and widely quoted, a two-compartment open model analysis of literature data is provided in Table V for the sake of completeness and comparison. These data should not be interpreted to represent actual pharmacokinetic parameters.

Cephaloridine—Cephaloridine, 1-[[2-carboxy-8-oxo-7-[2-(2-thienyl)acetamido]-5-thia-1-azabicyclo[4.2.0]oct-2-en-3-yl]methyl]pyridinium hydroxide inner salt (VII), has a qualitative spectrum of antibacterial activity almost identical to that described for cephalothin, although there are differences in the degree of activity against specific bacterial genera (103, 116, 144, 167, 171–182).

When the acetoxy group of cephalothin is replaced with pyridine, the resulting compound, cephaloridine, is more stable both *in vitro* and *in vivo* (77, 142, 143, 183, 184).

Cephaloridine was reported to be 15% (79) and 8–31% (185) protein bound in human serum. Other reports give protein binding values within this range; however, most values appear to be less than 20% (42, 167, 186).

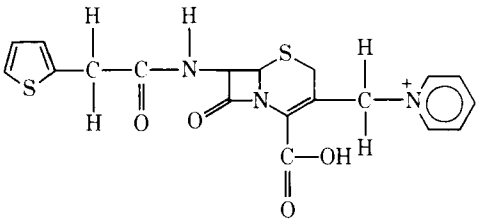
Absorption—Cephaloridine is readily absorbed after intramuscular injection and poorly absorbed from the GI tract (86, 173). A 250-mg intramuscular dose gave a peak serum concentration of about 15 μg/ml (103, 187), while 500-mg and 1-g doses gave peak blood levels of approximately 30 and 40 μg/ml, respectively (103, 188). Slightly lower levels also were reported (48, 108, 116, 126, 173, 179, 186, 189).

After intramuscular administration to children 2 months to 14 years of age, peak serum concentrations (approximately 45 min after administration) were 7, 16, and 32 μg/ml after a 50-, 100-, and 200-mg/kg dose, respectively (190). Similar results were reported in another study (275).

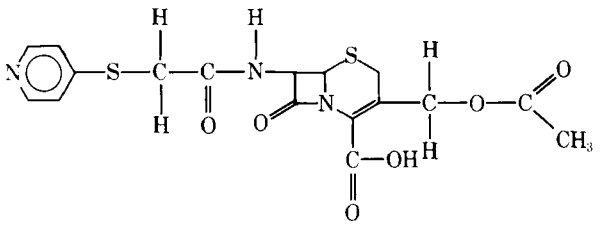
Distribution—Biliary excretion was found to be a minor pathway of elimination. A biliary concentration of 10 μg/ml was found when the serum concentration was 24 μg/ml (189, 191).

Cephaloridine concentrations in the spinal fluid of patients with uninfected meninges were 6–12% of those in the blood and serum (188, 189, 192, 193). However, poor penetration of the drug into the cerebrospinal fluid was reported (194).

Cephaloridine concentrations of about 12 μg/ml were found in fresh wounds 1 hr after a 15-mg/kg intravenous dose (185). The antibiotic levels decreased as the wound age increased.



VII: cephaloridine



VIII: cephapirin

Cephaloridine was found to diffuse into peritoneal and pleural fluids (193, 195). The drug distributed well into the liver, spleen, stomach wall, and lung but was found in a much smaller amount in the cerebral cortex (189).

Cephaloridine injected subconjunctivally was found to penetrate aqueous humor and subretinal fluid effectively (196, 197). Low but effective subretinal levels were reported (198).

Records (199) was unable to detect antibacterial activity in the aqueous humor after a 1-g dose of cephaloridine. However, levels of 7–28 μg/ml were found in the secondary aqueous humor which refills the eye following aspiration of the primary fluid.

Prakash *et al.* (200) injected 1 g of cephaloridine intramuscularly every 12 hr for two doses to 60 women undergoing amniotomy for induction of labor to study the absorption and transfer of the antibiotic to the fetus as protection against intrapartum infections. The drug provided inhibitory concentrations in maternal serum, amniotic fluid, and umbilical cord blood. Similar results were found in another study (275).

Metabolism and Excretion—Urine specimens, both animal and human, which were examined by chromatographic and bioautographic techniques, showed that no other microbiologically active metabolites were present and that cephaloridine was excreted unchanged in the urine (183, 201).

Renal excretion of this drug is considered to be largely the result of glomerular filtration, but a 1.2-fold increase in cephaloridine serum concentration was found after probenecid administration (173, 179, 202, 203) and appears to indicate some tubular secretion. Excretion was found to be pH dependent, *i.e.*, increasing with decreasing pH (277).

Kidney disease resulting in impaired kidney function results in increased serum half-lives up to 23 hr (54, 105, 167, 189, 192, 203–207). The relationship between renal function and biological half-life was discussed (192).

Hemodialysis reduced the serum half-life in uremic patients to 3–5 hr (105, 167, 189, 204). An extraction ratio of 0.23–0.35 was reported (148, 192). Peritoneal dialysis resulted in a daily loss of 125–500 mg of drug from the body.

Urinary concentrations of 150 μg/ml or greater after a 0.5- or 1-g dose were maintained in all but anuric patients (192).

Urinary recovery after intramuscular and intravenous dosing was 60–80% (44, 116, 126, 172, 173, 179, 186, 187). Several investigations found 85% of a 500-mg dose in the urine (44, 105, 108).

Renal clearances were reported to be 146–280 ml/min (108, 173). A plasma clearance of 167 ml/min/1.73 m^2 and a renal clearance of 125 ml/min/1.73 m^2 were reported, indicating that approximately 75% of the drug was removed from the body by the kidneys (44, 108).

A serum half-life of approximately 1.1–1.5 hr was reported for cephaloridine (108, 126, 166, 167, 192, 205); however, a value of 48 min also was found (173). The volume of distribution was 16 liters (108).

Pharmacokinetic Considerations—Extensive pharmacokinetic analysis of literature data is not possible for this drug, since appropriate intravenous data have not been published. Most studies performed with cephaloridine involved intramuscular administration and were not designed for pharmacokinetic analysis. This drug is primarily administered *via* the intramuscular route.

Since its physicochemical properties are not significantly different from the other cephalosporins, the pharmacokinetics of this drug should be similar to those of the other cephalosporins; *i.e.*, its time course in the body is biexponential. Like the other cephalosporins, its half-life (1 hr) is short in relation to the normal dosing interval (6 hr), so the pharmacokinetics of this drug can be described adequately by a one-compartment open model.

Cephapirin—Cephapirin, 3-(hydroxymethyl)-8-oxo-7-[2-(4-pyridylthio)acetamido]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylate acetate (ester) (VIII), is a semisynthetic cephalosporin for parenteral use. It is derived from 7-aminocephalosporanic acid. Cephapirin has a spectrum of antimicrobial activity similar to that of cephalothin (208). Cephapirin is effective against Gram-positive bacteria. Except for two strains of *Staphylococcus epidermidis* and *S. aureus*, cephapirin is effective at concentrations of 3.1 μg/ml or less against all susceptible strains of Gram-positive bacteria. Gram-negative bacteria were found to be less sensitive to the effects of the drug (208).

The sodium salt of cephapirin is extremely soluble in water; at 25°, the solubility is greater than 500 mg/ml (209). The pH of a reconstituted solution of cephapirin (sodium salt) is between 5.7 and 7.3 (209). Cephapirin is stable at 25° when reconstituted in most commonly used parenteral solutions. When reconstituted in sterile water, only 6% of the initial activity of the drug is lost in a 24-hr period. A complete study of the stability of cephapirin has been published (209).

Cephapirin is 44–50% bound to human plasma proteins (210). The addition of cephapirin to serum had little effect in lowering the MIC of this drug to *S. aureus* (208).

Absorption—Since cephapirin is not absorbed orally, it is only administered through the intramuscular and intravenous routes. Cephapirin seems to be relatively well absorbed after intramuscular injection. In one study in which 1 g of cephapirin was administered intramuscularly, peak levels in the serum of 24.2 μg/ml were attained at 0.5 hr after injection (211). Peak levels of cephapirin after intramuscular

administration of 0.5- and 1.0-g doses were 8 and 15 μg/ml, respectively (210). Peak levels were attained between 0.5 and 1 hr after administration.

After intravenous administration, a level of 72.6 μg/ml was reported at 0.5 hr after injection (211). These levels fell to 6.06 μg/ml at 1 hr after administration. Another study (210) found much lower levels after intravenous administration of 1.0 g of cephapirin. A peak level of about 15 μg/ml was attained.

Excretion and Metabolism—Cephapirin is metabolized to desacetylcephapirin *in vivo* (212). Approximately 41% of the recovered antibiotic is in the desacetylcephapirin form. Desacetylcephapirin was approximately 54% as potent as the unchanged drug against *S. lutea* (212).

Cephapirin is eliminated from the body largely through renal processes. Less than 1% of the original dose was recovered in the bile (210).

After an intravenous dose of 1 g, urinary levels of 2560 ± 503 μg/ml were found. Recoveries after 1-g intravenous and intramuscular doses were 72 ± 7.5 and 54 ± 18.6%, respectively (211).

The reported half-lives of cephapirin after intravenous and intramuscular administrations to normal volunteers were 21 and 47 min, respectively (211).

Since the elimination of cephapirin is largely due to renal excretion, the half-life of cephapirin is extended in patients with renal dysfunction. A study using azotemic patients found that the serum half-life of cephapirin was 95.9 min (212). These patients were not undergoing any form of dialysis treatment during the study. The average creatinine clearance was 12.6 ml/min. These patients excreted 19.5% of the initial dose in the urine.

The effect of dialysis on the half-life of cephapirin was studied, and the half-life in patients undergoing dialysis was about 107 min (212). Dialysis removed 22.8% of the dose.

A review of the use of cephapirin in children was prepared (213). Intramuscular injections of 20 mg/kg produced peak levels of 14.5 μg/ml, and injections of 12.5 mg/kg produced peak levels of 7.6 μg/ml. The antibiotic concentration in the urine after an intramuscular dose of 20.0 mg/kg was 1600 μg/ml. This recovery was 53% of the initial dose.

Pharmacokinetic Considerations—A very limited number of studies report serum levels of cephapirin (211, 214). Due to the paucity of information, plus the fact that the drug is metabolized and assayed microbiologically, it is difficult to discuss the pharmacokinetics of cephapirin. Only one study (211) described the pharmacokinetics of this drug. The reported half-life was 21 min after intravenous injection. This value was probably calculated by a log-linear regression using all points. Graphical estimation of the terminal portion of the curve, however, results in a half-life of approximately 42 min.

These data (211) are shown in Fig. 6. It can be seen that the curve appears to be biexponential. For the sake of completeness, the data were fitted to a two-compartment open model (Table VI). The half-life of the β-phase is about 45 min, almost twice that reported but identical to that obtained from the terminal portion of Fig. 6. The rate constants in Table VI should be considered to be apparent due to the fact that the drug is metabolized to a less active compound and assayed *via* a microbiological procedure. A more detailed discussion appears in the *Cephalothin* section.

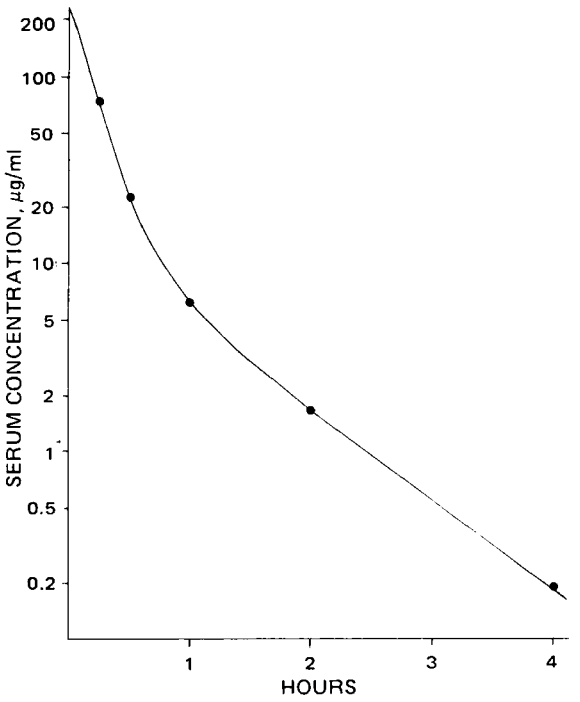


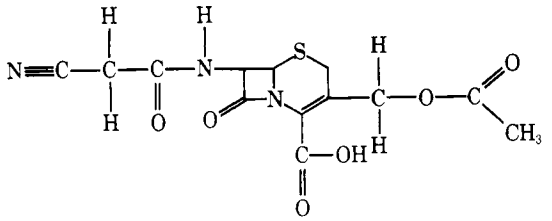
Figure 6—*Serum levels for cephapirin administered intravenously (rate constants for data generation from Table VI).*

Cephacetrile—Cephacetrile, 7-(2-cyanoacetamido) - 3 -(hydroxymethyl)-8-oxo-5-thia-1-azabicyclo-[4.2.0]oct-2-ene-2-carboxylate acetate (ester) (IX), is a bactericidal drug whose mode of action is similar to the β-lactam antibiotics (215). It is less active than cephaloridine against penicillin-susceptible strains of *S. aureus* but is more active than cephaloridine or cephalexin against β-lactamase-producing strains. Cephalothin is more active in this regard than cephacetrile. Cephacetrile was reported to be relatively stable to β-lactamase degradation (180). It is also less active than cephaloridine and more active than ceph-

Table VI—Pharmacokinetic Parameters for Cephapirin Administered Intravenously (Literature Data)[a]

Parameter	Literature Reference 211	214	Mean ± *SD*
α, hr^{-1}	5.353	5.290	5.321 ± 0.045
β, hr^{-1}	0.926	1.082	1.004 ± 0.110
K_{12}, hr^{-1}	0.890	1.090	0.990 ± 0.141
K_{21}, hr^{-1}	1.177	1.570	1.374 ± 0.278
K_{el}, hr^{-1}	4.213	3.710	3.960 ± 0.360
$T_{1/2}\alpha$, hr	0.129	0.132	0.131 ± 0.002
$T_{1/2}\beta$, hr	0.749	0.669	0.709 ± 0.057
V_c, liters	6.166	12.379	9.273 ± 4.393
Vd_{ss}, liters	10.830	20.916	15.873 ± 7.132
Vd_β, liters	28.055	41.829	34.942 ± 9.739
Vd_{ext}, liters	108.888	118.517	113.703 ± 6.809

[a] Analysis of data using a two-compartment open model (Scheme I).



IX: cephacetrile

alothin against certain Gram-negative organisms (215). Similar results were reported in other studies (216–219). Cephacetrile was 33–36% bound to serum (215, 220). A binding range of 23–34% also was reported (221). Metabolism of the drug appears to be negligible based on chromatographic work (215, 220). It also appears to be less nephrotoxic than cephaloridine (222–224).

Absorption—Cephacetrile is absorbed after intramuscular injection. In one study, peak serum concentrations of 22.7 μg/ml were achieved 1 hr after administration of a 1-g dose and declined to an average of 1.65 μg/ml 4 hr after dosing (225). Similar results were reported (226), but some lower peak levels also have been found (221, 227, 228). Peak levels of 17.1 μg/ml at 30 min after injection were reported (229).

Dvoracek *et al.* (228) used a one-compartment open model to fit their data and found an intramuscular absorption half-life of 0.3 hr. This value was one-fifth the elimination half-life of 1.4 hr. This drug apparently is rapidly absorbed without a significant lag time (221, 228). Patients with decreased renal function exhibited prolonged peak times (229).

Distribution—Distribution to duodenal juice was low after a 1-g intramuscular injection (227). The maximal concentrations ranged from 1.0 to 3.1 μg/ml and were attained at the 1st and 2nd hr after administration. Higher levels (2.9–7.0 μg/ml) were reported in patients with renal failure (227).

Cephacetrile was administered to healthy pregnant women during labor by intravenous infusion and multiple bolus dosing (230). Maternal serum and amniotic fluid concentrations were determined as a function of time, and cord serum concentrations were determined after delivery. In the maternal serum, a mean steady state of 30 μg/ml was obtained. Cord serum and amniotic fluid concentrations reached 14 and 8–9 μg/ml, respectively, after intravenous administration and 19 and 22–25 μg/ml, respectively, after intramuscular administration. Maurice *et al.* (221) reported cerebrospinal fluid, fetal cord, amniotic fluid, bone, and prostatic tissue levels.

Excretion—Peak bile levels were observed between 1 and 4 hr after a 1-g intramuscular dose of the drug in cholecystectomized subjects (227, 231). The biliary concentration was low, with the peak concentration ranging from 1.0 to 6.8 μg/ml. In normal patients, the 1-hr peak concentrations were 14.7–19.4 μg/ml (227, 231).

Cephacetrile was reported to be 60–100% excreted in the urine (215, 220–222, 225, 226, 228, 229, 232–234).

The renal and plasma clearances of this drug were found to be approximately 230–240 ml/min (228). A plasma and renal clearance of 361 ml/min/1.73 m^2 was reported (226), which is further evidence that the drug is not significantly metabolized (228, 233, 235).

The serum half-life was reported to range between 0.5 and 1.5 hr (215, 220, 221, 226, 227, 229, 235, 236).

The pharmacokinetics of this drug were analyzed using a two-compartment open model (228). A β-phase half-life of 1.2 hr was found after intravenous injection. The rate constants are shown in Table VII. The intramuscular data were analyzed *via* a one-compartment open model, and a half-life of 1.4 hr was found.

Altered renal function was accompanied by a drop in the urinary elimination of the drug (219, 221, 222, 226–228, 231, 233–235, 237). In patients with renal failure, the serum concentrations of the antibiotic rose significantly (40.6 ± 8.1 μg/ml) 1 hr after injection (227). An increase in the half-life from 2.2 to 30 hr was noted (221, 226, 227, 229, 231, 233–238), depending on the severity of the renal function.

The relationships between renal function and the drug's half-life of elimination from the body have been studied (221, 231, 233–237, 239). Suggested doses in the case of renal insufficiency have been recommended (221, 229, 234, 240).

Hemodialysis in one study reduced the serum half-life by a factor of eight, with a mean extraction ratio of 32% (236). Fillastre *et al.* (229) found a twofold de-

Table VII—Pharmacokinetic Parameters for Cephacetrile Administered Intravenously (Literature Data)[a]

	Literature Reference				
Parameter	221	228 241	228[b] 241	232 236 242	Mean ± *SD*
α, hr^{-1}	2.410	3.880	4.44	2.470	2.920 ± 0.830
β, hr^{-1}	0.536	0.530	0.57	0.550	0.540 ± 0.010
K_{12}, hr^{-1}	0.658	1.540	1.80	0.680	0.960 ± 0.500
K_{21}, hr^{-1}	1.010	1.370	1.66	1.050	1.140 ± 0.200
K_{el}, hr^{-1}	1.280	1.510	1.55	1.290	1.360 ± 0.130
$T_{1/2\alpha}$, hr	0.287	0.180	0.16	0.280	0.250 ± 0.060
$T_{1/2\beta}$, hr	1.294	1.300	1.24	1.270	1.290 ± 0.020
V_C, liters	11.239	8.967	9.221	11.169	10.458 ± 1.292
Vd_{ss}, liters	18.578	19.005	—	18.444	18.676 ± 0.293
Vd_{β}, liters	26.921	25.380	—	26.376	26.226 ± 0.780
Vd_{ext}, liters	44.747	35.805	—	42.952	41.168 ± 4.730

[a] Analysis of data using a two-compartment open model (Scheme I). [b] Literature values.

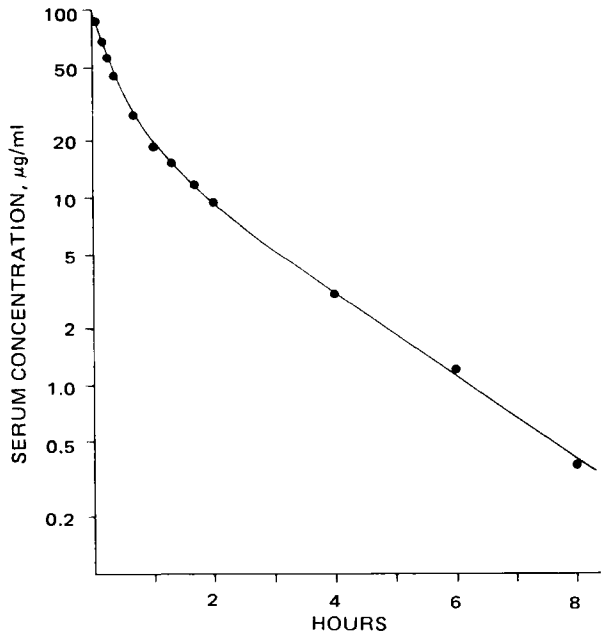


Figure 7—*Serum levels for cephacetrile administered intravenously (rate constants for data generation from Table VII).*

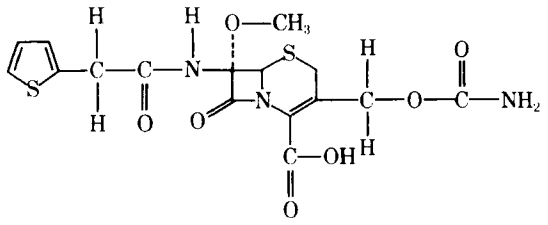
crease in serum levels compared to controls, with an extraction percentage of 27–33%. Similar results also were reported (226).

Evidence was presented that cephacetrile is actively secreted by the kidney and that probenecid can compete for the secretory route (233).

Pharmacokinetic Considerations—As with other cephalosporins, cephacetrile appears to follow two-compartment open model kinetics (Fig. 7). Investigators, with one exception (228), analyzed their data *via* a one-compartment open model. Shown in Table VII are the pharmacokinetic parameters obtained by analyzing literature data according to a two-compartment open model (220, 221, 228, 232, 236, 241). The analysis by Dvoracek *et al.* (228) is included for comparison and is similar to the other studies shown in Table VII. The half-life of the β-phase is within the range of reported values. Dvoracek *et al.* (228) reported a value of 9542 ml as the volume of the tissue compartment. The sum of $V_c + V_t$ is Vd_{ss} and is equal to 18,763 ml, in agreement with the Vd_{ss} values calculated using data from other studies (Table VII). The only other volume of distribution term reported was 0.3–0.5 liter/kg or 21,000–35,000 ml (221). The method of calculation was not clear, but the graphical data suggest that the reported value was actually Vd_{ext}.

In spite of the good fits of cephacetrile data using a two-compartment open model, little practical advantage is gained with this type of analysis for the reasons stated in the *Cephalexin* section.

Cefoxitin—Cefoxitin, 3-(hydroxymethyl)-7-methoxy-8-oxo-7-[2-(2-thienyl)acetamido]-5-thia-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid carbamate (ester) (X), is a semisynthetic cephamycin antibiotic (243). The 7-methoxylated cephalosporins possess marked resistance to the action of β-lactamases from Gram-positive and Gram-negative organisms (244). Of the Gram-negative microorganisms generally susceptible to cephalothin, *i.e.*, *E. coli*, *P. mirabilis*, and *Klebsiella*, cefoxitin was significantly more active than cephalothin (243, 245). The Gram-positive microorganisms, on the other hand, were less susceptible to cefoxitin than to cephalothin (243, 245).



X: cefoxitin

Cefoxitin does not appear to be orally absorbed; however, the drug appears rapidly in the blood after intramuscular injection (246). After 0.5- and 1.0-g doses, approximately 90% was recovered in the urine. After a 2-g iv dose, 99% was recovered in the urine, indicating that no extrarenal excretion occurred. Probenecid was found to affect the elimination of cefoxitin in mice (245), suggesting that this drug is eliminated *via* glomerular filtration as well as tubular secretion. This finding was also reported in humans (247). The drug was also found to be widely distributed in soft tissue (245).

The biological half-life of cefoxitin was calculated to be 45 min (246) compared to 25 min for cephalothin. The serum concentration *versus* time curve (Fig. 8) for this drug, as well as other cephalosporins, appears to exhibit biphasic decay. Analysis of the data of Kosmidis *et al.* (246) *via* a two-compartment open model analysis is shown in Table VIII. Since a paucity of published data exists concerning this antibiotic, it is not possible to draw further conclusions relative to its pharmacokinetic characteristics. The main advantage of this drug seems to be its resistance to degradation by β-lactamases.

Cephanone—Cephanone, 3-(5-methyl-1,3,4-thiadiazol-2-ylthiomethyl)-7-[2-(3-sydnone)acetamido]-

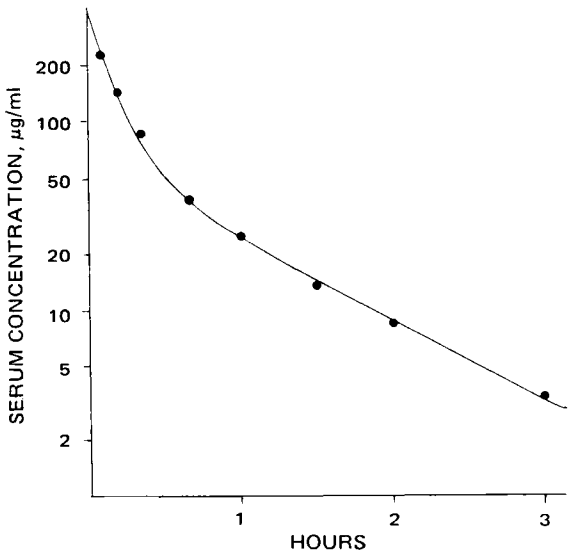


Figure 8—*Serum levels for cefoxitin administered intravenously (rate constants for data generation from Table VIII).*

Table VIII—Pharmacokinetic Parameters for Cefoxitin Administered Intravenously (Literature Data)[a]

Parameter	Literature Reference, 246
α, hr^{-1}	6.316
β, hr^{-1}	1.041
K_{12}, hr^{-1}	2.191
K_{21}, hr^{-1}	2.274
K_{el}, hr^{-1}	2.892
$T_{1/2\alpha}$, hr	0.110
$T_{1/2\beta}$, hr	0.666
V_c, liters	6.883
Vd_{ss}, liters	13.516
Vd_β, liters	19.120
Vd_{ext}, liters	29.463

[a] Analysis of data using a two-compartment open model (Scheme I).

Table IX—Pharmacokinetic Parameters for Cephanone Administered Intravenously (Literature Data)[a]

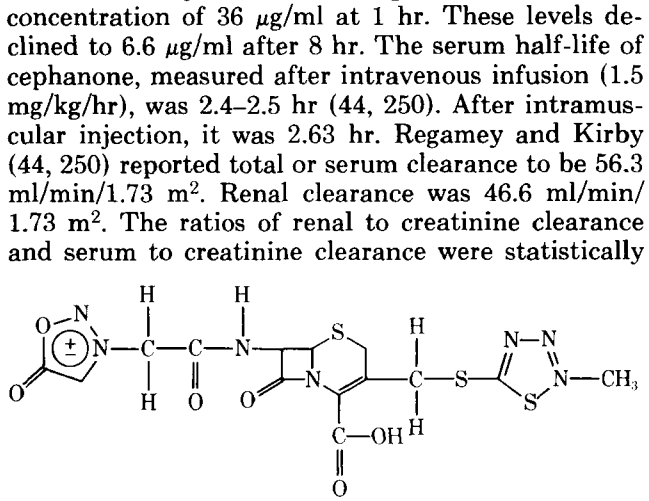
	Literature Reference		
Parameter	249	250	Mean ± *SD*
α, hr^{-1}	2.100	0.641	1.370 ± 1.032
β, hr^{-1}	0.282	0.224	0.252 ± 0.041
K_{12}, hr^{-1}	0.377	0.106	0.241 ± 0.191
K_{21}, hr^{-1}	1.645	0.360	1.002 ± 0.909
K_{el}, hr^{-1}	0.360	0.398	0.379 ± 0.027
$T_{1/2\alpha}$, hr	0.330	1.080	0.705 ± 0.532
$T_{1/2\beta}$, hr	2.457	3.097	2.776 ± 0.453
V_c, liters	10.400	5.184	7.792 ± 3.688
Vd_{ss}, liters	12.782	6.713	9.747 ± 4.291
Vd_β, liters	13.275	9.226	11.250 ± 2.863
Vd_{ext}, liters	13.870	15.867	14.868 ± 1.412

[a] Analysis of data using a two-compartment open model (Scheme I).

3-cephem-4-carboxylic acid (XI), is a semisynthetic cephalosporin derivative with an antibacterial spectrum similar to that of cephalothin (44, 248, 249). This drug was withdrawn from clinical trials due to suspected carcinogenicity, but it is included in this review for the sake of completeness. The drug is 86–88% protein bound (44, 250) and is relatively stable to β-lactamase degradation (180).

The average drug concentration in serum 0.5 hr after intramuscular administration of 1 g of cephanone was reported to be 38 μg/ml (249). Peak concentrations were attained at 1 hr. After 6 hr, the blood level declined to 22.1 μg/ml; after 12 hr, the blood level was still relatively high: 5.3 μg/ml. This level exceeds the MIC for most susceptible organisms (249). The half-life was 174 min; however, after intravenous administration, the half-life was 126 min, indicating that intramuscular administration does not provide, as with cefazolin and cephalexin, a pure elimination phase. After a 1-g iv injection, the blood levels ranged from 81.2 to 8 μg/ml after 8 hr. At 12 hr postadministration, drug levels were not detectable (249).

Regamey and Kirby (250), however, found higher blood levels after both intramuscular and intravenous injections. After intravenous administration of 250 mg of cephanone, a peak serum concentration of 31.9 μg/ml was present at the end of the 1st hr. An intramuscular injection of 500 mg resulted in a peak concentration of 36 μg/ml at 1 hr. These levels declined to 6.6 μg/ml after 8 hr. The serum half-life of cephanone, measured after intravenous infusion (1.5 mg/kg/hr), was 2.4–2.5 hr (44, 250). After intramuscular injection, it was 2.63 hr. Regamey and Kirby (44, 250) reported total or serum clearance to be 56.3 ml/min/1.73 m^2. Renal clearance was 46.6 ml/min/1.73 m^2. The ratios of renal to creatinine clearance and serum to creatinine clearance were statistically



XI: cephanone

identical, *i.e.*, 0.40–0.47. These ratios suggest that cephanone leaves the body chiefly through the kidneys.

The volume of distribution was reported to be 12 liters/1.73 m^2 (44) and 13.6 liters (250). These values were obtained after constant infusion and are analogous to the Vd_β of a two-compartment open model analysis.

Urinary excretion of cephanone appears to be high; however, variable results were reported (249). During a 24-hr period, all of a 1-g intravenous dose was excreted, but only 38% was found in the urine after a 500-mg injection. Intramuscular injection resulted in 50% of the administered (1-g) dose being excreted in the urine. Urinary drug concentrations were high. After administration of 1 g intramuscularly or intravenously, urine concentrations were 1.7 and 5.0 mg/ml, respectively, after 8 hr.

Regamey and Kirby (250) found 92% of a 500-mg im dose excreted in the urine. Similar results were obtained after intravenous infusion. Urine concentrations of cephanone were 900, 200, and 20 μg/ml in collections 0–4, 4–8, and 8–24 hr after administration, respectively.

After intravenous administration, the decline in cephanone serum levels appears to be biexponential (Fig. 9). It was possible to fit these data using a two-compartment open model analysis (Table IX). It can be seen that the half-life of the β-phase is in reasonable agreement with the literature using a one-compartment open model analysis. The volume of distribution terms are relatively low, probably due to the high degree (86–88%) of protein binding. This partially contributes to the relatively high blood levels seen with this drug. The large degree of protein binding probably results in the high and prolonged serum levels of cephanone.

Cefamandole—Cefamandole, 7-D-mandelamido-3-[[(1-methyl-1*H*-tetrazol-5-yl)thio]methyl]-8-oxo-5-thio-1-azabicyclo[4.2.0]oct-2-ene-2-carboxylic acid (XII), is a semisynthetic cephalosporin intended for parenteral use. The sodium salt has a molecular weight of 484.5. The spectrum of antibacterial activity is similar to that of other cephalosporins (251).

Since cefamandole is one of the newest cephalosporins, no pharmacokinetic studies have appeared.

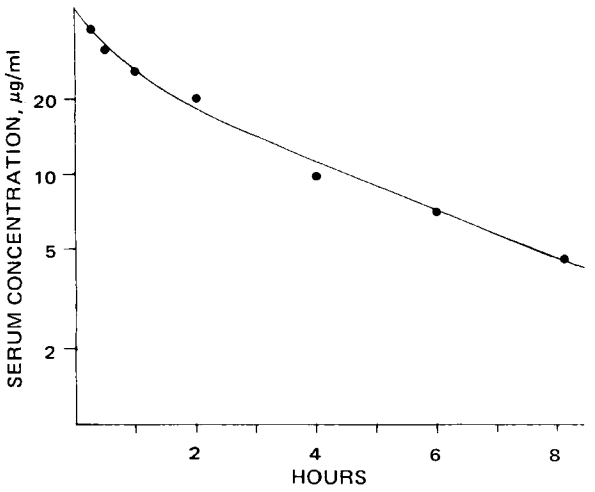
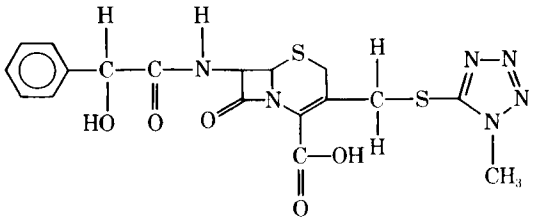


Figure 9—*Serum levels for cephanone administered intravenously (rate constants for data generation from Table IX).*

CLINICAL USE

Appropriate antibiotic usage requires an accurate knowledge of the antimicrobial susceptibility pattern of the drug to be given, sound clinical reasoning as to why the drug is being given, and knowledge of whether it will reach the site of the pathogen in adequate concentrations without producing serious side effects. This portion of the review is an attempt to provide an overview of the appropriate clinical use of cephalosporin antibiotics from the standpoint of an infectious disease physician.

General Considerations for Cephalosporin Administration—Cephalosporins are usually effective for infection from Gram-positive bacteria such as staphylococci (*S. aureus* and *S. epidermidis*), group A *Streptococcus*, anaerobic streptococci, and *Streptococcus pneumoniae* ("pneumococcus") and from Gram-negative organisms such as *E. coli*, *P. mirabilis*, *Hemophilus* species, and *K. pneumoniae*. However, once one of these bacteria has been identified as the causative organism of an infectious disease process, usually other antibiotic agents are preferable, either because of a greater degree of microbiological activity or because of a smaller likelihood of producing a suprainfection. For instance, the antibiotic of choice for group A *Streptococcus*, the "pneumococcus," nonpenicillinase-producing staphylococci, and anaerobic streptococci is penicillin G; ampicillin is the preferred antibiotic for *Hemophilus* infection and often has equal or greater activity toward *E. coli* and *P. mirabilis*; and oxacillin is a more logical choice for penicillinase-producing *S. aureus*. Cephalosporins have a greater degree of activity against *S. epidermidis* (about 80–85% sensitive to oxacillin, almost all sensitive to cephalosporins); but if a strain is sensitive to oxacillin, it is a better drug to give because of its narrower spectrum as compared to a cephalosporin.



XII: cefamandole

It is only with respect to *Klebsiella* that cephalosporins are clearly more active than ampicillin, penicillin G, or oxacillin. Although more strains of *Klebsiella* are sensitive to aminoglycosides, such as gentamicin and kanamycin, one usually prefers a cephalosporin in a situation where an isolate is sensitive to both classes of antibiotics due to the greater potential toxicity of the aminoglycosides.

The enterococci, indole-positive *Proteus*, *Enterobacter* species, *Serratia*, *Pseudomonas*, *Herellea*, and *Citrobacter* are usually resistant to cephalosporins.

The cephalosporin popularity stems mainly from their reasonably broad antimicrobial coverage, their relatively low incidence of serious adverse drug reactions, the small frequency of cross-reaction in patients with a history of penicillin hypersensitivity, and their common usage to prevent the development of infection following surgical procedures involving the insertion of prosthetic devices such as vascular grafts, artificial heart valves, and orthopedic hardware.

Appropriate Use of Cephalosporins by Parenteral Route—*Prevention of Infection on Prosthetic Devices*—Few subjects create more lively debate than the use of antibiotics for prophylactic purposes. As seen by Jawetz (252), there is no place for prophylactic antibiotics unless the prophylaxis is directed against one particular or small group of microorganisms.

All other attempts at more general prophylaxis with antibiotics usually have been unsuccessful. Chemoprophylaxis has never proven significant in reducing the likelihood of a patient developing an infection in clinical situations where multiple organisms may invade, as is the case in most GI surgical procedures, pancreatitis, or the insertion of an in-dwelling bladder catheter. In fact, general prophylaxis has often been detrimental by favoring colonization and infection by microbes resistant to the antibiotic given. Since most compromised hosts, such as patients with leukemia or lymphoma, develop infection from organisms on their skin or from their genitourinary tract, and since it is impossible to sterilize the entire patient, antibiotic prophylaxis has also been of no value in these cases.

In view of these comments, it would be logical to administer a cephalosporin antibiotic only in clinical situations where one can predict the likely pathogen with a high degree of certainty and the particular pathogen is easily eradicated by this type of antibiotic. The administration of a cephalosporin to prevent infection following the insertion of artificial material (*e.g.*, prosthetic heart valves, Dacron or Teflon artificial grafts, and orthopedic hardware) follows this dictum, since after the placement of these foreign bodies about 90% of the infections develop from a single group of organisms, namely staphylococci, either coagulase negative (*S. epidermidis*) or coagulase pos-

itive (*S. aureus*). As already mentioned, cephalosporin antibiotics are usually active against all types of staphylococci whereas the isoxazole antibiotics, such as oxacillin, are usually ineffective against 15–20% of coagulase-negative staphylococci. Because of this situation, cephalosporins have emerged as the most appropriate agents to use in the prevention of infections on prosthetic material.

Some clinical situations represent "gray zones" in prophylactic antibiotic usage because more than one organism, but usually not more than five, may produce the infection. An open comminuted fracture represents a situation where infection commonly occurs from a relatively small number of microbes. Traumatic wound infections above the waist are often from skin bacteria, such as *S. aureus* or group A *Streptococcus*, while those below the waist are usually caused not only by these common skin microbes but also by *E. coli* and *P. mirabilis* (indole-negative *Proteus*), probably derived from bacterial "fallout" from the GI tract. As a result, oxacillin, which exhibits its activity against these Gram-positive bacteria, and cephalosporins, which are bactericidal toward both these Gram-positive and Gram-negative organisms, are the logical choices for prophylaxis against wound infection above and below the waist, respectively.

Two major problems have developed with the use of cephalosporins for prophylaxis. One is that they are often administered too long, even in appropriate situations, and the other is that they are often given illogically in clinical problems such as bowel surgery where they can be detrimental by the replacement of susceptible organisms with resistant ones.

Various investigators have demonstrated clearly that maximum protection from infection from a surgical procedure occurs only if adequate tissue levels are present at the time of the surgical procedure (253) and that the continuation of antibiotics beyond the operative period has no value. This situation was nicely illustrated in a study where a single dose of cephalothin administered immediately prior to the insertion of prosthetic heart valves was as effective in the prevention of infection as was the use of additional dosages after the operation (254).

The tendency of physicians to be reluctant to discontinue completely antibiotics by the intravenous route after they no longer are necessary has been a traditional problem with all antibiotics, particularly with the use of cephalosporins for prophylaxis. For instance, it is quite common for a surgeon to administer a cephalosporin in a ritualistic sequence: first intravenously, then intramuscularly, and finally orally for 1 to many weeks following an operative procedure. In fact, one often wonders why the physician does not go next to the intradermal or intraocular route! Unfortunately, this gradual weaning process has been a major reason for the increase in cost for this class of antibiotics, because it not only increases the amount of antibiotic administered but also enhances the chances of toxic drug reactions or superinfection, resulting in greater morbidity and prolonged hospital stay.

Therapy of Lung Infections Acquired in the Community—If the sepsis appears to be from a pulmonary infection produced by bacteria acquired from outside the hospital, and especially if the patient has a history of chronic lung or other debilitating disease, a cephalosporin antibiotic seems to be the wisest choice. The usual pathogens in this setting are *K. pneumoniae*, *Strep. pneumoniae*, *Hemophilus influenzae*, *S. aureus*, and group A *Streptococcus* organisms and are almost always sensitive to this antimicrobial agent if given in adequate dosage. There is some hesitancy about relying on ampicillin or penicillin alone, since most *Klebsiella* organisms and many staphylococci are resistant to both penicillin and ampicillin (about 80% of the *S. aureus* acquired outside the hospital now produces penicillinase).

It usually is possible to use penicillin or ampicillin as the sole antibiotic only in healthy patients with mild respiratory infections, since the physician can usually exclude the *Staphylococcus* or *Klebsiella* organism in such a clinical setting because these organisms almost always produce very serious disease.

Therapy of Lung Infections Acquired in Hospitals—Pulmonary infection acquired in a hospital setting is a difficult clinical problem because of the rapid colonization of the upper respiratory tract by common as well as bizarre bacteria. In a recent epidemiological survey (255) (Table X), 10.7 and 15.3% of the hospital-acquired pneumonias were produced by *Pseudomonas aeruginosa* and *K. pneumoniae*, respectively, followed in decreasing frequency by *E. coli* (10.6%), *Strep. pneumoniae* (9.5%), *Enterobacter* species (8.0%), and *S. aureus* (6.6%). Thus, in patients who develop hospital-acquired pneumonias, a combination of a cephalosporin and gentamicin appears to make sense, since it affords a more clinically proven coverage against *S. aureus* and *Klebsiella* than may be obtained with gentamicin and ampicillin. Moreover, the *Bacteroides* species usually involved in hospital-acquired pneumonias come from the upper respiratory tract where they are usually sensitive to cephalosporins.

Although most strains of *S. aureus* are susceptible to gentamicin *in vitro*, there is extremely little clinical experience for the use of this antibiotic in patients with overwhelming staphylococcal disease. Moreover, the enterococcus is rarely, if ever, involved

Table X—Major Organisms Associated with Suspected Hospital-Acquired Pneumonia from October 1970 to October 1973

Organism	Number	Percent
Klebsiella pneumoniae	138	15.3
Pseudomonas aeruginosa	97	10.7
Escherichia coli	96	10.6
Streptococcus pneumoniae	86	9.5
Enterobacter species	73	8.0
Staphylococcus aureus	60	6.6
Proteus species	45	4.9
Serratia species	17	1.9
Candida	17	1.9
Herellea	14	1.5
Citrobacter	3	0.3
Enterococcus	1	0.1
Other organisms	256	28.3
Total	903	100.0

in producing pneumonia; hence, the absence of coverage for this organism with the combination of a cephalosporin and gentamicin is of no great concern. For instance, in a review of hospital-acquired pneumonias at Hartford Hospital (255), from 1970 to 1973 there was only a single equivocal case of pulmonary infection caused by the enterococcus (Table X).

Inappropriate Use of Cephalosporins by Parenteral Route—*Treatment of Central Nervous System Infections*—Although cephalothin is often an effective antibiotic in many types of infection, especially pulmonary infections, its poor penetration into the cerebrospinal fluid is often not appreciated. In humans without meningitis, no detectable levels of this antibiotic were achieved after the intravenous administration of cephalothin, whereas even in patients with meningitis, low levels of 0.16–0.31 μg/ml were obtained (155). After the rapid intravenous infusion of 2–4 g of cephalothin in 14 patients, either as a single dose or at 4–6-hr intervals for several days and, in one case, for 3 weeks, Lerner (256) found cerebrospinal cephalothin levels of 0.155–25 μg/ml only in patients with cerebrospinal fluid protein content in excess of 50 mg/100 ml. When the protein concentration was less than 50 mg/100 ml, no measurable drug activity could be found in the cerebrospinal fluid.

Brown *et al.* (257) treated 12 patients with meningococcal meningitis with intravenous cephalothin at a dosage of 100–200 mg/kg/day. Clinical results were variable, and they concluded that this antibiotic could not be recommended as an effective alternative to penicillin or ampicillin. Mangi *et al.* (258) recently summarized eight cases of meningitis that developed during cephalothin therapy from organisms sensitive to this antibiotic, indicating dramatically its poor penetration into the cerebrospinal fluid.

Empirical Therapy of Infections from the Genitourinary or GI Tract—Since infection derived from the genitourinary and GI tracts are also usually from Gram-negative organisms with a wide variety of antimicrobial susceptibility patterns, the antibiotic "coverage" must be very broad. Combination of ampicillin and gentamicin meets this requirement better than a cephalosporin with gentamicin. Gentamicin sulfate is an aminoglycoside which has *in vitro* activity against practically all important Gram-negative and Gram-positive bacteria including *Ps. aeruginosa*, *Serratia marcescens*, and *S. aureus*. This antibiotic, however, is usually not effective against *Strep. pneumoniae*, group D *Streptococcus* (enterococcus), and anaerobes such as *Bacteroides* species. By adding ampicillin, protection also is created against the group D *Streptococcus* and *Strep. pneumoniae*, but there is only moderate defense against *Bacteroides*.

There is usually no need for antibiotic coverage of anaerobes, *Strep. pneumoniae*, group A *Streptococcus*, and *S. aureus* in the urinary tract, since these organisms are essentially of no clinical importance in this area. Thus, since cephalosporins have little activity against group D *Streptococcus*, and since ampicillin has a high degree of activity against this organism, the latter antibiotic seems to be the more appropriate drug to combine with gentamicin, especially when the suspected source of the bacteremia is from the urinary tract, an area where the enterococcus commonly resides.

Table XI—Organisms Isolated from Blood in Patients with Bacteremia *Definitely* Related to Urinary Tract Instrumentation

Organism	Number of Cases	Cephalosporin Activity[a]
Escherichia coli	9	±
Enterococcus	4	—
Enterobacter species	3	—
Proteus mirabilis	3	+
Proteus vulgaris	2	—
Citrobacter freundii	2	—
Proteus morganii	1	—
Klebsiella species	1	+
Serratia marcescens	1	—
Pseudomonas aeruginosa	1	—
Total	27	

[a] + = high degree of activity, ± = moderate activity, and — = no activity.

Table XI records the organisms that were definitely involved in bacteremia following urinary tract manipulation from 1970 to 1973 (255). Until these epidemiological data were shown to physicians, particularly to urologists, it was customary to administer a cephalosporin as a sole antibiotic in patients who suddenly became septic following instrumentation of the urinary tract. As can be seen from this table, over half of the organisms were not covered by this antibiotic; a combination of gentamicin and ampicillin would have provided appropriate protection against all of these pathogens.

Table XII (255) shows the 10 most common organisms producing bacteremias on a general surgical service during the same period. It is again apparent that over half of the organisms would not have been covered if a cephalosporin was used as the only antibiotic.

Additional Therapeutic and Economic Considerations in Selection of Cephalosporins for Parenteral Administration—From an inspection of Table XIII and Fig. 10, it is apparent that cephalothin and cephapirin are very similar from a pharmacokinetic standpoint. After the administration of 1 g of either antibiotic, results from sequential determinations of blood levels are very similar. Moreover,

Table XII—"Top Ten" Bacteremias in General Surgery (1970–1973)

Organism	Number of Isolates	Cephalosporin Activity[a]
Escherichia coli	23	±
Pseudomonas	12	—
Bacteroides	12	—
Klebsiella	11	+
Staphylococcus aureus	8	+
Enterococcus	7	—
Proteus indole +	6	—
Proteus indole —	5	+
Enterobacter	4	—
Serratia	3	—
Total	91	

[a] + = high degree of activity, ± = moderate activity, and — = no activity.

Table XIII—Comparison of Pharmacokinetic Parameters of Cephalosporin Antibiotics

Parameter	Cephalexin[a]	Cephradine	Cephaloglycin[a]	Cefazolin	Cephalothin	Cephaloridine	Cephapirin	Cephacetrile	Cefoxitin	Cephanone
Serum clearance, ml/min	248 ± 11	435	—	62 ± 6	472	167	—	230—240	—	56.3
Renal clearance, ml/min	252 ± 5	367	—	64 ± 6	274	125	—	230—240	—	46.6
Renal excretion (24 hr), %	80—100	79—96	18—25	100	52	75	41	60—100	90—100	92
Half-life, hr	0.9	0.8	1.5	1.8	0.6	0.6—1.5	0.7	1.3	0.67	2.8
Half-life after hemodialysis, hr	—	—	—	6.9—9.3	3.3	3—5	1.8	—	—	—
Percent protein bound	6—10	8—20	24	73—86	65	8—31	44—50	23—36	—	86—88
Peak serum levels (0.5 g), μg/ml	18	46	0.9—2.0	11—70	6—8	30	8	—	10.9	36.0
Peak serum levels (1.0 g), μg/ml	32	86	—	38—75	15—20	40	15	17—22	22.5	81.2
Vd_{β}, liters	23	22	—	10	22	—	35	26	19	11
Vd_{ext}, liters	32	31	—	13	40	—	113	41	29	15

[a] Oral administration.

cephapirin shares essentially the same antibacterial spectrum (208) as cephalothin with similar therapeutic results from infections such as pneumoniae (259, 260), septicemia, empyema, osteomyelitis, soft tissue infections, and urinary tract infections (148, 213, 261, 262). Adding even further to the similarities is the conversion of both antibiotics to a desacetyl metabolite, which results in appreciable loss of antibiotic activity (263).

A double-blind study (264) comparing the phlebitis produced by cephapirin and cephalothin showed that neither the incidence nor the degree of phlebitis was significantly different with the two drugs. Although cephalothin and cephapirin are not generic equivalents, there appears to be no reason why these antibiotics cannot be considered equivalent, making it acceptable and safe for a patient receiving 1 g of cephalothin to be given 1 g of cephapirin in its place or vice versa.

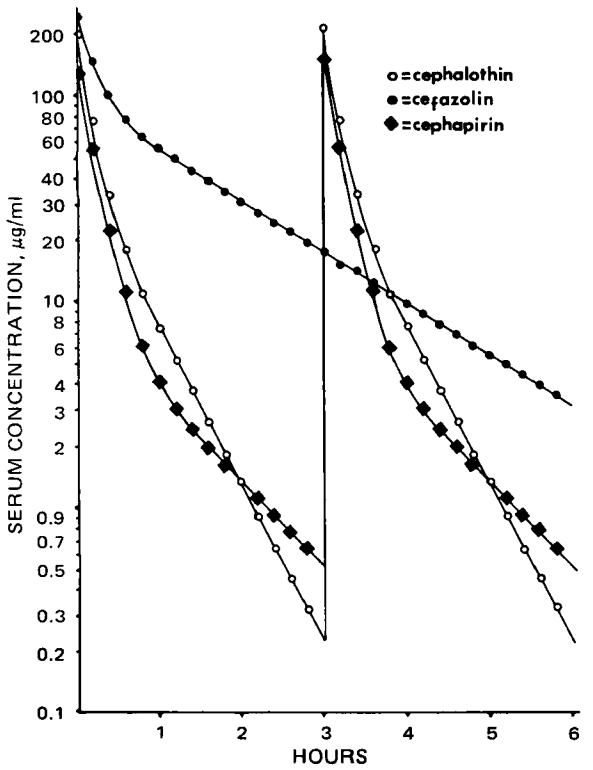


Figure 10—*Comparison of serum levels after intravenous administration of a single 1-g dose of cefazolin* versus *1 g of cephalothin or cephapirin every 3 hr (rate constants for data generation reported in Tables IV–VI).*

Cephaloridine has little justification for use in clinical medicine any longer since it is the only parenteral cephalosporin associated with significant nephrotoxicity (265–268) in humans and has no greater therapeutic effectiveness than any of the other three available cephalosporins for use by the parenteral route. With the recent availability of cefazolin, the logic for the continued usage of cephaloridine has been reduced even further.

Of the cephalosporins presently available for administration by the parenteral route, cefazolin is the preferred agent on both a therapeutic and economic basis. This impression is based on the following reasoning:

1. Cefazolin provides serum levels two to three times as high as cephalothin or cephapirin (see pharmacological and pharmacokinetic sections) (Table XIII and Fig. 10).

2. Biliary tract excretion of cephalosporins in humans is highest with cefazolin (approximately 17, 7, and 1 μg/ml for cefazolin, cephaloridine, and cephalothin, respectively) (269).

3. Cefazolin can be given comfortably and safely by either the intravenous or intramuscular route. In a dosage greater than 0.5 g, cephalothin is poorly tolerated.

4. Unlike cephalothin or cephapirin, cefazolin is not metabolized into a less active compound (see sections on pharmacology and pharmacokinetics).

5. Clinical studies demonstrated that cefazolin has therapeutic effectiveness at least as adequate as cephalothin and cephapirin (44).

6. Toxicology studies in animals and clinical experience with cefazolin in humans indicate that it is as safe as cephapirin and cephalothin (110, 274).

7. Cefazolin has the longest half-life of all cephalosporins (cephalothin, 0.6 hr; cephapirin, 0.7 hr; cephaloridine, 0.6–1.5 hr; and cefazolin, 1.8 hr). As a result, it can be administered less frequently (Table XIII).

8. Since cefazolin can be given less frequently and in a smaller total daily dosage (approximately half as frequently and/or half the number of grams), appreciable reduction in pharmacy cost can be predicted.

9. The microbiological activity of cefazolin is essentially similar to that of cephalothin and cephapirin except for its slightly greater activity against *E. coli* and *Enterobacter* species (111, 270).

10. To increase the economic savings for both the hospital and the patient, advantage should be taken of cefazolin's ability to be administered without appreciable discomfort by the intramuscular route. Such administration will reduce the cost of intravenous administration and lessen the demands on the intravenous therapist. The levels achieved by 1 g of cefazolin by the intramuscular route are high; peak levels in 1 hr are about 70 μg/ml, with appreciable levels (8–10 μg/ml) still present at 8 hr. These levels are certainly adequate since most Gram-positive bacteria such as *S. epidermidis, S. aureus,* the pneumococcus, and group A *Streptococcus* require less than 2–3 μg/ml, and the average MIC of cefazolin for Gram-negative bacteria such as *E. coli, P. mirabilis, Salmonella* species, *K. pneumoniae, H. influenzae,* and *Shigella* species are 1.6, 6.2, 4.0, 2.0, 4.0, and 8.0 μg/ml, respectively (104).

11. More frequent use of intramuscular medication will reduce the incidence of hospital deaths associated with bacteremia from prolonged in-dwelling intravenous polyethylene catheters.

12. Cefazolin is manufactured by two different pharmaceutical companies, allowing for competitive bidding.

In brief, cefazolin appears to combine the best features of cephalothin, cephapirin, and cephaloridine.

Cephalosporins for Oral Administration—Cephalexin, cephradine, and cephaloglycin are semisynthetic analogs of cephalosporin C that can be administered by the oral route. Their antibacterial spectrum *in vitro* is very similar to the parenteral cephalosporins; as expected, the enterococci, indole-positive *Proteus, Enterobacter, Serratia,* and *Pseudomonas* organisms are usually resistant. These oral agents often have less activity against staphylococci than any of the parenteral cephalosporins or the penicillinase-resistant penicillins. Cephalexin and cephradine have become the favorite oral cephalosporins since, in contrast to cephaloglycin which is relatively poorly absorbed from the GI tract and produces effective concentrations only in the urine (271), they are well absorbed and result in adequate antibiotic levels throughout most of the body (68, 79, 272).

Although cephalexin and cephradine have been used successfully in many infections of the respiratory tract, soft tissue, and urinary tract (63, 273), there is no evidence that their results are any better in the treatment of these illnesses from organisms that are sensitive to much cheaper oral drugs such as penicillin, ampicillin, oxacillin, dicloxacillin, or erythromycin. Their main use probably should be in the followup management of patients who are initially treated with parenteral cephalosporins or in the initial treatment of patients who have infections of the urinary tract from organisms (usually *Klebsiella)* resistant to other oral antibiotics or of patients with a history of penicillin hypersensitivity.

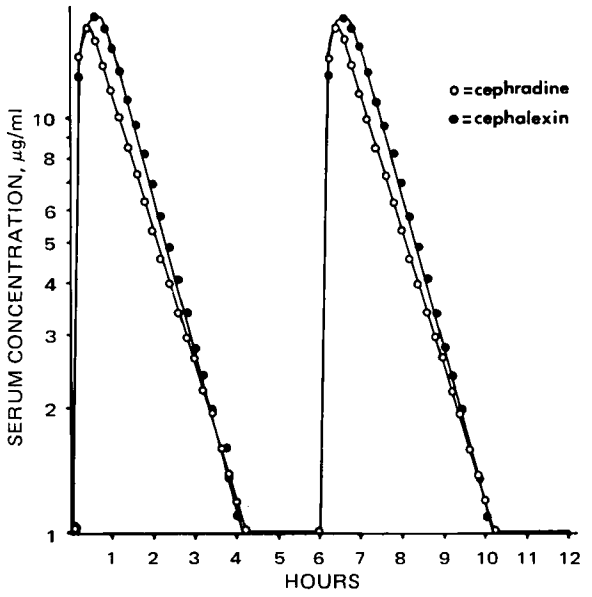


Figure 11—*Serum concentration of cephradine and cephalexin after 500 mg of each drug by the oral route (rate constants for data generation reported in Tables II and III).*

Cephalexin and cephradine, although different drugs, can also be considered similar in terms of pharmacokinetics and therapeutic equivalence (66) and can be interchanged in a fashion analogous to cephalothin and cephapirin. Figure 11 depicts the almost identical serum levels of cephradine and cephalexin following the administration of 500 mg of each drug. Moreover, inasmuch as cephradine and cephalexin are produced by different pharmaceutical companies, competitive bidding should result in a significant reduction in cost for the hospital and patient.

Conclusion—In brief, an explosion in the number of commercially available cephalosporins has occurred as the potential market for the sale of this class of antimicrobial agents has grown appreciably. To prevent excessively high hospital pharmacy cost with the resultant increase in the cost of a patient's hospital stay, the present cephalosporins and each new one that appears—which will be numerous—must not be automatically looked upon as "unique" antibiotics, even though they are not exact generic equivalents. The important issue is whether they are equivalent from pharmacokinetic and therapeutic standpoints, since the former determines the mode of administration and the likelihood of an adverse drug reaction, while the latter decides whether a patient will survive an infectious disease process.

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Inhibition of Drug Metabolism by Hydroxylated Metabolites: Cross-Inhibition and Specificity

DAVID M. SODA and GERHARD LEVY ˣ

Abstract □ Inhibition of drug metabolism was studied in adult male Sprague–Dawley rats. A hydroxylated metabolite of phenylbutazone (oxyphenbutazone) inhibited the elimination of phenytoin, which is metabolized by oxidative pathways. The biotransformation of a relatively polar and only slightly plasma protein-bound drug, antipyrine, was subject to product inhibition by a hydroxylated metabolite, 4-hydroxyantipyrine. Neither oxyphenbutazone nor 4-hydroxyantipyrine measurably affected the elimination kinetics or metabolic fate of a drug (sulfanilamide) that is not metabolized by oxidative pathways.

Keyphrases □ Metabolism—oxyphenbutazone, phenytoin, antipyrine, sulfanilamide, effect of hydroxylated metabolites, rats □ Oxyphenbutazone—effect on metabolism of phenytoin and sulfanilamide, rats □ Antipyrine—biotransformation, effect of 4-hydroxyantipyrine, rats □ Sulfanilamide—biotransformation, effect of oxyphenbutazone and 4-hydroxyantipyrine, rats □ Phenytoin—biotransformation, effect of oxyphenbutazone, rats □ 4-Hydroxyantipyrine—effect on metabolism of antipyrine and sulfanilamide, rats

Several studies have shown that the hydroxylated metabolites of certain drugs can inhibit the biotransformation of their precursors. Such inhibitory effects were demonstrated *in vitro* with 5-(*p*-hydroxyphenyl)-5-phenylhydantoin (hydroxyphenytoin) and 2-hydroxydesmethylimipramine (1, 2), the metabolites of phenytoin and desipramine, respectively. *In vivo* studies revealed the inhibitory effect of injected hydroxyphenytoin and oxyphenbutazone on the elimination of phenytoin and phenylbutazone, respectively (3, 4). Accumulation of endogenously formed hydroxyphenytoin in the body by blocking its glucuronidation also has caused inhibition of phenytoin elimination (5). Computer simulations indicate that product inhibition may account for the dose-dependent change in the half-life of some drugs (6).

More recently, it was shown *in vitro* that the hydroxylated metabolite of one drug can inhibit the biotransformation of another drug. Such cross-inhibition was reported for the following metabolite–drug systems: hydroxyphenytoin–phenylbutazone (7), oxyphenbutazone–phenytoin (7), hydroxyphenytoin–hexobarbital (8), hydroxyphenytoin–ethylmorphine (8), hydroxyphenytoin–zoxazolamine (9), and hydroxyphenytoin–aniline (9). It also has been shown that hydroxyphenytoin prolongs hexobarbital sleeping time in mice (8) and zoxazolamine paralysis time in rats (9), but there has been no direct pharmacokinetic demonstration of cross-product inhibition *in vivo*.

The purposes of this investigation were to determine: (*a*) if a product inhibitory effect can be demonstrated for a relatively hydrophilic and negligibly protein-bound drug (antipyrine), (*b*) if hydroxylated metabolites with a demonstrated inhibitory effect on the biotransformation of their precursors can affect the elimination of a drug metabolized by other than oxidative pathways (sulfanilamide), and (*c*) if the hydroxylated metabolite of one drug (oxyphenbutazone) can inhibit the *in vivo* elimination of another drug metabolized by oxidative pathways (phenytoin).

EXPERIMENTAL

All studies were performed on male Sprague–Dawley rats[1], 300–450 g. A two-piece cannula of silicone rubber–polyethylene was implanted in the right jugular vein under light ether anesthesia (3, 10), 3 days before the first experiment. Crossover experiments were carried out 2 weeks after the first experiment. Blood samples were obtained through one outlet of a three-way stopcock after withdrawal of 0.5 ml of blood (which was subsequently reinjected) to ensure against artifacts due to sample dilution by heparin or blood trapped in the cannula (3).

4-Hydroxyantipyrine–Antipyrine Experiments—Three rats received 4-hydroxyantipyrine[2], 100 mg/kg iv, 30 min before an intravenous injection of ^{14}C-antipyrine, 70 mg/kg, and additional doses of 4-hydroxyantipyrine, 50 mg/kg, every 45 min during the experiment. The hydroxylated metabolite was dissolved in a small volume of 1.2 *M* NaOH, which was diluted immediately with 0.2 *M* phosphate buffer, pH 7.4, to yield a 100-mg/ml solution of pH

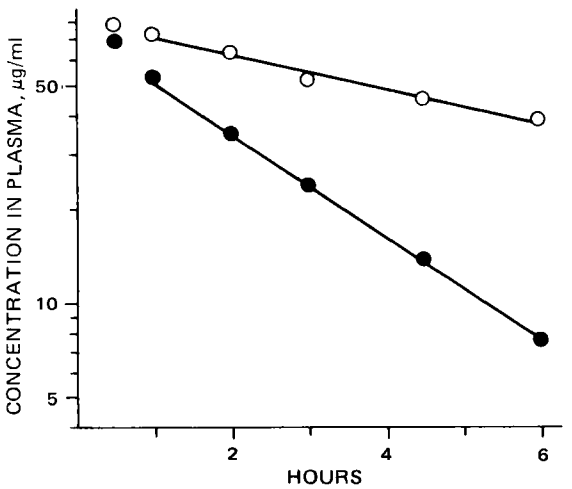

Figure 1—*Effect of 4-hydroxyantipyrine on the elimination of antipyrine, 70 mg/kg iv, in Rat 2-I. Key:* ●, *control experiment; and* ○, *with 4-hydroxyantipyrine, 100 mg/kg, 30 min before antipyrine injection and 50 mg/kg every 45 min thereafter for eight doses.*

[1] Blue Spruce Farms, Altamont, N.Y.
[2] Aldrich Chemicals Co., Milwaukee, Wis.

Table I—Effect of 4-Hydroxyantipyrine on the Elimination of Antipyrine in Rats

Rat	Half-Life, hr	
	Control	With 4-Hydroxyantipyrine
1-I	1.80[a]	4.05
2-I	1.70	5.20[a]
3-I	1.83	13.0[a]
4-I	1.60[a]	4.70
5-I	1.46[a]	5.60
6-I	1.30	4.58[a]
Mean	1.65	6.18
SD	0.23	3.38
Statistical significance of difference[b]	$p < 0.02$	

[a] First experiment in the crossover study. [b] Paired *t*-test.

9.5–10. This solution was injected slowly (3–5 min) to prevent irritation and precipitation. Three control animals received the solvent only. Crossover studies were performed on all six rats. The antipyrine injection solution was prepared by adding 5 μCi of ^{14}C-antipyrine, 12.1 mg/mCi labeled in the *N*-methyl position[3], to 0.5 ml of a 70-mg/ml antipyrine[2] solution in water.

Blood samples (0.5 ml) were obtained at 0.5, 1.0, 2.0, 3.0, 4.5, and 6.0 hr after antipyrine injection. A 200-μl portion of plasma with 0.05 ml of 1 *M* NaOH was extracted with 2 ml of chloroform by shaking on a reciprocating shaker (200 cpm) for 10 min. The phases were separated by centrifugation at 400×*g* for 5 min, and the aqueous phase was removed by aspiration. A 1.8-ml portion of the chloroform phase was evaporated to dryness in 2-ml vials[4] under nitrogen at room temperature. The sides of the vessel were washed twice with chloroform which was also evaporated.

The residue was redissolved in 50 μl of chloroform and applied to a TLC plate of silica gel G with fluorescent indicator F-254[5], which had been activated (1 hr at 100°) immediately before use. The vial was rinsed twice with 20-μl portions of chloroform, which were also applied on the plate. In addition to the plasma extracts, a chloroform solution of "cold" antipyrine was spotted as an R_f reference. The plate was developed immediately with chloroform–ethanol (9:1) for 45–60 min in a chamber which had been equilibrated with the solvent system (11).

The spots on the dried plate were visualized under UV light,

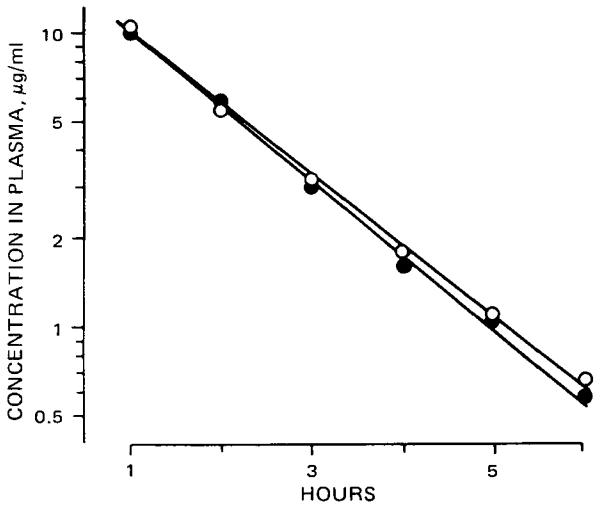

Figure 2—*Effect of 4-hydroxyantipyrine on the elimination of sulfanilamide, 20 mg/kg iv, in Rat 1-II. Key: ●, control experiment; and ○, with 4-hydroxyantipyrine, 100 mg/kg, 30 min before sulfanilamide injection and 50 mg/kg every 45 min thereafter for eight doses.*

[3] New England Nuclear Corp., Boston, Mass.
[4] Reactivials.
[5] EM Laboratories, Elmsford, N.Y.

Table II—Effect of 4-Hydroxyantipyrine on the Elimination of Sulfanilamide in Rats

Rat	Half-Life, hr	
	Control	With 4-Hydroxyantipyrine
1-II	1.15	1.26[a]
2-II	1.32[a]	1.38
3-II	1.17	1.22[a]
4-II	0.86[a]	0.85
Mean	1.13	1.18
SD	0.19	0.23
	N.S.	

[a] First experiment in the crossover study.

scraped off, and placed into vials containing 10 ml of scintillation fluid for subsequent counting in a spectrometer with an external standard. Recovery of antipyrine from spiked samples was 88 ± 3.7% (mean ± *SD*) and was not affected by the presence of 4-hydroxyantipyrine and its metabolite(s) (plasma from rats given 4-hydroxyantipyrine).

4-Hydroxyantipyrine–Sulfanilamide and Oxyphenbutazone–Sulfanilamide Experiments—Crossover studies were carried out on four rats for each of the two hydroxylated metabolites. The same dosage schedules used in the other experiments were used in this study. A control blood sample (for determination of blank value) was obtained immediately before injection of sulfanilamide, 20 mg/kg iv. A solution of the latter was prepared by dissolving 100 mg in 0.5 ml of 1 *M* NaOH, adjusting the pH to 8 with hydrochloric acid, and adding water to yield 5 ml.

Blood samples (0.5 ml) were obtained at 1, 2, 3, 4, 5, and 6 hr after sulfanilamide injection. The animals were housed individually in plastic metabolism cages for 2 days. Sulfanilamide in plasma and urine and sulfanilamide metabolites in urine were assayed by a modification of the Bratton–Marshall method (12). Two hundred microliters of plasma was shaken with 1 ml of 15% trichloroacetic acid and 0.5 ml of concentrated hydrochloric acid for 3 min. The precipitated proteins were separated by centrifugation at 400×*g*. To 1 ml of the supernate was added 0.1 ml of 0.2% sodium nitrite solution. After 15 min, 0.1 ml of 10% ammonium sulfamate solution was added. Five minutes later, 0.1 ml of 0.1% naphthylethylenediamine dihydrochloride solution was added; after 20 min, the absorbance was determined at 545 nm, using microcells.

Assays of urine were carried out with 2 ml of appropriately diluted urine to which 5 ml of 15% trichloroacetic acid and 1 ml of concentrated hydrochloric acid had been added. Sulfanilamide metabolites were assayed by determining the difference between the concentration of total and unmetabolized drug. Total sulfonamide was determined after hydrolysis of the urine–trichloroacetic

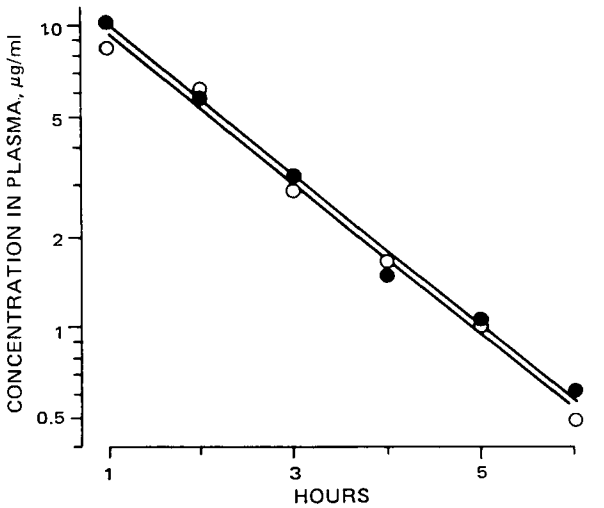

Figure 3—*Effect of oxyphenbutazone on the elimination of sulfanilamide, 20 mg/kg iv, in Rat 4-III. Key: ●, control experiment; and ○, with oxyphenbutazone, 80 mg/kg, at −1 hr and 40 mg/kg at +2 hr relative to the time of sulfanilamide injection.*

Table III—Effect of Oxyphenbutazone on the Elimination of Sulfanilamide in Rats

Rat	Half-Life, hr: Control	Half-Life, hr: With Oxyphenbutazone
1-III	1.23	1.12[a]
2-III	1.15[a]	1.12
3-III	1.35	1.38[a]
4-III	1.21[a]	1.19
Mean	1.24	1.20
SD	0.08	0.12
	N.S.	

[a] First experiment in the crossover study.

Table IV—Effect of Oxyphenbutazone and 4-Hydroxyantipyrine on the Metabolic Fate of Sulfanilamide in Rats

Experiment	Recovery in 48-hr Urine, % of Dose[a]: Sulfanilamide	Recovery in 48-hr Urine, % of Dose[a]: Metabolites
Oxyphenbutazone		
Without	22.4 ± 3.4	46.4 ± 5.4
With	25.8 ± 4.7[b]	49.9 ± 3.6[b]
4-Hydroxyantipyrine		
Without	23.9 ± 3.6	45.5 ± 7.7
With	25.2 ± 8.2[b]	46.3 ± 1.9[b]

[a] Mean ± *SD* for four animals in each group. [b] Not statistically significantly different from control value.

acid–hydrochloric acid mixture at 100° for 1 hr. Reference curves were based on absorbance data obtained with solutions of known sulfanilamide concentrations in plasma and water. Recovery of drug from spiked plasma samples from rats given 4-hydroxyantipyrine or oxyphenbutazone did not differ from the recovery from plasma of unmedicated animals.

Oxyphenbutazone–Phenytoin Experiments—Oxyphenbutazone[6], 80 mg/kg, was given intraperitoneally to 15 of 29 rats 1 hr before intravenous injection of ^{14}C-phenytoin, 10 mg/kg. A 40-mg/kg dose of oxyphenbutazone was administered 3 hr after the first dose of that drug. The control rats received only solvent instead of oxyphenbutazone. Six of the 29 rats were studied in crossover fashion.

Blood samples (~250 μl) were obtained at 10, 20, 40, 60, 80, 100, 120, 150, 180, and 210 min after phenytoin injection. Preparation of the injection solutions and analytical methodology were described previously (3, 4).

Plasma Protein Binding of Antipyrine—Plasma from unmedicated rats, with antipyrine added to yield concentrations from 7.4 to 74 μg/ml, was dialyzed at 37° for 3 hr against isotonic sodium phosphate buffer solution, pH 7.4, in dialysis cells separated by a cellophane membrane. Preliminary studies showed that dialysis equilibrium is attained in less than 3 hr under the experimental conditions.

Pharmacokinetic Analysis—Half-life values were obtained from the slope of the terminal linear portion of individual plots of the logarithm of drug concentration *versus* time, fitted to the data by the method of least squares.

RESULTS

The results of the crossover study of the effect of 4-hydroxyantipyrine on the elimination of antipyrine are summarized in Table I; a typical example of the detailed data is shown in Fig. 1. Treatment with 4-hydroxyantipyrine resulted in an almost fourfold increase in the average half-life of antipyrine.

Protein binding of antipyrine in rat plasma was 13.1 ± 1.5% (mean ± *SD*, n = 23), with no apparent concentration dependence in the 7.4–74-μg/ml range.

[6] Ciba-Geigy, Summit, N.J.

Table V—Effect of Oxyphenbutazone on the Elimination of Phenytoin in Rats

Type of Comparison	Half-Life ($t_{½\beta}$)[a], min: Control	Half-Life ($t_{½\beta}$)[a], min: With Oxyphenbutazone	Statistical Significance of Difference (p Value)
14 animals in control group, 15 in oxyphenbutazone group	57 ± 37	147 ± 93	<0.005[b]
Six animals in crossover study	47 ± 24	109 ± 97	<0.05[c]

[a] Mean ± *SD*. [b] Student t test. [c] Sign test for paired data.

Crossover experiments with and without 4-hydroxyantipyrine showed that this compound had no apparent effect on the elimination kinetics of sulfanilamide (Table II and Fig. 2). The same lack of effect was found when the plasma half-life of sulfanilamide was determined with and without oxyphenbutazone (Table III and Fig. 3). Neither 4-hydroxyantipyrine nor oxyphenbutazone had any apparent effect on the metabolic fate of sulfanilamide, as reflected by the composition of urinary excretion products (Table IV).

The results of the study of the effect of oxyphenbutazone on the elimination of phenytoin are summarized in Table V; detailed data obtained in crossover experiments on one animal are shown in Fig. 4 as a typical example. Administration of oxyphenbutazone with phenytoin caused an approximately 2.5-fold average increase in the plasma half-life of the latter. There was a strong (r = 0.95) and statistically significant (p < 0.005) positive correlation between the individual half-life values of phenytoin in control and oxyphenbutazone experiments in the group of six rats used for crossover studies.

DISCUSSION

The results of this investigation demonstrate directly *in vivo* that the hydroxylated metabolite of one drug can inhibit the elimination of another drug metabolized by an oxidative pathway. On the other hand, neither of the two hydroxylated metabolites with demonstrated inhibitory activity on oxidative biotransformation had any effect on the elimination of sulfanilamide, which is removed from the body mainly by acetylation and glucuronidation (*i.e.*, nonoxidative processes) and renal excretion (13). These results are consistent with the *in vitro* observations of others, which

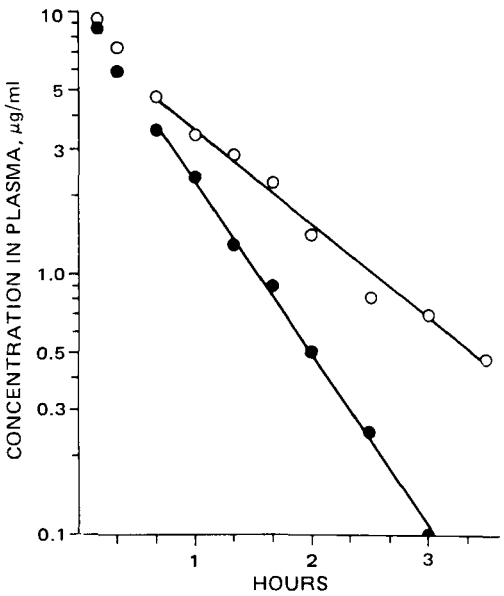

Figure 4—*Effect of oxyphenbutazone on the elimination of phenytoin, 10 mg/kg iv, in Rat 13-IV. Key:* ●, *control experiment; and* ○, *with oxyphenbutazone, 80 mg/kg, at −1 hr and 40 mg/kg at +2 hr relative to the time of phenytoin injection.*

suggested that product inhibition (including cross-product inhibition) of drug biotransformation processes may be due to an interaction of the inhibitory agents with cytochrome P-450 (8, 14). Specifically, a competition between the hydroxylated metabolite and the drug for binding sites on cytochrome P-450 was suggested (14).

The observation of product inhibition in the elimination of antipyrine is of particular interest because this drug differs from the others studied so far in that it is very hydrophilic and negligibly bound to plasma proteins. The metabolite 4-hydroxyantipyrine may be a potentially useful inhibitor of drug elimination in human drug therapy (analogous to the use of probenecid as an inhibitor of renal excretion of drugs). However, since its biological half-life in humans is very short[7], it may be most useful as an inhibitor of "first-pass" metabolism. This possibility remains to be explored.

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[7] Semilogarithmic plots of antipyrine concentration in plasma and 4-hydroxyantipyrine excretion rate *versus* time after administration of antipyrine to human subjects yield essentially identical half-life values (15), showing thereby that the biological half-life of 4-hydroxyantipyrine is much shorter than that of antipyrine.

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Solid-State Decomposition of *para*-Substituted Salicylic Acids

PAKDEE POTHISIRI and J. T. CARSTENSEN [x]

Abstract □ A series of *para*-substituted salicylic acids, all crystallizing monoclinically and decomposing by decarboxylation, were shown to adhere to Bawn-type kinetics, and the decomposition rate constants were shown to adhere approximately to a Hammett-type relation. The value of the reaction parameter was −8. Both *para*-substituted salicylic and benzoic acids occur as dimers in the crystalline state. The mechanism whereby the latter decomposes is speculated to be intermolecular in the sense that the substituent from one dimer interacts with the carboxyl group of a neighboring dimer. In the same sense of the word, the substituted salicylic acids decompose by an intramolecular mechanism, *i.e.*, within the dimer unit.

Keyphrases □ Salicylic acids, *para*-substituted—decomposition in the solid state, intramolecular mechanism, Bawn kinetics, Hammett relation □ Solid-state decomposition—*para*-substituted salicylic acids, intramolecular mechanism, Bawn kinetics, Hammett relation □ Decomposition—solid state, *para*-substituted salicylic acids, intramolecular mechanism, Bawn kinetics, Hammett relation

The theory of the kinetics of solid-state decomposition, where a solid decomposes into a liquid and a gas, was developed by Bawn (1); experimental data supporting the theory were reported by Carstensen and Musa (2). In this type of reaction, there are (at least) two parallel decomposition paths: (*a*) the solid itself decomposes *via* a first-order decay route with rate constant k_s in reciprocal time units, and (*b*) the compound saturates the liquid decomposition layer and here decomposes in solution with a rate constant k_l in reciprocal time units. It is assumed that the reaction products do not participate actively in the decomposition in any other way than supplying the liquid vehicle in which part of the overall reaction takes place.

BACKGROUND

As long as there is solid present, the fraction decomposed, x, will accelerate exponentially as shown in Fig. 1; as time progresses, there will be an increasing amount of liquid phase and a decreasing amount of solid phase present. At time t_S, the last solid compound disappears and the solubility, S (moles per mole), of the compound in its decomposition product can be determined from the fraction decomposed, x_S, at time t_S. At times later than t_S, the decomposition will be a conventional first-order decomposition with the shape denoted by BC in Fig. 1, so the overall curve (ABC in Fig. 1) will be of the frequently reported sigmoid shape.

One assumption made is the first-order decomposition in the solid phase; this point has been justified both theoretically and ex-

suggested that product inhibition (including cross-product inhibition) of drug biotransformation processes may be due to an interaction of the inhibitory agents with cytochrome P-450 (8, 14). Specifically, a competition between the hydroxylated metabolite and the drug for binding sites on cytochrome P-450 was suggested (14).

The observation of product inhibition in the elimination of antipyrine is of particular interest because this drug differs from the others studied so far in that it is very hydrophilic and negligibly bound to plasma proteins. The metabolite 4-hydroxyantipyrine may be a potentially useful inhibitor of drug elimination in human drug therapy (analogous to the use of probenecid as an inhibitor of renal excretion of drugs). However, since its biological half-life in humans is very short[7], it may be most useful as an inhibitor of "first-pass" metabolism. This possibility remains to be explored.

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[7] Semilogarithmic plots of antipyrine concentration in plasma and 4-hydroxyantipyrine excretion rate *versus* time after administration of antipyrine to human subjects yield essentially identical half-life values (15), showing thereby that the biological half-life of 4-hydroxyantipyrine is much shorter than that of antipyrine.

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Solid-State Decomposition of *para*-Substituted Salicylic Acids

PAKDEE POTHISIRI and J. T. CARSTENSEN [x]

Abstract □ A series of *para*-substituted salicylic acids, all crystallizing monoclinically and decomposing by decarboxylation, were shown to adhere to Bawn-type kinetics, and the decomposition rate constants were shown to adhere approximately to a Hammett-type relation. The value of the reaction parameter was −8. Both *para*-substituted salicylic and benzoic acids occur as dimers in the crystalline state. The mechanism whereby the latter decomposes is speculated to be intermolecular in the sense that the substituent from one dimer interacts with the carboxyl group of a neighboring dimer. In the same sense of the word, the substituted salicylic acids decompose by an intramolecular mechanism, *i.e.*, within the dimer unit.

Keyphrases □ Salicylic acids, *para*-substituted—decomposition in the solid state, intramolecular mechanism, Bawn kinetics, Hammett relation □ Solid-state decomposition—*para*-substituted salicylic acids, intramolecular mechanism, Bawn kinetics, Hammett relation □ Decomposition—solid state, *para*-substituted salicylic acids, intramolecular mechanism, Bawn kinetics, Hammett relation

The theory of the kinetics of solid-state decomposition, where a solid decomposes into a liquid and a gas, was developed by Bawn (1); experimental data supporting the theory were reported by Carstensen and Musa (2). In this type of reaction, there are (at least) two parallel decomposition paths: (*a*) the solid itself decomposes *via* a first-order decay route with rate constant k_s in reciprocal time units, and (*b*) the compound saturates the liquid decomposition layer and here decomposes in solution with a rate constant k_l in reciprocal time units. It is assumed that the reaction products do not participate actively in the decomposition in any other way than supplying the liquid vehicle in which part of the overall reaction takes place.

BACKGROUND

As long as there is solid present, the fraction decomposed, x, will accelerate exponentially as shown in Fig. 1; as time progresses, there will be an increasing amount of liquid phase and a decreasing amount of solid phase present. At time t_S, the last solid compound disappears and the solubility, S (moles per mole), of the compound in its decomposition product can be determined from the fraction decomposed, x_S, at time t_S. At times later than t_S, the decomposition will be a conventional first-order decomposition with the shape denoted by BC in Fig. 1, so the overall curve (ABC in Fig. 1) will be of the frequently reported sigmoid shape.

One assumption made is the first-order decomposition in the solid phase; this point has been justified both theoretically and ex-

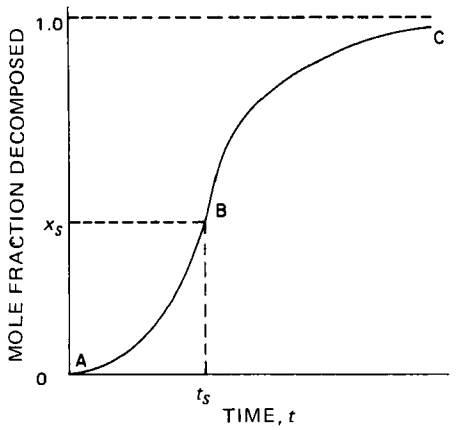

Figure 1—*Typical sigmoid-shaped curve for fraction decomposed in a solid as a function of time, when the solid decomposition produces a gas and a liquid. AB is the acceleratory period where both parent solid and liquid decomposition products are present, and BC is the part of the curve where only the liquid phase is present. The time scale is linear.*

perimentally by the authors (3). The assumption that the liquid reaction product is inert has been justified by consistency checks of the values of k_l obtained from the AB and BC portions of the curve in the case of substituted benzoic acids (2).

For a series of *para*-substituted benzoic acids, Carstensen and Musa (2) showed that the substituent σ-values have to be more negative than −0.3 for the compound to decompose below its melting point. Although the log k_s *versus* σ plot did not give a good linear fit, the correlation coefficient was high (0.9) and the reaction parameter, ρ, was positive. There have been other attempts reported in the literature to tie in reaction rates with substituent σ-values in solid-state reactions. Clark (4–6) found no such effect in a series of malonic acids, and Dorko *et al.* (7, 8) found no systematic correlation in decomposition reactions of tosylates. On the other hand, Meyers *et al.* (9) studied the reaction of benzoic acid derivatives with their salts at elevated temperatures under vacuum and obtained a systematic relation between the reactivities and the substituent σ-values of the compounds.

The aim of the study reported here was to determine whether a chemical substituent effect exists for k_s in a Bawn-type decomposition. A series of *para*-substituted salicylic acids was chosen because they were found experimentally to crystallize in the same crystal system (monoclinic) and they exhibited a clean decarboxylation decomposition without side reactions. These compounds, furthermore, have been studied in solution in quinoline (10, 11); in such solutions, they exhibited a linear free energy relationship for substituents with σ-values of less than +0.3.

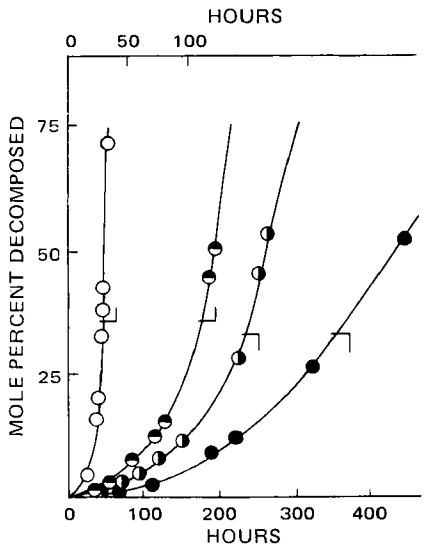

Figure 2—*Typical experimental decomposition curves. The data are* p-*ethoxysalicylic acid at 384°K (●), 399°K (◑), 404°K (◓), and 414°K (○).*

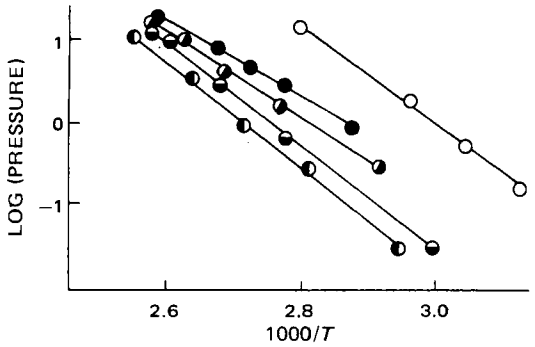

Figure 3—*Vapor pressure curves of five substituted salicylic acids. Key:* ○, p-*amino;* ◒, p-*diethylamino;* ◐, p-*dimethylamino;* ◑, p-*ethoxy; and* ●, p-*hydroxy.*

EXPERIMENTAL

Samples[1] of *p*-dimethylaminosalicylic acid, *p*-diethylaminosalicylic acid, *p*-hydroxysalicylic acid, and *p*-ethoxysalicylic acid were purified by recrystallization from anhydrous methanol. The crystals were reduced by grinding in a porcelain mortar and pestle to a particle size of 8–10 μm as measured by permeamitry[2]. Sample preparation and gas analysis methods were as described elsewhere (2, 3). As in the quoted cases dealing with *para*-substituted benzoic acids, the volatility of the compounds prohibits the use of conventional vacuum setups (12) since they would cause sublimation of the compound from the heated reaction site to the cooler analysis portion of the apparatus. Therefore, the break-seal technique described by Carstensen and Musa (2) was used; sample sizes were 300 mg.

The amount of decomposition was monitored by the number of moles of gas produced. The remaining solid was titrated and the amount of titer lost was shown to equal the number of moles of gas evolved, so the reactions are stoichiometric and of the form: $XC_6H_3(OH)COOH \rightarrow XC_6H_4OH + CO_2$. Since the compounds tested are somewhat volatile at the temperatures used in the study, their vapor pressures were determined as described elsewhere, as were the gas phase decomposition rate constants (2). The value of t_S was determined (to within 15 min) by visual observation of the break-seal tubes in each kinetic run.

The σ-values for one compound, *p*-diethylaminosalicylic acid, is

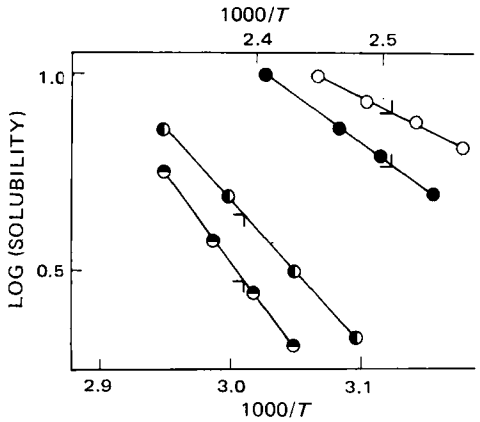

Figure 4—*Solubility–temperature plots of four* para-*substituted salicylic acids. Key:* ◒, *diethylamino;* ◐, *dimethylamino;* ●, *ethoxy; and* ○, *hydroxy.*

[1] Aldrich Chemical Co., Milwaukee, WI 53233

[2] Fisher sub-sieve sizer, Catalog No. 14-311, Instrument Division, Fisher Scientific, Pittsburgh, Pa.

Table I—Substituent σ-Values for Some *para*-Substituents

Substituent X	pKa of XC_6H_4COOH	σ[a]	pKa of $XC_6H_3(OH)COOH$	pKa (H) − pKa (X)
H	4.21	0	2.90[d]	0
OC_2H_5	4.45	−0.24	3.16[b]	−0.26
OH	4.59	−0.37	3.23[b]	−0.33
$N(CH_3)_2$	5.05 (6.01[c])	−0.84 (−1.88)	3.76[b]	−0.86
$N(C_2H_5)_2$	(6.19[c])	(−1.98)	3.84[b]	−0.94

[a] References 14 and 15. [b] Values obtained in this laboratory at 25 ± 0.1°. [c] Reference 16. [d] Reference 17.

unreported in the literature. The pKa value of each compound was determined potentiometrically according to the method of Albert and Sergeant (13).

RESULTS AND DISCUSSION

All of the compounds exhibited decomposition curves of the type shown in Fig. 1; the decomposition curves for *p*-ethoxysalicylic acid are shown in Fig. 2. The results from the vapor pressure determinations are shown in Fig. 3. The linearity gives some assurance (although it does not guarantee) that the compounds do not undergo polymorphic transformation in the temperature range studied. The decomposition rate of the compounds in the gaseous state was of such a magnitude that it would contribute less than 1% of the total decomposition in an ordinary solid run; in the following discussion, it is assumed that vapor phase decomposition can be neglected.

As mentioned, the solubility of the *para*-substituted salicylic acids in their corresponding *meta*-substituted phenols can be determined by removing the break-seal tube at the time of complete

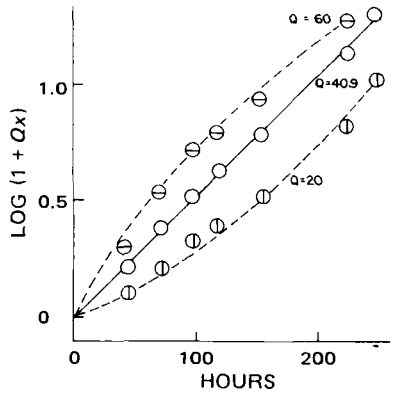

Figure 5—*Plot of ln [1 + Qx] versus time (Eq. 1) for varying values of* Q. *The data relate to the solid-state decomposition of* p-*ethoxysalicylic acid at 399°K.*

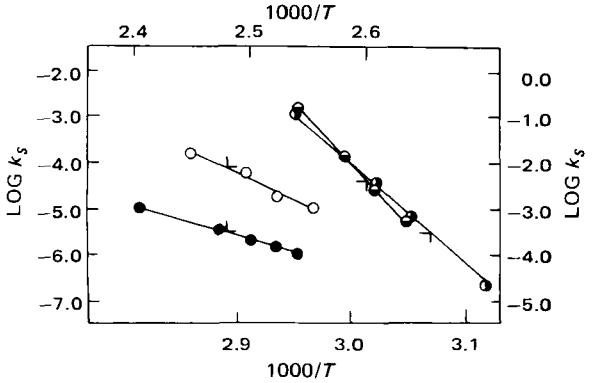

Figure 6—*Temperature dependence of decomposition rate constants in the solid state for* para-*substituted salicylic acids. Key:* ◑, *diethylamino;* ◒, *dimethylamino;* ●, *ethoxy; and* O, *hydroxy.*

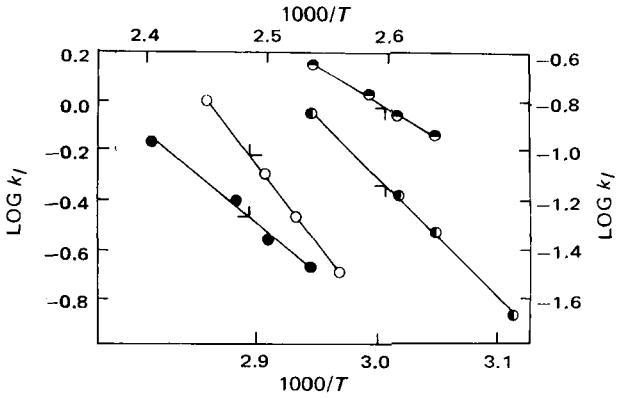

Figure 7—*Temperature dependence of decomposition rate constants in the liquid state for* para-*substituted salicylic acids in their decarboxylated analogs. Key:* ◒, *diethylamino;* ◐, *dimethylamino;* ●, *ethoxy; and* O, *hydroxy.*

liquefaction, t_S, and determining the mole fraction, x_S, decomposed. The solubility is then determined as $S = (1 - x_S)/x_S$ moles per mole. The solubilities are shown in Fig. 4, and the fact that log S is linear in $(1/T)$ is an indication (although no guarantee) that no polymorphic transformation takes place in the temperature range of the kinetic study.

The pKa values determined are shown in Table I, as are a series of reported σ-values for the substituents in question. The last column, denoted pKa (H) − pKa (X), is the difference between the pKa of the unsubstituted salicylic acid and the pKa of the X-substituted salicylic acid. The σ-value for the diethylamino substituent based on the pK value of the diethylaminobenzoic acid (6.19) appears to be of an abnormally high negative value. Since values of pKa (X) − pKa (H) for the salicylic acid series in the case of ethoxy, hydroxyl, and dimethylamino substituents are identical to reported σ-values, the value of pKa (H) − pKa (X) for diethylaminosalicylic acid was used in this study as was the σ-value for the diethylamino substituent.

If a solid decomposes into 1 mole of gas and 1 mole of a liquid decomposition product, then the mole fraction decomposed, x, is given by (1–3, 18, 19):

$$\ln [1 + Qx] = Qk_s t \qquad \text{(Eq. 1)}$$

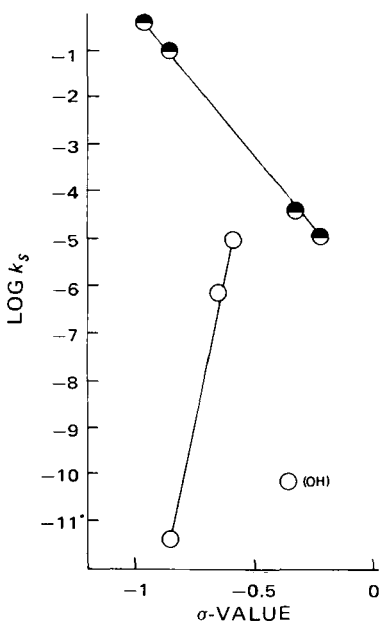

Figure 8—*Hammett-type plot of a series of* para-*substituted salicylic acids (◒) and benzoic acids (O). (The latter are from Ref. 2.)*

Table II—Kinetic Parameter Values for Decomposition of *para*-Substituted Salicylic Acids in the Solid State

Parameter	Substituent			
	OH	OC_2H_5	$N(CH_3)_2$	$N(C_2H_5)_2$
σ	−0.33	−0.25	−0.86	−0.94
log k_s (100°)[a]	−4.45	−4.84	−0.89	0.59
log k_l (100°)[b]	−2.23	−1.27	0.97	0.89
E_a (solid), kcal/mole[a]	52.5 ± 1.7	33.0 ± 0.3	100 ± 3	119 ± 2
E_a' (liquid), kcal/mole[b]	27.1 ± 0.2	19.2 ± 0.8	19.0 ± 0.7	13.2 ± 0.3
log Z (solid)[a], hr^{-1}	26.1	14.4	61.6	73.6
log Z_l (liquid)[b], hr^{-1}	13.6	9.9	12.1	8.6
Melting point, T_m, °K	498.6	426.1	413.1	409.9

[a] $k_s = Ze^{-Ea/RT}$. [b] $k_l = Z_l e^{-Ea'/RT}$.

where:

$$Q = k_l S - k_s S - k_s \qquad \text{(Eq. 2)}$$

Q is found by iteration and is the value which in Eq. 1 imparts linearity to the data. The data in Fig. 5 are data from Fig. 2 plotted in the form of Eq. 1. Knowing Q allows calculation of k_s from the best slope, and then knowing S allows calculation of k_l from Eq. 2. The value of k_l can also be obtained from the part of the decomposition curve beyond the inflection point ($t > t_S$), and k_l values found in the latter fashion agree with the former to within ±25%. Values of k_s and k_l (from Eq. 2) are plotted logarithmically *versus* reciprocal temperature in Figs. 6 and 7; the Arrhenius relationship holds well in both cases.

Gomes (20) reported that there is an inherent difficulty in assigning thermodynamic parameters to data from solid-state reactions. This difficulty is primarily due to the facts that such reactions frequently are dictated and controlled by physical phenomena (*e.g.*, strain and diffusion) and that the solid-state reaction rate constants are not comparable to conventional constants in solution kinetics. In topochemical reactions, for instance, rate constants have dimensions of square centimeters per second where square root laws pertain (21, 22). In contrast, the k_s in this study is of a usual first-order dimension; the data are summarized in Table II. The k_s values[3] are plotted *versus* σ-substituent values in Fig. 8. There appears to be good linearity, and the general correlations imply that the reactions are controlled by chemical factors rather than (or in addition to) physical factors. That the liquid layer may preferentially dissolve a solid at points of strain has been suggested (3) and may minimize the physical factors in a Bawn-type reaction.

As seen in Fig. 8, the trend in a series of *para*-substituted benzoic acids is opposite in sign to that of *para*-substituted salicylic acids. The explanation for this result is sought in the crystallographic arrangement of the two series. Pothisiri (19) described the molecular packing arrangement in crystals of *p*-aminosalicylic acid, *p*-hydroxysalicylic acid, and *p*-aminobenzoic acid (Figs. 9 and 10). *p*-Aminobenzoic acid is built up in dimeric units; hydrogen bonding through the hydrogen of the carboxyl group with the carbonyl oxygen of the neighboring molecule forms links between

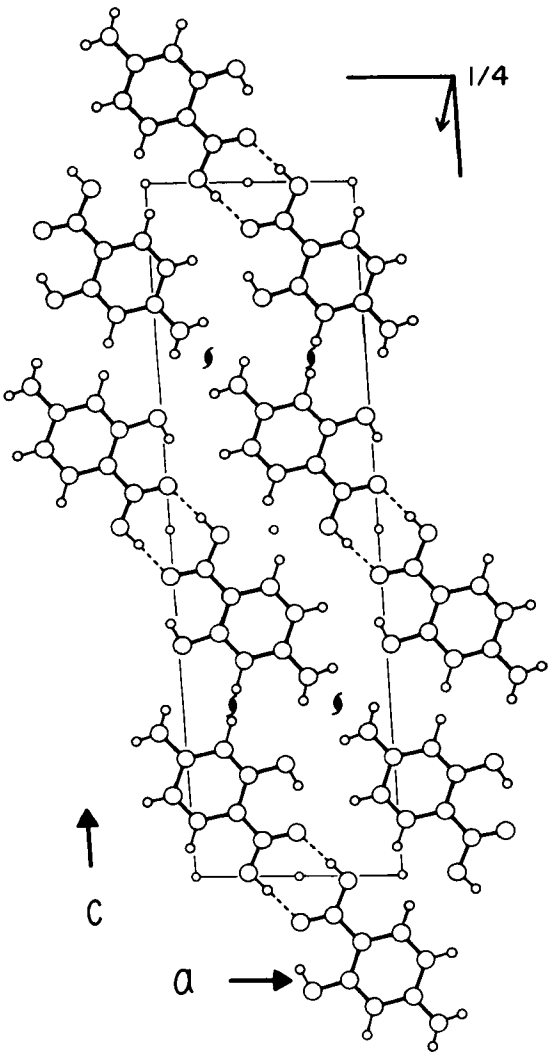

Figure 9—*Packing diagram of* p-*aminosalicylic acid in the [010] projection.*

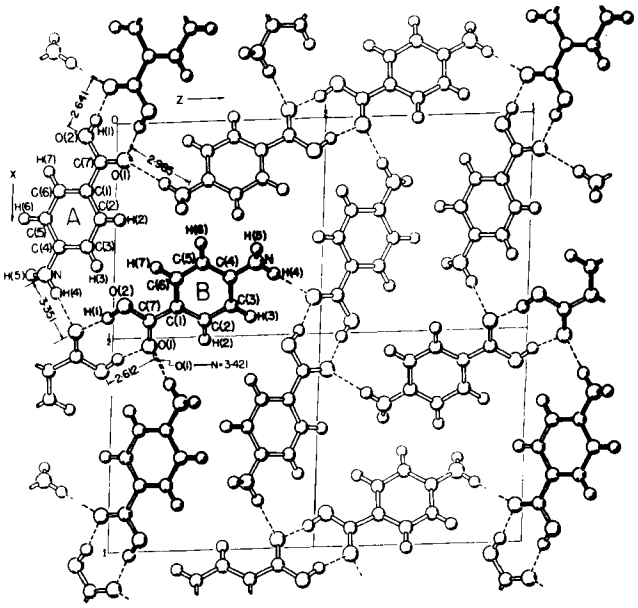

Figure 10—*Packing diagram of* p-*aminobenzoic acid in the [010] projection.*

[3] The k_l values were not plotted in this fashion, since each point corresponds to a different solvent.

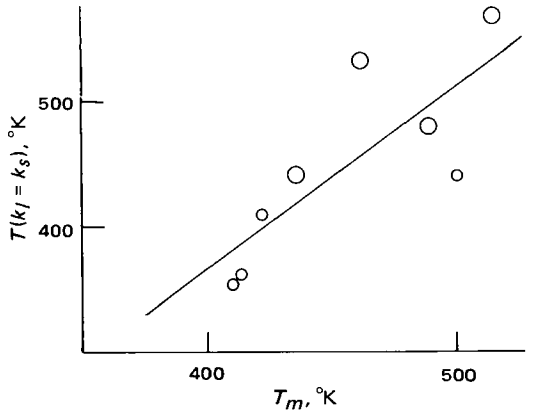

Figure 11—*Plot of the temperature where liquid and solid decomposition rate constants are equal* versus *the melting temperature.*

dimers. The arrangement is such, however, that there is a fairly close proximity (2.985 Å, Fig. 10) between the substituent X of one dimer pair and one carboxyl group of the neighboring pair.

In the case of substituted salicylic acids, however, Fig. 9 shows that the molecular unit is a dimer through hydrogen bonding between carboxyl groups and that a relatively strong intramolecular hydrogen bond exists between the hydroxyl group in the 2-position and the carboxyl oxygen atom. Pothisiri (19) showed that the O(3)—H(2) . . . O(2) distance is 2.610 Å and that there is no hydrogen bonding between the *para*-substituent from one molecule to the carboxyl oxygen of a neighboring molecule (dimer). Therefore, one explanation to the opposite signs of the reaction parameters would be that the *para*-substituted benzoic acid series decomposes *via* an intermolecular mechanism in the sense that there is an interaction between the substituent from one dimer pair with the carboxyl group of a neighboring dimer whereas, in the same sense of the word, compounds in the substituted salicylic series decompose *via* an intramolecular route.

No attempt was made here to seek further substantiation; the proposal should not be construed as a model but rather as a feasible explanation. Several kinetic characteristics (ρ, $\Delta S^{\ddagger}$, and E_a[4]) are of larger magnitude than in solution kinetics. Moreover, some points in the data treatment are not unambiguously resolved; there is, for instance, the possibility of a finite volume of activation which (since the pressure changes during the reaction) could change k_l and k_s as the reaction progresses. Furthermore, although the k_l values from the three-phase part of the curve equal those from the two-phase part[5] to within ±25%, the solution kinetics still could be carbon dioxide dependent, and this may influence the calculated k_s values. The sorting out of such parameters will have to await the results of a systematic kinetic study of *para*-substituted salicylic acids in their corresponding *meta*-substituted phenols at various carbon dioxide pressures.

[4] $\Delta S^{\ddagger}$ is the entropy of activation; it can be calculated from the data in Table II. It is based on a different standard state (moles per mole) than is customary in solution kinetics.

[5] During the latter part of the decomposition, only the liquid and gas phase are present.

The data in Table II allow calculation of the temperatures where k_l and k_s are equal. Such a calculation was performed on the *para*-substituted benzoic acid data reported by Carstensen and Musa (2). When these temperatures are plotted *versus* melting point, a straight line (with correlation coefficient of 0.9) results (Fig. 11). The general significance of this relation (and whether it will extend to other series of compounds) is left undiscussed here.

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Kinetics and Mechanisms of Drug Action on Microorganisms XXII: Effects of Aminosidine with and without Organism Pretreatment with Bacteriostatic Agents

EDWARD R. GARRETT ˣ and JOHN K. LEWIS

Abstract □ *Escherichia coli* generation in the logarithmic growth phase was inhibited in peptone broth USP at pH 7.0 without kill below 3.0 μg/ml of aminosidine. Above this value, the logarithms of the number of viables of the drug-treated culture ultimately decreased linearly with time and the slopes of these plots were independent of concentration. A concentration-dependent lag in the time of attainment of the cidal action was observed, and the extent of this lag was related to the ease of emergence of resistant organisms. The minimal concentration for cidal action increased with increasing concentrations of nutrients and with decreasing pH. Pretreatment of the cultures with novobiocin and tetracycline lessened the minimum bactericidal concentration of aminosidine whereas chloramphenicol pretreatment increased it. Tetracycline pretreatment inhibited the emergence of aminosidine-resistant organisms.

Keyphrases □ Aminosidine—kinetics and mechanisms of action, microorganisms, with and without pretreatment □ Antibiotics—aminosidine, kinetics and mechanisms of action, microorganisms, with and without pretreatment □ *Escherichia coli*—kinetics and mechanisms of aminosidine action, with and without pretreatment

Aminosidine is an oligosaccharide antibiotic isolated from *Streptomyces chrestomyceticus* (1). Structural studies indicated that the major component of aminosidine is identical with that of paromomycin and that aminosidine belongs to the neomycin group of aminoglycoside antibiotics (2). The nature of aminosidine or paromomycin activity indicates that it is closely related to streptomycin in its mode of action (3) in causing misreading of messenger-RNA. The bactericidal action may be due primarily to an irreversible binding to the ribosomes (3, 4).

The purpose of this work was to study the effects of aminosidine on a balanced growth culture of *Escherichia coli* as part of ongoing research into the kinetics and mechanisms of antibiotic action on microbial generation (5–7).

EXPERIMENTAL

Bacterial Cultures—The test organism, *Escherichia coli* (ATCC 12407) (5, 6), and the antibiotic medium 3[1] rehydrated to peptone broth USP (6) were as described previously. Studies were also conducted at twice and half the normal nutrient composition. The pH of the autoclaved medium, normally 7.0, was adjusted between 6.1 and 7.8 with sterile filtered 1 *N* HCl and 1 *N* NaOH for studies on pH effects

An aliquot (10 ml) of culture medium was inoculated from a fresh agar slant, and the culture generated for 15 hr at 37.5°. An aliquot (0.5 ml) was then diluted into 49.5 ml of fresh culture medium and generated to 2×10^7 *E. coli*/ml. An aliquot (0.5 ml) of this culture was treated similarly, and an aliquot of this final culture was diluted 100-fold into bulk broth. The suspension was dispensed by a sterile 49.5-ml pouring head into separate, sterile, loosely capped conical culture flasks maintained at 37.5 ± 0.01° in a constant-temperature water bath equipped with a shaker. After 25 min, when the organisms were in the logarithmic growth phase, 0.50 ml of an appropriate stock solution of antibiotic was added. In addition, the antibacterial effects of aminosidine[2] were studied at various inoculum concentrations of organisms.

The generation of viable and total *E. coli* as affected by aminosidine sulfate[2] concentrations was monitored by previously described methods (5–7); typical examples are shown in Figs. 1 and 2. Similar studies were conducted with paromomycin sulfate[3].

Bacterial Resistance to Aminosidine Action—Cultures of resistant organisms were prepared by overnight growth in the presence of 4.0, 5.0, and 6.0 μg/ml of aminosidine. Aliquots (0.5 ml) were diluted 100-fold with drug-free medium and subcultured twice at 1×10^7 organisms/ml. The presumed resistant organisms, inoculated at 1×10^5 organisms/ml in the logarithmic growth phase, were permitted to grow in fresh medium. At 60 min, drug concentrations ranging from 3.5 to 6.0 μg/ml were effected in different flasks and the generation rates were monitored. The various flasks were filtered at 1×10^9 organisms/ml, and the filtrates were stored at 4° for 17 hr. They then were inoculated with logarithmically generating naive organisms at 37.5°, and the generation rates were monitored.

Aminosidine Combinations with Bacteriostatic Agents—The effects on generation rates of aminosidine combinations with growth-inhibiting concentrations of the bacteriostatic chloramphenicol[4], tetracycline[5], or novobiocin[6] at pH 7.00 were studied by adding aminosidine subsequent to the establishment of drug-affected steady-state generation rates with one of the former.

Cell Size Distribution—The size–frequency distributions and total counts of the generating organisms in the presence of aminosidine were recorded with time using a pulse height analyzer[7]. This instrument had been calibrated with 1.305-μm diameter polytoluene latex beads.

Aminosidine pKa Values—Nitrogen-purged aminosidine sulfate solutions (10 ml containing 3 mg), to which a measured quantity of 1 *N* HCl had been added, were potentiometrically titrated[8] with 0.10 *N* NaOH. Volumes without drug and treated similarly were also titrated.

RESULTS AND DISCUSSION

Effect of Aminosidine on Microbial Generation—The numbers of *E. coli* as a function of time as affected by various concentrations of aminosidine are plotted semilogarithmically in Figs. 1 and 2. At bactericidal concentrations, the action of the drug demonstrated an initial lag period with a subsequent increasing diminution of generation rate. The numbers of viables tended to increase to a maximum and then decrease to approach an apparent first-order rate of dying, $k_{app} = -5.36 \times 10^{-4}$/sec at 37.5°, which was independent of drug concentration above the minimum necessary to exercise killing action. However, there was a relationship between the time required to achieve this first-order process of dying and drug concentration in the medium (Figs. 1 and 3), which

[1] Difco Laboratories, Detroit, Mich.

[2] Lot 515, Farmitalia Research Laboratories, Milan, Italy.
[3] Lot EPP96-I, Parke-Davis and Co., Detroit, MI 48232
[4] Lot H22254, Parke-Davis and Co., Detroit, MI 48232
[5] Lot U5965 (HCl) 4335R, The Upjohn Co., Kalamazoo, Mich.
[6] Albamycin U-6591, Lot 1FT33, The Upjohn Co., Kalamazoo, Mich.
[7] Coulter Channelyzer, Coulter Electronics Inc., Hialeah, FL 33010
[8] Radiometer TTTlc titrator with SBR2C titrigraph and SBU1a syringe burette, The London Co., Westlake, Ohio.

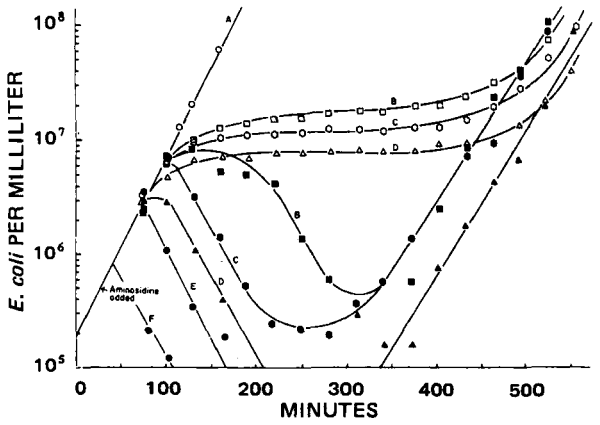

Figure 1—*Drug-affected death rates of* E. coli *in the presence of aminosidine sulfate at pH 7.00 in peptone broth USP. Open symbols are total counts, and solid symbols are viable counts. Key: A, 0.0 μg/ml; B, 4.0 μg/ml; C, 4.5 μg/ml; D, 5.0 μg/ml; E, 5.5 μg/ml; and F, 15 μg/ml.*

can be assigned to concentration-dependent processes of rates of drug permeation into the cell and reaction with receptor sites.

The fact that, after a definitive period of organism kill, there is a subsequent generation of viable organisms (Fig. 1) indicates: (*a*) development of drug-resistant organisms, (*b*) drug degradation in the culture with time, or (*c*) production of antagonists by the culture. The inoculation of fresh medium with these filtered organisms demonstrated generation rates ($k_o = 5.80 \times 10^{-4}$/sec) equivalent to those of naive organisms in the absence of drug, even at drug concentrations more than twice the minimum inhibitory concentrations for these organisms. When the filtrates of these cultures were inoculated with naive organisms, the action of aminosidine was the same as when equivalent amounts of fresh drug were added to fresh cultures.

It was concluded that negligible aminosidine degradation or antagonist production occurred *in situ* and that the later generation of organisms in the presence of bactericidal concentrations of drug was due to the production of resistants. The fact that extrapolation of the semilogarithmic plots for the generation of these resistant organisms (Fig. 1) gave different intercepts for the same inoculum size implied that resistant organisms developed during the generation observed in the presence of drug prior to the establishment of the first-order rate of kill. The longer the time at the lower bactericidal concentrations to obtain this maximum kill rate, the greater was the rate of production of resistant organisms.

Studies on aminosidine-affected microbial generation conducted at drug concentrations below the minimum (3.0 μg/ml) necessary to effect killing of *E. coli* at pH 7.0 demonstrated inhibition of generation rates with no significant kill. The evidence was the coincidence of viable and total counts of organism with time in these dose ranges.

There was a significant difference in the minimum concentration necessary to effect killing at pH 7.0 with various nutrient concentrations of medium. At twice the normal concentration of the nutrient medium, the value was 14 μg/ml but diminished to less than 3.0 μg/ml at half the normal nutrient concentration. This finding is strong evidence for antibiotic binding to the constituents of the medium.

Size distribution plots indicated that at the low drug concentrations studied, the mean size of the organisms decreased from 1.5 to 0.75 μm^3 as the population of the culture approached 1×10^8 *E. coli*/ml. This finding is consistent with the size distribution data observed for drug-free cultures.

At the higher aminosidine sulfate concentrations (*e.g.*, 15 μg/ml), which correspond to the plasma concentrations reached in humans under the recommended therapeutic regimen (8), the mean size remained the same after drug addition but the dispersion increased.

The organism appears to have an ability (Fig. 2) to survive with lessened generation rate until a critical concentration of aminosidine is reached in its biophase, at which time and concentration the process of dying becomes irreversible.

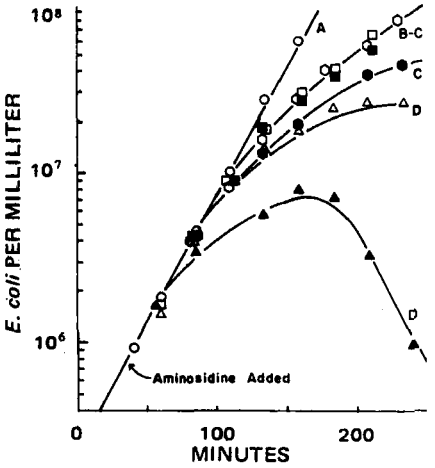

Figure 2—*Drug-affected generation of* E. coli *in the presence of aminosidine at pH 7.00 in peptone broth USP. Open symbols are total counts, and solid symbols are viable counts. Key: A, 0.0 μg/ml; B, 2.75 μg/ml; C, 3.0 μg/ml; and D, 3.25 μg/ml.*

There was no significant difference between the aminosidine- and paromomycin-affected generation curves for *E. coli* for all drug concentrations at pH 7.0 in the normal medium.

pH Effects of Aminosidine Action—Decreasing the pH of the culture medium from 7.8 to 6.1, which had no effect (9) on the generation rate of *E. coli*, increased the time necessary to reach the aminosidine-affected first-order rate of dying (Fig. 3) and increased the minimum concentrations of aminosidine necessary for observation of death from 3.0 to 12 μg/ml. The rate of death at bactericidal concentrations of aminosidine was independent of pH. These facts imply that the degree of protonation of the polyamine aminosidine decreases its rate of permeation into the cell, which may be dependent on the concentrations of species that are least ionized.

Analysis of the potentiometric titration curve (Fig. 4) of aminosidine by the difference method of Parke and Davis (10) permitted estimation of the pKa′ values for each of the five amino groups: 5.47, 6.24, 7.17, 8.15, and 9.07. These pKa′ values were estimated from the pH values at the midpoints of the five equal segments of the difference in titer necessary to achieve the same pH value for 10 ml of the aminosidine solution (0.300 mg/ml) and an equal volume of blank solution. The stoichiometric titer demonstrated that the active aminosidine was 706 μg/mg of the aminosidine sulfate used, which was within 5% of the estimated purity by the manufacturer's microbial assay.

Effects of Aminosidine Combinations with Bacteriostatic

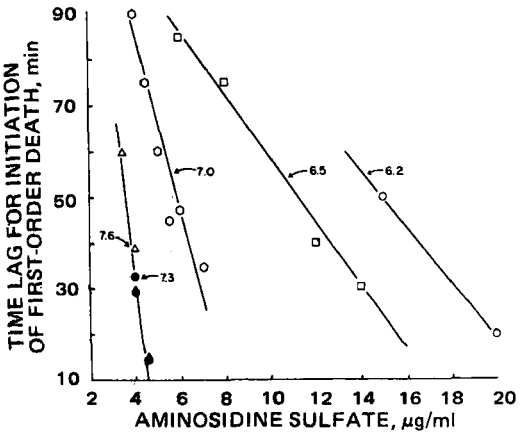

Figure 3—*Dependence of time lag to achieve first-order decay of* E. coli *on aminosidine concentrations. The lag was estimated from the intersection of the extrapolated linear segment of the decreasing log (numbers of organisms) against time with the semilogarithmic plot of the generation of organisms in the absence of drug. Curves are labeled with pH values of the medium.*

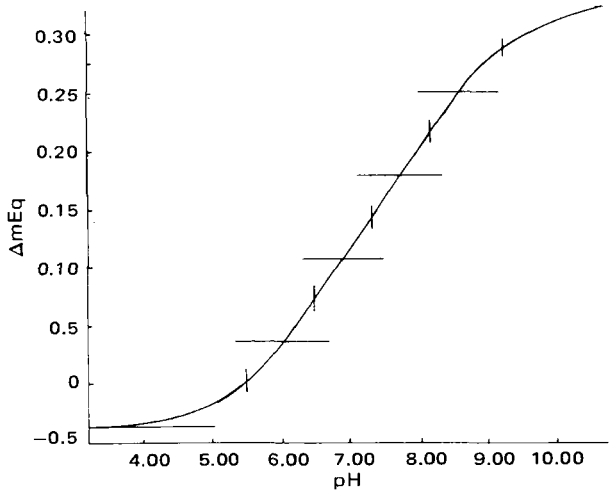

Figure 4—*Plots of the difference in milliequivalents of titer, ΔmEq, necessary to achieve a given pH for 10 ml of 0.300 mg/ml of aminosidine and an equal volume of blank solution against pH. The designated values are apparent pKa′ values at midpoints of five equal segments of the stoichiometric titer.*

Agents—The curves labeled A in Figs. 5–7 represent the generation rate of *E. coli* in the absence of any drug. The curves labeled B represent the action of the bacteriostatic agent alone. The curves labeled C, D, *etc.*, represent the action of aminosidine in combination with that same amount of bacteriostatic agent. The amount of aminosidine increased in the alphabetical order of the labeled curves. The minimum concentration for the ultimate killing rate of aminosidine alone on *E. coli* was 3.5 μg/ml, but it diminished to 1.0 μg/ml in the presence of 90 μg/ml of novobiocin (Fig. 5) and to 2.5 μg/ml in the presence of 0.3 μg/ml of tetracycline (Fig. 6); these combinations may be considered synergistic.

Only in the tetracycline case did combinations with 2.5, 3.0, and 3.5 μg/ml of tetracycline not show the development of resistant organisms on the following day. However, concentrations as high as 4.5 μg/ml of aminosidine in the presence of 1.98 μg/ml of chloramphenicol had less significant effects on *E. coli* generation (Fig. 7), and even this combination demonstrated the ultimate emergence of resistant organisms.

Rationalizations of Aminosidine Action—A toxicity model, similar to that proposed by Jusko (11), can rationalize the ultimate first-order dependency of the aminosidine-affected death rate, $-dN/dt$ (Fig. 1).

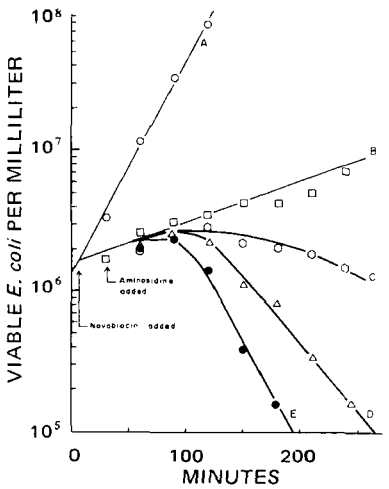

Figure 5—*Generation curves for combinations of aminosidine sulfate and 90 μg/ml of novobiocin. Curve A is for the control culture, and curve B is for novobiocin alone. Curves C, D, and E are for novobiocin combinations with 0.5, 1.0, and 2.0 μg/ml of aminosidine sulfate, respectively. All showed the development of drug-resistant* E. coli *on the following day.*

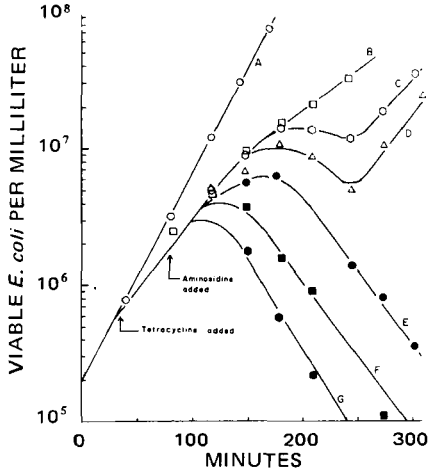

Figure 6—*Generation curves for combinations of aminosidine sulfate and 0.3 μg/ml of tetracycline hydrochloride. Curve A is for the control culture, and curve B is for tetracycline hydrochloride alone. Curves C, D, E, F, and G are for tetracycline hydrochloride combinations with 1.0, 2.0, 2.5, 3.0, and 3.5 μg/ml of aminosidine sulfate, respectively. The cultures of curves E, F, and G showed no subsequent development of resistant organisms.*

If the number of organisms dying per unit of time is proportional both to the net degree of complete inhibition, ΔI, of the biological or metabolic action necessary for organism survival and to the number of organisms, N, then:

$$-dN/dt = k_D(\Delta I)N = k_D(I - I_m)N \qquad \text{(Eq. 1)}$$

when $I > I_m$. The net degree of complete inhibition, ΔI, is a finite value when the degree of inhibition, I, exceeds a minimum value, I_m, below which the repair or homeostatic capacity of an individual organism permits the biological processes to continue at a level that maintains survival. If I is less than I_m, $-dN/dt = 0$ in the absence of generation.

These postulates include the premises that the repair capacity in every individual organism is the same and that a larger net degree of inhibition results in death in a shorter time than a smaller net degree of inhibition. The inhibitory process eventually results in the critical depletion of some biologically necessary product or effect, and thus death results ultimately in all organisms where $I > I_m$.

It follows that for $I > I_m$:

$$\int_{N_{t_1}}^{N} dN/N = \int_{t_1}^{t} k_D(I - I_m)dt \qquad \text{(Eq. 2)}$$

where N_{t_1} is the number of organisms alive at time t_1, and N is the number of organisms alive at time t. Thus:

$$\ln N/N_{t_1} = -\int_{t_1}^{t} k_D(I - I_m)dt \qquad \text{(Eq. 3)}$$

and the fraction, f_A, of organisms at time t_1 alive at any time t is:

$$f_A = N/N_{t_1} = e^{-\int_{t_1}^{t} k_D(I-I_m)dt} \qquad \text{(Eq. 4)}$$

where all ultimately die if $I > I_m$ and $f_A = 0$ at infinite time. Also $f_A = 1$ for all values of $I \leq I_m$ for all times.

If the net degree of inhibition is maintained constant after $t = t_1$, as by a constant concentration of aminosidine in the biophase of the organism, Eq. 4 reduces to:

$$\log N = -[k_D(I - I_m)/2.303](t - t_1) + \log N_{t_1} \qquad \text{(Eq. 5)}$$

which is consistent with the linear plot of log N against time after a time t_1 observed previously for other bactericidals (7) and for all bactericidal concentrations of aminosidine in Fig. 1 when the data are corrected for the emergence of resistant organisms. Thus, for aminosidine at 37.5°:

$$k_{app} = k_D(I - I_m) = 5.36 \times 10^{-4}/\text{sec} \qquad \text{(Eq. 6)}$$

The degree of inhibition of the necessary biological process should be related to a concentration, C', of the toxic agent in the

biophase. In an occupied receptor site model with a finite number of receptor sites, the degree of inhibition can be expressed in terms of the fraction of receptor sites occupied, which is a function of this concentration (12), so that:

$$I = aC'/(1 + aC') \qquad \text{(Eq. 7)}$$

and if the concentration in the biophase is proportional to the concentration in the nutrient medium, $C = C'/\alpha$:

$$I = a\alpha C/(1 + a\alpha C) = a'C/(1 + a'C) \qquad \text{(Eq. 8)}$$

Thus, at very high concentrations, $I = 1$; at low concentrations, $I = a'C$.

Substitution of forms such as Eq. 8 into Eq. 6 results in:

$$k_{app} = k_D \frac{a'C}{1 + a'C} - k_D \frac{a'C_m}{1 + a'C_m} \qquad \text{(Eq. 9)}$$

where C_m is the concentration in the nutrient medium that gives the steady-state concentration C_m' in the biophase that results in the inhibition, I_m, that does not cause death.

At high concentration, the maximum apparent mortality rate constant is:

$$\lim_{c \to \infty} k_{app} = (k_{app})_\infty = k_D - k_D \frac{a'C_m}{1 + a'C_m} \qquad \text{(Eq. 10)}$$

Thus, on consideration of Eqs. 9 and 10:

$$1/[(k_{app})_\infty - k_{app}] = 1/k_D + (a'/k_D)C \qquad \text{(Eq. 11)}$$

and a plot of the reciprocal of the difference between the maximum mortality rate constant, $(k_{app})_\infty$, and the constant, k_{app}, against the concentration where the latter was observed gives a slope of a'/k_D and an intercept, $1/k_D$, from which the values of k_D and a' can be calculated. These calculated values plus the known $(k_{app})_\infty$ permit the calculation of C_m from a rearrangement of Eq. 10:

$$C_m = [k_D - (k_{app})_\infty]/a'(k_{app})_\infty \qquad \text{(Eq. 12)}$$

Thus, from Eq. 12, when $C_m = 0$, $k_D = (k_{app})_\infty$, and from Eq. 9 or 11:

$$1/k_{app} = [1/a'(k_{app})_\infty](1/C) + 1/(k_{app})_\infty \qquad \text{(Eq. 13)}$$

and a plot of the reciprocal of the constant, k_{app}, against the reciprocal of the concentration gives a slope of $1/a'(k_{app})_\infty$ and an intercept of $1/(k_{app})_\infty$, from which the values of a' and $(k_{app})_\infty$ can be calculated when the latter equals k_D. The apparent linear dependency of kill rate on bactericidal concentrations of penicillin, kanamycin, and rifampin (7) at their studied concentrations implies that $a'C$ and $a'C_m \ll 1$, so Eq. 9 can be modified in these cases to:

$$k_{app} = k_D a'(C - C_m) \qquad \text{(Eq. 14)}$$

Aminosidine (Figs. 1 and 2) and also sulfisoxazole (13) are bacteriostatic below a minimum value, but the organisms demonstrate an invariant ultimate first-order dying rate above a certain minimum bactericidal concentration. These observations imply that the saturation of receptor sites, resulting in complete inhibition of the generation rate, results in a statistical probability of dying with organisms inhibited by these agents. Ribosomal binding of aminosidine that results in the misreading of the messenger-RNA that interferes with proper protein synthesis (4) must be initially reversible or else viable and total counts would not be coincident in the subbactericidal concentration range. An additional irreversible process must occur with aminosidine when generation is completely inhibited so as to account for the bactericidal action.

The fact that the time of achievement of the terminal first-order rate of dying decreases with increasing aminosidine concentrations (Fig. 3) implies a concentration-dependent process of aminosidine transfer from the medium into the biophase to effect antibacterial action. This process is confirmed further by the number of viables achieving a smaller maximum with increasing drug concentrations. At aminosidine sulfate concentrations of 15 μg/ml, the mean organism size did not increase after drug addition, implying a more abrupt halt to metabolic activity. This phenomenon is graphically shown in Fig. 8, where the ratio, N_p/N_o, of total organisms (N_o) produced prior to the emergence of resistants with bactericidal concentrations (see plateau values on plots of total organisms against time in Fig. 1) to the inoculum size at drug addition (N)

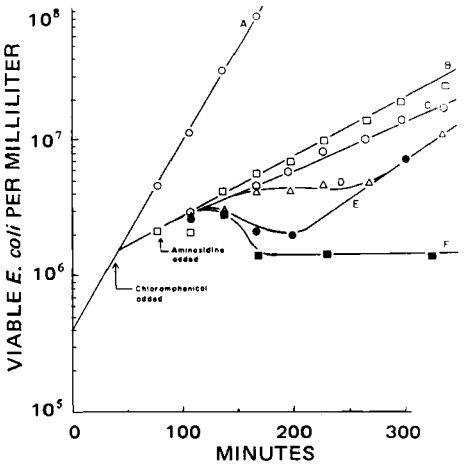

Figure 7—*Generation curves for combinations of aminosidine sulfate and 1.9 μg/ml of chloramphenicol. Curve A is for the control culture, and curve B is for chloramphenicol alone. Curves C, D, E, and F are for chloramphenicol concentrations with 3.0, 3.5, 4.0, and 4.5 μg/ml of aminosidine, respectively. All cultures showed the development of drug-resistant* E. coli *on the following day.*

decreases with aminosidine concentration. This ratio decreases with increasing inoculum size, which may indicate a possible drug binding or drug deactivation dependent on numbers of organisms or of structural mutants present at the time of drug addition. The hypothesis of drug inactivation appears to be negated by the fact that antibiotic-containing medium filtered from drug-affected organisms appeared to maintain its potency against naive organisms. An inoculum size effect of similar small magnitude was shown previously for many organisms (11).

The fact that other bacteriostatic agents, such as tetracycline and novobiocin, decrease the minimum bactericidal concentration of aminosidine implies that generation in their absence produces more receptor sites than necessary to be affected by aminosidine or that the generation process permits repair of the insult engendered by aminosidine.

Aminosidine (4) and tetracycline (3) both bind to the 30S ribosome. The action of novobiocin is not antagonized by tetracycline but is antagonized by chloramphenicol and other 50S binding inhibitors of protein synthesis (7). Thus, by analogous reasoning, it is not unexpected that chloramphenicol lessens the bactericidal activity of aminosidine, and it would be of interest to ascertain if other 50S binding antibiotics, such as erythromycin or lincomycin, act similarly.

The fact that aminosidine acts on organisms affected by such bacteriostatic agents denies a general premise (15) that bactericidals will not be active on organisms whose generation rate is inhib-

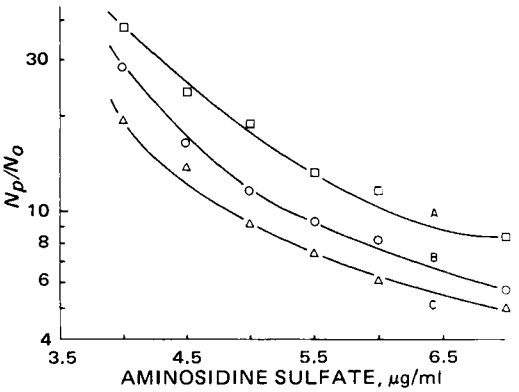

Figure 8—*Effect of inoculum size on aminosidine action. The ratio,* N_p/N_o, *of the plateau value,* N_p, *in total counts to the population,* N_o, *of* E. coli *at the time of drug addition is used as a measure of drug action. The various inoculum sizes were, in* E. coli *per milliliter: A,* 2.8×10^5; *B,* 6.4×10^5; *and C,* 1.29×10^6.

ited by such agents. In fact, the decreased emergence of resistants and lessened minimum concentrations of a drug to affect bactericidal action may indicate the advantages of such combinations.

The emergence of resistant organisms at earlier times with lower bactericidal concentrations of aminosidine (Fig. 1) implies that resistance is acquired in the presence of subbactericidal concentrations of the drug. It is most probably a property exhibited by generating organisms with aminosidine-lessened rates of generation and is consistent with the argument given (4) that altered ribosomes may be produced only by growth in the presence of drug. Aminosidine concentrations of 15 and 25 μg/ml, typical of the plasma levels achieved by the recommended therapeutic regimens (8), caused a rapid initiation of microbial death and did not permit that growth of resistants within 24 hr observed for concentrations below 7 μg/ml. This phenomenon was also observed (7) when penicillin or kanamycin was added to organisms previously treated with bacteriostatic agents, although the final killing rate was also not modified by such prior treatment.

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ANDRÉ GÉRARDIN [x], FRANÇOISE ABADIE, and JOËLLE LAFFONT

Abstract □ A GLC determination of carbamazepine in plasma and urine is described. It is performed by injecting the cyano derivative, which, in contrast to carbamazepine, has good GLC properties. The method is suitable for pharmacokinetic studies, since it is specific and accurate down to 0.27 μg/ml.

Keyphrases □ GLC—analysis, carbamazepine derivative, plasma and urine □ Anticonvulsants—carbamazepine derivative, GLC analysis, plasma and urine □ Carbamazepine—derivatization, GLC analysis, plasma and urine

Many GLC methods have been described for the determination of anticonvulsants in plasma or serum. Most of them are concerned with the monitoring of the effects of antiepileptic therapy. Some of these methods are suitable for the determination of carbamazepine[1] (I), which is injected either directly or after derivatization (1–12).

Reports on the GLC behavior of I indicate that its thermal stability is weak (6, 8, 9, 13, 14). As a result, some workers have used derivatives such as silylated I (6), dibenz[*b,f*]azepine, which is the thermal decomposition product of I (9), or its methylated derivative, obtained by flash reaction with a quaternary ammonium hydroxide (10). However, flash-reaction techniques sometimes require very precise conditions and may lead to poorly reproducible results. Moreover, these derivatives apparently cannot be obtained in a good state of purity (9). Recently, dimethylformamide dimethylacetal was reported to react with I, giving a derivative suitable for GLC (12).

These techniques generally meet clinical requirements, since the plasma concentrations in patients under continuous therapy (usually 2–10 μg/ml) do not require a high sensitivity. A method for the determination of I in plasma by mass fragmentography has been reported (14). The structure of the selected internal standard is closely related to I to compensate for its loss by thermal decomposition by an equivalent loss of the internal standard.

This paper describes a GLC method for the accurate and sensitive determination of I in plasma and urine, suitable for pharmacokinetic studies and bio-

[1] Tegretol, Geigy.

ited by such agents. In fact, the decreased emergence of resistants and lessened minimum concentrations of a drug to affect bactericidal action may indicate the advantages of such combinations.

The emergence of resistant organisms at earlier times with lower bactericidal concentrations of aminosidine (Fig. 1) implies that resistance is acquired in the presence of subbactericidal concentrations of the drug. It is most probably a property exhibited by generating organisms with aminosidine-lessened rates of generation and is consistent with the argument given (4) that altered ribosomes may be produced only by growth in the presence of drug. Aminosidine concentrations of 15 and 25 μg/ml, typical of the plasma levels achieved by the recommended therapeutic regimens (8), caused a rapid initiation of microbial death and did not permit that growth of resistants within 24 hr observed for concentrations below 7 μg/ml. This phenomenon was also observed (7) when penicillin or kanamycin was added to organisms previously treated with bacteriostatic agents, although the final killing rate was also not modified by such prior treatment.

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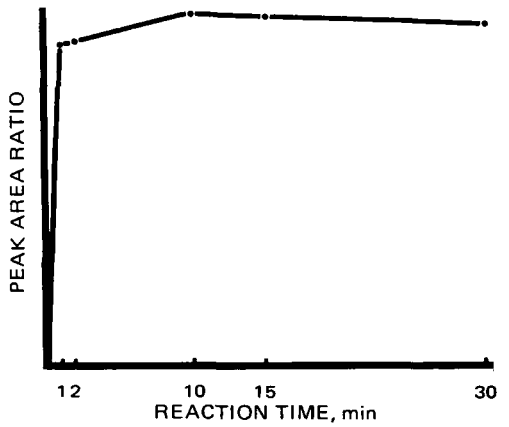


Figure 1—*Kinetics of the reaction according to Scheme I and the conditions given in* Experimental. *The internal standard used was a saturated hydrocarbon.*

availability assays. It involves the formation of 5-cyano-5*H*-dibenz[*b*,*f*]azepine, which is a stable derivative of low polarity, and does not require such expensive equipment as GLC–mass spectrometry.

EXPERIMENTAL

Reagents and Materials—The solvents[2] were analytical grade. Carbon disulfide and methylene chloride were used without further purification. All other solvents were distilled before use. Trifluoroacetic anhydride[3] and triethylamine[3] were used as 10% (v/v) solutions in methylene chloride. Fresh solutions were prepared daily. 10-Methoxycarbamazepine[4] was the internal standard. A 2.5-μg/ml stock solution in methylene chloride was prepared and stored at 4°.

Instrumentation—The GLC determinations were carried out with a gas chromatograph[5] equipped with a flame-ionization detector. The columns were 2-m long glass tubes packed with 5% cyanopropylphenylmethyl silicone[6] on hexamethyldisilazane-treated Chromosorb W (90–115 mesh). The column temperature was 270°. The retention time of the I derivative was about 5 min. Its relative retention volume by comparison with the internal standard was 0.62.

The glassware was cleaned in the usual way and immersed in an ultrasonic bath of chloroform for 15 min. The columns used in the purification step were made up from Pasteur pipets (capillary tips cut off), packed (3 cm high) with silica gel for adsorption column chromatography[7] (activity grade 2). They were immersed in a mixture of ether–pentane (1:1), and the bubbles were drawn off by suction. The prepared columns were stored at a temperature lower than 22°.

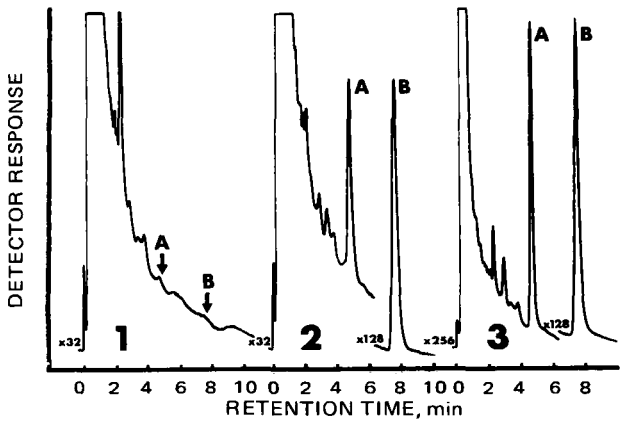


Figure 2—*Examples of chromatograms. Key: 1, plasma blank; 2, 0.2 μg of I/ml of plasma; 3, 2 μg of I/ml of plasma; A, I derivative; and B, internal standard derivative.*

[2] E. Merck AG, Darmstadt, Germany.
[3] Fluka AG, Buchs, Switzerland.
[4] Ciba-Geigy, Basle, Switzerland.
[5] Fractovap model 2400 T, Carlo Erba, Milan, Italy.
[6] Applied Science Laboratories, State College, Pa.

Table I—Recovery Experiments[a]

Added, μg/ml	*n*	Mean Recovered, μg/ml	Mean (±*SD*) Recovery, %
0.2	3	0.21	104 (±5.8)
0.5	2	0.48	96
1.2	8	1.22	102 (±4.0)
3.0	2	3.07	102
6.0	2	5.95	99
		Overall	101.3 (±4.7)

[a] The plasma samples were added to known amounts of I.

Procedure—One milliliter of plasma (or urine), 2 ml of ether, 1 ml of internal standard solution in methylene chloride, and 0.25 ml of 30% sodium hydroxide were introduced into a 10-ml ground-glass-stoppered centrifuge tube. The mixture was shaken for 5 min and centrifuged, and the organic phase was removed and evaporated under nitrogen.

The residue was dissolved in 200 μl of ether–pentane (1:1) and transferred to a small column packed with silica gel (described previously). Four milliliters of ether–pentane (1:1) was eluted and discarded, followed by 4 ml of acetone which was collected in a 10-ml glass centrifuge tube and evaporated to dryness under nitrogen.

The residue was dissolved in 100 μl of triethylamine, and 50 μl of trifluoroacetic anhydride was added. The stoppered tube was allowed to stand for 10 min and then 2 ml of hexane and 1 ml of 0.25 *M* sodium hydroxide were added. The mixture was shaken for 3 min and centrifuged. The organic phase was transferred to a conical glass tube and evaporated under nitrogen. The residue was dissolved in 100 μl of carbon disulfide, and 1–5 μl was injected into the gas chromatograph.

The concentrations were read from a calibration curve obtained from methylene chloride solutions containing known amounts of I and the same amount of internal standard as that used for the samples. These solutions were evaporated to dryness under nitrogen, and the residues were derivatized as described.

RESULTS AND DISCUSSION

Derivatization—The I derivative was synthesized (Scheme I) on a preparative scale and identified as Compound II by mass spectrometry.

Anal.—Calc. for $C_{15}H_{10}N_2$: C, 82.54; H, 4.62; N, 12.84. Found: C, 82.32; H, 4.66; N, 12.84.

The internal standard undergoes a similar reaction. A series of samples, containing known amounts of I, was prepared to determine the optimal reaction time. The results (Fig. 1) indicate that the formation of II was completed within a few minutes. The yield,

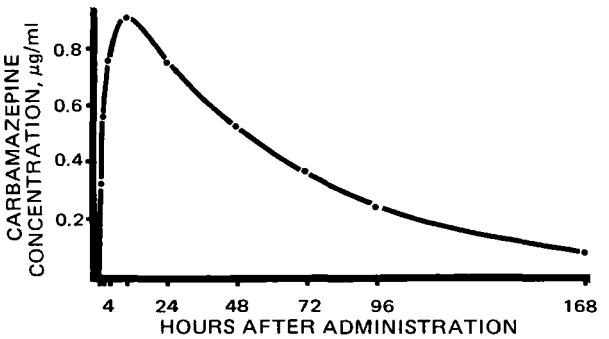


Figure 3—*Plasma concentrations of carbamazepine* versus *time in Subject 6 after the oral administration of a single 100-mg dose.*

[7] M. Woelm, Eschwega, Germany.

Table II—Cross-Check of Methods[a]

Plasma			Urine		
Hours	Isotope Dilution, μg/ml	GLC, μg/ml	Hours	Isotope Dilution, μg/ml	GLC, μg/ml
24	4.28	4.01	0–24	1.28	1.28
48	2.85	3.05	24–48	1.00	1.02
72	2.21	2.25	48–76	0.71	0.72

[a] A volunteer was given a single 400-mg dose of ^{14}C-carbamazepine orally. Samples of blood and urine were collected at the indicated times.

as measured with ^{14}C-carbamazepine, was 85% and did not depend on the amount of carbamazepine. Compound II is stable and can stand as a dry residue for hours before being injected.

GLC—The thermal decomposition of I was thoroughly checked before its derivative was investigated. Various conditions recommended in the literature were carefully reproduced. In addition to the presence of a small peak with the same retention time as dibenzazepine, it was observed that the tailing of the solvent peak was smaller when the pure solvent was injected. This result might imply a partial decomposition of I during GLC. It was confirmed by injecting a larger amount of I (1 μg), which permitted the tailing of the solvent peak to be reduced by increasing the attenuation of the signal. Under these conditions, the chromatogram showed a different baseline level before and after the peak, which is characteristic of this kind of decomposition.

When II was injected, there was no difference in the baseline levels. The peak corresponding to 5 ng was nearly symmetrical, and the response of the flame-ionization detector proved to be linear between 5 ng and 0.5 μg.

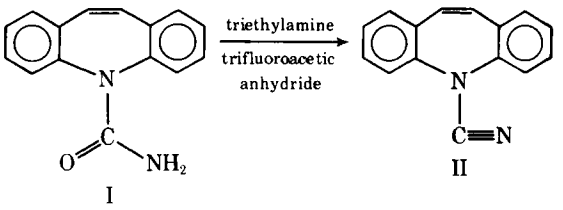
Extraction and Purification—Upon extraction of I, the yield was measured at a plasma concentration of 1 μg/ml and found to be 95%. The crude extracts must be purified to remove cholesterol and other plasma components of low polarity. The absolute yield of the purification step was 87% as measured by adding the internal standard after column chromatography and making the comparison with a pure solution of the two compounds in equal amounts. Experiments showed that the loss was exactly compensated for because the internal standard was lost to the same extent. A series of plasma samples, containing known amounts of I, was prepared to determine a calibration curve; this curve proved to be linear. Therefore, the overall yield of the procedure is not dependent on the concentration of I in the plasma.

Calculations—The calculations were made on the basis of the areas of the peaks. Their ratio remained constant whether I and the internal standard were added to plasma, urine, water, or an organic solution. Furthermore, the ratio remained the same when the internal standard was introduced into the solvent instead of the plasma. Therefore, for convenience, it was decided to use a solution of the internal standard as the extractive solvent and to make the calculations by comparison to a calibration curve obtained from organic solutions containing the same amount of internal standard and known amounts of I.

Recovery Experiments—Plasma samples containing known amounts of I were analyzed according to the described method. Examples of chromatograms are given in Fig. 2. In the blank, the small peak with nearly the same retention time as II corresponds to less than 20 ng/ml of I. The results (Table I) indicated that recovery of I from plasma is quantitative down to 0.2 μg/ml.

Specificity Experiments—A volunteer was given 400 mg of ^{14}C-carbamazepine orally. Samples of blood and urine were collected and analyzed by both inverse isotope dilution and GLC. The results (Table II) indicated a good agreement between the two methods.

The fate of carbamazepine-10,11-epoxide (the main metabolite of I in plasma) during the analytical procedure was investigated. It was shown by elemental analysis and mass spectrometry to undergo a reaction similar to I.



Scheme I—Derivatization of carbamazepine for GLC determination

Anal.—Calc. for $C_{15}H_{10}N_2O$: C, 76.91; H, 4.30; N, 11.96. Found: C, 76.75; H, 4.41; N, 11.87.

However, no peak was obtained with these GLC conditions.

Suitability for Pharmacokinetic Studies—Six healthy volunteers were given I orally in single doses. Blood was collected at different times in heparinized tubes, and plasma samples obtained by centrifugation were kept frozen until analysis. An example of the plasma concentration curves calculated from the results by a digital computer is shown in Fig. 3. The deviation of the experimental points from the calculated curve remains, in all cases, small. Each determination was made in duplicate. The results corresponding to the lowest plasma concentration were 0.084 and 0.078 μg/ml. An extensive study of the pharmacokinetic behavior of I in humans is underway and will be the subject of a future paper.

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[x] To whom inquiries should be directed.

Antimalarial Amino Alcohols II: Anthraceneaminoethanols and Anthraceneaminopropanols (1- and 9-Substituted)

J. T. TRAXLER ˣ, M. J. MOATS, E. P. LIRA, and C. W. HUFFMAN

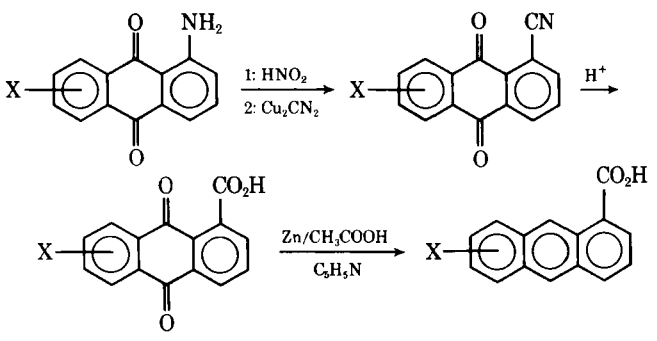
Abstract □ The syntheses of seven anthracene amino alcohols with one, two, or three additional substituents are described. These compounds include three 1-aminoethanols, two 9-aminoethanols, and two 9-aminopropanols, prepared from substituted anthraquinones or from 10-chloro-9-anthraldehydes. The antimalarial activity of these compounds, as well as tentative structure–activity relationships, is discussed in the light of previously published work.

Keyphrases □ Amino alcohols—synthesis of substituted 1- and 9-anthraceneaminoethanols and aminopropanols □ Anthraceneaminoethanols and aminopropanols—synthesis from anthraquinones or anthraldehydes

A previous report (1) summarized earlier investigations into the antimalarial activity of anthracene amino alcohols. Considerably enhanced activity is induced in anthracene-9-aminoethanols if a halogen (preferably chlorine) is substituted for the 10-hydrogen on the anthracene nucleus. Thus, 9-(di-*n*-heptylamino-1-hydroxyethyl)anthracene hydrochloride (2) was curative at 320 mg/kg (two cures out of five[1]) against *Plasmodium berghei* in mice, while the 1-chloro analog (1) was curative (two cures out of five) at 80 mg/kg in the same screen.

Further synthetic work by Stogryn (3), who prepared a series of halogen-substituted anthracene-1-, 2-, and 9-aminoethanols, indicated higher activity (four cures out of five at 80 mg/kg) for 9-(2-di-*n*-heptylamino-1-hydroxyethyl)-4,5-dichloroanthracene. Significant activity (two cures at 320 mg/kg) also was shown (3) by the 1-aminoethanol analog, *i.e.*, 1-(2-di-*n*-heptylamino-1-hydroxyethyl)anthracene, and this activity was increased to three cures out of five at 160 mg/kg by the substitution of chlorine at the 4- and 8-positions. Stogryn's work also showed that anthracenes with the aminoethanol moiety at the 2-position were active but not curative at the highest treatment level used (640 mg/kg).

In an effort to extend these studies and to derive increased antimalarial activity and/or useful structure–activity relationships, the synthesis of additional halogen-substituted anthracene 1- and 9-amino alcohols was undertaken.



Scheme I

RESULTS AND DISCUSSION

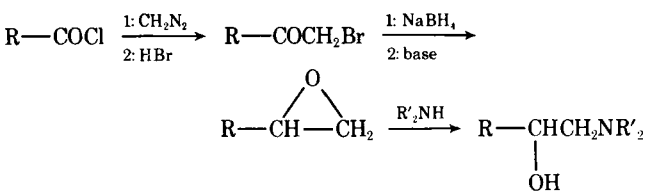
The anthracene-1-aminoethanols were prepared from commercially available 1-aminoanthraquinones by a diazonium salt–Sandmeyer combination to give the 1-cyanoanthraquinone derivatives. This preparation was followed by hydrolysis and reduction to the corresponding anthroic acids (Scheme I), which permitted entry into the now classical sequence (4, 5) for the conversion of carboxylic acids to aminoethanols (Scheme II).

Considerable isolation difficulties and poor yields were encountered in the preparation of 6,7-dichloro-1-cyanoanthraquinone until it was realized, on the basis of IR and TLC evidence, that the crude product contained at least one cyanohydrin, formed presumably by reaction of hydrocyanic acid with the anthraquinone carbonyl(s). Oxidation of the crude product with chromium trioxide in acetic acid resulted in a fairly facile isolation procedure for the desired product.

The reduction of 6,7-dichloroanthraquinone-1-carboxylic acid to the corresponding anthroic acid with zinc in ammonium hydroxide resulted in low yields (20–40%) of difficult-to-purify products. It was then found that reduction of the anthraquinone proceeded efficiently if carried out with zinc and acetic acid in pyridine (6).

Because of the finding (3) that several anthracene 1- and 2-amino alcohols could be efficiently prepared by the displacement of the bromine in anthracene 1- and 2-bromomethyl ketones, but not 9-bromomethyl ketones (7), this alternative route to target aminoethanols also was investigated. Reaction of pure (by IR and TLC) 1-bromoacetyl-6,7-dichloroanthracene with di-*n*-butylamine in tetrahydrofuran or dimethylformamide gave the desired amino ketone, but the yield was poor because of the formation of large amounts of di-*n*-butylammonium-6,7-dichloro-1-anthroate[2]. Scarcity of starting material induced the adoption of the sequence given in Scheme II for the preparation of further target anthracene-1-aminoethanols.

This sequence of reactions (Schemes I and II) also was carried out with 1-amino-5-chloroanthraquinone. In this case, however, preparation of the 5-chloro-1-anthroic acid by reduction of the an-

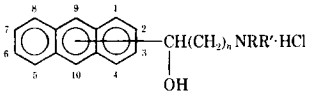


Scheme II

[1] R. E. Strube, Amino Alcohol Antimalarial Conference, Walter Reed Army Institute of Research, Washington, D.C., May 1969.

[2] Presumably arising through a Favorski- or haloform-type reaction on the bromomethyl ketone. Water was not rigorously excluded from the reaction solution.

Table I—Antimalarial Activity of Anthracene Amino Alcohols



Compound	Position: Side Chain	Position: Substituent	R[a]	R'[a]	n	Antimalarial Activity[b,c]: 20	40	80	160	320	640
Ia	1	5-Cl	C_4	C_4	1	0.1	0.5	1.1	3.5	5.1	6.1*
Ib	1	5-Cl	C_7	C_7	1	0.1	1.7	2.7	4.9	5.9	9.9*
Ic	1	6,7-Cl_2	C_4	C_4	1	0.1	3.9	5.1	7.7*	9.9*	11.9*
IIa	9	2,3-Cl_2	C_4	C_4	1	1.1	2.7	4.1	5.9	8.5*	3C[d]
IIb[e]	9	10-Br, 2,3-Cl_2	C_4	C_4	1	—	—	—	—	—	—
IIIa	9	10-Cl	C_4	H	2	0.3	0.3	0.7	4.9	10.5*	12.7*
IIIb	9	10-Cl, 3-CF_3	C_4	H	2	1.9[f]	3.7	7.3*	10.1*	—	—

[a] All *n*-alkyl groups. [b] For details of test procedure, see Ref. 19. Test data were supplied by the Walter Reed Army Institute of Research. [c] Increase in mean survival time (IMST) for mice infected with *Plasmodium berghei* after treatment at indicated dosage (in milligrams per kilogram). IMST >6.0 days = active (*); IMST >60 = cure (C). [d] Out of five. [e] Insufficient sample (14.3 mg); not tested. [f] Dosage = 10 mg/kg.

thraquinone with zinc and acetic acid in pyridine gave 5-chloro-1-anthroic acid and 5-chloro-9,10-dihydro-1-anthroic acid in a ratio of about 2:1. Conversion of the anthroic acid to the aminoethanols proceeded *via* Scheme II as expected.

An attempt to prepare other substituted anthracene-1-aminoethanols from benzanthrones (*i.e.*, 7-oxo-7*H*-benz[*d,e*]anthracenes) also was undertaken. The route envisioned involved the preparation of a suitably substituted benzanthrone, followed by oxidation to the anthraquinone-1-carboxylic acid, thus permitting entry into Scheme II after reduction to the corresponding 1-anthroic acid.

The reduction of 6-iodoanthraquinone-1-carboxylic acid [prepared by iodination of benzanthrone (8), followed by oxidation with chromium trioxide in acetic acid] with the classical zinc–ammonium hydroxide procedure resulted, not unexpectedly, in displacement of iodine to give 1-anthroic acid. Use of the much milder basic borane solution (prepared *in situ* from sodium borohydride and boron trifluoride) of Sanchorawala *et al.* (9) gave only a trace of the expected 6-iodo-1-anthroic acid after several experimental modifications. The original plan was to prepare 6-trifluoromethyl-1-anthroic acid by treatment of 6-iodo-1-anthroic acid with trifluoromethyl copper according to McLaughlin and Thrower (10, 11); but in view of the discussed difficulties and of the instability of the trifluoromethyl group to anthraquinone reduction conditions[3], this route was not pursued further.

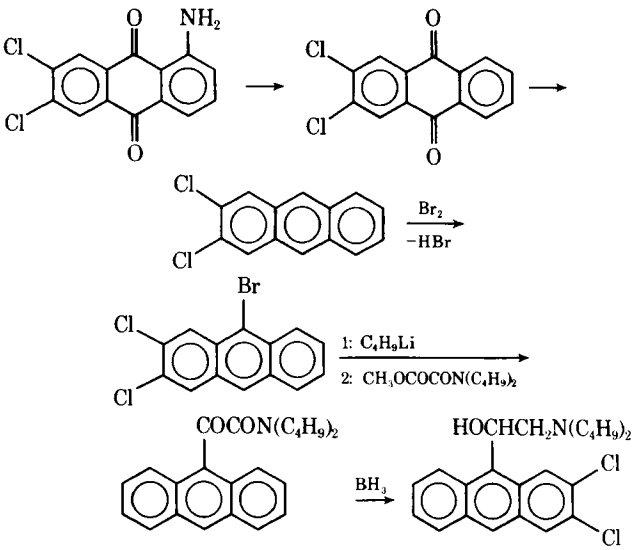
The preparation of anthracene-9-aminoethanols was achieved from 1-amino-6,7-dichloroanthraquinone by diazo deamination, reduction to the anthracene, and bromination–dehydrobromination to give 9-bromo-2,3-dichloroanthracene (Scheme III). The conversion of this material to 9-(2-di-*n*-butylamino-1-hydroxyethyl)-2,3-dichloroanthracene was carried out by the convenient procedure of Temple *et al.* (12). Good recoveries of starting materials resulted from treatment of *N,N*-dibutyl-2,3-dichloro-9-anthrylglyoxylamide and 6,7-dichloro-1-anthroic acid (13, 14) with bromine in acetic acid, as well as from treatment of the glyoxylamide and of 6,7-dichloro-1-anthroyl chloride with bromine in carbon tetrachloride. Reaction of the glyoxylamide with two equivalents of *N*-bromosuccinimide, however, gave a product from which, after reduction with borane, a small amount of 9-(2-di-*n*-butylamino-1-hydroxyethyl)-10-bromo-2,3-dichloroanthracene could be isolated.

The preparation of anthracene-9-aminopropanols was undertaken because of the possibility of increased antimalarial activity, since, with the same substitution pattern and *N*-alkyl groups in the phenanthrenes, the aminopropanols are more active than the aminoethanols[4] (15). The present efforts involved 10-chloro-9-anthraldehydes as starting materials because of their availability (1). Attempts to prepare *N,N*-dibutyl-1-(10-chloro-9-anthryl)-2-hydroxypropionamide *via* a modified Reformatsky procedure (15), as well as the preparation of lithio-*N,N*-dibutylacetamide (for subsequent reaction with 10-chloro-9-anthraldehyde), were unsuccessful.

In view of the higher antimalarial activity of phenanthrene monoalkyl aminoethanols compared to phenanthrene dialkyl aminoethanols (16) and the reactivity of monoalkyl acetamide dianions with phenanthraldehydes (17), attention was turned toward the conversion of the available 10-chloro-9-anthraldehydes to monoalkyl aminopropanols by reaction with dilithio-*N*-butylacetamide, followed by reduction of the amide with borane in tetrahydrofuran. These reactions proceeded generally as expected in the two cases indicated but with poor yields of very crude products. In other instances (*i.e.*, 10-methyl-, 3,10-dichloro-, and 3,6-dibromo-10-chloro-9-anthraldehydes), the presence of the expected crude products was indicated by IR and TLC, but none of the anticipated anthraceneaminopropanols could be isolated after reduction with borane. The complications in the latter three cases may have been caused by the presence of reactive fragments arising from the recently elucidated (18) degradation of tetrahydrofuran by butyl lithium, since excess butyl lithium was used.

The antimalarial activity of these compounds (19) is shown in Table I. The data bear out and extend tentative structure–activity relationships obtained earlier (1, 3) for anthracene amino alcohols. Thus, the *n*-heptyl alkyl groups lend slightly more activity than *n*-butyl (Ia *versus* Ib) (1, 3). With the side chain attached to C_1, monohalogen substitution removed from steric interference with the side chain [*e.g.*, C_5—Cl; Ia and Ib or C_3—Cl (3)] is more active than proximal halogen [*e.g.*, C_8—Cl (3)], and, furthermore, symmetrical [*e.g.*, C_4— and C_8—Cl (3) *versus* Ic] double chlorine substitution is more active than either. Chlorine at C_3, however, is the most active to date in the C_1-side-chain series (3).

The presence of the dialkyl amino alcohol side chain at C_9 on the unsubstituted anthracene nucleus already has been shown[1] to



Scheme III

[3] J. T. Traxler, unpublished observations.

[4] R. E. Strube, Walter Reed Army Institute of Research, personal communication.

be slightly more active than at C_1 (2, 3) and much more active than at C_2 (3). The presence of chlorine and, to a lesser extent, bromine, at C_{10} markedly increases activity (1). Unsymmetrical double halogen substitution (II*a*) is moderately active (curative), but symmetrical double halogen substitution [*e.g.*, 4,5-dichloro (3)] presently is the most active substitution pattern in the anthracene amino alcohols.

The anthracene-9-monoalkyl aminopropanols are much less active than the dialkyl aminoethanols [III*a versus* 9-(2-di-*n*-butylamino-1-hydroxyethyl)-10-chloroanthracene (1)], contrary to the indications cited for the phenanthrenes. As expected, the presence of C_3—CF_3 (III*b*) increases the activity of the 9-monoalkyl aminopropanols but not the level available in the halogen-substituted 9-dialkyl aminoethanols.

EXPERIMENTAL[5]

6,7-Dichloro-1-cyanoanthraquinone—To a stirred solution of 29.2 g (0.10 mole) of 1-amino-6,7-dichloroanthraquinone in 200 ml of sulfuric acid at 5° was added 7.6 g (0.11 mole) of solid sodium nitrite in small portions. The resulting yellow-orange solution was added to about 1.2 liters of ice, stirred well, and filtered. The damp salmon-colored solid was suspended in 200 ml of water and added to a solution of sodium cuprous cyanide [prepared by adding a solution of 38 g (0.77 mole) of sodium cyanide in 100 ml of water to a solution of 46 g (0.184 mole) of cupric sulfate pentahydrate in 200 ml of water and adjustment of the solution pH to 6–7 with sulfuric acid] in a 4-liter beaker at 25° over 2–3 min.

The foamy suspension was warmed to 70° (steam bath) for 2 hr and allowed to stand at 25° overnight. The resulting brown solid was filtered, washed with water, and partially air dried. This material was slurried in 500 ml of acetic acid, and 6.0 g of chromium trioxide in 10 ml of water was slowly added at 25° to oxidize the cyanohydrin. After 0.5 hr under reflux, the acetic acid solution was cooled to 25°, filtered, washed, and air dried to give 24.5 g (81.2%) of red-brown solid, mp 314–318°. A sample of this material was purified by recrystallization from chlorobenzene (two times) followed by sublimation at 200°/0.05 mm to give an analytical sample, mp 326–328°; IR: ν_{max} 2220 (CN) and 1670 (CO) cm^{-1}; TLC (benzene): R_f 0.82.

Anal.—Calc. for $C_{15}H_5Cl_2NO_2$: C, 59.52; H, 1.68; N, 4.64. Found: C, 59.15; H, 1.59; N, 4.35.

6,7-Dichloroanthraquinone-1-carboxylic Acid—To a suspension of 6.0 g (0.02 mole) of 6,7-dichloro-1-cyanoanthraquinone in 20 ml of water was added 75 ml of sulfuric acid in a thin stream with good stirring. Reflux for 0.5 hr gave a dark-brown solution. The reaction mixture was added at about 40° to 600 ml of ice, and the resulting suspension was stirred for 0.5 hr and filtered. Then the precipitate was washed with 50 ml of water and air dried to give 4.5 g of gray solid.

This solid was recrystallized from 50 ml of acetic acid (treated with charcoal[6]) to give 2.65 g of red-brown crystals, mp 275–279° [lit. (20) mp 275–276°]. An additional 0.56 g of product was obtained by concentration of the acetic acid filtrate, giving a total of 3.21 g (50%); IR: ν_{max} 2640 (OH), 1705 (CO), 1675 ($C_{9,10}O$), and 938 (OH) cm^{-1}; TLC (benzene): R_f 0.06.

6,7-Dichloro-1-anthroic Acid—To a solution of 4.1 g (12 mmoles) of 6,7-dichloroanthraquinone-1-carboxylic acid and 15.3 g of zinc dust (0.24 mole) in 150 ml of pyridine, at reflux, was added 65 ml of 80% aqueous acetic acid over 5 hr. The resulting yellow solution was stirred under reflux for an additional 0.5 hr, kept at 25° overnight, and added to a cold (10°) solution of 200 ml of concentrated hydrochloric acid in 1300 ml of water. The mixture was stirred for 15 min and filtered, and the yellow solid was washed with water and dried in a vacuum oven at 80° to give 3.4 g (97.2% crude) of product, mp 302–309°.

Recrystallization from 750 ml of acetic acid gave 1.73 g of product, mp 316–321°. An earlier analytical sample had a melting point of 320–321°. An additional crop of product was obtained by concentration of the acetic acid filtrate, giving a total yield of 2.13 g (61%); IR: ν_{max} 2640 (OH), 1670 (CO), 936 (OH), and 904 ($C_{9,10}H$) cm^{-1}; TLC [benzene–ether (1:2)]: R_f 0.85.

Anal.—Calc. for $C_{15}H_8Cl_2O_2$: C, 61.88; H, 2.78. Found: C, 62.28; H, 2.74.

6,7-Dichloro-1-bromoacetylanthracene—A suspension of 6,7-dichloro-1-anthroyl chloride (1.5 g, 4.8 mmoles, prepared in 65% yield by treatment of 10 mmoles of the acid in benzene with 30 mmoles of oxalyl chloride and recrystallization from methylcyclohexane, mp 193–195°) in 60 ml of tetrahydrofuran was cooled to 0°, and into this magnetically stirred mixture was distilled about 3.0 g of diazomethane (generated from 21.4 g, 0.01 mole, of *p*-tolylsulfonylmethylnitrosamide[7]) in ether over 45 min. Solution was complete after the addition of about one-third of the diazomethane. The reaction solution was allowed to warm to 25° and then was concentrated to 100 ml with a stream of nitrogen at 25°; to the resulting yellow solution at 10° was added 2.0 ml of 48% HBr dropwise.

The stirred solution was allowed to reach 25° and kept at that temperature overnight. The yellow-brown reaction mixture was added to 350 ml of ice water, filtered, washed with water, and air dried to give 1.71 g (96% crude) of yellow solid, mp 150–155°. A sample of this material was recrystallized four times from benzene to give an analytical sample, mp 162–163°; IR: ν_{max} 1675 (CO) and 904 ($C_{9,10}H$) cm^{-1}; TLC (benzene–petroleum ether): R_f 0.91.

Anal.—Calc. for $C_{16}H_9BrCl_2O$: C, 52.21; H, 2.45. Found: C, 52.33; H, 2.81.

5-Chloroanthraquinone-1-carboxylic Acid and Methyl Ester—A solution of 1-amino-5-chloroanthraquinone (39 g, 0.15 mole) in 200 ml of concentrated sulfuric acid was treated portionwise with good magnetic stirring at 5–10° with 12.5 g (0.15 mole) of solid sodium nitrite. The brown solution was stirred at about 5° for 0.25 hr, added to 500 ml of ice, filtered, and dried to a wet cake. The salmon-colored solid was added in portions at 25° to a solution of sodium cuprous cyanide, prepared according to Vogel (21) from 42.0 g of cupric sulfate pentahydrate. The resulting brown foamy mixture was heated for 1 hr on the steam bath and kept at about 60° overnight.

This mixture was filtered, the brown solid was washed with 200 ml of water, and the partially air-dried product was slurried with 600 ml of hot 3% NaOH. The insoluble solid was filtered and washed with 200 ml of hot 3% NaOH, followed by excess water. The partially air-dried 5-chloro-1-cyanoanthraquinone was then added to 800 ml of 65% H_2SO_4 at 25°, and the mixture was heated slowly to reflux, ~125°. After heating for 2 hr at 115–125°, the mixture was cooled to 25° and filtered through a fritted filter. The solid was washed with water and dried to a wet cake on the funnel. Then the filter cake was added to 1200 ml of 20% NH_4OH, and the mixture was heated and stirred at 60° for about 20 min and then filtered to remove 14.55 g of insoluble black solid.

The red filtrate was cooled in an ice bath and acidified with concentrated hydrochloric acid. The resulting yellow-brown solid was filtered, washed, and dried *in vacuo* at 80° to give 9.90 g of a brownish-black solid, mp 304–314° [lit. mp 306° (22) and mp 312° (23)]. An additional 0.60 g of product was obtained by reextracting the brownish-black solid with 750 ml of 20% NH_4OH, giving a total crude yield of 10.5 g (24.7%); IR: ν_{max} 2660 (OH), 1680 (CO), 1670 ($C_{9,10}O$), and 940 (OH) cm^{-1}; TLC (5% ether in benzene): R_f 0.56.

This combined material was very difficult to purify by recrystallization because of its low solubility, so the methyl ester was prepared in 63% yield from methyl iodide by the method of Shaw *et al.* (24) to give a melting point of 177–180° after recrystallization from methanol [lit. (22) mp 181°]; IR: ν_{max} 1725 (CO) and 1275 (OCH_3) cm^{-1}; TLC [benzene–ether (3:1)]: R_f 0.97.

5-Chloro-1-anthroic Acid and 5-Chloro-9,10-dihydro-1-anthroic Acid—Excess zinc dust (12.1 g) was added to a solution of 2.6 g (92 mmoles) of 5-chloroanthraquinone-1-carboxylic acid in 125 ml of pyridine, and the mixture was heated to reflux with mag-

[5] The chemicals were used as obtained from chemical supply houses without purification unless otherwise noted. Melting points were determined with a Thomas-Hoover capillary melting-point apparatus and are not corrected. Most reactions and purification procedures were followed by TLC on Gelman SG (silica gel) sheets. Visualization was accomplished with short- or longwave UV, 0.2% $KMnO_4$ in 1.0% Na_2CO_3, 0.4% bromphenol blue in methanol (for bases; adjust to pH ~8 for acids), iodine vapor, or 0.04% 2,4-dinitrophenylhydrazine in 2 *N* HCl. IR spectra were determined in mineral oil mulls (unless otherwise indicated) with a Beckman IR-5A prism instrument. NMR data were determined at 60 MHz in dimethyl sulfoxide-d_6 with a Varian A-60D instrument by Sadtler Research Laboratories, Philadelphia, Pa. Elemental analyses were carried out with a Hewlett-Packard model 185 carbon, hydrogen, and nitrogen analyzer by the IMC Organic Analysis Group under the direction of Mr. L. Ferrara.

[6] Darco G-60, Atlas Chemical Division of ICI America.

[7] Diazald, Aldrich Chemical Co.

netic stirring. To this refluxing solution was added dropwise, over 5.5 hr, 50 ml of 80% acetic acid. The red solution changed to yellow after about 0.5 hr; after the addition was complete, the clear yellow solution was stirred under reflux for an additional 0.5 hr and kept at 25° overnight. The excess zinc (2.3 g) was removed by filtration, and the filtrate was added to a solution of 150 ml of concentrated hydrochloric acid.

The mixture was stirred and filtered, and the solid was washed and air dried to give 1.4 g of yellow-brown solid, mp 255–285°. The product was recrystallized from 80 ml of acetic acid to give, after treatment with charcoal[6] and standing overnight at 25°, 0.50 g of yellow crystals, mp 285–289°. The filtrate was concentrated on a rotary evaporator to ~70 ml and kept at 10° overnight to give 0.20 g of brown crystals, mp 243–249°. An additional 0.10 g of material was obtained by concentration of this filtrate to 10 ml after standing overnight at 10°.

The higher melting-point product was recrystallized three times more from acetic acid to give an analytical sample, mp 293–294°, of 5-chloro-1-anthroic acid; IR: ν_{max} 2640 (OH), 1660 (CO), 938 (OH), and 901 ($C_{9,10}H$) cm^{-1}; TLC (10% ether in benzene): R_f 0.73; NMR: δ 7.26–7.86 (m, 3H, aromatic), 8.03–8.51 (m, 3H, aromatic), 8.94 (s, 1H, $C_{10}H$), 9.74 (s, 1H, C_9H), and ~13.0 (COOH).

Anal.—Calc. for $C_{15}H_9ClO_2$: C, 70.19; H, 3.53. Found: C, 70.02; H, 3.56.

The lower melting-point solid was recrystallized six times from acetic acid to give an analytical sample of 5-chloro-9,10-dihydro-1-anthroic acid, mp 250–252°; IR: ν_{max} 2640 (OH), 1675 (CO), and 918 (OH) cm^{-1}; TLC (10% ether in benzene): R_f 0.86; NMR: δ 4.08 (broad s, 2H, $C_{10}H_2$), 4.38 (broad s, 2H, C_9H_2), 7.15–7.85 (m, 6H, aromatic), and ~12.7 (b, 1H, COOH).

Anal.—Calc. for $C_{15}H_{11}ClO_2$: C, 69.65; H, 4.28. Found: C, 69.68; H, 4.35.

6-Iodoanthraquinone-1-carboxylic Acid—3,9-Diiodobenzanthrone [1.0 g, 2 mmoles, prepared in 55% yield from benzanthrone by the method of Gotoh and Li (8)] was dissolved in 20 ml of refluxing acetic acid with magnetic stirring. A solution of 2.0 g of chromium trioxide in 25 ml of 25% aqueous acetic acid was added dropwise at reflux over 35 min. The dark-green mixture was stirred under reflux for 2 hr (iodine fumes), 40 ml of warm water was added in a thin stream, and the mixture was allowed to stir while cooling at 25°.

The resulting solid (0.34 g, mp 307–310°) was removed by filtration, and the filtrate was chilled to give a second crop (0.01 g). The chromium salts were neutralized by the addition of 3.7 ml (6.8 g) of concentrated sulfuric acid; and by reducing the volume of the resulting liquid, an additional 0.19 g of product was obtained, giving a total yield of 0.54 g (71%). The combined product was recrystallized from acetic acid to give 0.40 g of light-yellow crystals, mp 309–311°.

A small sample was dissolved in hot ammonium hydroxide and filtered, and the solution was acidified. The resulting solid was recrystallized from acetic acid (three times) to give an analytical sample, mp 314–315°; IR: ν_{max} 2660 (OH), 1700 (shoulder, CO), 1680 ($C_{9,10}O$), and 910 (OH) cm^{-1}; TLC [benzene–ether (1:1)]: R_f 0.44.

Anal.—Calc. for $C_{15}H_7IO_4$: C, 47.64; H, 1.86. Found: C, 47.24; H, 1.96.

9-Bromo-2,3-dichloroanthracene—A suspension of 2.4 g (0.01 mole) of 2,3-dichloroanthracene (prepared in 41% overall yield by diazo deamination of 1-amino-6,7-dichloroanthraquinone followed by zinc–ammonia reduction) in 75 ml of acetic acid at 25° was treated dropwise with 1.6 g (0.01 mole) of bromine in 2 ml of acetic acid. The mixture was stirred at about 30° for 18 hr (hydrogen bromide), heated to boiling, and cooled to 10°. Filtration gave 2.67 g (83.5%) of yellow solid, mp 162–172°, which was recrystallized from toluene (after treatment with charcoal[6]) to give 1.80 g (56.4%) of product, mp 175–182°. An analytical sample was prepared by successive recrystallization of this material from toluene, acetone, and cyclohexane, mp 192–194°; TLC (petroleum ether): R_f 0.96.

Anal.—Calc. for $C_{14}H_7BrCl_2$: C, 51.58; H, 2.17. Found: C, 51.51; H, 2.03.

1-(2-Di-*n*-butylamino-1-hydroxyethyl)-5-chloroanthracene Hydrochloride (Ia)—5-Chloro-1-anthroic acid (3.39 g, 13.4 mmoles) was suspended in 300 ml of benzene, and 3.5 ml (5.06 g, 40 mmoles) of oxalyl chloride was added dropwise with good magnetic stirring. The mixture was stirred at reflux for 2.5 hr (solution after 2 hr) and kept at 25° overnight. A small amount of insoluble brown material was removed by filtration, and the yellow solution was concentrated on a rotary evaporator to give 3.6 g (100%) of yellow crude 5-chloro-1-anthroyl chloride, mp 147–155°; IR: ν_{max} 1750 (CO) and 901 ($C_{9,10}H$) cm^{-1}.

To a solution of 3.6 g (13.4 mmoles) of 5-chloro-1-anthroyl chloride in 100 ml of tetrahydrofuran at 0° was added about 3.0 g (70 mmoles) of diazomethane, generated from 21.4 g of *p*-tolylsulfonylmethylnitrosamide[7]. After stirring at 0° (yellow solid formed) for 0.75 hr, the reaction mixture was concentrated at 25° with a stream of nitrogen to about 100 ml, and 3 ml of 48% HBr was added dropwise with good stirring at 25°. The yellow reaction solution was stirred for 1 hr, added to 600 ml of ice water, and filtered. The yellow solid was washed and air dried to give a golden-yellow solid, 4.07 g, mp 126–132°, 92.7% yield of crude 1-bromoacetyl-5-chloroanthracene; IR: ν_{max} 1665 (CO) and 906 ($C_{9,10}H$) cm^{-1}; TLC (petroleum ether): R_f 0.16.

A solution of crude 1-bromoacetyl-5-chloroanthracene (4.0 g, 12.4 mmoles) in 50 ml of tetrahydrofuran and 30 ml of anhydrous ethanol was treated dropwise at 25° with excess (1.0 g) sodium borohydride in 30 ml of anhydrous ethanol. The solution was stirred at 25° for 0.5 hr and a solution of 3.0 g of sodium hydroxide in 10 ml of water was added dropwise at about 10° with good magnetic stirring, resulting in the separation of a white viscous liquid phase. After the mixture was stirred for 2.5 hr at about 20° (light-yellow solid after 15 min), the reaction mixture was filtered to give, after washing and air drying, 0.36 g of light-yellow product, mp 141–144°; TLC (2% methanol–benzene): R_f 0.94, one major spot; IR: ν_{max} 974 and 842 (epoxide) and 900 ($C_{9,10}H$) cm^{-1}.

The filtrate was added to 1 liter of ice water and stirred well for 0.5 hr; then the solid was washed and air dried to give 2.51 g of light-yellow solid, mp 106–124°. This material was recrystallized from 155 ml of methanol to give an additional 1.34 g of yellow-brown crystalline epoxide, mp 130–134°, shown by TLC and mixed melting point to be slightly less pure than the previous material. The total yield of 5-chloro-1-anthrylethylene oxide was 1.70 g (54.6%).

A mixture of 1.0 g (4.0 mmoles) of 5-chloro-1-anthrylethylene oxide and 7.0 ml of di-*n*-butylamine was stirred at 100° under nitrogen for 18 hr (light-brown solution in about 15 min). The solution was concentrated (rotary evaporator) to give 1.60 g of viscous brown oil, which was dissolved in 300 ml of anhydrous ether and chilled in an ice bath. A saturated solution of hydrogen chloride in ether was added dropwise with good magnetic stirring until a strongly acidic reaction was obtained with moist pH paper. The mixture was kept at 10° for 2 hr, and the light-brown solid was filtered to give 1.10 g, mp 117–130°.

An additional 0.37 g of crude sticky product was obtained by concentration of the ethereal filtrate. The combined product was recrystallized twice from isopropanol to give 1.01 g (58.8%), mp 194–197°. A sample of this material was further recrystallized for analysis from isopropanol (Table II); IR (KBr): ν_{max} 3210 (OH), 2900 and 2855 (CH_2), 2550 (R_3N^+), and 904 ($C_{9,10}H$) cm^{-1}; TLC (free base, 2% methanol–benzene): R_f 0.56.

1-(2-Di-*n*-heptylamino-1-hydroxyethyl)-5-chloroanthracene Hydrochloride (Ib)—A mixture of crude 5-chloro-1-anthrylethylene oxide (0.70 g, 2.67 mmoles, mp 135–145°) and 5.0 ml of di-*n*-butylamine was stirred under nitrogen at 100° for 18 hr (cloudy solution after about 4 hr). The reaction solution was transferred to a 65-mm Hickman still with tetrahydrofuran, and excess amine was removed at 100°/0.02 mm. A small amount of unidentified (nonproduct) yellow sublimate was deposited on the walls of the still. The viscous oily residue in the still was transferred with benzene and concentrated on the rotary evaporator to give 1.0 g of oily product which was dissolved in 250 ml of anhydrous ether. Then a few milliliters of ether was removed by distillation (to dry), the solution was chilled in ice water, and excess hydrogen chloride in ether was added with good magnetic stirring.

The solution (no precipitate formed) was concentrated on a rotary evaporator, and the oily residue was recrystallized from 50 ml of hot methylcyclohexane to give, after standing at 25° and then at 0° overnight, 0.92 g (66.6%) of pink crystals, mp 62–67°. A sample of this material was recrystallized from methylcyclohexane containing a few drops of ethereal hydrogen chloride to give an analytical sample (Table II); IR (KBr): ν_{max} 3210 (OH), 2900 and 2855 (CH_2), 2535 (R_3N^+), and 916 ($C_{9,10}H$) cm^{-1}; TLC (free base) (2% methanol–benzene): R_f 0.84.

1-(Di-*n*-butylamino-1-hydroxyethyl)-6,7-dichloroanthra-

Table II—Anthracene Amino Alcohols

Compound	Yield, %	Melting Point	Formula	Analysis, % Calc.	Found
Ia	59[a]	197–200°	$C_{24}H_{31}Cl_2NO$	C 68.57	68.72
				H 7.43	7.44
				N 3.33	3.25
Ib	67[a]	66–68°	$C_{30}H_{43}Cl_2NO$	C 71.41	71.09
				H 8.59	8.75
				N 2.77	2.71
Ic	24[b]	232–235°	$C_{24}H_{30}Cl_2NO$	C 63.35	63.44
				H 6.65	6.78
				N 3.08	2.99
IIa	13[c]	220–223°	$C_{24}H_{30}Cl_3NO$	C 63.35	63.57
				H 6.65	6.93
				N 3.08	2.89
IIb	8[c]	241–242°	$C_{24}H_{29}BrCl_2NO$	C 53.99	53.95
				H 5.47	5.80
				N 2.62	2.53
IIIa	6[d]	238–239°	$C_{21}H_{25}Cl_2NO$	C 66.68	66.49
				H 6.65	6.80
				N 3.70	3.70
IIIb	5[d]	185–190°	$C_{22}H_{24}Cl_2F_3NO$	C 59.19	58.99
				H 5.42	5.39
				N 3.13	3.02

[a] From epoxide and R_2NH. [b] From bromomethyl ketone and R_2NH. [c] From 10-bromo-2,3-dichloroanthracene. [d] From the aldehyde.

cene Hydrochloride (Ic)—To a solution of 6,7-dichloro-1-bromoacetylanthracene (2.15 g, 5.85 mmoles) in 35 ml of tetrahydrofuran was added 1.6 g (11.7 mmoles) of di-*n*-butylamine in 5 ml of tetrahydrofuran, dropwise, with good stirring. The resulting solution was stirred at 25° for 1.5 hr and concentrated on a rotary evaporator, and the resulting brown semisolid was extracted with about 50 ml of petroleum ether (bp 60–110°). After filtration, the solid was washed with about 100 ml of petroleum ether. The air-dried solid, 1.60 g, represented 130% recovery of the calculated amount of di-*n*-butylammonium bromide (see *Results and Discussion*).

The combined petroleum ether solutions were concentrated (rotary evaporator) to give 2.2 g of a yellow-brown viscous oil, which was dissolved in 40 ml of tetrahydrofuran and 20 ml of anhydrous ethanol. To this solution was added a solution of 0.44 g (11.6 mmoles) of sodium borohydride in 10 ml of anhydrous ethanol. The cloudy mixture was stirred at 25° overnight and concentrated on a rotary evaporator. The viscous semisolid residue was dissolved in 110 ml of ether and washed three times with water (100, 50, and 50 ml), and the ether layer was concentrated and dried azeotropically with acetone to give 1.7 g of clear brown viscous oil. This material was dissolved in 250 ml of ether and cooled to 0°, and excess hydrogen chloride in ether was added dropwise with good magnetic stirring.

After stirring at 0° for 0.5 hr, the mixture was filtered to give 0.82 g of light-tan solid, mp 195–205°. Concentration of the ether solution gave 1.0 g of a viscous oil, the IR of which showed carbonyl absorption and no hydroxy or tertiary amine functions. This oil could not be identified by treatment with sodium borohydride, aqueous methanolic sodium hydroxide, or di-*n*-butylamine.

The light-tan solid was recrystallized several times from isopropanol to give a melting point of 232–235°. An analytical sample was recrystallized again from isopropanol (Table II); IR (KBr): ν_{max} 3230 (OH), 2920 and 2852 (CH_2), 2600 (R_3N^+), and 912 ($C_{9,10}H$) cm^{-1}; TLC (free base, 2% methanol–benzene): R_f 0.90.

9-(2-Di-*n*-butylamino-1-hydroxyethyl)-2,3-dichloroanthracene Hydrochloride (IIa)—To a solution of 7.0 g (21.5 mmoles) of crude 9-bromo-2,3-dichloroanthracene in 500 ml of anhydrous ether (nitrogen atmosphere) at 0° was added 10.0 ml of 15% butyl lithium in hexane dropwise over 10 min. The yellow cloudy solution was stirred at 0° for 15 min and cooled to −60° with an isopropanol bath. A solution of 5.1 g (24 mmoles) of methyl-*N,N*-dibutyl oxamate (12) in 75 ml of anhydrous ether was added dropwise at −60°. The resulting reddish solution was stirred for 0.5 hr at −60°, allowed to warm to 25°, and then stirred for 17 hr. The reaction was then quenched by the addition of 250 ml of water in a thin stream with good magnetic stirring.

The layers were separated after filtration of 1.35 g of insoluble material (starting material and 2,3-dichloroanthracene by IR), and the aqueous layer was extracted twice more with 50 ml of ether. The combined ether layers were washed with water (2 × 50 ml), dried with phase separation paper, and concentrated on a rotary evaporator at 65° and then on a vacuum pump at 50°/0.10 mm to give 7.35 g (85.4% crude) of red-brown semisolid, 9-(2,3-dichloroanthryl)-*N,N*-di-*n*-butylglyoxylamide. The crude material could be purified with difficulty by recrystallization from 95% ethanol to give a yellow solid, mp 110–114°; IR: ν_{max} 1670 and 1630 (CO) cm^{-1}; TLC (5% ether–petroleum ether): R_f 0.58.

The crude anthrylglyoxylamide was dissolved in 75 ml of dry (refluxed over and distilled from sodium hydride) tetrahydrofuran and added dropwise to 100 ml of 1 *M* BH_3–tetrahydrofuran, diluted with 75 ml of dry tetrahydrofuran, at 0–5° over 20 min. The yellow solution was stirred for 0.5 hr at 0°, allowed to warm to 25°, stirred under reflux for 1 hr, and allowed to stand at 25° overnight. TLC indicated the absence of starting material, so the solution was cooled to 10° and treated cautiously with 120 ml of 6 *N* HCl.

After no more hydrogen evolution, the tetrahydrofuran was evaporated (steam bath, 1 atm) and the mixture was stirred under reflux for 0.5 hr to hydrolyze the boron complex. To the cooled mixture was added 150 ml of chloroform, the pH was adjusted to 6.5–7.0 with solid sodium bicarbonate, the layers were separated, and the aqueous layer was extracted with chloroform. The combined organic layers were washed (three times) with 75 ml of water and dried with phase separation paper, and the red-brown solution was concentrated on a rotary evaporator to give 6.2 g of red-brown semisolid, to which was added 175 ml of ether. The resulting yellow insoluble solid (0.81 g) was removed by filtration, and the filtrate was diluted with 125 ml of ether and cooled to 10°. An excess of hydrogen chloride in ether was added dropwise with good stirring. After standing (10°) overnight, the yellow sticky solid was filtered and dissolved in 25 ml of isopropanol.

The product was obtained in two crops, mp 213–219° and 212–215°, with a total yield of 1.48 g (15.2%). Recrystallization from isopropanol gave 1.10 g, mp 222–225°. A sample of the product from an earlier exploratory preparation was recrystallized from isopropanol (Table II); IR (KBr): ν_{max} 3190 (OH), 2920 and 2850 (CH_2), ~2540 (R_3N^+), and 900 ($C_{10}H$) cm^{-1}; TLC (free base, 5% ether–petroleum ether): R_f 0.23.

9-(2-Di-*n*-butylamino-1-hydroxyethyl)-10-bromo-2,3-dichloroanthracene Hydrochloride (IIb)—A solution of recrystallized 9-(2,3-dichloroanthryl)-*N,N*-di-*n*-butylglyoxylamide (1.9 g, 4.4 mmoles) in 40 ml of carbon tetrachloride and 0.80 g (4.4 mmoles) of *N*-bromosuccinimide was stirred under reflux for 20 hr, at which time all insoluble material was on top of the solution, but TLC indicated the presence of considerable starting material. An additional 0.80 g (4.4 mmoles) of *N*-bromosuccinimide was added, and the solution was stirred under reflux for an additional 24 hr. Filtration of the cooled solution gave 0.84 g (97.8%) of suc-

cinimide. Concentration of the filtrate on a rotary evaporator gave 2.01 g of crude product, which was recrystallized from anhydrous ethanol to give, after filtration, a yellow-brown insoluble material, mp 140–150°. This material showed one major spot on TLC (5% ether–petroleum ether) at R_f 0.74; IR (film): ν_{max} 2950 and 2850 (CH_2) and 1655 and 1630 (CO) cm^{-1}.

The crude bromodichloroglyoxylamide (2.0 g, about 3.9 mmoles) in 20 ml of dry (refluxed over and distilled from sodium hydride) tetrahydrofuran was added to a solution of 30 ml of 1 *M* BH_3 in tetrahydrofuran in 20 ml of pure tetrahydrofuran at 0–3° over 20 min under nitrogen. The reaction mixture was stirred for 0.5 hr at 0°, allowed to reach 25°, and refluxed for 2.5 hr (monitored for disappearance of starting material by TLC). Then the reaction mixture was cooled to 5°, and 40 ml of 5 *N* HCl was added dropwise with good stirring.

After decomposition of excess borane, the tetrahydrofuran was evaporated on the steam bath and the mixture was stirred under reflux for 0.5 hr. Chloroform (60 ml) was added to the cooled hydrolysis mixture, which was then neutralized with solid sodium bicarbonate to about pH 7 (pH paper). The layers were separated, and the aqueous layer was extracted once with chloroform. The combined organic layers were washed with water, dried with phase separation paper, concentrated on the rotary evaporator, and dried at 60°/0.01 mm to give 1.6 g of brown viscous liquid.

To this material was added 300 ml of ether, 0.25 g of insoluble material (nonproduct) was removed by filtration, and the chilled (0°) solution was treated with excess hydrogen chloride in ether. The resulting mixture was kept at 0° for 2 hr and filtered to give 0.23 g of air-dried brown solid, mp 205–215°. Further concentration of the filtrate gave 0.12 g of a mixture of 2,3-dichloro- and 9-bromo-2,3-dichloroanthracene as well as 0.94 g of an unidentifiable brown viscous oil.

Recrystallization of the product (0.23 g) twice from isopropanol gave light-yellow crystals, mp 241–242°. A sample of this material was recrystallized from isopropanol for analysis (Table II); IR (KBr): ν_{max} 3200 (OH), 2940 and 2860 (CH_2), and ~2550 (R_3N^+) cm^{-1}; TLC (free base) (2% methanol–benzene): R_f 0.84.

9-(3-*N*-*n*-Butylamino-1-hydroxypropyl)-10-chloroanthracene Hydrochloride (IIIa)—A solution of *N*-*n*-butylacetamide (2.76 g, 24 mmoles) in 100 ml of dry tetrahydrofuran was cooled to about −5° under nitrogen. To this vigorously stirred solution was added dropwise a solution of 1.6 *M* butyl lithium in hexane (30 ml, 48 mmoles). The resulting cloudy solution was stirred at from −5 to −10° for 2 hr and then treated dropwise with a solution of 10-chloro-9-anthraldehyde (3.61 g, 15 mmoles) in 150 ml of dry tetrahydrofuran; it was then stirred at −5° for 2 hr.

The reaction mixture was stirred at room temperature overnight and then at reflux for 1.5 hr. The yellowish-red solution was cooled to 2–5° and then treated with acetic acid (3.5 ml) in tetrahydrofuran (20 ml). The solution was stirred for 15 min, water was added, and the solution was stirred for an additional 30 min. Then the mixture was stripped to near dryness and partially taken up with chloroform (500 ml) and water (200 ml). The solid at the interface was isolated by filtration to yield the crude product, 1.8 g (21.5%), mp 208–210° dec. This sample was recrystallized from acetone to yield 1.15 g, mp 208–210° dec., one spot on TLC (50% ether–benzene); IR: ν_{max} 3310 (OH) and 1600 (CO) cm^{-1}.

The chloroform solution was washed with aqueous sodium carbonate and then water, dried, reduced in volume by one-half, and cooled to yield the pure product, 1.4 g (26%), mp 208–210° dec. The combined product was used in the following borane reduction.

To a −5° solution of 1 *M* BH_3 (12 ml, 12 mmoles) in tetrahydrofuran (15 ml) under a nitrogen atmosphere was added dropwise a solution of *N*-*n*-butyl-3-(10-chloro-9-anthryl)-3-hydroxypropionamide (2.3 g, 6.08 mmoles) in 90 ml of tetrahydrofuran. The reaction solution was maintained at −5° for 2 hr, at room temperature overnight, and then at 60° for 23 hr. More borane (10 ml, 10 mmoles) was then added, and the solution was heated for 2.5 hr at 60°.

The solution was cooled and treated cautiously with water and then evaporated to dryness *in vacuo* to give an off-white solid. This solid was taken up in methanol (60 ml) and 10% H_2SO_4 (9 ml) and then heated at reflux for 3 hr. The solution was cooled in ice, and the pH was adjusted to 10.5 with 1 *N* NaOH. The precipitated solid was partitioned between ether and water.

The resulting three-phase system was then filtered to give 0.66 g, mp 151–153° (starting material and unidentified by-products by IR), the layers were separated, and the ether layer was concentrated on a rotary evaporator to give, after azeotropic drying with benzene, 1.5 g of a yellow semisolid. The semisolid was dissolved in about 400 ml of ether, the solution was cooled in an ice bath, and excess hydrogen chloride in ether was added dropwise. The mixture was stirred at 0° for 1 hr, kept at 10° overnight, and filtered to give a light-pink solid, mp 190–213° dec.

Recrystallization of this solid from isopropanol (color change to yellow upon heating) gave 0.82 g of a yellow solid, mp 240–242° dec. Several smaller crops were obtained by concentration and by addition of ether to the filtrates, giving a total yield of 0.98 g. The combined product was suspended in ether–tetrahydrofuran (2:1) and treated with 25 ml of 2 *N* NaOH. The layers were separated and the organic layer was washed with water to pH 7, concentrated on a rotary evaporator, and dried with benzene to give 0.86 g of light-yellow solid. This solid was dissolved in 400 ml of ether and treated with excess hydrogen chloride–ether dropwise in an ice bath. The resulting pink precipitate (0.62 g, mp 211–214°) was recrystallized from 20 ml of isopropanol to give 0.55 g of a yellow solid, mp 234–236° dec. An analytical sample was prepared by recrystallizing this material from isopropanol and then from chloroform–ethanol–cyclohexane (Table II); IR (KBr): ν_{max} 3300 (OH), 2900 and 2840 (CH_2), and 2750 (R_2NH^+) cm^{-1}; TLC (free base) [methanol–benzene (1:1)]: R_f 0.80.

9-(3-*n*-Butylamino-1-hydroxypropyl)-10-chloro-3-trifluoromethylanthracene Hydrochloride (IIIb)—To a solution of *N*-*n*-butylacetamide (1.19 g, 10.3 mmoles) in 43 ml of dry tetrahydrofuran under a nitrogen atmosphere, cooled to about −5°, was added dropwise a solution of 1.6 *M* *n*-butyl lithium in hexane (12.9 ml, 20.7 mmoles). After the resulting cloudy solution was stirred for 3 hr at from −5 to −10°, a solution of 10-chloro-3-trifluoromethyl-9-anthraldehyde (2.00 g, 6.47 mmoles) in 60 ml of dry tetrahydrofuran was added dropwise at −5°. The cooling bath was slowly allowed to rise to 25° while the reaction mixture was stirred overnight.

The reaction mixture was refluxed on a steam bath for 1.5 hr and subsequently found to contain no unreacted starting material *via* TLC, using 10% ether in hexane on silica gel sheets. The dark-brown solution was cooled in ice and hydrolyzed by the dropwise addition of 10% ammonium acetate (130 ml). The pH was adjusted to 7 with acetic acid, and the layers were separated. The aqueous phase was extracted with 4 × 25 ml chloroform, and the combined organic layers were dried over anhydrous sodium sulfate.

Evaporation of the solvent gave a brown oil, which yielded 2.0 g of yellow-brown solid, mp 120–135°, on trituration with hexane. TLC (10% ether–benzene) showed the amide to have R_f 0.33 and to be contaminated with slight impurities at the origin and at R_f 0.8; IR: ν_{max} 3280 (OH), 1600 (CO), and 1168 and 1120 (CF_3) cm^{-1}. The crude *N*-*n*-butyl-3-(10-chloro-3-trifluoromethyl-9-anthryl)-3-hydroxypropionamide was used in the subsequent reduction.

To a stirred solution of 10 ml of 1 *M* BH_3–tetrahydrofuran in 21 ml of dry tetrahydrofuran, cooled to −5° under nitrogen, was added dropwise a solution of *N*-*n*-butyl-3-(10-chloro-3-trifluoromethyl-9-anthryl)-3-hydroxypropionamide (2.0 g, 4.72 mmoles) in 85 ml of dry tetrahydrofuran. The mixture was allowed to stir overnight, then 20 ml of 1 *M* BH_3–tetrahydrofuran was added, and the mixture was refluxed for 24 hr. At the end of the reflux period, no carbonyl absorption could be detected in the IR.

The yellow reaction mixture was cooled in ice and hydrolyzed by the dropwise addition of 5 ml of water–tetrahydrofuran (1:1). The solution was stripped to dryness under vacuum, and the yellow semisolid was refluxed with a solution of 7 ml of 10% H_2SO_4 in 30 ml of methanol for 4 hr. The solution was then cooled in ice, and the pH was adjusted to 10–11 with 1 *N* NaOH. The solution and precipitate were extracted with 3 × 50 ml of ether, and the ether extracts were washed with water and dried over anhydrous sodium sulfate. The ethereal solution was evaporated to a small volume and then diluted to 500 ml with ether. This process was repeated an additional time, and the resulting solution of amine in 500 ml of ether was used to prepare the hydrochloride.

The ethereal solution of amine was cooled in an ice bath, and 50 ml of hydrogen chloride in ether was added dropwise with stirring. The precipitated hydrochloride salt was filtered and washed with ether. The salt was dissolved in 4 ml of isopropanol and chilled to give the yellow hydrochloride, mp 185–190° dec. Concentration of the ethereal filtrate gave an oil, which yielded additional hydrochloride on crystallization from isopropanol–hexane.

A total of 150 mg (7.1%) of the hydrochloride was isolated. Recrystallization from isopropanol gave an analytical sample (Table II); TLC (free base) (5% methanol–benzene): R_f 0.44; IR (KBr): ν_{max} 3300 (OH), 2930 and 2850 (CH_2), 2750 (R_2NH^+), and 1165 and 1120 (CF_3) cm^{-1}. On standing, the melting point of the salt dropped to 120–130°, although TLC showed a single spot with the same R_f. TLC in two additional systems also showed a single component. The decrease in melting point may have been due to crystalline polymorphism.

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Synthesis and Biological Evaluation of Potential Hypoglycemic Agents I: Carnitine Analogs

SHARON G. BOOTS and MARVIN R. BOOTS [x]

Abstract □ A series of 4-dialkylamino-3-hydroxybutyric acid hydrochlorides and methochlorides, analogs of carnitine, was synthesized by treatment of *tert*-butyl 3,4-epoxybutyrate with the appropriate amine or amine hydrochloride in methanol followed by mild acid hydrolysis. The compounds had no effect on blood glucose or serum fatty acid levels in rats.

Keyphrases □ Carnitine analogs—potential hypoglycemic agents, synthesis, biological evaluation, IR and NMR spectra □ Hypoglycemic agents, potential—carnitine analogs, synthesis, biological evaluation, IR and NMR spectra

For years, diabetes mellitus has been described as a disorder of carbohydrate metabolism and the changes observed in lipid metabolism were thought to be secondary. Recently, however, Randle (1) suggested that the primary event in the development of diabetes mellitus might be an abnormality of glyceride metabolism.

Randle suggested a possible mechanism involving an increased release of free fatty acids. This release, in turn, produces a resistance to the hypoglycemic action of insulin, thereby causing a rise in fasting glucose levels and eventual exhaustion of the pancreatic β-cells. As a result of the increased serum free fatty acid levels, a corresponding increase in the rate of fatty acid oxidation is noted. This increase causes the tissue concentration of acetyl coenzyme A to rise, which results in a disruption of carbohydrate metabolism (2) through inhibition of several enzymes (pyruvate dehydrogenase, phosphofructokinase, and hexokinase).

If this hypothesis is correct, a possible approach to the treatment of diabetes mellitus would involve the design of agents that would decrease the high rate of fatty acid oxidation and increase glucose oxidation. Evidence in support of this approach is provided by the hypoglycemic activity of α-bromopalmitic acid (3), a known inhibitor of fatty acid oxidation (4).

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Table I—Properties of Compounds III*a*–III*d* and IV*a*–IV*d*

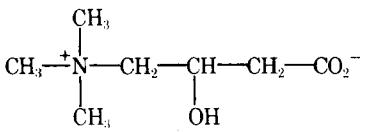
Compound	R_1	R_2	Yield, %	Physical Description (Melting Point when Crystalline)	IR Data, cm^{-1}	NMR Data, ppm	Formula	Analysis, % Calc.	Found
III*a*	CH_3CH_2	CH_3CH_2	41	Pale-yellow liquid[a]	Described under *Experimental*[b]		—	—	
III*b*	$(CH_2)_4$		81	Pale-yellow liquid[a]	3420 (OH) 1730 (C=O)	3.80–4.30 (m, 1H, —CH<)	—	—	
III*c*	$(CH_2)_5$		69	Pale-yellow liquid[a]	3420 (OH) 1730 (C=O)	3.80–4.15 (m, 1H, —CH<)	—	—	
III*d*	O<(CH$_2$)$_2$ / (CH$_2$)$_2$		49	Pale-yellow liquid[a]	3420 (OH) 1730 (C=O)	3.80–4.30 (m, 1H, —CH<)	—	—	
IV*a*	CH_3CH_2	CH_3CH_2	87	109–111°[c]	Described under *Experimental*[d]		$C_8H_{18}ClNO_3$	C 45.4 H 8.5 N 6.6	45.5 8.4 6.5
IV*b*	$(CH_2)_4$		92	83–88°[c]	1718 (C=O)	—	$C_8H_{16}ClNO_3$	C 45.8 H 7.6 N 6.7	45.8 7.6 6.5
IV*c*	$(CH_2)_5$		79	118–122°[c]	1718 (C=O)	—	$C_9H_{18}ClNO_3$	C 48.3 H 8.0 N 6.3	48.1 8.1 6.1
IV*d*	O<(CH$_2$)$_2$ / (CH$_2$)$_2$		85	168–170°[c]	1718 (C=O)	—	$C_8H_{16}ClNO_4$	C 42.6 H 7.1 N 6.2	42.8 7.1 6.1

[a] Attempted distillation of III*a*–III*d* led to extensive decomposition; therefore, no boiling-point or analytical data are reported. The spectral data are reported because this method was used for identification of these products. [b] IR spectra for III*a*–III*d* were run as liquid films. NMR spectra for III*a*–III*d* were run in deuterochloroform. [c] IV*a*–IV*d* were recrystallized from absolute ethanol–acetone to yield white prisms. [d] IR spectra for IV*a*–IV*d* were run as mineral oil mulls. NMR spectra for IV*a*–IV*d* were run in deuterium oxide.

and Hanley (5) suggested several sites where control of fatty acid metabolism is feasible. One possible point of pharmacological control would involve the inhibition of entry of long chain (>10 carbons) fatty acid coenzyme A derivatives into the mitochondria where oxidation occurs. Since long chain fatty acid coenzyme A derivatives cannot diffuse through the mitochondrial membrane, a transport system is required if they are to reach the enzymes involved in β-oxidation. This mechanism is called carnitine transport, and it is dependent on the presence of (−)-carnitine (I) and carnitine acyl transferases to transport long chain fatty acids into the mitochondria. The activity of this system has been shown to increase in livers of diabetic rats (6) and appears to be rate limiting for fatty acid oxidation.

Thus, the goals of this study were the design and synthesis of analogs of carnitine that would act as inhibitors of this transport system. Relatively few compounds have been studied as inhibitors of carnitine acyl transferases (7). Norcarnitine acts as a substrate in the absence of carnitine and as a competitive inhibitor in the presence of carnitine. Other related compounds, (*RS*)-β-hydroxy-γ-aminobutyric acid, *tert*-butylacetic acid, (*RS*)-β-hydroxy-γ-dimethylaminobutyramide, and choline, failed to act as inhibitors or substrates in this system.

A weak competitive inhibitor (3-deoxycarnitine) resulted when the β-hydroxyl group of carnitine is removed (7). The observed reduction of the affinity of



I

3-deoxycarnitine for the enzyme may result from a loss of the hydroxyl group as a binding site. Alternatively, the loss of the hydroxyl group may produce a change in the conformation of the inhibitor molecule, which alters its ability to bind to the enzyme.

These preliminary data indicate that the distance between the carboxyl group and the cationic function may be important. Therefore, initial synthetic efforts were directed toward compounds having the same chain length found in carnitine.

Thus, a series of dialkylaminohydroxybutyric acid hydrochlorides (IV*a*–IV*d*) and methochlorides (VI*a*–VI*c*), analogs of carnitine, was synthesized and evaluated with regard to their effects on blood glucose and serum free fatty acid levels.

DISCUSSION

tert-Butyl 3,4-epoxybutyrate (II) (8) was used as the starting material for the synthesis of the carnitine analogs IV*a*–IV*d* and VI*a*–VI*c* (Scheme I). The epoxy ester II readily reacted with the four secondary amines shown in Table I in methanol to provide the desired dialkylaminohydroxy esters (III*a*–III*d*). The yields were variable, and the major side reaction that occurred will be discussed in a future publication. The NMR spectral data afforded evidence that nucleophilic attack by the amine occurred at the desired 4-position and not at the 3-position. Hydrolysis of III*a*–III*d* with a dilute hydrochloric acid solution yielded the desired dialkylaminohydroxybutyric acid hydrochlorides (IV*a*–IV*d*) (Table I).

The synthesis of VI*a*–VI*c* was patterned after the synthesis of (*RS*)-carnitine chloride (8). It was found that II could be converted directly into the *tert*-butyl dialkylamino-3-hydroxybutyrate methochlorides (V*a*–V*c*) by treatment with the appropriate tertiary amine hydrochloride in methanol (Table II).

Hydrolysis of the esters V*a*–V*c* to the desired free acids VI*a*–VI*c* was again carried out by treatment with dilute hydrochloric acid. Although VI*a*–VI*c* (Table II) were all extremely hygroscopic (as were V*a*–V*c*), satisfactory NMR spectral data were obtained for all compounds. Since VI*c* could not be obtained in crystalline form, analytical data were obtained for the tetraphenylboronate salt (9).

Table II—Properties of Compounds Va–Vc and VIa–VIc

Compound	R_1	R_2	R_3	Yield, %	Physical Description (Melting Point when Crystalline)	NMR Data, ppm	Formula	Analysis, % Calc.	Found
Va	CH_3	CH_3	CH_3CH_2	90	Pale-cream semisolid[a,b]	Described under *Experimental*[c]	—	—	—
Vb	CH_3	CH_3CH_2	CH_3CH_2	67	Pale-yellow viscous liquid[a,b]	2.57 (d, J = 6 Hz, 2H, $-CH_2CO_2-$)	—	—	—
Vc	CH_3	CH_3	$CH_3CH_2CH_2CH_2$	81	Pale-yellow viscous liquid[a,b]	2.57 (d, J = 6 Hz, 2H, $-CH_2CO_2-$)	—	—	—
VIa	CH_3	CH_3	CH_3CH_2	44	White prisms[d]	Described under *Experimental*[c]	$C_8H_{18}ClNO_3$	C 45.4 H 8.5 N 6.6	45.2 8.4 6.4
VIb	CH_3	CH_3CH_2	CH_3CH_2	57	White prisms, 133–137°[e]	2.70 (d, J = 6 Hz, 2H, $-CH_2CO_2-$)	$C_9H_{20}ClNO_3$	C 47.9 H 8.9 N 6.2	48.1 8.8 6.3
VIc	CH_3	CH_3	$CH_3CH_2CH_2CH_2$	62	Thick viscous oil, 133–134°[f]	2.65 (d, J = 6 Hz, 2H, $-CH_2CO_2-$)	$C_{34}H_{42}BNO_3$	C 78.0 H 8.0 N 2.7	78.0 8.1 2.6

[a] Attempted purification of Va–Vc by crystallization was unsuccessful; therefore, no analytical data are reported. [b] IR spectra for Va–Vc and VIa–VIc could not be run because these compounds were extremely hygroscopic. [c] NMR spectra for Va–Vc and VIa–VIc were run in deuterium oxide. [d] Although satisfactory analytical data were obtained for this compound, it was too hygroscopic to obtain a melting point. [e] VIb was recrystallized from absolute ethanol–acetone. [f] VIc was analyzed as the tetraphenylboronate salt (Ref. 9) because the chloride could not be obtained in crystalline form.

RESULTS

Hypoglycemic Activity in Rats—Three control and three treated male rats, 140–160 g, from the local Harlan colony were fasted for 18 hr. All rats were given 100 mg of glucose in water subcutaneously, followed immediately by a single oral administration of the test compound in 5% aqueous acacia. Control rats received an equal volume of the vehicle. Blood samples were taken from the tail vein of all animals at 2 and 5 hr after administration of the test compound and were assayed for glucose with an automatic analyzer[1] using a modification of the method of Hoffman (10). Results for blood glucose are reported as the percent difference of the mean value for the treated animals from the mean value for the control animals.

No significant differences in the blood glucose levels relative to controls were observed for IVa–IVd and VIa–VIc when administered orally at a dose of 100 mg/kg.

Serum Free Fatty Acids—Three control and three treated male rats, 140–160 g, from the local Harlan colony were fasted for 18 hr. A single oral administration of the test compound in 5% aqueous acacia was given to the treated rats. Control rats received an equal volume of the vehicle. Blood samples were taken from the orbital sinus of all animals 1 hr after administration of the test compound for determination of serum free fatty acids. Serum free fatty acids were extracted using the method of Dole and Meinertz (11) and were assayed with an automatic analyzer[1] using the method of Baird *et al.* (12). Results for serum free fatty acids are reported as the percent difference of the mean value for the treated animals from the mean value for the control animals.

No significant differences in the serum free fatty acid levels relative to control levels were observed after IVa–IVd and VIa–VIc were administered orally at a dose of 100 mg/kg.

EXPERIMENTAL[2]

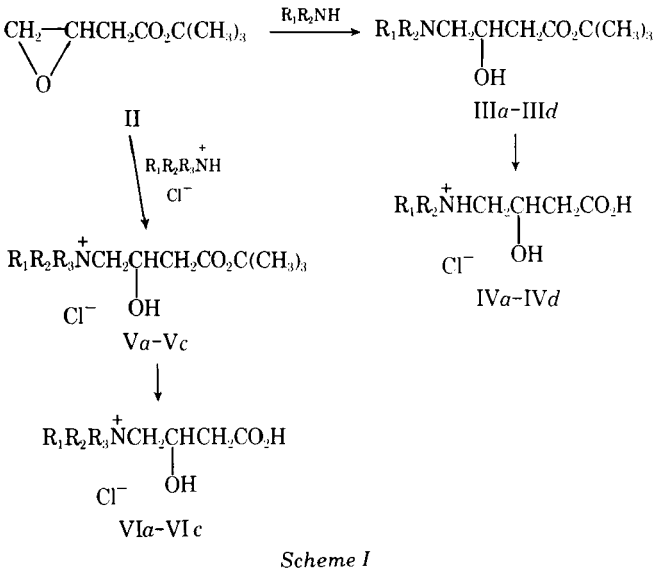
***tert*-Butyl 4-Diethylamino-3-hydroxybutyrate (IIIa)**—This example typifies the method used to prepare IIIa–IIId (Table I). To a solution of 1.58 g (0.010 mole) of *tert*-butyl 3,4-epoxybutyrate (II) (8) in 6 ml of methanol was added 1.3 ml (0.013 mole) of distilled diethylamine. The pale-yellow solution was stirred at 26° for 21 hr under a dry nitrogen atmosphere. The solvent was removed under reduced pressure to afford 1.91 g of a yellow liquid. This liquid was added to ether and an ice-cold 5% hydrochloric acid solution.

The acidic, aqueous extract was adjusted to pH 12 with 5% sodium hydroxide and then was extracted with ethyl acetate. The organic phase was washed with water and then a saturated sodium chloride solution. The organic phase was dried over anhydrous sodium sulfate, and the solvent was removed under reduced pressure to afford 0.943 g (41%) of IIIa as a pale-yellow liquid.

Attempted distillation of IIIa, as well as IIIb–IIId, resulted in extensive decomposition, so these products were identified by spectral methods only; IR (liquid film): 3420 (OH) and 1730 (ester C=O) cm^{-1}; NMR (deuterochloroform): δ 1.04 (t, J = 7 Hz, 6H, CH_3 in ethyl groups), 1.48 [s, 9H, $-C(CH_3)_3$], 2.32–2.83 [m, 8H, $(-CH_2)_2NCH_2-$, $-CH_2CO_2-$], 3.82 (s, 1H, —OH), and 3.75–4.73 (m, 1H, >CH—) ppm.

4-Diethylamino-3-hydroxybutyric Acid Hydrochloride (IVa)—This example typifies the method used to prepare IVa–IVd (Table I). A solution of 0.943 g (0.004 mole) of IIIa in 20 ml of 7% hydrochloric acid was stirred at 25° for 20 hr under a nitrogen atmosphere. The water was removed under high vacuum at 50° to afford 0.863 g of a pale-yellow oil, which crystallized upon trituration with acetone followed by storage at 5° for 3 days.

The acetone was removed from the resulting white crystalline solid and the solid was dried, affording 0.723 g (83%) of IVa, mp



Scheme I

[1] Technicon Autoanalyzer.

[2] Melting points were determined with a Thomas-Hoover capillary melting-point apparatus and are uncorrected. Elemental analyses were obtained from Galbraith Laboratories, Knoxville, Tenn. IR spectra were determined using a Perkin-Elmer model 237 spectrophotometer. NMR spectra were determined on a Perkin-Elmer model R-24 spectrometer in deuterochloroform, using tetramethylsilane as the internal reference, or in deuterium oxide, using sodium 2,2-dimethyl-2-silapentane-5-sulfonate as the internal reference.

104–106°. An analytical specimen of IV*a*, mp 109–111°, was prepared by recrystallization from absolute ethanol–acetone; IR (mineral oil): 1718 (CO_2H) cm^{-1}; NMR (deuterium oxide): δ 1.28 (t, J = 7 Hz, 6H, CH_3 in ethyl groups), 2.65 (d, J = 6 Hz, 2H, $—CH_2CO_2—$), 3.05–3.50 [m, 6H, $(—CH_2)_2NCH_2—$], and 4.20–4.80 (m, 1H, >CH—) ppm.

***tert*-Butyl 4-Ethylmethylamino-3-hydroxybutyrate Methochloride (V*a*)**—This example typifies the method used to prepare V*a*–V*c* (Table II). A mixture of 0.656 g (4.2 mmoles) of *tert*-butyl 3,4-epoxybutyrate (II) (8), 0.361 g (3.30 mmoles) of dimethylethylamine hydrochloride, and 2.0 ml of anhydrous methanol was allowed to stand at 25° for 6 days in a glass-stoppered flask. The methanol was removed under reduced pressure, and the residue was added to ether and water. The organic phase was washed an additional time with water, and the aqueous extracts were combined. The water was removed under high vacuum at approximately 30–40°, and then the traces of water remaining were removed by azeotropic distillation with four 10-ml portions of *tert*-butyl alcohol under reduced pressure.

The resulting oil was dried for 19 hr at 25° under high vacuum (a pale-cream semisolid resulted after drying). All attempts at further purification by recrystallization failed, since all compounds listed in Table II were extremely hygroscopic; NMR (deuterium oxide): δ 4.30–4.60 (m, >CH—, no integration on this proton could be obtained because it was located underneath the HOD peak), 3.32–3.75 (m, 4H, $—CH_2NCH_2—$), 3.12 [s, 6H, $(CH_3)_2N—$], 2.57 (d, J = 6 Hz, 2H, $—CH_2CO_2—$), 1.45 [s, 9H, $—C(CH_3)_3$], and 1.35 (t, J = 6 Hz, 3H, CH_3— in ethyl group) ppm.

4-Ethylmethylamino-3-hydroxybutyric Acid Methochloride (VI*a*)—This example typifies the method used to prepare VI*a*–VI*c* (Table II). A solution of 0.760 g (2.86 mmoles) of V*a*, 50 ml of water, and 2.4 ml of concentrated hydrochloric acid was stirred at 25° for 17 hr. The water was removed at 30–40° under high vacuum, and then four 30-ml portions of *tert*-butyl alcohol were added as described for V*a* to remove azeotropically the remaining water. The resulting pale-yellow viscous oil (0.550 g, 91%) was triturated with acetone and a few drops of absolute ethanol. The white gummy oil crystallized slowly after standing at 5° for 5 days.

The resulting white prisms were dried under high vacuum at 25° for 1 day. The yield was 0.266 g (44%) of VI*a*, which was too hygroscopic to obtain melting-point or IR data; NMR (deuterium oxide): δ 4.55–4.82 (m, >CH—, no integration on this proton could be obtained because it was located underneath the HOD peak), 3.30–3.70 (m, 4H, $—CH_2NCH_2—$), 3.12 [s, 6H, $(CH_3)_2N—$], 2.67 (d, J = 6 Hz, 2H, $—CH_2CO_2—$), and 1.35 (t, J = 6 Hz, 3H, CH_3— in ethyl group) ppm.

Anal.—Calc. for $C_8H_{18}ClNO_3$: C, 45.4; H, 8.5; N, 6.6. Found: C, 45.2; H, 8.4; N, 6.4.

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[x] To whom inquiries should be directed.

Alkaloids of *Vinca rosea* L. (*Catharanthus roseus* G. Don) XXXVI: Isolation and Characterization of New Dimeric Alkaloids

S. TAFUR ˣ, W. E. JONES, D. E. DORMAN, E. E. LOGSDON, and G. H. SVOBODA

Abstract □ In a continuing effort to study thoroughly the alkaloids of *Catharanthus roseus*, new dimeric alkaloids were isolated and characterized. Structures are proposed for leurocolombine and vinamidine based on UV, IR, PMR, high-resolution mass spectrometry, and CMR. Pseudovincaleukoblastine diol was identified by PMR and mass spectrometry. Leurocolombine exhibited antimitotic activity and marginal antitumor activity against the Ridgeway osteogenic sarcoma.

Keyphrases □ Alkaloids—isolation of three from *Catharanthus roseus*, characterization by UV, IR, NMR, and mass spectra □ *Vinca rosea*—isolation of three dimeric alkaloids, characterization by UV, IR, NMR, and mass spectra □ *Catharanthus roseus*—isolation of three dimeric alkaloids, characterization by UV, IR, NMR, and mass spectra □ Leurocolombine—isolation from *Catharanthus roseus*, UV, IR, NMR, and mass spectral structure identification □ Vinamidine—isolation from *Catharanthus roseus*, UV, IR, NMR, and mass spectral structure identification □ Pseudovincaleukoblastine diol—isolation from *Catharanthus roseus*, UV, IR, NMR, and mass spectral structure identification

In continuing efforts to study thoroughly the alkaloids of *Catharanthus roseus* G. Don[1], three new dimeric alkaloids were isolated: leurocolombine (I), vinamidine (II), and pseudovincaleukoblastine[2] diol (III). Postvincaleukoblastine–preleurocristine (1) production-scale chromatographic column cuts were subjected to preparative high-pressure liquid chromatography (HPLC), gradient pH purification (2), or gradient pH followed by preparative HPLC or preparative TLC. Structure elucidation studies of the three new dimeric alkaloids are reported in this paper.

Leurocolombine demonstrated antimitotic activity[3] at 2×10^{-2} μg/ml and produced 27% inhibition against the Ridgeway osteogenic sarcoma at 15 mg/kg. Vinamidine was not toxic to Swiss white mice at 30 mg/kg and showed no antimitotic activity, so *in vivo* testing was not attempted. Pseudovincaleukoblastine diol did not show antimitotic activity, and insufficient material was available for further testing.

EXPERIMENTAL

Isolation of Leurocolombine (I)—Twenty grams of a production-scale postvincaleukoblastine–preleurocristine chromatographic column cut was subjected to gradient pH purification. Leurocolombine cocrystallized from methanol with vincaleukoblastine, leurosine, and other trace impurities from pH 3.90 and 4.40 as the free base. Each pH fraction was subjected to sulfate formation in the usual manner[4]. Sulfates from pH 3.90, 4.40, and 4.90 were shown by TLC on silica gel[5] in ethyl acetate–ethanol (3:1) to contain vincaleukoblastine, leurosine, desacetylvincaleukoblastine, leurocolombine, and trace quantities of unidentified compounds.

The mixed sulfates were combined and dissolved in 2 liters of hot ethanol for recrystallization. Upon cooling to room temperature, a white amorphous precipitate deposited and was harvested to give 2.2184 g of first crop material. Three subsequent crops of amorphous sulfates, totaling 0.7023 g, were harvested from the chilled concentrated mother liquor. All crops in the attempted recrystallization of the mixed sulfates were amorphous mixtures, and none was appreciably enriched in I. Consequently, the second, third, and fourth crops of mixed sulfates were converted to their free bases for preparative TLC.

Between 80 and 100 mg of bases was applied to 20 × 20-cm silica gel preparative plates[6], which were developed in ether–diethylamine–toluene–methanol (100:5:5:5). Bands were distinguished by their UV-quenching effect and removed and eluted with chloroform and methanol. This method yielded 105 mg of I. The first crop sulfate (2.2184 g) was converted to its free base (1.98 g) and subjected to gradient pH purification to enrich fractions prior to preparative TLC. Leurocolombine was again found in pH 3.90, 4.40, and 4.90 (total weight 1.07 g) and cocrystallized with vincaleukoblastine and other trace impurities as both the free base and sulfate. Preparative TLC of all mixtures yielded an additional 150.6 mg of I.

Leurocolombine had a typical indole–dihydroindole UV spectrum[7]: λ_{max} 217 (a_m = 51,091) and 265 (a_m = 15,666) nm with shoulders at 290 and 298 nm. The IR spectrum[8] had a strong band at 3570 cm^{-1} (OH) in addition to the other typical bands found in vincaleukoblastine and leurosine (3). Leurocolombine has pKa values of 5.05 and 6.3. The mass spectrum[9] indicated a molecular ion at *m/e* 826.

Acetylation of Leurocolombine (I)—To a solution of 21.9 mg of I in 0.5 ml of dry pyridine, excess acetic anhydride was added. The reaction mixture was allowed to stand at room temperature overnight. After evaporating to dryness *in vacuo*, the mixture was shown by TLC on silica gel in ethyl acetate–ethanol (3:1) to contain unreacted I and one reaction product. Preparative separation afforded leurocolombine acetate (M^+ 868).

Isolation of Vinamidine (II)—Ten grams of a production-scale chromatographic preleurocristine fraction was fractionated in a series of preparative HPLC runs. Chromatography was carried out in stainless steel columns, using neutral alumina packing[10] and ethyl acetate–methylene chloride (25:75). The initial run was made on a 2.5-cm × 7-m column at 900 psi with a consequent flow rate of 66 ml/min. Eluent was subdivided based on the profile generated by a 280-nm UV flow monitor.

TLC of fractions on silica gel in ether–diethylamine–toluene–methanol (100:5:5:5) revealed a fraction containing a new alkaloid. This fraction was further purified by three sequential runs on an 8-mm × 7-m column at 320 psi with a 7-ml/min flow rate. Final purification was completed in an 8-mm × 2-m column using meth-

[1] Plant material obtained from Meer Corp. and Madis Labs.

[2] Pseudovincaleukoblastine is desacetoxyvincaleukoblastine. See previous paper in series, XXXV: N. Neuss, A. J. Barnes, and L. L. Huckstep, *Experientia*, in press.

[3] Metaphase arrest was observed using Chinese hamster ovary cells. Vincaleukoblastine was active at 2×10^{-3} μg/ml.

[4] See U.S. pat. 3,225,030 (Dec. 21, 1965).

[5] Precoated silica gel 60 F-254, 0.25-mm plates, E. Merck, Darmstadt, Germany.

[6] Quantum Industries, Fairfield, NJ 07006

[7] Recorded on a Cary 15 UV spectrophotometer in ethanol.

[8] Recorded on a Perkin-Elmer 457A IR spectrophotometer in chloroform.

[9] Recorded on a Varian MAT model 731 double-focusing spectrometer. Samples were inserted directly into the ion source by direct probe.

[10] Woelm W-200 (60–180 μm).

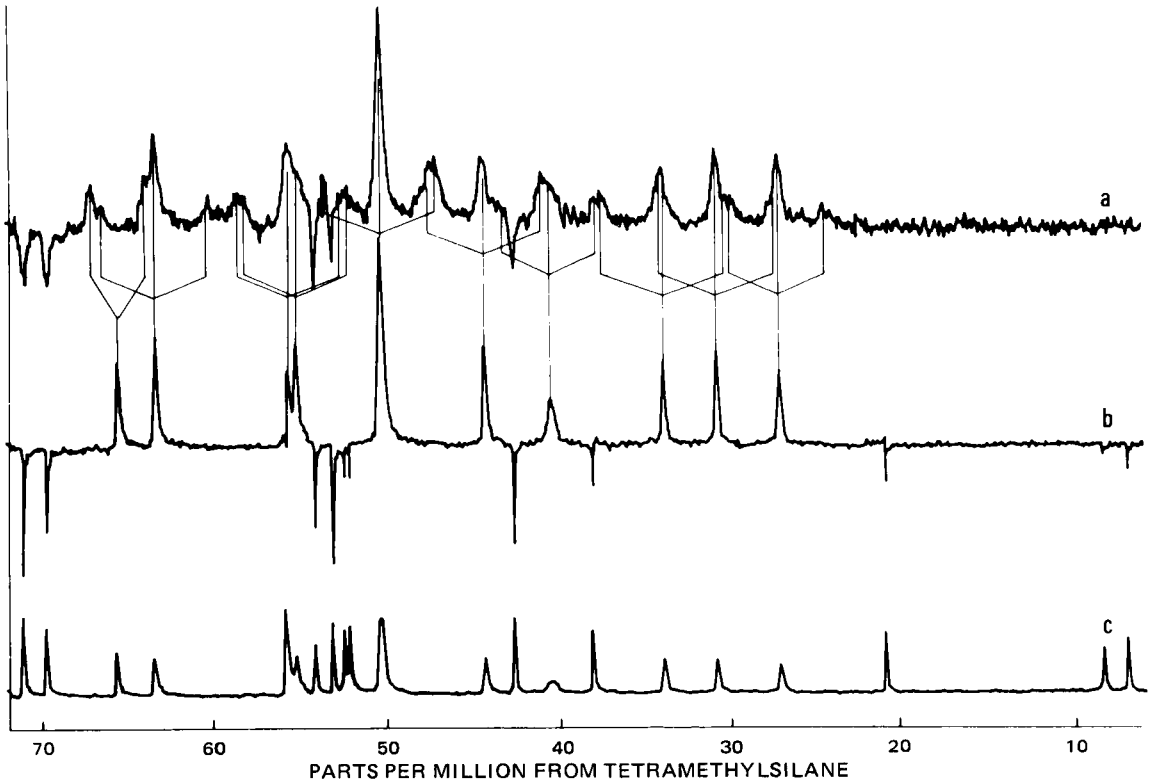


Figure 1—*Upfield portion of ^{13}C spectrum of leurocolombine. Key:* a, *off-resonance decoupled spectrum at τ = 0.4 sec;* b, *proton decoupled spectrum at τ = 0.4 sec; and* c, *proton decoupled spectrum.*

yl acetate–methylene chloride (60:39) at 1 atm of pressure and a flow rate of 1.5 ml/min. A total of 152 mg of II was isolated, being equivalent to 19 ppb of *C. roseus* leaf.

Vinamidine exhibited λ_{max} 216 (a_m = 57,278) and 268 (a_m = 16,815) nm with shoulders at 285, 295, and 311 nm. The IR spectrum exhibited an intense band at 1660 cm^{-1}, which is typical of an amide. The mass spectrum indicated a molecular ion at *m/e* 824.

Acetylation of Vinamidine (II)—Vinamidine acetate (M^+ 866) was prepared in the manner described for I.

Sodium Borohydride Reduction of Vinamidine (II)—Thirty milligrams of vinamidine base was dissolved in anhydrous methanol. An excess of sodium borohydride was added slowly with stirring. After stirring at room temperature for 0.5 hr, 10 ml of water was added and the methanol was removed *in vacuo*. The aqueous suspension was then extracted with chloroform; the chloroform extract was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness *in vacuo* to yield a one spot reaction product (M^+ 826).

Acetylation of Vinamidine Reduction Product—The vinamidine reduction product was acetylated in the manner described for I to yield two reaction products, subsequently identified as a monoacetate (M^+ 868) and a diacetate (M^+ 910) of the vinamidine reduction product.

Isolation of Pseudovincaleukoblastine Diol (III)—To isolate III, 11.75 g of a postvincaleukoblastine–preleurocristine production-scale chromatographic column cut was subjected to gradient pH purification. After sulfates were made in the usual manner, mixed sulfates containing vincaleukoblastine, leurocristine, and a third component were obtained from pH 5.40 and 5.90. A 100-mg sample of sulfate from pH 5.40 was fractionated in a preparative HPLC run on a stainless steel 0.793-cm × 6-m column packed with neutral alumina[11] using a linear gradient of 0–5% ethanol in methylene chloride.

The weighed sample was dissolved in 5 ml of water, and ammonium acetate was added to pH 9.0. The precipitate was centrifuged, the supernate was decanted, and the precipitate was dissolved in 3 ml of methylene chloride for application to the column. The run was made at 1100 psi (uncorrected) with a consequent flow rate of 180 ml/hr[12]. Fractions were collected every 3 min once peaks were observed on a UV profile. Compound III (M^+ 768) was located in fractions 30–32 by TLC on silica gel in ether–diethylamine–toluene–methanol (100:5:5:5). The remainder of the mixed sulfates containing the compound was subjected to preparative TLC in the same system since better yields were obtained than with the preparative HPLC run. After two additional preparative TLC runs, a total of 25.2 mg of III was isolated.

Acetylation of Pseudovincaleukoblastine Diol (III)—Compound III was acetylated in the manner described previously to yield its diacetate (M^+ 852) as the major reaction product with traces of a monoacetate (M^+ 810).

DISCUSSION

In the structure analyses of the new dimeric alkaloids, it is helpful to relate the unknown structures to the known structure of vincaleukoblastine (4). The vindoline portion of the dimers can readily be identified by characteristic chemical shifts in the PMR spectrum (5), by characteristic fragments in the mass spectrum (6), and by characteristic carbon resonances in the CMR spectrum (7).

Leurocolombine (I)—The mass spectrum of I indicated a molecular weight of 826, and high-resolution measurements verified a molecular formula of $C_{46}H_{58}N_4O_{10}$ (Table I). Deuterium exchange in combination with mass spectrometry disclosed that I had one more exchangeable proton than vincaleukoblastine, indicating that the additional oxygen was most likely a hydroxy group. The vindoline half of the dimer appeared intact, as evidenced by peaks at *m/e* 121, 122, 135, 282, 469, and $M^+ - C_6H_7O_5$ (6). Since typical indole fragments at *m/e* 143 and 144 were present, the extra hydroxy was assumed to be in the alicyclic portion of the indole half of the molecule.

In vincaleukoblastine, *m/e* 154 is characteristic for the alicyclic portion of the velbanamine portion of the molecule (8). If the extra hydroxy was in this portion, a peak at *m/e* 170 would be expected; indeed, a weakly intense peak did appear, but high resolution indicated it was a mass triplet. One component did correspond to the

[11] Woelm N-18 (18–30 μm).

[12] Lapp LS-30 Pulsafeeder pump.

Table I—High-Resolution Mass Spectral Measurements for Leurocolombine

Formula	Observed *m/e*	Calculated *m/e*
$C_{46}H_{58}N_4O_{10}$	826.41613	826.41530
$C_{44}H_{55}N_4O_8$	767.40267	767.40199
$C_{40}H_{51}N_4O_5$	667.38395	667.38595
$C_{26}H_{33}N_2O_6$	469.22936	469.2338
$C_{14}H_{20}NO_5$	282.13126	282.13415
$C_{12}H_{12}N$	170.09755	170.09697
$C_9H_{16}NO_2$	170.11731	170.11810
$C_{11}H_{10}N$	156.08161	156.08132
$C_8H_{14}NO_2$	156.10182	156.10245
$C_9H_{14}NO$	152.10906	152.10754
$C_{10}H_{10}N$	144.08187	144.08132
$C_{10}H_9N$	143.07408	143.07350

desired molecular formula for the additional hydroxy while the other components were determined to be the indole portion of the molecule together with attached carbon and nitrogen atoms.

A peak at *m/e* 152 had an empirical formula corresponding to loss of water from the $C_9H_{16}NO_2$ component at *m/e* 170. This region of the mass spectrum was further complicated by a mass doublet at *m/e* 156, the more intense component together with its associated ^{13}C isotope peak corresponding to the alicyclic portion of the molecule and containing two oxygens. Deuterium exchange in this region showed an increase in intensity at *m/e* 157 with no other discernible significant changes.

Inspection of the PMR spectrum of I showed few differences from that of vincaleukoblastine. The resonances that are usually identified as vindoline protons were present with few changes. The aromatic resonances, H-9 and OCH_3-11 closest to the bond linking the two monomers, were broadened, suggesting possible restriction of rotation around that bond. In addition, a doublet at δ 4.16 was resolved, with a coupling constant of 15 Hz characteristic of geminal coupling of a methylene proton attached to a heteroatom.

The key to placement of the extra hydroxy in I came from CMR studies[13]. The initial experiment involved running the proton-decoupled CMR spectrum to get chemical shift values for as many of the carbons as possible. Overlap made it difficult to distinguish all 46 carbon resonances, and additional experiments were performed to resolve the resonances and to gain data useful for making assignments.

From relaxation time (T_1) measurements on vincaleukoblastine and other dimeric alkaloids, the nonprotonated carbons with long T_1 values were easily distinguished from protonated carbons that have shorter T_1 values (9). Average T_1 values were 1–3 sec for nonprotonated carbons; 0.5 sec for methyl carbons attached to carbon, oxygen, or nitrogen; 0.1 sec for methine carbons; and 0.05 sec for methylene carbons. The T_1 values of methines and methylenes were not always clearly differentiated, so a procedure using the inversion-recovery (180–τ–90) method (10) in conjunction with off-resonance decoupling (11) was used to distinguish these two types of carbons.

In the inversion-recovery method, resonances are inverted by a 180 R_f pulse and allowed to relax only partially before being measured by a 90 pulse. A resonance having a relaxation time, T_1, is nulled if an interval (τ) of ln $2T_1$ (0.69 T_1) is used. By using an interval of 0.35 sec (0.69 × 0.5), the methyl resonances were nulled in vincaleukoblastine. Leurocolombine required a τ value of 0.4 sec. The resulting spectrum showed nonprotonated carbon resonances as inverted peaks and the methines and methylenes as upright peaks (Fig. 1*b*). At this point, nonprotonated carbons and methyl carbons were clearly identified, but methine and methylene carbon resonances were not yet differentiated. The inversion-recovery spectrum was subsequently recorded with off-resonance decoupling to produce methines as "doublets" and methylenes as "triplets" (Fig. 1*a*).

The ^{13}C resonances of the vindoline half of vincaleukoblastine have been reported (7). These resonances were subjected to the inversion-recovery/off-resonance decoupling procedure to test both the assignments and the procedure. Excellent agreement was obtained. Resonances in I with chemical shifts similar to the assigned vindoline resonances in vincaleukoblastine were checked by the procedure to confirm their assignments in I (Table II).

[13] Recorded on a JEOL PS-100 spectrometer in Fourier transform mode operating at 25 MHz with repetition times of 2–3 sec. Chemical shifts are reported relative to internal tetramethylsilane, using concentrations of 50–200 mg/2 ml deuterated chloroform, depending on availability of sample.

Table II—^{13}C Chemical Shifts of Vindoline Half of Vincaleukoblastine and Leurocolombine

	Vincaleukoblastine	Leurocolombine
Acetate C=O	171.6	171.5
Ester C=O	170.8	170.8
C-11	158.0	158.6
C-13	152.5	153.6
C-15	129.9	129.8
C-14	124.4	124.4
C-9	123.5	123.6
C-8	122.6	122.3
C-10	121.1	120.0
C-12	94.2	94.6
C-2	83.3	83.2
C-16	79.7	79.4
C-17	76.4	76.2
C-21	65.5	65.7
OCH_3	55.7	55.9
C-7	53.2	53.2
$COOCH_3$	52.1	52.2
C-3	50.2	50.4
C-5	50.2	50.4
C-6	44.6	44.3
C-20	42.7	42.6
$N\text{-}CH_3$	38.3	38.1
C-19	30.8	30.8
$OCOCH_3$	21.1	21.0
C-18	8.3	8.3

By schematically representing the I spectrum (Fig. 2*a*) and subtracting the vindoline resonances (Fig. 2*b*), only those resonances corresponding to the velbanamine-like half of the dimer remained (Fig. 2*c*). This procedure reduced the complexity of the total spectrum.

The aromatic carbons of the indole half and the ester carbonyl and methoxy were readily assigned by comparison of chemical shifts to vincaleukoblastine (7), and these assignments were confirmed by the inversion-recovery/off-resonance decoupling procedure (Table III). This procedure confirmed the deduction based on mass and PMR spectra that the indole portion of the molecule was unsubstituted. From the remaining resonances, it was observed that I contained three "singlets," *i.e.*, nonprotonated carbons, rather than two as in vincaleukoblastine. One singlet corresponded well to C-20′ of vincaleukoblastine, and one was in the range of C-16′. The third singlet resonated at 71.2 ppm in the range of carbons singly bonded to oxygen (12) and thus had the extra hydroxy. Since the methine doublet corresponding to C-14′ in vincaleukoblastine is absent in I, the hydroxy must be placed at C-14′. These data are accommodated by Structure I.

Since vincaleukoblastine and I have identical C-18′ chemical shifts, it may be concluded that the configuration at C-20′ is the same in I as in vincaleukoblastine. At present, no configuration has been determined for C-14′.

A hydroxy at C-14′ could possibly sterically hinder rotation around the bond linking the two monomers, and this would ex-

Table III—^{13}C Chemical Shifts of Selected Carbons of Indole Half of Vincaleukoblastine and Leurocolombine

	Vincaleukoblastine	Leurocolombine
Ester C=O	174.9	174.3
C-13′	134.9	135.1
C-2′	131.3	130.4
C-8′	129.4	129.2
C-10′	122.1	122.3
C-11′	118.7	118.9
C-9′	118.4	118.4
C-7′	117.0	117.1
C-12′	110.4	110.4
C-20′	69.4	69.9
C-16′	55.7	54.2
$COOCH_3$	52.1	52.5
C-18′	6.9	6.9

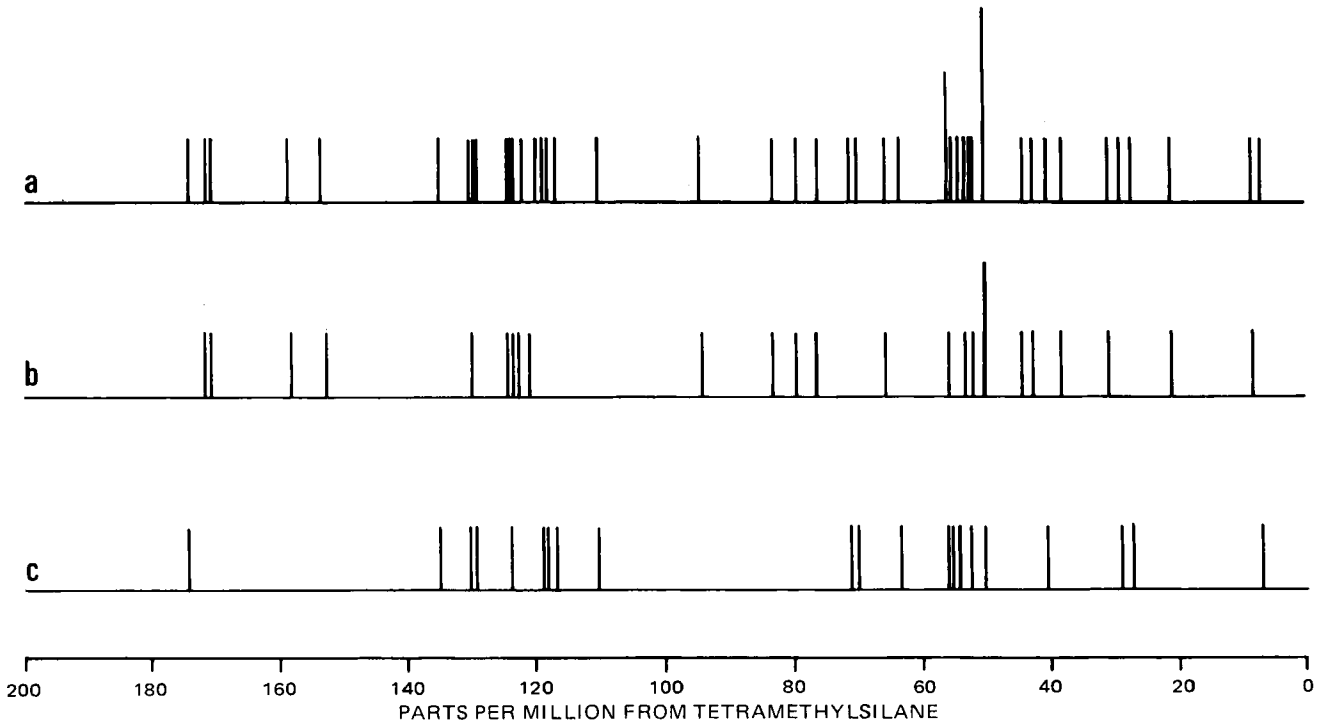


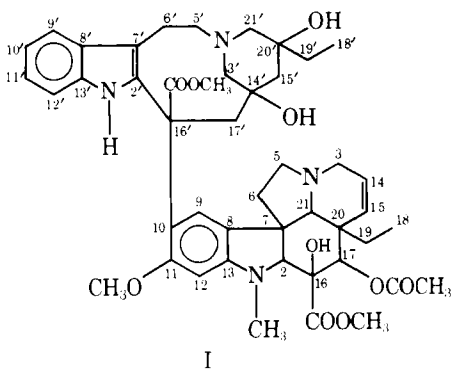
Figure 2—*Schematic diagrams of* ^{13}C *spectra. Key:* a, *total leurocolombine spectrum;* b, *vindoline spectrum; and* c, *indole half of leurocolombine.*

plain the broadened signals observed in the PMR spectrum. Both C-16′ and C-10 showed 1-ppm shifts in the CMR spectrum, which could reflect an effect on that bond.

Vinamidine (II)—The mass spectrum of II indicated a molecular weight of 824, and high resolution established a molecular formula of $C_{46}H_{56}N_4O_{10}$, which is isomeric with leurocristine (13). The IR spectrum had an intense band at 1660 cm^{-1}, characteristic of an amide.

In the PMR spectrum, the characteristic chemical shifts for the vindoline half of the molecule were observed. Likewise, the carbon resonances for the vindoline half of vinamidine agreed well with the assigned chemical shift values for the vindoline carbons in vincaleukoblastine and were checked by the inversion-recovery/off-resonance decoupling procedure. Consequently, an *N*-formyl group in the vindoline half was eliminated as the source of the intense amide band in the IR spectrum.

The CMR studies indicated the presence of a ketone at 210.4 ppm (12) in the indole half of the molecule. Sodium borohydride reduction resulted in the disappearance of the ketone resonance, with a new signal appearing at 68.4 ppm, indicative of an alcohol (12). The vinamidine reduction product formed a diacetate, while the parent compound formed a monoacetate. The CMR spectrum also had a resonance at 163.4 ppm, which appeared as an upright doublet in the inversion-recovery/off-resonance decoupling procedure. This downfield chemical shift, coupled with the fact that the carbon bears one proton, suggested a formamide. An N_b-formyl group would be consistent with the observed CMR resonance as well as the IR band. The reduction reaction affected neither this carbon resonance nor the IR band.

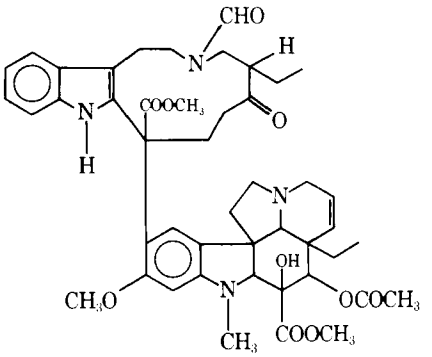


I

The ketone was placed at C-15′, although C-14′ is the biogenetically preferred position. Despite C-20′ being nonoxygenated, the chemical shift of C-18′ in II is quite similar to C-18′ in vincaleukoblastine. A ketone at C-15′ rather than C-14′ would exert a shielding effect (14) on C-18′ to cause a slight upfield shift and effectively compensate for any downfield shift due to no oxygenation at C-20′. From these data, Structure II is proposed for vinamidine.

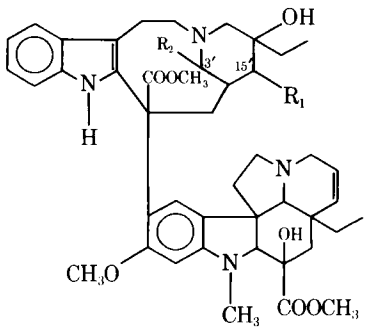
The structure of catharine, another *Catharanthus* indole–dihydroindole alkaloid, was reported recently[14]. It differs from II by having a double bond between C-20′ and C-21′ and a ketone at C-14′ rather than C-15′. The hydrogenation products of the two compounds were not identical, thus indicating that II was not merely dihydrocatharine. Sodium borohydride reduction of catharine produced a lactone between the carbomethoxy carbonyl and the reduced ketone, but II formed only the alcohol to substantiate again different placement of the ketone.

Pseudovincaleukoblastine Diol (III)—The mass spectrum of III indicated a molecular ion of M^+ 768, which corresponds to the molecular weight of desacetylvincaleukoblastine; however, the remainder of the spectrum showed that the compounds differed. The characteristic vindoline fragments of *m/e* 469 and 282 were shifted



II

[14] Presented by P. Potier at the IUPAC Natural Products Symposium in Ottawa, Canada, July 1974.



$R_1 = OH$ $R_2 = H$ or $R_1 = H$ $R_2 = OH$

III

58 mass units to *m/e* 411 and 224, respectively. Similarly, the usual $M^+ - 160$ (5) was shifted to $M^+ - 102$, which indicated a desacetoxy rather than desacetyl compound. Comparison to the mass spectrum of pseudovincaleukoblastine[2] showed identical fragments of *m/e* 411, 224, and $M^+ - 102$. The peaks at *m/e* 154 and 355, which are characteristic of the velbanamine half of vincaleukoblastine (8), were shifted to *m/e* 170 and 371 in III to indicate an extra hydroxy group in the alicyclic portion of the indole half of the dimer. These two peaks are also seen in the mass spectrum of vincadioline[15].

Inspection of the PMR spectrum of III showed the compound lacked an acetate peak. In the PMR of desacetyl compounds of the vindoline-containing dimeric alkaloids, H-17 is shifted to δ 4.075 (5) and the vinyl protons coincide in a multiplet around δ 5.85 (15). Compound III had no signal near δ 4.0 assignable to H-17 and had a broad multiplet at δ 5.46–5.78, which was clearly different from the multiplet observed in a simple desacetyl derivative.

Compound III formed a diacetate with one acetate group in the vindoline half and the other acetate in the indole half as evinced by the peak at *m/e* 371 shifting to *m/e* 413. More specifically, the peak at *m/e* 170 is shifted to *m/e* 212 to locate the acetate group in the alicyclic portion. The two acetates were observed in the PMR spectrum at δ 1.88 and 1.90. The peak at δ 1.90 was assigned to the acetate at C-16 in the vindoline half, which is identical to the chemical shift of the C-16 acetate in desacetoxyvincaleukoblastine acetate[2]. The acetate peak at δ 1.88 is quite similar to the one observed C-15′ acetate in vincadioline diacetate at δ 1.85[15]. The acetate derivatives of both III and vincadioline exhibited a new one-proton singlet at δ 4.47–4.48, assigned to the proton at the C-15′ acetate. In the parent compounds, this proton is hidden but, upon acetylation, shifts downfield to be clearly identified.

From these data, Structure III, where R_1 is OH, is proposed for pseudovincaleukoblastine diol. There is a possibility that the hydroxy could be placed at C-3′ or C-21′ since a carbinolamine could form an acetate under the conditions used. The most likely placement, however, of the hydroxy is at C-15′, based on comparison to vincadioline. The small amount of compound precluded further evidence of the structure.

[15] Vincadioline is 15′-hydroxyvincaleukoblastine, a minor alkaloid from *C. roseus;* unpublished results from Lilly Research Laboratories.

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Absorption of ^{3}H-Benzocaine from Ointments following Rectal Administration in Rats

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Abstract □ Total radioactivity in the blood of rats for 5 hr following rectal administration of ^{3}H-benzocaine in oleaginous, absorption, emulsion (water-in-oil and oil-in-water), and water-soluble ointment vehicles was measured. The release was greatest from the water-soluble vehicle and followed the same relative order as seen in an earlier *in vitro* experiment. No intact benzocaine was found in the blood using radiochromatography. *In vitro* hydrolysis of benzocaine by rat blood did not occur as determined with the techniques of this experiment.

Keyphrases □ Benzocaine—absorption from ointments, rectal administration, rats □ Anesthetics, local—benzocaine, absorption from ointments, rectal administration, rats □ Ointments—benzocaine absorption, rectal administration, rats

Benzocaine (ethyl *p*-aminobenzoate) is an extensively used local anesthetic, commercially available in semisolid dosage forms in concentrations varying from 0.5 to 20%. Different vehicles are used in the multitude of products listing benzocaine as an active ingredient. The *in vitro* release of benzocaine from oleaginous, absorption, emulsion (water-in-oil and oil-in-water), and water-soluble ointment vehicles through a cellulose membrane to an aqueous sink was recently studied (1). It was shown that vehicle composition markedly affected the rate of release of the active ingredient.

Benzocaine is often included in hemorrhoidal products for the treatment of pain and pruritis. The drug must leave the vehicle and pass into the mucous membranes in order to act as a local anesthetic by blocking nerve conduction. After penetration, the drug is removed from the rectal tissues *via* the hemorrhoidal veins. The lower hemorrhoidal veins bypass the liver and carry absorbed drug directly into the general circulation; the upper hemorrhoidal vein connects with the portal vein leading to the liver (2).

The rate of appearance and amount of drug in the blood after rectal application should be a useful measure of the rate and amount of drug transfer from a dosage form to the sensory nerve endings in the rectal mucosa. Absorption of some local anesthetics has been reported to be dependent on the mucous surface involved (3). The rate of absorption largely depends upon the blood supply to the tissue (4).

The absorption of detectable amounts of benzocaine after application to abraded abdominal skin in dogs (3) and the ability of various benzocaine-containing preparations to obtund the itch and burning of electrical stimulation in humans (5) were reported. No comparisons of absorption of benzocaine from various formulations of semisolids through mucous membranes have been published.

The metabolic fate of local anesthetics has important clinical significance (4), but no metabolic studies of benzocaine have been reported. It may, however, follow the metabolic pathways of procaine (6, 7), which is also a local anesthetic ester. Although detoxification of most local anesthetics is accomplished almost entirely by the liver (4), procaine is rapidly hydrolyzed in human plasma. The enzymes involved are nonspecific (4) and hydrolyze different local anesthetics at various rates. For example, piperocaine was hydrolyzed 6.1 times faster than procaine, and tetracaine was hydrolyzed only 0.22 times as fast as procaine (7).

Hydrolysis of the local anesthetics in the plasma of laboratory animals was shown to be very slow (8) and dependent on the animal plasma used. In another experiment using isolated, perfused rat liver, procaine was not hydrolyzed in the rat blood but was hydrolyzed by the liver (9). Hydrolysis studies of benzocaine by the enzymes in rat blood have not been reported.

The purposes of the present investigation were to: (*a*) compare the effects of various ointment bases, with markedly different physical–chemical properties, on the rate of release of benzocaine after rectal administration in rats; (*b*) relate these results to a previously reported *in vitro* study (1); and (*c*) investigate the stability of benzocaine in whole rat blood *in vitro*.

EXPERIMENTAL

Reagents and Equipment—^{3}H-Benzocaine[1] (general label) was used as obtained. An internal standardization method was used to determine the activity of the drug as 0.067 mCi/mmole. A liquid scintillation spectrophotometer[2] was used to count all samples.

Other reagents and equipment used were: dioxane[3] (scintilAR), naphthalene[3] (purified), 70% perchloric acid[3], 30% hydrogen peroxide[3], benzene[3], dioxane[3], 2,5-diphenyloxazole (scintillation grade)[4], 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene (scintillation grade)[4], polyethylene glycol 1000[5], polyethylene glycol 4000[5], white petrolatum[6], pentobarbital sodium[7], heparin sodium injection[8] (1000 USP units/ml), polyethylene tubing[9], commercial fluor[10], acetic acid[11], chromatographic plates[12], absorption vehicle[13], water-in-oil emulsion[14], and oil-in-water emulsion[15].

Pharmacology—Female Sprague–Dawley rats, 100–280 g, were

[1] Calatomic, Los Angeles, Calif.
[2] Packard Tri-Carb liquid scintillation spectrometer model 2405 or 3375, Packard Instrument Co., Downers Grove, Ill.
[3] Mallinckrodt Chemical Works, St. Louis, Mo.
[4] Packard Instrument Co., Downers Grove, Ill.
[5] J. T. Baker Chemical Co., Phillipsburg, N.J.
[6] Matheson, Coleman and Bell, Los Angeles, Calif.
[7] City Chemical Corp., New York, N.Y.
[8] Eli Lilly Co., Indianapolis, Ind.
[9] Clay Adams, New York, N.Y.
[10] Omnifluor, New England Nuclear, Boston, Mass.
[11] Dupont.
[12] Uniplate (precoated with silica gel GF, 250 μm), Analtech, Inc.
[13] Aquaphor, Duke Laboratories, South Norwalk, Conn.
[14] Aquaphor–water (1:1).
[15] Neobase, Burroughs Wellcome and Co., Research Triangle Park, N.C.

used. Pentobarbital sodium (55 mg/kg ip) was used for induction and maintenance of general anesthesia. The trachea was intubated [polyethylene tubing, 0.139 cm (0.055 in.) i.d., 0.191 cm (0.075 in.) o.d.] to facilitate respiration. A midventral incision was made into the abdominal cavity extending from the xyphoid process to the midabdominal region. The inferior vena cava was cannulated [polyethylene tubing, 0.076 cm (0.030 in.) i.d., 0.122 cm (0.048 in.) o.d.], and the abdominal incision was closed with wound clips.

The ointments were inserted rectally from a U-80 insulin syringe with the barrel cut off at the 10-U mark. The dosage form was followed by a short, blunt, glass rod, which served as a plug to prevent leakage. A 0.1-ml blood sample was withdrawn *via* the inferior vena cava cannula before the drug was inserted and at 5, 10, 20, 40, 60, 90, 120, 240, and 300 min after insertion.

A 0.1-ml heparinized normal saline solution (7.5 units of heparin/ml) was injected through the cannula immediately after each blood sample was obtained. All blood samples were placed directly into the counting vials. When necessary, additional pentobarbital sodium (2.75 mg/kg ip) was injected to maintain anesthesia.

Sample Analysis—Total radioactivity in each blood sample was determined using a wet oxidation method (10) as follows. First, 0.1 ml of blood was placed in each counting vial. Then 0.2 ml of 70% perchloric acid was added to each sample vial for digestion, and the contents were agitated until the acid was well mixed with the sample. Then 0.4 ml of 30% hydrogen peroxide was added, and the contents were mixed again.

The vial was tightly capped to minimize evaporation of fluid during digestion and then placed in a water bath at about 70° for 60 min or until the contents of the vial became clear and colorless. After the samples were cooled, 8 ml of 2-methoxyethanol and 10 ml of toluene scintillation solution were added. The vial was capped and shaken, and a clear solution was formed.

The toluene scintillation solution consists of 4 g of a commercial fluor[10] in enough toluene to make 1 liter. All samples were counted on a liquid scintillation spectrometer[2] using the ^3H quick set and a preset time of 10 min. The counting efficiency was determined by use of external standardization (AES ratio).

Metabolism—TLC was used to separate radioactive benzocaine from its metabolites after rectal administration of 20% ^3H-benzocaine in polyethylene glycol. At 30, 60, 120, 180, 240, and 300 min, 0.1 ml of blood was withdrawn through the cannula, immediately placed in a small test tube containing 1 drop of 50% sodium arsenite solution and 1 drop of 10% saponin solution, and mixed well. The sample was then spotted on commercially available plates[12] and developed (11) in benzene–dioxane–acetic acid (90:75:8).

After solvent evaporation, the plate was examined under UV light for spot separation. Then the entire chromatographic band, from the origin to the solvent front, was divided into 1-cm successive zones, and the adsorbent of each zone was removed with a razor blade and placed in counting vials. 2-Methoxyethanol (8 ml) and commercial fluor[10] (10 ml) in toluene scintillation fluid were added and mixed. After the adsorbent completely settled in the bottom of the vials, each vial was counted for 10 min.

For the *in vitro* study of benzocaine hydrolysis, 2 ml of blood was freshly withdrawn from the inferior vena cava of the rat and placed in a 10-ml beaker containing 1 drop of heparin solution (1000 units/ml). Two drops of ^3H-benzocaine solution (50 mg/ml in alcohol) were added. At 0, 5, 30, and 60 min, 0.1 ml of blood was taken from the beaker and immediately placed in a test tube. The tube contained 1 drop of 50% sodium arsenite solution to inhibit enzyme activity (6) and 1 drop of 10% saponin solution to lyse the red blood cells, and the solution was mixed well.

The sample was then spotted as a row of small spots on a 10 × 20-cm precoated thin-layer plate[12] and developed with the three-component solvent system. The chromatoplates were examined under UV light to locate the benzocaine and its metabolites. The plates were divided and counted as already described. The same procedure was repeated with a saturated solution of ^3H-benzocaine in water and 50 mg of ^3H-benzocaine/ml in polyethylene glycol 4000. The blood was analyzed after 60 min only in the latter study, and the blood was maintained at 37° in a water bath.

Dosage Form Preparation—^3H-Benzocaine was incorporated in the vehicle with the use of a spatula and an ointment slab by geometric dilution. The ointment was loaded into a plastic disposable U-80 insulin syringe and stored for no more than 1 week in the refrigerator. The end of the syringe was cut off at the 10-U mark, and the contents were inserted directly from the modified syringe into the rectal cavity.

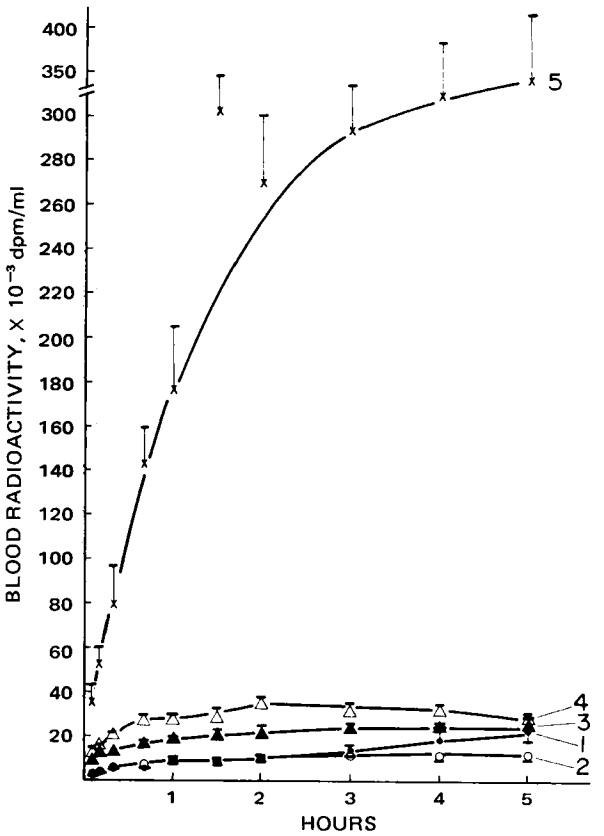

Figure 1—*Blood radioactivity after the application of 20% ^3H-benzocaine in different ointment vehicles. Key: 1, white petrolatum; 2, absorption vehicle; 3, water-in-oil emulsion; 4, oil-in-water emulsion; and 5, polyethylene glycol. The standard error of the mean is shown on one side of each point.*

RESULTS AND DISCUSSION

The rectal absorption in rats of radioactivity from 20% ^3H-benzocaine in five different ointment bases (white petrolatum, absorption vehicle, oil-in-water emulsion, water-in-oil emulsion, and water-soluble vehicle) was compared. The means of at least five animals for each formulation are shown in Fig. 1. The results of a point-by-point comparison of the means obtained at each sample time was made using the Student *t* test.

The experimental values obtained were significantly different (95% confidence level) for almost every sample time when comparing all products except Formulation 1 *versus* 2. Statistical analysis using an unequal variance technique showed that less than 5% of the variation in blood level radioactivity among different rats receiving different formulations was due to rat weight variation. No pharmacokinetic analysis was attempted since total radioactivity was measured and the method is not specific for benzocaine.

In fact, as will be seen later, no intact benzocaine was detected *in vivo*, and the radioactivity represents metabolized drug. However, total radioactivity can be used to indicate the relative rate of absorption of the drug from the various formulations, since all radioactivity in the blood originated as intact radioactive drug in the dosage forms.

For a drug to be absorbed from the rectum, it must be released from the vehicle and be distributed through the surrounding fluids where it is absorbed through the rectal mucosa. The rectum contains fluids in which water-soluble vehicles can dissolve. However, the quantity of this fluid is not sufficient to effect rapid dissolution of a suppository (12).

The release of drug from the suppository vehicle depends on the relative affinities of drug for the vehicle and the rectal fluid. Low affinity of the drug for the vehicle or ready solubility of drug in the

aqueous rectal fluid favors rapid release of drug. Conversely, high affinity of drug for the vehicle and poor drug solubility in aqueous rectal fluid decrease the rate of released drug (13). Benzocaine is known to have a low affinity for, or a low solubility in, aqueous fluids.

The five types of vehicles chosen for this experiment represent a wide range of physical–chemical properties and relative affinities for benzocaine. The relative release profiles in Fig. 1 show that the semisolid polyethylene glycol solution released the drug much more rapidly than the semisolid suspensions, even though the polyethylene glycol vehicle has a much greater affinity for the drug than the other vehicles. These results support the contention that one primary rate-limiting step in drug absorption from suppositories is the partitioning of the dissolved drug from the vehicle rather than the dissolution of the drug in the body fluids (2).

Although it is desirable that the partition coefficient of the drug favor solubility in the rectal fluids rather than in the vehicle, a drug that is very insoluble and suspended in the vehicle will be absorbed slowly due to the small concentration of dissolved drug available to partition into the rectal fluid. It follows that the onset of the local anesthetic action of benzocaine applied rectally as a suspension is likely to be slower and perhaps less intense than when applied as a solution, even though the solubilizing vehicle may have a relatively high affinity for the drug. Theoretically, the suspensions will continue to release the drug over a longer time period than the polyethylene glycol solution, but this has no significance during the first 5 hr and the significance beyond that time is unknown (Fig. 1). The relative release rates *in vivo* are in agreement with an earlier *in vitro* study (1) which measured the relative release rates of benzocaine from these vehicles.

When the radioactivity of a sample is counted, the total radioactivity is the sum of the total number of radioactive molecules of any chemical form in the sample. Generally, each metabolite formed from the drug has its own rate of formation, rate of elimination, and volume of distribution and their pharmacokinetic parameters are different from each other and from the original drug. Therefore, a thin-layer radiochromatographic method of separating benzocaine from its metabolites was investigated to determine if the total radioactivity was due to intact ^{3}H-benzocaine.

Procaine, which is structurally similar to benzocaine, has been reported (6) to be rapidly hydrolyzed by human serum cholinesterase and rat liver (9) to *p*-aminobenzoic acid. Since hydrolysis of benzocaine would also lead to *p*-aminobenzoic acid, a TLC system (11) that separated benzocaine (R_f 0.85) from *p*-aminobenzoic acid (R_f 0.70) was chosen. The R_f values of both substances were high enough to leave room to detect more polar metabolites, since *p*-aminobenzoic acid is reported to be converted to various polar conjugates (6, 11).

Periodic blood samples were collected to measure the *in vivo* degradation of benzocaine. The 30-min blood sample did not show any radioactivity above background in the zone where benzocaine should be located. The counts were high in the zone where the R_f value was approximately 0.70, the R_f value of *p*-aminobenzoic acid. For the 60-, 120-, 180-, 240-, and 300-min samples, the R_f value of the zone where the highest count was located was approximately 0.60–0.66. Another radioactive zone with an R_f value of 0.40–0.50 was present. The R_f values obtained in this study can only be approximations, because the exact positions of the spots could not be determined using scintillation. Oxidation of the silica gel to release absorbed radioactive molecules increased the counts but did not provide any further information concerning the hydrolysis of benzocaine.

The possibility that the prolonged blood level of radioactivity (Fig. 1) was due to formation of tritiated body water due to exchange of ^{3}H from the drug or its metabolites was ruled out in the following manner. Whole blood (1.0 ml) collected at different times from five rats was treated with 1 drop of 10% saponin solution to lyse the red blood cells and microdistilled at atmospheric pressure to collect 0.1 ml of aqueous material. The distillate contained less than 8% of the radioactivity of an equal volume of whole blood, indicating that little or none of the radioactivity in whole blood was due to tritiated water formed by exchange with the tritiated drug or its metabolites. From these results, it is probable that benzocaine is hydrolyzed *in vivo* in rats to *p*-aminobenzoic acid, which is then further metabolized.

An attempt was made to determine if benzocaine was being hydrolyzed by blood enzymes in the rat. Various amounts of ^{3}H-benzocaine in different solvents (see *Experimental*) were introduced into heparinized blood. At specified time intervals, the blood enzymes were inactivated, the cells were lysed, and the samples were chromatographed. The chromatoplates were scraped off and counted in a liquid scintillation spectrometer, but the distribution of the total radioactivity was not well delineated. Some zones on the plate, below the benzocaine zone, did show slightly higher counts than background but represented an extremely small amount of, if any, metabolites. Scintillation counting showed much higher counts than background only in the zone for benzocaine as detected with UV light.

Therefore, no *in vitro* hydrolysis of benzocaine was found with the method used in this study. The reason could be that (*a*) benzocaine is not hydrolyzed in rat blood, (*b*) the enzyme was inactive under the *in vitro* conditions utilized, or (*c*) the method used was not sensitive enough to detect the amount that was hydrolyzed. The first reason for the results is the most probable (7, 8). Benzocaine is clearly degraded *in vivo*, however, and the degradation probably occurs in the liver (9).

CONCLUSIONS

1. The rectal absorption and blood concentration of total radioactivity in rats following insertion of ^{3}H-benzocaine in various ointment vehicles were determined.

2. The total radioactivity absorbed rectally from the vehicles was in the following decreasing order: polyethylene glycol > oil-in-water emulsion > water-in-oil emulsion > absorption base and white petrolatum. The absorption base and white petroleum did not give significant differences in the blood level curves.

3. Relative *in vivo* results for the formulations studied here correlated well with results of an *in vitro* method reported earlier.

4. No intact benzocaine was found in the blood at the time periods studied following rectal administration of the drug.

5. *In vitro* hydrolysis of benzocaine by whole rat blood was not found. More research is needed to determine the mechanism of benzocaine degradation.

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Synthesis of 2-Pyridyl-α-toluenesulfonates as Antimalarials

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Abstract □ A series of substituted 2-pyridyl-α-toluenesulfonates was synthesized for antimalarial testing. They were prepared by treating various 2-pyridinols with α-toluenesulfonyl chlorides in the presence of an alkali. In tests against *Plasmodium berghei* in mice at 640 mg/kg, only 3,5-dichloro-2-pyridyl-α-toluenesulfonate was considered active, *i.e.*, doubled the mean survival time.

Keyphrases □ 2-Pyridyl-α-toluenesulfonates—synthesis, screened for antimalarial activity □ Antimalarials, potential—synthesis and screening of 2-pyridyl-α-toluenesulfonates

Some time ago, a series of alkanesulfonate esters of variously substituted 2-pyridinols was prepared in these laboratories (1). In connection with this earlier study, some α-toluenesulfonates also were prepared. One of these, 3,5-dichloro-2-pyridyl-α-toluenesulfonate (I), was found to be active against *Plasmodium berghei* in mice. The mean survival time (MST) for mice treated with I at 640 mg/kg was 15.8 days; for controls, it was 6.1 days. Consequently, a series of substituted 2-pyridyl-α-toluenesulfonates was prepared for antimalarial testing[1].

DISCUSSION

These compounds were prepared mainly by treatment of a 2-pyridinol[2] with an α-toluenesulfonyl chloride in toluene at 25° in the presence of a small excess of triethylamine (Scheme I). The yields were generally in the range of 30–90%. In two instances, the insolubility of the pyridinol in the toluene and triethylamine mixture necessitated the use of 1% KOH or 1% NaOH in the Schotten–Baumann procedure (Table I, XXV and XXIX).

Sulfonylation of the 2-pyridinols occurred at the oxygen in all but one case. The single exception was the product from the reaction of 5-carbethoxy-3-chloro-2-pyridinol with α-toluenesulfonyl chloride. The insolubility of this pyridinol also required the use of 1% KOH. In this case, only the *N*-substituted product was isolated, as evidenced by its IR and UV spectra (see *Experimental*).

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Four of the 2-pyridinols employed have not previously been reported (Table II). The product resulting from the chlorination of 3-methoxy-2-pyridinol at 25° in 10% hydrochloric acid (Table II, XXXII) proved to be the 6-chloro isomer, as evidenced by its NMR spectrum. Chlorination of 4-methyl-2-pyridinol [from diazotization and hydrolysis (3) of 2-amino-4-picoline] gave the previously unreported 3,5-dichloro-4-methyl-2-pyridinol (Table II, XXXIII). The intermediate 5-carbethoxy-3-chloro-2-pyridinol (Table II, XXXIV) was synthesized *via* chlorination of the reported (4) 5-carbethoxy-2-pyridinol. Attempts at chlorinating the acid (6-hydroxynicotinic acid) prior to esterification were unsuccessful.

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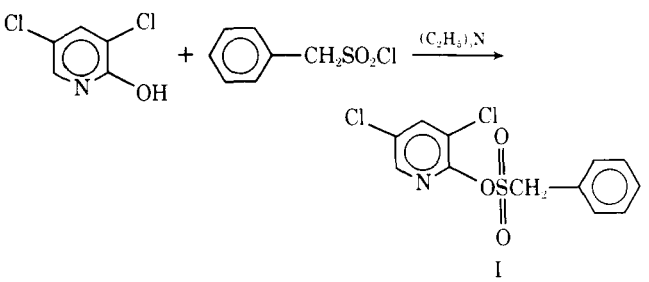

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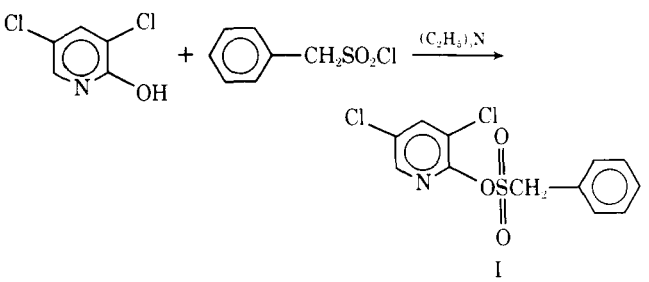

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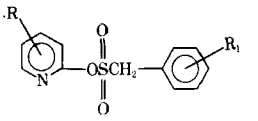


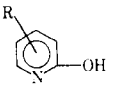
Compound	R	R_1	Melting Point	Recrystallization Solvent[a]	Yield, %	Molecular Formula	Analysis, % Calc.	Found
I	3,5-Cl_2 [b]	H	118–119°	A	61	$C_{12}H_9Cl_2NO_3S$	C 45.30	45.28
							H 2.85	3.05
							N 4.40	4.31
II	3,5-Cl_2	2-Cl	86.5–88°	B	32	$C_{12}H_8Cl_3NO_3S$	C 40.87	40.65
							H 2.29	2.32
							N 3.97	3.80
III	3,5-Cl_2	3-Cl	111.5–113.5°	B	68	$C_{12}H_8Cl_3NO_3S$	C 40.87	40.65
							H 2.29	2.28
							N 3.97	3.80
IV	3,5-Cl_2	4-Cl	69.5–71°	C	51	$C_{12}H_8Cl_3NO_3S$	C 40.87	40.55
							H 2.29	2.39
							N 3.97	3.85
V	3,5-Cl_2	2-Br	103–104.5°	B	71	$C_{12}H_8BrCl_2NO_3S$	C 36.30	36.43
							H 2.03	2.15
							N 3.53	3.33
VI	3,5-Cl_2	3-Br	106.5–107.5°	B	92	$C_{12}H_8BrCl_2NO_3S$	C 36.30	36.03
							H 2.03	2.19
							N 3.53	3.30
VII	3,5-Cl_2	4-Br	83.5–84.5°	B	24	$C_{12}H_8BrCl_2NO_3S$	C 36.30	36.07
							H 2.03	1.87
							N 3.53	3.36
VIII	3,5-Cl_2	2-F	82–83.5°	A	56	$C_{12}H_8Cl_2FNO_3S$	C 42.87	43.03
							H 2.40	2.69
							N 4.17	4.12
IX	3,5-Cl_2	3-F	120.5–121°	B	56	$C_{12}H_8Cl_2FNO_3S$	C 42.87	42.90
							H 2.40	2.61
							N 4.17	4.01
X	3,5-Cl_2	4-F	80–88° [c]	A	15	$C_{12}H_8Cl_2FNO_3S$	C 42.87	42.70
							H 2.40	2.47
							N 4.17	4.14
XI	3,5-Cl_2	2-CF_3	65–66.5°	A	52	$C_{13}H_8Cl_2F_3NO_3S$	C 40.43	40.19
							H 2.09	2.36
							N 3.63	3.47
XII	3,5-Cl_2	3-CF_3	84–86°	B	62	$C_{13}H_8Cl_2F_3NO_3S$	C 40.43	40.61
							H 2.09	2.06
							N 3.63	3.61
XIII	3,5-Cl_2	4-CF_3	97–105° [c]	A	60	$C_{13}H_8Cl_2F_3NO_3S$	C 40.43	40.27
							H 2.09	2.08
							N 3.63	3.58
XIV	3,5-Cl_2	2,4-Cl_2	102–103°	B	57	$C_{12}H_7Cl_4NO_3S$	C 37.24	37.09
							H 1.82	1.87
							N 3.62	3.56
XV	3,5-Cl_2	2,6-Cl_2	126–127°	B	36	$C_{12}H_7Cl_4NO_3S$	C 37.24	37.08
							H 1.82	1.82
							N 3.62	3.50
XVI	3,5-Cl_2	3,4-Cl_2	137–138°	B	61	$C_{12}H_7Cl_4NO_3S$	C 37.24	37.14
							H 1.82	1.82
							N 3.62	3.53
XVII	3,5-Cl_2	2,4,5-Cl_3	129.5–131°	A	38	$C_{12}H_6Cl_5NO_3S$	C 34.19	34.30
							H 1.44	1.67
							N 3.32	3.35
XVIII	3,5-Cl_2	2,3,4,5,6-F_5	117–118°	B	66	$C_{12}H_4Cl_2F_5NO_3S$	C 35.30	35.16
							H 0.99	1.23
							N 3.43	3.49
XIX	3,5-Cl_2	3-CH_3	69–71°	A	62	$C_{13}H_{11}Cl_2NO_3S$	C 47.00	47.10
							H 3.34	3.35
							N 4.22	4.11
XX	3,5-Cl_2	4-CH_3	80–80.5°	A	45	$C_{13}H_{11}Cl_2NO_3S$	C 47.00	47.10
							H 3.34	3.38
							N 4.22	4.19
XXI	3-Br, 5-Cl	H	117–118°	B	48	$C_{12}H_9BrClNO_3S$	C 39.75	39.91
							H 2.50	2.48
							N 3.86	3.84
XXII	5-Br, 3-Cl [d]	H	121–122°	A	66	$C_{12}H_9BrClNO_3S$	C 39.75	39.70
							H 2.50	2.55
							N 3.86	3.67
XXIII	3,5-Br_2	H	126–126.5°	A	34	$C_{12}H_9Br_2NO_3S$	C 35.40	35.53
							H 2.23	2.36
							N 3.44	3.35
XXIV	5-Cl, 3-CH_3	H	135.5–136°	D	72	$C_{13}H_{12}ClNO_3S$	C 52.44	52.53
							H 4.06	3.96
							N 4.70	4.71

Table I— *Continued*

Compound	R	R_1	Melting Point	Recrystallization Solvent[a]	Yield, %	Molecular Formula	Analysis, % Calc.	Found
XXV	6-Cl, 3-OCH_3 [e]	H	131.5–132.5°	D	17	$C_{13}H_{12}ClNO_4S$	C 49.77 H 3.86 N 4.47	49.69 3.95 4.58
XXVI	3-Cl, 5-NO_2	H	102.5–103°	A	24	$C_{12}H_9ClN_2O_5S$	C 43.85 H 2.76 N 8.52	43.96 2.89 8.59
XXVII	5-Cl, 3-NO_2	H	103–104°	A	71	$C_{12}H_9ClN_2O_5S$	C 43.85 H 2.76 N 8.52	43.90 2.88 8.46
XXVIII	3,5-Cl_2, 4-CH_3 [f]	H	134–135°	A	61	$C_{13}H_{11}Cl_2NO_3S$	C 47.00 H 3.34 N 4.22	47.34 3.28 4.31
XXIX	3,5-Cl_2, 6-CH_3 [g]	H	110.5–111.5°	—[h]	17	$C_{13}H_{11}Cl_2NO_3S$	C 47.00 H 3.34 N 4.22	46.92 3.43 4.11
XXX	3,5,6-Cl_3	H	123–124°	A	76	$C_{12}H_8Cl_3NO_3S$	C 40.87 H 2.29 N 3.97	41.20 2.32 3.77

[a] A = isopropanol, B = cyclohexene, C = cyclohexane, and D = ethyl acetate. [b] NMR ($CDCl_3$): δ 4.93 (s, 2H, CH_2), 7.47 (m, 5H, phenyl), 7.85 (d, 1H, pyridyl, J_{AB} = 2.5 Hz), and 8.52 (d, 1H, pyridyl, J_{AB} = 2.5 Hz) ppm. [c] Single spot on TLC (silica gel, hexane). [d] NMR ($CDCl_3$): δ 4.91 (s, 2H, CH_2), 7.43 (m, 5H, phenyl), 7.95 (d, 1H, pyridyl, J_{AB} = 2.5 Hz), and 8.52 (d, 1H, pyridyl, J_{AB} = 2.5 Hz) ppm. [e] Prepared using 1% NaOH as the base: NMR ($CDCl_3$ + CD_3SOCD_3): δ 3.86 (s, 3H, OCH_3), 4.90 (s, 2H, CH_2), 7.45 (m, 5H phenyl), 7.45 (d, 1H, pyridyl, J_{AB} = 2.5 Hz), and 7.90 (d, 1H, pyridyl, J_{AB} = 2.5 Hz) ppm. [f] NMR ($CDCl_3$): δ 2.51 (s, 3H, CH_3), 4.91 (s, 2H, CH_2), 7.47 (m, 5H, phenyl), and 8.24 (s, 1H, pyridyl) ppm. [g] Prepared using 1% KOH as the base; NMR ($CDCl_3$): δ 2.62 (s, 3H, CH_3), 4.93 (s, 2H, CH_2), 7.44 (m, 5H, phenyl), and 7.80 (s, 1H, pyridyl). [h] Purified on silica gel column, eluting with benzene–hexane (1:1).

Table II—Unreported 2-Pyridinols



Compound	R	Melting Point	Recrystallization Solvent[a]	Yield, %	Molecular Formula	Analysis, % Calc.	Found
XXXI	5-Br, 3-Cl	199–201°	A	73	$C_5H_3BrClNO$	C 28.81 H 1.45 N 6.72	28.81 1.62 6.69
XXXII	6-Cl, 3-OCH_3 [b]	196–197°	B	48	$C_6H_6ClNO_2$	C 45.16 H 3.79 N 8.78	44.97 3.76 8.83
XXXIII	3,5-Cl_2, 4-CH_3 [c]	192–193.5°	B[d]	53	$C_6H_5Cl_2NO$	C 40.48 H 2.83 N 7.87	40.70 2.66 7.91
XXXIV	5-$CO_2C_2H_5$, 3-Cl[e]	178–178.5°	C	39	$C_8H_8ClNO_3$	C 47.66 H 4.00 N 6.95	47.89 4.15 6.92

[a] A = isopropanol, B = ethanol, and C = butanone. [b] NMR ($CDCl_3$ + CD_3SOCD_3): δ 3.8 (s, 3H, CH_3), 6.7 (d, 1H, pyridyl), 7.0 (d, 1H, pyridyl), and 13.2 (diffuse, 0.6H, N—H) ppm. [c] NMR ($CDCl_3$): δ 2.48 (s, 3H, CH_3), 7.47 (s, 1H, pyridyl), and ~12.5 (diffuse, 1H, N—H) ppm. [d] Analytical sample was recrystallized from 95% ethanol; previously from ethyl acetate. [e] NMR ($CDCl_3$): δ 1.37 (t, 3H, CH_3), 4.32 (q, 2H, CH_2), 8.11 (d, 1H, pyridyl), and 8.15 (d, 1H, pyridyl) ppm.

EXPERIMENTAL

Activity—This series of compounds was tested[3] for blood schizonticidal antimalarial activity according to a literature method (12).

Only one compound, 3,5-dichloro-2-pyridyl-α-toluenesulfonate (I), was considered active, *i.e.*, gave an increase in mean survival time of at least 100% (+6.1 days) in mice infected with *P. berghei*. It gave an increase in mean survival time of 9.7 days at 640 mg/kg. Two other compounds, 3,5-dichloro-2-pyridyl-4-chloro-α-toluenesulfonate (Table I, IV) and 3,5-dichloro-4-methyl-2-pyridyl-α-toluenesulfonate (Table I, XXVIII), caused significant prolongation of life with infected mice, but the increases were not sufficient for them to be considered active. They increased the mean survival time by 5.6 (92%) and 4.9 (80%) days, respectively, at 640 mg/kg.

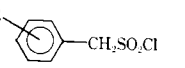
Chemistry—A single detailed example is given for the preparation of each of the two types of intermediates and also of an α-toluenesulfonate ester and an *N*-sulfonated product. In cases where the experimental procedure differed markedly from the examples given, the details are given in the table footnotes.

3-Bromo-α-toluenesulfonyl Chloride—To a boiling solution of 3-bromobenzyl bromide[4] (20.0 g, 0.08 mole) in 50 ml of 95% ethanol was added a solution of sodium sulfite, 10.1 g (0.08 mole), in 50 ml of water, dropwise with stirring over about 0.5 hr. The mixture was heated at reflux for 64 hr and cooled. The crystals were removed by filtration, washed with benzene, and dried under vacuum over phosphorus pentoxide, giving 13.7 g (63%) of dried sodium salt. All of this material (0.05 mole) was treated with 40 ml of

[3] Rane Laboratory, University of Miami.

[4] In most of the cases, the benzyl chloride was used.

Table III—Unreported α-Toluenesulfonyl Chlorides



Compound	R	Melting Point	Recrystallization Solvent[a]	Yield, %	Molecular Formula	Analysis, % Calc.	Found
XXXV	3-Br	88.5–89.5°	A	62	$C_7H_6BrClO_2S$	C 31.20	31.38
						H 2.24	2.28
XXXVI	4-Br	108–109°	A	49	$C_7H_6BrClO_2S$	C 31.20	31.11
						H 2.24	2.58
XXXVII	4-F	25–30°[b]	—	14	$C_7H_6ClFO_2S$	C 40.29	39.89
						H 2.90	2.83
XXXVIII	2-CF_3	37–39°	A	43	$C_8H_6ClF_3O_2S$	C 37.14	36.75
						H 2.34	2.15
XXXIX	3-CF_3	98–100°	—	62	$C_8H_6ClF_3O_2S$	C 37.14	36.79
						H 2.34	2.37
XL	4-CF_3	57–58°	A	59	$C_8H_6ClF_3O_2S$	C 37.14	36.83
						H 2.34	2.44
XLI	3-CH_3	50°	B	70[c]	$C_8H_9ClO_2S$	C 46.91	46.90
						H 4.43	4.50
XLII	4-CH_3	33°	B	33[c]	$C_8H_9ClO_2S$	C 46.91	46.93
						H 4.43	4.44
XLIII	2,6-Cl_2	47–50°	A	43	$C_7H_5Cl_3O_2S$	C 32.40	32.49
						H 1.94	1.90
XLIV	2,3,4,5,6-F_5	103° (3.5)[d]	—	16	$C_7H_2ClF_5O_2S$	C 29.96	30.09
						H 0.72	0.63

[a] A = isopropanol, and B = cyclohexane. [b] Distilled at 120° (2.5 mm). [c] Treatment of the sodium α-toluene sulfonate with phosphorus oxychloride was carried out at 60° instead of the usual 105°. [d] Boiling point (mm).

phosphorus oxychloride by heating at reflux for 18 hr.

The excess phosphorus oxychloride was removed under vacuum, and the residue was treated with ice and then extracted with ether. The ether extract was washed with water and then with sodium bicarbonate solution and dried over magnesium sulfate. The ether was removed by evaporation, and the residue was recrystallized from 2-propanol, giving 8.5 g (62%) of white solids, mp 88.5–89.5°.

5-Bromo-3-chloro-2-pyridinol—Into a solution of 5-bromo-2-pyridinol (20.8 g, 0.12 mole) in 70 ml of 12% hydrochloric acid was bubbled chlorine (8.5 g, 0.12 mole) over 30 min. Water was added to keep the mixture stirrable as solids precipitated. After the addition, the mixture was stirred another hour, and the solids were filtered, washed with water, and dried *in vacuo* to give 18.2 g (73%) of white solids, mp 195–200°.

Two recrystallizations from isopropanol raised the melting point to 199–201.5°.

3,5-Dichloro-2-pyridyl-α-toluenesulfonate—α-Toluenesulfonyl chloride (31.5 g, 0.165 mole) in 200 ml of toluene was added to a solution of 3,5-dichloro-2-pyridinol (27.0 g, 0.165 mole) in 100 ml of toluene and triethylamine (20.0 g, 0.20 mole) at 25° with cooling in 15 min. The mixture was stirred at 25° for 1 hr and filtered to remove triethylamine hydrochloride, which was washed with fresh toluene (150 ml). The washings were collected with the filtrate.

Excess triethylamine was removed by distilling about 375 ml, and this volume of fresh toluene was added back before washing, first with 4% sodium hydroxide and then with 1% acetic acid. The toluene was removed under vacuum, and the residue was stirred with 100 ml of cyclohexane after crystallization commenced. The mixture was filtered, and the solids were dried and purified by recrystallization from ethanol, giving 32.2 g (61%) of white crystals, mp 117–118°. The analytical sample was purified by another recrystallization from ethanol, mp 118–118.5°.

5-Carbethoxy-3-chloro-1-(α-toluenesulfono)-2-pyridone—α-Toluenesulfonyl chloride (3.4 g, 0.02 mole) in 80 ml of toluene was added to a solution of 5-carbethoxy-3-chloro-2-pyridinol (3.6 g, 0.02 mole) in 1% potassium hydroxide solution over 10 min at 22°. The mixture was then stirred for an additional 0.5 hr, while sufficient 5% potassium hydroxide solution was added to maintain the pH at 9–10. The reaction mixture was then filtered to remove unreacted 2-pyridinol (0.5 g, 14%). The aqueous layer was separated and acidified, yielding an additional 1.0 g (28%) of starting material.

The toluene layer was washed once with dilute sodium hydroxide and then with cold water until neutral and evaporated under vacuum. The crude solids left on evaporation were purified on a silica gel column by eluting first with hexane–benzene (3:1) to remove unreacted α-toluenesulfonyl chloride, then with mixtures of these solvents progressively richer in benzene, and finally with benzene alone to yield 0.7 g (11%) of white solids, mp 130–131°; NMR (deuterochloroform): δ 1.32 (t, 3H, CH_3), 4.29 (q, 2H, CH_2), 5.13 (s, 2H, CH_2), 7.35 (s, 5H, phenyl), 8.04 (d, 1H, pyridone, J_{AB} = 2.5 Hz), and 8.27 (d, 1H, pyridone, J_{AB} = 2.5 Hz, J_{AX} = 14 Hz) ppm; IR (potassium bromide): λ_{max} 5.8, 5.9, 7.1, 7.35, 7.85, 8.6, 11.2, and 13.2 μm; UV (methanol): λ 265 (ε 12.3 × 10^3) and 300 (ε 5.9 × 10^3) nm.

Anal.—Calc. for $C_{15}H_{14}ClNO_5S$: C, 50.64; H, 3.97; N, 3.94. Found: C, 51.07; H, 4.09; N, 3.88.

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[x] To whom inquiries should be directed.

Determination of Tolmetin in Human Plasma by GLC and Spectrophotometric Procedures

WILLIAM A. CRESSMAN ˣ, BARBARA LOPEZ, and DARRELL SUMNER *

Abstract □ GLC and spectrophotometric methods for the determination of tolmetin in plasma were developed. The methods can detect tolmetin in concentrations above 0.5 μg/ml. The plasma levels obtained with a 600-mg dose of tolmetin to a human subject are reported.

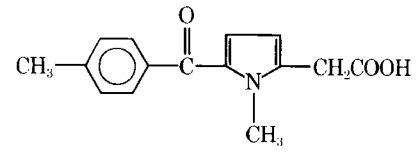
Keyphrases □ Tolmetin—GLC and spectrophotometric analyses, plasma □ GLC—analysis, tolmetin in plasma □ Spectrophotometry—analysis, tolmetin in plasma

The pyrrole derivative 1-methyl-5-*p*-toluoylpyrrole-2-acetic acid, tolmetin[1] (I), is a new anti-inflammatory agent currently undergoing clinical trials in rheumatoid arthritis (1, 2). GLC and spectrophotometric assays were developed to evaluate the absorption, distribution, and excretion of tolmetin in human subjects. These procedures were used to evaluate dose–response relationships, bioavailability, and the potential drug interaction between tolmetin and aspirin. The spectrophotometric assay has primary utility in bioavailability and other studies conducted under controlled conditions. The GLC assay has broader utility for monitoring blood levels of tolmetin in clinical therapy.

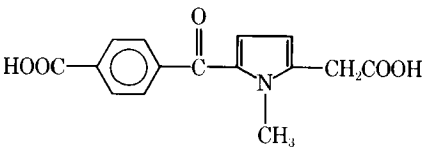
The major tolmetin metabolite in humans (3) is 5-(*p*-carboxybenzoyl)-1-methylpyrrole-2-acetic acid (II).

EXPERIMENTAL

Reagents—Citric acid buffer was prepared by dissolving 210 g of citric acid in 1000 ml of water and adjusting the pH to 2.5 with 5 *N* NaOH. Phosphate buffer was prepared by dissolving 69 g of monobasic sodium phosphate in 1000 ml of water and adjusting to pH 7.0 with 5 *N* NaOH.



I



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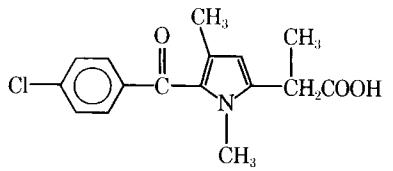

III

[1] Tolectin, McNeil Laboratories.

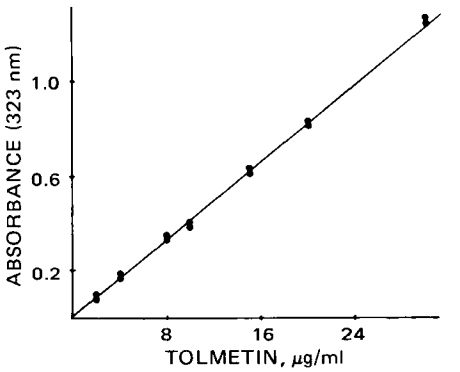

Figure 1—*Standard curve for the spectrophotometric determination of tolmetin in plasma (blank absorbance = 0.03 absorbance unit).*

The GLC internal standard (III) was dissolved in ether to give a concentration of 0.5 μg/ml. Diazomethane was prepared by adding 50 mg of *N*-nitrosomethylurea to 5 ml of ether and 3 ml of 40% KOH. The resulting diazomethane–ether solution was dried over potassium hydroxide pellets prior to use.

Spectrophotometric Assay—*Step 1*—To a 3-ml plasma sample in a 30-ml (1-oz) disposable glass screw-capped bottle with a polyethylene-lined cap were added 1 ml of water, 3 ml of pH 2.5 citric acid buffer, and 20 ml of 1% isopentyl alcohol in methylcyclohexane. The bottle was shaken for 20 min on a wrist-action shaker.

Step 2—Eighteen milliliters of the 1% isopentyl alcohol–methylcyclohexane layer was transferred to a clean 30-ml (1-oz) bottle, 2.5 ml of pH 7.0 phosphate buffer was added, and the mixture was shaken for 20 min.

Step 3—The 1% isopentyl alcohol–methylcyclohexane layer was removed and discarded, 2 ml of ether was added to the phosphate buffer layer, and the mixture was shaken for 15 min.

Step 4—The ether layer was then removed and discarded. An additional 2 ml of ether was added, the mixture was shaken for 15 min, and the new ether layer was removed and discarded.

Step 5—The absorbance of the pH 7.0 phosphate buffer layer was read at 323 nm against a pH 7.0 phosphate buffer blank. A plasma blank was also run, and each sample absorbance reading

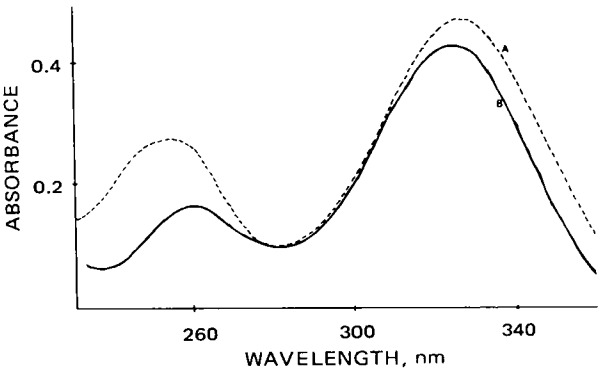

Figure 2—*Spectrum of 10-μg/ml solutions of tolmetin (B) and the tolmetin metabolite (A) in pH 7.0 buffer.*

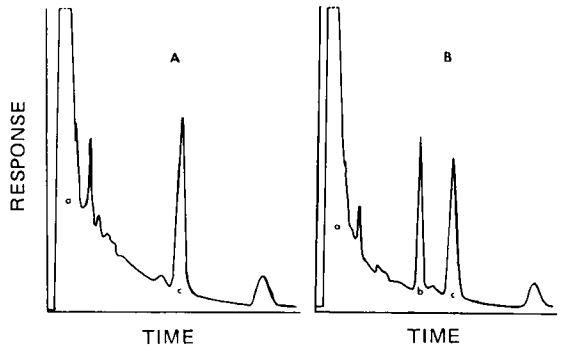

Figure 3—*Chromatograms of a blank human plasma sample (A) and a plasma sample containing 16 μg/ml of tolmetin (B). Key: a, solvent front; b, tolmetin methyl esther peak; and c, internal standard (III) methyl ester peak.*

was corrected for the blank (absorbance observed was usually 0.01–0.02 absorbance unit).

GLC Assay—*Instrumental Conditions*—A gas chromatograph[2] equipped with a flame-ionization detector was used. The column was a 1.8-m (6-ft) × 7-mm o.d. (4-mm i.d.) glass coil packed with 3% OV-1 on 60 – 80 - mesh Gas Chrom Q[3]. The injection port was maintained at 265°, the column was maintained at 200°, and the detector was maintained at 310°. Nitrogen carrier gas was used at a flow rate of 60 ml/min. The column was silylated[4] regularly each morning prior to use.

Under these conditions, the following retention times were observed: tolmetin (I) (methyl ester), 6 min; internal standard (III) (methyl ester), 8.5 min; and tolmetin metabolite (II) (dimethyl ester), > 13 min.

Sample Preparation—The first four steps of sample preparation are similar to those noted for the spectrophotometric assay with the following minor changes. In Step 1, only 1 ml of plasma was required; in Step 2, 5 ml of buffer was used; and in Steps 3 and 4, 3 ml of ether was used. The assay was then continued as follows.

The ether layer was removed and discarded; 4.5 ml of the aqueous layer (pH 7 phosphate buffer) was transferred to a clean 30-ml (1-oz) bottle, and the pH was adjusted to ~1 with 3.0 ml of 1 *N* HCl. Then 20 ml of the ethereal internal standard solution was added and shaken for 20 min.

Eighteen milliliters of the ether layer was removed and evaporated to about 2 ml. The sample was placed in a hood, and 2–3 ml of the anhydrous, ethereal diazomethane solution was added (sample should retain a yellow color). Additional reagent was added if the yellow color was dissipated, and the mixture was allowed to stand for 10 min or until the bubbling stopped. The excess diazomethane was evaporated with nitrogen in a hood until the sample turned colorless. The sample was evaporated to dryness using nitrogen and a warm water bath (30–50°). At this point, the sample was stored in a refrigerator.

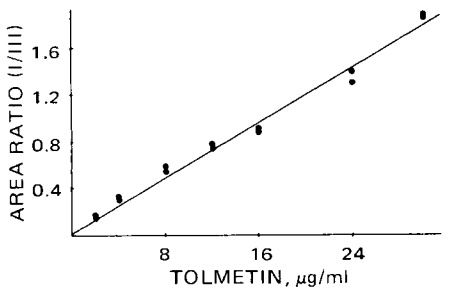

Figure 4—*Standard curve for the GLC determination of tolmetin in plasma.*

[2] Perkin-Elmer model 900.
[3] Applied Science Labs., State College, Pa.
[4] Silyl-8, Pierce Chemical Co., Rockford, Ill.

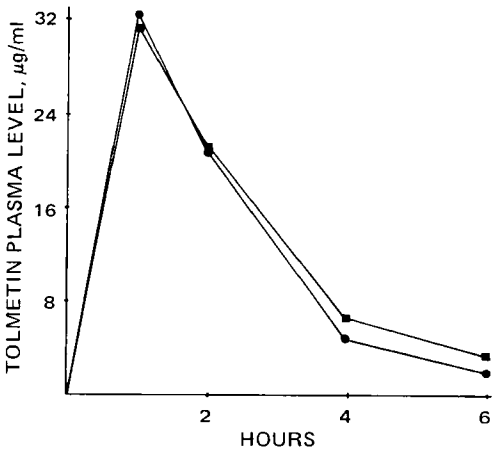

Figure 5—*Plasma and urine levels of tolmetin in a human subject administered 600 mg of tolmetin as an oral solution. Key: ●, GLC assay; and ■, spectrophotometric assay. One hundred percent of the radioactivity from an oral dose of ^{14}C-tolmetin is excreted in the urine within 24 hr.*

The dried residue was dissolved in 30 μl of methanol, and the sides of the tube were washed down with methanol. One-tenth of the sample (3 μl) was injected into the gas chromatograph using a 1-μl methanol plug in the syringe behind the sample. The appropriate attenuation setting for a 30-μg/ml plasma sample is 1×10^{-10} amp full-scale deflection.

In these studies, with this and other GLC plasma assays, the results were always more reproducible when the internal standard was added in the last step prior to evaporation or derivatization, *i.e.*, to monitor derivative formation and/or injection variables. In most cases, addition of the internal standard in earlier steps resulted in increased variability, which appears to increase with the number of extraction steps involved in the assay.

Standard Curves—Standard samples were prepared by dissolving tolmetin sodium dihydrate in water at appropriate concentrations so that 1 ml of the seeded solution could be used in place of the 1 ml of water that is added to each sample in Step 1 of the assay. The standard samples were prepared using heparinized human plasma from an untreated subject.

RESULTS AND DISCUSSION

Spectrophotometric Assay—A typical standard curve for the spectrophotometric assay, using duplicate seeded samples, is shown in Fig. 1. The lower limit of detection of the assay is 0.5 μg/ml. Samples of urine carried through the procedure gave low blank absorbance values (0.02 absorbance unit at 323 nm). Since II (the major metabolite of tolmetin in human subjects) also shows an absorption maximum at 323 nm with approximately the same absorptivity (Fig. 2), the potential interference of this compound was evaluated.

Less than 2% of the initial amount of II was recovered in the final assay layer. Studies with ^{14}C-tolmetin have shown that plasma levels of II are always less than 50% of the levels of tolmetin and that the plasma half-life of II is similar to that of tolmetin[5]. Therefore, no significant interference from II is expected.

GLC Assay—Using the chromatographic conditions described, tolmetin has a retention time of 6 min. A typical gas chromatogram of plasma extracted with and without the addition of tolmetin is shown in Fig. 3. The use of larger volumes of plasma to increase the sensitivity of the procedure was explored, but this procedure was accompanied by an equivalent increase in background "noise" and/or minor interfering peaks and was not pursued further. The dimethyl ester of II has a retention time greater than 13 min and, therefore, should not interfere in the assay. In addition, only 2% of the II seeded into plasma was carried through the assay.

A typical standard curve for the GLC assay, using duplicate seeded samples, is shown in Fig. 4. The lower limit of detection

[5] Data on file at McNeil Laboratories, Inc.

Table I—Urine Levels (Percent of Dose)

Method	0–12 hr	12–25 hr
GLC	2.9	0.31
UV	2.4	0.25

used in this procedure is 0.5 μg/ml. A similar GLC assay designed to measure both I and II was reported recently (4).

Interference of Aspirin and Salicylic Acid—Since tolmetin often could be used with large doses of aspirin, the interference of aspirin or salicylic acid in the spectrophotometric and GLC assay for tolmetin was evaluated. Plasma was seeded with 500 μg/ml[6] of aspirin or 500 μg/ml of salicylic acid and assayed by both procedures.

No interference was observed from either aspirin or salicylic acid with the GLC assay, but significant interference was observed in the spectrophotometric assay. For 500 μg/ml of aspirin, the spectrophotometric assay gave an absorption equivalent to ~10 μg/ml of tolmetin. For 500 μg/ml of salicylic acid, the assay gave absorption equivalent to 50 μg/ml of tolmetin. Since the levels of tolmetin at therapeutic doses (400–600 mg) are between 10 and 30 μg/ml, the spectrophotometric assay would not be suitable for the determination of plasma levels of tolmetin in patients who are simultaneously receiving aspirin products.

With the spectrophotometric assay, one can easily assay 36 samples/day. With the GLC assay, using a dual-column gas chromatograph and manual injection, analytical productivity is reduced by approximately half. Therefore, the spectrophotometric assay offers significant economic advantages over the GLC assay in controlled situations, *e.g.*, bioavailability studies (6). Because of the greater specificity of the GLC procedure, it offers greater utility in the assay of plasma levels of tolmetin in clinical practice (6).

[6] The 500-μg/ml level of aspirin or salicylic acid is at least two to three times the level expected from a 4.5-g dose of aspirin per day (5).

Comparison of the Two Analytical Procedures—To evaluate the applicability of the assay procedures, plasma and urine samples from a fasted subject dosed with 600 mg of tolmetin as an oral solution were assayed using both methods. The results are shown in Fig. 5. Studies with ^{14}C-tolmetin (3) showed that 100% of the administered dose of radioactivity is excreted in the urine within 24 hr. These radioactive studies also demonstrated that only 2–3% of the administered dose is excreted in the urine as intact drug. Therefore, the urine assay (Table I) provides a severe test of both assay procedures. The agreement of the urine and plasma assays by both methods, as well as the agreement of the results with data from other studies using radioactive and nonradioactive tolmetin (3, 4), validates the utility of both assay procedures.

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GLC Determination of Procainamide in Biological Fluids

K. J. SIMONS and R. H. LEVY [x]

Abstract □ A GLC method for the determination of procainamide in biological fluids is presented. By using a dipropyl analog of procainamide as an internal standard, both compounds can be chromatographed directly, yielding linear calibration curves and a sensitivity that allows quantitative determination of concentrations as low as 0.1 μg/ml. The extraction procedure was carefully modified to avoid hydrolysis of *N*-acetylprocainamide, a major metabolite of procainamide. The usefulness of the procedure is demonstrated by following the disappearance of procainamide from the plasma and urine of human subjects treated with the drug.

Keyphrases □ Procainamide—GLC analysis, biological fluids, therapeutic range □ GLC—analysis, procainamide in biological fluids

The effectiveness of procainamide[1] (I) in the prevention or treatment of cardiac arrhythmias has been firmly established (1, 2). Careful clinical studies demonstrated that plasma levels of procainamide may be correlated with pharmacological responses. The range of therapeutic plasma concentrations is 4–8 μg/ml (2–4).

Due to individual differences in absorption and elimination of procainamide, dosages necessary for therapeutic effects may vary widely (2). Several methods are presently available for the determination of procainamide plasma concentrations within the therapeutic range.

The most commonly used method of determining procainamide in biological fluids is a colorimetric method (1) where, following complexation with *N*-(1-naphthyl)ethylenediamine, absorbance is read at 550 nm. This method has been used by many investigators (2, 4–11) and has been shown to have satisfactory precision above 2–3 μg. However, its specificity recently has been questioned. Gibson *et al.* (12) reported that *N*-acetylprocainamide, the major metabolite in humans (5), is hydrolyzed to procainamide under the conditions of the procedure of Mark *et al.* (1).

Spectrophotofluorometric detection is also possible

[1] Pronestyl, E. R. Squibb & Sons. The chemical name is *p*-amino-*N*-[2-(diethylamino)ethyl]benzamide hydrochloride.

Table I—Urine Levels (Percent of Dose)

Method	0–12 hr	12–25 hr
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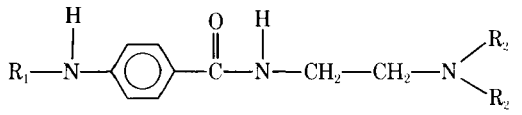

I: procainamide, $R_1 = H$, $R_2 = -CH_2CH_3$
II: internal standard, $R_1 = H$, $R_2 = -CH_2CH_2CH_3$
III: *N*-acetylprocainamide, $R_1 = CH_3C(=O)-$, $R_2 = -CH_2CH_3$

since procainamide fluoresces at 385 nm following excitation at 295 nm at pH 11 (13). This method has been useful (14–17) and successfully compared to the colorimetric method (2), but it retains the limitations of sensitivity and specificity.

A GC method for procainamide determinations was developed (18) but shows nonlinearity in the calibration curve at low concentrations as a result of adsorption to active sites on the column and/or packing material. Karlsson (14) used GC for the determination of procainamide in urine, but the method has not been published.

Sterling *et al.* (19) developed a GC method using polyamide incorporated onto the packing to reduce adsorption. They also developed a fluorometric method which involves coupling with fluorescamine to enhance the fluorescence of procainamide. Both methods were tested with pooled human plasma spiked with procainamide to concentrations as low as 2 μg/ml and with plasma from patients chronically maintained on procainamide.

However, to conduct safe, low-dose pharmacokinetic studies with procainamide in normal subjects (where many blood samples have to be drawn), it is necessary to have an assay that requires 1 or at most 2 ml of plasma and is sensitive at levels below 1 μg/ml. A GLC method was developed in this laboratory in which adsorption is minimized to yield linearity and sensitivity to levels as low as 0.1 μg/ml. Furthermore, the extraction procedure is modified to eliminate the hydrolysis of *N*-acetylprocainamide.

EXPERIMENTAL

Reagents—Procainamide and the internal standard, a dipropyl analog of procainamide, *p*-amino-*N*-[2-(dipropylamino)ethyl]-benzamide hydrochloride (II), mp 188–190°, were used[2]. Stock solutions of procainamide contained 2, 5, 10, 15, and 20 μg/ml for plasma and 10, 25, 50, 75, and 100 μg/ml for urine in water. Stock solutions of the internal standard contained 5, 10, 25, and 50 μg/ml in water. Solutions of phthalate buffer (pH 3) (20) and 2 *N* Na_2CO_3 buffer (pH 10.8) also were used.

All solvents were reagent grade, except for chloroform which was spectral grade. *N*-Acetylprocainamide (III), mp 189–190°, was synthesized according to the method of Dreyfuss *et al.* (6). GC and colorimetric analysis showed it to be 95% pure with 5% unchanged procainamide.

Extraction of Plasma Standards and Specimens from Dosed Subjects—*Control Plasma Standards*—The extraction procedure is a modification of the approach proposed by Mather and Tucker (21) for basic amine drugs. To 1 ml of pooled control plasma samples in 15-ml Pyrex culture tubes, fitted with Teflon-lined screw caps, were added 0.5 ml of aqueous procainamide solution, 0.5 ml of the appropriate internal standard solution, and 0.5 ml of carbonate buffer (pH 10.8).

The samples were extracted with 8 ml of ether by shaking at high speed on a reciprocating shaker for 5 min. Then sufficient sodium chloride was added to saturate the aqueous layer completely. The samples were shaken again at a low speed for 30 sec and at high speed for 5 min, followed by centrifugation at 2000 rpm for 20 min. This shaking procedure minimizes emulsion formation from the plasma protein–ether mixture.

The samples were then frozen in an acetone–carbon dioxide bath, the ether layers were poured into 15-ml test tubes, and the aqueous layer was discarded. After the addition of 0.2 ml of phthalate buffer (pH 3), each sample was mixed[3] for 60 sec and centrifuged as before for 5 min. The samples were frozen again, and the ether layers were discarded. Then the samples were heated in a water bath at 50° for 10 min to thaw the aqueous layer and evaporate remaining traces of ether.

The aqueous layers were poured from the test tubes into 1-ml conical centrifuge vials[4]. To each vial were added 0.1 ml of carbonate buffer (pH 10.8) and sufficient sodium chloride to saturate the aqueous layer; then the vials were mixed[3] for 30 sec. Samples were extracted with 10 μl of water-saturated chloroform by high speed shaking for 20 min, followed by centrifugation at 2000 rpm for 20 min. From the chloroform bead formed in the conical tip of the vial, 1–3 μl was injected directly into the gas chromatograph.

Plasma Samples from Dosed Subjects—Appropriate aliquots (0.5–2 ml) of plasma were pipetted into 15-ml tubes. Following addition of the internal standard, the samples were extracted by the same procedure as was used for the plasma standards.

Extraction of Urine Standards and Specimens from Dosed Subjects—*Control Urine Samples*—Procainamide concentrations in urine are about 10 times that found in plasma, and significant protein is not usually present. Therefore, the plasma assay was simplified for urine samples by omitting the ether extraction. Calibration curves were prepared by adding 0.1-ml samples of both procainamide and internal standard stock solutions and 0.1 ml of carbonate buffer (pH 10.8) directly into the 1-ml vials[4]. Sufficient sodium chloride was added to saturate the aqueous layer, followed by mixing[3] for 30 sec. Samples were then extracted with 10 μl of water-saturated chloroform by high speed shaking for 20 min, followed by centrifugation at 2000 rpm for 20 min. From the chloroform bead formed in the conical tip of the vial, 1–3 μl was injected directly into the gas chromatograph.

Urine Samples from Dosed Subjects—A 0.1-ml sample of urine, suitably diluted to contain between 1 and 10 μg of procainamide, was pipetted directly into the 1-ml vials[4]. To each sample was added 0.1 ml of internal standard solution and then the same extraction procedure was used as for the urine standards.

GLC—GLC was carried out on a gas chromatograph[5] equipped with a flame-ionization detector. Glass columns (coiled 0.91 m × 0.63 cm, 2 mm i.d.), packed with 10% OV-7-coated Gas Chrom Q[6] (60–80 mesh), were maintained for isothermal chromatography at 245° with the injector at 280° and the detector at 300°. Helium, the carrier gas, was maintained at 40 ml/min, with hydrogen and air at 30 and 450 ml/min, respectively. Under these conditions, procainamide, the internal standard, and *N*-acetylprocainamide have retention times of ~3, ~4, and ~9 min, respectively (Fig. 1).

To prolong column life, temperatures were reduced at night. Prior to raising temperatures at the beginning of a run and before igniting, the columns were conditioned with 30 μl of a trimethylsilyl mixture[7]. With these precautions, columns were used for up to 8 months.

The first sample each day was injected two or three times to ensure reproducibility and column stability. Where possible, samples were usually injected in order of decreasing drug concentration, and larger volumes were injected into the gas chromatograph as drug concentrations decreased.

Calculations—Peak height ratios were calculated by dividing the height of the procainamide peak by the height of the internal standard peak. Calibration curves were constructed daily from the

[2] Provided by Dr. T. Q. Spitzer, E. R. Squibb and Sons, New Brunswick, N.J.

[3] Vortex mixer, Scientific Products, American Hospital Supply Corp., Evanston, Ill.

[4] Solu-vials, Chemical Research Services, Addison, IL 60101

[5] Aerograph 200, Varian Aerograph, Walnut Creek, CA 94598. It was equipped with a Speedomax W recorder, Leeds and Northrup, Philadelphia Pa.

[6] Applied Science Labs, State College, PA 16801

[7] Silyl 8, Pierce Chemical Co., Rockford, IL 61105

Table I—Calibration Curve and Precision Data for Procainamide Determination from Spiked Plasma Samples

Procainamide Added, μg[a]	Peak Height Ratio[b]	Procainamide Found, μg[b]
1.0	0.225 ± 0.022	0.98 ± 0.09
2.5	0.572 ± 0.021	2.50 ± 0.09
5.0	1.137 ± 0.054	4.97 ± 0.23
7.5	1.717 ± 0.112	7.50 ± 0.49
10.0	2.282 ± 0.132	9.96 ± 0.58

[a] $n = 6$. [b] Mean ± SD.

results of spiked pooled control plasma samples by plotting peak height ratios against concentration of procainamide (micrograms per milliliter).

GLC–Mass Spectrometry—A gas chromatograph–mass spectrometer[8], fitted with a Gohlke separator, was used to check the identity and purity of the procainamide, internal standard, and *N*-acetylprocainamide peaks in a control aqueous solution and in plasma and urine samples. The ionizing energy was 70 ev.

RESULTS AND DISCUSSION

GLC—Figure 1 shows typical chromatograms obtained after addition of 1 and 10 μg of procainamide to pooled plasma samples and a chromatogram of a patient urine sample showing procainamide, internal standard, and *N*-acetylprocainamide peaks. Procainamide and the internal standard gave well-resolved, symmetrical peaks with no tailing. The *N*-acetylprocainamide peak, although well resolved, was broad and tailing, which makes quantification more difficult under these chromatographic conditions.

The identity and purity of the peaks were confirmed by mass spectrometry. The spectra and fragmentation patterns were comparable to those reported previously (17, 18).

The linearity of the system was established by analyzing the results of six calibration curves assayed simultaneously (Table I). The calibration curve constructed from the mean values had the linear regression equation $y = 0.229x - 0.002$ ($r = 1.000$). The precision of this method was determined from these data; for amounts ranging from 1 to 10 μg, coefficients of variation ranged from 3.6 to 9.5%. The standard deviation of the slope of nine routine calibration curves over 8 months was 4.0%.

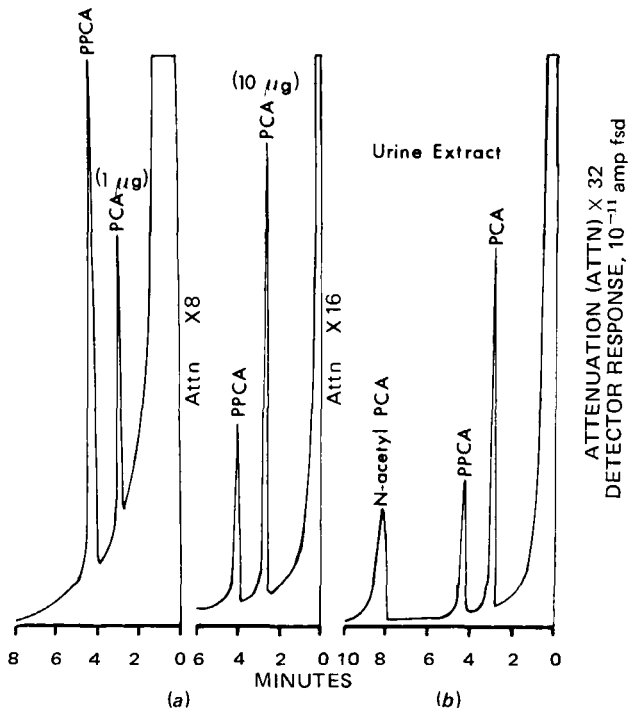

Figure 1—(a) *Gas chromatograms of procainamide (PCA) and internal standard (PPCA), showing 1 and 10 μg of drug extracted from spiked human plasma samples.* (b) *Chromatogram of a urine sample from a patient given 3.5 mg/kg iv showing procainamide (PCA), internal standard (PPCA), and* N*-acetylprocainamide* (N*-acetyl PCA*).

[8] Finnigan model 3000.

Table II—Recovery of Procainamide from Spiked Plasma and Urine Samples

	Plasma (Acid/Base Wash)		Urine (Single Extraction)	
Procainamide Added, μg/ml[a]	Procainamide Found, μg/ml	Recovery, %	Procainamide Found, μg/ml	Recovery, %
1.0	0.35	34.67	0.86	85.67
2.5	0.98	38.68	2.29	91.72
5.0	1.82	36.34	3.74	74.80
7.5	2.80	37.29	6.55	87.33
10.0	3.95	39.47	7.49	74.93
		37.29 ± 1.90		82.89 ± 7.65

[a] $n = 2$.

The standard deviation of the peak height ratio of the 1-μg sample of the same nine calibration curves over 8 months was 7.2%. The absolute peak height of a 1-μg sample from 1 ml of plasma was as high as 90 mm. Levels as low as 0.1 μg/ml, using 2-ml aliquots, could be measured. This sensitivity was made possible by using a small volume (10 μl) of chloroform in the final extraction. Although amounts of 0.2–0.5 μg were not included in the daily calibration curve, experience with duplicate determinations showed a variability of less than 10%. Duplicate calibration curves using 1 and 2 ml of plasma had the same slope, demonstrating that the extraction efficiency was not affected by increasing the plasma volume to 2 ml.

The recovery of procainamide from spiked plasma samples was determined to be 37.3%, and the recovery from urine samples was 82.8% (Table II). The low recovery in the double-extraction procedure is due to several factors: (*a*) the unfavorable solvent ratio of 8 ml of ether to 0.2 ml of phthalate buffer (pH 3), (*b*) the transfer of this small volume from test tubes to the vials, and (*c*) the necessity of two extractions at pH 10.8 [procainamide pKa 9.23 (2)] to prevent hydrolysis of *N*-acetylprocainamide.

However, in spite of the low absolute recovery, the overall precision of the method was satisfactory and comparable to that obtained by other GLC methods (18, 19). The variability of recovery shown in Table II is larger than that suggested by Table I, because in this instance the internal standard was not added until the final extraction step was completed and the extraction steps were not made quantitative.

Chromatographic Adsorption—The problem of procainamide adsorption onto the column and/or support during GLC is adequately documented (18, 19). Two approaches generally are used to prevent drug adsorption: (*a*) formation of a derivative of the drug, by which means the active sites on the drug are protected; and (*b*) use, if possible, of a combination of column supports and stationary phases that eliminates active sites for adsorption.

In the present work, it was possible to form derivatives of both procainamide and the internal standard with dimethylformamide dimethyl acetal[9], but the resulting derivatives still showed adsorption characteristics. Benzoyl derivatives, synthesized and chromatographed, also revealed that the adsorption problem was not resolved. Attempts were made to use base-washed column supports and amine packings, including 10% Apiezon L plus 2% KOH[10] and 28% Penwalt 223 plus 4% KOH[6]. When using these stationary phases, it was not possible to develop narrow peaks even at maximum stationary phase temperatures.

The approach of Meijer (22) was then used in which the percent of the stationary phase (of optimum polarity) is increased, the par-

[9] Methyl-8, Pierce Chemical Co., Rockford, IL 61105
[10] Supelco, Inc., Bellefonte, PA 16823

Table III—Hydrolysis of *N*-Acetylprocainamide

N-Acetylprocainamide Added, μg/ml	Condition	Hours	Procainamide Found, μg/ml	Hydrolysis, %
	At 50°[a]			
80	1 *N* HCl	8	60.29	77.48
80	pH 1.3	10	11.75	11.03
80	pH 2.8	32	0.0	0.0
80	pH 4.95	40	0.0	0.0
80	pH 7.0	40	0.0	0.0
80	pH 9.0	32	0.0	0.0
80	pH 9.98	20	0.0	0.0
80	1 *N* NaOH	8	64.31	82.97
Procainamide Added, μg/ml				
80	1 *N* HCl	8	79.26	0.92
80	1 *N* NaOH	8	80.31	0.0
N-Acetylprocainamide Added, μg/ml	At 25°[a]			
5	1 *N* HCl	18	1.44	26.49
10	1 *N* HCl	18	2.90	26.66
20	1 *N* HCl	18	5.62	25.70
				26.28 ± 0.51

[a] Determined by measuring the appearance of procainamide using this GLC method for samples at 50° and the colorimetric method (4) for samples at 25°.

ticle size of the support is also increased, thereby decreasing the surface area, and the column length is shortened. The percent of OV-17[6] was increased from 3 to 10% on 80–100-mesh support, yielding improved peaks and a reduced solvent front but little effect on adsorption. Ten percent OV-17[6] on 60–80 mesh also gave little improvement. Increasing the percent of this phase to 20% on 60–80-mesh[6] support yielded no improvement over the 10%, and the column temperature had to be raised to obtain usable peaks.

A switch to an OV-1[6] phase allowed the use of lower temperatures, and adsorption was decreased when column length was reduced to 0.91 m. A 10% OV-7[6] phase showed optimum polarity and reduced adsorption to the extent that the calibration curves became linear with negligible intercepts (less than 0.1 μg) (Table I).

Specificity—Elson *et al.* (23) found that patients maintained on procainamide therapy often have plasma levels of *N*-acetylprocainamide several times higher than those of procainamide. In addition, Gibson *et al.* (12) reported on the potential of hydrolysis of *N*-acetylprocainamide during the colorimetric analysis of procainamide using the method of Mark *et al.* (1). Therefore, an assessment of the specificity of any procedure is dependent on an understanding of the conditions of hydrolysis of *N*-acetylprocainamide.

A study of the hydrolysis of *N*-acetylprocainamide in 1 *N* HCl revealed 77.5% hydrolysis in 8 hr at 50° and 26.3% hydrolysis in 18 hr at 25°. In 1 *N* NaOH, 83.0% hydrolysis of *N*-acetylprocainamide was found in 8 hr at 50°. Control studies on procainamide showed no hydrolysis in either 1 *N* HCl or 1 *N* NaOH in 8 hr at 50°. There was, however, no hydrolysis of *N*-acetylprocainamide between pH 3 and 10 for up to 40 hr at 50° (Table III). Therefore, buffers at pH 3 (phthalate buffer) and pH 10.8 (2 *N* Na_2CO_3) were substituted for the 2 *N* HCl and 2 *N* NaOH solutions, respectively, used in the procedure of Mather and Tucker (21). Analysis of solutions of *N*-acetylprocainamide by the proposed method showed no more procainamide present than control solutions of *N*-acetylprocainamide injected directly into the gas chromatograph without extraction.

Application—This method was used to follow the disappearance of procainamide from the plasma and urine of seven normal human subjects given 4 mg/kg iv. The plasma concentration *versus* time curves showed that all plasma procainamide concentrations following the first 0.5 hr postadministration were less than 1 μg/ml and that values fell to below 0.1 μg/ml in less than 6 hr after the dose.

CONCLUSIONS

This GLC method for the determination of procainamide in biological fluids minimizes the adsorption problem so that concentrations as low as 0.1 μg/ml can be quantitatively determined. Also, it has the added advantage of specificity, thus eliminating the interference of *N*-acetylprocainamide. With respect to monitoring procainamide concentrations in the therapeutic range, this procedure is slightly more tedious than other currently available GLC methods. However, with its added sensitivity, it is useful when only small volumes of plasma are available.

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Molecular Connectivity I: Relationship to Nonspecific Local Anesthesia

LEMONT B. KIER *x, LOWELL H. HALL *§, WALLACE J. MURRAY *, and MILAN RANDIĆ ‡

Abstract □ A very significant linear correlation was found between a recently proposed connectivity index and molecular polarizability, cavity surface areas calculated for water solubility of alcohols and hydrocarbons, and biological potencies of nonspecific local anesthetics. The simplicity of calculation of the index from the connectivity in the molecular skeleton, together with the very significant correlation, indicates its practical value.

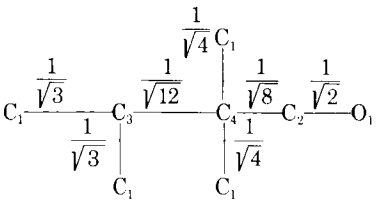
Keyphrases □ Molecular connectivity—correlation with polarizability, cavity surface areas, and biological potencies, hydrocarbons, alcohols, nonspecific local anesthetics □ Polarizability—correlation with molecular connectivity, hydrocarbons, alcohols, nonspecific local anesthetics □ Cavity surface area—correlation with molecular connectivity, hydrocarbons, alcohols, nonspecific local anesthetics □ Anesthetics, nonspecific local—correlation between molecular connectivity and polarization, cavity surface areas, and biological potencies

Recent studies in these laboratories on fundamental aspects of drug–receptor interactions have considered, as part of the total energy, the long-range forces of dispersion and polarization (1–4). These forces are predicted to be dominant in interaction phenomena involving molecules with nonpolar to moderately dipolar structures. The predicted energies of interaction involving these forces between model compounds, simulating all or parts of the drug and receptor molecules, were shown to relate to the quantitative biological response of several series of compounds (1–4).

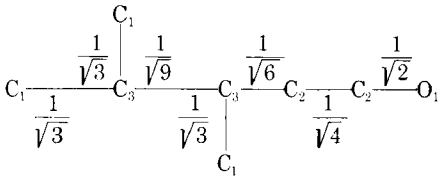
A fundamental property governing the dispersion and polarization energies of interaction is the polarizability of atoms in a molecule (5). The polarizability of bonds between these atoms is derived from this property (6). Close relationships have indeed been found directly between calculated bond and molecular polarizabilities and the extent of a biological response (1, 7).

The atomic polarizability relates to the volume of the atom; hence, the total molecular polarizability should bear a relationship to some topological property of a molecule (8). In a recent study, Randić (9) devised an index relating to a topological property of a hydrocarbon: the degree of branching or connectivity in the molecule. He showed that this index has a linear relationship to certain physical properties in a series of molecules, including boiling points, the Kovats constant, and empirical parameters dealing with vapor pressure in the Antoine equation.

It is possible that this simply derived index may be fundamental to a topological property governing physical and, in turn, biological properties, which in some areas may be influenced by molecular volume or surface. The purpose of this study was to test the relationship between this index and a biological property relating to polarizability; a very significant correlation was found. Subsequent papers in this series will show that a very close relationship exists with other molecular properties such as water solubility of organic compounds, partition coefficients, molar refractivity, parachor, and physicochemical and thermodynamic properties of the linear free energy type.



2,2,3-trimethylbutanol, $\chi = 3.504$



3,4-dimethylpentanol, $\chi = 3.681$

Scheme I—Calculation of connectivity indexes for two isomers of heptanol

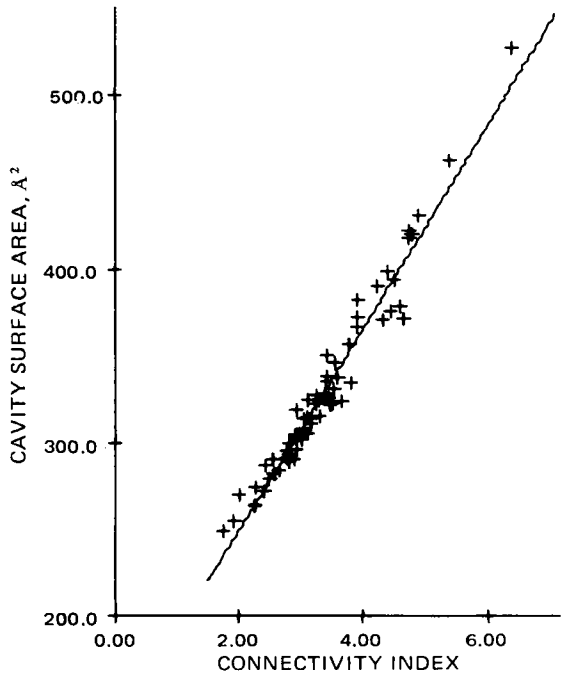


Figure 1—*Computer-drawn plot of the cavity surface area* versus *the connectivity index for 51 aliphatic alcohols and 18 nonaromatic hydrocarbons.*

Table I—Cavity Surface Area (CSA) and Connectivity Index (χ)

Compound	χ	CSA	CSA Predicted
1-Butanol	2.414	272.1	273.8
2-Methyl-1-propanol	2.269	263.8	265.6
2-Butanol	2.269	264.1	265.6
1-Pentanol	2.914	303.9	303.1
3-Methyl-1-butanol	2.769	291.4	294.9
2-Methyl-1-butanol	2.807	289.4	297.2
2-Pentanol	2.769	295.9	294.9
3-Pentanol	2.807	293.5	297.2
3-Methyl-2-butanol	2.641	284.3	287.2
2-Methyl-2-butanol	2.561	282.5	282.5
1-Hexanol	3.414	335.7	332.4
2-Hexanol	3.270	327.7	324.2
3-Hexanol	3.307	325.3	326.5
3-Methyl-3-pentanol	3.122	305.8	315.4
2-Methyl-2-pentanol	3.061	314.3	311.9
2-Methyl-3-pentanol	3.179	314.3	318.9
3-Methyl-2-pentanol	3.179	311.3	318.9
2,3-Dimethyl-2-butanol	2.943	301.2	304.8
3,3-Dimethyl-1-butanol	3.061	307.5	311.9
3,3-Dimethyl-2-butanol	2.943	296.7	304.8
4-Methyl-1-pentanol	3.269	323.0	324.2
4-Methyl-2-pentanol	3.124	314.9	315.4
2-Ethyl-1-butanol	3.346	308.6	311.9
Cyclohexanol	2.893	290.5	301.9
1-Heptanol	3.914	367.5	361.7
2-Methyl-2-hexanol	3.561	346.1	341.2
3-Methyl-3-hexanol	3.622	337.7	344.7
3-Ethyl-3-pentanol	3.683	324.4	348.2
2,3-Dimethyl-2-pentanol	3.481	323.8	336.5
2,3-Dimethyl-3-pentanol	3.504	321.8	337.6
2,4-Dimethyl-2-pentanol	3.416	328.6	332.9
2,4-Dimethyl-3-pentanol	3.552	331.7	340.6
2,2-Dimethyl-3-pentanol	3.481	326.1	336.5
3-Heptanol	3.807	357.1	355.8
4-Heptanol	3.807	357.1	355.8
1-Octanol	4.414	399.4	391.0
2,2,3-Trimethyl-3-pentanol	3.811	335.2	355.8
2-Octanol	4.269	391.0	382.8
2-Ethyl-1-hexanol	4.345	371.3	387.5
1-Nonanol	4.914	431.2	420.3
2-Nonanol	4.769	423.2	412.1
3-Nonanol	4.807	420.8	414.4
4-Nonanol	4.807	420.8	414.4
5-Nonanol	4.807	420.8	414.4
2,6-Dimethyl-4-heptanol	4.520	394.0	397.4
3,5-Dimethyl-4-heptanol	4.628	379.3	403.9
2,2-Diethyl-1-pentanol	4.743	372.5	406.8
7-Methyl-1-octanol	4.769	418.7	388.0
3,5,5-Trimethyl-1-hexanol	4.454	376.6	393.3
1-Decanol	5.414	463	449.6
1-Dodecanol	6.414	527	508.2
n-Butane	1.914	255.2	244.7
2-Methylpropane	1.732	249.1	234.0
n-Pentane	2.414	287.0	274.0
2-Methylbutane	2.270	274.6	265.6
3-Methylpentane	2.807	300.1	297.0
Neopentane	2.000	270.1	249.7
2,2-Dimethylbutane	2.561	290.8	282.6
2,4-Dimethylpentane	3.125	324.7	315.7
2,2,4-Trimethylpentane	3.417	338.9	332.8
2,2,5-Trimethylhexane	3.917	373.0	362.1
Cyclohexane	2.500	279.1	279.0
Methylcyclohexane	2.893	304.9	302.1
1-*cis*-2-Dimethylcyclohexane	3.304	315.5	326.2
Cycloheptane	3.000	301.9	308.3
Cyclooctane	3.500	322.6	337.6
n-Hexane	2.914	319	303.3
n-Heptane	3.414	351	332.6
n-Octane	3.914	383	361.9

EXPERIMENTAL

Calculation of Connectivity Index—The index is derived from the numerical extent of branching or connectivity in the molecular skeleton. The first row atoms of a molecule are dealt with in an identical manner. The hydrogen atoms are suppressed. The degree of bond saturation is ignored in this study but will be considered later.

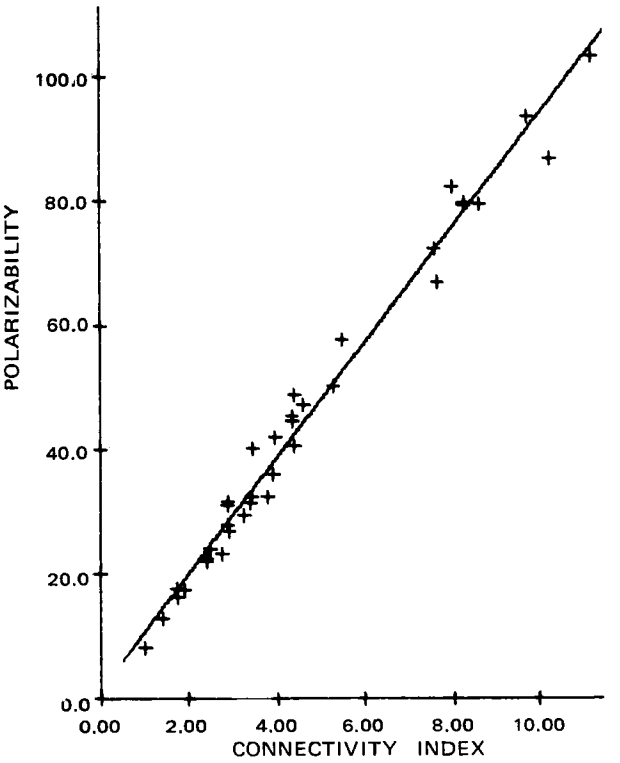


Figure 2—*Computer-drawn plot of the molecular polarizability* versus *the connectivity index for 36 compounds used in a nonspecific anesthesia study.*

The structure is drawn out in skeletal form. Each atom is assigned a number corresponding to the number of atoms attached, *i.e.*, 1, 2, 3, or 4 in the case of a carbon atom. Hydrogen atoms are not included in the count. A number is derived for each bond by calculating the product of the numbers associated with the two

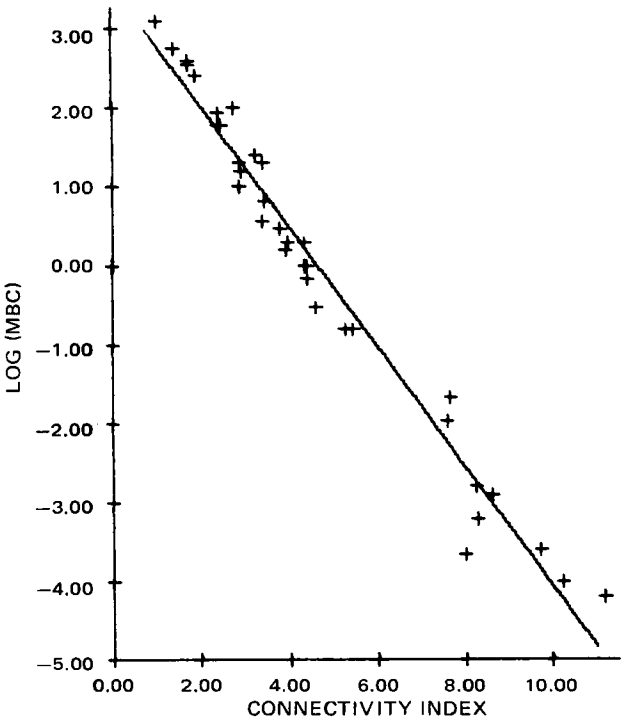


Figure 3—*Computer-drawn plot of the log of the minimum blocking concentration* versus *the connectivity index for 36 compounds used in a nonspecific anesthesia study.*

Table II—Polarizability (α), Minimum Blocking Concentration (MBC), and Connectivity Index (χ) of Local Anesthetics

Anesthetic	χ	α	Predicted α	Log MBC, mM	Predicted Log MBC
Methanol	1.000	8.2	10.9	3.09	2.79
Ethanol	1.414	12.9	14.7	2.75	2.47
Acetone	1.732	16.2	17.7	2.6	2.23
2-Propanol	1.732	17.6	17.7	2.55	2.23
1-Propanol	1.914	17.5	19.3	2.4	2.09
Urethan	2.769	23.2	27.3	2.0	1.44
Ether	2.414	22.5	24.0	1.93	1.71
Butanol	2.414	22.1	24.0	1.78	1.71
Pyridine	2.500	24.1	24.8	1.77	1.65
Hydroquinone	3.288	29.4	32.1	1.4	1.05
Aniline	2.893	31.6	28.4	1.3	1.35
Benzyl alcohol	3.432	32.5	33.4	1.3	0.935
Pentanol	2.914	26.8	28.6	1.2	1.33
Phenol	2.893	27.8	28.4	1.0	1.35
Toluene	2.893	31.1	28.4	1.0	1.35
Benzimidazole	3.466	40.2	33.7	0.81	0.901
Hexanol	3.414	31.4	33.2	0.56	0.949
Nitrobenzene	3.804	32.5	36.8	0.47	0.651
Quinoline	3.966	42.1	38.3	0.3	0.528
8-Hydroxyquinoline	4.376	44.7	42.1	0.3	0.215
Heptanol	3.914	36.0	37.9	0.2	0.567
2-Naphthol	4.359	45.4	42.0	0.0	0.228
Methyl anthranilate	4.423	48.9	42.6	0.0	0.182
Octanol	4.414	40.6	42.5	−0.16	0.186
Thymol	4.608	47.3	44.3	−0.52	0.038
o-Phenanthroline	5.448	57.8	52.1	−0.8	−0.602
Ephedrine	5.253	50.2	50.3	−0.8	−0.453
Procaine	7.668	67.0	72.6	−1.67	−2.29
Lidocaine	7.578	72.5	71.8	−1.96	−2.22
Diphenhydramine	8.270	79.5	78.2	−2.8	−2.75
Tetracaine	8.629	79.7	81.5	−2.9	−3.03
Phenyltoloxamine	8.254	79.9	78.1	−3.2	−2.74
Quinine	9.705	93.8	91.5	−3.6	−3.85
Physostigmine	7.969	82.4	75.4	−3.66	−2.52
Caramiphen	10.224	87.0	96.3	−4.0	−4.24
Dibucaine	11.182	103.6	105.2	−4.2	−4.97

atoms of the bond. The reciprocal of the square root of this number is then computed and becomes the bond value. Finally, the bond values are summed to give a number, called the connectivity index (χ) for the molecule. Two examples of this process are illustrated in Scheme I.

The two molecules, isomers differing only in their branching, yield different connectivity indexes, which are also different from the index for 1-heptanol, 3.914. It appears, then, that the degree of branching is also related to the connectivity index.

Cyclic structures possess one additional bond for each ring relative to the chain isomer. The value of one bond must be subtracted for each ring. For cyclohexane, the summation yields 3.000, from which $1/\sqrt{2 \times 2}$ must be subtracted to yield $\chi = 2.500$. Aromatic molecules are treated in the same way in this study. Thus, benzene has $\chi = 2.500$ while acetanilide has $\chi = 4.287$. Molecules that can ionize are considered in their neutral state.

Correlation of Solvent Cavity Surface Area with Connectivity Index—In studies of hydrocarbon solubility in water, Hermann (10) developed a method for calculating the surface area inside the idealized solvent cavity required to accommodate the hydrocarbon solute. The calculated cavity surface contains the centers of the water molecules in the first layer around the solute. Since this cavity area has the properties of an envelope of enclosure, and hence depends upon the connectivity of the molecular skeleton, it was felt that it may be related to the connectivity index.

These studies found a highly significant correlation between the calculated cavity surface area (CSA) and the connectivity index for 18 hydrocarbons (branched or straight chain). Cyclohexanol is the only cyclic alcohol included. In computing the connectivity index, the quantity 0.5 is subtracted for each ring in the cyclic compounds; *e.g.*, for methylcyclohexane, $\chi = 3.393 - 0.500$.

The regression equation, correlation coefficient, and standard deviation are:

$$\text{CSA} = 133.4 + 58.24\chi \qquad \text{(Eq. 1)}$$
$$r = 0.978 \quad s = 11.2 \quad n = 69$$

The plot of the cavity surface area *versus* the connectivity index is shown in Fig. 1, and the supporting data are presented in Table I.

The second paper in this series discusses the relationship between the connectivity index and the water solubility of hydrocarbons and aliphatic alcohols (11), a property whose fundamental relationship to the cavity surface area was explored by Hermann (10).

Correlation of Molecular Polarizability with Connectivity Index—The polarizability (α) of a molecule may be derived as a sum of atomic refraction constants (7). To explore further how the connectivity index may be related to physical properties of biological importance, the dependence of polarizability on the connectivity index was considered, and a high degree of correlation between these quantities was found.

In Fig. 2 the polarizability is plotted against the connectivity index, and the supporting data are displayed in Table II. The regression equation, correlation coefficient, and standard deviation are:

$$\alpha = 1.60 + 9.26\chi \qquad \text{(Eq. 2)}$$
$$r = 0.990 \quad s = 3.59 \quad n = 36$$

Because of the high degree of correlation found between the connectivity index and polarizability, the relationship of the connectivity index to nonspecific anesthesia, a property thought to be related to molecular polarizability (7), was examined.

Correlation between Local Anesthetic Potency and Connectivity Index—Agin *et al.* (7) compiled and obtained experimentally the minimum blocking concentrations (MBC) of almost 40 molecules on isolated nerve or muscle fiber. They measured the minimum concentration required to excite cells in a standardized set of conditions (Table II). The relationship between the log MBC and the connectivity index is shown in Fig. 3. The regression equation, correlation coefficient, and standard deviation are:

$$\log \text{MBC} = 3.55 - 0.762\chi \qquad \text{(Eq. 3)}$$
$$r = 0.983 \quad s = 0.390 \quad n = 36$$

Acetanilide and aminopyrine were not included from the Agin *et al.* data, because they made an unexplained correction in the activ-

ity for these molecules. Chloroform was not included because of some uncertainties about the chlorine parameter.

DISCUSSION

Two observations come boldly into view: the correlations of the connectivity index with cavity surface area, polarizability, and biological activity are very good, suggesting a fundamental significance for the connectivity index; and the connectivity index value for each molecule is very simply derived, almost by inspection, from no more than four numbers (1, 2, 3, and 4) describing connections.

The mathematical derivation of the connectivity index is extremely simple and is based on discrimination of bond types in a molecule according to the number of its nearest neighbors. The physical significance of the connectivity index is more fundamental than may appear. The individual connectivity index values reflect molecular connectivity and present ultimate fragmentation of the molecular skeleton. Any function of a molecule can be represented as a power expansion of bond contributions. At this initial stage, attention was confined to the linear term of such bond additive schemes.

One may look at connectivity index values as distinctive weighting of bond contributions. It is very significant that the weighting is based solely on the valency of vertexes of molecular graphs and, hence, is nonempirical.

It is plausible to expect that the connectivity index relates to such molecular properties as enclosure volume and surface area. The predictive value of this simply computed index is demonstrated to the biological scientist. In subsequent papers, additional correlations will be described.

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Molecular Connectivity II: Relationship to Water Solubility and Boiling Point

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Abstract □ The connectivity index, easily computed by arithmetic and based upon the degree of connectedness at each vertex in the molecular skeleton, is shown to give highly significant correlations with water solubility of branched, cyclic, and straight-chain alcohols and hydrocarbons as well as with boiling points of alcohols. These correlations are superior to those based on well-founded theory relating to solvent cavity surface area. An empirical modification to the connectivity index gave an improved correlation for both solubilities and boiling points.

Keyphrases □ Molecular connectivity—correlation with water solubility and boiling point, hydrocarbons, alcohols □ Solubility—correlation with molecular connectivity, hydrocarbons, alcohols □ Boiling point—correlation with molecular connectivity, hydrocarbons, alcohols

It has long been known that branched- and straight-chain organic molecules differ in their properties. For example, branched-chain alcohols and hydrocarbons generally have lower boiling points and higher solubilities than the corresponding straight-chain isomers. However, until the recent suggestion by Randić (1), there was no simply computed quantitative scheme for correlating physicochemical data with such topological characteristics as branching. The earlier ideas of Hosoyea (2) and Smolenski (3) have not received acceptance as means for correlating properties. The Hosoyea index shows only a qualitative correlation, and the Smolenski additivity function becomes complex to apply.

Since the manner in which organic groups are connected to form a molecule appears to influence molecular properties, the relationship between a connectivity index and various physicochemical and biological properties was studied. Success in correlating the connectivity index (χ) with polarizability and biological activity of certain nonspecific local anesthetics was reported in Part I (4).

The great utility of a simply computed index that could be used to predict physicochemical properties led to the study of the water solubilities of aliphatic alcohols and hydrocarbons. By applying the significant structure theory to the solubilities of these compounds, several studies showed that the log of the solubilities correlates well with the calculated surface area of the cavity required to accommodate the solute in the solvent water (5, 6).

In Part I, a highly significant correlation of the cavity surface area to the connectivity index was shown (4). This paper explores the correlation of the

ity for these molecules. Chloroform was not included because of some uncertainties about the chlorine parameter.

DISCUSSION

Two observations come boldly into view: the correlations of the connectivity index with cavity surface area, polarizability, and biological activity are very good, suggesting a fundamental significance for the connectivity index; and the connectivity index value for each molecule is very simply derived, almost by inspection, from no more than four numbers (1, 2, 3, and 4) describing connections.

The mathematical derivation of the connectivity index is extremely simple and is based on discrimination of bond types in a molecule according to the number of its nearest neighbors. The physical significance of the connectivity index is more fundamental than may appear. The individual connectivity index values reflect molecular connectivity and present ultimate fragmentation of the molecular skeleton. Any function of a molecule can be represented as a power expansion of bond contributions. At this initial stage, attention was confined to the linear term of such bond additive schemes.

One may look at connectivity index values as distinctive weighting of bond contributions. It is very significant that the weighting is based solely on the valency of vertexes of molecular graphs and, hence, is nonempirical.

It is plausible to expect that the connectivity index relates to such molecular properties as enclosure volume and surface area. The predictive value of this simply computed index is demonstrated to the biological scientist. In subsequent papers, additional correlations will be described.

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Molecular Connectivity II: Relationship to Water Solubility and Boiling Point

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Abstract □ The connectivity index, easily computed by arithmetic and based upon the degree of connectedness at each vertex in the molecular skeleton, is shown to give highly significant correlations with water solubility of branched, cyclic, and straight-chain alcohols and hydrocarbons as well as with boiling points of alcohols. These correlations are superior to those based on well-founded theory relating to solvent cavity surface area. An empirical modification to the connectivity index gave an improved correlation for both solubilities and boiling points.

Keyphrases □ Molecular connectivity—correlation with water solubility and boiling point, hydrocarbons, alcohols □ Solubility—correlation with molecular connectivity, hydrocarbons, alcohols □ Boiling point—correlation with molecular connectivity, hydrocarbons, alcohols

It has long been known that branched- and straight-chain organic molecules differ in their properties. For example, branched-chain alcohols and hydrocarbons generally have lower boiling points and higher solubilities than the corresponding straight-chain isomers. However, until the recent suggestion by Randić (1), there was no simply computed quantitative scheme for correlating physicochemical data with such topological characteristics as branching. The earlier ideas of Hosoyea (2) and Smolenski (3) have not received acceptance as means for correlating properties. The Hosoyea index shows only a qualitative correlation, and the Smolenski additivity function becomes complex to apply.

Since the manner in which organic groups are connected to form a molecule appears to influence molecular properties, the relationship between a connectivity index and various physicochemical and biological properties was studied. Success in correlating the connectivity index (χ) with polarizability and biological activity of certain nonspecific local anesthetics was reported in Part I (4).

The great utility of a simply computed index that could be used to predict physicochemical properties led to the study of the water solubilities of aliphatic alcohols and hydrocarbons. By applying the significant structure theory to the solubilities of these compounds, several studies showed that the log of the solubilities correlates well with the calculated surface area of the cavity required to accommodate the solute in the solvent water (5, 6).

In Part I, a highly significant correlation of the cavity surface area to the connectivity index was shown (4). This paper explores the correlation of the

Table I—Boiling Points of Alcohols and Water Solubilities of Alcohols and Hydrocarbons *versus* Connectivity Index (χ)

Compound	χ	Tb_{obs}[a]	Tb_{calc}[b]	S_{obs}[a], molal	S_{calc}[c], molal
1-Butanol	2.414	117.7°	116.6°	1.006	0.748
2-Methylpropanol	2.269	107.9°	111.0°	1.023	1.079
2-Butanol	2.269	99.5°	100.0°	1.068	1.805
1-Pentanol	2.914	137.8°	135.8°	0.26	0.211
3-Methylbutanol	2.807	131.2°	130.3°	0.311	0.305
2-Methylbutanol	2.769	128.7°	131.7°	0.347	0.277
2-Pentanol	2.769	119.0°	119.2°	0.53	0.510
3-Pentanol	2.807	115.3°	120.6°	0.615	0.463
3-Methyl-2-butanol	2.641	111.5°	114.3°	0.667	0.705
2-Methyl-2-butanol	2.561	102.0°	104.6°	1.403	1.170
1-Hexanol	3.414	157.0°	155.0°	0.0614	0.0597
2-Hexanol	3.269	139.9°	138.4°	0.136	0.144
3-Hexanol	3.307	135.4°	139.9°	0.160	0.131
3-Methyl-3-pentanol	3.122	122.4°	126.2°	0.436	0.283
2-Methyl-2-pentanol	3.061	121.4°	123.9°	0.327	0.331
2-Methyl-3-pentanol	3.179	126.5°	134.9°	0.2	0.181
3-Methyl-2-pentanol	3.179	134.2°	134.9°	0.194	0.181
2,3-Dimethyl-2-butanol	2.943	118.6°	119.3°	0.427	0.446
3,3-Dimethylbutanol	3.061	143.0°	141.5°	0.075	0.146
3,3-Dimethyl-2-butanol	2.943	120.0°	125.9°	0.244	0.329
4-Methylpentanol	3.269	151.8°	149.5°	0.102	0.0861
4-Methyl-2-pentanol	3.124	131.7°	132.8°	0.163	0.208
2-Ethylbutanol	3.345	146.5°	152.4°	0.0616	0.0710
Cyclohexanol[d]	3.393	161.0°	143.2°	0.383	0.373
1-Heptanol	3.914	176.3°	174.2°	0.0155	0.0169
2-Methyl-2-hexanol	3.561	142.5°	143.1°	0.0843	0.0934
3-Methyl-3-hexanol	3.622	142.4°	145.4°	0.104	0.0801
3-Ethyl-3-pentanol	3.683	142.5°	147.7°	0.147	0.0687
2,3-Dimethyl-2-pentanol	3.481	139.7°	140.0°	0.135	0.114
2,3-Dimethyl-3-pentanol	3.504	139.0°	140.9°	0.144	0.108
2,4-Dimethyl-2-pentanol	3.416	133.0°	137.5°	0.117	0.135
2,4-Dimethyl-3-pentanol	3.552	138.8°	149.3°	0.0607	0.0704
2,2-Dimethyl-3-pentanol	3.481	136.0°	146.5°	0.0711	0.0843
3-Heptanol	3.807	156.8°	159.1°	0.041	0.0370
4-Heptanol	3.807	155.0°	159.1°	0.0409	0.0370
1-Octanol	4.414	195.2°	193.4°	4.51×10^{-3}	4.76×10^{-3}
2,2,3-Trimethyl-3-pentanol	3.811	152.5°	152.7°	0.0533	0.0500
2-Octanol	4.269	179.8°	176.8°	8.6×10^{-3}	1.15×10^{-2}
2-Ethylhexanol	4.345	184.6°	190.8°	6.76×10^{-3}	5.67×10^{-3}
1-Nonanol	4.914	213.1°	212.6°	1.0×10^{-3}	1.34×10^{-3}
2-Nonanol	4.769	198.5°	196.0°	1.8×10^{-3}	3.25×10^{-3}
3-Nonanol	4.807	194.7°	197.5°	2.2×10^{-3}	2.95×10^{-3}
4-Nonanol	4.807	193.0°	197.5°	2.6×10^{-3}	2.95×10^{-3}
5-Nonanol	4.807	195.1°	197.5°	3.2×10^{-3}	2.95×10^{-3}
2,6-Dimethyl-3-heptanol	4.517	178.0°	186.3°	3.1×10^{-3}	6.14×10^{-3}
3,5-Dimethyl-4-heptanol	4.628	187.0°	190.6°	5.0×10^{-3}	4.64×10^{-3}
1,1-Diethylpentanol	4.683	192.0°	186.2°	3.8×10^{-3}	5.47×10^{-3}
7-Methyloctanol	4.769	206.0°	207.1°	3.2×10^{-3}	1.94×10^{-3}
3,5,5-Trimethylhexanol	4.454	193.0°	195.0°	3.12×10^{-3}	4.30×10^{-3}
1-Decanol	5.414	—	—	2.0×10^{-4}	6.35×10^{-5}
1-Dodecanol	6.414	230.2°	231.9°	2.3×10^{-5}	3.04×10^{-5}
Butane	1.914	—	—	2.34×10^{-3}	1.74×10^{-3}
2-Methylpropane	1.732	—	—	2.83×10^{-3}	2.75×10^{-3}
1-Pentane	2.414	—	—	5.37×10^{-4}	4.91×10^{-4}
2-Methylbutane	2.269	—	—	6.61×10^{-4}	7.06×10^{-4}
3-Methylbutane	2.307	—	—	1.48×10^{-4}	1.82×10^{-4}
Neopentane	2.000	—	—	7.48×10^{-4}	1.40×10^{-3}
2,2-Dimethylbutane	2.561	—	—	2.14×10^{-4}	3.38×10^{-4}
2,4-Dimethylpentane	3.125	—	—	4.07×10^{-5}	8.13×10^{-5}
2,2,4-Trimethylpentane	3.417	—	—	7.48×10^{-5}	3.91×10^{-5}
2,2,5-Trimethylhexane	3.917	—	—	8.95×10^{-6}	1.10×10^{-5}
Cyclohexane[d]	3.000	—	—	6.61×10^{-4}	3.95×10^{-4}
Methylcyclohexane[d]	3.393	—	—	1.41×10^{-4}	1.46×10^{-4}
1,2-Dimethylcyclohexane[d]	3.804	—	—	5.38×10^{-5}	5.17×10^{-5}
Cycloheptane[d]	3.500	—	—	3.05×10^{-4}	1.12×10^{-4}
Cyclooctane[d]	4.000	—	—	7.05×10^{-5}	3.17×10^{-5}
Hexane	2.914	—	—	1.11×10^{-4}	1.39×10^{-4}
Heptane	3.414	—	—	2.93×10^{-5}	3.95×10^{-5}
Octane	3.914	—	—	5.79×10^{-6}	1.11×10^{-5}
Cyclopentanol	2.893	140.85°	124.0°	—	—
Cycloheptanol	3.893	185.0°	162.4°	—	—
1-Ethylcyclohexanol	4.269	166.0°	170.3°	—	—
2-Ethylcyclohexanol	4.341	181.0°	179.6°	—	—
1-Methylcyclohexanol	3.708	155.0°	148.7°	—	—
2-Methylcyclohexanol	3.803	165.0°	158.9°	—	—
3-Methylcyclohexanol	3.803	174.5°	158.9°	—	—
4-Methylcyclohexanol	3.803	173.5°	158.9°	—	—
1,3,5-Trimethylcyclohexanol	4.494	181.0°	178.9°	—	—
Ethanol	1.000	78.5°	78.3°	—	—

(*continued*)

Table I—*Continued*

Compound	χ	Tb_{obs}[a]	Tb_{calc}[b]	S_{obs}[a], molal	S_{calc}[c], molal
2-Propanol	1.732	82.4°	79.4°	—	—
1-Propanol	1.914	97.4°	97.5°	—	—

[a] Solubility (S), expressed as molality, and boiling points (Tb), expressed in degrees centigrade, were taken from Refs. 5 and 6 and "Handbook of Chemistry and Physics," 51st ed., Chemical Rubber Co., Cleveland, Ohio, 1971, and the references cited therein. [b] Calculated boiling points are based on Eq. A3. [c] Calculated solubilities are based on Eq. A2. [d] For these cyclic compounds, the connectivity index is modified as described in Part I (4): a value of 0.500 is subtracted from the computed connectivity index for the solubility study only.

connectivity index with the water solubility of aliphatic alcohols and hydrocarbons. Furthermore, since boiling points tend to show a variation with structure similar to that of solubilities, the relationship between the connectivity index and the boiling point was also developed.

EXPERIMENTAL

Formulation of Connectivity Index—The connectivity index (χ) (4) is obtained from the molecular skeleton, which is also called the skeletal graph or, more simply, the graph. Hydrogen atoms are suppressed. The degree of connectivity, δ_i, is assigned to each vertex of the skeletal graph. The value of $\delta_i = 1, 2, 3$, or 4, the number of connections at the ith vertex. Then the connectivity index is the sum of connectivities, C_k, of each connection (or edge) of the graph between vertexes i and j:

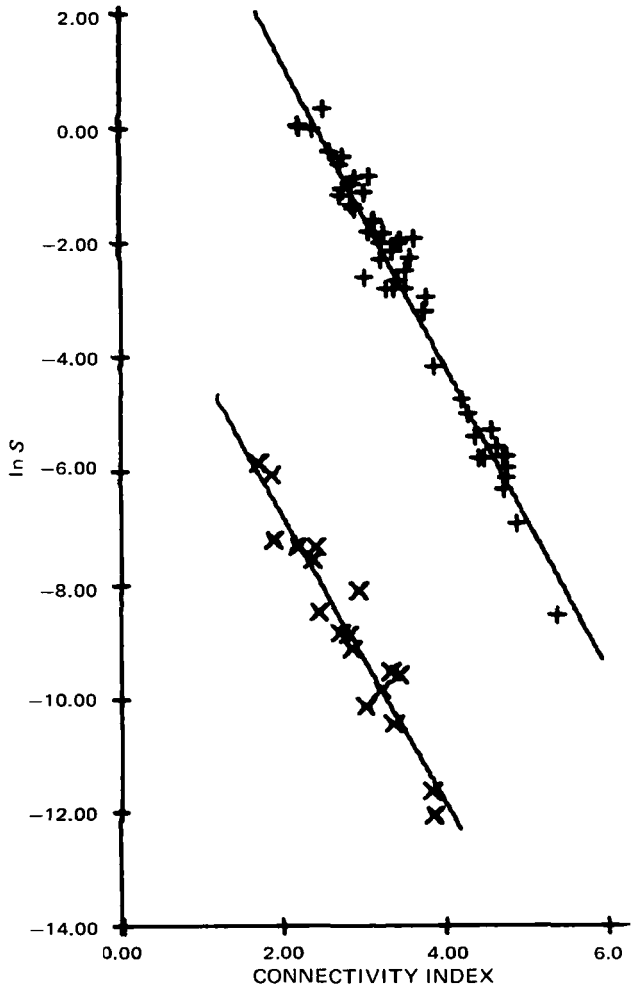

Figure 1—*Computer-drawn plot of the natural logarithm of solubility* versus *the connectivity index for 51 alcohols* (+) *and 18 hydrocarbons* (×) *with the corresponding regression lines.*

$$\chi = \sum_{k=1}^{N} C_k = \sum_{k} \frac{1}{(\sqrt{\delta_i \delta_j})_k} \qquad \text{(Eq. 1)}$$

where N is the number of edges and χ is then simply a sum of terms, each of which is a connectivity contribution for each bond in the molecular skeleton. Illustrations of the calculation of the connectivity index were given in Part I (4). Only simple arithmetic is required: the summation of reciprocal square roots.

Correlation of Water Solubility with Connectivity Index—The correlation of the connectivity index with the water solubility of the hydrocarbons (5, 6) and alcohols (6) was determined. The regression equations, correlation coefficients, and standard errors are as follows (Table I).

For alcohols:

$$\ln S = 6.702 - 2.666\chi \qquad \text{(Eq. 2)}$$

$$r = 0.978 \quad s = 0.455 \quad n = 51$$

For hydrocarbons:

$$\ln S = -1.505 - 2.533\chi \qquad \text{(Eq. 3)}$$

$$r = 0.958 \quad s = 0.511 \quad n = 18$$

where S is solubility expressed as molality (see *Appendix*).

The correlations are of a general high quality (Fig. 1). It is useful to consider the predictive power of this relationship based on connectivity. Table II presents measured and predicted solubilities for three solid alcohols. The solubilities are for the supercooled liquid (6).

The obvious utility of the connectivity index in correlating and predicting solubility is the extraordinary simplicity of its calculation by simple arithmetic from the integers 1, 2, 3, and 4, representing the degrees of connectedness in the molecular skeleton.

Correlation of Boiling Point with Connectivity Index—The correlation between the connectivity index and the boiling points of two sets of alcohols was determined. The regression equations, correlation coefficients, and standard errors are as follows.

For 51 alcohols (from Ref. 6):

$$bp = 10.25 - 39.22\chi \qquad \text{(Eq. 4)}$$

$$r = 0.964 \quad s = 8.68 \quad n = 51$$

For 63 alcohols (from Ref. 6 plus the last 12 compounds in Table I):

$$bp = 11.60 - 39.13\chi \qquad \text{(Eq. 5)}$$

$$r = 0.970 \quad s = 9.35 \quad n = 63$$

It can be clearly seen from Fig. 2 that the use of the connectivity index is excellent both by virtue of its great simplicity and its superior correlation in the case of boiling points. Furthermore, this study included 10 cyclic alcohols in addition to straight-chain and variously branched alcohols.

An empirical improvement to the correlation may be obtained if the degree of connectivity of the hydroxyl group is allowed to vary and its value is determined by multiple regression analysis of alcohol solubility (see *Appendix*).

CONCLUSIONS

A simple connectivity index, easily calculated by arithmetic, based on the degree of connectedness at each vertex in a molecular graph which depicts the molecular skeleton, gives highly significant correlations with the water solubility of hydrocarbons and alcohols as well as with boiling points of alcohols. These correlations

Table II—Observed and Predicted Solubilities for Three Solid Alcohols

Compound	S_{obs}, molal	S_{calc}, molal	S_{calc}[a], molal	χ	c_{OH}
1-Tetradecanol	2.84×10^{-6}	2.12×10^{-6}	2.43×10^{-6}	7.414	0.707
1-Pentadecanol	1.02×10^{-6}	0.56×10^{-6}	0.69×10^{-6}	7.914	0.707
1-Hexadecanol	4.55×10^{-7}	1.47×10^{-7}	1.94×10^{-7}	8.414	0.707

[a] Based on Eq. A2.

are superior to those based on well-founded theory which relates these properties to the surface area of the cavity required to accommodate the solute in water.

The great appeal of the connectivity index lies both in the utter simplicity of its computation and in its fundamental relationship to molecular connectivity. At the heart of chemistry is the concept that an important characteristic of a molecule is the manner in which its substituent parts are connected. The evidence of the first two papers in this series strongly suggests that the connectivity index expresses the fundamental connectedness of organic molecules.

The ability to correlate water solubility with the connectivity index strongly suggests a possible correlation of the connectivity index with the partition coefficients studied by Hansch *et al.* (7). These correlations, as well as those with related biological activity, are presented in Part III.

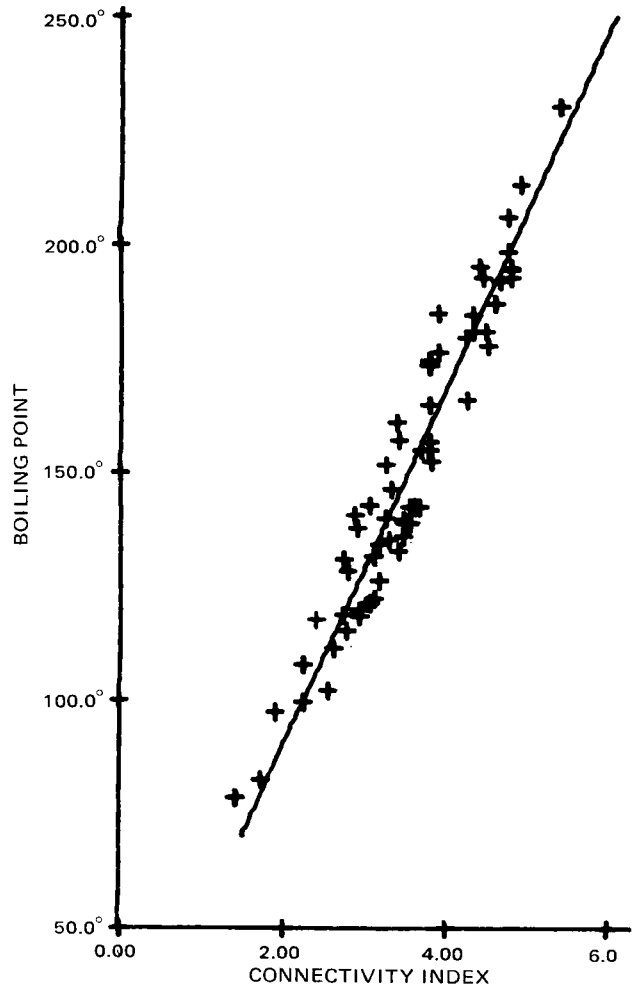

Figure 2—*Computer-drawn plot of the boiling point* versus *the connectivity index for 63 alcohols with the corresponding regression line.*

APPENDIX

The multiple regression equation in this additional study is:

$$\ln S = b_0 - b_1\chi - b_2 c_{OH} - b_3 Q \qquad \text{(Eq. A1)}$$

where b_i terms are regression coefficients, c_{OH} is the contribution of the C—OH bond graph edge to the connectivity index calculated with the OH degree of connectivity equal to 1, and Q has a value of 1 for an alcohol and of 0 for a hydrocarbon. Hence, solubility data for 51 alcohols and 18 hydrocarbons were merged for this multiple regression analysis.

The results of the analysis are:

$$\ln S = -1.516 - 2.528\chi - 3.961 c_{OH} - 10.13Q \qquad \text{(Eq. A2)}$$

$$r = 0.994 \qquad s = 0.383 \qquad n = 69$$

As can be seen, this modification provides a significant improvement in correlation, with a 16% decrease in the standard error of the log of alcohol solubility from 0.455 to 0.383. The calculated solubilities (Table I) are based on the multiple regression equation (Eq. A2).

Based on this improved equation (Eq. A2), the predicted solubilities for the three solid alcohols mentioned earlier are given in Table II.

A similar approach to modification of the connectivity index was applied to the boiling points of the studied alcohols, and significant improvement was also achieved. The regression equation and associated parameters are:

$$\text{bp} = -36.23 - 38.41\chi - 2.216 c_{OH} \qquad \text{(Eq. A3)}$$

$$r = 0.982 \qquad s = 6.47 \qquad n = 63$$

The calculated boiling points in Table I are based on Eq. A3. This and related modifications are currently under study and will be reported later.

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Molecular Connectivity III: Relationship to Partition Coefficients

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Abstract □ The molecular connectivity index is shown to be linearly related to the octanol–water partition coefficients of a variety of monofunctional chemical classes including esters, alcohols, ketones, ethers, carboxylic acids, amines, and hydrocarbons. A modification of the connectivity index, taking into account the valency or degree of unsaturation of an atom, merges the data for all compounds except hydrocarbons. The connectivity index is also shown to be useful for correlating biological activity. These studies indicate that partition coefficients represent empirical quantities having intermediate significance between biological data and the more fundamental property of molecular connectivity.

Keyphrases □ Molecular connectivity—relationship to octanol–water partition coefficients of various chemical classes □ Partition coefficients—octanol–water, relationship to molecular connectivity of various chemical classes

Parts I and II showed the relationship of molecular connectivity, given by χ, to polarizability, cavity surface area, local anesthetic action (1), water solubilities, and boiling points (2). The connectivity index appears to be a topological index in that the calculation is fundamentally dependent on the way constituent atoms of a molecule are attached to one another. Seemingly encoded in the connectivity index, which results from a simple mathematical operation (1), is the molecular structure of a chemical species. It might be expected that any physicochemical or biological property that is a function of the topological structure of a molecule, in this case its connectedness, is going to be related in some fashion to the connectivity index. Having shown the high correlation of a linear relationship between water solubilities and the connectivity index (2), it seems logical to examine the relationship of the connectivity index to partition coefficients.

The work of Hansch *et al.* (3, 4) and Nys and Rekker (5) on the partition coefficients of various compounds in an octanol–water system provided partition data (log P) for comparison. From their work were selected log P values for 45 hydrocarbons, 24 esters, nine carboxylic acids, 49 alcohols, 28 amines, 16

Table I—1-Octanol–Water Partition Coefficients (log P) and Molecular Connectivity Index (χ)

Compound	χ^a	Log P^b	Predicted Log P^c
Methanol	1.000	−0.66	−0.57
Ethanol	1.414	−0.32	−0.17
Propanol	1.914	0.34	0.31
Butanol	2.414	0.88	0.80
Pentanol	2.914	1.40	1.28
Hexanol	3.414	2.03	1.76
Heptanol	3.914	2.34	2.25
Octanol	4.414	2.84	2.73
Nonanol	4.914	3.15	3.21
2-Propanol	1.732	0.14	0.14
2-Methyl-1-propanol	2.270	0.61	0.66
2-Methyl-2-propanol	2.000	0.37	0.40
3-Methyl-1-butanol	2.563	1.14	0.94
2-Methylbutanol	2.807	1.14	1.17
1-Methylbutanol	2.770	1.14	1.14
3-Pentanol	2.807	1.14	1.17
3-Methyl-2-butanol	2.642	0.91	1.14
2-Methyl-2-butanol	2.561	0.89	0.94
2,2-Dimethyl-1-propanol	3.270	1.36	0.94
2-Hexanol	3.270	1.61	1.62
3-Hexanol	3.307	1.61	1.66
3-Methyl-3-pentanol	3.121	1.39	1.48
2-Methyl-2-pentanol	3.061	1.39	1.42
2-Methyl-3-pentanol	3.180	1.41	1.54
3-Methyl-2-pentanol	3.180	1.41	1.54
4-Methyl-2-pentanol	3.125	1.41	1.48
2,3-Dimethyl-2-butanol	2.944	1.17	1.31
3,3-Dimethyl-1-butanol	3.061	1.86	1.42
3,3-Dimethyl-2-butanol	2.944	1.19	1.31
2-Methyl-2-hexanol	3.561	1.87	1.90
3-Methyl-3-hexanol	3.620	1.87	1.96
3-Ethyl-3-pentanol	3.680	1.87	2.02
2,3-Dimethyl-2-pentanol	3.480	1.67	1.83
2,3-Dimethyl-3-pentanol	3.505	1.67	1.85
2,4-Dimethyl-2-pentanol	3.417	1.67	1.77
2,4-Dimethyl-3-pentanol	3.387	1.71	1.74
2,2-Dimethyl-3-pentanol	3.481	1.69	1.83
2,2,3-Trimethyl-3-pentanol	3.811	1.99	2.15
Cyclohexanol	2.893	1.23	1.26
4-Penten-1-ol	2.520	1.04	0.90
3-Penten-2-ol	2.398	0.81	0.78
1-Penten-3-ol	2.433	0.81	0.82
1-Hexen-3-ol	2.933	1.31	1.30
2-Hexen-4-ol	2.935	1.31	1.30
2-Methyl-4-penten-3-ol	2.806	1.11	1.18
Benzyl alcohol	2.471	1.10	0.85
2-Phenylethanol	2.971	1.36	1.34
3-Phenyl-1-propanol	3.471	1.88	1.82
Diphenylcarbinol	3.975	2.67	2.30
Diethyl ether	2.414	1.03	1.02
Methyl butyl ether	2.914	1.53	1.50
Methyl *sec*-butyl ether	2.770	1.33	1.36
Methyl isobutyl ether	2.801	1.33	1.40
Methyl *tert*-butyl ether	2.561	1.06	1.16
Ethyl propyl ether	2.914	1.53	1.50
Ethyl isopropyl ether	2.770	1.33	1.36
Dipropyl ether	3.414	2.03	1.99
Propyl isopropyl ether	3.270	1.83	1.85
Methyl propyl ether	2.414	1.03	1.02
Methyl isopropyl ether	2.270	0.83	0.88
Ethyl cyclopropyl ether	2.432	1.24	1.04
Ethyl formate	2.023	0.23	0.30
Propyl formate	2.523	0.73	0.80
Methyl acetate	1.914	0.23	0.19
Ethyl acetate	2.414	0.73	0.69
Propyl acetate	2.914	1.23	1.19
Isopropyl acetate	2.770	1.03	1.05
Butyl acetate	3.414	1.73	1.69
sec-Butyl acetate	3.270	1.53	1.54
Methyl propionate	2.475	0.73	0.75
Methyl butyrate	2.975	1.23	1.25
Ethyl hexanoate	4.475	2.73	2.74
Ethyl heptanoate	4.975	3.23	3.24
Ethyl octanoate	5.475	3.73	3.74
Ethyl nonanoate	5.975	4.23	4.24
Ethyl decanoate	6.475	4.73	4.74
Ethyl propionate	2.975	1.23	1.25
Ethyl butyrate	3.475	1.73	1.75
Ethyl isobutyrate	3.358	1.53	1.63

Table I—*Continued*

Compound	χ^a	Log P^b	Predicted Log P^c
Pentyl acetate	3.914	2.23	2.19
Butyl pentanoate	4.975	3.23	3.24
Benzyl acetate	3.616	1.96	1.74
Methyl 3-phenyl-propionate	4.032	2.32	2.30
Methyl 2-phenyl-acetate	3.532	1.83	1.80
Methyl 4-phenyl-butyrate	4.532	2.77	2.80
Acetic acid	1.354	−0.17	−0.15
Propionic acid	1.915	0.25	0.37
Butyric acid	2.415	0.79	0.83
Hexanoic acid	3.415	1.88	1.76
Decanoic acid	5.915	4.09	4.07
2-Phenylacetic acid	2.971	1.41	1.34
3-Phenylpropionic acid	3.471	1.84	1.81
4-Phenylbutyric acid	3.971	2.42	2.27
2,2-Diphenylacetic acid	5.041	3.05	3.26
Methylamine	1.000	−0.57	−0.54
Ethylamine	1.414	−0.13	−0.13
Propylamine	1.914	0.48	0.36
Butylamine	2.414	0.75	0.84
Pentylamine	2.914	1.49	1.33
Hexylamine	3.414	1.98	1.82
Heptylamine	3.914	2.57	2.31
Isobutylamine	2.270	0.73	0.70
sec-Butylamine	2.270	0.74	0.70
2-Aminooctane	4.307	2.82	2.69
Cyclohexylamine	2.893	1.49	1.31
Isopropylamine	1.732	0.26	0.18
Methylethylamine	1.914	0.15	0.36
Dipropylamine	3.414	1.67	1.82
Triethylamine	3.346	1.44	1.76
Dibutylamine	4.414	2.68	2.80
Diethylamine	2.414	0.57	0.84
Propylbutylamine	3.914	2.12	2.31
Methylbutylamine	2.914	1.33	1.33
Piperidine	2.500	0.85	0.93
Ethylisopropylamine	2.770	0.93	1.19
Propyl-*sec*-butylamine	3.807	1.91	2.20
Propylisobutylamine	3.769	2.07	1.77
Trimethylamine	1.732	0.27	0.18
Dimethylbutylamine	3.270	1.70	1.68
Dimethylbenzylamine	3.327	1.98	1.74
Benzylamine	2.471	1.09	0.90
Phenethylamine	2.971	1.41	1.39
Acetone	1.354	−0.21	−0.28
2-Butanone	1.915	0.29	0.27
2-Pentanone	2.414	0.79	0.76
3-Pentanone	2.475	0.79	0.82
3-Methyl-2-butanone	2.298	0.59	0.64
2-Hexanone	2.914	1.29	1.25
3-Hexanone	2.975	1.29	1.31
3-Methyl-2-pentanone	2.835	1.09	1.17
4-Methyl-2-pentanone	2.770	1.09	1.11
2-Methyl-3-pentanone	2.859	1.09	1.20
2-Heptanone	3.414	1.79	1.74
3-Heptanone	3.475	1.79	1.80
2,4-Dimethyl-3-pentanone	3.240	1.39	1.57
5-Nonanone	4.475	2.79	2.78
2-Nonanone	4.414	2.79	2.72
Acetophenone	3.014	1.58	1.35
Pentane	2.414	2.50	2.54
2-Methylbutane	2.270	2.30	2.41
2-Methylpentane	2.770	2.80	2.85
3-Methylpentane	2.807	2.80	2.88
Hexane	2.914	3.00	2.98
Heptane	3.414	3.50	3.42
2,4-Dimethylpentane	3.125	3.10	3.17
Octane	3.914	4.00	3.89
Cyclopentane	2.000	2.05	2.17
Cyclohexane	2.500	2.46	2.62
Methylcyclopentane	2.398	2.35	2.52
Cycloheptane	3.000	2.87	3.06
Methylcyclohexane	2.893	2.76	2.96
Cyclooctane	3.500	3.28	3.50
1,2-Dimethylcyclohexane	3.304	3.06	3.33
1-Pentyne	1.850	1.98	2.04
1-Hexyne	2.350	2.48	2.48

Table I—*Continued*

Compound	χ^a	Log P^b	Predicted Log P^c
1-Heptyne	2.850	2.98	2.93
1-Octyne	3.350	3.48	3.37
1-Nonyne	3.850	3.98	3.81
1,8-Nonadiyne	3.286	3.46	3.31
1,6-Heptadiyne	2.284	2.46	2.42
1-Pentene	2.023	2.20	2.20
2-Pentene	2.025	2.20	2.20
1-Hexene	2.523	2.70	2.64
2-Heptene	3.025	3.20	3.08
1-Octene	3.523	3.70	3.52
4-Methyl-1-pentene	2.379	2.50	2.51
1,6-Heptadiene	2.633	2.90	2.73
1,5-Hexadiene	2.133	2.40	2.29
1,4-Pentadiene	1.633	1.90	1.85
Cyclopentene	1.649	1.75	1.86
Cyclohexene	2.149	2.16	2.31
Cycloheptene	2.649	2.57	2.75
Toluene	2.410	2.73	2.54
Ethylbenzene	2.906	3.15	2.98
Isopropylbenzene	3.199	3.66	3.23
Propylbenzene	3.411	3.68	3.42
Diphenylmethane	4.528	4.14	4.41
1,2-Diphenylethane	5.028	4.79	4.85
Biphenyl	4.071	4.04	4.01
p-Xylene	2.821	3.25	2.90
Benzene	1.999	2.13	2.17
Naphthalene	3.403	3.37	3.42
Phenanthrene	4.814	4.46	4.66

[a] The connectivity index was calculated using valency modification described in text. [b] Partition coefficients in a 1-octanol–water system were taken from Refs. 3, 5, and 7 and references cited therein. [c] Calculated log P values were derived from the equations developed for each functional group series compiled in Table II.

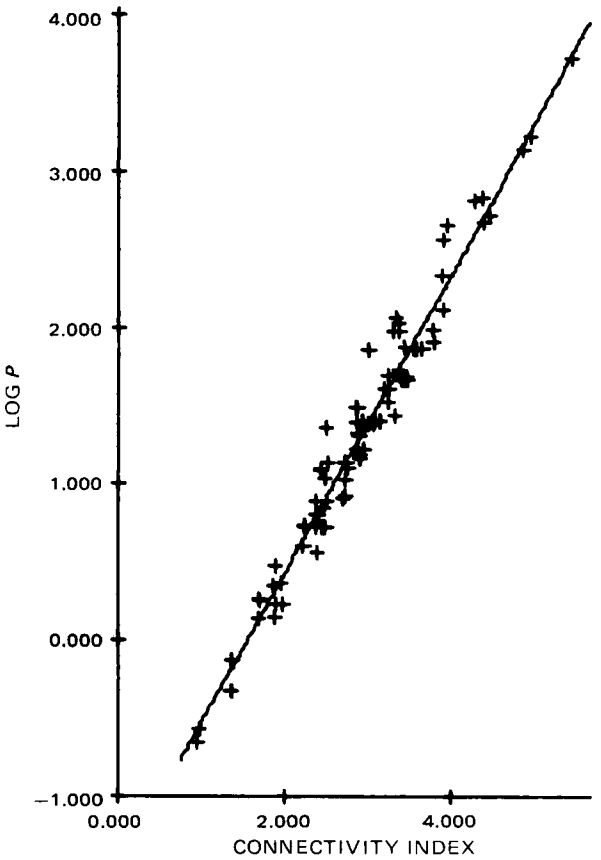

Figure 1—*Computer-drawn plot of the logarithm of the partition coefficient* versus *the molecular connectivity index for 24 esters, nine carboxylic acids, 49 alcohols, 28 amines, 16 ketones, and 12 ethers with the corresponding regression line.*

Table II—Summary of Regression Equations, Correlation Coefficients, and Standard Deviations

Class of Compounds	n	Slope	Intercept	r	s
Carboxylic acids	9	0.927 ± 0.03	−1.41 ± 0.11	0.996	0.122
Esters	24	0.996 ± 0.01	−1.71 ± 0.04	0.999	0.060
Ethers	12	0.964 ± 0.07	−1.30 ± 0.19	0.976	0.080
Alcohols	49	0.966 ± 0.03	−1.53 ± 0.09	0.997	0.151
Amines	28	0.977 ± 0.04	−1.51 ± 0.12	0.979	0.179
Ketones	16	0.982 ± 0.03	−1.16 ± 0.09	0.993	0.094
Hydrocarbons	45	0.884 ± 0.03	0.406 ± 0.09	0.975	0.160
All compounds except hydrocarbons	138	0.950 ± 0.01	−1.48 ± 0.04	0.986	0.152

ketones, and 12 ethers (Table I). In addition to the functional group differences, each series provides compounds with structural diversity, *i.e.*, branched, straight-chain, cyclic, unsaturated, and aromatic hydrocarbon skeletons. This selection provides sufficient breadth of structure to demonstrate that a linear relationship between log P and the connectivity index is more than simply fortuitous.

EXPERIMENTAL

The molecular connectivity (χ) was calculated in the usual manner (1, 2). The molecular skeleton was drawn and each carbon atom in the skeleton was assigned a number, 1, 2, 3, or 4, corresponding to the number of nonhydrogen atoms connected to the carbon atom. The isosteric relationship of $-CH_3$, $-OH$, and $-NH_2$ was utilized so hydroxyl and amino functionalities were assigned the number 1, an ether oxygen was assigned the number 2, and the nitrogen atom of substituted amines, RNH— and R_2N—, were assigned connectivities of 2 and 3, respectively. Initially, the valency of atoms was ignored so that, for example, a carbonyl oxygen was assigned a connectivity of 1 and unsaturated carbon atoms, RHC=, were given connectivity numbers based solely on the number of nonhydrogen attachments.

Plots of log P *versus* the connectivity index were computed, and families of parallel lines were obtained, each having approximately the same slope but different intercepts. These families of curves appear to be related to the degree of unsaturation inherent in each functional group or in the hydrocarbon skeleton.

Table III—Molecular Connectivity Index (χ) *versus* Selected Biological Activities[a]

Compound	χ[b]	V-17[c], pC obs.	V-18[d], pC obs.	V-19[d], pC obs.
Methanol	1.000	−0.14	−0.20	−0.14
Ethanol	1.414	0.28	0.21	0.28
Propanol	1.914	0.79	0.98	0.98
2-Propanol	1.732	0.92	—	—
Butanol	2.414	1.46	1.72	1.67
Pentanol	2.914	1.84	—	—
Hexanol	3.414	2.41	3.17	2.99
Heptanol	3.914	3.02	3.70	3.57
Octanol	4.414	3.62	4.12	3.97
2-Methyl-1-propanol	2.270	1.54	—	—
2-Butanol	2.270	1.16	—	—
2-Methyl-2-propanol	2.000	0.98	—	—
3-Methyl-1-butanol	2.770	1.86	2.18	2.09
2-Methyl-2-butanol	2.561	1.34	—	—
Benzyl alcohol	2.971	2.15[e]	—	—

[a] Biological activities (pC, obs.) were obtained from Ref. 9. [b] The connectivity index was calculated using valency modification described in text. [c] Biological activity is barnacle larvae narcosis (10). [d] Biological activity is I_{100}, movement of 2.5-day-old and 12-day-old tadpoles at 18° (11). [e] Not included in the derivation of regression equation.

It is assumed that a π-bond increases the connectivity of a carbon or oxygen atom by one unit, *e.g.*, CH_2= has connectivity 2, RHC= has connectivity 3, R_2C= connectivity 4, O= connectivity 2, and HC≡ connectivity 3. The valency modification merges the data for all classes of compounds (except the hydrocarbons) onto one line (Fig. 1). Table II summarizes the regression equations, correlation coefficients, and standard deviations for each class of compounds.

This refinement, *i.e.*, the use of valency, is not always necessary. In the linear relationship between the connectivity index and polarizability (1), a correlation coefficient of 0.990 was obtained, taking into account only attachments. The modification does, however, appear to be necessary in correlating the connectivity index to log P and solubility (2).

DISCUSSION

The molecular connectivity index, suggested by Randić (6), correlates significantly with a number of physicochemical properties (1, 2). It is now shown to have a high degree of correlation with the partition coefficients (log P) of a variety of compounds, encompassing several monofunctional groups and having structural diversity.

The importance of log P as a measure of lipophilicity and its use in structure–activity relationships are well known (7–9). It seems obvious that since the connectivity index is linearly related to log P, it should correlate in the same fashion as log P to biological activity. This hypothesis was tested on several systems where log P had been correlated with biological activity (9). Table III summarizes the data. These systems were all linearly related to log P and the connectivity index. For these systems, the following relationship and statistics were obtained.

For Study V-17 [narcosis, larvae, barnacle (10)]:

$$pC = -1.167(\pm 0.011) + 1.073(\pm 0.041)\chi \qquad \text{(Eq. 1)}$$

$$r = 0.991 \quad s = 0.141 \quad n = 15$$

For Study V-18 [I_{100}, movement 2.5-day-old tadpoles, 18° (11)]:

$$pC = -1.541(\pm 0.115) + 1.328(\pm 0.040)\chi \qquad \text{(Eq. 2)}$$

$$r = 0.997 \quad s = 0.127 \quad n = 8$$

For Study V-19 [I_{100}, movement 12-day-old tadpoles, 18° (11)]:

$$pC = -1.41(\pm 0.088) + 1.294(\pm 0.030)\chi \qquad \text{(Eq. 3)}$$

$$r = 0.998 \quad s = 0.097 \quad n = 8$$

These results compare very favorably with relationships to log P (9). Current studies are extending this exploration of the direct correlation of molecular connectivity to biological activity. The relationship of the connectivity index to other parameters such as Taft's steric parameter, E_s (12), used in structure–activity relationships is also being investigated.

Apparently, the partition coefficient represents an experimental quantity of intermediate significance between biological data and the more fundamental property of molecular connectivity, expressed as χ.

The tremendous utility of the connectivity index lies in the simplicity of its computation; no tables are needed for the calculations. Simply by assigning the degree of connectedness and the valency of an atom in a molecule and by performing a simple arithmetic computation, the connectivity index may be calculated. Simultaneously, the connectivity index appears to be related to the

fundamental notion that molecular structure, or connectedness, plays an important role in physicochemical and, hence, biological properties. The relation of the connectivity index to other constitutive, additive properties is being pursued.

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Size Distribution Effects in Multiparticulate Dissolution

PETER VENG PEDERSEN [x] and K. F. BROWN

Abstract □ The evaluation of models for single-particle dissolution, based on multiparticulate dissolution data, is complicated by the distribution effect present when the particles are not truly monodispersed. By using simulated data, it is shown that remarkably good linearity can be obtained with log-normal powders using an incorrect model. It is suggested that particle-size analysis is necessary to enable calculation of the distribution effect and to prevent this type of misinterpretation. The change in particle-size distribution during dissolution is calculated and shows potential for distinguishing between two, but not all three, of the models investigated. Four theoretical rules for multiparticulate dissolution are stated and discussed. The concept of "time scaling" is presented. By using this procedure, it should be possible to reduce considerably computational errors arising from nonlinear dissolution data. It is demonstrated that dissolution profiles can be transformed to a standard form, enabling the distribution effect to be evaluated without interference from rate or particle-size parameters.

Keyphrases □ Dissolution, multiparticulate—size distribution effects, log-normal powders, three single-particle models investigated □ Powders, dissolution—size distribution effects, log-normal distribution profile, three single-particle models investigated □ Particle dissolution—size distribution effects, log-normal powders, three single-particle models investigated

The dissolution profile of a powder is determined by its particle-size distribution and the way the single particles dissolve. Several mathematical models have been presented to describe single-particle dissolution (1–4), but none of these has yet received complete acceptance. Experimental evaluation of the models on the basis of multiparticulate dissolution data is complicated by the distribution effect present when the powder is not truly monodispersed. Such powders are impossible to obtain in most cases (5). Processes such as sieving, precipitation, and grinding do not yield completely uniform particles. This situation is particularly true for fine powders which are of greatest pharmaceutical importance.

In recent years, there has been increasing interest in evaluating the distribution effect in multiparticulate dissolution (6–9). A previous paper (9) discussed the theory and mathematics of multiparticulate systems in relation to single-particle dissolution and the initial size distribution. The present paper evaluates distribution effects for log-normal powder systems; three single-particle dissolution models from the literature are considered. By using simulated dissolution data and particle-size distributions, the possibility of distinguishing between the models is investigated.

THEORY

Let:

$$w = g(w_0,t) \qquad \text{(Eq. 1)}$$

and:

$$w_0 = g^{-1}(w,t) \qquad \text{(Eq. 2)}$$

describe the dissolution equation and inverse dissolution equation, respectively, for a single particle, where w and w_0 are the particle weights at time t and 0, respectively. Further, let $l_0(a_0)$ denote the initial ($t = 0$) particle-size density ("probability") distribution. By assuming that particles are spherical and remain so during dissolution, the particle weight, w, is related to the diameter, a, at any time by $w = \rho\pi a^3/6$, where ρ is the particle density.

By using a technique similar to the one used in a previous paper (9), the following equation can be derived which rigorously describes the particle-size distribution, $l(a)$, at any time if the initial distribution, $l_0(a_0)$, is known together with the particle dissolution function, g:

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$$l(a) = \frac{l_0\left(\left[\frac{6}{\pi\rho}g^{-1}\left(\frac{\pi\rho}{6}a^3,t\right)\right]^{1/3}\right)\frac{d}{da}\left[\frac{6}{\pi\rho}g^{-1}\left(\frac{\pi\rho}{6}a^3,t\right)\right]^{1/3}}{\int_{L_1}^{L_2} l_0(a)\,da} \quad \text{(Eq. 3)}$$

for:

$$P\left[\frac{6}{\pi\rho}g\left(\frac{\pi\rho}{6}d_0^3,t\right)\right]^{1/3} \leq a \leq P\left[\frac{6}{\pi\rho}g\left(\frac{\pi\rho}{6}D_0^3,t\right)\right]^{1/3}$$

and $l(a) = 0$ elsewhere.

The integration limits L_1 and L_2 depend on time as follows:

$$L_1 = d_0 \quad \text{for } t \text{ such that } Pg\left(\frac{\pi\rho}{6}d_0^3,t\right) > 0 \quad \text{(Eq. 4a)}$$

$$L_1 = \left[\frac{6}{\pi\rho}g^{-1}(0,t)\right]^{1/3} \quad \text{for } t \text{ such that } Pg\left(\frac{\pi\rho}{6}d_0^3,t\right) = 0 \quad \text{(Eq. 4b)}$$

$$L_2 = D_0 \quad \text{for } t \text{ such that } Pg\left(\frac{\pi\rho}{6}D_0^3,t\right) > 0 \quad \text{(Eq. 4c)}$$

$$L_2 = \left[\frac{6}{\pi\rho}g^{-1}(0,t)\right]^{1/3} \quad \text{for } t \text{ such that } Pg\left(\frac{\pi\rho}{6}D_0^3,t\right) = 0 \quad \text{(Eq. 4d)}$$

where d_0 and D_0 denote the initial diameters of the smallest and largest particles, respectively. The operator P has been introduced to make the expression generally applicable (9). It is defined to be equal to one in the time period before the operand becomes zero and is equal to zero beyond that time. The lower integration limit, L_1, changes value at $g[(\rho\pi/6)d_0^3,t] = 0$, that is the critical time, when the particles start to disappear. The time at which $g[(\rho\pi/6)D_0^3,t] = 0$ corresponds to the disappearance of the last particle and marks the completion of the dissolution process.

Many powders have size distributions that are approximately log-normal (5). Consider such a powder distributed such that $\ln a_0$ approximates a normal distribution with mean μ and standard deviation σ truncated at $\ln d_0 = \mu - i\sigma$ and $\ln D_0 = \mu + j\sigma$, where i and j are truncation parameters. The initial particle-size distribution, $l_0(a_0)$, is then given by (9):

$$l_0(a_0) = \frac{a_0^{-1}N(\ln a_0,\mu,\sigma)}{\int_{d_0}^{D_0} a_0^{-1}N(\ln a_0,\mu,\sigma)da_0} \qquad d_0 \leq a_0 \leq D_0 \quad \text{(Eq. 5)}$$

where $N(\,)$ is the normal distribution with $\ln a_0$ as the variable.

The change in the particle-size distribution during dissolution depends on the way the individual particles dissolve. Three widely known models for single-particle dissolution are considered. When written in the same form as Eq. 1, the Hixson–Crowell "cube root law" (3) can be expressed as:

$$w = (w_0^{1/3} - k_3t)^3 \quad \text{(Eq. 6)}$$

In a similar way, the equation presented by Niebergall *et al.* (2) can be written simply:

$$w = (w_0^{1/2} - k_2t)^2 \quad \text{(Eq. 7)}$$

and the model proposed by Higuchi and Hiestand (1) can be written:

$$w = (w_0^{2/3} - k_1t)^{3/2} \quad \text{(Eq. 8)}$$

For simplicity and because their evaluation is not important to the theoretical discussion, the constants k_1, k_2, and k_3 are used in place of the original time coefficients which included parameters such as the shape factor, particle density, and diffusion coefficient. In the following discussion, Eqs. 8, 7, and 6 will be referred to as Models 1, 2, and 3, respectively.

By having defined the initial size distribution, $l_0(a_0)$ (Eq. 5), and the dissolution function (Eqs. 6–8), the size distribution at time t, $l(a)$, can be expressed applying Eq. 3:

$$l(a) = \frac{a^{\left(\frac{3}{m}-1\right)}(a^{\frac{3}{m}} + Kt)^{-1}N[\ln(a^{\frac{3}{m}} + Kt)^{\frac{m}{3}},\mu,\sigma]}{F\left(\frac{T_2-\mu}{\sigma}\right) - F\left(\frac{T_1-\mu}{\sigma}\right)} \quad \text{(Eq. 9)}$$

for:

$$P(e^{\frac{3}{m}(\mu-i\sigma)} - Kt)^{\frac{m}{3}} \leq a \leq P(e^{\frac{3}{m}(\mu+j\sigma)} - Kt)^{\frac{m}{3}}$$

and $l(a) = 0$ elsewhere. Also:

$$T_1 = \mu - i\sigma \quad \text{for } \frac{m}{3}\ln(Kt) < \mu - i\sigma \quad \text{(Eq. 10a)}$$

$$T_1 = \frac{m}{3}\ln(Kt) \quad \text{for } \frac{m}{3}\ln(Kt) \geq \mu - i\sigma \quad \text{(Eq. 10b)}$$

$$T_2 = \mu + j\sigma \quad \text{for } \frac{m}{3}\ln(Kt) < \mu + j\sigma \quad \text{(Eq. 10c)}$$

$$T_2 = \frac{m}{3}\ln(Kt) \quad \text{for } \frac{m}{3}\ln(Kt) \geq \mu + j\sigma \quad \text{(Eq. 10d)}$$

and:

$$K = (6/\rho\pi)^{\frac{1}{m}}k \quad \text{(Eq. 11)}$$

Equation 9 describes the size distribution for all three models. For Model 1, $m = 3/2$; for Model 2, $m = 2$; and for Model 3, $m = 3$. The constants k and K for each model should be k_1, k_2, and k_3 and K_1, K_2, and K_3, respectively. The function $F(\,)$, the commonly tabulated area under the standard normal curve function, is defined as:

$$F(x) = \int_{-\infty}^{x}(1/\sqrt{2\pi})e^{-\frac{x^2}{2}}\,dx \quad \text{(Eq. 12)}$$

The mean particle size (diameter), $\bar{a}$, can be obtained by applying the usual integration approach used in mathematical expectation:

$$\bar{a} = \frac{\int_R a^{\frac{3}{m}}(a^{\frac{3}{m}} + Kt)^{-1}N[\ln(a^{\frac{3}{m}} + Kt)^{\frac{m}{3}},\mu,\sigma]da}{F\left(\frac{T_2-\mu}{\sigma}\right) - F\left(\frac{T_1-\mu}{\sigma}\right)} \quad \text{(Eq. 13)}$$

The integration interval R in Eq. 13 is the same as the interval for a defined in Eq. 9. Equation 13 considers Models 1 and 2 ($m = 3/2$ and $m = 2$, respectively). The mean particle size for the third model ($m = 3$) simplifies further to:

$$\bar{a} = \frac{F\left(\frac{T_2-\mu}{\sigma} - \sigma\right) - F\left(\frac{T_1-\mu}{\sigma} - \sigma\right)}{F\left(\frac{T_2-\mu}{\sigma}\right) - F\left(\frac{T_1-\mu}{\sigma}\right)}e^{\mu+\sigma^2/2} - K_3t \quad \text{(Eq. 14)}$$

where T_1 and T_2 are still defined as in Eqs. 10a–10d. The exact dissolution profile of a log-normal powder with single particles dissolving according to each of the three models can be derived using Eq. 13 presented in the previous paper (9):

$$\frac{W}{W_0} = \sum_{n=0}^{m}\binom{m}{n}(-Kt)^{(m-n)} \times \frac{F\left(\frac{T_2-\mu}{\sigma} - \frac{3n\sigma}{m}\right) - F\left(\frac{T_1-\mu}{\sigma} - \frac{3n\sigma}{m}\right)}{F(j-3\sigma) - F(-i-3\sigma)} \times e^{\frac{3}{m}(n-m)[\mu+\frac{3}{m}(n+m)\sigma^2/2]} \quad \text{(Eq. 15)}$$

where W_t and W_0 are the amounts of undissolved powder at time t and 0, respectively. Equation 15 with $m = 3$, although presented in more compact form, is identical to Eq. 18 in the previous paper (9). Equation 15 is not defined for Model 1 ($m = 3/2$), which must be considered separately:

$$\frac{W}{W_0} = \frac{\int_{R_1}^{R_2}(w^2 - K_1t)^{3/2}w^{-1}N(\ln w,\mu,\sigma)\,dw}{F(j-3\sigma) - F(-i-3\sigma)}e^{-3\mu-9\sigma^2/2} \quad \text{(Eq. 16)}$$

where:

$$R_1 = e^{\mu-i\sigma} \quad \text{for } (K_1t)^{1/2} < e^{\mu-i\sigma} \quad \text{(Eq. 17a)}$$

$$R_1 = (K_1t)^{1/2} \quad \text{for } (K_1t)^{1/2} \geq e^{\mu-i\sigma} \quad \text{(Eq. 17b)}$$

$$R_2 = e^{\mu+j\sigma} \quad \text{for } (K_1t)^{1/2} < e^{\mu+j\sigma} \quad \text{(Eq. 17c)}$$

$$R_2 = (K_1t)^{1/2} \quad \text{for } (K_1t)^{1/2} \geq e^{\mu+j\sigma} \quad \text{(Eq. 17d)}$$

The derivations of these equations are based on two assumptions: (*a*) that the particles in the multiparticulate system dissolve

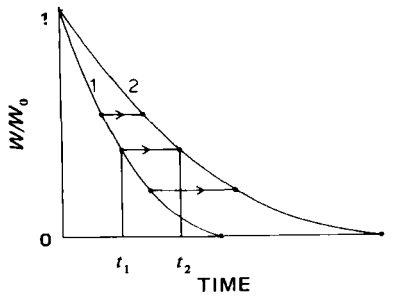

Figure 1—*Two dissolution curves having the same intrinsic dissolution profile. Curve 1 can be brought into curve 2 by a time-scaling factor,* t_2/t_1. *Rule 2 states that* $k_{(1)} = (t_2/t_1)k_{(2)}$, *where* $k_{(1)}$ *and* $k_{(2)}$ *are the rate parameters in the single-particle dissolution equation for Systems 1 and 2 having the same particle-size distribution. These statements also include plots where* W/W_0 *is raised to any other exponent.*

independently of each other, which will be approximated well under sink conditions; and (*b*) that they dissolve according to the same single-particle dissolution model having fixed parameters (for these cases, k_1, k_2, and k_3 are the same for all particles and do not vary during the dissolution). If these conditions exist, then it is possible to make some general rules concerning the dissolution process. These rules are explained in relation to what will be termed "the intrinsic dissolution profile," which can be defined in the following way. Dissolution curves have the same intrinsic dissolution profile if, by a suitable scaling of time, they can be brought into each other in a W/W_0 *versus* time plot (Fig. 1).

It should be clear from observation of Eqs. 12 and 13 in the previous paper (9) that the coefficient of time in an expression correctly defining the multiparticulate dissolution profile originates directly from the coefficient of time in the single-particle dissolution equation. Thus, a different value of the rate parameter, that is, a different coefficient of time in the single-particle dissolution equation, has the same effect as a different scaling of time. Therefore, the intrinsic dissolution profile will still be the same. The following rule can thus be stated:

1. *The intrinsic dissolution profile is independent of the value of the rate parameter, that is, the coefficient of time in the single-particle dissolution equation.*

According to this rule, the rate parameters k_1, k_2, and k_3 (Eqs. 6–8) have no influence on the intrinsic dissolution profile. Furthermore, there will always be a proportional relationship between the coefficient of time in the multiparticulate dissolution equation and the rate parameter [note, for example, that K in Eqs. 15 and 16 is related to k by $K = (6/\rho\pi)^{1/m}k$ as seen in Eq. 11]. The following rule can therefore be stated:

2. *In two systems having identical particle-size distributions, the time-scaling factor that brings one dissolution curve into another is equal to the factor with which the rate parameters are proportionally related in the two systems* (Fig. 1).

Consider two particle-size distributions that on a logarithmic scale are distributed $l_1(\log a)$ and $l_2(\log a)$, having the same shape. Then, for any diameter, a (Fig. 2) $l_1(\log a) = l_2(\log a + \log K_a)$;

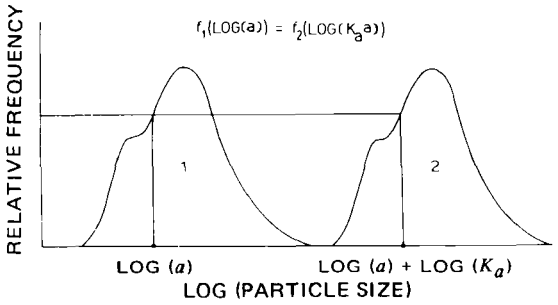

Figure 2—*Two particle-size distributions having the same shape on a logarithmic scale. According to Rule 3, these distributions result in dissolution curves with the same intrinsic dissolution profile (for example, curves 1 and 2 of Fig. 1).*

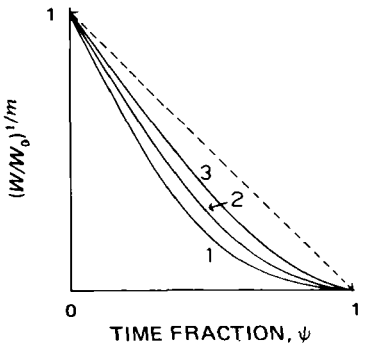

Figure 3—*Normalized dissolution profiles of powders initially log-normal* ($\sigma = 0.2$, i = j = 2), *calculated (Eqs. 16 and 19) to dissolve and plotted according to Models 1, 2, and 3: Model 1,* m = 3/2; *Model 2,* m = 2; *and Model 3,* m = 3. *The stippled line represents monodispersed powders* ($\sigma = 0$).

i.e., $l_1(\log a) = l_2(\log K_a a)$, where $\log K_a$ is a measure of the distance between the logarithmic distributions. Therefore, if the particles in System 2 are scaled (measured) in units of K_a, the resulting distribution is the same as that for System 1. Such a scaling of the particle sizes has an effect on the calculated dissolution profile.

However, if the coefficient of time in the single-particle dissolution equation is dimensionally dependent on length, as in a model that is not first order, then a scaling in length, as in the above transformation of the particle-size distribution, has the same effect as a scaling in time. If the single-particle dissolution is a first-order process, then the dissolution profile is completely independent of the particle-size distribution, because the fraction of each particle that dissolves in a given time is the same, independent of its size. Therefore, it can be concluded that Systems 1 and 2 have the same intrinsic dissolution profile, and the following rule can be given:

3. *Two powders dissolving according to the same single-particle dissolution model have the same intrinsic dissolution profile if their particle-size distributions are of the same shape on a logarithmic scale* (Fig. 2).

It follows from this rule that it is not the "position" of the distribution, that is, not the actual size of the particles, but the shape of the distribution that affects the intrinsic dissolution profile. Thus it can be stated that:

4. *The intrinsic dissolution profile does not depend on the actual sizes of the particles but on the shape of their distribution.*

RESULTS AND DISCUSSION

According to Rule 2, the concept of time scaling in dissolution studies should have some practical application. It already has been stated that it is impossible to prepare completely monodispersed powders; therefore, some nonlinearity always is present in the dissolution profile due to the distribution effect (9). This nonlinearity

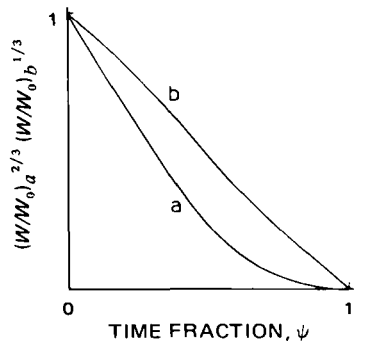

Figure 4—*Normalized dissolution profiles of powders initially log-normal* ($\sigma = 0.14$, i = j = 2), *calculated (Eq. 16) to dissolve and plotted according to Model 1 (curve a). Curve b is the same data plotted according to Model 3, resulting in almost complete cancellation of the size distribution effect observed in curve a.*

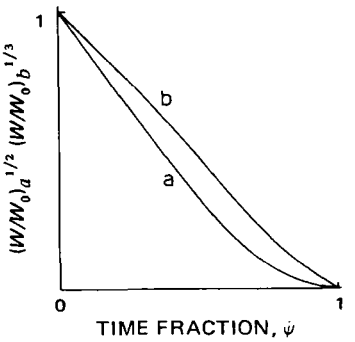

Figure 5—*Normalized dissolution profiles of powders initially log-normal (σ = 0.12, i = j = 2), calculated (Eq. 19, m = 2) to dissolve and plotted according to Model 2 (curve a). Curve b is the same data plotted according to Model 3, resulting in almost complete cancellation of the size distribution effect observed in curve a.*

introduces errors into determination of the influence of factors such as the stirring rate, temperature, and vehicle composition on the rate parameter (k_1, k_2, or k_3) using conventional "initial slope" or "line of best fit" computational techniques.

However, Rule 2 provides an alternative approach where this type of error is negligible. The new technique simply involves finding a time-scaling factor that brings one curve into the other or, more correctly, minimizes the separation of the curves. This factor is then equal to the factor by which the two rate parameters are proportionally related. Possibly the best criterion is to minimize the squared horizontal differences between the curves.

According to Rules 1 and 3, it should be possible to normalize the calculated dissolution profiles for log-normal powders by appropriate scaling of time to a form that does not depend on either the rate parameter (k_1, k_2, or k_3) or the actual sizes of the particles. One approach is to scale the time in the W/W_0 *versus* time plots as the time fraction, ψ, defined as the fraction of the time necessary for complete dissolution. The expression defining the resulting normalized dissolution profile can be obtained in the following way, using Models 2 and 3 as examples. The time for complete dissolution, t_0, is given by $(m/3)\ \ln\ (Kt_0) = \mu + j\sigma$ (Eqs. 9 and 15); then since $\psi = t/t_0$, it follows that:

$$\psi = Ke^{-\frac{3}{m}(\mu + j\sigma)}t \qquad \text{(Eq. 18)}$$

Accordingly, the Kt terms in Eq. 15 can be substituted by

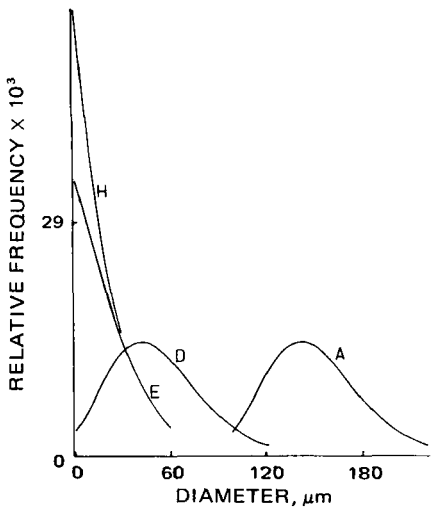

Figure 6—*Size distribution change with time for a powder initially log-normal (μ = 5, σ = 0.2, i = j = 2), calculated (Eq. 9, m = 3) to dissolve according to Model 3. Distributions are labeled in chronological order. Key: A, initial distribution; and D, distribution at critical time.*

$\psi^{3(\mu+j\sigma)/m}$, which causes the μ terms to cancel out. After rearrangement, Eq. 15 can be written:

$$\frac{W}{W_0} = \sum_{n=0}^{m}\binom{m}{n}(-\psi)^{(m-n)}\frac{F\left(j - \frac{3n\sigma}{m}\right) - F(A)}{F(j - 3\sigma) - F(-i - 3\sigma)} \times e^{\frac{3}{m}(n-m)(-j\sigma + \frac{3}{m}(n+m)\sigma^2/2)} \qquad \text{(Eq. 19)}$$

where:

$$A = i - \frac{3n\sigma}{m} \qquad \text{for } 0 \le \psi < e^{-\frac{3}{m}(i+j)m} \qquad \text{(Eq. 20)}$$

and:

$$A = j + \frac{m \ln \psi}{3\sigma} - \frac{3n\sigma}{m} \qquad \text{for } e^{-\frac{3}{m}(i+j)m} \le \psi \le 1 \qquad \text{(Eq. 21)}$$

It is seen that the normalized dissolution profile (Eq. 19, m = 2 or m = 3) does not contain any rate terms (k_3 or k_2 from Eq. 6 or 7) or any term (μ) representing the sizes of the particles[1]. Scaling of time according to Eq. 18 has brought all dissolution curves originating from distributions with the same "logarithmic shape" (which is completely defined by parameters σ, i, and j) into one single curve (Eq. 19) which does not depend on the sizes of the particles or the rate parameter from the single-particle equation. This finding confirms Rules 1 and 3.

The transformation has essentially normalized all possible systems having the same intrinsic dissolution profile into one single curve. This curve is unique in that it makes it possible to evaluate the isolated distribution effect. This evaluation is best done by plotting in a way that linearizes the underlying single-particle dissolution equation (Eqs. 6–8), by using $(W/W_0)^{1/m}$ instead of W/W_0 in the plot. Such a plot will be linear with slope = −1 for a true monodispersed system. Any deviation from this linearity and slope will be due solely to the distribution effect.

Figure 3 shows such normalized dissolution profiles, calculated according to Eqs. 16 and 19, for powders initially log-normal, having distribution (shape) parameters σ = 0.2 and $i = j = 2$, for particles dissolving according to each of the three models[2] (Eqs. 6–8). The fraction of undissolved powder, W/W_0, is raised to the powers of 1/3, 1/2, and 2/3 for the reason just given. The distribution effect is smallest for Model 3 and greatest for Model 1. Remarkably good linearity is observed for the first part of the dissolution process for all three models. This result does not mean, however, that the distribution effect is negligible in the beginning, as is seen from the fact that the slopes differ considerably from −1.

It is obvious that good linearity in such plots is no necessary guarantee that the powder is monodispersed or that there is no distribution effect. This error has been made frequently in investigations where the dissolution process is not followed to the end or very near the end. Furthermore, the validity of a particular dissolution model cannot always be assessed solely from the linearity of a plot of data according to that model *even when the dissolution is followed to the very end.* This fact is clearly demonstrated in Figs. 4 and 5.

Curve a in Fig. 4 shows a normalized dissolution profile of log-normal powders (σ = 0.14, $i = j = 2$) calculated (Eq. 16) to dissolve and plotted (W/W_0 to power of 2/3) according to Model 1. The size distribution effect is clearly reflected in the nonlinearity of the curve. By plotting the same data according to an incorrect model, Model 3 (W/W_0 to power of 1/3), the size distribution effect is almost entirely canceled and surprisingly good linearity is obtained *that extends to the very end of the dissolution process* (curve b). Figure 5 shows the same phenomenon for powders (σ = 0.12, $i = j$ = 2) where the particles are calculated to dissolve according to Model 2 (Eq. 19, m = 2).

A judgment based solely on the linearity of such plots will often

[1] The normalized dissolution profile can also be obtained directly from Eq. 15 or 16 by choosing an arbitrary value for μ and calculating W/W_0 for different Kt values, ranging from 0 to $e^{3(\mu+j\sigma)/m}$, corresponding to a set of ψ values (Eq. 18). This technique was applied for Model 1 using Eq. 16. The computations were done for a wide range of μ values and showed independence of this parameter, in agreement with theory.

[2] A CDC Cyber 76 digital computer, equipped with calcomp plotter, was used for calculations and plots. Numerical evaluations were tested to six digits.

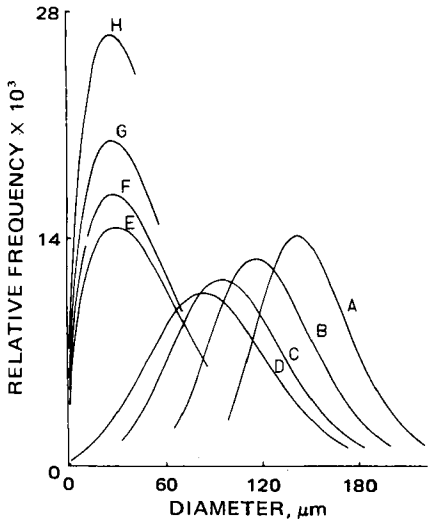

Figure 7—*Size distribution change with time for a powder initially log-normal ($\mu = 5$, $\sigma = 0.2$, i = j = 2), calculated (Eq. 9, m = 2) to dissolve according to Model 2. Distributions are labeled in chronological order. Key: A, initial distribution; and D, distribution at critical time.*

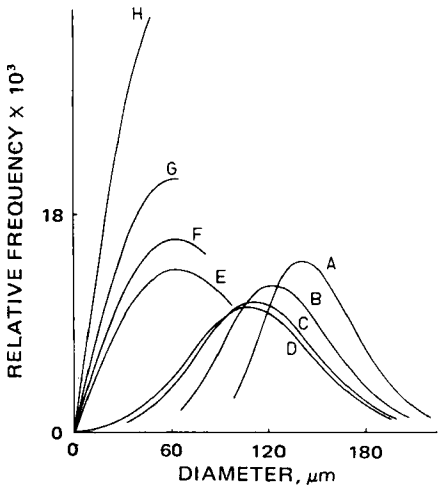

Figure 8—*Size distribution change with time for a powder initially log-normal ($\mu = 5$, $\sigma = 0.2$, i = j = 2), calculated (Eq. 9, m = 3/2) to dissolve according to Model 1. Distributions are labeled in chronological order. Key: A, initial distribution; and D, distribution at critical time.*

lead to false conclusions about the validity of the model, even where dissolution is followed to completion, unless an analysis of particle size is made. The phenomena demonstrated in Figs. 4 and 5 are not special cases for the particular values of σ chosen but were observed to apply for a wide range of σ values.

These findings clearly emphasize the importance of particle-size analysis in investigations of dissolution kinetics. It is of interest to see how the particle-size distribution changes during dissolution. The distribution of a powder that is initially log-normal ($\mu = 5$, $\sigma = 0.2$, $i = j = 2$) was calculated at various times according to Eq. 9 and plotted in Figs. 6, 7, and 8, illustrating dissolution according to Models 3, 2, and 1, respectively. The distributions are labeled in chronological order from A to H. Curves A and D represent the distributions initially and at critical time, respectively.

When particles dissolve according to Model 3 (Fig. 6), then the shape of the size distribution remains constant before critical time, consistent with isotropic dissolution ($da/dt = -K_3$). This is not the case for dissolution according to Models 1 and 2 where the absolute rate of change in size of the particles, da/dt, increases with time. For Model 1:

$$da/dt = -\frac{K_1}{2}(a_0^2 - K_1 t)^{-1/2} \qquad \text{(Eq. 22)}$$

and for Model 2:

$$da/dt = -\frac{2}{3}K_2(a^{3/2} - K_2 t)^{-1/3} \qquad \text{(Eq. 23)}$$

As a result, the distribution broadens before critical time (Figs. 7 and 8) and is particularly affected at the small particle end as zero is approached where da/dt takes extreme values. Because of the latter effect, near the end of the dissolution process the relative frequency of the very small particles increases with increasing size for Models 1 and 2 (Figs. 8 and 7) while it decreases for Model 3 (Fig. 6). This information should be of value for distinguishing Model 3 from the two others. However, from the distribution change alone, it is difficult to distinguish between Models 2 and 3.

It appears from this discussion that it is not possible to distinguish clearly between all three models from dissolution data or from particle-size measurements alone. It would be of interest to determine whether this could be done if both types of information are combined quantitatively. One convenient combination seems to have potential for discriminating between the models. It will be called the dispersion product, s, and is defined as:

$$s = \left(\frac{\bar{a}}{\bar{a}_0}\right)^3 \times \frac{W_0}{W} \qquad \text{(Eq. 24)}$$

where $\bar{a}$ and $\bar{a}_0$ are the mean particle diameters at times t and 0, respectively. The dispersion product is readily obtained, requiring only information for the fraction dissolved and simple averages from particle-size measurements. Time-consuming evaluations of distribution parameters are not required. It is a dimensionless variable that depends only on the initial distribution parameter, σ (for fixed i and j), and the single-particle dissolution equation[3]. The variation of s during the dissolution period should for this reason reflect the basic equation.

Figure 9 shows this variation for powders initially log-normally distributed ($\sigma = 0.2$, $i = j = 2$). The curves representing the three dissolution models are significantly different. The basic shape of the curves remains the same for varying values of σ, although the minima shift to the right and to higher values for very narrow distributions. All three curves approach $s = 1$ (stippled line) when σ approaches zero as expected for a completely monodispersed powder. The values of the three minima remain approximately con-

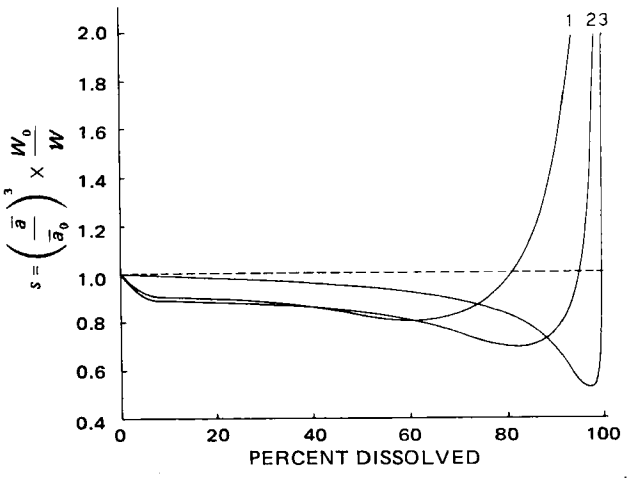

Figure 9—*Variation of the dispersion product (Eq. 24), with progress of dissolution of powders initially log-normal ($\sigma = 0.2$, i = j = 2), calculated (Eqs. 13, 14, 16, and 19) to dissolve according to Models 1, 2, and 3. The stippled line represents a monodispersed powder ($\sigma = 0$).*

[3] The numerical evaluation of the dispersion product was made using several widely different arbitrarily chosen values for μ and confirmed that this parameter cancels out in the calculations as expected. It is clear from previous discussions that s does not depend on the value of the rate parameter (k_1, k_2, or k_3 in Eqs. 6–8).

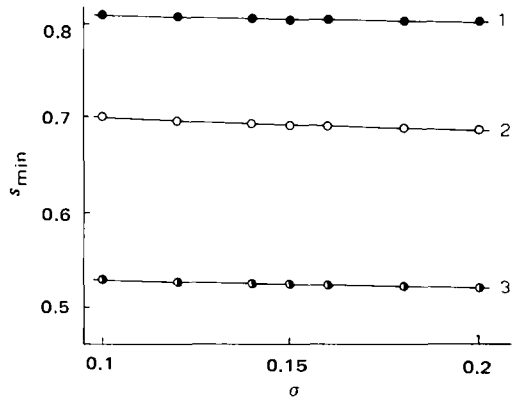

Figure 10—*Variation of dispersion product minima, s_{min}, with the initial distribution parameter, σ, for dissolution according to Models 1, 2, and 3* (i = j = 2).

stant when σ ranges from somewhat less than 0.1 to at least 0.2 (Fig. 10), which should encompass most fine powder distributions encountered in practice. Theoretically, the considerable difference between the s_{min} values should make it possible to distinguish clearly between the three models.

The dispersion product profile (s *versus* W/W_0 or percent dissolved) has some powerful properties, since it is a dimensionless quantity dependent only on the *shape* of the distribution and the *form* of the single-particle dissolution equation. Any change in the coefficient of time k_1, k_2, or k_3 (Eqs. 6–8) during the dissolution will have no effect on the s profile, because s is essentially parametrically represented by the time, provided the change is the same for all particles irrespective of their size. Thus, if changes in such conditions as temperature, agitation, or vehicle composition during dissolution have identical influence on all particles, these changes will have no effect on the s profile. Alternatively, if the external conditions are maintained constant, then s should indicate whether the coefficients are independent of particle size and, hence, whether such parameters as the interfacial concentration gradient, shape factor, and interfacial reaction rate are independent of particle size. The dispersion product should, therefore, be a valuable tool in dissolution kinetic studies.

The extent to which these mathematical models can be applied to describe the dissolution of a "real" powder depends on three assumptions.

1. As mentioned earlier, it was assumed that the particles dissolve independently of each other. This should be approximated well under sink conditions.

2. It was assumed that the dissolution of each particle in the powder can be described by an equation having the same parameter value (k) for all of the particles. In practice, this assumption is rarely valid because of differences in individual particle shapes, crystal structure, and interaction with the vehicle. However, these types of effects probably can be averaged to produce a parameter value for the single-particle dissolution model which, when used in the multiparticulate dissolution equation, results in a good approximation of the actual multiparticulate dissolution behavior.

3. It was assumed that the initial particle-size distribution can be approximated by a truncated log-normal distribution. Although it is generally accepted that most powders are approximately "log-normal," it is likely that the log-normal distribution function only provides a coarse approximation of the actual particle-size distribution, which usually contains a number of irregularities.

This paper has dealt only with log-normal powders and three dissolution models. However, Eq. 3, together with Eq. 13 from the previous paper (9), makes it possible to calculate dissolution characteristics for a powder of any initial size distribution, or a sieve fraction of such a powder, considering any single-particle dissolution equation of explicit form.

SYMBOLS

w = weight of dissolving particle at time t
w_0 = initial weight of particle
$W = \sum w$ = weight of undissolved powder at time t
$W_0 = \sum w_0$ = initial weight of powder
a = diameter of dissolving particle at time t
a_0 = initial diameter of particle
$\bar{a}$ = mean particle diameter at time t
$\bar{a}_0$ = initial mean particle diameter
t = time
$N(x,\mu,\sigma) = (1/\sigma\sqrt{2\pi})e^{(x-\mu)^2/2\sigma^2}$ = normal distribution function
μ = mean of normal distribution
σ = standard deviation of normal distribution
i, j = lower and upper truncation parameters for the initial size distribution that is log-normal and truncated at $\ln a_0 = \mu - i\sigma$ and $\ln a_0 = \mu + j\sigma$
d_0 = min a_0 ($\ln d_0 = \mu - i\sigma$)
D_0 = max a_0 ($\ln D_0 = \mu + j\sigma$)
$F(x) = \int_{-\infty}^{x} (1/\sqrt{2\pi})e^{-x^2/2}\,dx$ = area under standard normal distribution function
$w = g(w_0,t)$ = particle dissolution function (for example, Eqs. 6–8)
$w_0 = g^{-1}(w,t)$ = inverse particle dissolution function
$l(a)$ = particle-size density distribution at time t
$l_0(a_0)$ = initial particle-size density distribution
Model 1 = $w = (w_0^{2/3} - k_1 t)^{3/2}$
Model 2 = $w = (w_0^{1/2} - k_2 t)^2$
Model 3 = $w = (w_0^{1/3} - k_3 t)^3$
ψ = time fraction = fraction of time necessary for complete dissolution
m = 3/2, 2, and 3 for Models 1, 2, and 3, respectively
k_i = parameter in model, i = 1–3
$K_i = (6/\rho\pi)^{1/m} k_i$
$s = (\bar{a}/\bar{a}_0)^3 \times W_0/W$, dispersion product
$\binom{m}{n} = m!/[n!\,(m-n)!]$, binomial coefficient
P = operator that is equal to 1 in the time period before the operand becomes equal to zero and is equal to zero after that time

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Elementary Osmotic Pump

FELIX THEEUWES

Abstract □ The elementary osmotic pump is a new delivery system for drugs or other active agents; it delivers the agent by an osmotic process at a controlled rate. Control resides in the: (*a*) water permeation characteristics of a semipermeable membrane surrounding the formulated agent, and (*b*) osmotic properties of the formulation. In its simplest embodiment, the system is constructed by coating an osmotically active solid agent with the rate-controlling, semipermeable membrane. This membrane contains an orifice of critical size through which solubilized agent is dispensed. The system can contain the agent in solid form at loadings higher than 90% of the total volume, and the agent can be delivered at rates several orders of magnitude higher than can be achieved by solution diffusion through polymeric membranes. The delivery rate, the fraction of total content delivered at zero order, and the system's delivery portal size have been calculated for delivery of a single compound. Experimental work verified the theory. The release rate from the system was found to be independent of outside agitation when the system is not deformed by shaking action, the pH of the environment, and delivery portal size for sizes within a specified range. The delivery rate from this system *in vitro* and in the GI tract of dogs was found to be equal.

Keyphrases □ Delivery systems, drug—elementary osmotic pump, controlled delivery rate, release characteristics, equations □ Drug delivery systems—elementary osmotic pump, controlled delivery, release rates, equations □ Osmotic pump—osmotic process, controlled rate drug delivery system, release rates, equations □ Membrane permeation—osmotic process, controlled rate drug delivery system

To achieve controlled administration of active agents, delivery mechanisms that can provide desired temporal patterns of the delivery rate have attracted great interest. The mechanism of delivering the active species by solution diffusion through a rate-controlling barrier has been found to be flexible and dependable (1) but limited in magnitude [maximum on the order of 0.2 μg/(cm hr)] unless microporous barriers are used. To overcome these low rates and to permit the delivery of water-soluble species, an osmotic system was developed which delivers at membrane-controlled rates that can be several orders of magnitude higher than the drug diffusional rates. This new system is called the elementary osmotic pump (2). This paper describes such systems and gives examples of their characteristics for single-compound delivery.

The principles discussed here are applicable to systems using water or other solvents, but the reported work involved only water.

The elementary osmotic pump consists of an osmotic core containing the drug, surrounded by a semipermeable membrane with a delivery orifice. A cross section of the system is shown in Fig. 1.

When exposed to water, the core imbibes water osmotically at a controlled rate, determined by the membrane permeability to water and by the osmotic pressure of the core formulation. For a system at a constant internal volume, the device delivers, in any time interval, a volume of saturated solution equal to the volume of solvent uptake. The rate of solute delivery by the system is constant as long as excess solid is present inside the device, but the rate declines parabolically toward zero once the concentration falls below saturation. The solution volume and mass solute delivery rate from the elementary osmotic pump can be predicted from the physicochemical parameters defining the system, as will be discussed. A typical release rate obtained from this system is illustrated in Fig. 2.

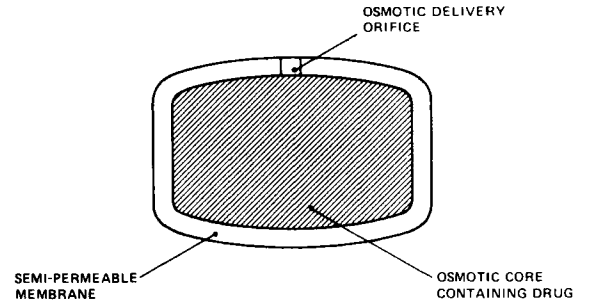

Figure 1—*Elementary osmotic pump cross section.*

THEORETICAL

Delivery Rate—The delivery of agent from the system is controlled by the solvent influx across the semipermeable membrane, which in turn carries the agent to the outside (Fig. 1). Liquid transport by osmosis was qualitatively discussed by Starling (3), who identified the osmotic and hydrostatic pressure differences across capillary membranes as important factors governing transcapillary fluid transport. The process was rigorously treated in the field of nonequilibrium thermodynamics; Eq. 1, which describes the volume flux, dV/dt, across semipermeable membranes has been basic to the field of reverse osmosis (4):

$$\frac{dV}{dt} = \frac{A}{h} L_p(\sigma \Delta\pi - \Delta P) \qquad \text{(Eq. 1)}$$

where $\Delta\pi$ and ΔP are the osmotic and hydrostatic pressure differences, respectively, between the inside and outside of the system; L_p is the mechanical permeability; σ is the reflection coefficient; A is the membrane area; and h is the membrane thickness.

Equation 1 also describes the water flux into the elementary os-

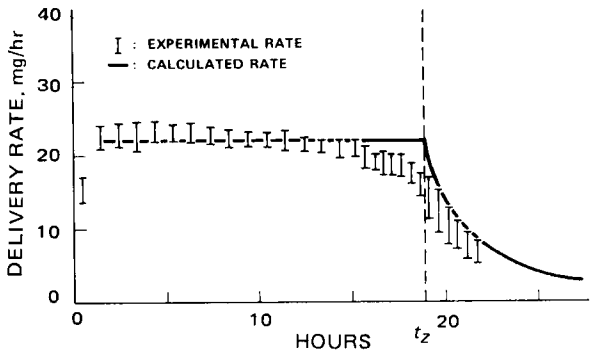

Figure 2—In vitro *release rate of potassium chloride from elementary osmotic pumps in water at 37°. Key:* I, *range of experimental data obtained from five systems; and* —, *calculated release rate.*

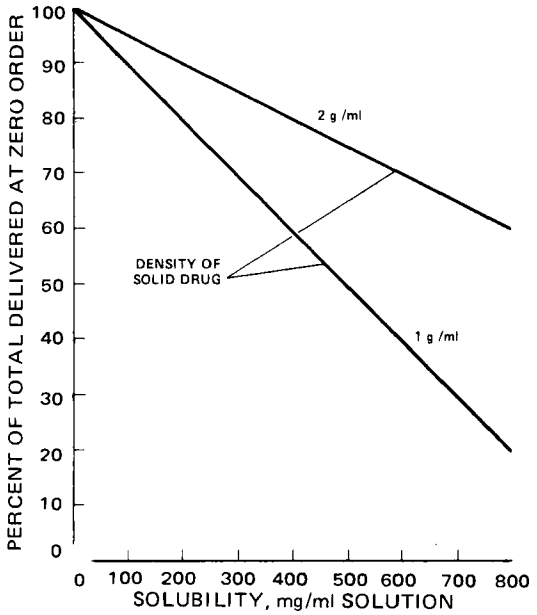

Figure 3—*Fraction of drug content delivered from the elementary osmotic pump at constant rate.*

motic pump (Fig. 1). The general expression for the solute delivery rate, dm/dt, obtained by pumping through the orifice is described by:

$$\frac{dm}{dt} = \frac{dV}{dt} C \qquad \text{(Eq. 2)}$$

where C is the concentration of compound in the dispensed fluid expressed per unit volume of solution.

Substituting Eq. 1 into Eq. 2 results in Eq. 3, which most broadly describes the solute delivery rate:

$$\frac{dm}{dt} = \frac{A}{h} L_p(\sigma \Delta\pi - \Delta P)C \qquad \text{(Eq. 3)}$$

As the delivery orifice increases, hydrostatic pressure inside the system is minimized as expressed by the condition $\Delta\pi \gg \Delta P$.

When the osmotic pressure of the formulation (π) is large compared to the osmotic pressure of the environment, π can be substituted for $\Delta\pi$. Equation 3 then reduces to a much simpler expression in which the constant k replaces the product $L_p\sigma$:

$$\frac{dm}{dt} = \frac{A}{h} k\pi C \qquad \text{(Eq. 4)}$$

Zero-Order Delivery Rate—The release rate from the elementary osmotic pump is zero order from $t = 0$ until a time t_z, at which time all of the solid in the core has dissolved and is described by:

$$\left(\frac{dm}{dt}\right)_z = \frac{A}{h} k\pi_s S \qquad \text{(Eq. 5)}$$

where S is the solubility, and π_s is the osmotic pressure at saturation.

The rate of dissolution of a single compound within the system is much larger than the rate of pumping as given by Eq. 5. For this reason, the concentration, C, can be replaced by the component solubility, S, from time $t = 0$ to $t = t_z$.

Nonzero-Order Release Rate—The nonzero-order release rate from the system (Eq. 4) is obtained by describing the concentration, C, as a function of time. For simplicity, the volume flux into the system is replaced by the symbol F:

$$F = \frac{A}{h} k\pi \qquad \text{(Eq. 6)}$$

and F_s represents the flux during the zero-order time and is related to F by:

$$\frac{F_s}{F} = \frac{\pi_s}{\pi} = \frac{S}{C} \qquad \text{(Eq. 7)}$$

By substituting Eq. 7 into Eq. 4, the nonzero-order release rate as a function of concentration is given by:

$$\frac{dm}{dt} = \frac{F_s}{S} C^2 \qquad \text{(Eq. 8)}$$

Beyond t_z, the mass, m, of component dissolved into the elementary pump volume, V, is given by:

$$m = CV \qquad \text{(Eq. 9)}$$

The change in mass at constant volume, V, causes a concentration change, dC/dt, given by:

$$\frac{dm}{dt} = -V\frac{dC}{dt} \qquad \text{(Eq. 10)}$$

The delivery rate, dm/dt, can be eliminated between Eqs. 8 and 10 as shown by:

$$-\frac{dC}{dt} = \frac{F_s}{VS} C^2 \qquad \text{(Eq. 11)}$$

The concentration, C, inside the system is obtained by integrating Eq. 11 from time t_z to t, when the concentration changes from S to C:

$$-\int_S^C \frac{dC}{C^2} = \frac{F_s}{VS}\int_{t_z}^t dt \qquad \text{(Eq. 12)}$$

Solving Eq. 12 and rearranging terms result in an expression for the concentration as a function of time:

$$C = \frac{VS}{V + F_s(t - t_z)} \qquad \text{(Eq. 13)}$$

Substituting Eq. 13 into Eq. 8 gives the release rate as a function of time, indicating the parabolic decline:

$$\frac{dm}{dt} = \frac{F_s S}{\left[1 + \frac{F_s}{V}(t - t_z)\right]^2} \qquad \text{(Eq. 14)}$$

The nonzero-order release rate can also be expressed as a fraction of the zero-order rate:

$$\frac{dm}{dt} = \frac{(dm/dt)_z}{\left[1 + \frac{1}{SV}\left(\frac{dm}{dt}\right)_z (t - t_z)\right]^2} \qquad \text{(Eq. 15)}$$

The delivery rate discussed in this section is the rate from the elementary osmotic pump when most of the contents are delivered by pumping. When the membrane is not ideally semipermeable, a fraction of the agent is delivered by diffusion through the membrane. The case involving both pumping and diffusion is treated in the *Appendix*.

Mass Delivered at Zero Order, m_z, and Zero-Order Delivery Time, t_z—For a total mass, m_t, contained in the core of the elementary osmotic pump, only an amount m_z is delivered at zero order, and an amount m_{NZ} is delivered at a parabolically declining rate given by Eq. 14. The amount m_{NZ} is the mass that just fills the internal volume of the system with a saturated solution, as shown by:

$$m_{NZ} = SV \qquad \text{(Eq. 16)}$$

The internal volume, V, of the system containing a pure component is related to the total mass, m_t, by the density, ρ, of the core by:

$$m_t = \rho V \qquad \text{(Eq. 17)}$$

The fraction not delivered at zero order is obtained from Eqs. 16 and 17 and given by:

$$\frac{m_{NZ}}{m_t} = \frac{S}{\rho} \qquad \text{(Eq. 18)}$$

Since the sum of m_{NZ} and m_z is equal to m_t, the fraction of the total mass delivered at zero order is given by:

$$\frac{m_z}{m_t} = 1 - \frac{S}{\rho} \qquad \text{(Eq. 19)}$$

The fraction of total device content expressed in percent calculated from Eq. 19 is shown in Fig. 3 for two different compound densities as a function of compound solubility.

The time t_z at which the mass m_z is delivered for an ideal system, with zero startup time, is obtained from:

$$\frac{m_z}{t_z} = \left(\frac{dm}{dt}\right)_z \qquad \text{(Eq. 20)}$$

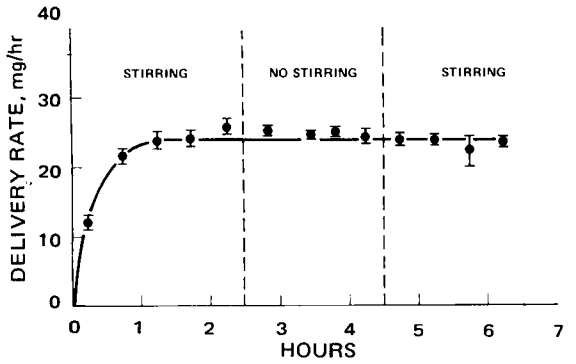

Figure 4—In vitro *release rate of potassium chloride from elementary osmotic pumps in water at 37°. The vertical dashed lines indicate the time at which the systems were transferred from a stirred to a stagnant medium and back to a stirred medium.* ⧳ *represents the range of experimental data obtained from five systems.*

Combining Eqs. 19 and 20 gives:

$$t_z = m_t \left(1 - \frac{S}{\rho}\right) \frac{1}{(dm/dt)_z} \qquad \text{(Eq. 21)}$$

Size of Delivery Orifice—The size of the delivery orifice must satisfy two conditions:

1. It must be smaller than a maximum size, A_{max}, to minimize the contribution to the delivery rate made by solute diffusion through the orifice.

2. It must be sufficiently large, above a minimum size, A_{min}, to minimize hydrostatic pressure inside the system that would affect the zero-order release rate in the following ways. Hydrostatic pressure within the system not only decreases the osmotic influx as seen from Eq. 1, but also it can increase the volume of the system. During the time that the system volume is increasing, the outflow would be smaller than the inflow, resulting in a depressed delivery rate.

Mathematically, these two conditions can be expressed by $A_{min} \leq A_0 \leq A_{max}$, where the cross-sectional area of the orifice, A_0, is larger than or equal to a minimum value and smaller than or equal to a maximum value.

The minimum cross-sectional area can be estimated from Poiseuille's law (4):

$$A_{min} = 5 \left(l \frac{dV}{dt} \frac{\eta}{\Delta P_{max}}\right)^{1/2} \qquad \text{(Eq. 22)}$$

where dV/dt is the volume flux through the orifice, l is the length of the orifice, η is the viscosity of the dispensed solution, and ΔP_{max} is the maximum hydrostatic tolerated pressure difference between the inside and outside of the device. The ΔP_{max} is the pressure at which deformation of the membrane housing occurs or that is significant with respect to the osmotic driving pressure, whichever is smallest.

The maximum cross-sectional area allowed, A_{max}, is obtained by imposing the condition that the diffusional contribution to the release rate must be a factor, F, smaller than the zero-order pumping rate as defined by Eq. 5. This condition is expressed by:

$$A_{max} = \frac{l}{F} \left(\frac{dm}{dt}\right)_z \frac{1}{DS} \qquad \text{(Eq. 23)}$$

in which D is the diffusion coefficient of the compound being delivered in the solvent within the orifice.

In practice, perfect membrane-controlled osmotic delivery has been obtained when $F \geq 40$, as will be discussed.

EXPERIMENTAL

Potassium Chloride Elementary Osmotic Pump Fabrication—Systems were prepared containing 500 mg of potassium chloride each, described by the following parameters: $V = 0.25$ cm^3, $h = 0.025$ cm, $A = 2.2$ cm^2, $k\pi_s = 0.686 \times 10^{-3}$ cm^2/hr, $P = 0.122 \times 10^{-3}$ cm^2/hr, $S = 330$ mg/ml, and $\rho = 2$ g/ml.

The total mass was compressed in a hard tablet and the volume, V, was calculated assuming the density, ρ (5). The area, A, was calculated from the tablet geometry, and the membrane thickness

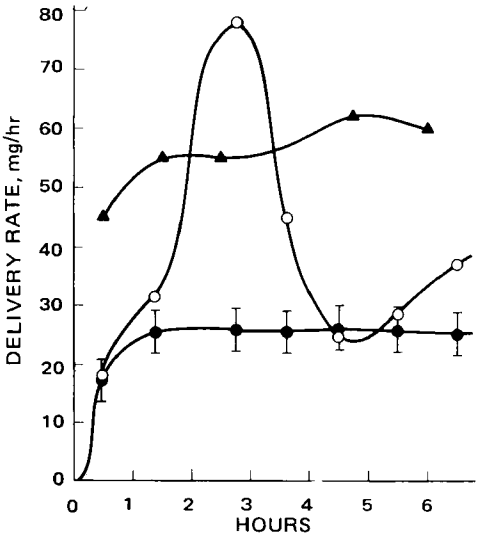

Figure 5—In vitro *release rate of potassium chloride from elementary osmotic pump systems in water at 37°. Key:* ⧳, *range of experimental data obtained from systems with cylindrical orifices of diameters 75, 128, 190, and 274 μm;* ○, *system with 435-μm orifice diameter; and* ▲, *system with 368-μm orifice diameter.*

was measured using a standard thickness gauge. The solubility, S, at 37° was determined by electrical conductance measurements after dilution of the filtered saturated solution into the concentration range where a calibration curve was constructed.

The permeability coefficient, P, and the product, $k\pi_s$, were determined in a permeation experiment (4) where the membrane was placed in a diffusion cell separating water from the stirred saturated solution of potassium chloride. The permeability coefficient, P, was calculated, by measuring the salt transported across the membrane by electrical conductance, from an equation of the form of the second term of Eq. A3. The product, $k\pi_s$, was calculated from Eq. 6, where the volume flow was determined by the displacement of the meniscus of the saturated solution in a graduated cylinder mounted on the cell half containing the saturated solution during a time interval, Δt, measured with a stopwatch.

***In Vitro* Delivery Rate Measurements**—The release rate from the systems was obtained by transferring each at regular, usually hourly, intervals from one test tube to the following and measuring the amount released in each test tube. All experiments were carried out at 37° in the fluids indicated on the figures. The potassium chloride systems were agitated with a stroke of 2.5 cm at a frequency of 0.5 stroke/sec for the experiments shown in Figs. 2, 4, 5, and 6. The larger phenobarbital sodium elementary osmotic pumps referred to in Fig. 7 were manually transferred to sequential flasks in which the liquid was stirred constantly.

Potassium chloride concentrations were measured by electrical conductance. Phenobarbital sodium concentrations were measured spectrophotometrically.

***In Vivo* Delivery Rate Measurements**—Color-coded 500-mg

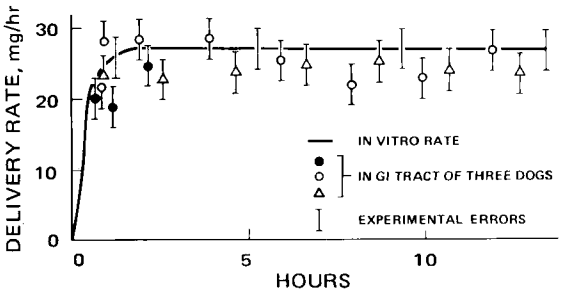

Figure 6—In vitro *and* in vivo *release rate of potassium chloride from elementary osmotic pumps. Key:* —, *average* in vitro *rate from systems of the same batch; and* △, ○, ●, *average release rate of one system in the GI tract of Dogs 1, 2, and 3, plotted at the total time period each system resided in the dog.*

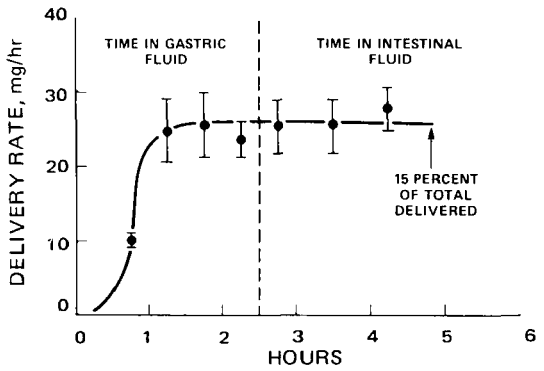

Figure 7—In vitro *release rate of phenobarbital sodium from elementary osmotic pump systems in gastric and intestinal fluid USP without enzymes. The vertical dashed line indicates the time at which the systems were transferred from gastric to intestinal fluid.* ⧳ *represents the range of experimental data obtained from three systems.*

potassium chloride elementary osmotic pump systems were administered to three dogs at recorded time intervals. The dogs were sacrificed, the devices were retrieved, and the *in vivo* rates were determined gravimetrically from the initial and final dry weights of each individual system.

RESULTS AND DISCUSSION

Predictability of Delivery Rate—For elementary osmotic pump systems containing 500 mg of potassium chloride and prepared as already described, the predicted pumping rate, as calculated from Eq. 5, was 20 mg/hr. By correcting for the 20% diffusional contribution, as shown by Eq. A4 (*Appendix*), the release rate was found to be 24 mg/hr.

When assuming one adjustable parameter, $(dm/dt)_z$ = 22 mg/hr, as the observed experimental rate, the zero-order time, t_z = 19 hr, was calculated from Eq. 21. When using the experimental zero-order rate, the nonzero-order tail was calculated from Eq. 15 (solid line in Fig. 2). An identical curve was obtained by accounting for the diffusional contribution (treated in *Appendix*) by calculating the nonzero-order rate from Eqs. A5 and A13. The data are represented within the limits of experimental error by these equations. For simplicity, the system is well described by the equations applying to pumping when the diffusional contribution is not more than 20%.

Because of its osmotic action, the system exhibits two important characteristics which are described in the next two subsections.

Delivery Rate Is Independent of Outside Agitation—The release rates of five similar potassium chloride systems were measured sequentially in (*a*) stirred, (*b*) stagnant (no stirring), and (*c*) stirred media. The release rate was measured by the mass delivered during about 30-min intervals (Fig. 4). During stirring, the systems were moved up and down with a stroke of 2.5 cm and a frequency of 0.5 stroke/sec in water at 37°. The devices were kept immobile during the stagnant period. As can be seen in Fig. 4, the release rate under both conditions was identical.

Delivery Rate Is Independent of pH—Because of the semipermeable characteristics of the membrane, ions are not readily exchanged across it and the formulation contained within the device can be programmed at a pH independent from its environment. A compound with a solubility that is highly pH dependent can be incorporated in the water-soluble salt form. During operation, the internal core of the elementary pump keeps the pH created by this salt form as its *in situ* created formulation. The delivery rate from the system, as explained previously, is governed by the osmotic pressure of the formulation and the water permeability of the membrane and is therefore independent of the pH of the environment.

As an example, the average release rate from three elementary osmotic pump systems delivering phenobarbital sodium in artificial gastric (pH 2) and intestinal fluid (pH 7.5) (without enzymes) is shown in Fig. 7. The release rate was independent of the pH of the delivery medium.

Delivery Rate Is Independent of Delivery Port Sizes within Predictable Limits—According to $A_{min} \leq A_0 \leq A_{max}$, the delivery rate should be independent of the delivery orifice size for sizes within the range expressed by Eqs. 22 and 23. For pharmaceutical dosage forms where the orifice length is on the order of the membrane thickness, $l = h \simeq 25 \times 10^{-4}$ cm, η = 1 cps, $(dV/dt) \simeq 0.1$ ml/hr, and $\Delta P \simeq 1$ atm, the minimum orifice area calculated from Eq. 22 is on the order of $A_{min} \simeq 13 \times 10^{-8}$ cm^2. For a cylindrical hole, the diameter is then 4×10^{-4} cm—difficult or impossible to be drilled.

To find an operational delivery orifice, it is of practical importance to calculate only the upper size, A_{max}, as defined by Eq. 23. The sizing factor, F, has been experimentally determined by measuring the release rate as a function of the hole size. In Fig. 5, the release rate from 500-mg potassium chloride elementary osmotic pumps is plotted for various hole sizes. Complete membrane-controlled delivery was observed for all hole diameters, 75, 128, 190, and 274×10^{-4} cm. At 368×10^{-4} cm and above, control over the delivery rate was lost. The transition from membrane control to the occurrence of diffusion and probably convection within the orifice was dramatic. No systematic trend in delivery was observed within the hole size range from 75 to 274 μm.

The sizing factor, F, was calculated from Eq. 23 assuming A_{max} as the orifice area corresponding to the 274-μm diameter hole. The length of the passageway through the membrane was $l = 25 \times 10^{-4}$ cm; $(dm/dt)_z$ = 25 mg/hr, as seen from Fig. 5; S = 330 mg/ml; and diffusion coefficient $D = 2 \times 10^{-5}$ cm^2/sec (5). The sizing factor was found to be F = 40.

Delivery Rate in GI Tract Is Equal to *In Vitro* Rate—The elementary pump system can be broadly applied to controlled delivery. A number of pharmaceutical applications such as implants, inserts in body cavities, or oral delivery come to mind, since all of these applications provide an environment of constant water activity.

The system is of special interest in oral application because high, predictable delivery rates can be obtained independent of GI motility and the pH of luminal fluids. An additional benefit is the system's delivery of the drug in solution ready for absorption. Thus, the elementary osmotic pump is an *in situ* prepared liquid dosage form.

In the example shown in Fig. 6, the *in vivo* functionality of the system is demonstrated in the GI tract of dogs. Each experimental point represents the average *in vivo* release rate from each system during its total residence time in the GI tract. The data points are plotted at the total residence time observed for the device. The solid line is the *in vitro* release rate.

The *in vitro* delivery profile was measured for systems of the same batch in water at 37°. The average *in vivo* delivery rate was found to be systematically lower by about 10%. The *in vivo* data agree with the *in vitro* data within the combined experimental error of 20% (6).

CONCLUSIONS

1. The mode of operation of the elementary pump is well understood, and the *in vitro* delivery rate from the system can be accurately predicted.
2. The fraction of drug delivered at zero order can be predicted from the compound solubility and core density.
3. The delivery rate is independent of: (*a*) the pH of the environment, (*b*) the agitation of the environment, and (*c*) the size of the orifice for orifices within the predictable range.
4. The *in vivo* delivery rate from the system is essentially equal to the predictable *in vitro* delivery rate.

APPENDIX

The release rate from the elementary osmotic pump is the total mass delivered per unit time from this system. In practice, three mechanisms contribute to the delivery: (*a*) delivery by pumping, $(dm/dt)_P$, which was described previously; (*b*) delivery by diffusion through the orifice, $(dm/dt)_{DO}$; and (*c*) delivery by diffusion through the membrane, $(dm/dt)_{DM}$. The total delivery rate is then given by:

$$\left(\frac{dm}{dt}\right)_t = \left(\frac{dm}{dt}\right)_P + \left(\frac{dm}{dt}\right)_{DO} + \left(\frac{dm}{dt}\right)_{DM} \qquad \text{(Eq. A1)}$$

By design of the system, the diffusion through the orifice was selected to be negligible, as expressed by $A_{min} \leq A_0 \leq A_{max}$, and Eq. A1 reduces to:

$$\left(\frac{dm}{dt}\right)_t = \left(\frac{dm}{dt}\right)_P + \left(\frac{dm}{dt}\right)_{DM} \quad \text{(Eq. A2)}$$

For most agents that have a high molecular weight and/or are ionic, the membrane will appear as ideally semipermeable and a negligible amount of agent will be delivered by diffusion through it.

When delivering low molecular weight substances and when the membrane is sufficiently solvated, the second term in Eq. A2 can become important. The total release rate (Eq. A2) is controlled by the same membrane and, as before, a zero-order and nonzero-order pattern will be observed.

Zero-Order Delivery by Pumping and Diffusion—The total zero-order rate is given by the sum of two terms: the pumping rate expressed by Eq. 5 and the diffusional term expressed by Fick's law:

$$\left(\frac{dm}{dt}\right)_{t,z} = \frac{A}{h} k\pi_s S + \frac{A}{h} PS \quad \text{(Eq. A3)}$$

where P is the permeability coefficient. Equation A3 reduces to:

$$\left(\frac{dm}{dt}\right)_{t,z} = \frac{A}{h} S(k\pi_s + P) \quad \text{(Eq. A4)}$$

which is of the same form as Eq. 5 for the ideal case.

In practice, the zero-order rate can be calculated from Eq. A4 or 5 after defining an effective $k\pi_s$ or solubility number.

Nonzero-Order Delivery by Pumping and Diffusion—The nonzero-order delivery rate is given by the sum of the pumping rate, Eq. 8, and the diffusional term:

$$\frac{dm}{dt} = \frac{F_s}{S} C^2 + \frac{A}{h} PC \quad \text{(Eq. A5)}$$

To express dm/dt as a function of time, the concentration, C, inside the system must be expressed as a function of time. This can be done by solving the differential Eq. A6 derived from Eq. A5 by substituting again Eq. 10 for dm/dt:

$$-\frac{dC}{dt} = \frac{F_s}{VS} C^2 + \frac{AP}{Vh} C \quad \text{(Eq. A6)}$$

Then substituting:

$$R = \frac{F_s}{VS} \quad \text{(Eq. A7}a\text{)}$$

$$Q = \frac{AP}{Vh} \quad \text{(Eq. A7}b\text{)}$$

into Eq. A6 results in:

$$\frac{dC}{C(RC + Q)} = -dt \quad \text{(Eq. A8)}$$

Equation A8 can be integrated by fractions after identifying the coefficients α and β as shown in:

$$\frac{1}{C(RC + Q)} = \frac{\alpha}{C} + \frac{\beta}{RC + Q} \quad \text{(Eq. A9)}$$

Equalizing the coefficients of equal powers of C results in:

$$\alpha = \frac{1}{Q} \quad \text{(Eq. A10)}$$

$$\beta = -\frac{R}{Q} \quad \text{(Eq. A11)}$$

Substituting Eqs. A10 and A11 into Eq. A9 and substituting Eq. A9 into Eq. A8 result in:

$$\frac{dC}{QC} - \frac{R}{Q}\frac{dC}{(RC + Q)} = -dt \quad \text{(Eq. A12)}$$

Equation A12 can be integrated from time t_z to t when the concentration changes from S to C. Also, replacing R and Q by their values given in Eq. A7 results in:

$$t - t_z = \frac{Vh}{AP} \ln \frac{\left(F_s \frac{C}{S} + \frac{AP}{h}\right)}{\left(F_s + \frac{AP}{h}\right)\frac{C}{S}} \quad \text{(Eq. A13)}$$

Equation A13 does not give the concentration, C, as an explicit function of time for substitution into Eq. A5. From Eq. A13, C and C^2 can be tabulated as a function of time, and from these values the rate can be calculated using Eq. A5.

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New and Expedient Determination of Atenolol in Biological Samples

J. O. MALBICA ˣ and K. R. MONSON

Abstract □ A method is described for the GLC determination of atenolol BP in plasma and urine. Extraction is accomplished under dehydrating conditions, and interfering impurities are removed by using an acidified cyclohexane–isopropanol mixture (2:1) and charcoal-treated paper disks. The drug thus isolated appears to react more efficiently with heptafluorobutyric anhydride, increasing the sensitivity of GLC electron-capture analysis. Concentrations as low as 0.02 μg/ml were measured using 0.5-ml aliquots of plasma or 0.1 ml of urine. Amino alcohols such as atenolol may form hydrates or alcoholates, precluding complete derivatization with heptafluorobutyric anhydride.

Keyphrases □ Atenolol—GLC analysis, biological samples □ GLC—analysis, atenolol, biological samples □ Adrenergic agents—atenolol, GLC analysis, biological samples

Atenolol[1] [4-(2-hydroxy-3-isopropylaminopropoxy)phenylacetamide] (I) is a new β-adrenergic blocking agent. The results of animal studies indicate that atenolol differs from other β-blocking drugs currently available for study in humans because of its cardioselectivity, absence of intrinsic sympathomimetic activity, and membrane-stabilizing activity (1–4). The compound may be valuable as an antihypertensive agent in humans (5, 6).

A GC method for the determination of atenolol in whole blood, serum, tissue, and urine was recently reported (7). The method required an initial aqueous–solvent extraction followed by purification steps involving back-extraction techniques. This extraction procedure was tedious, and a great chance for error resulted from the number of transfer steps involved. As a result, a simplified extraction procedure was developed.

The extraction procedure for both plasma and urine, as developed in this laboratory, is performed under dehydrating conditions. It is both expedient and efficient, requiring only 0.5 ml of plasma or blood and 0.1 ml or less of urine. The method is of sufficient sensitivity (0.02 μg/ml) to determine plasma levels of atenolol in humans after a single 25-mg oral dose.

EXPERIMENTAL

Reagents—Ethyl acetate[2], cyclohexane[2], isopropyl alcohol[2], and acetone[2] were glass-distilled, reagent grade solvents. Acetic acid[3], ammonium hydroxide[3] (14.8 *N*), anhydrous sodium sulfate[3], and anhydrous ether[3] were all reagent grade, ACS certified.

Heptafluorobutyric anhydride was freshly prepared from heptafluorobutyric acid[4] (sequenation grade) by refluxing over excess phosphorus pentoxide[5] for 3 hr. The mixture was then fractionally distilled through a 25.4-cm (10-in.) Vigreux column, and the anhydride was collected at 107–108°. The acidic wash solvent was prepared by adding 128 ml of isopropyl alcohol to a mixture of 250 ml of cyclohexane and 5 ml of acetic acid.

Table I—Effect of Acetone in Removing Solvate from Atenolol Residue prior to Reaction with Heptafluorobutyric Anhydride[a]

Original Solution	Relative GLC Peak Area	
	Control	Acetone
Water	0.589	0.955
Absolute ethanol	0.603	0.955

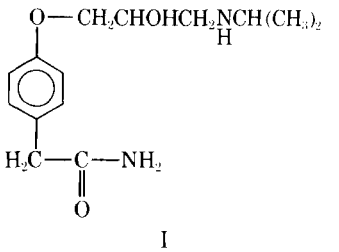
[a] Concentration of atenolol was 0.4 μg/ml. Conditions were as described in the text.

Plasma Level Study—Atenolol was administered to two healthy male volunteers[6]. Subject 1 (24 years, 75.5 kg) received a 25-mg tablet[7], and Subject 2 (31 years, 71.9 kg) received a 100-mg tablet[7]. Blood was withdrawn at various time intervals from an indwelling needle attached to a plastic catheter[8] in a superficial vein of the forearm. The blood was collected in oxalated containers and centrifuged, and the plasma was transferred and stored in plastic vials at −10°. Urine samples were also collected during 24-hr intervals within a 72-hr period and pooled.

Extraction of Atenolol—A premeasured volume of anhydrous sodium sulfate (1.0 ml) was added to a 15.0-ml graduated conical centrifuge tube containing 0.5 ml of plasma. The tube was mixed[9], and 2.5 ml of the acidic wash solvent was added to the resulting paste. The tube was again mixed until the solid sodium sulfate was dispersed and then was centrifuged at 2000 rpm in an angular head, clinical centrifuge for 3 min.

The organic phase was removed and discarded using a disposable Pasteur pipet. One drop (0.05 ml) of 14.8 *N* NH_4OH was added to the residue, followed by 4.0 ml of freshly distilled ethyl acetate. The tube was mixed, and two 6.5-mm diameter charcoal-treated paper disks[10] were added. After mixing for 30 sec, the tube was centrifuged as before.

The ethyl acetate layer was carefully transferred, using a Pasteur pipet, into a tube and flash evaporated *in vacuo*[11] to dryness. To the residue was added 0.4 ml of heptafluorobutyric anhydride–ether (1:4). After reacting at ambient temperature for 5 min, the contents of the tube were dried under a nitrogen stream (using an in-line activated silica gel desiccant filter) for 15 min. The residue was dissolved in 1.0 ml, or some other suitable volume, of freshly distilled ethyl acetate. Atenolol extracts from plasma or urine that



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[1] British Pharmacopoeia approved name.
[2] Burdick-Jackson Labs., Inc., Bodman Chemicals, Narberth, Pa.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] Eastman Kodak, Rochester, N.Y.
[5] Fisher Scientific Co., Fairlawn, N.J.
[6] Plasma and urine samples were obtained from Dr. R. F. Maronde, University of Southern California School of Medicine.
[7] Preliminary experimental formulations.
[8] Butterfly-19, Abbott Laboratories.
[9] Vortex Genie, Fisher Scientific Co.
[10] Made with a paper punch-out from S & S charcoal-treated filter paper No. 505.
[11] Evapo-Mix, Buchler Instruments, Division of Searle Analytic Inc., Fort Lee, N.J.

Table II—Peak Area (GLC) of Atenolol Extracted from Plasma at Different Concentrations and Times of Day over a 3-Day Period[a]

Atenolol, μg/ml	Day 1		Day 2		Day 3	
	am	pm	am	pm	am	pm
0.2	0.338	0.295	0.283	0.233	0.368	0.341
0.3	0.383	0.435	0.379	0.421	0.391	0.398
0.4	0.559	0.477	0.447	0.453	0.435	0.455
0.6	0.724	0.702	0.628	0.571	0.622	0.556

[a] Each value represents a separate plasma extraction and is the average of a duplicate run on GLC. The maximum volume of atenolol used to spike plasma was 25 μl.

were not reacted immediately after extraction were stored in a desiccator over phosphorus pentoxide.

Urine—A fresh solution of urease[12] was prepared just before use (25 mg/ml, approximately 3500 units/g in 1 *M* KH_2PO_4 buffer, pH 7.4). To 0.5 ml of diluted urine (1:5 dilution with distilled water) in a 15-ml conical centrifuge tube was added 0.2 ml of the urease solution. The mixture was then incubated at 30° for 10 min. After incubation, the mixture was analyzed as previously described for plasma except that 1.2 ml of premeasured anhydrous sodium sulfate was used instead of 1.0 ml.

Electron-Capture GLC—The derivatized samples were analyzed using a gas chromatograph[13] equipped with a ^{63}Ni-electron-capture detector. A glass column (1.2 m × 6 mm o.d.) was packed with a 3.8% UCW 98 on Gas Chrom Q, 80–100 mesh. The injection port and oven temperatures were maintained at 225 and 208°, respectively. The detector temperature was set at 320° and operated with a pulse interval of 150 μsec. The carrier gas was argon with 5% methane at a flow rate of 60 ml/min. Under these conditions, the retention time of the atenolol derivative was approximately 3 min. The injection volume was 2 μl.

Calculations—A stock solution of atenolol in absolute ethanol (1.0 mg/ml) was used to prepare daily working standards (0.02–1.0 μg/ml) by spiking plasma or urine obtained from undosed controls. From these standards, a calibration curve of concentration *versus* peak area was determined. The overall recovery of atenolol from plasma or urine was determined by comparing standards extracted from plasma or urine by the described procedure with nonextracted acetone-stripped standards. The latter procedure involved flash evaporating a standard solution *in vacuo*[11], then adding 5.0 ml of acetone, and flash evaporating once again. This procedure with acetone was repeated one more time. The stock solution of atenolol was stored at 5°, protected from light, and was stable for several months.

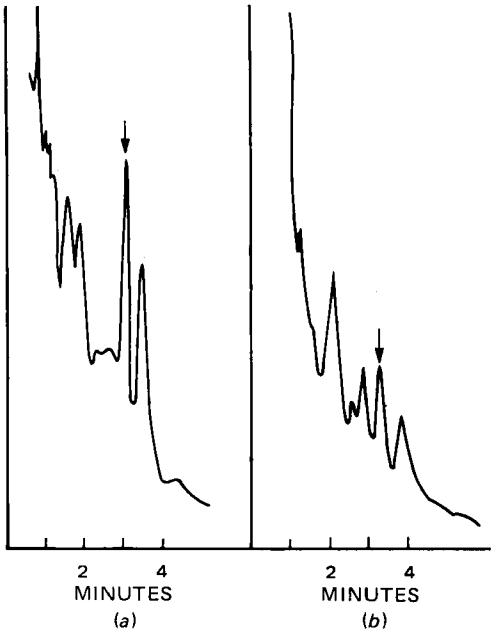

Figure 1—*(a) GLC trace of extract from plasma containing 0.10 ng/μl of atenolol. (b) GLC trace of extract from urease-treated urine containing 0.05 ng/μl of atenolol. (Position of atenolol is designated by the arrow.)*

[12] Sigma, Type III.
[13] Hewlett-Packard HP7620A.

Table III—Slope and Intercept Variations within the Day and from Day to Day

Time	Day 1	Day 2	Day 3
am:			
Slope	1.02	0.85	0.65
Intercept	0.12	0.11	0.21
pm:			
Slope	0.98	0.79	0.54
Intercept	0.11	0.12	0.24
Average:			
Slope	1.00	0.82	0.59
Intercept	0.11	0.12	0.22

RESULTS AND DISCUSSION

The GLC peak of the heptafluorobutyrate derivative of atenolol isolated from human plasma and urine is shown in Fig. 1. The GLC recorder trace for the blank plasma and urine is shown in Fig. 2. The electron-capture detector response was very high for this derivatized amino alcohol. The structure of the atenolol heptafluorobutyrate derivative was confirmed by mass spectrometry (7), which showed that the secondary alcohol was esterified and the amino group was acylated. The amide group on the phenyl ring was dehydrated on reaction with heptafluorobutyric anhydride to form the nitrile.

The relative GLC peak area was diminished significantly if an aqueous or ethanol solution of atenolol was reacted with heptafluorobutyric anhydride without prior removal of solvate from residue with acetone, as described under *Experimental*. Typical data are shown in Table I; the GLC peak area was enhanced as much as

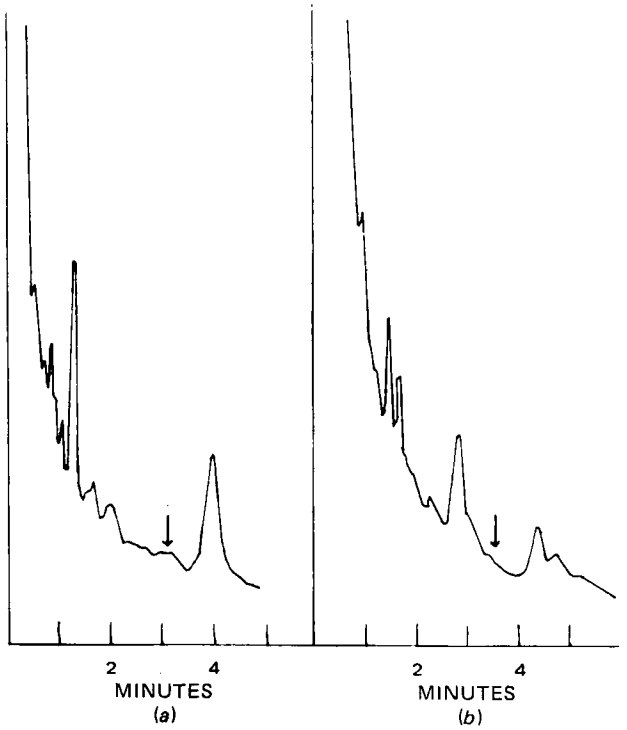

Figure 2—*(a) GLC trace of extract from plasma blank. (b) GLC trace of extract from urine blank. (Position of atenolol is designated by the arrow.)*

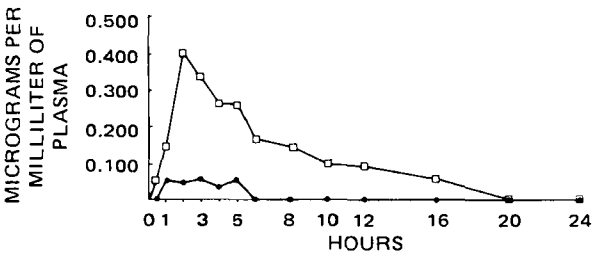

Figure 3—*Plasma concentrations of atenolol in two subjects administered a single oral dose. Key:* ●, *Subject 1, 25 mg; and* □, *Subject 2, 100 mg.*

38% by using acetone to remove solvate.

The overall recovery of atenolol extracted from plasma or urine was 60 ± 4% over the concentration range of 0.05–1.0 μg/ml[14]. This range is comparable to what was previously reported (7). A consistent 27–30% of the drug, independent of the concentration, was lost in the initial wash step. Attempts to minimize this loss by using solvents of different polarities resulted in an increase in interfering impurities on GLC. Preextraction with the acidic wash solvent resulted in the removal of some plasma components which, if carried to the derivatization step, would elute from the GLC column approximately 50 min after an initial sample injection. Other interfering plasma impurities were found to absorb preferentially onto charcoal-coated paper disks.

The somewhat lower recoveries (approximately 40–50%) obtained using the aqueous–solvent extraction procedure previously reported (7) may be attributed to an apparent hydrated form of the amino alcohol and also possibly to a less efficient extraction procedure.

Plasma and urine samples containing atenolol (0.05 and 0.2 μg/ml) and stored at −10° were stable for at least 1 month, with no significant change in concentration noted.

The high and variable concentration of urea in urine interfered with the heptafluorobutyric anhydride derivatization step. Pretreating a urine aliquot with urease and incubating for the prescribed period eliminated this interference.

Within-day and day-to-day variation and reproducibility of the extraction procedure were evaluated at four different concentrations of atenolol in plasma (Table II). From these data, the slope and intercept variations within the day and from day to day were obtained (Table III). As described, the am and pm slopes within the day were not significantly different. However, the day-to-day slope and intercept variation were significantly different ($p < 0.01$). For this reason, a fresh control plasma standard calibration curve was prepared daily. Calibration plots were strictly linear over the range of 0.0–1.0 ng injected onto the column.

[14] Based on aqueous standard, dried *in vacuo*, and stripped with acetone as described in the text prior to reacting with heptafluorobutyric anhydride.

Table IV—Atenolol Excreted in Urine after a Single Oral Dose

Subject	Dose, mg	Atenolol Excreted, mg			Percent of Dose
		0–24 hr	24–48 hr	48–72 hr	
1	25	7.15	0.39	0.06	30.4
2	100	25.15	0.92	0.13	26.2

Plasma levels of atenolol (preliminary experimental formulation) administered to two human volunteers following a single oral dose are shown in Fig. 3. The amount of atenolol excreted in urine over 72 hr is shown in Table IV. A 25-mg oral dose resulted in plasma levels close to the limit of detection (0.02 μg/ml).

The method described is sensitive for the determination of atenolol concentrations as low as 0.02 μg/ml in plasma or urine. It offers several advantages over aqueous–solvent extractions, employing back-extraction techniques. First, the number of transfer steps is kept to a minimum by extracting under dehydrating conditions. Second, under these same conditions, an apparent hydrate of atenolol is prevented from forming, thus favoring a more complete reaction with heptafluorobutyric anhydride.

Acetone was effective in removing apparent bound water or alcohol to drug molecules when prepared for standard use. Preliminary data indicate that this phenomenon may be peculiar only to amino alcohols when used for derivatization by heptafluorobutyric anhydride.

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Comparative Pharmacokinetics of Coumarin Anticoagulants XV: Relationship between Pharmacokinetics of Dicumarol and Warfarin in Rats

AVRAHAM YACOBI, CHII-MING LAI, and GERHARD LEVY ˣ

Abstract □ The distribution, elimination, and anticoagulant effect of dicumarol and warfarin were determined in adult male rats following intravenous injection of single doses of these drugs in crossover experiments. The biological half-life of dicumarol ranged from 5 to 28 hr; that of warfarin ranged from 9 to 30 hr. There was a statistically significant correlation between the following pharmacokinetic characteristics of dicumarol and warfarin in individual animals: biological half-life, apparent volume of distribution, total plasma clearance, and concentration in plasma eliciting one-half the maximum anticoagulant effect (effective concentration). The mean ratio of the respective biological half-lives (warfarin/dicumarol) was 1.42, and that of the apparent volumes of distribution was 1.50. The ratio of the effective plasma concentrations (dicumarol/warfarin) was correlated negatively with the half-life of dicumarol and positively with the ratio of the half-life values (warfarin/dicumarol) in individual animals. Additional studies with serum samples from other rats showed pronounced interindividual differences in the serum protein binding of both dicumarol and warfarin and a strong correlation between the protein binding of these two drugs in serum of individual animals. The results of this study, together with the results of previous studies in this series, indicate that serum protein binding is the major determinant of interindividual differences in the pharmacokinetics of dicumarol and warfarin in rats under these experimental conditions.

Keyphrases □ Dicumarol—distribution, half-life, elimination, anticoagulant effect determined, compared to warfarin, rats □ Warfarin—distribution, half-life, elimination, anticoagulant effect determined, compared to dicumarol, rats □ Coumarin anticoagulants—comparative pharmacokinetics of dicumarol and warfarin, rats □ Anticoagulants—comparative pharmacokinetics of dicumarol and warfarin, rats

The relationship between the elimination kinetics of different drugs in the same subjects has been the subject of a number of recent studies (1–4). The purposes of these studies were to explore the possible existence of common biotransformation pathways and genetic influences and to assess the feasibility of determining the elimination kinetics of one drug and using the results to predict the elimination kinetics of one or more other drugs in the same individual. The cited studies were limited generally to determinations of the biological half-life of the drugs. The study reported here was designed to obtain more comprehensive information about the relationship between the pharmacokinetic characteristics of two major anticoagulants, dicumarol and warfarin, in the same animals. Crossover studies were carried out in rats to determine the apparent volume of distribution, biological half-life, serum protein binding, and anticoagulant effect of these two drugs.

Table I—Biological Half-Life of Dicumarol (D) and Warfarin (W) in Individual Rats

Rat	Half-Life, hr		Ratio of Half-Lives (W/D)	Half-Life of Dicumarol in Screening Experiment, hr
	Dicumarol	Warfarin		
1[a]	5.12	9.72	1.90	4.75
2	6.54	8.89	1.36	5.96
3[a]	6.79	10.1	1.49	7.99
4[a]	11.6	16.7	1.44	11.3
5[a]	13.5	24.5	1.82	15.2
6	15.4	15.4	1.00	10.7
7	15.9	19.6	1.23	13.1
8	27.9	30.5	1.09	23.8
Mean			1.42	

[a] These animals received dicumarol first.

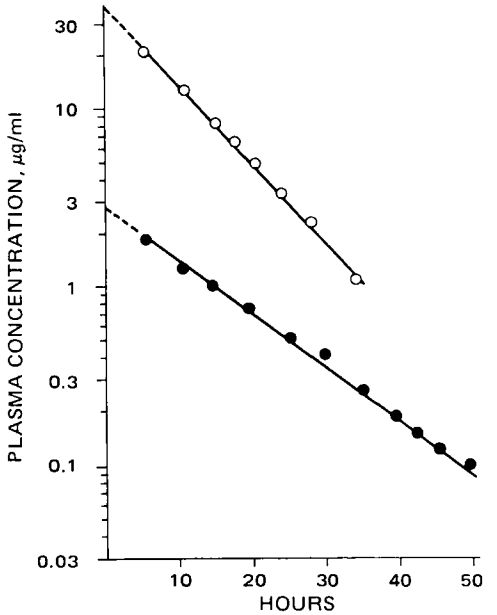

Figure 1—*Concentrations of dicumarol (○) and warfarin (●) in the plasma of a rat (Rat 3) as a function of time after administration of dicumarol, 6 mg/kg iv, and warfarin, 0.6 mg/kg iv, in separate experiments 3 weeks apart.*

EXPERIMENTAL

Thirty male Sprague–Dawley rats[1], 270–390 g, with unrestricted access to food[2] and water at all times, received ^{14}C-dicumarol, 6 mg/kg iv, in an initial screening experiment. The biological half-life of dicumarol was determined from the time course of drug concentrations in plasma (5). Eight animals, with dicumarol half-lives of 4.8–23.8 hr, were selected for further study 3 weeks later. The purpose of this selection process was to obtain a group of animals with a wide and relatively even distribution of dicumarol half-lives.

A crossover experiment was carried out in which four rats received dicumarol, 6 mg/kg iv, including about 30 μCi/kg of ^{14}C-dicumarol (10.9 μCi/mg). The other four rats received warfarin, 0.6 mg/kg iv, including about 26 μCi/kg of ^{14}C-warfarin (71 μCi/mg).

[1] Blue Spruce Farms, Altamont, N.Y.
[2] Charles River formula 4RF.

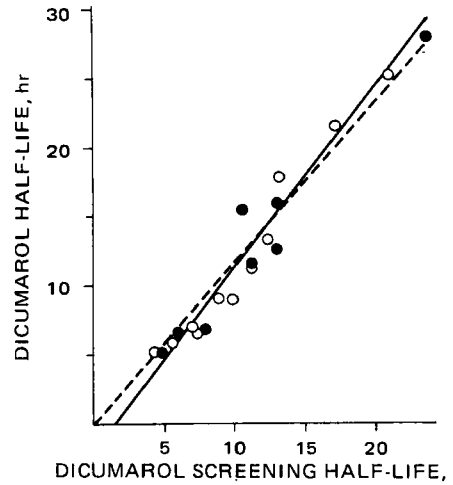

Figure 2—*Relationship between the biological half-life of dicumarol in the initial screening experiment and in the subsequent crossover study on the same animals (●). The open circles represent data for control animals from another study under the same conditions (to be published) (r = 0.967, p < 0.001). The continuous regression line was obtained by double regression analysis (11), and the stippled line was forced through the origin in this figure and in Figs. 3, 4, and 7.*

Three weeks later, the first group of animals received warfarin and the second received dicumarol. Serial blood samples (0.45 ml) were obtained after injection until the prothrombin complex activity returned to 65–100% of normal. Determinations of prothrombin complex activity, dicumarol and warfarin concentrations, and pharmacokinetic calculations were carried out as previously described (5–7).

Serum protein binding of dicumarol and warfarin was determined by equilibrium dialysis at 37° (8). Portions of serum from unmedicated individual rats were spiked with ^{14}C-warfarin (about 1 μg/ml) or ^{14}C-dicumarol[3] (about 20 μg/ml). A total of 21 rats was used. Warfarin concentrations were determined by scintillation counting after extraction and TLC (6, 7). Dicumarol concentrations were determined by scintillation counting after selective extraction (5) of samples from 10 rats and by scintillation counting after TLC of samples from the other 11 animals.

^{14}C-Dicumarol determinations by TLC were carried out by adding 0.1 ml of the serum or 1 ml of the dialysate sample to 0.1 ml of an aqueous solution of unlabeled dicumarol (400 μg/ml) in 0.15 *M* isotonic phosphate buffer, pH 7.4. This solution was acidified with 1 ml of 0.5 *N* hydrochloric acid and extracted into 2.5 ml of ethylene dichloride. After shaking for 45 min, the phases were separated by centrifugation at 1000×*g* for 3 min and the aqueous phase was removed by aspiration.

A 2-ml portion of the organic phase was placed in a vial[4] and evaporated at room temperature to dryness under a flow of nitrogen. The sides of the vial were washed down three times with chloroform, which was also evaporated. The residue was dissolved in 25 μl of chloroform and spotted on a silica gel TLC sheet[5] which had been activated (30 min at 100°) immediately before use. The vial was rinsed three times with 25-μl portions of chloroform, which were also applied to the TLC sheet. The sheet was developed immediately with toluene–ether–acetic acid (20:5:1) in a chamber which had been equilibrated with the solvent system. The development was stopped when the solvent front had advanced about 10 cm.

The dicumarol spot on the dried plate was visualized under UV light and identified as such by its R_f value (0.65). It was scraped off the TLC sheet and placed in a vial with 10 ml of scintillation fluid, and the radioactivity was determined[6] at 15° using an external standard. The recovery of dicumarol from serum samples con-

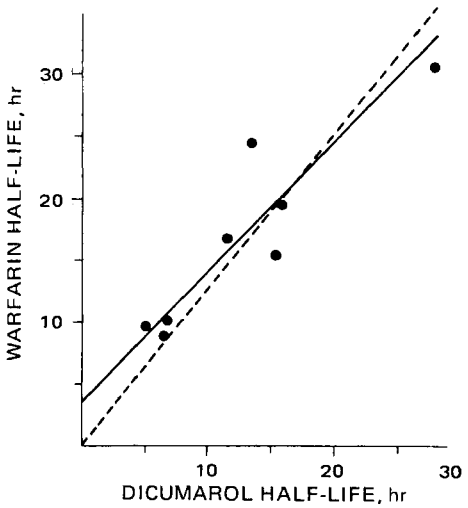

Figure 3—*Relationship between the biological half-life of dicumarol and warfarin in individual rats in the crossover study (r = 0.910, p < 0.005).*

Table II—Apparent Volume of Distribution and Total Plasma Clearance of Dicumarol (D) and Warfarin (W) in Individual Rats

Rat	Apparent Volume of Distribution, ml/kg			Total Plasma Clearance, ml/hr/kg		
	Dicumarol	Warfarin	Ratio (W/D)	Dicumarol	Warfarin	Ratio (W/D)
1	177	309	1.75	24.0	22.1	0.92
2	182	323	1.77	19.1	25.2	1.32
3	155	230	1.48	15.8	15.8	1.00
4	164	185	1.13	9.79	7.66	0.78
5	140	172	1.23	7.18	4.86	0.68
6	171	234	1.37	7.66	10.5	1.37
7	134	199	1.49	5.83	7.06	1.21
8	106	197	1.82	2.64	4.47	1.69
Mean			1.50			1.12

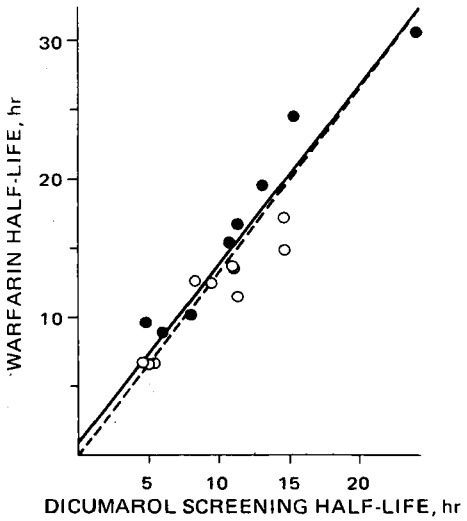

Figure 4—*Relationship between the biological half-life of dicumarol in the initial screening experiment and the biological half-life of warfarin in the subsequent crossover study on the same animals (●). The open circles represent data from another study (7) carried out under similar conditions except that the warfarin dose was 12 mg/kg (r = 0.936, p < 0.001).*

[3] Specific activity of 61.7 μCi/mg.

[4] Reacti-Vial, Pierce Chemical Co., Rockford, Ill.
[5] Silica gel F-254 TLC sheets, EM Laboratories, Elmsford, N.Y.
[6] Packard scintillation spectrometer, model 3320.

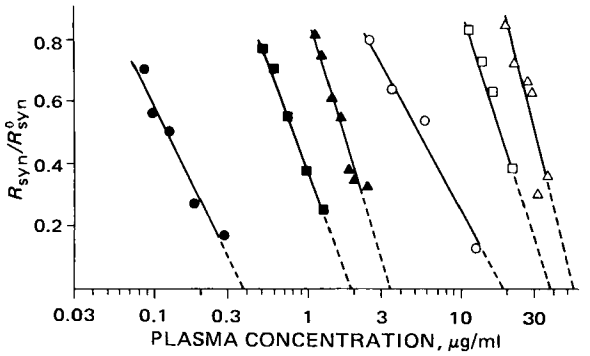

Figure 5—*Relationship between the anticoagulant effect (relative synthesis rate of prothrombin complex activity, expressed as R_{syn}/R^0_{syn}) and the concentrations of dicumarol (open symbols) and warfarin (solid symbols) in Rat 1 (circles), Rat 4 (squares), and Rat 8 (triangles).*

taining 0.01–48 μg/ml was independent of drug concentration and averaged 90.8 ± 2.4% (mean ± *SD*, n = 14). In preliminary experiments, excellent agreement was found between results when dicumarol-spiked serum samples were analyzed by both the selective extraction and TLC methods.

RESULTS

Figure 1 shows the time course of dicumarol and warfarin concentrations observed in one animal in separate experiments, 3 weeks apart. The biological half-life ($t_{1/2}$) of dicumarol in the individual rats ranged from 4.75 to 23.8 hr in the initial screening experiment and from 5.12 to 27.9 hr in the crossover study, while the $t_{1/2}$ of warfarin ranged from 8.89 to 30.5 hr (Table I). The apparent volume of distribution (V_d) of dicumarol in the crossover study ranged from 106 to 182 ml/kg, and the V_d of warfarin ranged from 172 to 323 ml/kg (Table II). There was a statistically significant positive correlation between the apparent first-order elimination rate constant (k_{el}) and V_d for dicumarol (r = 0.721, $p < 0.05$) and warfarin (r = 0.878, $p < 0.005$).

Figure 2 shows the relationship between the $t_{1/2}$ of dicumarol in the screening experiment and in the subsequent crossover study on the same animals. Included in the figure are 11 data points from control rats of another study. The correlation was very strong, and the average ratio of the individual $t_{1/2}$ values (crossover experiment/screening experiment) was 1.09. There was a strong and statistically highly significant correlation between the $t_{1/2}$ for dicumarol and warfarin in the crossover study (Fig. 3) and between the $t_{1/2}$ for dicumarol in the screening study and the $t_{1/2}$ for warfarin in the subsequent crossover study on the same animals (Fig. 4). The ratio of the warfarin $t_{1/2}$ to the dicumarol $t_{1/2}$ in the individual rats ranged from 1.00 to 1.90, with a mean of 1.42 (Table I).

The V_d of warfarin was 50% larger than that of dicumarol on the average (Table II), and the difference was highly statistically significant ($p < 0.005$ by paired t test). The correlation coefficient between the individual V_d values for dicumarol and warfarin was 0.696, but the correlation was not statistically significant in the eight animals. There was a strong correlation between the total plasma clearances of dicumarol and warfarin (r = 0.930, $p <$ 0.001); the mean ratio of the clearance values (warfarin/dicumarol) was 1.12 (Table II).

Figure 5 shows the relationship between the anticoagulant effect and the plasma concentrations of dicumarol and warfarin for three animals in this study. The regression lines were characterized by a slope (m) and by the plasma concentration at which the synthesis rate of prothrombin complex activity (R_{syn}) was one-half of its normal value (R^0_{syn}). That concentration is designated as $C_{50\%}$. The $-m$ and $C_{50\%}$ values for dicumarol and warfarin in all eight animals are listed in Table III.

There was a statistically significant correlation between $-m$ and $t_{1/2}$ for dicumarol (r = 0.920, $p < 0.001$) but not for warfarin (r = 0.470, $p > 0.2$). There also was a statistically significant correlation between $C_{50\%}$ and $t_{1/2}$ for dicumarol (r = 0.809, $p < 0.02$) and warfarin (r = 0.950, $p < 0.001$). Furthermore, there was a statistically significant negative correlation between $C_{50\%}$ and V_d with dicumarol (r = −0.789, $p < 0.02$) as well as warfarin (r = −0.707, $p <$ 0.05).

Table III—Relationship between Anticoagulant Effect (Inhibition of Prothrombin Complex Activity Synthesis Rate) and Plasma Concentration of Dicumarol (D) and Warfarin (W) in Individual Rats

Rat	—Slope[a] ($-m$), %/Day		Ratio of m Values (D/W)	Plasma Concentration when R_{syn}/R^0_{syn} = 0.5 μg/ml		Ratio of Concentrations (D/W)
	Dicumarol	Warfarin		Dicumarol	Warfarin	
1	0.932	1.02	0.91	5.41	0.123	44.0
2	0.899	1.11	0.81	5.62	0.177	31.8
3	0.854	1.52	0.56	8.19	0.217	37.7
4	1.63	1.38	1.18	18.8	0.813	23.1
5	1.46	1.42	1.03	28.5	0.830	34.3
6	1.41	1.69	0.83	14.0	0.599	23.4
7	1.66	1.13	1.47	13.9	0.725	19.2
8	2.06	1.63	1.26	30.5	1.63	17.6

[a] Slope of a plot of R_{syn}/R^0_{syn} *versus* log plasma concentration (m).

The average ratio of the individual m values (dicumarol/warfarin) was 1.01 ± 0.29, and there was no apparent correlation with the $t_{1/2}$ of either drug. This finding permits a determination of the relative potency of the two drugs on the basis of the ratio of their equieffective plasma concentrations ($C_{50\%}$). The ratio of the $C_{50\%}$ values (dicumarol/warfarin) ranged from 17.6 to 44.0 (Table III) and varied systematically with the $t_{1/2}$ of dicumarol (Fig. 6). This ratio also correlated with the ratio (warfarin/dicumarol) of the $t_{1/2}$ for the two drugs (r = 0.828, $p < 0.01$). There was a strong positive correlation between the $C_{50\%}$ values of dicumarol and warfarin in individual rats (r = 0.911, $p < 0.005$).

For technical reasons, serum protein binding of the two anticoagulants was determined in another group of 21 animals. These animals did not receive either dicumarol or warfarin; the drugs were added directly to the individual serum samples. There were pronounced interindividual differences in the serum protein binding of dicumarol and warfarin, but there was a strong correlation (r = 0.978, $p < 0.001$) between the free fraction (100 × free fraction = percent free) of the two drugs in the serum of the individual animals (Fig. 7). At the concentrations used, the ratio of free fraction values (warfarin/dicumarol) was 53.3 ± 7.0 (mean ± *SD*).

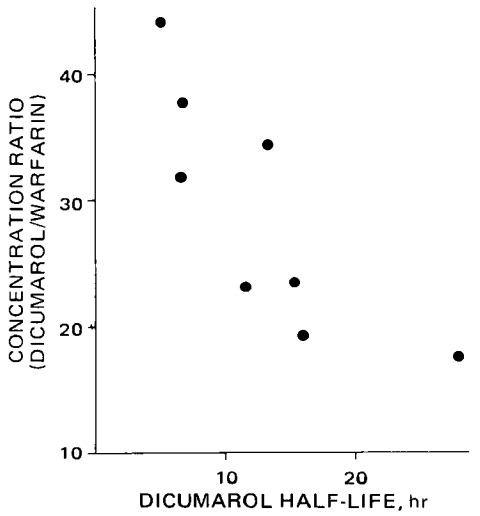

Figure 6—*Relationship between anticoagulant potency ratio (warfarin/dicumarol) [expressed as the ratio (dicumarol/warfarin) of plasma concentrations at which the rate of synthesis of prothrombin complex activity is 50% of normal] and biological half-life of dicumarol in individual rats (r = −0.800, $p < 0.02$).*

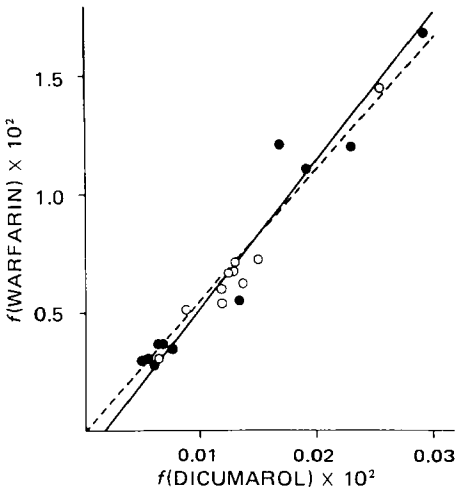

Figure 7—*Relationship between protein binding of dicumarol and warfarin in the serum of individual rats. Plotted are the free fractions (f) determined by equilibrium dialysis at 37°. Key: ●, data from 11 animals with dicumarol assayed by TLC; and ○, data from 10 other animals with dicumarol assayed by selective extraction method (5) (r = 0.978, p < 0.001). Dicumarol was 99.97–99.995% bound and warfarin was 98.3–99.7% bound to serum protein.*

DISCUSSION

The results of this investigation reveal a very strong correlation between dicumarol and warfarin with respect to their $t_{1/2}$, V_d, total plasma clearance, and serum protein binding characteristics in individual rats. The $t_{1/2}$ of warfarin was 42% longer than that of dicumarol, and the V_d of warfarin was 50% larger than that of dicumarol on the average. Consequently, the total plasma clearance of the two drugs was almost identical (Table II). The excellent reproducibility of the dicumarol $t_{1/2}$ values found in this investigation also was observed in a previous study (9); the $t_{1/2}$ of warfarin also showed excellent reproducibility in individual animals (7).

We have found that the interindividual differences in the elimination kinetics of warfarin are related to (and presumably caused by) differences in serum protein binding of the drug (8, 10). The higher the free fraction of warfarin, the shorter is the $t_{1/2}$ and the larger is the V_d of the drug (8). The results of this study suggest that the same relationship holds true for dicumarol (although the evidence is only indirect) and that the correlation of pharmacokinetic characteristics of the two drugs in individual animals is a consequence of the strong correlation of their serum protein binding characteristics.

Because of the relative constancy of the individual $t_{1/2}$ and V_d ratios, it is possible to compare the two drugs with respect to $t_{1/2}$ and V_d, despite pronounced interindividual variations in the value of these constants. It is much more difficult to compare the relative potency of dicumarol and warfarin. The ratio of equieffective plasma concentrations (dicumarol/warfarin) is higher in rapid metabolizers than in slow metabolizers of these drugs (Table III). The reasons for this systematic variation are complex and relate in part to the nonlinear serum protein binding characteristics of dicumarol[7], the different potency of the two enantiomers of warfarin (7), and the time-dependent change in the enantiomer concentration ratio following administration of racemic warfarin (7).

It is interesting, however, that in this group of rats warfarin was *approximately* 30 times as potent as dicumarol on the basis of the mean ratio of their equieffective concentrations[8], while the non-protein-bound fraction of warfarin was *approximately* 50 times larger than that of dicumarol. Thus, the two anticoagulants were almost equipotent in the rat if compared on the basis of their free (non-protein-bound) concentrations.

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[x] To whom inquiries should be directed.

[7] To be published.

[8] This refers to the concentrations at which R_{syn} is one-half of normal (*i.e.*, $C_{50\%}$). Moreover, the equieffective concentration ratio of dicumarol and warfarin differs in rapid and slow metabolizers of these drugs, and it is generally advisable to focus on individual values rather than on the average value of this ratio.

Selective Assay of Benzoyl Peroxide in Lotions and Creams

ROBERT E. DALY ˣ, JOSEPH J. LOMNER, and LESTER CHAFETZ

Abstract □ A selective titrimetric method for the determination of benzoyl peroxide in lotions and creams was developed. It is based on the work of Horner and Jürgens, in which diacylperoxides, dialkylperoxides, peracids, and alkylhydroperoxides can be determined selectively by iodometry and acidimetry. The proposed assay is stability indicating with respect to peracids. Good recovery data were obtained.

Keyphrases □ Benzoyl peroxide—titrimetric, TLC, and spectrophotometric analyses compared, lotions and creams □ Titrimetric analysis—benzoyl peroxide in lotions and creams, compared to TLC and spectrophotometric analyses □ TLC—analysis, benzoyl peroxide in lotions and creams, compared to titrimetric and spectrophotometric analyses □ Spectrophotometric analysis—benzoyl peroxide in lotions and creams, compared to titrimetric and TLC analyses

Gruber and Klein (1) reported that the iodometric titration generally used for the assay of benzoyl peroxide and its pharmaceutical preparations is nonspecific. They proposed a spectrophotometric method based on the measurement of iodine liberated by the oxidation of iodide ion by benzoyl peroxide, and they compared the spectrophotometric and titrimetric procedures with a polarographic assay for benzoyl peroxide. In this laboratory, the Gruber and Klein (1) spectrophotometric assay provides essentially the same results as the titrimetric method, a finding that is not surprising in view of the identity of the chemistry of both methods. Moreover, the polarographic method used by Gruber and Klein (1) could not be reproduced in this laboratory.

Horner and Jürgens (2) described an assay scheme wherein diacylperoxides, dialkylperoxides, peracids, and alkylhydroperoxides can be determined by iodometry and acidimetry with the aid of phenyl sulfide and triethylarsine. Because the simple formulations investigated would not be expected to contain any alkylhydroperoxides or dialkylperoxides, a procedure was developed, based on the Horner and Jürgens work (2), which is selective for benzoyl peroxide in the presence of perbenzoic acid. The procedure is a simple modification of the presently used iodometric titration, requiring only the addition of phenyl sulfide before titration to destroy any perbenzoic acid present.

Table I—Rate of Destruction of Perbenzoic Acid by Phenyl Sulfide

Minutes after Addition	Milligrams Found as Benzoyl Peroxide
0	11.2
2	0
4	0
6	0
8	0
10	0

Table II—Effect of Phenyl Sulfide on Benzoyl Peroxide

Minutes after Addition	Milligrams Taken	Milligrams Found	Percent Recovery
0	24.0	23.4	97.5
3	24.0	22.9	95.5
5	24.0	22.9	95.5
10	24.0	22.9	95.5

The proposed assay was compared with the titrimetric method, the Gruber and Klein spectrophotometric assay (1), and a quantitative thin-layer method. The proposed selective titrimetric method gave results comparable to quantitative TLC values. The modified procedure is presented and the results obtained in the assay of benzoyl peroxide formulations are described in this report.

EXPERIMENTAL[1]

Equipment and Supplies—Phenyl sulfide[2], acetone, sodium thiosulfate, potassium iodide, benzene (all ACS grade or equivalent), and silica gel GF plates[3] (20 × 20 cm, 250 μm) were used.

Quantitative TLC—*Standard Preparation*—Dissolve 250 mg of recrystallized benzoyl peroxide, accurately weighed, in exactly 50 ml of acetone.

Assay Preparation—Transfer a quantity of lotion or cream equivalent to about 250 mg of benzoyl peroxide, accurately weighed, to a 50-ml volumetric flask. Dissolve in, or uniformly disperse in, 30 ml of acetone and dilute to volume with acetone.

Procedure—Streak 0.5 ml (5 × 100 μl followed by a 100-μl rinse of the micropipet with acetone) of the assay preparation and the standard preparation on separate silica gel GF_{254} plates (20 × 20 cm, 250 μm), which had been previously activated for 15–30 min at 105° and which had been prechromatographed in the developing solvent. Develop the plates in a standard unequilibrated chromatography tank (27 × 7 × 28 cm) to within 2–3 cm of the top, employing benzene as the developing solvent.

Locate the benzoyl peroxide streak under shortwave UV light, scrape the streak off, and transfer it into a 125-ml conical flask through a powder funnel. Quantitatively rinse the funnel with 30 ml of acetone. Stir the mixture for about 5 min with a magnetic stirrer and filter quantitatively[4] into a 100-ml beaker, rinsing the flask and funnel with 4 × 5 ml of acetone.

Add 5 ml of a 20% solution of potassium iodide to the filtrate, cover with a watch glass, and place in the dark for about 5 min. Then titrate the liberated iodine against a white background with

[1] Spectrophotometric assays were carried out in a Beckman DU fitted with the Gilford model 222 photometer.
[2] Catalog No. 619, Eastman.
[3] Brinkmann.
[4] Whatman No. 1 filter paper or equivalent.

Table III—Assay for Benzoyl Peroxide in Commercial[a] Products by Four Methods

Sample	Lot	Benzoyl Peroxide, %				
		Claimed	Titrimetric	Spectrophotometric	Proposed	Quantitative TLC
Acne lotion	A	5.0	5.03, 5.16, 4.87	5.02, 5.06	4.34, 4.24, 4.26	4.3
Cream	A	5.0	5.10, 5.09, 5.20	5.40, 5.38	4.91, 4.95, 4.90	3.6
Sulfur cream	A	10.0	10.5, 10.4, 10.5	11.2, 11.2	9.81, 9.67, 9.67	10.5
Cream	B	5.0	4.23, 4.12, 4.15	4.28, 4.25	3.97, 3.99, 3.97	4.4
Cream	C	5.0	5.24, 5.27, 5.26	5.51, 5.53	4.92, 4.85, 4.84	4.9
Lotion	B	5.0	5.30, 5.42, 5.20	5.61, 5.52	4.72, 4.72, 4.70	4.7
Lotion	C	5.0	5.60, 5.60, 5.60	5.69, 5.73	4.52, 4.52, 4.52	4.5

[a] Persadox lotion or cream, Texas Pharmacal Co.

0.01 *N* sodium thiosulfate from a 5-ml buret to the disappearance of the yellow iodine color. Calculate the percent benzoyl peroxide with:

$$\% \text{ benzoyl peroxide} = \frac{\text{ml thiosulfate (assay preparation)}}{\text{ml thiosulfate (standard preparation)}} \times \frac{\text{concentration of standard (g/50 ml)}}{\text{sample weight (g)}} \times 100 \quad \text{(Eq. 1)}$$

Selective Titrimetric Method—*Assay Preparation*—Accurately weigh a sample of cream or lotion equivalent to about 250 mg of benzoyl peroxide into a 100-ml beaker. Add 30 ml of acetone and stir the mixture until a solution or uniform dispersion results. Quantitatively transfer the mixture to a 100-ml volumetric flask with the aid of at least 4 × 10 ml of acetone. Then dilute the mixture to volume with acetone.

Procedure—Transfer 10.0 ml of the assay preparation to a 125-ml glass-stoppered conical flask, add 0.2 ml of phenyl sulfide, and let stand for 4 min. Add 2 ml of a 20% potassium iodide solution and let stand, protected from light, for 15 min. Add 25 ml of acetone and titrate the liberated iodine against a white background with standard 0.01 *N* sodium thiosulfate to the disappearance of the yellow color. Each milliliter of 0.01 *N* sodium thiosulfate is equivalent to 1.211 mg of benzoyl peroxide.

RESULTS AND DISCUSSION

To validate the method, perbenzoic acid was synthesized according to a literature method (3). A solution of perbenzoic acid in chloroform thus obtained, containing 610 mg/ml, was diluted to represent a concentration equivalent in iodide-oxidizing capacity to about 10 mg of benzoyl peroxide/sample aliquot. Samples were assayed at various time intervals after the addition of 0.2 ml of phenyl sulfide (Table I).

The effect of phenyl sulfide on benzoyl peroxide was found to be minimal (Table II). The reduction in assay value after addition of phenyl sulfide may be attributable to the presence of some perbenzoic acid in the sample employed.

Mixtures of benzoyl peroxide with perbenzoic acid were prepared to contain 90.0 and 50.0% of the labeled amount of benzoyl peroxide. Assays of these samples by the proposed method provided results of 90.1 and 50.2%, respectively, as the average of triplicate determinations.

Results of assays of commercial products by the proposed method, a quantitative TLC method, the titrimetric method (same as proposed method without phenyl sulfide), and the Gruber and Klein spectrophotometric method (1) are presented in Table III. A standard of recrystallized benzoyl peroxide, mp 104.8–105.5°, was used in the quantitative TLC method.

Results obtained by the proposed procedure on commercial products were in fair agreement with those obtained by TLC and, in every case, were lower than those obtained by the titrimetric or spectrophotometric method (1). Results obtained by the spectrophotometric method (1) are in good agreement with those obtained by the titrimetric method.

The proposed method is also applicable to the determination of purity of benzoyl peroxide raw materials.

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[x] To whom inquiries should be directed.

Colorimetric Determinations of Chlorpheniramine Maleate, Ephedrine Hydrochloride, and Guaiacolsulfonate Potassium in a Cough Syrup

V. DAS GUPTA ˣ and ANA J. L. de LARA *

Abstract □ Colorimetric methods for the quantitative determinations of chlorpheniramine maleate, ephedrine hydrochloride, and guaiacolsulfonate potassium in a cough syrup containing color (amaranth) are reported. Chlorpheniramine maleate can be assayed using the cyanogen bromide method as reported in the literature. Ephedrine hydrochloride can be assayed using a dye method in which interference from chlorpheniramine maleate is taken into consideration. Guaiacolsulfonate potassium can be assayed by coupling it with 4-aminoantipyrine (the method is similar to the one for phenylephrine hydrochloride). All of the methods are simple, accurate, and precise. The application of the guaiacolsulfonate potassium assay method to commercial dosage forms is reported.

Keyphrases □ Chlorpheniramine maleate—colorimetric determination in cough syrups □ Ephedrine hydrochloride—colorimetric determination in cough syrups □ Guaiacolsulfonate potassium—colorimetric determination in cough syrups

Many cough syrups contain an antihistamine such as chlorpheniramine maleate, a bronchodilator such as ephedrine hydrochloride, and an expectorant such as guaiacolsulfonate potassium. The USP method (1) for the analysis of chlorpheniramine maleate in elixir and the NF methods for the analysis of ephedrine hydrochloride (2) and guaiacolsulfonate potassium (3) are not applicable for quantitative determinations due to interference of one active ingredient with the other. The purposes of these investigations were to develop a colorimetric method for the quantitative determination of guaiacolsulfonate potassium (I) and to modify calculations in the previously reported dye method (4) for the analysis of ephedrine hydrochloride (II). These modifications were necessary due to the interference from chlorpheniramine maleate (III), since it could be accurately determined by the cyanogen bromide method (5).

A colorimetric method for the determination of guaiacolsulfonate potassium (6) was not used because of a lack of reproducibility. Another colorimetric method (7) was not preferred due to a lack of sensitivity, since it is important to dilute the syrup as much as possible before the analysis to prevent interference from the coloring agent. The method developed in these investigations is based on oxidizing I with potassium ferricyanide in an alkaline borate buffer solution and then coupling it with 4-aminoantipyrine; the method is similar to that reported for the determination of phenylephrine hydrochloride (8).

Table I—Assay Results on Cough Syrup

Experiment	Assay Results, % of Claim		
	I	II	III
1	100.87	98.45	99.16
2	100.34	99.66	99.56
3	100.00	99.12	100.04
Average	100.40	99.08	99.59
Average deviation	±0.44	±0.42	±0.30

Table II—Assay Results on Guaiacolsulfonate Potassium in Commercial Dosage Forms

Product	Percent of Label Claim Found[a]
Expectorant I	101.38
Expectorant II	99.23
Expectorant III	122.35[b]
Expectorant IV	101.77

[a] Average of three results. [b] Results are high due to interference from phenylephrine hydrochloride.

EXPERIMENTAL

Chemicals and Reagents—All chemicals and reagents were USP, NF, or ACS grade. Chlorpheniramine maleate[1], ephedrine hydrochloride[2], guaiacolsulfonate potassium[2], cherry flavor[3], and coloring agent (amaranth[4]) were purchased and used without further purification.

Preparation of Cough Syrup—The cough syrup was prepared from two stock solutions. The aqueous stock solution contained 4.0 g of guaiacolsulfonate potassium, 200 mg of chlorpheniramine maleate, 500 mg of ephedrine hydrochloride, 50 ml of distilled water, and 190 ml of syrup USP. The alcoholic stock solution contained 12.5 mg of methylparaben, 5 mg of propylparaben, 25 mg of amaranth, 1.0 ml of chloroform, 0.6 ml of cherry flavor, and 2.5 ml of alcohol. The aqueous and alcoholic stock solutions were mixed together and brought to a volume of 250 ml with distilled water.

Preparation of Solutions—The following standard solutions were prepared: 40.0 μg/ml of III in approximately 0.25 *N* HCl; 4.0 μg/ml of III in distilled water; 10.0 μg/ml of II in distilled water; 10.0 μg/ml of II plus 4.0 μg/ml of III in distilled water; and 150.0, 200.0, 250.0, and 300.0 μg/ml of I in distilled water.

The cyanogen bromide solution was prepared by decolorizing bromine TS with 10% potassium cyanide solution.

A buffer solution containing sulfanilic acid and sodium acetate for the analysis of III was prepared according to a reported procedure (5), as was a 1×10^{-4} *M* solution of bromothymol blue in phosphate buffer solution (pH 6.2) (4).

The following aqueous solutions were prepared fresh daily: a 2% solution of sodium borate (buffer for the analysis of I), a 3% solution of 4-aminoantipyrine, and a 4% solution of potassium ferricyanide. Assay solutions for the analysis of I and II were prepared by diluting 10.0 ml of the cough syrup to 100.0 ml with water and then diluting 5.0 ml of the 1:10 dilution to 100.0 ml with water. For III, 5.0 ml of cough syrup was diluted to 100.0 ml with approximately 0.25 *N* HCl.

Preparation of Calibration Curve for Guaiacolsulfonate Potassium—A 2.0-ml quantity of each standard solution was mixed with 1 ml of potassium ferricyanide solution in a 50-ml volumetric flask. The volume was brought to approximately 48 ml

[1] Schering Corp., Bloomfield, N.J.
[2] Merck & Co., Rahway, N.J.
[3] Dolco cherry No. 5225 imitation, Dodge & Olcott, Inc., New York, N.Y.
[4] Hartman Leddon Co., Philadelphia, Pa.

with sodium borate buffer solution, and the solution was mixed. A 1-ml aliquot of 4-aminoantipyrine solution was then added, and the mixture was brought to volume with borate buffer and mixed. The absorbance of each clear solution was determined[5] at 500 nm against a reagent blank prepared by substituting water for the standard solution. Beer's law was followed.

Assay Procedure for Guaiacolsulfonate Potassium—The same procedure was followed except that 5.0 ml of the assay preparation was used for the analysis instead of 2.0 ml of the standard solution. Since Beer's law was followed, the results (Table I) were calculated using the following equation:

$$\frac{A_a}{A_s} \times 10 = \mu g/ml \text{ of I} \qquad \text{(Eq. 1)}$$

where A_a = absorbance of the assay solution, and A_s = absorbance (0.426) of a standard solution that provides 10 μg/ml of I in a 50-ml volumetric flask.

Assay Procedure for Chlorpheniramine Maleate—A 1.0-ml quantity of the standard solution (in 0.25 *N* HCl) and 1.0 ml of the assay preparation were mixed (separately) with 7.0 ml of the buffer solution and 3.0 ml of cyanogen bromide solution and then allowed to stand for 30 min. The absorbance of each solution was measured at 480 nm against a reagent blank prepared by substituting 1.0 ml of 0.25 *N* HCl for the standard solution. Since Beer's law was followed, the results (Table I) were calculated as follows:

$$\frac{A_a}{A_s} \times 100 = \% \text{ of label claim} \qquad \text{(Eq. 2)}$$

where A_a = absorbance of the assay solution, and A_s = absorbance of the standard solution.

Assay Procedure for Ephedrine Hydrochloride—To five different separators, each containing 5.0 ml of bromothymol blue solution and 10.0 ml of chloroform, the following solutions were added: 5.0 ml of distilled water (reagent blank), 5.0 ml of standard aqueous solution of II (A_s), 5.0 ml of standard aqueous solution of II plus III (A_{sc}), 5.0 ml of standard aqueous solution of III (A_c), and 5.0 ml of assay preparation (A_a). Each separator was shaken for 60 sec, layers were allowed to separate, and the absorbance of each clear chloroform layer was measured at 420 nm against the reagent blank.

The absorbance of A_{sc} was equal to $A_s + A_c$. The corrected absorbance value (A_{cc}) due to chlorpheniramine maleate in the assay preparation was determined as follows:

$$A_{cc} = A_c \times \frac{\% \text{ of III present (from Table I)}}{100} \qquad \text{(Eq. 3)}$$

The A_{cc} was subtracted from the A_a value to get the corrected absorbance value (A_{ac}) due to ephedrine hydrochloride in the assay preparation. Since Beer's law was followed (5), the results (Table I) on II were calculated as follows:

$$\frac{A_{ac}}{A_s} \times 100 = \% \text{ of label claim} \qquad \text{(Eq. 4)}$$

Application of Assay Procedure for Guaiacolsulfonate Potassium to Commercial Dosage Forms—The assay procedure for guaiacolsulfonate potassium was tested on four commercial products.

Expectorant I[6] contained, per 5 ml: 10 mg of codeine sulfate, 0.5 mg of menthol, 3.75 mg of bromodiphenhydramine hydrochloride, 8.75 mg of diphenhydramine hydrochloride, 80 mg of guaiacolsulfonate potassium, 80 mg of ammonium chloride, and 5% alcohol.

Expectorant II[7] contained, per 5 ml: 10 mg of codeine phosphate, 5 mg of promethazine hydrochloride, 0.01 ml (0.17 minims) of ipecac fluidextract, 0.015 ml (0.25 minims) of chloroform, 44 mg of guaiacolsulfonate potassium, 66 mg of citric acid, 197 mg of sodium citrate, and 7% alcohol.

Expectorant III[8] contained 5 mg/5 ml of phenylephrine hydrochloride in addition to the ingredients contained in Expectorant II. Expectorant IV[8] (a pediatric product) contained 7.5 mg/5 ml of dextromethorphan hydrobromide instead of 10 mg of codeine phosphate as in Expectorant II.

For the analysis of guaiacolsulfonate potassium, 4.0 ml of Expectorant I was diluted to 250 ml while 7.0 ml each of the other expectorants was diluted to 250 ml with water. A 2.0-ml quantity of the diluted expectorant was mixed with 1 ml of potassium ferricyanide solution in a 50-ml volumetric flask. The rest of the procedure was the same as reported under *Preparation of Calibration Curve for Guaiacolsulfonate Potassium*. In separate experiments, it was determined that there was no interference from other ingredients except phenylephrine hydrochloride, which was present in Expectorant III with codeine. The results, calculated using Eq. 1, are presented in Table II.

DISCUSSION

The results indicate that all three active ingredients of a cough syrup, guaiacolsulfonate potassium, ephedrine hydrochloride, and chlorpheniramine maleate, can be assayed accurately (Table I) using colorimetric methods. All three methods are simple, accurate, and precise. There is no interference from the other active or inactive ingredients, except from chlorpheniramine maleate in the assay procedure for ephedrine hydrochloride. This interference can be taken into consideration easily as explained under *Experimental*.

The new technique for the analysis of guaiacolsulfonate potassium (coupling with 4-aminoantipyrine) follows Beer's law and is rapid (10 min/assay after the standard curve is prepared). The assay method for the analysis of guaiacolsulfonate potassium can be applied to a number of commercial dosage forms (Table II) containing other ingredients such as codeine, menthol, dextromethorphan, promethazine, bromodiphenhydramine, diphenhydramine, and ammonium chloride. Phenylephrine hydrochloride does interfere, which was expected (8).

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[5] A Bausch & Lomb Spectronic 20 was used.

[6] Ambenyl, Parke, Davis & Co., Detroit, Mich.

[7] Phenergan, Wyeth Laboratories, Philadelphia, Pa.

[8] Phenergan VC, Wyeth Laboratories, Philadelphia, Pa.

Time Course and Dose Dependence of Antacid Effect on Urine pH

MILO GIBALDI ˣ, BARBARA GRUNDHOFER, and GERHARD LEVY

Abstract □ Daily administration of a proprietary magnesium and aluminum hydroxides suspension, 15 ml four times a day, to normal adult volunteers resulted in a statistically significant increase in urine pH on the 1st day of treatment. The urine pH's on the 2nd and subsequent days of treatment were statistically significantly higher than on the 1st day. A 7.5-ml dose of the antacid suspension, taken four times a day, had only a small and not statistically significant effect on urine pH, while 30 ml four times a day increased urine pH by approximately the same magnitude as the 15-ml doses. The effect of the antacid on urine pH persisted for at least 1 day after discontinuation of dosing.

Keyphrases □ Antacid—magnesium and aluminum hydroxides suspension, effect on urine pH □ Magnesium and aluminum hydroxides suspension—effect on urine pH □ Aluminum and magnesium hydroxides suspension—effect on urine pH □ pH, urine—effect of magnesium and aluminum hydroxides suspension

Regular administration of certain antacids can increase urine pH sufficiently to have pronounced effects on the kinetics of elimination of some acidic and alkaline drugs (1). A widely used proprietary antacid product containing magnesium and aluminum hydroxides[1] was the most effective of the products tested in increasing urine pH. To extend the information obtained in the initial study (1), an investigation was undertaken to determine the time course and dose dependence of the effect of this antacid on urine pH.

EXPERIMENTAL

The study panel consisted of eight healthy males, 23–38 years of age, who by education and experience (graduate students in pharmaceutics) were capable of giving their informed consent to participate in this investigation. The subjects were under no dietary restrictions but took no drugs during the study. They were instructed to empty their bladders and to collect the urine from 8:00 am to 10:00 am, 10:00 am to 2:00 pm, and 2:00 pm to 4:00 pm. The urine pH was determined immediately after voiding. All measurements were carried out by the same individual on a pH meter[2], which was routinely standardized with buffer solutions of pH 4.0, 7.0, and 9.0.

To determine the time course of the antacid effect on urine pH, seven subjects collected urines on the 1st and 2nd days and took 15 ml of the antacid suspension at 8:00 am, 1:00 pm, 6:00 pm, and 11:00 pm from the 3rd to 8th day. Urine samples were collected until the 10th day, except on Days 6 and 7.

To determine the effect of antacid dose on urine pH, eight subjects took 0, 7.5, 15, or 30 ml of the antacid suspension at 8:00 am, 1:00 pm, 6:00 pm, and 11:00 pm on a Sunday and Monday according to a Latin-square experimental design. Urine samples were obtained on Mondays and Tuesdays, *i.e.*, the 2nd day of antacid administration and the day after antacid was discontinued.

The daily mean pH values reported in the tables were obtained by averaging as such the pH of the three urine samples obtained from every subject.

[1] Maalox, William H. Rorer, Inc., Fort Washington, Pa.
[2] Beckman Zeromatic II.

Table I—Time Course of Urine pH Change Caused by Administration of Magnesium and Aluminum Hydroxides Suspension in Healthy Adults

Day	Antacid[a]	Mean Urine pH ± *SD*[b]	Significance of Difference from Control pH (*p* Values)[c]
1	—	5.78 ± 0.42	—
2	—	5.83 ± 0.51	—
3	+	6.16 ± 0.40	<0.025
4	+	6.94 ± 0.41	<0.0025
5	+	6.46 ± 0.29	<0.0125
6	+	—[d]	—
7	+	—[d]	—
8	+	6.76 ± 0.28[e]	<0.0005
9	—	6.07 ± 0.41[f]	N.S.
10	—	5.80 ± 0.66[e]	N.S.

[a] Fifteen milliliters of Maalox Suspension at 8:00 am, 1:00 pm, 6:00 pm, and 11:00 pm. [b] Mean of seven subjects except where indicated. [c] Paired *t* test, compared to average pH on Days 1 and 2. [d] Not determined (Saturday and Sunday). [e] Mean of six subjects (one subject discontinued antacid on Day 6 due to a respiratory infection). [f] Mean of five subjects (a second subject was unable to collect urine samples on that day).

RESULTS AND DISCUSSION

The time course of urine pH change caused by administration of 15 ml of the antacid four times a day for 6 days is summarized in Table I. A small but statistically significant increase in urine pH was noted on the 1st day of antacid administration. There was no increase in urine pH with time on that day (mean pH 6.28 ± 0.62, 6.09 ± 0.54, 6.14 ± 0.64 for the first, second, and third collection periods, respectively). The previous study had shown that there are no significant differences in control urine pH for the three urine collection periods (1).

The mean urine pH on the 2nd, 3rd, and 6th days of antacid administration was almost 1 unit higher than during the control period and was also statistically significantly higher than during the 1st day of antacid administration. There was no systematic change

Table II—Relationship between Dose of Magnesium and Aluminum Hydroxides Suspension and Urine pH in Healthy Adults

Dose[a], ml	Day[b]	Mean Urine pH ± *SD*[c]	Significance of Difference from Control pH (*p* Values)[d]
0	2	5.73 ± 0.42	—
0	3	5.74 ± 0.38	—
7.5	2	6.08 ± 0.54	N.S.
0	3	6.14 ± 0.61	N.S.
15	2	6.56 ± 0.40	<0.0025
0	3	6.17 ± 0.45	<0.025
30	2	6.44 ± 0.57	<0.01
0	3	6.45 ± 0.34	<0.0025

[a] Volume of Maalox Suspension taken at 8:00 am, 1:00 pm, 6:00 pm, and 11:00 pm. [b] Antacid was taken on Days 1 and 2 except in the control experiment. [c] Mean of eight subjects. [d] Paired *t* test, compared to average pH of Days 2 and 3 of the control period.

in mean urine pH with time from the 2nd to the 6th day of antacid administration. The mean pH on the 1st day after discontinuation of the antacid was somewhat above the control value but not statistically significantly so. Urine pH was in the normal control range on the following day.

The results of the experiments to determine the relationship between the dose of the antacid and urine pH are summarized in Table II. Urine pH was determined on the 2nd day of treatment in view of the results obtained in the first part of this investigation. A dose of 7.5 ml four times a day had only a small and not statistically significant effect on urine pH *on the average*.

However, it was noted previously that the magnitude of the antacid effect on urine pH is inversely proportional to the urine pH during the control period (1). The same tendency was apparent in this study; some individuals with low control pH values showed an increase of more than 0.5 pH unit with the 7.5-ml dosing regimen. The 15-ml dosing regimen increased urine pH by more than 0.8 unit on the average, a highly statistically significant difference from the control value. No further increase was observed when the dose of the antacid was raised to 30 ml. Urine pH on the 1st day after discontinuation of the 15- and 30-ml doses was significantly above the control value; this residual effect was particularly pronounced after the 30-ml dose.

The results of this study show that a pharmacokinetically significant increase in urine pH is produced within 24 hr of administration of regular therapeutic doses of a widely used antacid and that this effect persists for about 24 hr after the antacid has been discontinued. The residual effect on urine pH is probably due to the retention of antacid in the GI tract for some time. It is not known if the lack of difference between the effect of the 15- and 30-ml dosage regimens on urine pH is paralleled by a similar lack of dose–response relationship with respect to antacid efficacy.

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(1) M. Gibaldi, B. Grundhofer, and G. Levy, *Clin. Pharmacol. Ther.*, **16**, 520(1974).

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GLC Determination of Underivatized Carbamazepine in Whole Blood

MICHAEL SHEEHAN [x] and RAYMOND E. BEAM [*]

Abstract □ This report describes a rapid GLC assay for carbamazepine in blood. Carbamazepine is chromatographed directly using phenyl methyl silicone gum as the stationary phase. The calibration curve is linear up to 50 μg/ml, with a lower limit of sensitivity of 1 μg/ml. Other anticonvulsants may be measured simultaneously, and the procedure works equally well with plasma or blood.

Keyphrases □ Carbamazepine—GLC analysis, whole blood □ Anticonvulsants—GLC analysis of carbamazepine in whole blood □ GLC—analysis, carbamazepine in whole blood

Carbamazepine is used for the relief of pain from trigeminal neuralgia (1) and in the treatment of epilepsy (2–4). Frequently, patients with complicated seizure disorders are given several other anticonvulsants (*e.g.*, phenytoin, primidone, and phenobarbital) as well as carbamazepine (the usual case in this hospital).

Methods of analysis for carbamazepine that rely on spectrophotometry (5) are complicated by interfering absorbances when mixtures of drugs are analyzed. For such mixtures, GLC is usually the preferred method, and several GLC methods for measuring carbamazepine in serum or plasma recently were reported (6–13). Because of various drawbacks (*e.g.*, tedious extraction requirements, necessity of derivative formation, and inability to use whole blood), none of these methods is considered suitable.

This paper describes a GLC method for the assay for carbamazepine levels in whole blood. This method is simple and rapid and does not require derivative formation. Furthermore, other anticonvulsants can be measured simultaneously.

EXPERIMENTAL

Reagents—Reagent grade chloroform, methanol, and acetic acid were used without further purification. Authentic iminostilbene was obtained commercially[1].

Standards—A stock solution equivalent to 1.0 mg/ml of heptabarbituric acid was prepared by dissolving exactly 108.4 g of dry heptabarbital sodium[2] in 0.9% saline, which had been made alkaline with 5.0 ml of 0.45 *N* sodium hydroxide. The final volume of the solution was adjusted to 100 ml with 0.9% saline. A 20.0-μg/ml working internal standard was prepared by diluting 10.0 ml of the stock solution to 500 ml with 0.9% saline. The 16.3-μg/ml carbamazepine blood standard was prepared by dissolving 4.80 mg of carbamazepine[2] in 10.0 ml of anhydrous methanol and then diluting a 0.34-ml aliquot of this solution with 100 ml of expired blood bank blood. Blood standards of other concentrations were prepared from the carbamazepine stock solution as needed.

Apparatus—The GLC analyses were performed on a gas chromatograph[3] equipped with dual flame-ionization detectors. The chromatograph was fitted with 1.83-m (6-ft) long, U-shaped, glass columns (4.0 mm i.d.) packed with 1.5% phenyl methyl silicone gum (OV-17) on Gas Chrom G HP[4] (80–100 mesh). For the combined analysis of primidone and carbamazepine, a column packed with 1.5% OV-225 on Gas Chrom G HP[4] (80–100 mesh) also was used. The columns were conditioned before use by heating at 290° (260° for OV-225) for 24 hr with 5-ml/min carrier gas flow.

[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] Ciba Pharmaceutical Co., Summit, N.J.
[3] Varian model 2100.
[4] Applied Science, State College, Pa.

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EXPERIMENTAL

Reagents—Reagent grade chloroform, methanol, and acetic acid were used without further purification. Authentic iminostilbene was obtained commercially[1].

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[1] Aldrich Chemical Co., Milwaukee, Wis.
[2] Ciba Pharmaceutical Co., Summit, N.J.
[3] Varian model 2100.
[4] Applied Science, State College, Pa.

Table I—Effect of Temperature on the Carbamazepine–Iminostilbene Ratio

Initial Column Temperature[a]	Peak Height Ratio[b], Carbamazepine to Iminostilbene
180°	1.02
	1.25
	1.48
190°	0.96
	1.24
	2.46
200°	0.82
	2.10
	3.60
210°	1.03
	3.43
	1.67
220°	1.39
	1.03
	1.40
230°	2.66
	1.20
	1.48
240°	3.24
	1.06
	1.80
250°	1.62
	1.65
	1.23
260°	2.50
	0.97
	1.91

[a] Column temperature was programmed to 266° at 6°/min. [b] Measurements were made from chromatograms of extracts of a 10.0-μg/ml carbamazepine standard.

The chromatography settings for the OV-17 column were as follows: nitrogen as carrier gas, 30 ml/min; air, 300 ml/min; hydrogen, 30 ml/min; injection port and detector temperatures, 270°; initial column temperatures, 180°; and program rate, 6°/min up to 266° followed by isothermal heating at 266° for 10 min. For the OV-225 column, the initial column temperature was 230° and the program rate was 10°/min up to 270°.

The mass spectral analysis was performed on a gas chromatograph–mass spectrometer[5] under the following conditions: electron energy, 60 ev; emission current, 450 μamp; electron multiplier[6], 2.2 kv; preamplifier range, 10^{-7} amp-v^{-1}; and GLC conditions, same as for OV-17 column.

Extraction Procedure—To a 25-ml glass vial were added 2.0 ml of whole blood (ethylenediaminetetraacetic acid as anticoagulant), 1.0 ml of the 20.0-μg/ml heptabarbital working standard, and 10 drops of acetic acid. The vial was swirled gently for a few seconds, and 10 ml of chloroform was added. After being capped tightly, the vial was shaken vigorously for 5 min mechanically.

The solution was allowed to settle for 30 sec, and the chloroform layer was separated (the congealed blood cells usually adhered to the sides of the vial) and evaporated to dryness under a gentle stream of air in a 60° water bath. (For plasma, the solution was first filtered through 1 g of anhydrous sodium sulfate.) The residue was reconstituted with 50 μl of chloroform, and 5 μl of this solution was injected into the chromatograph.

RESULTS AND DISCUSSION

Figure 1 shows a chromatogram of an extract from 2 ml of blood containing 16.3 μg/ml of carbamazepine to which 20 μg of heptabarbital had been added. As previously reported (10–13), a substantial percentage of the carbamazepine had decomposed to a second compound with a relative retention time of 0.85. This peak also was found when a pure sample of carbamazepine was chromatographed directly.

It has been reported (11, 13) that carbamazepine undergoes on-column decomposition to give iminostilbene; under the given GLC conditions, an authentic sample of iminostilbene had the same relative retention time as this peak (0.85 relative to heptabarbital). Likewise, the mass spectra of authentic iminostilbene and the decomposition product were identical (base peak and M^+ both at *m/e* 193). From these findings it was concluded that the decomposition product was iminostilbene.

Table I shows that this transformation was not strictly a function of temperature. That is, the ratio of carbamazepine to iminostilbene was variable at all temperatures studied. Even with derivatization (10, 13), the problem of iminostilbene formation still exists, although the authors claim that accurate quantitation of carbamazepine can still be achieved. In view of these results, it was felt that any GLC assay for carbamazepine should also account for the formation of iminostilbene.

[5] Finnigan model 1015D.
[6] Bendix.

Table II—Replicate Chromatograms of a 16.3-μg/ml Carbamazepine Blood Standard

Run	Relative Response[a]		
	Iminostilbene	Carbamazepine	Total
1	0.68	0.96	1.64
2	0.87	0.79	1.66
3	0.70	0.96	1.66
4	0.81	0.86	1.67
5	0.43	1.20	1.63
6	0.44	1.22	1.66
7	0.63	0.96	1.59
8	0.87	0.82	1.69
9	0.53	1.17	1.70
10	0.39	1.24	1.63
11	0.54	1.12	1.66
12	0.51	1.14	1.65
13	0.56	1.08	1.64
14	0.52	1.12	1.64
15	0.54	1.03	1.57
16	0.45	1.24	1.69
17	0.64	1.06	1.70
18	0.54	1.17	1.71
19	0.53	1.15	1.68
20	0.59	1.14	1.73
		Mean =	1.66
		SD =	±0.04
		CV =	2.3%

[a] Relative response = height of peak/height of internal standard. Concentration of internal standard = 10.0 μg/ml.

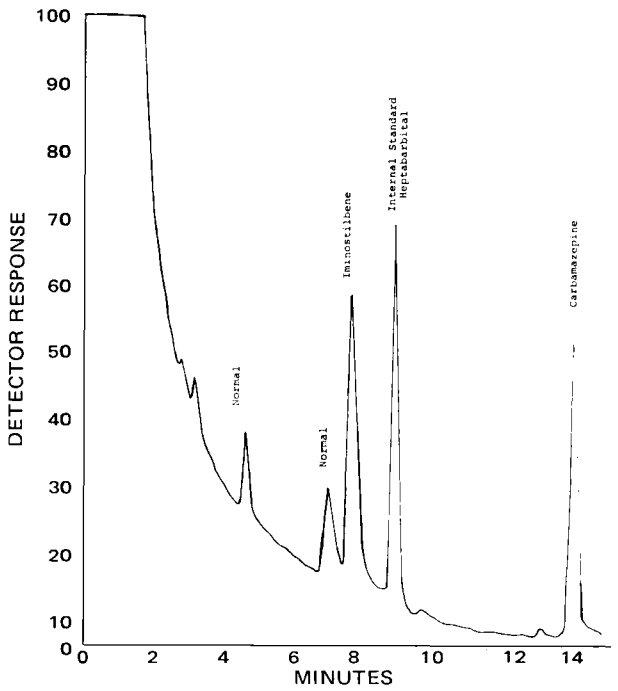

Figure 1—*Chromatogram of extract from 2 ml of blood containing 3.3 μg of carbamazepine.*

Table III—Recovery Study

Amount of Drug Added to Blood Pool, μg/ml	Amount of Drug Recovered, μg/ml	Relative Recovery, %
2	1.8	90
5	5.7	114
10	9.3	93
20	18.0	90
40	40.5	101
80	86.0	107
100	101.0	101

Table IV—Relative Retention Times of Various Anticonvulsants

Drug or Metabolite	OV-17[a]	OV-225[a]
Ethosuximide	0.08	—
Methsuximide (as metabolite)	0.50	0.46
Iminostilbene	0.85	0.70
Phenylethylmalonamide	0.86	0.93
Phenobarbital	0.89	—
Heptabarbital (internal standard)	1.00	1.00
Primidone	1.58	—
Carbamazepine	1.63	1.83
Phenytoin	1.68	2.41

[a] See *Experimental* for GLC conditions.

It seemed likely that whereas the ratio of carbamazepine to iminostilbene might vary from one run to the next for any given concentration of carbamazepine, the sum of the relative responses of these two drugs might not vary; and if the sum of the relative responses did not vary, then carbamazepine could be quantitated by simply comparing the sum of the areas (peak heights) of the two peaks to that of an internal standard. This assumption proved to be correct: the ratio of carbamazepine to iminostilbene varied from 2.5 to 0.9 for 20 consecutive runs of a 16.3-μg/ml blood standard whereas the sum of the relative responses for this standard did not vary by more than 2.5% from the mean (Table II). Thus, the carbamazepine concentration was linearly related to the sum of the relative responses of carbamazepine and iminostilbene over the range investigated (up to 50 μg/ml). Recovery of carbamazepine from whole blood ranged from 90 to 114% (Table III), and the between-run standard deviation was only 0.4 μg/ml, with a lower limit of sensitivity of approximately 1 μg/ml.

The other anticonvulsants that can be determined simultaneously with this method are listed in Table IV. For the combination of primidone and carbamazepine, the phenylethylmalonamide metabolite is not completely resolved from iminostilbene on the OV-17 column. For a patient on these drugs, it is necessary to estimate and subtract the amount of phenylethylmalonamide in the combined peak by taking advantage of the fact that phenylethylmalonamide and iminostilbene are completely resolved on an OV-225 column (see *Experimental* for GLC conditions). The blood extract is chromatographed on both OV-17 and OV-225 columns, and the amount of phenylethylmalonamide (relative to the standard) is estimated from the latter chromatogram (the ratio of phenylethylmalonamide to internal standard did not vary from one run to another).

Various therapeutic ranges for carbamazepine in serum or plasma have been reported (3–8), with consensus being a range of 4–13 μg/ml. Since this assay used whole blood, it was necessary to compare blood with plasma or serum levels of carbamazepine to determine whether therapeutic ranges could be correlated. This precaution was felt to be necsssary due to the well-known (14) dilutional effect of red cells on the concentration of some drugs. Accordingly, whole blood specimens from several patients were obtained, and one portion of each was analyzed as blood and the other portion was spun down and analyzed as plasma. *No* significant differences for carbamazepine levels were observed between the two different specimens for these patients.

To determine the linearity of this comparison, whole blood standards of carbamazepine were prepared and analyzed in the same manner, and again no appreciable differences were found up to the highest concentrations studied (50 μg/ml). In addition, this comparison shows that either plasma or whole blood can be used in this assay. From the limited analyses carried out to date (less than 10 patients), a therapeutic range has not been determined. Levels between 2 and 8 μg/ml have been found, but other anticonvulsants as well as carbamazepine were being administered to these patients. Further clinical studies are necessary to determine the therapeutic range or ranges of carbamazepine in combination with various other anticonvulsants.

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Quantitative Analysis of Tribromsalan in Blood and Urine

H. S. CHUN HONG ˣ, R. J. STELTENKAMP, and N. L. SMITH

Abstract □ Tribromsalan can be quantitatively measured in whole blood and urine by a technique involving extraction with ethyl acetate, treatment with silica gel, separation by TLC, and quantitative measurement by fluorescent spectrophotometry. This method has a sensitivity down to 125 ng (25 ppb in 5.0 ml of sample) of free tribromsalan and shows an average 90% recovery of tribromsalan in blood and urine with standard deviations of 9.7 and 7.4%, respectively.

Keyphrases □ Tribromsalan—analysis, blood and urine, TLC, fluorometry □ TLC—analysis, tribromsalan, blood and urine □ Fluorometry—analysis, tribromsalan, blood and urine □ Bacteriostatic agents—tribromsalan, analysis, blood and urine, TLC, fluorometry

Tribromsalan[1] is a bacteriostat which was widely used in soap and detergent products. It is effective against common Gram-positive bacteria (*e.g., Staphylococcus* type) (1–3).

Since recent studies demonstrated the absorption of hexachlorophene through human skin into the blood (4–6), other bacteriostats used in skin products must also be examined for skin absorption. Such study requires detection methods sensitive to parts per billion in blood. Tribromsalan was analyzed in toilet soaps by rapid liquid chromatography (7) with a detection limit of 100 ng. This method was not applicable to tribromsalan in blood or urine because of extracted interference.

A method is now presented for quantitatively determining tribromsalan in blood. It involves extraction of tribromsalan with ethyl acetate, isolation by TLC, and measurement through fluorescence spectrophotometry.

EXPERIMENTAL

Reagents—Tribromsalan was 99% pure as analyzed by the manufacturer. The solvents used were ethyl acetate, acetic acid, and absolute methanol, all reagent grade. Silica gel G-325[2] was used.

Procedure—The standards (25–1000 ppb) were prepared by adding known amounts of tribromsalan stock solution (1 μg/10 μl of ethanol) to each 5.0-ml blood sample; they were treated the same way as other samples to be described.

A 5.0-ml blood sample (5 mg of sodium citrate or 0.2 mg of heparin/ml of blood was added as anticoagulant) was frozen in an ethylene glycol–ether–dry ice bath (−20°) for 10 min for complete hemolysis and then thawed. Extraction was made with 15 ml of ethyl acetate on a mixer[3] for 2 min followed by centrifuging at 3500 rpm for 5 min.

Ten milliliters of the supernate was transferred to a 15-ml graduated centrifuge tube and evaporated to 3.0 ml on a water bath[4] at 60°. Approximately 100 mg of silica gel G was added, mixed well, and centrifuged. The supernate, 2.5 ml, was then transferred to a 2.5-ml centrifuge tube and evaporated to exactly 0.2 ml. If the final volume became less than 0.2 ml, it was adjusted with additional ethyl acetate.

Sixty microliters of this aliquot was spotted on a diethylaminoethylcellulose-coated glass plate[5]. At least three standards of tribromsalan were spotted on each plate along with unknown samples. The plate was developed to the top three times with absolute methanol to remove the interfering background from the original tribromsalan spot. Finally, the plate was developed with 7.5% acetic acid in methanol to approximately one-third of the plate (7–8 cm, R_f value for tribromsalan = 0.43). The plate was air dried thoroughly (see *Results and Discussion*).

The tribromsalan spot was located visually under UV light (350 nm). The plate was run on a fluorescent spectrophotometer[6] with a TLC scanner for quantitative measurements. A No. 390-nm cutoff filter was used before the emission monochromator with a 0.5 × 0.5-cm scanning slit. Spectra for tribromsalan were obtained at an excitation wavelength of 362 nm and an emission wavelength of 422 nm.

Spectra were photocopied, cut out, and weighed for quantitative measurement. Since the instrument setting for the TLC scanner can be varied from one reading to another, peak height measurement alone was not satisfactory. For area measurement, a photocopying and weighing method was convenient and as accurate as measuring height times width at half-height.

For the analysis of urine samples, the procedure was followed as described for blood samples with these exceptions:

1. The pH of urine samples was adjusted to 6.8 with monobasic or dibasic potassium phosphate before extraction with ethyl acetate, since the natural pH of urine samples varies a great deal.
2. The samples were not frozen in dry ice, since they did not need hemolysis.
3. The silica gel treatment was omitted, and the 10-ml aliquot of the ethyl acetate extract was evaporated to a final volume of 0.3 ml before spotting.

RESULTS AND DISCUSSION

The average recovery of tribromsalan from human blood was 90.6 ± 9.7% (14 samples). This value was established by extracting blood samples containing known amounts of tribromsalan (100–2500 ppb) and comparing with ethyl acetate solutions of known amounts of tribromsalan.

The linearity of the method was confirmed with human blood, rabbit blood, and human urine samples through standard curves. Correlation coefficients (r) for linearity were calculated (8) for eight different sets of experiments with three to five points for each set. They were found to be 0.976, 0.983, 1.000, 0.996, 0.992, and 1.000 for blood samples (25–1000 ppb) and 0.982 and 0.960 for urine samples (100–1000 ppb). Greater background interferences were noticed for urine samples than for blood samples. With urine, the lower limit of sensitivity was 100 ppb.

The precision of the method was determined by 10 replicate analyses of a human blood sample containing 500 ppb of tribromsalan. These blood samples were prepared by adding 50 μl of an ethanolic tribromsalan solution (1 μg of tribromsalan/μl of alcohol) to 100 ml of human blood. After thorough mixing, 10 5-ml samples of this blood were analyzed on the same TLC plate. The relative standard deviation was 7.4%.

Lowering the pH of blood to 5.9 using 10% acetic acid did not affect the recoveries. Since the minor variations (pH 7.0–7.4) that

[1] 3,4′,5-Tribromosalicylanilide, TBS, Fine Organics, Inc.
[2] Research Specialties Co.
[3] Vortex Genie, Scientific Industries.
[4] N-EVAP-A water bath with fitted test tube rack which has an air flow device on the top, Organomation, Inc.
[5] S&S material, 100–125 μm thickness, 20 × 20 cm.
[6] Hitachi model MPF-2A with TLC scanner (an accessory for model MPF-2A), Perkin-Elmer Corp.

occur with human or animal blood would not be expected to have any effect, no buffering was used. Urine samples, however, vary significantly in pH and need pH adjustment to 6.8.

The character of the fluorescent spectrum changed as the plate dried. The peak became larger and more defined at the given excitation and emission wavelengths (362 and 422 nm, respectively). There was also a significant shift of both the excitation and emission maximum wavelengths to 330 and 480 nm, respectively, when tribromsalan was in 7.5% acetic acid in methanol. Spectra obtained with wet plates resembled those in 7.5% acetic acid in methanol. The change of fluorescence in the presence of acid was also observed by other investigators with aspirin (9). Drying the plate for more than 24 hr in the air provided peaks with consistent intensities without a shift of maximum wavelengths on the fluorescent spectrophotometer.

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pH-Dependent Secretion of Procainamide into Saliva

JEFFREY R. KOUP, WILLIAM J. JUSKO [x], and ALLEN L. GOLDFARB

Abstract □ The relationship between serum and stimulated, mixed saliva concentrations of procainamide was determined in 12 chronically medicated patients. Samples were obtained at times chosen to approximate the maximum and minimum serum concentrations of the drug during a dosing interval. Marked intersubject variability was found in the ratio of saliva to serum concentration of the drug (0.27–8.93). There was no correlation between the dose (milligrams per kilogram per day) and the minimum serum or saliva concentration of procainamide. Saliva pH ranged from 6.3 to 8.0 in eight subjects. The ratio of saliva to serum concentration of procainamide increased with decreasing pH. This result can be largely explained by the pH-dependent ionization and distribution of procainamide, a weak base.

Keyphrases □ Procainamide—pH-dependent secretion into saliva, ratio of saliva to serum concentration, humans □ Saliva—concentration of secreted procainamide, pH dependent, compared to serum concentration, humans

Procainamide, an antiarrhythmic compound, demonstrates marked intersubject variability in absorption and elimination (1). The range of therapeutic serum concentrations, however, has been defined (1). Mean plasma concentrations ranging from 4 to 8 μg/ml are generally effective in controlling arrhythmias, while toxicity is likely to occur at concentrations above 12 μg/ml. Thus, serum concentration measurements are useful in monitoring therapy in individual patients. However, procainamide has a short half-life, approximately 3–6 hr, which necessitates multiple venipunctures to characterize the peak and minimum serum concentrations during a dosing interval and during continued therapy. These requirements may be excessively traumatic for routine clinical application. The purpose of this study was to assess whether saliva concentrations of procainamide can be used to monitor drug concentrations in the plasma of patients receiving the drug.

The salivary secretion of other drugs has been the subject of several investigations. Good correlation between either total or unbound serum and mixed saliva concentrations was demonstrated in humans for theophylline (2), lithium (3), digoxin (4), acetaminophen (5), salicylate (at low concentrations) (6), phenytoin (7), and several sulfonamides (8).

EXPERIMENTAL

Twelve patients in an acute coronary care unit[1], who had been receiving a fixed dose of procainamide hydrochloride[2] for at least 2 days, were studied. Daily doses, dosage intervals, and times of sample collection in relation to dose are listed in Table I for the individual patients. The clinical records indicated that renal and liver functions were essentially normal in all subjects.

Blood and mixed, stimulated saliva were collected simultaneously before and after a regularly scheduled dose of procainamide. Saliva flow was stimulated by having the patients chew on waxed film[3]. For Patients K and L, only one sample set was obtained. A third sample set was obtained at approximately 1 week after the initial samples for Patients A and E. All samples were obtained between 10 and 11 am and between 1 and 3 pm to avoid mealtimes and to minimize the possible effects of diurnal variation in saliva composition (9).

Saliva pH was determined immediately after sample collection for Patients E–L. The serum and saliva samples were refrigerated at 4° and analyzed within 2 days. Serum and saliva concentrations of procainamide were assayed by a double-extraction, spectrophotofluorometric method (1, 10).

[1] At the Millard Fillmore Hospital.

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[3] Parafilm.

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[2] Pronestyl.

[3] Parafilm.

Table I—Procainamide Dosage Regimens and Serum and Saliva Concentrations

Patient	Dosing Regimen: Dose, mg/kg/day	Dosing Regimen: Dosing Interval, hr	Sample 1: Hours before Dose	Sample 1: Saliva Concentration, μg/ml	Sample 1: Saliva pH	Sample 1: Serum Concentration, μg/ml	Sample 2: Hours after Dose	Sample 2: Saliva Concentration, μg/ml	Sample 2: Saliva pH	Sample 2: Serum Concentration, μg/ml
A[a]	41.3	4	0.50	16.6	—[b]	16.3	1.5	23.7	—[b]	23.6
B	39.8	3	0.50	10.8	—[b]	3.4	1.0	9.7	—[b]	3.5
C	27.0	6	0.25	19.3	—[b]	4.9	2.0	22.3	—[b]	7.1
D	23.1	6	0.50	15.0	—[b]	2.4	2.0	32.5	—[b]	4.4
E[a]	25.6	4	0.12	4.4	7.2	1.6	2.0	13.6	6.8	1.9
F	36.7	4	0.25	4.4	8.0	3.1	1.5	7.4	7.5	4.7
G	28.3	4	0.25	4.1	7.1	2.1	1.5	0.7	7.1	2.6
H	41.3	4	0.50	8.7	7.1	4.4	1.0	10.9	7.0	4.1
I	36.7	3	0.12	18.8	6.7	2.5	1.0	22.8	6.6	2.6
J	47.6	4	1.0	12.7	6.2	9.4	1.0	47.5	6.3	8.2
K	47.5	3	0.25	18.9	6.4	4.8	—	—	—	—
L	30.0	3	0.25	13.1	6.3	3.4	—	—	—	—

[a] Third samples were also collected for these patients. [b] Saliva pH was not measured.

RESULTS

The serum and saliva procainamide concentrations measured in the 12 subjects are listed in Table I. There was no correlation between the dose (milligrams per kilogram per day) and either the minimum serum or saliva concentration of procainamide. No attempt was made to correlate the drug dose with the maximum concentrations since a single sample taken 1–2 hr after the dose would not be expected to identify accurately the actual peak concentration. These observations are in agreement with the findings of Koch-Weser and Klein (1), who noted a substantial degree of intersubject variability in serum concentrations of procainamide among patients receiving similar doses.

The saliva–serum concentration ratio of procainamide is plotted *versus* the serum concentration of the drug in Fig. 1. Pronounced intersubject variation was observed in this ratio, with values ranging from 0.27 to 8.93. Substantial intrasubject variation was also observed. A notable example is Patient E, in whom the ratio varied from 2.75 to 7.16 to 3.28 on three different occasions.

One possible reason for this variation was thought to be the differences among patients with respect to saliva pH. Procainamide is a weak base with a pKa of 9.4. The unionized form of the drug is more lipophilic than the cation and would be expected to diffuse more freely across mucosal membranes and the epithelial cells lining the salivary ducts. The relationship between the saliva–serum concentration ratio of procainamide and saliva pH is shown in Fig. 2.

The theoretical distribution ratio expected for the total concentration of an ionizable species found on two sides of a lipoid membrane where differences in pH exist is also shown in Fig. 2. This ratio is described by the following relationship derived from the Henderson–Hasselbach equation:

$$\frac{C_{sal}}{C_{ser}} = \frac{1 + 10^{(pKa - pH_{sal})}}{1 + 10^{(pKa - pH_{ser})}} \qquad \text{(Eq. 1)}$$

where the subscripts sal and ser refer to the respective concentration (C) or pH values for saliva and serum. Serum pH values of 7.2–7.6 were used, reflecting the lower and upper limits of the theoretical range. There was a significant correlation ($r = 0.57$, $p < 0.05$) between the actual and theoretical ratios (at a serum pH of 7.4).

Besides accounting for much of the intersubject variability in salivary distribution of procainamide, the differences in saliva pH also accounted for intrasubject changes in the distribution ratio. For each patient who exhibited differences in saliva pH between the two collection periods, the change in ratio was in the direction predicted by Eq. 1.

DISCUSSION

The measurement of procainamide concentrations in saliva is not a useful method for monitoring the plasma concentration of procainamide in patients. The variability in the saliva–serum concentration ratio is large and unpredictable. Even if saliva pH measurements were made for the purpose of applying a correction factor based on Eq. 1, the range of saliva concentrations found at any given pH is appreciable. For example, three patients had saliva pH values near 7.0 and the saliva–serum concentration ratio varied more than fourfold (Fig. 2).

The variability in salivary distribution of procainamide may be partly due to several other factors. Since the drug is only 15% bound to serum proteins (10), protein binding is not a likely factor. The contact time of procainamide with the buccal mucosa may be of slight importance. Meyers *et al.* (11) instilled buffered (pH 5–11) solutions of procainamide into the mouth and measured the loss of drug after 5 min. The absorption rate of procainamide was slow; only 4.5 and 14.3% absorption occurred at pH 5 and 11, respectively. The magnitude of these differences in absorption rate is much less than anticipated based on the degree of ionization of procainamide. However, these investigators did not measure the pH of the solution after equilibrium in the mouth. Saliva contains sufficient bicarbonate (40–50 mEq/liter) (12) to alter the pH of weakly buffered acidic solutions and possibly mute the pH differences.

A major factor which may affect the distribution ratio is the intrinsic and effective pH values at the site of saliva secretion. Serum pH may not adequately reflect the pH of the interstitial fluid on one side of the salivary glands while saliva pH, after mixing with the contents of the mouth, may differ from that which was formed by the gland and which contacts the epithelial cells within the gland. Thus, the theoretical curves shown in Fig. 2 may only approximate the effects of real pH differences across the salivary gland on a microscopic level. Finally, the rate of secretion of saliva affects the saliva concentration of some small molecules. For example, the concentration of urea in saliva decreases with the rate of salivary flow (13).

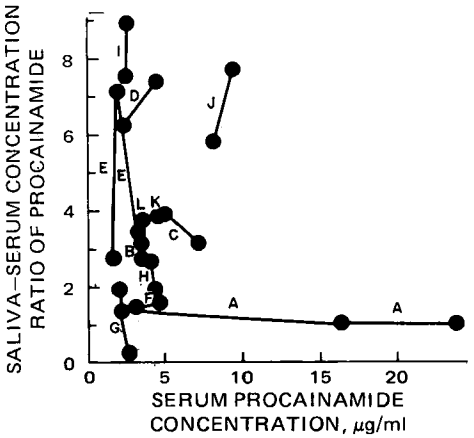

Figure 1—*Range of saliva–serum concentration ratios of procainamide as a function of serum concentration of procainamide in 12 patients during therapy with the drug. Lines connect data obtained repeatedly in individual patients. Letters identify data points for each patient in Table I.*

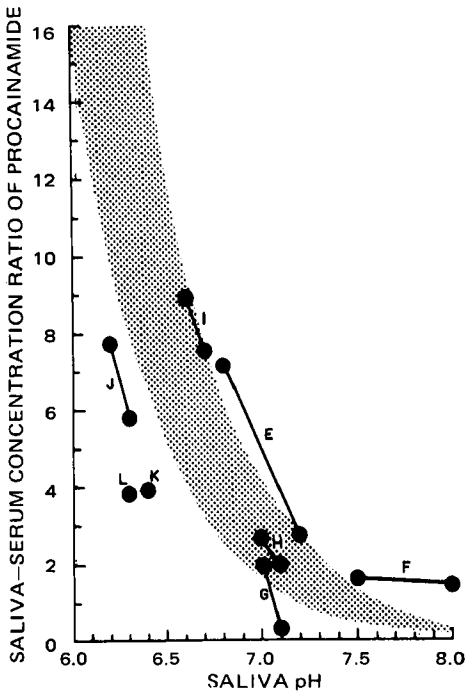

Figure 2—*Relationship between saliva–serum concentration ratios of procainamide and salivary pH in individual patients. The shaded area shows the theoretical concentration ratios calculated from Eq. 1, using a serum pH range of from 7.2 (lower edge) to 7.6 (upper edge).*

Examination of the effects of modifying pH on procainamide secretion into saliva in individual volunteer subjects does not appear feasible. Levy and Lampman (14) found that administration of large doses (5 g) of sodium bicarbonate did not significantly increase the pH of saliva of healthy subjects while urine pH changed appreciably. Doses of ascorbic acid or ammonium chloride were similarly without effect on saliva pH in single preliminary tests in this laboratory. Thus, the only reasonable approach to study the effect of pH on drug secretion into saliva is the present one, namely, a panel of subjects or patients with naturally different salivary pH values.

Other drugs have exhibited salivary secretion rates that were dependent on pH and protein binding. Gruneison and Witzgall (8) found a linear relationship between the saliva concentrations of several sulfonamides and the unbound drug in plasma of humans. Differences in ratios among the sulfonamides were consistent with the degree of ionization of these drugs. Sulfanilamide (pKa 10.4) produced the highest saliva–serum water concentration ratio (1.08), while sulfadiazine (pKa 6.28) yielded a ratio of 0.338. These studies were performed at only one salivary pH value (slightly lower than 7.4). However, the data of Rasmussen (15) on the same two drugs in cows and goats are of interest. These animals had a saliva pH of 8.2. Sulfanilamide, which would be essentially 100% unionized at pH 7.4 and 8.2, had a saliva–serum ultrafiltrate ratio of 1.0. Sulfadiazine, which would be 2% unionized at pH 8.2 and 11% unionized at pH 7.4, had a saliva–serum ultrafiltrate ratio of 1.3 in the animals.

Another weak base, trimethoprim (pKa 7.3), appears to behave like procainamide with respect to pH-dependent salivary secretion. Hansen *et al.* (16) reported a range in saliva-to-plasma concentration ratios of 1–8.3 in 14 patients. With the use of Eq. 1, the theoretical concentration ratios over a saliva pH range of 6.3–8.0 is 7.6 to 0.7, in close agreement with observed data.

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Fluorometric Determination of 2,3-Bis(*p*-methoxyphenyl)imidazo[1,2-*a*]pyrimidine in Biological Materials

DAVID G. KAISER ˣ and ARLINGTON A. FORIST

Abstract □ Interest in 2,3-bis(*p*-methoxyphenyl)imidazo[1,2-*a*]-pyrimidine as a potent, orally active, anti-inflammatory agent required a method for its determination in serum, urine, and feces to permit studies of its absorption, metabolism, and excretion. A simple, rapid, sensitive, and specific procedure was developed based on an ethyl acetate extraction of alkaline specimens and subsequent fluorometric analysis of ethanolic solutions of the extract residues. The method is sensitive to 0.2 μg/ml, 0.3 μg/ml, and 1.2 μg/100 mg of drug in serum, urine, and feces, respectively. The overall mean recovery and the standard deviation from biological samples are 99.8 ± 5.1%. The procedure has been successfully applied to absorption studies in the dog.

Keyphrases □ 2,3-Bis(*p*-methoxyphenyl)imidazo[1,2-*a*]pyrimidine—fluorometric analysis, biological materials, anti-inflammatory agent □ Anti-inflammatory agents—2,3-bis(*p*-methoxyphenyl)-imidazo[1,2-*a*]pyrimidine, fluorometric analysis, biological materials □ Fluorometry—analysis, 2,3-bis(*p*-methoxyphenyl)imidazo-[1,2-*a*]pyrimidine, anti-inflammatory agent, biological materials

In a continuing search for nonsteroidal anti-inflammatory drugs, 2,3-bis(*p*-methoxyphenyl)imidazo[1,2-*a*]pyrimidine (I), together with some structurally related compounds, was found to be a highly potent agent (1). Studies of the absorption, metabolism, and excretion of I required a method for its determination in serum, urine, and feces.

Previous reports described fluorometric procedures for the determination of indoxole [2,3-bis(*p*-methoxyphenyl)indole] (2) and 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile (3) in biological materials. These methods were successfully used in investigations in animals and humans (3–6).

Exploratory studies showed that I possessed useful fluorescence characteristics. Therefore, a simple, rapid, sensitive, and specific fluorometric procedure was developed for the determination of I in biological materials.

EXPERIMENTAL

Reagents and Materials—Aqueous sodium hydroxide (1 *N*) was stored in glass containers. Ethyl acetate was equilibrated with an equal volume of water prior to use in the extraction step. A solution of quinine sulfate[1] (2.25 μg/ml in 0.1 *N* aqueous sulfuric acid) was utilized as a spectrophotofluorometric standard. All pipets and centrifuge tubes were thoroughly rinsed with alcohol or acetone and air dried prior to use.

Instrumentation—A blender[2] and/or homogenizer[3] were used in the preparation of fecal specimens for extraction. A two-speed reciprocating shaker[4] was utilized for sample extractions in the horizontal position. Fluorometric measurements were made with a spectrophotofluorometer[5] equipped with a xenon lamp dc power supply[6], a compact xenon arc lamp (150 w)[7], a potted photomultiplier tube (1P28)[5], and a two-axis recorder[8].

Table I—Lower Limits of Detection for I in Rat and Dog Serum, Urine, and Feces

Specimen	Detection Limit[a]
Rat serum	0.06 μg/ml
Dog serum	0.18 μg/ml
Rat urine	0.30 μg/ml
Dog urine	0.24 μg/ml
Rat feces	1.20 μg/100 mg
Dog feces	0.88 μg/100 mg

[a] Based on a sample response twice that of the appropriate specimen blank.

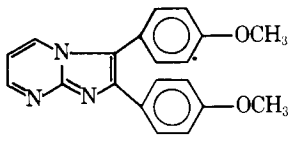
Standards—A stock solution of I was prepared by dissolving 5–10 mg in 100 ml of 95% ethanol with shaking. Working standards were prepared by dilution of the stock solution with 95% ethanol to give final concentrations of approximately 0.05, 0.1, 0.2, 0.4, 0.6, and 0.8 μg/ml. No special storage conditions were required since I is photochemically stable in acidic, neutral, or basic ethanolic solutions for at least 24 hr.

Collection and Storage of Specimens—Blood samples were allowed to clot at room temperature (27°) for 1 hr and then were centrifuged at 2000 rpm for 10 min, and the serum was harvested. Urine specimens were collected with 1 ml of 37% formaldehyde added for each 50 ml. All serum, urine, and fecal specimens were stored at −18° until analyzed.

Extractions—In a 15-ml glass-stoppered centrifuge tube were placed 1 ml of serum or urine, 0.05 ml of 1 *N* aqueous sodium hydroxide, and 3 ml of ethyl acetate. The mixture was shaken in a horizontal position for 10 min and then centrifuged for 10 min at 2000 rpm. A 1-ml aliquot of the ethyl acetate layer was transferred to a separate glass-stoppered centrifuge tube and evaporated to dryness in a stream of nitrogen. The residue was reconstituted in 5 ml of 95% ethanol for fluorometric analysis.

For rat feces, the frozen specimen was weighed and placed in the homogenizer[3]. A volume of water equivalent to twice the sample weight was added, and the mixture was homogenized at high speed for 1 min. A 100-mg aliquot of the slurry was weighed into a 15-ml glass-stoppered centrifuge tube, and 1 ml of water, 0.05 ml of 1 *N* aqueous sodium hydroxide, and 3 ml of ethyl acetate were added. The extraction was completed as described for serum and urine.

For dog feces, the total specimen was weighed and placed in the blender[2]. A volume of water equivalent to twice the sample weight was added, and the mixture was homogenized at high speed for 1 min. An aliquot (about 20 ml) was removed and homogenized[3] at high speed until a fine slurry was produced. A 100-mg aliquot was weighed into a 15-ml glass-stoppered centrifuge tube, and 1 ml of water, 0.05 ml of 1 *N* aqueous sodium hydroxide, and 3 ml of ethyl



I

[1] Matheson, Coleman and Bell, Milwaukee, Wis.
[2] Model PB-5A, Waring Products Corp., Winsted, Conn.
[3] Virtis 45, Virtis Co., Gardiner, N.Y.
[4] Eberbach & Sons, Ann Arbor, Mich.
[5] Aminco-Bowman, American Instrument Co., Silver Spring, Md.
[6] Sola Electric Co., Elk Grove, Ill.
[7] Engelhard Hanovia, Inc., Newark, N.J.
[8] Autograf, F. L. Moseley Co., Pasadena, Calif.

Table II—Recovery of I from Aqueous Solution and Biological Materials

Specimen	I Added[a]	I Found[b]	Recovery, %
Water	0.50	0.49	98.0
	1.00	0.98	98.0
	1.99	2.00	100.5
		Mean ± *SD*	98.8 ± 1.4
Rat serum	0.50	0.55	110.0
	1.00	0.95	95.0
	1.99	2.00	100.5
		Mean ± *SD*	101.8 ± 7.6
Dog serum	0.50	0.49	98.0
	1.00	1.03	103.0
	1.99	2.00	100.5
		Mean ± *SD*	100.5 ± 2.6
Rat urine	0.50	0.52	104.0
	1.00	1.03	103.0
	1.99	1.98	99.5
		Mean ± *SD*	102.2 ± 2.4
Dog urine	0.50	0.48	96.0
	1.00	1.00	100.0
	1.99	2.02	101.5
		Mean ± *SD*	99.1 ± 2.8
Rat feces	5.0	4.3	86.0
	10.0	9.8	98.0
	19.9	20.7	104.0
		Mean ± *SD*	96.0 ± 9.2
Dog feces	5.0	4.7	94.0
	10.0	10.0	100.0
	19.9	20.6	103.5
		Mean ± *SD*	99.1 ± 1.8

[a] Values for water, serum, and urine are expressed as micrograms per milliliter; values for feces are expressed as micrograms per 100 mg. [b] Corrected for appropriate specimen blank.

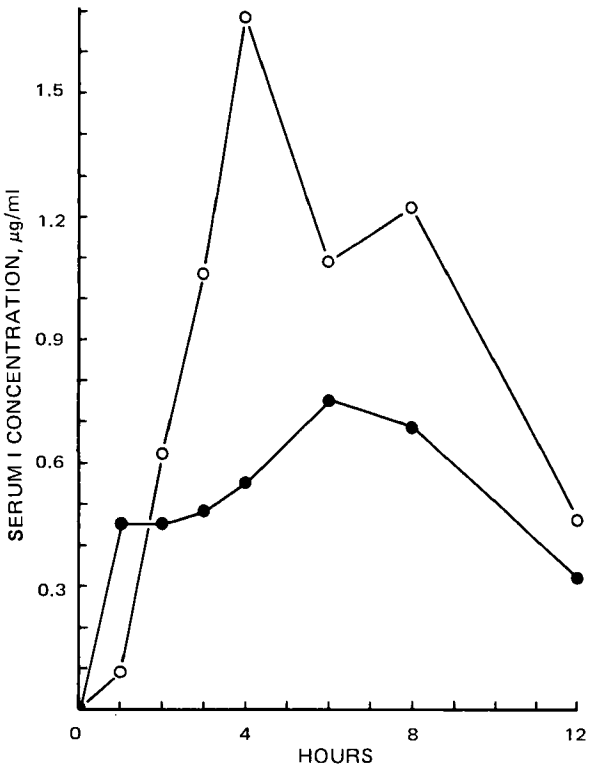


Figure 1—*Serum concentrations of I in dogs after single-dose oral drug administration in polysorbate 80. Key:* ○, *Dog BL-73 (male), 43.3 mg/kg; and* ●, *Dog 366 (male), 37.3 mg/kg.*

acetate were added. The extraction was completed as described for serum and urine.

Fluorometric Analysis—The spectrophotofluorometer was standardized with the quinine sulfate solution (60% transmission at 0.3 meter multiplier setting)[5], using the excitation (370 nm) and emission (470 nm) maxima for I. Fluorescence intensities of the extract residue solutions were measured at these wavelength settings. Compound I concentrations were calculated from a standard curve (working standards) with appropriate corrections for serum, urine, or feces blanks.

Preparation of Animals—In studies designed to determine the utility of the analytical methodology, three male beagle dogs, 10.5 ± 1.4 kg, were fasted for 16 hr prior to single-dose oral drug administration. Two dogs received ~40 mg of I/kg in polysorbate 80, and the remaining animal served as a vehicle-treated control. All animals were fasted for an additional 4 hr after drug administration and were then allowed free access to food and water.

Blood specimens were withdrawn from the jugular veins at 0, 1, 2, 3, 4, 6, 8, and 12 hr after drug administration. Urine specimens were collected at predetermined time intervals during the first 24 hr after drug administration. All specimens were stored at −18°.

TLC—All chromatography was conducted on thin layers (250 μm) of silica gel F_{254}[9], ascendingly developed in a solvent system of acetone–*n*-heptane–concentrated ammonium hydroxide (200:200:1 v/v). The separated materials were visualized by (*a*) irradiation of the plates with short wavelength (254 nm) and long wavelength (366 nm) UV lamps, or (*b*) spraying with 50% (v/v) aqueous sulfuric acid and heating at 110° for 20 min. Under these conditions, intact I had an R_f value of 0.18.

RESULTS AND DISCUSSION

Earlier studies with indoxole (2) and 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile (3) showed those compounds to be susceptible to photochemical degradation. By contrast, I is stable in acidic, neutral, or basic ethanolic solutions in the presence of light for at least 24 hr. Therefore, no special precautions are necessary in the storage of specimen extracts.

The maximum fluorometric sensitivity for I in 95% ethanol is 0.005 μg/ml (meter multiplier setting of 0.003)[5]. However, the practical lower limits of detection in biological matrixes are controlled by the magnitude of the specimen blanks in addition to the inherent fluorescence emission of I. Calculated lower detection limits for I, based on sample responses twice those of the appropriate blanks, are shown in Table I. In serum, urine, and feces, I may be detected at levels of ≥0.2 μg/ml, ≥0.3 μg/ml, and ≥1.2 μg/100 mg, respectively.

Under the described conditions, a linear relationship between fluorescence response and concentration of I is obtained over the 0–0.8-μg/ml range in 95% ethanol. Direct quantification from a standard curve has been adequate.

To determine recoveries from biological samples, known amounts of I in 95% ethanol were evaporated to dryness in centrifuge tubes, and water or specimens of rat or dog serum, urine, or feces were added. The samples were thoroughly mixed and analyzed by the standard procedure. Recoveries were quantitative (overall mean and standard deviation of 99.8 ± 5.1%), justifying the use of a simple standard curve in routine analysis as described previously (Table II).

Utility of the method was established by a limited study in dogs. Following oral administration of approximately 40 mg of I/kg, peak serum I levels of 0.75–1.7 μg/ml were observed between 4 and 6 hr (Fig. 1). Measurable concentrations were present throughout the 12-hr sampling interval. These results are similar to those reported for indoxole (2, 5) and 4,5-bis(*p*-methoxyphenyl)-2-phenylpyrrole-3-acetonitrile (3) in dogs.

The present method detects materials that are extractable from an alkaline solution with ethyl acetate and that possess excitation and fluorescence emission characteristics in 95% ethanol similar to I. Interference by endogenous materials in most specimens is very low, as indicated by the lower limits of detection (Table I). However, metabolic transformations could give rise to compounds responding in the assay. Accordingly, excitation spectra were routinely obtained on serum extracts as a check on specificity meta-

[9] Brinkmann Instruments, Westbury, N.Y.

bolic changes would be expected to result in altered UV spectra and, therefore, fluorescence excitation spectra. No such changes were observed in the dog. Moreover, TLC of serum extracts showed that the major component (>85%) was similar to I, confirming the measurement of intact drug. Thus, the method possesses adequate sensitivity and specificity for drug absorption studies in animals.

Based on fluorometric and TLC analyses, less than 1% of the dose was excreted in urine as intact I by the dog during the 24-hr collection interval. Extensive metabolism and/or biliary secretion are indicated.

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Conformationally Constrained Analogs of Mescaline II

ROBERT J. WOLTERS [x], A. J. BEJ, and N. S. TANNER

Abstract □ The synthesis of methyl-2-(3,4,5-trimethoxyphenyl)-2-(2-piperidyl) acetate is described. In addition, preliminary pharmacological data comparing the compound with mescaline are given.

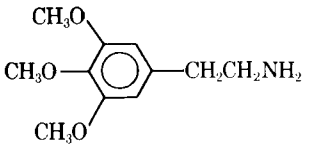
Keyphrases □ Mescaline—synthesis of conformationally constrained analog, methyl-2-(3,4,5-trimethoxyphenyl)-2-(2-piperidyl) acetate, IR and NMR spectra □ Methyl-2-(3,4,5-trimethoxyphenyl)-2-(2-piperidyl) acetate—analog of mescaline, synthesis, IR and NMR spectra

In an earlier publication (1), the synthesis of several conformationally constrained analogs of mescaline (I) was reported. This article reports the synthesis of methyl -2-(3,4,5-trimethoxyphenyl) -2- (2-piperidyl) acetate (II) as a modified mescaline analog. Compound II possesses the trimethoxyphenyl ring and the two-carbon chain of mescaline. The nonmethoxy analog of II, methylphenidate[1] (III), is a known central nervous system stimulant (2). It was anticipated that II would possess stimulant properties like mescaline and methylphenidate.

DISCUSSION

The synthetic route utilized for II was basically the same as that employed by Hartmann and Panizzon (3) for the synthesis of methylphenidate (Scheme I). The starting material, 3,4,5-trimethoxyphenylacetonitrile (IV), was synthesized according to the procedure of Telang and Smith (4).

Compound IV was reacted with 2-chloropyridine, using sodium hydride as the base, to form 2-(3,4,5-trimethoxyphenyl)-2-(2-pyridyl)acetonitrile (V). Compound V was hydrolyzed with sulfuric acid to form the amide VI. Methanolysis of VI gave the methyl ester VII. Catalytic hydrogenation of VII afforded the desired product II.



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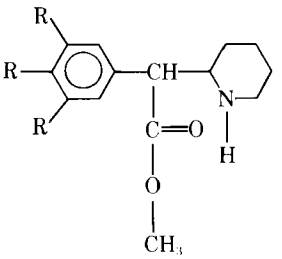

II: R = —OCH_3

III: R = —H

EXPERIMENTAL[2]

Pharmacology—The spontaneous activity of the test compound was compared to amphetamine, methylphenidate, and mescaline by means of activity chambers according to the procedure of Wolters *et al.* (1). The response of amphetamine, methylphenidate, and mescaline was reported previously (1).

The test compound at the 50-mg/kg dosage induced locomotor activity corresponding to that of saline in the first 40 min. Beyond 40 min, the rate of activity was comparable to mescaline, although the stereotyped behavior of mescaline scratching was not observed with the test compound at this dose.

Chemistry *2- (3,4,5-Trimethoxyphenyl) -2- (2-pyridyl)acetonitrile Hydrochloride (V-HCl)*—A solution of 75 ml of anhydrous dimethylformamide and 11.0 g (0.0531 mole) of IV was cooled to 0°. Then 3.0 g (0.060 mole) of sodium hydride was added. After the cessation of hydrogen evolution, a solution of 6.07 g (0.053 mole) of 2-chloropyridine in 50 ml of dimethylformamide was added drop-

[1] Ritalin Hydrochloride, Ciba Pharmaceutical Co., Summit, N.J.

[2] IR spectra were determined on a Perkin-Elmer model 337 spectrophotometer using potassium bromide pellets. NMR spectra were determined on the Varian model A-60A using tetramethylsilane as the internal standard and deuterochloroform as the solvent in approximately 30% concentration. The letter abbreviations used follow: s = singlet, 2s = two singlets, t = triplet, m = multiplet, and b = broad. Melting points were determined in open glass capillaries using a Thomas-Hoover Uni-Melt apparatus and are uncorrected. Microanalyses were performed by Alfred Bernhardt Mikroanalytishes Laboratorium, Fritz-Pregel-Strasse, West Germany.

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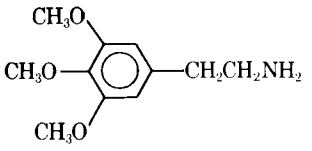
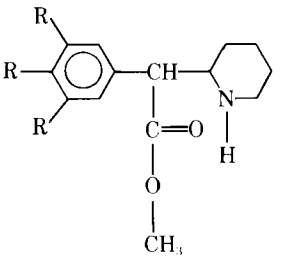
The synthetic route utilized for II was basically the same as that employed by Hartmann and Panizzon (3) for the synthesis of methylphenidate (Scheme I). The starting material, 3,4,5-trimethoxyphenylacetonitrile (IV), was synthesized according to the procedure of Telang and Smith (4).

Compound IV was reacted with 2-chloropyridine, using sodium hydride as the base, to form 2-(3,4,5-trimethoxyphenyl)-2-(2-pyridyl)acetonitrile (V). Compound V was hydrolyzed with sulfuric acid to form the amide VI. Methanolysis of VI gave the methyl ester VII. Catalytic hydrogenation of VII afforded the desired product II.



I



II: R = —OCH_3

III: R = —H

EXPERIMENTAL[2]

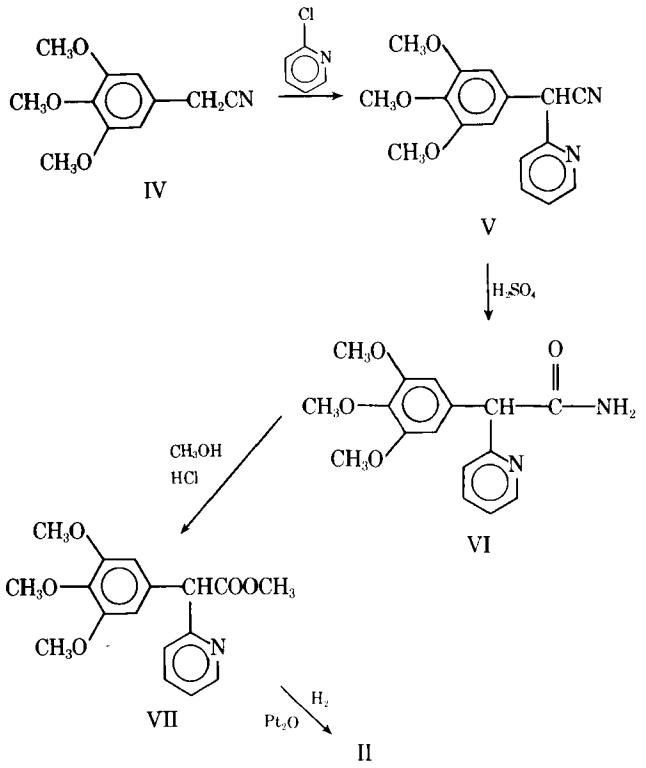
Pharmacology—The spontaneous activity of the test compound was compared to amphetamine, methylphenidate, and mescaline by means of activity chambers according to the procedure of Wolters *et al.* (1). The response of amphetamine, methylphenidate, and mescaline was reported previously (1).

The test compound at the 50-mg/kg dosage induced locomotor activity corresponding to that of saline in the first 40 min. Beyond 40 min, the rate of activity was comparable to mescaline, although the stereotyped behavior of mescaline scratching was not observed with the test compound at this dose.

Chemistry *2- (3,4,5-Trimethoxyphenyl) -2- (2-pyridyl)acetonitrile Hydrochloride (V-HCl)*—A solution of 75 ml of anhydrous dimethylformamide and 11.0 g (0.0531 mole) of IV was cooled to 0°. Then 3.0 g (0.060 mole) of sodium hydride was added. After the cessation of hydrogen evolution, a solution of 6.07 g (0.053 mole) of 2-chloropyridine in 50 ml of dimethylformamide was added drop-

[1] Ritalin Hydrochloride, Ciba Pharmaceutical Co., Summit, N.J.

[2] IR spectra were determined on a Perkin-Elmer model 337 spectrophotometer using potassium bromide pellets. NMR spectra were determined on the Varian model A-60A using tetramethylsilane as the internal standard and deuterochloroform as the solvent in approximately 30% concentration. The letter abbreviations used follow: s = singlet, 2s = two singlets, t = triplet, m = multiplet, and b = broad. Melting points were determined in open glass capillaries using a Thomas-Hoover Uni-Melt apparatus and are uncorrected. Microanalyses were performed by Alfred Bernhardt Mikroanalytishes Laboratorium, Fritz-Pregel-Strasse, West Germany.



Scheme I

wise and the reaction mixture was stirred for an additional hour. The mixture was allowed to equilibrate to room temperature and was stirred for an additional 12 hr.

Approximately 1.5 liters of water was added to the mixture, and the organic phase was separated and extracted with ether. Then the ether phase was extracted with several 25-ml portions of 10% hydrochloric acid. The combined acid extract was neutralized with 10% sodium hydroxide and extracted exhaustively with ether. The ether extract was evaporated on a steam bath to yield 8.9 g (61%) of viscous oil, and the oil was dissolved in anhydrous ether. Dry hydrogen chloride was bubbled into the ether solution to yield 10 g (59%) of crude product.

After recrystallization from a mixture of ethanol and anhydrous ether, the product melted at 162–180°. Additional recrystallizations did not appreciably reduce the melting-point range. The IR spectrum showed a peak at 2250 cm^{-1}, indicative of a nitrile group. The NMR spectrum of the free base gave the following: δ 3.9 (2s, 9H, methoxys), 6.5 (s, 1H, —CH), 7.1 (2s, 2H, aromatic), 7.5 (b, 3H, pyridine ring), and 8.2 (b, 1H, pyridine ring).

Anal.—Calc. for $C_{16}H_{16}N_2O_3 \cdot HCl$: C, 59.91; H, 5.34; N, 8.73. Found: C, 59.76; H, 5.50; N, 8.54.

2-(3,4,5-Trimethoxyphenyl)-2-(2-pyridyl)acetamide (VI)—To 35 ml of concentrated sulfuric acid was added 6.59 g (0.0232 mole) of V. The resulting solution was allowed to stand for 12 hr at room temperature. The reaction mixture was poured into crushed ice and neutralized with sodium carbonate, and the product was extracted with chloroform. The chloroform was removed on a steam bath, and the residue was recrystallized from ethyl acetate to yield 5.01 g (72%) of product, mp 144–146°.

The IR spectrum showed a peak at 1700 cm^{-1}, indicative of amide carbonyl. The NMR spectrum gave the following: δ 3.8 (s, 9H, methoxys), 5.0 (s, 1H, —CH), 6.8 (s, 2H, aromatic), 7.6 (b, 3H, pyridine ring, and 2H, amide), and 8.6 (b, 1H, pyridine ring).

Anal.—Calc. for $C_{16}H_{18}N_2O_4$: C, 63.56; H, 6.00; N, 9.27. Found: C, 63.59; H, 6.11; N, 9.08.

Methyl-2-(3,4,5-trimethoxyphenyl)-2-(2-pyridyl) Acetate (VII)—Hydrogen chloride gas was bubbled into a solution of 5.01 g (0.0166 mole) of VI and 150 ml of anhydrous methanol for 6 hr. After most of the methanol was removed, sodium carbonate was added to form the free base. The precipitate was filtered and recrystallized from 50% ethanol and then from ether, yielding 2.79 g (53%) of product, mp 101–104°.

The IR spectrum showed a carbonyl peak at 1740 cm^{-1}. The NMR spectrum gave the following: δ 3.8 (s, 3H, methoxys), 3.9 (s, 9H, methoxys), 5.2 (s, 1H, —CH), 6.8 (s, 2H, aromatic), 7.5 (b, 3H, pyridine ring), and 8.6 (b, 1H, pyridine ring).

Anal.—Calc. for $C_{17}H_{19}NO_5$: C, 64.34; H, 6.03; N, 4.41. Found: C, 64.26; H, 6.21; N, 4.25.

Methyl-2-(3,4,5-trimethoxyphenyl)-2-(2-piperidyl) Acetate Hydrochloride (II-HCl)—A mixture of 1.34 g (0.00423 mole) of VII, 10 ml of acetic acid, and 5 mg of platinum oxide was shaken with hydrogen (initial pressure of 55 psi) in a Parr hydrogenator for 20 hr. The platinum oxide was removed by filtration. The filtrate was diluted with three times its volume of water, and sodium carbonate was added to precipitate the free base, which was extracted with ether. The ether solution was dried over anhydrous magnesium sulfate, and the hydrochloride salt was made from the dried ether solution in the usual manner.

The crude product was recrystallized from methyl ethyl ketone, yielding 0.69 g (45%) of product, mp 202–203°. The NMR spectrum of the free base gave the following: δ 1.8 (b, 9H, piperidine ring), 3.6 (b, 1H, —NH), 3.8 (s, 3H, methoxys), 3.9 (2s, 9H, methoxys), 4.0 (b, 1H, —CH), and 6.6 (2s, 2H, aromatic).

Anal.—Calc. for $C_{17}H_{25}NO_5 \cdot HCl$: C, 56.76; H, 7.28; N, 3.89. Found: C, 56.84; H, 7.16; N, 4.00.

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Bioequivalency of Doxycycline Products

EDWARD J. ANTAL, JAMES M. JAFFE, ROLLAND I. POUST, and JOHN L. COLAIZZI ˣ

Abstract □ The bioavailability of three different brands and three different dosage forms of doxycycline was studied in normal subjects. Single doses, equivalent to 200 mg of doxycycline, were administered to six subjects in a crossover design as the innovator's intravenous solution given orally (Treatment A), the innovator's capsule product (Treatment B), a noninnovator's capsule product (Treatment C), the innovator's oral suspension product (Treatment D), and a second noninnovator's capsule product (Treatment E). All dosage forms contained doxycycline as the hyclate, except the suspension which contained the nonhyclate form. Serum levels were determined periodically over 48 hr, and cumulative urinary excretion was measured concurrently over a 120-hr collection period. No statistically significant differences were observed in any *in vivo* indicator of bioequivalence when the three capsule products were compared. Consequently, they were judged to be bioequivalent. When these capsule products were compared to the oral solution, no statistically significant differences were observed. However, when the capsules and the suspension were compared, statistically significant differences were found in the rate of absorption. *In vitro* dissolution tests were also conducted on the three brands of capsules, and times required to achieve 50% dissolution showed rank-order correlation with corresponding absorption rate constants.

Keyphrases □ Doxycycline products—bioavailability, three different brands and three different dosage forms □ Bioavailability—doxycycline products, three different brands and three different dosage forms

In recent years, numerous products introduced by pharmaceutical manufacturers have been chemical equivalents rather than innovator products. Consequently, the biological equivalency of the available solid, oral, multiple-source dosage forms of a given drug entity has become important and comparative bioequivalency studies have become a means of evaluating the quality and acceptability of such noninnovator products. The tetracycline analog, doxycycline, recently became available on a multiple-source basis. Since cases of bioinequivalence for some chemically equivalent products of the tetracycline antibiotics have been documented (1–4), bioequivalence of different sources of doxycycline cannot be merely assumed.

Two tetracycline antibiotics for which a substantial bioequivalency literature exists, tetracycline and oxytetracycline, were recently classified as "drugs whose solid oral dosage forms it is not possible at this time to state categorically there is, or is not, a potential for bioequivalence . . . problems" in a report of the APhA Academy of Pharmaceutical Sciences (5). In a recent study (6), the 50- and 100-mg capsules of one of the two noninnovator's brands of doxycycline capsule dosage forms were compared and determined to be bioequivalent to the innovator product.

The objectives of the present study were: (*a*) to evaluate the bioequivalency of the two currently commercially available, chemically equivalent, noninnovator brands of doxycycline capsules, using the innovator's capsule product as the standard; (*b*) to study the bioavailability of all three brands of capsules in comparison to equivalent doses of an oral solution and an oral suspension of the drug; and (*c*) to determine the correlation, if any, between the dissolution profiles of the capsule dosage forms and their relative bioavailability.

EXPERIMENTAL

***In Vivo* Bioavailability Study Protocol**—Six healthy, normal, adult male volunteers, 21–30 years old, with normal creatinine clearance values were employed as subjects. Five different dosage forms or brands of doxycycline were administered with approximately 240 ml of water. Each of the five treatments provided a dose of 200 mg of doxycycline, calculated as the base.

In Treatment A, reconstituted doxycycline hyclate for injection[1] was administered as an oral solution; Treatments B[2], C[3], and E[4] consisted of different brands of capsules, each containing doxycycline hyclate equivalent to 100 mg of doxycycline base. Treatment D consisted of an oral suspension[5], which contained 25 mg of doxycycline base, present as the monohydrate, per 5 ml after reconstitution.

Each treatment was administered using a complete crossover design, which was randomized to minimize any possible sequential effects. A time interval of at least 1 week elapsed between treatments, allowing adequate time for essentially complete doxycycline elimination. The subjects fasted at least 3 hr prior to dosing and continued to fast until 3 hr after drug administration.

Blood samples (4 ml) were collected in evacuated glass containers[6] just prior to dosing and at 1, 2, 3, 4, 7, 12, 24, 28, 34, and 48 hr after drug administration. Urine voids were collected at specific intervals and pooled for 120 hr postadministration.

***In Vitro* Dissolution Studies**—*In vitro* dissolution determinations were conducted on the three brands of capsules. Although doxycycline has no compendial monograph dissolution requirement, the general Method I of NF XIV (7) was used to establish and compare dissolution profiles. The mesh screen basket[7] was rotated at 25 rpm in a dissolution fluid of 0.1 *N* HCl. Samples were taken at 3-min intervals, filtered through a 0.22-μm membrane filter[8], and analyzed spectrophotometrically[9] at a wavelength of 268 nm. Doxycycline concentrations in solution were determined from a standard calibration plot prepared using doxycycline base[10] as a standard.

Potency Determinations of Test Products—Maximum concentrations of doxycycline achieved in the dissolution medium were employed as an indicator of the actual potency for each brand of capsule, thus providing information concerning content uniformity. Six oral suspension and intravenous solution products of identical lot number were analyzed by the method used in serum

[1] Vibramycin Intravenous, Lot 28286, Pfizer Laboratories. (This solution also contained 480 mg of ascorbic acid.)
[2] Vibramycin Hyclate Capsules, Lot 31570, Pfizer Laboratories.
[3] Doxychell Hyclate Capsules, Lot 55301E3, Rachelle Laboratories.
[4] Doxy II Hyclate Capsules, Lot 5570563, USV Pharmaceutical Corp.
[5] Vibramycin Monohydrate for Oral Suspension, Lot 27387, Pfizer Laboratories.
[6] Vacutainers, silicone-coated interior, Becton, Dickinson and Co., Rutherford, N.J.
[7] Model 53 stirring motor, rotating-basket assembly, 65-212, Hansen Research Corp., Van Nuys, Calif.
[8] Millex disposable filter unit, SLGS02805-0.22, Millipore Corp., Bedford, Mass.
[9] DB-G spectrophotometer, Beckman Instruments, Fullerton, Calif.
[10] A sample of doxycycline base, Lot 70681-S8052, was generously donated by Pfizer Laboratories.

Table I—Derived Pharmacokinetic Parameters from Both Serum Level and Urinary Excretion Data

Parameter	Treatment					Statistics[a]
	A	B	C	D	E	
Peak value of mean serum concentration–time curve, μg/ml	3.65	3.57	3.53	4.12	3.51	N.S.[b]
Mean value of peaks of individual serum concentration–time curves, μg/ml	3.86	3.68	3.55	4.29	3.69	$p < 0.05$
Mean time of peak values of individual serum concentration–time curves, hr	2.50	2.83	2.83	2.67	3.00	N.S.
Mean of area under individual serum concentration–time curves, μg/ml × hr	80.89	79.08	93.39	94.96	77.97	N.S.
Mean of individual total urinary excretion of unchanged drug, mg	97.69	92.93	85.20	100.16	90.21	N.S.

[a] Analysis of variance for repeated measures design. [b] N.S. = not statistically significant at 0.05 level of significance ($p > 0.05$).

and urine concentration determinations because of excipient interference in the UV spectral analysis.

Fluorometric Determination of Doxycycline in Biological Samples—The analytical technique employed for the determination of doxycycline in urine and serum was based on the fluorometric[11] method of Kohn (8). This method yields results comparable in accuracy and reproducibility to microbiological methods (9).

Statistical Evaluation of Results—Serum concentrations at each time period, peak serum concentrations, areas under the serum level–time curves, and total urinary excretion of doxycycline were analyzed by analysis of variance for a crossover design, using a computerized statistical program (10). This program utilizes a repeated measures design to allow for treatment interactions in the same subject, which would not be seen if each treatment was given separately to different subjects (11).

Any statistical differences found among treatments were further compared to determine differences between treatments by Tukey's Allowable Difference Test (12). Differences in calculated absorption rate constants for each treatment were tested for statistical significance using the Student t test for each possible combination of treatment pairs.

RESULTS

A summary of the average serum concentrations of the six subjects for each treatment at each of the 10 sampling times after drug administration is shown in Fig. 1. The insert in the figure depicts these same values but on an expanded time scale from 0 to 12 hr postadministration. Derived pharmacokinetic parameters from both serum levels and urinary excretion data appear in Table I. The results of the analysis of variance among treatments are recorded in the final column of this table. A significant difference was found among treatments for the average peak values of the individual serum concentration–time curves (Table I) and also at the 2-hr postadministration time (Fig. 1) when the mean serum concentrations were evaluated.

These differences among treatments were further compared by means of a multiple-range test, Tukey's Allowable Difference Test, to determine significant differences between treatments at the 0.05 level of significance (12). At 2 hr postadministration, a statistical difference was noted between the serum levels produced by Treatment D, the oral suspension, and Treatment E, a noninnovator capsule product. A borderline difference was also observed between the suspension serum levels and Treatment C, the other noninnovator capsule product. All other paired comparisons did not produce statistically different results.

When comparing the mean peak values of the individual serum concentration–time curves, a statistically significant difference was observed between Treatments D and C. Borderline differences were noted between the suspension and both Treatment B, the innovator's capsule, and Treatment E. No statistically significant differences were observed in other paired comparisons.

Absorption rate constants, k_a, were calculated for each treatment by fitting the mean serum level data for six subjects to the classical one-compartment pharmacokinetic model (13), using a nonlinear least-squares regression computer program (14, 15) adapted for use on the PDP-10 system[12]. The k_a values determined were as follows: Treatment A, 1.38 hr^{-1}; Treatment B, 0.91 hr^{-1}; Treatment C, 0.87 hr^{-1}; Treatment D, 1.32 hr^{-1}; and Treatment E, 0.88 hr^{-1}. No statistical significance was observed when these values were compared using a two-tailed Student t test at the 0.05 level of significance.

A summary of the *in vitro* test results is provided in Table II. Six capsules of each manufacturer's product were studied, and the dissolution profiles of the three capsule products are presented in Table II. The mean cumulative amounts of doxycycline dissolved are reported for each time up to one interval past the maximum amount dissolved. The time to achieve 50% dissolution was calculated after the dissolution data were fitted to a model describing the dissolution profiles of conventional capsule dosage forms (16).

Table II also shows the actual amount of active drug present in each capsule dosage form. These values were derived from the dissolution profiles. The maximum amounts of drug in solution were averaged for each of the six capsules used to study a certain manufacturer's product. For the oral solution and suspension, six containers with identical lot numbers were assayed by the method previously described, and the average amounts of active drug present in the solution and suspension dosage forms were 107.2 ± 3.4 and 112.9 ● 3.2 mg, respectively.

DISCUSSION

Bioavailability involves both the rate and extent of drug absorption. In this study, the bioavailability of two chemically equivalent capsule formulations was compared to a recognized standard, the formulation of the innovator whose efficacy has been documented by clinical experience. Chemically equivalent products that achieve the same bioavailability profile as measured by appropriate *in vivo* parameters are generally assumed to produce the same therapeutic effects (17).

Parameters used to compare the extent of bioavailability or the amount of drug absorbed from the formulations are the area under the serum concentration–time curve and the amount of unchanged drug excreted in the urine. According to an evaluation of these two parameters, the three different brands of doxycycline capsules did not produce any statistically significant differences. Therefore, the amount of drug absorbed from each was essentially equal for the subjects tested.

[11] Model 110 fluorometer, Turner Associates, Palo Alto, Calif.

[12] Digital Equipment Corp., Maynard, Mass. (The assistance of the University of Pittsburgh computer staff is acknowledged.)

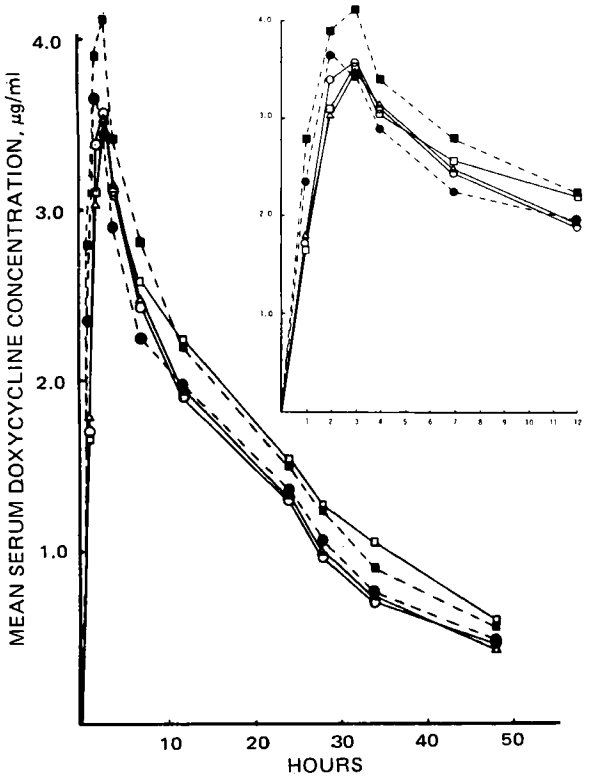

Figure 1—*Mean serum concentrations for six subjects following five treatments (0–48 hr). Insert shows expanded 0–12-hr data. Key: ●- - -●, Treatment A; ○——○, Treatment B; □——□, Treatment C; ■- - -■, Treatment D; and △——△, Treatment E.*

To be effective, doxycycline must be present in the blood and tissues at certain concentrations for certain durations of time. In comparing the three brands of capsules, no statistically significant differences were observed in serum concentrations, including the peak serum concentrations, at any sampling time period studied. The peak serum concentrations were compared both for the means of the six subjects and also on an individual subject basis, with no observed statistical difference.

Differences in rate of absorption can be compared by evaluating differences in peak times. The insert in Fig. 1 shows that mean serum concentration plots for the six subjects result in peak times of 3 hr for the three brands of capsules. Analysis of mean peak times of the individual serum concentration–time curves for the three capsule formulations tested revealed no statistically significant differences for this parameter. Therefore, on the basis of bioavailability profiles, these three capsule brands were judged to be similar in both rate and extent of absorption and may be termed bioequivalents.

A conclusion of bioequivalency for doxycycline products was also determined in another recent study (6) involving the 50- and 100-mg capsules of the innovator and one noninnovator[13]. Plasma levels were determined by a microbiological assay in 12 volunteers from 0 to 12 hr postadministration of a 100-mg dose of the drug. Each capsule was administered after a 1-hr fast in a randomized crossover design. No statistically significant differences were observed in mean plasma levels, areas under the plasma level curves, and the times for attaining the peak plasma levels (6). Although there were differences in the protocols and the observed peak times, both studies show bioequivalence for the brands of doxycycline capsule products studied. Differences in the protocols of the two studies included the number of subjects tested, assay methodology, biological samples tested, sampling times and duration, fasting period, dose of drug given, and number of different brands and dosage forms tested.

In comparing the bioavailabilities of the capsule dosage forms to those of the oral solution (Treatment A) and oral suspension (Treatment D), the parameters evaluated were the same as those used in determining the bioequivalence between capsule products. Statistically significant differences ($p < 0.05$) were observed for two of these parameters. The first parameter occurred at the 2-hr postadministration sampling time, with a statistically higher mean serum concentration produced by the oral suspension (Treatment D) as compared to one generic capsule (Treatment E). Differences at this early sampling time, which is prior to achieving the peak concentration, reflect differences in the rates of drug absorption. Since a drug must undergo dissolution before it can be absorbed, the suspension may present the drug in a form that permits it to undergo more rapid dissolution, leading to a higher serum concentration in the prepeak time periods. Another possible explanation for this result may be related to the fact that the suspension dosage form contained the nonhyclate (nonsalt) form of doxycycline, whereas all of the other dosage forms in this study contained the hyclate (hydrochloride salt) form.

The second statistically different parameter resulted from the peak serum concentrations produced in the individual subjects. Statistically higher ($p < 0.05$) concentrations were produced by the suspension (Treatment D) than by one noninnovator capsule (Treatment C). Although the peak serum levels of Treatment D showed a trend toward higher levels as compared to the other two capsule products (Treatments B and E), these differences were just short of statistical significance. These differences could be related to the different forms (salt *versus* nonsalt) of doxycycline involved or to other formulation differences between the capsules and the suspension. Since there were no differences in the compared areas under the serum concentration–time curves between capsules and suspension, a faster rate of absorption might have been responsible for producing the higher peaks, rather than differences in the active amounts of doxycycline present in the different dosage forms.

When the dissolution rates, as measured by the time required to achieve 50% dissolution, and the corresponding absorption rate constants of each capsule formulation were compared, rank-order correlation was observed. The innovator's capsule formulation (Treatment B) corresponded to the shortest time to reach 50% dissolution and the greatest absorption rate, as indicated by the k_a. The noninnovator's formulations followed in numerical ranking, with Treatment C corresponding to the longest time to reach 50% dissolution and the smallest absorption rate constant. However, since there were no statistically significant differences among any of the *in vivo* parameters studied, including the calculated rate constants of absorption, for the three capsule products, dissolution data could presumably represent a range of acceptable values.

This study was undertaken to ascertain the bioequivalency of

Table II—*In Vitro* Dissolution Data for Three Manufacturers' Capsule Dosage Forms of Doxycycline

Minutes	Mean Cumulative Amount Dissolved for Six Capsules (mg ± *SD*)		
	Manufacturer 1[a]	Manufacturer 2[b]	Manufacturer 3[c]
3	1.5 ± 0.9	3.1 ± 1.3	1.9 ± 1.3
6	30.6 ± 2.7	40.4 ± 6.4	35.9 ± 8.8
9	50.8 ± 5.4	67.9 ± 5.3	58.2 ± 10.4
12	63.0 ± 6.7	87.7 ± 10.0	72.2 ± 13.0
15	72.3 ± 8.5	99.8 ± 7.5	81.0 ± 13.4
18	82.4 ± 9.8	104.4 ± 7.9	88.7 ± 14.1
21	88.6 ± 10.6	106.0 ± 6.1	92.7 ± 12.7
24	94.4 ± 11.2	104.9 ± 6.1	96.5 ± 11.1
27	97.7 ± 9.9	—	99.2 ± 10.2
30	100.3 ± 8.6	—	101.6 ± 8.7
33	101.1 ± 6.6	—	102.6 ± 7.0
36	102.9 ± 5.7	—	102.6 ± 6.9
39	102.4 ± 5.7	—	102.0 ± 6.1
Time (minutes) for 50% dissolution	9.33	7.12	7.77
Mean capsule potency, mg ± *SD*	103.4 ± 6.2	106.1 ± 6.0	103.3 ± 6.5

[a] Rachelle Laboratories. [b] Pfizer Laboratories. [c] USV Pharmaceutical Corp.

[13] Doxy II Hyclate Capsules, USV Pharmaceutical Corp.

the three currently commercially available capsule dosage forms of doxycycline hyclate and to compare their bioavailability profiles with those obtained from oral administration of an equivalent amount of doxycycline hyclate in solution form or an equivalent dose of doxycycline base in suspension form. Through the use of serial serum determinations and cumulative urinary excretion data collected in a single-dose study of crossover design, *in vivo* parameters were compared.

No statistically significant differences among the three capsule products were found, and the products were judged to be bioequivalent. This finding corroborates one other study (6) which concluded bioequivalency between two of the three brands employed in this study. When these same capsules were compared to the oral solution and suspension, differences were found in one prepeak mean serum level. *In vitro* dissolution tests were conducted on the three capsule products; time for 50% dissolution showed rank-order correlation with corresponding absorption rate constants.

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[x] To whom inquiries should be directed.

Stability of Aspirin in Liquid and Semisolid Bases V: Polyglycerol Esters

C. W. WHITWORTH [x] and A. F. ASKER

Abstract □ The stability of aspirin in decaglycerol tetraoleate, decaglycerol octaoleate, and decaglycerol decaoleate was studied at 4, 26, and 45°. Degradation of aspirin in these polyglycerol esters was temperature dependent. Aspirin demonstrated the greatest stability in decaglycerol octaoleate and the lowest stability in decaglycerol tetraoleate at all temperatures studied. The hydroxyl value and the viscosity of the polyglycerol ester appeared to influence the stability of aspirin.

Keyphrases □ Aspirin—solubility and stability in polyglycerol esters, effect of temperature, hydroxyl value, viscosity □ Polyglycerol esters—as solvents for aspirin, stability, effect of temperature, hydroxyl value, viscosity □ Stability—aspirin in decaglycerol tetraoleate, decaglycerol octaoleate, and decaglycerol decaoleate

In previous reports (1–4), the stability of aspirin in various liquid and semisolid bases was investigated. The decomposition of aspirin in polyethylene glycols was due, at least in part, to a transesterification reaction between aspirin and polyethylene glycols (5). Blocking free hydroxyl groups on the polyethylene glycols retarded the decomposition of aspirin (1).

Polyglycerol esters are synthetic products and may be hydrophilic or lipophilic, depending on the number of hydroxyl groups that are reacted with the fatty acids and/or oils in question (6). The consistency of these esters varies from waxy solids and semisolids to liquids (6). Feeding studies (7) indicated that such esters are completely nontoxic and are degraded fully by the body to yield glycerol and the fatty acid. The Food and Drug Administration approved the use of polyglycerol esters ranging from 2 to 30 moles of glycerin and placed no limits on the amounts for use (6).

Decaglycerol tetraoleate, decaglycerol octaoleate, and decaglycerol decaoleate are liquid at room temperature and possess viscosity values of 6000–8000,

the three currently commercially available capsule dosage forms of doxycycline hyclate and to compare their bioavailability profiles with those obtained from oral administration of an equivalent amount of doxycycline hyclate in solution form or an equivalent dose of doxycycline base in suspension form. Through the use of serial serum determinations and cumulative urinary excretion data collected in a single-dose study of crossover design, *in vivo* parameters were compared.

No statistically significant differences among the three capsule products were found, and the products were judged to be bioequivalent. This finding corroborates one other study (6) which concluded bioequivalency between two of the three brands employed in this study. When these same capsules were compared to the oral solution and suspension, differences were found in one prepeak mean serum level. *In vitro* dissolution tests were conducted on the three capsule products; time for 50% dissolution showed rank-order correlation with corresponding absorption rate constants.

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Keyphrases □ Aspirin—solubility and stability in polyglycerol esters, effect of temperature, hydroxyl value, viscosity □ Polyglycerol esters—as solvents for aspirin, stability, effect of temperature, hydroxyl value, viscosity □ Stability—aspirin in decaglycerol tetraoleate, decaglycerol octaoleate, and decaglycerol decaoleate

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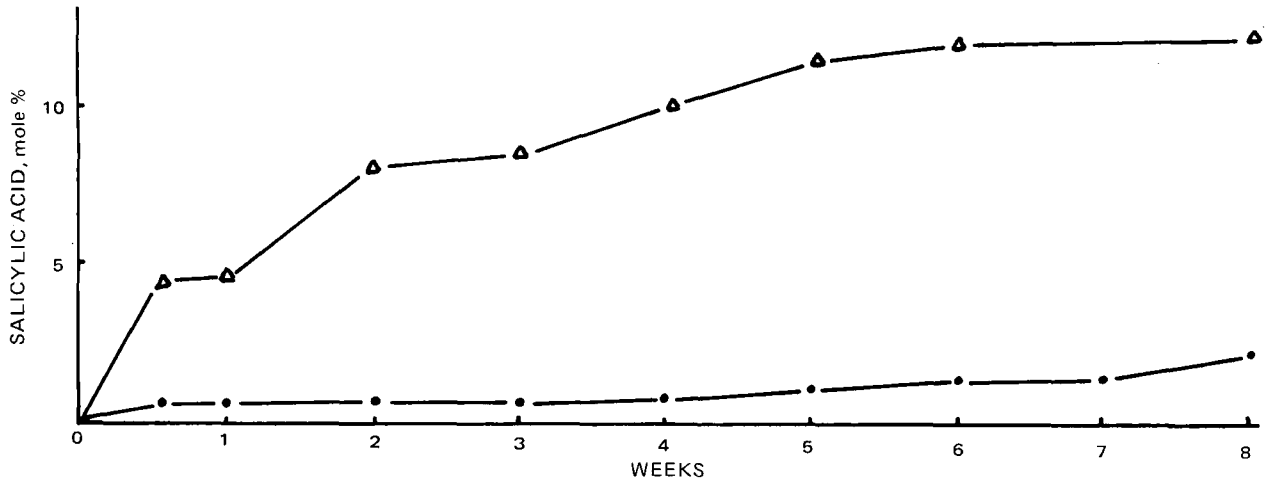

Figure 1—*Appearance of salicylic acid* versus *time for aspirin suspensions in polyglycerol esters at 4°. Key: △, decaglycerol tetraoleate; and ●, decaglycerol decaoleate.*

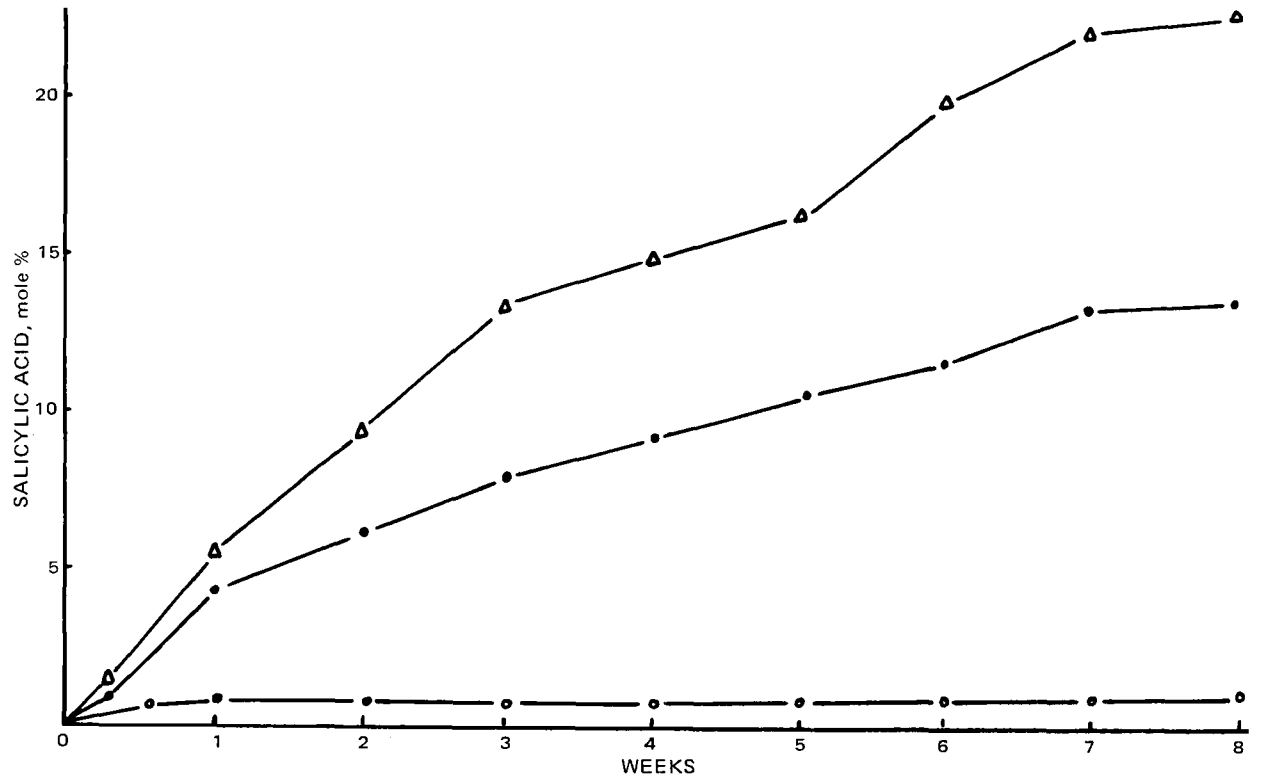

Figure 2—*Appearance of salicylic acid* versus *time for aspirin suspensions in polyglycerol esters at 26°. Key: △, decaglycerol tetraoleate; ●, decaglycerol decaoleate; and ○, decaglycerol octaoleate.*

800, and 400 cps, respectively (8). The hydroxyl values of these esters are 210–250 for the tetraoleate, 70–90 for the octaoleate, and 25–50 for the decaoleate (6, 8). The moisture content of these esters runs about 10%. The solubility of aspirin in the vehicles was determined and found to be 2.76, 2.19, and 2.01 mg/100 mg of vehicle for the tetraoleate, octaoleate, and decaoleate, respectively.

EXPERIMENTAL

Materials—Aspirin[1] USP and salicylic acid[1] were used as received. The polyglycerol esters[2] were supplied by the manufacturer. Chloroform[3] was spectrograde.

Sample Preparation—The samples were prepared by suspending 0.3 g of aspirin in 9.7 g of the polyglycerol ester contained in airtight amber glass vials with screw caps. The suspensions were stored at 4, 26, and 45°.

Analytical Method—UV spectrophotometric analysis, as reported by Tinker and McBay (9), was employed to measure aspirin and salicylic acid. Samples were taken at specific intervals and dissolved in 100 ml of chloroform containing 1% acetic acid. A further appropriate dilution was made, and the absorbance of the dilution was read[4] at 280 nm for aspirin and at 310 nm for salicylic acid.

[1] Merck and Co., Rahway, N.J.

[2] PVO International Inc., Boonton, N.J.
[3] J. T. Baker Chemical Co., Phillipsburg, N.J.
[4] Cary model 118 spectrophotometer.

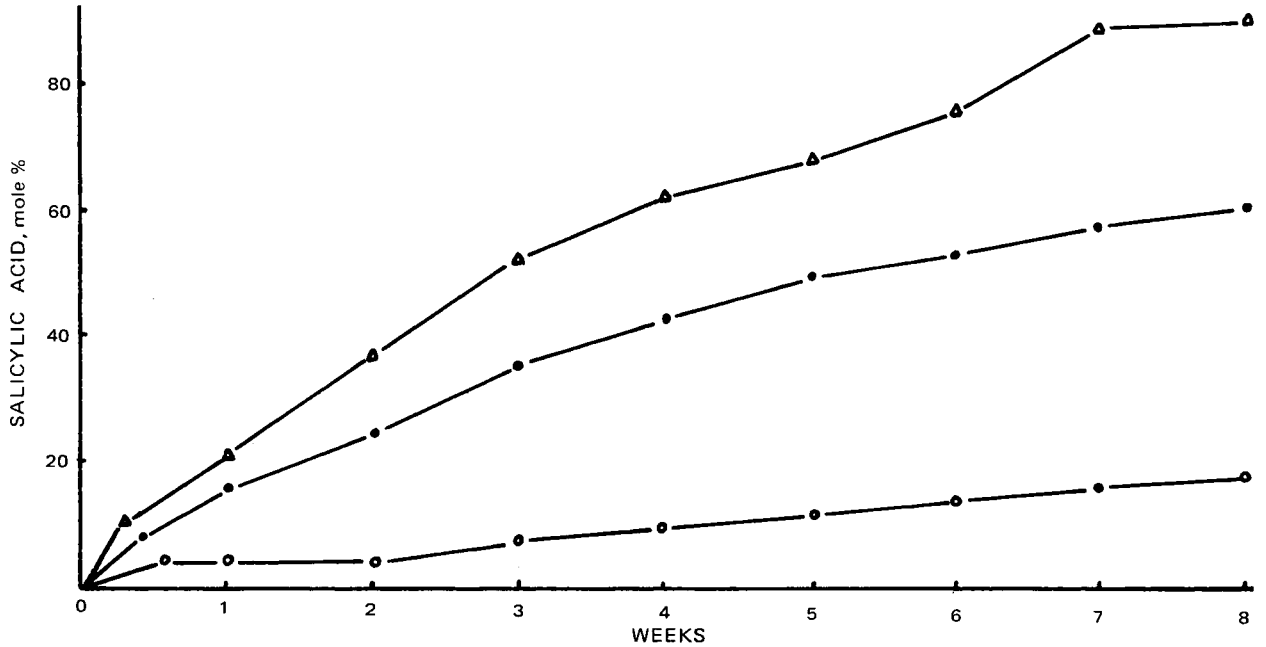

Figure 3—*Appearance of salicylic acid* versus *time for aspirin suspensions in polyglycerol esters at 45°. Key:* △, *decaglycerol tetraoleate;* ●, *decaglycerol decaoleate; and* ○, *decaglycerol octaoleate.*

Standard curves were prepared for aspirin and salicylic acid at these wavelengths. The quantification of aspirin and salicylic acid was based on the standard method of simultaneous spectrophotometric determinations.

Solubility Determination—The solubility of aspirin in each liquid was determined at 26° by adding 1 g of drug to 5 ml of vehicle and mixing with a mechanical stirrer for 24 hr. The mixture was then transferred to a test tube and centrifuged at 5000 rpm for 30 min. Three drops of the supernate were transferred to a 10-ml volumetric flask and accurately weighed.

The sample was dissolved by adding a 1% solution of acetic acid in chloroform, and the absorbance of a diluted sample of this solution was read at 280 and 310 nm. Three samples were used in each determination. The concentration of aspirin was determined by the standard method of simultaneous spectrophotometric determinations.

RESULTS AND DISCUSSION

The overall profiles of aspirin degradation in decaglycerol tetraoleate, decaglycerol octaoleate, and decaglycerol decaoleate at 4, 26, and 45° are shown in Figs. 1–3. Aspirin demonstrated the least stability in decaglycerol tetraoleate at all temperatures studied. Decaglycerol tetraoleate had the highest hydroxyl value (210–250) of the polyglycerol esters studied.

A previous publication (5) reported that the decomposition of aspirin in polyethylene glycols was due, at least in part, to a transesterification reaction and that the blocking of free hydroxyl groups retarded aspirin decomposition (1). Therefore, it appears that the lowest stability of aspirin in decaglycerol tetraoleate could be due to the highest hydroxyl value possessed by this vehicle. Moreover, decaglycerol tetraoleate is dispersible in water, while the octaoleate and the decaoleate are immiscible (6). Therefore, water that might have contaminated the aspirin powder or the decaglycerol tetraoleate during sample preparation would be distributed throughout the vehicle to react with aspirin particles.

Aspirin in decaglycerol octaoleate showed no detectable decomposition at 4°, about 1% at 26°, and 18% at 45° after 8 weeks. Although decaglycerol octaoleate has a higher hydroxyl value than that of decaglycerol decaoleate, aspirin was more stable in the former vehicle. This finding could be explained on the basis that the degradation-enhancing effect due to the higher hydroxyl value would be outweighed by the stabilizing effect due to the higher viscosity possessed by decaglycerol octaoleate. This higher viscosity would hinder diffusion of any moisture that might have contaminated the aspirin powder during sample preparation and, therefore, retard the degradation reaction. The solubility of aspirin in the vehicles was very similar for all three liquids and does not explain the differences in stability.

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Microbial Transformation of Aspidospermine

SHEN-KAI LIN *, M. TIN-WA, and E. H. TAYLOR ˣ

Abstract □ Thirty actinomycetes were isolated from soil samples and screened for their ability to modify the structure of aspidospermine. One actinomycete culture converted aspidospermine into *O*-demethylaspidospermine but failed to modify *N*-deacetylaspidospermine, *N*-ethyl-*N*-deacetylaspidospermine, 7-methoxyindole, and 7-methoxytryptophan.

Keyphrases □ Aspidospermine—structural transformation (demethylation) by actinomycetes, UV, IR, and NMR spectral identification of conversion products □ Alkaloids—structural transformation by actinomycetes, UV, IR, and NMR spectral identification of conversion products □ Actinomycetes—structural transformation of aspidospermine, UV, IR, and NMR spectral identification of conversion products

The enzyme systems of microorganisms have been used to transform numerous organic compounds including steroids and other biologically active substances such as alkaloids, flavonoids, terpenoids, and antibiotics. Although interest in the modification of alkaloid structures by microorganisms seems to be increasing, the volume of work in this field is still not large. Typical alkaloids that have been been converted microbiologically include conessine (1), agroclavine (2), 14β-bromocodeinone (3), sparteine (4), nicotine (5), and indoles such as yohimbine (6) and vindoline (7).

These successes in modifying alkaloid structures and the unique advantages of microbial conversions (*e.g.*, reactions at chemically inactive positions, stereospecific substitutions, coupling of several reactions, and mild reaction conditions) led to the investigation of microbial transformations of other alkaloids.

Thirty actinomycete cultures were screened for their ability to modify indole alkaloids. One effectively modified aspidospermine, and the major transformation product was isolated and characterized. This report is the first in the literature concerning the cleavage of a phenyl methyl ether bond of an alkaloid by a microorganism.

EXPERIMENTAL[1]

Materials and Methods—The 30 actinomycetes employed in the screening work were isolated by the usual method (8) from soil samples collected locally. The pure cultures were maintained on sucrose nitrate slants. A crude aspidospermine fraction, previously obtained in this laboratory from the bark of *Aspidosperma quebracho-blanco* Schlecht (Apocynaceae) (9), was purified by recrystallization from ethanol.

N-Deacetylaspidospermine was prepared by acid hydrolysis of aspidospermine (10). *N*-Ethyl-*N*-deacetylaspidospermine was prepared from *N*-deacetylaspidospermine using ethyl iodide (10). 7-Methoxyindole was purchased[2].

Screening Procedures—The microorganisms were grown in 125-ml erlenmeyer flasks in a medium composed of 3% glycerol, 0.35% casamino acid[3], 0.1% K_2HPO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.05% KCl, and a trace of ferrous sulfate heptahydrate. The flasks were incubated on a rotary (240 rpm) shaker at 28°. After 2 days, 1 ml of each culture was transferred to new medium and incubated for 2 days.

Aspidospermine (I) was added to each culture (0.2 mg/ml), and the reaction was allowed to proceed for 48 hr. The culture broths were extracted for alkaloids in the usual (acid–base shakeout) manner, and the crude alkaloid mixtures were chromatographed on silica gel GF_{254} TLC plates and developed with benzene–acetone (1:1). After drying, the plates were sprayed with ceric ammonium sulfate reagent (11), followed by Dragendorff's reagent (12).

One isolate, designated as Culture 14823[4], gave a conversion product (different R_f value and chromogenic reaction) and was used for the larger scale production of this conversion product. Control experiments were performed in which no cultures were added. No transformation took place in these control experiments, indicating that the experiment with Culture 14823 was indeed an enzyme-mediated reaction.

Conversion Procedure in Fermentor—Culture 14823 was grown in a 14-liter stirring fermentor. Aspidospermine (200 mg/liter) was added to the culture fluid, and incubation was continued under the cultivating conditions. Samples of 50 ml each were taken at 8-hr intervals, extracted for alkaloids, and analyzed by TLC. The ratio of the amount of the product formed to that of the substrate remaining was determined semiquantitatively by observing the size and color depth of the spots after spraying with Dragendorff's reagent.

When the conversion appeared maximal (48 hr), the alkaloids were extracted and then purified on a silica gel PF_{254}[5] column by eluting with benzene–acetone (1:1). The major conversion product (32% yield) isolated exhibited the same R_f values in three different TLC solvent systems as did the product obtained from the small flask fermentation. Several additional experiments carried out in the fermentor yielded similar results. A few other fermentation products were detected, but the yields were too minute and no further isolation work was performed.

RESULTS AND DISCUSSION

Identification of Conversion Product—The UV spectrum of the major conversion product showed absorptions at λ_{max} (ethanol): 220 (log ϵ 4.39), 258 (3.93), and 293 (3.54) nm (indicative of a dihydroindole moiety) (9). A bathochromic shift to 308 nm (log ϵ 3.90) in 0.1 *N* KOH in ethanol suggested the presence of a phenolic group.

An IR spectrum of this conversion product taken in potassium bromide showed absorptions at ν 2930, 2780, 1635, 1601, 1580, 1475, 1445, 1380, 1360, 1262, 1182, 1132, 1106, 1062, 935, 770, and 740 cm^{-1}. The mass spectrum of the conversion product showed a molecular ion, M^+, at *m/e* 340 (25); other significant ions were observed at *m/e* 339 (20.0), 312 (8.3), 160 (4.1), 152 (5.4), 146 (3.3), and 124 (100.0). Thus, the conversion product showed the same base peak at *m/e* 124 as well as a common peak at *m/e* 152 as the substrate aspidospermine (13), indicating that the nonindole portion of both molecules is the same.

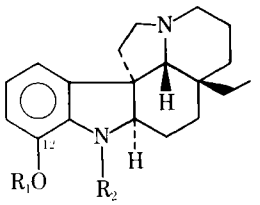
[1] The fermentor used was "Magnaferm" model MA-100 from New Brunswick Scientific Co., New Brunswick, N.J. UV spectra were taken using a Beckman model DB-G grating spectrophotometer, and spectra were examined from 200 to 340 nm. IR spectra were taken using a Beckman IR 18A spectrophotometer, and spectra were recorded from 4000 to 600 cm^{-1}. Mass spectra were determined using a single-focusing Hitachi Perkin-Elmer model RMU-6D mass spectrometer, operating at 70 ev. Proton NMR spectra were taken using a Varian A-60 instrument operating at 60 MHz.

[2] Aldrich Chemical Co. Inc., Milwaukee, Wis.

[3] Difco Laboratories, Detroit, Mich.

[4] Culture 14823 was identified by Dr. T. G. Pridham, Agricultural Research Service, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill., as a strain of subspecies of *Streptomyces griseus* (Krainsky) Waksman and Henrici.

[5] E. Merck, Darmstadt, West Germany.



I: $R_1 = CH_3$, $R_2 = COCH_3$
II: $R_1 = H$, $R_2 = COCH_3$
III: $R_1 = CH_3$, $R_2 = H$
IV: $R_1 = CH_3$, $R_2 = CH_2CH_3$

The molecular ion of the conversion product was at 14 mass units less than that observed for aspidospermine. This finding and the bathochromic shift observed in the UV spectrum suggested that the methoxy at C-12 of aspidospermine was converted to a hydroxyl. If so, the remaining peaks at *m/e* 339, 312, 160, and 146 can be readily identified to be the corresponding peaks of *m/e* 353, 325, 174, and 160, respectively, observed in the spectrum of aspidospermine (13).

An NMR spectrum in deuterochloroform, with tetramethylsilane as an internal standard, confirmed the conversion product to be *O*-demethylaspidospermine (II). An intramolecularly hydrogen-bonded phenolic proton at C-12 appeared as a singlet at δ 10.89, three aromatic protons appeared as a multiplet centered at δ 6.90, the proton at C-2 appeared as a quartet at δ 4.15, the *N*-acetyl group appeared as a three-proton singlet at δ 2.35, and the three protons of the ethyl side chain appeared as a triplet at δ 0.67. The methoxy proton present in the NMR spectrum of aspidospermine at δ 3.89 was absent in the spectrum of the conversion product.

The IR and NMR spectra were superimposable with those of semisynthetic *O*-demethylaspidospermine, prepared according to the method of Witkop and Patrick (10) from aspidospermine.

It appears that the enzyme systems in Culture 14823 cleave the phenyl methyl ether linkage without affecting the neighboring acetyl group. To determine if this type of reaction could be applied to other compounds with the phenyl methyl ether moiety, the substrate specificity of the enzyme system was investigated. Culture 14823 was tested for its ability to transform the following compounds utilizing the same screening procedure: 7-methoxyindole, 7-methoxytryptophan[6], *N*-deacetylaspidospermine, and *N*-ethyl-*N*-deacetylaspidospermine. No significant transformations occurred.

SUMMARY

Aspidospermine was demethylated during the fermentation process by Culture 14823. The phenyl methyl ether bond is stable and requires drastic chemical means for fission, *e.g.*, heating with aluminum chloride at 140° or fusion with sodium hydroxide above 240° (10). However, the cleavage of this bond can be achieved by enzymatic action under very mild conditions.

This culture failed to demethylate *N*-deacetylaspidospermine, *N*-ethyl-*N*-deacetylaspidospermine, 7-methoxyindole, and 7-methoxytryptophan. Since these substances all lack the acetyl group on the indole nitrogen, this group may be essential for this enzymatic reaction to take place.

A literature search revealed that only a few examples of the microbial cleavage of phenyl methyl ether bonds have been reported, *e.g.*, demethylation of griseofulvin by fungi (14), of vanillic acid by bacteria (15), and of 7-methoxyisoflavone by *Penicillium cyclopium* (16).

[6] Supplied by Dr. A. Kalir, Department of Organic Chemistry and Pharmacology, Israel Institute for Biological Research, Ness-Zionn, Israel.

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Antineoplastic and Cytotoxic Components of Desert *Baileya*

Keyphrases □ *Baileya multiradiata*—six antineoplastic and cytotoxic components, isolated and evaluated □ Antineoplastic agents, potential—isolated from *Baileya multiradiata*, biological evaluation □ Cytotoxic agents—isolated from *Baileya multiradiata*, biological evaluation

To the Editor:

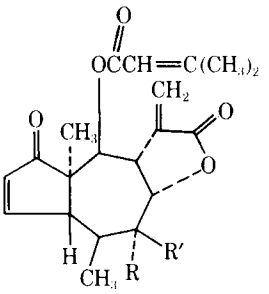
The bright-yellow ray-florets of *Baileya multiradiata* Harv. and Gray (family Compositae)[1] are commonly seen in the southwestern United States from Texas to California. The flowering portion is attractive to sheep, and ingestion has led to the death of many such range animals (1).

In 1966, we collected *B. multiradiata* in Arizona for evaluation in respect to possible antineoplastic components (2). Later, an ethanol extract of the complete plant was found to reach confirmed active status in the National Cancer Institute's murine lymphocytic leukemia P-388 screen. A cell line derived from this PS system suitable for *in vitro* measurements was studied as a bioassay method for isolating the antineoplastic constituents of *B. multiradiata*.

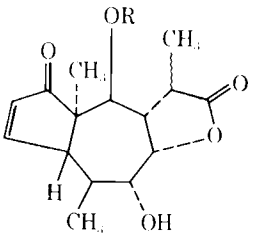
We now wish to report the isolation of six cytotoxic and antineoplastic constituents of *B. multiradiata* and that the new P-388 *in vitro* screening technique[2] proved very efficient for this purpose. The results of this bioassay are expressed as ED_{50}, namely, that concentration of material (in micrograms per milliliter) that produces 50% inhibition of growth in a 48-hr incubation period.

A chloroform-soluble fraction (5.98 g) prepared from *B. multiradiata* Harv. and Gray was partially separated by chromatography on cross-linked dextran gel[3]. The fraction (4.8 g) eluted with methanol–chloroform (6:4) was subjected to careful gradient (benzene to ethyl acetate) elution chromatography on silica gel. The following were obtained (in order of isolation): fastigilin C (I*b*, 0.13 g, ED_{50} 0.004)[4] (4), fastigilin B (II*a*, 0.09 g, ED_{50} 0.078) (4), baileyin (III, 0.05 g, ED_{50} 0.47) (5), and pleniradin (IV*a*, 2.03 g, ED_{50} 5.7), characterized as the monoacetate derivative IV*b* (ED_{50} 2.9) (6).

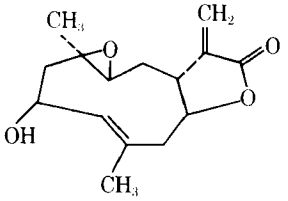
A new pseudoguaianolide, designated multiradiatin (I*a*, ED_{50} 0.02, crystals from acetone–heptane, 0.19 g, mp 226–230°) (4), as well as radiatin (II*b*, 0.67 g, ED_{50} 0.39) (6), was isolated by analogous large-scale chromatography (161 g of chloroform fraction). The structural assignment for multiradiatin was confirmed by the identity of this substance with the ketone obtained by chromic acid (Jones reagent) oxidation of fastigilin C. Both fastigilin C and baileyin (III) were isolated previously from *B. multiradiata* by Waddell and Geissman (5).



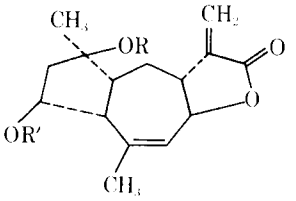
I*a*: R = R′ = O
I*b*: R = OH, R′ = H



II*a*: R = COCH=C(CH$_3$)$_2$
II*b*: R = COC(=CH$_2$)CH$_3$



III



IV*a*: R = R′ = H
IV*b*: R = H, R′ = COCH$_3$

In vivo biological studies against several tumors of the National Cancer Institute's screening systems are now in progress. At present, consistent *in vivo* activity has been obtained, for example, with fastigilin C (I*b*), which in the PS screen leads to a 50% increase in survival time at a dose of 3 mg/kg. Recently, we (7) summarized analogous and encouraging biological activity for the related pseudoguaianolide helenalin. Thus, results of the present study suggest that the pseudoguaianolide lactone of the fastigilin, helenalin, and radiatin type possesses (in addition to the usual cytotoxic properties of such lactones) carrier groups that fall within requirements for *in vivo* antineoplastic activity. Also, the cytotoxicity displayed by the sesquiterpene lactones of *B. multiradiata*, while correlated with the antineoplastic activity, may suggest that these components are responsible for sheep poisonings attributed to this plant.

[1] The plant material was identified by Mrs. E. Lehto, Herbarium curator, Department of Botany, Arizona State University. Voucher specimens are preserved in our Institute and in the University's Herbarium.

[2] For results of a study employing L-1210 cells *in vitro* as a cytotoxicity assay, refer to Ref. 3.

[3] Sephadex LH-20.

[4] Spectral data (mass, IR, and PMR) and elemental analyses were consistent with each structural assignment. Reported yields correspond to the recrystallized (from benzene–ethyl acetate unless otherwise noted) product.

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Germination and Morphine Content of *Papaver somniferum* Plants Produced from Commercially Available Poppy Seed

Keyphrases □ *Papaver somniferum*—germination and cultivation from commercial poppy seeds, morphine content, TLC, column chromatography, IR and mass spectra □ Morphine—isolated from *Papaver somniferum* germinated and cultivated from commercial poppy seeds, TLC, column chromatography, IR and mass spectra □ Narcotics—morphine, isolated from *Papaver somniferum* germinated and cultivated from commercial poppy seeds, TLC, column chromatography, IR and mass spectra

To the Editor:

There are no legal constraints to the possession of seeds of *Papaver somniferum*, the opium poppy. However, it is illegal to cultivate this plant in the United States. This paradox is interesting since *Cannabis sativa* (marijuana) seeds must be sterilized prior to sale. The narcotic regulations do not require sterilization of *P. somniferum* seeds.

Because *P. somniferum* could be illicitly cultivated in the United States as a source of codeine and morphine, the present study was carried out to answer the following questions. What is the botanical identification of commercially available poppy seeds? What is the germination rate of such seeds? And will plants produced from commercial poppy seeds grow and produce morphine in a temperate climate such as we have in the United States?

Poppy seeds (100-g samples) were obtained from two commercial bakeries in Chicago as well as from three supermarkets in the area. The seeds were soaked for 2 min in a dilute solution of sodium hypochlorite, washed thoroughly with water, and germinated on wet vermiculite. Approximately 60% of the seeds from each of five lots germinated.

After the seedlings developed well-expanded cotyledons, they were transplanted to potting soil. The plants were grown in a greenhouse in 10.2-cm (4-in.) diameter clay pots. Flowering occurred about 8 weeks after germination. Greenhouse plants were similar in appearance to fieldgrown plants, although they were smaller[1].

Immature capsules of one seed lot (I-9786) were pierced with a suitable needle daily, and the dried latex was scraped from the capsules and accumulated. A total of 24 mg of dried latex was collected in this manner from several capsules.

When the plants were fully mature, the entire plants were dried and then subjected to alkaloid extraction. The latex (24 mg), which had been scraped from the immature capsules, was dissolved in 15 ml of 1% HCl and filtered. After the filtrate was made alkaline with ammonium hydroxide, it was extracted three times with 15-ml volumes of chloroform. The combined chloroform extracts were taken to dryness to yield a 10-mg residue, which was used in the TLC analyses.

TLC of the extract on silica gel G plates, when compared with an authentic morphine sample, indicated morphine to be present in the alkaloid extract in addition to several other alkaloids.

Chromatography of the alkaloid extract over a small column (disposable pipet) containing 1.5 g of silica gel PF_{254}, using chloroform–methanol–ammonium hydroxide (90:10:1) as the eluent, resulted in the isolation of a compound (2 mg) whose R_f on silica gel PF_{254} plates, using the same solvent system, was 0.71.

The mass spectrum of the compound indicated a molecular ion M^+ at *m/e* 285 nm, and the fragmentation pattern was consistent with that reported for morphine (1).

To confirm this identification, additional capsules and foliage (8.38 g) were processed for alkaloids as already described. We wanted to determine if a larger quantity of the compound, tentatively identified as morphine, could be isolated and more definitively identified. TLC analysis of the resulting alkaloid extract indicated the same pattern as was observed for the alkaloid extract prepared from the latex.

Column chromatography of this extract, using 10 g of silica gel PF_{254}, resulted in the isolation of 7.6 mg of the compound. The UV spectrum of the sample (in ethanol) showed a maximum absorption at 284 nm,

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Thermodynamics of Gaseous Anesthesia of Mice

Keyphrases □ Anesthesia—gaseous, thermodynamics of equilibrium process, evaluation of Ferguson principle, mice □ Thermodynamics—gaseous anesthesia, evaluation of Ferguson principle, mice □ Ferguson principle—applicability to thermodynamics of gaseous anesthetics

To the Editor:

Evidence was presented recently indicating that the gaseous anesthesia of mice is largely a gas–biophase equilibrium process (1). This evidence consists principally of the observations that gaseous anesthetic potencies can be correlated against gas–oil distribution coefficients (1) but not against oil–water or 1-octanol–water partition coefficients (2). Since gaseous anesthesia has the characteristics of an equilibrium process, it is appropriate to establish the apparent thermodynamic variables pertaining to the anesthetic process in mice. This report presents such an attempt. The results indicate that the Ferguson principle (3), as presently formulated, is in error. A revised expression of the Ferguson principle is a direct outcome of this study.

The Clausius–Clapeyron equation for the distribution of a gas with a condensed phase is given by:

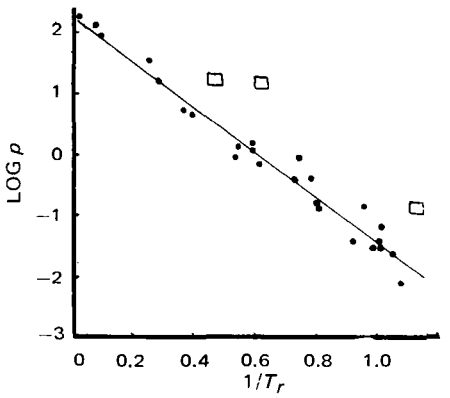

Figure 1—*Graph of anesthetic pressure against the reciprocal of the physiological reduced temperature. Compounds not following the relationship (fluoromethane, perfluoromethane, and perfluoroethane) are identified by the symbol* □.

$$\left(\frac{\partial \ln a_2}{\partial T}\right)_p = \left(\frac{\partial \ln p}{\partial T}\right)_p = \frac{\Delta H_v}{RT^2} = \frac{\Delta S_v}{RT} \qquad \text{(Eq. 1)}$$

where p is the partial pressure of the gas (presumed ideal) at an absolute temperature, T, and pressure, P, and a_2 is the thermodynamic activity of the gas solubilized in the condensed phase.

Equation 1 is appropriate for the determination of the apparent enthalpy and entropy of vaporization, ΔH_v and ΔS_v, at each temperature T from the slopes of graphs of log p against $1/T$ or log T, respectively. However, if the distribution of a number of gases with a condensed phase is of interest, one must either determine the partial pressure variation with temperature for each gas or, following the Principle of Corresponding States, approach the problem in terms of reduced thermodynamic variables so that the same equation of state will apply to each gas.

The reduced thermodynamic variables, P_r, V_r, and T_r, for a substance are obtained by dividing the critical values, P_c, V_c, and T_c, for the substance into the corresponding thermodynamic variables, P, V, and T. Critical values define the point where a gas can no longer be condensed into a liquid phase. When con-

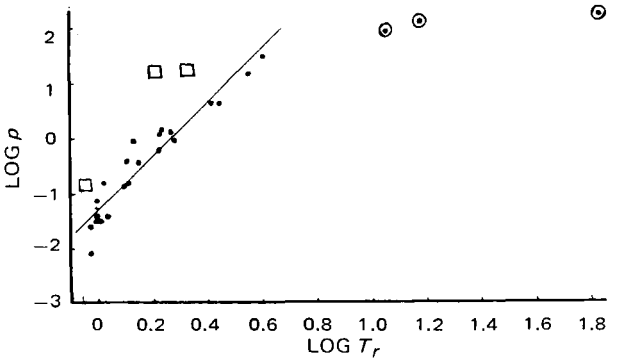

Figure 2—*Graph of anesthetic pressure against the logarithm of the physiological reduced temperature. Compounds not following the relationship (fluoromethane, perfluoromethane, and perfluoroethane) are identified by the symbol* □. *Anesthetics whose boiling points are near 0°K (helium, hydrogen, and neon) and which may be expected to depart from general trends are identified using the symbol* ⊙.

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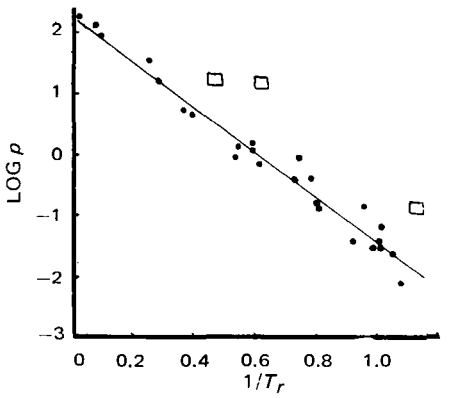

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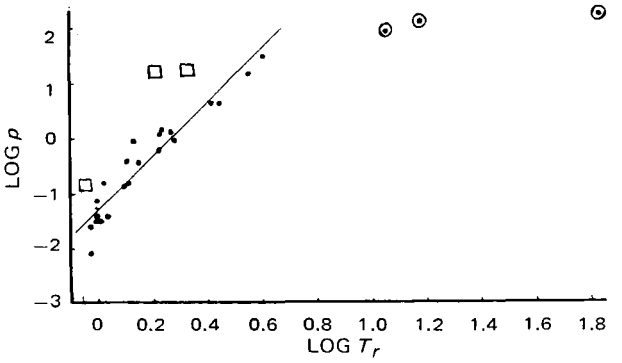

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Table I—Minimum Anesthetic Pressures to Inhibit the Righting Reflex of Mice and the Physiological Reduced Temperatures for the Gases

Gas	Log p[a]	T_{BP}, °K[b]	$T_r = \frac{T}{T_{BP}} = \frac{310}{T_{BP}}$
Helium	2.28	4.5	68
Hydrogen	2.14	20.6	15.0
Neon	1.94	27.1	11.4
Nitrogen	1.52	77.3	4.01
Hexafluoroethane	1.19	194.1	1.60
Tetrafluoromethane	1.24	145.4	2.13
Argon	1.18	87.5	3.54
Methane	0.66	111.6	2.77
Krypton	0.65	121.1	2.56
Nitrous oxide	0.18	184.6	1.68
Ethylene	0.15	169.4	1.83
Ethane	0.11	184.5	1.68
Xenon	−0.02	165.1	1.88
Propane	−0.05	231.0	1.34
Acetylene	−0.15	189.5	1.63
Dichlorodifluoromethane	−0.40	243.3	1.27
Propylene	−0.40	225.4	1.37
Cyclopropane	−0.80	239.6	1.29
Trichloromonofluoromethane	−0.82	296.8	1.04
Fluoromethane	−0.85	351.3	0.88
Chloromethane	−0.85	249.1	1.24
Iodomethane	−1.15	315.5	0.98
Ethyl chloride	−1.40	285.4	1.08
Ethyl bromide	−1.40	311.5	0.99
Ether	−1.52	307.7	1.01
Dichloromethane	−1.52	313.8	0.99
1,1-Dichloroethane	−1.59	330.5	0.94
Chloroform	−2.08	334.4	0.93

[a] From Refs. 1 and 5. [b] Boiling points at 1 atm. (from "Handbook of Chemistry and Physics," R. C. Weast and S. M. Selby, Eds., Chemical Rubber Co., Cleveland, Ohio, 1967, pp. D113–D137).

sidering gases at 1 atm, the critical temperature, T_c, can be replaced by the boiling point, T_{BP}, in determining reduced temperature, since the temperatures of substances at their boiling points are within 0.66 of their critical temperatures, provided hydrogen bonding or other association effects do not occur in the gaseous or condensed phases (4).

Gases at the physiological temperature of mice, 37° (310°K) (5), differ in relation to their physiological reduced temperature, as obtained from the relation $T_r = 310/T_{BP}$. Table I shows the physiological reduced temperatures for the gaseous anesthetics studied by Miller *et al.* (1, 6). The Clausius–Clapeyron relation (Eq. 1), expressed in terms of physiological reduced temperature, may thus be used to compare the partial pressures of gases for the anesthesia of mice. The criterion of anesthesia is the loss of the righting reflex.

Figures 1 and 2 show the partial anesthetic pressures of gases toward mice plotted according to the two alternative integrated forms of the Clausius–Clapeyron equation. In each graph the fluorinated hydrocarbon, fluoromethane, and the perfluoro compounds, perfluoromethane and perfluoroethane, depart from the general linear trend, perhaps because of the unique solubility properties of fluorinated hydrocarbons. The complimentariness expected on the basis of Eq. 1 between Figs. 1 and 2 is generally real-

Table II—Estimation of the Thermodynamic Activities for Some Gaseous Anesthetics

Gas	Boiling Point, T_{BP}[a]	Enthalpy of Vaporization, ΔH_v[a]	Partial Pressure Pure Liquid, log p^0	Anesthetic Partial Pressure, log p	Thermodynamic Activity, log (p/p^0)
Helium	4.2	20	1.025	2.28	1.255
Hydrogen	20.4	216	2.158	2.14	−0.018
Neon	27	450	3.317	1.94	−1.377
Nitrogen	77	1330	2.826	1.52	−1.306
Tetrafluoromethane	145	2650	2.358	1.24	−1.118
Hexafluoroethane	195	3860	1.598	1.19	−0.408
Argon	87	1560	2.808	1.18	−1.628
Methane	112	1960	2.438	0.66	−1.778
Krypton	121	2160	2.369	0.65	−1.719
Nitrous oxide	184	3960	1.905	0.18	−1.725
Ethylene	169	3240	1.894	0.15	−1.744
Ethane	184	3510	1.688	0.11	−1.578
Xenon	165	3020	1.863	−0.02	−1.883
Propane	231	4490	1.076	−0.05	−1.026
Propylene	225	4400	1.167	−0.40	−1.567
Cyclopropane	114	4536	5.470	−0.80	−6.270
Chloromethane	249	5150	0.884	−0.85	−1.734

[a] From J. H. Hildebrand and R. L. Scott, "The Solubility of Nonelectrolytes," Dover, New York, N.Y., 1964, pp. 435–439.

ized, except in Fig. 2 where the points for helium, hydrogen, and neon are far from the line. Since these compounds have boiling points close to 0°K, aberrant behavior with respect to other compounds is to be expected in a graph involving log T.

Least-squares regression of the points contained in Figs. 1 (omitting those for the deviant fluorocarbons) and 2 (omitting those for the deviant fluorocarbons, helium, hydrogen, and neon) provides the slopes from which, respectively, apparent enthalpies and entropies of vaporization can be estimated. The derived relationships are expressed by:

$$\Delta H_v = 16.81(\pm 0.32)T_r^2 \quad \text{(Eq. 2)}$$

$$\Delta S_v = 22.31(\pm 1.56)T_r \quad \text{(Eq. 3)}$$

In principle, at a reduced temperature of 1, the constants appearing in Eqs. 2 and 3 should be identical. The fact that they are not, but are nearly so, may be attributed to the use of a biological end-point, the loss of the righting reflex, in making partial pressure assignments for the anesthetic gases. Nevertheless, given the uncertainties in the partial pressure values, the agreement found is remarkable.

Inspection of Table I shows that the reduced temperatures for many important gaseous anesthetics are generally not far from 1, *e.g.*, chloroform, $T_r = 0.93$; ether, $T_r = 1.01$; and cyclopropane, $T_r = 1.29$. The apparent entropy of vaporization of these substances from the biophase responsible for anesthesia in the mouse is thus on the order of 20 cal/mole-°K. This finding may be contrasted with the Trouton's rule value of 21 cal/mole-°K for the entropy of vaporization of nonassociating substances. These anesthetics thus appear to be dissolving in a nonpolar phase; and since the process is universal to the gaseous anesthetics under consideration, the same nonpolar biophase must be involved for them all. No special character to anesthesia is imparted by having electronegative atoms on carbon-bearing hydrogen atoms, as was conjectured recently (2).

A particularly important conclusion can be drawn from the finding of a nonzero slope to the lines shown in Figs. 1 and 2, since this finding designates that the Ferguson principle must be restated. The Ferguson principle as presently accepted states that, under equilibrium or near equilibrium conditions, compounds must accumulate to a certain level in an organism or tissue to elicit an equivalent biological response and that this level of accumulation is directly measured by the thermodynamic activities of the compounds in the phase external to the organism or tissue.

This statement signifies that all gaseous anesthetics would have the same thermodynamic activities at the biological end-point measured as the loss of the righting reflex of mice. If this was indeed true, then the slope of the data presented in Figs. 1 and 2 would have to be zero according to Eq. 1. That is, ΔH_v and ΔS_v would be zero, signifying that gaseous anesthetics behave as if they form an ideal solution with the biophase of mice. The nonzero slope to the line in Figs. 1 and 2 shows that the "solution" formed by gaseous anesthetics in a biophase is not ideal. Hence, *the presently accepted statement of the Ferguson principle applies only if the properties of drugs in a biophase are similar to those of an ideal solution.*

The nonideal behavior of the biosolubilization of gaseous anesthetics provides a basis for understanding the origins of linear free energy relationships in relation to anesthesia. Were the properties of drugs in a biophase to be in accord with the Ferguson principle, no linear free energy relationships could exist, since there would be no variation in enthalpy or entropy. However, the Ferguson principle, as revised to apply to the ideal case, is invalid for gaseous anesthesia. Hence, there are variations in enthalpy and entropy that can be related to corresponding variations in model systems.

In further support of the conclusion that the Ferguson principle is invalid for gaseous anesthesia, the thermodynamic activities for some gaseous anesthetics were determined. The relationship given by Eq. 4 was used, where the vapor pressure of the pure liquid, p^0, was determined using Eq. 5, as suggested by Hildebrand and Scott (7):

$$\log (p/p^0) = \log a_2 \quad \text{(Eq. 4)}$$

$$\log p^0 = \frac{\Delta H_v}{2.3R}\left(\frac{T - T_{BP}}{TT_{BP}}\right) \quad \text{(Eq. 5)}$$

Table II shows the results of these determinations. Log p^0 at the physiological temperature of 37° (310 °K) was relatively constant and of much greater magnitude than the partial pressures of anesthesia given by log p. The calculated thermodynamic activities were found to be roughly constant, due principally to the relatively constant value of log p^0, the anesthetic potencies being small in comparison. There is thus gained the illusion that anesthetics in a biophase behave ideally, since for ideal solutions $a_2 = 1/p_2^0$ when the vapor pressure is measured at 1 atm. Therefore, the Ferguson principle has been founded on an observation that by its very nature excludes the variation in anesthetic partial pressure.

The thermodynamic description of the gaseous anesthesia of mice resulting from this study has led to a restatement of the Ferguson principle. All of the observations made by directly applying thermodynamic principles to the anesthetic potencies are in full accord with the gas–oil distribution studies conducted by Miller *et al.* (1). The distinction here is that thermodynamics have been applied solely to the biological observations rather than in relating the biological observations to the behavior of a model system.

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Relationship between Quinidine Plasma and Saliva Levels in Humans

Keyphrases □ Quinidine—plasma and saliva concentrations compared, humans □ Saliva—determination of quinidine levels, correlated with plasma levels

To the Editor:

Increasing applications of the principles of pharmacokinetics to clinical situations (1) have emphasized the need for rapid, noninvasive techniques for monitoring drug concentrations in biological fluids, especially with pediatric patients or whenever a large number of serial samples is indicated. Since several drugs such as salicylate (2), sulfonamides (3), barbiturates (4), tetracyclines (5), phenytoin (6), theophylline (7), and digoxin (8) are secreted in saliva, this measurement appears to have potential use, especially when these levels can be correlated with plasma levels in patients.

There are no previous reports concerning salivary concentrations of quinidine. However, blood level monitoring of quinidine is occasionally useful clinically, since the drug exhibits an extremely narrow range between a usually effective serum level (2 μg/ml) and a usually toxic serum level (8 μg/ml) (9).

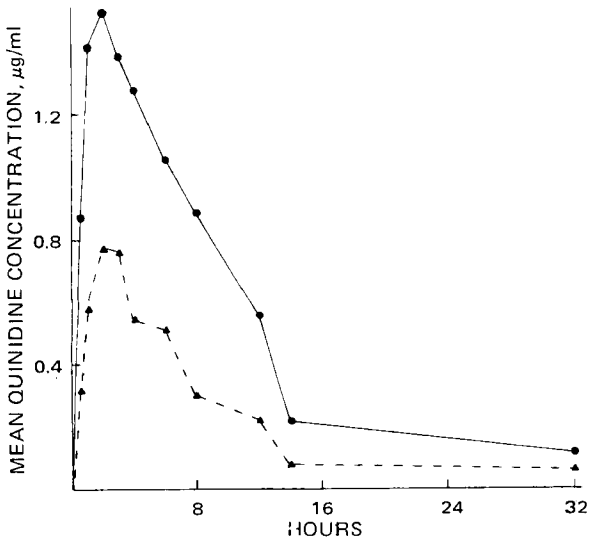

Figure 1—*Mean plasma and saliva concentrations as a function of time for Subject 1. Each concentration is the mean of two determinations. Key: ●, plasma concentrations; and ▲, saliva concentrations.*

Table I—Relationship between Plasma and Saliva Concentrations of Quinidine in Three Subjects

Subject	Mean Plasma–Saliva Ratio (±*SD*)	Correlation Coefficient	Statistical Significance
1	2.29 (±0.89)[a]	0.885	$p < 0.001$
2	1.55 (±0.70)[a]	0.681	$p < 0.01$
3	2.35 (±0.70)[b]	0.825	$p < 0.001$

[a] Eighteen determinations. [b] Nineteen determinations.

Thus, a technique that would obviate the need for collecting serial blood samples would be of value.

The data reported here on saliva levels of quinidine were obtained in conjunction with a larger study on the bioequivalency of various commercially available quinidine sulfate tablets in humans. The specific value of this study relates best to the possible use of saliva in bioavailability studies rather than for clinical monitoring.

Three adult, normal, male subjects involved in the bioequivalence study were selected randomly and, in addition to providing blood samples (by venipuncture), were requested to expectorate into 10-ml test tubes from approximately 5 min before until 5 min after each blood collection or until 3–5 ml of mixed saliva had been obtained. Blood and saliva quinidine levels were determined at 0.5, 1, 2, 3, 4, 6, 8, 12, 24, and 32 hr after administration of two 200-mg tablets of any one of four brands of quinidine sulfate. These same subjects repeated this procedure a second time with at least a 1-week interval between administrations. The values reported in Table I for each subject were pooled from the two different runs and involve 18 or 19 determinations/subject since saliva levels were not always sufficiently high to be determined at the 24- and 32-hr time periods.

Aliquots (0.5 ml) of plasma or saliva (centrifuged to remove sputum) at each time period were assayed by the fluorometric method of Cramer and Isaksson (10) with only slight modifications. Blank specimens of serum and saliva were assayed in the same manner as the samples, and the appropriate blank corrections were applied. Figure 1 shows a plot of plasma and saliva quinidine concentrations as a function of time for one of the three subjects (Subject 1 of Table I).

Although a significant correlation exists between plasma and saliva levels of quinidine, differences in the extent of correlation are evident among the three subjects (Table I). While a number of studies (2, 6) showed an even better correlation between plasma and saliva levels of other drugs, the present study nonetheless suggests that quinidine can be detected in human saliva in measurable concentrations and that these concentrations are related to their corresponding plasma levels. The pH of the saliva samples was not measured, although fluctuations in saliva pH could possibly account for the observed intrasubject variability in plasma saliva ratios for the weak base quinidine (11).

Plasma samples in this study consisted of both bound and unbound drug, and better correlations might have resulted if unbound quinidine in plasma

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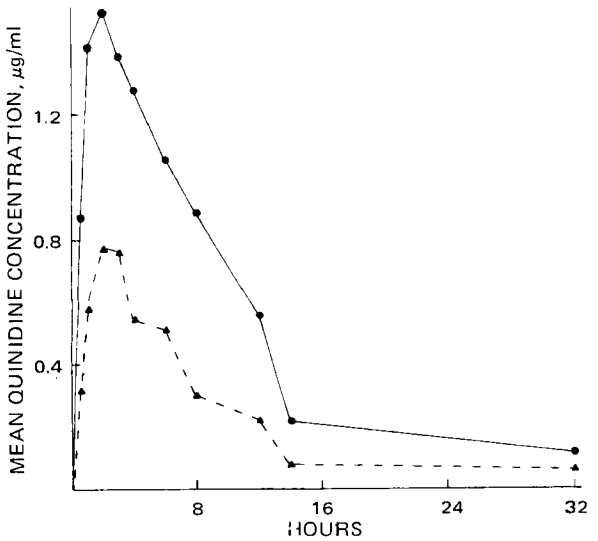

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Dissolution of Model Gallstones in Bile Acid Solutions I: Implications for T-Tube Infusion Treatment of Retained Common Duct Stones with the Cholate System

Keyphrases □ Dissolution—gallstones, *in vitro*, T-tube infusion treatment with sodium cholate □ Gallstones—*in vitro* dissolution, T-tube infusion treatment with sodium cholate □ Sodium cholate—infusate for *in vitro* dissolution of gallstones by T-tube infusion

To the Editor:

The problem of retained common bile duct stones affects about 5% of the patients undergoing cholecystectomies (1–3). Considerable effort has been directed by clinicians[1] (2–6) to find a nonsurgical solution to the problem which will save the patient a high risk operation.

One study (2) showed that retained common bile duct stones can be dissolved using a T-tube infusion; sodium cholate solution was used as the infusate. The investigators were able to dissolve retained stones in 12 of 22 patients within 14 days of continuous infusion at a rate of 30 ml/hr. With the same technique and a similar formula, retained stones were dissolved in five of six patients within 5 days (3).

However, recent controlled T-tube infusion studies were less successful[1]. Only two of six patients showed dissolution of the retained stones. This difference in results between the studies was not expected since there was only a 25% difference in the cholate concentration levels between the formulations tested.

This communication presents a probable physical–chemical explanation for the differences observed. This explanation is based upon the surface resistance to the cholesterol dissolution in bile acid media and the influence of electrolytes upon it.

Compressed cholesterol monohydrate pellets were used as model cholesterol stones. Infusion media were prepared according to Way *et al.* (2) and La-Russo *et al.*[1], using a purified grade of cholic acid[2]. The pH of the media was adjusted to pH 7.5 with sodium hydroxide.

Dissolution experiments were conducted using the apparatus and procedures reported previously (7). Equilibrium solubilities were measured after allowing excess amounts of ^{14}C-cholesterol monohydrate to equilibrate with the infusion media with continuous shaking at 37°.

Dissolution rates and solubility values are shown in Table I. The resistance to dissolution, R, was calculated from the equation:

$$\frac{J}{A} = \frac{C_s}{R} \qquad \text{(Eq. 1)}$$

where J/A is the dissolution rate per unit surface area, and C_s is the solubility.

The data show that the rate of cholesterol dissolution in the Way *et al.* (2) and Lansford *et al.* (3) infusion medium is about nine times faster than that in the La Russo *et al.*[1] medium. This large difference in the rate of dissolution cannot be explained on the basis of a diffusion-controlled mass transport mechanism, because the solubility increases by only about a factor of two (Table I) and the diffusivities are not expected to vary significantly.

These results, therefore, point to the likely importance of surface kinetic factors in the dissolution process. A significant interfacial resistance has been shown (7–11) to govern the rate of cholesterol monohydrate and cholesterol gallstone dissolution in bile media generally, and it is proposed that interfacial resistance is the primary factor responsible for the difference in cholesterol dissolution and, hence, the

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Table I—Dissolution Rates, Solubilities, and Resistances to Dissolution of Cholesterol Monohydrate in Sodium Cholate Infusion Media

Infusion Medium	$(J/A)\ 10^4$, mg/(cm² sec)	C_s, mg/ml	$R \times 10^{-3}$, sec/cm
Way *et al.* (2) and Lansford *et al.* (3) (100 m*M* sodium cholate + 154 m*M* sodium chloride)	2.038	1.440	7.066
La Russo *et al.*[a] (75 m*M* sodium cholate + 77 m*M* sodium chloride)	0.236	0.710	30.085
75 m*M* sodium cholate	0.061	0.452	74.10
100 m*M* sodium cholate	0.201	1.032	51.34
100 m*M* sodium cholate + 77 m*M* sodium chloride	0.682	1.315	19.282
75 m*M* sodium cholate + 154 m*M* sodium chloride	0.744	0.974	13.091

[a] See Footnote 1.

T-tube infusion efficacy between the two solutions.

Previous investigations in our laboratories (11–13) showed that the interfacial barrier to cholesterol transport in bile acid systems may be highly sensitive to electrolytes. It is proposed, therefore, that the difference in the electrolyte levels of the two formulations is primarily responsible for the efficacy difference in retained stones dissolution.

Additional data supporting this interpretation can be seen in Table I. For example, in 75 m*M* sodium cholate, the cholesterol dissolution rate increased fourfold when 77 m*M* sodium chloride was added and an additional threefold when the sodium chloride concentration was doubled.

The results of this study should open the door for a much broader systematic investigation of the formulation–activity relationships of solutions for T-tube infusion in patients with retained common duct stones.

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Vehicle for Stabilizing Aluminum Hydroxide Gel

Keyphrases □ Aluminum hydroxide gel—vehicles for stabilization □ Stability—aluminum hydroxide gel, vehicles investigated □ Antacids—vehicles for stabilizing aluminum hydroxide gel

To the Editor:

A recent research article (1) demonstrated that aluminum hydroxide gel loses acid reactivity depending on the vehicle used for dilution and the degree of dilution. A significant loss of acid reactivity occurred when the gel was diluted with double-distilled water. Dilution with dioxane or mother liquor had no effect on reactivity. It was suggested that a carefully designed diluting solution could minimize the loss of acid reactivity normally observed and result in a dosage form with increased efficacy and a longer shelf-life. We now wish to demonstrate the stabilizing effect of a vehicle suitable for use in aluminum hydroxide gel dosage forms.

An aluminum hydroxide gel was prepared as previously described (1) by the reaction of aluminum chloride, sodium bicarbonate, and sodium carbonate at pH 6.5. The gel was divided into four portions and diluted to 3.3% Al_2O_3. One portion was diluted with a saturated aqueous solution of potassium chloride (equivalent to 72 mEq/15 ml). The second portion was diluted with propylene glycol. The third portion was diluted with distilled water saturated with carbon dioxide, and the fourth portion was diluted with double-distilled water to serve as the control.

The acid reactivity was monitored by an automated[1] pH-stat technique similar to the method described by Steinberg *et al.* (2). An appropriate volume of water was added to the reaction vessel and brought to pH 3.0. A volume of sample containing 38 mg of aluminum oxide was then added. The volume of water was adjusted for each sample to produce a reaction volume of 22 ml. The instrument was set to maintain pH 3.0 by adding 1.0 *N* HCl from the autoburet. The instrument was activated simultaneously with the sample injection. The recorder plotted milliliters of 1.0 *N* HCl added *versus* time. The acid reactivity of each sample is expressed as T_{50}: the time re-

[1] PHM 26; TTT 11; ABU 12, 2.5 ml; TTA 3; SBR 2; Radiometer, Copenhagen, Denmark.

Table I—Dissolution Rates, Solubilities, and Resistances to Dissolution of Cholesterol Monohydrate in Sodium Cholate Infusion Media

Infusion Medium	$(J/A)\ 10^4$, mg/(cm² sec)	C_s, mg/ml	$R \times 10^{-3}$, sec/cm
Way *et al.* (2) and Lansford *et al.* (3) (100 m*M* sodium cholate + 154 m*M* sodium chloride)	2.038	1.440	7.066
La Russo *et al.*[a] (75 m*M* sodium cholate + 77 m*M* sodium chloride)	0.236	0.710	30.085
75 m*M* sodium cholate	0.061	0.452	74.10
100 m*M* sodium cholate	0.201	1.032	51.34
100 m*M* sodium cholate + 77 m*M* sodium chloride	0.682	1.315	19.282
75 m*M* sodium cholate + 154 m*M* sodium chloride	0.744	0.974	13.091

[a] See Footnote 1.

T-tube infusion efficacy between the two solutions.

Previous investigations in our laboratories (11–13) showed that the interfacial barrier to cholesterol transport in bile acid systems may be highly sensitive to electrolytes. It is proposed, therefore, that the difference in the electrolyte levels of the two formulations is primarily responsible for the efficacy difference in retained stones dissolution.

Additional data supporting this interpretation can be seen in Table I. For example, in 75 m*M* sodium cholate, the cholesterol dissolution rate increased fourfold when 77 m*M* sodium chloride was added and an additional threefold when the sodium chloride concentration was doubled.

The results of this study should open the door for a much broader systematic investigation of the formulation–activity relationships of solutions for T-tube infusion in patients with retained common duct stones.

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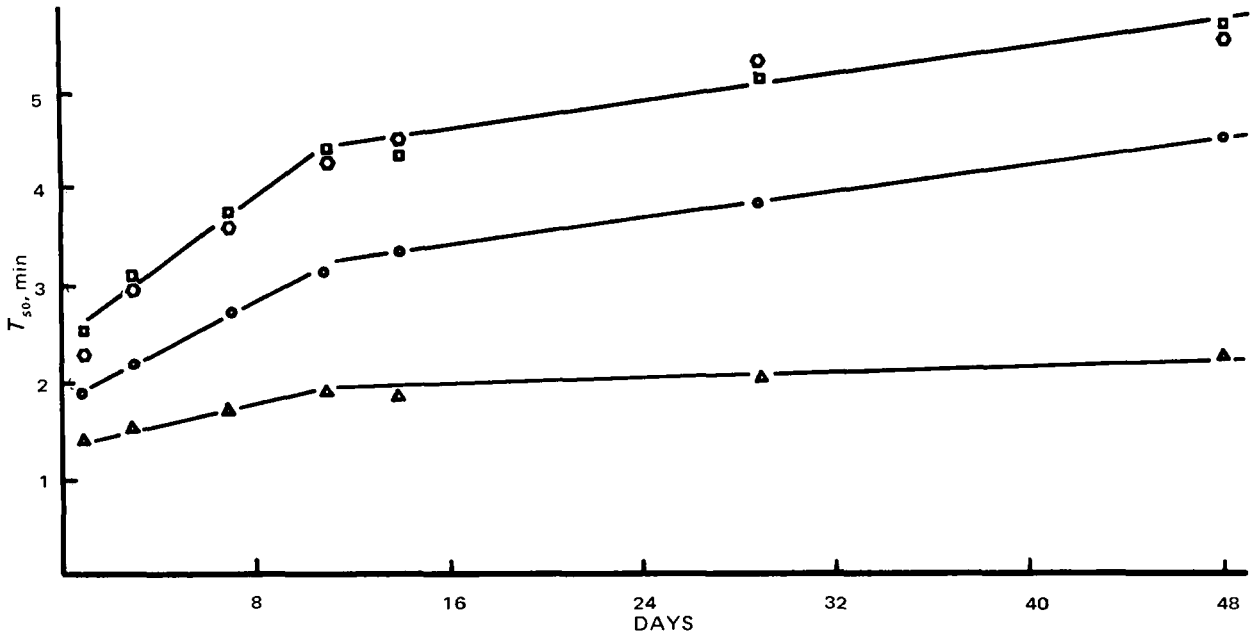

Figure 1—*Effect of diluting vehicle on acid reactivity of aluminum hydroxide gel aged at 25°. Gels were diluted to 3.3% Al_2O_3 with: □, distilled water; ○, carbon dioxide-saturated distilled water; ○, propylene glycol; and △, saturated aqueous solution of potassium chloride.*

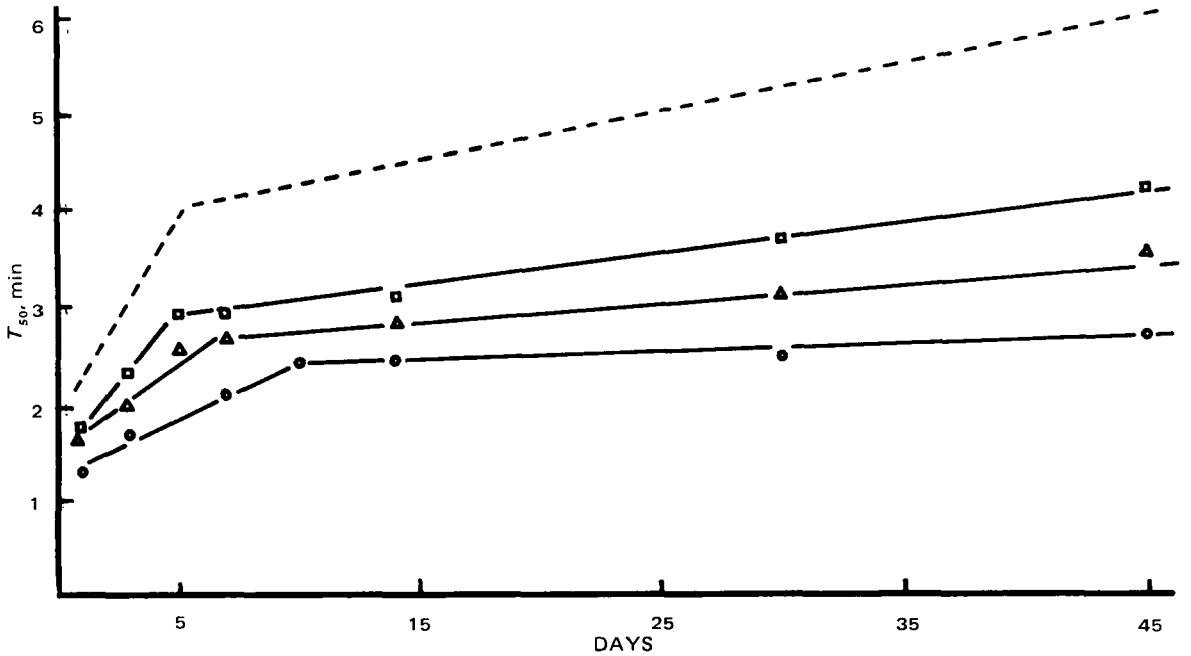

Figure 2—*Stabilization of aluminum hydroxide gel by dilution with potassium chloride solutions. Gels were aged at 25° and diluted to 3.6% Al_2O_3 with: ○, 40 mEq of potassium chloride/15 ml; △, 20 mEq of potassium chloride/15 ml; □, 10 mEq of potassium chloride/15 ml; and - - -, distilled water.*

quired to add 50% of the total 1.0 *N* HCl needed to neutralize the aluminum hydroxide gel at 25°.

As seen in Fig. 1, the acid reactivity of the gel diluted with saturated potassium chloride was virtually unchanged. The sample diluted with propylene glycol was more stable than the portions diluted with distilled water or carbon dioxide-saturated water.

Lower levels of potassium chloride also significantly improve the stability of aluminum hydroxide gel. A second aluminum hydroxide gel was prepared as before, divided into three portions, and diluted to 3.6% Al_2O_3 with solutions equivalent to 40, 20, and 10 mEq of potassium chloride/15 ml. The stabilization by the potassium chloride solution was directly related to the potassium chloride concentration of the diluting solution (Fig. 2).

The samples continue to be monitored, and at 120 days the correlation coefficient for the linear portion commencing at 7 days was 0.999, 0.998, and 0.987 for the gels diluted with 10, 20, and 40 mEq of potassium chloride/15 ml, respectively. The 10-mEq/15-ml dilution resulted in a 30% improvement in T_{50} after 120

days at 25° compared to dilution with distilled water.

The 10 mEq of potassium chloride/15-ml dilution does not have an objectionable taste and could be suitably flavored to produce an acceptable pharmaceutical product. The OTC Antacid Review Panel report (3) stated that potassium presents no problem to normal persons, but that products containing 25 mEq or more of potassium per maximum daily dose must include a warning to patients with kidney disease. Thus, a vehicle designed to maintain the stabilizing ions in the aluminum hydroxide gel appears to be a feasible approach to improving the stability of aluminum hydroxide gel.

(1) N. J. Kerkhof, J. L. White, and S. L. Hem, *J. Pharm. Sci.*, **64**, 940(1975).

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(3) *Fed. Reg.*, 38 (65), 8714(Apr. 5, 1973).

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BOOKS

REVIEWS

Alicyclic Chemistry, Volume 2—A Review of the Literature Published during 1972. Senior Reporter, W. PARKER. The Chemical Society, Burlington House, London WIV OBN, England, 1974. 470 pp. 14 × 22 cm. Price $35.

This book is part of a series of 32 Specialist Periodical Reports designed, according to the Chemical Society, to give an in-depth coverage of the whole field of chemistry. Ultimately some 40 titles are contemplated. The first volume was published in three reports covering aliphatic, alicyclic, and saturated heterocyclic chemistry from January 1970 to December 1971. Other reports of interest to medicinal chemists are: Aliphatic Chemistry, Saturated Heterocyclic Chemistry, Aromatic and Heteroaromatic Chemistry, Organic Compounds of Sulfur, Selenium and Tellurium (all Volume 2), and Organophosphorous Chemistry, already up to Volume 5.

The present volume consists of four chapters. Chapter 1 covers three- and four-membered rings. The first 10 pages are devoted to theoretical and structural considerations including X-ray studies, MO calculations, and energetics. The remainder of the chapter is divided into syntheses and reactions of three- and four-membered carbocyclic compounds. The coverage is thorough. The reactions considered include those with electrophiles, nucleophiles, thermal reactions, cycloadditions, rearrangements, eliminations, functional group modifications, and radicals. Chapter 2 views five- and six-membered rings in light of structural and conformational considerations, followed by an in-depth overview of their reactivities. A 10-page section also considers fused rings. Thus, the reactions of decalyl tosylates and amines are described in terms of acetolyses, deaminations leading to twist conformers, and ring contractions. Bicyclo[4.3.0]nonane and bicyclo[4.2.0]octane systems are also discussed.

Chapter 3 is devoted to medium and large ring compounds. It has 16 sections. Energies and conformations of seven- to 10-membered rings are covered, followed by synthetic routes to such systems. Intramolecular photochemical electrocyclic and cycloaddition reactions, as well as intermolecular photochemical reactions, are surveyed. Transannular reactions, ring contractions, and ring-opening reactions are briefly covered. Finally there is a section on general reactions, *e.g.*, of cycloalkenes, cyclic ketones, amines, alcohols, and esters. Organometallic derivatives complete the chapter.

The final chapter is titled Bridge Carbocyclics. A lengthy introductory section discusses physical methods and energy calculations. This is followed by a section on bicyclic and polycyclic structures. Of particular interest to medicinal chemists are some references to bornane chemistry and extensive coverage (11 pages) of adamantanes. Extensions of known methods of syntheses for this carbon skeleton are reported. The chapter further details cycloadditions, photo- and organometallic chemistry, and solvolytic reactions. Finally, several miscellaneous reactions, including a new stereospecific reducing agent (lithium tris-s-butyl borohydride) and an enzymatic reduction (horse liver dehydrogenase and NADH), are referred to. An author index is included.

The book is profusely endowed with structural formulas; the reader need not fumble with vast quantitites of IUC nomenclature. Its value to the medicinal chemist would be mainly as an adjunct reference source for new developments in those alicyclic ring systems of potential biological interest either as pharmacophores (adamantanes) or as useful carriers for such groups.

Reviewed by Alex Gringauz
Brooklyn College of Pharmacy
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If there has been a criticism of *Roger's Inorganic Pharmaceutical Chemistry*, perhaps this was due chiefly to the fact that the contents of the work were not organized to stress biological activity or medicinal use. If there has been a criticism of inorganic pharmaceutical chemistry, perhaps it has been that the material the discipline treats is somehow supposed to not be of a great deal of therapeutic or clinical importance.

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To go into greater detail, the first three chapters are summaries of general chemical principles together with appropriate pharmaceutical exemplifications. This statement also applies to Chapter 4 although the proportion of "practical" information in this chapter is greater. Thus in Chapter 1, an account of the electronic structure of atoms and molecules is presented. A somewhat more specialized discussion of coordination chemistry together with descriptions of specific pharmaceutically and therapeutically significant chelating agents follows. Chapter 2 is a survey of group properities of elements. Chapter 3 treats solutions and solubility. The first portion of this chapter treats concentration and solubility expressions and their calculation. Thereafter, material mainly dealing with the physical chemistry of the solution process is presented. Chapter 4 first treats concepts of acidity and basicity, expressions of acidity and basicity in water, and buffer theory, together with appropriate practical pharmaceutical exemplification. The second portion of the chapter deals with antioxidants—first theory, then practical applications. The chapter concludes with a treatment of pharmaceutically significant properties of water and of glass.

There is a greater emphasis on the pharmacological and clinical aspects commencing in Chapter 5, Major Intra- and Extracellular Electrolytes, and continuing in Chapter 6, Essential and Trace Ions. (In these two chapters there are excellent biochemical treatments of the inorganic agents as well.) The other chapters are as follows: Chapter 7, Nonessential Ions; Chapter 8, Gastrointestinal Agents; Chapter 9, Topical Agents; Chapter 10, Dental Products; Chapter 11, Radiopharmaceuticals and Contrast Media; and Chapter 12, Miscellaneous Inorganic Pharmaceutical Agents (Inhalants, Antidotes, Emetics, *etc.*).

Overall, in these chapters, I was impressed with the entertaining and still scholarly style. These chapters also impressed with their conciseness, balance, and logical ordering. Notably, I thought the interplay and balance between the biological and chemical portions was very well struck.

I did detect some minor omissions of biochemical functions of trace ions, some biological actions of nonessential ions, some therapeutic uses of topical agents, and certain agents in the miscellaneous group, but I feel that in most of the cases it would be difficult not to make such omissions in condensing material of such breadth into textbook form.

In summary, I found this book to be a well-balanced blend of the theoretical, the practical, the biological, and the therapeutic aspects of inorganic pharmaceutical chemistry. To return to the comments which opened this review and to place the emphasis a little differently, I sincerely believe that this book *per se* is a clear affirmation of the critical importance of inorganic pharmaceutical chemistry and of courses in which inorganic pharmaceutical chemistry is taught, both to modern pharmaceutical education and to modern pharmaceutical practice. I also believe that this book affords a literate, flexible, compact, and extremely valuable textbook for inorganic pharmaceutical chemistry courses.

Reviewed by Eugene I. Isaacson
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The Alkaloids. Volume 4. A Specialist Periodical Report. J. E. SAXTON, Senior Reporter. The Chemical Society, Burlington House, London W1V OBN, England, 1974. xxii + 443 pp. 13.5 × 21.5 cm. Price £14 (about $40.00).

This book is divided into 17 chapters containing up-to-date information on various aspects of alkaloid studies. It is well written and is a very comprehensive review of the literature on alkaloids.

Chapter 1 updates existing information on the biosynthesis of various type of alkaloids as well as specific alkaloids. The contents of the book consist of chapters on the phytochemistry of pyrrolidine, piperidine, and pyridine; tropane; pyrrolizidine; indolizidine; quinolizidine; quinoline, quinazoline, acridone, and related alkaloids; β-phenethylamines and the isoquinoline; aporphine, *Amaryllidaceae*, and related compounds; *Erythrina* and related compounds; indole; *Lycopodium*; diterpenoid; steroidal and miscellaneous alkaloids.

Of particular interest to this reviewer is the fact that pharmacological aspects of certain of the classes of alkaloids are discussed as well as the detailed discussions on spectral interpretations as given by M. Shamma and S. S. Salgar in their chapter on the aporphine alkaloids.

For investigators specialized in alkaloid or students of natural products chemistry, this book is a highly recommended item. One disconcerting aspect of the book is the relatively high cost for a book that is obviously produced by the offset printing process.

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Isolation and Identification of Drugs (in Pharmaceuticals, Body Fluids and Post-mortem Material), Vol. 2. Edited by E. G. C. CLARKE. The Pharmaceutical Press, 17 Bloomsbury Square W.C.-1A2NN, London, England (U.S. Distributor: Rittenhouse Book Distributors, 253 South 24th St., Philadelphia, Pa.), 1975. xi + 385 pp. 14.8 × 22.6 cm. Price £13.50 (about $35.00).

Clarke's 1969 presentation by the same title was the first large-scale collation of data of greatest interest to toxicologists, biochemists, pharmacists, pathologists, and forensic and pharmaceutical scientists. He drew into monograph form, in a beautifully produced book, data on drug identification and separation methods, properties, metabolism, and toxicology. It rapidly and most deservedly became a standard text.

This new volume carries forward the same format to a wealth of supplementary material. New monographs for about 250 drugs not covered in the 1969 opus are the heart of the volume, with a cumulative index for both. Most of the notable omissions of drug entries, already evident at the time of publication of the first volume, have been corrected. Not all drug substances, not even all official drugs, are included, but such an enormous task is not called for in a work devoted to the restrictions inherent in the book's title.

Previous chapters on drug metabolism, the three chromatographies (PC, TLC, and GLC) used in the monographs, and spectroscopic and color tests have all been updated by the original authors to maintain the generally high quality established before. The chapter on screening tests for common drugs has been recast entirely and greatly enlarged and is a major plus for the new volume.

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Of particular interest to this reviewer is the fact that pharmacological aspects of certain of the classes of alkaloids are discussed as well as the detailed discussions on spectral interpretations as given by M. Shamma and S. S. Salgar in their chapter on the aporphine alkaloids.

For investigators specialized in alkaloid or students of natural products chemistry, this book is a highly recommended item. One disconcerting aspect of the book is the relatively high cost for a book that is obviously produced by the offset printing process.

Reviewed by Harry H. S. Fong
College of Pharmacy
University of Illinois at the Medical Center
Chicago, IL 60612

Isolation and Identification of Drugs (in Pharmaceuticals, Body Fluids and Post-mortem Material), Vol. 2. Edited by E. G. C. CLARKE. The Pharmaceutical Press, 17 Bloomsbury Square W.C.-1A2NN, London, England (U.S. Distributor: Rittenhouse Book Distributors, 253 South 24th St., Philadelphia, Pa.), 1975. xi + 385 pp. 14.8 × 22.6 cm. Price £13.50 (about $35.00).

Clarke's 1969 presentation by the same title was the first large-scale collation of data of greatest interest to toxicologists, biochemists, pharmacists, pathologists, and forensic and pharmaceutical scientists. He drew into monograph form, in a beautifully produced book, data on drug identification and separation methods, properties, metabolism, and toxicology. It rapidly and most deservedly became a standard text.

This new volume carries forward the same format to a wealth of supplementary material. New monographs for about 250 drugs not covered in the 1969 opus are the heart of the volume, with a cumulative index for both. Most of the notable omissions of drug entries, already evident at the time of publication of the first volume, have been corrected. Not all drug substances, not even all official drugs, are included, but such an enormous task is not called for in a work devoted to the restrictions inherent in the book's title.

Previous chapters on drug metabolism, the three chromatographies (PC, TLC, and GLC) used in the monographs, and spectroscopic and color tests have all been updated by the original authors to maintain the generally high quality established before. The chapter on screening tests for common drugs has been recast entirely and greatly enlarged and is a major plus for the new volume.

Inorganic Medicinal and Pharmaceutical Chemistry. By J. H. BLOCK, E. B. ROCHE, T. O. SOINE, and C. O. WILSON. Lea & Febiger, Philadelphia, PA 19106, 1974. 472 pp. 15.5 × 24 cm. Price $18.50.

If there has been a criticism of *Roger's Inorganic Pharmaceutical Chemistry*, perhaps this was due chiefly to the fact that the contents of the work were not organized to stress biological activity or medicinal use. If there has been a criticism of inorganic pharmaceutical chemistry, perhaps it has been that the material the discipline treats is somehow supposed to not be of a great deal of therapeutic or clinical importance.

The present book, the successor to *Roger's Inorganic Pharmaceutical Chemistry*, lays to rest the first criticism and gives the lie to the second. This is not to state that general principles of chemistry are dispensed with. On the contrary, I feel that they are thoroughly and succinctly covered, principally in Chapters 1–4. It is simply that, thereafter, in Chapters 5–12, the emphasis is on the role of inorganic agents in biology and medicine, and the fact that inorganic agents have a great deal of biological and therapeutic significance is most entertainingly and effectively brought home.

To go into greater detail, the first three chapters are summaries of general chemical principles together with appropriate pharmaceutical exemplifications. This statement also applies to Chapter 4 although the proportion of "practical" information in this chapter is greater. Thus in Chapter 1, an account of the electronic structure of atoms and molecules is presented. A somewhat more specialized discussion of coordination chemistry together with descriptions of specific pharmaceutically and therapeutically significant chelating agents follows. Chapter 2 is a survey of group properities of elements. Chapter 3 treats solutions and solubility. The first portion of this chapter treats concentration and solubility expressions and their calculation. Thereafter, material mainly dealing with the physical chemistry of the solution process is presented. Chapter 4 first treats concepts of acidity and basicity, expressions of acidity and basicity in water, and buffer theory, together with appropriate practical pharmaceutical exemplification. The second portion of the chapter deals with antioxidants—first theory, then practical applications. The chapter concludes with a treatment of pharmaceutically significant properties of water and of glass.

There is a greater emphasis on the pharmacological and clinical aspects commencing in Chapter 5, Major Intra- and Extracellular Electrolytes, and continuing in Chapter 6, Essential and Trace Ions. (In these two chapters there are excellent biochemical treatments of the inorganic agents as well.) The other chapters are as follows: Chapter 7, Nonessential Ions; Chapter 8, Gastrointestinal Agents; Chapter 9, Topical Agents; Chapter 10, Dental Products; Chapter 11, Radiopharmaceuticals and Contrast Media; and Chapter 12, Miscellaneous Inorganic Pharmaceutical Agents (Inhalants, Antidotes, Emetics, *etc.*).

Overall, in these chapters, I was impressed with the entertaining and still scholarly style. These chapters also impressed with their conciseness, balance, and logical ordering. Notably, I thought the interplay and balance between the biological and chemical portions was very well struck.

I did detect some minor omissions of biochemical functions of trace ions, some biological actions of nonessential ions, some therapeutic uses of topical agents, and certain agents in the miscellaneous group, but I feel that in most of the cases it would be difficult not to make such omissions in condensing material of such breadth into textbook form.

In summary, I found this book to be a well-balanced blend of the theoretical, the practical, the biological, and the therapeutic aspects of inorganic pharmaceutical chemistry. To return to the comments which opened this review and to place the emphasis a little differently, I sincerely believe that this book *per se* is a clear affirmation of the critical importance of inorganic pharmaceutical chemistry and of courses in which inorganic pharmaceutical chemistry is taught, both to modern pharmaceutical education and to modern pharmaceutical practice. I also believe that this book affords a literate, flexible, compact, and extremely valuable textbook for inorganic pharmaceutical chemistry courses.

Reviewed by Eugene I. Isaacson
College of Pharmacy
Idaho State University
Pocatello, ID 83209

The Alkaloids. Volume 4. A Specialist Periodical Report. J. E. SAXTON, Senior Reporter. The Chemical Society, Burlington House, London W1V OBN, England, 1974. xxii + 443 pp. 13.5 × 21.5 cm. Price £14 (about $40.00).

This book is divided into 17 chapters containing up-to-date information on various aspects of alkaloid studies. It is well written and is a very comprehensive review of the literature on alkaloids.

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The updated chapters are generally adequate for the purpose, but strong exception must be taken to the claim that no significant advances in TLC have taken place since the first volume. In seven years? We need consider only the recent instrumentation introduced for both quantitative and multiple-development applications to turn back such an argument. Has familiarity bred contempt?

Two new chapters deal with radioimmunoassays and mass spectrometry. Both techniques have had an explosive growth in clinical applications and the inclusion of those topics is clearly necessary. Neither, however, appear in the drug monographs. Because of the limited number of drugs for which radioimmunoassay is in general use, treatment within the background chapter is preferred, but the lack of further mass spectral data is puzzling. Both new chapters are strongly practical rather than theoretical in presentation and this is desirable for the intended audience.

One notable development in drug analysis has been ignored and that is high-pressure liquid chromatography. Sufficient data are available, and at least a general chapter could have been expected at this time.

In recommending heartily the supplementing of a copy of Clarke 1 with this new Clarke 2, it is however time to take stock of the broader situation. Drug analysis and identification currently have available several important compilations—Macek on TLC, Sunshine's editing of analytical toxicology data, the recent Zweig and Sherma handbooks of chromatography, Stahl's compilations—all of which compile data in other than monograph style, but nevertheless, the data are readily retrievable. Viewed against these more detailed compilations, Clarke's volumes sometime suffer in the competition. The hardest blow is delivered when these monographs are compared for content with *Analytical Profiles of Drug Substances*, Volumes 1–4, K. Florey, Ed., which is becoming the primary nonofficial work in drug analysis (but as yet covers but a minority of drugs and not all manufacturers). This is not to say that Clarke 1 and 2 do not fulfill an important function, and well, but rather that the working analyst has the joy of being able to resort to more than one leading source for data before plunging into the primary literature for the late news.

Reviewed by Lee T. Grady
Drug Research and Testing Laboratory
United States Pharmacopeial Convention, Inc.
Rockville, MD 20852

Applications of High-Speed Liquid Chromatography. By N. DONE, J. H. KNOW, and J. LOHEAC. Wiley, 605 Third Avenue, New York, NY 10016, 1975. 238 pp. 16 × 24 cm. Price $19.95.

As with any emerging analytical technology, there have been a sizable number of books published in the area of high-speed liquid chromatography (HSLC). All too often these first volumes provide much in the way of theory and generalized approaches to problems but little practical information that can be helpful to the research scientist with a specific problem to solve. Happily these authors have achieved a balance between theory and practice.

In the first part of the book the authors talk of HSLC theory, equipment, and principles. The discussion is neither too simple nor too detailed and serves as a good refresher to the scientist who has had a more intense orientation in the not too distant past. While much could be done to improve the content, such changes would necessitate depth which is beyond the scope of the book.

Part two provides a listing of approximately 150 chromatograms and related chromatographic data compiled from the literature for the period 1969–1973. Although significant numbers of the chromatograms deal with the typical hydrocarbon separations so often presented in the sales literature for commercial instruments, a good percentage of these chromatograms can be useful to an individual interested in pharmaceuticals and related compounds. Each chromatogram is well presented and includes most pertinent data needed to reproduce the chromatogram which is also given. Each also contains the direct literature reference for further detailed information.

Overall, the volume seems to achieve its objectives. There are a few comments that must be mentioned by way of criticism. First is the basic fact that the field of HSLC is moving so very rapidly that, by the time the book was received for review, so much had evolved in the field that the contents of the volume seemed somewhat dated. This is particularly noticeable in the second part of the book when one compares separations reported there with more recent publications using small particle columns. Finally, the value of the book must be weighed against the price charged by the publisher. Although the book is well done, the price of $19.95 seems excessive considering the somewhat dated nature of the contents.

Reviewed by Arthur F. Michaelis
Sandoz Inc.
East Hanover, NJ 07936

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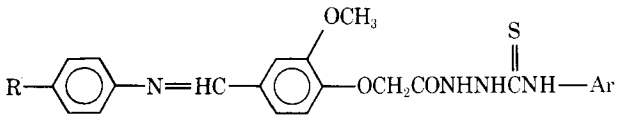
Sandoz Inc.
East Hanover, NJ 07936

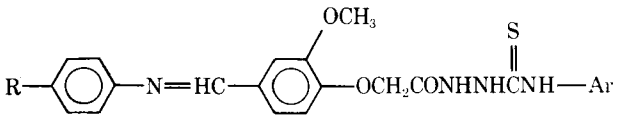
ERRATA

In the article titled "Synthesis of Substituted Anilino-[3-methoxy- 4- (4-arylthiosemicarbazidocarbonylmethyleneoxy)]benzylidenes: Correlation between Anticonvulsant Activity and Monoamine Oxidase Inhibitory and Antihemolytic Properties" (1), the following corrections should be made:

On page 1125, column 1, the structure for IX–XXIV in Scheme I should be:



On page 1126, Table II, the structure should be:



(1) C. Dwivedi, R. D. Harbison, B. Ali, and S. S. Parmar, *J. Pharm. Sci.*, **63,** 1124(1974).

In the article titled "Microbiological Diffusion Assay I: Operations Studied with Cooper Equation" (1), the following correction should be made:

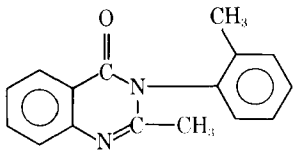
On page 1459, column 2, Eq. 2 should read:

$$X^2 = 4DT_0 \ln (m_0/m') + 4Dh \ln (m_0/m')$$

(1) F. W. Kavanagh, *J. Pharm. Sci.*, **63,** 1459(1974).

In the article titled "Hydrolytic Degradation of Methaqualone" (1), the following correction should be made:

On page 1785, column 1, the structure for methaqualone in Scheme I should be:



(1) J. J. Zalipsky, D. M. Patel, and N. H. Reavey-Cantwell, *J. Pharm. Sci.*, **63,** 1784(1974).

In the article titled "Dose–Response Predictability of Urinary Bladder Hyperplasia by *N*-2-Fluorenylacetamide Feeding in Mice: Its Modification by Sex" (1), the following corrections should be made:

On page 1947, column 1, the material pertaining to Eqs. 2–5 should read:

$$\mathrm{var}(Y) = 1/Snw + x^2/Snwx^2 + 2xz/Snwxz + z^2/Snwz^2 \quad \text{(Eq. 2)}$$

$$x = X - \bar{X} \quad \text{(Eq. 3)}$$

$$z = Z - \bar{Z} \quad \text{(Eq. 4)}$$

By replacing $Y = \bar{Y} + bx + cz$:

$$X = \bar{X} + \frac{-B \pm \mathrm{root}(B^2 - 4AC)}{2A} \quad \text{(Eq. 5)}$$

where:

$$K = \bar{Y} + cz - Y_0$$
$$A = b^2 - t^2/Snwx^2$$
$$B = 2(K - t^2z/Snwxz)$$
$$C = (K^2 - t^2/Snw - t^2z^2/Snwz^2)$$

(1) T. J. Haley, G. Schieferstein, W. E. Jaques, J. Farmer, C. Frith, and R. W. Sprawls, *J. Pharm. Sci.*, **63,** 1946(1974).

In the article titled "Cholelithiasis Chemotherapy: An *In Vitro* Approach" (1), the following corrections should be made:

On page 363, Table I, the last two entries under the first column should read "Benzyldimethyl[(octadecylcarbamoyl)methyl]ammonium chloride[e]" and "Cholesteryl-3-α-amine hydrochloride hydrate[g]."

(1) D. Mufson, K. Triyanond, and L. J. Ravin, *J. Pharm. Sci.*, **64,** 362(1975).

In the article titled "Mathematical Formulation for Nonuniform Multiple Dosing" (1), the following corrections should be made:

On page 464, column 1, Eq. 2 should read:

$$\dot{\mathbf{x}} = K\mathbf{x}$$

On page 464, column 2, Eq. 15*a* should read:

$$\dot{x} = -kx + \delta(t - t_1)$$

On page 465, column 1, Eq. 20 should read:

$$\dot{\mathbf{x}} = K\mathbf{x} + \begin{pmatrix} a_1\delta(t - t_1) + a_2\delta(t - t_2) \\ 0 \\ 0 \end{pmatrix}$$

On page 466, column 1, the first line after Eq. 27 should read "where $\mathbf{b} = P^{-1}\mathbf{x}_0$" instead of "$b = P^{-1}\mathbf{x}_0$."

(1) J. R. Howell, *J. Pharm. Sci.*, **64,** 464(1975).

In the article titled "Drug–Biomolecule Interactions: Interactions of Mononucleotides and Polybasic Amino Acids" (1), the following correction should be made:

On page 476, column 2, the abscissa label in Fig. 3 should read "adenosine 5′-monophosphate concentration" instead of "guanosine 5′-monophosphate concentration."

(1) J. C. Lacey, Jr., and K. M. Pruitt, *J. Pharm. Sci.*, **64,** 473(1975).

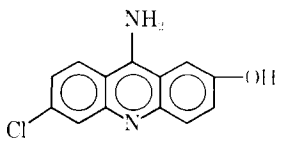
In the article titled "Effect of Surfactants on Absorption through Membranes III: Effects of Dioctyl Sodium Sulfosuccinate and Poloxalene on Absorption of a Poorly Absorbable Drug, Phenolsulfonphthalein, in Rats" (1), the following correction should be made:

Throughout the article, "poloxamer 188" should be substituted for "poloxalene."

(1) S. N. Malik, D. H. Canaham, and M. W. Gouda, *J. Pharm. Sci.*, **64,** 987(1975).

In the article titled "Facile Preparation of 6-Chloro-9-amino-2-hydroxyacridine, a Urinary Metabolite of Quinacrine and Quinacrine Mustard" (1), the following correction should be made:

On page 1418, column 2, Structure II in Scheme I should be:



(1) K. C. Tsou, S. Ledis, E. Steiger, and R. Nietrzeba, *J. Pharm. Sci.*, **64,** 1418(1975).